Organophosphorus Compounds in the German Baltic Coastal Area

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Method Development and Analysis

Cumulative dissertation

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by M.Sc. Constantin Lohrer (né Recknagel)
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1. Referee:
Prof. Dr. Detlef E. Schulz-Bull
Leibniz Institute for Baltic Sea Research Warnemünde
Department: Marine Chemistry

2. Referee:
Prof. Dr. Jonas Bergquist
Uppsala University
Department: Chemistry – BMC, Analytical Chemistry

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List of Abbreviations

^{1}H-NMR	^{1}H-Nuclear magnetic resonance
^{13}C-MPn	^{13}C-Methylphosphonic acid
^{13}C-^{15}N-AMPA	^{13}C-^{15}N-Aminomethylphosphonic acid
^{14}C-CO_2	^{14}C-Carbon dioxide
2-AEP	2-Aminoethylphosphonic acid
2D-NMR	Two-dimensional nuclear magnetic resonance
^{31}P-NMR	^{31}P-Nuclear magnetic resonance
ACN	Acetonitrile
AMPA	Aminomethylphosphonic acid
ATP	Adenosine triphosphate
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
CE-UV	Capillary electrophoresis-ultraviolet detection
D_3-MPN	D_3-Methylphosphonic acid
DNA	Deoxyribonucleic acid
DW	Dry-weight
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia*
ESI	Electrospray ionisation
EU	European Union
FID	Flame ionization detection
FLD	Fluorescence detector
FMOC	Fluorenylmethyloxycarbonyl
FPD	Flame photometric detector
GC-FID	Gas chromatography flame ionization detection
GC-MS	Gas chromatography-mass spectrometry
h	Hours
H_2O	Water
HELCOM	Helsinki Commission
HILIC	Hydrophilic interaction chromatography
HMW-DOP	High molecular weight dissolved organic phosphorus
HPLC-FLD	High-performance liquid chromatography-fluorescence detector
HPLC-ICP-SF-MS	High-performance liquid chromatography inductively coupled plasma sector field mass spectrometry
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
IC-MS/MS	Ion-chromatography mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry

InsP	Inositolphosphate
$InsP_4$	Inositoltetrakisphosphate
$InsP_5$	Inositolpentakisphosphate
$InsP_6$	Inositolhexakisphosphate
LMW-DOP	Low molecular weight dissolved organic phosphorus
LOD	Limit-of-detection
MPn	Methylphosphonic acid
MRM	Multiple reaction monitoring
MTBSTFA	*N*-Methyl-*N*-*tert*-butyldimethylsilylfluoroacetamide
NaOH	Sodium hydroxide
$(NH_4)_2CO_3$	Ammonium carbonate
NH_4HCO_3	Ammonium bicarbonate
OPA-SH	*o*-Phthalaldehyde-thiol
Org-P	Organic phosphorus
P	Phosphorus
RE	Relative error
RSD	Relative standard deviation
SAX	Strong-anion exchange
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
t	Tons
TBDMS	*tert*-Butyldimethylsilyl
TMS	Trimethylsilyl
TOC	Total organic carbon
TOF-MS	Time-of-flight mass spectrometry
UV	Ultraviolet

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1. Aim of this Thesis

The significance of organophosphorus compounds for the aquatic environment is well known and was also shown for the Baltic Sea [1] and the German Baltic coastal Area [2]. However, there is still an open discussion on the role of single organophosphorus compounds. Among others, this is due to the lack of analytical methods for the quantification of these substances in the different aquatic samples (*e.g.* water or sediment). Several organophosphorus compounds (*e.g.* nucleotides, inositol phosphates, phosphonates) were mentioned which may account for a large share of the total organic phosphorus [3]. For some organophosphorus compounds like adenosine triphosphate (ATP) or phospholipids at least some data on their concentrations in the aquatic system "Baltic Sea" exist [4,5]. However, for other organophosphorus compound groups like inositol phosphates or natural phosphonates, there are no data available for this system and so their role remains unclear. Due to this gap of knowledge, these compound groups were chosen to be the subject of this thesis. This is because a literature review indicates that also these groups are part of the total organic phosphorus and may have important roles in the aquatic environment. As the two groups contain several substances, representatives of the inositol phosphates (*myo*-inositol hexakisphosphate) and the natural phosphonates (methylphosphonic acid and 2-aminoethylphosphonic acid) which are discussed to have important functions in the aquatic nature, were further studied [6,7]. The goal of this thesis was to detect these substances in samples collected from the German Baltic coastal area and to determine their fraction on the total organic phosphorus. For this, two objectives were defined. As there is a lack of suitable analytical methods for quantification of the target substances in the studied matrices, these methods had to be developed within this thesis (Objective 1). These methods should then be used to determine the target substances in the collected samples (Objective 2).

1.1. Objective 1: Methodological Aspects of the Target Compounds

Within this objective, suitable analytical methods for the determination of the target analytes had to be established. All these methods were based on chromatographic separation coupled to mass spectrometric detection. The main challenge was the fact that all of the target analytes show ionic character in the aquatic matrix which makes the separation difficult. As the three chosen compounds differ in their chemical behaviour, specific methods for each compound had to be developed.

For the analysis of inositol phosphates in sediment samples, an existing quantification method based on ion-chromatography tandem mass spectrometry (IC-MS/MS) had to be

optimized. Because previous studies of aquatic sediments showed that *myo*-inositol hexakisphosphate was the main representative of this group [8,9] and because it was shown that $InsP_6$ can be mobilized *e.g.* by aquatic cyanobacteria [10], this compound was chosen as an exemplary compound. Furthermore, it was mentioned that *myo*-$InsP_6$ accounts for a high proportion of phosphorus stored in plant seeds [11,12]. The goal was to optimize the recently published IC-MS/MS-method [8,9], especially in the reduction of matrix effects and to provide a reliable quantification method.

Liquid chromatography was also used for the analysis of 2-aminoethylphosphonic acid (2-AEP). As there was no current analytical method for the determination of 2-AEP described in the literature, the aim was to develop a method using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) for the quantification of this important phosphonate in saltwater samples. As it was shown during this study that saltwater has a strong influence on the chromatographic behaviour of the herbicide glyphosate, this phenomenon was studied for 2-AEP as well.

In the literature, several analytical methods for the determination of methylphosphonic acid (MPn) based on liquid as well as gas chromatography are described. However, there is a lack of methods which can analyse samples from the marine system. So, a suitable gas chromatographic method with several pre-treatment steps had to be established within this thesis. In the course of this method development, a special focus was made on the extraction of the ionic compound MPn from environmental water samples with special focus on high salt containing marine samples.

1.2. Objective 2: Quantification of the Target Compounds in Environmental Samples

It was the second objective to analyse and quantify the target analytes in environmental samples. With this, first insights on the importance of these compounds for the aquatic environment in the area of Rostock, Germany should be achieved. Until now there were no data for the concentration of the target analytes in this region ($InsP_6$) or even for aquatic systems at all (MPn). As a review of the corresponding literature indicated different environmental behaviour of the target analytes, different sample types (sediment, water) were studied for them within this thesis. There, *myo*-$InsP_6$ was quantified in several sediment samples collected at the river Warnow in the area of Rostock and the Darss-Zingst-Bodden chain. In contrast to this, MPn was studied in water samples because of its described role in the marine methane paradox.

2. Introduction

2.1. The Baltic Sea

The Baltic Sea is a semi-closed and shallow sea in Northern Europe. With a surface area of 420 000 km^2 it is smaller compared to other semi-closed seas like the Mediterranean Sea (2 500 000 km^2). However, it is one of the biggest brackish water systems in the world [13]. With only a narrow connection to the North Sea, it is a rather isolated water system. This is the reason why the salinity varies between 0 and 18, which is characteristic of brackish waters. Saltwater is introduced into the Baltic Sea during inflow events which can have a large influence on the ecological state as it happened *e.g.* at the end of 2014 with an estimated inflow volume of 198 km^3 [14]. Beside the saltwater input from the North Sea, freshwater is introduced by numerous rivers, with the river Neva being the largest one [15]. Per year, the rivers introduce a total amount of water of 479 km^3 and so they represent a main source of pollution. The high amount of freshwater results in the characteristic salinity gradient with higher salt concentrations in the southwestern part and low salt concentrations in the northern part.

The Baltic Sea is surrounded by nine countries (Denmark, Sweden, Finland, Russia, Estonia, Latvia, Lithuania, Poland and Germany) and the catchment area includes five additional countries (Norway, Belarus, Ukraine, Czech Republic, Slovakia). In total, the catchment area has a size of 1 720 000 km^2 with around 85 million inhabitants [13]. In Germany, four federal states are part of the catchment area, namely Mecklenburg-West Pomerania, Schleswig-Holstein, Brandenburg and Saxony with a total area of 29 000 km^2 [16]. 72 % of the German catchment area is in agricultural use which has a high influence on the phosphorus input into the Baltic Sea [17]. The catchment area of the whole Baltic Sea is characterized by a high proportion of agricultural use in the south compared to a low proportion in the north [18].

This causes high environmental pressure on the Baltic Sea. One example is the high eutrophication which affects over 95 % of the Baltic Sea [13]. One of the reasons for this is the high load of nutrients (*e.g.* phosphorus) coming from the land. Since people became aware of this problem, several actions were implemented. With this, it was possible to significantly reduce the amount of nutrient input from land since the 1990s (see Figure 1). However, the effects of the excessive input are still observed, *e.g.* by the high degree of eutrophication as already mentioned.

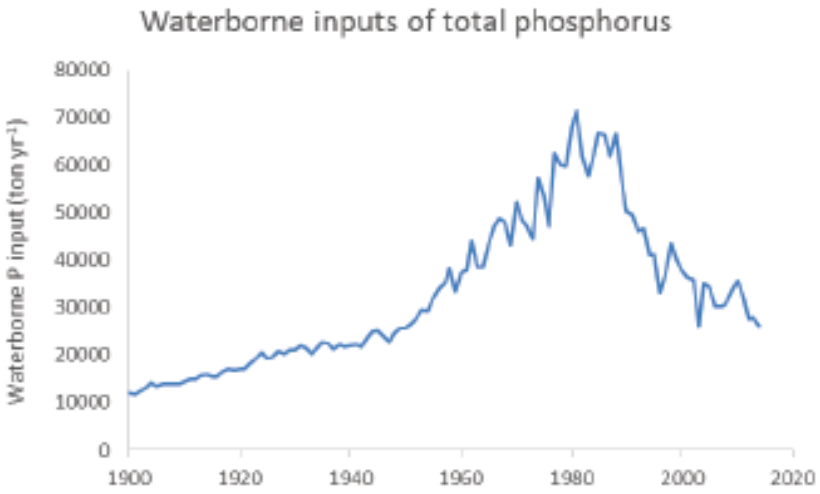

Figure 1: Development of the waterborne inputs of total phosphorus into the Baltic Sea since 1900. The figure is taken from the HELCOM report "State of the Baltic Sea" 2017 [13]

2.2. The Study Site — A German Baltic Coastal Area

The main sources of phosphorus in the Baltic Sea are rivers and so they are of high interest when evaluating the phosphorus input. Compared to the large European rivers entering the Baltic Sea such as the Neva or the Vistula, the main rivers in Germany are quite small, despite the river Odra, which enters the Baltic Sea in the cross-region of Germany and Poland. One of the German rivers entering the Baltic Sea is the Warnow which is located in Mecklenburg-West Pomerania (Figure 2). The catchment area of the Warnow is the second largest one in Mecklenburg-West Pomerania which discharges into the Baltic Sea [19]. In comparison with the Neva, the Warnow is much smaller as the mean annual runoff as well as the catchment area of the Neva is ~100 times higher [15,20]. However, with a phosphorus load of ~ 40 t/a (mean value for the time 2014-2017, [21]) the Warnow has a large influence on the situation of the Baltic coastal area of eastern Germany.

The other aquatic system, studied within this thesis, is the Darss-Zingst Bodden Chain, a highly eutrophic lagoon system at the German Baltic coastal area (Figure 2). The size of this coastal water system is 197 km^2 and the catchment area is 1578 km^2 [22]. As several connections between the Bodden and the Baltic Sea were closed in the past, the exchange of water is only possible in the eastern part of the chain. This limited water exchange, combined with an intensification of agriculture, led to very high eutrophication of the Bodden chain.

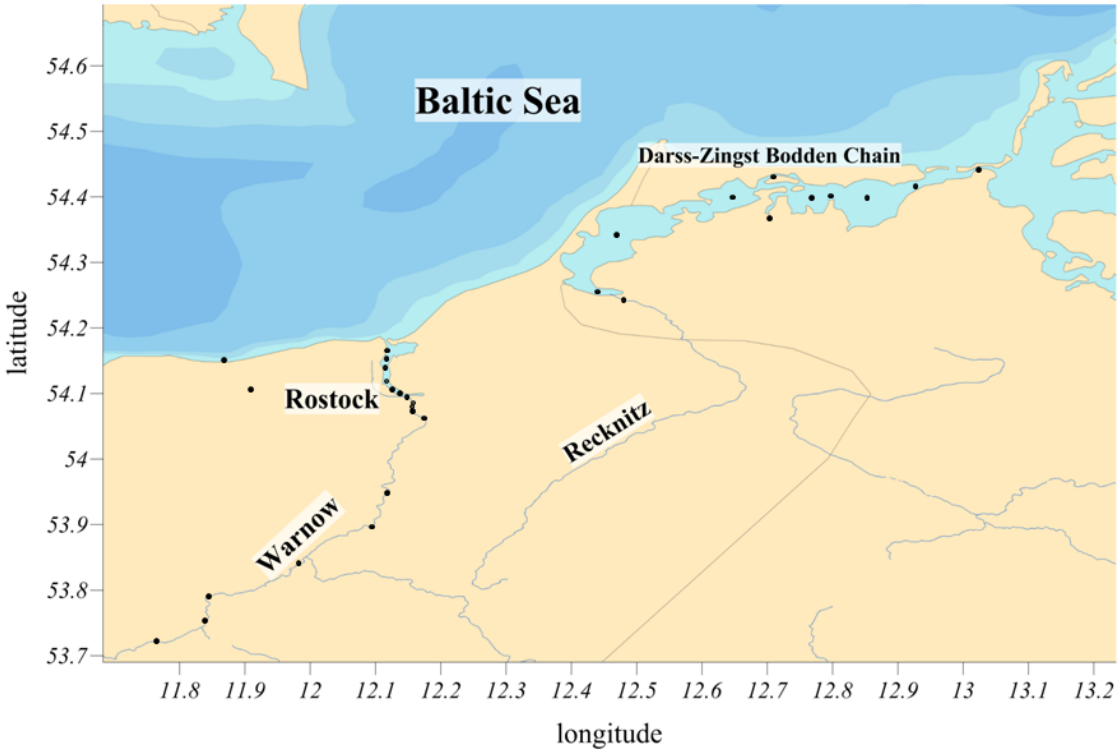

Figure 2: The study site of this thesis in Mecklenburg-West Pomerania, Germany. The sampling stations are marked with dots for all type of samples.

2.3. Phosphorus and its Organic Compounds — Occurrence, Classification and Importance

Phosphorus (P) is the 13th most abundant element in the earth crust where it exists in different forms of phosphorus oxides. Phosphorus is essential for all type of living systems with different chemical forms and functions. For example, phosphorus is part of bones or deoxyribonucleic acid (DNA) and the daily need of phosphorus for a human adult is between 1 and 1.2 g [23]. Furthermore, phosphorus belongs to the three main elements in agricultural fertilizers (besides nitrogen and potassium) and therefore it is a key factor in the worldwide feeding with worldwide consumption of mineral phosphorus fertilizers of around 40 million tons [24]. However, high amounts of phosphorus are also one of the reasons for ecological problems in aquatic systems when they are washed out from fields. A more detailed description of this topic is done in Section 2.4. As phosphorus is a non-substitutable resource, it became of special interest in the last years and was put on the list of the 27 critical raw materials in 2017 by the European Union (EU). This is because 100 % of the used phosphorus has to be imported from outside the EU [25]. Even there is still a discussion about the worldwide reserves-to-production ratio (2012: 324 years [24]), it is important to study the possibilities of recycling and saving phosphorus. In contrary to crude oil or natural gas, phosphorus is not lost by chemical conversion. However, phosphorus is lost when it is

transported to the sea and distributed finely, which makes it difficult and expensive to mine it again.

Numerous phosphorus compounds exist in nature, which can be divided into two groups, namely inorganic phosphorus compounds and organic phosphorus compounds, based on their chemical bonding status. Both groups contain a wide variety of different compounds which will be discussed in the following.

Inorganic phosphorus exists mainly in the form of orthophosphate in minerals with calcium phosphate minerals (apatite) being the most common ones in the earth crust. Around 300 different minerals of phosphorus are described, however, apatite makes up around 95 % [26]. Organic phosphorus names a group of chemical compounds containing phosphorus and carbon. Within this group, four main subgroups exist in nature, namely the orthophosphate monoesters (*e.g.* inositol phosphates), the orthophosphate diesters (*e.g.* DNA), the polyphosphate monoesters (*e.g.* adenosine triphosphate) and the phosphonates (*e.g.* methylphosphonic acid). Despite the natural phosphorus compounds, anthropogenic phosphorus compounds play an important role as well, *e.g.* as pesticides. An overview of different organic phosphorus compound groups is presented in Figure 3. One can see that in most of the natural compound groups the linkage between phosphorus and the organic rest of the molecule is via an oxygen-ester bond. The only exceptions are the phosphonates, as they are characterized by a direct carbon-phosphorus bond. This has a large influence on the chemical behaviour of these compounds, which will be discussed in more detail in Section 2.4.2.

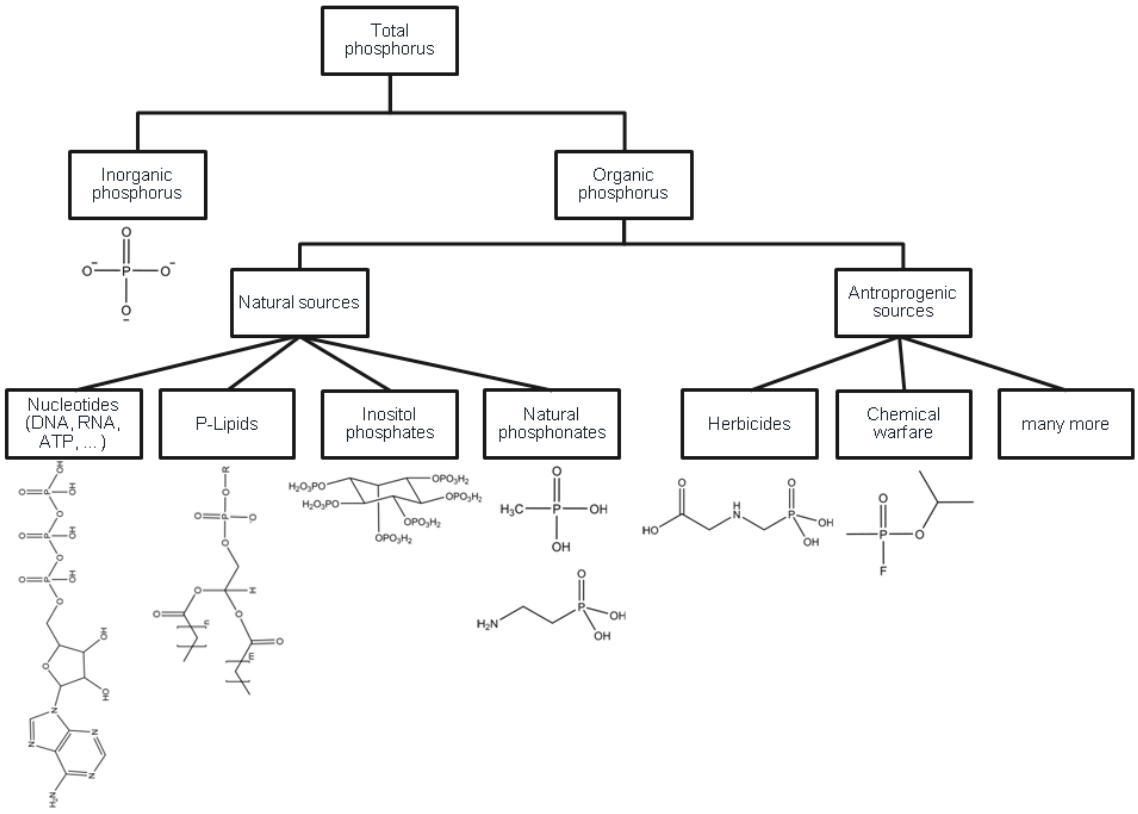

Figure 3: Classification of phosphorus compounds with a focus on organophosphorus compounds. Chemical structures for exemplary compounds are shown for natural and anthropogenic organic phosphorus compounds (adenosine triphosphate, phospholipids, *myo*-inositol hexakisphosphate, methylphosphonic acid and 2-aminoethylphosphonic acid; glyphosate; sarin).

2.4. Phosphorus as a Key Factor in the Aquatic Environment

In aquatic systems, phosphorus is an important nutrient as well and here it often represents the limiting factor of primary production. This is because nitrogen, another important nutrient, can be fixed from the atmosphere by some aquatic organisms, especially cyanobacteria [27]. This is not possible for phosphorus and so this element is often controlling the primary production. As there are no relevant natural gaseous phosphorus compounds, the atmospheric input of phosphorus into aquatic systems is relatively low compared to nitrogen. However, phosphorus is introduced via the atmosphere into aquatic systems as well. This is due to fine particles originated for example from wind erosion [28]. Svendsen *et al.*, 2015, calculated the portion of atmospheric phosphorus on total phosphorus introduced into the Baltic Sea in 2012 to be ~ 7 % (nitrogen: ~ 27 %) [29]. However, Berthold *et al.*, 2019, recently showed, that the atmospheric input of phosphorus into the Darss-Zingst-Bodden chain was in the same range as riverine input [30].

As already mentioned above, high nutrient-concentrations cause eutrophication resulting in ecological problems. Therefore, programs were started to reduce the amount of phosphorus in aquatic systems, *e.g.* the Baltic Sea Action Plan by the Helsinki Commission (HELCOM) in 2007. With this plan, it was possible to reduce the load of phosphorus by ~ 19 % in the years 2012 – 2014 compared to the reference period 1997-2003 [13]. However, still most of the areas of the Baltic Sea are not in good status with regard to phosphorus concentrations. A reduction of phosphorus concentrations was also achieved in the two aquatic systems studied within this thesis. In the case of the Warnow, the average total phosphorus concentration decreased from 8.23 µM (1980-1990) to 1.73 µM (2000-2010) at a sampling station close to the Baltic Sea. This was due to the reduction of the so-called “point sources” (*e.g.* sewage plants) [31]. However, there was still a load of 41 t P/year between 2001 and 2005. For the Darss-Zingst-Bodden chain, the situation is slightly different. A significant reduction of phosphorus introduced by the three main rivers entering the Bodden chain system (Saaler Bach, Barthe and Recknitz) of 75 % was achieved as well since the 1980s. In the case of the Barther Bodden, this led to a reduction of the annual average concentration of total phosphorus from 4.37 µM (1980/1990) to 3.02 µM (2000/2010).

In the aquatic environment, phosphorus exists in different forms. The total phosphorus can be divided into four groups: dissolved inorganic phosphorus, particulate inorganic phosphorus, dissolved organic phosphorus and particulate organic phosphorus. The existence of an organic phosphorus fraction was described for the first time by Matthews in 1916 [32]. The interest in organic phosphorus was aroused when it became clear that also this group, not only inorganic phosphorus, is acting as a phosphorus source during primary production. In the Baltic Proper, Nausch *et al.,* 2014, detected 0.21 – 0.32 µM dissolved organic phosphorus between 2008 and 2012 [33]. Organic phosphorus is introduced into the Baltic Sea by two main sources. One of them is the phytoplankton which transforms inorganic phosphorus into organic phosphorus during growth. When this particulate matter is dead, it can be dissolved, and so particulate organic phosphorus is transferred into dissolved organic P. Furthermore, organic phosphorus is introduced by rivers entering the Baltic Sea. Nausch *et al.*, 2017, recently studied the sub-basin of the river Warnow and showed that 14 – 46 % of the total phosphorus was organic phosphorus, either in the dissolved or in the particulate form [34].

The biological and ecological importance of organic phosphorus in the aquatic environment was neglected for many years and only inorganic phosphorus was expected to be bioavailable [11]. Stepanauskas *et al.*, 2002, were the first to show that a significant fraction of dissolved organic phosphorus in several rivers entering the Baltic Sea was bioavailable for bacterioplankton. However, in the studied rivers the fraction of bioavailable phosphorus differed strongly between 4 and 131 % [35]. Nausch and Nausch, 2006, conducted an

incubation experiment with water samples from four stations located in the Baltic Proper and investigated the change in the dissolved organic phosphorus concentration during a period between four and six days. The difference of the dissolved organic phosphorus concentration at the beginning and the end of the experiment was assumed to be the bioavailable P. As a result, a sustainable fraction of dissolved organic phosphorus was evaluated as bioavailable depending on the station and sampling date with the highest amount of around 60 % [1]. In another study, Qin *et al.*, 2015, investigated fractions of bioavailable phosphorus in wastewater effluents of two wastewater treatment plants in the United States [36]. Unlike Nausch and Nausch, 2006, they separated the dissolved organic phosphorus by chromatographic methods into a hydrophobic and a hydrophilic fraction followed by incubation experiments. Up to 75 % of the total phosphorus was found to be bioavailable in both samples. However, clear differences between the two sampling sites were shown regarding the ratio between hydrophobic and hydrophilic dissolved organic phosphorus fractions as well as the bioavailability in these subfractions. All of these studies show that a substantial fraction of dissolved organic phosphorus in different aquatic systems is bioavailable. However, the exact composition of these phosphorus pools is unclear. The fraction of bioavailable phosphorus on total organic phosphorus differed within the mentioned studies, which implies that the different organic phosphorus pools had different chemical properties and, thus, different ecological potential. This underlines the necessity to investigate the molecular composition of dissolved organic P.

Particular importance on the aquatic phosphorus cycle is also attached to the aquatic sediments. They can either act as a sink or, under certain conditions, as a phosphorus source. Phosphorus is buried for example by sinking particulate matter or by the formation of insoluble minerals like iron phosphates. However, under hypoxic conditions, phosphorus can be released again and so sediments act as a source in this case [37].

Baldwin, 2013, and Karl and Björkman, 2015, reviewed organophosphorus compounds in the aquatic environment and classified the predominant classes which are the orthophosphate monoesters, orthophosphate diesters, polyphosphate monoesters and phosphonates [3,11]. However, there are only rare data on the type and concentrations of individual natural organic phosphorus compounds in aquatic systems. Karl and Björkman, 2015, mentioned that less than half of the marine dissolved organic phosphorus pool is chemically characterized [3]. Therefore, the focus of this work was on inositol phosphates and phosphonates which are introduced in the following sections.

2.4.1. Inositol Phosphates in the Aquatic Environment

Inositol phosphates (InsP) are a group of compounds derived from the sugar alcohol inositol and belong to the phosphate monoesters. They exist with different degrees of phosphorylation and different isomeric structures. Thereby the *myo*-isomer is the most common form in nature with *myo*-inositol hexakisphosphate ($InsP_6$, also called phytic acid, see Figure 4) being the most abundant one [38]. In nature, InsPs take over different functions. For example, $InsP_6$ plays an important role as a phosphorus storage compound in plant seeds, where it can represent up to 75 ± 10 % of the total phosphorus [11,12]. During seed germination, it is degraded by the enzyme phytase to release phosphorus [39]. However, other InsPs take up different functions in biology as well. For example, inositol trisphosphate acts as a second messenger for the mobilisation of calcium(II) ions [40]. Within this process, the so-called phosphatidylinositols, a class of phospholipids, act as a preliminary stage [41]. More detailed information on this biochemical process can be found in a review by Irvine and Schell, 2001 [42].

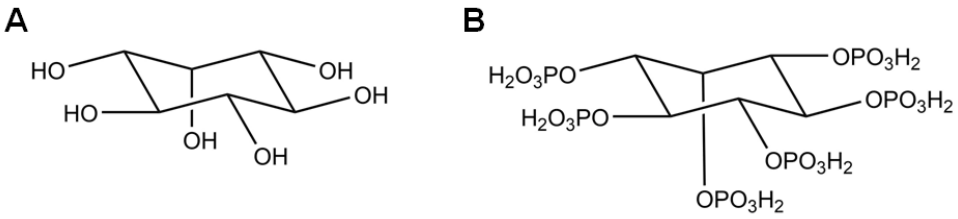

Figure 4: Chemical structures of *myo*-inositol (A) and *myo*-inositol hexakisphosphate (B)

Knowledge regarding the introduction of inositol phosphates into the aquatic environment is rather limited. Several sources for InsP are discussed and were presented in a conceptual cycle by Turner *et al.*, 2002 (see Figure 5) [38]. Based on the importance of InsP, especially $InsP_6$, in the terrestrial environment, terrestrial sources like soils and manures from monogastric animals are discussed as a substantial part of the total InsP-input [6]. Furthermore, there are also some aquatic plants which can synthesise $InsP_6$ and so they might be a source of aquatic $InsP_6$ and other InsPs as well. This synthesis was described *e.g.* for the slime mould *Dictyostelium* [43] and the floating duckweed *Spirodela polyrhiza* L. [44].

