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PhD Thesis
by
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PREFACE

This PhD thesis entitled "Development of analytical methods for arsenic speciation and their application to novel marine feed resources" is submitted in fulfillment of the requirements for obtaining the *Doctor of Philosophy* (PhD) degree at the National Food Institute, Technical University of Denmark. The PhD work was part of the project "Metals, arsenic and arsenic species in novel marine feed resources" (Project No. 15333), which was funded by the Ministry of Trade, Industry and Fisheries (NFD), Norway. The project was carried out under the auspices of the Institute of Marine Research (IMR), Bergen, Norway and National Food Institute, Technical University of Denmark (DTU Food) from April 2019 to October 2022. The research work was performed under the supervision of Professor Jens Jørgen Sloth (DTU Food) as principal supervisor, and Senior Scientist Heidi Amlund (DTU Food) and Scientist Veronika Sele (IMR) as co-supervisors.

Jojo Samson Tibon

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To my father, Jose Tibon (1958-2021), and father-in-law, Allan Oane (1965-2021), who never got to witness the completion of this PhD.

-Jojo

SUMMARY

By 2050, the world's population is expected to surpass nine billion. Aquaculture has a huge potential to augment the demand for safe and nutritious food by the growing public. However, this would entail an increase in seafood supply, which translates to an additional feed volume requirement. In Norway, while the current composition of Atlantic salmon feed is predominantly plant-based ingredients, many have realized that the agricultural sector has turned into one of the biggest contributors of greenhouse gases. Thus, current research is directed towards novel marine feed resources which can alleviate aquaculture's carbon footprint. Much focus has been given recently to marine resources at low-trophic levels due to their abundance and suitable nutritional composition, e.g. high in proteins and essential fatty acids. Primary producers and consumers such as microalgae, blue mussels, and mesopelagic organisms are currently considered as future ingredients for salmon feed.

Before novel marine resources can be fully utilized, it is necessary to document the levels of undesirable substances. Within the European Union (EU), maximum limits (MLs) are established for undesirable substances in feed and feed ingredients (Directive 2002/32 EC and amendments), which include toxic elements such as mercury, cadmium, lead, and arsenic. Arsenic (As) has over 100 naturally occurring As species in the marine environment. It is mainly recognized for its toxic properties associated with its inorganic forms, i.e. arsenite (As(III)) and arsenate (As(V)). In contrast, the non-toxic arsenobetaine (AB) is the predominant As compound in most marine organisms. Macrolagae, commonly known as seaweeds, contain significant proportions of arsenosugars (AsSug). In fatty fish, lipid-soluble As species, i.e. arsenolipids (AsLipids), are abundant. Recent studies have reported AsSug and AsLipids as potentially toxic compounds. Considering the varying toxicities of As species, the European Food Safety Authority (EFSA) recognizes the need for more As speciation data, which can only be realized when analytical methods for As speciation have been established.

In this PhD project, analytical methods for determining water-soluble As species in marine matrices were developed. A 2^{7-3} fractional factorial design was performed to optimize the extraction procedure. Extraction temperature and the type of extraction solution were identified as significant factors. Arsenic speciation analysis was carried out using ion-exchange

high-performance liquid chromatography coupled to inductively coupled plasma-mass spectrometry (HPLC-ICP-MS). The mobile phase composition was also optimized by investigating the effects of mobile phase buffer and pH on the retention of analytes. Furthermore, the response of ICP-MS was enhanced by the addition of organic solvent in the mobile phase. The methods underwent single-laboratory validation using several marine certified reference materials (CRMs). Overall, satisfactory method performance characteristics were achieved.

The developed methods were applied to novel marine feed resources, i.e. mesopelagic organisms, blue mussels, and microalgae. The overall conclusion was that primary producers such as micro- and macroalgae, which are at the base of the aquatic food pyramid, do not contain AB but only the precursors. These precursors are then metabolized by higher-trophic animals to form AB. The effects of feed processing on As speciation was also studied through a lab-scale set-up with mesopelagic biomass as the starting raw material. An overall dilution effect was noted for total As (tAs) and most As species in mesopelagic meal and oil. However, the study also demonstrated the transfer of potentially toxic AsLipids from the biomass to the resulting mesopelagic meal, and further up-concentrated in mesopelagic oil.

The uptake and biotransformation of As in low-trophic marine food chain was likewise investigated by conducting an exposure and feeding experiment involving microalgae and blue mussels. The exposure of *D. lutheri* to higher iAs concentrations resulted to increased levels of iAs, DMA, and MA. However, at 10 µg/L, iAs accumulated, suggesting that the methylation threshold has been breached, and that detoxification mechanisms are overwhelmed.

Overall, novel marine feed resources will likely comply with current MLs for As (and iAs) in feed materials. However, since low-trophic marine organisms contain significant proportions of AsSug and, presumably, AsLipids, they will likely cause variation in As speciation compared to traditional feed raw materials, e.g. forage fish where AB is predominant. Studies on bioavailability in fish and possible accumulation of these compounds in final fish products should be endeavored to gain solid basis for risk assessment in terms of feed and food safety.

RESUMÉ

I 2050 forventes verdens befolkning at overstige ni milliarder mennesker. Akvakultur har et kæmpe potentiale til at imødekomme den voksende befolknings behov for sunde og nærende fødevarer. Dette vil medføre en stigning i udbuddet af fisk og skaldyr, hvilket igen betyder at behovet for foder øger. I Norge, hvor den nuværende sammensætning af foder til Atlantic laks overvejende er plantebaseret, har mange indset at landbrugssektoren er blevet en af de største bidragsydere til drivhusgasser. Forskning er således rettet mod nye marine foderressourcer, som kan reducere CO_2-fodaftrykket fra akvakulturen. På det seneste har der været et stort fokus på marine ressourcer fra lavtrofiske niveauer på grund af deres volumen og gunstige ernæringsmæssige sammensætning, f.eks. højt indhold af proteiner og essentielle fedtsyrer. Primærproducenter og –konsumenter såsom mikroalger, blåmuslinger og mesopelagiske organismer anses for at være fremtidige ingredienser i laksefoder.

Før nye marine ressourcer kan udnyttes fuldt ud er det nødvendigt at dokumentere niveauerne af uønskede stoffer. Den Europæiske Union (EU) har fastsat grænser for maksimumsindhold (MLs) af uønskede stoffer i foder og foderingredienser (Direktiv 2002/32 EF, og senere ændringer), og disse omfatter toksiske elementer som kviksølv, cadmium, bly og arsen. I det marine miljø findes der mere end 100 naturligt forekommende former af arsen (As). Arsen er kendt for sine toksiske egenskaber forbundet med dets uorganiske former, dvs. arsenit (As(III)) og arsenat (As(V)). Arsenobetain (AB) er den fremherskende As-forbindelse i de fleste marine organismer, og er, i modsætning til de uorganiske As-forbindelser, ikke toksisk. Makroalger, almindeligvis kendt som tang, indeholder betydelige andele af sukkerholdige As-forbindeler (AsSug). I fede fisk er fedtopløselige As-forbindelser, eller arsenolipider (AsLipider) udbredte. Nylige studier har rapporteret at AsSug og AsLipider er potentielt toksiske. I betragtning af As-forbindelsernes varierende toksicitet, har den Europæiske Fødevareautoritet (EFSA) set behovet for flere data på forekomsten af de forskellige As-forbindelser. Dette kun kan ske når analytiske metoder for As speciering er etableret.

I dette PhD projekt er der udviklet analytiske metoder til bestemmelsen af vandopløselige As-forbindelser i marine matricer. For at optimere ekstraktionsproceduren blev et 2^{7-3}

fraktioneret faktorielt (fractional factorial) design benyttet. Ekstraktionstemperatur og ekstraktionsopløsning blev identificeret som væsentlige faktorer. Arsen specieringsanalyser blev udført ved hjælp af ionbytter højtydende væskekromatografi koblet til induktivt koblet plasma massespektrometri (HPLC-ICP-MS). Sammensætningen af mobilfasen blev også optimeret ved at undersøge effekten af mobilfasens buffer og pH på retentionen af analytter. Endvidere blev responsen i ICP-MS'en forstærket ved tilsætning af organisk opløsningsmiddel i mobilfasen. Metoderne blev valideret i et enkelt laboratorium ved hjælp at flere marine certificerede referencematerialer (CRM). Samlet set blev der opnået acceptable præstationskriterier for metoden.

De udviklede metoder blev anvendt på nye marine foderressourcer, dvs. mesopelagiske organismer, blåmuslinger og mikroalger. Den overordnede konklusion var, at primærproducenter som mikro- og makroalger, som findes i bunden af den akvatiske fødepyramide, ikke indeholder AB men kun forløbere til AB. Disse forløbere metaboliseres derefter af højere trofiske dyr til AB. Effekten af forarbejdning af foder på As-speciering blev også undersøgt i laboratorieskala med mesopelagisk biomasse som råmateriale. En samlet fortyndingseffekt blev observeret for total as (tAs) og de fleste As-forbindelser i mesopelagisk mel og olie. Undersøgelsen viste midlertidig også, at potentielt toksiske AsLipider blev overført fra biomassen til mesopelagisk mel og yderligere opkoncentreret i mesopelagisk olie. Optag og biotransformation af As i lavtrofisk marine fødekæde blev ligeledes undersøgt ved at udføre et eksponerings- og fordringsforsøg, der involverede mikroalger og blåmuslinger. Eksponering af *D. lutheri* for højere koncentrationer af iAs resulterede i øgende niveauer af iAs, DMA og MA. Ved 10 µg/L blev iAs akkumuleret, hvilket tyder på at afgiftningsmekanismerne er overvældet.

Samlet set, så vil nye marine foderressourcer sandsynligvis overholder gældende MLs for As (og iAs) i foder og fodermaterialer. Men da lavtrofiske marine organismer indeholder betydelige andele af AsSug og formodentlig AsLipider, vil de sandsynligvis variere i As-speciering sammenlignet med traditionelle foderråvarer, f.eks. fisk, hvor AB er fremherskende. Undersøgelser af biotilgængelighed i fisk og eventuel akkumulering af disse forbindelser i endelige fiskeprodukter bør tilstræbes for at kunne få et solidt grundlag for risikovurdering af foder- og fødevaresikkerhed.

LIST OF PUBLICATIONS

The PhD thesis is based on the publications listed below:

Paper I **Tibon, J.**, Silva, M., Sloth, J. J., Amlund, H., & Sele, V. (2021). Speciation analysis of organoarsenic species in marine samples: method optimization using fractional factorial design and method validation. Analytical and Bioanalytical Chemistry, 413(15), 3909–3923. https://doi.org/10.1007/s00216-021-03341-4

Paper II **Tibon, J.**, Amlund, H., Gomez-Delgado, A. I., Berntssen, M. H. G., Silva, M. S., Wiech, M., Sloth, J. J., & Sele, V. (2022). Arsenic species in mesopelagic organisms and their fate during aquafeed processing. Chemosphere, 302, 134906. https://doi.org/10.1016/j.chemosphere.2022.134906

Paper III **Tibon, J.***, Gomez-Delgado, A. I.*, Aguera, A., Strohmeier, T., Silva, M. S., Lundebye, A. K., Larsen, M. M., Sloth, J. J., Amlund, H., & Sele, V. Arsenic speciation in low-trophic marine food chain – an arsenic exposure study on microalgae (*Diacronema lutheri*) and blue mussels (*Mytilus edulis* L.) (in preparation)

**These authors contributed equally to this work.*

Paper IV Wiech, M., Silva, M., Meier, S., **Tibon, J.**, Berntssen, M. H. G., Duinker, A., & Sanden, M. (2020). Undesirables in Mesopelagic Species and Implications for Food and Feed Safety—Insights from Norwegian Fjords. *Foods*, *9*(9), 1162. https://doi.org/10.3390/foods9091162

OTHER PUBLICATIONS (NOT COVERED IN THIS THESIS)

Paper A Gomez-Delgado, A. I., **Tibon, J.**, Silva, M. S., Lundebye, A. K., Aguera, A., Rasinger, J. D., Strohmeier, T., & Sele, V. Seasonal variations in mercury, cadmium, lead and arsenic species in Norwegian blue mussels (*Mytilus edulis* L.) – assessing the influence of biological and environmental factors. (Under review in Journal of Trace Elements in Medicine and Biology)

Paper B Silva, M. S., **Tibon, J.**, Sartipiyarahmadi, S., Remø, S. C., Sele, V., Søfteland, L., Sveier, H., Wiech, M., Philip, A. J. P., & Berntssen, M. H. G. Feed-to-fish transfer of arsenic and arsenic species in Atlantic salmon fed on diets containing Norwegian farmed blue mussel and kelp. (In preparation)

LIST OF ABBREVIATIONS AND ACRONYMS

AB	Arsenobetaine
AC	Arsenocholine
CAN	Acetonitrile
ANF	Antinutritional factors
As(III)	Arsenite
As(V)	Arsenate
AsFA	Arsenic-containing fatty acids
AsHC	Arsenic-containing hydrocarbons
AsLipids	Arsenolipids
AsPC	Arsenophosphatidylcholine
AsPE	Arsenophosphatidylethano-lamine
AsPL	Arsenic-containing phospholipids
AsSug	Arsenosugar
AsSug OH	Glycerol arsenosugar
AsSug PO4	Phosphate arsenosugar
AsSug SO3	Sulfonate arsenosugar
AsSug SO4	Sulfate arsenosugar
BMDL	Benchmark dose lower confidence limit
Bw	Body weight
CCA	Chromium-copper arsenate
CE	Capillary electrophoresis
CONTAM Panel	EFSA Panel on Contaminants in the Food Chain
CRM	Certified reference material
CV-AFS	Cold vapour atomic fluorescence spectroscopy
DCM	Dichloromethane
DDMAA	5'-deoxy-5'-dimethylarsinoyl-adenoside
DHA	Docosahexaenoic acid
DMA	Dimethylarsinate
DMAA	Dimethylarsinoyl acetate
DMAE	Dimethylarsinoyl ethanol
DMAP	Dimethylarsinoyl propionate
DOE	Design of experiments
Dw	Dry weight
EC	European Commission
EEZ	Exclusive economic zone
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
ETAAS	Electrothermal atomic absorption spectroscopy
EU	European Union
GAC	Green Analytical Chemistry
GC	Gas chromatography
GF-AAS	Graphite furnace atomic absorption spectroscopy
HG-AAS	Hydride generation atomic absorption spectrometry
HG-AFS	Hydride generation atomic fluorescence spectrometry
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IARC	International Agency for Cancer Research
iAs	Inorganic arsenic
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IE	Ionization energy
IEC	Ion-exchange chromatography
INAA	Instrumental neutron activation analysis
IUPAC	International Union of Pure and Applied Chemistry
LB	Lower bound
LC-PUFA	Long-chain polyunsaturated fatty acids
LC-HRMS	Liquid chromatography high resolution mass spectrometry

LOD	Limit of detection
LOQ	Limit of quantification
m/z	mass-to-charge ratio
MA	Methylarsonate
MeOH	Methanol
ML	Maximum levels/limits
MS	Mass spectrometry
MTBE	Methyl *tert*-butyl ether
OFAT	One-factor-at-a-time
PAH	Polycyclic aromatic hydrocarbons
PC	Phosphatidylcholine
PCB	Polychlorinated biphenyls
PE	Phosphatidylethanolamine
PP	Polypropylene
PTE	Potentially toxic elements
RF	Radio frequency
RP-LC	Reversed-phase liquid chromatography
RSM	Response surface modelling
RT	Retention time
SAM	S-adenosylmethionine
SEC	Size exclusion chromatography
SPE	Solid-phase extraction
tAs	Total arsenic
TETRA	Tetramethylarsonium ion
TFA	Trifluoroacetic acid
TMAO	Trimethylarsine oxide
TMAP	Trimethylarsoniopropionate
USA	United States of America
WHO	World Health Organization
ww	Wet weight

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CHAPTER 1: INTRODUCTION

1.1 Project rationale

The global population is expected to reach nine billion by 2050 [1]. Food production systems must keep up with the growing public. Aquaculture has the capacity to meet the foreseen demand for safe and nutritious food [1, 2]. Seafood are rich in vitamins, minerals, proteins, and long-chain polyunsaturated fatty acids (LC-PUFA), which can alleviate malnutrition or micronutrient deficiency [3]. However, to increase seafood production, additional aquafeed volume is also required [2]. Traditionally, fish feed contains mainly marine ingredients such as fish oil and fish meal [4]. While significant efforts have been made to replace conventional marine-based ingredients [5], the use of plant-based raw materials may introduce new risks to aquaculture due to antinutritional factors (ANFs) and undesirable substances [6, 7]. The agricultural sector also ultimately contributes to increasing greenhouse gas emissions [8, 9]. Thus, research is now focused on novel sources of protein and essential fatty acids for aquaculture feed [2]. To lessen the dependence on pelagic fish, the European Commission recommends utilization of marine resources from low-trophic levels [10]. In line with this, feed ingredients from primary producers and consumers, such as microalgae, blue mussels, and mesopelagic organisms, are now considered as candidates for future salmon feeds [2]. Compared to traditionally used pelagic fish species, these low-trophic organisms have not been widely used in feed production to date [2, 4]. If harnessed responsibly, their use can contribute to fulfillment of United Nations Sustainable Development Goal 14, which aims for conservation and sustainable utilization of aquatic resources.

Before novel marine resources can be used as feed ingredients, the levels of undesirable substances should be documented. Within the European Union (EU), maximum levels (MLs) are established for undesirable substances in feed and feed ingredients (Directive 2002/32 EC and amendments), which include potentially toxic elements (PTEs) such as mercury (Hg), cadmium (Cd), lead (Pb), and arsenic (As) [11]. The prevalence of PTEs in the environment can be attributed to both anthropogenic and natural sources [12, 13]. For some elements, the chemical form (i.e. chemical species) is an important consideration since the toxicity depends on the species, e.g. methylmercury and inorganic As are more toxic than other forms of these

elements [14, 15]. Arsenic is a metalloid which exists as different species, especially in the marine environment [16]. There are large differences in toxicity among As species. Inorganic As (iAs) is classified as a carcinogen by the International Agency for Cancer Research (IARC) [17], while the organic compound arsenobetaine (AB) is considered non-toxic to both human and animal [17-20]. In marine fish, AB is generally the most abundant As species, accounting for at least 70% of total As (tAs) [21-23], whereas arsenosugars (AsSug) are more common in marine algae [24]. Lipid-soluble As species, commonly referred to as arsenolipids (AsLipids), have been identified in oils of marine organisms, while these are rather absent in the terrestrial environment [25, 26]. AsSug and AsLipids are regarded as potentially toxic due to studies showing neurotoxicity and cytotoxicity [27, 28]. Due to the varying toxicities of As species, the European Food Safety Authority (EFSA) aims to gather more inorganic and organic As data [29], thus, publishing a tender for an extensive literature search on organic As in food and a call for continuous collection of chemical contaminants occurrence data [30, 31]. More As speciation data can be generated when analytical methods for As speciation have been established.

Arsenic speciation analysis typically utilizes mild extraction conditions to preserve the integrity of As species [23, 32, 33]. Water-soluble As species are usually extracted using aqueous-based extraction solvents [34-37] with the aid of heating and/or agitation equipment (e.g. water baths, mechanical shakers, etc.) [36-39]. Lipid-soluble As species are extracted using organic solvents with varying polarities, mostly hexane and methanol (MeOH) [40-42], occasionally followed by a clean-up step [43, 44]. Separation of As species is mostly carried out by high-performance liquid chromatography (HPLC) using ion-exchange and reversed phase columns for water-soluble and lipid-soluble As species, respectively [33]. Inductively coupled plasma mass spectrometry (ICP-MS) is still the most employed detection system due to its high sensitivity and compatibility with HPLC [45]. Attributing to their different polarities, a single extraction method has not yet been devised for all As species in all foodstuffs. In addition, optimum HPLC-ICP-MS settings need to be established since separation and detection is influenced by factors such as mobile phase composition and pH [33]. Hence, it is important to optimize extraction and instrument parameters tailored for the matrices of interest [33, 46, 47].

Global seafood supply from aquaculture is expected to increase by 26 million metric tons by 2030, which entails an additional feed volume of 40 million metric tons [2]. Alternative marine feed ingredients derived from low-trophic species are expected to augment this requirement. However, several studies have reported occasional high levels of As in these samples. Blue mussels (*Mytilus edulis*) collected from Norwegian fjords were found to contain elevated levels of tAs (as high as 13.8 mg/kg ww) and iAs (up to 5.8 mg/kg ww), which was linked to microalgae as their diet [48]. Similarly, blue mussels collected from an As-contaminated harbor in Canada had high tAs and iAs concentrations. Several reports also noted spatio-temporal variations for tAs in blue mussels [49, 50]. Among the low-trophic species are mesopelagic organisms which include both fish and other invertebrates such as shrimps and crustaceans – an unexploited resource with a global biomass amounting to 10 billion tons [7, 51-53]. In an earlier study, high concentrations of tAs were reported for mesopelagic organisms, with some exceeding the MLs set in feed regulations [7, 54]. It was also observed that processing of mesopelagic biomass into fish meal and oil altered the levels of As in the final products [55]. However, so far, no studies have looked into the effect of processing on As species. In a recent review of novel marine feed ingredients which included microalgae, insects, zooplanktons, and mesopelagic fish among others, nutritional composition (e.g. protein, fatty acids) and levels of some undesirable substances (e.g. fluoride, Cd, wax esters) were examined [2]. Arsenic and As species were not discussed, highlighting a knowledge gap. Since future aquafeed resources are likely primary producers and consumers, knowledge on the uptake and biotransformation of As at the base of the marine food chain is important to gain a better insight on the levels of tAs and prevalence of As species in these samples.

1.2 Aims and objectives

Currently, knowledge regarding the occurrence and source of different As species in marine matrices is limited. Considering their varying toxicities, the overall aim of the thesis was to document the presence of As species in novel marine resources. The results will be useful for future risk assessment in terms of feed and/or food safety. To achieve this overarching goal, the PhD project had the following specific objectives:

- **Objective 1**: Develop, optimize, and validate a method for determining organically bound water-soluble As species in marine samples.

- **Objective 2**: Quantify the levels of As and As species in novel marine feed resources, including mesopelagic organisms, microalgae, and blue mussels.

- **Objective 3**: Describe the fate of As species during aquafeed processing.

- **Objective 4**: Investigate how iAs is taken up and biotransformed into other As species by conducting exposure and feeding experiments in microalgae and blue mussels.

1.3 Structure of the PhD thesis

The PhD thesis is divided into chapters with several corresponding sections. Chapter 1 describes the project rationale, aims and objectives, and structure of the thesis. Chapter 2 gives background information on properties, occurrence, biotransformation, and toxicity of As and As species, as well as existing legislations. Chapter 3 elaborates on analytical method development and validation. Chapter 4 presents and tackles the occurrence of As and As species in novel marine feed resources, bioaccumulation and biotransformation of As species in low-trophic marine organisms, effects of feed processing on As speciation, and overall assessment in regard to feed safety. Lastly, Chapters 5 and 6 sum up the work with the conclusions and future perspectives.

CHAPTER 2: BACKGROUND

2.1 Arsenic – chemistry, sources, and occurrence

When the practice of alchemy flourished, the same period was characterized by a stark division between science and religion. The German philosopher Albertus Magnus was one of those who believed that the two can co-exist harmoniously. Later canonized and became known as St. Albert the Great, he is also recognized for discovering As in the 13th century [56]. In his book *De Mineralibus*, he mentioned how pure As was derived by heating orpiment (As_2S_3) with soap [57]. Orpiment is a yellow/golden arsenic sulfide mineral which was used in ancient civilizations as a dye, medicine, or poison [56, 57]. Today, As is known as a metalloid which belongs to Group 15 in the Periodic Table of Elements, occurs naturally as a monoisotopic element, and has an atomic number of 33 and atomic mass of 74.922 Da [57-59]. Arsenic has five electrons in the outer shell of its atom, and in comparison with other elements under Group 15, e.g. nitrogen (N) and phosphorus (P), As has a higher oxidation potential which enables it to readily exhibit +3 and +5 oxidation states [57, 58]. Through covalent bonding with oxygen, As^{3+} and As^{5+} forms AsO_3^{3-} (arsenite; As(III)) and AsO_4^{3-} (arsenate; As(V)) [58, 59]. Having both metallic and non-metallic properties, As can act as a non-metal and bind with metals in the form of As(III) and As(V). Likewise, it can behave as a metal to form chlorides, oxides, and sulfides [57, 58]. It can also easily bind with carbon to produce organoarsenic compounds [57]. Table 1 and Fig. 1 present the acronyms, chemical formulas and structures of the As compounds covered in this thesis.

Table 1. Acronyms and chemical formulas of the different arsenic compounds covered in this thesis.

Arsenic compound	Acronym	Chemical formula
Arsenite	As(III)	$As(O^-)_3$
Arsenate	As(V)	$O{=}As(O^-)_3$
Methylarsonate	MA/MA(V)	$CH_3AsO(O^-)_2$
Dimethylarsinate	DMA/DMA(V)	$(CH_3)_2AsO(O^-)$
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$
Trimethylarsoniopropionate	TMAP	$(CH_3)_3As^+CH_2CH_2COO^-$
Tetramethylarsonium ion	TETRA	$(CH_3)_4As^+$
Arsenocholine	AC	$(CH_3)_3As^+CH_2CH_2OH$
Arsenobetaine	AB	$(CH_3)_3As^+CH_2\ COO^-$
Glycerol arsenosugar	AsSug OH*	$C_{10}H_{21}AsO_7$
Sulfonate arsenosugar	AsSug SO3*	$C_{10}H_{21}AsO_9S$
Sulfate arsenosugar	AsSug SO4*	$C_{10}H_{21}AsO_{10}S$
Phosphate arsenosugar	AsSug PO4*	$C_{13}H_{28}AsO_{12}P$

*In **Paper 1**, AsSug OH, -SO3, -SO4, and -PO4 were referred to as AsSug 328, -392, -408, and -482, respectively.

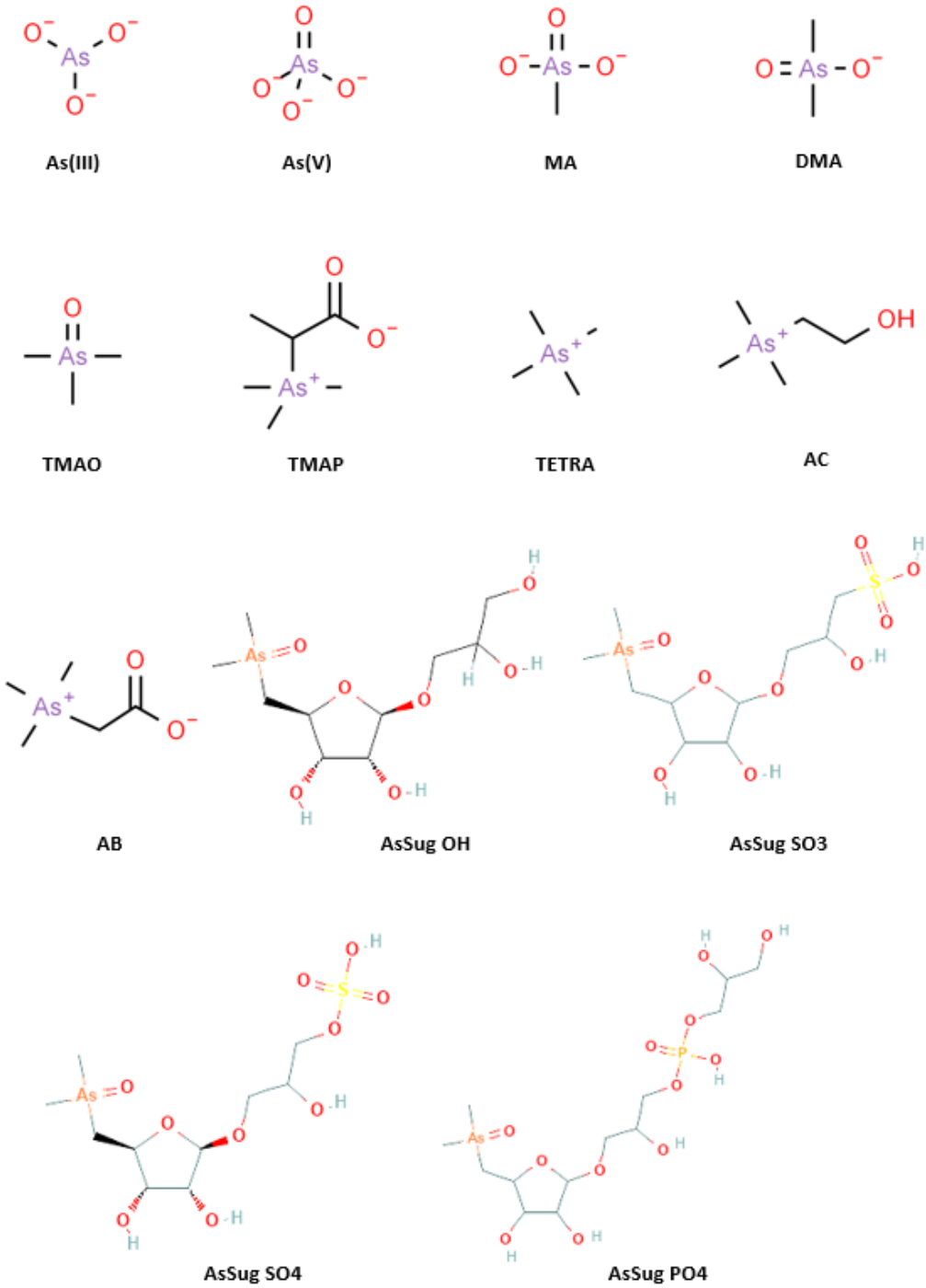

Fig. 1. Chemical structures of the different arsenic compounds covered in this thesis. *Chemical structures were retrieved from ChemSpider and PubChem.*

2.1.1 Natural sources of arsenic

Arsenic is regarded as the 20th most abundant element in the earth's crust [57, 60], with estimated average concentrations ranging from 1.8 mg/kg to 5 mg/kg [56, 58, 61]. Arsenic originates from both natural and anthropogenic sources. Natural processes which influence

the prevalence of As in the environment include volcanic activities, weathering, hydro- and geothermal phenomena among others [61]. There are over 320 As-containing minerals, e.g. arsenopyrite, orpiment, and realgar, which are formed beneath the earth's surface under anoxic conditions [59, 61]. Natural weathering of these mineral deposits contribute to the presence of As(III) and As(V) in groundwater and in the marine environment [58]. Hydrothermal vents also release As-rich fluids and particulates, which contribute to As background concentration in the ocean [57]. Likewise, geothermal systems exacerbate As contamination when geothermal fluids reach water reservoirs used for irrigation and drinking water supply [62].

2.1.2 Anthropogenic sources

Minor quantities of As are currently used in glass and textiles production, and electronics and alloys manufacturing [63]. Due to its demand, mining of As is an anthropogenic activity which hugely contributes to As contamination. Arsenic which leaches from ores enters the soil and may thereby contaminate the groundwater. Uncontrolled release due to mining site accidents and improper waste treatment may worsen the situation [58, 61, 62]. Combustion of coal is responsible for As release to the atmosphere; not to mention the possible dissipation of As from the fly ash produced by the combustion process [57]. The released As may then return to the ground through condensation [62]. In the past, As compounds were traditionally used as pesticides, feed additives, and wood preservatives. However, due to potential exposure to As from contaminated crops, food, and products, their use has been forbidden in several countries. China, one of the world's biggest suppliers of poultry and swine products, has banned the use of phenylarsonic feed additives in 2019, joining the United States of America (USA), the EU, and many others who have disallowed the use of As-based poultry drugs years earlier [64]. The use of chromium-copper arsenate (CCA) as wood preservative was also discontinued in the USA since 2003, and similarly in Japan, Australia, and others [65]. While measures are in place to mitigate As exposure, there are still countries which allow the use of roxarsone, for example, as a feed additive [66, 67]. In addition, long-term use of roxarsone caused enrichment of As species, mainly iAs, in surface soils around chicken farms in China [68]. Disposal of CCA-treated woods through burning was also reported to produce bottom

ashes and leachate with considerable amounts of As [65]. Hence, while the industrial use of As compounds has been reduced, the threat of exposure to As is still present.

2.1.3 Arsenic in the terrestrial environment – As in groundwater and rice

Arsenic is so ubiquitous that it can be found in most parts of the biosphere. Arsenic contamination in groundwater is now a global concern, with almost 108 countries with As levels in drinking water exceeding the provisional guideline value of 10 µg/L by the World Health Organization (WHO) [69]. One of the worst affected areas is the Bengal Basin covering Bangladesh and India. In a survey commissioned by UNICEF in 2001, 66% of the 317,000 tubewells from Bangladesh had groundwater with As concentrations above 50 µg/L, which is the permissible limit in Bangladesh [70]. This translated to roughly 35 million people drinking As-contaminated water. Rice cultivated and cooked in As-contaminated groundwater is also seen to exacerbate dietary exposure to As [71, 72]. Arsenic levels as high as 2.05 mg/kg dw in raw rice were reported in Bangladesh, where rice is the staple food [72]. Other terrestrial plants have As concentrations from 0.2 to 0.4 mg/kg [73], although values can rise up to several hundreds mg/kg dw in contaminated areas [74, 75]. In contrast, As in marine matrices is generally higher than terrestrial samples, though levels can vary widely [76].

2.1.4 Arsenic in the marine environment

The tAs concentration in the ocean ranges from 0.5 to 3 ug/L, with an average value of 1.7 ug/L [77, 78]. Marine sediments from coastal areas have lower As levels (5 to 15 mg/kg dw) compared to those obtained further offshore (average value of 40 mg/kg), but industrial and agricultural run-off may result to elevated values [79]. Arsenic levels in aquatic flora and fauna usually range from 5 to 100 mg/kg dw [21]. Macroalgae can contain As ranging from 2.15 to 149 mg/kg dw [80]. Among marine macroalgae, brown algae usually have higher As concentrations compared to red or green algae [81, 82]. Arsenic levels ranging from 0.3 to 1.9 mg/kg ww were found in a variety of clams [35], while crustaceans can contain several tens of mg/kg ww [81, 83, 84]. Fish species such as Northeast arctic cod and tusk have larger variation in concentrations, with levels ranging from 0.38 to 100 mg/kg dw and 0.33 to 89 mg/kg dw, respectively [85].

2.2 Arsenic species

2.2.1 Definitions and comparison between terrestrial and marine environments

Arsenic exists as various compounds in the environment. These distinct chemical forms of As are referred to as 'species'. The last few decades have seen a growing interest in measuring elemental species instead of total elemental concentrations [86]. To have a common reference within the analytical community, the International Union of Pure and Applied Chemistry (IUPAC) released a guideline which elaborates the terms pertaining to chemical speciation and fractionation of elements [87]:

IUPAC definitions:

- ***Chemical species*.** Chemical elements: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.
- ***Speciation analysis*.** Analytical chemistry: analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.
- ***Speciation of an element; speciation*.** Distribution of an element amongst defined chemical species in a system.

It is estimated that over 100 As species exist in the environment [88]. In terrestrial systems, As compounds are mainly limited to As(III), As(V), DMA, and MA [89], as found in rice grains [90]. However, rice plants (including roots, straws, and grains) collected from paddy fields near mining sites contained AB and AC as well [91]. Similarly, terrestrial invertebrates such as slugs, ants, and spiders were reported to contain trace quantities of TMAO, AC, DMA, MA, and AB, with As(III) and As(V) being the major compounds [92]. In contrast, As compounds in the marine environment are more complex and varied [89]. Arsenic in seawater primarily exists in inorganic forms, but in marine flora and fauna, organic forms generally dominate [21, 81, 93]. Marine organisms are harnessed both as food and feed ingredients. Either way, they contribute to the human diet, which highlights the importance of As speciation for risk assessment in terms of As exposure. Table 2 gives an overview of the levels of tAs and As species in major groups of marine matrices, and their occurrence are discussed in detail in the next sections.

Table 2. Levels of total arsenic and arsenic species (mg/kg) in major groups of marine matrices reported in selected works.

Total As/ As species	Levels (in mg/kg*)						Reference
	Finfish	Bivalves	Crustaceans	Cephalopods	Microalgae	Macroalgae	
tAs	*0.039-7.6*	*0.75-2.7*	*0.094-22*				[94]
	1.4-35	8.3-25	1.2-2.3				[95]
	15	0.74-6.9	2.1	1.5			[96]
		0.34-1.6					[35]
	0.35-0.60	3.7	0.13-10.3		0.47	26	[97]
						1-105	[98]
iAs	*0.001*	*0.017-0.065*	*0.001-0.14*				[94]
		0.08-0.35	0.033-0.060				[95]
	<0.003	0.15-0.26	<0.003	<0.003			[96]
		0.006-0.073					[35]
		0.20			0.27		[97]
						0.014-54	[98]
AB	*0.030-3.8*	*0.012-0.68*	*0.002-20*				[94]
	0.86-33	7.9-16	0.61-2.2				[95]
	14	0.58-5	2.1	1.3			[96]
		0.13-1.5					[35]
	0.17-0.47	0.49	0.010-9.5				[97]
						0.017-0.36	[98]
MA		*0.001*	*0.001-0.027*				[94]
	0.010-0.014	0.08-0.13	0.012-0.08				[95]
	<0.012	<0.012	<0.012	<0.012			[96]
						0.012-0.32	[98]
DMA	*0.001-0.019*	*0.001-0.020*	*0.002-0.011*				[94]
	0.012-0.24	0.07-0.25	0.06				[95]
	<0.006	0.7	<0.006				[96]
		0.013-0.12					[35]
	0.013-0.025	0.12	0.003-0.019		0.011	0.68	[97]
						0.049-2.9	[98]
TMAO	*0.002*		*0.002-0.093*				[94]
	0.008-0.07	0.06					
						0.017-0.11	[95]
TMAP	*0.002-0.036*	*0.003-0.021*	*0.003-0.80*				[94]
						0.023-0.24	[98]
AC	*0.001-0.009*		*0.003-0.004*				[94]
	0.02-0.07	0.02-0.29	0.005-0.016				[95]
						0.012-0.17	[98]
TETRA	*0.001-0.088*	*0.01-0.06*	*0.002-0.065*				[94]
AsSug OH		*0.012-0.092*	*0.001-0.047*				[94]
		0.53				6.8	[97]
						0.043-11	[98]
AsSug SO3			*0.003*				[94]

		0.064				5.5	[97]
						0.018-53	[98]
AsSug SO4		*0.01*	*0.008-0.009*				[94]
						11	[97]
						0.051-9.4	[98]
AsSug PO4		*0.011-0.17*	*0.002-0.054*				[94]
		0.74			0.14	1.5	[97]
						0.053-22	[98]
AsHC	*0.006-0.20*	*0.050-0.075*	*0.025-0.064*	*0.019-0.023*			[99]
						0.022-0.41	[100]
AsFA	*0.001-0.059*	*0.006-0.012*	*0.003-0.006*	*0.002-0.006*			[99]

*Italicized values are given in wet weight (ww).

2.2.2 Inorganic arsenic species

Inorganic forms of As, i.e. As(III) and As(V), predominate in seawater and marine sediments, where As(V) is usually higher in concentration than As(III) [101, 102]. In marine organisms, the prevalence of As(III) and As(V) tends to vary. In clams harvested in China, As(V) were present in all samples while As(III) were only identified in nearly half of those [35]. In seafood samples bought from a market in the USA, As(III) was only found in oyster, while As(V) was found in scallops and squid [96]. Inorganic arsenic, which is usually reported as the sum of As(III) and As(V) [103], generally comprises a minor fraction of tAs in marine organisms [104]. Levels of iAs in seafood samples from Belgium, Norway, Brazil, Spain, and western Arabian gulf ranged from 0.005 to 0.71 mg/kg ww [38, 83, 95, 105]. The proportion of iAs was at most 3.3% of tAs. In contrast, elevated levels of iAs were recorded in 10 species of seaweed, ranging from 2.8 to 20 mg/kg ww [106]. Samples of hijiki (*Sargassum fusiforme*/*Hizikia fusiformis*) were found to have an average As(V) concentration of 107 mg/kg dw [107]. As seen in Table 2, macroalgae can contain higher concentrations of iAs than other marine matrices. Blue mussels in Norway were also reported to have high levels of iAs, reaching up to 5.8 mg/kg ww [48].

2.2.3 Arsenobetaine

Since its first identification in 1977 by Edmonds et al. [108], AB has been well-acknowledged as the predominant As species in most marine organisms [23, 104, 109]. In finfish, it accounts for approximately 70% to 80% of tAs [38, 96, 109], whereas in shellfish, AB represents 60%

on average [35, 38]. The prevalence of AB in marine organisms has been linked to its chemical structure similarity to glycine betaine – a compatible solute known to aid osmolytic processes [109, 110]. With increasing salinity, cells need more of this osmolyte. And since AB is structurally similar, cells are unable to distinguish between the two, which results in accumulation of AB. Uptake of AB in blue mussels was also reported to increase with higher salinity, which could explain the higher concentration of AB in marine organisms compared to freshwater animals [22]. In marine macroalgae, AB was also detected, albeit, in low concentrations (less than 1.2 mg/kg) [111-113]. However, it remains unclear whether AB was synthesized by the macroalgae or originated from the attached epiphytes [111, 114]. Arsenobetaine was also found in marine sediments (up to 0.02 mg/kg) [115] and in seawater (0.5 to 10 ng/kg) [116].

2.2.4 Methylated arsenic species

Marine organisms are capable of biotransforming iAs through reduction and methylation mechanisms, producing methylated As species. In most marine samples, MA and DMA are present as minor species [105] (Table 2). In samples of shark, shrimp, squid, oyster, and scallops from a market in the USA, MA was below limit of quantification (LOQ), while DMA was present in quantifiable levels only in oyster [96]. It appears that DMA is more abundant than MA in the marine environment [97]. Species with higher degree of methylation, such as TMAO, are usually present in fish species in trace amounts [95], though it has been reported as well in blue mussels [117]. In crab samples, TMAP was present in elevated concentrations [94], as also observed for other crustaceans in Table 2. TETRA was seen as significant species in clams [38] and the second prevalent species in the mollusk *Tectus pyramis* [118].

2.2.5 Arsenocholine

Generally, only trace levels of AC are found in marine samples (Table 2), accounting for less than 1% of tAs [93]. Its limited occurrence has been linked to immediate transformation to AB or break down to other organic As species [23, 81, 119]. In contrast, AC was found to be the most prevalent As compound in muscle tissues of grunts (*Haemulon* sp.) and lane snappers (*Lutjanus synagris*), and in the shrimp *Farfantepenaeus notialis* [120]. Likewise, AC was one of the predominant As species in some types of sea anemones [121].

2.2.6 Arsenosugars

First isolated in brown kelp in 1981 [122], AsSug are ribofuranosides, usually with a dimethyl- or trimethylarsinoyl moiety [23, 119]. The molecular forms of AsSug differ depending on the side chain attached at the C-1 position [24] and the most common forms are given in Table 1. In the marine environment, macroalgae are known to contain high levels of AsSug [23, 24, 109, 119] (Table 2), which can account for over 80% of tAs [82, 98]. The prevalence of a particular AsSug is influenced by algal taxonomy. To illustrate, AsSug SO3 is the major AsSug in kombu [98, 112, 123] and kelp [37, 98], AsSug OH in wakame [98, 112], AsSug SO4 in hijiki [98, 124], and AsSug PO4 in nori [98, 123]. Aside from macroalgae, AsSug were also found in crustaceans and bivalves [34, 119], and in marine microalgae [125] (Table 2).

2.2.7 Arsenolipids

Morita and Shibata [126] were the first to isolate and identify an AsLipid from wakame (*Undaria pinnatifida*), a type of brown seaweed. To date, several structural groups of AsLipids have been identified which include As-containing hydrocarbons (AsHC), As-containing fatty acids (AsFA), and arsenosugar phospholipids (AsPL) as the most studied groups [127]. In a market basket survey conducted in Japan which covered 17 different food groups, AsHC, AsFA, and AsPL were only found in marine samples, ranging from 4.4 to 233 µg/kg ww [128]. Fish and shellfish had AsFA as the major AsLipids. In contrast, AsPL were more common in seaweeds [128]. Seaweeds are known to contain AsLipids [129]. The major species in samples of wakame were AsPL, while in hijiki, AsHC were more prevalent [100]. In the Mediterranean mussel *Mytilus galloprovincialis*, 36 AsLipids were identified, majority of which were AsFAs [130]. Phytoplanktons from the North Atlantic Ocean also contained AsLipids, where it was observed that samples from high-nutrient locations had less AsPL [131]. This suggests that the distribution of AsLipids in phytoplanktons can be linked to nutrient status of the waters [131]. Products of marine origin also contain AsLipids. In fish meal from capelin [44] and commercial fish oils [132], the main AsLipids found were AsHC, while AsFA were present in minor concentrations [44]. With further research, more AsLipid groups are being identified such as arseno-ether phospholipid [130], a methoxy-sugar AsLipid which contains phytol [130, 131], arsenic-containing phosphatidylcholines [133], and trimethylarsenio fatty alcohols [41] among others.

2.3 Biotransformation of arsenic species in the marine environment

2.3.1 Uptake and transformation of inorganic arsenic species

Inorganic As is the major form of As in seawater, where As(V) is more prevalent in oxygen-rich environment while As(III) exists in anoxic conditions [14, 72]. In microalgae, the uptake of As(V) is attributed to its chemical and structural similarity to phosphate, and the two compounds are said to compete with each other [93, 134]. Low-phosphate conditions have led to higher uptake of As(V), while phosphate-rich environment caused limited uptake [79, 134]. The acquired As(V) tends to interfere with phosphorylation, so it needs to be excreted or transformed to prevent cell damage [14, 79, 93]. Due to its poor mobility, As(V) is instead enzymatically reduced to As(III) in the cytosol facilitated by As(V)-reductase or thru non-enzymatic reaction with glutathione, as observed in bacteria [135], microalgae [136], and several aquatic organisms [14, 84]. For example, species of microalgae (*Dunaliella* sp., *Closterium aciculare*) were reported to reduce As(V) to As(III) [14]. In contrast, a few microalgae were observed to oxidize As(III) to As(V) [135]. This process generates energy which is used by microorganisms for photosynthetic and chemolithoautotrophic processes [137].

2.3.2 Methylation of arsenic species

Inorganic As is biotransformed to organoarsenic species through methylation, which is said to be a detoxification mechanism among bacteria, phytoplanktons, plants, animals, and humans [14, 72, 84, 135, 138]. The Challenger pathway was among the first elucidations of the methylation process [139] (Fig. 2a). Inorganic arsenic undergoes oxidative methylation facilitated by S-adenosylmethionine (SAM) (methyl donor) and catalyzed by ArsM (enzyme), producing MA(V), which is then reduced to MA(III). This then acts as a substrate for another oxidative methylation step, producing DMA(V) and subsequently reduced to DMA(III) [119, 138, 140] (Fig. 2a). Other As species such as TMAO and TETRA may arise following the Challenger pathway. It has been debated whether As methylation can be considered a detoxification process since it also yields trivalent methylated arsenicals which are highly toxic [79, 137, 138]. Nonetheless, these trivalent species are thermodynamically unstable and the conversion of MA(III) to DMA(V) is faster compared to the reduction of DMA(V) to DMA(III).

This explains why the trivalent intermediates are rarely detected and why their pentavalent counterparts are usually present in marine samples [14, 119, 138].

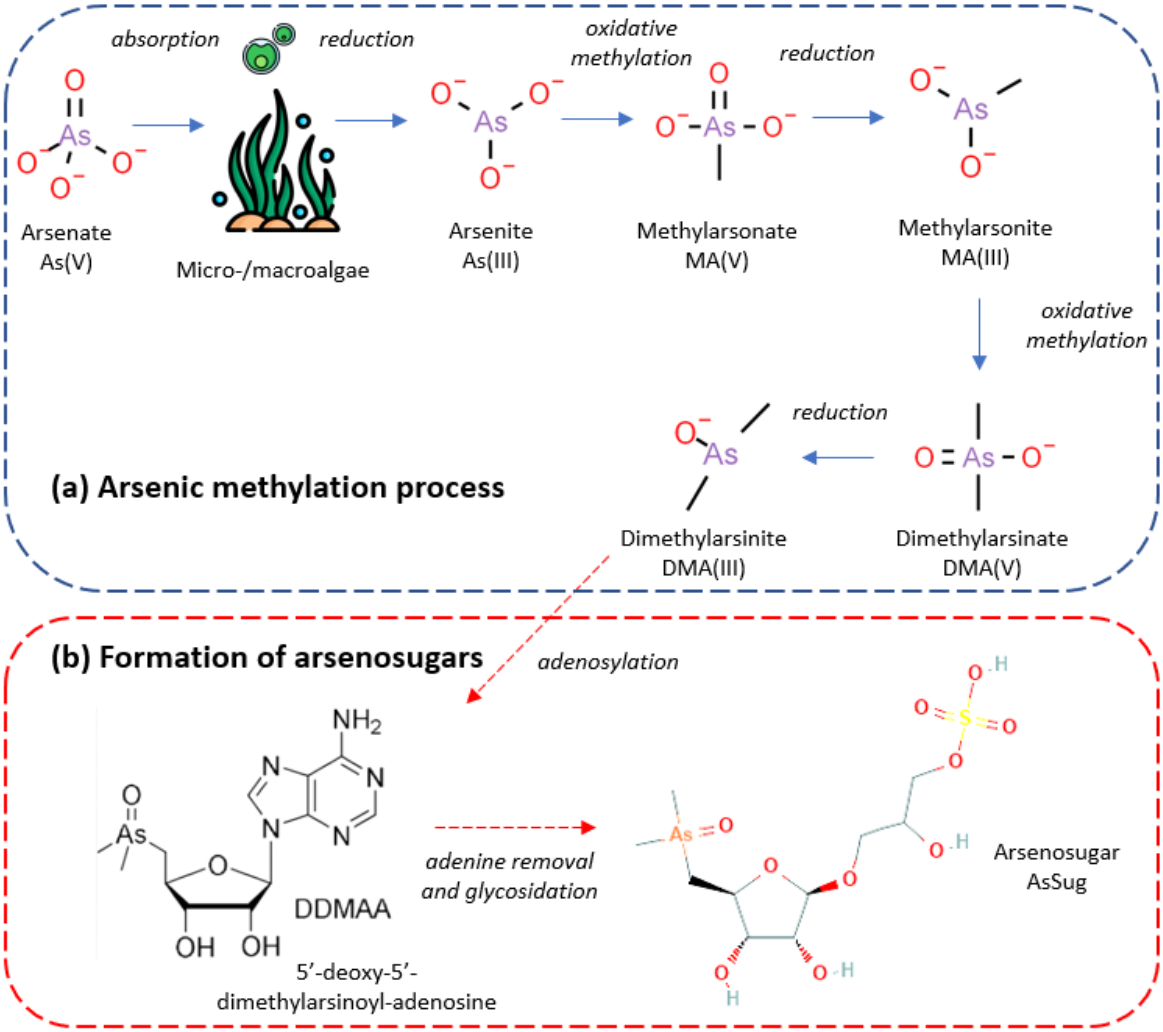

Fig. 2. An illustration of the (a) arsenic methylation process following the Challenger pathway and (b) the subsequent formation of arsenosugars. *Chemical structures were retrieved from ChemSpider and PubChem, and icons from Flaticon.*

In the marine environment, microalgae are among the well-studied matrices in regard to As methylation [14, 125, 135, 141]. They serve as a major food source for several marine organisms, highlighting their role in biotransformation and trophic transfer of As in the aquatic environment. In microalgae, As(V) is taken up from the water and usually converted to more complex organoarsenic species [72]. The formation of methylated As species is dependent on several factors which include algal taxonomy, ambient As concentration, temperature, salinity, growth phase, and nutrient availability among others [14, 72, 119, 135, 136]. Methylated As species are released in the water by microalgae when exposed to higher

As concentrations and longer exposure periods [14]. Aside from methylation, demethylation of arsenic species has been reported in microalgae and in bacteria isolated from marine and freshwater environments [119].

2.3.3 Formation of arsenosugars

Arsenosugars are mainly produced by micro- and macroalage by uptake of iAs from seawater via phosphate uptake mechanism, followed by methylation and subsequent adenosylation [72]. Edmonds and Francesconi [140] first proposed a plausible pathway for AsSug synthesis. Following the Challenger pathway for As methylation, the adenosyl group of the methylating agent (SAM) attaches to the As atom of DMA(III), forming an intermediate (Fig. 2b). This is then succeeded by enzymatic and hydrolytic removal of adenine and glycosidation, leading to the synthesis of AsSug [140]. This pathway was supported by a recent study which characterized the ArsS enzyme involved in AsSug formation [142]. It was reported that DMA(III) undergoes a radical SAM-mediated alkylation (also called adenosylation) with the aid of the ArsS enzyme, producing the intermediate now identified as 5'-deoxy-5'-dimethylarsinoyl-adenoside (DDMAA) [142] (Fig. 2b). DDMAA was reported in the kidney of the giant clam *Tridacna maxima*, which also contained significant amount of AsSug [143].

Phytoplanktons serve as the first vehicle for As transfer from seawater to higher trophic animals [144]. The exposure of the unicellular green alga *Chlamydomonas reinhardtii* to As(V) for 24 hours resulted to formation of AsSug OH and AsSug PO4 [145]. The formation of AsSug is affected by phosphate concentrations in the environment [134]. *Dunaliella tertiolecta* produced more AsSug PO4 under phosphate-rich conditions, while low-phosphate setup yielded more AsSug OH [134]. Different species of microalgae also have distinct biotransformation efficiencies, thereby resulting to varying occurrence of As species [72] For example, chlorophytes are known to produce AsSug OH and AsSug PO4, while heterokontophytes yield AsSug OH, AsSug SO4, and AsSug PO4 [125]. Other factors which affect As speciation in microalgae include ambient As concentration, culture regime, and exposure period [14].

2.3.4 Formation of arsenobetaine

It has been hypothesized that AsSug are the precursors of AB in the marine food chain [72, 138, 140]. Edmonds and Francesconi [140] first proposed that AsSug can be converted to AC by cleaving the C-3-C-4 bond of the sugar residue, followed by oxidation at the C-4 position, then undergoing further reduction and methylation to form AB. Currently, four possible pathways have been extensively discussed in literature, as shown in Fig. 3. The first pathway involves the degradation of trimethylated AsSug to form AC, followed by oxidation to AB [23, 114]. Second, dimethylated AsSug can be degraded to dimethylarsinoylethanol (DMAE), then reduced/methylated to form AC. AC can then be oxidized to form AB [114]. The third pathway still involves degradation of dimethylated AsSug to DMAE, followed by oxidation and decarboxylation to produce dimethylarsinoylacetate (DMAA). This only requires further reduction/methylation to form AB [93, 114]. The fourth pathway suggests a reaction of DMA(III) with glyoxylate to form DMAA [23, 114, 141], which could account for the presence of AB in terrestrial organisms [141].

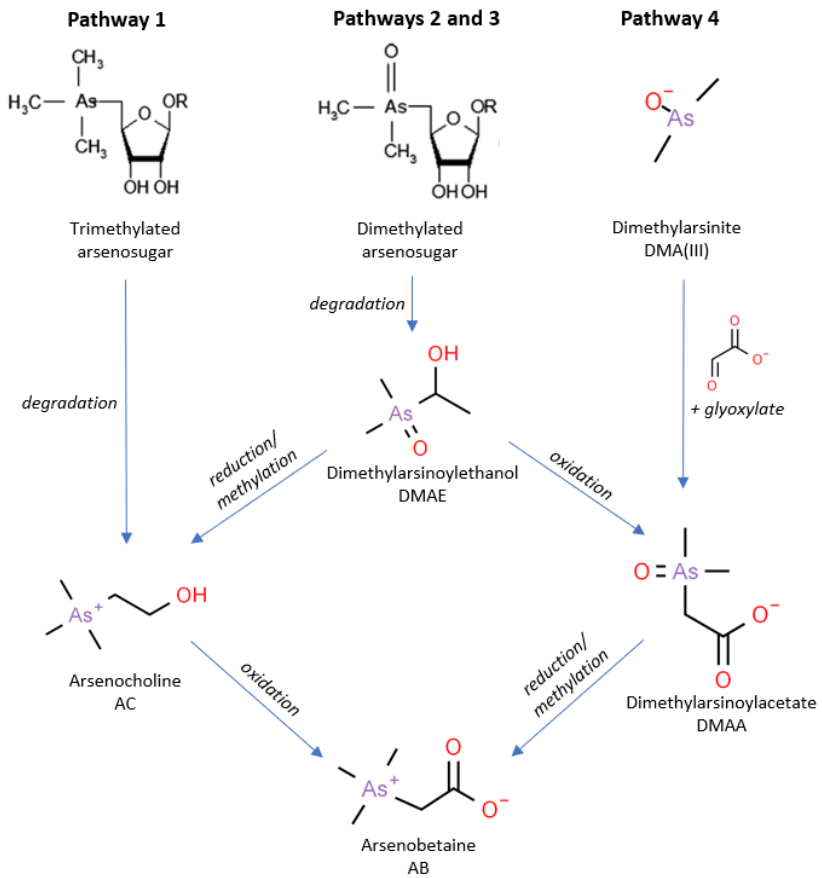

Fig. 3. An illustration of the four proposed pathways for the formation of arsenobetaine. *Adapted from Caumette et al. [141] and Popowich et al. [114]. Chemical structures were retrieved from ChemSpider.*

In a review by Caumette et al. [141], it was proposed that phytoplanktons do not contain AB but mostly AsSug and iAs. They only provide the precursors for AB formation in higher-trophic animals [144]. For example, intermediate As species such as DMAA and DMAE were detected in lipid and cytosolic fractions of *D. tertiolecta* and *Thalassiosira pseudonana* [125, 144]. The first signs of AB appear in herbivorous zooplanktons, though only as minor compound; AsSug were the major species [141]. It is in carnivorous zooplanktons where AB becomes the dominant As species [141]. This supports that AB is synthesized by low-trophic organisms and efficiently accumulated by carnivorous animals [114, 125]. Marine organisms from benthic environments also contained AB [146]. This is despite having no available phytoplanktons as food source which can provide AsSug as precursors to AB. It is presumed that chemolithoautotrophic bacteria from the surrounding hydrothermal vents facilitated the formation of AB or provided the precursors [114, 146]. Bacteria are also known to synthesize AB. Such is the case for *Pseudomonas* sp. which was shown to be capable of biotransforming DMAA to AB [111, 114].

2.3.5 Formation of arsenolipids

The formation of AsLipids is believed to be due to 'biochemical promiscuity' [147]. Due to their inability to differentiate As species from their analogous compounds, cells mistakenly incorporate As in its processes [132]. For example, As replaces either phosphorus (P) or nitrogen (N) in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) during phospholipid biosynthesis resulting to formation of arsenophosphatidylethanolamine (AsPE) or arsenophosphatidylcholine (AsPC) [14, 148]. The presence of AsFA in marine oil can also be due to fortuitous incorporation of As to essential fatty acids [132].

Formation of AsLipids has been observed in both micro- and macroalgae. In *D. tertiolecta*, AsPL increased with increasing phosphate concentration [134]. The opposite was seen for the brown seaweed *Ectocarpus*, where low-phosphate conditions favored the biosynthesis of AsPL [16]. The contradicting results suggest species-specific differences in how micro- and macroalgae biotransform As. Moreover, it was observed that AsLipids in marine algae were bound similarly as AsLipids found in higher trophic marine animals [25]. This implies that

AsLipids are synthesized in primary producers and eventually transferred along the food chain [25].

2.4 Toxicity of arsenic species

2.4.1 Toxicity of iAs

Among As species, iAs is the most studied in terms of exposure-related health effects. The IARC classified iAs as carcinogenic [17]. In 2009, EFSA released a 'Scientific Opinion on Arsenic in Food', where the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) evaluated the exposure of the European population to As via dietary intake, as well as associated health risks [149]. In this report, benchmark dose lower confidence limit ($BMDL_{01}$) values between 0.3 and 8 µg/kg body weight (bw) per day were identified and used for risk characterization for iAs [149]. However, occurrence data were mainly reported as tAs (98% of occurrence data), so the contribution of iAs was only estimated based on certain assumptions. In line with this, the CONTAM panel emphasized the need for speciation data [149]. In 2014, EFSA published a scientific report on 'Dietary exposure to inorganic arsenic in the European population' [150]. Majority of the occurrence data was still on tAs, but other As species were reported sparingly such as iAs, MA, DMA, As(V), As(III), and AB. In 2021, EFSA released an updated report on 'Chronic dietary exposure to inorganic arsenic'. Similar to the findings in the 2014 report, the highest dietary exposure was foreseen in infants, toddlers, and other children due to consumption of rice- and grain-based products as part of their diet [151]. The mean dietary exposure estimates at the lower bound (LB) were generally below the $BMDL_{01}$ values. In comparison to the 2014 report, lower exposure estimates to iAs were calculated, primarily due increased availability of measured iAs data, which allowed more realistic dietary exposure assessment [151].

