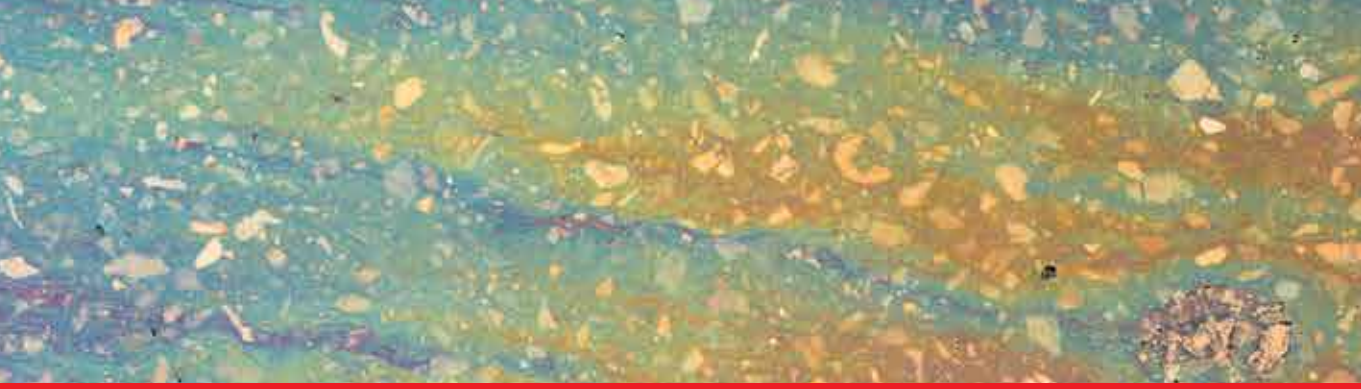

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Emerging Pollutants in the Environment

Current and Further Implications

Edited by Marcelo L. Larramendy and Sonia Soloneski

EMERGING POLLUTANTS IN THE ENVIRONMENT - CURRENT AND FURTHER IMPLICATIONS

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and **Sonia Soloneski**

Emerging Pollutants in the Environment - Current and Further Implications
http://dx.doi.org/10.5772/59332
Edited by Marcelo L. Larramendy and Sonia Soloneski

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Notice

First published in Croatia, 2015 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Emerging Pollutants in the Environment - Current and Further Implications
Edited by Marcelo L. Larramendy and Sonia Soloneski

p. cm.

ISBN 978-953-51-2160-2

eBook (PDF) ISBN 978-953-51-5404-4

Meet the editors

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Contents

Preface

In 2008, Farré and collaborators introduced the concept of **Emerging Pollutants** as those compounds that are not currently covered by existing water-quality regulations and which may be candidates for future regulation, depending on their toxicity, potential health effects, occurrence in various environmental matrices, and public perception, because they represent a potential threat to environmental ecosystems and human health and safety.

The concept of emerging pollutants primarily refers to those contaminants for which no regulations currently require monitoring or public reporting of their presence in our water supply or wastewater discharges. Many congeners described as emerging pollutants are made up of a wide variety of chemicals like pharmaceuticals and household chemicals, which may enter the environment through human and animal urine and feces, the flushing of unused medications, household uses, or bathing and may result in concentrations as low as nanograms per liter to as high as micrograms per liter. Many contaminants are increasingly detected due to improvements in detection methods, an important aspect for advancing the overall understanding of emerging pollutants. It is worth mentioning that the release of pharmaceuticals from manufacturing facilities is regulated in most developed countries and is therefore not a major contributor of these constituents to the environment, though this is not the case over the majority of our planet.

The most important sources of environmentally relevant emerging pollutants are, as expected, primarily wastewater treatment plant effluents and, secondarily, terrestrial runoffs, e.g., from roofs, pavements, roads, and agricultural land, including atmospheric deposition, among others. Furthermore, it has been observed that one of the major characteristics of some types of these contaminants is that they do not need to be persistent in the environment to exert negative effects. It is well known, at least for the majority of them, that the high transformation/removal rates they possess are compensated by their continuous introduction into the environment. Accordingly, for most emerging pollutants, risk assessment and ecotoxicological data are still an open question. It is therefore difficult to predict which jeopardizing health effects may be exerted not only for humans but also for other terrestrial and aquatic organisms and the ecosystems where those organisms are immersed.

Emerging pollutants reach our environment through various anthropogenic sources and activities, and are distributed throughout different environmental matrices. It is well known that these contaminants are commonly derived from municipal, agricultural, and industrial wastewater sources. These recently recognized contaminants represent a shift in traditional thinking, since many of them are produced by industries and then dispersed to the environment through domestic, commercial, and industrial applications. During the last decades, great advances have been achieved not only in the detection but also the analysis of trace

pollutants worldwide. However, due to the continued development of new and more refined and specific methodologies, a wide array of previously undetected pollutants with emerging environmental concern require determination and quantification in various environmental components as well as in different biological matrices and different compartments of living exposed organisms.

An increasing number of solid field studies designed to provide basic scientific information concerning the occurrence and potential transport of contaminants in the environment are being continuously conducted worldwide to identify which contaminants enter the environment, at what concentrations, and in what combinations. Research has been focused on contaminants of priority and the most well-known pollutants worldwide, such as pesticides, toxic metals, and radionuclides, among others, though fortunately, in recent years the attention of the scientific community has shifted to emerging contaminants. Therefore, a major challenge for our scientific community nowadays is to identify and further characterize those chemicals that will potentially become dangerous to the environment in the near future.

Caution should be taken with emerging pollutants. These xenobiotics may be mobile and persistent in air, water, soil, sediments, and a large battery of ecological receptors, even at very low concentrations. Important data on their fate and behavior in the environment as well as on threats to both ecological and human health are documented; however, robust and comprehensive analyses are still lacking. Furthermore, the ecotoxicological significance of a large number of emerging pollutants, including those so-called micropollutants, remains largely unknown since convincing and/or satisfactory data to determine their risk often do not exist, or that risk has been only partially suggested or demonstrated.

Our awareness and scientific understanding of the potential effects and risk of emerging pollutants is improving. Research is being documented with increasing frequency, showing that many chemical and microbial constituents that have not historically been considered as contaminants are present in the environment on a worldwide scale. This book will provide the latest findings on those pollutants and their possible effects, not only on our environment but also on living organisms, including human beings. Finally, we hope this book will answer them or at least provide some clues to the inherent potential risk of the presence of these types of contaminants in our environment.

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Section 1

Emerging Pollutants in the Environment - Current and Further Implications

Chapter 1

Ecological Risk and Distribution of Polychlorinated Biphenyls in Fish

T. Brázová, V. Hanzelová and P. Šalamún

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60405

Abstract

The distribution and concentrations of polychrolinated biphenyls (PCBs) were determined in nine freshwater fish species and the parasite *Acanthocephalus lucii* in Zemplínska šírava, a heavily polluted water reservoir in Slovakia. The study performed at two different time points five years apart (2004 and 2009) revealed excessive PCB contamination of the fish muscle tissue and significant interspecies as well as tissue-specific differences in PCB uptake by fish. Total PCBs broadly correlated with the trophic position of individual fish species within a food chain ($P < 0.01$). The concentrations were particularly high in predatory fish species, perch, pike and pike-perch (108.0, 90.1, 113.0 $mg.kg^{-1}$ lipid wt, respectively), but comparable PCB values were also found in non-predatory detritivorous freshwater bream (128.0 $mg.kg^{-1}$ lipid wt). Tissue-specific differences in PCB concentrations were observed: liver > adipose tissue > muscles > hard roe > bones > brain. Maximum concentrations of PCBs were recorded in the liver of northern pike (214.0 $mg.kg^{-1}$ lipid wt) and the hepatopancreas of freshwater bream (163.0 $mg.kg^{-1}$ lipid wt). Individual congeners were not distributed homogeneously within the investigated organs and the adipose tissue. PCB 153 was present in the higher concentrations than other congeners in all fish organs as well as the adipose tissue. Acanthocephalans absorbed significantly higher concentrations of PCBs ($P<0.001$) than the muscles, liver, kidney, brain and adipose tissue of their host. About 20-fold lower PCB levels was detected in the liver and almost three times in the muscles of infected perch. Data on PCB accumulation in perch infected with acanthocephalans demonstrated a decline of PCB values in all organs as well as the adipose tissue compared to uninfected fish. The study has shown that the fish species, its feeding habit and specific conditions of the habitat are mutually interrelated factors that are responsible for significant variations in fish body burdens.

Keywords: polychlorinated biphenyls, pollution, fish, parasites, water reservoir Zemplínska šírava

1. Introduction

The ability of persistent organic pollutants (POPs) to spread through long distances and remain in the environment has resulted in their presence worldwide. They have a tendency to accumulate within the food chain and, as a result, pose a high risk to human and animal health. The risks caused by industrial POPs are best illustrated by polychlorinated biphenyls (PCBs), extensively used in a wide variety of applications from the early 20th century. PCBs became ubiquitous contaminants of various biotic and abiotic environments worldwide due to their massive and uncontrolled use in industry and agriculture [1, 2].

PCBs are highly persistent compounds in the environment, especially in aquatic sediments, which act as a stable reservoir from which PCBs can continue to be released over a long period of time, because of their low solubility in water and low volatility [3]. The involvement of PCBs in the food chain occurs through the incorporation of suspended particles in phytoplankton and zooplankton, at the base of the food chain. Bottom feeders and other aquatic organisms ingest, accumulate and pass PCBs upward in the food chain [4, 5]. Fish near the top of the aquatic food web have a relatively long life span and concentrate high amounts of PCBs [6]. The concentration of poorly metabolized chemicals accumulated in fish can thus reflect the degree of pollution in an aquatic system [7, 8].

Biomonitoring is a vital and rapidly growing field that uses several biological groups, such as phytoplankton, macrophytes, invertebrates and fish, as bioindicators [9]. This ecological methodology is increasingly used to assess the level of pollution in aquatic environments [10–13]. Numerous studies have also documented the close relationship between aquatic pollution and parasitism [e.g. 13, 14]. Certain parasitic organisms have the ability to concentrate high quantities of pollutants in their tissues and organs and thus can provide information about the chemical state of the environment [15, 16]. These studies have mainly focused on intestinal parasites, mostly acanthocephalans, as indicators of heavy-metal pollution [14, 17, 18], but data on organic pollutants, including PCBs and their bioaccumulation in parasitic organisms, are still scarce.

The Zemplínska šírava water reservoir (eastern Slovakia; Fig. 1) is one of the most PCB-contaminated sites in Europe [19, 20]. Large amounts of PCB compounds have been released to the reservoir from a chemical factory in the nearby town of Strážske without treatment (decontamination) or preventive measures, leading to heavy contamination of soil, superficial and underground water and subsequently food chains in this area [21]. The PCBs in the reservoir and the inflowing Šíravský canal thus belong to the so-called old environmental burdens, and their amount varies from tens to hundreds of milligrams per kilogram of sediment dry weight. At least 40, 000 tons of PCB-contaminated sediments are assumed to be

http://dx.doi.org/10.5772/60405

still present in the reservoir, effluent canal and Laborec River [22]. Studies over the past 20 years have indicated that elevated concentrations of PCBs in the reservoir pose a potential hazard to human and aquatic health [19, 23].

2. Material and methods

2.1. Study area

The Zemplínska šírava reservoir (48°47′32″N; 22°0′42″E), one of the largest artificial water-storage sites in Slovakia, is located in the eastern part of the country (Fig. 1).

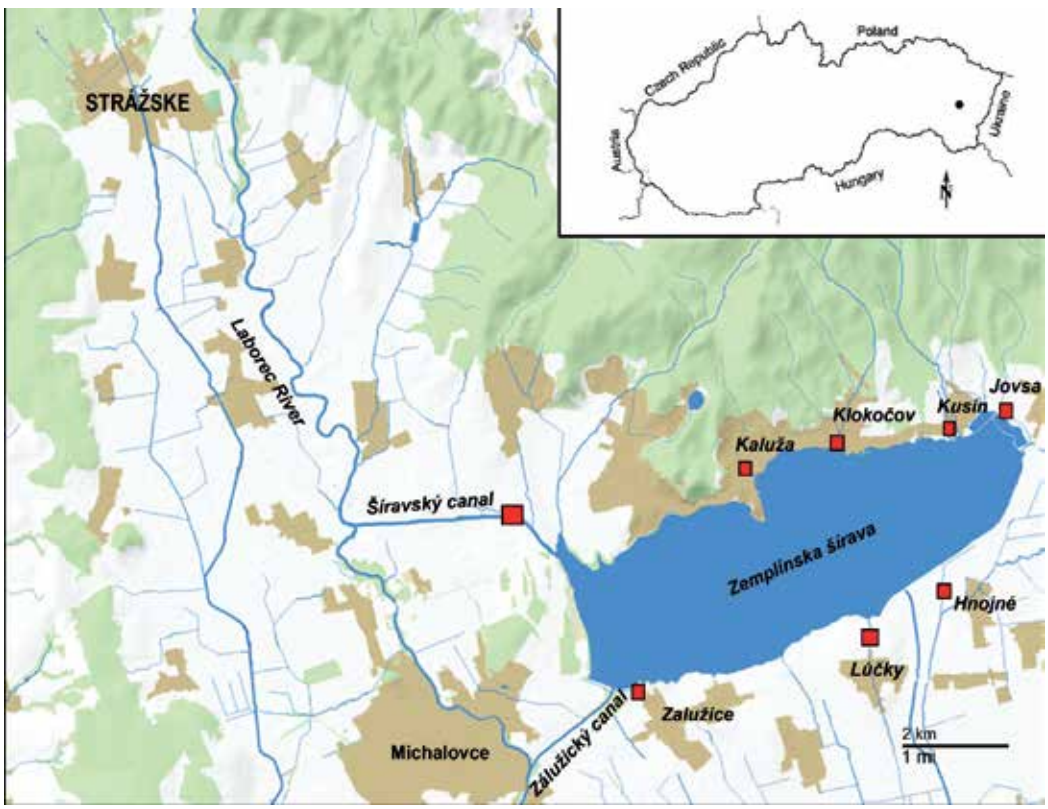

Figure 1. Location of the sampling sites in the Zemplínska šírava reservoir.

It was built between 1961 and 1965 for regulating flood overflows from the Laborec River and partly for irrigation purposes in the area. The reservoir is 11 km long, ca. 3.5 km wide and covers an area of 33 km². PCB compounds from the chemical factory in Strážske were released into the Strážsky (effluent) canal to the Laborec River and from there by the Šíravský (influent) canal to the Zemplínska šírava reservoir (Fig. 1). The reservoir has abundant ichthyofauna,

represented predominantly by dense populations of common carp, *Cyprinus carpio* L.; freshwater bream, *Abramis brama* L.; pike-perch, *Sander lucioperca* L.; northern pike, *Esox lucius* L.; European perch, *Perca fluviatilis* L.; Wels catfish, *Silurus glanis* L. and goldfish, *Carassius auratus* L. The PCBs in fish were monitored twice, first in 2004 and then in 2009, at eight sites in the reservoir (Fig. 1).

2.2. Fish and parasite collection

A total of 50 fish of nine species belonging to five families (Anguillidae, Cyprinidae, Esocidae, Percidae and Siluridae) were collected. The distribution and concentrations of PCBs were determined in predatory (*P. fluviatilis*, *E. lucius*, *S. lucioperca* and *S. glanis*) and non-predatory (*C. carpio*; the European eel, *Anguilla anguilla*; *Ab. brama*; *C. auratus* and the roach, *Rutilus rutilus*) species. The fishes were transported to the laboratory alive, weighed, measured and divided into feeding guilds [24, 25] (Table 1).

Fish species	*N*		Major food item	Trophic level*	Number of samples						
	2004	2009			Musc	Liv	Kidn	Hroe	AT	Bone	Brain
Predators											
European perch	0	16	Zoobenthos, nekton	3.2–4.4	6	5	5	–	3	–	5
Northern pike	1	2	Nekton	3.8–4.5	2	2	2	1	1	1	2
Pike-perch	2	3	Nekton	4.3	3	3	3	–	3	–	2
Wels catfish	0	3	Nekton	4.3–4.4	2	2	2	1	1	1	2
Non-predators											
Common carp	2	1	Zoobenthos	2.1–3.1	1	1	1	–	1	1	1
European eel	7	0	Zoobenthos	2.3–3.5							
Freshwater bream	5	1	Zoobenthos	2.9–3.1	1	1	–	1	1	1	1
Goldfish	1	5	Zoobenthos	2.0	5	5	5	1	–	2	5
Roach	1	0	Zoobenthos, plants	2.3–3.4	1	–	–	–	–	–	–

N, number of fish examined.

* Trophic level was assessed as in [25].

Table 1. Number of fish and tissue/organ samples examined, food items and trophic levels. *Abbreviations*: Musc, muscle; Liv, liver; Kidn, kidney; Hroe, hard roe; AT, adipose tissue (Data from [26, 27]).

Only muscle tissues (19 samples) were collected in 2004. A more detailed study in 2009 focused on the tissue-specific distribution of PCBs to assess the temporal variations of PCBs in the

reservoir. Samples of the liver (hepatopancreas in cyprinids), kidney, adipose tissue, hard roe, bone and brain were collected from 31 fish belonging to seven species [26].

Only perch were infected with parasites and so received special attention. Twelve of 16 perch in a single catch were small, and therefore the tissue/organ samples from these fish were pooled by the weight of the fish: up to 10 g (n=7) and 14–30 g (n=5). The four remaining fish weighed 60–130 g and were analysed separately. A total of 24 samples of muscles, livers, kidneys, adipose tissues and brains were examined for the presence of PCBs (Table 1). Screening of the digestive tracts for parasites using a stereomicroscope found only one helminth species, the acanthocephalan *Acanthocephalus lucii*. The two largest perch (>120 g) contained 50 and 19 acanthocephalans. The remaining fish were either free of acanthocephalans or their infections were low, mostly 1–2 acanthocephalans per fish in the 10–30 g group. Perch weighing 60 g contained a maximum of five parasites. Perch harbouring such low parasite burdens were considered as uninfected for the comparison of PCB accumulation in infected versus uninfected perch. The parasites were washed in saline, frozen and subsequently examined for PCBs. A total of 95 tissue/organ samples of all fish species (Table 1) and two samples of acanthocephalan parasites were analysed spectrophotometrically [27]. The scientific and common names of the fish match those in the FishBase database [25].

2.3. Analytical procedure

The extraction and clean-up of the samples followed the methods described by Himberg et al. and Fisher and Ballschmiter [28, 29], with slight modifications. Briefly, the fish and parasite samples were homogenized in anhydrous sodium sulfate and extracted with a mixture of petroleum ether (90%) and acetone (10%) using a separation funnel. The extracts were concentrated in a rotary evaporator and then purified using a Florisil chromatographic column according to STN EN 12393-2 (2001). The final extracts were analysed on a 6890 gas chromatograph (Hewlett-Packard) equipped with and electron capture detector. The HP-5 capillary column was 30 m in length, with an i.d. of 0.25. and having a film thickness of 0.25 μm. The chromatograph was operated at an injector temperature of 250°C and a detector temperature of 300°C and used helium as the carrier gas at a flow rate of 1.4 ml min^{-1}. The following oven temperature program was used: start temperature 80°C for 1 min, 80–180°C at 30°C min^{-1} and maintained for 1 min, 185–205 °C at 6°C min^{-1} and maintained for 15 min and 205–290 °C at 20°C min^{-1} and maintained for 7.5 min. Individual PCB congeners were identified by their comparison with the retention times of known standards and qualified by comparing the peak areas to the appropriate peaks in the standard mixture (PCB Mix C-SCA-06). The extracts were injected under a splitless mode. The recovery rates of the PCBs in the spiked samples were 80–95%, whereas the detection limits were 1 μg kg^{-1} based on wet weight. Six PCB indicator congeners – PCB 28, 52, 101, 138, 153 and 180 – were analysed. All PCB concentrations in the biological samples are given in mg kg^{-1} lipid weight (lipid wt).

2.4. Statistical analysis

The data obtained did not meet the requirements for parametric statistical tests (normality). So they were analysed non-parametrically or were log-transformed [ln (x + 1)] to satisfy the

assumptions (normal distribution and homogeneity of variances) of analyses of variance (ANOVAs). Differences in PCB load between years and between fish tissues/organs and parasites were determined by *t*-tests. The effects of trophic strategy (predatory and non-predatory fish) and tissue/organ type on PCB levels were tested with two-way ANOVAs. Main-effect ANOVAs were used instead of factorial ANOVAs because no significant interactions were confirmed between the factors. The data were consequently divided into two groups, predatory and non-predatory fish, and were processed separately. The concentrations are expressed as mean ± SD (standard deviation). Differences in mean concentrations (mg kg^{-1} lipid wt) of the congeners and total PCBs in the fish tissues/organs (muscle, liver, kidney, brain, adipose tissue and bone for predatory/non-predatory fish) were tested with a non-parametric Kruskal-Wallis ANOVA with post-hoc multiple comparison. The analyses were performed in Statistica for Windows, version 9.0 [30].

3. Results and discussion

3.1. PCB distribution associated with fish species and feeding habit

The total PCB concentrations (the sum of the six PCB congeners) in the muscle tissues varied greatly in the nine fish species collected in 2004 and 2009 from the Zemplínska šírava reservoir (Fig. 2).

Total PCB concentrations were generally associated with the trophic position of the species within a food chain. In the predatory species, pike and pike-perch, the concentrations in both years were particularly high, which was expected for the highest trophic level (Table 1). The bottom-feeding and detritivorous non-predatory freshwater bream that obtains nutrients directly from the sediments exposed to PCBs, however, had comparable PCB concentrations. In contrast, the common carp that also feeds from the bottom sediments surprisingly had the lowest PCB concentrations, along with the European eel with a benthic feeding habit and a high body-fat content (Fig. 2).

PCBs clearly tended to bioaccumulate in the muscle tissues of some species (freshwater bream, pike and pike-perch) during the five years (2004–2009) between analyses (Fig. 2). PCB concentrations increased significantly (t=-2.43, df=13, P<0.05) in these species and more than two-fold in freshwater bream. The opposite, but not significant, trend was seen in two cyprinids (common carp and goldfish), in which the PCB concentrations were lower in 2009 than in 2004 (Fig. 2).

3.2. PCB distribution associated with fish tissues/organs

The concentrations of the PCB congeners in the tissues/organs of both predatory and non-predatory fish were high (Table 2).

The liver was the major target organ for PCBs in the predatory fish (Table 2), and it received the largest proportion (24%) of the overall contaminant burden. The PCB concentrations (lipid

http://dx.doi.org/10.5772/60405

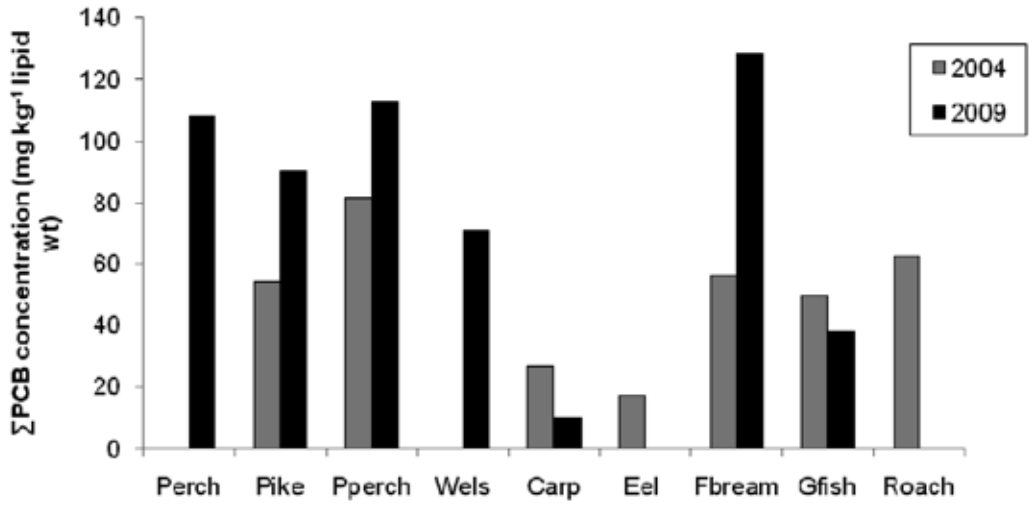

Figure 2. Total PCB concentrations (∑PCB) in the muscles of the various fish species from the Zemplínska šírava reservoir in 2004 and 2009. Perch and Wels catfish for 2009 and eels for 2004 were not analysed. *Abbreviations*: Carp, common carp; Eel, European eel; Fbream, freshwater bream; Gfish, goldfish; Perch, European perch; Pike, Northern pike; Pperch, pike-perch; Wels, Wels catfish [26].

wt) in the tissues/organs decreased in the order: liver > muscles > adipose tissue > hard roe > kidney > bones > brain, with congener 153 markedly dominant in all tissues/organs of the predatory fish. The concentrations of all PCB congeners were significantly lower in the brain and in the liver and muscles, which had the highest concentrations, except for congener 28 in the muscles.

Hard roe had the highest PCB accumulation capacity in the non-predatory fish, followed by adipose tissue, bones, liver, muscles, kidney and brain. Similarly to the predators, the non-predators had high mean concentrations of congener 153 in all tissues/organs. Despite the considerable variation in PCB concentrations in the various tissues/organs, the observed differences were not significant in the non-predatory fish (P>0.05; Table 2).

The two-way main-effect ANOVA indicated that the feeding habits of the fish [F(6, 77)=9.10, P<0.00001] and particularly the individual fish tissues/organs [F(36, 340.89)=1.77, P<0.01] affected PCB bioaccumulation. PCB concentrations detected in the muscles and liver were nearly two-fold higher in the predators, which prefer a diet of live fish, compared to the non-predatory fish (Table 2). The concentrations markedly exceeded the acceptable PCB limits established for fish by the Food Codex [31] in the muscle tissues for both groups of fish.

The tissue/organ analysis performed in 2009 showed that fish accumulated PCBs selectively. The liver (hepatopancreas in cyprinids) had the highest PCB burden, with maximum concentrations recorded in northern pike (214.0 mg kg^{-1} lipid wt) and freshwater bream (163.0 mg kg^{-1} lipid wt) (Table 3).

	Muscle	Liver	Kidney	Brain	Hard roe	Adipose tissue	Bones	Limit[3]
Predators[1]								
PCB 28	$2.60^{a,b} \pm 0.82$	$3.63^{b} \pm 1.62$	$2.95^{a,b} \pm 1.97$	$1.17^{a} \pm 1.24$	1.91 ± 0.290	$2.15^{a,b} \pm 1.28$	0.79 ± 0.13	0.2
PCB 52	$8.63^{b} \pm 5.91$	$8.23^{b} \pm 6.07$	$4.04^{a,b} \pm 2.76$	$1.49^{a} \pm 1.30$	11.0 ± 7.44	$5.51^{ab} \pm 3.37$	3.62 ± 3.70	0.2
PCB 101	$13.4^{b} \pm 6.45$	$14.3^{b} \pm 11.2$	$7.73^{a,b} \pm 8.72$	$2.09^{a} \pm 1.45$	9.43 ± 1.77	$13.2^{b} \pm 9.80$	1.95 ± 2.12	0.2
PCB 138	$24.7^{b} \pm 13.3$	$29.9^{b} \pm 28.4$	$14.7^{a,b} \pm 17.1$	$4.35^{a} \pm 5.74$	18.6 ± 9.26	$23.5^{b} \pm 18.7$	5.64 ± 2.31	0.3
PCB 153	$29.5^{b} \pm 14.8$	$37.2^{b} \pm 34.1$	$20.6^{a,b} \pm 24.1$	$7.96^{a} \pm 9.64$	23.4 ± 8.54	$26.6^{a,b} \pm 20.9$	5.44 ± 5.77	0.3
PCB 180	$20.5^{b} \pm 11.7$	$26.4^{b} \pm 25.0$	$11.3^{a,b} \pm 13.2$	$2.80^{a} \pm 2.75$	15.0 ± 6.03	$22.1^{b} \pm 21.4$	3.41 ± 1.46	0.2
∑PCB	**$99.3^{b} \pm 49.9$**	**$120^{b} \pm 104$**	**$61.4^{ab} \pm 63.0$**	**$19.9^{a} \pm 20.1$**	**79.4 ± 18.5**	**$93.1^{a,b} \pm 71.5$**	**20.9 ± 15.5**	
Non-Predators[2]								
PCB 28	1.31 ± 0.93	1.14 ± 1.02	1.33 ± 1.13	0.84 ± 0.93	1.52 ± 0.110	0.86 ± 0.55	2.05 ± 1.89	0.2
PCB 52	4.31 ± 2.71	4.03 ± 2.94	3.52 ± 2.99	1.51 ± 2.18	4.53 ± 0.24	2.28 ± 2.04	4.29 ± 5.31	0.2
PCB 101	5.82 ± 4.15	4.87 ± 4.84	4.56 ± 3.82	1.60 ± 1.98	8.68 ± 6.30	6.73 ± 7.93	8.81 ± 12.2	0.2
PCB 138	8.17 ± 12.1	10.6 ± 15.4	1.51 ± 0.82	1.73 ± 1.51	23.2 ± 19.7	20.2 ± 25.2	3.33 ± 4.84	0.3
PCB 153	16.0 ± 15.1	15.4 ± 18.2	9.25 ± 7.99	3.67 ± 4.26	28.7 ± 24.5	24.3 ± 30.5	20.8 ± 33.1	0.3
PCB 180	11.3 ± 11.9	12.0± 15.0	6.90 ± 5.55	1.93 ± 2.32	21.6 ± 19.8	18.4 ± 23.1	14.6 ± 23.9	0.2
∑PCB	**46.9 ± 42.2**	**48.1 ± 54.7**	**26.1 ± 20.4**	**11.3 ± 11.6**	**88.2 ± 69.9**	**72.7 ± 89.4**	**53.8 ± 80.2**	

Significant differences (P<0.05) between tissues/organs for mean congener concentrations are indicated by different letters. Means followed by the same letters in the same row are not significantly different (post-hoc multiple comparison, P=0.05).

[1] Means without letters (hard roe and bones) are not included in the comparisons, because these samples were too small.

[2] No significant differences were confirmed for the non-predatory group.

[3] Acceptable limits established for food [31].

Table 2. Mean (± SD) concentrations (mg kg^{-1} lipid wt) of congeners and total PCBs in fish tissues/organs (Data from [27]).

The storage capacity of the muscles was slightly lower, but in some cases, PCB concentrations were higher in the muscles than in the liver, as in the European perch (108.0 and 80.5 mg kg^{-1} lipid wt in muscles and liver, respectively) and goldfish (38.0 and 32.6 mg kg^{-1} lipid wt, respectively). PCBs accumulated to a lesser degree in the kidney and brain than in the muscle tissue and liver (Table 3), with a significant difference only for the brain (P<0.05).

Most organic pollutants in aquatic ecosystems tend to accumulate in organisms, in which they can reach levels hundreds or thousands of times higher than the levels in water. Fish are at the top of the aquatic food web and concentrate high amounts of contaminants, including PCBs [6]. Ecotoxicological studies that have used fish as bioindicators have highlighted that the bioaccumulation of organic pollutants is affected substantially by biological factors, such as

species, age, size, physiological condition and also parasitism, which was revealed recently [32, 33]. The position of fishes in a food chain and their lipid contents represent other important predictors of organochlorine accumulation [34, 35].

Fish species	Muscle	Liver	Kidney	Brain
Predators				
European perch	108.0	80.5	75.9	27.3
Northern pike	90.1	214.0	14.3	13.6
Pike-perch	113.0	128.0	110.0	31.1
Wels catfish	71.1	71.1	13.1	3.74
Non-predators				
Common carp	10.2	10.2	3.80	1.44
Freshwater bream	128.0	163.0	-	1.73
Goldfish	38.0	32.6	30.5	15.2

Table 3. Mean concentrations (mg kg^{-1} lipid wt) of PCBs in individual tissues/organs collected in 2009 [26].

Fish are exposed to PCBs both via direct contact with the water and via food uptake by the membranes of the gastrointestinal tract [36]. Most of the PCBs, which have low solubility in water and fish, are likely derived from food rather than the ambient environment. Predatory fish, by eating other contaminated fish, accumulate higher amounts of organochlorine pollutants than fish with other feeding habits. Some authors [37], however, have arrived at opposite conclusions, and some studies have reported only slight differences between predators and bottom feeders [38, 39]. The results obtained in the present study are not entirely unequivocal; PCB concentrations were highest in the predatory perch, pike and pike-perch, but the non-predatory freshwater bream had comparable concentrations. The feeding behavior of bream is characterized by permanent direct contact with the sediments and a small radius of migration. Bream were thus once considered an important transfer vector for PCBs and one of the best organisms for monitoring freshwater contamination [40]. Our results support this finding.

European eel is commonly classified as a predaceous carnivorous fish, although its position in the trophic scale is lower than that of typical predators such as pike and pike-perch [25]. PCBs are lipophilic compounds prone to high bioaccumulation in eels, which have a high fat content. Accordingly, PCB concentrations in eels are usually higher than in other freshwater fish species [34, 41]. In our study, the muscles of the European eel had an unexpectedly low level of total PCBs (17.1 mg kg^{-1} mean lipid wt), which is the second lowest level in the nine species examined. Due to the interrupted natural flow of groundwater, we assumed that the sedentary eels from the reservoir do not use their energy reserves to migrate upstream, and so may have stored PCBs for long periods in their metabolically inactive adipose tissues. Adipose tissue can retain PCBs for a long time, or PCBs may accumulate in other fat-rich

tissues/organs, most probably the liver. We could not confirm the latter hypothesis, as we did not capture any eels in 2009, when the tissue-specific accumulation was investigated.

The closely related cyprinids (carp, freshwater bream, goldfish and roach) with similar ecologies and food preferences [25] differed markedly from each other in PCB content. PCB concentrations were relatively similar in these fishes in 2004, ranging between 27.2 mg kg^{-1} lipid wt in carp and 62.5 mg kg^{-1} lipid wt in roach, but varied widely in 2009. Freshwater bream had extraordinarily high PCB concentrations in 2009 (128.0 mg kg^{-1} lipid wt). Species-specific differences in PCB concentrations were also found in the individual tissues/organs of the cyprinids. PCBs preferentially accumulated in the hepatopancreas in freshwater bream, but the concentrations were equal or nearly equal in the hepatopancreas and muscles of carp and goldfish, respectively (Table 3). These differences could not be attributed to different diets, because these species feed on similar foods (bottom-dwelling invertebrates, plankton in the deeper parts of water bodies, plants and detritus). In addition to biological indices, the environmental conditions prevailing at a particular field site must be considered, because even slight environmental perturbations can induce a high degree of heterogeneity in biochemical markers [42].

Among the potential causative factors responsible for the high variation of PCB levels in our fish, particularly the tissues/organs in the same species (compare pike, pike-perch, carp and bream in 2004 and 2009), the highly varying hydrological conditions in the reservoir may have played a large role. The regular monitoring of the reservoir from 1999 to 2007 [43, 44] revealed large variability in the deposition of sediments at different sites and even the movement of sediments (so-called bottom waves) during seasonal floods, which released PCBs from the sediments and became more available to aquatic organisms. The concentrations of PCB indicator congeners in the bottom sediments increased substantially between 1999 and 2003 (467 and 1633 μg kg^{-1} dry wt, respectively) but were much lower at the same sites in 2005 and 2007 (428 and 692 μg kg^{-1} dry wt, respectively) [43, 44]. The study by Šalgovičová and Zmetáková [20] is consistent with a role for hydrological conditions in PCB accumulation. PCB concentrations in their study showed a decreasing tendency in non-predatory fish species. The levels of PCBs in goldfish reached 133.7 mg kg^{-1} in 2002 but had decreased nine-fold by 2004.

Most information about PCB compounds in aquatic organisms is from the muscles of fish and piscivorous birds [19, 45, 46], and very few data are available for the PCB contents in other organs. More attention has only recently been paid to the tissue-specific distribution of PCBs in fish [47].

The amount of total PCBs in a tissue or organ depends on the lipid content [19, 48]. The most important depot sites for PCBs in the predatory and non-predatory fish examined in the present study, next to the intestinal fat with obviously high lipid content (ca. 50%) [49], were the liver, hard roe and muscles. Other studies [50–52] have also reported higher PCB concentrations in the liver than in other organs, which could be due to its high fat content.

Ovaries are also rich in fat and serve as important depots of contaminants such as PCBs [51]. The lipid and consequently PCB contents in hard roe, unlike in the liver, however, can vary considerably. During maturation of female fish, lipids pass from somatic to gonad tissue,

accompanied by an increase in PCBs in the hard roe. In contrast, lipid-rich eggs released during spawning represent a major route of PCB loss in female fish [53]. The high PCB concentrations we found in the hard roe in both fish groups may thus be associated with the spawning period, because the majority of the fish in this study were caught in May, shortly before the peak of the spawn.

As shown by our results, the distribution of lipophilic compounds is not always directly proportional to the lipid content in individual tissues/organs. The brain, for example, with a relatively high fat content [54], appears to be much better protected against the accumulation of PCBs than other tissues/organs. This protection may be due to the efficient blood–brain barrier, as indicated by the relatively low concentrations of all investigated PCB congeners in our study, ranging from 0.84 (PCB 28 in non-predators) to 8.0 (PCB 153 in predators) mg kg^{-1} lipid wt. Several other conditions/factors, including species, age, size, feeding habits, metabolic activity of individual organs or the complex PCB transport in an organism may control PCB accumulation [26, 55].

3.3. Concentrations and profiles of the PCB congeners in fish

The levels of six indicator congeners, 28, 52, 101, 180, 138 and 153 (in order of increasing concentrations), were high. All the congeners reached their upper acceptable limit in both predators and non-predators (Table 2). The various congeners were not distributed homogeneously within the tissues/organs. PCB 153 was present in higher concentrations than the other congeners in all tissues/organs, representing 31% and 34% of the mean total PCB concentrations in predators and non-predators, respectively (Table 2). PCB 138 and 180 accounted for approximately 25% and 21% of the total amount of PCBs in predators and non-predators, respectively.

Despite the differences in PCB concentrations in individual tissues/organs, the relative proportions of the congeners within the major target tissues/organs, the liver, hard roe, muscle, adipose tissue and kidney, were relatively similar in both fish groups. PCB 153 and 138, the congeners most frequently found in animal samples [56], were also predominant in our assays. Congeners 138, 153 and 180 are less hydrophobic and not as tightly bound to sediments as the more highly chlorinated octa-, nona- and deca-PCBs and are thus more readily available to aquatic organisms [57]. In addition, these congeners with chlorine atoms in positions 2, 4 and 5 in one (PCB 138) or both (PCB 153 and PCB 180) rings may be more resistant to metabolism and elimination from the organism than the congeners with fewer chlorine atoms, such as 28, 52 and 101 [58, 59]. The very high proportion of PCB 138, 153 and 180 found in our tissue/organ samples (76% and 79% of all PCB congeners in predators and non-predators, respectively) may reflect their low rates of biotransformation and resistance to metabolism.

3.4. PCB concentrations in perch and parasites

The accumulation of PCBs was studied in the perch–*A. lucii* host–parasite system. Acanthocephalans attached to the intestinal mucosa accumulated significantly higher concentrations of PCBs (single-means *t*-test, $t<-4.2$, $P<0.001$) than did the tissues/organs of their hosts (Fig. 3 and Table 4).

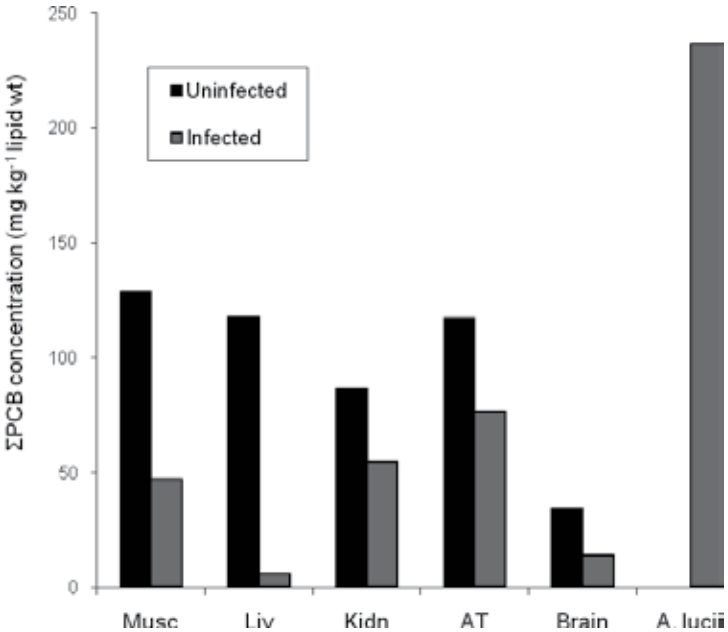

Figure 3. PCB concentrations in the tissues/organs of infected and uninfected fish and in *Acanthocephalus lucii* (Parasite). *Abbreviations*: Musc, muscle; Liv, liver; Kidn, kidney; AT, adipose tissue [27].

PCB 153 predominated in acanthocephalans (as in the fish), followed by PCB 138 and 180 (Table 4). Notably, the concentration of PCB 28 was approximately 10-fold higher in the parasites than in the muscles, liver and adipose tissue of the perch (Table 4).

	Muscle	Liver	Adipose tissue	Kidney	Brain	Parasite
PCB 28	2.9	2.9	2.0	5.0	3.2	20.9
PCB 52	6.3	2.5	5.4	4.1	4.6	7.2
PCB 101	16.8	10.2	20.0	11.7	14.7	36.2
PCB 138	26.9	17.5	22.9	15.9	20.8	61.4
PCB 153	31.4	28.4	24.6	22.1	26.6	63.6
PCB 180	23.8	19.0	21.9	17.1	20.5	47.2
∑PCB	108.1	80.5	96.9	75.9	90.3	237.0

Table 4. Mean and total (∑PCB) concentrations (mg kg^{-1} lipid wt) of the congeners in various tissues/organs of perch (*P. fluviatilis*) and in its intestinal parasite (*A. lucii*) [27].

The numbers of fish tissue/organ samples were too low for reliable statistical analysis (10 samples from infected and 14 samples from uninfected fish), but the results indicated much lower quantities of PCBs in all tissues/organs from the fish infected with acanthocephalans. The concentrations of PCBs were approximately 20- and 3-fold lower in the liver and muscles, respectively, of the infected perch (Fig. 3).

Any kind of pollution represents environmental stress that influences host–parasite interactions and could cause outbreaks of fish diseases [60]. The immune system of fish is severely impaired by exposure to contaminants that consequently leads to an increase of parasitism. Sagerup et al. [61] found a positive correlation between PCB contamination of the habitat and intensity of nematode infection in gulls (*Larus hyperboreus*). Immunosuppression induced by PCBs was confirmed experimentally [62]. European eels infected with the swim-bladder nematode *Anguillicola crassus* were exposed to sublethal concentrations of PCB 126, which completely suppressed the antibody response in the eels. The fish exposed to the PCBs were consequently more easily infected than unexposed fish. A combination of anthropogenic stressors (e.g. PCB pollution) and natural stressors (e.g. parasitic infection) usually has a more detrimental effect on fish than each stressor alone [63].

Parasites are able to accumulate pollutants and consequently reduce their incorporation in the hosts [64, 65], particularly heavy metals. Supporting data for this role of parasites in fish PCB contamination, however, remain scarce. *Pisidium amnicum*, a freshwater clam, infected with larval stages of digeneans and exposed to organic pollutants (2,4,5-trichlorphenol (TCP) and benzo(a)pyrene) contained approximately 12% and 40% less TCP and benzo(a)pyrene, respectively, than uninfected clams [66]. Persson et al. [67], however, reported a slightly lower concentration of PCBs (1.92 ng g^{-1} lipid wt) in the tapeworm *Eubothrium crassum* than in their salmon (*Salmo salar*) hosts (2.65 ng g^{-1} lipid wt), which was attributed to the absence of an alimentary canal in all developmental stages of the tapeworms.

The present study provides the first evidence of the ability of fish parasites (acanthocephalans) to accumulate relatively high levels of PCBs. Our data demonstrated lower PCB concentrations in all tissues/organs of infected relative to uninfected perch under natural conditions. These data indicate that some parasitic organisms may have a positive influence on their hosts in PCB-contaminated environments, as previously demonstrated for heavy-metal pollution [14, 65].

4. Conclusions

Our results demonstrated a persistent problem with the "old environmental heritage" of PCBs, leading to high risks mainly for predatory fish species in aquatic ecosystems, and for the human populations living near these regions. The commercial production of PCBs has been banned or severely restricted in Slovakia since the 1980s, but the fish in the Zemplínska šírava reservoir continue to accumulate these chemicals from the sediments polluted in the previous decades. Critical PCB levels recorded in fishes and the tendency of bioaccumulation in some species in the reservoir are essential for predicting which food webs are at risk for higher rates of bioaccumulation that endanger the health of upper-trophic predators, including humans living in the area. This study indicated that the different fishes, their dietary intakes and the chemical properties of the pollutants were interrelated factors, all of which were important in the tissue/organ burdens. Fish parasites, due to their good properties of bioaccumulation and position in the food chain, should be carefully considered in any ecotoxicological research.

Acknowledgements

We thank Dr. Ľudmila Turčeková for her cooperation in collecting fish samples and Dr. William Blackhall for his editorial comments on the text. We gratefully acknowledge the funding of this study by the Slovak Research and Development Agency, project No. LPP-0151-07, Grant Agency of the Ministry of Education of the Slovak Republic, and Slovak Academy of Sciences (VEGA), project No. 2/0080/10. The publication has been realized as part of the project of the Centre of Excellence for Parasitology (Code ITMS: 26220120022) supported by Operational Programme "Research & Development" funded by the European Regional Development Fund (rate 0.9).

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Chapter 2

Heavy Metals — Soil Communities at Ecological Risk

P. Šalamún, V. Hanzelová and T. Brázová

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60404

Abstract

This article outlines an effect of the different kinds of environmental pollution on the soil nematode communities at two industrial regions (Krompachy and Jelšava) of eastern Slovakia. At the locality Krompachy, soil nematode communities were significantly affected by increased concentrations of As, Cd, Cu, and Zn. At the other location Jelšava, despite low concentrations of heavy metals, long-term emissions of magnesium dust had indirect impact on soil environment through altered soil conditions, mainly by alkaline pH. Pollution effects at the community structure (trophic and c-p groups) of soil nematodes were found to be the highest near the pollution sources, where communities with low population densities consisted mainly of disturbance-tolerant taxa from c-p 1 and c-p 2 groups. With an increasing distance from the pollution sources, the nematode communities were more structured and complex, with higher proportions of sensitive c-p 4 and 5 nematodes, composed mainly of predators and omnivores. Ecological indices (H′, MI2-5, SI) also clearly showed deteriorating environmental conditions towards the pollution sources. The greatest difference in the nematode community structure between the two localities was found in the proportion of c-p 1 and c-p 2 bacteriovorous nematodes. At the locality Krompachy characterised by critical heavy metal load, c-p 2 nematodes dominated, while c-p 1 nematode group prevailed at under extremely alkaline soil conditions in Jelšava. Results also showed that the free-living nematodes are useful tools for bioindication of industrial contamination and could be used as an additional source of information to the common approaches based on chemical methods.

Keywords: Soil nematodes, Industrial pollution, Heavy metals, Magnesium, Salinity bioindication

1. Introduction

The quality of human life depends on the chemical composition of the environment, including food, water, and many other factors necessary for life. The development of human society, however, has brought substantial increases in ecological problems and contamination of the environment [1]. Some parts of the environment (e.g. atmosphere and hydrosphere) are able to partially remove the contaminants or to disperse them over wide areas and eliminate their potentially ecotoxic effects. The pedosphere, however, cannot, and important "hot-spots" with extremely high levels of a variety of pollutants have developed concurrently with human society in recent decades [2]. Soil is one of the most important factors for terrestrial life through the production of food, so its degradation could reduce the diversity or abundance of various valuable organisms necessary for maintaining balanced ecological functions [3].

Understanding the impact of pollution on important soil processes, where soil organisms play key roles, requires observing not only the quantity of the pollutants in a system, but also their qualitative effects and ability to change the environmental conditions for soil biota [4]. Various approaches using different soil communities were thus developed for biomonitoring potentially endangered ecosystems. The wide variety of communities, either directly in the soil or above it, tightly linked to soil processes makes the selection of an appropriate bioindicator challenging. Soil mesofauna, composed of various invertebrate communities, are the most suitable [5]. Soil invertebrate communities are able to buffer short-term resource shortages without significant losses in abundance or diversity [6], but they also have relatively rapid and identifiable reactions to contamination, because they are integrators of the physical, chemical, and biological properties related to their food resources [5]. Within the mesofauna, soil nematodes [7], springtails [8], and mites [9] have been considered for use as reliable bioindicators. Nematodes may be the most promising candidates for systems of bioindication, because more information on their taxonomy and ecology [10] is available than for other animal groups.

1.1. Nematodes as bioindicators

Nematodes are amongst the most abundant multicellular organisms in nearly all soil ecosystems, occupying key positions at most trophic levels in the soil food web [11]. They are involved in organic matter mineralisation, plant growth, and crop productivity by their regulation of detritivore populations and thus contribute to the stability of the food web [12, 13, 14]. Their life cycles have been widely studied, partly because some taxa are pests [15]. The nematode *Caenorhabditis elegans* is a key model for genetic studies, which could significantly improve the bioindicating potential of nematodes on molecular and gene-expression levels.

The introduction of the maturity index by [7], one of the best-known indicators of soil health [15], was a crucial breakthrough in nematode bioindication. Nematodes are classified in this system along a coloniser-persister (c-p) continuum and assigned to c-p groups ranging from 1 (colonisers) to 5 (persisters). Similar to the r/K dichotomy, colonisers are characteristic of early successional stages, and persisters are found mainly in more developed and complex ecosystems. The advantage of the c-p system is that nematodes only need to be identified to the generic level, which does not affect the resolution of the indication.

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Several other derived or new ecological indices have been developed from the maturity index. Ferris et al. [11] provided a framework for determining the enrichment (enrichment index, EI) and structure (structure index, SI) of food webs based on the relative weighted abundance of the functional guilds of nematodes. A matrix of feeding habits, with life history characteristics embodied in the c-p classification, is used to enhance the resolution of nematode analyses and to connect the functional groups with the enrichment and structure of the food web and channels of decomposition.

We applied these approaches at two locations, Kovohuty JSC Krompachy and Slovak Magnesite Works JSC Jelšava, with soils contaminated by dust fallout containing high amounts of potential ecotoxic elements. The levels of contamination at these sites have been analytically determined, but little is known about the soil communities and their interactions or about the general health of the soil. The aims of this study were thus to improve our knowledge of the polluted sites and to better understand the biotic processes under environmental pressure and the consequences of industrial pollution for soil food chains.

The specific objectives were

i. To clarify the impact of contamination on the quality of soil abiotic and biotic components.

ii. To evaluate the ability of soil nematode communities to detect ecosystemic disturbances.

iii. To define the suitability of this system of bioindication for the detection of soil degradation.

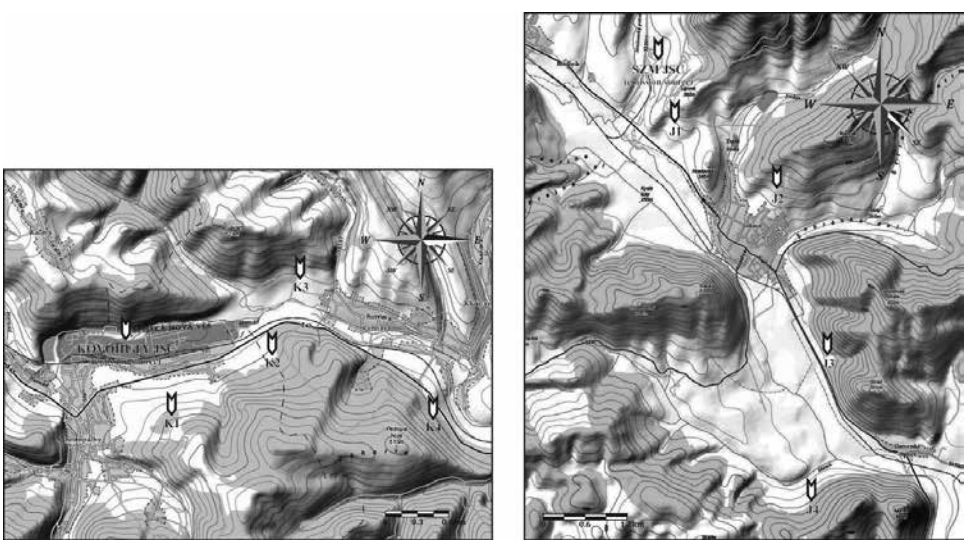

Figure 1. Maps of the study areas with indication of the emission source and sampling points (white arrows - K1-K4 for Krompachy, J1-J4 for Jelšava); prevailing winds are indicated in the compass rose.

2. Material and methods

Study areas

The study was performed in the model towns of Krompachy and Jelšava that have important emission sources, smelters Kovohuty JSC Krompachy and Slovak Magnesite Works Jelšava (SMZ JSC). Krompachy has been known for its processing of iron and copper ore since the 14th century, and copper has been distributed all over Europe. The magnesite industry in Jelšava has flourished since the end of the 19th century. Soil contamination in both areas is caused by the fallout of solid particles containing various elements during the mining and processing of the ores [16]. More than an estimated 1500 tonnes of solid particles yearly containing accessorial elements (Cd, Cr, Cu, Pb, and Ni) from Kovohuty JSC Krompachy, and as many as 165, 000 tonnes of emissions containing magnesium in total from SMZ JSC were emitted to the atmosphere between the 1960s and the 1990s [17].

Sampling and data analysis

Soil samples were collected in April 2010 for Krompachy and in May 2009 for Jelšava at four sampling sites each along transects downwind of the emission sources (Figure 1). Four 1 kg replicates (each consisting of four subsamples) were collected from the surface horizons (0-20 cm) of permanent grasslands at each sampling site by quadrat sampling. Each replicate was stored in a plastic bag at 4 °C until analysed. The soil samples were analysed for geochemical properties and nematode community structure.

2.1. Geochemical properties and heavy metals

The soil samples were processed as follows:

1. Soil moisture was measured gravimetrically by drying the replicates to a constant weight at 105 °C [18].
2. Organic matter content (C_{org}) was determined by titration with $K_2Cr_2O_7/H_2SO_4$ [18].
3. NH_4^+ was determined spectrophotometrically using Nessler reagent [18].
4. NO_3^- was determined by an ion-selective electrode [18].
5. pH was determined in a solution of $CaCl_2$ [18].
6. The total and mobilisable concentrations of heavy metals (As, Cd, Cu, Cr, Mg, Ni, Pb, and Zn) were determined by inductively coupled plasma mass spectrometry on an Agilent 7500 C (Agilent Technologies, USA) following the manufacturer's instructions. The replicate samples were air-dried before analysis in an oven at 30 °C for 48 h or until constant weight, ground, and sifted through a 0.2-mm sieve. Metals were extracted by 2M HNO_3 (10 g of soil in 50 mL of HNO_3) for 6 h for total content and by 0.05M Na_2EDTA neutral solution (5 g of soil in 50 mL of Na_2EDTA) for mobilisable content [19].

Steps 1-5 were performed by the Laboratory of the Central and Testing Institute in Agriculture in Košice, Slovakia according to certified methods [18].