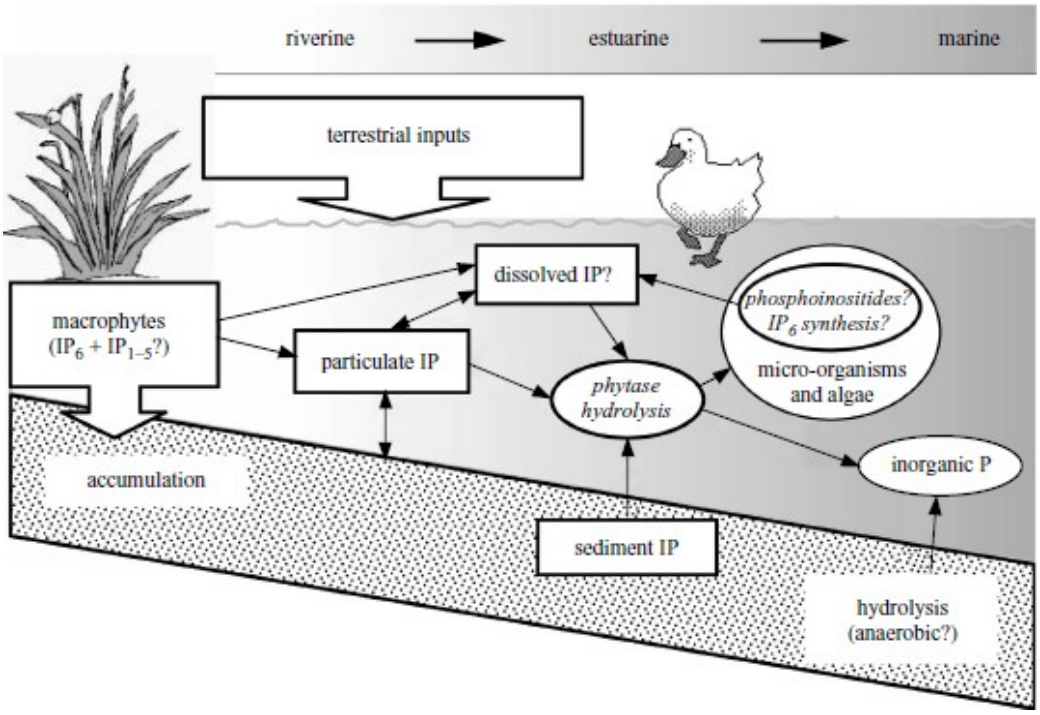

Figure 5: Conceptual biogeochemical cycle of InsPs (here named IP) in the aquatic environment [38] (Republished with permission of Royal Society (Great Britain, from Inositols in the environment, Turner *et al.*, Vol: 357, Issue: 1420, Year: 2002; permission conveyed through Copyright Clearance Center, Inc.)

It was discussed by Turner *et al*, 2002, that InsPs make up a substantial proportion of the total phosphorus compounds in aquatic sediments [38]. However, published data on the amount of InsP in sediments show a more differentiated picture on this (see Table 1). Suzumura and Kamatani, 1995, detected $InsP_6$-amounts of low µg InsP-P/g in sediments from the coastal area of Tokyo Bay representing only ~1 % of the total organic phosphorus [45]. Despite that, Turner and Weckström, 2009, quantified $InsP_6$ in a range of 10 – 77 µg InsP-P/g in a sediment core from an embayment in Helsinki, Finland. In this case, the fraction of the total organic phosphorus was between 7 and 24 %. Interestingly, such different InsP-conditions were detected in one single sediment core. This might be due to different ecological situations in different periods [46]. The highest amounts of $InsP_6$ in sediments were reported by Jørgensen *et al.*, 2011, who studied the $InsP_6$-concentrations in 15 Danish lakes. In some of them, they found $InsP_6$-concentrations of up to 320 µg InsP-P/g which made up to 90 % of the total organic P. However, in other lakes $InsP_6$ was not detected at all [47]. These three studies show that the abundance of InsP highly differs in different aquatic systems.

Table 1: Reported data for inositol phosphates in aquatic sediments ([a] Average of three different extraction and purification methods; [b] Fraction on the sum of monoester and diester P)

Publication	InsP [µg InsP-P/g DW]	Sediment origin	Method	%Org-P
Suzumura and Kamatani, 1995 [45]	0.3 – 3.1 2.2 – 20.5	Coastal area River and estuary	Hypobromite oxidation, anion-exchange chromatography, GC	0.1 – 1.2 1.2 – 10.2
De Groot and Golterman, 1993 [48]	24 – 149	Marshes, drainage ditch, lake	Enzymatic degradation, molybdenum-blue method	8.3 – 26.9
Jørgensen *et al.*, 2011 [47]	30 – 320	Lake (15 lakes)	^{31}P-NMR	12.5 – 90.9[b]
Paraskova *et al.*, 2015 [8]	73.3	Lake	HPLC-ESI-MS/MS	Not determined
Turner and Weckström, 2009 [46]	10.6 – 77.0	Embayment	^{31}P-NMR	7 – 24.3
Weimer and Armstrong, 1977 [49]	46[a]	Lake	Ion-exchange chromatography	11.4

For some time there has been the commonly held view that $InsP_6$ occurs only in the aquatic sediments and not as a dissolved compound in the water column [6]. However, some studies reported data on $InsP_6$ in water samples from rivers, lakes and estuaries [50-54]. One has to notice, that in none of these studies $InsP_6$ was detected directly. This shows that the dynamics of InsP in the aquatic environment are more complicated and further research is necessary. As the current literature still indicates that the sediment has a higher importance in the InsP-cycle than the water column, the focus in this thesis was on sediments.

2.4.2. Phosphonates in the Aquatic Environment

Van Mooy *et al.*, 2015, reported about the importance of a special group of dissolved organic phosphorus, the phosphonates [7]. This group is part of intensive environmental research as its origin and function are still unknown in many cases. Harbouring a C-P bond, their biochemical utilization requires a higher demand of energy for cleavage. Furthermore, another enzymatic machinery is required for this compared to the oxygen-containing C-O-P phosphate group which is the major phosphorus group for current life with respect to energy production or informational storage [55]. Organosphosphonate compounds are considered as ancient molecules, which were *e.g.* detected on the Murchison meteorite indicating their prebiotic origin [56]. Considering the reducing atmosphere of early earth life, organophosphonates are assumed to be the previous form of phosphate in this form of life.

However, even though contemporary life depends on phosphate esters, the phosphonates did not vanish. They might be still advantageous to life in some cases and so they are still present in the environment. The carbon-phosphorus bond is resistant both to the action of phosphatases and to chemical hydrolysis [55]. Therefore, as part of structural elements such as membranes, the presence of organophosphonates might confer higher resistance to microbial attack. They are also products of secondary metabolism such as the antibiotic fosfomycin isolated from the species Streptomyces [55].

There are several phosphonates from natural sources which exist in aquatic systems [57]. Due to van Mooy *et al.*, 2015, two of these substances, which may have a large impact on the marine environment, are methylphosphonic acid (MPn) and 2-aminoethylphosphonic acid (2-AEP) (see Figure 6).

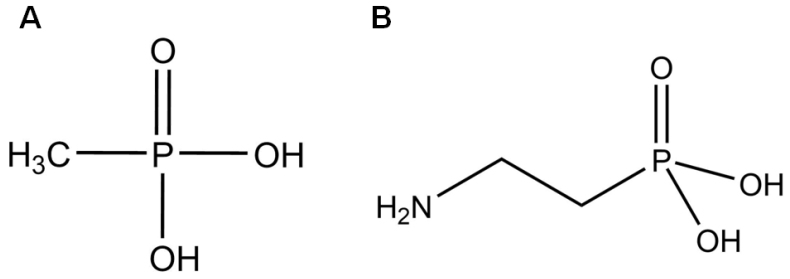

Figure 6: Phosphonates studied in this thesis. A: Methylphosphonic acid; B: 2-Aminoethylphosphonic acid

The ability to utilize 2-AEP as a phosphorus source was shown for aquatic bacteria, for example by Chin *et al.*, 2018 [58]. They could even show that 2-AEP may also act as a nitrogen source. In 1959, 2-AEP was the first phosphonate detected in living nature [59]. Since then it was found in a lot of different animals and plants as a free molecule or as part of bigger molecules like structural lipids [60]. In the marine environment, 2-AEP was detected

for example in different species of phytoplankton where it represented the main aminophosphonic acid and made up to 3.2 % of the total phosphorus [61]. An overview of several organisms in which 2-AEP was identified, is given by Hildebrand and Henderson, 1983 [60]. Recently, Shinohara *et al.*, 2018 detected 2-AEP in suspended particles originated from an ultraoligotrophic lake in Japan. However, based on their measurements using nuclear magnetic resonance (NMR), they concluded that the concentration of 2-AEP in the analysed samples was extremely low [62].

Methylphosphonic acid might have different roles in the marine environment. Many publications mention MPn as the final product of the hydrolysis of different chemical warfare agents like the nerve agents sarin or soman [63]. Furthermore, it was shown in several publications that microbial degradation of MPn resulted in the release of methane and so MPn was discussed as one explanation for the marine methane paradox [64-67]. In a recent publication Repeta *et al.*, 2016 showed that dissolved organic matter from the North Pacific contains polysaccharide, which incorporated MPn-esters. Within their study, they incubated seawater samples with the purified polysaccharides and detected an increase in the methane concentration. This led them to the assumption, that the degradation of these esters might be an explanation for the mentioned marine methane paradox [68]. Within this process, phosphonate-P is utilized by breaking the carbon-phosphorus bond releasing methane as a side product (see reviews by Quinn *et al.*, 2007 [69] and McGrath *et al.*, 2013 [55]). For this cleavage, two pathways exist in bacteria, namely the C-P-lyase and the phosphonatase pathway [70] which are also present in aquatic organisms [71].

As a source of MPn in marine waters, microorganisms like the cyanobacteria *Trichodesmium* are discussed as well [72]. Recently Born *et al.*, 2017, showed that the enzyme methylphosphonate synthase produces methylphosphonic acid. Markers of this enzyme were found in several marine microbes and so this supports the hypothesis that there is biosynthesis of MPn in the marine environment [73].

Until now, only the total amount of phosphonates in the marine environment was studied and no data on the concentrations of single natural phosphonates exist. For example, Clark *et al.*, 1999, reported that the share of phosphonates on the total high molecular weight dissolved organic phosphorus pool (HMW-DOP, > 1000 DA) was ~ 25 % in samples from the Pacific Ocean [74]. With an improved method, Young and Ingall, 2010, isolated the low molecular weight dissolved organic phosphorus (LMW-DOP) as well and detected a share of phosphonates on this between 5 and 10 % [75]. Despite the dissolved organic phosphorus Pool, also the particulate organic phosphorus pool was analysed for the occurrence of phosphonates. For example, sinking particles were collected across a redox boundary by Benitez-Nelson *et al.*, 2004 [76]. The share of phosphonates on the total particulate phosphorus, in that case, ranged from 3 – 18 %.

2.5. Analytical Methods for the Quantification of Phosphorus Compounds

For many scientific questions, it is sufficient to determine phosphorus in several groups (*e.g.* reactive phosphorus, organic phosphorus) as bulk parameters which is often done with the molybdenum-blue method [77-79]. However, in some cases a more specific analysis of the total phosphorus pool is necessary. For example, organic phosphorus compounds show differences in their bioavailability and so a bulk analysis of total organic phosphorus is not enough to assess the whole potential of this pool. To answer this question, compound-specific methods can comprise more detailed information. Several possibilities are mentioned in the literature, but there is still a lack of analytical methods for a lot of important phosphorus compounds (see *e.g.* reviews by Baldwin, 2013 [11] or Karl and Björkman, 2015 [3]). So, it is the goal of this thesis to provide new and improved ways for determination of selected organic phosphorus compounds in samples derived from the aquatic environment.

Particularly for samples from the marine environment, a preconcentration step is necessary when analysing organic phosphorus compounds. Due to the matrix, this is a challenging task as it contains high amounts of salt (*e.g.* oceanic water: 35 g/L). The main organic phosphorus compounds mentioned above are highly polar or even ionic compounds in seawater and so they cannot be extracted with liquid-liquid extraction using a non-polar solvent. Therefore, preconcentration or extraction of the corresponding phosphorus compound from the water matrix might be difficult as it contains a high amount of ions which have to be separated from the target compounds. In freshwater systems, the matrix effects based on the salt content decrease and so analysis might be easier. However, concentrations of *e.g.* dissolved organic carbon are often higher in these systems which also might exert matrix effects. As the salt concentration in the Baltic Sea varies from south to north as well as in the depth with higher salt concentrations in the deeper water layers, no general comment can be given on the applicability of published freshwater methods for this special aquatic system. Furthermore, concentrations of organic phosphorus compounds in water samples originated from rivers or the Baltic Sea are expected to be higher than in the open ocean and so less sample volume has to be concentrated to reach the analytical sensitivity of the methods.

An overview of published methods for the analysis of selected phosphorus compounds is given in several reviews, *e.g.* by Baldwin, 2013 [11], Karl and Björkman, 2015 [3] or Worsfold *et al.*, 2008 [80]. There, the most common technique for the analysis of environmental samples is ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR). For example, this technique was used by Cade-Menun *et al.*, 2006, to characterize the phosphorus composition of water samples [81] or by Turner *et al.*, 2009, to quantify the amount of inositol

phosphates in samples of different origin [46,82-84]. One of the reasons that ^{31}P-NMR is the most widely used technique for studying organic phosphorus in environmental samples is the fact, that it is much more compound-specific than other techniques described before. As an illustration, it is possible to quantify phosphonates and inositol phosphates side by side. The second group can even be characterized by its different isomers [83]. A review on the use of ^{31}P-NMR for the analysis of phosphorus in agricultural and environmental samples was given by Cade-Menun, 2005 [85]. However, ^{31}P-NMR has several disadvantages as, *e.g.*, it requires a long measuring time per sample (~16 – 20 h). Furthermore, it is characterized by low sensitivity (LOD ~ 0.05 mg/mL) and so an intensive sample preconcentration is necessary.

It was mentioned, that there is a lack of knowledge in the characterization of the marine dissolved organic phosphorus pool. To overcome this, investigation of the molecular composition of organic phosphorus is necessary and therefore suitable analytical techniques have to be developed in a first step. For this, there are different methodological possibilities available which will be discussed in the following in more detail for the target compounds of this thesis.

2.5.1. Analytical Determination of Inositol Phosphates

Analysis of inositol phosphates is most widely done using ^{31}P-NMR, especially when environmental samples like water [81], sediments [46,47,86] or soils [82,83] are studied. This is because this technique enables the direct determination of the different inositol phosphates without separation or conversion. As shown *e.g.* by Turner *et al.*, 2012, or Hill and Cade-Menun, 2009, it is even possible to differentiate between the different isomers of InsP [83,87].

Another methodological set-up for the determination of InsPs is the use of the molybdenum-blue method. As InsP will not react directly with the used reagent, one has to hydrolyse it to molybdate-reactive phosphorus before. As an example, enzymatic hydrolysis using the enzyme phytase was used for this when analysing the amount of $InsP_6$ in water samples [88,89]. However, commercial phytase hydrolyses other organophosphorus compounds as well and so InsP can be overestimated. Another technique to transfer InsP into molybdate-reactive phosphorus is the chemical conversion, *e.g.* by alkaline persulphate like it was used by McKelvie *et al.*, 1993 [51]. Other methods based on this conversion were reviewed by Turner *et al.*, 2002 and McKelvie, 2007 [6,38].

To gain more insights into the composition of the different inositol phosphates, separation from other matrix components is necessary. For this, chromatographic methods were used for a long time. Numerous studies were published using size-exclusion [51] or ion-exchange

chromatography for analysing InsP in different matrices like food, biological samples or soil [90-94]. Furthermore, this technique was used to analyse the content of InsP in environmental water and sediment samples, however, there is a lower number of publications compared to the matrices mentioned before [8,9,49,95,96]. The critical point when using these methods is the detection of InsP which should be as sensitive as possible when low amounts in environmental samples are analysed. As InsPs are not directly detectable with spectrophotometric methods like colourimetry or fluorimetry, post-column reactions were used [93,94,97]. However, in some cases, these methods are not sufficiently sensitive to analyse the InsP-concentrations present in the aquatic environment. Therefore, methods based on mass spectrometric detection were developed in the last years (see Table 2). Except for the method of March *et al.*, 2001, who analysed derivatized inositol after enzymatic degradation of $InsP_6$ by gas chromatography-mass spectrometry (GC-MS), all these methods are based on liquid chromatography which is due to the chemical behaviour of the inositol phosphates. There three different separation modes were used, namely anion-exchange, hydrophilic interaction (HILIC) and reversed-phase based on the ion-pairing mode. The sensitivity of these methods is in the low µmol/L-level, however, the values for the limit-of-detection reflect the instrumental sensitivity. These values are achieved by injection of standard substances not taking into account the effect of the different sample matrices. Samples collected from the aquatic environment were only studied by the group of Per Sjöberg (Paraskova *et al.*, 2015 [8] and Sjöberg *et al.*, 2016 [9]).

Table 2: Analytical methods based on mass spectrometry for the quantitative analysis of inositol phosphates

Publication	Separation mode	Detection method	Limit-of-detection (LOD)	Matrix
Paraskova *et al*, 2015. [8]	Anion-exchange	HPLC-MS/MS	0.03 µmol/L (injected solution)	Sediments
Sjöberg *et al.*, 2016 [9]	Anion-exchange (three different columns)	HPLC-MS/MS	0.03-0.16 µmol/L (37-99 ng P/g DW), (injected solution)	Sediments, soil manure
March *et al.*, 2001 [98]	Gas chromatography (derivatized inositol)	GC-MS	0.01 µmol/L (injected solution)	Biological samples
Liu *et al*, 2009. [91]	Anion-exchange	HPLC-MS/MS	0.0625 µmol/L (41.25 µg/L), (injected	Biological samples

			solution)	
Ito *et al.*, 2018 [99]	Hydrophilic interaction (HILIC)	HPLC-MS/MS	0.04-0.10 µmol/L (injected solution)	Biological samples
Rougemont *et al*, 2016. [100]	Reversed-phase (ion-pair mode)	HPLC-MS/MS	Not given	Whole blood
Tur *et al.*, 2013 [101]	Reversed-phase (ion-pair mode)	HPLC-MS/MS	0.05-0.12 µmol/L (injected solution)	Plasma samples
Lee and Mitchell, 2019 [102]	Anion-exchange	HPLC-MS/MS	0.1-0.3 µmol/L (injected solution)	Raw almonds
Duong *et al.*, 2017 [103]	Anion-exchange	HPLC-MS/MS	1 µmol/L (injected solution)	Almonds
Helfrich and Bettmer, 2004 [104]	Reversed-phase (ion-pair mode)	HPLC-ICP-SF-MS	230 ng/g	Food samples
Rugova *et al.*, 2014 [105]	Anion-exchange	HPLC-ICP-MS	0.3 µmol/L (injected solution)	Soil solution, plant extract
Zhang *et al.*, 2017 [106]	Reversed-phase (ion-pair mode)	HPLC-MS/MS	0.5-5 µmol/L (injected solution)	Plant samples

2.5.2. Analytical Determination of Natural Phosphonates

Natural phosphonates in the aquatic environment were often determined as a compound group using different analytical techniques. Again, one possibility is the use of ^{31}P-NMR which for example was done by Clark *et al.*, 1999 when analysing the fraction of phosphonates in the high molecular weight fraction of the oceanic dissolved organic matter. In their study, the HMW-fraction was obtained by tangential-flow ultrafiltration [74]. In a more recent study, combined electrodialysis/reverse osmosis was used to isolate the HMW- as well as LMW-fraction followed by ^{31}P-NMR detection [75]. Due to the different chemical bonding situation of the phosphorus atom in phosphonates compared to phosphates, a differentiation in the NMR-spectra is possible as the chemical shift is highly different [107]. Another set-up to quantify total phosphonates in seawater was used by Cembella and Antia, 1986. They implemented chemical fractionation with acid- and enzymatic hydrolysis (alkaline

phosphatase) and stated that the difference between this quantified phosphorus fraction and the total phosphorus is phosphonate-based phosphorus [108]. However, this set-up seems to be problematic as one has to take care that all other phosphorus compounds in the sample are hydrolysed. Otherwise, the content of phosphonate-P would be overestimated.

2.5.2.1. Analytical Methods for the Quantification of Methylphosphonic Acid

As already described in Section 2.4.2, methylphosphonic acid is suspected to play an important role in the marine phosphorus cycle. However, there are no data available regarding actual concentrations of MPn in oceanic as well as in other aquatic systems. Though, there are several methods described in the literature related to the analysis of MPn in water samples (see Table 3). The context of all these methods was the analysis of chemical warfare agents. This is because MPn is the degradation product of several chemical warfare agents belonging to the group of organophosphorus nerve agents (*e.g.* sarin).

Table 3: Methodical aspects of selected publications regarding the analysis of MPn in environmental water samples

Publication	MPn preconcentration	Detection method	Limit-of-detection (LOD)	Matrix
Kataoka *et al.*, 2000 [109]	Macroporous strong anion-exchange resin	GC-MS	0.18 µg/g (soil)	*e.g.* seawater, soil
Nassar *et al.*, 1999 [110]	-	CE-UV	1-2 µg/L (art. seawater)	*e.g.* estuarine water, artificial seawater
Baygildiev *et al.*, 2017 [111]	-	HPLC-MS/MS	10 µg/L	Natural water, groundwater, tap water
Rodin *et al.*, 2015 [112]	Rotary evaporation	HPLC-MS/MS	0.1 µg/L	Well water, river water, tap water
Singh *et al.*, 2015 [113]	Magnetic strong anion-exchange resin	GC-MS	0.1 µg/L	Rainwater, tap water, muddy water
Maddah *et al.*, 2010 [114]	Anion-exchange resin (SPE)	GC-FID	0.5 µg/L	Doubly-distilled deionized water

Analytical separation of MPn is mainly done by gas and liquid chromatography followed by mass spectrometric detection in most cases. However, other methods were mentioned in the literature as well. For example, capillary electrophoresis with different detectors [110,115-117] or ^{1}H- and ^{31}P-NMR spectroscopy [118] were used. Recently, Repeta *et al.*, 2016, characterized dissolved organic matter from the North Pacific by ^{31}P-NMR and high-resolution mass spectrometry and were able to identify methylphosphonate esters of polysaccharides [68].

As already mentioned, most gas chromatographic methods use mass spectrometry for the detection of MPn. However, other detectors like flame photometric detection (FPD) and flame ionization detection (FID) were used as well [119]. Regardless of the detection mode, all these methods have in common that MPn has to be derivatised before gas chromatographic analysis which is due to the non-volatile behaviour of MPn. There are several compound groups to which MPn can be derivatised to make it suitable for GC analysis, namely methyl esters, silyl esters and pentafluorobenzyl esters (see review by Black and Muir, 2003 [63]).

As the method developed within this thesis uses silylation for MPn-derivatisation, only this compound group will be briefly described here. The silylation is mostly done with different reagents reacting to trimethylsilyl (TMS) or *tert*-butyldimethylsilyl (TBDMS) esters [63]. A comparison of different reagents for the TBDMS-esterification was done by Purdon *et al.*, 1989, using different reaction conditions as well as different detectors [119]. The use of GC-MS for the analysis of silylated phosphonic acids is described in several publications with *N*-Methyl-*N*-*tert*-butyldimethylsilylfluoroacetamide (MTBSTFA) [120-125] as well as *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) [113,126,127] used for derivatisation.

Furthermore, MPn can be analysed by methods based on liquid chromatography. Despite to GC, derivatisation of MPn is not necessary and most published methods do not use this additional sample preparation step [111,128-137]. However, derivatisation can improve the performance of the analytical method in some cases and so it was used in some publications as well [112,138]. Thereby separation was done by using different reversed-phase as well as normal-phase columns. For detection, different MS detectors like time-of-flight mass spectrometry (TOF-MS) [128-130], tandem MS [112,131-136] and inductively coupled plasma mass spectrometry (ICP-MS) [137] were used in general. Other detection methods like ultra-violet (UV) or conductivity detection were used as well but without high significance [139,140].

One important aspect of all analytical methods is the sample preparation. Because the different silylation reactions are sensitive to water, it is necessary to separate the analytes from the water matrix or to remove the water. As MPn exists in its ionic form in environmental water samples, it is not possible to do liquid-liquid extraction with nonpolar organic solvents.

Several possibilities on how to do this separation are described in the literature. Some methods use a rotary evaporator or a stream of nitrogen to dry water- [112,124,125] or human-urine-samples [141]. However, as the authors used these techniques only for a small volume of spiked samples (~ 1 mL), they are not suitable for real water samples especially when water samples with a high salt content are analysed. Another technique which is intensively used for separating MPn from the sample matrix is solid-phase extraction (SPE). Thereby different sorbent materials were used with strong-anion exchange materials (SAX) being the most important ones. However, when seawater samples are analysed, the high ionic strength of the samples can lower the recovery of the SPE, because the matrix ions compete with the target ions on the active centre of the SPE-material. Furthermore, the ionic sample matrix can elute the target ions from the SPE-material. Though, Kataoka *et al.*, 2000, investigated the use of a SAX-material for the analysis of different spiked samples, *e.g.* a seawater matrix, and achieved a recovery for MPn of ~ 60 % at a sample volume of 2 mL [109]. This SPE-material was also used for the analysis of aqueous soil extracts [121,123] and human plasma as well as urine [122]. Sega *et al.*, 1997, compared two anion exchange columns, aminopropyl and a quaternary amine SPE-column, for their recovery when analysing distilled and artificial groundwater. Thereby the highest recovery for MPn achieved in groundwater samples was ~ 50 % [142]. Wang *et al.*, 2005, investigated the recovery of four different SPE-materials when spiked water and human plasma samples were analysed. The best recovery (~ 60 %) for MPn was achieved with a strong-anion exchange material [126]. A recovery rate of ~ 100% was achieved by Maddah *et al.*, 2010, with the use of a self-synthesized high capacity anion-exchange resin [114]. Among these methods based on strong anion-exchange materials, Owens and Koester, 2009, published a method for extracting MPn from different beverages. They used Strata®-X cartridges, a polymeric reversed-phase material. Under optimum conditions, they achieved a recovery rate of 52 % for MPn in reagent-water. For other matrices, the recovery rates were even lower [133].

2.5.2.2. Analytical Methods for the Determination of 2-Aminoethylphosphonic Acid

As already mentioned above, 2-aminoethylphosphonic acid (2-AEP) was the first phosphonate detected in nature. However, there is a lack of analytical methods described in the literature, especially for water samples. Analysis of 2-AEP using gas chromatography was done by Karlsson, 1970 [143], Kataoka *et al.*, 1989 [144] and Alhadeff and Daves, 1970 [145]. There, Karlsson, 1970, converted 2-AEP into its trimethylsilyl derivate and analysed the resulting compound with GC-FID and GC-MS [143]. Kataoka *et al.*, 1989, used isobutylchloroformate as derivatisation agent and used GC-FPD as a detector for analysing animal tissues [144]. Alhadeff and Daves, 1970, derivatized 2-AEP to dimethyl-2-acetamidoethylphosphonate and used GC-FID for analysis of human brain tissue [145].

Another method was published by Kameyama and Kitaoka, 1971, who used the phenol-sodium hypochlorite method which is based on optical density measurement with a photometer [146]. Indirect determination of 2-AEP was done by Czerkawski and Faulds, 1974 [147] as well as by Cembella and Antia, 1986 [108]. There, 2-AEP was separated from other phosphorus compounds either by ion-exchange chromatography [147] or by fractionation [108] and subsequent analysis as inorganic phosphorus. Other methods used an amino acid analyser for the detection of 2-AEP [148,149].

One example of the analysis of 2-AEP in samples from the aquatic environment was published by Quin and Quin, 2001. They studied the occurrence of phosphonates in different marine animals with ^{31}P-NMR and found 2-AEP to be present in all samples [150]. In 2018, two studies were published related to the occurrence and analysis of 2-AEP in environmental water samples. Shinohara *et al.*, 2018, detected 2-AEP qualitatively in suspended particles of an ultraoligotrophic lake by using two-dimensional nuclear magnetic resonance (2D-NMR) [62]. A more methodological work was published by Wang *et al.*, 2018. They developed a method based on an HPLC-separation equipped with a reversed-phase column and detection by a fluorescence detector (FLD). During sample preparation, 2-AEP was derivatized with *o*-phthalaldehyde-thiol (OPA-SH) to achieve a fluorescent compound. To test the method, different water matrices like lake water or artificial seawater were spiked and analysed. The method was characterized by an LOD of 7.2 µg/L for pure water and 12.0 µg/L for artificial seawater [151].