For animals, toxicity studies have also been primarily based on iAs. The adverse effects tend to vary depending on the animal and As exposure levels [152-155]. For cattle, horses, and pigs, the common clinical signs of As toxicity include diarrhea, salivation, ataxia, muscle weakness/incoordination, and depression [152, 153]. For poultry animals, usual symptoms are reduced feed intake, decreased egg production, and some neurological symptoms [152, 154]. For fish, iAs exposure may lead to disruption of proper gene expression and enzymatic

activities, increased hepatic metallothionein levels, immune system failure, and reduced growth rate [152, 155].

2.4.2 Toxicity of other As species

Among As species, AB is regarded as non-toxic [156, 157]. In contrast, the IARC classified the methylated arsenicals MA(V) and DMA(V) as possibly carcinogenic [17]. However, the trivalent methylated As species, MA(III) and DMA(III), are more toxic compared to their pentavalent counterparts and iAs [14, 158]. In comparison to iAs and methylated As species, AsSug are less toxic [27, 159]. However, when ingested by humans, they can be metabolized, consequently forming cytotoxic As species [23, 24, 27, 119, 138]. In an *in vitro* study, it was demonstrated that the AsSug metabolites DMA(V) and its sulfur analogue, thio-DMA(V), were more toxic than As(III) [27]. This was supported by a report which showed that consumption of seaweed, which is known to contain high levels of AsSug, resulted to detection of DMA(V), thio-DMAE, thio-DMAA, and thio-DMA in urine samples [123]. Initial studies on the toxicity of AsLipids also showed adverse effects. An *in vitro* study involving cultured human bladder and liver cells reported high potential cytotoxicity of AsHC [160]. This was followed up by an *in vivo* study using the fruit fly *Drosophila melanogaster* as a model organism, where the toxicity of AsHC was confirmed [161]. It was reported the AsHC can be five times more toxic than As(III) and may induce neurotoxic effects, while AsFA are less toxic compared to AsHC [28, 162]. Based on these studies, the toxicity of As evidently varies among its chemical species. Hence, risk assessment based on tAs alone is not enough, emphasizing the need for As speciation data. Also, in comparison to iAs, studies on the toxicity of these organic As compounds are scarce, which warrants further research.

2.5 Regulatory aspects of arsenic in relation to food and feed safety

In the EU, food safety is safeguarded following a ‘farm to fork’ approach. This entails implementation of EU legislations and standards across the food production and processing chain, with the overall goal of ensuring a high level of protection of human, animal, and plant health. One of the focus areas of the EU Food Safety Policy is the monitoring of contaminants

and residues in food and animal feed, which includes As. For foodstuffs, MLs for contaminants are specified in Commission Regulation (EC) No 1881/2006 and amendments [163]. Table 3 contains the applicable MLs for iAs in specific commodities within the EU. For comparison, MLs imposed in some countries outside the region, as well as recommended MLs by Codex Alimentarius, are also provided. Most MLs are for drinking water, rice, and rice-based products (Table 3). In terms of marine food products, Canada, Australia, New Zealand, and Hong Kong have MLs for tAs and iAs [164-167]. The MLs for iAs range from 0.1 mg/kg for fish and fish oil in Hong Kong [167] to 2 mg/kg for fish and crustaceans in Australia and New Zealand [166]. It is quite common to have MLs which are different from country to country since they are based on As data from commodities which are available in the specific area [166]. However, the varying MLs can also be a trade barrier, preventing exchange of goods across regions.

Table 3. Maximum levels and guideline values for total arsenic and inorganic arsenic in different commodities as imposed/recommended in various jurisdictions.

Region/Country	Commodity	Total As	Inorganic As	Reference
EU	Non-parboiled milled rice (polished or white rice)		0.2 mg/kg	[163]
	Parboiled rice and husked rice		0.25 mg/kg	
	Rice waffles, rice wafers, rice crackers and rice cakes		0.30 mg/kg	
	Rice destined for the production of food for infants and young children		0.10 mg/kg	
USA	Bottled water	10 ppb		[164]
	Infant rice cereal		100 ppb[a]	
	Apple juice		10 ppb[b]	
Canada	Fish protein	3.5 ppm		[165]
	Edible bone meal	1 ppm		
	Beverages, except fruit juice, fruit nectar, grape juice and grape nectar	0.1 ppm		
	Water in sealed containers	0.01 ppm		
	Husked (brown) rice		0.35 ppm	
	Polished (white) rice		0.2 ppm	
	Rice-based foods intended specifically for infants and young children		0.1 ppm	
	Fruit juice, except grape juice; Fruit nectar, except grape nectar		0.01 ppm	
	Grape juice; Grape nectar		0.03 ppm	
Australia and New Zealand	Seaweed and molluscs		1 mg/kg	[166]
	Fish and crustacea		2 mg/kg	
	Cereals (including rice)	1 mg/kg		
	Salt	0.5 mg/kg		

Hong Kong	Vegetables	0.5 mg/kg		[167]
	Cereals, other than rice	0.5 mg/kg		
	Meat of animal	0.5 mg/kg		
	Edible offal of animal	0.5 mg/kg		
	Meat of poultry	0.5 mg/kg		
	Edible offal of poultry	0.5 mg/kg		
	Edible fats and oils, other than fish oil	0.1 mg/kg		
	Fat spreads and blended spreads	0.1 mg/kg		
	Salt, food grade	0.5 mg/kg		
	Bottled or packaged drinking water, other than natural mineral waters	0.01 mg/kg		
	Natural mineral waters	0.01 mg/kg		
	Husked rice		0.35 mg/kg	
	Polished rice		0.2 mg/kg	
	Aquatic animals, other than fish		0.5 mg/kg	
	Fish		0.1 mg/kg	
	Fish oil		0.1 mg/kg	
	Seaweed		1 mg/kg	
Codex Alimentarius	Edible fats and oils		0.1 mg/kg[c]	[168]
	Fat spreads and blended spreads		0.1 mg/kg[c]	
	Natural mineral waters		0.01 mg/kg[c]	
	Rice, husked		0.35 mg/kg[c]	
	Rice, polished		0.20 mg/kg[c]	
	Salt, food grade		0.5 mg/kg[c]	

[a]Guidance for industry, i.e. not required, but only a recommendation.
[b]Draft action level proposed in 2013 to encourage industries to reduce iAs levels in apple juices.
[c]Recommended maximum level by the Codex Alimentarius Commission.

Feed safety is equally important to protect animal welfare and to ensure animal-derived food products are safe for human consumption. For animal feed and feed materials, Commission Directive 2002/32/EC and amendments define the MLs for tAs depending on the type of product [11]. While the use of As-based feed additives has been banned in the EU, USA, China, and many other countries [64, 169], this mainly concerns poultry and pig farms. For aquaculture, the use of aquafeed and its changing composition over the years [5] require monitoring. For marine-based feed and feed ingredients, the MLs for tAs range from 10 to 40 mg/kg (Table 4). If required by authorities, it should also be possible to demonstrate that the iAs content is less than 2 mg/kg [11]. In May 2022 [170], the European Commission (EC) issued a recommendation for member states and feed producers to monitor iAs in feed following the availability of a CEN method for iAs analysis [171].

Table 4. Maximum levels for total arsenic in different feed products derived from marine sources as stated in Directive 2002/32/EC and amendments [11].

Products intended for animal feed	Maximum total As content (mg/kg) relative to a feed with a moisture content of 12%.
Feed materials	
- fish, other aquatic animals and products derived thereof	25[a]
- seaweed meal and feed materials derived from seaweed	40[a]
Complementary feed for pet animals containing fish, other aquatic animals and products derived thereof and/or seaweed meal and feed materials derived from seaweed	10[a]
Complete feed for fish and fur animals	10[a]
Complete feed for pet animals containing fish, other aquatic animals and products derived thereof and/or seaweed meal and feed materials derived from seaweed	10[a]

[a]Upon request of the competent authorities, the responsible operator must perform an analysis to demonstrate that the content of inorganic As is lower than 2 ppm.

With recent reports showing potential toxicity of other As species, the EFSA has requested for more occurrence data and commissioned an extensive literature review of organic As species in both food and feed [29-31]. The goal is for EFSA to gather sufficient information to be able to conduct a comprehensive risk assessment, which can then form the basis of the EC whether MLs are necessary. This PhD project aims to contribute by developing methods for As speciation and providing occurrence data of organic As species in novel marine feed resources.

CHAPTER 3: METHOD DEVELOPMENT

*This chapter focuses on the work related to analytical method development (**Paper I**). A brief overview is given for each step involved in As speciation analysis, followed by a discussion on the implemented strategies to optimize and validate the method. The main findings in **Paper I**, in addition to some unpublished results, are presented.*

3.1 Arsenic speciation analysis – overview

Over the years, different approaches have been proposed to determine As species in marine matrices. Table 5 presents selected works which demonstrate variation in how As speciation analysis has been conducted in the last decade (2012-2022). In general, As speciation analysis encompasses an extraction step, followed by separation of species, and then detection [32, 33]. A complementary characterization or identification step is also employed to verify the identity of a species aside from retention-time matching [33]. A clean-up procedure can likewise be performed to reduce matrix effects or remove co-extracted compounds [33, 88]. The variety of implemented extraction procedures, separation columns, and detection systems demonstrate the complexities associated with As speciation analysis. The suitability of the method largely depends on the matrices and analytes of interest, which then calls for a targeted approach [33, 46]. In this PhD work, analytical steps were optimized mainly for water-soluble As species in marine matrices.

3.2 Extraction

3.2.1 Overview

The extraction step is a critical point in As speciation analysis since it largely influences the quality of analytical results [33]. Well-resolved chromatographic peaks and sensitive detection systems can only be useful when analytes are extracted properly from the matrix. It is desirable to obtain high extraction efficiency, but it is also necessary to preserve the original chemical properties of the species throughout the analysis to ensure accurate quantification [32, 88]. Mild extraction techniques are usually employed, complemented by spiking tests to verify the stability of As species during sample preparation [88]. The

differences in polarities of As compounds also require extraction solvents and procedures adapted specifically to the target analytes [32, 46].

The most common extraction solvents for water-soluble As species include pure water [35, 172-175], or its mixture with MeOH in different proportions [36, 37, 102, 176-178] (Table 5). Water is a known soft extractant, which reduces the likelihood of species conversion. Its mixture with MeOH targets the less polar As compounds, which are not easily released from the matrix by using water alone [173]. A 50:50 mixture is usually employed, since use of greater proportions of MeOH was reported to decrease extraction efficiency [179, 180]. The use of diluted acids, such as nitric acid (HNO_3) [95, 96, 101, 174, 181, 182], hydrochloric acid (HCl) [124], phosphoric acid (H_3PO_4) [183, 184], and trifluoroacetic acid (TFA) [38, 185], has also been applied to extract hydrophilic As species. Mildly acidic solutions improved extraction efficiencies in plants and animal tissues [45], but can cause transformation of As species, as observed in the degradation of AsSug to DMA in seaweed [186]. Diluted acids as extractants may also produce apparent higher recoveries, but this may be due to breakdown of AsLipids [23]. An important consideration for extraction solvents, which are also often the final diluent of the analyte, is their compatibility with the detection system [33].

Extraction of As species is typically carried out with the aid of agitation and heating equipment, such as mechanical shakers and vortex mixers [36, 37, 101, 187-189], water bath [38, 39, 117, 190, 191], ultrasonic bath and probes [102, 172, 178, 192, 193], microwave [35, 95, 174, 177, 183, 194], heating block [96, 173, 176, 195], and oven [175, 182] (Table 5). Extraction times vary from as fast as five minutes in a microwave under controlled temperature and pressure [196], to 12 hours in a shaker [197], or overnight when left to stand at room temperature [188]. As observed in extracting As species in seafood using microwave and ultrasonic baths [96], harsher equipment settings may lead to conversion of species. Thus, optimization is necessary.

Table 5. List of selected works from 2012 to 2022 on arsenic speciation analysis in marine matrices employing different approaches for extraction, separation, and detection.

As species	Matrix	Extraction approach	Extraction solvent	Separation	Column	Detection	Reference
As(III), As(V), DMA, MA, AsSug, AB, TETRA, TMAO	Seaweed	Microwave-assisted	Water	HPLC	PRP-X100, PRP-X200	HG-AFS	[196]
iAs, AB, DMA, AC, TETRA	Oyster	Microwave-assisted	MeOH	HPLC	Supelco SAX1, Supelco SCX	GF-AAS	[179]
As(III), As(V), AB, DMA	Benthic and pelagic rays	Water-bath heating	MeOH:water	HPLC	PRP-X100, Zorbax 300-SCX	HG-AFS	[198]
AsSug	Seaweed	Vortex mixing, sonication	MeOH:water	HPLC	PRP-X100	INAA	[186]
DMA, AB, TMAO, AC, TETRA, iAs	Bivalves, cuttlefish, shrimp, finfish	Water-bath heating	Diluted TFA with peroxide, MeOH:water	HPLC	PRP-X100 IonoSpher 5C	ICP-MS	[38]
As(III), As(V), DMA, MA, AB	Sardines and anchovies	Vortex mixing, sonication	Diluted di-ammonium hydrogen phosphate	HPLC	PRP-X100	ICP-MS	[199]
As(III), As(V), AB, AC, DMA, DMAA, DMAE, DMAP, MA, TETRA, TMAO, TMAP, AsSug	Cod, haddock, mackerel, crab, shrimp, geoduck clam, oyster, kombu	Vortex mixing, use of heating block	Water	HPLC	PRP-X100, Metrosep C6	ICP-MS	[34]
AB, AC, As(III), As(V), MA, DMA	Shrimp	Microwave-assisted, sonication	Water, diluted CH_3COOH, TFA, HNO_3	HPLC	PRP-X100	ICP-MS	[193]
AsSug	Seaweed	Use of rotating wheel and heating block, sonication	Water, diluted HCl with pepsin	HPLC	Thermo AS14a, ACE phenyl-hexyl, CAPCELL PAK C18 MG	ICP-MS	[124]
iAs, AB, DMA, AsSug	Microalgae, macroalgae, bivalves, clam, crustaceans, finfish	Vortex mixing, oven extraction	Water, diluted HNO_3	HPLC	PRP-X100	ICP-MS	[97]
iAs, MA, DMA, AsSug, AB, AC	Seaweed	Microwave-assisted	Water	HPLC	IonPac AS7	ICP-MS	[200]

Less common extraction techniques include enzymatic and microextraction procedures [39, 201-203]. The use of trypsin and pepsin showed good extraction recoveries for AB, DMA, MA, As(III), and As(V) in seafood [39, 201], but the long incubation time and high cost of enzymes render enzymatic extraction a less practical approach [173, 185]. Microextraction procedures, such as matrix solid phase dispersion techniques, have also been applied to extract As(III), As(V), MA, DMA, AB, and AC in fish and mollusks [202]. The use of microextraction procedures require less solvent, offering a more environment-friendly approach [203]. Nowadays, analytical method development incorporates the concept of Green Analytical Chemistry (GAC), which aims to prevent wastes, promote use of safer chemicals, and strive for energy-efficient operations [204]. In line with GAC principles, extraction procedures should attempt to reduce usage of solvent and other laboratory consumables, as well as extraction times [205, 206].

3.2.2 Optimization of the extraction procedure

The wide range of polarities of As species entails a targeted sample treatment tailored for the matrices and analytes of interest [33, 46, 47]. Extraction parameters, such as composition of extraction solvent, equipment settings, etc., need to be optimized to ensure maximum extraction efficiency while preserving the state of the As species. Traditionally, method optimization is performed following a 'one-factor-at-a-time (OFAT)' approach, which may necessitate repetitive, time-consuming, and costly experiments [204]. An alternative strategy is to utilize chemometric tools such as 'design of experiments' (DOE), which facilitates multivariate optimization by performing a reduced number of experiments yet obtaining as much information as possible [207, 208]. The application of DOE has been effective in optimizing extraction procedures for element speciation analysis, e.g. As, Hg, selenium (Se), zinc (Zn), and chromium (Cr), in different matrices [209-213].

In **Paper I**, a 2^{7-3} fractional factorial design was performed to optimize the extraction procedure for a wide range of As species in marine certified reference materials (CRMs) using blue mussel as a test matrix. Table 6 shows the seven factors investigated and the corresponding low-level and high-level settings. In total, 16 experiments were required. Significant factors were identified based on tAs as the response, which was quantified by ICP-

MS after microwave digestion (**Paper I**). Following the conditions per experiment (Table 1 in **Paper I**), a sample weight of either 0.2 g or 0.5 g of blue mussel was uesd, followed by addition of 5 or 15 mL of water or 30 mM HNO_3 as extraction solvent. H_2O_2 was also added in some experiments. Samples were subjected to vortex mixing, then tubes were heated in a shaking water bath at a temperature of 25 or 90 °C for an extraction time of 30 or 60 min. Thereafter, some tubes were ultrasonicated. All tubes were centrifuged, followed by filtration of extracts and subsequent analysis for tAs.

Table 6. Factors tested and their corresponding low- and high-level settings in the 2^{7-3} fractional factorial design devised in **Paper I.**

Factors	Low-level setting	High-level setting
Sample weight (g)	0.2	0.5
Type of extraction solvent	Water	30 mM HNO_3
Volume of extraction solvent (mL)	5	15
Addition of H_2O_2	No	Yes
Extraction temperature (°C)	25	90
Extraction time (min)	30	60
Use of ultrasonication	No	Yes

From the Pareto chart of standardized effect estimates (Fig. 2 in **Paper I**), the two significant factors were the extraction temperature and the type of extraction solvent (**Paper I**). Extraction temperature has a positive effect, which means a higher extraction temperature yields better recoveries (**Paper I**). This was similarly observed in method optimization studies in As speciation in rice [212] and soil [214]. The type of extraction solution has a negative effect, which implies that the lower setting (i.e. water) is preferred to have higher response (**Paper I**). A fractional factorial design can be supplemented by response surface methodology (RSM), which further optimizes the method by creating a model and specifying factor settings which will yield the highest response [208]. The employed high-level setting for extraction temperature in **Paper I** was 90 °C in a water bath. Higher temperature setting would necessitate the use of an oil bath. Hence, due to equipment limitation, the extraction temperature was kept at 90 °C. The type of extraction solvent, which was the remaining factor to optimize, was fine-tuned by comparing extraction efficiencies in blue mussel and some CRMs by using either pure water or aqueous MeOH (50%, v/v) (**Paper I**). Based on *t*-test results ($p < 0.03$), generally higher extraction efficiencies were obtained when using aqueous

MeOH (**Paper I**). Similarly, previous studies have shown that aqueous MeOH solutions were effective in extracting water-soluble As species in marine matrices [36, 37, 102, 176], and the increase in recoveries was attributed to the incorporation of MeOH, which solubilizes less polar As compounds [173].

Based on the results of optimization experiments, maximum extraction efficiency can be obtained by employing the following extraction conditions: 0.2 g of sample material, 5 mL of aqueous MeOH solution (50%, v/v) as extraction solvent, and water-bath heating at 90 °C for 30 min (**Paper I**). The non-significant extraction parameters (e.g. extraction solvent volume, extraction time) were maintained at their low-level settings (Table 6), requiring less energy and chemical usage, thereby adhering with GAC principles [204].

3.2.3 Application of multivariate optimization in other element speciation studies

In this PhD work, the choice of factors to optimize were mainly based on literature, i.e. which factors have been reported to significantly influence extraction efficiencies for As in marine matrices. In other element speciation studies, the selected factors varied depending on the target analytes, matrix, and analytical approach [209-213]. Similar to **Paper I**, previous studies working on solid matrices typically optimized factors such as extraction temperature, extraction time, choice of extraction solvent, or solvent volume [211, 212]. However, the optimum factor settings also varied depending on the element species analyzed. Improved extraction efficiencies were obtained when high extraction temperature was employed in As speciation analysis in rice [212], while lower temperature worked better for Zn speciation analysis in fish feed [211]. In contrast, for liquid matrices, it is usually the preconcentration step which is optimized to obtain concentrations which are aligned with the detection limits of the detector [209, 210]. For inorganic Se speciation in Argentinean beverages [209], an on-line preconcentration procedure using solid-phase extraction (SPE) was optimized. Thus, flow rates (sample, reagent, acid, and buffer) and concentrations (acid, reagent, and buffer) were chosen as factors. A study on Cr speciation in water samples using dispersive liquid-liquid microextraction optimized sample pH and ionic liquid volume to improve extraction efficiency [210]. The two aforementioned studies utilized inductively coupled plasma optical emission spectrometry (ICP-OES) and electrothermal atomic absorption spectrometry (ETAAS),

respectively, which are less sensitive than ICP-MS. Alternatively, the instrument settings of the detection system can also be optimized to improve sensitivity, as carried out in Hg speciation analysis in food using HPLC coupled to UV-cold vapor atomic fluorescence spectrometry (HPLC-CV-AFS) [213].

3.2.4 Extraction of lipid-soluble As

The optimized extraction procedure was mainly targeted for water-soluble As species. In contrast, AsLipids are lipid-soluble compounds, thus, requiring a different extraction approach [40, 173]. In **Paper II**, AsLipids were estimated based on an earlier work [130] using MeOH and methyl *tert*-butyl ether (MTBE) as extraction solvent, followed by mixing using a test-tube rotator, collecting the organic phase, then evaporating under heating (40 °C) and a stream of nitrogen. The remaining lipid pellet was subsequently analyzed for tAs. Other extraction strategies which have been used in the past include sequential extraction using hexane and MeOH [40] and extraction by shaking using a dichloromethane (DCM):MeOH mixture (2:1, v/v) [173]. The sequential extraction is often used to separate non-polar AsLipids from the polar AsLipids [129]. However, caution should be taken as the order of extraction can result to differences in concentrations [215]. AsLipids extraction using DCM:MeOH without prior extraction using water will lead to co-extraction of AB and other similarly soluble As compounds, thereby overestimating the AsLipids fraction [34, 215]. This was hypothesized to be the reason for the higher recoveries obtained when As mass balance was verified in **Paper II**.

3.3 Separation

3.3.1 Overview

Separation of As species can be carried out using analytical techniques such as HPLC, gas chromatography (GC), and capillary electrophoresis (CE) among others [33, 88, 203]. Table 7 presents some of the advantages and disadvantages of the different separation techniques commonly employed for As speciation. Aside from the ability to effectively separate the analytes of interest, one of the primary considerations for the separation technique is its compatibility with the detector in terms of coupling [33]. While CE entails relatively low cost to operate, it has poor detection limits and its coupling with usual detection systems is not as

straightforward compared to HPLC [33, 88]. The use of GC is also limited since most As species are non-volatile [33]. To a great extent, HPLC is the most employed separation technique for As speciation analysis [33, 216, 217] (Table 5).

Table 7. Advantages and disadvantages of commonly employed separation techniques in As speciation analysis [33, 88, 203].

Separation technique	Advantages	Disadvantages
HPLC	• Can separate water-soluble and lipid-soluble As species • Can be easily interfaced with various detection systems • Can handle different matrices • Availability of different analytical columns and types of mobile phase	• Possible co-elution of species which have similar physicochemical properties • Requires standards for retention-time matching/species identification
GC	• Separates volatile As species • Can be easily interfaced with various detection systems • Overcomes complications related to use of organic solvents in HPLC-ICP-MS (e.g. AsLipids analysis)	• Most As species are non-volatile and thermolabile • Requires derivatization to form arsines, but most As species do not form arsines
CE	• Relatively simple and cheap to operate • Requires less sample volume (in nL) • Rapid analysis • Anionic and cationic As species can be separated simultaneously	• Relatively poor sensitivity • Can be challenging to interface with various detection systems • Limited to As species which have ionic charge (i.e. not suitable for AsLipids) • Limited to simple matrices

One of the main advantages of HPLC is the availability of different mobile phases and analytical columns, giving flexibility to adjust HPLC conditions to improve selectivity of the method [216, 217]. There are several modes of separation, which include ion-exchange chromatography, reversed-phase liquid chromatography (RP-LC), hydrophilic interaction liquid chromatography (HILIC), and size-exclusion chromatography (SEC) [88, 216]. By far, ion-exchange chromatography (IEC) is the most applied technique to separate water-soluble As species, primarily because hydrophilic As compounds exist in different ionic forms depending on the pH [33]. In IEC, the analytical column, which is coated with surface ions, acts as ion exchanger, thereby retaining the As species. The mobile phase contains oppositely charged ions with higher affinity to the stationary phase, facilitating the elution of As species by competitive ion exchange [33]. Arsenic species have diverse physicochemical properties, specifically pK_a values. Depending on the pH, some compounds can exist as anions, while others as cations (Fig. 4), hence, requiring two separation modes: cation-exchange and anion-

exchange. Consequently, two analytical columns should be used complementarily to quantify anionic and cationic As species properly.

As species	pKa values	pH (1 2 3 4 5 6 7 8 9 10 11 12 13 14)
As(III)	pKa1 = 9.23 pKa2 = 12.13 pKa3 = 13.4	H_3AsO_3 | $H_2AsO_3^-$ | $HAsO_3^{2-}$ | AsO_3^{3-}
As(V)	pKa1 = 2.19 pKa2 = 6.98 pKa3 = 11.53	H_3AsO_4 | $H_2AsO_4^-$ | $HAsO_4^{2-}$ | AsO_4^{3-}
DMA	pKa = 6.2	$(CH_3)_2AsO(OH)$ | $(CH_3)_2AsO_2^-$
MA	pKa1 = 4.1 pKa2 = 8.7	$CH_3AsO(OH)_2$ | $CH_3AsO_2(OH)^-$ | $CH_3AsO_3^-$
TMAO	pKa = 3.6	$(CH_3)_3AsO$ | $(CH_3)_3As^+OH$
TMAP	-	$(CH3)_3As^+(CH_2)_2COO^-$
AC	None	$(CH_3)_3As^+(CH_2)_2OH$
TETRA	None	$(CH_3)_4As^+$
AB	pKa = 2.2	$(CH_3)_3As^+CH_2COOH$ | $(CH_3)_3As^+CH_2COO^-$

Fig. 4. List of pKa values for the different arsenic species and their corresponding forms depending on the pH; pKa values obtained from Reid et al. [88] and Feldmann et al. [218].

3.3.2 Chromatographic columns

Several HPLC columns have been used in previous studies for the speciation of As (Table 5). Cationic As species, such as AB, TMAO, TMAP, AC, and TETRA, can be separated using cation-exchange columns such as Metrosep C6 [34], IonoSpher 5C [36, 38, 40, 219], Zorbax 300 SCX [95, 220], or PRP-X200 [117, 188, 196]. Separation of anionic As species, such as DMA, MA, and As(V), can be carried out using PRP-X100 [37, 95, 102, 189, 195], IonPac AS7 [174, 202], or Supelco SAX1 [179] among others.

Table 8. HPLC columns tested during the method development phase.

Type	Product	Dimensions	Packing material	pH range	Max P (bar)
Cation-exchange	IonoSpher 5 C	100 x 3.0 mm, 5 µm	Derivatized silica material containing sulfonate functional groups	2.5 to 6.5	200
	Metrosep C6	250 x 4.0 mm, 5 µm 150 x 2.0 mm, 5 µm	Silica gel with carboxyl groups	2 to 7	200
Anion-exchange	PRP-X100	250 x 4.6 mm, 5 µm 150 x 4.6 mm, 5 µm	Polystyrene-divinylbenzene (PSDVB) copolymer with quaternary ammonium groups	1 to 13	345
	IonPac AS7	250 x 2 mm, 10 µm	Alkyl quaternary ammonium	0 to 14	275
Multimode (reversed phase and cation exchange)	RSpak NN-414	150 x 4.6 mm, 10 µm	Polyhydroxy methacrylate gel with a small amount of cation-exchange group	2 to 12	20

Table 8 presents an overview of the different analytical columns tested during the method development phase. In **Paper I**, IonoSpher 5C and Metrosep C6 were tested for the analysis of cationic As species. IonoSpher 5C (100 x 3.0 mm, 5 µm) allowed a faster separation of analytes (15 min) compared to Metrosep C6 (250 x 4.0 mm, 5 µm) (23 min), but poor reproducibility in retention time (RT) was noted for IonoSpher 5C (**Paper I**). This was also observed in earlier studies which determined several As species in marine samples [36, 173]. Metrosep C6, which has a column material made of silica gel containing carboxyl groups, was reported to separate several As species [173]. In **Papers I, II, and III**, at most seven As species were separated using Metrosep C6. DMA, which is usually analyzed using the anion exchange method, is also retained in Metrosep C6, though eluting quite close to the void (Fig. 5a; also in Fig. 4b of **Paper I**). DMA has a minor co-elution with AsSug PO4 using the anion exchange method (Fig. 5b; also in Fig. 4a of **Paper I**), so its separation using Metrosep C6 is helpful in verifying its concentration. A shorter version of the Metrosep column with smaller diameter (150 x 2.0 mm, 5 µm) was also tested to reduce the run time, but relatively poor chromatographic resolution was obtained even after trying several gradient profiles. It could be that the column was too short, which meant less time for the analytes to interact with the stationary phase. For the analysis of anionic As species, PRP-X100 was unanimously selected (**Paper I**) among the tested columns due to its wide applicability in several studies [95, 102, 195]. A shorter version of PRP-X100 (150 mm) was also tested, but better separation was observed when using 250 mm. PRP-X100 contains quaternary ammonium functional group as stationary phase [88].

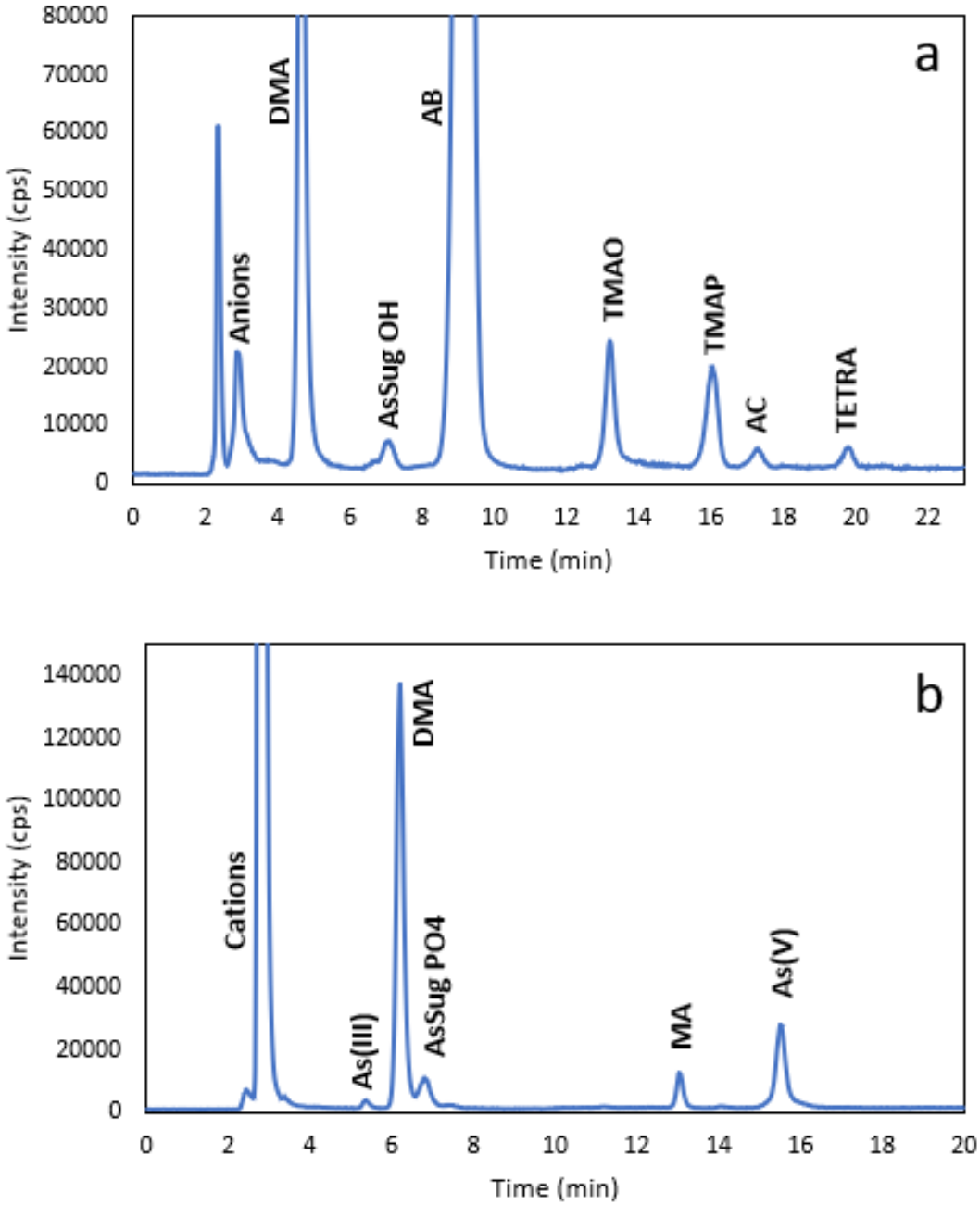

Fig. 5. Chromatograms of arsenic species in a TORT-3 extract using (a) cation- and (b) anion-exchange HPLC-ICP-MS.

3.3.3 Mobile phase selection and effect of pH

The retention of analytes is largely influenced by the mobile phase composition, concentration, and pH [33]. Most cation exchange methods utilized pyridine-based mobile phases [36, 40, 95], while anionic As species are usually eluted using phosphate- [189, 221], carbonate- [105, 187], and nitrate-based [101, 117, 190] solutions. While the use of phosphate salts produced satisfactory results [96, 172, 196], it is non-volatile and may cause buildup of deposits on the sampler and skimmer cones in the ICP-MS [222]. It also causes

signal suppression in electrospray ionization (ESI), which is a potential drawback in future characterization studies of unknown As species [223]. In **Papers I, II, and III**, pyridine-based and carbonate-based mobile phases were used for the determination of cationic and anionic As species, respectively. Although the use of ammonium carbonate may cause carbon contamination of the ICP-MS system [224], it leaves less residue on the ICP-MS compared to phosphate-based mobile phases [222].

The pH of the mobile phase and pK_a of the analytes are key factors which dictate the electrostatic interactions between the functional groups of the stationary phase and ionic species [33]. As shown in Fig. 4, As compounds such as MA (pK_{a1} = 4.1, pK_{a2} = 8.7), DMA (pK_a = 6.2), and As(V) (pK_{a1} = 2.19, pK_{a2} = 6.98, pK_{a3} = 11.53) exist as anions under neutral pH [88], while TMAO (pK_a = 3.6), AC, and TETRA are permanently cations [33, 225]. AB (pK_a = 2.2) has zwitterionic properties [33]. The extent of ionization, and consequently, retention, of these compounds can be modified by adjusting the pH of the eluent. Pyridine-based mobile phases for cationic separation usually employ a pH of 2.3 to 2.8 to facilitate complete protonation of analytes [32]. For anionic separation, the pH tends to vary depending on mobile phase composition and pK_a of target analytes. Phosphate-based eluents normally have pH between 5 to 6, while carbonate-based mobile phases typically have pH between 8 to 11 [32].

In **Paper I**, carbonate-based eluents having either pH 9.3 or 10.3 were tested for the separation of anionic As species. The use of pH 9.3 yielded better retention and separation of analytes, while pH 10.3 led to early elution. Ammonium carbonate has dissociation constants characterized by pK_{a1} = 6.35 and pK_{a2} = 9.25. The mobile phase is composed of almost equal proportion of bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}) at pH 9.3, while CO_3^{2-} is the predominant form at pH 10.3. By competitive ion exchange, CO_3^{2-} ions have stronger affinity to the stationary phase and effectively elutes the analytes. The use of pH 10.3 also resulted in co-elution of As(III) and AsSug PO4 (Fig. 3 in **Paper I**). As(III) has its first pK_a value at 9.23 [88], so when pH 10.3 was used, $H_2AsO_3^-$ ions dominated and resulted to longer retention. The co-elution was confirmed by spiking with As(III) (**Paper I**). For AsSug, the presence of aglycone moieties yields varying degree of retention [33]. AsSug such as AsSug PO4, AsSug SO3, and AsSug SO4 are better retained at pH above 5, so separation is usually by anion-exchange. AsSug OH is slightly retained in a cation-exchange column [33]. In **Papers I, II, and**

III, the final mobile phases utilized were pyridine-based solutions with pH 2.7 for cationic separation and carbonate-based eluents with pH 9.3 for anionic separation, both employing gradient elution. Fig. 6 presents examples of chromatograms obtained by applying the optimized HPLC parameters, showing separation of up to 17 As cationic and anionic species in a sample of blue mussel.

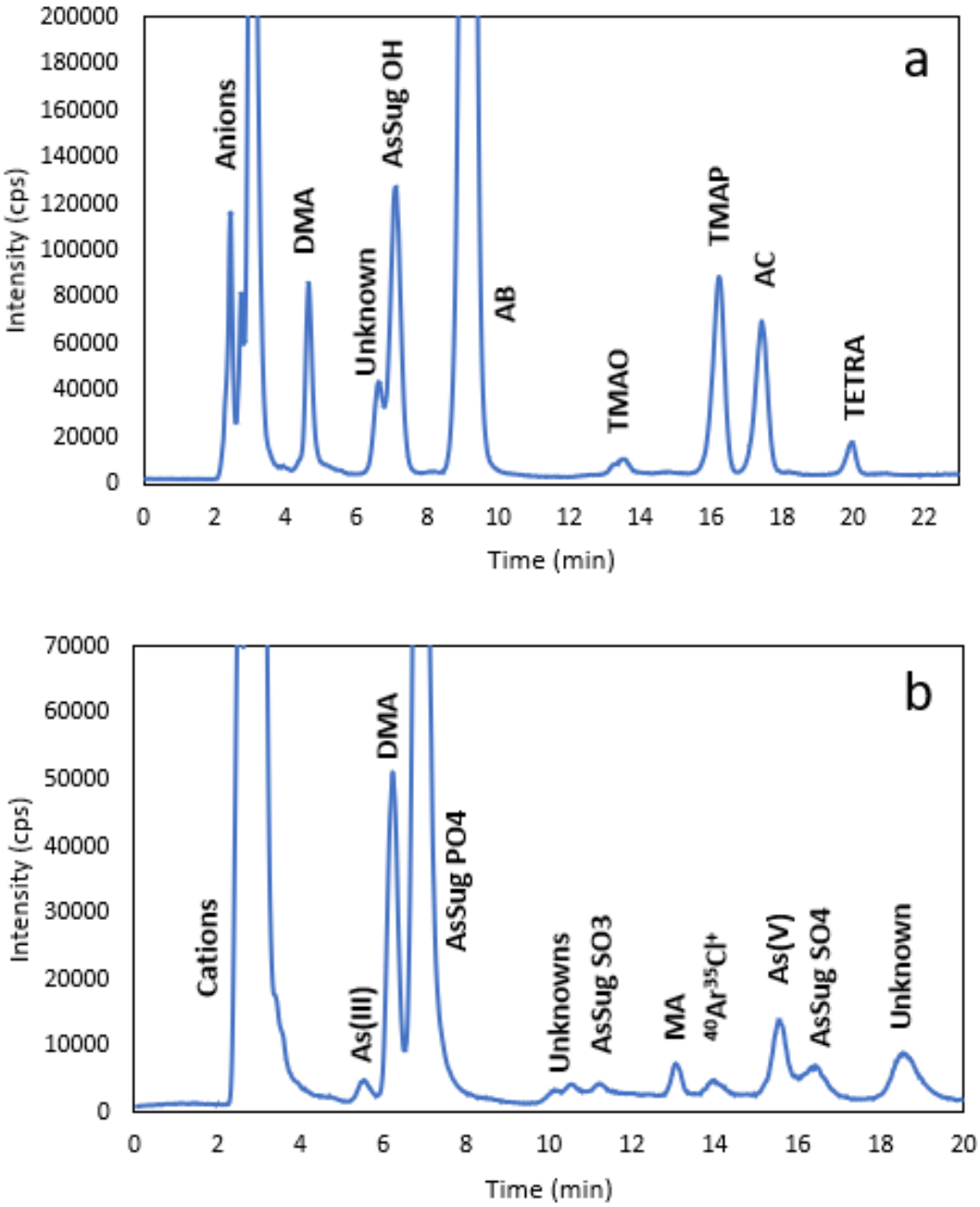

Fig. 6. Chromatograms of arsenic species in a blue mussel extract using (a) cation- and (b) anion-exchange HPLC-ICP-MS. *Adapted from Tibon et al. [226] under the CC BY license (https://creativecommons.org/licenses/by/4.0/).*

3.3.4 Co-elution and presence of unknown As species

The proposed chromatographic settings were developed using available analytical standards, i.e. gradient was adjusted to ensure best chromatographic resolution of the known peaks. However, co-elution can still occur, especially when unknown As species are present. In Fig 6a, an unknown peak was observed to co-elute with AsSug OH when blue mussel was analyzed using cation-exchange (**Paper I**). Similarly, in **Papers I and III**, a prominent unknown peak was observed eluting after AsSug SO4 when blue mussel was analyzed using anion-exchange (Fig. 6b). This was discovered after series of injections resulted to poorly shaped chromatographic peaks in the middle of the gradient. Apparently, this was due to carryover from previous injections of blue mussel extracts. The gradient had to be prolonged to fully elute the unknown anionic As species. Hence, it should be emphasized that co-elution with the proposed method (**Paper I**) cannot be fully disregarded. Modifying the gradient may further reveal unknown peaks in other matrices not covered in this work. Nonetheless, identification of the unknown As species via LC-high resolution mass spectrometry (LC-HRMS) is relevant for future work.

3.4 Detection

3.4.1 Overview

There are several detection techniques for As speciation analysis, which include hydride generation atomic absorption spectrometry (HG-AAS), hydride generation atomic fluorescence spectrometry (HG-AFS, and inductively coupled plasma mass spectrometry (ICP-MS) [33, 88, 203], among others. Table 9 presents some of the advantages and disadvantages of commonly employed detection systems for As speciation analysis. HG-AAS and HG-AFS offer traditional approaches for As speciation, but their application can be limited since not all As species can readily generate hydrides [33, 88]. Since its first introduction in 1980 [227], ICP-MS has become the most extensively used detection technique for As speciation due to its high sensitivity, good selectivity, wide linear dynamic range, and compatibility with separation instruments, especially HPLC [32, 45, 217]. Compared to atomic spectroscopy techniques, its low detection limits and its ability to eliminate interferences with the use of reaction/collision cell technology make ICP-MS the most preferred instrument for speciation analysis [228].

Table 9. Advantages and disadvantages of commonly employed detection systems in As speciation analysis [33, 229].

Detection system	Advantages	Disadvantages
ICP-MS	• Compatibility with most separation and sample introduction systems • High sensitivity • Wide linear dynamic range • Can quantify concentrations down to ng/L • High sample throughput • Simple sample preparation needed • Less sample volume needed • ICP-MS/MS can overcome interferences	• More expensive to acquire and operate than traditional detection systems • Requires gases with high purity • High level of staff expertise needed • May suffer from polyatomic and isobaric interferences • Use of organic solvents (e.g. AsLipids analysis) may require special ICP-MS configurations
HG-AFS and HG-AAS	• Relatively simple to operate • Less expensive to acquire and operate • Reduced matrix effects • Can quantify concentrations down to µg/L levels	• Limited number of organoarsenic species forming hydrides • Low sample throughput • Requires relatively high sample volume

3.4.2 Brief introduction to ICP-MS

From the HPLC, the mobile phase containing the analytes goes through the nebulizer and into the spray chamber where a fine aerosol is created (Fig. 7). The aerosol is atomized and ionized by the high-temperature argon plasma, thereby generating ions, which are extracted by sampler and skimmer cones. Under high vacuum, the ion beam is guided by ion optics into the quadrupole mass analyzer, where the ions of interest are separated from the rest through their mass-to-charge ratio (*m/z*). The ions are then measured by the detector through conversion of ion flux intensity to an electronic signal [229, 230]. While the ICP-MS offers many advantages over traditional detection systems, certain challenges need to be addressed, especially when used for As speciation analysis. These include the relatively poor ionization of As and possible spectral interferences.

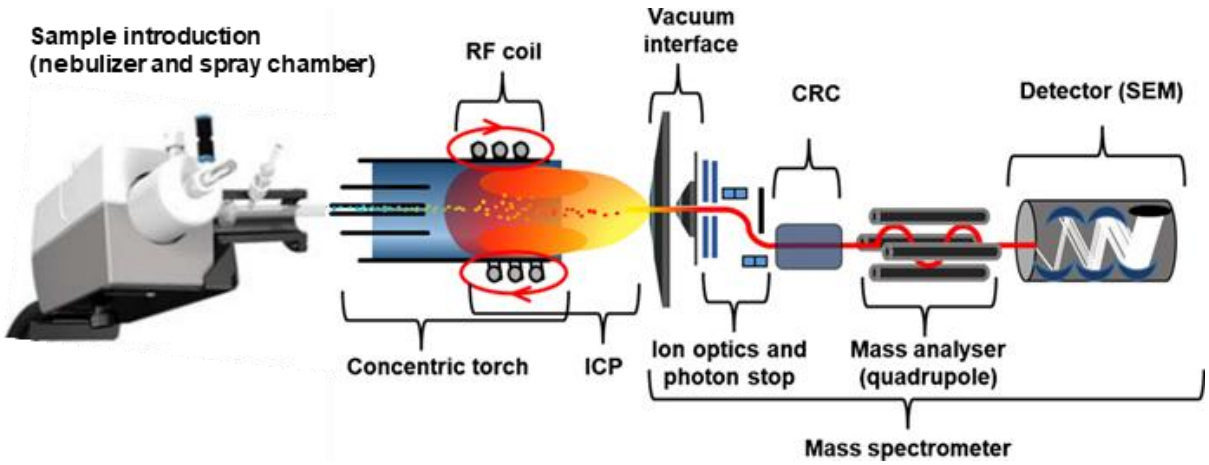

Fig. 7. Overview of the different parts of the ICP-MS involved in the nebulization of the sample, ionization of the analytes, and subsequent detection. *Adapted from Clases and Gonzales De Vega [231] under the CC BY license (https://creativecommons.org/licenses/by/4.0/).*

3.4.3 Carbon-induced signal enhancement

Arsenic has a high ionization potential (ionization energy, IE = 9.82 eV), which classifies it as one of the hard-to-ionize elements under standard ICP-MS conditions [224]. With a degree of ionization of only 52% [224], the ICP-MS detection of As may suffer from poor sensitivity. The addition of organic solvent, either through the mobile phase or directly into the spray chamber, was reported to enhance the ICP-MS signal [32]. Larsen and Stürup [224] proposed the inclusion of 3% (v/v) MeOH in the mobile phase to achieve maximum signal enhancement for As. The addition of MeOH results to an increase in population of C^+ ions in the plasma. The abundance of C^+ ions facilitates charge transfer (electrons) from elements with slightly lower ionization potential than carbon, effectively improving the degree of ionization [225]. Carbon has an ionization potential of 11.26 eV and elements such as As and Se (IE = 9.75 eV) benefit the most from this signal enhancement effect [224].

In **Paper I**, carbon-induced signal enhancement was investigated by aspirating As(V) standard solutions with varying proportions of acetonitrile (ACN) and MeOH. Metrosep C6 is lined with carboxyl groups, and the presence of alcohol in the mobile phase may result to esterification of ion-exchange sites [232], thus, ACN was chosen for cation-exchange. Maximum signal enhancement was observed when using 0.5% (v/v) ACN and 3% (v/v) MeOH (**Paper I**), which agrees with the findings of Larsen and Stürup [224] for MeOH. The amplification of signal at low percentage of ACN may be attributed to its two carbon atoms and a nitrogen atom,

compared to MeOH which only has one carbon atom. Also, the introduction of nitrogen gas was reported to improve the signal in laser ablation ICP-MS [233], and the same mechanism may be responsible for the signal enhancement observed when incorporating ACN in the mobile phase (**Paper I**). When proportions greater than 0.5% ACN and 3% MeOH were aspirated into the ICP-MS, signal enhancement became less pronounced (**Paper I**). Instead of improving the signal, suppression was observed for ACN proportions greater than 3.5%. The reduced intensity may be due to the cooling effect of plasma. Higher proportions of organic solvent require more energy from the ICP for decomposition, which lowers the plasma temperature and ionization efficiency [234, 235].

3.4.4 Influence of mobile phase composition on ICP-MS sensitivity

The mobile phase composition (i.e. buffer used) and the gradient elution program can also affect the ICP-MS response. This was verified by analyzing a mixed standard solution containing DMA, AB, and TETRA through cation-exchange HPLC, using mobile phases (a) without added ACN, (b) with 0.5% (v/v) ACN and (c) 3% (v/v) ACN. Using setting (b), peak heights for DMA and AB had a two-fold increase compared to setting (a) (Fig. 8a). In contrast, the effect on TETRA was close to negligible, which may be due to the gradient since TETRA was eluted by 100% mobile phase B (50 mM pyridine). To verify, two sets of 5 µg/L As(V) standard solutions with different ACN proportions were prepared. The first set did not contain pyridine, while the other had a concentration of 50 mM pyridine. The result showed that the enhancement brought about by ACN was countered by the presence of pyridine (Fig. 8b). The increased carbon load from both pyridine and ACN precluded the efficient ionization of analytes, resulting to very little signal enhancement. Using setting (c), all three species experienced signal suppression, wherein TETRA was severely affected, having a decrease in signal of approximately 70% (Fig. 8a). This is further supported by Fig. 8b, wherein a 5 µg/L As(V) standard solution with 50 mM pyridine and 3% ACN suffered from signal suppression of similar magnitude. The combined effect of high proportion of ACN and increased eluent concentration saturated the plasma with carbon, thereby causing a cooling effect, as also reported in earlier studies [234, 235]. Furthermore, an increase in proportion of ACN shortened the RT, most noticeably for AB and TETRA (Fig. 8a). This could be explained by the presence of organic solvent altering the dielectric constant of the mobile phase and

consequently reducing the available sites for ion exchange in the stationary phase, as suggested by others [236].

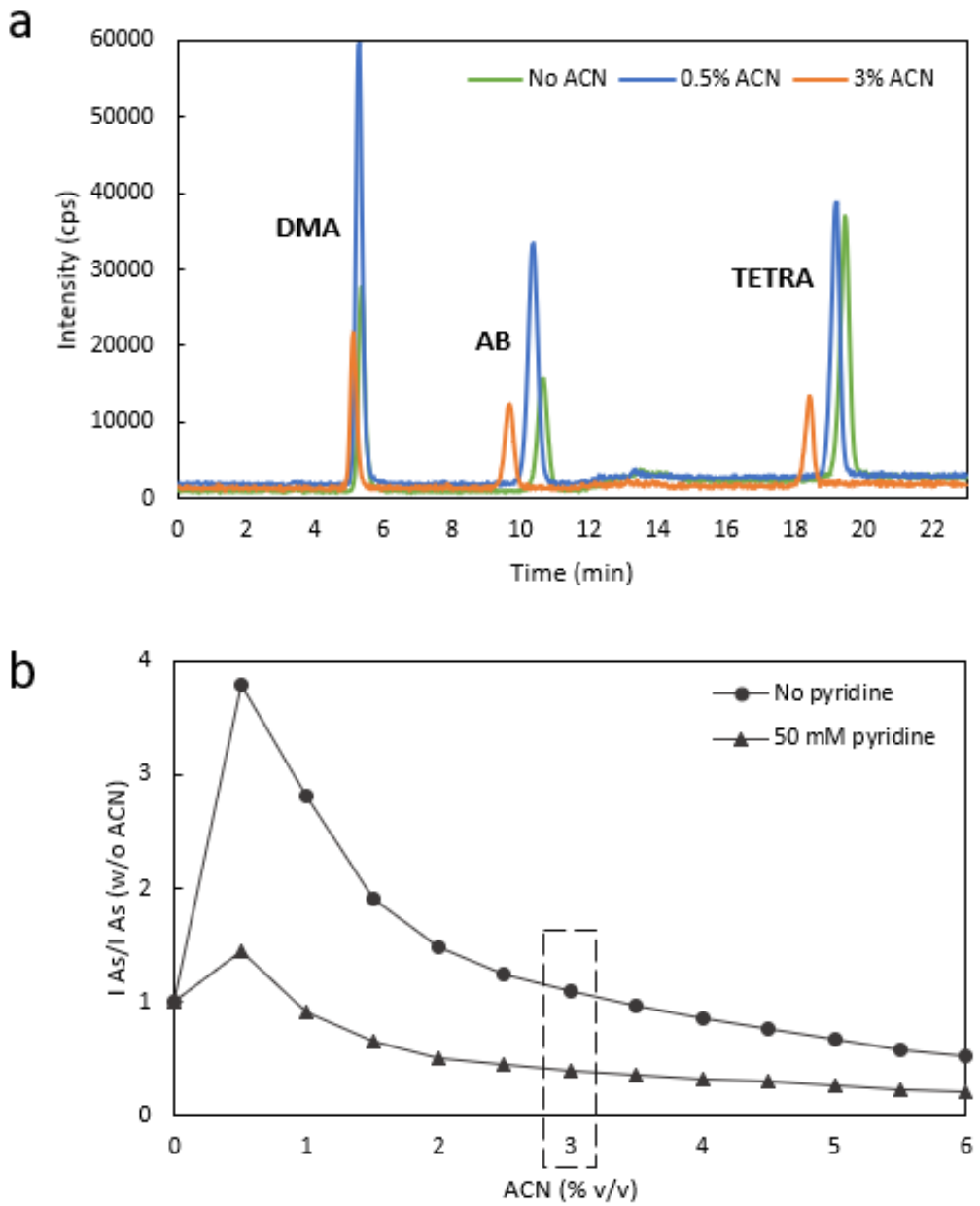

Fig. 8. (a) Overlaid cation-exchange chromatograms of a 1 µg/L mixed standard solution of DMA, AB, and TETRA under different ACN concentrations. (b) Relative ICP-MS response for a 5 µg/L standard solution of As(V) in different concentrations of pyridine and acetonitrile. I As – intensity of ^{75}As for a standard solution with a certain % of ACN; I As (w/o ACN) – intensity of ^{75}As for a standard solution without ACN. Solutions were prepared at 5 µg/L As(V).

3.4.5 Common issues encountered with ICP-MS

The ICP-MS, with its low detection limits and ease in coupling with HPLC, is an effective tool for As speciation. However, it may also be subject to polyatomic interferences such as the formation of $^{40}Ar^{35}Cl^{+}$ ion [32, 217, 225]. This can be resolved by using a triple quadrupole ICP-MS in oxygen reaction mode, where the $^{75}As^{+}$ ion is oxidized to from $^{75}As^{16}O^{+}$, which can then be measured by monitoring mass-to-charge ratio (*m/z*) of 91 [33]. Alternatively, the corresponding peak for $^{40}Ar^{35}Cl^{+}$ (RT: 14.6 min) can be separated chromatographically from anionic As species, as seen in **Paper I**, where it elutes between MA (RT: 13.4 min) and As(V) (RT: 16.3 min) (Fig. 9). Also, the use of organic solvents in the mobile phase can cause buildup of carbon deposits on the cones, which may interfere with efficient ionization of analytes [32, 33]. This is not much of an issue for the determination of water-soluble As species, but for AsLipids analysis, a special ICP-MS configuration and oxygen introduction to the plasma may be necessary [33]. Lastly, ICP-MS employs hard ionization, which results to loss of molecular information. In the presence of unknown As species, complementary techniques which utilize soft ionization are needed, such as electrospray ionization mass spectrometry (ESI-MS) [32, 33, 88]. Alternatively, simultaneous elemental and molecular information can be obtained by splitting the flow from the HPLC, wherein one part goes to the ICP-MS and the other to HRMS [44]

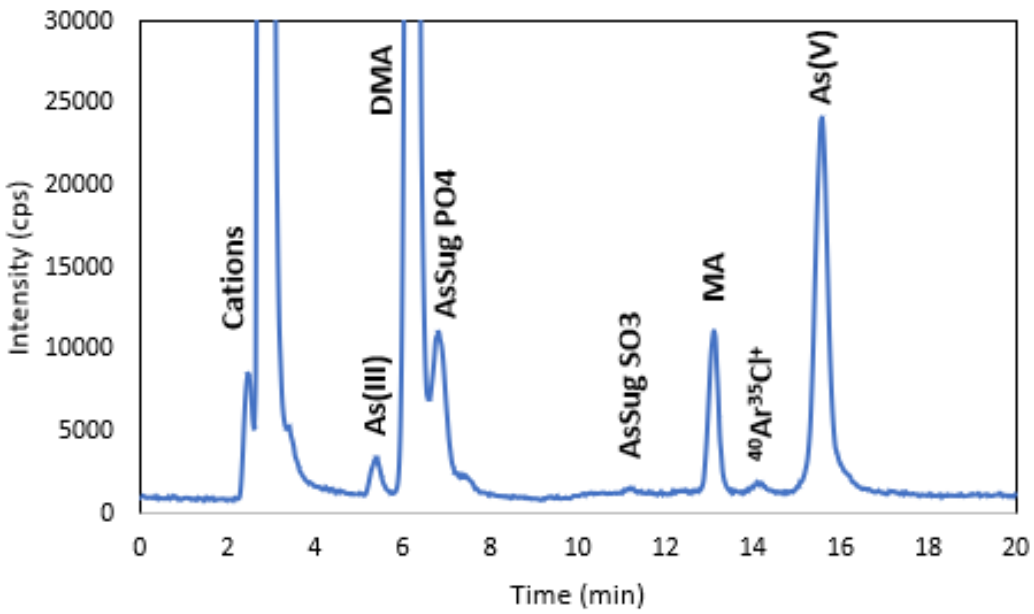

Fig. 9. Anion-exchange chromatogram of a DORM-4 extract showing that the polyatomic ion $^{40}Ar^{35}Cl^{+}$ (retention time: ~14 min) is chromatographically separated from MA and As(V).

3.5 Quantification

Arsenic species are identified by matching the RTs with available standards [88, 216]. The response is measured based on chromatographic peak areas and quantification is based on external calibration curves generated from standard solutions [32], as applied in **Papers I, II, and III**. Commercially available analytical standards do not exist for some As compounds (e.g. AsSug). The ICP-MS is an element-specific detector, which allows the use of the calibration curve of the nearest eluting standard, with the assumption that ICP-MS response is consistent throughout the run and does not vary depending on the species [32]. In contrast, Larsen and Stürup [224] observed that signals for organic As species varied when using a first-generation ICP-MS. The perceived variation was attributed to the molecular species by which As enters the ICP-MS. Grotti et al. [237] reported that instrument configurations, e.g. spray chamber temperature and sample uptake, may also contribute. In the method developed in **Paper I**, the uniformity of ICP-MS response was verified based on the slopes of the calibration curves of the different species, which were found to be similar. The method employs gradient elution, and it is important that the gradient does not alter the ICP-MS sensitivity drastically. Alternatively, changes in signal intensity due to the gradient or instrumental drifts are sometimes compensated by using suitable internal standards [32], e.g. Se [172] and germanium (Ge) [41, 238].

3.6 Method validation

3.6.1 Optimum extraction procedure and HPLC-ICP-MS settings

Based on the optimization results, the final extraction procedure implemented in **Papers I, II, and III** was as follows. Approximately 0.2 g of sample was weighed into polypropylene (PP) tubes, followed by addition of 5 mL of aqueous MeOH (50%, v/v). Samples were subjected to vortex mixing for a few seconds, then tubes were placed in a water bath set at 90 °C for 30 min (shaking speed: 100 rpm). Tubes were allowed to cool and subsequently centrifuged (3800 rpm for 10 min). The extracts were filtered (0.45 µm) and transferred to another PP tube. An aliquot of the extract was transferred to a 1-mL PP HPLC vial, then diluted accordingly prior to analysis by HPLC-ICP-MS. The optimum operating parameters for HPLC-ICP-MS are given in Table 10.

