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Environmental monitoring and molecular mechanistic insights on pesticides in waters and in the bivalve *Scrobicularia plana*, from Mondego and Tagus estuaries and Ria Formosa Lagoon

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AUTHOR STATEMENT

This Thesis includes six articles published in international journals, which are presented as Chapter 2-6, and 8 according to the references:

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All the works that contributed to this Thesis were made by the candidate in close cooperation/co-authorship with supervisors and other researchers. The conception and full draft of the Introduction and the Final Remarks were the sole responsibility of the candidate, being subject to revision by the supervising team. In all other Chapters, the candidate made substantial contributions to conception, design, data acquisition, and data analysis and interpretation. The candidate drafted all Thesis Chapter and approved their final versions.

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Resumo

O principal objectivo desta Tese foi identificar e quantificar pesticidas de três sistemas estuarinos Portugueses, de forma a realizar um diagnóstico ambiental, tendo em conta os regulamentos Europeus, e estimar eventuais impactos nas cadeias tróficas. Devido à sua localização, os estuários e outros importantes sistemas aquáticos costeiros (como as rias) são sujeitos a concentrações significativas de contaminantes, provenientes de diversas atividades antropogénicas, criando uma "sopa tóxica" para a biota local; e até influenciar humanos. Esta Tese reporta concentrações de cinquenta e seis pesticidas, conforme avaliados em três ecossistemas e três matrizes, tendo em conta possiveis flutuações espaço-temporais.

Em 2010 e 2011 foram recolhidas amostras de águas da Ria Formosa e dos estuários dos Rio Mondego e Rio Tejo, abrangendo todas as estações do ano, de forma a realizar uma primeira avaliação nestes sistemas estuarinos. As amostras (500 mL) foram pré-concentradas 2500 vezes, por extração de fase sólida, e analizadas por cromatografia gasosa acoplada a espectrometria de massas (GC-MS). Foram quantificados quarenta e sete pesticidas na Ria Formosa, atingindo somas médias totais de 11000 ng/L; 17% dos compostos quantificados excederam os valores médios anuais de qualidade ambiental, para substâncias prioritárias e outros poluentes, definidos pela Diretiva Europeia 2013/39/EU. Foram quantificados quarenta e sete biocidas no estuário do Mondego e cinquenta e quatro no Tejo, atingindo montantes totais médios de 5750 ng/L e 2800 ng/L em cada ecossistema; comparando o total das concentrações médias com as normas de qualidade ambiental da Diretiva 2013/39/UE, 19% e 14% dos compostos estavam acima dos níveis europeus estabelecidos para as águas de transição.

Como em todas as situações se verificaram quantidades substanciais de pesticidas, sugerindo um impacto considerável, foi executada uma segunda campanha (2012-2013), para analisar com maior detalhe estes compostos, em três matrizes diferentes; duas delas — fase aquosa dissolvida (DAP) e material particulado em suspensão (SPM) — foram recolhidas de águas superficiais, enquanto a terceira visou o bivalve *Scrobicularia plana*, como modelo biológico para estudos ambientais. A espécie, como filtradora detritívora e séssil, é indicativa de padrões de bioacumulação locais que podem afetar níveis tróficos superiores, atingindo também os seres humanos, por consumo direto/indireto.

Na Ria Formosa quantificaram-se quarenta e oito, trinta e um, e cinquenta e quatro pesticidas nas matrizes DAP, SPM, e bivalves, respetivamente. O perfil de contaminação entre matrizes foi marcado por somas totais médias de 1800 ng L (DAP), de 12,7 mg/kg (SPM), e 0,7 mg/kg (bivalves); várias amostras exibiram valores acima dos níveis da Diretiva 2013/39/UE.

No estuário do Rio Tejo foram quantificados dezanove pesticidas na matriz DAP, trinta e seis na matriz SPM, e cinquenta e três nas amostras de bivalves. Foram registados somas totais médias de 1750 ng/L, 22,3 mg/kg e 1,0 mg/kg, na matriz DAP, SPM, e nas amostras de bivalves. Considerando as médias anuais, 53% das amostras DAP e SPM e 64% das amostras de bivalves, excederam os níveis estabelecidos pela Directiva 2013/39/UE. Considerando os pesticidas detetados e quantificados procedeu-se ao cálculo de quocientes de risco teóricos onde foram estimados potenciais riscos para os organismos aquáticos, nomeadamente invertebrados.

Para entender melhor os mecanismos subjacentes à ação dos pesticidas em invertebrados, particularmente em bivalves, foram realizados estudos usando a *S. plana*. A função do receptor nuclear e a sua ligação a xenobióticos começou a ser estudada. O domínio de ligação ao ligando foi isolado pela primeira vez e identificado como NR1Jβ. Os ensaios de transactivação foram usados como uma ferramenta para a avaliação e comparação entre o receptor X do pregnano humano e o NR1Jβ da *S. plana*. Nesta primeira abordagem foram utilizados três compostos — dois pesticidas (esfenvalerato e triclosan), usando como composto de referência uma toxina natural (ácido ocadaico) — levando a diferentes respostas de transactivação. Os resultados indicam distintas potências de ligação e de eficácia, explicados tanto pela natureza e estrutura dos compostos-alvo como pelas concentrações testadas.

Em suma, desenvolveram-se metodologias analíticas eficazes que permitiram a identificação/quantificação de pesticidas em três grandes sistemas aquáticos costeiros portugueses, tendo em conta fatores espaciais e temporais, em três matrizes, estimando-se possíveis impactos dos poluentes. Os resultados mostram que a poluição por pesticidas existe e é bastante relevante. O trabalho subsequente iniciado por técnicas de biologia molecular permitiu inferir novos mecanismos e efeitos de pesticidas em bivalves, abrindo novas "portas" para serem exploradas em pesquisas futuras.

Abstract

The main objective of this Thesis was to identify and quantify pesticides on three Portuguese brackish water systems, so to make an environmental diagnose of the situation, namely in view of European regulations, and to estimate eventual impacts across trophic levels. Because of their location, estuaries and other key costal water systems (like lagoons) are loaded with significant amount of contaminants, from diverse anthropogenic activities, creating a "toxic soup" for local biota; humans can be struck too. The Thesis portrays the concentrations of fifty-six pesticides in three ecosystems and matrices, with consideration to possible spatial and temporal fluctuations.

Water samples from Ria Formosa Lagoon and from Mondego and Tagus River estuaries were collected during 2010-2011, covering all year seasons, to evaluate the primary status of these estuarine systems. Samples (500 mL) were pre-concentrated 2500 times by solid phase extraction and analyzed by gas chromatography-mass spectrometry (GC-MS). Forty-seven pesticides were quantified at Ria Formosa Lagoon, attaining total average sums of 11000 ng/L; 17% of the quantified compounds exceeded the annual average environmental quality standards (EQS) set for priority substances and certain other pollutants, as defined by the European Directive 2013/39/EU. In the Mondego and Tagus River estuaries, forty-seven and fifty-four biocides were quantified, respectively, reaching total average sums of 5750 ng/L and 2800 ng/L in each aquatic system; comparing the total average concentrations with the EQS set by the 2013/39/EU Directive, 19% and 14% of the compounds were above the European levels established for transitional waters.

Because all scenarios demonstrated the presence of substantial amounts of pesticides and were strongly suggestive of impacts, a second campaign (2013-2013) was conducted, to analyze these compounds more in-depth, in three different matrices; two of them — dissolved aqueous phase (DAP) and suspended particulate matter (SPM) — were collected from surface waters, while the third one applied the bivalve *Scrobicularia plana* as a biologic model for the environmental studies. The species, as a surface deposit and suspension feeder and sessile animal, can indicate local bioaccumulation patterns that may affect higher trophic levels, up to humans, by in/direct consumption.

Analyses at Ria Formosa Lagoon quantified forty-eight, thirty-one, and fifty-four pesticides in DAP, SPM, and bivalves matrices, respectively. The contamination profile among matrices was marked by total average sums of 1800 ng/L (DAP), 12.7 mg/kg (SPM), and 0.7 mg/kg (bivalves), respectively; with several samples exhibiting loads above the concentrations defined by the 2013/39/EU Directive.

In the Tagus River estuary, nineteen pesticides were quantified in DAP, thirty six in SPM, and fifty three in bivalve matrices. Total average sums of 1750 ng/L, 22.3 mg/kg, and 1.0 mg/kg were registered for DAP, SPM, and bivalve samples. Considering annual averages, 53% of the DAP and SPM samples and 64% of bivalve samples exceeded the defined levels established by the 2013/39/EU Directive. Considering the detected pesticides and their amounts, theoretical risk quotients pointed out potential hazards for aquatic organisms, mainly for invertebrates.

To better understand the mechanisms underlying the pesticide action in invertebrates, particularly in bivalves, further studies were carried out, using *S. plana*. The nuclear receptor function and its connection to xenobiotics started to be studied. The ligand-binding domain was first isolated and identified as NR1Jβ. Transactivation assays were applied as a tool for evaluation and comparison between the human pregnane X receptor and the NR1Jβ. In this first approach three compounds — two pesticides (esfenvarelate and triclosan) using as reference compound the natural toxin (okadaic acid) — were used, leading to different transactivation responses. The results indicate distinct ligand potency and efficacy, linked closely to the nature and structure of the target compounds and to their tested concentrations.

In sum, the development of effective analytical methodologies allowed the identification/quantification of pesticides in three major Portuguese aquatic costal systems, considering spatial and temporal factors, in various matrices, and estimation of their potential impacts. Data show that pesticide pollution of importance exists. Going a step further, molecular work was done to gains insights on mechanisms of pesticide/xenobiotic effects in bivalves, opening new exploratory “doors” for future research.

Background and goals

I. Persistent Organic Pollutants (POPs) – General Review

Since the industrial revolution and continuing with after World War II industrial advancements and economic growth, the production of chemicals have continuously enlarged; more recently, it was found that solely in the European market approximately 100 000 substances exist, from which 30 000 have an annual production over 1 tonne [1, 2]. The chemical industry employs over 1.2 million people and contributed to the economy with 527€ billion in 2013 [3]. In 2012, from the total worldwide chemical sales, Europe represented 22% (673€ billion), the entire Asia 55% (1 724€ billion), North and South America 21% (670€ billion), and the rest of the world 2% (60€ billion) [4].

The marketed substances are used for various purposes from the industrial area and disease control, through crop production until different consumer needs [2]. However, some of these compounds brought negative unexpected effects to the environment and human health [5-8]. Due to their chemical' nature, many of these organic substances are persistent and subject to accumulation in organisms, while having characteristics prone to impose noxious effects to human health [7, 9]. According to their molecular structure and atoms' nature, the substances present different properties [10], being some of them considered Persistent, Bioaccumulative and Toxic substances (PBTs) [11].

The Persistent Organic Pollutants (POPs) are defined as a subclass of PBTs with the following characteristics: (*i*) long life-span in soil, air and biota; (*ii*) easily transported by air, water and migratory species; (*iii*) toxic; and that (*iv*) bio-accumulate in terrestrial and aquatic ecosystems through the food chain, causing adverse environmental and human health effects [12]. POPs are carbon-containing and often halogenated chemical substances, characterized by low water solubility (hydrophobicity) and high lipid solubility (lipophilicity), leading to their bioaccumulation in fatty tissues [13]. Due to these characteristics and cross-border problems, international initiatives

have been taken to promote an effective regulation and management of POP compounds.

The United Nations Economic Commission for Europe (UNECE), funded at 1947, adopted in 1979 at the Convention on Long Range Transport of Air Pollution (CLRTAP) identifies the general principles for international cooperation on air pollution abatement and provides an institutional framework, bringing together science and policy [14]. However, only in 1990 the Executive Body of the Convention agreed to establish a task force on POPs. A work plan was adopted in 1995 and a list of selected substances was created taking in consideration the following criteria:

1. Evidence of environmental persistence (compounds with low vapour pressure (P), or showing more than 2 days of half-life in the atmosphere), and low biodegradability (*i.e.,* 30% of the compound still exist after 28 days of its release or present in remote areas);
2. Prioritization scoring based on bioconcentration factors or octanol-water partition coefficient (K_{ow}) and mammalian or aquatic toxicology;
3. Risk assessment.

From an initial list of 107 substances, 16 substances were identified for initial inclusion in the protocol (11 pesticides, 2 industrial products and 3 unintentional by-products; see Table 1) [11].

Subsequently, the Intergovernmental Forum on Chemical Safety (IFCS) and the International Programme on Chemical Safety (IPCS) prepared an assessment of the 12 worst POPs, known as the "*dirty dozen*". In May 2001, the Stockholm Convention on POPs — where the United States of America together with more 90 countries — agreed to reduce or eliminate the production, use, and/or release of 12 key POPs [15].

On 18 December 2009, seven more substances (see Table 1) were included and the obligations for DDT, heptachlor, hexachlorobenzene and PCBs, as well as the emission limit values (ELVs) from waste incineration were revised [16, 17].

Table 1: List of the priority substances (POPs) that have to be eliminated or substantially reduced.

Industrial products	
Hexabromobiphenyl	*a*
Polychlorinated biphenyls (PCBs)	*a,b*
Octabromodiphenyl ether (OBDE)	*d*
Pentabromodiphenyl ether	*d*
Perfluorooctane sulfonates (PFOS)	*d*
Polychlorinated naphthalenes (PCN)	*d*
Short-chain chlorinated paraffins (SCCPs)	*d,e*
Pesticides	
Aldrin	*a*
Chlordane	*a*
Chlordecone	*a*
Dieldrin	*a*
Endrin	*a*
Heptachlor	*a*
Mirex	*a*
Toxaphene	*a*
DDT	*a,b*
Hexachlorocyclohexane (HCH)	*a,b*
Hexachlorbenzene (HCB)	*a,c*
Pentachlorobenzene (PeCB)	*d*
Unintentional by-products of combustion and industrial processes	
Polycyclic aromatic hydrocarbons (PAHs)	*c*
Polychlorinated dibenzo-p-dioxins (PCDDs)	*c*
Polychlorinated dibenzofurans (PCDFs)	*c*
Hexachlorobutadiene (HCBD)	*d*

a-substances scheduled for elimination
b-substances scheduled for restriction use
c-substances scheduled for emission reduction by the use of best available technology (BAT)
d-substances included on the protocol ECE/EB.AIR/2009/14
e-substances that meet the EPA definition

Focusing on pesticides, the main goal of this review is to compile a significant amount of representative data, mainly from Europe, and discuss the published results taking in consideration factors, such as matrix, pesticide category, and the European Directive limits. The matrices herein discussed involve total or partially aquatic systems, which are our focus.

II. Pesticides

1. Definition and Nomenclature of the Pesticide

As mentioned before, chemicals are substances (natural or human-made) that serve many human purposes (industrial, health and agricultural field). A pesticide is a substance or mixture of substances that prevent, destroy, repel or mitigate any pest [18]. Since pesticides have different physico-chemical properties, chemical structure, application, and toxicity, they can be divided into different subclasses.

According to EXIOPOL (an integrated project funded by the European Commission under the 6th framework programme, priority 6.3 Global Change and Ecosystems), pesticides are divided in acaricides, algaecides, bactericides, fungicides, herbicides, insecticides, molluscicides, nematicides, rodenticides and others [19].

Depending on the nature of the pesticides, they can be: botanic — obtained from plants, antibiotics, and synthetics — compounds produced by man [20].

In Europe, 1331 active substances exist and according regulation (EC) No 1107/2009 only 482 of them are approved (Figure 1). Thousands of commercial formulations can be prepared from them or their residues, however these ones must be innocuous for people and animal health, and with no harm to environment [21, 22].

