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DOCTORAL THESIS

DEVELOPMENT OF ANALYTICAL METHODS FOR THE DETERMINATION OF EMERGING CONTAMINANTS IN THE AQUATIC ENVIRONMENT BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ΑΝΑΠΤΥΞΗ ΜΕΘΟΔΩΝ ΠΡΟΣΔΙΟΡΙΣΜΟΥ ΝΕΩΝ ΡΥΠΩΝ ΣΤΟ ΥΔΑΤΙΚΟ ΠΕΡΙΒΑΛΛΟΝ ΜΕ LC-MS/MS

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ΧΗΜΙΚΟΣ

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DOCTORAL THESIS

Development of Analytical Methods for the Determination of Emerging Contaminants in the Aquatic Environment by Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Benzotriazoles, benzothiazoles and benzosulfonamides are three classes of organic compounds which have been considered as emerging contaminants in the aquatic environment.

In this thesis, we developed an integrated LC-(ESI+)MS/MS analytical methodology for the simultaneous determination of four benzotriazoles and four benzothiazoles in wastewater (both dissolved phase and particulate phase) and sewage sludge. The target benzotriazoles include 1H-benzotriazole, 1-hydroxy-benzotriazole, tolyltriazole (the mixture of isomers 4-methyl-1H-benzotriazole and 5-methyl-1H-benzotriazole), and xylyltriazole (or 5,6-dimethyl-1H-benzotriazole) while the target benzothiazoles are benzothiazole, 2-hydroxy-benzothiazole, 2-methylthio-benzothiazole, and 2-amino-benzothiazole). A solid-phase extraction (SPE) protocol and an ultra-sonication procedure were optimized for the extraction of the compounds from the dissolved phase and biosolid samples, respectively. Quantitative mean relative recoveries (> 89 %) with good precision (< 14%) were obtained for all the benzotriazoles and benzothiazoles in both dissolved and biosolid samples, except for 2-methylthiobenzothiazole with mean relative recoveries of 71% and 64% in dissolved and biosolid samples, respectively. The limits of detection (LODs) ranged from 0.08 (2-methylthio-benzothiazole) to 17 ng L^{-1} (benzothiazole) in dissolved samples and from 0.04 (2-methylthio-benzothiazole) to 13 ng g^{-1} (benzothiazole) in biosolid samples. This analytical approach was proven to be highly selective and sensitive and was applied successfully for the determination of the target compounds in wastewater and sludge.

We also developed a method for the quantification of three benzosulfonamides (Benzenesulfonamide, ortho-toluenesulfonamide and para-toluenesulfonamide) in wastewater based on pseudo-selected reaction monitoring (Pseudo-SRM) mass spectrometry. A solid phase extraction procedure was optimized for pre-concentration and extraction of the compounds from wastewater. Extracts were analyzed with LC-(ESI-)MS/MS in Pseudo-SRM mode. Absolute recoveries of the extraction method ranged from 83 to 118%. Method limits of detection (LODs) were 0.01 μg L^{-1} for p-toluenesulfonamide and 0.09 μg L^{-1} for both benzenesulfonamide and o-toluenesulfonamide in wastewater. Good precision of less than 13% for all the benzosulfonamides was achieved with the method. The developed method was employed for the determination of the three benzosulfonamides in wastewater.

Finally, we studied the occurrence of the target benzotriazoles, benzothiazoles and benzosulfonamides in raw wastewater, treated wastewater and sludge samples from sewage treatment plants of Athens; as well as their removal and fate during treatment processes. The highest mean concentrations of 3028 ng L^{-1} for tolyltriazole and 254 ng L^{-1} for benzothiazole were found in treated wastewater for both classes of benzotriazoles and benzothiazoles, respectively. A significant part, ranging up to 14% for 1H-benzotriazole and 20% for benzothiazole, was found in the particulate phase of wastewater influent. In sewage sludge, the highest mean concentrations were observed for tolyltriazole (146 ng g^{-1} dw) among the benzotriazoles and, for 2-hydroxylbenzothiazole (52 ng g^{-1} dw) among the benzothiazoles. 1-hydroxybenzotriazole, benzothiazole and 2-methythio-benzothiazole were highly removed during wastewater treatment whereas tolyltriazole, 1H-benzotriazole and 2-hydroxybenzothiazole were only partially removed. Biodegradation/biotransformation was the major mechanism responsible for the removal of benzotriazoles and benzothiazoles during wastewater treatment.

Among the benzosulfonamides, highest concentrations were found for p-toluenesulfonamide (up to 1473 ng L^{-1}) in both influent and effluent wastewater samples. Benzenesulfonamide was largely removed, p-toluenesulfonamide was partially removed and o-toluenesulfonamide was formed during wastewater treatment.

This study demonstrates the need for thorough control and environmental monitoring of the target benzotriazoles, benzothiazoles and benzosulfonamides in the aquatic environment as a result of their ubiquitous occurrence in wastewater and sludge, and incomplete removal during wastewater treatment.

SUBJECT AREA: Environmental Analytical Chemistry

KEYWORDS: Wastewater, sewage sludge, benzotriazoles, benzothiazoles, benzosulfonamides, LC-MS/MS

ΠΕΡΙΛΗΨΗ

Οι βενζοτριαζόλες, βενζοθειαζόλες και τα βενζοσουλφοναμίδια ειναι τρεις κατηγορίες οργανικών ενώσεων, οι οποίες θεωρούνται αναδυόμενοι ρύποι στο υδάτινο περιβάλλον.

Στη παρούσα εργασία, έγινε ανάπτυξη μιας ολοκληρωμένης αναλυτικής μεθοδολογίας LC-(ESI+)MS/MS για τον ταυτόχρονο προσδιορισμό τεσσάρων βενζοτριαζολών και τεσσάρων βενζοθειαζολών σε υγρά απόβλητα (διαλυτή και σωματιδιακή φάση) και σε ενεργό ιλύ. Οι βενζοτριαζόλες περιλάμβαναν την 1Η-βενζοτριαζόλη, 1-υδροξυ-βενζοτριαζόλη, τολυλτριαζόλη (μείγμα των ισομερών 4-μεθυλο-1Η-βενζοτριαζόλη και 5-μεθυλο-1Η-βενζοτριαζόλη) και την ξυλυλτριαζόλη (ή 5,6 διμεθυλο-1Η-βενζοτριαζόλη), ενώ στις βενζοθειαζόλες περιελάμβανονταν οι βενζοθειαζόλη, 2-υδροξυ-βενζοθειαζόλη, 2-μεθυλοθειο-βενζοθειαζόλη, και η 2-αμινο-βενζοθειαζόλη. Για την εκχύλιση των ενώσεων από τη διαλυτή και τη στερεή φάση χρησιμοποιήθηκε εκχύλιση στερεάς φάσης και λουτρό υπερήχων, αντίστοιχα. Μέσες σχετικές ανακτήσεις (>89%) με ικανοποιητική ακρίβεια (<14%) παρουσίασαν όλες οι βενζοτριαζόλες και βενζοθειαζόλες σε διαλύτη και στερεή φάση, εκτός της 2-μεθυλοθειο-βενζοθειαζόλη με μέσες σχετικές ανακτήσεις 71% και 64% σε διαλύτη και στερεή φάση, αντίστοιχα. Τα όρια ανίχνευσης κυμαίνονταν απο 0,08 (2-μεθυλοθειο-βενζοθειαζόλη) έως 17 ng L^{-1} (βενζοθειαζόλη) στη διαλυτή φάση, και από 0,04 (2-μεθυλοθειο-βενζοθειαζόλη) έως 13 ng g^{-1} (βενζοθειαζόλη) στη στερεή φάση. Η αναλυτική μέθοδος κρίθηκε ότι ήταν εκλεκτική και με ικανοποιητική ευαισθησία και εφαρμόστηκε για τον προσδιορισμό των αναλυτών σε υγρά απόβλητα και ιλύ.

Επίσης, αναπτύχθηκε μέθόδος για τον ποσοτικό προσδιορισμό τριών βενζοσουλφοναμιδίων (βενζενοσουλφοναμίδιο, ορθο-τολουολοσουλφοναμίδιο και παρα-τολουολοσουλφοναμίδιο) σε υγρά απόβλητα με υγροχρματογραφία-φασμοτομετρία μάζας, με την τεχνική ψευδο-SRM. Η εκχύλιση στερεάς φάσης (SPE) βελτιστοποιήθηκε για την προσυγκέντρωση και παραλαβή των ενώσεων απο τα δείγματα. Τα εκχυλίσματα αναλύθηκαν με LC-(ESI-)MS/MS με ψευδο-SRM. Οι απόλυτες ανακτήσεις από την SPE κυμαίνονταν απο 83% εώς 118%. Τα όρια ανίχνευσης της μεθόδου ήταν 0,01 μg L^{-1} για το παρα-τολουολοσουλφοναμίδιο και 0,09 μg L^{-1} για το βενζενοσουλφοναμίδιο και ορθο-τολουολοσουλφοναμίδιο σε υγρά απόβλητα. Η αναπαραγωγιμότητα της μεθόδου (%RSD$_R$) ήταν μικρότερη από 13%. Η μέθοδος εφαρμόστηκε για τον προσδιορισμό των βενζοσουλφοναμιδίων σε υγρά απόβλητα από το Κέντρο Επεξεργασίας Υγρών Λυμάτων της Ψυττάλειας (ΚΕΛΨ).

Τέλος, έγινε μελέτη της ύπαρξης των βενζοτριαζολών, βενζοθειαζολών και των βενζοσουλφοναμιδίων σε ανεπεξέργαστα και επεξεργασμένα υγρά απόβλητα και δείγματα ιλύος από το ΚΕΛΨ Αθήνας, καθώς επίσης μελετήθηκε η απομάκρυνση και η «τύχη» τους κατά τη διάρκεια της επεξεργασίας. Οι μέσες υψηλότερες συγκεντρώσεις βρέθηκαν για την τολυλοτριαζόλη (3028 ng L^{-1}) και τη βενζοθειαζόλη (254 ng L^{-1}) στα επεξεργασμένα απόβλητα για την ομάδα των βενζοτριαζολών και βενζοθειαζολών, αντίστοιχα. Σημαντικό ποσοστό έως 14% για την 1Η-βενζοτριαζόλη και 20% για τη βενζοθειαζόλη ανιχνέυτηκε στη σωματιδιακή φάση των εισερχόμενων αποβλήτων. Στην ενεργό ιλύ, οι υψηλότερες μέσες συγκεντρώσεις παρατηρήθηκαν για τη τολυλτριαζόλη (146 ng g^{-1} dw) από τις βενζοτριαζόλες και για τη 2-υδροξυ-βενζοθειαζόλη (52 ng g^{-1} dw) από τις βενζοθειαζόλες. Η 1-υδροξυ-βενζοθειαζόλη, η βενζοθειαζόλη και η 2-μεθυλοθειο-βενζοθειαζόλη απομακρύνονταν σημαντικά κατά τη διάρκεια της επεξεργασίας, ενώ η τολυλοτριαζόλη, η 1Η-βενζοτριαζόλη και η 2-υδροξυ-βενζοθειαζόλη απομακρύνονταν μερικώς. Η βιοαποικοδόμηση ήταν ο κύριος μηχανισμός απομάκρυνσής τους κατα τη διάρκεια της επεξεργασίας.

Από τα βενζοσουλφοναμίδια σε υψηλότερη συγκέντρωση ανιχνεύτηκε στο π-τολουλοοσουλφοναμίδιο (εώς 1473 ng L^{-1}) σε εισερχόμενα και εξερχόμενα υγρά απόβλητα. Το βενζενσουλφοναμίδιο απομακρύνεται σε μεγάλο βαθμό, το π-τολουολοσουλφοναμίδιο απομακρύνεται μερικώς, ενώ το ο-τολουολοσουλφοναμίδιο σχηματίζεται κατά τη διάρκεια της επεξεργασίας.

Η εργασία αυτή υποδεικνύει την ανάγκη για ενδελεχή και συνεχή έλεγχο και περιβαλλοντική παρακολούθηση των ενώσεων αυτών στο υδάτινο περιβάλλον, λόγω της ύπαρξης τους σε επεξεργασμένα υγρά απόβλητα και λάσπη λόγω της μερικής απομάκρυνσης τους κατά τη διάρκεια της επεξεργασίας.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Περιβαλλοντική Αναλυτική Χημεία

Λέξεις-Κλειδία: Υγρά απόβλητα, ενεργός ιλύ, βενζοτριαζόλες, βενζοθειαζόλες, βενζοσουλφοναμίδια, LC-MS/MS

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

In recent years, ‘emerging contaminants’ has become a highly relevant topic in environmental science and technology because of their increasing release into the environment, potential adverse effects on human health or ecosystem safety and the need for development of suitable analytical methods for their determination in the aquatic environment.

The main points of collection and subsequent release of these emerging contaminants into the environment are wastewater treatment plants, where they enter via domestic and hospital sewages or industrial discharges. Some of them are even likely to accumulate in sewage sludge due to their physicochemical properties. With the rising need to study the occurrence, fate and transport of these emerging contaminants in the environment, it is necessary to unambiguously identify and quantify these compounds with sensitive analytical methods.

The objectives of this thesis were to develop sensitive and selective analytical methods for the determination of three classes of emerging contaminants (benzotriazoles, benzothiazoles and benzosulfonamides) in wastewater and sewage sludge by LC-MS/MS, as well as to study the occurrence, removal and fate of these compounds in sewage treatment plant.

The thesis comprises the description and definitions of emerging contaminants, brief discussion on some emerging contaminants most commonly detected in the aquatic environment, the state of the art of analysis of emerging contaminants and structures, properties, uses and toxicity of benzotriazoles, benzothiazoles and benzosulfonamides. An elaborate review of the existing analytical methodologies, occurrence in the environment, and removal in sewage treatment plants is undertaken for benzotriazoles, benzothiazoles and benzosulfonamides. Experimental sections detail the instrumentation and chemicals used throughout the experiments, as well as the experimental procedures for (1) simultaneous determination of benzotriazoles and benzothiazoles in wastewater and sludge (2) determination of benzosulfonamides in wastewater and (3) occurrence, removal and fate of benzotriazoles, benzothiazoles and benzosulfonamides in sewage treatment plant. The results of the findings are thoroughly discussed and areas of further research in the future are suggested.

It is hoped that these studies will provide a major step forward in better understanding methods of determination, occurrence and fate of these compounds of emerging concern in the aquatic environment.

Athens, February 2013

CHAPTER 1
EMERGING CONTAMINANTS

Every day, industries, agricultural practices and the general population are using water and releasing many compounds into wastewaters and eventually, into other environmental water compartments. Indeed, agricultural practices, industrial discharges and human beings play an important role on the issue of pollutants in wastewater and other aquatic environments. All these practices have generated various pollutants and altered the water cycle causing a global concern linked to their eventual impact on wildlife and human health [1, 2]. A wide range of these man-made chemicals which are designed for use in industry, agriculture, and as consumer goods, and chemicals unintentionally formed or produced as by-products of industrial processes or combustion, are potentially of environmental concern [3–5]. In addition to already recognized environmental pollutants, numerous new chemicals are still being synthesized and released into the environment with little or no iota of unforeseen adverse environmental consequences.

Moreover, more than 180 million organic compounds are known at present. Consequently, even if only a small portion of these substances exhibit properties leading to environmental problems, the number of organic contaminants is still high and, in principle seems unlimited, as the development of new substances continues [6]. Approximately 2,000 substances are presently estimated to be environmentally relevant, of which 100-300 compounds are summarized in lists of substances to be treated with priority by various international organizations, such as the EU (EU-Water Framework Directive; EU-WFD) and OSPAR Commission(for the protection of the Marine Environment of the North-East Atlantic [7].

For decades, environmental scientists have focused on the study of chemicals whose presence in the environment has been regulated through the various list of criteria or priority pollutants included in the different legislations. Traditionally hydrophobic *persistent organic pollutants (POPs)*, that have very high potential to accumulate in sediments and enrich along food chains, used to be the focus of extensive studies [6, 8]. However, the perspective on contamination of aqueous environment by anthropogenic trace pollutants has experienced a remarkable change in the past few years. Numerous studies combining chemical and biological approaches to hazard assessment of complex environmental mixtures have indicated that priority pollutant concentrations are a poor indicator of toxicity. It is therefore, evident that the aim to assess and forecast the impact of chemical pollution cannot be met on the basis of priority pollutant analysis alone [9–11]. Meanwhile, there is already an increase in awareness that polar contaminants may also pose significant problems to water quality, especially if they are not well degradable [6,12–15]. In recent years, new classes of aquatic pollutants have received attention from environmentalists, scientists and regulators due to their introduction into the environment, unforeseen effects associated with them, or enhanced analytical techniques presently capable of detecting them [16]. Several research articles have reported the presence of these new compounds, called "emerging pollutants" in wastewater and other aquatic environments [17–19]. The introduction of new, more sensitive analytical equipment for the detection and quantification of these new compounds in complex sample matrices on the one hand, and a growing knowledge about their ecotoxicological effects on the other hand, have drawn keen attention to these new compounds which have been largely outside the scope of monitoring and regulation [3,8, 20–24]. For most of these new chemicals, occurrence, risk assessment and ecotoxicological data are not available and, therefore, it is difficult to predict what health effects they may have on humans and aquatic organisms. Potential threats from these emerging pollutants continue to be a driving force -if not a rallying cry- for innovation in environmental research [8, 25].

1.1. Description and Definitions of Emerging Contaminants

While "classical" POPs are mostly non polar, lipophilic compounds and belong to a few compound classes only- such as chlorinated hydrocarbons (PCB, DDT, dioxins, etc.) or polycyclic aromatic hydrocarbons (PAHs)- new, emerging pollutants exhibit properties which are different from "classical" pollutants [6]. The new compounds are often more polar, less volatile and belong to many quite different compound classes and origin from a great number of different applications.

"*Emerging contaminants*" is a phrase commonly used to broadly classify chemicals that do not fall under standard monitoring and regulatory programmes but may be candidates for future regulation once more is known about their toxicity and health effects. They are not currently covered by existing water-quality regulations, have not been studied before, and are thought to be potential threats to environmental ecosystems and human health and safety. Little is known about them, from their production volumes, to their physical-chemical properties, to their effects on humans and the environment, to how best to regulate the unknown risks they pose [1, 9, 20, 21, 25, 26]. According to the network of reference laboratories for monitoring of emerging environmental pollutants (NORMAN), emerging contaminants are defined as contaminants that are currently not included in routine monitoring programmes and which may be candidates for future regulation, depending on research on their ecotoxicity, potential health effects, public perception, and on monitoring data revealing their occurrence in different environmental compartments [27].

The term '*emerging*' can be misinterpreted as an indication that the chemical's presence in the environment is new, when in fact it means the chemical has just recently gained the interest of scientific and regulatory communities. For instance, chemicals such as polybrominated diphenyl ether (PBDE) flame retardants, musk fragrances, and pharmaceuticals have been present in the environment since their first use decades ago [28−31] but only recently have they been considered and measured due to advances in monitoring techniques and the increased understanding of their toxicological impact. On the other hand, other chemicals such as nanomaterials, can truly be defined as emerging, that is 'new' because though they have been present in research laboratories since the early 1980s, it has only been since the early 2000s that nanomaterials have been produced in sufficient quantities for consumer use [32, 33].

It has been reiterated that the definition of an emerging contaminant is a bit elusive, because what is emerging is a matter of *perspective* as well as *timing* [34]. The US Geological Survey (USGS) has defined emerging contaminants (ECs) as any synthetic or naturally occurring chemicals or microbial constituents that have not historically been considered to be contaminants [35]. These synthetic or naturally occurring chemicals or microorganisms are not commonly monitored in the environment but have the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects.

A contaminant may also be 'emerging' because of the discovery of a new source or technology, a new pathway to humans, or a new method of detection [36]. Many review articles have also tried to articulate different definitions, but most articles agree that emerging contaminants are unregulated chemicals, which have become of environmental concern either through advances in analytical techniques to detect them, their association with a newly reported effect, or through their recent introduction into the environment [3,16,37–39].

The term ‘emerging contaminants’ does not necessarily refer to ‘new substances’ only, i.e. newly introduced chemicals and their degradation products and/or metabolites or by-products but also refers to naturally occurring compounds, with previously unrecognized adverse effects on ecosystems [3]. In some cases, release of chemical or microbial contaminants to the environment has likely occurred for a long time, but may not have been recognized until new, more sensitive detection methods were developed. In other cases, synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging contaminants.

The term “emerging” is also used to describe not the pollutant itself, but rather a new “emerging concern” about the contaminant, i.e. newly demonstrated toxic effect and/or mechanism of action of an old pollutant [40−42]. As such, emerging contaminants are often referred to as ‘chemicals of emerging concern’ or ‘contaminants of emerging concern’. This approach is highly legitimate and is often favoured among toxicologists in comparison to classifications and definitions based on chemical entities. Fawell and Ong even suggested that a better description of these compounds would be ‘contaminants of emerging concern’ [23]. In reality, however, scientists and regulators will have to deal with both the terms “emerging contaminants” and “emerging concerns”, and this artificial partition is certainly not critical for principal understanding of the problem and its possible solutions.

1.2. Environmental issues of emerging contaminants

In 2004, the environmental campaign group World Wide Fund (WWF) tested the blood of government ministers (Environment and health ministers) from 13 European Union (EU) Member States for chemicals that can negatively affect human health and wildlife. WWF found on average 37 out of the 103 tested substances in the ministers’ blood [41,43]. It was clear that the EU citizens were concerned. Also, in a survey, the impact of chemicals used in everyday products came fifth in a list of 15 environmental issues of concern. When asked about which issue they feel they lack information, citizens cited chemicals first [44]. Do they have reason(s) to be concerned? Undoubtedly, the answer is positive – the overview of the “chemical world” concentrated mainly to today’s synthetic emerging contaminants, clearly suggests that there are real human and environmental health problems that have to be addressed. Considering the issue of chemical contamination, all critical parties – regulators, risk managers, industry sector, politicians, and, finally, scientists need to offer answers and solutions needed for citizens to be less concerned.

Moreover, the contamination of natural waters by emerging chemicals is a major concern in many parts of the world, and as new chemicals are introduced, or others find new applications, and improvement in analytical methods, the occurrence of these previously undetected chemicals in wastewaters and receiving waters is frequently reported [45]. Much attention has focused on compounds known to exhibit biological activity at low concentrations, in particular pharmaceuticals, steroid estrogens and other endocrine disruptors. The occurrence of these pharmaceutical compounds began to receive attention during the 1990s when they were linked to toxicological effect in fish [46]. However, consumer products such as washing powders containing detergents, bleaching agents and other ingredients such as corrosion inhibitors, are used in high volumes than pharmaceuticals and may therefore also contribute to the wide dispersive occurrence of xenobiotics in wastewater and other environmental water compartments [47, 48].

The threat of emerging contaminants lies in the fact that the environmental and human toxicology of most of these compounds have not yet been studied extensively and also in the fact that many of these compounds are not or cannot be tested for in municipal water systems [42]. In addition, when these contaminants pass through wastewater treatment systems or water drinking systems, transformation products are sometimes generated whose chemical properties are as yet undetermined. The danger inherent in our inadequate knowledge with regards to these compounds is underscored by the Handbook of Environmental Chemistry (2008) which states: “*Every day new potential emerging contaminants are discovered and new disinfection by- products are also generated during treatment, with a total ignorance of their potential toxicity or effect on human health*” [41, 42].

1.2.1 Determination at trace level

The need to understand the processes controlling emerging contaminant sources, transport and fate in the environment, and ecologic and human health effects has increased the need to study environmental occurrence of these compounds down to trace levels. There is therefore, need for sensitive analytical methods that can detect and quantify accurately this range of emerging contaminants in the aquatic environment. Though methods have been developed to enhance capabilities for measuring emerging chemical and microbial contaminants and their associated degradation products in the environment, more intensive efforts are still needed in this area in order to determine these compounds at lower and lower levels within the realm of possibility. Therefore, since there is a great myriad of these emerging chemicals of concern, prioritization of compounds investigated requires evaluation for the potential of their environmental occurrence and persistence, potential health effects, and the appropriate level at which they should be measured. Criteria for establishing priorities for methods development include the quantity produced and used, pathways for release to the environment, anticipated environmental behaviour, information of human and environmental effects, potential use as an environmental indicator or tracer or stakeholder priorities [49,50].

1.2.2. Issues on sources and occurrence

Evidence has been accumulating, depicting that many emerging contaminants occur at environmentally significant levels in wastewaters and natural waters, these compounds appear in natural waters through municipal, agricultural and industrial waste water discharges [12, 51–55]. Pharmaceuticals and personal care products, synthetic and natural estrogens, industrial by-products and other substances are commonly found in waste water as well as in receiving waters around the world.

Emerging pollutants can reach the environment by being transported and distributed via different routes. The physico-chemical properties of these chemicals (e.g., water solubility, vapour pressure and polarity) and other environmental characteristics determine their behaviour in the environment. The major sources of environmentally relevant emerging contaminants can be divided into point-sources and diffuse sources of pollution [20,49,56]. Point-pollution originates from discrete locations whose input into aquatic systems can often be defined in a spatially discrete manner. The spatial extent or plume of pollution is therefore generally constrained. Important examples include municipal wastewater treatment plant effluents, industrial effluents, waste disposal sites and buried septic tanks. Diffuse pollution, in contrast, originates from poorly defined, diffuse sources; examples include agricultural run-offs, storm-water and urban run-off and diffuse aerial deposition.

Several extensive national and multinational monitoring programmes have been launched in the last few years in order to provide comprehensive reconnaissance of the occurrence of various emerging pollutants with a special emphasis on polar organic wastewater contaminants [4, 13, 37, 51, 57, 58]. All these studies have confirmed the presence of complex mixtures of unregulated emerging contaminants, having various origins, and raised concern about their potential interactive effects.

1.2.3. Issues on fate and transport

The way that organic compounds enter the environment depends on their pattern of usage and mode of application (e.g. disposal of municipal, industrial and agricultural wastes, excretion of pharmaceuticals and accidental spills). Once in the environment, they can be widely distributed right from their production through their use and final disposal. Because many of these emerging pollutants are from human use, their emissions have become a serious issue for some wastewater processes. Therefore, the study of occurrence and fate of emerging contaminants in wastewater treatment plants (WWTP) is important [20]. Reports on the fate of emerging chemicals in sewage treatment plants (STP) have shown that they may be partially removed during conventional treatment [59, 60]. Treated effluents (and in some cases raw sewage) are discharged into receiving waters, including small streams, rivers, lakes and drinking water supplies. In this way, there is an increase in the number of reports dealing with emerging contaminant levels in natural water as a result of wastewater discharges [51, 53, 59–63].

1.2.4. Issues on ecotoxicology

There is a growing concern that contaminated water could have a negative impact on wildlife through the spread of these so called emerging contaminants [64, 65]. Some of these emerging compounds have the potential to interfere on normal endocrine functions, modulating or disrupting hormonally regulated mechanisms in living organisms as well as producing adverse effects at trace and ultra-trace levels [1, 66, 67].

1.3. GROUPS OF EMERGING CONTAMINANTS

The several types of emerging contaminants are generally grouped by their initial use but sometimes by structure or their mechanism of action. Emerging pollutants encompass a diverse group of compounds, such as pharmaceuticals, drugs of abuse (Illicit drugs), personal-care products (PCPs), steroids, and hormones, surfactants, perfluorinated compounds (PFCs), brominated flame retardants, industrial additives, polychlorinated naphthalenes (PCNs), polychlorinated alkanes (PCA), synthetic musks, quaternary ammonium compounds, (QACs), bisphenol A (BPA), nanomaterials, drinking water and swimming pool disinfection by-products, sunscreen/UV filters, benzotriazoles, benzothiazoles, benzosulfonamides, dioxane, siloxanes, pesticides transformation products and new pesticides, perchlorate, algal toxins, microorganisms as well as their transformation products (TPs) [8,9,16,20,21,39,56,68,69].

In the following sub-sections, some selected groups of emerging contaminants among the large variety of compounds most commonly detected in environmental samples are briefly dicussed.

1.3.1. Pharmaceuticals and personal care products (PPCPs)

Pharmaceuticals and personal care products (PPCPs) represent a category of emerging pollutants that are released into the environment through personal activities. Small but cumulative usage of these products by a large number of individuals results in widespread but significant pollution, which is difficult to control [16]. The term “pharmaceuticals” covers a wide-ranging class of compounds with substantial variability in structures, function, behaviour, and activity. Developed to elicit a biological effect, they are used in both humans and animals to cure diseases, fight infection, and/or reduce symptoms [70–73]. The most common pharmaceuticals belong to the categories of antibiotics, analgesic and anti-inflammatory. However, other groups include diuretics, antidiabetics, anticoagulants, psychiatric drugs, lipid regulators [74–76], anti-epileptic drugs [76, 77], disinfectants, antidepressants, antiseptics, hormones [76], vascodilators, barbiturates, anticancers, anticonvulsants [78], anti-hypersensitives and antilipidemics [79]. Among pharmaceuticals, antibiotics have become of special concern in recent years. The reason is that these substances are continuously being introduced into the environment and may spread and maintain bacterial resistance in the different compartments [80]. Personal care products include synthetic musk fragrances (nitro and polycyclic musk fragrances), antimicrobials (triclosan and its metabolites and triclocarban), sunscreen agents (ultraviolet filters), insect repellents (N,N diethyl-m-tolueneamide, known as DEET) and parabens (p-hydroxybenzoic esters), which are basically substances used in soaps, shampoos, deodorants, lotions and toothpaste [81]. Pharmaceuticals and personal care products (PPCPs) enter into the environment through individual human activity and as residues from manufacturing, agribusiness, veterinary use, and hospitals and community use. Individuals may add PPCPs to the environment through waste excretion or bathing, as well as by direct disposal of unused medications into septic tanks, sewers, or trash containers [23,74,82]. Because pharmaceuticals are often the active ingredients in medicines, they are engineered to be bioavailable and potent and may result in adverse environmental effects at low concentrations [16]. The potential for PPCPs to cause a variety of physiological responses in non-target species has raised concerns for possible impacts on the environment [41].

Individual PPCPs have also distinct chemical and physical properties that suggest potentially different mechanisms and locations for removal in a wastewater treatment plant(WWTP). PPCPs can have octanol–water partition coefficients (Kow) or water solubility values that vary up to 7–8 orders of magnitude. Many of the PPCPs are ionizable chemicals and it may not be appropriate to assess their lipophilicity based only on the Kow value [74,83].

With hundreds of different PPCPs in the market, WWTPs represent a key potential point source to the aquatic environment, but at the same time a major opportunity for centralised removal processes [75]. Due to the continous input of pharmaceuticals and other PPCPs into the aquatic media through WWTPs, they are considered to be ‘pseudo-persistent’ [84]. Their presence has also been identified and quantified in surface waters [76], drinking water [85], groundwater [86], biosolids [87], biota [88] and agricultural manures [89].

LC-MS/MS is the preferable method of analysis for PPCPs, due to its versatility, specificity and selectivity, gradually replacing GC-MS. GC-MS can still be successfully applied in some cases, especially for nonpolar and volatile pharmaceutical compounds, however it still requires a time-consuming derivatization step, during which there are risks of analyte losses [37, 90, 91]. Among LC-MS/MS techniques, triple quadrupole (QqQ) and ion trap (IT) instruments are in common use, and they permit the detection of pharmaceuticals at the ng L^{-1} range. More recent approaches in LC-MS/MS are linear ion traps (LITs), new generation triple quadrupoles and hybrid instruments, such as quadrupole–time of flight (QqTOF) and quadrupole-linear ion trap (QqLIT) [92]. QqTOF instruments have been used for the elucidation of structures proposed for their transformation products [93–95].

The removal rates of PPCPs from wastewater treatment systems may vary considerably even for the same PPCP and inter-comparisons are most of the time problematic due to not only different definitions of removal rates but also to different sampling strategies (integrated vs grab samples) [74,78].

PPCPs may have unpredicted and unknown side effects on different organisms, particularly after long-term exposure to low concentrations. The toxicological implications of PPCPs in the environment have been examined and it has been found that low concentrations of these compounds can elicit adverse effects on aquatic life [78,96,97]. These findings have engendered public concerns as to the possible effects the compounds may exert on both human health and waterway ecology [98]. The transformation products of PPCPs may be environmentally persistent [82, 99]. For instance, the environmental persistence of the main active metabolite of the blood lipid regulators (clofibrate, erofibrate and theofibrate), clofibric acid, is 21 years [100]. However, the potential effects of many metabolites of PPCPs, in organisms of the ecosystem, are still unknown [2].

1.3.2. Illicit drugs

In the last few years, the scientific community started paying more attention to the occurrence of drugs of abuse or illicit drugs in various environmental compartments. Illicit drugs are those for which nonmedical use is prohibited by the national and international laws [101,102]. Compounds of interest in this category belong to the groups of stimulants, hallucinogens, opiods, dissociative anaesthetics, etc. [74,103] whilst key target chemicals are cocaine, heroin, nicotine, amphetamine, methamphetamine, opiates or cannabis, among others, and their metabolites, e.g. benzoylecgonine (BE), ecgonine methyl ester, norbenzoylecgonine, norcocaine and cocaethylene just to name few [103–106]. The main source of these compounds in the aquatic environment is sewage treatment plant (STP) effluents, as direct disposal in natural streams is not likely. Some classes of illicit drugs, such as cannabinoids, are highly hydrophobic, with log K_{OW} between 5 and 7.6, and can therefore be expected to bind to sewage sludge or particulate matters [80]. Conversely, other drugs of abuse, such as amphetamine and related compounds, and opioids, present medium to polar characteristics, being thus expected to partition to a higher extent in the aqueous phase.

Both gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) have been used to detect illicit drugs, but solid phase extraction, SPE (mixed reversed phase cation exchange cartridges) for sample preparation followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) has widely been accepted as technique of choice for determination of illicit drugs and metabolites from environmental matrices [102,107–109]. However, determination of illicit drugs and metabolites in water by SPE, followed by derivatization and gas chromatography ion trap tandem mass spectrometry has also been proposed [110]. The determination of these drugs in wastewater and surface water can be used for environmental forensic investigations, which is possible due to the high sensitivity of the analytical methods [109].

The disposal or excretion of products of illicit drugs consumed in a given area is mainly into local wastewater and then on to receiving surface water, soil, and sediments [57,102,111]. The detection of illicit drug residues in wastewater is purported to provide evidence-based real-time information on the nature and magnitude of community-wide illicit drug use [74,108,112].

The residues of illicit drugs found in consumers' urine that enter the sewage network with wastewater are only partially removed by STPs [102,113]. In urine drugs are generally detected in the mg L^{-1} range, but in wastewater and surface waters their concentration is much lower - at µg L^{-1} levels [114]. The decrease in concentration of illicit drugs and their metabolites in sewage effluents and surface waters depends on the technologies used in various wastewater or sewage treatment plant processes (WWTP/STP), concentrations of drugs, and the nature of the particular compound.

The information on ecotoxicity of illicit drugs in the scientific literature is scanty and not systematic. Only few reports are available until now on ecotoxicity of amphetamine, cocaine, and morphine on aquatic organisms [102]. Pal and co-workers were of the opinion that from the available reports the stimulatory or inhibitory effects of illicit drugs depend on the chemical and the test organism [102].

1.3.3. Perfluorinated Compounds

Perfluorinated compounds have been manufactured for more than 50 years and they have been released into the environment following production and use [69, 80, 84, 109]. Perfluorinated substances such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) have been widely used as building blocks in dirt-repellent coatings, non-stick coatings such as grease-repellent coatings and sprays for leather and textiles, polytetrafluoroethylene (PTFE) non-stick cookware, and fire-fighting foams [23]. PFCs are unusual chemically, in that they are both hydrophobic (repel water) and lipophobic (repel lipids/grease), and they contain one of the strongest chemical bonds (C-F) known. Because of these properties, they are highly persistent in the environment and are found in water, often groundwater, where they can accumulate [23,69].

PFCs reach the aquatic environment either through their release into rivers or via wastewater discharge into receiving waters [84]. In contrary to most brominated flame retardants (BFRs), PFCs are not efficiently eliminated along a wastewater treatment process. In fact, some authors observed a phenomenon of negative removal or in-plant production [74,115,116].

Potential health concerns of PFCs include developmental toxicity, cancer, and bioaccumulation [23, 69]. Perfluorinated compounds have been reported in surface waters throughout Europe [4,117–119) and they pose a particular problem for groundwater, where they can remain for a very long time, even if the source is stopped.

1.3.4. Artificial Sweeteners

Artificial sweeteners are used worldwide as sugar substitutes in remarkable amounts in food, beverages, and also in drugs and sanitary products, such as mouthwashes. They provide no or negligible energy and thus are ingredients of dietary products [120,121]. They are water contaminants that are highly specific to wastewater and have been considered in environmental sciences only recently. Some of the popular artificial sweeteners commonly found in aqueous environmental samples include sucralose (SUC), acesulfame (ACE), aspartame (ASP), cyclamate (CYC), neotame (NEO), neohesperidine dihydrochalcone (NHDC), saccharin (SAC).

Owing to the ionic nature of the weakly acid artificial sweeteners and the high sensitivity and selectivity of tandem mass spectrometry, most methods of analysis for the determination of artificial sweeteners at trace levels are based on the following coupling techniques: liquid chromatography–tandem mass spectrometry (LC-MS/MS) [122–125], LC–high-resolution mass spectrometry (HRMS) [126], and ion chromatography–MS/MS [127].

ACE, CYC, SAC, and SUC were detected in wastewater treatment plants in high microgram per liter concentrations. Per capita loads of individual sweeteners can vary within a wide range depending on their use in different countries. Whereas CYC and SAC are usually degraded by more than 90% during wastewater treatment, ACE and SUC pass through wastewater treatment plants mainly unchanged [120].

There are a few studies on the ecotoxicological impact of artifificial sweeteners and the studies have mainly focused on SUC [120,124,128,129], which was detected first among the artificial sweeteners in the environment. SUC is considered safe for human consumption but its effects in the ecosystem are not yet studied in depth. So far, SUC has low bioaccumulation potential and negligible acute/chronic toxicity in aquatic organisms [129,130] and does not appear toxic to plant growth [124].

1.3.5. Brominated Flame Retardants

Brominated flame retardants are a group of organic flame retardants that contain bromine. They are applied to prevent electronics, clothes, and furniture from catching fire. Brominated flame retardants include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), brominated cyclohydrocarbons, decabromodiphenyl ethers (*De*BDEs), hexabromocyclododecanes (HBCDs), and tetrabromobisphenol A (TBBPA) [131,132]. Because of their widespread presence in the environment and their potential toxicity to humans and animals, as well as their presence in locations where they are produced or used, increasing concern has prompted many countries to ban some of them [39,69,132]. As far as brominated FRs are concerned, polybrominated diphenyl ethers (PBDEs) are the most studied class [74].

The problem related to the use of brominated flame retardants (BFRs) is the recycling of wastes containing those chemicals, which are resistant to biodegradation and create the potential for long-range transport and bioaccumulation in the lipids of biota [133]. They are environmentally persistent and lipophilic and bioaccumulate in animals and humans [69].

Due to the hydrophobic characters of brominated flame retardants, GC/negative chemical ionization (NCI)-MS, GC/high resolution-MS, and GC/ion trap-MS are the most commonly used techniques for their determination, and newer techniques, such as fast tandem-GC/MS and LC/MS, have improved the analysis for higher molecular weight PBDEs [69,132].

Assessment of health risks associated with human exposure and accumulation of PBDEs is complicated and has not been adequately characterized. However, the potential risks associated with exposure to the most bioactive congeners (tri- to octa-BDE) include thyroid hormone disruption, neuro-developmental defects and cancer. Several studies have shown that PBDEs share similar general properties to organo-halogenated compounds as in vivo exposure of rodents resulted in reduction of serum total and free thyroid hormone (thyroxine T4) levels [99,134,135]. Altered thyroid hormone function, particularly during development, is profound and has been hypothesized to lead to disrupted brain development and permanent neurological damage [99,136].

1.3.6. Nanomaterials

Nanomaterials are 1 to 100 nm in size and can have unique properties, including high strength, thermal stability, low permeability, and high conductivity [69,137]. Because of their unique properties they are increasingly used in a wide range of scientific, industrial, and medical applications [23, 32, 137]. The chemical structures of nanomaterials are highly varied, including fullerenes, nanotubes, quantum dots, metal oxanes, TiO_2 nanoparticles (NPs), nanosilver, nanogold and zerovalent iron NPs.

Quantitative data on the environmental occurrence of nanomaterials is still very scarce. In addition to the possible disturbance of the state of NPs during sampling and sample preparation, the separation steps and the instrumental LODs of the approaches available are the main reasons [23,137]. Most of quantitative procedures for nanomaterials in environmental samples have been devoted to carbon-based nanomaterials. For the quantification of low-concentration fullerenes, liquid chromatography (LC) is used most [138] , combined with MS [137,139], tandem mass spectrometry (MS^2) [140], time-of-flight (TOF)-MS [141] and tandem TOF-MS (TOF-MS^2) [142]. Other mass spectrometry techniques used for measuring nanomaterials include ICP-MS and single particle-IC-PMS (for metal-containing nanomaterials) and ESI- andAPPI-MS/MS for fullerenes [69].

Almost no direct data relevant to the fate and behavior of manufactured nanomaterials in aquatic ecosystems currently exist. However, the main identified possible sources of engineered NPs in the aquatic systems are production facilities, production processes, wastewater-treatment plants and accidents during the transport and intentional releases [23,137]. In order to investigate transport and interaction of natural NPs and engineered NPs, there is a need for efficient analytical methods able to investigate real environmental conditions. In a recent study, a laser-nephelometer prototype was developed, incorporating a low-volume, flowthrough cell in order to provide a continuous record of the elution of particles from water columns [143].

Despite the success of nanotechnology, there is a lack of information about the potential health and environmental risks associated with nanomaterials [144,145]. Because of their widespread use in consumer products, it is expected that engineered NPs (ENPs) will find their way into the environment where their fate and behavior are largely unknown [137,146,147]. The small size and large surface areas of these materials increase their potential to cross cell membranes. In addition, NPs are similar in size to cellular components (e.g., ribosome) and, as such, may allosterically interact with proteins and nucleic acids.These interactions could potentially disrupt vital processes (e.g., enzyme function, and gene translation and transcription). However, their large surface area increases chemical reactivity, so they could act as carriers for other contaminants, thereby providing rapid, long-range transport [137]. Overall, the ecotoxicology studies on NPs and NMs are still very few, toxic effects have been identified in a range of fish and invertebrates that raise sufficient concern that NPs in the environment could have adverse effects on wildlife, if present at high enough levels. There are still several significant knowledge gaps in the ecotoxicology of nanomaterials [148].

1.3.7. Benzotriazoles, benzothiazoles and benzosulfonamides

These classes of compounds are the focus of our study and so only a brief discussion will be undertaken under this general group of emerging contaminants. More details about these groups of emerging contaminants are discussed in chapters three and four.

Benzotriazoles are complexing agents that are mainly used as corrosion inhibitors in dishwasher detergents, aircraft anti-icing fluids, automotive anti-freeze formulations, industrial cooling systems, metal-cutting fluids, brake fluids and as antifoggants in photography [22, 69]. The two common forms, benzotriazoles and tolyltriazole, are soluble in water, resistant to biodegradation and only partially removed in wastewater treatment systems [15,149].

Because of their water solubility, LC/MS and LC/MS/MS methods have been recently developed for their measurement in environmental waters though GC-MS and GC-MS/MS methods have also been developed [22, 69]. While reports of benzotriazoles in the aquatic environment are fairly recent (approximately last 8 years), studies indicate that they are likely ubiquitous environmental contaminants [69].

Benzotriazole has been reported to be toxic to aquatic organisms that can cause long-term adverse effects, There is also new evidence for estrogenic effects *in vitro* but, so far, not *in vivo*, in recent fish studies [69, 150].

Benzothiazole and its derivatives are also an important class of industrial chemicals. Benzothiazoles are mainly used as vulcanization accelerators in the manufacture of rubber, as pesticides, as corrosion inhibitors in antifreeze formulations, and as photosensitizers in photography [22,109,151].

Benzothiazoles have been analyzed by LC or GC coupled to different detectors, including mass spectrometry. Analytical methods based on SPE followed by GCxGC-TOF-MS [109,152] and LC-LTQ-FT Orbitrap MS [153] allow the detection of benzothiazole in natural and surface waters down to concentrations of 5 and 10 ng L^{-1}, respectively.

Benzosulfonamides are widely used as plasticizers and intermediates in the synthesis of sweeteners and can be metabolites of corrosion inhibitor [109,154].

They have been detected in wastewater and other aquatic environmental compartments. Studies on the determination and occurrence of benzosulfonamides in the aquatic environment until now are still very few.

CHAPTER 2

2.0. ANALYSIS OF EMERGING CONTAMINANTS IN THE AQUATIC ENVIRONMENT

The first step in understanding the potential chemical and biological impacts of the emerging contaminants in the environment is to identify and quantify the types of emerging contaminants that are present [155]. To do so, innovative sampling methodologies need to be coupled with analytical techniques that can confirm the identity of targeted and unknown chemicals at trace concentrations in complex environmental samples. This chapter is, therefore, devoted to a more elaborate discussion of the state of the art of analytical procedures involved in the determination of emerging contaminants in the aquatic environments.

As discussed in the preceeding chapter, emerging contaminants (ECs) are a highly relevant topic in environmental science and technology [1,15]. The analysis of environmental samples for organic pollutants is often a complicated procedure involving many steps. These steps often culminate in the use of chromatographic separation coupled to a suitable detector [156]. The occurrence of many emerging organic contaminants in environmental samples is still in trace amounts at the microgram per litre (ppb) or nanogram per litre (ppt) levels for most of the contaminants [157]. These compounds can have very different polarities and chemical properties. Screening for low levels of this large variety of compounds requires high performance from analytical instruments as well as sample preparation techniques.

In general, environmental water samples cannot be analyzed without some preliminary sample preparation because they are too dilute and too complex for an efficient separation by the chromatographic column at low detection levels. Sample pre-treatment is therefore an essential part of the whole chromatographic procedure. Its objective is to provide a sample fraction enriched in all the analytes of interest, and as free as possible from other matrix components. Moreover, before implementing any strategy, it is important to consider the strong interdependence of the various steps of the whole analytical procedure: i.e. sample handling, separation and detection. There is no unique strategy for the sample pretreatment of organic compounds in waters. It mainly depends on the nature of the solutes to be determined (e.g. volatility, polarity, molecular weight), on the nature of the matrix and on the level of concentration required. Interference removal is a critical step which depends strongly on the concentrations of the analytes of interest and of the nature of aqueous media. In other words, the strategy for determining an organic compound below the microgram per litre in drinking water will be different from the one to be used for polluted river water. It will also be guided by the separation, and more importantly the method of detection. The sample pre-treatment is still the weakest link and the time-determining step in the whole analytical procedure and the primary source of errors and discrepancies between laboratories.

It is noteworthy that the number of emerging organic pollutants found in the environment is expected to increase with continually increasing applications of synthetic organic chemicals [158]. In this context, a general framework for sampling and analytical strategies is desirable in meeting the demands for routinely monitoring of organic pollutants in the environment.

2.1. Sampling Strategies

Obtaining a sample of the matrix of interest is an often-overlooked but vital component of any environmental monitoring program. Failure to properly collect a sample can invalidate any results subsequently obtained. The main objective in any sampling strategy is to obtain a representative portion of the sample. This requires a detailed plan of how to carry out the sampling [155]. Therefore planning of the sampling strategy is an important part of the overall analytical procedure as the consequences of a poorly defined sampling strategy, could well lead to getting the wrong answer, as well as costing both time and money. A successful sampling strategy must begin with a thorough plan and established protocols. Questions that need to be addressed while planning the sampling trip include (1) selection of the sampling method to obtain a representative sample, (2) determination of the sample quantity needed to meet the minimum limits of quantification of the analytical method, (3) identification of quality control (QC) measures to be taken to address any bias introduced by the sample collection, (4) identification of safety measures that need to be taken, and (5) determination of sampling objectives.

While natural water would appear to be homogeneous this is not in fact the case. Natural water is heterogeneous, both spatially and temporally, making it extremely difficult to obtain representative samples. Stratification within oceans, lakes and rivers is common with variations in flow, chemical composition and temperature. Variations with respect to time (temporal) can occur, for example because of heavy precipitation (snow, rain) and seasonal changes. The most common method for collecting surface water samples is taking grab or spot samples. This may involve taking a single sample or a composite sample representative of a width- and depth-integrated profile. Collecting a sample by hand directly into the shipping sample container is the easiest method, especially in small, wadeable streams. In deeper water such as lakes and reservoirs, samples are often taken using bailers or thief samplers. Groundwater samples are generally collected from existing supply wells or monitoring wells. The sampling methods vary depending on water depth and well size. Because monitoring wells are generally small, sampling is less frequent. Automated samplers are often used due to their ease of use at multiple sites. Wastewater sampling is generally performed by one of two methods, grab sampling or composite sampling. Grab samples are discrete samples per discharge event while composite samples are individual discrete samples collected at regular intervals over a period of time (usually 24 hours) and will therefore represent the average performance of a wastewater treatment plant for the collection period.

2.2. Sample Preparation

Trace analysis of organic contaminants in environmental samples is always challenging due to the complexity and diversity of sample matrices. Because matrix effects might exert a detrimental impact on important method parameters as limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision, sample pre-treatments involving isolation of analytes, purification of extracts and preconcentration are required [159]. In addition, concentrations of emerging contaminants(ECs) found in the water samples are typically below the $\mu g\ L^{-1}$ range, making extraction, pre-concentration, and cleanup prior to detection very important steps [155]. There are several goals of sample extraction prior to instrument analysis [132,160,161]. Firstly, it is often necessary to separate the target organic compounds from a complex aqueous matrix such as sewage and marine water samples to remove interfering components. Secondly, enrichment of the target analytes is important, especially when analytes in trace levels are to be determined. Sample extraction and concentration steps are applied to improve determination sensitivity. Finally, the compatibility between the sample matrix and the instrumental analysis must be considered. For example, generally aqueous samples are not immediately analyzed with GC analysis in which a solvent extraction procedure is normally applied. In this regard, the shortcomings of liquid–liquid extraction (LLE) are well known (such as the consumption of huge amounts of potentially toxic organic solvents), and have been the motivation toward developing new, alternative procedures. Solid-phase extraction (SPE) employing an adsorptive solid extractive phase in the form of a cartridge or a disk has, on the other hand, significantly reduced the amount of solvents utilized compared with LLE. Therefore, SPE has been shown to be a better choice in many extraction situations and has gained popularity in spite of its high-price products. Nevertheless, LLE has also been used for the extraction of emerging organic contaminants.

2.2.1. Liquid-Liquid Extraction

Liquid-Liquid Extraction is a traditional technique to extract organic compounds from aqueous samples. The fundamental principle for liquid-liquid extraction (LLE) lies in the partition of target analytes in two immiscible liquid phases [162]. A review of the organic-group interactions that can be used to identify the desirable functional group(s) in a solvent for any given solute and screen potentially suitable solvents was carried out by Robbins [163]. In general, a high distribution coefficient, good selectivity and little miscibility with the sample matrix under consideration are the preferable attributes that a solvent should possess. Due to its tedious procedure and large amount of organic solvent consumed, LLE is being replaced by other extraction techniques.

2.2.2. Solid Phase Extraction

Because many hydrophilic emerging contaminants do not partition into an organic solvent from the aqueous phase, resulting in poor extraction efficiencies, SPE rather than LLE are often used. Compared to LLE, SPE is a modern extraction technique and has become the most common sample preparation technique in environmental analysis [164,165]. SPE offers lower solvent consumption, shorter processing times, automation options, higher recoveries, and simpler procedures than LLE. Other advantages of SPE over LLE are elimination of emulsions, improved selectivity, specificity and reproducibility. Since direct sampling in the field is an option for SPE, the need for transport and storage of large sample volumes of water to the laboratory can be avoided [166,167]. Field-portable SPE can reduce the possibility of degradation of target analytes during sample holding times after sample collection. Solid phase extraction (SPE) techniques have been developed to replace many traditional liquid-liquid extraction methods for the determination of organic analytes in aqueous samples. An excellent review of the use of SPE has been written by Liska and co-workers [168]. SPE methods utilize bonded silica or resin solid sorbents packed into disposable plastic or glass cartridges or imbedded into Teflon or glass fibre disks. The SPE method requires a measured volume of the aqueous sample to be passed through a cartridge tube packed with a suitable solid adsorbent material. The organic pollutants in the sample are adsorbed onto the solid surface from which they are eluted by a properly selected solvent. The sample is applied at the top of the tube and drawn through the bed by a syringe or vacuum, maintaining a flow rate of 1–2 drops/second. Alternatively, larger pore size particles may be used to allow fast flow rates for large volume samples. The tube is washed with a nonpolar solvent for polar analytes, and with a polar solvent for non-polar analytes. Finally, the analytes are eluted out of the column by a suitable solvent. Polar solvents should be used for polar analytes, and nonpolar solvents for nonpolar analytes. The sample extracts may be concentrated further by evaporation of the solvent.

The choice of sorbent in SPE is critical since it controls selectivity, affinity and capacity. The selection of adsorbent-packing material is based on the polarity of the pollutants to be analyzed. The nonpolar hydrophobic adsorbents retain the nonpolar analytes and allow the polar substances to pass through the column. The hydrophilic adsorbents adsorb the polar components, allowing the nonpolar materials to pass through. Classical SPE sorbents include chemically bonded silica with the C8 or C18 organic group, carbon, and ion-exchange materials. More recently, polymeric materials, immunosorbents, molecularly-imprinted polymers, and restricted access materials have been introduced to improve the extraction efficiency of target compounds [169,170].

2.2.3. Other Extraction techniques

Other extraction techniques used for the extraction of organic contaminants in the aquatic environment include liquid-phase microextraction (LPME), dispersive Liquid-

liquid microextraction (DLLME), solid phase microextraction (SPME), stir bar sorptive extraction (SBSE). Details about these extraction techniques are described in these references [161,171,172].

2.3. Detection techniques

Most detection techniques for emerging contaminants are based on mass spectrometry, which has become the preferred method in environmental analysis due to the inherent complexity of most environmental samples. Chromatography, including gas chromatography (GC) and high performance liquid chromatography (HPLC) is the dominant modern analytical technique. Although both liquid chromatography and gas chromatography continue to be the leading techniques for the separation of pollutants in the aquatic environment, liquid chromatography is becoming more and more the focus of research with detection almost exclusively based on MS, especially MS/MS, in order to fulfil the requirements for unequivocal confirmation of target compounds [3,20,173]. This rapid rise in the use of LC-MS is due to the capability of LC-MS for the determination of polar analytes without the need for any prior derivatization and subsequent to this advantage; LC-MS is particularly attractive when simultaneously analyzing compounds belonging to structurally distinct groups whose determination by GC-MS would involve more than one derivatization reaction.

In GC analysis, by comparing their mass spectra with mass spectral libraries, the full scan mode of MS is employed for identifying compound structures, based on interpreting abundant fragment-ion patterns. For quantification, selective ion mode (SIM) of MS can be applied to achieve high sensitivity. More recently, to improve determination selectivity and sensitivity, triple quadrupole (QqQ) MS has been introduced in GC analysis. For unbiased determination and confirmation of chemical structures of metabolites, biodegradation, and transformation products, high-resolution MS is also employed [132].

LC-MS/MS techniques, mainly triple quadrupole and to a lesser extent ion trap (IT), are today prevalent choices for reliable determination of emerging polar organic compounds in the environment [3, 159,173]. The extremely high selectivity and sensitivity of MRM techniques allow trace constituents of complex mixtures to be determined. Among the possible ionization techniques, electrospray ionization (ESI) is by far the most widely used as compared with atmospheric pressure chemical ionization (APCI) or the more recent atmospheric pressure photo ionization (APPI). More advanced MS techniques, such as time of flight MS (TOF-MS), etc. have also been introduced, making a new powerful identification tool available; one new hybrid quadrupole-time of flight mass spectrometer (QqToF) permits the acquisition of full-scan product ion spectra, with the accurate mass of the product ions. Based on the product ion spectra, structure elucidation of unknown compounds as well as identification of target compounds can be obtained with a much higher degree of certainty [132,175]. Using liquid chromatography combined with mass spectrometry, a vast number of reliable analytical methods have been developed for the determination of emerging pollutants and studies of their occurrence in the environment.

Due to the highly sensitivity and selectivity of liquid chromatography- tandem mass spectrometry for the analysis of emerging organic contaminants in complex environmental matrices, LC-MS/MS instrumental technique was adopted in this study and the basics of the technique will be discussed in the following section.

2.4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The heart of the HPLC system is the column where the actual separation occurs. A mobile phase is pumped from the reservoir, through an injector, into the column, and out to the detector. A sample dissolved in the mobile phase or a similar solvent is injected into the flowing mobile phase on the column, separation occurs that is specific for that of column and the separated peaks elute flowing into the detector causes a signal to be sent to the data system.

A block diagram of an HPLC system, illustrating its major components is shown in Fig. 2.1.

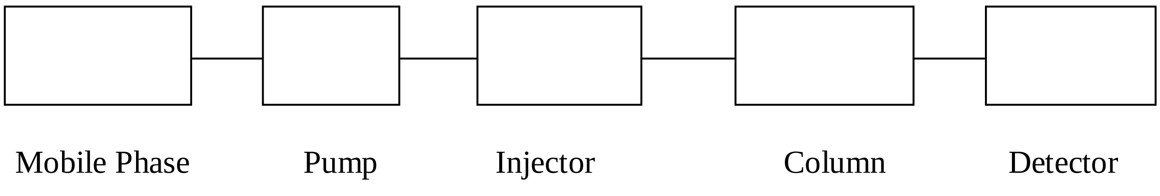


Fig 2.1: Block diagram of a typical HPLC system

2.4.1. Separation Mechanisms in Liquid Chromatography

A useful classification of the various LC techniques is based on the type of distribution mechanism applied in the separation.

2.4.1.1. Normal Phase HPLC

Normal phase HPLC is suitable for the analysis of relatively non-polar compounds. The retention of the components in a mixture increases with the increasing polarity of the compounds .Silica gel is used as an adsorbent in most applications and the mobile phase solvent can be arranged in a so-called elutropic series going from weak eluents such as pentane and hexane, through moderate eluents, such as chloroform and dichloromethane, to polar eluents, such as propanol and methanol. The retention of the analyte can be adjusted by the mixture of a polar solvent with a moderate to weak solvent and the mechanism of the retention of the analytes is well described by Snyder [176].

2.4.1.2. Reversed Phase HPLC

Reversed phase HPLC is the most commonly used LC method, it accounts for around 80% of all HPLC separations. Reversed-phase LC is ideally suited for the analysis of polar and ionogenic analytes, and as such is ideally suited to be applied in LC-MS. The non-polar stationary phase is a chemically modified silicabase, i.e. the silanol groups on the surface are modified by reaction with different alkyl, and aryl group side chains to produce a range of selective stationary phases. The most commonly used is the octadecyl (C18) or octyl (C8) functional group with a range of column diameters and lengths. Due to the steric hindrance of these functional groups, there is still a free silanol group present and it will interact with the analytes causing peak tailing. To minimize this effect end capping is introduced to cover the silanol group [177]. Other non polar packing materials, such as styrene-divinylbenzene copolymers or hybrid silicon-carbon particles are also used. The retention decreases with increasing polarity of the analyte. Mixtures of water or aqueous buffers and an organic modifier (methanol, acetonitrile, or tetrahydrofuran (THF)) are used as eluent. The percentage and type of organic modifier is the most important parameter in adjusting the retention of nonionic analytes.

2.4.2. Tandem Mass Spectrometry

For this research work, we used a tandem mass spectrometer for the detection and quantification of compounds of study. It is therefore worthwhile to describe briefly this range of mass spectrometric techniques. Tandem mass spectrometry (MS/MS) is a term which covers a number of techniques in which one stage of mass spectrometry, not necessarily the first is used to isolate an ion of interest and a second stage is then used to probe the relationship of this ion with others from which it may have been generated or which it may generate on decomposition [178].

A triple quadrupole is probably the most widely used MS/MS instrument and consists of three sets of quadrupole rods in series. The second set of rods is not used as a mass separation device but as a collision cell, where fragmentation of ions transmitted by the first set of quadrupole rods is carried out, and as a device for focussing any product ions into the third set of quadrupole rods. Both sets of rods may be controlled to allow the transmission of ions of a single *m/z* ratio or a range of *m/z* values to give the desired analytical information. Other types of MS/MS being used in conjunction with LC include quadrupole-time -of -flight instrument, tandem mass spectrometry on the Ion-trap, the hybrid mass spectrometer.

