Universidade de Aveiro Departamento de Biologia
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Fabiana Encarnação Pinto Freitas

Compreensão do metabolismo de arsenolípidos: no organismo modelo *Mytilus galloprovincialis*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor António J. A. Nogueira, Professor Catedrático no Departamento de Biologia, Universidade de Aveiro (Portugal) e do Doutor Kevin A. Francesconi, Professor Catedrático no Institute of Chemistry, NAWI Graz, University of Graz (Áustria).

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I dedicate this work to all the living beings that gave their life
for the sake of science

o júri

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palavras-chave

Arsenolípidos, *Mytilus galloprovincialis*, Privação de alimento, Ingestão, Microbioma.

resumo

Arsenolípidos, lípidos que contêm arsénio, são encontrados em organismos marinhos, mas sua origem, transferência de cadeia alimentar e valor bioquímico permanecem desconhecidos. *Mytilus galloprovincialis*, é um organismo exclusivamente filtrador e representativo de um nível trófico intermediário, sendo o organismo escolhido como modelo para investigar o metabolismo dos arsenolípidos.

Os arsenolípidos foram extraídos usando a metodologia MTBE:MeOH. A sua quantificação e sua determinação molecular foi feita usando HPLC acoplado a espectrometria de massa molecular de alta resolução e elementar. A diversidade e abundância de arsenolípidos foram investigados sob condições de privação de alimento, ingestão do ambiente e sob condições de decomposição.

M. galloprovincialis mostraram ter uma grande diversidade de arsenolípidos, com um perfil diferente entre o manto e a glândula digestiva, com remobilização destes compostos entre órgãos. Os arsenolípidos presentes nos mexilhões provavelmente resultam da ingestão direta destes compostos e não de biogênese. A privação de alimento causou variações no teor de arsenolípidos semelhantes às dos lípidos não contendo arsénio. Observou-se a depuração dos arsenolípidos através de fezes e tendo-se inferido a biogénese dos arsenolípidos por bactérias entéricas. A ingestão de arsenolípidos do ambiente é rápida e os organismos não apresentaram resiliência à absorção e armazenamento dos mesmos. Verificou-se a bioacumulação de arsenolípidos de cadeia de carbono muito longa, assumimos que seja por serem moléculas funcionais ou pela incapacidade do organismo de os depurar. Em condições de decomposição o perfil de arsenolípidos sofreu alterações, com aumento de compostos de cadeia mais curta. As metodologias de deteção e quantificação, existentes e usadas nesta tese, ainda não foram capazes de identificar todos os compostos presentes com indícios de subestimação do arsenolípidos de cadeia de carbono muito longa. A diversidade e abundância dos arsenolípidos parecem estar intimamente relacionadas com a matriz biológica em estudo. O metabolismo dos arsenolípidos no *M. galloprovincialis*, tem compostos que parecem estar intimamente relacionados com o comportamento lipídico e outros que aparentemente são apenas armazenados.

Permanecem ainda desconhecidas as várias características do seu possível papel em vias celulares, a relevância dos efeitos toxicológicos e os efeitos das moléculas bioacumuladas.

keywords Arsenolipids, *Mytilus galloprovincialis*, Starvation, Uptake, Microbiome

abstract

Arsenolipids, lipids containing arsenic, are found in marine organisms, but their origin, food chain transfer and biochemical value remain unknown. *Mytilus galloprovincialis*, is a filter feeder organism and representative of an intermediate trophic level, where it was chosen as the model organism to investigate the metabolism of arsenolipids.

Arsenolipids were extracted using the MTBE:MeOH methodology. Their quantification and molecular determination were performed using HPLC coupled to high resolution and elementary molecular mass spectrometry. Diversity and abundance of arsenolipids were investigated under conditions of food deprivation, ingestion from the environment and under decomposing conditions.

M. galloprovincialis showed a great diversity of arsenolipids, with a different profile between the mantle and the digestive gland under natural conditions, with exchange of these compounds between organs. The arsenolipids present in mussels probably result from direct intake of these compounds and not from biogenesis. Food deprivation caused variations in arsenolipid content similar to those of lipids not containing arsenic. Arsenolipid depuration by faeces was observed and the biogenesis of arsenolipids by enteric bacteria was inferred. The ingestion of arsenolipids from the environment is rapid and the organisms were not resilient to their absorption and storage. Arsenolipids with a very long carbon chain have bioaccumulated, it was inferred that was because they are functional molecules or the organism is unable to excrete them. Under decomposition conditions the profile of arsenolipids has changed, with an increase in shorter chain compounds. The detection and quantification methodologies, existing and used in this thesis, have not yet been able to identify all compounds present with evidence of underestimation of very long chain carbon arsenolipids. The diversity and abundance of arsenolipids seem to be closely related to the biological matrix under study. Arsenolipid metabolism in *M. galloprovincialis* includes compounds that appear mirror the lipid behaviour and others that are apparently only stored. The various characteristics of their possible role in cell pathways, the relevance of toxicological effects and the effects of bioaccumulated molecules remain unknown.

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Introduction

Arsenic: the element

Since the Bronze Ages, arsenic has been used by mankind (Charles 1967), from China to Egypt, Greece, ancient Persia and the Roman Empire. Arsenic was used in antiquity for colouring and in beauty pigments, such as orpiment and realgar, in bronze production and in mirrors, and more recently in pesticides, herbicides, fertilizers and in medicine (Nriagu 2002; Henke and Atwood 2009).

Although arsenic was already a well-known element, its official discovery as As(0), has been attributed to Albert Magnus, who around 1250 was the first to provide convincing evidence of its existence (Nriagu 2002). Native arsenic, As(0), is unstable; it is formed by low-temperature epithermal mineralization (50-200°C) under sulfur-deficient and strongly reducing conditions rather than from weathering processes (Nordstrom and Archer 2003). Arsenic is in group 15 of the periodic table; it has one stable (nonradioactive) naturally occurring isotope (^{75}As) and the most common valence states are −3, 0, +3, and +5.

There are over 320 minerals that contain arsenic, but only about 10 are relatively common in the Earth's sediments, soils, and crustal rocks. The most common arsenic minerals are: orpiment (As_2S_3), realgar (As_4S_4), arsenolite (As_2O_3), arsenopyrite (FeAsS) and enargite (Cu_3AsS_4) (Foster 2005; Henke and Hutchison 2009). Arsenic dissolved in natural waters occurs mostly as As^{3+} and As^{5+} usually bond with oxygen to form arsenite and arsenate, respectively. As(III) mostly exists in low-oxygen groundwaters and hydrothermal waters, and, depending on pH, mainly exists as $H_3AsO_3^0$, H_2AsO3^-, $HAsO_3^{2-}$, or AsO_3^{3-}. As(V) is more common in oxidizing groundwaters and surface waters, and typically occurs as $H_3AsO_4^0$, $H_2AsO_4^-$, $HAsO_4^{2-}$, or AsO_4^{3-}, also, depending on pH (Henke and Hutchison 2009).

Natural presence and concentrations

Arsenic is the 20th most abundant element on the earth's surface (Cullen and Reimer, 1989), with an estimated average concentration of 0.05 μg g^{-1} in the silicate portion, and 1.7 μg g^{-1} for the bulk Earth

(McDonough 2014). In marine waters, arsenic is the 25th most abundant element with an estimated averaged value of 3.7 μg L^{-1} (Lide 2013), whereas surface open waters have a lower total arsenic concentration of about 1.5 μg L^{-1} (Reimann et al. 2009; McDonough 2014). Estuaries are major recipients of both natural and anthropogenic drainages, and their arsenic values can range from 4 μg L^{-1} in a pristine environment up to 16 μg L^{-1} in a contaminated one (Smedley and Kinniburgh 2002). In rivers and lakes, arsenic concentrations are highly variable; in uncontaminated rivers values typically range from 0.1 to 0.8 μg L^{-1}, but when contamination occurs, such as from hydrothermal vents drainage, values can reach up to 1 000 mg L^{-1} (Smedley and Kinniburgh 2002). Lakes have similar values to rivers, and thus are normally <1 μg L^{-1}, but following arsenic contamination from volcanic springs and rocks, for example in Lake Mono (Ca., USA), extremely high arsenic concentrations up to 10 000 – 20 000 μg L^{-1} have been reported (Smedley and Kinniburgh 2002). Groundwater with levels of arsenic more than 10 μg L^{-1} are not uncommon, and can result from both natural processes and by human contamination (Henke 2009).

Natural remobilization of arsenic occurs mainly from weathering, erosion, sedimentation, volcanic activity, and drifting from thermal fluids and hydrothermal vents. Arsenic remobilization is also increased by human activities such as mining, coal utilization, ore smelting, industrial waste, products enhanced with arsenic, agriculture (Henke and Atwood 2009) and underground water exploitation (*e.g.* Bangladesh (Dhar et al. 2011). Without any preventive measures, arsenic concentrations present in the environment will likely increase in the future.

A toxic element and society

Arsenic lethal toxicity was noticeable for centuries. During the Middle Ages in Europe, arsenolite was being produced and commercialized, with their major use in paints and dyes, medicinal applications, embalming fluids, and in agriculture as a pesticide and herbicide (Nriagu 2002; Henke and Atwood 2009). The easy access to arsenic encouraged its use as a popular aid to murder and suicide. The earliest murder record in history involved Nero, who assassinated Britannicus to secure the throne of the Roman Empire, in AD 55 (Nriagu 2002). Other historical famous cases include the many deaths caused by the Medici and Borgia families, who often used arsenic to eradicate rivals, and the death of Napoleon Bonaparte, which some conspiracy theorists claim was a political assassination involving arsenic (Henke and Atwood 2009; Hughes et al. 2011). Arsenic's role as an undetectable poison was over in 1832, when Dr Marsh, a forensic scientist in England, developed a test that was able to identify and quantify low concentrations of arsenic (Cullen 2008). Arsenic was also the first chemical for which carcinogenic properties were understood; as early as 1879, high rates of lung cancer in miners were attributed in part to inhaled arsenic (Smith et al. 2002; Reimann et al.

2009). In stark contrast to its role as a poison, arsenic was also widely used as medicine till the 19th century, because it was believed to have curative properties for many diseases, such as cancer, malaria, diabetes, syphilis and even rabies (Nriagu 2002). Despite the acknowledged arsenic toxicity, new applications have been developed in the 20th century, including: chemical weapons, paints and dyes, livestock application (livestock dips and poultry food additive), and wood preservatives, the latter of which were phased out by 2003 (Taylor et al. 2003; Wasson et al. 2005; Henke and Atwood 2009). Arsenic is still being used today, for instance in medicine as a drug for treating leukaemia (Soignet et al. 1998; Zhu et al. 2019) or in the development of semiconductors (Henke and Atwood 2009).

In the natural environment, the water soluble inorganic arsenic forms, arsenate (AsV) and arsenite (AsIII), are the most toxic (Eisler 1988; Hughes et al. 2011), causing impaired health (Lin et al. 1995), health chronic conditions, as diabetes cardiovascular diseases and hypertension (Navas-Acien et al. 2008; Abhyankar et al. 2012; Moon et al. 2012), various cancers (Huang et al. 2004) and even death (Hughes 2002). These forms are prevalent in freshwater and therefore pose a great danger to human health as drink water is a major source of exposure. The World Health Organization (WHO) in 1993 recommended a maximum allowable level for inorganic arsenic present in drink water of 10 $\mu g\ L^{-1}$, rectifying a previous higher level of 50 $\mu g\ L^{-1}$. However, many countries continue to retain the 50 $\mu g\ L^{-1}$ limit, mainly for pragmatic reasons because they lack the analytical equipment, sampling programs and funding to enforce a lower concentration limit (Henke and Atwood 2009).

Groundwater bodies are responsible for the most severe cases of exposure to these forms of arsenic - by the year 2018 it was estimated that about 140 million worldwide were exposed to water with inorganic arsenic above 10 $\mu g\ L^{-1}$ (Ravenscroft et al. 2009; WHO 2018). The most serious situations were registered in Taiwan, China, Bangladesh, India, Hungary, Argentina, Chile, Mexico and parts of United States of America, with concentrations in water ranging from 50 up to 5 000 $\mu g\ L^{-1}$ (Smedley and Kinniburgh 2002; WHO 2018).

From element to compounds

As an element of group 15 from the periodic table, arsenic shares chemical properties with its neighbours phosphorus and nitrogen (O'Day 2006; Sun 2010). Arsenic can form and integrate into many compounds including inorganic and organic forms (Rahman et al. 2012). In the presence of surface and near-surface aerated water, arsenide and arsenosulfide minerals oxidize to more water-soluble arsenates. Several water-soluble organic compounds containing arsenic are known: methylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AB), arsenochlorine (AC),

trimethylarsine oxide (TMAO) and tetramethyl arsonium ion (TETRA). Edmond and collaborators (1977) were the first to discover an organoarsenic compound in biota, namely arsenobetaine in rock lobster (*Panulirus cygnus* George, 1962). This discovery prompted studies on organoarsenic compounds in nature, investigating their diversity, toxicity and characterization (Phillips 1990; Akter et al. 2005; Francesconi 2010). These organo-compounds are known generally to be less toxic than inorganic arsenic, but some of them can pose a slight chronic toxicity (Andrewes et al. 2004).

Arsenic is a prevalent element on the environment, and the study of its organic forms, their concentrations and diversity, their behaviour and their metabolism are essential to fully comprehend arsenic's behaviour and fate in the environment. Of all the types of organo-arsenicals, the arsenolipids, lipid compounds that incorporate an arsenic atom, are the most recently discovered and least understood, and thus there is a need to fully investigate these compounds.

Arsenolipids were first shown to exist in cod liver in 1928 by Sadolin (1928). Technological advances in analytical methods, particularly in mass spectrometry, allowed the complete molecular identification of several arsenolipids in 2008, by Rumpler et al. (2008). Studies concerning arsenolipids have been focused on compound diversity, concentration and prevalence in biota, and toxicity to humans. The arsenolipids identified so far have all been from marine sources such as algae and fish tissues including muscle, liver oils and eggs (Taleshi et al. 2008, 2010, 2014; Rumpler et al. 2008; Amayo et al. 2011, 2013; García-Salgado et al. 2012; Raab et al. 2013; Lischka et al. 2013; Viczek et al. 2016). Although one study reported arsenolipids in human milk, a fish-based diet was the likely source of the compounds (Stiboller et al. 2017). Known arsenolipid compounds are made up of seven lipid classes: As-fatty acids (Rumpler et al. 2008), As-hydrocarbons (Taleshi et al. 2008), As-fatty alcohols (Amayo et al. 2013), arsenosugar-phospholipids (Morita and Shibata 1988; García-Salgado et al. 2012), As-phosphatidylcholines and As-phosphatidylethanolamines (Viczek et al. 2016) and, the most recent, an arsenosugar-phytol (Glabonjat et al. 2017) and As-triacylglycerols (Řezanka et al. 2019). Although these compounds are known to exist in several organisms, their effect on the cells' metabolism is still far from being understood. Fatty acids and hydrocarbon compounds that contain arsenic have been shown to have cytotoxic properties (Meyer et al. 2014, 2015); with As-hydrocarbons having the ability to cross to blood-brain barrier (Niehoff et al. 2016).

Arsenolipids seem to be formed in biota by lipid metabolism infidelity (Rumpler et al. 2008), with arsenic been integrated into these complex molecules by the same mechanisms as for non-arsenic molecules. Given the lipid class heterogeneity and diversity of lipid compounds, it is likely that there are many new arsenolipids still to be discovered and identified. Arsenolipids are thought to be formed at the lower trophic levels such as algae (Wrench and Addison 1981; Xue et al. 2014) and then

transferred to higher trophic levels represented so far only by fish. There have been no studies to date looking at arsenolipids in other types of marine organisms.

Overview and aim of the thesis

Filter feeding molluscs, such as the *Mytilus* genus species, represent an intermediate trophic level, and their investigation might shed light on the arsenolipid transformations taking place along a food chain. The main objective of this thesis was to access if arsenolipids content and profile can be affected by different ecological scenarios (*i.e.*, starvation, uptake and degradation conditions) using the Mediterranean mussel, *Mytilus galloprovincialis*, as model organism. These investigations provide the first studies for: arsenolipid profile in mussels (diversity of compounds), information on their possible role as an energy source (starvation - using as reserves), their uptake (trophic transfer), and the bacterial influence on their profile (environmental cycle).

To achieve this goal a stepwise approach was used. *Mytilus edulis* obtained from the local market (collected from a natural area), were screened for total arsenolipid content. Various methodologies for extracting arsenolipids from *M. edulis* were compared (Chapter 1), and the method providing the best outcomes was then applied to the samples of *M. galloprovincialis* investigated in the subsequent parts of this thesis work. A profile of arsenolipids present in the digestive gland and mantle of *M. galloprovincialis* was obtained by using HPLC/mass spectrometry (Chapter 2). The effect of food depletion (starvation) on the arsenolipid profile and on the total lipid content of *M. galloprovincialis* was then examined (Chapter 3). The ability of these organisms to recover from the starvation effects on arsenolipid changes was tested in an *in-situ* uptake experiment conducted with starved mussels (Chapter 4). The possible role of bacteria on arsenolipid content or profile changes in *M. galloprovincialis* was also investigated (Chapter 5).

Limitations

The studies presented were conducted with one species and for the first time in Bivalvia. Therefore, further elaboration of a general nature of arsenolipid metabolism should be done with caution. Moreover, analysis of the non-arsenic containing lipids was not conducted, and thus it was not possible to compare the metabolism of arsenolipids and their non-arsenic homologs. Although the recent technological advances in extraction protocols and in mass spectrometry enabled the determination of the many compounds identified in this thesis, the identification of all compounds present in a particular sample was still not possible.

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Scope of the Thesis

Chapter one

*Quantification of arsenolipids and lipids extracted from blue mussels (*Mytilus edulis*): using a MTBE:MeOH extraction*

Fine-tuning methodologies for arsenolipids and lipid extraction using MTBE:MeOH methodology is compared to the previously used with a DCM:MeOH extraction. Advantages are stated for the MTBE:MeOH extraction.

Chapter two

Lipids that contain arsenic in Mediterranean mussels, Mytilus galloprovincialis

Arsenolipids screening was conduct in digestive glands and mantle of mussels, with 36 identifiable compounds, of where 22 were newly found. This compound screen made a useful contribution to the known diversity of the arsenolipids and can prompt more studies in mussels for arsenolipid identification.

Chapter three

Changes to the arsenolipid profile in the Mediterranean mussel, Mytilus galloprovincialis*, held under starvation conditions*

Determine the depletion of arsenolipids from organisms in food deprivation (starvation) for 15 days. Arsenolipid content changes were observed for digestive gland and mantle, and correlations were established between total lipids and arsenolipids content.

Chapter four

Uptake of arsenolipids by starved Mytilus galloprovincialis*: results from an* in-situ *experiment*

Starved mussels were exposure to field conditions to access uptake of arsenolipids from food sources (plankton). Excretion of arsenolipids during starvation and the *in-situ* recovery time for lipid and arsenolipids content were monitored. Eight new arsenolipids were found.

Chapter five

Microbial influence on arsenolipid profiles in digestive glands of Mytilus galloprovincialis *maintained under post-mortem conditions*

Digestive gland with different bacteria communities were exposed over a period of 30 days to analyse arsenolipid abundance and diversity changes associated with bacterial communities. Four new compounds were found.

Chapter six

Arsenolipids diversity: an overall analysis

A review on the diversity of arsenolipids in biota is presented with their possible relation by a cluster analysis. Conclusions were drawn from the samples studied in this thesis and in conjugation of all known biota analysed till date of publication

Chapter 1

Quantification of arsenolipids and lipids extracted from blue mussels (*Mytilus edulis*): using a MTBE:MeOH extraction

Abstract

Arsenolipid extraction methodology is not standardized for all biological matrices, as it requires adjustments of standard lipid extraction protocols depending on the sample type. In this work we compare the established methodology for arsenolipids using DCM:MeOH with MTBE:MeOH extraction protocols previously reported for non-arsenic lipids. This work establishes the methodology adopted in the further works conducted on the same sample material (mussel tissue). The MTBE:MeOH extraction was shown to be the best methodology for arsenolipid extraction for this sample material, with better extraction values for lipid and arsenolipids, particularly for the latter eluting compounds. In both protocols, an organic phase water-wash resulted in lower extraction yield owing to some of the arsenolipids partitioning into the aqueous phase. The MTBE:MeOH protocol was the more efficient method, it was less time consuming, did not suffer from Cl interference on the arsenic signal, experienced less degradation of arsenolipid compounds and is less toxic than DCM. For these reasons, the MTBE:MeOH protocol was adopted as the extraction procedure for the experiments with mussels performed in this thesis work.

Introduction

Lipid extraction methods based on a methanol/chloroform were developed by Folch et al. (1957) and Bligh and Dyer (1959). Owing to chloroform's toxicity (WHO 2004; Vilanova et al. 2005), new methodologies were developed whereby chloroform was replaced by a less toxic organic solvent such as dichloromethane (Cequier-Sánchez et al. 2008) or methyl tertiary-butyl ether (MTBE, $(CH_3)_3COCH_3$) (Matyash et al. 2008).

For arsenolipid extraction no specific nor standardized methodology exists. Rather, the methods used are based on lipid extractions with minor modifications, which take into consideration the different biological matrices. For some examples, when a screen on arsenolipids is target, extraction is done by partitioned between hexane and aqueous methanol (Taleshi et al. 2008; Rumpler et al. 2008; Amayo et al. 2013); or conducting an extraction using DCM:MeOH after the hexane partition (Raab et al. 2013; Amayo et al. 2014); when the total lipid content is also target extraction is conducted using a DCM:MeOH extraction without an hexane partition (Glabonjat et al. 2014; Viczek et al. 2016). In fact, Glabojant et al. (2014) tested different organic solvents. The optimization of arsenolipids output was done for a certified reference material NMIJ 7405-a Hijiki (*Sargassum fusiforme,* brown macroalgae), being the most effective a DCM:MeOH mixture. Additional steps can be added to remove non-target compounds, *e.g.*: in biologic matrices that are known to contain high concentrations of non-lipid arsenic, a water partition is usually conducted on the final organic fraction (Viczek et al. 2016); and for the removal of non-arsenic lipids a standard procedure of silica column clean is performed (see Glabonjat et al. 2014, for details). Preliminary experiments have shown that silica clean-up protocol does degrade long chain lipids containing arsenic present in blue mussel's lipid extract (personal observation). However, when a silica cleanup is not conducted on a DCM:MeOH recipe extraction it often enhances the polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As).

This work aims to test if the methodology developed by Matyash and co-workers (2008) for normal lipids is also suitable for extraction of lipids containing arsenic, and to compare that method with the method using DCM:MeOH (2:1). The sample used to test the method was whole mussel tissue from the blue mussel (*Mytilus* sp.), which presents a matrix very similar to that of the Mediterranean mussel, the subject of later research in this thesis. The Matyash´s method avoids the Cl^- interference and it presents other advantages such as: it simplifies and reduces sampling handling, it is proved to deliver similar or better recoveries of lipids when compared to the method of Folch et al. (1957) or Bligh and Dyer (1959). Additionally, MTBE is non-toxic and non-carcinogenic, and is also a non-corrosive and chemically stable solvent that does not form peroxides during storage, which could lead to degradation of labile lipids.

Methods

Biological matrix

Mytilus edulis were purchased from the local market in Graz (Austria) (samples origin: Denmark, caught by dredging). Organisms were dissected for the whole tissue and freeze-dried to constant mass. The bulk sample was ground to a powder with a mortar and pestle and sieved (pore: 15 µm). Total arsenic concentration was found to be 10.1 ± 0.7 mg kg^{-1} (n = 8).

Chemicals and Standards

Water (>18 MΩ cm) used throughout the investigation was from a Milli-Q Academic water purification system, (Millipore GmbH, Vienna, Austria). The following chemicals, all of analytical grade, were used in the study: nitric acid 68%, p.a., further purified by using a MLS duoPUR sub-boiling unit (MLS GmbH, Leutkirch, Germany); methanol (≥99.8%, MeOH) purchased from ChemLab (Zedelgum, Belgium); and purchased from Carl Roth GmBH (Karlsruhe, Germany) were: dichloromethane (≥99.9%, DCM), methyl-tert-butyl ether (99.5%, MTBE), ammonium acetate (97%), ammonia (25%), formic acid (≥98%) and the single element standards (^{75}As, ^{74}Ge, ^{115}In and ^{125}Te). The reference material DOLT-3, certified for arsenic, was obtained from the National Research Council Canada. Standard arsenolipid compounds: fatty-acids (AsFA362, AsFA418, AsFA388) and hydrocarbons (AsHC332, AsHC360, AsHC444) were synthesized in-house, with 99% purity accessed by NMR (Nuclear Magnetic Resonance) and HPLC-MS (High-Performance Liquid Chromatography/Mass Spectrometry) (Taleshi et al. 2014).

Total arsenic quantification

Quantification of total arsenic was conducted on an Inductively Coupled Plasma Mass Spectrometer (ICPMS 7900 from Agilent Technologies, Waldbronn, Germany), equipped with an Ari Mist HP nebulizer (Burgerner, Mississauga, Canada). Total arsenic was determined for a portion of 10 mg (weighed to 0.1mg) of the original sample, from fractions of the lipid extraction (1 mL aliquots of organic and water fractions) plus the remaining pellet (all was used); and a 100 µL aliquot of the lipid fraction prior to HPLC-MS analysis. Samples were mineralized by microwave-assisted acid digestion using 2 mL water + 2 mL HNO_3 in an Ultraclave IV microwave system (MLS GmbH, Leutkirch, Germany); using the following program of temperatures: 80°C (10 min); 150°C (15 min); 250°C (15 min); 250°C (30 min). After sample mineralization samples were made ready for ICPMS analysis by diluting them to a final volume of 10 mL, with 10% of internal standard solution (100 µg L^{-1} Ge, In and Te). Arsenic calibration concentrations were 0, 0.1, 0.5, 1, 5, 10, and 50 µg L^{-1}. The calibration curve was validated by an in-house water reference solution with a known arsenic

concentration of 20.0 µg L^{-1} ± 0.50, measurements 20.1 ± 0.27 (n=3). In order to mitigate polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As), measurements were conducted in collision cell mode with a flow of helium gas; further ICPMS feature parameters are summarized in Table 1-1. Total arsenic measurement stability and precision were ensured by the measurement of the 1 µg L^{-1} calibration standard after every 20 samples; all were within the 2% of the target value. Limit of quantification (LOQ) and detection (LOD) for total arsenic were 0.02 and 0.06 µg L^{-1}, respectively. Accuracy of the total arsenic measurements in a biological matrix was checked against the reference material DOLT-3 (certified [As] value: 10.20 µg g^{-1} ± 0.50), which returned a mean value of 10.2 µg g^{-1} ± 0.3 (n = 3).

Table 1-1: Quantification parameters for total arsenic ICPMS 7900

ICPMS	**Agilent 7900**
Mode	Spectrum
RF power	1550
Carrier gas	0.83 L min^{-1}
Optional gas	12% CO_2
Reaction mode	4 mL min^{-1} flow of He
Masses recorded	*m/z* 74(Ge), *m/z* 75(As), *m/z* 77(Se), *m/z* 78(Se), *m/z* 115(In), *m/z* 125(Te)

Lipid extraction procedures

For all extraction procedures, about 50 mg (weighed to 0.1mg) of freeze-dried tissue was used. Lipid extraction was conducted in triplicates, using 15 ml propylene tubes (CellStar®Tubes, Greiner bio-one).

DCM:MeOH:

Method I: Lipid extraction was done on one set of triplicates. Sample was weighed into a tube and 5 mL of DCM:MeOH (2:1) solution was added, tubes were vortexed for 10 seconds and shaken on a rotatory shaker arm for an hour at room temperature. After the mixture was centrifuged (3500 g, 20 min), the DCM:MeOH fraction was collected. Another 5 mL DCM:MeOH (2:1) solution was added and procedure was repeated once more, with a longer time in the vortex mixer to allow the total disintegration??miscegenation of the pellet. The two organic fractions were combined, and phase separation was attained by adding 3.5 mL of water and gently mixing by hand (5 turns). After collecting the water fraction, another 3.5 mL of water was added to the organic phase and the process repeated. The combined water fraction was washed by adding 5 mL DCM:MeOH (2:1), and mixing

gently by hand, and the organic fraction was collected and added to the previous combined organic fractions.

MTBE:MeOH:

Method II: Lipid extraction followed the recipe described in Matyash et al. (2008) on one set of triplicates. Sample was weighed directly into to a 15 mL polypropylene tube, 1.5 mL of MeOH was added and the mixture vortexed for 10 seconds before addition of 5 mL of MTBE. The tube was shaken on rotatory shaker arm for an hour at room temperature. Phase separation was attained by adding 1.25 mL of water, without any shaking of tube; after 10 minutes the mixture was centrifuged at 3500 g (20 min). The upper organic phase was collected and the remaining layer and pellet were re-extracted with 2 mL of a mixture of MTBE/MeOH/H_2O (10:3:2.5 v/v/v); after 10 min, the mixture was centrifuged at 3500 g (20 min). The organic phase was collected and combined with the previous one. In a separate experiment, the extraction procedure above was repeated, in triplicate, but on this occasion the solvent volumes were doubled (a 50 mL polypropylene tube was used). No increase in the amount of lipid and arsenolipid extracted was noted. Therefore, all subsequent extractions were performed using the sample/solvent ratio described above, which also corresponded to the volumes used by Matyash et al. (2008).

Method IIa: The water partition when concerns arsenolipids is very important step as it removes water soluble compounds and reduces the void signal on the chromatography results. To infer on the water partition efficiency and effects on the MTBE/MeOH method output, lipid extraction was conducted on one set of triplicates as described above except for water partition. Water was added and shaking by hand was done as described in the DCM:MeOH recipe (5 turns, bottom-up turns).

MTBE:MeOH recipe:

Standard extraction and Spiked samples: Using Method II, lipid extraction was conducted on 1 mL of a solution of the 6 in-house arsenolipid standards, each with a concentration of 10 µg As L^{-1} (**II-StD**). Additionally, mussels´ sample were spiked with the same a mixture of standards aiming for a 2 µg L^{-1} increment of each compound (**II-Spk**). These two procedures were each done with three replicates.

Arsenolipid samples for HPLC-MS analysis

All solvent fractions (organic and water) were removed on a speed dry vacuum concentrator (temperature maintained at < 30°C) (Christ RVC 2-33 CD plus, Martin Christ GmbH, Osterode am Hartz, Germany). To aid the evaporation process, 200 µL of MeOH was added after 25 min of evaporation. The dried pellets were weighed and re-dissolved in 500 µL of MeOH. To aid the pellet dissolution,

samples were sonicated for 15 min and afterwards centrifuged (3500 g, 20 min). Samples were filtered using a cellulose acetate membrane syringe filter (0.20 µm, 30 mm, VWR International, USA) prior to analysis. Arsenolipid quantification was conducted on the organic fractions, and on one replicate of the water fractions.

Arsenolipids: Quantification and Speciation

Compound separation was conducted with an Agilent 1260 series HPLC using an ACE Ultra Core SuperC18 column (5 µm, 250x4.6 mm Advanced Chromatography Technologies Ltd, UK) under a gradient elution (ICPMS and HPLC parameter details are given in Table 1-2. Chromatography mobile phases were water and MeOH, both with 20 mM of ammonium acetate (pH=9.2, adjusted with ammonia); flow rate was 1 mL min^{-1}, with the following MeOH gradient: 0 min, 50%; 0-25 min – up to 100%; 25-42 min –100%; 42.1-50 min – down to 50%. The detection of the arsenic signal was by introducing an effluent of 10% from the HPLC to the ICPMS using a passive splitter (Analytical Scientific Instrument, Richmond, USA). Sample injection volume was 50 µL mixed online with 50 µL of water. Internal standard solution of 20 µg L^{-1} each, of Ge, In and Te, was introduced using a T-piece after the splitter at an isocratic flow of 8 mL min^{-1}. A continuous flow of 10% MeOH solution was introduced, providing the carbon compensation as described by Raber et al. (2010). Arsenolipid calibration was done with a mixture of the 6 in-house compounds at concentrations of 0, 1, 5 and 10 µg As L^{-1}. Stability and precision of measurements were monitored by measuring the mixture of 5 µg L^{-1} standards every 5 samples. Method blanks were conducted for all lipid extraction procedures. Limit of quantification (LOQ) and detection (LOD) were 0.24 and 0.80 µg L^{-1}, respectively (LOQ and LOD were defined as 3 times and 10 times the standard deviation from method blanks). Data analysis was conducted in Mass Hunter Version B.01.01 (Agilent, Waldbronn, Germany). Quantification was determined by integration of peak areas against external calibration with standards.

Results and Discussion

Bulk mussel sample gave a total arsenic concentration value of 10.1 ± 0.7 µg g^{-1} (n = 5). Arsenic mass balance from the different fractions ranged between 85-95% (mean 89 ± 5%). HPLC of the arsenolipids was conducted with a total arsenic recovery that ranged between 83-97% (mean 89 ± 6%), calculated against the total arsenic value in the injection solution with integration of the full chromatogram, with the void peak included.

Methodology comparison

The Method I (DCM:MeOH) and II (MTBE:MeOH) were about equally efficient at extracting the total lipid. However, there is an increase of 10% towards the Method II, there was a considerable reduction for Method IIa (MTBE:MeOH with shaking). For arsenolipids, however, the extraction efficiency is 50% higher for Method II compared to the Method I (Table 1-3). Therefore, MTBE:MeOH was a more suitable extraction solvent and is possible to inferring a possible reduction of arsenolipid degradation , as stated by Matyash et al. (2008) for lipids. The front peak (void signal) with Method II increase 37-fold compared to the Method I. The water partitioning with shaking is effective for removing non-target arsenic compounds as it only increases the void volume by factor of 5, compared to Method I. The shaking (Method IIa), does not affect the total output of the arsenolipids. However, it shows an increase of arsenolipids in the water fraction (Table 1-3). Arsenolipids were thereby found to migrate to the water fraction. It can be hypothesized that MeOH solubility in water imparts "solubility" to lipids, or it assists micelle formation (Moelbert et al. 2004). In the study of Glabonjat et al. (2014), no water partitioning was conducted and therefore this behaviour was not observed. This migratory event is possibly correlated to the sample matrix and lipid class ratios, since the presence of amphipathic compounds enhances micelle formation (Moelbert et al. 2004). The concentration of these lipids in the water fraction seems to be enhanced also by agitation, given that Method I and IIa had higher values for arsenolipids concentration on the water fraction (Table 1-3).