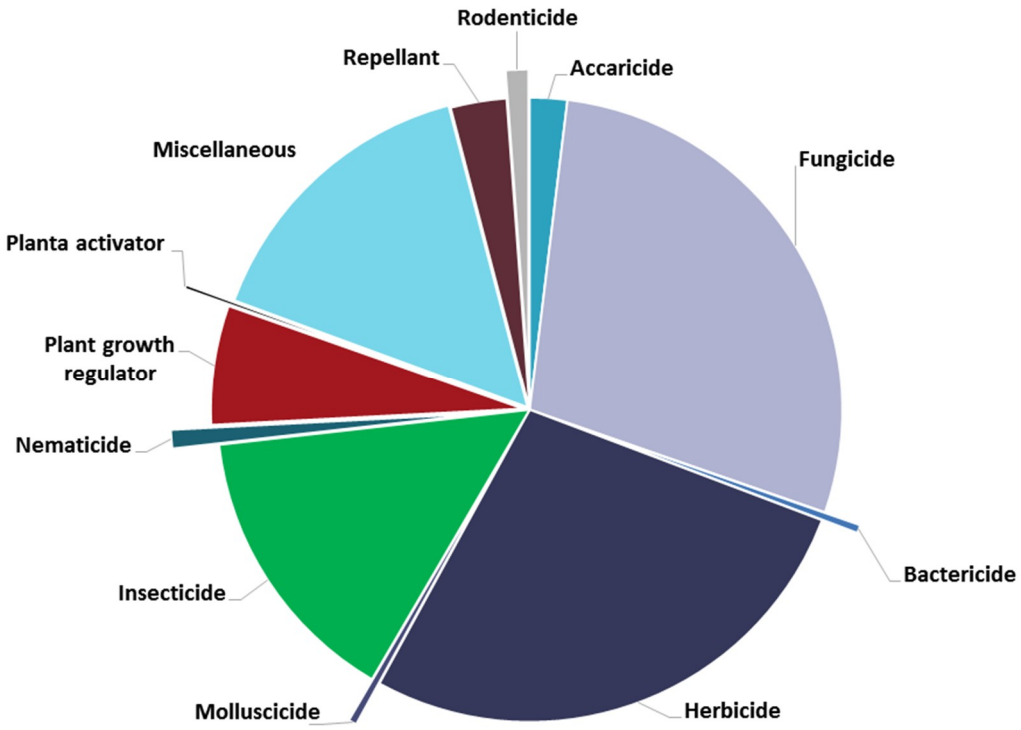

Figure 1: Percentage of the different activity classes of 482 substances, as approved, by the European Union.

The European Chemicals Agency (ECHA) defines, evaluates, and regulates, through the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), the potential biological risks of chemical substances [23].

To distinguish their toxicological degree, pesticides can be grouped in five classes: extremely hazardous (class Ia); highly hazardous (class Ib); moderately hazardous (class II); slightly hazardous (Class III); unlikely to present acute hazard (U). This classification is based on the identification of a risk component that is present in a chemical substance, based on the LD_{50} for rats [24].

Chemically, pesticides can be classified as inorganic and organic (which can be divided into synthetics and naturals)[25]. The discovery of synthetic organic products have permitted the rising of diverse products that are classified in 42 classes: organochlorines, clorophosphates, organophosphorus, carbamates, pyrethroids, sulfonylureas, triazines, and others [26, 27].

2. Sources and Pathways of Pesticides in the Environment

The current overuse and careless application of pesticides may impact diverse ecosystems, depending on: the type of usage (localized or widespread), spreading methodology (conventional, aerial), and usage intensity [28].

Environmentalists and scientists are aware of the hazardous effects pesticides may cause in the long-run. They can circulate through various mechanisms, becoming an additional source of contamination and economic loss, among other consequences. Due to these facts, contamination can be specific, in case of storage leaks, occasional drainage, improper containers and disposal procedures, or unspecific, when a widely polluted area provokes water contamination (wastewater draining into ground water) and/or drift of pesticides in the air [29].

i. Industrial Production

The main percentage of pesticides used is synthetic. In Europe, approximately 540 companies produce and/or distribute these compounds [30].

Accidental seepages and insufficient wastewater treatment plant (WWTP) procedures are the main sources of pesticide output into the environment. According to the European legislation, each Member State must maintain an inventory that includes emissions, discharges, and losses of regulated substances, but no limits are established by the legal document [31].

ii. Agriculture and Human Use

The exigent economic sector demands an intensive and modern agriculture. For these reasons, pesticides serve as important tools for this sector. Conversely, several adverse effects are known such as pesticide degradation, absorption and desorption in the soils, secondary pest growth, plant and insect resistance, agricultural seepage, and food contamination [32].

A lack of knowledge combined with non-conscious application, may lead to an excessive use of these compounds, increasing the concentrations in soil and crops. Additionally, the contamination of raw materials leads to a biomagnification processes since many of them are used in the feeding of

farmed animals. In the last European Food Safety Authority (EFSA) report (2013), more than 80 000 samples were analysed, from which 45.4% had measurable residues and 2.6% of them had values above the established MRL [33]. Additionally, other studies confirmed the presence of persistent pesticides in diverse animal products, such as meat, milk, and eggs consumed in Europe [27, 34-36].

3. Fate and Occurrence of Pesticides in the Environment

Pesticides are commonly used for a specific purpose, usually linked to agronomical production. Besides the specific application, these compounds are released into the environment by evaporation, leaching, water runoff, and uptake by plants and organisms.

i. Water

The contamination of the main water bodies by anthropogenic pollutants, namely pesticides, can be resultant of surface water run-off, waste water discharge, accidental seepage, soil erosion, and/or leach from treated fields [29].

Due to their chemical structure, pesticides, when in contact with water, are susceptible to hydrolysis processes [9]. This reaction is measured by the half-life of the main compounds along time [9]. The half-life of a pesticide may be affected by temperature, pH, and other particles or compounds present in the water. In addition to possible hydrolysis processes, pesticides may be subject to microbial degradation [37]. As well as in soil, this degradation process is affected by the same parameters described in *item 3.i* [37]. The hydrolysis half-life value is an essential aspect to estimate the persistence of a compound in water and help us to evaluate the impact on the aquatic habitats.

The groundwater ubiquity score (GUS index) estimates the potential of pesticides to contaminate groundwater. This parameter can be calculated through the formula: GUS = log (pesticide half-life) x [4 - log (K_{OC})]. With this

potential indicator of pollution pesticides classify as having extremely low (<-1) to very high potential (>4) to move toward groundwaters [38, 39].

ii. Soils/sediments

Intense and frequent use of pesticides led to an accumulation and persistence in soils. As these compounds are mostly non-polar and hydrophobic, they tend to be less soluble and highly stable in this matrix [40]. Several phenomenon may occur when these compounds are in contact with the soil:

Sorption is the phenomenon that describes the affinity of these compounds into the physical structure of a matrix, in this case the soil. This process is affected by organic matter content, humidity, texture [29] but also by size, hydrophobicity, charge, capacity to form hydrogen bonds, and structure arrangement [41].

The content of organic matter in the soil is linked with the amount of pesticide adsorbed (K_d). This parameter, expressed by L/kg, can be determined by the ratio beteween the amounts of pesticides measured in the soil (mg/kg) per their amount in water (mg/L). A low K_d ratio indicates more pesticide in the solution and a higher value that the pesticide is more strongly sorbed to soil.However, in order to normalize K_d coefficient, it should be divided by the organic matter content of the soil (sorption coefficient; K_{OC}) [42]. Values of $K_{OC} \leq 300$ indicate higher potential of pesticides to leach or move with surface runoff [29].

The adsorption of pesticides also depends on physical and/or chemical characteristics, such as van der Waals forces and chemical bonds, established between pesticides and soils [41].

Microbial degradation by fungi, bacteria, and other microorganisms also affects the availability of these substances. Also, physicochemical environmental conditions such as pH, temperature, soil moisture, and aeration are important for the degradation and breakdown processes [43].

Additionally, vapour drift may also contribute to the loss of pesticides; the higher the value of the Henry's law constant (K_h) the greater the tendency to pesticides volatilize from the soil.

Considering these facts and based on half-life of these compounds, pesticides may be classified according to their persistence: low (less than 30 days), moderate (between 30-100 days), and high persistence (greater than 100 days) [44].

iii. Aquatic Organisms

Since the 1990s, the world is aware of the harmful effects of pesticides. However, the overuse of these compounds still affects the ecosystem, bringing devastating consequences for organisms. Due to their characteristics, low solubility in water and high persistence, pesticides tend to accumulate in the biota.

Definitions, like bioconcentration (uptake of a chemical available in water), and bioaccumulation (uptake from water and food), are important to understand the biomagnification processes [11].

The bioaccumulation factor (BAF) is an important attribute to evaluate the concentrations found in a living organism, when compared to concentrations found in the habitat [45]. BAF may be influenced by several factors, as for instance, the chemical characteristics of the pesticide, lipid content and metabolism of the organism, as well as the habitat conditions (salinity, temperature, water currents, and dissolved oxygen) [42]. This means the BAF is higher in case of a non-polar compound with low solubility in water (high K_{OW}) and greater half-life values. On the other hand, the organism has to have an elevated capacity to uptake, high lipid content, slow metabolism, and a deficient capacity to metabolize the parent compound and its metabolites [46].

Based on laboratory experiences and mathematical models, REACH defined the maximum values of BAF as being >2000 L/Kg for PBTs and >5000 L/Kg for very persistent and very bioaccumulative compounds (vPvBs) [23]. The use of mathematical models is a good approach but still needs to be improved. Characteristics, like the maximum diameter and molecular length of a compound, and the octanol solubility (K_{OW}), should be considered in the models to have a better linearity between the experimental results and the mathematical models for a broad range of pesticides [45, 47].

III. European Legislation for Pesticides

To establish regulatory limits for substance residues, solid bases (credible data) are required supported by measurable quantification limits and toxic hazard effects. Depending on the matrix, the regulatory limits are different.

1. Water

Due to these characteristics, each country must define their own regulatory limits, based on their economic and technological situation. Nonetheless, European Union (EU) countries must follow, at least, the directives implemented by the Council of European Union.

The water intended for human consumption has restricted maximum levels, set by the Directive 98/83/EC; a maximum concentration of 0.1 μg/L and 0.5 μg/L were defined for individual and total pesticides, respectively, excluding aldrin, dieldrin, heptachlor, and heptachlor epoxide, for which limits were set to 0.03 μg/L [48].

For aquatic environments, the limits are based on Environmental Quality Standards (EQS) [31] as defined by the Directive 2008/105/EC. Nonetheless, the EU Member States are only compelled to follow the EQS values for surface waters. Concerning the bathing water quality, no legislation is available considering the pesticides levels [49].

Meanwhile, the 2013/39/EU, as an amending of 2000/60/EC and 2008/105/EC directives, demonstrate the importance of a strict control in other matrixes, such as soil and biota [50, 51] (Table 2).

Table 2: Environmental quality standard values for pesticides (µg/L) – Directive 2013/39/EU.

Pesticides	Annual average		Maximum allowable concentration	
	Inland surface waters	*Other surface waters*[(b)]	*Inland surface waters*	*Other surface waters*
Aclonifen	0.12	0.01	0.12	0.01
Alachlor	0.3	0.3	0.7	0.7
Atrazine	0.6	0.6	2	2
Bifenox	0.01	1.20E-03	0.04	4.00E-03
Chlorfenvinphos	0.1	0.1	0.3	0.3
Chlorpyrifos	0.03	0.03	0.1	0.1
Cybutryne	2.50E-03	2.50E-03	0.02	0.02
Cyclodiene pesticides [(a)]	Σ0.01	Σ0.005	na	na
Cypermethrin	8.00E-05	5.00E-06	6.00E-04	6.00E-05
4,4'-DDT	0.01	0.01	na	na
DDT total	2.50E-02	2.50E-02	na	na
Dichlorvos	6.00E-04	6.00E-04	7.00E-04	7.00E-05
Dicofol	1.30E-03	3.20E-05	na	na
Diuron	0.2	0.2	1.8	1.8
Endosulfan	5.00E-03	5.00E-03	0.01	0.01
Heptachlor and heptachlor epoxide	2.00E-07	1.00E-08	3.00E-04	3.00E-05
Hexachlorobenzene (HCB)	0.01	0.01	0.05	0.05
Hexachlorocyclohexane (HCH)	0.02	2.00E-03	0.04	0.02
Isoproturon	0.3	0.3	1	1
Octylphenol	0.1	0.1	na	na
Pentachlorobenzene (PeCB)	7.00E-03	7.00E-04	na	na
Pentachlorophenol	0.4	0.4	1	1
Quinoxyfen	0.15	0.02	2.7	0.54
Simazine	1	1	4	4
Terbutryn	0.07	0.01	0.34	0.03
Tributylin compounds	2.00E-04	2.00E-04	1.50E-03	1.50E-03
Trichlorobenzenes	0.4	0.4	na	na
Trifluralin	0.03	0.03	na	na

([a]): Aldrin, Dieldrin, Endrin, Isodrin; ([b]) **other surface waters:** transitional, coastal and territorial waters; **na:** not applicable

2. Soils and Sediments

Several data have been published concerning soil contamination in Europe [52-55]. As no specific and official EU legislation exists, leading authors refer to the "Dutch List" (Table 3) [54, 56-59].

Table 3: Target and intervention values, for soil/sediment remediation for pesticides, according to the Dutch List.

Pesticides	Target values	Intervention values
	mg/kg dry matter	
ΣDDT/DDD/DDE (total)	0.01	4
Aldrin	0.00006	-
Dieldrin	0.0005	-
Endrin	0.00004	-
ΣDrins	0.005	4
HCH (alpha)	0.003	-
HCH (beta)	0.009	-
HCH (gamma)	0.00005	-
ΣHCH	0.01	2
Atrazine	0.0002	6
Carbaryl	0.00003	5
Carbofuran	0.00002	2
Chlordane	0.00003	4
Endosulfan	0.00001	4
Heptachlor	0.0007	4
Heptachlor epoxide	0.000002	4
Maneb	0.002	35
MCPA	0.00005	4
Organotin compounds	0.001	2.5

3. Food and Biota

In Europe the amount of pesticide residues in food must be kept under a safe threshold for all consumers; these amounts are defined by the maximum residue levels (MRLs), which are the highest levels of a pesticide residue that is legally tolerated in food or feed. When a specific pesticide MRL is not mentioned, a MRL of 0.01 mg/kg is applied [60].

As being aquatic organisms/biota, the new directive (2013/60/EU) established EQS of 10, 33, and 0.0067 µg/kg wet weight (ww), for hexachlorobenzene, dicofol, and the sum of heptachlor and heptachlor epoxide, respectively [51]. For the other pesticides a general concentration of 10 µg/kg ww was adopted.

IV. Database Analyses – An Up-to-Date Perspective.

This review focuses on three matrices (water–soil–aquatic organisms) to better understand the interactions between them. Since a great amount of data is available, we select a period of 15 years (from 2000 to 2016) to analyse results before and after the "*dirty dozen*" law [15]. All the available data – minimum (min), maximum (max), and average concentrations (av) – were collected and expressed as ng/L (water) and ng/g (soils and organisms). Additional information, such as the number of samples used in each study were also noted. Data was grouped by pesticide category. Europe is used as the main pillar of this study, in comparison with other continents, because is the continent with more available information about this issue. For biota data, such as species, scientific name, and identification of the used matrix, were also collected. Online databases, as Web of Science (Thomson Reuters) and PubMed (NCBI), served to access to the indexed articles used in this work.