3. Methodological Approach

Individual methods were established in this study to analyse the target compounds $InsP_6$, MPn and 2-AEP. Furthermore, the studied matrix varied for the different analytes. The phosphonates MPn and 2-AEP were analysed in water and $InsP_6$ was determined in sediment samples. This led to different sample preparation procedures for each analyte which can be seen in a schematic overview in Table 4. For the two phosphonates, it was necessary to derivatise them before instrumental analysis (see Figure 7 for the chemical reactions). All methods were based on chromatographic separation coupled to mass spectrometric detection and examples for typical chromatograms are shown in Figure 8.

Table 4: Schematic overview on the methods developed within this study

Target analyte	*myo*-$InsP_6$ **(Publication 1)**	MPn **(Publication 2)**	2-AEP **(Publication 3)**
Sample type	Sediment	Water	Water
Pretreatment	Lyophilization	(Electrodialysis)	
Used standard type	Standard addition (*myo*-$InsP_6$)	Internal standard (D_3- and ^{13}C-MPn)	Internal standard (^{13}C-^{15}N-AMPA)
Extraction	aq. NaOH/EDTA (1 M/0.2 M)	SPE (weak-anion exchange)	-
Derivatisation (see Figure 7)	-	MTBSTFA + 1% TBDMSCl	FMOC-Cl
Stationary phase	Ion-exchange	Non-polar	Reversed-phase
Mobile phase	Gradient (MilliQ-H_2O and aq. $(NH_4)_2CO_3$ (600 mM))	Helium	Gradient (NH_4HCO_3 (2 mM, pH 9), ACN)
Instrumental analysis	LC-MS/MS	GC-MS	LC-MS/MS
Detection mode	MRM	SIM	SRM

The quantification of $InsP_6$ was based on a previously published method using IC-MS/MS [9] with slight modifications on the extraction and the quantification procedure. A detailed description of this method development was published in Recknagel *et al.*, 2018, Talanta 188: 192-198. (**Publication 1**) [152].

Analysis of MPn in water samples was done using a GC-MS-method which was originally based on a method published by Richardson and Caruso, 2007 [124]. However, several modifications were worked out within this study and were published in Lohrer *et al.*, 2020, Talanta 211, 120724 (**Publication 2**) [153].

2-AEP has a similar chemical structure as aminomethylphosphonic acid (AMPA), which is the main degradation product of the herbicide glyphosate. Therefore, the analytical method for the analysis of 2-AEP was based on the chromatographic method for the analysis of these two compounds which was previously published [154]. Because there was no isotopically-labelled 2-AEP commercially available, isotopically-labelled AMPA (^{13}C-^{15}N-AMPA) was used as the internal standard for 2-AEP quantification. Analytical details of the method can be found in Skeff *et al.*, 2016, J. Chromato. A 1475, 64-73 (**Publication 3)** [155].

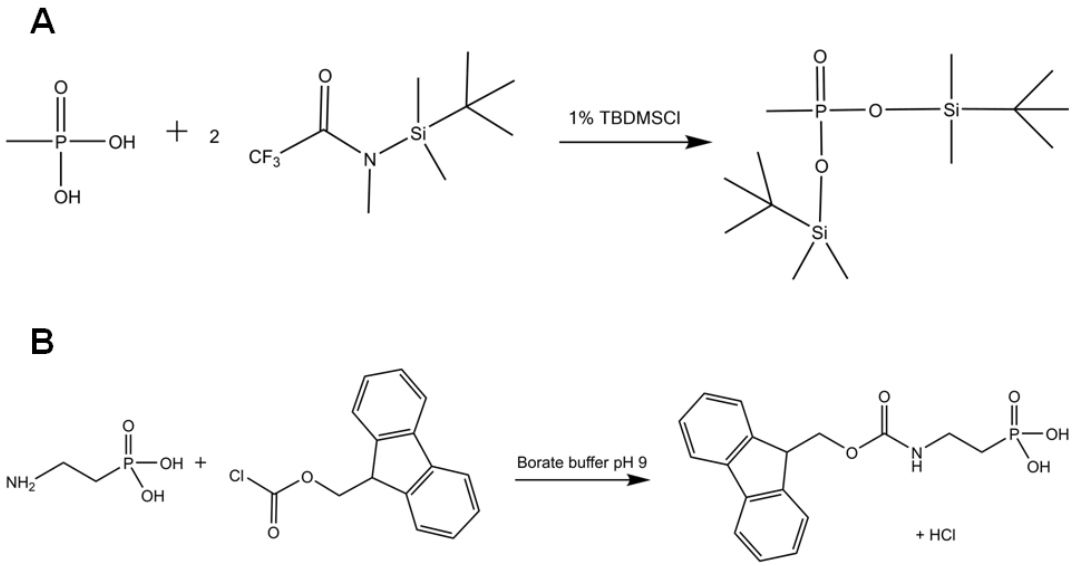

Figure 7: Chemical equations for the derivatisation reactions used in this study.
A: Derivatisation of methylphosphonic acid with MTBSTFA;
B: Derivatisation of 2-aminoethylphosphonic acid with 9-fluorenylmethylchloroformate (FMOC-Cl)

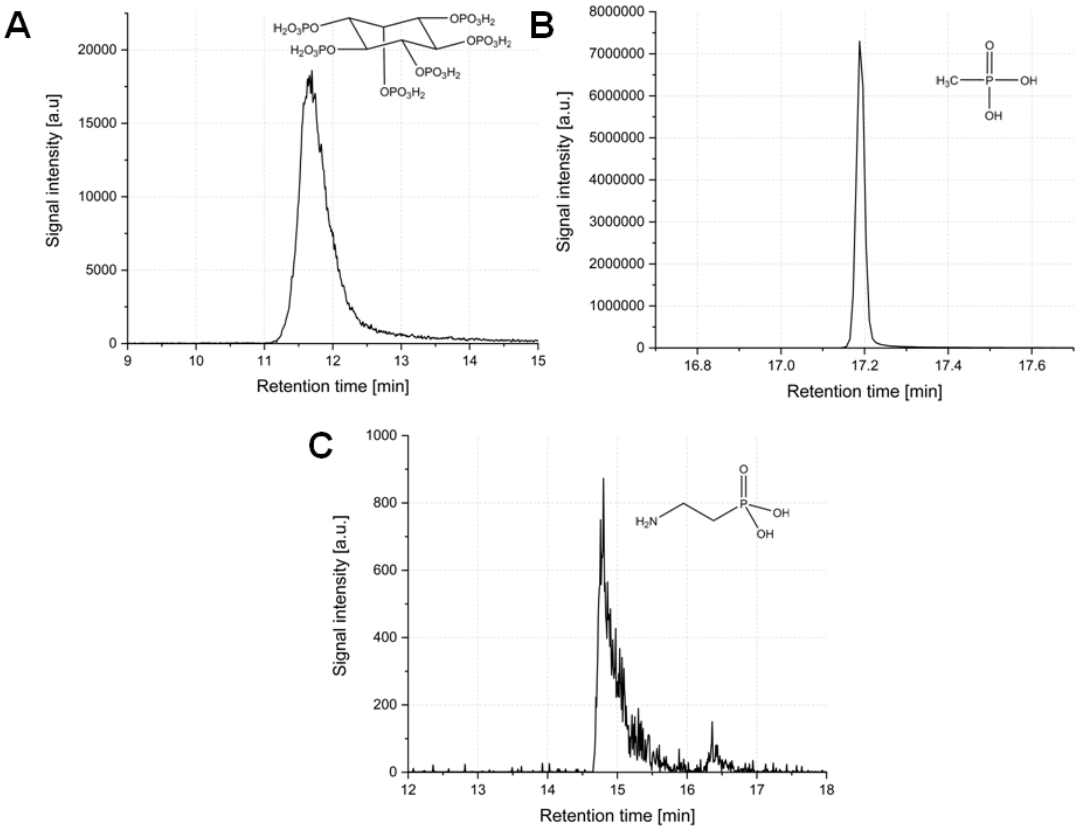

Figure 8: Examples for chromatograms from standard substances of the target analytes used within this study. A: Chromatogram of $InsP_6$ in MilliQ-water; B: Chromatogram of MPn in MilliQ-water; C: Chromatogram of 2-AEP in HPLC-grade water

4. Summary of the Results and Discussion

The main goal of this work was to develop suitable analytical methods for the quantification of selected organophosphorus compounds with possibly high relevance for the aquatic phosphorus cycle. This study focused on three representatives of this group, namely the inositol phosphates, methylphosphonic acid and 2-aminoethylphosphonic acid. As these compounds are chemically different, it is not useful to quantify them with one single analytical method. So, different chromatographic methods were developed which reflect the properties of the target compounds as well as the corresponding environmental matrix with the highest significance for the respective substances. In summary, reliable analytical methods were developed or optimized for all selected compounds within this study. Additionally, two of the selected compounds were studied in environmental samples to receive first insights into their abundance in sediments (inositol phosphates) and water samples (methylphosphonic acid).

With this, the presented work represents a contribution to the elucidation of the role of organic phosphorus compounds in the aquatic environment.

4.1. Inositol Phosphates

This section summarizes the results of the work on inositol phosphates which are published in

> Recknagel *et al.*, 2018, “Using standard additions to improve extraction and quantification of inositol hexakisphosphate in sediment samples by ion chromatography electrospray ionization mass spectrometry“, Talanta 188, pp. 192-198 [152].

4.1.1. Methodological Aspects (Objective 1)

As already mentioned in section 2.5.1, several methods for the analysis of inositol phosphates based on mass spectrometry are described in the literature (Table 2) [8,9,91,99-101,106]. In this study [152] an IC-MS/MS-method was used to analyse $InsP_6$, which was developed in previous works by Paraskova *et al.*, 2015 [8] and Sjöberg *et al.*, 2016 [9] for the analysis of different inositol phosphates in sediment samples. In these studies, InsPs were quantified based on an external calibration. However, with further studies it became obvious, that matrix effects strongly influence the mass spectrometric detection. This can either cause overestimation or underestimation of the corresponding InsP-content in the samples. As there are no isotopically-labelled InsPs commercially available, a different method for reliable quantification had to be utilized. Within this project, standard addition response curves in different matrices were obtained to study the influence of these matrix effects. As it was

shown by Paraskova *et al.*, 2015 [8] and Sjöberg *et al.*, 2016 [9], $InsP_6$ was the dominant representative of the inositol phosphates in sediment samples. Therefore, this compound was studied as a model substance within this work. The study showed that even for MilliQ-water and the extraction solvent (NaOH/EDTA (0.25 M/0.05 M)) as sample matrices, matrix effects were observed (see also Fig. 1 in Recknagel *et al.*, 2018, [152]). As a consequence, quantification using external calibration (*e.g.* in MilliQ-water) may lead to false results when sediment extracts are analysed. So a quantification method based on standard addition was developed for the analysis of $InsP_6$ [152]. Despite the matrix effects on the mass spectrometric detection, also matrix effects on the extraction efficiency of the used aqueous NaOH/EDTA-solution due to the composition of the different sediment samples were observed. There, sediments with a higher total organic carbon (TOC)-content had a lower extraction efficiency than *sandier* sediments which is due to stronger matrix effects. This is a critical fact as no internal standard could be used to correct for insufficient extraction and so a significant underestimation of the $InsP_6$-content would have been the consequence. Based on these results, a quantification procedure with a standard correction for all types of matrix effects during the whole procedure was necessary. In the present case, the standard addition set-up was chosen. Because of the matrix effects of the extraction efficiency, standard addition had to be done on the sediment samples before the extraction.

Furthermore, it was shown that, especially for sediment samples characterized by a higher TOC-content, insufficient extraction efficiency was achieved when Sjöberg's extraction protocol was used [9]. Therefore, several extraction conditions (extraction time, sediment: extraction solvent ratio and concentration of the extraction solvent ingredients) were evaluated and optimized. With this, the extraction efficiency increased strongly as it can be seen in Figure 9 (for more details see Fig. 3-5 in [152]). For the highly organic sediment sample, the extraction yield was increased by 720 % and for the sandy sediment sample by 18 % [152]. There, changes in the sediment: extraction solvent ratio and in the concentration of the extraction solvent ingredients had the largest effects. This might be due to the case that the strength of the extraction solvent increased and so extraction of more strongly bound $InsP_6$ was possible. As natural $InsP_6$-concentrations in aquatic sediments are partially low, this improvement enables the possibility to even detect $InsP_6$ at lower concentrations.

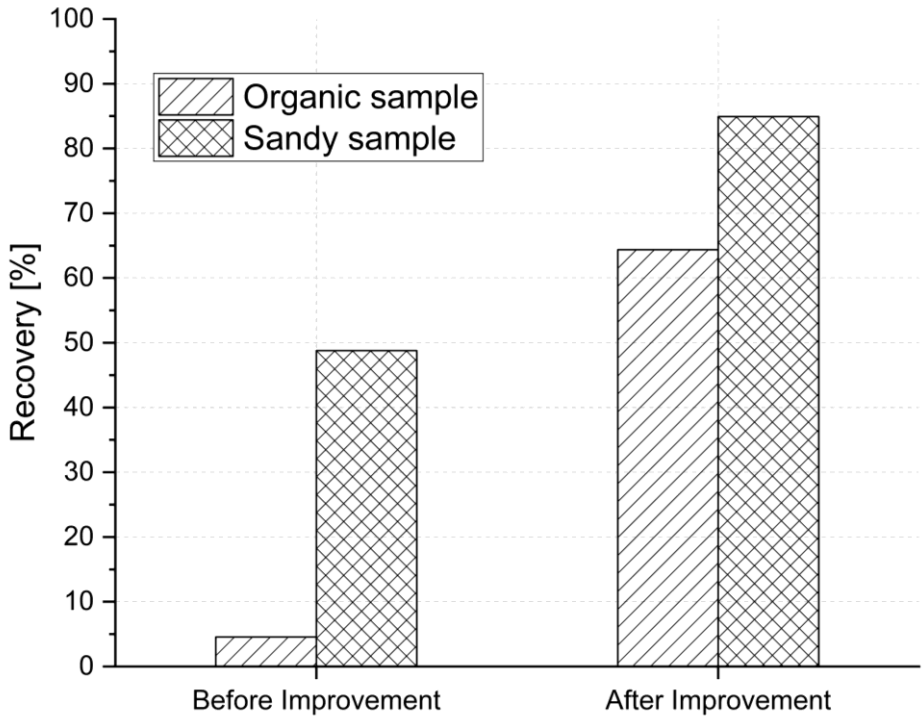

Figure 9: Comparison of the extraction efficiency of the extraction conditions before and after improvement (*Before*: Sediment:solvent ratio: 1:10; extraction time: 4 h; NaOH/EDTA: 0.25 M/0.05 M; *After*: 1:15;overnight (16 h);1 M/0.2 M). The effects on two types of sediment are shown. (obtained and modified from Recknagel *et al.*, 2018 [152])

Finally, the method was tested on spiked sediment samples with different matrix composition, indicated *e.g.* by a TOC-content of 1, 5 and 10 %. For all these matrices, reliable results for quantification of $InsP_6$ were achieved, shown by an accuracy of -6 – 12 RE% and a precision of 3 – 11 RSD% [152]. With these results, one can see that a robust and reliable method for the quantification of $InsP_6$ in different sediment samples was presented with this work.

4.1.2. Environmental Samples (Objective 2)

The developed method was then used to analyse several surface sediment samples collected at the river Warnow in the urban area of Rostock and the highly eutrophic lagoon system Darss-Zingst Bodden Chain, located at the German Baltic coastal area in Mecklenburg-West Pomerania, Germany for their concentration of $InsP_6$ (for a map of the sampling sites see Fig 6 in Recknagel *et al.*, 2018 [152]). There, it was detectable at six sampling stations with concentrations between 2.3 and 15.2 µg $InsP_6$-P/g DW. Interestingly, $InsP_6$ was not detectable in any sample collected at the different Bodden systems from the Darss-Zingst Bodden Chain. Compared to other previously published data for $InsP_6$ in aquatic sediments, the contents in the collected samples are rather low as $InsP_6$-concentrations of up to 320 µg InsP-P/g DW were reported before, *e.g.* by Jørgensen *et al.*,

2011 [47] (see Table 1). However, the values of this study are similar to the InsP-data published by Suzumura and Kamatani, 1995, for the coastal area of Tokyo Bay [45]. This sampling area is comparable to the ones shown in this present study, as all are influenced by the marine environment. Based on the data shown in Figure 10 one can assume that the load of InsP in aquatic systems is higher in lakes than in coastal or oceanic systems.

It was reported before that InsP may account for a substantial part of the total organic phosphorus in sediments. For example, Turner and Weckström, 2009, detected an $InsP_6$-fraction of up to 24 % on the total organic phosphorus in lower sublayers of a sediment core from an embayment located in Helsinki, Finland with narrow connections to the Baltic Sea (see Table 1, [46]). The sampled embayment was described to have little exchange to the open ocean but still a salinity of 4.8. The surface sediment showed an $InsP_6$-concentration of 14.74 µg $InsP_6$-P/g DW with a fraction of the total organic phosphorus of 6.7 % which is comparable to the values found for the river Recknitz within this study [46,152]. The sediment samples analysed by Jørgensen *et al.*, 2011, which were collected at 15 different Danish lakes, showed a fraction of $InsP_6$ on all phosphorus monoester- and phosphorus diester-compounds of up to 91 % [47]. However, other reported values show a different situation with InsP being only a small part of the total organic phosphorus (see Table 1). Samples analysed within this study showed low fractions of $InsP_6$ on the total organic phosphorus of only 0.5 – 3.1 % (see Table 2 in [152]). Again, these data are comparable to the ones published by Suzumura and Kamatani, 1995 [45]. Figure 10 compares the results achieved for the samples analysed within the present study with selected data from the literature. Large differences in the content of $InsP_6$ as well as in its fraction on the total organic phosphorus for different study sites were found. However, Figure 10 indicates that sediments in aquatic systems, influenced by the marine environment, have lower significance for $InsP_6$-storage. This could be either due to a reduced input of $InsP_6$ or due to mobilization, probably induced by saltwater. In this context, the results determined by Turner and Weckström correlated with the changing nutrient loading by wastewater [46]. Gardolinski *et al.* showed that organic phosphorus was released from freshwater sediments when they were incubated with seawater with a salinity > 10. However, they did not characterise the organic phosphorus and so it remains unclear whether $InsP_6$ is mobilised under these conditions as well [156]. In summary, there are only a few studies and, thus, more work is necessary to further elucidate the role of InsP in aquatic systems.

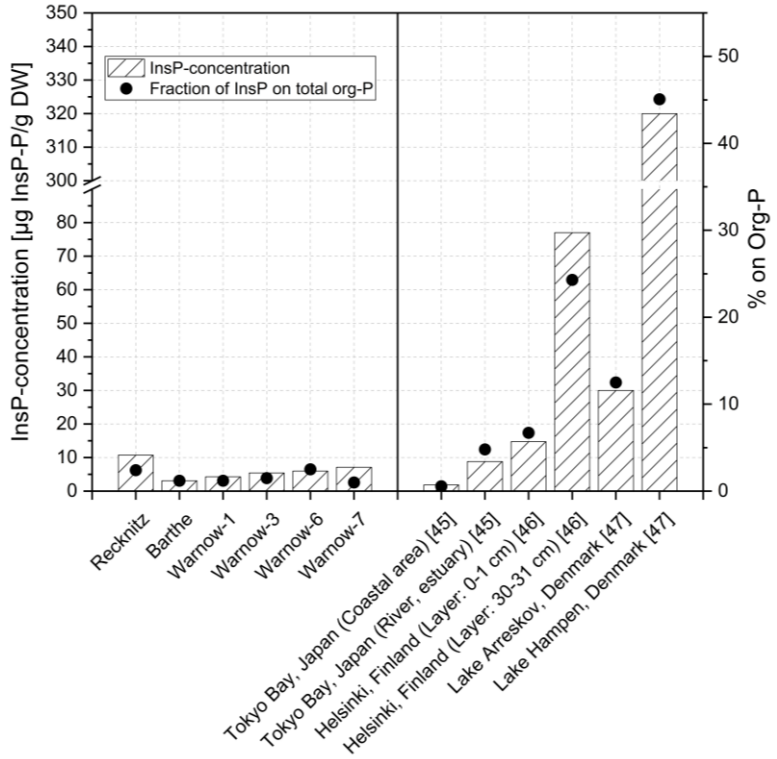

Figure 10: Comparison of data for InsP and the corresponding fraction on org-P. Data on the left side were collected within this study (Recknagel *et al.*, 2018 [152]). Data on the right side were collected from literature data and partly converted. For all sampling sites, the first centimetre of the sediment is shown unless otherwise stated. (Suzumura and Kamatani, 1995 [45]: Mean values of all shown data)

4.1.3. Outlook

With the published data for $InsP_6$ in two aquatic systems in the coastal area of the Baltic Sea in eastern Germany, it was shown, that inositol phosphates are present in sediments in this area as well, even though the contents were low compared to other reports. Therefore, a more intensive environmental study should be performed. In this, the sampling areas could be expanded to the sources of the river as well as to the Baltic Sea to follow the way of inositol phosphates in these systems and to identify the sources of InsPs. Here it might also be interesting to analyse suspended particles in the overlying water bodies as well as the water samples. Furthermore, it was shown by Turner and Weckström that $InsP_6$-concentrations, as well as their fraction on total organic phosphorus, varied strongly in different layers of a sediment core [46]. Therefore, sediment cores from the two systems studied within this project should be analysed for $InsP_6$-variations as well.

Within the present study, $InsP_6$ as the most important representative of the inositol phosphates was taken into account for the evaluation of the standard addition method. However, the samples analysed within this work were also qualitatively analysed for other inositol phosphates which were not detectable at all. Though, Sjöberg *et al.*, 2016, detected other isomers of $InsP_6$ as well as $InsP_5$ and $InsP_4$ in a sediment extract from Lake Erken,

Denmark with the same method used here [9]. Therefore, it might be interesting to expand the quantitative method by other $InsP_6$-isomers as well as by other inositol phosphates. As it was shown that there are instrumental matrix effects based on the composition of the mobile phase, the use of *myo*-$InsP_6$ for standard addition in the determination of other InsPs might not work properly. So, more studies on the quantification of these compounds are necessary to overcome this problem. One option might be standard addition using a mixed standard like it was used by Sjöberg *et al.*, 2016 [9].

Additionally, one has to address that this study only focused on the abundance of $InsP_6$ in sediments. However, the concentration of $InsP_6$ in environmental water samples is of interest as well as McKelvie, 2007, reported an indicative concentration of 27 µg $InsP_6$-P/L in the overlying water of a sediment sample and up to 281 µg $InsP_6$-P/L in the pore water [6]. These concentrations made up to 66 and 85 % of the total filterable P. This clearly shows that there is some interaction of $InsP_6$ between the sediment and the water and the water layer should be studied as well. As the concentrations in other aquatic systems might be significantly lower, the sensitivity of the presented method has to be improved. Therefore a preconcentration step will be necessary, *e.g.* by solid-phase extraction or gel filtration [84]. This preconcentration will be a critical task, especially for brackish and marine water samples due to the high salt content and the fact that $InsP_6$ is an ionic compound at environmental conditions.

4.2. Methylphosphonic Acid

This section summarizes the results of the work on methylphosphonic acid published in

> Lohrer *et al.*, 2020, “Methodological Aspects of Methylphosphonic Acid Analysis: Determination in River and Coastal Water Samples“ Talanta 211, 120724 (pp. 1-8) [153].

Methylphosphonic acid has been the subject of several studies regarding its relevance in the marine environment as a source of methane (see Section 2.4.2). However, there is no study in which the concentration of MPn in aquatic systems was determined. One of the reasons for this is the lack of suitable analytical methods for this sample matrix. There are several methods for the analysis of MPn in different matrices (*e.g.* water, urine, human plasma) which were published in the context of chemical warfare analysis. This is because MPn is the degradation product of nerve agents like sarin or soman (see Table 3 and reviews by B’Hymer, 2019 [157] and Hoojschuur *et al.*, 2002 [158]). On this occasion, seawater was used as a model substance for method development for example by Kataoka *et al.*, 2000, [109] and Nassar *et al.*, 1999 [110]. Within the present study, a method for the determination of MPn in environmental water samples, including saline samples, was developed [153].

Furthermore, the established method was applied to water samples, collected from the river Warnow and the German Baltic Coastal area at Heiligendamm in Mecklenburg-West Pomerania, Germany, to receive first insights into the relevance of MPn in these systems.

4.2.1. Methodological Aspects (Objective 1)

Within this study, MPn was determined with a method based on gas chromatography-mass spectrometry. To enable the analysis of water samples, several sample preparation steps were included in the method procedure. As it was shown that the recovery of the used SPE-material (weak anion-exchange material) decreases strongly with increasing sample salinity (see Fig. 3 and 4 in Lohrer *et al.*, 2020 [153]), a pretreatment step with electrodialysis was implemented when saline samples were analysed. With this, the desalination of the sample is achieved, which is of high importance for the further method procedure. The evaluation of the electrodialysis for MPn-containing samples was linked to a study by Wirth *et al.*, 2019 [159]. In this study, an electrodialysis method for, among others, several organophosphorus pesticides was developed. To evaluate the use of electrodialysis for determination of MPn, Lohrer *et al.*, 2020 spiked a coastal water sample, characterized by a salinity of 12.8, with MPn and recorded the recovery during electrodialysis at different salinities. It was shown, that up to a salinity of 2.7 an MPn-recovery of > 90 % was achieved (see also Fig. 5A in Lohrer *et al.*, 2020 [153]). However, if the salinity is decreased even more, the MPn-recovery decreases as well which is due to the ionic behaviour of MPn. Summarising, this means that environmental water samples containing high salt concentrations can be pretreated with electrodialysis which resulted in a better sensitivity of the method.

As MPn cannot be derivatised with MTBSTFA directly in the aqueous matrix, an extraction method using SPE was developed within this study. For this, two different SPE-materials were tested. Strata®-X-AW is a weak-anion exchange material which interacts with the ionic groups of MPn to retain it. Generally, this interaction seems to be beneficial, as MPn is an ionic compound in water at natural pH-conditions. However, the high salt-containing matrix of marine samples may have negative effects on the retention of MPn. Due to this, a second SPE-material was tested during method development. Strata®-X is a reversed-phase material which can interact with the methyl-group of MPn and which was used by Owens and Koester for extracting MPn from beverages [133]. This interaction is much weaker than the ionic interaction, however, the influence of the salt-containing matrix might be reduced. For this, the sample was acidified to pH 1 to transfer MPn into a non-ionic state. Results for the recoveries of the two SPE-materials with MilliQ- as well as saltwater matrices are shown in Figure 11. For both materials, the recovery decreased strongly when saltwater was used as sample matrix. As for Strata®-X the recovery was lower than for Strata®-X-AW, it was excluded from further method development.

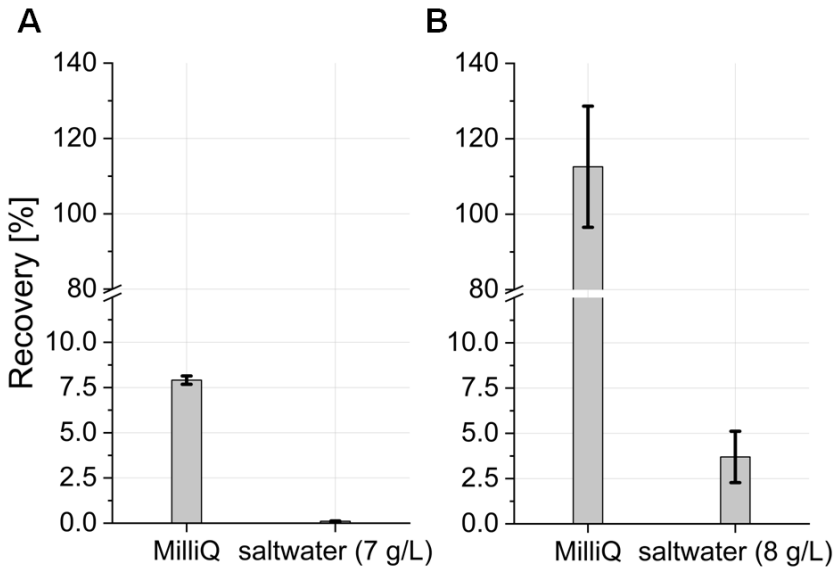

Figure 11: Comparison of the recovery of two SPE-materials on the recovery of MPn in MilliQ- and saltwater. A: Strata®-X (reversed-phase material); B: Strata®-X-AW (obtained and modified from Lohrer *et al*. [153])

One can see that the MPn-recovery of the used SPE-materials strongly depended on the concentration of salt in the different water samples. Therefore, the use of an internal standard is necessary to correct for losses of the analyte during SPE. Within this study, two isotopically-labelled MPn compounds were tested, namely ^{13}C-MPn and D_3-MPn. In Figure 12A one can see that the use of ^{13}C-MPn as internal standard resulted in a non-linear calibration curve. This is due to interferences from the mass fragments of the labelled and non-labelled compounds in the standard materials and results from the only 1 Dalton mass difference between both. Using a non-linear calibration method like it was published by Rule *et al.*, 2013 [160] resulted in correct results for MPn-quantification. However, D_3-MPn showed better characteristics in terms of the quantification and so it is the preferred internal standard (see Figure 12 and section 3.1 in Lohrer *et al.*, 2020 [153]).