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Krompachy	Sampling site			
	K1	**K2**	**K3**	**K4**
GPS position	48°55'01"N, 20°53'07"E	48°55'17"N, 20°54'13"E	48°55'52"N, 20°54'35"E	48°54'56"N, 20°55'55"E
Distance (m)	750	1660	2250	3820
Altitude (m)	460	380	407	404
pH	4.90±0.12	5.75±0.21	5.70±0.37	5.15±0.25
C_{org} (%)	2.52±0.87	3.92±0.40	3.56±0.41	2.90±0.38
NO_3^- (mg.kg^{-1})	4.03±1.77	5.83±0.53	5.63±1.81	4.53±1.94
NH_4^+ (mg.kg^{-1})	8.20±1.37	12.10±2.01	8.78±2.76	19.73±8.12
$N_{inorg.}$ (mg.kg^{-1})	12.23±2.93	17.93±2.13	14.40±4.56	24.25±9.87
Moisture (%)	18.87±3.17	28.43±1.35	19.47±0.73	22.77±3.11
Jelšava	**J1**	**J2**	**J3**	**J4**
GPS position	48°38'39"N, 20°13'40"E	48°38'05"N, 20°14'46"E	48°36'44"N, 20°15'26"E	48°35'49"N, 20°15'13"E
Distance (m)	487 m	2196 m	4630 m	6096 m
Altitude (m)	338 m	326 m	260 m	261 m
pH	> 9.0	7.4±0.1	7.5±0.1	6.9±0.1
C_{org} (%)	2.4±0.1	2.0±0.4	2.3±0.4	1.9±0.2
NO_3^- (mg.kg^{-1})	4.3±0.9	4.8±3.0	5.5±1.0	2.3±0.2
NH_4^+ (mg.kg^{-1})	9.0±1.5	15.0±2.3	12.2±3.0	14.0±2.2
$N_{inorg.}$ (mg.kg^{-1})	13.4±2.3	19.8±5.0	17.7±3.5	16.3±2.3
Moisture (%)	22.0±5.5	23.3±1.0	18.7±1.2	19.9±0.9

* - by Slovak Soil Science and Conservation Research Institute

Table 1. Study sites characteristics [1, 53].

Loamy Cambisols with intense stony skeletons dominated in both areas. Despite some variations in soil properties, the basic characteristics of the soils were similar (Table 1), with soil moistures ca. 20% and inorganic nitrogen contents ($N_{inorg.}$) between 10 and 20 mg kg^{-1}. The Krompachy soils were richer in C_{org} and were more acidic than the Jelšava soils (soil pH >9).

2.2. Analysis of nematode community structure

Nematode communities were isolated by extraction from 100 g of each replicate soil sample using a modified Baermann procedure [20]. The extracted nematodes were fixed in Ditlevsen's solution (formalin/acetic acid/alcohol) [21] and were counted using a microscope. All nematodes in the samples were classified to order, family, and genus. The nematode communities were analysed for (i) total abundance (number of individuals per 100 g of soil), (ii) trophic structure (bacterial feeders; fungal feeders; plant feeders; omnivores; and predators) [22], and (iii) the ecological indices (1) MI2-5 [23] with Nemaplex c-p values, (2) the Shannon-Weaver index (H′) for generic diversity [24], (3) generic richness, expressed as mean number of genera at a site, (4) EI based on the proportion of opportunistic bacterivorous and fungivorous nematodes, (5) SI based on the relative weighted abundance of guilds representing the structure of the food web, and (6) the channel index (CI) [11].

Trace elements	Sampling site				
	K1	K2	K3	K4	Limit
	Total concentration (HNO_3 extraction)				
Arsenic (As)	78.654±15.287[a]	19.574±5.402[b]	24.377±5.685[b]	5.609±1.306[c]	5
Cadmium (Cd)	1.494±0.181[a]	1.061±0.478[b]	0.339±0.062[c]	0.161±0.013[c]	0.3
Chrome (Cr)	5.781±7.019[a]	1.458±0.159[a]	1.475±0.206[a]	1.012±0.021[a]	10
Copper (Cu)	257.643±38.452[a]	175.398±80.216[b]	65.796±14.021[c]	18.322±2.129[c]	20
Nickel (Ni)	2.617±0.262[b]	3.598±0.349[a]	0.627±0.314[c]	1.021±0.027[c]	10
Lead (Pb)	25.140±8.380[a]	29.038±11.551[a]	8.216±3.761[b]	5.195±0.089[b]	30
Zinc (Zn)	80.62±18.019[a]	60.253±29.127[a]	8.067±4.449[b]	3.201±0.290[b]	40
	Mobilisable fraction (Na_2EDTA extraction)				
Arsenic (As)	4.779±0.688[a]	2.870±0.690[b]	1.074±0.494[c]	0.105±0.021[d]	n.a.
Cadmium (Cd)	0.407±0.101[a]	0.266±0.097[b]	0.066±0.016[c]	0.023±0.003[c]	n.a.
Chrome (Cr)	0.081±0.02[a]	0.065±0.031[a]	0.073±0.036[a]	0.010±0.003[b]	n.a.
Copper (Cu)	76.660±23.554[a]	46.945±18.585[b]	14.320±3.788[c]	4.380±0.011[c]	n.a.
Nickel (Ni)	0.54±0.115[a]	0.568±0.112[a]	0.167±0.197[b]	0.101±0.008[b]	n.a.
Lead (Pb)	18.565±2.744[a]	16.293±7.951[a]	5.413±1.681[b]	4.055±0.337[b]	n.a.
Zinc (Zn)	63.285±17.193[a]	26.945±12.502[b]	3.600±1.377[c]	0.643±0.085[c]	n.a.

[abcd] Means followed by the same letters on the same rows are not statistically different according to Least Significant Difference Test ($P<0.05$)

Limit - limits posted by The Decree of the Ministry of Land Management of the Slovak Republic No. 531/1994-540 on the admissible values of harmful substances in uncontaminated soil. n.a. – not available

Table 2. Total and mobilisable concentration of trace elements in sampling sites from Krompachy (mg.kg-1) [1].

2.3. Data analysis

Spearman's nonparametric correlation coefficient (r_s) was calculated using STATISTICA v. 9.0 to test the relationships between the characteristics of a nematode community and the concentrations of mobilisable heavy metals at a site [25]. Correlations at $P<0.05$ and $P<0.01$ were considered significant. Differences in the mean traits and indices of a community amongst sites were tested by Duncan's tests. We used a constrained ordination redundancy analysis (RDA) in CANOCO 5 to analyse the ecological distances between sites (nematode community and soil parameters). The significance of an axis was tested by Monte Carlo permutation [25].

The effects of contamination on soil ecosystems can be categorised as direct or indirect. Alterations in the soil communities near Kovohuty JSC Krompachy were likely due to direct toxicity from the high levels of heavy metals in the soil samples. The contamination acted mostly indirectly near SMZ JSC, altering the basic soil properties. We will present and discuss the results from these two areas separately.

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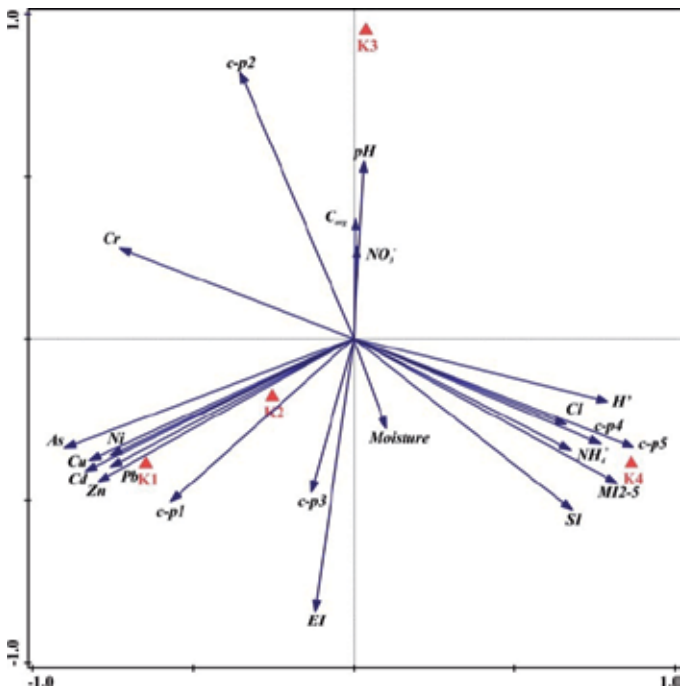

Figure 2. Redundancy analysis (RDA) performed on physicochemical soil properties, trace elements and selected indices in relation to sampling sites with data explained 73.1 % of the variation in the first two axes; F=10.9; P=0.002 (Krompachy) [1].

3. Direct toxic effects of industrial emissions – A case study at Kovohuty JSC Krompachy

3.1. Results

3.1.1. Heavy metals

The total heavy metal content (except Pb and Ni) along the Krompachy transect was highest at the K1 site and decreased downwind (Table 2). The patterns of the decreases in concentration, however, varied amongst the metals. As, Cd, and Cu decreased significantly (P<0.05) and relatively continuously towards K4. The concentrations of Ni, Pb, and Zn were relatively high at sites K1 and K2 but were significantly lower at K3 (P<0.05). Cr content did not vary significantly (P>0.05) along the transect. The mobile fractions of all metals had similar decreasing trends (Table 2). As, Cd, Cu, and Zn had high total concentrations, exceeding the limits for uncontaminated soils in Slovakia [26] (Table 2). C_{org} and pH were likely the most important factors influencing the mobility of the metals, and the RDA (Figure 2) indicated that they were negatively correlated with the concentrations of all heavy metals except Cr.

3.1.2. Composition of the nematode fauna: trophic and c-p groups

A total of 58 genera were identified, including 20 bacterivores, five fungivores, 10 omnivores, eight predators, and 15 plant feeders (Table 3). The most common genera in the communities were *Acrobeloides*, *Aphelenchoides*, *Geocenamus*, *Helicotylenchus*, *Paratylenchus*, *Pratylenchus*, and *Rhabditis* (Table 3). The mean number of genera (17.75) in the replicates was lowest near the industrial plant (K1), which also had the lowest mean population density of 386.5 per 100 g of soil (Table 4). Both parameters increased with distance from the plant to 35.75 and 833.25, respectively, at K4. $N_{inorg.}$ was significantly correlated (P<0.01) with generic richness, and soil pH (P<0.01) and $N_{inorg.}$ (P<0.05) were significantly correlated with nematode abundance.

The industrial complex affected the densities of the trophic groups. The trophic structure differed at each sampling site (Table 4). Plant feeders were the most abundant trophic group, with a proportion in the community near 66% at the most contaminated site, K1. Bacterivores were the second most common group, representing more than 50% of the total nematode populations at K2 and K3. This group was positively correlated with C_{org} and pH (P<0.01) (Table 5). Fungivores, considered relatively insensitive to disturbances, including heavy metal pollution, occurred in relatively low densities, except at K2 with 8.98% of the total population. Omnivores and predators were most affected by the pollutants, with very low densities at K1-K3. The low resistance of these two groups to disturbances is in agreement with their negative correlation (P<0.01) with Cr (predators) or with all heavy metals (omnivores). Both trophic groups were also negatively correlated with $N_{inorg.}$, and soil moisture (Table 5). The distribution of the c-p groups near the plant was consistent with their life strategies and survival abilities. General opportunistic nematodes, c-p 2, were most common at the most polluted sites (K1-K3), followed by c-p 1. The other three c-p groups represented only ca. 10% of the identified nematodes. The numbers of c-p 4 and 5 nematodes were significantly higher at the most remote site, K4, where representatives of c-p 4 were the most abundant within the nematode community (Table 4).

3.1.3. Ecological indices

Mean H′ and generic richness were highest at K4, indicating the richest composition of nematode taxa (Table 4). MI2-5 and SI, measures of ecosystemic maturity, showed analogous development (Figure 2), with stable values from K1 towards K3 and steep increases at K4 (Table 4). EI values indicated that K2 stored the highest amounts of organic matter available for degradation, but K1 had the highest rate of degradation, as indicated by the lowest CI value (P<0.05) (Table 4).

Nematode genera		K1	K2	K3	K4
	c-p	Abundance	Abundance	Abundance	Abundance
			Bacterial feeders		
Acrobeloides	2	44.00±26.15	84.25±25.13	202.3±39.8	30.00±13.04
Alaimus	4	1.00±2.00	0.50±0.58	0.75±1.50	15.25±12.74

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Nematode genera		K1	K2	K3	K4
	c-p	Abundance	Abundance	Abundance	Abundance
Amphidelus	4	-	-	-	7.75±15.50
Anaplectus	2	-	-	-	0.75±1.50
Aulolaimus	3	-	0.50±1.00	-	-
Cephalobus	2	7.50±4.04	44.75±6.65	82.75±36.79	32.00±26.77
Diplogaster	1	-	-	-	2.50±4.36
Eucephalobus	2	3.25±4.72	16.00±18.49	-	9.50±10.66
Eumonhystera	1	-	-	-	1.50±1.73
Geomonhystera	1	-	-	-	0.75±0.96
Heterocephalobus	2	2.00±2.83	31.25±29.62	36.00±25.34	3.00±2.58
Mesorhabditis	1	7.25±13.20	-	-	1.25±1.50
Monhystera	1	1.50±3.00	22.50±11.59	8.25±7.54	-
Panagrolaimus	1	1.00±0.82	-	-	9.25±13.84
Plectus	2	5.25±3.30	20.00±16.19	23.00±10.68	27.75±3.40
Prismatolaimus	3	1.50±2.38	7.00±10.92	0.25±0.50	9.25±6.34
Protorhabditis	1	4.75±9.50	-	-	-
Rhabditis	1	32.75±18.30	286.5±63.2	41.75±24.51	55.00±16.25
Teratocephalus	3	10.00±9.90	11.00±12.68	5.25±3.77	0.25±0.50
Wilsonema	2	-	2.25±1.71	0.50±0.58	0.75±1.50
			Fungal feeders		
Aphelenchus	2	0.25±0.50	83.75±43.66	3.75±4.27	15.00±7.79
Diphtherophora	3	-	-	3.50±3.00	-
Dorylaimoides	4	-	-	-	4.50±9.00
Filenchus	2	1.25±1.89	3.00±6.00	-	11.25±10.18
Tylencholaimus	4	-	-	-	4.25±7.85
			Omnivores		
Aporcelaimellus	5	-	0.75±1.50	4.00±4.83	37.00±9.31
Aporcelaimus	5	1.00±2.00	-	0.75±1.50	21.25±25.99
Campydora	4	-	-	-	2.75±5.50
Dorylaimus	4	2.00±2.83	4.50±6.45	11.25±9.60	45.75±30.61
Enchodelus	4	-	-	-	0.75±1.50
Epidorylaimus	4	-	-	4.00±4.69	-
Eudorylaimus	4	-	1.25±2.50	13.50±19.36	34.50±7.33

Nematode genera		K1	K2	K3	K4
	c-p	Abundance	Abundance	Abundance	Abundance
Mesodorylaimus	5	-	-	1.50±3.00	2.00±1.63
Microdorylaimus	4	-	-	-	3.75±2.63
Prodorylaimus	5	-	-	-	1.00±2.00
			Predators		
Anatonchus	4	-	0.50±1.00	-	2.25±3.30
Clarkus	4	-	4.50±9.00	0.25±0.50	14.25±12.50
Mononchus	4	1.75±2.36	30.25±23.77	3.25±4.03	0.75±1.50
Mylonchulus	4	-	1.75±2.87	-	8.75±5.32
Nygolaimus	5	-	-	-	1.00±2.00
Oxydirus	5	-	-	-	17.25±16.09
Prionchulus	4	-	5.25±8.54	-	0.25±0.50
Tripyla	3	-	2.00±2.45	0.75±1.50	15.75±7.41
			Plant feeders		
Aglenchus	2	5.50±1.73	27.50±21.55	6.00±3.74	31.50±21.58
Aphelenchoides	2	8.75±7.80	147.5±74.9	68.75±23.43	10.50±13.10
Axonchium	5	1.00±2.00	-	0.75±1.50	-
Boleodorus	2	10.00±9.06	20.75±9.64	15.00±10.10	17.25±3.30
Coslenchus	2	-	-	0.50±1.00	1.25±1.89
Criconema	3	-	0.75±0.96	16.25±30.51	1.25±2.50
Geocenamus	3	149.8±129.8	2.00±2.83	5.00±3.92	2.75±1.26
Helicotylenchus	3	1.25±0.50	73.75±100.1	34.00±34.81	218.8±208.6
Heterodera	3	1.25±2.50	-	-	20.75±25.18
Macrotrophurus	3	-	-	-	0.50±1.00
Malenchus	2	0.50±1.00	2.25±4.50	2.25±3.86	7.50±11.21
Paratylenchus	2	14.50±14.15	31.25±30.61	159.0±177.9	21.25±19.10
Pratylenchus	3	66.00±69.33	94.00±54.30	89.00±94.70	53.00±52.35
Rotylenchus	3	-	-	-	0.50±1.00
Tylenchus	2	-	-	0.50±1.00	0.50±1.00

Table 3. C-p values, the average abundance (±SD), and dominance of nematode genera in individual sampling sites from Krompachy [1].

Several significant relationships between the heavy metal contents in the soil and the ecological indices were observed across all sampled sites (Table 5). As an indicator of diversity, generic

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richness was negatively correlated (P<0.05 or <0.01), with nearly all heavy metals except Ni, similar to H′ (except Ni and Pb). SI and MI2-5 were negatively correlated (P<0.01) with Cr content.

Indices, trophic & c-p groups	Sampling sites			
	K1	K2	K3	K4
	Trophic & c-p groups			
Bacterial feeders	31.87±7.32^{a}	50.25±5.60^{b}	51.83±14.57^{b}	26.12±8.59^{a}
Fungal feeders	0.36±0.44^{b}	8.98±5.10^{a}	0.98±0.48^{b}	4.21±0.87^{b}
Omnivores	1.19±1.30^{c}	0.79±1.00^{c}	3.35±2.43^{c}	17.90±5.71^{b}
Predators	0.71±1.21^{c}	4.00±2.20^{bc}	0.47±0.67^{c}	7.50±2.99^{ab}
Plant feeders	65.86±8.88^{b}	35.99±9.23^{a}	43.37±12.62^{a}	44.27±12.60^{a}
c-p 1	33.86±10.47^{c}	46.80±6.56^{d}	10.42±4.68^{a}	15.63±4.84^{ab}
c-p 2	51.93±7.77^{cd}	42.67±6.24^{bc}	79.76±11.88^{e}	29.73±10.60^{ab}
c-p 3	8.39±5.75^{b}	3.00±2.06^{ab}	2.04±1.49^{a}	5.78±2.73^{ab}
c-p 4	5.39±7.82^{b}	7.42±4.01^{b}	6.51±5.40^{b}	31.19±12.30^{a}
c-p 5	0.43±0.86^{c}	0.12±0.23^{c}	1.26±1.11^{c}	17.67±5.19^{b}
	Ecological indices			
Genera richness	17.75±2.06^{b}	22.25±4.27^{b}	23.25±3.86^{b}	35.75±2.36^{a}
Abundance	386.5±228.8^{a}	1063.75±290.2^{b}	844.25±385.0^{ab}	833.25±261.0^{ab}
Enrichment Index	71.10±10.57^{a}	82.21±4.1^{a}	34.30±13.04^{b}	69.96±3.49^{a}
Structure Index	42.86±15.39^{b}	44.81±13.53^{b}	30.20±20.45^{b}	88.48±5.97^{a}
Channel Index	1.11±1.30^{c}	6.67±2.85^{ab}	3.05±3.3^{bc}	9.24±2.52^{a}
Maturity Index 2-5	2.31±0.17^{c}	2.34±0.14^{c}	2.22±0.176^{c}	3.43±0.291^{b}
Shannon-Weaver Index	2.01±0.22^{b}	2.34±0.15^{b}	2.25±0.148^{b}	2.85±0.366^{a}

^{abc} Means followed by the same letters on the same rows are not statistically different according to Least Significant Difference Test (P<0.05)

Table 4. Percentage of individual nematode trophic groups and average of ecological indices values calculated for Krompachy sampling sites [1].

3.2. Discussion

Heavy metals can both directly and indirectly affect soil environments, directly by modulating the physiology and behaviour of the soil fauna and flora, or indirectly by altering environmental conditions such as pH or resource availability [27, 28]. The solubility of heavy metals in soil, for which substrate pH is the main driver, determines their toxicity to soil biota [3]. The soil conditions (mostly pH) in the Krompachy area suggest that the solubility of heavy metals

could be relatively low, but only the solubilities of As and Cr were low. The solubility and thus the accessibility of the other heavy metals with >20% potential mobility at all sites (K1-K4) could be considered a consequence of chronic contamination of the soil profile and/or a low ability of the soil to bind these elements. The values of the soil parameters and the trend of heavy metal contamination along the transect ruled out the impact of natural geological and/or physicochemical properties. Kovohuty JSC Krompachy was thus the most likely source of the higher heavy metal levels. This conclusion is in agreement with previous data collected from this area [17, 29, 30].

The influence of polymetallic pollution is evident in all aspects of soil ecosystems, including communities of soil nematodes and their trophic structures. Bacterivores and plant feeders were the most resistant to the adverse effects of pollution, consistent with the findings of a previous study in this area [31]. On the other hand, acidic soil combined with heavy metal pollution can positively influence mostly fungi and decrease bacterial densities [32, 33], but our results do not support these effects. We found relatively low proportions of fungivores and high proportions of bacterivores in the acidic soils at most sites. Numerous studies [34, 35, 36, 37] have described differences in the survival of soil nematodes with similar ecological and life strategies under harsh abiotic circumstances, including heavy metal contamination. Fungivores, such as representatives of the genus *Aphelenchus* tolerant to contamination [38, 39], should thus be more abundant under such environmental contamination. The density of *Aphelenchus*, however, was significantly higher only at K2, which could indicate that interacting environmental factors (e.g. moisture and texture) might have an important impact on the population density of this trophic group [40].

Indices, trophic & c-p groups	***As***	***Cd***	***Cr***	***Cu***	***Ni***	***Pb***	***Zn***	***pH***	***Moisture***	$N_{inorg.}$	C_{org}
Bacterial feeders	0.162	0.135	0.338	0.141	0.138	0.124	0.035	0.664**	0.1	-0.088	0.699**
Fungal feeders	-0.384	-0.321	-0.215	-0.313	-0.022	-0.19	-0.409	0.34	0.802**	0.611*	0.352
Omnivores	-0.753**	-0.846**	-0.693**	-0.857**	-0.748**	-0.831**	-0.796**	-0.178	-0.069	0.475	-0.035
Predators	-0.469	-0.41	-0.723**	-0.44	-0.074	-0.28	-0.475	0.03	0.522*	0.628**	0.276
Plant feeders	0.303	0.329	0.003	0.35	0.209	0.297	0.444	-0.54*	-0.529*	-0.479	-0.600*
c-p 1	0.576*	0.629**	0.185	0.612*	0.768**	0.662**	0.588*	0.012	0.479	0.074	0.102
c-p 2	0.329	0.318	0.612*	0.276	-0.1	0.191	0.297	0.275	-0.509*	-0.609*	0.169
c-p 3	-0.015	0.112	-0.232	0.091	0.226	0.079	0.206	-0.494	-0.212	-0.176	-0.727**
c-p 4	-0.615*	-0.679**	-0.674**	-0.671**	-0.506*	-0.532*	-0.656**	-0.143	0.224	0.594*	0.116
c-p 5	-0.804**	-0.746**	-0.854**	-0.777**	-0.507*	-0.737**	-0.712**	-0.153	-0.092	0.433	-0.221
Genera richness	-0.836**	-0.736**	-0.845**	-0.771**	-0.409	-0.601*	-0.765**	0.144	0.267	0.656**	0.085
Abundance	-0.329	-0.182	-0.162	-0.168	-0.029	-0.018	-0.176	0.680**	0.444	0.529*	0.297
Enrichment Index	0.382	0.468	0.018	0.468	0.753**	0.521*	0.444	0.059	0.662**	0.288	0.104
Structural Index	-0.447	-0.474	-0.750**	-0.459	-0.25	-0.341	-0.453	-0.303	0.2	0.553*	-0.081

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Indices, trophic & c-p groups	*As*	*Cd*	*Cr*	*Cu*	*Ni*	*Pb*	*Zn*	*pH*	*Moisture*	$N_{inorg.}$	C_{org}
Channel Index	-0.564*	-0.542*	-0.338	-0.514*	-0.281	-0.416	-0.612*	0.103	0.614*	0.581*	0.118
Maturity Index 2-5	-0.456	-0.485	-0.741**	-0.479	-0.259	-0.359	-0.468	-0.285	0.259	0.597*	-0.046
Shannon Weaver Index	-0.632**	-0.568*	-0.688**	-0.635**	-0.347	-0.432	-0.638**	0.169	0.382	0.579*	0.338

* and ** represent significant level at P<0.05 and P<0.01, respectively

Table 5. Correlations among heavy metal concentrations, soil characteristics, nematode community structure, and selected ecological indices from Krompachy [1].

Omnivores and predators are generally considered the most sensitive to any disturbances and stresses [41, 42, 43]. Our data supported the high sensitivity of these two trophic groups and their preference for more eco-friendly conditions over polluted environments. In addition, only these two groups were strongly negatively correlated (P<0.01) with heavy metal pollution. Specifically, omnivores correlated negatively with all heavy metals (P<0.01), and predators strongly correlated negatively (P<0.01) with Cr. Nagy et al. [44] reported similar findings from a field experiment in Hungary, where the lowest concentration of the Cr mobile fraction that produced an observable effect was approximately 0.5 mg kg^{-1}. The presence of the more toxic and mobile Cr^{6+}, which our assay could not detect, may have been responsible for the significant influence of Cr on these two trophic groups, despite the lower Cr concentrations in our study. A synergistic contribution of other heavy metals may also have been responsible for the stress response of these trophic groups.

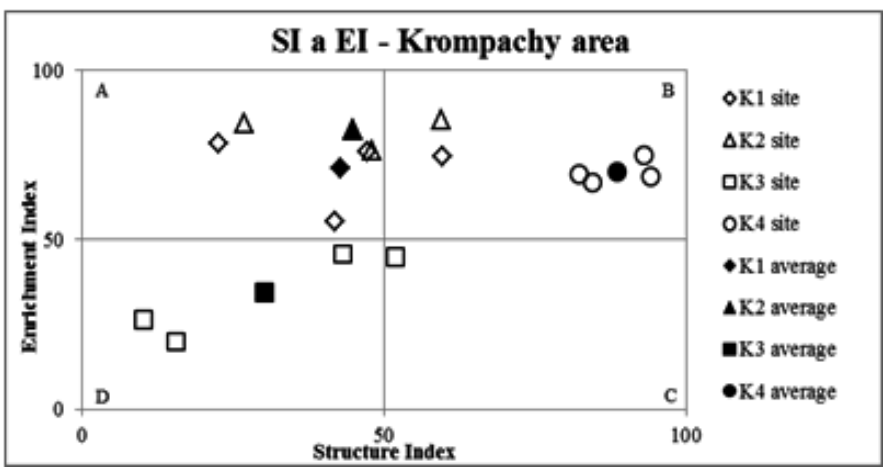

Figure 3. Structure and Enrichment Index graphic representation (Krompachy) [1]. A - disturbed food web, bacterial decomposition; B - maturing food web, balanced decomposition; C - structured food web, fungal decomposition; D - degraded food web, fungal decomposition [11].

Community characteristics and ecological indices (H′, MI2-5, and SI) indicated that the soil conditions at our sites were significantly stressed by the contaminants. Generic richness and H′ were lower under higher loads of heavy metals. The lightly contaminated K4 site had the highest diversity of nematode genera, belonging to the entire c-p scale, including opportunists (e.g. *Rhabditis*, *Acrobeloides*, and *Aphelenchus*) and persisters (e.g. *Dorylaimus*, *Oxydirus*, and *Nygolaimus*). The proportion of persisters with higher c-p values rapidly decreased with increasing pollution, which consequently led to lower nematode diversity and a simplification of the food chain. Diversity was mostly negatively correlated with the degree of pollution, but nematode abundance increased at moderately contaminated K2 and K3, but rapidly decreased under a heavy load at K1. Sánchez-Moreno et al. [39] reported similar findings in southern Spain after the Aznalcollar mining spill. A higher generic diversity reflects a higher resilience of ecosystems and complexity of relationships at all trophic levels [45, 46]. A decrease in generic diversity may cause an ecosystem to regress to an earlier successional stage or even to collapse. On the other hand, an increase in total density under a low or moderate degree of pollution does not necessarily indicate an uncontaminated environment but rather better survival conditions, as in our study (the growth in the population of opportunistic nematodes). Contamination can in some cases act as a direct or indirect stimulus to a particular component of the ecosystem and lead to inaccurate assessments of the actual environmental state [4]. Nevertheless, the generic composition of the nematode communities and several of the ecological indices (EI, SI, and MI2-5) suggest that a certain amount of regression is occurring in the study area closer to the pollution source.

Georgieva et al. [42] have studied the combined effect of heavy metals. The maturity of an ecosystem, measured by MI2-5, was negatively affected more under significantly lower polymetallic contamination than if the heavy metals were applied separately. A nematode community was highly insensitive to Cd, showing no significant changes, at soil concentrations up to 160 mg kg^{-1} [47]. Similar insensitivities in nematode communities were also described for increased levels of As [48]. The toxicity of some elements, though, can increase in combination with other heavy metals and may have negative impacts on nematodes [42]. This synergistic effect could thus be responsible for the decrease in MI2-5 and SI towards the pollution source in our study, despite their nonsignificant correlation with the heavy metals.

The analysis of SI and EI at Krompachy indicated significant differences amongst the sites in structural complexity and resource availability of the ecosystem. The graphic representation of the SI and EI parameters (Figure 3) mapped K4 in quadrat B (maturing ecosystem with low or moderate disturbance), K1 and K2 in quadrat A (ecosystem under high disturbance and with a disturbed food web), and K3 in quadrat D (stressed ecosystem with a degraded food web). These results indicated differences in the composition of the structural component (c-p groups 3-5) rather than the enrichment component (c-p 1) [49]. The shift in the ecosystem in our study under heavy metal pollution corresponded well with the conditions reported by [50, 51, 52].

The decomposition of organic matter can also have a large impact on the development of a soil ecosystem and its maturity. The rate of breakdown of organic matter depends on soil conditions and the participation of a variety of decomposers [11]. We studied decomposition using

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CI and found that the breakdown of organic matter followed distinct degradation pathways at the different sites. EI indicated high levels of energy resources at all sites except K3, but CI indicated that the quality of these resources (C:N ratio) differed: K1 and K3 had mainly bacterial pathways of degradation, dominant in N-enriched conditions, but the other sites had a slower breakdown, with an increasing importance of fungi breaking down organic matter with a higher C:N ratio. The change from fast to slow decomposition was also apparent in the nematode community structure and the substitution of bacterial enrichment opportunists (mainly *Rhabditis*) by more efficient general opportunists (*Cephalobus* and *Acrobeloides*).

4. Indirect effects of industrial emissions – A case study of SMZ JSC Jelšava

4.1. Results

4.1.1. Heavy metals

The total concentrations of heavy metals in the soil were relatively low and did not exceed the limits for uncontaminated soils (Table 6) set by [26]. Only the concentration of Mg increased, where the total and mobilisable concentrations were more than ten-fold higher at J1 (568.2 and 423.9 mg kg^{-1}, respectively) than at J4 (46.03 and 16.4 mg kg^{-1}, respectively). The increasing Mg gradient, but also for other heavy metals (Cr, Cu, Ni, and Zn) towards the pollution source (J1) is shown in Figure 4.

4.1.2. Composition of the nematode fauna: trophic and c-p groups

A total of 52 nematode genera were identified (Table 7). The abundance of most genera, e.g. *Helicotylenchus*, *Aphelenchoides*, *Axonchium*, and *Oxydirus*, increased with distance from the pollution source, but some genera, e.g. *Acrobeloides* and *Rhabditis*, dominated only at the most polluted site, J1. The generic richness was approximately two-fold higher at J2-J4 than at J1 (Table 8) and, as with abundance, was significantly (P<0.01) limited by pH (Table 9).

Trace elements	Sampling site				
	J1	J2	J3	J4	Limit
	Total concentration (HNO_3 extraction)				
Arsenic (As)	0.01±0.07^{a}	0.06±0.04^{a}	0.03±0.02^{a}	0.07±0.05^{a}	5
Cadmium (Cd)	0.03±0.01^{a}	0.031±0.01^{a}	0.03±0.01^{a}	0.02±0.01^{a}	0.3
Chrome (Cr)	0.19±0.05^{b}	0.04±0.01^{a}	0.03±0.004^{a}	0.12±0.15^{ab}	10
Copper (Cu)	7.15±1.34^{a}	3.73±1.17^{a}	2.73±0.29^{a}	1.85±0.27^{a}	20
Magnesium (Mg)	568.18±98.13^{c}	68.25±8.06^{a}	105.40±2.83^{ab}	46.03±3.46^{a}	n.a
Nickel (Ni)	0.95±0.36^{b}	0.35±0.02^{a}	0.66±0.08^{ab}	0.50±0.22^{a}	10
Lead (Pb)	2.34±1.06^{ab}	5.78±1.15^{c}	1.56±0.06^{a}	1.73±0.20^{a}	30
Zinc (Zn)	0.37±0.10^{d}	0.14±0.03^{ab}	0.22±0.03^{bc}	0.12±0.06^{a}	40
	Mobilisable fraction (Na_2EDTA extraction)				

Trace elements	Sampling site				
	J1	J2	J3	J4	Limit
Arsenic (As)	0.02±0.01[a]	0.05±0.03[a]	0.02±0.02[a]	0.02±0.002[a]	n.a.
Cadmium (Cd)	0.014±0.005[bc]	0.023±0.002[d]	0.008±0.001[a]	0.013±0.002[b]	n.a.
Chrome (Cr)	0.10±0.03[c]	0.02±0.004[a]	0.01±0.01[a]	0.02±0.004[ab]	n.a.
Copper (Cu)	5.13±2.27[c]	2.32±1.29[ab]	1.02±0.08[a]	1.64±0.26[a]	n.a.
Magnesium (Mg)	423.88±183.43[c]	60.13±6.12[ab]	47.75±14.50[a]	16.43±8.58[a]	n.a.
Nickel (Ni)	0.52±0.09[d]	0.30±0.02[b]	0.20±0.02[a]	0.34±0.02[bc]	n.a.
Lead (Pb)	0.48±0.14[a]	0.91±0.04[b]	0.75±0.11[ab]	0.72±0.42[ab]	n.a.
Zinc (Zn)	0.21±0.04[d]	0.12±0.03[bc]	0.06±0.01[a]	0.09±0.01[ab]	n.a.

[abcd] Means followed by the same letters on the same rows are not statistically different according to Least Significant Difference Test (P<0.05)

Limit - limits posted by The Decree of the Ministry of Land Management of the Slovak Republic No. 531/1994-540 on the admissible values of harmful substances in uncontaminated soil. n.a. – not available

Table 6. Total and mobilisable concentration of trace elements in sampling sites from Jelšava (mg.kg^{-1}) [53].

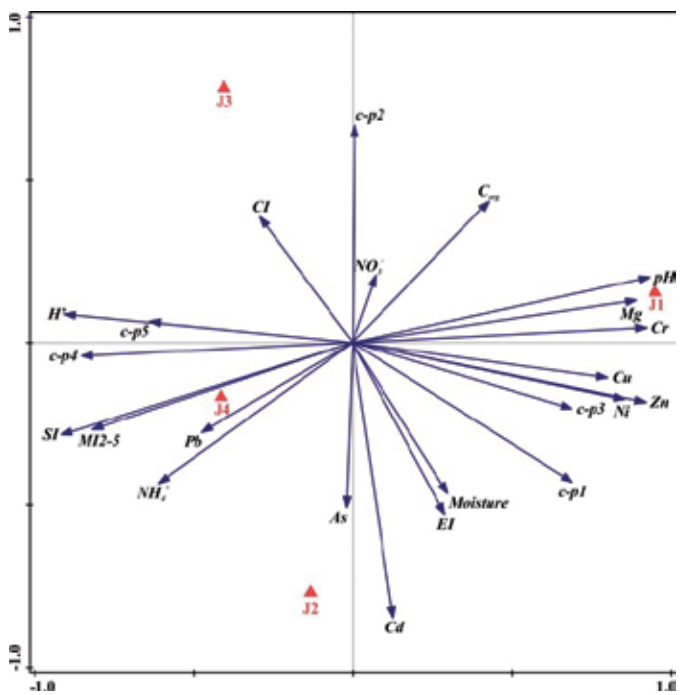

Figure 4. Redundancy analysis (RDA) performed on physicochemical soil properties, trace elements and selected indices in relation to sampling sites with data explained 64.0 % of the variation in the first two axes; F=7.1; P=0.002 (Jelšava) [53].

The soil pH and concentrations of Mg, Cr, Cu, Ni, and Zn strongly (P<0.01) influenced all trophic groups, predators and omnivores in particular (Tables 8 and 9). Bacterivores, with more

than 90% proportion in community at J1, were the only trophic group with a positive correlation (P<0.01) with pH, Cu, Mg, and Zn (Table 9). The proportion of plant feeders, the second most abundant trophic group, was relatively low (8.1%) near the pollution source, while fungivores reached a proportion of only 0.41%, omnivores 0.22%, and predators were absent in community (Table 8).

The distribution of the c-p groups amongst the sites supported their mapping according to r/K characteristics. The group most tolerant to pollution, c-p 1, dominated under strong contamination (Table 8) and was able to survive even at the higher concentrations of Mg, Zn, and Cu (Table 9). The abundance of c-p 3 was positively correlated only with Cr and Zn (Table 9) and was highest at J1 (Figure 4), mostly due to a high frequency of bacterivores (*Prismatolaimus* and *Teratocephalus*) and plant feeders (*Pratylenchus*) (Table 7). In contrast, c-p 4 and 5 were sensitive to Cu, Mg, and Zn contamination and alkaline soil (Table 9), and occurred predominantly at the sites farthest from the pollution source. Representatives of c-p 2 were positively influenced mainly by C_{org}.

4.1.3. Ecological indices

The ecological indices indicated degradation of the soil ecosystem at J1, most likely due to the exceptionally high alkalinity and Mg content (Figure 4). J3 had the highest diversity, as shown by H′, which decreased towards J1 (Table 8). MI2-5 and SI supported this trend, with significant changes (P<0.01) in values between J1 and J2 (Table 8).

The amount of available food resources in the soil system identified by EI was similar at all sites, but J3 had slightly less favourable conditions (Table 8). A graphic representation of EI and SI suggested a higher level of ecological disruption at J1 relative to the other sites (Figure 5). The trend in CI values reflected the increasing importance of fungi in the decomposition of organic matter with distance from the pollution source. The high concentration of Mg; higher levels of Cu, Zn, and C_{org}; and soil alkalinity were the most important factors influencing the structure and maturity of the nematode communities (Table 9, Figure 4).

Nematode genera		J1	J2	J3	J4
	c-p	Abundance	Abundance	Abundance	Abundance
			Bacterial feeders		
Acrobeles	2	-	1.00±2.00	-	-
Acrobeloides	2	39.75±9.91	24.50±23.16	27.00±13.14	11.50±7.85
Alaimus	4	0.25±0.50	2.75±2.50	3.00±3.56	6.75±4.99
Anaplectus	2	-	-	-	2.75±3.59
Aulolaimus	3	-	-	0.50±1.00	2.00±4.00
Cephalobus	2	4.00±3.83	21.00±14.49	21.75±14.73	11.50±3.11
Cervidellus	2	6.50±4.51	-	-	-

Nematode genera		J1	J2	J3	J4
	c-p	Abundance	Abundance	Abundance	Abundance
Diplogaster	1	-	1.25±2.50	2.25±2.87	-
Eucephalobus	2	8.75±4.11	8.25±5.50	8.25±9.54	1.25±1.89
Heterocephalobus	2	3.25±3.77	-	-	0.50±1.00
Chiloplacus	2	4.00±5.66	-	-	-
Mesorhabditis	1	11.50±7.72	4.75±9.50	-	-
Monhystera	2	-	1.00±2.00	1.00±0.82	1.50±1.73
Panagrolaimus	1	5.00±5.48	-	-	-
Plectus	2	0.50±0.58	5.00±4.08	9.50±5.80	7.25±1.89
Prismatolaimus	3	5.25±3.40	6.25±7.59	-	-
Rhabditis	1	176±76.6	286±132.4	69.75±58.01	144.3±114.4
Teratocephalus	3	11.75±6.55	-	-	-
			Fungal feeders		
Aphelenchus	2	1.25±1.89	9.00±3.56	19±8.04	19.75±9.67
Diphtherophora	3	-	1.50±1.73	2±1.83	0.5±0.58
Dorylaimoides	4	-	0.5±1	-	-
Filenchus	2	-	2.75±5.5	-	3.75±3.3
			Omnivores		
Aporcelaimellus	5	0.5±1	18±5.89	11.25±9.91	7.50±3.11
Dorylaimus	4	-	47±36.52	18.5±26.8	37±12.03
Epidorylaimus	4	-	11.25±8.06	0.5±0.58	0.5±0.58
Eudorylaimus	4	0.25±0.5	2.25±2.63	4±1.83	3.5±1.29
Mesodorylaimus	5	-	1.00±0.82	-	11.25±4.92
Prodorylaimus	5	-	6.75±6.8	9.75±9.84	12.75±11.67
			Predators		
Anatonchus	4	-	0.25±0.50	6.25±2.75	2±1.41
Clarkus	4	-	0.5±1	-	15.50±9.61
Discolaimus	5	-	-	1.25±1.26	-
Miconchus	4	-	2.00±2.31	2.00±2.16	0.25±0.50
Mononchus	4	-	0.50±0.58	0.50±1.00	-
Mylonchulus	4	-	4.50±2.89	7.25±4.11	3.00±4.24
Nygolaimus	5	-	0.25±0.50	-	-
Oxydirus	5	-	16.50±22.22	17.25±9.18	93.75±88.28

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Nematode genera		J1	J2	J3	J4
	c-p	Abundance	Abundance	Abundance	Abundance
Prionchulus	4	-	0.25±0.50	-	-
Tripyla	3	-	12.25±15.52	0.75±1.50	-
			Plant feeders		
Aglenchus	2	1.00±2.00	12.00±14.24	0.25±0.50	0.50±1.00
Aphelenchoides	2	7.00±6.78	22.50±11.70	38.50±40.34	6.50±1.73
Axonchium	5	-	22.75±29.03	11.50±8.70	210±64.10
Boleodorus	2	-	10.75±13.79	2.75±4.19	7.00±5.60
Coslenchus	2	-	-	-	0.50±1.00
Ditylenchus	2	0.25±0.50	-	-	-
Geocenamus	3	0.25±0.50	0.50±1.00	-	-
Helicotylenchus	3	1.00±1.41	88.25±109.2	30.75±32.94	64.00±35.80
Malenchus	2	-	14.75±16.76	-	3.75±5.68
Paratylenchus	2	0.25±0.50	25.00±38.43	11.25±13.40	3.75±1.71
Pratylenchus	3	15.50±17.90	6.75±5.68	0.25±0.50	2.75±4.19
Rotylenchus	3	-	21.50±32.13	13.25±16.68	24.25±13.87
Tylenchus	2	-	16.50±14.06	7.00±6.48	18.75±22.97
Xiphinema	5	-	24.00±28.32	-	-

Table 7. C-p values and the average abundance (±SD) of nematode genera in individual sampling sites from Jelšava [53].

4.2. Discussion

In this case study, the long-term industrial emission of magnesite has affected the soil ecosystem, mostly by changing the basic soil conditions. Such high loads of Mg in the soils as recorded in our study are rare, so the impact of Mg emissions on soil fauna is not often studied and is not yet understood [53]. Similar steep increases in soil alkalinity as a direct consequence of industrial inputs of Mg as was found in our assay described also Machín and Navas [54]. Most studies have investigated the importance of Mg deficiency in higher plants as a trace element important in photosynthesis. Hronec [17] described a decrease in soil fertility and the replacement of native plant species by tolerant halophytes in an area with high Mg input. Moreover, the long-term fallout of Mg dust (>600 t km^{-2} y^{-1}) may degrade natural ecosystems and create corrosive crusts that could affect natural interactions in the soil even more [17].

The absence of sensitive nematode groups, likely caused by an ineffective regulation of osmotic pressure by their cuticles due to changes in the concentrations of various ions in the soil pore water, can be a direct effect of altered soil conditions [55]. The concentrations of ions in soil solutions under extreme changes in pH may negatively affect the regulation of water by

organisms [56], thereby disrupting homeostasis. A change in the concentrations of soil ions may thus have been an important factor responsible for the decrease in the higher c-p groups (mainly predators and omnivores with highly permeable cuticles) at the sites with high pH and Mg concentration. The indirect influence of contamination was likely a restriction in the availability of resources and the interactions necessary for survival or an effect on the abiotic attributes of the environment [32].

Indices, trophic & c-p groups	Sampling sites			
	J1	J2	J3	J4
	Trophic & c-p groups			
Bacterial feeders	91.31±6.28^{a}	50.47±12.29^{bc}	38.98±7.86^{cd}	25.20±13.23^{d}
Fungal feeders	0.41±0.52^{a}	3.02±3.49^{ab}	6.16±2.38^{b}	3.19±0.77^{ab}
Omnivores	0.22±0.26^{a}	11.32±3.05^{a}	11.24±9.64^{a}	10.09±1.87^{a}
Predators	0±0^{a}	4.38±3.22^{ab}	12.67±9.17^{ab}	14.94±8.21^{b}
Plant feeders	8.06±6.18^{a}	30.80±14.86^{ab}	30.95±14.16^{ab}	46.59±5.97^{b}
c-p 1	67.96±5.10^{a}	58.85±7.00^{ab}	25.66±16.27^{c}	33.42±20.39^{bc}
c-p 2	25.57±5.57^{a}	15.14±4.42^{a}	38.96±19.95^{a}	15.35±3.55^{a}
c-p 3	6.11±1.54^{a}	3.81±3.81^{a}	1.04±0.90^{a}	0.94±1.75^{a}
c-p 4	0.22±0.44^{a}	14.06±4.71^{b}	16.58±7.91^{b}	17.64±3.58^{b}
c-p 5	0.13±0.25^{a}	8.14±3.10^{a}	17.75±8.25^{ab}	32.65±19.04^{b}
	Ecological indices			
Genera richness	14.50±0.58^{a}	28.75±2.22^{b}	25.25±3.50^{b}	27.50±1.00^{b}
Abundance	303.75±104.6^{a}	764±376.06^{a}	358.75±169.6^{a}	742.00±224.8^{a}
Enrichment Index	91.33±2.30^{a}	93.94±2.01^{a}	68.96±27.63^{a}	87.50±8.31^{a}
Structure Index	38.12±7.22^{a}	88.25±4.03^{b}	80.73±11.18^{b}	93.24±4.87^{b}
Channel Index	0.16±0.20^{a}	1.47±1.70^{a}	20.42±32.01^{a}	5.35±3.10^{a}
Maturity Index 2-5	2.22±0.050^{a}	3.39±0.25^{b}	3.21±0.47^{b}	3.93±0.44^{b}
Shannon-Weaver Index	1.56±0.08^{a}	2.33±0.24^{b}	2.57±0.13^{b}	2.29±0.11^{b}

^{abcd} Means followed by the same letters on the same rows are not statistically different according to Least Significant Difference Test (P<0.01).

Table 8. Percentage of individual nematode trophic groups and average of ecological indices values calculated for Jelšava sampling sites [53].

In areas with neutral soil pHs (as at J4), strong shifts in soil composition are usually expected after an increase in pH. Alterations in edaphic diversity after pH manipulation have been confirmed experimentally and by field studies. Earthworms, enchytraeids, and nematodes were more abundant after liming [57, 58], but Acari preferred acidic conditions [59, 60]. The altered soil conditions in our study as a consequence of Mg contamination and an extremely

http://dx.doi.org/10.5772/60404

high pH have led to direct and indirect effects on the nematode communities and most likely influenced the composition of the entire soil fauna. The plant feeders showed the first signs of the indirect effects of high Mg levels. A fluctuation in plant feeder density is generally considered a sign of changes in primary production [61]. Excessive concentrations of Mg under alkaline conditions induce a deficiency of essential nutrients (e.g. Ca and P) and impede the development of plant root systems [62]. Relatively impoverished phytocoenoses composed mainly of halophytes such as *Puccinellia distans* or *Agrostis stolonifera* are usually found near pollution sources [63]. A shift towards bacterivorous nematodes in the community was another indication of an indirect influence of pollution in our study, because bacteria prosper more than fungi at alkaline pHs [63].

Indices, trophic & c-p groups	***As***	***Cd***	***Cr***	***Cu***	***Mg***	***Ni***	***Pb***	***Zn***	***pH***	***Moisture***	$N_{inorg.}$	C_{org}
Bacterial feeders	0.116	0.168	0.349	0.779**	0.888**	0.471	-0.162	0.642**	0.750**	0.253	-0.438	0.433
Fungal feeders	-0.266	-0.479	-0.653**	-0.467	-0.472	-0.696**	0.24	-0.794**	-0.361	-0.396	0.542*	-0.052
Omnivores	0.108	0.303	-0.513*	-0.306	-0.437	-0.381	0.620*	-0.462	-0.711**	-0.032	0.414	-0.432
Predators	-0.056	-0.301	-0.535*	-0.641**	-0.839**	-0.545*	0.225	-0.681**	-0.603*	-0.384	0.446	-0.539*
Plant feeders	0.019	0.078	-0.259	-0.726**	-0.862**	-0.406	0.2	-0.499	-0.789**	-0.074	0.465	-0.471
c-p 1	0.221	0.41	0.491	0.788**	0.738**	0.606*	-0.126	0.793**	0.555*	0.482	-0.403	0.188
c-p 2	-0.361	-0.576*	0.013	-0.047	0.406	-0.091	-0.279	-0.141	0.550*	-0.321	-0.221	0.727**
c-p 3	0.118	0.351	0.653**	0.319	0.619*	0.481	-0.274	0.700**	0.526*	0.425	-0.322	0.329
c-p 4	-0.16	-0.126	-0.495	-0.596*	-0.681**	-0.457	0.265	-0.726**	-0.735**	-0.263	0.375	-0.271
c-p 5	-0.081	-0.244	-0.526*	-0.646**	-0.791**	-0.563*	0.257	-0.651**	-0.617*	-0.33	0.419	-0.526*
Genera richness	0.175	0.406	-0.500*	-0.543*	-0.527*	-0.491	0.618*	-0.396	-0.725**	0.018	0.359	-0.632**
Abundance	0.043	0.394	-0.247	-0.313	-0.475	-0.13	0.215	-0.18	-0.715**	0.171	-0.012	-0.613*
Enrichment Index	0.458	0.661**	0.228	0.476	0.288	0.306	0.194	0.553*	0.055	0.597*	0.012	-0.303
Structural Index	0.08	0.121	-0.422	-0.429	-0.732**	-0.338	0.353	-0.421	-0.753**	-0.121	0.365	-0.753**
Channel Index	-0.241	-0.445	-0.501*	-0.581*	-0.720**	-0.593*	0.104	-0.753**	-0.478	-0.313	0.583*	-0.148
Maturity Index 2-5	0.1	0.105	-0.451	-0.415	-0.729**	-0.35	0.359	-0.439	-0.744**	-0.144	0.359	-0.736**
Shannon Weaver Index	-0.162	-0.292	-0.679**	-0.829**	-0.456	-0.779**	0.362	-0.777**	-0.426	-0.379	0.421	-0.295

* and ** represent significant level at P<0.05 and P<0.01, respectively.

Table 9. Correlations among heavy metal concentrations, soil characteristics, nematode community structure, and selected ecological indices from Jelšava [53].

An abundance of food for bacteria often increases the proportion of c-p 1 bacterivores, as confirmed experimentally by [58]. Representatives of the genus *Rhabditis* were the most abundant nematodes along the entire transect in our study. The survival and dominance of *Rhabditis* and other bacterivores in this hostile environment could, however, be a complex phenomenon, comprising not only food resources, but also behavioural and physiological adaptations to the hostile conditions [64]. For example, *C. elegans* can withstand high pH and salinity and survive under extreme osmotic pressure [55, 65]. Moreover, c-p 1 nematodes can produce inactive dauer larvae, which increase the chances of withstanding harmful or limited conditions. Other studies [1, 4] have shown that c-p 2 nematodes are also able to withstand severe ecological conditions and comprise the dominant c-p group in highly disturbed ecosystems [66]. Our results only partially supported these findings. More than 25% of the nematodes at J1 belonged to the c-p 2 group, and of these, species of *Cervidellus* and *Chiloplacus* were the most tolerant. The proportion of c-p 2 nematodes within the community, however, was significantly lower than that of c-p 1 nematodes. The discrepancy between the hypothetical dominance of stress-tolerant c-p 2 nematodes and our results was likely due to various physiological and ecological aspects of c-p 1 and 2 nematodes and their population dynamics (e.g. reproduction rate, length of life cycle, and resource availability). The abundance of c-p 1 nematodes in the soil may also be due to, aside from soil fertility reported by [11], the changes in soil pH. Further study is necessary to confirm these possibilities.

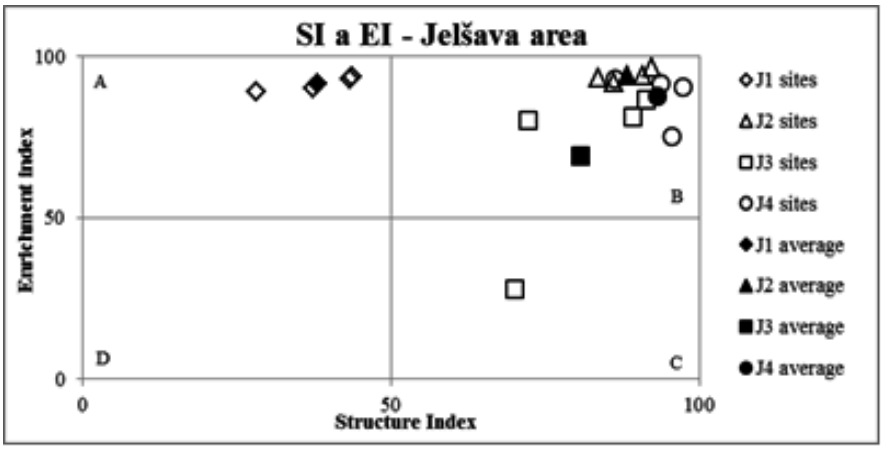

Figure 5. Structure and Enrichment Index graphic representation (Jelšava) [53]. A - disturbed food web, bacterial decomposition; B - maturing food web, balanced decomposition; C - structured food web, fungal decomposition; D - degraded food web, fungal decomposition [11].

This study has demonstrated the negative ecological impact of contamination by quantifying different ecological indices (e.g. SI, MI2-5, and H′) and measuring key parameters of nematode communities (generic richness and abundance). Not all communities traits showed a clear response to stress in the ecosystem, and some responded inconsistently, e.g. abundance similar as in the Krompachy case study did not reflect the stress of the ecosystem. Total abundance

http://dx.doi.org/10.5772/60404

should thus not be used as an indicator for either heavy metals, as suggested by [42], or physical or chemical changes in soil properties such as pH. Generic richness and H′, though, indicated substantial differences in species diversity in the ecosystems with diverse contamination and soil salinity, in accordance with diversity shifts observed in other nematode communities [58] and in other edaphic groups after pH manipulation [59, 67]. SI and MI2-5 were the best tools in our study for showing the impact of high Mg concentrations and soil salinity on nematode community structure and the maturity of the ecosystem. SI combined with EI identified J1 as severely disturbed (quadrat A), and the other sites showed only a low degree of stress. A decrease in the intensity of stress and an increase in the complexity of soil community structure with distance from a pollution source have been reported in several other areas of intensive industry [1, 44]. Based on low CI values in our study, indicating predominant bacterial pathway of decomposition, c-p 1 enrichment opportunists were eliminated from maturity assessment (MI2-5), as they respond more to the food availability than pollution [47]. Eliminating the influence of enriched conditions provided us with clear gradual increase in environment stability and maturity from the site J1 onwards.

