

## Report

# Natural and anthropogenic organic P compounds - inositolphosphates, phospholipids and glyphosate

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# 1 Introduction and Goals of the Project

Baltic Sea eutrophication is frequently observed even if inorganic P is very low. It was demonstrated that also the organic P pool is partially bioavailable and, thus, might contribute as nutrient source (Nausch and Nausch, 2006; Nausch and Nausch, 2007).

The majority of the organic P pool presumably derives from phosphate esters (Figure 1), the biochemical backbone of contemporary life, which are highly abundant as nucleotides for energy production (e.g., ATP) or informational storage (e.g., DNA) and structural integrity of membranes (phospholipids). Less is known about the natural occurring organophosphonates (Figure 1). Harboring a C-P bond their biochemical utilization requires higher energy demand for cleavage and a different enzymatic machinery for organisms than cleaving the oxygen containing C-O-P phosphate group (McGrath et al., 2013). Considering the reducing atmosphere of early Earth life organophosphonates are considered as the preceding form of phosphate in early life forms.

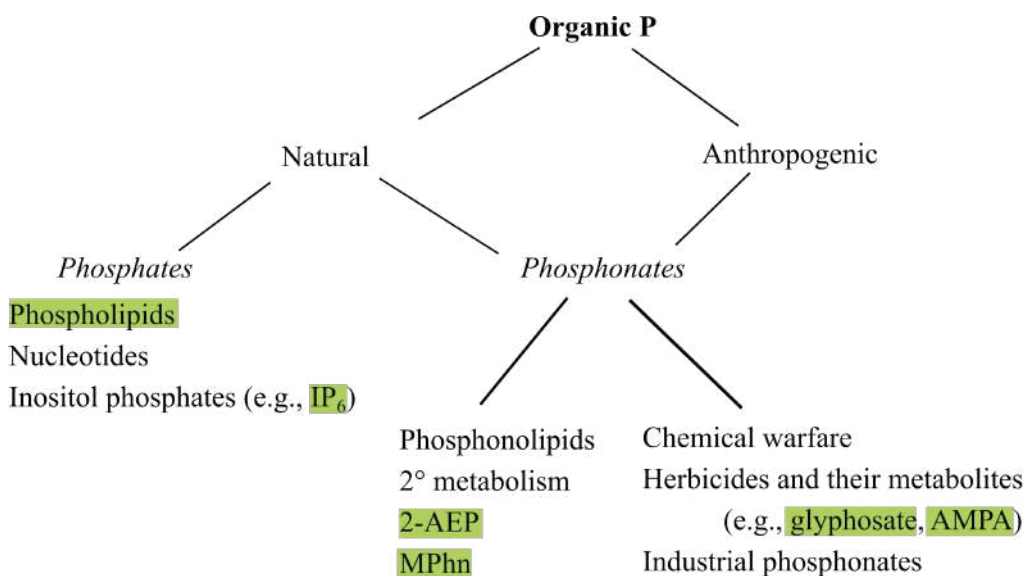


Figure 1: Classification of organic P compounds in the environment. This project focuses on the labelled compounds. see page 4 for abbreviations

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 through  $^{31}\text{P}$  NMR analysis that phosphonates make up about 25 % of the high molecular weight dissolved organic phosphate pool (Clark et al., 1999) and some marine species were shown to be potential sources for phosphonates in the marine envi-

ronment (Clark et al., 1999; Dyhrman et al., 2009). Therefore, organophosphonate compounds presumably relevantly contribute to the marine biogeochemical P cycle.

Also anthropogenic organic P compounds (Figure 1), such as the herbicide compound glyphosate which is one of the major herbicides applied in Germany contribute to the environmental organic P pool.