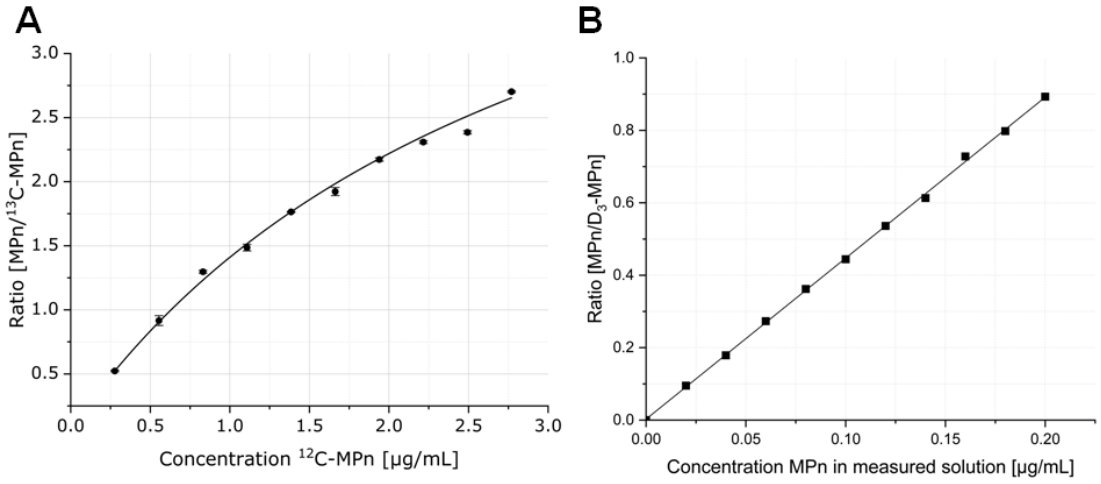

Figure 12: Calibration curves for MPn using different internal standard materials. A: ^{13}C-MPn; B: D_3-MPn (obtained and modified from Lohrer *et al.*, 2020 [153])

With the use of an internal standard, correct quantification of MPn was possible even so the SPE-recovery differed. This was shown by the validation of the method using spiked MilliQ- and river water. Values for the precision of 1.6 – 8.9 RSD% and for the accuracy of -5.8 – 14.1 RE% at different concentrations and sample volumes were achieved (see also Table Suppl. 3 in Lohrer *et al.*, 2020 [153]). As a result, the method was characterized by an instrumental LOD of 8 µg/L and by a method LOD of 0.4 µg/L which is in the same range as other methods for water analysis published before (Table 3). Overall, this study presents a method which is suitable for the analysis of environmental water samples including brackish waters and so it enables data collection for natural MPn-concentrations.

4.2.2. Environmental Samples (Objective 2)

Within this work, the first results for MPn-concentrations were achieved by analysing several environmental water samples. MPn was detectable in three samples from the river Warnow with concentrations close to the method LOD (0.4 – 0.6 µg/L, see Figure 13). Furthermore, it was possible to detect MPn in a coastal water sample after electrodialysis at a salinity of 5.6 (original sample salinity: 10.4). With further electrodialysis and decreasing salt concentration, the measured MPn-value in the sample decreased as well as it was described in the methodological aspects. One has to mention that the internal standard was added to the samples after electrodialysis due to methodological reasons. In the non-electrodialysed sample, MPn was not detectable because of its high salinity and the resulting decrease of SPE-recovery. However, an exponential correlation ($R^2 = 0.9486$) for the decrease of quantified MPn based on the continued electrodialysis was observed and so an MPn-value of 1.8 µg/L was proposed for this coastal water sample (see also Fig. 5B, C in Lohrer *et al.*, 2020 [153]). In several other samples, analysed within this study, MPn was not detectable at all.

To the best knowledge, these values were the first data for MPn-concentrations in environmental water samples. As the estimated amount of MPn in the coastal water sample was three times higher than the values collected for the river, this might be an indication that MPn plays a more important role in the coastal area. However, more samples have to be studied to give a more precise statement on the environmental significance of MPn. It was already mentioned that marine microbes contain the enzyme methylphosphonate synthase and so it seems to be likely that MPn is part of the marine phosphorus cycle [73]. This assumption is also supported by the described linkage of MPn and methane production in several incubation experiments [67,68]. However, there are no data for marine MPn concentrations available.

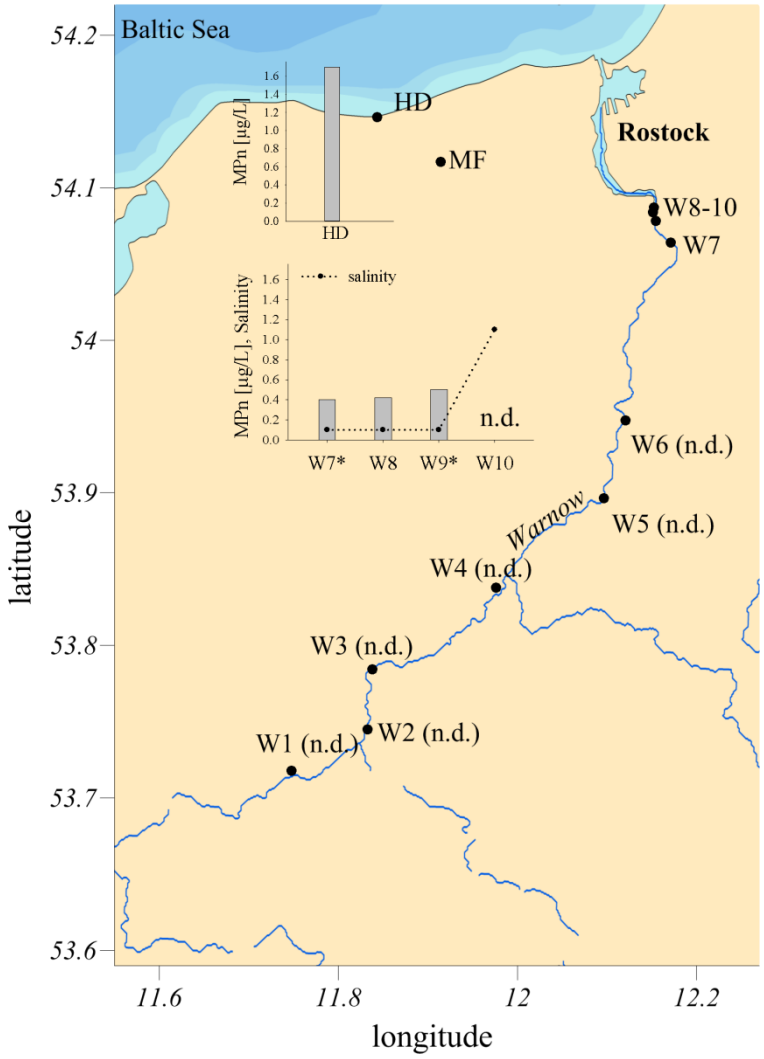

Figure 13: Sampling stations at the German Baltic coastal area in Mecklenburg-West Pomerania, Germany for MPn-analysis. Obtained MPn data for the sampling stations HD and W7-W10 are shown as bar diagrams (* data obtained in winter samples). MPn was not detectable at the other sites. The MPn-concentration at station HD was achieved by exponential correlation (obtained and modified from Lohrer *et al.*, 2020 [153]).

4.2.3. Outlook

The published method opens the possibility of various further studies. On the one hand, more work is necessary to further improve the analytical method; on the other hand, it is now possible to study several environmental aspects with the presented method.

On the methodological side, the electrodialysis method should be further improved and fully validated in the aspect of MPn-quantification. It was already mentioned that the internal standard was added after electrodialysis and so it cannot correct for the effects of this sample preparation step. However, it was shown that electrodialysis has a strong influence on the recovery of MPn. The internal standard was not added due to the high sample volume (3 L). In this case, a high amount of the internal standard would have to be used which is

highly cost-intensive (~ 550 €/sample). This problem might be solved by concentrating the sample after electrodialysis, such as through reverse osmosis or evaporation using *e.g.* a rotary evaporator. Furthermore, one could evaluate electrodialysis systems which require smaller sample volumes.

On the environmental side, the method enables the possibility to study the role of MPn in the aquatic environment. Several incubation experiments showed that MPn can be utilized as a phosphorus source by marine microorganisms and that methane was released during this process [65-67]. Thereby, the correlation between MPn and methane was shown either by the use of ^{14}C-MPn and determination of the produced ^{14}C-methane as ^{14}C-CO_2 [66] or by direct methane production. With our method, it is possible to show the increase of methane directly linked to the suspected decreasing MPn-concentration. Furthermore, the relevance of this process in nature remains unclear as little is known about the abundance of MPn at different marine areas, *e.g.* areas with high methane concentrations in the surface water. In the Baltic Sea, this supersaturation was for example detected in the eastern part of the Gotland Basin in the central Baltic Sea [161]. Furthermore, Teikari *et al.*, 2018 recently showed that cyanobacteria (*Nodularia spumigena)* collected at the Gulf of Finland were able to use MPn as a phosphorus source when inorganic phosphorus was depleted. Several phosphonates were tested within their study and the highest growth was observed in the presence of MPn [71]. So the analysis of MPn in different regions of the Baltic Sea as well as in different seasons is an important task for future works. Here, samples should be taken along the river, at the coastal line as well as at the open sea.

4.3. 2-Aminoethylphosphonic Acid

This section summarizes the results of the work on 2-aminoethylphosphonic acid (2-AEP) which are published in

> Skeff *et al.*, 2016, “The influence of salt matrices on the reversed-phase liquid chromatography behavior and electrospray ionization tandem mass spectrometry detection of glyphosate, glufosinate, aminomethylphosphonic acid and 2-aminoethylphosphonic acid in water“, J. Chromato. A 1475, pp. 64-73 [155].

4.3.1. Methodological Aspects (Objective 1)

As described before, 2-AEP is the first phosphonate detected in organisms [59]. Even though it is said that 2-AEP is widespread in nature [7,69,162], there is limited knowledge regarding environmental concentrations, especially in the aquatic environment. This is due to a lack of analytical methods for water and sediment samples. To overcome this problem, an analytical method for the analysis of 2-AEP in water samples based on HPLC-MS/MS was developed

within this study. As the chemical structure of 2-AEP is very similar to the one of AMPA, the main degradation product of the herbicide glyphosate, a combined method for these analytes was implemented [155]. Before instrumental analysis, 2-AEP was derivatised using fluorenylmethyloxycarbonyl chloride (FMOC-Cl) (see Figure 7B). This is a common derivatisation reaction for the analysis of glyphosate and AMPA [163], however, it was not yet described for the analysis of 2-AEP. As one can see in Figure 7B the FMOC-molecule reacts with the amine-group of 2-AEP, which results in a more hydrophobic compound. The relatively fast reaction (~2 h, [155]) can be performed in the LC-vial and so it fulfils the requirements of a good derivatisation reaction [164]. As no isotopically-labelled 2-AEP was commercially available, the ability to use isotopically-labelled AMPA (^{13}C-^{15}N-AMPA) for the quantification of 2-AEP was investigated. As a result, good linearity (R^2 = 0.992, see Figure 14) was achieved for a water matrix containing 4 g/L artificial sea salt within a concentration range of 2.5 – 30 µg/L. The method was characterized by an LOD of 2.6 µg/L for 2-AEP in a water matrix containing 4 g/L artificial sea salt. Recently, Wang *et al.*, 2018, published a method for the analysis of 2-AEP in seawater as well [151]. In contrary, this method was based on derivatisation using *o*-phthalaldehyde-ethanethiol followed by instrumental analysis with HPLC-FLD. It is characterised by an LOD of 7.2 µg/L for pure water and 12.0 µg/L for artificial seawater. No internal standard was used in this study.

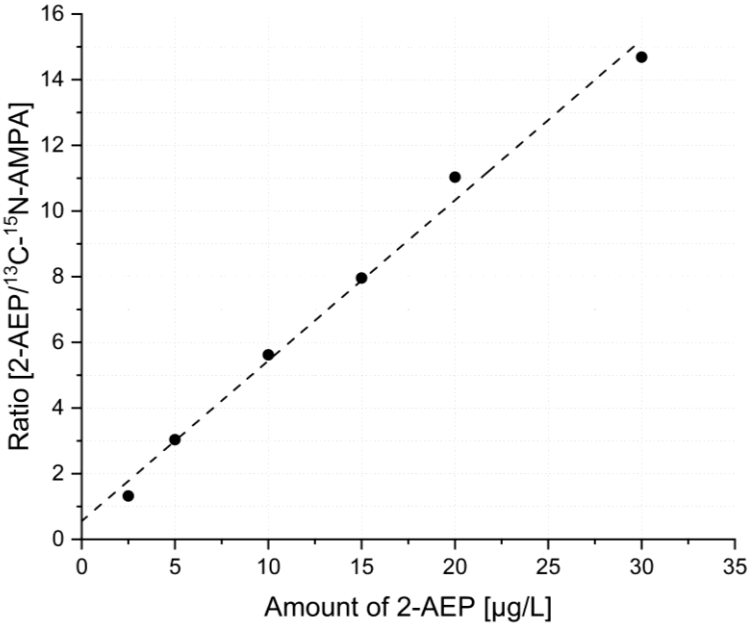

Figure 14: Calibration curve of 2-AEP in saltwater (4 g/L) using ^{13}C-^{15}N-AMPA as internal standard (obtained and modified from Skeff *et al.*, 2016, [155])

Furthermore, the present study showed, that the salt concentration of the matrix had a high influence on the retention of glyphosate on the reversed-phase columns, probably resulting from the formation of a glyphosate-metal-complex (see Figure 15). It was shown, that metal cations with a charge of +2 (*e.g.* magnesium(II)) yielded in a different retention time of

glyphosate or made it impossible to detect this compound at all. At certain concentrations of the metal ions, it was even possible to detect two peaks for glyphosate. In contrary, metal cations with a charge of +1 (*e.g.* sodium(I)) and +3 (*e.g.* iron(III)) did not show this effect (see Section 3.1 in Skeff *et al.*, 2016, [155]). As this phenomenon had a strong influence on the quantitative analysis, it was evaluated for 2-AEP as well. However, matrix effects were negligible for 2-AEP as well as for the other studied compounds AMPA and the herbicide glufosinate under all conditions tested (see Figure 15).

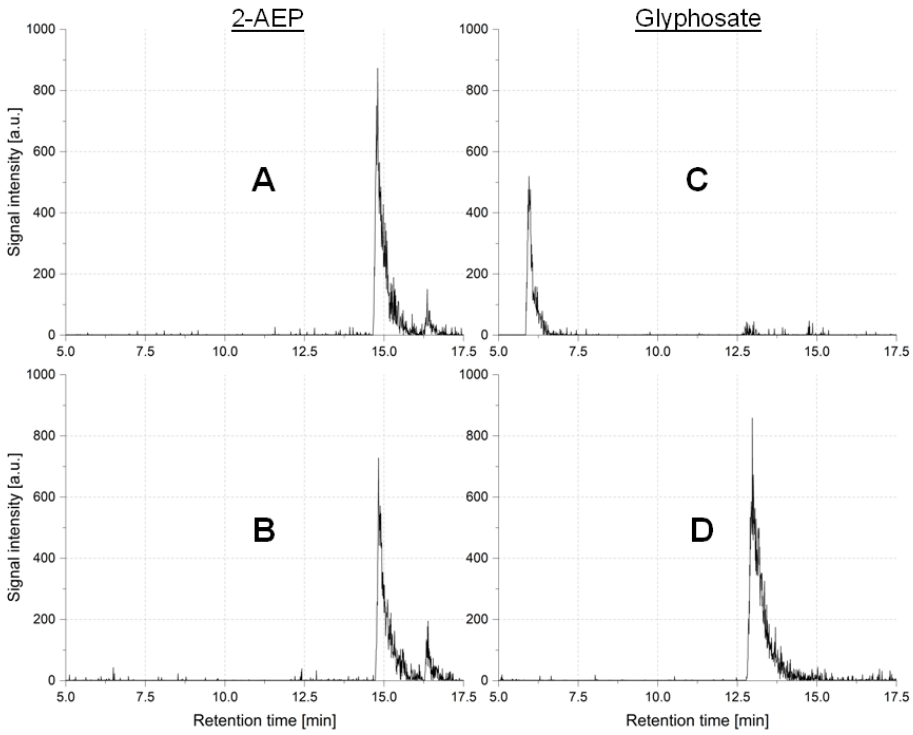

Figure 15: Chromatogram of 2-AEP in HPLC-grade- (A) and in saltwater (4 g/L) (B) compared to glyphosate in HPLC-grade- (C) and in saltwater (4 g/L) (D). (Obtained and modified from Skeff *et al.*, 2016, [155])

4.3.2. Outlook

To the best knowledge, the presented method is the first method for the direct analysis of free 2-AEP in water samples containing different amounts of salt. This method enables the possibility to study the significance of 2-AEP in aquatic systems as a phosphorus source during inorganic phosphorus starvation conditions. In several studies, it was described that marine microorganisms can use 2-AEP as a phosphorus source [71,165]. However, the actual role of this phosphonate in the aquatic environment remains unclear until now due to missing data. With the presented method, one could fill this gap by analysing several samples from different aquatic systems. As the concentrations of dissolved organic phosphorus compounds may be in the range of low µmol/L, it might be necessary to increase the sensitivity of the presented method. A possible solution is the addition of a

preconcentration-step using solid-phase extraction. As the derivatisation is performed in water, there are two possibilities regarding this step. On the one hand, the SPE-step can be performed before the derivatisation. In this case, one has to use a material with ionic interactions, as 2-AEP shows ionic behaviour at natural water conditions. This can be a challenging task in salt-containing samples like it was described in Section 4.2.1 for the analysis of MPn. An example of this set-up was published by Delmonico *et al*, 2014. who analysed the amount of glyphosate and AMPA in tap water [166]. On the other hand, it is possible to implement the SPE-step after the derivatisation. In this case, a more hydrophobic material needs to be used. Here the matrix effect of the sample matrix would be lower. However, one has to derivatise a large amount of sample which would lead to high consumption of chemicals, especially of the derivatisation agent FMOC-Cl. This set-up was used for example by Hanke *et al.*, 2008, who studied ultra-trace-levels of glyphosate, AMPA and glufosinate in different water samples from Switzerland [167]. Toss *et al.*¸ 2017, recently modified this method for the analysis of glyphosate and AMPA [168]. It has to be noted, that none of these methods contained 2-AEP in their analytical scope. However, as it was shown within the present study, the analysis of 2-AEP is similar to AMPA-analysis and so a transfer of these methods to 2-AEP is promising.

Furthermore, another study within this work showed that up to 80 % of the added 2-AEP is adsorbed by different sediments (mud, silt and sand) derived from the Baltic Sea [169]. The results show, that 2-AEP is adsorbed by the mud type sediment sample and by the silt type sediment sample up to 50 %. For the sandy sample, no adsorption of 2-AEP was observed. Additionally, the pH value as well as the salt content of the overlying water influence the adsorption behaviour and so studies for the occurrence of 2-AEP in different aquatic sediment samples are of high interest. For this an analytical method including an extraction method is necessary. Finally, one has to take into account that 2-AEP is often a side group of compounds like exopolysaccharides, glycoproteins or phosphonolipids [60]. So, a hydrolysation step might be necessary to be able to quantify the amount of 2-AEP linked to bigger compound groups as well.

5. General Outlook

The origin, distribution and significance for the aquatic environment of several organophosphorus compounds are still an open question. So, in the present study, three analytical methods for selected compounds were developed which allow determining them in environmental samples. These methods might help to get more insights into the organic phosphorus pool. Even though the developed methods were suitable for quantitative analysis of environmental samples, further improvement is necessary for all of them as described for the specific compounds in the section before. One has to notice that the studied compounds are a selection of possible relevant organophosphorus compounds. However, other phosphorus compounds are part of the organic phosphorus pool and so method development for these compounds is necessary as well to fully understand the organic phosphorus pool and its significance for the global phosphorus cycle.

For all selected compounds one sample type was evaluated as a starting point (sediment or water samples). As a future task, these methods should be expanded for the other sample type. Further specific tasks for method development were mentioned in each section before.

To study the ecological significance of the selected compounds one has to design several sampling campaigns. In this process, the water column, as well as the sediments, should be studied. In the course of this also seasonal changes should be evaluated as well as different regions of aquatic systems (*e.g.* rivers, estuaries, open sea). This could be done in the studied environment, the German Baltic coastal area and should be expanded to the central Baltic Sea. First results for inositol phosphates and methylphosphonic acid showed that they were present in samples from the study site, however, in lower concentrations. Here more work is necessary to give a more precise statement on the ecological role of these compounds. For this, a sampling campaign as mentioned before should be done. Furthermore, the selected compounds were studied only in sediments (InsP) or in water samples (MPn, 2-AEP). Here the analysis of the other sample class is interesting as well. Finally, specific scientific questions could be further studied. As an example, the role of MPn for the marine methane cycle is mentioned.

6. References

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Contributions to the manuscripts

The contributions to the manuscripts are indicated as follows:

Recknagel, C., Thelin, P., Abraham, M., Schulz-Bull, D. and Sjöberg, P. J. R., Using standard additions to improve extraction and quantification of inositol hexakisphosphate in sediment samples by ion chromatography electrospray ionization mass spectrometry, Talanta, 188 (2018), 192-198

Specific contributions: CR took the samples, designed the experiments together with PS and did most of the lab work, with the exception of the first detection of matrix effects. Furthermore, CR created the concept and wrote the manuscript. PT did the experiments with the postcolumn infusion technique. PS supported during the experiments and when writing the manuscript. MA supported during the manuscript writing process. Revision of the manuscript was done by PT, PS, MA and DSB.

Project principal investigator: D. E. Schulz-Bull

The work of Constantin Lohrer (né Recknagel) to this publication accounts for approximately 90 %.

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Specific contributions: CL took the samples in winter 2018, designed the experiments together with MK and did most of the lab work, with the exception of the work on electrodialysis (MW and PC) and some measurements for method validation (PC). MK and MW supported during the manuscript writing process. Revision of the manuscript was done by PC, MW, DSB and MK.

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The work of Constantin Lohrer to this publication accounts for approximately 75 %.

Skeff, W., Recknagel, C. and Schulz-Bull, D. E., The influence of salt matrices on the reversed-phase liquid chromatography behavior and electrospray ionization tandem mass spectrometry detection of glyphosate, glufosinate, aminomethylphosphonic acid and 2-aminoethylphosphonic acid in water, J. Chromato. A, 1475 (2016), 64-73

Specific contributions: WS and CR designed the experiments and evaluated the results. The lab work was mostly done by CR. WS evaluated the measurements and wrote the manuscript. Revision of the manuscript was done by CR and DSB.

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Publication 1

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Using standard additions to improve extraction and quantification of inositol hexakisphosphate in sediment samples by ion chromatography electrospray ionization mass spectrometry

Constantin Recknagel[a,*], Pernilla Thelin[b], Marion Abraham[a], Detlef Schulz-Bull[a], Per J.R. Sjöberg[b]

[a] Leibniz Institute for Baltic Sea Research Warnemuende, Department of Marine Chemistry, Seestrasse 15, 18119 Rostock, Germany
[b] Department of Chemistry – BMC, Uppsala University, P.O. Box 599, S-75124 Uppsala, Sweden

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Abbreviations:
InsPnInositol phosphates
TOCTotal organic carbon
TPTotal phosphorus
TOPTotal organic phosphorus
DWDry weight

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ABSTRACT

Several key aspects for the analysis of inositol hexakisphosphate ($InsP_6$) have been investigated in order to establish a suitable method for the study of sediment samples from different aquatic systems. Apparent matrix effects for the ion chromatography electrospray ionization tandem mass spectrometric detection (IC-ESI-MS/MS) method were accounted for with a standard addition approach, which also compensated for variation in extraction efficiency. Several parameters of the extraction method were optimized to improve the extraction efficiency for different sediment types. We observed an improvement in the extraction efficiency between 18% and 720%. Finally, the method was used to gain first insights into the relevance of $InsP_6$ in two aquatic systems located at the German Baltic coastal area. $InsP_6$ was detected in several sediment samples with concentrations between 2.3 and 15.2 µg $InsP_6$-P/g dry weight (DW).

1. Introduction

Phosphorus (P) is a key nutrient in the aquatic environment during eutrophication. Inorganic P is easily bioavailable, however, organic forms of P have been shown to contribute to eutrophication as well [1]. There are a wide number of different organic P-compounds (OP), and they are normally determined as total organic P (TOP) [2]. Due to the fact that different OP-compounds show different bioavailability, it seems to be necessary to determine the different OP-compounds separately.

One important group of OP-compounds are the inositol phosphates (InsP). The most abundant natural representative of this compound group is *myo*-inositol hexakisphosphate (*myo*-$InsP_6$), also referred as phytic acid [3]. *Myo*-$InsP_6$ was identified to be a key component of the total organic P in most lake sediments [3]. However, the fraction of *myo*-$InsP_6$ on the total P in sediments varies considerably between evaluated aquatic systems. In earlier studies, it ranged from less than 1% [4] up to more than 50% [5].

The method mostly used for determination of inositol phosphates in sediments and soils is based on ^{31}P NMR [6–10]. Other methods are based on liquid chromatography using different separation modes such as size exclusion chromatography [11], ion-exchange chromatography [12–18] or reversed-phase chromatography, mostly with the ion-pairing mode [19–22]. Several detector strategies are utilized such as post column derivatization enabling colometric or fluorimetric detection [23,24]. Recent methods use a mass spectrometer for detection [12–14,19,21].

In a previous study [13] an LC-MS method was presented to separate and quantify different inositol phosphate isomers present in environmental samples. The method had a simple sample preparation protocol with a single NaOH-EDTA extraction step with subsequent LC-MS/MS analysis. Good separation, reliable quantitative performance, and good extraction recovery and precision were achieved for the studied samples. However, since the introduction of this method, more samples have been analyzed, and some concerns regarding matrix effects have arisen. To overcome those, the use of SPE was tested in our former studies with unsatisfactory results though [13]. Additionally, matrix effects could also influence the extraction and result in different extraction efficiencies. Therefore, it is important to use a calibration method which corrects for matrix effects during the whole procedure. Using an internal standard seems to be problematic. There is currently no isotopically-labelled $InsP_6$ commercially available which would be

* Corresponding author.
E-mail address: constantin.recknagel@io-warnemuende.de (C. Recknagel).

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the optimal internal standard.

Therefore, the aim of this study is to further explore matrix effects during extraction and analysis of $InsP_6$ from environmental samples, to utilize the standard addition method to overcome these matrix related uncertainties and to develop a suitable method for the quantification of $InsP_6$. Our study is based on an approach for analyzing polychlorinated biphenyls (PCB) presented by Mechlińska et al. in which the standard was added to the sediment before extraction in a larger volume to ensure its homogeneous distribution in the sediment sample [25].

Finally, our proposed method was tested on sediment samples from two estuarine systems which locate close to the German Baltic Sea Coast in the federal state of Mecklenburg-West Pomerania to gain first insights into the relevance of $InsP_6$ in these systems.

2. Experimental

2.1. Chemicals and reagents

Ammonium carbonate ($(NH_4)_2CO_3$) and 2-propanol were of analytical grade and purchased from Sigma-Aldrich (Germany). Milli-Q®-water was used for preparing aqueous solutions as well as for preparing the eluents for the HPLC. A stock solution of $InsP_6$ with a concentration of 2.54 mM was prepared by dissolving an appropriate amount of *myo*-inositol hexakis(dihydrogen phosphate) dipotassium salt (Sigma-Aldrich) in Milli-Q®-water. The concentration of $InsP_6$ in this InsP-salt was determined by calibrating the system with an in-house standard as the used salt consists of $InsP_6$ and $InsP_5$ (c = 0.64 mM, approx. 20%, Fig. S1 in the supplementary material). Details regarding the preparation and characterization of this in-house standard can be found in our previous work [13]. Working solutions were prepared from the stock solution by dilution with Milli-Q®-water. The extraction solvents were prepared by dissolving an appropriate amount of sodium hydroxide (NaOH, p.a. EKA Bohus-Sweden) and ethylenediaminetetraacetic acid disodium salt (EDTA disodium salt, Merck) in Milli-Q®-water.

