Polar Herbicides in the German Baltic Estuaries

Analysis, Occurrence and Effects

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Abstract

The Baltic Sea is considered to be one of the most polluted seas in the world. The rivers transport very of large amounts of herbicides which enter the marine environment. The majority of research and monitoring programs on pesticide pollution in the Baltic Sea and its estuaries focuses on persistent organic pollutants (POPs) such as organochlorine pesticides. On the other hand, very little attention has been paid to the occurrence of other classes of pesticides such as mid and highly polar pesticides. For a proper risk assessment of polar pesticides, detailed information is required about their transport to the Baltic Sea.

Monitoring trace polar contaminants in water samples require sensitive analytical methods. Mass spectrometric $(MS)^n$ techniques have become an increasingly valuable tool in environmental analysis. In this work, a comparison between two analytical techniques, gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), for analysis of nine polar compounds in water samples was conducted. The target compounds were six polar herbicides glyphosate, MCPA, mecoprop, isoproturon, bentazon and chloridazon and three of their metabolites aminomethylphosphonic acid (AMPA), chloridazon-desphenyl (CD) and chloridazon-methyl-desphenyl (CDM).

HPLC-MS/MS was preferred over GC-MS for their analysis. The differences in the physico-chemical properties of the target analytes such as polarity and water solubility required two HPLC-MS/MS analytical methods for obtaining good analytical results. The first method is a direct HPLC-MS/MS analysis of the compounds MCPA, mecoprop, isoproturon, bentazon, chloridazon and CDM. The second method is an HPLC-MS/MS analysis of the compounds glyphosate and AMPA after their derivatization with 9H-Fluoren-9-ylmethyl chloroformate (FMOC-Cl). In both methods the separation of the target analytes was achieved on reversed phase columns.

The analytical methods were developed and validated. Their development was based on HPLC and MS/MS parameters. The validation parameters included linearity, limit of detection and quantification, precision, accuracy, analytes and system stability. Quantitative analysis of the target compounds was carried out using selected reaction monitoring (SRM) via a heated electrospray ionization (HESI) interface in order to obtain best detector sensitivity. The standard addition method was used for the quantitative analysis in order to avoid matrix effect problems.

The analytical methods were applied in order to study the potential transport of the target compounds into the Baltic Sea based on their occurrence in some German Baltic estuaries. Water samples were collected from ten German Baltic estuaries in Mecklenburg-Vorpommern in the period between May and September 2012. The collection of samples was carried out under both wet and dry weather conditions. The samples were analyzed with the HPLC-MS/MS methods described above.

Of all the target compounds, the most frequently detected compounds in the investigated stations were the metabolite AMPA and its parent herbicide glyphosate. All investigated estuarine sampling sites were found to be contaminated with AMPA and nine of them with glyphosate. Moreover, glyphosate and AMPA have the highest concentrations which reached up to the microgram per liter range in some samples. Based on these results, data are needed to evaluate the effects of glyphosate and AMPA on non-target estuarine and marine organisms.

In this work, the effect of the herbicide Roundup®, the commercial formulation of glyphosate, and the metabolite AMPA on the growth of *Nodularia spumigena* was studied. *Nodularia spumigena* is a blue-green algae commonly observed in brackish water. It is one of the dominant cyanobacteria observed during the summer bloom in the Baltic Sea. In the experiments, the growth measurements were based on chlorophyll-*a*, cell density and particulate organic carbon. *Nodularia spumigena* showed tolerance to both toxicants when exposed to concentrations between 1-500 µg/L. *Nodularia spumigena* was found to be unable to degrade AMPA under to the experimental conditions used.

Zusammenfassung

Die Meeresumwelt der Ostsee als Binnenmeer ist stark durch Einträge von Schadstoffen und Nährstoffen aus Flusseinträgen und der Atmosphäre belastet. Herbizide und Pestizide aus der Landwirtschaft werden durch das sehr große Einzugsgebiet der Ostsee in bedeutenden Mengen und teilweise hohen Konzentrationen ins Ökosystem der Ostsee eingetragen. Die persistenten organischen Schadstoffe, wie PCB, PAH und DDT sind in den laufenden HELCOM-Monitoring Programmen gut untersucht. Dies gilt nicht für die polaren, besser wasserlöslichen „neuen" Schadstoffe. Für diese Spurenstoffe müssen erst Methoden zur Anreicherung und Messung im Meerwasser getestet und etabliert werden sowie Bestimmungen in der Meeresumwelt durchgeführt werden, bevor diese Substanzen in die Monitoring Programme aufgenommen werden können.

Als erste Zielsetzung der Promotionsarbeit wurden die analytischen Techniken zur Messung von sechs polaren Herbiziden (Glyphosat, Mecoprop, 2-Methly-4-Chlorphenoxy-essigsäure, Isoproturon, Bentzon, Chloridazon) sowie von 3 Metaboliten (Aminomethyl-phosphonsäure (AMPA), Desphenyl-Chloridazon und Methyl-Desphenyl-Chloridazon) in Meerwasser weiter entwickelt. Die gaschromatographische Bestimmungs-methode mit Massenspektrometrie zur Detektion wurde mit einem Verfahren basierend auf der Hochdruckflüssigskeitschromatographie als Trennmethode und ebenfalls massens-pektrometrischer Detektion verglichen. Hierbei wurde eine Derivatisierungsmethode mit 9-Fluorenyl-Methyl-Chloroform eingesetzt. Beide Methoden wurden erfolgreich getestet, die Messungen mit der HPLC stellten bei der Reproduzierbarkeit, der Linearität und der erreichbaren Bestimmungsgrenze als geeigneter heraus. Zur Quantifizierung wurde eine Standardaddition durchgeführt, um die Matrix-Effekte zu kompensieren.

Die entwickelte Methode zur Anreicherung und Bestimmung von polaren Pestiziden ermöglichte es die Konzentration der Schadstoffe in Ästuaren entlang der Küste Mecklenburg-Vorpommerns zu bestimmen. Es konnten wichtige Eintragsquellen identifiziert werden. Die höchsten Konzentrationen mit bis zu 1690 ng/L und 4156 ng/L wurden für das Pestizid Glyphosat und sein Abbauprodukt AMPA gefunden.

Im letzten Kapitel der Arbeit werden die Ergebnisse der experimentellen Untersuchungen zu Effekten des technischen Produkts Roundup mit Glyphosat als Wirkstoff und seines Metaboliten AMPA auf die Cyanobakterie *Nodularia spumigena* beschrieben. Diese stickstofffixierende Blaualge führt im Sommer in der zentralen Ostsee zu extremen Algenblüten. In den Experimenten wurde gezeigt, dass *Nodularia spumigena* durch Konzentrationen im Bereich von 1–500 µg/L Glyphosat nicht negativ beeinträchtigt wird, aber auch keine Aufnahme/ Abbaureaktion von Glyphosat zu messen ist.

Preface

This thesis has three main objectives. The first objective is a comparison of two analytical techniques including gas and liquid chromatography tandem mass spectrometry for the analysis of polar herbicides and their metabolites in water samples. The second aim of this thesis is to study the occurrence of selected herbicides and metabolites in German Baltic estuaries as an indicator for their transport into the Baltic Sea. The third goal of this thesis is to study the effect of the compounds most observed in the Baltic estuaries on *Nodularia spumigena*, the dominant cyanobacterium observed in summer blooms in the Baltic Sea. The thesis is subdivided into four chapters

Chapter 1 is an introduction into key aspects relevant for the importance and better understanding of the present work. The introduction includes basic information about the Baltic Sea region and its pollution problem by pesticides, herbicide pathways to the surface water and their effects on the aquatic ecosystem. In addition, it includes information on pesticides classification and the German pesticide market. Furthermore, the motivation for the selection of polar herbicides for this study as well as literature information about their physico-chemical properties, chemical structures, uses, modes of action, environmental fate and toxic effects on aquatic organisms are described. Lastly, it involves a brief description of the most common techniques used for polar herbicides analysis in environmental samples.

Chapter 2 describes the methodology part of the thesis which includes: chemicals and materials, the final gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) methods applied in this thesis, sampling sites, sample collection, treatment and the experimental part of the toxic effect study as well as a brief overview of the methods applied for the determination of chlorophyll-*a*, particulate organic carbon and cell numbers.

Chapter 3 outlines "Results and Discussion" and it is subdivided into three main sections. The first section describes a comparison between the two techniques GC-MS and HPLC-MS/MS for the analysis of the herbicides glyphosate, mecoprop, MCPA, isoproturon, bentazon and chloridazon and the three metabolites aminomethylphosphonic acid (AMPA), chloridazon-desphenyl (CD), and chloridazon-methyl-desphenyl (CDM) in water samples. Furthermore, it represents development and validation of two HPLC-MS/MS analytical methods in order to study the occurrence of the selected compounds in estuarine water samples. The first method is a direct HPLC-MS/MS analysis of the compounds mecoprop, MCPA, isoproturon, bentazon, chloridazon and CMD in water samples. The second method is an HPLC-MS/MS method for the analysis of glyphosate and AMPA in water samples after

pre-derivatization with fluorenylmethyloxycarbonyl chloride (FMOC-Cl). The second section is an application of the analytical method for the determination of the selected herbicides and metabolites in ten of the German Baltic estuaries and their transport into the Baltic Sea. In the third section, results obtained from testing the response of the cyanobacterium *Nodularia spumigena* when exposed to different concentrations of the herbicide glyphosate (Roundup®) and the metabolite AMPA were shown.

The general conclusion and a future perspective are given in **Chapter 4.**

1 Introduction

1.1 The Baltic Sea

The Baltic Sea is a semi-closed sea located in Northern Europe. It is considered a small sea with a catchment area of approximately 1 720 000 km^2 and an average depth of 52 m. On the other hand, it is one of the largest isolated bodies of brackish water in the world (Andersen et al. 2010, Schiewer et al. 2004). The Baltic Sea is shared by 14 countries. Almost 95% of the catchment area of the Baltic Sea belongs to nine countries; Germany, Sweden, Russia, Poland, Latvia, Finland, Estonia, Lithuania and Denmark. The remaining 5% relates to five countries; Norway, Belarus, Ukraine, the Czech and Slovak Republics (Figure 1.1) (Burkhardt et al. 2005). Germany makes up almost 4% (28600 km^2) of the catchment area of the Baltic Sea (HELCOM 2004). The salinity is much lower than ocean salinity (35) and it varies throughout geological epochs.

Figure 1.1: The Baltic Sea region [modified from stepmap.de].

The inflow of freshwater and water exchange between the Baltic Sea and the North Sea are considered to be the reason of salinity variation (Carlsson M. 1997).

The Baltic Sea is connected to the North Sea via the Danish straits which includes three straits, the Great Belt, the Little Belt and the Sound (She et al. 2007). River runoff to the Baltic Sea is considered to be a main factor effecting the ecosystem of the Baltic Sea (Bergström et al. 2000). The seven largest rivers flowing to the Baltic Sea are the Neva, Vistula, Neman, Daugava, Oder, Göta älv, and Kemijoki which form almost half of the riverine fluxes into the Baltic Sea. The long term average riverine fluxes into the Baltic Sea are estimated to be approximately 479 km^2 per year at rate of 15 190 m^3/s (Burkhardt et al. 2005).

Four German federal states share the catchment area with the Baltic Sea with 16 720 km^2 in Mecklenburg-Vorpommern, 5 940 km^2 in Brandenburg, 5 250 km^2 in Schleswig-Holstein and 880 km^2 in Sachsen (BUND Mecklenburg-Vorpommern 2012). The Baltic drainage basin is generally characterized by agriculture in the south (Bergström et al. 2000). 72% of the German Baltic catchment area consists of agricultural areas and only 15% is woodland, therefore Germany has the highest agricultural activities compared to other Baltic countries (BUND Mecklenburg-Vorpommern 2012). The German riverine flow into the Baltic Sea forms a small portion of the total flow and is limited to a large number of small rivers (BUND Mecklenburg-Vorpommern 2012, Burkhardt et al. 2005). Table 1.1 shows the average flow, discharge per unit area, basin area and length of some German rivers flowing into the Baltic Sea through the federal states of Mecklenburg-Vorpommern and Schleswig-Holstein.

Table 1.1: The average flow, discharge per unit area, basin area and length of ten German rivers draining into the Baltic Sea through the federal states of Mecklenburg-Vorpommern (M-V) and Schleswig-Holstein (S-H) (Bachor et al. 2002, BERNET CATCH 2006).

River	Average flow (m^3/s)	Discharge per unit area ($L/s.km^2$)	Basin area (km^2)	Length (km)	Federal state
Peene	23.4	4.7	5127	124.3	M-V
Warnow	13.4	4.4	3304	155.2	M-V
Ucker	7.61	3.1	2439	44.5	M-V
Trave	7.58	10.44	1807	113	S-H
Stepenitz	5.71	8.21	762.76	55.8	M-V
Recknitz	4.43	6.71	668.73	76.8	M-V
Schwentine	4.38	9.58	726	70	S-H
Barthe	2.23	6.9	342.76	34.2	M-V
Wallensteingraben	1.18	17.8	158.17	17.8	M-V
Ryck	0.77	5.5	239.99	26.5	M-V

1.2 Pesticides, classification and the German herbicides market

Pesticides are substances used directly to control pest populations or to prevent or reduce pest damage. Pesticides can be classified into groups according to their purpose:

- The group of pests controlled (e.g. herbicides, insecticides, fungicides)
- Their chemical composition (organic, inorganic, biological pesticides)
- The target pest (e.g. gypsy moth insecticide)
- Use patterns (e.g. foliar vs. soil fungicides)

Mostly, pesticides are classified according to the pests controlled (Waxman 1998).

Germany is the second largest consumer of pesticides in Europe after France (Zhang et al. 2011). The total amount of pesticides used in 2012 in Germany was 46 326 tons. In 2012, herbicides made up 43% of totally used pesticides in Germany (Figure 1.2) (BVL. 2012). Sales of herbicides on the German market have increased from 2003 to 2012 by approximately 23% (Figure 1.3) (BVL. 2012).

The intensive application of pesticides causes growing in two areas. First, Pesticides may contaminate foods which have been handled with pesticides (Van der Hoff et al. 1999). Second, the aquatic environment will be contaminated with pesticides with potential toxic effects to non-target aquatic organisms (Palma et al. 2009).

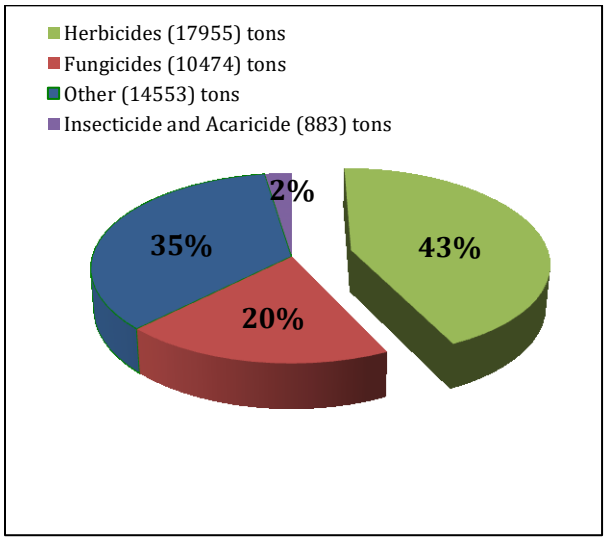


Figure1.2: Application of pesticides in 2012 in Germany (BVL 2012) .

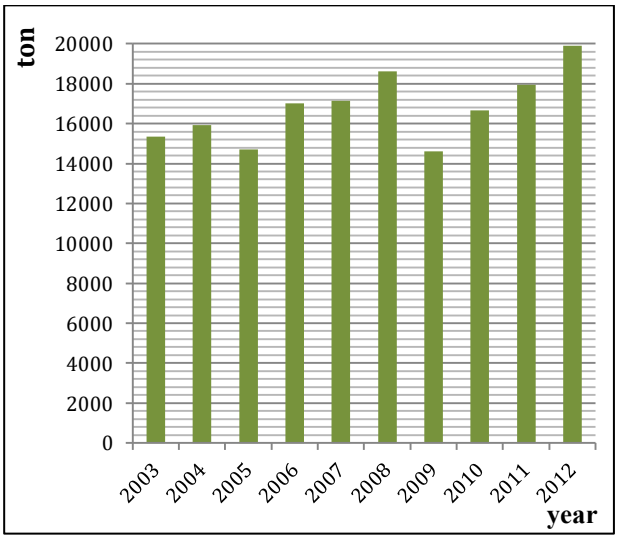


Figure 1.3: The annual German sales of the herbicides from 2003 to 2012 (BVL 2012).

1.3 Pesticide contamination of the Baltic Sea and its estuaries

A large population of about 80 million people lives in the catchment areas of the Baltic Sea. Renewal of the water of the Baltic Sea through water exchange with the North Sea takes about 30 years. Due to these characteristics, the Baltic Sea is considered to be one of the worst polluted sea in the world (HELCOM, 2003).

The Baltic Sea is exposed to various problems such as pollution, eutrophication, overfishing, construction (e.g. dumping of dredged material) and the introduction of alien species. Pollution by pesticides is considered to be one of the main problems in the Baltic Sea which has a major impact on its biological diversity (Rheinheimer, 1998; Walday and Kroglund, 2002).

The majority of research and monitoring programs on the pollution of the Baltic Sea are focusing on persistent organic pollutants (POPs) such as dioxins, polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs) and organochlorine pesticides (OCPs) such as dichlorodiphenyl-trichloroethane (DDT), hexachlorobenzene (HCB), hexachlorocyclo-hexanes (HCHs), dieldrin, chlordanes, toxaphene, heptachlor and heptachlor epoxide (Allsopp et al., 2001; Falandysz et al., 2004; Pikkarainen, 2007; Strandberg et al., 1998).

However, very little attention has been paid to the occurrence of other classes of pesticides (e.g. mid and highly polar) and their metabolites in the Baltic estuaries and their transport to the Baltic coast. There is the general assumption that most polar pesticides degrade fast and do not reach the marine environment (Barceló and Hennionb, 1997). Thus, many investigations have been pursued on the occurrence of these pesticides and their transformation products in fresh surface water, drinking and ground water (Brouwer et al., 1995; Loos et al., 2007; Loos et al., 2010).

1.4 Pathways of herbicides to the surface water

Upon herbicides application they become distributed among for major compartments: soil, water, air and biota (living organisms) (Linde, 1994). Herbicides can enter the surface water by point and nonpoint source. The point source contamination is derived from farmyard activities including filling containers, cleaning pesticide application equipment, leaks from storage containers, pesticide spills and unsuitable discarded herbicides and their containers. The nonpoint source or diffusion is the contamination that arises from surface and subsurface runoff, soil erosion from treated areas, application drift, deposition and precipitation after volatilization (Tiryaki and Temur, 2010; Wenneker et al., 2010).

The mobility of herbicides into the surface water is strongly affected by their physico-chemical properties such as water solubility and partition coefficients (Gramatica and

Guardo, 2000), the nature and properties of the soil components such as organic matter content, pH, surface area, clay content, cation exchange capacity and moisture content (Heatwole et al., 1992), herbicides management, type of plants as well as other environmental factors including weather and topography (Kreuger and Tomqvis, 1998).

Runoff induced by precipitation or irritation, is considered to be the most important non-point source of surface water contamination through herbicides (Dabrowski et al., 2002; Neumann et al., 2002). In runoff water, both the dissolved and the particle-bound pesticides can be transferred to the surface water (Wu et al., 2004). In general, medium and highly polar herbicides with high water solubilities are more likely to be in the dissolved phase (Steen, 2002). Once herbicides reach the river streams, they are transported via estuaries to the coastal areas. Rivers transport the largest amount of herbicides entering estuaries and the marine environment (Olsson et al., 2012).

When herbicides enter the marine environment, they distribute between the dissolved and particulate phases. The distribution process depends on the sorption and desorption equilibrium with the suspended particulate matter present in the water. The pesticides may sorb to particles and organisms such as phytoplankton and subsequently they are transferred from the surface waters to the depth by sinking particles and by zooplankton vertical migration (Quante et al., 2011).

1.5 The effect of herbicides on the aquatic ecosystem

Toxic effects of herbicides do not exert only in the applied areas, but also in places distant from their application as they are transported to aquatic ecosystems and cause negative effects on marine organisms. Most studies of herbicide effects in aquatic ecosystems have been focused on the herbicide atrazine.

However, when herbicides enter aquatic ecosystems, they may affect the ecosystem both directly and indirectly (Nielsen and Dahllof, 2007). Direct impacts of herbicides include the structural or functional level (Fairchild et al., 1998). The structural level involves static measures of different parameters such as biomass, cell numbers, community diversity or species composition (Pérez et al., 2011). The functional level is the assessment of changes of rates of processes occurring in living organisms such as oxygen evolution, carbon uptake, nutrient cycling, enzyme activity, population growth rates or changes in system metabolism (Pérez et al., 2011). The indirect effects of herbicides are the impacts on consumer populations such as fish and invertebrates due to negative impacts on primary producers such as macrophytes and algae (Pérez et al., 2011).

As herbicides are synthesized to inhibit weeds growth, aquatic plants and algae are potential non-target organisms sensitive to herbicides. Aquatic plants and algae are the

essential components of the aquatic systems (Scheffer et al., 1993). Several ecotoxicological surveys have shown that fresh and marine plants and algae are sensitive to herbicides and are affected by the exposure to herbicides. Magnusson et al. (2010) showed that the herbicides diuron, tebuthiuron, atrazine, simazine, and hexazinone and the herbicide breakdown products (desethyl-atrazine (DEA) and 3,4-dichloroaniline (3,4-DCA)) caused photosystem II-inhibition to the tropical benthic microalgae *Navicula sp*., *Cylindrotheca closterium*, *Nephroselmis pyriformis* and *Phaeodactylum tricornutum*. The green algae *Nephroselmis pyriformis* showed higher sensitivity to the selected herbicides than diatoms. Lewis et al. (2009) reported that the herbicide levels in riverine flood plumes flowing to the Great Barrier Reef lagoon can cause negative effects on some marine organisms. Subsequently, this may lead to a change in the community structure of seagrass, mangrove and coral reef ecosystems. Bester et al. (1995) studied the effects of the herbicide atrazine on marine phytoplankton from the German Bight (North Sea). The results showed that atrazine reduced photosynthesis accompanied by lower chlorophyll concentrations and reduced primary production.

Aquatic bacteria also play an important role in the aquatic food web. They are essential players in the decomposition process of dead material and in recycling carbon and nutrients as well as in the mineralization processes and chemical transformation of elements between reduced and oxidized forms (Bertoni, 2011; Tang et al., 2012). Herbicides can affect aquatic bacteria. Anand et el. (2012) demonstrated that the activity of 50 strains of 250 tested marine bacteria strains were affected by exposure to herbicides, whereas eight strains of them showed 90% inhibition at very low concentration of 5 µg/L of the herbicides.

Invertebrates include a large group of aquatic species such as zooplankton, insects, worms and snails with a high variety in shape and size, which play a necessary role in the aquatic ecosystem (Pérez et al., 2011). Studies on acute toxicity tests LC_{50}/EC_{50} of the herbicides atrazine to estuarine/marine invertebrates (e.g. mysid shrimp and eastern oyster) categorized atrazine as "highly toxic on an acute exposure basis" (EPA, 2006). Contardo-Jara et al. (2009) demonstrated a considerable increase in enzyme activities of the worm *Lumbriculus variegatus* upon exposure to the herbicide glyphosate.

Fish have an important value in human nutrition and medicine. They are used for the treatment of vector-borne diseases like malaria and schistosomiasis. They have an important function in the aquatic ecosystem in nutrient cycling, control of algae and macrophytes, reduction of waste and regulation of food web dynamics (Holmlund and Hammer, 1999). A study conducted on the toxicity of the three herbicides alachlor, atrazine and diuron on turbot flatfish revealed that the herbicide alachlor is highly toxic to *Psetta maxima*. The larvae were more sensitive than turbot embryos to the three herbicides. Toxicity symptoms were observed through malformations (embryos), clear decrement in

hatching success, pericardial oedema and skeletal deformation (larvae) (Lazhar et al., 2012). Another study showed that the exposure of zebrafish to the herbicide atrazine at a concentration lower than 5 µg/L affected the swimming behavior of the fish (Steinberg et al., 1995).

1.6 Studied herbicides and their physicochemical properties

For this study six polar herbicides and three of their metabolites have been chosen. The herbicides are the organophosphuros herbicide glyphosate and its metabolite aminomethyl-phosphonic acid (AMPA), the phenoxyacetic acid herbicides MCPA and Mecoprop, the phenylurea herbicide isoproturon, the tiodiazine herbicide bentazon and the pyridazinone herbicide chloridazon and its metabolites chloridazon-desphenyl (CD) and chloridazon-methyl-desphenyl (CDM). The physico-chemical properties of the six selected herbicides are shown in Table 1.2.

The compounds were chosen based on their presence in watercourses of Mecklenburg-Vorpommern, Germany according to the special report of the Agency for the Environment, Nature Conservation and Geology Mecklenburg-Vorpommern, in 2008 (Bachor et al., 2008) and on sales on the German market. The used amount of the selected six herbicides in Germany in 2012 is shown in Table 1.3. Less is known about their occurrence in the estuaries and their transport to the marine environment such as the Baltic Sea. However, for including these compounds in the Baltic monitoring programs and for proper risk assessment, detailed information is required about their occurrence in the Baltic estuaries and their transport to the Baltic Sea.

Table 1.2: The physicochemical properties of the six studied herbicides glyphosate, MCPA, mecoprop, bentazon, isoproturon and chloridazon (IUPAC, 2013).

Property	Glyphosate	MCPA	Mecoprop	Bentazon	Isoproturon	Chloridazon
CAS No.	1071-83-6	94-74-6	7085-19-0	25057-89-0	34123-59-6	1698-60-8
Chemical formula	$C_3H_7NO_5P$	$C_9H_9ClO_3$	$C_{10}H_{11}ClO_3$	$C_{10}H_{12}N_2O_3S$	$C_{12}H_{18}N_2O$	$C_{10}H_8ClN_3O$
Molecular mass (g moL^{-1})	168.07	200.62	214.65	240.3	206.28	221.6
Water Solubility (20 °C mg/L)	10500	29390	250000	570	70.2	422
Dissociation constant (pKa) at 25 °C	2.34	3.73	3.11	3.28	-	3.38
Vapor pressure at 25 °C (mPa)	0.0131	0.4	1.6	0.17	5.50×10^{-03}	1.0×10^{-06}
Henry's Law Constant (Pa.m3/mol at 25 °C)	2.10×10^{-07}	5.50×10^{-05}	2.20×10^{-04}	7.20×10^{-05}	1.46×10^{-05}	5.30×10^{-10}
Octanol/Water Partition Coefficient (Log K_{OW}) at pH 7, 20 °C	-3.2	-0.81	-0.19	-0.46	2.5	1.19
Sorption Partition Coefficient, K_{OC}	1435	-	47	55.3	-	120
Soil degradation (days) aerobic	12	24	8.2	13	12	31

Table 1.3: The used amount of selected herbicides in Germany in 2012 (BVL, 2012).

Herbicides	Amount (tons)
Mecoprop	100-250
Chloridazon	100-250
MCPA	250-1000
Isoproturon	1000-2500
Bentazon	1000-2500
Glyphosate	2500-10000

1.6.1 Glyphosate

Glyphosate [*N*-(phosphonomethyl)-glycine] (Figure 1.4), is the active ingredient in the commercial product Roundup®. It is a broad spectrum, non-selective and post-emergent herbicide, developed in 1971 by the Monsanto company (Cox, 2004). Glyphosate uses are not limited only to the agricultural area but it is also used to control aquatic weeds (Carlisle and Trevors, 1987). It is extensively used in parks, gardens, yards, forest, lawns, roadsides, railway tracks, industrial areas and other non-agriculture areas (Cox, 2004; Miller et al., 2010). Glyphosate controls weeds by inhibiting the activity of the enzyme 5-enolpyruvyl-shikimic acid-3-phosphate synthase (EPSP) which is necessary for the formation of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. For animals those aromatic amino acids are essential (Carlisle and Trevors, 1987; Miller et al., 2010; Steinrticken and Amrhein, 1980).

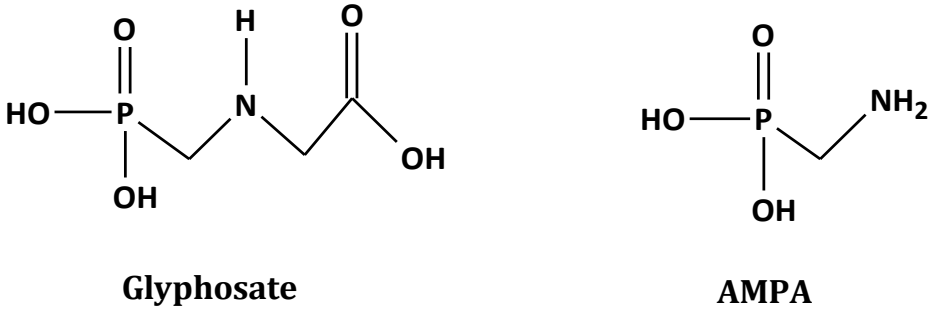


Figure 1.4: The chemical structures of glyphosate and its metabolite aminomethylphosphonic acid (AMPA).

Glyphosate can be degraded in soil, water and plant (Battaglin et al., 2005). Two different pathways have been demonstrated for the metabolism of glyphosate: the aminomethyphosphonic acid (AMPA) pathway and the sarcosine pathway (Malik et al., 1989). Glyphosate degrades primarily by microbial metabolism forming the product AMPA (Rueppel et al., 1977). AMPA (Figure 1.4) is the most frequently detected metabolite of glyphosate in soil, water and plants (Carlisle and Trevors, 1987; Dick and Quinn, 1995; Van der Hoff and Van Zoonen, 1999). Strong adsorption to soil particles decreases the degradation rate of glyphosate (Mandy et al., 2011). The half-life of glyphosate in soil varies from a few days to several years depending on the adsorption process and the level of microbial activity (Carlisle and Trevors, 1987). AMPA has a lower water solubility (5.8 g/L at 25°C) than the parent glyphosate and it is more persistent, more mobile and has a longer half-life in soil; between 76 to 240 days (Coupe et al., 2012)

Glyphosate binds to the soil particles rapidly during the first hour following application and slowly after that (Sprankle et al., 1975). Sorption, leaching and degradation of glyphosate can be very different from soil to soil depending on soil composition and properties (Borggaard and Gimsing, 2008). Glyphosate unlike most water soluble herbicides has an

extremely high ability to adsorb onto soil particles (Mandy et al., 2011). The adsorption of glyphosate increases with increasing clay content like humic substances (Piccolo et al., 1996), cation exchange capacity (Glass, 1987) and decreasing phosphor content (Vereecken, 2005) and soil pH (Gimsing et al., 2004). The competition of glyphosate with phosphate and a decreasing soil pH restrict the adsorption of glyphosate on soil particles and leads to a large mobility of glyphosate into drains, ground and surface water (Vereecken, 2005).

Generally, those commercial formulations of glyphosate (e.g. Roundup®) including surfactants showed a higher toxicity to aquatic organisms than technical glyphosate (Cedergreen and Streibig, 2005; Sobrero et al., 2007; Tsui and Chu, 2003). Aquatic plants and microalgae showed a higher sensitivity to the herbicide glyphosate than other organisms such as bacteria, protozoa, invertebrates, fish and amphibians due to the mode of action of glyphosate interfering with plant metabolisms (Pérez et al., 2011).

1.6.2 Mecoprop and MCPA

Mecoprop and MCPA are Chlorophenoxy herbicides (Figure 1.5). They are selective herbicides for post emergence control of a wide variety of broad-leaved weeds (RED, 2004; RED, 2007). Mecoprop and MCPA can cause auxin-like responses in broadleaf plants and kill them by disrupting nutrient transport and growth (Brent et al., 2004).

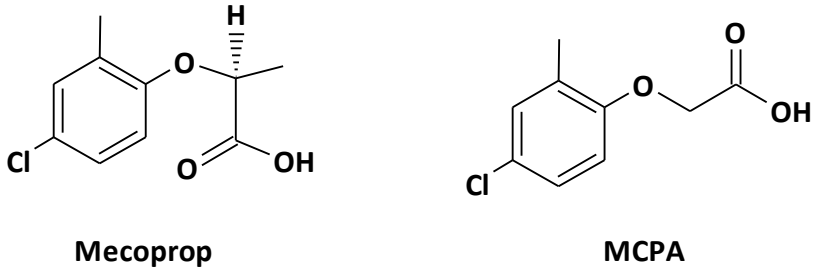


Figure 1.5: The chemical structures of the herbicides mecoprop and MCPA.

Due to the polar nature of MCPA and mecoprop and their high water solubility, they can be easily transferred to the surface and ground-water (Cserhati and Forgacs, 1998). MCPA is used in different commercial formulations such as MCPA acid, MCPA DMAS and MCPA 2-EHE. Acute aquatic toxicity studies have considered MCPA 2-EHE to be the highest toxic formulation of them, moderately to highly toxic, to the tested estuarine/marine non-vascular plants (*Skeletonema costatum*), invertebrates, and fish (RED, 2004). Mecoprop is also used in several traded formulations such as technical grade mecoprop (MCPP), mecoprop-p (MCPP-p), dimethyl-amine salts of mecoprop (MCPP DMA) and mecoprop-p (MCPP-p DMA) (Johnson et al., 2007). However, Mecoprop is toxic to non-target aquatic

organisms such as bacteria, diatom and plankton (Cox, 2004). Macrophytes and algae are more sensitive to the herbicide mecoprop than other taxa (Johnson et al., 2007).

1.6.3 Isoproturon

Isoproturon, also known by trade names such as Arelon®, Alon®, Graminon ®and Hytane®, is a selective, systemic herbicide (Figure 1.6) used for pre- and post-emergence control of germinating broadleaf weeds and grasses in crops, such as vegetables, beans, corn, alfalfa, fruits, cotton, cereals and nuts (Mallat et al., 2001; Paris-Palacios et al., 2010). Isoproturon inhibits photosynthesis in plants by blocking electron transport in photosystem II (Fobbe et al., 2006).

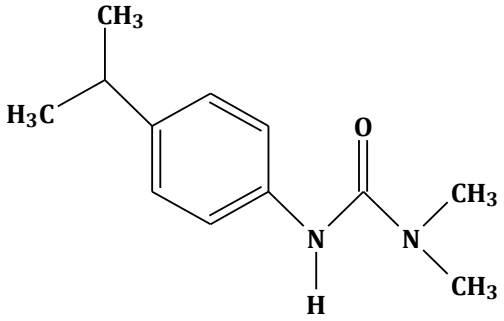
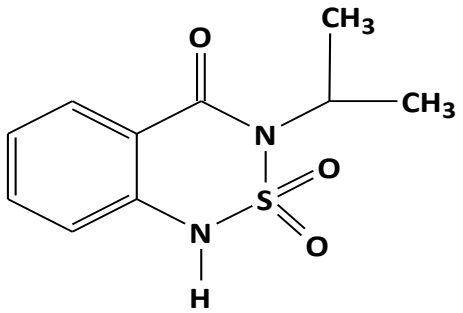


Figure 1.6: The chemical structure of the herbicide isoproturon.

The intensive use of isoproturon and its properties such as water solubility, low chemical and biochemical degradation rates and low adsorption to soils causes it to be easily released into the aqueous environment. It is often detected in ground and surface water (Bachor et al., 2008; Irace-Guigand et al., 2004; Reddy et al., 2012; Spliid and Koppen, 1998). Many toxicity studies of the herbicide isoproturon on algae showed that isoproturon can cause adverse effects on the growth of algae such as *Scenedesmus subspicatus, Lemna minor, Scenedesmus obliquus, Scenedesmus vacuolatus, isochrysis galbana and Chaeloceros calcilrans* (Dewez et al., 2008; His and Seaman, 1993; Nitschke et al., 1999; Vallotton et al., 2009). A study on the toxic effects of isoproturon on periphyton communities demonstrated that the biomass production of the algal community was reduced in response to a higher concentration of isoproturon (Schmitt-Jansen and Altenburger, 2005). Isoproturon was observed to have a strong effect on the growth of *Crassostrea gigas* larvae and less an effect on larvae of oysters during nine days of exposure of these organisms to the herbicide isoproturon (His and Seaman, 1993). Isoproturon can accumulate in worm tissues and it has a moderate effect on tubifex worm (Paris-Palacios et al., 2010).

1.6.4 Bentazon

Bentazon is a selective, contact herbicide (Figure 1.7) manufactured by BASF Corporation. It is marketed under several trade names such as Basagran® and Basamais® (FAO, 1999; Hourmant et al., 2009; RED, 1994). It is absorbed by plant leaves and acts as a photosynthetic electron transfer inhibitor for the plants. It is used for the control of broad-leaved weeds and sedges in rice, pepper, corn, mint, beans, peanuts, and others (FAO, 1999; RED, 1994). Bentazon has a high water solubility and a low octanol/water partitioning coefficient (K_{ow}), therefore it is found to be quit mobile in soil (Bach et al., 2010; Boesten and Van der Pas, 2000). It enters the surface water (e.g. rivers and streams) and is subsequently transported through estuaries to the marine environment.



Bentazon

Figure 1.7: The chemical structure of the herbicide bentazon.

Studies on the effect of bentazon and its formulation Basamais on the growth and physiological responses in the marine diatom *Chaetoceros gracilis* showed that the toxicity of Basamais is higher than bentazon itself. The study concluded that after exposure of the herbicide bentazon to *C. gracilis* for three days a decrease in the cell density was observed (Hourmant et al., 2009). Another performed work on the effect of bentazon on growth and maximum quantum yield of photosystem II (Fv/Fm) in cells of the marine diatom *Skeletonema costatum* demonstrated that bentazon rapidly leads to Fv/Fm decrease, while effects on algal growth were detected after 24 h of exposure (Macedo et al., 2008). Bentazon is considered a slight toxin to aquatic invertebrate and nontoxic to both coldwater and warmwater fish (Kamrin, 1997).