2.4.3. MS/MS Techniques

2.4.3.1. Precursor- Ion Scan: In this scan, the second stage of mass spectrometry (MS_3) is set to transmit a single *m/z* ratio, namely that of the product (fragment) ion of interest, while the first stage (MS_1) is set to scan through the mass range of interest, with the fragmentation of ions passing through MS_1 being again carried out in MS_2, the collision cell. A signal is seen at the detector only when ions are being transmitted by both MS_1 and MS_3, i.e. when an ion, being transmitted by MS_1, fragments to give the desired ion.

2.4.3.2. Product-Ion Scan: the first stage of mass spectrometry MS_1 is used to isolate an ion of interest – in LC–MS, this is often the molecular species from the analyte. Fragmentation of the ion is then effected; the means by which this is achieved is dependent on the type of instrument being used but is often by collision with gas molecules in a collision cell, i.e. MS_2 in the triple quadrupole. The second-stage mass spectrometer MS_3 is scanned to provide a mass spectrum of the ions formed in the collision cell, i.e. the product (fragment) ions.

2.4.3.3. Selected Reaction Monitoring: the fragmentation of a selected precursor ion to a selected product ion is monitored. This is carried out by setting each of the stages of mass spectrometry to transmit a single ion, i.e. the precursor ion by MS_1 and the product ion by MS_3.

CHAPTER 3

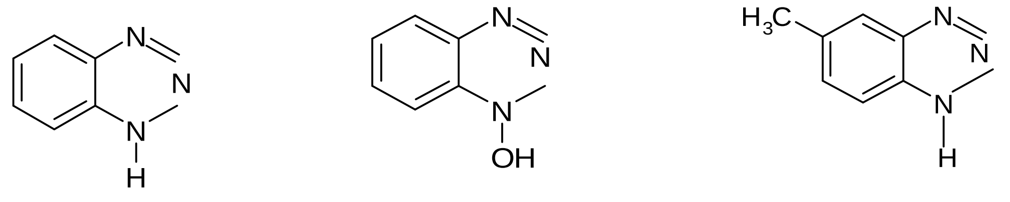
BENZOTRIAZOLES, BENZOTHIAZOLES AND BENZOSULFONAMIDES AS EMERGING CONTAMINANTS

3.1. BENZOTRIAZOLES: STRUCTURES, PROPERTIES, USES AND TOXICITY

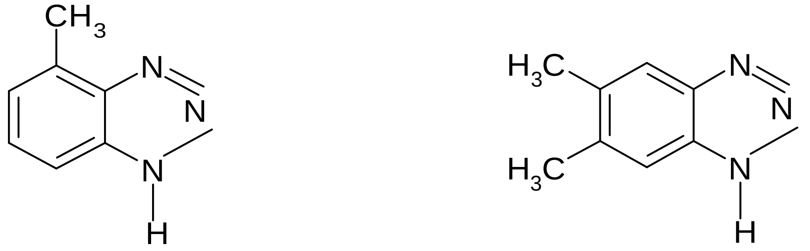
With a large number of applications of synthetic chemicals in human daily lives, as well as in industrial and agricultural production, various emerging contaminants are continually being identified [179]. Benzotriazole (1H-BTR) is a typical emerging contaminant [20]. Benzotriazole and its methyl substituted compounds are regarded as potential hazards to aquatic organisms, and can cause long-term adverse effects in aquatic environments [180,181]. Benzotriazole first became a concern because of the contamination caused by aircraft deicing fluids of which the primary agents were benzotriazole and its derivatives [182]. Benzotriazole and its derivatives are anti-corrosive agents used in aircraft deicing and antifreeze fluids (ADAFs), which are critical to the safety and smooth running of airports in cold climates. As such, ADAFs are used in extremely large quantities. It was estimated that 3,785 L are used each time a large passenger jet is de-iced, and 8,000,000 L of these chemicals are used per year in Canada alone [183]. In addition, the annual production of benzotriazole is approximately 9000 tons worldwide [149,179].

3.1.1. Structures of benzotriazoles

Benzotriazole compounds are derivatives of a benzene ring on which a vicinal pair of carbon atoms covalently bond to three nitrogen atoms in a five membered ring [184]. Benzotriazole compounds are heterocyclic compounds, with the parent compound benzotriazole having the formula $C_6H_4N_3H$. As shown in the structures in Fig 3.1, there are three lone-pairs of electrons in the triazole ring and these electrons polarise the molecule. Some of the derivatives of benzotriazole include 5-Methylbenzotriazole, 1-Hydroxybenzotriazole, 5,6-Dimethylbenzotriazole



1H-benzotriazole 1-Hydroxybenzotriazole 5-Methyl-1H-benzotriazole



4-Methyl-1H-benzotriazole 5,6-Dimethyl-1H-benzotriazole

Fig. 3.1 : Structures of benzotriazoles

3.1.2. Properties of benzotriazoles

Benzotriazoles are polarized molecules with three lone pairs of electrons on three nitrogen atoms. They are generally stable to heat, ultraviolet radiation, and chemical or bacterial attack which makes them persistent xenobiotics under environmental conditions. They are significantly soluble in water, highly soluble in methanol and possess very low vapour pressure [184,185]. They possess N-H acidity which gives them the capability to function as complexing agents [184]. The two common forms, benzotriazole and tolytriazole, are soluble in water, resistant to biodegradation, and are only partially removed in wastewater treatment [12,14,186,187]. Although benzotriazoles have been shown to have high affinity for an organic phase, they still possess polar character and this property makes them unusual and stems from their strong permanent dipole moments [184]. Benzotriazoles have relatively low values of $\log K_{ow}$ as shown in Table 3.1 and so are expected to be highly mobile in surface water, groundwater and soil [188].

Table 3.1: Properties of Benzotriazoles

Compound	**Benzotriazole**	**1-Hydroxybenzotriazole**	**5-Methylbenzotriazole**	**5,6,-Dimethylbenzotriazole**
CAS No.	95-14-7	2592-95-2	136-85-6	4184-79-6
Other names	1,2,3-Benzotriazole; Aminoazophenylene; Aziminobenzene; Cobratec 99	1H-benzotriazole, 1-hydroxy; 1H-1,2,3-Benzotriazol-1-ol; Benzotriazolol	Tolyltriazole; 1H-Benzotriazole, 5-methyl-;5-Methyl-1H-benzotriazole; tolutriazole; Cobratec tt-100	1H-1,2,3-benzotriazole, 5,6-dimethyl; 5,6-Dimethyl-1H-benzotriazole; 5,6-Dimethyl-1H-benzotriazole
Mol. formula	$C_6H_5N_3$	$C_6H_5N_3O$	$C_7H_7N_3$	$C_8H_9N_3$
Mol. Weight	119.12	135.13	113.15	147.17
Log K_{ow}	1.44	0.11	1.71	2.26
pK_a	8.4	7.39	8.66	9.28
Water Solubility (g L^{-1})	19.8	70.7	3.06	0.9
Henry's Law constant (Pa* m^3/mole)	0.015	1.8 $\times 10^{-5}$	0.016	0.0016

(from http//:www.syrres.com/escphysdemo.htm)

3.1.3. Uses of Benzotriazoles

The production and use of benzotriazoles have increased to the point that the United States Environmental Protection Agency (USEPA) has classified them as High Production Volume Chemicals [189]. They have been used in various applications, the three primary uses being corrosion inhibitors in dishwasher detergents, aircraft anti-icing fluids, automotive anti-freeze formulations, industrial cooling systems, metal-cutting fluids, brake fluids, UV-light stabilizer for plastics, and anti-foggant in photography [22]. They have been reported to be the primary additives of environmental concern in aircraft de-icing/anti-icing fluids (ADAF) which are used to reduce flammability hazards and to protect electronic components in the fuselage [182,184,190−192]. As a result of the N-H acidity of benzotriazoles which gives them the capability to function as complexing agents [184], benzotriazole and tolyltriazole are used in the metal finishing industry, in semiconductor industry [193] and in milk processing [194].

3.1.4. Toxicity of benzotriazoles

Benzotriazole has been reported to be toxic to aquatic organisms that can cause long-term adverse effects. It is hydrophilic, not biodegradable in a municipal waste treatment plant and no evidence of biodegradation by elective culture and continuous enrichment with bacteria [195,196] even though there are reports of its photodegradation [197,198]. Some of the first toxicity data produced regarding benzotriazole and its derivatives involved isolation of a number of ADAF fractions to determine their contribution towards microtox activity using the Microtox bioassay [183]. Acute toxicity assays have revealed that after 96h, the median lethal concentration (LC_{50}) was 65 mg L^{-1} of benzotriazole for the fathead minnow (*Pimephales promelas*), with a corresponding no-observable-adverse-effect (NOEC) concentration of 46 mg L^{-1} whereas benzotriazole was less sensitive to the water flea (*Ceriodaphnia dubia*), with a LC_{50} of 102 mg L^{-1} [181]. These concentrations are very high and would suggest that benzotriazoles are likely to pose an acute threat to the wildlife. However, very little chronic toxicity data is available to base a sound ecological risk assessment. A chronic 21 day reproduction toxicity test using *Daphnia magna* resulted in a NOEC value of 3 mg L^{-1} and based on these data, a predicted no-effect concentration (PNEC) of 0.06 mg L^{-1} was calculated for benzotriazole [195,199,200].

Benzotriazole has been described as possessing clear antiestrogenic properties *in vitro* assays conducted using a recombinant yeast but no evidence of such antiestrogenic activity was observed *in vivo* study with fathead minnow (*Pimephales promelas*) [150]. The developmental effects of benzotriazole to the marine invertebrate *Ciona intestinalis* (Chordata, Ascidiae) has also been assessed [201]. The 24-hour NOEC and LOEC (Lowest observable effect concentration) values based on embryo morphological development were 100 and >100 mg L^{-1} respectively while after 48h, the NOEC and LOEC values were 10 and 32 mg/L, respectively. The structural similarity of benzotriazoles with naturally occurring substances (such as adenine, guanine, indole) suggests that they could affect nervous and endocrine systems and inhibit the synthesis of proteins, enzymes and RNA in mammalian systems [185,190].

Benzotriazoles have also been identified as possible carcinogenic substances and have been shown to induce toxic responses in fish, invertebrates, marine and soil bacteria, at relatively low concentrations [185,202–204]. Benzotriazole has been found to induce VTG and CYP19a gene expression, but inhibit the CY1A1 gene expression [205]. These results imply that benzotriazole has adverse potential on the marine (seawater) medaka’s endocrine system, which may lead to adverse effects, including compromised growth and reproduction, altered development, abnormal behaviour, and alterations in hormone biosynthesis.

The toxic effects of benzotriazoles in vegetation are not totally understood but due to their possession of some similarity to natural plant growth regulators such as auxin and cytokinin, they can cause damage to the root system [206]. Benzotriazoles concentrations greater than 100 mg L^{-1} in the aqueous phase could be toxic to vegetative systems [207]. A damage to the root system leading to growth inhibition was the main effect observed. In consideration of its widespread use, high solubility and persistence in water and high bioaccumulation, concerns about the occurrence, accumulation and risks of benzotriazole and its derivatives to aquatic organisms have been raised.

From the available ecotoxicity data of benzotriazoles and its derivatives, their entry into the aquatic environment could present negative effects on the aquatic organisms

3.2. BENZOTHIAZOLES: STRUCTURES, PROPERTIES, USES AND TOXICITY

3.2.1. Structures of benzothiazoles

Benzothiazoles form a part of xenobiotic, heterocyclic, molecular structures comprising a benzene ring fused with a thiazole ring.

Benzothiazole 2-Hydroxybenzothiazole

2-Aminobenzothiazole 2-(Methylthio)benzothiazole

Fig 3.2: Structures of benzothiazoles

3.2.2. Properties of benzothiazoles

The benzothiazoles covered in this study are moderately polar and slightly hydrophobic compounds (Table 3.2). Therefore, some (BTH, 2-OH-BTH, 2-Amino-BTH) are expected to be moderately soluble in water. Some of the compounds have also been observed to be resistant to biodegradation and only partially removed during wastewater treatment [208,209].

3.2.3. Uses of Benzothiazoles

2-substituted benzothiazoles are a group of widely used industrial chemicals, primarily as vulcanization accelerators in tire production and are present in all kinds of rubber products [210]. They are also used as bio-corrosion inhibitors in cooling systems, in paper manufacturing and as ingredients in anti-freeze [211]. Aminobenzothiazole (2-AminoBTH) in particular is used in the manufacturing of some disperse azo dyes, which are prepared by diazotization of aminobenzothiazoles followed by coupling with N,N-dialkylaniline and for the preparation of pesticides.

Table 3.2: Properties of benzothiazoles

Compound	**Benzothiazole**	**2-Hydroxybenzothiazole**	**2-Aminobenzothiazole**	**2-(Methylthio)benzothiazole**
CAS No.	95-16-9	934-34-9	136-95-8	615-22-5
Other names	1,3-Benzothiazole; Benzothiazol; Benzosulfonazole	1,3-Benzothiazol-2-ol; 2-Hydroxy-1,3-benzothiazole; S-Orthophenylene thiocarbamate	1,3-Benzothiazol-2-Amine;2-Benzothiazolamine; o-Aminobenzothiazole	2-(methylthio)-1,3-benzothiazole; 2-(methylsulfanyl)-1,3-benzothiazole; 2-(Methylmercapto)benzothiazole
Mol. formula	$C_7H_5N_6$	C_7H_5NOS	$C_7H_6N_2S$	$C_8H_7NS_2$
Mol. Weight	135.19	151.19	150.2	181.28
Log K_{ow}	2.01	1.76	2.0	3.15
pK_a	-	-	-	-
Water Solubility (g L^{-1})	4.3	2.35	1.48	0.125
Henry's Law constant (Pa* m^3/mole)	3.7×10^{-2}	3.9×10^{-3}	1.32×10^{-5}	1.9×10^{-3}

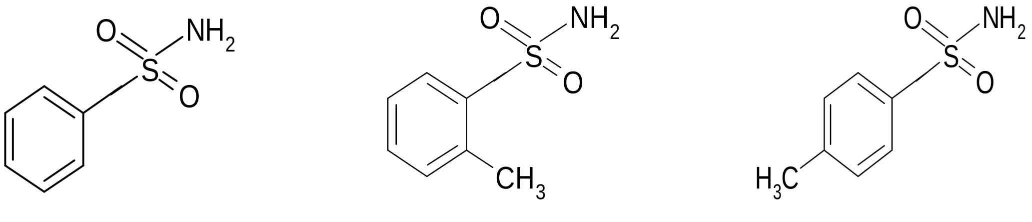
3.2.4. Toxicity of Benzothiazoles

The toxicological and ecotoxicological effects of benzothiazioles derivatives were described by Reemtsma et al. [212] concerning bacteria (*Vibro fischeri*) and inhibition of nitrification. Luminescence inhibition (EC_{50}) of *V. fischeri* was measured and was found to be 6 μg L^{-1} for Thiocyanomethylthiobenzothiazole and 3 mg L^{-1} for benzothiazole. They also found an inhibition of nitrification in sediment columns at concentrations of 0.1-0.3 mg L^{-1} for the benzothiazoles. Hendricks et al. tested 2-(methylthio)benzothiazole and benzothiazole for acute toxicity on *Daphnia magna* and found EC_{50} values of 10 and 50 mg L^{-1} for 2-methylbenzothiazole and benzothiazole respectively [213]. Reemtsma and co-workers concluded that 2-substituted benzothiazoles are not completely removable by biological wastewater treatment and are of concern for aquatic environment due to their limited biodegradability and potential toxicity [212].

3.3. BENZOSULFONAMIDES: STRUCTURES, PROPERTIES, USES AND TOXICITY

3.3.1. Structures of benzosulfonamides

p-TSA is formed by reaction of *p*-toluenesulfonyl chloride with ammonia [214]. All processes are in a closed system except for drying and packaging. The structures of benzenesulfonamide and its derivatives (o-TSA and p-TSA) are displayed in Fig 3.3



Benzenesulfonamide o-Toluenesulfonamide p-Toluenesulfonamide

Fig. 3.3: Structures of benzosulfonamides

3.3.2. Properties of benzosulfonamides

Benzosulfonamides (BSA, o-TSA and p-TSA) are a group of relatively high polar compounds as depicted by their respective low octanol-water partition coefficient (Table 3.3). They are therefore expected to be mobile in the aquatic environments, p-toluenesulfonamide has been shown to decrease significantly during wastewater treatment processes [154,215].

Table 3.3: Properties of Benzosulfonamides

Compound	**Benzenesulfonamide**	**o-Tolunesulfonamide**	**p-Toluenesulfonamide**
CAS No.	98-10-2	88-19-7	70-55-3
Other names	Benzolsulfonamide; Benzosulfonamide; Benzolsulfonamid	2-Methylbenzenesulfonamide; Benzenesulfonamide, 2-methyl;2-Toluenesulfonamide; Toluene-2-sulphonamide	4-Methylbenzenesulfonamide; 4-Toluenesulfonamide; p-Tosylamide
Mol. formula	$C_6H_7NO_2S$	$C_7H_9NO_2S$	$C_7H_9NO_2S$
Mol. Weight	157.19	171.22	171.22
Log K_{ow}	0.31	0.84	0.82
pK_a	10.1	-	10.2
Water Solubility (g L^{-1})	4.3	1.62	3.16
Henry's Law constant (Pa* m^3/mole)	4.22×10^{-2}	4.7×10^{-2}	4.7×10^{-2}

(from www.syrres.com)

3.3.3. Uses of Benzosulfonamides

Benzene- and toluenesulfonamides (BTS), especially *p*-Toluenesulfonamide (*p*-TSA), o-Toluenesulfonamide (*o*-TSA) and Benzenesulfonamide (BSA) have a broad application as industrial chemicals. *p*-TSA is used as a plasticizer, an intermediate for pesticides and drugs, and is the primary degradation product of the disinfectant chloramine-T (*N*-sodium-*N*-chloro-*p*-toluenesulfonamides) in water. Chloramine-T is used as an antimicrobial agent in the food industry to disinfect surfaces, instruments and machinery and also a therapeutic drug for bacterial gill diseases of fish species, and for bacterial disease of swine and poultry [154,216,217]. The main application for *o*-TSA is its use for the production of the artificial sweetener saccharin. BSA is used for synthesizing dyes, photo chemicals and disinfectants [216,218]. *p*-TSA is used in the formulation of toluenesulfonamide/formaldehyde resin (TSFR), which is used in fingernail polishes and enamels at concentrations up to 10% [216].

3.3.4. Toxicity of Benzosulfonamides

Studies on ecotoxicology of benzosulfonamides are very scarce at present. However, the toxicological relevance of p-TSA was tested by the German Federal Environment Protection Agency (UBA) and the UBA recommends a maximum allowed concentration of 0.30 $\mu g\ L^{-1}$ for drinking water. For o-TSA and BSA the toxicological relevance has not been tested yet and the drinking water limit of 0.10 $\mu g\ L^{-1}$ for unknown substances can be applied. It has also been shown that o-TSA and BSA are not removed during waste- and drinking water treatment [154,215,219].

The LD50 for *p*-TSA in mice was found to be 400 mg L^{-1} and in rats, the oral LD50 was reported to be greater than 2000 mg L^{-1} [216].

CHAPTER 4

REVIEW OF ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF BENZOTRIAZOLES, BENZOTHIAZOLES AND BENZOSULFONAMIDES IN THE AQUATIC ENVIRONMENT

4.1. INTRODUCTION

Benzotriazoles, benzothiazoles and benzosulfonamides are classes of organic compounds that belong to groups of compounds generally referred to as *emerging contaminants* [109,159]. It is therefore imperative to review the different analytical approaches present in the literature for their determination and the concentration levels at which the compounds occur in the aquatic environment. In addition, their removal rate in wastewater treatment plants is also reviewed.

The issue of emerging contaminants is closely related to analytical performance concerning their monitoring in various environmental compartments. With the development of sophisticated and sensitive analytical apparatus and procedures, more and more emerging pollutants can be detected at trace levels in the environment [9,220]. Consequently, a number of new or previously ignored and/or unrecognized contaminants have been brought under scrutiny. However, it is still necessary to further improve rapid and sensitive analytical procedures, in particular in two directions: on the one hand, high sensitivity at ultra-trace levels and on the other hand ,versatility in simultaneous screening for a wide variety of compounds with large differences in physicochemical properties (e.g. log K_{ow}, high water solubility, zwitterionic form according to pKa) [221]. Continual improvements in analytical equipment and capabilities have brought within the realm of possibility the detection and quantification of virtually any contaminant including benzotriazoles, benzothiazoles and benzosulfonamides at lower and lower levels in the environment [49].

Mass Spectrometry (MS) has revolutionalized environmental analytical chemistry by allowing the analysis of complex organic mixtures for trace amounts of analytes due to confirmation capability. Currently, the detection and quantification of organic contaminants at trace levels in environmental water samples, often require the use of chromatographic techniques (gas chromatography and liquid chromatography) hyphenated to mass spectrometry using several types of analyzers or hybrid instruments. Gas chromatography-mass spectrometry methods have greatly contributed to the characterization of small apolar (except when derivatization step is used) contaminants in water whereas liquid chromatography mass spectrometry methods have been more recently utilized to extend the investigation of water contaminants to non-volatile, (highly) polar and thermally labile compounds thus allowing the detection of analytes that were not routinely analyzed in the past, many of which are new emerging pollutants [3,9,15,23,34,39,58,222].

Nowadays, the major challenge is the analysis of highly polar compounds at trace concentration levels in the aqueous environmental samples. This challenge is as a result of the shift towards the use of more hydrophilic compounds in industrial and consumer applications. Thus, the development of appropriate analytical methods that can deal with specific properties and also a multitude of different chemicals with differing properties, is urgently required in the environmental analysis of emerging pollutants [15,222].

Reports on the fate of emerging chemicals like benzotriazoles, benzothiazoles and benzosulfonamides in sewage treatment plants (STP) showed that they may only be partially removed during conventional treatment [14,55,59,60,149,154,223,224]. Evidence has been accumulating, depicting that these emerging chemicals appear in natural waters through municipal, agricultural and industrial wastewater discharges. Treated effluents (and in some cases raw sewage) are discharged into receiving waters, including small streams, rivers, lakes and drinking water supplies [53,55,59,62,63]. Due to widespread occurrence of benzotriazoles, benzothiazoles and benzosulfonamides in the aquatic environment, they have been considered emerging contaminants. In the following sections and subsections, the reviews of literatures on their analytical methodologies of determination, occurrence in the environment and removal efficiencies in the wastewater treatment plants will be undertaken.

4.2. ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF BENZOTRIAZOLES IN THE AQUATIC ENVIRONMENT

There has been an increasing interest in the determination and occurrence of benzotriazoles which is a class of emerging contaminant. For many years chemicals of the benzotriazole group have commonly been used as industrial chemicals with versatile applications for technical and commercial production. Benzotriazoles contain a five-membered ring with three nitrogen atoms directly bonded to one another as substituents on a benzene ring (Fig 3.1). In other words, they are derivatives of a five-member heterocyclic compound containing three nitrogen atoms and a vicinal pair of carbon atoms.

The two common forms (benzotriazole and tolytriazole) are soluble in water, resistant to biodegradation, and are only partially removed in wastewater treatment [12,14,186, 187]. Because of their water solubility and poor biodegradability in wastewater treatment plants, these emerging contaminants have been detected in various environmental water samples including wastewaters, surface water and groundwater [4,12,14,15,153,223,225]. The compounds have also been found to occur at environmental level concentrations in soil, sediment and sludge [226,227,228]. More details about the properties, uses and occurrence of these compounds in the environment have been discussed in Chapter 3.

Therefore, due to the widely acknowledged persistence, high resistance to biodegradation, ubiquitous occurrence, and potential acute and chronic toxicity of benzotriazole and its common derivatives in the environment, it is worthwhile to review the current analytical methodologies, so far available, for the determination of these compounds in the aqueous and solid environmental samples and to determine the concentration levels at which they appear in the aquatic environment.

4.2.1. Extraction of Benzotriazoles from Aqueous Samples

In very dilute water samples, preconcentration procedure is indispensable. Solid phase extraction is widely used in environmental analytical field because it extracts and pre-concentrates in a single step. Solid phase extraction, so far, is the most widely and frequently used sample pretreatment and preconcentration procedure for the determination of benzotriazole and its common derivatives in environmental water samples. In few cases, other extraction procedures, such as liquid-liquid extraction (LLE), mixed mode solid phase extraction, dispersive liquid-liquid microextraction (DLLME) have also been exploited [12,153,180,229,230]. The most common SPE sorbents used for the analysis of benzotriazoles in environmental water samples are polymeric sorbents due to their intrinsic advantages over silica-based sorbents. The inherent advantages include stability over a wider pH range, capability to retain larger quantity of analytes, different modes of separation mechanisms. Nevertheless, this technique (SPE) also presents some disadvantages such as increased chances of losses during sample handling and the requirement of large volume samples [231].

On-line SPE strategies coupled to an LC system appear to solve the drawbacks exhibited by the classical SPE procedure and it has been used for the extraction and preconcentration of benzotriazoles in environmental water samples [232]. The benefits of this on-line extraction system versus the conventional SPE methodology are numerous and important. First, the tedious conventional SPE procedure is automated, and the operator only needs to place the sample vial in the apparatus and this automation minimizes sample loss or contamination during sample handling and improves the reproducibility of the analysis. In addition, the reduction of both the required sample volume and time of the analysis is achieved.

Moreover, since modern trends in analytical chemistry are approached towards the simplification of sample preparation, microextraction techniques are playing an important role in the determination of different pollutants. A novel microextraction technique named dispersive liquid-liquid microextraction (DLLME) was developed by Assadi and co-workers [233] and the technique was adopted for the extraction and preconcentration of benzotriazoles and benzothiazoles in environmental water samples by Pena and co- researchers [230]. DLLME offers several important advantages over the classical solvent extraction methods, which include faster operation, no need of large amount of organic extraction solvents, low time and cost and easier linkage to most analytical methods.

Moreover, some researchers have also used either direct injection of water samples or liquid-liquid extraction (LLE) methods but these methods were characterized with low sensitivity, high limits of quantification and occurrence of matrix effect especially when mass spectrometry was used as detection method [12,234].

Weiss and Reemtsma carried out an extraction of benzotriazole and the two isomers of tolyltriazole (4 and 5-tolyltriazole) in environmental waters using 60-mg Oasis HLB cartridges [12]. Recoveries were determined at two concentration levels (10 and 50 ng L^{-1}) and ranged from 95 to 113%. No significant difference in recovery was observed between these two concentration levels and no significant influence of the sample matrix on the recovery could be observed. Another research group also reported the use of 60-mg Oasis HLB cartridges for the determination of benzotriazole and tolyltriazole in municipal wastewater and river water [180,223]. Better recoveries were obtained (99% for benzotriazole and 97% for tolyltriazole), the study suggested that the eluting solvent (1.5 mL of dichloromethane containing 3% methanol) may have stronger affinity for the compounds than the eluting solvent 5 mL of methanol/acetone (6/4) as used by Weiss and Reemtsma [12].

Two different commercially available cartridges (Oasis HLB, 5mL, 200mg glass cartridges and Oasis HLB, 6mL, 500mg polypropylene cartridges) were compared by van Leerdam et al. for preconcentration of six benzotriazoles and four benzothiazoles in water samples [153]. The effects of the amount of adsorbent and solvent composition for the elution of the SPE-cartridges were investigated. Three different compositions of elution solvents (acetonitrile, methanol and 50:50 acetonitrile/methanol) and three different evaporation temperature values during evaporation step of the sample extracts (37, 45 and 56°C) were assessed. They also examined the influence of pH (neutral and acidic-7.8 and 2) on the recovery. The recovery of all benzotriazoles at neutral pH was less than 50%, whereas the recovery at pH of 2 was in the range of 50-110%. The results also showed that the recoveries of the target compounds were higher than 68% when using 500 mg adsorbent and a mixture of acetonitrile/methanol as elution solvent. It was observed that every other combination of the parameters investigated resulted in a recovery below 34% for one or more compounds. The temperature of the evaporation step did not influence significantly the recovery of the analytes and so for practical reasons, a temperature of 45ºC for the evaporation was selected.

The same research group developed two different SPE protocols for the preconcentration of the emerging contaminants including pharmaceuticals, illicit drugs and benzotriazoles in environmental water samples [222]. Benzotriazoles were pre-concentrated using Oasis HLB 200mg 5mL glass cartridges, the sample pH was adjusted to 2 and the compounds were eluted with 3 x 2.5mL of acetonitrile. Two internal standard compounds (fenuron and chloroxuron) were used to check the SPE procedure and enable the calculation of the SPE recoveries. For majority of the benzotriazoles, the method recoveries were > 70% in drinking, surface and ground water.

The influence of the matrix on the SPE breakthrough volume of benzotriazoles in ultrapure water, river water and raw wastewater was examined by Jover et al. [152]. 250, 500, 1000 and 2000 mL of each matrix were pre-concentrated. In addition, they tested two sample pH values (3 and 11) on spiked samples of ultrapure water. The best recoveries were obtained at pH 3 because the compounds were kept in their neutral form at this pH which enabled a better interaction and absorption with the SPE polymeric phase. With Strata-X 100mg polymeric cartridge (modified pyrrolidone divinylbenzene co-polymeric surface) chosen as adsorbent at this pH of 3, recoveries ranged from 92.8% to 99.6% in ultrapure water, from 85% to 115% in river water and from 78% to 98% in wastewater. For ultrapure water, breakthrough was not achieved at 2000 mL, but was exceeded at 2000 mL for river water and at 1000 mL for raw wastewater. Dominguez et al. also applied the SPE protocol developed by Jover et al. as the sample extraction procedure for the determination of benzothiazoles and benzotriazoles in wastewater using ionic liquid stationary phases in gas chromatography mass spectrometry [209]. The same recoveries range of 78% to 98% for wastewaters was observed. Matamoros and co-workers adopted the same SPE procedure for studying the occurrence and fate of benzothiazoles and benzotriazoles in constructed wetlands in Spain [224].

Bond Elut ppl cartridges (200mg, 3ml) were employed by Kiss and Fries [235] for enrichment of benzotriazole, 5-methylbenzotriazole and 4-methylbenzotriazole in river water. The recoveries for the analytical procedure were 62% for benzotriazole and 70% for 5-methylbenzotriazole.

Matamoros et al. expolited Strata–X SPE sorbent (100mg, 6 mL) as the preconcentration stationary phase for the simultaneous extraction of 97 organic contaminants, including 5 benzotriazoles and benzothiazoles in river water [236]. Two sample pH values (2 and 7) were evaluated on spiked river samples. It was observed that non-acidic compounds such as the benzotriazoles yielded similar recoveries in acidic or neutral pH. A sample pH of 2 was finally selected for subsequent extraction of the analytes from the river water. Recoveries of benzotriazole and 4-methylbenzotriazole in the developed analytical method were 66% and 102% respectively.

Benzotriazole and tolyltriazole were enriched in wastewaters, river waters and tap water samples from the UK by solid phase extraction using Oasis cartridges and the samples adjusted to pH 3 [48]. The eluting solvent used was 5 mL of dichloromethane containing 3% methanol. The method recoveries of benzotriazole and tolyltriazole from wastewaters ranged from 83 to 101% and 86 to 118%, respectively, whereas the recoveries in spiked Milli-Q water samples for benzotriazole and tolyltriazole were 91% and 101%, respectively.

The input and occurrence of benzotriazole and tolyltriazole as aquatic contaminants in Swiss rivers and lakes were studied by Giger et al. [180]. Water samples from the rivers and lakes were initially filtered through 0.7µm glass fibre filters, the pH of the filtrates was adjusted to 2.5-3.0 and pre-concentrated by solid phase extraction procedure using Oasis HLB Cartridges (60mg, 3mL). Recoveries of benzotriazole and tolyltriazole were 99% and 97%, respectively.

Solid phase extraction procedure was employed for the extraction of benzotriazoles and ultraviolet filters from aqueous samples by Liu and co-workers [228]. They evaluated the SPE method by testing four SPE cartridges (Oasis HLB, 6mL 500 mg; Supelco ENVI-18, 6mL 500 mg; Strata X-C-33µm, 6mL 500 mg, Selby Biolab C18, 6mL 500mg), five elution solvents (methanol, dichloromethane, acetonitrile, methanol/acetonitrile 50/50, methanol/dichloromethane 50/50), four elution volumes (1 x 2mL, 2 x 2mL, 3 x 2mL, 4 x 2mL for elution solvent methanol/dichloromethane 50/50) and two pH values (2 and 7). The optimized method involved the use of Oasis HLB, 6 mL 500mg cartridges, 3 x 2mL of methanol/dichloromethane 50/50 as elution solvent. The recoveries for the benzotriazoles were much lower at pH 7 (< 50%) than at pH 2 (79-110%), which was in agreement to the study of van Leerdam et al. [153] where the sample pH adjusted to an acidic pH yielded better recoveries for the compounds than at a neutral pH. They subsequently adjusted the pH of water samples to 2 prior to SPE of the target compounds. Oasis HLB cartridges gave the best recoveries among the cartridges as a result of better interactions of the polar compounds with hydrophilic-lipophilic balanced reversed phase sorbent than C18 or charcoal in the other cartridges. They also applied the developed SPE protocol to investigate the occurrence and removal of benzotriazoles and UV filters in a municipal wastewater treatment plant in South Australia [237]. The method recoveries of most target compounds ranged between 75% and 133% in influent and effluent samples.

Carpinteiro et al. developed an improved selectivity method for the simultaneous determination of four benzotriazoles and six benzothiazoles in aqueous matrices by mixed-mode solid-phase extraction followed by liquid chromatography-tandem mass spectrometry [229]. They exploited the advantage of a mixed-mode SPE sorbent which combines ionic exchange and reversed-phase mechanisms for the extraction of the compounds. Parameters affecting the extraction process such as the type and amount of sorbent/elution solvent were thoroughly investigated. Under optimal conditions, Oasis MAX cartridges and a 5 mL of methanol/acetone (7:3) as elution solvent were selected. This type of sorbent retains basic and neutral species and improves the retention of the acidic compounds by strong anionic exchange with protonated amino moieties existing in the structure of the sorbent. By considering the same elution mixture as for Oasis HLB, MAX Cartridges provided clear extracts with a lower complexity. A second cartridge was even connected in series to check the sample breakthrough and no signal was observed in the second cartridge. Recoveries between 80% and 118% were obtained for influent, effluent and river water. The SPE methodology provided an enhanced selectivity compared to the conventional reversed-phase SPE, allowing the determination of the compounds in surface water and effluent samples using simply external standard calibration.

Loos and co-workers analyzed 59 selected organic compounds including benzotriazoles (benzotriazole and methyl benzotriazole) in ground water samples from 23 European countries [118]. The ground water samples were extracted by solid phase extraction using Oasis HLB (200mg, 6 mL) cartridges and 6mL of methanol as elution solvent. The method recoveries for benzotriazole and methylbenzotriazole were 56% and 47% respectively. The research group also applied the SPE protocol for the determination of 34 polar water-soluble organic contaminants including benzotriazole and tolyltriazole in the dissolved liquid water phase of river water samples from the river Danube and its major tributaries [239]. Generally, whereas 400 mL water samples were pre-concentrated in the case of groundwater, 1L samples were used for the river water.

Kovalova et al. developed an automated online SPE-HPLC-MS/MS analytical method for the determination of 68 target analytes including benzotriazole and 4/5 methylbenzotriazole mixture in hospital wastewater in order to study the performance and efficiency of membrane bioreactor for the elimination of the compounds [232]. The in-house filled online SPE cartridge contained Isolute ENV+ and Oasis HLB solid phase materials. To provide satisfactory results for majority of the compounds, they considered conditions such as SPE material or material combination, pH of the sample at loading, suitable SPE eluent which serves also as part of the HPLC mobile phase, SPE cartridge washing solvent and the time of valve switching. In total, seven different SPE materials in eight different combinations and at three different pH values (pH 3, 7 and 9) were investigated. The optimized method used a layer of Isolute ENV+ and Oasis HLB, pH of 3 and methanol as elution solvent. The relative recoveries of the final analytical method ranged between 78% to 129% for benzotriazole and 135% to 227% for 4/5 methylbenzotriazole mixture in hospital wastewater (raw and treated wastewater) and municipal wastewater (influent and effluent).

A simple and rapid dispersive liquid-liquid microextraction (DLLME) method was developed by Pena and co-workers for the determination of eleven benzotriazoles and benzothiazoles in water samples [230]. They evaluated the influence of the different experimental variables (type of dispersant and extraction solvent, volume of dispersant and extraction solvent, pH, ionic strength) on the performance of the sample preparation step. For the optimized DLLME method, an aliquot of 9 mL water sample adjusted to a pH of 6 containing 2g of NaCl was placed in a 10 mL volumetric flask. A rapid injection of 0.5 mL MeOH (as disperser solvent) containing 100 µL Tri-n-butylphosphate (TBP) as extraction, was performed. TBP provided higher extraction efficiency than chlorinated solvents and ionic liquids. The extraction process was efficient with recoveries ranging from 67% to 97%. The extraction yield underwent a significant reduction with the increase in the complexity of the sample. It was noted that although microextraction techniques provide great advantages such as miniaturization, reduction of organic solvent consumption, and improvement in the selectivity of the extraction compared to SPE and LLE, the developed DLLME exhibited similar recovery values to those obtained by conventional extraction processes such as LLE and SPE. The optimized method exhibited a precision level (RSD < 9%) similar or even better than those obtained by LLE and SPE methods. Additionally, the use of TBP provided some advantages such as reduction of exposure to toxic solvent, possibility of obtaining more reproducible results since evaporation of extractant was not required and obtaining a directly analyzable extract in a short-single step. Moreover, extraction was carried out in a few seconds in DLLME, while SPE or LLE involved multi-step processes such as activation of cartridges and additional evaporation process, increasing the overall sample treatment time.

Liquid-liquid extraction (LLE) procedure was exploited by Pervova et al. for the extraction of 1,2,3 benzotriazole from aqueous solution (sewage water) [234]. The extraction was performed with toluene which was purified with concentrated H_2SO_4 and washed with water to a neutral pH value, dried with anhydrous Na_2SO_4 and distilled. The extraction method involved the acetylation of benzotriazole with acetic anhydride in an aqueous solution in the presence of sodium hydrogen carbonate with the simultaneous extraction of the forming N- acetylbenzotriazole into toluene. In studying the extraction of aqueous solutions of N-acetylbenzotriazole with toluene, it was shown that for the ratio between the aqueous and organic phases 100:1, the recovery was about 80%. The incompleteness of the extraction of benzotriazole as N-acetylbenzotriazole was, however, taken into account for the calculation. One notable observation was that benzotriazole was poorly extracted from aqueous media thus, the distribution ratio between water and ether was only 3.8.

4.2.2 Extraction of Benzotriazoles from Solid Samples

Solid samples are often subject to removal of water first and then ground into powders. Apparently, the goal of modifying solid samples to fine solids is to allow subsequent extraction to proceed more effectively. Freeze-drying and oven-drying are commonly- used methods for removing water from solid samples [239]. The analytical procedures used in the determination of emerging contaminants (such as benzotriazoles, benzothiazoles and benzosulfonamides) in environmental solid samples involve a sample preparation step that includes sampling, extraction, clean-up and occasionally, derivatization to enhance the analytical response of these compounds [240]. For determination at trace level, analyses are normally carried out by chromatographic methods combined with detection techniques preferably able to confirm analyte identity.

So far, very few studies have been undertaken for the determination of benzotriazoles in environmental solid samples (sludge, sediment and soil). In general, the methods have largely involved a preliminary extraction of the solid samples with either liquid-solid extraction (LSE) or pressurized liquid extraction (PLE) followed by pre-concentration or clean-up by Solid phase extraction (SPE) and the extracts analyzed by liquid chromatography-mass spectrometry or gas chromatography [188,226,227, 228].

Soil and groundwater samples were collected at an abandoned airport in order to determine the persistence of benzotriazole as a de-icing additive at the abandoned airport [188]. Soil samples were extracted with methanol, diluted with water and analyzed with liquid chromatography and mass chromatography detector.

McNeill and Cancilla analyzed soil samples from three USA airports representing low, mid and large volume users of de-icing fluids (ADAFs) by LC-MS/MS for the presence of benzotriazoles [226]. The soil samples were vortex-mixed for 10 min with two 20 mL aliquots of a 1:1 acetonitrile: water (2 mM ammonium acetate) solution and then allowed to sit for 10 min. Samples were then centrifuged for 10 min at 3,500 rpm, after which the supernatant was removed and filtered. The two 20mL extractions from each sample were then analyzed individually by LC-MS/MS and the values from the two measurements summed to determine the concentrations of the benzotriazoles. The method recovery values in fortified soil samples for benzotriazole and 5-methylbenzotriazole were 30.8% and 32.1% respectively. As a result of these low recovery values in fortified samples, the authors categorically stated that it was likely the concentrations measured in the environmental soil samples were underestimated.

Zhang et al. determined benzotriazole in sewage sludge and sediments by LC-MS/MS [227]. 5 mL of methanol were added to the samples as extracting solvent, shaken for 30 min and centrifuged at 4000 rpm for 4 min and the solvent layer was transferred into a new polypropylene tube. The extraction was repeated twice and the extracts were combined. The extract was evaporated under a gentle stream of nitrogen to approximately 0.5 mL and Milli-Q water was added; after thorough mixing, extracts were passed through a solid phase extraction (SPE) cartridge (Oasis HLB, 500mg, 6ml). The compounds were eluted with 6 mL of 15% methanol in ethyl acetate. The extraction procedure was optimized by testing extracting efficiencies of various solvents (methanol, ethyl acetate, dichloromethane, a 1:1 mixture of methanol/acetyl acetate). Methanol provided the greatest recoveries (≥70% for sludge and ≥80% for sediment). The eluting solvent for the clean-up step was also optimized and 6 mL of 15% methanol in ethyl acetate was finally used.

An overview of all the extraction techniques expoited so far is presented in Table 4.1.

Table 4.1: Overview of Analytical Methodologies for determination of benzotriazoles in the environment *

Analytes	Environmental Media	Sample Preparation Technique/Cartridges/Solvents	Analytical Technique	Column/Mobile Phase	Analytical Parameters	Ref.
BTR, TTR	Groundwater	SPE/C18 Column/Dichloromethane for elution	GC-MS		LOD= 0.1 µg L^{-1}	[188]
BTR, 4TTR, 5TTR, XTR	Municipal wastewater, Surface & groundwater	SPE /Oasis HLB (60 mg) Cartridge, MeOH/Acetone (6:4 v/v) for elution. Direct Injection	LC-MS/MS	Pursuit Diphenylcolumn (2mm x 150mm, 3µm)/gradient elution with H_2O/MeOH (10:90v/v) modified with 0.1% HCOOH	Rec_{METHOD}= 95-113%, LOQ_{SPE}=15,25 ng L^{-1}, LOQ_{DI}= 100 ng L^{-1}	[12]
BTR, 5TTR, XTR	River water, Wastewater	SPE/Strata X (100 mg) cartridge, Ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30m x 0.25mm, 0.25µm)	Rec_{METHOD}=78-115%, LOD =48-112 ng L^{-1}, LOQ =73-198 ng L^{-1}	[152]
BTR, 5TTR	Influent and Effluent	Direct Injection	LC-MS/MS	Pursuit Diphenyl column (2mm x 150mm, 3µm)/ Isocratic elution with H_2O/MeOH (10:90 v/v) modified with 0.1% HCOOH	LOQ= 100 ng L^{-1}	[225]
1H-BTR	Tank water	SPE/Oasis HLB (60 mg/3mL) column, MeOH/Dichloromethane (3:97 v/v) for elution	LC-MS/MS	Endcapped C8 125 x 2.1mm, 5µm, column/ Isocratic elution with a mixture of MeOH/H_2O/HCOOH (70:30:0.4)	Rec_{METHOD} = 99%, LOD = 8 ng L^{-1}, LOQ = 30 ng L^{-1}	[150]
BTR, TTR	River water, Lake water	SPE/Oasis HLB (60mg/3mL) column, MeOH/Dichloromethane	LC-MS/MS	Endcapped C8 125 x 2.1mm, 5µm,	Rec_{METHOD}= 9%,97%, LOD= 8 ng L^{-1},3 ng L^{-1},	[180]

		(3:97 v/v) for elution		column/ Isocratic elution with a mixture of MeOH/H_2O/HCOOH (70:30:0.4)	LOQ= 25 ng L^{-1}, 10 ng L^{-1}	
BTR , 4TTR, 5TTR	Municipal Wastewater	SPE/ Oasis HLB (60 mg) Cartridge, MeOH/Acetone (6:4 v/v) for elution Direct Injection	LC-MS/MS	Pursuit Diphenyl column (2mm x 150mm, 3μm)/gradient elution with H_2O/MeOH (10:90 v/v) modified with 0.1% HCOOH	LOQ_{SPE}= 10 ng L^{-1}, LOQ_{DI} = 100 ng L^{-1}	[149]
BTR,5TTR ,OH-BTR,XTR	Surfacewater, Drinking water, Wastewater	SPE/Oasis HLB (200mg/5mL) glass column & HLB (500mg/6mL) propylene column; ACN/MeOH (50:50) for elution	LC-LTQ-FT-MS	Phenomenex Aqua C_{18}–Column (250mm x 4.6mm, 5μm)/gradient elution with ACN/H_2O/HCOOH	Rec_{METHOD}= 68-10%, LOD =1-5 ng $L^{-1,}$ LOQ = 2-11 ng L^{-1}	[153]
BTR,4TTR, 5TTR	Wastewater Groundwater	SPE	LC-MS/MS			[14]
BTR, TTR	Municipal wastewater River water	SPE/Oasis HLB (60mg/3mL) column, MeOH/Dichloromethane (3:97 v/v) for elution	LC-MS/MS	Endcapped C8 125 x 2.1mm, 5μm, column/ Isocratic elution with a mixture of MeOH/H_2O/HCOOH (70:30:0.4)	Rec_{Method}= 99%,97%, LOQ=30 ng L^{-1}, 10 ng L^{-1}	[223]
BTR,4TTR,5TTR, XTR	Wastewater	SPE/Strata X Polymeric cartridge, hexane/ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30m x 0.25mm, 0.25μm)	LOD= 5-56 ng L^{-1}; LOQ= 35-99 ng L^{-1}	[224]
BTR, TTR	River water	SPE/ Oasis HLB (200mg, 6 mL) cartridges, MeOH for elution	LC-MS/MS	Hypersil Gold column (Thermo Electron Corp., 100 x 2.1 mm,	Rec_{SPE}= 56%,47% LOD= 1 ng L^{-1}	[4]

				3μm),gradient elution with ACN/H_2O (acidified with 0.1% acetic acid)		
BTR,4BTR,5BTR	River water	SPE/ Bond Elut ppl (200mg, 3 mL) cartridges, methanol as elution solvent	GC-MS	DB-5 ms (30 m x 0.25mm x 0.5μm, Agilent Technologies	Rec_{Method}= 62%,70% LOD =12 ng L^{-1}, 8 ng L^{-1}, LOQ =36 ng L^{-1}, 24 ng L^{-1}	[235]
BTR,4BTR,5BTR	Drinking water, Surface water, Groundwater	SPE/Oasis HLB (200mg,5 mL)glass cartridges; ACN for elution	LC-LTQ-FTMS	Omnisphere C18 (100mm x 2.0mm, 3μm) column/ gradient elution with ACN/H_2O	Rec_{METHOD} > 70%, LOQ= 10-30 ng L^{-1}	[222]
BTR,TTR	Groundwater	SPE	LC-MS/MS		1.0 ng L^{-1}	[118]
BTR	Sewage water	LLE/Acetylation followed by extraction with Toluene	GC-FID	Fused_silica capillary column ZB_5 (phenyl–Methyl silicone, 5% of phenyl groups) 30 m x 0.25 mm, 0.25 μm)	Rec_{LLE} = 80% LOQ = 10000 ng L^{-1}	[234]
BTR, TTR	River water, Sea water	SPE/Oasis HLB (500 mg/60μm), MeOH for elution	HPLC-MS/MS	A Phenomenex Synergi Hydro RP 80A column, MeOH/0.1% HCOOH in H_2O	Rec_{Method} = 53%, 69% LOQ = 1.2 ng L^{-1}, 0.4 ng L^{-1}	[241]
BTR,TTR	Influent, Effluent	SPE/ Bond Elut PPL cartridges (200 mg/3mL), MeOH for elution	LC-MS/MS	Kinetex PFP column (100mm x 2.1mm; 2.6μm), Elution with H_2O/MeOH both modified with 2Mm ammonium	LOD =10 ng L^{-1}	[123]

				carbonate		
BTR, TTR	Tap water, River water, Effluent	SPE/Oasis cartridges, MeOH/Dichloromethane(3:97 v/v) for elution	LC-MS/MS	Phenomenex (7cm x 2mm i.d. Synergi 4µm Hydro-RP80A), H_2O/MeoH both mixed with 0.4%HCOOH for gradient elution	Rec_{Method}=83-18% LOQ = 0.2 ng L^{-1}	[48]
BTR,TTR,XTR , ClBTR	Tap water, Effluent	SPE/ Oasis HLB(500mg/ 6 mL) cartridges, MeOH/Dichloromethane(50:50 v/v) for elution	GC-MS/MS	HP-5MS column (30mm x 0.22mm, 0.25µm thickness)	Rec_{SPE}=79-110%, Rec_{Method}= 75-133% LOD=4.2-14.1 ng L^{-1}, LOQ= 14-47 ng L^{-1} Precision(%RSD) =0.5-9.3	[228]
BTR, TTR, XTR	River water, Urban Influent & Effluent, Ind. Influent & Effluent	Mixed mode –SPE/ Oasis HLB, MAX Cartridges, MeOH/Acetone(7:3 v/v) for elution	LC-MS/MS	Zorbax SB-phenyl (100mm x 2.1mm, 3.5µm) column/ gradient elution with H_2O/MeOH both modified with 1mM ammonium acetate	Rec_{RW}= 87-102% Rec_{Inf} = 50-65% Rec_{Eff} = 81-91% LOQ_{RW} =2-5 ng L^{-1} LOQ_{Inf} = 20-50 ng L^{-1} LOQ_{Eff} = 11-28 ng L^{-1} Precision(%RSD)=3-12 $R^2 > 0.99$ Range = LOQ to 10 µg L^{-1}	[229]
BTR,5TTR, XTR	Influent, Effluent	SPE/Strata X (100 mg) cartridge, Hexane/ethyl acetate(1:1 v/v) for elution	IL-GC-MS/MS	SLB-IL159 Column	Rec_{method} =78-98%, LOD =25-562 ng L^{-1}, LOQ =60-815 ng L^{-1} Precision(%RSD) <10 $R^2 > 0.993$ Range=31-3000ng mL^{-1}	[209]

BTR, 4TTR, 5TTR, ClBTR, 4OHBTR	Tap water, River water, Raw Wastewater, Industrial effluent	DLLME/0.5 mL MeOH (as disperser solvent) containing 100 µL Tri-n-butylphosphate (TBP) for extraction	HPLC-FL/UV, LC-ESI-MS/MS	C_{18} Column (250 mm x 4.6 mm, 5µm Waters)/ gradient elution with ACN/H2O modified with 0.05%HCOOH	Rec_{DLLME} = 67-97%, Rec_{Method} = 74-86% LOD_{Fl}= 40-2200 ng L^{-1}, LOQ_{Fl} =100-7300 ng L^{-1} Precision(%RSD)= 4.1-6.5	[230]
BTR, TTR, XTR, ClBTR,	Influent, Effluent	SPE/ Oasis HLB (500mg/ 6 mL) cartridges, MeOH/Dichloromethane (50:50 v/v) for elution	GC-MS/MS	HP-5MS column (30mm x 0.22mm, 0.25µm thickness	Rec_{Method}=75-133%, LOD_{Inf}= 16.3 ng L^{-1}, LOD_{Eff}= 14.1 ng L^{-1}	[237]
BTR, 4/5TTR	Hospital Wastewater	Online SPE/ Isolute ENV+ and Oasis HLB cartridges, MeOH for elution	HPLC-MS/MS	Atlantis T3 Column	Rec_{Method} =78-129%, 135-227% LOQs= 730 ng L^{-1}, 63 ng L^{-1}	[232]
BTR, 4TTR	River water	SPE/Strata X Polymeric cartridge, ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30m x 0.25mm, 0.25µm)for 1st dimension,TRB-50HT (2m x 0.1mm, 0.1µm) for 2nd dimension	Rec_{Method} = 66%, 102% LODs= 6 ng L^{-1}, 38 ng L^{-1} LOQs =3 ng L^{-1}, 73 ng L^{-1} R^2 = 0.9979, 0.9835 Precision(%RSD)= 10,12	[236]
BTR, 4TTR, 5TTR	Soil	LSE/ACN:H_2O in 2Mm Ammonium acetate (1:1 v/v) as extraction solvent	LC-MS/MS	Zorbax Eclipse XDB- C18 (150mm x 4.6mm, 5µm)	Rec_{method} = 31%,32% LOQs = 3.1 ng g^{-1}, 2.2 ng g^{-1}	[226]
BTR, 5-TTR	Sediment, Sludge	LSE with MeOH followed by SPE / Oasis HLB(500mg,6mL) cartridge, MeOH/ethyl acetate(15:85 v/v) for elution	LC-MS/MS	Thermo Betasil C18 (100mm x 2.1mm, 5µm), MeOH/H_2O for gradient elution	Rec_{Method} = 81%,72% LOD_{sed} = 0.067 ng g^{-1}, LOD_{sludge} = 0.67 ng g^{-1}, LOQ_{sed} = 0.22 ng g^{-1}, LOQ_{sludge} =2.2 ng g^{-1}	[227]

BTR, 5-TTR, XTR, Cl-BTR	Sludge	PLE/ASE 300 Accelerated solvent extraction system (Dionex, USA), MeOH/Dichloromethane (50:50 v/v) as extraction solvent	GC-MS/MS	HP-5MS column (30 m× 0.22 mm, 0.25 µm thickness)	Rec_{method} =66-133%, LOD = 0.9-4.1 ng g^{-1}, LOQ = 2.9-13.8 ng g^{-1}	[228]
BTR, TTR	Soil, Sediment	LSE/MeOH as extraction solvent	LC-MS		LOD =100 ng g^{-1}	[188]

[*]Abbreviations

BTR: benzotriazole; 1BTR: 1H-benzotriazole; TTR: tolyltriazole; 4TTR: 4, tolyltriazole; 5TTR: 5,tolyltriazole; XTR: xylyltriazole; OH-BTR: 1-hydroxybenzotriazole; ClBTR: chlorobenzotriazole; 4/5TTR: 4/5 tolyltriazole; 4OHBTR: 4-hydroxybenzotriazole; SPE: Solid Phase Extraction; DI: Direct Injection; Rec_{SPE}: SPE Recovery; Rec_{LLE}: LLE Recovery; Rec_{DLLME}: DLLME; Recovery Rec_{METHOD}: Method Recovery; Rec_{RW}: Method Recovery for river water; Rec_{Inf} : Method Recovery for influent; Rec_{Eff}: Method Recovery for effluent; LOQ_{RW}: LOQ for river water; LOQ_{Inf}: LOQ for influent; LOQ_{Eff}: LOQ for effluent; LOQsed: LOQ for sediment; LOQ_{sludge}: LOQ for sludge; LOQ_{Fl}: LOQ for fluorescence detection; LOQ_{SPE}: Method LOQ for SPE, LOQ_{DI}: Method LOQ for Direct injection; LOD_{Fl}: LOD for fluorescence detection; LOD_{Inf} : LOD for influent; LOD_{Eff} : LOD for effluent; LOD_{sed}: LOD for sediment; LOD_{sludge} : LOD for sludge

4.2.3. Instrumental methods of analysis for benzotriazoles

Benzotriazoles are rather polar compounds, thus, liquid chromatography (LC) has been the preferred instrumental technique for their determination in the aquatic environment. To avoid time-consuming extraction and cleaning steps, the determination of benzotriazoles in directly injected river waters and waste water samples was applied successfully using LC-ESI-MS/MS [12,149].

Gas chromatography-mass spectrometry, combined with common extraction methods like SPE and LLE, was demonstrated as an appropriate tool to analyze benzotriazoles in airports-influenced water samples [182] and also in river water [235]. Benzotriazoles have also been determined in river water by using two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC x GC-TOF-MS) and gas chromatography-tandem mass spectrometry [152,228]. However, most authors have demonstrated the combination of LC with mass spectrometry (MS) using electrospray ionization (ESI) and different types of analyzers [12,153,229].

Weiss and Reemtsma developed the first LC-ESI-MS^2 method for the determination of benzotriazoles in environmental water samples [12]. Four benzotriazoles (benzotriazole, 5-tolytriazole, 4-tolyltriazole and xylyltriazole) expected to occur in the aquatic environment were analyzed. Extraction and preconcentration of the compounds from water samples was carried out by SPE. Analysis was performed on a HPLC system coupled to a triple stage quadrupole mass spectrometer, the separation was performed using a Pursuit Diphenyl column (2mm x 150mm) with 3µm particle size at 40ºC. Both isocratic and gradient elution were used separately. In the isocratic run, the two isomers (4-methylbenzotriazole and 5-methylbenzotriazole) were separated and detected whereas in the gradient elution, the two isomers co-eluted. The compounds were detected with tandem mass spectrometry using multiple reaction monitoring (MRM) in the positive ion mode. The mean recoveries ranged from 95% to 113%. With an extracted sample volume of 100ml, the LOQ for ground water was 10ng/L for all analytes while the LOQs were 15 and 25 ng L^{-1} for xylyltriazole in WWTP effluents and influents respectively. The method could not allow a reliable determination of LOQs for benzotriazole and tolyltriazole in municipal wastewaters because their concentrations in effluents were orders of magnitude above the LOQ. The analytical method was also employed for carrying out a long-term study on the occurrence and removal of benzotriazoles in a large WWTP [149]. A few ozonated WWTP effluents were analyzed after enrichment by solid-phase extraction (SPE) as already described, while all other samples were analyzed by direct injection. Limit of quantification (LOQ) was 0.1µg/L for direct injection and 10 ng L^{-1} after SPE extraction. For SPE extraction of groundwater samples the LOQ was approximately 5 ng L^{-1}.

In order to investigate the environmental occurrence of benzotriazole and tolyltriazole in Swiss rivers and lakes, an analytical method based on SPE-LC-MS/MS was developed by Giger and co-workers [180]. Separation was achieved on a 125 x 2.1mm endcapped C8 column in an isocratic elution with a mixture of methanol/water/formic acid (70:30:0.4) as a mobile phase at a flow rate of 0.2 mL/min. The benzotriazoles were detected with electrospray ionization in positive mode and using multiple reaction monitoring (MRM). Their method did not achieve separation of the 4- and 5-methyl benzotriazole isomers and as a consequence, the results were reported as the sum of the two isomers. The analytical parameters of the method are presented in Table 4.1. Moreover, the method was applied for the determination of benzotriazole and tolyltriazole in municipal wastewater and river water by the same group [223].

In a research work to demonstrate that benzotriazole is antiestrogenic *in vitro* but not *in vivo*, an analytical method was developed for determining benzotriazole in tank water [150]. The method involved enrichment by Solid Phase Extraction using Oasis HLB cartridges, the extracts were analyzed for benzotriazole by liquid chromatography-tandem mass spectrometry. Separation was accomplished with an end-capped C8 column (125 x 2.1mm, 5µm particle size) and by an isocratic elution with a mixture of methanol/water/formic acid (70:29.6:0.4) as mobile phase at a flow rate of 0.2 mL/min. Detection of the analytes was accomplished with electrospray ionization in positive mode and using multiple-reaction monitoring. Recovery was 99% and the limit of detection was 8 ng L^{-1} while the limit of quantification was 30 ng L^{-1}.