5. Conclusions

Heavy metal contamination of soil is long-term ecological problem. High levels of pollutants may lead to losses in production, breaches in the otherwise strict sanitary limits for agricultural products, and health-related problems for local inhabitants. This study demonstrated that the background geochemical heavy metal content was significantly exceeded, with measurable consequences for the local environments. The sensitive groups of soil nematodes, mainly omnivorous and predacious nematodes of the higher c-p groups, were significantly suppressed in the ecosystems, and tolerant groups, with better physiological and behavioural adaptations to harsh conditions, survived. Such alterations in community structure may have far-reaching consequences, because some of the ecological regulation or environmental feedbacks provided by nematodes may be suppressed in, or eliminated from, the ecosystem.

Despite the recent decrease in emissions and the application of various treatments to prevent their leakage at both metallurgical plants, the potential threat of further environmental deterioration remains relatively high. The high concentrations of possible toxic elements bound to the soil matrix and temporally unavailable to the soil biota are the main threat in the Krompachy area. The release of the pollutants to the soil solution by sudden shifts in local soil conditions, e.g. an increase in soil acidity by acid rain, could lead to their increased toxicity to soil communities or to groundwater contamination. The threat from pollution to the ecosystem around Jelšava is less imminent, because the levels of toxic elements were not significantly higher. The high Mg concentrations and soil salinity, and consequently the extreme soil alkalinity, however, are the most important problems in this area. Under these conditions, only a few organisms with adaptations for surviving in such an environment are able to prosper, as our results clearly showed. In both case studies, the nematode community structures collapsed near the pollution sources, with the domination of nematodes able to survive inhospitable soil conditions.

Acknowledgements

The authors acknowledge the support of the Slovak Research Development Agency (project LPP-0085-09) (0.6), VEGA (Grant No. 2/0193/14) (0.3), and the project "Application Centre to protect humans, animals and plants against parasites" (Code ITMS: 26220220018) with the support of the Operational Program "Research and Development" funded by the European Regional Development Fund (0.1).

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Chapter 3

Perfluorinated Chemicals in Sediments, Lichens, and Seabirds from the Antarctic Peninsula — Environmental Assessment and Management Perspectives

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60205

Abstract

Antarctica is one of the last frontiers of the planet to be investigated for the environmental transport and accumulation of persistent organic pollutants. Perfluorinated contaminants (PFCs) are a group of widely used anthropogenic substances, representing a significant risk to wildlife and humans due to their high biomagnification potential and toxicity risks, especially in food webs of the northern hemisphere and Arctic. Because the assessment of PFCs in the Antarctic continent is scarce, questions linger about the long-range transport and bioaccumulation capacity of PFCs in Antarctic food webs. To better understand the global environmental fate of PFCs, sediment, lichen (*Usnea aurantiaco-atra*), and seabird samples (southern giant petrel, *Macronectes giganteus*; gentoo penguin, *Pygoscelis papua*) were collected around the Antarctic Peninsula in 2009. PFC analytes were analyzed by LC/MS/MS, revealing the detection of PFHpA in seabirds' feather and fecal samples, and PFHxS in lichens. PFBA and PFPeA were detected in 80% and 60% of the lichens, and PFTA in 60% of sediment samples. While oceanic currents and atmospheric transport of PFCs may explain the ubiquitous nature of these contaminants in the Antarctic Peninsula, military bases and research stations established there may also be contributing as secondary sources of PFCs in the Antarctic ecosystem.

Keywords: Perfluorinated compounds, biota, sediment, Antarctic Peninsula, global transport

1. Introduction

Past research shows that legacy persistent organic pollutants (POPs) such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), and hexachlorocyclohexanes (HCHs) pose substantial problems related to environmental and ecosystem health on a global scale [1–5]. POPs can be transported over very long distances, biomagnify in food webs, and cause adverse health effects in high trophic level species such as birds and marine mammals. Cold regions that are typically isolated from anthropogenic activity, such as the Arctic and the Antarctic, are particularly vulnerable to POPs because of the global distillation phenomenon, which causes many pollutants to concentrate in these regions [6, 7]. The Arctic Monitoring Assessment Program (AMAP), in association with the United Nations Environment Programme (UNEP) Stockholm Convention on Persistent Organic Pollutants, has played a key role in documenting the fate, transport, and effects of these pollutants in the Arctic, and has promoted global initiatives to monitor, manage, and control these substances [6, 8]. Despite enhanced understanding of POP contamination in the Arctic, limited information exists on the state of pollution in Antarctic food webs. Researchers have identified a lack of comparative data between the polar regions of the world, where many efforts have been directed toward understanding POP contamination in the high latitudes of the Northern Hemisphere such as the Canadian Arctic and Greenland [8–12].

Ongoing research has identified emerging contaminants of concern, including perfluorinated contaminants (PFCs), which are expected to pose significant risks to the environment and wildlife, particularly in the Arctic and the Antarctic [13–15]. Although PFCs have been detected in some Antarctic ecosystems and biota, the environmental transport and bioaccumulation patterns of PFCs, mainly perfluoroalkyl acids (PFAAs) such as perfluorinated carboxylates (PFCAs) and perfluorinated sulfonates (PFSAs), remain relatively unexplored within Antarctica. PFCs are highly fluorinated anthropogenic compounds, often utilized as repelling agents, with applications including coatings for paper or food packaging and textiles, industrial surfactants, insecticides, and historically, aqueous film-forming foams [16,17]. Due to their widespread use, PFCs are now considered environmentally ubiquitous substances, found in all areas around the world. In response, numerous measures have been taken to reduce the adverse impacts of PFCs on local and global scales [8].

PFCs are extremely persistent, can travel long distances (predominantly via ocean currents), bioaccumulate in food webs, and achieve highest concentrations in marine mammals and birds. PFCs are of particular ecological and toxicological concern due to their tendency to biomagnify in food webs and cause adverse health effects, including reproductive damage, immunotoxicity, and hepatotoxicity [18]. Of further interest is the unique physicochemical nature of PFCs. Whereas many legacy POPs are lipophilic and therefore accumulate in fatty tissues, PFCs tend to accumulate primarily in protein-rich tissues, such as the liver. Two PFAAs, perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), represent the most commonly investigated PFCs of significant risk to wildlife and humans due to their ubiquitous nature, global fate and transport, high biomagnification potential, and toxicity risks, especially in aquatic and marine food webs of the northern hemisphere and Arctic [18–21]. Phase out

http://dx.doi.org/10.5772/60205

programs designed to eliminate the production of PFOS were established for some regions in the early 2000s, followed by the addition of PFOS to the list of restricted POPs under the Stockholm Convention on Persistent Organic Pollutants in 2009 [8]. Despite these initiatives, production of PFOS, PFOA, and several other PFAAs still take place around the world, including several developing countries [22–25]. One of the priority actions under the Antarctic Treaty is the assessment and monitoring of POPs, including PFCs, in Antarctica. Considering that assessments of PFCs in the Antarctic are limited, questions linger about the long-range environmental transport of these substances to the Southern Hemisphere, and the capacity of these substances to bioaccumulate in Antarctic food webs.

PFCs have been detected in various Antarctic environmental media and biota, typically in the pg/g to ng/g range, though many samples return nondetectable levels or levels below the minimum level of quantification [9]. Recent studies show that levels of many PFCs in Arctic environments have been increasing, with concentrations of several PFCs equivalent to or surpassing that of DDT, PCBs, PBDEs, and other organochlorine pesticides [19]. Similar patterns are anticipated for PFCs in Antarctic environments as they are continuously delivered from other geographic locations via long-range transport.

Although some PFCs are already categorized as POPs, the majority of these substances are not subject to global or local controls. To ensure that potential impacts of pollutants on Antarctic wildlife are considered in the global environmental agenda and throughout negotiations on commercial chemical production and use, it is important that a high-quality research program is developed on the fate and effects of contaminants in Antarctic ecosystems and wildlife. As part of an ongoing scientific initiative and collaboration between the Ecuadorian Antarctic Institute (INAE), Simon Fraser University (Canada), the Institute of Ocean Sciences (IOS, Fisheries and Oceans Canada, DFO), and the Escuela Superior Politecnica del Litoral (Ecuador), a study to investigate and monitor PFCs was initiated in Peninsula Antarctica around the surrounding areas of the Ecuadorian Station "Pedro Vicente Maldonado" during the 2009 Ecuadorian–Antarctic expedition. In this chapter, we provide one of the primary findings on PFCs in sediments and biotic matrices, including lichens as well as feces and feathers from the southern giant petrel (*Macronectes giganteus*) and gentoo penguin (*Pygocelis papua*), and evaluate the use of noninvasive techniques to monitor emerging organic contaminant of concern in the Antarctic environment.

2. Materials and methods

2.1. Study Area and sampling

The Ecuadorian Research Station "Pedro Vicente Maldonado" (Maldonado Station, hereafter) is located at Fort William Point, Greenwich Island (62°31'S; 59°46'W; Figure 1). The study area encompassed the Barrientos Island (62°24'01"S; 59°43' 52"W), Dee Island (62º25'48.5" S; 59º47'69.6" W), Punta Ambato (62º26'33" S; 59º47'28.8" W), and the surroundings of the Maldonado Station (62º27'59"S; 59º43'32.5"W), as illustrated in Figure 1. Sampling was conducted using three tracks established by the Maldonado Station to access the coastline of

Fort Williams, which enclose two sampling zones: Ensenada Guayaquil and Bahia Chile. These sectors are only used by technical and military personnel that work at the Station and visiting scientists that come to the island for research purposes. Barrientos Island is used principally as a tourist stopover for cruise ships where tourists land and walk around the island for bird-watching. In Dee Island and Punta Ambato, sampling was deployed around the coastline. All sampling was done during the Austral summer and seabird breeding seasons of 2009. The collection of abiotic and biotic samples is described as follows.

2.1.1. Sediments

Sediment samples were collected from three locations in the Antarctic Peninsula including Dee Island (n = 1 site), Maldonado Station (n = 2 sites), and Punta Ambato (n = 2 sites) (Figure 1). Sediment samples were directly collected using 100 mL centrifugation tubes, stored at < 4°C until transportation to the laboratory in Canada.

2.1.2. Seabirds

Gentoo penguins (*Pygoscelis papua*) and southern giant petrels (*Macronectes giganteus*), two species of seabirds that inhabit the Antarctic Peninsula, were identified as potential bio-indicators of PFCs contamination. The main reason for selecting seabirds is based on studies showing that bird populations are most affected by contaminants, specifically POPs, among wildlife species (see [26] for a review). Bird species have the greatest capacity to biomagnify chemicals because of their highly energy-efficient metabolic system and also because of their high trophic position within the food web. Bird populations are therefore often at high risk from bioaccumulative substances, and can act as the "canary in the coal mine" for the larger Antarctic ecosystem.

In this context, we conducted a noninvasive sampling technique to minimize or completely avoid the impacts of lethal or invasive sampling on the local bird populations. Sampling focuses on the collection of shed/molted feathers and excreted fecal matter from nesting sites. Because of the very high affinity of PFCs for protein, feathers are good noninvasive sampling media for PFCS, as they consist mainly of protein matter (i.e., keratin, a high molecular weight protein). Feathers have also been used to successfully monitor mercury in seabird populations such as brown skuas, *Catharacta lonnbergi*, chinstrap penguins, *Pygoscelis antarctica*, and gentoo penguins, *P. papua*), in our study area [27], as well as PFCs in the feathers of aquatic and marine birds, including grey heron (*Ardea cinerea*) and herring gull, (*Larus argentatus*) from the Northern Hemisphere [28]. Fecal matter is known to contain some of the highest concentration of contaminants due to the gastrointestinal magnification that occurs in the intestinal tract of consumer organisms. In addition, the contaminant concentrations in fecal matter are related to compounds absorbed by the organism, such that they can provide a measure of accumulated concentrations. The low capacity to migrate to the gaseous phase (i.e., air) and high octanol-air partition coefficient (K_{OA}) of the analytes (Table 1) cause minimal losses of the contaminants from feces or feathers to the air after feathers or fecal matter have been dropped. This means that the concentrations of the chosen analytes can remain a measure of bird exposure levels long after the feces have been excreted or feathers have been shed.

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Molted feathers were collected randomly in and around nests and colonies of petrels surrounding the Maldonado's Station and stored in ziploc-type plastic bags ($n = 5$). Only one bag of feather samples for gentoo penguin was collected from the Maldonado's Station. Fecal matter samples from gentoo penguins were collected from nesting sites and colonies around the Maldonado's Station ($n = 9$), Barriento Island ($n = 7$), and Dee Island ($n = 3$). All feces samples were placed into 20 mL glass vials. Both feather and fecal samples were stored in coolers and transported by airplane with dry ice (–20°C) until transportation to the lab in Canada.

2.1.3. Lichens

Lichen (*Usnea aurantiaco-atra*) samples (n = 5) were collected from rocky areas around the surroundings of the Maldonado's Station and wrapped with clean, sterile aluminum foil and stored in ziploc plastic bags until further transportation to the lab. The rationale to select lichens is based on the premise that this biological matrix can be used as a potential monitor and indicator of global atmospheric transport of some PFCs to the Antarctic Peninsula.

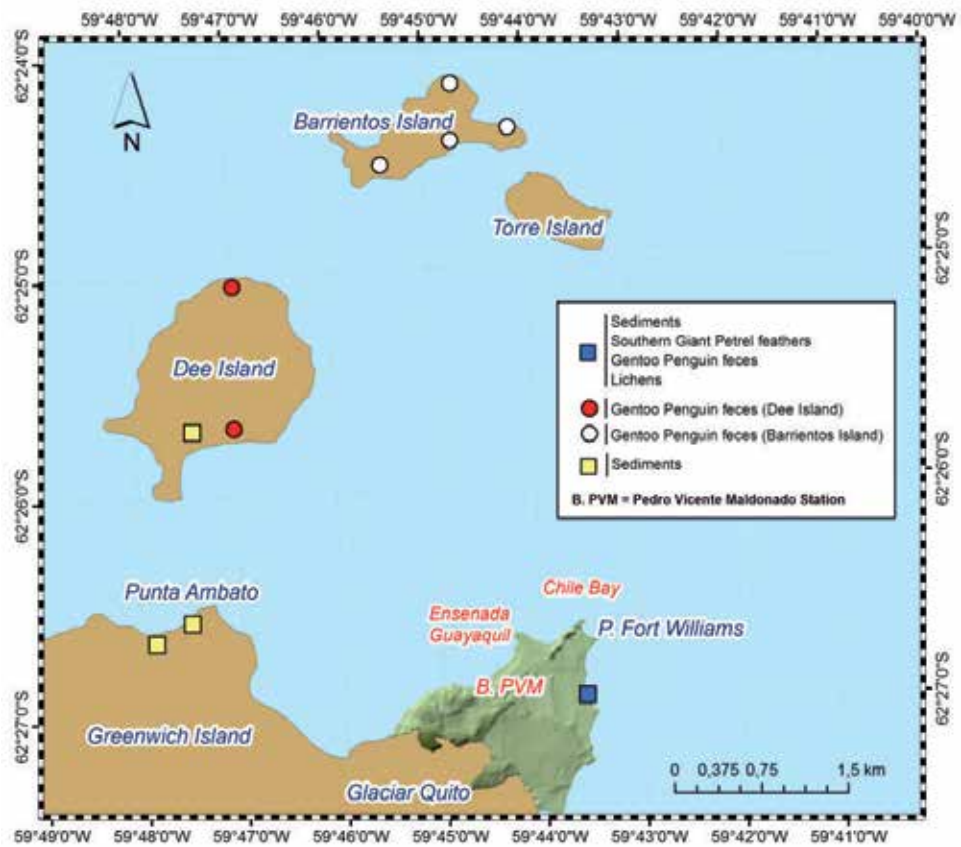

Figure 1. Geographical location of the study area and sampling sites in the Antarctic Peninsula

2.2. PFC physical – Chemical properties

Table 1 summarizes the compiled physical–chemical properties for the various PFCs studied, including molecular weights (MW), log octanol–water partition coefficients (log K_{OW}), log octanol–air partition coefficients (log K_{OA}), and log D values. Because the physicochemical properties of PFCs are considerably different from that of many other legacy POPs (i.e., they can be ionized at environmentally relevant pH), it is important to recognize that relationships applicable to other POPs may be less relevant when applied to PFCs and other ionizable compounds. For instance, many organic compounds of concern, including numerous agricultural and pharmaceutical compounds, are lipophilic in nature, and will tend to accumulate in fatty tissues [3]. The octanol–water partition coefficient (K_{OW}) has become a common property used to describe the tendency of a substance to partition into lipid, as the behavior of octanol and lipid are quite similar. Octanol thus serves as a suitable surrogate for lipid, particularly within predictive bioaccumulation models [29]. However, K_{OW} describes the lipophilicity of neutral compounds, and is not necessarily applicable to ionizable organic compounds (IOCs) such as PFCs, where the measure of lipophilicity is pH-dependent [30]. Many PFCs are almost completely ionized at environmentally relevant pH [31]. A more applicable indicator for predicting the lipophilicity of ionizable substances is log D, where both the neutral and the ionic species of the compound are accounted for [30].

2.3. PFC analysis: Extraction and quantification

Sediment and biological samples were extracted and analyzed at the Institute of Ocean Sciences (IOS), Fisheries and Oceans Canada (DFO), Sidney, British Columbia, Canada. PFC concentrations were analyzed by liquid chromatography tandem mass spectrometry with double mass detectors (LC/MS/MS), as described elsewhere [19]. Analyte concentrations were determined with respect to the mass labeled quantification and internal standards using isotope dilution method. Fifteen PFCs were examined in this study (Table 1). High purity (>95%) analytical standards, including perfluorobutane sulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), PFOS, perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotetradecanoic acid (PFTA), and perfluorooctanesulfoamide (PFOSA), were used. Mass-labeled internal standards included six PFCs ($^{13}C_2$ PFOA, $^{13}C_2$ PFDA, $^{13}C_2$ PFDoA, and $^{13}C_4$ PFOS, $^{13}C_4$-PFOA). Calibration curves were constructed from the analysis of calibration standard solutions (range 0.08–5.0 ng/mL).

Various calibration standards and standard additions were prepared and used as quality assurance/quality control (QA/QC). QA/QC measures included initial method validation work, consisting of analyte recovery experiments of native PFCs in clean sediments and biota. The method of detection limit (MDL) was set equal to the concentration of the method's level of quantification (MLOQ) for samples and subtracted from quantified concentrations of each analyte (Table 2). Only corrected data above the MLOQ are reported

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in this work. Concentrations of PFCs were expressed on a wet weight basis (ng/g ww). Extraction methods are briefly described as follows.

2.3.1. Sediment

Sediment samples (≈ 10 g wet weight) were added to 50 mL polypropylene centrifuge tubes and spiked with internal surrogate spiking solution (360 ng of $^{13}C_2$ PFOA, 120 ng of $^{13}C_2$ PFDA, 120 ng of $^{13}C_2$ PFDoA, and 120 ng of $^{13}C_4$ PFOS; Table 1). After 20 min, 10 mL of 0.1% acetic acid in MeOH was added, and samples were extracted on a shaker table for 16 h. After extraction and centrifugation, 1 mL was pipetted into 1.5 mL Ependorf vial containing 25 mg of activated carbon. Then the vial was subject to centrifugation for 30 min at 14,000 rpm; 300 μL of supernatant was taken and combined with 300 μL of water and 50 μL of 20 ppb of recovery standard and centrifuged again for 15 min at 14,000 rpm. Then, 300 μL of supernatant was used for LC/MS/MS analysis (i.e., injection volume=100 μL for LC/MS/MS).

2.3.2. Feathers

Approximately 0.74 g of feather was weighed, and then homogenized by adding first HNO_3 (e.g., 4 × 0.9 mL, 2 × 0.9 mL) with a series of vortexing steps until the whole particulates completely disappeared within 3 h. Samples were set up for digestion at room temperature (RT) for 12 h. Afterwards, 15 mL of 5 M NaOH prepared in water was added to samples and shaken on a shaker table for 5 min. The pH was measured to ensure the sample was acidic enough (i.e., pH~3–4) prior to direct injection in LC/MS/MS (large volume injection). After neutralization and extraction with 2.5 mL MeOH for a total volume 27.5 mL, ion suppression was found from recoveries; therefore, additional dilution (10×) was done until ion suppression was reduced (i.e., injection volume = 200 μL).

2.3.3. Feces

Penguin fecal matter (~0.65 g of feces) was weighed and homogenized with HNO_3 (e.g., 4 × 0.9 mL, 2 × 0.9 mL and vortexing). Samples were set at RT for digestion during 12 h. After digestion, samples were neutralized to pH equal to 3.2–4.2, brought up to 50 mL and centrifuged at 6000 rpm for 25 min; 100 μL of recovery standard (^{13}C4-PFOA) was added to an aliquot of 400 μL and injected into LC/MS/MS (i.e., injection volume= 200 μL).

2.3.4. Lichen

Lichen (2 g) was extracted based on the methodology described in reference [32]. After extraction, 4 mL of solution was blown down to 2 mL, followed by collecting 1 mL aliquot and added into 1.5 mL Ependorf vial containing 25 mg of activated carbon. The vial was subject to centrifugation for 30 min at 14,000 rpm and 300 μL of supernatant was obtained and combined with 300 μL water and 50 μL of 20 ppb recovery standard and centrifuged for 15 min at 14,000 rpm. Then, 300 μL of supernatant was used for LC/MS/MS analysis (i.e., injection volume=100 μL).

Chemical Name	Abbreviation	Formula	MW (g/mol)	Log* K_{OW}	Log* K_{OA}	Log** D
Target Analytes						
Perfluorobutanoic acid	PFBA	$C_3F_7COO–$	214.0	1.3	5.0	0.060
Perfluoropentanoic acid	PFPeA	$C_4F_9COO–$	264.0	2.1	5.3	0.54
Perfluorohexanoic acid	PFHxA	$C_5F_{11}COO-$	314.1	3.1	5.6	1.1
Perfluoroheptanoic acid	PFHpA	$C_6F_{13}COO–$	364.1	2.8	5.9	1.6
Perfluorooctanoic acid	PFOA	$C_7F_{15}COO–$	414.1	3.6	6.3	2.3
Perfluorononanoic acid	PFNA	$C_8F_{17}COO–$	464.1	4.5	6.6	2.9
Perfluorodecanoic acid	PFDA	$C_9F_{19}COO–$	514.1	5.4	6.8	3.5
Perfluoroundecanoic acid	PFUnA	$C_{10}F_{21}COO–$	564.1	6.4	7.1	4.2
Perfluorododecanoic acid	PFDoA	$C_{11}F_{23}COO–$	614.1	7.1	7.4	5.0
Perfluorotetradecanoic acid	PFTA	$C_{13}F_{27}COO–$	714.1	8.8	8	6.1
Perfluorobutane sulfonic acid	PFBS	$C_4F_9SO_{3-}$	300.1	N/A	N/A	-0.53
Perfluorohexane sulfonic acid	PFHxS	$C_6F_{13}SO_{3-}$	400.1	N/A	N/A	0.54
Perfluorooctane sulfonic acid	PFOS	$C_8F_{17}SO_{3-}$	500.1	4.3	7.8	1.7
Perfluorodecane sulfonic acid	PFDS	$C_{10}F_{21}SO_{3-}$	600.1	N/A	N/A	3.1
Perfluorooctane sulfonamide	PFOSA	$C_8F_{17}SO_2NH_2$	499.1	6.3	8.4	-
Mass Labeled Standards						
Perfluorooctanoic acid[a]	$^{13}C_2$–PFOA	-	-	-	-	-
Perfluorodecanoic acid[b]	$^{13}C_2$–PFDA	-	-	-	-	-
Perfluorododecanoic acid[c]	$^{13}C_2$–PFDoA	-	-	-	-	-
Perfluorooctane sulfonic acid[d]	$^{13}C_4$–PFOS	-	-	-	-	-
Perfluorooctanoic acid[e]	$^{13}C_4$–PFOA	-	-	-	-	-

MW: molecular weight

*Log K_{OW} and K_{OA} values of individual PFCs were compiled from published values calculated using SPARC general partitioning model [33].

**Log D values were calculated at pH = 7.5 and T = 21°C using SPARC.

[a] used to quantify PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA

[b] used to quantify PFDA and PFUnA

[c] used to quantify PFDoA and PFTA

[d] used to quantify PFBS, PFHxS PFOS, PFDS, PFOSA

[e] used to quantify recovery of mass labeled surrogates

Table 1. List of target perfluoroalkyl chemicals (PFCs) and radiolabeled surrogates monitored using LC/MS/MS.

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Analyte	Sample Type			
	Feather	**Feces**	**Lichen**	**Sediment**
	MLOQ (ng/g) for 0.74 g sample	MLOQ (ng/g) for 2 g sample	MLOQ (ng/g) for 2 g sample	MLOQ (ng/g) for 10 g sample
PFBA	0.81	2.16	0.73	2.04
PFPeA	1.22	5.86	4.11	4.34
PFHxA	1.10	2.51	3.33	1.75
PFHpA	0.57	3.25	0.77	0.55
PFOA	1.14	3.08	3.34	2.34
PFNA	0.80	4.04	1.04	1.23
PFDA	0.82	2.80	0.51	0.40
PFUnA	2.78	4.01	0.78	0.17
PFDoA	1.79	7.10	0.22	0.26
PFTA	1.30	10.86	0.57	0.25
PFBS	0.03	0.92	0.09	0.12
PFHxS	0.10	0.45	0.19	0.25
PFOS	0.30	0.88	0.75	0.33
PFDS	2.44	1.42	0.12	0.13
PFOSA	1.43	N/A	0.76	0.32
FHUEA	0.64	2.96	0.19	0.25
FOUEA	1.06	1.55	0.08	0.27
FDUEA	4.02	4.73	0.31	0.25

Table 2. Method's limit of quantification (MLOQ) for PFC analytes measured by LC/MS/MS.

3. Result and discussion

3.1. PFC concentrations

Several PFC compounds showed concentrations above the MLOQ, as shown in Table 3. Perfluorotetradecanoic acid (PFTA), a chemical with a high K_{OW} and high K_{OA} that will persist for decades in humans, was measured in 60% of sediment samples, but undetected or below the MLOQ in lichens, feces, and feathers. Perfluoroheptanoic acid (PFHpA) was detected in all seabird feather samples (range = 1.60–2.85 ww ng/g; Table 3), and in 47% of penguin feces, ranging 0.37–22 ng/g ww. All lichen samples exhibited concentrations of perfluorohexanesulfonate (PFHxS), ranging 0.20–1.20 ng/g ww, while perfluorobutyric acid (PFBA), perfluoro-n-pentanoic acid (PFPeA), and PFHpA were measured in 80%, 60%, and 60% of lichen samples, respectively. PFOA and PFOS were not quantified in most samples (i.e., < MLOQ or ND; Table

3), except for the detection of PFOS in two penguin feces samples (2.8 and 3.14 ng/g ww), and PFOA in a single fecal sample (2.0 ng/g ww) and lichen (4.7 ng/g ww). The lack of PFOS and PFHxS detection in Antarctic seabird feathers contrasts with the levels of PFOS and PFHxS found in feathers of grey herons (PFOS: 247 ng/g dw; PFHxS: ≈ 20 ng/g dw) and herring gulls (PFOS: 79 ng/g dw; PFHxS: > 30 ng/g dw) from the Northern Hemisphere (Flanders, Belgium) [28]. However, the absence of PFOA in our feather samples is consistent with the lack of detection of this compound in bird feathers from the same region [28]. For comparison purposes, the PFOA concentration detected in a sample of gentoo penguin feces was 14 times lower than the PFOA concentration (28.2 ng/g ww) detected in a single herring gull liver sample from Belgium [28]. Despite samples from other parts of the world that indicate a continued increase or no change in PFOS levels following the 2002 phase-out [34–37], a fast decline in PFOS concentrations has been observed in wildlife over the past decade [38,39]. PFDA, PFUnA, PFDoA, and PFOSA were not detected (ND) or < MDL. Except for the compound PFHpA, lack of detection of most analytes in samples and small sample sizes preclude undertaking robust statistical analyses for multisite or/and inter-species comparisons.

3.2. PFC patterns

Figure 2 shows the composition of PFCs observed in biotic and abiotic samples. PFHpA was the only compound detected in feathers of both petrels and penguins, accounting for 100% of total PFCs, while PFTA was equal to 100% of PFCs in sediment samples (Figure 2). PFHpA was also found in feces and lichen samples making up 24.5% and 23% of total PFCs, respectively. PFDS contributed to 54% and 17% of PFCs in feces and lichens, contrasting with PFPeA and PFHxS, which accounted for 3.4% and 50%, and for 4% and 21% of PFCs in feces and lichen samples, respectively. PFNA accounted for 38% of the PFCs in feces. These patterns clearly show that both perfluorinated sulfonates (PFSAs) and carboxylates (PFCAs) exhibit different fractions in seabirds, reflecting the potential role of biotransformation in shaping the accumulation of these compounds.

3.3. Bioaccumulation of PFCs

The biomagnification factor (BMF) [40] for PFHpA (i.e., BMF = C_B/C_D, where C_B is the PFHpA concentration detected in the predator, the giant petrel, and C_D is the PFHpA concentration observed in the diet/prey, gentoo penguin) was calculated using feather concentrations, as this was the only PFC compound readily detected in 100% of feathers samples. Hence, the concentrations of PFHpA in the petrel feathers (i.e., mean ±SD = 2.6 ± 0.60 ng/g ww; n = 5) and that of the penguin (1.60 ng/g ww; n =1; Table 3) were used as surrogates for concentrations in the tissues of the whole organism, assuming that the birds had been exposed to the compound for a sufficiently long time to allow the concentrations to reach steady state [40]. The criterion applied to indicate that PFHpA was biomagnified in petrels was a BMF > 1, such that a BMF greater than 1 indicates that the chemical is a bioaccumulative substance [41]. Here, we found that the BMF was close to 2 (i.e., 1.6), indicating that PFHpA biomagnifies in petrels. Although the concentrations of PFHpA in feces appear to be relatively higher than the concentrations found in lichen and feathers, comparisons of the PFHpA concentrations among

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Sample Type	Sample code	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFTA	PFBS	PFHxS	PFOS	PFDS	PFOSA	FHUEA	FOUEA	FDUEA
Feather	PETFEA1202MS1	N.D	N.D	N.D	2.85	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<0.30	<2.44	N.D	N.D	N.D	N.D
	PETFEA1202MS2	N.D	N.D	N.D	2.32	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	PETFEA1202MS3	N.D	N.D	N.D	3.54	N.D	N.D	N.D	N.D	N.D	N.D	<0.03	N.D	N.D	<2.44	N.D	N.D	N.D	N.D
	PETFEA1202MS4	N.D	N.D	N.D	2.24	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<0.30	N.D	N.D	N.D	N.D	N.D
	PETFEA1202MS5	N.D	N.D	N.D	1.93	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<0.30	<2.44	N.D	N.D	N.D	N.D
	GENFEA1002	N.D	N.D	N.D	1.63	N.D	<0.82	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<2.44	N.D	N.D	N.D	N.D
Feces	SF2801DI2	N/A	1.95	<2.51	<3.25	N.D	<4.0	N.D	N.D	N.D	N.D	1.42	4.90	<0.88	N.D	N.D	11.7	34.4	N.D
	SF2801DI1	N/A	N.D	<2.51	<3.25	<3.1	<4.0	N.D	N.D	N.D	N.D	N.D	<0.45	<0.88	<1.42	N.D	N.D	N.D	N.D
	SF2801DI3	N/A	N.D	<2.51	10.2	<3.1	9.50	N.D	N.D	N.D	N.D	0.33	<0.45	<0.88	3.33	N.D	N.D	N.D	N.D
	GENFEC0202MS1	N/A	2.63	0.94	N.D	<3.1	3.90	N.D	N.D	N.D	N.D	N.D	N.D	<0.88	108	N.D	N.D	32.7	<4.7
	GENFEC0202MS2	N/A	N.D	3.37	1.15	<3.1	<4.0	N.D	N.D	N.D	N.D	0.28	<0.45	<0.88	<1.42	N.D	N.D	N.D	N.D
	GENFEC0502MS1	N/A	N.D	2.30	4.38	<3.1	N.D	N.D	N.D	N.D	N.D	2.39	2.67	2.80	117	N.D	<2.96	37.8	0.56
	GENFEC0502MS2	N/A	N.D	3.27	0.37	<3.1	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	10.5	N.D	N.D	N.D	N.D
	GENFEC0502MS3	N/A	N.D	<2.51	0.16	<3.1	N/A	N.D	N.D	N.D	N.D	0.32	<0.45	N.D	N.D	N.D	N.D	N.D	N.D
	GENFEC0502MS4	N/A	N.D	N.D	N.D	<3.1	N/A	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<1.42	N.D	N.D	N.D	N.D
	GENFEC0502MS5	N/A	9.65	1.33	11.09	2.03	7.23	N.D	N.D	N.D	N.D	1.52	1.77	3.14	130	N.D	<2.96	37.9	0.07
	GENFEC3101BI1	N/A	N.D	<2.51	<3.25	<3.1	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	<1.42	N.D	N.D	N.D	N.D
	GENFEC3101BI2	N/A	N.D	N.D	<3.25	<3.1	5.50	N.D	N.D	N.D	N.D	N.D	<0.45	<0.88	<1.42	N.D	N.D	N.D	N.D
	GENFEC3101BI3	N/A	N.D	N.D	21.9	<3.1	1.23	N.D	N.D	N.D	N.D	N.D	N.D	<0.88	<1.42	N.D	N.D	N.D	N.D
	GENFEC3101BI4	N/A	N.D	N.D	N.D	<3.1	5.01	N.D	N.D	N.D	N.D	0.38	N.D	N.D	<1.42	N.D	N.D	N.D	N.D
	GENFEC3101BI5	N/A	N.D	N.D	<3.25	<3.1	5.81	N.D	N.D	N.D	N.D	N.D	<0.45	N.D	23.1	N.D	N.D	N.D	N.D
	GENFEC3101BI6	N/A	N.D	N.D	N.D	<3.1	<4.0	N.D	N.D	N.D	N.D	N.D	<0.45	<0.88	<1.42	N.D	N.D	N.D	N.D
	GENFEC3101BI7	N/A	9.65	<2.51	2.41	<3.1	N/A	N.D	N.D	N.D	N.D	N.D	N.D	N.D	117	N.D	N.D	32.7	<4.7
	PENFEC2601MS1	N/A	3.80	<2.51	17.61	<3.1	<4.0	N.D	N.D	N.D	N.D	N.D	N.D	<0.88	75.5	N.D	N.D	32.4	<4.7
	PENFEC2901MS2	N/A	N.D	N.D	N.D	<3.08	N.D	N.D	N.D	N.D	N.D	0.54	<0.45	N.D	N.D	N.D	N.D	N.D	N.D
Lichen	L1002MS1	2.91	4.06	N.D	5.28	N.D	N.D	N.D	N.D	<0.22	<0.57	<0.09	1.05	N.D	<0.12	<0.76	<0.19	N.D	N.D
	L1002MS2	<0.73	<4.1	N.D	N.D	N.D	<1.04	N.D	N.D	<0.22	<0.57	<0.09	0.80	N.D	0.32	<0.76	<0.19	N.D	N.D
	L1002MS3	0.55	6.05	N.D	1.77	<3.3	<1.04	N.D	N.D	<0.22	<0.57	<0.09	0.11	<0.75	N.D	<0.76	<0.19	N.D	N.D
	L1002MS4	1.30	5.29	<3.3	0.87	4.69	<1.04	N.D	N.D	<0.22	<0.57	N.D	1.16	<0.75	<0.12	<0.76	<0.19	N.D	<0.31
	LB2601MS1	1.22	<4.1	<3.3	N.D	<3.3	<1.04	N.D	N.D	<0.22	<0.57	N.D	0.19	<0.75	0.07	<0.76	N.D	N.D	<0.31
Sediment	Control	N.D	N.D	N.D	N.D	<2.3	<1.2	<0.40	<0.17	<0.26	0.12	N.D	N.D	<0.33	N.D	N.D	N.D	N.D	<0.25
	S2801DI1	N.D	N.D	N.D	N.D	<2.3	N.D	<0.40	<0.17	<0.26	N.D	N.D	N.D	<0.33	<0.13	<0.32	<0.25	<0.27	<0.25
	S0502MS1	N.D	N.D	N.D	N.D	<2.3	N.D	N.D	<0.17	N.D	N.D	N.D	N.D	<0.33	<0.13	<0.32	<0.25	<0.27	<0.25
	S0502MS2	N.D	N.D	N.D	N.D	<2.3	<1.2	<0.40	<0.17	<0.26	0.14	N.D	<0.25	N.D	<0.13	<0.32	N.D	N.D	<0.25
	S2701PA1	<2.04	N.D	N.D	N.D	<2.3	N.D	<0.40	<0.17	<0.26	0.11	N.D	N.D	N.D	<0.13	<0.32	N.D	N.D	<0.25
	S2701PA2	N.D	<4.3	N.D	N.D	<2.3	N.D	<0.40	<0.17	<0.26	0.11	N.D	N.D	N.D	<0.13	<0.32	N.D	N.D	<0.25

ND means that the compound of interest is not detectable with the current method; no visible peak in the ion chromatogram.
Values showing the less than sign (<) are equal to <MLOQ (see Table 2). <MLOQ means that the compound of interest is detectable with the current.
Method, but unable to quantify due to the low concentration in the sample; there is a visible peak in the ion chromatogram, but not quantifiable.
N/A means not applicable, identification uncertainly due to chromatographic interference.

Table 3. Quantification data of PFCs (ng/g ww) in feather, feces, lichen, and sediment samples collected in the Antarctic Peninsula. Data taken and modified from reference [63].

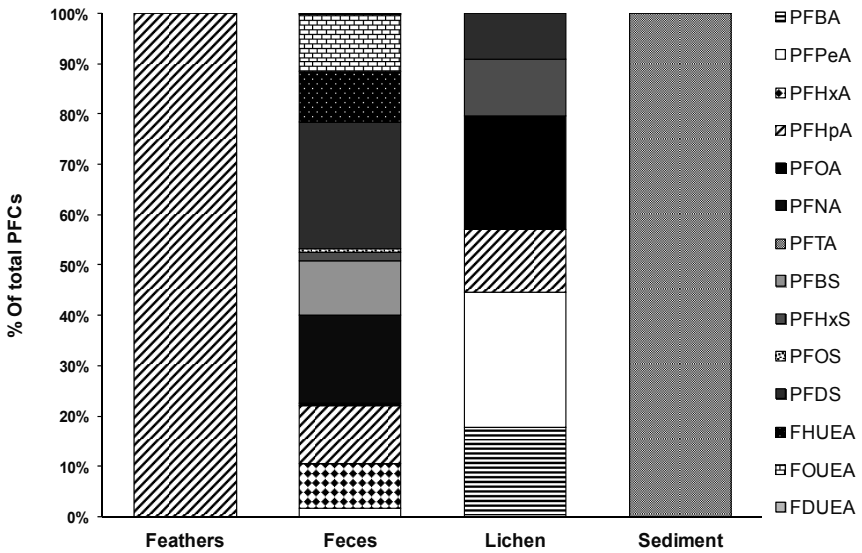

Figure 2. Composition pattern of PFC compounds detected in biotic (feathers and feces of seabirds, and lichen) and abiotic (sediment) samples from the Antarctic Peninsula. Abbreviations for PFC chemical names are defined in Table 1.

biota samples show lack of significant differences (Welch's ANOVA, $p > 0.05$; Tukey–Kramer HSD (honest significant difference) test, $p > 0.05$), as shown in Figure 3.

To further illustrate the behavior of PFC concentrations in these samples, detected PFC compounds were plotted as a function of log *D* and log K_{OA}, as shown in Figure 4. The majority of PFCs concentrations observed in biotic samples (i.e., feces and lichens) fall within log *D* values between 0 and 3, as seen in Figure 4A. While concentrations of PFCs tend to increase with increasing log *D* values from log *D* of 0 to log *D* of 3 in feces, PFC concentrations appear to decrease as the log *D* increases within the same range of log *D* values in lichens (Figure 4A). This observation may be an indication that both ionized and unionized forms of PFC compounds with low log *D* values (i.e., PFBA, PFPeA, PFHxA, PFHxS, PFHpA, PFOS, PFOA, PFNA, PFDS) are present in some organisms residing in this region and prone to potential transportation by oceanic currents (e.g., Antarctic Circulation Current) from either continental/regional or local sources (i.e., international military bases and research stations) to the Antarctic Peninsula. Similarly, most PFCs concentrations observed in these samples, especially in lichens, fall within log K_{OA} values of 5.0 and 6.5 (Figure 4B). Although concentrations for some PFC compounds show a tendency to decrease with increasing log K_{OA} in feces (i.e., PFBS, PFHxS, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFOS), concentrations for similar PFCs seem to increase as log K_{OA} increases in lichens (i.e., PFDS, PFHxS, PFBA, PFPeA, PFHpA, PFOA), as seen in Figure 4B. These trends may support the notion that low molecular weight compounds (e.g., 214-414 g/mol) with low log K_{OA} are likely to be subject to long-range atmospheric transport and potentially reaching the region, where these compounds accumulate in biotic compartments, mainly in natural air samplers such as lichens and secondary in air-breathing organisms such as seabirds (petrels, penguins).

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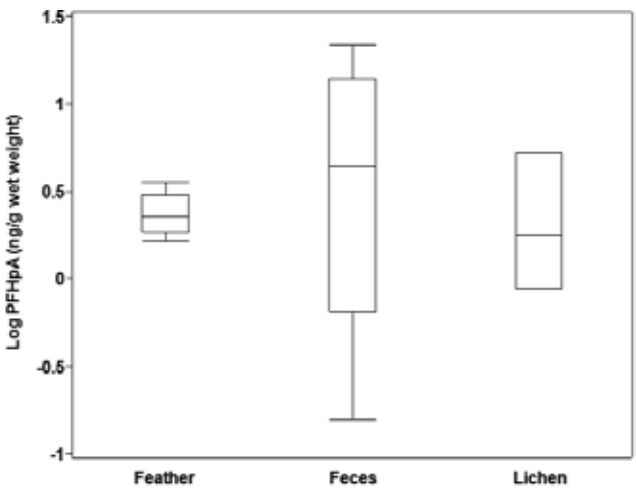

Figure 3. Box plots showing log transformed concentrations of PFHpA (ng/g ww) detected in lichen (n =3), and feather (n =6) and feces (n =9) of seabirds from the Antarctic Peninsula. The internal line across the box is the median; the ends of the box are the 25% and 75% quartiles; and the whisker bars are the minimum and maximum values. Because of unequal variances (i.e., heteroscedasticity; Bartlett test, $p < 0.005$), a Welch's ANOVA, followed by a Tukey–Kramer HSD test, was used for the multicomparison, showing no significant differences in PFHpA concentrations among the biotic samples ($p > 0.05$).

3.4. PFC health risks

Concentrations of PFCs detected in feathers and feces of the two seabird species studied here are well below the toxicological reference value (TRV) of PFOS (600 ng/g ww), calculated as an exposure threshold value for birds in nature, especially for apex avian predators [42]. This comparison indicates that gentoo penguins and petrels are not at risk by PFOS toxic effects.

3.5. Transport mechanisms, global and local sources of PFCs to the Antarctic

There is still a degree of uncertainty surrounding the dominant pathway of PFC movement to the Antarctic, though researchers have highlighted two primary mechanisms generally accepted as the major modes of PFC transportation to the Antarctic: atmospheric and oceanic. Neutral, volatile precursor compounds, such as perfluorinated sulfonamide alcohols (FOSEs), perfluorooctane sulfonamides (FOSAs), and fluorotelomer alcohols (FTOHs), referred to as "flyers", are capable of being delivered to the Antarctic via fast, direct transport of contaminated wind, as opposed to cold trapping, as is common for many legacy POPs [12, 43–45]. Following deposition, these compounds are degraded via oxidation to form ionic PFCs, including PFSAs and PFCAs [12, 44–48]. Evidence supporting this mechanism of travel includes measurements of FTOHs from Europe to the Antarctica showing declining concentrations in the atmosphere with increased distance from sources in the Northern Hemisphere [12]. Given the far distances PFCs must travel to reach the Antarctic, in combination with short atmospheric residence times (ranging on average from 10 to 50 days), the level of effectiveness associated with atmospheric delivery of PFCs is relatively low. Additionally, the yield of ionic

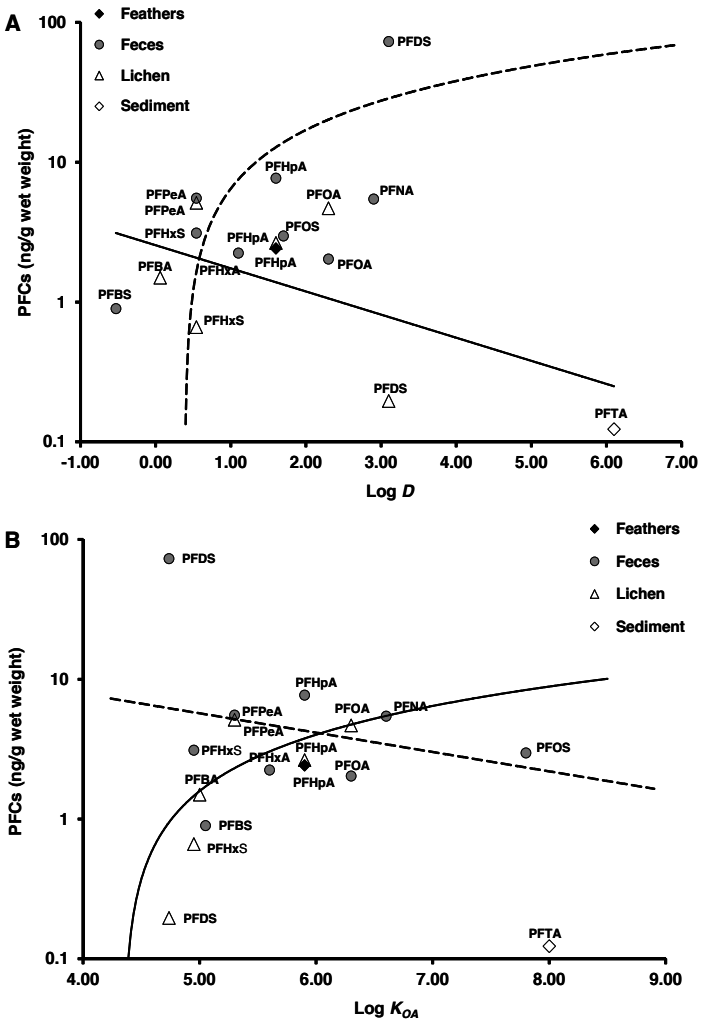

Figure 4. Concentrations of PFC compounds (logarithmic scale in ng/g wet weight) measured in sediments, lichens, and seabird feces and feathers from the Antarctic Peninsula as a function of log *D* (A) and log K_{OA} (B). In Figure 4A, the solid line shows the behavior of detected PFC concentrations versus log *D* in lichen; the dashed line indicates the trend of detected PFC concentrations versus log *D* values in feces. In Figure 4B, the solid line indicates the behavior of detected PFC concentrations versus log K_{OA} in lichen, while the dashed line shows detected PFC concentrations versus log K_{OA} in feces. Abbreviations for PFC chemical names are defined in Table 1.

PFCs produced via oxidation of precursor compounds once transported to the Antarctic is often low [10, 11, 43–45, 47].

It is therefore expected that most PFCs are delivered to the Antarctic in their ionic, water-soluble state via the oceans [12, 14, 49]. Oceanic transport functions on a slower time scale for the Antarctic (in the order of decades, compared to days or weeks for atmospheric transport) because of the circulation patterns of the Southern Ocean, protecting the Antarctic from immediate fluxes in PFC concentrations as they are released elsewhere in the world. As time

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progresses, however, contamination from oceanic sources is anticipated to increase [10, 11, 45]. Slow oceanic transport is cited as the reason for increasing PFC concentrations in the Arctic since the 1950s [14]. Models designed for Arctic research show that if oceanic transport to the Arctic ceased, the quantity of PFCs and their precursors delivered to the Arctic via atmospheric transport could not account for the concentrations measured in water, and thus marine transport is considered to be more important than atmospheric transport [15, 43]. It is also important to note that atmospheric and oceanic transport may be difficult or impossible to discern. For instance, PFCs found in the ocean are made up of three inputs: direct emissions to water, atmospheric deposition into water, and precursor compounds into water followed by degradation to ionic PFCs [14].

Among the compounds found in this study, PFHpA, PFBA, and PFPeA are byproducts of stain/grease-proof coatings on food packaging, couches, and carpets, while PFHxS was used in fire-fighting foams and carpet treatments and phased out of consumer products along with PFOS and PFOA by the major manufacturer (3M Co.) in the early 2000s due to health risks. While long-range atmospheric and oceanic transport of PFCs may partially explain the ubiquitous nature of these contaminants in the Antarctic Peninsula, military bases and infrastructure of nations established there may also be contributing sources of PFCs in Antarctic ecosystems. Atmospheric long-range transport of PFAAs as marine aerosols and degradation of PFCA and PFSA precursors such as low molecular weight FTOHs and acrylates/acids (FTAs) or perfluoroalkyl sulfonamids (FASA) and sulfonamido ethanols (FASE), which are more volatile and released to the atmosphere during fluoropolymer production processes, can be considered as other major pathways [11, 14, 44, 50, 51] to reach and deposit on the Antarctic Peninsula.

Additional and potential sources of PFAAs in the Antarctic Peninsula include aqueous film-forming foams (AFFF) and emissions of a current use insecticide, sulfluramid (N-ethyl perfluorooctane sulfonamide), to control leaf-cutting and fire ants in South America [20, 52; J. Benskin, pers. comm., June 2012). AFFF formulations have consisted of perfluoroalkyl sulfonates (PFHxS, PFOS, PFDS) and more recently, fluorotelomer sulfonamide-based surfactants. While these latter materials can degrade down to short-chain perfluoroalkylcarboxylates (typically C4, C5, C6 PFCAs), sulfluramid can degrade to PFOS, FOSA, and PFCAs by abiotic and/or biological processes [53, 54]. Sulfluramid is manufactured in Brazil (≈30 tons/year in 2007), and, in 2006, about 12 tons was exported to 13 other Central and South American countries [23, 55]. Because this insecticide is a semivolatile substance, it could be transported atmospherically to the Antarctic. Sulfluramid degradation products include PFOSA, PFOS, and potentially PFOA [52]. Despite high concentrations of PFOS, PFOA, and PFOSA measured off the Atlantic coast of South America (South Atlantic), increasing from Brazil to near Rio de la Plata (Argentina–Uruguay), attributed to the use of this substance [52], PFOS and PFOA were not detected in these Antarctic samples, with the exception of two feces samples and a lichen sample. This indicates that these two compounds have not yet fully reached the Antarctic Peninsula region, or local sources are not significant. The detection of several PFCA compounds in the present study is of particular importance as increasing trends of PFCA precursors (i.e., FTOHs) was observed in the Arctic with doubling times of 2.3–3.3 years from 2006 to 2012 [6].

4. Impact assessment, environmental management, and monitoring implications

The Ecuadorian Pedro Vicente Maldonado Scientific Station has been operated since 1988 shortly after Ecuador signed the Antarctic Treaty System (ATS) in 1987. In 1988, Ecuador became an associated member of the Scientific Committee for Antarctic Research (SCAR), and in November 1990 became a consultative member of the ATS [56, 57]. To accomplish this task, Ecuador fulfilled the Antarctic Treaty of "peaceful purposes" and "freedom of scientific investigation" [58]. The commitment to the protection of the Antarctic environment requires being in compliance with the Madrid Protocol, which since 1991 is the prime basis for environmental management of the Antarctic terrestrial and near-shore environments. At the Maldonado Station, the Antarctic environmental management program deploys and integrates a range of generic and international tools, including environmental impact assessments (EIAs), monitoring of pollutants in the marine environment, species and habitat protection, following the environmental principles of the Madrid Protocol, and the administrative and procedural mechanisms of the Committee for Environmental Protection (CEP) [58]. The Ecuadorian Antarctic Institute (INAE) has established good environmental practices, and trained their staff and visitors with a conduct code according to the Madrid Protocol. During the 2010–2011 period, an EIA was performed by the INAE [59] to establish the baseline conditions of the military base and research station, including its area of influence, developing the Environmental Management Plan for the activities taken place at the Maldonado Station.

Considering that local activities and maritime traffic can pollute the surroundings of the Maldonado Station and Antarctic Peninsula, results from impact assessments and monitoring of water quality and potential contaminants have revealed the presence of other anthropogenic pollutants such as hydrocarbons and pesticides in the marine environment [59]. For instance, analyses of total hydrocarbons (THCs) were performed in water samples at the Guayaquil Bay, where concentrations ranged from 0.3 mg/L in sites near the Maldonado Station to 0.85 mg/L at Chile Bay [59]. Pesticide concentrations at several sites of Chile Bay revealed the presence of the organochlorine insecticides, including lindane or γ-hexachlorocyclohexane (γ-HCH) (i.e., 0.335 mg/L) and β-hexachlorocyclohexane (β-HCH or beta-BHC) (i.e., 0.00072 mg/L), as well as traces of the organophosphate malathion and the herbicide atrazine [59]. The long-range atmospheric transport associated with direct deposition or precipitation of volatile organic chemical is now recognized as a major pathway by which pesticides can be transported and deposited in surface waters and ice of Antarctica thousands of kilometers far from their sources [60–62]. Although relatively low concentrations of some PFCs were observed in biota and sediments samples of the remote western Antarctic Peninsula environment and local sources associated with scientific stations and military bases appear to not be significant sources of PFCs, this study gives further evidence of background concentrations around the Antarctic. Results from this study are consistent with research showing that volatile PFCs are subject to atmospheric long-range transport to remote regions, contributing to the contamination of persistent PFCA and PFSA compounds in the Antarctic [11, 44].

Despite the limited sampling and the need for replication to confirm the findings of this study, the biological (lichens, feathers, feces) and abiotic (sediments) samples assessed in this work can be used as environmental matrices to track the fate of PFCs at various temporal and spatial

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scales in Antarctica. Of particular importance is the detection of several PFC compounds in seabird feces and lichens, which can be used as matrices for noninvasive sampling and long-term monitoring programs of PFCs in the Antarctic Peninsula. Long-term air monitoring and sampling of volatile PFCs in the Antarctica Peninsula is also recommended to elucidate atmospheric sources to the Maldonado's Station and surrounding environment.

Acknowledgements

We are in debt with the staff and military personnel of the Ecuadorian Antarctic Institute (Instituto Antártico Ecuatoriano, INAE), especially CPNV José Olmedo Morán, for the logistics and coordination for the 2009 Ecuadorian–Antarctic expedition. Special thanks to Dr. Patricia Ruano and Peter Olaya for their support to elaborate the map and to Dr. Jonathan Benskin for providing preliminary insights to this work.

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Chapter 4

Impact of Oil Spills on Marine Life

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60455

Abstract

Petroleum contamination is a growing environmental concern that harms both terrestrial and aquatic ecosystems. However, the public and regulatory and scientific communities have given more attention to the contamination of marine habitats. This is because marine oil spills can have a serious economic impact on coastal activities, as well as on those who exploit the resources of the sea. Thus, communities that are at risk of oil disasters must anticipate the consequences and prepare for them.

The deliberate release of around 6 million barrels of oil during the 1991 Gulf War in the marine environment is the largest oil spill in history. In the Gulf of Mexico, the BP Deepwater Horizon (DWH) oil spill on 20th April, 2010, which lasted over approximately three months, is the second largest in human history.

When oil is spilled at sea it initially spreads out and moves on the water surface as a slick. It is a few millimetres thick and moves with the wind and current. At the same time, it undergoes a number of chemical and physical changes. The spreading of marine oil spills is affected by the action of winds, waves, water currents, oil type and temperature. Marine oil spills enhance the evaporation of volatile fractions such as low molecular weight alkanes and monoaromatic hydrocarbons. The natural actions, which are always at work in aquatic environment, include weathering, evaporation, oxidation, biodegradation and emulsification.