1.6.5 Chloridazon

Chloridazon is the active ingredient of the herbicide pyramine®. It is a selective systemic herbicide (Figure 1.8) which can be used pre- and post-emergence to control broad-leaved weeds in fodder beet, sugar beet, and beetroot (Buttiglieri et al., 2009; Kucharski et al., 2012). It works by blocking electron transport in photosystem II in green plants, thereby

inhibiting photosynthesis (Bisewska et al., 2012). Chloridazon-desphenyl (CD) and chloridazon-methyl-desphenyl (CMD) (Figure 1.8) are two degradation products of chloridazon observed in the environment (Buttiglieri et al., 2009). Chloridazon was found to be mobile and persistent in different types of soil, subsequently it can be transported to ground and surface water causing contamination of the water resources of certain areas with the tendency to persist (RED, 2005). Thus, many studies have verified the occurrence of chloridazon and its metabolites CD and CMD in surface water in Germany (Bachor et al., 2008; Buttiglieri et al., 2009).

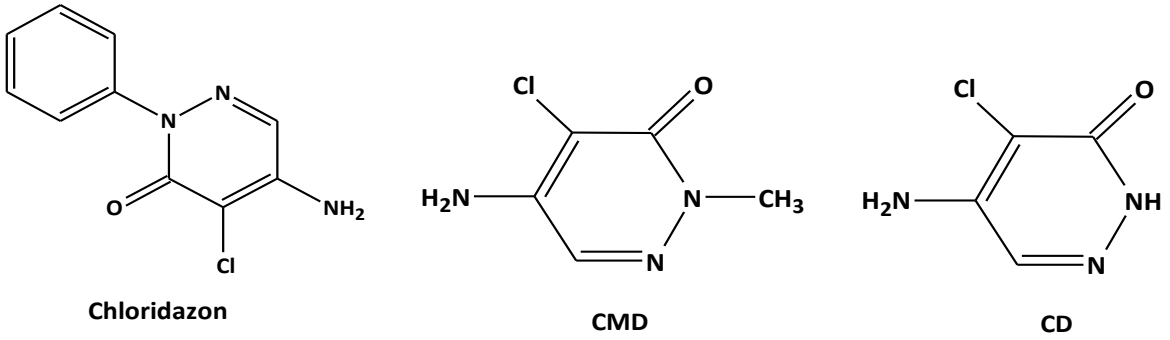


Figure 1.8: The chemical structures of the herbicide chloridazon and its metabolites Chloridazon-desphenyl (CD) and chloridazon-methyl-desphenyl (CMD).

Chloridazon and its metabolites were detected in surface water samples in Mecklenburg-Vorpommern, Germany. The metabolite CD was most frequently detected with highest concentrations among them (Bachor et al., 2008). There is a lack of available data on acute and chronic effects of the herbicide chloridazon and its metabolites on aquatic organisms.

1.7 Analytical techniques for polar herbicide analysis

In recent decades many analytical techniques have been developed to identify organic contaminations such as pesticides in environmental matrices, which are often present at trace levels (Ferrer and Barceló, 1998). Due to the satisfactory separation capacity and versatility of chromatographic techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) their application is growing for the identification and quantification of pesticide residues in different organic and inorganic matrices (Cserhati et al., 2004). High polarity, low volatility and thermal liability are the three main reasons for the failure of direct GC/MS analysis of medium and highly polar herbicides and make HPLC the preferred analytical separation technique (Ferrer and Barceló, 1998; Queiroz et al., 2004).

Many detectors have been used in the HPLC analysis of polar herbicides, including common ultraviolet (UV) (Tran et al., 2007), diode-array (Mills, 1998), single mass spectrometry (MS) (Volmer and Levsen, 1994; Wu et al. 2002) and tandem mass spectrometry (MS/MS) (Fillatre et al., 2011b). Selected ion monitoring (SIM) in single-stage quadrupole and selected reaction monitoring (SRM) in triple-stage quadrupole are the most common modes used for quantitative analysis of pesticide residues (Fillatre et al, 2011a; Tadeo et al., 2000). Mass spectrometry can couple to HPLC using different interfaces as thermospray, particle beam, electrospray ionization (ESI) and atmospheric pressure chemical ionization (Kollroser and Schober, 2002). Reversed phase chromatography is the most popular separation technique used for herbicide analysis (Tadeo et al., 2000).

High performance liquid chromatography tandem to mass spectrometry (HPLC-MS/MS) with triple quadrupole (QqQ) analyzers operating in the selected reaction monitoring (SRM) via electrospray ionization (ESI) interface has proven to have a high sensitivity and selectivity for the determination of polar pesticides in aqueous samples (Bossi et al., 2002; Giordano et al., 2009; Postigo et al., 2010; Steen et al., 1999).

1.8 Objectives of the thesis

Monitoring of Baltic Sea pollution and risk assessment programs is focusing on persistent organic pollutants (POPs). On the other hand, very little attention has been paid to the monitoring of other classes of pollutants such as polar organic pesticides. To include polar organic pesticides in the monitoring programs of the Baltic Sea, information is required about their potential transport into the Baltic Sea. These substances should be measured with appropriate analytical methods. In this study, six polar herbicides and three of their metabolites were selected. The compounds are glyphosate and its metabolite aminomethyl-phosphonic acid (AMPA), the herbicides MCPA and Mecoprop, the phenylurea herbicide isoproturon, the tiodiazine herbicide bentazon and the pyridazinone herbicide chloridazon and its metabolites chloridazon-desphenyl (CD) and chloridazon-methyl-desphenyl (CDM). The main objectives of this study were as following:

(a) A comparison between two analytical techniques GC-MS and HPLC-MS/MS for the analysis of selected polar compounds. Subsequently, development and validation of analytical methods using this suitable technique for their determination in water samples at trace levels.
(b) The application of these analytical methods in order to investigate the potential transport of the target compounds into the Baltic Sea through their occurrence in some of the German Baltic estuaries.
(c) Study the effect of two of the target compounds (i.e. Roundup® and AMPA) on the growth of the cyanobacterium *Nodularia spumigena,* the dominant cyanobacteria species observed in cyanobacteria Baltic Sea eutrophication.

2 Materials and Methods

In this chapter:

Materials, preparation of the solutions and the HPLC-MS/MS eluents, the derivatization reactions, the final GC-MS and HPLC-MS/MS methods, sampling time and locations, sample collection and preparation as well as a brief overview of the experimental part of the toxic effect experiment which includes algae and culture conditions and determination of each of chlorophyll-*a*, cell number, particulate organic carbon and toxicants concentrations in the treated cultures is given.

2.1 Chemicals and reagents

The following chemicals were obtained from Dr. Ehrenstorfer GmbH (Ausburg, Germany) at concentrations of 100 ng/µL:
Pure standards of glyphosate (N-(Phosphonomethyl) glycine), Aminomethylphosphonic acid (AMPA), mecoprop (methyl chlorophenoxypropionic acid), MCPA ((4-chloro-2-methyl-phenoxy) acetic acid), isoproturon (3-(4-isopropyl- pheneyl)-1,1-dimethylurea), bentazon (3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one2,2-dioxide), chloridazon (5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone), chloridazon-desphenyl (5-amino-4-chloro-3(2H)-pyridazione), and chloridazon-methyl-desphenyl (5-amino-4-chloro-2methyl-3(2H)-pyridazinone) in addition to the internal standards which involve isotope-labeled glyphosate 1,2-C^{13} N^{15} and AMPA-C^{13} N^{15} and deuterium labeled MCPA-D_6, mecoprop-D_6, isoproturon-D_6, bentazon-D_6 and chloridazon-D_5 (in water for glyphosate, AMPA and their labeled compounds and in acetonitrile for the other compounds). All standard solutions were stored in capillary bottles (Certan®, LGC Promochem) at 5 °C in the dark. The herbicide Roundup® 66935 Speed which includes 7.2 g/L glyphosate as isopropylamine salt 9.7 g/L and 9.55 g/L pelargonic acid was purchased from a commercial source (Baumarket, Germany). HPLC water and the chemicals used in f/2 medium $NaNO_3$, Na_2HPO_4, $Na_2SiO_3x9H_2O$, $ZnSO_4xH_2O$, $CuSO_4xH_2O$, $CoSO_4x7H_2O$, $MnSO_4xH_2O$, $FeCl_3x6H_2O$, $Na_2EDTAx2H_2O$, vitamins B12 and Biotin were obtained from VWR (Germany). The derivatization reagents trifluoroacetic anhydride (TFAA), trifluoroethanol (TFE) and 9H-Fluoren-9-ylmethyl chloroformate (FOMC-Cl ≥ 99.0%) and sodium tetraborate decahydratec which was used in preparation of borate buffer as well as acetic acid and formic acid used in the HPLC mobile phase were purchased from Sigma-Aldrich (Germany). The solvent ethyl acetate (SupraSolv®) was obtained from Merck KGaA (Germany).

Ethanol 96%, HPLC-optigrade acetonitrile and methanol were purchased from Walter-CMP GmbH (Germany). The sea salt was obtained from Tropic Marin®.

2.2 Preparation of working solutions, buffer solutions and eluents

Stock solutions (100 ng/µL) of the all target analytes were purchased and kept in a fridge at 5 °C in the dark. Working solutions were prepared by accurate dilution of the stock solutions. Polypropylene vessels were used during glyphosate and AMPA preparations due to their high polarity and their possible adsorption onto glassware. On the other hand, glassware was used for the analytes MCPA, mecoprop, bentazon, isoproturon, chloridazon, CD and CMD because of their lower polarity.

Borate buffer pH 9 was prepared by dissolving 1g of sodium tetraborate decahydratec in 50 mL Milli-Q water.

The eluent of 2 mM NH_4HCO_3 pH 9 was prepared by dissolving 158 mg of ammonium bicarbonate in 1 liter of water. Then, a volume of 100 µL of ammonium solution was added in order to adjust to pH 9. The eluents of 0.1% acetic acid in water and 0.1% formic acid in methanol were prepared by adding 1 ml of acetic acid and 1 ml of formic acid to 1 L water and 1 L methanol, respectively.

2.3 TFAA and TFE derivatization for GC-MS analysis

A 10 µL volume of each stock standard solutions of glyphosate, AMPA, mecoprop, MCPA, isoproturon, bentazon, chloridazon, CD and CMD as well as their labeled compounds glyphosate 1,2-C^{13} N^{15}, AMPA-C^{13} N^{15}, mecoprop-D_6, MCPA-D_6, isoproturon-D_6, bentazon-D_6, and chloridazon-D_5 were placed in 0.9 mL clear crimp vials. The solvent was evaporated under a stream of clean air at 20 °C for 30 minutes using a TurboVap LV Evaporator (Caliper Life Sciences, USA). 150 µL trifluoroacetic anhydride (TFAA) and 150 µL trifluoroethanol (TFE) were added to the residues. The vials were closed with ultraclean aluminum crimp caps. The vials were shaken and heated at 90 °C for 1 hour. The samples were allowed to cool at room temperature for about 30 min. The derivatized solution was evaporated to dryness. The vials were rinsed with 100 µL ethyl acetate to minimize loss of the derivatives and subsequently the solvent was evaporated to dryness for 15 min. The residues were redissolved in 100 µL ethyl acetate and the solutions were analyzed by GC-MS.

2.4 FMOC-Cl derivatization for HPLC-MS/MS analysis of glyphosate and AMPA

The derivatization process of glyphosate and AMPA was achieved by placing 800 µL of mixed standard solutions of glyphosate and AMPA in water into 2 mL Safe-Lock-tubes (Eppendorf, Germany). 100 µL of 0.07 M borate buffer of pH 9 was added to the solution and vigorously shaken. 100 µL of 1 mM FMOC-Cl in acetonitrile was added to each tube containing the solutions. The tubes were closed, shaken well and kept at room temperature for 4 hours for the derivatization reaction to occur. After 4 hours the samples were subjected to a filtration step by passing through a 0.45 µm Phenex-Rc 15 mm syringe filter (Phenomenex, Germany). Finally, the samples were analyzed by HPLC-MS/MS.

2.5 GC-MS instrument and operating conditions for analysis of all the target analytes

Derivatized standards with TFAA and TFE were analyzed by a Hewlett Packard 6890 Gas Chromatograph coupled with a Hewlett Packard 5973 Mass Spectrometer Detector (MSD) and an Agilent 6890 autosampler. The gas chromatographic system was equipped with a programmed temperature vaporizer injector (PTV) containing a glass liner (Gerstel, Germany). A DB-5ms non-polar capillary column (30 m length, 250 µm inner diameter and 0.25 µm film thickness 5%-phenyl- 95% dimetheylpolysiloxane (J & W Agilent, USA)) was used for the gas chromatographic separation. Instrument operating and data processing was managed with MS ChemStation software (Agilent, USA). A 2 µL volume of the sample were injected into the PTV. The injector temperature was 60 °C before injection and increased directly after the injection to 280 °C at a rate of 12 °C per second and held at this temperature for 3 minutes. Helium was used as carrier gas. The carrier gas flow in the column was at a constant flow at 1.2 mL per minute, the pressure was 0.703 bar and the average velocity was 40 cm per second. The injector was operating in splitless mode. After 2 minutes the split valve was opened in order to avoid solvent tailing and to sweep any vapors remaining in the liner. The split flow was adjusted to 11.8 mL/min during the sample transfer and increased to 15.5 mL/min during injector cleaning. The oven temperature program for the effective separation of the herbicides and metabolites is shown in Figure 2.1.

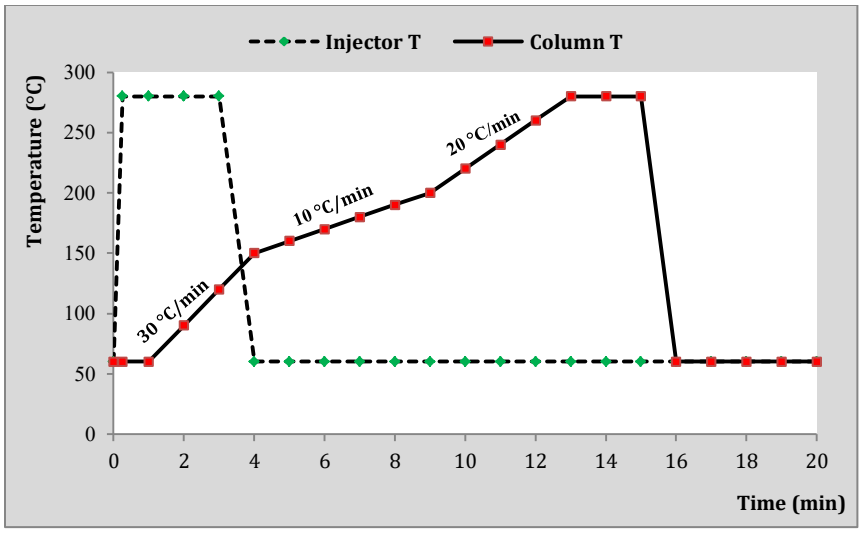


Figure 2.1: Temperature program of GC-MS injector and oven for the separation of the herbicides compounds.

For detection, the transfer line temperature was set at 280 °C. Ionization was achieved by electron impact at 70 eV at an ion source temperature of 230 °C and the temperature of the MS quadropule was 150 °C. Autotune was usually achieved before measurements were taken in order to apply voltages on the source elements. The calibration substance used in the tune process was perfluorotributylamine (PFTBA) on the masses 69, 219 and 502. The electron multiplier voltage (EM) was between 1642-1800 eV. Full scan mode was used in order to determine the fragmentations and the retention times of the analytes. The obtained mass spectra of the target analytes and their labeled compounds were in the mass range from 15 to 550 u with a scan rate of 2.73 scans per second.

2.6 HPLC-MS/MS instrument and operating conditions for direct analysis of MCPA, mecoprop, isoproturon, bentazon, chloridazon and its metabolites CD and CMD

The analysis was achieved using an LC-MS-MS (Thermo Fisher Scientific). The HPLC system consisted of an Accela autosampler (Series: 750477), Accela pump (Series: 700862) and Maylab Mistra Switch model 886 (Series: 100027). A TSQ vantage triple quadrupole mass analyzer (Thermo Fisher Scientific Series-Nr.: TQU 02725) equipped with a heated electrospray ionization source (HESI) interface was used for detection of the analytes. For initial method setup, the MS system was calibrated and tuned using polytyrosine 1, 3, 6 standard solution. The ionization was achieved in both negative and positive mode.

The chromatographic reversed phase separation was performed on a Kinetex 2.6 µm C_{18} column (50 x 2.1) mm from Phenomenex (Germany) at 20 °C. The used mobile phase A was Milli-Q water with 0.1% acetic acid and the mobile phase B was a methanol with 0.1% formic acid. The gradient elution program was as follows: 0-14 min gradient from 90% A and 10% B to 10% A and 90% B until separation of all the target compounds was achieved, 14-16 min isocratic 10% A and 90% B, 16-17 min gradient 10% A and 90% B to 90% A and 10% B, 17-20 min isocratic 90% A and 10% B at a flow rate of 250 µL/min. A volume of 50 µL samples were directly injected into the HPLC-MS/MS system.

The optimal HESI-MS-MS conditions operating in negative and positive modes were set as follows: capillary temperature 300 °C, vaporizer temperature 300 °C, spray voltages in positive and negative polarity were ±3500 V, sheath gas pressure 20 psi; auxiliary gas flow rate 10 arbitrary units (a.u.); sweep gas pressure 0 psi. The collision gas used was argon and the collision gas pressure was set to 32 psi. The instrument was controlled by Xcalibur software (Version 2.1).

2. 7 HPLC-MS/MS operating conditions for glyphosate and AMPA analysis after derivatization with FMOC-Cl

The analysis was performed through the HPLC-MS/MS system described in section 2.6. Reversed phase separation was achieved using a (150 x 2.0) mm, 3 µm particle size, Gemini-NX C_{18} with a Gemini-NX Security Guard cartridge (4 x 2.0) mm, both supplied by Phenomenex, Germany. The oven temperature was set to 20 °C. Chromatography separation was achieved through gradient elution with a 2 mM ammonium bicarbonate buffer at pH 9 (mobile phase A) and pure acetonitrile (mobile phase B) at a flow rate of 100 µl/min. The elution steps were as follow 0-2 min isocratic at 99% A and 1% B, 2-15 min gradient from 99% A and 1% B to 37% A and 63% B, 15-17 min isocratic at 37% A and 63%, 17-19 min gradient from 37% A and 63% B to 5% A and 95% B, 19-27 min isocratic at 5% A and 95% B, 27-30 min gradient from 5% A and 95% B to 99% A and 1% B. The volume of sample injections into HPLC-MS/MS was 50 µL.

The analytes were detected with the previously described TSQ vantage triple quadrupole mass analyzer equipped with the heated electrospray ionization source (HESI) interface using the optimized MS parameters. For the initial method setup, the MS system was calibrated and tuned using polytyrosine 1, 3, 6 standard solution. The ionization was done in negative mode. The optimal HESI-MS/MS conditions operating in negative mode were set as follows: capillary temperature 300 °C, vaporizer temperature 200 °C, spray voltage -3500 V, sheath gas pressure 20 psi; auxiliary gas flow rate10 arbitrary units (a.u.); sweep gas pressure 0 psi; S-lens offset 62 V and 43 V for glyphosate and AMPA, respectively. The

used collision gas was argon and the collision gas pressure was set to 32 psi. The collision energy was 21 and 20 eV for glyphosate and AMPA, respectively. Data processing was managed using Xcalibur (Version 2.1).

2.8 Sampling sites and period

Water samples were taken from ten different estuarine stations (1-10) and one from the Baltic Sea station (11). The sampling stations are distributed along the Baltic Sea coastline of Mecklenburg-Vorpommern, Germany (Figure 2.2).

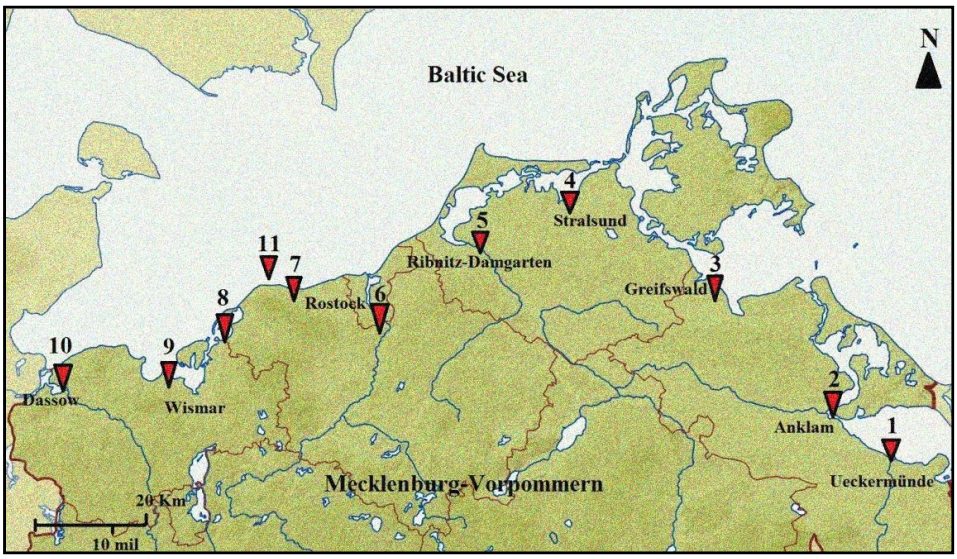


Figure 2.2 Location of the sampling stations in Mecklenburg-Vorpommern, Germany.

Two parameters including salinity and temperature were monitored during sample collection. The names of sampling stations, sampling areas, sampling numbers, coordinates, and the minimum and maximum values of the measured salinities and temperatures are presented in Table 2.1.

The samples were collected in 2012 during the period of pesticide application between May and September. The samples were taken once a month in May, June, August and September from the estuarine sites (1-6, 8-10) and at rate of one sample in May, three in June, two in July, two in August from May to September from the stations (7) and (11).

Table 2.1: Numbers, names, coordinates, and the minimum and maximum measured salinity and temperature in the collected samples.

Station		Sampling number	Coordinates		Salinity		Temperature (°C)	
Number	Name	N	Latitude	Longitude	Min	Max	Min	Max
1	Uecker river	4	53° 43' 55.54'' N	14° 02' 52.31'' E	0.1	0.4	12.3	20.9
2	Peene river	4	53° 51' 56.91'' N	13° 49' 40.11'' E	0.9	1.3	12.8	22.6
3	Ryck river	4	54° 05' 36.54'' N	13° 26' 54.90'' E	1.3	6.1	13.7	21.4
4	Barthe river	4	54° 21' 58.78'' N	12° 41' 14.97'' E	1,2	4.1	13.0	21.0
5	Recknitz river	4	54° 14' 50.68'' N	12° 28' 01.82'' E	0.3	2.6	11.8	20.9
6	Warnow river	4	54° 03' 50.96'' N	12° 10' 16.72'' E	0.1	0.3	12.9	20.6
7	Mühlenfließ	9	54° 08' 50.20'' N	11° 52' 08.70'' E	0.1	0.8	11.7	20.2
8	Hellbach	4	54° 03' 39.03'' N	11° 37' 15.18'' E	0.2	0.3	11.2	17.1
9	Wallensteingraben canal	4	53° 54' 05.84'' N	11° 28' 18.84'' E	0.0	0.3	13.2	21.5
10	Stepenitz river	4	53° 54' 25.24'' N	10° 58' 01.10'' E	0.9	3.1	12.0	20.0
11	Baltic Sea coast in Heiligendamm	9	54° 08' 46.55'' N	11° 50' 36.07'' E	9.0	15.7	10.4	17.1

Min: Minimum, **Max**: Maximum, **Mühlenfließ**: Muehlen stream, **Hellbach**: Hell stream

2.9 Sample collection, treatment and data analysis

1 L water samples were collected from the selected stations in amber glass bottles in order to analyze the compounds MCPA, mecoprop, isoproturon, bentazon and chloridazon and its metabolite CMD, while they were collected in polypropylene bottles for glyphosate and AMPA analysis due to their high polarity and possible adsorption onto the glass bottle wall. The bottles were cleaned prior to sampling by rinsing with the water to be sampled. The bottles were filled to the top with as little air as possible remaining and sealed tightly. The samples were transported to the laboratory and stored at 5 °C in the dark within 3 days for glyphosate and AMPA analysis and a week for other herbicides analysis.

Matrix effects through other unwanted components in samples are a major problem in the quantitative analysis of environmental samples using high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS). This may lead to suppression or enhancement of the analyte signals (Patel, 2011). Standard addition is a powerful method to correct for sample matrix effects (Kalivas, 1987). Therefore, the samples were prepared according to the standard addition method for quantitative analysis of all target compounds.

In order to analyze glyphosate and AMPA, 40 mL of each sample were distributed into four 10 mL polypropylene volumetric flasks. Increasing concentrations of the mixed standard solutions of glyphosate and AMPA were added to three of the flasks while the last was without addition. A volume of 800 µL of each sample was inserted into an Eppendorf tube together with 100 µL of 0.07 M borate buffer at pH 9, and 100 µL 1 mM FMOC-Cl were added and vigorously shaken. The samples were kept for 4 hours at room temperature to

allow the derivatization reaction to proceed to completion. The samples were filtered using 0.45 µm Phenex-Rc 10 mm syringe filters (Phenomenex, Germany) to remove particulate matter. The samples were analyzed according to the developed HPLC-MS/MS method (section 2.7).

For analysis of the compounds MCPA, mecoprop, isoproturon, bentazon, chloridazon and CMD a 40 mL of each sample were filtrated using 0.45 µm Phenex-Rc 10 mm syringe filters. The filtered 40 mL sample was split into 4 fractions of 10 mL into four 10 mL glass volumetric flasks. Three increasing concentrations of the mixed stock solution of all analytes were added to three of them. A 1 mL volume from each volumetric flask was inserted into 1.5 mL glass vial and measured according to the developed HPLC-MS/MS method mentioned earlier (section 2.6).

The dilution process of the natural samples was achieved when the estimated concentrations of the samples were out of the concentration range of the calibration curves. The data were transferred to an Excel worksheet and plotted with the added standard concentration on the x-axis and the peak areas on the y-axis, and the unknown concentrations were determined as minus the estimated x-intercept.

2.10 Algae and culture conditions

The cyanobacterium *N. spumigena* was provided by Dr. Monika Nausch and maintained at the Leibniz Institute for Baltic Sea Research in 2 L batch cultures in f/2 medium. The used equipment, glassware and medium were sterilized using an autoclave. *N. spumigena* was cultured at 15 °C with a cycle of 16:8 h light/dark (cool, white fluorescent light, 100 µmol photons m^{-2} s^{-1}). 200 mL medium containing *N. spumigena* cells were distributed in polycarbonate bottles containing 800 mL liquid f/2 medium. The initial algal density based on chlorophyll-*a* was set to be about 10 µg/L. Many treated plants with glyphosate have not shown symptoms for treatment for 7-10 days until the depletion of aromatic amino acid which subsequently reduce rates of protein synthesis (Cobb and Reade, 2010). Therefore, the toxicity experiment period was designed to be a relatively long period of 26 days exposure to Roundup® and AMPA. The test started by adding Roundup® and AMPA at different concentrations 1, 10, 50, 100 and 500 µg/L. The added volumes of Roundup® and AMPA were sterilized using 0.2 µm filter. Triplicates of control and treated cultures were grown under the same conditions of temperature and photoperiod. Samples were measured at different intervals during 26 days of Roundup® and AMPA exposure.

2.11 Chlorophyll-*a*, cell count, particulate organic carbon and AMPA analysis

Samples of 30 mL volume were taken from each culture during the experiment at different intervals and immediately filtered through Whatman GF/F filters for chlorophyll-*a* (Chl-*a*) analysis. The filters were stored at -80 °C. Then, the samples were extracted with 10 mL of 96% ethanol. Chl-*a* fluorescence was measured with a Turner fluorometer (10-AU-005) at an excitation wavelength of 450 nm and an emission of 670 nm. The concentrations of Chl-*a* and Phaeopigments were calculated according to JGOFS protocol (Knap et al., 1996).

In order to measure the cell density of *N. spumigena*, samples of 50 mL were taken at different intervals and preserved by adding 250 µL of acetic Lugol's (KCl) solution. The analysis was achieved by the Utermöhl's method using an inverted microscope (Utermöhl, 1958).

Particulate organic carbon (POC) was measured on Whatman GF/F filters by filtering 50 mL of the samples. The filters were kept at -20 °C. Samples were analyzed for POC using CE Instruments Elemental Analyzer EA 1110 in the laboratory of the Leibniz Institute for Baltic Sea Research Warnemuende (IOW) according to a standard method.

AMPA concentrations in the cultures were measured by the HPLC-MS/MS analytical method after derivatization using FMOC-Cl described earlier (section 2.7).

3 Results and Discussion

3.1 GC-MS and HPLC-MS/MS for analysis of the selected polar herbicides and metabolites

The contamination of the aqueous environment by anthropogenic trace pollutants has clearly changed in the past ten to fifteen years from persistent, not easily degradable contaminants to more polar, thermo-labile and less volatile compounds (Fobbe et al., 2006). Nowadays, many analytical techniques have been developed for determining pesticide residues in environmental samples (Ferrer and Barceló, 1998). However, gas chromatography (GC) and high performance liquid chromatography (HPLC) are the predominant analytically sufficient techniques used for identification and quantification of pesticide residues in environmental matrices (Cserhati et al., 2004).

The six polar herbicides glyphosate, isoproturon, mecoprop, MCPA, bentazon, chloridazon and three of their metabolites AMPA, CD and CMD were chosen for this study. The selected compounds have different functional groups such as carboxyl, hydroxyl, carbonyl, amine and amide giving rise to diverse chemical properties (see figures 1.4, 1.5, 1.6, 1.7 and 1.8).

Generally, organic compounds containing functional groups with "active" hydrogen atoms (e.g. -COOH,-OH,-NH,-SH) are hardly measurable by gas chromatography due to their polarity, low volatility and thermal decomposition (Liska and Slobdnik, 1996). On other hand, the development of derivatization techniques has rendered various polar compounds accessible to gas chromatography (Fobbe et al., 2006). Derivatization reactions for GC analysis comprise acylation, alkylation, esterification, and silylation reactions (Drozd, 1981). Numerous reagents have been used for the derivatization of the selected herbicides. Available data are summarized in Table 3.1.

Diazomethane is the most used derivatization reagent for the selected compounds with a yield of up to 100% for the acidic herbicides such as MCPA and mecoprop. On other hand, diazomethane is toxic, carcinogenic, mutagenic, irritant and explosive above 90°C.

Glyphosate and its metabolite AMPA have the highest polarity of the selected herbicides and metabolites. Trifluoroacetic anhydride (TFAA) and trifluoroethanol (TFE) are the common derivatization reagents of glyphosate and AMPA for GC analysis (Deyrup et al., 1985; Roy et al., 1989; Stalikas et al., 2000).

Table 3.1: Some used reagents in the derivatization reactions of the selected herbicides and metabolites for their GC analysis.

Reagents	Glyphosate AMPA	MCPA Mecoprop	Bentazon	Isoproturon
1	Diazomethane and chloroformate (Kataoka et al., 1996)	Diazomethane (Ngan and Ikesaki, 1991)	Diazomethane (Cessna, 1985)	Diazomethane (Morais et al., 2011)
2	Trifluoroacetic anhydride and trifluoroethanol (Deyrup et al., 1985)	Dimethyl sulfate (Catalina et al., 2000)	Trimethylsilyldiazo methane (Moy and Brumely, 2003)	Heptafluorobutyric anhydride (Brinkman et al., 1984)
3	*N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide (Hori et al., 2003)	Pentafluorobenzyl bromide (Vink and Vander Poll, 1996)	Pentafluorobenzyl bromide (Vassilakis et al., 1998)	Pentafluorobenzyl bromide (Scheyer et al., 2005)
4		Benzyl bromide (Nilsson et al., 1998)	Trimethylanilinium hydroxide (Ogierman, 1990)	Iodomethane (Scott, 1993)
5		Butylchloroformate (Henriksen et al., 2001)		
6		Sodium hydride /dimethyl sulphoxide /methyl iodide (Crespo-Corral et al., 2008)		

The application of liquid chromatography in order to analyze polar compounds is increasing due to their high efficiency and sensitivity when it is tandem to mass spectrometry with atmospheric pressure ionization (Fobbe et al., 2006). Furthermore, the possibility of direct injection of a large volume of aqueous samples into the HPLC system has been confirmed to be an adequate technique for sensitive, selective and rapid determination of polar herbicides in aqueous samples (Sancho et al., 1996). Most of HPLC separations of herbicides were achieved utilizing reversed phase (RP) chromatography (Tadeo et al., 2000). The direct HPLC analysis of glyphosate and its metabolite AMPA are difficult, whereas a satisfied determination was successful only after derivatization processes (Bauer et al., 1999; Stalikas and Konidari, 2001). 9-Fluorenylmethyl chloroformate (FMOC-Cl) is the most used reagent for pre-column derivatization of glyphosate and AMPA in combination with HPLC-MS/MS because it has a high reactivity towards the amino groups in both glyphosate and AMPA, it is highly non-polar, fluorescent, has the possibility of performing derivatization reaction in aqueous media, is commercially and available in pure form (Hanke et al., 2008; Stalikas and Konidari, 2001).

The objectives of this section are:

(1) to compare between the two analytical techniques GC-MS and HPLC-MS/MS for analysis of six polar herbicides and three of their metabolites in water samples.
(2) to further develop and validate analytical methods for identification and quantification of the selected nine compounds in water samples using the appropriate technique (i.e. GC-MS and/or HPLC- MS/MS) for their analysis.

3.1.1 GC-MS and HPLC-MS/MS for direct analysis of the selected compounds

The objective of this experiment was the investigation of the possibility of direct analysis of the six polar herbicides glyphosate, MCPA, mecoprop, isoproturon, bentazon, and chloridazon as well as their three metabolites AMPA, CD, and CMD by both analytical techniques: GC-MS and HPLC-MS/MS.
Mixture standards of all the target compounds in addition to their labeled compounds were prepared at a concentration of 10 ng/µL in ethyl acetate solvent and directly analyzed by GC-MS. The labeled compounds were used as internal standards for GC-MS analysis. The full scan mode was used for recording source mass spectra of interest in which all or most of produced ions are present. A chromatogram obtained from direct GC-MS analysis of 10 ng/µL standard solution of the target analytes using full scan mode is shown in Figure 3.1.

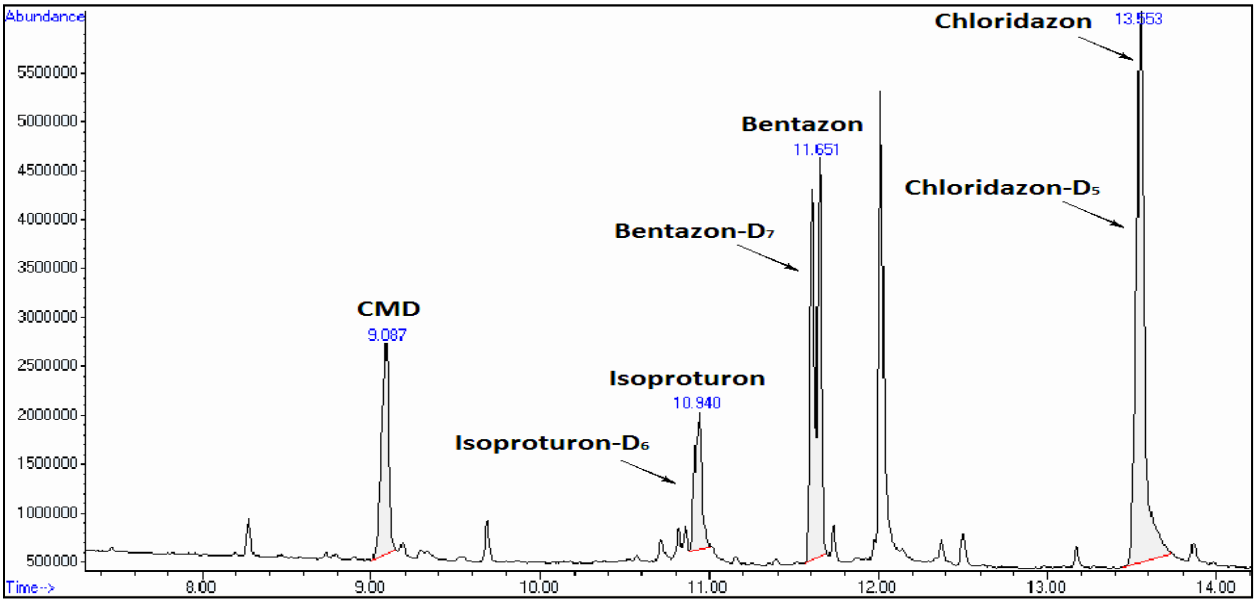
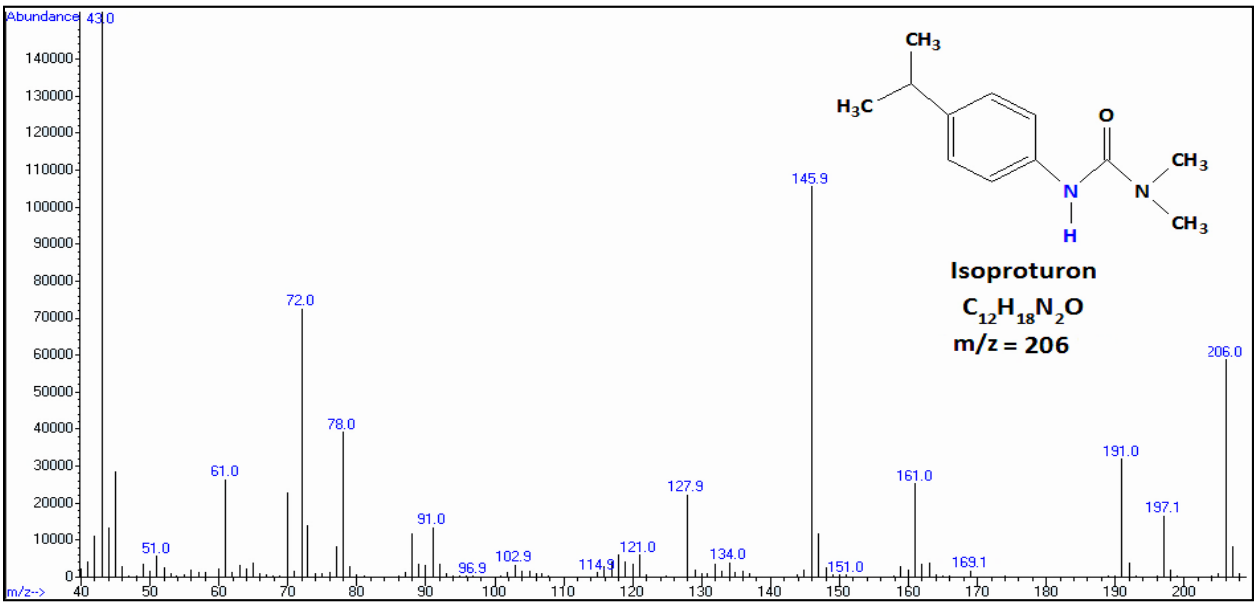
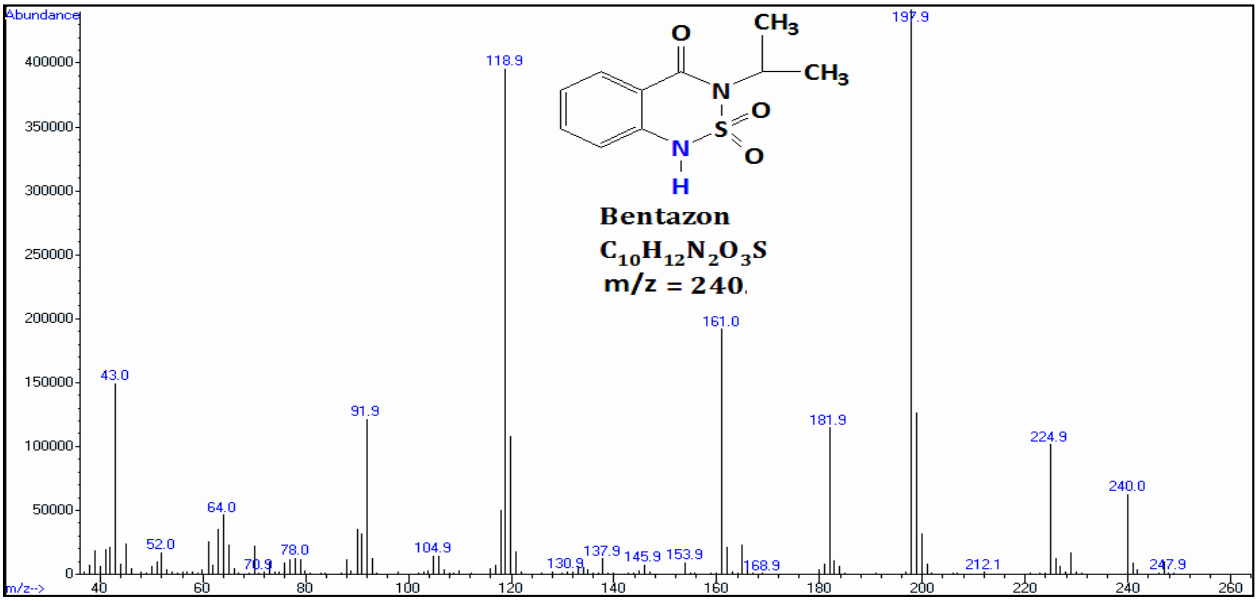


Figure 3.1: Chromatogram obtained from direct GC-MS analysis of 10 ng/µL mixture standard solutions of the nine target analytes using full scan mode.