1. Water

A total of 78 articles were collected and compiled in Table 4. During data selection were prioritized surface waters and dissolved aqueous phase matrices, representing a total of 78% and 7% of the collected data, respectively. This was done to reach a better correlation between matrices (sediments and aquatic organisms) that are further discussed in this review. 63% of the analysed data belong to Europe, the rest being divided between Africa, Asia (each with 15%), followed by South America and Oceania. No data was found for North America with the above presented requirements (Table 4); so, when citing herein "worldwide", the continent will not appear. More than 46 aquatic systems were studied in Europe, from which Spain stands out with a total of 13 aquatic systems published in 12 journals.

Table 4: Pesticide concentrations (minimum, maximum and average values; ng/L) in water samples, displayed by continent, country, and aquatic system; the number of quantified pesticides and sampling year were also added.

	Pesticides No.	Sampling year	min	max	average	References
			ng/L			
Africa						
Egypt						
Manzala Lake	13	1993	0.1	0.2	0.1	[61]
Nile River	12	19;93	0.0	0.0	0.0	[61]
Ghana						
Bosomtwi Lake	4	2004	0.3	0.9	0.1	[62]
Nigeria						
Ikoro River	4	na	405.5	431.0	420.3	[63]
Ogba River	7	na	536.7	571.6	554.7	[63]
Ovia River	7	na	490.0	525.3	509.0	[63]
Owan River	14	na	-	-	190.0	[64]
South Africa						
Buffalo River	15	2002	-	-	35.2	[65]
Leiskamma River	14	2002	-	-	44.6	[65]
Lourens River	4	1999	25.0	135.0	77.9	[61]
Swartkops River	15	2002	-	-	40.7	[65]
Tyhume River	14	2002	-	-	47.3	[65]
Asia						
China						
Beijing Guanting reservoir	30	2003	3.2	27.9	9.7	[66]
Jinjiang River	9	na	3.0	4.6	3.5	[67]
Minjiang River	17	1999	9.7	126.7	46.6	[68]
Qiantang River	13	2005	0.3	29.1	4.8	[69]
Tonghui River	18	2002	14.0	246.6	41.4	[70]
Yangtze River	13	2005	0.2	8.5	1.5	[71]
Yellow Sea	5	2006	240.0	922.0	512.0	[72]
India						
Bay of Bengal	10	2011	0.0	2.2	0.2	[73]
Vasai creek	13	2009	41.0	127.5	87.0	[74]
Macau						
Pearl River	18	2001	0.8	3.5	1.6	[75]
Russia						
Obskaya bay	7	2005	-	-	0.1	[76]
Yenisei River	7	2003	-	-	0.0	[76]
Europe						
Central and Eastern Europe						
Danube River	9	2007	-	24.1	6.3	[77]
Belgium						
Escaut-Lys River	7	2002	-	-	312.1	[78]
Scheldt River	6	2004	-	-	48.4	[79]
Bulgaria						
Strymonas River	8	na	6.6	10.4	5.3	[80]
France						
Beillant River	3	2010	-	-	26.9	[81]
Bretagne River	2	2007	-	-	32.5	[82]
Jauron River	19	2003	317.4	636.8	466.3	[83]
Rhône-Alpes	4	2007	81.7	94.3	88.4	[84]

(continued)

	Pesticides No.	Sampling year	min	max	average	References
			ng/L			
Europe						
Save River	12	2008	-	727.3	140.3	[85]
Seine River	6	2006	90.0	3451.7	566.7	[86]
Germany						
Elbe and Weiβe Elster rivers	12	2001	-	-	3.7	[87]
Elbe River	19	2001	-	-	10.5	[87]
Modau River	1	2003-2006	4.0	3070.0	580.0	[88]
Schwarzbach	1	2003-2006	4.0	250.0	60.0	[88]
Weiβe Elster River	17	2001	-	-	9.1	[87]
Weschnitz	1	2003-2006	4.0	5600.0	540.0	[88]
Winkelbach	1	2003-2006	4.0	550.0	30.0	[88]
Greece						
Amvrakia lake	23	2007-2008	-	170.4	19.6	[89]
Axios River	9	1996-1998	<LOQ	47.3	34.8	[90]
Evros River	11	1996-1998	<LOQ	49.5	33.7	[90]
Kalamas River	3	2000	47.3	124.0	99.3	[91]
Nestos River	9	1996-1998	<LOQ	29.2	25.0	[90]
Pamvotis Lake	9	1998-1999	11.6	803.3	49.9	[92]
Strimonas River	9	1996-1998	<LOQ	39.9	31.6	[90]
Hungary						
Danube River	2	2010-2011	-	-	417.1	[93]
Poland						
Warka-Grójec region	5	2002-2003	525.4	1322.6	42.0	[94, 95]
Oder River	8	2003-2004	1.3	55.6	8.5	[96]
Portugal						
Alqueva dam	14	2006-2007	5.9	125.2	31.2	[97]
Douro River	39	2010-2011	-	-	134.7	[98]
Lake Vela	8	2004	-	-	3288.1	[99]
Mondego River	56	2010-2011	4.0	550.5	89.3	[100]
Ria Formosa Lagoon	54	2010-2011			137.6	[101]
Ria Formosa Lagoon	18	2012-2013	-	-	39.7	[102]
Tagus River	53	2010-2011	8.8	555.0	63.4	[103]
Romania						
Mures, Tarnava Mare and Tarvana Mica	7	2004-2005	8.3	9.8	37.1	[104]
Spain						
Anoia River	9	2010	<LOQ	35.8	9.6	[105]
Barcelona	5	2000	28.1	61.7		[106]
Cadiz	4	2007	-	-	15.0	[107]
Catalan rivers	45	2007-2008	6.1	448.7	66.0	[108, 109]
Duero River	5	2001	10.0	218.0	4.0	[110]
Ebro River	36	2006	10.0	947.0	103.6	[39, 55, 111]
Girona River	11	1996-1997	8.5	99.2	18.6	[112]
Guadalquivir River	13	2010	58.4	61.8	5.0	[113]
Guadalquivir River	11	2005	-	-	1125.5	[114]
Llobregat River	3	2009-2010	-	12.7	7.1	[105]
Llobregat River	19	2003-2004	-	196.3	34.3	[115]
Llobregat River	28	2000	26.7	50.3	-	[106]

(continued)

	Pesticides No.	Sampling year	min	max	average	References
			ng/L			
Europe						
Miño River	4	2001	17.5	180.0	35.1	[110]
Tinto/Odiel River	1	2005	-	-	940.0	[114]
Netherlands						
Several aquatic systems	13	2008	34.6	79.2	43.8	[116]
Oceania						
Australia						
Proserpine, O'Connell, and Pioneer rivers	6	2002	138.3	2680.0	759.1	[117]
Tully–Murray Basin	7	2006	11.0	3398.6	326.9	[118]
South America						
Argentina						
Buenos Aires southeast basin	8	2012	28.3	139.6	53.5	[119]
Brazil						
São Lourenço River	10	1999	4.9	40.1	12.9	[120]
Batalha and Vargem Limpa River	11	2005	18.1	50.6	23.6	[121]

Symbols (na) and (-): *no data found/available*

Overall, the data collected between 1993 and 2012 shows average concentrations ranging from 0.002 to 7984 ng/L (Table 4). Among the selected articles, 136 compounds were detected and quantified in Europe, 28 in Africa, 40 in Asia, 8 in Oceania, and 25 in South America.

On a Worldwide scale, the insecticides prevail (59%) in terms of available and quantified data when compared with both herbicides and fungicides. Per continent, the percentage of insecticides increases more than 90% in Africa and Asia, assuming approximately 40–60% in Europe and South America. No cases were observed in North America, Oceania, and Antarctica (Figure 2). The percentage of insecticides in Asia may be related to the high cereals production (more than 13 x 10^8 tonnes) on the continent, while in Africa it can be linked to cereals and pulse production, plague control, and vector-borne diseases control [122-124]. The diverse percentage between categories, in Europe and South America, may be a response to diverse agriculture practices and industrial needs [6].

Looking at the nature of the matrix, most studies use surface water as a model (75%) the rest taken by groundwater (10%), dissolved aqueous phase (7%), and others (Figure 2).

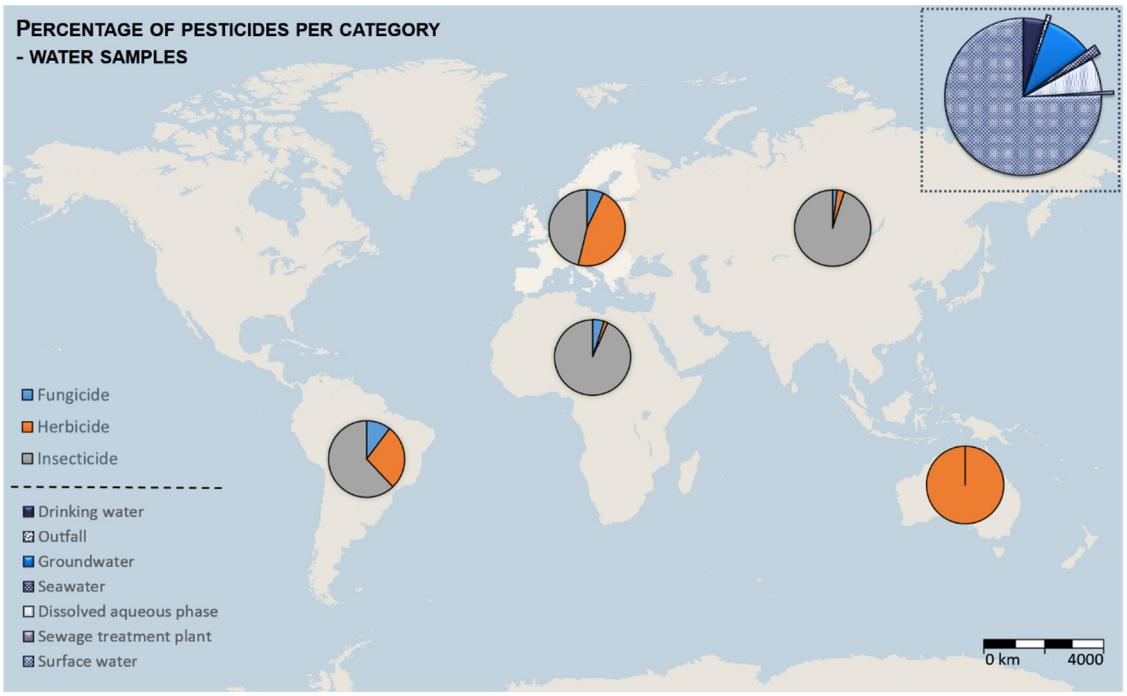

Figure 2: Representation of the quantified pesticides in water samples (%), per category, on each continent; the right upper corner figure represents the type of matrices found worldwide.

In spite of these facts, we should be aware that these results are dependent on the authors' selection, which may not correspond entirely to what is present in the aquatic systems. The same is applied to the number of field samples. Here, the highest frequencies are associated to a higher number of field samples, as the probability of getting a contaminated sample increases; based on this we may assume that, in most cases, the number of samples are not sufficient to get representative data (Table 5).

The quantified pesticides data are also compared to the levels set by Directive 2013/39/EU (Table 5). Considering the pesticides with concentrations above these levels (n), many cases (n = 45) are registered in Africa and Asia for insecticides, while Europe has similar number of cases, for insecticide (n = 87) and herbicide (n = 107) categories. Few cases are observed for other continents.

In Europe, pesticides levels average between 5 and 562 ng/L, where Portugal (39%), Spain (26%), and Greece (11%) were the top three countries with published articles (from a total of 42 publications), represented in more than

260, 170 and 73 measured pesticides in different aquatic systems. Proportionally, Portugal (n = 80) followed by Spain (n = 46) and Greece (n = 24) had several quantifications above the directive limits [39, 89, 90, 100-102, 105-108, 110-115].

Table 5: Pesticides average concentrations (ng/L) in water samples, displayed by continent and pesticide category; Europe is presented with more detailed information; the number of field samples, as well as the number of samples above 2013/39/EU Directive levels, were also included; references are only defined for the samples above the 2013/39/EU Directive, per category.

	Average Amounts (ng/L)	Field samples No.	Samples above 2013/39/EU	References
Africa				
Fungicide	32.5	6	6	[65, 125]
Herbicide	210.0	2	1	[64]
Insecticide	121.0	115	44	[61-65, 125]
Asia				
Fungicide	4.1	3	3	[66, 76]
Herbicide	6.1	5	2	[66]
Insecticide	37.0	152	53	[66, 68-76]
Europe				
Fungicide				
Bulgaria	9.1	1	1	[80]
France	127.6	9	-	-
Greece	31.8	8	2	[89, 90]
Portugal	115.4	24	8	[100-103]
Spain	562.4	5	-	-
Netherlands	120.0	2	-	-
Herbicide				
Central/Eastern Europe	7.1	9	4	[77]
Belgium	201.6	12	8	[78, 79]
France	383.9	30	7	[81-83, 85, 86]
Germany	32.0	49	15	[87, 88]
Greece	35.4	15	8	[89, 91, 92]
Hungary	417.1	2	-	-
Portugal	201.3	80	30	[97-103, 126]
Romania	113.5	2	1	[104]
Spain	126.7	113	34	[39, 105-108, 110-115]
Netherlands	23.8	8	-	-
Insecticide				
Belgium	56.0	1	1	[78]
Bulgaria	4.7	7	4	[80]
France	140.7	7	3	[83-86]
Germany	13.6	3	3	[87]
Greece	32.1	50	14	[89-92]
Poland	19.7	13	6	[94-96]
Portugal	219.1	164	42	[97-103]
Romania	6.5	5	2	[104]
Spain	84.7	57	12	[39, 105, 107-109, 111-114, 127]
Netherlands	120.0	1	-	-

(continued)

	Average Amounts (ng/L)	Field samples No.	Samples above 2013/39/EU	References
Oceania				
Herbicide	526.4	13	5	[117, 118]
South America				
Fungicide	39.3	3	-	-
Herbicide	15.1	8	5	[119, 120]
Insecticide	32.1	18	5	[119-121]

Symbol (-): *absence of samples above 2013/39/EU Directive*

Since the number of compounds observed are quite different between the published articles, the most frequent pesticides (more than 10 observations, *i.e.* quantification of pesticides in different aquatic systems or countries) were analysed to compare the average concentrations between continents with a relevant amount of data. We observed that the most quantified pesticides (Table 6) belong to the priority list cited before (Table 1); this data once more indicates the attention, of researchers, to these illegal compounds.

The fungicide HCB is present on three continents, with similar average concentrations in Africa and Europe (40 ng/L), and lower amounts in Asia (4 ng/L). Considering the category of the herbicides, atrazine and simazine are being measured in Europe, Oceania, and South America. However, atrazine is reported with values 10 times higher in Oceania than in the other continents. By the contrary, simazine concentrations range between 45 and 95 ng/L for Oceania and Europe, presenting lower values in South America (9 ng/L). Amongst insecticides, ∑DDT, ∑endosulfan, and ∑HCH residues are the most frequent in Africa, Asia, and Europe.

Comparing the total average sum of these insecticides (∑), Africa presents higher concentrations (~1500 ng/L) than Asia, Europe (~410 ng/L), and South America (~200 ng/L).

The ratios parent compound/residues are calculated for DDT, endosulfan, and heptachlor. Results demonstrate an active use of DDT in Asia, Europe, and Africa, from which the first one stands out with a ratio of 1.4. Endosulfan presents high ratio values in Africa (6.5), Europe (3.5), and South America (5.5). The same is observed for heptachlor in Asia (3.1) and Europe (2.4). These results intend to show that countries in these continents maintain the usage of banned pesticides.