Generally, the effects of oil toxicity depend on a multitude of factors. These include the oil composition and characteristics (physical and chemical), condition (i.e., weathered or not), exposure routes and regimen, and bioavailability of the oil. If the levels exceed the threshold concentration, the additive toxic effect of hydrocarbons can lead to mortality. PAHs are the major contributors to toxicity. They have different metabolic pathways that produce metabolites. These have oxidative and carcinogenic properties due to their ability to attack and bind to DNA and proteins. Inhaling

hydrocarbons can cause respiratory tract irritations, as well as narcosis in mammals and birds. This is due to the volatile nature of hydrocarbons. Oil dispersants, which are a common tool used after oil spills, are also toxic and threaten pelagic and benthic organisms, as well as fish.

In recent years, written studies have provided a considerable amount of information regarding the impact of oil spills and contamination of the seawater by hydrocarbons. The impact on marine life is impaired by the toxicity and tainting effects resulting from the chemical composition of oil, as well as by the diversity and variability of biological systems and their sensitivity to oil pollution. Marine life may also be affected by clean-up operations or, indirectly, by the physical damage to the habitats in which plants and animals live.

Communities that are threatened by marine oil spills have developed their own plans and policies to counteract the risk of marine oil contamination. These range from permitting or prohibiting increased oil transport volumes, to developing the capacity to respond to and recover from potential oil spill disasters. Considering that approximately half of the world's oil production comes from the Gulf States and passes through the Gulf, its liability to pollution is about 48 times that of any other similar area on earth. Thus, the Gulf is possibly the most chronically oil-polluted marine area in the world. Due to the different anthropogenic activities relating to oil spills, in addition to the natural environmental stresses of the Gulf, a number of socio-economic impacts are predicted. The multi-million dollar fish industry will be threatened, as well as the desalination of plants that supply most of the Gulf population's freshwater. Furthermore, people with careers in scuba diving will lose their jobs. As a result, the scuba diving tourism sector will also come under threat.

Keywords: Contamination, Crude, Hydrocarbon, Impact, Life, Marine, Oil, Petroleum, Spill, Toxicity, Water

1. Introduction

Environmental pollution caused by petroleum is of great concern. This is because petroleum hydrocarbons are toxic to all forms of life and harm both aquatic and terrestrial ecosystems. The pollution of marine habitats has caught the attention of researchers and environmentalists. This is due to the serious impact of oil spills on marine life, as well as on people whose career relies on the exploitation of the sea's resources. Additionally, marine life may be affected by clean-up operations. It may also be indirectly affected by the physical damage to the habitats in which plants and animals live in.

Petroleum marine fuel spills, which result from damage, transportation accidents and various other industrial and mining activities, are classified as hazardous waste [1]. They are considered to be the most frequent organic pollutants of aquatic ecosystems [2-3].

In recent years, there have been numerous studies regarding the levels of contamination of the seawater by hydrocarbons. The majority of these studies were conducted following the Gulf War of 1991 [4-11] and after, the BP Deepwater Horizon (DWH) oil spill on 20th April, 2010 [12-14].

In the current manuscript, we discuss the effects of petroleum oil spills on marine life. It incorporates different sections that outline the major petroleum oil spills associated with the marine environment, chemical composition of crude oil, toxicity of oil and oil dispersants. We discuss the major petroleum oil spills associated with the chemical and physical states of crude oil and their impact on microbial, plant and animal marine life. We also consider the economic impact of oil spills on coastal activities and on the people who exploit the resources of the sea.

2. Major oil spills related to marine environments

Saltwater bodies are referred to as 'marine environments', with the oceans covering about 70% of the earth. Statistics estimate that 3.2 million tonnes of oil per year are released from all sources into the environment. The majority of this oil is due to general shipping and industrial activities [15]. During the Iran-Iraq war (1980-1988), approximately 2 million barrels of oil were discharged into the Arabian Gulf sea water. These included 1.5 million barrels from the Nawruz blow-out in 1983 [16]. Following the Gulf War in 1991, between 4 and 8 million barrels (1,000 tonnes = 7,500 barrels) were released into the Gulf and the Kuwaiti Desert, making this the largest oil spill in history at that time [17]. When compared to other major spills, the size of this spill attracted global attention. For example, the *Amoco Cadiz* off the coast of Brittany (France), spilling 200,000 tonnes (1.5 million barrels); the *Torrey Canyon*, *Braer*, *Sea Empress* and the super tanker *Breaf* off the coast of Shetland (UK) in 1993, spilling a maximum of 84,000 tonnes (607,300 barrels); the *Exxon Valdez* in Prince William Sound or Alaska (US), which was approximately 36,224 tonnes (261,904 barrels) [18]. Of the recorded oil spills, more than 80% were less than 1,000 tonnes (7,500 barrels) and only 5% were greater than 10,000 tonnes. An accepted average sample size of an oil spill is about 700 tonnes (5,061 barrels) [15]. Previous observations indicated that the number of large spills (>700 tonnes) has decreased significantly over the last 30 years (Table 1).

Year	7-700 tonnes	>700 tonnes	Quantity Spilled x 10^3 tonnes
1970-1974	189	125	1114
1975-1979	342	117	2012
1980-1984	221	41	570
1985-1989	124	48	513
1990-1994	165	48	907
1995-1999	108	25	194
2000-2009	182	-	213
2010-2013	28	-	22

Table 1. Number of oil spills over 7 tonnes from 1970 to 2013 (http://www.itopf.com/stats.html).

During the 1990s, the average number of large spills per year was about a third of the amount that was witnessed during the 1970s. When looking at the quantities of oil spilled, it should be noted that 1, 133, 000, 213, 000 tonnes of oil were lost in the 1990s and 2000s, respectively. In a four-year period, 2010-2013, there were 22,000 tonnes of oil lost. Table 2 shows a brief summary of the top 22 major oil spills since 1967.

Ship name	Year	Location	Spill (10^3) tonnes
Torrey Canyon	1967	Scilly Isles, UK	119
Sea Star	1972	Gulf of Oman	115
Jakob Maersk	1975	Oporto, Portugal	88
Urquiola	1976	La Coruna, Spain	100
Hawaiian Patriot	1977	300 nautical. miles off Honolulu	95
Amoco Cadiz	1978	Off Brittany, France	223
Atlantic Empress	1979	Off Tobago, West Indies	287
Independenta	1979	Bosphorus, Turkey	94
Irenes Serenade	1980	Navarino Bay, Greece	100
Castillo de Bellver	1983	Off Saldanha Bay, South Africa	252
Novo	1985	Off Kharg Island, Iran	70
Odyssey	1988	700 nautical. miles off Nova Scotia, Canada	132
Khark 5	1989	120 nautical. miles off Atlantic coast of Morocco	70
Exxon Valdez	1989	Prince William Sound, Alaska, USA	37
ABT Summer	1991	700 nautical miles off Angola	260
Haven	1991	Genoa, Italy	144
Aegean Sea	1992	La Coruna, Spain	74
Katina P.	1992	Off Maputo, Mozambique	67
Braer	1993	Shetland Islands, UK	85
Sea Empress	1996	Milford Haven, UK	72
Prestige	2002	Off Galicia, Spain	63
Hebel Spirit	2007	South Korea	11

Table 2. Top 22 major oil spills since 1967 (http://www.itopf.com/stats.html).

The BP Deepwater Horizon (DWH) oil spill on 20th April, 2010, initiated the discharge of more than 2.6 million gallons (over 800 million litres) of oil into the Gulf of Mexico (Figure 1) over approximately three months. This oil spill was the second largest in human history [19-21]. During the 1991 Gulf War, the deliberate release of over 6 million barrels of oil [22] into the marine environment was considered as the largest in history.

http://dx.doi.org/10.5772/60455

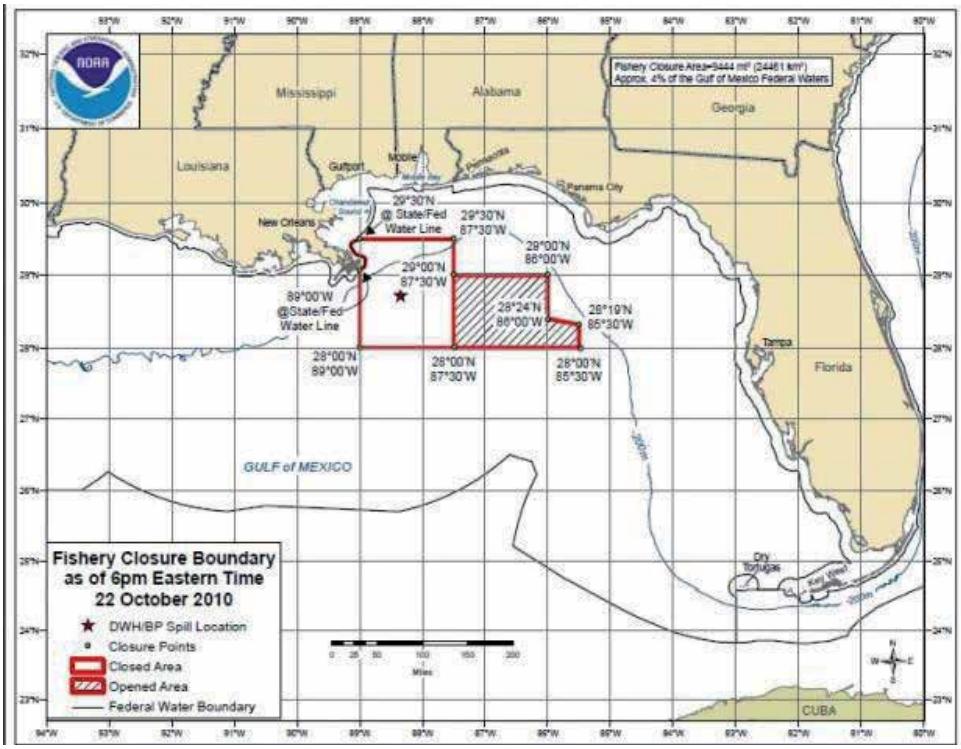

Figure 1. The Site of the Gulf Spill: Closed Areas as of 22nd October, 2010 [23].

3. Crude oil and its properties

Crude oil is a complex mixture of organic compounds. These mainly consist of hydrocarbons, in addition to heterocyclic compounds and some heavy metals. The different hydrocarbons that make up crude oil come in a wide range of molecular weights and structure compounds. These compounds include methane gas, high molecular weight tars, asphaltenes, resins, waxes and bitumens. They also include straight and branched chains, single or condensed rings and aromatic rings such as the monocyclic (benzene, toluene, ethylbenzene and xylene). They additionally include polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, anthracene and phenanthrene. Examples of the chemical structure of some common components of crude petroleum are shown in Figure 2.

3.1. Toxicity of oil

The general effects of oil toxicity depend on a multitude of factors. These include the oil composition and characteristics (physical and chemical), condition (i.e., weathered or not), exposure routes and regimen, and the bioavailability of the oil [24]. One major effect of oil is

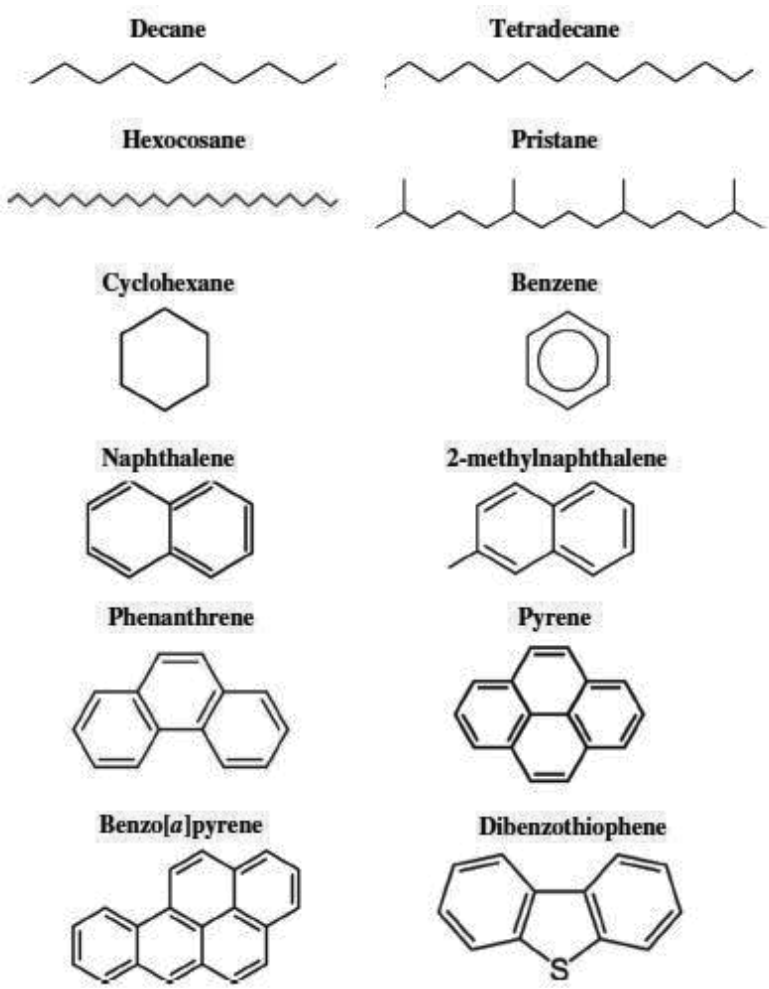

Figure 2. Structure of selected components of petroleum [13].

narcosis, a reversible anaesthetic effect caused by the oil partitioning into the cell membrane and nervous tissue. This causes dysfunctions of the central nervous system [25].

The additive toxic effect of hydrocarbons can lead to mortality, if the levels exceed the threshold concentration [24]. When oil hydrocarbons are ingested by marine animals, they travel to the liver where enzymes activate PAHs to become more toxic and reactive products. The metabolites of polycyclic aromatic hydrocarbons (PAHs) and aliphatic hydrocarbons can be highly toxic and carcinogenic [26]. In particular, PAHs are the major contributors to toxicity, with different metabolic pathways producing metabolites. These have oxidative and carcinogenic properties due to their ability to attack and bind to DNA and proteins [24]. Hydrocarbons have a volatile nature and, therefore, inhalation of them results in respiratory tract irritation and narcosis of mammals and birds.

Physical contact is the major route of exposure and usually affects birds and furred mammals. These animals rely on their outer coats for buoyancy and warmth. Consequently, they often succumb to hypothermia, drowning and smothering when oil flattens and adheres to the outer layer [24]. A second general exposure route is through the ingestion or inhalation of the hydrocarbon by organisms that reside on the surface [24]. Exposure by these routes leads to absorption into the bloodstream via the gastrointestinal or respiratory tracts.

3.2. Toxicity of oil dispersants

Oil dispersants (57 chemical ingredients approved for use by the US EPA) are a common tool used after oil spills in marine environments. They break up oil slicks on the water surface and increase the oil's rate of biodegradation. Oil dispersants are quickly used when other means, such as oil containment and removal, are insufficient. However, consequences of the toxicity of oil spill dispersants alone or in the presence of oil must be evaluated. Generally, undispersed oil poses the greatest threat to shorelines and surface dwelling organisms. However, most dispersed oil remains in the water column where it mainly threatens pelagic and benthic organisms [27]. A complete and updated list of used oil dispersant is available from the US EPA at the website http://www2.epa.gov/emergency-response/alphabetical-list-ncp-product-schedule-products-available-use-during-oil-spill.

Several studies have compared the toxicity of oil spill dispersants alone or in the presence of oil. Analyses of tests conducted on a variety of species of aquatic life showed that crustaceans are more sensitive to oil dispersant exposure, compared with fish [28]. A study by [29] indicated that the species with the least amount of protective shell or external tissue is the most sensitive to oil dispersant exposure.

It has been shown that the use of oil dispersants increases the exposure and uptake of PAHs by fish. This is particularly the case with fish that live throughout the water column of coastal areas, the ocean and lakes. Researchers found that 'the risk of PAH toxicity... especially to sensitive life stages, such as eggs and larvae, is enhanced by chemical dispersion' [30]. In addition, 'concentrations of LMWPAHs and HMWPAHs (low and high molecular weight PAHs) were found to be higher in the water column following the application of chemical dispersants to the surface slicks' [31]. 'For example, see [31]'. They reported that chemical dispersants mobilize PAHs to toxic concentrations as the biomarker ethoxyresorufin-O-deethylase (EROD) activity is increased after exposing newly-hatched mummichog (*Fundulus heteroclitus*) for 96 h to crude oil and chemically dispersed crude oil.

Reference [29] compared the toxicity of a new dispersant, Superdispersant-25 (SD-25), to Corexit 9527 using four species of marine invertebrates at 15 C. The most sensitive species was the snakelocks anemone *Anemonia viridis* with a 48-hour LOEC of 20 ppm (nominal). This was followed by mussel (*Mytilus edulis*) feeding rate (50 ppm), seagrass (*Zostera marina*) photo synthetic index (80 ppm), burrowing amphipod (*Corophium volutator*) (mortality 175 ppm) and mussel lethality (250 ppm).

Moreover, in a study by [32], adults of four species of wild-caught Newfoundland nearshore fishes were exposed for four days in flow-thru conditions to the dispersant Corexit 9527 alone,

water accommodated fraction (WAF) of Hibernia light crude oil alone and dispersed Hibernia crude oil. Test toxicants (20 to 50 ml) were added daily to 300 L tanks for four days, followed by up to six weeks in clean water. The investigators did not report exposure temperatures or toxicant concentrations except to note that initial daily concentrations were 50-100 ppb for Hibernia water-accommodated fraction (WAF). On the first day, the caplein responded to the dispersant by swimming erratically. On the second and subsequent days, they responded by death accompanied by hemorrhaging of the gill lamellae.

4. Fate of oil spills in the marine habitats

After oil is spilled at sea and with the effect of wind and water current, the oil spreads out and moves on the water surface as a slick a few millimetres thick. At the same time, it undergoes a series of chemical and physical changes [13]. These processes are collectively termed 'weathering'. Weathering causes the spilled oil to break down and become heavier than water. Some of these processes, like the natural dispersion of oil into water, lead to the removal of the oil from the sea surface and facilitate its natural breakdown in the marine environment. Others, particularly the formation of water-in-oil emulsions, cause the oil to become more persistent and remain at sea or on the shoreline for prolonged periods of time. The speed and relative importance of these processes depend on a number of factors. These include the quantity spilled, the oil's initial physical and chemical characteristics, weather and sea conditions and whether the oil remains at sea or is washed ashore. Ultimately, the marine environment usually eliminates spilled oil through the long-term process of biodegradation. (http://www.itopf.com/knowledge-resources/data-statistics/statistics/).

The natural actions, which are always at work in aquatic environment, are summarized in the US EPA archive document (http://www.epa.gov/oem/docs/oil/edu/oilspill_book/chap1.pdf). These include weathering, evaporation, oxidation and biodegradation.

4.1. Impact of oil spills on marine organisms

Ultimately, the impact of oil on marine organisms depends on the fate of the oil. As previously described, when oil is present in the environment, it is either dispersed in the top layer of the water (littoral zone) or remains on the surface and, consequently, on the coastal areas. If the oil is not dispersed, it remains on the surface. In this case, currents bring the oil towards coastal areas which harms coastal organisms like invertebrates, mammals and birds. However, if the oil is dispersed, organisms, such as fish, plankton and larvae, are immediately subjected to oil toxicity.

4.2. Impact of oil spills on planktonic organisms

Zooplankton is a particularly important food resource, especially for baleen whales. It can influence or control the primary productivity by top-down effects [33] in return. Its population dynamic change can influence the biomass of other marine animals like fish by bottom-up effects [34]. Some zooplankton, such as copepods, euphausiids and mysids, assimilate

hydrocarbons directly from seawater and by ingesting oil droplets and oil contaminated food [35 as cited by 36]. The ingestion of oil by these organisms often causes mortality, while surviving organisms often show developmental and reproductive abnormalities [37].

The impact of petroleum pollution on marine plankton has been a great cause for concern. Reference [37] summarized the reports regarding the toxic effects of oil water accommodated fraction (WAF) on marine phytoplankton, zooplankton and the early life stages of animals. Generally, oil WAF toxicity enhances with increasing carbonic chain length and benzene ring number. The paper summarized the research results regarding the influence of oil WAF on marine plankton. It also suggested future study points to further promote the quantified evaluation of the damage by oil pollution to marine ecology.

For the oil WAF, [37] reported that plankton are capable of accumulating PAH due to their great lipophilic abilities. They, therefore, stimulate various harmful effects. The investigators reported that marine plankton is highly sensitive to the petroleum WAF, as the order of median effective/lethal concentration is as low as lg/L or mg/L. Examinations of the toxicity effect of 10 polycyclic aromatic hydrocarbons associated with the Prestige fuel oil spill on adult copepods (*Oithona davisae*) revealed that the PAHs had narcotic effects on these organisms [38].

4.3. Impact of oil spills on benthic organisms and invertebrates

Benthic invertebrates and higher forms, such as the sand eel and *Ammodvtes americanus* (a main food resource of Atlantic humpback whales) [39 as cited by 36], may accumulate petroleum hydrocarbons from water, contaminated sediments and food [40 as cited by 36]. Thus, these whales are adversely affected.

Bivalve molluscs tend to accumulate petroleum hydrocarbons to higher concentrations and retain them longer than other taxa [41-42 as cited by 36]. This is essentially due to the lack of a mixed function oxygenase (MFO) system [43 as cited by 36] that makes them unable to metabolize the compounds to execrable polar metabolites. Thus, they are likely to transfer them to their predators. Marine mammals that rely heavily on bivalve molluscs for food, such as the walrus and otter, share a higher risk of ingesting petroleum hydrocarbons [44 as cited by 36]. Benthic amphipods are quite sensitive to spilled oil. They are among the first marine animals killed and the slowest to recover [45 as cited by 36]. However, most marine crustaceans have a well-developed MFO system [43 as cited by 36]. As a result, they are able to metabolize and excrete accumulated hydrocarbons quite rapidly.

Previous studies have explored the recovery of the invertebrate populations after oil spills [46-47]. In the intertidal habitat, the biological recovery of the exposed shores is faster than the sheltered shores. This is because strong wave action promotes the removal of contamination and the animals and plants of exposed shores tend to be severely affected. Thus, they are better able to re-colonize an impacted shore quickly. Sublittoral habitats are generally contaminated by sedimentation of oiled particulate material and clean-up for these habitats is not practiced. As a result, the recovery of subtidal communities impacted by oil spills usually takes a longer time. For example, the *Abra alba* bivalve sand community in the Bay of Morlaix, Brittany was severely affected by the *Amoco Cadiz* oil spill (1978) [47]. After the spill in 1978, the biomass

values for the sand community immediately fell. However, within two years, they recovered to pre-spill levels. Productivity also showed similar trends.

Invertebrate populations, such as the amphipod sand hopper, *Ampelisca*, are quite sensitive to oil pollution and, for various reasons, are slow to re-populate and 'recover'. For example, the initial impact of the spill in the Bay of Morlaix, following the *Amoco Cadiz* spill in 1978, was to kill off populations of the amphipod sand hopper, *Ampelisca*, which dominated the community [46]. Although the sediment was rapidly purged of the contaminating oil, it has been noted that *Ampelisca* was back to its pre-spill population density after 10 years. The standing crop biomass and productivity was restored much more rapidly as the ecological niches had been occupied by other opportunists like the bivalve *Abra* and the worm *Nephtys.* These had quickly filled the place left by the *Ampelisca* [46].

In other studies on oil spill accidents, it has been shown that six months after the Prestige oil spill and clean-up campaign, invertebrate populations of the exposed sandy beaches, notably the isopod *Eurydice*, the spionid polychaete *Scoleleoius squamata*, nemerteans and Diptera, were significantly reduced. Furthermore, their abundance inversely related with the oil pollution gradient. The number of taxa was reduced but not the diversity values. The only clam on these sandy beaches was *Donax.* Before the spill and clean-up, it occurred in six beaches but afterwards, only in one. Upper dry sand communities were particularly reduced due to both oil toxicity and extensive beach grooming that also removed seaweed wrack [48-49].

Marine invertebrates are unique living organisms in different aspects. They have been explored as a model for several biological markers as a result of oil spill pollution. Reference [50] surveyed the marine invertebrate mussels *(Mytilus edulis)* exposed in situ to the oil that came ashore after the wreck of the '*Erika*' tanker on the Brittany (France) coast in December, 1999. The mussel response was assessed using a set of biomarkers (acetylcholinesterase (AChE), glutathione S-transferase (GST), catalase (CAT), malondialdehyde (MDA) and deoxyribonucleic acid (DNA) adducts, related to the metabolism of the organic contaminants. The results of a series of validation tests revealed that there were no significant reductions in the GST or CAT levels. Six months immediately following the accident, observations indicated that the DNA adducts and MDA levels were high and the levels of AChE were significantly lower during the first year of the survey. This suggested a general stress. A simple multivariate graphic method - the integrated biomarker response index - was used to combine four of the five validated biomarkers and quantify the degree of impact on mussels at different sites. The results indicated that mussel populations were affected by the oil spill during only the first year after the accident.

4.4. Impact of oil spills on coral reefs

In addition, recreational attractions for divers, coral reefs are considered to be important constituents of marine ecosystems. This is because they are important nurseries for shrimp, fish and other animals [51]. The aquatic organisms that live within and around the coral reefs are at risk of exposure to the toxic substances within oil, as well as smothering. They are rapidly deteriorating because of a variety of environmental and anthropogenic pressures. Thus, they

are suffering significant changes in diversity, species abundance and habitat structure worldwide [52].

Oil dispersants are potentially harmful to marine life including coral reefs [53]. In a study using coral nubbins in coral reef ecotoxicology testing, [54] found that dispersed oil and oil dispersants are harmful to soft and hard coral species at early life stages. The investigators also employed a 'nubbin assay' on more than 10, 000 coral fragments to evaluate the short- and long-term impacts of dispersed oil fractions (DOFs) from six commercial dispersants (Slickgone, Petrotech, Inipol, Biorieco, Emulgal and Dispolen) and the dispersants and water-soluble-fractions (WSFs) of Egyptian crude oil on two Indo-Pacific branching coral species, *Stylophora pistillata* and *Pocillopora damicornis* [53]. They found that the dispersant concentrations recommended by the manufacturer were highly toxic and resulted in mortality of all nubbins. The dispersed oil and the dispersants were significantly more toxic than the crude oil WSFs. As corals are very sensitive to oil detergents and dispersed oil, the results of these assays indicated the negative and harmful effect of using any oil dispersant in coral reefs and in the area closely around them. These dispersants were rated based on their eco-toxicological impacts on the corals. Observations indicated a scale from the least to the most harmful agent, as follows: Slickgone > Petrotech > Inipol > Biorieco > Emulgal > Dispolen.

4.5. Impact of oil spills on fish

Due to the well-developed hepatic mixed function oxygenase (MFO) system, in addition to the reactivity of the metabolites that would not be released in a toxic form during digestion and absorption, most fish, even in heavily oil-contaminated environments, do not accumulate and retain high concentrations of petroleum hydrocarbons. Thus, they are not likely to transfer them to predators. Thus, no serious threat is predicted [55 as cited by 36].

Generally, marine carnivores are inefficient assimilators of petroleum compounds in food. For this reason, and because all prey species are able to release hydrocarbons from their tissues [41 as cited by 36], the marine food chain biomagnification does not occur. Thus, there is an indirect correlation between a marine mammal's trophic level and the concentration of residues that it might consume. In fact, as top carnivores that feed on large pelagic fish and seals, polar bears and killer whales are less likely to be exposed to petroleum in their food than other species, such as walrus and baleen whales, which feed on zooplankton and benthic invertebrates.

Experiments that explain the effect of oil spills on the early stages of fish were demonstrated by [56]. They found that chronic exposure of juvenile pink salmon (*Oncorhynchus gorbuscha*) to Alaska North slope crude oil resulted in a variety of responses. These included melanosis, erratic swimming, loss of equilibrium, reduced mobility and startled response. Interestingly, the effects were not enhanced by the phototoxicity from UV irradiation, presumably of the highly pigmented nature of the fish's skin (in contrast to phototoxicity in translucent early life stages of marine invertebrates). When they examined the phototoxicity of the water accommodated fractions of weathered crude oil, they found that pink salmon may be at less risk of photoenhanced toxicity, compared to the more translucent early-life stages of several other Alaskan species.

4.6. Impact of oil spills on seabirds

As one of the major routes of exposure, physical contact usually affects birds. For example, thousands of African penguins (*Spheniscus demerus*) were oiled following the 2000 Treasure oil spill in South Africa.

An evaluation of the impact of oil spills on seabirds has not been fully appreciated during incidents, despite pressure from the public concern, media and other interested parties for precise and up-to-date information on the damage. Consequently, the approximate numbers of seabird casualties involved in many major spills have only been estimated, while impacts at the population level have been difficult to determine. Natural variation and the huge range of factors that influence bird population statistics make it difficult to assess the impact of oil spill on sea birds.

In reference [57], in their efforts to assess the impact of oil spills on seabirds in Europe and in North America, the investigators reported that there are two inter-linked aspects to dealing with oiled seabirds during major spills. The first is a relatively expensive and logistically complicated process. This involves birds found alive and the humanitarian efforts made to clean, rehabilitate and successfully release them into the wild. The second involves assessing the likely impact of the spill on the populations of those species affected.

The relation between the size of an oil spill and the number of seabird casualties is not directly proportional. Moreover, estimates of the number of seabird deaths from oil slicks are highly speculative. This is because an unknown number of oiled birds may die at sea and not reach the coast. For example, following the *Exxon Valdez* oil spill, over 35,000 seabird carcasses were recovered in the northern Gulf of Alaska [58]. However, after the Braer accident [59], even though the Braer spill (85,000 tonnes) was almost two and a half times as large as that of the Exxon Valdez (Table 3), only 1,500 dead birds were counted.

Researchers argued that the rapid recovery of the murre breeding colonies in Alaska and the number of dead birds might be lower than was estimated. They also suggested that breeding pairs may have been replaced by younger birds that came in from the open sea [60]. On the other hand, [61] argued that the impacts of the spill may have been diffused over a large area, permitting local recovery and making it difficult to detect any changes in local abundance or habitat occupancy. Nine years after the Exxon Valdez oil spill, in their long-term effects observation, [62] claimed that the populations of most bird species have not recovered and others still show potential population effects. However, the report is at variance with other findings [63-64].

During the winter season of 2003, approximately 64,000 tonnes of Prestige heavy fuel oil spilled off Galacia, Spain. As a result, 10 % of the European shag (cormorants, *Phalacrocorax aristotellid*) were killed. In turn, this resulted in a 50% reduction in the 2003 breeding success of oiled colonies, compared to the unoiled colonies [65]. Reference [66] found that the kidneys of 32 oiled shag had extensive tissue damage and haemorrhaging. They further found that the oiled shag were heavily infected with an eimeriorin Apicomplex coccidian, which under normal circumstances is not pathogenic.

Species group	Alaskan spill	Shetland spill
Sea ducks (eiders, etc.)	1440	167
Mergansers	121	1
Loons	395	14
Grebes	462	0
Heron	1	3
Geese/swans	9	0
Gulls	696	74
Kittiwakes	1225	133
Cormorants/shags	836	864
Shearwaters	3399	0
Fulmars	870	31
Guillemots/murres	20562	220
Other auks	2174	29
Bald eagles	125	NA
Other birds	3152	0
Total	35467	1536

Table 3. Comparison of the number of dead seabirds recovered following the *ExxonValdez* and *Braer* oil spills [47].

4.7. Impact of oil spills on marine mammals

Marine mammals include bottlenose dolphins, fins, humpbacks, rights, sei whales, sperm whales, manatees, cetaceans, seals, sea otters and pinnipeds. As previously indicated, the physical contact of oil with furred mammals usually affects these animals because they rely on their outer coats for buoyancy and warmth. Consequently, these animals often succumb to hypothermia, drowning and smothering when oil flattens and adheres to the outer layer [24].

As part of their activities, all marine mammals spend a considerable amount of time at the surface. Here, they swim, breathe, feed or rest. Thus, the possibility of their contact with a surface slick, water-in-oil emulsion, or tar balls, is high. In heavy pelage marine mammals, such as fur seals, sea otters and polar bears, this contact may lead to fouling. Polar bears and otters groom themselves regularly as a means of maintaining the insulating properties of the fur and may, thereby, ingest oil. Animals with smooth surfaces or relatively little to no pelage, such as whales, dolphins, manatees and most seals, have an advantage as oil would have fewer tendencies to adhere to their surface [36].

Some baleen whales are skim-feeders, i.e., they eat at the surface [67 as cited by 36]. When in an area of slick or tar balls, this behaviour can lead to foul in the feeding apparatus. Tarry residues, in particular, can coat the baleen plates.

Animals, such as narwhals, belugas, ringed seals, walruses and polar bears in Polar Regions, spend most of their time at the ice edge in leads, polynyas and breathing holes. This is where spilled oil tends to accumulate.

Oil that contaminates a shore is likely to severely affect pinnipeds. Pinnipeds require such areas for nursery and, to a lesser extent, otters and bears. Some of the oil is eventually returned in subtidal sediments, where it may transfer to gray whales, walrus and some seals. Such species feed heavily on benthic animals.

When marine mammals encounter fresh oil, they are likely to inhale volatile hydrocarbons evaporating from the surface slick. These volatile fractions contain toxic monoaromatic hydrocarbons (benzene, toluene and xylenes) and low molecular weight aliphatics with anaesthetic properties. The inhalation of these volatile hydrocarbon compounds is potentially harmful [68-69 as cited by 36]. The inhalation of concentrated petroleum vapours can cause the inflammation of and damage to the mucus membranes of airways, lung congestion or even pneumonia [70 as cited by 36]. Volatile benzene and toluene, which can be inhaled, can be transferred rapidly from the bloodstream into the lungs. Furthermore, they can accumulate from the blood into the brain and liver, causing neurological disorders and liver damage [71 as cited by Neff 36].

Marine mammals are probably poor accumulators to oil directly from the solution or dispersion in the water column. This is because the skin of cetaceans is relatively impermeable to oil [72 as cited by 36]. Additionally, most marine mammals do not drink large volumes of seawater. Thus, a significant accumulation of hydrocarbons by this route is unlikely to occur.

There is an extensive and diverse database regarding subjects that deal with effects of oil on marine mammals and those aspects of an animal's life history vulnerable to exposure of spilled oil. This database is summarized in a publication by the Department of Interior/Minerals Management Service (MMS 88-0049)/Atlantic OCS region, Canada [72]. 'For example, see [36]', described the effects of oil on marine mammal populations. At the same time, [73] described the physiological and toxicological effects of oil on each of the marine mammal groups.

Reference [73], in his study of 'Physiologic and Toxicologic Effects on Pinnipeds', indicated that pinnipeds are inappropriately sensitive to the harmful properties of oil. Incidental ingestion during feeding, exposure to vapour concentrations and surface contamination with relatively fresh oil does not appear to cause a disaster. However, Pinnipeds trapped near the source of a spill or those which are forced to emerge in heavy accumulations of oil in leads and around rookeries exhibit the most severe effects. Experimental studies by the same investigators on fur seals indicated that surface fouling decreases the insulation value of the pelt. This can potentially lead to thermal and energetic stress. Furthermore, the sensitivity to the effects of oil exposure may be high in species and groups that are compromised by pre-existing disease, or stressed by pressures of an unfavourable habitat, intra-specific competition or unusual environmental conditions. [73].

http://dx.doi.org/10.5772/60455

4.8. Impact of oil spills on marine plants

In several aspects, aquatic plants are important to the functioning of ecosystems. These include the fact that they are oxygen producers, their ability to sequester carbon and for their base position in aquatic food chains. In addition, they serve as nursery, feeding and breeding habitats for a variety of animal and plant species, including recreationally and commercially important fish.

Plants and animals are affected by the oil in which they come into contact with as a result of an oil spill. In their review of toxicities of oils, dispersants and dispersed oils to algae and aquatic plants, [74] summarized the reported phytotoxicities of oils, dispersants and their combinations to aquatic plants. They assessed the ability of the reviewed database to support toxicity predictions and evidence-based risk assessments. The phytotoxicity database mainly includes the results of research conducted after oil spills to marine waters. The toxicity of at least 41 crude oils and 56 dispersants were recorded. At least 107 response parameters were monitored for 85 species of unicellular and multicellular algae, 28 wetland plants, 13 mangroves and nine seagrasses. Due to experimental diversity, the effect concentrations available from this toxicity database are varied and diverse. As a result, there are restricted phytotoxicity predictions and identification of sensitive species, life stages and response parameters. Thus, the impact of toxicity of petrochemicals and dispersants on aquatic plants was not supported by this database.

Reference [74] recommended a proactive and experimentally-consistent approach to provide the threshold toxic effect concentrations for sensitive life stages of aquatic plants inhabiting diverse ecosystems.

The effects of heavy fuel oil contamination on the growth and the development of *Salicornia fragilis* Ball and Tutin, a salt-marsh edible species, were studied as greenhouse experiments by [75]. Phytotoxicity assessments and PAH shoots assays were followed to measure the impact of petroleum on plant development. Syptoms like chlorosis, yellowing, growth reduction and perturbations in developmental parameters were visually observed. In this study, shoot coating appeared to be less than through soil and the investigators observed more marked effects on plants as an indication of the degree of pollution. However, a significant bioaccumulation of PAHs in shoot tissues was also found, even at very low levels of contamination. These highly related to the conditions of exposure to oil. This strong relationship between the PAH contents of *Salicornia* plants and growth reduction suggest a chemical toxicity of fuel oil. The investigators concluded that the type and degree of fuel oil contamination are two important factors in controlling the impact of fuel oil on *S. fragilis*.

In 1986, more than 8 million litres of crude oil spilled into a complex region of mangroves, seagrasses and coral reefs, just east of the Caribbean entrance to the Panama Canal [76]. Intertidal mangroves, sea grasses, algae and associated invertebrates were covered by oil and died soon after. Investigators reported that seedlings of red mangrove, *Rhizophoram angle*, which were transplanted to heavily oiled sites, did not produce new leaves. This contrasted the transplants at an unoiled site. Entire beds of intertidal seagrass *Thalassiat estudinum* were killed on some heavily oiled reef flats, as shown by the abundant oil-covered dead leaves

washed ashore, as well as the dead, but intact, root-rhizome mats. In contrast, the subtidal *Thalassia* survived everywhere after the spill. Having said this, in the heavily oiled areas, the leaves of the subtidal *Thalassia* became brown and heavily fouled by algae for several months.

Another example of the oil spill effect on mangrove plants is December, 2000. In this case, 500 mangrove saplings in a 6.34 ha reserve in Hong Kong were subjected to a smuggled fuel oil spill. More than 80% died in root-zone sediments containing 60 - 80 ug/g total petroleum hydrocarbons (TPH). However, one year later (December, 2001), the injured survivors had recovered and re-grown, with root-zone sediment TPH concentrations approaching urban 'background' values of 40 ug/g TPH [77].

4.9. Impact of oil spills on cyanobacteria and other microorganisms

There are numerous factors that determine the microbial response to marine oil spills. These include the oil composition and degree of weathering, as well as the environmental conditions, particularly temperature and nutrient concentrations. Reference [13, 78-80], for example, reviewed the different factors affecting the biodegradation of the petroleum hydrocarbons by microorganisms and how environmental and biological factors could determine both the rate at which and extent to which hydrocarbons are removed from the environment.

When crude oil is introduced into seawater, the microbial community changes and consists of multiple co-existing species. These can be explained by resource sharing [81]. In reference [13], in their review paper, reported that the diverse array of hydrocarbons present in crude oil requires resource partitioning by microbial populations, as well as microbial modification of oil components and the surrounding environment, will lead to temporal succession. The reviewers observed a network of direct and indirect interactions within and between species, even when just one type of hydrocarbon is present. They also provided a schematic illustration (Figure 3) of some of the interactions involved in hydrocarbon biodegradation. Elements of these interactions were stated in several studies reviewed by them.

Multiple co-existing species and/or temporal succession are typically observed when *Alcanivorax* spp., which degrade straight-chain and branched alkane, are increased and found abundantly [82-88]. This is followed by *Cycloclasticus* spp., which degrade PAHs [82-86, 89-92]. Other genera of obligate alkane degraders, such as *Oleibacter* sp. [87, 93] and *Oceanospirillales* sp. [94], have been abundantly detected in other oil-rich marine environments [95-96].

At the molecular level, studies by the microarray analysis of 16S rRNA genes have revealed that Gamma-proteobacteria were enriched in marine environments that are historically exposed to frequent, episodic, natural 'oil spills' [97]. This analysis also revealed that marine water exposed to oil spills enriches indigenous oil degrading bacteria, where members of the order *Oceanospirillales* comprised more than 90% of the bacterial community, compared to 5% of the uncontaminated sample [97]. Experiments on the incubation and mixing of marine sediment with phenanthrene and bromodeoxyuridine (BDU), followed by the analysis of BDU-labelled DNA, revealed diverse groups of PAH degraders belonging to the genera *Shewanella*, *Exiguobacterium*, *Methylomonas*, *Pseudomonas* and *Bacteroides*. It also revealed

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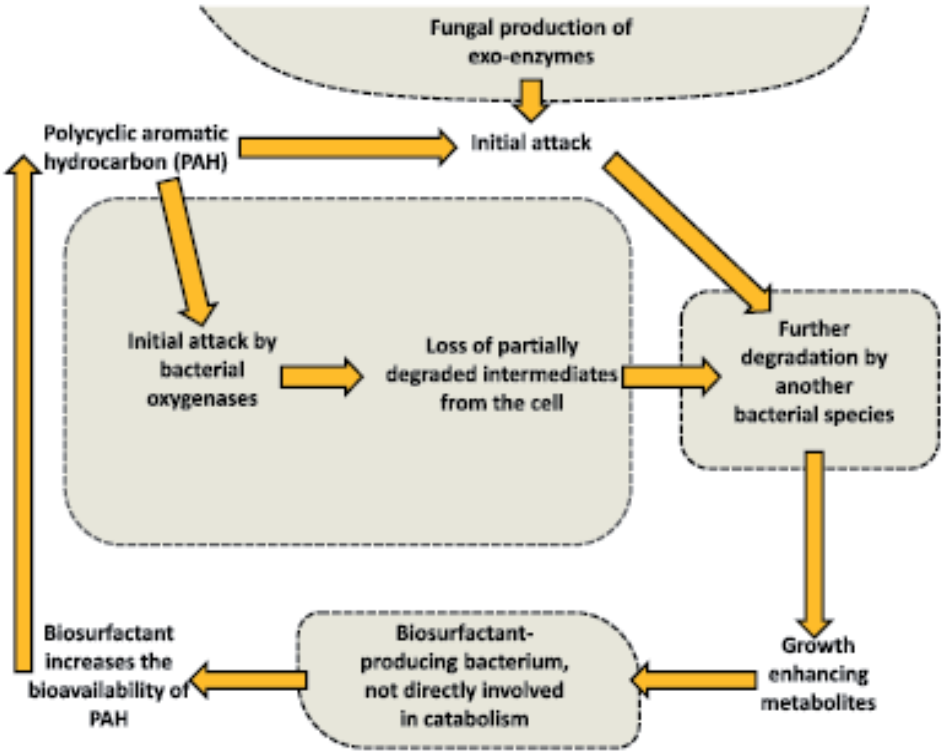

Figure 3. Schematic illustration of some of the interactions involved in hydrocarbon biodegradation [13].

Gammaproteobacteria and Deltaproteobacteria, which were not closely related to the cultivated organisms [98].

Following the 1991 Gulf War environmental disaster, an extensive formation of cyanobacterial mats was observed to colonize most of the oil polluted shores. Although most of the intertidal cyanobacterial mats were severely affected by the oil spill, these organisms were the first to re-colonize the destroyed habitats [99]. This initial massive growth of cyanobacteria, especially on sites where they did not occur before the oil spill, indicated the preference of the cyanobacterial mats to the absence of bioturbation (i.e., destabilizing the sediment surface caused by crabs and polychaetes) of the sediment for their growth. Cyanobacteria do not usually occur where bioturbation has been carried out and - together with the grazing pressure by benthic animals - prevents the establishment of cyanobacterial mats. The extensive growth of cyanobacteria following the oil pollution of the shores can be explained by destroying most of the crab colonies in the mudflats and immediately stopping the bioturbation process, as well as grazing by gastropods. Reference [100] explained this colonization by three different processes. The first one is the desiccation, cracking and peeling of the cyanobacterial mats. This removes the uppermost part of the oiled sediment. The second is the resettlement of burrowing macrofauna like crabs and benthic animals, such as gastropods, which outcompete the cyanobacteria again. The third is further extensive growth of cyanobacteria building thick

laminated mats. These layers completely seal the surface and hence, produce an anaerobic zone which inhibits oil degradation. As long as such a bloom of cyanobacteria exists, microbial oil degradation will be prevented. They will also prevent any resettlement by macrofauna.

5. Communities at risk of marine oil spills/anticipation and preparation

The threatening of marine environments with the petroleum oil spills has caught the attention of many communities, encouraging them to develop their own plans and policy issues. These have ranged from permitting or prohibiting increased oil transport volumes, to developing the capacity to respond to and recover from potential spill disasters.

A comprehensive literature review was conducted by [101], who covered 300 academic, governments and industry papers and reports relating to oil spills and their environmental and societal consequences, with emphasis on economic impacts. Reference [14], in their report of the 'Consequences of Oil Spills from Tanker Accidents', provided a review and structured framework that supports the efforts of such communities to anticipate the spectrum of issues, factors, stakeholders and strategies that may be involved. The review presented an initial and important input into the larger process of addressing the risk of oil spill disasters. The research was based on two premises. The first was that, although previous disasters provide an essential information source for anticipating future events, not all lessons may be transferrable across locales and the key to successful planning and learning from experience is that it be based on systematic assessment activities' [102]. The second premise was that developing realistic expectations of oil spill consequences requires an understanding of the full range of impacts and interactions within and across the affected systems. These include the marine ecosystems and socioeconomic systems.

The purpose of the summary literature review and overview framework provided by [14] was to help communities systematically consider the factors and linkages that would influence consequences of a potential oil spill. Studying previous oil spill disasters has assisted communities to focus on several main domains of interest. These include the nature of the oil spill itself, how to manage the disaster, the physical marine environment, marine biology, human health, economy and policy. Key factors that influence the severity of the impact were identified and significant interactions between variables were recognized. By using this framework, it is suggested that communities can clarify the complexity of oil spill impacts, develop experience for planning from other oil spill disasters and develop the capacity to respond to and recover from potential spill disasters. Furthermore, such a framework encourages debates about risk analysis and policy to understand and reduce the susceptibility of their localities to potential spill disasters. The investigators concluded that a comprehensive overview can help clarify the complexity of oil spill disasters, make comparisons across events, identify data gaps and develop planning scenarios in preparation for future oil spill disasters.

Local communities that depend on the fishing industry, aquaculture and tourism should realize that the impact of an oil spill is governed by complex factors. These include the oil spill's volume and location relative to fishing/cultivation areas, currents, tides and wave action. Other

factors include whether species harvested in the region are sedentary or mobile, as well as government decisions relating to fishing bans and compensation schemes.

6. Economic impact of oil spills in the Gulf

During the 1930s and 1940s, the discovery of oil in the Gulf led to a massive increase in shipping. This discovery is principally responsible for the huge economic wealth and strategic importance associated with the region today. Thus, the socio-economic development of the Gulf region is highly dependent on its marine environmental quality.

In 1991, the second Gulf War led to the largest oil spill in human history. Around 6 million barrels of oil were discharged into the Gulf [22]. Moreover, 770 km of coastline from southern Kuwait to Abu Ali Island (Saudi Arabia) were smothered with oil and tar, erasing most of the local plant and animal communities. Many of these communities were internationally significant. For example, a number of bird species, green and hawksbill turtles and dugongs are endemic to the region. Such species were harmed and disturbed [100].

Considering that approximately 49% of the world's oil production comes from the Gulf States and passes through the Gulf, its liability to pollution is about 48 times that of any other similar area on earth [103]. Hence, the Gulf is possibly the most chronically oil-polluted marine area in the world, even before the war [104].

Due to the different anthropogenic activities relating to oil spills, in addition to the natural environmental stresses of the Gulf, i.e., enclosed and shallow nature, a number of socio-economic impacts are predicted:

a. Fisheries as a multi-million dollar industry and the artisanal fisheries as a resource of great social significance are threatened. This is because oil spills are harmful to coral reefs, mangrove areas and seagrass. These organisms provide a support and grounds for a number of commercially significant fish and shrimp species.

b. Desalination plants that provide most of the population's freshwater supply for the Gulf region are threatened.

c. Disruption to the fishing industry and a reduction in scuba diving tourism [105]. Consequently, people who have a career in this industry will lose their jobs, which they have had for a long time.

d. Disruption of the coastal and marine environments which are internationally significant for a number of bird species, green and hawksbill turtles and dugongs. These are endemic to the region.

7. Conclusion

Marine oil spills can have a serious impact on marine life, as well as on the economic coastal activities and the communities that exploit the resources of the sea. Generally, the effects of oil

toxicity depend on a multitude of factors, including the oil composition and characteristics (physical and chemical), condition (i.e., weathered or not), exposure routes and regimen, and bioavailability of the oil. Oil dispersants, which are a common tool used after oil spills, are also toxic and threaten pelagic and benthic organisms, as well as fish. Marine life can also be affected by clean-up operations or indirectly through the physical damage to the habitats in which plants and animals live. Communities that are threatened by marine oil spills have realized the risk and have, therefore, developed their own plans and policy issues to counteract the risk of marine oil contamination. Due to the different anthropogenic activities relating to oil spills, in addition to the natural environmental stresses of the Gulf, a number of socio-economic impacts are predicted. These are summarized by the threatening of the fish industry and desalination plants that supply most of the populations' freshwater for the Gulf region, in addition to the scuba diving tourism.

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Acknowledgements

The author would like to thank the University of Sharjah for its administrative support. Special thanks are also extended to Dr. Ammira Sarah Akil, for reading the manuscript.

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Chapter 5

Veterinary Antibiotics in the Environment

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60847

Abstract

In recent years, pharmaceutical pollution in the environment has been a great concern due to the potential effects on the human and animal health. Some of the most used classes such as antibiotics, which are used to prevent and treat bacterial infections and promote the growth of livestock, deserve to be highlighted since their intensive use has contaminated environmental matrices such as soil, water, sediment, plants, and animals with effects on the biota. To better understand the potential ecological risk of antibiotics in environments and to develop management strategies for these substances searching to reach the reduction of these compounds in aquatic systems, one of the most important steps is to determine the environmental concentrations of these compounds in the environments through analytical methods and evaluate their effects on the biota. The goal of this chapter is contribute with information about the effects of these compounds on the biota as well as its environmental behavior and bacterial resistance in additional to the main techniques for samples preparation and quantitative and confirmatory methods for its determination in the environment.

Keywords: Antibiotic, chromatography methods, environmental contamination, sample preparations

1. Introduction

1.1. Concept and main classes

Antibiotics (ATBs) are natural or synthetic chemical agents that belong to the group of drugs that play a major role in the prevention and treatment of diseases in human and veterinary medicine [1] inhibiting (bacteriostatic) or killing (bactericidal) microorganisms such as bacteria, fungi, and protozoa [2], besides acting as animal growth promoters [1].

ATBs differ by their chemical structure and mechanism of action, two characteristics that allow these compounds to be grouped into several classes, such as β-lactams, quinolones, tetracyclines, macrolides, sulfonamides, and chloramphenicol, among others. Some of the main ATBs classes used in veterinary medicine, as well as some examples of compounds belonging to them, are shown in Table 1.

Class	Compounds
β-Lactams	Amoxillin, piperacillin
Tetracyclines	Oxytetracycline, chlortetracycline
Macrolides	Erythromicin, tylosin
Sulfonamides	Sulfamethazine, sulfadiazine
Amphenicol	Florfenicol, chloramphenicol
Fluoroquinolones	Ciprofloxacin, enrofloxacin

Table 1. Important classes and examples of veterinary ATBs.

ATBs may be of natural or synthetic origin. The first ATB, penicillin, which is produced by fungi of the Penicillium genus, that is, of natural origin, was discovered in 1929 by the physician and bacteriologist Alexander Fleming. Currently, the ATBs, that are small molecules with molecular weight of less than 1000 Da, are produced by chemical synthesis or by chemical modification of naturally occurring compounds [1].

2. The use of antibiotics in veterinary medicine, characteristics, and environmental contamination

The use of ATBs in the veterinary sector has been for many years an effective method used in animal husbandry, as these chemical agents promote animal growth, besides prevention and therapy against microorganisms [1]. Virtually, livestock activities, such as cattle, pigs, goats, and aquaculture, among others, make the use of these molecules to ensure animals' good quality and well-being, and in the case of activity for commerce, ensure product quality and market competitiveness.

Once ATBs are used and subsequently absorbed by animals, such compounds are metabolized. The metabolism degree depends on the type of substance and the treated species, as well as its age and health condition. If the compound is not metabolized, it will be eliminated in the feces and urine, reaching the environment (mainly soil and water) [3]. According to Kümmerer [1], 80% to 90% of ATBs are excreted as parent compounds in the environment, i.e., compounds that have not undergone metabolism in the animal body. According to Katz [4], the amount of eliminated ATBs varies according to the type of ATB, the dosage, and the type and age of the animal.

http://dx.doi.org/10.5772/60847

In the case of farm animals such as cattle, pigs, goats, and sheep, after reaching the soil through feces and urine, this group of drugs may be leached or suffer runoff to aquatic environments and still be in the soil [5]. Three other important soil and water introduction pathways to be mentioned are the packaging disposal in inappropriate places, especially on small farms, where the educational level of most farmers is low, the use of animal excreta for fertilization [6] and water direct contamination through aquaculture. The ATBs may also contaminate the environment by emissions during their fabrication process, although researchers consider this introduction pathway to be less relevant than those described above [7]. Thus, concern about the effects of these compounds in the environment has increased in recent years, which puts them as major environmental concern contaminants. Figure 1 exemplifies the main contamination pathways of veterinary ATBs in the environment.

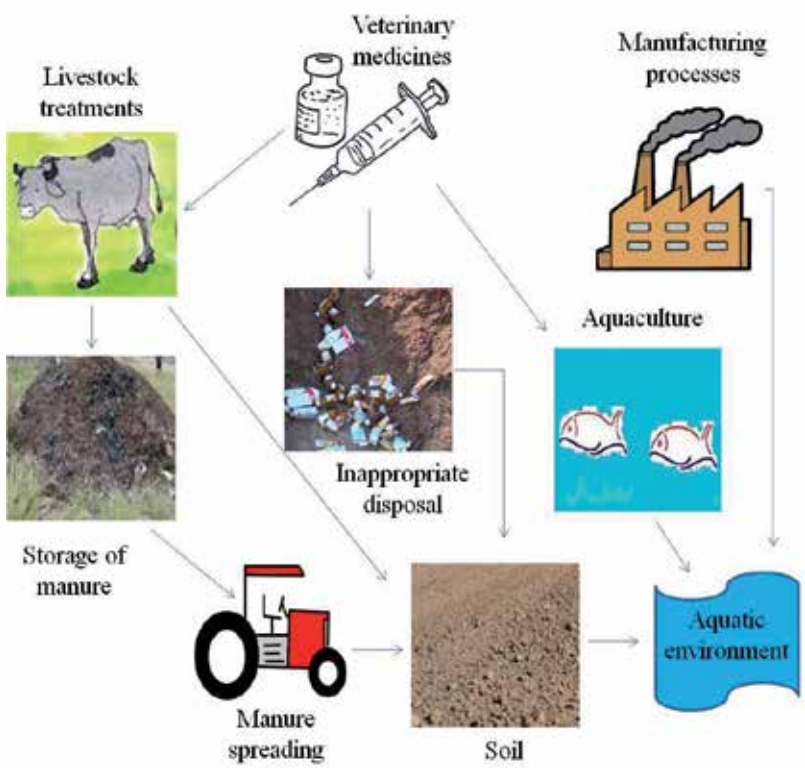

Figure 1. Introduction pathways of veterinary antibiotics in the environment. Adapted from Boxall et al. [7].