A sensitive, reliable and robust method was developed for the trace determination of six polar benzotriazoles and four benzothiazoles in drinking water, surface water and effluents of two sewage treatment plants in the Netherlands on the basis of solid-phase extraction and Liquid Chromatography-Electrospray Mass Spectrometry using a linear ion trap-Orbitrap hybrid instrument (LTQ FT Orbitrap) [153]. Separation of the compounds was performed using a 250mm x 4.6mm i.d. Phenomenex Aqua C_{18} –Column with 5µm particles and a pre-column 4.0 x 3.0 mm i.d. Phenomenex Security Guard column. A linear gradient elution was used at a flow rate of 1.0 mL/min. A linear ion trap-Fourier Transform (LTQ-FT) Orbitrap mass spectrometer was used for the detection and quantification. In order to investigate the effect of the matrix on the response of the mass spectrometer, the signal suppression was calculated for all compounds in sample extracts compared to a standard in ultrapure water. The total recovery in ultrapure water ranged from 68% for 1-Hydroxybenzotriazole up to 97% for 5, 6-dimethylbenzotriazole. In surface water, the recoveries ranged from 57% for 1-Hydroxybenzotriazole to 125% for 4-methylbenzotriazole. It was stated that the relatively high recovery of 4-methylbenzotriazole could be explained by signal enhancement due to matrix effects. The reproducibility of the total procedure was good with relative standard deviations of 3-7% in ultrapure water, 4-8% in drinking water and 2-13% in surface water at a concentration level of 0.2 µg L^{-1}. Quantification and detection limits were determined using a signal-to-noise approach. The limit of quantification was estimated for the concentration of compound that results in a signal-to-noise ratio of 10:1 while the limits of detection corresponds to the concentration that results in a signal-to-noise ratio of 3:1. In drinking water, limits of detection ranged from < 1 ng L^{-1} to 4 ng L^{-1} and LOQ ranged from 2 ng L^{-1} to 9 ng L^{-1} whereas in surface water LOD ranged from 1 ng L^{-1} to 5 ng L^{-1} and LOQ from 2 ng L^{-1} to 11 ng L^{-1}.

The research group of van Leerdam also used LTQ FT Orbitrap MS-MS for screening some emerging organic contaminants, including benzotriazoles, in drinking, surface and groundwater [222]. The LC system consisted of a 100mm x 2.0mm i.d. column packed with 3µm Omnisphere C18 material, the guard column was 4.0 mm x 2.0 mm i.d. packed with pellicular C18 material,25-35µm. The analytical column and the guard column were maintained at a temperature of 21ºC in a column thermostat. A linear gradient of acetonitrile and water (as elution solvents) at flow rate of 0.3 mL/min was used. Recoveries were greater than 70% in drinking, surface and groundwater, the quantification limits were between 0.01 and 0.03 µg L^{-1}. The concentration levels in drinking water were 0.1 µg L^{-1} for both benzotriazole and methyl-benzotriazole and 0.01 µg L^{-1} for dimethylbenzotriazole.

A GC x GC-TOF-MS approach was proposed and developed, for the first time, for the simultaneous determination of three classes of organic pollutants classified as emerging pollutants in aqueous matrices [152]. The target contaminants include four benzotriazoles, three benzothiazoles and five benzosulfonamides; the method was designed to be suitable for complex aqueous matrices such as river water and raw sewage and suitability of GC x GC-time-of -flight (TOF) MS for identifying and quantifying emerging pollutants in complex aqueous matrices. The two dimensional gas chromatography improved separations, enabling identification of minor components that might be overlooked with other methods. Pre-concentration was carried out by solid phase extraction. Recoveries were calculated by analyzing a river sample and raw sewage in duplicates, limits of detection (LODs) were calculated as the content of the blank plus three times its standard deviation. Limits of quantification (LOQs) were calculated as the level of the blank plus ten times its standard deviation or the lowest concentration of the calibration plot (whichever was higher). The use of the lowest calibration concentration as LOQ is a more conservative method that considers both sensitivity and linearity criteria. Quantification was carried out using an external calibration corrected by the internal standard. The method performance parameters are presented in Table 4.1. In comparison, the LOD values obtained were similar to those of LC-MS/MS, but lower than those of GC-MS, 4-tolyltriazole and 5-tolyltriazole isomers were resolved at LOD and LOQ values comparable to those of LC-MS/MS [12,188].

Loos and co-workers developed an analytical method for the determination of 35 selected compounds, including benzotriazole and tolyltriazole, in river water samples from 27 European countries [4]. They used solid phase extraction followed by reversed-phase liquid chromatography (RP-LC) electrospray ionization (ESI) mass spectrometry (MS). Detection was accomplished in a multiple reaction monitoring mode (MRM) and positive ionization. The benzotriazoles were quantified with the internal standard carbamazepine d_{10}. The limits of detection (LODs) were calculated from the mean concentration of the blank of real water samples plus three times the standard deviation and the LOD was 1 µg L^{-1} for both benzotriazole and tolyltriazole.

An analytical procedure by LC-MS/MS was developed by McNeill and Cancilla for the determination of triazoles in soil samples from three USA airports [226]. Triazoles, specifically benzotriazole, 4-methylbenzotriazole and 5-methylbenzotriazole were determined. Soil samples were extracted by liquid-solid extraction and the extracts analysed directly. The compounds were separated using a Zorbax Eclipse XDB-C18 (4.6mm x 150mm, 5µm particles and involved a gradient elution. The mass spectrometer was operated in positive ion mode and multiple reactions monitoring (MRM) scan type. Method detection limits (MDL) were 3.1 µg Kg^{-1} benzotriazole and 2.2 µg Kg^{-1} for both 4-methylbenzotriazole and 5-methylbenzotriazole. Recovery values for benzotriazole and 5-methylbenzotriazole in fortified soil samples were 30.8% and 32.1%, respectively.

In order to evaluate the removal efficiency and aquatic mass discharge rate of benzotriazoles and benzothiazoles in a conventional activated sludge and two constructed wetlands, Matamoros et al. [224] analyzed a suite of benzotriazoles and benzothiazoles in wastewater.

Enrichment was carried out by solid phase extraction (SPE). The compounds in the eluates were analyzed by two multidimensional gas chromatography coupled to time of flight mass spectrometry (GC x GC-TOF-MS). As a first dimension, a column of 30 x 0.25mm ID coated with 0.25µm film thickness TRB5-MS(5% diphenyl-polydimethylsiloxane) was used whereas a second dimension of 2 m x 0.10mm ID coated with 0.10µm film thickness TRB-50 HT(high temperature 50% diphenyl-polydimethylsiloxane) was installed. Limit of detection (LOD) of the method ranged from 5 to 56 ng L^{-1} while limit of quantification (LOQ) ranged from 35 to 99 ng L^{-1}.

Matamoros et al. developed a method based on comprehensive two dimensional gas chromatography (GC x GC) coupled with time of flight mass spectrometry for the simultaneous determination of 97 organic contaminants including 5 benzothiazoles and benzotriazoles at trace level concentration in river water [236]. Preconcentration was performed by solid phase extraction (SPE). Limit of detection (LOD) was calculated as the content of the blank plus three times its standard deviation while limit of quantification (LOQ) was calculated as the level of the blank plus 10 times its standard deviation. The method performance parameters are shown in Table 4.1.

Kiss and Fries developed an analytical procedure for the determination of benzotriazole, 5-methylbenzotriazole and 4-methylbenzotriazole in river water by SPE followed by gas chromatography/mass spectrometry (GC/MS) [235]. The column used was a DB-5 MS (30 m x 0.25mm x 0.5µm, Agilent Technologies). Recoveries were 62% for benzotriazole and 70% for 5-methylbenzotriazole. LOD was defined as three times the peak-to-noise and the values were 12 ng L^{-1} for benzotriazole and 8 ng L^{-1} for 5-methylbenzotriazole. Limits of quantification (LOQs) were set at three times the LOD and were 36 ng L^{-1} for benzotriazole and 24 ng L^{-1} for 5-methylbenzotriazole.

Zhang et al. developed an analytical methodology for the determination of benzotriazole and 5-methylbenzotriazole, along with five other benzophenone derivatives, in sediment and sludge samples based on liquid-solid extraction followed by SPE- liquid chromatography-tandem mass spectrometry [227]. They used a Thermo Betasil C18 (100mm x 2.1mm, 5µm) chromatographic column serially connected with a guard column Thermo Electron Corporation, Bellenfonte, PA, USA (20 x 2.1mm, 5µm). Separation was carried out at 300µL/min using a gradient elution. Mass spectrometry was operated at negative ionization mode using multiple reactions monitoring (MRM). Instrumental limits of detection (LOD) were calculated from the signal-to-noise ratio(S/N >3) of 3 for the pure standard solutions injected onto the column while the entire method limits of quantification (LOQ) were determined by the signal-to-noise ratio (S/N> 10) for the injected extracts. The method LOD and LOQ values for sediment analyzed were 0.067 ng g^{-1} and 0.22 ng g^{-1} for benzotriazole and 5-methylbenzotriazole respectively, while the method LOD and LOQ values for sludge samples were 0.67 and 2.2 ng g^{-1} for benzotriazole and 5-methylbenzotriazole respectively. Recoveries of target compounds spiked at 50- and 100 ng levels in sediment and sludge passed through the entire analytical procedure ranged from 70% to 116%. Recoveries of the compounds from matrix spikes ranged from 71.2% to 115%.

Janna and co-researchers developed an analytical methodology which involved solid phase extraction(SPE), followed by separation and quantification by liquid chromatography-tandem mass spectrometry for the determination of benzotriazole and tolyltriazole in wastewaters, river waters and tap water in UK [48]. The analytes were separated on a 7cm x 2mm i.d. Synergi 4µ Hydro-RP80A (Phenomenex, Macclesfield, UK) at a flow rate of 0.2 mL/min in a gradient elution. The compounds were detected and quantified by mass spectrometry with a multiple reaction monitoring in the positive ion mode. The two isomers of tolyltriazole; 4- and 5- methyl benzotriazoles, were not separated by the chromatography and so were reported together as tolyltriazole.

A method using gas chromatography-tandem mass spectrometry (GC-MS/MS) was developed and validated by Liu et al. for simultaneous determination of four benzotriazoles and six UV filters in tap water, effluent and biosolid samples [228]. The four benzotriazoles determined were benzotriazole, 5-methylbenzotriazole, xylyltriazole and chlorobenzotriazole. They used solid phase extraction (SPE) and pressurized liquid extraction (PLE) as the preconcentration method for water samples (ground water and effluent) and biosolid samples, respectively. The target compounds were separated on a HP-5MS column (30mm x 0.22mm, 0.25µm thickness) with helium as carrier gas at a flow rate of 1.656 mL/min, the GC oven temperature was programmed from 80ºC (hold 2min) to 280 º C (hold 6 min) at a rate of 15º C/min. Mass spectrometric analyses were performed by tandem mass spectrometry that operated in electron impact (EI) mode. Quantification of the target compounds was performed in the multiple reaction monitoring (MRM) mode by recording two pairs of MRM ions per compound. Recoveries of the compounds in samples of tap water and effluent ranged between 70% and 122% while for the biosolids, recoveries ranged between 66% and 133%. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the signal to noise ratio (SNR). LOD is defined as three times of SNR, and LOQ ten times of

SNR. The LODs for the benzotriazoles in the tap water, effluent and biosolid samples were 2.9-11 ng L^{-1}, 4.2-14.1 ng L^{-1} and 0.9- 4.1 ng g^{-1} respectively while the LOQs in the tap water, effluent and biosolid were 9.8- 36.7 ng L^{-1}, 14-47 ng L^{-1} and 2.9-13.8 ng g^{-1} respectively.

Loos and co-researchers undertook a study to provide the first pan-European survey on the occurrence of selected polar organic persistent pollutants in groundwater and analyzed 59 selected organic compounds, including benzotriazoles (benzotriazole and methylbenzotriazole), by reversed-phase liquid chromatography (RP-LC) followed by electrospray ionization(ESI) tandem mass spectrometry(MS) detection[118]. Recoveries of benzotriazole and methylbenzotriazole were 56% and 47% respectively. The method limit of detection was 1ng/L for the two compounds.

An investigation of the occurrence, distribution and fluxes of benzotriazoles along the German large river basins into the North sea was carried out by Wolschke et al. [241]. This study happened to be the first report on the occurrence of benzotriazoles in the marine environment. The sample extracts were analyzed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) in electrospray positive ionization mode. A Phenomenex Synergi Hydro RP 80A column and a suitable guard column (Phenomenex Synergi 2 Hydro RP Mercury 20 2mm) were used for separation. The separation of tolyltriazole isomers was not possible and thus, the isomers were considered as sum. Detection and quantification of the benzotriazoles were carried out by triple-quadrupole mass spectrometer with an electrospray ionization interface in positive ionization mode. Recovery rates of the mass labelled internal standard 1H-Benzotriazole-d_4 in river water samples ranged between 53% for the winter season and 40% for the summer season while the recovery for the seawater samples was 69%. The method quantification limits (MQL) were calculated based on a signal-to-noise ratio of ten. The resulting MQLs were 1.2 ng L^{-1} and 0.4 ng L^{-1} for benzotriazole and tolyltriazole respectively.

Liu et al. applied a previously developed method [228] to investigate the occurrence and removal of four benzotriazoles (benzotriazole, 5-methylbenzotriazole, 5-chlorobenzotriazole and 5,6-dimethylbenzotriazole) and six UV filters in a full scale municipal waste treatment in South Australia [237]. Wastewater samples were extracted by solid phase extraction while the sludge samples were extracted by pressurized liquid extraction. The extracts were analyzed by gas chromatography-tandem mass spectrometry. Repeatability for the target compounds varied from 0.5 to 5.1%, while reproducibility ranged from 0.9 to 9.3%.

Scheurer et al. carrried out a study to investigate the correlation of six anthropogenic markers in wastewater, surface water, bank filtrate, and soil acquifer treatment [123]. Six trace contaminants including benzotriazole and tolyltriazole were traced from wastewater treatment plants to receiving water and further to riverbank filtration wells to evaluate their prediction power as potential wastewater markers. Benzotriazole and 4-methylbenzotriazole were preconcentrated by solid phase extraction (SPE) and analyzed by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry. The compounds were separated by HPLC on a Kinetex PFP column (100mm x 2.1mm; 2.6µm) and run in a gradient elution programme. The compounds were detected and quantified by mass spectrometer in positive ionization mode. Limits of detection for the compounds were 10 µg L^{-1}.

An automated online SPE-HPLC-MS/MS analytical method was developed for the determination of 68 target analytes including benzotriazole and 4/5 methylbenzotriazole mixture in hospital wastewater in order to study the performance and efficiency of membrane bioreactor for the elimination of the compounds [232]. The method consisted of an in-house built SPE, an HPLC column Atlantis T3 for chromatographic separation, and triple quadrupole mass spectrometer detection. Electrospray ionization in polarity switching mode (ESI +/-) was used. MS/MS data were acquired in time selective reaction monitoring (SRM) mode. Limit of quantification was determined by extrapolation from signal to noise ratio of an unspiked sample peak intensity. The relative recoveries of the final analytical method ranged between 78% to 129% for benzotriazole and 135% to 227% for 4/5 methylbenzotriazole mixture in hospital wastewater (raw and treated wastewater) and municipal wastewater (influent and effluent). Limits of quantification for benzotriazole and 4/5 methylbenzotriazole in wastewater effluents were 0.73 µg L^{-1} and 0.063 µg L^{-1}. In their study, sample dilution played an important role for getting reliable results which were little affected by the matrix and therefore improved the accuracy of the method by decreasing the background noise but led to increase in the quantification limit. Generally, the higher the dilution factor the better the recoveries of the compounds.

A simple and rapid dispersive-dispersive liquid-liquid microextraction was developed for the determination of 11 benzotriazoles and benzothiazoles [230]. Analytical determinations were carried out by high- performance liquid chromatography with fluorescence and UV detection and liquid chromatography-tandem mass spectrometry. Chromatographic separations were performed with a C_{18} Column (250 mm x 4.6 mm, 5µm Waters) and a Phenomenex C_{18} guard cartridge and optimized by means of computer-assisted method development with a chemometrics tool (PREGA). Detection was performed at selected fluorescence programming for each compound to obtain good sensitivity. Regarding UV detection, the benzotriazoles were analyzed at 254 nm. For mass spectrometric detection, benzotriazoles and benzothiazoles were determined by operating the ESI source in the negative and positive ionization modes. The HPLC-Flu-UV method exhibited instrumental LOD values between 0.3 and 23.2 µg L^{-1} whereas values between 0.1 and 101.3 µg L^{-1} were achieved by LC-ESI-MS/MS. Fluorescence detection provided good sensitivity for benzotriazoles, obtaining LODs (between 0.3 and 1.1 µg L^{-1}) comparable to those obtained by LC-ESI-MS/MS (between 0.1 and 2.4 µg L^{-1}) for the same compounds.

Carpinteiro and co-workers developed an improved selectivity method for the simultaneous determination of four benzotriazoles and six benzothiazoles in aqueous matrices by mixed-mode solid phase extraction followed by liquid chromatography-tandem mass spectrometry [229]. Compounds were separated using a Zorbax SB-phenyl (100mm x 2.1mm, 3.5µm) column and a C18 guard cartridge in a gradient elution. Compounds were recorded in the multiple reaction monitoring (MRM) positive ionization mode, using two transitions per compound. Absolute recoveries between 80% and 118% were obtained for effluent, influent and river water. The precision of the method, in terms of RSD, ranged between 1% and 12%, the LOQs of the method ranged from 0.002 to 0.4 ng mL^{-1} for river water, from 0.11 to 0.222 ng mL^{-1} for effluent and from 0.0125 to 0.286 ng mL^{-1} for influent.

An analytical procedure was developed for the determination of benzotriazole in aqueous solution by gas chromatography [234]. The proposed method involved a preliminary derivatization with acetic anhydride in an aqueous solution in the presence of sodium bicarbonate with the simultaneous extraction of the forming N-acetyl-benzotriazole with toluene. The toluene extract was analyzed by GC-FID and the structure of the reaction product confirmed by GC-MSD. In the analysis of N-acetylbenzotriazole solutions within the concentration range of 0.002 – 1 mg L^{-1}, the perfect linearity of the FID response was observed; the retention time was constant, the determination limit was 0.001 mg mL^{-1}.

Dominguez and co-workers reported the first application of ionic liquid stationary phases for the determination of benzotriazoles and benzothiazoles in wastewaters by in gas chromatography mass spectrometry [209]. They examined the selectivity and suitability of five new commercially available ionic liquid (IL) stationary phases for GC-MS, in order to obtain the best separation between the various target analytes as well as the highest peak symmetry. The optimum stationary phase (SLB-IL159) was used for the separation and quantification by GC-MS. The linearity range was 0.031 to 3 µg L^{-1} with the correlation coefficients of the calibration curves greater than 0.993. The LODs and LOQs, which are compound dependent, were in the range of 0.025 to 0.562 µg L^{-1} and 0.060 to 0.815 µg L^{-1}, respectively.

4.3. OCCURRENCE OF BENZOTRIAZOLES IN THE AQUATIC ENVIRONMENT

On account of their low sorption capacity [184], benzotriazoles occur mostly in the aquatic environment [14,48,149,235,242]. However, although the mobility of these polar compounds in the environment is still not well characterized, their presence has already been reported in ground water [182,238], bank filtrate [14], and drinking water [22,48,153]. Reported concentrations of benzotriazoles in the environment are summarized in Table 4.2

In the environment, benzotriazoles were first reported in subsurface water close to airports as potential source in concentrations of 128 mg L^{-1} of benzotriazole, 17 mg L^{-1} of 4-tolyltriazole, 198 mg L^{-1} for total tolyltriazole [182,241]. Because of the broad applications, benzotriazole and tolyltriazole have high loads in wastewater treatment plants (WWTPs). Mean influent concentrations of 12 µg L^{-1} for benzotriazole, and 4.4 µg L^{-1} for total tolyltriazole isomers were observed in WWTPs of Berlin [12]. In another study, the mean concentrations in untreated wastewaters of WWTPs of Berlin were 12 µg L^{-1}, 1.3 µg L^{-1}, 2.1 µg L^{-1} for benzotriazole, 4-tolyltriazole and 5-tolyltriazole respectively [149,225].

In addition, the median concentration values for benzotriazole in primary and secondary effluents from 24 municipal WWTPs in Switzerland were 18 and 10 µg L^{-1} respectively [223]. In the effluents of two sewage treatment plants from the Netherlands, of the benzotriazoles determined, the maximum concentrations were observed for benzotriazole (8 µg L^{-1}) and methyl benzotriazole (summed concentration of the two isomers: 3 µg L^{-1}) [153]. The results were in accordance with a study on the occurrence of benzotriazoles and benzothiazoles in different samples from the Berlin region, reported by Weiss and Reemtsma [12]. From a domestic WWTP in Spain, benzotriazole was found to occur at a concentration of 7292 ng L^{-1} in the domestic wastewater influent and a concentration of 2443 ng L^{-1} in the effluent [152] but the two tolyltriazole isomers were not detected in the domestic wastewater influent and effluent. The concentrations of benzotriazole, 4-tolyltriazole and 5-tolyltriazole in industrial effluents were found to be 8221, 6958 and 17153 ng L^{-1}, respectively [152]. These high concentrations in industrial wastewater showed that industrial wastewater was the main pathway by which these compounds enter the aquatic environment in Spain. In sewage effluents of the UK were concentrations up to 3600 ng L^{-1} for benzotriazole and 5700 ng L^{-1} for tolyltriazole [48]. The concentrations of the benzotriazoles in the effluent samples from Bolivar sewage treatment plant, South Australia ranged from 77.2 to 2206 ng L^{-1} [228]. The concentrations of benzotriazole, 5-methylbenzotriazole, 5-chlorobenzotriazole and xylyltriazole in primary effluent were 5706 ng L^{-1}, 6758 ng L^{-1}, 1133 ng L^{-1} and 1313 ng L^{-1} respectively in two sampling campaigns [237]. The concentrations in secondary effluent were still up to 4164 ng L^{-1} for benzotriazole and 1589 ng L^{-1} for 5-methylbenzotriazole. In the final effluent after tertiary treatment including waste stabilization lagoon and DAFF, the target compounds were detected at the concentrations of 2439 ng L^{-1} for benzotriazole, 610 ng L^{-1} for 5-methylbenzotriazole, 182 ng L^{-1} for 5-chlorobenzotriazole and 161 ng L^{-1} for xylyltriazole. It was observed that the concentrations of benzotriazole and 5-methylbenzotriazole were comparable to those found in other studies [149,180,223].

Concentrations of benzotriazoles in raw wastewater were found to range from 0.06 to 36.2 µg L^{-1} by Matamoros and co-workers [224] with highest concentrations measured for benzotriazole. Carpinteiro et al. observed concentration levels of up to 10 ng mL^{-1} for benzotriazole in influent samples and 0.21 ng mL^{-1} for tolyltriazole in river [229]. Dominguez et al. also detected benzotriazoles at a concentration range from 1000 ng L^{-1} to 1700 ng L^{-1} in influent and 650 ng L^{-1} to 1400 ng L^{-1} in effluent [209]. Benzotriazoles were found in raw wastewater and industrial effluent at concentration range from 1600 to 17,600 ng L^{-1} by Pena and co-researchers [230].

Giger et al. observed a maximum concentration value of 6.3 µg L^{-1} in the Glatt river for benzotriazole and a range of 0.1 to 1.2 µg L^{-1} for benzotriazole in the lakes [180]. Concentrations of benzotriazole and tolyltriazole in surface water, influenced by WWTP effluents in Berlin, were found to be in the range of 0.2- 2 µg L^{-1} [12] and the concentrations of benzotriazole, 4-tolyltriazole and 5-tolyltriazole in a river water (Spain) were found to be 1080 ng L^{-1}, 925 ng L^{-1} and 1561 ng L^{-1} respectively [152]. Loos et al. detected benzotriazole and tolyltriazole in 94% and 81% of 122 river water samples distributed over the European Union (EU) with mean concentrations of 493 ng L^{-1} and 617 ng L^{-1} for benzotriazole and tolyltriazole, respectively [4]. In addition, benzotriazole and tolyltriazole were among the single compounds with the highest maximum concentrations; tolyltriazole (20 µg L^{-1}) and benzotriazole (8 µg L^{-1}). In fact, benzotriazole and tolyltriazole were ranked 2nd and 3rd in the median concentration of the 34 analytes covered in the campaign. Moreover, benzotriazole and tolyltriazole were among the compounds detected at low concentrations in the most pristine river samples (clean water samples) which showed their ubiquitous occurrence in even remote areas [9]. The concentrations of benzotriazole in three German rivers ranged from 38-1474 ng L^{-1}, between 25 ng L^{-1} and 281 ng L^{-1} for 5-methylbenzotriazole and from 25 to 952 ng L^{-1} for 4-methylbenzotriazole. Median concentrations of benzotriazole, 5-methylbenzotriazole and 4-methylbenzotriazole were lower in river Main than in river Hengstbach [235].

Also in an EU-wide study on groundwater, the average concentrations of benzotriazole and methylbenzotriazole were 24 ng L^{-1} and 20 ng L^{-1} respectively [118]. Benzotriazole (1.0 µg L^{-1}) was one of the chemicals that were detected at the highest single concentration levels. Benzotriazole, with a median concentration of 185 ng L^{-1}, was one of the most relevant polar compounds identified in the Danube River basin [239]. A river survey in the UK demonstrated that benzotriazole and tolyltriazole were present in all samples, including those from the small tributaries, with the highest concentration observed upstream of a sewage treatment plant being 118 ng L^{-1}, indicating sources other than sewage effluents, such as industrial or urban runoff [48]. The concentrations of benzotriazoles in ground water, South Australia ranged from 97.5 to 280 ng L^{-1} [228]. Concentration levels observed in surface water in the Netherlands were between 0.1 and 1.0 µg L^{-1} for benzotriazole, methyl-benzotriazole and dimethylbenzotriazole [222].

Wolschke et al. investigated the occurrence, distribution and fluxes of benzotriazoles along the German large river basins into the North sea [241]. This study happened to be the first report on the occurrence of benzotriazoles in the marine environment. The total concentrations of benzotriazoles ranged from 1.7 ng L^{-1} to 40 ng L^{-1} in the North sea in coastal areas. Concentrations in rivers ranged from 200 ng L^{-1} to 1250 ng L^{-1}.

In drinking water samples from the Netherlands, the concentration levels of benzotriazoles ranged from 0.01 µg L^{-1} for the compounds 5-methylbenzotriazole, 5,6-dimethylbenzotriazole, 5-chlorobenzotriazole to 0.2 µg L^{-1} for benzotriazole and 4-methylbenzotriazole [153,222]. Concentrations of benzotriazole in tap water samples in the UK ranged from 0.6 to 79.4 ng L^{-1} with an average of 30.9 ng L^{-1} while the concentrations of tolyltriazole ranged from <0.5 to 69.8 ng L^{-1}, with an average of 15.1 ng L^{-1} [48].

Benzotriazole and xylyltriazole were not detected in biosolid (sludge) samples from Bolivar sewage treatment plant, South Australia, whereas the concentrations of

5-methylbenzotriazole and chlorobenzotriazole in the biosolid (sludge) samples were 18.7 and 21.4 ng g^{-1} respectively [228]. Zhang et al. observed mean concentrations of benzotriazole and 5-methylbenzotriazole in sludge samples to be 142 and 71.5 ng g^{-1} respectively [227].

The concentration of the benzotriazoles in soil samples from three USA airports ranged from 2.35 to 424.19 µg Kg^{-1}, with the highest concentrations recorded for 4-methylbenzotriazole in the soil samples [226]. Benzotriazole was not detected in most of the soil samples. The concentration ratio of 4-methylbenzotriazole to 5-methylbenzotriazole in the airport soils generally followed a pattern established in surface and ground water studies, where 4-methylbenzotriazole was more recalcitrant and 5-methylbenzotriazole was more biodegradable. However, in one of the soil samples, a higher level of 5-methylbenzotriazole was measured. Degradation of 5-methylbenzotriazole differs depending on the aerobic or anaerobic conditions of the soil [243].

4.4. REMOVAL EFFICIENCY AND FATE OF BENZOTRIAZOLES IN WASTEWATER TREATMENT PLANTS

As a consequence of insufficient removal during treatment of wastewater released from industries and households, different classes of organic micropollutants are nowadays detected in surface and drinking water [244]. Polar compounds may occur in WWTPs effluents because they are truly persistent under the conditions of an activated sludge process or because their microbial degradation was not fast enough to be completed within a low hydraulic retention time (HRT). An incomplete degradation implies, however, that these compounds could be further degraded after its discharge in the receiving water body. The concentration of a metabolite may increase in activated sludge treatment even though it is degradable, provided that its formation proceeds faster than its further transformation [187].

Moreover, in recent years, an increasing attention has been paid to the removal of various emerging contaminants including benzotriazoles in wastewater treatment plants, mainly due to increasing environmental awareness and wastewater reuse requirements [180,193,223,224]. WWTPs were initially designed to remove/decrease conventional pollution parameters (BOD_5, COD, total suspended solids, etc) from the wastewater stream, so that the final effluents did not constitute a new source of pollution for the water body receiving it.

Table 4.2: Reported concentrations of benzotriazoles in environmental samples and removal in wastewater treatment plants

Environmental Sample	**Reported Concentrations of Benzotriazoles**	**Removal in WWTP**	**Ref**
WWTP influent, WWTP effluent, surface water from Berlin, Germany	12 µg L^{-1} for 1H-BTR in influent 4.4 µg L^{-1} for total TTR in influent 0.02 µg L^{-1} for XTR in influent 9.6 µg L^{-1} for BTR in effluent 2.0 µg L^{-1} for 5-Me-BTR in effluent 2.1 µg L^{-1} for 4-Me-BTR in effluent 0.013 µg L^{-1} for XTR in effluent 0.2 to 2 µg L^{-1} for BTR and TTR in surface water	19% for 1H-BTR 7% for TTR	[12]
Untreated wastewater from WWTPs Berlin	12 µg L^{-1} for 1H-BTR 1.3 µg L^{-1} for 4-TTR 2.1 µg L^{-1} for 5-TTR	37% for 1H-BTR 11% for 5-TTR -6% for 4-TTR	[149]
Municipal Wastewater from 10 WWTPs, Switzerland Glatt river, Switzerland	< 10 to100 µg L^{-1} for 1H-BTR and TTR in influent and effluent 0.64 to 3.69 µg L^{-1} for 1H-BTR in river 0.12 – 0.63 µg L^{-1} for TTR in river	13-62% for BTR 23-74% for TTR	[223]
Drinking water, Surface water, Effluents from STPs Netherland	Up to 8 µg L^{-1} for BTR in effluent Up to 3 µg L^{-1} for total TTR in effluent 0.01 to 0.2 µg L^{-1} in drinking water 0.1 to 1 µg L^{-1} in surface water		[153]
Wastewater influent and effluent from domestic and industrial WWTPs, river water ,Spain	7.29 µg L^{-1} for 1H-BTR in domestic influent 2.44 µg L^{-1} for 1H-BTR in domestic effluent 8.22 µg L^{-1} for 1H-BTR in industrial effluent 6.96 µg L^{-1} for 4-TTR in industrial effluent 17.2 µg L^{-1} for 5-TTR in industrial effluent Up to 1.08 µg L^{-1} for 1H-BTRin river Up to 0.925 µg L^{-1} for 4-TTR in river Up to 1.562 µg L^{-1} for 5-TTR in river		[152]
Sewage effluents , river water, tap water, UK	0.84 to 3.61 µg L^{-1} for 1H-BTR in effluent 2.68 to 5.70 µg L^{-1} for TTR in effluent Up to 0.118 µg L^{-1} in river 0.006 to 0.079 µg L^{-1} for BTR in tap water <0.005 to 0.0698 µg L^{-1} for TTR in		[48]

	tap water		
Effluents from Bolivar STP, ground water, sludge, South Australia	0.077 to 2.21 µg L^{-1} for BTRs in Effluent 0.098 to 0.28 µg L^{-1} for BTRs in ground water 18.7 ng g^{-1} for 5-Me-BTR in sludge 21.4 ng g^{-1} for 5-Cl-BTR in sludge		[228]
Influents and Effluents from municipal STP, South Australia	5.71 µg L^{-1} for 1H-BTR in influent 6.76 µg L^{-1} for 5-Me-BTR in influent 5.13 µg L^{-1} for 5-Cl-BTR in influent Up to 4.16 µg L^{-1} for 1H-BTR in effluent Up to 1.59 µg L^{-1} for 5-Me-BTR in effluent	>85% for 5-Me-BTR and XTR < 75% for BTR and 5-Cl-BTR	[237]
Raw wastewater, Barcelona Spain	0.06 to 36.2 µg L^{-1} for BTRs	65-70% for BTRs	[224]
Influents, River water	Up to 10 µg L^{-1} for BTR in Influent Up to 0.21 µg L^{-1} for TTR in river water		[229]
Influents, effluents	1.0 to 1.7 µg L^{-1} for BTRs in influent 0.65 to 1.4 µg L^{-1} for BTRs in effluent		[209]
Raw wastewater, Industrial effluents	1.6 to 17.6 µg L^{-1} for BTRs		[230]
River water, Lake water	Up to 6.3 µg L^{-1} for 1H-BTR in river water 0.1 to 1.2 µg L^{-1} for 1H-BTR in lake		[180]
River water, EU	Up to 8 µg L^{-1} for 1H-BTR Up to 20 µg L^{-1} for TTR		[4]
River water samples, Germany	0.038 to 1.47 µg L^{-1} for 1H-BTR 0.025 to 0.28 µg L^{-1} for 5-Me-BTR 0.025 to 0.95 µg L^{-1} for 4-Me-BTR		[235]
Groundwater, EU	Up to 1.0 µg L^{-1} for 1H-BTR Average 0.02 µg L^{-1} for TTR		[118]
River Basin, Danube	Median 0.185 µg L^{-1} for 1H-BTR		[239]
Surface water, Netherlands	0.1 to 1.0 µg L^{-1} for 1H-BTR,TTR & XTR		[153]
Sea water and river water, Germany	0.0017 to 0.04 µg L^{-1} for BTRs in sea water 0.20 to 1.25 µg L^{-1} in river		[241]
Sludge	Average 142 ng g^{-1} for 1H-BTR Average 71.5 ng g^{-1} for 5-Me-BTR		[227]
Soil	2.35 to 424 ng g^{-1} for BTRs		[226]

They were not specifically designed for the removal of emerging contaminants such as benzotriazoles. However, it was soon found out that the wastewater organic loads included high levels of variety of hazardous organic pollutants and thus additional treatment steps and control measures became necessary [74].

The elimination efficiency of benzotriazoles from wastewater treatment plants showed wide variations [241] and available reports on their removal are presented in Table 4.2. According to Weiss et al., elimination of benzotriazole, 4-tolyltriazole and 5-tolyltriazole in waste treatment plants appear to be poor [12,149] and can be followed through a water cycle from treated municipal wastewater through surface water to bank filtrate used for drinking water production. The removal efficiencies of benzotriazole and total tolyltriazoles during wastewater treatment were 19% and 7% respectively. It was suggested that the removal of tolyltriazole was solely due to 5-tolyltriazole, whereas the 4-tolyltriazole concentration was not significantly decreased [12]. The 4-tolyltriazole and 5-tolyltriazole isomers showed markedly different biodegradation behaviour with 4-tolyltriazole being the more stable. Removal in sludge was 37% for benzotriazole, but almost no removal of 4-tolyltriazole was observed.

In comparison to a Swiss study [223], there was 13-60% removal for benzotriazole and 23-74% removal for total tolyltriazole in wastewater samples of 10 WWTPs. The elimination efficiencies of benzotriazole and tolytriazole in WWTPs were relatively low, the significant removal of tolyltriazole was remarkable. However, since there was no differentiation between the two isomers in that study and the differences in wastewater quality and treatment as compared to this study were not known, these differences in the findings could not be explained. In two of the WWTPs, the concentrations even increased from the primary to secondary effluent. They assumed this increase effect can either be caused by an input variation or a formation during the treatment, though, for the latter to take place, there would be a need for a precursor chemical.

Benzotriazole was reduced by 37% on average in the membrane bioreactor system (MBR) [225]. Of the two tolyltriazole isomers, 5-tolyltriazole was more amenable to biodegradation [149] and was removed by 11% in the Conventional activated sludge (CAS) system and by 61% in the MBR. 4-tolyltriazole showed no removal in the CAS system, while a weak but insignificant removal of 14% was observed for the MBR. In the cases of benzotriazole and 5-tolyltriazole the MBR effluent quality was significantly better than the CAS effluent.

Matamoros et al. reported the removal efficiency of benzotriazole to range from 65% to 70% in conventional WWTP and from 89 to 93% in constructed wetlands [224]. The higher performance of constructed wetlands in the removal of these compounds was attributed to the contribution of biodegradation, photodegradation and plant uptake.

The analysis of the distribution profile of benzotriazoles in the North Sea, revealed that the decrease of the concentration was mostly caused by dilution and that the benzotriazoles were poorly degradable in the North Sea [241]. Benzotriazoles were removed at various elimination rates in Bolivar WWTP [237]. High aqueous phase removals (> 85%) were achieved for 5-tolyltriazole and xylyltriazole, while low to moderate removals (< 75%) were achieved for benzotriazole and chlorobenzotriazole. It was concluded that biological degradation played a significant role in the removal of the benzotriazoles.

Scheurer et al. observed in their study that benzotriazole appeared to be more degradable than 4-methylbenzotriazole under the operating conditions of the WWTPs [260].

4.5. ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF BENZOTHIAZOLES IN THE AQUATIC ENVIRONMENT

Another crucial class of chemicals of emerging environmental concern investigated in our studies are benzothiazoles. The determination of benzothiazole and its homologues in wastewater requires a sensitive analytical method suitable for complex matrices. Liquid-liquid extraction (LLE) and, more frequently, solid phase extraction (SPE) have been applied to extract benzothiazoles from industrial and municipal wastewater [252]. In these previous studies, benzothiazoles have been analyzed either by liquid chromatography (LC) or gas chromatography (GC) coupled to different detectors including mass spectrometry [152,153, 245, 246,247].

4.5.1. Sample Preparation and Extraction Procedures of Benzothiazoles from aqueous Samples

An analysis of organic pollutants in wastewater requires procedures that are both flexible and capable of detecting specific pollutants. However, the behaviour and performance of the different extractions materials, even those successfully for the extraction of water samples may change on account of the nature of the sample matrix, and the pollutants of interest. Liquid-liquid extraction (LLE) and, more frequently, solid phase extraction (SPE) have been applied to extract benzothiazoles from industrial and municipal wastewaters. Some authors have also employed dispersive liquid-liquid microextraction (DLLME) and stir bar sorptive extraction (SBSE) for the extraction of benzothiazoles from water samples.

The use of LLE, was investigated by Warner et al. who employed dichloromethane, but the study was limited to only thiocyanomethylthiobenzothiazole and benzothiosulfonic acid from spiked distilled water; recoveries of these benzothiazoles from wastewater were poor (or low) [248]. Fiehn et al. developed a method for the extraction of methylbenzothiazole, methylthiobenzothiazole, thiocyanomethylthiobenzothiazole from industrial wastewater using ethyl acetate and toluene [249]. Liquid-liquid extraction was compared with solid-phase extraction (SPE). For liquid-liquid extraction, the efficiencies of different extracting solvents at various pH values were examined. Ethyl acetate was used as the optimum extracting solvent at optimum pH 8.5. They also tested solid phases of different polarity for SPE at the optimum pH 8.5. From the results obtained, it was inferred that SPE appeared to be of limited value for the parallel extraction of the benzothiazoles even though a method for the simultaneous quantitative extraction of these benzothiazoles by solid phases would be of value for the monitoring of surface waters.

Kloepfer and co-workers extracted benzothiazoles from municipal wastewater by solid phase extraction using a polymeric sorbent (Oasis HLB, 200 mg) and 7 mL of methanol/acetone (6:4) as elution solvent [208,246]. The recovery for 2-aminobenzothiazole, benzothiazole-2-sulfonic acid and 2-hydroxybenzothiazole ranged from 85 to 105%, the high recovery of the two most polar analytes (benzothiazole-2-sulfonic acid and 2-aminobenzothiazole) proved that the capacity of the SPE cartridges was not exceeded and no breakthrough occurred. Recovery of benzothiazole, 2-mercaptobenzothiazole and 2-(methylthio)benzothiazole remained limited to 60-70%. It was observed that for 2-mercaptobenzothiazole, a certain extent of oxidation cannot be excluded even though glutathione was added to preserve the thiol moiety. For 2-(methylthio)benzothiazole and benzothiazole, some loss to evaporation might have occurred, as they are the least polar and most volatile benzothiazoles in this series.

Céspedes et al also employed SPE for the extraction of benzothiazoles from surface water, influent and effluent samples [250]. Filtered samples were preconcentrated upon Lichrolut RP-18 (500 mg, 6mL cartridges) and the compounds eluted with 2 x 5mL of MeOH : CH_2Cl_2 (9:1). Recoveries of 62% for benzothiazole, 81% for 2-hydroxybenzothiazole and 64% for 2-(methylthio)benzothiazole were obtained.

A set of six benzothiazoles was extracted from riverine runoff water samples by liquid-liquid extraction (LLE) [247]. Recoveries of benzothiazole and methylthiobenzothiazole in matrix spiked samples were 53 and 75% respectively.

van Leerdam et al used SPE for the simultaneous extraction of benzothiazoles and benzotriazoles from water samples as described under the extraction of benzotriazoles [153]. For the benzothiazoles in their study, the recovery of only 2-aminobenzothiazole decreases from >50% to < 30% when the pH was lowered to 2 due to the basic properties of the compound. The recovery of the other benzothiazoles was not significantly affected by the pH (difference of less than 5%). Recoveries of benzothiazoles ranged between 73 and 132%. In another study, benzothiazoles were enriched from water samples adjusted to pH 6 by solid-phase extraction (SPE) on Oasis HLB 200 mg cartridges and eluted with a mixture of methanol and acetone (60/40, v/v) [251]. Extraction of freeze-dried sludge samples was also accomplished by pressurized liquid extraction (PLE) using a mixture of methanol and water

(50/50, v/v) as extraction solvent followed by SPE.

Bayona and co-workers have also demonstrated the efficiency of polymeric sorbents in SPE for the extraction of benzothiazoles as described in section 4.2.1 for the extraction of benzotriazoles [152,224,236]. Fries et al., preconcentrated benzothiazole in river water, wastewater influent and effluent samples by SPE using Bond Elut ppl cartridges and methanol as the eluting solvent [252]. Mean recoveries of benzothiazole in river water and wastewater influent were 91% and 77% respectively.

Fries also used polar phase stir bar sorptive extraction (SBSE) for the extraction of only benzothiazole from untreated wastewater and river water [253]. The effects of ionic strength and extraction time on the response was studied and optimized. A sodium chloride concentration of 30% and an extraction time of 240 min were selected as optimum parameters. Benzothiazole can be volatilized during extraction procedure resulting in low recoveries for LLE and SPE. Thus, one main advantage of SBSE is that losses via volatilization during extraction are omitted because extraction is performed in sealed vials. The author concluded that SBSE is a solvent free technique that clearly performs better than all previously used extraction techniques for the analysis of benzothiazole in wastewater in terms of easy use, sample throughput and analytical cost.

Pena and co-researchers have also demonstrated the simplicity and efficiency of dispersive liquid-liquid microextraction for the simultaneous extraction of benzothiazoles and benzotriazoles from river, treated and untreated wastewater [230]. Details have been described in section 4.2.1 for the extraction of benzotriazoles. Recoveries ranged between 80.1 % and 97.1%. Carpinteiro et al. also demonstrated the applicability of mixed mode SPE for the simultaneous extraction of benzotriazoles and benzothiazoles from aqueous matrices [229] as already described in section 4.2.1. Absolute recoveries for the benzothiazoles in their study ranged from 87 to 101% in river water, 80 to 102% in effluent and 70 to 90% in influent samples.

A summary of the extraction methods for benzothiazoles from aqueous samples is presented in Table 4.3.

Table 4.3: Overview of Analytical Methodologies for determination of benzothiazoles in the environment *

Analytes	Environment alMedia	Sample Preparation Technique/Cartridges/Solvents	Analytical Technique	Column/Mobile Phase	Analytical Parameters	Ref.
BTH, SH-BTH, Me-S-BTH, TMBTH	Municipal wastewater	LLE/Ethyl acetate as extracting Solvent, SPE	LC-UV			[249]
BTH, SH-BTH, Me-S-BTH, TMBTH	Industrial wastewater	LLE/ Ethyl acetate as extracting Solvent	HPLC-UV			[212]
BTH, Amino-BTH, OH-BTH, SH-BTH, BTHSA, Me-BTH, TMBTH	Raw wastewater, Effluent	Dilution, Direct Injection	LC-ESI-MS/MS	Eurosphere C18(3 x 125 mm ,5µm), gradient elution MeOH/H_2O modified with 0.1%formic acid	LODs= 10-200 ng L^{-1}, 250 ng L^{-1}	[245]
BTH, SH-BTH, OH-BTH	Municipal wastewater	SPE/ Oasis HLB, 200mg, MeOH/Acetone (6:4 v/v) as elution solvent	LC-ESI-MS/MS	Eurosphere C18(3 x 125 mm ,5µm), gradient elution MeOH/H_2O modified with 0.1%formic	LOQs = 25-200 ng L^{-1},	[246]
BTH, Amino-BTH, BTHSA, OH-BTH, SH-BTH, Me-S BTH	Effluent	SPE/ Oasis HLB, 200mg, MeOH/Acetone (6:4 v/v) as elution solvent	LC-MS/MS		LOQs =25-200 ng L^{-1}	[208]
BTH, OH-BTH, Me-S-BTH	Wastewater, River water	SPE/ Lichrolut RP-18(500mg,6ml) column, MeOH/CH_2Cl_2(9:1 v/v) for elution	LC-MS	Lichrospher 100RP-18 (250mm x 4mm, 5µm) Column, gradient elution with MeOH/H_2O	Rec$_{method}$=81,62, 64% LODs = 10,10,100 ng L^{-1}	[250]
BTH, Me-S-BTH,	Riverine runoff water	LLE/CH_2Cl_2:MeOH(1:1) as extraction solvent	GC-MS	DB-5 Column(30m x 0.25mm, 0.25µm)	Rec$_{method}$= 69,71% LOD = 5 ng L^{-1}	[247]
BTH, Amino-	Drinking water,	SPE/Oasis HLB (200mg/5ml)	LC-LTQ-FT-	Phenomenex Aqua	Rec$_{method}$ = 62-111%,	

BTH, OH-BTH, Me-S-BTH	Surface water	glasscolumn & HLB (500mg/6ml) propylene column; ACN/MeOH (50:50) for elution	MS	C_{18}column (250 mm x 4.6mm, 5µm)/gradient elution with $ACN/H_2O/HCOOH$	LOQ = 1-33 ng L^{-1}	[153]
BTH,OH-BTH, Me-S-BTH	River water, Effluent	SPE/Strata X (100mg) cartridge, Ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30m x 0.25mm, 0.25µm)	Rec_{method} =68-90% LOD = 5-36 ng L^{-1} LOQ = 35-146 ng L^{-1}	[152]
BTH, OH-BTH, Me-S-BTH, BTHSA, MoBTH	Influent, Effluent, Groundwater	SPE/ Oasis HLB (200 mg,30µm) cartridges, MeOH/Acetone(6:4 v/v) for elution	LC-(ESI/APCI)-MS/MS	Synergi Fusion-RP 80 A column (150 x3mm, 4µm)/gradient elution with 10 mM ammonium formate/ACN modified with 0.1% HCOOH	$Rec_{ESI-Inf}$ = 84-24% $Rec_{ESI-Eff}$= 42-109% Rec_{ESI-GW}=91-101% $Rec_{APCI-Inf}$ =70-97% $Rec_{APCI-Eff}$ =72-99% $Rec_{APCI-GW}$=102-15%, LOQ_{Inf} = 10-200 ng L^{-1},	[251]
BTH, Me-S-BTH	River	SPE/Strata X Polymeric cartridge, ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30 m x 0.25 mm, 0.25µm)for 1st dimension,TRB-50HT (2 m x 0.1 mm, 0.1µm) for 2nd dimension	Rec_{Method} = 07%,81% LODs=4 ng L^{-1},6 ng L^{-1} LOQs = 6 ng L^{-1}, 10 ng L^{-1} R^2 = 0.9957, 0.9992 Precision(%RSD)= 10,3	[236]
BTH	Untreated wastewater	SBSE /30% NaCl solution for 240 min	TDS-GC-MS	DB-Wax capillary column (30 m x0.25 mm, 0.5µm)	LOD 256 ng L^{-1} R^2 = 0.999 Precision(%RSD) =9.8	[253]
BTH, OH-BTH, Me-SH-BTH, SH-BTH	Influent, Effluent	SPE/Strata X (100mg) cartridge, Hexane/ethyl acetate(1:1 v/v) for elution	IL-GC-MS/MS	SLB-IL159 Column	Rec_{method} = 69-80% LOD =25-562 ng L^{-1}, LOQ =60-815 ng L^{-1} Precision (%RSD) < 10 R^2 > 0.993 Range=31-3000 ngmL^{-1}	[209]
BTH	River water, Influent,	SPE/Bond Elut ppl (100 mg) cartridges, methanol as elution	GC-MS	DB-5 ms (30 m x 0.25 mm x 0.5µm,	Rec_{RW} = 91% Rec_{Inf} <77%	

	Effluent	solvent		Agilent Technologies	LOD =15 ng L^{-1} Precision(%RSD) = 4.4%	[252]
BTH, OH-BTH, Amino-BTH, Me-BTH, Me-S-BTH	River water, Urban Influent & Effluent, Ind. Influent & Effluent	Mixed mode –SPE/ Oasis HLB, MAX Cartridges, MeOH/Acetone(7:3 v/v) for elution	LC-MS/MS	Zorbax SB-phenyl (100 mm x 2.1 mm, 3.5µm) column/ gradient elution with H_2O/MeOH both modified with 1 mM ammonium acetate	Rec_{RW} = 96-100% Rec_{Inf} =68-95% Rec_{Eff} =83-110% LOQ_{RW}= 2-40 ng L^{-1} LOQ_{Inf} =36-286 ng L^{-1} LOQ_{Eff} = 28-222 ng L^{-1} Precision(%RSD)=1-12 $R^2 > 0.99$ Range=LOQ –10 µg L^{-1}	[229]
BTH, OH-BTH, Amino-BTH, Me-S-BTH, SH-BTH	Tap water, River water, Industrial effluent, treated and untreated wastewater	DLLME /100µL TBP as extraction solvent using MeOH as disperser solvent	HPLC-Flu-UV, LC-ESI-MS/MS	C_{18} Column (250 mm x 4.6 mm, 5µm)/gradient elution with ACN/H2O modified with 0.05%HCOOH	Rec_{method} = 80-95% LOD_{UV} = 300-1300 ng L-1 LOQ_{UV}= 1000-9000 ng L^{-1}, Precision(%RSD)= 4.1-6.5 $R^2 > 0.999$ Range =3-536 µg L^{-1}	[230]
BTH, OH-BTH, Me-S-BTH	Wastewater	SPE/Strata X Polymeric cartridge, hexane/ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30m x 0.25mm, 0.25µm)	LOD=5-56 ng L^{-1} LOQ=35-99 ng L^{-1}	[224]
BTH, Me-S-BTH, BTHSA, OH-BTH, MoBTH	Activated sludge	PLE/H_2O/MeOH (50:50 v/v) as extraction solvent, followed by SPE/Oasis HLB (200 mg,30µm) cartridges, MeOH/Acetone(6:4 v/v) for elution	LC-(ESI/APCI)-MS/MS	Synergi Fusion-RP 80 A column (150 x 3 mm, 4µm)/gradient elution with 10 mM ammonium formate/ACN modified with 0.1% HCOOH	Rec_{PLE} = 15-63% $Rec_{ESI-Sludge}$=89-105% $Rec_{APCI-Sludge}$ = 67-104% LOQ_{ESI} = 2.5-100 ng g^{-1} LOQ_{APCI} = 5-100 ng g^{-1}	[251]

*Abbreviations

BTH: benzothiazole; SH-BTH: 2-mercaptobenzothiazole, Me-S-BTH: 2-methylthiobenzotriazole, TMBTH: thiocyanomethylthiobenzothiazole; Amino-BTH: 2-aminobenzothiazole; BTHSA: benzothiazolesulfonic acid; OH-BTH: 2-hydroxybenzothiazole; MoBTH: 2-(4-morpholinyl)benzothiazole; Rec_{method}: method recovery; PLE: pressurized liquid extraction; Rec_{PLE}: recovery for PLE; $Rec_{ESI-Inf}$: method recovery for ESI in influent; $Rec_{ESI-Eff}$: method recovery for ESI in effluent; $Rec_{APCI-Inf}$: method recovery for APCI in influent; $Rec_{APCI-Eff}$: method recovery for APCI in effluent; Rec_{ESI-GW}: method recovery for ESI in groundwater; $Rec_{APCI-GW}$: method recovery for APCI in groundwater; $Rec_{ESI-Sludge}$: method recovery for ESI in sludge; $Rec_{APCI-Sludge}$: method recovery for APCI in sludge; Rec_{RW}: method recovery in river water; Rec_{Inf}: method recovery in influent; Rec_{Eff}: method recovery in effluent; LOQ_{Inf}: LOQ in influent; LOQ_{Eff}: LOQ in effluent; LOQ_{RW}: LOQ in river water; LOQ_{UV}: LOQ for UV detection; LOQ_{ESI}: LOQ for ESI; LOQ_{APCI}: LOQ for APCI; LOD_{UV}: LOD for UV detection

4.5.2. Instrumental methods of analysis for benzothiazoles

So far, development of analytical methods for benzothiazoles has been focused on extraction techniques for the analysis of benzothiazoles in complex aqueous samples such as wastewater. Benzothiazoles have been analyzed by LC or GC coupled to different detectors including mass spectrometry. The overview of the developed analytical methods for the determination of benzothiazoles in the environment is presented in Table 4.3.

Reemtsma developed a method for the analysis of 2-substituted benzothiazoles from wastewater by reversed-phase liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) [245]. Raw wastewater samples were diluted 1:5 prior to analysis in the negative mode. In all other cases, effluent samples were analyzed without dilution. Chromatograhic separation for the positive mode was achieved with a 3 x 125 mm Eurosphere C18 column and a 3 x 5 mm pre-column. Eluent A was H_2O/MeOH (80:20) and B was H_2O/MeOH (10:90), both containing 0.1% formic acid using a linear gradient. Chromatographic separation in the negative method, a 3 x 125 mm Super-sphere 100 C18 column (4µm) with a 3 x 5 mm pre-column of the same material. In this case, eluent A was H_2O/MeOH (85:15), eluent B was H_2O/MeOH (10:90), both with 10mM NH_4OAc. Quantitative analyses were performed in the MS/MS mode by multiple reaction monitoring (MRM). Limits of detection ranged from 10 ng L^{-1} for 2-aminobenzothiazole up to 200 ng L^{-1} for 2-hydroxybenzothiazole with 100 µL injection volume. Benzothiazole was far less sensitive, the low sensitivity was ascribed probably to the lack of any ionic or easily ionized moiety in this molecule, resulting in a detection limit of 2,500 ng L^{-1}.

Kloepfer et al. developed a method for the determination of benzothiazoles (benzothiazole, 2-aminobenzothiazole, 2-(methylthio)benzothiazole, 2-hydroxybenzothiazole, benzothiazole-2-sulfonic acid, 2-mercaptobenzothiazole) from municipal wastewater and surface waters by liquid chromatography-mass spectrometry after an extraction procedure by SPE using a polymeric sorbent [246].The solid phase extraction was carried out with a 200 mg Oasis HLB cartridges, and elution performed with 7 mL methanol/acetone (6:4). Two separate chromatographic runs were performed; one for the MS positive ionization mode and the other for the negative ion mode. For the negative ionization mode separation was carried out on a 3 mm x 125 mm Super-Sphere C18e column with 4 µm particle size while for the positive ion mode, a Eurosphere C18 column (3 mm x 125 mm, 5µm) was used. Recovery of benzothiazole, 2-mercaptobenzothiazole and 2-(methylthio)benzothiazole remained limited to 60-70%. For 2-(methylthio)benzothiazole and benzothiazole, some loss to evaporation might have occurred, as they are the least polar and most volatile benzothiazoles in this series. Reliable quantification with RSD below 25% was achieved. The LOQs of 25- 200 ng L^{-1} for benzothiazoles in treated municipal wastewater were fully satisfactory, but may be too high to allow detection of benzothiazoles from surface waters.

Weiss and Reemtsma developed a method for simultaneous determination of benzotriazoles and benzothiazoles (benzothiazole, 2(methylthio)benzothiazole and 2-aminobenzothiazole) in aqueous environmental samples. Instrumental detection limits for benzothiazole, 2-aminobenzothiazole and 2-(methylthio)benzothiazole, were 50 pg, 15 pg and 35 pg respectively [12]. Details of the method development have already been described in section 4.2.2.

Cespedes et al. undertook a study for the estimation of toxicants like benzothiazoles in surface water samples (along the Ter River and two of its tributaries) [250]. Influent and effluent waters and particulate matter from five sewage treatment plants (STPs) that discharge into these rivers were also studied. They used solid phase extraction (SPE) followed by LC-ESI-MS for the unequivocal identification of different compound classes of pollutants including three benzothiazoles (benzothiazole, 2-(methylthio)benzothiazole and 2-hydroxybenzothiazole). Filtered samples were preconcentrated upon Lichrolut RP-18(500 mg, 6mL cartridges) and the compounds eluted with 2 x 5 mL of MeOH : CH_2Cl_2 (9:1), Chromatographic separation was carried out on a 250 x 4mm, 5µm column (LiChrospher 100 RP-18) preceeded by a guard column (4 x 4 mm i.d., 5µm) of the same packing material from Merck. Two separate separations were carried out, one for the set of compounds detected in positive ionization mode and the other set detected in negative ion mode. Benzothiazole and 2-(methylthio)benzothiazole were detected in the positive ionization mode while 2-hydroxybenzothiazole was detected in the negative ionization mode. Gradient elution was used in both cases. Quantitative analysis was performed in selected ion monitoring (SIM) mode using external standard calibration. Recoveries were 62% for benzothiazole, 81% for 2-hydroxybenzothiazole and 64% for 2-(methylthio)benzothiazole. Limit of detection for benzothiazole was 0.1 µg L^{-1} while LOD for both 2-(methylthio)benzothiazole and 2-

hydroxybenzothiazole was 0.01 µg L^{-1}. A method was developed by van Leerdam and co-researchers for simultaneous determination of benzothiazoles (benzothiazole, 2-Aminobenzothiazole, 2-hydroxybenzothiazole and 2-(methylthio)benzothiazole) and benzotriazoles in environmental water samples by LC-LTQ FT Orbitrap mass spectrometry [153]. Details of the method development have already been described in section 4.2.2 under instrumental method for analysis of benzotriazoles. In addition, recoveries of the benzothiazoles at 0.2 µg L^{-1} spiking concentration ranged from 45% for 2-(methylthio)benzothiazole to 77% for 2-aminobenzothiazole in ultrapure water, 62% for 2-hydroxybenzothiazole to 93% for benzothiazole in drinking water, 67% for 2-hydroxybenzothiazole to 111% for benzothiazole in surface water. The limits of quantification ranged between 3 ng L^{-1} and 17 ng L^{-1} in ultrapure water, between 1 ng L^{-1} and 25 ng L^{-1} in drinking water and between 1 ng L^{-1} and 33 ng L^{-1} in surface water.

A comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry was used by Jover et al. for simultaneous characterization of benzothiazoles, benzotriazoles and benzosulfonamides in aqueous matrixes after a preliminary enrichment by solid phase extraction [152]. Details are described in section 4.2.2 under instrumental methods for analysis of benzotriazoles. Limits of quantitation in river water ranged from 5 ng L^{-1} for benzothiazole to 18 ng L^{-1} for 2-hydroxybenzothiazole and in wastewater ranged from 10 ng L^{-1} to 36 ng L^{-1}.

Wick and co-workers developed a multi-residue method for the determination of 36 emerging organic pollutants including five benzothiazoles in raw and treated wastewater, activated sludge and surface water using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [251]. The targeted benzothiazoles were benzothiazole, 2-(methylthio)benzothiazole, benzothiazole-2- sulfonic acid, 2-hydroxybenzothiazole and 2-(4-morpholinyl)benzothiazole. They assessed and compared matrix effects in the ESI and APCI interfaces in positive and negative ionization mode. The target analytes were enriched from water samples adjusted to pH 6 by solid-phase extraction (SPE) on Oasis HLB 200 mg cartridges and eluted with a mixture of methanol and acetone (60/40, v/v). Extraction of freeze-dried sludge samples was accomplished by pressurized liquid extraction (PLE) using a mixture of methanol and water (50/50, v/v) as extraction solvent followed by SPE. Chromatographic separation was carried out on a Synergi Fusion-RP 80 A column (150 x 3 mm, 4µm) with a SecurityGuard pre-column (4 mm x 3 mm). Two different LC-MS/MS methods were developed, one for positive ionization mode and the other for negative ionization mode. For the benzothiazoles, positive ionization mode was used except 2-hydroxybenzotriazole. Gradient elution was used for the separation.