Considering the 2013/39/EU Directive for transitional waters, all continents documented average concentrations 15- to 40-times higher than the legal concentrations (consult Table 3).

Table 6: Average values (ng/L) of the most frequent pesticides, quantified in water samples, displayed by category and continent; data based on the references cited in Table 3; references organized by pesticide category.

Average amounts (ng/L)	Africa	Asia	Europe	Oceania	South America	References
Fungicides						
HCB	32.5	4.1	43.0			[65, 66, 76, 80, 90, 98, 100, 101, 103, 125]
Herbicides						
Alachlor		1.7	506.7		11.0	[39, 64, 66, 77-79, 81-83, 85, 87, 89, 91, 92, 94, 97-99, 101, 102, 104-106, 108, 110-114, 117-120, 126]
Atrazine	150.0		74.7	674.3	17.0	
DEA			26.6	65.2		
Diuron			266.1	1768.3		
Simazine			95.0	45.0	9.0	
Insecticides						
ΣDDT	868.5	215.7	134.1		105.0	[39, 61-70, 72-76, 78, 80, 83-85, 87, 90, 94-99, 101, 102, 104, 107-109, 111, 112, 120, 121, 125]
2,4´-DDD	152.7	25.1	31.0			
2,4´-DDE	50.0	0.0	1.4			
2,4´-DDT	212.7	63.8	4.0		6.0	
4,4´-DDD	119.1	0.9	16.6		41.0	
4,4´-DDE	170.4	65.4	22.3		36.0	
4,4'-DDT	163.6	60.5	58.8		22.0	
DDT/DDE+DDD	*0.8*	*1.4*	*0.9*		*0.4*	
Σcyclodiene	318.6	42.3	424.9			
Aldrin	279.6	20.7	392.1			
Endrin	39.0	21.6	32.7			
Σendosulfan	166.7	59.1	122.5		38.5	
Endosulfan (alpha)	116.7	17.0	97.5		16.0	
Endosulfan (beta)	50.0	42.0	25.0		22.5	
Endosulfan sulfate	25.7	61.5	34.7		7.0	
Σendosulfan/endosulfan sulfate	*6.5*	*1.0*	*3.5*		*5.5*	
ΣHCH	525.5	156.9	137.6		60.0	
HCH (alpha)	85.08	61.0	24.7		13.0	
HCH (beta)	76.86	19.3	39.1		37.0	
HCH (gamma)	331.08	75.4	73.8		10.0	
HCH (sigma)	32.52	1.2				
ΣHeptachlor, Heptachlor epoxide	475.00	39.1	57.9			
Heptachlor	45.00	29.6	41.1			
Heptachlor epoxide	430.00	9.5	16.81			
heptachlor/heptachlor epoxide	*0.10*	*3.1*	*2.4*			
Σ	1560.7	431.6	394.2		203.5	

The pesticide names in bold are in the 2013/39/EU Directive ; the ratio parent/residues is presented in italic style

In Europe (Portugal, Spain, France, Netherlands, and Greece), in spite of the different number of quantified pesticides per category, the average concentrations of fungicides, herbicides and insecticides being reported are in the same order [39, 46, 81-86, 89-92, 97-103, 105-116]. This possibly indicates similar agricultural practices among these countries, leading to comparable pollution degree. However, different toxic effect may occur in each aquifer system.

The most frequent pesticides (equal or more than 10 quantifications in different aquatic systems or countries) were selected and grouped by category (Table 7), reaching a total of 22 compounds; eleven of them are above the MRLs set by 2013/39/EU Directive. The range of concentrations (min-max) was assessed to display the most substantial differences between countries. Seven pesticides (alachlor, chlortoluron, diuron, metolachlor, terbuthylazine, aldrin, and dieldrin) stand out with higher ranges (numbers in bold, Table 7). Alachlor is present in the Iberian Peninsula at levels above the 2013/39/EU Directive limits, which may relate to a regional application of this herbicide [97-103, 126]; the same was observed for diuron, in Spain, France, and Belgium [78, 83, 86, 105, 108, 111, 114, 127]. The cyclodiene pesticides (∑aldrin and dieldrin) were above the annual average concentrations (∑= 5 ng/L) set by the same directive for all registered cases, presenting extremely high amounts in Portugal (∑cyclodienes 2363 ng/L), demonstrating an abusive and illegal use of these compounds [98-101, 103].The herbicides, chlortoluron and terbuthylazine were quantified, in France, at concentrations above 300 ng/L, indicating an abusive application and/or improper waste treatment [83, 85].

Table 7: Average values (ng/L) of the most frequent pesticides, quantified in water samples, displayed by European country; referring to the most frequent pesticides.

Average amounts (ng/g)	Central and Eastern Europe	Belgium	Bulgaria	France	Germany	Greece	Poland	Portugal	Romania	Spain	Netherlands	min-max
References	[77]	[78, 79]	[80]	[81-86]	[87, 88]	[89, 90]	[94-96]	[97-103]	[104]	[105-109, 112-114]	[116]	
Alachlor				41.0	36.4	66.4		795.6		380.3		**36.4-795.6**
Atrazine	2.0	213.7		95.6	18.6	67.9		136.4	77.0	32.6		2.0-213.7
DEA	11.0			38.1	11.1	45.3		25.0		26.3		11.0-45.3
Chlortoluron				340.5	3.3			7.8		7.4	20.0	3.3-340.5
Cyanazine					0.3			72.2		5.2		0.3-72.2
Diuron	3.0	820.0		740.0	7.1			49.5		225.4		**3.0-820.0**
Isoproturon	2.0	270.0		144.0	13.1			3.3		3.9	40.0	2.0-270.0
Metolachlor		327.0		96.4	4.1			53.4		9.7		**4.1-327.0**
Simazine		71.9			7.7	2.7		33.5		168.8		2.7-168.8
Terbuthylazine	11.0	36.0		1950.0	4.1			65.4		414.0		**4.1-1950.0**
Terbutryn					203.4			37.4		0.6		0.6-203.4
ΣDDT			3.7	180.5		65.8	14.1	131.6		23.6		3.7-180.5
4,4'-DDD				84.0			7.5	11.0		7.6		7.5-84.0
4,4'-DDE						30.8	0.0	24.6		3.6		0.0-30.8
4,4'-DDT			3.7	96.5		35.0	6.6	96.0		12.5		3.7-96.5
Σcyclodiene			5.6			43.0	6.0	2363.2		6.4		**5.6-2363.2**
Aldrin			5.6			23.9	1.0	832.1		3.1		**1.0-832.1**
Dieldrin						19.2	5.0	1531.1		3.2		**3.2-1531.1**
Chlorpyriphos						2.5		29.1		1.9		1.9-29.1
Diazinon						93.1		62.4	20.0	9.0		9.0-93.1
Dimethoate						5.2		92.0		23.1		5.2-92.0
Endosulfan sulfate						19.1		47.2				19.1-47.2
Fenitrothion						3.3		77.9		151.5		3.3-151.5
HCH (gamma)		56.0	12.8	200.0	1.3	25.7	83.4	165.4	2.6	15.7		1.3-200.0

The pesticide names in bold are in the 2013/39/EU Directive; the highest range differences are represented in bold

From the pesticides quantified in Europe, the ones identified by the Stockholm Convention were selected by sampling year as displayed in Table 8.

Nine priority pesticides and their residues were quantified from 1996 to 2012, from which almost all were reported in 1996 and in the last three years. Similar average concentrations reveal a continuous use of these pesticides along these years, even with the lack of information between 2001 and 2008. In 2004, the highest amounts of two cyclodiene pesticides (aldrin and dieldrin) were registered in the same aquatic system (Lake Vela, Portugal; see Table 3) [99]. Again, these results prove the abusive use of biocides.

The parent/residues ratio, described previously for DDT and heptachlor, reveals that values both cases are almost always above 1, which indicates an active utilization of these pesticides; at least until 2012.

Table 8: Average values (ng/L) of the priority listed pesticides quantified in water samples, collected in Europe, and displayed by sampling year; referring to the most frequent pesticides

	1996	2001	2002	2003	2004	2006	2007	2008	2009	2010	2012
References	[90, 112]	[87]	[94]	[39, 83, 96]	[99]	[97]	[84, 107-109]	[85]	[98]	[100-103]	[102]
ΣDDT	55.3						126.1		132.8	*141.0*	70.3
DDT/DDE+DDD	*0.5*						*1.0*		*1.3*	4.0	*15.7*
2,4´-DDD							31.0				
2,4´-DDE	1.8						1.0				
2,4´-DDT							4.0				
4,4´-DDD	3.2						27.8		25.4	7.2	4.2
4,4´-DDE	30.8						3.6		31.4	21.1	
4,4'-DDT	19.5						58.8		76.0	112.6	66.1
ΣCyclodiene	36.6			43.0	7533.0				321.7	146.8	79.6
Aldrin	19.7			1.0	2377.3				167.1	23.7	
Dieldrin	13.9			5.0	5155.8				87.8	79.5	79.6
Endrin	3.0			37.0					66.8	43.6	
ΣChlordane										4.6	
Chlordane (gamma)										4.6	
ΣHCHs	112.7	1.30	56.00	58.7	10.60	1.07	5.4	119.1	779.0	62.0	26.3
HCH (alpha)	33.8			7.0	5.80						
HCH (beta)	57.2			4.0	2.20						
HCH (gamma)	21.8	1.30	56.00	47.7	2.60	1.07	5.4	119.1	779.0	62.0	26.3
Heptachlor	13.2			1.0					188.2	32.3	15.9
Heptachlor epoxide	13.6			0.2					63.8	6.5	
Heptachlor/Heptachlor epoxide	*1.0*			*5.9*					*3.0*	*5.0*	
Mirex										25.9	1.5
PeCB										28.5	127.5
$\overline{X}$	26.9	1.3	56.0	19.8	2153.8	1.1	21.9	119.1	155.0	40.5	40.9

The pesticides in bold are on the Stockholm convention list; the ratio parent/residues is presented in italic style; the dashed line represents the separation before and after the Stockholm Convention (2009).

2. Soils/sediments

Most of the works use sediments as a preferable matrix (74%), tailed by suspended solids (18%) and soils (8%).

Information from 6 continents, mainly Europe (40%) and Asia (31%), followed by Africa (16%), North and South America (6%) and Antarctica (1%) was gathered. No data was available for Oceania (Table 9) so, when citing herein "worldwide", the continent will not appear.

Twenty-two aquatic systems were studied along Europe and 10 of them centred in Portugal (Table 9). Comparing with the water matrix, there are half the number of aquatic systems studies; this may reflect a lack of concern on the pesticide effects present in these matrices (soils and sediments). Average concentrations range from 0.4 to 543.6 ng/g. In Europe, Portugal presents the highest percentage (67%) of the number of pesticides detected/quantified (124 of 184 quantifications) when compared to others countries (2–8%).

Table 9: Pesticide concentrations (minimum, maximum and average values) in sediment samples, displayed by continent, country, and aquatic system; the number of quantified pesticides and sampling year were also added.

	Pesticides No.	Sampling year	min	max	average	References
			ng/g			
Africa						
Egypt						
Maryut Lake	7	2005	-	10.5	1.7	[128]
Manzala Lake	14	1993	0.6	45.2	4.9	[125]
Nile River	13	1993	7.5	8.8	8.0	[125]
Ghana						
Bosomtwi Lake	6	2004	3.6	10.2	4.9	[62]
Nigeria						
Owan River	15	na	-	-	1097.3	[64]
South Africa						
Buffalo River	15	2002	11.4	92.5	45.8	[65]
Lourens River	5	1999	3.3	166.8	49.5	[61]
Antarctica						
Antarctic						
King George Island	4	2009-2010	-	-	0.1	[129]
Asia						
China						
Beijing Guanting reservoir	30	2003-2004	0.3	1.2	0.4	[66]
Hangzhou bay	18	2000-2001	0.5	9.7	4.0	[130]
Jinjiang River	18	na	3.0	8.4	4.2	[67]

(continued)

	Pesticides	Sampling year	min	max ng/g	average	References
Asia						
Minjiang River	18	1999	0.7	4.0	2.0	[68]
Qiantang River	13	2004	1.0	26.6	7.1	[69]
Tonghui River	16	2002	0.1	1.1	0.4	[70]
Yangtze River	8	?	0.6	7.2	2.4	[131]
Macau						
Pearl River	21	2001	0.0	0.5	0.3	[75]
Europe						
Belgium						
Scheldt River	4	2000	-	-	4.0	[132]
France						
Moselle River	4	2008	0.3	0.7	0.4	[133]
Greece						
Pamvotis Lake	4	1998-1999	-	403.5	129.0	[92]
Italy						
Lambro River	4	2001	-	-	9.7	[134]
Poland						
Gulf of Gdańsk	2	2002	3.9	23.0	20.1	[135]
Vistula River	4	2005	2.9	6.3	4.3	[136]
Portugal						
Ave River	11	2007-2008	-	-	1.8	[137]
Cávado River	14	2007-2008	-	-	2.4	[138]
Douro River	11	2007-2008	-	-	1.3	[137]
Ria Formosa Lagoon	2	2007	-	-	1.7	[137]
Ria Formosa Lagoon	18	2012-2013	-	-	543.6	[102]
Lake Vale	5	2004	-	-	9.2	[99]
Lima River	11	2007	-	-	0.7	[137]
Minho River	7/11	2007-2008	-	-	1.4	[137]
Ria de Aveiro Lagoon	1	2011	-	-	3.6	[139]
Sado River	11	2007	-	-	3.4	[137]
Romania						
Danube River	2	2001	-	-	2.3	[140]
Mures, Tarnava Mare and Tarvana Mica River	4	2004-2005	20.0	48.7	29.3	[104]
Serbia						
Danube River	12	2002	-	-	2.3	[141]
Slovakia						
Hron River	7	na	6.3	77.4	10.2	[142]
Spain						
Ebro River	5	2004	-	-	10.7	[143]
Girona River	4	1996-1997	0.9	2.4	1.5	[112]
Guadalquivir River	5	2010-2011	1.5	38.0	5.6	[113, 114]
North America						
California						
Salton Sea	15	2000-2001	3.1	9.1	5.8	[144]
Canada						
Des Prairies River	4	na	-	-	22.0	[145]
USA						
Idaho/Maine/Wisconsin River	10	2009	-	34.7	5.7	[146]
South America						
Brazil						
Batalha/Vargem Limpa River	17	2005	0.2	0.7	0.3	[121]
São Lourenço River	8	1999-2000	0.6	2.1	2.1	[120]

Symbols (na) and (-): *no data found/available*

Data collected between 1993 and 2013, averaged between 0.02 and 1097 ng/g (Table 9). Overall 79 pesticides are quantified Worldwide, the highest number observed in Europe and Asia (more than 40), followed by Africa (32), North and South America (20), and Antarctica (4).

The highest average concentrations and standard deviations (SD) were measured in Africa (253 ng/g; SD 495), then Europe and North America (7 ng/g; SD 17), Asia (2 ng/g; SD 4), and finally South America and Antarctica (less than 1 ng/g, SD 1). In Africa the concentrations of pesticides were at least 36 times higher than in the rest of the world; the highest values are quantified in the Owan River (15 insecticides), Manzala Lake (4,4'-DDE), and Buffalo River (Chlordane (gamma)) [64, 65, 125]. These are independent aquatic systems, so the concentrations are not a local problem, but a consequence of an excessive and improper use of these insecticides over a wide geographical area, *i.e.* from Egypt to South Africa.

Grouping data by pesticide category, it is observed a predominance of insecticides (88%) over the fungicides (7%), and herbicides (5%). The same pattern is applicable per continent, with the exception of North America that presents a similar percentage for insecticides and fungicides (40%) (Figure 3). No comparisons can be made with North America, Antarctica, and Oceania, due to insufficient data.