2.2. Sampling

Sediment samples were collected at different stations from the Darss-Zingst Bodden Chain and the river Warnow in Mecklenburg-West Pomerania, Germany (see Table S1 in the supplementary material). Samples were collected in March 2016 with a bottom sampler according to van Veen (Hydro-Bios, Kiel, Germany) and in May 2016 (Darss-Zingst Bodden Chain) and June 2016 (Warnow) with a sediment corer (Uwitec, Mondsee, Austria). All samples taken with the corer were sliced into pieces of one cm immediately after sampling and stored at −20 °C before freeze-drying. The Oder Bank sample was taken with a multi-corer during cruise MSM50 in January 2016 and treated in the same way as the other sediment cores. For $InsP_6$ analysis the upper 2 cm were used.

2.3. Preparation of spiked sediment samples and extraction of $InsP_6$ from sediment

Two different modes of spiking sediment samples were used depending on whether the extraction efficiency (results presented in Section 3.2) or the method performance (results presented in Section 3.3) was investigated.

In-house reference sediment samples were prepared for evaluation of extraction efficiency by first adding 750 µL Milli-Q®-water containing a known amount of $InsP_6$ to approximately 100 mg of dry sediment, and incubated by shaking overnight in a 2.0 mL micro centrifuge tube (VWR International LLC, Randor, PA) to allow complete adsorption of $InsP_6$. The samples were then extracted using aqueous NaOH containing EDTA with a ratio of 5:1 at different concentrations. 1.5 mL of this extraction solvent was added to the sediment and the mixture was shaken overnight unless otherwise mentioned, using a Multi Reax (Heidolph Instrument, Schwabach, Germany). The samples were centrifuged at 10,000 rpm for 20 min (Spectrafuge 7 M, Labnet International Inc. Edison, NJ) and the solution was transferred to a 1.5 mL glass vial. Standard addition was done by transferring 270 µL of the extract into a 1.5 mL glass vial and spiking with 30 µL of $InsP_6$ in Milli-Q®-water at four different concentration levels. When the extraction was done with NaOH/EDTA (1 M/0.2 M) the solution was diluted 1:1 before analysis.

The performance of the method was tested by standard addition to the sediment before the extraction to correct for incomplete extraction (Section 3.3). Therefore, four samples at approximately 100 mg sediment were weighed into 2.0 mL tubes. The four subsamples were spiked with different $InsP_6$ concentrations, i.e. ~ 0.1 µmol $InsP_6$/g sample as the spiking concentration and 0%, 50%, 100% and 150% of this as standard addition. The samples were shaken overnight to allow complete adsorption. Afterwards the samples were extracted overnight with NaOH/EDTA (1 M/0.2 M). Before instrumental analysis the extracts were diluted 1:1 with Milli-Q®-water.

Samples from the Warnow and the Darss-Zingst Bodden chain were analyzed with a qualitative screening before quantification to check for occurrence of $InsP_6$. The samples were extracted as described before and the extract was then injected directly into the LC-system. All samples where $InsP_6$ was detected were then conducted to further quantitative analysis using the described standard addition method. However, no dilution of the NaOH/EDTA-solution (1 M/0.2 M) was done after extraction as it was shown that there was no negative influence of the high NaOH-concentration on the performance of the column and the mass spectrometer.

2.4. Instrumentation

Samples were analyzed for $InsP_6$ using a 3200 Q-Trap MS/MS system (AB Sciex, Concord, ON, Canada) equipped with a 1260 Infinity LC system (Agilent Technologies, Waldbronn, Germany). The LC-MS/MS method was basically the same as previously reported [13].

Long term stability of the chromatographic column was improved by incorporating an injection program. After elution of $InsP_6$ Milli-Q®-water (10 µL), NaOH-EDTA (50 µL: 0.25 M/0.05 M) and formic acid (50 µL, 0.1%) were injected for cleaning the column. Long term stability for the MS-detection was achieved by reducing the turbo gas flow to 10 psi and turning off the nebulizer gas and high voltage after $InsP_6$ elution.

Evaluation of the data was done with the Analyst® 1.4.2 software (AB Sciex). $InsP_6$ was detected by using the multiple reaction monitoring mode (MRM) as reported previously [13] and all MRM transitions were summed before manual peak integration.

The instrumental method was carefully characterized in terms of linearity and reproducibility. Furthermore, the limit-of-detection (LOD) and the limit-of-quantification (LOQ) were evaluated as described in the supplementary materials. As a result, the method was characterized by a LOD of 0.2 µM $InsP_6$ and a LOQ of 0.6 µM $InsP_6$.

3. Results and discussion

3.1. Method characteristics

The work presented in this paper is based on our previously published work [13]. When the method was applied to a larger set of samples, matrix effects became of concern for certain types of samples. Matrix effects can be caused by a multitude of reasons, and two main strategies are used to reveal if they are present: namely the post-extraction addition technique; and the postcolumn infusion technique [26]. One potential problem could be co-elution of components that in the end influence the detection of the analytes of interest. To investigate this, we adopted the postcolumn infusion technique that was originally presented by Bonfiglio et al. [27]. By continuous infusion of a standard solution containing $InsP_6$ (5 µL/min, 250 µM) and mixing it with the LC-

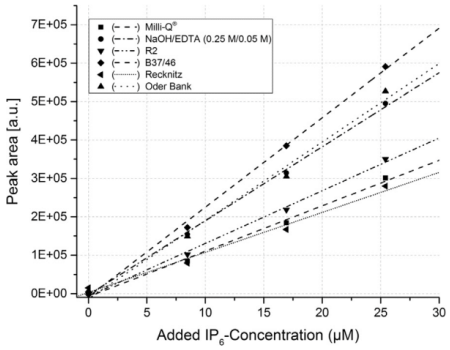

Fig. 1. Standard addition response curves for four samples (Oder Bank, R2, B37/46, Recknitz) and two standards (Milli-Q®-water, extraction solvent (NaOH/EDTA (0.25 M/0.05 M))). The addition of the different $InsP_6$-levels was done after extraction of the samples. The peak area is the sum of all recorded MRM transitions for $InsP_6$.

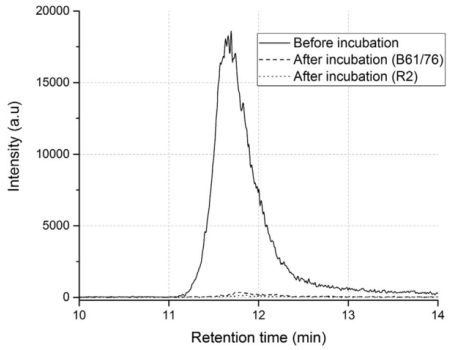

Fig. 2. Chromatogram of the spiking solution before and after incubation with the R2 and the B61/76 sediment. The concentration of $InsP_6$ in the solution was ~30 µM. The intensity is the sum of all recorded MRM transitions for $InsP_6$.

eluent from a sample extract, it should be possible to reveal if any severe matrix effects are present. Firstly, the results showed a quite large signal suppression due to the mobile phase gradient when it was altered from 3 to about 55% mobile phase B (600 mM aqueous $(NH_4)_2CO_3$). About 90% decrease in signal intensity was observed at the corresponding retention time for $InsP_6$ (~ 11.6 min). A similar signal decrease was observed when a sample extract originating from a top sediment collected at river Recknitz was injected (see Fig. S3).

As the decrease in signal intensity due to the LC gradient was quite large, it could be suspected that suppression effects by the sample matrix might be difficult to detect. Thus, the use of standard addition was explored for different sediment extracts.

To sediments with low (~ 0.1 − 1%, B37/46 and Oder Bank), intermediate (~ 10%, R2) and high TOC (~ 20%, Recknitz) different $InsP_6$ concentrations were added and the resulting extracts were analyzed (Fig. 1). It can be seen that the slopes of the different calibration curves differ, indicating that matrix effects are present. Furthermore, the slopes for the standards prepared in Milli-Q®-water and the extraction solvent (in this case NaOH/EDTA (0.25 M/0.05 M)) have a steeper slope for $InsP_6$ in the extraction solvent. This indicates that detection sensitivity is quite strongly affected by the composition of the injected solution. Furthermore, the sediment extracts show different sensitivities, where some have a relatively similar slope to $InsP_6$ in Milli-Q®-water, and some to $InsP_6$ in NaOH/EDTA. Sample parameters that influence the response are not yet well characterized. One observation is that the extracts of the *sandier* samples (B37/46 (TOC ≈ 1%) and Oder Bank (TOC ≈ 0.1%)) are comparable to $InsP_6$ in the NaOH/EDTA solution and the samples with a higher organic content R2 (TOC ≈ 10%) and Recknitz (TOC ≈ 20%)) show a similar slope like $InsP_6$ in Milli-Q®-water. Presumably, there are more matrix components in the extracts in the case of the high TOC containing sediments which cause higher matrix suppression during the measurement. Another aspect is a potential signal enhancement due to the NaOH/EDTA mixture. This can be seen with the higher slope of $InsP_6$ in this matrix compared to $InsP_6$ in Milli-Q®-water. As the extracts of the R2- and the Recknitz-sample (nearly black) were much darker than the B37/46- and the Oder Bank-sample (slightly brown), multiple reasons could have caused these observations.

This finding strongly implies that using external calibration might lead to insufficient accuracy and therefore, it is not appropriate for quantification. Due to the lack of suitable internal standard compounds, standard addition seems to be the best choice for accurate quantification. This set-up is promising, as good linearity was achieved for all calibration curves (Fig. 1, R^2 = 0.985 − 0.998).

Another factor that can contribute to accuracy problems are possible variations in extraction efficiency due to the sediment composition which will be discussed in the next section.

3.2. Improvement of the extraction

A common method for $InsP_6$ extraction from soil and sediments is using a mixture of NaOH and EDTA, which was studied for organic P previously by e.g. Zhang et al. for river sediments [28]. In our study spiked sediment samples were used to analyze the recovery of $InsP_6$ through variation of the extraction conditions, in particular extraction time, sample to solvent ratio and extractant concentrations. The standard addition method after the extraction was used for sediments with low (B61/76 (TOC ≈ 1%)) and intermediate (R2 (TOC ≈ 10%)) TOC. In these sediments natural $InsP_6$ was not detectable. So, spiked sediment samples were used to calculate the recovery of $InsP_6$ using the different conditions. First, to test the adsorption behavior of $InsP_6$ on the different sediments, the spiking solution was measured before and after overnight incubation with the different sediments. Fig. 2 shows that the peak for $InsP_6$ nearly completely disappeared after incubation with the sediments for both tested samples. This indicates that $InsP_6$ is adsorbed completely and the extraction efficiency test can be performed after treating sediments that way.

Fig. 3 shows the effect of extraction time on the recovery of $InsP_6$ from the two spiked sediment samples when using a NaOH/EDTA concentration of 0.25 M/0.05 M. It can be seen that there is a slight increase for all sediments tested when extracting overnight especially for the more sandy sediment B61/76. As a result, further experiments in this study were performed by extracting overnight (16 h). This measurement also shows that the extraction efficiency is not satisfactory especially for the R2 sample, where the recovery is ≤ 5%.

Another evaluated parameter was the sediment to extraction solvent ratio. We extracted three different amounts of spiked R2-samples (50, 100 and 150 mg $InsP_6$) with 1.5 mL NaOH/EDTA (1 M/0.2 M and 0.5 M/0.1 M) and evaluated the extraction efficiency (Fig. 4). A clear trend is observed where the efficiency increases when the amount of extraction solvent is increased. This indicates that the extraction solvent is "consumed" by the sediment matrix, presumably in particular when it consists of a high organic carbon content. However, the total amount of $InsP_6$ is lower when less sediment is used and therefore the absolute peak area decreases which can lead to a bad traceability. Based on the results shown in Fig. 4 we suggest using a sample to solvent ratio of 1:15 with a NaOH/EDTA-concentration of 1 M/0.2 M.

In Fig. 4 it can also be seen that different concentrations of NaOH

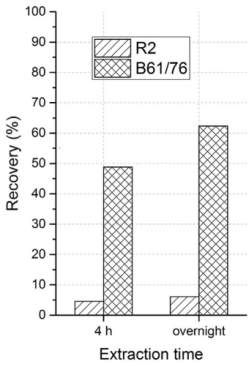

Fig. 3. Effect of the extraction time on the recovery of $InsP_6$ from different spiked sediment samples (0.25 M NaOH/0.05 M EDTA).

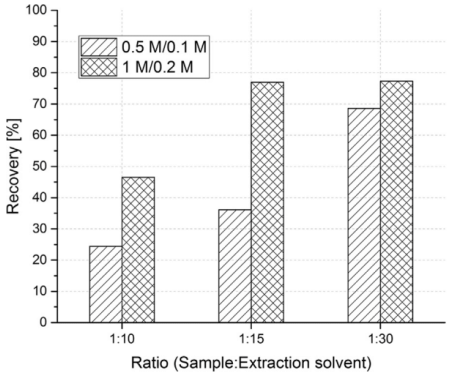

Fig. 4. Effect of the sample to extraction solvent ratioon the recovery of $InsP_6$ from the R2 sample using 0.5 M NaOH/0.1 M EDTA and 1 M NaOH/0.2 M EDTA (diluted 1:1 before measurement)).

and EDTA in the extraction solvent result in different recoveries of $InsP_6$. To evaluate these effects and to improve the efficiency of the extraction, three different sediments (R2, R86/88 and B61/76, Table Suppl. 2) were spiked with $InsP_6$ and extracted with three different concentrations of NaOH and EDTA. Here, the sample R86/88 (TOC ≈ 5%) was added to the experiment to test a third sediment with a TOC-level in between the two tested before. Fig. 5 shows the results of this experiment and the best recovery was achieved for all sediments when samples were extracted with 1 M NaOH and 0.2 M EDTA. Compared to the initial extraction conditions the extraction efficiency was improved between 18% for the B61/76-sample and 720% for the R2-sample. Furthermore, the extraction efficiency for the different sediment samples differed also when using the strongest extraction solvent. This shows that there is a strong matrix effect on the extraction resulting in varying recoveries for different sediment types. If the described set-up with standard addition of $InsP_6$ after the extraction is used, it is possible to underestimate the total $InsP_6$-content in the evaluated sediments. Thus, comparing $InsP_6$-contents of samples from different sediment types may lead to incorrect conclusions.

Even though extraction efficiency was largely improved, none of the

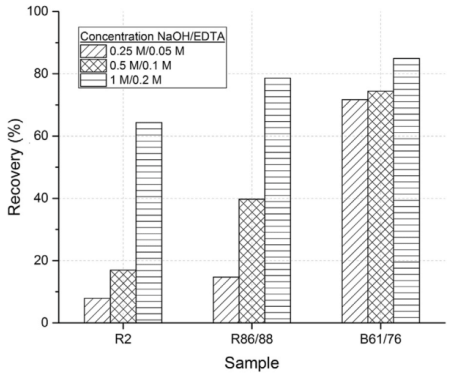

Fig. 5. Recovery of $InsP_6$ when extracting with different concentrations of NaOH/EDTA for different spiked sediment samples. The spiked concentration was~ 0.1 µmol/g and the extraction time was overnight. Samples extracted with a concentration of 1 M/0.2 M were diluted 1:1 before injection. Quantification was done by standard addition before extraction. Sediment:solvent ratio: 1:15.

Table 1
Validation of the standard addition before extraction method using three different sediments and a spiked $InsP_6$-concentration of ~ 0.1 µmol/g. Accuracy was calculated by comparing the achieved result with the spiked value. Precision was calculated by triplicate injection and represents the instrumental precision of one batch analysis.

Sample	Accuracy [RE%]	Precision [RSD%]
R2	1	11
R86/88	12	8
B61/76	− 6	3

tested sediments showed a recovery of 100%. This might be attributable to incomplete extraction which again emphasizes the need for a quantification approach robust against matrix effects and compensating different extraction recoveries of $InsP_6$ from field samples. However, enzymatic degradation might also be considered in view of the incomplete recovery of $InsP_6$. Phytases are abundant in the environment and likely to be present in the sediment samples which may hydrolyze some $InsP_6$ during overnight incubation. This phenomena should be studied in more detail e.g. by autoclaving the sediments before spiking.

3.3. Standard addition before extraction

As described before complete extraction of $InsP_6$ from the evaluated sediments was not achieved and differences in extraction efficiency of different sediments were obtained. The ionic character of $InsP_6$ and the high amount of ions in the extraction solution makes it difficult to extract $InsP_6$ and so preconcentration of $InsP_6$ before analysis is not promising. Clean up the extract with a reversed-phase SPE was tested in our previous study without satisfying results [13]. A repeated extraction of the sediment does not seem to be useful as the obtained fractions need to be merged and so the corresponding dilution would result in low sensitivity. Therefore, we tested the standard-addition approach in which the sediments were spiked with different amounts of standard ($InsP_6$) before extraction. For this set-up the three different sediment samples R2, R86/88 and B61/76, which were free of natural $InsP_6$, were used (Table 1). It can be seen that the accuracy (RE) for all

sediments ranged from 1% to 12% and precision (RSD) from 3% to 11% (see Equations S2 and S3 in the supplementary material). This shows that the standard addition set-up leads to satisfactory quantitative results, which enables matrix independent quantification and thus, comparative analysis of sediment samples with different characteristics.

3.4. Sediment samples

The evaluated method was tested on sediment samples from the German coastal Baltic Sea area - the Darss-Zingst Bodden Chain connecting four lagoons with the Baltic Sea and, therefore, characterized by a strong salinity gradient and the river Warnow in the city of Rostock (see Tables S1 and S2). $InsP_6$ was qualitatively detectable in four samples obtained from the river Warnow and in two samples from the Darss-Zingst Bodden Chain area; hence these samples were studied in more detail.

Fig. 6 gives an overview on the sampling stations and the $InsP_6$-content which was detected in the corresponding samples. The results for the individual samples are also shown in Table 2 and compared with the total P-content as well as the organic P-content in the sample. The organic P-content is assumed to be the difference between total P and HCl-extractable P, as described by Pardo et al. [29] and Slomp et al. [30]. In their study, Slomp *et al.* showed that this procedure yielded in good agreement with a sequential extraction method for most of their sediment samples. Due to this, we use the term "organic P" for the amount of non-HCl-extractable P in the following. Previously reported $InsP_6$-contents were up to several hundred of µg/g DW $InsP_6$ for sediments from lakes and rivers [4,8]. However, the estuarine sampling sites of our study located close to the Baltic Sea and obtained $InsP_6$-contents are in the same order of magnitude as those obtained for other marine sites such as Tokyo Bay for which $InsP_6$-contents ranged from 0.01 to 0.1 µmol/g DW (corresponding to 39.6–396 µg $InsP_6$-P/g DW). The facts that for the river Warnow the highest amount of $InsP_6$ was found at the station Warnow-7 which is the one furthest away from the Baltic Sea and for the Darss-Zingst Bodden Chain it was only detectable in the two rivers entering this system (sites Recknitz and Barthe) support previous discussions that terrestrial sites are the predominant sources for $InsP_6$ in the marine environment [3]. Moreover, the low fraction of $InsP_6$-P on the total organic P also supports previous findings that in marine sediments $InsP_6$ is not a substantial part of the organic P pool which is in contrast to other aquatic systems [3,4].

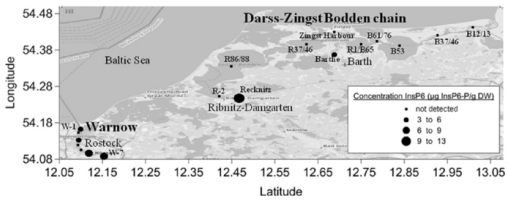

Fig. 6. Sampling stations at the two aquatic systems at the German Baltic coastal area in Mecklenburg-West Pomerania, Germany, analyzed in this manuscript. The size of the dots symbolizes the amount of $InsP_6$ detected at this station (mean of the two layers). For the river Warnow only the station names Warnow-1 (W-1) and Warnow-7 (W-7) are shown.

Interestingly, for a number of sampling sites in our study $InsP_6$ could not be detected at all which might be also attributed to the sediment characteristics. Therefore, we evaluated obtained $InsP_6$ data with respect to the organic carbon and total P fraction at the respective sites and observed linear correlations (see Fig. 7 A and B). This indicates that the deposition of $InsP_6$ in the sediment is influenced by the amount of TOC and TP. This is also supported by our findings during method improvement that sediments with higher TOC content required stronger extraction conditions than sediments with lower TOC content (see Fig. 5). In this light, many of the sampling sites within the Darss-Zingst Bodden Chain exhibit a low TOC, but high sand content (Table S2 in the supplementary material) which presumably hinders $InsP_6$ deposition here. This might also explain the observation that for some sites in the river Warnow no $InsP_6$ could be detected such as at Warnow-5.

However, for some sites the absence of $InsP_6$ cannot be explained solely by the sediment characteristic, so there must be other factors influencing the abundance of $InsP_6$ in the sediments. In this regard, the ephemeral nature of $InsP_6$ in the marine environment was identified before [4] and factors such as environmental conditions or enhanced microbial increasing $InsP_6$ mobility were discussed [3,4].

Table 2
$InsP_6$-concentration in samples from Warnow and Darss-Zingst Bodden Chain. The results are compared to the total P-concentration and the "org. P"-concentration (non-HCl-extractable P) in the sample. Further selected parameters of the sediments can be found in the supplementary data.

Sample	$InsP_6$-concentration [µg P/g DW]	Total P [µg P/g DW]	Fraction of $InsP_6$-P on total P [%]	"org. P" [µg P/g DW]	Fraction of $InsP_6$-P on "org. P" [%]	Total organic carbon [%]
Recknitz						
0–1 cm	10.7	4340	0.2	448	2.4	18.8
1–2 cm	15.2	4846	0.3	487	3.1	19.0
Barthe						
0–1 cm	3.1	1017	0.3	255	1.2	7.9
1–2 cm	4.9	777	0.6	232	2.1	6.4
Warnow-1						
0–1 cm	4.3	1726	0.2	345	1.2	5.0
1–2 cm	2.3	1511	0.2	457	0.5	4.4
Warnow-3						
0–1 cm	5.4	2023	0.3	362	1.5	7.5
1–2 cm	3.7	2092	0.2	379	1.0	7.1
Warnow-6						
0–1 cm	6	1939	0.3	238	2.5	9.8
1–2 cm	7.9	1963	0.4	300	2.6	10.4
Warnow-7						
0–1 cm	7.1	1843	0.4	731	1.0	12.0
1–2 cm	7.9	2031	0.4	918	0.9	13.0

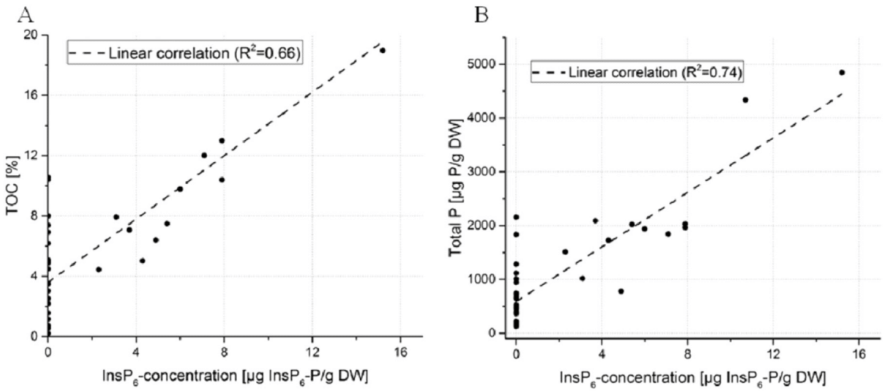

Fig. 7. Correlation of the $InsP_6$-content and the corresponding side parameters for all samples analyzed in this study: A: Correlation of TOC- and $InsP_6$-content, B: Correlation of TP- and $InsP_6$-content. A linear fit is included to show the linear correlation.

4. Conclusion

The presented method enables quantification of $InsP_6$ in different sediment types correcting for different matrix effects which arise from the sediment matrices using a standard addition approach. Extraction efficiency was increased for all types of investigated sediments, especially for sediments with higher organic carbon content. We think that this method could also be used to analyze other inositol phosphates.

Finally, $InsP_6$ could be detected at trace concentration levels in sediment samples from the German Baltic Sea coastal region. Here, further work is necessary to answer the question whether the input of InsPs in these systems is low, or if mobilization of InsPs takes place. In this case, InsPs could be an important phosphorus source in these systems.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.05.072.

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Publication 2

Methodological Aspects of Methylphosphonic Acid Analysis:
Determination in River and Coastal Water Samples

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Methodological aspects of methylphosphonic acid analysis: Determination in river and coastal water samples

Constantin Lohrer*, Paul P. Cwierz, Marisa A. Wirth, Detlef E. Schulz-Bull, Marion Kanwischer

Leibniz Institute for Baltic Sea Research Warnemuende, Department of Marine Chemistry, Seestrasse 15, 18119, Rostock, Germany

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ABSTRACT

Methylphosphonic acid (MPn) is suspected to play an important role in aquatic systems like rivers or the open ocean. To gain more insights into the importance of MPn, e.g., for the aquatic phosphorus cycle, an analytical method for its quantitative determination was developed. The method is based on the use of an isotopically-labelled internal standard and sample preparation including solid-phase extraction (SPE). Instrumental detection was done using GC-MS after derivatisation of MPn with *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). The study compares different isotopically-labelled compounds as well as different SPE-materials. As water samples with high salt content decrease the recovery of the chosen SPE-material, a desalting procedure using electrodialysis was implemented. Finally, water samples from different aquatic systems located at the German Baltic Sea coastal area were analysed to gain first insights into the relevance of MPn in these systems. MPn-concentrations in the low µg/L-range were detected.

1. Introduction

Naturally occurring organophosphonates comprise a class of P organic compounds, whose origin and function still is a puzzling subject of environmental research. Since organophosphonates contain a C–P bond, organisms require a different enzyme apparatus and more energy for their biochemical utilization compared to the oxygen-containing C–O–P phosphate ester group, which is the fundamental biochemical backbone for current life [1].

Organophosphonates are considered as the preceding form of phosphate in early life forms. However, they remained at particular sites at which their presence presumably is advantageous to life. In this regard, it was shown through ^{31}P nuclear magnetic resonance (^{31}P NMR) analysis that phosphonates make up 25% of the marine high molecular weight dissolved organic phosphorus pool [2]. Therefore, organophosphonate compounds presumably relevantly contribute to the marine biogeochemical P-cycle. Metabolic pathways for C–P cleavage have been conserved among a number of organisms in particular for those of the marine environment. It was shown that under P starvation conditions, in particular, methylphosphonic acid (MPn) was utilized by marine bacteria; *vice versa* for some marine microorganisms, the presence of an MPn synthesis was shown, too [3,4]. In this regard, MPn receives great interest, as it is discussed in view of the methane paradox in the aquatic environment [4–7].

However, there is limited information on concentrations of this small organophosphonate compound in the environment, which is probably due to the ionic behaviour conferred through the phosphonate group and, therefore, the high solubility in water.

There are several analytical methods for the determination of MPn in different sample matrices. These methods are mostly described in the context of chemical warfare analysis, as MPn is also a degradation product of organophosphorus nerve agents [8], and most of them base on gas chromatography (GC) [8–11] or liquid chromatography (LC) [12–14] coupled to mass spectrometry (MS). For MPn analysis through GC, a derivatisation step, e.g. methylation or silylation [15], is required to confer sufficient volatility for GC separation. Methods based on LC can also involve a derivatisation step, either to change the chromatographic behaviour [12] or to increase the sensitivity of the detection method [16].

When using silylation reactions for derivatisation, the separation of MPn from the water matrix is necessary, as silyl reagents are sensitive to water. However, a direct extraction with an organic solvent is not possible due to the ionic behaviour of MPn. To accomplish this, selected methods dry the sample with a stream of nitrogen gas or employ a rotary evaporator [8,12,17]. This is only useful when small amounts of water sample are used, and also if other matrix components are not disturbing the derivatisation of MPn. Another useful technique for sample preparation is solid-phase extraction (SPE). There are several

* Corresponding author.
E-mail address: constantin.lohrer@io-warnemuende.de (C. Lohrer).

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methods which exploit strong anion-exchange materials [11,18–22]. However, only Kataoka et al. investigated the influence of saltwater on the recovery of MPn with anion exchange-materials and found a recovery of ~60% [19]. Owens and Koester used a reversed-phase SPE method and reported a recovery of 16–60% for MPn in different beverages [13]. The use of liquid-liquid extraction with nonpolar organic solvents is not possible due to the ionic behaviour of MPn in water and its low solubility in nonpolar organic solvents [23].

Many of these methods have in common that the high amount of salt in the studied water samples may cause negative effects during SPE enrichment and sample analysis (e.g. chromatographic behaviour, derivatisation yield). Therefore, desalination of the matrix before further analysis should improve the performance of the analytical methods. Such a reduction of the salt matrix can be achieved by subjecting the samples to electrodialysis [24–26].