Table 10. The operating parameters for HPLC-ICP-MS. *Adapted from Tibon et al. [226] under the CC BY license (https://creativecommons.org/licenses/by/4.0/).*

Instrument settings		
HPLC-ICP-MS settings	**1260 HPLC and 7900 ICP-MS**	
RF Power	1550 W	
Nebulizer gas flow	1.03 L/min	
Plasma gas flow	15.0 L/min	
Spray chamber temperature	2 °C	
Isotopes monitored	^{75}As, ^{35}Cl	
Integration time	1 s	
	Cation-exchange	**Anion-exchange**
Guard column	Metrosep C 6 Guard (4.0 mm)	PRP-X100 Guard cartridge, PEEK
Analytical column	Metrosep C 6 (250 x 4.0 mm, 5 um)	PRP-X100 (250 x 4.6 mm, 5 um)
Mobile phase	A: 0 mM pyridine, 0.5% ACN, pH 2.7 B: 50 mM pyridine, 0.5% ACN, pH 2.7	A: 0.5 mM $(NH_4)_2CO_3$, 3% MeOH, pH 9.3 B: 60 mM $(NH_4)_2CO_3$, 3% MeOH, pH 9.3
Gradient	0-8 min (10% B), 8-10 min (10% to 100% B), 10-20 min (100%B), 20-23 min (10% B)	0-6 min (20% B), 6-17 min (100%B), 17-20 (20% B)
Flow rate	0.9 mL/min	1 mL/min
Injection volume	50 µl	50 µl

3.6.2 Method validation parameters

To demonstrate that the developed method is fit for purpose, a single-laboratory validation was conducted following Eurachem's recommendations [239] (**Paper I**). Blue mussel and several marine certified reference materials were used, namely BCR 627 (tuna fish tissue), ERM CE278k (mussel tissue), ERM-CD200 (seaweed), DORM-4 (fish protein), DOLT-5 (dogfish liver), SQID-1 (cuttlefish), and TORT-3 (lobster hepatopancreas), CRM-7405b (hijiki) and SRM 1566b (oyster tissue). The investigated method validation parameters include selectivity, limits of detection and quantification (LOD and LOQ), linearity and working range, trueness, precision, and measurement uncertainty. Trueness was assessed based on recoveries of certified values of CRMs and spiking tests. Good recoveries were achieved, suggesting that the integrity of the species was preserved throughout the analysis. Overall, satisfactory method performance characteristics were obtained (**Paper I**).

3.6.3 Analytical quality control – CRMs, analytical standards, and As mass balance

Method validation is often an overlooked component in 'method' papers on As speciation. Ardini et al. [32] reported that out of the 200 reviewed papers on As speciation analysis in environmental samples, approximately 25% performed method validation, 64% used CRMs and only one third of those utilized CRMs for method validation. In **Papers I, II, and III**, CRMs

were always included in the analyses. Table 11 presents the CRMs used and the corresponding certified and information values for As species, together with the obtained concentrations (**Paper I**). Among the CRMs used, only CRM 7405-b is certified for As(V), AsSug OH, and AsSug SO4. All other CRMs were only certified for AB (and DMA, in the case of BCR 627), while some information values were given. Several review articles emphasized the lack of suitable CRMs for As species [32, 33, 109, 217]. Nonetheless, the use of relevant CRMs, even though without assigned values for the analytes of interest, should be endeavored as the results would provide good basis for comparison within the research community [240]. Alternatively, the availability of robust and reproducible As speciation methods, which can be easily implemented in other laboratories, may help in the establishment of certified or information values through interlaboratory studies and proficiency tests.

Table 11. Certified reference materials (CRM) used in **Paper I** alongside certified and information values for total arsenic and arsenic species. Obtained concentrations are also reflected for comparison (mean ± SD, *n* = 5).

CRM	Analytes with certified values			Analytes with information values		
	Analyte	Certified value (mg/kg)	Obtained concentration (mg/kg)	Analyte	Information value (mg/kg)	Obtained concentration (mg/kg)
BCR 627 Tuna fish tissue	Total As	4.8 ± 0.3	4.4 ± 0.1			
	AB	3.9 ± 0.2	3.9 ± 0.1			
	DMA	0.15 ± 0.02	0.155 ± 0.004			
CE278k Mussel tissue	Total As	6.7 ± 0.4	6.7 ± 0.1			
DORM-4 Fish protein	Total As	6.87 ± 0.44	6.95 ± 0.08			
SQID-1 Cuttlefish	Total As	14.1 ± 2.2	16.4 ± 0.2	DMA	0.03	0.032 ± 0.003
	AB	13.96 ± 0.54	13.59 ± 0.25	As(III)	0.019	0.020 ± 0.004
				As(V)	0.028	0.032 ± 0.006
DOLT-5 Dogfish liver	Total As	34.6 ± 2.4	31.7 ± 1.0			
	AB	24.2 ± 0.8	26.1 ± 1.7			
TORT-3 Lobster hepatopancreas	Total As	59.5 ± 3.8	64.7 ± 2.0			
	AB	54.9 ± 2.5	48.5 ± 1.0			
CRM 7405-b Hijiki	Total As	49.5 ± 1.0	48.2 ± 1.0	DMA	0.24	0.29 ± 0.01
	As(V)	24.4 ± 0.7	24.3 ± 0.6	AsSug PO4	0.2	0.313 ± 0.004
	AsSug SO4	1.41 ± 0.04	1.360 ± 0.03	AsSug SO3	0.16	0.18 ± 0.010
	AsSug OH	0.44 ± 0.02	0.415 ± 0.002			

Similar to CRMs, the lack of well-characterized standards for other As species precludes analytical traceability in As speciation analysis [33, 88, 216]. In **Papers I, II, and III**, quantification and RT matching were based on standard solutions prepared from available As analytical standards. Those without available standards, including unknown As species, were

quantified using the calibration curve of the nearest eluting compound with available analytical standard. Lastly, when performing method development studies, it is essential to check extraction efficiency, column recovery, and overall As mass balance to verify the reliability of As speciation data. In **Paper I**, mass balance showed that As recovery ranged from 104 to 118%. Based on the results of the method validation, the optimized extraction procedure and HPLC-ICP-MS parameters facilitated accurate quantification of As species in marine matrices, demonstrating that the methods are fit for purpose.

CHAPTER 4: ARSENIC SPECIATION IN NOVEL MARINE FEED RESOURCES

*This chapter presents As speciation data in novel marine feed resources, which were analyzed using the methods discussed in Chapter 3/**Paper I**. A brief background on future aquafeed ingredients is provided, followed by presentation and discussion of the As species found. Special focus is given on the bioaccumulation and biotransformation of As species and the effects of feed processing on As speciation. The chapter concludes with an evaluation of As speciation data in light of feed safety. Relevant findings from **Papers I, II, III, and IV** are discussed.*

4.1 Novel marine feed resources and the need for As speciation data

The socioeconomic concerns related to the use of wild, edible fish as aquafeed ingredients and increasing prices of fish meal and fish oil highlight the need for alternative feed ingredients [1, 241]. In Norway, while the current salmon feed composition is dominated by plant-derived protein and oils [242], these 'green' raw materials may contain ANFs and undesirable substances (e.g. pesticides and mycotoxins) which can negatively impact fish health and food safety [6]. In addition, cultivation and processing of plant ingredients, both as food and feed, ultimately contribute to increasing greenhouse gas emissions [8, 9]. Hence, research is directed towards novel sources of protein and essential fatty acids for aquaculture feed [2]. Some of the future feed resources identified include insects, animal by-products, and other plant-based by-products, but much attention has been given recently to unexploited marine organisms at lower trophic levels due to their abundance, suitable nutritional composition, and reduced carbon footprint associated with their usage [2, 10, 243]. The use of low-trophic marine organisms as aquafeed ingredients has high potential, but at the same time, comes with inherent risks and challenges which need to be fully addressed. These include ecological impact and sustainability, economic feasibility, supply availability, and the presence of ANF and undesirable substances [2, 6, 243].

4.1.1 Mesopelagic organisms

Mesopelagic organisms are marine species which thrive between 200 to 1000 m beneath the sea surface [244]. With global biomass estimates amounting to 10 billion tons, their

abundance makes them one of the biggest marine resources worldwide [51, 245]. Their presence extends from the Arctic to Antarctic [246], where notable populations were found in the northeast Atlantic Ocean (including the Norwegian exclusive economic zone (EEZ)) [245], continental shelfs in the Pacific and Indian Oceans [244], and the Gulf of Oman and off North Africa [247]. There are at least 31 families of mesopelagic organisms, including species of lanternfish, krill, shrimps, and jellyfish [7, 244, 246, 247]. They are rich in essential amino acids, vitamins (A and B12), and minerals (Ca, Se, Fe, and iodine (I)) [7, 52, 248]. They also contain significant amounts of LC-PUFA, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which can augment the requirement for LC-PUFA in salmon feed [2, 52, 53]. However, mesopelagic organisms were reported to contain variable amounts of wax esters, potentially affecting digestion and absorption of nutrients in fish [2]. Likewise, elevated levels of fluoride, Cd, and As have been reported [7, 53, 246]. In **Papers II and IV**, six of the most abundant mesopelagic species in Norwegian fjords were analyzed for As and its species, including (i) the helmet jellyfish *Periphylla periphylla*, (ii) the glacier lanternfish *Benthosema glaciale*, (iii) the silvery lightfish *Maurolicus muelleri*, (iv) the Northern krill *Meganyctiphanes norvegica*, (v) the decapods *Pasiphaea* sp., and (vi) the Arctic red prawn *Eusergestes arcticus* (Fig. 10).

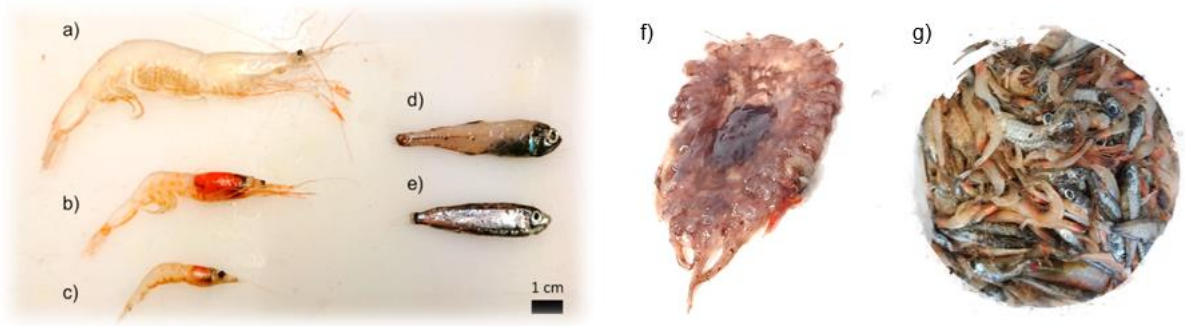

Fig. 10. Mesopelagic organisms and mesopelagic biomass in Norwegian fjords: (a) the decapods *Pasiphaea* sp., (b) the Arctic red prawn *Eusergestes arcticus*, (c) the Northern krill *Meganyctiphanes norvegica*, (d) the glacier lanternfish *Benthosema glaciale*, (e) the silvery lightfish *Maurolicus muelleri*, (f) the helmet jellyfish *Periphylla periphylla*, and (g) the mesopelagic biomass composed of *M. norvegica* and *M. muelleri*. *Adapted from Alvheim et al. [52] under the CC BY license (https://creativecommons.org/licenses/by/4.0/).*

4.1.2 Blue mussels (*Mytilus edulis* L.)

Blue mussels are rich in essential amino acids and LC-PUFA [2]. The inclusion of blue mussel in diets of Japanese flounders (*Paralichthys olivaceus*) was shown to improve the organism's

growth when compared to diets which did not contain blue mussel [249]. The cultivation of blue mussels can be incorporated in integrated multitrophic aquaculture (IMTA) schemes. They can be grown next to salmon farms and can feed on minute suspended organic matter (e.g. from feces and feed), which was observed to improve their growth and fatty acids composition [250]. Concomitantly, mussels and other shellfish are known to sequester carbon, improving aquaculture's climate footprint [251]. However, the full potential of blue mussels as feed ingredient is hampered by expensive production and downstream processing [2]. Likewise, blue mussels are known to accumulate undesirable substances (e.g. PTEs and organic pollutants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs)) from the surrounding environment [243, 252], including As and its species [117]. In **Paper I**, pooled blue mussel samples from the Norwegian surveillance program [253] and the CRM ERM CE278k were analyzed for As and its species. Similarly, blue mussels from the As exposure experiment in **Paper III** were examined and included in the analysis.

4.1.3 Microalgae

The use of microalgae both as food and feed has been regarded as a more sustainable alternative since its biomass production emits less greenhouse gases and requires less water and space [254, 255]. Microalgae are rich in lipids, proteins, and elements beneficial for fish growth [2]. Some of the microalgal strains currently explored as aquafeed component include *Nannochloropsis* sp., *Chlorella* sp., *Tetraselmis* sp., and *Arthrospira* sp. (popularly known as *Spirulina*) [2, 243, 256]. The inclusion of microalgae in fish diets resulted to increased LC-PUFA in Atlantic salmon (*Salmo salar*), improved protein digestibility in rainbow trout (*Oncorhynchus mykiss*), and overall enhanced nutritional quality in Nile tilapia (*Oreochromis niloticus*) [257]. However, microalgae are also known to accumulate PTEs (e.g As, Cr, Hg, Pb, Cd) [258, 259], which can negatively impact food and feed safety. Microalgae are at the base of the marine food chain, hence, they serve as vectors for the transfer of undesirable substances to higher forms of aquatic animals (e.g. zooplanktons, shrimp, and other shellfish) [256], which include As and its species [125, 141]. In this work, the microalgae *Diacronema lutheri* (Fig. 11), together with blue mussels, were used to investigate the trophic transfer, bioaccumulation, and biotransformation of As species at the bottom of the aquatic food

pyramid (**Paper III**). *D. lutheri* has been customarily used as feed in bivalve aquaculture [260], and the occurrence of As species in these types of samples are hereby documented.

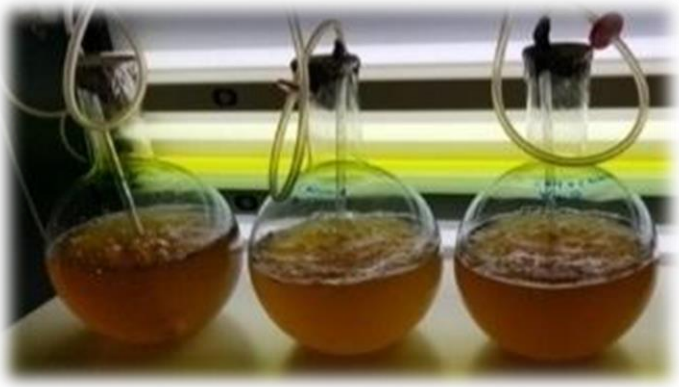

Fig. 11. Cultivation of the microalgae *Diacronema lutheri* under constant light and aeration.

4.1.4 Macroalgae

Macroalgae, commonly referred to as seaweeds, are known to contain amino acids, vitamin K, the essential element I, LC-PUFA, and other bioactive components which make them suitable as food/food supplement and feed ingredient [2, 106, 261]. Initial studies on the incorporation of seaweed meals in fish diets reported improved growth, lipid metabolism, and stress response among others [262]. However, macroalgae are also known to have high concentrations of essential and toxic elements, such as I and As, respectively [261]. Regarded as hyperaccumulators of As, macroalgae contain higher As levels than most terrestrial products, though much of it is in the form of AsSug [106]. However, certain species of macroalgae, e.g. hijiki and oarweed (*Laminaria digitata*), have shown consistently high concentrations of iAs, while other species contain barely detectable levels [98, 263]. The wide variation in the occurrence of As and its species in different types of macroalgae highlight the relevance of As speciation analysis in this type of matrix. In the current work, it should be emphasized that macroalgae was not one of the main novel marine feed resources investigated. Nonetheless, a hijiki CRM 7405-b, produced from wild hijiki harvested from the sea near Japan, was analyzed for As and its species in **Paper I** as a point of comparison.

4.2 Total arsenic and arsenic species in novel marine feed resources

Among the undesirable substances, PTEs are a major concern due to the bioaccumulation ability of low-trophic marine organisms [252, 258, 264, 265]. Special focus should be given to As and its species due to their varying toxicities and prevalence among marine organisms [109]. The following sub-sections discuss the levels of tAs and As species found in novel marine feed resources studied in this PhD project. Table 12 contains the details of the samples.

Table 12. Samples of novel marine feed resources covered in this PhD work.

Classification	Scientific name/identification	Common name/remarks	Number of samples (*n*)
Mesopelagic organism	*Benthosema glaciale*	Glacier lanternfish	7[a]
	Maurolicus muelleri	Silvery lightfish	4[a]
	Meganyctiphanes norvegica	Northern krill	4[a]
	Pasiphaea sp.		3[a]
	Eusergestes arcticus	Arctic red prawn	4[a]
	Periphylla periphylla	Helmet jellyfish	2[a]
Blue mussel	BM – NSP (*Mytilus edulis*)	Blue mussel from the Norwegian surveillance program	10[b]
	ERM CE278k (*Mytilus edulis*)	Certified reference material	5[c]
	BM – EE (*Mytilus edulis*)	Blue mussel from the As exposure experiment	15[d]
Microalgae	*Diacronema lutheri*	*D. lutheri*	5[e]
Macroalgae	CRM 7405-b *(Sargassum fusiforme/Hizikia fusiformis)*	Certified reference material	5[c]

[a]Pooled samples; at least 27 specimens per pooled sample
[b]Analytical replicates for a pooled sample containing at least 50 individual samples
[c]Analytical replicates
[d]Pooled samples; nine specimens per pooled sample
[e]Pooled samples; at least three batches of microalgae per pooled sample

4.2.1 Total As

Total As levels varied widely among novel marine feed resources (Fig. 12). For mesopelagic organisms, the mesopelagic crustaceans (i.e. *M. norvegica*, *Pasiphaea* sp., and *E. arcticus*) had considerably higher tAs concentrations than the fish species (i.e. *B. glaciale* and *M. muelleri*). *M. norvegica* generally had the highest tAs concentrations, ranging from 38 to 160 mg/kg dw (**Paper IV**). Results are comparable to the levels found in similar species collected from the Northeast Atlantic and Mediterranean Sea [266, 267]. *Pasiphaea* sp. had slightly lower tAs levels, i.e. between 32 to 136 mg/kg dw (**Paper IV**). *B. glaciale* had tAs levels ranging from 6.9 to 19 mg/kg dw (**Paper IV**), similar to what was found in *Benthosema pterotum* sampled off

the Gulf of Oman [246]. *M. muelleri* had tAs concentrations between 15 to 17 mg/kg dw, while the jellyfish *P. periphylla* contained the least As, ranging from 1.9 to 3.2 mg/kg dw (**Paper IV**).

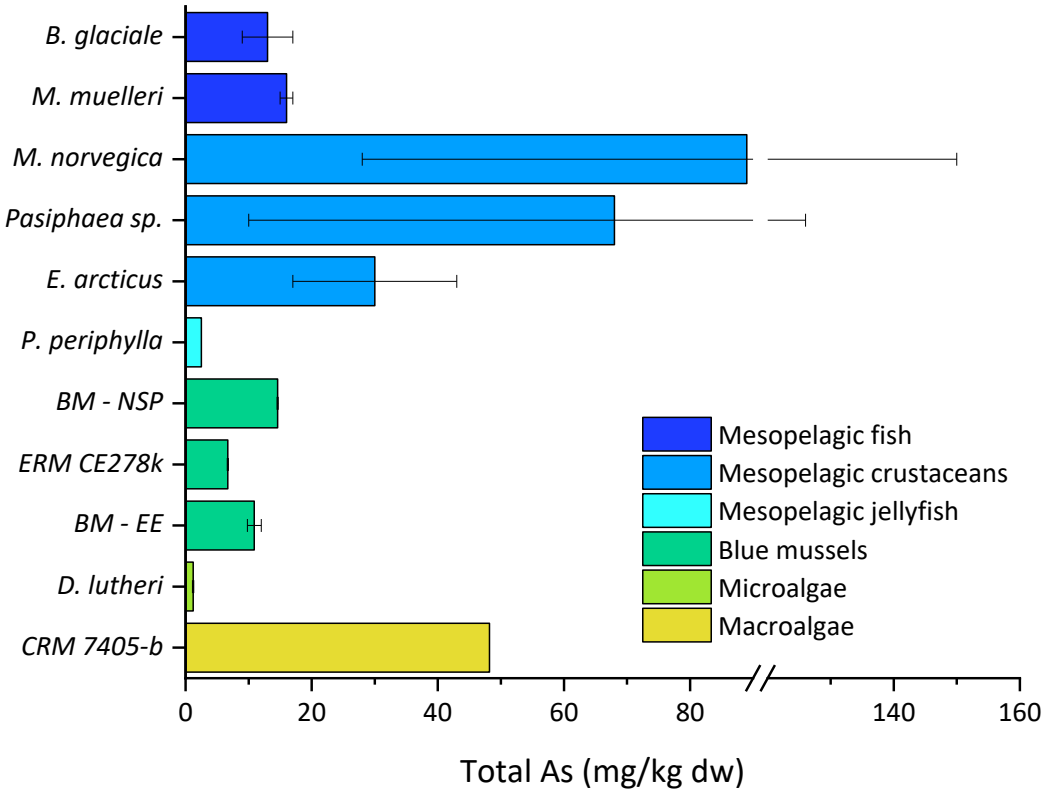

Fig. 12. Levels of total arsenic (mg/kg dw) in novel marine feed resources.

Mesopelagic hauls are usually composed of biomass containing a mixture of different species [7, 53], and their utilization as an aquafeed raw material will most likely be as biomass [55]. Hence, it would be more relevant to determine tAs levels in biomass samples instead of individual species. In **Paper II**, the average tAs level in mesopelagic biomass composed of *M. muelleri* and *M. norvegica* was 11.4 mg/kg dw, equivalent to 3.0 mg/kg ww. The result is comparable to tAs concentrations in mesopelagic hauls from the Mid-Atlantic Ridge, ranging from 1.2 to 7.2 mg/kg ww [53]. The relatively high tAs levels for some of the biomass were attributed to hauls consisting mainly of krill. Similarly, an earlier study involving mesopelagic biomass sampled off Norwegian fjords reported tAs concentrations between 15 and 30 mg/kg dw, and elevated tAs levels were also linked to high proportion of crustaceans in the haul [7]. The wide variation in tAs among individual species, as seen in **Paper IV**, will result to inconsistent and unpredictable tAs levels in mesopelagic hauls. It was discussed in **Paper IV** that species-specific data can be used in estimating the levels of undesirable substances in a

haul provided that the catch composition is known. However, this is far from reality as mesopelagic hauls are often comprised of several organisms. To have better control with the level of As (and undesirable substances, in general) in mesopelagic biomass, selective trawl systems are necessary to capture only the species of interest, but this would require further investments [53].

The blue mussel samples had similar tAs levels as mesopelagic fish but were remarkably lower than mesopelagic crustaceans (Fig. 12). Pooled blue mussel samples from the Norwegian surveillance program had a tAs concentration of 14.6 mg/kg dw, while the blue mussel ERM CE278k had a tAs level of 6.7 mg/kg dw (**Paper I**). In **Paper III**, the blue mussels from the control group had tAs concentrations ranging from 9.5 to 12.2 mg/kg dw, equivalent to 1.8 to 2.3 mg/kg ww. Results are comparable to blue mussels collected along the Norwegian coast from 2004 to 2006, where tAs levels were generally below 3.5 mg/kg ww [48]. However, in the same study, an extremely high value of 13.8 mg/kg ww was also obtained in one of the samples. The variation in tAs levels was attributed to microalgae as part of the mussels' diet, which tends to vary depending on the location and season [48]. In another study, elevated levels of tAs in blue mussels from a contaminated marine harbor were correlated with the extent of contamination in the area [117]. Spatio-temporal variations of metals in mussels have been extensively studied [49, 50, 268]. Due to their sessile nature, high bioaccumulation ability, and limited biotransformation capacity, mussels are used as environmental sentinels, acting as bioindicators for monitoring of several pollutants (e.g. Mussel Watch programs) [252, 269]. The same characteristics, however, pose challenges to the use of blue mussels as aquafeed ingredients. To minimize the accumulation of undesirable substances, careful site selection is necessary [243].

Among the novel marine feed resources investigated in this work, microalgae had the lowest As content (Fig. 12). In **Paper III**, the tAs concentration of *D. lutheri* from the control group was only 1.2 mg/kg dw. Microalgae species which are intended as animal feed ingredient generally have low tAs levels [270-272]. Microalgal biomass of *Chlorella vulgaris*, *Nannochloris bacillaris*, *Tetracystis* sp., and *Micractinium reisseri* isolated from freshwater samples had tAs concentrations below quantification limits (<0.5 mg/kg) [270]. Similarly, *Spirulina* sp. had tAs levels less than 0.01 mg/kg [271], while *Tetraselmis* sp. grown in

industrial photobioreactors contained less than 0.8 mg/kg [272]. In contrast, whole cells and co-product of *Nannochloropsis oculata*, which were used to replace fishmeal in the diet of Nile tilapia, had tAs concentrations of 5.9 mg/kg and 0.2 mg/kg, respectively [255]. Even with slightly higher tAs level in whole cells, the inclusion of *N. oculata* in experimental diets yielded tAs concentrations which were below detection limits both in the formulated feed and fillet of Nile tilapia after 84 days of feeding [255]. Due to their bioactive components, microalgae-based products are commonly used as food supplements [273]. Hence, elemental composition data in literature are more available for microalgae as functional food to assess product safety [273, 274]. A study which compared *C. reinhardtii* with commercially purchased *Chlorella* and *Spirulina* powder reported tAs concentrations of 0.02 mg/kg dw, 0.85 mg/kg dw, and 0.89 mg/kg dw, respectively [274]. Another study which determined essential and toxic elements in *Chlorella*- and *Spirulina*-based food supplements generally found tAs concentrations less than 0.1 mg/kg [273]. Aside from human consumption, these microalgae species, i.e. *C. reinhardtii*, *Chlorella* and *Spirulina*, are also used in animal feed formulations [2, 243].

In comparison to microalgae, more occurrence data are available in literature for elemental composition in macroalgae, primarily due to their inclusion in the human diet and superior ability to bioaccumulate elements [270, 275]. In the EU, monitoring data on their levels have also been requested to support risk assessment and possible establishment of MLs [276]. In recent years, macroalgae have also been considered as a novel aquafeed ingredient because of their nutritional value and metabolites (e.g. peptides, fatty acids, phlorotannins, carotenoids) which are beneficial for fish health [262, 277, 278]. In **Paper I**, CRM 7405-b had a tAs concentration of 48.2 mg/kg dw, which is in agreement with the certified value. In a study involving 180 seaweed samples from South Korea, hijiki had the highest tAs concentration at 145 mg/kg [113], which was also observed in another report [275]. While hijiki is not commonly used as a feed ingredient, other macroalgae species currently explored as novel feed resources were reported to contain similarly high levels of tAs [279]. *L. digitata* harvested along the coast of Norway had a tAs concentration of 120 mg/kg dw [261]. Hijiki and *L. digitata* are classified as brown algae, which are known to accumulate high levels of As [261, 280]. Red and green algae have lower tAs concentrations [113, 261, 275]. The bioaccumulation ability of macroalgae appears to be species- and origin-specific [262, 277].

Thus, identifying the most suitable species and cultivation site should be among the primary considerations when harnessing macrolagae as feed ingredient.

4.2.2 Arsenobetaine

In most marine organisms, AB is acknowledged as the predominant As species, representing at least 70% of tAs [109]. This was verified in **Paper I**, where AB comprised 76% to 82% of tAs in several marine CRMs. In novel marine feed resources, levels of AB varied widely (Fig. 13). Mesopelagic crustaceans had average AB concentrations between 18.1 and 71.2 mg/kg dw, though large standard deviations were noted, suggesting considerable variation within individual species (Fig. 13 and **Paper II**). Mesopelagic fish had more consistent AB levels, ranging from 6.4 to 6.7 mg/kg dw (Fig. 13 and **Paper II**). AB concentrations in the pooled blue mussel sample from the Norwegian surveillance program and the ERM CE278k were 6.46 mg/kg dw and 2.24 mg/kg dw, respectively (**Paper I**). Despite having higher tAs concentrations than ERM CE278k, blue mussel samples from the As exposure experiment only had an average AB concentration of 1.9 mg/kg dw (**Paper III**). Concentration of AB was positively correlated with tAs in mesopelagic organisms (Fig. S1 in **Paper II**), which was also noted in an earlier study on different seafood [94]. However, the same was not observed in blue mussel samples in **Paper III**.

In **Paper II**, AB in mesopelagic crustaceans and fish accounted for approximately 70% and 50% of tAs, respectively. The difference in proportions of AB was linked to the organisms' feeding habits (**Paper II**). For the blue mussels, AB only comprised 18% to 45% of tAs (**Papers I, II, and III**). AB usually represents 30% of tAs in blue mussels [281], though lower percentages (i.e. 4% to 21%) were obtained in mussels from a contaminated area, where iAs was the most abundant species [117]. Contrastingly, AB was not detected in the microalgae *D. lutheri* (**Paper III**), which agrees with earlier studies on other microalgae species [144, 282]. Similarly, AB was not found in the hijiki CRM 7405-b (**Paper I**). Although AB was detected in some macroalgae in previous reports [106, 112, 113], its presence has been associated with epiphytes and bacteria which could be difficult to remove when cleaning the macroalgae samples [111].

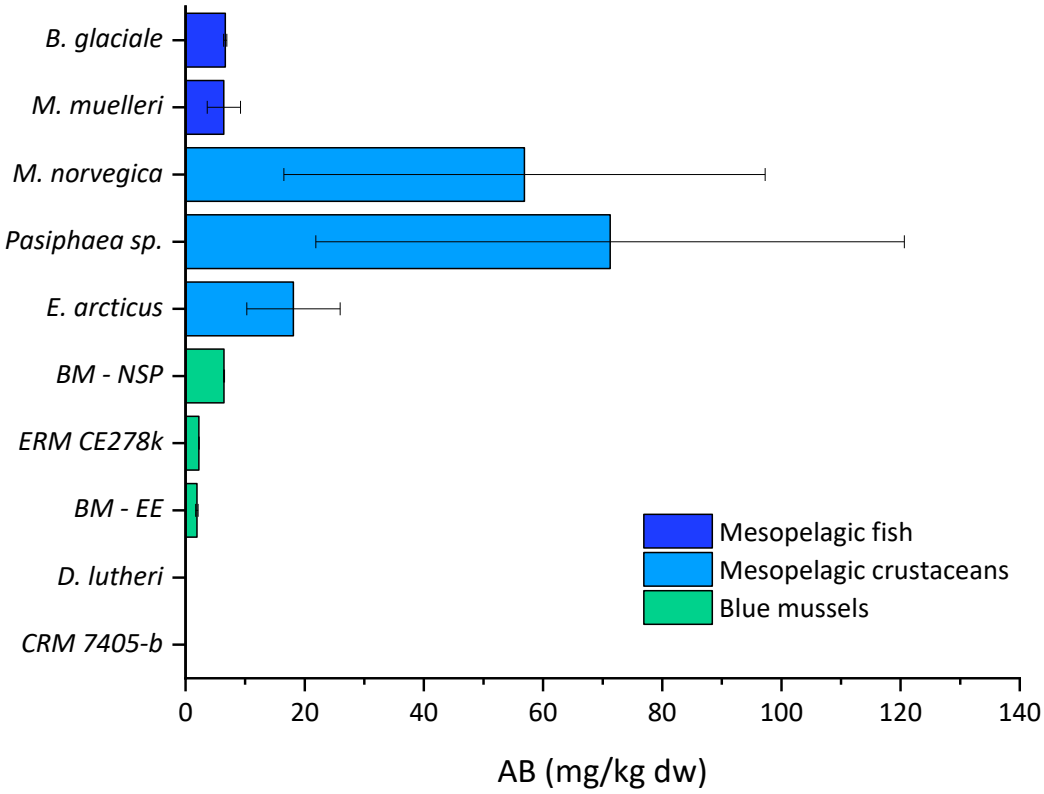

Fig. 13. Levels of arsenobetaine (AB; mg/kg dw) in novel marine feed resources.

4.2.3 Other methylated As species and arsenocholine

Methylated As species, e.g. MA, DMA, TMAO, TETRA, and TMAP (Table 1), are generally present in marine organisms in low levels [119]. In mesopelagic organisms and blue mussels, these compounds accounted for less than 10% of tAs (**Papers I, II, and III**). DMA was present in all mesopelagic organisms, where the highest level was found in the fish species *M. muelleri* at 0.80 mg/kg dw, comprising approximately 6% of tAs (Fig. 14 and **Paper II**). The rest of the mesopelagic organisms had DMA levels which were one magnitude lower (Fig. 14). MA was only detected in *M. muelleri* and the decapods *Pasiphaea* sp., and levels were close to LOQ (Fig. 14). TMAO was more abundant in mesopelagic fish species (Fig. 14 and **Paper II**). In contrast, TMAP was present at higher concentrations in mesopelagic crustaceans (Fig. 14), i.e. between 0.14 and 0.58 mg/kg dw (**Paper II**), which corroborate the levels found in shrimp in another study [94]. AC was present at slightly higher concentrations than majority of the simple methylated species in mesopelagic organisms, while TETRA was mostly below LOQ (Fig. 14 and **Paper II**).

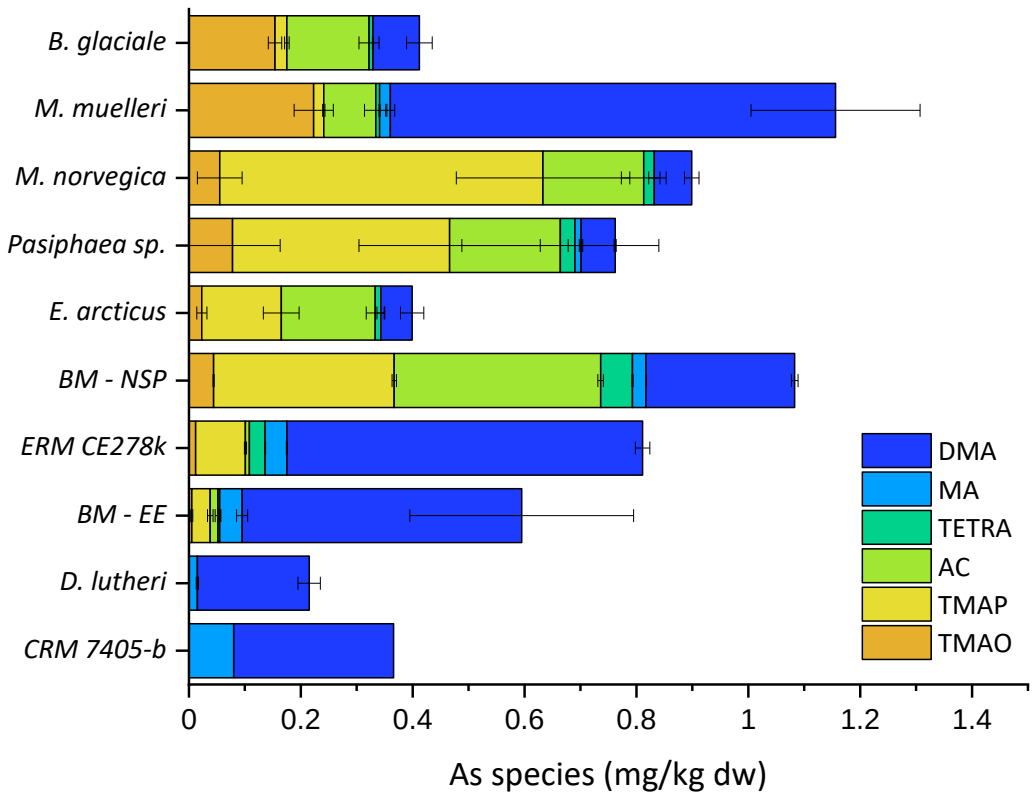

Fig. 14. Levels of methylated arsenic species (mg/kg dw) in novel marine feed resources.

In blue mussels, DMA concentrations were within the range of 0.27 to 0.66 mg/kg dw (**Papers I and III**). In contrast to mesopelagic organisms, MA in blue mussels were present in quantifiable concentrations, ranging from 0.02 to 0.04 mg/kg dw (**Papers I and II**). TMAO, TMAP, and TETRA were generally detected in trace levels (**Papers I and III**), though the pooled blue mussel sample from the Norwegian surveillance program contained a relatively high amount of TMAP (0.32 mg/kg dw) (Fig. 14 and **Paper I**). Similarly, AC was quite prominent in the pooled blue mussel sample (0.37 mg/kg dw), whereas concentrations were mostly below LOQ in ERM CE278k and blue mussels from the As exposure experiment (Fig. 14 and **Papers I and III**).

The microalgae *D. lutheri* had a totally different As species profile compared to mesopelagic organisms and blue mussels (Fig. 14). Among the methylated As species, only MA and DMA were detected (Fig. 14 and **Paper III**). This agrees with findings in earlier studies on *D. tertiolecta* [144] and *T. pseudonana* [282]. The control group of microalgae (i.e. cultivated in f/2 medium-enriched seawater) in **Paper III** had DMA and MA concentrations of 0.20 and 0.02 mg/kg dw, accounting for 17% and 1% of tAs, respectively. In the hijiki CRM 7405-b, the

anionic species DMA and MA were present as minor components (<1% of tAs) (Fig. 14 and **Paper I**). The organic As species TMAO, TMAP, AC, and TETRA were not detected in hijiki, similar to *D. lutheri* (Fig. 14 and **Paper III**). The results are in agreement with another study which analyzed the same CRM [98]. In a previous report, trace levels of TMAP, TMAO and AC were found in few samples of hijiki, dulse, kombu, and laver, but TETRA remained undetected [98].

4.2.4 Arsenosugars

Arsenosugars are commonly found in macroalgae, though they can also be present in mollusks and crustaceans [119]. Both mesopelagic crustaceans and fish contained AsSug OH, with levels ranging from 0.14 to 0.62 mg/kg dw (Fig. 15 and **Paper II**). It is the second most abundant water-soluble As species next to AB in *B. glaciale*, *M. norvegica*, and *E. arcticus*, though only comprising at most 2% of tAs in *B. glaciale*. AsSug PO4 was found in all samples except *B. glaciale*, while AsSug SO3 was only detected in *E. arcticus*, albeit at very low concentration (Fig. 15 and **Paper II**). Contrastingly, AsSug SO4 was not seen in any of the mesopelagic organisms (Fig. 15 and **Paper II**).

In blue mussels, AsSug PO4 was the predominant AsSug (Fig. 15), representing up to 13% of tAs, though slightly lower percentage was noted in ERM CE278k (~4%) (**Papers I and III**). Pooled blue mussel samples from the Norwegian surveillance program and blue mussels from the As exposure experiment had comparable AsSug OH concentrations (Fig. 15), between 0.5 to 0.7 mg/kg dw (**Papers I and III**). AsSug SO3 was also detected in trace levels in the two sets of samples, while it was not found in ERM CE278k (Fig. 15). Similar to mesopelagic organisms, AsSug SO4 was not detected in blue mussels from the As exposure experiment (**Paper III**) and ERM CR278k (**Paper I**), though it was present in minor amounts in the pooled blue mussel samples from the Norwegian surveillance program (**Paper I**) (Fig. 15).

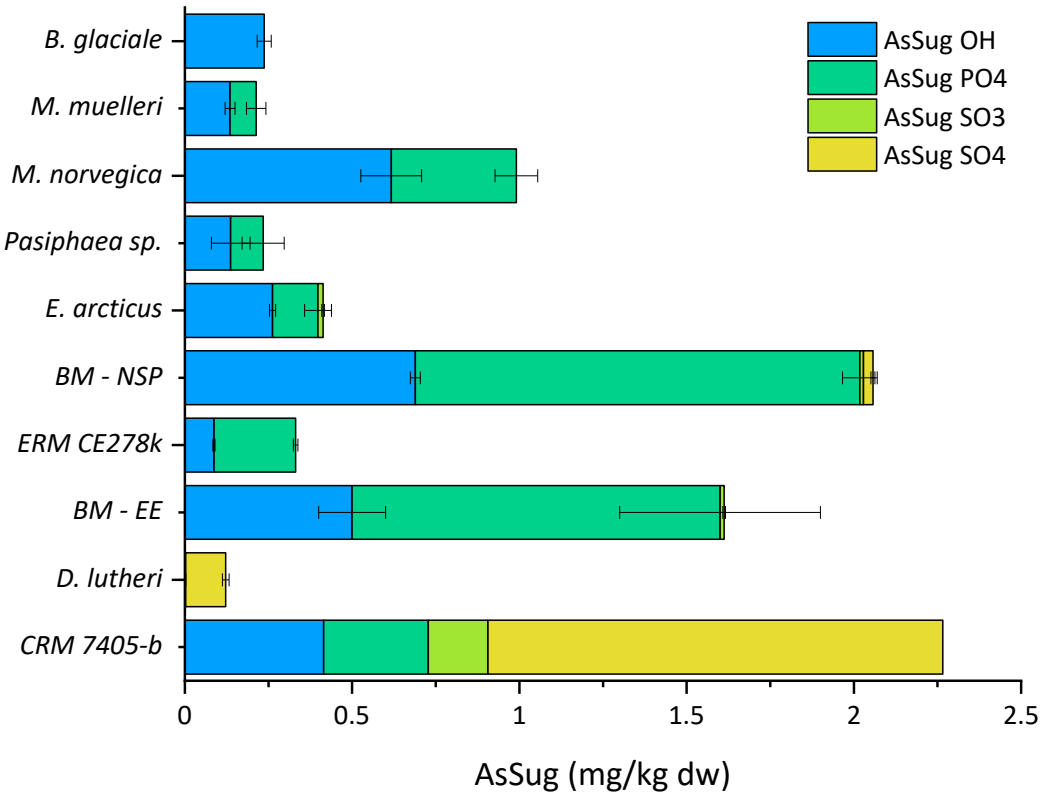

Fig. 15. Levels of arsenosugars (AsSug; mg/kg dw) in novel marine feed resources.

The microalgae *D. lutheri* from the control group contained AsSug SO4 (Fig. 15), accounting for 12% of tAs, while AsSug OH was below LOQ (**Paper IIII**). In contrast to mesopelagic organisms and blue mussels, AsSug PO4 and AsSug SO3 were not detected in *D. lutheri* (Fig. 15 and **Paper III**). In the macroalgae CRM 7405-b, AsSug SO4 was the second most abundant water-soluble As species (Fig. 15), next to iAs, comprising approximately 3% of tAs (**Paper I**). The prevalence of AsSug SO4 in hijiki samples was also observed in another study, where three samples of hijiki from Korea and Japan were analyzed alongside other types of macroalgae [98]. The occurrence of AsSug tends to vary across different taxonomic groups of macroalgae [106], e.g. AsSug OH was abundant in wakame and Irish moss, while AsSug SO3 was most prevalent in oarweed, kombu, and arame [98]. AsSug PO4 was the major AsSug in dulse, laver, and nori [98]. The proportion of AsSug also varied, ranging from 2% to 84% of tAs [98]. The variable accumulation of AsSug has been attributed to inherent genetic and enzymatic differences in biotransformation capabilities of macroalgae species [98], though local growing conditions and season may affect as well [283]. Likewise, the occurrence of AsSug in microalgae depends on several factors, such as the type of microalgae and culture conditions [125, 282, 284].

4.2.5 Arsenolipids, unextracted arsenic, and unknown As compounds

Lipid-soluble As was determined in **Paper II** based on a modified extraction procedure by Freitas et al. [130] followed by tAs determination of the lipid fraction. Mesopelagic crustaceans had higher and more varying AsLipids concentrations, ranging from 7.1 to 23.5 mg/kg dw, while the fish species had similar levels (both at 4.3 mg/kg dw) (**Paper II**). Proportion-wise, lipid-soluble As accounted for 33% to 37% of tAs in the fish species, whereas it was approximately 20% for the crustaceans (**Paper II**). *B. glaciale* and *M. muelleri* had fat contents which were two to three times higher than mesopelagic crustaceans, which could account for the higher fraction of AsLipids.

In blue mussels from the As exposure experiment (**Paper III**), a large portion of the As was unextracted, approximately 50%. In comparison, the pooled blue mussel samples from the Norwegian surveillance program and ERM CE278k had extraction efficiencies of 74% and 66%, respectively (**Paper I**). Similar extraction efficiencies in blue mussels were reported in earlier studies [117, 285, 286]. Since the method employed was mainly devised to target water-soluble As species (Chapter 3/**Paper I**), it can be presumed that AsLipids comprised the unextracted As. AsLipids were detected in *Mytillus galloprovincialis* [130], a close relative of blue mussels.

The microalgae *D. lutheri* from the control group (**Paper III**) also had a high fraction of unextracted As, representing 49% of tAs. It is likewise possible that AsLipids comprised the unextracted part since *D. lutheri* was reported to contain 29% lipids [287], and AsLipids in some microalgae species accounted for at least 50% of tAs [288]. Alternatively, it may be the case that some As was bound in the cells, which necessitates a more aggressive extraction approach, as also observed in previous reports [144, 289]. In the macroalgae CRM 7405-b, an extraction efficiency of only 57% was obtained (**Paper I**). In a study on several commercially purchased seaweeds in the USA, extraction efficiency ranged from 25% to 101% [98]. The unextracted fraction was linked to As attached to cell components and proteins [98]. Microalgae and macroalgae are structurally unique matrices due to the presence of cellular components (e.g. cell wall), which make it difficult to efficiently extract As [98]. Harsher

extraction conditions may be employed, but this may lead to degradation/conversion of As species to other forms [33].

Unknown As-containing peaks were detected in the studied matrices except for microalgae (Fig. 6 in section 3.3.3; **Papers I, II, and III**). In **Paper I**, as many as 17 unknown As peaks were noted in different CRMs. In blue mussel alone, 8 unknown As peaks were detected (**Paper I**). A notable unknown As compound was observed to be strongly retained in the anion-exchange column, eluting at a RT close to 19 min (Fig. 6 in section 3.3.3). The compound was chromatographically well-resolved from the nearest eluting peak (AsSug SO4), so it was easy to isolate and collect the compound through chromatography. An attempt to characterize/identify this unknown compound was done by injecting the isolated extract in LC-HRMS (Orbitrap-MS), however, no clear signal was obtained. It may be the case that the isolated extracted needed additional pre-concentration to produce more intense signals in the Orbitrap-MS. Earlier studies have also reported strongly anionic As compounds in mussels, and these were described as thio-analogues of AsSug [290, 291]. It is possible that one of these thio-AsSug is responsible for the prominent unknown peak detected in blue mussels in **Papers I and III**.

4.2.6 Inorganic arsenic

In mesopelagic organisms, iAs was present in trace levels, mostly below LOQ (**Paper II**). The highest iAs concentration was found in *M. norvegica* at 0.25 mg/kg dw. In blue mussels, iAs levels were similar at approximately 0.4 mg/kg dw, though one of the samples contained 2.2 mg/kg dw (**Papers I and III**). Other studies also reported unusually high levels of iAs in blue mussels from Norwegian fjords (as much as 5.8 mg/kg ww) ([48] and *Paper A*) and from a contaminated harbor (33 mg/kg dw) [117]. In the study by Sloth et al. [48], iAs was positively correlated with tAs. However, this was not observed in blue mussels analyzed in **Papers I and III**.

The highest concentration of iAs (24.3 mg/kg dw) was seen in hijiki CRM 7405-b (**Paper I**). Hijiki and other *Sargassum* species are known to accumulate high levels of iAs [98, 107, 113, 124, 263]. Cultivated oarweed was also reported to have high iAs concentrations [263]. In

contrast, other types of macroalgae such as dulse, kombu, laver, nori, and wakame had relatively lower iAs levels [98, 112, 113]. The varying prevalence of iAs in macroalgae has been generally attributed to taxonomic differences in biotransformation abilities [292], though other external factors such as local environmental conditions may also contribute [98]. Similarly, iAs was the predominant water-soluble As compound in the microalgae *D. lutheri* from the control group at 0.26 mg/kg dw, accounting for 22% of tAs (**Paper III**). However, iAs levels tend to be higher when microalgae are exposed to higher As concentrations [125, 293], as demonstrated in **Paper III** where iAs concentration increased eight-fold when *D. lutheri* was exposed to 10 µg/L As(V).

4.3 Bioaccumulation and biotransformation of As species in low-trophic marine organisms

The As speciation data in novel marine feed resources suggest that aside from species-specific differences in bioaccumulation and biotransformation capabilities, the occurrence of As species is primarily dictated by their diet/feeding habits and the local environmental/culture conditions.

4.3.1 Arsenic speciation as influenced by the diet

In **Paper II**, the difference in tAs levels between mesopelagic crustaceans and fish can be linked to their diet preferences. The fish species, *B. glaciale* and *M. muelleri* mainly rely on zooplanktons such as copepods, amphipods, and krill [294]. The crustaceans have more diverse food options, as they are omnivores, and may scour the benthic zone in search of copepods and phytoplanktons (Fig. 16), which make up the majority of their diet [295]. Microalgae contribute to the phytoplankton biomass in the world's oceans [296], and as seen in **Paper III**, they can accumulate As. The preference of mesopelagic crustaceans for phytoplanktons as food [295] may partly explain the higher tAs levels compared to mesopelagic fish. Similarly, the prevalence of AB in crustaceans (**Paper II**) is supported by another study wherein the shrimp *Crangon crangon* retained almost half of the AB acquired through diet-borne exposure [297]. The relatively lower levels of AB in mesopelagic fish may be due to their dependence on copepods [294], which was reported to contain only trace

amounts of AB [298]. Copepods were reported to contain significant levels of AsSug SO4 [298]. However, AsSug SO4 was not detected in mesopelagic fish (**Paper II**) even though copepods were part of their diet [294], suggesting possible biotransformation. Another possibility is that AsSug SO4 was excreted unchanged. To the best of my knowledge, no study has, so far, investigated AsSug uptake, metabolism, accumulation, and excretion in fish. At least for mammals, AsSug are converted to different metabolites after ingestion, as seen in studies involving human and sheep after seaweed consumption [123, 299, 300]. For mesopelagic crustaceans, the differing prevalence of AsSug may be due to variation in phytoplanktons as food source. Phytoplanktons, such as microalgae, are known to have significant variation in AsSug due to taxonomic differences [125]. In **Paper III**, the microalgae *D. lutheri* was found to contain notable proportions of AsSug SO4. However, after feeding the blue mussels with *D. lutheri* for 25 days (**Paper III**), AsSug SO4 was not detected in any of the blue mussels, suggesting possible biotransformation. AsSug PO4 was the most abundant AsSug in blue mussels, yet their diet (*D. lutheri*) did not contain the same AsSug. In contrast, it may be the case that the large fraction of unextracted As in blue mussels (possibly AsLipids) was transferred from *D. lutheri*, which was also noted to contain significant proportions of unextracted As (**Paper III**). *D. lutheri* has been reported to have high lipid content [287], which has been correlated to presence of AsLipids [25, 99].

In **Paper III**, blue mussels were fed with As(V)-exposed *D. lutheri* (diet-borne As exposure). However, there were no significant differences both between groups and within groups (Tukey test; $p < 0.05$). Using linear mixed models, it was discovered that As concentrations were decreasing through time, resembling a depuration process (**Paper III**). It may be the case that the blue mussels were exposed to As sources with higher concentrations in their natural environment. But when they were relocated to the experimental tanks, the As concentrations in *D. lutheri* might have still been considerably less than what was available to the blue mussels in their previous habitat.

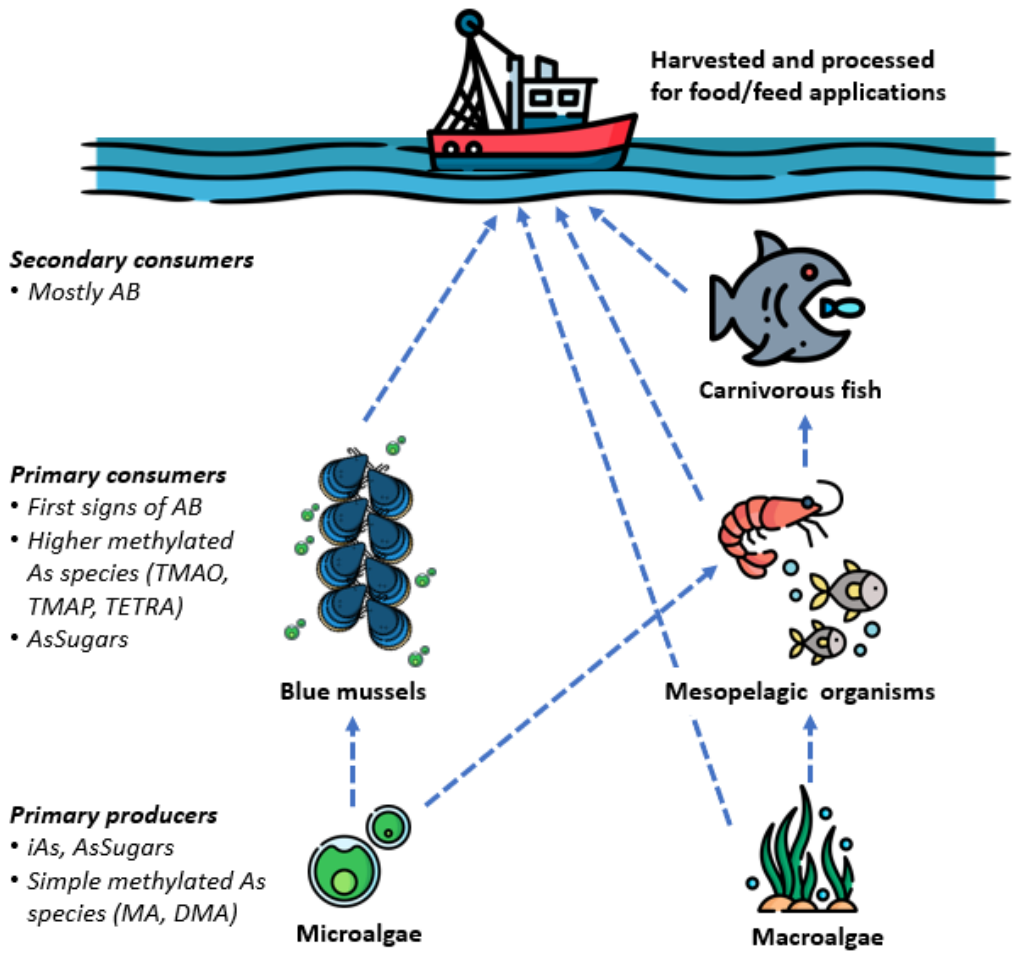

Fig. 16. Example of a low-trophic marine food chain showing predator-prey relationships and the occurrence of arsenic species in each trophic level.

Based on the preceding discussions, the trophic position of an organism largely influences the As speciation. For those at the bottom of the marine food chain, e.g. primary producers such as micro- and macroalgae (Fig. 16), the main As compounds include iAs, AsSug, and simple methylated As species (i.e. MA and DMA) (Fig. 17). For primary consumers such as mesopelagic organisms and blue mussels, AB is detected and becomes more prevalent (Fig. 16 and 17). Higher methylated As species (e.g. TMAO, TMAP, TETRA) and AsSug are also present as minor components (Fig. 17). From Fig. 17, the unextracted fraction, which is possibly comprised of AsLipids, also seems to decrease in higher-trophic animals. One key takeaway from these findings is that, when harnessing low-trophic marine organisms as aquafeed resources, As speciation will vary in the resulting feed material. In contrast to traditional fish meals which utilize forage fish where AB is the predominant As species [215], fish meals derived from low-trophic marine resources will likely comprise less AB and more of AsSug , AsLipids, and other methylated As species.

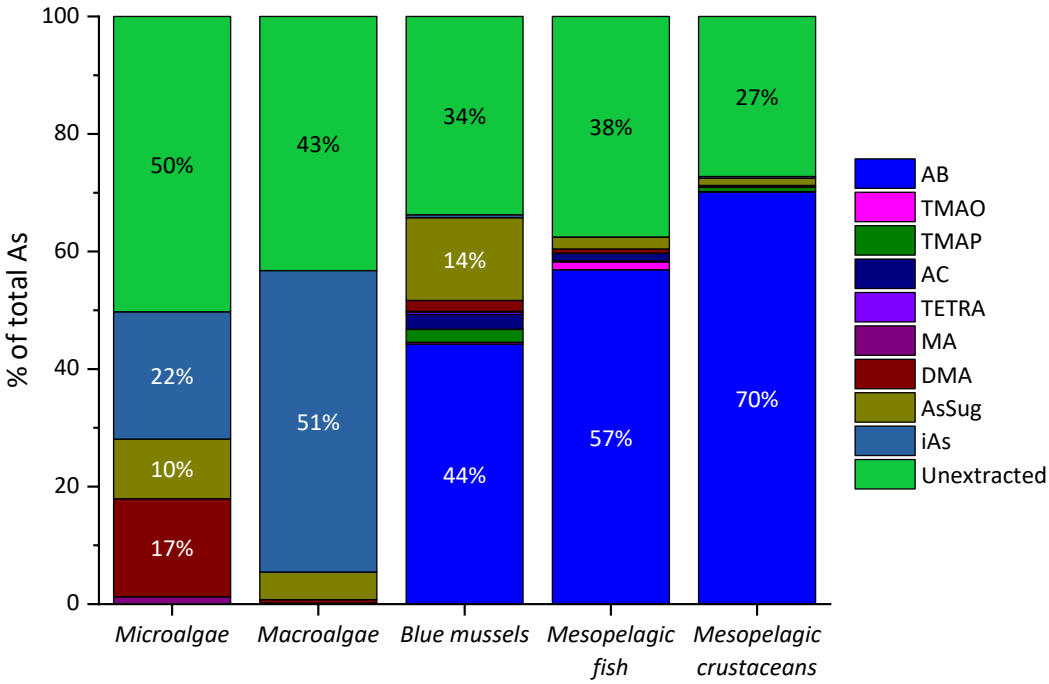

Fig. 17. Proportions of arsenic species in novel marine feed resources.

4.3.2 Arsenic speciation as influenced by culture/environmental conditions

Aside from the diet (food-borne), As uptake may also take place through the dissolved phase, e.g. via the gills (water-borne). In **Paper III**, blue mussels were exposed to approximately 5 µg/L As(V) in seawater. However, there were no significant differences both between groups and within groups, suggesting that As was not accumulated (**Paper III**). These results agree with a previous report where higher (20 times) exposure concentrations were employed [301]. Studies on other organisms which reported change in tAs levels after As exposure utilized unrealistically high As concentrations (up to 4000 times higher than the ones used in **Paper III**) which resulted to mortalities [302, 303]. In these reports, high levels of exposure resulted to accumulation of iAs as the predominant species. The idea behind the As exposure experiment in **Paper III** was to utilize As concentrations which mimic the natural marine environment (0.5 to 3 µg/L) [77, 78], thereby providing more useful insights on As bioaccumulation in uncontaminated areas.

Exposure to higher iAs concentrations in the culture media resulted to increased iAs levels in *D. lutheri* (**Paper III**), suggesting that methylation systems are overwhelmed [125]. The

methylation of As, as described by the Challenger pathway [139], is believed to be a detoxification mechanism by organisms to address the toxicity of iAs, which is the predominant species in seawater [101, 102]. In **Paper III**, exposure to higher iAs concentrations (i.e. 5 and 10 µg/L As(V)) led to higher levels of MA and DMA in *D. lutheri*. As seen in the matrices studied in this work, concentrations were always lower for MA than DMA (**Papers I, II, and III**). The production of MA and its biotransformation to DMA were reported to occur swiftly in *C. reinhardtii* [145], which supports the lower MA concentrations found in the current work (**Paper III**). At an exposure concentration of 10 µg/L As(V), it may be the case that the methylation threshold has been surpassed, resulting to similar DMA levels in *D. lutheri* exposed to 5 and 10 µg/L As(V) (**Paper III**). This was more evident for AsSug SO4 and AsSug OH, where significantly lower levels were obtained for *D. lutheri* exposed to 10 ug/L As(V) (**Paper III**). The microalgae *D. lutheri* did not contain other organic species such as TMAO, TMAP, TETRA, and AC (**Paper III**). Similarly, these compounds were not found in the macroalgae hijiki CRM 7405-b (**Paper I**). Lastly, the absence of AB in *D. lutheri* (**Paper III**) and the hijiki CRM 7405-b (**Paper I**) suggest that micro- and macroalgae only provide the precursors for AB formation in higher forms of aquatic animals [141].

4.4 Effects of feed processing on As speciation in novel marine feed resources

The effects of feed manufacturing techniques on nutrient composition, digestibility, and levels of undesirable substances should not be overlooked [241]. A recent study demonstrated that processing of novel marine resources modified the levels of undesirable substances in resulting feed products, e.g organic pollutants such as PCBs were up-concentrated in the oil fraction, while As was diluted in the meal fraction [55]. In **Paper IV**, it was estimated that current MLs for As in feed ingredients (Directive 2002/32/EC and amendments) will be exceeded when mesopelagic organisms are processed. This was based on a theoretical worst-case approach, and the assumption was As will end up completely with the protein fraction. However, it was subsequently verified that As partitioned both in the meal and oil products [55].