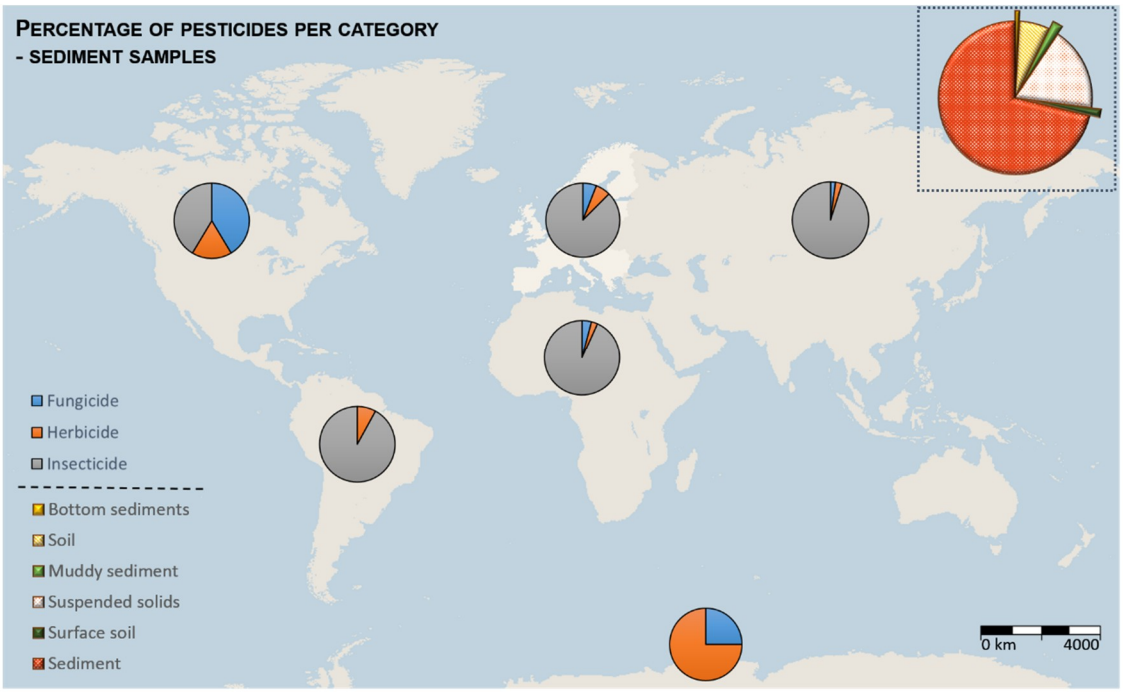

Figure 3: Representation of the quantified pesticides in sediment/soil samples (%), per category, on each continent; the right upper corner figure represents the type of matrices found Worldwide.

The sediment and soil data (Table 10) were evaluated according to the Dutch List standards [59]. The highest frequencies of quantified pesticides were attained for the insecticide category; similar to water fraction, their frequencies seem to be related to the number of field samples. For this category, Europe, Asia, and Africa present a higher number of quantified results (86, 70, and 40, respectively) above the Dutch List optimum levels (Table 10). While for Antarctica, North and South America each has 2, 4, and 10 results above these limits, respectively. Worldwide, 50% of the quantifications were above the optimum levels for insecticides, showing once more an abusive usage of this category of pesticides.

By country, the optimum levels were exceeded with most cases in Portugal (69 samples), followed by China (59 samples), Egypt (15 samples), and Brazil (10 samples) (data not shown) [66, 68-70, 99, 102, 130, 131, 137, 138].

Table 10: Pesticide average concentrations (ng/g) in sediments/soils, displayed by continent and pesticide category, as well as the number of field samples, and the samples above the Dutch List standards; references are only defined for the samples above the Dutch List limits, per category.

	Average amounts ng/g	Field samples No.	Samples above Dutch List	References
Africa				
Fungicide	8.1	3	-	-
Herbicide	1070.0	2	1	[64]
Insecticide	225.5	69	39	[61, 62, 64, 65, 125, 128]
Antarctica				
Fungicide	0.1	1	-	-
Insecticide	0.1	3	2	[129]
Asia				
Fungicide	0.6	3	-	-
Herbicide	0.1	4	-	-
Insecticide	2.3	135	70	[66-70, 75, 130, 131]
Europe				
Fungicide				
Belgium	0.9	1	-	[102, 113, 132, 134, 139, 142, 143]
Italy	5.9	1	-	
Portugal	3.6	5	1	
Slovakia	2.1	1	-	
Spain	6.9	3	-	
Herbicide				
Greece	148.3	3	1	[92, 99, 102, 135]
Poland	20.1	2	-	
Portugal	14.1	7	2	
Insecticide				
Belgium	5.1	3	1	[92, 99, 102, 104, 112-114, 132-134, 136-138, 140-143]
France	0.4	4	1	
Greece	71.0	1	-	
Italy	11.0	3	1	
Poland	4.3	4	2	
Portugal	1.8	112	66	
Romania	18.5	5	2	
Serbia	2.3	12	8	
Slovakia	11.6	6	1	
Spain	6.0	11	4	
North America				
Fungicide	5.1	12	-	-
Herbicide	18.2	5	2	[144, 145]
Insecticide	6.5	12	4	[144]
South America	0.9			
Herbicide	0.6	2	-	-
Insecticide	0.9	23	10	[120, 121]

Symbol (-): *absence of samples above Dutch List*

By taking the most frequent quantified pesticides, equal or more than 10 observations, into consideration, data were grouped per category and continent (Table 11). Excluding methoxychlor, all other pesticides are on the Dutch List. The fungicide HCB ranged from 0.1 to 8.1 ng/g, and it was

present on almost all continents (Table 11). From eleven insecticides, ∑DDT and ∑drins were the most frequent (more than 20 published cases).

To compare between continents, mutual pesticides (∑DDT, ∑drins, ∑HCH, and heptachlor) were summed (Table 11). The highest concentrations were measured in Africa (4436 ng/g) and lowest in South America (4 ng/g). Here, discrepant concentrations between Africa and the rest of the continents are once more observed, reflecting a neglected application/treatment of these compounds. No comparisons are made with North America and Antarctica due to the lack of common data.

Table 11: The average values (ng/g) of the most frequent pesticides, quantified in sediment/soil samples, displayed by category and continent; referring to the most frequent pesticides.

Average amounts (ng/g)	Africa	Asia	Europe	North America	South America	Antarctica	References
Fungicide							
HCB	8.1	0.6	6.1	1.2		0.1	[65, 66, 125, 129, 130, 132, 134, 139, 142-144]
Insecticide							
ΣDDT	317.7	15.0	18.4	24.1	4.6		[61, 62, 64-70, 75, 99, 102, 104, 112, 120, 121, 125, 128-134, 136-138, 140-144, 147]
2,4´-DDT	41.0		4.5	1.1	2.2		
4,4´-DDD	35.0	3.7	1.8	1.8	0.1		
4,4´-DDE	59.0	6.8	5.9	21.2	1.7	0.0	
4,4´-DDT	182.7	4.5	6.2		0.6	0.3	
DDT/DDE+DDD	*2.4*	*0.4*	*1.4*	*0.0*	*1.6*	*7.8*	
Σdrins	814.8	5.0	7.1	5.0	0.4		
Aldrin	334.1	1.5	1.2		0.1		
Dieldrin	244.8	2.3	4.2	5.0	0.2		
Endrin	235.9	1.1	1.8		0.1		
Endosulfan (alpha)	541.3	0.4	1.5				
ΣHCH	1326.3	6.6	2.5	2.6	0.6		
HCH (alpha)	542.4	1.0			0.3		
HCH (beta)	467.1	2.7			0.1		
HCH (gamma)	316.8	2.9	2.5	2.6	0.1		
Heptachlor	357.6	3.3	2.9	1.6	0.1		
Heptachlor epoxide	1070.0	2.7	0.5		0.0		
Heptachlor / heptachlor epoxide	*0.3*	*1.2*	*5.4*		*1.6*		
Methoxychlor		1.1	2.7				
Σ	4435.9	33.4	194.4	35.64	4.1	0.4	

The pesticides in bold are on the Dutch List; the ratio parent/residues is presented in italic style

The most frequent pesticides (equal or more than 10 quantification of pesticides in different aquatic systems or countries) in Europe are compiled in Table 12. A total of 10 pesticides are being consistently registered in 9 countries, from which Portugal and Serbia have at least one occurrence of each one of them. Pesticides like 4,4'-DDT, dieldrin, and endosulfan (alpha) have the highest range of concentrations, when compared to the other pesticides; these ones were observed in Portugal (Table 12, ranges in bold). With exception of endosulfan (alpha), all the other cases are listed as priority pesticides to elimination. Between countries, ∑DDT, aldrin, dieldrin, endrin, and lindane (HCH gamma) presented concentrations above the optimum levels referred in the Dutch List. Overall, the most frequently quantified pesticides are insecticides –listed either as illegal or with usage restriction based on the Stockholm Convention list – presenting concentrations above the threshold values set by the Dutch List for sediments and soils.

Table 12: Average values (ng/g) of the most frequent pesticides, quantified in sediment/soil samples, displayed by category and European country; referring to the most frequent pesticides.

Average amounts (ng/g)	Belgium	France	Italy	Poland	Portugal	Romania	Serbia	Slovakia	Spain	min-max
References	[132]	[133]	[134]	[135, 136]	[99, 101, 137, 138]	[140]	[141]	[142]	[143]	
ΣDDT	15.2	0.7	33.0	16.0	155.4	46.4	12.1	26.6	27.4	0.8-155.4
4,4'-DDD	6.1	0.5	0.6	3.5	8.8		0.9	2.7	2.6	0.5-8.8
4,4'-DDE	6.2	0.2	19.2	1.3	1.2	11.4	6.4	20.5	19.1	0.3-20.5
4,4'-DDT	2.9		13.2	11.3	145.3	35.0	4.9	3.4	5.7	**2.9-145.3**
Aldrin					1.1		0.6		2.6	0.6-2.6
Dieldrin					49.6	33.0	0.6			**0.6-49.6**
Endrin					1.8		2.0		1.6	1.6-2.0
Endosulfan (alpha)					38.2		0.6			**0.6-38.2**
HCH (gamma)				1.3	2.3	1.8	5.6			1.3-5.6
Heptachlor					2.9		2.0			2.0-2.9
Heptachlor epoxide					0.5		0.7			0.5-0.7

The pesticides in bold are on the Dutch List; the highest range differences are represented in bold

The quantified Stockholm Convention pesticides were also grouped by sampling year (Table 13).

In Europe, 9 pesticides and their residues were quantified between 1996 and 2012. The highest number of pesticides quantified were registered between 2002 and 2008. Identical range of concentrations (9 ng/g) were registered between 1996 and 2011, but 2012 stands out with extremely high concentrations (413 ng/g); only Africa registered the same level of concentrations [61, 64, 65]. These last values were quantified in Portugal (Ria Formosa Lagoon) in suspended particulate matter [102]. As few information is available about this specific fraction, no comparisons were made with other European countries.

The parent/residues ratio of DDT and heptachlor (Table 13) were mostly always above 1, demonstrating an active use of both pesticides. These last pesticides were addressed by the Stockholm list in 2009, however there is no sufficient information available from the following years to take conclusions about their use.

Table 13: Average values (ng/g) of the priority listed pesticides quantified in sediment/soil samples, collected in Europe, and displayed by sampling year; referring to the most frequent pesticides.

	1996	1998	2000	2001	2002	2004	2005	2007	2008	2011	2012
References	[112]	[92]	[112, 132]	[134]	[133]	[99, 104, 143]	[136]	[137]	[138, 141]	[139]	[102]
ΣDDT	**1.9**		**15.2**	**24.8**	**12.1**	**46.5**	**16.0**	**5.2**	**3.6**		**1500.8**
2,4'-DDD									0.5		
2,4'-DDE									0.2		
4,4'-DDD	1.1		6.1	0.6	0.9	4.1	3.5	1.0	0.9		70.8
4,4'-DDE			6.2	11.0	6.4	19.5	1.3	1.2	1.1		
4,4'-DDT	0.8		2.9	13.2	4.9	22.8	11.3	3.0	1.3		1430.0
DDT/DDE+DDD	*0.7*		*0.2*	*1.1*	*0.7*	*1.0*	*2.4*	*1.3*	*0.5*		*20.2*
ΣDrins	4.1				3.2	12.2		4.3	3.8		529.7
Aldrin	2.6				0.6			0.9	2.3		
Dieldrin					0.5	12.2		1.6	1.5		529.7
Endrin	1.6				2.0			1.8			
Atrazine		193.0									143.5
DEA		235.0									
Atrazine/DEA		0.8									
HCB			0.8	5.9		18.3				3.6	
HCH (gamma)				1.8	5.6		1.3	2.5	1.3		
Heptachlor					2.0			2.8	3.3		
Heptachlor epoxide					0.7			0.5			
Heptachlor/Heptachlor epoxide					2.9			5.7			
Mirex											64.5
PeCB						1.2					217.2
$\bar{X}$	1.5	214.0	4.0	7.2	2.4	14.5	4.3	1.7	1.4	3.6	392.0

The pesticides in bold are on the Stockholm convention list; the ratio parent/residues is presented in italic style; the dashed line represents the separation before and after the Stockholm Convention (2009).

3. Aquatic Organisms

A total of 37 studies were used, discussing 1039 cases considering the type of pesticides, organisms and aquatic systems (Table 14). The continent about which exists a higher percentage of available results (quantified pesticides in different organisms) is Africa (36%), followed by Europe (29%), Asia (16%), and others (8-9%).

Table 14: Pesticide concentrations (minimum, maximum and average values; ng/g) in aquatic organisms, presented by continent, country, and aquatic system; the number of quantified pesticides and sampling year were also added.