Herein, we report on further method development for the analysis of MPn in river and coastal water based on sample purification and enrichment through SPE with subsequent silylation and GC-MS analysis. We present data on the usage of two types of internal standard for MPn quantification and show our investigations on two different SPE-materials and their performance using salt-containing samples. The resulting method was validated in terms of accuracy and precision. Its analytical performance was described with figures of merit and accuracy studies were conducted using the elliptic joint confidence region (EJCR) test. In addition, we tested electrodialysis for sample processing in order to reduce the saline matrix load for coastal marine water. Finally, the developed method was tested for the analysis of MPn in riverine and coastal water samples in Mecklenburg-Western Pomerania, Germany.

2. Experimental

2.1. Chemicals and reagents

Methylphosphonic acid (98% purity) was purchased from VWR International GmbH (Hannover, Germany) and stock and working solutions were prepared using MilliQ-water (18.2 MΩ, Merck Millipore, Schwalbach, Germany). Deuterated methylphosphonic acid (D_3-MPn, 98% purity, 100 µg/mL in methanol, Sigma-Aldrich, Taufkirchen, Germany) was used as internal standard (IS) and working solutions were prepared with MilliQ-water as well. ^{13}C-methylphosphonic acid (99 atom-% ^{13}C, 98% purity, Sigma-Aldrich, Taufkirchen, Germany) was prepared by dissolving 10 mg in 10 mL LC-MS grade water (VWR International GmbH, Hannover, Germany) resulting in a 1 g/L-stock solution and tested as internal standard during method development.

Acetonitrile (ACN) and methanol (MeOH) were purchased from Walter-CMP (Kiel, Germany) and were of LC-MS grade. Barrelled n-hexane was obtained from Mallinkrodt Baker B. V and was purified through distillation. Ammonia solution 32% (v/v) in water was purchased from VWR International GmbH. *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-Butyldimethylchlorosilane (TBDMSCl) (assay ≥95%) was obtained from VWR. Sodium sulphate (Merck KGaA) and GC vials were combusted at 450 °C for 15 h. Artificial seawater for method development and electrodialysis was prepared using Tropic Marin® Sea Salt Classic (Tropic Marin AG, Hünenberg, Switzerland).

2.2. Sampling

Sampling was conducted in summer 2017 and 2018 as well as in winter 2018/2019 from freshwater, brackish and coastal waters. Surface water samples were collected from the Mühlenfließ (MF), a small tributary to the Baltic Sea, and the river Warnow in the German federal state Mecklenburg-Western Pomerania (Table 1). The Warnow is characterised by a large lowland catchment area discharging into the Baltic Sea (for a detailed description see Bitschofsky and Nausch, [27]). Samples were obtained from the upper freshwater course (W-1 to W-9) and from the brackish water part influenced through Baltic Sea coastal water (W-10). Baltic Sea coastal water samples were obtained from the pier at the site Heiligendamm (HD). The water samples were collected from a river bank or from bridges and filled into pre-rinsed polypropylene bottles. They were stored at −20 °C until further analysis.

2.3. Sample preparation

The final sample preparation included an enrichment and purification step on Strata-X-AW (500 mg/3 mL, Phenomenex, Aschaffenburg, Germany) cartridges with subsequent derivatisation of MPn to its silylated derivative. For this, the cartridges were conditioned with 5 mL methanol and water each. A volume of 5 mL water sample was spiked with the internal standard and directed through the column with a flow rate of approx. 1–2 mL/min. The column was washed with 2 mL MilliQ-water and methanol each and dried thereafter with a stream of nitrogen. Elution was conducted with 10 mL of 5% (v/v) NH_3 in methanol. The eluates were evaporated to dryness at 40 °C under a stream of clean-air (TurboVap, LV, Zymark, USA).

Derivatisation was done according to previously published protocols [8,9,28] with slight modifications. Thus, 100 µL ACN and 50 µL MTBSTFA were added to the dried sample and the reaction mixture was incubated for 2 h at 60 °C in a water bath. To extract the derivatisation products, 100 µL MilliQ-water and 250 µL *n*-hexane were added, the suspension was vortexed and centrifuged for phase separation (10 min, 1000 rpm). The above hexane layer was shortly dried on sodium sulphate and transferred into a GC-vial for GC-MS analysis.

During method development, sample preparations varied. For those data, methodological differences are stated in the further descriptions.

2.4. Instrumentation and GC-MS analysis of MPn-Derivatives

Analysis of derivatised methylphosphonic acid was conducted on a Trace-DSQ-GC-MS system (Thermo Fisher Scientific, Waltham, USA) equipped with a TriPlus autosampler. A DB-5MS capillary column (60 m × 0.25 mm I.D., 0.25 µm film thickness, Agilent, Waldbronn, Germany) was used for gas chromatographic separation. A sample volume of 1 µL was injected into the heated injector (50 °C) operating in splitless mode with a purge time of 1 min and a purge flow of 50 mL/min. Helium was used as carrier gas with an initial flow of 1.5 L/min. The oven temperature program started at a temperature of 50 °C for 1 min following a temperature ramp of 10 °C/min to a temperature of 220 °C and with a second ramp of 20 °C/min to 280 °C with a final hold for 20 min. The transfer line was set to 250 °C and the MS source to 240 °C. Electron impact (70 eV) was used for ionization.

Full scan mode (m/z = 50–550) was used to identify the retention times of the analytes and to determine the characteristic mass fragments, which were m/z = 267 and 309 for MPn and m/z = 270 and 312 for D_3-MPn. However, as the mass fragments m/z = 309, 312 had an abundance of only 4.5% of the more intensive fragments, we excluded them from the observation. Quantitative analysis was performed in the SIM mode with a dwell time of 100 ms for the monitored fragments.

Operation of the GC-MS system and evaluation of the data was done with XCalibur 3.0 (Thermo, USA). Only data with a signal-to-noise ratio (S/N) above 3 were used for further evaluation.

2.5. Calibration and quantification

D_3-MPn was used as internal standard (IS). Within this study, different concentration ranges of MPn were studied. For precise quantification including the low concentration range, calibrations for three concentration ranges were obtained. For each calibration range, a different amount of IS was used (Table Suppl. 2).

For validation, precision (RSD%) and accuracy (RE) were determined as described in Equations Suppl. 1 and 2.

Table 1
List of stations for the sampling of surface water including the corresponding sampling dates, the salinity and the detected MPn-concentration (n.d.: not detected, *: result achieved by exponential extrapolation).

Station	Coordinates	Sampling date	Salinity S [PSU scale]	MPn [µg/L]
Mühlenfließ (MF)	N54° 07.02 E11° 54.87	November 22, 2018	0.3	n.d.
Heiligendamm (HD)	N54° 08.78 E11° 50.60	September 25, 2018 /February 26, 2019	12.8 /10.4	1.8*
Warnow-1 (W1)	N53° 43.07 E11° 44.87	August 02, 2017	0.25	n.d.
Warnow-2 (W2)	N53° 44.70 E11° 49.95	August 02, 2017	0.26	n.d.
Warnow-3 (W3)	N53° 47.06 E11° 50.29	August 02, 2017	0.25	n.d.
Warnow-4 (W4)	N53° 50.27 E11° 58.55	August 01, 2017	0.26	n.d.
Warnow-5 (W5)	N53° 53.79 E12° 05.79	August 01, 2017	0.28	n.d.
Warnow-6 (W6)	N53° 56.85 E12° 07.25	August 01, 2017	0.29	n.d.
Warnow-7 (W7)	N54° 03.85 E12° 10.27	July 31, 2017 /December 07, 2018	0.29 /0.1	n.d. /0.41
Warnow-8 (W8)	N54° 04.69 E12° 09.26	December 07, 2018	0.1	0.47
Warnow-9 (W9)	N54° 05.03 E12° 09.08	July 31, 2017 /December 07, 2018	0.29 /0.1	n.d. /0.57
Warnow-10 (W10)	N54° 05.22 E12° 09.13	December 07, 2018	1.1	n.d.

2.6. Electrodialysis

The water sample collected at station Heiligendamm (HD) was subjected to electrodialysis (ED), in order to reduce their salt content and possibly improve the recovery of the subsequent SPE step. The procedure was conducted with an ED system from Deukum GmbH (Frickenhausen, Germany) as described by Wirth et al. [26]. In an electrodialysis cell, anion- and cation exchange membranes are alternatingly positioned between a set of electrodes. The sample (diluate) and a receiving solution (concentrate) are circulated through the alternating interspaces between the membranes. An electric field is applied perpendicular to the membrane surfaces, which causes salt ions in the diluate to be transported through the membranes and into the concentrate. A third solution (electrode rinse) is circulated along the electrodes to carry off oxygen and hydrogen gas formed due to water splitting at the electrodes.

The ED system was carefully cleaned with aqueous HCl (pH 2, 3%) and MilliQ water before sample processing. Afterwards, the filtered sample (GF/F filters Ø 47 mm, 0.7 µm; Whatman GmbH, Dassel, Germany) was filled into the diluate channel. The concentrate was a 0.2 g/L salt solution prepared with artificial sea salt in MilliQ water. The electrode rinse was a 5 g/L Na_2SO_4 solution prepared in MilliQ water. During the ED run, the diluate and concentrate were circulated in the system at a flow rate of 50 L/h, while the electrode rinse was circulated at 125 L/h. Desalination of the sample is achieved through the electric field provided by the laboratory power supply. The supplied current was adjusted so that it never exceeded 80% of the limiting current. In order to maintain the concentration gradient along with the membrane stack, ~80% of the concentrate was replaced with MilliQ water whenever concentrate conductivity was 2 mS/cm above diluate conductivity.

Subsamples from the diluate tank were taken at the beginning, as well as at conductivities of 15, 10, 5, 2.5, 1, 0.5 and 0.2 mS/cm. At 25 °C, this corresponds to salinities of 8.7, 5.6, 2.7, 1.3, 0.5, 0.2 and 0.1. After the final subsamples were taken, the current was switched off, the solutions were drained from the system and their residual volume was measured. Subsamples taken during the ED run were stored frozen at −20 °C until analysis.

The determined MPn concentrations were corrected for sample volume loss that occurred during the ED process. Small amounts of water (~0.2–0.4 L) are transported through the membranes alongside the salt. Therefore, the theoretical sample volume was corrected as described in Wirth et al. [26]. MPn recoveries were calculated using the determined concentrations and the sample volume, which was corrected for water loss and subsample removal.

Initially, to test the retention of MPn during the ED process, a coastal Heiligendamm water sample (Table 1) was spiked with MPn and processed in triplicate (MPn concentration in the ED start sample about 1 mg/L, see Wirth et al. [26]). During the ED, subsamples were taken at distinct sample conductivities and analysed for MPn.

3. Results and discussion

3.1. Instrumental calibration and validation

It was the aim to quantify MPn through the use of an isotopically-labelled internal standard. The ^{13}C-MPn and the D_3-MPn are currently commercially available. The ^{13}C-MPn has a mass difference of only 1 Da to the natural MPn. However, within our studies, we evaluated using ^{13}C-MPn as an internal standard as well. For this, a calibration in the range of 0.27–2.77 µg/mL was prepared (MPn-concentration in the measured solution, Fig. 1) and a non-linear course of the calibration curve, even at small MPn amounts, was observed. A statistical assessment revealed that a non-linear regression model fits best (Mandel: TV = 18.48, F(f1 = 1, f2 = N-3, P = 99%) = 12.25). It was discussed before by Rule et al. [29] that when using an isotopically-labelled compound as internal standard, interferences between the analyte and the internal standard may occur, leading to this non-linear behaviour. In the present case, the mass difference between the analyte and the internal standard was only 1 Da and, therefore, such interferences might play a crucial role.

Examination of the obtained mass spectra (Fig. 1) revealed an isotopic peak for natural MPn (m/z = 268), which overlays with the mass fragment of the ^{13}C-MPn used for quantification. Moreover, the signal of the mass fragment m/z = 266.9 of ^{13}C-MPn contributes to one of the characteristic mass fragments of ^{12}C-MPn. This is what Rule et al. described as the basic underlying cause for the interference [29]. Therefore, the non-linear fitting curve of the calibration data was calculated according to their study with Equation Suppl. 3 and compared to a linear fit. Obtained R^2-values for both regression models indicate a higher correlation to the fitted non-linear regression (R^2 = 0.997) than for the linear (R^2 = 0.967). As it can be seen in Fig. 1, using a linear regression model might cause overestimation of the concentration levels in the low and high concentration ranges and underestimation in the middle concentration range, which is in agreement with the results obtained by Rule et al. [29].

Since the deuterated MPn (D_3-MPn) internal standard has a mass difference of 3 Da to the natural MPn, linearity over a wide MPn mass range can be assumed. We obtained calibrations with the D_3-MPn as internal standard up to 2 µg/mL of MPn (Fig. 2, Table Suppl. 2) and the linearity test (F-test) revealed that a linear regression model fits best for all measured concentration ranges (see Table Suppl. 2 for further figures of merit of the calibration).

To further characterize the method, the instrumental limit of detection (LOD, resp. detection capability L_D [30]) was assessed from the calibrations as described by Miller and Miller (Equation Suppl. 4) [31] and was 0.008 µg/mL respectively 8 µg/L (MPn-concentration in the final measured solution). This is in the same range as another method published before by Baygildiev et al., which was based on LC-MS/MS and was characterised by a LOD of 10 µg/L [14]. Compared to other

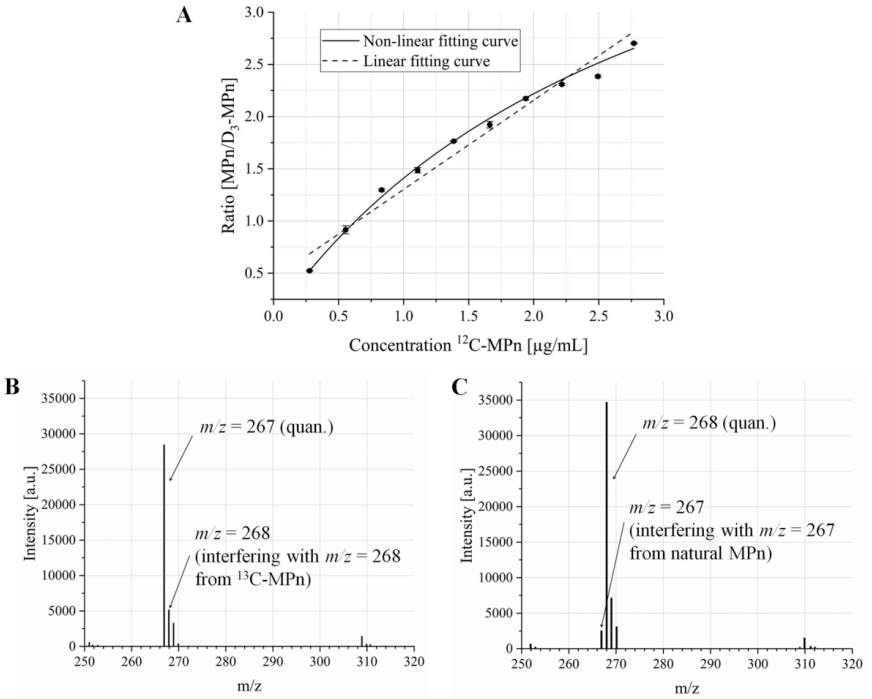

Fig. 1. A: Calibration of MPn using ^{13}C-MPn as internal standard (c = 0.333 µg/mL in measured solution). A linear fit as well as a non-linear fit are shown. Here, the samples were derivatised at 80 °C for 2 h in ACN/MTBSTFA (100 µL/50 µL). Afterwards the solution was diluted 1:10 with ACN and directly conducted to GC-MS-analysis. Each data point was achieved by triplicate injection; B: Mass spectra of the derivatised natural ^{12}C-MPn standard in the range of *m/z* = 250–320 (c = 0.243 µg/mL in measured solution); C: Mass spectra of the derivatised ^{13}C-MPn standard in the range of *m/z* = 250–320 (c = 0.666 µg/mL in measured solution).

GC-MS-based methods by Richardson and Caruso (LOD = 5 µg/L, [8]) or by Singh et al. (LOD = 0.1 µg/L, [11]), our method showed a slightly higher LOD [8,11]. For this current study, we decided to continue with the D_3-MPn as internal standard compound.

For quality assurance, MPn control samples were regularly included into sample sequences to verify the instrumental calibration (e.g., Table Suppl. 3A). The obtained data show that the described method leads to satisfying results for MPn-analysis for the tested combinations. Accuracy was mostly below 20%; however, in the lower concentration range, higher uncertainties must be considered. With RSD of less than 15%, the method can be considered as precise.

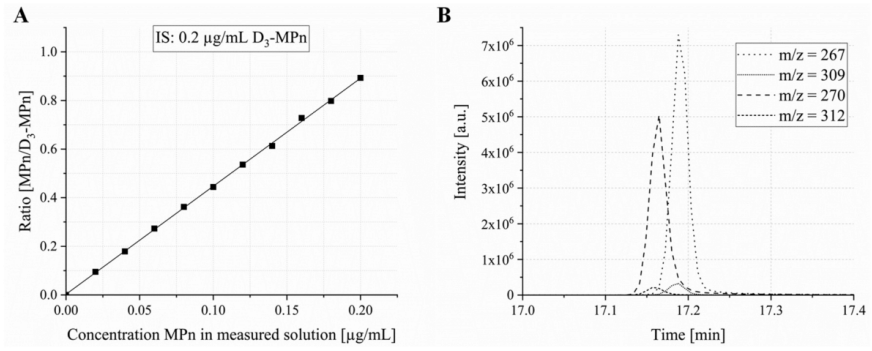

Fig. 2. A: Calibration curve for MPn using D_3-MPn as internal standard. Each data point represents a single measurement; B: Chromatograms of MPn (1 µg/mL) and D_3-MPn (0.8 µg/mL). For each substance, both recorded mass fragments are shown.

3.2. Development of sample enrichment through SPE

Solid-phase extraction of MPn is necessary in order to change the sample matrix, as the silylation reaction cannot be conducted in water. Evaporation of the water matrix with a stream of nitrogen or through rotary evaporation, as it was done by Richardson and Caruso [8], was not successful, probably due to the salt residue which was obtained even in samples with low salinity (S ~0.1, data not shown), inhibiting subsequent derivatisation.

Two different SPE materials were tested for their ability to extract MPn from brackish water samples. Strata-X is a polymeric reversed-phase material, which presumably interacts with the methyl group of MPn. This material was used by Owens and Koester for analysis of MPn in beverages with a recovery of about 30% for most matrices [13]. The second SPE material investigated was the Strata-X-AW material, which is a weak anion-exchange material. Both materials were previously used for MPn analysis [21,32]. However, the influence of salt-containing matrices on the recovery is poorly studied [19].

Our results (Fig. 3) show that MPn is retained to different degrees by the tested SPE materials when MPn was applied in MilliQ-water, with superior recovery with the ion-exchange material (Fig. 3C). Upon using salt-containing matrices, MPn was hardly detectable with the reversed-phase material (Fig. 3A and B), which might result from salt precipitations interfering with the derivatisation procedure. To mitigate this, a washing step with 2 mL of MilliQ-water (pH 1) was added after the sample was loaded onto the SPE cartridge. With the washing step, the salt residue in the eluent was reduced. However, the recovery also for MilliQ-water samples clearly decreased (Fig. 3A and B). This implies that the interaction between MPn and the reversed-phase material is too weak and, therefore, we precluded this material for further use.

Upon using the weak anion-exchange material, high recovery (about 100%) of MPn was obtained for MilliQ-water as the matrix (Fig. 3C), which is in good agreement with earlier studies [20]. However, for salt-containing water samples, a clear decrease in the recovery from about 100% to 4% was observed (Fig. 3C). We think that this is due to the matrix anions which probably compete with the target analyte for the active sites of the sorbent. Furthermore, the matrix

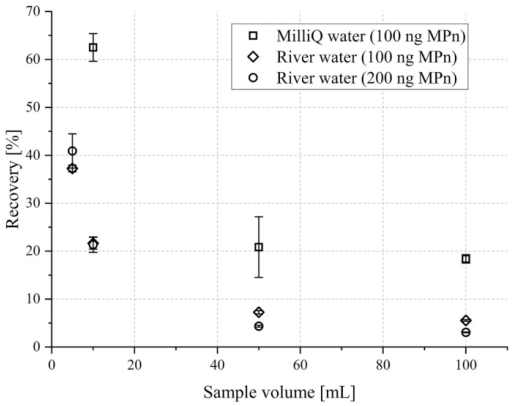

Fig. 4. Recovery of MPn after SPE from different matrices depending on the sample volume. The given amount of MPn is the total amount added to the different sample volumes prior the SPE processing.

cations might increase the eluting strength of the matrix. Therefore, we conclude that this SPE material should be used only for low salinity, i.e. fresh and brackish water samples.

To investigate the operational capability of the entire method, the SPE step was further characterised, i.e. with respect to the influence of the low salinity matrix, sample volume and MPn concentration. For this, we utilized MilliQ as matrix-free sample and a river sample of low salinity (river Mühlenfließ, Table 1) spiked with MPn. Before the analysis, the samples were analysed for MPn, which was below LOD for both.

Fig. 4 shows the obtained recovery for MPn in the spiked MilliQ- and river samples. A clear difference between the recovery of MPn in 10 mL of MilliQ- (~60%) and river water (~20%) was obtained. The fact that the salinity of the river sample was only 0.3 indicates that not

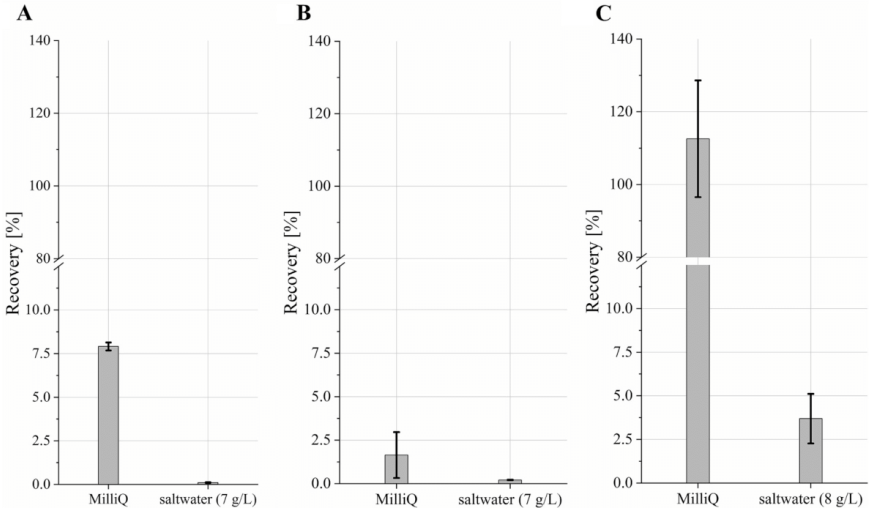

Fig. 3. Comparison of two different SPE materials and recovery of MPn from different water matrices (2 mL each). A: Strata-X (500 mg/3 mL, Phenomenex, Aschaffenburg, Germany) without washing step (Conditioning: 10 mL MeOH, 10 mL pH 1 water /loaded sample acidified to pH 1/elution: 10 mL ACN) B: Strata-X incl. a washing step C: Strata-X-AW incl. a. washing step. Error bars present triplicate injections.

only the salt matrix interferes with the SPE, but also other matrix components. With increasing sample volume, MPn recovery decreased further for MilliQ as well as for river water. Therefore, only small sample volumes of river water should be used for quantitative analysis, because even if the internal standard corrects for losses during SPE fortification, a low recovery has a large impact on the sensitivity of the method. Thus, for the river sample, a sample volume of 5 mL was additionally tested. The results show that the recovery for 5 mL is twice as high as for 10 mL. Based on these results and to keep matrix effects to a minimum, we suggest using sample volumes of not more than 5–10 mL, depending on the sample type and, in particular, the sample matrix.. Furthermore, Fig. 4 indicates that the total amount of MPn in the river sample is not affecting the recovery of MPn, when a total amount of up to 200 ng is added to the SPE-cartridge. On the basis of these results, we conclude that the recovery of MPn from the SPE depends on sample matrix and also largely on the sample volume. Hence, MPn fortification through SPE is strongly limited.

Moreover, the method was validated for MilliQ and river water samples which were processed through SPE in terms of accuracy (RE) and reproducibility (RSD%) by triplicate analysis at two different MPn-levels (Table Suppl. 3B). With obtained data for RSD% and RE% below 15%, the results indicate that the method is valid even with decreased recovery at a larger sample volume of 100 mL.

Finally, considering the determined instrumental LOD of 8 µg/L MPn and a sample volume of 5 mL, the entire method is characterised by a method LOD of 0.4 µg/L in the initial water sample. This is comparable to other methods published previously [12,14,33].

To test whether the instrumental calibration is also valid for the samples after their processing through SPE as well as in view of possible matrix effects, we conducted matrix-matched calibration in 10 mL of MilliQ and river water, obtained from Mühlenfließ, which were spiked with MPn up to 500 ng and D_3-MPn (200 ng) as internal standard. MPn concentrations were determined using the instrumental calibration and compared to the spiked concentration (Figure Suppl. 1). For statistical analysis of the method accuracy, the elliptic joint confidence region (EJCR) for the true slope and intercept of the linear regression were calculated (Figure Suppl. 1) [34]. The ideal point (0; 1 (intercept; slope)) is within the ellipse for river water, but not for MilliQ-water. This results from the larger area of the ellipse for river water, which is due to the lower precision of the river water data compared to the MilliQ-water data. However, this analysis shows that for matrix affected environmental samples the analytical method results in accurate quantitative data.

3.3. Matrix reduction through electrodialysis

As the sample matrix was identified to be an obstacle for MPn sample preparation, electrodialysis was tested to reduce the seawater matrix load and to analyse the effect of reducing salinity on the efficiency of the solid-phase extraction. In a recent study by Wirth et al. it was shown that at a final salinity of 0.1 not more than 30% of initially added MPn could be recovered [26]. However, aiming at optimising the method for MPn analysis, we analysed MPn recovery during the electrodialysis course in more detail (Fig. 5A). Basically, the recovery of MPn during the ED follows an exponential course with a sample salinity of about 1.4 as the critical point at which MPn is lost through the electrodialysis membrane, which is probably due to the ionic behaviour of MPn. With a $\log K_{OW}$ of −2.28, MPn is a very hydrophilic compound [35]. In addition, during electrodialysis, pH usually slightly increased up to a pH of about 7.8, so that MPn partially was twice negatively charged [26,36]. This presumably led to an increasing loss during electrodialysis with decreasing salinity, as it was described for other hydrophilic compounds such as glyphosate [26]. However, our data show that MPn recovery stays above 90% until a salinity of 1.4, which implies that until this point, the sample matrix can be reduced without major MPn loss. However, the influence of sample matrices on sample preparation may vary significantly from one sample to another. Therefore, for MPn analysis, the electrodialysis terminating point is a compromise of MPn recovery and salt tolerance of subsequent

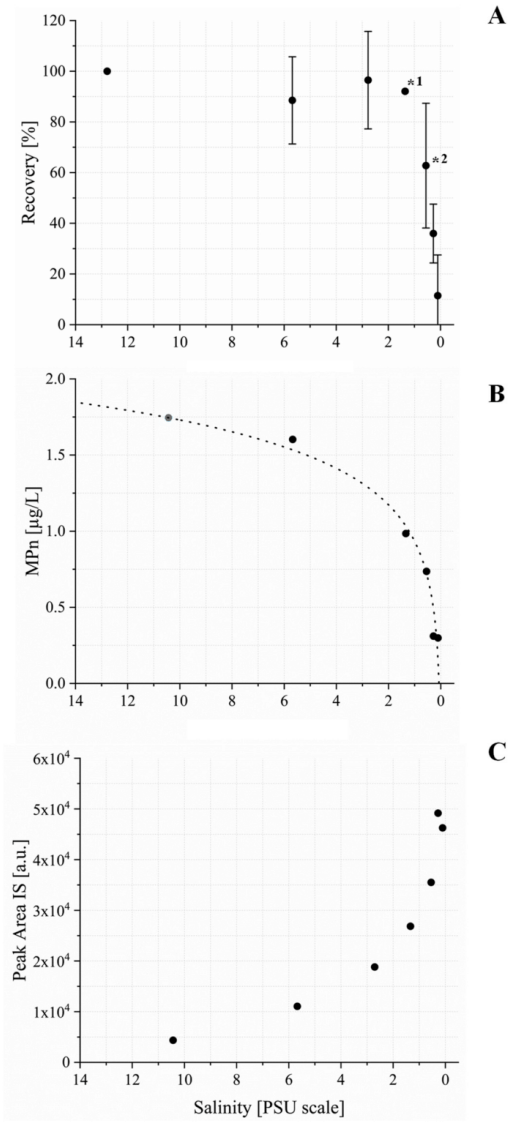

Fig. 5. A: Three batches of the same coastal water sample which were spiked with MPn were electrodialysed and subsamples at distinct sample conductivities were analysed for MPn. All measured values were corrected by the MPn-blank of the individual run (*1 single analysis, *2 duplicate analysis, see also [26]) B: A coastal water sample from the same site (without MPn addition) was electrodialysed and subsamples were analysed for MPn (bright circle: calculated MPn concentration of 1.8 µg/L upon an exponential correlation of the obtained MPn data). All measured values were blank-corrected. C: The IS peak area of the MPn measurements in B increased exponentially.

processing steps as it was discussed before [26]. In this regard, the application of an internal standard before electrodialysis processing seems essential.