Of all animal husbandry activities, fish farming may be the one that contributes with the largest share of direct contamination of ATBs in the environment. In general, these compounds are administered in fish farming through three forms: inclusion in food (the most practiced and used in tank-nets crops), baths (restricted to water-soluble compounds and administered in tanks with interrupted water renovation during treatment), and finally through hypodermic injection (high cost). According to Shao [8], ATB inclusion in the feed is the most convenient

way due to lower amounts of drug required in comparison with administration through water, for example. Consequently, the number of such molecules that enter the aquatic environment would be lower, according to this author.

Cravedi [9] reported that about 7% to 9% of the ingested oxytetracycline was absorbed during the passage through the gastrointestinal tract of rainbow trout, and thus 93% to 99% polluted the environment. Rogstad et al. [10] observed absorption of less than 1% after 24 h of oxytetracycline administration and of 2.6% after 72 h. Among sick fish, low intake rate is common due to reduced palatability of the diet. Thinking of decreasing the contamination of aquatic environments, hypodermic injection or vaccine has been used in many fish farms.

Even with the use of ATBs in aquaculture facilities, limited data on types and amount used of these products are not available. In the study of Sapkota et al. [11], a list of 26 antibiotics used in the 15 countries that more practiced aquaculture by the year of 2005 according to FAO was presented, which comprises representatives of the class of sulfonamides, tetracyclines, penicillins, quinolones, nitrofurans, macrolides, aminoglycosides, and chloramphenicols.

In general, ATBs used in aquaculture are oxytetracycline, florfenicol sarafloxacin, erythromycin and sulfonamides potentiated with trimethoprim or ormetoprim [12]. In Brazilian fish farms, only florfenicol-based ATBs are approved by the Ministry of Livestock Supply (MAPA) for use in tilapia [13], although others, such as oxytetracycline, are also used. Both are representatives of the classes of chloramphenicols and tetracycline, respectively, and the main form of use of these drugs has been through inclusion in the feed. Florfenicol is a fluorinated analog of thiamphenicol [14], which binds to 50S and 70S ribosome subunits [15], inhibiting protein synthesis transpeptidation of Gram-negative and Gram-positive bacteria [16]. Oxytetracycline is an antibacterial agent, which is effective in the treatment of infections caused by Gram-positive and Gram-negative bacteria, mycoplasmas, and large viruses. It inhibits protein synthesis by preventing the association of aminoacyl-tRNA to the bacterial ribosome [3].

As with most chemical agents, the destination and behavior of ATBs in the environment is influenced by the physical and chemical characteristics of the compounds (molecular structure, size, shape, solubility, hydrophobicity) and of the soil (pH, texture field organic), besides the climatic conditions (temperature, precipitation) [17] and biological factors (microbial degradation). ATBs that have high potential of sorption (K_d) to the soil particles, for example, tend to accumulate and persist in this matrix, unlike those who have low K_d value, which are easily transported to aquatic environments [18]. According to Regitano and Leal [19], in general, compounds with K_d <5 L kg^{-1} values and half-life of less than 21 days, such as sulfonamides (K_d = 0.2 to 2.0 L kg^{-1}), for example, have relative persistence and can be leached into groundwater, unlike those that have K_d > 5 L kg^{-1} and half-life of more than 21 days, which tend to accumulate in the soil surface layers, as is the case of compounds belonging to the group of tetracyclines and fluoroquinolones (K_d = 70 at 5.000 L kg^{-1}). K_d values may vary considerably for certain compounds in different types of soil [20].

According to Tolls [21], ATBs sorption may also be influenced by cation exchange processes, by adsorption to the surfaces of clay minerals, by complexing reactions with metal ions and by hydrogen bridges. For example, in the study of Sassman and Lee [22], the main mechanism involved in the sorption of tetracyclines was cation exchange, and sorption potential was

influenced by the environment pH and by the cation exchange capacity of clay minerals prevailing in the soil matrix.

ATBs, which are mostly complex molecules, may have different functionalities within the same molecule, which causes that under different pH conditions they can be neutral, cationic, anionic, or zwitterionic. Due to different functions within a single molecule, its physicochemical and biological characteristics, such as the log Kow [23], sorption behavior, photo reactivity, antibiotic activity, and toxicity, can change with pH. Other factors that are pH dependent are solubility, hydrophobicity, and log Kow. Regarding drugs solubility being pH dependent, this can affect not only destination and transport, but also the assessment of environmental effects, which includes toxicological assessments [24], which are going to be portrayed in this chapter.

Class	Antibiotic	pK_a [a]	Log Kow[a]	Molecular weight	Molecular formula
Tetracycline	Oxytetracycline	3.27	–0.9	460.45	$C_{22}H_{24}N_2O_9$
	Chlortetracycline	3.3	–0.62	478.88	$C_{22}H_{23}C_lN_2O_8$
	Tetracycline	3.3	–1.30	444.43	$C_{22}H_{24}N_2O_8$
	Doxycycline	na[b]	–0.02	444.44	$C_{22}H_{24}N_2O_8$
Fluoroquinolones	Nalidixic acid	8.6	1.59	232.23	$C_{12}H_{12}N_2O_3$
	Oxolinic acid	6.87	0.94	261.23	$C_{22}H_{24}N_2O_8$
	Flumequine	na	1.6	261.25	$C_{13}H_{11}NO_5$
Amphenicol	Chloramphenicol	na	1.14	323.13	$C_{11}H_{12}C_{12}N_2O_5$
	Thiamphenicol	na	–0.33	356.22	$C_{12}H_{15}C_{12}NO_5S$
	Florfenicol	na	na	358.21	$C_{12}H_{14}C_{12}FNO_4S$
Macrolides	Erythromycin	8.88	3.06	733.92	$C_{37}H_{67}NO_{13}$
	Roxithromycin	na	2.75	837.05	$C_{41}H_{76}N_2O_{15}$
	Josamycin	na	3.16	827.99	$C_{42}H_{69}NO_{15}$
	Spiramycin	na	1.456	843.05	$C_{43}H_{74}N_2O_{14}$
Sulfonamides	Sulfaguanidine	11.25	–1.22	214.24	$C_7H_{10}N_4O_2S$
	Sulfacetamide	7.59	–0.96	214.24	$C_8H_{10}N_2O_3S$
	Sulfamethazine	8.43	0.89	278.33	$C_{12}H_{14}N_4O_2S$
	Sulfapyridine	6.36	0.35	249.29	$C_{11}H_{11}N_3O_2S$
	Sulfadiazine	6.5[c]	–0.09	250.28	$C_{10}H_{10}N_4O_2S$
	Sulfadimethoxine	5.9[c]	1.63	310.33	$C_{12}H_{14}N_4O_4S$
	Sulfametizole	5.5[c]	0.54	270.33	$C_9H_{10}N_4O_2S_2$
	Sulfamethoxazole	8.8[c]	0.89	253.27	$C_{10}H_{11}N_3O_3S$
	Sulfamiderazine	8.0[c]	0.14	264.30	$C_{11}H_{12}N_4O_2S$

[a]Values obtained from the United States National Library of Medicine: http://toxnet.nlm.nih.gov/.

[b]Not available

[c]Values from Białk-Bielinska [26].

Table 2. Physical and chemical characteristics of some veterinary ATBs.

Generally, pharmaceutical products are compounds characterized by a complex chemical structure that have very variable molar masses (172 at 916 g mol^{-1}), low volatilization potential, several ionizable functional groups (amphoteric molecules), different pK_a values, and low octanol–water partition coefficient values (log Kow), which indicates low bioaccumulation potential [25]. The log Kow indicates the tendency of an organic chemical product to partition into lipids or fats and adsorb to particles of soils, sediments, biomasses, and muds [23]. Table 2 shows some of these characteristics described above for some ATBs.

3. Occurrence in the environment

3.1. Surface water, groundwater, and sediment

As described previously, veterinary ATBs may contaminate the environment after their use, principally soil and water matrices, and also aquatic nontargeted organisms and sediments.

The first reported case of antibiotic contamination in surface waters happened in England more than two decades ago, when Watts et al. [27] found at least one compound belonging to the group of macrolides, tetracyclines, and sulfonamides in river water, in 1 μg L^{-1} concentrations. Subsequently, other studies, such as those of Richardson and Bowron [28], Pearson and Inglis [29], Ternes [30], and Hirsch et al. [31], have been developed, enabling the detection of other ATBs groups. Although the study of pharmaceutical residues in the environment is relatively a new topic, a lot of papers have already been published from the 1990s to the present day, as can be seen in Tables 3, 4, and 5, which describe ATBs and their reported concentrations in different environmental matrices in several parts of the world.

Antibiotics	Concentration (ng L^{-1})	Location	Reference
β-Lactams			
Amoxillin	200	River water, Australia	[32]
Cefaclor	200	River water, Australia	[32]
Penicillin G	250	River water, Australia	[32]
Penicillin V	10	River water, Australia	[32]
Fluoroquinolones			
Ciprofloxacin	1300	River water, Australia	[32]
	17.4–588.5	Po, Olona, and Lambro Rivers, Italy	[33]
	<10	Seine River, France	[34]
	370–9660	Arc River, France	[34]
	14.4–26.2	Po and Lambro Rivers, Italy	[35]
Danofloxacin	19	Seine River, France	[34]
Enoxacin	11	Seine River, France	[34]
Enrofloxacin	300	River water, Australia	[32]

Antibiotics	Concentration (ng L^{-1})	Location	Reference
Flumequine	32	Seine River, France	[34]
	0.79–3.70	Panjiakou Reservoir, China	[36]
Nalidixic acid	750	River water, Australia	[32]
	<10	Seine River, France	[34]
	<0.25–11.20	Panjiakou Reservoir, China	[36]
Oxolinic acid	19	Seine River, France	[34]
	0.31–2.70	Panjiakou Reservoir, China	[36]
Fluoroquinolones			
Norfloxacin	1150	River water, Australia	[32]
	163	Seine River, France	[34]
	251	Pearl River, Guangzhou, China	[37]
	24–48	Lake and river water, India	[38]
Ofloxacin	19.3–306.1	Po and Lambro Rivers, Italy	[35]
	8.1–634	Victoria Harbour, Hong Kong	[39]
	55	Seine River, France	[34]
	108	Pearl River, Guangzhou, China	[37]
	33.1–306.1	Po and Lambro Rivers, Italy	[35]
Lincosamides			
Clindamycin	10	River water, Australia	[32]
Lincomycin	50	River water, Australia	[32]
	1.9–17.3	Po, Olona, and Lambro Rivers, Italy	[33]
	24.4–248.9	Po and Lambro Rivers, Italy	[35]
Macrolides			
Clarithromycin	3.0–114.8	Po, Olona, and Lambro Rivers, Italy	[33]
	600–2330	Arc River, France	[34]
	190	River water, Germany	[40]
	1.6–20.3	Po and Lambro Rivers, Italy	[35]
Oleandomycin	20	River water, Australia	[32]
Roxithromycin	350	River water, Australia	[32]
	169	Pearl River, Guangzhou, China	[37]
	190	Lutter River, Germany	[40]
	<30–40	Elbe River and tributaries, Germany	[41]
	<0.13–3.90	Panjiakou Reservoir, China	[36]
Spiramycin	3.3–459.5	Po, Olona, and Lambro Rivers, Italy	[33]
	<1.9–58.81	Panjiakou Reservoir, China	[36]
Tylosin	9.8–74.2	Po and Lambro Rivers, Italy	[35]

Antibiotics	Concentration (ng L^{-1})	Location	Reference
	60	River water, Australia	[32]
Tetracyclines			
Chlortetracycline	600	River water, Australia	[32]
	160	Cache La Poudre, USA	[42]
	1–180	Choptank watershed, USA	[43]
	<0.17–22.13	Panjiakou Reservoir, China	[36]
Demeclocycline	120–440	Cache La Poudre, USA	[42]
Doxycycline	50–80	Cache La Poudre, USA	[42]
	13–146	Choptank watershed, USA	[43]
	400	River water, Australia	[32]
	0.19–13.69	Panjiakou Reservoir, China	[36]
Oxytetracycline	100	River water, Australia	[32]
	7.7–105.1	Po, Olona, and Lambro Rivers, Italy	[33]
	80–130	Cache La Poudre, USA	[42]
	1–388	Choptank watershed, USA	[43]
	110–680	Arc River, France	[34]
	2–7	Mess and Alzette Rivers, Luxembourg	[44]
	68000	River water, Japan	[45]
	0.20–19.93	Panjiakou Reservoir, China	[36]
	14–7993	Ilha Solteira Reservoir, Brazil	[46]
Tetracycline	80	River water, Australia	[32]
	60–140	River water, USA	[42]
	1–5	Choptank watershed, USA	[43]
	7–8	Mess abd Alzette, Luxembourg	[44]
	0.14–14.05	Panjiakou Reservoir, China	[36]
Sulfonamides			
Sulfadiazine	1.9–2312	Segre, Llobregat, and Anoia Rivers, Spain	[47]
	336	Pearl River, Guangzhou, China	[37]
	0.35–10.86	Panjiakou Reservoir, China	[36]
Sulfadimethoxine	50–90	Cache La Poudre, USA	[42]
	1–9	Choptank watershed, USA	[43]
	3.0	Mess and Alzette Rivers, Luxembourg	[44]
	1.5–182.4	Segre, Llobregat, and Anoia Rivers, Spain	[47]
	0.95–3.56	Panjiakou Reservoir, China	[36]

Antibiotics	Concentration (ng L^{-1})	Location	Reference
Sulfadimidine	323	Pearl River, Guangzhou, China	[37]
Sulfamethazine	220	Cache La Poudre, USA	[42]
	<10	Seina River, France	[34]
	1.7–6192	Segre, Llobregat, and Anoia Rivers, Spain	[47]
	0.21–3.40	Panjiakou Reservoir, China	[36]
Sulfamethoxazole	2000	River water, Australia	[32]
	50–120	Cache La Poudre, USA	[42]
	300	Rio Grande, New Mexico	[48]
	1–7	Choptank watershed, USA	[43]
	480	Lutter River, Germany	[40]
	193	Rio Pearl, Guangzhou, China	[37]
	<30–70	Elbe River and tributaries, Germany	[41]
	544	Seine River, France	[34]
	1–22	Mess and Alzette Rivers, Luxembourg	[44]
	6.4–1488	Segre, Llobregat, and Anoia Rivers, Spain	[47]
	<0.11–7.23	Panjiakou Reservoir, China	[36]
	47–96	Lake and water River, India	[38]
Sulfapyridine	1.2–12000	Segre, Llobregat, and Anoia Rivers, Spain	[47]
	<0.10–3.40	Panjiakou Reservoir, China	[36]
Sulfasalazine	30	River water, Australia	[32]
Sulfisoxazole	0.5–2.8	Segre, Llobregat, and Anoia Rivers, Spain	[47]
Sulfathiazole	40	River water, Australia	[32]
	1.5–332	Segre, Llobregat, and Anoia Rivers, Spain	[47]
	2	Mess River, Luxembourg	[44]
Chloramphenicol			
Chloramphenicol	266	Pearl River, Guangzhou, China	[37]
	0.29–7.15	Panjiakou Reservoir, China	[36]
Thiamphenicol	<0.13–45.00	Panjiakou Reservoir, China	[36]
Florfenicol	0.12–73.66	Panjiakou Reservoir, China	[36]
	7.0–425	Ilha Solteira Reservoir, Brazil	[46]

Table 3. Antibiotic concentrations reported in surface water in several regions of the world. Adapted from Fata-Kassinos et al. [24].

Antibiotics	Concentration (ng L^{-1})	Location	Reference
Lincosamides			
Lincomycin	320	18 States, USA	[49]
Sulfonamides			
N4-acetil sulfamethazine	2.7	Barcelona, Espain	[47]
	0.02–56.95	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfabenzamide	0.09–10.32	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfacetamide	1.77–3461	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfadimethoxine	0.2	Barcelona, Espain	[47]
Sulfadoxine	0.02–53.63	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfaguanidine	3.3–91.78	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfamiderazine	0.11–744.7	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfamethazine	360	18 States, USA	[49]
	0.03–106.8	Plana de Vic and La Selva, Catalonia, Spain	[50]
	>160	Agricultural areas, Germany	[31]
Sulfamethizole	0.22–9.29	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfamethoxazole	9.9	Barcelona, Espain	[47]
	1110	18 States, USA	[49]
	0.08–312.2	Plana de Vic and La Selva, Catalonia, Spain	[50]
	>410	Baden-Württemberg, Germany	[51]
	>470	Áreas agrícolas, Alemanha	[31]
Sulfametoxipiridazina	0.02–68.70	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfanitran	0.04–568.8	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfapyridine	0.07–72.45	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfaquinoxaline	0.01–112.1	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfathiazole	0.01–16.78	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfisomidine	0.01–64.40	Plana de Vic and La Selva, Catalonia, Spain	[50]
Sulfisoxazole	0.21–4.43	Plana de Vic and La Selva, Catalonia, Spain	[50]

Antibiotics	Concentration (ng L^{-1})	Location	Reference
Sulfadiazine	">17	Baden-Württemberg, Germany	[51]
Sulfadimidine	>23	Baden-Württemberg, Germany	[51]
Macrolides			
Erythromicyn-H_2O	>49.0	Baden-Württemberg, Germany	[51]
Roxithromycin	>26.0	Baden-Württemberg, Germany	[51]

Table 4. Antibiotic concentrations reported in groundwater in several regions of the world. Adapted from Fata-Kassinos et al. [24].

Antibiotics	Concentration (μg Kg^{-1})	Location	References
Sulfonamides			
Sulfadiazine	0.07–0.71	Huangpu River, China	[52]
	0.5	Yangtze Estuary, China	[53]
	2.07	Baiyangdian Lake, China	[54]
Sulfapyridine	6.6	Huangpu River, China	[52]
	9.1	Yangtze Estuary, China	[53]
	1.6–8.1	Dagu River, China	[55]
Sulfamethoxazole	0.05–0.6	Huangpu River, China	[52]
	9.7–14.7	Dagu River, China	[55]
	1.6	Cache La Poudre River, USA	[X]
Sulfathiazole	0.6	Huangpu River, China	[52]
Sulfamerazine	0.03–0.8	Huangpu River, China	[52]
Sulfamethazine	0.2–2.7	Huangpu River, China	[52]
	19.7	Pearl River, China	[57]
	5.2	Cache La Poudre River, USA	[56]
Sulfaquinoxaline	0.08–0.9	Huangpu River, China	[52]
Macrolides			
Erythromycin	1.5–24.6	Huangpu River, China	[52]
Roxithromycin	0.3–4.1	Huangpu River, China	[52]
Fluoroquinolones			
Enrofloxacin	8.9	Huangpu River, China	[52]
Ofloxacin	12.4	Huangpu River, China	[52]
Amphenicol			
Chloramphenicol	0.7	Huangpu River, China	[52]
Thiamphenicol	1.3	Huangpu River, China	[52]

Antibiotics	Concentration (μg Kg^{-1})	Location	References
	1.3	Huangpu River, China	[52]
Tetracyclines			
Tetracycline	21.7	Huangpu River, China	[52]
	10.4–22.0	Ilha Solteira Reservoir, Brazil	[46]
	17.9	Cache La Poudre River, USA	[56]
Oxytetracycline	0.6–18.6	Huangpu River, China	[52]
	11.5–7342.7	Reservatório de Ilha Solteira, Br Ilha Solteira Reservoir, Brazil	[46]
	14.8	Cache La Poudre River, USA	[56]
Chlortetracycline	6.3	Huangpu River, China	[52]
	16.1	Ilha Solteira Reservoir, Brazil	[46]
	10.8	Cache La Poudre River, USA	[56]

Table 5. Antibiotic concentrations reported in sediments of aquatic environments in several regions of the world. Adapted from Fata-Kassinos et al. (2011).

4. Antibiotic effects on the environment

Veterinary ATBs are designed to affect microorganisms found in animals. However, as discussed above, they are rapidly eliminated in its active form or as by-products, contaminating the environment. After contaminating the environment, such drugs have the potential to cause adverse effects to the aquatic and terrestrial biota of different trophic levels and also to humans, through consumption of contaminated food derived from aquaculture or through contact with contaminated water. ATBs transference in the body is determined by its ability to move through the lipid bilayer of epithelial cells. The most important properties affecting their permeation across biological membranes are lipophilicity, hydrogen bonding capacity, size, and charge [58].

To demonstrate the negative effects of these compounds, several authors have performed toxicity tests using a wide range of test organisms under controlled conditions [59–70].

Toxicity tests are divided into acute and chronic. The acute toxicity test is designed to evaluate the effects on organisms in a short period of exposure, with the goal of determining the concentration of a studied substance that produces deleterious effects in controlled conditions. When the test organism is fish, lethal effect is observed in most of the times, from which the concentration of the toxic agent that causes 50% mortality (LC50) is determined. On the other hand, for microcrustaceans, the observed effect can be lethality and also mobility, and in the latter case, the average effective concentration (EC50) that causes 50% immobility is calculated [71]. In chronic toxicity tests, organisms are continually exposed to the evaluated substance for a significant period of time of their life cycle, which can range from half to two thirds of

the cycle [71]. Depending on the tested substance characteristics, due to the long test period, it may be necessary to the test solutions to be renewed. In this test, sublethal effects, such as changes in growth and reproduction, changes in behavior (such as movement difficulty and increased lid opening frequency, in the latter case to fish), physiological, biochemical, and tissue alterations [72, 73], among others are evaluated. The chronic toxicity test depends directly on the results of the acute test, once sublethal concentrations are calculated from CL50 and CE50.

For choosing the test organism, the following selection criteria are often used: abundance and availability, significant ecological representation, cosmopolitanism, knowledge of its biology, physiology and diet, genetic stability and uniformity of their populations, sensitivity, commercial importance, ease of cultivation in the laboratory and, if possible, the species should be native, to a better representation of ecosystems [71].

The sensitivity of algae to ATBs varies widely. In a performed toxicity test, it was shown that the green alga *Selenastrum capricornutum* was less sensitive than *Microcystis aeruginosa* microalgae for most of the tested molecules. The growth of *M. aeruginosa* was inhibited when concentrations of less than 0.1 mg L^{-1} were exposed [66]. Blue-green algae (cyanobacteria) were also sensitive to several ATBs, such as amoxicillin, penicillin benzyl, spiramycin, tetracycline, among others. All these results are very worrying, once that algae are located at the base of the food chain, and a drop in the population of these organisms can disrupt aquatic ecosystems.

Reproductive effects have also been observed in aquatic organisms, such as *Artemia* sp. and *Daphnia magna* when exposed to ATBs [55, 61, 66, 74, 75]. It is important to consider that reproductive effects in any population of organisms can cause considerable damage to the natural balance since the organisms are directly related to each other in the trophic chain.

Numerous studies have evaluated the acute toxicity of ATBs for different aquatic organisms. For example, Wollenberger et al. [75] studied the acute toxicity of nine commonly used veterinary ATBs and reported lower acute toxicity ($CE50_{48h}$, mg L^{-1}) of the oxolinic acid (4.6) and higher toxicity to oxytetracycline (~1000). Previously, Dojmi di Delupis et al. [76] had reported moderate toxicity to aminosidine, bacitracin, erythromycin, and moderate lincomycin ATBs to *D. magna* microcrustacean, with CE5048h value of between 30 and 500 mg L-1, with bacitracin being the most toxic. In another study, Kolodziejska et al. [77] determined the toxicity of four veterinary ATBs for different aquatic organisms. In this study, oxytetracycline and florfenicol had stronger effects on *Lemna minor* (CE50 = 3.26 and 2.96 mg L-1, respectively) and on green alga *Scenedesmus vacuolatus* (CE50 = 40.4 and 18.0 mg L-1) than on the marine bacterium *Vibrio fischeri* (CE50 = 108 and 29.4 mg L-1) and on microcrustacean *D. magna* (CE50 = 114 and 337 mg L-1).

The chronic effects of ATBs to aquatic organisms were also studied. Kin et al. [78] evaluated the chronic toxicity of acetaminophen and lincomycin ATBs for two crustaceans species (*D. magna* and *Moina macrocopa*) and for the fish *Oryzias latipes*. To *D. magna*, acetaminophen ATB caused no significant effect on reproduction when exposed to the concentration of 5.72 mg L-1. Similar results were observed for survival and growth when microcrustaceans were exposed to the highest concentration of lincomycin (153 mg L-1). For fish, a significant reduction in

survival was observed 30 days after hatching, when exposed to 95 mg L-1 of acetaminophen and 0.42 mg L-1 of lincomycin. Several other studies were conducted to evaluate ATBs acute and chronic toxicity of different classes, using organisms of different trophic levels, as can be seen in Table 6.

Antibiotic taxonomic group	Species	Test duration/end point	Concentration (mg L^{-1})	References
Chlortetra-cycline				
Bacteria	Vibrio fischeri	5 min, luminescence inhibition EC50	>20.0	[80]
Bacteria	Vibrio fischeri	15 min, luminescence inhibition EC50	13.0	[80]
Alga	Microcystis aeruginosa	7 days, growth EC50	0.05	[66]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	3.1	[66]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	1.8	[81]
Duckweed	Lemna gibba	7 days, wet weight EC50	0.219	[82]
Duckweed	Lemna gibba	7 days, frond number EC50	0.318	[82]
Duckweed	Lemna gibba	7 days, chlorophyll a EC50	0.630	[82]
Duckweed	Lemna gibba	7 days, chlorophyll b EC50	0.650	[82]
Duckweed	Lemna gibba	7 days, carotenoids EC50	1.620	[82]
Invertebrate	Daphnia magna	24 h, immobilization EC50	380.1	[79]
Invertebrate	Daphnia magna	48 h, immobilization EC50	225.0	[79]
Invertebrate	Moina macrocopa	24 h, immobilization EC50	515.0	[79]
Invertebrate	Moina macrocopa	48 h, immobilization EC50	272.0	[79]
Fish	Oryzias latipes	48 h, survival LC50	88.4	[79]
Fish	Oryzias latipes	96 h, survival LC50	78.9	[79]
Oxytetra-cycline				
Bacteria	Vibrio fischeri	5 min, luminescence inhibition EC50	235.4	[79]
Bacteria	Vibrio fischeri	15 min, luminescence inhibition EC50	87.0	[79]
Bacteria	Vibrio fischeri	30 min, luminescence inhibition EC50	64.50	[83]
Bacteria	Vibrio fischeri	30 min, luminescence inhibition EC50	121.01	[84]
Rotifer	Brachionus calyciflorus	24 h, survival LC50	34.21	[83]
Rotifer	Brachionus calyciflorus	48 h, growth EC50	1.87	[83]
Algae	Chlorella vulgaris	48 h, growth EC50	6.4	[83]

Antibiotic taxonomic group	Species	Test duration/end point	Concentration (mg L^{-1})	References
Algae	Chlorella vulgaris	72 h, growth EC50	7.05	[86]
Algae	Chlorella vulgaris	72 h, growth EC50	<3.58	[86]
Algae	Microcystis aeruginosa	7 days, growth EC50	0.207	[87]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	4.18	[88]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	0.342	[86]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	0.17	[83]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	0.183	[86]
Algae	Pseudokirchneriella subcapitata	7 days, growth EC50	4.5	[87]
Duckweed	Lemna gibba	7 days, chlorophyll a EC50	1.179	[82]
Duckweed	Lemna gibba	7 days, chlorophyll b EC50	1.152	[82]
Duckweed	Lemna gibba	7 days, carotenoids EC50	>1,000	[82]
Duckweed	Lemna minor	7 days, growth EC50	4.92	[85]
Invertebrate	Ceriodaphnia dúbia	48 h, immobilization EC50	18.65	[83]
Invertebrate	Ceriodaphnia dúbia	7 days, population growth EC50	0.18	[83]
Invertebrate	Daphnia magna	24 h, immobilization EC50	831.6	[79]
Invertebrate	Daphnia magna	24 h, immobilization EC50	22.64	[83]
Invertebrate	Daphnia magna	48 h, immobilization EC50	621.2	[79]
Invertebrate	Daphnia magna	48 h, immobilization EC50	>200	[89]
Invertebrate	Daphnia magna	48 h, immobilization LOEC	100	[75]
Invertebrate	Daphnia magna	21 days, reproduction EC10	7.4	[75]
Invertebrate	Daphnia magna	21 days, reproduction EC50	46.2	[75]
Invertebrate	Hydra attenuata	96 h, survival LC50	>100	[90]
Invertebrate	Hydra attenuata	96 h, morphology NOEC	50	[90]
Invertebrate	Moina macrocopa	24 h, immobilization EC50	137.1	[90]
Invertebrate	Moina macrocopa	48 h, immobilization EC50	126.7	[79]
Invertebrate	Thamnocephalus platyurus	24 h, survival LC50	25.0	[83]
Fish	Danio rerio	96 h, survival NOEC	1,000	[83]
Fish	Oryzias latipes	48 h, survival LC50	215.4	[79]
Fish	Oryzias latipes	96 h, survival LC50	110.1	[79]
Sulfa-methazine				
Bacteria	Vibrio fischeri	5 min luminescence inhibition EC50	303.0	[91]

Antibiotic taxonomic group	Species	Test duration/end point	Concentration (mg L^{-1})	References
Bacteria	Vibrio fischeri	15 min, luminescence inhibition EC50	344.7	[91]
Algae	Pseudokirchneriella subcapitata	72 h, growth EC50	8.7	[81]
Duckweed	Lemna gibba	7 days, wet weight EC50	1.277	[82]
Duckweed	Lemna gibba	7 days, frond number EC50	>1.000	[82]
Duckweed	Lemna gibba	7 days, chlorophyll a EC50	>1.000	[82]
Duckweed	Lemna gibba	7 days, chlorophyll b EC50	>1.000	[82]
Duckweed	Lemna gibba	7 days, carotenoids EC50	>1.000	[83]
Invertebrate	Daphnia magna	24 h, immobilization EC50	133	[89]
Invertebrate	Daphnia magna	24 h, immobilization EC50	506.3	[79]
Invertebrate	Daphnia magna	48 h, immobilization EC50	174.4	[91]
Invertebrate	Daphnia magna	48 h, immobilization EC50	105	[89]
Invertebrate	Daphnia magna	48 h, immobilization EC50	185.3	[92]
Invertebrate	Daphnia magna	48 h, immobilization EC50	215.9	[79]
Invertebrate	Daphnia magna	48 h, immobilization EC50	202	[88]
Invertebrate	Daphnia magna	96 h, immobilization EC50	158.8	[91]
Invertebrate	Daphnia magna	96 h, immobilization EC50	147.5	[92]
Invertebrate	Daphnia magna	21 days, survival NOEC	30	[79]
Invertebrate	Daphnia magna	21 days, reproduction NOEC	30	[79]
Invertebrate	Daphnia magna	21 days, reproduction NOEC	1.563	[88]
Invertebrate	Daphnia magna	21 days, growth NOEC	1.563	[88]
Invertebrate	Moina macrocopa	24 h, immobilization EC50	310.9	[79]
Invertebrate	Moina macrocopa	48 h, immobilization EC50	110.7	[79]
Invertebrate	Moina macrocopa	7 days, survival NOEC	30	[79]
Invertebrate	Moina macrocopa	7 days, reproduction NOEC	30	[79]
Fish	Oryzias latipes	48 h, survival LC50	>100	[91]
Fish	Oryzias latipes	96 h, survival LC50	>100	[91]
Fish	Oryzias latipes	48 h, survival LC50	>500	[79]
Fish	Oryzias latipes	96 h, survival LC50	>500	[79]
Sulfathiazole				
Bacteria	Vibrio fischeri	5 min, luminescence inhibition EC50	>1000	[91]
Bacteria	Vibrio fischeri	15 min, luminescence inhibition EC50	>1000	[91]
Invertebrate	Daphnia magna	24 h, immobilization EC50	616.7	[79]
Invertebrate	Daphnia magna	48 h, immobilization EC50	149.3	[91]
Invertebrate	Daphnia magna	48 h, immobilization EC50	135.7	[92]

Antibiotic taxonomic group	Species	Test duration/end point	Concentration (mg L^{-1})	References
Invertebrate	Daphnia magna	48 h, immobilization EC50	160.8	[93]
Invertebrate	Daphnia magna	48 h, immobilization EC50	142.2.	[94]
Invertebrate	Daphnia magna	48 h, survival LC50	253.1	[95]
Invertebrate	Daphnia magna	96 h, immobilization EC50	85.4	[91]
Invertebrate	Daphnia magna	96 h, immobilization EC50	78.9	[92]
Invertebrate	Daphnia magna	21 days, survival NOEC	11	[79]
Invertebrate	Daphnia magna	21 days, reproduction NOEC	11	[79]
Invertebrate	Moina macrocopa	24 h, immobilization EC50	430.1	[79]
Invertebrate	Moina macrocopa	48 h, immobilization EC50	391.1	[79]
Invertebrate	Moina macrocopa	7 days, survival NOEC	35	[79]
Invertebrate	Moina macrocopa	7 days, reproduction NOEC	35	[79]
Fish	Oryzias latipes	48 h, survival LC50	>500	[91]
Fish	Oryzias latipes	96 h, survival LC50	>500	[91]
Erythromycin				
Bacteria	*Vibrio fischeri*	30 min, luminescence inhibition NOEC	100	[83]
Rotifer	*Brachionus calyciflorus*	24 h, survival LC50	27.53	[83]
Rotifer	*Brachionus calyciflorus*	48 h, growth EC50	0.94	[83]
Algae	*Chlorella vulgaris*	72 h, growth EC50	33.8	[86]
Algae	*Chlorella vulgaris*	72 h, growth NOEC	12.5	[86]
Algae	*Anabaena cylindrica*	144 h, growth EC50	0.035	[96]
Algae	*Anabaena cylindrica*	144 h, growth NOEC	0.0031	[96]
Duckweed	*Lemna gibba*	7 days, wet weight EC50	>1000	[82]
Duckweed	*Lemna gibba*	7 days, frond number EC50	>1000	[82]
Duckweed	*Lemna gibba*	7 days, chlorophyll a EC50	>1000	[82]
Duckweed	*Lemna gibba*	7 days, chlorophyll b EC50	>1000	[82]
Duckweed	*Lemna gibba*	7 days, carotenoids EC50	>1000	[82]
Invertebrate	*Thamnocephalus platyurus*	24 h, survival LC50	17.68	[83]
Invertebrate	*Ceriodaphnia dubia*	48 h, immobilization EC50	10.23	[83]
Invertebrate	*Ceriodaphnia dubia*	7 days, population growth EC50	0.22	[83]
Invertebrate	*Daphnia magna*	24 h, immobilization EC50	22.45	[83]
Invertebrate	*Daphnia magna*	24 h, survival LC50	388	[76]
Fish	*Danio rerio*	96 h, NOEC	1,000	[83]
Fish	*Oryzias latipes*	96 h, survival LC50	>100	[97]
Fish	*Oryzias latipes*	10 days, hatchability NOEC	1000	[98]

Table 6. Acute and chronic effects of antibiotics to aquatic organisms. Adapted from Ji et al. [79].

Genotoxic and enzymatic effects on aquatic organisms exposed to ATBs were also observed by several authors. For example, Botelho et al. (submitted manuscript) reported genotoxic effects of oxytetracycline and florfenicol ATBs in concentrations found in the water of a major Brazilian reservoir where fish farming activity is practiced with *Oreochromis niloticus* fish species. In this study, DNA damage was observed using the comet test when exposed to concentrations of 425 and 4000 ng L^{-1} of florfenicol and oxytetracycline, respectively.

Oliveira et al. [98] observed the inhibition of catalase activity in adult brain and gills of *Danio rerio* fish when exposed to higher concentrations of amoxicillin (50 and 100 mg L^{-1}). There was also a tendency for the induction of glutathione S-transferase (GST) enzyme at all concentrations of the same ATB. In this same study, a dose-dependent catalase was observed in the brain of *D. rerio* adults after oxytetracycline exposure, while GST activity increased after exposure to concentrations higher than 1 mg L^{-1} of oxytetracycline in muscle and liver samples.

Most of the studies related to ATBs effects on aquatic organisms refer to acute effects (mainly lethality) in a short period of time. Note that in the aquatic environment, due to the phenomenon of dilution, the concentrations of chemicals in general, including ATBs, are found at the levels of μg L^{-1} and ng^{-1}. Thus, the observed effects will be chronic, i.e., at a considerably longer period than that observed for acute effects. Thus, in toxicity evaluations, especially to aquatic organisms, the use of environmentally relevant concentrations should be taken into account since this way the effects will be more realistic and will portray in a more real way what happens in the environment if such chemical agents are present.

Soil plays important roles in ecosystems since it is the basis of nutrients and the animal and plants habitat, in addition to functioning as an immense bioreactor, where the degradation of pollutants and nutrients transformation occurs. However, as already seen in this chapter, the soil may also be the final destination of ATBs used in veterinary medicine originating from manure and sewage mud used to fertilize vegetables [99] or from package disposal. Due to the ecological importance of soil for the ecosystem, it is important to know whether or not ATBs have negative effects on the fauna.

As shown earlier in this chapter, once in the soil, depending on the physical and chemical characteristics of the ATBs and the soil, they may follow different pathways, such as being leached or carried superficially by rain, contaminating aquatic environments (low K_d values) or persisting in the soil (high K_d values).

In general, the effects of ATBs to aquatic organisms are higher than those of soil fauna, and thus little is known about the toxicity of these drugs for these organisms. According to Ding and He [100], once in the soil, ATBs can change the structure of the microbial community because even to those which have a broad spectrum of action, selective effects on several microorganism groups may occur. As a result, the relative abundance of microorganisms is changed, interfering with the interactions between different species.

The sorption of pollutants in general in the soil is one of the major mechanisms controlling toxicity, by reducing its availability [101]. Thus, in toxicity studies with chemical agents, the choice of a molecule with low K_d is recommended. In addition, toxicity to organisms in the soil decreases over time due to transformations the molecule undergoes over time through less

toxic secondary compounds and due to tolerance of some soil microorganisms to ATBs [102], such as some *Pseudomonas* species, for example [103].

Girard et al. [104] studied the effects of ciprofloxacin ATB on soil microbial communities and observed a reduction in soil microbial activity in the first 25 days of experiments, when exposed to concentrations ranging from 0.2 to 20 mg kg^{-1}. According to the authors, this behavior is due to this molecule being bacteriostatic. From this result, according to the authors, ciprofloxacin could interfere with the recycling of nutrients in the soil. In Table 7, some studies that were conducted with oxytetracycline ATB for three organisms that live in the soil can be observed.

Antibiotic taxonomic group	Species	Test duration/end point	Concentration	References
Springtail	*Folsomia fimetaria*	21 days, survival NOEC	≥5,000[a]	[105]
Springtail	*Folsomia fimetaria*	21 days, reproduction NOEC	≥5,000[a]	[105]
Pot-worm	*Enchytraeus crypticus*	21 days, survival NOEC	3,000[a]	[105]
Pot-worm	*Enchytraeus crypticus*	21 days, reproduction NOEC	2,000[a]	[105]
Earthworm	*Aporrectodea caliginosa*	21 days, survival NOEC	≥5,000[a]	[105]
Earthworm	*Aporrectodea caliginosa*	21 days, reproduction NOEC	3,000[a]	[105]
Earthworm	*Aporrectodea caliginosa*	21 days, growth NOEC	3,000[a]	[105]
Earthworm	*Aporrectodea caliginosa*	21 days, hatchability NOEC	≥5,000[a]	[105]

Table 7. Chronic effect of oxytetracycline antibiotic on organisms that represent the soil fauna.

Generally, veterinary ATBs can suffer abiotic or biotic degradation on soil–water compartment. However, some degradation products have similar toxicity to the parent compound [106]. Degradation can be affected by environmental conditions, such as temperature, humidity, season, soil type, pH, and characteristics of the molecule, such as size, among others. With respect to the season, for example, in winter, the degradation half-life of ivermectin is six times higher than in summer, and degradation was faster in sandy soil than in sandy loam soil [107, 108].

5. Microbial resistance

One of the biggest problems related to the use of ATBs, in addition to those already discussed in this chapter, is the development of bacterial strains resistant to ATBs in the environment, mainly due to the continuing use of these drugs at low concentrations. Bacterial resistance arises and is maintained by mutations in the bacterial DNA or by horizontal gene transfer mechanisms, which include conjugation with other bacteria, transduction with the bacteriophage, and free DNA uptake via transformation.[59] In the case of continuous and prolonged use of sublethal concentrations and the subsequent elimination of feces in the soil, they could

cause the sharing of resistant plasmids to nonresistant organisms [18, 99]. Another possibility of occurrence of bacterial resistance is that low concentrations of ATB residues transferred to the soil by the application of contaminated animal manure favor the selection of resistant populations [109]. However, the direct introduction of resistant microorganisms derived from feces of animals treated with ATBs seems to be more important to resistance [99] than induction due to the presence of ATB residues on the environment.

It is important to remember that there is a large reservoir of ATBs-resistant bacterial genes in the soil. However, according to Schmidt e al. [110], it is not known whether this occurs naturally or due to the use of veterinary ATBs. As an example, in a study by Esiobu et al. [102], isolated bacteria of a garden soil fertilized with dairy cattle manure showed 70% resistance to ampicillin, penicillin, tetracycline, vancomycin, and streptomycin ATBs.

The exposure intensity of bacteria to ATBs agents influences the amplitude of its resistance, and the exposure intensity usually depends on the origin of the treatments by which bacteria were submitted. Costanzo et al. [111] indicated that bacteria from a sewage treatment plant reactor were resistant to ciprofloxacin, tetracycline, ampicillin, trimethoprim, erythromycin, and sulfamethoxazole antibiotics, while bacteria isolated from the effluent receiver watercourse showed resistance to erythromycin and ampicillin. This same study showed that erythromycin, clarithromycin, and amoxicillin ATBs, at a concentration of 1.000 $\mu g\ L^{-1}$, decreased more significantly the rate of bacterial denitrifying.

In aquaculture, the intensive use of ATBs provides a selective pressure for the creation of bacteria resistant to drugs and genes resistant to transmitted pathogens of fish and other bacteria in the aquatic environment. From these resistant bacteria, resistance genes can be spread by horizontal gene transfer and transfer to human pathogens. Drug-resistant pathogens present in the aquatic environment can directly reach humans. The horizontal gene transfer can occur in the aquaculture environment, in the food chain, or in the human intestinal tract. Among ATBs commonly used in aquaculture, several are classified by the World Health Organization (WHO) as extremely important for use in humans. The occurrence of ATBs resistance in human pathogens severely limits the therapeutic options in human infections. Taking into account the rapid growth and the importance of the aquaculture sector in many regions of the world, due to the widespread, intense and often irregular use of ATBs in this animal production area, efforts are necessary to prevent the development and spread of bacterial resistance in order to reduce the risk to human health [112].

Another issue in aquaculture regarding bacterial resistance needs to be highlighted, that is, if bacterial populations are resistant to a certain ATB used in this sector, or the producer changes the ATB or increases the dose in anticipation of a more efficient control. However, these two practices make such microorganisms to become resistant to this new applied molecule over time. Increasing the dose may also have negative effects on native aquatic biota of where the creation is installed.

In the study of Akinbowale et al. [113], isolated bacteria from water samples and organisms used in aquaculture showed widespread resistance to ampicillin, amoxicillin, cephalexin, and

erythromycin ATBs; frequent resistance to oxytetracycline, tetracycline, nalidixic acid, and representatives of the sulfonamide group; and infrequent resistance to florfenicol, chloramphenicol, ceftiofur, oxolinic acid, gentamicin, and trimethoprim. In another study performed on the Ilha Solteira Reservoir, São Paulo, where one of the largest and most important aquaculture parks in Brazil is located and where fish farming is intense, Monteiro [46] studied the bacterial resistance in Nile tilapia kidneys, which is a species cultivated in this place, and observed bacterial resistance to sulfonamides, quinolones, and tetracyclines. These two examples from cited studies confirm that both aquaculture products and water from aquaculture environments have risks of transferring ATBs-resistant bacteria to humans through these product consumption and contact with water, as mentioned in the previous paragraph. Remember that in the case of the Ilha Solteira Reservoir, this environment is an important aquaculture redoubt where the population uses its waters for water sports, in addition to fishing and fish consumption.

As a direct consequence of bacterial resistance, there is the increased frequency of ineffective treatments, increased severity of infections, prolonged duration of diseases, increased frequency of bloodstream infection, increased hospitalization, and increased mortality. The prolongation of diseases has been demonstrated in case-control studies of Campylobacter resistant to fluoroquinolones, and the increased severity of infections of *Salmonella typhimurium* resistant to quinolones was also demonstrated, as well as increased morbidity or mortality also assigned to nontyphoidal *Salmonella* serotypes and to Campylobacter [112].

Several studies have reported the occurrence of bacterial resistance in environmental compartments, such as in wastewater, groundwater, surface water, sediments, and soils [114–119].

As discussed so far, bacterial resistance is a threat to the effectiveness of ATBs in animal husbandry and to the health of the environment. Therefore, the prudent use of these molecules in all livestock sectors seems to be the solution to combat or reduce this problem.

6. Detection and quantification of antibiotics into the environment

6.1. Sample preparation techniques

The determination of antibiotics in environmental samples is a difficult task due to the high complexity of the analyzed matrices and the low concentrations of these compounds in the samples [120].

The sample preparation step affects all the other steps of the test and therefore is critical for unambiguous identification, confirmation, and quantification of antibiotics. It includes the isolation and/or preconcentration of interest compounds from the matrix and also properly provides the compounds for the separation and detection. Sample preparation takes typically more than 70% of total analysis time.

Chromatographic methods are usually preferred in the analysis of organic molecules, which causes the need to have an initial sample preparation, a process of extraction, which is normally a liquid–liquid extraction, followed by a clean-up process, which is usually a solid-phase extraction (SPE). Comparing the analysis by ultrafast chromatography with the conventional, sample pretreatment processes are more laborious and time consuming, as it requires an even purer extract. For this reason, many new sample preparation techniques have been developed, and there is a continuing interest in this area.

A quick search in the scientific literature showed that more than 1300 articles on analysis of antibiotic residues were published during the period of 2004–2015, and liquid extraction (LE) and liquid–solid extraction (LSE) were the most popular sample treatment techniques, which were used in 30% and 60% of the reported studies, respectively. The LE includes all techniques based on liquids, such as liquid–liquid extraction (LLE), liquid–liquid microextraction, and pressurized liquid extraction (PLE). LSE includes solid-phase extraction (SPE) and all other procedures based on extraction absorbers, such as solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), restricted access material (RAM), turbulent flow chromatography (TFC), dispersive solid-phase extraction (DSPE), and matrix solid-phase dispersion (MSPD). Other techniques for some specific applications are the microwave-assisted extraction (MAE), the ultrasound-assisted extraction (UAE), extraction based on immune affinity, and the technique that use molecular imprinted polymers (MIP).

There have been many changes in sample preparation with the advent of mass spectrometry. Previously, methods of analysis were able to analyze residues of only a limited number of compounds (usually a single class of drugs); but with mass spectrometry, now there is the possibility of residue analysis of many compounds in a single analysis. Although mass spectrometry allows the use of simple and generic cleaning methods, the effective removal of matrix constituents is necessary since these may affect the performance of the mass spectrometer (MS), in particular, by ion suppression.

There was also the migration of manual sample preparation to faster techniques with automated processes. The automated preparation of samples can be made online (with sample preparation directly connected to the chromatographic system) or offline (sample preparation is automated, but the sample has to be manually transferred to the chromatographic system).

Most analytical methods developed for the antibiotic determination in water use offline solid-phase extraction (SPE) and LC-MS/MS [121–127]. Some studies, however, point toward developing methods with the SPE-LC-MS/MS system, which allows reduction in the sample amount, lower preparation time, and consequently, increase in productivity, in addition to less sample manipulation, decreasing contamination chances [128–132]. The SPE-online system has also been used for the determination of pesticides, hormones, explosives, pharmaceuticals, and personal care products [133–137].

Among the advantages of SPE-online method, it is possible to highlight the small sample volume requirements, making it easier to transport and store. As in most cases, the sampling sites are too distant from laboratories. Several sample preparation steps, such as evaporation

and reconstitution, are eliminated, and there is less need for sample handling and processing, which lead to a reduction in analysis time and analyst's interference, minimizing errors, losses, and sample contamination, which is reflected in better method accuracy and precision values, in addition to a significant reduction in the consumption of organic solvent, contributing to the "green chemistry" [138, 139].

Besides these, the automated preparation of samples has the advantage of performing the clean-up, concentration, and separation of the compound in a closed system. This reduces the sample preparation time, and the whole sample becomes available for analysis, leading to a reduction in detection limits. It also decreases the analyst procedural errors, thereby improving accuracy and reproducibility. Moreover, in automated sample preparation, the cost is also reduced, using less solvent and less personnel. Other advantages include reduced risk of sample contamination and elimination of analyte disposal losses by evaporation or degradation during sample preconcentration.

Automatic methods also have some disadvantages, such as increase in initial capital expenditure and risk of increased service downtime due to equipment breakdowns, which require parallel processes to be made in order to reduce the laboratory inactivity.

For the determination of antibiotics in environmental, soil, sediment, and manure solid matrices, among others, different procedures are performed, which involve several techniques, such as accelerated solvent extraction (ASE), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). The solvent choosing is critical to ensure selectivity and minimize the extraction of other matrix constituents. Better diffusion of the solvent in the matrix interstices by mixing the sample and quartz sand is essential for best performance. The correct use of pH also increases extraction efficiency, and pH acids are generally more indicated because they favor electrostatic repulsion between antibiotics and sediment surface, which are both protonated [140].

Prior to antibiotic chromatographic determination, postextraction sample purification is often necessary to remove interfering (e.g., coextracted organic matter or organic solvent) and to achieve lower quantification limits. The adoption of this strategy leads to substantial improvement in method selectivity, where the cleaning is carried out in most cases by solid-phase extraction (SPE). Used adsorbents differ in composition, chemical properties, and affinity with the analyte [140].

Currently, EAU followed by filtration or centrifugation is the most common procedure. Yang et al. [57] developed a method with EAU using a mixture of acetonitrile and citrate buffer (50:50 v/v) placed in an ultrasound bath for 15 min, repeating this process three times. The extract was then purified by using SPE cartridges in series, SAX (6 mL, 500 mg), and HLB (6 mL, 200 mg); the procedure obtained good recoveries for 14 antibiotics studied in the sediment of Pearl rivers in Guangdong province, China, and was also used by Zhou et al. [141], which increased the number of analyzed antibiotics.

Antibiotic extraction from sediment and the transfer of these to an aqueous solution can provide the use of the SPE-online system, increasing the sensitivity of the method, facilitating the preparation of samples, and reducing purification steps, as shown by Monteiro [46] in his

PhD study. In this study, the procedure proposed by Yang et al. [57] was used, but the purification step using SPE with HLB cartridges was replaced by SPE-online system, in which a semipreparative precolumn with C8 adsorbent was used, obtaining optimal results for antibiotics of the class of tetracyclines, fluoroquinolones, sulfonamides, and phenicols in sediments collected from tank-nets fish farms.

As for biological samples, such as fish and aquatic plants, for example, one of the main problems for quantitative analysis of pharmaceuticals is that the analyte is typically bound to proteins and peptides, with the consequent need for cleavage of these structures before analysis. Enzymatic digestion is widely accepted as a sample preparation method for analyzing compounds in biological matrices. However, these methods are labor intensive and significantly prolong the examination time [142]. Most methodologies use extraction procedures based on liquid–liquid extraction, with relatively polar solvents and subsequent extraction purification using solid-phase cartridges [143, 144].

Another technique that has been highlighted for the extraction and purification of antibiotics in biological matrices is QuEChERS (quick, easy, cheap, effective, rugged, and safe), which was initially developed by Anastassiades et al. [145] for the determination of pesticide residues in food. It has been adapted and has been used for the determination of other compounds, including antibiotics in fish [54, 146].

The extraction procedure should be appropriate to the intended analysis and the reality of laboratory, so factors such as reagents consumption, availability of skilled labor force, and equipment acquisition are crucial. Simple and rapid methods stand out in this context because they are less dependent on high investments [147, 148].

6.2. Confirmatory and quantitative methods

For the determination of medicaments, different analytical methods are reported in literature, which are primarily valid for biological matrices, such as blood and tissue [148–150], and some modifications in these methods may be sufficient to environmental samples. However, residual drug analysis in WWTP effluents, rivers, subsoils, sediments, soil, and sludge waters still require the development of more sensitive methods for the detection of concentrations in $\mu g\ L^{-1}$ and $ng\ L^{-1}$ range.

Separations in environmental chemistry generally involve the two most recognized chromatographic techniques: high-performance liquid chromatography (HPLC) and gas chromatography (GC).

Knowledge of physical and chemical properties of the analytes is of utmost importance to avoid problems in quantification, which can be related to side reactions, impurities, or degradation in their structure during the analytical method application. For example, tetracycline antibiotics may irreversibly interact with residual metal ions present in sorbents of solid-phase extraction cartridges based on modified silica with alkyl groups (C18, C8, etc.), and certain metals can catalyze the ring opening of β-lactams. This problem can be solved by adding a chelating agent to the matrix to be extracted (Na_2EDTA, for example) or by replacing the cartridges sorbent by polymeric material [57].

The solubility of the analytes in the environmental sample and in the eluting solvent as well as in the mobile phase to be used also deserves attention. For example, some antibiotics form water-insoluble lipophilic complexes in the presence of alkali metal cations. Furthermore, penicillin patterns undergo methanolysis when solubilized in methanol, and should be prepared in acetonitrile or another compatible solvent [57].

During the detection step, when using a mass spectrometer (MS), there may be some sort of fragmentation that is characteristic of the analyte, depending on its pH range. For example, the erythromycin in acidic solution has a mass loss of 18 Da, which corresponds to the loss of a water molecule [57].

One of the critical parameters to be observed during antibiotic determination is referred to the sample pH, because in many cases, the medium pH determines the chemical form of the analyte in solution and thus interferes in the extraction efficiency. For example, the low recovery percentage of quinolones extraction process was improved after acidifying the solution in 2.5 pH. However, it is important to choose a pH range in which degradation of the analytes will not occur [57].

Furthermore, the pH of the mobile phase needs adjustment, in which its value depends on the pK_a of the compounds to be analyzed. The recommended buffer concentration is in the range between 2 and 20 mmol L^{-1} to avoid solubility problems in the mobile phase and to facilitate the ionization mode when using the MS detector [128].

The most commonly used stationary phases in HPLC for separation of organic compounds are of reverse phase (RP) type, which are silica based with C18 groups. Stationary phases with C8 groups may be used for β-blockers and antibiotics (tetracyclines, penicillins, sulfonamides, and macrolides) [128], [57].

The mobile phases used in the RP-HPLC are mixtures of methanol–water (MeOH:H_2O) or acetonitrile–water (ACN:H_2O) with adjustments of the chromatographic strength and mobile phase selectivity to the obtainment of enough resolution to occur the separation of all chromatographic peaks in minimum analysis time. The addition of modifiers, such as formic acid, ammonium acetate, ammonia, etc., is performed in order to favor the process of analytes ionization by medium pH adjustment, improving their interactions with the mobile phase and the stationary phase. Medium pH control may also be performed by using buffered mobile phases.

When the mass spectrometer detector (MS) and the electrospray ionization process (ESI) are used, modifiers may also be used in order to favor the process of analytes ionization.