With the electrospray ionization (ESI) method, relative recoveries of the benzothiazoles ranged from 91% to 101% in groundwater, 42% to 109% in WWTP effluent, 84% to 124% in WWTP influent. With APCI, relative recoveries ranged from 102% to 115% in groundwater, 72% to 99% in WWTP effluent and 70% to 97% in WWTP influent. The relative recoveries in activated sludge ranged between 89% and 105% with ESI while it ranged between 67% and 104% with APCI. Limit of quantification with ESI in WWTP influent ranged from 10 ng L^{-1} for morpholinyl-benzothiazole to 200 ng L^{-1} for benzothiazole while LOQ with APCI in WWTP influent also ranged from 10 ng L^{-1} for 2-(methylthio)benzothiazole to 200 ng L^{-1} for 2-hydroxybenzothiazole. In activated sludge, LOQ with ESI and APCI ranged from 2.5 ng g^{-1} to 100 ng g^{-1} TSS and 5 ng g^{-1} to 100 ng g^{-1} TSS respectively [251].

Ninety seven organic contaminants including benzothiazoles were determined at trace concentration in river water by Matamoros et al. [236]. Limits of detection for benzothiazole and 2-(methylthio)benzothiazole were 4 ng L^{-1} and 6 ng L^{-1} respectively while the limits of quantification were 6 ng L^{-1} and 10 ng L^{-1} respectively.

Dominguez et al. also applied the SPE protocol developed by Jover et al. [152] as the sample extraction procedure for the determination of benzothiazoles and benzotriazoles in wastewaters using ionic liquid stationary phases in gas chromatography mass spectrometry [209]. The same recoveries range of 78% to 98% for wastewaters was also observed. Matamoros and co-workers also adopted the same SPE procedure for studying the occurrence and fate of benzothiazoles and benzotriazoles in constructed wetlands in Spain [224].

4.6. OCCURRENCE OF BENZOTHIAZOLES IN THE AQUATIC ENVIRONMENT

Due to widespread applications, persistence and toxicity, benzothiazole derivatives have remained an environmental concern. Several benzothiazoles such as benzothiazole and 2-methylthiobenzothiazole have been detected in street dust, atmospheric aerosols, street run-off, sediment and an industrial effluent from a tyre manufacturer. The occurrence of benzothiazoles was ascribed mainly to the automobile tyres and rubber manufacturing industries [247,254]. However, detection of benzothiazoles in other environmental compartments such as ground water, surface water, municipal wastewater and sludge [224,236,208,251], indicates that the emission of benzothiazoles is no longer restricted to the rubber industry.

The occurrence of benzothiazoles in wastewater of the tanning industry and their behaviour during biological treatment was studied by Reemtsma et al. [212]. In the wastewater samples of the two tanneries, 2-(mercapto)benzothiazole was the dominant benzothiazole accounting for 87% and 94% of the four benzothiazoles in the study. Benzothiazole and 2-(methylthio) benzothiazole were present, but remained minor constituents in the untreated wastewater while 2-(thiocyanomethylthio)benzothiazole, originally applied in the pickling step of the tanning process, was not detectable.

The mean concentrations of the benzothiazoles as determined by Reemtsma in the raw effluent and biologically treated wastewater, ranged from 0.1 µg L^{-1} to 746.9 µg L^{-1} and 0.1 µg L^{-1} to 76.8 µg L^{-1} respectively [245]. In a study by Kloepfer and co-workers to determine six benzothiazoles, all benzothiazoles except 2-aminobenzothiazole were detected, with concentrations ranging from 180 ng L^{-1} for 2-(methylthio)benzothiazole to 1700 ng L^{-1} for the most polar benzothiazole-2-sulfonic acid being the dominant compound of this class [246]. Concentrations of total benzothiazoles in the effluents of municipal wastewater treatment plants ranged from 1.9 µg L^{-1} to 6.7 µg L^{-1} on average were reported by Kloepfer et al. [208]. In all effluents, benzothiazole-2-sulfonate was the dominant compound with average concentrations between 1 µg L^{-1} and 2.3 µg L^{-1} (35 -70%) followed by benzothiazole, 2-hydroxybenzothiazole and 2-(methylthio)benzothiazole. Concentrations of 2-mercaptobenzothiazole and 2-aminobenzothiazole were below the LOQs (50 ng L^{-1} and 30 ng L^{-1}) respectively.

An overview of the occurrence of benzothiazoles in the aquatic environment is presented in Table 4.4.

Table 4.4: Reported concentrations of benzothiazoles in environmental samples and removal in wastewater treatment plants

Environmental Sample	**Reported Concentrations of Benzothiazoles**	**Removal in WWTP**	**Ref**
Raw and treated wastewater	0.1 to 747 µg L^{-1} for BTHs in raw wastewater 0.1 to 76.8 µg L^{-1} for BTHs in treated wastewater		[245]
Wastewater	0.18 to 1.7 µg L^{-1} for BTHs		[246]
Effluents	1.9 to 6.7 µg L^{-1} for BTHs	5-28% for BTHs	[208]
Influent and effluents	Up to 12.8 µg L^{-1} for 2-Me-S-BTH in influent Up to 6.18 µg L^{-1} for 2-Me-S-BTH in effluent 0.07 to 0.15 µg L^{-1} for 2-OH-BTH		[250]
River water	0.027 to 0.092 µg L^{-1} for BTH 0.015 to 0.305 µg L^{-1} for 2-Me-S-BTH		[236]
Raw wastewater	0.2 to 2.2 µg L^{-1} for BTHs	Up to 55% for BTH Up to 40% for 2-OH-BTH -20% for 2-Me-S-BTH	[224]
Influent, Effluent, surface water and activated sludge	0.01 to 1.49 µg L^{-1} for BTHs in influent 0.009 to 2.04 µg L^{-1} for BTHs in effluent 0.0008 to 2.8 µg L^{-1} for BTHs in surface water 5.3 to 326 ng g^{-1} for BTHs in activated sludge		[251]
Raw wastewater(domestic),Industrial Wastewater	13.2 µg L^{-1} for 2-Me-S-BTH in raw wastewater(domestic) 17.6 µg L^{-1} for 2-Me-SH-BTH in industrial wastewater		[230]
Influents, effluents	Average 0.4 µg L^{-1} for BTH in influent Average 0.7 µg L^{-1} for 2-OH-BTH in influent Average 0.15 µg L^{-1} for 2-Me-S-BTH in influent Average 0.3 µg L^{-1} for BTH in effluent Average 1.0 µg L^{-1} for 2-OH-BTH in effluent Average 0.1 µg L^{-1} for 2-Me-S-BTH in effluent		[209]
Influent, Effluent, river water	Up to 1.4 µg L^{-1} for 2-OH-BTH		[229]

Since concentrations of 2-aminobenzothiazole never exceeded the LOQ in any of the samples analyzed, it was considered as not environmentally relevant.

In a study by Cespedes et al., benzothiazole was not detected in all influent and effluent samples, 2-(methylthio)benzothiazole was one of the most ubiquitous compounds detected (68% of the samples analyzed) [250]. The highest levels of 2-(methylthio)benzothiazole (12.8 µg L^{-1} and 6.18 µg L^{-1}) were found in the STPs, lower concentrations of 0.07 – 0.15 µg L^{-1} of 2-hydroxybenzothiazole were also found in the STPs.

Benzothiazole was detected in the Dutch drinking water samples, 2-Aminobenzothiazole was not detected in surface water samples [153]. Concentrations of benzothiazole and 2-(methylthio)benzothiazole in four Catalonian rivers ranged from 27 to 92 ng L^{-1} and from 15 to 305 ng L^{-1} respectively [236]. In addition, concentrations of benzothiazoles in raw wastewater ranged from 0.2 to 2.2 µg L^{-1} [224].

Wick et al., in consistence with the study by Kloepfer et al. [208], detected some benzothiazoles in the high ng L^{-1} to the low µg L^{-1} range in influents and effluents, with benzothiazole-2-sulfonic acid being the most prominent analyte [251]. Concentrations of the benzothiazoles ranged from 10 ng L^{-1} for morpholinyl-benzothiazole to 1490 ng L^{-1} for benzothiazole-2-sulfonic acid in WWTP influent, 9 ng L^{-1} to 2040 ng L^{-1} in WWTP effluent, 0.8 ng L^{-1} to 2800 ng L^{-1} in surface water, 5.3 ng g^{-1} TSS to 326 ng g^{-1} TSS in sludge. Generally, the highest concentrations were found for benzothiazole-2-sulfonic acid while the lowest concentrations were found morpholinyl-benzothiazole. This was the first time benzothiazole, 2-(methylthio)benzothiazole, 2-hydroxybenzothiazole and benzothiazole-2-sulfonic acid were detected in activated sludge, as it was previously reported that no studies had reported the concentrations of 2-hydroxybenzothiazole and 2-mercaptobenzothiazole in sludge [99]. The concentration of the compounds in sludge is crucial to correctly assess their fate (sorption or biotransformation) in WWTPs.

Pena and co-researchers detected 2-mercapto-benzothiazole (17.6 µg L^{-1}) in an industrial wastewater and 2-methylthiobenzothiazole (13.2 µg L^{-1}) in raw wastewater [230]. None of the benzothiazoles was found in tap water, river water and treated wastewater. As determined by Dominguez et al. [209] the mean concentrations of benzothiazole, 2-hydroxybenzothiazole, and 2-methylthiobenzothiazole in influent samples were 0.4 µg L^{-1}, 0.7 µg L^{-1} and 0.15 µg L^{-1}, respectively, and their mean concentrations in effluent samples were 0.3 µg L^{-1}, 1.0 µg L^{-1} and 0.1 µg L^{-1}, respectively. Carpinteiro et al. also found 2-

hydroxylbenzothiazole, mercaptobenzothiazole and methylthiobenzothiazole in environmental matrices (river water, influent and effluent) in concentrations up to 1.4 µg L^{-1} of 2-hydroxylbenzothiazole [229].

4.7. REMOVAL EFFICIENCY AND FATE OF BENZOTHIAZOLES IN WASTEWATER TREATMENT PLANTS

The removal of micropollutants from wastewater during treatment occurs through abiotic transformation, biological degradation and/or sorption [224]. Among these mechanisms, sorption to suspended solids and biodegradation were reported to play dominant roles. Nevertheless, mechanisms of removal do not follow a general rule since their relative contribution depends on the physicochemical properties of the micro-pollutant, the origin and composition of the wastewater and the operational parameters of the wastewater treatment facility.

There are scarce data on the removal of benzothiazoles in biological wastewater treatment with contradictory reports. Moreover, most of these studies used grab samples and not proportional flow composite samples. Although benzothiazole and 2-hydroxylbenzothiazole are considered to be biodegradable, 2-methylthiobenzothiazole was recalcitrant. Benzothiazole was largely eliminated during aerobic treatment, whereas 2-methylthiobenzothiazole remained after passing through sewage treatment plants, or even formed during treatment [208,212,246].

The overall removal efficiency of total benzothiazoles in municipal wastewater treatment was reported to range from 5 to 28% [208]. The very limited removal was primarily due to benzothiazole-2-sulfonate and 2-methylthiobenzothiazole that were either hardly removed or even increased in concentration. This finding contrasted previous results for an industrial wastewater treatment, where an average elimination of 87% was observed for the same set of benzothiazoles [25].

Matamoros et al. studied the occurrence and fate of benzothiazoles and benzotriazoles in constructed wetlands and evaluated the removal efficiency and aquatic mass discharge rate of benzothiazoles (i.e. benzothiazole, 2-hydroxylbenzothiazole and 2-methylthiobenzothiazole) in one conventional activated sludge WWTP and two constructed wetlands [224]. The removal efficiencies of benzothiazoles ranged from zero to 80%, in the conventional WWTP, and from 83 to 90% in constructed wetlands. The higher performance of constructed wetlands in the removal of the compounds was attributed to the contribution of biodegradation, photodegradation and plant uptake.

4.8. ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF BENZOSULFONAMIDES IN THE AQUATIC ENVIRONMENT

Analytical methodologies for quantification of the benzosulfonamides (BSA, *p*-TSA and *o*-TSA) in environmental water matrices, including wastewater samples, are still limited. Only one quantitative analytical method exists at present for the simultaneous determination of the benzosulfonamides (BSA, *o*-TSA and *p*-TSA) in environmental water matrices [154]. An analytical methodology based on two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC x GC-TOF-MS) has also been demonstrated for the determination of some benzosulfonamides alongside with some benzotriazoles and benzothiazoles in wastewater samples [152].

In a study for the screenings of wastewater in Berlin, Hartig and Jekel detected p-TSA and o-TSA [256]. They developed a method for the simultaneous quantitative analysis of a large set of wastewater sulphonamides residues (antibiotics, herbicides and plasticizers) but eventually discarded p-TSA and o-TSA because of interference with other wastewater and surface water components. They did not, therefore, publish any actual values of p-TSA and o-TSA in environmental water samples. Both compounds were also detected in surface water by Hendricks et al. [213].

4.8.1. Sample Preparation and Extraction Procedures of Benzosulfonamides from aqueous Samples.

Until the period of this work, studies on the determination of benzenesulfonamide and toluenesulfonamides in environmental water samples are few. Solid phase extraction (SPE) has been the preconcentration method of choice for benzosulfonamides in environmental water samples [152,154].

Richter and co-workers extracted benzosulfonamides (benzenesulfonamide, p-toluenesulfonamide and o-toluenesulfonamide) from groundwater, surface water, drinking water and wastewater influents and effluents by SPE using a polymeric sorbent [154]. The elution solvent was 4 mL of methanol. The method recovery for each type of aqueous matrix ranged from 94-120% for all analytes.

Jover et al. also used SPE for simultaneous extraction of three classes of organic compounds (benzotriazoles, benzothiazoles and benzosulfonamides) from influent, effluent and river water samples [152]. The details of the extraction procedure and optimization have been described in section 4.2.1. under extraction procedures of benzotriazoles from aqueous samples. At the optimum SPE conditions used for the simultaneous extraction of the three classes of compounds, recoveries for benzosulfonamides were 92% and 71% in river water and wastewater respectively.

4.8.2. Instrumental methods of analysis for benzosulfonamides

An analytical method was developed for the sensitive and simultaneous determination of three benzosulfonamides (BSA, p-TSA and o-TSA) in wastewater, surface water, groundwater and drinking water [154]. The method was based on solid phase extraction coupled with reversed-phase liquid chromatography and tandem mass spectrometry. Liquid chromatographic separation was carried out using a Sunfire C18 column (150 mm x 2.1 mm, 3.5 µm) and a RP1 guard column (2 mm x 10 mm). For the separation of the isomeric analytes (*p*-TSA and *o*-TSA), isocratic elution was used. A tandem mass spectrometer equipped with electrospray ionization operated in the negative ionization mode was used. The recovery for each type of aqueous matrix was 94- 120% for all analytes. Acceptable recoveries for BSA, *p*-TSA and *o*-TSA were observed with surrogate correction despite the 22 min difference in retention time of BSA in respect to *p*-TSA and *o*-TSA. Limit of quantification in surface water, and in influent and effluent samples of the WWTPs could not be determined for all substances because of their ubiquitous occurrence in these matrixes. LOQs could only be calculated in purified water and drinking water, which were assumed to be valid for all matrices. LOQ for *p*-TSA was 0.01 µg L^{-1} and was 0.02 µg L^{-1} for both *o*-TSA and BSA.

Jover et al. developed a methodology based on GC x GC-TOF-MS which enabled the simultaneous characterization of three chemical classes (benzotriazoles, benzothiazoles and benzosulfonamides) of increasing environmental relevance in complex matrices such as river water and raw wastewater [152]. The compounds were preconcentrated by SPE using Strata X polymeric sorbent. Details about the methodology have been described in section 4.2.2. Recoveries for benzosulfonamides were 92% and 71% in river water and wastewater respectively. The LODs of the analytical method in river water and wastewater were 9 ng L^{-1} and 18 ng L^{-1} respectively, while the LOQs in river water and wastewater were 31 and 62 ng L^{-1} respectively.

An overview of the available analytical methodologies for the determination of benzosulfonamides in the aquatic environment is presented in Table 4.5.

4.9. OCCURRENCE OF BENZOSULFONAMIDES IN THE AQUATIC ENVIRONMENT

Hendricks et al. measured a single value of p-TSA in a semi-quantitative GC-MS screening of a large set of organic compounds in river Rhine water (Germany) and found the concentration of p-TSA to be 0.063 µg L^{-1} but they did not analyze o-TSA and BSA [213]. Richter et al. also detected p-TSA, o-TSA and BSA in surface water samples throughout Berlin concentrations up to 1.15, 0.8 and 0.52 µg L^{-1} respectively [154]. The occurrence and behaviour of wastewater residues p-TSA, o-TSA and BSA through wastewater treatment, surface water, bank filtration and drinking water treatment were also studied by Richter and co-workers [215]. All three compounds were ubiquitous in the aquatic environment of Berlin. p-TSA was found in high concentrations in the wastewater influent (2-15 µg L^{-1}), in the wastewater effluent (<0.15 – 2.34 µg L^{-1}) and in sewage farm ground water (<0.05 to 20 µg L^{-1}), bank filtrate (<0.05 to 0.30 µg L^{-1}) and drinking water (<0.05 to 0.54 µg L^{-1}). The concentration ranges of o-TSA and BSA in wastewater influents were 0.11 to 8 µg L^{-1} and <0.05 to 0.64 µg L^{-1}, respectively, while the values in wastewater effluents were 0.14 to 4 µg L^{-1} for o-TSA and 0.25 to 0.49 µg L^{-1} for BSA.

The concentrations measured in surface water, sewage farm ground water, bank filtrate and drinking water were <0.05 to 1.74 µg L^{-1} for o-TSA and <0.05 to 0.53 µg L^{-1} for BSA.

Table 4.5: Overview of Analytical Methodologies for determination of benzosulfonamides in the environment

Analytes	Environmental Media	Sample Preparation Technique/Cartridges/Solvents	Instrumental Technique	Column/Mobile Phase	Analytical Parameters	Ref.
BSA, o-TSA, p-TSA	Wastewater, Surface water, Ground water Drinking water	SPE/ SDB 1 Polymeric sorbent (200mg, 6ml), methanol as elution solvent	LC-MS/MS	Sunfire C18 (150 mm x 2.1mm, 3.5µm) Column/ Isocratic elution with 10%ACN/ 90%H_2O	Rec_{method} = 94-120% LOQ = 10 ng L^{-1}, 20 ng L^{-1} Precision (%RSD)= 4-18 $R^2 > 0.998$ Linear range= 20- 500 ng L^{-1}	[154] [215]
p-TSA	River water, Wastewater	SPE/Strata X (100 mg) cartridge, Ethyl acetate for elution	GC X GC-TOF/MS	TRB5-MS(30m x 0.25mm, 0.25µm	LOD_{RW} = 9 ng L^{-1} LOD_{WW} = 18 ng L^{-1} LOQ $_{RW}$ = 31 ng L^{-1} LOQ_{WW} = 62 ng L^{-1} $R^2 > 0.998$	[152]

Table 4.6: Reported concentrations of benzosulfonamides in environmental samples and removal in wastewater treatment plants

Environmental Sample	Reported Concentrations of Benzosulfonamides	Removal in STP	Ref.
Surface water, drinking water, Wastewater influent and effluent from Berlin	Up to 1150 ng L^{-1} for p-TSA in surface water, Up to 800 ng L^{-1} for o-TSA in surface water, Up to 520 ng L^{-1} for BSA in surface water, Up to 2700 ng L^{-1} for p-TSA in drinking water, Up to 90 ng L^{-1} for o-TSA in drinking water < 50 ng L^{-1} for BSA in drinking water, Up to 20,000 ng L^{-1} for p-TSA in wastewater influent, < 500 ng L^{-1} for o-TSA in influent and effluent, ~50 ng L^{-1} for BSA in influent, >350 ng L^{-1} for BSA in effluent	~90% for p-TSA, Formation of both o-TSA and BSA	154
Wastewater influent and effluent, Ground water(near sewage farm), Drinking water from Berlin	2000-15,000 ng L^{-1} for p-TSA in influent,<150 -2340 ng L^{-1} for p-TSA in effluent,<50-20,000 ng L^{-1} for p-TSA in ground water,1100-8000 ng L^{-1} for o-TSA in influent,140-4000 ng L^{-1} for o-TSA in effluent, 250-490 ng L^{-1} for BSA in effluent,<50-640 ng L^{-1} for BSA in influent,<50-1740 ng L^{-1} for o-TSA in groundwater,<50-530 ng L^{-1} for BSA in groundwater	~90% for p-TSA, Formation of BSA,	215

Jover et al. detected the concentrations of benzosulfonamides in a range of 49 to 256 ng L^{-1} in river water, <LOQ to 85 ng L^{-1} in municipal wastewater influent, <LOQ to 125 ng L^{-1} in municipal wastewater effluent and <LOQ to 2091 ng L^{-1} in industrial wastewater effluent [152].

4.10. REMOVAL EFFICIENCY AND FATE OF BENZENESULFONAMIDES IN SEWAGE TREATMENT PLANTS

According to Richter et al., p-TSA, o-TSA and BSA behaved differently during wastewater treatment and varied from one wastewater treatment plant to another [154,215]. p-TSA was considerably depleted during wastewater treatment (approximately 90% removal). Depending on the WWTP, o-TSA concentration may increase, decrease or remain stable during treatment. It was suggested that a site-specific process might be responsible for the formation of *o*-TSA during wastewater treatment. BSA could be found in small amounts (~ 0.05 µg L^{-1}) in the influent, but higher concentrations of above 0.35 µg L^{-1} in the effluent. BSA effluent concentrations were 4-6 times higher than the influent concentrations, BSA was clearly formed during treatment and was presumably caused by biodegradation or bioconversion. Knepper et al. described metabolism studies of phenylsulfonamides and the results of the degradation studies showed that *N*-butyl-*N*-phenylsulfonyl may undergo an ω-oxidation to form a glycine-N-(phenylsulfonyl) [257]. They therefore, assumed that BSA is a metabolite of sulphonamide with a higher molecular weight

Moreover, in another study Richter et al. investigated the fate of sulfonamides downgradient of a decommissioned sewage farm near Berlin, Germany [218]. They concluded that the presence of O_2 facilitates the degradation of p-TSA. When the redox conditions were anoxic, p-TSA was not degraded. o-TSA was exclusively degraded in the presence of O_2. BSA was not eliminated under oxic or anoxic experimental conditions. The high concentrations in the study area also showed that the sulphonamides could persist over decades under anoxic conditions in an acquifer environment even though they are rapidly degraded during aerated waste and drinking water treatment [215, 219].

4.11. Summary of Literature Survey on analytical methodologies for the determination of BTRs, BTHs and BSAs in the Aquatic Environment

From the literature survey above, it could be observed that majority of the studies involved in the determination of benzotriazoles, benzothiazoles and benzosulfonamides in the aquatic environments employed solid phase extraction (SPE) as preconcentration and extraction procedures probably due to the efficiency of polymeric sorbents for the extraction of organic compounds covering a range of wide polarity. Liquid-liquid extraction (LLE) was also employed in few cases. Some modifications of SPE such as mixed-mode solid phase extraction and modifications of LLE as Dispersive Liquid liquid microextraction (DLLME) have also been exploited for the extraction of benzotriazoles and benzothiazoles in water samples. Only one study demonstrated the efficiency of stir bar sorptive extraction protocol (SBSE) for the extraction of only benzothiazole from untreated wastewater. Few studies also utilized either direct injection or dilution before injection. In the very few studies of biosolid samples, sample preparation entailed liquid-solid extraction (LSE) or pressurized liquid extraction (PLE) followed by solid phase extraction as clean-up and preconcentration technique.

The preferred instrumental technique of analysis for the compounds was liquid chromatography in combination with mass spectrometry (LC-MS). Gas chromatography-mass spectrometry was also used in some cases. The gas chromatographic methods usually focused on the efficiency of separation and retention of the compounds in the column e.g. as in the choice of ionic liquids for stationary phases and the use of two dimensional gas chromatography (GC x GC). The most commonly used ionization source either in LC-MS or GC-MS was electrospray ionization (ESI) though few studies also used atmospheric pressure chemical ionization (APCI). In some studies, depending on the nature of each analyte separation was carried out in two different chromatographic runs, each chromatographic run for either positive ionization mode or negative ionization mode. Moreover, the most commonly used mass analyzer was triple quadrupole though to a less extent, other type of mass analyzers like time of flight, hybrid ion trap-orbitrap were also used. Finally, other less selective and sensitive detectors for liquid chromatography and gas chromatography such as UV, fluorescence, flame ionization detector were also exploited.

CHAPTER 5

SCOPE AND OBJECTIVES

5.1. PROBLEMS STATEMENT

Many studies have revealed the widespread occurrence of low level concentrations of several organic micro-pollutants which are often considered as ‘emerging contaminants’ in the aquatic environment. For many of the emerging contaminant classes like benzotriazoles, benzothiazoles and benzosulfonamides there are hardly enough environmental survey data, basically because they have not been regulated in the environment. Another reason for this insufficient environmental survey data of the compounds may partly be due to lack of suitable sensitive analytical methods for their determination, proper risk assessment and for monitoring in wastewater, surface and drinking water. Benzotriazoles, benzothiazoles and benzosulfonamides are three classes of organic compounds which have been considered as emerging contaminants in the aquatic environment.

Benzotriazoles and benzothiazoles are polar high production volume chemicals that find broad applications in various industrial processes and household applications. Benzosulfonamides are widely used as plasticizers and chemical intermediates in the synthesis of sweeteners and could even be metabolites of corrosion inhibiting agents and disinfectants.

It could be inferred from the literature survey that benzotriazoles, benzothiazoles and benzosulfonamides are rapidly gaining the attention of environmental scientists as a result of their perceived resistance to degradation, solubility in water, incomplete removal in wastewater treatment plants and consequently, their ubiquitous occurrence in the environment. Many of these compounds have also been shown to have long–term adverse effects on the aquatic organisms and bacteria. However, the mobility of these polar compounds in the environment is still not well characterized even though some studies have investigated and reported their presence in various environmental water compartments. In particular, until the period of this study, no study has exclusively focused on the determination of the compounds in both dissolved phase and particulate phase of wastewater as well as in sewage sludge in parallel. This neglect in the study of particulate phase and sludge could be attributed to more intensive and laborious efforts required in the development of selective and sensitive analytical methodologies for adequate qualitative and quantitative determination of the compounds, like many other organic micropollutants, in these challenging complex solid matrices. Consequently, apart from the possibility of analyte underestimation in the aquatic environment, this challenge has invariably posed some limitations in studying the fate and distribution of these compounds in sewage treatment plants and other environmental water compartments at large. Moreover, most studies of these compounds in the aquatic environment focused on the determination of only few homologous members of each class of the compounds either independently or in parallel determination alongside with other organic micropollutants. For example, benzotriazole and tolyltriazoles of the benzotriazoles class, benzothiazole and 2-methylthiobenzothiazole of benzothiazoles class have been mostly studied. Also, some of the studies have only determined benzotriazoles alongside with other organic micropollutants such as UV filters, pharmaceuticals and perfluorinated compounds. As a result of their determination in parallel with other classes of organic micropollutants, only optimum extraction and instrumental conditions were applied which invariably might not have been the best instrumental conditions for the determination of the individual class of compounds. Moreover, only very few studies have investigated both classes of benzotriazoles and benzothiazoles simultaneously in the aquatic environment. It is therefore, desirable to include more members of each class of the compounds in a simultaneous determination of both classes of the compounds in the aquatic environment. Furthermore, no study has been undertaken until the period of this study to investigate the determination and occurrence of these compounds (benzotriazoles, benzothiazoles and benzosulfonamides) in the Greek aquatic environment and especially their fate and distribution in the Greek sewage treatment plants.

The understanding of the occurrence, removal and fate of emerging contaminants in different wastewater treatment processes is a matter of high importance for ecosystem protection and wastewater reuse. However, very limited systematic data are available on the fate of benzotriazoles, benzothiazoles and benzosulfonamides in sewage treatment plants. This could be due to the fact that most environmental studies of these compounds are usually focused exclusively on aqueous phase concentrations and removal. So far, there are limited data on the contribution of sorption on primary and secondary sludge and the role of biotransformation processes on the removal of benzotriazoles, benzothiazoles and benzosulfonamides in wastewater treatment.

Additionally, to the best of our knowledge, only one study has reported the occurrence of benzothiazoles in sludge (activated) and no information is available on the distribution constants (K_d) of both benzotriazoles and benzothiazoles in sewage sludge.

5.2. RESEARCH OBJECTIVES AND SCOPE

In view of the issues discussed above in section 5.1, we aimed to develop and validate sensitive analytical methods for the determination of these compounds of emerging environmental concern (benzotriazoles, benzothiazoles and benzosulfonamides) in the aquatic environment by liquid chromatography-tandem mass spectrometry with a view to studying their occurrence, fate and distribution in the Greek sewage treatment plants. We particularly paid attention to the determination of the compounds in wastewater (dissolved phase and particulate phase) and sewage sludge from sewage treatment plants. To this effect, we aimed to: (a) develop and validate new analytical methods for the determination of benzotriazoles, benzothiazoles and benzosulfonamides in wastewater (dissolved phase and particulate phase) and sewage sludge of wastewater treatment plants by liquid chromatography-tandem mass spectrometry, (b) investigate the occurrence of benzotriazoles, benzothiazoles and benzosulfonamides in wastewater and sewage sludge of the sewage treatment plant of Athens, Greece, (c) study the removal, fate and distribution of benzotriazoles, benzothiazoles and benzosulfonamides in the sewage treatment plant of Athens, Greece.

The scope of the research work includes: (a) sampling and pretreatment of wastewater and sewage sludge samples from the sewage treatment plant, (b) optimization of solid phase extraction procedures for extraction and pre-concentration of benzotriazoles, benzothiazoles and benzosulfonamides in terms of sorbents, elution solvents and amount of elution solvent (c) extraction from biosolids (sludge and particulate phase) by liquid-solid extraction aided by sonication followed by clean-up and preconcentration by SPE, (d) development of a preliminary Liquid Chromatography-UV method for determination of benzotriazoles, benzothiazoles and benzosulfonamides in environmental aquatic matrices, (e) development and validation of new analytical methods by liquid chromatography-tandem mass spectrometry for the simultaneous determination of eight compounds: four benzotriazoles [1H-benzotriazole (1H-BTR), 5-methylbenzotriazole (5-Me-BTR), 1-hydroxybenzotriazole (1-OH-BTR), 5,6-dimethylbenzotriazole (XTR)] and four benzothiazoles [benzothiazole (BTH), 2-hydroxybenzothiazole (2-OH-BTH), 2-aminobenzothiazole (2-Amino-BTH) and 2-methylthiobenzothiazole (2-Me-S-BTH)] in wastewater and sewage sludge, (f) development and validation of a sensitive analytical method for the determination of three benzosulfonamides [Benzenesulfonamide (BSA), o-Toluenesulfonamide (o-TSA) and p-

Toluenesulfonamide(p-TSA)] in wastewater by liquid chromatography-tandem mass spectrometry, (g) application of the validated analytical methods for investigating the occurrence, removal efficiencies, fate and distribution coefficients of benzotriazoles, benzothiazoles and benzosulfonamides in sewage treatment plant of Athens, Greece.

Finally, we compared the obtained results from this study with existing data in the literature and suggested other areas where further studies should be focused in future research as far as these emerging environmental contaminants are concerned.

CHAPTER 6

INSTRUMENTATION AND MATERIALS

6.1. Instrumentation

In this present study, two liquid chromatographic systems were used: High Performance Liquid Chromatography with UV and Fluorescence detectors (Agilent 1100 Series) and Liquid Chromatography coupled with Tandem Mass Spectrometry (Thermo Electron TSQ Quantum Access with ESI and APCI sources and U-HPLC Thermo Accela pump and autosampler).

The HPLC-UV/FL (Agilent Series) is equipped with a Quat pump (DE43629811), solvent mixer, degasser (JP40720352), Column compartment (DE43643139), UV detector (JP43825677) and fluorescence detector (DE43606666) and system controller(computer) equipped with chemstation software.The system is connected to a printer (HP Deskjet 5740) Sample injection was performed via an injection valve with 20 µL loop. Detection was carried out by a UV detector. Degassing of the mobile phases was achieved by helium sparking in the solvent reservoirs by a degassing unit. The system was controlled and data analyzed on a computer equipped with LC solution software (Chemstation).

The LC-MS/MS system is equipped with UHPLC Accela pump, incorporating a column thermostat, a degasser an autosampler (San Jose, USA). The tandem mass spectrometer system was a Thermo TSQ Quantum Access triple quadrupole mass analyzer, the instrument is connected to an electronic computer with Xcalibur software for data processing.

UV/Visible spectrophotometer (HITACHI-U 2000 Spectrophotometer)

6.2. Laboratory Apparatus and materials

SPE 12-Port vacuum manifold column processor from Supelco (Supelco Visiprep^{TM}), SPE 24-Port vacuum manifold column processor (VAC ELUT SPS 24) from Varian, Supelco 12-port Mini-Vap concentrator/evaporator, Ultrapure water production unit: Millipore Direct-Q UV, apparatus for stirring (Vortex) (VELP$^{®}$ SCIENTIFICA), apparatus for sonication (BRANSON 2510), 15 and 50 mL polypropylene Eppendorf tubes (Sarstedt, Nümbrecht, Germany), Analytical weighing balance, ± 0.0001g (Sartorius-Basic B120).
Micropipettes: 10 µL, 100 µL, 1000 µL and 5000 µL for organic and aqueous solvents.

Glass fibre pre-filters 0.45 µm (Millipore, Bedford, MA, US)

Mini-UniPrep syringeless RC filter membranes 0.2 µm (Whatman, Brentford, Middlesex, UK)

Analytical columns experimented include : Zorbax SB C_{18} 150 mm x 0.46 cm, 5µm; Brand Xterra MS C_{18} 100 mm x 2.1mm, 3.5 µm; Lichrospher 100RP-18, 250 mm x 4mm, 5 µm ; Symmetry C_{18}, 150 mm x 3.9 mm, 5µm ; Zorbax XDB C_{18} 150 mm x 0.46 cm,5µm; Ascentis Express column C_{18}, 7.5cm x 2.1mm, 2.7µm from SUPELCO; Pathfinder Schimadzu column C_{18} EP,4.6mm x 150mm, 3.5 µm; Atlantis T_3 C_{18} 2.1 x 100mm, 3 µm from Water, Ireland.

Guard Column: Phenomenex C18 (4.0 mm × 2.0 mm, 5 µm)

SPE cartridges and sorbents evaluated: Oasis HLB (200mg/6mL, 30µm, 82Å) obtained from Waters USA; Strata X Polymeric RP (200mg/ 6mL, 33µm, 90 Å), obtained from Phenomenex, Germany; Evolute ABN (200mg/6mL, 50µm, 48Å), obtained from Biotage UK; Isolute C18 (500mg/ 6mL), obtained from Biotage UK; Polyclean Polymeric (200mg/6mL, 30 µm), obtained from Interchim, France).

6.3. Chemical Reagents

HPLC grade Acetonitrile, obtained from Merck (Darmstadt, Germany), HPLC grade Methanol, obtained from Merck (Darmstadt, Germany), LC/MS grade methanol, obtained from Merck (Darmstadt, Germany), LC/MS grade acetonitrile, obtained from Merck (Darmstadt, Germany), Formic acid (HCOOH) (98% v/v), obtained from Sigma-Aldrich (Steinheim, Germany). Ultrapure water, purified with a Milli Q water purification system from Millipore (Millipore, Bedford, MA, USA)

6.4. Analytical Standards

1H-Benzotriazole (bottle of 50g, ≥ 99%, CAS No : 95-14-7) purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany); 5-Methyl-1H-Benzotriazole (bottle of 10g, 98%, CAS No : 136-85-6) purchased from Acros Organics (New Jersey, USA); 4-Methyl-1H-Benzotriazole (bottle of 50mg, >90%) purchased from Sigma-Aldrich (Steinheim, Germany); 1-Hydroxy benzotriazoles hydrate (bottle of 10g, ≥ 98%, CAS No : 2592-95-2) purchased from Sigma-Aldrich (Steinheim, Germany); 5,6 Dimethylbenzotriazole (bottle of 5g, ≥99%, CAS No :4184-79-6) purchased from Acros Organics (New Jersey, USA); Benzothiazole (bottle of 50g, ≥ 96%, CAS No : 95-16-9) purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany); 2-Amino benzothiazole (bottle of 100g, 97%, CAS No:136-95-8) purchased from Acros Organics (New Jersey, USA); 2-Hydroxybenzothiazole (bottle of 5g, 98%, CAS No: 934-34-9), purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany); 2-(Methylthio) benzothiazole (bottle of 50g, 97%, CAS No : 615-22-5) purchased from Sigma-Aldrich (Steinheim, Germany); Benzenesulfonamide (98%, CAS No: 98-10-2) purchased from Acros Organics (New Jersey, USA); p-Toluenesulfonamide (99%, CAS No : 70-55-3) purchased from Acros Organics (New Jersey, USA); o-Toluenesulfonamide (99%, CAS No :88-19-7) purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany); Atrazine-d5 (10mg, pestanal grade, CAS No :163165-75-1) purchased from Sigma-Aldrich (Steinheim, Germany); 1H-Benzotriazole-d4 (1 mL, 50 μg mL^{-1} in acetone, CAS No: 1185072-03-0) purchased from Sigma-Aldrich (Steinheim, Germany).

CHAPTER 7

DETERMINATION OF BENZOTRIAZOLES AND BENZOTHIAZOLES IN WASTEWATER AND SLUDGE BY LC-MS/MS

7.1. HPLC-UV METHOD DEVELOPMENT FOR BENZOTRIAZOLES AND BENZOTHIAZOLES

To develop an efficient HPLC method, preliminary experiments were carried out with a view to optimizing the chromatographic conditions necessary for effective separation of the compounds: Benzotriazoles (Benzotriazole, 5-Methyl-1H-Benzotriazole, 1-Hydroxy benzotriazole, 5,6-dimethylbenzotriazole) and benzothiazoles (benzothiazole, 2-hydroxybenzothiazole, 2-Aminobenzothiazole, 2-(methylthio)benzothiazole).Optimization of chromatographic parameters was performed on Zorbax SB C_{18} (150 mm x 0.46 cm, 5µm) column. Chromatographic conditions, such as wavelength of detection, mobile phase composition, flow rate and column oven temperature were optimized. The optimization was achieved by changing the value of the parameters one at a time, while keeping other conditions constant. We tested several columns of different stationary phases for their efficiency in the separation of the compounds. Particular attention was paid to the optimization of mobile phase composition in order to achieve a chromatographic separation of all the compounds with acceptable resolution within a short elution time. Standard solutions at different concentrations ranging from 50 µg L^{-1} to 5 mg/L of the compounds were used for the optimization.

7.1.1. Preparation of Standard Solutions

Stock solutions of benzotriazoles and benzothiazoles were prepared at concentration level 1000 mg L^{-1} by dissolving an appropriate amount of each compound in methanol and stored at - 20°C. Working standard solutions were prepared from stock solutions by further dilution with solvent(s) similar in composition to the mobile phase used for chromatographic elution.

7.1.2. Optimization of UV absorbance wavelength for benzotriazoles and benzothiazoles

To develop a preliminary method for the determination of benzotriazoles and benzothiazoles with HPLC-UV, some preliminary experiments were carried out in order to determine the optimum absorption wavelength of each of the compounds with UV/Visible spectrophotometer. The absorption spectrum of each of the compounds was taken by running the standard solution of the compound on a UV/Visible spectrophotometer. 2 mg L^{-1} standard solution of each of the compounds, prepared in an approximate mobile phase composition intended to be used for liquid chromatographic separation, was put in a pre-cleaned cuvette (as the sample cell) and the mobile phase solvent in another cuvette (as the reference cell) for the spectra to be obtained. Both the sample and the reference cells were initially filled with the solvent (HPLC mobile solvent) to run a baseline spectrum, which was stored by the instrument computer system. The sample cell was then cleaned and filled with sample solution (standard solution of compound), while the reference cell containing the blank mobile phase was left unremoved. An optimum wavelength of detection was obtained for each of the benzotriazoles and benzothiazoles. A compromise optimum wavelength of 254 nm was selected for simultaneous detection of all the compounds with UV detector on HPLC.

7.1.3. Optimization of Chromatographic Parameters

Different analytical columns (Zorbax SB C18 150 mm x 4.6 mm, 5 µm; Zorbax XDB C18 150 mm x 4.6 mm, 5 µm; Lichrospher 100RP-18, 250 mm x 4mm, 5 µm) were tested for separation of the compounds. Based on the efficiencies of separation of the compounds on the different analytical columns tested, Zorbax SB C18 was subsequently chosen for the simultaneous separation of four benzotriazoles and four benzothiazoles. Moreover, mobile phase composition, flow rate, column oven temperature and elution programme were also optimized to achieve good resolution within a possible short elution time to avoid tailing of the peaks (especially the late eluting ones like benzothiazole and 2-methylthiobenzothiazole). In addition, the shorter the elution time, the faster will be the whole analytical process and consequently, higher throughput. For the optimized conditions with reversed-phase Zorbax SB C18, water-acetonitrile was used as mobile phase in a gradient elution programme at a flow rate of 0.5 mL/min, with 20°C as column oven temperature and wavelength of 254 nm. The gradient elution programme is as shown in Table 7.1.

Table 7.1: Chromatographic gradient elution programme for HPLC-UV separation of benzotriazoles and benzothiazoles

Time(min)	%A	%B
0	20	80
12	40	60
20	44	56
24	100	0
25	20	80

Mobile Phase: (A)-Acetonitrile, (B)-Water, Zorbax SB C18 Column

The chromatograms of separation of the compounds (four benzotriazoles and four benzothiazoles) are shown in the Fig 7.1.

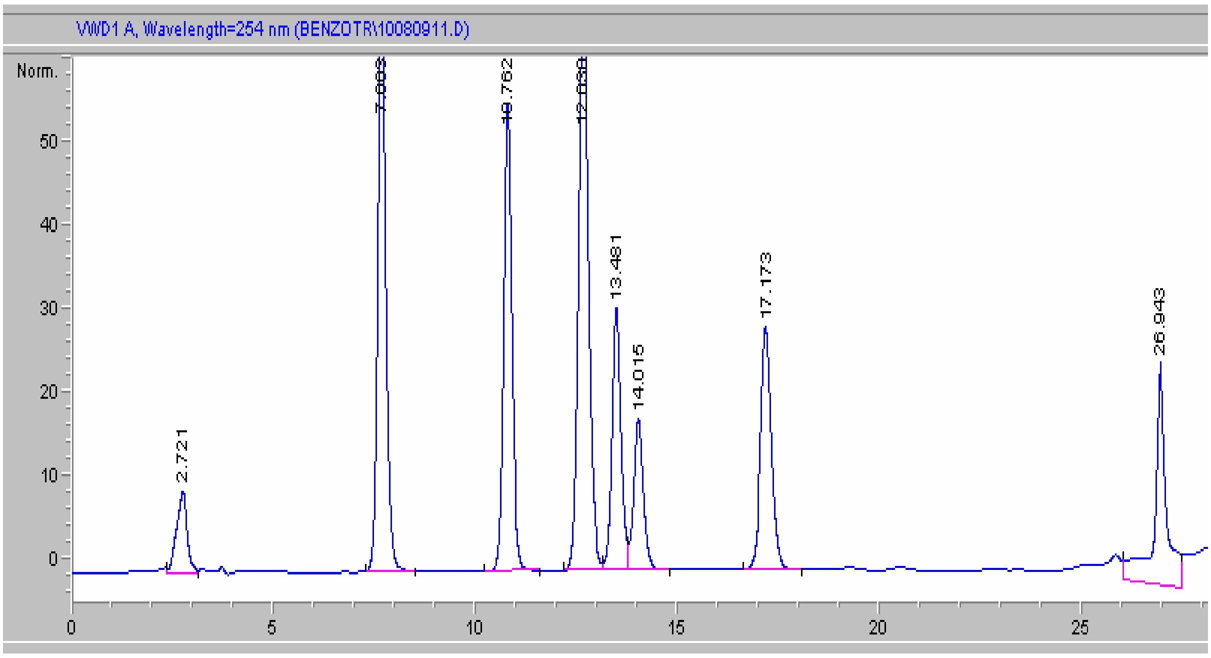


Fig 7.1: Chromatograms of separation of four benzotriazoles and four benzothiazoles with HPLC-UV

With these optimized chromatographic parameters on HPLC-UV, all the compounds were well separated with good retention and satisfactory resolution. The performance parameters of the chromatographic separation of the compounds are presented in Table 7.2.

Table 7.2: Performance parameters of chromatographic separation of benzotriazoles and benzothiazoles with HPLC-UV

Compound	**Rt (min)**	**Area**	**Symmetry**	**Width**	**Plates**	**Resolution**	**Selectivity**
1H-BTR	7.60	631	0.87	0.17	10338	12.79	2.59
1-OH BTR	2.72	101	1.79	0.25	743	-	-
5-Me-1H-BTR	10.76	534	0.92	0.10	16612	9.78	1.41
XTR	13.48	285	0.94	0.20	24462	2.05	1.06
BTH	17.17	682	0.93	0.27	22462	7.66	1.22
2-OH-BTH	14.02	162	0.78	0.21	23817	1.52	1.04
2-Amino-BTH	12.03	770	0.84	0.24	15463	5.19	1.18
2-Me-S-BTH	26.94	383	0.93	0.19	112248	25.08	1.57

Column: Zorbax SDB C18 (150mm x 4.6mm, 5µm), Flow rate :0.5ml/min, mobile phase (Acetonitrile/Water)

The instrumental limits of detection for the compounds ranged between 0.014 ng and 0.140 ng as depicted in Table 7.3.

Table 7.3: Instrumental Limits of Detection of benzotriazoles and benzothiazoles with HPLC-UV (20 µL injection)

Compound	**Instrumental LOD with HPLC-UV (ng)**
1H-BTR	0.034
1-OH-BTR	0.140
5-Me-1H-BTR	0.015
XTR	0.030
BTH	0.030
2-OH-BTH	0.014
2-Amino-BTH	0.041
2-Me-S-BTH	0.068

7.2. Development and optimization of a SPE protocol for extraction of benzotriazoles and benzothiazoles using purified water

In order to extract and pre-concentrate the compounds in aqueous samples, we developed and optimized a preliminary solid phase extraction (SPE) protocol using purified water. Three different SPE cartridges and sorbents (Oasis HLB, 200 mg/6mL; Evolute ABN, 200mg/6mL; and Strata X, 200mg/6mLwere tested for their performance in terms of recovery rates for the compounds. In addition, we compared the efficiency of the sorbents at two different sample pH values; one at a neutral pH (~7) and the other in acidic (~ 3). Different elution solvents (methanol, acetonitrile and 50:50 acetonitrile: methanol) and different elution volumes (3mL, 6mL, 10mL, 15mL) were also evaluated to obtain the optimum conditions for most of the compounds. The SPE procedure was as follows:

A mixture of standard solution of the compounds (BTH, TTR, 1-OH-BTR, XTR, BTH, 2-OH-BTH, 2-AminoBTH, 2-Me-S-BTH) at 2 mg L^{-1} level was prepared from 1000 mg L^{-1} stock solution. A set of purified water samples were adjusted to a pH of ~3 with 25%

hydrochloric acid and other set samples to a pH of ~7 with ammonia solution**.** The purpose of acidifying the water samples was to ensure that the analytes were less readily soluble since they are known to be moderately polar and weakly basic. 100 mL of the purified water samples (pH adjusted) was spiked with 2µg of each of the compounds (1 mL of 2 mg L^{-1} mixture) to give a 20 µg L^{-1} spike concentration level for all the compounds. The three cartridges/sorbents tested were conditioned with 10 mL methanol and then equilibrated with 10mL acidified water(purified) (pH ~3) for the experiments at acidic pH or 10 mL purified water at pH ~7 for the experiments at neutral pH. Both the acidified water at pH ~3 and purified water at pH ~7 were used in order to prepare the analytes in the sample for interaction with the sorbents and to wash off potential substances that could serve as interference. The purified water samples were loaded on the cartridges at a flow rate of approximately 5mL/min. The cartridges were later washed with 5 mL twice of acidified purified water (a total of 10 mL) in the case of the experiments in acidic pH or with 5 mL twice of purified water at neutral pH (a total of 10 mL) in the case of the experiments for pH 7. The flow rate during the washing was carefully controlled to ensure maximum elution of any interfering substance. The cartridges were left to dry under vacuum for about 60 minutes and the analytes eluted with 10 mL of 50/50 methanol/acetonitrile. The eluents were evaporated to dryness under a gentle stream of nitrogen gas at 45°C and the extract was reconstituted in 1 mL of 20:80 Acetonirile: water. The same procedure was repeated simultaneously for another set of samples at pHs of ~ 3 and ~7 without a prior spiking of the compounds before extraction but the corresponding concentrations of the compounds in the final extract were spiked after the evaporation step.

The reconstituted extracts were injected on HPLC-UV for analysis.

7.3. METHOD DEVELOPMENT FOR DETERMINATION OF BENZOTRIAZOLES AND BENZOTHIAZOLES IN WASTEWATER (DISSOLVED PHASE) BY LC-MS/MS

7.3.1. Sample Pretreatment for wastewater

A mixture of effluent wastewater samples from a sewage treatment plant was used for method development. The mixed effluent sample was filtered with a glass fibre pre-filters (Tullagreen Carrigtwohill co.) under vacuum and adjusted to a pH of ~3 with 25% hydrochloric acid.

7.3.2. Extraction and Preconcentration from wastewater (dissolved phase) by SPE

Extraction and preconcentration of the compounds in wastewater effluent was carried out by the optimized solid phase extraction procedures developed using purified water (section 7.2) with slight modifications. Strata-X (200mg/6mL) and Evolute ABN (200mg/6mL) cartridges/sorbents were compared for the SPE protocol. 50mL of mixed wastewater effluent was adjusted to an approximately pH of 3 with 25% hydrochloric acid. The effluent samples were spiked prior to extraction with the standard solution of the target compounds at three different levels of fortification; representing low, medium and high concentration levels (0.1 ng mL^{-1}, 0.5 ng mL^{-1} and 1.0 ng mL^{-1}; the concentrations in the final extracts were 5 ng mL^{-1}, 25 ng mL^{-1} and 50 ng mL^{-1} respectively). The effluent samples were also spiked with 1 ng mL^{-1} each of atrazine-d5 and benzotriazoles-d4 as internal standards. The remaining part of the SPE procedure was the same as reported for purified water in Section 7.2. The matrix matched samples were also carried through the same procedure, only that the sample extracts were spiked with the standard solution of the target compounds after the evaporation stage. The absolute recovery of the compounds with the SPE procedure was computed by comparing the response of each compound (concentration) in the spiked samples before extraction to the response (concentration) of the same compound in the spiked samples after extraction. The relative recovery of the compound was computed by comparing the response of each compound relative to the response of atrazine-d5 or benzotriazole-d4 response in spiked samples before extraction to the response of the same compound relative to the response of atrazine-d5 or benzotriazoles-d4 in spiked samples after extraction

7.3.3. Chromatographic Separation of Benzotriazoles and Benzothiazoles in wastewater (dissolved phase) by LC-MS/MS

In order to obtain an efficient chromatographic procedure for the separation of the analytes of interest with LC-MS/MS, the already optimized chromatographic parameters, with HPLC-UV for the compounds by using their standard solutions (Section 7.1.3), were exported and optimized into analogous values for the analytical column (dimension) and mobile phase composition used for LC-MS/MS.

7.3.3.1. Selection of Analytical column for LC-MS/MS

Analytical columns, Xterra MS C18 (100 mm x 2.1 mm; 5µm) and Atlantis T3 column (C18 2.1 x 100 mm, 3µm from Water, Ireland) were tested for the separation of the compounds on

LC-MS/MS. For all target compounds (BTRs and BTHs), with the exception of 2-Amino-BTH, slightly higher signal-to-noise ratios were achieved with Atlantis T3 than with XTerra MS C18, whereas slightly higher peak areas were achieved with XTerra MS C18 than with Atlantis T3. Atlantis T3 column was finally selected for the determination of the compounds in wastewater and sludge samples.

7.3.3.2. Selection of Mobile Phase and Modifier

Mobile phase composition was optimized for the separation of the compounds considering also the effects on their ionization in mass spectrometry. A combination of acetonitrile and water only, acetonitrile and 0.01% formic acid in water, acetonitrile and 0.05% formic acid in water, acetonitrile and 0.1%formic acid in water were evaluated separately for the separation of the compounds. Methanol in place of acetonitrile was also tested but acetonitrile produced constantly higher responses and better peak shapes.

Ionic strength and mobile phase pH can affect notoriously the efficiency of ionization in mass spectrometry. In this study, the mass spectrometer was operated in positive ionization mode and, since benzotriazoles and benzothiazoles are weakly basic, a proton donor such as formic acid or acetic acid would be a desirable additive for adjustment of mobile phase pH as it also enhances the ionization of the compounds. Thus, different concentration of formic acid (0 to 0.1%) in purified water were tested and it was shown that 0.1% formic acid gave the highest intensity for the compounds. 0.1 % formic acid was therefore, subsequently selected as the mobile phase modifier for the chromatographic separation. A combination of acetonitrile and 0.1% formic acid in water were finally applied as mobile phase solvents at a flow rate of 200 µL/min on LC-MS/MS.

The optimized chromatographic conditions finally used for the separation of the analytes of interest on LC-MS/MS are as follows:

Analytical Column: Atlantis T3 (100 mm × 2.1 mm, 3µm)
Mobile Phase composition: Acetonitrile and 0.1% formic acid in purified water using the gradient elution programme in Table 7.4
Flow rate: 200 µL/min
Column Oven Temperature: 25°C
Injection Volume: 5 µL
The gradient elution programme is shown in Table 7.4

Table 7.4: Gradient Elution Programme for Atlantis T3 column (100 mm × 2.1 mm, 3µm) with LC-MS/MS at 200µL/min

Time(min)	**%ACN**	**%HCOOH(0.1% in H_2O)**
0	5	95
6.5	40	60
10.5	100	0
14.5	100	0
16	5	95
21	5	95

7.3.4. Optimization of Mass spectrometric parameters for Benzotriazoles and benzothiazoles

Mass Spectrometry (MS) has revolutionalized environmental analytical chemistry by allowing the analysis of complex organic mixtures for trace amounts of analytes due to its intrinsic characteristics such as selectivity, sensitivity, and identification-confirmation capability. Once compounds have been separated into distinct chromatographic peaks, they need to be moved into mass spectrometer for analysis. This includes everything coming out of the column: separated compounds, elution solvents and volatile reagents. The optimization of mass spectrometric parameters is an inevitable aspect of mass spectrometric experiments if the ultimate goal is to obtain maximum sensitivity for the determination of the compounds of interest. The ionization techniques most widely used for liquid chromatography-mass spectrometry in environmental analysis are the so called ‘soft ionization’ techniques. Two examples of such ionization techniques are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), both of which are considered as atmospheric pressure ionization (API) techniques. In this work, we demonstrated the applicability of electrospray ionization (ESI) as ionization source for the mass spectrometry determination of the BTRs and BTHs due to the relatively high polarity and low molecular weight of most of the compounds (benzotriazoles and benzothiazoles) as depicted by their log K_{ow} values (Table 3.1 and Table 3.2), since ESI is well amenable for the determination of polar and low molecular weight compounds. The correct measurements of mass spectrometric parameters for ion source usually results in the improvement of the signals and sensitivity of the compounds under a given study.

Some of the major parameters to be optimized in mass spectrometry experiments include the tube lens, spray voltage, sheath gas, auxiliary gas, capillary temperature, micrometer and probe position. The tube lens is flow rate, mass (compound) and temperature – dependent; therefore, for maximum sensitivity, the tube lens should be optimized for every compound of interest using the approximate liquid chromatographic conditions to be used for the experiments. The position of the probe, capillary temperature, the flow rates of sheath gas and auxiliary gas should be optimized until a stable spray is obtained. The voltages on the ion optics components should also be optimized for the focussing of the analyte towards the analyzer. For MS/MS, parameters such as collision energy and collision pressure must be set. In this study, direct infusion was used for the optimization of tube lens for the analytes of interest (BTRs and BTHs). Liquid Chromatography with Tandem Mass Spectrometry (Thermo Electron TSQ Quantum Access) equipped with ESI and APCI sources and U-HPLC Thermo Accela pump and autosampler) was used throughout the experiments for the mass spectrometric determination of the compounds.

7.3.4.1. Optimization of ESI Source Parameters for benzotriazoles and benzothiazoles

The optimization of tube lens for each of the compounds was carried out by direct infusion using the electrospray ionization (ESI) ion source in the positive ionization mode. Positive ionization mode was selected because the compounds (benzotriazoles and benzothiazoles) are weakly basic and basic compounds are known to give the protonated molecular ion (positive ion) in low pH solution. Positive ion API may also be seen as a more general ionization mode since protons may be loosely associated to a molecule even when there are no obvious basic functional groups. Standard solutions of each of the compounds (1H-BTR, TTR, 1-OH-BTR, XTR, BTH, 2-OH-BTH, 2-Amino-BTH, 2-Me-S-BTH) were prepared at 2 mg L^{-1} concentration level from 1000 mg L^{-1} stock solution in Acetonitrile/0.1% formic acid(in MilliQ grade water) (1:1 v/v) as solvents which are similar in composition to the mobile phase intended for the LC separation. For efficient ionization, 0.1% formic acid (in purified water) was included as a mobile phase solvent so as to create an enabling environment for the production of positive ions (H^+) (proton donor) by adjusting the pH to below the pK_a of the compounds. Individual compound in solution was optimized separately for electrospray ionization (ESI) parameters. The parameters included in the optimization experiments are the spray voltage, sheath gas flow, auxiliary gas flow, capillary temperature, micrometer, and the position of the probe. The optimization was carried out by continuously injecting 10 µL standard solution of the analyte (via loop injection), changing one parameter at a time, until an optimum response for the compound was obtained. The experiments were carried out in MS using the flow rate (200 µL/min) intended to be used for the LC separation and compounds were dissolved in solvents similar in composition to the mobile phase (Acetonitrile and 0.1% formic acid) of the LC. The standard solutions of the compounds were initially prepared in three different compositions of solvents [H_2O/ACN (95:5 v/v), H_2O/ACN (80:20 v/v), ACN/0.1%HCOOH in H_2O (95:5 v/v)] and experimented for the optimization of ESI source parameters. ACN/0.1%HCOOH in H_2O was eventually used because it gave generally better sensitivity for all the compounds than any of the other combinations in the ESI source optimization experiments and since there was no significant difference in the chromatographic behaviour of the compounds of interest when ACN/0.1%HCOOH in H_2O was used in place of ordinary H_2O as mobile phase. The optimization of the ESI source parameters was performed in a continuous flow mode.

The optimized parameters for the electrospray ionization (ESI) in the positive ionization mode for the compounds of interest at 200 µL/min are presented in Table 7.5.

From the results obtained from the ESI source optimization experiments, a compromise value of each parameter was chosen for all the compounds in order to perform a single LC-MS/MS run for the simultaneous determination of the compounds.

The following optimized ESI source conditions were selected as the compromise values for subsequent experiments in MS:

Spray Voltage (V): + 4000

Sheath Gas pressure (psi): 23

Auxiliary Gas flow rate: 20

Probe position: Between B and C (V-position)

Micrometer (inches): 0.45

Capillary temperature (°C): 290

Collision gas (Ar) pressure (mTorr): 1.5

Table 7.5: Optimized ESI Source Parameters for Benzotriazoles and Benzothiazoles in positive ionization mode at 200µL/ min flow rate.

Compound	**Spray Voltage(V)**	**Sheath gas(psi)**	**Auxilliary gas**	**Capillary Temp(°C)**	**Probe Position**	**Micrometer**
1H-BTR	4500	25	22	275	B	0.5
1-OH-BTR	3500	23	21	290	B	0.45
5-Me-1H-BTR	4000	23	21	240	B	0.45
XTR	4000	23	21	240	B	0.45
BTH	4000	19	17	260	B	0.45
2-OH-BTH	3500	25	25	275	B	0.45
2-Amino-BTH	4000	23	21	280	B	0.45
2-Me-S-BTH	4000	23	21	310	C	0.45

7.3.4.2. Optimization of ESI/MS/MS Parameters for Benzotriazoles and Benzothiazoles

For MS^2 analysis, selected reaction monitoring (SRM) in positive ESI ionization mode was optimized for each of the compounds. Collision energy and tube lens were optimized for each of the compounds (1H-BTR, TTR, 1-OH-BTR, XTR, BTH, 2-OH-BTH, 2-Amino-BTH, 2-Me-S-BTH). Two SRM transitions of each compound from the precursor ion to product ions were monitored for quantification (transition 1) and confirmation (transition 2). The description of the precursor ions and product ions of the compounds of interest, with their respective collision energies is presented in Table 7.6.