This project aims a better understanding of the relevance of organic P compounds for the marine environment. In this regard organic P compounds were selected and it was the aim to implement suitable analytical methods to enable their analysis in samples from the Baltic Sea environment to study their relevance and behavior at marine sites. These compounds were the

- *myo*-inositolhexakisphosphate (IP<sub>6</sub>)
- phospholipids
- methyl phosphonate (MPhn)
- 2-aminoethylphosphonate (2-AEP)
- glyphosate and its metabolite aminomethylphosphonic acid (AMPA)

## 2 Material and Methods

### 2.1 Determination of 2-AEP

2-AEP was analyzed through LC-MSMS after derivatization with FMOC-Cl. Detailed information on the methodology can be obtained from Skeff et al. (2016).

### 2.2 Determination of MPhn

Stock solutions of MPhn (98% purity, VWR International GmbH, Hannover, Germany) and deuterated methylphosphonic acid (methyl-D<sub>3</sub>-MPhn, Sigma-Aldrich, Taufkirchen, Germany) were prepared in MilliQ. The methyl-D<sub>3</sub>-MPhn was used as internal standard during analysis.

Samples containing the internal standard were enriched on Strata-X-AW cartridges (500mg/3 mL, Phenomenex, Aschaffenburg, Germany) which were preconditioned

with methanol (LC-MS grade, Walter-CMP, Kiel, Germany) and MilliQ. The cartridges were quickly washed with 1 ml of MilliQ and methanol and subsequently eluted with 10 ml of a 5% (v/v) ammonia solution (VWR International GmbH, Hannover, Germany) in methanol. The eluate was concentrated to dryness at 40 °C under a stream of clean air. Derivatization was done according to protocols published before (e.g. Purdon et al., 1989; Richardson and Caruso, 2007) with slight modifications. Thus, 100  $\mu$ L acetonitrile (LC-MS grade, Walter-CMP, Kiel, Germany) and 50  $\mu$ L *N-tert*-butyldimethylsilyl-*N*-methyltriouoroacetamide containing 1% *tert*-butyldimethylchlorosilane (MTBSTFA, VWR International GmbH, Hannover, Germany) were added to the dried sample and the reaction mixture was incubated for 2 h at 60 °C in the water bath. To extract the derivatization products 100  $\mu$ L of MilliQ and 250  $\mu$ L of *n*-hexane were added. The suspension was vortexed and centrifuged for phase separation and the above hexane layer was collected and shortly dried on combusted sodium sulfate. Extracts were transferred to vials and conducted for GC-MS analysis on a Trace-DSQ-GC-MS system (Thermo Fisher Scientific, Waltham, USA). MPhn was separated on a DB-5MS column (60 x 0.25 mm, film thickness 0.25  $\mu$ m, Agilent, Waldbronn, Germany) and detected by mass spectrometry in the Selected Ion Mode (MPhn:  $m/z=266.9$  (quantifier), 208.9, methyl-D3-MPhn:  $m/z=269.9$  (quantifier), 311.95).

### 2.3 Determination of IP<sub>6</sub>

Extraction of IP<sub>6</sub> from sediment samples was conducted with NaOH/EDTA and extracts were analyzed with LC-MSMS. Detailed information on the used IP<sub>6</sub> methodology can be obtained from Recknagel et al., 2018.

## 3 Results and Discussion

### 3.1 Determination of IP<sub>6</sub> in sediments from the Baltic Sea coast

The group of inositol phosphates derives from inositol with different states of phosphorylation and diverse biochemical functions. The 6fold phosphorylated IP<sub>6</sub> predominantly derives from terrestrial sources and serves as P storage in plant seeds (Figure 2).



Figure 2: **A** Chemical structure of IP<sub>6</sub>. **B** During germination of plant seeds P is mobilized from IP<sub>6</sub>

For fresh water systems IP<sub>6</sub> contents ranging from 12 to 83% of total P were shown (Herbes et al., 1975; McKelvie et al., 1993); for lake sediments IP<sub>6</sub> contents ranged from 10 to 50% of total P (McKelvie et al., 1993; Weimer and Armstrong, 1977). IP<sub>6</sub> contents in sediments from Tokyo Bay were determined with 0.4% of total P (Suzumura and Kamatani, 1993). However, there is only little knowledge about IP<sub>6</sub> from the marine environment. Therefore, it was the goal of this part of the project to determine IP<sub>6</sub> in sediments from the Baltic Sea. As it is known that IP<sub>6</sub> predominantly derives from terrestrial sites IP<sub>6</sub> analysis was conducted at Baltic Sea coastal sites, i.e., at the Darss-Zingst-Boddendchain and the river Warnow in the city of Rostock in the federal state of Mecklenburg-West Pomerania, Germany (Figure 3).