6.3. Detectors for HPLC

Spectrophotometric absorption detectors in the ultraviolet range (UV) and by fluorescence were initially used in HPLC equipment for the analysis of compounds, which are absorbed in the ultraviolet region or are fluorescent. The UV detection was used for the determination of antibiotics from fluoroquinolones class in environmental matrices (hospital effluent) [149], and the fluorescence detection was used for the determination of antibiotics in water, sediment,

and fish farm plants samples since they are lower cost equipment comparing to HPLC-MS. However, the limits of detection values obtained for UV detectors are much higher, in the range of μg L^{-1} to ng L^{-1}, in comparison to the MS detector, that can achieve detection limits in the order of ng L^{-1} to pg L^{-1} when used in series (MS/MS), offering also the possibility of confirmation of the analyzed compounds. For environmental analyzes for the purpose of screening, with detection limits in the concentration range of μg L^{-1}, the UV detector can be optimally utilized, besides being used when the concentration of the analyte in the matrix is high, as is the case of the publications cited above [149, 151].

The wide use of HPLC-MS/MS in environmental chemistry is due to the fact that most USEPA official methods use this separation and detection mode [140] due to good limits of detection and the possibility of structural confirmation of the analyzed compounds, besides the robustness of the method.

The electrospray ionization process is the most used in the detection by mass spectrometry (MS) because it is a more versatile ionization form that works for analytes with median polarity to very polar and poorly volatile, as is the case for most drugs, or thermally labile analytes, such as certain antibiotics, when compared with the atmospheric pressure chemical ionization (APCI), which uses heating in the range of 300°C–400°C for analytes thermal desorption [57].

Several recent studies [152–155] used analytical methods based on HPLC separation with mass spectrometry detection in series with ESI ionization (HPLC-ESI-MS/MS) for the determination of antibiotics in aqueous matrices. This can be explained by the versatility of this ionization mode, which can be used for analytes with polarities ranging between medium and high, with better detectability by ESI.

The most used mass analyzers for analytes detection are the triple quadrupole (QqQ) for sequential mode (mass in series), the time of flight (TOF), and the ion trap. The TOF type analyzer was used in the determination of drugs (analgesics, antibiotics, β-blockers, and antiepileptics) in surface water, groundwater, and wastewater samples due to having higher detectability, linear dynamic range, and mass accuracy than triple quadrupole type analyzers (QqQ), although the best detection limits were found for the QqQ type analyzer [128].

Recently, the quadrupole time-of-flight hybrid analyzer type (Q-TOF) has been used for providing better resolution and detectability than the conventional quadrupole, thus being applied for identification and quantification of drugs unknown metabolites [57, 128, 139].

In recent years, capillary electrophoresis (CE) has become a popular technique because of its simplicity, high separation power, short analysis time, and low consumption of sample and solvents [138, 139]. Among different CE modes, micellar electrokinetic chromatography (MEKC) [140], which makes use of micellar solutions of ionic surfactants, has proved to be a very attractive technique for separating different medicament classes, including antibiotics, nonsteroidal and steroidal anti-inflammatory agents, and analgesics [141]. However, capillary electrophoresis with UV-Visible detection has not been applied for drug residue analysis in small parts levels per billion (μg L^{-1}) due to its lower intrinsic detectability [46].

6.4. Trends in liquid chromatography applied to the study of antibiotics

The trends in high-performance liquid chromatography applied to the study of antibiotics include the use of ultra-performance liquid chromatography (UHPLC), two-dimensional liquid chromatography (LC-LC), and hydrophilic interaction chromatography (HILIC).

The UHPLC is one of the latest advances in liquid chromatography, using stationary phases with smaller particle diameters (approximately 2μm) compared to those employed in HPLC. The use of these particles, in addition to high linear velocities of the mobile phase (MP), allows to reduce the analysis time while maintaining resolution and separation efficiency, providing less broad peaks (5–10 s), besides drastically reducing the analysis time to approximately 10 min or less [136].

An example of UHPLC applied to the study of antibiotics is the work done by Zhou et al. [156]. A robust and sensitive method with UHPLC-MS/MS was developed for the simultaneous determination of multiclass of antibiotic residues in several environmental matrices (surface water, pond wastewater, effluent, sediment, sludge, and manure). The analytical method applied SPE with HLB cartridges for water samples and ultrasound extraction for solid samples followed by cleanup using SAX-HLB cartridges. The method was successfully applied to the analysis of environmental samples collected from a WWTP of a swine farm. The detection of several antibiotics with high concentrations in the analyzed samples indicates that WWTP and animal farms are two major sources of antibiotic residues in the environment.

Two-dimensional liquid chromatography is a good alternative when performing analysis in complex samples, such as biological samples, for example, because with the increase of a dimension, there is also the increase of peak capacity, and subsequently the separation process is enhanced [157, 158]. A very interesting example of this technique was the method developed by Wang et al. [159], which analyzed 14 antibiotics in urine with two-dimensional liquid chromatography coupled with Q-TOF mass spectrometer detector, quadrupole time-of-flight hybrid analyzer, where detection limits of 0.04 to 1.99 ng mL^{-1} were obtained. The method was used to identify the antibiotics in urine of children, but it can be a great tool for use in environmental matrices, especially biological matrices such as fish, crustaceans, aquatic plants, and more.

The term HILIC was proposed by Alpert in 1990 as an acronym for "hydrophilic-interaction chromatography" for the separation of polar solutes. This technique has also been also called "hydrophilic-interaction liquid chromatography," and "aqueous normal phase." In a simple way, it can be said that HILIC is an HPLC form very similar to liquid chromatography "in normal phase," using a column in hydrophilic stationary phase ("normal"), but with an eluent comprising water, buffer, and a high concentration of organic solvent that is miscible with water (typical of a "reverse phase"). The elution order obtained in an HILIC system will be practically opposite to that obtained when employing the reversed phase (RP) mode. Retention is directly proportional to the polarity of the solute and inversely proportional to the polarity of the mobile phase [160].

The HILIC was applied to extract and quantify spectinomycin and lincomycin in manure supernatant liquid from swine and farmland erosion water treated with manure [161]. It was

also successfully applied in the determination of oxytetracycline in environmental waters [162], and the results showed that there was no irreversible adsorption of oxytetracycline in the stationary phase, which shows that the silica column may be used in the determination of tetracycline antibiotics in HILIC mode with no peaks distortion, providing a different understanding of what was previously found in the literature.

As prospects for the area of liquid chromatography applied to the study of antibiotics, the identification of metabolites and processing products as well as even lower detection ranges and quantification limits is highlighted.

HPLC is also closely linked to applied environmental legislation because today, for the vast majority of antibiotics, there is no maximum permissible concentration established by the legislation.

Studies on bioremediation and advanced processes of wastewater treatment, such as chemical and photochemical oxidation through advanced oxidation processes (AOP), ozonolysis, nanofiltration, reverse osmosis, membrane bioreactors, etc., are likely to use high-performance liquid chromatography as key tool in monitoring antibiotic degradation processes in artificial or natural environments.

7. Final remarks

Veterinary antibiotics have become an integral component in maintaining animal health, and although they have been used in large quantities for some decades, the existence of these substances in the environment has received little importance until recently. It is only in recent years that a more complex investigation of antibiotic substances has been undertaken in order to permit an assessment of the environmental risks they may pose.

This chapter showed that the fate of antibiotics in soil–water systems and their effects on plants, soil, and aquatic organisms have been heavily studied through chromatographic analysis and toxicity test with many species. The multianalyte methods using SPE, LC/MS, LC/MS/MS, ASE, and others described in the chapter have been used to show the occurrence and transport of antibiotics from their sources into the environment.

The environmental dissemination of antibiotic-resistant bacteria and their relationship with human health has also been the objective of this study and needs to be investigated in greater depths by health and regulatory bodies so that a compromise can be made when it comes to the prudent use of VAs and their risk to human health and the environment in general.

Acknowledgements

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2013/08143-5) and the National Council for Scientific and Technological Development (456720/2014-6) for the financial support.

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Chapter 6

Immunotoxicological Threats of Pollutants in Aquatic Invertebrates

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60216

Abstract

Immunology deals with the physiological activity of organisms to defend against pathogen and toxin invasion. Invertebrates residing in aquatic ecosystems often face toxicological threat arises from habitat pollution. The aquatic habitat of invertebrates is in the precarious risk of pollution caused by diverse groups of environmental toxins. Immunotoxins have been considered as a special group of pollutants capable of affecting the immunological profile of organisms. Invertebrates residing in water bear ecological, economical, medicinal, industrial, nutritional and biotechnological significance. Global aquatic bioresource is largely composed of invertebrates belonging to multiple Phyla. These organisms, including insects, snails, clams, mussels, crabs and sponges, are physiologically dependent on innate immunological response for defense against pathogen and environmental contaminants. External physicochemical barriers of invertebrates act as primary line of defen against toxin entry. Principal barriers have been identified as shell, tunic, test, carapace, mucus, etc., in diverse species. Toxin-induced morphological damage of specialized immunocytes of invertebrates has been reported. Toxin-induced shift in density, surface adhesion efficacy and aggregation of blood cells or haemocytes have been identified as major xenobiotic stress in invertebrates. Various environmental toxins are capable of initiating alteration in the innate phagocytic response and cytotoxicity of blood cells. Lysosomes of invertebrate haemocytes are functionally involved in intracellular destruction of environmental pathogens. Toxins like arsenic, pyrethroid pesticides, azadirachtin and washing soda were reported to increase the relative fragility of

lysosomal membranes of immunocytes. This often leads to impairment in the efficacy of invertebrates to destroy pathogen under the exposure of pollutants. Xenobiotics like pyrethroid pesticides have been recorded to affect apoptosis and necrosis of invertebrate immunocytes. Selected toxin-induced morphological damages of heart, gill, digestive gland, mantle and antennae may result in the overall impairment in homeostatic levels of invertebrates inhabiting the polluted environment. Global environment, in recent times, is under the serious threat of contamination by diverse chemical compounds of unknown or less known toxicity. A thorough ecotoxicological analysis at cellular and molecular levels needs to be carried out in invertebrates occupying the different realms of the planet in future.

Keywords: Pesticides, arsenic, washing soda, flow cytometry, respiratory burst, phagocytosis, nitric oxide, molluscs, crab, sponge

1. Introduction

Invertebrates constitute more than ninety-five per cent of the existing biodiversity of the world. They present an extreme range of variation in terms of their body architecture, adaptation, food preference, habitat preference, behaviour and physiological response against environmental stressors. Evolutionarily, they are an ancient group of animals which have survived an extreme range of environmental adversities and perturbations from the time of their origin. Invertebrates occupied diverse kinds of habitats including terrestrial, freshwater, marine and estuarine ecosystems. Aquatic invertebrates are usually distributed in the multiple spatial compartments of the water bodies from the surface to the bottom region called the benthic stratum. Aquatic invertebrates are also found in the conspicuous ecological areas of the mud–water interface and subsoil region at the floor of aquifers. Freshwater ponds of India serve as habitat for diverse aquatic organisms including members of Porifera, Mollusca and Arthropoda. Majority of the ecotoxicological research was carried out on *Eunapius carteri* (Porifera: Demospongiae: Spongillidae); *Bellamya bengalensis* (Mollusca: Gastropoda: Prosobranchia); *Pila globosa* (Mollusca: Gastropoda: Ampullariidae) and *Lamellidens marginalis* (Mollusca: Bivalvia: Eulamellibranchiata).

Aquatic invertebrates bear ecological, economical, nutritional and biotechnological significance and demand a special scientific attention. Many of them are the dietary source of nutrition for human and other organisms. Their importance as potential source of bioactive substances and pharmacologically active compounds cannot be denied. Current scientific reports indicate that the rapid and unrestricted contamination of freshwater and marine ecosystem by diverse pollutants poses serious ecotoxicological threat for the existence of invertebrates in their natural habitats. Various anthropogenic activities like habitat destruction and habitat contamination have been identified as the causative factors of dwindling of invertebrate species on earth. Continuous addition of various toxic chemical compounds into the global environment is a major environmental challenge encountered by human and other

http://dx.doi.org/10.5772/60216

organisms. Many of these environmental pollutants are of either industrial or agricultural origin and need a thorough toxicological screening in invertebrate models.

The overall evolutionary success and survival efficacy of invertebrates depended on several factors including the development of a highly advanced immunological system. Immunology deals with the typical strategies of biological defence against environmental toxins and pathogens. Components of immune system and their coherent functional attributes enabled the invertebrates to overcome the toxin-induced chemical stresses of the primitive and modern global environment (Figure 1). Many of the environmental pollutants of the current hydrosphere are less researched, with limited toxicological information. Invertebrates over a period of time are assumed to evolve novel and unique modes of immunological reactivities to defend against the toxic insults of environmental pollutants at the cellular and molecular levels. Aquatic invertebrates are relatively a neglected group of organisms with reference to their immunotoxicological status in a biounsafe environment. The principal immunotoxicological responsiveness of invertebrates centre around the reactivities of circulating immunocytes and selected effector organs like gill, digestive gland, intestine, labial palp, etc.

2. Immunotoxins and their impact on aquatic invertebrates

Immunotoxins are chemical compounds that can modulate the immune-related parameters and can adversely affect the biological and physiological functioning of the immune system of organisms including human. The freshwater ecosystem of India is being contaminated by diverse groups of xenobiotics of known and unknown chemistry. Ray et al. (2011) reported mineral acids, alkalis, detergents, metalloids and pesticides as major environmental contaminants of the Indian freshwater ecosystem [1]. Scientists reported [2] the toxic effects of pyrethroids, cypermethrin and fenvalerate in aquatic molluscan invertebrates and claimed them as immunotoxins for their adverse effect on the immune parameters of host animal. Toxicity of these pyrethroid pesticides on non-target aquatic invertebrates appears to be detrimental and can seriously affect their survival efficacy and reproductive success. Arsenic, another metalloid and a potent immunotoxin, bears toxic effects on aquatic invertebrates and can modulate the histopathology and immune parameters of freshwater mollusc [3] and estuarine mud crab [4]. Biopesticide azadirachtin has been recently identified as an immunotoxin due to its potential to affect the immunofunctioning of mussels. Moreover, during monsoon and flood, pesticide-laden agricultural runoff often contaminates the freshwater ecosystem and poses serious threat to its inhabitants. Washing soda, chemically known as anhydrous sodium carbonate, is an important aquatoxin that can alter the selected physiological parameters of diverse groups of invertebrates. Mukherjee et al. (2015b) reported the toxicity of washing soda in a freshwater sponge of India with reference to its phagocytic and cytotoxic status [5]. Altered functioning of the immune system may lead to opportunistic invasion of environmental pathogens and parasites into the body of host and increase the vulnerability of these biofilter species in polluted environment.

The nature and magnitude of immunotoxicity depend on multiple parameters including the concentration of toxin, type and span of exposure and route of entry to the host. Immunotoxins

are difficult to identify as they can cause a wide magnitude of adversity on the immune status of organisms. Immunotoxicology deals with the assessment of toxicological response in an organism by estimating the responsiveness and reactivity of its immunological parameters. Immunotoxicology of invertebrates, in recent times, has been gaining a special scientific attention for its efficacy in monitoring the health of environment – both aquatic and terrestrial. Accuracy and precision of selected toxicological responses of aquatic invertebrates enabled a few species to function as suitable biomonitoring agents of water pollution [6]. Several effective immunomarkers of aquatic pollution have recently been established in model invertebrates [7].

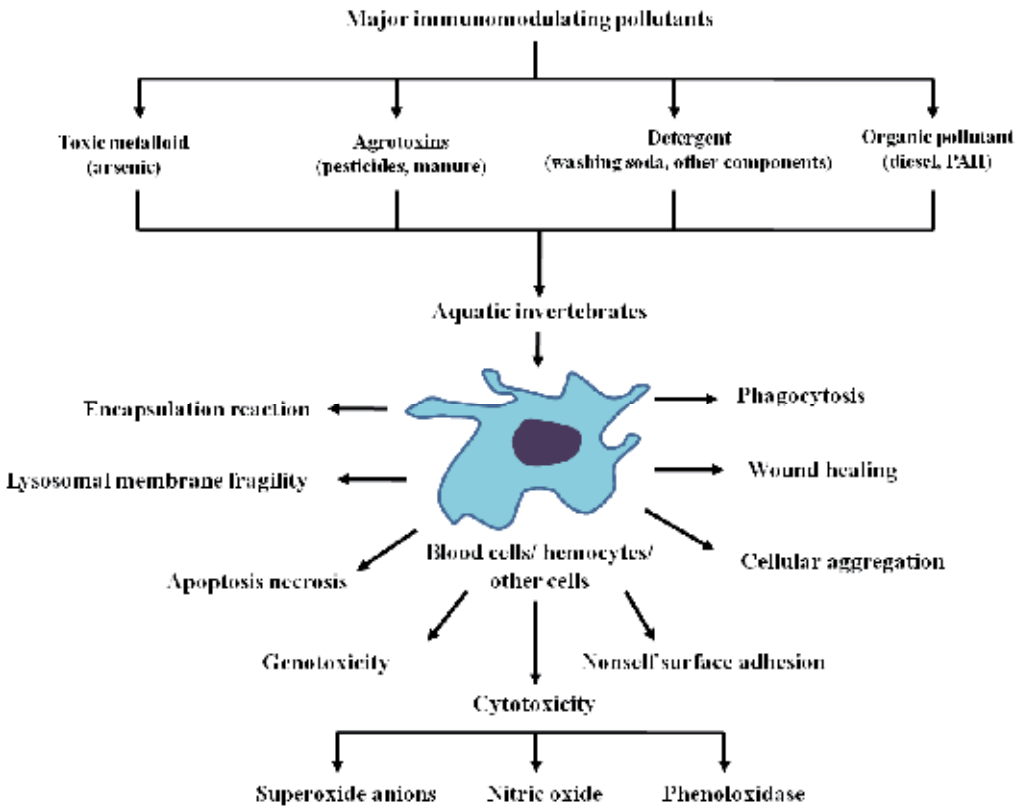

Figure 1. Attributes of cell-mediated immune responses of invertebrate immunocytes exposed to various aquatic pollutants.

3. External physicochemical barriers of invertebrates as first line of immune defence against pollutants

External physicochemical barriers of the invertebrates act as the first line of defence against the invasion of parasite, pathogen and toxin in a polluted aquatic environment. External

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physicochemical barriers of the invertebrates include shell, tunic, test, cuticle, carapace, pinacoderm and others. In mollusc, hard calcareous shell or valves provide the primary protection against entry of pathogens and toxins. However, in shell-less molluscs, thick external cuticular sheet known as mantle or pallium are assumed to play a significant physiological role against toxin and pathogen entry. Soft body wall of molluscs consists of cuticle, epidermis and muscles, which are thought to take an active part in the innate immune defence against environmental pathogens and toxins. In a polluted environment, where environmental pathogens and toxins are present in sufficient quantity, the two valves of the mussel are kept closed to minimize the entry of undesired agents. In addition to external shell, mucus secreted from the internal viscera provides another line of defence against the invasion of pathogens and toxins. Mucus acts as a protective barrier preventing the direct contact of toxins to epithelia. Secretion of mucus is reported to be an important detoxification and evasive mechanism of invertebrates. Pathogens are trapped within the mucus secreted by the organism leading to elimination. Calcareous shell made up of calcium carbonate and mucus is considered as external physiochemical barrier and plays an important role in the immune defence of aquatic invertebrates. Carapace, the external physiochemical barrier of crab provides the first line of defence in crustaceans. Corrosive toxins including mineral acids, alkalis, pesticides and detergents appear to be the potential threats of the invertebrate. Contamination of water bodies by these pollutants often results in breaches of the physicochemical barriers of external body surface, which leads to facilitation of invasion of toxic microorganisms and parasites into the viscera of target invertebrates. Components of innate immune response comprise physicochemical barrier against external pathogen and parasite entry. Cuticle covered with waxy material serves as a mechanical barrier of crab against parasitic infection. This chitinous exoskeleton or cuticle helps in the process of wound healing by preventing the fatal loss of haemolymph from body, maintenance of tissue architecture and prevention of opportunistic invasion of pathogen. Whenever this cuticle is damaged by injury or infection, the wound is rapidly sealed by clotting of immunocytes, preventing blood loss and pollutant entry. Once the clot is formed, wound is darkened and accumulation of melanin occurs. Melanin is reported to be involved in sealing of wound and synthesis of new cuticle. On the other hand, pinacoderm made up of flattened pinacocytes forms a continuous layer on the external surface of freshwater sponges and acts as a first line of defence against foreign invaders.

An experiment was performed to investigate the possible role of mucus as a primary barrier in removal of external particles. In this context, the shell of freshwater gastropod, *P. globosa* was drilled to create a micropore and a solution of cultured yeast (*Saccharomyces cerevisiae*) suspended in sterile phosphate buffered saline was injected into the body through a syringe (Figure 2). The mollusc was under scientific surveillance and the activity of the mollusc was photodocumented. After two hours of injection, hypersecretion of mucus was recorded by the experimental *P. globosa* within a time span of approximately thirty minutes. The expelled mucus contained more than ninety-five per cent of yeast particles, indicating the efficacy of mucus to act as external physicochemical barrier.

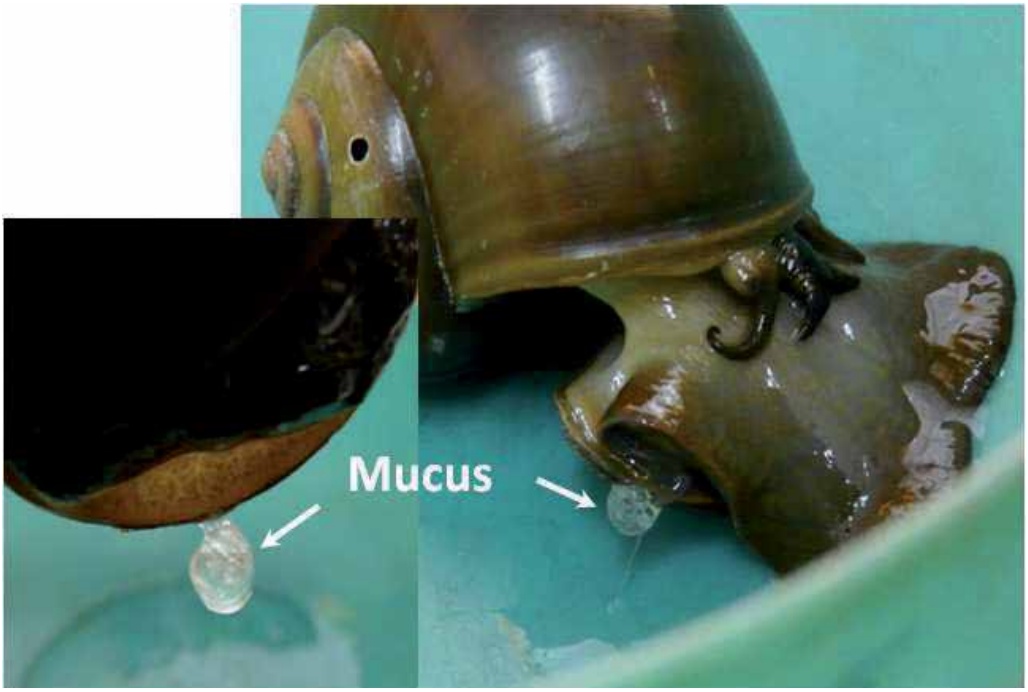

Figure 2. Rapid elimination of foreign particulates entrapped in the mucus of aquatic invertebrate, *P. globosa*.

4. Xenobiotic-induced shift in haemocyte density and morphological damages of blood cells

Haemocytes are the immunocompetent cells which are functionally responsive to various xenobiotics present in the aquatic environment. Homeostasis of total haemocyte density of aquatic invertebrates within the permissible physiological limit may be considered as an important immunological parameter [8] of cell-mediated immune response of molluscs [2]. Chakraborty et al. (2008) reported suppression in the total count of haemocytes of the fresh-water edible mollusc, *L. marginalis* under the sublethal exposure of sodium arsenite, an aquatic pollutant [9]. Mukherjee et al. (2006) reported modulation in the total cell density of the same specimen under the sublethal concentrations of azadirachtin, a neem-based pesticide, a common contaminant of pond water [10]. According to them, toxin-induced alteration in the total cell dynamics may lead to a gradual decline of this species in its natural habitat. The total count of haemocyte of *Villorita cyprinoides* was found to decrease under the exposure of copper [11]. Ray et al. (2013b) reported dynamics of the total haemocyte density of *B. bengalensis* and *L. marginalis* exposed to environmentally realistic sublethal concentrations of cypermethrin and fenvalerate, respectively [2]. Authors reported a significant increase in total haemocyte count following exposure to experimental concentrations of cypermethrin and fenvalerate to *B. bengalensis* and *L. marginalis*, respectively. Russo and Madec (2007) reported effect of

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pollutant fomesafen pesticide on the haemocyte density in the snail, *Lymnaea stagnalis* [12]. Authors reported the increase in total number of circulating haemocytes under the exposure of fomesafen as "facilitated cell turnover." The differential cell count, on the other hand, is also considered as an important immunotoxicological marker of environmental pollution [13]. Moreover, heterogenous populations of invertebrate blood cells have been reported to perform diverse physiological activities including nonself recognition, encapsulation and generation of cytotoxic molecules. Chakraborty et al. (2008) reported that haemocyte density can be considered as a potential biomarker of toxicity of sodium arsenite, a vital pollutant of the Sunderbans estuary of India [9]. Their experimental findings revealed the variation in the relative densities of granulocytes, agranulocytes, blast-like cells, hyalinocytes and asterocytes under the exposure of sodium arsenite. Ray et al. (2013b) reported a non-linear fluctuation in the differential cell density of *B. bengalensis* and *L. marginalis* following sublethal exposure of cypermethrin and fenvalerate, respectively [2]. According to them, aquatic pesticide-induced alteration in the differential haemocyte density was indicative to stress-induced loss of cell balance, which might alter the physiological homeostasis of these species distributed in pesticide-contaminated habitat. Qubella et al. (1993) proposed that fluctuation in the differential haemocyte density may be a result of reversible migration of haemocytes from tissues to haemolymph or *vice versa* [14].

Mukherjee et al. (2008) reported an increase in relative percentage of pro-haemocytes subpopulation in aquatic mollusc *L. marginalis* under the sublethal exposure of azadirachtin [15], a contaminant of water bodies. According to them, pesticide-induced depletion in the densities of granulocytes, agranulocytes, hyalinocytes and asterocytes was indicative to a possible impairment of haemocytic function of mussels distributed in polluted environment. Ray et al. (2013b) flow cytometrically identified (Figure 5a) five discrete subpopulations of haemocytes, that is, blast-like cells, round hyalinocytes, spindle hyalinocytes, round granulocytes, and granular asterocytes in the freshwater molluscs, *B. bengalensis* and *L. marginalis* [2]. Experimental exposure of 0.5 ppm of cypermethrin for 96 hours resulted in significant increase in the per cent populations of blast-like cells and round hyalinocytes and significant decrease in the subpopulation of granular asterocyte in *B. bengalensis* as compared to control. Exposure of 3 ppm of fenvalerate for 96 hours resulted in significant increase in the relative percentage of round granulocyte and significant decrease in the subpopulation of round hyalinocyte in *L. marginalis* as compared to control. Das et al. (2012) reported alteration of relative densities of haemocyte subpopulations of *L. marginalis* under sublethal exposures of cypermethrin [16]. Exposure to sublethal concentrations of cypermethrin yielded decrease in densities of agranulocytes and asterocytes and increase in densities of blast-like cells and granulocytes. Pyrethroids-induced alterations in the differential cell density of freshwater molluscs may lead to impairment of blood cell homeostasis in the experimental species distributed in polluted environment.

Morphological alterations of haemocytes under the exposure of environmental toxins play an important role in cell-mediated immune response of molluscs (Figure 3). Ray et al. (2013b) identified cytoplasmic hypervacuolation, rounding up of cell, alteration in cell shape, hypergranulation, increased cytoplasmic spreading, membrane disintegration and membrane

blebbing as principal aberrations in the haemocytes of *B. bengalensis* and *L. marginalis* under the exposure of pollutant like pyrethroid [2]. Cypermethrin- and fenvalerate-induced morphological damages in the circulating haemocytes of freshwater edible molluscs may lead to possible impairment of the functioning of cells and their immunological reactivity. Chakraborty and Ray (2009) reported dose- and time-dependent increase in the frequency of binucleated and micronucleated haemocytes and gill cells in the freshwater bivalve, *L. marginalis*, under the exposure of sodium arsenite [6]. Authors claimed arsenic-induced nuclear anomalies may be used as a biomarker of arsenic toxicity in freshwater ecosystems.

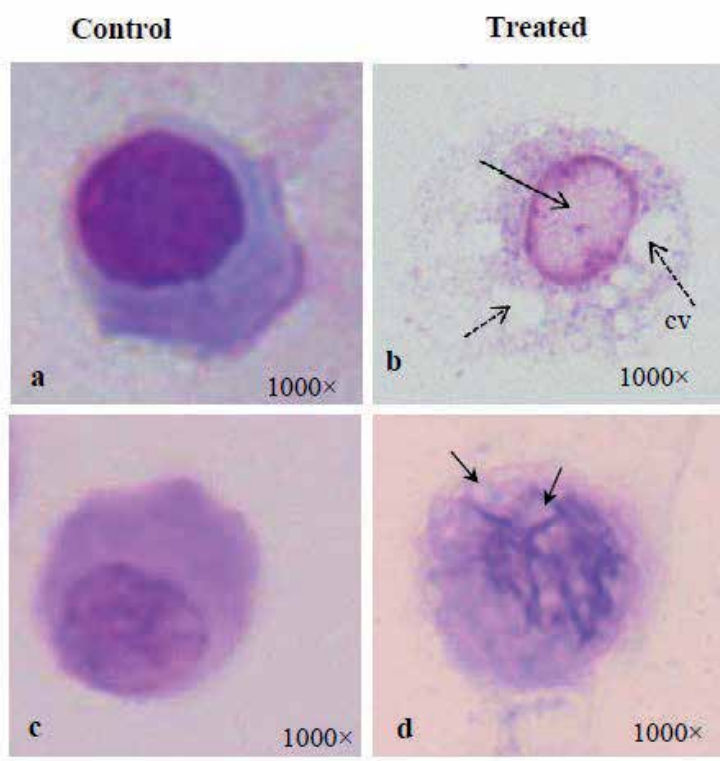

Figure 3. Photomicrographs of normal haemocytes of aquatic molluscs, *P. globosa* (a) and *L. marginalis* (c). Cypermethrin treatment (1.5 ppm/7 days) yielded intense cytoplasmic vacuolation (cv) (b) in the haemocytes of *P. globosa* and nuclear disintegration (d) in the haemocytes of *L. marginalis*.

5. Nonself surface adhesion and aggregation response of haemocytes

Nonself surface adhesion is considered as an important immunological mechanism which is fundamentally related to self–nonself discrimination. Cell adhesion and interaction between

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cell and the substratum play a pivotal role in the development, maintenance and immune recognition in multicellular animals. Mukherjee et al. (2007) studied the glass surface adhesion efficacy of haemocytes of *L. marginalis* under the sublethal concentrations of azadirachtin [17]. Authors reported a decrease in activity of surface adhesion property of haemocyte under prolonged exposure of pesticide. According to them, the data were indicative to impairment of immunological response of *L. marginalis* in its natural habitat leading to a possible dwindling of this biofilter species from aquatic ecosystem. Ghosh et al. (2008) studied the kinetics of nonself surface adhesion in the haemocytes of mud whelk, *Telescopium telescopium* exposed to diesel in the Sunderbans biosphere reserve [18]. Vehicle diesel is reported [19] to be a serious pollutant of Indian estuary. Authors reported diesel-induced inhibition in the nonself surface recognition efficacy and a shift in the kinetics of adhesion. Saha et al. (2008a) reported modulation in the nonself surface adhesion efficacy of haemocytes of edible mud crab, *Scylla serrata*, under the sublethal exposures of sodium arsenite [20]. Ray et al. (2012) reported arsenic-induced glass surface adhesion of haemocytes of juvenile mud crab [21]. According to them, arsenic-induced alteration in the reactivity of haemocyte may render the juvenile mud crab to become immunologically impaired in the parasite- and pathogen-contaminated natural habitat.

In the dynamic freshwater ecosystem, the inhabitants often compete for niche for their better survival and propagation. Overlapping in the niche leads to a state of acute predation and fighting among animals. As a result, the animals may encounter acute competition and subsequent physical damage and loss of body fluid. Cellular aggregation is a functional attribute offered by the haemocytes of invertebrates [22] to prevent the accidental blood loss by formation of biological plug at the site of wound and resist the entry of pathogenic microorganism. Selected pollutants like sodium arsenite, washing soda, pyrethroid, azadirachtin are reported to affect the aggregation response of many aquatic invertebrates (Figure 4). Hence, cell–cell aggregation is considered as an immunological response for host defence. Aggregation of haemocytes around invaded microorganisms is termed as "encapsulation response" and is considered as an important immunological response [23]. When successful encapsulation occurs, a host animal can restrict the proliferative and invasive property of a pathogen. Encapsulation reaction is mediated by specific population of immunoactive blood cells and is effective in cell-mediated immunity of invertebrates. Mukherjee et al. (2011) reported anticoagulant and carbohydrate-induced interference of cellular aggregation of mussel, *L. marginalis*, under experimental exposure of azadirachtin [24]. Persistent exposure of azadirachtin inhibited the cellular aggregation response in freshwater mussel. Workers apprehend such scenario in the natural environment may lead to a decline in the population of freshwater mussel and loss of freshwater biodiversity of India. Furthermore, a drastic increase in the occurrence of free cells was recorded against ethylene diamine tetraacetic acid and manure treatment, which was suggestive to possible role of these chemical agents as inhibitor of cellular aggregation. Ray et al. (2012) reported sodium-arsenite-induced inhibition in aggregation of haemocytes of juvenile mud crab, *S. serrata* [21]. According to them, arsenic-induced alteration of immune status may impart a state of vulnerability in juvenile crab inhabiting the arsenic-polluted environment.

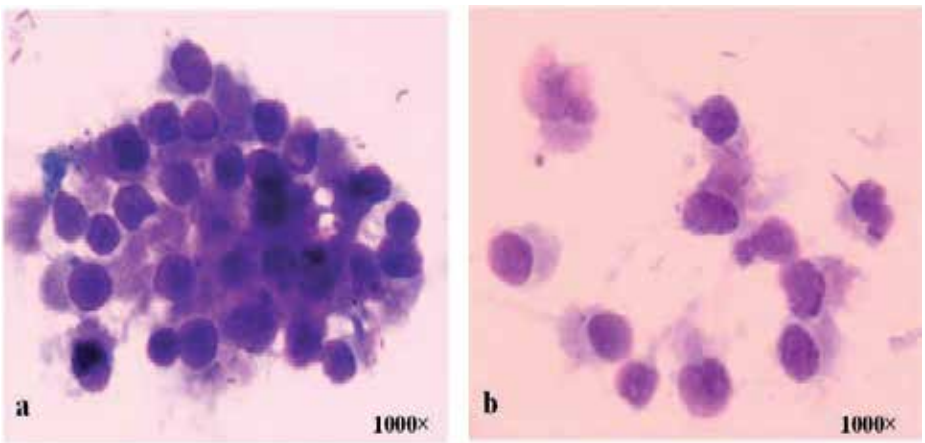

Figure 4. Typical aggregation response of haemocytes of a freshwater gastropod, *Pila globosa* (a). Cypermethrin is a contaminant of the habitat of *P. globosa* which inhibits the degree of haemocyte aggregation (b).

6. Phagocytic response in the face of environmental stressors

Phagocytosis, in general, is considered a classical innate immune response reported in the majority of the invertebrate Phyla. It is an established immunological response and is considered as a biomarker of aquatic pollution [13]. Phagocytic response enables invertebrates to combat against invading pathogens and pollutants of known and unknown chemistry. Haemocyte-mediated phagocytosis of nonself particles provides natural immunity in the bivalves [25]. Chakraborty et al. (2009) reported the inhibitory effect of sodium arsenite on the phagocytic response of *L. marginalis* under the challenge of yeast at various sublethal concentrations [7]. Inhibition in phagocytic response was also recorded under similar laboratory condition for the haemocytes of arsenic-treated *L. marginalis* when challenged with human red blood corpuscles [26]. According to them, impairment in the phagocytic potential of the haemocytes of arsenic-treated mussels may lead to compromisation of the immune status of the animals distributed in the contaminated habitat. Mukherjee et al. (2011) reported azadirachtin-induced suppression in the phagocytic potential of haemocytes of *L. marginalis* under the experimental challenge of charcoal particulate [27]. Azadirachtin-induced inhibition in the phagocytic response indicated a state of immune suppression of mussels. Ray et al. (2012) screened the immunotoxicological reactivity of haemocytes of juvenile mud crab, *S. serrata* of the Sunderbans biosphere reserve and reported sodium-arsenite-induced inhibition in the phagocytic potential under the challenge of yeast [21]. Arsenic-induced altered reactivity of haemocytes may affect the propagation and survival of mud crab population by increasing its vulnerability to higher rate of disease and parasite attack. Sponges, on the other hand, are non-selective filter feeders which depend on phagocytosis for the purposes of feeding and digestion (Figure 5b, c). Phagocytosis, in sponge, plays a dual physiological attribute in the form of food procurement and innate immune response [28]. Mukherjee et al. (2015b) reported washing-

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soda-induced inhibition in the phagocytic potential of cells of freshwater sponge, *E. carteri*, under the challenge of yeast [5]. According to them, decrease in phagocytic potential is suggestive to a possible impairment of both food capture efficiency and innate immune status, leading to suppression of immunological status of *E. carteri* distributed in detergent-contaminated natural habitat.

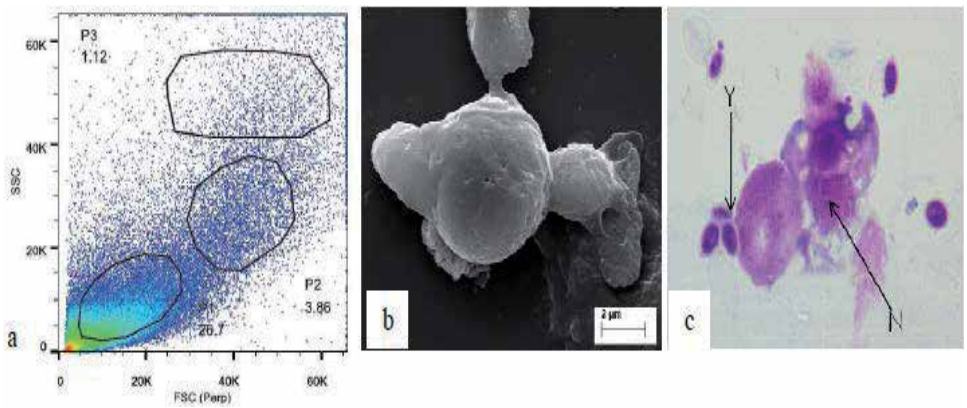

Figure 5. Flow cytometry of isolated cells of sponge, *E. carteri*, depicting their relative size and granularity (a). Phagocytosis of yeasts (Y) by the cells of *E. carteri* (b, c) exposed to washing soda. N = Nucleus.

7. Cytotoxicity of blood cells as an effective immune strategy

Cytotoxic molecules generated by the immunocompetent cells of invertebrates are reported to play an important role in the destruction and deactivation of foreign engulfed pathogens [29]. Authors reported superoxide anion, nitric oxide and phenoloxidase as established cytotoxic molecules of freshwater molluscs affected by various environmental pollutants. Contamination of natural habitat by toxic metals, metalloids, pesticides and washing soda results in a significant alteration in the cytotoxic status of invertebrates. Cytotoxic molecules are considered as an effective component of innate immune defence of invertebrates distributed in polluted environment. Nappi and Ottaviani (2000) reported nitric oxide and superoxide anions as potential "killing agents" of invertebrates [30]. Generation of reactive oxygen intermediate and reactive nitrogen intermediate by the phagocytic cells are mediated by NADPH oxidase and nitric oxide synthase (NOS), respectively. According to them, generation of superoxide anions can be correlated with increased respiratory burst activity in phagocytic cells. Mukherjee et al. (2012) reported a dose-dependent increase in the generation of superoxide anion in the cells of freshwater edible bivalve, *L. marginalis* under the exposure of azadirachtin, a neem-based pesticide [31]. According to them, this response of haemocytes may be considered as cellular stress under the sublethal and environmentally realistic concentrations of azadirachtin. Nitric oxide is considered as a signalling molecule generated in the biological system by the immunocytes of invertebrates as a potent cytotoxic agent for killing of invading microor-

ganisms. It is generated during the conversion of L-arginine to L-citrulline by nitric oxide synthase. Saha et al. (2008b) reported sodium-arsenite-induced increase in the generation of the intracellular nitric oxide in estuarine mud crab, *S. serrata* [32]. According to them, dose-dependent increase in the generation of nitric oxide may be considered as a biomarker of arsenic pollution in the Sunderbans delta of India. Chakraborty et al. (2009) reported inhibition in generation of nitric oxide in *L. marginalis* under prolonged exposure of arsenic [7]. Authors claimed this studied parameter as possible biomarker of arsenic toxicity in aquatic environment. Phenoloxidase, on the other hand, is considered as another cytotoxic molecule and is involved in the process of nonself recognition, phagocytosis and melanisation. The production of toxic quinoid derivative by phenoloxidase is an early step of biosynthesis of melanin for host defence. Chakraborty et al. (2010a) reported depletion in generation of cytotoxic molecule like nitric oxide and activity of phenoloxidase in the gill of freshwater bivalve, *L. marginalis*, under the sublethal exposures of sodium arsenite [33]. According to them, sodium-arsenite-induced alteration in the cytotoxic status in gill indicated a state of immunocompromisation in the animal. Chakraborty et al. (2013) reported inhibition in the generation of superoxide anions and nitric oxide and activity of phenoloxidase in the digestive gland of the same experimental bivalve under prolonged exposure of arsenic [34]. Generation of cytotoxic molecules like superoxide anions and nitric oxide and activity of phenoloxidase are important immunological responses offered by the cells of sponge under the exposure of pathogens and toxins [5]. Authors reported washing-soda-induced alteration in the generation of superoxide anion, nitric oxide and activity of phenoloxidase in the dissociated cells of freshwater sponge, *E. carteri.* Washing-soda-induced alteration in cytotoxic response of *E. carteri* may lead to an undesirable shift in the immune status of the animal distributed in detergent contaminated natural habitat.

8. Lysosomal membrane stability and activity of phosphatases

Lysosome is an important subcellular organelle involved in the process of degradation of foreign engulfed particulate. After phagocytosis, the phagocytic vacuole with engulfed foreign particles fuses with lysosome to form phagolysosome. As lysosome plays an important role in secretion of various digesting enzymes, maintenance of lysosomal membrane integrity has been gaining special scientific attention from the immunological point of view. Chakraborty and Ray (2009) reported impairment of lysosomal membrane integrity by neutral red retention assay in the haemocytes of *L. marginalis* exposed to sodium arsenite [6]. According to them, arsenic-induced fragility of lysosomal membrane may lead to leakage of lysosomal enzymes into the cytosol and subsequent destruction of the adjoining self cell or tissue. Ray et al. (2013b) reported lysosomal membrane integrity of haemocytes of *B. bengalensis* and *L. marginalis* under the experimental exposures of cypermethrin and fenvalerate, respectively [2]. Authors reported pyrethroid-induced lysosomal membrane fragility of haemocytes suggesting substantial damage and destabilization of lysosomal membrane (Figure 6). Screening of lysosomal membrane integrity of molluscan haemocytes by neutral red retention assay is considered as a possible biological marking of arsenic toxicity [6] and as an early warning tool

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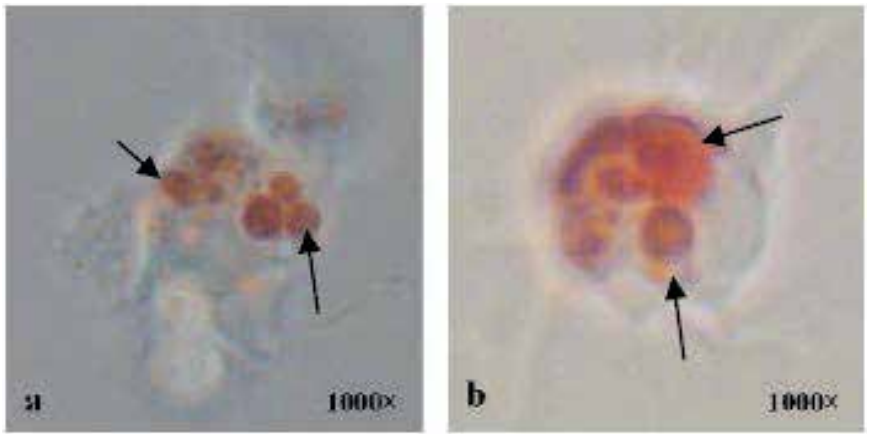

Figure 6. Cypermethrin-induced fragility of lysosome membrane of haemocytes of aquatic mollusc as evident from neutral red dye retention assay. Dye neutral red concentrated in the lysosomal compartments at zero minute of the assay (a). Complete diffusion of neutral red in the cytoplasm of cypermethrin-exposed haemocytes has been designated as "end point" of the assay (b).

of environmental pollution [35]. Phosphatases are principal lysosomal enzymes which are involved in pathogen destruction and are reported as markers of environmental stress [36]. Chakraborty et al. (2010a) reported suppression in the activity of hydrolytic enzymes, acid and alkaline phosphatases in the gill of freshwater edible bivalve, *L. marginalis* under the exposure of sodium arsenite [33]. According to them, inhibition in the activity of these enzymes might cripple the immune status and nutrient mobility in the gill of *L. marginalis.* Chakraborty et al. (2013) reported inhibition in the activity of phosphatases in the haemocyte and digestive tissue of edible bivalve under similar toxic insult by environmental arsenic [34]. Workers apprehend a state of immunocompromisation of the organisms under persistent exposure of arsenic. Saha et al. (2009) reported inhibition in the activity of phosphatases in the haemocytes of estuarine mud crab, *S. serrata* under the exposure of arsenic [37]. According to them, this situation in the natural environment might result in impairment of immunological activity and opportunistic invasion of parasite and pathogen into the body of the organism.

9. Pollutant-induced apoptosis and necrosis of invertebrate immunocytes

Apoptosis, the programmed cell death, is functionally involved in developmental and immunological processes of organisms. Moreover, apoptosis helps in the process of removal of damaged cells and thus is considered an important machinery of survival of host animal in polluted environment. It is involved in specific cell signaling mechanism, which is yet to be studied in invertebrates in detail. However, pesticides and other environmental xenobiotics are reported to affect the apoptotic and necrotic pathways of the immunoactive cells of invertebrates (Figure 7). Current scientific reports suggest apoptosis as an effective biomarker of aquatic pollution. Apoptosis presented changes in the morphological characteristics of cells including membrane blebbing, nuclear condensation, cytoplasmic shrinkage and membrane

asymmetry. Kiss (2010) reported the translocation of phosphatidylserine from inner leaflet of the plasma membrane to the outer leaftet, which was considered a hallmark of apoptosis [38]. Ray et al. (2013b) reported the apoptotic and necrotic cell deaths of haemocytes of *B. bengalensis* and *L. marginalis* under the sublethal exposures of cypermethrin and fenvalerate, respectively, employing flow cytometry [2]. According to them, cypermethrin and fenvalerate treatment yielded decrease in the percentage of apoptotic and necrotic haemocyte morphotypes of *B. bengalensis* and *L. marginalis.* Pyrethroid-induced apoptosis of molluscan haemocytes is considered as impairment of immunological status of *B. bengalensis* and *L. marginalis.*

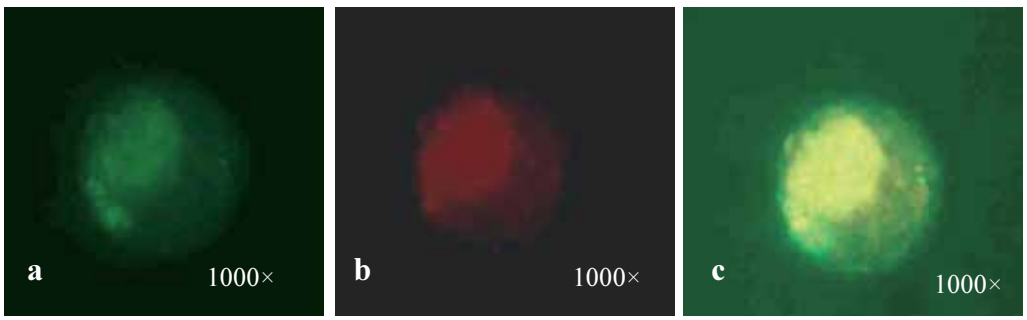

Figure 7. Immunofluorescent detection of apoptosis of haemocytes of pond snail, *B. bengalensis* exposed to sublethal (3000 ppm/7 days) concentrations of washing soda. (a) and (b) represent positive staining by Annexin V-FITC and propidium iodide, respectively; (c) depicts co-localization of fluorescent signals.

10. Toxin-induced damage of target organs and tissues

For filter feeding aquatic organisms, the gill, heart, digestive gland, mantle and antennae act as major target organs of common environmental xenobiotics (Figure 8). Mollusca bear a well-developed gill which serves as an organ of gaseous exchange, filter feeding and immunosurveillance. Molluscan gill is a thin membranous and vascularized organ that is in continuous contact with the environmental toxicants distributed in water. During the process of respiration and filter feeding, gill is exposed to various toxins of known and unknown chemistry. Exposure of gill to aquatic sodium arsenite, pyrethroid pesticide and washing soda of pond water yields a structural damage of lamellae. Chakraborty et al. (2010a) reported hyperchromatic anaplastic cells in the gills of *L. marginalis* exposed to sublethal concentrations of arsenic [33]. This is often associated with tissue rupture and formation of dense fibrosis. The heart, in general, acts as an organ of body fluid pumping. Exposure of cypermethrin results in severe histopathological damage of heart of mollusc. Similar kind of morphological damage was recorded due to exposure of aquatic mollusc to washing soda, a common pollutant of pond water. Digestive gland of mollusc bears immense physiological significance. The organ acts as an important site of diverse metabolic activities and biochemical detoxification. Exposure of sodium arsenite yields histopathological damage of the digestive gland of *L. marginalis* [34]. Toxin-induced morphological alteration of digestive gland of mollusc includes hyperinfiltration of haemocytes, vacuolation and inflammatory lysis.

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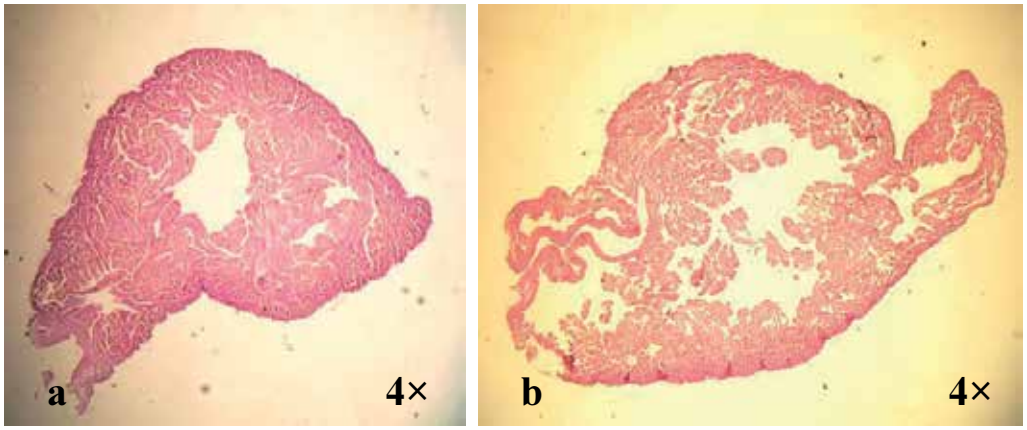

Figure 8. Cypermethrin (1.5 ppm/7 days)-induced histopathological damage of heart of mollusc, *P. globosa*.

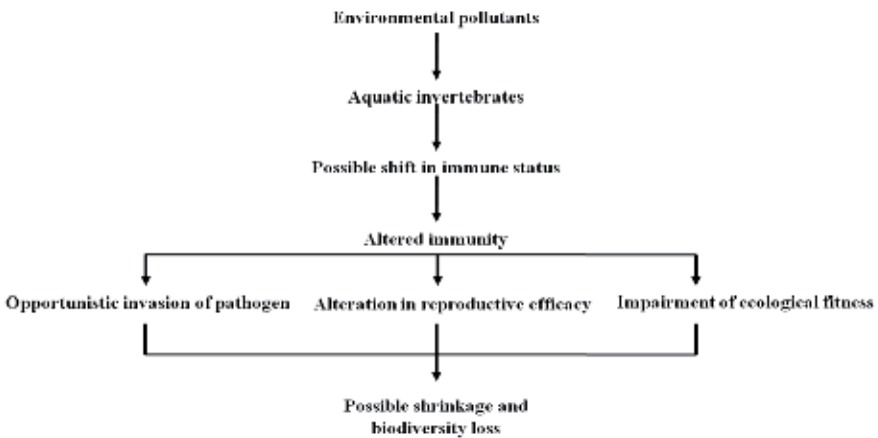

Figure 9. Ecophysiological consequences of environmental immunotoxins on aquatic invertebrates.

11. Discussion

Global environment in recent times is characterised by the presence of various xenobiotics of known, less known and unknown toxicity and chemistry. Information of immunological attributes of chemical compounds in aquatic invertebrates is limited in the current scientific literature. Limited but significant reports indicate a substantial impairment of the immunological status of invertebrates under the exposure of selected ecotoxins (Figure 9). Acute, subchronic and chronic exposure of common toxins like pesticides, arsenic and alkaline washing soda cause severe damage in the morphological and functional profiles of haemocytes, the chief immunoeffector cells of invertebrates and other organs and tissues. Sponges,

in general, are devoid of well-developed organ system. A variety of specialized cells of sponges are functionally involved in various immunological activities. Exposure of immunotoxins like washing soda largely affected the density dynamics of sponge cells as well as the cytotoxic and phagocytic status in freshwater sponge. Invertebrates of freshwater ecosystem act as important economical resource for developed and developing countries. A thorough toxicological analysis of the functional performance of target cells and tissues of invertebrates needs to be carried out for the purpose of conservation and culture of this species in their natural environment.

Acknowledgements

Authors gratefully acknowledge the scientific contributions of Suman Mukherjee, Sudipta Chakraborty and Sanjib Saha – the former members of the Aquatic Toxicology Laboratory. CRNN of University of Calcutta, UGC SAP DRS II and DST INSPIRE are acknowledged for financial and analytical support as well.

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Chapter 7

Endocrine Disrupting Compounds – Problems and Challenges

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60410

Abstract

In this chapter, information about some of the estrogenic compounds and their environmental fate and biological influence can be found. Special attention is paid to the review of the analytical approaches used at the stages of detection and determination of Endocrine Disrupting Compounds (EDCs) in the environmental samples. Also, a brief characterization of both cellular and non-cellular bioassays is presented.

The discovery of micropollutants occurring in the environment resulted in new methodologies being put into the analytical practice. These methodologies are developed in two different directions. The first is based on methodological solutions designed to detect, identify, and determine xenobiotics that occur in various environmental samples. For this purpose, instrumental methods such as gas and liquid chromatography with mass spectrometry detection are usually used. The second approach is to put into the analytical practice the new bioanalytical methodologies. These methodologies allow the estimation of the sample endocrine potential, but they do not provide the information about which of the sample ingredient is responsible for causing the toxic effect. These results can be the basis for estimating the endocrine potential of the environment exhibited by certain species. Moreover, bioanalytical techniques may be supplementary to the techniques of quantitative and qualitative determination of EDCs.