Table 7.6: Description of precursor ions and product ions of BTRs and BTHs monitored with their respective collision energies and Tube lens offset voltage

Compounds & Internal Stds	**Precursor ions $[M+H]^+$ (m/z)**	**Product Ions (m/z)**	**Collision Energy (V)**	**Tube Lens Offset (V)**
1H-BTR	120.0	65.4*	21	70
		92.2	16	70
1-OH-BTR	136.0	91.2*	20	64
		119.1	17	76
5-Me-1H-BTR	134.2	77.3*	26	76
		106.2	15	76
XTR	148.0	77.3*	27	76
		91.2	25	76
BTH	136.0	109.1*	25	76
		65.4	32	76
2-OH-BTH	152.0	92.2*	20	76
		80.4	29	76
2-Amino-BTH	151.0	109.1*	26	84
		124.1	33	84
2-Me-S-BTH	182.0	166.9*	22	76
		109	34	76
ATR-d5	221.0	178.9	17	60
1H-BTR-d4	124.2	69.2	21	84

[a] Quantification ion

7.3.4.3. Choice of internal standard

A serious drawback of LC-MS/MS methods is their susceptibility to matrix effects, such as the signal suppression or enhancement by matrix compounds entering the ion source at the same time [159,174,251]. Matrix effects can strongly vary with the environmental matrix and result in poor analytical accuracy and reproducibility. Stable isotope-labelled standards are usually used to compensate matrix effects in environmental analysis. In this respect, we tested the effectiveness of 1H-Benzotriazole-d4 and atrazine-d5 as internal standards for matrix effect compensation for the determination of BTRs and BTHs.

7.4. METHOD DEVELOPMENT FOR DETERMINATION OF BENZOTRIAZOLES AND BENZOTHIAZOLES IN SOLID SAMPLES (SLUDGE/PARTICULATE PHASE) BY LC-MS/MS

7.4.1. Extraction of benzotriazoles and benzothiazoles from solid samples by ultra-sonication

Extraction of the target compounds (BTRs and BTHs) from solid samples (particulate matter of wastewater and sewage sludge) was performed by ultrasonication. For the method development, a portion of 100 mg (± 10 mg) dried sludge was transferred into a 50 mL Eppendorf tube and spiked with the methanolic solution of the ISs (BTR-d4 and Atrazine-d5) and standard solution of target compounds and were left over-night or in a fume cupboard for 1hour in order to remove the organic solvent. Then, 5 mL of mixture of acidified MeOH/Milli-Q grade water (1:1 v/v) (adjusted with HCl solution to pH 3.00 ± 0.10) was added as the extraction solvent performing liquid-solid extraction by vortex-mixing for 1 min. Afterwards, ultra-sonication was carried out at 37 ± 2 °C for 45 min. The supernatant was collected after centrifuging (×1) at 4000 rpm for 10 min, and diluted to a final volume of 50 mL using acidified Milli-Q grade water (adjusted with 25% HCl solution to pH 3.00 ± 0.10), the supernatant was then passed through the SPE extraction procedure as described in section 7.3.2. In the case of the particulate matter derived from primary and secondary wastewater effluent samples, two or three (×3) successive centrifugations were required in order to collect a clean supernatant. Matrix match sample was also carried through the same procedure, the difference being that the spiking of target compounds was after the extraction.

For the optimized extraction method of BTRs and BTHs from solid matrices, as described above, we initially tested three different ultrasonication time (15, 30 and 45 min) in sludge matrix at a temperature of (37 ± 2) °C and no considerable differences were observed on the responses of the target compounds. No degradation of the target compounds (BTRs and BTHs) was observed from the application of heat; because after the centrifugation step, the sample was better dispersed and more transparent supernatant was obtained when 45 min time was applied, 45min ultrasonication time was subsequently chosen for the method protocol. As a consequence, maximum performance was achieved during SPE extraction, since better flow of the sample was achieved through the cartridge sorbent.

7.4.2. Chromatographic Separation and Mass Spectrometric determination of Benzotriazoles and Benzothiazoles in Solid matrices (Sludge/Particulate Phase) by LC-MS/MS

The developed method by LC-MS/MS, involving the optimization of chromatographic separation parameters and mass spectrometry ESI parameters for dissolved phase wastewater (as described in sections 7.3.3 and 7.3.4), was applied for the determination of the target compounds (BTRs and BTHs) in the solid matrices (sludge and particulate phase).

7.5. METHOD VALIDATION FOR THE DETERMINATION OF BENZOTRIAZOLES AND BENZOTHIAZOLES IN WASTEWATER AND SLUDGE

The developed methods by LC-MS/MS were validated in terms of analytical performance parameters as linearity, accuracy (recovery), precision (repeatability and reproducibility), limit of detection (LOD) and limit of quantification (LOQ).

7.5.1. Linearity

Linearity was determined by constructing calibration curves using internal standard calibration with Atrazine-d5 and 1H-Benzotriazole-d4 as internal standards. The concentrations of every target compound (BTRs and BTHs) (in the final 1 mL extract prior to analysis), used for the calibration curves ranged from 2.5 to 250 ng mL^{-1} while Atrazine-d5 and 1H-Benzotriazole-d4 were present at a concentration of 5 and 25 ng mL^{-1} respectively in every standard solution. Each concentration was analyzed in duplicate, the calibration curves were constructed using the peak area ratio of the target compounds to the internal standards (Atrazine-d5 and 1H-Benzotriazole-d4) separately versus the nominal concentrations of the target compounds. Least square linear regression analysis of the data gave slope, intercept, and correlation coefficient of determination. The responses for the target compounds in wastewater and sludge samples are presented in Table 7.8 and Table 7.9 respectively.

7.5.2. Limit of detection (LOD) and Limit of Quantification (LOQ)

LODs and LOQs are the most important criteria to evaluate the performance of a method [174]. The LOD corresponds to a minimal quantity or concentration, different from zero, which can be reliably detected with a certain degree of confidence while the LOQ depicts the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The LODs and LOQs were calculated by the use of the signal-to-noise ratio (S/N). The method LOD for each target compound was defined as the concentration of the compound in matrix that was equal to three times the average level of the baseline noise close to the respective peak, whereas the method LOQ for each target compound was defined as the concentration that was ten times the same noise level. The values for the method LODs and LOQs in both wastewater and sludge samples are shown in Table 7.10.

7.5.3. Accuracy/Recovery

The trueness of the methods was evaluated through recovery experiments at three fortification levels of the target compounds. To accomplish this, wastewater (50 mL) and sludge samples (about 100 mg) were spiked with the appropriate amount of the target compounds. The low, medium and high fortification levels for all compounds, except for 5-Me-1-BTR, were 25, 50 and 100 ng mL^{-1}, respectively. For 5-Me-1H-BTR, the low, medium and high fortification levels were 50,100 and 300 ng mL^{-1}, respectively. The results of the method recoveries in spiked wastewater and sludge samples are given in Table 7.11.

7.5.4. Precision (Repeatability and Reproducibility)

For both matrices (wastewater and sludge), precision was assessed by performing repeatability and reproducibility experiments. For repeatability experiments, a sample (either wastewater or sewage sludge) was spiked at three fortification levels of the target compounds, and three replicates (n=3) were prepared for each fortification level (intra-day precision). For reproducibility experiments, a sample (either wastewater or sewage sludge) was spiked at three fortification levels of the target compounds, and six replicates (n=6) were prepared in-between 2 different days (k=2) (inter-day precision). The low, medium and high fortification levels for all compounds, except for 5-Me-1H-BTR, were 25, 50 and 100 ng mL^{-1}, respectively. For 5-Me-1H-BTR, the low, medium and high fortification levels were 50, 100 and 300 ng mL^{-1}, respectively. For 5-Me-1H-BTR, repeatability and reproducibility experiments were realized at higher concentrations due to their high abundance in all environmental samples. Precision data of the method at three different fortification levels for the two matrices (wastewater and sludge) are presented in Table 7.11. In addition, the repeatability and reproducibility of the extraction procedures for both wastewater and sludge samples were determined. The precision data for extraction from wastewater and sludge samples are presented in Table 7.12 and Table 7.13 respectively.

7.6. APPLICATION TO ENVIRONMENTAL SAMPLES

In this study, we applied successfully the developed methods by LC-MS/MS for analyzing simultaneously four benzotriazoles (1H-BTR, 5-Me-1H-BTR, 1-OH-BTR, XTR and four benzothiazoles (BTH, 2-OH-BTH, 2-AminoBTH, 2-Me-S-BTH) simultaneously in wastewater (dissolved phase and particulate phase) and sewage sludge samples.

7.6.1. Sampling and Handling of Environmental Samples

Wastewater (treated and untreated) samples, Sludge (primary and secondary) samples, dewatered sludge samples and mixed liquor (from sequencing batch reactors) samples were collected from the sewage treatment plant Psittalia, Athens, Greece. The average sewage flow for the period of this study was 750 000m^3/day while the average production of dewatered sludge was 120 kg DS per day. The plant is equipped with primary sedimentation, activated sludge process with biological nitrogen and phosphorus removal and secondary sedimentation (activated sludge process). More details of the information about the operation parameters of the sewage treatment plant have been reported by Stasinakis et al. [258]

During a sampling campaign in December 2010, influent wastewater samples, effluent wastewater samples and sewage dewatered sludge samples were collected in seven consecutive days from the treatment plant. COD and BOD removal for this period were generally low in the samples collected at the treatment plant. All the sludge samples were dewatered anaerobic digested sludge for this campaign. In addition, another sampling was carried out in April, 2011; influent wastewater samples, effluent wastewater samples and dewatered sludge samples were collected in seven consecutive days. For the calculation of (K_d values) in section 9.1.2, samples of primary sludge and secondary sludge were taken in 5 consecutive days during this second sampling campaign.

All wastewater samples were collected in pre-cleaned polypropylene (PP) bottles. 50 mL of influent and effluent wastewater (24-h composite flow proportional) samples, 5 mL of primary sludge and 20 mL of secondary sludge were immediately filtered (with glass fibre pre-filters 0.45 μm) within 4 hour of their arrival in the laboratory, thereafter the pre-weighed filters (for particulate phase) were oven dried at 60 °C for 24 hours, and stored at −20 °C until analysis. From every dissolved phase matrix, 50 mL was obtained and acidified with hydrochloric acid (HCl) solution to pH 2.50 ± 0.10 and stored in the dark at 4 °C until analysis.

All dewatered sludge samples were collected in polypropylene (PP) bags. Within 12 hours of their arrival in the laboratory approximately 2 g from every homogenized sludge sample was oven dried at 60 °C for 24 hours, then grinded using a mortar and pestle, and stored at −20 °C until analysis.

7.6.2. Extraction and Preconcentration from wastewater (dissolved phase) samples

A volume of 50 mL of dissolved phase was transferred in a 50 mL Eppendorf tube and adjusted with 25% HCl to pH 3.00 (± 0.10) prior to the loading step of the SPE protocol. Zero matrix samples and samples analyzed were spiked only with a constant amount of ISs (atrazine-d5 and 1-H-BTR-d4). Spiked matrix samples and QC samples were spiked with the same constant amount of the ISs and an appropriate amount of target compounds. All samples prior to SPE were vortex mixed for 1 min to ensure sufficient distribution of the spiked compounds in the solution. Isolation of the compounds from the spiked wastewater samples was performed using Strata-X cartridges 33 μm Polymeric RP 6mL/200mg fitted on the vacuum apparatus. The cartridges were conditioned by 10 mL of MeOH and equilibrated by 10 mL of acidified Milli-Q gradewater (adjusted with HCl solution to pH 3.00 ± 0.10) without applying vacuum. Then, the acidic samples were passed through the cartridge, applying a vacuum of 0 (no vacuum) to -5 inHg. In order to remove any matrix interference, the cartridges were washed with 2×5 mL of acidified Milli-Q grade water (adjusted with HCl solution to pH 3.00 ± 0.10) and then dried under -5 inHg of vacuum for 60 min. The compounds were eluted with 10 mL of mixture of MeOH/ACN (1:1 v/v) and collected with a 15 mL Eppendorf tube. The eluents were set on a heated tray (45 ^{0}C) and were evaporated to a volume of 500 μL, under a gentle stream of nitrogen gas (N_2). Then, the eluents were diluted up to 1 mL with a mixture of MeOH/ACN (1:1 v/v), filtered and set to the auto-sampler tray for analysis. For the preparation of matrix match standard solutions, the ISs and target compounds were spiked at the received extract after the evaporation step. In every batch of samples prepared, the preparation of at least one procedural blank during the whole sample preparation procedure (including SPE process and evaporation step) was essentially carried out in order to detect any cross-contamination issues

7.6.3. Extraction and Preconcentration from Sludge (and particulate matter) samples

Extraction of the target compounds from solid samples (particulate matter of wastewater and sewage sludge) was performed by ultra-sonication. A portion of 100 mg (± 10 mg) dried sludge or a pre-weighed dried filter containing the particulate matter of wastewater (typical masses on the filters were: 10-20 mg for influent samples and 0.2-0.5 mg for effluent samples 30.0 mg for primary sludge and 110 mg for secondary sludge) was transferred in a 50 mL Eppendorf tube. The dried sludge samples (zero matrix samples, samples analyzed and spiked matrix or QC samples) were spiked with the methanolic solution of the ISs and target compounds when demanded, and were left over-night or in a fume cupboard for 1h in order to remove the organic solvent. Then, 5 mL of mixture of acidified MeOH/Milli-Q grade water (1:1 v/v) (adjusted with HCl solution to pH 3.00 ± 0.10) was added as the extraction solvent performing liquid-solid extraction by vortex-mixing for 1 min, thereafter ultra-sonication was carried out at 37 ± 2 °C for 45 min. The supernatant was collected after centrifuging (×1) at 4000 rpm for 10 min, and diluted to a final volume of 50 mL using acidified Milli-Q grade water (adjusted with HCl solution to pH 3.00 ± 0.10), the 50mL supernatant was extracted, according to the SPE procedure described in section 7.6.2. . In the case of the particulate matter derived from primary and secondary wastewater effluent samples, two or three (×3) successive centrifugations were required in order to collect a clean supernatant. Matrix match solutions and procedural blanks were prepared as described in the previous section (section 7.6.2).

7.6.4. LC-MS/MS measurements of Benzotriazoles and Benzothiazoles

Chromatographic separation was performed with Atlantis T3 C18 column and the column oven temperature was set at 25 $^{\circ}$C. The collision gas (Ar) pressure was set at 1.5 mTorr. Two characteristic fragment ions were selected; one for quantifying and the other for confirming each target compound. The precursor - product ions, the collision energies and the tube lens voltage values set for the method are as presented in Table 7.8.

Chromatographic analyses were carried out using a gradient elution program with ACN and Milli-Q grade water (acidified with 0.1% v/v HCOOH) as binary mobile phase mixture at a flow rate of 200 μL min^{-1}. The gradient elution started with 5 % v/v ACN and was increasing linearly up to the 6.5th min, reaching 40 % v/v ACN. A linear gradient elution followed from 40 % v/v ACN at 6.5th min to 100 % v/v ACN at 10.5th min. 100 v/v % ACN was held for a further 4.1 min (until 14.6th min). Then, a decreasing gradient elution followed, from 100 % v/v ACN at 14.6th min to 5 % v/v ACN at 15.0th min. A re - equilibration period (from 15.0th min to 21.0th min) at 5 % v/v ACN followed, to give a total run of 21.0 min.

The ESI was applied at a potential of +4.0 kV. The probe was fixed at 0.5 inches position of the micrometer (for Y-distance) and at probe position in-between positions B and C (for V-distance). The lateral probe adjustment value was fixed at position 0. The sheath gas (N_2) flow pressure was set at 23 psi, the auxiliary gas (N_2) flow rate was set at 20 A.U. and the ion transfer capillary temperature was set at 290°C. The skimmer offset potential was set at 0 V. The data acquisition values were set at 0.050 s for scan speed, 0.200 m/z for scan width, 0.70 FWHM for resolving power and 10 A.U. for the chromatography filter (noise reduction). The final in-vial composition of all environmental samples and standard solutions was MeOH/ACN (1:1 v/v), and were injected on column with partial-loop injection (5 μL).

7.7. RESULTS AND DISCUSSIONS

7.7.1. Optimization of SPE Protocol

The optimization of the extraction of the target compounds (BTRs and BTHs) from wastewater was based on the type of sorbents, the type/volume of organic solvents for elution and the pH of water sample.

The employed SPE sorbents were polymeric-based with hydrophobic and hydrophilic chemical moieties. The target BTRs are compounds that contain nitrogen atoms, whereas the target BTHs are compounds that contain nitrogen and sulfur atoms. Additionally, 1-OH-BTR and 2-OH-BTH contain one oxygen atom each. Thus, when the applied cartridge sorbent demonstrates hydrophilic chemical moieties, retention is theoretically favoured. Strata-X RP and Oasis HLB sorbents are made of poly (divinylbenzene-N-vinylpyrrolidone) and Evolute ABN sorbent is made of polystyrene divinylbenzene. Therefore, all three sorbents possess the ability to apply hydrophobic, π-π and hydrophilic intermolecular forces to the target compounds. However, due to the introduction of the polar group of N-vinylpyrrolidone in the sorbent of Strata-X RP and Oasis HLB, hydrophilic chemical moieties are theoretically stronger than in the simple polystyrene divinylbenzene sorbent of Evolute ABN.

Recovery experiments in dissolved phase of wastewater and particulate matter / sludge samples were achieved employing the already described sample preparation protocols (sections 7.6.2. and 7.6.3.) except for the different types of SPE cartridges applied. Oasis

HLB sorbent presented the same behaviour in recoveries for all target compounds in a similar manner as Strata X-RP sorbent, whereas slight differences were observed between Strata-X RP and Evolute ABN sorbents. In Table 7.7, results from the experiments on Strata-X RP and Evolute ABN are presented.

Table 7.7: SPE Recoveries of BTRs and BTHs % (RSD %, n =3 fortification level (25 ng mL^{-1} at final extract) × 2 replicates) from Evolute ABN and Strata X-RP.

Compounds	**Dissolved phase of wastewater**		**Particulate matter of wastewater**	
	Abs. Rec. values Evolute ABN / Strata X-RP	**Rel. Rec. values# Evolute ABN / Strata X-RP**	**Abs. Rec. values Evolute ABN / Strata X-RP**	**Rel. Rec. values# Evolute ABN / Strata X-RP**
1H-BTR	106.4 (15.2) / 94.8 (19.8)	96.6 (7.9) / 97.7 (8.8)	63.7 (6.3) / 69.9 (8.2)	95.5 (5.6) / 91.4(4.8)
1-OH-BTR	97.2 (17.5) / 103.5 (21.3)	98.2 (4.5) / 106.7 (8.5)	72.2 (16.7) / 76.6 (11.4)	95.7 (3.8) / 100.9 (6.9)
TTR	34.8 (17.0)*/ 73.3 (18.9)*	110.4 (12) / 98.6 (6.9)	63.7 (13.7) / 74.6 (19.3)	109.3 (8.3) / 104.0 (9.0)
XTR	79.6 (13.3) / 80.2 (17.7)	89.5 (12.5) / 94.4 (7.7)	68.5 (11.6) / 65.3(10.0)	99.5 (6.6) / 94.9 (8.1)
BTH	101.5 (17.1) / 100.2 (20.2)	103.4 (14.8) / 100.8 (8.2)	49.5 (22.5) / 60.4 (17.4)	92.1 (13.2) / 95.1(8.8)
2-OH-BTH	109.5 (8.3) / 100.6 (3.4)	103.3 (2.4) / 103.2 (6.4)	65.0 (14.3) / 68.7 (12.2)	100.5 (3.3) / 104.8 (9.6)
2-Me-S-BTH	52.6 (17.3) / 54.8 (12.2)	55.2 (8.9) / 55.0 (1.6)	35.9 (11.0) / 34.8 (10.4)	56.1 (1.3) / 55.1 (1.4)
2-Amino-BTH	97.6 (7.5) / 101.5 (8.7)	100.2 (9.9) / 100.6 (3.7)	82.4 (15.5) / 82.2 (15.7)	110.0 (5.4) / 102.1(8.9)

#ATR-d5 was used for calculation, * High blank value in dissolved phase

Taking into consideration only relative recovery values (%), due to their isolation from uncertainties derived mainly from instrumental instability of MS/MS detector, it was concluded that recoveries from dissolved phase samples were generally reported a bit higher with lower R.S.D (%) values in Strata-X RP than in Evolute ABN cartridge, whereas the opposite phenomenon was observed for recoveries obtained for particulate matter samples (Table 7.7). As far as recoveries from dissolved phase samples are concerned, the differences between the two sorbents were due to the stronger hydrophilic chemical moieties of Strata X-RP and its higher average pore size that increases retention capacity. In the case of particulate matter samples however, the observed differences in recoveries were attributed to the average particle diameter of the sorbents. It was acknowledged that this parameter stood as one of the most critical in particulate matter samples since Evolute ABN sorbent possesses an average particle diameter of 50 μm (33μm for Strata X-RP), minimizing the retention of endogenous materials with high molecular weight, and consequently achieving a more effortless flow of the sample through the sorbent during the loading step of the SPE protocol.

It is worthwhile to note that during the experiments on Evolute ABN, the application of vacuum was not needed under any case. On the contrary, Strata X-RP and Oasis HLB sorbent demanded extra care to obtain clean supernatant from the LSE step (sections 7.4.1. and 7.6.3.) in order to avoid clogging of the sorbent and/or the application of a by far more than -5 inHg of vacuum during the SPE protocol. This was due to the fact that Strata X-RP and Oasis HLB possess an average particle diameter ~ 1.7 times smaller than that possessed by Evolute ABN.

The relative recoveries of all target compounds, with the exception of 2-Me-S-BTH, were greater than 90%. These results show that the optimized solid phase extraction (SPE) conditions (Strata X-RP cartridge, 10mL of MeOH/ACN (50:50 v/v) as elution solvent, sample pH of 3) were suitable enough for efficient extraction of the target compounds from aqueous samples. The lower recovery of 2-Me-S-BTH could be attributed to its relatively high hydrophobicity (log Kow = 3.15) compared to other target compounds (Tables 3.1 and 3.2). Kloepfer et al. observed that pure methanol proved insufficient for the elution of 2-Me-S-BTH from a polymeric sorbent [246]. It is therefore suggested that 2-Me-S-BTH was retained so much strongly on the reversed phase sorbent that the elution solvents, (MeOH/ACN) which are polar and even stronger than pure methanol, could not elute the compound more effectively despite the use of up to 10mL of the solvents mixture.

Nevertheless, since internal standard calibration was used for quantification, both incomplete recoveries from the SPE and matrix effects were accounted for.

The relative recoveries of the compounds were similar to those obtained by van Leerdam et al. (50 -110%) when the extraction was performed with sample matrix pH of 2 [153] and a recovery of 78-98% in wastewater obtained by Jover and co-workers [152]. However, a better recovery for 2-Amino-BTH was obtained in this study (100% recovery) contrary to a recovery of < 30% obtained with sample matrix pH of 2 by van Leerdam et al. [153]. Considering the generally lower RSD (%) values of extraction recoveries obtained for the target compounds in both dissolved (wastewater) and particulate matters when internal standard ATR-d4 was added, a good precision for the extraction methods is justified. Therefore, the low recovery of 2-Me-S-BTH should be accepted since the precision in terms of RSD (%) value was less than 1.6% in both matrices when Strata X-RP sorbent was adopted for extraction.

7.7.2. Extraction from sludge matrix

Three ultra-sonication times (15, 30 and 45 min) in sludge matrix at a temperature of (37 ± 2) °C were tested and no considerable differences were observed on the response of the target compounds. Degradation of the target compounds was not observed from the application of heat. The application of 45 min of ultra-sonication with heating was chosen in the final method protocol, because the sample was better dispersed and therefore, after the centrifugation step, transparent supernatants were obtained. As a consequence, maximum performance was achieved during SPE extraction, since better flow of the sample was achieved through the cartridge sorbent, as needed.

However, it was observed that after 45 min of ultra-sonication, higher response intensity was obtained from an unknown highly abundant isobaric peak, eluted at approximately 7.48 min and observed at the SRMs of TTR (Figures A7.1-A7.3). Yet, this fact did not affect quantification of TTR. Under no case this unknown peak was a “ghost” peak, but a peak originated from sludge matrix. In wastewater samples, the same peak was observed in a by far lower abundance and, of course, it was not observed at all in standard solvent solutions.

The relative recoveries of extraction (involving LSE sonication and SPE) for all target compounds (except for 2-Me-S-BTH) from particulate phase (sludge) were greater than 91.4% (Table 7.7.). The lower recovery of 55.1% for 2-Me-S-BTH could be as a result of incomplete elution of the compound during the SPE procedure as explained above or in addition to this explanation, incomplete extraction from LSE/sonication step is also possible.

Nevertheless, since the RSD (%) value of recovery was 1.4% for 2-Me-S-BTH in particulate matter when Strata X-RP sorbent was adopted for SPE procedure, the low recovery of 2-Me-S-BTH should be accepted. Until now, there are limited studies of the target compounds in sludge. The results of the recoveries of benzotriazoles in sludge obtained in this study were comparable to those obtained by Liu et al. [228] who adopted pressurized liquid extraction (PLE) for the extraction of benzotriazoles from sludge (> 101%). However the recoveries of benzothiazoles in this work are much higher than recoveries reported by Wick and co-researchers for benzothiazoles in activated sludge (15% for 2-OH-BTH to 63% for 2-Me-S-BTH) when the PLE extraction was used [251]. Compared to the study of Zhang et al. who also extracted IH-BTR and 5-Me-BTR from sludge by LSE/SPE but utilized a large amount of 25mL methanol as extraction solvent and a rotary evaporator to concentrate the supernatant, better recoveries of the compounds were obtained in this study. Moreover, smaller volumes of organic solvent (only 5mL of MeOH/H_2O) employed in this study reduce the cost and shorten more even the time of analysis. In addition, loss of volatile compounds through the use of a rotary evaporator when a large volume of solvent is used was avoided in this study.

7.7.3. Selection of Analytical Column

The chromatographic conditions incorporated for the selection of the analytical column was as reported in section 7.6.4. For all target compounds, with the exception of 2-Amino-BTH, slightly higher signal-to-noise ratios were achieved with Atlantis T3 column, whereas slightly higher peak areas were achieved with XTerra MS C18. Thus, their sensitivity was considered rather constant between Atlantis T3 and XTerra MS C18. For 2-Amino-BTH in particular, it was clear that the sensitivity was better with Atlantis T3, since the signal-to-noise ratio and peak area was approximately 30 % higher than that obtained with XTerra MS C18. Yet, the major drawback of the hydrophobic XTerra MS C18 column was the fact that it maintained disturbed peak shapes for the two most polar compounds, 1-OH-BTR and 2-Amino-BTH (peaks with intense tailing).

This performance of Atlantis T3 in enhancing the retention of polar compounds such as that of 1-OH-BTR and 2-Amino-BTH is attributed to the design of a tri-functional C18 alkyl phase bonded at a ligand density [Atanltis T3 Column manual]. It has been reported that the proprietary T3 end-capping process of Atlantis T3 is more effective in minimizing silanol activity than traditional trimethyl silane (TMS) end-capping as in XTerra MS C18 [259]. The proprietary T3 end-capping procedure reacts with more silanols thereby dramatically improving peak shape. However, though XTerra MS C18 column presents a lower silanol activity, the latest elution time (10.97 min) was shorter than that obtained by Atlantis T3 column (11.71 min) thereby lead to an increase in throughput. The retention times (RT) of all target compounds with XTerra MS C18 column are presented in Table A7.1, whereas the retention times (RT) with Atlantis T3 column are given in Table A7.2.

7.7.4. Analytical Method Performance

7.7.4.1. Linearity

An internal standard calibration was carried out with 1H-BTR-d4, and ATR-d5 as ISs for concentrations ranging from 2.5 to 250 ng mL^{-1} (concentration at the final 1mL extract prior to analysis) for every target compound. 1H-BTR-d4 and ATR-d5 were present at a concentration of 25 and 5 ng mL^{-1} in every standard solution, respectively. As shown in Tables 7.8 and 7.9, the responses of MS/MS detector in wastewater and sludge were linear for all the BTRs and BTHs with coefficients of determination, $R^2 > 0.99$.

Table 7.8: Calibration equations and linearity for BTRs and BTHs in the dissolved phase of wastewater samples

Compound	**Calibration Equations**[a]		**Correlation Coefficients**[a] **(R^2)**	
	Relative Values with ATR-d5	**Relative Values With 1H-BTR-d4**	**Relative Values with ATR-d5**	**Relative Values with 1H-BTR-d4**
1H-BTR	y = 0.016x+0.202	y = 0.613x+15.76	0.999	0.996
1-OH-BTR	y = 0.008x-0.017	y = 0.293x+1.968	0.9996	0.996
TTR	y = 0.031x-0.802	y = 1.134x+21.25	0.994	0.994
XTR	y = 0.025x-0.169	y = 0.967x+1.199	0.999	0.998
BTH	y = 0.005x+0.035	y = 0.173x+4.178	0.999	0.994
2-OH-BTH	y = 0.002x-0.031	y = 0.069x-0.640	0.995	0.9999
2-Amino-BTH	y = 0.030x-0.149	y = 1.158x+3.775	0.9991	0.998
2-Me-S-BTH	y = 0.023x-0.014	y = 0.898x+8.351	0.998	0.996

[a] Calibration points: 2.5, 5, 10, 25, 50, 100, 150, 175, 250, 300 ng mL^{-1} at the final 1 mL extract.

Table 7.9: Calibration equations and linearity for BTRs ad BTHs in sewage sludge samples

Compound	**Calibration Equations[a]**		**Correlation Coefficients[a] (R^2)**	
	Relative Values with ATR-d5	**Relative Values with 1H-BTR-d4**	**Relative Values with ATR-d5**	**Relative Values with 1H-BTR-d4**
1H-BTR	y = 0.019x-0.115	y = 0.633x-1.796	0.9994	0.9998
1-OH-BTR	y = 0.011x+0.002	y = 0.347x+1.739	0.9991	0.9996
TTR	y = 0.039x-0.244	y = 1.254x-1.456	0.999	0.9999
XTR	y = 0.032x-0.185	y = 1.032x-2.593	0.9996	0.9997
BTH	y = 0.007x+0.058	y = 0.216x+3.226	0.9995	0.998
2-OH-BTH	y = 0.002x-0.018	y = 0.070x-0.712	0.996	0.995
2-Amino-BTH	y = 0.038x-0.047	y = 1.236x+3.332	0.996	0.998
2-Me-S-BTH	y = 0.038x-0.334	y = 1.243x-7.707	0.999	0.996

[a] Calibration points: 2.5, 5, 10, 25, 50, 100, 150, 175, 250 ng mL^{-1} at the final 1 mL extract

7.7.4.2. Limits of detection (LODs) and Limits of quantification (LOQs)

The method LOD for each compound (BTRs and BTHs) was defined in this study as the concentration of the compound in matrix that was equal to three times the average level of the baseline noise close to the respective peak, whereas the method LOQ for each target compound was defined as the concentration that was ten times the same noise level. The method LODs and LOQs are presented in Table 7.10.

Table 7.10: Method limits of detection (LODs) and quantification (LOQs) of BTRs and BTHs in wastewater and sludge samples

Compound	**Wastewater (Dissolved Phase)**		**Sewage Sludge**	
	LOD (S/N= 3, ng L^{-1})	**LOQ (S/N= 10, ng L^{-1})**	**LOD (S/N =3, ng g^{-1})**	**LOQ (S/N= 10, ng g^{-1})**
1H-BTR	0.77	2.54	0.69	2.27
1OH-BTR	3.05	10.00	7.62	25.10
TTR	0.13	0.42	0.06	0.21
XTR	0.34	1.13	0.26	0.85
BTH	16.7	55.00	12.50	41.30
2-OH-BTH	4.58	15.10	4.00	13.20
2-Amino-BTH	4.17	13.7	10.40	34.40
2-Me-S-BTH	0.08	0.28	0.04	0.14

The achieved LODs and LOQs were adequate for environmental monitoring of the target compounds and low enough, taking into consideration the complexity of the sample matrices. For liquid samples(wastewater), the LODs achieved in the present work for all target compounds, except for BTH, were at similar levels or lower than those obtained in previous studies (Tables 4.1& 4.3) with different instrumentations: LC-MS [250], LC-MS/MS [12,14,48,223,229,232,241,245,246,251,260,], LC-LTQ-FT-MS [153], GC–MS [235,252,253], GC–MS/MS [228,237], GC x GC-TOF-MS [152,224] and IL-GC-MS/MS [209]. Conspicuously, the LODs derived in this study were far lower than those obtained when a sample extraction method like LLE or DLLME was used; and when a less sensitive detection method like UV or Fluorescence detector was employed [230,234]. In sludge samples, LOD values of 0.9, 4.1 and 3.0 ng g^{-1} were reported by Liu et al. [228] for BTR, 5-Me-1H-BTR, and XTR, respectively, with a GC–MS/MS instrumentation. Zhang et al. [227] also reported LOD values of 0.067 ng g-1 for both 1H-BTR and 5-Me-1H-BTR in sediment and 0.67 ng g^{-1} in sludge samples with a LC–MS/MS instrumentation. Thus, in the present work, similar or lower LODs were also presented for 1H-BTR, XTR, and TTR (compared to 5-Me-1H-BTR) in sludge samples. Until the period of this study, only one study has reported the determination of some benzothiazoles in activated sludge [251], the LOQ values of 25 ng g^{-1} for 2-Me-S-BTH and 100 ng g^{-1} for both BTH and 2-OH-BTH were reported. Herein in this work, we obtained LOQ values of 0.14 ng g^{-1} for 2-Me-S-BTH, 13.2 ng g^{-1} for 2-OH-BTH and 41.3 ng g^{-1} for BTH. These LOQ values were generally lower than those obtained by Wick et al. [251]. From our point of view the high LOD values obtained in both liquid and solid matrices for BTH resulted from the high background in SRMs of the compound in both matrices. The higher background for BTH, in contrast to the rest target compounds (BTRs and BTHs), is evident in the chromatograms of both liquid and solid matrices (Fig.7.3, Fig. 7.4, Figures A7.5- A7.8). Fries also reported a higher LOD value of 256 ng L^{-1} for BTH in wastewater using stir bar sorptive extraction (SBSE) [253].

7.7.4.3. Method Recovery

The trueness of the method was evaluated through recovery experiments at two fortification levels of the target compounds that were realized in-between three different days (k = 3). To accomplish this, wastewater (50 mL) and sludge samples (about 100 mg) were spiked with the appropriate amount of the target compounds. The low and high fortification levels in wastewaters for all compounds, except for TTR, were 50 and 100 ng mL^{-1}, respectively. For TTR, the low and high fortification levels were 100 and 300 ng mL^{-1}, respectively. Similarly, the low and high fortification levels in sludge were 50 and 100 ng g^{-1} respectively. The results are given in Table 7.11. The relative recoveries of the compounds were good at both levels of fortification. Using ATR-d5 as internal standard, apart from 2-Me-S-BTH with an exceptionally low recovery (55%), the relative recoveries in wastewater ranged from 90% to 103% and ranged from 90% to 106% in sludge. Regarding 2-Me-S-BTH, the (%) relative recovery values were better in wastewater and sludge samples, when 1H-BTR-d4 was used as internal standard (Table 7.11). With 1H-BTR-d4, the relative recoveries in wastewater ranged from 71% for 2-Me-S-BTH to 121% for XTR and ranged from 64% for 2-Me-S-BTH to 116% for 2-Amino-BTH in sludge. These results from recovery experiments demonstrated the suitability of the two internal standards (ATR-d5 and 1H-BTR-d4) for accurate quantification of the target compounds in complex matrices as wastewater and sludge samples by LC-MS/MS.

Table 7.11: Mean recoveries (%, *n* = 6, k=3) of BTRs and BTHs in spiked (dissolved phase of) wastewater and sludge samples with relative standard deviation

Compounds	**Wastewater**				**Sludge**			
	(Rel. Rec. %) ± (R.S.D %, n=6) (with ATR-D5)		**(Rel. Rec. %) ± (R.S.D %, n=6) (with 1H-BTR-d4)**		**(Rel. Rec. %) ± (R.S.D %, n=6) (with ATR-D5)**		**(Rel. Rec. %) ± (R.S.D %, n=6) (with 1H-BTR-d4)**	
	Low Level	**High Level**	**Low Level**	**High Level**	**Low Level**	**High Level**	**Low Level**	**High Level**
1H-BTR	90.9±9.1	96.4±4.9	114±5.7	106±9.9	93±6.0	96.2±7.6	94.6±5.1	104.0±4.2
1-OH-BTR	96.0±5.2	89.7±4.4	106.2±9.5	98.4±10.0	93.9±8.7	95.4±11.3	106.6±4.3	104.1±1.7
TTR	97.1±6.9	99.1±5.3	121±4.3	107±12	99.6±6.9	90.1±9.7	99.3±4.8	101.2±9.0
XTR	109.2±2.8	96.6±3.7	121.8±2.0	102±10	100.1±6.3	92.9±9.2	99.5±3.8	104.2±8.2
BTH	106.2±8.5	90.5±5.4	118.4±7.1	102.7±9.3	90.3±5.7	90.7±6.9	94.3±5.7	93±7.7
2-OH-BTH	102.3±9.0	89.8±6.4	111.7±8.5	104.1±9.2	106±10	91.3±4.5	102.9±6.1	103.8±8.7
2-Me-S-BTH	58.6±5.8	54.9±5.0	70.5±4.7	71.6±5.9	49.9±3.5	51±4.5	63.8±5.4	64±6.6
2-Amino-BTH	103.3±5.8	98.9±4.2	119.9±4.3	112±11	106±10	103±10	111.3±4.7	116.4±8.7

*The low and high fortification level for all compounds, except for TTR, was 50 and 100 ng mL^{-1}, respectively. For TTR, the low and high fortification level was 100 and 300 ng mL^{-1}, respectively.

7.7.4.4. Method Precision

For both types of matrices, precision was assessed by performing repeatability and reproducibility experiments at three fortification levels as described in section 7.5.4. Precision data of the extraction procedure for the two types of samples are given in Tables 7.12 and 7.13. At low fortification level, the absolute values (%RSD) for intra-day precision (repeatability) and inter-day precision (reproducibility) in wastewater were up to 12.6% and 19.3% respectively. Relative to internal standard atrazine-d5 at low level fortification, intra-day precision and inter-day precision were less than 10% and 14.6% respectively, while relative to internal standard 1H-BTR-d4 intra-day precision and inter-day precision were less than 2.4 % and 12.4% respectively. At the low fortification level, the absolute values (%RSD) for intra-day precision (repeatability) and inter-day precision (reproducibility) in sludge were up to 18.5% and 22.2% respectively for 2-OH-BTH. Relative to internal standard atrazine-d5 at low level fortification, intra-day precision and inter-day precision in sludge were reduced to less than 10.5% and 11.8% respectively, while relative to internal standard 1H-BTR-d4 intra-day precision and inter-day precision were less than 11.4 % and 13.8% respectively.

The results showed satisfactory intra- and inter-day precision of the analytical procedure, both for wastewater and sludge samples. RSDs (%) were less than 15 % for all the compounds in both substrates when applying internal standardization method, indicating the excellent precision of the developed methods for both types of samples.

Table 7.12: Precision data of the extraction procedure for BTRs and BTHs in wastewater samples (dissolved phase).

Compounds	**Abs. values of Intra- (n=3) / Inter-day precision (n=6, k=2)**			**Rel. values with ATR-d5 of Intra-(n=3) / Inter-day precision (n=6, k=2)**			**Rel. values with 1H-BTR-d4 of Intra- (n=3) / Inter-day precision (n=6, k=2)**		
	Low level	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**
1H-BTR	11.7/11.9	3.5/3.5	2.2/7.7	6.6/7.7	7.3/9.1	5.8/6.6	9.3/12.4	5.6/6.6	8.2/11.2
1-OH-BTR	0.8/ 7.8	4.9/5.3	1.3/6.9	3.5/3.9	10.9/11.0	4.3/10.0	2.9/3.5	9.5/11.4	7.8/10.0
TTR	12.6/16.8	2.7/3.0	2.6/10.1	6.4/7.8	6.4/6.9	5.2/6.3	9.3/11.7	4.3/9.8	2.0/10.7
XTR	4.5/6.3	1.7/3.3	1.3/6.6	1.4/1.7	4.5/5.1	3.7/4.0	7.1/8.3	3.6/4.4	9.3/11.1
BTH	8.0/13.3	1.3/9.2	3.9/11.6	8.2/12.8	7.5/13.0	3.8/10.4	2.4/2.5	7.1/10.4	9.3/11.1
2-OH-BTH	16.3/19.3	5.9/6.1	4.2/4.5	10.0/14.6	8.9/11.4	6.3/7.7	4.6/7.1	2.2/10.7	4.5/9.2
2-Me-S-BTH	11.2/11.4	1.8/2.1	2.1/13.6	7.2/8.7	5.4/5.7	2.2/5.0	10.1/11.6	4.7/5.7	6.0/8.8
2-Amino-BTH	6.3/10.4	1.4/1.4	1.5/5.8	3.3/4.9	5.4/5.7	4.0/4.1	8.3/10.1	4.2/5.1	11.2/13.6

*The low, medium and high fortification levels for all compounds, except for TTR, were 25, 50 and 100 ng mL^{-1}, respectively. For TTR, the low, medium and high fortification levels were 50, 100 and 300 ng mL^{-1}, respectively.

Table 7.13: Precision data of the extraction procedure for BTRs and BTHs in sludge samples.

Compounds	**Abs. values of Intra- (n=3) / Inter-day precision (n=6, k=2)**			**Rel. values with ATR-d5 of Intra-(n=3) / Inter-day precision (n=6, k=2)**			**Rel. values with 1H-BTR-d4 of Intra- (n=3) / Inter-day precision (n=6, k=2)**		
	Low level	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**
1H-BTR	1.3/1.8	1.4/5.3	3.3/3.3	2.9/3.2	6.0/10.8	7.6/11.2	6.3/7.2	1.5/4.4	4.8/5.9
1-OH-BTR	4.4/ 5.8	8.3/12.4	2.2/3.2	4.0/5.7	10.0/11.0	9.7/11.7	9.4/11.0	9.0/11.8	3.9/4.6
TTR	6.5/7.1	1.2/3.7	1.5/2.3	6.1/6.6	6.8/6.8	8.0/9.7	11.4/13.8	0.2/2.5	3.1/3.8
XTR	3.8/4.0	1.1/3.0	2.1/2.9	6.3/7.2	6.3/12.0	9.2/11.6	4.6/5.6	0.4/1.9	3.8/4.7
BTH	8.5/8.9	2.4/7.8	2.4/2.7	10.5/11.3	5.7/9.8	6.8/11.9	9.9/12.0	1.3/6.5	4.2/5.1
2-OH-BTH	18.5/22.2	3.0/3.7	3.9/4.5	6.7/11.8	5.4/8.7	5.5/7.0	4.5/11.2	4.7/5.3	6.8/7.2
2-Me-S-BTH	14.2/16.7	5.8/6.9	7.7/9.6	7.8/8.9	5.9/8.7	4.7/7.8	9.2/11.8	4.9/5.6	6.8/9.8
2-Amino-BTH	2.3/2.8	2.5/3.3	1.5/2.6	1.0/1.1	6.8/10.7	9.6/10.3	6.8/7.7	1.6/2.1	3.4/4.1

*The low, medium and high fortification levels for all compounds, except for TTR, were 25, 50 and 100 ng mL^{-1}, respectively. For TTR, the low, medium and high fortification levels were 50, 100 and 300 ng mL^{-1}, respectively.

7.7.5. Choice of Internal standard for quantification

Quantification of all target compounds was performed with the internal standard method in all samples analyzed. We tested the effectiveness of two internal standards (1H-BTR-d4 and ATR-d5) for compensating matrix effects. The 1H-BTR-d4 IS was incorporated for the quantification of all BTRs and 2-Me-S-BTH, whereas the rest compounds were quantified with ATR-d5. However, as demonstrated in Tables 7.8 and 7.9, quantification of all target compounds was possible with both internal standards. In dissolved phase of wastewater samples, as observed in Table 3, best R^2 values for all target compounds, except for 2-OH-BTH, were achieved with ATR-d5. On the other hand, in sludge samples, as observed in Table 7.9, best R^2 values for all BTRs and 2-Amino-BTH were achieved with 1H-BTR-d4. Therefore, taking into consideration these analytical figures (Table 7.9), precision (Table 7.13), and recovery (Table 7.11) in sludge, quantification of all BTR derivatives with 1H-BTR-d4 presented less uncertainty than the values with ATR-d5. It seems that 1H-BTR-d4, as a deuterated analogue of 1H-BTR, maintained similar behaviour with all BTR derivatives, and thus it was extracted from the sludge matrix in a similar way during the solid / liquid partitioning step.

7.7.6. Matrix Effects

The matrix factor of the methods for every target compound was quantified by comparing the slope of the matrix match curve with the slope of the standard solution curve, and consequently the matrix effect (%) was calculated and presented in Fig. 7.2. It is evident from Fig. 7.2 that ion suppression occurred for all target compounds. However, based on the results of mean relative recoveries (Table 7.11) obtained for the compounds when internal standards ATR-d5 and 1H-BTR-d4 were used for quantification, the trueness of the method was not undermined. The internal standards effectively compensated for the matrix effects.

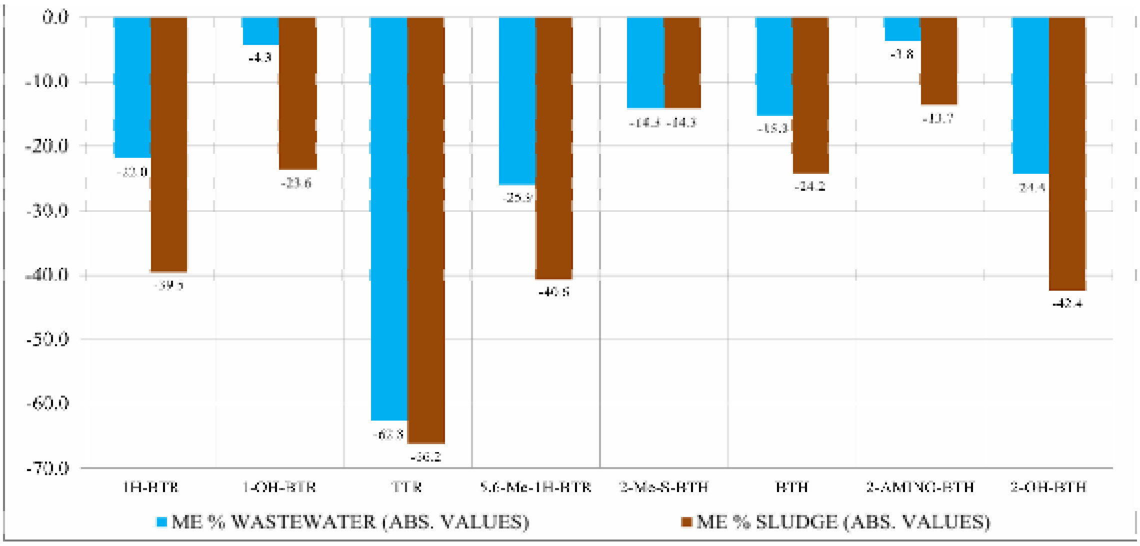


Fig.7.2: Matrix effect (%) for benzotriazoles and benzothiazoles in the dissolved phase of wastewater and in sludge.

7.7.7. Identification and Confirmation of target compounds

In mass spectrometry, it is better to have at least four identification points to ensure the correct identity of the compound [174]. The four characteristic parameters for LC-MS/MS include retention time, two transitions in multi-reaction monitoring mode (MRM) and the ratio between the two ionic signals.

In this study, the two fragment ions (quantification and confirmation ion) that were selected in the developed methods for all target compounds were the fragments with the highest abundance (Table 7.6). However, the confirmation ion of 1-OH-BTR and 2-Amino-BTH were set at 119.1 and 124.1 m/z (Table7.6), respectively, in order to avoid cross-talking issues (that were observed) between the two co-eluted compounds. 1-OH-BTR and BTH also maintained a common SRM (136.0 > 65.4 m/z), but since they were eluted with a difference of more than 4 min, monitoring of both compounds at this SRM did not pose a problem. For the monitoring of 1H-BTR-d4, its second ion fragment in abundance was selected (69.2 m/z) as best for the method, since the most abundant fragment of 96.0 m/z presented high background noise (Fig. A7.4).

The retention time (RT), relative retention time (RRT), and ion ratio (%) of all target compounds in dissolved phase of wastewater samples and in sludge samples are presented in Tables A7.2 and A7.3 respectively. These analytical parameters were calculated from data exported from the reproducibility experiments.

For indication, chromatograms of a spiked sample are presented at a fortification level of 100 ng mL^{-1} for TTR and 50 ng mL^{-1} for the rest target compounds (concentration at the final eluent prior analysis) in dissolved phase of wastewater (Fig. 7.3) and sludge (Fig. 7.4).

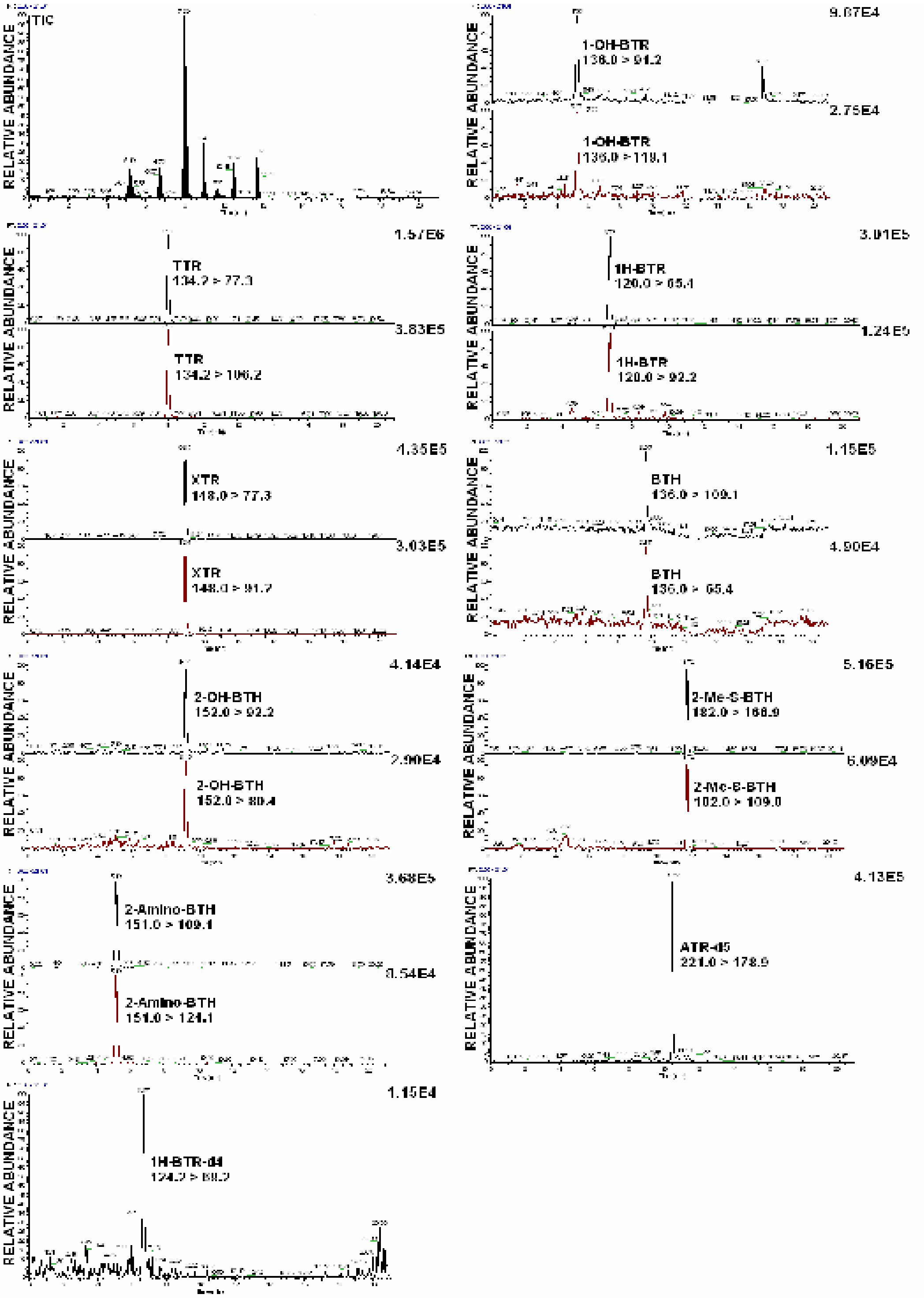


Fig. 7.3: SRM chromatograms of BTRs and BTHs in spiked wastewater sample (dissolved phase) at a fortification level of 100 ng mL^{-1} for TTR and 50 ng mL^{-1} for the rest target compounds (m/z transitions depicted).

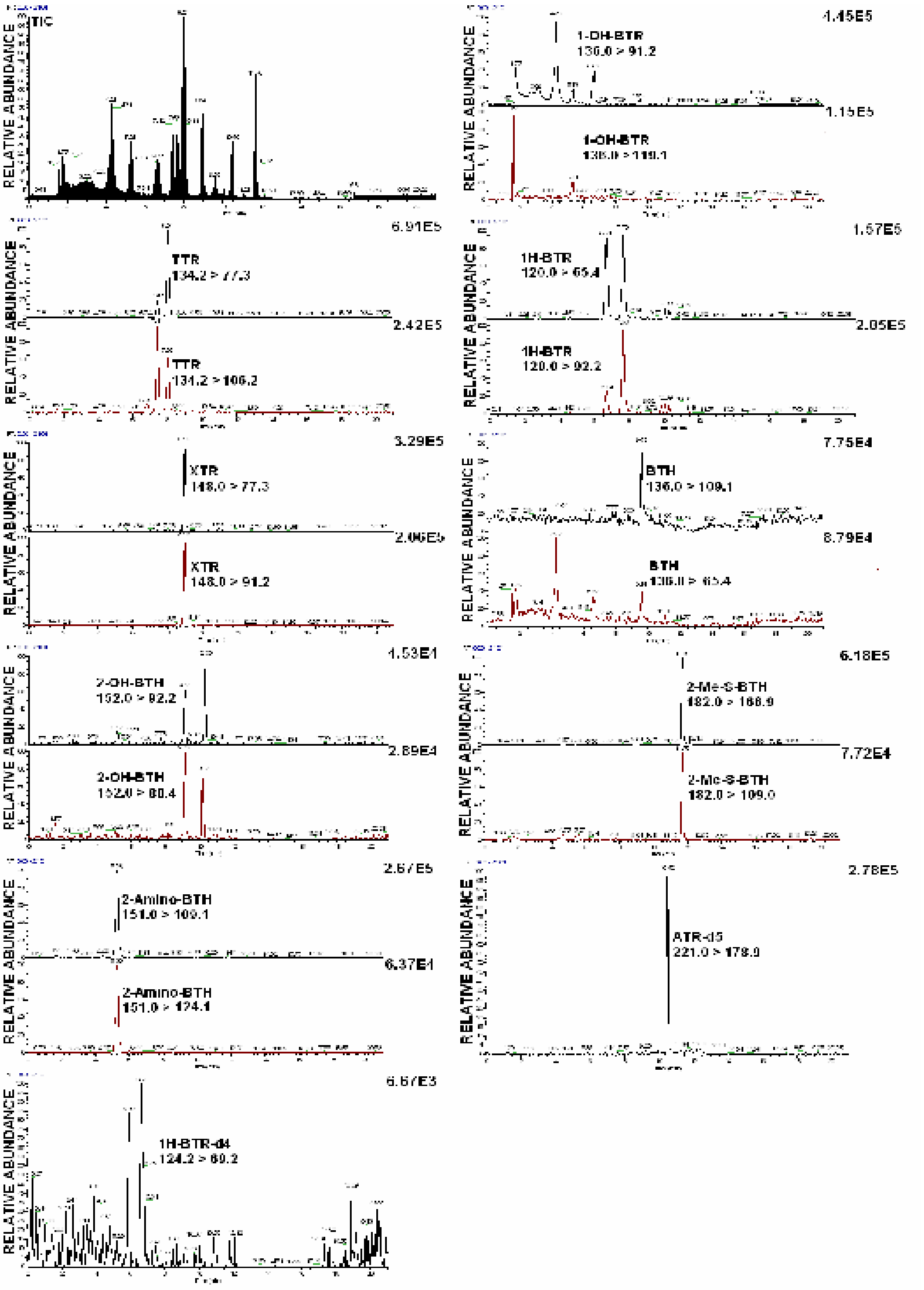


Fig. 7.4: SRM chromatograms of BTRs and BTHs in spiked sludge sample at a fortification level of 100 ng mL^{-1} for TTR and 50 ng mL^{-1} for the rest target compounds (m/z transitions depicted).

Moreover, indicative chromatograms of matrix match samples are demonstrated for wastewater (Fig. A7.5) and sludge (Fig. A7.6). The "blank" samples of wastewater and sludge that were used for spiking are equally presented in Fig. A7.7 and Fig. A7.8, respectively. As witnessed from the demonstrated chromatograms (Fig. 7.3, Fig 7.4, Figs. A7.5-A7.8), a number of other isobaric unknown endogenous compounds from the matrices exhibited the same SRM transitions as the target BTRs and BTHs, but eventually they did not interfere, due to adequate chromatographic separation. Retention times are presented in Tables A7.2 and A7.3, in order to clearly identify the target compounds from the demonstrated chromatograms.

The developed method does not separate the isomers 4-Me-1H-BTR and 5-Me-1H-BTR. However, we tested if the isomers exhibit the same sensitivity and we concur with the findings of Weiss and Reemtsma [12] that 4-Me-1H-BTR and 5-Me-1H-BTR exhibit equal response in MS/MS detection. All the analytical figures for both compounds were similar. Consequently, both isomers were quantified together as TTR in this work.

7.7.8. Environmental levels

The results of the environmental levels at which the target benzotriazoles and benzothiazoles are present in wastewater and sewage sludge from the sewage treatment plant of Athens are discussed in Section 9.1.

CHAPTER 8

DETERMINATION OF BENZOSULFONAMIDES IN WASTEWATER BY LC-MS/MS

8.1. HPLC-UV METHOD DEVELOPMENT FOR BENZOSULFONAMIDES

Preliminary experiments were carried out to optimize the chromatographic conditions necessary for effective separation of three benzosulfonamides compounds [Benzenesulfonamide (BSA), o-Toluenesulfonamide (o-TSA) and p-Toluenesulfonamide (p-TSA)]. Chromatographic conditions, such as wavelength of detection, mobile phase composition, flow rate and column oven temperature were optimized. The optimization was achieved by changing the value of the parameters one at a time, while keeping other conditions constant. We tested several columns of different stationary phases for their efficiency in the separation of the compounds. Particular attention was paid to the optimization of mobile phase composition in order to achieve a chromatographic separation of all the compounds especially the two isomers (o-TSA and p-TSA) with acceptable resolution. Standard solutions at different concentrations ranging from 100 µg L^{-1} to 50 mg L^{-1} of the compounds were used for the optimization.

8.1.1. Preparation of Standard Solutions of Benzosulfonamides

Stock solutions of benzosulfonamides (BSA, o-TSA and p-TSA) were prepared at concentration level of 1000 mg L^{-1} by dissolving an appropriate amount of each compound in methanol and stored at - 20°C. Working standard solutions were prepared from stock solutions by further dilution with solvent(s) similar in composition to the mobile phase used for chromatographic elution.

8.1.2. Optimization of UV absorbance wavelength for Benzosulfonamides

Some preliminary experiments were carried out to determine the optimum absorption wavelength of each of the compounds (BSA, o-TSA and p-TSA) with UV/Visible spectrophotometer. The absorption spectrum of each of the compounds was taken by running the standard solution of the compound on a UV/Visible spectrophotometer using 10 mg L^{-1} standard solution of each of the compounds, prepared in an approximate mobile phase composition intended to be used for liquid chromatographic separation. The measurement was carried out as described for benzotriazoles and benzothiazoles in section 7.1.2. An optimum wavelength of detection was obtained for each of the benzosulfonamides. Since the three compounds belong to the same class and therefore possess the same functional group (- SO_2NH_2), there was only very little difference in their optimum absorption wavelengths. A compromise optimum wavelength of 264 nm was selected for simultaneous detection of all the compounds with UV detector on HPLC.