	Pest n	Sampling year	Sample type	min	max	average	References
				ng/g			
Africa							
Egypt							
Manzala Lake	14	1993	F	1.1	8.2	4.1	[125]
Nile River	14	1993	C,F	6.3	7.6	130.2	[125]
Ethiopia							
Koka River	4	2011	F	4.3	27.2	17.1	[148]
Ghana							
Lake Bosomtwi	6	2004	F	1.6	2.8	1.6	[62]
Nigeria							
Ogba River	1	na	F	29.8	32.9	31.4	[63]
Ouémé River	11	2003	F	na	na	540.8	[149]
Ovia River	8	na	F	29.8	32.9	31.4	[63]
Owan River	13	na	F	na	na	476.1	[64]
Tunisia							
Bizerte Lagoon	7	2010	F	14.9	39.3	22.1	[150]
Asia							
China							
East China Sea	10	2003	F	na	na	0.7	[151]
Hong Kong	5	2005	C,F	na	na	1440.2	[152]
Taihu Lake	17	na	Mo	na	34.5	11.8	[153]
Yangtze Estuary	4	2005	C,Mo	2.5	12.8	5.6	[154]
Tibete							
Lhasa River	8	2005	F	na	na	1.0	[155]
Europe							
Baltic Sea							
Gulf of Gdańsk	3	2003	C,F,Mo	8.1	10.8	9.4	[156]
Belgium							
Scheldt River	2-3	2001	F,Mo	1.5	7.6	4.1	[35, 157]
Finland							
Gulf of Finland	5	2002	F	1.8	4.4	na	[158]
France							
Charente River	2	2001	F	0.2	0.5	0.3	[35]
Gironde River	2	2001	F	0.8	1.7	1.1	[35]
Loire River	2	2001	F	0.3	4.3	1.1	[35]
Moselle River	9	2008	F	0.4	0.7	0.4	[133]
Seine River	2	2001	F	0.2	1.5	0.6	[35]

(continued)

	Pest n	Sampling year	Sample type	min	max	average	References
					ng/g		
Europe							
Italy							
Garigliano River	5	2005	F	4.2	21.6	10.9	[159]
Italy coast	3	2002	C,F,Mo	0.9	2.8	1.5	[160]
Mediterranean sea	3	2010	F	6.9	9.6	8.2	[150]
Poland							
Oder River	8	2003-2004	F	na	na	0.3	[96]
Portugal							
Lake Vela	2	2004	F	na	na	0.2	[99]
Ria de Aveiro	1	2011	Mo	na	na	0.2	[139]
Ria Formosa Lagoon	54	2012-2013	Mo	7.6	27.2	14.9	[161]
Tagus River	53	2012-2013	Mo	4.6	72.0	18.6	*Chapter 7*
Romania							
Danube delta	6	2001	F,Z	188.3	278.4	220.3	[140]
Spain							
na	2	2011	Mo	10.3	9.0	8.0	[162]
Girona	4	1996-1997	F	0.4	2.5	1.2	[112]
Vigo	3	na	Mo	0.6	4.4	na	[163]
North America							
Californa							
Salton Sea	19	2001	F	1.5	25.2	7.2	[144]
Canada							
Kitimat river	20	1999-2000	F	0.7	2.5	1.5	[164]
Greenland							
na	6	1994-1995	F,Mo	na	na	5743.4	[165]
USA							
Missouri and Mississipi River	3	2004-2005	F	na	na	7.2	[166]
South America							
Brazil							
Cananeia	5	1996-2001	Ma	132.9	16351.9	4994.0	[167]
Piracicaba river	3	2006	F,Mo	30.1	135.1	99.8	[168]
Ponta Grossa Lake	29	2005	F	13.5	92.3	20.6	[169]
Rio de Janeiro coast	22	2009	Mo	0.1	0.1	0.1	[170]

Symbols (na) and (-): *no data found/available;* ***C, F, Ma, Mo, and Z****: crustaceans, fishes, mammals, molluscs, and zooplankton;*

The data collected between 1993 and 2013 averaged from 0.004 to 26 000 ng/g (Table 14). Europe is represented by eleven aquatic systems, while other continents do not have more than five. In spite of this, Africa have more observations (338) than Europe (299) and the other continents (between 83 and 167). Since the number of quantified pesticides are similar between continents, these differences are owning to the number of species used in each study, revealing a wide range in Africa. North America stands out with average concentrations of 1017 ng/g (SD 3802), followed by Asia, Africa, South America (294 ng/g; SD 1465), and Europe (62 ng/g; SD 282).

This scattered difference is mainly due to the average values in Greenland (Table 14).

When grouping pesticides by category, insecticides prevail in 89% of biologic analyses, leaving 5 and 7% for the herbicide, and fungicide categories, respectively. The same pattern is applicable among continents (Figure 4). No data are available for Oceania and Antarctica, so, when citing herein "worldwide", these continents will not appear.

Analysing data by matrix, the most common is fish (74%) and molluscs (21%); the rest is completed with crustaceans, zooplankton and mammals (Figure 4).

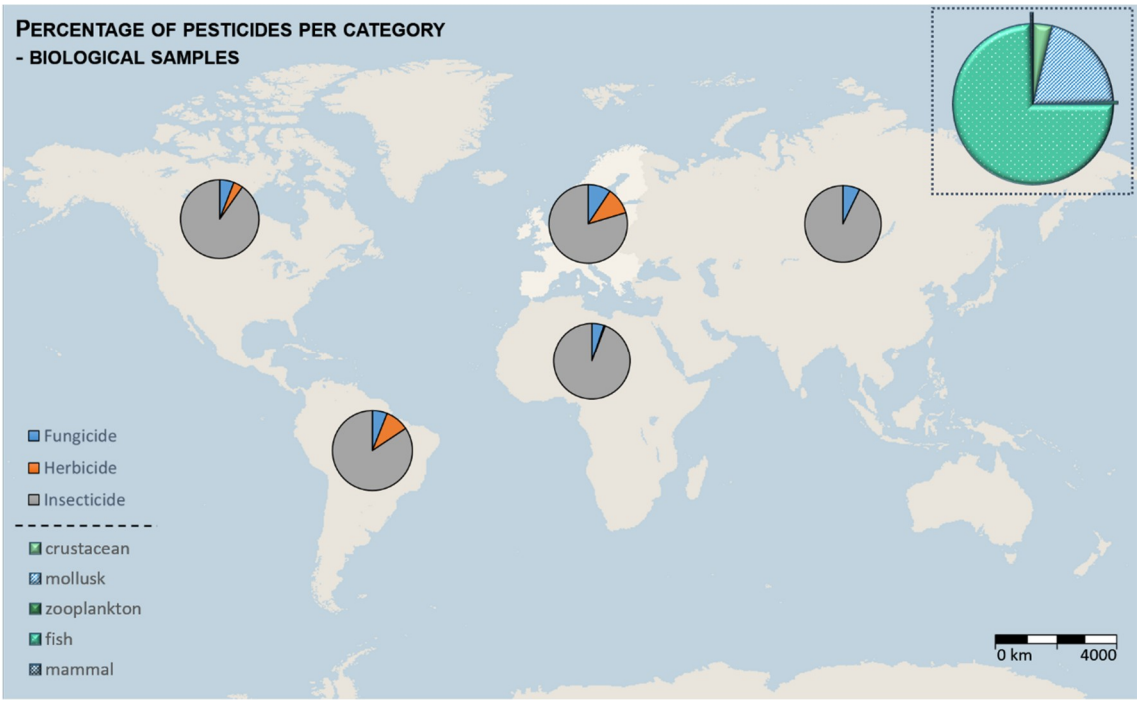

Figure 4: Representation of the quantified pesticides in organisms (%), per category, in each continent; the right upper corner pie chart represents the Metazoan lineages used worldwide.

By grouping the organisms, we can see that 75% of the quantified pesticides are done in vertebrates and the other 25% in invertebrates (Figure 5). While for the latter, 86% of the quantifications were done using the whole animal (86%), for vertebrates it is further divided; specific organs or tissues are used to quantify pesticides.

Many factors can influence these results. Invertebrates are small, less complex and as a food resource almost entirely eatable, while the same is not applicable to vertebrates. Besides that, it may also depend on the objective of the study (food control or environmental/toxicological studies) and on the different organs extracted; as the pesticide quantities are different when analysing muscle, liver, gonads, or gills. The bubbler tissue is only applicable for aquatic mammals (Figure 5).

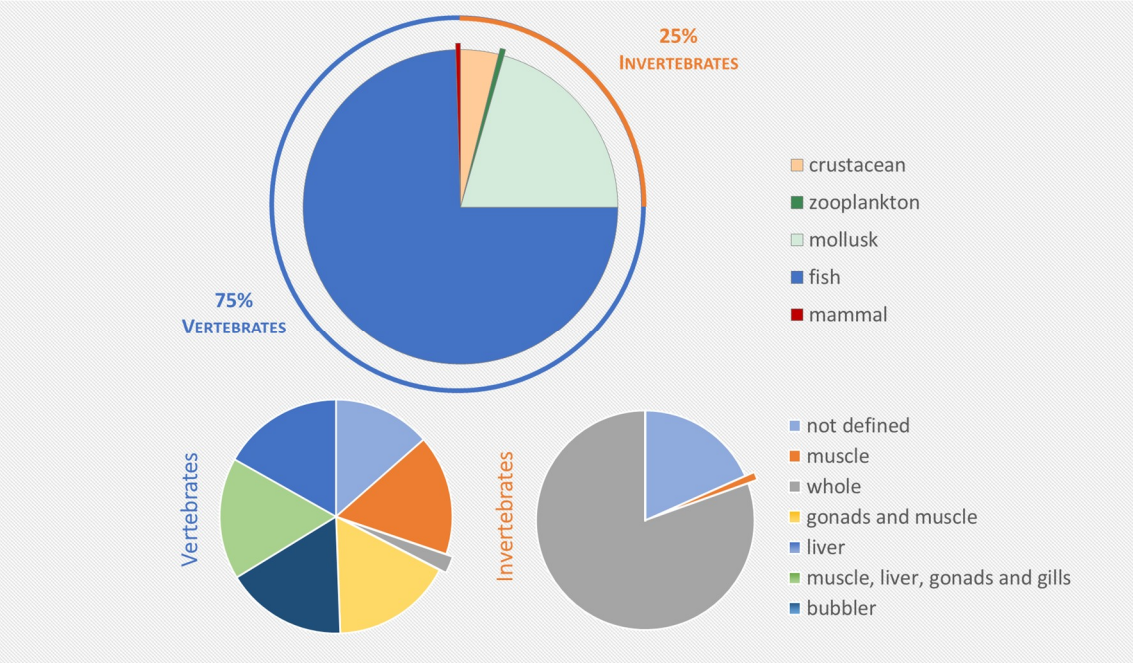

Figure 5: Representation of the quantified pesticides in organisms (%), per lineages of Metazoan, vertebrates and invertebrates, and matrices.

Results per Metazoan lineages (crustacean, fish, mammal, mollusc, and zooplankton) were assessed considering the averages concentrations and the number of quantifications (Figure 6). Average concentrations swing from 15 ng/g (zooplankton and mollusc) to 271 ng/g (crustacean and fish) and 5000 ng/g (aquatic mammal; Figure 6A). Such pattern is quite revealing about the bioaccumulative properties of pesticides.

Within continents, Europe presents the highest diversity on analysed organisms (4 Metazoan lineages) and the lowest concentrations (60 ng/g), comparing to others collected from the other continents; the highest concentrations were registered in North America (1020 ng/g; Figure 6B).

As for concentrations, the number of quantifications were quite different between the taxa; more cases were identified/quantified for fish and mollusc than for crustacean, zooplankton, and mammals. This fact may be influenced by the researcher purpose and sample availability/convenience.

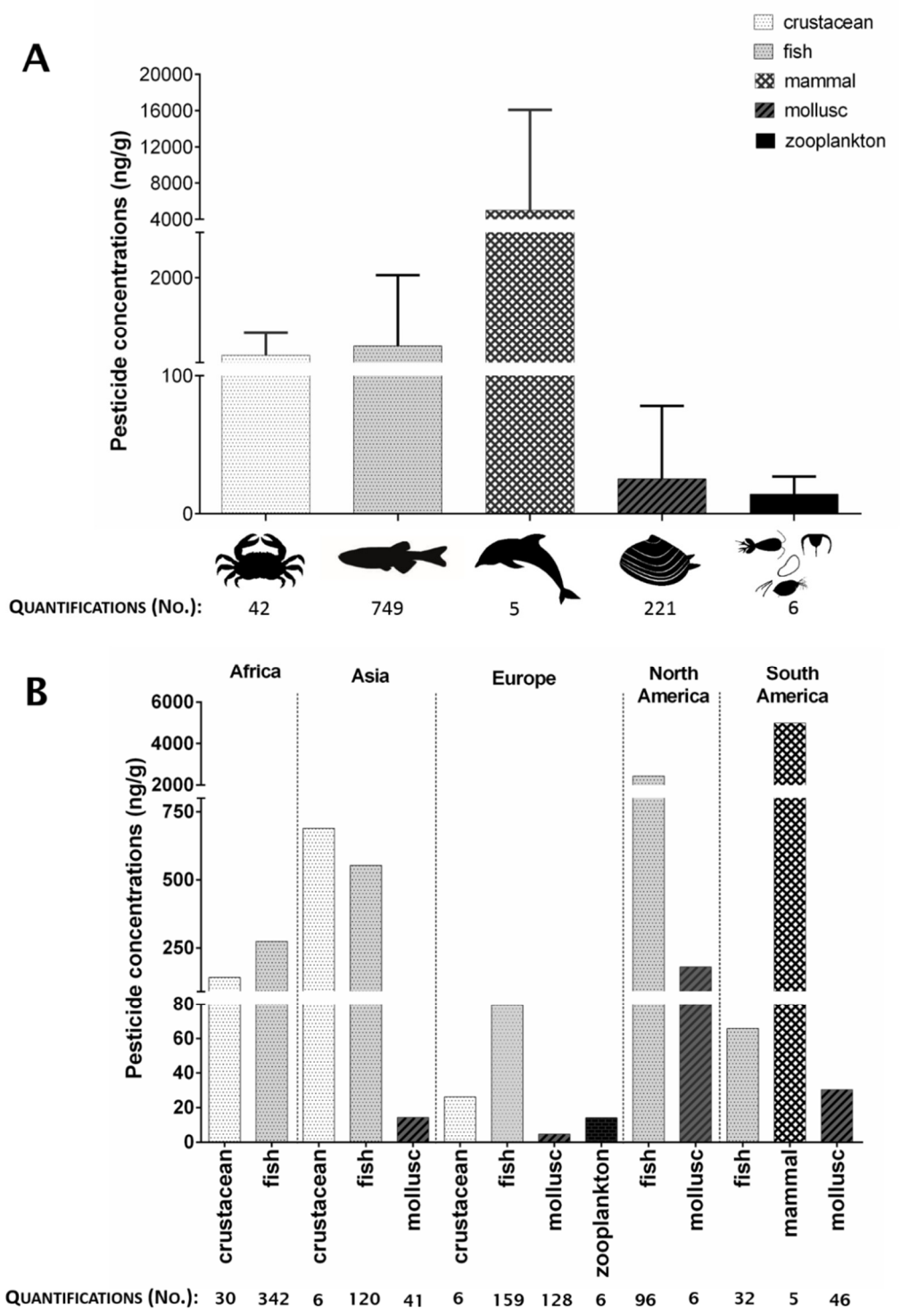

Figure 6: Average concentrations (ng/g) and number of quantifications per Metazoan lineage; Worldwide (A) and continent (B) representation.

Average concentrations are also evaluated according to the MRL values set by 2013/39/EU Directive (Table 15). On a global scale, insecticides have the highest average concentrations (314 ng/g) when compared to fungicides (117 ng/g) and herbicides (29 ng/g); these differences are a result of the high concentrations measured in Greenland, North America (1017 ng/g). We found no data for herbicides in Asia.

Using the European Directive as a standard reference, 67% of the quantified samples in Africa were above that MRLs, followed by Europe (48%) and the rest of the continents with similar percentages (30%).

Within Europe, more countries were able to quantify insecticides than other category of pesticides (Table 15). Among insecticides, Romania presents the highest average levels (261.6 ng/g) in comparisons to the rest of the European areas (6 ng/g). Fungicides and insecticides reached an average of 6 and 7 ng/g, respectively. In spite of these values being below 10 ng/g, half of the quantified samples are above 2013/39/EU levels; Romania and Portugal have almost all samples with concentrations above the maximum recommended levels.

Table 15: Pesticide average concentrations (ng/g) in aquatic organisms, displayed by continent and pesticide category, as well as the number of field samples, and the samples above the 2013/39/EU Directive standards; references are only defined for the samples above the 2013/39/EU Directive, per category.