Extraction of the 6 standard compounds, using the Method II, was successfully accomplished, although the recovery was lower ranging from 64-85% (Table 1-4). Degradation of these compounds was observed as a front peak was detected, with the averaged concentration of 0.50 ± 0.06 μg L^{-1} (peak not observed for method blanks). This loss can also be attributed to arsenolipids migration to the water fraction (detection was not conducted for these samples).

Table 1-2: Quantification parameters for arsenolipid analysis using HPLC-MS coupling

HPLC	**Agilent 1260**
Column	ACE SuperC18
Column temperature	40°C
Injection volume	50 μL sample + 50 μL water
Flow rate	1 mL min^{-1}
Mobile phases	A: Water 20 mM of ammonium acetate B: MeOH 20 mM of ammonium acetate (both pH=9.2, adjusted with ammonia);
Gradient	0 min: 50% 0-25min: up to 100%; 25-42min: 100%; 42.1-50min: down to 50%.

Splitter	10% to ICPMS
Support flow	20 µg L^{-1} Ge, In and Te
Gradient compensation	10% MeOH

ICPMS	**Agilent 7900**
Mode	Time Resolved Analysis
RF power	1550
Carrier gas	1 L min^{-1}
Masses recorded	*m/z* 74(Ge), *m/z* 75(As), *m/z* 77(Se), *m/z* 78(Se), *m/z* 115(In), *m/z* 128(Te)

Table 1-3: Comparative extraction of lipids and arsenolipids outputs, using: DCM:MeOH (Method I) and MTBE:MeOH (Method I and IIa). Averaged values are followed by the standard deviation. Arsenic speciation was done in triplicates for all methods, except the Method I with only one replicate.

	Method	**Total Lipids (%)**	**Total Arsenic (µg As L^{-1})**	**Void Signal (µg As L^{-1})**	**Arsenolipids (µg As L^{-1})**
Organic Fraction	**I**	16.8 ± 1.8 (n=3)	95.2 (n=1)	3.2 (n=1)	89.3 (n=1)
	II	18.7 ± 1.7 (n=3)	298.4 ± 26.6 (n=3)	118.5 ± 10.1 (n=3)	132.8 ± 11.5 (n=3)
	IIa	13.1 ± 0.5 (n=3)	154.0 ± 6.6 (n=3)	16.4 ± 1.3 (n=3)	131.8 ± 6.3 (n=3)
Water Fraction	**I**	-	232.7 (n=1)	-	15.9 (n=1)
	II	-	225.5 ± 12.1 (n=3)	-	2.5 ± 0.2 (n=3)
	IIa	-	445.4 ± 43.1 (n=3)	-	18.0 ± 0.9 (n=3)

Table 1-4: Concentrations (µg L^{-1}) of the standard arsenolipids compounds present at the biologic sample matrix with the different methods: DCM:MeOH (Method I) and MTBE:MeOH (Method II, IIa, II-Spk and II-StD. nd: not detected. Averaged values are followed by the standard deviation. Arsenolipids equivalent to 2 µg As L^{-1} were added to the spiked samples.

Method	**AsFA362**	**AsFA388**	**AsFA418**	**AsHC332**	**AsHC360**	**AsHC444**
I	0.8	1.3	1.1	1.4	nd	nd
II	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	3.1 ± 0.1	nd	nd
IIa	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	2.6 ± 0.5	nd	nd
II-Spk	2.7 ± 0.2	2.9 ± 0.2	2.9 ± 0.0	5.0 ± 0.2	2.4 ± 0.0	2.6 ± 0.3
II-StD	8.5 ± 0.2	7.9 ± 0.2	7.3 ± 0.1	6.2 ± 0.4	6.4 ± 0.3	6.7 ± 0.3

The arsenolipid profiles showed no qualitative change between the methods, with differences in the concentrations for the latter eluting compounds (Figure 1-1). Method II seems to better preserve the latter eluting arsenolipids, those above 20 min retention time. This difference is consistent with the total arsenolipid differences and the loss of AsHC332 in Method I (Table 1-4). Method II also is shown to be most efficient at extracting the other arsenolipid compounds, presenting always the highest values in comparison. Analysis of spiked samples showed that sample matrix does not alter the retention times (Figure 1-2) and the mean recovery was 98 ± 12% for all the spiked compounds.

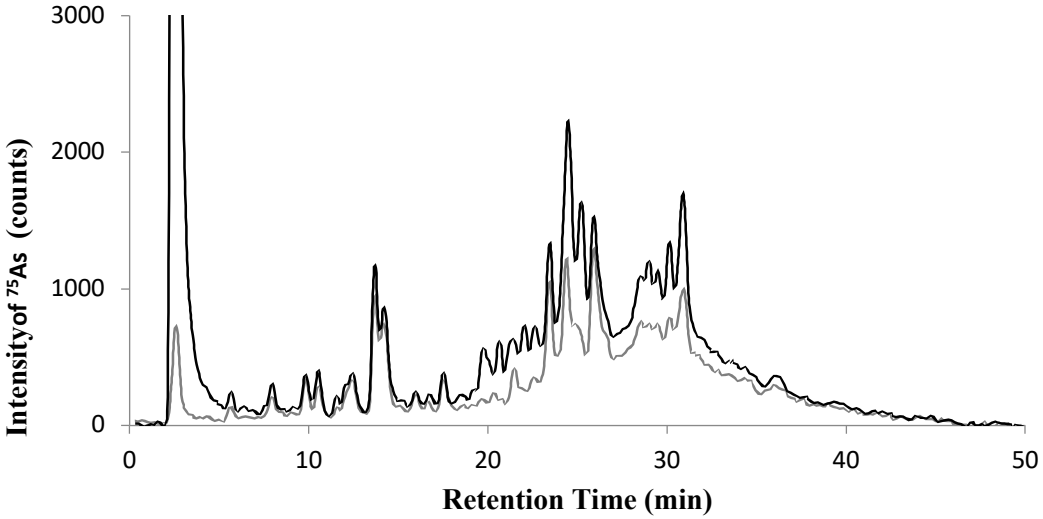

Figure 1-1: Chromatogram of arsenolipids type-profile (one replica), with Method I (grey line), Method II (black line).

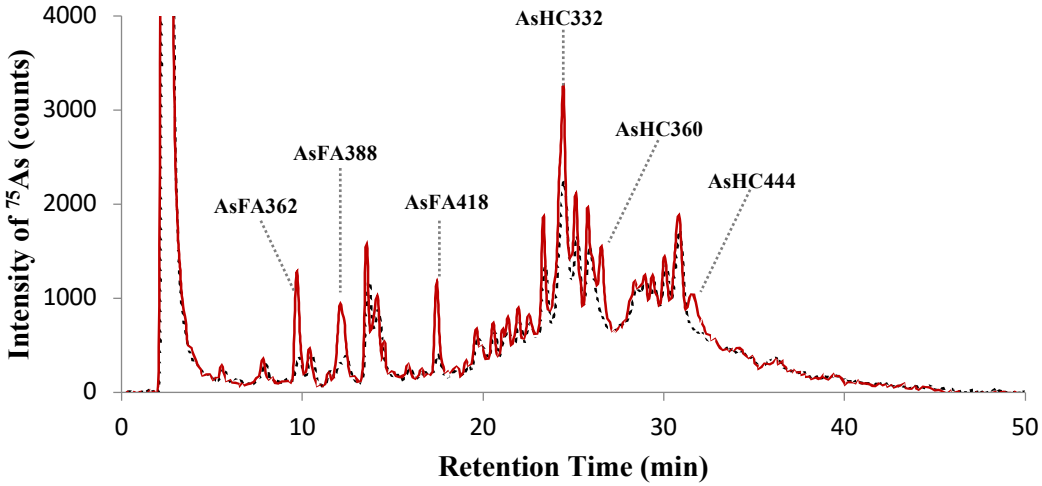

Figure 1-2: HPLC-MS chromatograms of arsenolipid profiles, with Method II (black dashed line) and for spiked samples (red line).

Conclusion

The methodology developed by Matyash and co-workers (2008) was found to be also suitable for arsenolipids extraction from blue mussels; it gave higher extraction efficiency of total lipids and for arsenolipids when compared to the method using DCM:MeOH.

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Chapter 2

Lipids that contain arsenic in Mediterranean mussels, *Mytilus galloprovincialis*

Abstract

Arsenic-containing lipids, arsenolipids, are widely found among marine organisms, but their origin and possible biochemical roles remain unknown This work describes the diversity and abundance of arsenolipids in the digestive gland and mantle of nine specimens of the Mediterranean mussel, *Mytilus galloprovincialis*. By using HPLC coupled to both elemental and high-resolution molecular mass spectrometry, we identified 36 arsenolipids including arsenic derivatives of fatty acids, hydrocarbons, arsenosugar-phospholipids and arsenosugar-phytol; 21 of these arsenolipids were identified for the first time and a new group of arsenolipids was identified comprising arseno-ether-phospholipids. The arsenic compounds in the mussels show distinct profiles depending on the tissue type, providing insight into arsenolipid origin. The results suggest that the presence of arsenolipids in the mussels is from direct uptake of the compounds, presumably from food, rather than biogenesis within the mussels.

Introduction

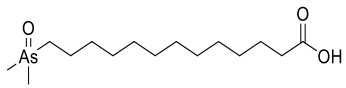
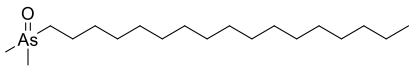
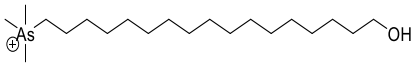
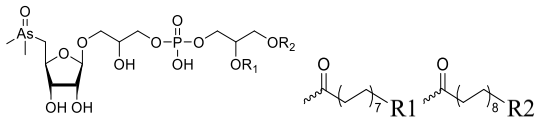
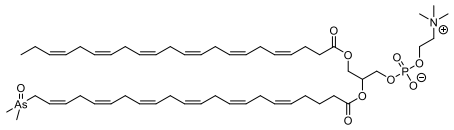
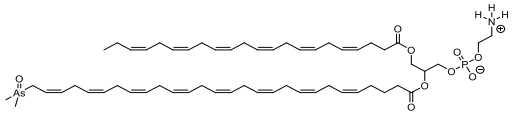
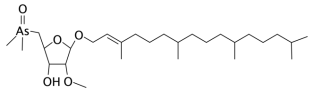
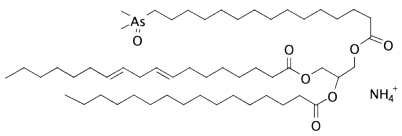
Lipids that contain arsenic, arsenolipids, were first noted by Sadolin (1928), but the identification of the first arsenolipid was not reported until 60 years later when Morita and Shibata (1988) identified an arsenosugar-phospholipid in a marine algae. It was another 20 years (Rumpler et al. 2008), however, before the field was revisited, this time with the help of vastly improved identification methods based on mass spectrometry, and in the following decade a further 100 or more arsenolipids have been reported from marine sources. There have been no reports of arsenolipids from terrestrial organisms.

Arsenolipids have been found in various organisms, including in algae (García-Salgado et al. 2012; Yu et al. 2018; Řezanka et al. 2019), and in fish and fish oil (Rumpler et al. 2008; Lischka et al. 2013; Amayo et al. 2013), fish roe (Viczek et al. 2016). The arsenolipids fall into several groups that correspond to groups of non-arsenic lipids commonly found in, and used by, marine organisms; the major groups are summarised in Table 2-1. In addition to the fundamental biochemical interest in lipids that contain arsenic, an element associated with highly toxic effects, the presence of arsenolipids in commercial seafood products also raises human health issues (Taylor et al. 2017). Toxicological studies over the last five years have demonstrated that some of the arsenolipids are cytotoxic (Meyer et al. 2014a, b), and that they have the ability to cross the blood-brain barrier (Niehoff et al. 2016).

On-going advances in the speciation analysis resulting from the combination of elemental mass spectrometry and high-resolution molecular mass spectrometry have facilitated the identification of arsenolipids, which in turn has expanded our knowledge of the distribution of arsenolipids in marine ecosystems and their possible role in organisms. The focus has been on fish and algae, with no studies on molluscs so far. Mussels (*Mytilus* spp.) are sentinel species, which, because of their widespread habitat and high rates of filtration, are commonly used in programs, such as the Mussel Watch Program, to monitor the environmental quality of aquatic ecosystems (Farrington and Tripp 1995). Arsenic has been routinely measured in these Mussel Watch Programs since their inception in the 1960s (Rodriguez y Baena and Thébault 2006); there are no data, however, on arsenolipids.

The present study investigates the arsenolipids in lipid extracts of the digestive gland and mantle of the Mediterranean mussel, *Mytilus galloprovincialis*, by using HPLC coupled to both elemental and molecular mass spectrometry. We provide an arsenolipid profile, with 36 identifiable compounds including 21 newly discovered species, in the mussel digestive gland and mantle, and briefly discuss the heterogeneity and abundance of the arsenolipids regarding the organs' function.

Table 2-1: Arsenolipids seven known classes, and the work where they can be found. The representative structures are given as an example.

Arsenolipid Class (abbreviation)	**A representative structure** Abbreviation (nominal mass of the compound)		**Reported in:**
Arsenic fatty acids (AsFA)		AsFA334	(Rumpler et al. 2008; Amayo et al. 2011, 2013; Arroyo-Abad et al. 2013, 2016; Lischka et al. 2013; Pereira et al. 2016)
Arsenic hydrocarbons (AsHC)		AsHC360	(Taleshi et al. 2008; Amayo et al. 2011; Arroyo-Abad et al. 2013)
As-fatty alcohols (TMAsFOH)		TMAsFOH375	(Amayo et al. 2013)
Arsenosugar phospholipids (AsPL)		AsPL986	(Morita and Shibata 1988; García-Salgado et al. 2012; Raab et al. 2013)
As-phosphatidylcholines (AsPC)		AsPC997	(Viczek et al. 2016; Řezanka et al. 2019)
As-phosphatidylethanolamines (AsPE)		AsPE1035	(Viczek et al. 2016; Řezanka et al. 2019)
Arsenosugars-phytol (AsPt)		AsPt546	(Glabonjat et al. 2017)
As-triacylglycerols (AsTAG)		AsTAG953	(Řezanka et al. 2019)

Methods

Collection and processing of mussels

Specimens of *Mytilus galloprovincialis*, known as the Mediterranean mussel, were sampled in summer 2015, at the mouth of Minho River, Portugal (GPS: 41º52'N, 8º52'W). Nine organisms were processed for arsenolipid analyses: 3 females, 5 males and 2 of indeterminate sex, with a mean length of 6.3 ± 2.9 cm, and a mean mass of 6.2 ± 1.3 g blot weight. The digestive gland and mantle were dissected from each mussel, individually freeze-dried to constant mass, and stored in polypropylene containers at 5°C. The samples were ground (mortar and pestle) to a homogeneous powder immediately before analysis.

Chemicals and Standards

Water (>18 MΩ cm) used throughout the investigation was from a Milli-Q Academic water purification system, (Millipore GmbH, Vienna, Austria). The following chemicals, all of analytical grade, were used in the study: nitric acid 68%, p.a., further purified by using a MLS duoPUR sub-boiling unit (MLS GmbH, Leutkirch, Germany); methanol (≥99.8%, MeOH) purchased from ChemLab (Zedelgum, Belgium); and methyl-tert-butyl ether (99.5%, MTBE), ammonium acetate (97%), ammonia (25%), formic acid (≥98%) and the single element standards (^{75}As, ^{74}Ge, ^{115}In and ^{125}Te), all purchased from Carl Roth GmBH (Karlsruhe, Germany). The reference material DOLT-3, certified for arsenic, was obtained from the National Research Council Canada. Three arsenic fatty acids (AsFA362, AsFA418, AsFA388) and three arsenic hydrocarbons (AsHC332, AsHC360, AsHC444) were used as standards for calibration and identification of arsenolipids; these standards (99% pure by NMR and HPLC/MS) were available in-house, previously synthesised by the method of Taleshi et al. (2014b).

Total arsenic quantification

Arsenic was measured by inductively coupled plasma mass spectrometry (ICPMS; 7900 from Agilent Technologies, Waldbronn, Germany) in a portion *ca.* 10 mg (weighed to 0.1 mg) of the original powdered tissue samples, and in the two fractions from the lipid extraction: pellet + water (all was used), and the organic phase (1 mL aliquot). After the samples had been mineralised by microwave-assisted acid digestion with 2 mL nitric acid + 2 mL of water in a Ultraclave III microwave system (MLS, Leutkirch, Germany). After the mineralisation, samples were made ready for ICPMS analysis by adding water and 1 mL of internal standard solution containing Ge, In and Te at 100 μg L^{-1} to a final volume of 10 mL. Arsenic calibration concentrations were 0, 0.1, 0.5, 1, 5, 10 and 50 μg L^{-1}.

Polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As) were mitigated by recording the measurements in collision cell mode with a flow of He, at 4 mL min^{-1}. Stability and precision of the ICPMS measurements were monitored by measuring, every 10 samples, a 1 µg L^{-1} calibration standard, which was always within 2 % of the target value over the course of the analyses. Limit of quantification (LOQ) and detection (LOD), calculated from the standard deviation of method blanks, were 0.006 µg L^{-1} (3 x SD) and 0.02 (10 x SD) µg L^{-1}, respectively. By using this mineralisation and measurement procedure, the CRM DOLT-3 (certified [As] = 10.20 µg g^{-1} ± 0.50) returned a value of 9.62 µg g^{-1} ± 0.54, n=4 using 10 mg of material.

Lipid extraction

Preliminary tests were carried out comparing two extraction methods: DCM:MeOH (2:1) (Cequier-Sánchez et al. 2008) and MTBE:MeOH (5:1.5) (Matyash et al. 2008). In the first method, the sample was extracted twice with 5 mL of DCM:MeOH (2:1), with one hour shaking at room temperature for each extraction. Water partitioning was done by adding 3.5 mL of water twice sequentially; phases were mixed by hand with a bottom-up turns (5 times). The MTBE:MeOH mixture was shown to be 50% more efficient at extracting the arsenolipids from the mussel samples, and hence this method was used for all subsequent samples as described below.

We weighed about 50 mg (weighed to 0.1mg) of freeze-dried tissue directly into a 15 mL polypropylene tube (CellStar®Tubes, Greiner bio-one), added MeOH (1.5 mL) and vortexed the mixture (10 seconds) before adding MTBE (5 mL). The tubes were shaken (rotating mechanical arm) for 1 hour at room temperature before we effected phase separation by adding water (1.25 mL). We let the samples stand for 10 min before we centrifuged them (3500 g, 20 min) and collected the upper organic phase. We re-extracted the remaining lower phase with a mixture of MTBE/MeOH/H_2O (2 mL; 10:3:2.5, v/v/v), as described above, and added the resultant upper layer to that from the first extraction. The combined organic phase was dried at 30°C on a vacuum concentrator (Christ RVC 2-33 CD plus, Martin Christ GmbH, Osterode am Hartz, Germany); to aid the evaporation process, we added MeOH (200 µL) to the solutions after 25 min of evaporation. The lipid pellet (total lipid content) was weighed and re-dissolved in MeOH (500 µL); solubilisation was aided by sonication. The sample was then centrifuged and filtered through a syringe filter (cellulose acetate membrane, 0.20 µm, 30 mm from VWR International) prior to HPLC-MS analysis.

Quantification of arsenolipids by HPLC/ICPMS

Compound separation was performed with an Agilent 1260 HPLC system using an ACE Ultra Core SuperC18 column (5 µm, 250 x 4.6 mm; Advanced Chromatography Technologies Ltd, UK) at 40ºC, under gradient elution conditions. The mobile phases were water and MeOH, both with 20 mM

ammonium acetate (pH=9.2, adjusted with ammonia); flow rate was 1 mL min^{-1}, with the following MeOH gradient: 0 min, 50%; 0-25 min, up to 100%; 25-42 min, 100%; 42.1-50 min, down to 50%. Sample injection volume was 50 µL mixed online with 50 µL of water. Arsenic was measured by transferring 10% of the column effluent directly to the ICPMS by using a passive splitter (Analytical Scientific Instrument, Richmond, USA). An internal standard solution of 20 µg L^{-1} each of Ge, In and Te was introduced using a T-piece after the splitter at an isocratic flow of 0.8 mL min^{-1}. An aqueous solution containing 10% MeOH was continuously introduced to the ICPMS spray chamber providing carbon compensation, as described by Raber et al. (2010). Calibration was done with a mixture of the 6 arsenolipid standards, with concentrations of 0, 1, 5 and 10 µg As L^{-1}. Stability of the HPLC-MS measurements was monitored by repeated injection of the 5 µg As L^{-1} standard every 10 samples. LOD and LOQ, calculated from 3*SD and 10*SD of the method blanks, respectively, were 0.09 µg As L^{-1} and 0.30 µg As L^{-1}. Data analysis was conducted with Mass Hunter Version B.01.01 (Agilent Technologies, Waldbronn, Germany). Quantification was based on peak areas against external calibration with the standards.

Identification of arsenolipids by high-resolution molecular mass spectrometry

Arsenolipids were identified by using a Dionex Ultimate 3000 HPLC system coupled to a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Sci., Erlangen, Germany) equipped with a heated electrospray ionization (HESI) source. The chromatography was performed using the same column and eluting conditions as described above for the Agilent system, with a flow rate of 1 mL min^{-1}, the column was held at 40°C, and the injection volume was 20 µL. Mass spectrometry was performed in the positive ion mode and source settings were: spray voltage 3700 V, capillary temp 300 °C, gas temp 500 °C, with flow rates of 65 (sheath) and 20 (aux) instrument units. The full scan range was 300 - 1100 m/z (mass/charge) with the resolution set to 35 000 (full width half-maximum, FWHM), an automatic gain control (AGC) target of $1E^6$ and a maximum injection time (IT) of 100 ms were used. Data dependent mass spectrometry (ddMS/MS) for the precursor ions was performed after recording full scans. Normalized collision energies (NCE) of 20, 35, 40 (stepped) instrument units were used for the fragmentation experiments. MS/MS settings were: maximum injection time 50 ms, AGC $2E^5$, resolution 17500 FWHM. An isolation window of 0.4 m/z was used and five MS/MS experiments were performed before a new full scan. The fragmented ions were subsequently excluded for 10 s. Samples (6 digestive glands and 6 mantles) from six individuals were analysed, and an additional four samples were subjected to the silica cleanup protocol (2 digestive glands and 2 mantles). Samples were initially run on ESMS under the same chromatographic conditions as for HLPC analysis (*i.e.* mobile phases of water and MeOH, both with 20 mM ammonium acetate, pH=9.2). The same samples were also run under acidic conditions (mobile phases: MeOH and water,

both with 0.1% FA). Because arsenolipid measurements by high-resolution molecular mass spectrometry had not previously been performed under alkaline HPLC conditions, the data collected from both acidic and alkaline conditions were cross-checked and confirmed for each arsenolipid compound.

Results and Discussion

Analytical aspects

Total arsenic mass balance for all fractions of the lipid extraction procedure ranged from 78 - 130% (mean 110 ± 20%, n = 9). The column recovery following HPLC of the lipid phase ranged from 80 - 130% (mean 108 ± 16%, n = 9). Some of this arsenic eluted at the solvent front and was unlikely to be bound into arsenolipids; probably it represents dimethylarsinic acid; front peak represents in average 56 ± 6% (n=19) of the total arsenic quantified by integration of the full chromatogram. The total amount of (true) arsenolipids was determined by integration of the chromatogram after the front peak (RT > 5 min). The large number of arsenolipids present in the extracts compromised the HPLC separation, and made it difficult to individually quantify many of the arsenolipids present; they could, however, be reliably identified by use of high-resolution mass spectrometry.

Total arsenic, lipid percentage, and arsenolipid content

No clear differences in lipid or arsenic contents between males, females and indeterminates were observed, so the data from the three groups were combined for the data analysis (Table 2-2). Total arsenic levels in the digestive gland were on average *ca* 14-fold higher than those in the mantle. Total lipid, as a percentage of mass, was marginally higher for digestive gland - 17.6 % compared to 13.9 % for mantle. The digestive gland also contained about 2-fold higher levels of arsenolipids compared to the mantle. For the mantle, the arsenolipid content correlated positively with total lipid content, linear regression $R^2 = 0.66$ (F-test (1, 7) = 13.5, p = 0.01), but no such correlation was observed for the digestive gland. Possibly, the positive correlation found for mantle was related to lipid and arsenolipid storage and use, whereas the digestive gland reflects lipid and arsenolipid uptake, digestion and accumulation (Martínez-Pita et al. 2012).

Table 2-2: Total lipids (%), total arsenic in raw sample and total arsenic in the organic fraction (lipid soluble) for digestive gland and mantle of *Mytilus galloprovincialis*, for males (M), females (F) and indeterminate sex (Ind). * denotes the selected organisms taken for ES MS analysis; for organisms 5 and 8, a silica cleanup was conducted.

			Digestive gland			Mantle		
Mussel	**Sex**	**Length** (mm)	**Lipids** (%)	**Total As** [μg g^{-1}]	**Lipid As** [μg g^{-1}]	**Lipids** (%)	**Total As** [μg g^{-1}]	**Lipid As** [μg g^{-1}]
1*	M	68.0	18.2	26.22	0.33	18.0	13.17	0.14
2*	M	62.9	17.7	32.49	0.30	10.1	12.34	0.14
3	M	60.5	18.3	25.23	0.26	10.3	13.47	0.10
4	M	58.8	16.3	41.91	0.23	10.2	9.54	0.13
5*	F	62.4	18.7	27.34	0.26	19.9	13.50	0.15
6*	F	63.0	14.9	30.05	0.26	14.8	14.66	0.19
7	F	62.2	17.2	31.19	0.34	11.7	15.61	0.09
8*	Ind	62.7	17.5	31.71	0.27	22.1	13.28	0.24
9*	Ind	68.0	19.4	68.03	0.19	7.81	10.23	0.09
Data summary	Min	58.8	14.9	25.23	0.19	7.8	9.54	0.09
	Max	68.0	19.4	68.03	0.34	22.1	15.61	0.24
	Mean	63.2	17.6	34.91	0.27	13.9	12.87	0.14
	SD	3.1	1.3	13.35	0.05	5.1	1.94	0.05

Identification of arsenolipids by HPLC-Orbitrap-MS: analysis by tissue type

A distinct arsenolipid profile was found for each of the two tissue types with digestive gland showing a much wider diversity of compounds (Figure 2-1). Little inter-individual variability was shown by the digestive gland of mussels, a result reflecting not only similar exposure to and consequent uptake of arsenolipids, but also their storage; this effect has previously been observed for non-As lipids (Martínez-Pita et al., 2012). The mantles, however, showed a larger individual variation in arsenolipid profiles, which is also consistent with the observation of lipids by Martínez-Pita et al. (2012); in the mantle, the lipid content can fluctuate widely as reserves are recruited to gametogenesis. The *M. galloprovincialis* tissues proved to be a rich source of arsenolipids. By use of high-resolution MS we were able to identify a total of 36 compounds, of which 21 are reported here for the first time (Table 2-3). The majority of the new identified compounds were fatty acids; it is likely that these compounds resulted from more complex arsenolipids such as phosphatidyl compounds and triacylglycerol derivatives (Taleshi et al. 2014a; Viczek et al. 2016; Řezanka et al. 2019), which were

hydrolysed to the fatty acids in the digestive gland. Also, among the newly discovered arsenolipids were one arsenic-hydrocarbon, two arsenosugar-phospholipids and one compound of a new class of ether-phospholipid.

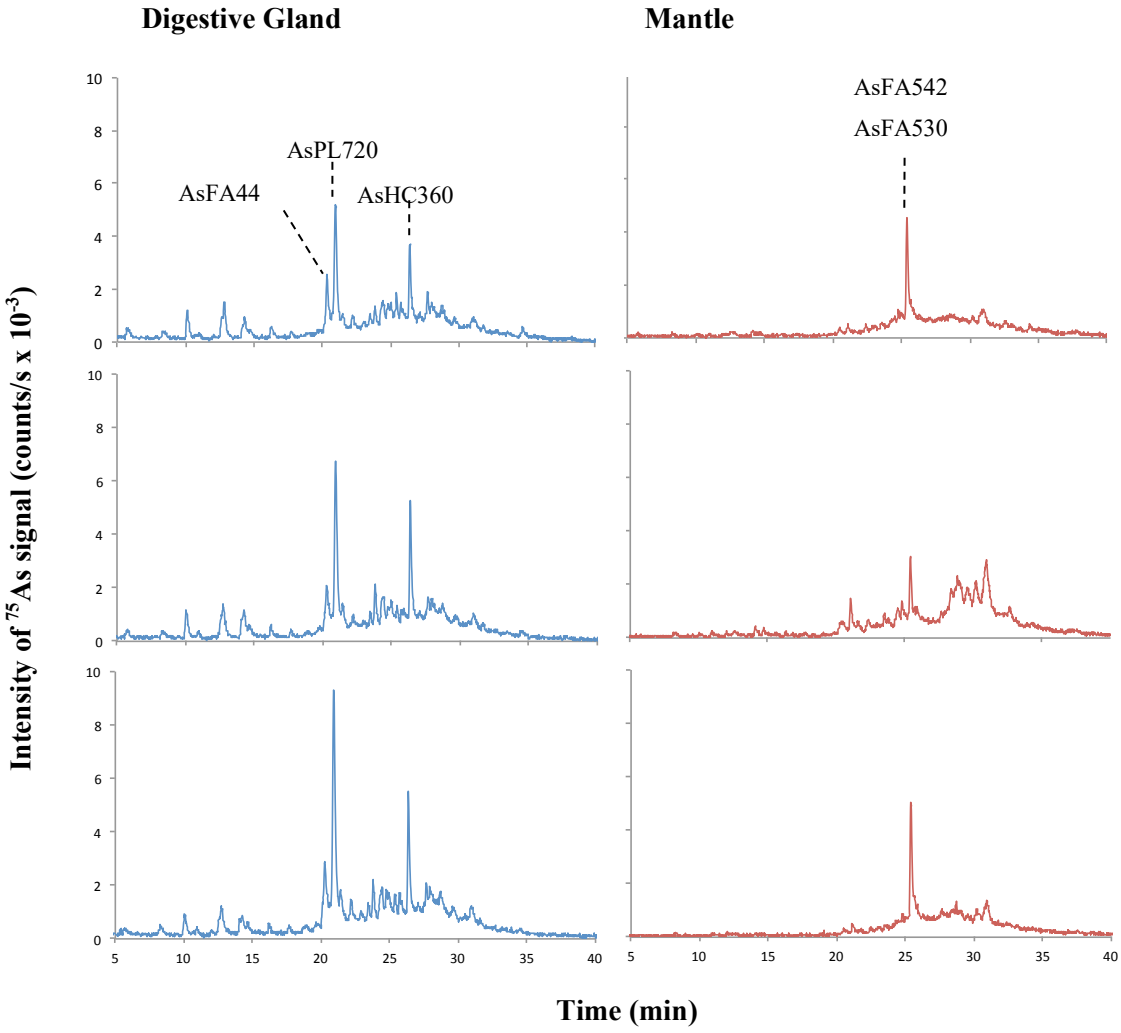
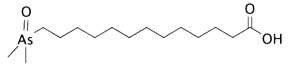
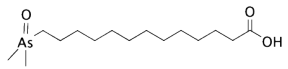
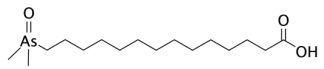
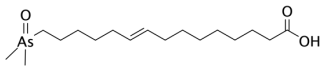
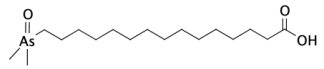
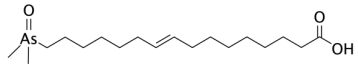
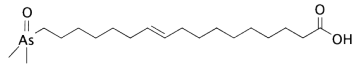
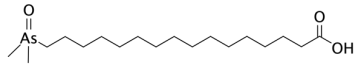
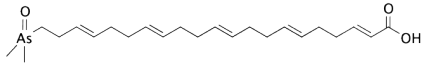
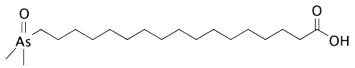
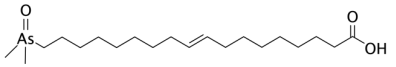
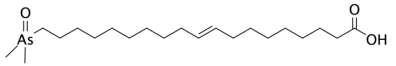
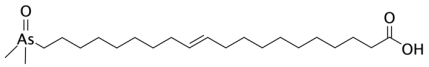
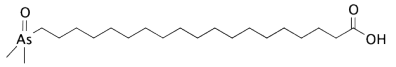
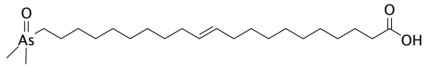
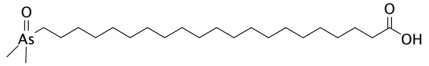
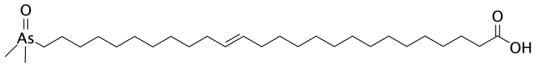
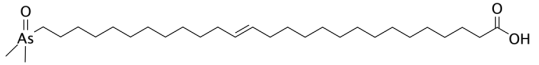
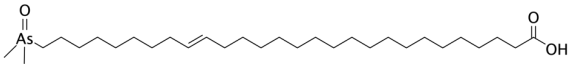
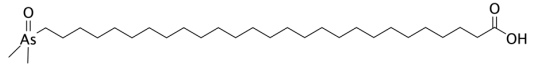
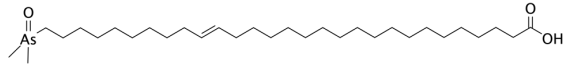
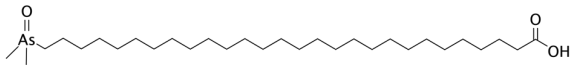
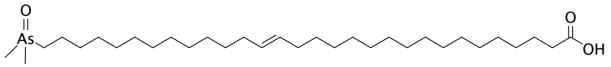
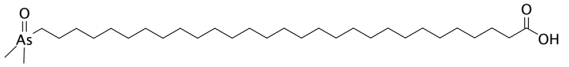
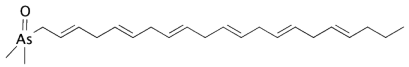
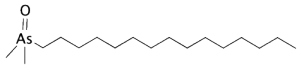
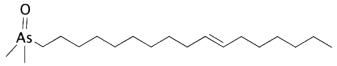
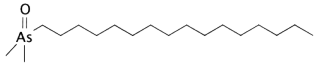
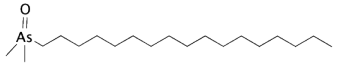
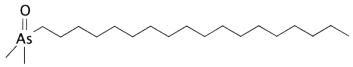
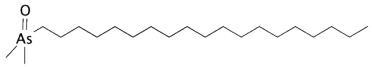
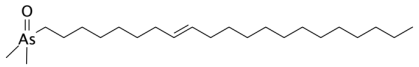


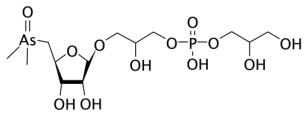
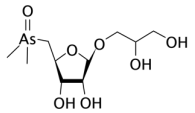
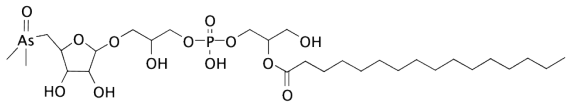
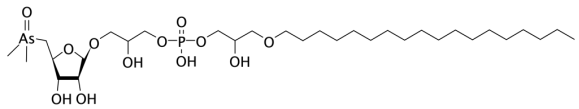
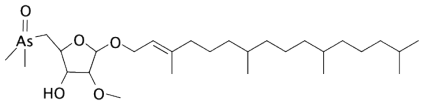
Figure 2-1: Chromatograms obtained by HPLC-MS coupling for digestive gland and mantle of three *Mytilus galloprovincialis* organisms.