Keywords: Endocrine Disrupting Compounds, xenohormones, trace organic pollutants, EDC milestones, biotests

1. Introduction

The development of new technologies, progressive urbanization, increasing consumerism, and industrial boom in developing countries has led to elevated pollution of the environment. The broad spectrum of pollutants produced and released to the environment has increased in the last few decades, including the agricultural, industrial, pharmaceutical, and plastic industries. These chemicals can be found in the individual elements of the environment, both living (biota) and non-living. Chemists very often pay attention only to chemical compounds, which are treated as substances foreign to the average chemical composition of individual elements of the environment or occur at levels higher than the so-called mean composition. However, attention should also be paid to legal aspects connected with the presence of specific pollutants in the individual elements of the environment, which are often defined as xenobiotics. Environmental research includes a broader spectrum of chemical individuals – xenobiotics – that need to be detected, identified, and determined. They can be divided into [1]:

- compounds that are already subjected to legal regulations because their physicochemical properties, as well as immediate and distant toxic effects (as a result of ecotoxicological tests), and appropriate methodologies are already available and it is possible to obtain reliable information about changes in the content of these analytes in various types of environmental samples. Thus, it was possible to propose appropriate standards defining the highest concentration of a given xenobiotic in a defined environmental element. These normative values are called the ***M**aximum **A**dmissible **C**oncentration* ***(MAC)*** in European countries and the ***T**hreshold **L**imit **V**alue* ***(TLV)*** in the United States [2, 3];
- compounds that are not subjected to legal regulations yet. This group includes xenobiotics detected in the environment because new analytical methodologies were introduced into the analytical practice, which make it possible to detect and determine analytes occurring in tested environmental samples at very low levels (so-called micropollutants). It is said that the determined compounds have been so far called **unidentified pollutants**. The group of pollutants, which are not subjected to legal regulations, includes the so-called newly emerging pollutants [4]. These compounds are introduced into the individual elements of the environment as a result of new manifestations of human pressure, e.g. a new technology of manufacturing products or consumer goods. As a result, it has not been possible yet to define the ecotoxicological properties or develop and validate appropriate analytical procedures, which could make it possible to obtain reliable information about the levels of these compounds in various environmental samples. The occurrence of some specific micropollutants (EDCs), which are not subjected to legal regulation yet, has become more and more concerning in the last years [5].

The examples of the EDC groups, whose presence in the environment are both regulated and non-regulated by legal aspects, are presented in the Figure 1.

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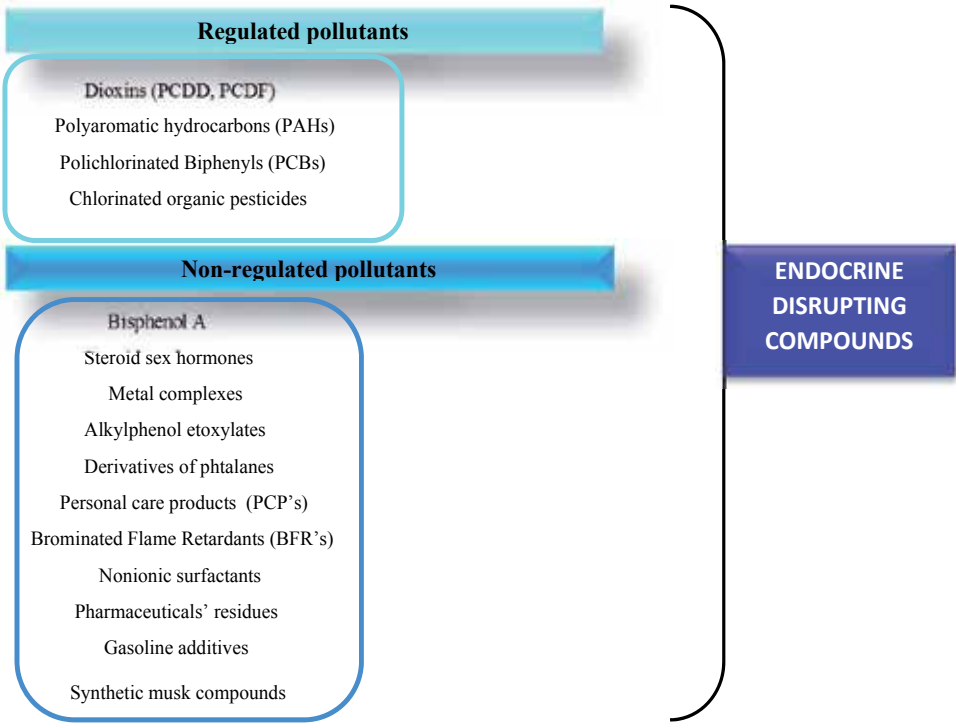

Figure 1. Groups of EDC pollutants subjected/not-subjected to legal regulations.

1.1. The basics of hormonal regulation

To understand endocrine disruption, the basic features and mechanisms of the endocrine system must be explained. The endocrine system consists of a number of ductless glands that secrete hormones directly into the circulatory system (to the blood) in order to regulate various functions of the body. In turn, a hormone is called a special signaling molecule that is produced by an endocrine gland. Hormone molecules travel through the blood to target distant cells and tissues to regulate physiological functions and behavior [6].

The endocrine system is made up by the following glands:

- the pituitary gland at the base of the brain;
- the thyroid gland in the neck;
- the adrenal glands in the abdomen next to the kidneys;
- the gonads (ovaries and testes) and certain parts of the pancreas;
- the parathyroid gland;
- the thymus.

Next to these specialized endocrine glands, many other organs and tissues have secondary endocrine functions and secrete hormones (e.g. heart, adipose tissue, muscle, liver, kidneys) [7, 8]. Just to make a story short, every system of internal secretion glands, hormones, and final organs sensitive to given stressors can be named the hormonal system. With such systems, the organism can reach and hold the homeostasis.

Hormones produce effects by acting on specialized proteins called receptors that attract and bind to specific hormones. Hormone receptors provide specificity to hormone actions, both in terms of the time and the place of hormone action. A receptor proteins superfamily consists of glucocorticoid, mineralocorticoid, androgen, estrogen, progesterone, retinoic acid, vitamin D, and thyroid receptors. Binding with ligand (antagonist or agonist) *in vivo* causes conformational changes, dimerization, and binding to a specific DNA sequence responding to characteristic receptor (see Figure 2) [1].

The target of the hormonal system is the activity of estrogenic, androgenic, thyroid, and glucocorticoids hormones. Classification of the hormones based on their structural properties is presented in Table 1. The modes of action of specific signaling systems are summarized in Figure 2 [1].

1.2. Endocrine disrupting compounds as xenohormones

EDCs are chemicals responsible for the occurrence of disturbances in the hormonal balance of the organism. This group includes both egzogenic and endogenic substances or their mixtures that impact the function of the natural hormones in the organism [13]. Taking into account complexity and importance of hormones played in organisms functioning, it must be stated that endocrine chemicals have versatile and almost unlimited cells at low concentration levels. EDCs, also called xenohormones, disturb natural hormonal balance by modifying the functioning of the hormonal system in numerous ways [13-15]. Below are specified selected processes through which EDCs may influence human beings [8, 13, 16]:

- modifying hormones synthesis pathways;
- hormones excretion mechanisms;
- cell/tissue transport of hormones in the organism;
- binding to receptors;
- hormones degradation pathways.
- EDCs have also imprinted in the specific mechanisms of modifying organisms functioning, just to mention [15]:
- mimicking the endogenous hormones' functioning;
- antagonism with synthesis of natural hormones or their metabolism;
- changes of level or activity of the hormonal receptors.
- EDCs can be assigned to one of two groups [17]:

- natural endocrine chemicals;
- chemicals emitted to the environment as a result of anthropopression.

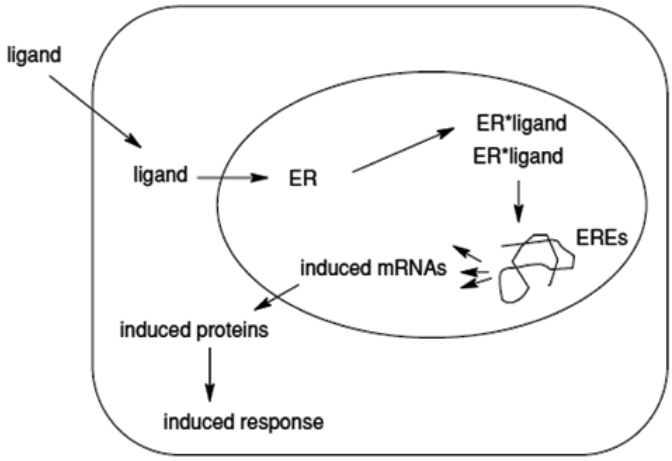

Figure 2. Mode of hormonal action in target receptor.

Type	Characteristics
Steroid hormones	They have lipophilic characteristics and contain fragments similar to cholesterol. These belong mostly in sex hormones such as estrogens, androgens, and progesterone. Both males and females produce all these hormones, but in different quantities.
Amino acids' derivatives	They have hydrophilic characteristics and are stored in endocrine cells until the moment it needs to be released. They connect with specific surface receptors and activate secondary signaling factors. Epinephrine is an example of such hormone.
Polypeptides	They contain amino acids varying from few to over 200 residues. These are water-soluble hormones such as insulin, growth hormone, prolactine and are stored in endocrine cells until they are needed, e.g., during metabolic regulations, lactation, growth, breeding.

Table 1. Division of the hormones based on their structural properties [1, 9-12].

1.2.1. A brief history of EDCs discovery

The first evidences of endocrine disruption in nature have been observed since the 1950s, but the source of the occuring phenomena was not known yet. Figure 3 shows the most important milestones in the development of the knowledge about endocrine disrupting micropollutants.

Currently, the studies on EDCs are spreading in all branches of science including analytical chemistry, toxicology, chemometrics (data treatment), modeling, chemical processing, *etc*. [30]. Their aim is to predict the environmental fate of these chemicals, EDCs' potential to cause observable deformations, identify newly emerging pollutants, and assess the efficiency of

novel sozotechnical methods being designed to reduce threats posed by EDCs. The next step – and the most difficult one – for sure will be validating and establishing legal frames to control the pollution level with EDC.

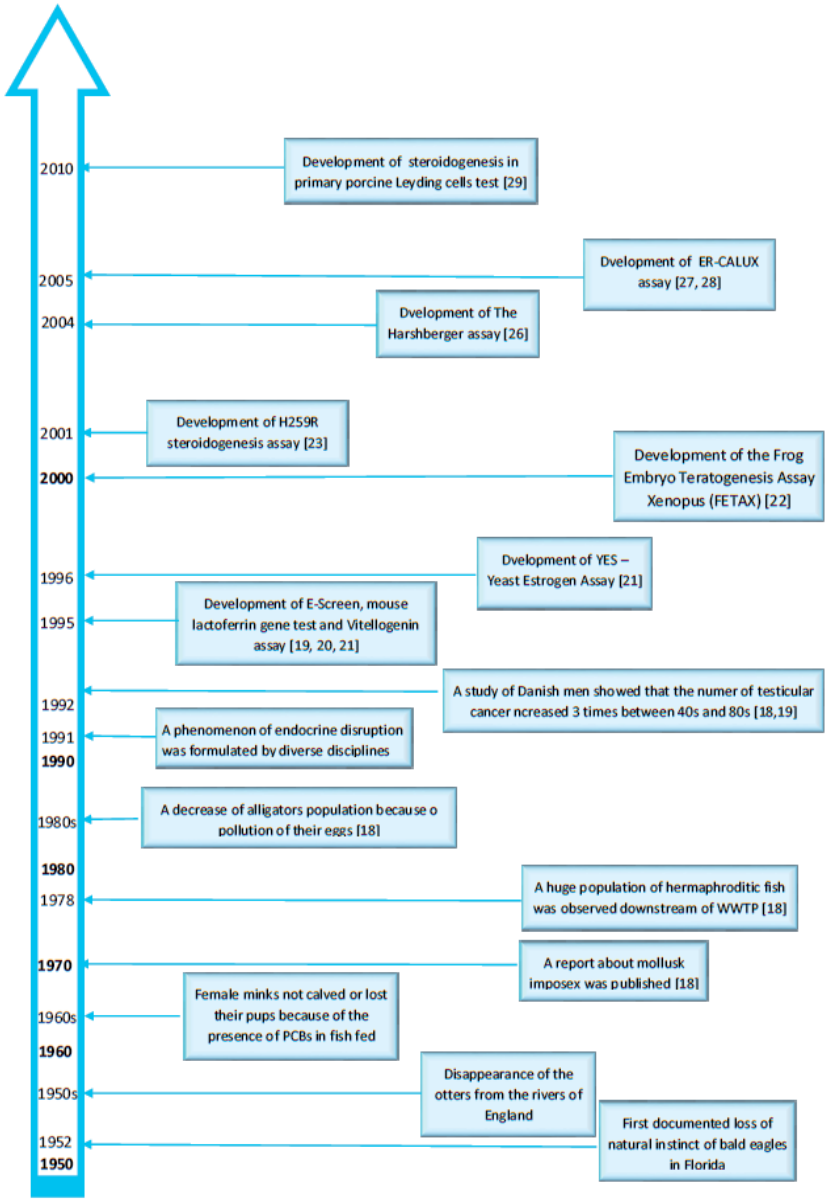

Figure 3. Selected milestones on Endocrine Disrupting Chemicals analysis and environmental issues.

Since the 1960s, the huge increase of the number of such scientific papers has been observed. The rate of this increase is presented in Figure 4.

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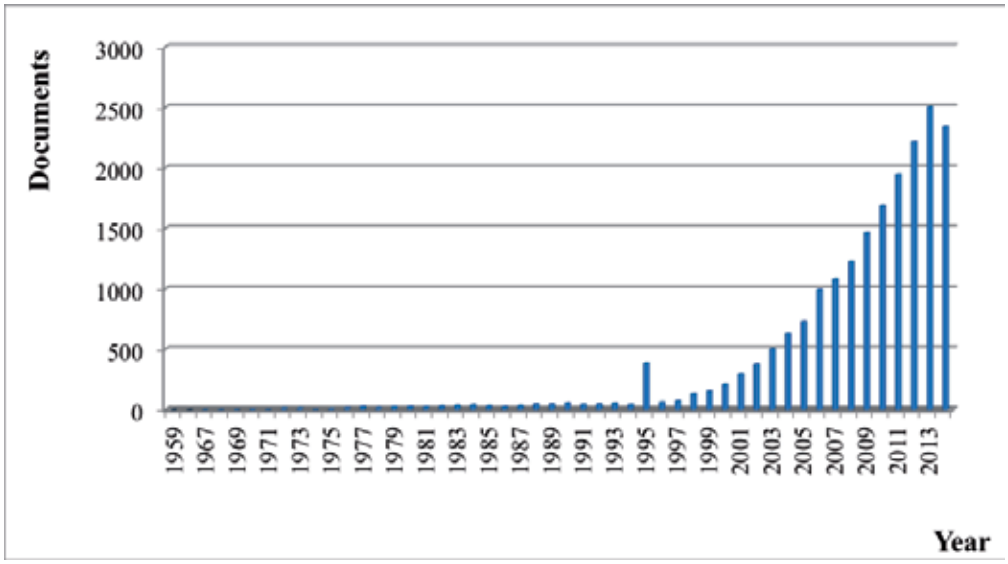

Figure 4. Increasing number of manuscripts on EDC over the years.

1.2.2. Scientific centers and laboratories dealing with the issue of EDC

There is still not enough knowledge about mechanisms, modes of action and the effects that endocrine disrupting compounds and their mixtures, which are present in the environment, have on single organisms and on whole ecosystems. That is the reason why researches in this field of expertise are being held in numerous scientific centers and laboratories all over the world. In Table 2, the above mentioned scientific units are presented. The studies conducted are aimed at:

- developing new analytical methodologies and their validation;
- the use of various procedures to obtain information about the content of various groups of xenobiotics in samples collected from various elements of non-living environment and biota samples.

2. Health effects

2.1. Harmful health effects of EDCs on vertebrates

2.1.1. Humans

Until now, there is no clear opinion in the scientific circles concerning the harmful effects of EDCs. However, it is hard to remain calm as the review of literature concerning the issues connected to environmental chemistry and ecotoxicology shows an increasing number of

Scientific center
Catalan Institute of Water Research (ICRA) Girona, Spain
University of Saskatchewan, Department of Veterinary Biomedical Sciences and Toxicology Centre, Saskatoon, Canada
Gdańsk University of Technology, Department of Analytical Chemistry
University of Arizona, Department of Chemical and Environmental Engineering, Tuscon United States
University of Exert, Bioscences Exeter, United Kingdom
Carleton University, Department of Chemistry, Ottawa, Canada
Institute of Molecular Science, Division of Molecular Environmental Endocrinology, Japan
Universiteit Antwerpen, Toxicological Center, Antwerpen, Belgium

Table 2. Information on selected scientific units conducting research on EDCs.

articles showing a relation between the presence of xenobiotics in the environment and the annually increasing rate of incidence of various kinds of neoplasms, distorted reproductive behavior, and an increasing level of feminization of specific populations at different levels of the food chain.

Even though the majority of those studies has been conducted on animals, there is evidence confirming the negative effect of even small doses of those substances have on humans [31]. The EDC group compounds are characterized by a similar structural construction to natural estrogens. Although the activity of many xenoestrogens has been estimated to be lower than the activity of the feminine sex hormone estradiol, numerous *in vitro* studies indicate its capability of binding with ERα, Estrogen Receptors, and aryl hydrocarbon and thyroid hormones receptors. These properties are the reason why estrogen is suspected to cause diseases resulting from hormonal disorders, including: fertility problems, heart diseases, circulatory problems, and diabetes [32]. The data published during the last few years more and more often indicates to a relation between identification and the growing levels of EDCs in various biological samples and the global problem of obesity, which occurs on an epidemic scale.

In addition, as the latest reports from the scientific world indicate, many of these compounds may have an influence on organisms not only through receptors. The epigenetic tests conducted have confirmed that these compounds influence the process of methylation of histone proteins, influencing alterations in the molecule expression. There are many concerns regarding the fact that these contaminations are capable of crossing the placenta barrier and the blood-brain barrier, and thus, they may have a negative influence on organisms since the early stages of their lives. This fact has been confirmed in many epidemiological studies and experiments, what indicates to a strong correlation between an exposure of the mother to the activity of

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xenobiotics and the occurrence of neurodevelopmental disorders of her offspring, such as ADHD, autism, or alterations in behavioural development as well as impairment of cognitive functions [33]. There are many indications that show that the compound may be also responsible for the initiation of carcinogenesis, that is why studies conducted in numerous research centers are aimed at finding the relations between the presence of xenoestrogens in the human body and the frequency of incidence of neoplasms of e.g., the testicles, prostate, uterus, ovaries, and breasts [34].

Humans may be exposed to the harmful effects of the EDCs *via* many ways as presented in Figure 5. [7].

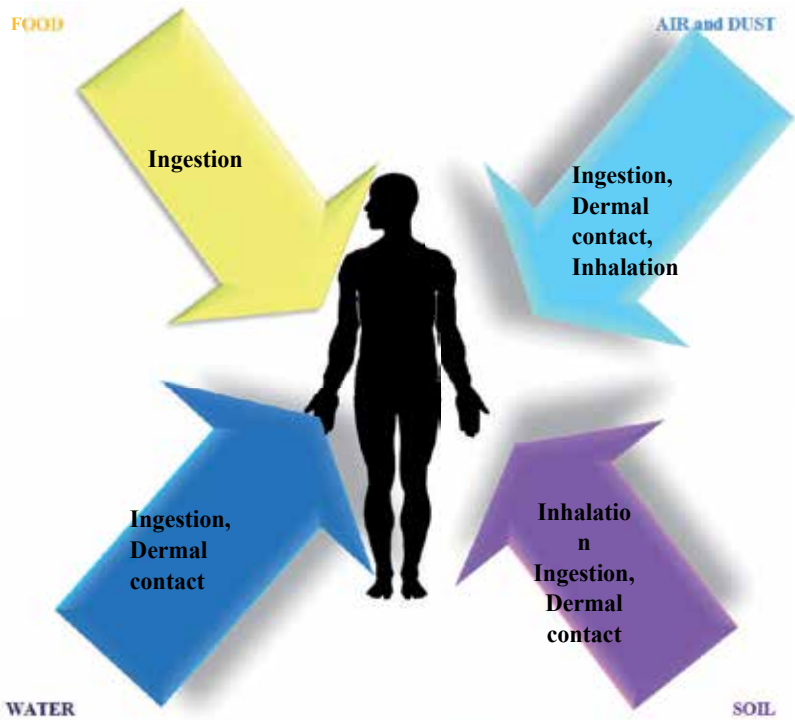

Figure 5. Routes of human exposure to EDCs.

2.1.2. Wildlife

Most data about the adverse effects of endocrine disruptors present in the environment on wild aimals come from Europe and North America. Observed changes vary from very subtle, such as small changes in the physiology and sexual behavior of some species to permanently altered sexual diferentiation. Most affected are aquatic species located on the top of the food chain, but some effects have also been observed in terrestial species. Table 3 provides information concerning some health effects induced by EDCs on wildlife [5].

Area of the harmful action	Examples	Chemicals responsible
Female reproductive health	Endometriosis	PCBs, phtalates, dioxins
	Fibroids	Phtalates
	Interferences in endocrine signallig of pubertal timing, fecundity, fertility and menopause	
Male reproductive health	Testicular cancer	
	Testis germ cel	
	Genital abnormalities in babies	
	Cryptorchidism	Diethylstilbestrol, pesticides
	Reduced semen quality	dioxins
	Hypospadias	Endocrine disrupting pesticides
	Feminization	Estrogenic chemicals
Sex ratio	Fewer male offsprings in human	Dioxin, 1,2-dibromo-3-chloropropane
	EDC-related sex ratio imbalances in wild fish and molluscs	
Thyroid-related disorders	Interferences in thyroid function, including pregnant women; reduced thyroid hormones levels in blood serum in rodents	PCBs, BPA, phtalates, perfluorinated chemicals
Hormone-related cancers	Breast, endometrial, ovarian, proostate cancers	Xenoestrogens (PCBs, pesticides, dioxins)
	Thyroid cancer	Pesticides,2,3,7,8-tetrachlorodibenzo-p-dioxin
Adrenal disorders in human and wildlife	Adrenocortical hyperplasia (Baltic Sea seals)	Mixture of DDT and PCBs and their methyl sulfone metabolites
	Interfering development of the fetal adrenal cortex	PCBs
	Induction delayed effects in the response to stress in animal	PCBs
Bone disorders	Bone disorders, decreased bone mineral density	PCBs, DDT, hexachlorobenzene
Metabolic disorders	Obesity, diabetes	BPA, PCBs, dioxins
Immune function and diseases in humans and wildlife	Prostate inflammation	Xenoestrogens
	Allergic sensitization	BPA
	Lymphoma and leukemia	-
	Autoimmune thyroid disease	PAHs, PCBs
	Endometriosis and allergies	Phtalates, dioxins
	Asthma	Phtalates

Table 3. Summarized information about the adverese health effects of EDCs on wildlife [7].

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2.2. Harmful health effects of EDCs on invertebrates

There is still not enough knowledge on endocrine effects on invertebrates; however, these organisms seem to be good intermediates in modeling hormonal potential toward higher organisms. There are some historical reports in which females have exhibited signs of masculinization, apparently in association with exposure to EDCs. Exposure of marine gastropods to Tributyltin (TBT), a biocide used in anti-fouling paints, provides the clearest example in invertebrates of an endocrine-mediated adverse effect caused by exposure to an environmental contaminant. Masculinization of marine gastropods exposed to TBT has resulted in worldwide declines of gastropods. The endocrine mechanism probably involves elevated androgen levels possibly through altered aromatase activity. Tributyltin-induced imposex in prosobranch female snails is a condition in which the penis "imposes" on the normal female reproductive anatomy. The associated development of the sperm duct can, in extreme cases, lead to the blockage of the oviduct of the female, resulting in sterility and population declines [1, 5, 7].

3. Environmental fate

Fate and transport data interpretation is a very challenging task to perform. Although the amount of information is sufficient, it is crucial to identify critical processes and transport pathways for prioritization and screening purposes.

Analyzing the ways the endocrine active compunds enter the enviornment, it can be distinguished as an nonpoint or a one point source of pollution. Areas affected with pollution are mainly stream downs from cornfields and farm areas where different types of plant protetction products and fertilizers are used, which can contain significant quantities of pharmaceutical residue. Smaller quantities can reach the ecosystems by precipitation.

There's no doubt that the main source of xenoestrogene emissions to the enviornment are one point pollutuion sources. A significant part of the EDC group compounds is reaching water ecosystems with sewage.

And with that occuring, surface waters and underground waters have higher levels of concentration of these substances than in air or soil.

Residue of pharamceuticals and other substances that are biologically active coming from sources such as houses, hospitals, and production plants head to the sewer plants where they undergo different processes of water purification. Unfortunately, due to their physicochemical properties, they are resistant to biodegradation processes. This results in significant quantities of residue are not eliminated and get across to water ecosystems or with sewage sludge to the soil, groundwaters, and drinking waters. The ineptitude of widely used water purification systems has caused all the elements of the environment to be polluted by endocrine compounds. Xenobiotics, after reaching water ecosystems, undergo many different changes in chemical processes in living organisms as well as the abiotic part of the environment.

There are three environmental processes that affect the environmental fate of EDCs (as well as other pollutants). They are defined as:

- Persistence – the tendency of a chemical substance or its degradation products to survive in the environment without being transformed into other forms, (measure: hydrolysis half-life, aerobic and anaerobic soil metabolism, and photolysis).
- Mobility – the tendency of a chemical substance to move within environmental media or between media (measure: volatility, Henry's law constant, K_d, K_{oc}, groundwater ubiquitous score, aged soil column leaching, and terrestrial field dissipation studies).
- Bioaccumulation – the capacity of a chemical to accumulate (be stored in tissue) in an organism as a result of uptake from all environmental sources (measure: octanol water partition coefficient, BCF, and animal metabolism).

The environmental fate of endocrine disruptors is shown schematically in Figure 6 [35].

Compounds interfereing in the endocrine balance can undergo biodegradation, photodegradation, sedimantation, elimination hydrolisys, or sorption on the matter particules suspended in water. The level on which they will be adsorbed depends on the physical and chemical properties and affinity to the particles present in water [35, 36].

Compounds included in this group, just like other types of xenobiotics, may undergo the bioaccumulation process in tissues and organs of organisms at higher trophic levels. This thesis is confirmed by data on toxaphene presented in Table 4. Toxaphene is an insecticide contained in over 670 products. Toxaphene is characterised by toxicity, stability, and ability to bioaccumulate in animals and to travel long distances. Toxaphene is poorly soluble in water, so it can be found in the air, soil, or sediments on the bottom of lakes and streams [37]. In the 1970s, toxaphene was one of the most commonly used pesticides in the world [38, 39].

Element of the environment	Concentration [ppb]
Air	0.0007
Snow	0.0009–0.002
Seawater	0.0003
Zooplankton	3.6
Arctic cod	14–46
Arctic char	44–157
Ringed seal oil	130–480
European sturgeon oil	1380–5780
Narwhal oil	2240–9160

Table 4. Toxaphene concentrations in samples from various parts of non-living environment and biota accumulated in the Arctic areas of Canada [41].

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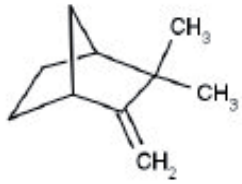


Toxaphene was used for fighting pest insects feeding on cotton, grain, fruits, nuts, and vegetables. In the 1970s, fishing and hunting agencies also used toxaphene for killing fish species that were considered undesirable. It was also used for fighting ticks and other acari in domestic animals and poultry. Toxaphene is currently banned in the USA and in 57 other countries worldwide, while in other 12 countries, its use is strictly restricted. At the beginning of the 1990s, toxaphene was produced in Africa and Latin America; it is estimated that it is used in the largest quantities in Africa [37, 40].

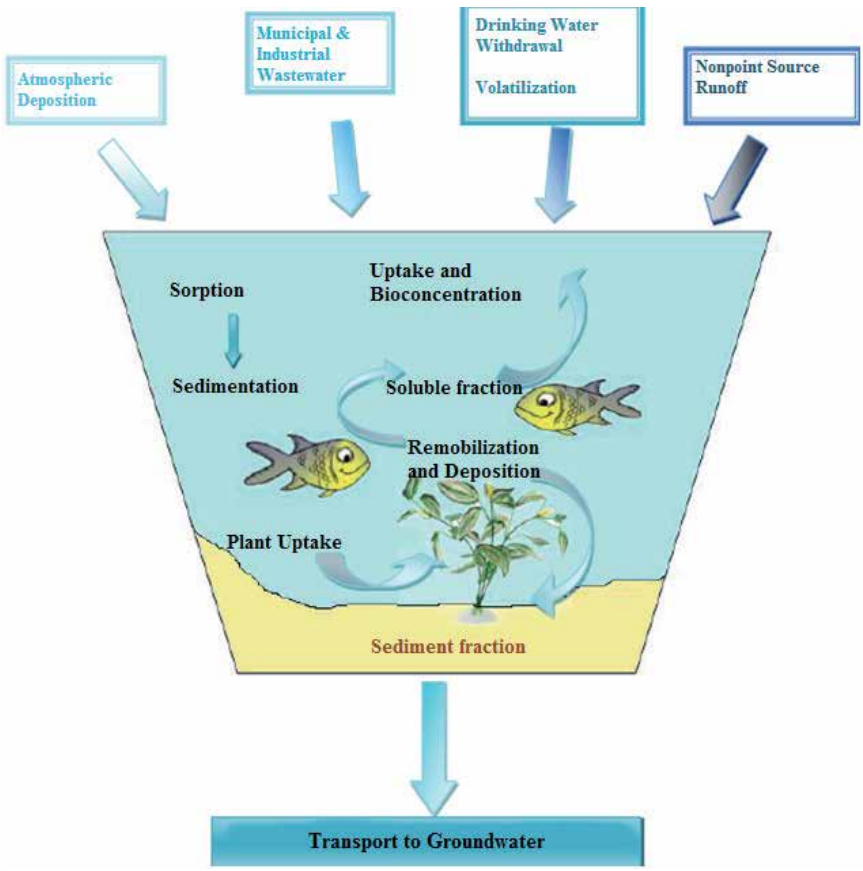
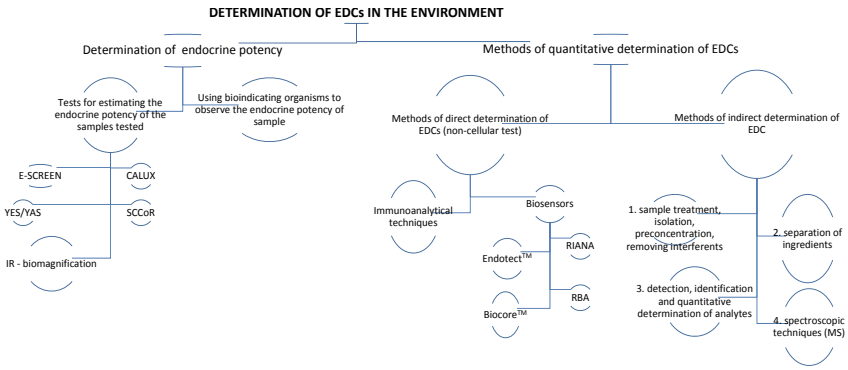


Figure 6. Schematic presentation of the environmental fate of endocrine disruptors.

4. Analysis and monitoring of EDCs in environmental samples

From a historical point of view, the instrumental techniques were first tools to determine trace organic pollutant concentration levels in the environment. With the run of time albo biological methods were introduced into the scientific routine to obtain more comprehensive and reliable information of the pollution levels of given environemtnal compartments. In Figure 7, (A and B below), basic instrumental and biological data together with their short description to present the development of tools in the field of endocrine potency determination with biological methods are presented.



(a)

E-SCREEN	• Presence of estrogen induces response of cells proliferation rate being proportional to estrogen concentration in the sample studied. The cells line being used are estrogen-defendant ones, e.g. cell lines of human breast cancer MCF-7. These cells are being incubated in the presence of sample testes while in the control sample either the 17 β-estradiol is present (positive control) or not (negative control). Comparison of cells proliferation rates in samples tested and controls ensures possibility of determining the estrogenicity potential [1,44-46].
CALUX	• Cells show sensitivity to given types of chemicals, these may be estrogenic, androgenic or xenoestrogenic substances (e.g. dioxin-like chemicals). Similarly to YES/YAS assay the main receptor gene is hyphenated with the reporting gene. If the sample tested poses the activity to proper group of cells the emission of light will take place (proportional to amount of active chemicals in the sample tested) [1,48,49].
YES/YAS	• The human estrogen receptor gene is introduced to the *Saccharomyces cerevisiae* (in case of YES test, for YAS it is androgenic gene) hyphenated to LacZ reporter gene [43,47]. Such cells are becoming estrogen activity controls. In case of substance's estrogenic potential it binds the estrogen receptor and is signaling presence of estrogenic chemical initiating the reporting gene activity. The reporting gene is coding synthesis of β-galactosidase which afterwards takes part in process of transforming the dye present in the sample solution from yellow to red one [1,48]. The intensity of red color is directly related to the sample estrogenic activity.
SCCoR	• There are numerous mammalian cell lines utilizable in the SCCoR (*Single Cell Coactivator Recruitment*). Genetically introduced ability of indicator to fluorescence enables unique possibility of distinguishing whether or not the analyte is agonist or antagonist of the estrogen receptor in the cell [50].
IR-biomagnification	• It is the only cell test not created with genetic engineering methods engagement or elaborating cells 'proliferation potency. Application of this test is based on fact that changes in cells functioning can be assessed due to changes in IR radiation changes caused by the cell organelles. The IR microscopy is used for this purpose. The IR radiation in the mid-IR range is characterized with sufficiently low energy not destroying the cell organelles [51]. The light dispersion is being measured with set of 128 sensors and the response is calibrated against naturally functioning cell.
Biocore™	• Utilizes the plasmon resonance phenomena) in order to assess reactivity between chemicals and estrogenic receptor at the detector surface being the golden plate. The microflow system enables of transporting the sample stream at the surface of golden plate and the system of optical detection enables measurement of the plasmon surface resonance. Numerical value of this parameter is directly proportional to the concentration of xenoestrogen tested. It is also possible to use other physical phenomena to detect the estrogenic activity e.g. the piezoelectric effect or chelation of the nickel atoms
Endotect™	• Based on activity of human estrogenic receptors (hER) which are bind to promoters enabling fluorescence measurable with new type of detector called evanescence-type detector. For this reason the hER and promoter are bound on the glass fiber and the total fluorescent response is being measured along the fiber in the process based on the evanescence phenomena.
RIANA	• Used to determine the estrogenic properties of selected analytes. Its usability has been tested for assessing presence of xenobiotics (atrazine, isoproturone, estrone) in the water samples [57]. The method utilizes laser to fluorescently induce the antibodies that is specifically bound to analyte. Similarly to Endotect™ the evanescent response around the glass fiber is obtained and quantitatively measured [35,42].
RBA	• Based on competition between radiolabelled estradiol and the tested EDC to bind to the estrogenic receptor active site [58]. The purified protein of human estrogenic receptor is being added to the sample of standards mixture – estradiol (of known concentration and labeled with tritium) and ligand being determined (with increasing concentration) and incubated. During this period the receptor-ligand complex settles on the surface of hydroxyapatite followed by flushing out the unbound ligand from the surface. The product bound to the surface is radiolabelled thus can be measured.

(b)

Figure 7. (a) Classification of analytical approaches used in order to detect and determine EDCs in the environmental samples and the endocrine potency of different samples. (b) Description of selected bioassays utilized for endocrine potency determination.4.1 BIOLOGICAL METHODS

4.1. Biological cellular tests used to assess the endocrine potency of the environmental samples

Cellular biotests are good alternative to traditional analytical procedures, as well as to immunotechniques and methods utilizing living organisms as biomarkers of exposure to EDC [91]. In these types of biotests, the yeast or human cells (e.g. cells of breast or kidney cancer) are used to determine disturbances in the run of hormonal signaling [92]. The cells can be used in unchanged form or altered with proper bioengineering methods to obtain the proper response of cells to the presence of specific chemicals belonging to EDC [93]. For example, the estrogen gene can be introduced to the yeast cells from human, fish, or other species genome. In such case, the term of estrogen equivalent concentration (EEC) finds its application in the form of the formula [42]:

$$EEC_i = C_i \cdot EEF_i \tag{1}$$

where:
C_i – concentration of particular EDC in the sample studiedEEF_i – numerical value of the endocrine equivalent factor

Numerical values of this factor determines in the relative way the endocrine character of given the chemical in relation to the endocrine potency of the reference chemical, most often estradiol or 17β-estradiol.

In this way, the endocrine potency can be described using the equation [32]:

$$EEC_t = \Sigma EEC_i \tag{2}$$

In Table 5, there are given numerical values of EFF of selected chemicals belonging to EDC.

COMPOUND	EEF
Estradiol	1
17α-etynyloestradiol	1
Estrone	0, 1–1
Bisphenol A	$5 \cdot 10^{-4}$–$6 \cdot 10^{-5}$
Nonylophenol	$2.3 \cdot 10^{-5}$–$9 \cdot 10^{-4}$ 7. $2 \cdot 10^{-7}$–$1.9 \cdot 10^{-2}$
Octylophenol	$1 \cdot 10^{-5}$–$4.9 \cdot 10^{-4}$

Table 5. Numerical values of EFF of selected chemicals belonging to EDC

In Table 6, the data concerning the analysis of various samples with bioassays is given.

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Concentration determined	References
1	Bisphenol A	Bottled water samples		ELISA	0.05 ng/cm^3	0.01–1.33 ng/cm^3	[59]
2	Estradiol and estrone	River water	Extraction on C-18 columns	Radioimmunoassay	0.3 ng/dm^3	1.2–9.4 ng/dm^3	[60]
	Testosterone				0.3 ng/dm^3	>0.4 ng/dm^3	
	Estriol			ELISA	0.1 ng/dm^3	>0.5 ng/dm^3	
	Ethinylestradiol				0.1 ng/dm^3	>0.2 ng/dm^3	
3	Alkylphenol ethoxylates	Wastewater	Filtration with a glass-fiber filter, SPE (HLB)	LC-MS/MS ELISA	20–1000 μg/dm^3	0.724–78.15 μg/dm^3	[61]
	Bisphenol A				5–500 μg/dm^3	0.08–1.55 μg/dm^3	
	17β-estradiol				0.05–1 μg/dm^3	0.57–1.73 μg/dm^3	
	17α-ethinylestradiol				0.12 ng/cm^3	0.5–1000 ng/cm^3	
	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]-2-benzopyran (HHCB)				1.3 ng/g	<LOQ–62.1 ng/g	
	Musk xylene				0.5 ng/g	<LOQ–13.0 ng/g	
	Norethindrone				15 ng/dm^3	-	
	Levonorgestrel				15 ng/dm^3		
	Nonylphenol				1.3 ng/dm^3	<LOD–118 ng/dm^3	
4	Testosterone	River water	SPE (C18)	YES	-	0.8–35.5 ng/dm^3	[62]
	Estrone, estradiol			Radioimmunoassay	-	3.2–4.3 ng/dm^3	
	Estradiol			ELISA	-	0.7–3.4 ng/dm^3	
	Ethinylestradiol				-	1.4–19.4 ng/dm^3	
5	Estrone	Sewage effluents	SPE (C18)	MCF-7	-	70 ng/dm^3	[63]
6	Estradiol	Cleaned wastewaters	SPE (C18)	YES	-	1.1–11.1 ng/dm^3	[64]
7	17 α-ethinilestradiol	Surface and sewage waters		ELISA	-	0.035±0.002 µg /dm^3	[65]
	Estradiol				-	0.085±0.010 µg/dm^3	

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Concentration determined	References
8	Estrone	River waters	SPE (C18)	RIANA	-	0.17–10.7 μg/dm^3	[57]
	Atrazine				-	0.35–1.47 μg/dm^3	
	Isoproturon				-	0.11–2,83 μg/dm^3	
9	17 β-Estradiol	Wastewaters	SPE (C18)	YES	-	8.1 ng/dm^3	[66]
	Estradiol				-	11.5 ng/dm^3	
	p-Nonylphenol				-	55 ng/dm^3	

Table 6. Concentrations of selected EDCs determined in environmental samples using biological and instrumental methods.

4.2. Analytical and instrumental methods used for detecting EDCs

Detection, identification, and quantitative determination of EDC-like chemicals are currently achieved mostly with chromatographic techniques. Prior to chromatographic separation and detection (mostly with mass spectrometry or time of flight detection), complex and time- and labor-consuming sample treatment are necessary as presented in Figure 8 (as exmple on the basis of data revision [67,68]).

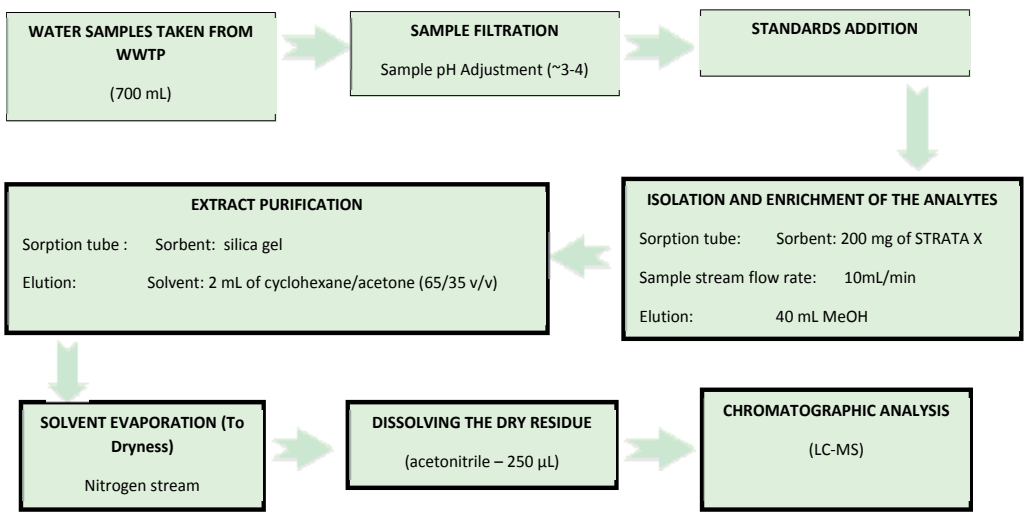


Figure 8. The schematic presentation of selected estrogen determination in sewage samples with LC-MS.

- Pre-treatment

Water and sewage samples collected for determination of endocrine disrupting compounds contain other various impurities. At this moment of sample treatment, the majority of samples is subjected to filtration to remove solid impurities. Pre-existing coagulation facilitates the

filtration. Then the sulfuric acid, hydrochloric acid, methanol, or formaldehyde can be added to the samples to obtain the appropriate pH. The addition of one of these compounds also prevents the degradation of the assayed analytes. Samples are stored in darkness and low temperature, usually <4°C, in bottles made of amber glass to avoid photodegradation of analytes [69, 70].

- **Extraction**

The usual method of trace organic pollutants extraction is solid-phase extraction (SPE). The first step is filling the column-conditioning of the sorbent. After conditioning, the column is percolated with test sample. The target analytes and other compounds absorb in the sorbent. The next step is the elution of interfering compounds from the column. At the end, the target compounds are eluted with proper solvent mixtures. Solid phase extraction can be either: on-line where the extraction is directly integrated into the system of the quantitative analysis; or it may be off-line where the extraction column is not connected in any way with the gas or liquid chromatograph. In on-line SPE, full automation of the process occurs and the method is characterized with ease of application of samples and a large throughput. However, despite the higher costs off-line SPE, it is often used because when combined with GC, water must be removed totally prior to eluting analytes [70].

When choosing the appropriate sorbent for the SPE column, one has to take into account the chemical and physical properties of assayed compounds. One of the most frequently used cartridge packing is Oasis HLB. It allows to obtain high recovery of both the acidic, basic, and neutral compounds. Recovery exceeds 70%. This sorbent can be used for the large range of pH of the samples, ranging from 2–7. Lichrolut ENV+ cartridges are used when the sample has a low pH and contains polar organic compounds or when sample contains neutral drugs and its pH is neutral. Columns packed with C-18 are suitable for non-polar or moderately polar compounds. The extraction process must then be optimized: sample volume, the volume of sorbent cartridge, percolation rate, type of eluent and its volume. The elution solvent is selected depending on the properties of the compounds eluted and its elution strength.

A less frequently used method is the Solid Phase Microextraction (SPME). It depends on the distribution of the analyzed chemical compounds between the sample and the sorbent. This method is fast, moderately new, and an easy method of extraction. SPME coupled with GC content allows the study of semivolatile, volatile, and non-polar analytes. More difficult is the combination of this technique with liquid chromatography. Non-volatile compounds are not totally desorbed during the thermal desorption. SPME has many advantages thus it is more attractive than the SPE method, but has a more restricted choice of sorbent and too little sorption capacity. Therefore, the parameters of this technique are still optimized for wider application and greater sensitivity. Samples after SPE or SPME are concentrated using evaporation under a gentle nitrogen stream [70].

Another common method of extraction is liquid-liquid extraction (LLE). LLE relies on shaking the sample with an organic solvent for a specified period of time. One can perform this operation several times. The organic phase is separated from the water, and mixture of all the extracts is obtained. The resulting solution is dried, for example, using anhydrous sodium

sulfite. When the sample volume is sufficiently small, determination of analytes can be started [71].

- **Determination of concentration levels of target analytes**

Liquid chromatography combined with tandem mass spectrometer is the most widely used analytical technique because it allows ion fragmentation that is needed for accurate and precise determination of the analytes. LC-MS/MS determines the compounds that have identical molecular weight but disparate product ions. Using MS/MS increases the selectivity and sensitivity of the method. Atmospheric pressure chemical and electrospray ionization (APCI and ESI) are modes of ionization interfaces that are the most widely used with LC-MS/MS. Low or medium polar compounds are determined by APCI, and the analysis of polar analytes is conducted using ESI. The main use of liquid chromatography is to determine non-volatile, polar, or degradable under high temperature substances. For example, beta-blockers and antibiotics can by analyzed using only LC-MS/MS [70].

One of the biggest difficulties with LC-MS/MS is interference in the matrix effects. This effect causes the strengthening or suppression of the analyte signal, thus producing erroneous results. When contaminated environmental samples are analyzed, for example wastewater, it is necessary to perform efficient clean up of samples. The process of optimizing the analytical methods, such as of liquid chromatography, involves making a series of studies to determine the parameters that give the best results for all determined substances. MS parameters are also optimized for each analyte by conducting the flow injection analysis (FIA). To obtain credible results, it is needed to optimize the separation of compounds by liquid chromatography and mass spectrometry parameters.

In case of GC-MS analysis, the matrix effects occur less frequently than during the analysis of LC-MS/MS. The disadvantage is that it is a more time-consuming technique and requires complex preparation of the sample in case of derivatization step.

As a result of derivatization of polar components, their analogs are less polar and more thermostable. It increases the sensitivity of analysis but also increases the loss of sample by performing additional operations. A negative aspect of derivatization is the use of carcinogenic and toxic reagents. Derivatization reaction should allow the detection of analytes that have polar functional groups. It is effective when the reaction occurs in a given time with 90% efficiency.

In literature there can be found many applications of GC-MS for the analysis of drugs, PAHs, PCBs, and other pollutants in water and wastewater samples [70].

In Table 7, there are presented examples of determining the EDCs in environmental samples, mainly in samples of river, drinking, surface, and sewage water. They are also determined in samples of food, air, in the tissues of the Chinese sturgeon, in house dust, and in human serum.

It can be stated that LC-MS/MS and GC-MS are the most often used techniques in determining EDCs. Other methods such as high performance liquid chromatography with fluorescence or diode array detector are less frequently used in the analysis of EDCs. The best results are given by the combination of LC-MS/MS with GC-MS.

On the basis of data collected in Table 1, it can be concluded that people and other living organisms are exposed to EDCs throughout their entire life. Even low concentrations levels of EDCs can have a significant impact on animal and human health and the existence/health state of entire populations. Many people do not realize that even these small amounts can be significantly harmful after long exposure. The concentration of some compounds from the EDC groups has decreased because their application was banned. Unfortunately, they have long half-lives, so trace amounts are present in the samples assayed decades after the release of specific chemicals.

Determination of EDCs poses many challenges and problems. Newer and more accurate analytical methods are required and need to be used by the scientific community. There are more and more articles/books about detection of EDCs in environmental samples and their harmfulness. Application of EDC should be reduced as far as possible because contamination of these compounds poses a huge risk to the environment.

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Measured concentration	REFERENCES
1	Testosterone	Human serum	LLE (diethyl ether)	HPLC-MS/MS	-	0.1 ng/cm^3	[72]
	17-hydroxyprogesterone				-	0.1 ng/cm^3	
	Cortisone and estradiol				-	0.1–50.0 ng/cm^3	
	Androstenedione				-	0.1 ng/cm^3	
2	DEET	River water	SPE	LC-MS/MS	11.6 ng/dm^3	1.49–29.9 ng/dm^3	[73]
	2,4-dichlorobenzoic acid				2.3 ng/dm^3	3.24–9.35 ng/dm^3	
	Erythromycin				13 ng/dm^3	3.08–134.5 ng/dm^3	
3	Bisphenol A	Drinking and surface water		Carbon nanotube-tyrosinase based amperometric enzymatic biosensors	0.02 μM	0.5 μg/dm^3	[74]
5	Bisphenol A	Natural water	LiChrolut RP-18 SPE	LC–ESI-MS	6.3 ng/dm^3	<LOD–0,007 μg/dm^3	[75]
	Estrone				2.5 ng/dm^3	<LOD–0,022 μg/dm^3	
	Desethylatrazine		LiChrolut RP-18 SPE	LC–APCI-MS	1.61 ng/dm^3	0.002–0.003 μg/dm^3	

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Measured concentration	REFERENCES
	Diuron				10.95 ng/dm^3	0.004 μg/dm^3	
6	Estriol	Surface, drinking, and waste waters	pH adjustment to 2 followed by SPE (HLB)	LC-MS/MS	5.0 ng/dm^3	8.9–25.0 ng/dm^3	[69]
	17α-ethynylestradiol				1.0 ng/dm^3	1.3 ng/dm^3	
	Estrone				1.0 ng/dm^3	1.7–36.0 ng/dm^3	
	Testosterone				1.0 ng/dm^3	1.1 ng/dm^3	
	DEET				1.0 ng/dm^3	2.0–69 ng/dm^3	
7	Polyfluorinated alkyls	Air samples		GC-MS		64–546 pg/m^3	[76]
8	12 perfluorinated surfactants	Surface and drinking water	SPE	HPLC-MS/MS		2–4385 ng/dm^3	[77]
10	Estrone	River water	SPE (HLB) Cartridge (polymer of N-vinylpyrrolidone and divinylbenzene)	HPLC-DAD GC-MS	44.0 ng/dm^3	<LOD–112.9 ng/dm^3	[78]
	Ethynylestradiol				18.0 ng/dm^3	<LOD–101.9 ng/dm^3	
	Daidzein				10.0 ng/dm^3	<LOD–888.4 ng/dm^3	
	4-nonylphenol				7.0 ng/dm^3	<LOD	
12	Estriol	Wastewater from a swine farm	SPE (N-vinylacetamide), pH adjusted to 3	LC-MS/MS LC-MS		5200–5400 ng/dm^3	[79]
	Estrone					2200–3000 ng/dm^3	
	17α-ethinylestradiol				0.12 ng/cm^3	0.5–1000 ng/cm^3	
	Estriol				0.006 ng/cm^3	0.35 ng/cm^3	
	Bisphenol A				0.02 ng/cm^3	0.47–0,54 ng/cm^3	
	17α-ethinylestradiol				0.1 ng/cm^3	3.57 ng/cm^3	
13	Hexachlorobenzene (HCB)	Liver, muscle, heart, gonad,	Soxhlet extraction (dichlorometha ne and	GC-MS	0.07 ng/g	1.6–525.0 ng/g	[80]

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Measured concentration	REFERENCES
	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT)	stomach, intestines, adipose, gill, pancreas, kidney, gallbladder, and roe from 13 female Chinese sturgeons	methanol mixture solution)		0.2 ng/g	<LOQ–480 ng/g	
14	Estrone	Wastewater	SPE (Oasis)	GC-MS	5.6 ng/dm^3	21–128.5 ng/dm^3	[81]
	17β-estradiol				11.2 ng/dm^3	10.9–224 ng/dm^3	
	Bisphenol-A				17.4 ng/dm^3	15–890 ng/dm^3	
	4-tert-Octylphenol				8.5 ng/dm^3	29–710 ng/dm^3	
15	Sulfadiazine	Waste water	SPE (Oasis HLB)	HPLC-MS/MS	1 ng/dm^3	6–50 ng/dm^3	[82]
	Estriol				5 ng/dm^3	4648–22633 ng/dm^3	
	17α- Ethynylestradiol				10 ng/dm^3	<487 ng/dm^3	
	Ethinylestradiol					5.7–30.8 ng/dm^3	
16	Atrazine	Wastewater	SPE (Oasis HLB)	LC-MS/MS		1118 ng/dm^3	[83]
17	Testosterone	Drinking water	SPE (HLB)	LC-MS-ESI		0.116–0.214 μg/dm^3	[84]
	Bis(2-ethylhexyl) phthalate					7–20 μg/dm^3	
	17α-ethynylestradiol		The CLLE extracts derivatization	GC-MS		0.073–0.831 μg/dm^3	
	Progesterone					0.11–0.199 μg/dm^3	

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Measured concentration	REFERENCES
	Sulfamethoxazole				1.8 ng/dm^3	<410 ng/dm^3	
18	4-Nonylphenol	Wastewater	SPE (C18)	HPLC-DAD HPLC-FLD GC-MS	0.09 ng/dm^3	3.39–169 ng/dm^3	[85]
19	4-nonylphenol	Surface water	LLE (CH_2Cl_2)	HPLC-FLD	0.075 μg/dm^3	0.08–0.39 μg/dm^3	[86]
	4-*tert*-octylphenol				0.05 μg/dm^3	<0.16 μg/dm^3	
20	4-nonylphenol	Indoor air		GC-MS		21–420 ng/m^3	[87]
	Diethyl phthalatec					130–4300 ng/m^3	
	Di-n-butyl phthalated					52–1100 ng/m^3	
	Bis(2-ethylhexyl) phthalate					77–1000 ng/m^3	
	Diisobuhtyl phthalate					11–990 ng/m^3	
	Methyl paraben					2.9–21 ng/m^3	
	4-nonylphenol	Household dust	Soxhlet extraction (6% diethyl ether in hexane)	GC-MS		2.58–8.68 μg/g	
	Nonylphenol monoethoxylate					3.36–15.6 μg/g	
	Benzyl butyl phthalate					3.87–1310 μg/g	
	Bis(2-ethylhexyl) phthalate					166.7–7700 μg/g	
	Metyl paraben					0.978–8.24 μg/g	
	Benzo[a]pyrene					0.712–18.1 μg/g	
21	17β-estradiol	Surface water, sewage sludge, and sediments.	SPE (C18)	HPLC-UV		1–35 ng/dm^3	[88]
	Ethinyl estradiol					0.001–2 ng/dm^3	

No.	ANALYTES	SAMPLE TYPE	EXTRACTION	DETECTION TECHNIQUE	LOD	Measured concentration	REFERENCES
	DEET	Surface and groundwater	SPE (Oasis HLB)	LC-MS		2.3–3.3 ng/dm^3	[89]
22	4-*tert*-octylphenol	Wastewater	SPE (C18)	GC-MS		85 ng/dm^3	[90]
	4-nonylphenol					329 ng/dm^3	
	Bisphenol A					457 ng/dm^3	
	Estrone					63 ng/dm^3	
	17α-ethynylestradiol					48 ng/dm^3	

Table 7. Concentrations of selected EDCs determined in environmental samples using instrumental methods.