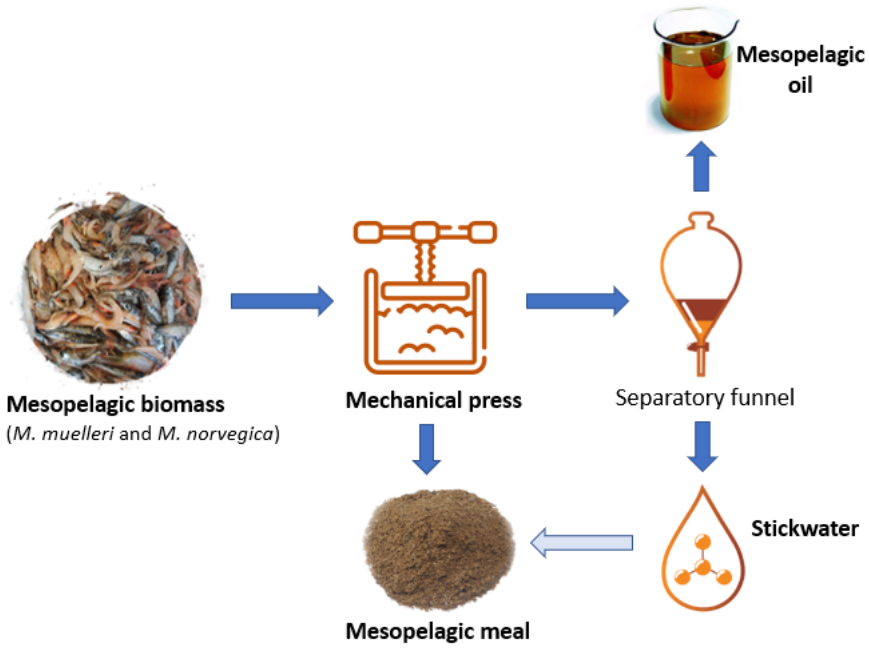

Fig. 18. Schematic diagram of the lab-scale feed processing study wherein mesopelagic biomass was processed into mesopelagic meal and oil.

In **Paper II**, several As species were studied in-depth following the processing of mesopelagic biomass into feed products (Fig. 18). The starting mesopelagic biomass (mixture of *M. muelleri* and *M. norvegica*) was mainly composed of AB, representing 57% of tAs. However, AB in the resulting mesopelagic meal only comprised 32% of tAs (**Paper II**). This is a marked contrast to AB in traditional fish meals, where AB comprised 71% to 93% of tAs [215]. It was lipid-soluble As that made up majority of the tAs in mesopelagic meal (45% of tAs) (**Paper II**), which was unexpected since AsLipids can be presumed to have affinity with the oil phase based on their lipophilic properties. Since a lab-scale feed processing setup was used, it is possible that the conditions were not able to replicate industrial settings where high-pressure extruders are employed with controlled manufacturing parameters (e.g. pressure, temperature). This could have resulted to less efficient extraction, causing a large portion of oil to remain with the meal. Lipid-soluble As was the predominant species in mesopelagic oil (96% of tAs), while AsSug were only present in mesopelagic meal and stickwater (**Paper II**). Minor compounds (i.e. MA and AsSug SO3) initially present in the mesopelagic biomass were below LOQ in the resulting fractions, suggesting possible degradation or transformation to other forms. Lastly, iAs levels in the resulting feed products were all below LOQ (**Paper II**).

Similarly, microalgal cultivation techniques can alter elemental concentrations in the resulting biomass. Studies have reported that the use of flue gas from coal combustion as a source of CO_2 resulted in increased levels of heavy metals [259, 304]. The use of chemical flocculants when harvesting microalgae may also introduce undesirable substances [305], while further processing techniques to improve protein digestibility (e.g. pasteurization, chemical treatments) may also alter the final concentrations of undesirable substances [257]. For macroalgae, simple processes such as washing, soaking, and cooking were noted to reduce the heavy metals content [243]. Noticeably, processing studies on microalgae and macroalgae have mostly accounted total elemental concentrations. Studies on processing effects on As speciation in these matrices (similar to what was done in **Paper II**) provides more details on the fate of As species in the final products, which is relevant information considering the varying toxicities of As species.

4.5 Feed safety

The discussion in this section is under the context that the novel marine feed resources will be used as aquafeed ingredients in the EU. Similar to the MLs stipulated in Directive 2002/32/EC and amendments, all concentrations mentioned in this section are expressed as 88% dry matter.

The utilization of novel marine resources will most likely be as feed materials. Based on Directive 2002/32/EC and amendments, MLs of 25 mg/kg and 10 mg/kg apply to feed materials and complementary/complete feed, respectively, when using mesopelagic organisms and blue mussels. Levels of tAs in individual species of mesopelagic crustaceans (i.e. *M. norvegica, Pasiphaea* sp., and *E. arcticus*) may exceed the MLs for feed materials (**Paper IV**). However, it is very unlikely that mesopelagic biomass consisting of a single species will be used as a feed ingredient. Hence, it is more relevant to assess the tAs levels in mixed mesopelagic biomass. In **Paper II**, the mixed mesopelagic biomass had an equivalent tAs concentration of 10 mg/kg, which is well below the 25 mg/kg ML for feed material. However, this can be problematic if included in complementary/complete feed. Nonetheless, mesopelagic biomass will most likely still be processed into mesopelagic meal prior to utilization. In **Paper II**, a dilution effect in tAs was noted in the resulting mesopelagic meal,

with an equivalent tAs concentration of 5.5 mg/kg. This is well within the MLs for both feed material and complementary/complete feed. Similarly, the pooled blue mussel sample analyzed in **Paper I** and some of the blue mussel samples in **Paper III** will comply with the ML for feed material. For macroalgae (seaweeds), an ML of 40 mg/kg and 10 mg/kg is applicable when used as feed material or included in complementary/complete feed, respectively. A different ML is applicable for calcareous marine algae (10 mg/kg), though none of the matrices in the current work fall under this category. Presently, there are no specified MLs imposed for tAs in microalgae as animal feed. In terms of iAs, all novel marine feed resources covered in this project are below 2 mg/kg. If required by authorities, it should be demonstrated that the iAs level in feed material is below 2 mg/kg [11]. Aside from iAs, there are currently no MLs for other As species.

Outside the EU, regulations on the use of macro- and microalgae as feed components vary between countries [277, 305]. Aside from interspecies variability, the prevailing environmental conditions in the cultivation site of macroalgae can cause significant differences in tAs levels [262, 277, 279]. Similarly, culture conditions and local site parameters in growing microalgae and blue mussels must be monitored to prevent accumulation of As and its species [259, 305]. As demonstrated in **Paper II**, it should be emphasized that levels of As and its species may change after processing [243, 279]. In addition, there is limited data on bioavailability of As in fish when novel marine feed resources are incorporated in feed formulations [259, 277]. From preceding discussions, low-trophic marine organisms contain significant proportions of AsSug and AsLipids, which are regarded as potentially toxic compounds [23, 24, 27, 160, 161]. Considering the varying toxicities of the different species, EFSA recognizes the need for more As speciation data (e.g. AsLipids, AsSug, methylated As species, thiolated iAs) for future risk assessment [30, 31]. This is particularly important since novel marine feed resources have different bioaccumulation and biotransformation capabilities, resulting to wide variation in As species profile (**Paper III**). The possible accumulation of these As species in the final fish product when novel marine feed resources are used should be investigated. Furthermore, an overall assessment of novel marine feed resources in regard to fish health, and consequently, food safety, is important.

CHAPTER 5: CONCLUSIONS

In this PhD project, analytical methods were developed for determining water-soluble As species in marine matrices (**Paper I**). The methods were subsequently applied to novel marine feed resources to document the occurrence of As species (**Papers II, III, and IV**). The uptake and biotransformation of As in low-trophic marine food chain was investigated by conducting exposure and feeding experiments involving microalgae and blue mussels (**Paper III**). The effects of feed processing on As speciation was also studied through a lab-scale set-up with mesopelagic biomass as the starting raw material (**Paper II**). The following conclusions were obtained:

Multivariate optimization proved to be an effective method development approach. The optimization of the extraction procedure using a 2^{7-3} fractional factorial design identified extraction temperature and type of extraction solution as significant factors. Non-significant factors, such as sample weight, extraction solvent volume, and extraction time, were maintained at their low-level settings to reduce chemical and energy usage. The optimum extraction parameters ensure high extraction yield while preserving the chemical state of As species, and concomitantly adhering to GAC principles.

The use of gradient-elution HPLC with Metrosep C6 and PRP-X100 as cation- and anion-exchange columns, respectively, allowed the separation of at most 33 known and unknown As species, which were quantified using ICP-MS. Analyte retention and chromatographic resolution were highly dependent on the mobile phase buffer and pH, as well as pKa values of the different As species. The addition of organic solvent in the mobile phase (3% MeOH and 0.5% ACN) improved the ionization of As and resulted to signal enhancement. Quantification was performed using external calibration curves generated from standard solutions. However, the lack of commercially available analytical standards for other As species (e.g. AsSug) is still seen as an issue which needs to be addressed. In this work, several unknown As compounds were detected, including a strongly retained anionic As species when injecting blue mussel extracts. The identification of these unknown As compounds need to be confirmed using HRMS.

A single-laboratory method validation was carried out using several marine CRMs representing different taxonomic groups. Overall, satisfactory method performance characteristics were achieved. However, most marine CRMs lack certified values for As species. The As speciation data generated in **Paper I** provide valuable information for qualitative and quantitative comparison of analytical data in future As speciation studies.

Novel marine feed resources varied widely in tAs content and As species. The mesopelagic crustaceans had the highest tAs levels (up to 160 mg/kg dw) while the microalgae *D. lutheri* contained the least As (1.2 mg/kg dw). The prevalence of As species varied depending on the trophic position of the organism. In primary producers such as microalgae and macroalgae, iAs, AsSug, and simple methylated species (i.e. MA and DMA) were most abundant. However, the unextracted fraction of tAs was quite high, comprising up to 50% of tAs. AB was not found in microalgae and macroalgae. For primary consumers such as blue mussels, AB was detected, accounting for approximately 30% of tAs. In addition to MA and DMA, higher methylated species such as TMAO, TMAP, and TETRA were also noted in minor concentrations in blue mussels. For mesopelagic organisms, AB was more predominant. TMAP and AC were seen in higher proportions in mesopelagic crustaceans than mesopelagic fish species. The unextracted fraction in mesopelagic fish was notable, accounting for approximately 40% of tAs. In general, the proportion of AsSug and simple methylated species seemed to decrease higher up the marine food chain, while proportion of AB increased. Primary producers, which are at the base of the aquatic food pyramid, do not contain AB but only the precursors (e.g. DMA, AsSug). These precursors are then metabolized by higher-trophic animals to form AB.

Aside from the organism's trophic position/feeding habits, its As speciation is also influenced by culture/local environmental conditions. The exposure of *D. lutheri* to 5 and 10 µg/L As(V) in f/2 medium-enriched seawater resulted to increased levels of iAs, DMA, and MA. However, at 10 µg/L, the DMA concentration was not significantly different than *D. lutheri* exposed to 5 µg/L. The AsSug levels were also notably lower. The results suggest that the detoxification mechanism is overwhelmed, i.e. the methylation threshold has been breached, which resulted to accumulation of iAs.

Processing mesopelagic biomass into feed ingredients was observed to alter the As content and speciation in the resulting meal and oil fractions. AB in mesopelagic meal only accounted for approximately 30% of tAs, whereas lipid-soluble As comprised 45% of tAs. The mesopelagic oil was mainly composed of AsLipids. An overall dilution effect was noted for tAs and most As species in mesopelagic meal and oil. However, the potentially toxic AsLipids were up-concentrated in mesopelagic oil.

In light of feed safety, among novel marine feed resources, individual species of mesopelagic crustaceans may exceed the MLs for As in feed materials. However, mixed mesopelagic biomass will likely be used and processed into feed ingredients. Thus, the resulting tAs in mesopelagic meal will be considerably lower and will comply with the applicable MLs. The levels of iAs were also generally low (or below LOQ). Low-trophic marine organisms contain notable proportions of AsSug and lipid-soluble As (AsLipids). Thus, harnessing these novel marine resources as feed ingredients will likely cause variation in As speciation compared to traditional feed raw materials, e.g. forage fish where AB is predominant. In this PhD project, it was demonstrated that potentially toxic As compounds, i.e. AsSug and AsLipids, can be transferred to the resulting feed products.

CHAPTER 6: FUTURE PERSPECTIVES

The limited time frame in which the PhD project was conducted presented several opportunities which can be explored for future work. Emphasis is also given on analytical challenges which need to be addressed to further advance the field of As speciation analysis.

Development of analytical methods for AsLipids determination

The methods developed in this PhD project were mainly targeted for water-soluble As species. Results showed that a significant fraction of the tAs was unextracted, especially for low-trophic marine organisms. Hence, to verify the composition of the unextracted fraction, future work should aim to optimize analytical methods for determination of AsLipids. While initial method development studies have been conducted in the past, it may be beneficial to apply multivariate optimization, e.g. for the extraction procedure, with the goal of using less solvent as possible yet maintaining a high extraction efficiency. At the same time, AsLipids analysis requires a special instrument configuration. Thus, it would also be interesting to test different columns and ICP-MS settings to optimize the chromatographic separation and ICP-MS response.

Identification of unknown As compounds by LC-HRMS

Several unknown As compounds were present in some matrices, most notably for blue mussels, where a strongly anionic compound eluting after AsSug SO4 was detected (RT: 19 min). An attempt to identify this compound was done by isolating the compound through chromatography and fraction collection, and then injecting the solution in an LC-HRMS instrument (Orbitrap). However, no conclusions could be drawn since no clear signal was obtained. For future work, the compound can again be collected by running series of injections, but this time, employing a pre-concentration step afterwards. This might produce stronger signals in the Orbitrap. Alternatively, flow-splitting can be explored, where the flow from the HPLC is split – one going to the ICP-MS and the other to HRMS, as employed in an earlier study [44]. This will provide elemental and molecular information of As species simultaneously. However, the proposed setup might pose logistical challenges since it will require the ICP-MS and HRMS to be situated close to each other, which is often not the case

in most laboratories. Similarly, the presence of buffers in the mobile phase from the chromatography step may affect the ionization in HRMS.

Analytical standards and certified reference materials

The lack of analytical standards and relevant CRMs with certified values for As species is still a major challenge in As speciation analysis. Low-trophic marine organisms contain significant proportions of AsSug, and even while AsSug have been studied for several decades, there are still no commercially available analytical standards for AsSug. Researchers have mostly relied on isolated AsSug (mostly from macroalgae) by other research groups. However, information on compound purity and stability are usually lacking. With the right demand, companies/laboratories working on chemical synthesis might be interested in commercial production of As species standards. This can only happen, though, if there is sustained interest in As speciation studies. Most available CRMs in the market have certified values for only a few As species (mostly AB; in rare occasions, DMA). Even without certified values, researchers should still strive to utilize relevant CRMs when available and publish As speciation data as these will help in analytical comparison in future studies. Also, it may be beneficial to pursue stronger collaboration between research groups performing As speciation analysis, as this may lead to possible ring/proficiency tests, which can then help in the establishment of reference values for As species.

As speciation data in a wider range of novel feed resources

The novel marine feed resources investigated in this project were limited to mesopelagic organisms, blue mussels, and microalgae. However, there are also on-going efforts to explore the potential of other marine resources such as copepods, Antarctic krill, polychaetes, and tunicates. Insect and bacterial meals have also been recently included in feed formulations. Future work should focus on gathering As speciation data in these novel feed resources, widening the scope to not just marine-derived but also other sources. The results will be useful for authorities in conducting risk assessment, both in terms of feed and food safety, and in potential establishment of MLs in future legislations. Currently, MLs only exist for tAs, with minor reference to iAs. With more studies highlighting the potential toxicity of some of the organoarsenic species (e.g. AsSug and AsLipids), it is possible that MLs for such compounds will be established in the future.

Studies on bioavailability and retention of As species in fish

Feed materials derived from novel marine resources are vectors for As transfer in fish. Since low-trophic marine organisms vary slightly in As speciation compared to e.g. forage fish as raw material for fish meal, their inclusion in feed formulations might lead to accumulation of potentially toxic As compounds (e.g. AsSug and AsLipids) in final fish products intended for human consumption, thereby introducing new risks in regard to food safety. Studies on bioavailability in fish and possible accumulation of these compounds in final fish products should be endeavored to gain solid basis for risk assessment in terms of feed and food safety. Concomitantly, a multi-sectoral study on potential ecological, environmental, and economic repercussions is necessary before low-trophic marine organisms can be fully utilized.

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APPENDICES

Paper I **Tibon, J.**, Silva, M., Sloth, J. J., Amlund, H., & Sele, V. (2021). Speciation analysis of organoarsenic species in marine samples: method optimization using fractional factorial design and method validation. Analytical and Bioanalytical Chemistry, 413(15), 3909–3923. https://doi.org/10.1007/s00216-021-03341-4

Paper II **Tibon, J.**, Amlund, H., Gomez-Delgado, A. I., Berntssen, M. H. G., Silva, M. S., Wiech, M., Sloth, J. J., & Sele, V. (2022). Arsenic species in mesopelagic organisms and their fate during aquafeed processing. Chemosphere, 302, 134906. https://doi.org/10.1016/j.chemosphere.2022.134906

Paper III **Tibon, J.***, Gomez-Delgado, A. I.*, Aguera, A., Strohmeier, T., Silva, M. S., Lundebye, A. K., Larsen, M. M., Sloth, J. J., Amlund, H., & Sele, V. Arsenic speciation in low-trophic marine food chain – an arsenic exposure study on microalgae (*Diacronema lutheri*) and blue mussels (*Mytilus edulis* L.) (in preparation)

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RESEARCH PAPER

Speciation analysis of organoarsenic species in marine samples: method optimization using fractional factorial design and method validation

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Abstract

Organoarsenic species in marine matrices have been studied for many years but knowledge gaps still exist. Most literature focuses on monitoring of arsenic (As) species using previously published methods based on anion- and cation-exchange high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS). These studies are often limited to few As species and/or only specific method performance characteristics are described. Most marine certified reference materials (CRMs) are only certified for arsenobetaine (AB) and dimethylarsinate (DMA), making it difficult to evaluate the accuracy of analytical methods for other organoarsenic species. To address these gaps, the main objective of this work was to develop and validate a method for speciation analysis of a broad range of organoarsenic species in marine matrices. Optimum extraction conditions were identified through a 2^{7-3} fractional factorial design using blue mussel as test sample. The effects of sample weight, type and volume of extraction solution, addition of H_2O_2 to the extraction solution, extraction time and temperature, and use of ultrasonication were investigated. The highest As recoveries were obtained by using 0.2 g as sample weight, 5 mL of aqueous methanol (MeOH:H_2O, 50% v/v) as extractant, extraction carried out at 90 °C for 30 min, and without ultrasonication. Anion- and cation-exchange HPLC-ICP-MS settings were subsequently optimized. The method detected a total of 33 known and unknown As species within a run time of 23 and 20 min for cation-exchange and anion-exchange, respectively. A single-laboratory validation was conducted using several marine CRMs: BCR 627 (tuna fish tissue), ERM-CE278k (mussel tissue), DORM-4 (fish protein), DOLT-5 (dogfish liver), SQID-1 (cuttlefish), TORT-3 (lobster hepatopancreas), and CRM 7405-b (hijiki seaweed). Method performance characteristics were evaluated based on selectivity, limits of detection and quantification, linearity, trueness, precision, and measurement uncertainty. This work proposes an extraction procedure which allowed satisfactory quantification of As species with low solvent and energy consumption, supporting "Green Chemistry" principles. The study also presents a new set of As speciation data, including methylated arsenic species and arsenosugars, in recently issued marine CRMs, which will be valuable for future speciation studies on As. This work is the first to report a total of 33 different As species in marine CRMs.

Keywords Arsenic speciation · HPLC · ICP-MS · Marine certified reference materials

Introduction

Marine organisms are known to accumulate arsenic (As) from their environment. The cycle usually starts with inorganic As (iAs) present in seawater, which is taken up by phytoplanktons and other organisms at lower trophic levels. These primary producers and consumers are preyed on by other marine animals, causing As to be transformed to organoarsenic species and biomagnified through the food chain [1]. Most monitoring studies report high total As concentrations in marine food products (8–22 mg/kg w.w.) [2], but only a small fraction (<1% of total As) exists as the toxic iAs [3] (sum of arsenite [As(III)] and arsenate [As(V)]). Some exceptions include hijiki (*Hizikia fusiforme*), a family of brown seaweed reported to have As(V) concentrations as high as 107 mg/kg d.w. [4], and blue mussels harvested from Norwegian fjords with

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unusual elevated levels of iAs (up to 5.8 mg/kg w.w.) [5]. Arsenobetaine (AB) is the predominant organoarsenic species found in most finfish and shellfish, typically accounting for more than 90% of the total As [6]. Seaweed is known to contain several arsenosugars (AsSug), as described in the analysis of edible algae samples [7]. Arsenolipids are prevalent in marine oils and fats [8] but were also reported in commonly consumed types of seafood [9]. Other methylated As species exist as minor components, with dimethylarsinate (DMA) being the most common [10]. Tetramethyl arsonium ion (TETRA) was observed to be the predominant species in some mollusks [11], while elevated levels of trimethylarsoniopropionate (TMAP) were found in crabs [2]. Trimethylarsine oxide (TMAO), methylarsonate (MA), and arsenocholine (AC) were observed in trace concentrations in most seafood [10]. The chemical structures of the most common As species can be found in an article by Luvonga et al. [11].

Based on the classification by the International Agency for Research on Cancer (IARC), iAs is a carcinogen, AB is generally regarded as non-toxic, while other methylated As species such as DMA and MA were classified as possibly carcinogenic [11, 12]. There are also discussions on the potential toxicity of arsenosugars and arsenolipids, with studies citing neurotoxic and cytotoxic effects [13–15]. The metabolism of these complex As species commonly found in seafood leads to formation of toxic dimethylated forms [16]. Considering the potential toxicity of the different organoarsenic species, it may not be sufficient to base the risk assessment on iAs alone. Hence, the European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM) emphasized the importance of As speciation data in different foodstuffs for a holistic evaluation of As exposure due to diet [17]. The recent findings highlight the need for robust, validated analytical methods for As speciation to contribute to the crafting of future food legislations, and subsequent routine monitoring and food control analysis. While European standard methods for iAs already exist [18, 19], a standardized method for organoarsenic species is still not issued.

In speciation analysis, mild extraction conditions are typically employed to liberate the analytes from the matrix while preventing conversion of species [1, 10, 20]. For the analysis of water-soluble As species, commonly used extraction solvents include pure water [21, 22], mixtures of methanol and water [23, 24], and mildly acidic solutions, e.g., nitric acid [25, 26]. An agitation and/or heating device is used to facilitate the extraction, e.g., a mechanical shaker/vortex mixer [23, 24], hotblock [21, 27], water bath [28], ultrasonic bath/probes [29], or microwave systems [22, 26]. By far, high-performance liquid chromatography (HPLC) using cation- and/or anion-exchange columns is still the most utilized technique in As speciation analysis. Inductively coupled plasma mass spectrometry (ICP-MS) is widely used as an arsenic-specific detection system due to its high sensitivity, good selectivity, and compatibility with separation instruments, especially HPLC [30].

Due to the distinct polarities of As species and complexities of the different matrices, a universal extraction procedure for all As species in all foodstuffs has not yet been developed. Thus, a targeted sample treatment has been recommended wherein extraction conditions are optimized specifically for the matrices and analytes of interest [20, 31]. Most method development studies are carried out using a univariate ("one-factor-at-a-time") strategy, but this approach is rather time-consuming and laborious. A recommended alternative approach is to use multivariate optimization wherein variables are changed simultaneously, thereby allowing maximum gain of information with as few experiments as possible [32]. The use of design of experiments (DoE), such as a two-level factorial design, is commonly used for evaluation of factors with significant effects and interactions [33]. If dealing with several factors and if resources are constrained, a more pragmatic approach is a fractional factorial design. The DoE as a chemometric tool for method optimization has previously been used in speciation analysis of arsenic [34, 35], zinc [36], selenium [37], chromium [38], and mercury [39] in a wide range of matrices.

In a recent review by Ardini et al. [1] covering literature on As speciation analysis of environmental samples published from 2004 to 2018, almost half of the papers were devoted to investigation in marine organisms. Only around 25% delved into method optimization. In addition, out of the 200 papers reviewed, only 60% used CRMs, and only a third utilized CRMs in their method validation. To bridge this gap, the aims of the present study were (1) to perform extraction optimization using fractional factorial design with blue mussel as the test matrix, (2) to optimize HPLC-ICP-MS conditions, (3) to perform a single-laboratory validation using several marine matrices, and (4) to apply the method to a range of marine CRMs with an overall goal of providing information values which can be used as reference for evaluation or comparison of future analytical methods.

Materials and methods

Reagents and standards

All reagents used were analytical grade and of high purity. Methanol (MeOH, ≥ 99.97%), pyridine (C_5H_5N, ≥ 99.5%), formic acid (HCOOH, ≥ 98%), nitric acid (HNO_3, 65%), hydrogen peroxide (H_2O_2, 30%), ammonia solution (NH_3, 25%), and ammonium carbonate ($(NH4)_2CO_3$, reagent grade) were purchased from Merck (Darmstadt, Germany). Nitric acid was further purified using a sub-boiling distillation unit (Savillex, Eden Prairie, MN, USA). Acetonitrile (CH_3CN/

ACN, ≥ 99.95%) was obtained from VWR Chemicals BDH (Fontenay-sous-Bois, France). Ultrapure water (18.2 MΩ-cm) was produced in-house using a Milli-Q water purification system (Merck Millipore, Burlington, MA, USA) and was used throughout the study.

Arsenite [As(III)] and arsenate [As(V)] solutions (1000 mg/L) were produced by Spectrascan Teknolab (Ski, Norway). Arsenobetaine (AB, ≥ 95%) and a sodium salt of dimethylarsinic acid (DMA, ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethyl arsonium iodide (TETRA, 97%) and trimethylarsine oxide (TMAO, 95%) were supplied by Toronto Research Chemicals (Toronto, Ontario, Canada). The standard solution of arsenocholine (AC, 19.77 mg/kg) was produced by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), while monomethylarsonic acid (MA, 99.5%) was sourced from Chem Service, Inc. (West Chester, PA, USA). Standard solutions of other methylated arsenic species such as trimethylarsoniopropionate (TMAP), dimethylarsinoyl acetate (DMAA), dimethylarsinoyl ethanol (DMAE), and dimethylarsinoyl propionate (DMAP), as well as the glycerol-arsinoylriboside (AsSug 328) and other arsenosugars (AsSug 392, 408, and 482), were procured from the University of Graz (Austria). Stock solutions were prepared by dissolving or diluting appropriate amounts of the standards in water. Accurate As concentrations were determined by ICP-MS.

Samples and reference materials

Blue mussel samples ($n = 50$) from the Norwegian surveillance programme for mussels in 2017 [40], led by the Norwegian Food Safety Authority, were pooled and homogenized using a food processor (Braun Multiquick 7 K3000, Kronberg im Taunus, Germany). These were subsequently freeze-dried for 72 h (Labconco FreeZone 18 L, Kansas City, MO, USA) and homogenized using a knife mill (Retch Grindomix GM 100, Haan, Germany). The resulting pooled sample served as an in-house quality control (QC) material and was analyzed for total As with 10 replicates. The average result was set as the target total As concentration. The blue mussel sample was used as test matrix for the extraction optimization using fractional factorial design. Blue mussel was chosen since previous studies reported the presence of several As species, including four to six unknowns [41, 42].

The certified reference materials (CRMs) utilized were tuna fish tissue (BCR 627), mussel tissue (*Mytilus edulis*, ERM-CE278k), and bladderwrack seaweed (*Fucus vesiculosus*, ERM-CD200) from the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre (IRMM, Geel, Belgium); fish protein (DORM-3 and DORM-4), dogfish liver (*Squalus acanthias*, DOLT-5), cuttlefish (*Sepia pharaonis*, SQID-1), and lobster hepatopancreas (TORT-3) from the National Research Council Canada (NRC, Ottawa, Ontario, Canada); hijiki seaweed (*Hizikia fusiforme*, CRM 7405-b) from the National Metrology Institute of Japan (NMIJ, Ibaraki, Japan); and oyster tissue (*Crassostrea virginica*, SRM 1566b) from NIST (Gaithersburg, MD, USA).

Experimental overview

In all experiments from the initial method development phase until validation, extraction efficiencies were evaluated by comparing the total As in the extracts and in the samples. Chromatographic recovery was assessed by comparing the sum of As species from HPLC-ICP-MS with the total As in the soluble extracts. Overall mass balance was checked to ensure that As in the different fractions were accounted for.

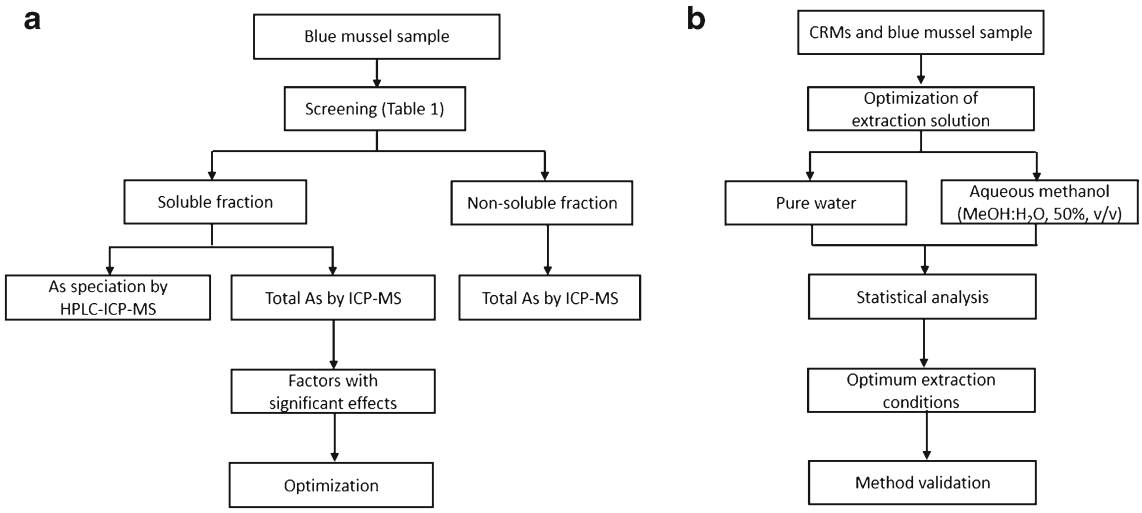

Fig. 1 A process flow chart of the (a) screening and (b) optimization experiments leading to method validation using blue mussel and CRMs

A process flow chart summarizing the experiments performed in this study is presented in Fig. 1.

Extraction optimization: screening of factors using fractional factorial design

Based on a review of extraction procedures used for As speciation in marine matrices [21, 26, 27], a total of seven factors were identified as the most important and were chosen for the experimental design: (A) sample weight (g), (B) type of extraction solution, (C) volume of extraction solution (mL), (D) addition of H_2O_2 in the extraction solution, (E) extraction temperature (°C), (F) extraction time (min), and (G) use of ultrasonication. A 2^{7-3} fractional factorial design was devised (resolution IV), with a total of 16 experiments performed in random order as described in Table 1. Total As concentrations in the soluble extracts were chosen as the response to optimize.

For the extraction, 0.2 g or 0.5 g of the blue mussel sample was weighed into 50-mL polypropylene tubes. Five or 15 mL of water or 30 mM HNO_3 was added. Depending on the experimental set-up (Table 1), H_2O_2 was added to the extraction solution to yield a concentration of 1% H_2O_2 (v/v). A vortex mixer (IKA, Staufen, Germany) was used for 10 s, and then the tubes were placed in a water bath (OLS200, Grant, Cambridge, UK) at 25 °C or 90 °C, and left shaking (100 rpm) for 30 or 60 min. Selected tubes were ultrasonicated afterwards (Table 1). Subsequently, the tubes were placed in a centrifuge (1780×*g*, 10 min; Eppendorf Centrifuge 5702, Hamburg, Germany). The extracts (soluble fraction) were filtered using a 5-mL single-use syringe (Henke-Sass Wolf, Tuttlingen, Germany) connected to a 0.45-μm syringe filter (Sartorius, Göttingen, Germany) and transferred to new polypropylene tubes. The tubes with the residues (non-soluble fraction) were placed in a drying oven (60 °C, Fisher Scientific, Ottawa, Ontario, Canada) and left to dry for 2 days. Both soluble and non-soluble fractions were analyzed for total As using ICP-MS, while a portion of the soluble fraction was diluted with water (1:4, v/v) in a 1-mL polypropylene HPLC vial, and analyzed for As speciation using HPLC-ICP-MS.

Optimization of factors with significant effects: extraction solution

To further optimize, extraction efficiencies of pure water and aqueous methanol (MeOH:H_2O, 50% v/v) were compared using the blue mussel sample and CRMs (BCR 627, ERM-CD200, DORM-3, and TORT-3). Briefly, 0.2 g of sample was

Table 1 2^{7-3} fractional factorial design (resolution IV). The tested factors were (A) sample weight (g), (B) type of extraction solution, (C) volume of extraction solution (mL), (D) addition of H_2O_2, (E) extraction temperature (°C), (F) extraction time (min), and (G) use of ultrasonication. Coded factor levels are denoted as "−1" or "+1" followed by the real factor setting in parenthesis. Total arsenic concentration in the blue mussel sample was 14.6 ± 0.1 mg/kg d.w. (mean ± SD, $n = 10$). Results for arsenic concentration in soluble extracts (mg/kg d.w., $n = 1$) are given in the rightmost column

Factors: coded (real)

Experiment	A Sample weight (g)	B Type of extraction solution	C Volume of extraction solution (mL)	D Addition of H_2O_2	E=ABC Extraction temperature (°C)	F=BCD Extraction time (min)	G=ACD Use of ultrasonication	As conc. (mg/kg d.w.)
1	−1 (0.2)	−1 (water)	+1 (15)	−1 (No)	+1 (90)	+1 (60)	+1 (Yes)	11.1
2	−1 (0.2)	+1 (30 mM HNO_3)	−1 (5)	+1 (Yes)	+1 (90)	−1 (30)	+1 (Yes)	10.4
3	−1 (0.2)	+1 (30 mM HNO_3)	−1 (5)	−1 (No)	+1 (90)	+1 (60)	−1 (No)	10.4
4	+1 (0.5)	+1 (30 mM HNO_3)	−1 (5)	−1 (No)	−1 (25)	+1 (60)	+1 (Yes)	10.1
5	+1 (0.5)	+1 (30 mM HNO_3)	−1 (5)	+1 (Yes)	−1 (25)	−1 (30)	−1 (No)	9.9
6	−1 (0.2)	−1 (water)	−1 (5)	+1 (Yes)	−1 (25)	+1 (60)	+1 (Yes)	10.2
7	+1 (0.5)	−1 (water)	−1 (5)	+1 (Yes)	+1 (90)	+1 (60)	−1 (No)	10.6
8	+1 (0.5)	+1 (30 mM HNO_3)	+1 (15)	+1 (Yes)	+1 (90)	+1 (60)	+1 (Yes)	10.5
9	−1 (0.2)	+1 (30 mM HNO_3)	+1 (15)	+1 (Yes)	−1 (25)	+1 (60)	−1 (No)	10.0
10	+1 (0.5)	−1 (water)	−1 (5)	−1 (No)	+1 (90)	−1 (30)	+1 (Yes)	10.3
11	+1 (0.5)	−1 (water)	+1 (15)	+1 (Yes)	−1 (25)	−1 (30)	+1 (Yes)	10.3
12	−1 (0.2)	−1 (water)	−1 (5)	−1 (No)	−1 (25)	−1 (30)	−1 (No)	10.4
13	+1 (0.5)	+1 (30 mM HNO_3)	+1 (15)	−1 (No)	+1 (90)	−1 (30)	−1 (No)	10.3
14	+1 (0.5)	−1 (water)	+1 (15)	−1 (No)	−1 (25)	+1 (60)	−1 (No)	10.1
15	−1 (0.2)	+1 (30 mM HNO_3)	+1 (15)	−1 (No)	−1 (25)	−1 (30)	+1 (Yes)	10.3
16	−1 (0.2)	−1 (water)	+1 (15)	+1 (Yes)	+1 (90)	−1 (30)	−1 (No)	10.8

weighed into a 13-mL polypropylene tube. Five milliliters of pure water or aqueous methanol (MeOH:H_2O, 50% v/v) was added, followed by vortex mixing. The tubes were placed in a shaking water bath (90 °C, 100 rpm) for 30 min and centrifuged (1780×*g*, 10 min). The soluble fraction was filtered using a 5-mL single-use syringe connected to a 0.45-μm syringe filter, transferred to new tubes, and analyzed for total As by ICP-MS and As speciation by HPLC-ICP-MS. Three replicates were performed for each sample.

Total As determination by ICP-MS

Total As was determined by microwave digestion followed by analysis in ICP-MS, as described by Julshamn et al. [43]. Briefly, 0.2 g of sample was weighed into quartz tubes and added with 2 mL HNO_3 and 0.5 mL H_2O_2. The tubes were capped and placed in a single-reaction-chamber microwave system (UltraWAVE, Milestone, Sorisole, Italy) for digestion. The digested solutions were allowed to cool then quantitively transferred to a 25-mL volumetric flask and diluted with water. The same digestion procedure was applied to the non-soluble and soluble fractions; only here, the sample weights were 0.2 g ± 0.1 g (mean ± standard deviation (SD), $n = 16$) for the non-soluble fraction (depending on how much residue was left) and 0.25 g for the soluble fraction. Total As analysis was carried out with an iCAP Q ICP-MS (Thermo Scientific, Waltham, MA, USA) equipped with an SC-4 DX autosampler (Elemental Scientific, Mainz, Germany). Daily instrument optimization was conducted following the manufacturer's instructions. A complete list of instrument settings is given in Table 2. Instrument control and data processing were carried out through the Qtegra software (v. 2.10, 2018, Thermo Scientific, Waltham, MA, USA). For analyte quantification, calibration standard solutions were prepared by serially diluting appropriate amounts of a stock solution of As with aqueous 5% HNO_3. The resulting calibration curve ranged from 0.5 to 25 μg/L. To compensate for possible instrumental drifts and matrix effects, online internal standard addition of germanium was employed. As part of quality control, TORT-3 and SRM 1566b were analyzed in duplicate in each analytical series and were used to evaluate method accuracy.

As speciation by HPLC-ICP-MS

As speciation was achieved using cation- and anion-exchange methods using a 1260 Infinity HPLC coupled to a 7900 ICP-MS (Agilent Technologies, Santa Clara, CA, USA). The cation-exchange settings were based on previous studies [21, 23] and were further optimized in this work. A Metrosep C 6 column (250 × 4.0 mm, 5 μm, Metrohm, Herisau Switzerland), filled with silica gel with carboxyl groups, and a corresponding guard column were used to separate the cationic species. For the mobile phase, appropriate amounts of pyridine were diluted in aqueous 0.5% (v/v) acetonitrile to the desired ionic strength and subsequently adjusted to pH 2.7 with formic acid. The anion-exchange conditions were also developed based on previous works [21, 27]. A PRP-X100 column (250 × 4.6 mm, 5 μm, Hamilton, Reno, NV, USA), filled with polystyrene-divinylbenzene copolymer with quaternary ammonium group, and a corresponding guard column were utilized. The mobile phase was prepared by dissolving appropriate amounts of ammonium carbonate in aqueous 3% (v/v) methanol to the desired ionic strength and adjusted to pH 9.3 with ammonia. Mobile phases were vacuum-filtered through a 0.45-μm PTFE filter (Agilent Technologies, Santa Clara, CA, USA) prior to use. Gradient elution was implemented

Table 2 The operating parameters for ICP-MS and HPLC-ICP-MS

Instrument settings		
ICP-MS settings	iCap Q	
RF power	1550 W	
Plasma gas flow	14.0 L/min	
Carrier gas flow	1.02 L/min	
Makeup gas flow	0.80 L/min	
Dwell time	0.1 s per isotope	
Isotopes monitored	^{75}As, ^{72}Ge (internal standard)	
HPLC-ICP-MS settings	1260 HPLC and 7900 ICP-MS	
RF power	1550 W	
Nebulizer gas flow	1.03 L/min	
Plasma gas flow	15.0 L/min	
Spray chamber temperature	2 C	
Isotopes monitored	^{75}As, ^{35}Cl	
Integration time	1 s	
	Cation-exchange	Anion-exchange
Guard column	Metrosep C 6 Guard (4.0 mm)	PRP-X100 Guard cartridge, PEEK
Analytical column	Metrosep C 6 (250×4.0 mm, 5 um)	PRP-X100 (250×4.6 mm, 5 um)
Mobile phase	A: 0 mM pyridine, 0.5% ACN, pH 2.7 B: 50 mM pyridine, 0.5% ACN, pH 2.7	A: 0.5 mM $(NH_4)_2CO_3$, 3% MeOH, pH 9.3 B: 60 mM $(NH_4)_2CO_3$, 3% MeOH, pH 9.3
Gradient	0–8 min (10% B), 8–10 min (10% to 100% B), 10–20 min (100% B), 20–23 min (10% B)	0–6 min (20% B), 6–17 min (100% B), 17–20 (20% B)
Flow rate	0.9 mL/min	1 mL/min
Injection volume	50 μL	50 μL

for both cation- and anion-exchange separations. The optimized HPLC-ICP-MS settings are also presented in Table 2.

For the quantification of analytes, mixed calibration standard solutions were prepared by serial dilution of appropriate amounts of stock solutions in aqueous methanol (MeOH:H_2O, 50% v/v). External calibration curves were generated, and chromatographic peak areas were used for the quantification. Chromatographic peaks for the sample extracts were identified by comparison of retention time (RT) with the standards. Unknown peaks were quantified using the calibration curve of the As species with closest retention time. For quality control, CRMs were included in every analytical series. Extraction blanks were also analyzed to check for possible contamination. Instrument control and data processing were facilitated through the MassHunter 4.5 Workstation software (v. C.01.05, Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis and data treatment

For the fractional factorial design, statistical significance of the main effects was evaluated using analysis of variance (ANOVA) with a 95% confidence interval. In comparing the extraction efficiencies of pure water and aqueous methanol (MeOH:H_2O, 50% v/v), a *t*-test was used to assess whether the results of the two extractants were significantly different. Statistica (v. 13.5.0.17, TIBCO, Palo Alto, CA, USA) was used in generating the experimental design and processing the corresponding analytical results. Microsoft Excel (Microsoft, Redmond, WA, USA) was used in statistical treatment of data and calculation of other analytical figures of merit. OriginPro 2020b (v. 9.7.5.184, OriginLab, Northampton, MA, USA) was used in creating figures.

Results and discussion

Total As in the pooled blue mussel sample and CRMs

The average total As concentration for the pooled blue mussel sample was 14.6 ± 0.1 mg/kg (mean ± SD, $n = 10$). This value was set as the target total As concentration and was used to calculate extraction efficiencies in the experimental design. Total As concentrations and extraction efficiencies for the different CRMs are given in Table 3. Based on *t*-test results, obtained total As concentrations were not significantly different from the certified values (95% confidence level).

Extraction optimization: screening of factors using fractional factorial design

As shown in Table 1, the soluble As concentration from the experiments ranged from 9.9 to 11.1 mg/kg, with experiments 5 and 1 posting the lowest and highest recoveries, respectively. These correspond to 68% to 76% of the total As concentration of the blue mussel sample (14.6 mg/kg). Figure 2 shows the Pareto chart of standardized effect estimates of the different factors. The critical *t*-value denoting statistical significance was 2.306 ($p = 0.05$). Factors with *t*-values above this limit have significant effects on the response (soluble As concentration). The significant factors were extraction temperature (E) and type of extraction solution (B), having positive and negative effects, respectively (Fig. 2). This suggests that extraction temperature should be kept at the "+1" setting, while the extraction solution at the "−1" setting. As shown in Table 1, the effect of extraction temperature is aliased by a three-factor interaction (i.e., E = ABC), which is a caveat of using fractional factorial design. However, by choosing a 2^{7-3} fractional factorial design, resolution IV was achieved. Main effects are only aliased with three-factor interactions and higher, which are often non-significant. This approach reduces the likelihood of making false interpretations [33].

Optimization of significant factors

When using multivariate techniques during method development, screening experiments are usually followed by further optimization using response surface methodology (RSM). The use of RSM models the relationship between the factors and the response/s, and identifies factor settings which will give the maximum (or minimum) response [33]. In the present work, the significant factors determined were extraction temperature and type of extraction solution. The high-level setting of extraction temperature in the screening experiments was already at 90 °C. Similar studies have explored applying up to 85 °C only for the extraction of arsenic [34, 35]. If boiling or higher temperatures are required, an oil bath would be more appropriate to use. Thus, due to equipment limitation and safety consideration, the extraction temperature was fixed at 90 °C.

With only one factor left to optimize, a univariate approach was implemented instead of RSM. Furthermore, the type of extraction solution is a non-numerical, discontinuous variable, so the use of RSM, which generates polynomials based on quantitative variables [32, 33], is not entirely applicable. From Fig. 2, low-level setting (pure water) was preferred for the extractant. Hence, other aqueous-based extraction solutions were considered. In this study, the extraction efficiencies of aqueous methanol (MeOH:H_2O, 50% v/v) and pure water were compared. As confirmed by *t*-test results, extraction efficiencies were significantly higher for BCR-627, DORM-3, and the blue mussel sample when using aqueous methanol (MeOH:H_2O, 50% v/v) (see Supplementary Information (ESM) Fig. S1). The highest increase was seen for DORM-3 with a 20% improvement. Most arsenic species in marine samples are water-soluble; however, the addition of methanol

Table 3 Arsenic concentrations in the CRMs and the blue mussel sample, soluble and non-soluble fractions, with calculated parameters for arsenic mass balance (mean ± SD, $n = 5$)

Arsenic species	BCR 627	CE278k	DORM-4	SQID-1	DOLT-5	TORT-3	CRM 7405-b	Blue mussel
Total As (mg/kg)	4.4 ± 0.1	6.7 ± 0.1	6.95 ± 0.08	16.4 ± 0.2	31.7 ± 1.0	64.7 ± 2.0	48.2 ± 1.0	14.6 ± 0.1
Certified value (mg/kg)	4.8 ± 0.3	6.7 ± 0.4	6.87 ± 0.44	14.1 ± 2.2	34.6 ± 2.4	59.5 ± 3.8	49.5 ± 1.0	
Soluble As (mg/kg)	4.3 ± 0.1	4.4 ± 0.1	5.68 ± 0.14	14.7 ± 1.3	32.6 ± 2.6	63.5 ± 0.8	27.3 ± 0.9	10.8 ± 0.6
Extraction efficiency (%)^	97 ± 1	66 ± 2	82 ± 2	90 ± 8	103 ± 8	98 ± 1	57 ± 2	74 ± 4
Sum of As species (mg/kg)*	4.3 ± 0.1	3.7 ± 0.1	5.49 ± 0.06	14.2 ± 0.3	29.6 ± 1.9	55.2 ± 1.1	27.6 ± 0.7	10.1 ± 0.2
Non-soluble As (mg/kg)	0.5 ± 0.1	2.9 ± 0.1	1.64 ± 0.07	3.5 ± 0.9	4.9 ± 0.4	8.4 ± 0.2	24.5 ± 1.2	4.3 ± 0.1
Sum As (mg/kg)¤	4.8 ± 0.1	7.3 ± 0.1	7.3 ± 0.2	18.2 ± 0.4	37.6 ± 2.9	71.9 ± 0.6	51.8 ± 0.4	15.1 ± 0.6
As recovery (%)¤¤	109 ± 2	109 ± 2	105 ± 3	111 ± 3	118 ± 9	111 ± 1	107 ± 1	104 ± 4

^ Extraction efficiency = (Soluble As/Total As) × 100

* Sum of As species = Sum of chromatographed peaks

¤ Sum As = Soluble As + Non-soluble As

¤¤ As recovery = (Sum As/Total As) × 100

increases the solubility of less polar arsenic species which are not extracted with water [44]. In contrast, there was a non-significant difference observed for extraction efficiencies for ERM-CD200 (seaweed) and TORT-3 (lobster hepatopancreas) (Fig. S1). An overall high extraction recovery was observed when using aqueous methanol (MeOH:H_2O, 50% v/v), with over 90% of total As extracted in most samples. The only exceptions were the blue mussel and seaweed CRM, having approximately 80% of the total As extracted. The non-extracted arsenic species are possibly lipid-soluble species and would require a different extraction strategy using more non-polar extraction solutions.

The applicability of MeOH:H_2O solutions in extracting water-soluble As species has been widely documented [23, 24, 29]. Aqueous methanol has also been used in extracting arsenolipids, although a higher percentage of methanol is often applied (e.g., MeOH:H_2O, 9:1 v/v) [45, 46]. In this regard, the use of methanol might co-extract polar arsenolipids causing an apparent increase in extraction efficiency but will subsequently be unquantified since they will elute with the void volume. To verify if this is the case, the chromatographic recoveries were checked to ensure that extracted As species are accounted for. The chromatographic recoveries obtained were between 84 and 103% (Table 3), suggesting that the extracted arsenic species were sufficiently quantified with the proposed method.

From the results of the screening and optimization experiments, the optimum extraction conditions were identified: 0.2-

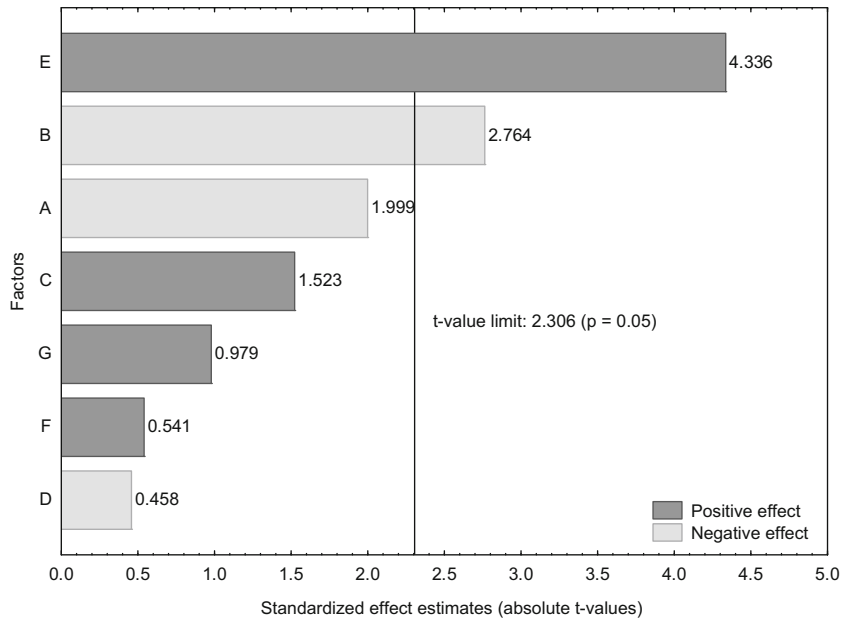

Fig. 2 Pareto chart of standardized effects with soluble As concentration as the response. The factors investigated were (A) sample weight (g), (B) type of extraction solution, (C) volume of extraction solution (mL), (D) addition of H_2O_2 in the extraction solution, (E) extraction temperature (°C), (F) extraction time (min), and (G) use of ultrasonication. The *t*-value limit was 2.306 ($p = 0.05$), above which signifies statistical significance. Bars in dark gray and light gray represent positive and negative effects, respectively

g sample weight, 5 mL of aqueous methanol (MeOH:H_2O, 50% v/v) as extraction solvent, extraction temperature of 90 °C, and extraction time of 30 min. The non-significant factors were kept at low levels in line with "Green Chemistry" principles [47].

Optimization of HPLC-ICP-MS parameters

Column selection

Water-soluble As compounds have different pK_a values which lead to formation of anionic or cationic species in aqueous solutions depending on the pH. Hence, a single chromatographic approach is usually not feasible, and the combined use of cation- and anion-exchange chromatography is consequently recommended [21, 26, 41, 45]. For cation-exchange, columns which were typically used in previous studies include IonoSpher 5C [23, 45], Zorbax 300 SCX [26], and Metrosep C 6 [21]. In the present work, IonoSpher 5C and Metrosep C 6 were explored since they have been reported to separate the largest number of cationic species [21, 23]. However, a shift in RT was observed for TMAO when IonoSpher 5C was used in between days. Similar poor reproducibility when using IonoSpher columns has previously been reported [23, 44]. The findings were attributed to both chemical properties of the compounds and endogenous matrix components. In line with these observations, Metrosep C 6 was chosen as the cation-exchange column for succeeding experiments. For anion-exchange, PRP-X100 was applied in the present work, which has been the most commonly used column for As speciation analysis in marine matrices [1].

Buffer selection and effect of pH

For the mobile phase, cationic As species are normally eluted by pyridine-based solutions [21, 23, 26]. For anionic As species, phosphate- [48], carbonate- [49], and nitrate-based eluents [25, 41] are utilized. In this work, ammonium carbonate was used as the mobile phase buffer for anion-exchange and pyridine for cation-exchange.

Ion-exchange chromatography relies on electrostatic interactions between functional groups of the stationary phase and the charged analytes, as influenced by the mobile phase pH and pK_a of the As compounds [20]. To evaluate the effect of pH on the retention of analytes, two pH values were tested for anionic separation using ammonium carbonate as buffer: 9.3 and 10.3. It was seen that analytes were more retained at pH 9.3, as shown in the comparison of two chromatograms of TORT-3 in Fig. 3. At this pH, carbonate ions exist primarily as HCO_3^-, whereas at pH 10.3, carbonic acid has reached its second dissociation equilibrium, causing an increase of CO_3^{2-} ions. Since CO_3^{2-} ions have stronger affinity to the quaternary ammonium groups in the stationary phase, anionic species were eluted more easily. It was also noted that the chromatographic peak for As(III) disappeared at pH 10.3 while the peak area for AsSug 482 slightly increased, suggesting a shift in RT for As(III). This was confirmed by a spiking experiment with As(III) to a TORT-3 extract (data not shown). This shift in RT can be explained by the first pK_a of As(III) being 9.23 [50]; hence, at pH 10.3, the dominant form is the deprotonated $H_2AsO_3^-$. The increase of negatively charged ions results in a stronger interaction with the stationary phase; thus, As(III) is more retained and elutes in the RT of AsSug 482 (Fig. 3). Other As species were not affected since their pK_a values are much lower. Due to the foregoing observations, pH 9.3 was identified as the optimum pH for a carbonate-based mobile phase. For the cationic separation, pH 2.7 was chosen for a pyridine-based eluent since this has been demonstrated to work well in previous studies [21, 23], and was also confirmed in the present study.

Carbon-induced signal enhancement

The effect of adding organic solvent to the mobile phase to improve ICP-MS sensitivity has been extensively described in the literature [51–53]. An increase in signal is desirable, particularly for As which has a high ionization potential and consequently not quantitatively ionized in the argon plasma of the ICP-MS [51]. Thus, the effect of addition of methanol and acetonitrile concentrations to the mobile phases was optimized in the present study. It has been stated that methanol, or alcohols, in general, should not be used with cation-exchange columns with carboxyl groups due to possible esterification of ion-exchange sites [54]. Hence, ACN was chosen as the added organic solvent for cation-exchange chromatography using Metrosep C 6. Two sets of 5 μg/L standard solutions of As(V) containing different fractions of organic solvent were aspirated into the ICP-MS. Highest signal enhancement was achieved at 0.5% (v/v) ACN and 3% (v/v) MeOH, with four- and fivefold increase, respectively (ESM Fig. S2). The nitrogen atom in ACN may be contributing to the signal enhancement, similar to the increased signal intensity brought about by nitrogen gas in laser ablation ICP-MS [55]. In addition, MeOH is more volatile than ACN and, hence, would require less energy from the ICP for decomposition [53]. This could possibly explain why the ICP can tolerate a higher proportion of MeOH. The identified optimum MeOH concentration of 3% (v/v) is in accordance with the findings of Larsen et al. [51]. At concentrations beyond 0.5% (v/v) ACN and 3% (v/v) MeOH, the magnitude of signal enhancement started to decline. In fact, at ACN > 3.5% (v/v), the obtained intensity was even less than that without added ACN, suggesting signal suppression. The decline in intensity after reaching a certain threshold for organic solvent is commonly attributed to the cooling effect on the plasma, which decreases the plasma

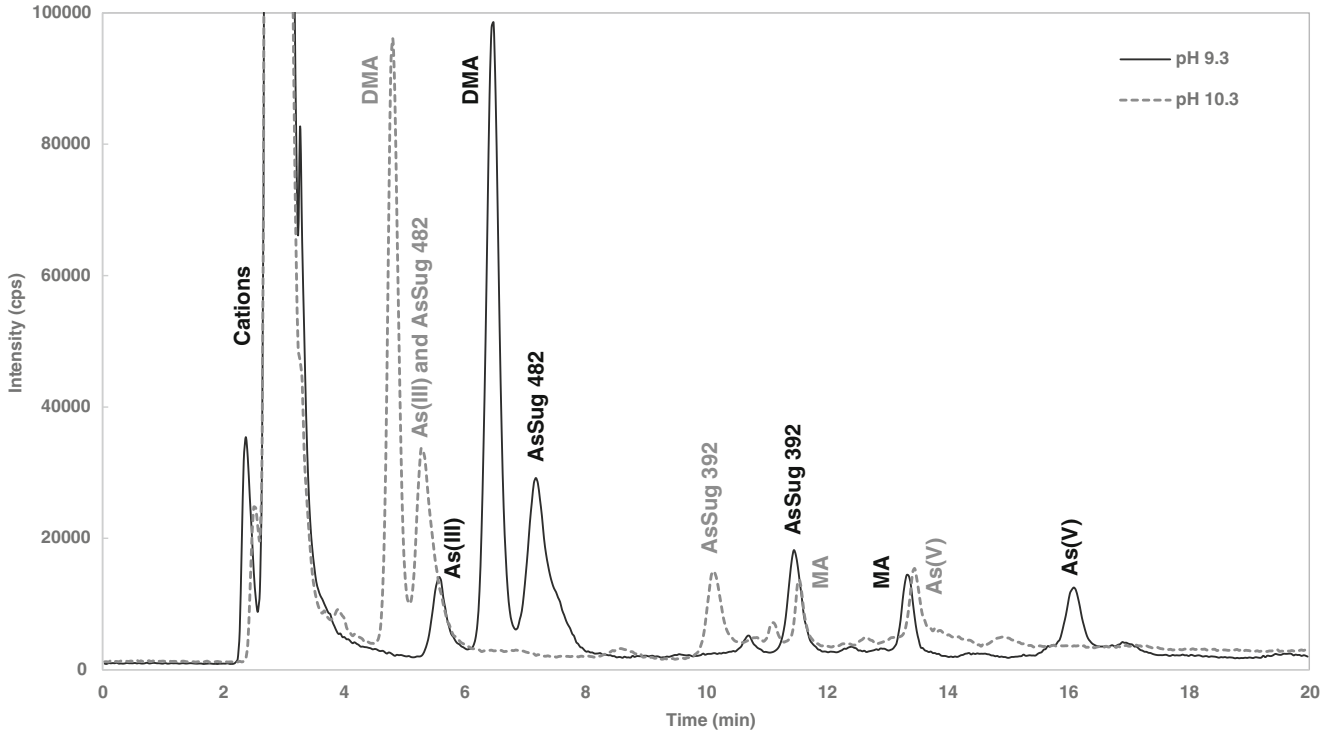

Fig. 3 Overlaid chromatograms of anion-exchange separation of TORT-3 using mobile phase with different pH 9.3 (solid line) and pH 10.3 (dotted line)

temperature and hampers the efficient ionization of analytes [52, 53].

Based on the experimental results, the optimum conditions for the pyridine-based mobile phase are pH 2.7 and 0.5% (v/v) ACN. For the carbonate-based eluent, pH 9.3 and 3% (v/v) MeOH were chosen. The optimized mobile phase compositions, together with the HPLC-ICP-MS settings (Table 2), allowed chromatographic separation of several As species, with peaks of sufficient intensity, and run time of less than 25 min. Sample chromatograms for DORM-4 and blue mussel are presented in Fig. 4. Chromatograms for the standard solutions can be found in the ESM (Fig. S3).

Method validation

To demonstrate the applicability of the developed method, a single-laboratory validation was carried out according to Eurachem's recommendations [56]. Due to limited availability of standards, some method validation parameters (i.e., working range, linearity, spiking recovery, and precision) could not be calculated for all methylated arsenic species and arsenosugars (i.e., DMAA, DMAP, AsSug 328, AsSug 392, AsSug 408, and AsSug 482).

In this study, spectral interference of $^{40}Ar^{35}Cl^{+}$ with As (*m/z* 75) was avoided by employing a gradient profile which chromatographically separated the chloride from the rest of the anionic As species. The retention time for chloride under anion-exchange settings was 14.6 min, while the closest eluting analytes were MA (13.4 min) and As(V) (16.3 min), hence, no coelution of chloride with the As species.

The limit of detection (LOD) was calculated as three times the SD of ten replicates of a 0.5 μg/L mixed standard solution subjected to the extraction procedure and analyzed with HPLC-ICP-MS, while the limit of quantification (LOQ) was set as ten times the SD. The LOQ values ranged from 0.005 to 0.025 mg/kg for the different species (ESM Table S1). The obtained LOD and LOQ values were comparable with those reported elsewhere [21, 26, 27].