Table 2-3: All arsenolipid compounds identified in *Mytilus galloprovincialis,* found the digestive gland (DG) and mantle (M). Compounds are order by the retention times (RT), these are given for the HLPC-MS analysis. Compounds molecular mass is given by $M+H^+$. Compound structures are given for all found compounds

ID	RT	$[M+H]^+$	Calculated $[M+H]^+$	Formula	Double bonds	$\lvert\Delta m/m\rvert$ [ppm]	Structure	Found	Reference
Arseno-Fatty-acids									
AsFA334	5.88	335.1564	335.1562	$C_{15}H_{31}O_3As$	0	0.26		DG/M	(Rumpler et al. 2008)
AsFA334	5.88	335.1564	335.1562	$C_{15}H_{31}O_3As$	0	0.26		DG/M	(Rumpler et al. 2008)
AsFA348	7.53	349.1727	349.1718	$C_{16}H_{33}O_3As$	0	0.28		DG	this work
AsFA360	8.11	361.1721	361.1718	$C_{17}H_{33}O_3As$	1	0.83		DG/M	this work
AsFA362	9.57	363.1874	363.1875	$C_{17}H_{35}O_3As$	0	0.31		DG/M	(Rumpler et al. 2008)
AsFA374	10.22	375.1895	375.1875	$C_{18}H_{36}O_3As$	1	0.14		DG	this work
AsFA388	12.09	389.2029	389.2031	$C_{19}H_{37}O_3As$	1	0.52		DG/M	(Rumpler et al. 2008)
AsFA376	13.56	377.2031	377.2031	$C_{18}H_{37}O_3As$	0	2.79		DG	this work
AsFA436	13.62	437.2029	437.2031	$C_{23}H_{37}O_3As$	5	0.11		DG/M	(Rumpler et al. 2008)

ID	RT	[M+H]$^+$	Calculated [M+H]$^+$	Formula	Double bonds	|Δm/m| [ppm]	Structure	Found	Reference
AsFA390	13.76	391.2189	391.2188	$C_{19}H_{39}O_3As$	0	0.20		DG/M	(Rumpler et al. 2008)
AsFA402	14.00	403.2192	403.2188	$C_{20}H_{39}O_3As$	1	0.96		DG	this work
AsFA416	15.69	417.2345	417.2344	$C_{21}H_{41}O_3As$	1	0.07		DG/M	this work
AsFA430	17.28	431.2514	431.2501	$C_{22}H_{43}O_3As$	1	0.20		DG/M	this work
AsFA418	17.42	419.2507	419.2501	$C_{21}H_{43}O_3As$	0	0.22		DG/M	(Rumpler et al. 2008)
AsFA444	18.60	445.2657	445.2657	$C_{23}H_{45}O_3As$	1	0.34		DG	this work
AsFA446	20.07	447.2826	447.2814	$C_{23}H_{47}O_3As$	0	0.64		DG/M	this work
AsFA514	24.11	515.3443	515.3440	$C_{28}H_{55}O_3As$	1	0.60		DG/M	this work
AsFA528^s	24.83	529.3593	529.3596	$C_{29}H_{57}O_3As$	1	0.74		DG/M	this work
AsFA542	25.56	543.3752	543.3753	$C_{30}H_{59}O_3As$	1	0.10		DG/M	this work
AsFA530	25.76	531.3738	531.3753	$C_{29}H_{59}O_3As$	0	1.86		DG/M	this work
AsFA556	26.27	557.3909	557.3909	$C_{31}H_{61}O_3As$	1	0.03		DG/M	this work

ID	RT	[M+H]$^+$	Calculated [M+H]$^+$	Formula	Double bonds	|Δm/m| [ppm]	Structure	Found	Reference
AsFA544	26.41	545.3920	545.3909	$C_{30}H_{61}O_3As$	0	1.09		DG/M	this work
AsFA570	26.74	571.4069	571.4066	$C_{32}H_{63}O_3As$	1	0.05		DG/M	this work
AsFA558	26.98	559.4061	559.4066	$C_{31}H_{63}O_3As$	0	0.29		DG/M	this work
Arseno-Hydrocarbons									
AsHC404	23.55	405.2133	405.2133	$C_{23}H_{37}OAs$	6	0.02		DG/M	(Taleshi et al. 2008)
AsHC332	24.13	333.2132	333.2133	$C_{17}H_{37}OAs$	0	0.43		DG/M	(Taleshi et al. 2008)
AsHC358	24.60	359.2290	359.2289	$C_{19}H_{39}OAs$	1	0.02		DG/M	(Amayo et al. 2013)
AsHC346	25.15	347.2290	347.2289	$C_{18}H_{39}OAS$	0	1.09		DG	(Amayo et al. 2013)
AsHC360	26.22	361.2446	361.2446	$C_{19}H_{41}OAs$	0	0.12		DG/M	(Taleshi et al. 2008)
AsHC374	27.13	375.2601	375.2602	$C_{20}H_{43}OAs$	0	0.52		DG	(Amayo et al. 2013)
AsHC388	27.86	389.2773	389.276	$C_{21}H_{45}OAs$	0	1.37		DG	(García-Salgado et al. 2012)
AsHC414	28.77	415.2918	415.2915	$C_{23}H_{46}OAs$	1	0.59		DG/M	this work

ID	RT	$[M+H]^+$	Calculated $[M+H]^+$	Formula	Double bonds	$\lvert\Delta m/m\rvert$ [ppm]	Structure	Found	Reference
Arsenosugars-phospholipids									
AsPL482	3.06	483.0606	483.0607	$C_{13}H_{28}O_{12}PAs$	-	0.21		DG/M	this work
AsPL328	3.15	329.0577	329.0576	$C_{10}H_{21}O_7As$	-	0.15		DG	this work
AsPL720	20.51	721.2899	721.2904	$C_{29}H_{58}O_{13}PAs$	-	0.26		DG/M	(Yu et al. 2018)
Arseno-ether-phospholipid									
AsEP734	25.10	735.3422	736.3502	$C_{31}H_{64}O_{12}PAs$	-	0.99		M	this work
Arsenosugar-Phytol									
AsPt546	27.52	547.3323	547.3338	$C_{28}H_{55}O_5As$	-	0.04		DG/M	(Glabonjat et al. 2017)

Arseno-ether-phospholipid, a new arsenolipid group:

The arseno-ether-phospholipid (AsEP734) (Figure 2-2) is a new type of arsenolipid not previously reported; it was found in 3 mantle samples in our study. It was identified based on the presence of the same fragments in the mass spectrum as shown for arsenosugar-phospholipids (m/z = 237.01 and 391.01). The non-As analogue of AsEP734, and related ether phospholipids, are widely distributed in the animal kingdom where they serve as major structural components of cell membranes (Nagan and Zoeller 2001; Dean and Lodhi 2018). In mammals, ether phospholipids are present in liver but at concentrations lower than those of other tissues (Dean and Lodhi 2018), which is consistent with the apparent absence from the digestive gland of the mussel.

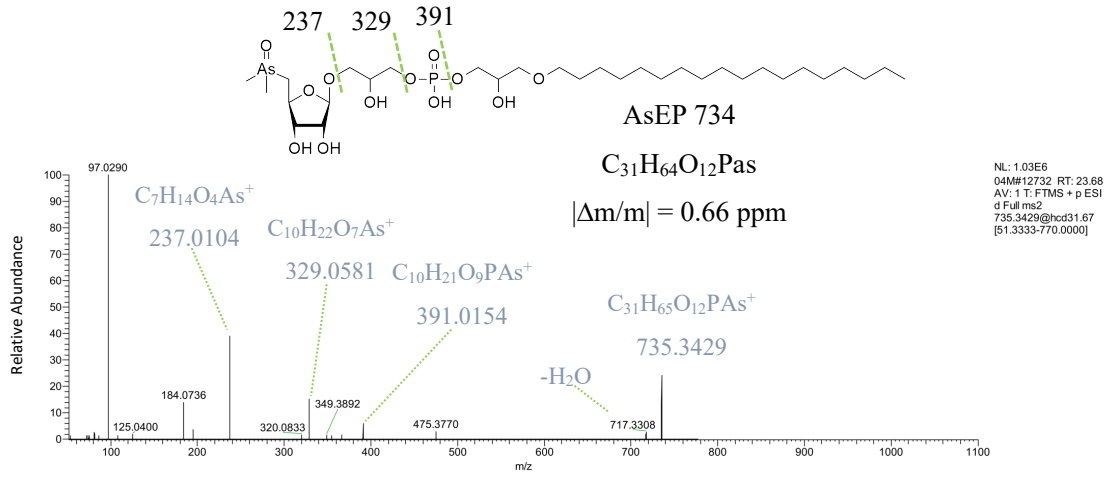


Figure 2-2: MS/MS spectrum and proposed structure for AsEP734

Diversity of arsenolipid compounds

AsFA376 and AsFA444 were found only in the silica clean samples, and the following were detected only in crude extracts: AsFA446, AsFA514, AsFA542, AsFA556, AsFA558, AsHC374, AsHC388, AsHC414, AsPL482 and AsEP738. The presence and relative abundance of the compounds are summarized in Table 2-4. The digestive gland contained a much more diverse range of arsenolipids than did the mantle. Arsenolipids that were exclusively found in the digestive gland were AsFA348, AsFA374, AsFA376, AsFA402, AsFA444, AsHC346, AsHC374, AsHC388 and AsPL328, whereas only the arseno-ether-phospholipid (AsEP734) was found exclusively in the mantle. The arsenosugar phospholipids have previously only had been found in algae (García-Salgado et al. 2012; Raab et al. 2013); their presence in *M. galloprovincialis* is probably due to algae uptake, and, for the mantle at least, might also be contributed to by symbiotic algae (Rodríguez et al. 2008). The arsenic phytol

derivative, AsPT546, was identified in the mussel digestive gland and mantle; its source was probably from diet as it occurs in unicellular algae (Glabonjat et al. 2017).

Table 2-4: Arsenolipids in *Mytilus galloprovincialis*, present in digestive gland and mantle, relative abundance based on ESMS intensity data, assuming a similar response in the mass spectrometer for the various compounds. Legend: T – trace (<1%); M – medium (1-5%); S – significant (5-10%): FA: fatty acids; HC: hydrocarbons; PL: sugars phospholipids; EP: etherphospholipids and Pt: phytol; ID represent the mussels sample identification (Table 2-3).

			Digestive gland						Mantle					
		ID	1	2	5	6	8	9	1	2	5	6	8	9
FA	AsFA334		T	T	T	T	T	T	T	T	T	T	T	T
	AsFA348		T		T	T	T	T						
	AsFA360		T	T	T	T	T	T		T	T	T	T	
	AsFA362		M	M	M	M	M	M	T	T	T	T	T	T
	AsFA374		T	T	T	T								
	AsFA376				T		T							
	AsFA388		M	M	M	M	M	M	T	T	T	T	T	T
	AsFA390		M	M	M	M	M	M		T		T	T	T
	AsFA436		M	M	T	M	M	M	T		T	T	M	
	AsFA402		T	T		T	T	T						
	AsFA416		T	M	M	T	M	M		T	T	T	T	T
	AsFA418		T	T	T	T	T	T				T	T	T
	AsFA430		T	T	T	T	T	T	T				T	
	AsFA444				T		T							
	AsFA446					T							T	
	AsFA514		T	T	T	T	T	T	T	T		T		
	AsFA528		T	T	T	T	T	T	T	T	T	T		
	AsFA530		T	T	T	T	T	T	M	T	T	T	T	T
	AsFA542		T	M	T	T	T	T	M	M	M	M	T	T
	AsFA544							T	T	T		T	T	
	AsFA556		T	T	T	T	T	T	T	T	T	T	T	T
	AsFA558		T	T	T	T	T		T	T	T	T	T	
	AsFA570			T		T						T		
HC	AsHC332		T	T	T	T	T	M		T	T	T	T	
	AsHC358		T	T	T	T	T	T				T		
	AsHC346		T	T	T	T	T	T						
	AsHC360		M	M	M	S	M	S	T	T	T	T	T	T
	AsHC374		T	T	T	T	T	T						
	AsHC388		T	T	T	T	T	T						
	AsHC404		M	S	M	M	M	S		T		T	T	T
	AsHC414		T	T	T	T	T	T				T		
PL	AsPL328				T		T	T						
	AsPL482			M		M			T	T	T	T	T	T
	AsPL720		T	T	T	T	T	T				T	T	
EP	AsEP734								T		T			T
Pt	AsPt546		M	M	M	M	M	M		T		T		T

Arsenolipid analogues in biota

Normal (non-As) fatty acids mostly contain 12 to 22 carbons with a prevalence of even number carbon of 16-20, whereas very long chain fatty acids with a chain longer than 20 carbon atoms are rare (Řezanka and Sigler 2009). In *M. galloprovincialis* mussels saturated or mono-saturated very long chain fatty acids containing arsenic were present in both organs. Therefore, we describe the very long chain fatty acids homologous to the arsenolipids found in this work on Table 2-5, we describe the very long chain fatty acids homologous to the arsenolipids found in this work. All compounds are reported in the marine environment and their presence in *M. galloprovincialis* organs can be inferred to be from uptake, as the elongation of fatty acids is unlikely to take place in mussels (Fearman et al. 2009). We note that the mono-saturated AsFA528 was found in most of the mussel samples in our study whereas its non-As homolog appears to be exclusive to bacteria (Řezanka and Sigler 2009). Mussels have the ability to take up lipids present in ingested bacteria (Jamieson and Wardlaw 1989; Conway and McDowell Capuzzo 1991), and thus bacteria are the likely source of AsFA528 found in the mussels.

Conclusion

Arsenolipids in organisms might be “purpose-built” and playing some specific biochemical role in the organism, or their presence might simply reflect an error in the biochemical workings of the organism whereby a dimethylated arsenic species is accidentally incorporated into the synthetic pathway for essential lipids. The wide diversity of arsenolipids in mussels reported in this study, and their structural similarity to non-arsenic lipids common to marine organisms, suggests accidental incorporation of arsenolipids. Nevertheless, and given the importance of lipids as structural and functional compounds, the presence of abnormal (wrong) compounds homologous to essential lipids could provoke impairment to the cell function and damage the organism. These aspects need further research

Table 2-5: Very long carbon chain lipids non-containing arsenic homologous to those containing arsenic found in this study for the *Mytilus galloprovincialis* digestive gland and mantle. Known homologous lipid are given with the biological matrix that has been found

Arsenolipid	Lipid analogue	Present in:	Reference
AsFA436	Docosapentaenoic acid DPA (22:5 n-3)	Intermediary between EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). Present in halophyte *Gracilaria salicornia* and alga *Ulva lactuca.* Marine fish (<0.3%).	(Kaur et al. 2011; Tabarsa et al. 2012; Byelashov et al. 2015; Dernekbaşı 2015)
AsFA444	Docosenoic acid (22:1 w11)	Copepoda wax, is a zooplankton biomarker	(Ackman et al. 1980a, b; Parrish 2013; George and Parrish 2015)
AsFA446	Saturated docosanoic acid (22:0)	Scarce reports in marine environment: fish liver (hake and whiting) sediment bacteria, in alga (*U. lactuce)* and as the major lipid in the marine sponge (*Siphonodictyon coralliphagum).* Biomarker for terrestrial plants.	(Williams 1965; Perry et al. 1979; Mishra and Sree 2009; El Ashry et al. 2011; Parrish 2013)
AsFA514	(C27:1)	Several marine organisms, including: sponges and unicellular algae	(Řezanka and Sigler 2009)
AsFA530	Octacosanoic acid (28:0)	Bacteria, mycobacteria and unicellular algae	(Řezanka and Sigler 2009)
AsFA528	C28:1	Only detected in bacteria	(Řezanka and Sigler 2009)
AsFA542	C29:1	Bacteria and unicellular algae	(Řezanka and Sigler 2009)
AsFA556	(30:1)	Unicellular algae	(Řezanka and Sigler 2009)
AsFA544	(29:0)	Clam, yeast, bacteria, unicellular algae, peat, sediment and compost	(Řezanka and Sigler 2009)
AsFA570	(31:1)	Mycobacteria, unicellular algae, pollen from the elder plant	(Řezanka and Sigler 2009)
AsFA558	Triacontanoic acid (30:0)	Fungi and peat	(Řezanka and Sigler 2009)

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Chapter 3

Changes to the arsenolipid profile in the Mediterranean mussel, *Mytilus galloprovincialis*, held under starvation conditions

Abstract

Arsenolipids, lipid compounds that incorporate arsenic, are ubiquitous in the marine environment with compounds discovered in algae, mussels and fish tissues. The role of arsenolipids on the lipid metabolism of organism is not known. This study with the Mediterranean mussel *Mytilus galloprovincialis* tracks the changes in the content of lipids and arsenolipids in the mussels during a starvation period. Arsenolipids have shown a similar behaviour to lipids under starvation conditions with more polar arsenolipids, decreased, presumably because they were consumed as an energy source, and less polar arsenolipids were preserved, maintained probably for their functional role. In digestive gland AsFA436, AsHC360 and AsHC404 had the most pronounced decreases. AsFA514, AsFA528, AsFA530, AsFA542 and AsFA558 compounds were exceptions, these have been preserved or increased during starvation. Remobilization of arsenolipids was found from digestive gland to the mantle.

Introduction

Arsenolipids, lipids that contain arsenic, are ubiquitous in marine organisms, being so far reported from algae (Řezanka et al. 2019), macroalgae (García-Salgado et al. 2012; Yu et al. 2018), fish and fish derivates (Rumpler et al. 2008; Sele et al. 2012; Lischka et al. 2013; Amayo et al. 2013). Given that starvation induces in all organisms physiological and biochemical adaptive responses, one being the consumption of their energetic reserves of carbohydrates, proteins and lipids (Wang et al. 2006; McCue 2010, 2012), insights into arsenolipid metabolism might be inferred under starvation conditions.

In species of the genus *Mytilus* sp., the first energetic reserves to be consumed are carbohydrates (mainly glycogen), followed by protein and lipids (Bayne and Thompson 1970; Bayne 1973; Freites et al. 2002; Pleissner et al. 2012). When *Mytilus* spp. are held with depleted food sources in natural or laboratory conditions, the use of their protein/lipid reserves is dependent on seasonal patterns, with protein reserves being favoured in winter and lipids in summer (Bayne 1973; Pleissner et al. 2012). In winter, lipids are preferentially allocated for gamete production for spawning in winter-spring (Prato et al. 2010; Irisarri et al. 2015), which could explain why proteins are used as the main energy resource under conditions of food deprivation. In bivalves, when lipids are used, triacylglycerols become the main energy source, hence phospholipids for their structural type functions are kept at constant concentrations, even during stressful conditions (*e.g.* nutrition, temperature or seasonal variation) (Freites et al. 2002). During short time starvation the total lipid content stays stable to some extent, suggesting autophagy of cells and their inherent remobilization of lipids (Thompson et al. 1978; McVeigh et al. 2006; Singh et al. 2009; Zaffagnini and Martens 2016). In extended starvation, digestive gland reserves rapidly remobilize to other organs, which is correlated to a loss of weight and to physiological changes, as digestive tubules are degraded under food deprivation (Thompson et al. 1974; Cajaraville et al. 1991).

In normal conditions, variability in total lipids is higher in the mantle, because of gametogenesis cycle, while in the digestive gland lipid content varies in a smaller range (Fearman et al. 2009; Martínez-Pita et al. 2012). The profile on lipidomic in *Mytilus* sp. shows a wide variability of compounds, with 16 identifiable lipid classes (Donato et al. 2018). For arsenolipids, the profiles trend to be more diverse in compounds in the digestive gland when compared to the mantle (Freitas et al. 2019 - Chapter 2), four arsenolipids classes were identified, with most of the lipid compounds containing arsenic as fatty acids.

We aimed to investigate if arsenolipid profiles change with starvation conditions and whether arsenolipids are consumed as a source of energy during starvation and if they follow the same known changes as lipids, using the digestive gland and the mantle from *Mytilus galloprovincialis*.

Arsenolipid profiles and arsenolipid diversity for both organs was already done by Freitas et al (2019 - Chapter 2) work. This is the first work conducted to infer arsenolipid consumption under starvation conditions.

Methods

Starvation

Mytilus galloprovincialis individuals were collected in summer 2015 from rocky shores at Minho Estuary (41.8N, -8.8W). Epibiota on the mussels' shells were removed by scrubbing the shells under running tap water. To establish the initial condition (day 0), 25 organisms were randomly selected and measurements of shell length, total wet weight, wet shell weight, and wet meat weight were recorded; this measurement procedure was also conducted for the other groups of mussels used in the starvation experiments. Starvation exposure was conducted for 14 days in an experimental life support system (ELSS) (Coelho et al. 2013). The room was acclimated to 19°C, photoperiod was set to 12/12 h (light/dark), with exposure to UV light for a 4-hour period (from 4^{th} to 8^{th} hour). Artificial salt water (salinity of 30) was prepared from reverse osmosis water (Aqua-win RO-6080 (Taiwan)) and artificial marine salt (Ocean Fish Prodac International S.r.l. (Italy)). Mussels were kept in 7 L aquariums (ratio of 1 L medium to organism (ASTM 1994)), with constant aeration, 50% water renewal every day and bottom of the aquariums clean daily. Abiotic factors of salinity, temperature and pH, using a multiparametric probe (YSI 556 MPS) and ammonia levels (Sera® Ammonium/ammonia-test (NH_4/NH_3) were monitored daily. Sampling of seven organisms was conducted on day 2, 4, 8 and 14. Mantle and digestive gland were dissected and freeze-dried to constant mass, with a loss in moisture of 77 ± 3.7% for digestive gland and of 84 ± 4.0% for mantle. Samples were stored in polypropylene containers at 5°C until analysis, and ground (mortar and pestle) to a homogeneous powder immediately before analysis. Condition index (CI) of organisms was assessed by dividing the digestive gland total freeze-dried mass (FDM) in (mg) by the total length (mm) (Lane 1986). Total lipids, total arsenic and arsenolipids were quantified and characterized for digestive gland and mantle.

Chemicals and Standards

Water (>18 MΩ cm) used in all sample experimental procedures was from Milli-Q Academic water purification system (Millipore GmbH, Vienna, Austria). Analytical grade chemicals were used: nitric acid 68%, p.a., further purified by using a MLS duoPUR sub-boiling unit (MLS GmbH, Leutkirch, Germany); methanol (≥99.9%, MeOH), methyl-tert-butyl ether (99.5%, MTBE), ammonium acetate

(97%), and ammonia (25%) and formic acid (≥98%, FA); purchased from Carl Roth GmBH (Karlsruhe, Germany). Single metal standards (^{75}As, ^{74}Ge, ^{115}In and ^{125}Te) were purchased from Carl Roth GmBH (Karlsruhe, Germany) and the reference material DOLT-3 was obtained from NRC-CNRC, Canada. Six arsenolipids were used as standards for calibration and identification: fatty-acids AsFA362, AsFA418, AsFA388 and hydrocarbons AsHC332, AsHC360, AsHC444; these compounds were synthesized in-house (Taleshi et al. 2014), with 99% purity assessed by NMR and HPLC/MS.

Total arsenic quantification

Arsenic was measured by inductively coupled plasma mass spectrometry (ICPMS; 7900 from Agilent Technologies, Waldbronn, Germany) in portions *ca.* 10 mg (weighed to 0.1 mg) of the original powdered tissue samples, and in the two fractions from the lipid extractions: pellet+water (all was used), and the organic phase (1 mL aliquot). Samples were measured by ICPMS after been mineralised by microwave-assisted acid digestion with nitric acid in a Ultraclave III microwave system (MLS, Leutkirch, Germany). After the mineralisation, samples were made ready for ICPMS analysis by adding water and 1 mL of internal standard solution containing Ge, In and Te at 100 µg L^{-1} to a final volume of 10 mL. Arsenic calibration concentrations were 0, 0.1, 0.5, 1, 5, 10 and 50 µg L^{-1}. Polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As) were mitigated by recording the measurements in collision cell mode with a flow of He, at 4 mL min^{-1}. Stability and precision of measurements were ensured by the measurement of a calibration standard of 1 µg L^{-1}, every 10 samples, with measurements always within 2% of the target value. LOD and LOQ were respectively 0.001 (3 * SD) and 0.003 (10 * SD) µg L^{-1}, using standard deviation of method blanks. Following the above mineralisation and measurement procedure, the CRM DOLT-3 (certified [As]= 10.20 µg g^{-1}, SD 0.50) returned a value of 9.62 µg g^{-1} SD 0.54, n = 4 using 10 mg of material.

Lipid extraction

Lipids were extracted from the mantle and digestive gland, using about 50 mg (weighed to 0.1 mg), following the MTBE:MeOH (5:1.5) lipid extraction method (Matyash et al. 2008). 1.5mL MeOH was added to samples and vortexed; after 5mL of MTBE was added and tubes were shaken for 1 hour at room temperature, phase separation was attained by adding 1.25 mL of water. After 10 min incubation, samples were centrifuged at 3500 g (20 min). The upper organic phase was collected, and the remaining phase was re-extracted with 2 mL of MTBE/MeOH/H_2O (10:3:2.5, v/v/v), followed by 10 min incubation and centrifugation. Organic phases were combined and dried at 30°C on a vacuum concentrator (Christ RVC 2-33 CD plus, Martin Christ GmbH, Osterode am Hartz, Germany). To aid the evaporation process 200 µL MeOH was added to the solution after 25 min of

centrifugation. The lipid pellets were weighed (total lipid content) and re-dissolved in 500 µL of MeOH; solubilisation was aided by 15 min sonication followed by centrifugation. Samples were filtered through syringe filters (cellulose acetate membrane, 0.20 µm, 30 mm from VWR International) prior to HPLC/MS analysis.

Quantification and speciation of lipids containing arsenic

Compound separation was conducted with an Agilent 1260 HPLC system using a ACE Ultra Core SuperC18 column (5 µm, 250 x 4.6 mm; Method Development Kit, Advanced Chromatography Technologies Ltd, UK) under gradient elution conditions. Mobile phases were water and MeOH, both with 20 mM of ammonium acetate (pH=9.2, adjusted both with ammonia); flow rate was set to 1 mL min^{-1}, with the following MeOH gradient: 0 min, 50%, 0-25 min, up to 100%; 25-42 min, 100%; 42.1-50min, down to 50%. Sample injection volume was 50 µL mixed online with 50 µL of water. Arsenic signal detection was measured by transferring 10% of the column effluent directly to the ICPMS by using a passive splitter (Analytical Scientific Instrument, Richmond, USA). An internal standard containing Ge, In and Te each at 20 µg L^{-1} was introduced to the ICPMS using a T-piece at an isocratic flow of 0.8 mL min^{-1}. An aqueous solution of 10% MeOH was continuously added to the ICPMS spray chamber providing carbon compensation, as described by Raber et al. (2010). Calibration was done with a mixture of the 6 arsenolipids, with concentrations of 0, 1, 5 and 10 µg As L^{-1}. Stability and precision of measurements were monitored by the measurement of a 5 µg $L^{-1,}$ standard every 10 samples. Limit of quantification (LOQ) and detection (LOD), calculated using the standard deviation of method blanks, were 0.18 (3 * SD) and 0.60 µg L^{-1} (10 * SD), respectively. Data analysis was done in Mass Hunter Version B.01.01 (Agilent, Waldbronn, Germany). Quantification was determined by integration of peak areas against external calibration with standards.

The arsenic lipid speciation was determined for up to 5 organisms on days: 0, 2, 4, 8 and 14, for digestive gland and mantle. Total percentage of arsenolipids (% AsL) on the total lipid content is given by the absolute amount of arsenolipids (AsL (µg)) in grams divided by the molecular mass of Arsenic (75 g mol^{-1}) and an estimated averaged mass value for one arsenolipid molecule (500 g mol^{-1}) using the following formula:

$$\%AsL = \left[\left(\frac{AsL(\mu g) \times 10^6}{75}(g/mol)\right) \times 500\ (g/mol)\right] / \ Lipids\ (g) \times 100$$

(Equation 1)

Identification of Compounds by high resolution mass spectrometry

For identification of arsenolipid compounds, a Dionex Ultimate 3000 HPLC system was coupled to a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Sci., Erlangen, Germany) equipped with a heated electrospray ionization (HESI) source. The chromatography was performed using the same column and elution conditions as described above for the Agilent system, with a flow rate of 1 mL min^{-1}, the column was held at 40°C, the injected sample volume was 20 µL. Mass spectrometry was performed in positive mode and source settings were: spray voltage 3700 V, capillary temp 300 °C, gas temp 500 °C, with flow rates of 65 (sheath) and 20 (aux) instrument units. The full scan range was m/z 300 - 1100 with the resolution set to 35 000 (full width half-maximum, FWHM), an automatic gain control (AGC) target of $1E^6$ and a maximum injection time (IT) of 100 ms was used. Data dependent MS/MS (ddMS/MS) for the precursor ions was performed after recording full scans. Collision energies (NCE) of 20, 35, 40 (stepped) instrument units were used for the fragmentation experiments. MS/MS settings were: maximum injection time 50 ms, AGC $2E^5$, resolution 17500 FWHM. An isolation window of 0.4 m/z (mass/charge) was used and five MS/MS experiments were performed before a new full scan. The fragmented ions were subsequently excluded for 10 s.

Digestive gland and mantle samples from 5 organisms were analysed for days: 0, 2, 4, 8 and 14. Samples were run under the same conditions as previously described for the reversed-phase chromatograms, followed by runs under acidic conditions changing the mobile phases to MeOH and water both with 0.1% FA. Chromatograms were compared and the peaks matched between the acidic and the alkaline conditions.

Data analysis

Data analysis was conducted using the R program (R Development Core Team 2011). Normality was tested using Shapiro Test and homoscedasticity with Bartlett Test. Linear modelling was conducted to identify trends. Statistical significance was set at α=0.05. When averaged data are presented, variability is expressed as standard deviation (SD).

Results

Organism condition

No mortality was registered during the experiment. Salinity was at 30.6 ± 0.2 g L^{-1}, temperature at 21 ± 0.8°C and pH at 8.2 ± 0.2. Ammonia levels averaged 0.6 ± 0.3 mg L^{-1}, with a noticeable decrease in values from day 12 onwards. Changes in condition index, and lipid and arsenolipid content were attributed to starvation alone, since abiotic factors did not change to any degree during the experiment

time. The digestive glands showed a 52% loss in condition index during starvation (Figure 3-1), whereas the mantles had negligible losses of <5% (data not shown).

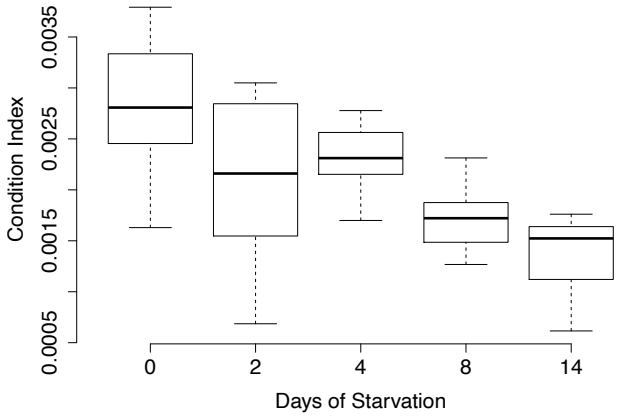

Figure 3-1: Condition Index for the digestive gland of the *Mytilus galloprovincialis* during the 14 days of starvation

Total arsenic measurements

At their initial conditions, digestive glands had higher amounts of arsenic when compared to the mantle, with a mean of 1.44 ± 0.14 µg compared to 0.74 ± 0.24 µg. From day 0 to 14, a decline in total arsenic of 37% was recorded in digestive gland. The loss was hypothesized to be depuration of water-soluble arsenic (speciation not measured). Since, in these samples total arsenolipids represents a small fraction of the total arsenic, averaged in 8.29 ± 2.3%.