This work resulted in the publication Recknagel et al., 2018 with the PhD student as the first author. The following presented results of this part of the project are obtained from this publication.

A method for the determination of IP<sub>6</sub> in sediments (Sjöberg et al., 2016) was further improved to analyze IP<sub>6</sub> in sediments from the Baltic Sea coast. In this work it was shown that extraction of IP<sub>6</sub> was largely dependent on the sediment characteristics; particularly the organic carbon content determines the extraction efficiency. In this regard, IP<sub>6</sub> extraction was largely improved. Moreover, the standard addition method was introduced in the procedure to enable quantification of IP<sub>6</sub> and to correct for the matrix dependend extraction defficiencies.

The improved method was successfully utilized to determine IP<sub>6</sub> in the Baltic Sea surface sediments from the Darss-Zings-Boddendchain and the river Warnow in the city of Rostock (Figure 3). Determined IP<sub>6</sub> contents ranged from 0.2 to 0.4% on total P which is in the same order of magnitude as it was shown before for marine sediments (Suzumura and Kamatani, 1993). The obtained IP<sub>6</sub> contents decline from the inbound sites to those close to the sea indicating the terrestrial origin of IP<sub>6</sub>.

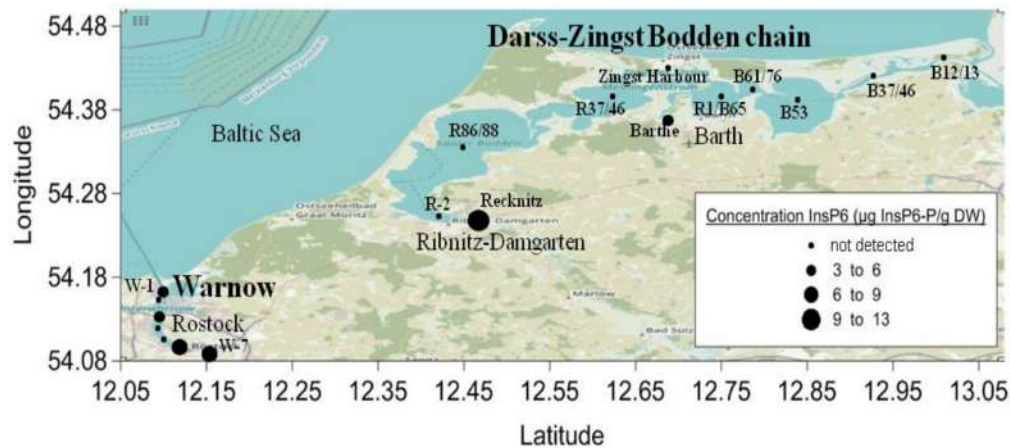


Figure 3: Analysis of IP<sub>6</sub> in surface sediments at coastal sites of the Baltic Sea (obtained from Recknagel et al., 2018). The size of the dots symbolizes the determined IP<sub>6</sub> contents.

Moreover, it was found that the determined sediment IP<sub>6</sub> content is largely dependent on the characteristics of the analyzed sediments such as the total organic carbon content (Figure 4).