8.1.3. Optimization of Chromatographic Parameters

Several analytical columns were tested for the separation of the compounds. The analytical columns tested include Zorbax SB C18 150 mm x 4.6 mm, 5 µm; Zorbax XDB C18 150 mm x 4.6 mm, 5 µm; Lichrospher 100 RP-18, 250 mm x 4 mm, 5 µm; Symmetry C18 150 mm x 3.9 mm, 5 µm; Ascentis Express C18 (Supelco) 75mm x 2.1 mm, 2.7 µm; Pathfinder Schimadzu C18 EP 150 mm x 4.6 mm, 3.5 µm). Based on the efficiencies of separation of the compounds as obtained for the different analytical columns tested, Symmetry C18 (150 mm x 3.9 mm, 5 µm) column was finally selected as it gave the best resolution for the separation of the two isomers (o-TSA and p-TSA). Moreover, mobile phase composition, flow rate, column oven temperature and elution programme were also optimized. For the optimized conditions with reversed-phase Symmetry C18 (150 mm x 3.9 mm, 5 µm) column, and in order to separate the two isomers, mobile phase composition with a high content of water and acetonitrile (80% water/ 20% acetonitrile) was employed in an isocratic elution at a flow rate of 0.5 mL/min, with 30°C as column oven temperature and 264 nm wavelength. Mobile phase composition as methanol only and water and methanol/acetonitrile and water were also tested. It was observed that the methanol generally produced broader peaks of the compounds and the two isomers were poorly separated. The performance parameters of the chromatographic separation with the Symmetry column were investigated using different percentage mobile phase composition (acetonitrile and water) and at different column oven temperature values.

The typical chromatograms of the compounds are shown in Fig 8.1.

The comparisons of chromatographic performance parameters using different percentage of mobile phase composition (acetonitrile and water) and at different values of column oven temperature are presented in both Tables 8.2 and 8.3. There was no significant difference in resolution, peak area and theoretical plates with 15% ACN and 10%ACN but a longer total elution time with 10%ACN. Also, no significant difference was observed in resolution and total elution time at both 30°C and 20°C of column oven temperature. However, the peak area and number of theoretical plates were higher at 30°C than at 20°C when 15%ACN was used. Motivated by this observation, we proceeded to testing higher values of column oven temperature but the results were worse than at 30°C indicating the optimum oven temperature for the performance of the column (Symmetry C18) in the separation of the compounds.

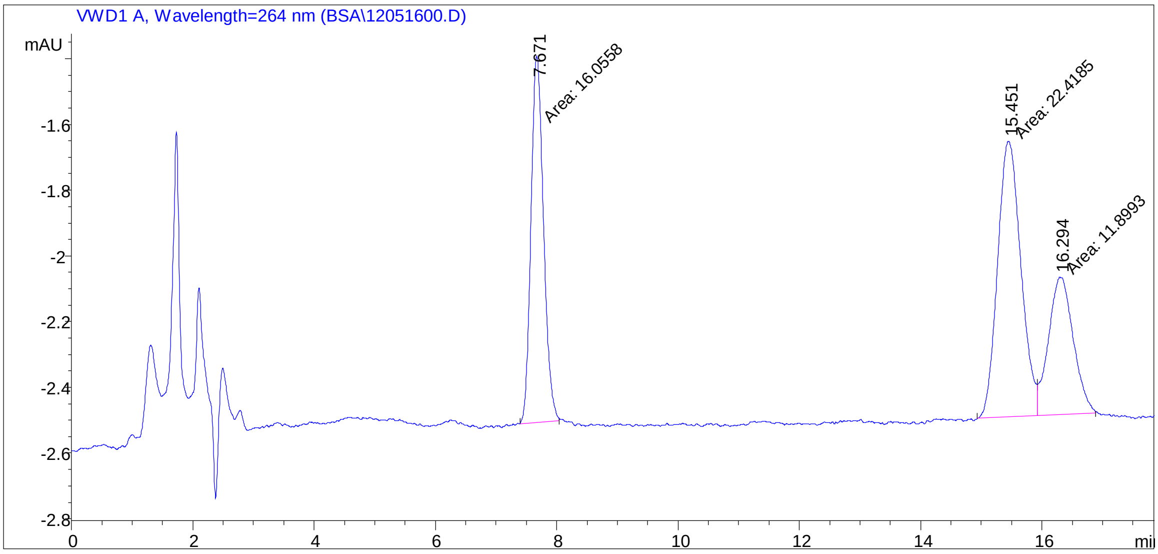


Fig 8.1: Chromatograms of separation of benzosulfonamides with HPLC-UV; 2 mg L^{-1} standard injection, Symmetry C18 column, Flow rate: 0.5 mL/min, 15/85 (ACN/H_2O)

The following chromatographic conditions were finally chosen for a satisfactory separation of the compounds on HPLC-UV:

Column (Stationary Phase): Symmetry C18 (150 mm x 3.9 mm, 5 µm)

Mobile Phase: 15%Acetonitrile/ 85%water in isocratic elution

Column oven temperature: 30°C

Flow rate: 0.5 mL/min

UV absorption wavelength: 264 nm

The instrumental limits of detection (LODs) with HPLC-UV for the compounds are presented in Table 8.1.

Table 8.1: Instrumental Limits of Detection of Benzosulfonamides with HPLC-UV

Compound	**Instrumental LOD (µg)**
BSA	6.23
o-TSA	8.08
p-TSA	6.25

20 µL injection with Symmetry C18 (150 mm x 3.9 mm, 5 µm) column

The comparisons of chromatographic performance parameters at different % ACN and different values of column oven temperature are presented in Table 8.2 and Table 8.3

Table 8.2: Comparison of HPLC-UV chromatographic performance parameters on symmetry C18 column with different % ACN at 30°C, (n=5)

Compound	**Rt (min)**	**Rt (min)**	**Peak Area**	**Peak Area**	**Symmetry**	**Symmetry**	**No of Plates**	**No of Plates**	**Resolution**	**Resolution**
	15% ACN	***10% ACN***	***15% ACN***	***10% ACN***	***15% ACN***	***10% CAN***	***15% ACN***	***10% ACN***	***15% ACN***	***10% ACN***
BSA	7.6±2.2	11.2±0.4	54.3±8.1	58.5±4.5	0.77±1.84	0.78±1.59	6423± 4	6684±2		
o-TSA	15.5±0.2	27.4±0.6	78.1±8.6	83.4±6.6	0.77±3.57	0.81±0.01	6561±4	6772±2	1.08±1.58	1.05±0.78
p-TSA	16.3±0.3	28.9±0.5	42.3±8.2	46.4±7.5	0.78±2.54	0.83±9.64	6179±4	5865±2	1.08±1.58	1.05±0.78

Table 8.3: Comparison of chromatographic performance parameters on Symmetry C18 column with 15%ACN at different values of column oven temperature, (n=5)

Compound	**Rt (min)**	**Rt (min)**	**Peak Area**	**Peak Area**	**Symmetry**	**Symmetry**	**No of Plates**	**No of Plates**	**Resolution**	**Resolution**
	30°C	**20°C**	**30°C**	**20°C**	**30°C**	**20°C**	**30°C**	**20°C**	**30°C**	**20°C**
BSA	7.6±2.2	8.0±0.1	54.3±8.1	33.6±3.3	0.77±1.84	0.76±2.00	6423±4	6202±1		
o-TSA	15.3±0.2	16.4±0.2	78.1±8.6	46.6±2.6	0.77±3.57	0.82±4.40	6561±4	6521±1	1.08±1.58	1.03±0.53
p-TSA	16.2±0.3	17.3±0.1	42.3±8.2	26.2±4.7	0.78±2.54	0.82±4.22	6179±4	6054±2	1.08±1.58	1.03±0.53

8.2. Development and optimization of SPE protocol for extraction and preconcentration of benzosulfonamides using Tap water

A preliminary solid phase extraction (SPE) protocol was developed and optimized for extraction and preconcentration using tap water. Five different SPE cartridges and sorbents; one C18 and four polymeric sorbents (Isolute C18 500 mg/6mL, Oasis HLB, 200 mg/6mL; Evolute ABN, 200mg/6mL; and Strata X, 200mg/6mL and Polyclean polymeric 200mg/6mL) were tested for their extraction efficiency. Different elution solvents (methanol, acetonitrile and 50:50 acetonitrile:methanol) and different elution volumes (3 mL, 6 mL, 10 mL) were also evaluated to obtain the optimum conditions for most of the compounds. The SPE procedure was as follows:

50 mL of Tap water sample was spiked at 200 µg L^{-1} level for the compounds (BSA, o-TSA and p-TSA) and adjusted to a pH of 3 with 25% HCl. The cartridge (sorbent) was preconditioned with 10 mL methanol, equilibrated with 10 mL acidified purified water (at pH 3) followed by loading of the acidified 50 mL tap water sample on the catridge. The cartridge was washed with 10 mL of acidified purified water (pH 3) and then left to dry for about 1 hour. The compounds were eluted with10 mL of a 50:50 mixture of methanol and acetonitrile. The eluent was evaporated to approximately 200 µL under a gentle stream of nitrogen gas at 35°C. The extract was then made up to 1 mL with 20/80 acetonitrile/water giving rise to a final concentration of 10 mg L^{-1}. The final extract was injected on both HPLC-UV and LC-MS/MS.

The procedure was carried out in a similar way for all the cartridges except in another experiment where the efficiency of Evolute ABN was compared with that of Polyclean polymeric at a lower spike concentration. In this case, tap water samples were spiked at 4 µg L^{-1} giving rise to a final concentration of 2 mg L^{-1}. It should be noted that we initially tested different types and volumes of elution solvents (methanol only, acetonitrile only, mixture of acetonitrile and methanol) with Evolute ABN. We eventually adopted 10 mL of 50:50 (methanol/acetonitrile). Recovery corresponding to SPE was calculated by comparing the concentration of each compound in the tap water samples spiked before extraction to the concentration in the tap water samples spiked after extraction. The total recovery was calculated by comparing the concentration of each compound in the tap water spiked before extraction to the concentration in the pure standard solution. Matrix effect was determined by comparing the concentration of each compound in tap water spiked after extraction to the concentration in the pure standard solution. The LC-UV chromatograms of the compounds in spiked tap water samples before and after extraction are presented in Figures 8.2 and 8.3.

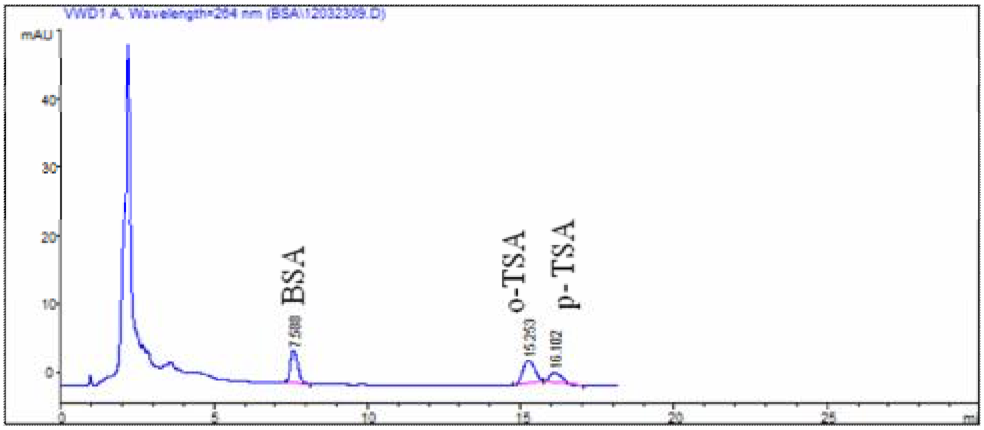


Fig. 8.2: LC-UV chromatograms of benzosulfonamides in spiked (before extraction) tap water at 10 mg L^{-1} fortification level; Symmetry C18 Column, Evolute ABN cartridge

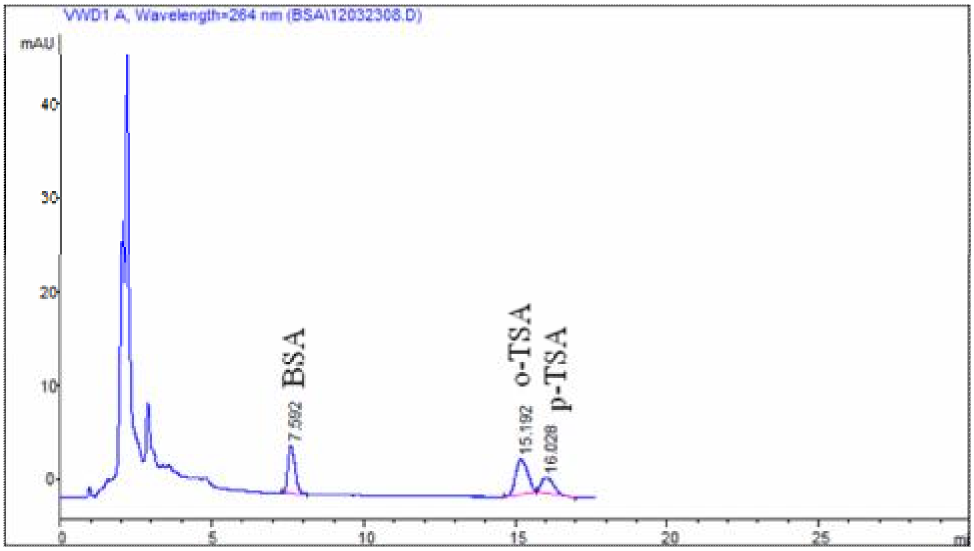


Fig. 8.3: LC-UV chromatograms of benzosulfonamides in spiked (after extraction) tap water at 10 mg L^{-1} fortification level; Symmetry C18 Column, Evolute ABN cartridge

The recoveries of the compounds (BSAs) with the different sorbents are presented in Tables 8.4- 8.6.

Table 8.4: Comparison of Recoveries (%) of Benzenesulfonamide in tap water with different cartridges (sorbents)

BSA						
	ISOLUTE C18 **10 mgL^{-1} final**	**OASIS** **10 mgL^{-1} final**	**EVOLUTE** **10 mgL^{-1} final**	**STRATA X** **10 mgL^{-1} final**	**EVOLUTE** **2 mg L^{-1}** **final**	**POLYCLEAN** **2 mgL^{-1}** **final**
SPE RECOVERY	9	103	95	99	67	94
MATRIX EFFECT	108	98	103	86	92	63
TOTAL RECOVERY	9	101	98	85	62	59

Table 8.5: Comparison of Recoveries (%) of o-Toluenesulfonamide in tap water with different cartridges (sorbents)

o-TSA						
	ISOLUTE **C18** **10 mgL^{-1} final**	**OASIS** **10 mgL^{-1} final**	**EVOLUTE** **10 mgL^{-1} final**	**STRATA X** **10 mgL^{-1} final**	**EVOLUTE** **2 mgL^{-1}final**	**POLYCLEAN** **2 mgL^{-1}** **final**
SPE RECOVERY	69	102	95	101	93	96
MATRIX EFFECT	103	100	104	84	87	75
TOTAL RECOVERY	71	102	99	85	81	72

Table 8.6: Comparison of Recoveries (%) of p-Toluenesulfonamide in tap water with different cartridges (sorbents)

p-TSA						
	ISOLUTE C18 10 mgL^{-1} final	**OASIS 10 mgL^{-1} final**	**EVOLUTE 10mgL^{-1} final**	**STRATA X 10mgL^{-1} final**	**EVOLUTE 2 mgL^{-1} final**	**POLYCLEAN 2 mgL^{-1} final**
SPE RECOVERY	64	102	98	99	97	99
MATRIX EFFECT	102	99	102	87	89	71
TOTAL RECOVERY	66	101	100	86	86	70

8.3. METHOD DEVELOPMENT FOR THE DETERMINATION OF BENZOSULFONAMIDES IN WASTEWATER (DISSOLVED PHASE) BY LC-MS/MS

8.3.1. Sample Pretreatment for wastewater

A mixture of effluent wastewater samples from a sewage treatment plant was used for the method development. The mixed effluent sample was filtered with a glass fibre pre-filters under vacuum and adjusted to a pH of ~3 with 25% HCl.

8.3.2. Extraction and Preconcentration from wastewater (dissolved phase) by SPE

Extraction and preconcentration of the compounds in wastewater effluent was carried out by the optimized solid phase extraction procedures developed with tap water with slight modifications. Polyclean polymeric (200mg/6mL) were used for the SPE protocol. 50mL of mixed wastewater effluent was adjusted to approximately pH of 3 with 25% hydrochloric acid. The effluent samples were spiked prior to extraction with the standard solution of the analytes at three different levels of fortification; representing low, medium and high concentration levels (0.5 µg L^{-1}, 1 µg L^{-1} and 5 µg L^{-1}); the final eluate concentrations were

25 µg L^{-1}, 50 µg L^{-1} and 250 µg L^{-1} respectively.The remaining part of the SPE procedure was the same as reported for tap water in Section 8.2. The matrix matched samples were also carried through the same procedure, only that the sample extracts were spiked with standard solution of the compounds after the evaporation stage. The recovery of the compounds with the SPE procedure was determined by comparing the response of each compound (concentration) in the spiked samples before extraction to the response (concentration) of the same compound in the spiked samples after the extraction.

8.3.3. Chromatographic Separation of Benzosulfonamides in wastewater (dissolved phase) by LC-MS/MS

For separation of the compounds with LC-MS/MS, the already optimized chromatographic parameters with HPLC-UV for the compounds by using their standard solution (Section 8.1) were used for LC-MS/MS determination. Since several analytical columns were tested on HPLC-UV and only Symmetry C18 (150 mm x 3.9 mm, 5µm) column proved satisfactory for the separation and good resolution of the two isomers (o-TSA and p-TSA), the column was also adopted for the separation of the compounds on LC-MS/MS.

8.3.3.1. Selection of Mobile Phase and Modifier

A combination of ACN and H_2O only, ACN and 0.05% (v/v) HCOOH in water, ACN and 0.1% (v/v) HCOOH in water, acetonitrile and ammonium formate (5 mM) were evaluated separately for the separation of the compounds. Methanol in place of acetonitrile was also tested but acetonitrile produced constantly higher responses, better resolution and better peak shapes.

For LC-MS/MS determination of BSAs, the mass spectrometer was initially tested in positive ESI ionization mode, but the intensity of the compounds in positive ionization was not satisfactory, even when a proton donor such as formic acid was added as modifier. We eventually tested negative ionization mode for ionization of the compounds and there was improvement in the intensity of the compounds compared to the positive ionization mode. Thus, a combination of acetonitrile and water only was finally applied as mobile phase solvents at a flow rate of 400 µL min^{-1} on LC-MS/MS.

8.3.4. Optimization of ESI/MS/MS Parameters for Benzosulfonamides

8.3.4.1. Selected Reaction Monitoring Method

For MS/MS analysis of the benzosulfonamides (BSA, o-TSA and p-TSA), selected reaction monitoring (SRM) in negative ESI ionization mode was initially optimized for each of the compounds. Collision energy and tube lens were optimized for each of the compounds (BSA, o-TSA and p-TSA). Two SRM transitions of each compound from the precursor ion to product ions were monitored for quantification (transition 1) and confirmation (transition 2). The optimization of tube lens for each of the compounds was carried out by direct infusion of standard solution of the compounds. The description of the precursor ions and product ions of the compounds of interest, with their respective collision energies and tube lens offset voltage is presented in Table 8.7.

Table 8.7: Precursor ions and product ions (SRM) of BSAs monitored with their respective collision energies and tube lens offset voltage

Compound	Precursor ion $[M-H]^-$ (m/z)	Product ions (m/z)	Collision Energy (V)	Tube Lens Offset (V)
BSA	156	78.9	28	52
		92.0	17	52
o-TSA	170	79.0	22	62
		106.0	19	62
p-TSA	170	79.0	22	62
		106.0	19	62

The breakdown curves of the optimization of tube lens for the compounds in the SRM method are given in Figures 8.4-8.6

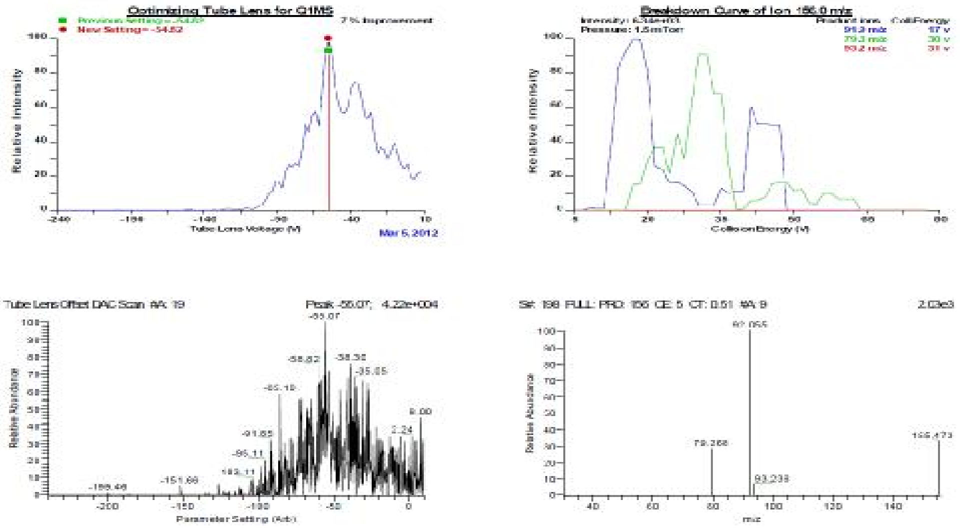


Fig. 8.4: BSA Breakdown curve of tube lens optimization for SRM method

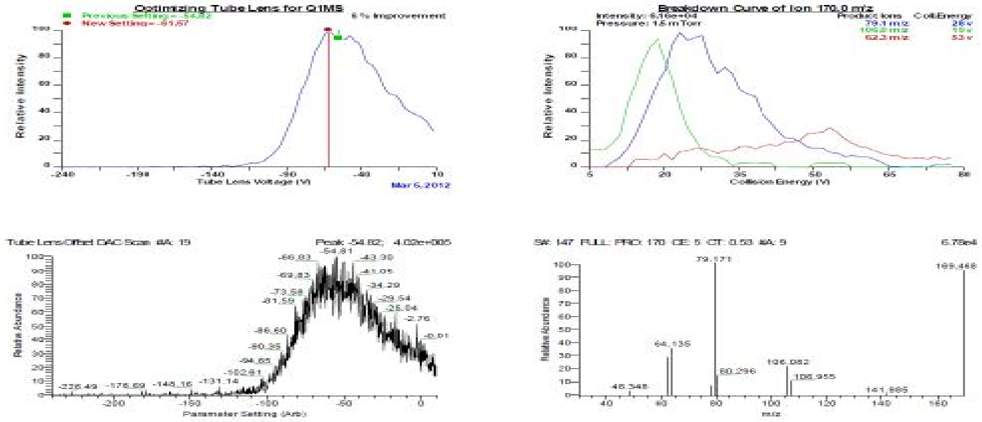


Fig.8.5: o-TSA Breakdown curve of tube lens optimization for SRM method

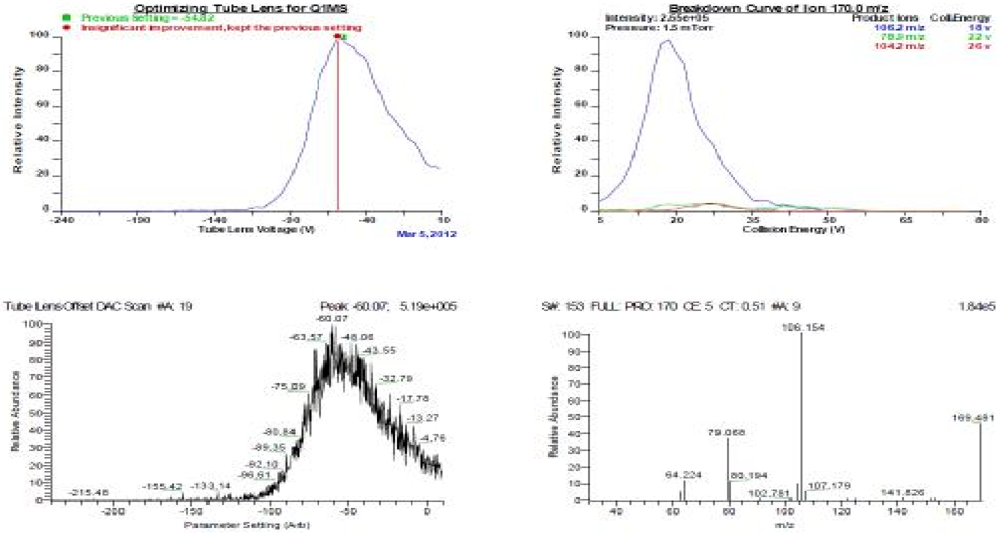


Fig. 8.6: p-TSA Breakdown curve of tube lens optimization for SRM method

Electrospray ionization source parameters, such as the spray voltage, sheath gas pressure, auxiliary gas flow, capillary temperature, distance from entrance (micrometer), and the position of the probe were optimized for the SRM method. The optimization was carried out by continuously injecting 10 µL of 1 mg L^{-1} standard solution of the analyte prepared in 20/80 acetonitrile/water via loop injection, changing one parameter at a time, until an optimum response for the compound was obtained. The experiments were carried out using 200 µL/min as flow rate. The standard solutions of the compounds were initially prepared in different compositions of solvents and tested for both the positive and negative ionization modes. The response of the compounds (BSAs) in the positive ionization mode was lower than in the negative ionization mode even when a proton donor such as formic acid was used in preparing the standard solutions. We subsequently carried out the optimization in negative ionization mode using acetonitrile/water only as the compound dissolving solvent. The optimized parameters for the electrospray ionization (ESI) in the negative ionization mode for the SRM method are presented in Table 8.8. It was observed that both o-TSA and p-TSA isomers presented similar behaviour (same transitions and similar ESI source parameters) but as a result of their good separation and resolution in the chromatographic experiments, the two isomers' peaks could easily be identified and differentiated.

Table 8.8: Optimized ESI Source Parameters of Benzosulfonamides for SRM method in negative ionization mode at 200 µL/ min flow rate

Compound	**Spray Voltage (V)**	**Sheath gas (Psi)**	**Auxilliary gas**	**Capillary Temp (°C)**	**Probe Position**
BSA	3500	30	20	250	C/D
o-TSA	4000	30	20	250	C
p-TSA	3500	30	20	250	C/D

The SRM method was applied for recovery experiments of the compounds in Tap water.

The following optimized ESI source conditions were selected as compromise values for:

Spray Voltage (V): - 3500
Sheath Gas pressure (psi): 30
Auxiliary Gas flow rate: 20
Probe position: Between C and D (V-position)
Micrometer (inches): 0.45
Capillary temperature (°C): 250
Collision gas (Ar) pressure (mTorr): 1.8

8.3.4.2. Pseudo-Selected Reaction Monitoring Method

The developed SRM method was not sensitive enough to detect the compounds (BSAs) in wastewater due to limited fragmentation of the compounds as shown in Figures 8.4-8.6. Consequently, an alternative and simple approach method was developed using a technique where the two quadrupoles of a tandem mass spectrometry monitor the same m/z (usually the molecular ion) in a selected reaction monitoring. This type of SRM method is called Pseudo-selected reaction monitoring (Pseudo-SRM). The Pseudo-SRM method was developed in negative ionization mode of ESI source.

ESI parameters were optimized using 200 µL/min flow rate but in order to reduce the total elution time, 300 µL/min, 400 µL/min and 500 µL/min were also experimented separately. In addition, the optimization was carried out separately using acetonitrile/water only and acetonitrile/ammonium formate solution as compound dissolving solvents. Ammonium formate (5 mM) in place of only water was added to investigate if a better reponse with higher intensity would be produced. There was only small difference in the responses, when Acetonitrile/water only and Acetonitrile/ammonium formate were used as compound dissolving solvents. Acetonile/water only was selected instead of acetonitrile/ammonium formate as dissolving solvent for the purpose of column life span. Moreover, 400 µL/min flow rate was finally used as it produced adequate intensities and reduced the total elution time compared to 200 µL/min.

The description of the monitored ions of the compounds of interest, with their respective collision energies and tube lens offset voltage is presented in Table 8.9

Table 8.9: Description of monitored ions in Pseudo-SRM method of BSAs with their respective collision energies and Tube lens offset voltage

Compound	Q1 Ion $[M-H]^-$ (m/z/)	Q3 Ion (m/z)	Collision Energy (V)	Tube Lens Offset (V)
BSA	156	156	10	52
o-TSA	170	170	10	62
p-TSA	170	170	10	62

The following optimized ESI source conditions were selected for the Pseudo-SRM method at 400µL/min flow rate:

Spray Voltage (V):	- 3200
Sheath Gas pressure (psi):	30
Auxiliary Gas flow rate:	20
Probe position:	Between C and D (V-position)
Capillary temperature (°C):	250
Collision gas (Ar) pressure (mTorr):	1.0

The Pseudo-SRM method was also initially applied for recovery experiments of the compounds in tap water and finally evaluated by using wastewater samples.

8.4. METHOD VALIDATION FOR THE DETERMINATION OF BENZOSULFONAMIDES IN WASTEWATER

The developed methods by LC-MS/MS were validated in terms of analytical performance parameters as linearity, accuracy (recovery), precision (repeatability and reproducibility), limit of detection (LOD) and limit of quantification (LOQ).

8.4.1. Linearity

Linearity was determined by constructing calibration curves using standard addition calibration. The standard solution of every target compound was spiked into wastewater samples before SPE extraction. The expected concentrations of every target compound (BSA, o-TSA and p-TSA) (in the final 1 mL extract prior to analysis), used for the calibration curves ranged from 5 to 250 ng mL^{-1}. The calibration curves were constructed using the peak areas of target compounds versus the nominal concentrations of the target compounds. Least square linear regression analysis of the data gave slope, intercept, and correlation coefficients of determination. The responses for the target compounds are presented in Table 8.10

8.4.2. Limit of detection (LOD) and Limit of Quantification (LOQ)

The LODs and LOQs of the Pseudo-SRM method were determined via the calibration curves obtained by spiking wastewater samples at low concentration level.

The variation (standard deviation) of the response of the successive injections of six replicates of a spiked wastewater sample at the low concentration was multiplied by a factor of 3 and 10 for calculating LODs and LOQs respectively.

The values for the method LODs and LOQs are shown in Table 8.10.

8.4.3. Accuracy/Recovery

The trueness of the method was evaluated through recovery experiments at three fortification levels of the target compounds that were realized in-between three different days (k = 3). To accomplish this, effluent (50 mL) spiked with the appropriate amount of the target compounds. The low, medium and high fortification levels for the compounds were 0.5, 1.0 and 5 ng mL^{-1}, respectively. The results are given in Table 8.11.

8.4.4. Precision (Repeatability and Reproducibility)

Precision of the Pseudo-SRM method was assessed by performing repeatability and reproducibility experiments. For repeatability experiments, a wastewater sample was spiked at three fortification levels of the target compounds, and three replicates (n=3) were prepared for each fortification level (intra-day precision). For reproducibility experiments, a wastewater sample was spiked at a fortification level of the target compounds, and six replicates (n=6) were prepared in-between 2 different days (k=2) (inter-day precision). The low, medium and high fortification levels for all compounds were 0.5, 1.0 and 5 ng mL^{-1}. Precision data of the method are given in Table 8.11.

8.5. APPLICATION TO ENVIRONMENTAL SAMPLES

We applied successfully the developed Pseudo-SRM method for determination of the three benzosulfonamides in wastewater (dissolved phase)

8.5.1. Sampling and Handling of Environmental Samples

Wastewater (treated and untreated) samples were collected from the sewage treatment plant Psittalia, Athens, Greece.

During a sampling campaign in April 2012, influent and effluent wastewater samples were collected on seven consecutive days from the treatment plant. All wastewater samples were collected in pre-cleaned polypropylene (PP) bottles. 50 mL of influent and effluent wastewater (24-hour composite flow proportional samples), were immediately filtered (with glass fibre pre-filters 0.45 μm) within 4 hour of arrival in the laboratory. From every dissolved phase matrix, 50 mL was obtained and acidified with hydrochloric acid (HCl) solution to pH 3.0 ± 0.10 and stored in the dark at 4 °C until analysis.

8.5.2. Extraction and Preconcentration from wastewater (dissolved phase) samples

A volume of 50 mL of dissolved phase was transferred in a 50 mL Eppendorf tube and adjusted with 25% HCl to pH 3.00 (± 0.10) prior to the loading step of the SPE protocol. Spiked matrix samples and QC samples were spiked with an appropriate amount of target compounds. All samples prior to SPE were vortex mixed for 1 min to ensure sufficient distribution of the spiked compounds in the solution. Isolation of the compounds from the spiked wastewater samples was performed using Polyclean polymeric cartridges (200mg/6mL, 30 μm) fitted on the vacuum apparatus. The cartridges were conditioned by 10 mL of MeOH and equilibrated by 10 mL of acidified Milli-Q gradewater (adjusted with HCl solution to pH 3.00 ± 0.10) without applying vacuum. Then, the acidified samples were passed through the cartridge, to remove any matrix interference, the cartridges were washed with 2×5 mL of acidified Milli-Q grade water (adjusted with HCl solution to pH 3.00 ± 0.10) and then dried under -5 inHg of vacuum for 45 min. The compounds were eluted with 10 mL of mixture of MeOH/ACN (1:1 v/v) and collected with a 15 mL Eppendorf tube. The eluents were set on a heated tray (35 ^{0}C) and were evaporated to a volume of 200 μL, under a gentle stream of nitrogen gas (N_2) and then made up to 1 mL with a mixture of ACN/H_2O (20:80 v/v). The extract was filtered and set to the auto-sampler tray for analysis. For the preparation of matrix match standard solutions, the target compounds were spiked into the final extract after the evaporation step. In every batch of samples prepared, the preparation of at least one procedural blank during the whole sample preparation procedure (including SPE process and evaporation step) was essentially carried out in order to detect any cross-contamination.

8.5.3. LC-MS/MS measurements of Benzosulfonamides

Chromatographic separation was performed with Symmetry C18 (150 mm x 3.9 mm, 5µm) column and the column oven temperature was set at 25 °C. The collision gas (Ar) pressure was set at 1.0 mTorr. The monitored ions for the compounds, the collision energies and the tube lens voltage values set for the Pseudo SRM method are presented in Table 8.10.

Chromatographic analyses were carried out using an isocratic elution 15%ACN and 85% Milli-Q grade water as binary mobile phase mixture at a flow rate of 400 µL min^{-1}.The ESI was applied at a potential of -3.2 kV. The probe was set at a position in-between positions C and D (for V-distance). The sheath gas (N_2) flow pressure was set at 30 psi, the auxiliary gas (N_2) flow rate was set at 20 A.U. and the ion transfer capillary temperature was set at 250°C. The skimmer offset potential was set at 0 V. The data acquisition values were set at 0.20 s for scan speed, 0.200 m/z for scan width, 0.70 FWHM for resolving power and 10 A.U. for the chromatography filter (noise reduction). The final in-vial composition of all wastewater samples and standard solutions was in ACN/H_2O (20:80 v/v), and injection was carried out with partial-loop injection (10 µL). Quantification of the compounds in wastewater samples was carried out with standard addition method. One of the wastewater samples was spiked before extraction at different concentration levels ranging from 5 to 250 ng mL^{-1} in the final extracts prior to analysis. The spiked samples were carried through the whole analytical procedure as the samples and the peak areas of the spiked samples were used for constructing calibration curves. The spiked sample calibration was necessary for quantification since no internal standard was added to evaluate the method performance. Chromatograms of the target compounds in dissolving solvents only (standard solution), wastewater samples spiked with the target analytes prior to extraction (pre-extraction matrix spikes) and wastewater samples spiked with target analytes after extraction (post-extraction matrix spikes) are presented in Figures 8.2-8.4.

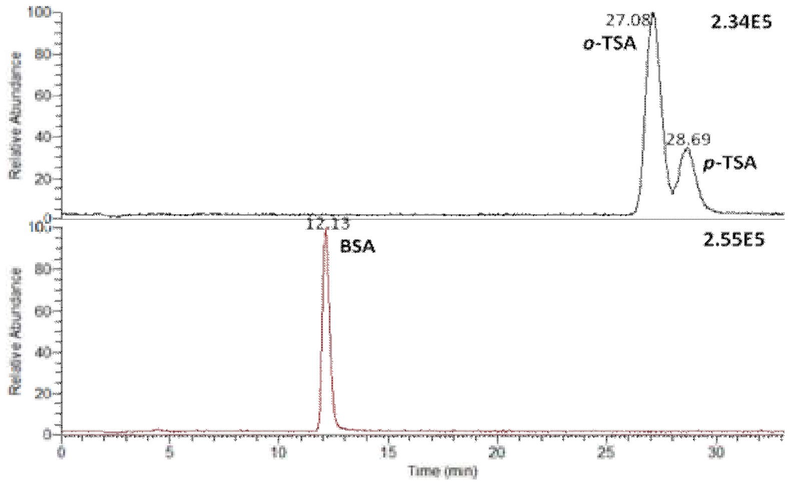


Fig. 8.7: LC-MS/MS Chromatograms of benzosulfonamides in standard solution (250 ng mL^{-1})

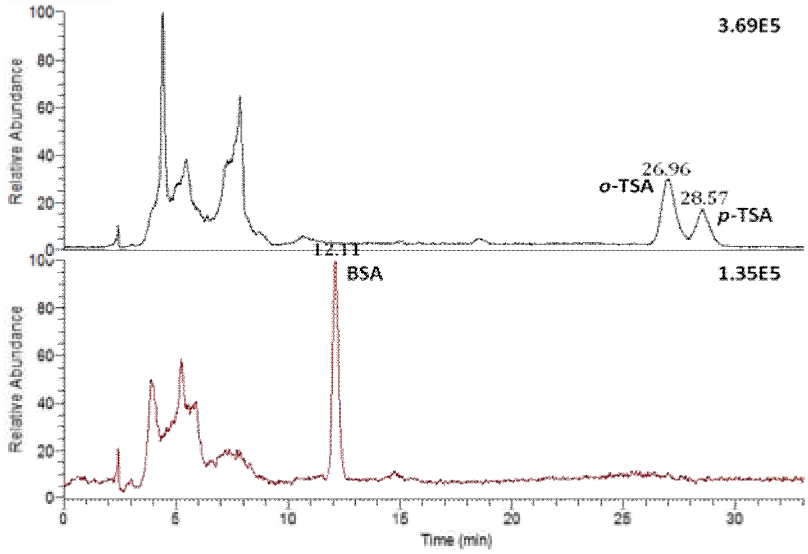


Fig. 8.8: LC-MS/MS Chromatograms of benzosulfonamides in matrix-matched (after extraction) wastewater sample at a fortification level of 250 ng mL^{-1}

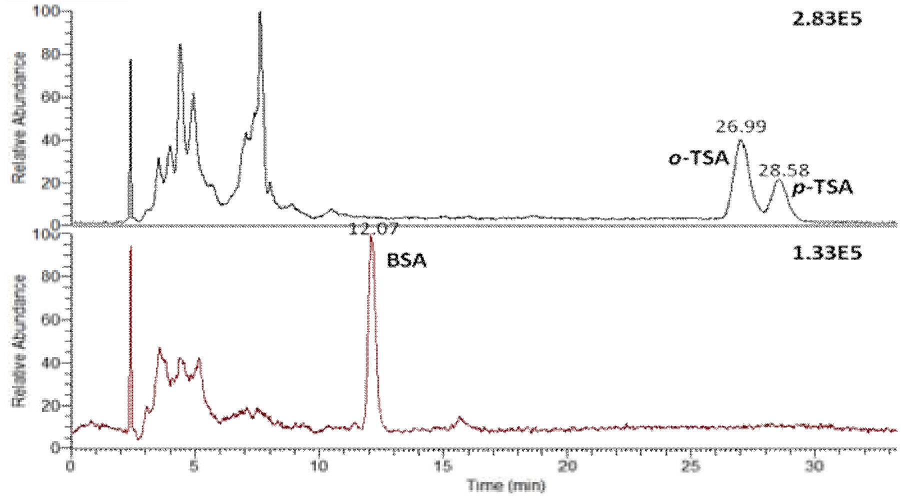


Fig. 8.9: LC-MS/MS Chromatograms of benzosulfonamides in spiked (before extraction) wastewater sample at a fortification level of 250 ng mL^{-1}

8.6. RESULTS AND DISCUSSION

8.6.1. Extraction from wastewater

The recovery percentage corresponding to the extraction step was assessed by comparing the responses obtained for samples spiked before and after the SPE steps. Five different SPE sorbents (cartridges) were initially tested and evaluated for their extraction and preconcentration efficiencies for the compounds in tap water. One of the sorbents is a C18 material (Isolute C18 500 mg/6mL) while the remaining four sorbents are polymeric materials (Oasis HLB, 200 mg/6mL; Evolute ABN, 200mg/6mL; and Strata-X, 200mg/6mL and Polyclean polymeric 200mg/6mL). With the Isolute C18, an exceptionally low recovery of only 9% was obtained for BSA. Recoveries of 69% and 64 % were obtained for both o-TSA and p-TSA respectively. The generally low recoveries of benzenesulfonamides with Isolute C18 were mainly due to the highly hydrophilic properties of the compounds. Of the three compounds, BSA is the most polar with $\mathrm{LogK_{ow}}$ value of 0.3, while the $\mathrm{Log\ K_{ow}}$ values of o-TSA and p-TSA are 0.84 and 0.82 respectively (Table 3.3). It is widely known that C18 sorbents possess non-polar surfaces; as a result the compounds could not be strongly retained by the Isolute C18 cartridge. The extent of non-retention increases with the polarity of the compounds, therefore, responsible for the very low recovery obtained for BSA. On the other hand, all the polymeric sorbents yielded high recoveries for all the compounds in tap water (> 93%) as depicted in Tables 8.4-8.6. The high recoveries were attributed to the presence of both hydrophilic and hydrophobic chemical moieties in each of the polymeric sorbents.We subsequently adopted Polyclean polymeric (200mg/6mL) cartridges for the extraction and preconcentration of the compounds in wastewater at three different fortification levels (0.5µg $\mathrm{L^{-1}}$, 1.0 µg $\mathrm{L^{-1}}$ and 5.0 µg $\mathrm{L^{-1}}$). Recoveries for the three compounds at the three fortification levels ranged from 83 to 118% (Table 8.11) indicating an excellent performance of the sorbents for the extraction and preconcentration of the compounds in a complex environmental matrix as wastewater.

8.6.2. Analytical Method Performance

8.6.2.1. Linearity

The linearity of the method for the compounds was determined as described in section 8.4.1 within a concentration range of 5 µg $\mathrm{L^{-1}}$ and 250 µg $\mathrm{L^{-1}}$. As shown in Table 8.10, the responses of MS/MS detector in wastewater were linear for all the target compounds with coefficients of determination, R^2 of 0.9946 for BSA, 0.9966 for o-TSA and 0.9993 for p-TSA.

8.6.2.2. Limits of detection (LODs) and Limits of quantification (LOQs)

The LODs and LOQs of the method in wastewater were determined as described in section 8.4.2 and values given in Table 8.11. The method LOD was 0.09 µg L^{-1} for both BSA and o-TSA while the LOD value was 0.01 µg L^{-1} for p-TSA. The method LOQ was 0.30 µg L^{-1} for both BSA and o-TSA while the LOQ value was 0.04 µg L^{-1} for p-TSA. Richter et al. also calculated LOQ value of 0.01 µg L^{-1} for p-TSA and LOQ value of 0.02 µg L^{-1} for both BSA and o-TSA in purified and drinking water using the signal to noise ratio approach [154].

Table 8.10: Some analytical characteristics of Pseudo-SRM method for the determination of benzosulfonamides in wastewater: Calibration Equations, Coefficients of determination (R^2) for concentrations range between 5 µg L^{-1} and 250 µg L^{-1}, Method limits of detection (LODs) and quantification (LOQs) in wastewater

Compound	**Calibration Equations**	**R^2**	**LOD (µg L^{-1})**	**LOQ (µg L^{-1})**
BSA	y = 9673.3x - 150628	0.9946	0.088	0.290
o-TSA	y = 17067x - 184106	0.9966	0.089	0.300
p-TSA	y = 9032.5x - 127474	0.9993	0.011	0.035

They could not determine LOQs of the compounds in wastewater samples because of their ubiquitous occurrence in the matrices and therefore assumed the LOQs determined for drinking water to be valid also for wastewater. Considering the ubiquitous occurrence of these compounds (BSAs) in wastewater samples, the LOD and LOQ values obtained in this study are low enough for adequate and accurate quantification of the compounds in the matrices.

8.6.2.3. Method Recovery

The trueness of the method was evaluated through recovery experiments at three fortification levels of the target compounds. To accomplish this, wastewater (50 mL) spiked with the appropriate amount of the target compounds. The low, medium and high fortification levels for the compounds were 0.5, 1.0 and 5 ng mL^{-1}, respectively. The method recovery was determined by comparing the response of each compound in the spiked wastewater samples before extraction to the response of the compound in pure standard solution. The results are given in Table 8.11.

The absolute recoveries of all three compounds were low at the three fortification levels. The low overall recoveries of the compounds were predominantly as a result of the significant ion suppression experienced (Fig. 8.2) since we did not experience significant incomplete extraction during the SPE extraction step as substantiated by the results of SPE recoveries (Table 8.11). It was therefore necessary to employ the method of standard addition for accurate quantification of these compounds in the wastewater samples. Spiked sample calibration curves were used for quantification since no internal standard was added to evaluate the method performance.

8.6.2.4. Method Precision

Precision of the pseudo-SRM method was assessed by performing repeatability and reproducibility experiments as described in section 8.4.4. At low fortification level (0.5 µg L^{-1}), the absolute values (%RSD) for intra-day precision (repeatability) were 4.1%, 2.7% and 0.4% for BSA, o-TSA and p-TSA respectively whereas the absolute values for inter-day precision (reproducibility) were 9.3%, 11.2% and 13.6% respectively. The results showed satisfactory intra- and inter-day precision of the analytical procedure.

Table 8.11: Recoveries and precision of Pseudo-SRM method for determination of benzosulfonamides in wastewater

Compound	**SPE recovery**			**Overall recovery**			**Intra-day precision RSD (%)**			**Inter-day precision RSD (%)**
	0.5 µg L^{-1}	**1.0 µg L^{-1}**	**5.0 µg L^{-1}**	**0.5 µg L^{-1}**	**1.0 µg L^{-1}**	**5.0 µg L^{-1}**	**0.5 µg L^{-1}**	**1.0 µg L^{-1}**	**5.0 µg L^{-1}**	**0.5 µg L^{-1}**
BSA	118	84	105	38	21	40	4.1	6.7	5.1	9.3
o-TSA	101	115	106	38	23	37	2.7	6.8	5.9	11.2
p-TSA	83	95	94	35	35	54	0.4	10.2	6.3	13.6

8.6.3. Matrix Effects

The matrix factor of the method for every target compound was quantified by comparing the slope of the matrix match curve with the slope of the standard solution curve, and consequently the matrix effect (%) was calculated and presented in Fig. 8.1. It was evident from Fig. 8.1 that ion suppression occurred for all target compounds. Comparing the matrix effects for each compound in both tap water and wastewater, it was obvious that the ion suppression phenomenon occurred at a higher level in wastewater than in tap water due to the presence of more interfering components. Standard addition method was therefore incorporated to quantify the compounds.

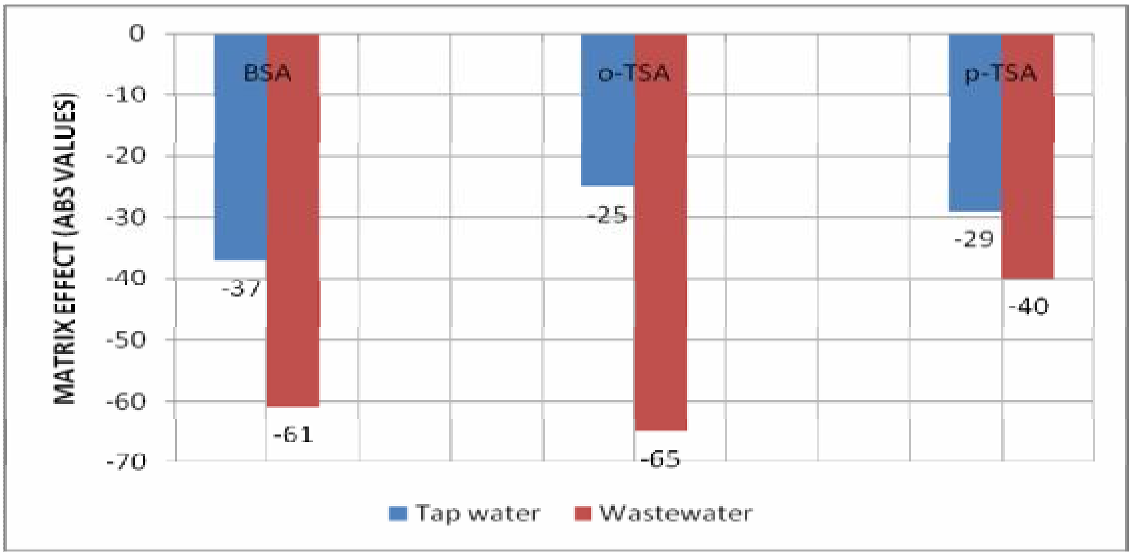


Fig. 8.10: Matrix effects (%) for benzosulfonamides in the dissolved phase of wastewater and tap water

8.4. Environmental Levels of benzosulfonamides

The results of environmental levels at which the benzosulfonamides appear in wastewater influent and effluent from STP of Athens are treated and discussed in detail in section 9.2.

CHAPTER 9

OCCURRENCE AND FATE OF BENZOTRIAZOLES, BENZOTHIAZOLES AND BENZOSULFONAMIDES IN SEWAGE TREATMENT PLANT OF ATHENS

The understanding of the occurrence, removal and fate of emerging contaminants in different wastewater treatment processes is a matter of high importance for ecosystem protection and wastewater reuse. The amounts of the compounds found in effluent or sludge depend on the removal efficiency of the treatment plant and the physicochemical properties of the compounds. In particular, the effluent and sludge concentrations are important from the environmental point of view since the compounds eventually find their way into the environment through discharges of treated wastewater (effluents) into rivers or disposal of sludge for agricultural and forest land.

9.1. Occurrence and fate of benzotriazoles and benzothiazoles in Athens STP

In order to study the occurrence and distribution of benzotriazoles and benzothiazoles in raw wastewater, treated wastewater and sewage sludge, seasonal and daily variations in concentration levels, distribution between dissolved and particulate phase were studied. Mass balances were applied to investigate the role of sorption and biotransformation on the removal of the compounds and to quantify the contribution of primary and secondary sedimentation on their elimination.

9.1.1. Sampling and Analysis of benzotriazoles and benzothiazoles

Wastewater influent, effluent and sewage sludge were collected from Athens STP in two different sampling campaigns and analyzed as described in sections 7.2.1 - 7.2.4. In brief, two sampling campaigns were performed in December 2010 (05.12.10-11.12.10) and April 2011 (03.04.11-09.04.11). Twenty four-hour flow-proportional composite samples of sewage influents and secondary effluents as well as grab dewatered sludge samples were taken in 7 consecutive days during each sampling campaign. No rain events were noticed in both sampling periods. For the calculation of (K_d values), samples of primary sludge and secondary sludge were taken in 5 consecutive days during the second sampling campaign and concentrations of the target compounds were analyzed in the dissolved and particulate phase. The analysis of benzotriazoles and benzothiazoles was carried out by LC-MS/MS after extraction and preconcentration by SPE (sections 7.2.2 – 7.2.4).

9.1.2. Calculations of removal efficiency, distribution coefficients and mass balances

The removal efficiency of the compounds during wastewater treatment was calculated from the mass flows of each compound in raw sewage water (M_{inf}) and secondary effluent (M_{eff}) according to Equation 9.1:

$$Removal\ (\%) = \frac{(M_{inf}) - (M_{eff})}{(M_{inf})} \text{ x } 100 \qquad (9.1)$$

The mass load of target compounds that was lost in STP due to the sum of all transformation processes ($W_{lost,STP}$) was calculated according to Equation 9.2:

$$W_{lost,STP} = (Q_{inf} \text{ x } C_{inf}) - (Q_{eff} \text{ x } C_{eff}) - (M_{dew.sludge} \text{ x } C_{dew.sludge}) \qquad (9.2)$$

where Q_{inf} and Q_{eff} are the flow rates (m^3 d^{-1})of influent wastewater and effluent wastewater respectively, C_{inf} and C_{eff} are total concentrations (dissolved + particulate) of compounds in influent and effluent wastewater (mg m^{-3}), $M_{dew.sludge}$ is the mass of dewatered sludge (Kg dw d^{-1}) and $C_{dew.sludge}$ the compound concentration in dewatered sludge (mg Kg^{-1} dw).

The mass load of compounds lost in activated sludge process ($W_{lost,AS}$) was calculated using Equation 9.3:

$$W_{lost,AS} = (Q_{inf} \text{ x } C_{inf}) - (W_{prim.sludge}) - (Q_{eff} \text{ x } C_{eff}) - (W_{sec.sludge}) \qquad (9.3)$$

where $W_{prim.sludge}$ and $W_{sec.sludge}$ are the mass outputs (mg d^{-1}) of compounds in primary and secondary sludge respectively. These values were calculated using Equations 9.4 and 9.5:

$$W_{prim.sludge} = (Q_{prim.sludge} \text{ x } C_{inf,w}) + \frac{(M_{prim.sludge} \text{ x } K_{d(prim.sludge)} \text{ x } C_{inf,w})}{1000} \qquad (9.4)$$

$$W_{sec.sludge} = (Q_{sec.sludge} \text{ x } C_{eff,w}) + \frac{(M_{sec.sludge} \text{ x } K_{d(sec.sludge)} \text{ x } C_{eff,w})}{1000} \qquad (9.5)$$

where $Q_{prim.sludge}$ and $Q_{sec.sludge}$ are the flow rates (m^3 d^{-1}) of primary and seconadary sludge, $C_{inf,w}$ and $C_{eff,w}$ the dissolved concentrations(mg m^{-3}) of the compounds in influents and effluents, $M_{prim.sludge}$ and $M_{sec.sludge}$ are the mass outputs of primary and secondary sludge (Kg d^{-1} dw) while $K_{d(prim.sludge)}$ and $K_{d(sec.sludge)}$ are the solid –water distribution coefficients(L Kg^{-1}) in primary and secondary sludge respectively.

The values of K_d were estimated for primary and secondary sludge using Equation 9.6:

$$K_d = \frac{C_s}{C_w} \qquad (9.6)$$

9.1.3. Occurrence of benzotriazoles and benzothiazoles in wastewater

During the two sampling campaigns, twenty eight samples were collected altogether: fourteen influent samples and fourteen effluent samples. The data obtained are presented in Table 9.1. For the untreated wastewater all the benzotriazoles (except XTR) and benzothiazoles (except 2-Amino-BTH) were detected in all samples. For most of the compounds, concentrations determined in this study fall into the range reported in the literature or even lower/higher in few cases [48,209,224,228,229,230,237,245,246,250,251]. It should be noted that in this work, the employed analytical method for the determination of these compounds does not separate the isomers of 4-Me-1H-BTR and 5-Me-1H-BTR (Chapter 7) and therefore were quantified together as TTR. Lower mean concentrations were observed for 1H-BTR in this study compared to some other studies [12,14,152,230] whereas much higher concentrations were detected in this study for TTR [13,153]. However, higher maximum concentrations up to 100 µg L^{-1} and 8 µg L^{-1} in wastewater were detected for 1H-BTR in Switzerland and the Netherlands respectively [153,223]. Though the concentration of 2-Me-S-BTH was noticeably high (maximum concentration of 4522 ng L^{-1} in influent wastewater), Pena et al. also found a much higher concentration up to 13,200 ng L^{-1} for the compound in raw wastewater.

Considering seasonal variation, there was no stastistically significant differences in the concentration levels of the compounds between different sampling campaigns indicating a rather uniform use during the year. Statistically significant higher concentrations were observed only for BTR and XTR in the first campaign and for TTR and 2-OH-BTH in the second campaign.

Table 9.1: Occurrence of BTRs and BTHs in Influent and secondary effluent wastewater (in ng L^{-1}), (n=14)

Compound			**Influent**					**Effluent**				
	LOD	**LOQ**	**Frequency (%)**[a]	**Mean**	**Median**	**Min**	**Max**	**Frequency (%)**[a]	**Mean**	**Median**	**Min**	**Max**
1H-BTR	0.77	2.5	100	1175	829	516	2632	100	300	325	14	548
1-OH-BTR	3.1	10	100	240	224	129	448	53	47	38	<LOD	182
TTR	0.13	0.42	100	7270	6307	2833	15971	100	3028	2824	352	5773
XTR	0.34	1.1	64	11	9	<LOD	27	79	11	9	<LOD	30
BTH	17	55	100	1096	1117	528	1452	100	254	161	89	616
2-OH-BTH	4.6	15	100	503	420	256	908	100	251	264	94	514
2-Amino-BTH	4.2	14	14	<LOD	<LOD	<LOD	18	21	<LOQ	<LOQ	<LOD	31
2-Me-S-BTH	0.085	0.28	100	719	398	219	4522	100	112	86	36	368

[a] Frequency of detection: Percentage of samples with concentration higher than limit of quantification of the method

Comparison of concentrations determined between different days of each sampling campaign showed that there was no significant correlation between specific day of the week and higher concentrations. However, the ratio of maximum to minimum concentration during a week was only significant for 2-Me-S-BTH in the first sampling campaign where a ratio higher than 10 was calculated (Fig 9.1) indicating that occasional use and/or no continuous sources could be responsible for its release into the sewerage system. For the remaining BTRs and BTHs, the concentrations were much more stable during the week (Fig 9.1) probably due to their wide use in several every-day products.

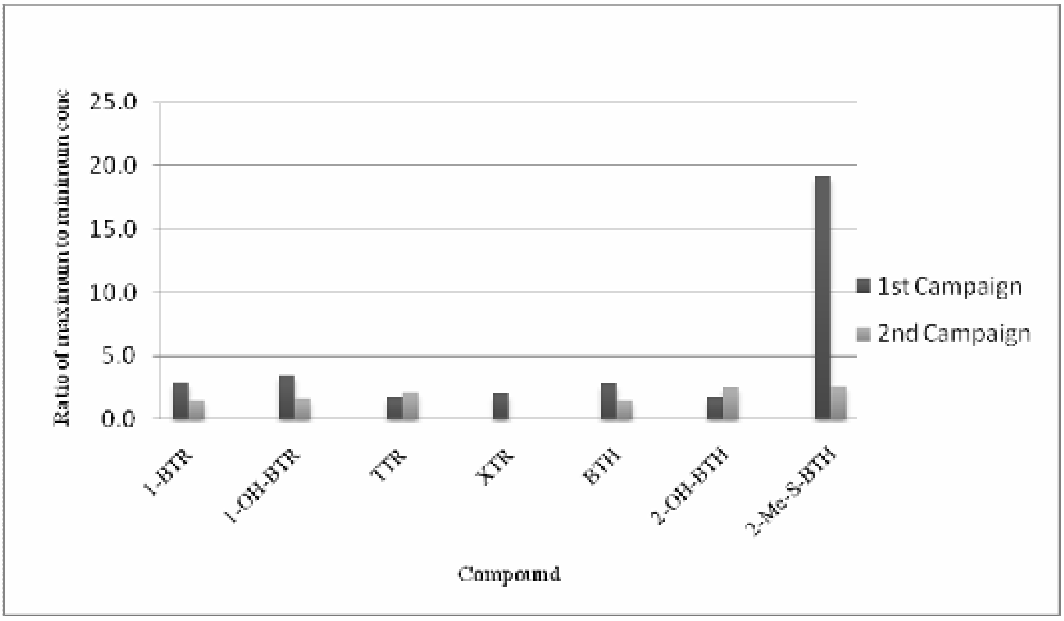


Fig 9.1: Ratio of maximum to minimum concentration of individual BTRs and BTHs during a week. Data are given for both sampling campaigns and for the compounds that all concentrations were above the method LOQ.