Lipids and arsenolipids variation

At their initial conditions, total lipids percentages for digestive gland averaged 17.7 ± 0.9% and 15.4 ± 4.8% for mantle. A pattern for total lipids was present in both organs: from day 0 to 4 a decrease, follow by a recovery at day 8, and by a slight decrease till day 14 (Figure 3-2). Overall total lipids percentage losses were 20% and 10%, respectively for digestive gland and mantle. Arsenolipid percentages of total lipids (estimated by Equation 1), represent a small fraction with values always below 0.02%. Linear modelling trend lines were computed (Figure 3-2), data normality and homoscedasticity were met (mantle data was ln transformed). Sex was not considered, due to few individuals. Digestive gland linear models showed that total arsenolipids had the same trend as lipids did during the starvation period with a decrease over time, and with total lipids presenting a smaller recovery for day 8. For the mantle, despite the low correlation value, an arsenolipid prompt remobilization to

the mantle can be observed when compared to total lipids. Arsenolipid remobilization to the mantle had a faster rate than lipids, given by a positive slope value (Figure 3-2). When the contents from digestive gland and mantle were combined, changes on lipids and arsneolipids contents were less evident.

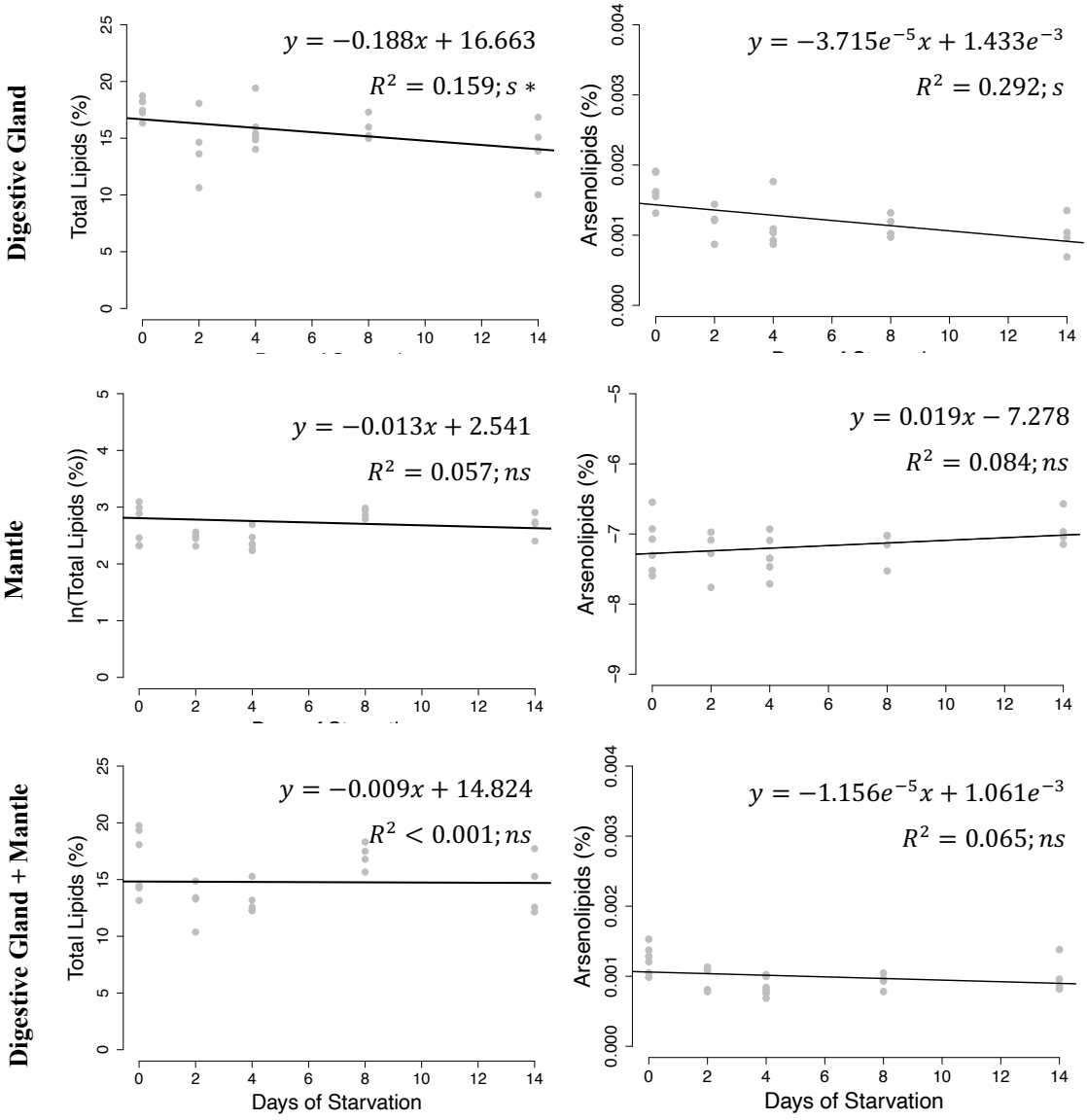

Figure 3-2: Total Lipids (%) and Arsenolipids (%, by Eq. 1) in *Mytilus galloprovincialis* digestive gland (DG), mantle (M) and for both organs (DG+M, combined), under 14 days of starvation. GLM are given for each distribution; note: mantle data were ln transformed; ns: not statistically significant; s: statistically significant. * p = 0.053

Arsenolipid speciation

Digestive gland and mantle chromatogram profiles were clearly different at their initial condition (Day 0). Digestive glands show a diverse range of compounds with a greater relative amount of polar arsenolipids (early eluting on the HPLC-MS conditions), while in the mantles the less polar arsenolipids predominate (latter eluting on the HPLC-MS conditions) (Supplementary Data – Figure 3-1). The differences in arsenolipid profiles between organs decreased as starvation continued; the mantle profile remained fairly constant over the days, while the digestive gland profile came to resemble the profile for the mantle (Supplementary Data – Figure 3-1). This effect for the digestive gland resulted from the continuous decrease of the polar compounds while the less polar ones decreased initially but then stabilized (Figure 3-3). Apparently, no new arsenolipid compounds were formed during the starvation.

In HPLC-MS chromatograms the visible independent and quantifiable peaks were chosen to describe starvation interferences (Table 3-1). Depletion of compounds during starvation were evident to: AsFA362, the group of AsFA376/AsFA436/ AsFA390/AsFA402, AsFA416, AsFA418 and AsHC404. Preserved compounds in digestive gland were within the groups: AsFA514/AsHC332 and AsFA542/530, with the latter group showing an accentuated increase in the mantle. Using the intensity data from ESMS, identified compounds concentration was calculated (Table 3-2). We observe that several of the identified compound were depleted or decrease for both organs, with most pronounced decreases for AsFA436, AsHC360 and AsHC404 in digestive gland. The few exceptions of compounds that remained stable or increase were: AsFA514, AsFA528 (mantle), AsFA530, AsFA542 and AsFA558, note that AsFA514 and AsFA530 increase for the mantle. In mantle, the arsenosugar phospholipids AsPL482 was not depleted, which evidences for algae activity (García-Salgado et al. 2012).

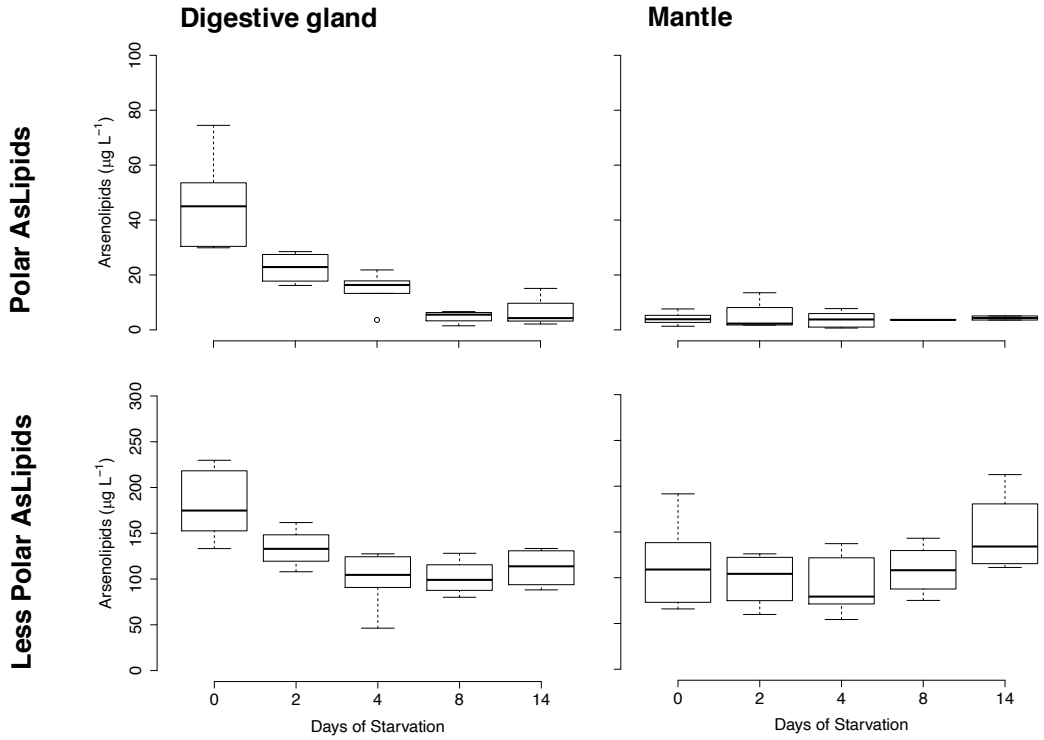

Figure 3-3: Variation on digestive gland and mantle of *Mytilus galloprovincialis* arsenolipids concentration (µg As L^{-1}), for polar (early eluting on HPLC-MS conditions: 0-20min), and for less polar (latter eluting on HPLC-MS conditions: 20-40min) compounds. Boxplot whiskers shows maximum and minimum, box for 1st and 3rd quartile and bar for median value.

Table 3-1: Concentration for identifiable compounds peaks (µg L^{-1}), data is presented in averaged values and their respective standard deviation, for digestive glands (DG) and mantle (M) for *Mytilus galloprovincialis*. RT is for retention times in minutes the HPLC-MS analysis. Concentration values below LOQ (0.6 µg L^{-1}) were not taken into the calculations.

	RT	9.57	12.09	13.56	15.69	17.42	20.00	20.51	23.55	24.11	25.56	26.22
		AsFA362	AsFA388	AsFA376/ 436/390/402	AsFA416	AsFA418	AsFA446	AsPL720 +others	AsHC404	AsFA514 AsHC332	AsFA542/ 530	AsHC360
	Day											
DG	0	3.41±0.85	6.30±1.24	7.63±2.92	1.19±0.29	1.07±0.25	6.56±3.05	17.87±7.60	5.07±4.57	3.80±0.48	2.64±0.66	13.30±3.10
	2	1.12±0.57	1.63±0.95	1.48±0.76	0.60	0.78	2.03	5.46±2.70		4.76±1.58	2.99±0.57	4.17±1.66
	4	1.06±0.70	1.87±1.85	1.19±0.83	1.03		1.41	2.80±0.80		3.63±1.28	2.41±0.72	1.84±0.81
	8		0.60				1.20	2.36±1.02		2.83±0.69	3.09±0.22	
	14		0.86±0.16				1.70	2.09±1.51		3.18±1.07	3.07±1.23	1.13
M	0		0.64	1.03±0.29			1.16±0.42	1.50±1.10	0.71±0.07	1.56±0.62	7.46±3.68	0.84
	2			1.04			1.00	1.55±1.32		1.56±0.57	6.97±2.65	
	4			0.65				1.49±1.11		1.06±0.70	8.48±4.59	
	8									0.94	8.33±2.47	
	14									1.20±0.70	10.49±1.73	

Table 3-2: Max concentration (µg L^{-1}) for arsenolipids for each day of starvation, based on intensity data from ESMS assuming similar response in the mass spectrometer for the various compounds. Concentration values under 0.18 µg L^{-1}(LOD) were not taken in consideration. Trend for arsenolipids variation on concentration is given when values are above 0.60 µg L^{-1}(LOQ), stable values were set for variations under 50%; (↗=increase, ↘=decrease, and ⇆=stable).

		Digestive gland				Mantle			
		Day 0	Day 8	Day 14	trend	Day 0	Day 8	Day 14	trend
FA	AsFA334	1.6	0.3	0.3	↘	0.3			
	AsFA348	0.3							
	AsFA360	0.4							
	AsFA362	4.4	0.6	0.6	↘	0.4			
	AsFA374	0.3							
	AsFA376	0.8			↘				
	AsFA388	8.7	1.3	1.0	↘	0.8			↘
	AsFA390	6.9	1.0	0.7	↘	0.3			
	AsFA402	0.4							
	AsFA416	4.2	1.0	0.4	↘	0.5			
	AsFA418	2.0	0.5	0.2	↘	0.7			↘
	AsFA430	1.5	0.6	0.3	↘	0.2			
	AsFA436	14.0	0.2	1.0	↘	2.5			↘
	AsFA444	0.3							
	AsFA446	0.2				0.5			
	AsFA514	1.9	1.8	2.9	⇆	0.9	0.2	2.7	↗
	AsFA528	1.0	0.3		↘	1.4	0.2	1.2	⇆
	AsFA530	1.3	1.6	0.7	⇆	1.8	0.9	6.1	↗
	AsFA542	3.3	4.3	4.7	⇆	2.1	1.6	1.4	⇆
	AsFA544		0.2					0.4	
	AsFA556	0.5				0.5		0.3	
	AsFA558	0.6	0.5	0.7	⇆	0.6	0.3	0.4	⇆
	AsFA570	0.2	0.4						
HC	AsHC332	2.3			↘				
	AsHC346	0.3							

	AsHC358	1.2			↘				
	AsHC360	16.5	0.6	0.4	↘	1.0			↘
	AsHC374	0.3							
	AsHC388	1.0			↘				
	AsHC404	20.8			↘	1.2			↘
	AsHC414	0.2							
PL	AsPL328	1.2			↘				
	AsPL482	6.2			↘	0.9	0.5	0.3	↘
	AsPL720	1.3			↘	0.2			
EP	AsEP734					0.4		0.4	
Pt	AsPT546	9.4			↘	0.9			↘

Discussion

Arsenolipid profiles are clearly different between organs, inferring an allocation of arsenolipids according to the organ's function. Digestive gland presents more diversity of arsenolipids, probably reflecting the environment uptake. Mantle was more homogenous on diversity, given its function devoted to reproduction, with storage of energetic reserves and gametes production (Fearman et al. 2009; Martínez-Pita et al. 2012). Total lipid content changes match what is described for starvation condition modelling (Freites et al. 2002). Arsenolipids show a behaviour similar to that described for lipids in starvation conditions, with shorter chain (polar) lipids being consumed, presumably to be used in metabolic pathways, with longer chain (less polar) lipids are preserved during starvation because they of their structural function (Freites et al. 2002). The preservation of less polar arsenolipids might be related to their similar function to their homologous or the inability to depurate them. Some arsenolipids seem to be consumed during starvation, it is possible that arseno-metabolites might result and can pose toxicity to the organism, which is already dealing with a stressful starving situation.

In digestive gland the reduction of total lipid content is consistent with the loss of the organism's condition during starvation; this change emphasises the known cellular autophagy to comply with the food restriction and the remobilization of its reserves to other organs (Thompson et al. 1974; Cajaraville et al. 1991). The cells autophagy and inherent remobilization is probably responsible for the increases in concentration of lipids during the later days of starvation (Thompson et al. 1978; McVeigh et al. 2006; Singh et al. 2009; Zaffagnini and Martens 2016). The fluctuations of

arsenolipids between both organs observed in the current study are indicative of remobilization processes from digestive gland to the mantle. Remobilization is the most likely process, since the *de novo* synthesis of lipids and arsenolipids in mantle is quite unlikely, as these organisms have a limited ability to elongate fatty acid precursors (in Fearman et al. 2009). Given the lipids and arsenolipids remobilization, starvation studies must comply analysis to different organs, a whole organism analysis might induce into a non-change of contents result.

Conclusion

When *Mytilus galloprovincialis* are maintained under starvation conditions, arsenolipids show a behaviour similar to that described for non-arsenic lipids. This observation suggests that arsenolipids are being used as "normal" lipids by *M. galloprovincialis*, and follow the same metabolic pathways as non-arsenic containing lipids. In the present work, *M. galloprovincialis* does not appear to have a cellular selection mechanism capable of distinguishing between normal lipids and those that contain arsenic. The use of arsenolipids as energy source and their possible toxicity are still unknown.

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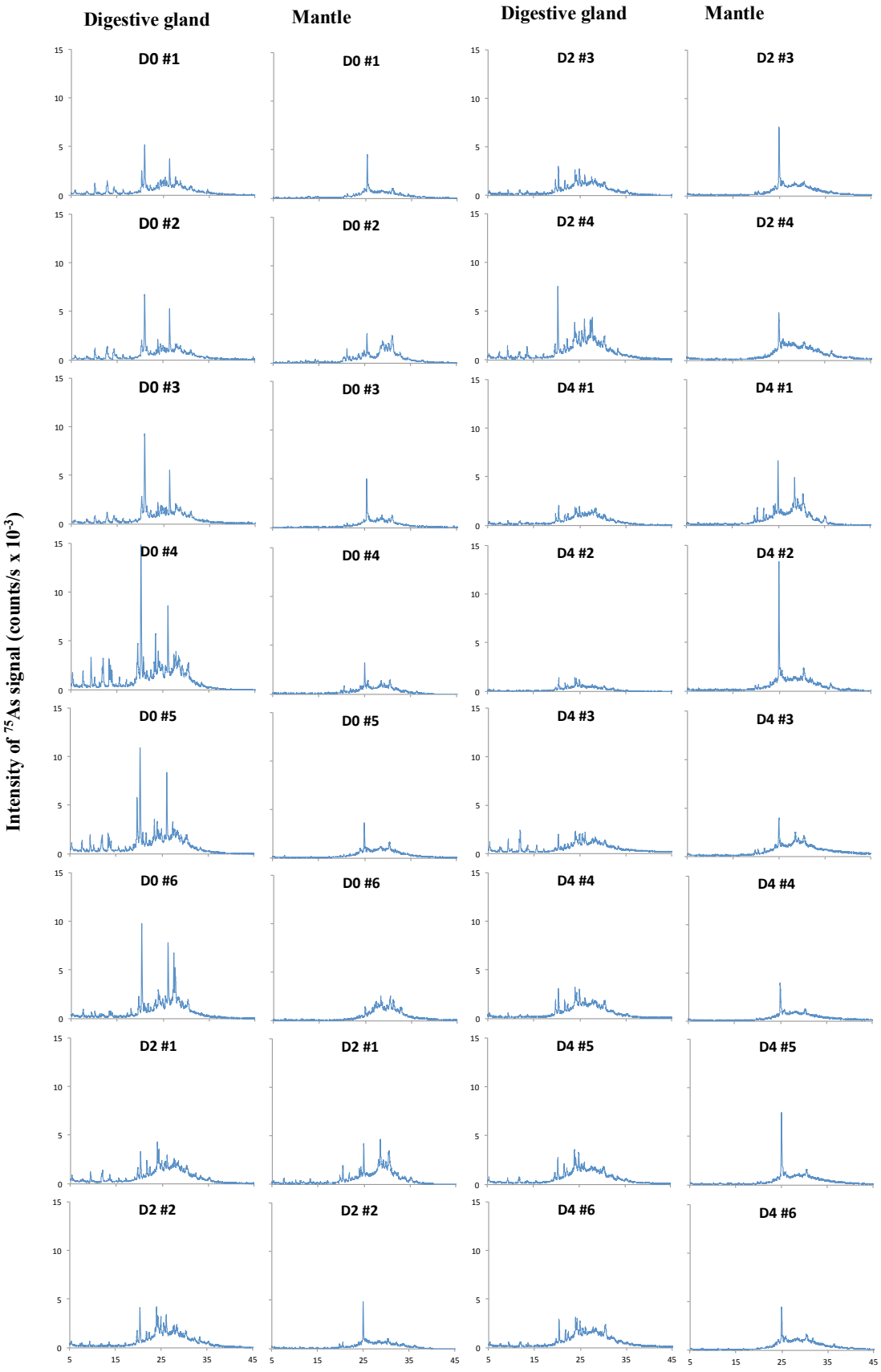
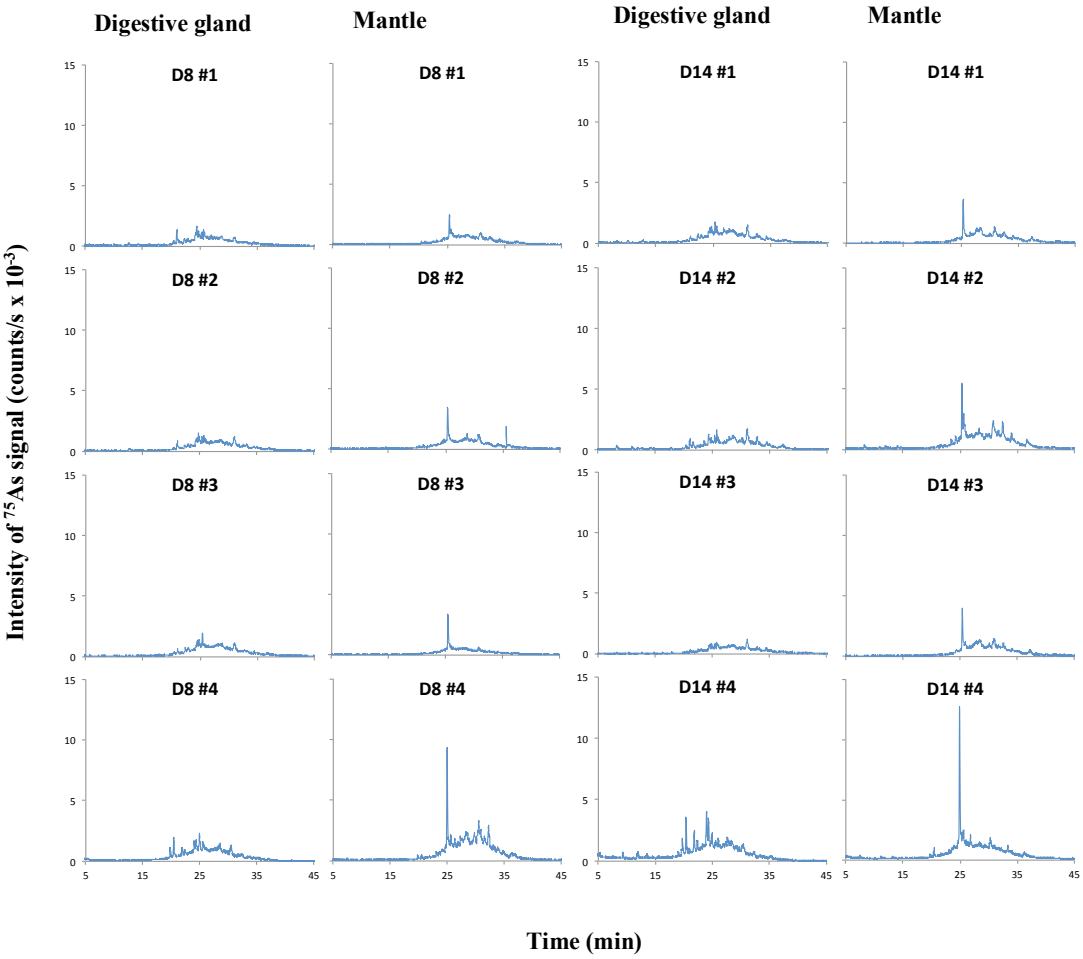
Supplementary Data





Supplementary Data - Figure 3-1: Chromatograms of all the samples for Digestive gland and Mantle. Day is set above each chart (from Day 0 to Day 14) with the number of each organism sample (#).

Chapter 4

Uptake of arsenolipids by starved *Mytilus galloprovincialis*: results from an *in-situ* experiment

Abstract

Arsenolipids are abundant and diverse in the digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. The origin of these compounds, however, is not known. In this study we aimed to determine if arsenolipids in *M. galloprovincialis* digestive gland come mainly from direct uptake through food (plankton). We used mussels previously starved in aquaria, because it lowers the content of polar arsenolipids, and then transferred them to their natural environment where they were able to feed on plankton. The arsenolipids in the plankton and in the mussels were monitored by HPLC/mass spectrometry. Additionally, during the starvation phase, the depuration of arsenolipids compounds by the mussels was monitored, by analysing the ambient water and the mussels' faeces. Arsenolipids were present in the faeces, indicating that mussels can depurate some arsenolipids. The greater diversity of arsenolipids in mussels compared with plankton might suggest that the source of arsenolipids in mussels is more than just plankton, and could reflect a combination of uptake, bioaccumulation and biosynthesis in the animal from simpler arsenic species. In support of this view was the observation that the digestive gland and faeces of mussels contained, between them, 8 arsenolipids not previously reported in plankton or algae.

Introduction

Arsenolipids, lipids that contain arsenic, are widespread among marine organisms (Sele et al. 2012; García-Salgado et al. 2014; Yu et al. 2018). It was stipulated that these compounds are to be produced to some extent into organism by biosynthesis infidelity, and are present and widely distributed in marine organism (Wrench and Addison 1981; Rumpler et al. 2008).

Lipids provide the densest form of energy in marine ecosystems, with two-thirds more energy per gram than proteins or carbohydrates. Lipid energy is transferred up the food chain from algae to vertebrates, via zooplankton (Parrish 2013). The lipid content of an animal is well correlated to its condition, for instance for reproduction energy allocation, act as a carrier for fat-soluble vitamins or for organic contaminants (Marshall et al. 1999, 2000; Laender et al. 2010). The better understanding of lipid transfer in food chain is of pivotal importance for models of population dynamics, bioaccumulation of chemicals and understanding their role and importance in organism metabolism.

The planktonic communities play the major role in remobilization of elements and compounds to higher trophic levels, being a main food source for filtering organisms (Harrison et al. 1990); however, uptake of lipids is also relevant from bacteria intake (Jamieson and Wardlaw 1989).

Previous studies have described the arsenolipid profile for the mussel *Mytilus galloprovincialis* digestive gland and mantle and the short periods of starvation have shown to influence the arsenolipid profile (Freitas et al. 2019 - Chapter 2 and 3). In the mussel's tissues were identified lipids containing arsenic homologous to non-common very long chain fatty acids, it was suggested that these compounds were uptake (Freitas et al. 2019 - Chapter 2), and not produced by synthesis infidelity. The present work investigates arsenolipids in natural plankton from two locations, and the uptake of arsenolipids by artificially starved *M. galloprovincialis*, when kept under natural conditions at these two locations. The data are discussed in terms of the source of arsenolipids in the mussels.

Methods

Experimental design

Mytillus galloprovincialis were collected from rocky shores Minho Estuary (GPS: 41º52'N, 8º52'W), on July 6, 2016. 125 individuals were selected in the field for sizes between 60-80 mm and were immediately transported to the lab facilities under dry conditions. In the lab, shells were cleaned of epi-biota by scrubbing them under tap water. 120 organisms were maintained without food for 21 days in a 60 L aquarium with 75% water renewed every day. This starvation was conducted in an acclimated room to 19°C, photoperiod was 15h/9h under natural light but without direct exposure to

sunlight. Artificial salt water was prepared with reverse osmosis water (Aqua-win RO-6080 (Taiwan)) and marine salt (Ocean Fish Prodac International S.r.l. (Italy)). Abiotic parameters of salinity, temperature, pH and oxygen were registered with a multiparametric probe YSI 556 MPS; and ammonia levels were monitored daily using the Sera® Ammonium/ammonia-test (NH_4/NH_3). Five individuals were sampled at day 0 (environmental condition) and 21 (starved condition); length and total wet weight were measured, and digestive glands were dissected and freeze-dried. Additionally, for total arsenic and arsenolipid quantification, water and faeces samples were collected on day 1, 2, 3, 4, 7, 14 and 21.

No mortality was registered till the end of the starvation experiment. Salinity was kept at 30.0 ± 0.1, temperature 19.6 ± 0.3 °C and pH 7.9 ± 0.1, ammonia levels averaged 0.5 ± 0.3 mg L^{-1}. On the 21st day of starvation mussels were assembled in 8 tubular bags made of nylon net (mesh of 10 mm), which was flexible thereby allowing the mussels' valves to open. Each bag contained 7 mussels, which were further compartmentalized by placing a plastic string between each organism. This plastic string was designed to increase foraging efficiency by preventing the byssus developing over other mussels, which would otherwise restrain their ability to filter water.

The bags containing the mussels were transferred to two locations in the Aveiro Coastal Lagoon Estuary (GPS: 40º64'N, 8º74'W). This estuarine system, 45 km long and 10 km wide, has a very irregular and complex geometry. Despite, several small rivers contributing to the freshwater input, the hydrologic circulation is essentially dominated by tidal forcing (Lopes and Dias 2007). The two locations were selected based on different ocean exposure (Figure 4-1): the one close to the estuary mouth (Location 1) and other upstream (Location 2). The greatest depth is about 7 meter at the channel mouth, maintained by regular dredging activities, but the overall system has an average depth of just 1 m (Lopes and Dias 2007). 4 bags were place at each location; one bag was removed at days 1, 7, 14 and 21. At each sampling event, 7 mussels from natural conditions at the same location were also sampled for comparison proposes. Days 1 and 14 corresponded to neap tides, and days 7 and 21 to spring tides. Mussels length averaged 67.9 ± 4.1 mm and 65.5 ± 2.4 mm, for location 1 and 2, respectively. Condition Index (CI) was calculated by dividing the total mass of freeze-dried digestive gland by shell total length (Lane 1986). Plankton sampling was conducted during the high tide: 100 L was sampled for location 1 and 60 L for location 2. Water was pre-filtered through a 500 μm mesh and phytoplankton collected in a mesh net of 30 μm. Salinity was measured at the highest tide amplitude at each sampling event: location 1, 35.7 ± 0.4 and location 2, 35.1 ± 0.7. Samples for bacterioplankton were collected in membrane filters (pore: 0.20 μm) from the plankton-filtered water using volumes of 1 L and 0.5 L, for location 1 and 2 respectively.

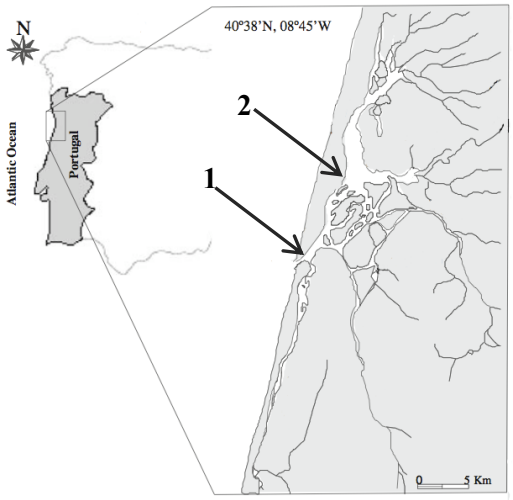


Figure 4-1: Aveiro Coastal Lagoon Estuary sites for the *in-situ* exposure for Location 1 (near estuary mouth) and Location 2 (up-stream). (Figure adapt from Pereira et al. 2009)

Chemicals and Standards

Water (18 MΩ cm) used throughout this investigation was from a Milli-Q Academic water purification system (Millipore GmbH, Vienna, Austria). The following chemicals, all analytical grade, were used in the study: nitric acid 68%, p.a., further purified by using a MLS duoPUR sub-boiling unit (MLS GmbH, Leutkirch, Germany); methanol (≥99.8%, MeOH) purchased from ChemLab (Zedelgum, Belgium); methyl-tert-butyl ether (99.5%, MTBE), ammonium acetate (97%), ammonia (25%), formic acid (≥98%, FA), ammonium hydrogen carbonate (≥99%, p.a.), pyridine (≥99% for synthesis) and purchased from Carl Roth GmBH (Karlsruhe, Germany). Single element standards (^{75}As, ^{74}Ge, ^{115}In and ^{125}Te) were purchased from Carl Roth GmBH (Karlsruhe, Germany). For calibration and identification of arsenolipids, three arsenic fatty acids (AsFA362, AsFA388, AsFA418) and three arsenic hydrocarbons (AsHC332, AsHC360, AsHC444) standards were available in-house (99% purity assessed by NMR and HPLC/MS), previously synthesized by the method described by Taleshi et al. (2014b). The reference material DOLT-3, certified for arsenic, was obtained from NRC-CNRC, Canada.

Lipid extraction:

Extraction was conducted in digestive glands using MTBE:MeOH methodology (Matyash et al. 2008). We weight about 50 mg (weighed to 0.1mg) of freeze-dried tissue directly into a 15ml propylene tubes (CellStar®Tubes, Greiner bio-one), added MeOH (1.5 mL) and vortexed the mixture

(10 seconds) before adding MTBE (5 mL). The tubes were shaken (rotating mechanical arm) for 1 hour at room temperature before we effected phase separation by adding water (1.25 mL). We let the samples stand for 10 min before we centrifuged them (3500 g, 20 min) and collected the upper organic phase. We re-extracted the remaining lower phase with a mixture of MTBE/MeOH/H_2O (2 mL; 10:3:2.5, v/v/v), as described above, and added the resultant upper layer to that from the first extraction. The combined organic phase was dried at 30°C on a vacuum concentrator (Christ RVC 2-33 CD plus, Martin Christ GmbH, Osterode am Hartz, Germany); to aid the evaporation process, we added MeOH (200 µL) to the solutions after 25 min of evaporation. The lipid pellet (total lipid content) was weighed and re-dissolved in MeOH (500 µL) of MeOH; solubilisation was aided by sonication (15 min) followed by centrifugation 3500 g (20 min).