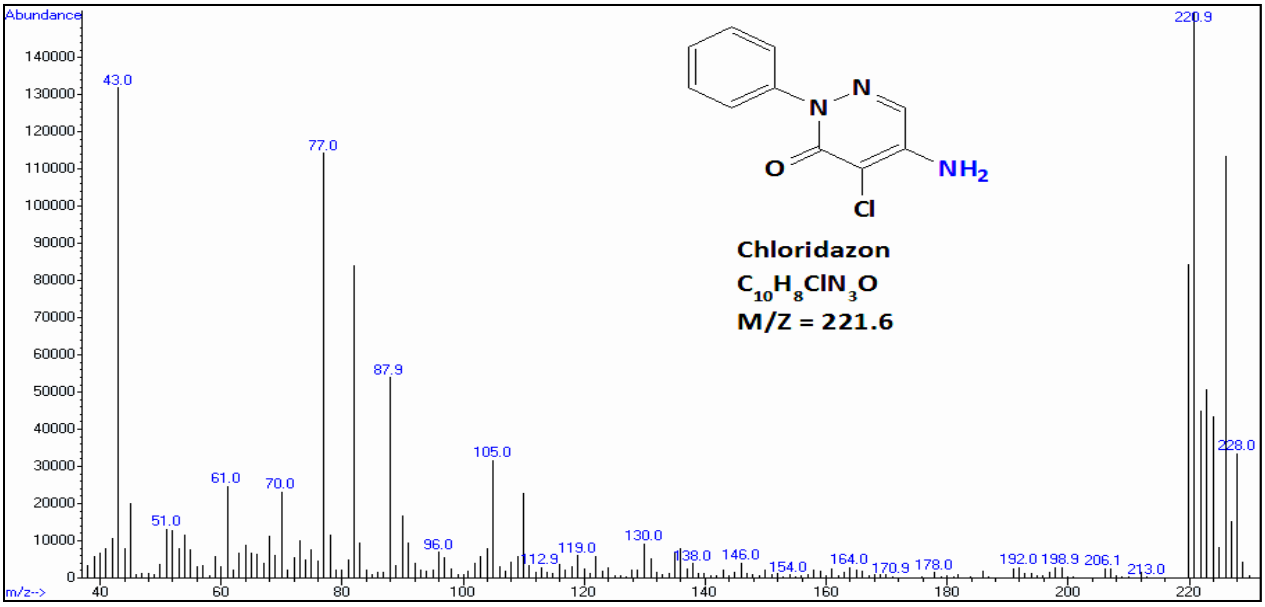
According to mass spectral analysis, four compounds isoproturon, bentazon, chloridazon and CMD were found in the chromatogram obtained from direct GC-MS analysis. The mass spectra of the components isoprouron, bentazon, chloridazon and CMD are shown in Figure 3.2 and these of their labeled compounds are shown in Appendix 1-3.



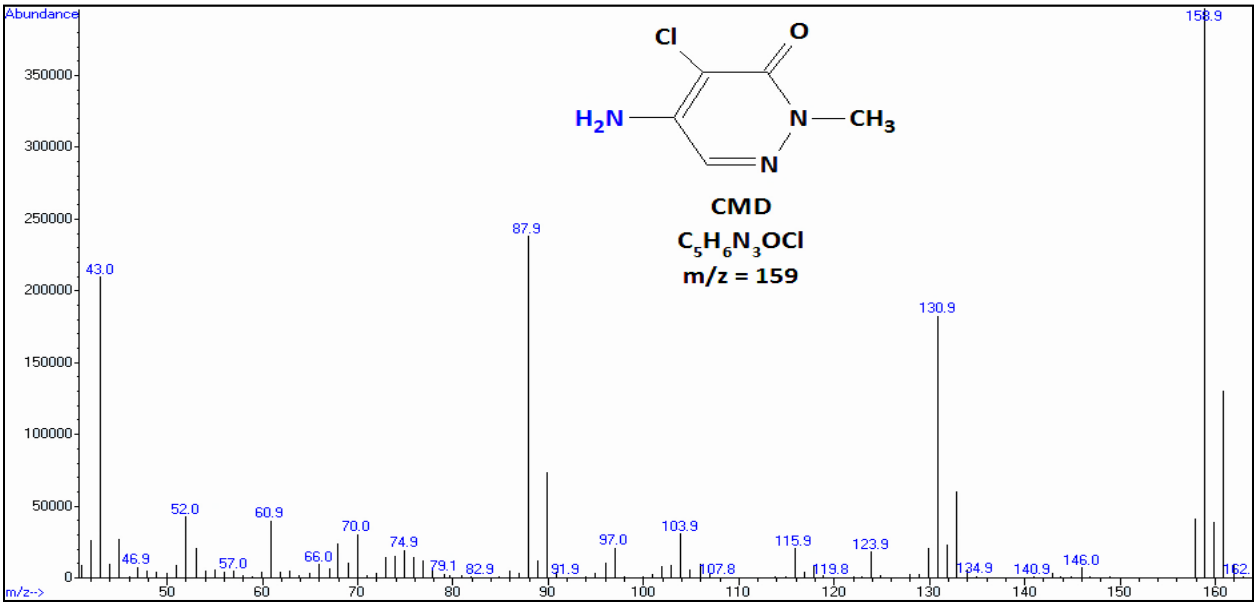
(A)



(B)



(C)



(D)

Figure 3.2: The mass spectra obtained from direct GC-MS analysis of the compounds (**A**) isoproturon, (**B**) bentazon, (**C**) chloridazon and (**D**) CMD.

Despite the observation of the compounds isoproturon, bentazon, chloridazon and CMD in the chromatogram obtained from direct GC-MS analysis, their analysis has failed because of unstable and unrepeatable measurements with low precision (Appendix 4). The

unrepeatability of their measurements can be explained by thermal instability of these four compounds due to their polar nature resulting from polar groups such as amide and amine groups. Very poor peaks of the metabolite CD were found only in some chromatograms obtained from direct GC-MS analysis after several measurements. This fluctuation and instability of CD detection due to thermal instability and low volatility can be explained by the fact that CD is a relatively small molecule and has amide and amine polar groups which create strong intermolecular attractions between the polar groups, and/or by polar interactions by hydrogen bonds which subsequently lead to the low volatility and thermal instability of CD (Drozd, 1981). The components glyphosate, MCPA, mecoprop and AMPA were undetectable by direct GC-MS analysis. Glyphosate and its metabolite AMPA were expected to be undetectable by direct GC-MS analysis due to their very high polarity. These four compounds contain carboxylic and/or hydroxyl groups which lower their volatility due to intermolecular forces such as ionic interactions or hydrogen bonds. According to these results, all the selected compounds are unsuitable for the direct GC-MS analysis. Therefore, derivatization is a necessary technique for making them suitable for GC-MS analysis.

In order to test the direct HPLC-MS/MS analysis of the selected analytes, dissolved standards of each compound were prepared at a concentration of 500 µg/L. The standard solutions were directly infused to tune the instrument in both positive and negative electrospray ionization (ESI) in order to determine the parent and the product ions of interest. Reversed phase chromatography is the most popular separation technique used for herbicide analysis due to its versatility and ability to resolve a number of different types of compounds (Tadeo et al., 2000). Therefore, reversed phase was aimed to be tested for the separation of the target analytes. A Kinetex 2.6 µm C_{18} column (50 x 2.1) mm was used for this purpose. Standards at concentrations of 1 µg/L of the target analytes in water were measured by HPLC-RP-MS/MS.

As shown in Figure 3.3 seven compounds could be separated on the C_{18} reversed phase column and were detected using MS/MS detector. These compounds are MCPA, mecoprop, isoproturon, bentazon, chloridazon and its metabolites CD and CMD.

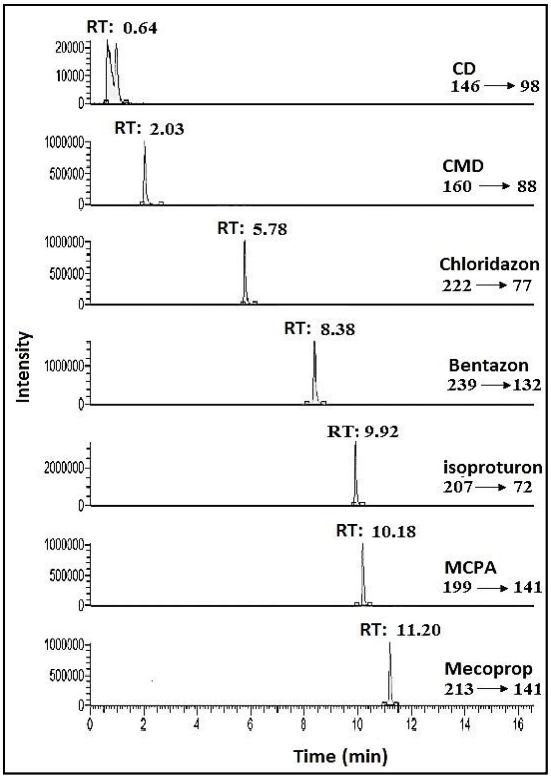


Figure 3.3: Peaks of seven compounds CD, CMD, chloridazon, bentazon, isoproturon, MCPA and mecoprop resulting from their direct analysis using HPLC-RP-HESI-MS/MS.

The detected seven compounds have lower polar properties compared to glyphosate and AMPA. The herbicide glyphosate and its metabolite AMPA were unsuitable for reversed phase separation on a C_{18} silica column because of their ionic characteristic, high polarity, high water solubility and low organic solvent solubility. These results are in agreement with references which have confirmed the requirement of the derivatization technique for performing HPLC-RP-MS/MS analysis of glyphosate and AMPA due to their difficult physicochemical properties (Martins-Júnior et al., 2011; Vreeken et al., 1998). A comparison of GC-MS and HPLC-RP-MS/MS for direct analysis of the nine target compounds indicates that HPLC-RP-MS/MS is a suitable technique for direct analysis of the mid-polar analytes but not suitable for reversed phase separation of very high polar compounds such as glyphosate and AMPA, while GC-MS is unsuitable for direct analysis of all the selected compounds.

A direct HPLC-RP-MS/MS analytical method was developed and validated in order to identify and quantify the herbicides MCPA, mecoprop, isoproturon, bentazon, chloridazon and its metabolites CD and CMD in water samples. Method development was based on the optimization of HPLC and MS/MS parameters in combination with direct injection of 50 µL water samples without pre-concentration processes such as the solid phase extraction and clean-up steps.

3.1.2 HPLC-MS/MS method optimization for direct analysis of MCPA, mecoprop, isoproturon, bentazon, chloridazon CD and CMD

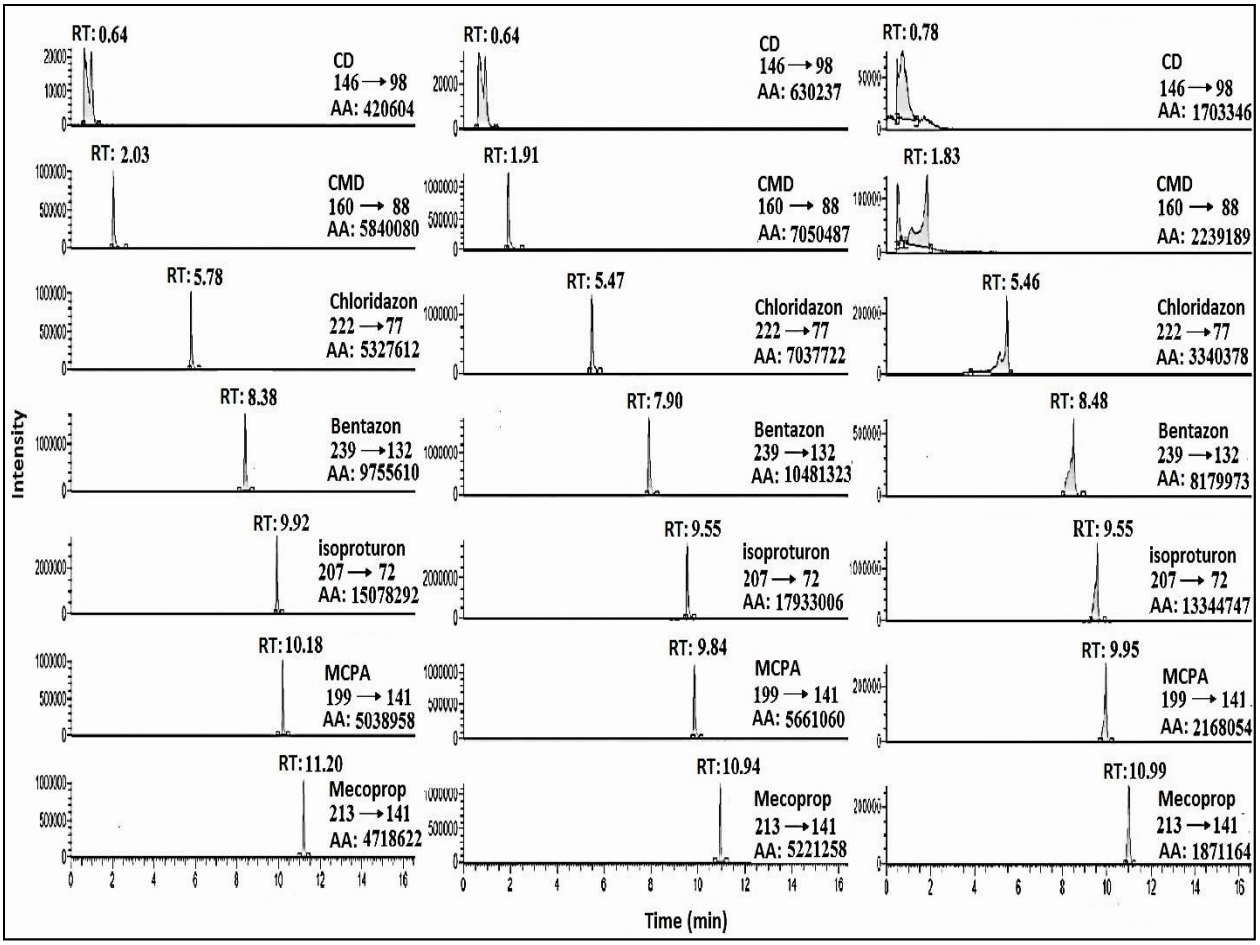
3.1.2.1 Mobile phase composition

Methanol and acetonitrile were tested as organic eluents in the analytical method. Methanol was preferred over acetonitrile because it showed better peak shapes and separation performance for all target compounds. Methanol was chosen as eluent B. Generally, addition of acid as acetic and formic acids to the HPLC mobile phase gave some advantages such as improving the chromatographic separation when running on a non-polar stationary phase (Xu, 2013). Three mobile phases involving three different compositions of acetic and formic acid were tested (Table 3.2). The obtained chromatograms from direct HPLC-MS/MS analysis of 5 µg/L mixed standard with regards to the three tested mobile phases are shown in Figure 3.4.

Table 3.2: The three tested mobile phase compositions.

Mobile phase	Eluent A	Eluent B
A	Water with 0.1% formic acid	Methanol with 0.1% formic acid
B	Water with 0.1% acetic acid	Methanol with 0.1% formic acid
C	Water with 0.1% acetic acid	Methanol with 0.1% acetic acid

The mobile phase (B) offered best HPLC-MS-MS performance including better peak shapes, shorter retention times and higher mass spectrometry sensitivity for all the target analytes than any other mobile phases tested. Therefore, water with 0.1% acetic acid and methanol with 0.1% formic acid were chosen as the mobile phase in the final analytical method. The metabolite CD was found in the range of dead time in both tested eluent methanol and acetonitrile. This result reflects poor interaction of CD with the reversed phase C_{18} column and may be due to its polarity resulting from the amide group and its high solubility in water. Many efforts were spent in order to achieve the analysis of CD. The attempts included different sample solvents, different mobile phase pH solutions, different mobile phase gradients and flow rates. No improvement was observed in the interaction of CD with the stationary phase. CD was unsuitable according for this analytical method. Therefore, it was excluded from the analytical method.



(**A**) (**B**) (**C**)

Figure 3.4: Chromatograms obtained from direct HPLC-MS/MS analysis of 5 µg/L mixed standard by the three tested mobile phases (**A**), (**B**) and (**C**) which are shown in Table 3.2.

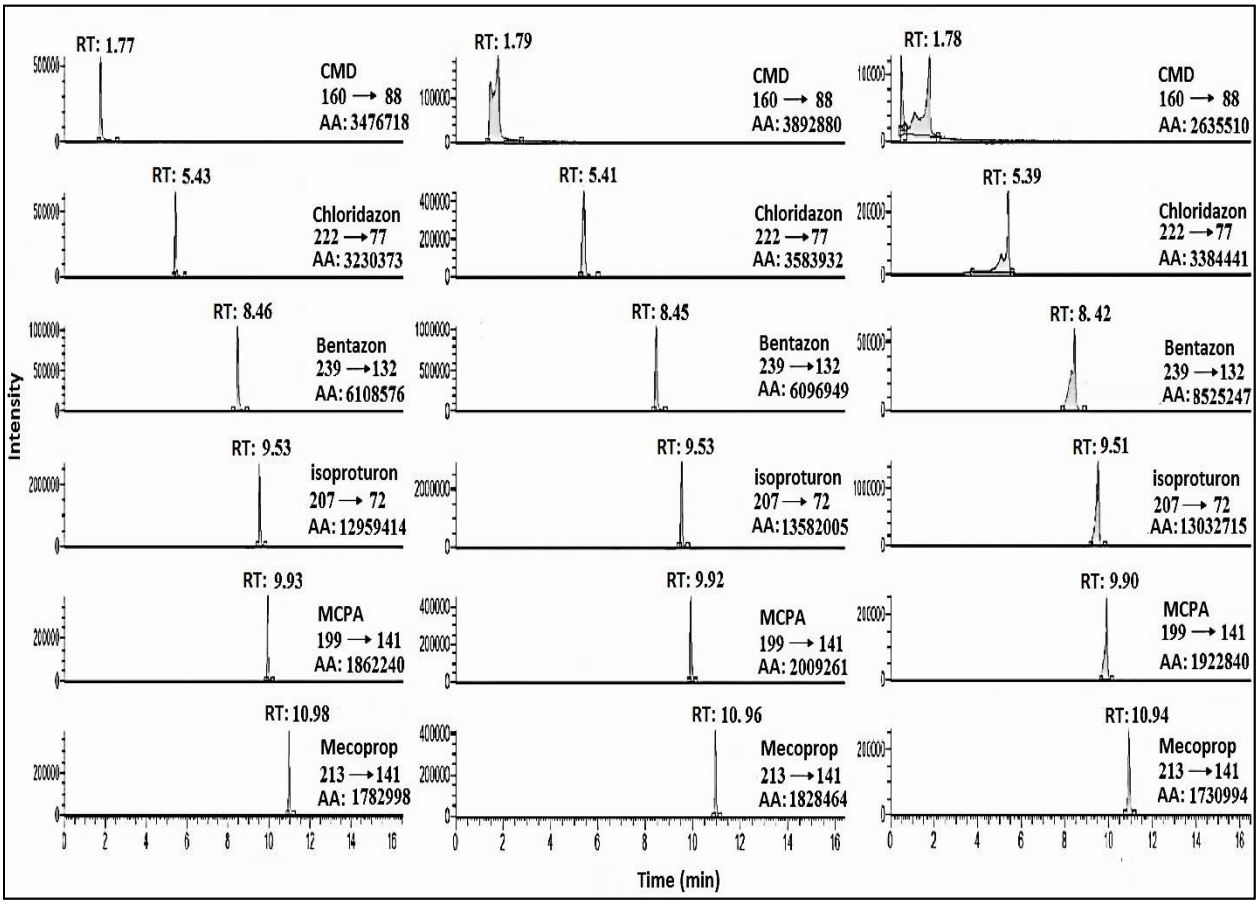
3.1.2.2 Comparison of three sample solvents

Standards of the analytes were prepared at a concentration of 1 µg/L in three different solvents:

1- water
2- water/methanol (50/50)
3- methanol

The chromatograms obtained from direct HPLC-MS/MS analysis of the three tested sample solvents are shown in (Figure 3.5). A comparison of the obtained chromatograms regarding retention times, sensitivities and peak shapes of the target analytes was made. Retention times of all the compounds were almost stable when the sample solvents were used. Methanol is an inadequate solvent for all the target analytes because of peak tailing. The water/methanol (50/50) solvent offered the highest sensitivities for most of the target

analytes but a poor peak shape for the metabolite CMD. Water as sample solvents showed good sensitivities and peak shapes for all the target analytes. Therefore, water was used as a sample solvent in the final methods.



(**A**) (**B**) (**C**)

Figure 3.5: Chromatograms obtained from direct HPLC-MS/MS analysis of 1µg/L mixed standard in (**A**) water, (**B**) water/methanol (50/50) and (**C**) methanol.

3.1.2.3 MS/MS optimization

Optimization of MS/MS parameters were achieved by the infusion of each compound at a concentration of 500 µg/L into the mass spectrometer in positive and negative ionization mode at a flow rate of 5 µL/min. Full scan mass spectra were recorded in order to select the most abundant mass-to-charge ratio (m/z). The relative intensity for the most abundant m/z was used to evaluate the performance of each ionization mode. The most intense product ion for each target analyte was chosen for quantification, and a secondary product

ion was used as a qualifier ion for confirmation. The parent ions, product ions, SRM collision energies, start times, stop time, S-lenses and polarities for each analyte are shown in Table 3.3. Additionally, the MS/MS parameters such as capillary and vaporizer temperatures, spray voltages, sheath and auxiliary gas pressures were also optimized. For the final analysis, optimal parameters have been applied as described in section 2.6.

Table 3.3: The parent ions, product ions, SRM collision energy, start time, stop time, S-lens and polarity for the analytes.

Analyte	Parent	product	Collision energy	start	stop	S-lens	polarity
CMD	160	88	31	1.00	3.00	76	+
	160	117	23	1.00	3.00	76	+
Chloridazon	222	77	33	5.00	6.50	79	+
	222	92	24	5.00	6.50	79	+
Bentazon	239	132	28	7.00	8.50	98	-
	239	197	22	7.00	8.50	98	-
Isoproturon	207	64	16	9.00	10.10	77	+
	207	72	17	9.00	10.10	77	+
MCPA	199	105	29	9.00	10.20	60	-
	199	141	17	9.00	10.20	60	-
Mecoprop	213	105	32	10.10	12.00	53	-
	213	141	18	10.10	12.00	53	-

3.1.3 HPLC-MS/MS Method validation for direct analysis of MCPA, mecoprop, isoproturon, bentazon, chloridazon and CMD

In the validation of the analytical method and in the quantitative analysis, the HPLC-MS/MS system was operated in selected reaction monitoring (SRM) mode in which a limited number of parent and product ions can be monitored and subsequently the sensitivity for detection of each target analyte can be increased. The parameters involved in validation of the analytical method were linearity, accuracy, precision (repeatability), limit of detection (LOD) and quantification (LOQ), analytes and system stability.

3.1.3.1 Linearity

The performance verification of the mass spectrometry detector was conducted by determining the linearity of the detector response. A volume of 50 µL of mixed standards of the analytes at ten different concentrations (i.e. at a concentration range between 10 ng/L and 2000 ng/L) were injected to the HPLC-MS/MS and analyzed using the optimized method. The calibration curves obtained for all analytes (n = 3) are shown in Figure 3.6. Linear relationships ($R^2 > 0.99$) were established in the concentration ranges of 10-2000 ng/L for bentazon, isoproturon and CMD, of 50-2000 ng/L for MCPA and mecoprop and of 100-2000 ng/L for chloridazon (Table 3.4). The obtained good linearity reflects a strong relationship between the concentrations of the analytes and detector response.

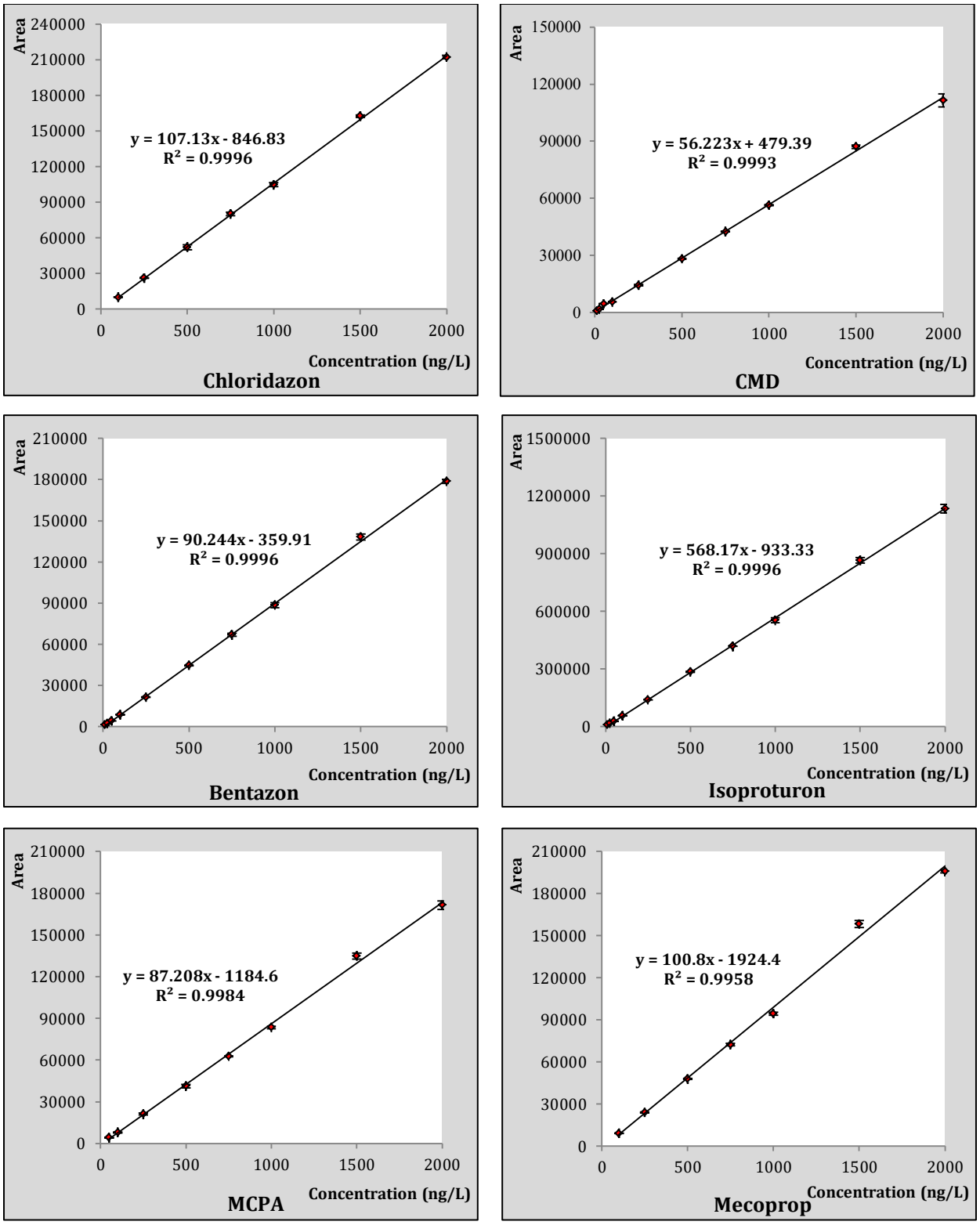


Figure 3.6: The obtained calibration curves of the compounds MCPA, mecoprop, bentazon, isoproturon, chloridazon and CMD after their direct analysis using HPLC-RP-MS/MS.

3.1.3.2 Accuracy

The accuracy of a method is a measurement of the systematic error or bias and is defined as the deviation between the measured values and the true values. Accuracy is best reported as percentage bias (relative error RE %) that is calculated from the following expression (Pinto et al. 1999):

$$\text{Accuracy \%} = \left[\frac{\text{measured value - true value}}{\text{true value}} \right] \text{x } 100 \qquad (1.1)$$

Some of the possible error sources causing biased measurements are: errors in sample preparation or errors in sample analysis. The accuracies were calculated for all concentrations which were used in the calibration curves in triplicate. The range of calculated accuracy for each analyte is shown in Table 3.4. The results indicate an acceptable accuracy of the analytical method with a maximum error below 12% for all six analytes (Table 3.4). The highest error of 11.4% and 11.1% were calculated for bentazon and CMD, respectively, at concentration levels close to their LOQs. Better accuracy values with errors below 7% were obtained at concentration levels over 100 ng/L for all the target analytes.

3.1.3.3 Precision (Repeatability)

The precision of a method is a measurement of random errors and is defined as the difference between replicate measurements of the same sample. It is expressed as the relative standard deviation (RSD%) of replicate measurements and it is calculated from the following expression (Pinto et al. 1999):

$$\text{Precision (R.S.D.) \%} = \left[\frac{\text{absolute standard deviation}}{\text{Mean}} \right] \text{x } 100 \qquad (1.2)$$

The instrument precision was evaluated in terms of the RSD% by performing 3 injections of a standard solution containing the analytes in the calibration curves. The range of evaluated precision of each analyte is shown in Table 3.4. Data obtained from precision experiments showed values ranged from 0.1% to 10% which assure satisfactory HPLC-MS/MS precision for the tested analytes.

3.1.3.4 Limit of detection and limit of quantification

The limit of detection (LOD) of an analytical method is the lowest amount of the target compound in the sample that can be detected, but not necessarily quantified as an accurate value using the experimental conditions. The limit of quantification (LOQ) is the lowest amount of the target analytes in the sample that can be quantified in the experimental conditions. LODs and LOQs of this analytical method were experimentally estimated according to the signal to noise ratios (S/N) of 3 and 10, respectively. According to the analytical method, LODs and LOQs of the target analytes were at concentration ranges of 0.5-10 ng/L and of 4-90 ng/L, respectively (Table 3.4). The herbicide isoproturon has the lowest LOD and LOQ values of 0.5 ng/L and 4 ng/l, respectively.

Table 3.4: Quality control parameters of the analytical method (n=3) including: linearity (R^2), accuracy (RE %), precision (RSD %), LODs (ng/L) and LOQs (ng/L).

Compound	Linearity (R^2)	RE%	RSD%	LOD (ng/L)	LOQ (ng/L)
CMD	0.9996	0.1-11.1	0.5-10.0	10	25
Chloridazon	0.9993	0.4-1.6	0.6- 9.0	10	90
Bentazon	0.9996	0.2-11.4	0.4-3.2	3.0	6.0
Isoproturon	0.9996	0.3-9.4	0.1-5.0	0.5	4.0
MCPA	0.9984	0.4-5.4	0.7-4.9	10	50
Mecoprop	0.9958	0.6-6.6	0.7-8.2	10	50

3.1.3.5 Analytes and system stability

In order to study the analytes stability, two standard solutions of all the analytes were prepared at concentrations of 1µg/L. One of them was kept at ambient laboratory temperature of 21 °C and the other stored in a fridge at 5°C. Samples of the two solutions were analyzed daily for one week by HPLC-MS-MS in order to test the stability of the analytes. The results showed that the analytes are stable during the tested period. No additional peaks were observed and the changes in peak areas of the all analytes were less than 5%.

System suitability is defined as checking the system used before or during analysis of unknowns, to ensure system performance. Mixed standard solutions at concentration of 1 µg/L were injected into the HPLC-MS/MS system in order to check its stability. Cleaning of the mass spectrometry system was a necessary step, when a remarkable decrease in the sensitivities was observed, especially because the samples were analyzed without pre-concentration (e.g. extraction and clean-up).

3.1.4 TFAA and TFE derivatization for GC-MS analysis of polar herbicides and metabolites

The goal of this experiment was to study the possibility of GC-MS analysis of the six polar herbicides and their three metabolites in a single run after their derivatization with TFAA and TFE. Glyphosate and its metabolite AMPA are the most difficult compounds for GC-MS analysis compared to the other selected compounds due to their physicochemical characteristics such as their high polarity, no volatility and low molecular weight (Martins-Júnior et al., 2011). TFAA and TFE were chosen as derivatization reagents in this study. Their selection was based on their predominant use in glyphosate and AMPA derivatization reactions for GC-MS analysis due to their satisfactory efficiencies (Deyrup et al., 1985; Roy and Konar, 1989; Stalikas et al., 2000). Samples of mixed standard solutions of the target analytes and their labeled compounds were prepared at concentrations of 10 ng/µL. The samples were derivatized as described in section 2.3 and then analyzed by GC-MS. The chromatogram obtained from GC-MS analysis of 10 ng/µL of the derivatized standard mixture using the full scan mode is shown in Figure 3.7. Seven compounds were derivatized with TFAA and TFE and separated on DB-5ms columns. These compounds are glyphosate, AMPA, MCPA, mecoprop, chloridazon, CD and CMD. These components were identified by their mass spectra and their fragmentation patterns. The mass spectra of the analytes glyphosate, AMPA, MCPA and mecoprop are shown in figure 3.8 and the mass spectra of their labeled compounds are shown in Appendix 5-8.