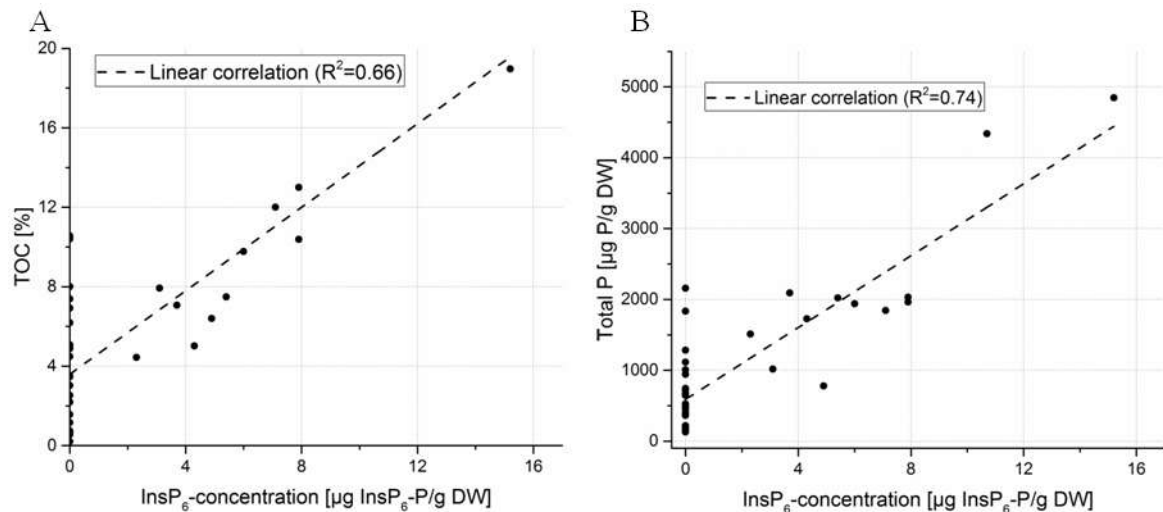


Figure 4: The IP<sub>6</sub> sediment content correlates to **A** the TOC content and **B** the total P content of the sediment (obtained from Recknagel et al., 2018).

### 3.2 Phospholipids

Lipid methodology was implemented comprising lipid extraction and separation of the neutral and polar lipids with solid phase extraction. Fatty acids were analyzed by GC-MS as their fatty acid methyl esters after transmethylation. However, further separation of the polar lipids was not sufficiently achieved. Due to instrumental limitations this part of the project was suspended and will be pursued later.

### 3.3 Method development for MPhn for the analysis in samples from the Baltic Sea estuary

Analysis of organophosphonate compounds of previous studies often bases on  $^{31}\text{P}$  NMR which allows to give insights into the abundance of the compound class on the total organic P pool. However, there is limited information on concentrations of particular organophosphonate compound species in the environment. It was shown that under P starvation conditions in particular MPhn can be utilized by marine bacteria; MPhn is also currently discussed in view of the methane paradox in the aquatic environment (e. g., Metcalf et al., 2012; Yao et al., 2016).

During this part of the project it was the goal to develop a method for the determination of MPhn in water to analyze their concentrations in the aquatic environment. However, due to the strong polar characteristic of MPhn so far solid phase extraction (SPE) is limited to water samples in the very low salinity range.

The developed method includes an enrichment step on SPE column with subsequent derivatization and GC-MS analysis. Quantification was conducted with D3-methyl-MPhn as internal standard (Figure 5).

With the obtained method an LOD of about 10 ng was determined. Comparing MPhn samples of different concentrations in MilliQ and river water with the instrumental calibration revealed that determination of MPhn is matrix independent and proves the use of the internal standard as suitable quantification method (Figure 5 C).

Analysis of natural samples from the Baltic Sea estuary revealed preliminary data for MPhn ranging from 100 to 150 ng/l.