Investigation of the distribution of the compounds between dissolved and particulate phase of influent wastewater showed that the major part of BTRs and BTHs were in the dissolved phase (Fig 9.2). Nevertheless, even though the compounds are known to be highly or moderately polar (Tables 3.1 and 3.2), a significant part, ranging up to 14% for 1H-BTR and 20% for BTH, was still found in the particulate phase. These findings therefore, reflect the importance of analysis of the compounds both in the dissolved phase and particulate phase of influent wastewater in order to avoid their underestimation in STPs.

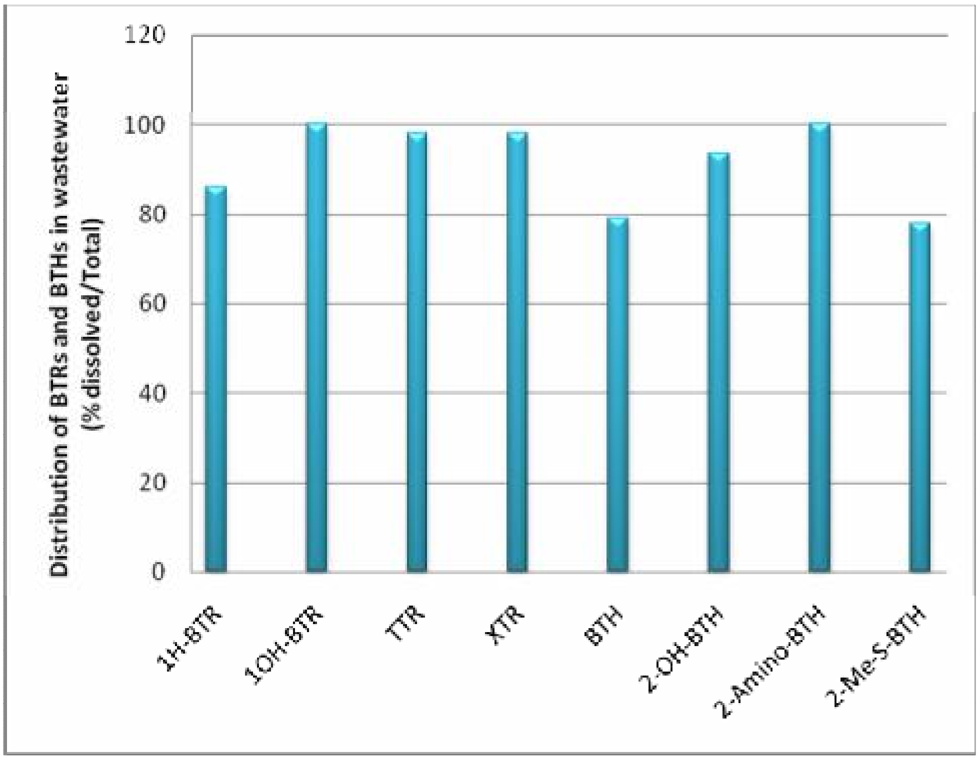


Fig. 9.2: Distribution of BTRs and BTHs between dissolved and particulate phase in influent wastewater.

Regarding treated wastewater (effluents), the highest mean concentrations of 3028 ng/L for TTR and 254 ng L^{-1} for BTH were found for both classes BTRs and BTHs respectively. Calculation of the average daily loads of the compounds showed that almost 2500 g d^{-1} of BTRs, of which 90% is due to TTR, are discharged via treated wastewater to the aquatic environment. TTR is a technical mixture of 4-methyl benzotriazole and 5-methyl benzotriazole with low acute toxicity on aquatic organisms in the range of mg L^{-1}. Very little information about its chronic toxicity is presently available [44,181]. Having in mind the amounts of these compounds that are discharged into the aquatic environment as well as their toxicological characteristics, the adoption of advanced wastewater treatment processes seems to be necessary to achieve further removal before final wastewater disposal.

9.1.4. Occurrence of benzotriazoles and benzothiazoles in sewage sludge

A total of fourteen dewatered sludge samples were collected from STP of Athens during this study. The results are demonstrated in Table 9.2. The highest mean concentrations among the BTRs and BTHs were observed for TTR (146 ng g^{-1} dw) and 2-OH-BTH (52 ng g^{-1} dw) respectively.

Table 9.2: Occurrence of BTRs and BTHs in sewage sludge (in ng g^{-1} dw), (n=14)

Compound			**Sewage Sludge**				
	LOD	**LOQ**	**Frequency (%)**[a]	**Mean**	**Median**	**Min**	**Max**
1H-BTR	0.69	2.3	100	114	87	76	412
1-OH-BTR	7.6	25	0	<LOD	<LOD	<LOD	<LOD
TTR	0.06	0.21	100	146	154	72	205
XTR	0.26	0.85	43	4	<LOD	<LOD	22
BTH	13	41	93	86	82	<LOD	174
2-OH-BTH	4	13	100	189	231	33	312
2-Amino-BTH	10	34	0	<LOD	<LOD	<LOD	<LOQ
2-Me-S-BTH	0.042	0.14	100	52	54	23	77

[a] Frequency of detection: Percentage of samples with concentration higher than limit of quantification of the method.

The concentrations of BTRs in sludge were in the same range with those reported in the literature [227,228]. Until the period of this work, no systematic study on the occurrence of benzothiazoles in dewatered sewage sludge has been undertaken. Wick et al recently detected some BTHs in activated sludge samples taken from aerated tank of a German STP [251].

9.1.5. Removal of benzotriazoles and benzothiazoles during wastewater treatment processes

The removal efficiency of the compounds during wastewater treatment and their fate in STP were investigated using Equations 9.1 and 9.2 respectively. The results are shown in Fig. 9.3. According to the results, three of the compounds presented high removal (> 70%), three partially removed (30 -70%) while one was slightly removed.

The compounds with high removal efficiencies were BTH, 2-Me-S-BTH and 1-OH-BTR. High removal efficiency has also been reported for BTH [13]. So far, there are limited and contradictory observations for the removal of 2-Me-S-BTH during wastewater treatment [208,224] as it is considered to be formed via methylation of mercaptobenzothiazole during wastewater treatment system and in the sewerage system [208].

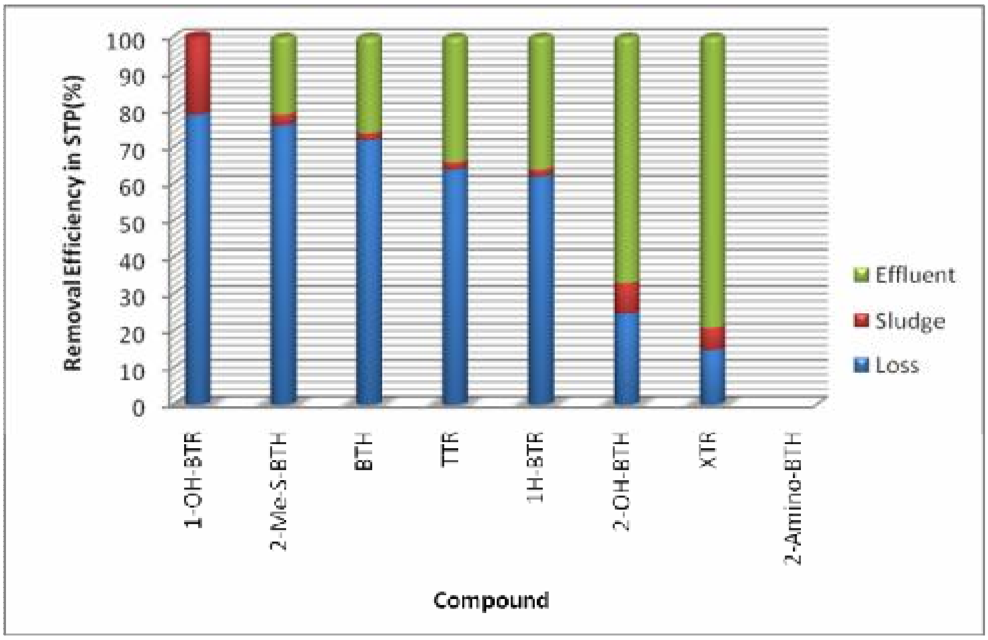


Fig. 9.3: Removal efficiency of BTRs and BTHs during wastewater treatment and their fate in STP

Regarding 1-OH-BTR, to the best of our knowledge this study happens to be the first where its removal was systematically studied in a STP.

The compounds that were partially removed (30- 70%) were TTR, 1H-BTR and 2-OH-BTH. The extent of removal of 1H-BTR and TTR in this study is in agreement with the removal efficiencies earlier reported for these compounds [149,223]. XTR was only slightly removed in this study.

9.1.6. Fate of BTRs and BTHs during primary and secondary wastewater treatment

In order to clarify the role of primary and secondary sedimentation of the compounds, sludge samples were taken from primary and secondary clarifiers and K_d values calculated for the compounds that presented significant removal during wastewater treatment (Fig. 9.3). The results are given in Table 9.3.

The K_d values for BTRs and BTHs were much low, not exceeding 227 L Kg^{-1} for (2-OH-BTH and in secondary sludge). Again, to the best of our knowledge, this is the first study in which the study of distribution coefficients of BTRs and BTHs is undertaken. The calculated K_d values were used in the corresponding mass balances for studying their removal during primary and secondary treatment.

For BTRs and BTHs, the role of sorption seems to be of minor importance (Fig 9.4). Benzotriazole are polar compounds (Table 3.1) and at neutral pH are expected to be in non-ionic form

Table 9.3: Distribution coefficients values K_d (in L Kg^{-1}) for BTRs and BTHs in primary and secondary sludge.

Compound	**Primary Sludge**			**Secondary Sludge**		
	C_w (ng L^{-1})	**C_s (ng g^{-1})**	**K_d (L Kg^{-1})**	**C_w (ng L^{-1})**	**C_s (ng g^{-1})**	**K_d (L Kg^{-1})**
1H-BTR	2882 ± 1419	16 ± 5	7 ± 3	391 ± 224	32 ± 7	133 ± 104
1-OH-BTR	<LOD[a]	<LOD	NC[b]	<LOD[a]	<LOD	NC
TTR	19324 ± 6761	14 ± 2	0.8 ± 0.2	5623 ± 858	31 ± 15	6 ± 4
BTH	10705 ± 5897	29 ± 10	3 ± 2	121 ± 110	24 ± 6	147 ± 63
2-OH-BTH	6880 ± 4428	63 ± 44	27 ± 23	193 ± 58	39 ± 12	227 ± 120
2-Me-S-BTH	1115 ± 586	22 ± 10	21 ± 3	211 ± 57	39 ± 21	181 ± 56

[a] <LOD, not detected. [b] NC, not calculated.

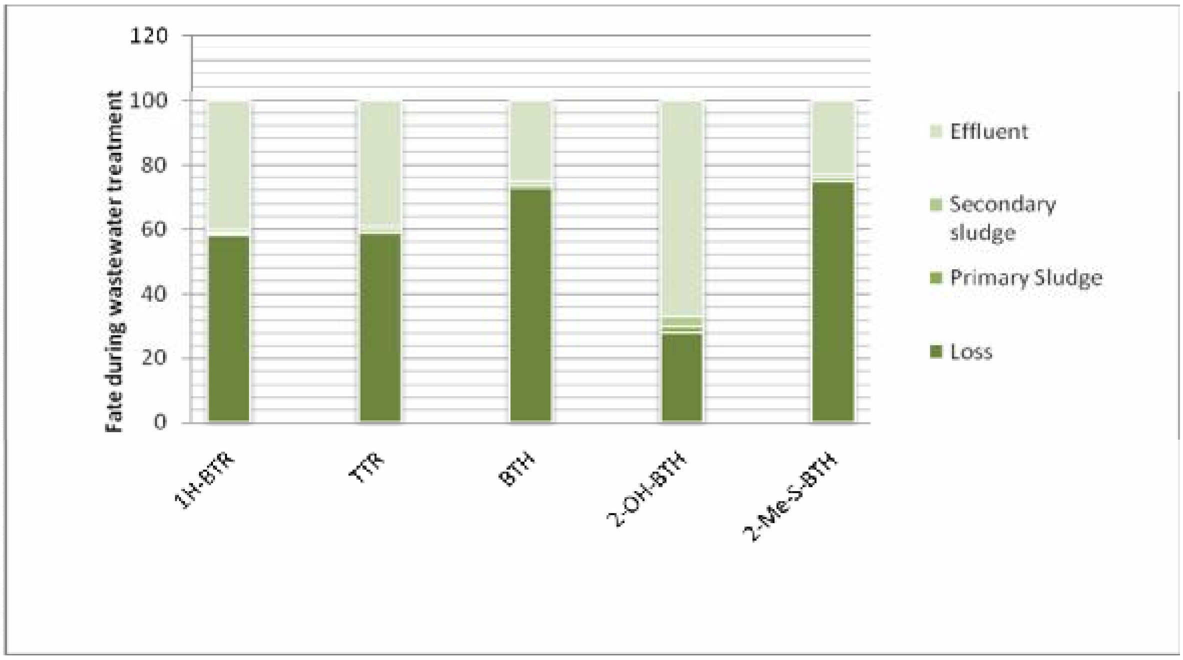


Fig. 9.4: Fate of BTRs and BTHs during wastewater treatment

Figure 9.4 shows that a significant part of all the compounds was lost through biologigal wastewater treatment ranging from 28% for 2-OH-BTH to 75% for 2-Me-S-BTH. Due to the fact that none of the compounds is very volatile and photodegradation seems unlikely to be of high importance in the conditions usually found in biological reactors (high depth, existence of suspended solids and turbid liquor), the major mechanism responsible for their removal is biotransformation/biodegradation. So far, there are some laboratory- scale studies which have reported the ability of biological treatment to remove these compounds from wastewater [212,261]. However, the existing knowledge on the biotransformation pathways of the compounds is limited which requires more studies in the future.

9.2. Occurrence and Removal of benzosulfonamides in wastewater from Athens STP

For the purpose of investigation of the occurrence of benzosulfonamides in wastewater, fourteen samples comprising seven wastewater influent and seven wastewater effluent samples were collected from STP of Athens during a sampling campaign in April, 2012. The samples were taken in seven consecutive days (24-hour composite flow), treated and analyzed as described in sections 8.51-8.53. The results are presented in Table 9.4.

All the benzosulfonamides (BSA, o-TSA and p-TSA) were detected in all the wastewater influent samples. Regarding wastewater effluents, the two isomers (o-TSA and p-TSA) were found in all samples, while BSA was detected in only 43% of the effluent samples giving a hint of its removal during wastewater treatment processes. Among the three benzosulfonamides, highest concentrations were observed for p-TSA both in the influent and effluent samples. The concentration levels in wastewater influents ranged from 734 -1473 ng L^{-1} for p-TSA, 349- 664 ng L^{-1} for o-TSA and 387 – 1228 ng L^{-1} for BSA. In wastewater effluents, concentration levels ranged from 353-937 ng L^{-1} for p-TSA, 442 – 935 ng L^{-1} for o-TSA and <LOD to 339 ng L^{-1} for BSA.

Until the period of this study, very limited studies have focused on the determination of these compounds in wastewater. To the best of our knowledge, only Richter et al determined the three compounds (BSA, o-TSA and p-TSA) simultaneously in wastewater. Jover et al included only p-TSA among the benzosulfonamides in their study. Consequently, there are very limited data to compare our results with. In agreement with our study, Richter et al also found highest concentrations for p-TSA in influent samples [154,215]. In composite wastewater influent and effluent samples that were collected over 24 hour, concentrations of p-TSA ranged from 5000 -8000 ng L^{-1} in the influent samples and from 500-1000 ng L^{-1} in the effluent samples [154]. Noticeably, these concentrations of p-TSA in the effluents were much higher than the levels obtained in this study.

Table 9.4: Occurrence of BSAs in Influent and secondary effluent wastewater (in ng L^{-1}), (n=7)

Compound			**Influent**					**Effluent**				
	LOD	**LOQ**	**Frequency (%)**[a]	**Mean**	**Median**	**Min**	**Max**	**Frequency (%)**[a]	**Mean**	**Median**	**Min**	**Max**
BSA	88	290	100	673	652	365	1228	43	<LOQ	<LOQ	<LOD	339
o-TSA	89	300	100	484	441	349	664	100	648	639	442	935
p-TSA	11	35	100	998	1074	734	1473	100	627	520	353	937

[a] Frequency of detection: Percentage of samples with concentration higher than limit of quantification of the method

However, the concentrations of p-TSA in the effluents (through which most organic micropollutants are discharged into the aquatic environment) were at similar levels as the ones obtained in our study (Table 9.4). Jover et al obtained a much lower concentration of 94 ng L^{-1} for p-TSA in effluent from an industrial WWTP [152]. In this work, although o-TSA was detected in lower amounts than p-TSA in the influents, the concentrations of o-TSA in all the effluents exceeded the concentrations in the corresponding influent samples. This same phenomenon of formation of o-TSA during treatment processes was also observed by Richter et al [154,215] and suggested that a site specific process might be responsible for the formation of o-TSA during wastewater treatment. The concentration levels of o-TSA obtained in this study (median concentration of 442 ng L^{-1}) lie within the same order of magnitude (median concentration of < 500 ng L^{-1}) as detected by Richter et al. [154,215].

BSA was found in wastewater influents at concentration levels comparable to p-TSA (365-1228 ng L^{-1}) but much lower concentration levels than p-TSA appeared in the effluent samples.

The trends of removal rates of the compounds during wastewater treatment over the week of sampling are displayed in Fig. 9.5. Effluent concentrations of o-TSA during the week were higher than corresponding concentrations in influent. o-TSA was noticeably formed during wastewater treatment processes (Fig. 9.5). Richter et al also observed the formation of o-TSA during wastewater treatment [215]. This process of formation of o-TSA is presumably caused by biodegradation or bioconversion of a sulfonamide with higher molecular weight [257]. Richter and co-workers assumed in their study that BSA is a metabolite of a sulfonamide because it was formed during wastewater treatment and they suggested that the formation was due to biodegradation or bioconversion of a sulfonamide with a higher molecular weight.

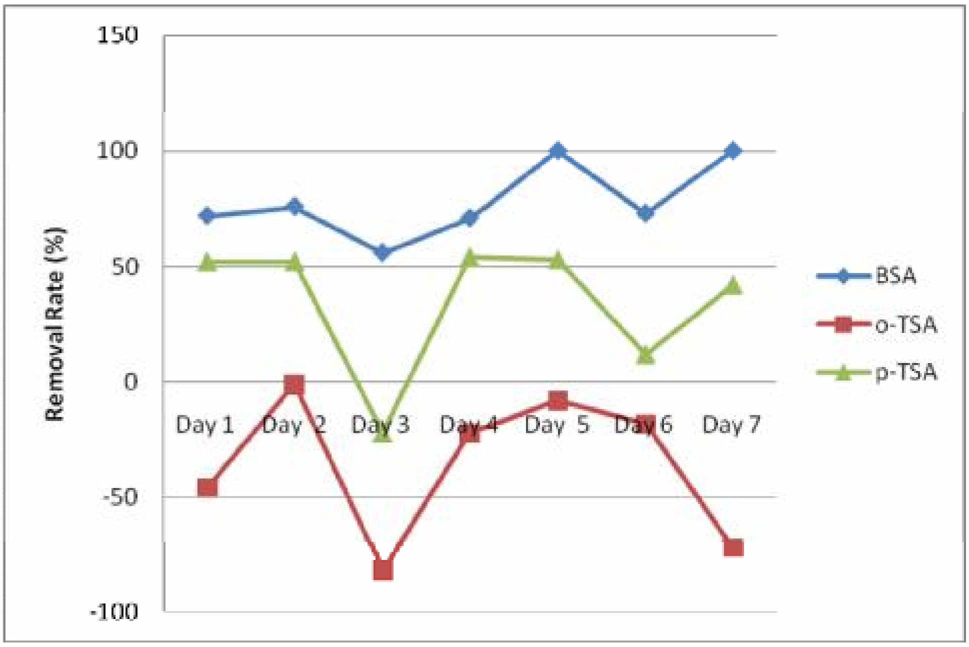


Fig 9.5: Trends of removal of BSAs over the week during wastewater treatment

On the contrary, in our studies, BSA removal was significant through out the week indicating that the compound was largely eliminated during treatment. This significant removal of BSA does not undermine its possible formation during treatment (as suggested by Richter et al), it is possible that the rate of biotransformation of a higher molecular weight sulfonamide to BSA was slower than the rate of BSA biodegradation [187] or absence of the higher molecular weight sulfonamide, hence, rendering its removal significant. In this study, p-TSA was partially removed during wastewater treatment, thus serving as a potential source of entry into surface water and groundwater compartments.

It should be noted that apart from the physicochemical properties of organic micropollutants, there are other STP operational factors that could affect their removal during wastewater treatment [244], these factors are site specific and may lead to variation in rates of removal of a micropollutant from one STP to another. Some of the factors include sludge retention time, biomass concentration, temperature and pH value during treatment.

Moreover, more studies have to be carried out to investigate the fate of these compounds (BSAs) during wastewater treatment. To this effect, future investigation should include the analysis of particulate phase of wastewater and sludge for comprehensive study of the role of sorption on the removal of the compounds even though they are hydrophilic (Table 3.3) and their sorption to solid matrices might seem negligible.

Finally, the fact that p-TSA and o-TSA are still present in high concentrations even in the effluent samples raises concerns. Further investigations on the processes controlling their occurrence, behaviour and degradation during wastewater treatment are therefore essential even though very little is known at present about their toxicological relevance.

CHAPTER 10

CONCLUSIONS

Our current study focused on the development of analytical methods for the determination of three classes of emerging contaminants in wastewater and sewage sludge by liquid chromatography-tandem mass spectrometry; and application of the developed methods in studying the occurrence, removal rate and fate of these compounds in the sewage treatment plant of Athens, Greece.

An integrated method based on LC-(ESI+)MS/MS was developed for the simultaneous determination of four benzotriazoles (1H-BTR, 1-OH-BTR, TTR and XTR) and four benzothiazoles (BTH, 2-OH-BTH, 2-Amino-BTH and 2-Me-S-BTH) in wastewater (dissolved phase and particulate phase) and sewage sludge. A solid-phase extraction protocol was optimized for the extraction of the target compounds from dissolved phase of wastewater while an ultra-sonication procedure was employed for the extraction of the compounds from sludge and particulate phase of wastewater. The analytical performance parameters of the developed method, in terms of linearity, recoveries, limits of detection (LODs) and limits of quantification (LOQs) showed its suitability for accurate identification and quantification of the target compounds in wastewater and sewage sludge. Limits of detection of the compounds in this study were at similar levels or lower than those reported in previous studies. This analytical approach was proven to be highly selective and sensitive due to the incorporation of a tandem mass detector and the incorporation of a suitable chromatographic system. The developed method was successfully applied for the determination of the target compounds in wastewater and sludge samples from the sewage treatment plant of Athens, Greece.

Secondly, an analytical methodology was also developed for quantification of three benzosulfonamides (benzenesulfonamide, o-toluenesulfonamide and p-toluenesulfonamide) in wastewater based on pseudo-selected reaction monitoring (pseudo-SRM) mass spectrometry. A solid phase extraction procedure was developed for preconcentration and extraction of the compounds from wastewater. Extracts were analyzed with LC-(ESI–)MS/MS in Pseudo-SRM mode. Method limits of detection (LODs) of 0.01 µg L^{-1} for p-toluenesulfonamide and 0.09 µg L^{-1} for both benzenesulfonamide and o-toluenesulfonamide were obtained in wastewater. To the best of our knowledge, this is the first study where limits of quantification (LOQs) were really determined for the compounds in wastewater based on LC-MS/MS. Richter et al. [154] could not determine LOQs of the compounds in wastewater samples because of their ubiquitous occurrence in the matrices and therefore assumed the LOQs determined for drinking water to be valid also for wastewater. Considering the ubiquitous occurrence of these compounds (BSAs) in wastewater samples, the LOD and LOQ values obtained in this study were low enough for adequate and accurate quantification of the compounds in the matrices. The method was employed for the determination of the compounds in wastewater from the sewage treatment plant of Athens, Greece.

Finally, the occurrence of the target benzotriazoles, benzothiazoles and benzosulfonamides in raw wastewater, treated wastewater and sludge samples from sewage treatment plants of Athens; as well as their removal and fate during treatment processes were systematically studied. Distribution coefficients were estimated, for the first time, for benzotriazoles and benzothiazoles in primary and secondary sludge. Mass balances were applied to investigate the role of sorption and biotransformation on the removal of the compounds and to quantify the contribution of primary and secondary sedimentation on their elimination.

For the untreated wastewater all the benzotriazoles (except XTR) and benzothiazoles (except 2-Amino-BTH) were detected in all samples. For most of the compounds, concentrations determined in this study fall into the range reported in the literature or even lower/higher in few cases. Investigation of the distribution of the compounds between dissolved and particulate phase of influent wastewater showed that a significant part, ranging up to 14% for 1H-BTR and 20% for BTH, was found in the particulate phase thus, necessitating their analysis also in the particulate phase to avoid their underestimation in wastewater.

In sewage sludge, the highest mean concentrations among the BTRs and BTHs were observed for TTR (146 ng g^{-1} dw) and 2-OH-BTH (52 ng g^{-1} dw) respectively. Until the period of this work, no systematic study on the occurrence of benzothiazoles in dewatered sewage sludge had been undertaken. Wick et al only detected some BTHs in activated sludge samples taken from aerated tank of a German STP [251].

During wastewater treatment, 1-hydroxybenzotriazole, benzothiazole and 2-methythio-benzothiazole were highly removed whereas tolyltriazole, 1H-benzotriazole and 2-hydroxybenzothiazole were only partially removed. Biodegradation/biotransformation was the major mechanism responsible for the removal of benzotriazoles and benzothiazoles during wastewater treatment.

Among the benzosulfonamides, highest concentrations were found for p-toluenesulfonamide (up to 1473 ng L^{-1}) in both influent and effluent wastewater samples. Benzenesulfonamide was largely removed, p-toluenesulfonamide was partially removed and o-toluenesulfonamide was formed during wastewater treatment.

Moreover, more studies should be carried out to investigate the fate of these compounds (BSAs) during wastewater treatment. To this effect, future investigation should include the analysis of particulate phase of wastewater and sludge for comprehensive study of the role of sorption on the removal of the compounds even though they are hydrophilic and their sorption to solid matrices might seem negligible.

Having in mind the amounts of these compounds (benzotriazoles, benzothiazoles and benzosulfonamides) that are discharged into the aquatic environment as well as their toxicological characteristics, the adoption of advanced wastewater treatment processes seems to be necessary to achieve further removal before final wastewater disposal. The existing knowledge on the biotransformation pathways of these compounds of emerging concern is limited and requires more studies in the future.

Finally, this study underscores the need for thorough control and environmental monitoring of the target benzotriazoles, benzothiazoles and benzosulfonamides in the aquatic environment.

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ABBREVIATIONS-ACRONYMS

List of Abbreviations

H-BTR	1H-Benzotriazole
1H-BTR-d4	Benzotriazole-d4
1-OH-BTR	1-hydroxybenzotriazole
2-Amino-BTH	2-amino-benzothiazole
2-Me-S-BTH	2-methylthio-benzothiazole
2-Me-SH-BTH	2-mercaptobenzothiazole
2-OH-BTH	2-hydroxybenzothiazole
4-Me-1H-BTR	4-methylbenzotriazole
4TTR	4-tolyltriazole
5-Cl-BTR	5-Chlorobenzotriazole
5-Me-1H-BTR	5-methylbenzotriazole
5TTR	5-tolyltriazole
XTR	Xylyltriazole/5,6-dimethylbenzotriazole
ACN	Acetonitrile
ADAFs	Aircraft deicing and antifreeze fluids
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
APPI	Atmospheric Pressure Photo Ionization
ASE	Accelerated solvent extraction
ATR-d5	Atrazine-d5
BFRs	Brominated Flame Retardants
BOD	Biochemical Oxygen demand
BSA	Benzenesulfonamide
BSAs	Benzosulfonamides
BTH	Benzothiazole
BTHs	Benzothiazoles
BTR	Benzotriazole
BTRs	Benzotriazoles
CAS	Conventional activated sludge
COD	Chemical Oxygen Demand
DDT	Dichlorodiphenyltrichloroethane
DLLME	Dispersive Liquid-liquid microextraction
ECs	Emerging Contaminants
EI	Electron Impact
ESI	Electrospray Ionization
EU-WFD	European Union-Water Framework Directive

FID	Flame Ionization Detector
Fl	Fluorescence
GC	Gas chromatography
GC x GC-TOF/MS	Two dimensional gas chromatography-time of flight mass spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography-Tandem Mass Spectrometry
GC-MSD	Gas chromatography-mass selective detector
H_2O	Water
HCl	Hydrochloric acid
HCOOH	Formic acid
HPLC	High Performance Liquid Chromatography
HPLC-UV	High performance Liquid Chromatography-ultraviolet detection
HRMS	High Resolution Mass Spectrometry
ICP-MS	Inductive Coupled Plasma-Mass Spectrometry
IL	Ionic liquid
IS	Internal standard
IT	Ion Trap
LC	Liquid chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of detection
LOEC	Low observable effect concentration
LOQ	Limit of Quantification
LPME	Liquid Phase Microextraction
LSE	Liquid solid extraction
LTQ-FT	Linear ion trap-Fourier Transform
m/z	Mass to charge ratio
MBR	Membrane bioreactor system
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^2	Tandem mass spectrometry
NMs	Nanomaterials
NOEC	No observable effect concentration
NPs	Nanoparticles
o-TSA	Ortho-toluenesulfonamide
PAHs	Polycyclic Aromatic Hydrocarbons
PBBs	Polybrominated Biphenyls
PBDE	Polybrominated Diphenyl Ether

PCB	Polychlorinated Biphenyls
PFCs	Perfluorinated Compounds
PFOA	Perfluorooctanoic Acid
PFOS	Perfluoro octane sulfonate
PLE	Pressurized liquid extraction
PNEC	Predicted no effect concentration
POPs	Priority Organic Pollutants
PPCPs	Pharmaceuticals and Personal Care Products
Pseudo-SRM	Pseudo-selected reaction monitoring
p-TSA	Para-toluenesulfonamide
QC	Quality Control
QqQ	Triple Quadrupole
Rec	Recovery
Ref.	Reference
RNA	Ribonucleic acid
RP-LC	Reversed phase-liquid chromatography
RRT	Relative retention time
RSD	Relative standard deviation
RT	Retention time
SBSE	Stir bar sorptive extraction
SIM	Selective ion mode
SNR	Signal to noise ratio
SPE	Solid Phase Extraction
SRM	Selected reaction monitoring
STP	Sewage Treatment Plant
TOF	Time-of-Flight
TTR	Tolyltriazole
USGS	United State Geological Survey
UV	Ultraviolet
WWF	World Wide Fund
WWTP	Wastewater Treatment Plant

APPENDIX 1

Tables and Figures for Chapter Seven

TableA7.1: Average (n=3) retention times (RT) BTRs and BTHs with XTerra MS C18 column.

Compounds	**RT (min)**
1H-BTR	5.83
1-OH-BTR	4.27
TTR	7.15
XTR	8.08
BTH	8.87
2-OH-BTH	8.18
2-Me-S-BTH	10.97
2-Amino-BTH	4.41

Table A7.2: Retention Times (RT), Relative Retention Times (RRT), and Ion Ratio % of BTRs and BTHs in dissolved phase of wastewater samples

Compounds	**RT ± RSD % (n=6)**			**RRT with ATR-D5 ± RSD % (n=6) / RRT with 1H-BTR-d4 ± RSD % (n=6)**			**Ion Ratio % ± RSD % (n=6)**		
	Low level	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**
1H-BTR	6.72±0.21	6.73±0.19	6.74±0.07	0.64±0.26 / 1.01±0.39	0.64±0.19 / 1.01±0.31	0.64±0.07 / 1.01±0.43	46.57±3.12	45.31±3.12	45.27±2.25
1-OH-BTR	5.32±0.59	5.32±0.45	5.32±0.19	0.51±0.62 / 0.80±0.63	0.51±0.46 / 0.80±0.28	0.51±0.19 / 0.80±0.53	13.06±12.41	12.12±14.07	9.27±13.98
TTR	7.99±0.07	8±0.07	8.01±0.10	0.76±0.02 / 1.2±0.16	0.76±0.13 / 1.2±0.25	0.76±0.10 / 1.2±0.43	23.4±0.76	23.32±3.07	23.42±1.8
XTR	8.97±0.06	8.96±0.11	8.96±0.16	0.85±0.12 / 1.35±0.27	0.85±0.15 / 1.34±027	0.85±0.16 / 1.35±0.49	68.44±2.4	64.97±5.14	63.23±3.41
BTH	9.64±0.06	9.64±0.1	9.65±0.10	0.92±0.11 / 1.45±0.26	0.92±0.10 / 1.45±0.23	0.92±0.10 / 1.45±0.46	39.56±11.77	40.09±17.86	44.22±10.12
2-OH-BTH	9.15±0.15	9.15±0.14	9.15±0.16	0.87±0.2 / 1.38±0.34	0.87±0.22 / 1.37±0.21	0.87±0.16 / 1.37±0.44	111.05±22.39	92.89±7.52	92.39±4.4

2-Me-S-BTH	11.71±0.08	11.71±0.04	11.71±0.05	1.12±0.08 / 1.76±0.25	1.11±0.06 / 1.76±0.22	1.11±0.05 / 1.76±0.36	12.13±3.43	11.95±3.60	13.01±5.52
2-Amino-BTH	5.19±0.35	5.19±0.25	5.19±0.36	0.49±0.36 / 0.78±0.50	0.49±0.34 / 0.78±0.22	0.49±0.36 / 0.78±0.72	21.01±2.65	22.36 ± 5.55	23.18±1.0

*The low, medium and high fortification level for all compounds, except from TTR, was 25, 50 and 100 ng mL^{-1}, respectively. For TTR, the low, medium and high fortification level was 50, 100 and 300 ng mL^{-1}, respectively.

Table A7.3: Retention times (RT), Relative Retention Times (RRT), and Ion Ratio % of BTRs and BTHs in sludge samples.

Compounds	**RT ± RSD % (n=6)**			**RRT with ATR-D5 ± RSD % (n=6) / RRT with 1H-BTR-d4 ± RSD % (n=6)**			**Ion Ratio % ± RSD % (n=6)**		
	Low level	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**	**Low level**	**Medium level**	**High level**
1H-BTR	6.71±0.14	6.71±0.07	6.71±0.14	0.64±0.22 / 1.01±0.74	0.64±0.08 / 1.01±0.44	0.64±0.23 / 1±0.55	48.42±7.02	47.27±3.36	44.70±2.57
1-OH-BTR	5.33±0.22	5.32±0.32	5.31±0.24	0.51±0.21 / 0.80±0.82	0.51±0.37 / 0.80±0.42	0.51±0.21 / 0.79±0.27	13.40±19.34	11.66±15.03	11.13±8.46
TTR	8.02±0.06	8.02±0.18	8.01±0.06	0.76±0.1 / 1.2±0.58	0.76±0.12 / 1.2±0.47	0.76±0.09 / 1.2±0.51	27.49±6.16	24.49±1.74	23.68±3.02

XTR	8.95±0.17	8.96±0.06	8.95±0.11	0.85±0.13 / 1.34±0.38	0.85±0.15 / 1.35±0.47	0.85±0.1 / 1.34±0.38	64.01±5.81	64.46±2.20	62.15±2.63
BTH	9.63±0.18	9.63±0.1	9.63±0.05	0.92±0.22 / 1.44±0.63	0.92±0.04 / 1.45±0.48	0.92±0.16 / 1.44±0.46	47.75±4.83	45.51±2.31	44.76±3.50
2-OH-BTH	9.15±0.12	9.15±0.11	9.13±0.17	0.87±0.05 / 1.37±0.75	0.87±0.01 / 1.37±0.26	0.87±0.13 / 1.37±0.57	76.04±8.64	84.16±7.45	85.25±7.13
2-Me-S-BTH	11.70±0.08	11.70±0.18	11.70±0.16	1.12±0.14 / 1.75±0.37	1.12±0.19 / 1.75±0.46	1.12±0.09 / 1.75±0.22	11.24±8.13	10.04±7.13	11.08±6.97
2- Amino BTH	5.27±0.50	5.29±0.36	5.30±0.24	0.5±0.58 / 0.79±0.86	0.5±0.47 / 0.79±0.26	0.51±0.12 / 0.79±0.45	22.21±6.96	22.46±3.53	21.99±1.99

*The low, medium and high fortification level for all compounds, except from TTR, was 25, 50 and 100 ng mL^{-1}, respectively. For TTR, the low, medium and high fortification level was 50, 100 and 300 ng mL^{-1}, respectively.

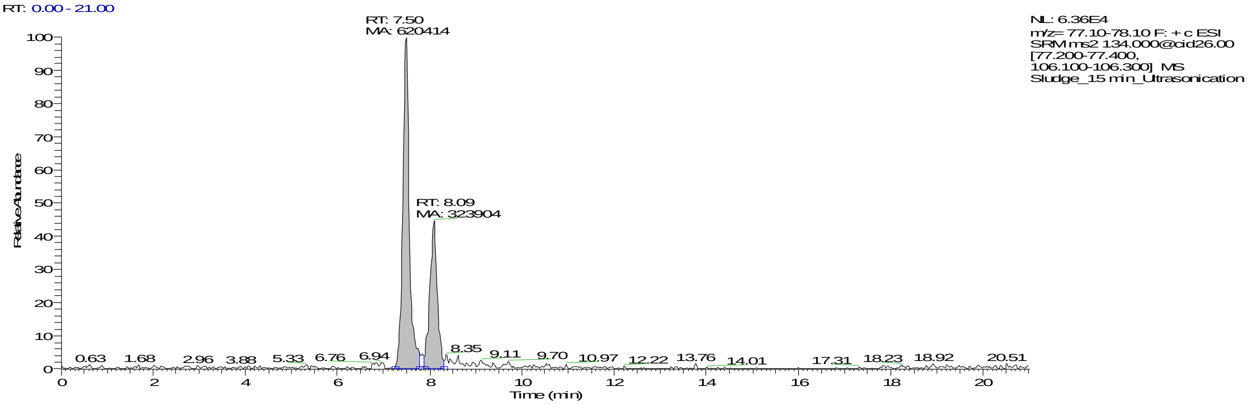

Figure A7.1.: Quantification SRM of TTR (RT= 8.09 min) from sludge sample after 15 min of ultra-sonication at 37 ± 2 °C.

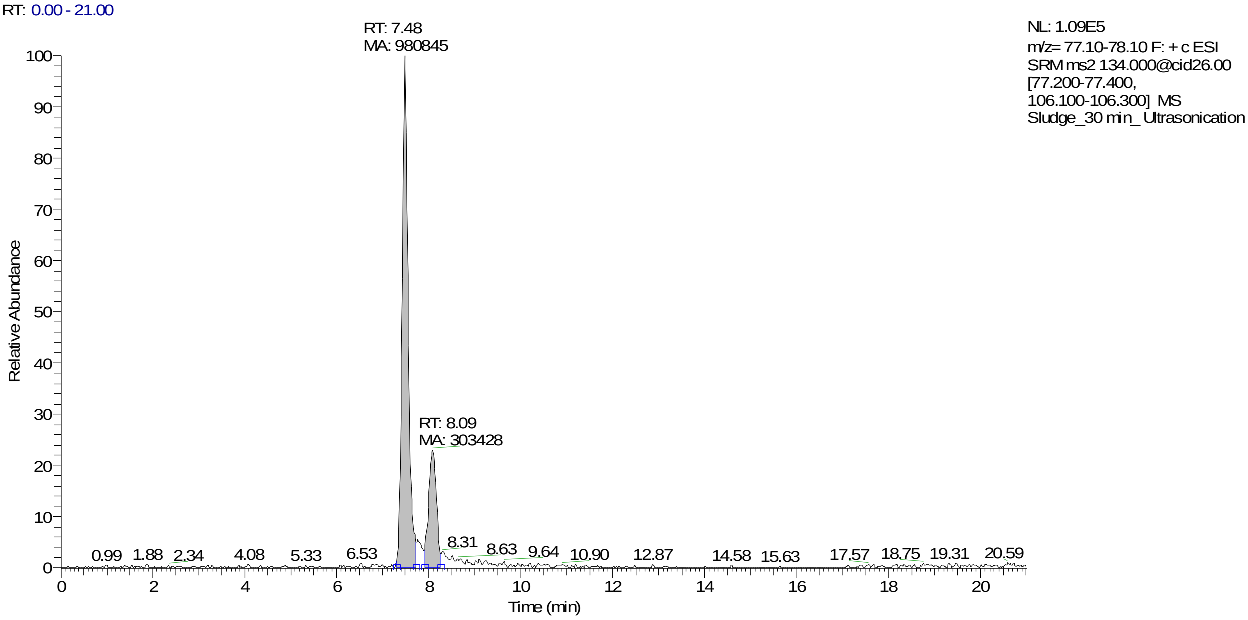

Figure A7.2.: Quantification SRM of TTR (RT= 8.09 min) from sludge sample after 30 min of ultra-sonication at 37 ± 2 °C.

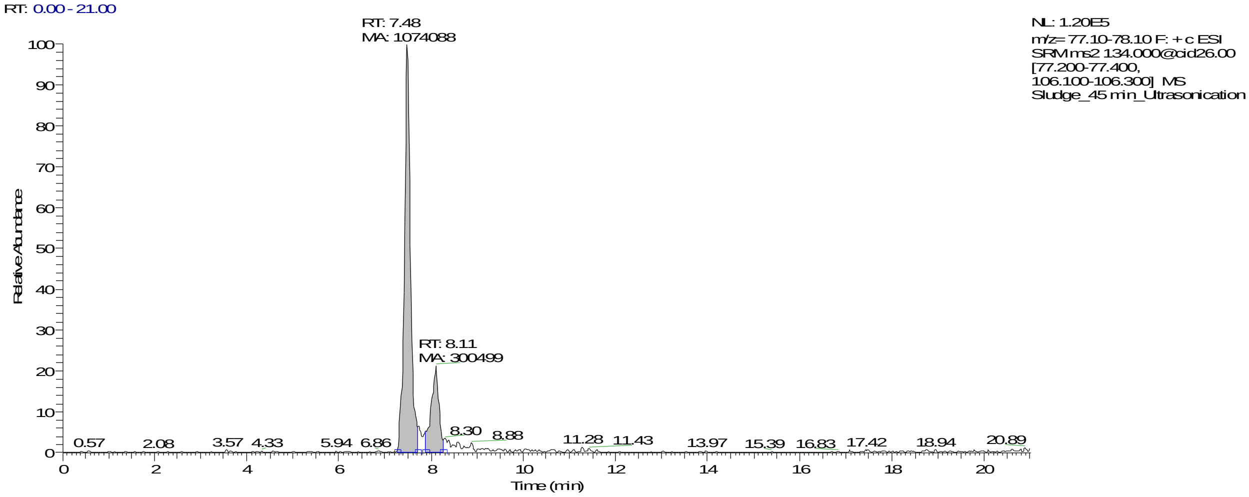

Figure A7.3. Quantification SRM of TTR (RT 8.11 min) from sludge sample after 45 min of ultra-sonication at 37 ± 2 °C.

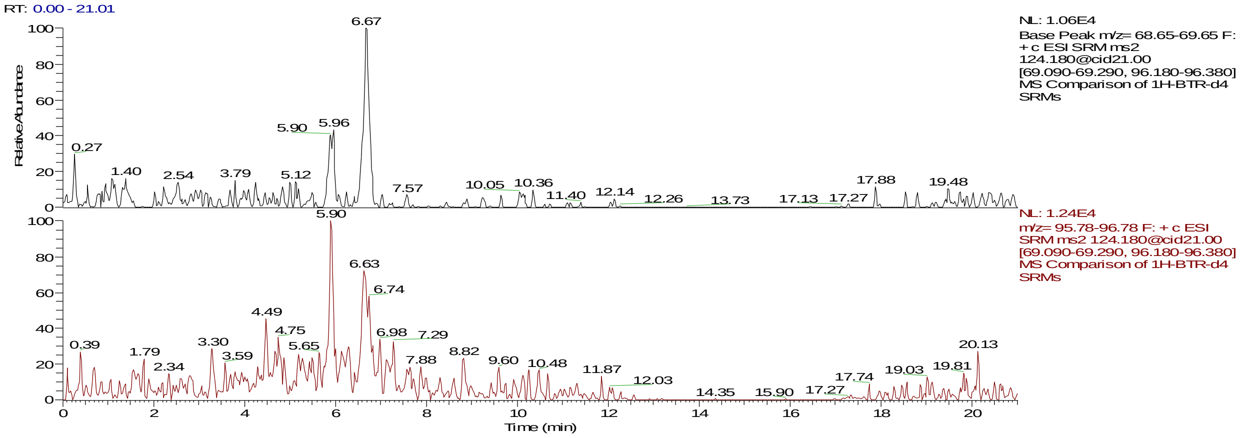

Figure A7.4.: SRMs of 1H-BTR-d4 (RT=6.67 min) at a fortification level of 25 ng mL^{-1} in solvent solution.

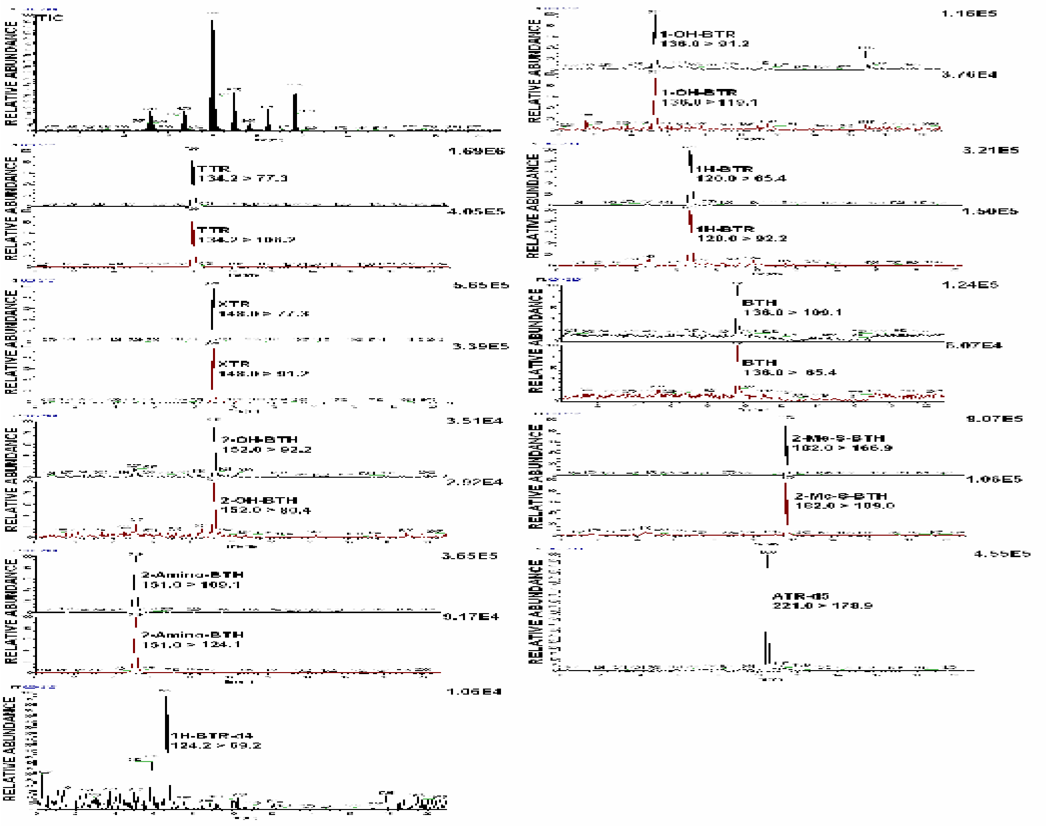

Figure A7.5: SRM chromatograms for BTRs and BTHs of matrix-matched wastewater sample at a fortification level of 100 ng mL^{-1} for TTR and 50 ng mL^{-1} for the rest target compounds (m/z transitions depicted).

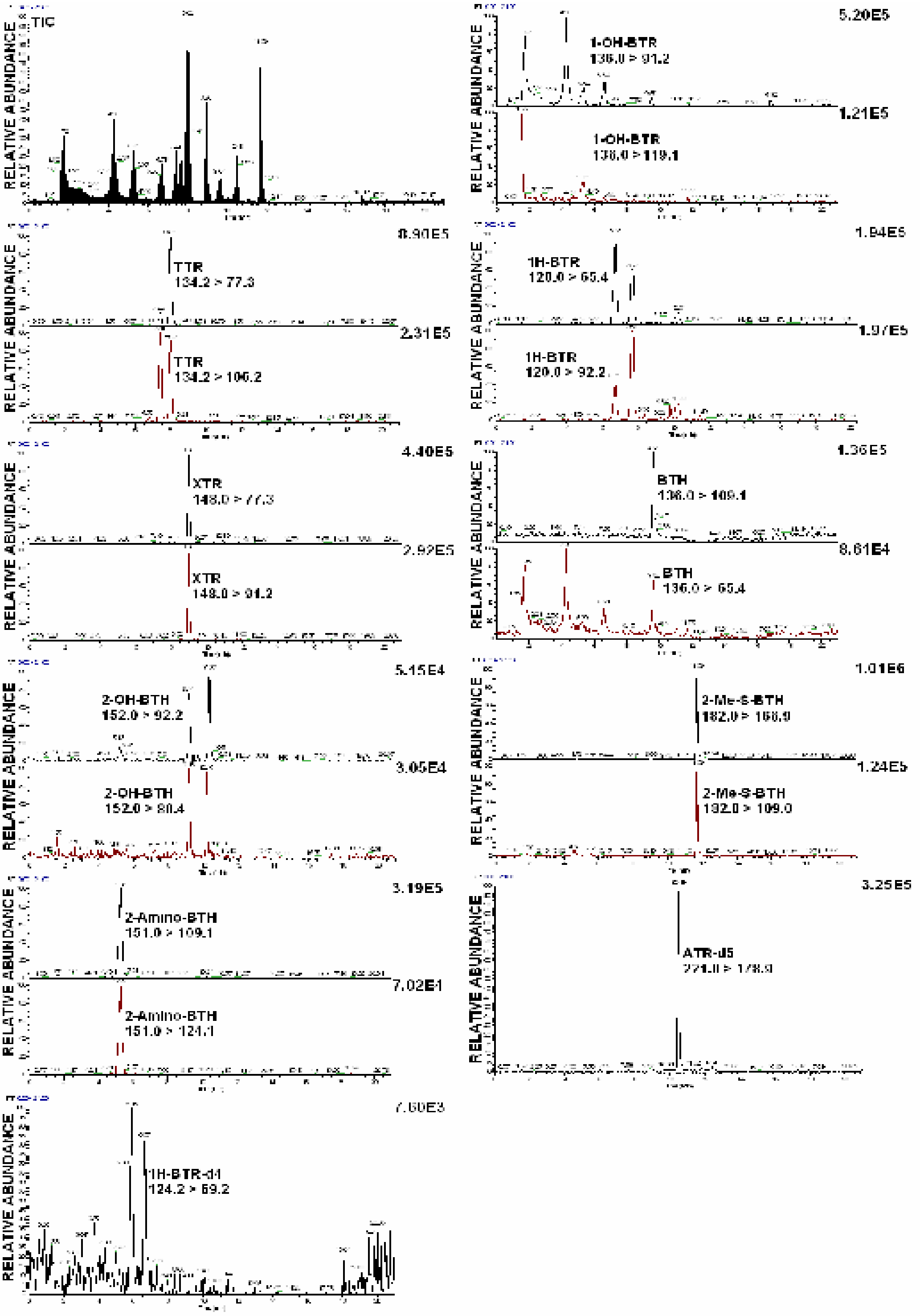

Figure A7.6: SRM chromatograms for BTRs and BTHs of a matrix match sludge sample at a fortification level of 100 ng mL^{-1} for TTR and 50 ng mL^{-1} for the rest target compounds (m/z transitions depicted).

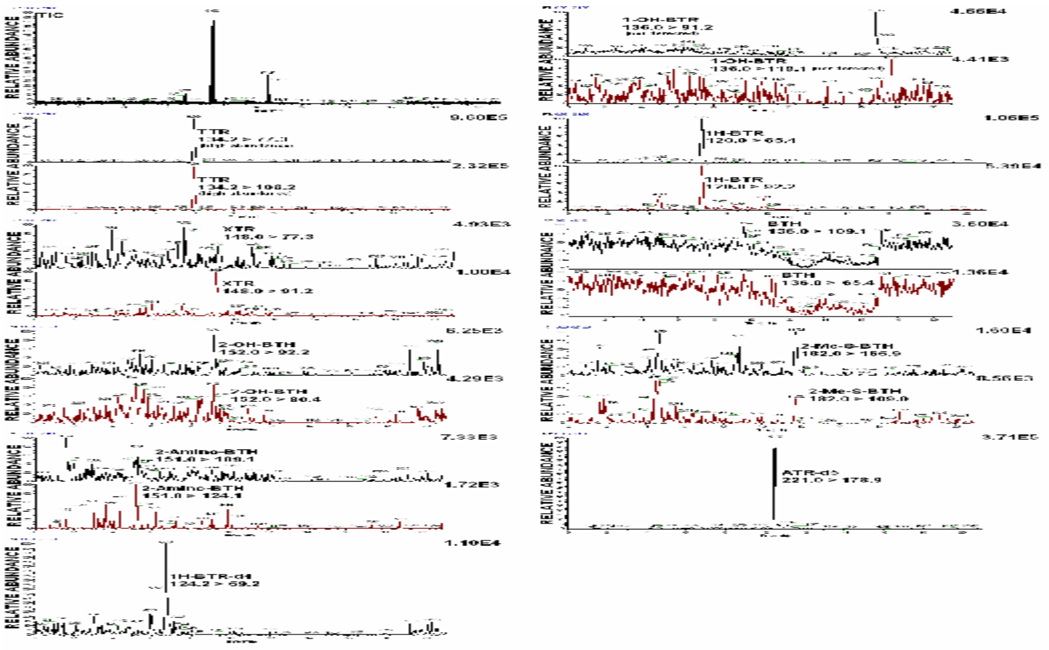

Figure A7.7: SRM chromatograms for BTRs and BTHs of a “zero” (not spiked) dissolved phase of wastewater sample (m/z transitions depicted).

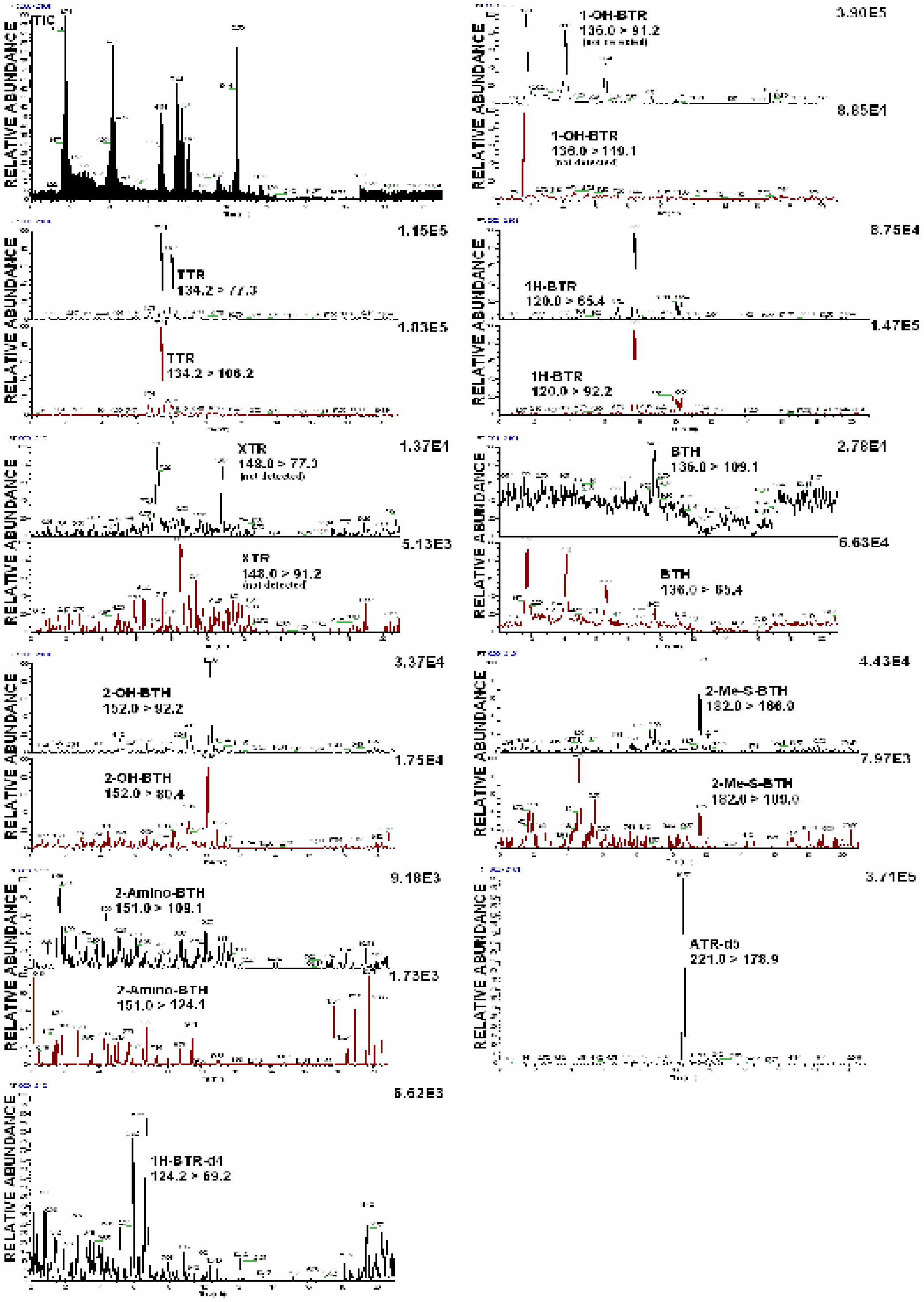

Figure A7.8: SRM chromatograms for BTRs and BTHs of a “zero” (not spiked) sludge sample (m/z transitions depicted).

REFERENCES

1. T. Deblonde, C. Cossu-Leguille, and P. Hartemann, Emerging Pollutants in Wastewater: A review of the literature, *International Journal of Hygiene and Environmental Health,* vol. 214, 2011, pp. 442-448.
2. M. D. Hernando, A. Rodríguez, J. J. Vaquero, A. R. Fernández-Alba and E. García, Environmental Risk Assessment of Emerging Pollutants in Water: Approaches Under Horizontal and Vertical EU Legislation, *Critical Reviews in Environmental Science and Technology,* vol.41, no.7, 2011, pp. 699-731.
3. M. Petrovic and D. Barceló, Liquid chromatography-mass spectrometry in the analysis of emerging environmental contaminants, *Analytical and Bioanalytical Chemistry,* vol.385, 2006, pp.422-424
4. R. Loos, B. M. Gawlick, G. Locoro, E. Rimaviciute, S. Contini and G. Bidoglio, EU-wide survey of polar organic persistent pollutants in European river waters, *Environmental. Pollution,* vol.157, 2009, pp.561-568.
5. R. Rodil, J. B. Quintana, E. Concha-Graña, P. López-Mahía, S. Muniategui-Lorenzo and D. Prada-Rodríguez, Emerging pollutants in sewage, surface and drinking water in Galicia (NW Spain), *Chemosphere,* vol. 86, 2012, pp.1040-1049.
6. N. Theobald, Emerging Persistent Organic Pollutants in the Marine Environment *Persistent Pollution- Past, Present and Future*, M. Quante et al., eds., Springer-Verlag Berlin Heidelberg, 2011, pp. 365-381.
7. OSPAR, 2005, Synergies in assessment and monitoring between OSPAR and European Union. Analysis of synergies in assessment and monitoring of hazardous substances, eutrophication, radioactive substances and offshore industry in the North-East Atlantic *OSPAR Publication,* no. 230, 2005.
8. M. J. Lopez-de Alda, S. Diaz-Cruz, M. Petrovic, and D. Barcelo, Liquid chromatography-(tandem) mass spectrometry of selected emerging pollutants (steroid sex hormones, drugs, and alkylphenolic surfactants) in the aquatic environment, *Journal of Chromatography A,* vol.1000, 2003, pp.503-526.
9. M. –H. Dévier, P. Mazellier, S. Aït-Aïssa, and H. Budzinski, New challenges in environmental analytical chemistry: Identification of toxic compounds in complex mixtures, *C.R. Chimie,* vol. 14, 2011, pp. 766-799.
10. M. W. Jacobs, J. A. Coates, J. J. Delfino, G. Bitton, W. M. Davis, and K. L. Garcia, Comparison of sediment extract microtox toxicity with semi-volatile organic priority

pollutant concentrations. *Archives of Environmental Contamination and Toxicology,* vol.24, 1993, pp. 461-468.