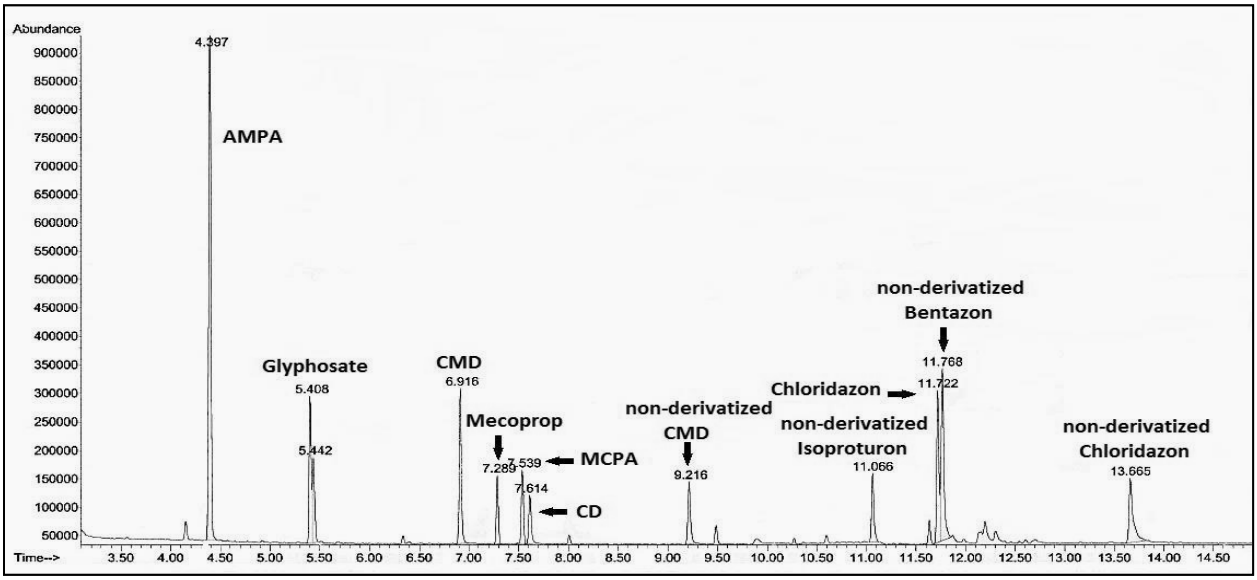
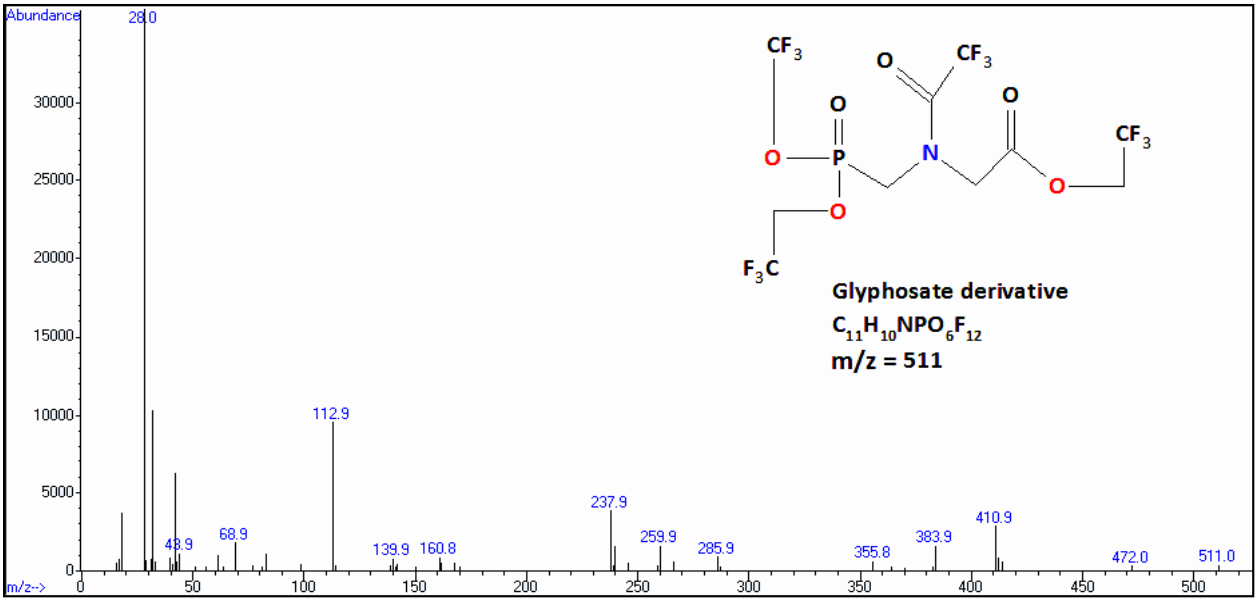
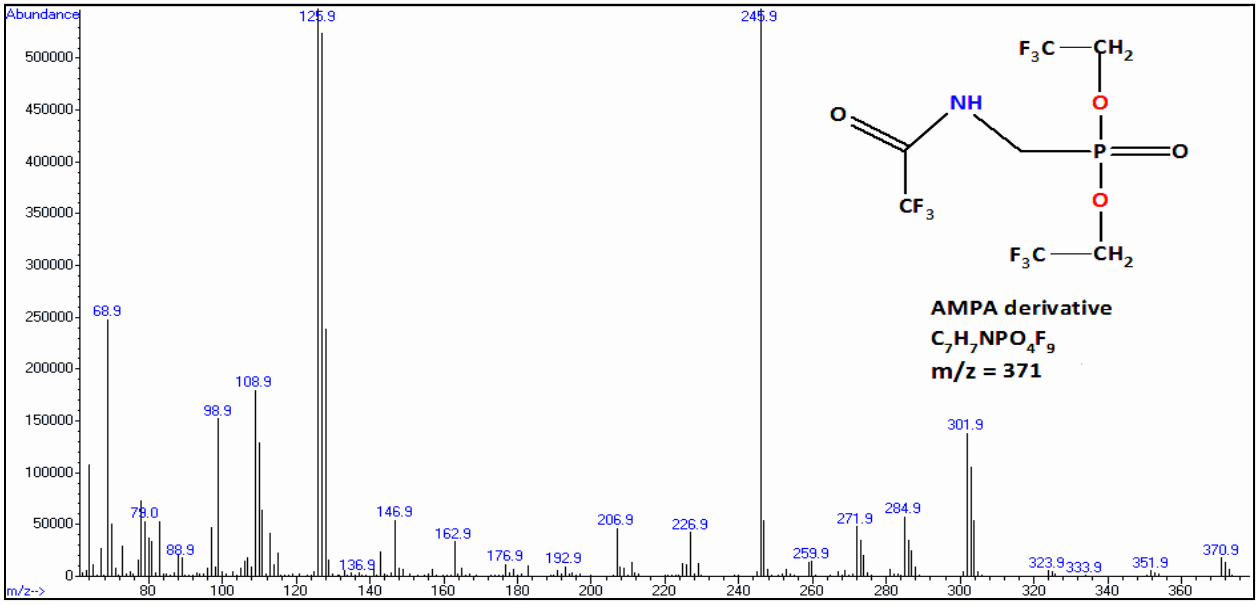


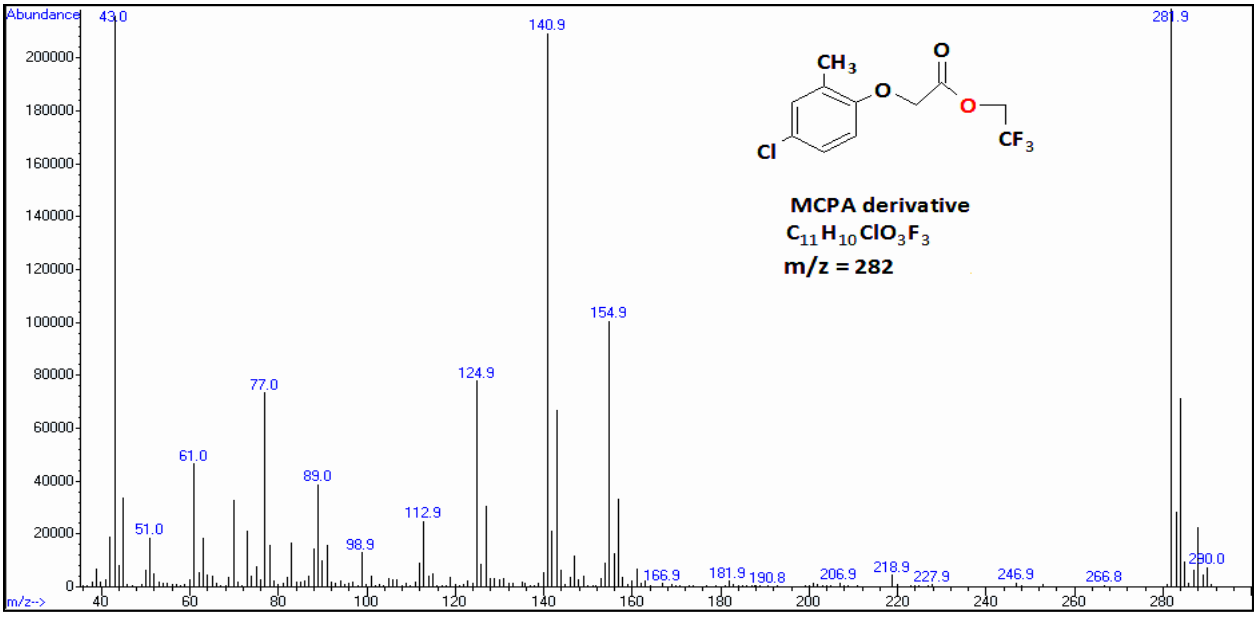
Figure 3.7: Chromatogram obtained from GC-MS analysis of the nine target analytes at concentrations of 10 ng/µL after derivatization by TFAA and TFE using full scan mode.


Abundance
28.0
112.9
237.9
410.9
68.9
43.9
139.9
160.8
259.9
285.9
355.8
383.9
472.0
511.0
m/z-->
Glyphosate derivative
C11H10NPO6F12
m/z = 511

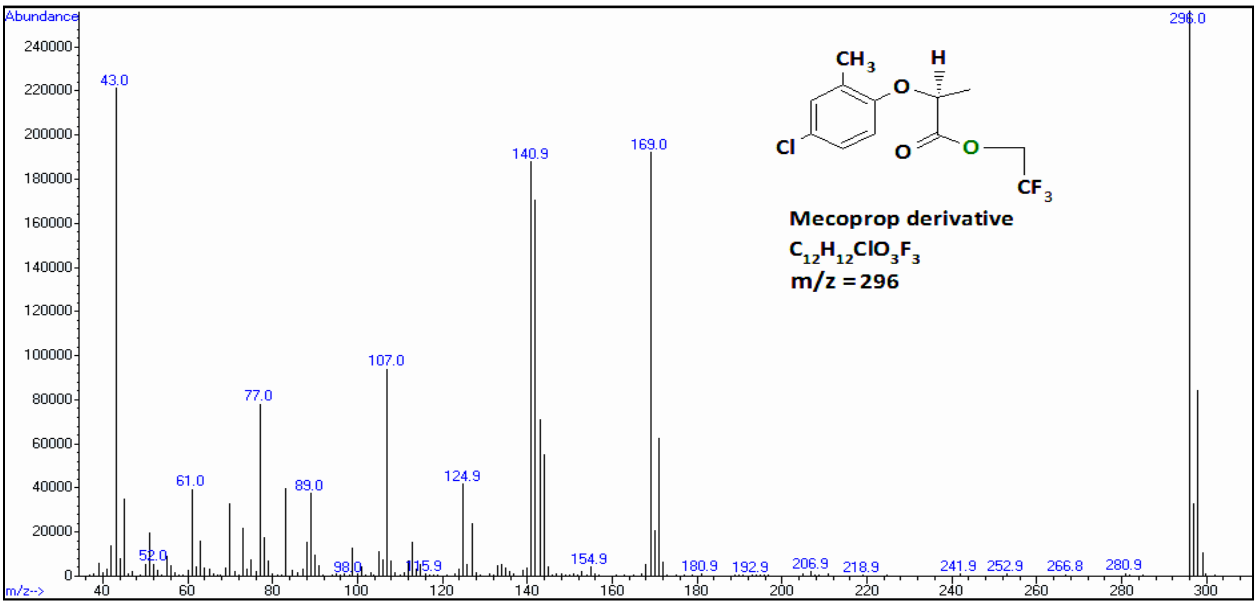
(A)


Abundance
125.9
245.9
68.9
108.9
98.9
301.9
79.0
146.9
162.9
206.9
226.9
271.9
284.9
88.9
136.9
176.9
192.9
259.9
323.9
333.9
351.9
370.9
m/z-->
AMPA derivative
C7H7NPO4F9
m/z = 371

(B)



(C)



(D)

Figure 3.8: The obtained mass spectra of the derivatized compounds (**A**) glyphosate, (**B**) AMPA, (**C**) MCPA and (**D**) mecoprop by GC-MS analysis.

Derivatization reactions of TFAA and TFE with glyphosate, AMPA, MCPA and mecoprop are shown in Figure 3.9. The derivatization reactions with their labeled compounds are shown in Appendix 9 and 10.

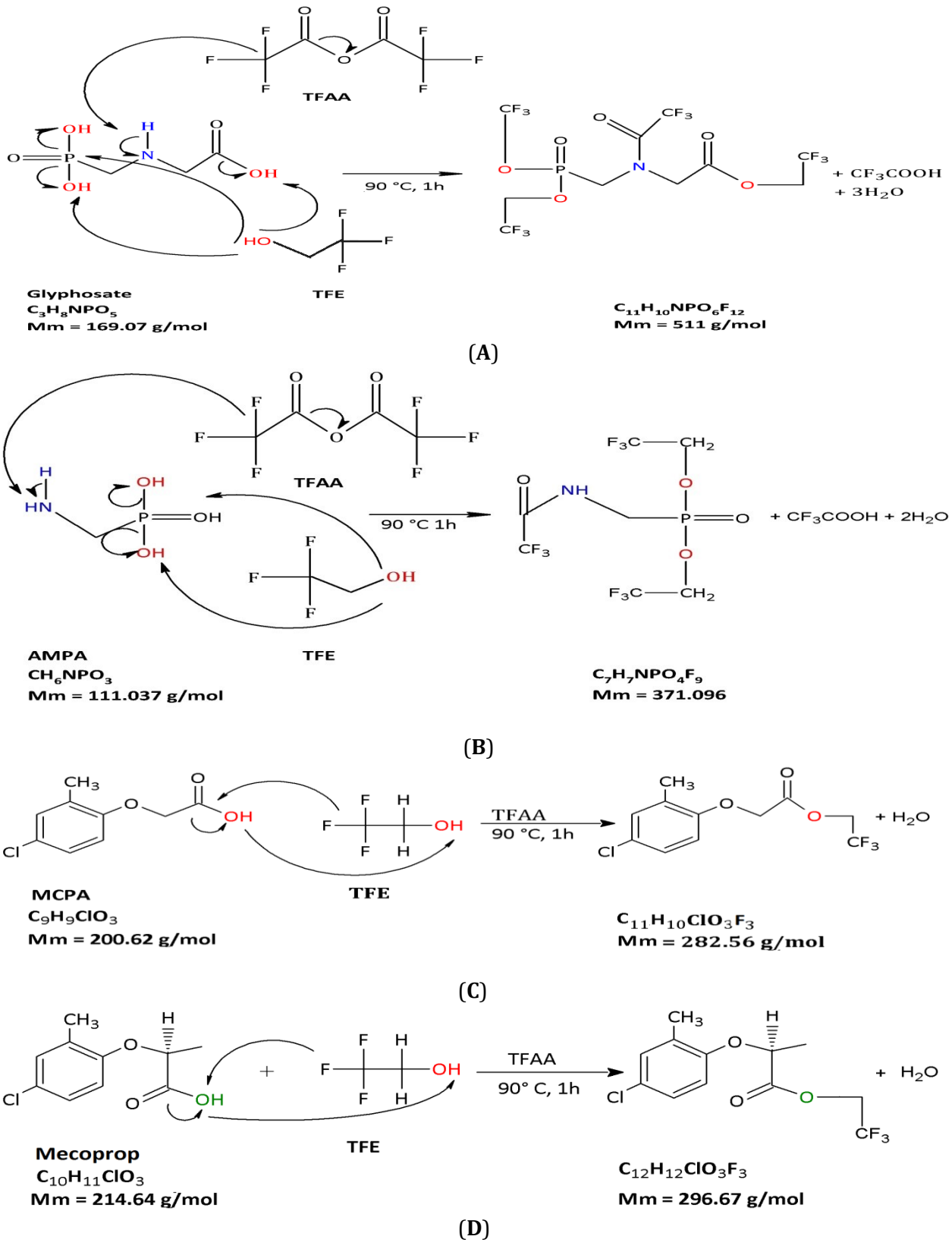
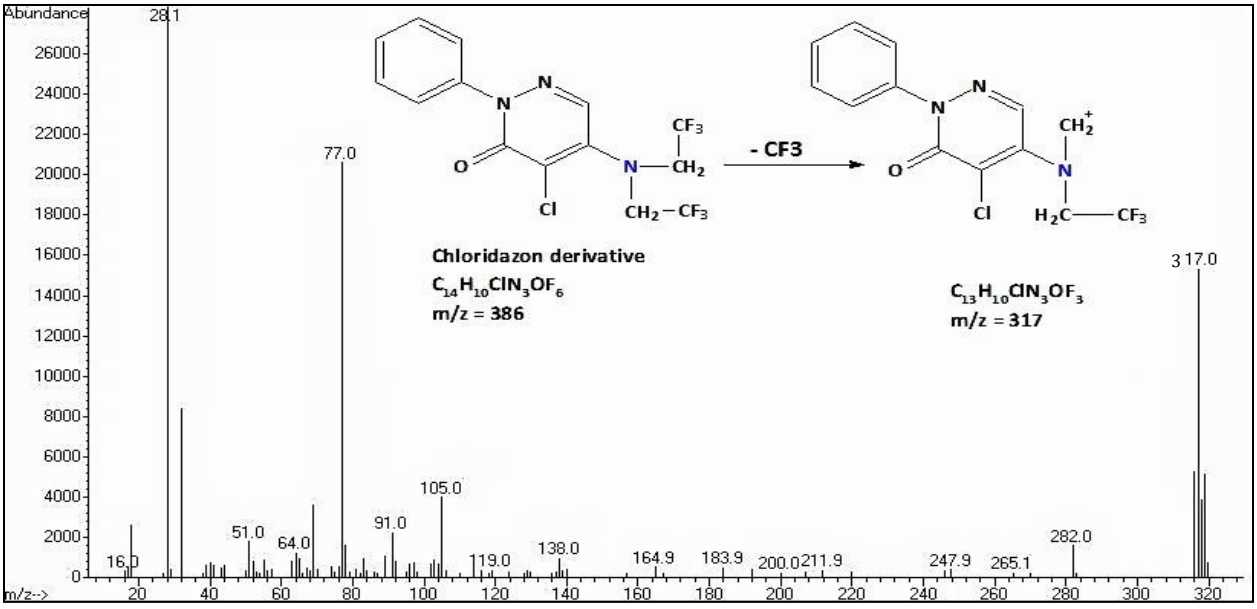


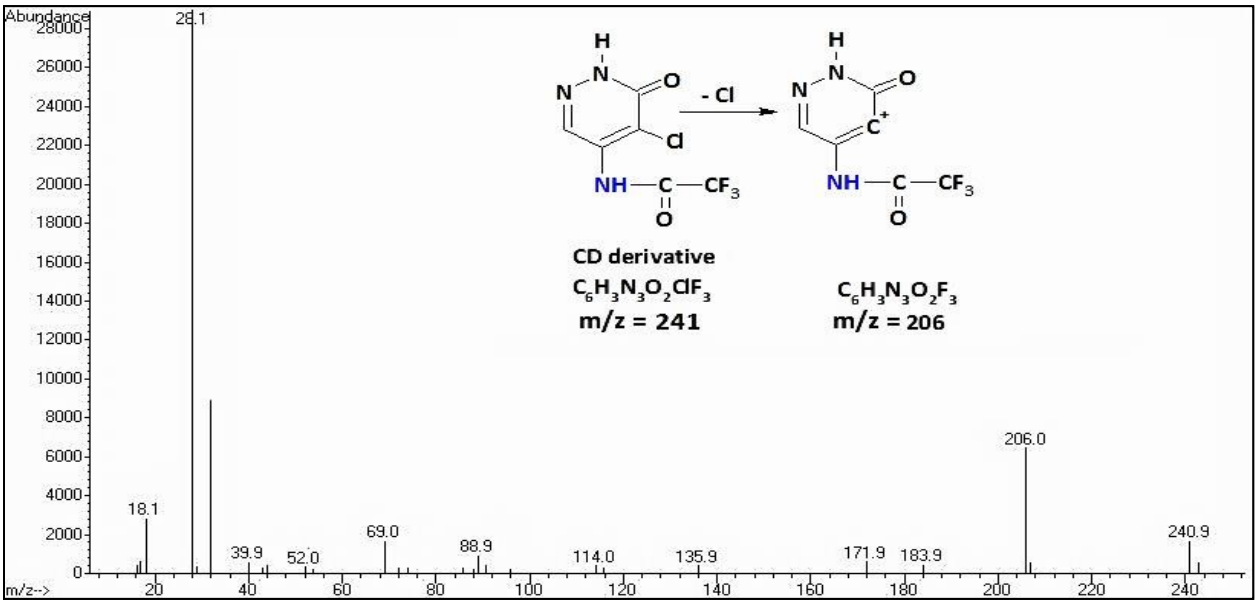
Figure 3.9: The derivatization reactions of (**A**) glyphosate, (**B**) AMPA, (**C**) MCPA and (**D**) mecoprop with TFAA and TFE (Deyrup et al., 1985).

Glyphosate, AMPA, MCPA and mecoprop produced molecular ions of m/z 511, 371, 282 and 296, respectively. According to the analysis of their mass spectra, derivatization of these compounds occurred through acylation and/or esterification reactions. Derivatization of glyphosate and AMPA occurred through the esterification of phosphonic and carboxylic groups and the acylation of amino groups. Derivatization reactions of MCPA and mecoprop took place only with the reagent TFE through the esterification of their carboxylic groups. The derivatization reactions of MCPA and mecoprop with TFE did not take place in the absence of TFAA. Therefore, the reagent TFAA plays a catalytic role in these derivatization reactions. These results indicate that derivatization of the compounds glyphosate, AMPA, MCPA and mecoprop with TFAA and TFE decreased their polarity and increased their volatility which subsequently made them suitable for GC-MS analysis.

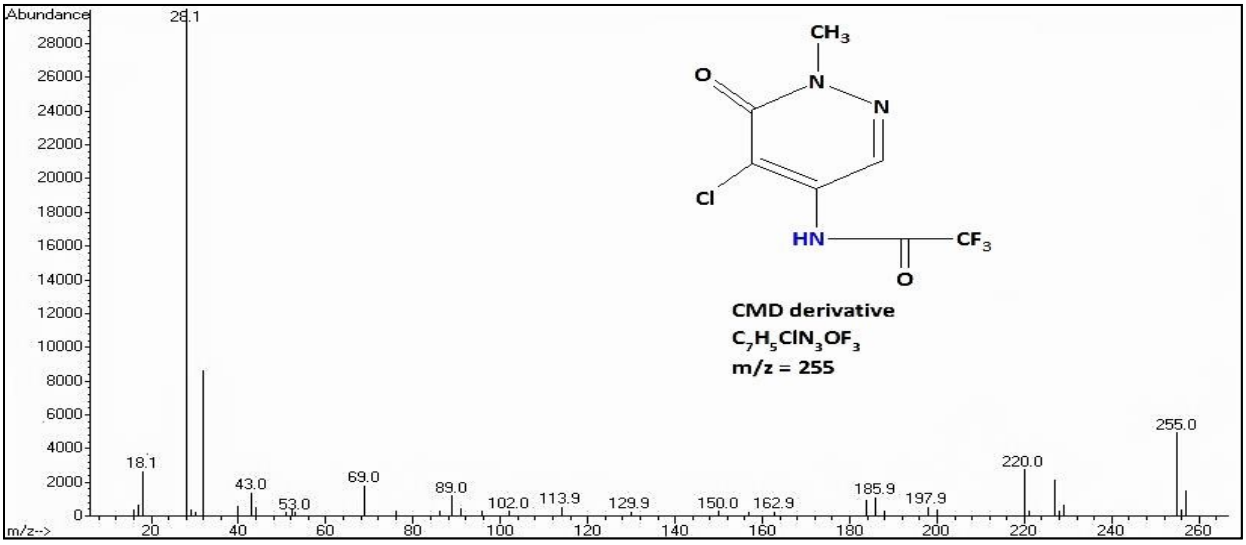
Five peaks were found in the chromatogram obtained from GC-MS analysis after derivatization of the herbicide chloridazon and its metabolites CD and CMD with TFAA and TFE (Figure 3.7). Three peaks of them related to derivatized chloridazon, CD and CMD, respectively, and the other two peaks resulted from underivatized chloridazon and CMD. Mass spectra of derivatized chloridazon, CD and CMD are shown in Figure 3.10. Derivatization of chloridazon, CD and CMD took place through acylation of their functional amino groups. The observation of underivatized chloridazon and CMD peaks can be explained by the fact that derivatization reactions of these two compounds are uncompleted and/or by the instability of these derivatives. A poor peak of the CD derivative was found in the chromatograms. A significant decrease in peak sensitivities of CD was observed with increasing the number of injections. Furthermore, the peaks of the CD derivative were missing in many chromatograms. The unrepeatability of these results displays instability and degradation of the CD derivative.


Abundance
m/z-->
28.1
77.0
105.0
91.0
51.0
64.0
16.0
119.0
138.0
164.9
183.9
200.0
211.9
247.9
265.1
282.0
317.0
- CF3
Chloridazon derivative
C14H10ClN3OF6
m/z = 386
C13H10ClN3OF3
m/z = 317

(A)


Abundance
m/z-->
28.1
18.1
39.9
52.0
69.0
88.9
114.0
135.9
171.9
183.9
206.0
240.9
- Cl
CD derivative
C6H3N3O2ClF3
m/z = 241
C6H3N3O2F3
m/z = 206

(B)



(**C**)

Figure 3.10: Mass spectra of GC-MS analysis of derivatized (**A**) chloridazon, (**B**) CD and (**C**) CMD.

Different temperatures, reaction times and amounts of the reagents TFAA and TFE were examined in order to solve this analytical problem and to find the best conditions for derivatization of chloridazon, CD and CMD. The derivatization parameters of volumes of 150 µL of TFAA and 150 µL of TFE and a temperature of 90 °C showed best derivatization conditions but the intial problem in their analysis still existed. Therefore, TFAA and TFET were considered to be inadequate reagents for use in derivatization of chloridazon, CD and CMD for GC-MS analysis.

Peaks of isoproturon and bentazon derivatives not observed in the chromatograms indicate that these two compounds did not react with TFAA and TFE which may be due to the large size of their molecules (i.e. steric effects) which made the reaction of TFAA and TFE with the amino groups difficult. The results of GC-MS analysis for all the target compounds, having different polar functional groups showed that TFAA and TFE were unsuitable derivatization reagents for the five compounds isoproturon, bentazon, chloridazon, CD and CMD. Consequently, it is impossible to analyze all the selected compounds in a single run after their derivatization with TFAA and TFE by GC-MS. However, two or more derivatization processes maybe required to make them suitable for GC-MS analysis. Moreover, many solid phase extraction processes were expected to be required for extracting them from water samples due to the difference of their properties, especially the ionic characteristic of glyphosate and its metabolite AMPA which made their extraction from saltwater samples very difficult (Corbera et al., 2006, Fritzsche, 2013). For the mentioned reasons, analysis of the nine selected compounds by GC-MS was difficult, tedious and both time and chemicals consuming.

3.1.5 Derivatization of glyphosate and AMPA with FMOC-Cl for HPLC-MS/MS analysis

As seen in section 3.1.1, glyphosate and its metabolite AMPA were inadequate for direct HPLC-RP-MS/MS analysis. Therefore, derivatization of glyphosate and AMPA was a necessary technique for making them suitable for HPLC-MS/MS analysis. Fluorenylmethyl-chloroformate (FMOC-Cl) was chosen as the derivatization reagent in this study. The selection of FMOC-Cl was based on the following factors: its high reactivity towards the amino groups in both glyphosate and AMPA, it is a high non-polar molecule, it has the possibility of performing the derivatization reaction in aqueous medium, it is available commercially in pure form and (Hanke et al., 2008; Stalikas and Konidari, 2001).

Derivatization of glyphosate and AMPA with FMOC-Cl was previously optimized regarding the amount of FMOC-Cl, organic solvent content and reaction time by Hanke et al. (2008). Therefore, the optimization of this analytical method was based on HPLC and MS/MS conditions in order to optimize the resolution, peak shapes and sensitivity of the detector. Derivatization of glyphosate and AMPA with FMOC-Cl was carried out as described in the experimental section 2.4. The derivatization reactions of glyphosate and AMPA with FMOC-Cl are shown in Figure 3.11.

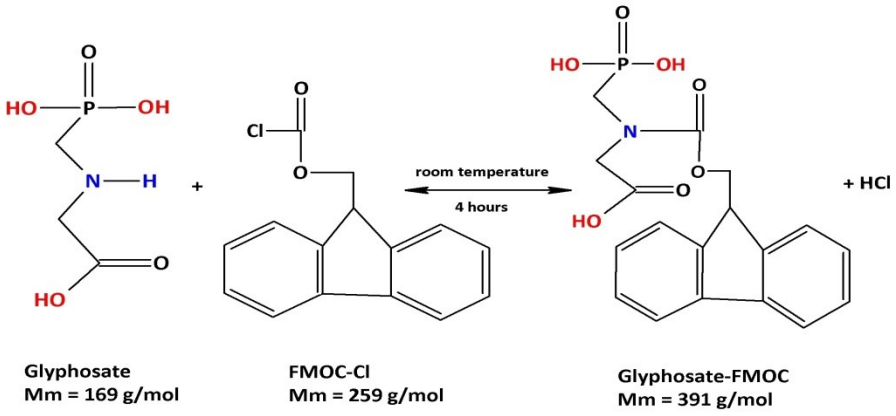


Figure 3.11: Derivatization reactions of (**A**) glyphosate and (**B**) AMPA with FMOC-Cl (Bernal et al., 2012).

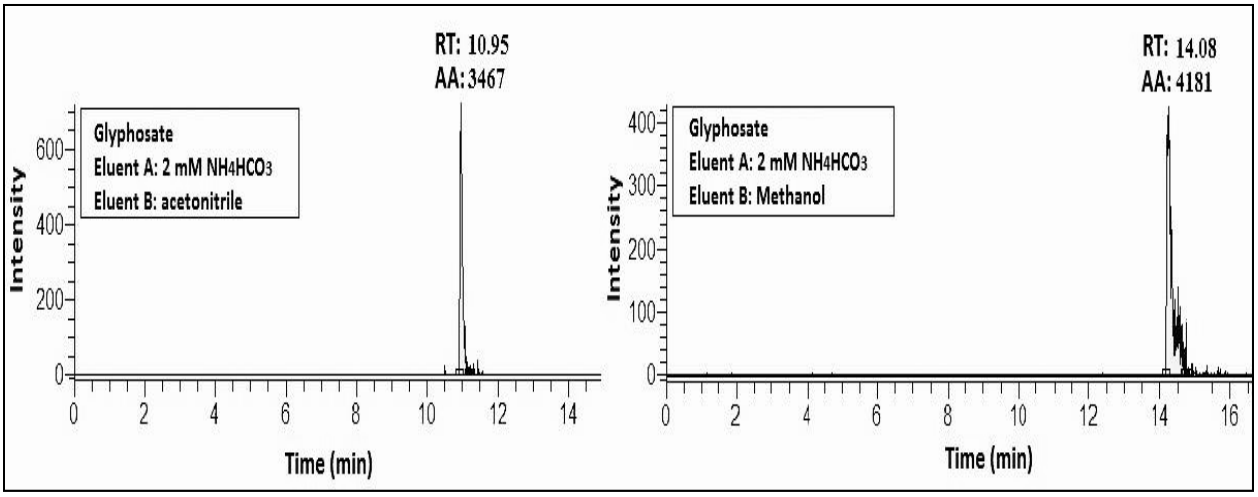
3.1.6 HPLC-MS/MS method optimization for analysis of glyphosate and AMPA after their derivatization with FMOC-Cl

The extraction of the herbicide glyphosate and its metabolite AMPA from saltwater samples is still a drawback in analytical chemistry. As reported by some researches salt concentrations in the analyzed sample caused a significant decrease of glyphosate and AMPA recoveries extracted using solid phase extraction techniques (Corbera et al., 2006; Fritzsche, 2013). Therefore, analysis of glyphosate and AMPA was conducted without pre-concentration steps and their derivatization process with FMOC-Cl was carried out in the aqueous medium. The development of the analytical method was focused on the optimization of HPLC and MS/MS parameters for direct injection of water samples into HPLC-MS/MS system. Detection of glyphosate and AMPA derivatives was performed by selected reaction monitoring mode (SRM). The SRM method was optimized using two SRM transitions for each analyte. For quantification, the transitions 390->168 and 332->110 were utilized for glyphosate-FMOC and AMPA-FMOC, respectively. The selected transitions 390->150 and 332->136 were used for the qualification of glyphosate-FMOC and AMPA-FMOC, respectively.

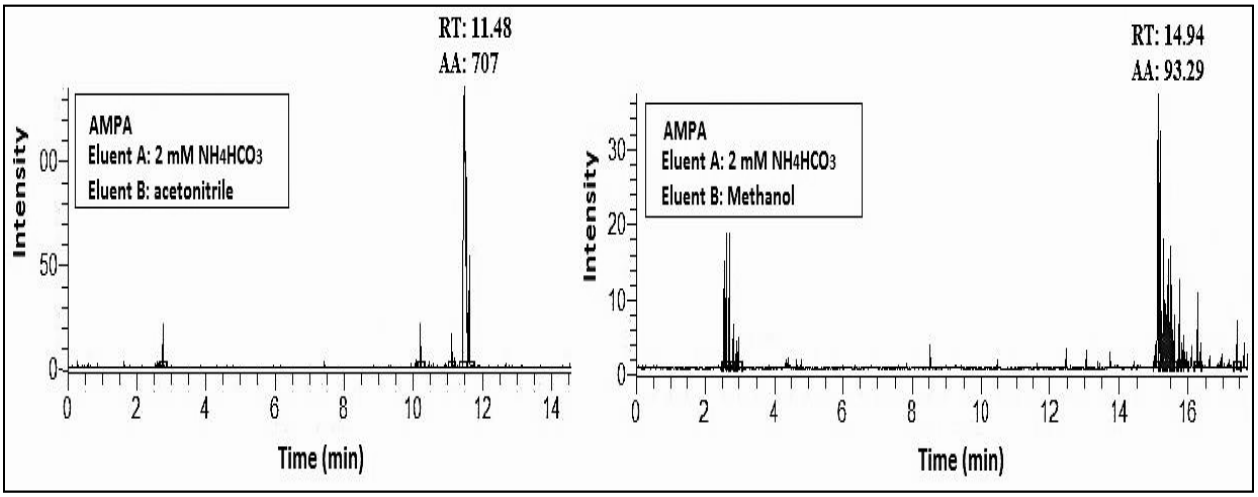
3.1.6.1 Comparison of the two different solvents methanol and acetonitrile

The mobile phase was optimized in order to reach good detection sensitivities, resolution and peak shapes. Most commonly used organic solvents as mobile phases in reversed chromatography are acetonitrile and methanol. A 2 mM ammonium bicarbonate buffer at pH 9 was used as eluent A and acetonitrile and methanol were tested as eluent B.

Chromatograms obtained from analyzing 500 ng/L of glyphoase-FMOC and AMPA-FMOC are shown in Figure 3.12. Shorter retention times, symmetrical peaks and highest sensitivities were observed for both compounds when acetonitrile was used as eluent. This result was expected due to the advantages of acetonitrile over methanol such as lower polarity, better kinetics, higher elution strength and lower back pressure resulting from mixing of acetonitrile with water (Kromidas, 2000).



(**A**)



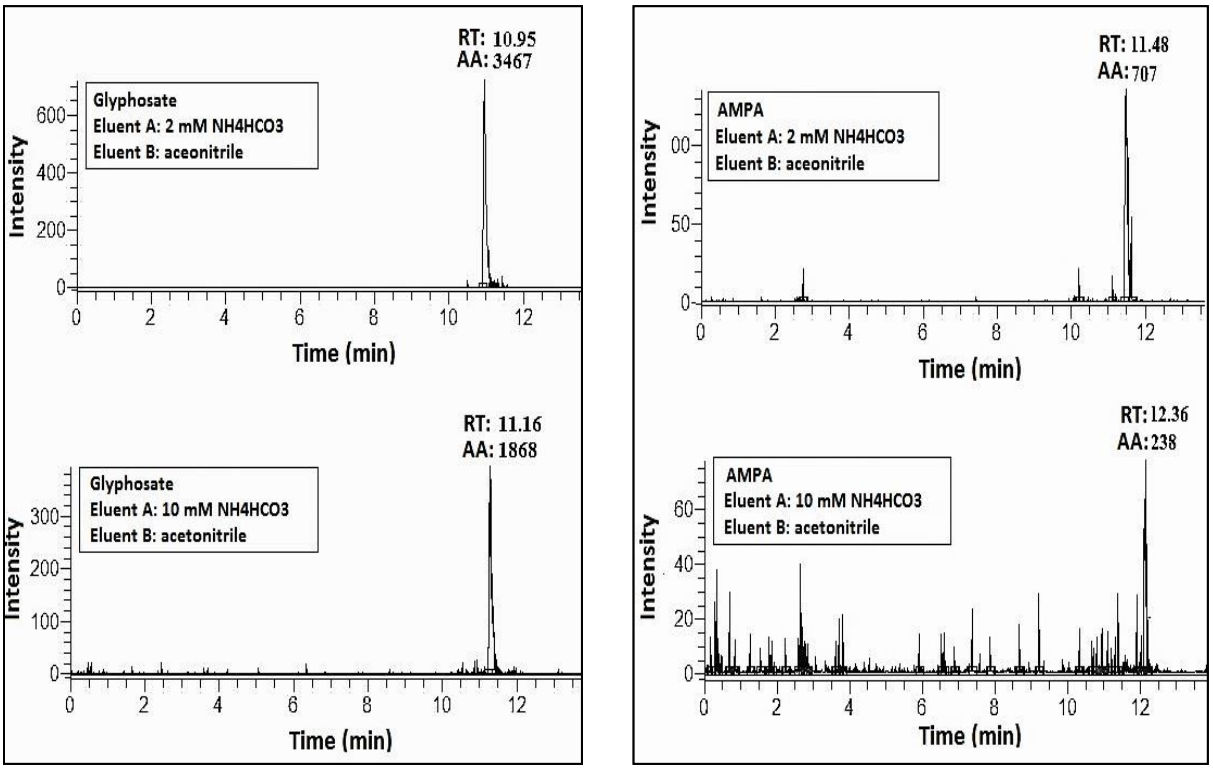
(**B**)

Figure 3.12: Chromatograms obtained from measuring 500 ng/L of glyphosate-FMOC (**A**) and AMPA-FMOC (**B**) with acetonitrile and methanol as eluent B.

The high peak area of glyphosate-FMOC was obtained due to peak tailing and integration error when methanol was used. The peak tailing of glyphosate and AMPA may be due to hydrogen bonding interaction between methanol and the underivatized polar groups in the analytes as hydroxyl groups. According to these results, a 2 mM ammonium bicarbonate buffer pH 9 as eluent A and acetonitrile as eluent B were chosen for the next optimization process.

3.1.6.2 Comparison of two buffer concentrations

Two different concentrations of 2 mM and 10 mM of ammonium bicarbonate buffer in water were tested as eluent A. Chromatograms obtained from measuring 500 ng/L of mixed glyphosate-FMOC and AMPA-FMOC are shown in Figure 3.13. The result showed that 2 mM of ammonium bicarbonate offered better chromatographic performance such as shorter retention times on the reversed phase column, less noise, better peak shapes and higher sensitivities than the eluent with a concentration of 10 mM for both target analytes. Therefore, 2 mM ammonium bicarbonate concentration at pH 9 was used as eluent A in the mobile phase in the final method.