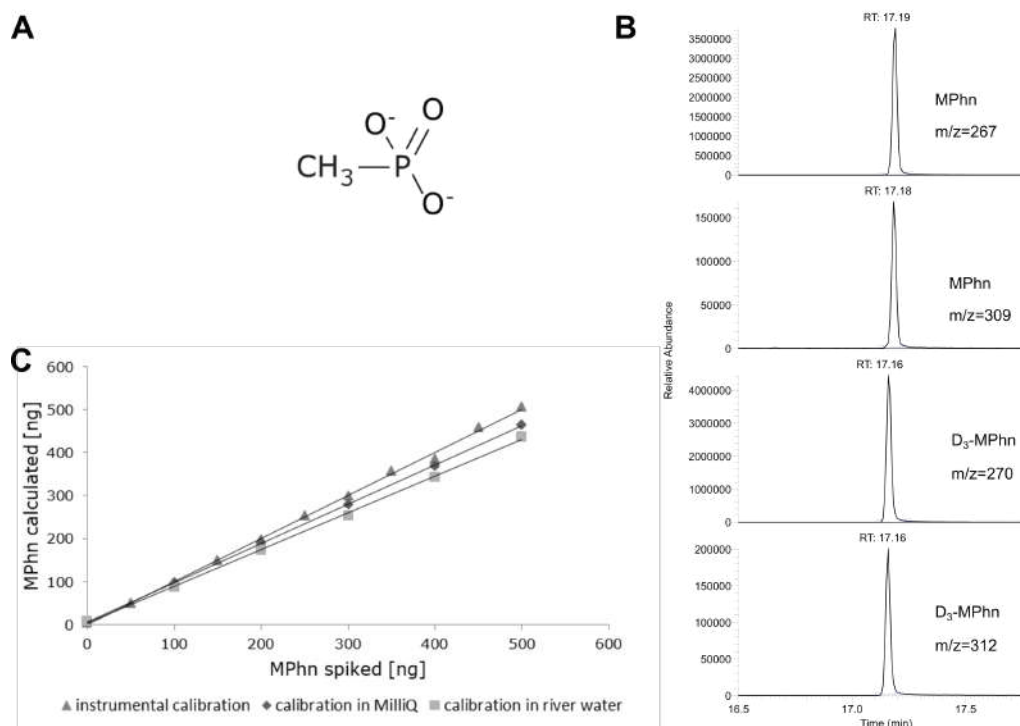


Figure 5: **A** Chemical structure of MPhn. **B** Derivatized MPhn and the internal standard D<sub>3</sub>-MPhn were determined by GC-MS analysis with two compound specific mass fragments. **C** Recovery functions: Calibrations of MPhn in MilliQ and river water comparing to the instrumental calibration.

Work on this part of the project is in progress to verify the obtained data for the natural water samples. Moreover, the data are currently compiled for peer review publication (Recknagel *et al.*, 2019, in preparation).

### 3.4 Method development for the analysis 2-AEP

2-AEP is considered as a predominant biogenic phosphonate compound. It was found in many marine species as lipid or peptide conjugates in different tissues suggesting a wide range of function for 2-AEP (McGrath *et al.*, 2013).

Within this part of the project a derivatization method with subsequent LC-MSMS analysis was developed for the determination of 2-AEP. The method is published in Skeff *et al.*, 2016, which also includes a study on the influence of the sample matrix on the chromatographic separation and mass spectrometric detection of 2-AEP, glyphosate, glufosinate and AMPA.

### 3.5 Adsorption study with glyphosate and AMPA

Glyphosate is one of the major broad spectrum herbicides applied in Germany. Previous research demonstrated its presence in the Baltic Sea estuaries and, thus, transport from the place of application to the marine sites (Skeff et al., 2015).

To further elucidate the fate in the marine environment an adsorption study was conducted to reveal the partitioning of glyphosate and its metabolite AMPA between the water and particulate phase. Adsorptions were studied on different marine sediment types (mud, silt, sand) at different temperature, salinity and pH. Highest adsorption coefficients were found for glyphosate followed by AMPA. Adsorption was highest on sediments with a high organic carbon fraction (mud); on sandy sediments adsorption was detectable only for glyphosate.

This work was published in Skeff et al., 2018.

## 4 Appendix

### Research stay at international Collaborators' laboratories

The PhD student visited the research group of Dr. P. Sjöberg at Uppsala University, Sweden from January to April 2017. During his stay C. Recknagel improved the method for the extraction and determination of IP<sub>6</sub> from sediments which was published by Sjöberg et al., 2016 for the analysis in sediments from the Baltic Sea coast.