Linearity was assessed by analyzing in triplicate a blank and six different concentration levels of As standard solutions. The response (peak area) was plotted against concentration and appropriate regression statistics were calculated. Obtained correlation coefficients (*r*) were at least 0.999 (ESM Table S1). Statistical analysis of residuals also demonstrated random distribution, hence, confirming good linearity of the method. The concentration levels used for the linearity experiments also represent the method working range (ESM Table S1).

Trueness was evaluated in two ways: (i) analysis of CRMs and (ii) analysis of spiked samples. As shown in Table 4, good agreement was found for the experimental results compared with certified and information values, with recoveries in the range of 88 to 109% of the certified concentrations. In addition, BCR 627, DORM-4, and the blue mussel sample were spiked at three concentration levels (0.3, 0.5, and 1 mg/kg for AB and DMA; 0.1, 0.3, and 0.5 mg/kg for others) in duplicate. The average spiking recoveries for the three sample types were in the range of 83 to

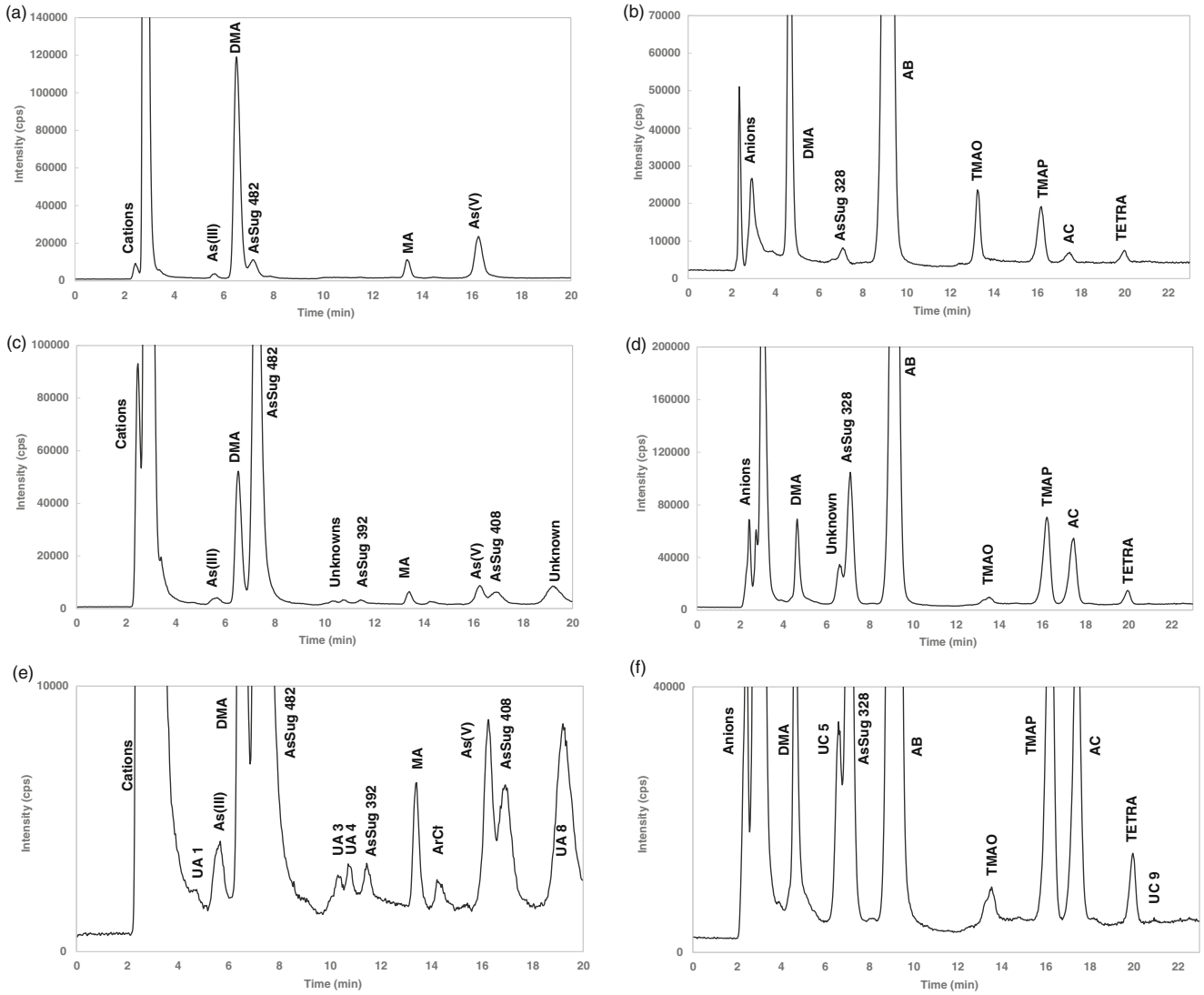

Fig. 4 Chromatograms of arsenic species in a DORM-4 extract using (a) anion- and (b) cation-exchange HPLC-ICP-MS. Chromatograms of arsenic species in a blue mussel extract using (c) anion- and (d) cation-exchange HPLC-ICP-MS. Enlarged chromatograms of (e) panel c and (f) panel d

120%, demonstrating that the integrity of species has been maintained throughout the analytical procedure. Wolle et al. [21] reported poor recoveries (<50%) for TMAO, DMAA, DMAP, DMAE, and As(III) in (non-freeze dried) cod, haddock, and shrimp which were attributed to the binding and interconversion of species due to endogenous matrix components. The problem was resolved with the addition of N-ethylmaleimide.

Precision was evaluated in terms of repeatability by performing five replicate analyses for the blue mussel sample and CRMs. The calculated RSD values for the obtained As species concentrations ranged from 1 to 28%. Concentrations close to LOQ registered the highest RSDs. Average RSDs for the spiked concentration levels were also calculated and were in the range of 0.1 to 10.7% for BCR 627, DORM-4, and the blue mussel sample. The general trend was that higher spiking concentrations yielded better precision.

Measurement uncertainty was estimated using the simplified approach proposed by Barwick et al. [57], wherein results from trueness and precision studies were used to calculate the standard uncertainty. The expanded uncertainty was obtained by multiplying the standard uncertainty by a coverage factor ($k = 2$; 95% confidence interval). Calculated expanded uncertainties were in the range of 2 to 67%, where the highest expanded uncertainties were associated with analytes in concentrations close to LOQ.

Arsenic species in certified reference materials

Good chromatographic recoveries (84 to 103%) were achieved for all CRMs and the blue mussel sample when using the speciation method developed. The obtained concentrations for the As species in the CRMs and the blue mussel

Table 4 Concentrations of arsenic species in the CRMs and the blue mussel sample using the validated method, alongside certified and information values for comparison (mean ± SD, $n = 5$)

Arsenic species	BCR 627	CE278k	DORM-4	SQID-1	DOLT-5	TORT-3	CRM 7405-b	Blue mussel sample
Anions								
As (III)	<0.025	0.064±0.002	<0.025	<0.025 (0.019)	0.125±0.008	0.361±0.012	0.429±0.006	0.043±0.004
DMA	0.155±0.004 **(0.15±0.02)**	0.636±0.013	0.618±0.006	0.032±0.003 (0.03)	1.870±0.120	1.181±0.030	0.286±0.005 (0.24)	0.266±0.006
DMAA	<0.017	0.162±0.007	0.055±0.004	0.055±0.006	0.166±0.009	0.278±0.028	-	0.091±0.005
AsSug 482	0.041±0.002	0.244±0.007	0.068±0.001	0.026±0.003	0.234±0.017	0.545±0.013	0.313±0.004 (0.20)	1.329±0.052
AsSug 392	-	-	-	-	-	0.195±0.019	0.178±0.010 (0.16)	<0.011
MA	<0.011	0.039±0.001	0.046±0.003	<0.011	0.100±0.007	0.131±0.011	0.080±0.005	0.024±0.001
As (V)	0.035±0.001	0.037±0.010	0.110±0.004	0.032±0.006 (0.028)	0.093±0.004	0.270±0.024	24.3±0.6 **(24.4±0.7)**	0.032±0.005
AsSug 408	-	-	-	-	-	0.195±0.035	1.36±0.03 **(1.41±0.04)**	0.028±0.006
Sum of unknown anions	–	0.024±0.001 (2 unknowns)	0.007±0.001 (1 unknown)	-	0.071±0.004 (2 unknowns)	0.105±0.002 (3 unknowns)	-	0.101±0.001 (4 unknowns)
Cations								
AsSug 328	0.008±0.001	0.087±0.003	0.027±0.002	0.020±0.002	0.118±0.009	2.315±0.064	0.415±0.002 **(0.44±0.02)**	0.689±0.015
DMAP	-	<0.007	-	-	-	-	0.013±0.001	<0.007
AB	3.94±0.09 **(3.9±0.2)**	2.24±0.04	4.32±0.05 **(3.95±0.36)**	13.6±0.3 **(13.96±0.54)**	26.1±1.7 **(24.2±0.8)**	48.5±1.0 **(54.9±2.5)**	-	6.46±0.08
TMAO	<0.012	<0.012	0.091±0.003	0.020±0.003	0.156±0.017	0.161±0.006	-	0.044±0.001
TMAP	0.023±0.001	0.089±0.002	0.068±0.001	0.347±0.032	0.338±0.024	0.308±0.008	-	0.323±0.004
AC	0.016±0.002	<0.007	0.017±0.001	0.033±0.003	0.115±0.009	0.037±0.002	-	0.369±0.005
TETRA	0.033±0.001	0.028±0.001	<0.018	<0.018	0.086±0.012	0.138±0.003	-	0.057±0.001
Sum of unknown cations	0.022±0.001 (1 unknown)	0.017±0.001 (4 unknowns)	0.016±0.001 (3 unknowns)	0.017±0.001 (3 unknowns)	0.069±0.004 (2 unknowns)	0.455±0.009 (4 unknowns)	0.208±0.004 (2 unknowns)	0.198±0.002 (4 unknowns)
Sum of As species (mg/kg)	4.3±0.1	3.7±0.1	5.49±0.06	14.2±0.3	29.6±1.9	55.2±1.1	27.6±0.7	10.1±0.2
Soluble As (mg/kg)	4.3±0.1	4.4±0.1	5.68±0.14	14.7±1.3	32.6±2.6	63.5±0.8	27.3±0.9	10.8±0.6
Chromatographic recovery (%)	100±3	84±1	96±1	97±9	88±4	88±2	103±4	94±5

Bolded numbers in parenthesis are certified values. Underlined numbers in parenthesis are information values

Chromatographic recovery = (Sum of As species/Soluble As) × 100

DMAE was not detected in any of the samples analyzed

"-", not detected

sample, together with certified and information values, are shown in Table 4. Due to its presence in the market for more than 20 years, BCR-627 is one of the most utilized CRMs in As speciation. In this study, the obtained AB and DMA concentrations were 3.94 ± 0.09 mg/kg and 0.155 ± 0.004 mg/kg, respectively. These are in accordance with the certified values for AB and DMA of 3.9 ± 0.2 mg/kg and 0.15 ± 0.02, respectively. For BCR 627, literature values for AB generally range from 3.6 to 3.9 mg/kg, while DMA results normally vary from 0.13 to 0.15 mg/kg. For DORM-4, the concentrations found for AB and DMA were 4.32 ± 0.05 mg/kg and 0.618 ± 0.006 mg/kg, respectively. These agree with the certified value for AB which is 3.95 ± 0.36 mg/kg, and with literature values ranging from 3.74 to 4.02 mg/kg for AB, and 0.54 to 0.94 mg/kg for DMA [21, 58–60]. Other CRMs were just recently introduced; hence, limited amount of data is

available. To date, Wolle et al. [21] have reported the most extensive work by quantifying as many as 35 known and unknown As species in two CRMs and a range of seafood samples. The present work aims to augment this effort by reporting the concentrations of different As species in recent versions of the CRMs.

As shown in Fig. 5, with the exception of CRM 7405-b, AB was the predominant As species in the majority of the tested CRMs, contributing as much as 77% of the total As. While arsenobetaine is mainly found in fish, it can also exist as major As species in e.g. crab and clam samples, and in minor proportions in shrimp [2]. DMA was also a major As species, although accounting for less than 10% of the total As. Other organoarsenic species such as MA, AC, TMAO, TMAP, AC, and TETRA were present in minor amounts (< 5%). In contrast, TETRA exists as a major species in mollusks [11]. Arsenosugars were quantified in all samples, but they were notable especially in the blue mussel sample, ERM CE278k (also a blue mussel), TORT-3, and CRM 7405-b (hijiki). Arsenosugars are not exclusively found in macroalgae, as they also appear in higher concentrations in clams, mollusks, and oyster tissue, and in trace levels in kelp [11]. The highest concentration of an arsenosugar was found in TORT-3 with 2.32 ± 0.06 mg/kg (AsSug 328), which is comparable to available literature data of 2.71 mg/kg [21]. Aside from arsenosugars, marine macroalgae are also known to contain elevated levels of iAs. In the present study, an As(V) concentration of 24.3 ± 0.6 mg/kg was found in CRM 7405-b, which is in accordance with the certified value (Table 4). Our results, as supplemented by available literature data, confirm that As exists in several forms and in various concentrations in a broad variety of marine matrices. Trace levels of As species can be found in matrices where one As form is predominant, but this does not imply cross-contamination. In addition, blank samples were regularly included throughout the analytical run and no "memory effects" were observed.

In the present work, most number of As peaks were detected in the blue mussel samples, with a total of 23 As peaks, where 8 peaks are unknown. A total of 33 peaks, 17 unknown (ESM Table S2) and 16 known arsenic species, were detected in the CRMs and the blue mussel sample analyzed. It should be clarified, however, that coelution with our approach cannot be completely ruled out, and further optimization of the chromatography may reveal additional unknown peaks.

Conclusions

In this work, an extraction procedure for water-soluble As species in marine samples was optimized using a 2^{7-3} fractional factorial design. Extraction temperature and the type of extraction solution were identified as factors with significant effects. Based on recoveries for total As content, the optimum conditions were 0.2-g sample weight, 5 mL of aqueous methanol (MeOH:H_2O, 50% v/v) as extractant, and extraction carried out at 90 °C for 30 min. Together with the optimized anion- and cation-exchange HPLC-ICP-MS parameters, these conditions allowed for satisfactory quantification of As

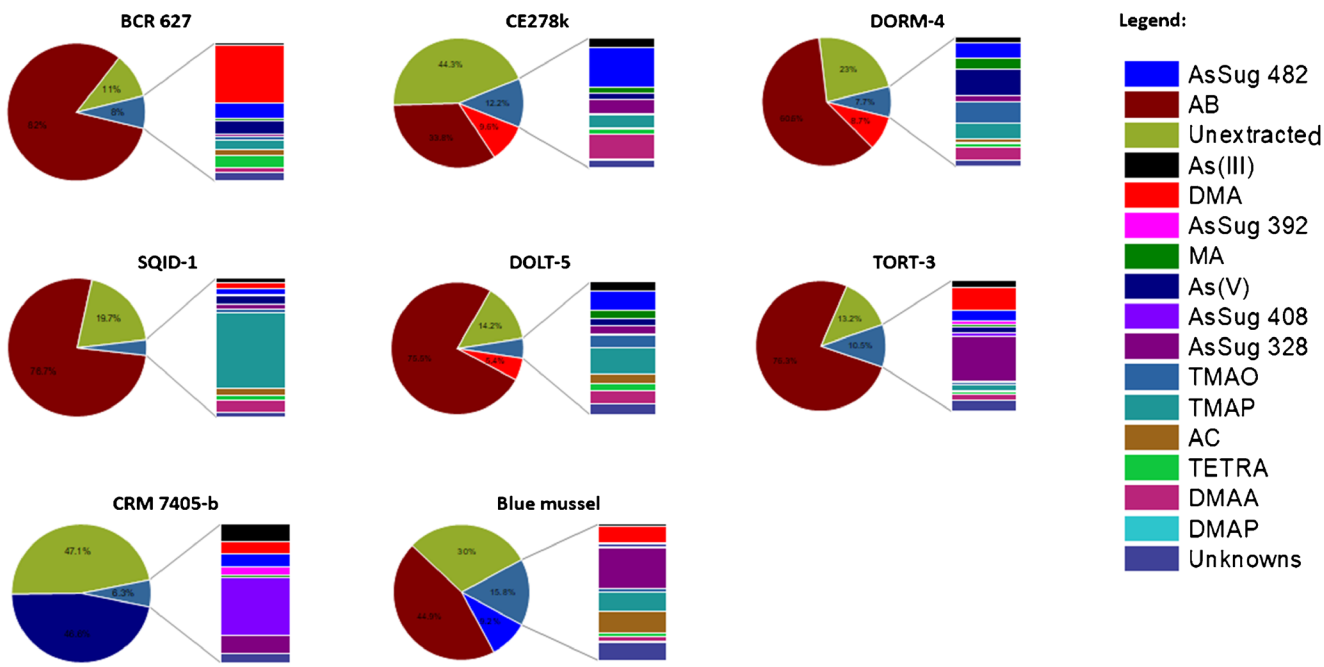

Fig. 5 Arsenic species profile in the CRMs and the blue mussel sample analyzed. Arsenic species fraction, % = (concentration of As species/total As) × 100

species with low solvent and energy consumption. A single-laboratory validation was performed to demonstrate the applicability of the developed method. Different marine CRMs were used as test samples and satisfactory method performance characteristics were achieved. With a total of 33 known and unknown water-soluble species quantified, this study produced a new set of As speciation data which serves as indicator values for succeeding speciation studies.

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Availability of data and material All data and material are available.
Code availability Not applicable

Author contribution All authors contributed to the study conception and design. Data collection and analysis were performed by Jojo Tibon. Results were reviewed and agreed upon by all authors. The first draft of the manuscript was written by Jojo Tibon and all authors participated in the review and editing process. All authors read and approved the final manuscript. Research funding was acquired by Veronika Sele.

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Declarations

Conflict of interest The authors declare no competing interests.

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Supplementary Information

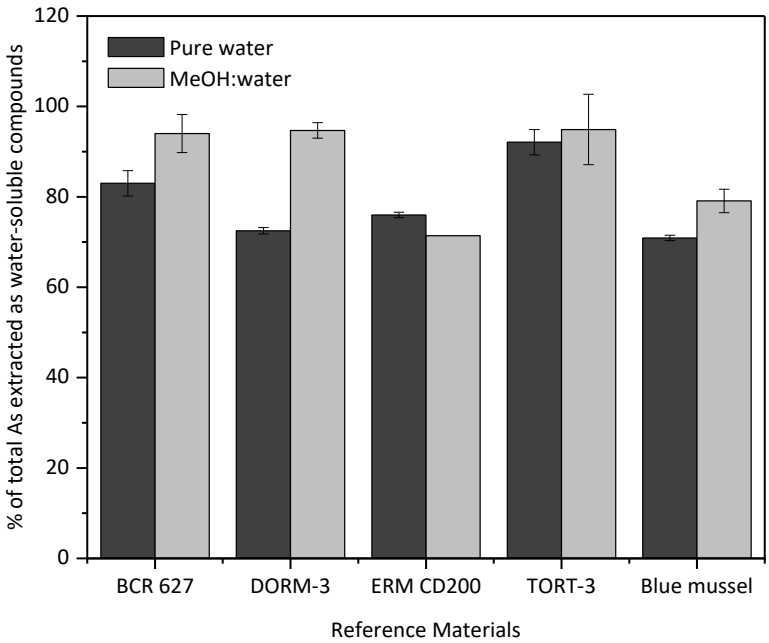
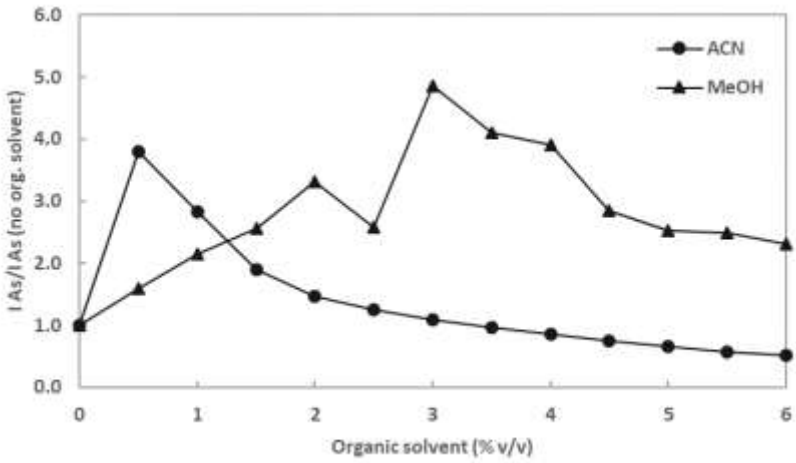


Fig. S1 Extraction efficiencies (EE) using pure water and aqueous methanol (MeOH:H_2O, 50% v/v) as extractant in the blue mussel sample and CRMs (mean ± SD, *n* = 3); EE = (total As in extract/total As in sample) x 100



- **I As** – intensity of ^{75}As for a std solution with a certain % of organic solvent
- **I As (no org. solvent)** – intensity of ^{75}As for a std solution without org solvent

Fig. S2 Relative ICP-MS response for a 5 µg/L standard solution of As(V) in different proportions (%) of acetonitrile and methanol

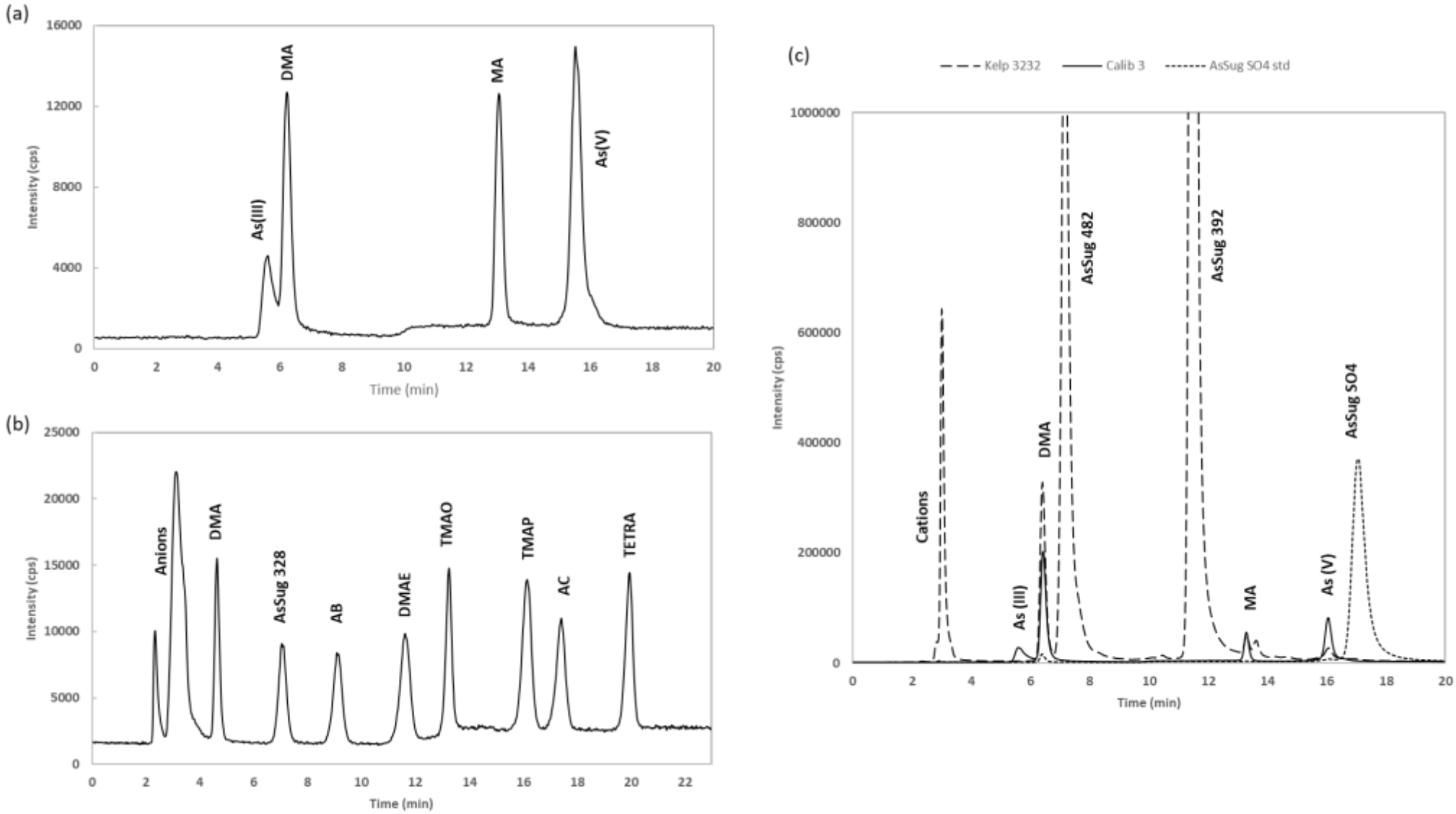


Fig. S3 Chromatograms of arsenic species in mixed standard solutions (~0.5 µg/L) using (a) anion- and (b) cation-exchange HPLC-ICP-MS. Due to limited/unavailable standard solutions of arsenosugars, overlaid anion-exchange chromatograms of SRM 3232 Kelp powder, an anion calibration standard, and a standard solution of AsSug SO4 are also provided to demonstrate retention times (c)

Table S1 LOD and LOQ values, alongside the working ranges and correlation coefficients (r) for the different arsenic species

Species	LOD (mg/kg)	LOQ (mg/kg)	Working range (μg/L)	r
As(III)	0.008	0.025	0.3-4.8	1.0000
DMA	0.005	0.017	1.0-15.7	0.9996
DMAA	0.005	0.017		
AsSug 482	0.005	0.017		
AsSug 392	0.003	0.011		
MA	0.003	0.011	0.3-5.3	0.9996
As(V)	0.004	0.013	0.3-4.8	0.9991
AsSug 408	0.004	0.013		
AsSug 328	0.002	0.007		
DMAP	0.002	0.007		
AB	0.003	0.010	18.9-66.1	0.9992
DMAE	0.006	0.019	0.6-5.7	1.0000
TMAO	0.004	0.012	0.4-4.4	0.9998
TMAP	0.001	0.005	0.5-5.5	0.9999
AC	0.002	0.007	0.5-4.5	0.9999
TETRA	0.005	0.018	0.5-4.5	0.9999

Table S2 Approximate concentrations of unknown arsenic species in the CRMs and the blue mussel sample using the validated method (mean ± SD, *n* = 5)

Species	RT (min)	BCR 627	CE278k	DORM-4	SQID-1	DOLT-5	TORT-3	CRM 7405-b	Blue mussel
Anions									
UA 1	4.5	-	-	-	-	-	0.055 ± 0.002	-	0.004 ± 0.001
UA 2	7.8	-	-	0.007 ± 0.001	-	-	-	-	-
UA 3	10.4	-	-	-	-	-	-	-	0.004 ± 0.001
UA 4	10.7	-	-	-	-	-	0.036 ± 0.003	-	0.006 ± 0.001
UA 5	12.5	-	-	-	-	-	0.014 ± 0.001	-	-
UA 6	15.4	-	-	-	-	0.032 ± 0.001	-	-	-
UA 7	18.0	-	0.009 ± 0.001	-	-	0.039 ± 0.007	-	-	-
UA 8	19.3	-	0.015 ± 0.002	-	-	-	-	-	0.087 ± 0.004
Cations									
UC 1	3.9	-	0.007 ± 0.001	0.007 ± 0.001	0.005 ± 0.001	0.035 ± 0.002	-	0.026 ± 0.003	0.005 ± 0.001
UC 2	4.4	-	-	-	-	-	0.169 ± 0.023	-	-
UC 3	5.2	-	-	-	-	-	0.149 ± 0.006	-	-
UC 4	5.3	-	-	-	-	-	-	0.182 ± 0.004	-
UC 5	6.6	0.022 ± 0.001	-	0.005 ± 0.001	0.004 ± 0.001	-	-	-	0.185 ± 0.005
UC 6	12.6	-	0.003 ± 0.001	0.004 ± 0.001	-	-	0.086 ± 0.004	-	-
UC 7	14.0	-	0.002 ± 0.001	-	-	-	-	-	-
UC 8	14.8	-	0.005 ± 0.001	-	-	-	-	-	0.004 ± 0.001
UC 9	21.0	-	-	-	0.007 ± 0.001	0.034 ± 0.006	0.051 ± 0.004	-	0.004 ± 0.001

UA: unknown anion
UC: unknown cation
'-': not detected

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Arsenic species in mesopelagic organisms and their fate during aquafeed processing

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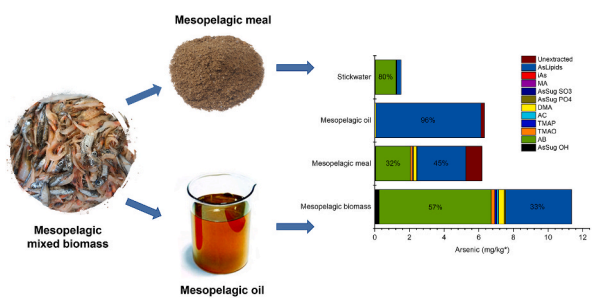
HIGHLIGHTS

- Up to 12 arsenic species were detected in mesopelagic samples.
- Arsenobetaine comprised 70% and 50% of total As in crustaceans and fish, respectively.
- Mesopelagic mixed biomass comprised mainly of arsenobetaine and arsenolipids.
- Arsenolipids were transferred to meal and up-concentrated in oil when processed.
- Inorganic arsenic was <0.007 mg/kg ww in most samples.

GRAPHICAL ABSTRACT



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ABSTRACT

A responsible harvest of mesopelagic species as aquafeed ingredients has the potential to address the United Nations Sustainable Development Goal 14, which calls for sustainable use of marine resources. Prior to utilization, the levels of undesirable substances need to be examined, and earlier studies on mesopelagic species have reported on total arsenic (As) content. However, the total As content does not give a complete basis for risk assessment since As can occur in different chemical species with varying toxicity. In this work, As speciation was conducted in single-species samples of the five most abundant mesopelagic organisms in Norwegian fjords. In addition, As species were studied in mesopelagic mixed biomass and in the resulting oil and meal feed ingredients after lab-scale feed processing. Water-soluble As species were determined based on ion-exchange high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS). This was supplemented by extracting arsenolipids (AsLipids) and determining total As in this fraction. The non-toxic arsenobetaine (AB) was the dominant form in mesopelagic crustaceans and fish species, accounting for approximately 70% and 50% of total As, respectively. Other water-soluble species were present in minor fractions, including carcinogenic inorganic As, which, in most samples, was below limit of quantification. The fish species had a higher proportion of AsLipids, approximately 35% of total As, compared to crustaceans which contained 20% on average. The feed processing simulation revealed generally low levels of water-soluble As species besides AB, but considerable fractions of potentially toxic AsLipids were found in the biomass, and transferred to the mesopelagic meal and oil. This study is the first to report occurrence data of at least 12 As

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species in mesopelagic organisms, thereby providing valuable information for future risk assessments on the feasibility of harnessing mesopelagic biomass as feed ingredients.

1. Introduction

The United Nations (UN) declared 2021–2030 as the Decade of Ocean Science, promoting sustainably harvested oceans as one of its goals. While the usage of traditional marine-based ingredients in aquafeed production has been reduced (Aas et al., 2019), plant-based raw materials can contain antinutritional factors and undesirable substances (e.g. pesticides and mycotoxins), introducing new risks to aquaculture (Glencross et al., 2020; Olsen et al., 2020). Alternative marine-based ingredients are now being explored. In lieu of pelagic fish as raw materials in the aquafeed industry, sustainable capture at low-trophic levels has been recommended (European Commission, 2017). The mesopelagic ecosystem is presumed to consist of species thriving between 200 and 1000 m below the sea surface – a biomass regarded as an unexploited resource for aquafeed production (Alvheim et al., 2020; Grimaldo et al., 2020; Olsen et al., 2020). However, prior to large-scale extraction of mesopelagic species, a holistic assessment is needed in terms of its impact on biodiversity and carbon sequestration (St. John et al., 2016).

Mesopelagic species were found to be high in proteins (Olsen et al., 2020), rich in omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Nordhagen et al., 2020), a good source of Vitamin A and B_{12}, and also rich in calcium, selenium, iron, and iodine (Alvheim et al., 2020; Nordhagen et al., 2020). Among the potentially toxic elements, cadmium (Cd) and arsenic (As) have been reported at levels above the maximum limits (MLs) set in the European legislation for feed and feed materials, especially when the haul is mostly comprised of crustaceans (Olsen et al., 2020). Processing of mesopelagic biomass into aquafeed has the potential to modify the concentrations of undesirable substances, including As and Cd, in mesopelagic products, i.e. meal and oil (Wiech et al., 2020; Berntssen et al., 2021). Wiech et al. (2020) assumed in a theoretical worst-case approach that the total amount of As could end up in the protein fraction and consequently would exceed MLs defined in Directive 2002/32 EC and amendments (European Commission, 2002). However, in their succeeding study involving lab-scale feed processing of mesopelagic biomass, this was found to be not the case since As partitioned both in the oil and meal fractions and resulted in a dilution effect (Berntssen et al., 2021).

Arsenic is an element which is highly abundant in the marine environment, predominantly existing in marine animals as the non-toxic arsenobetaine (AB) (Francesconi and Edmonds, 1997). However, other forms are also present such as inorganic As (iAs) and the methylated species (methylarsonate (MA) and dimethylarsinate (DMA)), classified by the International Agency for Research on Cancer (IARC) as carcinogenic and possibly carcinogenic, respectively (IARC Working Group, 2012). Among available literature, iAs concentrations in mesopelagic species have, so far, only been reported by Wiech et al., 2020. While relatively low levels of iAs were reported (max 0.16 mg/kg ww), the species comprising the major remaining fraction of total As were neither identified nor quantified. However, this is of high relevance since other organic forms such as arsenosugars (AsSug) and arsenolipids (AsLipids), including their metabolites, were shown to exhibit neurotoxic and cytotoxic activity, and are frequently classified as potentially toxic As species (Feldmann and Krupp, 2011; Leffers et al., 2013; Witt et al., 2017). In addition, no study has been conducted yet regarding the fate of As species during feed processing. Such study is beneficial to verify if any up-concentration, dilution, or transformation to more toxic forms occur. This complex chemical nature of As highlights the importance of obtaining speciation data as basis for further risk assessment.

The present study aims to determine the organic As species, which were not included in earlier reports on total As in mesopelagic species (Wiech et al., 2020) and processed mesopelagic biomass (Berntssen et al., 2021). The specific objectives were to (1) provide occurrence data of As species in mesopelagic organisms and (2) give insight on the fate of As species during aquafeed processing, with an overarching goal of providing initial information for future risk assessments on the feasibility of harnessing mesopelagic biomass as feed ingredients.

2. Materials and methods

2.1. Sample collection and processing

2.1.1. Mesopelagic samples grouped by species

The mesopelagic single-species samples analyzed in this study were composed of fish species (1) glacier lanternfish (*Benthosema glaciale*) and (2) silvery lightfish (*Maurolicus muelleri*); also crustaceans including (3) the decapod genus *Pasiphaea* (*P. sivado*, *P. multidentata*, and *P. tarda*, (4) another decapod species *Eusergestes arcticus*, and (5) the euphausiid *Meganyctiphanes norvegica*, commonly known as the Northern krill. The samples were collected in December 2018 from three fjords on the western coast of Norway. At least 27 specimens of the same species were collected from each sampling location. These were pooled to form a composite sample (i.e. one pooled sample representing one sampling location). For each mesopelagic species, three pooled samples were analyzed (i.e. taken from three different sampling locations). The pooled samples were immediately homogenized after sorting the catch on board the research vessel 'Johan Hjort', distributed into different tubes, and stored at −20 °C. These were then freeze-dried upon arrival on shore and analyzed for water-soluble As, AsLipids, and iAs (Fig. 1a). Total As levels were presented and discussed in our earlier work (Wiech et al., 2020). Additional details regarding the samples were extensively described in Alvheim et al. (2020) and Wiech et al. (2020).

2.1.2. Mesopelagic mixed biomass of M. muelleri and M. norvegica and its processing

This study utilized a total of four mesopelagic biomass samples obtained from four different stations in the North Atlantic during a research cruise on board 'MS Birkeland' from September to November 2019. In an earlier work, biomass composed of *M. muelleri* and *M. norvegica* underwent lab-scale feed processing (Berntssen et al., 2021). Mixed biomass were either mechanically pressed or centrifuged, producing a liquid phase and a solid phase (mesopelagic meal) (Fig. 1b). The liquid phase was transferred to a separatory funnel and divided into mesopelagic oil and stickwater. The starting biomass and the resulting fractions from this previous study were subsequently analyzed for water-soluble As, AsLipids, iAs, and total As.

2.2. Analytical methods

2.2.1. Determination of iAs

The iAs concentration was determined by anion-exchange high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) based on EN 16802:2016 (CEN, 2016) and the work of Julshamn et al. (2012). Briefly, 0.2 g of freeze-dried sample or 1.0 g of wet material was weighed into a 13-mL polypropylene tube, followed by addition of 10 mL of 0.1 mM HNO_3 in 3% H_2O_2 (HNO_3, 65%; H_2O_2, 30%; Merck, Darmstadt, Germany). A sub-boiling distillation unit (Savillex, Eden Prairie, MN, USA) was used to further purify the HNO_3. The tubes were then subjected to vortex-mixing (MS 1, IKA, Staufen, Germany) and left to stand overnight. Thereafter, the tubes were placed in a shaking water bath

(OLS200, Grant, Cambridge, UK) set at 90 °C for 1 h (shaking speed at 100 rpm) and centrifuged for 10 min (1780×*g*; 5702, Eppendorf, Hamburg, Germany). Approximately 1 mL of the supernatant was collected using a 5-mL syringe (Henke-Sass Wolf, Tuttlingen, Germany) affixed with a needle, then filtered (0.45-μm PTFE; Sartorius, Göttingen, Germany) and transferred to an HPLC vial. Quantification was performed using a 1260 Infinity HPLC coupled to a 7900 ICP-MS (Agilent Technologies, Santa Clara, CA, USA) with an anion-exchange column (IonPac AS7, 2 × 250 mm; Dionex, Sunnyvale, CA, USA). Isocratic elution was carried out using 50 mM $(NH_4)_2CO_3$ in 3% CH_3OH adjusted to pH 10.3 with NH_3 ($(NH_4)_2CO_3$, reagent grade; CH_3OH, ≥ 99.97%; NH_3, 25%; Merck, Darmstadt, Germany). Quantification was based on chromatographic peak areas using an external calibration curve from an arsenate (As (V)) standard solution (1000 mg/L; Spectrascan Teknolab, Ski, Norway). Accuracy of results was verified using a rice certified reference material (ERM-BC211) and an in-house control sample of tuna fish tissue (BCR-627) (IRMM, Geel, Belgium). Data processing was performed using MassHunter 4.5 Workstation Software (v. C.01.05, Agilent Technologies, Santa Clara, CA, USA).

2.2.2. Determination of water-soluble As species

The quantification of water-soluble As species was carried out by cation- and anion-exchange HPLC-ICP-MS based on our earlier study (Tibon et al., 2021). Briefly, 0.2 g of sample was weighed into a 13-mL polypropylene tube, followed by addition of 5 mL of aqueous methanol (CH_3OH: H_2O, 50% v/v). The tubes were subjected to vortex-mixing and subsequently placed in a shaking water bath set at 90 °C for 30 min (shaking speed at 100 rpm). Afterwards, the tubes were placed in a centrifuge (1780×*g*) for 10 min. The supernatant was poured into a 5-mL syringe connected to a 0.45-μm PTFE filter and transferred to another 13-mL polypropylene tube. An aliquot was transferred into an HPLC vial and diluted accordingly with aqueous methanol (CH_3OH: H_2O, 50% v/v). Arsenic speciation was carried out using a 1260 Infinity HPLC coupled to a 7900 ICP-MS. A Metrosep C6 cation-exchange column (250 × 4.0 mm, 5 μm; Metrohm, Herisau, Switzerland) was used to separate cationic As species by employing gradient elution using pyridine-based mobile phases (0 and 50 mM at pH 2.7, 0.5% acetonitrile). For anionic As species, separation was performed using a PRP-X100 anion-exchange column (250 × 4.6 mm, 5 μm; Hamilton, Reno, NV, USA) and a corresponding gradient elution with carbonate-based mobile phases (0.5 and 60 mM at pH 9.3, 3% methanol). Quantification was achieved by preparing mixed standard solutions of As compounds (Tibon et al., 2021) and integrating chromatographic peak areas to generate external calibration curves. Certified reference materials of tuna fish tissue (BCR-627) and fish protein (DORM-4; National Research Council Canada, Ottawa, Ontario, Canada) were included in the analytical series for quality control. MassHunter 4.5 Workstation Software was used for data processing.

2.2.3. Extraction of AsLipids

AsLipids were estimated based on the approach of Freitas et al. (2020). Approximately 50 mg of oil/freeze-dried sample or 200 mg of wet material was weighed into a borosilicate glass tube (13 × 100 mm; DWK, Mainz, Germany), followed by addition of 1.5 mL of methanol and vortex-mixing for 5 s. Five mL of methyl *tert*-butyl ether (MTBE, HPLC grade; Merck, Darmstadt, Germany) was subsequently added. The glass tubes were capped and placed in a test-tube rotator (LD-79, LABINCO, Breda, the Netherlands) for 1 h to allow sufficient contact time between solvent and matrix. Thereafter, 1.25 mL of water (ultrapure quality with resistivity of 18.2 MΩ*cm) was added to the tubes and were left to stand for 10 min. The tubes were then centrifuged (1780×*g*) for 10 min. The upper layer (organic phase) was collected using glass Pasteur pipettes (150 mm; DWK, Mainz, Germany) with a rubber bulb and transferred to quartz digestion tubes (ultraWAVE, Milestone, Sorisole, Italy). The tubes were subsequently placed in a heated nitrogen evaporator (40 °C; Reacti-Therm, Thermo Fisher Scientific, Waltham, MA, USA) until a lipid pellet was obtained. These were then analyzed for total As as described in the succeeding section.

2.2.4. Total As analysis

Total As analysis was carried out by ICP-MS, as elaborated by Julshamn et al. (2007). Two mL of HNO_3 was added to quartz digestion tubes containing the lipid pellets or 0.2 g of freeze-dried sample in 500 μL of water. This was then followed by microwave digestion (UltraWAVE, Milestone, Sorisole, Italy). The heating program which lasted for 62 min involved gradual increase of temperature to 260 °C and 25 min of cooling. After allowing to cool, the digested solutions were quantitively transferred to a 25-mL volumetric flask, diluted to volume with water, and transferred to 50-mL centrifuge tubes. Analysis was performed using an iCAP Q ICP-MS (Thermo Fisher Scientific, Waltham,

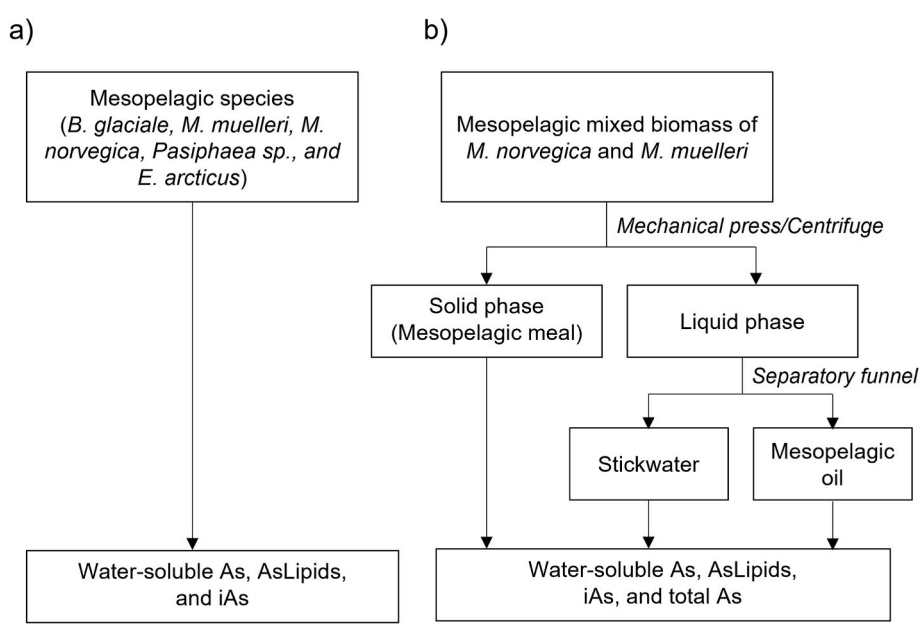

Fig. 1. An overview of the workflow, divided into (a) analysis of mesopelagic single-species samples and (b) processing and analysis of mesopelagic mixed biomass and resulting fractions (As – arsenic; AsLipids – arsenolipids; iAs – inorganic arsenic).

MA, USA) equipped with an SC-4 DX autosampler (Elemental Scientific, Mainz, Germany). Quantification was achieved by generating an external calibration curve from mixed standard solutions containing As, and online internal standard addition of germanium (Spectrascan Teknolab, Ski, Norway). To evaluate accuracy of results, certified reference materials of lobster hepatopancreas (TORT-3; National Research Council Canada, Ottawa, Ontario, Canada) and oyster tissue (SRM 1566b; National Institute of Standards and Technology, Gaithersburg, MD, USA) were analyzed in duplicate for each analytical series. Data processing was facilitated through the Qtegra software (v. 2.10, 2018, Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Quality assurance and control

The methods for the determination of iAs and total As are routine analyses at the Institute of Marine Research (Bergen, Norway) and are accredited by the Norwegian accreditation body according to NS-EN ISO/IEC 17025:2017. The method for water-soluble As species has been validated and method performance characteristics were presented in our previous work (Tibon et al., 2021). In the current study, analyses were done using either two or three replicates. An extraction blank was always included, and one of the calibration standards was injected periodically and at the end of the series to check for instrument drifts. The measured concentrations for the certified reference materials (CRM) were in good agreement with the certified values (Table S1). Results were within twice the standard deviation for the certified values, which is the acceptable limit in statistical control charts.

2.4. Processing factors

Processing factors (PF) were calculated based on the approach of Berntssen et al. (2021) and patterned after the European Food Safety Authority's (EFSA) definition (Scholz et al., 2018). EFSA uses PFs to give insight on the transfer of pesticide residues from raw agricultural commodities to processed products. Following the same approach, PF in this study will be expressed as the ratio of the concentration (in mg/kg) of the As species in produced mesopelagic meal (dw) or oil (ww) and the concentration (in mg/kg) of the As species in the raw mesopelagic biomass as starting material (dw). Mathematically, this is shown as:

$$PF = \frac{c_{meal\ (dw)\ or\ oil\ (ww)}}{c_{mesopelagic\ biomass\ (dw)}}$$

3. Results and discussion

3.1. Arsenic speciation in mesopelagic species

3.1.1. Total As

At least three pooled samples per species were analyzed and the average concentrations varied greatly, ranging from 2.2 to 28 mg/kg ww. The crustaceans (*M. norvegica*, *Pasiphaea* sp., and *E. arcticus*) generally had higher concentrations compared to the fish species (*B. glaciale* and *M. muelleri*), with one of the pooled samples of *M. norvegica* containing a total As of 52 mg/kg ww. However, since As species vary in toxicity, total As levels do not always give sufficient information, thus, calling for As speciation data.

3.1.2. Arsenobetaine

The mesopelagic samples contained at least 12 As species, of which AB was the most predominant form (Table 1). The fish species *B. glaciale* and *M. muelleri* had lower average AB concentrations at 2.2 ± 0.1 and 2.4 ± 1.0 mg/kg ww, respectively, while the crustaceans *M. norvegica* and *Pasiphaea* sp. had higher mean values at 14.2 ± 10.1 and 15.5 ± 10.7 mg/kg ww, respectively. This trend in AB concentration among the samples is very similar to total As concentration. Indeed, it was verified that a positive correlation ($R^2 = 0.996$, $p < 0.001$) exists between total As and AB (Fig. S1), similar to an earlier report for various types of seafood (Wolle et al., 2019b). When excluding the two highest points in Fig S1, the resulting R^2 is 0.965.

In several surveys of As species in seafood, it was observed that low-trophic marine animals such as shrimps contain higher AB concentrations than fish species which are positioned higher in the food chain (Ruttens et al., 2012; Wolle et al., 2019b; Luvonga et al., 2021). This has been generally attributed to their diet, habitat, and metabolic abilities (Kato et al., 2020). In the present work, while all samples were collected from the mesopelagic zone, the variation in As and AB concentrations could be explained by their differences in feeding behaviors. The fish species *B. glaciale* and *M. muelleri* are zooplanktivores, depending mostly on copepods, amphipods, and krill (García-Seoane et al., 2013). In contrast, crustaceans, such as *M. norvegica,* are omnivores which have been observed to scavenge the seabed for copepods and phytoplanktons (Schmidt, 2010), which can be significant sources of As. This is supported by another study which reported that elevated levels of total As and AB found in the shrimp *Metapenaeopsis palmensis* were due to their dependence on benthic food present in sediments (Zhang et al., 2018).

Table 1
Concentrations of total As and As species in mesopelagic samples (mg/kg ww, mean ± SD, $n = 3$).

As species	Fish species		Crustaceans		
	B. glaciale	*M. muelleri*	*M. norvegica*	*Pasiphaea* sp.	*E. arcticus*
Water-soluble species					
AsSug OH	0.077 ± 0.007	0.049 ± 0.005	0.15 ± 0.02	0.03 ± 0.01	0.077 ± 0.003
AB	2.2 ± 0.1 (2.1–2.2)[c]	2.4 ± 1.0 (1.6–3.5)[c]	14.2 ± 10.1 (8.0–25.8)[c]	15.5 ± 10.7 (7.8–27.7)[c]	5.3 ± 2.3 (4.4–7.9)[c]
TMAO	0.050 ± 0.004	0.08 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.007 ± 0.003
TMAP	0.007 ± 0.001	0.007 ± 0.001	0.14 ± 0.04	0.08 ± 0.04	0.042 ± 0.009
AC	0.048 ± 0.006	0.034 ± 0.007	0.05 ± 0.01	0.04 ± 0.04	0.049 ± 0.005
TETRA	<0.003	<0.003	0.005 ± 0.003	0.006 ± 0.003	<0.003
DMA	0.027 ± 0.008	0.29 ± 0.06	0.017 ± 0.003	0.013 ± 0.001	0.016 ± 0.006
AsSug PO4	<0.005	0.03 ± 0.01	0.09 ± 0.02	0.02 ± 0.01	0.04 ± 0.01
AsSug SO3	<0.003	<0.003	<0.003	<0.003	0.004 ± 0.001
MA	<0.003	0.007 ± 0.003	<0.003	0.002 ± 0.001	<0.003
iAs	<0.007	<0.007	0.06 ± 0.09	0.013 ± 0.007	<0.007
Unknowns	0.001 ± 0.001	0.007 ± 0.002	0.069 ± 0.003	0.04 ± 0.02	0.022 ± 0.002
AsLipids	1.4 ± 0.1	1.6 ± 0.3	2.8 ± 1.4	5.1 ± 4.7	2.1 ± 0.4
Sum[a]	3.8 ± 0.1	4.5 ± 0.8	17.5 ± 11.5	20.9 ± 15.5	7.6 ± 2.7
Total As	3.8 ± 0.1	5.1 ± 1.2	20.2 ± 13.6	21.2 ± 15.3	7.9 ± 3.0
Recovery (%)[b]	99 ± 3	88 ± 7	87 ± 2	99 ± 2	97 ± 4

[a] Sum = Sum of water-soluble As + AsLipids.
[b] Recovery = (Sum/Total As) *x* 100.
[c] Range of values.

It is well-known that AB is the most abundant form of As in marine organisms (Molin et al., 2015; Chen et al., 2020; Luvonga et al., 2020), usually accounting for at least 70% of total As (Francesconi and Raber, 2013). In the present study, the proportion of AB ranged from 45% to 75% of total As (Fig. 2). The crustaceans generally had higher percentage of AB (~70%) compared to the fish species (~50%). This agrees with an earlier report involving the North Pacific krill (*Euphausia pacifica*), a close relative of *M. norvegica*, wherein AB was the major As species (Shibata et al., 1996). In the same study, it was found that copepods (*Calanus* sp.) contained very little AB (0.21 mg/kg dw). Copepods are one of the principal food sources of *B. glaciale* (García-Seoane et al., 2013), which could explain the relatively lower fraction of AB in the fish species. As for the crustaceans, a study involving the common shrimp *Crangon crangon* showed that 42% of AB acquired through food was retained (Hunter et al., 1998). In contrast, exposure to water-borne AB only resulted in a small increase in concentration. AB has been detected in seawater, although at very low levels (0.5–10 ng/kg) (Glabonjat et al., 2018). This suggests that AB is mainly acquired from dietary sources rather than uptake from seawater.

3.1.3. Other water-soluble As species

Other water-soluble species were present in minor concentrations in the mesopelagic samples, accounting for less than 10% of total As (Table 1, Fig. 2). Inorganic As levels were generally less than 0.007 mg/kg ww (LOQ), with the highest concentration found in *M. norvegica* at 0.06 ± 0.09 mg/kg ww. Among the water-soluble As species, DMA was the second most abundant in *M. muelleri* at 0.29 ± 0.06 mg/kg ww, accounting for approximately 6% of total As. Methylarsonate was only found in *M. muelleri* and *Pasiphaea* sp., albeit at very low levels. Trimethylarsoniopropionate (TMAP) was found in higher concentrations in crustaceans compared to the fish species, ranging from 0.042 ± 0.009 to 0.14 ± 0.04 mg/kg ww. These levels are comparable to those obtained by Wolle et al. (2019b) for different shrimp species, where TMAP concentrations ranged from 0.003 to 0.037 mg/kg. Also, in their study, elevated levels of TMAP (as high as 0.8 mg/kg) were found in several species of crab. It is, however, difficult to conclude whether TMAP is characteristic of crustaceans since only a handful of As speciation studies have measured TMAP (Sloth et al., 2003; Leufroy et al., 2011; Wolle et al., 2019b). Most studies focus on the most common As species such as As (III), As (V), DMA, MA, and AB (Ruttens et al., 2012; Schmidt et al., 2018), hence, the current work hopes to bridge this gap.

Trimethylarsine oxide (TMAO) was slightly higher in *B. glaciale* and *M. muelleri*, while arsenocholine (AC) and tetramethyl arsonium ion (TETRA) were present in trace levels or below LOQ (Table 1). A few unknown peaks were also detected in the chromatograms (Fig. S2) and their concentrations were estimated using the calibration curve of the nearest eluting standard. As for the AsSug, glycerol-arsinoylriboside (AsSug OH) was detected in all samples, ranging from 0.03 ± 0.01 to 0.15 ± 0.02 mg/kg ww. Phosphate-arsinoylriboside (AsSug PO4) was also found in all samples except for the fish species *B. glaciale*. Sulfonate-

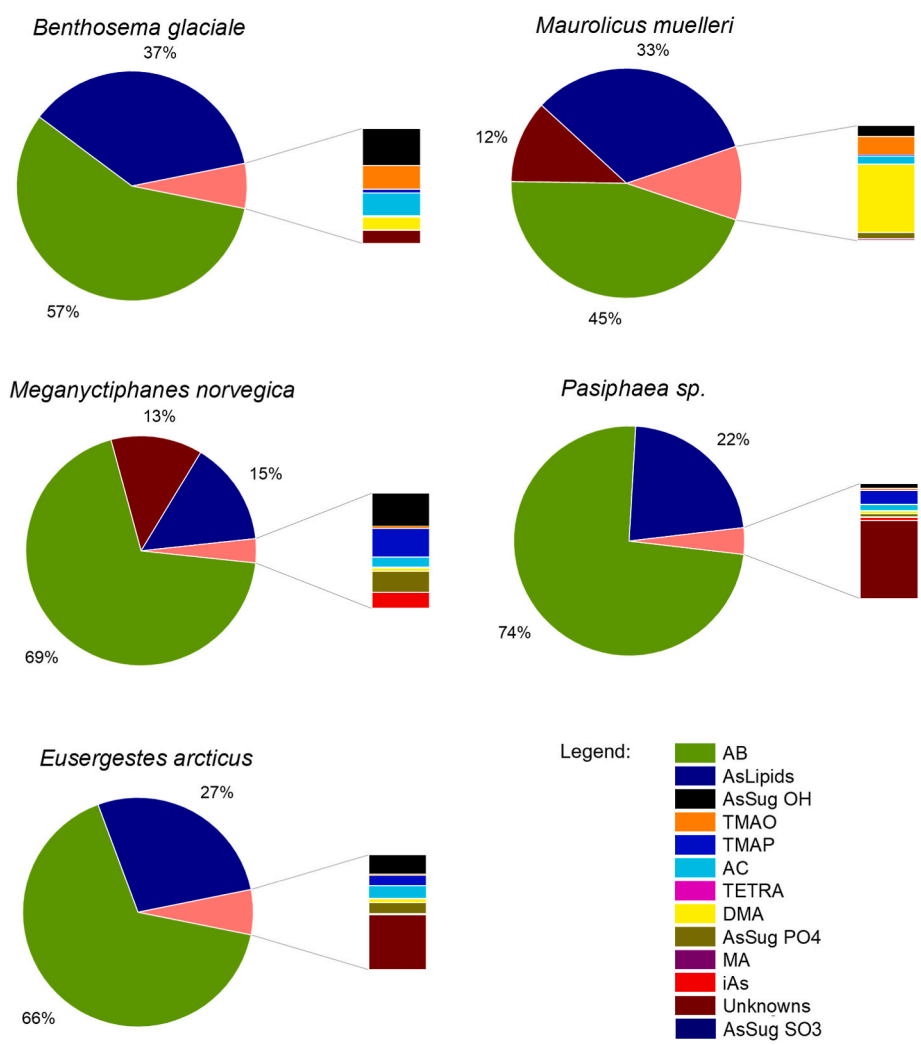

Fig. 2. Arsenic species profile in the mesopelagic samples. Arsenic species fractions are given in % = (concentration of As species/total As) *x* 100. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

arinoylriboside (AsSug SO3) was only detected in the crustacean *E. arcticus*. Sulfate-arsinoylriboside (AsSug SO4) was not found in any of the samples, which was unexpected. Glacier lanternfish (*B. glaciale*) are known to prey on copepods which are rich in AsSug SO4 (Shibata et al., 1996). The absence of AsSug SO4 in the mesopelagic fish samples could suggest that this AsSug is biotransformed to other As forms in the mesopelagic food web. The varying presence of AsSug in crustaceans could be attributed to their preference for different phytoplanktons as food. In contrast, Wolle et al. (2019b) did not find any AsSug in shrimp samples, while they were found in crabs and clams.

3.1.4. AsLipids

Notable concentrations of As in the lipid fraction, corresponding to AsLipids, were found in all samples. Lowest mean concentration was found in the fish species *B. glaciale* at 1.4 ± 0.1 mg/kg ww (Table 1), while the highest level was observed in the crustacean *Pasiphaea* sp. at 5.1 ± 4.7 mg/kg ww. In general, higher concentrations of AsLipids were found in crustaceans (*M. norvegica*, *Pasiphaea* sp., *E. arcticus*) compared to mesopelagic fish species (*B. glaciale* and *M. muelleri*). However, looking at the proportion of AsLipids relative to total As (Fig. 2), *B. glaciale* and *M. muelleri* contained 33%–37% AsLipids, while the crustaceans only had around 20% AsLipids. AsLipids are usually associated with 'fatty' fish such as herring (Lischka et al., 2013) and blue whiting (Taleshi et al., 2014), among others. In the work by Wiech et al. (2020), the mean fat contents for *B. glaciale* and *M. muelleri* were 14% and 18%, respectively, which were two to three times higher than the fat content in the crustaceans. The higher fat content in the fish species could hence explain the larger proportion of AsLipids compared to the crustaceans.

AsLipids can occur as arsenic-containing hydrocarbons (AsHC), arsenic-containing fatty acids (AsFA), and AsSug phospholipids (AsPLs), among others (Sele et al., 2012; Luvonga et al., 2020). AsHCs were the major AsLipids in salmon (*Salmo salar*), specifically oxo-analogs of AsHC 332, AsHC 360, and AsHC 404 (Xiong et al., 2022). In contrast, AsFA 362, AsFA 448, and AsFA 528 are more common in tuna fillet, and AsFA 360 and AsFA 422 in kelp (Liu et al., 2021). Planktons collected from the North Atlantic revealed prevalence of AsPLs, mainly AsPL 958, AsPL 978, and AsPL 1006 (Glabonjat et al., 2021). In a study involving the Mediterranean mussel (*M. galloprovincialis*), traces of AsPLs and arsenic-phytol derivatives (AsPT) were found (Freitas et al., 2020). These AsLipids were initially reported in algae, which suggest that transfer of AsLipids occur through the diet (Freitas et al., 2020). It would be interesting to conduct further studies on mesopelagic samples involving identification of AsLipids since they can provide an insight regarding the distribution of AsLipids at the bottom of the food chain.