Plankton and faeces samples had seawater salt residue that needed to be removed because of Cl interference with the arsenic signal. Lipids were extracted using a piridin based method (Raber, 2017, personal communication). Whole mass plankton samples and *ca.* 120 mg faeces samples were washed with 10mL of 20 mM of ammonium hydrogen carbonate in 15 ml propylene tubes (CellStar®Tubes, Greiner bio-one), and after centrifuge (3500 g, 20 min) supernatant was removed. Pellet samples were dried at room temperature to constant mass. Lipid content was after extracted with 1 mL of piridin, tubes were shacked for 2 hours at room temperature, followed by centrifugation at 3500 g (20 min). Supernatant was collected and 100 µL were removed for total arsenic determination.

Lipid extraction from the water samples follow the methodology described in Khan et al. (2016). For the 50 mL of sampled water, fractions of 10 mL were extracted twice with 5 mL of DCM, using 50 ml propylene tubes (CellStar®Tubes, Greiner bio-one). Organic phases were combined and dried at 30°C on a vacuum concentrator. Pellets were re-dissolved in 500 µL of MeOH; solubilisation was aided by sonication (15 min) followed by centrifugation 3500 g (20 min).

Quantification methods for total arsenic

Arsenic was measured by inductively coupled plasma mass spectrometry (ICPMS; 7900 from Agilent Technologies, Waldbronn, Germany) in a portion of *ca.* 10 mg (weighed to 0.1 mg) of the original powdered tissue samples and in 1 mL for water samples (aquaria water); also in the two fractions from the lipid extraction pellet+water (whole was used), and the organic phase (1 mL aliquot). Samples had been mineralised by microwave-assisted acid digestion with 2 mL of nitric acid + 2 mL of water in a Ultraclave III microwave system (MLS, Leutkirch, Germany). After the mineralisation, samples were made ready for ICPMS analysis by adding 1 mL of internal standard solution containing Ge, In and Te at 100 µg L^{-1}, solutions were made up to 10 mL. Arsenic calibration concentrations were 0, 0.1, 0.5, 1, 5, 10 and 50 µg L^{-1}. Polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As) were mitigated by recording the measurements in collision cell mode

with a flow of He, at 4 mL min^{-1}. Stability and precision of the ICPMS measurements were monitored by measuring, every 10 samples, a 1 µg L^{-1} calibration standard, which was always within 2 % of the target value over the course of the analyses. Limit of quantification (LOQ) and detection (LOD), calculated from the standard deviation of blanks, were 0.04 µg L^{-1} (3*SD) and 0.12 (10*SD) µg L^{-1}, respectively. Using the microwave mineralisation and measurement procedure, the CRM DOLT-3 (certified [As]= 10.20 µg g^{-1}, SD 0.50) returned an average value of 11.04 µg g^{-1} ± 0.52, n = 4, using 10 mg of material.

Quantification of arsenolipids by HPLC/ICPMS

All samples were centrifuged and filtered through syringe filters (cellulose acetate membrane, 0.20 µm, 47 mm from VWR International) prior to HPLC/MS analysis. Compound separation was conducted with an Agilent 1260 HPLC system using an ACE Ultra Core SuperC18 column (5 µm, 250 x 4.6 mm; Method Development Kit, Advanced Chromatography Technologies Ltd, UK) under gradient elution conditions. The mobile phases were water and MeOH, both with 20 mM ammonium acetate (pH=9.2, both adjusted with ammonia); flow rate was 1 mL min^{-1}, with the following MeOH gradients, for digestive gland: 0 min, 50%; 0-25 min, up to 100%; 25-42 min, 100%; 42.1-50 min down to 50%; and for faeces and plankton: 0 min, 20%; 0-4min up to 70% and then from 4.1-20 min up to 100%; 20.1-30 min at 100%; 30.1-35 down to 20%. Sample injection volume was of 50 µL mixed online with 50 µL of water. Arsenic was measured by transferring 10% of the column effluent directly to the ICPMS by using a passive splitter (Analytical Scientific Instrument, Richmond, USA). An internal standard solution of 20 µg L^{-1} (each of Ge, In and Te) was introduced using a T-piece after the splitter at an isocratic flow of 0.8 mL min^{-1}. An aqueous solution containing 10% MeOH was continuously introduced to the ICPMS spray chamber providing carbon compensation, as described by Raber et al. (2010). Calibration was done with a mixture of the 6 arsenolipids standards, with concentrations of 0, 1, 5 and 10 µg As L^{-1}. Stability of the measurements was monitored by repeated injection of the 5 µg L^{-1} standard every 10 samples. LOQ and LOD, calculated from 3*SD and 10*SD of the method blanks, respectively, were 0.18 µg L^{-1} and 0.58 µg L^{-1}; concentration values below LOQ were replaced by 0. Data analysis was conducted with Mass Hunter Version B.01.01 (Agilent, Waldbronn, Germany). Quantification was determined by integration of peak areas against external calibration with the standards.

Identification of arsenolipids by high-resolution molecular mass spectrometry

Arsenolipid were identified by using a Dionex Ultimate 3000 HPLC system coupled to a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Sci., Erlangen, Germany) equipped with a heated electrospray ionization (HESI) source. The chromatography was performed using the same column and

eluting conditions as described above for the Agilent system, with a flow rate of 1mL min^{-1}, the column was held at 40°C, the injected sample volumes were 20 µL. Mass spectrometry was performed in the positive modus and source settings were: spray voltage 3700 V, capillary temp 300 °C, gas temp 500 °C, with flow rates of 65 (sheath) and 20 (aux) instrument units. The full scan range was m/z 300 - 1100 with the resolution set to 35 000 (full width half-maximum, FWHM), an automatic gain control (AGC) target of $1E^6$ and a maximum injection time (IT) of 100 mses were used. Data dependent MS/MS (ddMS/MS) for the precursor ions was performed after recording of full scans. Collision energies (NCE) of 20, 35, 40 (stepped) instrument units were used for the fragmentation experiments. MS/MS settings were: maximum injection time 50 msec, AGC $2E^5$, resolution 17500 FWHM. An isolation window of 0.4 m/z (mass/charge) was used and five MS/MS experiments performed before a new full scan. The fragmented ions were subsequently excluded for 10 secs.

Data analysis

Data analysis was conducted using the R program (R Development Core Team 2011). Significant statistic was set for a $p<0.05$. Averaged data are followed by the standard deviation (SD).

Results and Discussion

Starvation:

In starvation, total arsenic in water shown a reduction 70%, stabilizing around the 1 µg L^{-1} (Figure 4-2). No arsenolipids detected in water, levels of arsenic in water evidences the ability to depurate arsenic-water-soluble compounds. The 5 organisms analysed showed loss of condition index during starvation, with digestive gland decreasing *ca.* 50% in mass. Digestive gland total arsenic did decrease 21%, until day 21 of starvation. The lipid content remained stable (Thompson et al. 1978; McVeigh et al. 2006; Singh et al. 2009; Zaffagnini and Martens 2016); and total arsenolipids concentration did not shown major alterations (Table 4-1). From day 1 to 21, mass of faeces from 120 organisms reduced by 80%; total arsenic decreased 80%, total lipids 60% and total arsenolipid 70%. In faeces, during the starvation, total arsenic and lipids and arsenolipids had their higher peak on day 3, followed by decreasing values until Day 21(Table 4-1).

Day 0 (environmental condition) organisms already had low values for polar arsenolipids (*i.e.* those eluting early from the HPLC column), when compared to previous studies. However, it was still evident that the starvation profile changes were similar to those reported in (Freitas et al. 2019 - Chapter 2 and 3), where the polar arsenolipids decreased in concentration while the less polar (latter

eluting) arsenolipids were preserved or increased their concentration (Supplementary Data – Figure 4-1).

Figure 4-2: Variation of total arsenic in the aquarium water during starvation experiment, from day 1 to 21, for a total of 120 individuals of *Mytilus galloprovincialis*

Table 4-1 Differences in freeze dry mass, total arsenic, lipid percentage and arsenolipid concentration, for *Mytilus galloprovincialis* digestive gland from environmental condition (Day 0) and end of starvation (Day 21); and for mussel's faeces collected during the starvation experiment.

	Days	Total mass (mg)	Total Arsenic (µg g^{-1})	Lipids (%)	Arsenolipids (µg g^{-1})
Mussels	0	112 ± 19	23.3 ± 3.4	9.2 ± 2.2	0.16 ± 0.03
n=5	21	60 ± 17	18.4 ± 2.5	9.0 ± 1.0	0.18 ± 0.04
Faeces	1	665	13.8	6.6	0.20
n=1	2	573	10.2	7.0	0.26
	3	478	15.2	9.6	0.36
	4	275	12.5	7.9	0.27
	7	238	9.0	4.4	0.13
	14	150	10.2	4.7	0.08
	21	144	2.7	2.2	0.06

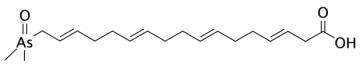
Newly found arsenolipids

In total 22 arsenolipids were identified on the various samples (presented ahead), with eight newly found arsenolipid as: two fatty acids and one phytol-containing-arsenic in mussels' faeces; four fatty acids and one hydrocarbon on digestive gland (Table 4-2). No new compounds were identified for plankton samples.

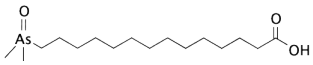
Table 4-2: Newly found arsenolipids on digestive gland (DG) and faeces (F) from *Mytilus galloprovincialis*, DB = is for double bonds in the structure. RT retention time in minutes for the HPLC-MS analysis.

Compound (Abbreviation)	**RT** (min)	**Mass** $[M+H]^+$	**Formula**	**DB**	**|Δm/m|** [ppm]	**Sample**
AsFA382	8.17	383.1562	$C_{19}H_{31}O_3As$	4	0.03	DG
AsFA370	8.65	371.1830	$C_{16}H_{34}O_3As$	3	0.28	DG
AsFA408	10.24	409.1718	$C_{21}H_{33}O_3As$	5	0.31	DG
AsFA410	11.04	411.1881	$C_{21}H_{35}O_3As$	4	0.56	DG
AsFA460	15.28	461.2979	$C_{24}H_{49}O_3As$	0	0.14	F
AsFA548	16.99	549.3281	$C_{31}H_{53}O_3As$	5	0.21	F
AsPt576	24.61	577.3810	$C_{30}H_{61}O_5As$	-	0.27	F

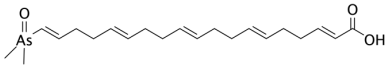
AsFA382



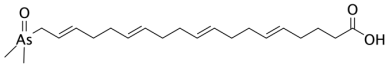
AsFA370



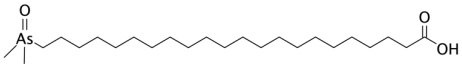
AsFA408



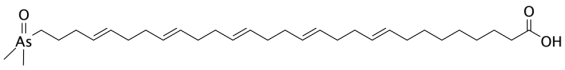
AsFA410



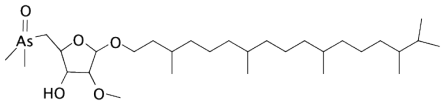
AsFA460



AsFA548



AsPt576



Arsenolipids compounds

During starvation in digestive glands, the decrease in concentration was most noticeable for peaks containing among AsFA446 and AsPL720 compounds (note that: together with these are other unidentified arsenolipids compounds). From the identifiable arsenolipids in digestive glands in the starvation experiment, only five were common with faeces samples: AsFA514/HC332; AsFA542; AsFA558 and AsPt546. On the other hand, six arsenolipids, namely AsFA460, AsFA548, AsPT576, AsHC374 and AsHC444 and one unknown compound, were exclusively found in the faeces (Table 4-3), their presence can be due to microbiome origin, or from depuration of undetected concentrations in the digestive gland. The AsPt546 does show high increases on faeces without reduction for digestive gland. Compounds that were preserved during starvation do not appear in faeces. Arsenosugar-phospholipid compounds have been previously found in mussel digestive gland (Freitas et al. 2019 - Chapter 2); the presence of AsPL482 and AsPL328 in faeces might indicate that the source is primary production on faeces (García-Salgado et al. 2012; Xue et al. 2014). Although AsFA446 and AsPL720 shown major decreases during the starvation period in digestive gland, these were not excreted in the faeces. The AsPL720 is also associated with algae, therefore its decrease is associated with reduction of algae uptake during the starvation experiment (Freitas et al. 2019 - Chapter 3); the AsFA446 was possibly consumed as source of energy. The decrease in AsHC332/AsFA514 and AsFA558 in digestive gland was related to an increase in faeces (Table 4-3) indicative of depuration process.

Table 4-3: Arsenolipid compounds peaks identified in mussel's digestive gland before and after starvation and for their bulk faeces sampled during the starvation period. Concentration is given in µg L^{-1}, RT denotes the retention times for each compound or set of compounds for HPLC-MS analysis.

		Organism				Faeces						
	RT	D0	D21		RT	D1	D2	D33	D44	D7	D14	D21
				AsPL482/328	4.4	2.2	2.9	3.7	3.2	2.3	0.7	
AsFA348	8.5	1.1±0.5	0.8±0.6									
AsFA362	10.4	0.1±0.3	0.2±0.4									
AsFA408	11.1	0.2±0.3	0.2±0.4									
AsFA410	12.1	0.5±0.7	0.2±0.4									
AsFA388	13.7	0.3±0.7	1.0±1.2									
AsFA436/390	14.1	1.6±0.5	1.2±0.5									
AsFA446	20.0	6.3±3.0	1.6±0.9									
AsPL720	20.6	17.4±7.9	3.5±0.9									
				AsFA460	17.3	8.3	10.9	16.8	10.2	4.2	1.7	
				AsFA548	19.2	5.5	7.2	9.7	8.1	3.4	2.2	
AsHC332/AsFA514	24.4	7.6±2.5	3.2±1.8	AsHC332/AsFA514	19.9	3.6	4.8	6.5	5.0	2.6	1.8	
				AsUKN – F1	20.8			2.2	1.9	1.6	1.7	
AsHC358	25.2	2.2±0.6	3.4±1.2									
AsFA528s	25.4	3.4±0.9	2.8±1.9									
AsFA542/530/HC360	26.4	1.0±0.6	0.9±1.0	AsFA542/530/HC360	21.2	2.1	2.7	3.6	2.5	2.1	1.4	
AsFA570	27.4	1.7±0.6	0.1±0.3									
AsFA558	27.7	5.5±1.7	0.5±0.3	AsFA558/HC374	22.7	12.7	16.6	23.9	17.0	7.7	3.1	
AsHC388/AsPT546	28.4	4.0±1.9	7.6±2.8	AsPT546	23.1	23.2	30.3	45.4	31.5	11.8	4.3	0.8
AsHC414	29.3	0.9±0.6	3.6±5.3									
AsUKN – D1	30.6	6.6±3.6	14.3±6.8									
				AsPT576	26.2	3.7	4.8	5.2	5.9	4.0	2.3	1.0
				AsHC444	26.5	4.6	6.1	8.2	6.9	3.2	0.8	

Uptake: plankton to mussels

Sampled plankton bulk mass was highly variable, and does not provide a factor for food uptake, as the sediment particles were not removed nor was the carbon content determined. At location 1 on Day 21, the plankton sample mass increase was due to harbour dredging activities (personal observation) (Table 4-4). Total arsenic was higher for spring tides at both locations (day 7 and 21). In plankton, arsenolipids were found at their highest concentrations for Day 1, with 0.11 µg g^{-1} and 0.07 µg g^{-1}, for Location 1 and 2, respectively. These high values for arsenolipids at both locations were followed by abrupt decreases. This relates to changes in the planktonic communities present, since the whole sample was extracted (Supplementary Data – Figure 4-2). Factors that provoke arsenolipids changes had a lesser influence on total lipid content. Arsenolipids were not detected in bacterioplankton samples (data not shown).

Table 4-4: Plankton variations on freeze dry mass, total arsenic, lipids percentage and concentration of arsenolipids; for all 4 samplings during the *in-situ* exposure. Day 1 and 14 were neap tides and day 7 and 21 spring tides.

	Total mass (mg)		Total Arsenic (µg g^{-1})		Lipids (%)		Arsenolipids (µg g^{-1})	
Days	**L1**	**L2**	**L1**	**L2**	**L1**	**L2**	**L1**	**L2**
1	167	612	2.4	3.1	4.8	1.8	0.11	0.07
7	193	513	5.9	11.1	3.7	1.1	0.02	0.02
14	128	163	3.2	9.1	7.3	0.9	0.02	0.02
21	528	366	4.9	11.9	1.9	2.6	0.02	0.02

For day 1, *in-situ* starved mussels had a lower condition index than field ones, and although their condition index increased during the 21 days, it never reached that of the field mussels (Figure 4-3). Starved organisms did recover to their previous environmental condition index (averaged in 0.002 ± >0.001), which infers an ability to catch up to previous starvation conditions.

Environmental exposure induced a strong recovery in total lipids for starved mussels, with values exceeding those of the field mussels (Figure 4-3). This could relate to a gorging behaviour after food deprivation, inducing a rapid increase of lipid reserves, as previously reported for crustacea (Hervant et al. 1999) and mice (Kliewer et al. 2015)). For both field and starved mussels, lipid content was higher for location 1, which might reflect better foraging conditions. Total arsenolipids recovery for starved organisms was noticeable, however, without reaching field values, excepting for day 7,

location 1 (Figure 4-3). At both locations, arsenolipids had higher increments on day 7 and 21 (spring tides days), this did not correlate to total lipid increment.

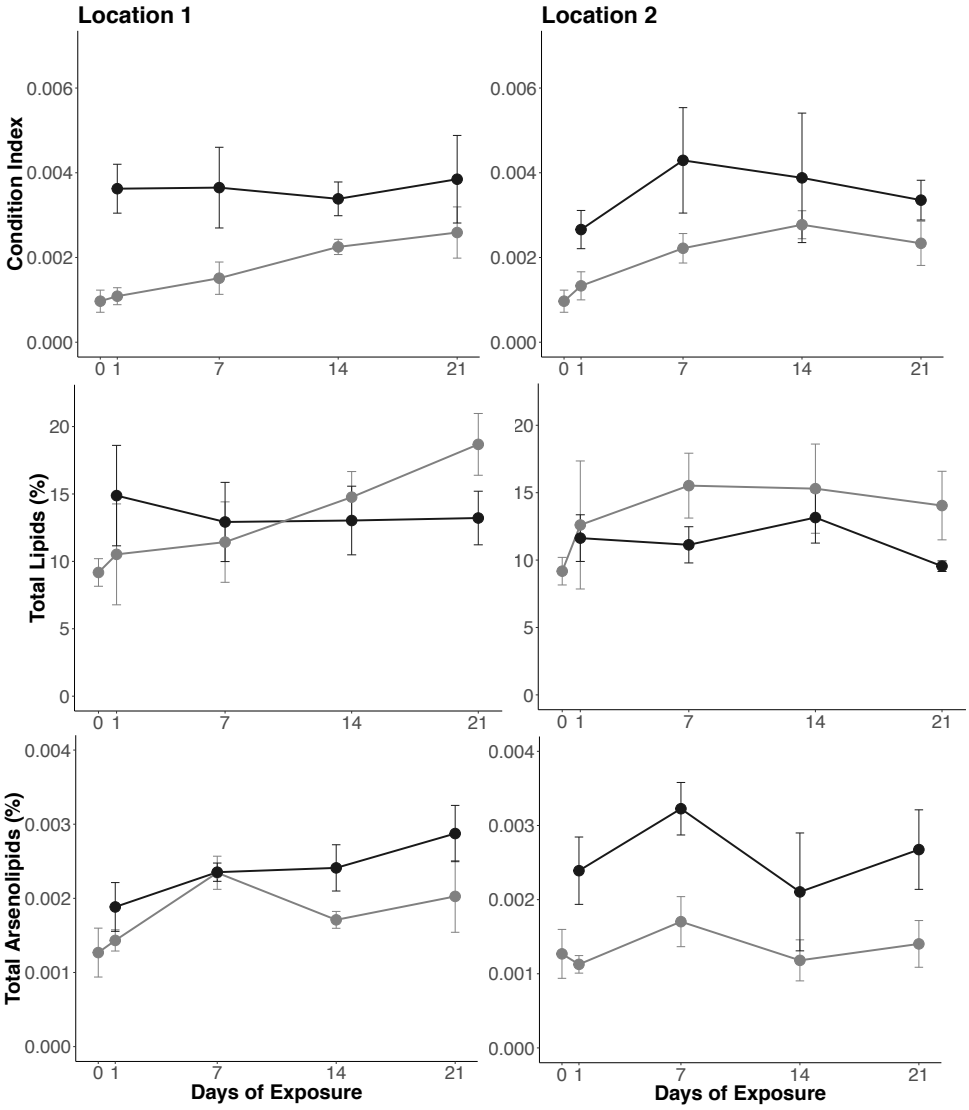


Figure 4-3: **Variation during the *in-situ* exposure for starved (grey) and field (black) mussel's digestive gland for Condition Index, Total Lipids (%) towards total mass and Total arsenolipids (%) towards the total lipids in sample.**

Arsenolipids in plankton and mussels: qualitative comparative analysis

In this study, two unidentified compounds, AsHC404, AsPL482 and AsPL328 were here exclusive to plankton samples (Tables 4-5 and 4-6). The arsenosugar-phospholipids and AsHC404 have been already found in mussel digestive gland (Freitas et al. 2019 - Chapter 2). The compounds present in plankton are the same in both locations, but at different concentrations (Tables 4-5 and 4-6). Both

plankton samples were less diverse in compounds than those present in digestive gland, apart from the two unknown compounds all other compounds were present in mussels. Plankton lacked the identification for 11 arseno-fatty acids and 2 arseno-hydrocarbons that were identified in digestive glands. These compounds could be absent or at undetectable concentrations and bioaccumulated in *M. galloprovincialis*.

The AsFA362, AsFA388, AsFA436, AsFA390 and AsHC358, AsHC444 are not exclusive for mussels and were previously found in other biological samples (Rumpler et al. 2008; Amayo et al. 2013; Taleshi et al. 2014a). AsFA348 AsFA408, AsFA410, AsFA430, AsFA528, AsFA570 and AsFA558 have been only found in mussel tissues (Freitas et al. 2019 - Chapter 2 and 3); and AsFA410 was exclusive to mussels from the sampling site in Minho Estuary, before the starvation experiment and after *in-situ* exposure. *M. galloprovincialis* had the presence of very long carbon chain fatty acids (C > 20) containing arsenic and their presence was inferred to be from food source (*i.e.*, AsFA436, AsFA444, AsFA446, AsFA514, AsFA530, AsFA528s, AsFA542, AsFA556, AsFA544, AsFA570 and AsFA558) (Řezanka and Sigler 2009, Freitas et al. 2019 - Chapter 2). On this survey this can apply to AsFA446, detected on plankton.

During the *in-situ* exposure, starved organisms at Location 1 shown in their arsenolipid profile the presence of polar arsenolipids (early eluting HPLC peaks), these were not relevant for the other organisms in this experiment (field mussels at Location 1 or for field and starved mussels at Location 2) (Supplementary Data – Figures 4-3 and 4-4). The less polar arsenolipids compounds presence seems to be a combination of an intraspecific metabolic difference between mussels in arsenolipid-biogenesis, and foraging from ocean origin. Another hypothesis, if we assume that the arsenolipid-biogenesis is less likely to occur in mussels (in Fearman et al. 2009), their presence can be from undetectable concentrations, in plankton, bacterioplankton or associated with sediment particles (Shi et al. 2001). The less-polar (latter eluting HPLC peaks) arsenolipids were preserved during starvation and present after exposure in starved and field organisms from both locations. Their continuous presence in organisms might be associated to their preservation for similar functions to homologous lipids, *i.e.*, for their structural functionality and not for energetic value. Another more likely possibility, is that they are simply being accumulated, not being enrolled into any metabolic pathway or perform any cellular role and these organisms are not able to depurate them.

Table 4-5: Arsenolipid identified peaks concentrations (µg L^{-1}) at Location 1, for plankton (n=1) and starved and field *Mytilus galloprovincialis* for the sampling days (1, 7, 14 and 21). Starved organisms n = 4, and Field organisms n = 3; RT is retention time in minutes for the HPLC-MS analysis

	Plankton						Starved organisms					Field organisms			
	RT	D1	D7	D14	D21		RT	D1	D7	D14	D21	D1	D7	D14	D21
AsPL482/328	4.4	4.5	1.3	2.0	2.8										
						AsFA348	8.5	3.6±0.5	3.8±1.5	1.9±1.0	3.2±1.1	0.2±0.4		0.9±0.9	0.4±0.7
						AsFA362	10.4		1.6±1.0	1.2±0.5	1.4±0.4	0.3±0.5		0.6±1.1	0.3±0.6
						AsFA408	11.1	2.3±0.1	2.9±1.2	1.2±0.4	2.3±0.9			0.7±0.7	0.3±0.5
						AsFA410	12.1	1.5±0.1	0.9±0.6		0.2±0.5				
						AsFA388	13.7	0.9±0.2	1.2±0.8	0.7±0.5	1.3±0.3	0.6±1.1	0.4±0.4	1.0±0.9	0.4±0.7
						AsFA436/390	14.1	4.7±1.7	7.6±4.5	3.0±0.5	5.6±2.8	1.4±2.4	1.4±0.8	3.4±2.3	2.8±0.9
						AsFA430	16.8	0.2±0.3	0.3±0.6				0.3±0.5		0.3±0.5
AsFA446	16.0	3.6	0.6	1.2		AsFA446	19.9	2.3±1.1	9.8±2.0	6.2±2.6	4.5±0.6	4.5±0.8	7.3±2.2	5.5±2.2	7.1±3.1
AsPL720	16.6	1.0				AsPL720	20.6	4.1±1.2	18.3±4.5	13.7±2.0	9.1±1.2	2.1±0.5	7.7±1.6	9.3±1.4	11.0±5.0
AsUNK – P1	18.1	6.2		1.0	0.6										
AsHC404	19.4	6.3	1.1	1.3	0.6										
AsHC332	19.9	25.1	3.4	2.4	1.2	AsHC332/ AsFA514	24.4	3.1±0.8	5.8±1.9	3.8±1.1	2.3±0.9	3.6±2.4	3.0±0.4	4.8±1.2	4.6±1.6
						AsHC358	25.1	3.5±1.7	1.9±0.9	2.2±0.5	3.1±1.9	3.2±0.5	2.7±0.9	3.5±1.1	4.6±3.2
						AsFA528s	25.4	7.0±3.9	4.9±1.1	3.6±1.2	4.4±1.7	1.6±0.8	2.1±0.5	3.0±1.5	3.3±0.7
AsHC360	21.8	2.2	1.5	0.8	0.6	AsFA542/530/ HC360	26.4	0.2±0.5				0.8±0.7	0.5±0.4	0.5±0.8	0.5±0.8
						AsFA570	27.4	0.3±0.6	0.4±0.8	0.9±0.6	0.4±0.5		0.3±0.5	0.5±0.4	
						AsFA558	27.7	0.5±0.6	1.1±0.9	4.1±1.5	1.9±0.4	1.9±0.8	1.6±0.6	1.9±1.0	2.6±1.2
AsPT546+ukn	23.1	4.1	1.3	0.9	0.8	AsHC388/ AsPT546	28.4	11.5±4.1	20.1±3.5	9.0±0.6	17.5±3.8	4.7±1.6	8.5±2.9	8.7±6.4	11.4±1.7
AsHC414+ukn	24.7	17.3	1.9	1.6		AsHC414	29.3	0.5±0.6	10.3±2.7	6.3±1.1	10.8±6.9	2.1±0.5	5.5±2.8	4.8±2.7	7.0±1.5
AsUNK – P2	25.8	8.3	2.0	4.1											
						AsUKN – D1	30.6	14.3±7.6	32.6±5.7	13.4±3.1	26.0±11.0	10.5±6.1	19.6±9.2	18.6±11.5	29.5±4.4

						AsHC444	31.1	1.7±1.5	0.3±0.6	0.3±0.6	0.4±0.45				1.0±0.1

Table 4-6: Arsenolipid identified peaks concentrations (µg L^{-1}) at Location 2, for plankton and starved and field *Mytilus galloprovincialis* for the sampling days (1, 7, 14 and 21). Starved organisms n= 4, and Field organisms n = 3; RT is retention time in minutes for the HPLC-MS analysis

	Plankton						Starved organisms					Field organisms			
	RT	**D0**	**D7**	**D14**	**D21**		**RT**	**D0**	**D7**	**D14**	**D21**	**D0**	**D7**	**D14**	**D21**
AsPL482/328	4.4	3.7	2.3		1.9										
						AsFA348	8.5			0.2±0.4					
						AsFA436/390	14.1	0.4±0.5	0.4±0.4	0.8±0.7	0.2±0.4	0.5±0.5	0.2±0.4	0.9±0.3	
AsFA446	16.0	2.2	1.0			AsFA446	19.9	3.1±1.6	14.5±7.6	4.1±1.2	11.4±2.7	5.0±0.6	10.1±4.9	3.5±1.1	12.9±7.6
AsPL720	16.6	1.6	0.8			AsPL720	20.6	2.2±1.1	9.1±4.9	2.6±0.6	6.6±1.4	2.2±0.6	5.2±3.8	1.7±0.5	4.6±3.0
AsUNK – P1	18.1	4.6													
AsHC404	19.4				1.3										
AsHC332	19.9	5.1	1.4		1.3	AsHC332/ AsFA514	24.4	1.4±0.6	1.2±0.9	3.5±0.5	0.9±0.8	3.3±1.6	4.6±1.9	4.9±2.8	1.7±0.3
						AsHC358	25.1	1.7±0.8	1.4±0.4	1.2±0.5	1.7±0.9	3.8±0.2	4.0±2.1	5.0±2.0	1.8±0.4
						AsFA528s	25.4	3.9±2.3	2.2±1.7	0.9±0.7	1.2±0.2	1.6±0.3	0.9±0.8	1.4±0.6	3.1±0.3
AsHC360	21.8		1.7	2.4	0.9	AsFA542/530/ HC360	26.4	0.4±0.5	0.7±0.1	2.4±1.0	0.4±0.7	2.2±1.0	0.8±0.9	1.8±0.8	1.5±0.4
						AsFA570	27.4	0.2±0.4	1.1±1.5	1.1±1.4	0.5±0.8		1.1±1.1		
						AsFA558	27.7	0.7±0.5	1.1±1.4	3.6±1.6	1.5±0.3	1.4±0.3	0.4±0.7	0.9±0.9	1.5±0.9
AsPT546+ukn	23.1	11.0	3.4	4.7	1.8	AsHC388/ AsPT546	28.4	7.6±2.4	12.3±5.6	7.5±1.9	9.0±1.6	14.3±5.6	16.4±6.3	6.6±1.6	12.6±3.7
AsHC414+ukn	24.7	13.7	2.4	3.0	1.5	AsHC414	29.3	1.7±0.4	8.6±3.2	3.5±1.2	5.7±1.2	2.9±1.0	5.3±2.2	3.5±1.1	5.5±2.9
AsUNK – P2	25.8	17.1	2.1	3.8	0.6										
						AsUKN – D1	30.6	7.3±3.3	16.1±6.1	8.4±2.7	14.4±5.7	16.7±5.5	31.6±10.9	13.6±2.8	25.5±2.7
						AsHC444	31.1	0.9±1.2	0.3±0.5	0.5±0.4	0.3±0.5	0.2±0.4		0.2±0.4	0.3±0.4

Conclusion

Mussels can depurate some arsenolipid through faeces, when in starvation. New compounds exclusive to faeces suggests that microbiome might influence arsenolipids production. Environmental conditions induced a fast recovery of arsenolipids and compounds present in plankton were taken up. The arsenolipids presence in *Mytilus galloprovincialis* seems to be a combination of food uptake, bioaccumulation and arsenolipid-biogenesis by biosynthesis infidelity.