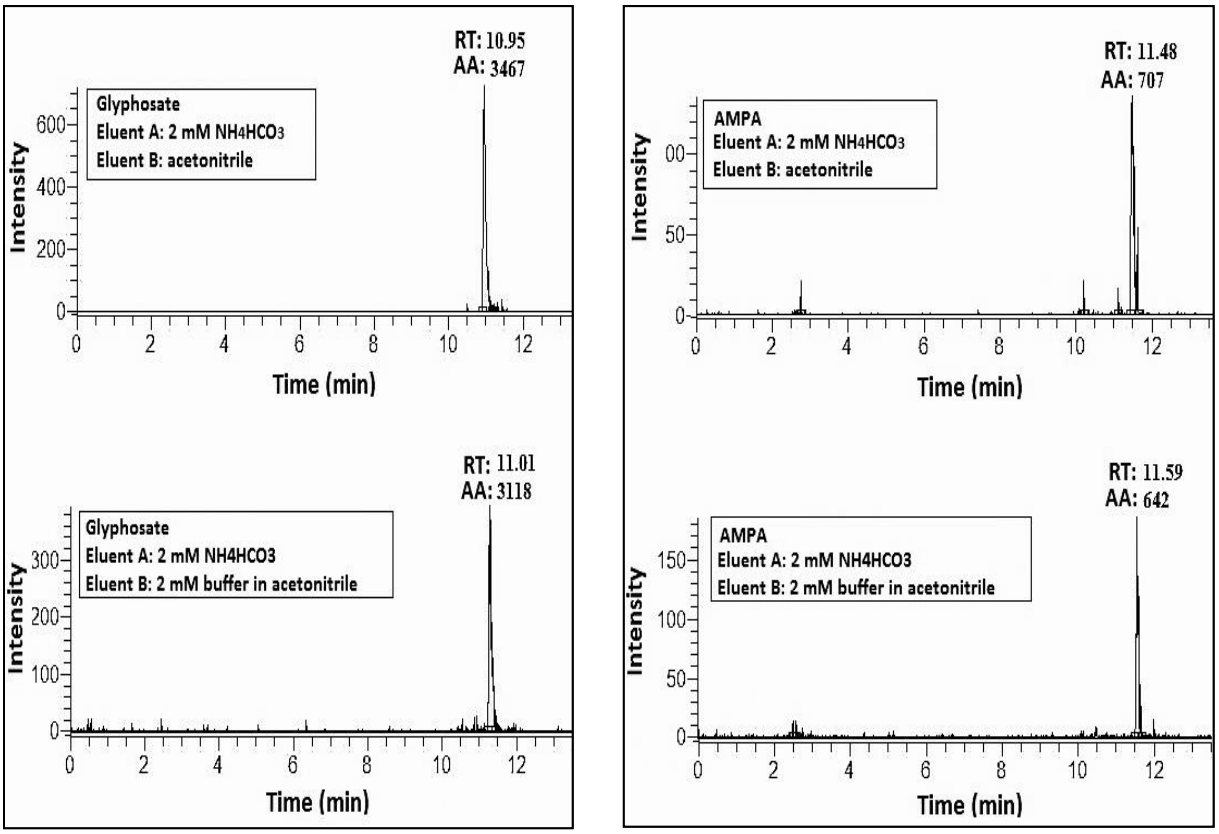
(**A**) (**B**)

Figure 3.13: Chromatograms of 500 ng/L of glyphosate left (**A**) and AMPA-FMOC right (**B**) with 2 mM and 10 mM buffer concentrations.

3.1.6.3 Comparison of pure acetonitrile and acetonitrile with buffer salts

For this optimization step, 2 mM ammonium bicarbonate at pH 9 was used as eluent A and the two different solvents pure acetonitrile and acetonitrile with 2 mM ammonium bicarbonate salt at pH 9 were tested as eluent B, respectively. As seen in Figure 3.14, the

use of a buffer in the organic eluent did not significantly affect retention times and sensitivities for both analytes. However, pure acetonitrile showed better chromatographic performance than acetonitrile with buffer. Therefore, pure acetonitrile was used as eluent B in the final method.



(**A**) (**B**)

Figure 3.14: Chromatograms of 500 ng/L of (**A**) glyphosate-FMOC and (**B**) AMPA-FMOC with pure acetonitrile and acetonitrile with 2 mM buffer salt.

3.1.6.4 Optimization of the mobile phase flow rate

In this optimization step the effect of the mobile phase flow rate on HPLC performance was studied. Samples containing 2 µg/L of glyphosate-FMOC and AMPA-FMOC were measured using different flow rates of 100, 150, 200, 250 and 300 µL/min. 2 mM ammonium bicarbonate buffer at pH 9 as eluent A and acetonitrile as eluent B were used as the mobile phase. As shown in Figure 3.15, the applied mobile phase flow rates showed minor effects on the resolution of both compounds in the reversed phase because desorption from the hydrophobic surface is not affected by the flow rate. On the other hand, the mobile phase flow rate showed a significant effect on the retention times of both analytes. Increasing the

flow rate shorted their retention times because the organic eluent acetonitrile carried them through the column faster after desorption. Moreover, the tested flow rates displayed a notable impact on the detection sensitivity. Peak areas of both components were decreased with increasing flow rate (Figure 3.16). Peaks were not found for either target compound due to high back pressure when a flow rate of 300 µL/min was applied. According to these results, a mobile phase flow rate of 100 µg/L was applied in the final method.

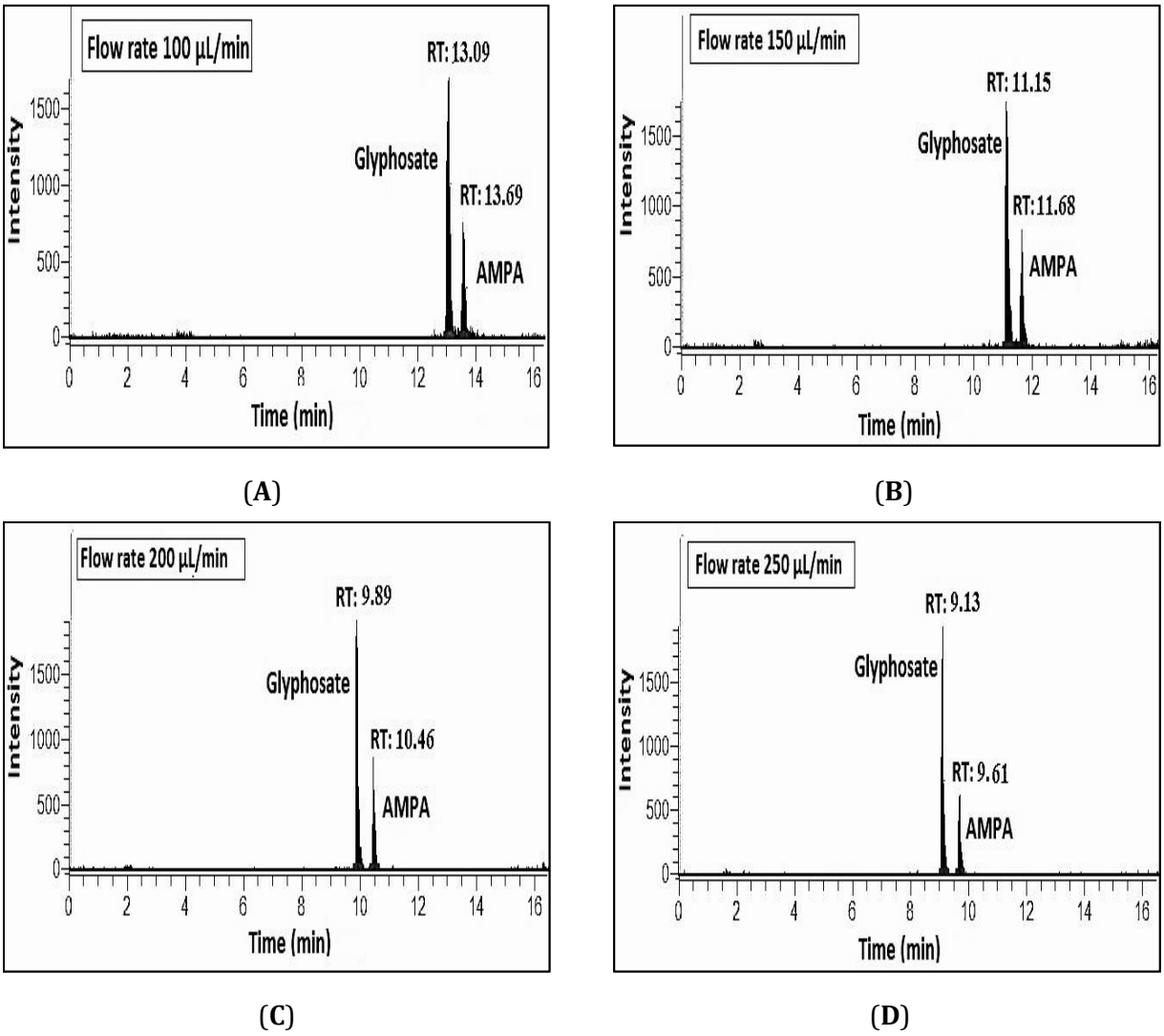


Figure 3.15: Chromatograms of HPLC-MS/MS analysis of 2 µg/L of glyphosate-FMOC and AMPA-FMOC obtained from different mobile phase flow rates (**A**) 100 mL/min, (**B**) 150 mL/min, (**C**) 200 mL/min and (**D**) 250 mL/min.

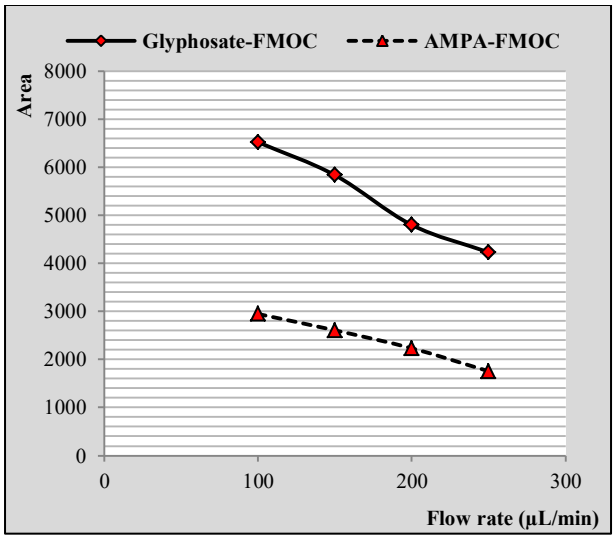


Figure 3.16: Obtained peak areas of analysis 2 µg/L of glyphosate-FOMC and AMPA-FMOC with mobile phase flow rates of 100, 150, 200, 250 µL/min.

3.1.6.5 Optimization of gradient elution

Glyphosate-FMOC and AMPA-FMOC at concentrations of 2 µg/L were measured with four gradient protocols in order to achieve the best resolution for glyphosate–FMOC and AMPA-FOMC. The four tested gradient tables are shown in Appendix 11. The resolution factors of glyphosate-FMOC peaks and AMPA-FMOC peaks were calculated from the four obtained chromatograms (Figure 3.17). The resolution factors were 0.71, 1.48, 0.81 and 1.62 according to gradient elution 1, 2, 3 and 4, respectively. Thus, gradient protocol 4 showed the best resolution with a resolution factor of 1.62 and therefore the gradient elution protocol 4 was applied in the final method.

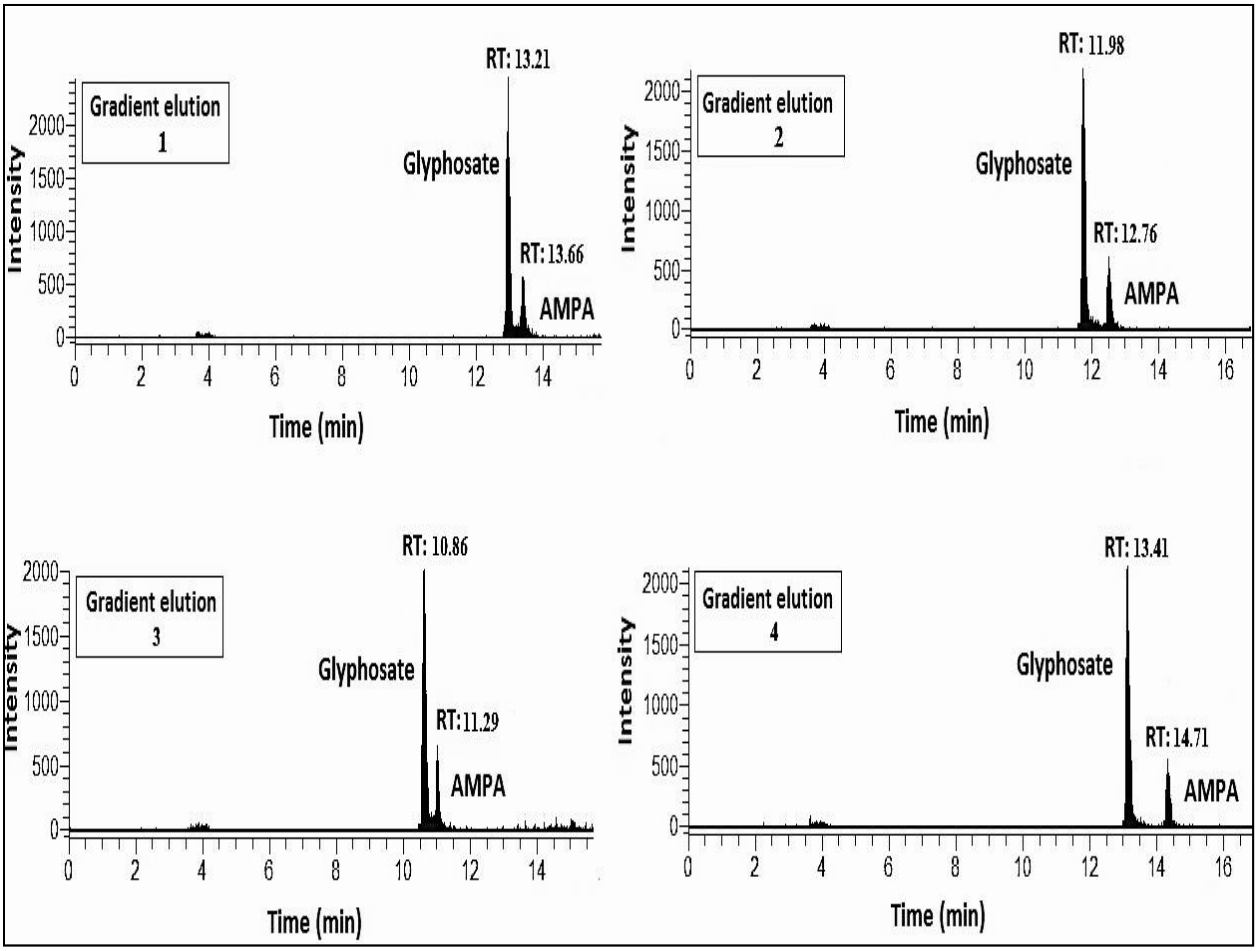


Figure 3.17: Chromatograms of HPLC-MS/MS analysis of 2 µg/L of glyphosate-FMOC and AMPA-FMOC obtained from different gradient elution protocols.

3.1.6.6 Optimization of MS/MS conditions

The optimization of HESI-MS/MS parameters and the selection of the appropriate ions were conducted by analysis of glyphosate and AMPA derivatives in order to monitor the MS conditions which would give the highest sensitivities for both compounds. Optimization of the MS/MS conditions was achieved by a variety of parameters such as vaporizer temperature, capillary temperature, sheath gas pressure, auxiliary gas flow rate and spray voltage. MS/MS parameters were adjusted to maximize the intensity of the product ions m/z 168 and 110 for glyphosate-FMOC and AMPA-FMOC, respectively. The mobile phase was 2 mM ammonium bicarbonate at pH 9 as eluent A and acetonitrile as eluent B. The flow rate was set to 100 µL/min of gradient 4. Separation was achieved on a Gemini 3 µm NX-C_{18} 110 A° HPLC column (150 x 2.0 mm) at 20 °C.

Four vaporizer temperatures 200, 250, 300 and 350 °C were tested. As shown in Figure 3.18(**A**), highest sensitivities were obtained at a vaporizer temperature of 200°C.

The second optimization step was to compare the effect of different capillary temperatures of 200, 250, 300 and 350 °C on the sensitivities. Highest sensitivities were obtained at a capillary temperature of 300 °C for glyphosate-FMOC, while two capillary temperatures of 250 °C and 300 °C offered the highest sensitivity for AMPA-FMOC 3.18(**B**). Therefore, a capillary temperature of 300 °C was chosen in the method.

Optimization of sheath gas pressure and auxiliary gas flow rate was achieved. Three sheaths gas pressures of 15, 20 and 25 psi and three auxiliary gas flow rate of 10, 15 and 20 arbitrary units were tested. As shown in Figure 3.18(**C**) and (**D**), highest sensitivities were achieved by a sheath gas pressure of 20 psi and an auxiliary gas flow rate of 15 arbitrary units for both the target compounds glyphosate-FMOC and AMPA-FMOC. A clear effect of the auxiliary gas flow on the sensitivities appeared especially for glyphosate-FMOC, whereas a double peak area was obtained by applying an auxiliary gas flow rate of 15 arbitrary units compared to pressures of 10 and 20 arbitrary units.

Lastly, the spray voltage was optimized in order to increase glyphosate-FMOC and AMPA-FMOC sensitivities. Four spray voltages of 2500, 3000, 3500 and 4000 volt were examined. As shown in Figure 3.18(**E**), spray voltages of 3000, 3500 and 4000 volt showed almost the same sensitivities for AMPA-FMOC. The highest sensitivity for glyphosate-FMOC was achieved by spray a voltage of 4000 volt. As a result, a spray voltage of 4000 volt was chosen in the final method.

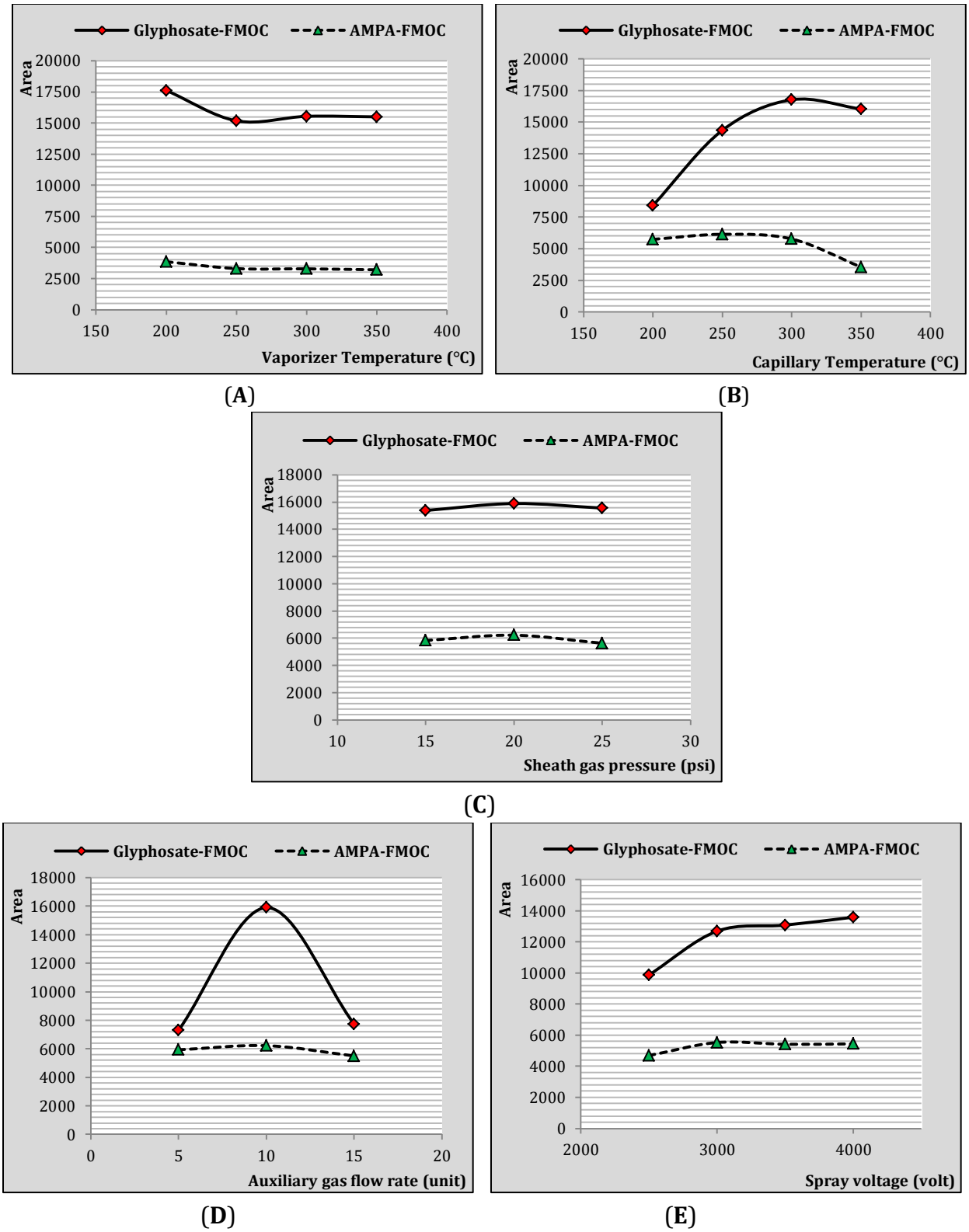


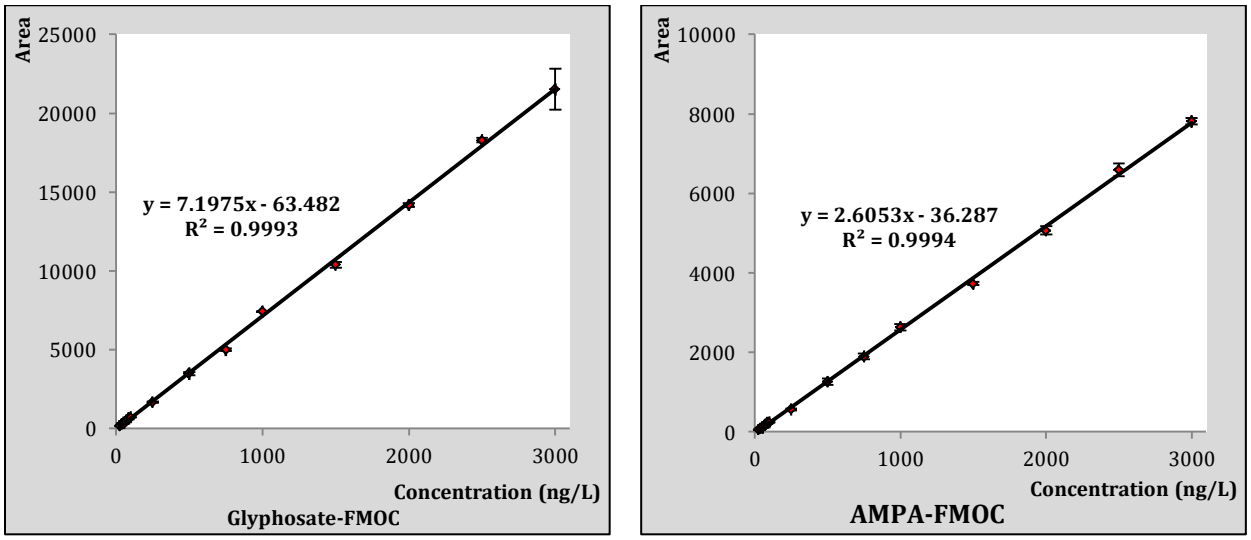
Figure 3.18: Effect of some developed parameters in the analytical method: (**A)** vaporizer temperature, (**B**) capillary temperature**,** (**C**) sheath gas pressure, (**D)** auxiliary gas pressure and (**E)** spray voltage on the MS/MS signal resulting from analysis of 2 µg/L concentrations of glyphosate-FMOC and AMPA-FMOC.

3.1.7 HPLC-MS/MS method validation for analysis of glyphosate and AMPA after their derivatization with FMOC-Cl

The developed method for glyphosate and AMPA analysis was validated. Method validation of the procedure was performed including the following parameters: linearity, limit of detection and quantification, precision, accuracy, matrix effect, analytes and system stability. HPLC-MS/MS system in the selected reaction monitoring mode was used for quantitative analysis and validation of the analytical method.

3.1.7.1 Calibration curves and linearity

The calibration curves of glyphosate-FMOC and AMPA-FMOC were created using 14 concentration levels ranging from 30 to 3000 ng/L (Figure 3.19). Linearity of the method was investigated by calculating of the regression line of the method and expressed by the correlation coefficient (R^2). Calibration curves showed high linearity over the measured concentration ranges with correlation coefficients 0.9993 and 0.9994 for glyphosate-FMOC and AMPA-FMOC, respectively. Obtained calibration curves for glyphosate-FMOC and AMPA-FMOC indicate a strong relationship between concentrations and peak areas.



(**A**) (**B**)

Figure 3.19: Calibration curves of (**A**) glyphosate-FMOC and (**B**) AMPA-FMOC at concentration ranges from10 ng/L to 3000 ng/L.

3.1.7.2 Limit of detection and quantification

Limit of detection (LOD) and quantification (LOQ) were estimated from the calibration curves according to DIN 32 645 using SQS 2000 (version 2.01). LOD and LOQ of glyphosate-

FMOC were determined to be at 9 ng/L and 27 ng/L, respectively and for AMPA-FMOC at11 ng/L and 32 ng/L, respectively.

3.1.7.3 Precision

Precision (repeatability) was expressed as the ability of re-analysis of interest with low standard deviation. The precision was conducted by HPLC-MS/MS measurements of standard solutions from the calibration curves in triplicates. The calculated RSDs % ranged from 0.2% to 11.6% for glyphosate-FOMC and from 1.0% to 11.1% for AMPA-FMOC (Table 3.5). The highest RSD% values of both glyphosate-FOMC and AMPA-FMOC were found at the low concentration levels. The repeatability of the method was confirmed by a good precision at concentration levels over 100 ng/L with RSDs lower 7% for both target analytes.

3.1.7.4 Accuracy

Accuracy (relative error RE %) is the difference between true values and the measured values. This deviation between the true value and the measured values were calculated for six concentrations in low, middle and high concentration ranges. The calculated accuracy of glyphosate-FMOC was in the range of -0.4% to -11.1% and in the range of 0.1% to 11.2% for AMPA-FMOC (Table 3.5). The method provided satisfactory accuracy for both glyphosate and AMPA especially at concentration levels over 100 ng/L.

Table 3.5: The calculated precision (RSD%) and accuracy (RE%) resulting of HPLC-MS/MS analysis of glyphosate-FMOC and AMPA-FMOC (n = 3) at concentrations lie between 25 ng/L and 3000 ng/L.

Concentration (ng/L)	glyphosate-FMOC		AMPA-FMOC	
	RSD%	RE%	RSD%	RE%
25	2.3	4.0	3.0	11.2
55	6.4	-11.1	10.9	-8.9
70	8.4	2.1	11.1	5.7
85	11.6	3.6	10.7	5.7
100	1.3	-1.1	3.4	-4.1
250	2.3	3.4	3.6	-1.6
500	3.0	1.4	6.7	2.4
750	1.8	-4.7	3.7	0.4
1000	0.2	4.9	3.3	3.2
1500	1.9	-2.9	1.4	-3.3
2000	0.8	-1.1	2.0	-2.1
2500	0.8	1.7	2.5	1.5
3000	6.0	-0.4	1.0	0.1

RSD%: Relative standard deviation
RE%: Relative error

3.1.7.5 Analytes stability

Two standard solutions of glyphosate and AMPA at a concentration of 1µg/L were prepared for the stability test. One of the solutions was kept at 5 °C while the other was kept at an ambient laboratory temperature of 21 °C. Samples were withdrawn from the solutions and analyzed at intervals of zero to 300 hours in order to monitor degradation of the target analytes. Changes in glyphosate-FMOC and AMPA-FMOC peak areas at the two selected temperatures are shown in Figure 3.20(**A**) and (**B**).

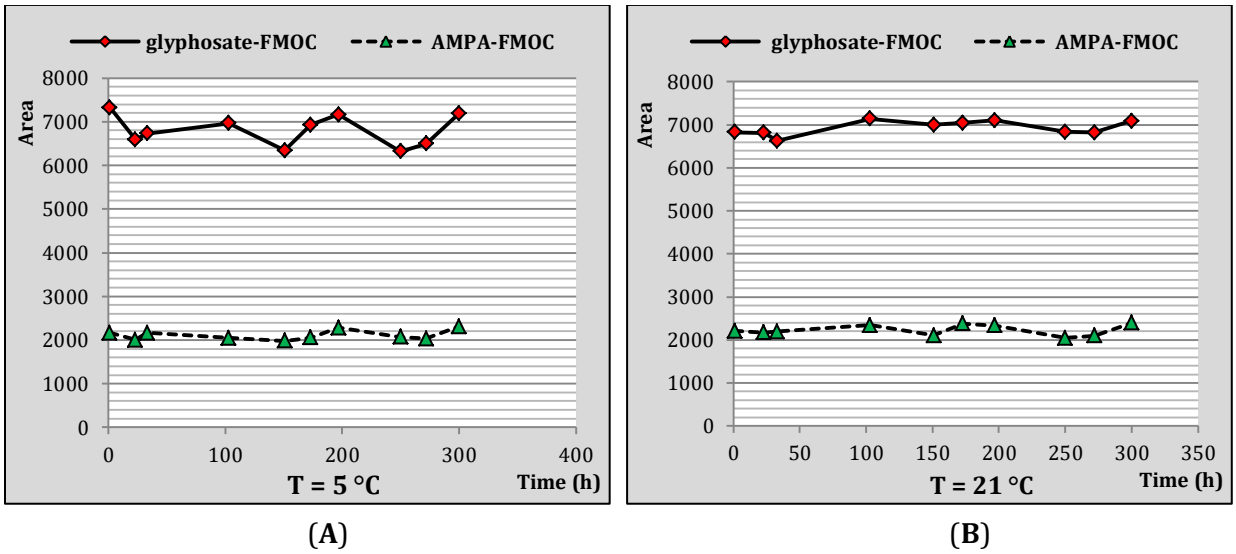


Figure 3.20: Stability testing of 1 µg/L glyphosate-FMOC and AMPA-FMOC at (**A**) 21 °C laboratory temperature and (**B**) 5 °C within 300 hours.

Changes in peak areas of both analytes were less than 6% during the tested period. Moreover, no additional peaks were observed in the obtained chromatograms. This result reflects satisfied stability of glyphosate-FMOC and AMPA-FMOC during the tested period of 300 hours.

3.1.7.6 System stability and blank samples

System stability is defined as the checking of a system before and during analysis of unknowns, to ensure system performance. For this aim, samples of standard solutions of glyphosate-FMOC and AMPA-FMOC in Milli-Q water at concentration of 1 µg/L were measured before and during measurements for controlling the HPLC-MS/MS system. System stability was a critical indicator to check the HPLC-MS/MS system for problems which occurred from precipitation of the excessive FMOC-Cl onto the column and the

contamination of TSQ vantage, especially in the case of natural samples which were measured without clean-up and extraction processes. However, cleaning the TSQ vantage, washing the column and changing the used security guard cartridge system were important steps in solving these problems and maintaining a the good efficiency of the HPLC-MS/MS system. Furthermore, blank samples were measured during each analysis in order to identify errors or contaminations which may have occurred through sample preparation and analysis.

Gas chromatography (GC) and high performance liquid chromatography (HPLC) tandem mass spectrometry $(MS)^n$ were the predominant analytical techniques used for the analysis of contaminants in the environment. In this section, a comparison of these two techniques was carried out for the analysis of six polar herbicides with different functional groups and three of their metabolites in water samples. The nine selected compounds were the herbicides glyphosate, MCPA, mecoprop, isoproturon, bentazon and chloridazon and the metabolites AMPA, CD and CMD. All the target compounds were found to be unsuitable for direct GC-MS analysis. Furthermore, GC-MS was incapable of analyzing all the target analytes in one single run after derivatization with TFAA and TFE. More than one derivatization process was found to be required in order to achieve their analysis by GC-MS which subsequently increasing numbers of sample preparation steps such as numbers of collected samples, filtration, solid phase extraction and derivatization. GC-MS analysis was found to be difficult, tedious and both time and chemical consuming.

Six compounds MCPA, mecoprop, isoproturon, bentazon, chloridazon and CMD were found to be able for direct HPLC-RP-MS/MS analysis. Glyphosate and its metabolite AMPA were found to be suitable for HPLC-RP-MS/MS analysis after derivatization with FMOC-Cl. The metabolite CD was excluded from the analytical method due to a peak tailing problem and very poor interaction with the C_{18} reversed phase column. The HPLC-RP-MS/MS technique was preferred to GC-MS and it was found to be a good approach for the analysis of the target compounds in water samples. Two rapid analytical methods have been developed and validated in order to achieve the analysis of the selected compounds in water samples using HPLC-RP-MS/MS. Table 3.6 shows a summary of GC-MS and HPLC-MS/MS analysis of the target compounds.

Table 3.6: Comparison between GC-MS and HPLC-MS/MS for the analysis of the nine target compounds.

Compound	Direct GC-MS	GC-MS after TFFA + TFE derivatization	Direct HPLC-MS/MS	HPLC-MS/MS after FMOC-Cl derivatization
Glyphosate	No	Yes	No	Yes
AMPA	No	Yes	No	Yes
MCPA	No	Yes	Yes	-
Mecoprop	No	Yes	Yes	-
Isoproturon	No	No	Yes	-
Bentazon	No	No	Yes	-
Chloridazon	No	No	Yes	-
CD	No	No	No	-
CMD	No	No	Yes	-

The first method is direct HPLC-RP-MS/MS analysis of the five herbicides MCPA, mecoprop, isoproturon, bentazon and chloridazon and the metabolites and CMD in water samples. The second method is HPLC-MS/MS analysis of the herbicide glyphosate and AMPA after pre-derivatization with FMOC-Cl in water samples. Optimization of the two analytical methods was conducted according to several HPLC and MS/MS parameters without pre-concentration steps. Satisfactory validation parameters of these analytical methods including linearity, accuracy, precision, system and analytes stability were obtained as shown in Table 3.7.

Table 3.7: Correlation coefficients (R^2), linearity, limits of detection (LODs) and of quantification (LOQs), precisions (RSDs %) and accuracies (RE %) of the developed HPLC-MS/MS methods for the analysis of the target compounds in water samples.

Compound	R^2	Linearity (ng/L)	LODs (ng/L)	LOQs (ng/L)	RSDs (%)	RE (%)
Glyphosate	0.9993	25-3000	9	27	0.2-11.6	0.4-11.1
AMPA	0.9994	25-3000	11	32	1.0-11.1	0.1-11.2
MCPA	0.9984	50-2000	10	50	0.7-4.9	0.4-5.4
Mecoprop	0.9958	50-2000	10	50	0.2-8.2	0.6-6.6
Isoproturon	0.9996	10-2000	0.5	4	0.1-5.0	0.3-9.4
Bentazon	0.9996	10-2000	3	6	0.4-3.2	0.2-11.4
Chloridazon	0.9996	100-2000	10	90	0.6-9.0	0.4-1.6
CMD	0.9993	10-2000	10	25	0.5-10	0.1-11.1

3.2 Occurrence of polar herbicides and some of their metabolites in the German Baltic estuaries

The contamination of the marine environment by anthropogenic trace pollutants has changed in the past ten to fifteen years from persistent and not easily degraded contaminants to more polar, thermo-labile and less volatile compounds (Fobbe et al., 2006). The Baltic Sea is one of the worst polluted seas in the world (HELCOM, 2003). Generally, rivers play an essential role in transporting pesticides into the marine environment (Olsson et al., 2012). The German riverine flow into the Baltic Sea forms a small portion of the total flow and constitutes a large number of small rivers (BUND, 2012; Burkhardt et al., 2005). On the other hand, Germany has the highest agricultural activities compared to other Baltic countries (BUND, 2012).

Polar herbicides such as glyphosate, MCPA, mecoprop, isoproturon, beantazon, and chloridazon are utilized in large amounts in Germany (BVL, 2012). Many studies have proven the contamination of European fresh surface waters by the polar herbicides mentioned here. In Switzerland, glyphosate and its metabolite AMPA were observed in the Rhine river with concentrations ranging from 25 ng/L to 55 ng/L of glyphosate and from 55 ng/L to 65 ng/L of AMPA. Additionally, these two compounds were detected in two Swiss lakes, where glyphosate concentrations were around 15 ng/L in Murtensee lake and around 35 ng/L in Greifensee lake and AMPA concentrations were around 60 ng/L in both lakes (Hanke et al., 2008). Glyphosate and AMPA were found in two rivers (Boele and Orge) examined studies in France under both dry and wet weather conditions. The concentrations of glyphosate were between <100 and 1.08 ng/L in the Boele river and between <100 and 0.2 ng/L in the Orge river. AMPA was detected at concentrations higher than glyphosate in both rivers and ranged from 0.34 to 1.93 ng/L in the Boele river and from 0.23 to 0.79 ng/L in the Orge river (Botta et al., 2009). The German Agency for the Environment, Nature Conservation and Geology Mecklenburg-Vorpommern reported that glyphosate and AMPA were detected in 58% and 82% respectively of the collected fresh surface water samples. Their frequency of detections with concentration higher than 100 ng/L were 22% and 46% of glyphosate and AMPA, respectively (Bachor et al., 2008).

Mecoprop and MCPA were detected in water samples collected from Greifen lake and a wastewater treatment plant (WWTP) in Switzerland. Mecoprop concentrations were in the range 30-50 ng/L in Greifen lake and 20-400 ng/L in WWTP, while MCPA concentrations ranged from 10 ng/L to 25 ng/L in Greifen lake and from undetectable to 100 ng/L in WWTP (Ollers et al., 2001). MCPA and mecoprop were observed in surface water samples of Mecklenburg-Vorpommern in Germany with maximum concentrations exceeding 1 µg/L (Bachor et al., 2008).