3.1.5. Arsenic mass balance

Arsenic recovery was calculated by comparing the sum of water-soluble As and AsLipids with the total As. Overall, good recoveries were obtained for the mesopelagic samples, ranging from 87 ± 2% to 99 ± 3% (Table 1). This suggests that the method for water-soluble As species was nicely complemented by the extraction technique used for the estimation of AsLipids.

3.2. Processing of mesopelagic biomass into aquafeed

3.2.1. As species

The distribution of As species in mesopelagic biomass and resulting fractions is presented in Fig. 3 (see Table S2 for tabulated values). The discussion on total As and its compliance to existing MLs in animal feed and feed materials (Directive, 2002/32 EC and amendments) was already presented in an earlier work (Berntssen et al., 2021). The focus of this section will therefore be on the organic As species. The main As compound found in the initial mesopelagic biomass was AB, accounting for 57% of total As (Fig. 3). The same can be observed for the stickwater wherein AB attributes for 80% of total As. In contrast, the mesopelagic meal only had approximately 32% of total As in the form of AB. This is a notable difference compared to fish meals produced from herring and blue whiting where the water-soluble As accounted for 71%–93% of total As (Pétursdóttir et al., 2018). In the present study, the majority of As in the mesopelagic meal was found as AsLipids (45% of total As). It can be assumed that AsLipids would partition mostly with the mesopelagic oil. However, the results show that AsLipids tend to bind also with the solid phase after the extrusion process. This agrees with an earlier study wherein residual lipids were found in fish meal processed from another species of lanternfish (*Benthosema pterotum*) (Haque et al., 1981). Fish meal typically end up having varying lipid content after production, which also dictates which type of fish protein concentrate it will be classified under (Einarsson et al., 2019; Hilmarsdottir et al., 2020). It can be presumed that AsLipids contribute to the total lipid content in fish meal. The final lipid content is highly dependent on the quality of the raw material and process parameters (Hilmarsdottir et al., 2020), which could also dictate the distribution of AsLipids in the produced meal and oil. In fish meals produced from capelin, Amayo et al. (2011) found AsHC 332, AsHC 360, AsHC 404 as the major AsLipids. Another study on herring and blue whiting fish meals reported the same set of AsLipids as the dominant species (Pétursdóttir et al., 2018).

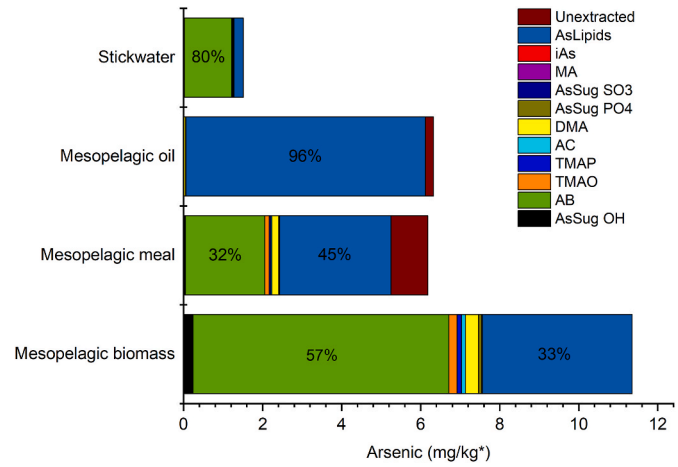

Fig. 3. Bar graph showing the fractions of As species (% = (concentration of As species/total As) *x* 100) in the mesopelagic biomass and produced mesopelagic meal, oil, and stickwater after feed processing (*Unit is in mg/kg dw for mesopelagic biomass and meal; mg/kg ww for mesopelagic oil and stickwater). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

As expected, mesopelagic oil was mostly comprised of AsLipids (~96%). Minor concentrations of DMA and MA were found, which can just be degradation products of AsLipids, as seen in previous studies (Amayo et al., 2014; Pétursdóttir et al., 2018). The presence of AsLipids in different types of fish oil has been described extensively in literature. Fish oil from Peruvian anchoveta (*Engraulis ringens*) contained AsHC 332 and AsHC 360 as major AsLipids, and AsFA 250, AsFA 278, AsFA 292 as minor species (Pereira et al., 2016). Similarly, Sele et al. (2014) found AsHC 332, AsHC 360, and AsHC 404 as the prevalent As compounds in commercial fish oil samples of blue whiting and anchovy. In contrast, krill oil mostly contained AsFA 362, AsFA 390, and AsFA 436 among others (Liu et al., 2021). Comparing the AsLipids found in fish meal (Pétursdóttir et al., 2018) and fish oil (Sele et al., 2014), both from blue whiting, it can be observed that the same set of AsLipids were present as dominant species. It appears that the partitioning of AsLipids does not follow a specific pattern (e.g. AsHC in fish meals and AsFA in fish oils).

As for the stickwater, AsLipids were the second most abundant species, accounting for approximately 16% of total As. The presence of AsLipids in stickwater is expected since stickwater was still found to contain lipids (approximately 2%) after centrifugation in a commercial fish meal production (Hilmarsdottir et al., 2020). In aquafeed ingredient

processing, stickwater is usually further concentrated by evaporating the water and then centrifuged, producing an oil and another concentrate. The oil is normally added to the first oil extracted, while the dried concentrate is mixed with the press cake, and eventually further dried until the final fish meal is produced (Hilmarsdottir et al., 2020).

Among the hydrophilic As species, DMA was the second most abundant in mesopelagic biomass and meal, representing 3% of total As. AsSug OH was also present in the biomass, meal, and stickwater, ranging from 0.8% to 2.5% of total As. AsSug PO4 was only found in the biomass and meal, while AsSug SO3 was only detected in the starting biomass (Table S2). The absence of AsSug in the mesopelagic oil suggests that AsSug only have affinity with the solid and aqueous phases. Other organic As forms were found in trace levels in the mesopelagic biomass and resulting fractions. Inorganic As concentrations were all less than 0.007 mg/kg ww (LOQ). Most of the As species present in mesopelagic biomass were also observed in mesopelagic meal. Arsenic species detected as trace compounds in mesopelagic biomass, specifically MA and AsSug SO3, seem to have degraded and/or transformed to other As forms as they were not detected in quantifiable levels in the resulting fractions. As for the As mass balance, good recoveries were obtained overall, ranging from 85 ± 3% to 119 ± 8% (Table S2). The higher recoveries obtained could be due to overestimation of the AsLipids fraction, e.g. that some water-soluble As species were co-extracted with the AsLipids.

It should be clarified that a process mass balance for As, i.e. As in the starting mesopelagic biomass is equal to the sum of the As in the meal, oil, and stickwater fractions, was not possible to calculate due to lack of data on the weights of the resulting fractions.

3.2.2. *Processing factors*

PFs were calculated following the approach of Berntssen et al. (2021) to verify possible up-concentration (PF > 1) or dilution/removal/degradation (PF < 1) of As species during aquafeed ingredients processing (Table 2). For the mesopelagic meal, results suggest that all compounds were diluted/removed/degraded after processing. In contrast, an up-concentration was observed for AsLipids in mesopelagic oil wherein a PF of 1.4 was calculated. The opposite was seen in stickwater where AsLipids were diluted. This is expected due to their different polarities, with stickwater being mostly water with some particles. In the mesopelagic meal, the dilution effect was more pronounced for the AsSug, having low PFs (0.2). In particular, AsSug SO3 was detected in low levels in the starting mesopelagic biomass but was absent in the resulting fractions. While not completely conclusive, a dilution in AsSug could suggest transformation to other forms. In a study involving macroalgae, AsSug were found to degrade to DMA, MA, and As (V) (Duncan et al., 2015). Similarly, Wolle et al. (2019a) reported a matrix-induced transformation of spiked AsSug to their thiolated counterparts in finfish and crustaceans. Degradation of AsSug is possible with the aid of marine microbes, through acid or base hydrolysis, or exposure to gastric-type conditions (Chen et al., 2020; Luvonga et al., 2020). If indeed the AsSug underwent transformation in this study, this could explain the slightly higher PFs in mesopelagic meal for the simple methylated arsenicals (i.e. DMA, MA).

Table 2
Processing factors of As species indicating up-concentration (PF > 1) or dilution/removal/degradation (PF < 1) during processing; expressed as median (range), $n = 4$.

	Mesopelagic meal	Mesopelagic oil	Stickwater
AB	0.3 (0.3–0.4)		0.19 (0.16–0.22)
TMAO	0.6 (0.5–0.6)		0.10 (0.05–0.14)
TMAP	0.4 (0.3–0.4)		0.18 (0.17–0.19)
DMA	0.6 (0.5–0.8)	0.2 (0.1–0.4)	0.02 (0–0.03)
MA	0.4 (0.2–0.8)	0.2 (0.1–0.4)	
AC	0.2 (0.2–0.2)		0.12 (0.08–0.14)
AsSug OH	0.2 (0.2–0.2)		0.10 (0.08–0.12)
AsSug PO4	0.2 (0–0.4)		
AsSug SO3			
AsLipids	0.8 (0.6–0.9)	1.4 (1.2–2.6)	0.06 (0.04–0.09)

Normally, PFs are applied to processes which remove or reduce compounds due to exclusion of certain parts of the commodity, e.g. removal of rice hull and non-edible parts of the fruit (Scholz et al., 2018). The use of PFs in this study is not conventional but was applied to provide an indicative value of how levels of As species are affected during aquafeed processing.

4. Feed and food safety implications

Due to their abundance and nutritional composition, the sustainable harvest of mesopelagic species has the potential to address micronutrient deficiency and contribute to food and feed security (Alvheim et al., 2020; Nordhagen et al., 2020; Olsen et al., 2020). However, a thorough risk assessment is needed due to inherent undesirable substances, including As. The negative reputation associated with As in terms of toxicity is mainly due to its inorganic forms, arsenite (As (III)) and arsenate (As (V)), classified by IARC as carcinogenic (IARC Working Group, 2012). The current EU food legislation (Commission Regulation (EC) No 1881/2006) only has set maximum limits for iAs, specifically in rice and products derived therefrom (European Commission, 2006). As for products intended for animal feed, Directive 2002/32/EC only imposes limits for total As, but the legislation also specifies that it should be possible to demonstrate that the iAs content is below 2 ppm (European Commission, 2002). The current study revealed low levels of iAs in all samples, showing compliance to applicable regulations.

Arsenobetaine, on the other hand, is generally considered non-toxic (Kaise et al., 1985; Sabbioni et al., 1991). AB was the most abundant As compound in the mesopelagic single-species samples and was present in high concentrations especially among crustaceans. So despite it was reported in our previous study that total As concentrations for some crustaceans exceeded the limits in feed legislation (Wiech et al., 2020), the current work shows that AB made up majority of the As species and does not pose a toxicological concern based on latest assessment (IARC Working Group, 2012). On the other hand, AsLipids were present in significant proportions in *B. glaciale* and *M. muelleri*. The lab-scale feed processing study demonstrated that a considerable fraction of AsLipids bound to the mesopelagic meal. While levels were generally low, potential neurotoxicity and cytotoxicity were reported in *in-vitro* and *in-vivo* studies where AsHCs were observed to exhibit similar or stronger toxicity than iAs, while AsFAs were generally less toxic than AsHCs (Meyer et al., 2014a, 2014b; Witt et al., 2017; Muller et al., 2018). A limitation of our study was that only estimates of bulk AsLipids concentration were provided. Since the toxicity of AsLipids also vary per species (Witt et al., 2017; Muller et al., 2018), future work should focus on complete analytical characterization of individual AsLipid compounds present in mesopelagic samples by HPLC-ICP-MS or coupling HPLC to high-resolution mass spectrometry (HPLC-HRMS).

An up-concentration of AsLipids was seen in the mesopelagic oil. The mesopelagic biomass used to produce the oil contains Northern krill, among others (Berntssen et al., 2021). Krill oil from Antarctic krill is currently considered as an alternative source of EPA and DHA and has gained approval from EFSA as a novel food ingredient (EFSA, 2009). In the EFSA Scientific Opinion, while it was reported that iAs is < 0.1 mg/kg, the Working Group recognized the need for organic arsenic data (EFSA, 2009). The data from the present study show that mesopelagic oil from Northern krill (and silvery lightfish) comprises mainly of potentially toxic AsLipids and does not contain AB, contrary to what was suggested in previous reports (EFSA, 2009). This corroborates an earlier study which detected the presence of AsFAs in krill oil (Liu et al., 2021). These findings, again, highlight the importance of As speciation, especially in novel ingredients intended for human and animal consumption. In the aquafeed industry, the produced fish oil usually undergoes decontamination procedures to remove organic contaminants such as

dioxins and dioxin-like PCBs (Knutsen et al., 2017). These additional processing steps can further reduce the level of certain AsLipids in the final fish oil product, as Sele et al. (2013) reported.

The AsSug were present in trace levels and their distribution varies among mesopelagic organisms. The feed processing experiment also suggests possible transformation of AsSug to other forms. Arsenosugars in their native forms are considerably less toxic than iAs (Leffers et al., 2013). However, these are bio-accessible to human and could yield metabolites which were demonstrated to induce cytotoxic effects (Feldmann and Krupp, 2011; Taylor et al., 2017; Chen et al., 2020; Luvonga et al., 2020).

5. Conclusion

Arsenic species were found in varying concentrations in the mesopelagic single-species samples, processed mesopelagic meal and oil. The non-toxic AB was the major As compound in mesopelagic fish and crustaceans, while AsLipids were also found in significant concentrations. Other As species, including the carcinogenic iAs, were present in low levels. The feed processing study demonstrated transfer of potentially toxic AsLipids from the mesopelagic biomass to both mesopelagic meal and oil, providing a novel insight regarding the partitioning of As during aquafeed processing. Due to the prevalence of AB, it can be presumed that the use of mesopelagic resources as feed ingredients will not pose any arsenic-related hazards. However, the possible adverse effects of AsLipids cannot be neglected and needs to be further studied. An overall assessment regarding the suitability of mesopelagic species whether as food or feed ingredient in light of As compounds is challenging due to (1) lack of occurrence data for As species, (2) lack of toxicological data for the less common As species, partly due to unavailability of compound standards which hampers toxicity studies, and (3) the surrounding issues on exploitation of mesopelagic resources, specifically impacts on biodiversity and carbon sequestration. A holistic evaluation is needed, and the current study aims to contribute by providing valuable As speciation data which can be used for future risk assessments on the feasibility of harnessing mesopelagic biomass as feed ingredients.

Author contributions statement

Jojo Tibon: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Heidi Amlund:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Ana I. Gomez-Delgado:** Investigation, Writing – review & editing. **Marc H. G. Berntssen:** Resources, Writing – review & editing. **Marta S. Silva:** Resources, Writing – review & editing. **Martin Wiech:** Resources, Writing – review & editing. **Jens J. Sloth:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Veronika Sele:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.134906.

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Table S1. Measured concentrations and certified values of certified reference materials (CRM) for arsenic (As) species and total As (mg/kg dw, mean ± SD, $n \geq 3$).

CRM	Sample type	Analyte	Certified values	Measured concentration
BCR-627	Tuna fish tissue	AB	3.9 ± 0.2	3.6 ± 0.1
		DMA	0.15 ± 0.02	0.14 ± 0.01
		iAs[a]	21 ± 3[b]	21.5 ± 0.3
DORM-4	Fish protein	AB	3.95 ± 0.36	4.21 ± 0.04
SRM 1566b	Oyster tissue	Total As	7.65 ± 0.65	7.60 ± 0.14
TORT-3	Lobster hepatopancreas	Total As	59.5 ± 3.8	64.9 ± 0.3
ERM-BC211	Rice	iAs[a]	124 ± 11	119 ± 1

[a]Unit is µg/kg
[b]In-house established reference values

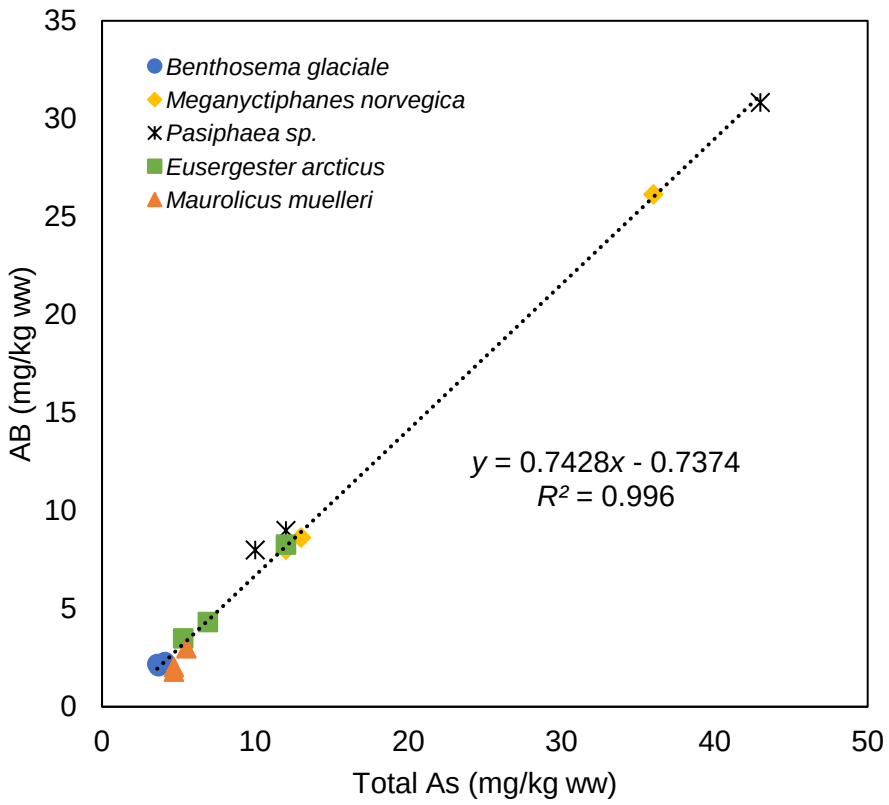

Fig. S1. Plot of total arsenic (As) versus arsenobetaine (AB) concentrations in mesopelagic single-species samples (R^2 = 0.996, p < 0.001).

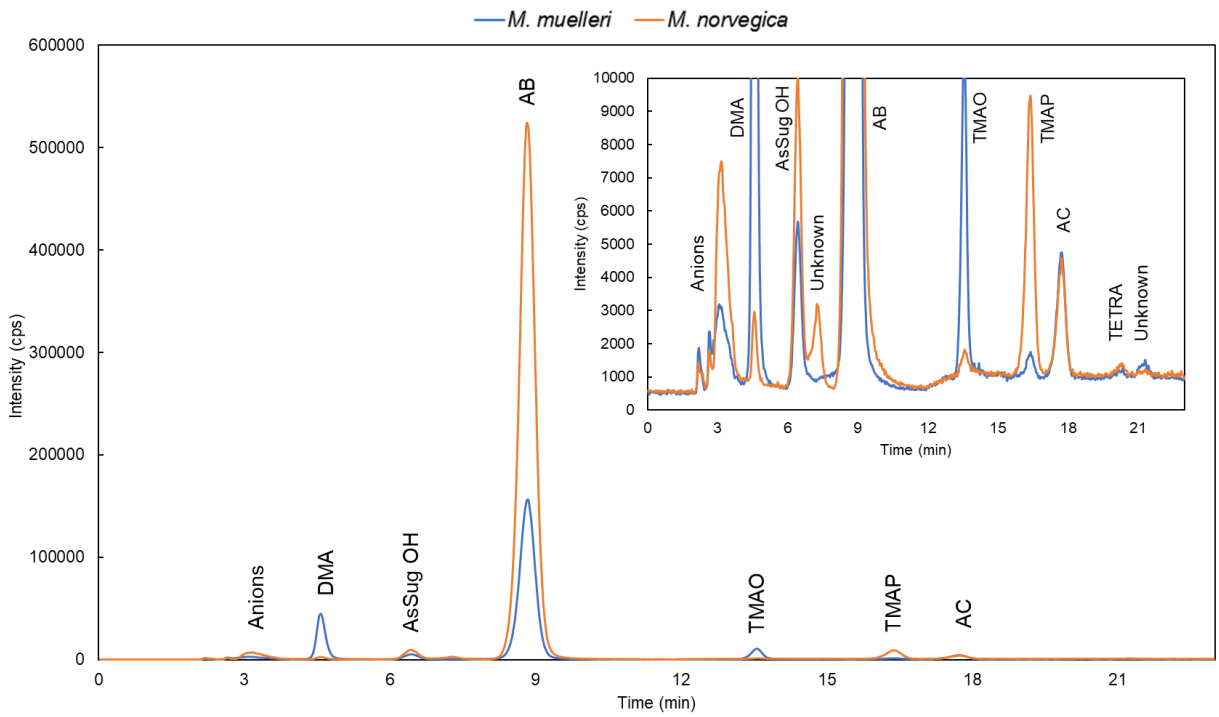

Fig. S2. Overlaid chromatograms of *M. muelleri* and *M. norvegica*, analyzed by cation-exchange HPLC-ICP-MS method described in Tibon et al. (2021).

Table S2. Concentrations of total arsenic (As) and As species in mesopelagic biomass and resulting fractions after feed processing (mg/kg dw, mean ± SD, *n* = 4).

As species	Mesopelagic biomass (mg/kg dw)	Mesopelagic meal (mg/kg dw)	Stickwater (mg/kg ww)	Mesopelagic oil (mg/kg ww)
Water-soluble species				
AsSug OH	0.3 ± 0.2	0.05 ± 0.04	0.03 ± 0.02	< 0.002
AB	6.5 ± 2.9	2.0 ± 0.6	1.2 ± 0.4	< 0.003
TMAO	0.21 ± 0.07	0.11 ± 0.04	0.02 ± 0.01	< 0.004
TMAP	0.1 ± 0.1	0.04 ± 0.03	0.02 ± 0.02	< 0.001
AC	0.11 ± 0.05	0.03 ± 0.01	0.01 ± 0.01	< 0.005
DMA	0.3 ± 0.2	0.18 ± 0.07	< 0.017	0.06 ± 0.01
AsSug PO4	0.08 ± 0.03	0.02 ± 0.01	< 0.005	< 0.005
AsSug SO3	< 0.011	< 0.003	< 0.003	< 0.003
MA	< 0.011	< 0.011	< 0.003	< 0.011
iAs	< 0.007	< 0.007	< 0.007	< 0.003
AsLipids	3.8 ± 0.9	2.8 ± 0.3	0.24 ± 0.07	6.1 ± 2.0
Sum[a]	11.4 ± 3.5	5.3 ± 0.8	1.5 ± 0.5	6.1 ± 2.0
Total As	9.6 ± 2.8	6.2 ± 1.0	1.3 ± 0.3	6.3 ± 1.9
Recovery (%)[b]	118.6 ± 8.0	84.9 ± 2.5	114.4 ± 9.4	96.7 ± 4.1

[a]Sum = Sum of water-soluble As + AsLipids
[b]Recovery = (Sum/Total As) *x* 100

Arsenic speciation in low-trophic marine food chain – an arsenic exposure study on microalgae (*Diacronema lutheri*) and blue mussels (*Mytilus edulis* L.)

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Abstract

Low-trophic marine organisms such as microalgae and blue mussels are currently considered as novel marine feed ingredients. However, they are known to accumulate undesirable substances from the environment, including arsenic (As). Microalgae can biotransform inorganic As (iAs) to more complex organoarsenic species and transfer these compounds to blue mussels. Knowledge on As uptake, biotransformation, and trophic transfer is important in regard to feed and food safety since As species have varying toxicities. In the current work, exposure experiments were conducted in two parts. The first part involved the exposure to 5 and 10 µg/L As(V) in seawater of the microalgae *Diacronema lutheri*. The second part comprised of exposure to As of blue mussels through the diet by feeding with *D. lutheri* exposed to 5 and 10 µg/L As(V), and through the dissolved phase by exposing to 5 µg/L As(V)

in seawater. The results showed that *D. lutheri* can take up As from seawater and transform it to methylated As species and arsenosugars (AsSug). However, exposure to higher iAs concentrations resulted to accumulation of iAs and less production of methylated As species, suggesting that detoxification mechanisms are overwhelmed. Exposure of blue mussels to 5 µg/L As(V) in seawater showed no accumulation of As. Similarly, no accumulation was noted after feeding the blue mussels with As-exposed *D. lutheri*. Both *D. lutheri* and blue mussels contained notable proportions of simple methylated As species and AsSug. Arsenobetaine (AB) was not detected in *D. lutheri*. The findings suggest that low-trophic marine organisms consist mostly of methylated As species and AsSug, which are the precursors for AB formation in higher-trophic aquatic animals. The use of low-trophic marine organisms as feed ingredients require further studies since AsSug are regarded as potentially toxic, which may introduce new risks in regard to feed and food safety

1. Introduction

Due to their nutritional composition and less carbon footprint associated with their production, blue mussels (*Mytilus edulis* L.) and microalgae are both currently considered as future feed ingredients for farmed salmon (Torres-Tiji et al., 2020; Albrektsen et al., 2022; Tamburini et al., 2022). Blue mussels are active suspension feeders and clear seawater of seston, which comprises plankton (including microalgae), and inorganic and organic particulate matter (detritus) in suspension (Strohmeier et al., 2012; Schöne and Krause, 2016; Beyer et al., 2017). Concomitantly, blue mussels may also take up undesirable substances both in the dissolved phase and bound to particulates present in the area, including arsenic (As). Knowledge on different As compounds in microalgae and blue mussels is essential in terms of feed and food safety since As species have varying toxicities (Francesconi and Raber, 2013).

In the marine environment, As exists as different inorganic and organic compounds (i.e. species). Seawater mainly contains inorganic forms of As (iAs), with As(V) usually more abundant than As(III) (Neff, 2002). In contrast, the non-toxic arsenobetaine (AB) is the predominant As compound in most marine animals (Francesconi, 2010). Seaweeds usually contain high proportions of arsenosugars (e.g. AsSug OH, AsSug PO4, AsSug SO3, AsSug SO4) (Feldmann and Krupp, 2011; Taylor et al., 2017; Luvonga et al., 2020), though elevated levels of iAs have been found in some species of seaweed (Maulvault et al., 2015; Park et al., 2019; Wolle et al., 2021). Arsenolipids (AsLipids) are the main As compounds in marine fats and oils (Sele et al., 2012), but are also present in seaweeds (Pétursdóttir et al., 2019), bivalves (Freitas et al., 2020), and phytoplankton (Glabonjat et al., 2021). Clearly, there is a large variation in the occurrence of As species in marine organisms. This has been usually attributed to environmental factors (e.g. salinity, nutrient availability, etc.), the organism's feeding mode or position in the marine food chain, and ability to take up, biotransform, and eliminate As species (Azizur Rahman et al., 2012; Zhang et al., 2022).

Being at the base of the marine food chain, microalgae play a key role in As cycling. These microscopic, unicellular marine algae serve as the first interface for As transfer by taking up mostly iAs from seawater (Duncan et al., 2010). As a detoxification mechanism, iAs is biotransformed by microalgae to other organic As species through a series of methylation and reduction processes first described by Challenger (1945) (Fig. 1). With the aid of a methyl donor S-adenosylmethionine (SAM) and the enzyme ArsM, arsenite (As(III)) undergoes oxidative methylation, forming methylarsonate (MA(V)), which is eventually reduced to methylarsonite (MA(III)). The MA(III) can then undergo oxidative methylation, forming dimethylarsinate (DMA(V)), which can subsequently be reduced to dimethylarsinite (DMA(III)) (Edmonds and Francesconi, 1987; Chen et al., 2020). More complex organic As

species can arise from the Challenger pathway, such as the formation of AsSug after adenosylation and glycosidation (Edmonds and Francesconi, 1987). Earlier studies revealed that As uptake and biotransformation in microalgae are affected by culture (environmental) conditions (Glabonjat et al., 2018; Papry et al., 2019; Hussain et al., 2021) and species-specific differences in biotransformation efficiencies (Azizur Rahman et al., 2012; Duncan et al., 2015). As primary producers, microalgae have the potential to transfer As to primary consumers such as blue mussels (Van Der Spiegel et al., 2013).

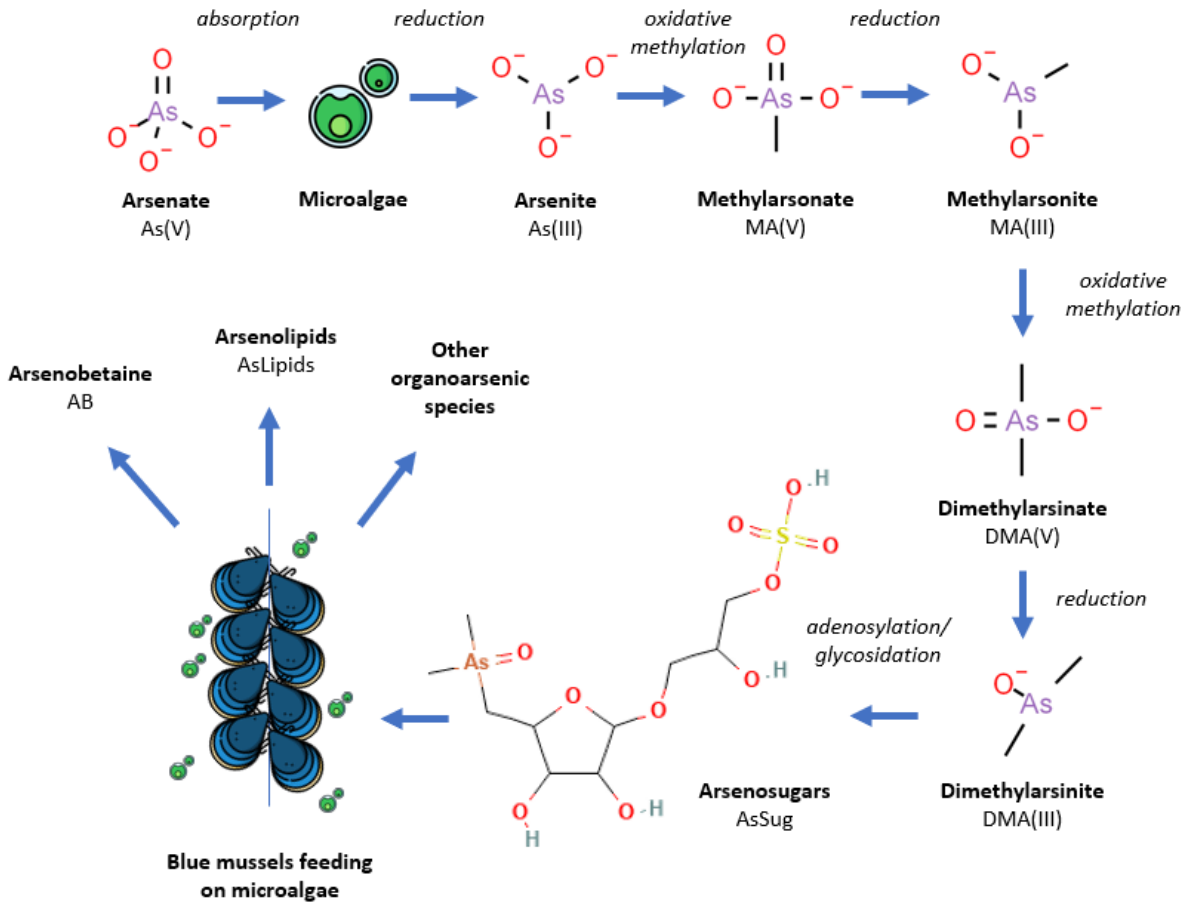

Fig. 1. Pathway for the biotransformation of inorganic arsenic (iAs) to more complex organoarsenic species in microalgae and blue mussels. *Chemical structures retrieved from ChemSpider and PubChem, and icons from Flaticon.*

Elevated levels of iAs (up to 5.8 mg/kg ww) were reported in blue mussels, which was hypothesized to be due to microalgae as part of their diet (Sloth and Julshamn, 2008). Levels of total As (tAs) and iAs in blue mussels were also positively correlated with the degree of As contamination in the area (Whaley-Martin et al., 2012), suggesting that As was taken up by blue mussels from the sediments and water column. Based on these reports, As uptake in blue mussels may occur through the dissolved phase as diffusion via e.g. gills, or through ingestion of particulates (Schöne and Krause, 2016; Beyer et al., 2017). While several studies in blue mussels have investigated the uptake of different As compounds via the dissolved phase, concentrations of As used (as high as 100 μg/L) were magnitudes higher than what is naturally found in seawater (around 2 μg/L) (Gailer et al., 1995; Francesconi et al., 1999; Clowes and Francesconi, 2004). Moreover, there is limited knowledge on how As species are accumulated and biotransformed by blue mussels via ingestion of microalgae, mimicking the natural low-trophic transfer of As and As species. To the best of our knowledge, a controlled trophic study on the uptake, transfer, and biotransformation of As species from seawater to microalgae, and from microalgae to blue mussels, has not yet been performed.

Hence, the present work aims to investigate (1) how microalgae take up and biotransform iAs, (2) how blue mussels accumulate and biotransform As species through the diet after feeding with microalgae exposed to iAs, and (3) how blue mussels take up and biotransform As species through the dissolved phase after exposure to iAs in seawater. Overall, this study aims to give an insight into As biotransformation at the base of the marine food chain.

2. Materials and Methods

2.1 Design of the study

In this study, exposure experiments were divided into two parts (Fig. 2). The first part involved the exposure to iAs of microalgae. Three different experimental cultures were produced: (i) one control culture with no addition of As(V) ('Microalgae Control'), (ii) one culture spiked at 5 µg/L ('Microalgae 5'), and (iii) one culture spiked at 10 µg/L ('Microalgae 10'). The second part of the exposure experiments comprised of exposure to As of blue mussels through the diet and the dissolved phase. Twelve tanks were randomized into four exposure groups (3 replicate tanks per exposure): the control group (Group A), mussels fed with 'Microalgae 5' (Group B), mussels fed with 'Microalgae 10' (Group C), and mussels exposed to 5 µg/L As(V) in seawater (Group D). Mussels in Groups A and D were fed with 'Microalgae Control'. For Group D, seawater was spiked with a standard solution of As(V) to arrive at a nominal concentration of 5 µg/L.

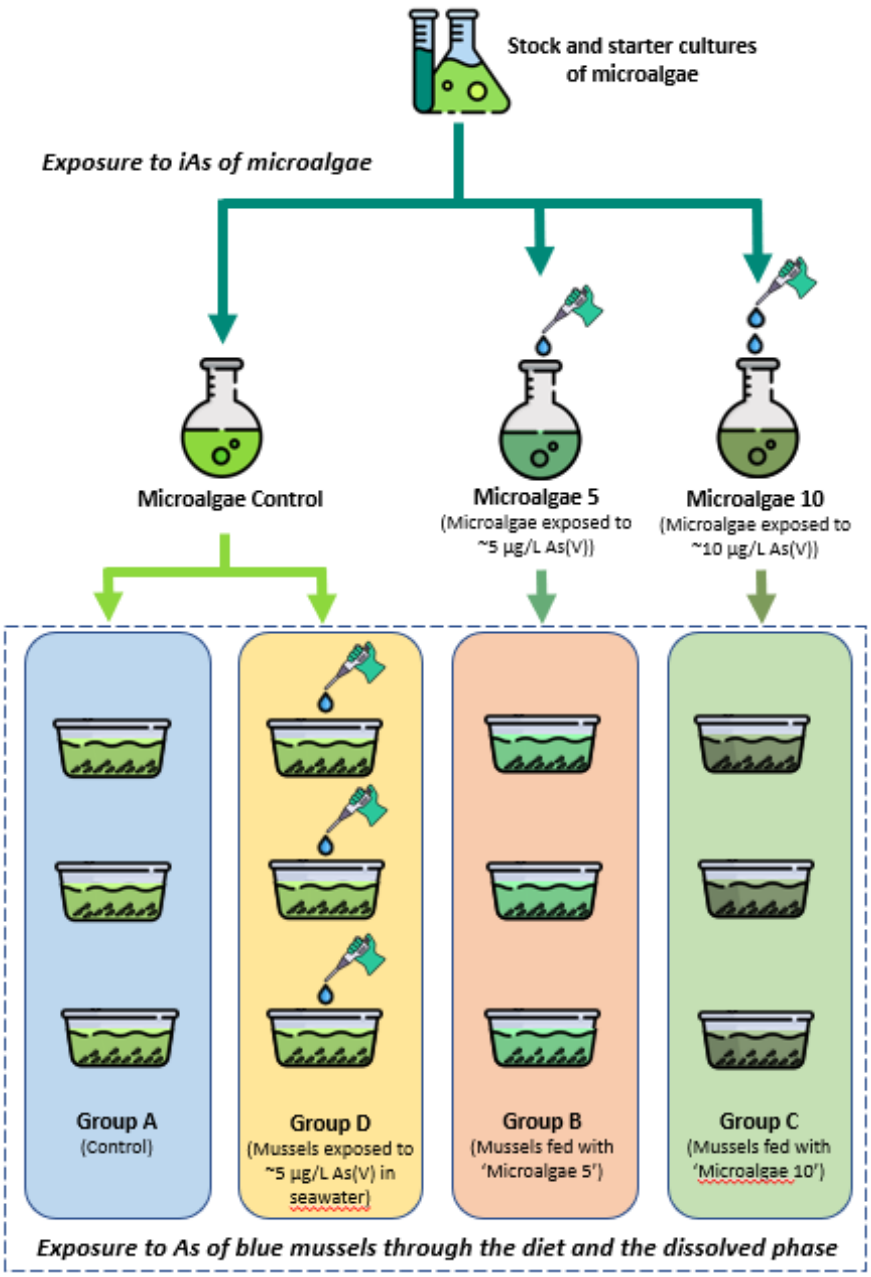

Fig. 2. Experimental overview of the study divided into exposure to inorganic arsenic of microalgae (top) and blue mussels exposed to arsenic through the diet and the dissolved phase (enclosed in dashed lines). *Icons retrieved from Flaticon*.

2.2 Specimens

2.2.1 Microalgae

Diacronema lutheri (Bendif et al., 2011), formerly known as *Pavlova lutheri*, was the microalgae species chosen for the experiments due to its use as aquaculture feed, usually for

bivalves, as a source of essential long-chain polyunsaturated fatty acids (Ponis et al., 2008). Axenic stock cultures of *D. lutheri* were sourced from the Norwegian Institute for Water Research (NIVA). The strain is part of the Norwegian Culture Collection of Algae (NORCCA) with identification number NIVA-4/92. To produce the starter culture (inoculum), 300 mL of the stock culture was grown in 4-L round-bottom flasks with filtered (2 μm), diluted, sterile seawater (20% ultrapure water) enriched with f/2 medium (Guillard, 1975) to a final volume of 1.8 L. Starter cultures were maintained under continuous light and aeration. The growth curve was monitored by daily measurements of cell density using a Z2 Coulter Particle Count and Size Analyzer (size range: 3.4 – 8 μm; Beckman Coulter, Brea, CA, USA), by which stationary phase was noted after five days. Starter cultures were sub-cultured aseptically once a week to maintain the starter culture line.

2.2.2 Blue mussels

Specimens of blue mussels, with shell length ranging from 3.5 to 4.8 cm ($n \approx 1000$), were collected from Hardangerfjord in western Norway between August and September 2020. Mussels were transported to the Austevoll research station of the Institute of Marine Research (IMR) where they were acclimated for two weeks (initial acclimation) in lantern nets submerged 3 m from the water surface. Thereafter, a total of 672 mussels were randomly selected and cleaned of epiphytes and other impurities. For acclimation under laboratory conditions (second acclimation), mussels were then randomly and equally divided into 12 tanks (i.e. 56 mussels per tank), initially containing 40 L of filtered natural seawater pumped from a depth of 160 m. The laboratory acclimation period lasted for another two weeks, wherein mussels were subjected to a 10 h light:14 h dark photoperiod and continuous aeration.

2.3 Chemicals and solutions

Solutions of 10 and 40 mg/L were prepared by diluting quantitatively an aliquot of a 1000 mg/L standard solution of As(V) (Spectrascan Teknolab, Ski, Norway) using ultrapure water (18.2 MΩ-cm).

2.4 Microalgae exposure experiment

2.4.1 Microalgae production and exposure to iAs

Batches of microalgae were produced twice a week and covered 3 to 4 days of feeding. First, a sample of the starter culture was checked for cell viability under a microscope and for cell density. Considering the cell density of the starter culture, the required experimental culture volume, and the target initial cell density of $2x10^6$ cells per L, the required volume of the starter culture was calculated, and transferred aseptically to an appropriate volume of diluted sterile seawater (20% ultrapure water) with f/2 medium in 4-L round-bottom flasks. Three different experimental cultures were produced (Fig. 2). The cultures with 5 and 10 μg/L concentrations of As(V) were prepared by adding appropriate volumes of 10 and 40 mg/L As(V) solutions, respectively. Similar growing conditions as the starter culture were applied (e.g. use of f/2 medium, light regime, aeration, etc.).

After 5 to 8 days of incubation (corresponding to stationary growth phase), a sample of experimental culture was checked for cell viability and cell density. Taking into account the cell density, number of tanks, the volume of seawater in each tank, and the number of mussel feeding times, an appropriate volume of the experimental culture was transferred to 50-mL centrifuge tubes. These were then centrifuged for 5 min at 4000 rpm and 15 °C (Centrifuge 5810 R; Eppendorf, Hamburg, Germany). The supernatant was subsequently removed from each tube using a Pasteur pipette. Cells were resuspended in 50 mL of diluted sterile seawater

(20% ultrapure water). Cell densities were again measured, which formed the basis for the volume of microalgae suspension to be added to each mussel tank to arrive at a cell concentration of $6x10^6$ cells per L.

2.4.2 Microalgae sample preparation

For each experimental culture, 200 to 400 mL was transferred to 50-mL centrifuge tubes. These were then centrifuged followed by removal of supernatant. The precipitates were washed with 5 mL diluted sterile seawater (20% ultrapure water), pooled into one tube, and filled with diluted sterile seawater (20% ultrapure water) up to 50 mL. The tube was subsequently centrifuged, and supernatant was removed. Samples were freeze-dried prior to analysis. Microalgae from the same treatment groups were pooled corresponding to the feeding periods of mussels.

2.5 Blue mussel exposure experiment

2.5.1 Blue mussel exposure to As

Blue mussels were exposed to As either through the diet (*D. lutheri*) or the dissolved phase (seawater). There were four exposure groups with 3 replicate tanks each (Fig. 2). Feeding with microalgae was done three times a day with interval of 2 to 3 hours. The food ration was maintained at $6x10^6$ cells per L each feeding time. A portable particle counter (PAMAS S4031 GO; PAMAS, Rutesheim, Germany) was used to measure particles in the tanks before and after each feeding to have an overview of the feed intake. The exposure experiment lasted for 25 days. During the exposure period, salinity, temperature, and pH were recorded daily. Check for mortality was also part of the routine.

2.5.2 Sampling of blue mussels

Mussels (*n* = 9) were collected from each tank before the first feeding of the day at exposure days 0, 2, 6, 12, 18, and 24, corresponding to time points t0, t1, t2, t3, t4, t5, respectively. Mussels from t0 were collected before As exposure started (i.e. after second/laboratory acclimation). Seawater was replaced every second day and volume was adjusted corresponding to the remaining number of mussels. Throughout the study, a total of 54 samples were collected per tank.

Blue mussels were cleaned prior to analysis. After allowing the samples to dry, shell length, height, and width were measured using a caliper. Whole body weights were then measured, followed by removal of soft tissue. The empty shells were weighed. After a few minutes of draining with tissue paper, soft tissues were also weighed. Subsequently, soft tissues of the nine mussels collected in each tank per time point were pooled, homogenized in a blender, and freeze-dried (FreeZone 18L Freeze Dryer; Labconco, Kansas, MO, USA). A second homogenization was performed afterwards (GRINDOMIX GM 200; Retsch, Haan, Germany). Using the biometric parameters obtained, condition indices (CI) were calculated following the description of Lundebye et al. (1997).

2.6 Sampling of seawater

During the start-up of microalgae production, the following samples were collected: filtered natural seawater, seawater with f/2 medium, and seawater with f/2 medium spiked at approximately 5 and 10 μg/L. This was to verify the As contribution from seawater and f/2 medium, and to measure the actual exposure concentration. During harvesting of cells, samples of the supernatant (culture medium) were also collected after centrifugation to verify if As has indeed been taken up by the microalgae.

Seawater samples were collected during laboratory acclimation of blue mussels. Similarly, throughout the exposure period, seawater samples were taken from the inlet (i.e. directly from the tube providing the seawater pumped from a depth of 160 m) every time the seawater in the tanks was changed. For all tanks, seawater samples were collected before blue mussels were sampled (i.e. at t0, t1, t2, t3, t4, t5). For Group D tanks, seawater collection was more frequent: daily, and before and after seawater was changed, to monitor if there were any changes in As concentration during the exposure period and if correct spiking was done. Seawater samples from replicate tanks (same treatment) were pooled.

2.7 Chemical analysis

2.7.1 Determination of total As

Total As in solid samples was determined after microwave digestion by inductively-coupled plasma mass spectrometry (ICP-MS) as described elsewhere (Julshamn et al., 2007). Briefly, approximately 0.2 g of sample was weighed into quartz digestion vessels followed by addition of 2 mL of concentrated (65%) HNO_3 (purified by sub-boiling distillation; Merck, Darmstadt, Germany). Samples were placed in the UltraWAVE system (Milestone, Sorisole, Italy) and the final clear solution was diluted with ultrapure water to 25 mL. Solutions were analyzed for tAs with iCAP Q ICP-MS (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a helium collision cell and FAST SC 4Q DX autosampler (Elemental Scientific, Omaha, NE, USA). Quantification was carried out using an external calibration curve generated from multielement standard solutions and online addition of germanium as the internal standard (both from Spectrascan Teknolab, Ski, Norway).

2.7.2 Determination of inorganic As

Determination of iAs was carried out by anion-exchange high performance liquid chromatography (HPLC) coupled to ICP-MS (HPLC-ICP-MS) based on EN 16802:2016 (CEN, 2016) and Julshamn et al. (2012). Approximately 0.2 g of sample was weighed into 13-mL polypropylene (PP) tubes. Thereafter, 10 mL of 0.1 M HNO_3 in 3% H_2O_2 (30%; Merck, Darmstadt, Germany) was added, followed by vortex mixing (MS 1; IKA, Staufen, Germany). Samples were left to stand overnight. The tubes were then placed in a water bath (OLS200; Grant, Cambridge, UK) for 1 h at 90 °C while shaking at 100 rpm. After allowing to cool, tubes were centrifuged for 10 min at 3800 rpm (Centrifuge 5702; Eppendorf, Hamburg, Germany). The supernatant was filtered using a 5-mL syringe (Henke-Sass Wolf, Tuttlingen, Germany) with disposable filters (0.45-µm PTFE; Sartorius, Göttingen, Germany) into 1-mL PP HPLC vials. Quantification was achieved using a 1260 Infinity HPLC coupled to a 7900 ICP-MS (Agilent Technologies, Santa Clara, CA, USA), equipped with an anion-exchange IonPac AS7 column (2 x 250 mm) and an IonPac AG7 guard column (2 x 50 mm) (both from Dionex, Sunnyvale, CA, USA). Isocratic elution was carried out using 50 mM $(NH_4)_2CO_3$ in 3% CH_3OH (pH 10.3) as mobile phase. An external calibration curve from As(V) standard solutions (Spectrascan Teknolab, Ski, Norway) was used for the quantification of iAs.

2.7.3 Determination of water-soluble As species

Water-soluble As species were determined by anion- and cation-exchange HPLC-ICP-MS as described elsewhere (Tibon et al., 2021). Approximately 0.2 g of sample was weighed into 13-mL PP tubes and 5 mL of extraction solution (CH_3OH:H_2O, 50% v/v) (CH3OH ≥ 99.97%; Merck, Darmstadt, Germany) was added. This was followed by vortex mixing for a few seconds and water bath heating for 30 min (90 °C, shaking speed at 100 rpm). The samples were allowed to cool and then centrifuged at 3800 rpm for 10 min. The supernatant was

transferred into a 5-mL syringe and filtered (0.45 µm) into another 13-mL PP tube. An aliquot of the supernatant was transferred into a 1-mL HPLC vial and diluted accordingly with the extraction solution. Analysis was carried out using HPLC-ICP-MS (same setup used for iAs determination) but with two different instrument methods. Cationic As species were separated using a Metrosep C6 column (250 x 4.0 mm, 5 µm; Metrohm, Herisau, Switzerland) by gradient elution with pyridine-based mobile phases. For anionic As species, a PRP-X100 column (250 x 4.6 mm, 5 µm; Hamilton, Reno, NV, USA) was used, and gradient elution was employed with carbonate-based mobile phases. Concentrations were determined based on an external calibration curve from mixed standard solutions of As compounds. For simplification, MA(V) and DMA(V) will be referred to as MA and DMA from hereon in this manuscript.

2.7.4 Analysis of seawater samples

Seawater samples were analyzed for tAs using atomic fluorescence (P S Analytical Millennium Excalibur, Kent, UK), after pre-reduction of As(V) to As(III) by 1:1 mixture of seawater with a solution containing 0.5% KI and 0.25% ascorbic acid (both Merck pro analysis grade, Darmstadt, Germany) in 7 M HCl (Merck suprapure grade, Darmstadt, Germany) for 30 min at 25 ºC. This was followed by hydride generation using in-line mixture of the reduced seawater in a 1.2 M HCl carrier stream with 0.7% sodium tetrahydroborate ($NaBH_4$) dissolved in 0.1M NaOH. The generated gaseous arsine was separated in a gas-liquid separator by an Ar-gas stream and passed through a Perma Pure dryer before being introduced to the fluorescence chamber in a hydrogen-flame, excited with an As hollow cathode lamp (Photron, Victoria, Australia) perpendicular to the measured fluorescence signal (P S Analytical APP017). Quantification was done with an external calibration curve of 0.5 to 10 µg/L solutions from the Agilent Multi Element Calibration Standard 2A (Agilent, Santa Clara, California, USA).

2.7.5 Quality assurance and control

Certified and in-house reference materials were included in every analysis and results were in good agreement with certified values. Some of the reference materials used were BCR-627 (tuna fish tissue), ERM CE278k (mussel tissue) (both from IRMM, Geel, Belgium), DORM-4 (fish protein), and NASS-6 and CASS-5 (seawater) (all from National Research Council Canada, Ottawa, Ontario, Canada). For each sample, two or three technical replicates were analyzed. Extraction blanks were also included, and one calibration standard was injected periodically throughout the run to monitor possible instrument drifts. Both iAs and tAs methods are accredited according to ISO/IEC 17025:2017, while the method for water-soluble As species has undergone single-laboratory validation (Tibon et al., 2021).

2.8 Statistics, equations, and software used

2.8.1 Bioconcentration factor

Bioconcentration factor (BCF) is expressed as the ratio of tAs concentration in the microalgae (µg/kg) to the tAs concentration in the dissolved phase or medium (µg/L) (Huang et al., 2021). Mathematically, this is shown as:

$$BCF = \frac{tAs_{microalgae}\ in\ \mu g/kg}{tAs_{medium}\ in\ \mu g/L}$$

2.8.2 Linear mixed models

The tAs content in blue mussels during the experiment was analyzed using linear mixed models. Tank ID was used as random variable to account for the repeated sampling over time in each tank. After data exploration, a Gamma distribution with log link was used. Statistical analyses were performed using R v4.1.2 (RCore Team, 2020) in RStudio (RStudio Team, 2019), with the packages lme4 and lmertest. The initial model had ‘treatment’, ‘CI’, ‘log-

transformed time', and their interactions as fixed effects. Model selection was based on Akaike information criteria (AIC). Model performance and fit were also evaluated by model residuals plots (Zuur et al., 2009). Final model predictions along a combination of covariates were used to further explore the underlying mechanism in As uptake in blue mussels.

2.8.3 Software

Microsoft Excel, Microsoft Powerpoint (Microsoft, Redmond, Washington, USA), OriginPro 2020b (v. 9.7.5.184, OriginLab, Northampton, Massachusetts, USA), and R v4.1.2 (R Core Team, 2020) in conjunction with RStudio (RStudio Team, 2015) were used for statistical analysis and creating figures.

3. Results

3.1 Total As concentrations in media and microalgae

The mixture of seawater and f/2 medium had a tAs concentration of 1.7 μg/L (n = 1), while the seawater with f/2 medium spiked to 5 and 10 μg/L As(V) showed tAs concentrations of 9.7 and 12.8 μg/L (n = 1), respectively. Considering the background As from seawater, the concentrations for seawater with f/2 medium spiked to 5 and 10 μg/L As(V) were acceptable, suggesting that target As exposure concentrations were achieved. For the microalgae samples, 'Microalgae Control' had an average tAs concentration of 1.2 ± 0.1 mg/kg (n = 5), while 'Microalgae 5' had 6.6 ± 0.4 mg/kg (n = 5). Tukey test showed significant difference for the two results ($p < 0.05$). The exposure group 'Microalgae 10' had a similar average tAs concentration as 'Microalgae 5' at 6.6 ± 0.4 mg/kg (n = 5). For BCFs, the values calculated for 'Microalgae Control' and 'Microalgae 5' were comparable at 688 and 678 L/kg, respectively, whereas 'Microalgae 10' had a BCF of 514 L/kg.

3.2 As species in microalgae

Five different As species were detected in *D. lutheri* (Fig. 3). Among the extracted As species in 'Microalgae Control', iAs was predominant at 0.26 ± 0.07 mg/kg (n = 5), accounting for 22% of tAs (Fig. 4). Other major species were DMA and AsSug SO4, with average concentrations of 0.20 ± 0.02 mg/kg and 0.12 ± 0.01 mg/kg (n = 5), respectively. The profile seemed to change when *D. lutheri* was exposed to ~5 μg/L As(V). The main As species found in 'Microalgae 5' was AsSug SO4 at 2.2 ± 0.1 mg/kg (n = 5), comprising 33% of tAs (Fig. 3 and 4). DMA was the second most abundant at 1.38 ± 0.07 mg/kg (n = 5). When *D. lutheri* was exposed to ~10 μg/L As(V), iAs was the most prevalent species at 2.0 ± 0.3 mg/kg (n = 5), though not markedly higher than AsSug SO4, which had an average concentration of 1.7 ± 0.1 mg/kg (n = 5) (Fig. 3). There were two AsSug detected in *D. lutheri*, namely AsSug SO4 and AsSug OH. In all exposure groups, AsSug SO4 was more abundant, approximately two magnitudes higher in concentration than AsSug OH (Fig. 3). In this study, AB was notably not detected in *D. lutheri*, and a relatively high fraction of tAs was unextracted (~20 to 49%).

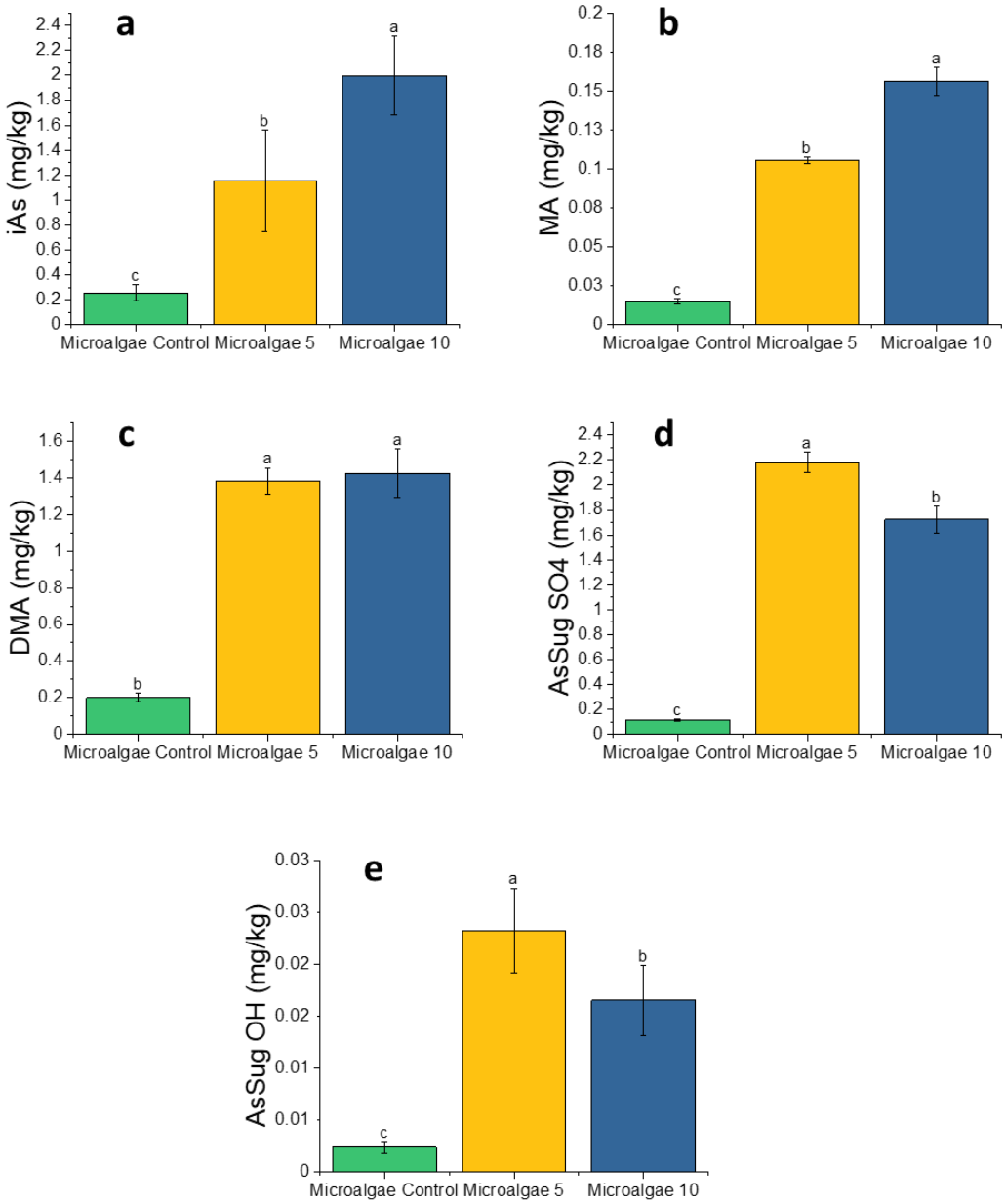

Fig. 3. Concentrations of (a) inorganic arsenic (iAs), (b) methylarsonate (MA), (c) dimethylarsinate (DMA), (d) sulfate-arsenosugar (AsSug SO4), and (e) glycerol-arsenosugar (AsSug OH) in *Diacronema lutheri* grown under different treatments. Different lower-case letters denote statistically significant difference between exposure groups (Tukey test; $p < 0.05$). Legend: Microalgae Control – *D. lutheri* cultured without added As(V), Microalgae 5 – *D. lutheri* exposed to 5 µg/L As(V), Microalgae 10 – *D. lutheri* exposed to 10 µg/L As(V).

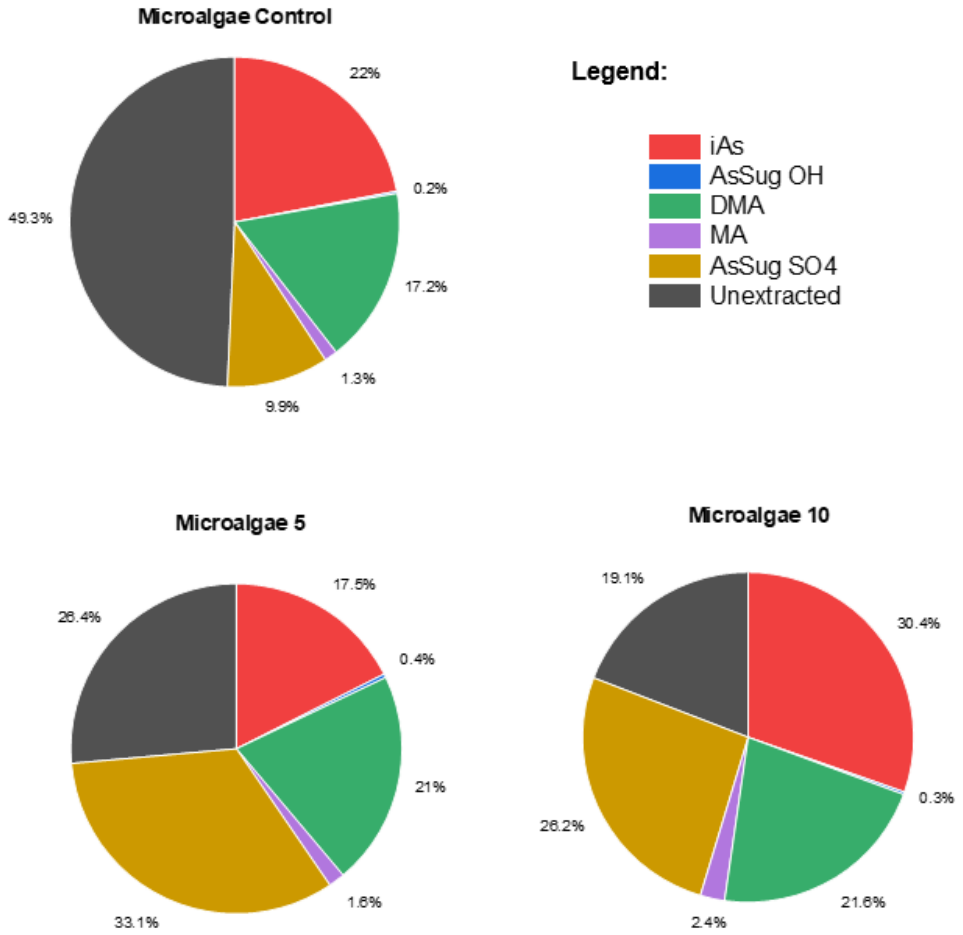

Fig. 4. Arsenic species profile in *Diacronema lutheri* grown under three different treatments. Arsenic species fraction, % = (concentration of As species/total As) x 100. Legend: iAs – inorganic arsenic, MA – methylarsonate, DMA – dimethylarsinate, AsSug SO4 – sulfate-arsenosugar, AsSug OH – glycerol-arsenosugar, Microalgae Control – *D. lutheri* cultured without added As(V), Microalgae 5 – *D. lutheri* exposed to 5 µg/L As(V), Microalgae 10 – *D. lutheri* exposed to 10 µg/L As(V).