However, this analysis also showed that MPn is detectable at the initial marine water salinity if concentrations are high enough. Thus, the matrix *per se* does not inhibit MPn recovery from SPE, but it is determined, on the one hand, by the sample's MPn concentration and, on the other hand, by the sample matrix.

3.4. Environmental samples

The described method was then tested by analysing several samples from the river Warnow, located in Mecklenburg-Western Pomerania, Germany (Section 2.6, Table 1). Analysis of the samples W1 – W7 and W9 from the freshwater part of the Warnow collected in summer 2017 and from the river Mühlenfließ in December 2018 revealed MPn concentrations below LOD. However, we observed MPn concentrations between 0.4 and 0.6 µg/L for the freshwater samples W7 – W9 collected in winter 2018. MPn was not detectable at the adjacent site W10 which might be derived from matrix dependent lower SPE recovery due to the higher salinity of 1.1 at this site compared to W7 – W9. The samples W7 and W9 were analysed twice (Table Suppl. 3C) and the results indicate satisfactory values for the precision of the method.

Several publications indicate the possible role of MPn in the marine methane system [5,37,38]. However, there are hardly any data for MPn in marine water samples available, which we attribute to missing analytical methods for this challenging matrix. It was already shown above, that the SPE-method is sensitive to the salt matrix resulting in decreased recovery. To test if a reduction of the seawater matrix might enable MPn analysis in these sample types, a Baltic Sea coastal water sample (Heiligendamm, salinity 10.4, Table 1) was electrodialysed and subsamples at distinct sample conductivities were analysed for MPn (Fig. 5B). Interestingly, at sea salt reduction to a salinity of 5.6, MPn was already detectable. At lower salinities, MPn concentrations decreased exponentially, as described above for the spiked water sample (Fig. 5A and B). Based on the determined MPn concentration at the salinity of 5.6 and the exponential course of MPn loss during the ED, an exponential extrapolation was used to determine an approximate value for the MPn-concentration in the initial sample. Based on this, we propose an MPn concentration of about 1.8 µg/L in the initial water sample (exponential correlation: $R^2 = 0.9486$). At this point, it is certainly preferable to determine the MPn-concentration by direct quantification, i.e., through addition of the internal standard before the electrodialysis. However, for the herein utilized electrodialysis system a sample volume of at least 3 L has to be processed which would be very costly. However, our results indicate that the approach through extrapolation results in reliable semi-quantitative data which might provide a first indication on the concentration range of MPn in marine water samples. Here, more work is necessary for reliable MPn quantification. In this regard, a reduction of the sample volume after the electrodialysis, e.g. through reverse osmosis, might further improve the sensitivity of the method [39].

The improvement of the sensitivity of the sample preparation method through reduction of the sample matrix can also be viewed through the determined peak area of the IS, which was added in same concentrations to the subsamples after electrodialysis (Fig. 5C). It increased exponentially with decreasing sample salinity, which is due to the higher recovery of the SPE-method at lower values for salinity.

Based on our obtained data, we postulate different ranges of MPn concentrations for the analysed river- and coastal-water samples. Our data indicate that MPn is with a concentration above 1 µg/L by far higher concentrated in coastal water than in river water, which was mostly below LOD. This implies a particular relevance of MPn for the marine environment, which was discussed previously [5,37,38].

4. Conclusion

This study presents an analytical method for the quantitative determination of methylphosphonic acid in environmental water samples. The critical extraction of MPn from different water samples with sufficient recovery was done by using a weak-anion exchange SPE-material with sufficient recovery. We show that recovery of MPn using SPE decreases with increasing salt concentration and, consequently, the analysis of brackish as well as marine samples results in lower sensitivity. To overcome this problem, the use of electrodialysis for desalting of water samples was successfully tested for a brackish water sample. Finally, MPn was detected at a low µg/L-level in water samples from the German river Warnow and the German Baltic Sea coastal area.

The presented method enables the possibility to quantify MPn in different aquatic systems. Therefore, the results of this work may help to study the role of MPn in these systems. As described before, an important role of MPn in aquatic systems was often assumed [4,5]. Our results indicate that MPn is present in river as well as coastal waters. However, more work is necessary to fully understand sinks and sources of MPn, as well as regional and seasonal changes of its concentration.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.120724.

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Publication 3

The influence of salt matrices on the reversed-phase liquid chromatography behavior and electrospray ionization tandem mass spectrometry detection of glyphosate, glufosinate, aminomethylphosphonic acid and 2-aminoethylphosphonic acid in water

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The influence of salt matrices on the reversed-phase liquid chromatography behavior and electrospray ionization tandem mass spectrometry detection of glyphosate, glufosinate, aminomethylphosphonic acid and 2-aminoethylphosphonic acid in water

Wael Skeff*, Constantin Recknagel, Detlef E. Schulz-Bull

Leibniz Institute for Baltic Sea Research Warnemuende, Department of Marine Chemistry, Seestrasse 15, 18119 Rostock, Germany

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ABSTRACT

The analysis of highly polar and amphoteric compounds in seawater is a continuing challenge in analytical chemistry due to the possible formation of complexes with the metal cations present in salt-based matrices. Here we provide information for the development of analytical methods for glyphosate, glufosinate, AMPA, and 2-AEP in salt water, based on studies of the effects of salt matrices on reversed-phase liquid chromatography–heated electrospray ionization-tandem mass spectrometry (RP-LC-HESI–MS/MS) after derivatization of the target compounds with FMOC-Cl. The results showed that glyphosate was the only analyte with a strong tendency to form glyphosate-metal complexes (GMC), which clearly influenced the analysis. The retention times (RTs) of GMC and free glyphosate differed by approximately 7.00 min, reflecting their distinct RP-LC behaviors. Divalent cations, but not monovalent (Na^+, K^+) or trivalent (Al^{3+}, Fe^{3+}) cations, contributed to this effect and their influence was concentration-dependent. In addition, Cu^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} prevented glyphosate detection whereas Ca^{2+}, Mg^{2+}, and Sr^{2+} altered the retention time. At certain tested concentrations of Ca^{2+} and Sr^{2+} glyphosate yielded two peaks, which violated the fundamental rule of LC, that under the same analytical conditions a single substance yields only one LC-peak with a specific RT. Salt-matrix-induced ion suppression was observed for all analytes, especially under high salt concentrations. For glyphosate and AMPA, the use of isotopically labeled internal standards well-corrected the salt-matrix effects, with better results achieved for glufosinate and 2-AEP with the AMPA internal standard than with the glyphosate internal standard. Thus, our study demonstrated that Ca^{2+}, Mg^{2+}, and Sr^{2+} can be used together with FMOC-Cl to form GMC-FMOC which is suitable for RP-LC-HESI–MS/MS analysis.

1. Introduction

Glyphosate (N-phosphonomethyl-glycine) and glufosinate (ammonium dl-homoalanin-4-(methyl) phosphinate) are post-emergence, non-selective, broad-spectrum organophosphorus herbicides intensively used for agricultural and non-agricultural purposes [1]. Their application is growing very rapidly worldwide, mainly due to the development of crops resistant to these herbicides [2]. Aminomethylphosphonic acid (AMPA) is the main metabolite of glyphosate detected in different environmental compartments, such as soil, water, and plants [3]. It is also a key metabolite that can be formed during the degradation of industrial phosphonates used in detergents, laundry agents, and the textile industry, such as ATMP (aminotrimethylene phosphonic acid) and DTPMP (diethylenetriamine pentamethylene-phosphonic acid) [4,5]. The phosphonic acid moiety of these compounds enables their strong adsorption on soils, leading to their low mobility and low potential for contaminating aquatic resources [6–8]. However, despite their high adsorption tendencies, these substances have been detected in different aqueous environments, where they have caused water contamination [1,8,9]. 2-aminoethylphosphonic acid (2-AEP) is a biogenic phosphonate. It has been identified in many types of plankton feeders and marine invertebrates [10]. However,

* Corresponding author.
E-mail addresses: wael.skeff@io-warnemuende.de, drei-stein@hotmail.com (W. Skeff).

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information on the presence of 2-AEP in waters is scarce because of the lack of suitable analytical methods.

The physicochemical characteristics of these analytes, such as their low molecular weight, high polarity, low volatility, thermal liability, high solubility in water, and low solubility in non-polar organic solvents make their analysis using liquid chromatography (LC) more suitable than gas chromatography (GC). Due to the absence of requisite chemical groups (e.g., chromophores and those absorbing UV) their detection using conventional detectors is inadequate [1]. Liquid chromatography coupled with electrospray ionization interface and mass spectrometry (LC-ESI–MS/MS) is a common analytical technique used in environmental samples, due to its high sensitivity, selectivity, and throughput [1,11,12]. Several different LC columns have been employed for the analysis of glyphosate, glufosinate, and AMPA in water, including C18 reversed-phase, anion exchange, mixed mode (C18/anion exchange), cation exchange, –NH2, IonPac, and Hypersil APS [3]. Reversed-phase (RP) in combination with LC-ESI–MS/MS after prederivatization of the compound of interest with 9-fluorenylmethyl chloroformate (FMOC-Cl) is the predominant analytical method for these highly polar and amphoteric compounds in different water matrices, including ground water, drinking water, rain, riverine, and estuarine waters [1,9,11–13]. However, matrix effects caused by the elution of other compounds with the analytes of interest are a major obstacle in the development of analytical LC-ESI–MS/MS methods [14]. The majority of studies of these matrix effects have focused on the interface between LC and MS (e.g., ESI). Their results showed the suppression or enhancement of analyte signals, leading to inaccurate and imprecise quantitative analyses [15]. In addition, Fang et al. [16] showed that the sample matrices can clearly alter the LC behavior of the analytes of interest, such that they are incorrectly identified. These matrix effects are highly diverse and depend on the nature of the matrix, the analytes, the sample preparation methods, and the instruments used in the analysis [17,18]. The amphoteric compounds glyphosate, glufosinate, AMPA, and 2-AEP, with functional groups such as phosphonate, amino, and carboxylate, are expected to be more vulnerable to sample-matrix effects due to the possible formation of pH-dependent complexes with metals ions and mineral surfaces [19–21]. Consequently, their analysis in samples containing metal ions, such as occurs in seawater samples, can be very difficult.

Studies of the presence of glufosinate and 2-AEP in seawater have yet to be published and only a very few have investigated glyphosate and AMPA. In the study of German estuaries that feed into the Baltic Sea, Skeff et al. [12] reported the detection of glyphosate and AMPA at concentrations up to μg/L level, sufficient to contaminate estuarine waters. However, that method was not sensitive enough to determine the levels of these compounds in open seawater samples (i.e. lower concentrations and higher salt contents). The objective of the present work was to obtain information on the influence of salt matrices in analyses of glyphosate, glufosinate, AMPA, and 2-AEP using RP-LC-HESI–MS/MS after derivatization of the analytes with FMOC-Cl in water. Matrix effects on the identification and quantification of these compounds were investigated. The results enabled the formulation of methodological approaches that resolve the problems posed by salt-matrix effects in LC–MS/MS studies.

2. Experimental

2.1. Chemicals and reagents

Stock solutions of glyphosate, a glyphosate internal standard (1-2-$^{13}C_2$ ^{15}N glyphosate), AMPA, and an AMPA internal standard (^{13}C ^{15}N AMPA), each at a concentration of 100 ng/μL in water, were supplied together with glufosinate ammonium, at a concentration of 100 ng/μL in methanol, by Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2-AEP was purchased from Sigma-Aldrich (Taufkirchen, Germany). A stock solution of 2-AEP, at a concentration of 100 ng/μL, was prepared by dissolving 10 mg in 100 mL LC–MS-grade water (VWR international GmbH, Darmstadt, Germany). All standards were stored at 5 °C in the dark. Borate buffer (pH 9) was prepared by dissolving 1 g of sodium tetraborate decahydrate (Sigma-Aldrich) in 50 mL of Milli-Q water (Merck KGaA, Darmstadt, Germany). A stock solution of FMOC-Cl (purity 99.0%, Sigma-Aldrich) was prepared by dissolving 1 g in 58 mL of acetonitrile (Walter-CMP GmbH, Kiel, Germany), yielding a final concentration of 66.6 mM. The FMOC-Cl solution was stored in a brown glass flask in the dark at 5 °C. A working solution was prepared daily by diluting the appropriate amount of the stock solution in acetonitrile using a glass syringe. Eluent A (2 mM NH_4HCO_3) was prepared by first dissolving 158 mg of ammonium bicarbonate (Sigma-Aldrich) in 1 L of LC–MS-grade water and then adding 100 μL of ammonia solution (32% v/v; Sigma-Aldrich) to adjust the pH to 9. Artificial sea salt was purchased from Tropic Marin®, Germany. The following salts were dissolved in LC–MS-grade water based on the required concentrations: $CaCl_2$ (AppliChem; Darmstadt, Germany), $AlCl_3$ (Alfa Aesar; Karlsruhe, Germany), and $ZnSO_4{}^*H_2O$ (Sigma-Aldrich). $CuSO_4{}^*5H_2O$, $MgCl_2$, NaCl, and $SrCl_2$ were obtained from Merck, and $CoSO_4{}^*7H_2O$, $MnSO_4{}^*H_2O$, $FeCl_3$ and KCl from VWR.

2.2. Sample preparation and derivatization

Working solutions of the target compounds, including the internal standards, were prepared in LC–MS-grade water at a concentration of 100 μg/L. An appropriate volume of the working solution was diluted with LC–MS-grade water, either alone or with the different dissolved salts, to the required concentration. 800 μL of each sample was then transferred to a 2 mL Eppendorf vial, adjusted to pH 9 by adding 100 μL of borate buffer, and treated with 100 μL of 3.33 mM FMOC-Cl in acetonitrile to allow their derivatization. After 2 h of incubation at the lab temperature of 21 °C, the derivatized samples were filtered through a 0.45 μm Phenex-RC 15 mm syringe filter (Phenomenex, Germany) and subjected to LC–MS/MS.

2.3. Instrumentation

The target substances were analyzed on a LC–MS/MS system (Thermo Fisher Scientific; Dreieich, Germany) comprising an Accela autosampler (series: 750477), an Accela pump (series: 700862), and a Maylab MistraSwitch column oven (model 886, series: 100027). The TSQ Vantage triple-quadrupole mass analyzer (series: TQU 02725) was equipped with a heated electrospray ionization source interface (HESI). Instrument operation as well as data processing and evaluation were managed using XCalibur® version 2.1 (Thermo Fisher). Chromatographic separation was carried out at 20 °C on a Gemini-NX C18 column (150 × 2.0 mm, 3 μm) coupled to a 4 × 2.0 mm Gemini-NX Security Guard cartridge (Phenomenex, Germany). The LC and HESI–MS/MS parameters were developed in previous work, in which glyphosate and AMPA in estuarine water were analyzed [12]. All analytes were ionized in negative ionization mode. The collision energy and S-lens values were optimized for each compound. Data were acquired in selected reaction monitoring (SRM) mode. Two transitions per substance, including the internal standards, were chosen. The most abundant transition was used for quantification and the other transition for confirmation. Details of the parent ions, product ions, quantifier ions (Qn), qual-

Table 1
The parent ions, product ions, quantifier ions (Qn), qualifier ions (Ql), collision energy (CE), and applied S-lens values of the target analytes.

Analyte	Parent (m/z)	Product (m/z)	Ion	CE (eV)	S-lens (V)
Glyphosate	390	168	Qn	16	90
	390	150	Ql	26	90
Glyphosate-IS	393	171	Qn	17	93
	393	153	Ql	31	93
Glufosinate	402	180	Qn	17	80
	402	206	Ql	17	80
AMPA	332	110	Qn	14	52
	332	136	Ql	21	52
AMPA-IS	334	112	Qn	18	53
	334	138	Ql	20	53
2-AEP	346	124	Qn	16	63
	346	79	Ql	52	63

IS: internal standard.

ified ions (Ql), collision energy, and S-lens values of the selected analytes are provided in Table 1.

2.4. Statistical analysis

Data regarding the effect of salt on the derivatization reaction (Section 3.2) are represented as mean ± standard deviation (SD) of three independent experiments, each measured in triplicates. One-way ANOVA followed by Holm-Sidak post-hoc test was used to compare different levels among the different treatment groups. The test was carried out using SigmaPlot software (version 13.0, Systat software Inc.). A P-value of less than 0.05 was expressed as statistically significant. The lack of fit test was applied for the validation of the linear calibration curves (triplicate injections) according to the guideline of the Analytical Method Committee [22]. This test was performed using the OriginPro 2016 software (OriginLab Corporation).

3. Results and discussion

3.1. Influence of the salt matrix on the RP-LC behaviors of the target analytes

The effect of a salt matrix on the RP-LC behavior of glyphosate, glufosinate, AMPA, and 2-AEP in water and, consequently, on the identification of these compounds was investigated. The four compounds as well as the internal standards, each at a concentration of 5 μg/L, were prepared in two different matrix solutions: LC–MS-grade water, presumably free of metal ions, and an ionic solution of artificial sea salt dissolved in LC–MS-grade water, yielding a final salt concentration of 4 g/L. Samples taken from both solutions were derivatized with FMOC-Cl at pH 9 (Fig. 1A) and analyzed under the same LC–MS/MS conditions. The transition ions of the selected analytes (Table 1) were monitored using the SRM mode from 3.00 min until the end of the procedure (30.00 min), which allowed the detection of all potential peaks formed by the target transitions.

Two different RP-C18 columns were tested for analyte separation: a Gemini-NX C18 column (150 × 2.0 mm, 3 μm) and a Kinetex® EVO C18 column (150 × 2.1 mm, 2.6 μm). SRM chromatograms obtained from the analysis using the Gemini-NX column are shown in Fig. 2 and those obtained using the Kinetex® EVO column in Supplementary data Fig. S1. The results showed important effects of the salt matrix on the glyphosate RT whereas the effects on the RTs of glufosinate, AMPA, and 2-AEP were negligible, on the order of few seconds. For both columns, the glyphosate RTs measured in the salt samples were increased by >6.00 min compared to those measured in LC–MS-grade water. This was attributed to the interaction of glyphosate with the metal ions present in the salt matrix, resulting in the formation of stable glyphosate-metal complexes (GMC) at pH 9, the pH used for the derivatization reaction. When run on the Gemini-NX column, glyphosate was eluted as a free analyte at RT ≈ 6.00 min and as a complex at RT ≈ 13.00 min.

The elution of glyphosate at different RTs can be explained by the diverse RP-LC behaviors of free glyphosate and GMC, which in turn may reflect differences in the interaction forces of these compounds with the stationary phase of the column and the different eluting strengths of the mobile phase. Due to the different molecular masses of GMC and free glyphosate, diverse parent ions should be formed in the ionization source. The formation of same parent ion (m/z 390) by the two analytes may have been caused due to the thermal liability of GMC derivative in the HESI interface during the ionization process (vaporizer temperature 200 °C). This may have induced derivatized free glyphosate formation [FG-FMOC − H]$^-$ and, consequently, the production of the same product ions (m/z 150 and 168) in the second quadrupole (Q2). Glyphosate differs from glufosinate, AMPA, and 2-AEP mainly by its secondary amine as well as the inclusion in its chemical structure of different functional groups, such as a hydroxyl group in the case of glufosinate and carboxyl groups in the case of AMPA and 2-AEP (Fig. 1). Interactions of glufosinate and AMPA with metal ions that result in the formation of pH-dependent complexes have been reported [23–25]. At pH 9, only the analysis of glyphosate was altered by the salt matrix, suggesting its tendency to form GMCs probably coordinated via the oxygen in the phosphonic and carboxylic groups of glyphosate and the metal cations (Fig. 1B) [25,26]. Our results suggest that the hydrogen atom of the amino group of glyphosate does not contribute in GMC formation at pH 9, the condition allowing glyphosate to be derivatized with FMOC-Cl but in this case yielding derivatized GMC (Fig. 1B).

The influence of GMC formation on glyphosate analyses has been investigated in only a few studies [11,20,27], which reported negative effects of GMC formation either during the derivatization reaction with FMOC-Cl, the enrichment step, or during detection. To the best of our knowledge, the present work is the first to examine the influence of GMC formation on RP-LC behavior and MS/MS-based detection. A basic principle employed in LC–MS aimed at identifying unknown compounds in sample matrices is a comparison of the RTs resulting from their analysis in standard solutions with the RTs obtained from their analysis in sample matrices, in which one chemical compound forms one LC-peak with a reliable RT. Our results show that sample matrices such as a salt matrix can clearly influence the LC-behavior of analytes, by yielding LC-peaks at RTs different than those obtained in standard solutions. If this is the sole method of identification or if automation software is used for this purpose then errors may occur, such that the target compounds will be incorrectly identified.

In seawater, dissolved metal cations are major or minor, depending on whether they are present at concentrations greater or less than 1 mg/L, respectively. Major cations, such as Na^+, Mg^{2+}, Ca^{2+}, K^+, and Sr^{2+}, generally behave conservatively; minor cations include Zn^{2+}, Fe^{3+}, Al^{3+}, Cu^{2+}, Mn^{2+}, and Co^{2+} [28]. To determine which cations contributed to the drift of the glyphosate RT, the influence of each one was independently investigated by spiking 5 μg of the analytes/L with LC–MS-grade water supplemented with the cations, representative of their concentrations in 4 g/L sea salt matrix. As seen in Table 2, the two main divalent cations, Mg^{2+} and Ca^{2+}, participated in the glyphosate RT shift by forming GMC. This result is in agreement with those of other studies showing that Mg^{2+} and Ca^{2+} are the cations that will most likely coordinate with glyphosate in GMC formation [25,29]. The two complexes, glyphosate-Ca^{2+} and glyphosate-Mg^{2+}, showed the same RP-LC behavior, yielding a single LC-peak at RT ≈ 13.00 min. None of the

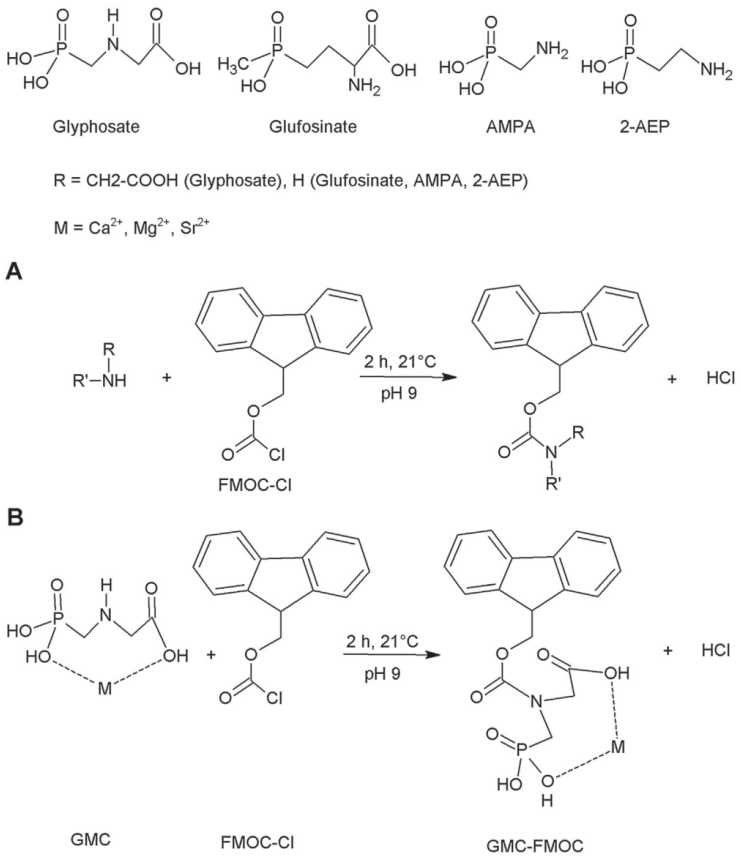

Fig 1. FMOC-Cl derivatization of A. glyphosate, glufosinate, AMPA, and 2-AEP and B. glyphosate-metal complexes (GMC). R' is the rest of the molecule after bounding R to the nitrogen atom.

Table 2
The retention times (RTs) of glyphosate based on its analysis in solutions supplemented with different concentrations of the major cations (Na^+, Mg^{2+}, Ca^{2+}, K^+, Sr^{2+}), six minor cations (Zn^{2+}, Fe^{3+}, Al^{3+}, Cu^{2+}, Mn^{2+}, Co^{2+}), or a mixture of the major (Mix. major) and minor (Mix. minor) cations. The RTs of glyphosate and glyphosate-IS are represented as mean ± SD based on three measurements of each sample.

Cation	Concentration	Glyphosate	Glyphosate-IS	Elution form
	(mg/L)	RT ± SD (min)	RT ± SD (min)	
Na^+	1234.3	5.95 ± 0.01	5.95 ± 0.02	Free
Mg^{2+}	147.4	13.08 ± 0.02	13.07 ± 0.03	Complex
Ca^{2+}	47.0	13.04 ± 0.02	13.03 ± 0.03	Complex
K^+	44.8	5.99 ± 0.01	5.99 ± 0.01	Free
Sr^{2+}	0.9	5.98 ± 0.00	5.98 ± 0.01	Free
Mix. major	1474.4	13.07 ± 0.05	13.06 ± 0.04	Complex
Zn^{2+}	5.7×10^{-4}	5.98 ± 0.01	5.98 ± 0.01	Free
Fe^{3+}	3.9×10^{-4}	5.99 ± 0.01	5.98 ± 0.00	Free
Al^{3+}	1.1×10^{-4}	5.99 ± 0.01	5.99 ± 0.01	Free
Cu^{2+}	1.0×10^{-4}	5.98 ± 0.01	5.98 ± 0.00	Free
Mn^{2+}	4.6×10^{-5}	5.98 ± 0.01	5.97 ± 0.01	Free
Co^{2+}	4.6×10^{-6}	5.99 ± 0.01	5.99 ± 0.01	Free
Mix. minor	2.5×10^{-3}	5.98 ± 0.01	5.98 ± 0.00	Free

other cations, whether monovalent (Na^+, K^+), divalent (Sr^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}), or trivalent (Al^{3+}, Fe^{3+}), had a clear influence on the RP-LC behavior of glyphosate at the investigated concentration levels, instead eluting glyphosate at peaks very well comparable to those obtained with unsupplemented LC–MS-grade water. This may have been due either to the inability of these cations to form complexes with glyphosate at pH 9 or to their presence at suboptimal concentrations. A mixture of the five major cations yielded only one LC-peak of glyphosate at RT = 13.04 min, which suggested that free glyphosate reacted completely with the metal ions to form GMC. A mixture of the six minor cations had no influence on the LC-behavior of glyphosate. To determine whether the major cation Sr^{2+} and the minor cations are able to form GMCs and thereby influence the RT of glyphosate, they were prepared at higher concentrations. Thus, glyphosate and its internal standard, each at a concentration of 5 μg/L, were derivatized in LC–MS-grade water containing each of the above mentioned cations at a concentration of 1.17 mM. The resulting SRM chromatograms showed two important effects on the analysis of glyphosate. First, the glyphosate RTs obtained from the Al^{3+} and Fe^{3+} containing solutions were very comparable to those obtained from the corresponding standards (RT ≈ 6.00 min), indicative of the inability of these cations to form GMC at pH

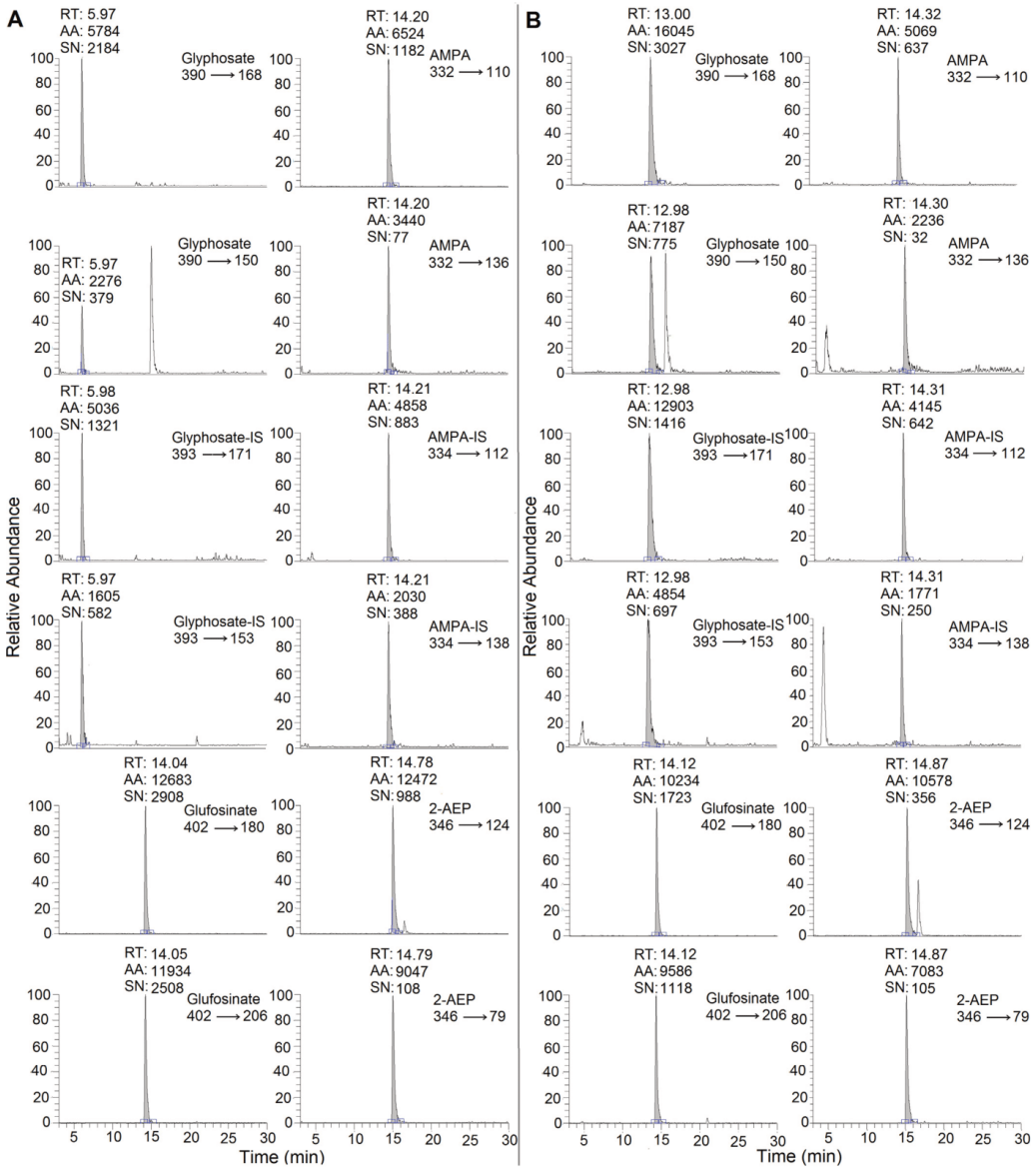

Fig. 2. Selected reaction monitoring (SRM) chromatograms obtained from the analysis of glyphosate, glyphosate-IS, glufosinate, AMPA, AMPA-IS, and 2-AEP, each at a concentration of 5 μg/L, and prepared in: A. LC–MS-grade water and B. artificial seawater with a salt concentration of 4 g/L. Reversed-phase chromatographic separation was achieved on a Gemini-NX C18 column (150 × 2.0 mm, 3 μm; Phenomenex, Germany). Two peaks (qualified and quantified ions) of each analyte are shown in the figure.