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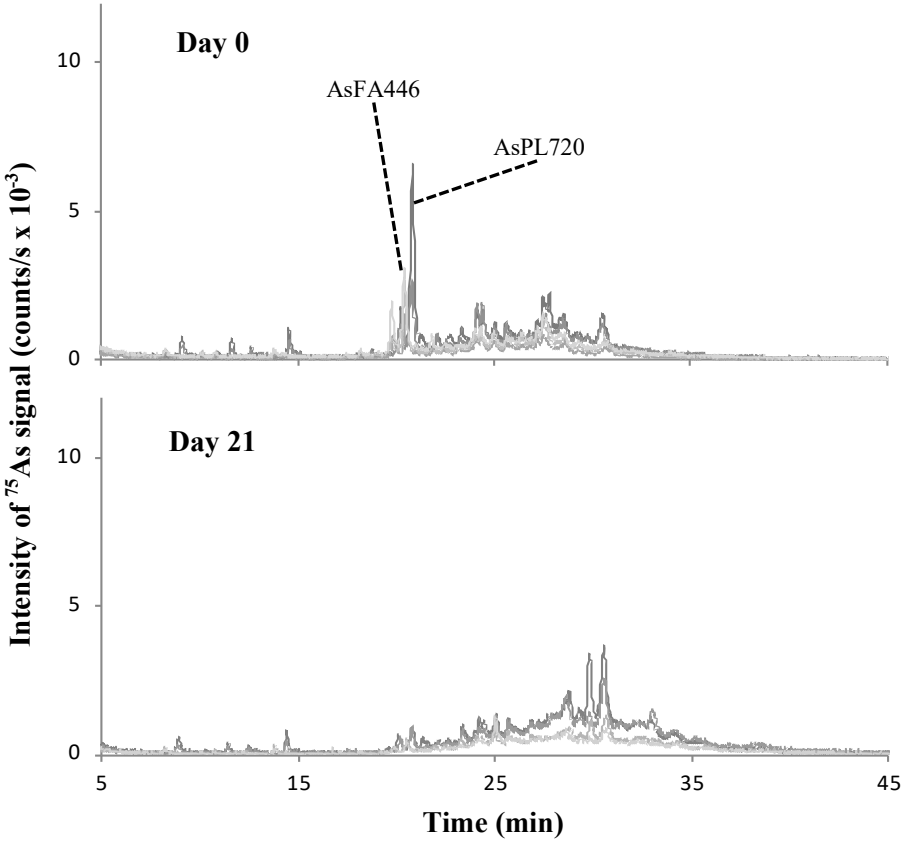
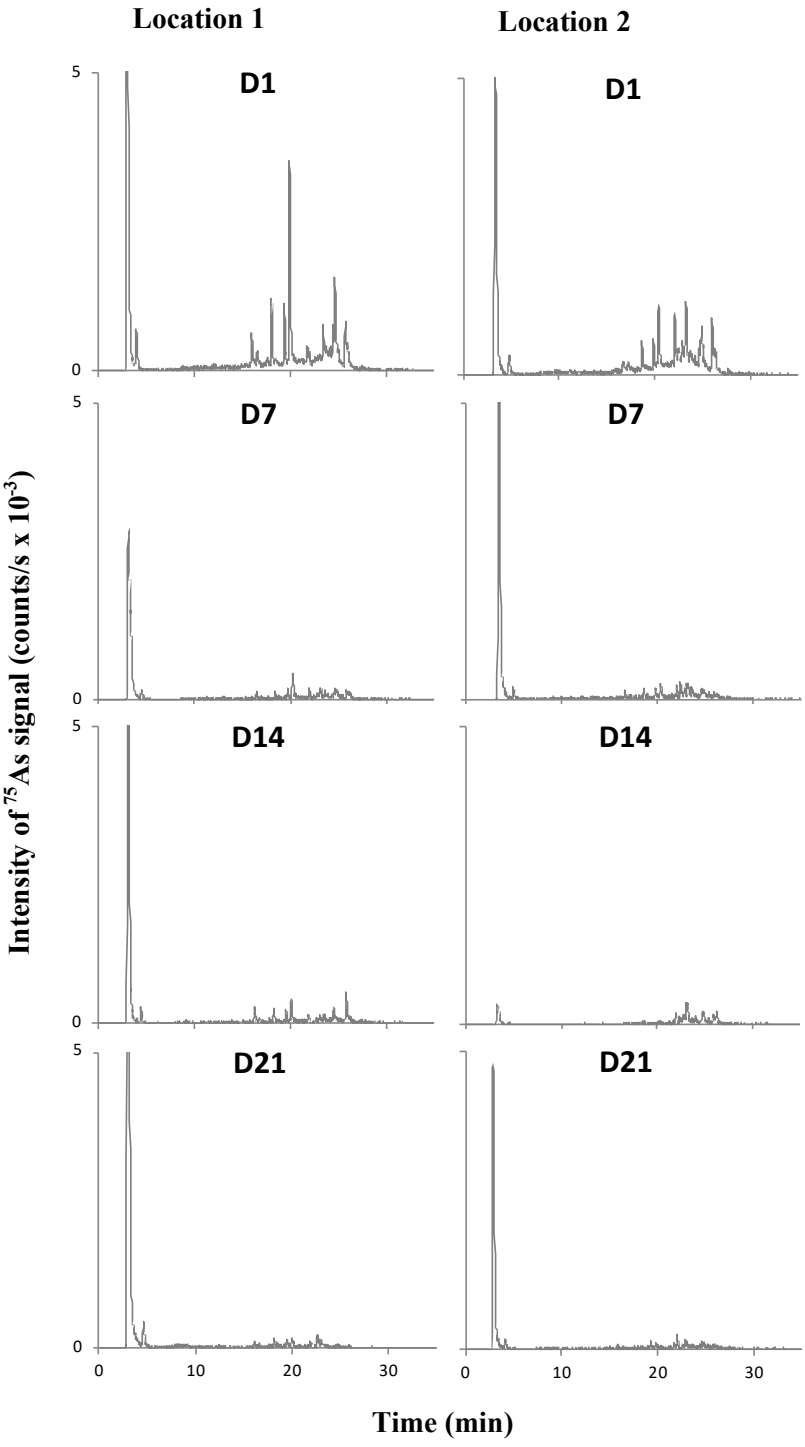
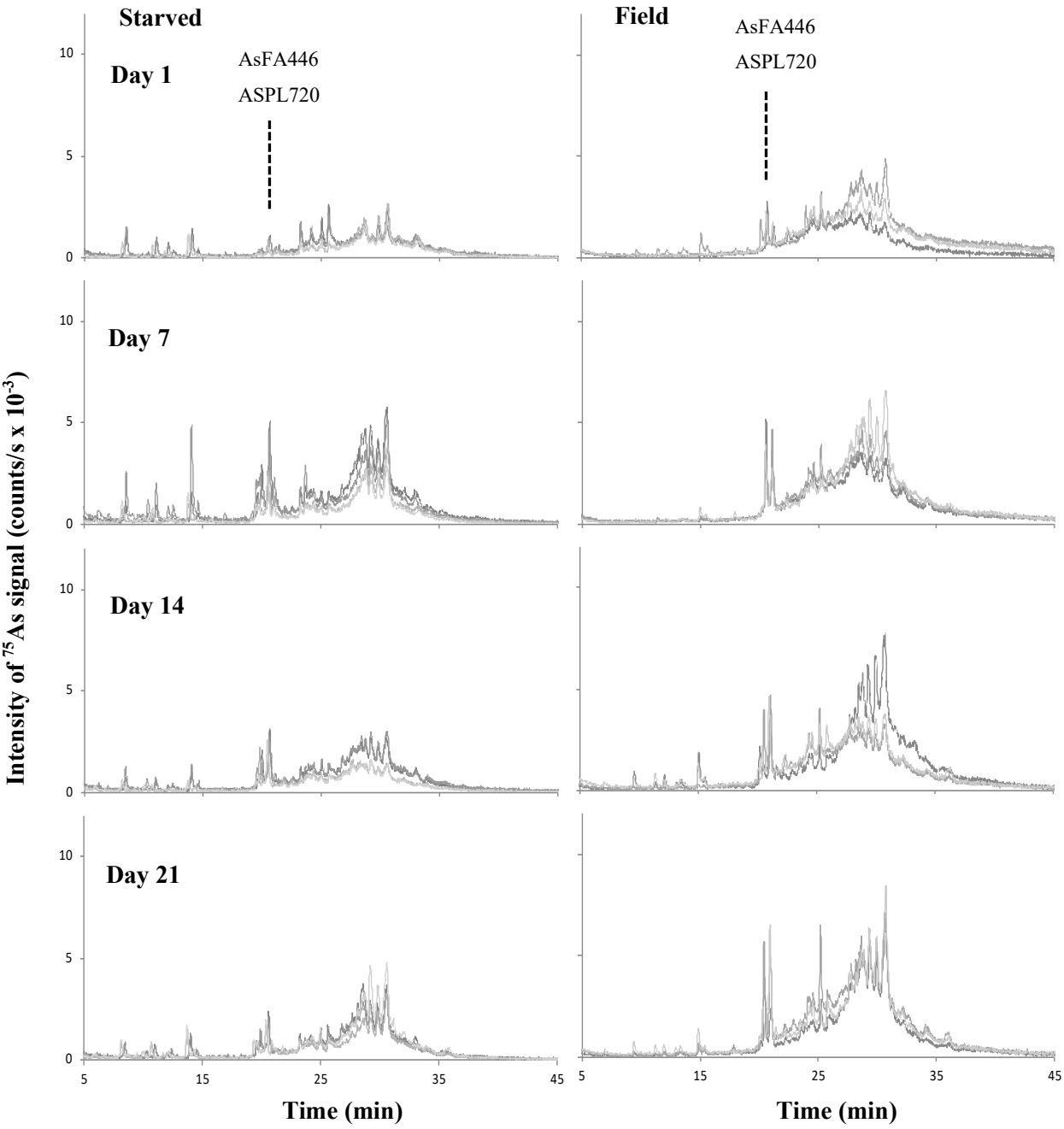
Supplementary data



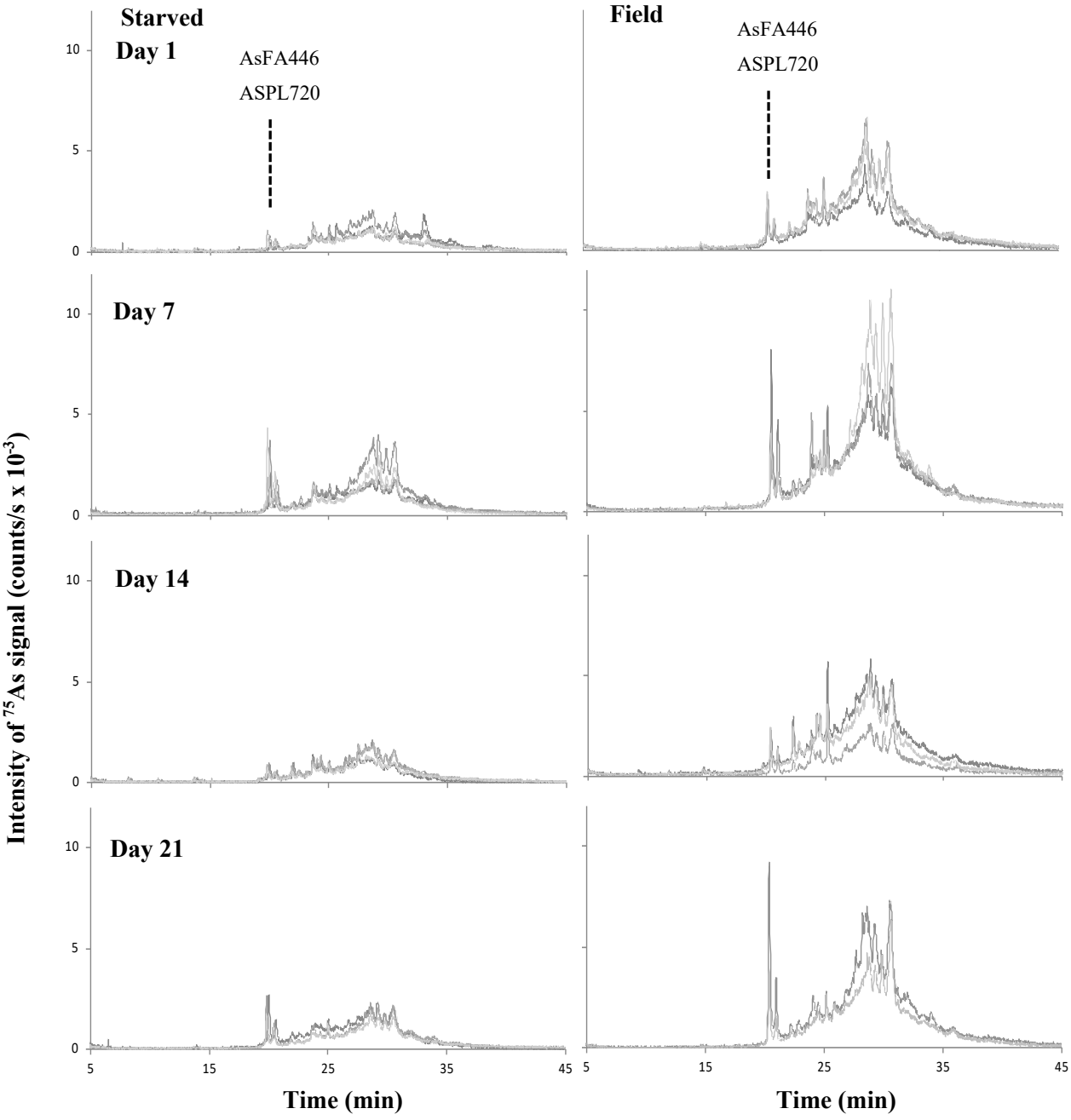
Supplementary Data - Figure 4-1: Chromatograms on arsenolipid content from environment condition (Day 0) and at end of starvation (Day 21). Front peak was removed.



Supplementary Data - Figure 4-2: Chromatograms for arsenolipid content for plankton samples, for both *in-situ* locations. Sampling on days: 1, 7, 14, 21. Front peak was not removed to show the arsenosugars-phospholipids peak.



Supplementary Data - Figure 4-3: Chromatograms of arsenolipid content in starved (n=4) and field (n=3) organisms for days 1, 7, 14 and 21, for Location 1.



Supplementary Data - Figure 4-4: Chromatograms of arsenolipid content in starved (n=4) and field (n=3) organisms for days 1, 7, 14 and 21, for Location 2

Chapter 5

Microbial influence on arsenolipid profiles in digestive glands of *Mytilus galloprovincialis* maintained under post-mortem conditions

Abstract

The changes to arsenolipid profiles from digestive glands of *Mytilus galloprovincialis* were monitored when different bacterial communities were present in a degradation time-line from day 0 to day 30. Bacteria communities activity, present as microbiome, does not seem to provoke major changes to the arsenolipids compounds present on digestive gland. 33 arsenolipid compounds were identified in this survey, with five arsenolipids been newly found. Digestive glands of *M. galloprovincialis* when exposed to simulated degradation processes, resulted in large changes to the arsenolipid profile with an increase in polar compounds (early eluting on HPLC) of +150;230%, most as arseno-fatty-acids. This increase is not proportional to the decrease on less polar (latter eluting on HPLC) (-10;20%).

Introduction

Bacterial communities in marine environments play pivotal roles in the transformation and cycling of nutrients and other compounds, and in the survival of organisms through the composition of their microbiome (Azam and Malfatti 2007; Kouzuma and Watanabe 2015; Sunagawa et al. 2015; Pierce and Ward 2018).

Arsenolipids, lipids that contain arsenic in their structure, are present mostly in the marine environment and throughout marine trophic levels; few examples are: microalgae (Glabonjat et al. 2017), plankton (Freitas et al. 2019 - Chapter 4); macroalgae (García-Salgado et al. 2012), mussels (Freitas et al. 2019 - Chapter 2), fish (Rumpler et al. 2008; Amayo et al. 2011, 2013) and fish roe (Viczek et al. 2016).

Bivalvia species are used as model organisms, mainly for their physiological ability to filter substantial volumes of water and to accumulate contaminants from the surrounding water (Farrington and Tripp 1995). Bivalve high filtration rate also reflects on their microbial community (microbiome), which correlates to that present on the surrounding environment (Gatesoupe 1999) and in higher concentration on the organisms than on surrounding water (Cavallo et al. 2009). Differences in the microbiome can exist between species, even those living at the same location (Vezzulli et al. 2018). Mussels are also able to degrade some bacteria (Birkbeck and McHenery 1982; McHenery and Birkbeck 1985), which enables the incorporation of their lipids by the mussels (Jamieson and Wardlaw 1989; Conway and McDowell Capuzzo 1991).

This exploratory work, tries to inferring if bacteria could produce or influence a different arsenolipid profile in a simulated decomposition condition (post-mortem degradation). As a model organism we used *Mytilus galloprovincialis*; as arsenolipids compounds presence and diversity in the digestive gland have been investigated (Freitas et al. 2019 - Chapter 2).

Methods

Mytilus galloprovincialis were collected from the Aveiro Coastal Lagoon Estuary, from two locations (Figure 5-1); 50 individuals were used from each location. Locations were chosen to provide the most difference on the microbiome, at this estuary compositional shifts on free bacteria were more relevant between the brackish and freshwater sections (Henriques et al. 2006); therefore, Location E (Espinheiro) is in the vicinity of inner river mouth, represents the interphase between freshwater and brackish; and Location O (Orbitur) is located in brackish water, where it receives a high percentage of sludge discharge. Mussel shells were cleared of epi-biota by brushing them under tap water. The digestive glands of all individuals per location were dissected, put together and

homogenised. Each homogenate was put into a sterilized plastic cup, both treatments were exposed to the same conditions in an acclimated room to 19°C under natural light, with a photoperiod of 15h/9h (light/dark). Replicates of 250 µL and 5 mL were taken at day 0, 5, 15 and 30, from the homogenates, for microbial community-level physiological profiling and arsenolipids detection and quantification, respectively.

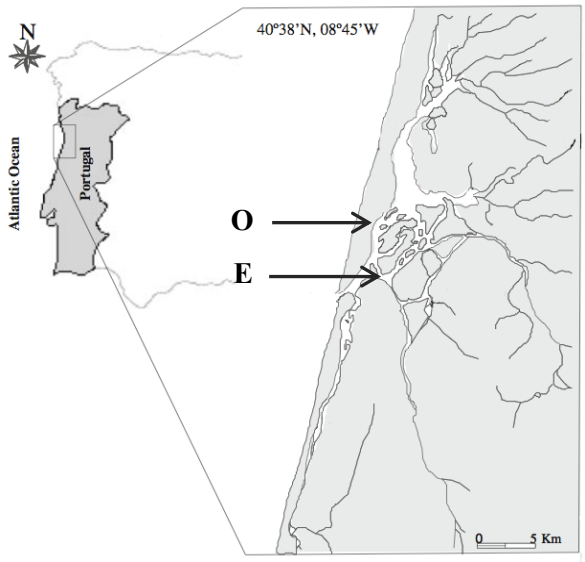


Figure 5-1: Aveiro Coastal Lagoon Estuary sampling sites for Location O (brackish water and sludge recipient) and Location E (interphase of freshwater and brackish) (Figure adapt from Pereira et al. 2009).

Chemicals and Standards

Water (18 MΩ cm) used throughout the investigation was from a Milli-Q Academic water purification system, (Millipore GmbH, Vienna, Austria). The following chemicals, all of analytical grade, were used in the study: nitric acid 68%, p.a., further purified by using a MLS duoPUR sub-boiling unit (MLS GmbH, Leutkirch, Germany); methanol (≥99.8%, MeOH) purchased from ChemLab (Zedelgum, Belgium); and purchased from Carl Roth GmBH (Karlsruhe, Germany): methyl-tert-butyl ether (99.5%, MTBE), ammonium acetate (97%), ammonia (25%), formic acid (≥98%) and the single element standards (^{75}As, ^{74}Ge, ^{115}In and ^{125}Te). The reference material DOLT-3, certified for arsenic, was obtained from NRC-CNRC, Canada. Three arsenic fatty acids (AsFA362, AsFA418, AsFA388) and three arsenic hydrocarbons (AsHC332, AsHC360, AsHC444) were used as standards

for calibration and identification of arsenolipids. These standards were synthesized in-house, with 99% purity assessed by NMR and HPLC/MS (Taleshi et al. 2014).

Total arsenic quantification

Arsenic was measured by inductively coupled plasma mass spectrometry (ICPMS; 7900 from Agilent Technologies, Waldbronn, Germany) in a portion (*ca.* 50 mg, weighed to 0.1 mg) of the original powdered tissue samples, and in the two fractions from the lipids extraction, the pellet+water (all was used), and the organic phase (1 mL aliquot) after the samples had been mineralised by microwave-assisted acid digestion with nitric acid in a Ultraclave III microwave system (MLS, Leutkirch, Germany). After the mineralisation, samples were made ready for the ICPMS analysis by adding water and 1 mL of internal standard solution containing Ge, In, and Te at 100 µg L^{-1} to a final volume of 10 mL. Arsenic calibration concentrations were 0, 0.1, 0.5, 1, 5, 10 and 50 µg L^{-1}. Polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$) on arsenic (^{75}As) were mitigated by recording the measurements in collision cell mode with flow of He, at 4 mL min^{-1}. Stability and precision of the ICPMS measurements were ensured by the measurement every 10 samples of a 1 µg L^{-1} calibration standard, which was always within 2 % of the target value over the course of the analyses. Limit of quantification (LOQ) and detection (LOD), calculated from the standard deviation of method blanks, were 1 µg L^{-1} (3 x SD) and 2 (10 x SD) µg L^{-1}, respectively. By using this mineralisation and measurement procedure, the CRM DOLT-3 (certified [As]= 10.20 µg g^{-1}, SD 0.50) returned a value of 9.62 µg g^{-1} ±0.54SD, n=4 using 10 mg of material.

Lipid extraction

Lipid extraction was conducted using the MTBE:MeOH method described in (Matyash et al. 2008), with adjustments. About 50 mg (weighed to 0.1mg) mass of freeze-dried tissues directly into a 15mL of polypropylene tube (CellStar®Tubes, Greiner bio-one), added 1.5mL MeOH and vortex the mixture (10 sec); after MTBE (5 mL) was added. Tubes were shacken (rotating arm) for 1 hour at room temperature. To induce the phase separation, we added 1.25mL of water. Samples stand for 10 min before centrifuge (3500 g, 20 min), the upper organic phase was collected. We re-extracted the remain lower phase with a mixture of MTBE/MeOH/H2O (2 mL, 10:3:2.5, v/v/v) as described above. Organic phases (upper layer) were combined and dried at 30°C on a vacuum concentrator (Christ RVC 2-33 CD plus, Martin Christ GmbH, Osterode am Hartz, Germany). To aid the evaporation process 200 µL MeOH was added to the solutions after 25 min of centrifugation. The lipid pellets (total lipid content) was weight and re-dissolved in MeOH (500 µL); solubilisation was aided by 15 min sonication. Samples were filter through syringe filters (cellulose acetate membrane, 0.20 µm, 47

mm from VWR International) prior to HPLC/MS analysis. Total lipids were measure gravimetrically.

Quantification of arsenolipids by HPLC/ICPMS

Compound separation was performed with an Agilent 1260 HPLC system using an ACE Ultra Core SuperC18 column (5 µm, 250 x 4.6 mm; Method Development Kit, Advanced Chromatography Technologies Ltd, UK) under gradient elution conditions. The mobile phases were water and MeOH, both with 20 mM ammonium acetate (pH=9.2, adjusted both with ammonia); flow rate was 1 mL min^{-1}, with the following MeOH gradient: 0 min, 50%; 0-25 min, up to 100%; 25-42 min, 100%; 42.1-50 min, down to 50%. Sample injection volume was of 50 µL mixed online with 50 µL of water. Arsenic was measured by transferring 10% of the column effluent directly to the ICPMS by using a passive splitter (Analytical Scientific Instrument, Richmond, USA). An internal standard solution of 20 µg L^{-1} (each of Ge, In and Te) was introduced using a T-piece after the splitter at an isocratic flow of 0.8 mL min^{-1}. An aqueous solution containing 10% MeOH was continuously introduced to the ICPMS spray chamber providing carbon compensation, as described by Raber et al. (2010). Calibration was done with a mixture of the 6 arsenolipids standards, with concentrations of 0, 1, 5 and 10 µg As L^{-1}. Stability of the measurements was monitored by repeated injection of the 5 µg L^{-1} standard. LOD and LOQ, calculated from 3*SD and 10*SD of the methods blanks, respectively, were 0.09 µg As L^{-1} and 0.30 µg As L^{-1}. Data analysis was conducted with Mass Hunter Version B.01.01 (Agilent, Waldbronn, Germany). Quantification was determined by integration of peak areas against external calibration with the standards.

Identification of arsenolipids by high-resolution molecular mass spectrometry

Arsenolipid were identified using a Dionex Ultimate 3000 HPLC system was coupled to a Q-Exactive Orbitrap Mass Spectrometer (Thermo Fisher Sci., Erlangen, Germany) equipped with a heated electrospray ionization (HESI) source. The chromatography was performed using the same column and eluting conditions as described above for the Agilent system, with a flow rate of 1 mL min^{-1}, the column was held at 40°C, the injected sample volumes were 20 µL. MS was performed in the positive modus and source settings were: spray voltage 3700 V, capillary temp 300 °C, gas temp 500 °C, with flow rates of 65 (sheath) and 20 (aux) instrument units (IS). The full scan range was m/z 300 - 1100 with the resolution set to 35 000 (full width half-maximum, FWHM), an automatic gain control (AGC) target of $1E^6$ and a maximum injection time (IT) of 100 mses were used. Data dependent MS/MS (ddMS/MS) for the precursor ions was performed after recording of full scans. Collision energies (NCE) of 20, 35, 40 (stepped) instrument units were used for the fragmentation experiments. MS/MS settings were: maximum injection time 50 msec, AGC $2E^5$, resolution 17500 FWHM. An

isolation window of 0.4 m/z (mass/charge) was used and five MS/MS experiments performed before a new full scan. The fragmented ions were subsequently excluded for 10 secs.

Community-level physiological profiling (CLPP)

The microbial communities were differentiated by the use of different carbon sources, using microplates from BIOLOG® EcoPlates™ (Biolog, Inc., Hayward, Canada) (Garland and Mills 1991). Each plate has 96 wells, and contained a blank well followed by 31 different carbon sources in three replicates. The carbon utilization by the microorganism is evidenced by the change in colour that results from the reduction of tetrazolium violet redox dye (Garland and Mills 1991). Absorbance (ABS) was measured by spectrophotometer (Jenway, UV-VIS 6505). Isolation of bacteria for the microplate incubation was done with 250 µl of raw sample of the homogenised digestive gland. The raw sample was removed and stored at 4°C for a maximum of 24 h before they were processed for analysis. Prior to extraction, the 250 µl samples were macerated in a sterilized blender and treated to reduce turbidity. Turbidity was reduced based on Button et al. (2016) work, and conducted as follow: whole sample was diluted in 20 mL of autoclaved solution of 0.9% NaCl and vortexed; to remove fragments of tissues, samples were centrifuged 700 g (5 min at 4°C) and supernatant was collected (this step was repeated twice). The combined supernatant was centrifuged at 200 g (20 min, at 4°C), to sediment the bacteria to a pellet. The bacterial community was recovered by re-suspending the pellet in 10 mL of NaCl solution (0.9%). Inoculation was done using 100 µl of bacterial suspension in each well. After the first absorbance measurement (t_0) at 590 nm, plates were incubated at 19 °C and fingerprint followed measurements every 24 h for seven days. Optical density (ODi) results were corrected for each well by subtracting the ABS value of the first absorbance measurement (t_0) of each corresponding well (Insam and Goberna 2008). ABS values above 0.1 were consider for analysis and values higher than 2 were put as 2, according to the limit of the reader. Optimum microbial activity in each microplate at each given time was expressed as the absorbance averaged values of all wells in the microplate, the Average Well Colour Development (AWCD) (Zak et al. 1994), with the optimum incubation hours defined as when growth was exponential.

Community profiling was also based on the carbon substrates used, which represented eight compound groups: 7 carbohydrates, 7 carboxylic acids, 2 phosphorylated chemicals, 2 aromatic compounds, 1 ester, 6 amino acids, 2 amines and 4 polymers. PCA analysis was conducted to discern differences between microbial communities by the eight carbon groups. Substrate richness values and evenness were calculated by the number of utilized carbon substrates, using Shannon indices for diversity (H) and evenness ($E=H/H_{max}$) (Zak et al. 1994). Shannon diversity (H) and the Principal Component Analysis (PCA) were conducted in Past3 software (Hammer et al. 1999).

Results

Total Arsenic, Lipids and Arsenolipids

Lipid extraction arsenic mass balance ranged between 88 to 104%. HPCL column recovery ranged between 88 and 106%, with total arsenic in the lipid phase been quantified by the ICPMS analysis. Total arsenolipid concentration was determined by integrating the chromatogram minus the front peak. Total arsenic was stable for the whole period of the experiment, 27.1 ± 1.4 μg g^{-1} and 30.2 ± 1.5 μg g^{-1}, for location O and E, respectively (n = 4, *i.e.*, day 0, 5, 15 and 30). On location O total lipids content decreased and then stabilised, the arsenolipids increased till day 15, followed by a stabilization. For location E, both lipids and arsenolipids followed the same pattern with a constant decrease till day 15 follow by a recovery (Table 5-1).

Table 5-1: Variation on total lipids and arsenolipid content for Digestive gland homogenate samples over the degradation time period, from sampling locations Location O (brackish and sludge recipient) and Location E (interface between freshwater and brackish). Data is given for a single replica (n=1) taken for each homogenate on each sampling day (0, 5, 15 and 30).

	Location O				Location E			
Day	0	5	15	30	0	5	15	30
Lipids (%)	21	15	13	13	14	13	11	18
Arsenolipids (μg g-1)	0.25	0.25	0.33	0.27	0.33	0.27	0.15	0.36

Newly found arsenolipids

33 arsenolipids compounds were identified on this survey in digestive glands from *Mytilus galloprovincialis*, with five newly found fatty acids (Table 5-2).

Table 5-2: Newly found arsenolipid compounds on samples and their respective structures. RT is retention times in minutes for the HPLC-MS analysis.

ID	RT	Mass $[M+H]^+$	Calculated Mass $[M+H]^+$	Formula	Double bonds	$\|\Delta m/m\|$ [ppm]
AsFA464	20.24	465.2344	465.2344	$C_{25}H_{41}O_3As$	5	0.03
AsFA486	22.37	487.3123	487.3127	$C_{26}H_{51}O_3As$	1	-0.71
AsFA502	18.12	503.2484	503.250	$C_{28}H_{43}O_3As$	7	0.31
AsFA520	21.49	521.2974	521.2970	$C_{29}H_{49}O_3As$	5	2.00
AsFA540	24.66	541.3595	541.3596	$C_{30}H_{57}O_3As$	2	1.42

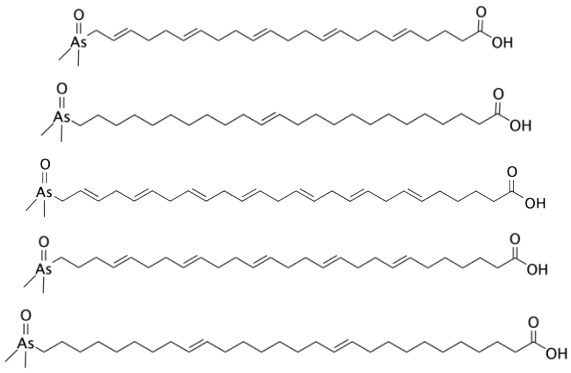
AsFA464

AsFA486

AsFA502

AsFA520

AsFA540



Arsenolipids profile changes

During the post-mortem time, arsenolipids profile shown a loss of less polar compounds, with an increase of the polar ones (Figure 5-2), excepting day 15 for location E. Compounds present and absent and their relative abundance towards the total arsenolipid content, showed that: AsPL720 was present only at day 0; AsFA390 and AsFA402 were present after day 5; AsFA486 and AsFA528s were present only after day 15; AsFA570 was only detected after day 5 for location E and on day 30 for location O. The newly found AsFA486, was in the present study the only one exclusively related to degradation processes, as it was not present for environmental conditions (Day 0) and present for day 15 and 30 (Table 5-3), other compounds not present for Day 0, were already found for other biological matrices. Single and groups of compounds identified on the chromatograms have shown an increase in their concentration from day 0 to 30 (Table 5-4). During the time of post-mortem, most had the same trend as the bulk arsenolipid content variation. Compound AsFA416 had the most pronounced increase, from values <0.6 up to 5 μg L^{-1} for both locations. The group of compounds AsFA436/390/402 were the dominant group of compounds reaching the highest concentration of *ca.* 20 and 30 μg L^{-1}, respectively for location O and E. The combined increase for polar compounds (early eluting) had a value of 150 and 230%, and the decrease for less polar (latter eluting) compounds was up to 10 and 20%.