5. Summary

The poor state of knowledge about the mechanisms of action and effects of EDC chemicals has forced the interdisciplinary scientific teams to intensify their work in the subject. Nowadays, many institutes are carrying out research focused on exploring the properties and metabolic pathways of EDCs and their mixtures in the environment. Good knowledge about the environmental fate, endocrine potential, and distant toxic effects of ecoestrogens will allow to estimate the levels of the pollution and minimal exposure on certain compounds. Moreover, this knowledge can be applied for upgrading the common tools used to detect and perform quantitative determination of EDCs, and can be the basis for the development of new techniques that will provide information about the composition of the sample and about its endocrine potential [94,95].

The discovery of micropollutants occurring in the environment resulted in new methodologies being put into the analytical practice. These methodologies are developed in two different directions. The first is based on methodological solutions designed to detect, identify, and determine xenobiotics that occur in various environmental samples. For this purpose, instrumental methods such as gas and liquid chromatography with mass spectrometry detection are usually used. These techniques provide reliable information about the presence, quantity, and influence of EDCs.

The second approach is to put into the analytical practice new bioanalytical methodologies. These methodologies allow estimation of the sample endocrine potential, but they do not provide information on which of the sample ingredient is responsible for causing the toxic effect. The results of the analysis of this biological response are valuable source of information for chemists and ecotoxicologists. These results can be the basis for estimating the endocrine potential of the environment exhibited by certain species. Moreover, bioanalytical techniques may be supplementary to the techniques of quantitative and qualitative determination of

endocrine disrupting chemicals. It is not possible to estimate the environmental risk of EDC presence based only on the information about the sample composition. It is necessary to determine both the magnitude and how in particular the endocrine homeostasis may be impacted by xenobiotics. These tasks can be realized only by using a well-chosen bioassays battery. In the recent years there has been a significant increase in the importance of the biological methodologies in environmental research because of their numerous advantages. It is reflected in the research literature and in the increase in the number of scientific publications on this subject.

In this chapter the information about some of the estrogenic compounds, their environmental fate, and biological influence can be found. Special attention was given paid to the review of the analytical approaches used at the stage of detection and determination of EDCs in the environmental samples. Also a brief characterization of both the cellular and non-cellular bioassays is presented, as well as the information regarding the changes occurring in the bioindicators as results of being exposed to a specific ecotoxins.

Acknowledgements

The work has been co-financed by the Polish Ministry of Science and Higher Education grant no. IP2011 028071.

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Chapter 8

The Relevance of ATR-FTIR Spectroscopy in Semiconductor Photocatalysis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60887

Abstract

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy has a high potential for investigating a wide range of samples and systems. In photocatalysis, various interfacial phenomena can be studied using this technique, including pH-dependent adsorption and photodegradation of probe molecules. The analysis of the processes occurring at the interface of thin particle films deposited on the surface of an ATR crystal, either in the liquid or the gas phase, is perhaps the best way to elucidate the mechanism of adsorption and heterogeneous photocatalytic reactions. This chapter summarizes the recent advances and applications of ATR-FTIR techniques in semiconductor photocatalysis. A brief outlook at some of the possible investigations in this area is provided and the different proposed adsorption and photocatalytic degradation mechanisms are discussed.

Keywords: Adsorption, attenuated total reflection Fourier-transform infrared (ATR-FTIR), photocatalysis, semiconductor, spectroscopy, thin films, TiO_2

1. Introduction

The expanding interest in environmental and energy issues led to the consideration of heterogeneous photocatalysis as one of the most promising advanced oxidation processes. The interest in this scientific field has increased in the last decade since photocatalysis is assumed to be a powerful tool for the destruction and remediation of highly toxic pollutants, the

purification of polluted water and air, the development of self-cleaning surfaces coated with semiconducting metal oxide materials, and the conversion of solar energy into chemical energy [1–3]. Many semiconductor materials have been tested as photocatalysts, nevertheless, due to its low cost, abundance, high activity, and stability under a variety of conditions. Titanium dioxide (TiO_2) is the most reliable and widely used material [4, 5]. Accordingly, there has been a tremendous amount of research on diverse aspects of TiO_2 (nano)materials, ranging from their synthesis, characterization, and applications to atomic scale, to experimental and theoretical investigations of their fundamental physical and chemical properties [1, 5–7]. Despite these investigations, there remains a need to better understand the reaction mechanisms of the transformation of organic molecules occurring during TiO_2 photocatalysis.

Surface science plays a prominent role in mechanistic investigations concerning the photocatalytic process, providing a unique approach to understand bulk, surface, and interfacial phenomena occurring at the TiO_2 surface [6, 8, 9]. According to several proposed photocatalytic mechanisms [10, 11], an important point for the conversion of the molecules on TiO_2 is the physical and electronic structure of the adsorbed state of the molecules. How a molecule binds onto the TiO_2 surface influences its electronic structure, as well as its redox properties. *Inter alia*, coverage, thermal stability, and reactivity, adsorption structure and site, are all important factors [8]. The interest in surface techniques to investigate liquid-solid and gas-solid interfacial chemistry has grown due to the importance of the information they provide. Few surface spectroscopic techniques are adequate to perform *in situ* analyses of interfacial interactions. For example, sum-frequency vibrational spectroscopy is restricted to planar solid-solution interfaces [12], infrared ellipsometry is considered mainly as a technique for the analysis of thin solid films rather than of interfacial species [13], and surface enhanced Raman spectroscopy (SERS) requires the presence of metals on the surface. Thus, this complicates the more widespread applicability of SERS [14, 15].

IR spectroscopy is the oldest and most commonly used method for identifying both organic and inorganic chemicals, as well as for providing specific information on molecular structure, chemical bonding, and molecular environment. Being a powerful tool for qualitative and quantitative studies, it can be applied to study solids, liquids, or gaseous samples [16]. Recently, IR spectroscopy has been applied *in situ* to study surface reactions on immersed solids such as oxides. This has been achieved with particle films via internal reflection or attenuated total reflection (ATR-FTIR) methods. Investigating several metal oxide solid particles in suspensions or deposited as thin films on ATR crystals, these developments have led to *in situ* ATR-FTIR studies of adsorption and chemical reactions on a variety of solid-liquid and/or solid-gas interfaces in the photocatalysis context [17–24]. The ATR-FTIR technique has proved to be a powerful tool for probing binding mechanisms and for characterizing the adsorption of organic molecules onto metal oxide surfaces in liquid media.

This book chapter focuses on the application of this technique in the above-mentioned context. An overview of the investigations that have been performed to date will be given, analyzing the different experimental procedures, and summarizing the performed investigation of

http://dx.doi.org/10.5772/60887

surface interactions. An in-depth analysis of the different proposed adsorption and photocatalytic reaction mechanisms on TiO_2, as well as on other metal oxides also employed in photocatalysis will be given. To complement this overview, results and interpretations of quantum chemical calculations will also be presented.

2. History and brief overview of ATR-FTIR spectroscopy

Infrared absorption spectroscopy (IR) has contributed for more than fifty years to the molecular view on a wide variety of systems. The selection rule for a vibrational mode of a molecule to be IR active is that there is a change of the electric dipole moment of the molecule upon absorption of light. The absorption of infrared light due to the excitation from the ground vibrational energy level to a higher energy level provides information concerning molecular structure and molecular interactions [15, 25, 26]. Due to the existence of the wide absorption spectra database in the mid-infrared region (4000–400 cm^{-1}), infrared spectroscopy is considered as a universal technique since many molecules have strong absorbances in this region [27].

Fourier-transform infrared (FTIR) spectroscopy is a well-established technique based on the idea of the interference of radiation between two beams to yield an interferogram. The latter is a signal produced as a function of the change of path length between the two beams. The two domains of distance and frequency are interconvertible by the mathematical Fourier transformation method.

ATR spectroscopy was introduced simultaneously by Harrik [28] and Fahrenfort [29] based upon the total internal reflection phenomena. In this approach, IR spectra are recorded for a sample material that is in contact with an internal reflection element (IRE). The IR beam is focused onto the edge of the IRE, reflected through the IRE, and then directed to the detector (cf. Figure 1) [26, 27]. In this case, all the light reflects off the internal surface of the IRE, hence explaining the term total internal reflection [27]. The internal reflection element (IRE) or ATR crystal has, in most cases, a higher refractive index (n_1) as compared to the sample (n_2). Another important parameter is the incidence angle θ that can be determined from the refractive indexes of the sample (n_2) and the IRE (n_1):

$$\theta = \sin^{-1}\left(\frac{n_2}{n_1}\right) \tag{1}$$

The major applications of the ATR method are in the mid-IR region. However, the range has been extended to the near-IR, the far-IR, as well as to the UV and visible spectral regions. Therefore, it is of great importance to choose a suitable ATR crystal for a given application. The most common ATR crystals with their respective refractive indexes and some other relevant properties are summarized in Table 1.

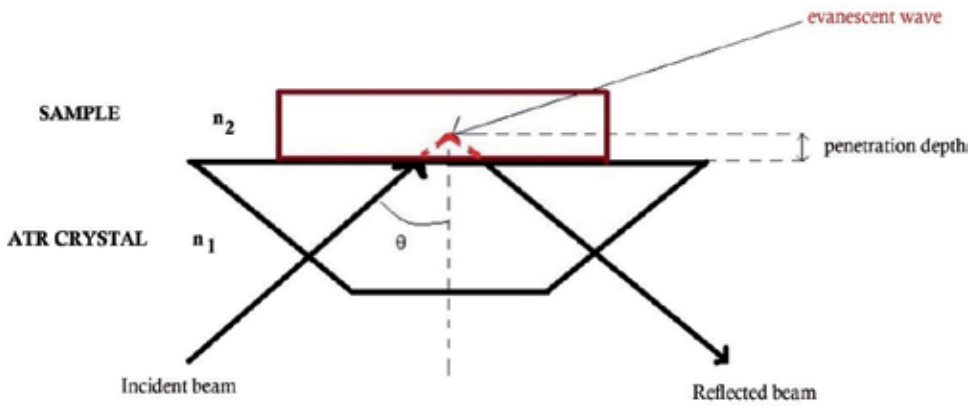

Figure 1. Schematic diagram of a horizontal ATR sampling accessory illustrating the important parameters [16].

Material	Refractive index	Wavenumber range (cm^{-1})	d_p (μm)	References
Diamond	2.4	45000–2500	1.35–1.66	[30][31][32]
Germanium (Ge)	4	5500–870	0.65–0.73	[30][31][32]
Zinc Selenide (ZnSe)	2.41	20000–650	1.22–1.66	[30][31][32]
AMTIR (As/Ge/Se glass)	2.5	11000–750	1.46	[30]
Silicon (Si)	3.4	-	0.84–1.17	[30][32]
Thallium bromoiodide (KRS-5)	2.37	20000–250	1.22–1.73	[30][32]
Cd telluride (CdTe)	2.67	10000–450	-	[31]
Saphire (Al_2O_3)	1.74	25000–1800	-	[31]
Zinc Sulfide (ZnS)	2.2	17000– 950	2.34	[31][32]
Cubic Zirconia (ZrO_2)	2.15	25000–1800	-	[31]

Table 1. Relevant properties of some common ATR crystals.

Since the IR beam should penetrate the sample, the penetration depth (d_p) is one of the important parameters in ATR-FTIR spectroscopy. The measure of the depth that the infrared beam enters into the sample is defined by equation (2):

$$d_p = \frac{1}{\left[2\pi W n_1 \left(\sin^2\theta - n_{21}^2\right)^{\frac{1}{2}}\right]} \tag{2}$$

where d_p is the depth of penetration, W the wavenumber, n_1 the refractive index of the ATR crystal, θ the angle of incidence, and n_{21} the fraction $\frac{n_2}{n_1}$.

Each of the parameters mentioned above has important messages to teach us about the ATR technique and its application. Readers interested in details of the theory of ATR should consult the respective literature [16, 25, 27].

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3. Experimental processing

One of the advantages of the ATR-FTIR technique is that an experiment can be easily conducted to study the interactions between a chosen probe molecule and the surface of different metal oxides. The whole procedure consists in the preparation of a thin film of nanoparticles of the chosen metal oxide on the ATR crystal. This thin film should be stable, at least during the experiment, and its thickness should allow the penetration of the IR beam to reach the interface, e.g., the sample solution above the oxide layer. A thin homogeneous layer of the nanoparticles on the ATR crystal is generally produced from their suspension in an adequate solvent. This suspension is carefully drop-casted on the IRE material. Examples of the preparation of these thin layers, especially those made of TiO_2, can be found elsewhere [18, 19, 23, 33].

It is worth noting that the contact between the probe molecule and the layer can lead to a change in some operational parameters such as pH, temperature, and ionic strength of the supernatant solution. Therefore, studies on adsorption phenomena are better carried out employing flow cell reactors either in the liquid or the gas phase (see Figure 2a) where the solution or the dispersant circulate continuously over the layer. This allows the control of the above-mentioned parameters and the monitoring of the evolution in time of the system under different conditions. As an alternative, a sample batch system can also be employed where the inlet and outlet are closed (Figure 2b) [23].

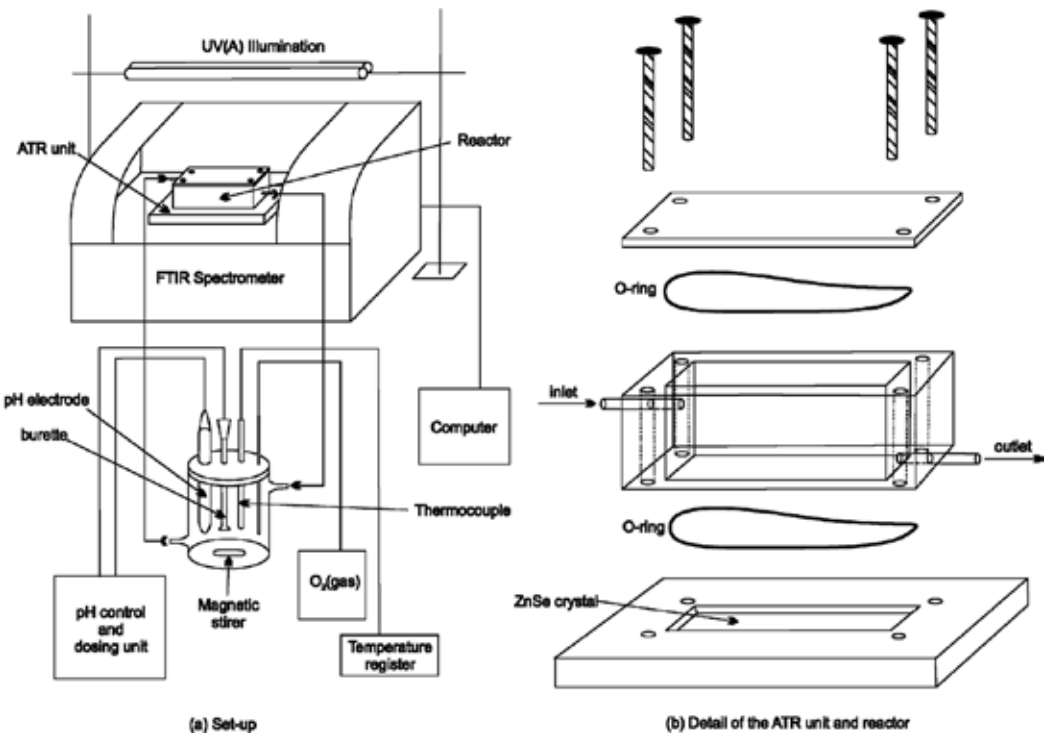

Figure 2. Flow cell reactor for ATR-FTIR spectroscopic studies (Reproduced from [23] with permission of the PCCP Owner Societies).

Prior to coating the ATR crystal, a spectrum of the blank ATR crystal is collected for spectral processing. Mainly, two different approaches can be used for the spectral processing. The first one is the normalization of the spectra of the ligand to that of the matrix (solvent at the pH of interest in the liquid phase or dispersant in the gas phase) from which a spectrum is collected. The probe molecule is then introduced and the corresponding spectrum is collected. The spectrum of the probe molecule is then referenced to the background spectrum (solvent/dispersant). The second approach is as follows: after preparing the thin film, a spectrum of the solvent at the pH of interest (or of the dispersant in the gas phase) is collected; the probe molecule is introduced and a spectrum is collected; the single beam spectrum of both solvent/dispersant and of the probe molecule in the solvent/dispersant is referenced to the blank ATR crystal to obtain the absorbance spectra of each. Subsequently, the absorbance spectrum of the solvent/dispersant is subtracted from the spectrum of the probe molecule. To collect spectra for the probe molecule alone the same experimental process is used but without the nanoparticle thin layer [30].

4. Probing interfacial reactions by ATR-FTIR investigations

4.1. Metal oxide-water interface

Considering its relevance to semiconductor photocatalysis, water splitting, and other important applications, the interaction of water with metal oxide surfaces, especially TiO_2, has been the focus of several experimental and theoretical investigations over the last decades [3, 4, 6, 8, 34–36]. Molecular, dissociated, and undissociated states of water adsorbed at a solid surface have been suggested. In addition to that, a mixture of these adsorption states is possible.

ATR-FTIR spectroscopy is one of the suitable techniques to investigate the adsorption of water molecules on a metal oxide surface under a wide range of conditions [37–39]. From many perspectives, numerous experimental and theoretical water adsorption studies have been conducted by means of ATR-FTIR spectroscopy [17, 37, 40, 41].

Figure 3 depicts the typical spectra of water adsorbed on TiO_2 (anatase/rutile Evonik-Degussa Aeroxide TiO_2 P25) [38]. The broad absorption band at around 3600–2800 cm^{-1} and the small peak at 3696 cm^{-1} are well-known to be the stretching vibration modes of the H_2O molecules, which have complex interactions through hydrogen bonds, and the end part of polymerically chained H_2O molecules without hydrogen bonds, respectively. The broad band contains not only the components of the H_2O molecules with different numbers of hydrogen bonds but also the Fermi resonance attributed to the overtone absorption of the bending mode δ (H_2O) at 1637 cm^{-1}. Therefore, it is difficult to analyze the detailed adsorption state of the polymerically chained H_2O molecules on metal oxide surfaces only from FTIR (mid-infrared) measurements [38]. However, based on the information obtained from such IR spectra, ATR-FTIR spectroscopy has been used for the characterization and identification of intermediate mechanisms involved in environmental interfaces [42], mainly during photocatalytic oxidation processes induced at the TiO_2-water interface [37,40].

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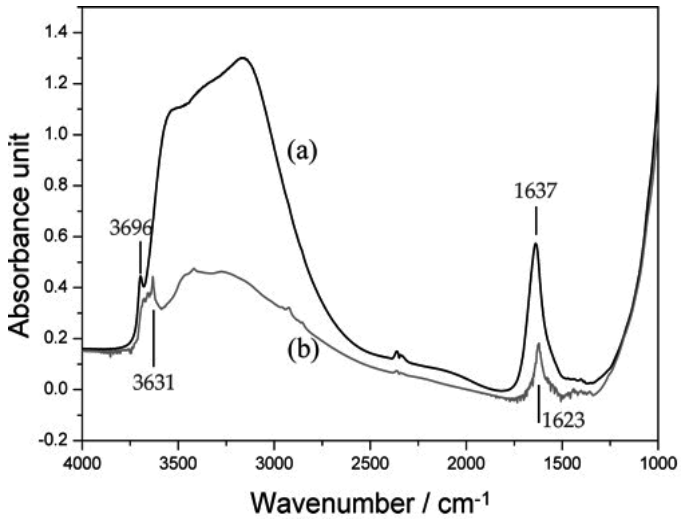

Figure 3. FT-IR (MIR) absorption spectra of TiO_2 (Evonik-Degussa Aeroxide TiO_2 P25) in air (a) and after evacuation at room temperature for 1 h (b) (Reprinted with permission from Takeuchi M, Martra G, Coluccia S, Anpo M. Investigations of the Structure of H_2O Clusters Adsorbed on TiO_2 Surfaces by Near-Infrared Absorption Spectroscopy. Journal of Physical Chemistry B; 109(15):7387–91. Copyright (2005) American Chemical Society).

Starting from the hypothesis that adsorbed H_2O changes its conformation due to the co-adsorption of cyclohexane on TiO_2 (anatase, Sachtleben Hombikat UV100), Almeida et al. [40] have shown with the help of additional DFT (Density Functional Theory) calculations, yielding the adsorption energy and the structure of the water molecule at different hydration levels (Figure 4), that at least three layers of water are formed during the adsorption process. The first layer includes only chemisorbed H_2O molecules. The second hydration level includes physisorbed (H-bonded) H_2O molecules on surface OH sites, and the highest hydration level contains an additional adsorbed water layer. The dissociative chemisorption of water is assumed to be energetically favored. In addition to that, dissociative chemisorption of water generates at least two different Ti-OH groups. At least one of these two new OH sites contains an oxygen atom originally originating from the TiO_2 lattice structure [40]. This finding allowed the authors to provide a spectral and structural interpretation of the mode of adsorption of cyclohexanone on the hydrated TiO_2 surface [40].

Besides of that, several research reports have identified and specified the different bending modes and structures of water on the TiO_2 surface during, before, and after UV light irradiation. It has been reported that UV irradiation induces a structural ordering of the adsorbed water layer [43], or results in an increase in the amount of surface OH groups, thus increasing the hydrophilicity of the TiO_2 surface [44]. Mendive et al. [37] have revealed by ATR-FTIR studies that the disaggregation of particle agglomerates plays an important role in UV illuminated aqueous TiO_2 nanoparticulate systems.

However, it should be noted here that the exact nature of the adsorption of water is still a matter of discussion in the field of metal oxide (especially of TiO_2) photocatalysis. This is a

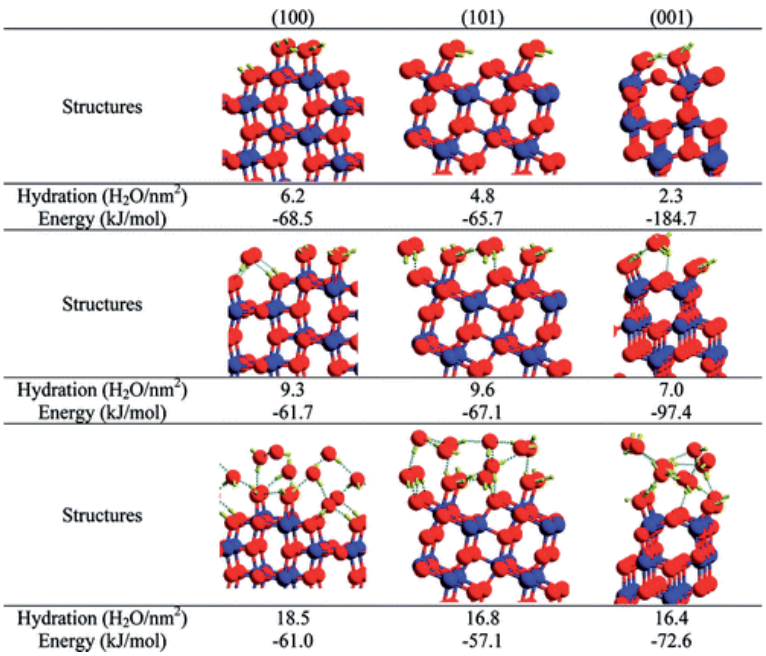

Figure 4. Adsorption energies and structures of H_2O on TiO_2 (100), (101), and (001) facets, at different hydration levels (Reprinted with permission from Almeida A, Calatayud M. Combined ATR-FTIR and DFT Study of Cyclohexanone Adsorption on Hydrated TiO_2 Anatase Surfaces. Journal of Physical Chemistry C; 115(29):14164–14172. Copyright (2011) American Chemical Society).

consequence of the diverse possibilities of interpretation arising from the combination of experimental results obtained by ATR-FTIR spectroscopy and by other techniques. Obviously, there is not yet a general consensus on the mechanism of adsorption of water on TiO_2.

4.2. Interactions of probe molecules with the metal oxide surface

ATR-FTIR spectroscopy yields important insight into the surface speciation of probe molecules adsorbed on nanomaterials [30]. Chemical or inner sphere adsorption is generally studied when it is expected that the probe molecule is able to coordinate with the metal ions of the substrate covering the ATR crystal [15].

Investigations of the interaction of a large number of ligands on metal oxide, metal hydroxide, and metal oxyhydroxide systems have been performed employing ATR-FTIR spectroscopy [26]. The objective of these investigations is to obtain an insight into the chemical nature of these interactions, being either qualitative such as the mode of adsorption and the surface speciation, or quantitative such as the kinetics and the surface coverage.

It is worth to note that TiO_2 nanoparticles are much more extensively used as substrates as compared with other metal oxides. The adsorption of organic compounds bearing common functional groups such as acids [23, 45, 46], amino acids [47], phenolic compounds [11, 48], and a few complex heteroaromatic compounds [49–52] has been studied in detail (cf. Table 3).

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As an example, a typical ATR-FTIR spectrum of an aqueous solution of the herbicide imazapyr in the absence and presence of a TiO_2 layer is presented in Figure 5. The reliability of information obtained from the IR spectra is dependent mainly upon the correct assignment of the vibrational modes by comparison with published spectroscopic data [15, 30]. Mudunkotuwa et al. have presented a summary of several common IR absorption band frequencies (Table 2) [30]. Furthermore, the infrared spectral data collected for coordination compounds [53] are very useful when interpreting the spectra of adsorbates, which mostly resemble those of ligands of coordination compounds [15]. In addition to that, the interpretation of the increase in the intensities of the bands of functional groups, as well as the shifting of these bands either to the blue or to the red spectral regions also provide important information concerning the type of interaction between adsorbate and surface. The interpretation of IR bands is very helpful for a qualitative analysis, e.g., concerning the points of interactions, the modes of adsorption, and the molecular speciation, respectively.

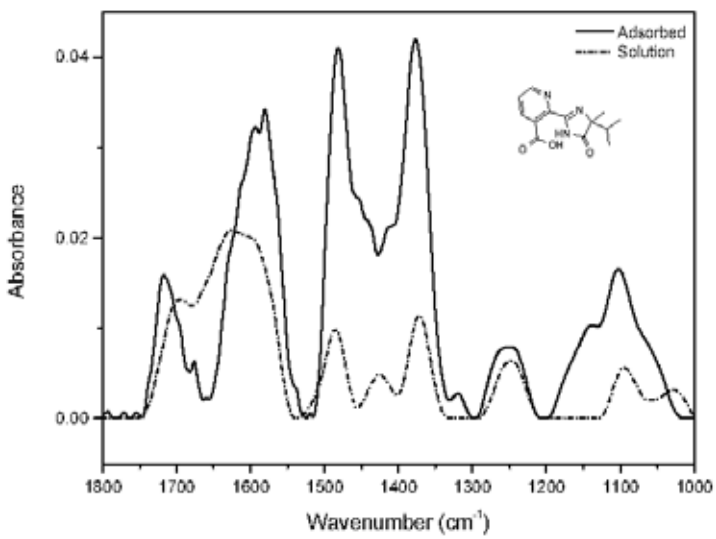

Figure 5. ATR-FTIR spectra of 8×10^{-3} mol L^{-1} imazapyr aqueous solution at pH 3 (dashed lines); and 2×10^{-3} mol L^{-1} imazapyr aqueous solution in contact with a TiO_2 film (solid lines). Reference spectra were of water in contact with the bare ZnSe prism and of the bare TiO_2 film respectively [54].

As mentioned above, the complexity of the obtained IR spectra usually requires the combination of different techniques to enable their interpretation. Generally, the deductions resulting from the analysis of the IR spectra have to be supported by the results of other experimental techniques and/or by theoretical calculations. Several experimental and theoretical studies on the adsorption of aliphatic mono- and di-carboxylic acids on metal oxide surfaces have been performed [46]. It is assumed that the binding of carboxylates at the solid metal oxide surface occurs in several ways such as physisorption through electrostatic attraction and hydrogen bonding, and chemisorption in different modes including monodentate, bridged bidentate, and chelating bidentate adsorbed structures [55–57]. These different binding modes can be

Vibrational Mode	Wavenumber (cm^{-1})
ν(C=O)	1730–1720
$\nu_{asym}(COO^-)$	1620–1590
$\nu_{sym}(COO^-)$	1410–1390
$\delta_{asym}(NH_3^+)$	1630
$\delta_{sym}(NH_3^+)$	1571
$\nu_{asym}(CH_2)$	2920
$\nu_{sc}(CH_2)$	1442–1438
$\nu_w(CH_2)$	1400–1200
$\nu_r(CH_2)$	730–720
ν_{sym}(NH)	3300–3100
$\nu_{sym}(C=O)^{major} + \nu_{sym}(C-N)^{minor}$	1700–1600 (Amide I)
$\nu_{sym}(C-N) + \delta\ (N-H)_{out\ of\ phase}$	1580–1510 (Amide II)
$\nu_{sym}(C-N) + \delta\ (N-H)_{in\ phase}$	1400–1200 (Amide III)

Table 2. IR absorption frequencies of common organic functional groups (Adapted from [30] with permission of The Royal Society of Chemistry).

distinguished in an infrared spectrum by the difference $\Delta\nu_{a\text{-}s}$ of the frequencies of the asymmetric and the symmetric mode of the carboxylate stretching vibration. By comparing the $\Delta\nu_{a\text{-}s}$ of free aqueous carboxylate, $\Delta\nu_{a\text{-}s}$ (free), to the $\Delta\nu_{a\text{-}s}$ (adsorbed) values measured in transition metal complexes, the following correlations were found [46, 56, 58]:

$\Delta\nu_{a\text{-}s}$ (adsorbed) > $\Delta\nu_{a\text{-}s}$ (free): monodentate coordination

$\Delta\nu_{a\text{-}s}$ (adsorbed) < $\Delta\nu_{a\text{-}s}$ (free): bidentate chelating or bridging

$\Delta\nu_{a\text{-}s}$ (adsorbed) << $\Delta\nu_{a\text{-}s}$ (free): bidentate chelating, unless short metal-metal bonds are present

DFT calculations have been performed by Vittadini et al. for several possible adsorption conformations of formic acid and sodium formate on the anatase surface to support the interpretation of ATR-FTIR spectra measured of formic acid adsorbed on the TiO_2 surface [59]. The comparison of the calculated results with this experimental information enabled the identification of seven different surface species (see Figure 6). On the hydrated surface, both HCOOH and HCOONa preferentially form inner-sphere adsorption complexes. HCOOH as monodentate adsorbate dissociates due to the interaction with a nearby water molecule, while HCOONa prefers a bridging bidentate structure [59].

Mono-carboxylic acids, i.e., formic and acetic acid, were found to bind on ZrO_2 and Ta_2O_5 surfaces in both protonated and deprotonated carboxylic acid forms indicating a bridging bidentate adsorption. Under the experimental conditions of this work no adsorption of formic acid onto TiO_2 and Al_2O_3 was observed [46].

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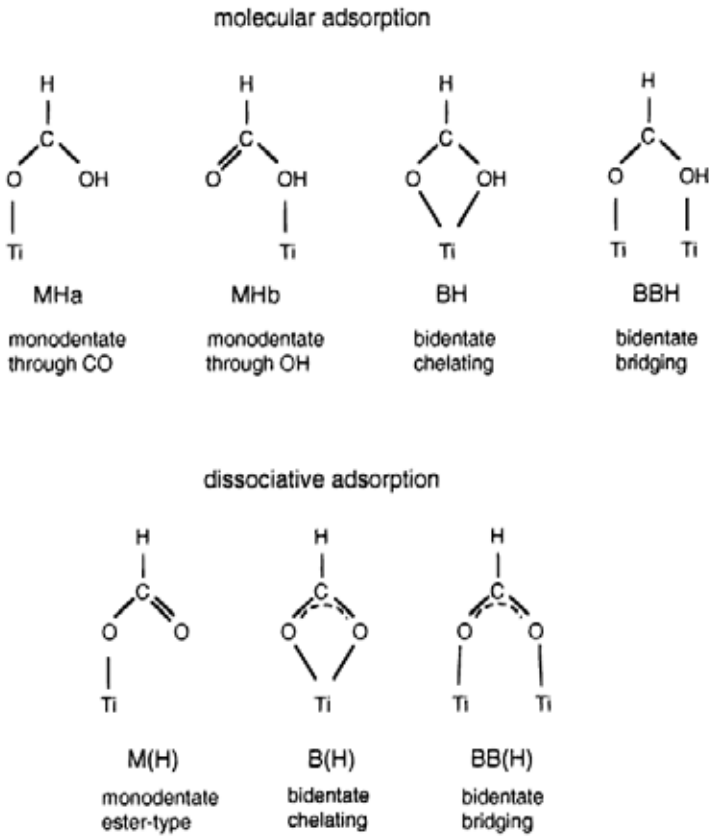

Figure 6. Possible configurations for HCOOH and $HCOO^-$ species bound to metal cations (Reprinted with permission from Vittadini A, Selloni A, Rotzinger FP, Grätzel M. Formic Acid Adsorption on Dry and Hydrated TiO_2 Anatase (101) Surfaces by DFT Calculations. Journal of Physical Chemistry B; 104(101):1300–1306. Copyright (2000) American Chemical Society).

Dicarboxylic acids adsorb much more strongly to oxide surfaces than mono-carboxylic acids due to both electrostatic and chemical interactions.

Oxalic acid is one of the most investigated molecules in this regard [18, 45, 58, 60, 61]. Based on a series of spectra recorded at varying different experimental parameters (concentration, pH, and ionic strength), and supported by the comparison of these spectra with those of the aqueous $[Fe(Ox)_y]_z$ complex, Hug et al. [18] described several surface complexes formed during the adsorption of oxalic acid at the TiO_2 P25 surface. The obtained data strongly support the assumption that oxalate forms specific inner-sphere coordination complexes with surface Ti^{4+} sites. These complexes are formed through bidentate bridging or monodentate bending modes.

Mendive et al. have published several papers presenting experimental results of their investigation of the TiO_2-oxalic acid system using both pure anatase and rutile phases. In addition to that, data of quantum chemical calculations using Modified Symmetrically Orthogonalized

Intermediate Neglect of Differential Overlap (MSINDO) have been presented to yield a complete insight into the TiO_2-oxalate system [23, 24, 62, 63]. A detailed analysis of the experimental ATR-FTIR data and the data obtained from theoretical calculations (IR spectra (Figure 7) and calculated bending energies) has led to the suggestion of different adsorbate structures of oxalic acid either on anatase or on rutile nanoparticles (Figure 8). By comparison between both TiO_2 phases (anatase and rutile), the difference as well as the similarity in the adsorption of oxalate can be explained either by the mode of adsorption, the structure of the surface complexes, the surface speciation of either TiO_2 phases, or the adsorption energies.

Young et al. [45] have published the results of an ATR-FTIR study focused on the adsorption-desorption kinetics of oxalic acid on the anatase TiO_2 surface. The measured spectra were not found to be well resolved. However, based on the absorbance versus time behavior, the authors were able to extract the pseudo-first-order rate constants corresponding to the three expected adsorbed species of oxalic acid at the TiO_2 surface.

Furthermore, Mendive et al. [61] have proposed the mechanism of the photocatalytic degradation of oxalic acid with the help of the above mentioned experimental and theoretical investigations [24, 63]. The possible pathways for the formation of oxalic acid photoproducts, as well as the role of the TiO_2 surface as active surface have been discussed in detail [61]. An example of the proposed degradation pathways of the oxalic acid surface complexes is depicted in Figure 9.

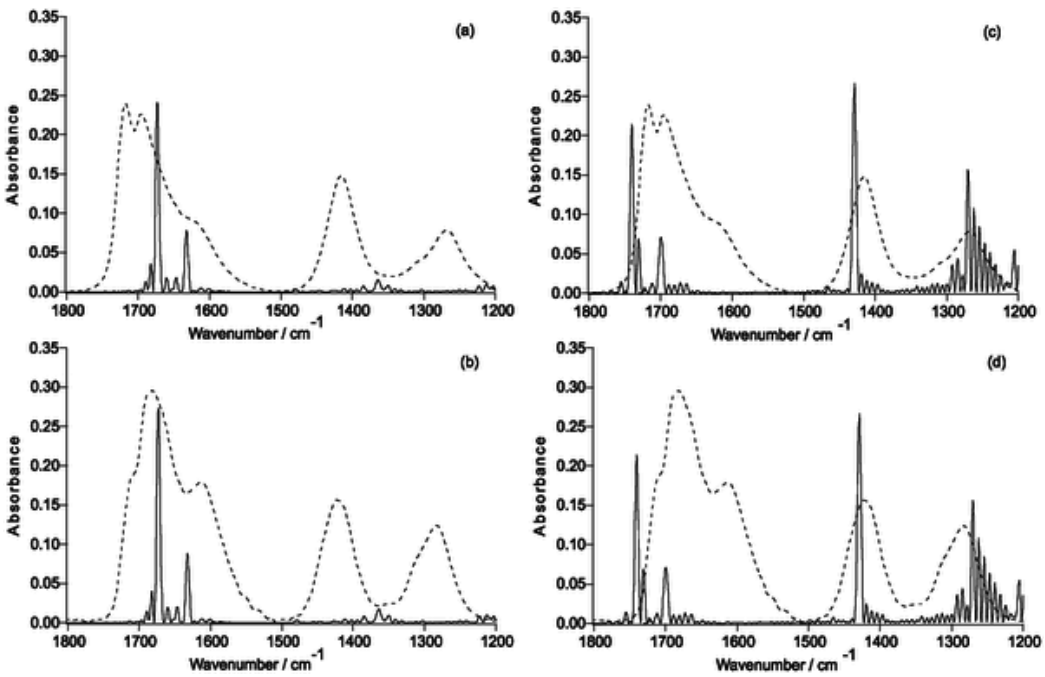

Figure 7. Experimental and calculated FTIR spectra of oxalic acid on anatase (Reproduced from [23] with permission of the PCCP Owner Societies).

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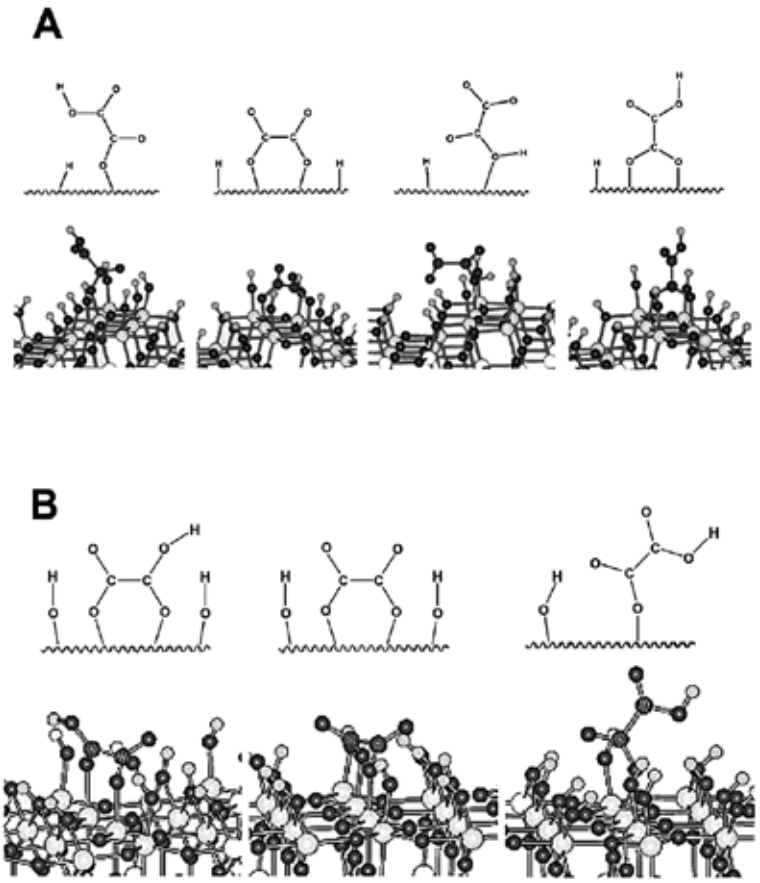


Figure 8. Adsorbed structures of oxalic acid on anatase (A) (Reproduced from [24] with permission of the PCCP Owner Societies) and Rutile (B) (Reproduced from [63] with permission of the PCCP owner Societies) in equilibrium in the dark. A scheme of every structure is provided. Ti, O, H and C atoms are represented by large light, dark, small light and dark-dashed spheres respectively.

As mentioned in the introduction of this chapter, the direct evidence for the formation of structurally different surface complexes is an important step in the understanding of metal oxide photocatalysis, especially of TiO_2 photocatalysis. This is due to the fact that the reactivity and the pathways for product formation are determined by the structures of the formed surface species during the dark adsorption. Recently, Montoya et al. [11] have investigated the interaction of the TiO_2 surface with three probe molecules, *e.g.*, formic acid, benzene, and phenol employing ATR-FTIR spectroscopy. Based upon the analysis of the IR spectra (Figure 10), assumptions have been made concerning the physisorption of benzene (no changes have been observed in the spectra with and without the TiO_2 layer), the strong chemisorption of formic acid, and also the role of the solvent (water or acetonitrile) for the adsorption mode of phenol. Based on these results, the authors provided an insight into the mode of interaction of the probe molecules with the TiO_2 surface (chemisorption or physisorption) (Figure 11). In addition to that, they discussed the photocatalytic oxidation mechanism induced either by the

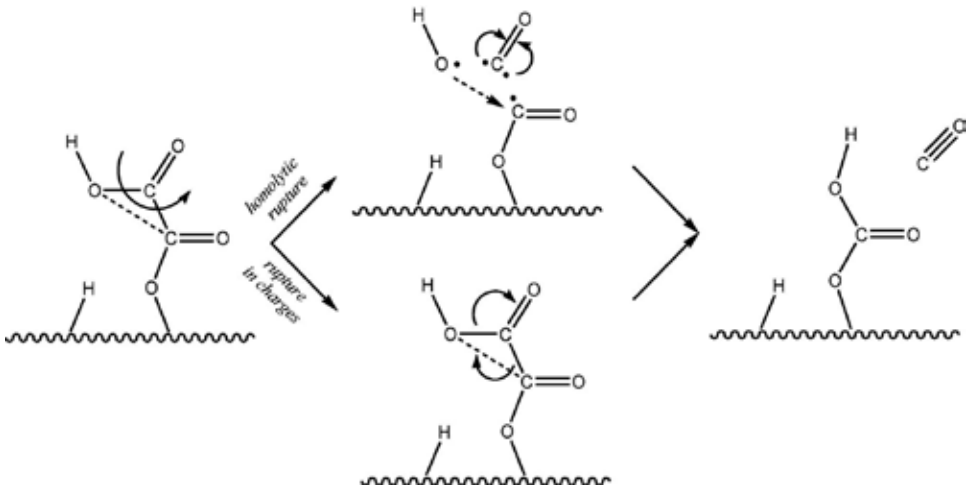
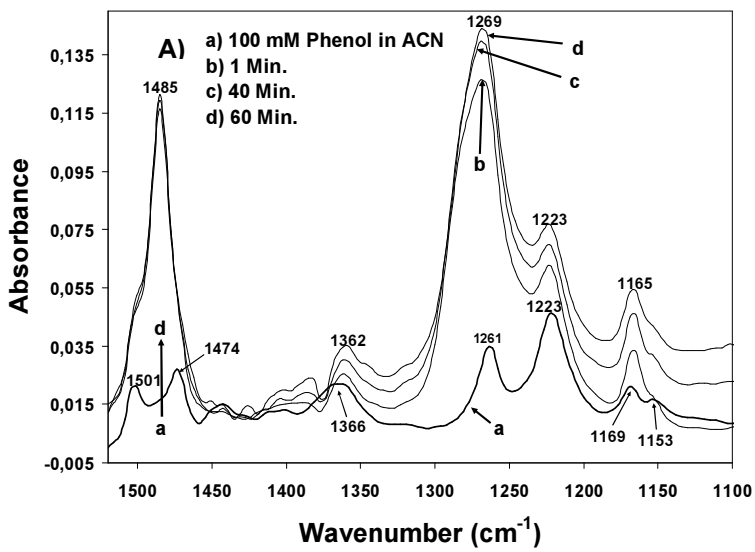


Figure 9. Possible photocatalytic degradation pathways of species adsorbed on anatase (Reprinted from Oxalic Acid at the TiO_2/Water Interface under UV(A) Illumination: Surface Reaction Mechanisms, Cecilia B. Mendive, Thomas Bredow, Jenny Schneider, Miguel Blesa, Detlef Bahnemann. Journal of Catalysis 2015, 322:60-72, Copyright (2015), with permission from Elsevier).

reaction of surface trapped holes with the adsorbate (direct pathway) or the reaction of photocatalytically generated ˙OH radicals with the physisorbed molecules (indirect pathway). The authors concluded that formic acid is directly oxidized due to its strong chemisorption onto the TiO_2 surface, while physisorbed benzene is indirectly oxidized. For phenol the authors suggested a combination of both pathways [10, 11].



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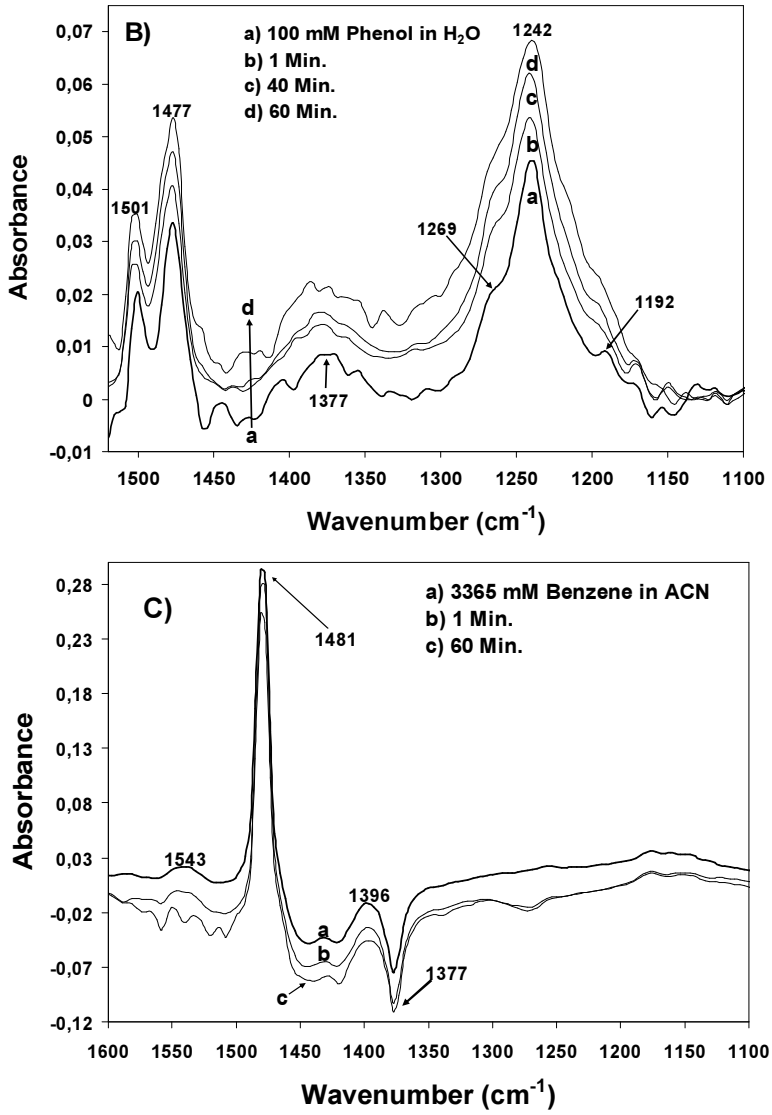

Figure 10. ATR-FTIR spectra of: A) a 100 mM solution of acetonitrile dissolved phenol, in the absence (a) and in the presence of an anatase film under different TiO_2-Phenol contact times (b-d); B) a 100 mM solution of water dissolved phenol (pH 3) in the absence (a) and in the presence of a TiO_2 anatase film, under different TiO_2-Phenol contact times (b-d); C) a 3365 mM solution of acetonitrile dissolved benzene in absence (a) and presence of a TiO_2 anatase film under different TiO_2-Benzene contact times (b-c) (Reprinted with permission from Montoya JF, Atitar FM, Bahnemann DW, Peral J, Salvador P. Comprehensive Kinetic and Mechanistic Analysis of TiO_2 Photocatalytic Reactions According to the Direct-Indirect (DI) Model: II) Experimental Validation. Journal of Physical Chemistry C; 28(118):14276–14290. Copyright (2014) American Chemical Society).

In addition to the presented examples, several experimental and theoretical studies have been performed concerning the adsorption and various photocatalytic reactions by means of the ATR-FTIR technique. Table 3 presents a survey of published data on the adsorption as well as

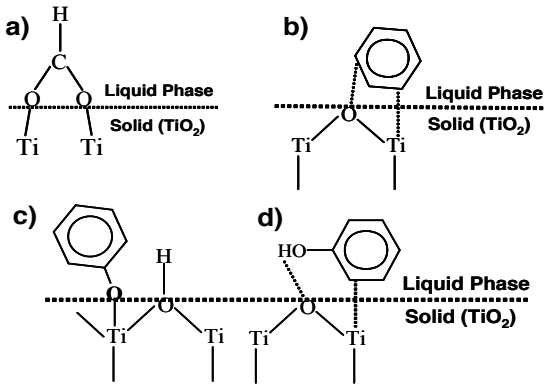

Figure 11. Interaction modes of benzene, formic acid and phenol, model organic compounds with the TiO_2 surface (Reprinted with permission from Montoya JF, Atitar FM, Bahnemann DW, Peral J, Salvador P. Comprehensive Kinetic and Mechanistic Analysis of TiO_2 Photocatalytic Reactions According to the Direct-Indirect (DI) Model: II) Experimental Validation. Journal of Physical Chemistry C; 28(118):14276–90. Copyright (2014) American Chemical Society).

the photooxidation of aqueous organic compounds on metal oxide surfaces studied by means of ATR-FTIR spectroscopy.

Adsorbate or reactant	Material	Study	Ref
Acetic acid	Rutile	Adsorption	[64]
Acetate	Rutile	Adsorption	[65]
Acrylic acid	P25	Adsorption	[66]
Poly(Acrylic acid)	Hematite	Adsorption	[67]
L-α-alanine	P25	Adsorption	[68]
Amino acid	P25	Photo-Oxidation	[69]
	Au/TiO_2		
p-Arsanilic acid	Iron-(Oxyhydr)Oxides	Adsorption	[70],[71]
Aspartic acid	TiO_2 (synthesis)	Adsorption	[72]
Benzoic acid / benzoate	Aluminum Hydroxide	Adsorption	[73]
Boric acid	Hydrous Ferric Oxide	Adsorption	[74]
Catechol	P25	Photo-Oxidation	[75]
		Adsorption	[76]
	TiO_2 (synthesis)	Adsorption	[48]
	Cr_2O_3	Adsorption	
	MnO_2	Adsorption	
	Fe_2O_3	Adsorption	
4-Chlorocatechol	P25	Adsorption	[77]
Citric acid	Rutile	Adsorption	[64]

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	Anatase		[78]
m-Cresol	P25	Adsorption	[76]
o-Cresol			
Cyclohexane	UV100	Adsorption	[33],[79]
		Photo-Oxidation	
Cyclohexanone	TiO_2 (synthesis)	Adsorption	[80]
Cysteine	P25	Adsorption	[47]
Dextrin	Anatase	Adsorption	[22]
Dicarboxylates	Fe(III)(hydr)oxides	Photo-Oxidation	[81]
α-hydroxydicarboxylates			
Dihydroxyphenylalanine	Rutile	Adsorption	[82]
Dimethylarsinic acid	Iron-(Oxyhydr)Oxides	Adsorption	[70]
E. Coli	P25	Photo-Oxidation	[83]
Ethanol	TiO_2 (synthesis)	Photo-Oxidation	[84]
Formic acid	Rutile	Adsorption	[64]
	TiO_2 (synthesis)		[55]
Formate	Rutile	Adsorption	[65]
Fumaric acid	Hematite	Adsorption	[85]
Gallic acid	P25	Photo-Oxidation	[86]
Glutamic acid	TiO_2 (synthesis)	Adsorption	[72]
Glyoxylic acid	P25	Adsorption	[87]
Isopropyl	P25	Adsorption	[88]
Methylphosphonofluoridate		Photo-Oxidation	
Lactic acid	P25	Adsorption	[66]
Maleic acid	Hematite	Adsorption	[85]
Malonate	Rutile	Adsorption	[58]
	Anatase		
Malonic acid	Au/TiO_2 (synthesis)	Photo-Oxidation	[89]
	P25		[90]
Nicotinic acid	TiO_2 (synthesis)	Adsorption	[51]
Nitrate	Al_2O_3	Adsorption	[91]
o-Phthalic acid	Hematite	Adsorption	[92]
Oxalate	Anatase	Adsorption	[58]
	Rutile		
Oxalic acid	Anatase	Photo-Oxidation	[61]
	Rutile	Adsorption	[45]
	Anatase		

Phenol	Anatase	Adsorption	[11]
	P25	Photo-Oxidation	[93]
4,4'-Bis(2-sulfostyryl)biphenyl	Rutile	Photo-Oxidation	[94]
	Anatase		
	Hematite		
	δ-Alumina		
	Lepidocrocite		
β-Picoline	TiO_2 (synthesis)	Adsorption	[51]
Polyacrylates	Hematite	Adsorption	[95]
1,2-propylene glycol	P25	Photo-Oxidation	[93]
Pyridine	P25	Adsorption	[96]
Pyruvic Acid	P25	Adsorption	[66]
Pyridine-3-carbaldehyde	TiO_2 (synthesis)	adsorption	[97]
			[51]
Ru-bpy	TiO_2 (synthesis)	Adsorption	[98]
Succinate	Rutile	Adsorption	[58]
	Anatase		
	Lepidocrocite		
Succinic acid	Hematite	Adsorption	[85]
	P25	Photo-Oxidation	[90]
Toluene	TiO_2 (synthesis)	Photo-Oxidation	[99]
	P25-TiO_2		[100]

Table 3. Selection of previously published ATR-FTIR studies concerning the adsorption and photooxidation of common ligands on metal oxides surfaces.

5. Concluding remarks

The ATR-FTIR technique offers the chance to obtain novel information concerning interfacial processes *in situ*. This information can be used to explain surface reaction mechanisms. Hence, ATR-FTIR studies are becoming increasingly popular as an investigative technique and may now be considered as one tool of choice in the field of interfacial chemistry when compared to other techniques, particularly in obtaining data under ambient conditions. This is due to the major advantages of ATR-FTIR spectroscopy such as the *in situ* data collection, as well as the high sensitivity, simplicity, and rapidity of the measurements.

The use of ATR-FTIR in the area of photocatalysis is of great importance since it provides both qualitative and quantitative molecular insight into interfacial processes occurring in the dark (adsorption) and under UV illumination (adsorption/desorption and chemical reactions). The information obtained will often be the key for a deeper understanding of the mechanisms occurring in metal oxide photocatalysis.

http://dx.doi.org/10.5772/60887

Acknowledgements

The present study was performed within the project "Establishment of the Laboratory of Photoactive Nanocomposite Materials" (No. 14.750.31.0016) supported by a Grant from the Government of the Russian Federation. M. Faycal Atitar gratefully acknowledges a scholarship from the DAAD in the Frame of a Sandwich-Program.

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Edited by Marcelo L. Larramendy and Sonia Soloneski

This edited book, Emerging Pollutants in the Environment Current and Further Implications, includes overviews by significant researchers on the topic of emerging pollutants toxicology, which covers the hazardous effects of common emerging xenobiotics employed in our every day anthropogenic activities. We hope that this book will meet the expectations and needs of all those who are interested in the negative implications of several emerging pollutants on living species.

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