### Presentations

Constantin Recknagel, Marion Abraham, Detlef E. Schulz-Bull: Natural and Anthropogenic Organic P Compounds - Inositol Phosphates, Phospholipids and Glyphosate, Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Mar 2015, Talk

Carina Deich, Lina Westphal, Marion Abraham, Wael Skeff, Bernd Lennartz, Detlef Schulz-Bull: Method Development for the Extraction of particulate Glyphosate, Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Mar 2015. Poster

Wael Skeff, Christine Neumann, Marion Abraham, Detlef Schulz-Bull: Development and Validation of an HPLC-MS/MS Method for the Determination of Glyphosate and AMPA in Estuarine Water, Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Mar 2015, Poster

Wael Skeff, Christine Neumann, Marion Abraham and Detlef Schulz-Bull: Occurrence of Glyphosate and AMPA in the German Baltic Estuaries, Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Mar 2015, Poster

Constantin Recknagel, Marion Abraham, Detlef E. Schulz-Bull: Natural and Anthropogenic Organic P Compounds - Inositol Phosphates and Phospholipids, Workshop of the Leibniz ScienceCampus Phosphorus Research Rostock, Jun 2015, Talk

Constantin Recknagel, Marion Abraham, Detlef E. Schulz-Bull: Natural Organic P Compounds - Phosphonates and Inositol Phosphates, Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Mar 2016, Talk

Constantin Recknagel, Per Sjöberg, Marion Abraham, Detlef Schulz-Bull: Determination of Inositol Phosphates in Aquatic Systems of the German Baltic Coastal Area, 8th International Phosphorus Workshop (IPW8), Sep 2016, Poster

Wael Skeff, Constantin Recknagel, Detlef Schulz-Bull: Development and Validation of an Analytical Method for the Determination of Glyphosate, Glufosinate, AMPA and 2-AEP in Seawaters, 8th International Phosphorus Workshop (IPW8), Sep 2016, Poster

Constantin Recknagel, Marion Abraham, Detlef Schulz-Bull: Natural Organic P Compounds - Phosphonates and Inositol Phosphates, Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Apr 2017, Talk

Constantin Recknagel, Marion Abraham, Detlef Schulz-Bull, P. Sjöberg.: Natural Organic P Compounds – Phosphonates and Inositol Phosphates, Seminar of the analytical chemistry group, Uppsala University, Sweden, Ma 2017, Talk

Constantin Recknagel, Marion Abraham, Udo Kragl, Detlef E. Schulz-Bull: Natural Organic P Compounds – Phosphonates and Inositol Phosphates. Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock, Nov 2017, Talk

Constantin Recknagel, Marion Abraham, Udo Kragl, Detlef E. Schulz-Bull: Natural Organic P Compounds – Phosphonates and Inositol Phosphates. Symposium of the Leibniz ScienceCampus Phosphorus Research Rostock Rostock, Nov 2017, Poster

## Resulting Publications (to date)

Wael Skeff, Constantin Recknagel, Detlef Schulz-Bull (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. Chromatogr.* 1475: 64-73, doi: 10.1016/j.chroma.2016.11.007

Wael Skeff, Constantin Recknagel, Yvonne Düwel, Detlef Schulz-Bull (2018): Adsorption behaviors of glyphosate, glufosinate, aminomethylphosphonic acid,

and 2-aminoethylphosphonic acid on three typical Baltic Sea sediments. *Marine Chemistry* 198: 1-9, doi: 10.1016/j.marchem.2017.11.008

Constantin Recknagel, Pernilla Thelin, Marion Abraham, Detlef Schulz-Bull, Per J. R. Sjöberg (2018). Using standard additions to improve extraction and quantification of inositol hexakisphosphate in sediment samples by ion chromatography electrospray ionization mass spectrometry. *Talanta* 188: 192-198, doi: 10.1016/j.talanta.2018.05.072

## Theses

Yvonne Düwel: Adsorption von Glyphosat, AMPA und Glufosinat an Sedimenten aus der Ostsee, University of Rostock, Master thesis, 2017

Carina Deich: Entwicklung einer Methode zur Bestimmung von an Partikeln gebundenem Glyphosat in der marinen Umwelt, University of Rostock, Bachelor thesis, 2016

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