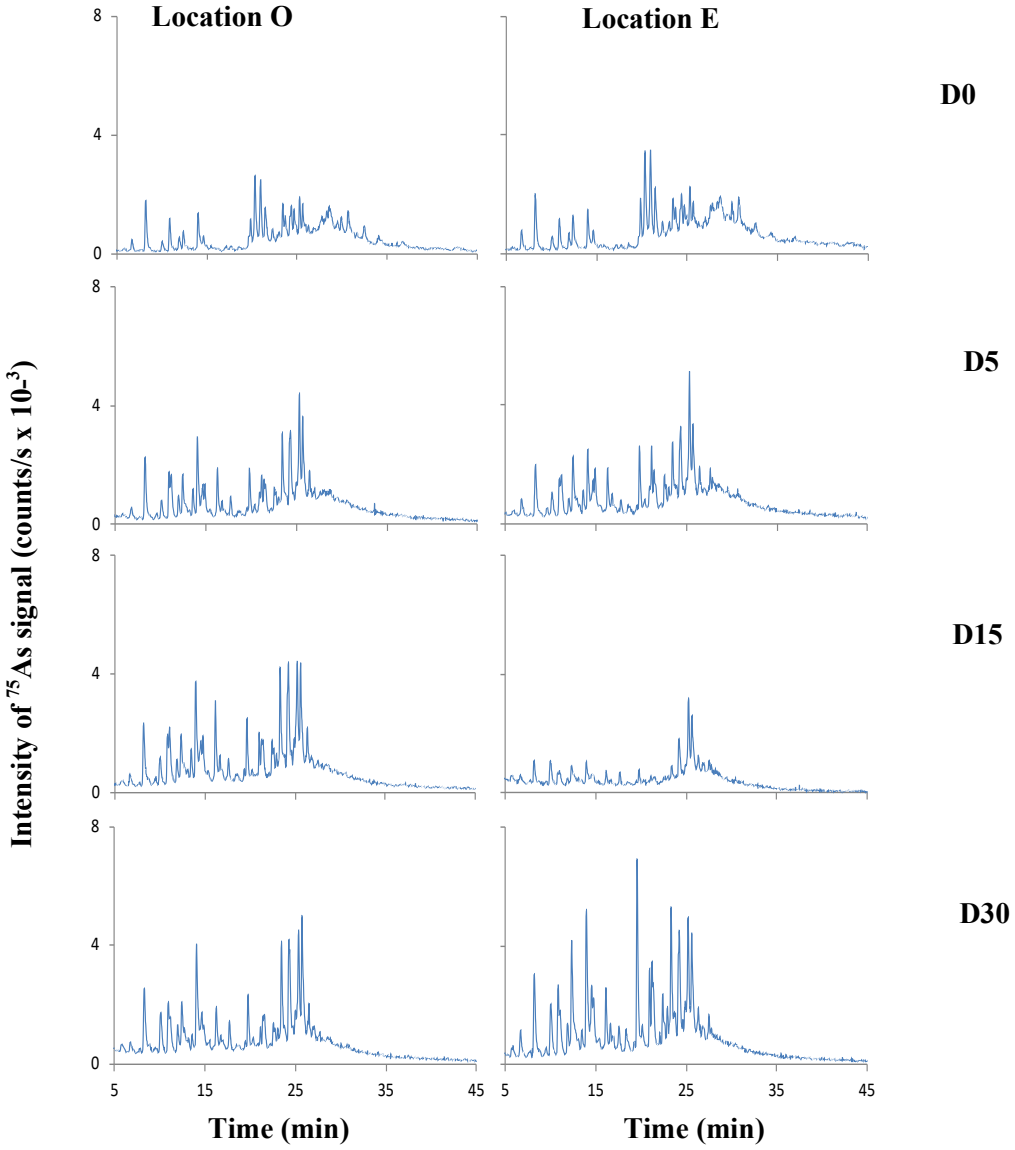


Figure 5-2: Chromatograms for arsenolipid content in Digestive gland homogenate samples for sampled days during the post-mortem time-line: day 0, 5, 15 and 30. from both sampling locations Location O (brackish and sludge recipient) and Location E (interface between fresh-water and brackish)

Table 5-3: Arsenolipids presence and relative abundance towards to total concentration of arsenolipids, based on intensity data from ESMS, assuming a similar response in the mass spectrometer for the various compounds, for the digestive gland homogenate for Location O (brackish water and sludge recipient) and Location E (interphase between freshwater and brackish), and for the sampling days (0, 5, 15 and 30). Legend: T – trace (<1%); M – medium (1-5%); S – significant (5-20%) - higher value *ca.* 15%: relative abundance increase is also represented by a grey scale; FA: fatty acids; HC: hydrocarbons; PL: sugars phospholipids; and Pt: phytol.

		Location O				Location E			
		D0	D5	D15	D30	D0	D5	D15	D30
FA	AsFA334	T	T	T	T	T	T	T	T
	AsFA348	T	T	T	T	T	T	T	T
	AsFA362	T	T	M	M	T	T	M	M
	AsFA382	S	M	M	M	M	M	M	M
	AsFA388	T	M	T	M	T	T	T	T
	AsFA402		T	T	T		T	T	T
	AsFA408	S	S	S	S	M	M	M	S
	AsFA410	M	M	M	M	M	M	M	M
	AsFA416	T	T	T	T	T	T	T	T
	AsFA418	T	T	T	M	T	T	T	T
	AsFA422	M	S	S	S	M	S	M	S
	AsFA430	M	M	M	M	T	M	M	M
	AsFA436	S	S	S	S	M	S	M	S
	AsFA390		T	T	T		T	T	T
	AsFA448	M	M	M	M	M	M	M	M
	AsFA446	T	T	T	T	T	T	T	T
	AsFA464	T	T	T	M	T	T	T	T
	AsFA486			T	T			T	T
	AsFA502	T	M	M	M	T	M	T	M
	AsFA514	M	M	M	S	T	M	S	M
	AsFA520	M	M	M	M	T	T	T	M
	AsFA528	S	S	S	S	M	S	M	S
	AsFA528s			T	T			T	T
	AsFA530	T	T	T	T		T	T	T
	AsFA540	T	T	T	T	T	T	T	T
	AsFA542	M	S	S	S	M	M	S	M
	AsFA548	S	S	S	S	M	M	M	S
	AsFA556	M	M	M	M	M	M	M	S
	AsFA558	T	T	T	T	T	T	T	T
	AsFA570				T		T	T	T
HC	AsHC360	M	T	T	T	T	M	T	T
	AsHC404	M	M	M	M	M	M	T	M
PL	AsPL720	T				T			
Pt	AsPt546	M	T	T	T	M	M	M	M

Table 5-4: Compounds concentration at the sampled post-mortem days, for *Mytilus galloprovincialis* sample from Location O and Location E. Variation (Var.) increase (+) or decrease (–) are for day 0 to 30th. RT indicates the retention time for each arsenolipid, for the HPLC-MS analysis.

Location O

Compounds	RT (min.)	Arsenolipid concentration (μg L^{-1}) Day 0	Day 5	Day 15	Day 30	Var. (%) 0-30
AsFA382	8.35	5.19	6.66	7.16	7.66	+ 50
AsFA362	10.01	1.15	2.18	3.73	5.30	+ 360
AsFA408/410	10.76	3.73	8.78	10.74	8.76	+ 140
AsFA422	12.00	1.36	2.06	2.26	2.73	+ 100
AsFA388	12.56	2.20	6.09	7.34	8.21	+ 270
AsFA436/390/402	14.00	7.47	14.60	22.55	20.83	+ 180
AsFA416	16.02	0.30	4.69	7.33	4.62	+ 1400
AsFA418	17.66	0.69	2.49	3.61	4.82	+ 600
AsFA548/HC404	23.50	6.45	8.86	12.61	11.93	+ 90
AsFA542/530	25.70	3.69	9.23	11.61	14.11	+ 280
AsHC360/FA570	26.94	0.91	1.54	2.49	2.56	+ 180
Early eluting	0- 20	38.22	70.16	96.54	94.76	+ 150
Late eluting	20-40	166.09	137.64	148.32	130.29	- 20

Location E

Compounds	RT (min.)	Arsenolipid concentration (μg L^{-1}) Day 0	Day 5	Day 15	Day 30	Var. (%) 0-30
AsFA382	8.35	5.65	5.89	2.18	8.59	+ 50
AsFA362	10.01	1.81	2.67	3.05	6.53	+ 260
AsFA408/410	10.76	3.10	7.74	2.51	10.64	+ 240
AsFA422	12.00	1.52	1.42	0.71	3.12	+100
AsFA388	12.56	4.76	8.59	3.19	17.30	+260
AsFA436/390/402	14.00	8.42	16.47	5.05	31.66	+280
AsFA416	16.02	0.30	4.26	1.19	5.46	+ 1700
AsFA418	17.66	0.97	2.44	1.77	4.86	+ 400
AsFA548/HC404	23.50	6.38	7.87	1.90	17.74	+ 180
AsFA542/530	25.70	3.62	10.02	7.53	12.56	+ 250
AsHC360/FA570	26.94	1.37	1.19	1.59	2.71	+ 100
Early eluting	0- 20	42.61	87.34	40.69	140.11	+ 230
Late eluting	20-40	200.76	151.47	75.99	178.90	- 10

Microbiome community differences

Both locations have shown an initial active microbiome, with similar AWCD values. Location E was the first to perish under the degradation conditions, with an abrupt decrease on activity. Location O showed a more resilient community with high values till day 15, with a complete die-off on day 30 (Figure 5-3). These observations are congruent with Shannon diversity and evenness indices for the carbon sources consumption (Table 5-5). For samples from both locations, itaconic acid and the aromatic compounds were never used. Carbon sources within the polymer and carbohydrates groups were among the most used carbon sources. Amino acids were promptly used in communities from location E, followed by a decrease; in Location O they were not used at their initial condition but on the following days, its use could infer enzymatic activity on protein production (Table 5-5). Microbiome community differences by carbon group use was plotted into a PCA, with components 1 and 2 explaining 89% of variance; these two components were highly characterized by E0 and O0 communities. Component 1 is mainly defined by esters, polymers and carbohydrates in its positive axis, with aromatic compounds and carboxylic acids presenting influence the negative axis of component 1. Community E0 and O0 present community differences, although being defined by the carbohydrates and polymer carbon groups consumption; O0 had a strong association to ester source. As post-mortem condition progressed the communities, E5, O5 and O15 became more related to O0. The E15, O30 and E30, communities present residual to null values, having no relevant influence (Figure 5-4). The relevant communities present a high variability on the carbon sources within each carbon group, with the various carbon sources been scattered over the PCA distribution.

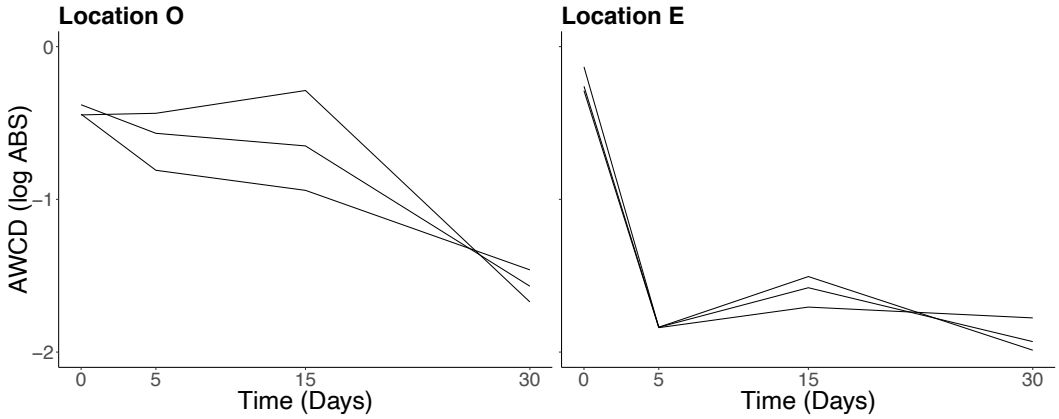


Figure 5-3: AWCD (average well colour density) for the 3 replicas with ABS values log_{10} transformed for the evolution of bacteria communities from location O and E, on the degradation time period 0, 5, 15 and 30 days.

Table 5-5: ABS values, average and standard deviation (n = 3), for the bacteria communities from both locations: Location O (brackish water with sludge recipient) and Location E (interphase between freshwater and brackish), of all the carbon sources present within each carbon group during the degradation time period 0, 5, 15 and 30 days.

	Location O				Location E			
Carbon Sources	**D0**	**D5**	**D15**	**D30**	**D0**	**D5**	**D15**	**D30**
Ester								
Pyruvic Acid Methyl Ester	1.15 ±0.51		0.19 ±0.33		0.55 ±0.96			
Polymer								
Tween 40	1.54 ±0.57	0.47 ±0.21	0.42 ±0.37		1.34 ±0.13	0.27 ±0.03	0.10 ±0.08	
Tween 80	0.54 ±0.50	0.66 ±0.06	0.81 ±0.23		0.91 ±0.12	0.38 ±0.08		
α-Cyclodextrin	0.55 ±0.96	0.15 ±0.25			0.59 ±1.02			0.04 ±0.07
Glycogen	0.70 ±1.12	0.40 ±0.04	0.04 ±0.06		2.00 ±0.00	0.34 ±0.03		
Carbohydrate								
D-Cellobiose	0.67 ±1.15	1.27 ±0.66	1.18 ±0.91		1.33 ±1.15	0.49 ±0.16		
α-D-Lactose		0.18 ±0.17			0.55 ±0.95	0.15 ±0.05		
β-Methyl-D-Glucoside		0.45 ±0.59	0.67 ±1.15			0.25 ±0.09		
D-Xylose		0.55 ±0.19	0.56 ±0.63			0.25 ±0.08		
i-Erythritol	0.10 ±0.17				0.29 ±0.41			
D-Manitol	1.95 ±0.04	0.77 ±0.20	0.48 ±0.55		1.47 ±0.50	0.54 ±0.10		
N-Acetil-D-Glucosamine	0.75 ±1.09	0.98 ±0.59	0.90 ±0.96		0.67 ±1.15	0.62 ±0.02		
Carboxylic Acids								
D-Glucosaminic Acid	0.05 ±0.09	0.22 ±0.21	0.29 ±0.51		0.04 ±0.07			
D-Galactonic Acid γ-Lactone			0.04 ±0.06					

	Location O				Location E			
Carbon Sources	**D0**	**D5**	**D15**	**D30**	**D0**	**D5**	**D15**	**D30**
D Galacturonic Acid		0.11 ±0.19	0.94 ±1.00					
γ-Hdroxybutyric Acid	0.21 ±0.36				0.44 ±0.44			0.12 ±0.21
Itaconic Acid								
α-Ketobutyric Acid		0.07 ±0.12						
D-Malic Acid		0.25 ±0.44	0.46 ±0.79					
Phosphorylated Compound								
Glucose-1-Phophate	0.24 ±0.24		0.18 ±0.31					
D,L-α-Glycerol Phosphate	0.18 ±0.31	0.37 ±0.36	0.35 ±0.46		1.26 ±0.21			
Aromatic compound								
2-Hydroxy Benzoic Acid								
4-Hydroxy Benzoic Acid								
Amino Acids								
L-Arginine		0.32 ±0.28	0.24 ±0.32		0.34 ±0.40	0.21 ±0.03		
L-Asparagine		0.22 ±0.19	0.10 ±0.17		1.33 ±1.15	0.13 ±0.11		
C-Phenylalanine	0.06 ±0.10	0.08 ±0.13						
L-Serine		0.25 ±0.22	0.11 ±0.20		0.46 ±0.79	0.11 ±0.10		
L-Threonine					1.57 ±0.48			
Glycyl-L-Glutamic Acid					1.06 ±0.76			
Amines								
Phenylethylamine	0.09 ±0.16							

Carbon Sources	Location O				Location E			
	D0	D5	D15	D30	D0	D5	D15	D30
Putrescine		0.34 ±0.13	0.04 ±0.08		0.87 ±1.02	0.26 ±0.00		
Shannon Diversity (H)	2.71	3.00	2.94	-	2.94	2.57	0.00	0.69
Shannon Evenness (E)	0.78	0.86	0.85	-	0.85	0.74	0.00	0.20

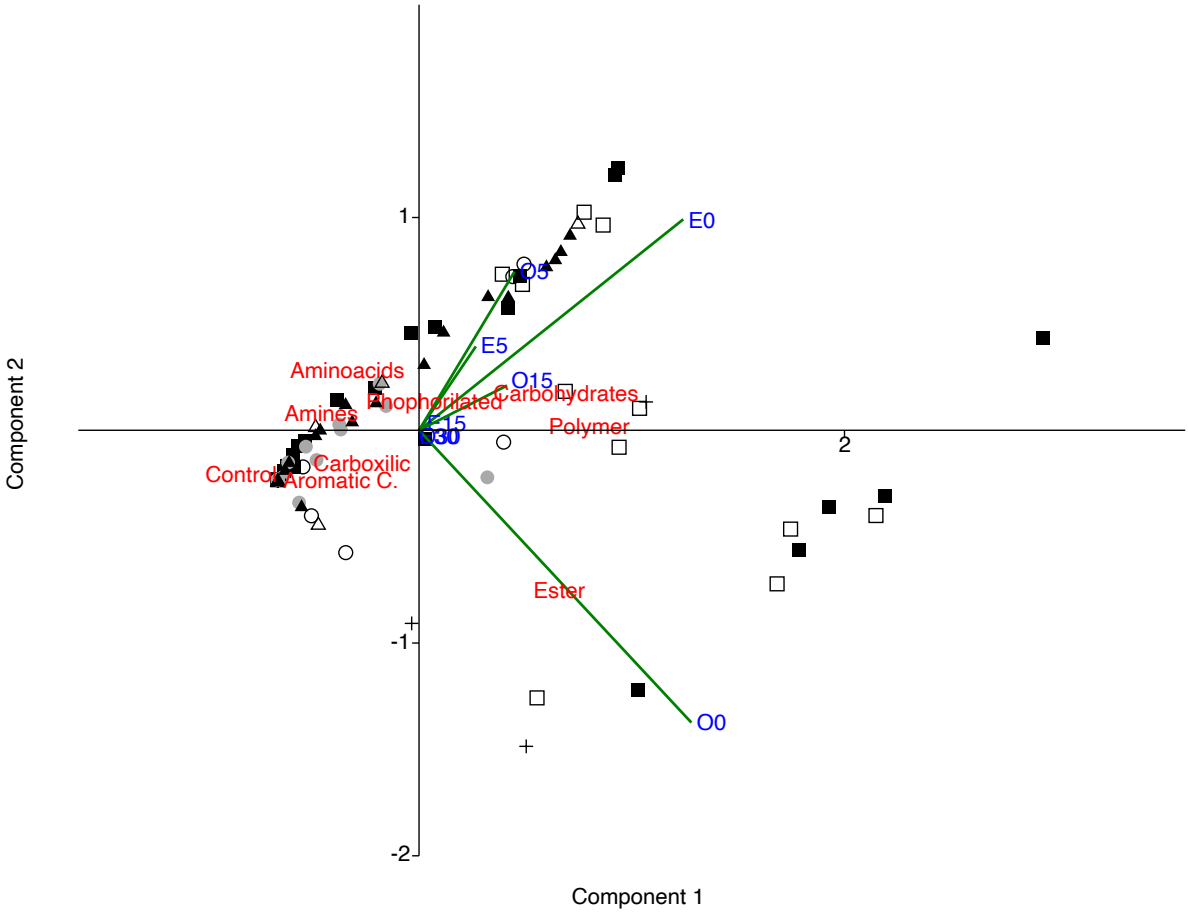


Figure 5-4: Principal Component Analysis (PCA) for carbon sources consumption, from the different microbial communities within the post-mortem time line for Location O (O0, O5, O15 and O30) and for Location E (E0, E5, E15 and E30). Component 1 (E0) explains 69% and component 2 (O0) 20%. PCA computation was done using variance-covariance, between the carbon groups. Carbon groups: Control (–), Ester (+); Polymer (□); Carbohydrates (■); Carboxylic acid (●); Phosphorylated compounds (○); Aromatic Compounds (◇); Amino Acids (▲) and Amines (△).

Discussion

Microbiome activity of *Mytilus galloprovincialis* digestive gland differs according to the surrounding environment (Gatesoupe 1999), as organism present a spatial variability within the same estuary, corroborating the data from Henriques et al ((2006)) on variability of free bacteria variability present in the sampled estuary (Ria de Aveiro Coastal Lagoon). The spatial differences between the two microbiomes analysed did not input any obvious differences for arsenolipid concentration nor diversity of compounds. Microbiome present in these organisms' digestive gland seems to have little to no influence on the overall arsenolipid diversity and profile.

The microbiome changed dramatically from its initial condition as post-mortem progressed. The bacteria communities under degradation conditions have turned similar amongst each other over time. Microbial activity was more active at the location close to sludge discharge, inferring a more opportunistic and resilient community to harsh conditions; in contrast to a more perishable community, from the river mouth. The experimental procedure led to a die-off of communities.

The AsFA486 is until now a newly found compounds only for post-mortem conditions. AsFA390, AsFA402, AsFA528s, AsFA570 compounds were not detected at the initial condition, have been previously found in organisms that did not went through any decomposition process, for example: previous *M. galloprovincialis* (Freitas et al. 2019 - Chapter 2) and for fish roe (Viczek et al. 2016)). The AsPL720, seems to be correlated to organism metabolism or forage consumption, since this compound was only found at the initial condition. Also, 2-O-methyl-riboside-arsenic (AsPt576), that was previously found in mussels' faeces (Freitas et al. 2019 - Chapter 4), and hypothesized to be of microbial origin was not found here, however, the same group compound AsPt546 was found. Therefore, their production could be related to algae and/or bacterial activity. In both bacteria communities, post-mortem conditions prompted differences into arsenolipid profiles. From the decrease of less polar compounds with an unproportioned increase of polar ones. The increase in arsenolipid concentration when microbial activity was null, might be explained by the degradation of more complex compounds, by degradation of triglycerides into fatty acids. Since concentration differences from later to early eluting compounds on HPLC do not match; it is possible, that the complex arsenolipids (later eluting/less polar), were underestimated and perhaps some non-polar arsenolipids were not recorded under the used HPLC-MS conditions.

Conclusion

When decomposition of digestive gland of *Mytilus galloprovincialis* is in place the arsenolipid profile changes to show more early eluting compounds (long carbon chain) in detriment of the later

eluting ones (very long carbon chain). The contribution of degradation conditions on arsenolipids cycle should be take into consideration.

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Chapter 6

Arsenolipids diversity: an overall analysis

Abstract

Arsenolipids overall determination and distribution on marine samples is still recent field of research and the full extent of these compounds is still been study. A review for arsenolipids present in biological was compiled, with a separated section on the arsenolipids found for *Mytilus galloprovincialis*, their faces and plankton (data from this thesis). Datasets were analysed for arsenolipids profile similarities by a cluster analysis. Given the exploratory nature of this analysis conclusion are taken with caution. In *M. galloprovincialis* arsenolipids diversity shown to be influenced by spatial factor. Arsenolipids presence differs based on samples biological origin, with three distinct groups; 1 - macroalga, 2 - fish and fish products (oils and fish meal), and 3 -blue mussels. So far, the biota origin of sample seems to be the major factor for arsenolipids profile.

Introduction

The arsenolipid group is still one of the least studied group of organic compounds containing arsenic, given that their accurate molecular structure was firstly known in 2008 (Rumpler et al. 2008) and the identification of these compounds requires an advance set of analytical chemistry instruments. Since then, several compounds have been found and described in literature and compounds were only found in marine organism, such as in fish muscle, marine oils from fish and fish liver, algae/macroalgae, plankton and in mussels (data compiled in Table 6-3). Arsenolipids have also been found in human milk from a Norwegian population (Stiboller et al. 2017), additional biological matrices have been already analysed, such as krill oil, tuna muscle and brain by Stiboller et al. (personal communication, data not yet published). Groups of arsenolipids have been established based onto their similarity towards non-arseno containing lipids. Up until now there are 8 arsenolipid groups known: arseno-fatty acids (AsFA), arseno-hydrocarbons (AsHC), arsenosugar-phospholipids (AsPL), Trimethylarsenio fatty alcohols (TMAsFOH), phosphatidylcholines and (AsPC), phosphatidylethanolamines (AsPE), 2-O-Methylriboside (AsPt), arseno-ether-phospholipids (AsEP). Several works have been conducted concerning these groups, most of them to describe their occurrence, *i.e.*, presence on biota, their artificial synthesis (Taleshi et al. 2014b; Arroyo-Abad et al. 2016; Guttenberger et al. 2016), and considerable important works on their possible toxicology effects (Meyer et al. 2014a, b; Niehoff et al. 2016).

The present work consists in an overall analysis of arsenolipids diversity in mussel *Mytilus galloprovincialis*, their faeces and plankton (data from previous chapters on this thesis); and for a more general analysis, of all compounds found in marine biologic matrices. This is an exploratory exercise to infer similarities of compounds occurrence between and within biological matrices and if there are compounds that trend to co-occur.

Methods

Data collection

Arsenolipid diversity found in *Mytilus galloprovincialis* tissues, and in their faeces, was compiled and summarized with all the compounds found presenting their structure and formula. Compounds diversity was distinguished between the biological matrices and different sampling locations, *i.e.*, *M. galloprovincialis* from Minho and Aveiro estuaries (mantle is presented only for Minho´s), mussels' faeces (Minho origin), post-decomposition samples (Aveiro origin), and plankton (Aveiro origin). Estuaries location for Minho (M) and Aveiro (Av) on Figure 6-1.

A review of all the arsenolipid known was conducted on June 2019 using the known specialized search engines (Scopus.com, Mendeley search and, for grey literature, Google Scholar. Only works that accurately described the compounds by ESMS analysis and from environmental collected samples were considered.

Statistical analysis

Past 3.25 was used for the statistical analysis of the data (Hammer et al. 1999). Cluster analysis (UPGMA) was used to group sites and to group arsenolipids using the Bray-Curtis index as the measure of similarity.

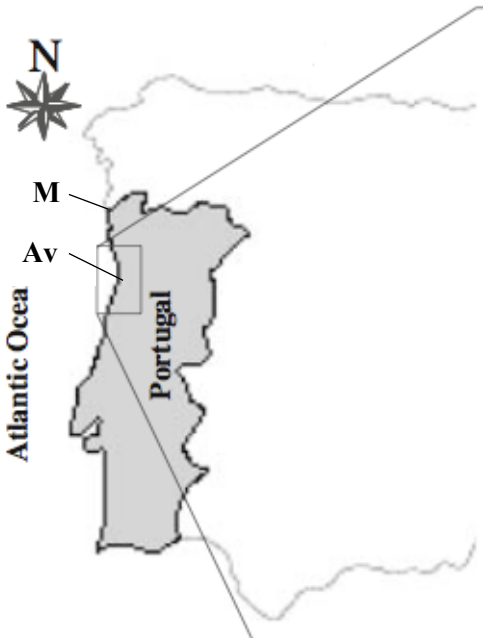


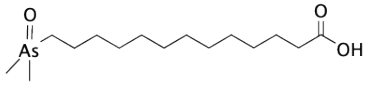
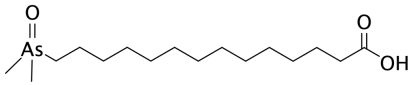
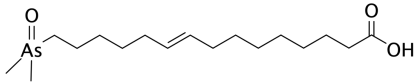
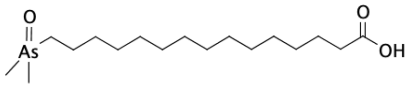
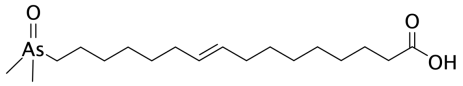
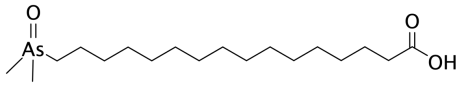
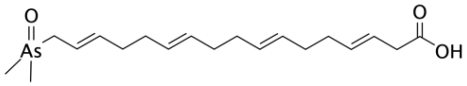
Figure 6-1: Estuaries location in Portugal, for samples of digestive gland and mantle of *Mytilus galloprovincialis*, their faces and Aveiro' plankton

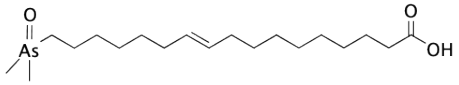
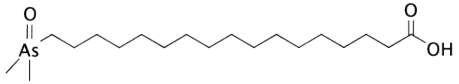
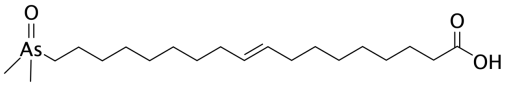
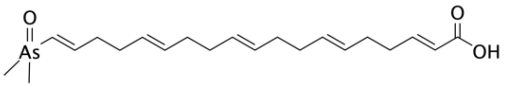
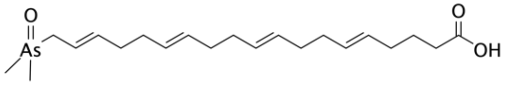
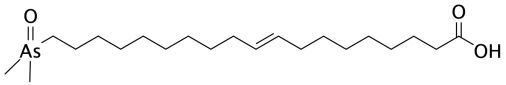
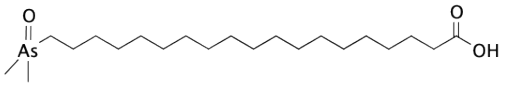
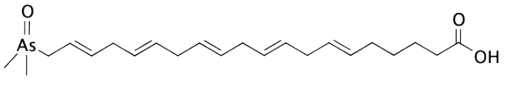
Results and Discussion

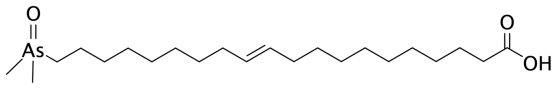
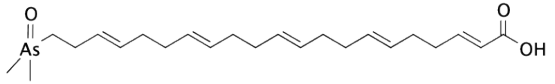
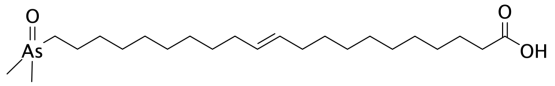
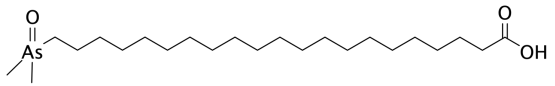
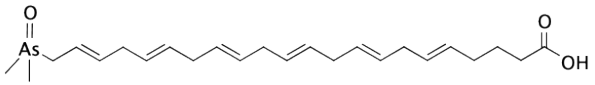
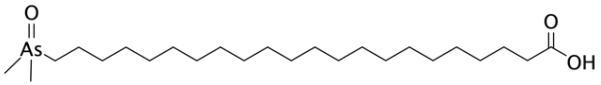
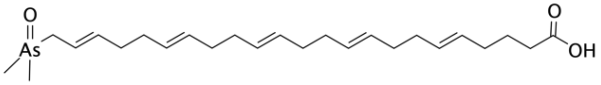
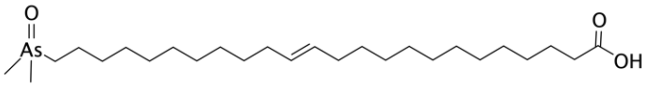
Mussels, faeces and plankton

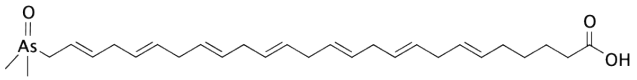
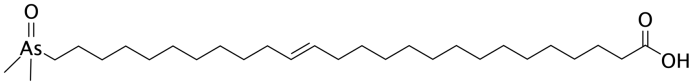
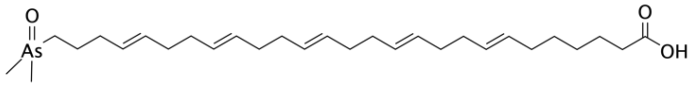
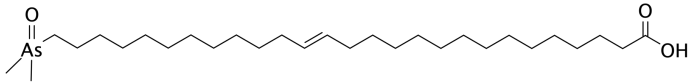
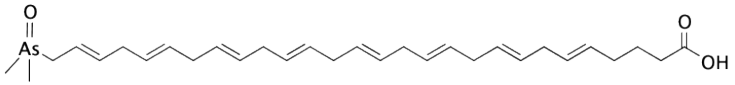
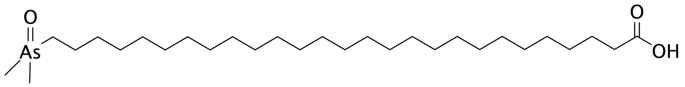
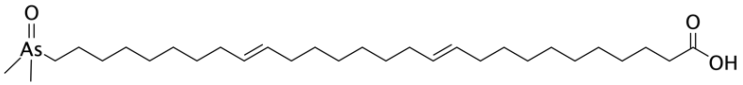
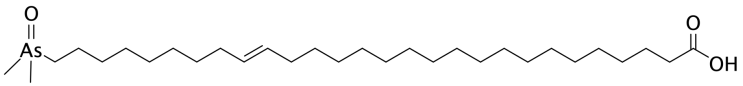
The full diversity of arsenolipid compounds found for *Mytilus galloprovincialis* samples and plankton are presented in Table 6-1, with their respective formula and molecular structure. A total of 50 different compounds was found on this survey, from these 32 were newly found compounds. *M. galloprovincialis* accounts for 46 compounds, with 19 were exclusive to the digestive glands, one to mantle (AsEP734) and 26 common to both organs, two compounds were exclusive to faeces, since AsFA548 was after found for digestive glands on mussels from Aveiro Coastal Lagoon Estuary, and one compound associated to decomposition conditions, AsFA486 (Table 6-2). No new compounds were found in plankton samples.

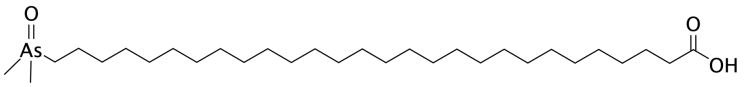
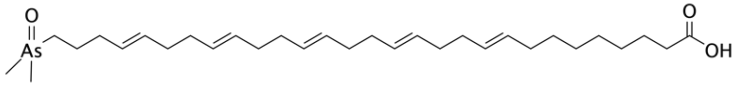
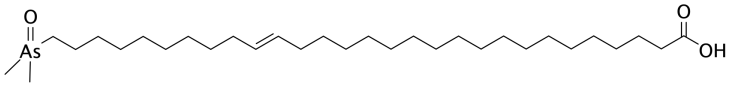
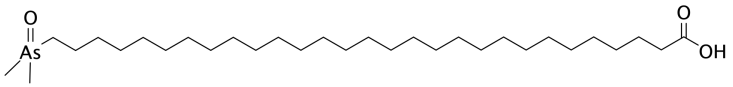
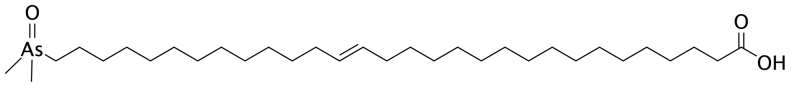
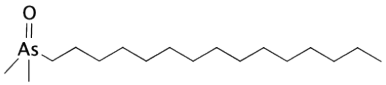
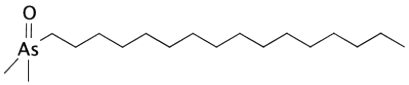
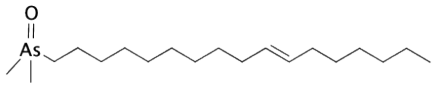
Table 6-1: Arsenolipids found in *Mytilus galloprovincialis*, with their respective formula and structure.