In France, isoproturon was detected in the Garonne river water at a concentration of 36 ng/L and at concentrations ranging from 53 ng/L to 58 ng/L in its Dropt tributary (Dupas et al., 1995). Isoproturon was detected at high concentration levels in Germany reaching 42 µg/L in effluents of rural wastewater treatment plants after biological treatment (Nitschke and Schussler, 1998).

The long-term Swedish national water quality monitoring program considered the herbicide bentazon to be the most commonly found substance in watercourses with detection frequencies of over 30% of all samples (period 1985-2005) (Törnquist et al., 2007). In water samples collected from the Main river in Germany, the 3-day average concentration maximum of bentazon at Würzburg station was 0.29 µg/L and the maximal weekly average at Schweinfurt station was 0.22 µg/L both exceeding the European drinking water standard of 0.1 µg/L for each pesticide. Bentazon was found in 67% of sewage treatment plant samples in concentrations above 0.1 µg/L with a maximum concentration of approximately 12 µg/L in one station (Bach et al., 2010).

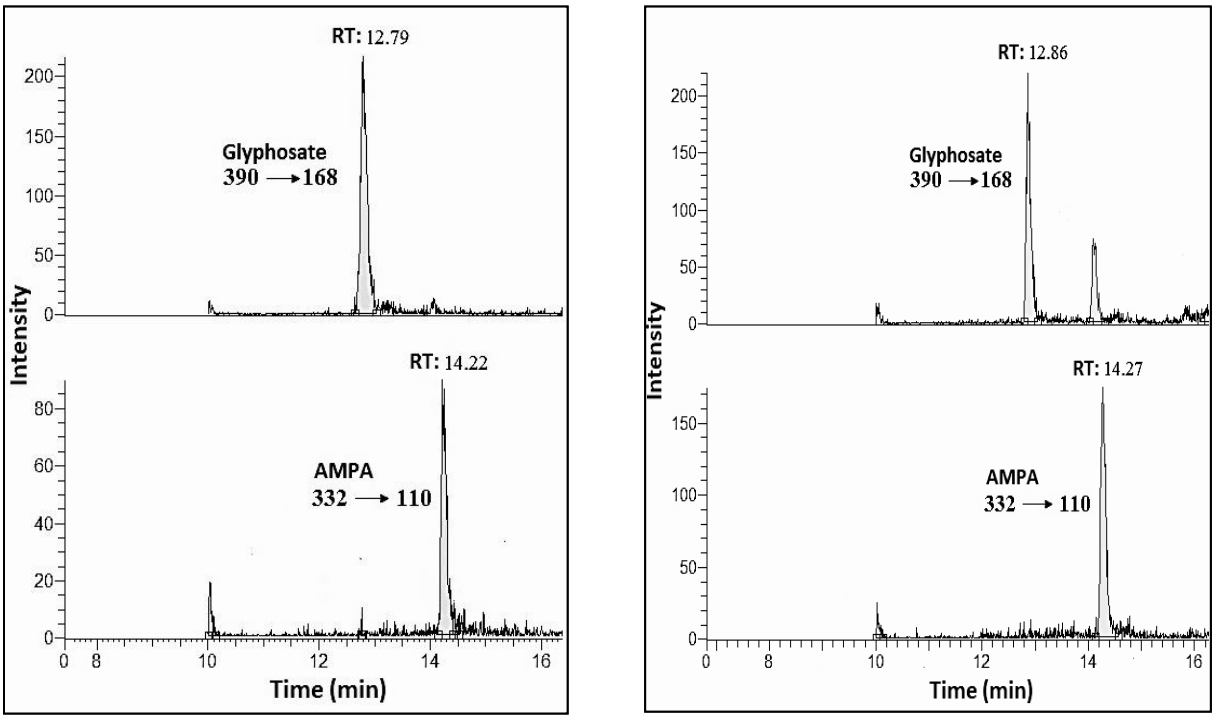
According to the monitoring program which was conducted following the environmental detection of the herbicide chloridazon in groundwater, surface water and waste water treatment plants (WWTP) in the Hesse region (Germany) during the year 2007, chloridazon was detected in surface water at concentrations below 1µg/L with a seasonal peak of 0.89 µg/L after its application in spring (Buttiglieri et al., 2009). Chloridazon and its metabolite CMD were also detected in freshwater samples in Mecklenburg-Vorpommern, Germany. (Bachor et al., 2008).

Estuaries receive great attention in studies on terrestrial regions as pollution contributors to the marine environment. Most studies and monitoring programs on the pollution of the Baltic Sea and its estuaries focused on persistent and non- and low polar pesticides (Bester and Hühnerfuss, 1993; Falandysz et al., 2004; Pikkarainen, 2007; Schulz-Bull et al., 1995; Schulz-Bull et al., 2013; Strandberg et al., 1998). However, very little attention has been drawn to the occurrence of other classes of pesticides (e.g. mid and highly polar pesticides) in the Baltic estuaries and their potential transport to the Baltic coast. Further data on the occurrence of the polar herbicides and metabolites listed above in Baltic estuaries and their transport to the Baltic Sea is important for the evaluation of their environmental fate (i.e. stability on their way to the sea) and for a comprehensive eco-toxicological risk assessment.

The objective of this section is to study the potential transport of the selected herbicides and metabolites into the Baltic Sea by studying their occurrence in some of the German Baltic estuaries.

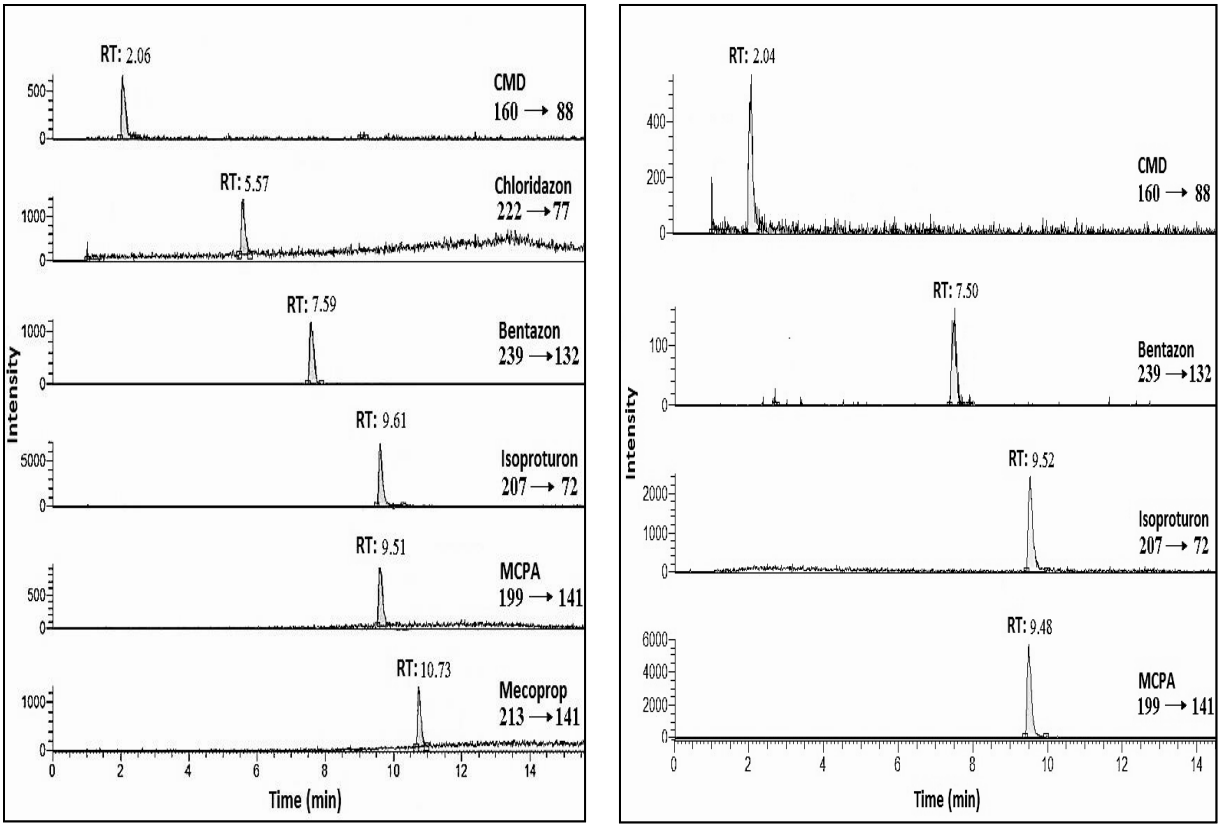
In order to study the potential transport of the six selected polar herbicides glyphosate, MCPA, mecoprop, isoproturon, bentazon, chloridazon and two of their metabolites AMPA and CMD into the Baltic Sea, water samples were taken from ten Baltic estuarine locations

and from the Baltic coast between May and September 2012. The sampling sites were located along the coast of the German federal state of Mecklenburg-Vorpommern. The samples were collected under different weather conditions (i.e. dry, wet and after rainfall events). All the collected samples were analyzed using the HPLC-MS/MS methods described in sections 2.6 and 2.7. The identification of target analytes in unknown samples was according to three parameters: quantifier ions, qualifier ions and the comparison of retention times of unknown peaks to peaks in the standard samples. The quantitative analysis was carried out in reference to the qualifier ions. Chromatograms from the analysis of glyphosate and AMPA standards and estuarine water samples are shown in Figure 3.21 and of the compounds CMD, chloridazon, bentazon, isoproturon, MCPA and mecoprop are shown in Figure 3.22.



(**A**) (**B**)

Figure 3.21: Chromatograms obtained from the analysis of (**A**) 250 ng/L of glyphosate and AMPA standards and (**B**) natural samples collected from the estuary of Mühlenfließ.



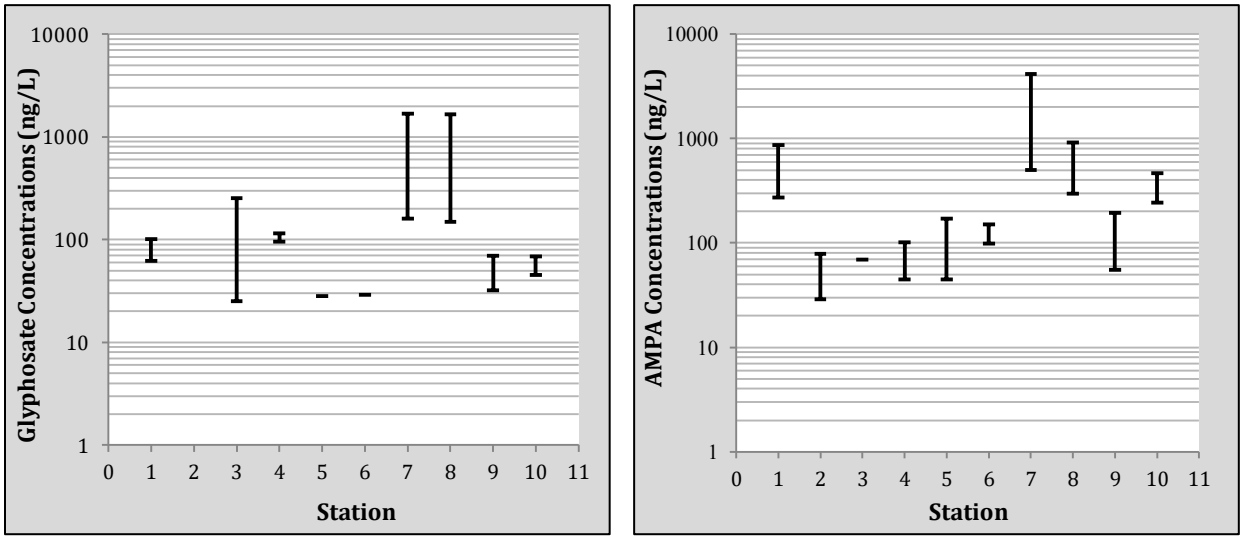
(**A**) (**B**)

Figure 3.22: Chromatograms from the analysis of (**A**) 100 ng/L of the analytes CMD, chloridazon, bentazon, isoproturon, MCPA and mecoprop and (**B**) natural sample collected from the estuary of Mühlenfließ.

The measured concentrations, salinities, temperature, sampling date and weather conditions are shown in Appendix 12.

3.2.1 Occurrence of glyphosate and AMPA

Glyphosate and its metabolite AMPA were found at the estuarine stations during the study period. As seen in Figure 3.23, all selected estuarine sampling sites were contaminated by the metabolite AMPA and nine of them by the parent herbicide glyphosate. The concentrations of AMPA ranged from 45 to 4156 ng/L, while glyphosate concentrations ranged from 28 to 1690 ng/L. The highest concentrations were observed at sampling sites (7 and 8) for glyphosate and at sampling site (7) for AMPA. Glyphosate and AMPA were observed at the estuarine stations under different weather conditions. Therefore, their transport to the Baltic Sea is not restricted by their runoff from agricultural areas after rainfall events.



(**A**) (**B**)

Figure 3.23: Concentration ranges of (**A**) glyphosate and (**B**) AMPA in the ten investigated estuarine stations during the study period in 2012.

The Mühlenfließ estuary (sampling site 7) was observed to be the site highest contaminated by glyphosate and AMPA but it does not necessarily have the highest input of them into the Baltic Sea due to its low water flow rate (0.77 m^3/s) compared to bigger rivers (StALUMM). A waste water-treatment plant was found as a potential significant source of glyphosate and AMPA for surface water. They can be released from suspended particles during waste water processing (Botta et al., 2009; Popp et al., 2008). The constant high concentration levels of glyphosate and AMPA observed in the estuary of Mühlenfließ may be due to the discharge of sewage water from the treatment plant in the area of Bad Doberan into the surface water of Mühlenfließ or/and due to high agricultural and non-agricultural use of glyphosate in the Mühlenfließ basin.

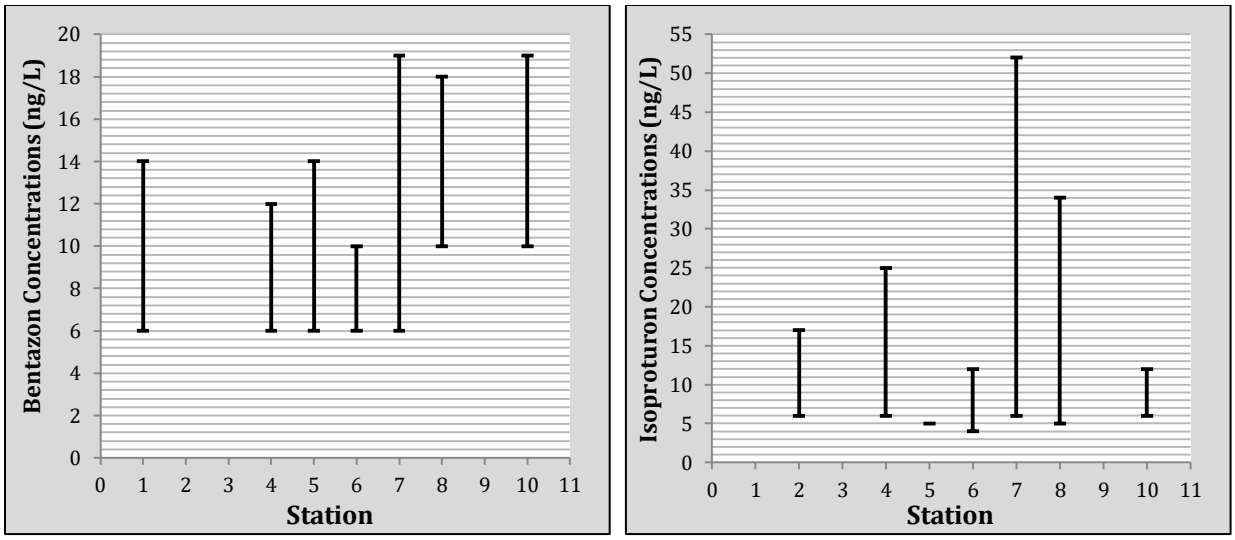
Frequencies of detection for the metabolite AMPA were higher than its parent herbicide glyphosate. Furthermore, the concentrations of AMPA were found to be higher than glyphosate concentrations in eight estuarine stations. This result can be explained by additional sources of the metabolite AMPA. As AMPA is not only the main metabolite of glyphosate, it is also the key metabolite formed through degradation of some phosphonates such as ATMP (aminotri-methylenephosphonic acid), EDTMP ((ethylenediaminetetra (methylene-phosphonicacid)), HDTMP (Hexamethylenediaminetetra-(methylenephos-phonate) and DTPMP (diethylenetri-aminepentamethylenephosphonic acid) used in laundry, detergents and industrial boilers and cooling media (Fürhacker et al., 2005; Jaworska et al., 2002).

Variations of glyphosate concentrations between different stations may result from different amounts of glyphosate applied and from different soil properties. Glyphosate

mobility is different from soil to soil and depends on soil properties (e.g. structure, clay content, iron dioxide, organic matter) and time of application (Vereecken, 2005). AMPA was detected in a frequency higher than glyphosate, indicating a higher mobility of AMPA compared to glyphosate. This result is in accordance with previous studies showing that the metabolite AMPA is more mobile than its parent herbicide glyphosate (Coupe et al., 2012). The reason for the observed high contamination by glyphosate and AMPA of the estuarine stations may not only be due to their surface runoff from agricultural areas, but also from urban regions (road and railway applications), where the use of glyphosate in urban areas has an essential impact on surface water contamination (Botta et al., 2009). Accordingly, the herbicide glyphosate and its metabolite AMPA were observed to have the highest detection frequency and highest concentrations. Unfortunately, no data were available with respect to the fate and transport of glyphosate and AMPA to the marine environment.

3.2.2 Occurrence of isoproturon and bentazon

The herbicides isoproturon and bentazon were detected in the collected water samples between May and September under different weather conditions (Appendix 12). Isoproturon and bentazon were frequently detected at the estuarine sampling stations. From ten investigated estuarine stations, eight of them (1, 2, 4-8, 10) were contaminated with isoproturon and seven of them (1, 4-8, 10) with bentazon. The measured concentrations ranged from 3 to 34 ng/L of isoproturon and 5 to 19 ng/L of bentazon (Figure 3.24). Isoproturon is relatively persistent in the soil environment, being broken down by up to 40% three months after its application (El-Sebai et al., 2005). Bentazon is low in persistence in soil with a half-life in filed soil ranging from 3 to 21 days and with an average of less than two weeks (Huber and Otto, 1994). Accordingly, the observed low concentrations of isoproturon and bentazon can be explained due to small amounts applied in Mecklenburg-Vorpommern and/or fast degradation in the soils of Mecklenburg-Vorpommern. These results obtained in this study illuminate that isoproturon and bentazon can be transported via most Mecklenburg-Vorpommern rivers and streams into the Baltic Sea but their transport was observed to be relatively low compared to glyphosate and AMPA.



(**A**) (**B**)

Figure 3.24: Concentration ranges of (**A**) bentazon and (**B**) isoproturon in the ten investigated estuarine stations during the study period in 2012.

Comparing these results to those of other areas such as Mediterranean deltas, German Baltic estuaries were highly contaminated with the herbicide isoproturon. Its concentrations in the Mediterranean deltas were below 10 ng/L in Ebro and Nile and it was undetectable in Rhone. On the other hand, the Mediterranean Ebro delta and the lagoons of the Rhone river (France) were highly contaminated with the herbicide bentazon in comparison to the studied German Baltic estuaries with a maximum concentration of bentazon of 1 µg/L in the Ebro delta and even higher than 1 µg/L in the lagoons of the Rhone river (Comoretto et al., 2007; Readman et al., 1995).

3.2.3 Occurrence of MCPA and mecoprop

The herbicides MCPA and mecoprop were detected in the water samples collected between May and August 2012. MCPA was detected in 4 sampling sites (1, 4, 7, 9). Obtained results for sampling site 1 are below the LOQ of the analytical method of 50 ng/L. Therefore, data are presented only for stations 4, 7 and 9 (Figure 3.26). Mecoprop was detected in sampling sites 1, 7 and 8, but below the LOQ of the analytical method of 50 ng/L. MCPA was measured under different weather conditions. Its maximum concentration of 747 ng/L was detected in a sample collected from Mühlenfließ estuary after a heavy rain event (Figure 3.25). These results indicate that MCPA and mecoprop are used only in some regions of Mecklenburg-Vorpommern and their low frequencies of detection may be explained due to small amounts applied or due to their high degradation rate. The detected concentrations of the herbicide mecoprop in the investigated German Baltic estuaries were in the same

range as those detected in the Ebro river delta. On the other hand, the German Baltic estuaries were less contaminated with the herbicides MCPA compared to the Ebro delta. The highest measured concentration of MCPA in the Ebro delta was 13.90 µg/L (Kuster et al., 2008).

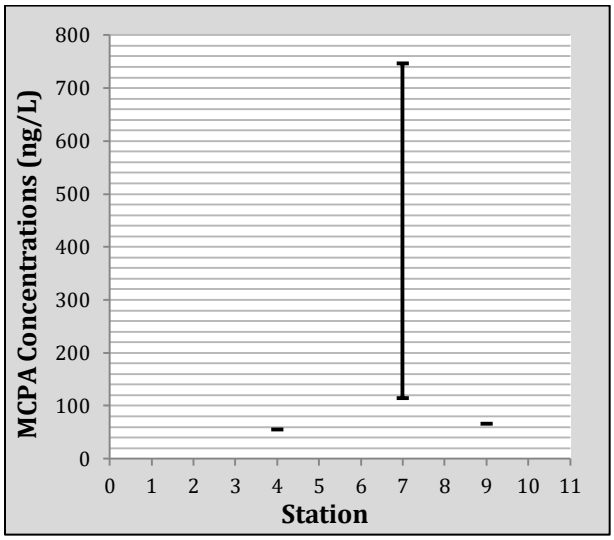


Figure 3.25: MCPA concentration ranges in the ten investigated estuarine stations during the study period in 2012.

However, high concentration of MCPA measured in the Mühlenfließ estuary may be due to rainfall that occurred after applying large amounts of MCPA in this area and the sewage treatment plant effluent which flows into the Mühlenfließ increases those concentrations. Many groups of organic contaminants (e.g. pesticides) can pass through sewage treatment plants, with entry to the system via storm water run-off and domestic or industrial sources (Kock-Schulmeyer et al., 2013). According to these results, transport of the herbicides MCPA and mecoprop into the Baltic Sea exists but their discharge amount is considered to be low compared to glyphosate and AMPA.

3.2.4 Occurrence of chloridazon and CMD

The herbicide chloridazon was not detected in any of the collected water samples. On the other hand, its metabolite CMD was detected in some of the estuarine sampling stations in May and June (Appendix 12). As seen in Figure 3.26, the metabolite CMD was quantified only in five water samples collected from two of the sampling sites (2 and 7). This result can be explained either by a low usage of chloridazon in the region of Mecklenburg-Vorpommern and consequently its transport into the Baltic Sea is in concentration levels lower than the limit of detection of the analytical method or there is no transport of chloridazon into the Baltic Sea due to its rapid degradation in soil, where the persistence of chloridazon in soil has been estimated to range from 13 days to 8 weeks (half-life)

depending on soil type (Buttiglieri et al., 2009). The measured concentrations of CMD ranged from 28 to 112 ng/L. The highest concentration of CMD of 112 ng/L was measured in the estuary of the Mühlenfließ after a rainfall event. According to these results, there is no important discharge of the herbicide chloridazon and its metabolite CMD into the Baltic Sea through the investigated rivers and streams. In different areas of Europe (Carafa et al., 2007) chloridazon was a frequently detected herbicide in Sacca di Goro lagoon and Adriatic Sea water samples with maximum concentrations of 102 ng/L and 50 ng/L, respectively.

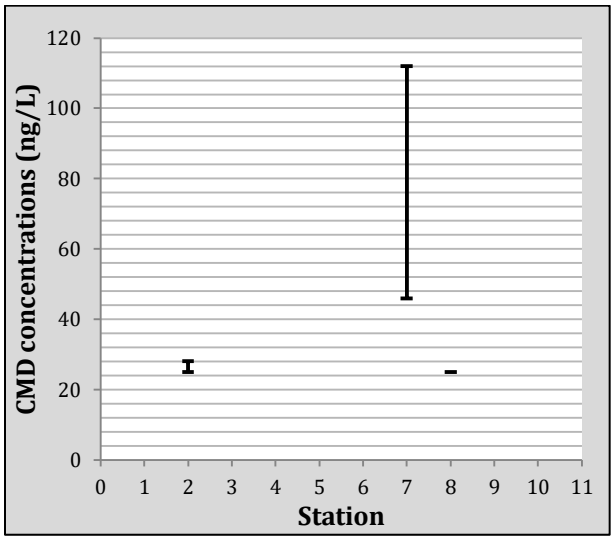


Figure 3.26: Concentration ranges of the metabolite CMD in the ten investigated stations during the study period in 2012.

A comparison of these results to those reported by the Agency for the Environment, Nature Conservation and Geology Mecklenburg-Vorpommern (LUNG) on the occurrence of pesticides in fresh waters of Mecklenburg-Vorpommern (Bachor et al., 2008) is shown in Appendix 13. However, the highest concentrations of the target compounds were found at areas not investigated by LUNG such as Mühlenfließ and Hellbach.

3.2.5 Detection frequencies of the selected compounds in the German Baltic estuaries

All estuarine stations were contaminated with at least one of the target compounds and maximally 7 of them. Figure 3.27 shows frequencies of detection of the target compounds in the collected estuarine water samples. The metabolite AMPA was the most detected compound in the estuarine water samples with 93% followed by bentazon, glyphosate and isoproturon with more than 70%. Low frequencies of detection of less than 23% were observed for the compounds MCPA, mecoprop and CMD. The herbicide chloridazon was not detected in any of the collected samples.

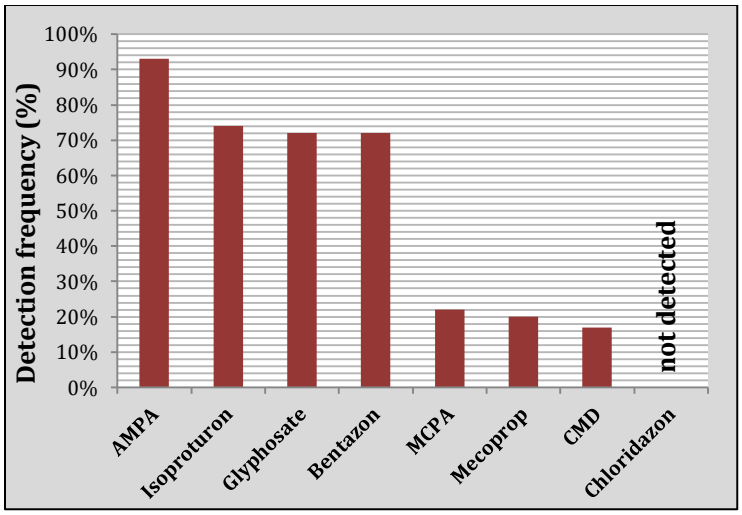


Figure 3.27: Detection frequencies of the herbicides glyphosate, isoproturon, bentazon, MCPA, mecoprop and chloridazon and their two metabolites AMPA and CMD in the collected estuarine water samples during the study period in 2012.

As seen in Figure 3.28, concentrations of the herbicides glyphosate and MCPA and the two metabolites AMPA and CMD exceeded the European quality standard for pesticides of 100 ng/L (Loos et al., 2010). The herbicide glyphosate and its metabolite AMPA were detected in the estuarine water samples at concentration levels exceeding this threshold value by 41% and 65%, respectively. Glyphosate and AMPA concentrations above 100 ng/L were observed in the sampling sites (1, 3, 4, 7, 8) and (1, 4-10), respectively.

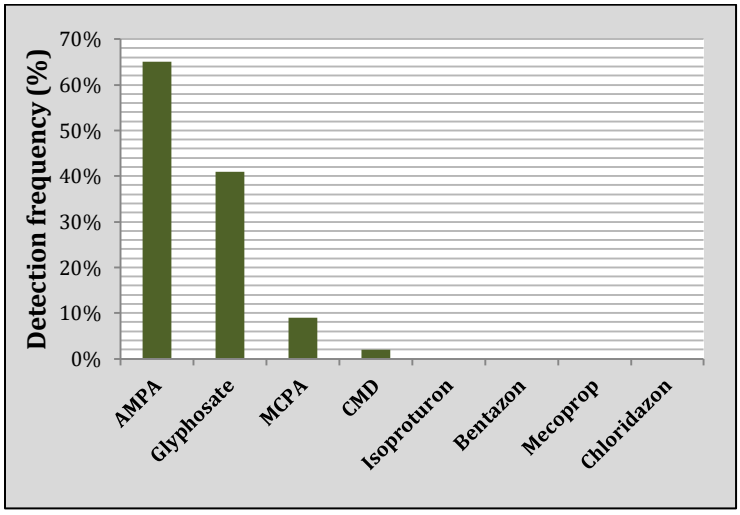


Figure 3.28: Detection Frequencies of the studied compounds with concentrations > 100 ng/L.

Glyphosate was always detected at concentration levels above 100 ng/L in sampling sites (7 and 8) and often in sampling site (4). AMPA exceeded the threshold value of 100 ng/L in all water samples collected from sites (1, 7, 8, 10) and in most samples collected from sites

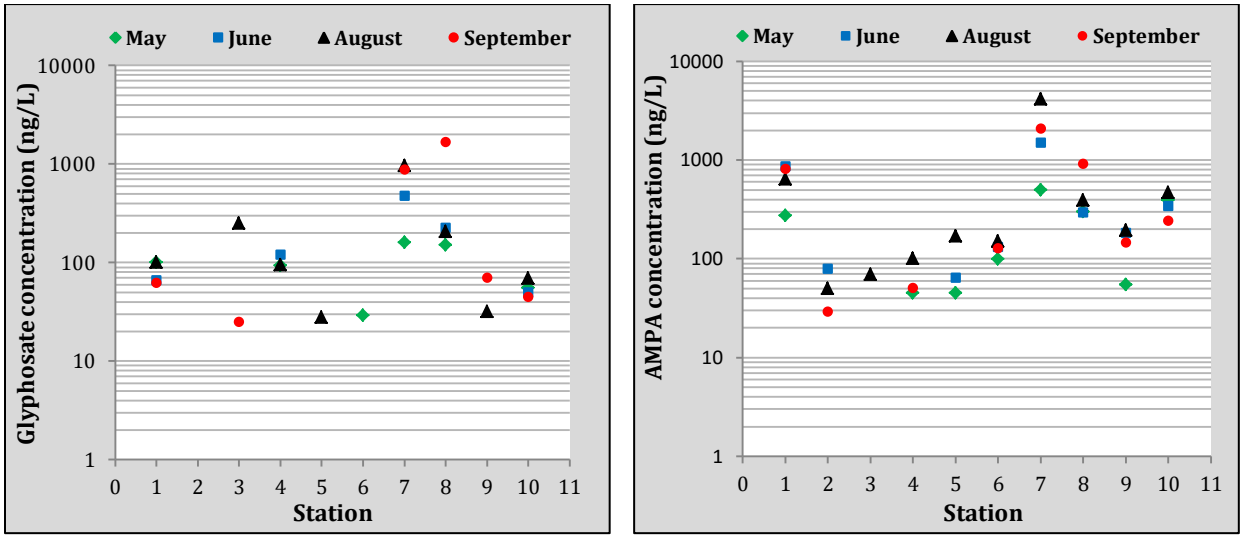
(6 and 9). Glyphosate was found at high concentration levels in the µg/L range in sites (7 and 8) while AMPA was found in this range in sampling site (7). The herbicide MCPA and the metabolite CMD were detected over the threshold value of 100 ng/L only twice and once, respectively. These concentrations of MCPA and CMD were observed in sampling site (7) after a rainfall event. According to these results, some rivers and streams in the federal state of Mecklenburg-Vorpommern are highly contaminated with the herbicide glyphosate and its metabolite AMPA, whereas they are less contaminated with other selected compounds. The sampling site of Mühlenfließ is the station highest contaminated with a high frequency of detection and high concentrations of the compounds glyphosate, AMPA, MCPA and CMD. The high contamination in the Mühlenfließ can be explained by high agricultural activities in this area or by the contribution of sewage treatment plants increasing the overall contamination level.

3.2.6 Spatial and time variations in transport of compounds with high detection frequencies to the Baltic Sea

As mentioned above, the metabolite AMPA and the three herbicides glyphosate, bentazon and isoproturon were detected over 70% in the estuarine water samples. In this study, a great spatial variation of glyphosate concentrations was registered during the survey period specially when the concentrations measured in the stream estuarine stations was compared with those measured in the estuaries of riverine stations (Figure 3.29(A)). Glyphosate concentrations were measured at microgram per liter levels in a few collected samples from the stream estuaries but its concentration did not exceed the nanogram per liter range in the river estuaries. The mean concentration of glyphosate at the estuaries of Mühlenfließ and Hellbach was 665 ng/L and 561 ng/L, respectively. On the other hand, their concentration in the river estuaries ranged between 10 and 82 ng/L. At most stations (3, 5, 7-10) the highest concentrations of glyphosate were observed in the end of summer (August) and in the first of autumn (September) because glyphosate is generally used at the agricultural areas as a post-emergent herbicide (Cox, 2004). At stations 1 and 10, glyphosate was found in the four studied months with very low monthly variations. Detection of glyphosate in May and June could be resulting from its use for non-agricultural purposes such as roadsides, railway tracks, industrial areas (Cox, 2004; Miller et al., 2010).

As shown in Figure 3.29(B), clear spatial variations of AMPA concentrations were observed, especially between station (7) and other stations. The mean measured concentration of AMPA was 1445 ng/L at station (7) and ranged from 360 ng/L to 650 ng/L in stations (1, 8, 10) and between 145 ng/L and 17 ng/L in stations (2-6, 9). The half-life of glyphosate in soil is varies from a few days to several years depending on the adsorption process and the level of microbial activity (Carlisle and Trevors, 1987). As in the case of glyphosate, the highest concentration of AMPA was observed in August and/or

September at most investigated stations (3-10) which reflect fast degradation of glyphosate in the soil of Mecklenburg-Vorpommern. The lowest concentrations of AMPA were found in May showing lower glyphosate activities in spring.



(A) **(B)**

Figure 3.29: Spatial and time variation of glyphosate (**A**) and AMPA (**B**) transport to the Baltic Sea through the ten investigated estuarine stations in May, June, August and September 2012.

However, it is difficult to assess the factors which cause these variations among different stations because many factors can lead to these observed variations. For example, application methods of glyphosate, dosage (agricultural or non-agricultural source), soil characteristics, weather conditions, behavior of glyphosate and AMPA during the transport process in rivers and estuaries, geological conditions and the mixing ratio of the contaminated freshwater with less contaminated seawater strongly influence the transport process.

In order to study the time variations in transport glyphosate and AMPA into the Baltic Sea through one station, nine water samples were collected between May and September from the estuary of Mühlenfließ (station 7). The variations in AMPA concentration were higher than that observed for glyphosate (Figure 3.30). In eight of the measured samples AMPA concentrations were higher than those of glyphosate could be due a higher mobility of AMPA than glyphosate as well as the additional source of AMPA such as the phosphonates. The highest concentration for both compounds was measured after rainfall events. Many factors can led to this variation such as weather conditions, the applied amount of glyphosate, an urban source of glyphosate and AMPA, the flow rate of rivers and stream and the dilution with the Baltic Sea. The observed variation reflects fluctuations of transport of glyphosate and AMPA through the German rivers and streams into the Baltic Sea.

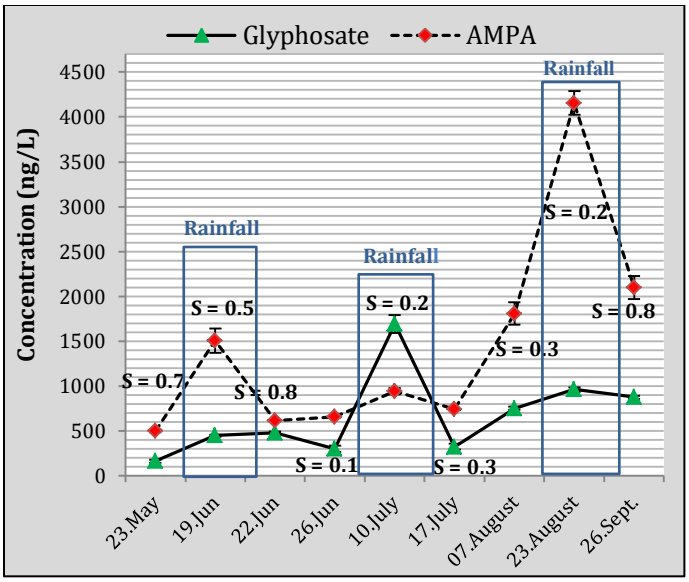
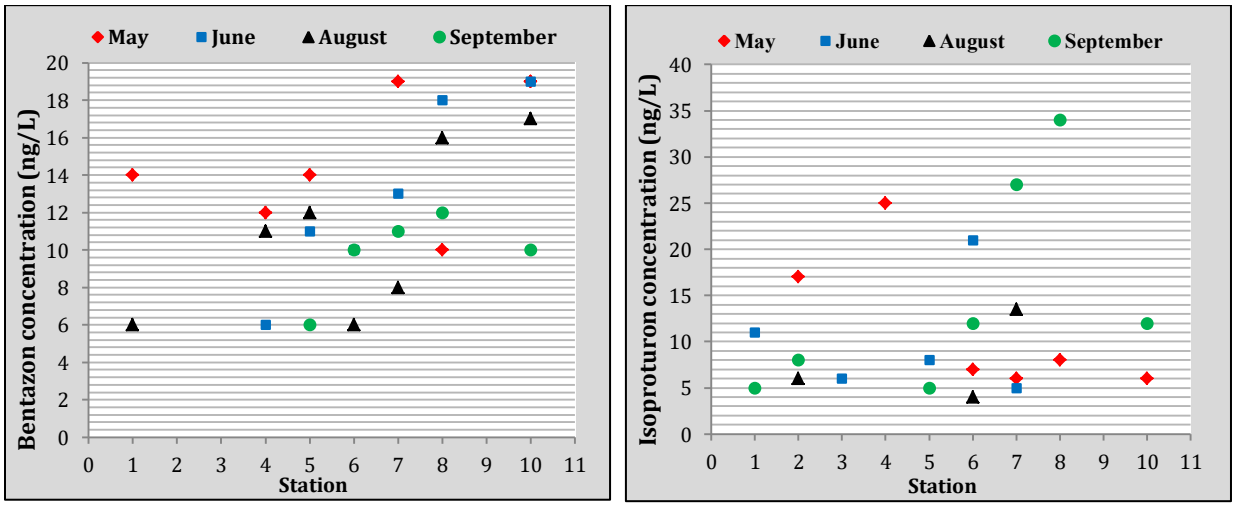


Figure 3.30: Time variation of glyphosate and AMPA transport to the Baltic Sea through the estuary of Mühlenfließ between May and September in 2012.