The accumulation of iAs and MA seemed to increase as the exposure concentration increased (Fig. 3), and the concentrations in the exposure groups were significantly different (Tukey test; $p < 0.05$). For DMA, the concentration found in 'Microalgae 5' was significantly higher (seven times) than in 'Microalgae Control' (Tukey; $p < 0.05$). DMA in 'Microalgae 10', even though having a slightly higher average concentration, was not significantly different from 'Microalgae 5'. Levels of AsSugs were significantly different between exposure groups (Tukey; $p < 0.05$). Comparing 'Microalgae Control' and 'Microalgae 5', a 19-fold difference was noted for AsSug SO4, while a 12-fold difference was observed for AsSug OH. Both AsSug in 'Microalgae 10' were significantly lower than in 'Microalgae 5'.

3.3 Total As concentrations in blue mussels

The total As concentrations ranged from 9.5 to 12.2 mg/kg dw for Group A mussels ($\bar{x}$ = 10.9 ± 0.8; n = 15), 9.3 to 13.0 mg/kg dw for Group B mussels ($\bar{x}$ = 11.0 ± 1.3; n = 15), 9.5 to 14.0 mg/kg dw for Group C mussels ($\bar{x}$ = 11.0 ± 1.1; n = 15), and 8.2 to 12.7 mg/kg dw for Group D mussels ($\bar{x}$ = 10.6 ± 1.2; n = 15) (Fig. 5 and 6). Two-way ANOVA results indicated that tAs concentrations were not significantly different between groups (different treatments) and within groups (different time points) (Tukey test; $p < 0.05$). Verification of PAMAS data showed that the mussels were feeding properly on the microalgae at a rate of approximately $6x10^5$ cells per h per mussel.

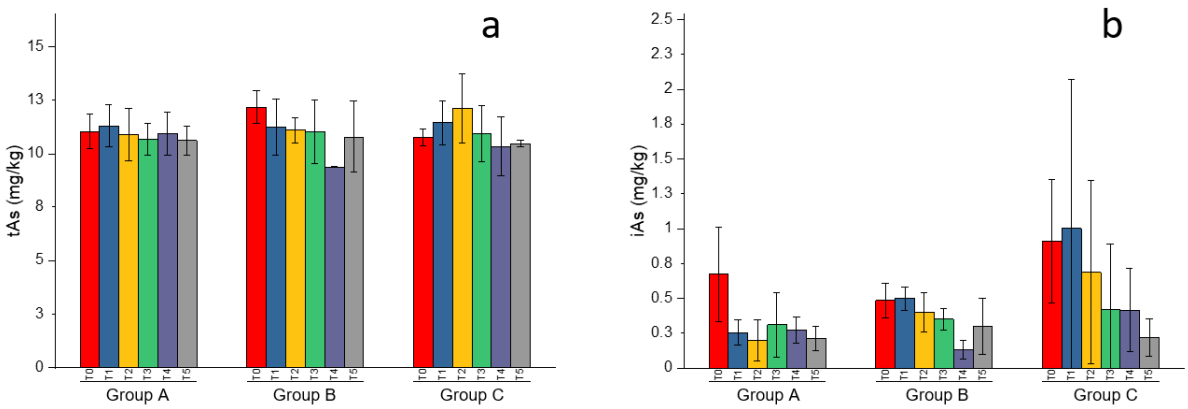

Fig. 5. Concentrations of (a) total arsenic (tAs) and (b) inorganic arsenic (iAs) in blue mussels belonging to Groups A (control), B (mussel fed with 'Microalgae 5'), and C (mussels fed with 'Microalgae 10') sampled at different time points. Time points t0, t1, t2, t3, t4, t5 correspond to exposure days 0, 2, 6, 12, 18, and 24, respectively.

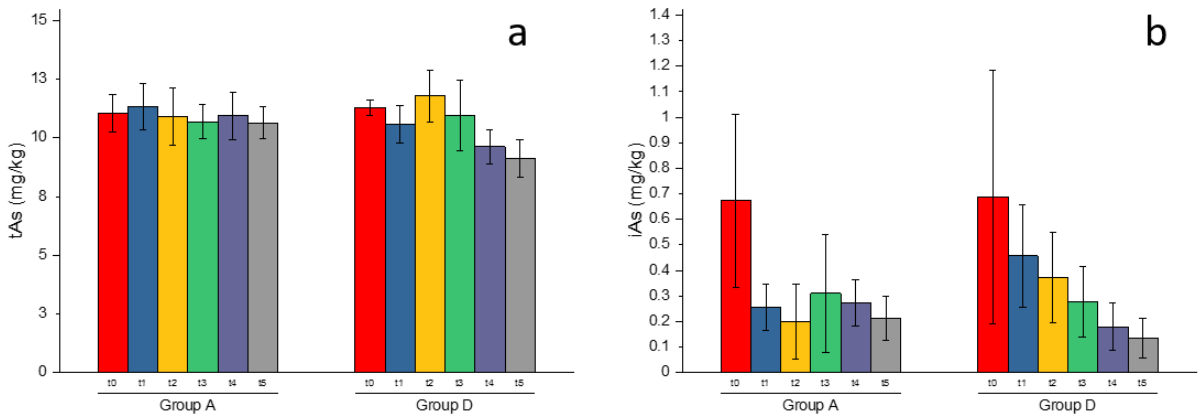

Fig. 6. Concentrations of (a) total arsenic (tAs) and (b) inorganic arsenic (iAs) for blue mussels belonging to Groups A (control) and D (mussels exposed to 5 µg/L As(V) in seawater) sampled at different time points. Time points t0, t1, t2, t3, t4, t5 correspond to exposure days 0, 2, 6, 12, 18, and 24, respectively.

3.4 As species in blue mussels

A total of 11 As species were detected in blue mussels. AB was the predominant species in all exposure groups, ranging from 1.4 to 2.4 mg/kg dw ($\bar{x}$ = 1.9 ± 0.2 mg/kg dw; n = 60), accounting for approximately 18% of tAs (Fig. 7). The second most abundant species, AsSug PO4, ranged from 0.6 to 1.9 mg/kg dw ($\bar{x}$ = 1.1 ± 0.3 mg/kg dw; n = 60), comprising approximately 13% of tAs. The other forms of AsSug found were AsSug OH and AsSug SO3, with average concentrations of 0.5 ± 0.1 mg/kg dw (n = 60) and 0.012 ± 0.004 mg/kg dw (n = 60), representing 4% and 0.1% of tAs, respectively. Low levels of other methylated As species, including DMA and MA, were found with a combined contribution of 7% of tAs. The concentrations of iAs ranged from 0.06 to 2.2 mg/kg dw ($\bar{x}$ = 0.4 ± 0.4; n = 60), which generally accounted for less than 8% of tAs. Unknown peaks were also detected in chromatograms (Fig. 8). The fraction of unextracted As comprised almost half of the tAs in blue mussel samples (Fig. 7). Two-way ANOVA results indicated that As species concentrations were not significantly different between groups (different treatments) and within groups (different time points) (Tukey test; $p < 0.05$).

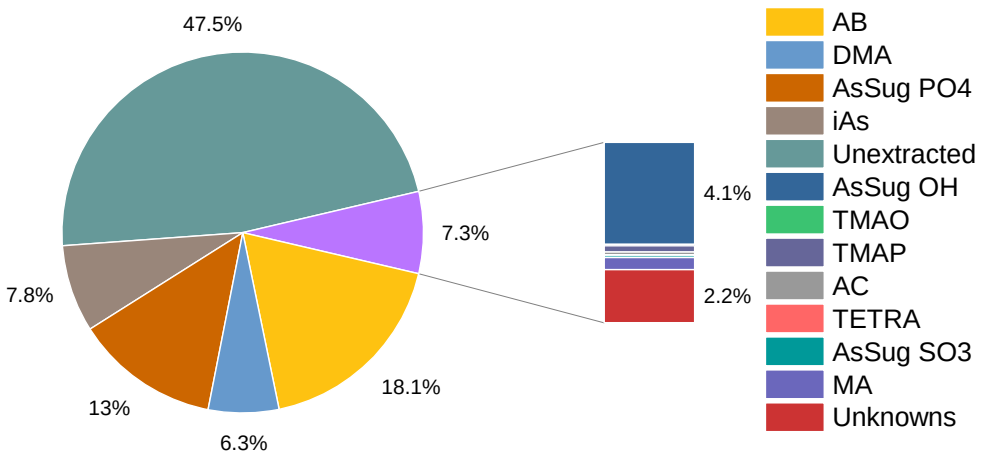

Fig. 7. An example of arsenic species profile in blue mussel from Group A (control). Arsenic species fraction, % = (concentration of As species/total As) x 100.

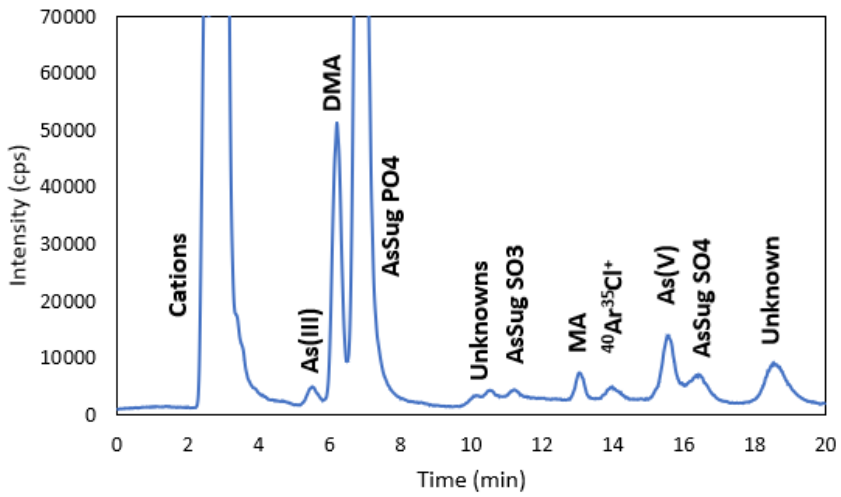

Fig. 8. Anion-exchange chromatogram of a blue mussel extract using HPLC-ICP-MS.

Linear mixed models found a significant effect of time in tAs concentrations in blue mussels. The use of predictions revealed that the tAs concentration decreased non-linearly throughout the exposure period.

3.5 Seawater

Total As concentrations of seawater samples are given in Table 1. The inlet seawater had an average tAs concentration of 2.5 ± 0.2 μg/L (n = 6) (salinity: 35.4 ± 0.1 psu; temperature: 12.5 ± 0.4 °C; pH: 8.03 ± 0.05), which agrees with estimates for tAs concentration in marine waters (0.5 to 3 μg/L) (Neff, 2002; Missimer et al., 2018). Seawater samples collected from Groups A, B, and C had tAs concentrations ranging from 1.0 to 4.0 μg/L ($\bar{x}$ = 2.2 ± 0.7 μg/L; n = 24) (salinity: 35.4 ± 0.3 psu; temperature: 11.7 ± 0.5 °C; pH: 8.05 ± 0.05). The tAs concentrations of the inlet seawater and those from the three exposure groups were not significantly different (Tukey test; $p < 0.05$). Seawater samples from Group D were significantly different from the rest, with tAs concentrations ranging from 4.0 to 7.5 μg/L ($\bar{x}$ = 6.1 ± 0.8 μg/L; n = 38). Verification of seawater As(V) concentrations after water change (to check if target exposure concentration was achieved) and before water change (to check if there were changes in concentration since the water was replaced) showed no significant difference throughout the exposure period.

Table 1. Total As concentrations (μg/L) of inlet seawater and seawater samples from the different exposure groups: Group A (control), Group B (mussel fed with 'Microalgae 5'), Group C (mussels fed with 'Microalgae 10'),a dn Group D (mussels exposed to 5 μg/L As(V) in seawater). Different lower-case letters denote statistically significant difference between treatments (Tukey test; $p < 0.05$).

Source/Treatment groups	tAs (μg/L)	*n*
Inlet seawater	2.5 ± 0.2[b]	6
Group A	1.8 ± 0.5[b]	8
Group B	2.7 ± 0.6[b]	8
Group C	2.2 ± 0.5[b]	8
Group D	5.3 ± 1.8[a]	8
Group D (before water change)	6.3 ± 0.7[a]	7
Group D (daily measurements/after water change)	6.1 ± 0.9[a]	23

4. Discussion

4.1 Exposure of *D. lutheri* to iAs

4.1.1 Arsenic uptake

The current work utilized exposure concentrations which try to replicate natural As concentrations found in seawater, e.g. 0.5 to 3 µg/L (Neff, 2002; Missimer et al., 2018). While extremely high levels of As can exist especially in contaminated sites (Whaley-Martin et al., 2012), the use of these lower concentrations gives a better insight on As biotransformation occurring naturally in unpolluted sites. In the present study, the concentrations used were notably lower than previous reports, where levels ranged from 100 µg/L to as high as 10 mg/L (Cullen et al., 1994; Miyashita et al., 2011; Wang et al., 2013; Huang et al., 2021). These studies employed different culture growing conditions which may have also affected the biotransformation capabilities of the microalgae (Duncan et al., 2013; Papry et al., 2019).

For the microalgae samples, the tAs concentration obtained in 'Microalgae Control' was significantly different from 'Microalgae 5', but the tAs concentration in 'Microalgae 10' was found to be similar to 'Microalgae 5'. BCFs were investigated to probe possible explanations. BCF is an indication of the bioaccumulation potential, usually of a compound or metal, in aquatic environments (DeForest et al., 2007). The calculated BCF value was lowest for 'Microalgae 10'. Earlier studies reported that BCFs for aquatic organisms have an inverse relationship with aqueous exposure concentrations (McGeer et al., 2003; DeForest et al., 2007), implying that BCF decreases as exposure concentration increases. The results suggest that As bioaccumulation of *D. lutheri* may not be as efficient at high exposure concentrations. After surpassing a certain tolerance limit, the organisms' ability to bioaccumulate metals may become restricted (Debelius et al., 2009; Huang et al., 2021; Das et al., 2022).

4.1.2 Arsenic speciation

Among the As species detected in *D. lutheri*, iAs was present in notable concentrations (Fig. 3). The prevalence of iAs has been reported in several microalgal species, and its accumulation in exposure studies has been usually related to unrealistically high exposure concentrations (Duncan et al., 2015). AsSug were also one of the most prevalent As compounds in the microalgae exposure groups. Microalgae are known to contain AsSug, and the occurrence varies e.g. depending on the type of microalgae (Duncan et al., 2015). AsSug OH and AsSug PO4 were found in chlorophytes, while heterokontophytes can contain AsSug SO4 in addition to AsSug OH and AsSug PO4 (Duncan et al., 2015). Also, the microalgal synthesis of AsSug can be influenced by factors such as nutrient medium, length of exposure period, and available As concentration (Duncan et al., 2013; Papry et al., 2022). In a study involving *Dunaliella tertiolecta* and *Thalassiosira pseudonana*, the use of f/2 medium favored the production of AsSug PO4 (Duncan et al., 2013). In contrast, AsSug PO4 was not detected in the current work even when f/2 medium was used, suggesting that different species of microalgae have inherent differences in biotransfomation abilities. The unextracted fraction of tAs was significant in this study (Fig. 4). As the analytical methods employed were mainly targeted to extract water-soluble As species, the unextracted fraction could be AsLipids. Some microalgal species contained AsLipids comprising at least 50% of tAs (Duncan et al., 2015). Although AsLipids were not determined in the current work, it is likely that they were present since *D. lutheri* was reported to have 29% lipid content (Mayer et al., 2022), and that lipid content has been usually correlated with AsLipid levels (Sele et al., 2012; Al Amin et al., 2020). Microalgae collected from the North Atlantic Ocean also contained AsLipids (Glabonjat et al., 2021). Alternatively, the unextracted fraction could be cell-bound iAs which requires a stronger extraction procedure to be released from the matrix, as seen in studies involving *D tertiolecta* (Duncan et al., 2010) and a strain of *Picocystis* (Glabonjat et al., 2020). The absence of AB in *D. lutheri* corroborates

observations in *D. tertiolecta* (Duncan et al., 2010) and *T. pseudonana* (Duncan et al., 2013). AB has not been found in microalgae to date. In a review which tried to elucidate AB transformation at the base of the marine food chain, traces of AB were first seen in herbivorous zooplankton which fed on microalgae (Caumette et al., 2012). It has been suggested that AsSug from microalgae is metabolized to AB in zooplankton indicating that microalgae contain precursors for AB formation in higher trophic aquatic organisms.

4.1.3 Arsenic biotransformation

Microalgae tend to take up iAs, mostly As(V), from seawater due to its chemical and structural resemblance to phosphate (Edmonds and Francesconi, 2003; Glabonjat et al., 2018). The ions exhibit a competitive behavior in terms of uptake via phosphate transporter systems, and an inverse relationship has been observed in some studies (Wang et al., 2014; Duncan et al., 2015; Wang et al., 2015). Phosphate-deficient conditions reportedly favored more efficient uptake of iAs in *Microcystis aeruginosa* (Wang et al., 2014). Contradicting results were seen in other species of microalgae, such as *Thalassiosira* sp. and *Chaetoceros* sp., where varying the P/As ratio did not produce significantly different results in terms of As uptake (Wang et al., 2015). Phosphate concentration was also observed to influence the formation of other As species. Plankton collected from high-nutrient waters contained less arsenosugar phospholipids (AsSug PL) compared to those found in low-nutrient waters (Glabonjat et al., 2021). In a study involving *D. tertiolecta*, DMA levels increased under reduced phosphate concentrations (Duncan et al., 2015). In contrast, levels of AsSug PO4 increased in *D. tertiolecta* and *T. pseudomonana* under increased phosphate concentrations (Duncan et al., 2015). The use of f/2 medium in this work yielded an average phosphate concentration of 26.0 ± 0.3 μM (n = 3) in the exposure groups, which is significantly higher than phosphate concentrations reported for the world's oceans (< 0.1 to 3.2 μM) (Glabonjat et al., 2021). Despite this, AsSug PO4 was not

detected in *D. lutheri*. Clearly, the effect of phosphate concentrations on As uptake and speciation is far more complex and involves interplay among several variables (Papry et al., 2022).

In the current work, higher As(V) exposure concentrations resulted in higher production of MA and DMA, with levels of MA considerably lower than DMA (Fig. 3). In a study involving *Chlamydomonas reinhardtii*, the production of MA was noted to occur rapidly within the first hour after exposure to 0.1 mM (~7.5 mg/L) As(V) (Miyashita et al., 2011). The concentration of MA eventually decreased after 24 h, with AsSug OH and DMA prevailing afterwards. A similar mechanism may have transpired in the current work and possibly explains the discrepancy between MA and DMA levels. Higher DMA and AsSug concentrations at higher exposure concentrations are in agreement with previous studies involving *D. tertiolecta* (Duncan et al., 2010) and *C. reinhardtii* (Miyashita et al., 2011). Previous studies have shown that when microalgae are exposed to elevated As levels, detoxification mechanisms may become overburdened, causing a reduced degree of methylation (Duncan et al., 2015). *P. peniculus* exhibited such behavior when exposed to 10 mg/L As(V), producing minimal levels of methylated As species (Cullen et al., 1994). A similar observation was noted in cyanophytes and chlorophytes after exposure to As(V) concentrations greater than 100 μg/L (Huang et al., 2021), suggesting deterioration of detoxification mechanisms. In the present study, exposure to 10 μg/L As(V) yielded significantly higher concentrations of MA in 'Microalgae 10' compared to 'Microalgae 5' (Fig. 3), as this is one of the first steps in the Challenger pathway (Fig. 1). However, comparing DMA in 'Microalgae 5 and 10', similar concentrations were obtained, which could suggest that biomethylation was inhibited. This was more evident for the AsSug, with lower levels found in 'Microalgae 10' than in 'Microalgae 5'. Since the formation of AsSug is one of the latter steps in the Challenger pathway, the results could imply

that biomethylation has become less efficient for the microalgae exposed to 10 μg/L. When detoxification mechanisms are overwhelmed, the microalgae will accumulate iAs instead (Duncan et al., 2015), which was observed in the current work. Higher proportion of iAs was noted in the 10 μg/L exposure group, comprising 30% of tAs, and could imply that detoxification mechanisms are affected (Fig. 4). It is uncertain, however, whether a 10-μg/L As exposure concentration is high enough to burden the detoxification mechanism of *D. lutheri*. This may be verified by investigating photosynthetic biomarkers such as chlorophyll (Chl *a*) content (Cabrita et al., 2018; Das et al., 2022), or oxidative stress biomarkers such as glutathione (GSH), glutathione S-transferase (GST), phytochelatin (PC), and catalase (CAT) (Koechler et al., 2016; Pikula et al., 2019; Tripathi and Poluri, 2021).

4.2 Exposure to As of blue mussels

4.2.1 Arsenic uptake through the dissolved phase

The tAs concentrations in blue mussels exposed to 5 μg/L As(V) in seawater (Group D) suggest no or very limited As accumulation via the dissolved phase. The results are in accordance with an earlier study involving blue mussels collected off the coast of Western Australia (Gailer et al., 1995), where a 10-day exposure to 100 μg/L As(III) and As(V) in seawater did not produce significantly different tAs concentrations. This concentration is substantially higher (20 times) than the exposure level in the current study. Yet even with considerably higher exposure concentrations, no significant As accumulation was observed. Similarly, Hunter et al. (1998) did not observe a distinct change in As concentrations after exposing the common shrimp *Crangon crangon* to 100 μg/L As(V) in seawater for 24 days. Zhang et al. (2012) had the same conclusions for the marine juvenile fish *Terapon jarbua* exposed to 100 μg/L As(V) and As(III) for 10 days. In contrast, the one-month water-borne exposure of the Bombay oyster *Saccostrea cucullata* to 1 to 20 mg/L As(III) and As(V) resulted in increased tAs levels proportional to the

exposure concentrations (Zhang et al., 2015). Also, significant difference in tAs concentrations was noted when the clam *Asaphis violascens* was exposed to 1 to 20 mg/L As(III) and As(V) in seawater (Zhang et al., 2019). All studies which reported increased tAs levels employed exposure concentrations which were 200 to 4000 times higher than the current study. In a study on oysters, Zhang et al. (2015) reported mortality rates of 20 to 30% after a 30-day exposure to 1 to 20 mg/L As(V), and 100% after only a 15-day exposure to 5 and 20 mg/L As(III), suggesting toxic exposure levels. It was further observed that organic As species were prevalent at low exposure concentrations while iAs prevailed at extremely high levels of exposure (Zhang et al., 2015; Zhang et al., 2019). An *in situ* study on blue mussels collected from a contaminated marine harbor reported similar findings (Whaley-Martin et al., 2012). Porewater samples registered tAs concentrations as high as 700 μg/L, which yielded tAs levels in blue mussels ranging from 34 to 109 mg/kg dw and iAs concentrations from 8 to 33 mg/kg (Whaley-Martin et al., 2012).

4.2.2 Arsenic uptake through the diet

While the two-way ANOVA suggested that there were no significant differences between and within exposure groups, the use of linear mixed models showed that 'time' seemed to be the only factor which was affecting the tAs levels in blue mussels. The tAs concentration appeared to decrease non-linearly throughout the exposure period. The result is somehow supported by Fig. 5, where mussels sampled on t5, noticeably, had less concentrations than the control, though not statistically significant. The trend was most obvious for iAs, but other As species (i.e. DMA and AsSug OH) also exhibited a similar pattern (data not shown). It appears that the blue mussels were losing As instead of gaining, resembling a depuration process. This can suggest that the acclimation period was not long enough or that the feeding rate was not sufficient for the mussels. A depuration process can occur when mussels are relocated from a

site with a higher degree of contamination to a location which is relatively less polluted, as seen when green oysters (*Crassostrea gigas*) and mussels (*Mytilus smarangdium*) were transferred from a copper-contaminated area to natural clean seawater (Han et al., 1993). Similarly, when Chilean blue mussels (*Mytilus chilensis*) were transferred from a contaminated setup to an uncontaminated seawater, the release of cadmium was characterized by an initial abrupt decrease in concentration followed by a slower efflux (Hervé-Fernández et al., 2010). In the present study, for the initial two-week acclimation where the mussels were kept close to a pier at 3 m depth, the mussels might have been exposed to higher As concentrations than when kept in tanks filled with seawater pumped from a depth of 160 m. It may be the case that there were differences in As concentrations in the water column (Cuong et al., 2008; Yuan et al., 2021), or other possible sources of As such as phytoplankton and detritus available to the blue mussels during the initial acclimation. This could suggest that the mussels were exposed to lower As concentrations than the ambient levels in the mussels' natural environment, which could explain the gradual decline in As concentrations, as described by the model.

A study on the flux of As through the Mediterranean mussel (*Mytilus galloprovincialis*) reported that As can also be released through the byssus (Ünlü and Fowler, 1979). Radiotracer techniques revealed that mussels were still excreting byssal threads which contained low but significant amounts of ^{74}As even after they have been removed from the radioactive seawater. Hence, this may also account for the apparent loss in As concentration in the current work. Radiotracer techniques are often used to develop toxicokinetic models which describe in detail the uptake, retention, and excretion of metals based on various biological and environmental factors (Ünlü and Fowler, 1979; Blackmore and Wang, 2003; Hervé-Fernández et al., 2010). The lack of appropriate radioisotopes and safety concerns on radioactive wastes limit the application of radiotracer techniques. For other elements, the use of a double stable isotope

method has been proposed, which combines the benefits of radiotracer techniques and stable isotopes (Lin et al., 2021). However, this cannot be applied for studying As as it only has one stable isotope (^{75}As).

4.2.3 Arsenic species profile

The As species profile of blue mussels from different treatment groups did not vary significantly. For representation, shown in Fig. 7 is the As profile of one of the blue mussels from the control group (Group A). The predominant species was AB but the percentage was relatively low, since AB typically comprises 30 to 40% of tAs in blue mussels (Molin et al., 2012; Tibon et al., 2021). Nonetheless, lower percentages were also reported, e.g. in mussels collected from a contaminated harbor in Canada, where AB only accounted for 4 to 21% of tAs and iAs was the predominant species, representing up to 36% of tAs in the mussels (Whaley-Martin et al., 2012). Similarly, Sloth and Julshamn (2008) found proportions of iAs as high as 42% of tAs in blue mussels collected from Norwegian fjords. In the present study, iAs generally comprised less than 5% of tAs.

The presence of AsSug in blue mussels have been reported in literature and their occurrence varies (Dahl et al., 2010; Whaley-Martin et al., 2012; Tibon et al., 2021). The relative abundance of AsSug PO4 in this study is in accordance with previous observations for blue mussels from Norway, where AsSug PO4 was present in higher concentrations than AsSug OH (Tibon et al., 2021). In contrast, Whaley-Martin et al. (2012) reported AsSug OH as more prevalent than AsSug PO4 in blue mussels obtained from an As-contaminated site. In the same study, AsSug SO4 was found in low concentration in only one sample, while in the current work, AsSug SO4 was not detected. The blue mussels fed on *D. lutheri*, which contained AsSug SO4 as one of its major species while AsSug PO4 was not detected. The absence of AsSug

SO4 in blue mussels in the current work may suggest that it was metabolized and may have been converted to other forms. The limited occurrence of other methylated species corroborates earlier studies (Molin et al., 2012; Tibon et al., 2021). Similar to the current work, unknown As species (chromatographic peaks) were found in blue mussels in previous studies (Dahl et al., 2010; Whaley-Martin et al., 2012). From Fig. 7, almost 50% of tAs was unextracted, which is comparable to extraction efficiencies reported in previous investigations (Dahl et al., 2010; Whaley-Martin et al., 2012). The unextracted As may include AsLipids, since the extraction method used was primarily developed for water-soluble As species. *D. lutheri* had considerable fractions of unextracted As (20 to 50%), and these may have carried over to the blue mussels. AsLipids have been reported in other species of mussels (*Mytilus galloprovincialis*) (Freitas et al., 2020).

5. Conclusion

Low-trophic marine organisms such as microalgae and blue mussels play a big role in As cycling in the environment. In this study, it was demonstrated that the microalgae *D. lutheri* can take up As from seawater and transform it to methylated As species and AsSug. However, exposure to higher concentrations of iAs can overwhelm detoxification mechanisms, resulting to inefficient methylation. When this happens, iAs can accumulate in the microalgae. The detoxification ability of a microalgae is dependent on several factors, such as nutrient medium, As exposure concentration, and taxonomic differences in biotransformation. Exposure of blue mussels to 5 μg/L As(V) in seawater showed no accumulation of As. This suggests that the dominant pathway for As accumulation in blue mussels is via ingestion of food. However, even after feeding the blue mussels with *D. lutheri* exposed to As(V), no As accumulation was noted. The use of linear mixed models showed that the blue mussels were slowly losing As, resembling a depuration process, which may be attributed to differences in As concentrations

in the mussels' natural environment and the exposure setups. Both *D. lutheri* and blue mussels contained notable proportions of simple methylated species (i.e. MA and DMA) and AsSug. The fraction of unextracted As was also quite significant. In contrast, AB only comprised 30% of tAs in blue mussels, while it was not detected in *D. lutheri*. The findings suggest that low-trophic marine organisms consist mostly of methylated As species and AsSug, which are the precursors for AB formation in higher trophic aquatic animals. The use of low-trophic marine organisms as feed ingredients can have potential implications in regard to feed and food safety since AsSug, together with AsLipids, are regarded as potentially toxic.

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Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Undesirables in Mesopelagic Species and Implications for Food and Feed Safety—Insights from Norwegian Fjords

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Abstract: The increase in the global population demands more biomass from the ocean as future food and feed, and the mesopelagic species might contribute significantly. In the present study, we evaluated the food and feed safety of six of the most abundant mesopelagic species in Norwegian fjords. Trace elements (i.e., arsenic, cadmium, mercury, and lead), organic pollutants (i.e., dioxins, furans, dioxin-like polychlorinated biphenyls, and polybrominated flame-retardants), and potentially problematic lipid compounds (i.e., wax esters and erucic acid) were analyzed and compared to existing food and feed maximum levels and intake recommendations. Furthermore, contaminant loads in processed mesopelagic biomass (protein, oil, and fish meal) was estimated using worst-case scenarios to identify possible food and feed safety issues. While most undesirables were low considering European food legislation, we identified a few potential food safety issues regarding high levels of fluoride in Northern krill, wax esters in glacier lanternfish, and long-chain monounsaturated fatty acids in silvery lightfish. Our estimates in processed biomass indicated high levels of undesirable trace elements in the protein fraction, frequently exceeding the maximum levels for feed ingredients. However, in fish meal, almost no exceedances were seen. In the oil fraction, dioxins and furans were above the maximum levels, given for food and feed ingredients. The present study is crucial to enable an evaluation of the value of these species; however, more data is needed before proceeding with large-scale harvesting of mesopelagic biomass.

Keywords: mesopelagic; contaminants; undesirables; trace elements; arsenic; fluoride; organic pollutants; wax esters; *Benthosema glaciale*; *Maurolicus muelleri*

1. Introduction

The global population is predicted to increase to 9.6 billion by 2050, demanding global food production to grow by 60% (WHO. Zero hunger—Hunger facts, http://www.fao.org/zhc/hunger-facts/en/). Seafood, being highly nutritious, has great potential to contribute to food security [1]. Marine resources can either be consumed directly by humans, processed before human consumption, or used as feed ingredients for aquaculture. Marine oils for human consumption and fish protein powder can be applied for food fortification and the production of value-added/functional foods. The continuous demand for alternative protein and oil sources for aquaculture, due to its short supply, high prices, and competition with human food, makes the exploration of new marine resources highly relevant.

However, the overfishing of commercially exploited fish stocks is still a growing issue [2]. It has been suggested that one way to reduce fishing pressure on already overfished stocks would be to

harvest from so far unexploited resources, preferentially from lower trophic levels, such as organisms from the mesopelagic zone [3].

Mesopelagic organisms are a prominent, almost totally unexploited resource. They are globally distributed and inhabit the ocean waters between 200 and 1000 m of depth. They are assumed to be one of the world's largest unexploited resources, with stock estimates of mesopelagic fish ranging from approximately one to ten billion metric tons [4–6]. In addition to fish species, the mesopelagic community also contains potentially exploitable species of crustaceans, jellyfish, and cephalopods.

Due to their extended deep-water zones, Norwegian fjords are a promising habitat for mesopelagic species, and it has been shown that considerable nutrient-dense biomass can be found there. The species variety is rather low, and the biomass consists of mainly six species: two species of mesopelagic fish, the glacier lanternfish (*Benthosema glaciale*), and the silvery lightfish (*Maurolicus muelleri*); the decapod *Eusergestes arcticus*; the decapod genus *Pasiphaea*; the euphausiid Northern krill (*Meganyctiphanes norvegica*); the scyphozoan helmet jellyfish (*Periphylla periphylla*) [7]. It has been shown that these species have the potential to contribute to global food and feed security being nutrient-rich with high levels of vitamin A_1, calcium, selenium, iodine, eicosapentaenoic acid, docosahexaenoic acid, and cetoleic acid, especially in fish [7].

However, more knowledge on the content of undesirables is needed to assess the suitability as food and feed ingredient, especially since some challenges have already been identified, such as high values of cadmium in some fish species [7–9] and fluoride in Northern krill [10,11] and high amounts of wax esters in *B. glaciale* [9,12].

The mesopelagic fisheries are so far in an experimental stage, and before exploiting the mesopelagic biomass as food or feed ingredient, it should be studied how this will impact the services of the mesopelagic organisms provide for the ocean and the climate with its function as carbon pump [13]. Therefore, the final development of the fisheries and final products and applications of the catch are not yet known. The species composition of mesopelagic catches can vary significantly, and at this point in time, we do not know yet if sustainable catches are possible, and if so, what the catches will be used for, and how the processing may influence the nutrient and contaminant composition. However, the first steps are needed to identify possible drawbacks regarding food and feed safety. Depending on the final product, different regulations might apply. In the European context, regulations setting maximum levels (MLs) for different types of contaminants are in place, both for food [14] and feed ingredients [15].

In the present study, we investigated the levels of the trace elements arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) in the most abundant mesopelagic species in western Norwegian fjords, whose genus or family are found widespread and highly abundant in mesopelagic ecosystems all around the globe [4,16–18]. Samples were also analyzed for organic legacy pollutants, including dioxins and dioxin-like polychlorinated biphenyls (PCBs), non-dioxin-like PCBs (PCB6), and the content of polybrominated diphenyl ethers ($PBDE_7$). Finally, the content of the indigestible wax esters and the fatty acid—erucic acid—was evaluated. Where applicable, the measured concentrations were compared to legal MLs. Furthermore, the contents of the analyzed contaminants in the protein concentrate fraction and marine oil fraction were roughly estimated using simple assumptions to enable the identification of possible food and feed risks.

2. Materials and Methods

2.1. Biological Material

The two fish species of glacier lanternfish, *Benthosema glaciale*, Pearlside, *Maurolicus muelleri*; the decapod species *Eusergestes arcticus*; the decapod genus *Pasiphaea*, including the three species *Pasiphaea multidentata*, *Pasiphaea sivado*, and *Pasiphaea tarda*; Northern krill, *Meganyctiphanes norvegica;* the jellyfish species helmet jellyfish, *Periphylla periphylla* were sampled in three different fjords of the Norwegian west coast—Osterfjorden, Bjørnafjorden, and Boknafjorden. Specimens were caught in a mesopelagic trawl between 5 and 9 December 2018, onboard the research vessel "Johan Hjort". Fish and

crustacean species were identified, and the standard length was measured for a representative number of animals from the catch ($n \geq 27$). For each species/genus from each location, a minimum one pooled sample was prepared. For the *B. glaciale*, *M. muelleri*, and *M. norvegica* samples from Osterfjorden, different size classes were sampled, and for *B. glaciale*, also different sexes were determined. *P. periphylla* was only sampled from Osterfjorden (n = 12) and Bjørnafjorden (n = 10), and total wet weight (w.w.) was used as size measurement. For the pooled sample, the jellyfish individuals were quartered. Samples were homogenized as soon as possible after the catch and distributed into different tubes for analysis. All samples were stored frozen at −20 °C until analysis, while samples for lipid analysis were stored frozen at −20 °C until 17 December 2018, and at −80 °C until analysis. A detailed overview of the samples (number of composite samples, specimens per composite samples, and the average length/weight) and images of the species are given in Alvheim et al. [7].

2.2. Chemical Analysis

2.2.1. Trace Elements

Pooled samples of the fish and crustacean species were homogenized and subsequently freeze-dried. Moisture content was determined by comparing the weight of the sample before and after freeze-drying. The freeze-dried sample material was homogenized before performing the analysis of trace elements. This determination was performed using inductively-coupled plasma mass spectrometry (ICP-MS), as described by Julshamn et al. [19]. The method is accredited by the Norwegian Accreditation Authority, according to NS-EN 17025. The accuracy of this method is assessed by using certified reference materials (i.e., lobster hepatopancreas (TORT-3; National Research Council Canada, Ottawa, ON, Canada) and oyster tissue (SMR1566b; National Institute of Standards and Technology, Gaithersburg, MD, USA)). In brief, approximately 0.2 g of sample material was digested using 2.0 mL of nitric acid (69% *w/w*) in an ultra wave digestion system (UltraWAVE, Milestone, Sorisole, Italy). The tubes were capped and placed in the ultra wave system in a container with 130 mL Milli-Q® (EMD Millipore Corporation, Billerica, MA, USA) water and 5 mL H_2O_2. The digested samples were diluted to 25 mL with Milli-Q® water. The tuning of the ICP-MS was performed following the manufacturer's instructions. A tuning solution (1 ppb tuning solution B, Thermo Fisher, in 2% HNO_3 and 0.5% HCl) was used prior to analyses. The concentrations of As, Cd, Hg, and Pb were determined by ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, MA, USA) equipped with an autosampler (FAST SC-4Q DX, Elemental Scientific, Omaha, NE, USA). Data were collected and processed using the Qtegra ICP-MS software (version 2.10, 2018, Thermo Scientific, Waltham, MA, USA). The dry weight-based limit of quantification (LOQd.w.) was set to 0.005 mg/kg d.w. with a standard sample size (0.2 g). The wet weight-based LOQ for each individual sample (LOQw.w.) was determined as: LOQw.w. = LOQd.w. × % dry $matter_{sample}$/100.

2.2.2. Inorganic Arsenic

The inorganic arsenic (iAs) concentration was performed, as previously described [20,21], based on an European Committee for Standardization method (NS-EN 16802:2016, European Committee for Standardization). Briefly, after freeze-drying samples, they were ground until a homogenous material was obtained. Approximately, 0.2 g of sample was weighed into a 13 mL propylene centrifuge tube (Sarstedt, Nümbrecht, Germany), and the 10 mL of extraction solution (0.1 M HNO_3 (trace select, ≥69.0% *w/w*) in 3% (*v/v*) H_2O_2 (Emsure® (Merck, Darmstadt, Germany) ACS, ISO, 30% *w/w*)) was added. The samples were placed in a water bath for 60 min at 90 °C, 100 rpm, and subsequently cooled down to room temperature and centrifuged during 10 min at 3800 rpm (Eppendorf® Centrifuge 5702, Hamburg, Germany). Prior to analysis, the soluble fraction was collected with a 5 mL disposable needle syringe and filtered through a disposable syringe filter (0.45 µm, Sartorius, Göttingen, Germany) into 1 mL polypropylene HPLC vials. During the extraction procedure, arsenite [As(III)] was oxidized to arsenate [As(V)], and the iAs concentration was determined as the sum of As(III) and As(V). This determination was done by using an external calibration curve of As(V) (Spectrascan TeknoLab,

Oppegaard, Norway) and using peak areas for quantification. The LOQ of this method was 0.01 mg·kg^{-1} d.w. Certified reference material of rice (ERM-BC211; Institute for Reference Materials and Measurements, IRMM, Geel, Belgium) was used to assess the accuracy of the method. The iAs concentration was determined using an HPLC-ICP-MS (1260 HPLC, 7900ICP-MS, Agilent Technologies, Wilmington, DE, USA) and anion-exchange column (IonPac AS7, 2 × 250 mm; Dionex, Sunnyvale, CA, USA) with respective guard column (IonPac AG7, 2 × 50 mm; Dionex, Sunnyvale, CA, USA). The mobile phase solution was prepared by dissolving an appropriate amount of $(NH_4)_2CO_3$ to reach the desired ionic strength (50 mM) in an aqueous 3% (*v*/*v*) MeOH solution (MeOH, LiChrosolv®, HPLC grade), followed by adjustment of pH to 10.3 with NH_3 (25% *v*/*v*). The instrument was tuned according to the manufacturer's instructions.

2.2.3. Fluoride

Total fluoride was analyzed according to Malde et al. [22]. Briefly, the fluorine content in 0.25 or 0.50 g sample material was determined by using a selective ion electrode (Orion 94–09, Thermo Orion ionpuls fluorine electrode, Beverly, MA, USA) after dry ashing in a muffle furnace (CSF 1100, Carbolite Furnaces, Bamford, Sheffield, England) at 550 °C with sodium hydroxide as an ashing aid, in order to aid the fluoride extraction as well as avoiding loss of fluoride during the ashing process. The dry-ashed samples were dissolved in distilled water (10–15 mL) and neutralized with hydrochloric acid to a pH of 7.2–7.5, in order to avoid hydroxide fluoride interference during determination. Aliquots of 5 mL were pH adjusted to pH 5.2–5.4 with 0.5 mL total ionic strength adjustment buffer III solution, which is the optimal pH-range for fluoride determination. Reagent blanks for blank determination and standard solutions (0.100, 1.000, and 10.000 mg F/L) were used for background and concentration determination. The precision of the method was assessed with certified reference material (i.e., oyster tissue, 1566a, NIST, Gaithersburg, MD, USA).

2.2.4. Crude Fat

The crude fat content was determined gravimetrically in wet homogenates using 30% isopropanol in ethyl acetate. The solution was filtered, the solvent evaporated, and the fat residue weighed. This method is accredited in accordance with ISO-EN 17025 and registered as a Norwegian Standard, NS 9402 [23].

2.2.5. Determination of Dioxins, Furans, Polychlorinated Biphenyls, and Polybrominated Flame-Retardants

The concentrations of dioxins and furans (PCDD/Fs) and non-ortho PCBs, mono-ortho PCBs, and PBDE were determined by using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS), according to Berntssen et al. [24,25]. Briefly, sample material was solvent extracted by pressure (80:20 dichloromethane:hexane for PPPBDE, hexane for all other substances (*v*/*v*)) with a Dionex ASE 300 solvent extractor (Dionex Sunnyvale, CA, USA). Acid-impregnated silica was added to the extraction cell for the on-line cleanup of NDL-PCBs and PBDEs. In an external clean-up procedure, co-extracted fat was removed by adding concentrated sulfuric acid to the extract. Prior to extraction, the following surrogate internal standards were added (^{13}C-labeled EDF-4147, 4097, 5999, 6999, 7999, 8999, 9999-3-4, 9999 for PCDD/F, PBDE 139 EO-5100 for PBDEs, EC-4935, 4979, 4937, 4976-3, 4976 for dioxin-like -PCBs, and PCB-53 for non-dioxin-like -PCBs (Cambridge Isotope Laboratories, Andover, MA, USA)). For PCDD/F and DL-PCBs, extracts were purified using H_2SO_4 on silica, multilayered silica, basic alumina, and carbon columns, respectively (FMS, Waltham, MA, USA, for solvent conditions see [26]). Following this, the samples were concentrated by pressurized evaporation (Turbovap II™ Zymark, Hopkinton, MA, USA). A mixture of ^{13}C-labeled performance standards (EDF 5999 for PCDD/F and EC-4979 for DL-PCBs, Cambridge Isotope Laboratories, Andover, MA, USA) was added prior to PCDD/F and DL-PCBs determination. High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS, MAT 95XL Thermo Finnigan, Bremen, Germany), equipped with a fused silica capillary column (30 m × 0.25 mm i.d. and 0.25

μm film thickness, RTX-5SILMS, Restek, Bellefonte, PA, USA), was used for analyses. According to the United States Environmental Protection Agency (USEPA) 1613 method [27], the quantification was performed according to the internal standard isotope dilution method using congener-specific relative response factors (RRFs) determined from three-point calibration standard runs (CS1–CS3, Cambridge Isotope Laboratories, Andover, MA, USA). Recovery values (%) were between 78 and 110%, and these values were calculated according to the USEPA methods [27], and PCCD/F and DL-PCB values are expressed as pg upper bound WHO-TEQ g^{-1} w.w. using the WHO-TEFs from 1998 [14]. The PCCD/F and DL-PCB under the limit of quantification (LOQ) are expressed as LOQ (upper bound). The LOQ for the other persistent organic pollutantss is given as <LOQ. Determination of NDL-PCBs was performed by GC-MS (TRACE GC Ultra™/DSQ™ Single Quadrupole GC/MS, Thermo Finnigan, Bremen, Germany) in negative chemical ionization SIM mode. The GC was equipped with a fused silica capillary column (30 m × 25 mm i.d. 25 μm film thickness HP-5MS Column, Agilent J&W, Santa Clara, CA, USA). The internal standard (IS) method was used for quantification, using congener-specific RRFs from a three-point linear external standard curve relative to the internal surrogate standard. Recovery for all congeners was validated by spiking each sample matrix with internal standards at three levels (recovery was 85–110% for NDL-PCBs). For OCPs, the extracts were purified on three sequenced solid-phase extraction (SPE) columns (Chem Elut™, BondElut® C18, and BondElut® Florisil columns, respectively, Varian Inc., Palo Alto, CA, USA, for solvent conditions see [26]) in an automated column system (ASPEC™ XL4, Gilson, Middleton, WI, USA). The PBDE extracts were analyzed by GC-MS (TRACE GC Ultra™/DSQ™ Single Quadrupole GC/MS, Thermo Finnigan, Bremen, Germany) equipped with an RTX-5MS capillary column (30 m × 0.25 mm i.d. 25 μm film thickness, Restek, Bellefonte, PA, USA). The recovery for PBDE and HBCD was between 81% and 118%, and quantification and recovery validation were performed, as described for the PCBs. All samples were run with one procedural blank and one in-house performance evaluation standard (homogenized salmon fillet) in batches of twelve, with a duplicate of the last sample. The LOQ was determined for each determination by using nine times the noise level (three times the limit of detection (LOD)). The LOD was statistically estimated as the analyte concentration, giving a peak signal of three times the background noise from an internal-surrogate standard-spiked procedural blank. The proficiency test, quantification quality, and assurance procedures were as validated by inter-laboratory tests (details are given by Berntssen et al. [26]). The trueness of the method was established by participating in proficiency tests of calibration material and spiked sample material (i.e., satisfactory trueness was set to $-2.0 \leq z\text{-score} \leq 2.0$ and repeatability as relative standard deviation RSD (%) of 10 % and better).

2.2.6. Wax Esters and Erucic Acid

Wax esters and erucic acid were analyzed by gas chromatography (HP-7890A Agilent, Santa Clara, CA, USA) coupled with a flame ionization detector (GC-FID), as described in Meier et al. [28], with the nonadecanoic acid (19:0) as an internal standard. For this, anhydrous methanol containing 2 N HCl was used as a methylation agent. The fatty acids methyl esters (FAME) were extracted using 2 × 2 mL hexane. Several of the samples contained wax esters, and nonadecanol (19:0 alk) was added in the hexane extracts as internal standard, and the FAME and fatty alcohols (FAOH) were separated using solid-phase column (500 mg aminopropyl-SPE, Supelco, Bellefonte, PA, USA. The FAME fraction was eluted with 3 mL hexane + 2 mL hexane:ethyl acetate 9:1 *v*/*v*), and the FAOHs were eluted with 4 mL chloroform. To obtain a suitable chromatographic response, the extracted hexane was diluted or concentrated so that the most abundant FAME/FAOH in the mixture was approximated 150 ng/μL. One μL was injected splitless with an injection temperature of 280 °C. A 25 m × 0.25 mm fused silica capillary, coated with polyethylene-glycol of 0.25 μm film thickness, CP-Wax 52 CB (Varian-Chrompack, Middelburg, The Netherlands) was used. Helium was used as the mobile phase at 1 mL/min for 45 min and then increased to 3 mL/min for 30 min. The temperature of the flame ionization detector was set at 300 °C. The oven temperature was programmed to hold at 90 °C for 2 min, then from 90 to 165 °C at 30 °C/min and then to 240 °C at 2.5 °C/min and held there for 35 min. Fifty-nine FAME peaks and fifteen

fatty alcohols peaks were selected in the chromatograms and identified by comparing retention times with a FAME standard (GLC-463 from Nu-Chek Prep. Elysian, MN, USA) and fatty alcohol standard (GLC-33-36A from Nu-Chek Prep. Elysian, MN, USA), and retention index maps and mass spectral libraries (http://www.chrombox.org/home/www.chrombox.org/index.html) were performed under the same chromatographic conditions as the GC-FID [29]. Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-463 mixture. The chromatograms were integrated using the EZChrom Elite software (Agilent Technologies, Santa Clara, CA, USA).

2.2.7. Estimation of Contaminant Levels in Processed Mesopelagic Biomass

To estimate contaminant levels, despite lacking specific knowledge on how the contaminants will be distributed in the oil and meal fraction after processing, assumptions were made, resulting in worst-case scenarios:

The total amount of As, iAs, F, Hg, Cd, and Pb would end up in the processed pure protein fraction and in fish meal, respectively. Fish meal was defined as total biomass adjusted to the fat content of 10% (crude fat).

The processing of fish oil was equally efficient as the here applied method for the estimation of the crude fat content. The total amount of here measured persistent organic pollutants (POPs), erucic acid, and wax esters followed the oil fraction.

The concentrations of trace elements in processed pure protein $\boldsymbol{C}^{Protein}_{Trace\ elements}$ with a dry matter content of 88%, as described in the EU directive 2002/32/EC [15], was estimated as:

$$\boldsymbol{C}^{Protein}_{Trace\ elements} = \frac{\boldsymbol{C}^{Meso}_{Trace\ elements}}{\boldsymbol{C}^{Meso}_{Protein}} \times \boldsymbol{dm}_{Meal} \quad (1)$$

with $\boldsymbol{C}^{Meso}_{Trace\ elements}$ being the dry weight-based concentration of trace elements in the whole mesopelagic organism, and $\boldsymbol{dm}_{Meal}$ being the dry matter content in the meal, set to 0.88 g/g.

The concentrations of the here measured POPs in fish oil $\boldsymbol{C}^{Oil}_{POPs}$ was estimated as:

$$\boldsymbol{C}^{Oil}_{POPS} = \frac{\boldsymbol{C}^{Meso}_{POPs}}{\boldsymbol{C}^{\boldsymbol{Fish}}_{Total\ fat}} \quad (2)$$

with $\boldsymbol{C}^{Meso}_{POPs}$ being the dry weight-based concentration of POPs in the whole mesopelagic organism, and $\boldsymbol{C}^{Meso}_{Total\ fat}$ being the dry weight-based fat content in the whole mesopelagic organism.

The concentration of trace elements in fish meal $\boldsymbol{C}^{Meal}_{Trace\ elements}$ adjusted to content of 0.88 g/g dry matter and fat content $\boldsymbol{fat}_{Meal}$ of 0.1 g/g was estimated as:

$$\boldsymbol{C}^{Meal}_{Trace\ elements} = \frac{\boldsymbol{C}^{Meso}_{Trace\ elements}}{(1 - \boldsymbol{C}^{Meso}_{Total\ fat})} \times \boldsymbol{dm}_{Meal} \times (1 - \boldsymbol{fat}_{Meal}) \quad (3)$$

The concentration of POPs in the fish meal $\boldsymbol{C}^{Meal}_{POPs}$ was estimated as:

$$\boldsymbol{C}^{Meal}_{POPs} = \boldsymbol{C}^{Oil}_{POPS} \times \boldsymbol{fat}_{Meal} \quad (4)$$

The calculations for erucic acid and wax esters were done in accordance with POPs, following formulas (2) and (4).

3. Results and Discussion

3.1. Trace Elements

The concentrations of As, iAs, Cd, Hg, Pb, F in the different species are shown in Table 1 based on dry weight (d.w.) and wet weight (w.w.).

Table 1. Concentrations of total arsenic (As), inorganic arsenic (iAs), cadmium (Cd), mercury (Hg), lead (Pb), fluoride (F) (Mean ± SD) on wet weight (w.w.) and dry weight (d.w.) basis in the most abundant mesopelagic species in western Norwegian fjords. An asterisk indicates upper bound mean concentrations.

Species	N	As		iAs		
		(g/kg w.w.)	(mg/kg d.w.)		(mg/kg w.w.)	(mg/kg d.w.)
		Mean ± SD		*N*	Mean ± SD	
Benthosema glaciale	7	4.0 ± 1.2 (2.2–6.0)	13 ± 4 (6.9–19)	3	<LOQ	<LOQ
Maurolicus muelleri	4	5.1 ± 0.5 (4.7–5.5)	16 ± 1 (15–17)	3	<LOQ	<LOQ
Meganyctiphanes norvegica	4	28 ± 19 (12–52)	89 ± 61 (38–160)	3	0.061 ± 0.086 (0.011–0.160)	0.244 ± 0.348 (0.042–0.646)
Pasiphaea sp.	3	22 ± 19 (10–43)	68 ± 58 (32–136)	3	0.014 ± 0.010 (0.007–0.025)	0.061 ± 0.032 (0.042–0.098)
Eusergestes arcticus	4	9.5 ± 4.2 (5.0–14)	30 ± 13 (16–44)	3	<LOQ	<LOQ
Periphylla periphylla	2	0.79 (0.59–1.0)	2.5 (1.9–3.2)	2	0.0022 (0.0021–0.0023)	0.046 (0.044–0.048)
Species	**N**	**Cd**		**Hg**		
		(mg/kg w.w.)	(mg/kg d.w.)		(mg/kg w.w.)	(mg/kg d.w.)
		Mean ± SD				
Benthosema glaciale	7	0.022 ± 0.014 (0.007–0.044)	0.069 ± 0.043 (0.022–0.14)		0.022 ± 0.012 (0.011–0.044)	0.069 ± 0.037 (0.035–0.14)
Maurolicus muelleri	4	0.033 ± 0.007 (0.026–0.041)	0.1 ± 0.02 (0.082–0.13)		0.026 ± 0.011 (0 011–0.035)	0.080 ± 0.033 (0.035–0.11)
Meganyctiphanes norvegica	4	0.016 ± 0.013 0.008–0.035)	0.051 ± 0.04 (0.025–0.11)		0.014 ± 0.007 (0 008–0.024)	0.044 ± 0.022 (0.025–0.076)
Pasiphaea sp.	3	0.26 ± 0.19 (0.14–0.47)	0.81 ± 0.58 (0.44–1.5)		0.038 ± 0.02 (0.022–0.060)	0.12 ± 0.06 (0.069–0.19)
Eusergestes arcticus	4	0.074 ± 0.042 (0.029–0.13)	0.23 ± 0.13 (0.092–0.41)		0.014 ± 0.007 (0.008–0.023)	0.043 ± 0.021 (0.025–0.073)
Periphylla periphylla	2	0.075 (0.064–0.085)	0.24 (0.20–0.27)		<LOQ	<LOQ
Species	**N**	**Pb**		**F**		
		(mg/kg w.w.)	(mg/kg d.w.)		(mg/kg w.w.)	(mg/kg d.w.)
		Mean ± SD				
Benthosema glaciale	7	0.016 ± 0.017 * (<LOQ–0.054)	0.049 ± 0.054 * (<LOQ–0.17)		-	-
Maurolicus muelleri	4	0.009 ± 0.001 * (<LOQ–0.010)	0.027 ± 0.004 * (<LOQ–0.032)		-	-
Meganyctiphanes norvegica	4	0.086 ± 0.075 (0.021–0.16)	0.27 ± 0.24 (0.066–0.51)		720 ± 160 (570–940)	3000 ± 500 (2700–3700)
Pasiphaea spp.	3	0.005 ± 0.002 * (<LOQ–0.006)	0.016 ± 0.005 * (<LOQ–0.019)		63 ± 8 (57–72)	300 ± 60 (240–360)
Eusergestes arcticus	4	0.01 ± 0.006 * (<LOQ–0.019)	0.032 ± 0.019 * (<LOQ–0.060)		27 ± 11 (18–42)	100 ± 60 (60–190)
Periphylla periphylla	2	<LOQ	<LOQ		8	168

* Upper bound concentration.

For comparison, the literature values on the investigated species are given in Table 2.

Table 2. Literature values of measured trace element concentrations (As, Cd, Hg, MeHg, Pb, and F) in the investigated species. The number of measured samples (N), the mean concentration in mg/kg based on either dry weight (d.w.) or wet weight (w.w.), the standard deviation (SD), and the range, whenever available, are given.