11. H. Hollert, M. Durr, R. Holtey-Weber, M. Islinger, W. Brack, H. Farber, L. Erdinger and T. Braunbeck, Endocrine Disruption of Water and Sediment Extracts in a Non-Radioactive Dot Blot/RNAse Protection-Assay Using Isolated Hepatocytes of Rainbow Trout(14pp). Defficiencies between bioanalytical effectiveness and chemically determined concentrations and how to explain them, *Environmental Science and Pollution Research,* vol.12, 2005, pp.347- 360.
12. S. Weiss and T. Reemtsma, Determination of benzotriazole corrosion inhibitors from aqueous environmental samples by liquid chromatography-electrospray ionization-tandem mass spectrometry, *Analytical Chemistry* ,vol.77, no.2, 2005, pp.7415- 7420.
13. T. Reemtsma, S. Weiss, J. Mueller, M. Petrovic, S. Gonzalez, D. Barcelo, F. Ventura, and T. P. Knepper, Polar Pollutants entry into the water cycle by municipal wastewater: A European perspective, *Environmental Science & Technology* ,vol.40, no.17, 2006, pp.5451-5458.
14. T. Reemtsma, U. Miehe, U. Duennbier, and M. Jekel, Polar pollutants in municipal wastewater and the water cycle: Occurrence and removal of benzotriazoles, *Water Research*, vol. 44, no.2, 2010, pp.596-604.
15. W. Giger, Hydrophilic and amphiphilic water pollutants: using advanced analytical methods for classic and emerging contaminants, *Analytical and Bioanalytical Chemistry,* vol. 393, 2009, pp. 37 - 44.
16. H. C. Poynton and C. D. Vulpe, Ecotoxicogenomics: Emerging Technologies for Emerging Contaminants, *Journal of the American Water Resources Association*, vol. 45, no.1, 2009, pp.83-96.
17. T. T. Pham and S. Proulx, PCBs and PAHs in the montreal urban community (Quebec, Canada) wastewater treatment plant and in the effluent plume in the St Lawrence river. *Water Res.,* vol. 31, no.8, 1997, pp. 1887-1896.
18. C. Vogelsang, M. Grung, T. G. Jantsch, K. E. Tollefsen and H. Liltved, Occurrence and removal of selected organic micropollutants at mechanical, chemical and advanced wastewater treatment plants in Norway, *Water Research,* vol. 40, 2006, pp. 3559-3570.
19. R. Rosal, A. Rodriguez, J. A. Perdigon-Melon, A. Petre, E. Garcia-Calvo, M. J. Gomez, A. Aguera and A. R. Fernandez-Alba, Occurrence of emerging pollutants in urban

wastewater and their removal through biological treatment followed by ozonation, *Water Research,* vol. 44, 2010, pp.578-588.

20. M. Farré, S. Pérez, L. Kantiani, and D. Barceló, Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment, *Trends in Analytical Chemistry*, vol. 27, no.11, 2008, pp. 991-1007.

21. K.Wille, H. F. De Brabander, E. De Wulf, P. Van Caeter, C. R. Janssen and L. Vanhaecke, Coupled chromatographic and mass-spectrometric techniques for the analysis of emerging pollutants in the aquatic environment, *Trends in Analytical Chemistry,* vol.35, 2012, pp. 87-108.

22. K. Fischer, K. Fries, W. Körner, C. Schmalz, and C. Zwiener, New developments in the trace analysis of organic water pollutants, *Applied Microbiology and Biotechnology*, vol. 94, 2012, pp. 11-28.

23. J. Fawell, and C. N. Ong, Emerging Contaminants and the Implications for Drinking Water*, International Journal of Water Resources Development*, vol. 28, no.2, 2012, pp. 247- 263.

24. A. Nikolaou and G. Lofrano, Detection of Transformation Products of Emerging Contaminants, *Green Technologies for Wastewater Treatment,* G. Lofrano, ed., SpringerBriefs in Green Chemistry for Sustainability, 2012, pp. 19-29.

25. H. P. H. Arp, Emerging Decontaminant, *Environmental Science & Technology*, vol. 46, 2012, pp. 4259-4260.

26. S. T. Glassmeyer, The cycle of emerging contaminants, *Water Resources IMPACT*, vol. 9, 2007, pp. 5-7.

27. NORMAN, 6th EU Framework Programme project NORMAN, Contract number 018486 http://www.norman-network.net/index_php.php?interface=1024

28. A. W. Garrison, J. D. Pope, and F. R. Allen, GC/MS analysis of organic compounds in domestic waters, *Identification and Analysis of Organic Pollutants in Water,* C.H. Keith, ed., Ann Arbor Science Publishers, 1976, pp. 517- 556.

29. C. Hignite and D. L. Azarnoff, Drugs and drug metabolites as environmental contaminants: Chlorophenoxyisobutyrate and salicyclic acid in sewage water effluent, *Life Science,* vol.20, 1977, pp.337-341.

30. T. Yamagishi, T. Miyasaki, S. Horri, and S. Kaneko, Identification of musk xylene and musk ketone in freshwater fish collected from the Tama River, Tokyo, *Bulletin of Environmental Contamination and Toxicology*, vol. 26, 1981, pp. 656-662.

31. C. A. De Wit, An overview of brominated flame retardants in the environment, *Chemosphere*, vol. 46, 2002, pp. 583- 624.

32. B. C. Englert, Nanomaterials and the environment: Uses, methods, and measurement, *Journal of Environmental Monitoring*, vol. 9, 2007, pp. 1154-1161.

33. S. D. Richardson, Water Analysis: Emerging Contaminants and Current Issues, *Analytical Chemistry*, vol. 81, 2009, pp. 4645- 4677.

34. J. A. Field, C. A. Johnson and J. B. Rose, What is "emerging"? *Environmental Science & Technology,* vol. 40, no.23, 2006, pp. 7105-7105.

35. USGS (2011), Toxic Substances Hydrology Program: Emerging Contaminants in the Environment (US Geological Survey), Available at: http://toxics.usgs.gov/regional/emc (accessed on 24/11/2012).

36. G. Murnyak, J. Vandenberg, P.J. Yaroschak, L. Williams, K. Prabhakaran, and J. Hinz, Emerging contaminants: Presentations at the 2009 Toxicology and Risk Assessment Conference, *Toxicology and Applied Pharmacology*, vol. 254, 2011, pp. 167-169.

37. D. W. Kolpin, E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, and H. T. Buxton, Pharmaceuticals, hormone and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance, *Environmental Science & Technology*, vol.36, no.6, 2002, pp. 1202-1211.

38. D. C. G. Muir and P. H. Howard, Are There Other Persistent Organic Pollutants? A Challenge for Environmental Chemists, *Environmental Science & Technology*, vol.40, 2006, pp. 7157-7166.

39. S. D. Richardson, Water Analysis: Emerging Contaminants and Current Issues, *Analytical Chemistry*, vol. 79, 2007, pp.4295- 4324.

40. C. G. Daughton, Non Regulated Water Contaminants: Emerging Research, *Environmental Impact Assessment Reviews*, vol. 24, 2004, pp.711- 732.

41. T. Smital, Acute and Chronic Effects of Emerging Contaminants, *Handbook of Environmental Chemistry*, D.Barceló and M. Petrovic, eds., Springer, 2008, vol.5, Part S/1, pp.105-142.

42. P. E. Rosenfeld and L. G. H. Feng, *Risks of Hazardous Wastes*, Elsevier, 2011, pp. 215-221.

43. WWF, Bad Blood? WWF reveals EU Lawmakers' Chemical Contamination, October 19, 2004, http://worldwildlife.org/press_releases/bad-blood-wwf-reveals-eu-lawmakers-chemical-contamination

44. European Commission, The attitudes of European citizens toward the environment, Special Eurobarometer, April 2005, http://europa.eu.int/comm/environment/barometer/index.htm

45. N. Bolong, A. F. Ismail, M. R. Salim and T. Matsuura, A review of the effects of emerging contaminants in wastewater and options for their removal, *Desalination*, vol. 239, 2009, pp.229-246.

46. S. Jobling, M. Nolan, C. R. Tyler, G. Brighty and J. P. Sumpter , Widespread sexual disruption in wild fish, *Environmental Science & Technology*, vol.32, 1998, pp.2498-2506.

47. T. Wind, U. Werner, M. Jacob and A. Hauk, Environmental concentrations of boron, LAS, EDTA, NTA and Triclosan simulated with GREAT-ER in the river Itter, *Chemosphere* vol.54, 2004, pp.1145-1154.

48. H. Janna, M. D. Scrimshaw, R. J.Williams, J. Churchley and J. P. Sumpter, From Dishwater to Tap? Xenobiotic Substances Benzotriazole and Tolyltriazole in the Environment, *Environmental Science & Technology*, vol.45, 2011, pp.3858-3864.

49. K. E. Murray, S. M. Thomas and A. A. Bodour, Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment, *Environmental Pollution*, vol.158, 2010, pp. 3462- 3471.

50. USGS, Toxicity Substances Hydrology Program: Analytical method development, available at http://toxics.usgs.gov/regional/emc/methods_devel.html (accessed on 24/11/2012)

51. T. A. Ternes, Occurrence of drugs in German sewage treatment plants and rivers, *Water Research*., vol.32, no.11, 1998, pp.3245-3260.

52. K. H. Chan, M. H. W. Lam, K. F. Poon, H. Y. Yeung and T. K. T. Chiu, Application of sedimentary fecal stanols and sterols in tracing sewage pollution in coastal waters, *Water Research*, vol. 32, no.1, 1998, pp.225-235.

53. T. Heberer, Tracking persistent pharmaceutical residues from municipal sewage to drinking water, *Journal of Hydrology,* vol. 266, 2002, pp. 175-189.

54. S. K. Khanal, B. Xie, M. L. Thompson, S. Sung, S. K. Ong, and J. H. van Leeuwen, Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems, *Environmental Science & Technology*, vol.40, no.21, 2006, pp.6537-6546.

55. F. F. Sodre, M. F. Locatelli and W. F. Jardim, Occurrence of Emerging Contaminants in Brazillian Drinking Waters: A Sewage-To-Tap Issue, *Water, Air & Soil Pollution*, vol. 206, 2010, pp. 57-67.

56. N. S. Thomaidis, A. G. Asimakopoulos and A. A. Bletsou, Emerging Contaminants: A tutorial mini-review, *Global NEST Journal,* vol.14, no.1, 2012, pp.70-77.

57. E. Zuccato, C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea and R. Fanelli, Cocaine in surface water: a new evidence-based tool to monitor community drug abuse, *Environmental Health Global*, vol. 4, 2005, pp.14–20.

58. S. Terzic, I. Senta, M. Ahel, M. Gros, M. Petrović, D. Barcelo, J. Müller, T. Kneeper, I. Martí, F. Ventura, P. Jovančić, and D. Jabučar, Occurrence and fate of emerging wastewater contaminants in Western Balkan Region, *Science of the Total Environment.,* vol.399, 2008, pp. 66-77.

59. T. A. Ternes, M. Stumpf, J. Mueller, K. Haberer, R.-D Wilken and M. Servos, Behaviour and occurrence of estrogens in municipal sewage treatment plants-1. Investigations in Germany, Canada and Brazil, *Science of the Total Environment,* vol. 225, 1999, pp.81-90.

60. M. Carballa, F. Omil, J. M. Lema, M. Llompart, C. Garcia-Jares, and I. Rodriguez, Behaviour of pharmaceuticals, cosmetics and hormones in a sewage treatment plant, *Water Research,* vol. 38, no.12, 2004, pp. 2918- 2926.

61. M. Auriol, Y. Filali-Meknassi, R. D. Tyagi, C. D. Adams and R. Y. Surampalli, Endocrine disrupting compounds removal from the wastewater treatment plant, a new challenge. *Process Biochemistry,* vol.41, no.3, 2006, pp.525-539.

62. X, Y. Yu, C. Tang, J. Tan, Q. Huang and Z. Wang, Occurrence of steroid estrogens, endocrine disrupting phenols, and acid pharmaceutical residues in urban riverine water of the Pearl River Delta, South China, *Science of the Total Environment,* vol.397, 2008, pp.158-166.

63. A. Arditsoglou and D. Voutsa, Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices, *Environmental Science and Pollution Research*, vol.15, no.3, 2008, pp. 228-236.

64. C. G. Daughton, ‘‘Emerging’’ Chemicals as Pollutants in the Environment: a 21st Century Perspective, *Renewable Resources Journal*, vol.23, no.4, 2005, pp.6-23.

65. R. P. Schwarzenbach, B. I. Escher, K. Fenner, T. B. Hofstetter, C. A. Johnson, U. von Gunten and B. Wehrli, The challenge of micropollutants in aquatic systems, *Science*, vol.313, no.5790, 2006, pp.1072-1077.

66. J. P. Sumpter and A. C. Johnson, Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment, *Environmental Science & Technology*, vol. 39, 2005, pp. 4321-4332.

67. J. P. Sumpter and A. C. Johnson, 10th Anniversary perspective: reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things inbetween), *Journal of Environmental Monitoring*, vol.10, 2008, pp.1476–1485.

68. D. J. Lapworth, N. Baran, M. E. Stuart and R. S. Ward, Emerging organic contaminants in groundwater: A review of sources, fate and occurrence, *Environmental Pollution*, vol.163, 2012, pp. 287-303.

69. S. D. Richardson, Environmental Mass Spectrometry: Emerging Contaminants and Current Issues, *Analytical Chemistry*, vol. 84, 2012, pp.747- 778.

70. H. R. Buser, T. Poiger and M. D. Muller, Occurrence and environmental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and wastewater, *Environmental Science & Technology*, vol. 33, 1999, pp. 2529-2535.

71. O. A. H. Jones, N. Voulvoulis and J. N. Lester, Human Pharmaceuticals in the Aquatic Environment a Review, *Environmental Technology*, vol.22, no.12, 2001, pp.1383-1394.

72. O. A. H. Jones, N. Voulvoulis and J. N. Lester, Human Pharmaceuticals in Wastewater Treatment Processes, *Critical Reviews in Environmental Science and Technology*, vol.35 no.4, 2005, pp.401-427.

73. J. G. M. Derksen, G. B. J. Rijs and R. H. Jongbloed, Diffuse pollution of surface water by pharmaceutical products, *Water Science and Technology,* vol.49, 2004, pp. 213–221.

74. N. Ratola, A. Cincinelli, A. Alves and A. Katsoyiannis, Occurrence of organic microcontaminants in the wastewater treatment process. A minireview, *Journal of Hazardous Materials,* vol.239-240, 2012, pp.1-18.

75. A. Jelic, M. Gros, A. Ginebreda, R. Cespedes-Sanchez, F. Ventura, M. Petrovic and D. Barcelo, Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment, *Water Research*, vol. 45, 2011, pp.1165–1176.

76. C. Miege, J. M. Choubert, L. Ribeiro, M. Eusebe and M. Coquery, Fate of pharmaceuticals and personal care products in wastewater treatment plants – conception of a database and first results, *Environmental Pollution*,vol.157, 2009, pp.1721–1726.

77. J. Radjenovic, M. Petrovic and D. Barcelo, Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment, *Water Research*, vol. 43, 2009, pp. 831–841.

78. K. M. Onesios, J. T. Yu, and E. J. Bouwer, Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review, *Biodegradation*, vol. 20, 2009, pp.441–466.

79. S. K. Behera, H. W. Kim, J. E. Oh and H. S. Park, Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea, *Science of the Total Environment,* vol.409, 2011, pp. 4351–4360.

80. E. Eljarrat, M. S. Díaz-Cruz, M. Farre´, M. J. López de Alda, M. Petrovic, and D. Barceló, Analysis of Emerging Contaminants in Sewage Sludge, *Emerging Organic Contaminants in Sludges: Analysis, fate and Biological Treatment*, T. Vicent et al.,

eds., Hdb Env Chem, DOI 10.1007/698_2012_155, Springer-Verlag Berlin Heidelberg, 2012.

81. A. Nikolaou and G. Lofrano, Detection of Transformation Products of Emerging Contaminants, *Green Technologies for Wastewater Treatment,* G. Lofrano, ed., SpringerBriefs in Green Chemistry for Sustainability, 2012, pp. 19-29.

82. K. Fent, A. A. Weston and D. Caminada, Ecotoxicology of human pharmaceuticals, *Aquatic Toxicology,* vol.76, 2006, pp.122-159.

83. M. J. M. Wells, Log Dow Key to understanding and regulating wastewater derived contaminants, *Environmental Chemistry,* vol.3, 2006, pp.439–449.

84. M. Petrovic, J. Radjenovic, C. Postigo, M. Kuster, M. Farre1, M. López de Alda and D. Barceló, Emerging contaminants in wastewaters: Sources and Occurrence**,** *Handbook of Environmental Chemistry*, D. Barceló and M. Petrovic, eds. Springer-Verlag Berlin Heidelberg, vol. 5, Part S/1 , 2008, pp.1-35.

85. A. Kumar, I. Xagoraraki, Pharmaceuticals, personal care products and endocrine-disrupting chemicals in U.S. surface and finished drinking waters: a proposed ranking system, *Science of the Total Environment*, vol.408, 2010, pp.5972–5989.

86. J. B. Ellis, Pharmaceutical and personal care products (PPCPs) in urban receiving waters, *Environmental Pollution*, vol.144, 2006, pp.184–189.

87. K. McClellan and R.U. Halden, Pharmaceuticals and personal care products in archived U.S. biosolids from the 2001 EPA national sewage sludge survey, *Water Research,* vol.44, 2010, pp.658–668.

88. H. Fromme, T. Otto, K. Pilz and F. Neugebauer, Levels of synthetic musks; bromocyclene and PCBs in eel (*anguilla anguilla*) and PCBs in sediment samples from some waters of Berlin/Germany, *Chemosphere*, vol. 39, 1999, pp.1723–1735.

89. M. Motoyama, S. Nakagawa, R. Tanoue, Y. Sato, K. Nomiyama and R. Shinohara, Residues of pharmaceutical products in recycled organic manure produced from sewage sludge and solid waste from livestock and relationship to their fermentation level, *Chemosphere,* vol. 84 2011, pp. 432–438.

90. A. Nikolaou and G. Lofrano, Detection of Transformation Products of Emerging Contaminants, *Green Technologies for Wastewater Treatment*, G. Lofrano, ed., SpringerBriefs in Green Chemistry for Sustainability, 2012, pp. 19-29.

91. S. Weigel, U. Berger, E. Jensen, R. Kallenborn, H. Thoresen and H. Huhnerfuss, Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites, *Chemosphere*, vol.56, 2004, pp.583–592.

92. S. Perez and D. Barcelo, Application of advanced MS techniques to analysis and identification of human and microbial metabolites of pharmaceuticals in the aquatic environment. *Trends in Analytical Chemistry*, vol.26, 2007, pp.494–514.

93. P. Eichhorn, P. L. Ferguson, S. Perez and D. S. Aga, Application of ion trap-MS with H/D exchange and QqTOF-MS in the identification of microbial degradates of trimethoprim in nitrifying activated sludge, *Analytical Chemistry*, vol.77, 2005, pp.4176–4184.

94. M.J. Gomez, O. Malato, I. Ferrer, A. Aguera and A.R. Fernandez-Alba, Solid-phase extraction followed by liquid chromatography-time-of-flight-mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study, *Journal of Environmental Monitoring*, vol. 9, 2007, pp.718-729.

95. A. A. M. Stolker, W. Niesing, E. A. Hogendoorn, J. F. M.Versteegh, R. Fuchs and U.A.T. Brinkman, Liquid chromatography with triple-quadrupole or quadrupole-time of flight mass spectrometry for screening and confirmation of residues of pharmaceuticals in water, *Analytical and Bioanalytical Chemistry*, vol.378, no.9, 2004, pp.955-963.

96. A. M. F. Choong, S. L-M. Teo, J. L. Leow, H. L. Koh and P. C. L. Ho , A preliminary ecotoxicity study of pharmaceuticals in the marine environment, *Journal of Toxicololgy and Environmental Health ,Part A*, vol.69, 2006, pp.1959–1970.

97. M. Crane, C. Watts and T. Boucard, Chronic aquatic environmental risks from exposure to human pharmaceuticals, *Science of the Total Environment*, vol.367, 2006, pp.23–41.

98. G. T. Ankley, B. W. Brooks, D. B. Huggett and J. P. Sumpter, Repeating history: pharmaceuticals in the environment, *Environmental Science & Technology*, vol.41, 2007, pp.8211–8217.

99. B. O. Clarke, and S. R. Smith, Review of ‘emerging’ organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids, *Environment International*, vol. 37, 2011, pp. 226-247.

100. M. S. Díaz-Cruz, M. J. López de Alda and D. Barceló, Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge, *Trends in Analytical Chemistry*, vol. 22, no. 6, 2003, pp.340-351.

101. W. Hall, L. Degenhardt and N. Sindicich, Illicit drug use and the burden of disease, *International encyclopedia of public health*, K. Heggenhougen and S. Quah, eds., Elsevier; 2008, p p. 523–530.

102. R. Pal, M. Megharaj, K. P. Kirkbride and R. Naidu, Illicit drugs and the environment-A review, *Science of the Total Environment*, 2012, In press, doi:10.1016/j.scitotenv.2012.05.086

103. D. R. Baker and B. Kasprzyk-Hordern, Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionisation tandem mass spectrometry, *Journal of Chromatography A*, vol.1218, 2011, pp.1620-1631.

104. M. R. Boleda, M.T. Galceran and F. Ventura, Behavior of pharmaceuticals and drugs of abuse in a drinking water treatment plant (DWTP) using combined conventional and ultrafiltration and reverse osmosis (UF/RO) treatments, *Environmental Pollution*, vol.159, 2011, pp.1584-1591.

105. A. L. N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M. L. de Alda, H. Neels, E. Zuccato, D. Barcelo and A. Covaci, Illicit drug consumption estimations derived from wastewater analysis: a critical review, *Science of the Total Environment*, vol. 409, 2011, pp.3564–3577.

106. C. Metcalfe, K. Tindale, H. Li, A. Rodayan and V. Yargeau, Illicit drugs in Canadian municipal wastewater and estimates of community drug use, *Environmental Pollution*, vol.158, 2010, pp.3179–3185.

107. S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli and R. Bagnati, Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry, *Analytical Chemistry*, vol. 78, 2006, pp.8421–8429.

108. S. Castiglioni, E. Zuccato, C. Chiabrando, R. Faneli and R. Bagnati, Mass spectrometric analysis of illicit drugs in wastewater and surface water, *Mass Spectrometry Reviews*, vol.27, 2008, pp.378-394.

109. S. D. Richardson and T. A. Ternes, Water Analysis: Emerging Contaminants and Current Issues, *Analytical Chemistry*, vol. 83, 2011, pp. 4614-4648.

110. I. González-Mariño, J.B. Quintana, I. Rodríguez and R. Cela, Determination of drugs of abuse in water by solid phase extraction, derivatisation and gas chromatography ion trap tandem mass spectrometry, *Journal of Chromatography A*, vol.1217, 2010, pp.1748–1760.

111. S. Castiglioni, E. Zuccato, C. Chiabrando, R. Faneli and R. Bagnati, Detecting illicit drugs and metabolites in wastewater using high performance liquid chromatography-tandem mass spectrometry, *Spectroscopy Europe*, vol.19, 2007, pp.7–9.

112. J. Bones, K.V. Thomas and B. Paull, Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals, *Journal of Environmental Monitoring*, vol.9, 2007, pp.701–707.

113. E. Zuccato and S. Castiglioni, Illicit drugs in the environment, *Philosophical Transactions of The Royal Society A*, vol. 367, 2009, pp.3965–3978.

114. J. C. Verster, Monitoring drugs of abuse in wastewater and air, *Current Drug Abuse Review,* vol. 3, 2010, pp.1-2.

115. J. Heidler and R. U. Halden, Meta-analysis of mass balances examining chemical fate during wastewater treatment, *Environmental Science & Technology*, vol.42, 2008, pp.6324–6332.

116. Y. Pan, Y. Shi, J. Wang and Y. Cai, Evaluation of perfluorinated compounds in seven wastewater treatment plants in Beijing urban areas, *Science China Chemistry*, vol.54, 2011, pp. 552–558.

117. L. Ahrens, S. Felizeter and R. Ebinghaus, Spatial distribution of polyfluoroalkyl compounds in seawater of the German Bight, *Chemosphere*, vol.76, 2009, pp. 179-184.

118. R. Loos, G. Locoro, S. Comero, S. Contini, D. Schwesig, F. Werres, P. Balsaa, O. Gans, S. Weiss, L. Blaha, M. Bolchi and B.M. Gawlic, Pan European survey on the occurrence of selected polar organic persistent pollutants in groundwater, *Water Research*, vol.44, no.14, 2010, pp. 4115-4126.

119. C. J. Kwadijk, P. Korytar and A.A. Koelmans, Distribution of perfluorinated compounds in aquatic systems in the Netherlands, *Environmental Science & Technology*, vol.44, 2010, pp. 3746–3751.

120. F. T. Lange, M. Scheurer and H-J. Brauch, Artificial sweeteners- a recently recognized class of emerging environmental contaminants: a review, *Analytical and Bioanalytical Chemistry*, vol. 403, no.9, 2012, pp. 2503-2518.

121. M. Kroger, K. Meister and R. Kava, Low-calories Sweeteners and Other Sugar Substitutes: A Review of the safety Issues, *Comprehensive Reviews in Food Science and Food Safety*, vol.5, 2006, pp.35–47.

122. I. J. Buerge, H.R. Buse, M. Kahle, M.D. Muller and T. Poiger, Ubiquitous Occurrence of the Artificial Sweetener Acesulfame in the Aquatic Environment: An Ideal Chemical Marker of Domestic Wastewater in Groundwater, *Environmental Science & Technology*, vol.43, no.12, 2009, pp.4381–4385.

123. M. Scheurer, H.J. Brauch and F.T. Lange, Analysis and occurrence of seven artificial sweeteners in German wastewater and surface water and in soil acquifer treatment (SAT), *Analytical and Bioanalytical Chemistry*, vol.394, no. 6, 2009, pp.1585–1594.

124. L. Soh, K.A. Connors, B.W. Brooks and J. Zimmerman, Fate of sucralose through environmental and water treatment processes and impact on plant indicator species *Environmental Science & Technology*, vol. 45, 2011, pp.1363-1369.

125. J. Minten, M. Adolfsson-Erici, B. Bjorlenius and T. Alsberg , A method for the analysis of sucralose with electrospray LC/MS in recipient waters and in sewage effluent subjected to tertiary treatment technologies, *International Journal of Environmental Analytical Chemistry*, vol.91, no. 4, 2011, pp.357-366.

126. I. Ferrer and E. M. Thurman, Analysis of sucralose and other sweeteners in water and beverage samples by liquid chromatography/time-of-flight-mass spectrometry, *Journal of Chromatography A*, vol.1217, 2010, pp.4127–4134.

127. D. R. Van Stempvoort, J.W. Roy, S.J. Brown and G. Bickerton, Artificial sweeteners as potential tracers in groundwater in urban environments, *Journal of Hydrology*, vol.401, 2011, pp.126–133.

128. M. Hjorth, J.H. Hansen and L. Camus, Short-term effects of sucralose on *Calanus finmarchicus* and *Calanus glacialis* in Disko Bay, Greenland, *Chemistry and Ecology*, vol.26, no.5, 2010, pp.385-393.

129. A. Lillicrap, K. Langford and K.E. Tollefsen, Bioconcentration of the intense sweetener sucralose in a multitrophic battery of aquatic organisms, *Environmental Toxicology and Chemistry*, vol. 30, no.3, 2011, pp.673-681.

130. M.G. Kokotou, A.G. Asimakopoulos and N.S. Thomaidis, Artificial Sweeteners as Emerging Pollutants in the Environment: Analytical Methodologies and Environmental Impact, *Analytical Methods*, vol. 4, 2012, pp.3057-3070.

131. A. Covaci, A.C. Gerecke, R.J. Law, S. Voorspoels, M. Kohler, N.V. Heeb, H. Leslie, C.R. Allchin and J. De Boer, Hexabromocyclododecanes (HBCDs) in the environment and humans: A review, *Environmental Science & Technology,* vol. 40, 2006, pp. 3679-3688.

132. J. Wu, L. Zhang and Z. Yang, A Review on the Analysis of Emerging Contaminants in Aquatic Environment, *Critical Reviews in Analytical Chemistry*, vol.40, no.4, 2010, pp. 234-245.

133. L. Kantiani, M. Llorca, J. Sanchís, M. Farré and D. Barceló, Emerging food contaminants: a review, *Analytical and Bioanalytical Chemistry*, vol. 398, 2010, pp. 2413-2427.

134. P. O. Darnerud, G. S. Eriksen, T. Johannesson, P. B. Larsen and M. Viluksela, Polybrominated diphenyl ethers: occurrences, dietary exposure, and toxicology, *Environmental Health Perspective*, vol. 109, 2001, pp.48–68.

135. T.A. McDonald, A perspective on the potential health risks of PBDEs, *Chemosphere*, vol.46, 2002, pp.745-755.

136. J. Legler and A. Brouwer, Are brominated flame retardants endocrine disruptors? *Environment International*, vol.29, 2003, pp.879–885.

137. M. Farré, J. Sanchís and D. Barceló, Analysis and assessment of the occurrence, the fate and the behavior of nanomaterials in the environment, *Trends in Analytical Chemistry*, vol. 30, no.3, 2011, pp.517-527.

138. J. D. Fortner, D.Y. Lyon, C.M. Sayes, A.M. Boyd, J.C. Falkner, E.M. Hotze, L.B. Alemany, Y.J. Tao, W. Guo, K.D. Ausman, V.L. Colvin and J.B. Hughes, C_{60} in

Water:Nanocrystal Formation and Microbial Response, *Environmental Science & Technology*, vol.39, no.11, 2005, pp. 4307-4316.

139. J.R. Deye, A.N. Shiveley, S.A. Oehrle and K.A. Walters, Separation of substituted fullerenes using non-aqueous reversed-phase liquid chromatography-mass spectrometry, *Journal of Chromatography A*, vol. 1181, 2008, pp.159-161.

140. M. Farré, S. Pérez, K. Gajda-Schrantz, V. Osorio, L. Kantiani, A. Ginebreda and D. Barceló, First determination of C60 and C70 fullerenes and N-methylfulleropyrrolidine C60 on the suspended material of wastewater effluents by liquid chromatography hybrid quadrupole linear ion trap tandem mass spectrometry, *Journal of Hydrology*, vol.383, 2010, pp. 44-51.

141. V. Kozlovski, V. Brusov, I. Sulimenkov, A. Pikhtelev and A. Dodonov, Novel experimental arrangement developed for direct fullerene analysis by electrospray time-of-flight mass spectrometry, *Rapid Communication in Mass Spectrometry*, vol.18, no.7, 2004, pp.780-786.

142. S. Ilchenko and R. J. Cotter, Collision energetics in a tandem time-of-flight (TOF/TOF) mass spectrometer with a curved-field reflectron, *International Journal of Mass Spectrometry*, vol. 265, 2007, pp.372-381.

143. R. B. Greswell, S. H. Rahman, M. O. Cuthbert and J.H. Tellam, An inexpensive flow-through laser nephelometer for the detection of natural colloids and manufactured nanoparticles, *Journal of Hydrology*, vol.388, 2010, pp.112-120.

144. R. Owen and M. Depledge, Nanotechnology and the environment: Risks and rewards, *Marine Pollution Bulletin*, vol. 50, no.6, 2005, pp.609-612.

145. R. Owen and R. Handy, Viewpoint: Formulating the Problems for Environmental Risk assessment of Nanomaterials, *Environmental Science & Technology*, vol.41, 2007, pp.5582-5588.

146. M. R. Wiesner, G. V. Lowry, P. Alvarez, D. Dionysiou and P. Biswas, Assessing the Risks of Manufactured Nanomaterials, *Environmental Science & Technology*, vol.40, 2006, pp.4336-4345.

147. A. D. Maynard, and R. J. Aitken, Assessing exposure to airborne nanomaterials: Current abilities and future requirements, *Nanotoxicology*, vol.1, no.1, 2007, pp. 26-41.

148. R. D. Handy, F. von der Kammer, J. R. Lead, M. Hassellöv, R. Owen and M. Crane, The ecotoxicology and chemistry of manufactured nanoparticles, *Ecotoxicology*, vol.17, 2008, pp. 287-314.

149. S. Weiss, J. Jakobs and T. Reemtsma, Discharge of three benzotriazole corrosion inhibitors with municipal wastewater and improvements by membrane bioreactor treatment and ozonation, *Environmental Science & Technol*ogy, vol.40, no.23, 2006, pp. 7193-7199.

150. C. A. Harris, E. J. Routledge, C. Schaffner, J. V. Brian, W. Giger, and J. P. Sumpter, Benzotriazole is antiestrogenic in vitro but not in vivo, *Environmental Toxicology and Chemistry*, vol. 26, no. 11, 2007, pp. 2367-2372.

151. B. G. Brownlee, J. H. Carey, G.A. MacInnis and I.T. Pellizzari, Aquatic environmental chemistry of 2-(thiocyanomethylthio) benzothiazole and related benzothiazoles. *Environmental Toxicology and Chemistry*, vol.11, 1992, pp.1153-1168.

152. E. Jover, V. Matamoros, and J.M. Bayona, Characterization of benzothiazoles, benzotriazoles and benzosulfonamides in aqueous matrixes by solid- phase extraction followed by comprehensive two- dimensional gas chromatography coupled to time- of-flight mass spectrometry, *Journal of Chromatography A*, vol.1216, no.18, 2009, pp. 4013-4019.

153. J. A. van Leerdam, A.C. Hogenboom, M.M.E. van der Kooi and P. de Voogt, Determination of Polar 1H-benzotriazoles and benzothiazoles in water by solid phase extraction and liquid chromatography LTQ FT Orbitrap mass spectrometry, *International Journal of Mass Spectrometry*, vol.282, no.3, 2009, pp.99-107.

154. D. Richter, U. Duennbier, G. Massmann, and A. Pekdeger, Quantitative and qualitative determination of three sulfonamides in environmental water samples using liquid chromatography coupled to electrospray tandem mass spectrometry. *Journal of Chromatography A*, vol.1157, 2007, pp.115-121.

155. D. A. Alvarez, and T. L. Jones-Lepp, Sampling and analysis of emerging pollutants, *Water quality concepts, sampling and analyses,*Y. Li and K. Migliaccio, eds., Taylor & Francis Group, 2011, pp. 199-226.

156. J. R. Dean, *Extraction methods for Environmental Analysis*, John Willey & Sons, 1998, pp. 1-22.

157. M. C. Hennion, Sample handling strategies for the analysis of organic compounds in environmental water samples, *Sample Handling and Trace Analysis of Pollutants: Techniques, Applications and Quality Assurance*, D. Barceló, ed., Elsevier Science, 2000, pp. 3-65.

158. S. Yan, S. Subramanian, R. Tyagi, R. Surampalli and T.C. Zhang, Emerging Contaminants of Environmental Concern: Source, Transport, Fate, and Treatment *Practice Periodical of Hazardous, Toxic and Radioactive Waste Management*, vol.14, no.1, 2010, pp. 2-20.

159. M. Farré, L. Kantiani, M. Petrovic, S. Pérez and D. Barceló, Achievements and future trends in the analysis of emerging organic contaminants in environmental samples by mass spectrometry and bioanalytical techniques, *Journal of Chromatography A*, vol. 1259, 2012, pp. 86-99.

160. J-Å. Jönsson and L. Mathiasson, Membrane-based techniques for sample enrichment, *Journal of Chromatography A*, vol. 902, no.1, 2000, pp. 205-225.

161. T. Barri and J-Å. Jönsson, Modern Techniques of Analyte Extraction, *Analytical Measurements in Aquatic Environments*, J. Namieśnik and P. Szefer, eds, Taylor &Francis Group, 2010, pp. 69-94.

162. L. Chimuka, E. Cukrowska, and J. A. Jonsson, Why liquid membrane extraction is an attractive alternative in sample preparation, *Pure and Applied Chemistry,* vol.76, 2004, pp.707–722.

163. L. A. Robbins, Liquid-liquid extraction: A pretreatment process for wastewater, *Chemical Engineering Progress*, vol.76, no.10, 1980, pp.58-61.

164. Y.–F. Cheng, D. J. Phillips, and U. Neue, Simple and rugged SPE method for the determination of tetracycline antibiotics in serum by HPLC using a volatile mobile phase, *Chromatographia,* vol.44, 1997, pp.187-190.

165. C. H. Galdiga and T. Greibrokk, Ultra-trace determination of fluorinated aromatic carboxylic acids in aqueous reservoir fluids using solid-phase extraction in combination with gas chromatography– mass spectrometry, *Journal of Chromatography A,* vol.793, 1998, pp.297-306.

166. L. I. Osemwengie and S. Steinberg, On-site solid-phase extraction and laboratory analysis of ultra-trace synthetic musks in municipal sewage effluent using gas

chromatography-mass spectrometry in the full-scan mode, *Journal of Chromatography A* vol.932, 2001, pp.107-118.

167. T. M. Primus, D. J. Kohler, M. Avery, P. Bolich, M. O. Way, and J. J. Johnston, Novel field sampling procedure for the determination of methiocarb residues in surface waters from rice fields, *Journal of Agricultural and Food Chemistry,* vol. 49, 2001, pp.5706-5709.
168. I. Liska, J. Krupcick, and A. Leclercq, The Use of Solid Sorbents for Direct Accumulation of Organic Compounds from Water Matrices - A Review of Solid-Phase Extraction Techniques *Journal of High Resolution Chromatography*, 1989, vol.12, no.9, 1989, pp.577-590.
169. D. M. Pavlovíc, S. Babíc, A. J. M. Horvat, and M. Kaštelan-Macan, Sample preparation in analysis of pharmaceuticals, *Trends in Analytical Chemistry,* vol. 26, 2007, pp.1062-1075.
170. P. Patnaik, *Handbook of Environmental Analysis- Chemical Pollutants in Air, Water, Soil and Solid Wastes*, Taylor and Francis Group, 2010, pp.49-56.
171. F. Sanchez-Rojas, C. Bosch-Ojeda, and J. M. Cano–Pavon, A review of stir bar sorptive extraction, *Chromatographia,* vol. 69, 2009, pp.79-94.
172. Y. Wang, Y. C. Kwok, Y. He, and H. K. Lee, Application of dynamic liquid-phase microextraction to the analysis of chlorobenzenes in water by using a conventional microsyringe, *Analytical Chemistry,* vol.70, 1998, pp.4610-4614.
173. A. Ballesteros-Gómez, and S. Rubio, Recent Advances in Environmental Analysis, *Analytical Chemistry,* vol. 83, 2011, pp. 4579-4613.
174. M. J. Capdeville, and H. Budzinski, Trace-level analysis of organic contaminants in drinking waters and ground waters, *Trends in Analytical Chemistry,* vol. 30, no.4, 2011, pp. 586-606.
175. M. Petrovic and D. Barceló, Application of liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QqTOFMS) in the environmental analysis, *Journal of Mass Spectrometry,* vol.41, 2006, pp .1259-1267.
176. L. R. Snyder, *Principles of adsorption Chromatography:the separation of nonionic organic compounds,* Marcel Dekker, 1968, pp. 1-143.

177. V. R. Meyer, *Practical High-Performance Liquid Chromatography*, John Wiley & Sons, 2010, pp. 8-12.

178. R. E. Ardrey, *Liquid Chromatography-Mass Spectrometry: An Introduction*, John Wiley & Sons, pp.33-74.

179. B. Xu, F. Wu, X. Zhao and H. Liao, Benzotriazole removal from water by Zn-Al-O binary metal oxide adsorbent: Behaviour, kinetics and mechanism, *Journal of Hazardous Materials,* vol.184 ,2010, pp.147-155.

180. W. Giger, C. Schaffner, and H. E. Kohler, Benzotriazole and Tolytriazole as Aquatic Contaminants. 1. Input and Occurrence in Rivers and Lakes, *Environmental Science & Technology,* vol. 40, 2006, pp. 7186-7192.

181. D. A. Pillard, J. S. Cornell, D. L. DuFresne and M. T. Hernandez, Toxicity of benzotriazole and benzotriazole derivatives to three aquatic species, *Water Research*, vol.35, 2001, pp. 557-560.

182. D. Cancilla, J. Martinez, and G. C. van Aggelen, Detection of aircraft deicing/antiicing fluid additives in a perched water monitoring well at an international airport, *Environmental Science & Technology*, vol. 32, 1998, pp. 3834-3835.

183. D. A. Cancilla, A. Holtkamp, L. Matassa, and X. Fang, Isolation and characterization of microtox-active components from aircraft deicing/anti-icing fluids, *Environmental Toxicology and Chemistry,* vol. 16, 1997, pp. 430-434.

184. D. S. Hart, L. C. Davis, L. E. Erickson, and T. M. Callender, Sorption and partitioning of benzotriazole compounds, *Microchemical Journal,* vol.77, 2004, pp. 9-17.

185. U.S. Environmental Protection Agency (USEPA), Investigation of selected potential environmental contaminants: Benzotriazoles, USEPA 560/2-77-001, Final Report, February, 1977.

186. S. D. Richardson and T.A. Ternes, Water Analysis: Emerging Contaminants and Current Issues, *Analytical Chemistry*, vol.77, 2005, pp.3807-3838.

187. G. Buttiglieri and T. P. Knepper, Removal of Emerging Contaminants in Wastewater Treatment: Conventional Activated Sludge Treatment, *Handbook of Environmental Chemistry*, M. Petrovic and D. Barceló, eds., Springer, vol. 5, Part S/2, 2008, pp.1-35.

188. G. D. Breedveld, R. Roseth, M. Sparrevik, T. Hartnik, and L. J. Hem, Persistence of the de-icing additive benzotriazole at an abandoned airport, *Water, Air & Soil Pollution: Focus*, vol.3, 2003, pp. 91-101.

189. U.S. Environmental Protection Agency (USEPA), Data Collection and development on high production volume (HPV) chemicals. USEPA, Federal Register, 65, 2000, 248. http://www.epa.gov/chemrtk/ts42213.pdf

190. S. Castro, L. C. Davis, and L. E. Erickson, Natural, Cost-Effective, and Sustainable Alternatives for Treatment of Aircraft Deicing Fluid Waste, *Environmental Progress*, vol.24 , no.1, 2005, pp. 26-33.

191. J. S. Cornell, D. A. Pillard, and M. T. Hernandez, Comparative measures of the toxicity of component chemicals in aircraft deicing fluid, *Environmental Toxicology and Chemistry*, vol. 19, 2000, pp. 1465-1472.

192. M. S. Switzenbaum, S. Veltman, D. Mericas, B. Wagoner and T. Schoenberg, Best management practices for airport deicing stormwater, *Chemosphere*, vol.43, 2001, pp.1051-1062.

193. J. Hollingsworth, R. Sierra-Alvarez, M. Zhou, K.L. Ogden and J. A. Field, Anaerobic biodegradability and methanogenic toxicity of key constituents in copper chemical mechanical planarization effluent of the semiconductor industry, *Chemosphere*, vol.59, 2005, pp.1219-1228.

194. V. Verheyen, A. Cruickshank, K. Wild, M.W. Heaven, R. McGee, M. Watkins and D. Nash, Soluble, semi-volatile phenol and nitrogen compounds in milk-processing wastewaters, *Journal of Dairy Science*, vol.92, 2009, pp.3484-3493.

195. L. J. Hem, M. Weideborg and E. Schram, Degradation and toxicity of additives to aircraft de-icing fluids; the effects of discharge of such fluids to municipal treatment plants, *Proceedings 2000 WEF and Purdue University Industrial Wastes Technical Conference*, Alexandria, VA. 2000, pp. 360-374.

196. G. Rollinson and A. G. Callely, No evidence for the biodegradation of benzotriazole by elective culture and continuous enrichment, *Biotechnology Letters*, vol.8, 1986, pp.303-304.

197. R. Andreozzi, V. Caprio, A. Insola, and G. Longo, Photochemical degradation of benzotriazole in aqueous solution, *Journal of Chemical Technology and Biotechnology*, vol. 73, 1998, pp. 93-98.

198. L. J. Hem, T. Hartnik, R. Roseth and G. D. Breedveld, Photochemical degradation of benzotriazoles, *Journal of Environmental Science and Health, A*, vol.38, 2003, pp.471-481.

199. L. J. Hem, and M. Weideborg, Degradation and toxicity of additives to deicing fluids. Test performed to see the effect of discharge of water containing deicing fluids to Gardemoen wastewater treatment plant, *Aquateam-Norwegian Water Technology Centre* A/S, Oslo, Norway, Report 99-016, 1999.

200. D. A. Cancilla, J. C. Baird, S. W. Geis, and S. R. Corsi, Studies of the environmental fate and effect of aircraft deicing fluids: Detection of 5-methyl-1H-benzotriazole in the fathead minnow (*Pimephales promelas), Environmental Toxicology and Chemistry,* vol. 22, 2003, pp. 134-140.

201. E. Kadar, S. Dashfield and T.H. Hutchinson, Developmental toxicity of benzotriazole in the protochordate *Ciona intestinalis* (Chordata, Ascidiae), *Analytical and Bioanalytical Chemistry,* vol.396, 2010, pp.641-647.

202. D. A. Pillard, Comparative toxicity of formulation glycol deicers and pure ethylene and propylene glycol to *Ceriodaphnia dubia* and *Pimephales promelas*, *Environmental Toxicology and Chemistry,* vol.37, 1995, pp.29-35.

203. D. J. Fisher, M. H. Knott, S. D. Turley, B. S. Turley, L. T. Yonkos, and G. P. Zeigler, The acute whole effluent toxicity of storm water from an international airport, *Environmental Toxicology and Chemistry*, vol. 14, 1995, pp. 1103-1111.

204. S. I. Hartwell, D. M. Jordahl, J. E. Evans, and E. B. May, Toxicity of aircraft deicer and anti-icing solutions to aquatic organisms, *Environmental Toxicology and Chemistry,* vol. 14, 1995, pp. 1375-1386.

205. H. Tangtian, L. Bo, L. Wenhua, P.K.S. Shin and R.S.S. Wu, Estrogenic potential of benzotriazole on marine medaka (*Oryzias melastigma*), *Ecotoxicology and Environmental Safety*, vol. 80, 2012, pp. 327–332.

206. D. A. Pillard and D. DuFresne, Toxicity of formulated glycol deicers and ethylene and propylene glycol to *Lactuca sativa, Lolium perenne, Selenastrum capricornutum* and

Lemma minor, Archives of Environmental Contamination and Toxicology, vol. 37, 1999, pp.29-35.

207. S. Castro, L. C. Davis, and L. E. Erickson, Plant-enhanced remediation of glycol-based aircraft deicing fluids, *Practice Periodical of Hazardous, Toxic, Radioactive Waste Management*, vol. 5, 2001, pp. 141-152.

208. A. Kloepfer, J.B. Quintana and T. Reemtsma, Operational options to reduce matrix effects in liquid chromatography-electrospray-mass spectrometry analysis of aqueous environmental samples, *Journal of Chromatography A*, vol.1067, 2005, pp.153-160.

209. C. Domínguez, C. Reyes-Contreras and J. M. Bayona, Determination of benzothiazoles and benzotriazoles by using ionic liquid stationary phases in gas chromatography mass spectrometry. Application to their characterization in wastewaters, *Journal of Chromatography A*, vol.1230, 2012, pp.117– 122.

210. H. De Wever, P. Besse, and H. Verachtert, Microbial transformations of 2-substituted benzothiazoles, *Applied Microbiology and Biotechnology*, vol. 57, 2001, pp. 620 – 625.

211. C. Reddy and J. G. Quinn, Environmental Chemistry of Benzothiazoles Derived from Rubber, *Environmental Science & Technology*, vol. 31, 1997, pp. 2847-2853.

212. T. Reemtsma, O. Fiehn, G. Kalnowski and M. Jekel, Microbial Transformation and Biological Effects of Fungicide-Derived Benzothiazoles Determined in Industrial wastewater, *Environmental Science & Technology*, vol.29, 1995, pp. 478-485.

213. A. J. Hendricks, J. L. Maasdiepeveen, A. Noordsu and M. A.Van der Gaag, Monitoring response of XAD-concentrated water in the Rhine delta: a major part of the toxic compounds remains unidentified, *Water Research*, vol.28, no.3, 1994, pp.581-598.

214. OECD (Organisation for Economic Co-operation and Development), Screening Information Data Set for High Production Volume Chemicals, *UNEP Chemicals*, vol. 2, 1994, p.28.

215. D. Richter, Duennbier and G. Massmann, Identification and significance of sulfonamides (p-TSA, o-TSA and BSA) in an urban water cycle (Berlin, Germany), *Water Research*, vol.42, 2008, pp.1369-1378.

216. K. E. Haneke, Chloramine-T [127-65-1] and p-toluenesulfonamide [70-55-3]-Review of Toxicological Literature, Integrated Laboratory Systems, 2002, pp. 1-68. http://ntp-server.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/ChloramineT.pdf

217. J. O. Harris, M. D. Powell, M. Attard and T. J. Green, Efficacy of chloramine-T as a treatment for amoebic gill disease (AGD) in marine Atlantic salmon (Salmo salar L.), *Aquaculture Research*, vol.35, no.15, 2004, pp.1448-1456.

218. D. Richter, G.Massmann, T. Taute and U. Duennbier, Investigation of the fate of sulfonamides downgradient of a decommissioned sewage farm near Berlin, Germany, *Journal of Contaminant Hydrology*, vol.106, 2009, pp.183-194.

219. D. Richter, G. Massmann and U. Duennbier, Behaviour of sulphonamides (p-TSA, o-TSA, BSA) during purification of contaminated groundwater used for the drinking water supply, *Chemosphere*, vol.71, 2008, pp.1574–1581.

220. A. M. Peck, Analytical methods for the determination of persistent, ingredients of personal care products in environmental matrices, *Analytical and Bioanalytical Chemistry*, vol.386, 2006, pp.907-939.

221. M. C. Pietrogrande and G. Basaglia, GC-MS analytical methods for the determination of personal-care products in water matrices, *Trends in Analytical Chemistry*, vol.26, 2007, pp.1086-1094.

222. A. C. Hogenboom, J. A. van Leerdam and P. de Voogt, Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-hybrid linear ion trap Orbitrap mass spectrometry, *Journal of Chromatography A*, vol. 1216, no.3, 2009, pp.510-519.

223. D. Voutsa, P. Hartmann, C. Schaffner and W. Giger, Benzotriazoles, Alkyphenols and Bisphenol A in Municipal Wastewaters and in the Glatt River, Switzerland, *Environmental Science and Pollution Research*, vol.13, no.5, 2006, pp.333-341.

224. V. Matamoros, E. Jover, and J. M. Bayona, Occurrence and fate of benzothiazoles and benzotriazoles in constructed wetlands, *Water Science and Technology*, vol.61, no.1, 2010, pp.191-198.

225. S. Weiss and T. Reemtsma, Membrane bioreactors for municipal wastewater treatment-A viable option to reduce the amount of polar pollutants discharged into surface waters? *Water Research*, vol. 42, 2008, pp. 3837- 3847.

226. K. S. McNeill and D.A. Cancilla, Detection of Triazole Deicing Additives in Soil Samples from Airports with Low, Mid, and Large Volume Aircraft Deicing Activities, *Bulletin of Environmental Contamination and Toxicology*, vol.82, 2009, pp.265-269.

227. Z. Zhang, N. Ren, Y. Li, T. Kunisue, D. Gao and K. Kannan, Determination of Benzotriazole and Benzophenone UV Filters in Sediment and Sewage Sludge, *Environmental Science &Technology,* vol.45, 2011, pp.3909-3916.

228. Y. S. Liu, G. G. Ying, A. Shareef and R. S. Kookana, Simultaneous determination of benzotriazoles and ultraviolet filters in ground water, effluent and biosolid samples using gas chromatography-tandem mass spectrometry, *Journal of Chromatography A*, vol. 1218, no.31, 2011, pp. 5328-5335.

229. I. Carpinteiro, B. Abuin, M. Ramil, I. Rodriguez, and R. Cela, Simultaneous determination of benzotriazole and benzothiazole derivatives in aqueous matrices by mixed-mode solid-phase extraction followed by liquid chromatography-tandem mass spectrometry, *Analytical and Bioanalytical Chemistry,* vol. 402, 2012, pp. 2471-2478.

230. M. T. Pena, X. Vecino-Bello, M. C. Casais, M. C. Mejuto and R. Cela, Optimization of a dispersive liquid-liquid microextraction method for the analysis of benzotriazoles and benzothiazoles in water samples, *Analytical and Bioanalytical Chemistry,* vol.402, 2012, pp.1679-1695.

231. S. Montesdeoca-Esponda, Z. Sosa-Ferrera, and J. J. Santana-Rodríguez, On-line solid-phase extraction coupled to ultra-performance liquid chromatography with tandem mass spectrometry detection for the determination of benzotriazole UV stabilizers in coastal marine and wastewater samples, *Analytical and Bioanalytical Chemistry,* vol.403, 2012, pp.867-876.

232. L. Kovalova, H. Siegrist, H. Singer, A. Wittmer and C. S. McArdell, Hospital Wastewater Treatment by Membrane Bioreactor: Performance and Efficiency for organic micropollutant Elimination, *Environmental Science & Technology,* vol.46, 2012, pp.1536-1545.

233. M. Rezaee, M. Assadi, M.R.M. Hosseini, E. Aghaee, F. Ahmadi and S. Berijiani, Determination of organic compounds in water using dispersive liquid–liquid microextraction, *Journal of Chromatography A,* vol.1116, 2006, pp. 1-9.

234. M. G. Pervova, V. E. Kirichenko and V. I. Saloutin, 2010. Determination of 1,2,3-benzotriazole in Aqueous Solutions and Air by Reaction-Gas-Liquid Chromatography, *Journal of Analytical Chemistry*, vol.65, no.3, 2010, pp.276-279.

235. A. Kiss and E. Fries, Occurrence of benzotriazoles in the rivers Main, Hengstbach, and Hegbach (Germany), *Environmental Science and Pollution Research,* vol.16, 2009, pp.702-710.

236. V. Matamoros, E. Jover and J.P. Bayona, Part-per-Trillion Determination of Pharmaceuticals, Pesticides, and Related Organic Contaminants in River Water by Solid-Phase Extraction Followed by Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry, *Analytical Chemistry*, vol.82, no.2, 2010, pp.699-706.

237. Y. S. Liu, G. G. Ying, A. Shareef and R. S. Kookana, Occurrence and removal of benzotriazoles and ultraviolet filters in a municipal wastewater treatment plant, *Environmental Pollution,* vol.165, 2012, pp. 225-232.

238. R. Loos, G. Locoro, S. Comero, S. Contini, D. Schwesig, F. Werres, P. Balsaa, O. Gans, S. Weiss, L. Blaha, M. Bolchi and B.M. Gawlic, Pan European survey on the occurrence of selected polar organic persistent pollutants in groundwater, *Water Research,* vol.44, no.14, 2010, pp. 4115-4126.

239. H. Ni, H. Zeng and E. Y Zeng, Sampling and Analytical framework for routine and environmental monitoring of organic pollutants, *Trends in Analytical Chemistry,* vol. 30, no.10, 2011, pp.1549-1559.

240. J. L. Tadeo, C. S□nchez-Brunete, B. Albero, A. I. García-Valc□rcel and R.A. Pérez, Analysis of emerging organic contaminants in environmental solid samples, *Central European Journal of Chemistry*, vol.10, no.3,2012, pp.480-520.

241. H. Wolschke, Z. Xie, A. Möller, R. Sturm, and R. Ebinghaus, Occurrence, distribution and fluxes of benzotriazoles along the German large river basins into the North Sea, *Water Research,* vol. 45, no.18, 2011, pp.6259- 6266.

242. A. Kiss and E. Fries, Seasonal source influence on river mass flows of benzotriazoles, *Journal of Environmental Monitoring*, vol. 14, no.2, 2012, pp. 697-703.

243. C. L. Gruden S. M. Dow and M. Hernandez, Fate and toxicity of aircraft deicing fluid additives through anaerobic digestion, *Water Environmental Research*, vol.73, 2001, pp.72-79.

244. M. Cirja, P. Ivashechkin, A. Schäffer and P. F. X. Corvini, Factors affecting the removal of organic micropollutants from wastewater in conventional treatment plants (CTP) and membrane bioreactors (MBR), *Reviews of Environmental Science and Biotechnology*, vol. 7, 2008, pp. 61-78.

245. T. Reemtsma, Determination of 2-substituted benzothiazoles of industrial use from water by liquid chromatography/electrospray ionization tandem mass spectrometry, *Rapid Communication in Mass Spectrometry*, vol.14, 2000, pp.1612-1618.

246. A. Kloepfer, M. Jekel and T. Reemtsma, Determination of benzothiazoles from complex aqueous samples by liquid chromatography-mass spectrometry following solid-phase extraction, *Journal of Chromatography A*, vol.10, 2004, pp.81-88.

247. H. Ni, F. Lu, X. Luo, H. Tian and E.Y. Zeng, Occurrence, Phase Distribution and Mass loadings of Benzothiazoles in Riverine Runoff of the Pearl River Delta, China, *Environmental Science & Technology,* vol.42, 2008, pp.1892-1897.

248. J. S. Warner, T.M. Engel and P.J. Mondron, Determination of MBTS and TCMTB in industrial and municipal wastewaters, *NTIS*, PB 85-189025 (1985)

249. O. Fiehn, T. Reemtsma and M. Jekel, Extraction and analysis of various benzothiazoles from industrial wastewater, *Analytica Chimica Acta*, vol.295, 1994, pp.297-305.

250. R. Céspedes, S. Lacorte, A. Ginebreda and D. Barceló, Chemical monitoring and occurrence of alkylphenols, alkylphenol ethoxylates, alcohol ethoxylates, phthalates and benzothiazoles in sewage treatment plants and receiving waters along the Ter River basin (Catalonia, N. E. Spain), *Analytical and Bioanalytical Chemistry*, vol. 385, 2006, pp. 992-1000.

251. A. Wick, G. Fink and T.A. Ternes, Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters, and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A.*, vol.1217, 2010, pp. 2088-2103.

252. E. Fries, T. Gocht, and J. Klasmeier, Occurrence and distribution of benzothiazole in the Schwarzbach watershed (Germany), *Journal of Environmental Monitoring*, vol 13, 2011, pp. 2838-2843

253. E. Fries, Determination of Benzothiazole in untreated wastewater using polar-phase stir bar sorptive extraction and gas chromatography-mass spectrometry, *Analytica Chimica Acta*, vol. 689, 2011, pp. 65-68.

254. G. A. Jungclaus, L. M. Games and R. A. Hites, Organic compounds in an industrial wastewater: A case study of their environmental impact, *Environmental Science & Technology,* vol.12, 1978, pp.88-96.

255. T. Reemtsma, B. Zywicki, M. Stueber, A. Kloepfer and M. Jekel, Removal of Sulfur-Organic Polar Micropollutants in a Membrane Bioreactor Treating Industrial Wastewater, *Environmental Science & Technology*, vol.36, 2002, pp.1102-1106.

256. C. Hartig, and M. Jekel, Analysis of aromatic sulphonamides in biological and aquatic matrices, *Vom Wasser*, vol. 96, 2001, pp. 131-158.

257. T. P. Knepper, F. Kirchhöfer, I. Lichter, A. Maes, and R-D .Wilken, Metabolism Studies of Phenylsulfonamides Relevant for Water Works, *Environmental Science & Technology*, vol.33, 1999, pp. 945-950.

258. A. Stasinakis, G. Gatidou, D. Mamais, N. S. Thomaidis and T. D. Lekkas, Occurrence and fate of endocrine disrupters in Greek sewage treatment plants, Water Research, vol. 42, 2008, pp. 1796-1084.

259. Atlantis T3 and Acquity UPLC HSS T3 Columns, pp.1-6, http://www.waters.com/webassets/cms/library/docs/720001887en.pdf last access on 27-11-12)

260. M. Scheurer, F. R. Storck, C. Graf, H. Brauch, W. Ruck, O. Lev and F. T. Lange, Correlation of six anthropogenic markers in wastewater, surface water, bank filtrate, and soil aquifer treatment, *Journal of Environmental Monitoring,* vol.9, 2011, pp.1154-1161.

261. Y. S. Liu, G. G. Ying, A. Shareef and R. S. Kookana, Biodegradation of three selected benzotriazoles under aerobic and anaerobic conditions, *Water Research*, vol.45, 2011, pp. 5005-5014.