As shown in Figure 3.31, small differences in the measured concentrations were observed among the stations for the herbicides bentazon and isoproturon. The mean concentration of bentazon in the investigated estuarine stations ranged from 1 ng/L to 16 ng/L and for isoproturon from 2 ng/L to 23 ng/L. The highest concentrations of isoproturon were observed in September in stations (1,5,6,8,10) and in May in stations (2,3) because of its application as a pre- and post-emergence herbicide (Mallat et al., 2001; Paris-Palacios et al., 2010).



(A) **(B)**

Figure 3.31: Spatial and time variation of (**A**) bentazon and (**B**) isoproturon transport to the Baltic Sea through the ten investigated estuarine stations in May, June, August and September 2012.

Bentazon is commonly used as an early post-emergence herbicide, therefore its highest concentration was found in May in stations (1,4,5,7,10) and its lowest concentration was observed in September in stations (1,4,5,10). According to these data, the spatial and monthly variation of the herbicides bentazon and isoproturon were minor compared to those of glyphosate and AMPA.

3.2.7 Fate of glyphosate and AMPA

In order to study the fate of the herbicide glyphosate and its metabolite AMPA, i.e. establish the relationship between their concentrations and salinity, water samples were collected from four different points with four different salinity values. The sampling stations were distributed along Mühlenfließ starting from a fresh water point (i.e. salinity 0.4) and ending in the Baltic Sea (salinity 12). As shown in Figure 3.32, glyphosate and AMPA concentrations decreased with increasing salinity. The measured concentrations of glyphosate and AMPA at a salinity value of 0.4 (3.5 km away from the mouth) were 2436 ng/L and 4434 ng/L, respectively. A significant decrease in the measured concentration of both compounds (over 50%) was found with an increasing salinity up to 1.7 (60 m away from the mouth) and over 62% with increasing salinity up to 1.9 in the mouth of Mühlenfließ. Glyphosate and AMPA were not detected in the Baltic Sea water sample which was collected 4 meters away from the mouth in a salinity of 12 due to the mixing of fresh water of Mühlenfließ and the less contaminated Baltic Sea water and consequently their concentrations are lower than the limits of detection of the method.

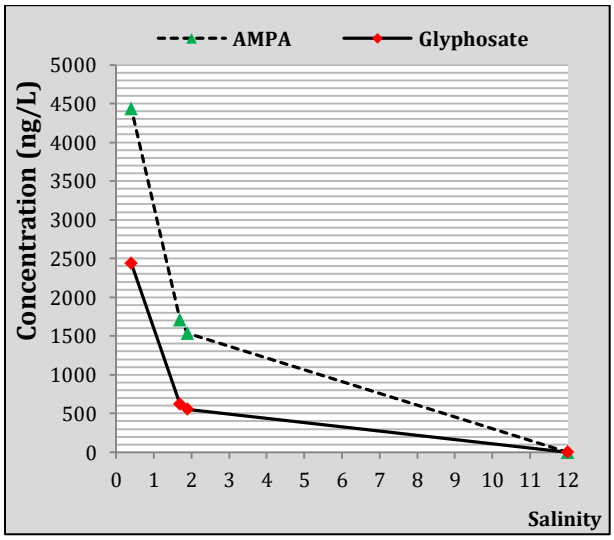


Figure 3.32: Mixing plot of glyphosate and AMPA in Mühlenfließ.

Compared to results from another study conducted on the fate of other pesticides such as diuron, atrazine and monuron, this result supports the previous study by also showing a decrease in pesticide concentration with an increasing salinity value (Steen et al., 2000). On the other hand, and according to our study, a decrease in glyphosate and AMPA concentrations was observed to be very high with a small increase in salinity. In addition to the dilution with sea water, the adsorption of compounds onto particles and subsequent sedimentation could be a reason for this significant decrease of concentration values.

3.2.8 Occurrence of the selected compounds in the German Baltic coast

Water samples were collected from the German Baltic coast in Heiligendamm (salinities > 9) between May and September, at a rate of two samples monthly, in order to study the potential occurrence of the selected herbicides and metabolites at the Baltic coast. None of the target analytes were detected in the collected water samples.
According to the transport data of the selected herbicides and metabolites, no detection of the herbicides chloridazon, MCPA and mecoprop and the metabolite CMD was expected because of the low and/or non-discharge into the Baltic Sea through German rivers. The herbicides isoproturon and bentazon were detected at high frequencies in the studied estuarine stations but their concentrations were lower than the low-ng/L level. Therefore, their occurrence at the Baltic Sea coast is expected to be at a concentration level lower than their limits of detection for the method used due to the dilution with the Baltic Sea.
In spite of the high detection frequency and comparatively high concentrations of the herbicides glyphosate and its metabolite AMPA in the estuarine stations, these compounds were not detected in water samples taken from the coast of the Baltic Sea. Several reasons may attribute to this result: glyphosate and AMPA could be present in the Baltic Sea in concentrations lower than the limit of detection of the analytical method, fast degradation, sedimentation, uptake by Baltic Sea plants or utilization by some microorganisms as the sole source of carbon, phosphorus or nitrogen.

In this section, the occurrence of six polar herbicides, glyphosate, isoproturon, bentazon, MCPA, mecoprop and chloridazon and two of their metabolites AMPA and CMD in the Baltic estuaries was examined by the analysis of water samples collected from ten German Baltic estuarine stations between May and September in 2012. The presence of the selected compounds in the Baltic estuaries was used as confirmation for their transport into the Baltic Sea.

All the target compounds were detected at the estuarine stations except for the herbicide chloridazon. The metabolite AMPA was the compound most detected at the estuarine stations followed by the herbicides isoproturon, glyphosate and bentazon. The metabolite

AMPA and its parent herbicide glyphosate were observed to have the highest detection frequency and concentrations ranged from a few nanograms per liter to a few micrograms per liter in some stations. Therefore, they can be considered to be the most important compounds transported through the German Baltic Estuaries into the Baltic Sea compared to other selected compounds in this study.

Even though isoproturon and bentazon were detected in many estuarine water samples, their highest measured concentrations were 52 ng/L and 19 ng/L, respectively, and consequently their transport into the Baltic Sea was considered to be less compared to glyphosate and AMPA. The compounds MCPA, mecoprop and CMD were detected at small values (< 23%) in the collected water samples. MCPA and CMD were quantified in some water samples, whereas the measured concentrations of mecoprop were below the LOQ of the analytical method of 50 ng/L. The highest concentrations of MCPA and CMD of 747 ng/L and 121 ng/L, respectively, were observed after a rainfall event. No or low transport (i.e. below the LOD of the analytical method of 10 ng/L) of the herbicide chloridazon into the Baltic Sea through the investigated stations was observed.

Clear local and temporal variations in transport of the herbicide glyphosate and its metabolite AMPA were observed between different stations and also in the same station over time. On the other hand, these variations were observed to be low for the herbicide isoproturon and bentazon. The compounds glyphosate, AMPA, isoproturon, bentazon, MCPA, mecoprop and CMD were found to be transported into the Baltic Sea under different weather conditions. AMPA was observed to be more mobile with higher transport rates after rainfall events as compared to glyphosate, whereas the difference in transport of bentazon and isoproturon in both wet and dry weather was negligible.

The two herbicides glyphosate and MCPA and the two metabolites AMPA and CMD were detected at high concentration levels exceeding the European ground water quality standard for pesticides of 0.1 µg/L. The frequencies of detection of AMPA and glyphosate at concentrations over 0.1 µg/L were 65% and 41%, respectively, followed by MCPA and CMD of 9% and 2%, respectively. The concentrations of the herbicides isoproturon, bentazon and mecoprop did not exceed this threshold. Regarding this threshold, the surface water in Mecklenburg-Vorpommern is highly contaminated with AMPA and glyphosate.

A significant decrease in glyphosate and AMPA concentrations were observed from the fresh water toward the Baltic Sea. None of the studied herbicides and metabolites was detected in the coast of the Baltic Sea in Mecklenburg-Vorpommern.

3.3 Response of *Nodularia spumigena* to the herbicide glyphosate (Roundup®) and its metabolite AMPA

A potential adverse impact of chemical pressure on coastal and estuarine marine microalgae is of major concern. Glyphosate and its metabolite AMPA were first detected in the German Baltic estuaries (this thesis, section 3.2.1). For a long period, Glyphosate was believed to be an "environmentally friendly" herbicide (Williams et al., 2000). However, many recent studies have given evidence that glyphosate and its formulations can have negative effects on aquatic organisms such as algae (Tsui and Chu, 2003), plants (Sobrero et al., 2007), invertebrates (El-Shenawy et al., 2009), and vertebrates (Glusczak et al., 2007; Salbego et al., 2010).

Glyphosate is usually used in different trade formulations with glyphosate being the basic ingredient in products such as Roundup®, Rodeo®, Vision®, Glyphos®, Duramax®, Durango®, etc. Roundup®, which includes surfactants, is the most common commercial name (Pérez et al., 2011). Due to the hydrophilic characteristic of glyphosate, its diffusion across the hydrophobic bilayers is limited. The addition of surfactants is to improve glyphosate transport into the plant tissue (Riechers et al., 1994). In general, the commercial formulations of glyphosate (e.g. Roundup®) have shown a higher toxicity to aquatic organisms than the technical glyphosate alone (Cedergreen and Streibig, 2005; Sobrero et al., 2007; Tsui and Chu, 2003). For example, Roundup® was approximately 4 times more toxic to the aquatic plant *Lemna minor L.* and the green alga *Pseudokirchneriella subcapitata* than the technical glyphosate (Cedergreen and Streibig, 2005). Another study conducted on the comparison of the sensitivities of different organisms to the herbicide glyphosate and some of its formulations has shown that Roundup® is approximately 4 times more toxic to *Selenastrum capricornutum*, 20 times to *Acartia tonsa*, 22 times to *Tetrahymena pyriformis* and 27 times to *Ceriodaphnia dubia* than technical glyphosate (Tsui and Chu, 2003).

Aquatic plants and microalgae are usually more sensitive to the herbicide glyphosate than other organisms such as bacteria, protozoa, invertebrates, fish and amphibians due to the mode of action with which glyphosate interferes with plant metabolisms (Pérez et al., 2011). Glyphosate controls plants by inhibiting the activity of the enzyme 5-enolpyruvyl-shikimic acid-3-phosphate synthase (EPSP) which is involved in the biosynthesis of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine, (Carlisle and Trevors, 1987; Miller et al., 2010; Steinrticken and Amrhein, 1980). Cyanobacteria are a group of prokaryotes which have the same photosynthetic mechanism as eukaryotic algae

and higher plants (Lipok et al., 2010). In a microcosms study on the influence of Roundup® on natural marine microbial communities it was demonstrated that Roundup® can disturb this ecosystem even at concentration of 1 µg/L (Stachowski-Haberkorn et al., 2008). However, still only little is known about the toxicity of the herbicide glyphosate and its metabolite AMPA to the marine algae especially blue-green algae.

It was shown that there are considerable differences in sensitivity in cyanobacteria to the herbicide glyphosate. For instance, growth of the cyanobacteria *aphanocapsa 6308, anabaena variabilis, nostoc strain mac* was completely inhibited when they were exposed to a glyphosate concentration of 10 mg/L, while the glyphosate concentration required for the inhibition of growth of *aphanocapsa 6714* was more than 100 mg/L (Hutber et al., 1979). Lipok et al. (2010) reported that the cyanobacteria *Anabaena catenula*, *Synechocistis aquatilis*, *Microcystis aeruginosa* and *Leptolynbya boryana* showed sensitivity to the herbicide glyphosate when they were exposed to a concentration of 0.07 mM, while *S. (Arthrospira) platensi*, *Arthrospira fusiformis* and *Nostoc punctiforme* were tolerant to the exposure to the same concentration of glyphosate.

Nodularia spumigena (*N. spumigena)* is a filamentous and heterocystous cyanobacterium commonly observed in brackish water (Voss et al., 2013). *N*. *spumigena* is one of the dominant cyanbacteria observed during the summer bloom in the Baltic Sea (Stal and Walsby, 2000; Wasmund, 1997). Mass occurrence of *N*. *spumigena* has a negative effect on human (e.g. tourism, recreation and fisheries) and on the aquatic ecosystem because it produces nodularin, a potent cyclic pentapeptide hepatotoxin, which can cause death in some organisms when it is present in high dosages (Grondahl, 2009; Mazur-Marzec et al., 2007; Stolte et al., 2002). However, *N*. *spumigena* as a nitrogen-fixing cyanobacterium plays an important role as primary producers by introducing both new carbon and nitrogen into the ecological system (Vintila et al., 2010).

The effect of both glyphosate in its formulation Roundup® and the metabolite AMPA on the growth of the non-target cyanobacterium *N. spumigena* was tested in this study. Three parameters Chl-*a*, total cell number and POC were measured to determine *N. spumigena* growth rates in the presence of various concentrations of Roundup® and AMPA (1, 10, 50, 100, 500 µg/L). Maximum tested concentrations of both toxicants in this study were 500 µg/L because higher than these concentration levels are not expected to occur in estuaries and in the marine environment due to the dilution process. Figure 3.33 presents the time changes in Chl-*a* content of *N. spumigena* cultures when exposed to different concentrations of Roundup® and AMPA.

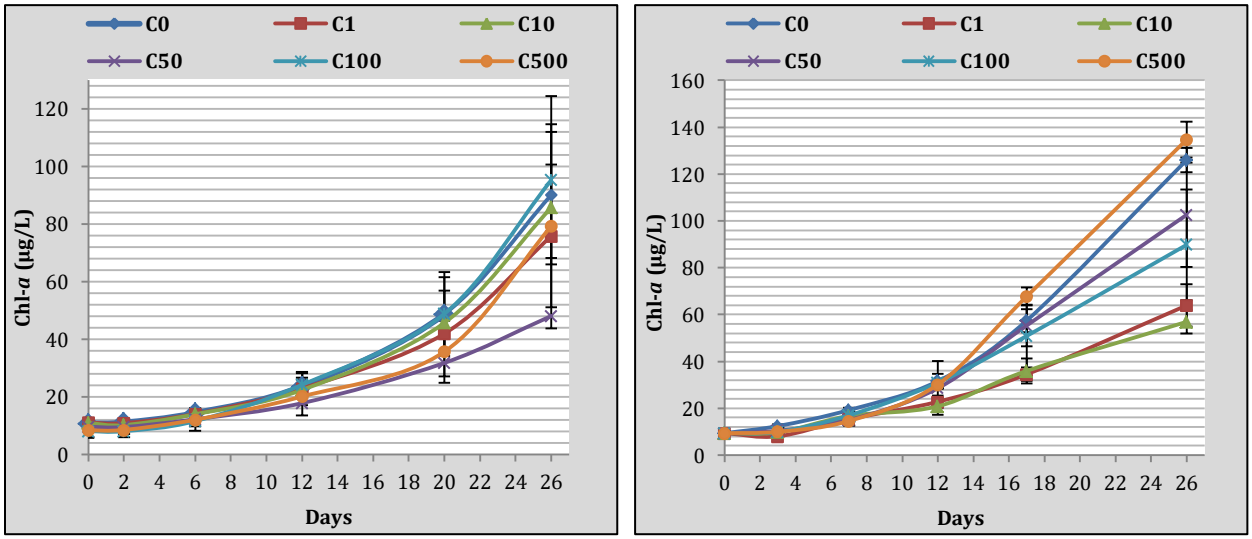
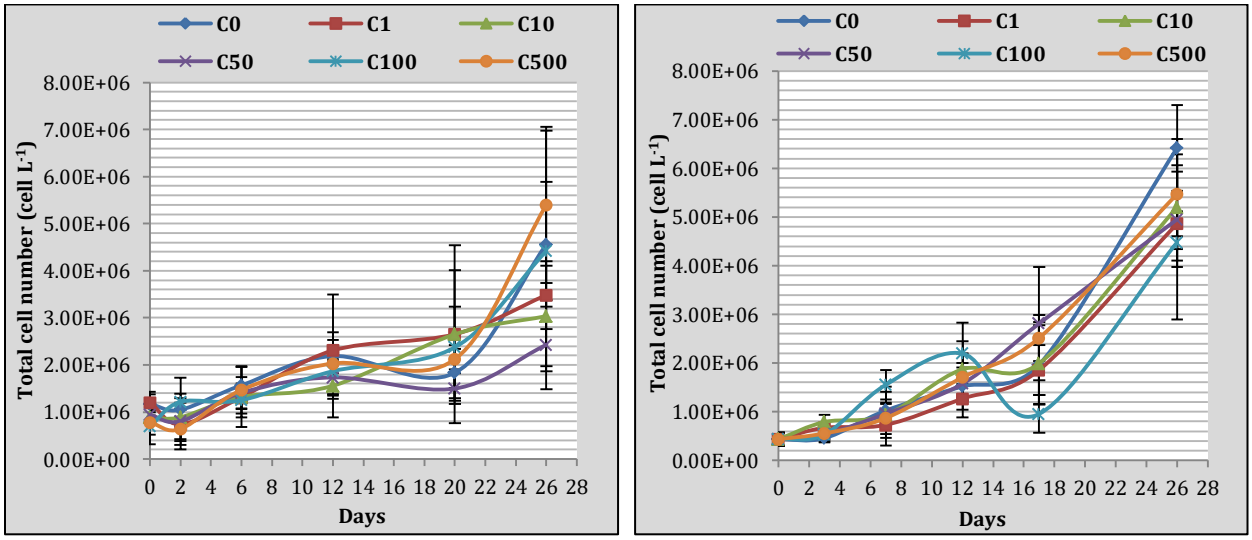


Figure 3.33: The Chl-*a* concentrations of control (C0) and treated cultures under different concentrations (C = 1, 10, 50, 100 and 500 µg/L) of (**A**) Roundup® and (**B**) AMPA during the toxicity experiment.

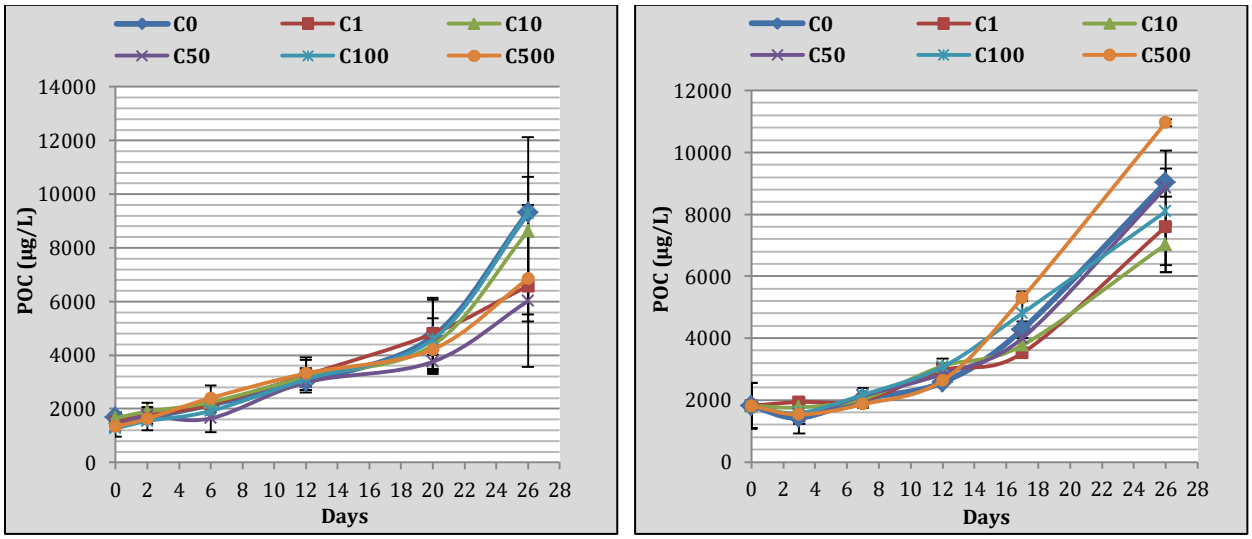
The results show that *N. spumigena* cultures treated with concentrations of Roundup® and AMPA between 1-500 µg/L produced a similar amount of Chl-*a* content as their controls. Increases in Chl-*a* content were observed in the cultures exposed to Roundup® and AMPA during the toxicity test period. At the highest exposure concentration of 500 µg/L, the Chl-*a* content in the culture exposed to Roundup® increased almost eightfold from 8.8 ± 1.9 µg/L (mean ± SD) on day 0 to 79.2 ± 35.4 µg/L on day 26 and increased almost fourteen-fold from 1.0 ± 0.8 µg/L on day 0 to 134.7 ± 7.7 µg/L on day 26 in case of AMPA. Slight increases in Chl-*a* content was observed during the first week in cultures exposed to Roundup® and AMPA. In the case of AMPA, low growth of *N. spumigena* was observed at cultures treated with the low tested concentration levels of 1 and 10 µg/l compared to their controls. This may be due to variations of light intensities and other inexplicable reasons. However, based on the highest exposure concentrations of 500 µg/L Roundup® and AMPA, no effect on the Chl-*a* synthesis was observed in regards to the tested concentrations of both compounds. This result does not mean that Roundup® and AMPA can't impact Chl-*a* synthesis of *N. spumigena* because the toxicity of chemicals depends on the dosage generally. Qiu el al. (2013) showed that growth of *Microcystis aeruginosa* based on Chl-*a* content was not affected when it was exposed to Roundup® concentrations below 1 mg P L^{-1} but it was significantly inhibited at exposure concentration of 5 mg P L^{-1} of Roundup®, for instance.
Based on the cell densities data, total cell numbers had increased in cultures treated with different concentrations of Roundup® and AMPA during the toxicity test of 26 days (Figure 3.34). In the case of 500 µg/L Roundup® exposure, the total cell number rose from 0.8 x

10^{+6} ± 0.3 x 10^{+6} cell L^{-1} on day 0 up to 5.4 x 10^{+6} ± 1.7 x 10^{+6} cell L^{-1} on day 26, showing almost a sevenfold increase. In the control culture on day 26, the total cell number increased up to 4.6 x 10^{+6} ± 1.3 x 10^{+6} cell L^{-1}.



(A) **(B)**

Figure 3.34: The total cell number of control (C0) and treated cultures under different concentrations (C = 1, 10, 50, 100 and 500 µg/L) of (**A**) Roundup® and (**B**) AMPA during the toxicity experiment.



(A) **(B)**

Figure 3.35: Time-related changes in POC of control (C0) and treated cultures with different concentrations (C = 1, 10, 50, 100 and 500 µg/L) of (**A**) Roundup® and (**B**) AMPA during the toxicity experiment.

In the case of 500 µg/L AMPA treatments, the total cell number increased from 0.4 x 10^{+6} ± 0.1 x 10^{+6} cell L^{-1} on day 0 up to 5.5 x 10^{+6} ± 1.1 x10^{+6} cell L^{-1} on day 26, showing almost a twelvefold increase. In the control culture on day 26, the total cell number increased up to 6.42 x 10^{+6} ± 0.88 x 10^{+6} cell L^{-1}. At the end of the experiment on day 26, there were no significant differences between cultures treated with 500 µg/L Roundup® and its control and also between cultures treated with 500 µg/L AMPA and its control, therefore statistical tests were not performed. The increase in the total cell number during the toxicity test was verified by POC data (Figure 3.35). No effect on cell densities was observed with increasing the toxicants concentrations to up to 500 µg/L level. Based on the results obtained in the present study, *N. spumigena* showed a high degree of tolerance to the herbicide Roundup® and the metabolite AMPA in regards to the tested concentrations in the range of 1-500 µg/L. Even though there are many studies on the contamination of AMPA in the environment (Coupe et al., 2012; Scribner et al., 2007), there is still a scarcity of literature on its toxicity on microorganisms, especially blue-green algae. Therefore, the general effect of AMPA on *N. spumigena* was difficult to be predicted. Cyanobacteria have shown remarkable adaptation to different kind of chemicals (Powell et al., 1991). Growth of the cyanobacteria *Spirulina platensis*, *Arthrospira fusiformis*, *Nostoc punctiforme*, *Leptolynbya boryana* and *Synechocystis aquatilis* was unaffected when they were exposed to Roundup® at concentration levels in the microgram per liter range (Lipok et al., 2010). Qiu el al. (2013) reported that Roundup® did not affect growth of *Microcystis aeruginosa* based on Chl-*a* content and cell density at concentration levels below 1mg P L^{-1}. Due to the ability of many cyanobacteria to adapt to the herbicide Roundup® stress and due to the relatively low tested concentrations of Roundup® (maximum 500 µg/L), these results were relatively anticipated.

Measurement of Glyphosate concentrations in the Roundup® formulation failed using HPLC-MS/MS because of an analytical problem. The reason of the analytical problem was a presence of high concentrations of isopropylamine salt in the Roundup® formulation which required high concentrations of FMOC-Cl for achieving glyphosate derivatization which subsequently caused a precipitation on the reversed phase column. Furthermore, presence of high concentrations of surfactants (pelargonic acid) in the Roundup® formulation could be an additional reason for this problem. However, numerous cyanobacteria as *Spirulina platensis*, *Anabaena* sp., *Leptolyng bya boryana*, *Microcystis aeruginosa* and *Nostoc punctiforme* are found to be able to degrade glyphosate in aqueous mediums and use it as source of phosphorus for their growth (Forlani et al., 2008; Lipok et al., 2007). Tolerance of some cyanobacteria to the ingredient glyphosate can be related to different mechanisms such as the production of a glyphosate-tolerant enzyme, overproduction of EPSP synthase and the ability of a cyanobacterium to degrade glyphosate and use it as a phosphorus source (Forlani et al., 2008; Powell et al., 1991).

Based on the measurement of AMPA concentrations in all the treated cultures (Figure 3.36), no significant changes in the concentrations were observed during 19 days of the toxicity test. This was probably due to the existence of PO_4^{-3} in the f/2 media which have prevented the cleavage of the C-P bond. The presence of PO_4^{-3} inhibits degradation and the use of AMPA as a source of phosphorus (Balthazor and Hallas, 1986).

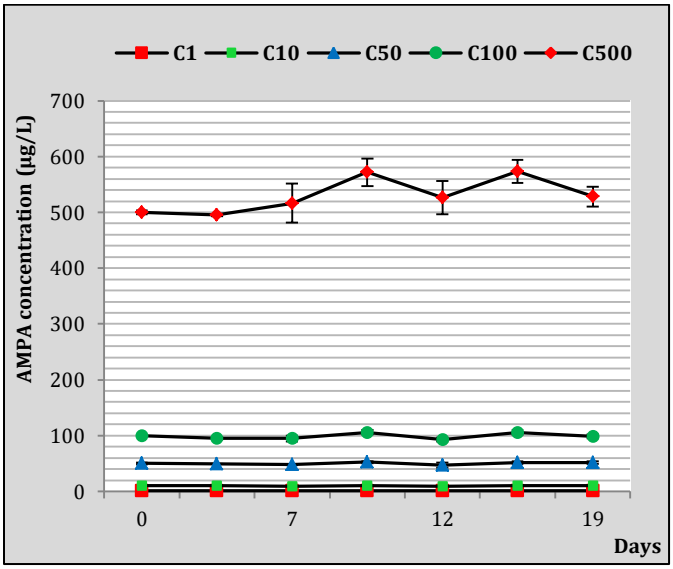


Figure 3.36: Change in AMPA concentrations (n=3) in the culture during 19 days of the toxicity experiment. The concentrations are C = 1, 10, 50, 100, 500 µg/L.

In order to study the ability of *N. spumigena* to degrade AMPA (i.e. estimation of the half time of AMPA) and to use it as source of phosphorus we suggest conducting the experiment in conditions of phosphorus limitation. Resistance of *N. spumigena* to the metabolite AMPA could be related to non-herbicidal activity of AMPA and/or to one of the resistance mechanisms of glyphosate. The results presented in this study provide new evidence on the tolerance of cyanobacteria to contaminants such as Roundup® and AMPA with respect to their tested dosages.

Nowadays there is limited information on the toxicity of the herbicide glyphosate (Roundup®) and its metabolite AMPA to marine algae. The 26 days growth of the marine blue-green algae *N. spumigena* based on Chl-*a*, cell densities and POC were unaffected when they exposed to concentrations levels 1-500 µg/L of Roundup® and AMPA. *N. spumigena* was unable to degrade AMPA in the f/2 medium during 19 days measurement. *N. spumigena* is added to the list of other cyanobacteria which showed tolerance to the herbicide Roundup® according to the treated concentrations such as *Chlorella vulgaris*, *Microcystis aeruginosa*, *Anabaena catenula*, Spirulina platensis, *Arthrospira fusiformis*, *Nostoc punctiforme*, *Leptolynbya boryana* and *Synechocystis aquatilis*. However, other types

of phytoplankton such as diatoms and dinoflagellates could be more sensitive to the herbicide Roundup® and maybe to its metabolite AMPA. Therefore, there is a need for further research in this field.

4. Conclusion and Outlook

The present thesis compared two analytical techniques GC-MS and HPLC-MS/MS for the analysis of six polar herbicides and three of their metabolites in water samples. The polar compounds are the six herbicides glyphosate, MCPA, mecoprop, isoproturon, bentazon and chloridazon and three of their metabolites aminomethylphosphonic acid (AMPA), chloridazon-desphenyl (CD) and chloridazon-methyl-desphenyl (CDM). As a result, HPLC-MS/MS was preferred over GC-MS for their analysis. GC-MS was found to be an unsuitable technique for their direct analysis. Moreover, it was found to be unable to achieve their analysis in one single run after their derivatization with the reagents TFAA and TFE. The technique HPLC-MS/MS was found to be suitable for the direct analysis of six of the substances. These compounds are MCPA, mecoprop, isoproturon, bentazon, chloridazon and CMD. Therefore, an analytical method was developed and validated for their direct analysis using HPLC-MS/MS. Due to the high polarity and water solubility of glyphosate and AMPA, they were unsuitable for the direct HPLC-MS/MS analysis but suitable after their derivatization with the reagent FMOC-Cl. Accordingly, another analytical method was developed and validated for the analysis of glyphosate and AMPA in water samples after their derivatization with FMOC-Cl. The metabolite CD was difficult to analyze by both techniques.

Both analytical methods were developed according to HPLC and MS/MS parameters in order to achieve the best chromatographic performance and detector sensitivity. Separation of both methods was achieved on a reversed phase column. The validation parameters included linearity, LODs and LOQs, precision, accuracy, matrix effect, analytes and system stability. Satisfied validation parameters were obtained as linearity, precision, accuracy. LODs and LOQs were at the low concentration level of nanograms per liter. A matrix effect problem was solved using the standard addition method.

The HPLC-MS/MS analytical methods were applied in order to study the occurrence of the selected compounds as evidence for their transport into the Baltic Sea. Water samples were collected from ten German Baltic Estuaries in 2012 between May and September and analyzed by HPLC-MS/MS. AMPA was often detected in the collected water samples (93%) followed by the herbicides isoproturon, glyphosate and bentazon over 70%. Frequency of detection of MCPA, mecoprop and CMD was less than 23%. The herbicide chloridazon was not detected in all estuarine water samples. The metabolite AMPA and its parent herbicide glyphosate had the highest frequencies of detection and concentration (up to µg/L in some

samples), therefore, they are considered to be the most important compounds transported through the German Baltic Estuaries into the Baltic Sea compared to other selected compounds in this study. All investigated sampling sites were observed to be contaminated with AMPA and nine of them with glyphosate.

The measured concentrations of isoproturon and bentazon lied in the range of 3-34 ng/L and 5-19 ng/L, respectively. MCPA was found in 4 sampling sites but in one of them at concentration levels below the LOQ of the analytical method of 50 ng/L. The maximum MCPA concentration measured was 747 ng/L. Mecoprop was detected in some sampling sites, but always below the LOQ of the analytical method of 50 ng/L. Detection frequencies of AMPA and glyphosate at concentration levels over 0.1 µg/L (the European ground water quality standard for individual pesticide) were 65% and 41%, respectively, following by MCPA and CMD of 9% and 2%, respectively. Mühlenfließ estuary was found to be the most contaminated sampling site compared to other investigated stations in this study. Urban origin such as wastewater treatment plants could be the main reason for the contamination of Mühlenfließ estuaries.

The compounds glyphosate, AMPA, isoproturon and bentazon were observed to be transported in wet and dry weather conditions. Clear local and temporal variations in transport of the herbicide glyphosate and its metabolite AMPA were observed. On the other hand, the variation was low for isoproturon and bentazon compared to glyphosate and AMPA. A significant decrease in glyphosate and AMPA concentrations were observed from the fresh water toward the Baltic Sea. The studied herbicides and metabolites were not analyzed in the coast of the Baltic Sea in Heiligendamm. However, further researches are required in order to study the fate of the detected contaminants in the Baltic Sea.

The effect of the herbicide Roundup® (the commercial formulation of glyphosate) and the metabolite AMPA on the growth of the marine cyanobacterium *Nodularia spumigena* was studied based on Chl-*a*, total cell number and POC. The algal cultures were grown in the presence of various concentrations of Roundup® and AMPA (1-500 µg/L). The incubation period was 26 days. As a result, no effect of both compounds on growth of *N. spumigena* regard to Chl-*a*, total cell number and POC was observed. *N. spumigena* showed a high degree of tolerance to the herbicide Roundup® and AMPA. *N. spumigena* was incapable to degrade AMPA according to the experimental conditions such as the presence of PO_4^{-3} which may inhibit its uptake and degradation.

For future investigations, the following suggestions could be of interest.

Study the role of the atmospheric precipitation in transport of glyphosate and AMPA into the Baltic Sea which was found to have a high adsorption capacity on the air particles.

Development of an extraction method for polar compounds from salt water (i.e. Baltic Sea samples).

Study fat and behavior of glyphosate and AMPA in the Baltic Sea as their degradation (i.e. half time), sedimentation and their utilization as a source of phosphorus and nitrogen for marine phytoplankton.

Study the toxicity of the detected compounds to different marine organism such as marine algae (e.g. diatoms and dinoflagellates) and plants, zooplankton and fish.

Study the potential transport of other polar contaminants classes into the Baltic Sea such as polar pharmaceutical.