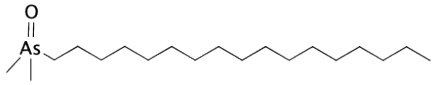
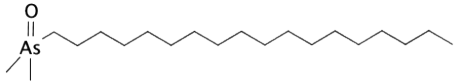
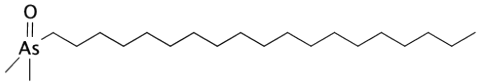
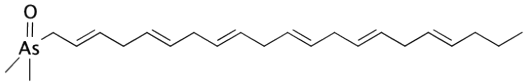
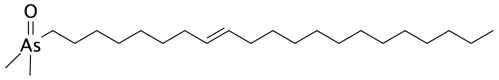
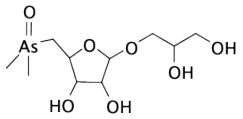
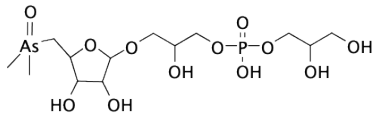
	Compound (ID)	Formula	Structure	Reference
1	AsFA334	$C_{15}H_{31}O_3As$		(Rumpler et al. 2008)
2	AsFA348	$C_{16}H_{33}O_3As$		This work - 1
3	AsFA360	$C_{17}H_{33}O_3As$		This work - 2
4	AsFA362	$C_{17}H_{35}O_3As$		(Rumpler et al. 2008)
5	AsFA374	$C_{18}H_{36}O_3As$		This work – 3
6	AsFA376	$C_{18}H_{37}O_3As$		This work – 4
7	AsFA382	$C_{19}H_{31}O_3As$		This work – 5

	Compound (ID)	Formula	Structure	Reference
8	AsFA388	$C_{19}H_{37}O_3As$		(Rumpler et al. 2008)
9	AsFA390	$C_{19}H_{39}O_3As$		(Rumpler et al. 2008)
10	AsFA402	$C_{20}H_{39}O_3As$		This work – 6
11	AsFA408	$C_{21}H_{33}O_3As$		This work – 7
12	AsFA410	$C_{21}H_{35}O_3As$		This work – 8
13	AsFA416	$C_{21}H_{41}O_3As$		This work – 9
14	AsFA418	$C_{21}H_{43}O_3As$		(Rumpler et al. 2008)
15	AsFA422	$C_{22}H_{35}O_3As$		(Raab et al. 2013)

	Compound (ID)	Formula	Structure	Reference
16	AsFA430	$C_{22}H_{43}O_3As$		This work – 10
17	AsFA436	$C_{23}H_{37}O_3As$		(Rumpler et al. 2008)
18	AsFA444	$C_{23}H_{45}O_3As$		This work – 11
19	AsFA446	$C_{23}H_{47}O_3As$		This work – 12
20	AsFA448	$C_{24}H_{37}O_3As$		(Amayo et al. 2011)
21	AsFA460	$C_{24}H_{49}O_3As$		This work – 13
22	AsFA464	$C_{25}H_{41}O_3As$		This work – 14
23	AsFA486	$C_{26}H_{51}O_3As$		This work – 15

	Compound (ID)	Formula	Structure	Reference
24	AsFA502	$C_{28}H_{43}O_3As$		This work – 16
25	AsFA514	$C_{28}H_{55}O_3As$		This work – 17
26	AsFA520	$C_{29}H_{49}O_3As$		This work – 18
27	AsFA528[s]	$C_{29}H_{57}O_3As$		This work – 19
28	AsFA528	$C_{30}H_{45}O_3As$		(Viczek et al. 2016)
29	AsFA530	$C_{29}H_{59}O_3As$		This work – 20
30	AsFA540	$C_{30}H_{57}O_3As$		This work – 21
31	AsFA542	$C_{30}H_{59}O_3As$		This work – 22

	Compound (ID)	Formula	Structure	Reference
32	AsFA544	$C_{30}H_{61}O_3As$		This work – 23
33	AsFA548	$C_{31}H_{53}O_3As$		This work – 24
34	AsFA556	$C_{31}H_{61}O_3As$		This work – 25
35	AsFA558	$C_{31}H_{63}O_3As$		This work – 26
36	AsFA570	$C_{32}H_{63}O_3As$		This work – 27
37	AsHC332	$C_{17}H_{37}OAs$		(Taleshi et al. 2008)
38	AsHC346	$C_{18}H_{39}OAS$		(Amayo et al. 2013)
39	AsHC358	$C_{19}H_{39}OAs$		(Amayo et al. 2013)

	Compound (ID)	Formula	Structure	Reference
40	AsHC360	$C_{19}H_{41}OAs$		(Taleshi et al. 2008)
41	AsHC374	$C_{20}H_{43}OAs$		(Amayo et al. 2013)
42	AsHC388	$C_{21}H_{45}OAs$		(García-Salgado et al. 2012)
43	AsHC404	$C_{23}H_{37}OAs$		(Taleshi et al. 2008)
44	AsHC414	$C_{23}H_{46}OAs$		This work – 28
45	AsPL328	$C_{10}H_{21}O_7As$		This work – 29
46	AsPL482	$C_{13}H_{28}O_{12}PAs$		This work – 30

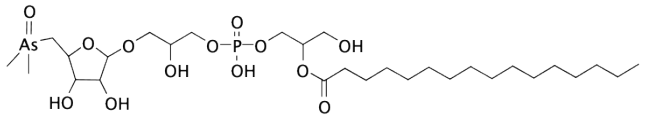
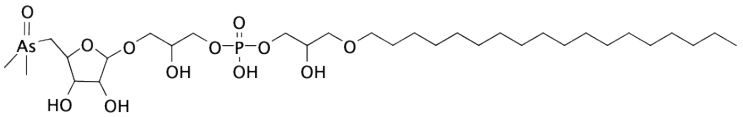
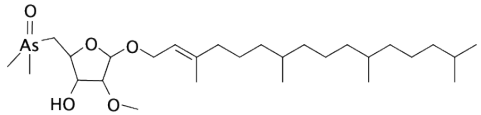
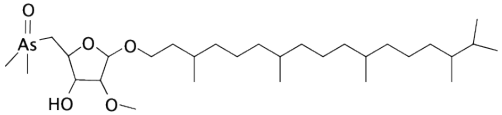
	Compound (ID)	Formula	Structure	Reference
47	AsPL720	$C_{29}H_{58}O_{13}PAs$		(Yu et al. 2018)
48	AsEP734	$C_{31}H_{64}O_{12}PAs$		This work – 31
49	AsPt546	$C_{28}H_{55}O_5As$		(Glabonjat et al. 2017)
50	AsPt576	$C_{30}H_{61}O_5As$		This work – 32

Table 6-2: Presence of arsenolipids on *Mytilus galloprovincialis*: M_dg = digestive gland, M_mt = mantle (data from Chapter 2), M_D0 = digestive gland at environmental conditions from Minho; M_D21 = digestive gland for environmental condition after 21 days on Aveiro Coastal Lagoon Estuary, M_fc = faeces, Pk_b = plankton bulk sample from Aveiro Coastal Lagoon Estuary (data on Chapter 4), for mussels from Aveiro Coastal Lagoon Estuary: Av_O = digestive gland from mussels inside the estuary, Av_E = digestive gland from mussels inside the estuary close to a river mouth, AvD_O = digestive gland after 30 days degradations and AvD_E = digestive gland after 30 days degradations. Compounds are given by their abbreviation on lipid group and follow the molecular mass.

		Chapter 2		Chapter 4				Chapter 5			
		Minho		Minho			Aveiro	Aveiro			
	ID	M_dg	M_m	M_D0	M_D21	M_fc	Pk_b	Av_O	Av_E	AvD_O	AvD_E
AsFA	AsFA334	X	X		X			X	X	X	X
	AsFA348	X		X	X			X	X	X	X
	AsFA360	X	X		X						
	AsFA362	X	X	X	X		X	X	X	X	X
	AsFA370			X							
	AsFA374	X									
	AsFA376	X									
	AsFA382				X			X	X	X	X
	AsFA388	X	X	X	X			X	X	X	X
	AsFA390	X	X	X	X		X			X	X
	AsFA402	X								X	X
	AsFA408				X			X	X	X	X
	AsFA410				X			X	X	X	X
	AsFA416	X	X		X			X	X	X	X
	AsFA418	X	X					X	X	X	X
	AsFA422							X	X	X	X
	AsFA430	X	X	X	X			X	X	X	X
	AsFA436	X	X		X			X	X	X	X
	AsFA444	X									

		Chapter 2		Chapter 4				Chapter 5			
		Minho		Minho			Aveiro	Aveiro			
	ID	M_dg	M_m	M_D0	M_D21	M_fc	Pk_b	Av_O	Av_E	AvD_O	AvD_E
	AsFA446	X	X					X	X	X	X
	AsFA448							X	X	X	X
	AsFA460					X					
	AsFA464							X	X	X	X
	AsFA486									X	X
	AsFA502							X	X	X	X
	AsFA514	X	X	X	X	X		X	X	X	X
	AsFA520							X	X	X	X
	AsFA528s	X	X					X	X	X	X
	AsFA528un									X	X
	AsFA530	X	X	X	X			X			
	AsFA542	X	X	X	X	X		X	X	X	X
	AsFA540							X	X	X	X
	AsFA544	X	X								
	AsFA548					X		X	X	X	X
	AsFA556	X	X					X	X	X	X
	AsFA558	X	X	X	X			X	X	X	X
	AsFA570	X	X		X					X	X
AsHC	AsHC332	X	X	X	X	X	X				
	AsHC346	X									
	AsHC404	X	X				X	X	X	X	X
	AsHC358	X	X								
	AsHC360	X	X	X	X	X	X	X	X	X	X
	AsHC374	X									
	AsHC388	X									
	AsHC414	X	X								
AsPt	AsPt546	X	X	X		X	X	X	X	X	X

		Chapter 2		Chapter 4				Chapter 5			
		Minho		Minho			Aveiro	Aveiro			
	ID	M_dg	M_m	M_D0	M_D21	M_fc	Pk_b	Av_O	Av_E	AvD_O	AvD_E
	AsPt576					X					
AsEP	AsEP734		X								
AsPL	AsPL482	X	X			X	X				
	AsPL328	X				X	X				
	AsPL720	X	X				X	X	X		
	Sum	**35**	**27**	**13**	**19**	**10**	**8**	**28**	**27**	**32**	**32**

Based on the *M. galloprovincialis* data set, the biological matrix of the sample influences the diversity of arsenolipids, with plankton and faeces samples showed a low similarity (*ca.* 35%) relative to the mussel's samples. The mussels' tissues (digestive glands and mantle) had a similarity of 55% (Figure 6-2). The arsenolipid diversity on mussel's digestive gland gives differences for a spatial factor, as organisms from Aveiro (Av_O and Av_E) differ from those of Minho' (M_dg and M_DO) (samples from same year). Exposition to different environmental conditions of same origin organism also influenced arsenolipids diversity, as samples from Minho origin, M_DO (environmental conditions) and M_D21 (after 21 days in Aveiro environmental conditions) differ on a higher level than Aveiro samples (Av_O and Av_E).

Compounds presence/absent shown several group associations (Figure 6-2). One group stands out as having the least similarity (Branch 1), with compounds only appearing once AsPt576/AsFA460 in faeces sample. Exclusive to degradation process were AsFA486 and AsFA528un (on Branch 2), note that AsFA528un was previously found for fish roe (see Table 6-3). The group from AsHC362 to AsFA388, (on Branch 2) comprise the most abundant compounds across samples. Samples of plankton and faeces dissimilarity toward other samples was due to their low abundance of compounds combined to low presence of arseno-fatty-acids. Compounds distribution amongst mussels from different locations does comply with a spatial factor for arsenolipids presence. A smaller branch (numbered 4), compounds were absent from the Minho origin samples (M), being therefore exclusive for Aveiro organisms (Av). On branch 3, all compounds (except AsFA530) are not present for Aveiro organisms.

Diversity of arsenolipids compounds: a review

Arsenolipid compounds for all known samples are described on Table 6-3; compounds occurrence was pull together by equal biologic sample matrix. Excluded from statistical analysis are compounds: AsPE1035 from Viczek et al. (2016) work and AsPt546 from Glabonjat et al. (2017) work; since is only appointed the of the compound and no full arsenolipid profile was given (compounds included on the review); also, samples from digestive gland on degradation conditions (excluding AsFA486), because they do not represent environmental samples. Excluded from this review is the survey on green algae *Coccomyxa* sp. from Řezanka et al. (2019) work, since samples were obtained from stains artificially grown in laboratory conditions exposed to an medium containing arsenic.

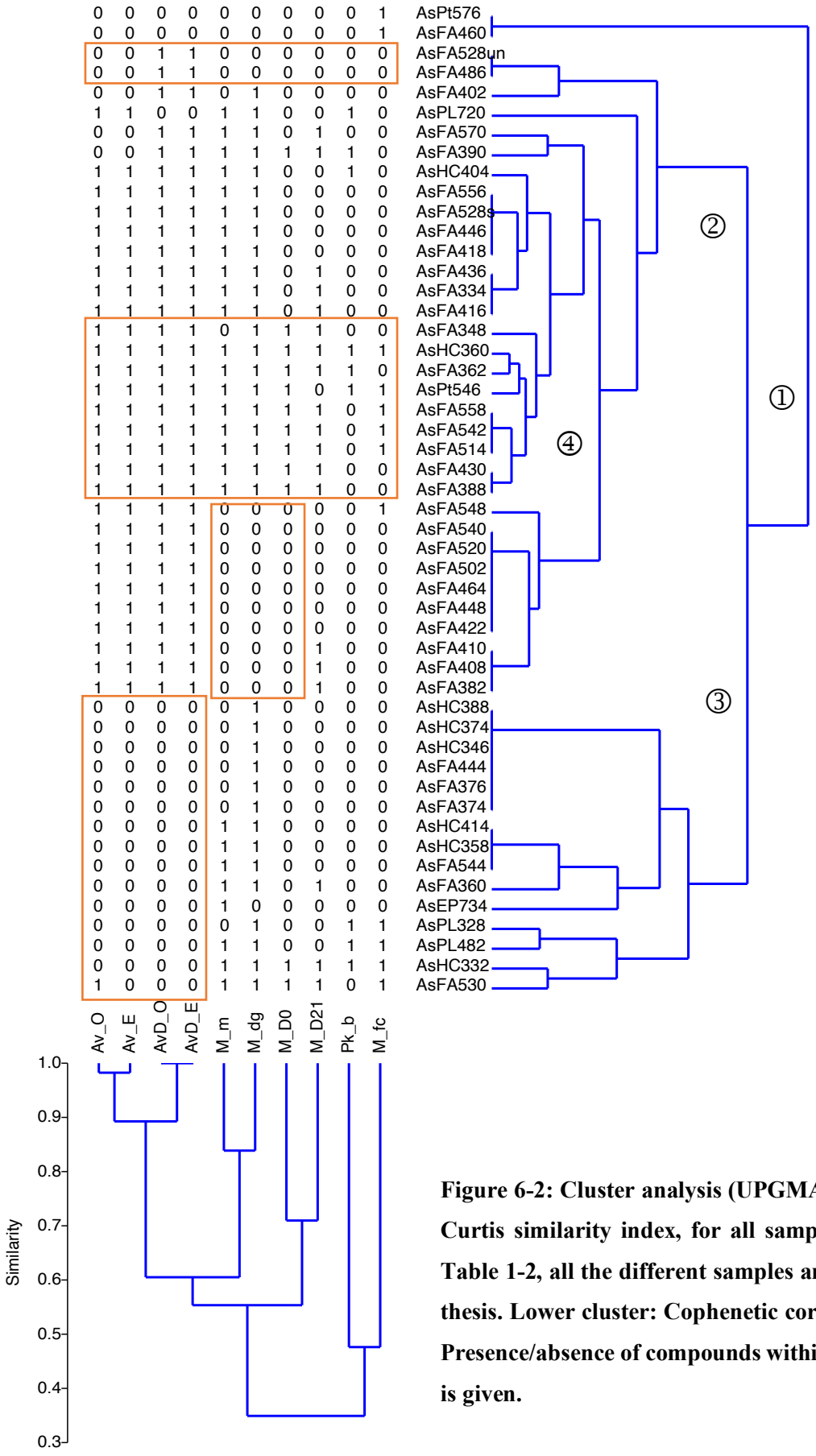


Figure 6-2: Cluster analysis (UPGMA) using Bray-Curtis similarity index, for all samples present in Table 1-2, all the different samples analysed on the thesis. Lower cluster: Cophenetic correlation: 0.94. Presence/absence of compounds within each sample is given.

Table 6-3: Review on the known arsenolipids and the biological matrices where they were found, compounds name are given by their abbreviation on lipid group and follow the molecular mass (ID). Samples are as follow: for Plankton Pk_b = plankton bulk sample and for *Mytilus galloprovincialis* as mussels samples: M_dg = digestive gland, M_mt = mantle and M_fc = faces (these found in this thesis work); for fish samples: H_m = muscle tissue of herring (*Clupea harengus*); H_c = fish roe of herring (*Clupea harengus*); T_m = Tuna muscle (*Thunus* sp.); for fish sub products: C_fm = fish meal from capelin (*Mallotus villosus*); C_o = oil extracted from capelin (*Mallotus villosus*); B_o = oil extracted from blue whiting (*Micromesistius poutassou*); CL_o = oil extracted from cod liver (*Gadus* sp.); and for macroalgae: H = Hiziki (*Hizikia fusiformis*); W = Wakame (*Undaria pinnatifida*); S = Sea belt (*Saccharina latissimi*); K = Kombu (*Saccharina japonica*). References are listed at the end of the table.

	P	Mussels samples			Fish samples			Fish sub products				Macroalgae				
ID	Pk_b	M_dg	M_mt	M_fc	H_m	H_c	T_m	C_fm	C_o	B_o	CL_o	H_a	W_a	S_a	K_a	Reference
AsFA264					X											9
AsFA276					X											9
AsFA302					X											9
AsFA304					X											9
AsFA316					X											9
AsFA328					X											9
AsFA334		X	X		X	X			X		X					1, 6, 9, 12, 15
AsFA348		X														15
AsFA356					X											9
AsFA360		X	X													15
AsFA362		X	X		X	X		X	X	X	X					1, 4, 6, 9, 7, 10, 12, 15
AsFA374		X														15
AsFA375		X														15
AsFA382		X														15
AsFA388		X	X		X	X			X	X	X					1, 6, 9, 10, 12, 15
AsFA390		X	X		X	X				X	X					1, 9, 10, 12, 15
AsFA402		X														15
AsFA408		X														15

	P	Mussels samples			Fish samples			Fish sub products				Macroalgae				
ID	Pk_b	M_dg	M_mt	M_fc	H_m	H_c	T_m	C_fm	C_o	B_o	CL_o	H_a	W_a	S_a	K_a	Reference
AsFA410		X														15
AsFA416		X	X													15
AsFA418		X	X								X					1
AsFA422														X		8
AsFA424														X		8
AsFA430		X	X													15
AsFA436		X	X		X	X		X	X	X	X					1, 4, 9, 7, 10, 12, 15
AsFA444		X														15
AsFA446	X	X	X													15
AsFA448					X	X		X	X							4, 9, 7, 12
AsFA460				X												15
AsFA464		X														15
AsFA486																15 (degradation)
AsFA502		X														15
AsFA514		X	X	X												15
AsFA520		X														15
AsFA528s		X	X													15
AsFA528un						X										12, 15 (degradation)
AsFA530		X	X	X												15
AsFA540		X														
AsFA542		X	X	X												15
AsFA544		X	X													15
AsFA548				X												15
AsFA556		X	X													15
AsFA558		X	X	X												15
AsFA570		X	X													15
AsHC304									X							7,

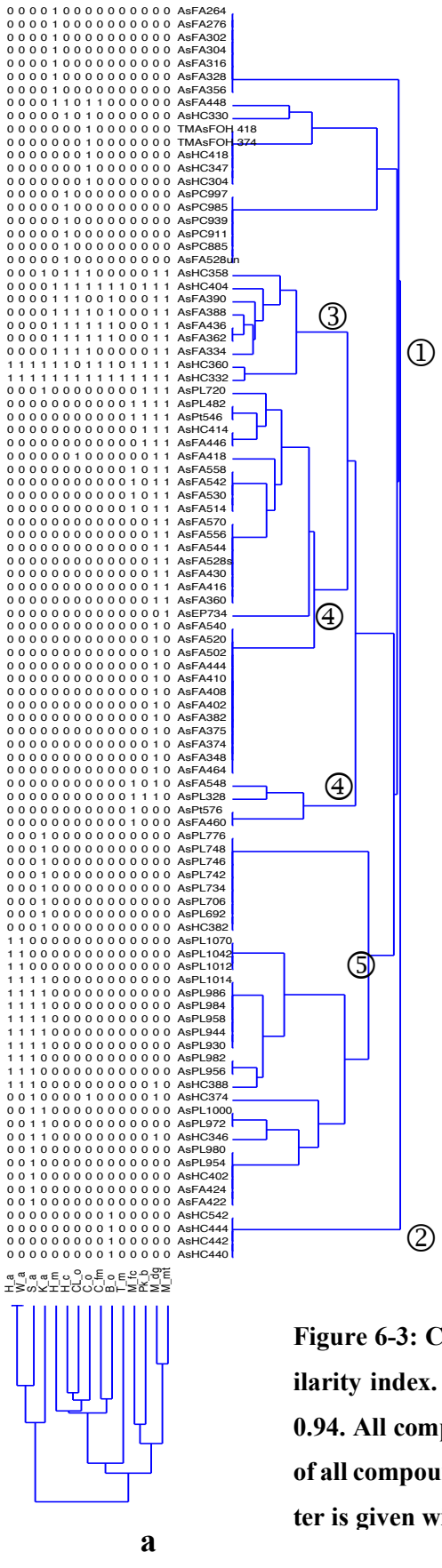
	P	Mussels samples			Fish samples			Fish sub products				Macroalgae				
ID	**Pk_b**	**M_dg**	**M_mt**	**M_fc**	**H_m**	**H_c**	**T_m**	**C_fm**	**C_o**	**B_o**	**CL_o**	**H_a**	**W_a**	**S_a**	**K_a**	**Reference**
AsHC330						X			X							7, 12
AsHC332	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15
AsHC346		X												X	X	8, 14, 15
AsHC347									X							7
AsHC358		X	X			X			X		X				X	6, 7, 12, 14, 15
AsHC360	X	X	X	X	X	X		X	X	X		X	X	X	X	2, 4, 5, 8, 7, 9, 10 11, 12, 14, 15
AsHC374		X							X					X		8, 7, 15
AsHC382															X	14
AsHC388		X										X	X	X		5, 8, 15
AsHC402														X		8
AsHC404	X	X	X		X	X	X	X	X	X	X					2, 3, 4, 6, 7, 9, 10,
AsHC414	X	X	X													15
AsHC418									X							7
AsHC440										X						10
AsHC442										X						10
AsHC444										X						10
AsHC542										X						10
TMAsFOH374									X							7
TMAsFOH418									X							7
AsPt546	X	X	X	X												13
AsPt576				X												15
AsEP734			X													15
AsPL328	X	X		X												15
AsPL482	X	X	X	X												15
AsPL692															X	14
AsPL706															X	14

	P	Mussels samples			Fish samples			Fish sub products				Macroalgae				
ID	Pk_b	M_dg	M_mt	M_fc	H_m	H_c	T_m	C_fm	C_o	B_o	CL_o	H_a	W_a	S_a	K_a	Reference
AsPL720	X	X	X												X	14, 15
AsPL734															X	14
AsPL742															X	14
AsPL746															X	14
AsPL748															X	14
AsPL776															X	14
AsPL930												X	X	X	X	5, 8, 14
AsPL944												X	X	X	X	5, 8, 14
AsPL954														X		8
AsPL956												X	X	X		5, 8
AsPL958												X	X	X	X	5, 8, 11, 14
AsPL972														X	X	8, 14
AsPL980														X		8
AsPL982												X	X	X		5, 8
AsPL984												X	X	X	X	5, 8, 14
AsPL986												X	X	X	X	5, 8, 11, 14
AsPL1000														X	X	8, 14
AsPL1012												X	X			5
AsPL1014												X	X	X	X	5, 8, 11, 14
AsPL1042												X	X			5, 11
AsPL1070												X	X			5, 11
AsPC885						X										12
AsPC911						X										12
AsPC939						X										12
AsPC985						X										12
AsPC997						X										12
AsPE1035																12 (in salmon cav-

	P	Mussels samples			Fish samples			Fish sub products				Macroalgae				
ID	**Pk_b**	**M_dg**	**M_mt**	**M_fc**	**H_m**	**H_c**	**T_m**	**C_fm**	**C_o**	**B_o**	**CL_o**	**H_a**	**W_a**	**S_a**	**K_a**	**Reference**
Sum	**9**	**43**	**27**	**12**	**16**	**17**	**2**	**6**	**16**	**11**	**9**	**14**	**14**	**20**	**21**	

References:

1 – (Rumpler et al. 2008)
2 – (Taleshi et al. 2008)
3 - (Taleshi et al. 2010)
4 – (Amayo et al. 2011)
5 – (García-Salgado et al. 2012)
6 – (Ruiz-Chancho et al. 2012)
7 – (Amayo et al. 2013)
8 – (Raab et al. 2013)
9 – (Lischka et al. 2013)
10 – (Taleshi et al. 2014a)
11 – (Glabonjat et al. 2014)
12 – (Viczek et al. 2016)
13 – (Glabonjat et al. 2017)
14 – (Yu et al. 2018)
15 – (Freitas et al. 2019) this thesis



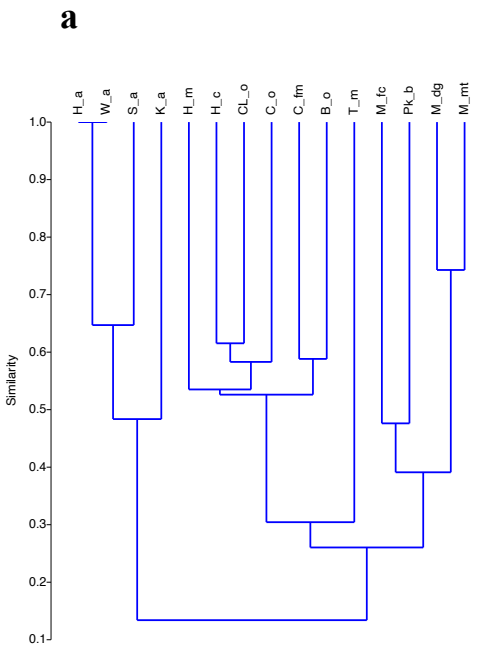


Figure 6-3: Cluster analysis (UPGMA) using Bray-Curtis similarity index. Lower cluster had a Cophenetic correlation of 0.94. All compounds described on Table 1-3. Presence/absence of all compound for biological matrices is given. The lower cluster is given with a better detail on a).

A total of 98 compounds are known for environmental samples, from mussels (*M. galloprovincialis*), mussels' faeces (*M. galloprovincialis*), plankton (bulk sample), two fish muscle (herring: *Clupea harengus* and Tuna: *Thunus* sp.), herring fish roe (*Clupea harengus*), commercial fish meal (capelin: *Mallotus villosus*), three fish oils (capelin: *Mallotus villosus*, blue whiting: *Micromesistius poutassou* and cod liver oil: *Gadus* sp.) and four macroalgae (Hijiki: *Hizikia fusiformis*, Wakame: *Undaria pinnatifida*, Sea belt: *Saccharina latissimi* and Kombu: *Saccharina japonica*). The *M. gallopronvincialis* digestive gland and mantle present the highest rank onto diversity of arsenolipid compounds, respectively having 43 and 27 different compounds; tuna muscle and capelin fish meal had the lowest diversity on arsenolipids; macroalgae had an intermedium diversity level. Cluster analysis evidences major dissimilarities between macroalgae and all the other samples (Figure 6-3). The rate of similarity among the macroalgae is about 50%, with Hijiki and Wakame being "identical". The tuna muscle (T_m) is set aside from this group as its diversity was very low with two compounds identified. Major three groups were: 1) the macroalga group (H_a, W_a, S_a and K_a), 2) the fish samples (muscle: H_m, caviar: H_c, fish meal: C_fm and oils CL_o; C_o; B_o), and the mussel group (M_dg, M_mt and M_fc), Note that herring caviar (H_c) was closer to fish oils extracts than to the same species fish muscle (H_m). On the two-way cluster analysis (Figure 6-3), top and bottom branches (branch 1 and 2) present the least similarities to other groups, as compounds remain exclusive to one sample, with the exception for AsFA448 and AsHC330, at the bottom branch (2) compounds were exclusive for blue whiting oil (B_o). Branch number 3 comprises the most prevalent compounds, with AsHC360 and AsHC332 being the most representative. Branches number 4 were defined by the compounds found on mussels' digestive gland and mantle (M_dg and M_mt). Compounds found in macroalgae define the branch 5, most compounds were arsenosugar-phospholipids. The macroalgae made one distinct group in terms of the arsenolipid diversity.

Conclusion

Spatial diversity is likely an influential factor of arsenolipid diversity on *Mytilus galloprovincialis*. Biologic matrix seems to be the major factor that influences the arsenolipids diversity. The biological samples, studied so far, are not representative of all marine diversity and definitive conclusions cannot be taken.

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Chapter 7

Final considerations

The main contribution

These were the first studies on mussels, *Mytilus galloprovincialis*, with a focus on the ecological and metabolic changes of arsenolipids.

M. galloprovincialis was shown here to be prolific on arsenolipids, with a total of 50 compounds being identified. Arsenolipids do present different profiles into different organs indicating that remobilization of these compounds is related to the organ. Arsenolipids presence in *M. galloprovincialis* was stipulated to be a conjugation from direct uptake from food sources, processes of bioaccumulation and, a less likely, *de novo* arsenolipid-genesis by molecular infidelity. A spatial factor was observed for the different diversity of compounds present.

Under starvation, *M. galloprovincialis* arsenolipids shown a similar variation to that stipulated to lipids non-containing-arsenic in starvation conditions, with compounds of shorter carbon chain being reduced and those of very long chain being preserved. Given some insight that some compounds might enrol on similar metabolic pathways. In *M. galloprovincialis* some arsenolipids were depurated trough faeces during the starvation, and some compounds were exclusive to faeces; inferring that microbiome might have a role on arsenolipids production. However, in *M. galloprovincialis* (microbiome) it had little to no influence on the overall arsenolipid profile on the organism. In artificial degradation conditions arsenolipids profile changes, with an increase of shorter compounds (polar); decomposition processes should be taken into consideration for arsenolipids environmental cycles.

Arsenolipids extraction methodology

The lipid extraction fine-tuning methodology, and consequently arsenolipid extraction, is no easy task and a standardized method for all biological matrix samples is not in place or advisable. In this work we state that an MTBE:MeOH extraction, is a more efficient method, reducing the Cl^- interference, with less steps and easier lab methodology, in comparison to the DCM:MeOH methodology. However, when samples had a high concentration of Cl^- (from NaCl) a pyridine extraction was preferred. The silica cleanup methodology is a good and standardized protocol, if the aim is to determine specific arsenolipid molecules, as it provides better results from ESMS analysis. However, we must not forget that this procedure provokes the loss of longer carbon chain arseno-fatty-acids (less polar compounds).

Summarizing, biological matrix and the aim of our study interferes with the selection of a method for arsenolipid extraction. The methodologies to be used need to be test previously to achieve the best desire outcome.

Arsenolipid diversity

The screening of arsenolipid diversity onto the different biological matrices is of pivotal importance, nor only due to the human health concern, but also for understanding their diversity and prevalence in the environment. This thesis work contributed with a total of 32 new compounds from a total of 50 identified arsenolipids compounds. A new group of arsenolipids has been identified within the group of non-arsenic containing ether-phospholipids. Most abundant compounds found in environmental matrices were AsHC360 and AsHC332. Arsenolipid diversity and abundance seems to be influence by the biological matrices. Being a recent field of study there is still not a representative array of species to make definitive statements.

New arsenolipid compounds are still being identified with new analytical methodologies still being develop, and the total extent of their diversity is still not completely unveiled.

Arsenolipids, ecological role

The distribution and abundance of arsenolipids differed between the *M. galloprovincialis* organs, mantle and digestive gland. Arsenolipids remobilization and accumulation in the organs might be related to their possible structural likeness to homologous lipids.

In starvation, arsenolipid content variations similar to non-arsenic lipids gave some insight for a similar metabolic pathway, and for process of remobilization of arsenolipids between organs. The

depuration of some arsenolipids, not all, was found trough faeces. Few arsenolipids were exclusive in faeces and lead to inferences on the microbiome influence onto the biogenesis of arsenolipids, independent form those present in the organism. Despite the evidences, this could not yet be proven and more research is needed.

In *M. galloprovincialis,* their role as a "normal" biological functional molecule is still not accurately define. However, it is likely that some compounds can be used by the cell or engage in some metabolic pathways (decrease of polar arsenolipids), and others can be stored for their functional role or have no cellular use and just been accumulated (preservation of less polar compounds). When arsenolipids were lowered in concentrations*, M. galloprovincialis* recovery was proven to be fast without any resilience on their accumulation and preservation. No cellular selection forward or against arsenolipid compounds seem to be in place, these compounds seemed to be uptake indiscriminately from environment, Also, it was noticeable the presence for compounds not present in plankton that prompts evidence for bioaccumulation of compounds present at undetectable concentrations in other food sources not analysed on this work.

Pin-point a common origin for arsenolipids for all organisms has proven to be a difficult task. We can, however, provide the most possible origins, 1) arsenolipid-genesis by molecular infidelity; 2) uptake from the environment, directly from food sources, 3) bioaccumulation of compounds from the environment present at untraceable concentrations and, at last 3) an origin from bacteria arsenolipid-genesis.

the future,

Arsenic and its organic forms are and will be a constant element into our environment, and as previously referred without any preventive measurement to arsenic remobilization, they will tend to increase in concentration. It is, therefore, important to understand the arsenolipids full behaviour in the cell, as a functional molecule, or just been bioaccumulated. Define the cellular burden from storing/using these compounds, known if the toxicological consequences for biota are relevant, and what are the ultimate consequences for human health. Additionally, is important to accurately determine the origin of these compounds, define their environmental cycle and their persistence into the environment.