	Average amounts ng/g	Field samples No.	Samples above 2013/39/EU	References
Africa				
Fungicide	17.3	20	13	[125, 150]
Herbicide	360.0	2	2	[64]
Insecticide	246.3	350	234	[62-64, 125, 148-150]
Asia				
Fungicide	625.7	12	1	[151, 152, 155]
Insecticide	308.2	155	46	[151-155]
Europe				
Fungicide				
Finland	3.2	1	1	
France	0.4	1	-	
Italy	1.8	1	-	[133, 139, 140,
Mediterranean sea	8.1	2	1	150, 158, 159]
Portugal	11.7	11	5	
Romania	13.6	12	5	

(continued)

	Average amounts ng/g	Field samples No.	Samples above 2013/39/EU	References
Europe				
Herbicide				
Belgium	1.0	2		[99, 171]
Portugal	13.1	31	17	
Insecticide				
Adriatic Sea	1.5	21		[35, 96, 112, 133, 140, 150, 156, 158-160, 162, 163, 171]
Baltic Sea	9.4	18	1	
Belgium	6.2	3	1	
Finland	2.7	4	4	
France	0.6	19		
Italy	13.1	4	2	
Mediterranean sea	8.2	6	2	
Poland	0.3	22	2	
Portugal	18.4	68	42	
Romania	261.6	60	57	
Spain	2.6	13	4	
North America				
Fungicide	2.6	6	-	-
Herbicide	1.7	4	-	-
Insecticide	1127.4	92	34	[144, 164-166]
South America				
Fungicide	6.1	5	2	[167, 169, 170]
Herbicide	27.5	8	3	[169]
Insecticide	371.7	70	18	[167-170]

Symbol (-): *absence of samples above 2013/39/EU Directive*

The most frequent pesticides (equal or more than 10 quantifications of pesticides in different aquatic systems or countries), are listed by category and continent in Table 16. The EQS, established by Directive 2013/39/EU, are used to provide a comparison with these data. From seven pesticides, six of them are listed in this directive; among them, only one is fungicide (HCB) and the rest are insecticides.

All the referred pesticides in Table 16 are reported, at least once, in concentrations that surpass the threshold levels set for biota in 2013/39/EU Directive. The insecticides ∑DDT and heptachlor were quantified – on all continents – above the reference levels of the directive. Analysing the data by continent, in Africa the average concentrations for all compounds were above the threshold limits referred by the EU Directive (2013/39/EU) (Table 16).

The highest cumulative amounts (Σ) were registered in North America (16 980 ng/g), followed by Africa and Asia (2800 ng/g), Europe (565 ng/g), and South America (172 ng/g).

In light of above, there are countries in all continents that have been exceeding the EQS values, demonstrating polluted aquatic environments capable to transfer these compounds into the biota. Among continents, Africa have constantly high concentrations—transposing the EU levels set for biota—which may affect dramatically the local and migratory fauna.

Table 16: The average values (ng/g) of the most frequent pesticides, quantified in organisms, displayed by category and continent.

Average amounts (ng/g)	Africa	Asia	Europe	North America	South America	References
Fungicide						
HCB	17.3	625.7	9.9	3.5	10.2	[125, 133, 139, 140, 144, 150-152, 155, 158, 159, 164, 167, 169, 170]
Insecticide						
ΣDDT	1602.4	648.2	462.2	8526.4	31.1	[35, 62-64, 96, 112, 125, 133, 140, 144, 148-156, 158-160, 162-165, 168-170]
DDT/DDE+DDD	*0.7*	*0.0*	*0.0*	*0.4*	*1.1*	
2,4'-DDD	90.2		0.3	3.3	10.7	
2,4'-DDT	62.0	1.2		2.3	0.1	
4,4'-DDD	286.8	2.5	142.3	1702.1	0.1	
4,4'-DDE	587.4	643.2	304.3	4198.2	4.0	
4,4'-DDT	576.0	1.2	15.2	2620.5	16.1	
Chlordane (alpha)	26.9	471.6		1.0	4.0	
Chlordane (gamma)	26.8	959.2	8.0	0.3	7.4	
ΣEndosulfan	535.8	0.6	17.7	0.3	7.3	
Endosulfan (alpha)	344.6	0.4		0.3	4.7	
Endosulfan (beta)	191.2	0.2	17.7		2.6	
ΣHCH	401.0	2.1	61.6	8442.8	68.2	
HCH (alpha)	140.6	0.5	43.4	6902.4	0.0	
HCH (beta)	208.2	1.2	0.4	718.7	0.2	
HCH (gamma)	52.2	0.5	17.7	821.7	67.9	
Heptachlor	292.6	6.2	5.7	6.5	44.1	
Σ	2902.8	2713.5	550.7	16980.8	172.2	

The pesticides in bold are in 2013/39/EU Directive; the ratio parent/residues is written in italics

The same study is done for Europe as well, providing detailed information by country (Table 17). The two insecticides, ∑DDT and ∑HCH, were the most frequent in all analysed samples. Romania have the highest concentrations (as detected in fish and zooplankton) for both DDT and HCH, being ca. forty fold higher than the average concentrations measured for these compounds in of other countries, mainly for fish. Since the Danube River, the second

longest river in Europe, passes through several countries and flows through Romania, the concentrations herein reported may be a consequence of the anthropogenic activities along its course. In fact, excluding Romania, in the other European countries the concentrations of these pesticides were in general low and, during mostly all time, inside the 2013/39/EU limits.

Table 17: The average values (ng/g) of the most frequent pesticides in European countries, quantified in aquatic organisms; referring to the most frequent pesticides.

Average amounts (ng/g)	Adriatic Sea	Baltic Sea	Belgium	Finland	France	Italy	Mediterranean sea	Poland	Portugal	Romania	Spain	min-max
References	[160]	[156]	[35]	[158]	[35,133]	[159,160]	[150]	[96]	Chapter 7	[140]	[112,162]	
ΣDDT	4.6	28.2	3.0	2.9	2.3	42.7	19.8		20.6	1216.0	14.5	*2.3-1216.0*
DDT/DDE+DDD	0.0	0.1		0.1	1.6	0.5			0.7	0.0		0.03-1.6
4,4'-DDD	3.6	0.9		0.6	0.2	7.7	9.3		7.0	385.1	2.5	0.2-385.1
4,4'-DDE	0.7	25.6		2.0	0.7	20.8	10.5		5.3	793.2	12.0	0.7-793.2
4,4'-DDT	0.2	1.7	3.0	0.3	1.4	14.2			8.3	37.6		0.2-37.6
ΣHCH			0.1	7.8	0.4		5.0	0.9		92.1	0.3	**0.1-92.1**
HCH (alpha)					0.1			0.3		57.7		0.1-57.7
HCH (gamma)			0.1	7.8	0.3		5.0	0.6		34.4	0.3	0.1-34.4

The pesticides in bold are in 2013/39/EU Directive; the ratio parent/residues is presented in italic style

The pesticides, addressed by the Stockholm Convention, are organized in Table 18 by sampling year. A total of ten pesticides were quantified between 1996 and 2012. Their concentrations ranged from 0.4 to 11 ng/g between years, with the exception for 2001 occasion when pesticide average values attained 194 ng/g, derived mainly from fish samples [35, 140]. In spite of this high concentration, similar or higher averages were registered on the other continents (Asia and North America) as well (261 and 1400 ng/g, respectively), in crustacean, fish, mollusc, and aquatic mammal samples. Repeatedly, these values are connected to 4,4'-DDE and 4,4'-DDD concentrations found in the Danube estuary in Romania [140].

Some studies were conducted on parent/residues ratio of DDT and heptachlor [35, 96, 112, 132, 133, 139, 150, 156, 158-160, 162]. While DDT did not present any ratio value above 1 along the years, heptachlor had two occurrences (years 2003 and 2012); for the rest of the years no data are available (Table 18).

The same range of concentrations were registered before and after the last Stockholm update (2009). This fact may indicate a continuous use of these pesticides along these years demonstrating an illegal usage of these compounds.

Table 18: Average values (ng/g) of the most frequent pesticides in aquatic organisms sampled in Europe, and displayed by sampling year; referring to the most frequent pesticides.

	1996	2001	2002	2003	2005	2008	2010	2011	2012
References	[112]	[132,35]	[160,158]	[156,96]	[159]	[133]	[150]	[139,109]	*Chaper 7*
ΣDDT	1.1	1205.4	4.3	28.2	42.7	1.5	19.8	16.0	20.6
DDT/DDE+DDD		*0.0*	*0.0*	*0.1*	*0.5*				*0.7*
2,4´-DDD						0.3			
2,4´-DDE						0.3			
4,4´-DDD	1.1	385.1	3.2	0.9	7.7	0.2	9.3	4.0	7.0
4,4´-DDE		793.2	0.9	25.6	20.8	0.7	10.5	12.0	5.3
4,4´-DDT		27.1	0.2	1.7	14.2				8.3
Σcyclodienes	2.9			1.0	9.8	0.2			24.7
Aldrin	0.4			0.2					10.5
Dieldrin	2.5			0.3	9.8	0.2			1.7
Endrin				0.4					12.5
Chlordane (gamma)									8.0
HCB		13.6	4.9		1.8	0.4	8.1	0.2	3.8
ΣHCH	0.3	82.0	7.8	1.3		1.3	5.0		
HCH (alpha)		57.7		0.3		0.1			
HCH (beta)				0.4		0.6			
HCH (gamma)	0.3	24.3	7.8	0.6		0.5	5.0		
Heptachlor				0.1					11.3
Heptachlor epoxide	1.2			0.1					7.0
Heptachlor/Heptachlor epoxide				*1.1*					*1.6*
Mirex									11.4
PeCB									5.0
$\overline{X}$	1.2	193.5	1.8	4.4	10.9	0.4	8.2	5.4	8.2

The pesticides in bold are on the Stockholm Convention list; the ratio parent/residues is presented in italic style; the dashed line represents the separation before and after the Stockholm Convention (2009)

V. Environmental and Human Risks

1. Half effective and lethal concentrations (EC_{50}/LC_{50}) for aquatic organisms

It is well established that all pesticides, at specific concentrations, are harmful to biota, affecting algae and plants, invertebrates and vertebrates [6]. Because of these negative impacts, databases like Pesticides Properties DataBase (PPDB) present information about physicochemical properties, environmental fate, human health, and ecotoxicological data of all active ingredients and approved pesticides [172].

In this work, and in order to evaluate the worst case situation, the mean of the maximum water concentrations measured in each continent were used and compared to PPDB documented values; acute and chronic concentrations for aquatic animals were taken into consideration (Table 19).

Table 19: Average of maximum environmental concentrations (MEC), per continent, and half effective and lethal concentrations of several pesticides at different aquatic trophic levels; data data expressed in mg/L.

	MEC	Fish	Invertebrate	Crustacean	Fish	Invertebrate
		96h LC_{50}	48h EC_{50}	96h LC_{50}	21 days NOEC	
Africa						
Azinphos-methyl	**2.4E-04**	-	-	2.2E-04	1.7E-04	-
Endosulfan	3.0E-04	-	-	-	1.0E-07	-
Asia						
Chlorpyrifos	**2.1E-04**	-	1.0E-04	4.0E-05	1.4E-04	-
Deltamethrin	**4.3E-06**	-	-	1.7E-06	-	4.1E-06
Endosulfan	1.5E-04	-	-	-	1.0E-07	-
Ethion	**2.1E-04**	-	5.6E-05	-	-	-
Europe						
Azinphos-methyl	**2.5E-04**	-	-	2.2E-04	1.7E-04	-
Chlorfenvinphos	1.4E-04	-	-	-	-	1.0E-04
Chlorpyrifos	**8.5E-05**	-	-	4.0E-05	-	-
Cyflurin	2.1E-04	-	-	-	1.0E-05	-
Cyhalothrin	**1.6E-03**	4.6E-04	-	-	-	-
Cypermethrin	4.3E-04	-	-	-	3.0E-05	-
Deltamethrin	**5.2E-03**	2.6E-04	5.6E-04	1.7E-06	3.2E-05	4.1E-06
Dichlorvos	**2.1E-04**	-	1.9E-04	-	-	-
Dieldrin	**8.0E-03**	1.2E-03	-	-	-	-
Endosulfan	5.5E-04	-	-	-	1.0E-07	-
Malathion	**1.1E-03**	-	7.0E-04	-	-	6.0E-05
Pyridaben	**6.1E-04**	-	-	5.4E-04	-	8.6E-05
South America						
Endosulfan	5.9E-05	-	-	-	1.0E-07	-

NOEC: *no-observed-effect-concentration; the dashed line represents the separation between acute and chronic assays*

From all continents, Europe reported the highest number of case studies above the half effective and lethal documented concentrations, followed by Asia, Africa, and South America; no reports were registered in North America and Oceania. However when data is proportional analysed by the number of analysed pesticides, the scenario changes. Respectively, Asia reported a higher number of quantified pesticides (18%), than Africa (14%), Europe (12%), and South America (6%).

On a global scale, 12 MEC of pesticides were present at concentrations above the acute response levels set for fishes, invertebrates, and crustaceans. These values at or up to nineteen times higher, are capable to cause immediate effects (in bold; Table 19).

2. Predictive aquatic risk assessment of pesticide mixtures

Despite of common occurrence of pesticides mixtures in the environment, laws, conventions and recommendations still focus on individual standard parameters. Modelling approaches, based on available ecotoxicological information, can be used to estimate the impact of mixtures in the biota, completing this lack of information [173].

Based on the European chemicals legislation REACH, the ecological Risk Quotient (RQ) is determined by the equation:

$$\mathrm{RQ}\left(\frac{MEC}{PNEC}\right) = \frac{\text{Measured Environmental Concentration (MEC; mg/L)}}{\text{Predicted No Effect Concentration (PNEC; mg/L)}}$$

PNEC is derived by selecting the most finest biotest organism (represented by the more sensitive trophic level – algae, crustacean or fish), and applying an appropriate assessment factor (AF) [172, 174]. The AF, also denoted as safety or uncertainty factor, considers intra- and inter-laboratory variation of the data, biological variance, and short-term to long-term exposures, presenting stipulated values for specific conditions [175, 176]; for instance, an AF = 100 should be applied considering the Maximum Acceptable Concentration–Quality Standards (MAC-QS) to assess short-term effects for each of the three trophic levels of the base set [175].

The RQ values, classified from <0.01 (negligible) to >1 (very high), indicate a range of potential risks for concern, but does not provide information about the individual toxicity (biological end point and organism) [173, 177]. Therefore, a second approach, which defines the most sensitive trophic level for the quantified environmental concentrations, should be applied [173]:

$$\text{RQ toxic units (TU)} = \frac{\text{MEC}}{\text{EC}_{50} \text{ or } \text{LC}_{50} \text{ per each trophic level}}$$

Afterwards, RQ_{TU} values are summed per trophic level (sum of the toxic units; RQ_{STU}) and the highest sum, among the selected trophic levels, is multiplied subsequently by AF. If $RQ_{(MEC/PNEC)}$ and RQ_{STU}>1, additional considerations are required [173]. Based on the two reference models–concentration addition (CA) and independent action (IA)–the RQ_{STU}/max_{TU} can be used to predict the second-tier, resulting in the maximum value from which CA may display higher toxicity values than IA [178].

In this work, the mean of the maximum measured concentration of pesticides in water samples were used to assess the potential risk per continent and on a worldwide scale (Table 20).

Table 20: Ecological risk assessment through the PNEC, using the maximum average concentrations of pesticides in water (mg/L), quantified in each continent and worldwide; based on Table 4 data and respective references.