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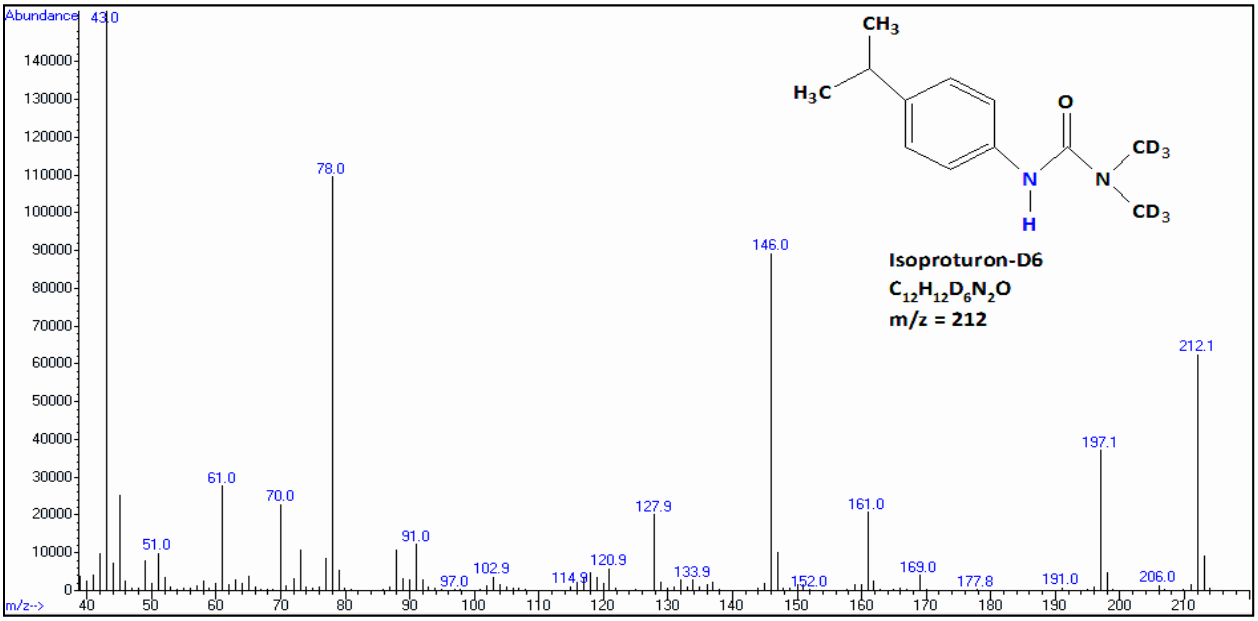
Abbreviations

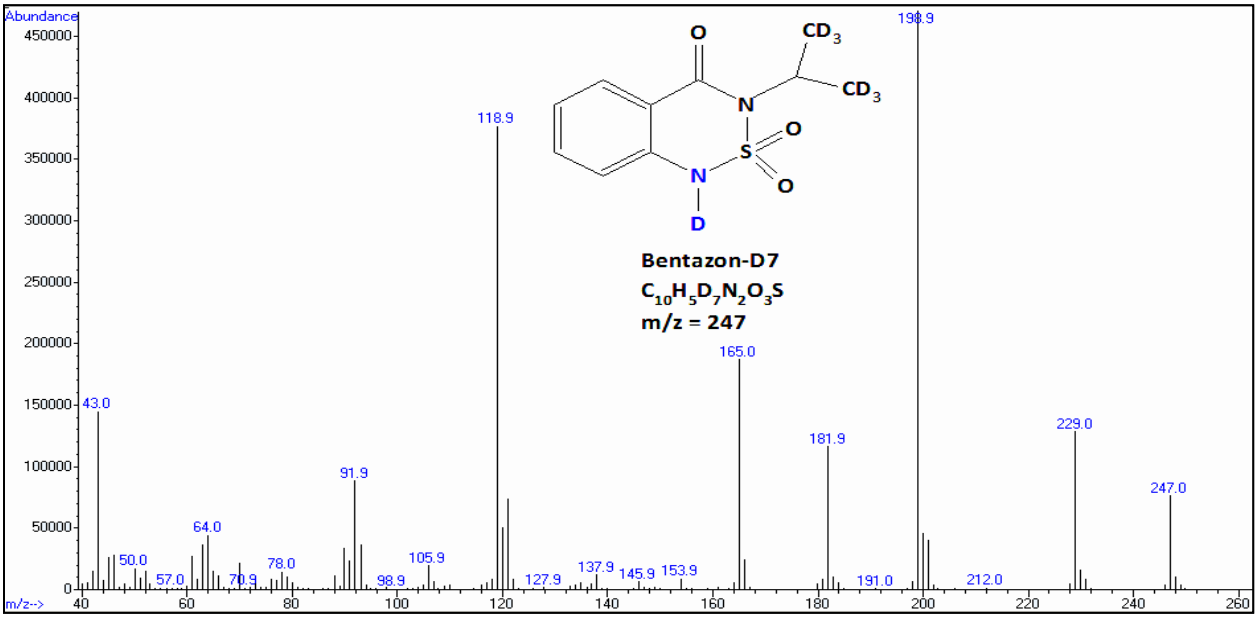
AA	Area
AMPA	Aminomethylphosphonic acid
AMPA-FMOC	Aminomethylphosphonic acid-9-fluorenylmethoxycarbonyl
ATMP	Aminotris (methylenephosphonate)
CD	Chloridazon-desphenyl
CMD	Chloridazon-methyl-desphenyl
DTPMP	Diethylenetriaminepenta (methylenephosphonate)
EDTMP	Ethylenediaminetetra (methylenephosphonate)
FMOC-Cl	9-fluorenylmethoxycarbonyl chloride
GC	Gas chromatography
Glyphosate-FMOC	Glyphosate-9-fluorenylmethoxycarbonyl
HDTMP	Hexamethylenediaminetetra (methylenephosphonate)
HESI	Heated Electrospray ionization
HPLC	High performance liquid chromatography
LC_{50}	Lethal concentration
LD_{50}	Median lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
M-W	Mecklenburg-Vorpommern
M/Z	mass-to-charge ratio
N. spumigena	*Nodularia spumigena*
PTV	Programmed temperature vaporizer
QqQ	Triple quadrupole
RE%	Relative error
RP	Reversed phase
RT	Retention time
RSD%	Relative standard deviation
S-H	Schleswig-Holstein
SRM	Selected reaction monitoring
TFAA	Trifluoroacetic anhydride
TFE	Trifluoroethanol
WWTP	Wastewater treatment plants

Appendices

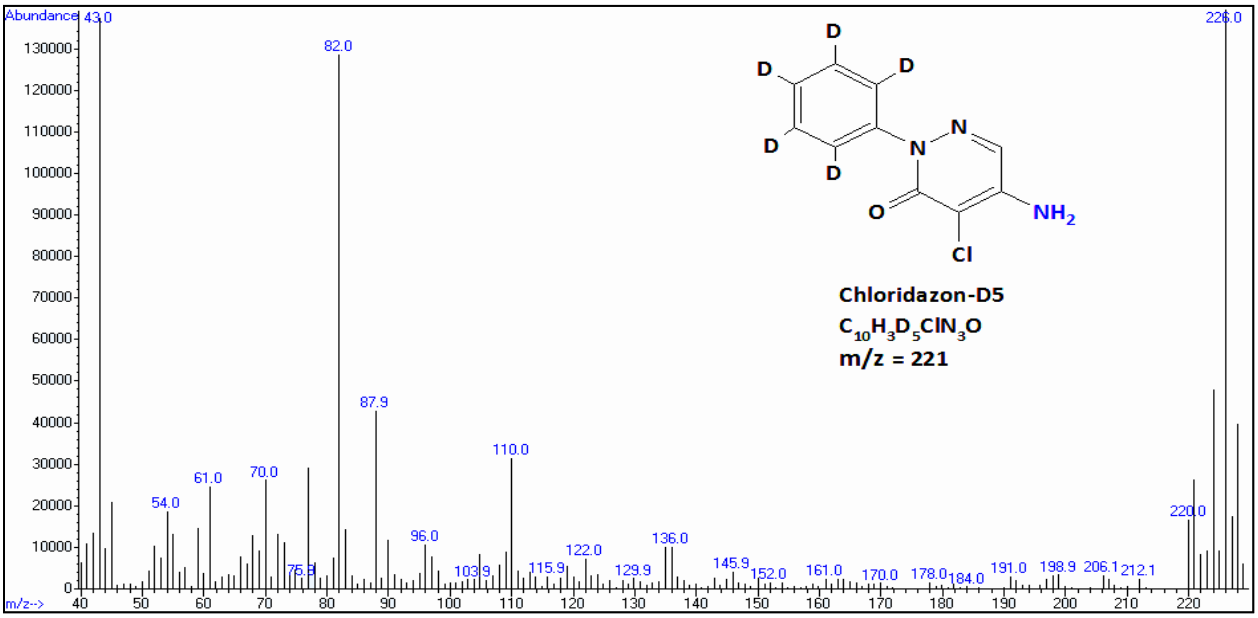
Appendix 1: Mass spectrum obtained from direct GC-MS analysis of isoproturon-D_6.



Appendix 2: Mass spectrum obtained from direct GC-MS analysis of bentazon-D_7.



Appendix 3: Mass spectrum obtained of direct GC-MS analysis of chloridazon-D_5.

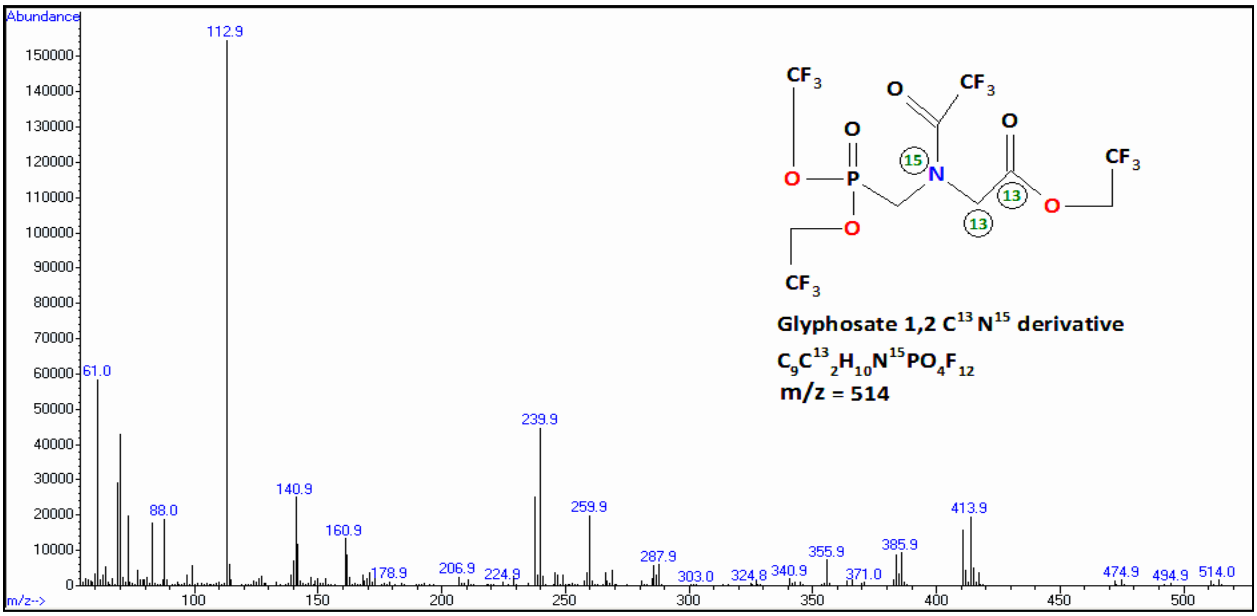


Appendix 4: Data of GC-MS analysis of four compounds CMD, isoproturon, bentazon, and chloridazon at masses range from 0.02 ng to 10 ng. The masses used of the labeled compounds chloridazon-D_5, isoproturon-D_6 and bentazon-D_7 are 10 ng.

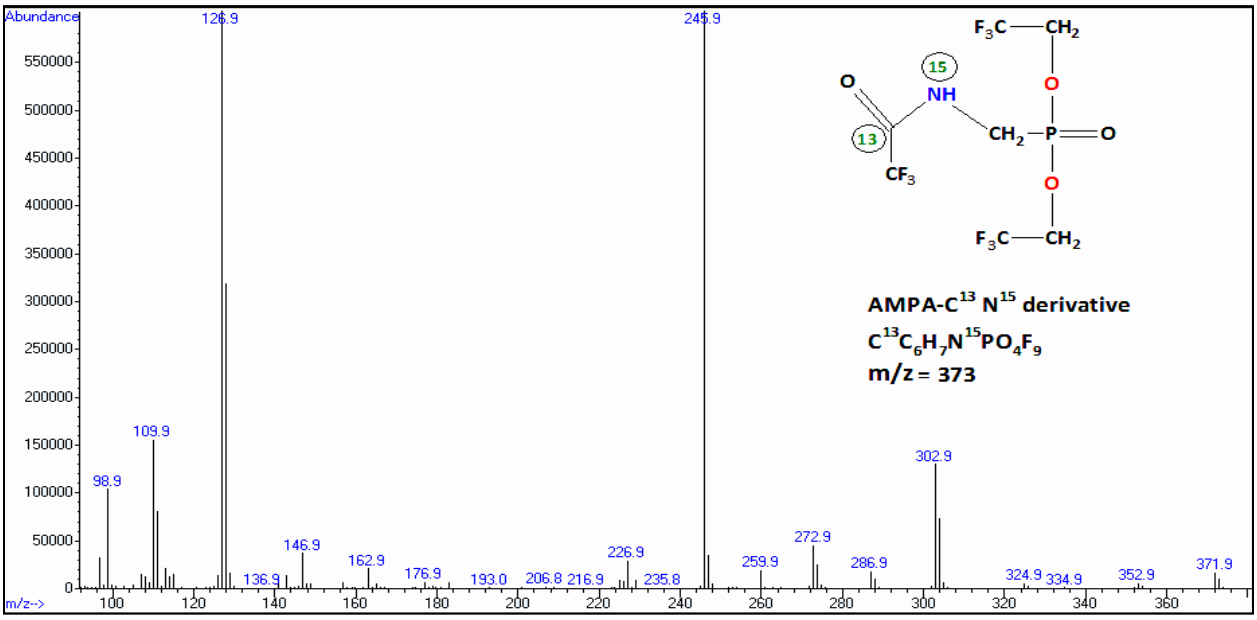
	CMD	Isoproturon	Bentazon	Chloridazon
Mass (ng)	0.02	0.02	0.02	0.02
Average area $_C$ / average area $_D$	0.002131294	0.001629093	0.229608049	0.00383936
SD	0.001657671	0.000725439	0.14487879	0.001660902
%RSD	77.8	44.5	63.1	43.3
Mass (ng)	0.05	0.05	0.05	0.05
Average area $_C$ / average area $_D$	0.002097398	0.005485502	0.037409322	0.003395247
SD	0.000524886	0.001090912	0.010375655	0.001108621
%RSD	25.0	19.9	27.7	32.7
Mass (ng)	0.1	0.1	0.1	0.1
Average area $_C$ / average area $_D$	0.003571168	0.012385331	0.026137975	0.008570284
SD	0.000636108	0.002290783	0.003885801	0.001321868
%RSD	17.8	18.5	14.9	15.4
Mass (ng)	0.15	0.15	0.15	0.15
Average area $_C$ / average area $_D$	0.005336568	0.016667014	0.032755344	0.011833652
SD	0.000709758	0.002103576	0.003098320	0.002348390
%RSD	13.3	12.6	9.5	19.8
Average area $_C$ / average area $_D$	0.007673289	0.021813658	0.040583672	0.012957742
SD	0.001347165	0.003691938	0.003216992	0.001913427
%RSD	17.6	16.9	7.9	14.8
Mass (ng)	0.3	0.3	0.3	0.3
Average area $_C$ / average area $_D$	0.016573372	0.031425186	0.052958821	0.021845987
SD	0.004485557	0.005787536	0.004834195	0.001711777
%RSD	27.1	18.4	9.1	7.8
Mass (ng)	0.5	0.5	0.5	0.5
Average area $_C$ / average area $_D$	0.021331183	0.053756893	0.080268427	0.040289006
SD	0.002286112	0.005134251	0.012548278	0.003635503
%RSD	10.7	9.6	15.6	9.0
Mass (ng)	1.0	1.0	1.0	1.0
Average area $_C$ / average area $_D$	0.037308077	0.132704793	0.153531377	0.074269236
SD	0.003042505	0.025669549	0.018252516	0.005869875
%RSD	8.2	19.3	11.9	7.9
Mass (ng)	2.0	2.0	2.0	2.0
Average area $_C$ / average area $_D$	0.120715284	0.258820003	0.295520205	0.168995324
SD	0.028497594	0.046267851	0.063554395	0.023761609
%RSD	23.6	17.9	21.5	14.1
Mass (ng)	5.0	5.0	5.0	5.0
Average area $_C$ / average area $_D$	0.281543827	0.495262219	0.754366947	0.445343239
SD	0.035919884	0.066046001	0.145558728	0.075487132
%RSD	12.8	13.3	19.3	17.0
Mass (ng)	7.0	7.0	7.0	7.0
Average area $_C$ / average area $_D$	0.390304267	0.804196826	1.214180242	0.539774559
SD	0.034280946	0.04488371	0.112772843	0.065093015
%RSD	8.8	5.6	9.3	12.1
Mass (ng)	10.0	10.0	10.0	10.0
Average area $_C$ / average area $_D$	0.524472651	1.22058545	1.820897949	0.858888373
SD	0.073979232	0.133268845	0.181331715	0.070703903
%RSD	14.1	10.9	10.0	8.2

SD: absolute standard deviation
%RSD: relative standard deviation
Average area $_C$ / average area $_D$: ratio of average of three measurements of the target compounds to three measurements of its labeled deuterium compound

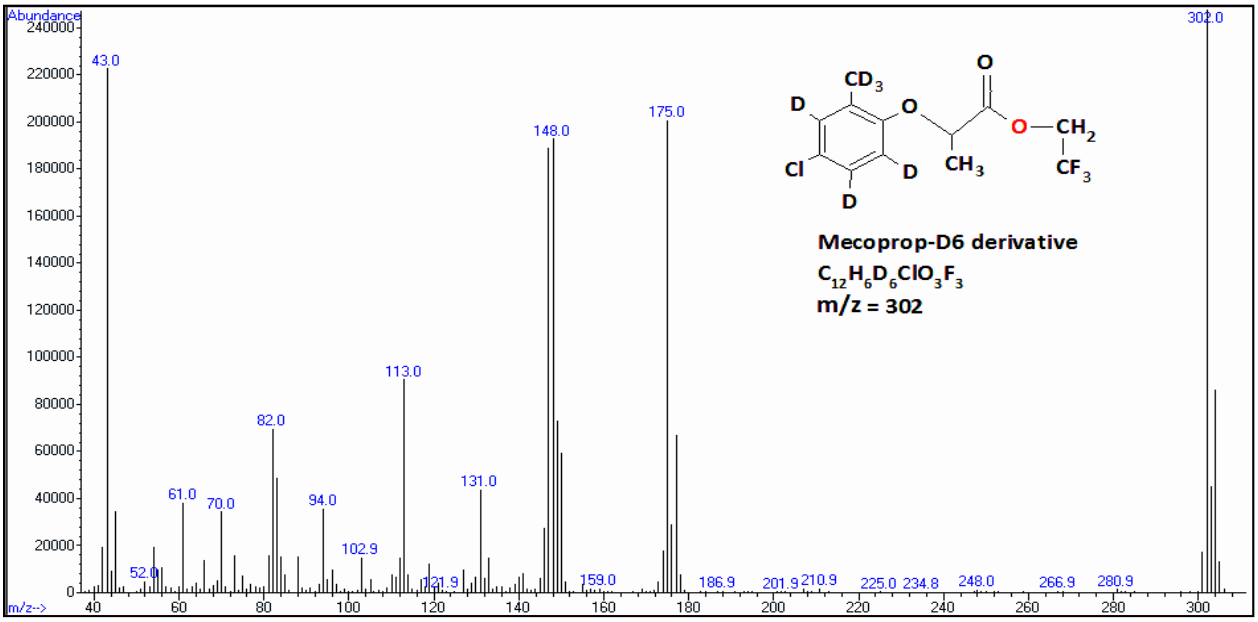
Appendix 5: Mass spectrum obtained of derivatized glyphosate 1, 2 C^{13} N^{15} with TFAA and TFE.



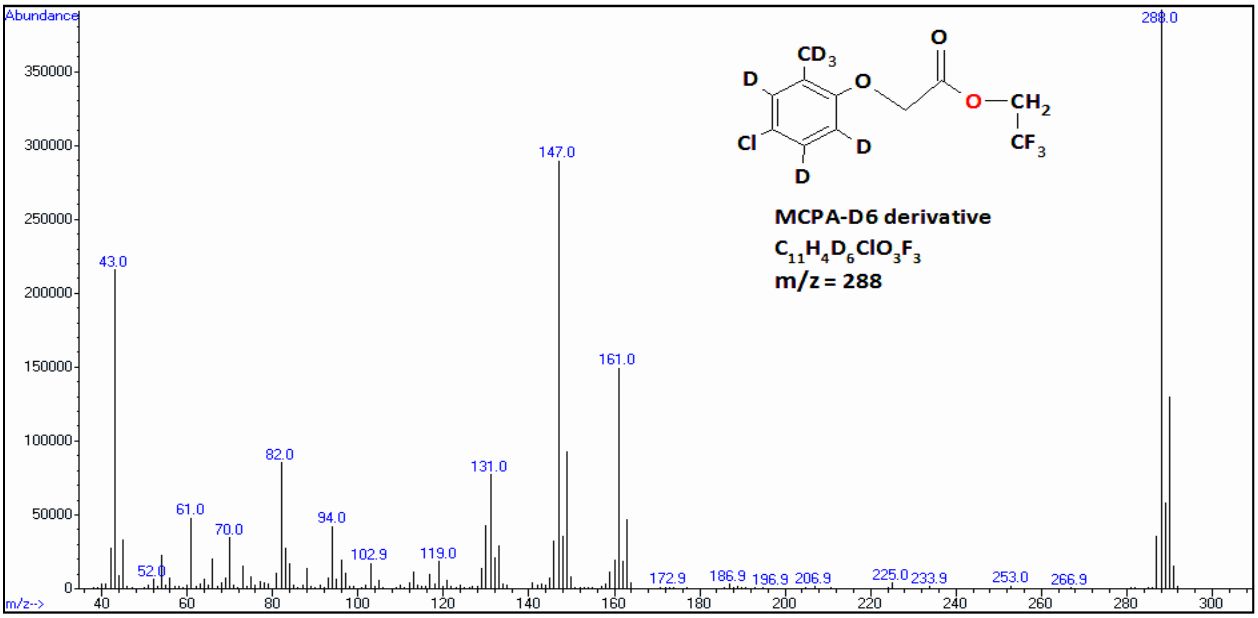
Appendix 6: Mass spectrum obtained of derivatized AMPA-C^{13} N^{15} with TFAA and TFE.



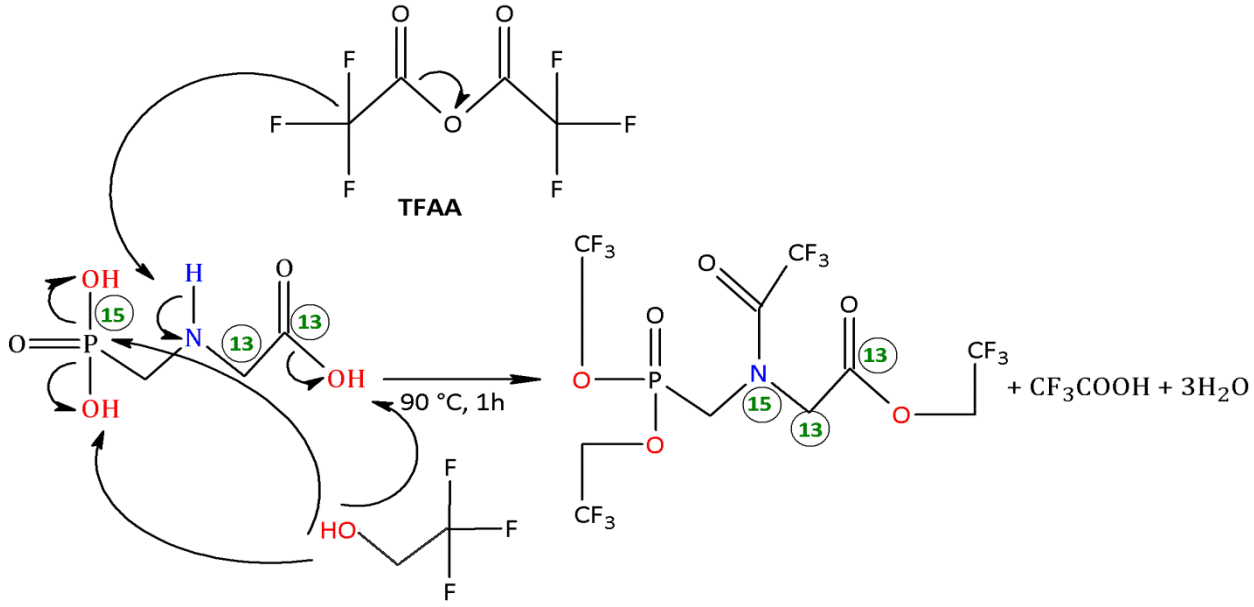
Appendix 7: Mass spectrum obtained of derivatized mecoprop-D_6 with TFAA and TFE.



Appendix 8: Mass spectrum obtained of derivatized MCPA-D_6 with TFAA and TFE.

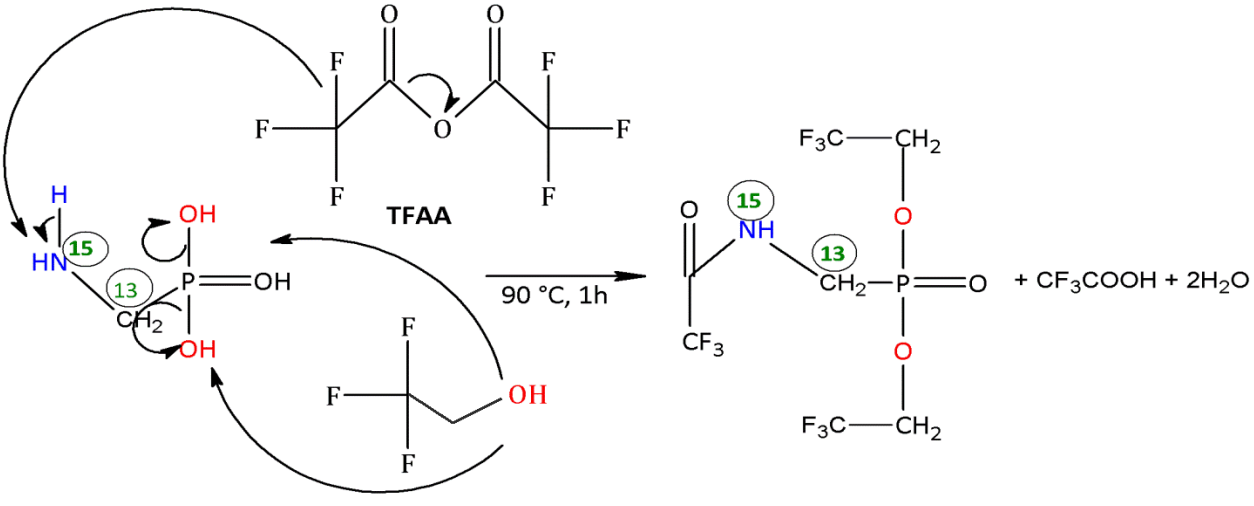


Appendix 9: Chemical reactions of labeled glyphosate 1, 2-C^{13} N^{15} and AMPA-C^{13} N^{15} with the reagents TFAA and TFE.



Glyphosate 1,2 C^{13} N^{15}
$C^{13}{}_2CH_8N^{15}PO_5$
Mm = 162.07 g/mol

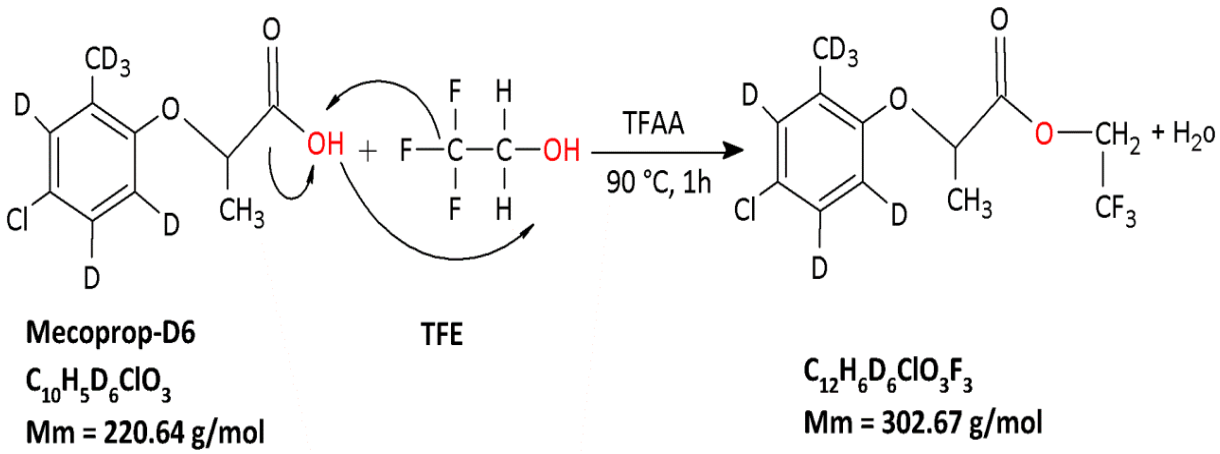
$C_9C^{13}{}_2H_{10}N^{15}PO_4F_{12}$
Mm = 514 g/mol



AMPA-C^{13} N^{15}
$C^{13}H_6N^{15}PO_3$
Mm = 113.03 g/mol

$C^{13}C_6H_7N^{15}PO_4F_9$
Mm = 373.096 g/mol

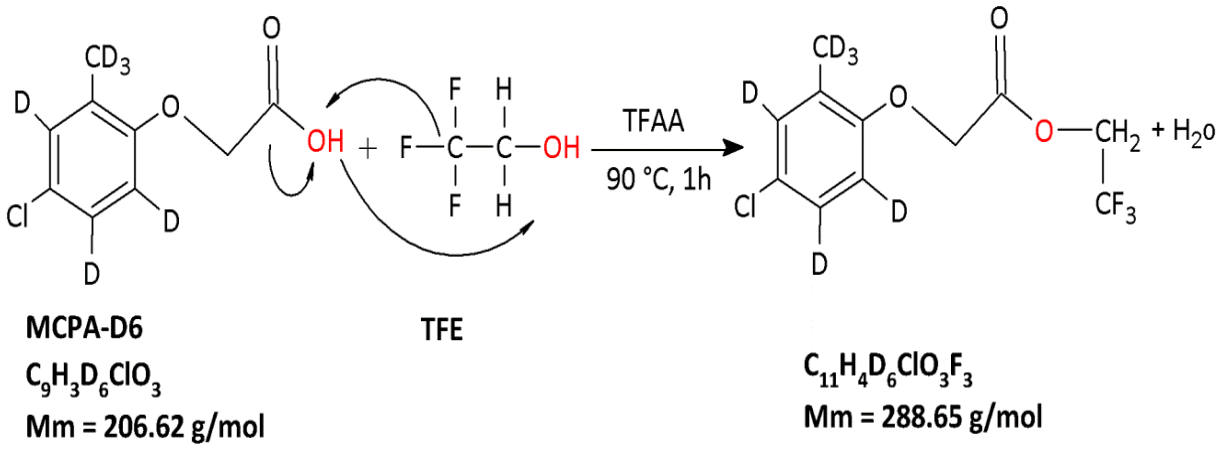
Appendix 10: Chemical reactions of labeled mecoprop-D_6 and MCPA-D_6 with the reagents TFAA and TFE.



Mecoprop-D6
$C_{10}H_5D_6ClO_3$
Mm = 220.64 g/mol

TFE

$C_{12}H_6D_6ClO_3F_3$
Mm = 302.67 g/mol



MCPA-D6
$C_9H_3D_6ClO_3$
Mm = 206.62 g/mol

TFE

$C_{11}H_4D_6ClO_3F_3$
Mm = 288.65 g/mol

Appendix 11: Four gradient protocols used for optimization of gradient elution in HPLC-RP- MS/MS analytical method for analysis glyphosate-FMOC and AMPA-FMOC.

Gradient elution **1**

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (µL/min)
0.00	99.00	1.00	100.00
4.00	99.00	1.00	100.00
10.00	37.00	63.00	100.00
11.00	5.00	95.00	100.00
24.00	5.00	95.00	100.00
26.00	99.00	1.00	100.00
30.00	98.00	1.00	100.00
	100.00	0.00	100.00

Gradient elution **2**

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (µL/min)
0.00	99.00	1.00	100.00
2.00	99.00	1.00	100.00
15.00	5.00	95.00	100.00
24.00	5.00	95.00	100.00
26.00	99.00	1.00	100.00
30.00	98.00	1.00	100.00
0.00	100.00	0.00	100.00
	99.00	1.00	100.00

Gradient elution **3**

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (µL/min)
0.00	99.00	1.00	100
2.00	99.00	1.00	100
10.00	5.00	95.00	100
24.00	5.00	95.00	100
26.00	99.00	1.00	100
30.00	98.00	1.00	100
0.00	100.00	0.00	100
	99.00	1.00	100

Gradient elution **4**

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (µL/min)
0	99.00	1.00	100
2	99.00	1.00	100
15	37.00	63.00	100
17	37.00	63.00	100
19	5.00	95.00	100
27	5.00	95.00	100
30	99.00	1.00	100
	100.00	0.00	100

Appendix 12: Data of the measured concentrations (ng/L) of the target compounds at the 11 sampling stations, salinities, temperature, sampling date and weather conditions. Source of the weather conditions (wettertopia.de)

Sta.	Gly.	AMPA	CMD	Chlo.	Bent.	Isop.	MCPA	Meco.	S	T (°C)	Date	Weather
1	101	273	nd	nd	14	nd	nd	nd	0.5	19.2	22.05.2012	dry
1	66	870	nd	nd	d	d	d	d	0.1	19.5	21.06.2012	dry
1	100	643	nd	nd	6	d	d	nd	0.3	20.9	22.08.2012	dry after rainfall
1	62	815	nd	nd	nd	5	nd	nd	0.1	12.3	25.09.2012	wet
2	nd	d	28	nd	nd	17	nd	nd	1.3	17.8	22.05.2012	dry
2	nd	79	25	nd	d	11	nd	nd	1.1	18.9	21.06.2012	dry
2	nd	50	nd	nd	d	6	nd	nd	0.9	22.6	22.08.2012	dry after rainfall
2	nd	d	nd	nd	d	8	nd	nd	1.1	12.8	25.09.2012	wet
3	d	nd	d	nd	nd	d	nd	nd	5.8	17.8	22.05.2012	dry
3	nd	nd	nd	nd	nd	nd	nd	nd	1.3	19.0	21.06.2012	dry
3	252	69	nd	nd	nd	d	nd	nd	5.7	21.4	22.08.2012	dry after rainfall
3	d	nd	nd	nd	nd	d	nd	nd	6.1	13.7	25.09.2012	wet
4	94	45	nd	nd	12	25	55	nd	1.2	18.2	22.05.2012	dry
4	120	d	nd	nd	6	6	nd	nd	1.2	19.1	21.06.2012	dry
4	95	101	nd	nd	11	nd	nd	nd	3.0	21.0	22.08.2012	dry after rainfall
4	nd	51	nd	nd	d	nd	nd	nd	4.1	13.0	25.09.2012	wet
5	nd	45	nd	nd	14	nd	nd	nd	0.5	17.2	22.05.2012	dry
5	nd	64	nd	nd	11	nd	nd	nd	0.5	20.3	21.06.2012	dry
5	28	171	nd	nd	12	nd	nd	nd	0.3	20.9	22.08.2012	dry after rainfall
5	nd	d	nd	nd	6	5	nd	nd	2.6	11.8	25.09.2012	wet
6	29	99	nd	nd	nd	7	nd	nd	0.3	16.6	22.05.2012	dry
6	nd	132	nd	nd	10	8	nd	nd	0.1	18.6	21.06.2012	dry
6	d	150	nd	nd	6	4	nd	nd	0.1	20.6	22.08.2012	dry after rainfall
6	nd	128	nd	nd	10	12	nd	nd	0.1	12.9	25.09.2012	wet

nd: Not detected
d: Detected but not quantified
St: Station
Gly: Glyphosate
Chlo: Chloridazon
Bent: Bentazon
Isop: Isoproturon
Meco: Mecoprop
S: Salinity
T: Temperature

Sta.	Gly.	AMPA	CMD	Chlo.	Bent.	Isop.	MCPA	Meco.	S	T (°C)	Date	Weather
7	160	497	d	nd	19	6	nd	nd	0.7	17.7	23.05.2012	dry
7	445	1502	112	nd	nd	11	147	d	0.5	18.5	19.06.2012	dry after rainfall
7	480	609	nd	nd	d	11	d	nd	0.8	20.2	22.06.2012	dry
7	300	656	nd	nd	13	41	155	d	0.1	14.1	26.06.2012	dry after rainfall
7	1690	940	nd	nd	8	52	747	d	0.3	17.6	10.07.2012	wet after rainfall
7	322	738	46	nd	6	31	121	d	0.3	17.5	17.07.2012	dry after rainfall
7	750	1808	nd	nd	nd	15	nd	d	0.3	18.0	07.08.2012	wet
7	960	4156	nd	nd	8	12	d	d	0.2	18.2	23.08.2012	dry after rainfall
7	874	2098	nd	nd	11	27	nd	nd	0.8	11.7	26.09.2012	wet
8	150	301	25	nd	10	8	nd	nd	0.4	17.1	23.05.2012	dry
8	225	296	nd	nd	18	5	nd	d	0.2	16.4	22.06.2012	dry
8	206	393	nd	nd	16	d	nd	d	0.2	16.9	23.08.2012	dry after rainfall
8	1664	912	nd	nd	12	34	nd	nd	0.1	11.2	26.09.2012	wet
9	nd	55	nd	nd	nd	nd	nd	nd	0.3	20.1	23.05.2012	dry
9	d	184	nd	nd	d	nd	nd	nd	0	20.1	22.06.2012	dry
9	32	195	nd	nd	nd	nd	nd	nd	0	21.5	23.08.2012	dry after rainfall
9	70	146	nd	nd	nd	nd	66	nd	0	13.2	26.09.2012	wet
10	55	391	d	nd	19	6	nd	nd	1.91	19.16	23.05.2012	dry
10	50	340	nd	nd	19	d	nd	nd	2.8	18.5	22.06.2012	dry
10	69	467	nd	nd	17	d	nd	nd	0.9	20.0	23.08.2012	dry after rainfall
10	45	243	nd	nd	10	12	nd	nd	3.1	12.0	26.09.2012	wet
11	nd	nd	nd	nd	nd	nd	nd	nd	11.7	10.4	23.05.2012	dry
11	nd	nd	nd	nd	nd	nd	nd	nd	11.8	14.9	19.06.2012	dry after rainfall
11	nd	nd	nd	nd	nd	nd	nd	nd	11.9	15.4	22.06.2012	dry
11	nd	nd	nd	nd	nd	nd	nd	nd	11.3	15.0	26.06.2012	dry after rainfall
11	nd	nd	nd	nd	nd	nd	nd	nd	9.4	14.8	10.07.2012	wet after rainfall
11	nd	nd	nd	nd	nd	nd	nd	nd	10.0	16.7	17.07.2012	dry after rainfall
11	nd	nd	nd	nd	nd	nd	nd	nd	9.0	16.6	07.08.2012	wet
11	nd	nd	nd	nd	nd	nd	nd	nd	9.1	17.1	23.08.2012	dry after rainfall
11	nd	nd	nd	nd	nd	nd	nd	nd	11.9	13.9	26.09.2012	wet

nd: Not detected
d: Detected but not quantified
St: Station
Gly: Glyphosate
Chlo: Chloridazon
Bent: Bentazon
Isop: Isoproturon
Meco: Mecoprop
S: Salinity
T: Temperature

Appendix 13: Comparison between the observed concentrations (ng/L) in the present study (PS) and to those found by (LUNG) in 2008 in freshwater samples (Bachor et al. 2008) regarding to the sampling stations (1, 2, 3, 5, 6).

Study	Sta. Nu.	Gly. May	Gly. June	AMPA May	AMPA June	MCPA	Meco.	Isop.	Bent.	Chlo.	CMD
PS	1	101	66	273	870	< 50	< 50	< 5	< 14	< 10	< 10
LUNG	1	100-1000	< 20	100-1000	< 10	< 20	< 20	< 10	< 10	< 10	< 50
PS	2	< 9	< 9	11-32	79	< 10	< 10	< 17	< 6	< 10	< 28
LUNG	2	100-1000	< 20	< 10	100-1000	< 20	< 20	< 10	< 10	< 10	< 50
PS	3	11-32	< 9	< 11	< 11	< 10	< 10	< 4	< 3	< 10	10-25
LUNG	3	< 20	< 20	>1000	< 10	< 20	< 20	< 10	< 10	< 10	< 50
PS	5	< 9	< 9	45	64	< 10	< 10	< 5	6-14	< 10	< 10
LUNG	5	< 20	< 20	< 10	< 10	< 20	< 20	< 10	< 10	< 10	< 50
PS	6	29	< 9	99	132	< 10	< 10	4-12	< 10	< 10	< 10
LUNG	6	< 20	< 20	100-1000	< 10	< 20	< 20	< 10	< 10	< 10	< 50

Sta. Nu: Station Number
Gly: Glyphosate
Meco: Mecoprop
Isop: Isoproturon
Bent: Bentazon
Chlo: Chloridazon

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Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den 05.05.2015

Wael Skeff