Species	Element	Location	N	Mean (mg/kg)	SD	Range	d.w./w.w.	Reference
Benthosema glaciale	As	North Atlantic	1 [c]	0.58			w.w.	[30] Grimaldo et al. in press
		N Norwegian Sea	25 [c]	1.4		1.2–1.8	w.w.	[9] Wiech et al., 2018
		Norwegian Coast	4 [c]	1.9		1.8–2.0	w.w.	[9] Wiech et al., 2018
		Mediterranean Sea	1	12.7			d.w.	[31] Fowler, 1986
	Cd	North Atlantic	1 [c]	0.090			w.w.	[30] Grimaldo et al. in press
		N Norwegian Sea	25 [c]	0.067		0.044–0.086	w.w.	[9] Wiech et al., 2018
		Norwegian Coast	4 [c]	0.009		0.006–0.018	w.w.	[9] Wiech et al., 2018
		Mediterranean Sea	9 [c]	0.71	0.15		d.w.	[31] Fowler, 1986
		Mediterranean Sea	4 [c]	0.19	0.08		d.w.	[31] Fowler, 1986
	Hg	North Atlantic	1 [c]	0.039			w.w.	[30] Grimaldo et al. in press
		N Norwegian Sea	25 [c]	0.019		0.014–0.024	w.w.	[9] Wiech et al., 2018
		Norwegian Coast	4 [c]	0.016		0.013–0.020	w.w.	[9] Wiech et al., 2018
		Mediterranean Sea	9 [c]	0.4	0.16		d.w.	[31] Fowler, 1986
		Mediterranean Sea	11 [c]	0.21	0.2		d.w.	[31] Fowler, 1986
	Pb	North Atlantic	1 [c]	<0.01			w.w.	[30] Grimaldo et al., in press
		N Norwegian Sea	25 [c]	0.021		0.007–0.089	w.w.	[9] Wiech et al., 2018
		Norwegian Coast	4 [c]	0.008		0.007–0.010	w.w.	[9] Wiech et al., 2018
Maurolicus muelleri	As	North Atlantic	2 [c]	1.6		1.2–1.9	w.w.	[30] Grimaldo et al. in press
		Norwegian Fjord	4 [c]	3.8		2.5–4.6	w.w.	[9] Wiech et al., 2018
	Cd	North Atlantic	2 [c]	0.38		0.31–0.44	w.w.	[30] Grimaldo et al., in press
		Norwegian Fjord	4 [c]	0.026		0.018–0.032	w.w.	[9] Wiech et al., 2018
	Hg	North Atlantic	2 [c]	0.026		0.022–0.030	w.w.	[30] Grimaldo et al., in press
		Norwegian Fjord	4 [c]	0.034		0.024–0.049	w.w.	[9] Wiech et al., 2018
		Azores	11	0.34		0.051–0.446	d.w.	[32] Monteiro et al., 1996
	Pb	North Atlantic	2 [c]	<0.05			w.w.	[30] Grimaldo et al., in press
		Norwegian Fjord	4 [c]	0.009		0.006–0.014	w.w.	[9] Wiech et al., 2018

Table 2. *Cont.*

Species	Element	Location	N	Mean (mg/kg)	SD	Range	d.w./w.w.		Reference
Meganyctiphanes norvegica	As	NE Atlantic	5	59.3	11.0		d.w.	[33]	Ridout et al., 1989
		NE Atlantic	8	42			d.w.	[34]	Leatherland et al., 1973
		Mediterranean Sea	1 [c]	55.8			d.w.	[31]	Fowler, 1986
	Cd	NE Atlantic	29	0.66		0.14–1.83	w.w.	[35]	P. S. Rainbow, 1989
		NE Atlantic	5	0.39	0.03		d.w.	[33]	Ridout et al., 1989
		NE Atlantic	29	1.6	1.2		d.w.	[33]	Ridout et al., 1989
		NE Atlantic	8	0.25			d.w.	[34]	Leatherland et al., 1973
		North Sea/Atlantic	18 [c]	0.54	0.10		d.w.	[36]	Zauke et al., 1996
		Greenland Sea	19 [c]	0.44	0.10		d.w.	[37]	Ritterhoff and Zauke, 1997
		Atlantic/Firth of Clyde	30	1.06		0.54–6.06	w.w.	[35]	P. S. Rainbow, 1989
		Mediterranean Sea	5 [c]	1.3			d.w.	[31]	Fowler, 1986
		Mediterranean Sea	2 [c]	0.12			d.w.	[38]	Fossi et al., 2002
		Mediterranean Sea	1	1.06			d.w.	[39]	Belloni et al., 1976
		Mediterranean Sea/Corsica	4	0.55	0.03		d.w.	[40]	Roméo and Nicolas, 1986
		Mediterranean Sea/Monaco	n.a.	0.74			d.w.	[41]	Fowler, 1977
		NE Pacific	9	2.8		0.8–5.5	d.w.	[42]	Martin and Knauer, 1973
	Hg	NE Atlantic	8	0.26			d.w.	[34]	Leatherland et al., 1973
		Mediterranean Sea	2 [c]	0.14			d.w.	[38]	Fossi et al., 2002
		Mediterranean Sea/Monaco	n.a.	0.35			d.w.	[31]	Fowler, 1986
		Mediterranean Sea	1	0.092			d.w.	[31]	Fowler, 1986
		Gulf of St Lawrence	6 [c]	0.60	0.05		d.w.	[43]	Lavoie et al., 2010
	Mehg	Gulf of St Lawrence	5 [c]	0.065	0.03		d.w.	[43]	Lavoie et al., 2010
	Pb	Greenland Sea	17 [c]	<0.3			d.w.	[37]	Ritterhoff and Zauke, 1997
		Mediterranean Sea/Corsica	4	4.65	2.11		d.w.	[40]	Roméo and Nicolas, 1986
		Mediterranean Sea/Monaco	n.a.	1.1			d.w.	[41]	Fowler, 1977
		Mediterranean Sea	2 [c]	0.50			d.w.	[38]	Fossi et al., 2002
		NE Pacific	9	2.4		1.0–10.9	d.w.	[42]	Martin and Knauer, 1973
	F-	W-Sweden/N-Kattegat	6	2153			d.w.	[10]	Adelung et al., 1987
		Norwegian Coast	2 [c]	1845		1330–2360	d.w. [f]	[11]	Soevik and Braekkan, 1979

Table 2. *Cont.*

Species	Element	Location	N	Mean (mg/kg)	SD	Range	d.w./w.w.		Reference
Pasiphaea spp.	Hg	Gulf of St Lawrence	2 [c]	0.11	0.02		d.w.	[43]	Lavoie et al., 2010
		Gulf of Maine	8 [c]	0.27	0.07	0.166–0.347	w.w.	[44]	Harding et al., 2018
	Mehg	Gulf of Maine	8 [c]	0.15	0.11	0.03–0.351	w.w.	[44]	Harding et al., 2018
Eusergestes arcticus	Cd	Mediterranean Sea	6 [c]	0.90		0.4–1.5	d.w.	[31]	Fowler, 1986
		Mediterranean Sea/Corsica	5	0.33	0.17	0.12–0.52	d.w.	[40]	Roméo and Nicolas, 1986
	Hg	Mediterranean Sea	1	0.31			d.w.	[31]	Fowler, 1986
	Pb	Mediterranean Sea/Corsica	4	2.13	0.36	1.71–2.38	d.w.	[40]	Roméo and Nicolas, 1986

[c] composite samples; [f] fat-free dry weight; n.a.: not available.

3.1.1. Arsenic and Inorganic Arsenic

The viability of mesopelagic species as an alternative food or feed source largely depends on compliance with existing legislation. In many fish and shellfish, the As concentrations can exceed the concentrations found in most terrestrial foods [45]. Consequently, seafood has been reported as one of the major sources of As in humans. However, there is no EU ML for As in seafood or marine oils intended for human consumption. In 2011, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) withdrew the provisional tolerable weekly intake (PTWI) for iAs in 2011 since it was no longer considered to be protective [46].

In terms of total As, most species had low average concentrations, ranging from 0.79 to 9.5 mg/kg w.w. The observed level for *B. glaciale* of 13 ± 4 mg/kg d.w. was comparable to the total As content reported by Fowler [31], and for another species of lantern fish—*Benthosema pterotum*— of 13.8 mg/kg d.w. [8]. The result for *M. muelleri* also corresponded to the total As found in the same species in another Norwegian fjord [9]. Higher concentrations were obtained for the northern krill *M. norvegica* and the shrimp *Pasiphaea* spp. at 28 and 22 mg/kg w.w., respectively. For both fish species, a high variation was observed in individual total As concentration, with few cases exceeding the ML of 25 mg/kg for fish-based feed ingredients Previous studies reported even higher concentrations in offshore samples of *M. norvegica*, more than twice the average concentration found in the present study [31,33]. The high total As concentrations found were comparable with a previous study on mesopelagic organisms, where crustaceans, such as krill and shrimp, were found to contain elevated concentrations of total As [47]. In the studied mesopelagic species, the toxic inorganic form only existed as a small portion of the total As (<2%). Fish species—*B. glaciale* and *M. muelleri*—and the shrimp *E. arcticus* had concentrations below LOQ. Quantifiable levels were observed in other species (*M. norvegica, Pasiphaea* spp., *P. periphylla*), with the highest value found in *M. norvegica* at 0.16 mg/kg w.w. The measured iAs concentrations found were well below 2 mg/kg, which could be required by competent authorities for fish meal (EU Directive 2002/32 and amendments [15]).

Arsenic occurs in different chemical forms, and they can be found in varying concentrations in fish and other marine organisms. It is well established that As toxicity is dependent on its chemical form. In marine organisms, the toxic iAs is usually present as less than 1% of the total As [20]. Thus, As exists mostly as organic species, with the relatively non-toxic form arsenobetaine being the predominant chemical species in most marine organisms, including fish, bivalves, and crustaceans [48,49]. The As speciation data obtained for the certified reference material MURST-ISS-A2 (Antarctic krill) showed that arsenobetaine, dimethylarsinate, trimethylarsoniopropionate, and oxo-arsenosugars were the major arsenical compounds found in the Antarctic krill sample [50]. Arsenobetaine concentration found corresponded to about 45% of the extractable As. Dimethylarsinate and trimethylarsoniopropionate were present as a minor but significant fraction (approximately 5% and 10% of the total extractable As, respectively), as well as oxo-arsenosugars (approximately 20% of the total extractable As) [50]. While there are no current MLs for organic As in food and feed, potential toxic effects have been reported for methylated species and arsenolipids [51]. This highlights the need for As speciation data, especially for mesopelagic species, which so far have not been investigated extensively. Further studies devoted to As speciation will provide the basis for proper risk assessment of mesopelagic species as a food or feed resource.

3.1.2. Cadmium

Rather high Cd concentrations were found in the here analyzed fish species, approaching but not exceeding the maximum levels in the food of 0.05 mg/kg w.w., given for fish intended to be consumed whole [14]. Considering the size of the here investigated species, consumption of whole fish was likely. Small fish consumed whole, including the head, and viscera are part of multiple food cultures [52,53], and the here investigated species have been shown to be nutrient-dense [7]. The concentrations of Cd were most likely so high, as whole individuals were analyzed. In fish, most Cd is located in the

kidney and liver, and crustaceans accumulate Cd in the hepatopancreas [54,55]. Therefore, the Cd concentration measured in whole individuals would generally be higher than in muscle samples.

Interestingly, in comparison to the here measured concentrations (0.022 ± 0.014 mg/kg w.w.), higher levels were seen in *B. glaciale* caught offshore in the Northern Norwegian Sea (0.09 mg/kg w.w.) [9] and in the North Atlantic (0.07 mg/kg w.w.) by Grimaldo et al. [30]. The concentrations of Cd reported in *B. glaciale* from the Mediterranean Sea were much higher with a large variation (0.19 ± 0.08–0.71 ± 0.15 mg/kg d.w.) [31]. For *M. muelleri*, the findings suggested a similar pattern with much higher concentrations offshore in the Atlantic [30] compared to the concentrations found in the fjords in the present study. Closer investigations are needed to test if the here investigated mesopelagic fish species, in general, contain higher levels of Cd offshore. However, the distribution pattern of Cd in seawater has long been recognized to have a strong correlation to nutrients, especially phosphate, and behaves similarly [56]. Meaning, Cd is depleted in the surface and enriched in deeper water, where organic matter is decomposed. Higher levels of Cd in deep-sea water explain the higher levels offshore, especially in *B. glaciale* inhabiting deeper waters than *M. muelleri*.

In crustaceans, the maximum limit in the EU regulation 1881/2006 of 0.5 mg/kg w.w. only applies to muscle meat from the abdomen, so the here presented concentrations could not directly be compared. However, the measured concentrations in *Pasiphaea* spp. appeared to be high, also compared to the other two species. The here measured concentrations in *M. norvegica* were low compared to the literature values being one magnitude higher. Cadmium levels in this species seemed to show large variations, also in the literature with large ranges of measurements.

Besides, Antarctic krill *Euphausia superba* from the Western Antarctic Peninsula contains contained higher values of Cd (0.29 mg/kg d.w.), and also other historical measurements have approved this trend [57].

For *E. arcticus*, the literature values from the Mediterranean Sea were comparable to our measurements. However, the comparison to literature values had some flaws. Factors, such as location, season, size, sex, and other physiological factors, might affect trace element content. In addition, the number of measured samples in the different studies was rather low, although mostly pooled samples were analyzed. Furthermore, differences in the used analytical approaches must be considered, especially for work done in the early years. Another factor facilitating differences between studies was the mobility of Cd during sample processing. It has been shown for other crustaceans that freezing and thawing are influencing the distribution of Cd within an animal [58], and as krill decomposes rather fast, a loss of Cd together with other fluids is not unlikely. To get a better understanding of the measured contaminant levels, fatty acid and stable isotope signatures might be analyzed and compared to understand the trophic niche of the different mesopelagic species and how and if the different contaminants are biomagnified in the food-web.

P. periphylla had values comparable to the crustaceans and thereby higher values than the fish species.

The JECFA set a provisional tolerable monthly intake (PTMI) for Cd of 25 µg/kg body weight per month, and the European Food Safety Authority (EFSA) announced an even lower tolerable weekly intake for Cd of 2.5 µg/kg body weight [59]. However, even considering the highest concentrations found in the here analyzed mesopelagic species, only unreasonably high consumption would cause health issues.

3.1.3. Mercury

The Hg levels in the fish species *B. glaciale* and *M. muelleri* were low compared to the maximum level of 0.5 mg/kg w.w. and most other commercially used fish species from the North-East Atlantic [60]. Measured concentrations were comparable to the literature values, except two measurements, one from the North Atlantic Ocean with 0.038 mg/kg w.w., and one exceptionally high measurement from the Mediterranean Sea clearly stood out. However, the reason for this could not be explained by the authors either, although local pollution could not be ruled out [31].

While our concentrations measured in the crustaceans *M. norvegica* and *E. arcticus* were considerably lower than the literature values, the concentrations in *Pasiphae* sp. were comparable with the literature values.

The JECFA revised the PTWI for methylmercury (MeHg) in 2007 and reduced it to 1.6 µg/kg body weight per week, and EFSA had set a lower TWI of 1.3 µg/kg body weight per week [61]. Even when assuming a high proportion of MeHg in the measured total Hg, the here measured levels in the mesopelagic organisms were low and not of food safety concern.

3.1.4. Lead

For the two measured fish species, Pb concentrations were low, also in the literature, and far below the EU maximum level in the muscle meat of fish and in whole fish, where fish are intended to be eaten the whole of 0.3 mg/kg w.w.

Moreover, the crustaceans were below the EU maximum level for muscle meat from appendages and abdomen in crustaceans of 0.5 mg/kg w.w., although whole individuals were analyzed. As there is evidence that Pb accumulates heavily in the hepatopancreas of marine shrimps [62–64], it can be assumed that the muscle meat of our investigated crustaceans also was below the EU maximum level. Recently, no TWI is in place for Pb, since EFSA in 2010 [65] and JECFA in 2011 [66] withdrew it. It was no longer considered to be protective as there is no evidence for a threshold for critical effects.

Compared to the literature values, our measured concentrations were rather low. However, the literature values for *M. norvegica* did vary much with mean concentrations between <0.3 and 4.65 mg/kg w.w.

3.1.5. Fluoride

Concentrations of fluoride measured in *M. norvegica* were high and comparable to the literature values and concentrations found in Antarctic krill *E. superba*. The concentrations in the other analyzed crustacean species and the jellyfish were much lower.

No maximum level for fluoride is given for foodstuffs in the EU; however, EFSA established a tolerable upper intake level (UL) of fluoride in different age classes [67]. Considering this UL, only low amounts of krill could be consumed, ranging from 2 to 10 g/day depending on the age (Table 3).

Table 3. Amount of the most common mesopelagic crustaceans from Norwegian fjords in grams that can be consumed before exceeding the daily tolerable upper intake level of fluoride (UL) proposed by the European food safety authority * in different age classes of consumers.

Age (*y*)	UL (mg/day)	*M. norvegica* (g)	*Pasiphaea* spp. (g)	*E. arcticus* (g)
1 to 3	1.5	2.1	24	56
4 to 8	2.5	3.5	40	93
9 to 14	5	6.9	81	185
≥15	7	9.7	113	259

* Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to the Tolerable Upper Intake Level of Fluoride [67].

In an existing exposure assessment on fluoride, two-year-old children and adults were estimated to exceed the UL, considering exposure from toothpaste, recommended use of dental tablets, the 95-percentile fluoride exposure from drinking water and of tea, and an estimated fixed value of 0.2 mg/day for other exposures [68]. Consequently, it cannot be recommended to consume considerable amounts of krill because of its high content of fluoride.

European Commission set a maximum level for fluorine in feed ingredients with a dry matter of 88% at 500 mg/kg. The only exception was krill, where the upper limit was set to 3000 mg/kg (88% dry matter). However, the final diet concentration must still be below 350 mg/kg (88% dry

matter). It was found that Atlantic salmon (*Salmo salar*) was highly tolerant to dietary fluoride given as krill meal with a concentration of fluoride up to 350 mg/kg diet, and that accumulation of fluoride from feeding diets containing krill meal did not lead to tissue accumulation in the fish, at least over a short period of time [69]. Fluorine uptake from krill (*Thysanoessa inermis* and *E. superba*) and the amphipod *Themisto libelulla* was evaluated in Atlantic salmon, Atlantic cod (*Gadus morhua*), rainbow trout (*Onchorhyncus mykiss*), and Atlantic halibut (*Hippoglossus hippoglossus*). Results showed no increase in fluorine levels of any organs and no effects in growth or health [70].

Most of the fluoride in Antarctic krill *E. superba* was found in carapace [10,11] and might be removed, which, however, is tedious, given the small size of krill. It has also been shown that fluoride leaks into the muscle meat of krill post mortem [71]. The fluoride content in krill was shown to be dependent on the molting stage of the krill [10] with much lower values right after ecdysis. In theory, this might be an opportunity to target freshly molted individuals if simultaneous molting takes place.

There is evidence that bioavailability of fluoride from Antarctic krill in mice is high [72] and that it can induce histopathology in livers, kidneys, and bones [73]. However, the actual bioavailability needs to be investigated further for *M. norvegica*. A high bioavailability paired with a high fluoride concentration may make exploitation of *M. norvegica* for direct consumption as food problematic.

3.1.6. Influence of Size, Location, and Sex on Trace Element Concentrations

For Hg in the fish species, we saw a clear indication of higher values of Hg at larger sizes. In *B. glaciale*, we compared three size classes (<40 mm, 45–55 mm, >60 mm) from the same fjord (Osterfjorden) and obtained Hg concentrations of 0.013, 0.025, and 0.044 mg/kg w.w., respectively. For *M. muelleri*, fish above and below 30 mm were compared, and the concentrations were 0.011 and 0.031 mg/kg w.w., respectively. As Hg is known to accumulate over time, this is not unexpected. A similar trend was found earlier in *B. glaciale* [9] and in other North-East Atlantic fish species [60].

For the crustaceans, only the samples of *M. norvegica* allowed a comparison of within the same fjord with individuals below and above 30 mm, and with concentrations of 0.013 and 0.024 mg/kg w.w., there was a clear indication for a size dependency as well.

For Cd, there were differences between the different size classes in both fish species, with higher concentrations in the smallest size classes (*B. glaciale*: <40 mm: 0.044 mg/kg w.w.; 45–55 mm: 0.008 mg/kg w.w., and *M. muelleri*: <30 mm: 0.041; >30 mm: 0.027 mg/kg w.w.). However, in *B. glaciale*, the largest fish (>60 mm) again had higher values than the medium-sized fish with 0.015 mg/kg w.w. No trends could be identified in *M. norvegica*, likely due to the limited amount of samples, as a clear negative correlation with size has been found earlier [35].

For the other trace elements, no clear trends could be identified. However, due to the low number of samples, further research would be desirable investigating the correlation between size and element concentrations, as there might be a potential for targeted harvesting of certain size classes to obtain lower concentrations of undesirable elements.

For *B. glaciale*, males and females were analyzed separately for the medium size class from Osterfjorden, and no trends could be found for any of the analyzed elements, and neither was there a visible trend in elements concentrations between the different fjords in any of the analyzed species.

3.2. Dioxins, Furans, PCBs, and Polybrominated Flame-retardants

The sum values of PCBs, dioxins, and furans, summed dioxins and furans (PCDD/F), summed dioxin-like PCBs (Sum dl-PCBs), the sum of these (PCDDF/F + dl-PCBs), the sum of six (PCB_6) and seven (PCB_7) indicator PCBs, respectively, and the sum of seven PBDEs ($PBDE_7$) in the analyzed samples are given in Table 4. The maximum levels are defined in terms of upper bound sum-parameters [14,74]. The sum-parameters regarding dioxins were measured in the TEQ pg/g w.w. scale (toxic equivalents), in effect, summing toxicities rather than their analytical concentrations, as specified in the regulation (EC) 1881/2006 [14].

Table 4. The sum values of PCBs, dioxins, furans, and polybrominated flame-retardants furans in the most abundant mesopelagic species in Western Norwegian fjords. Summed dioxins and furans (PCDD/F), summed dioxin-like PCBs (Sum dl-PCBs), the sum of these (PCDDF/F + dl-PCBs), the sum of six (PCB_6) and seven (PCB_7) indicator PCBs, respectively, and the sum of seven PBDEs ($PBDE_7$) (Mean ± SD; Min-Max) on wet weight basis are given. Maximum levels for certain contaminants in foodstuffs given in regulation EC1881/2006 are shown for comparison.

Species	N	Sum PCDD/F	Sum dl-PCBs	PCDD/F + dl-PCBs	PCB_6	PCB_7	$PBDE_7$
		(ng 2005-TEQ/kg w.w.)			(µg/kg w.w.)		
		Mean ± SD / (Min–Max)					
B. glaciale	5	0.77 ± 0.21 (0.46–1.03)	0.84 ± 0.44 (0.53–1.6)	1.6 ± 0.6 (1.1–2.6)	13 ± 11 (3.5–26)	15 ± 12 (4.1–31)	0.97 ± 0.68 (0.40–1.8)
M. muelleri	4	1.1 ± 0.6 (0.43–1.8)	0.97 ± 0.54 (0.42–1.6)	2.0 ± 1.0 (0.85–3.0)	13 ± 8 (5.4–25)	15 ± 10 (6.2–29)	1.0 ± 0.4 (0.63–1.5)
M. norvegica	3	0.29 ± 0.06 (0.23–0.35)	0.26 ± 0.17 (0.15–0.45)	0.54 ± 0.22 (0.38–0.79	5.8 ± 6.3 (1.9–13)	6.7 ± 7.2 (2.1–15)	0.42 ± 0.26 (0.25–0.72)
Pasiphaea spp.	3	0.37 ± 0.16 (0.22–0.55)	0.28 ± 0.13 (0.13–0.36)	0.66 ± 0.28 (0.35–0.90)	5.5 ± 5.6 (1.4–12)	6.3 ± 6.4 (1.6–14)	0.45 ± 0.27 (0.19–0.72)
E. arcticus	4	0.83 ± 0.32 (0.54–1.27	0.72 ± 0.35 (0.41–1.1)	1.6 ± 0.6 (0.94–2.2)	10 ± 8 (3.4–21)	12 ± 9 (3.9–24)	0.75 ± 0.39 (0.39–1.3)
P. periphylla	2	0.064 (0.038–0.089)	0.011 (0.011–0.012)	0.075 (0.048–0.10)	0.049 (0.042–0.056)	0.053 (0.046–0.061)	0.010 (0.008–0.011)
Maximum level		3.5	-	6.5	75	-	-

None of the measured values exceeded the maximum level for certain contaminants in foodstuffs given in EC1881/2006 [14]. Regarding PCDDF/F + dl-PCBs, it appeared that in all species, except the jellyfish *P. periphylla*, half of the burden was PCDD/F and the other half dl-PCBs.

Literature data on the here measured persistent organic pollutants is scarce for our analyzed species underlining the need for more data. For the fish species, only two studies could be identified for reporting values for reference. One study reported values for *B. glaciale* from a Norwegian fjord and the Northern Norwegian sea, and the values were comparable to our measured concentrations with a mean of 0.51 and 0.59 ng 2005-TEQ/kg w.w. sum PCDD/F, respectively [9]. However, the sum dl-PCBs was higher in our samples with a mean of 0.84 ng 2005-TEQ/kg w.w. compared to means of 0.51 in a Norwegian fjord and 0.42 ng 2005-TEQ/kg w.w. in the Northern Norwegian Sea, also after considering the dry matter content, which was comparable, and higher fat content. This also resulted in a higher value of PCDD/F + dl-PCBs. The mean values for the fish and krill species for the sum PCDD/F + dl-PCB in the present study, ranging from 0.55 to 2.0 ng 2005-TEQ/kg w.w., were similar, although with a higher range, compared to another pelagic species, the Norwegian spring-spawning (NSS) herring, with mean values per sampling position ranging between 0.45 and 1.2 1998-TEQ/kg w.w. [75].

Furthermore, the PCB_6 content in *B. glaciale* in our measured samples was much higher with a mean of 13 µg/kg w.w., compared to 5.0 and 2.7 µg/kg w.w. in a Norwegian Fjord and the Northern Norwegian Sea, respectively. The same pattern could be seen in $PBDE_7$ in *B. glaciale* with a mean of 0.97 µg/kg w.w. compared to 0.24 and 0.46 µg/kg w.w. in a Norwegian Fjord and the Northern Norwegian Sea, respectively. This indicated local differences, which also got evident when comparing our data from the three different fjords. Osterfjorden showed much higher concentrations of sum dl-PCBs, sum $PCB_{6/7}$, and $PBDE_7$ compared to Boknafjorden and Bjørnafjorden with rather similar values (Supplementary Table S1). The same trend was found for all other species, except the jellyfish, and thereby indicated a higher level of pollution due to a local source for these substances in Osterfjorden. The second set of literature values suggested low values in the North Atlantic with concentrations of 0.22 and 0.350 ng WHO 2005-TEQ/kg of PCDD/F and PCDDF/F + dl-PCBs [30]. Large differences in concentrations

were also found in a study investigating the accumulation of dioxins in deep-sea crustaceans in the Mediterranean. In *Pasiphaea multidentata*, they measured 0.90 ng WHO 1998-TEQ/kg of PCDD/F inside a submarine canyon, while outside 1.5 ng WHO 1998-TEQ/kg was found. Both values were rather high compared to our measured concentration of 0.44 ng WHO 1998-TEQ/kg in *Pasiphaea* spp. [76].

In *B. pterotum*, fished in the Gulf of Oman, the concentrations of PCDD/F and PCDD/F + dl-PCBs were measured to be 0.1 and 0.249 TEQ ng/kg d.w., respectively [8]; however, it is not given which TEQ was used. Our values measured in *B. glaciale* of 2.5 and 4.95 TEQ ng/kg dry weight, respectively, were much higher, and further investigations are needed to understand species differences in this closely related species.

Considering the different length classes from the same fjord analyzed in the two fish species, there was a clear indication of a positive relationship between the here measured persistent organic pollutants and size, similar to what was found for other organic pollutants in freshwater fish species [77]. We also observed a trend towards higher concentrations in females of *B. glaciale* having the same fat content as males, which could be caused by sexual growth dimorphism, with females of certain species investing more energy in reproduction, and thereby growing slower and having more time to accumulate persistent contaminants than males at the same size. However, sexual growth dimorphism was not found in this species in the Northwestern Atlantic [78] or at the Flemish cape [79], and neither the mercury concentrations were different between males and females, and further investigations are needed.

3.3. Lipid Compounds

The lipid contents are presented in Table 5, and fatty acid and fatty alcohol profiles are given in Supplementary Table S2. The fish species were the most lipid-rich of the studied organisms with 18 ± 8% fat in *M. muelleri* and 14 ± 4% fat in *B. Glaciale.* Followed by the shrimps with 9 ± 3% fat *in E. Arcticus* and 5 ± 3% fat in *Pasiphae* spp. Northern krill, *M. Norvegica* had 5 ± 1% fat, while the jellyfish—*P. periphylla*—was very lean, containing only 0.5 ± 0.2% fat.

Table 5. The content of fatty acids, fatty alcohols, wax esters, long-chain monounsaturated fatty acids (erucic acid, cetoleic acid), and total fat (Mean ± SD; Min–Max) in the most abundant mesopelagic species in Western Norwegian fjords.

Species	Fatty Acids	Fatty Alcohols	Wax Esters	Erucic Acid 22:1 (n-9)	Cetoleic Acid 22:1 (n-11)	Total Fat Content
	(µg/100 µg w.w.)		% of fatty acids	(µg/100 µg w.w.)		
	Mean ± SD / (Min–Max)					
Benthosema glaciale	6.8 ± 1.8 (3.1–7.8)	4.2 ± 1.2 (1.8–5.1)	76	0.05 ± 0.02 (0.02–0.07)	0.78 ± 0.24 (0.26–1.07)	13.7 ± 3.7 (6.1–16.0)
Maurolicus muelleri	14.5 ± 7.9 (5.3–21.1)	0.03 ± 0.01 (0.02–0.05)	<0.5	0.12 ± 0.08 (0.03–0.20)	3.1 ± 1.8 (0.7–4.6)	17.8 ± 8.1 (7.1–24.7)
Meganyctiphanes norvegica	4.2 ± 0.8 (3.3–4.9)	0.07 ± 0.02 (0.06–0.09)	<1.5	0.03 ± 0.02 (0.002–0.05)	0.26 ± 0.22 (0.012–0.54)	5.5 ± 0.6 (4.9–5.9)
Pasiphaea spp.	3.7 ± 1.8 (2.4–5.7)	0.02 ± 0.01 (0.01–0.03)	<0.5	0.03 ± 0.02 (0.013–0.05)	0.20 ± 0.15 (0.19–0.29)	5.4 ± 2.7 (3.3–8.4)
Eusergestes arcticus	5.3 ± 2.1 (2.6–7.8)	2.4 ± 1.0 (1.1–3.3)	46	0.04 ± 0.02 (0.01–0.05)	0.52 ± 0.23 (0.01–0.05)	9.4 ± 3.1 (4.9–12.1)
Periphylla periphylla	0.19 (0.15–0.22)	0.04 (0.01–0.08)	22	0.003 ± 0.001 (0.001–0.003)	0.027 ± 0.011 (0.011–0.035)	0.45 (0.34–0.56)

3.3.1. Wax Esters

Two of our investigated species—*B. glaciale* and *E. arcticus*—were storing energy as wax esters (long-chain fatty alcohols esterified to long-chain fatty acids), and the wax esters contributed with 64% and 46% to the total lipid, respectively. In *M. muelleri*, *M. norvegica*, and *Pasiphaea* spp., only traces

of wax esters were detected, constituting 0.2–1.5% of the total lipid. These originated most likely from calanoid specimens present in the stomach and digestion system. In *P. periphylla*, the wax esters contributed 22% to the total lipid; however, as the level of lipid was only 0.45% of the wet weight, it is also likely that these wax esters originated from *Calanus* prey.

Wax esters are common lipid in many mesopelagic invertebrates and fish, where it functions both as energy reserves and buoyancy regulator [80,81].

Since wax esters are not properly absorbed in the mammalian digestive tract [82], and a high intake can lead to oily diarrhea, also called keriorrhea [83], they do pose a food safety concern [84,85]. Keriorrhea has mainly been observed after the consumption of the two fish species—oilfish (*Ruvettus pretiosus)* and escolar (*Lepdocybium flavobrunneum).* Both have a high-fat content of about 20%, of which 90% are wax esters, resulting in a wax ester content in the fillet of up to 18% [85,86]. There have not been conducted any clinical studies on the effects of consumption of oilfish, but from volunteer testing, it has been reported that an intake of 140 g of escolar (corresponding to 25 g wax esters) could induce keriorrhea [85]. A portion of about 300 g whole *B. glaciale* would deliver a comparable amount of wax esters, so keriorrhea might be a problem if consuming a large amount of this fish. To our knowledge, no tolerable intake of wax esters has been established by any authority so far, and further studies are needed to get a better understanding of keriorrhea and if it can be induced by the consumption of mesopelagic fish-containing wax esters.

The safety of human consumption of wax ester rich oil from copepods (*Calanus finmarchicus*) was studied through clinical trials (randomized, double-blind, placebo-controlled), and long-term consumption (12 months) of 2 g/day did not show any negative effects on hematological and clinical chemistry parameters, including gastrointestinal-related effects [87,88]. Despite a large amount of wax ester, *calanus* oil has been suggested as a good source of the long-chain polyunsaturated fatty acids (PUFA), eicosapentaenoic acid, and docosahexaenoic acid for humans, and commercial products are available as dietary supplements [89,90].

The use of calanus oil as lipid sources for salmon feed has also been intensively studied [91]. Salmon has a limited ability to digest wax esters, and these lipids should not exceed 30% of the dietary lipid, so the high amount of wax ester in some of the mesopelagic species has to be taken into account when used for fish feed [84,92,93]. However, the mesopelagic species are also a good source of (n-3) PUFAs and can, therefore, be a valuable marine-based lipid source for fish feed [7,47].

3.3.2. Erucic Acid

Erucic acid is naturally present in the marine food chain, and the EFSA published a risk assessment of erucic acid (22:1n-9) in 2016, where a tolerable daily intake (TDI) for humans of 7 mg/kg body weight per day was established [94]. The concern about erucic acid in the human diet is focused on the consumption of plant oils, like mustard oil, in which erucic acid can make up to 50% of the total FAs. An ML for erucic acid in fish and seafood has not yet been considered [95]. Long-chain monounsaturated fatty acids (≥20 carbon) are poorly catabolized through normal β-oxidation in the mitochondria and induce instead peroxisomal β-oxidation, where the FAs are shortened into C18 MUFAs, which then can be further catabolized in mitochondrial β-oxidation [96]. Diets with high levels of erucic acid have been associated with myocardial lipidosis and cardiovascular diseases [94]. In marine sources, the (n-11) isomer is often dominating the (n-9)-isomers, and in the present mesopelagic marine species, the amount of cetoleic acid(22:1 (n-11) was 7–25 times higher than erucic acid. In contrast to the health concerns indicated for erucic acid, cetoleic acid has been reported to stimulate the synthesis of eicosapentaenoic acid and docosahexaenoic acid from α-linolenic acid in human HepG2 and of EPA in salmon hepatocytes in vitro and to increase whole-body retention of EPA + DHA in salmon [97].

M. muelleri had the highest levels of 22:1 acids, and consumption of around 400 g of this fish would give levels of erucic acid (480 mg) exceeding the EFSA TDI in a 70 kg person. However, further studies regarding its metabolism and its health effects in fish and humans are needed to improve

risk-benefit assessments since there also is evidence that the consumption of oily fish not necessarily is associated with negative effects on cardiovascular health [95,98].

3.4. Undesirables in Processed Mesopelagic Biomass

3.4.1. Estimates for Fish Meal and Protein Fraction

The results of our estimates of the concentration of undesirables in fish meal, assuming that they completely follow the protein fraction, are given in Table 6.

Assuming that the whole biomass will be processed to fish meal with 10% fat content, a meal consisting of *Pasiphae* sp. only would exceed the ML in fish feed for Hg, while a mixed catch without jellyfish would exceed the maximum level in fish feed for fluorine. If only the protein fraction is considered to be used for fish feed production, the maximum level for Cd would be exceeded in *Pasiphaea* spp. and *P. periphylla.* The As, Hg, and fluoride ML would be exceeded in all measured species. In addition, if the determination of iAs is required by competent authorities (EU Directive 2002/32 and amendments [15]), the iAs concentration would be exceeded in *P. periphylla* and in mixed catches with jellyfish. The Hg and fluoride maximum level would be exceeded in all measured species. Interestingly, due to its large contribution in the actual catches, the jellyfish *P. periphylla* is responsible for very high Cd values in the protein fraction only estimate, exceeding the maximum level 10-fold. However, as we do not have enough knowledge on the actual processing factors and if the protein fraction will be used in feed or for human consumption, the here estimated values have high uncertainty. We are aware that it is not likely that processed fish meal only will consist of the protein fraction, and some elements might even be eliminated during the processing or follow the oil fraction as described for Cd in marine oils from calanus [99]. The final complete fish feed will be composed of different ingredients, and the fish meal from the here investigated species will only contribute to a minor part of the feed composition. The results of the applied crude protein are also somewhat uncertain as we assumed a standard amino acid composition and that all measured nitrogen originates from protein. Future studies should take into account the amino acid profile to be able to calculate the true protein content [100,101]. The here calculated numbers are results of a worst-case scenario and were only used to identify possible issues.

3.4.2. Estimates for Fish Oil

The estimates of dioxins, furans, and PCBs in fish oil after processing, assuming that all these will end up completely in the oil fraction, are given in Table 7.

Oil produced from all species would exceed the maximum levels given in the EU Directive 2002/32/EC on undesirable substances in animal feed for PCDD/F (EU, 2002) and the Commission Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs [14]. The same would apply for oil produced from a bulk average catch with and without contribution from jellyfish in the investigated fjords (see Alvheim et al. [7] for catch composition). *P. periphylla* surprisingly showed the highest concentration of PCDD/F, probably due to its low-fat content. For PCDD/F + dl-PCB and PCB_6, none of the produced oils from the species nor the average catches would exceed the maximum level in animal feed. However, all would be above the maximum level in marine oil intended for human consumption. Nevertheless, many of the currently sold marine oils are cleaned before being sold, which may also be feasible for oils originating from mesopelagic species and grant them marked access.

Samples for this investigation have been taken in December, and there is evidence that the fat content also in mesopelagic species is varying with season [47], which, in turn, might influence the load of persistent organic pollutants and should, therefore, be investigated further.

Table 6. Estimated concentrations of trace elements (total arsenic (As), inorganic arsenic (iAs), cadmium (Cd), mercury (Hg), lead (Pb), fluoride (F)), dioxins, furans, dioxin-like polychlorinated biphenyls, polybrominated flame-retardants, erucic acid, and wax esters in processed mesopelagic biomass with a dry matter content of 88%, assuming that the respective trace elements will end up completely in protein/fish meal after processing. (A) shows the estimates for a fish meal with 10% fat content and (B) for the protein fraction only. Maximum levels given in EU Directive 2002/32/EC on undesirable substances in animal feed are given for comparison.

	(A) Fish Meal with 10% Fat and 88% Dry Matter								(B) Protein Fraction with 88% Dry Matter						
	As	**iAs**	**Cd**	**Hg**	**Pb**		**F**		**As**	**iAs**	**Cd**	**Hg**	**Pb**	**F**	
Species							**[mg/kg w.w.]**								
B. glaciale	12	0	0.065	0.065	0.046		0		82	0	0.45	0.44	0.003	0	
M. muelleri	15	0	0.094	0.074	0.025		0		110	0	0.75	0.55	0.002	0	
M. norvegica	99	0.21	0.057	0.049	0.30		2500		670	1.4	0.30	0.33	0.020	13000	
Pasiphaea spp.	84	0.054	0.99	0.15	0.019		240		620	0.40	5.2	1.1	0.001	1200	
E. arcticus	30	0	0.24	0.044	0.033		84		200	0	1.4	0.28	0.002	630	
P. periphylla	13	0.036	1.2	0.033	0.17		132		1500	4.2	22	3.8	0.19	3400	
Average catch [1] wo jellyfish	38	0.052	0.13	0.062	0.099		610 [2]		260	0.35	0.76	0.43	0.007	3200 [2]	
Average catch [1] w jellyfish	14	0.051	1.2	0.034	0.16		155 [2]		1500	4.0	21	3.7	0.18	3400 [2]	
Maximum level [3]	25	-	2	0.1	10		3000 [4]	500 [5]	25	-	2	0.1	10	3000 [4]	500 [5]

	Sum PCDD/F	**Sum dl-PCB**	**PCDD/F + dl-PCB**	**PCB_6**	**PCB_7**	**$PBDE_7$**	**Eurucic acid**	**Wax esters**
	[ng 2005-TEQ/kg w.w.]			**[µg/kg w.w.]**			**[µg/100 µg w.w.]**	
B. glaciale	0.56	0.61	1.2	9.5	1.1	7.1	0.04	3.8
M. muelleri	0.62	0.54	1.1	7.3	8.4	5.6	0.07	0.04 [6]
M. norvegica	0.53	0.47	0.98	11	1.2	7.6	0.06	0.03 [6]
Pasiphaea spp.	0.69	0.52	1.2	10	12	8.3	0.06	0.03 [6]
E. arcticus	0.88	0.77	1.7	11	13	8.0	0.04	2.6
P. periphylla	0.14	0.20	1.7	1.1	1.2	2.2	0.07	0.93
Average catch [1] wo jellyfish	0.61	0.59	1.2	9.6	11	7.2	0.05	2.0 [6]
Average catch [1] w jellyfish	1.4	0.26	1.6	1.5	1.7	2.5	0.07	9.8 [6]
Maximum level [3]	5.0	-	20	175	-	-		

[1] Average catch composition is shown in Alvheim et al. [7]; [2] Assuming the two fish species containing no fluoride at all; [3] Given in the EU Directive 2002/32/EC on undesirable substances in an5imal feed [15]; [4] Maximum level only applies to marine crustaceans, such as marine krill; [5] Maximum level applies to feed materials of animal origin except marine crustaceans; [6] Upper bound values.

Table 7. Estimated mean concentrations of summed dioxins and furans (PCDD/F), summed dioxin-like PCBs (Sum dl-PCBs), the sum of these (PCDDF/F + dl-PCBs), the sum of six (PCB_6) and seven (PCB_7) indicator PCBs, the sum of seven PBDEs ($PBDE_7$), erucic acid, and wax esters, assuming that the respective undesirables will end up completely in fish oil after processing. Maximum levels for non-human consumption (NHC) and human consumption (HC) given in EU Directive 2002/32/EC and Commission Regulation (EC) No 1881/2006, respectively, are given for comparison.

Species	N	Sum PCDD/F	Sum dl-PCBs	PCDD/F + dl-PCB	PCB_6	PCB_7	$PBDE_7$	Erucic Acid	Wax Esters
		(ng 2005-TEQ/kg w.w.)			(µg/kg w.w.)			(µg/100 µg w.w.)	
Benthosema glaciale	5	5.6	6.1	12	95	110	7.1	0.36	38
Maurolicus muelleri	4	6.2	5.4	11	73	84	5.6	0.67	0.41 [4]
Meganyctiphanes norvegica	3	5.3	4.7	9.8	110	120	7.6	0.55	0.27 [4]
Pasiphaea spp.	3	6.9	5.2	12	100	120	8.3	0.56	0.34 [4]
Eusergestes arcticus	4	8.8	7.7	17	110	130	8.0	0.43	26
Periphylla periphylla	2	14	2.0	17	11	12	2.2	0.67	9.3
Average catch [1] wo jellyfish		6.1	5.9	12	96	110	7.2	0.47	20 [4]
Average catch [1] w jellyfish		14	2.6	16	15	17	2.5	0.66	9.8 [4]
Maximum level [2]	NHC [2]	5.0	-	20	175	-	-		-
	HC [3]	1.75	-	6.0	200	-	-		-

[1] Average catch composition is shown in Alvheim et al. [7]; [2] Given in the EU Directive 2002/32/EC on undesirable substances in animal feed [15]; [3] Given in the EU Regulation 1881/2006 setting maximum levels for certain contaminants in foodstuffs [14]; [4] Upper-bound estimates.

3.5. General Discussion

In our study, we analyzed the contaminants in mesopelagic biomass on the species level. Catches of mesopelagic species have been shown to vary significantly in terms of species composition [30,47]. Our data showed a large variation in a load of undesirables of the species, and the contaminant load of a catch would vary accordingly. Therefore, our species-specific data is of high value as it can be used to predict the contaminant profile of a catch if the species composition of the catch is known. The commercial mesopelagic fishery is still under development, and for the moment, it is impossible to predict the main use of this resource. A targeted fishery for some more valuable species and/or fishing for bulk biomass for processing can be imagined. Regardless of the outcome, species-specific data on undesirables will be of significant value. As the genus or family of the here investigated species are found widespread and highly abundant in mesopelagic ecosystems all around the globe, our data allows predictions for other species and possible fisheries as well.

However, further investigations are needed to get a better understanding of the dynamics of contaminants, including nutrients in mesopelagic species, from different regions to assess spatial variation. Regarding harvesting the mesopelagic species, seasonal differences should especially be assessed to facilitate a targeted harvest of the most suitable biomass for food and feed. As feeding patterns vary throughout the year [102], differences in the body, species, and size composition of the catches are likely.

Our results showed that mesopelagic species from Western Norwegian fjords might be suitable for direct consumption with the exception of *M. norvegica* due to the high fluoride values. *B. glaciale* might have some limitations regarding the levels of wax esters and *M. muelleri* regarding erucic acid.

Considering our predictions of undesirables in the fish meal fraction, in the protein fraction, and oil fraction after processing, we were able to identify possible food and feed safety issues. Regarding fish meal, the predictions are less accurate, as we do not know enough about the actual processing. However, total As might be a challenge, but a better understanding of the processing, bioavailability, and speciation is needed before conclusions can be drawn. In the protein fraction, several undesirable elements showed concentrations above the MLs if the protein fraction was intended directly for human consumption. In virgin marine oils made of the here investigated mesopelagic species, the content

of PCDD/F would be likely to exceed the maximum level for non-human consumption (fish oil) and especially human consumption, and also the content of PCDD/F + dl-PCB would be too high for commercial trade for human consumption of marine oils. However, refining and cleaning methods are also applied in other marine oils to remove organic pollutant contamination. To be able to make more precise predictions, processing factors for the different relevant product scenarios should be established in future studies. In addition, other contaminants should be taken into consideration, like chlorinated pesticides or microplastics. As observed for other marine organisms (reviewed in Kögel et al. [103]), microplastic has also been reported in the digestive tract of mesopelagic fish species (*B. glaciale*, *M. muelleri*, and *Notoscopelus kroyeri*) in 11% of the individuals [104].

The knowledge created in the present study is crucial to enable an evaluation of the value of these species. The ecological role these animals might play in terms of carbon pumping is not fully understood yet, but there are clear indications that mesopelagic organisms are having a direct influence on the global CO_2 budget and thereby climate change [13,105]. Large-scale harvesting of mesopelagic biomass should, therefore, be postponed until we know what we actually can win or lose by harvesting the different species and applying different processing methods.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/9/1162/s1, Table S1: Concentrations of trace elements, dioxins, furans, PCBs, polybrominated flame-retardants, erucic acid, and wax esters in the most abundant mesopelagic species in Western Norwegian fjords on individual sample level., Table S2: Fatty acids and fatty alcohol profiles in the most abundant mesopelagic species in Western Norwegian fjords on the individual sample level.

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Supplementary Material to "Undesirables in mesopelagic species and implications for food and feed safety - Insights from Norwegian fjords"

Table S1. Concentrations of trace elements, dioxins, furans, polychlorinated biphenyls and polybrominated flame-retardants in the most abundant mesopelagic species in Western Norwegian Fjords on individual composite sample level.

Species	Area	N per sample	Size / weight	Length class	Sex	Dry matter	Total fat	As	Cd	Hg	Pb	Fluoride	PCDD/F	dl-PCBs	PCDD/F + dl-PCBs	PCB_6	PCB_7	$PBDE_7$
			[mm] / [g]	[mm]		[%]	[%]	[mg/kg w.w.]					[ng 2005-TEQ/kg w.w.			[µg/kg w.w.]		
Benthosema glaciale	Osterfjorden	27	63 ± 2	>60	17m 11f	32	15	4.7	0.015	0.044	0.009	-	-	-	-	-	-	-
		135	50 ± 3	45-55	m	32	16	3.6	0.007	0.021	0.01	-	0.79	0.89	1.7	22	26	1.6
		135	49 ± 3	45-55	f	32	15	3.7	0.008	0.028	0.011	-	1.0	1.6	2.6	26	31	1.8
		>50	24 ± 6	<40	immature	22	6.1	6	0.044	0.013	0.011	-	0.46	0.65	1.1	9.9	12	0.64
		15	50 ± 3	-	-	31		3.7	0.027	0.022	0.054	-	-	-	-	-	-	-
	Boknafjorden	>50	53 ± 6	-	-	32	15	2.2	0.034	0.011	< .007	-	0.80	0.56	1.4	3.8	4.4	0.4
	Bjørnafjorden	83	41 ± 11	-	-	34	16	4.1	0.018	0.013	< .007	-	0.78	0.53	1.3	3.5	4.1	0.41
Maurolicus muelleri	Osterfjorden	>50	24 ± 3	<30	-	23		5.5	0.041	0.011	0.01	-	0.43	0.42	0.85	11	13	0.63
		>50	45 ± 4	>30	-	31	16	5.5	0.027	0.031	< .007	-	1.2	1.6	2.8	25	29	1.5
	Boknafjorden	>50	48 ± 6	-	-	41	23	4.7	0.036	0.035	< .009	-	1.8	1.3	3.0	10	12	1.2
	Bjørnafjorden	>50	37 ± 9	-	-	38	25	4.7	0.026	0.025	< .008	-	0.81	0.61	1.4	5.4	6.2	0.68
Meganyctiphanes norvegica	Osterfjorden	>50	17 ± 2	<30	-	21		52	0.008	0.013	0.14	570	-	-	-	-	-	-
		>50	33 ± 2	>30	-	25	4.9	36	0.009	0.024	0.021	940	0.35	0.45	0.79	13	15	0.72
	Boknafjorden	>50	33 ± 2	-	-	25	5.9	12	0.035	0.011	0.021	660	0.29	0.17	0.45	2.6	2.9	0.29
	Bjørnafjorden	>50	30 ± 5	-	-	25	5.8	13	0.013	0.008	0.16	710	0.23	0.15	0.38	1.9	2.1	0.25
Pasiphaea spp.	Osterfjorden	>50	70 ± 11	-	-	24	3.3	43	0.14	0.060	< .006	72	0.35	0.36	0.71	12	14	0.72
	Boknafjorden	>50	82 ± 8	-	-	16	8.4	10	0.47	0.033	0.003	57	0.55	0.35	0.90	3.3	3.7	0.43
	Bjørnafjorden	>50	49 ± 20	-	-	25	4.6	12	0.16	0.022	< .006	60	0.22	0.13	0.35	1.4	1.6	0.19
Eusergestes arcticus	Osterfjorden	>50	32 ± 5	-	-	22	4.9	14	0.029	0.008	0.006	42	0.54	0.41	0.94	11	12	0.64
		>50	26 ± 5	-	-	31	12.1	12	0.075	0.023	< .007	18	0.87	1.1	2.0	21	24	1.3
	Boknafjorden	>50	50 ± 10	-	-	28	10.6	5	0.13	0.01	0.009	23	0.65	0.43	1.1	3.4	3.9	0.39
	Bjørnafjorden	>50	44 ± 7	-	-	29	10	6.9	0.063	0.014	0.019	23	1.3	0.95	2.2	5.6	6.4	0.67
Periphylla periphylla	Osterfjorden	12	575 ± 446	-	-	5	0.34	1	0.085	< .002	< .01	8	0.038	0.011	0.048	0.056	0.061	0.011
	Bjørnafjorden	10	952 ± 292	-	-	5	0.56	0.59	0.064	< .002	< .01		0.089	0.012	0.10	0.042	0.046	0.0083

Table S2. Fatty acids and fatty alchols profiles in the most abundant mesopelagic species in Western Norwegian Fjords on individual composite sample level.

	Benthosema glaciale (n=8)	Maurolicus muelleri (n=4)	Meganyctiphanes norvegica (n=4)	*Pasiphaea sp.* (n=3)	Eusergestes arcticus (n=4)	*Periphyalla periphylla* (n=2)
Amount FA (µg/100 µg sample weight))	**6.8 ± 1.7**	**14.5 ± 7.9**	**3.4 ± 1.7**	**3.7 ± 1.8**	**5.3 ± 2.1**	**0.19 ± 0.04**
Amount FAOH (µg/100 µg sample weight))	**4.3 ± 1.1**	**0.0 ± 0.0**	**0.05 ± 0.03**	**0.0 ± 0.0**	**2.4 ± 1.0**	**0.04 ± 0.05**
FA profile (% of total FAs)						
14:0	5.00 ± 0.52	7.05 ± 0.60	5.07 ± 1.03	2.42 ± 0.78	3.34 ± 0.75	3.03 ± 0.97
15:0	0.22 ± 0.02	0.52 ± 0.10	0.71 ± 0.09	0.37 ± 0.07	0.27 ± 0.05	0.28 ± 0.05
16:0	5.82 ± 0.51	16.14 ± 2.01	15.22 ± 0.41	15.92 ± 0.09	9.00 ± 0.78	9.64 ± 1.51
Iso 17:0	0.25 ± 0.02	0.24 ± 0.06	0.36 ± 0.06	0.36 ± 0.04	0.21 ± 0.02	0.29 ± 0.04
17:0	0.18 ± 0.04	0.28 ± 0.10	0.38 ± 0.13	0.36 ± 0.08	0.22 ± 0.05	0.35 ± 0.02
18:0	1.45 ± 0.29	1.66 ± 0.44	2.18 ± 0.27	2.80 ± 0.09	1.01 ± 0.20	4.43 ± 0.71
∑SFA	**13.04 ± 1.48**	**26.52 ± 2.40**	**24.73 ± 1.08**	**23.02 ± 0.84**	**14.49 ± 1.76**	**18.48 ± 1.83**
16:1 (n-9)	0.34 ± 0.03	0.20 ± 0.04	0.31 ± 0.09	0.25 ± 0.04	0.26 ± 0.02	0.12 ± 0.02
16:1 (n-7)	11.23 ± 0.87	5.55 ± 0.21	4.52 ± 0.38	3.66 ± 0.25	7.59 ± 0.29	3.45 ± 0.14
16:1 (n-5)	0.27 ± 0.05	0.27 ± 0.05	0.26 ± 0.06	0.16 ± 0.05	0.41 ± 0.09	0.15 ± 0.13
17:1 (n-8)	0.25 ± 0.24	0.31 ± 0.08	0.36 ± 0.07	0.43 ± 0.06	0.38 ± 0.05	0.36 ± 0.09
18:1 (n-11)	0.50 ± 0.13	0.34 ± 0.09	0.15 ± 0.08	0.18 ± 0.04	3.35 ± 2.21	0.41 ± 0.16
18:1 (n-9)	19.84 ± 3.13	9.40 ± 1.78	13.37 ± 3.45	21.76 ± 3.54	15.12 ± 4.03	16.57 ± 7.34
18:1 (n-7)	1.95 ± 0.15	2.01 ± 0.15	4.83 ± 0.65	5.79 ± 0.44	3.40 ± 0.72	3.13 ± 0.64
18:1 (n-5)	0.40 ± 0.04	0.35 ± 0.04	0.34 ± 0.04	0.41 ± 0.04	0.63 ± 0.08	0.67 ± 0.04
20:1 (n-11)	1.90 ± 0.28	1.02 ± 0.16	0.71 ± 0.19	0.97 ± 0.18	2.99 ± 0.80	1.44 ± 0.47
20:1 (n-9)	7.63 ± 1.08	9.99 ± 2.23	5.90 ± 3.22	4.71 ± 1.16	9.93 ± 2.32	9.70 ± 0.31
20:1 (n-7)	0.36 ± 0.05	0.22 ± 0.02	0.63 ± 0.11	0.45 ± 0.11	0.47 ± 0.11	3.22 ± 0.21
22:1 (n-11)	11.31 ± 1.45	20.40 ± 4.83	6.13 ± 4.05	4.91 ± 1.50	9.47 ± 2.10	14.39 ± 2.63
22:1 (n-9)	0.66 ± 0.11	0.81 ± 0.15	0.81 ± 0.42	0.73 ± 0.20	0.72 ± 0.16	1.42 ± 0.22
22:1 (n-7)	0.26 ± 0.03	0.24 ± 0.02	0.22 ± 0.04	0.32 ± 0.01	0.22 ± 0.02	0.31 ± 0.03
24:1 (n-9)	1.41 ± 0.27	1.27 ± 0.26	0.64 ± 0.06	0.85 ± 0.05	0.69 ± 0.29	0.63 ± 0.02
∑MUFA	**57.08 ± 5.83**	**52.72 ± 5.82**	**39.63 ± 6.57**	**46.10 ± 4.07**	**56.03 ± 4.05**	**56.67 ± 5.68**
16:2 (n-4)	0.85 ± 0.09	0.64 ± 0.09	0.46 ± 0.15	0.21 ± 0.10	0.52 ± 0.07	0.25 ± 0.02
18:2 (n-6)	1.81 ± 0.16	1.32 ± 0.09	2.75 ± 1.09	1.70 ± 0.43	2.19 ± 0.39	1.22 ± 0.02
20:2 (n-6)	0.24 ± 0.02	0.18 ± 0.02	0.33 ± 0.03	0.39 ± 0.02	0.37 ± 0.02	0.36 ± 0.00
20:4 (n-6)	0.56 ± 0.08	0.35 ± 0.10	1.24 ± 0.73	1.12 ± 0.42	0.70 ± 0.21	0.96 ± 0.11
22:4 (n-6)	0.09 ± 0.01	0.04 ± 0.01	0.09 ± 0.00	0.05 ± 0.00	0.06 ± 0.04	0.37 ± 0.05
22:5 (n-6)	0.17 ± 0.02	0.14 ± 0.04	0.40 ± 0.15	0.28 ± 0.07	0.19 ± 0.05	0.07 ± 0.00
18:3 (n-3)	1.42 ± 0.13	1.05 ± 0.18	1.26 ± 0.24	0.86 ± 0.27	1.52 ± 0.19	0.81 ± 0.13
18:4 (n-3)	2.26 ± 0.43	1.76 ± 0.32	1.55 ± 0.74	0.99 ± 0.62	1.63 ± 0.16	1.05 ± 0.26
20:3 (n-3)	0.15 ± 0.01	0.14 ± 0.03	0.39 ± 0.05	0.40 ± 0.08	0.26 ± 0.02	0.48 ± 0.03
20:4 (n-3)	0.98 ± 0.10	0.47 ± 0.11	0.47 ± 0.13	0.42 ± 0.09	1.03 ± 0.07	0.87 ± 0.20
20:5 (n-3)	6.17 ± 0.59	4.30 ± 0.76	10.23 ± 2.67	10.34 ± 1.11	8.85 ± 0.84	6.60 ± 1.60
22:4 (n-3)	0.08 ± 0.01	0.09 ± 0.03	0.04 ± 0.03	0.05 ± 0.01	0.10 ± 0.01	0.33 ± 0.04
22:5 (n-3)	0.90 ± 0.07	0.73 ± 0.07	0.81 ± 0.08	0.61 ± 0.06	0.99 ± 0.08	6.76 ± 1.72
22:6 (n-3)	10.39 ± 1.60	8.19 ± 2.45	14.19 ± 3.06	12.10 ± 1.18	9.05 ± 1.18	2.64 ± 0.26
24:5 (n-3)	0.40 ± 0.07	0.52 ± 0.09	0.32 ± 0.09	0.24 ± 0.01	0.50 ± 0.02	0.68 ± 0.15
20:2 NMI	0.17 ± 0.03	0.08 ± 0.03	0.23 ± 0.08	0.35 ± 0.07	0.19 ± 0.07	0.27 ± 0.15
∑PUFA	**27.14 ± 3.32**	**20.76 ± 3.65**	**35.64 ± 7.14**	**30.88 ± 3.56**	**29.48 ± 2.49**	**24.85 ± 3.85**
∑PUFA (n-6)	**3.23 ± 0.27**	**2.25 ± 0.23**	**5.03 ± 1.85**	**3.69 ± 0.79**	**3.80 ± 0.69**	**3.33 ± 0.32**
∑PUFA (n-3)	**22.51 ± 3.08**	**17.53 ± 3.47**	**29.69 ± 5.67**	**26.36 ± 2.95**	**24.32 ± 1.90**	**20.47 ± 4.30**
FAOH profile (% of total FAOHs)						
14:0 Alk	4.33 ± 0.74	3.19 ± 6.38	2.23 ± 1.56	4.07 ± 1.13	4.82 ± 0.23	2.74 ± 0.23
15:0 Alk	0.72 ± 0.07	0.00 ± 0.00	0.35 ± 0.26	0.17 ± 0.29	0.42 ± 0.07	0.39 ± 0.07
16:0 Alk	25.95 ± 1.74	14.62 ± 4.37	15.41 ± 7.10	14.44 ± 1.83	20.25 ± 5.65	24.42 ± 5.65
18:0 Alk	2.60 ± 0.45	21.92 ± 7.90	5.97 ± 6.93	3.55 ± 1.30	1.18 ± 0.24	1.76 ± 0.24
20:0 Alk	0.31 ± 0.03	3.38 ± 3.43	2.48 ± 3.46	2.06 ± 1.02	0.23 ± 0.11	0.50 ± 0.11
24:0 Alk	0.28 ± 0.03	2.73 ± 3.91	3.64 ± 3.77	2.97 ± 2.00	0.75 ± 0.10	0.65 ± 0.10
16:1 (n-7) Alk	3.11 ± 0.14	0.41 ± 0.82	1.96 ± 1.32	1.30 ± 0.99	5.08 ± 0.15	2.97 ± 0.15
18:1 (n-9) Alk	11.85 ± 2.56	3.37 ± 3.01	4.91 ± 4.45	4.29 ± 1.29	4.14 ± 1.58	2.48 ± 1.58
18:1 (n-7) Alk	3.32 ± 0.46	0.22 ± 0.43	1.62 ± 1.21	1.74 ± 0.30	1.99 ± 0.50	1.72 ± 0.50
18:1 (n-5) Alk	0.66 ± 0.02	2.57 ± 3.97	0.27 ± 0.26	6.53 ± 2.97	0.48 ± 0.01	0.41 ± 0.01
∑20:1 Alk (dominated by (n-9))	15.02 ± 1.68	20.95 ± 2.63	25.23 ± 10.72	24.78 ± 3.45	17.61 ± 2.21	19.85 ± 2.21
∑22:1 Alk (dominated by (n-11))	29.71 ± 3.29	23.04 ± 12.35	34.19 ± 11.59	31.98 ± 5.16	41.29 ± 5.82	39.87 ± 5.82
18:2 (n-6) Alk	1.01 ± 0.15	1.01 ± 1.22	0.60 ± 0.40	0.88 ± 0.22	0.87 ± 0.03	0.57 ± 0.03

Fatty acids that contribute to less than 0.3 % of the total FA profiles are not included in the table, but are still part of the sum of total FAs. This include iso 15:0, 20:0, 21:0, 22:0, 24:0, 14:1 (n-5), 16:1 (n-10)-7 me, 16:1 (n-11), 20:1 (n-5), 24:1 (n-7), 16:4 (n-1), 18:4 (n-1), 16:3 (n-4), 18:2 (n-4), 16:2 (n-6), 18:3 (n-6), 20:3 (n-6), 22:2 (n-6), 16:4 (n-3). These minor FAs contribute together with less than 2 % of the total FAs.