9. Second, glyphosate was not detected in samples containing 1.17 mM Cu^{2+}, Co^{2+}, Zn^{2+}, or Mn^{2+} [30,31]. The results of these experiments demonstrate that GMC with lower stability constants, including those with Ca^{2+} and Mg^{2+} (log K = 3.3) [30], are suitable for RP-LC-HESI–MS/MS analysis after FMOC-Cl derivatization. By contrast, GMC with higher stability constants, including those with Cu^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} (log K > 5.4) [30,31], are inadequate for the analysis when carried out using the applied HESI–MS/MS parameters. At a concentration of 1.17 mM Sr^{2+}, peaks corresponding to free glyphosate (RT ≈ 6.00 min) and GMC (RT ≈ 13.00 min) were observed in the same chromatogram. This was also the case for 0.75 mM Ca^{2+} (Fig. 3B). The observation of two LC-peaks of

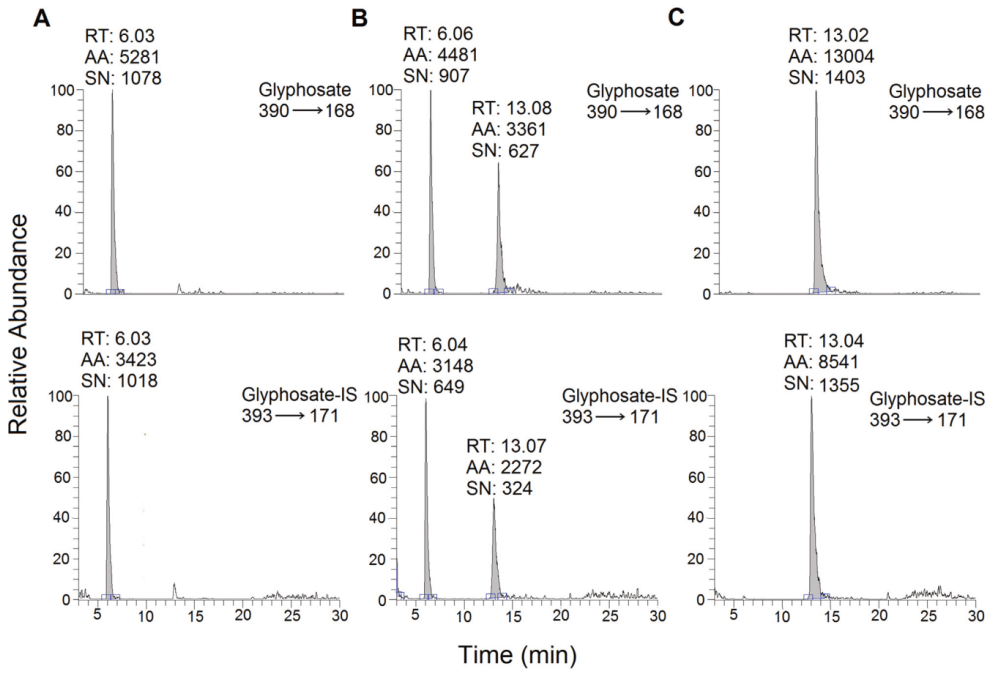

Fig. 3. SRM chromatograms obtained from the analysis of glyphosate and glyphosate-IS, each at a concentration of 5 μg/L, in solutions supplemented with different Ca^{2+} concentrations: A. 4 mg/L, B. 30 mg/L and C. 40 mg/L. The peaks are shown according to the quantified ions of glyphosate and glyphosate-IS.

glyphosate in the same chromatogram under the same analytical conditions violates the LC–MS/MS principle, that a chemical substance can yield only one LC-peak. Fang and co-workers [16] reported similar results for bile acids in urine samples from animals. In our study, Ca^{2+} and Sr^{2+}, each at a concentration of 1.17 mM, resulted in the elution of glyphosate as either one peak or two peaks, respectively. Thus, glyphosate interacted completely with Ca^{2+}, forming only one LC-peak representing a complex between these two compounds and eluting at RT ≈ 13.00 min (Fig. 3C). Sr^{2+}, however, only partly interacted with glyphosate, thus yielding two LC-peaks, one representing free glyphosate and the other the GMC. This clearly shows that: (i) GMC formation is cation- and concentration-dependent and (ii) the main divalent cations of sea salt (Ca^{2+}, Mg^{2+}, and Sr^{2+}), in their interactions with glyphosate, can act as additional derivatization reagents, besides FMOC-Cl. The interactions will depend on the cation concentrations in the matrix samples, such that glyphosate will elute in one peak, either as GMC (RT ≈ 13.00 min, high concentrations) or as free glyphosate (RT ≈ 6.00 min, low concentrations), or, when the cation concentration is not high enough to allow completion of the interaction, in two peaks (RT ≈ 6.00 min and ≈ 13.00 min).

The influence of sample matrices on the RP-LC behavior of the target analytes was then examined in environmental aqueous samples of rain, ground, drainage, riverine, estuarine, lagoon, Baltic Sea, and North Sea waters. The samples were collected at different sampling sites from Germany in 2016 and included different matrix and salt contents (Table 3). Each of the aqueous samples was spiked with each of the four analytes and the two internal standards, at a concentration of 5 μg/L. The samples were derivatized with FMOC-Cl at pH 9, filtered through 0.45-μm RC syringe filters (Phenomenex, Germany), and analyzed using LC–MS/MS. The elution of glufosinate, AMPA, and 2-AEP from these environmental samples was very well comparable to their elution in standard solution (data not shown). This result indicated the negligible influence of the sample matrices on the RP-LC behaviors of these three compounds and therefore on their identification. In the case of glyphosate and its internal standard (Table 3), they were eluted at RT ≈ 13.00 min in all analyzed samples with a maximum RTs variation of 0.3 min. The exception was the rainwater sample (RT ≈ 6.00 min), which because of the low ionic content of rainwater, glyphosate was eluted as free glyphosate. None of the environmental samples yielded two glyphosate elution peaks in the same chromatogram. Instead, the results showed the strong tendency of glyphosate in these samples to form complexes in different types of water, with the subsequent detection of those newly formed GMC.

3.2. *Influence of salt matrices on the HESI–MS/MS detection of target analytes*

Determination of the organic substances present in environmental samples at trace levels usually requires pre-concentration steps such as extraction, with solid-phase (SPE) often preferred over liquid-liquid extraction (LLE) for the pre-concentration of polar compounds from water, including glyphosate, glufosinate, and AMPA [1,11,27]. This is due to their high solubility in water and low solubility in organic solvents. SPE is also useful to minimize sample-matrix effects; these are usually the main obstacles in LC-ESI–MS/MS. The matrix effects of estuarine, lagoon, and seawater samples can primarily be attributed to the salt con-

Table 3
Aqueous samples collected at different sampling sites in Germany in 2016 were spiked with glyphosate and glyphosate-IS, each at a concentration of 5 μg/L. Sample names, geographical positions, salinity are presented. The salinity data were obtained using the WTW conductivity meter ProfiLine Cond 1970i. Glyphosate and glyphosate-IS RTs are expressed as mean ± SD according to triplicate measurements of each sample.

Aqueous sample	Sampling coordinates		Salinity	Glyphosate	Glyphosate-IS	Elution form
	Latitude	Longitude		RT ± SD (min)	RT ± SD (min)	
Rain	54° 10′ 46.13″ N	12° 04′ 53.65″ E	0.0	5.99 ± 0.03	5.98 ± 0.02	Free
Ground	54° 00′ 30.54″ N	12° 25′ 21.30″ E	0.0	13.05 ± 0.02	13.04 ± 0.04	Complex
Drainage	54° 00′ 30.54″ N	12° 25′ 21.30″ E	0.1	13.07 ± 0.02	13.08 ± 0.02	Complex
River	54° 08′ 21.80″ N	12° 15′ 41.14″ E	0.1	12.99 ± 0.02	13.01 ± 0.03	Complex
Estuary	54° 08′ 21.80″ N	11° 86′ 90.31″ E	0.4	13.08 ± 0.02	13.10 ± 0.02	Complex
Lagoon	54° 14′ 74.31″ N	13° 00′ 88.90″ E	15.7	13.14 ± 0.02	13.14 ± 0.03	Complex
Baltic Sea	54° 14′ 62.94″ N	11° 84′ 33.48″ E	11.0	13.15 ± 0.05	13.13 ± 0.03	Complex
North Sea	54° 17′ 35.23″ N	07° 89′ 35.30″ E	36.3	13.23 ± 0.04	13.24 ± 0.04	Complex

tent and the presence of particulate components as well as other impurities. However, even after the use of SPE remnants of the matrices can be still present in the samples, causing a change in the response of the analytes and therefore the erroneous reporting of sample concentrations [32]. Thus, in this experiment we used to investigate the influence of the salt matrix on the HESI–MS/MS response of the analytes glyphosate, glufosinate, AMPA, and 2-AEP. Salt solutions prepared at concentrations between 0 and 30 g/L were spiked with the same volumes of the working solution, to obtain a final analyte concentration of 5 μg/L. The samples were then derivatized and analyzed using LC-HESI–MS/MS. The peak areas of glyphosate, AMPA and their correction using internal standards, under the different salt concentrations, are shown in Fig. 4 and of glufosinate and 2-AEP, and a comparison of their correction using glyphosate-IS and AMPA-IS in Supplementary data Fig. S2. Signal enhancement/suppression due to matrix effects was considered low when it was ± 20%, moderate between ± 20% and ± 50%, and high more than ± 50% [33]. The results showed that the HESI–MS/MS response of glyphosate was clearly affected by the salt matrix. An increase of the salt concentration to 2 g/L caused a> 2-fold increase (181%) in the peak area of glyphosate. In the salt-free sample, glyphosate eluted as the free compound whereas in salt samples it eluted as GMC. The enhancement of the glyphosate signal has several possible explanations, including the different degrees of glyphosate and GMC ionization by HESI. Furthermore, the two compounds eluted at different RTs, separated by nearly 7.00 min. This means that they were ionized under different mobile phases and matrix compositions, which during HESI can greatly influence their ionization. Despite the peak areas obtained from 2 g/L salt concentrations were greatly higher than LC–MS-grade

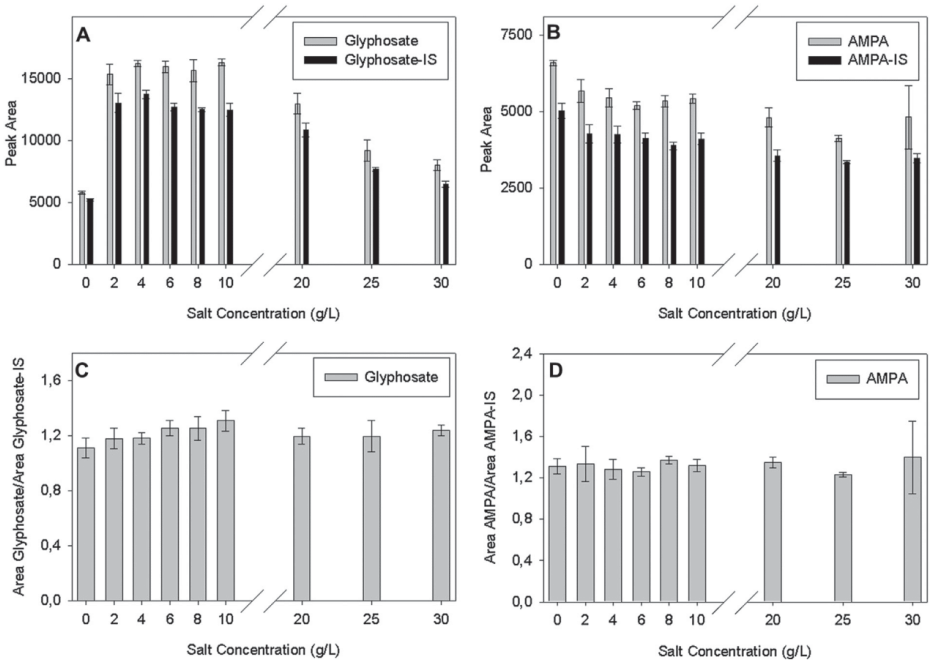

Fig. 4. Peak areas obtained from the analysis of A. glyphosate and glyphosate-IS, B. AMPA and AMPA-IS, each at a concentration of 5 μg/L, in the presence of salt concentrations between 0 and 30 g/L and rations of peak areas of C. Glyphosate/glyphosate-IS and D. AMPA/AMPA-IS. The peak areas of each analyte are expressed as mean ± SD according to triplicate measurements.

water, signal-to-noise ratios, however, were slightly higher which reflects the role of the injected salt in increasing the noise level. At salt concentrations of 2–10 g/L, the peak areas of glyphosate were highly stable (variation <5%), which demonstrated the similar effects of these salt levels on glyphosate ionization. However, the salt matrix caused a reduction of the peak signals of glufosinate, AMPA, and 2-AEP. Thus, at salt concentrations between 2 and 10 g/L, the amount of glufosinate and AMPA signal suppression was low, up to 8% and 15%, respectively, whereas suppression of the 2-AEP signal was medium, up to 29%. At higher salt concentrations (≥20 g/L), the peak areas of all analytes were further reduced but suppression was still considered to be moderate, reaching 30%, 44%, and 39% for glufosinate, AMPA, and 2-AEP, respectively, compared to their peak areas in LC–MS-grade water and up to 48% for glyphosate compared to its peak area in LC–MS-grade water supplemented with 2 g salt/L (i.e. peaks with the same RT ≈ 13.00 min). This was most likely due to the influence of salt on the efficiency of the formation of the desired parent ions in the HESI. The decrease in the peak signals of the analytes confirmed the general hypothesis of salt-matrix-induced ion suppression in LC–MS/MS [15].

The use of stable, isotopically labeled internal standards (SIL-IS) prepared in the same sample matrix is a well-established method for the correction of problems related to sample preparation and matrix effects [27,34]. However, because SIL-IS are not always commercially available, nearly identical chemicals are generally used as IS. In this study stable isotopically labeled glyphosate (glyphosate-IS) and AMPA (AMPA-IS) were used as internal standards for glyphosate and AMPA, respectively, and both internal standards were compared for their ability to compensate for the matrix effects of glufosinate and 2-AEP. As shown in Fig. 4A and B, glyphosate, AMPA, and their IS were similarly affected by the salt matrix. The variations in the glyphosate/glyphosate-IS and AMPA/AMPA-IS ratios under all tested salt concentrations were <15% (Fig. 4C and D). This demonstrated that glyphosate-IS and AMPA-IS can clearly compensate for salt-matrix effects on their analogous compounds. Like glyphosate, its IS could be eluted either as free analyte or as GMC, with significant differences in their RTs and peak areas. The peak area of the free glyphosate-IS (FG-IS) was always lower than that of GMC-IS (GMC-IS), resulting in an important difference in the signal ratios of glufosinate/FG-IS vs. glufosinate/GMC-IS (66%) and of 2-AEP/FG-IS vs.2-AEP/GMC-IS (71%). Under salt concentrations between 2 and 30 g/L, the glufosinate/GMC-IS and 2-AEP/GMC-IS ratios varied by as much as 39% and 47%, respectively. For glufosinate, this variation decreased greatly, to <6%, at salt concentrations between 2 and 20 g/L, with the exception of 4 g/L, whereas for 2-AEP a decrease to <15% at salt concentrations between 2 and 6 g/L was determined. This result demonstrated that glyphosate-IS can be used to correct for the salt-matrix effects on glufosinate and 2-AEP but only within a low salt concentration range. AMPA-IS yielded a better correction for glufosinate than glyphosate-IS, with a variation in the glufosinate/AMPA-IS peak area ratio of <15% for all samples and <7% when a salt concentration of 6 g/L was excluded. To our knowledge, this is the first study to report the use of glyphosate-IS to correct matrix effects on glufosinate. Hanke et al. [11] selected AMPA-IS for the quantification of glufosinate using RP-LC-ESI–MS/MS; however, why glufosinate was quantified with AMPA-IS not with glyphosate-IS even though the chemical structure of glyphosate is closer to that of glufosinate was not explained. Nonetheless, our data suggest that AMPA-IS is indeed a better choice than glyphosate-IS for glufosinate quantification due to their relatively similar RP-LC behaviors and the influences of sample matrices on their detection.

The influence of the salt matrix on the efficiency of derivatization with FMOC-Cl was also examined in this study. The reaction was carried out in three different matrix solutions spiked with the analytes at a final concentration of 10 μg/L. The first solution contained only LC–MS-grade water (i.e. ion-free), while the second and third solutions consisted of LC–MS-grade water supplemented with 4 g sea salt/L. Solutions 2 and 3 differed in the time of salt addition: before (solution 2) and after (solution 3) the derivatization reaction. The peak areas of the analytes are shown in Fig. 5A and their peak areas after correction with the IS in Fig. 5B. The statistical and percentage difference values of each analyte, which were prepared in the three mentioned solutions, are shown in Supplementary data Table S1. The peak areas of glyphosate and glyphosate-IS were significantly ($p < 0.05$, >83%) lower in solution 1 than in solution 2 or 3. However, the peaks from solution 1 were incomparable with those from solutions 2 and 3, due to their different LC behaviors and HESI–MS/MS detection. In contrast, higher peak areas of glufosinate, AMPA, AMPA-IS, and 2-AEP ($p < 0.05$, < 33%) were obtained with solution 1 than with solution 2 or 3. This may have been the result of ionization suppression rather than a decrease in the derivatization efficiency. The differences between the peak areas obtained in solution 2 and 3 varied between not significant ($p = 0.266$) for glufosinate and significant ($p < 0.05$, ≤12%) for glyphosate, glyphosate-IS, AMPA and AMPA-IS, and (<27%) for 2-AEP. This result can be explained that the ionization of each analyte could be diversely influenced by the salt matrix rather than the effect on the derivatization efficiency. The peaks observed of all analytes in solutions 2 and 3 clearly indicate to the possible performance of the derivatization reaction of all analytes with FMOC-Cl in salt matrices, at least for the metal concentrations tested. One explanation for this observation is that the hydrogen atoms of the amine groups of the analytes do not contribute in the complex formation, which allowed the derivatization reaction to take place before and after forming the complexes. As seen in Fig. 5B, the ratios of the peak area of the analytes to those of the IS were highly comparable for all three solutions, thus demonstrating that the derivatization reaction can be successfully performed in freshwater and also in saltwater. Freuze and co-workers [20] used HPLC separation and fluorescence detection after derivatization of the analytes with FMOC-Cl to study the effect of multivalent cations on the analysis of glyphosate and AMPA in water. They suggested that the formation of GMC prevents the reaction of the complexes with FMOC-Cl. However, according to our results, while high concentrations of multivalent cations such as Cu^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} may indeed negatively affect the reaction with FMOC-Cl, these cations are usually present in environmental aqueous samples as trace elements, and thus at very low concentrations, in which case they will not have important effects on glyphosate analysis. Moreover, major cations (Ca^{2+}, Mg^{2+} and Sr^{2+}), i.e., those present at higher concentrations, were found to improve glyphosate peak areas. Therefore, the limit of detection may greatly improve after decrease the noise levels in the chromatograms by minimizing the salt content injected into LC–MS/MS using SPE or clean-up steps. Many methods for the analysis of glyphosate and AMPA in water have added steps, such as sample acidification and the addition of ethylenediaminetetraacetic acid (EDTA), to obtain free glyphosate and therefore its precise investigation [11,27]. However, in these GMC-disrupting steps, metal contamination of the reaction may occur from several sources, such as gloves, solvent reservoirs, and the solvents used in the mobile phase. All of these can lead to a decrease in the free glyphosate peak area and favor the formation of the GMC peak.

An essential condition for a compound to be suitable for quantitative LC–MS/MS analysis is the linear relationship between its concentration and the detector response. To provide information for future method-development studies, we obtained calibration curves of the analytes in sea salt matrices with a salt concentration of 4 g/L. The calibration curves covered the concentration range from 2.5 to 30 μg/L for glyphosate, AMPA and 2-AEP, and from 2.5 to 20 μg/L for glufosinate. The obtained statistical parameters are summarized in Supplementary data Table S2. The lack of fit test

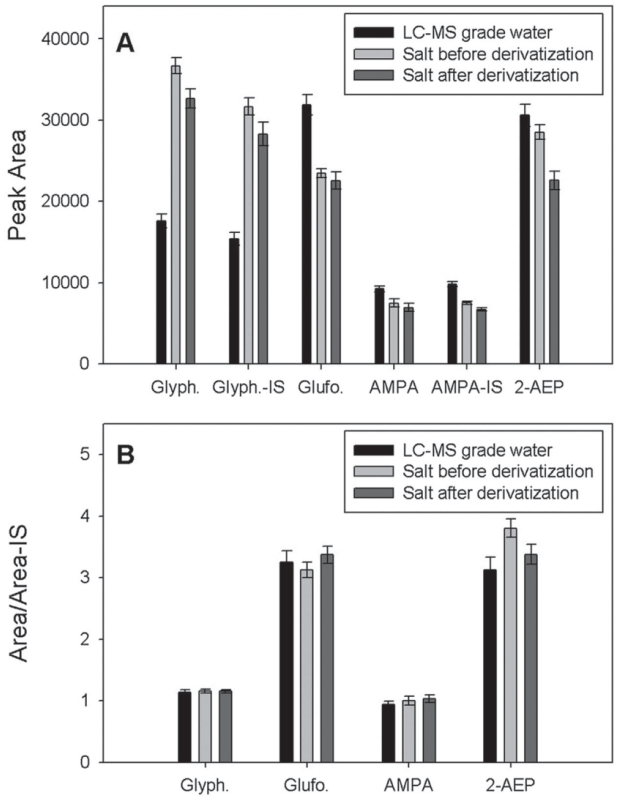

Fig. 5. A. Peak areas of glyphosate, glyphosate-IS, glufosinate, AMPA, AMPA-IS, and 2-AEP. B. Ratios of the peak areas of glyphosate/glyphosate-IS, glufosinate/AMPA-IS, AMPA/AMPA-IS, and 2-AEP/AMPA-IS obtained from the analysis of these compounds, each at a concentration of 10 μg/L, in three different solutions: black, LC–MS-grade water; gray, 4 g salt/L added to the LC–MS-grade water prior to the derivatization reaction; light black,derivatization in LC–MS-grade water followed by the addition of 4 g salt/L. The peak areas are represented as mean ± SD according to three independent experiments, each measured in triplicates.

was not significant with prob >F above 0.1 for all analytes which indicates a good degree of linearity. The obtained linearity clearly demonstrates the possible analysis of the mentioned compounds in saltwater matrix using internal standards as well as to the possible analysis of glyphosate in a complex form.

In future analyses of the studied compounds in environmental aqueous samples, based on our results we strongly recommend that the IS should be added to the sample matrices before performing any treatment of the samples. That allows the analogous compounds and the internal standards to behave similarly. Glyphosate can be analyzed either as a complex or a free compound. If the complex form is preferred, we suggest adding the cation Ca^{2+}, Mg^{2+} or Sr^{2+} at sufficient concentrations to allow GMC formation and to avoid the elution of glyphosate in two peaks. The derivatization reaction of the analytes with FMOC-Cl can be conducted before or after forming the complexes. Analysis of glyphosate in the free form has been described [11] by acidification of the samples using strong acid as HCl (pH 1) and adding EDTA. Sample contamination with metals should be avoided after using complexes-breaking steps. However, glyphosate analysis in water as complexes and free analyte should be compared in future work. In addition to the main aim of using SPE as a pre-concentration method, this process may also minimize the final salt contents injected into the LC–MS/MS and, subsequently, providing better detection and quantification levels by reducing the ion suppression and the noise levels present in the chromatograms. The influence of used elution solvents in SPE process on breaking GMC needs to be taken into consideration. In previous work [12] we used the standard addition method (SAM) for the quantification of glyphosate and AMPA in estuarine water samples. Since the analysis of the mentioned substances is highly dependent on sample matrices SAM can be also developed for glufosinate and 2-AEP. Then, both analytical quantitative strategies, IS and SAM, can be compared to ensure the quality of the quantitative results.

4. Conclusion

This study has shown that the analysis of nearly similar organic compounds can be differentially affected by sample matrices such as a salt matrix. We determined that glyphosate has a high tendency to form complexes with the salt matrix that differ from free glyphosate in their RP-LC behavior. By contrast, for glufosinate, AMPA and 2-AEP, the influence of the salt matrix on their RP-LC behavior was negligible. The formation of GMCs and thus

the altered RP-LC behavior of glyphosate depended on the type and concentration of the cation present in the samples. Whereas monovalent (Na^+, K^+) and trivalent (Al^{3+}, Fe^{3+}) cations had no influence on the RP-LC behavior of the analytes, high concentrations of divalent cations such as Cu^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} hindered the detection of glyphosate, while Ca^{2+}, Mg^{2+}, and Sr^{2+} greatly altered its RT. Moreover, particular tested concentrations of Ca^{2+} and Sr^{2+} caused glyphosate to be eluted in two peaks, thus violating the fundamental principle of LC, that a signal compound will form only one LC-peak when analyzed under the same analytical conditions. The derivatization reactions of all analytes with FMOC-Cl took place in the salt matrices which allow their analysis in different type of environmental aqueous samples including those with high salt contents such as seawater samples. In rain, ground, drainage, riverine, estuarine, lagoon, Baltic Sea, and North Sea water samples, glufosinate, AMPA and 2-AEP showed similar RP-LC behaviors, whereas the behavior of glyphosate in the rain water matrix was clearly different in comparison to other matrices. Ion suppression resulting from salt-matrix effects was observed for all analytes and it increased with increasing salt concentrations; however, a correction of these effects was achieved using glyphosate-IS and AMPA-IS. The influence of sample matrices on the chromatographic behavior and MS/MS detection of the mentioned compounds observed in this work strongly recommend taking this problem into the consideration during the development of LC–MS/MS methods, which can subsequently have great negative effects on the identification and quantification of the analytes of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.11.007.

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