	Africa	Asia	Europe	Oceania	South America	Worldwide	PNEC (mg/L)	Group
	MEC (mg/L)							
Fungicide								
Azoxystrobin			6.5E-04			6.5E-04	2.3E-03	Invert.
Benalaxyl			2.5E-05			2.5E-05	5.9E-03	Invert.
Cyproconazole			2.0E-05			2.0E-05	9.9E-04	Algae
Cyprodinil			3.5E-04			3.5E-04	2.2E-03	Invert.
Difenoconazol			5.1E-04			5.1E-04	3.2E-04	Algae
Epoxiconazol			1.7E-04		3.7E-05	1.7E-04	1.2E-02	Algae
Fenarimol			5.6E-06			5.6E-06	1.5E-02	Algae
Fenpropimorph			4.0E-06			4.0E-06	3.3E-03	Algae
Flusilazole			1.0E-05			1.0E-05	1.2E-02	Fish
HCB	8.5E-05	1.2E-05	1.3E-04			1.3E-04	1.0E-04	Algae
Metalaxyl			1.4E-04			1.4E-04	4.2E-03	Algae
Metconazole					4.8E-05	4.8E-05	1.7E-02	Algae
Oxadixyl			4.6E-04			4.6E-04	4.6E-01	Algae
PeCB			1.3E-04			1.3E-04	2.5E-03	Fish
Procymidone			1.0E-04			1.0E-04	1.8E-02	Invert.
Propiconazole			1.5E-04			1.5E-04	9.3E-04	Algae
Pyrimethanil			6.3E-05			6.3E-05	1.2E-02	Algae
Tebuconazole			3.1E-04		3.3E-05	3.1E-04	2.0E-02	Algae
Triadimefon			4.3E-06			4.3E-06	2.0E-02	Algae
Triadimenol			8.4E-06			8.4E-06	9.6E-02	Algae
Herbicide								
2,4,5-T			1.0E-05			1.0E-05	1.3E-02	Fish
2,4-D			1.5E-04	7.1E-04		7.1E-04	2.4E-01	Algae
Acetochlor		5.5E-07				5.5E-07	2.7E-06	Algae
Aclonifen			1.4E-04			1.4E-04	4.7E-03	Algae
Alachlor		1.7E-06	3.6E-03		1.1E-05	3.6E-03	9.7E-03	Algae
Ametryn				2.0E-04	2.0E-05	2.0E-04	3.6E-05	Algae
Atrazine	1.5E-04		5.3E-04	1.1E-03	2.5E-05	1.1E-03	5.9E-04	Algae
Atrazine-desethyl			9.9E-05	1.0E-04		1.0E-04	1.0E-03	Algae
Bentazone			5.7E-04			5.7E-04	1.0E-01	Algae
Bromacil			6.0E-05			6.0E-05	1.3E-04	Algae
Chloridazon			4.2E-05			4.2E-05	3.0E-02	Algae
Cyanazine			2.1E-04			2.1E-04	2.0E-03	Algae
Cyhalofop-butyl			1.8E-04			1.8E-04	7.9E-03	Fish
Dicamba			1.0E-05			1.0E-05	1.8E-02	Algae
Diuron			1.4E-03	2.1E-03		2.1E-03	2.7E-05	Algae
EPTC			1.0E-05			1.0E-05	5.5E-02	Algae
Fenuron			7.0E-06			7.0E-06	1.5E-02	Algae
Glyphosate	2.7E-04		2.5E-03			2.5E-03	4.4E-02	Algae
Hexazinone				5.7E-04		5.7E-04	1.5E-04	Algae
Imazapic					3.5E-05	3.5E-05	5.1E-04	Algae
Isoproturon			2.7E-04			2.7E-04	1.3E-04	Algae
MCPA			3.6E-04			3.6E-04	5.0E-01	Fish
MCPB			1.0E-05			1.0E-05	4.3E-02	Fish
Mecoprop			3.0E-05			3.0E-05	2.0E+00	Invert.
Metazachlor			1.0E-05			1.0E-05	1.6E-04	Algae
Metobromuron			8.0E-07			8.0E-07	6.3E-03	Algae
Metolachlor		2.3E-05	3.3E-04		5.0E-06	3.3E-04	3.9E-02	Fish
Metribuzin			4.8E-04			4.8E-04	2.0E-04	Algae
Monolinuron			4.4E-04			4.4E-04	1.0E-05	Algae

(*continued*)

	Africa	Asia	Europe	Oceania	South America	Worldwide	PNEC (mg/L)	Group
	MEC (mg/L)							
Herbicide								
Monuron			1.1E-06			1.1E-06	1.0E+00	Fish
Nitrofen		1.3E-06				1.3E-06	7.0E-02	Fish
Norflurazon			1.1E-03			1.1E-03	1.8E-04	Algae
Oxadiazon			1.8E-04			1.8E-04	4.0E-05	Algae
Pendimethalin			3.7E-04			3.7E-04	6.0E-05	Algae
Prometryn			4.5E-06			4.5E-06	2.0E-05	Algae
Propachlor			2.0E-05			2.0E-05	1.5E-04	Algae
Propanil			9.0E-05			9.0E-05	1.1E-03	Algae
Propazine			1.2E-04			1.2E-04	1.8E-03	Algae
Propyzamide			4.8E-05			4.8E-05	2.8E-02	Algae
Prosulfocarb			7.4E-05			7.4E-05	4.9E-04	Algae
Simazine			9.4E-04	4.5E-05	9.0E-06	9.4E-04	4.0E-04	Algae
Simetryn			6.5E-05			6.5E-05	9.8E-05	Algae
Terbumeton			1.1E-04			1.1E-04	9.0E-05	Algae
Terbuthylazine			4.5E-03			4.5E-03	1.2E-04	Algae
Terbutryn			5.8E-04			5.8E-04	2.4E-05	Algae
Trifluralin		4.5E-06	6.3E-04		7.0E-06	6.3E-04	1.2E-04	Algae
Insecticide								
ΣDDTs	2.7E-03	1.3E-03	4.0E-04		1.1E-04	2.7E-03	5.0E-05	Invert.
Aldrin	7.7E-04	7.5E-05	2.7E-03			2.7E-03	4.6E-05	Fish
Azinphos-methyl	2.4E-04		2.5E-04			2.5E-04	1.1E-05	Invert.
Carbofuran	3.0E-05		9.0E-06		2.5E-05	3.0E-05	9.4E-05	Invert.
Chlordane	9.0E-05	1.0E-05	6.3E-06			9.3E-05	9.0E-04	Fish
Chlorfenvin-phos			1.4E-04			1.4E-04	2.5E-06	Invert.
Chlorpyrifos		2.1E-04	8.5E-05			2.1E-04	4.0E-07	Invert.
Chlorpyrifos methyl			2.8E-06			2.8E-06	6.0E-06	Invert.
Cyfluthrine			2.1E-04			2.1E-04	1.6E-06	Invert.
Cyhalothrin (lambda)			1.6E-03			1.6E-03	4.6E-06	Fish
Cypermethrin		1.3E-06	4.3E-04			4.3E-04	3.0E-06	Invert.
Deltamethrin		4.3E-06	5.2E-03			5.2E-03	2.6E-06	Fish
Diazinon			1.6E-04			1.6E-04	1.0E-05	Invert.
Dichlorvos			2.1E-04			2.1E-04	1.9E-06	Invert.
Dicofol		9.1E-07	1.2E-04			1.2E-04	7.5E-04	Algae
Dieldrin	1.4E-04	7.1E-05	8.0E-03			8.0E-03	1.2E-05	Fish
Diethyltoluami-de					1.9E-04	1.9E-04	7.1E-01	Fish
Dimethoate			2.0E-04		3.5E-05	2.0E-04	2.0E-02	Invert.
Endosulfan	3.0E-04	1.5E-04	5.5E-04		5.9E-05	5.5E-04	2.0E-05	Fish
Endrin	6.0E-05	7.7E-05	6.7E-05			7.7E-05	7.3E-06	Algae
Ethion		2.1E-04	2.1E-05			2.1E-04	5.6E-07	Invert.
Fenamiphos			1.3E-04			1.3E-04	1.9E-05	Invert.
Fenitrothion			2.9E-04			2.9E-04	8.6E-05	Invert.
Fenvalerate		2.3E-06				2.3E-06	8.0E-07	Invert.
Fonofos			6.3E-05			6.3E-05	2.3E-05	Invert.
Lindane	7.9E-04	7.9E-04	7.8E-04		1.0E-05	7.9E-04	2.9E-05	Fish
Heptachlor	1.4E-04	1.0E-04	1.9E-04			1.9E-04	7.0E-05	Fish
Heptachlor epoxide	4.3E-04	3.3E-05	6.4E-05			4.3E-04	2.0E-04	Fish
Imidacloprid			2.4E-04			2.4E-04	1.0E-01	Algae
Isodrin			2.0E-06			2.0E-06	1.2E-04	Fish
Malathion			1.1E-03		4.2E-05	1.1E-03	7.0E-06	Invert.

(*continued*)

	Africa	Asia	Europe	Oceania	South America	Worldwide	PNEC (mg/L)	Group
	MEC (mg/L)							
Insecticide								
Methidathion			2.0E-06			2.0E-06	6.4E-05	Invert.
Methomyl			6.6E-04			6.6E-04	7.6E-05	Invert.
Methoxychlor		7.9E-05	2.7E-04			2.7E-04	5.2E-04	Invert.
Mirex			4.0E-05			4.0E-05	1.0E-03	Invert.
Oxamyl			1.4E-04			1.4E-04	3.2E-03	Invert.
Parathion-ethyl			6.2E-05			6.2E-05	2.5E-05	Invert.
Parathion-methyl			2.5E-04			2.5E-04	7.3E-05	Invert.
Permethrin			1.3E-05			1.3E-05	6.0E-06	Invert.
Phosmet			2.7E-04			2.7E-04	2.0E-05	Invert.
Pirimicarb			2.6E-05			2.6E-05	1.7E-04	Invert.
Pyridaben			6.1E-04			6.1E-04	7.0E-06	Fish
Tetrachlorvin-phos			5.4E-05			5.4E-05	2.0E-05	Invert.

Invert: *invertebrates*

From a total of 127 pesticides quantified in water samples, 109 were used for ecological risk assessment (Table 20); the rest, mostly isomers and metabolites, were not integrated due to lack of information about their EC_{50} and LC_{50} concentrations set for these trophic levels. The PNEC values ranged from 4.0E-7 to 2.0. In general, algae proved to be the most sensitive group to herbicides and fungicides, while invertebrates showed the highest sensitivity for insecticides (data not shown).

Globally (Figure 7), the $RQ_{(MEC/PNEC)}$ resulted in 43% of very high risk cases; grouping by category, the insecticides led this ranking (70%), followed by the herbicides (33%). Fungicides were the least worrisome category, as 50% of the cases presented negligible risks (Figure 7).

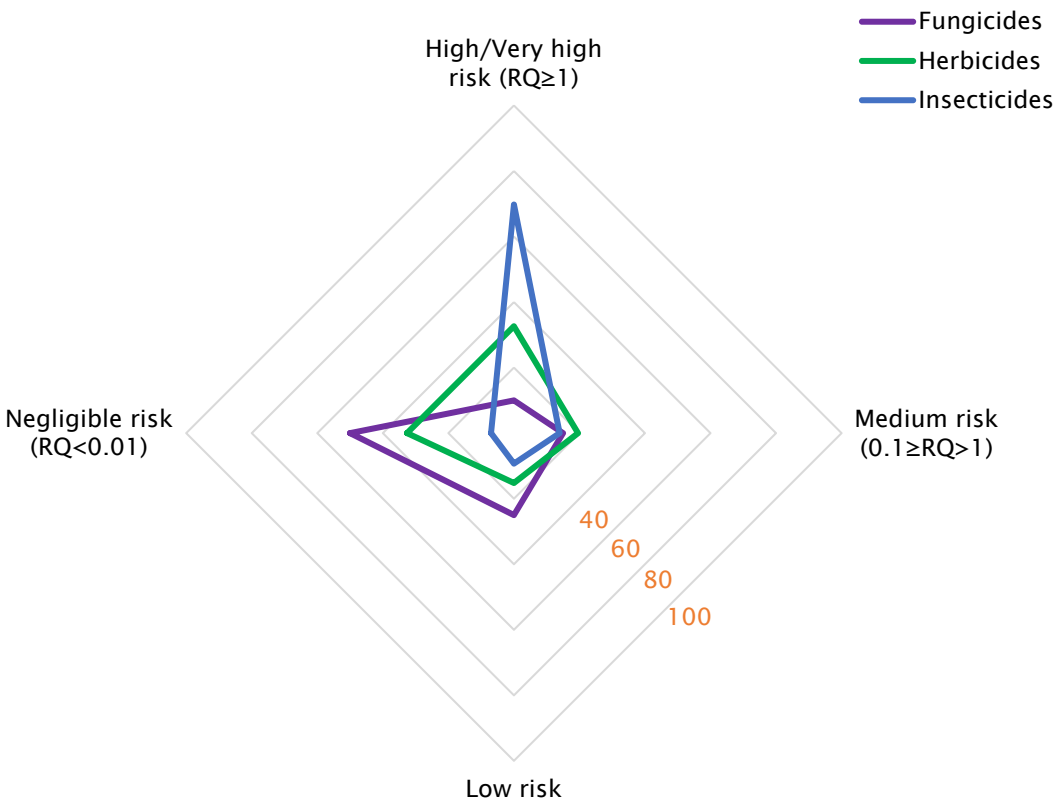

Figure 7: Worldwide distribution of pesticides in aquatic systems per category (%), according to $RQ_{(MEC/PNEC)}$ ranking.

The results presented above are a consequence of the highest values measured around the world. Since Europe was the continent with more values of $RQ_{(MEC/PNEC)}$, these results are mostly representative for this continent. However, this does not mean that concentrations measured on the other continents are innocuous. Proportionally to the number of compounds analysed per continent, Oceania and Africa presented the most disturbing scenarios (Figure 8).

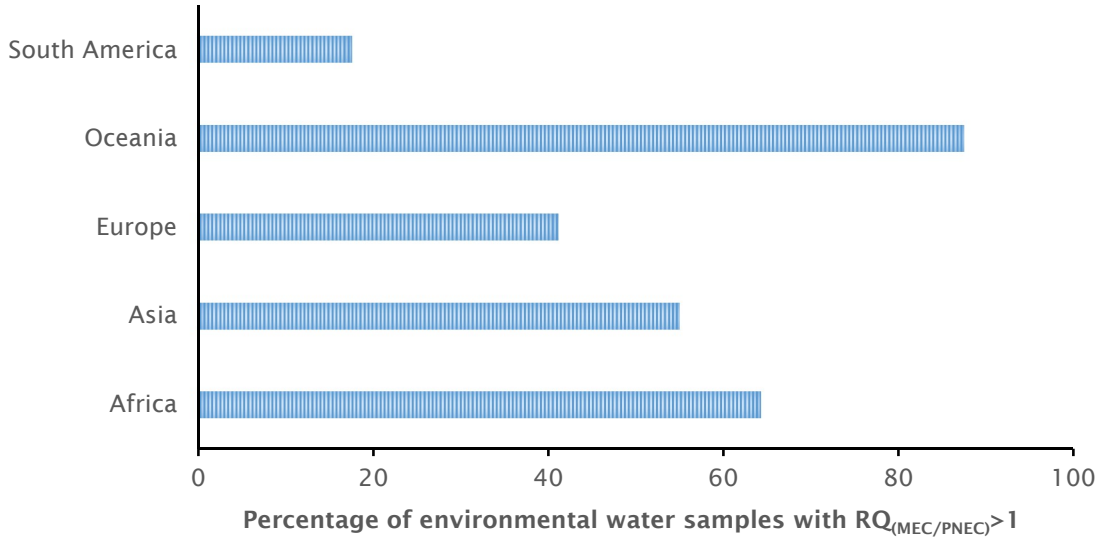

Figure 8: Percentage of $RQ_{(MEC/PNEC)}$ samples above 1, grouped by continent.

Subsequent to the $RQ_{(MEC/PNEC)}>1$ results, we follow up with the second approach in order to evaluate the effect of the maximum average concentrations found per each individual trophic level (RQ_{TU}), further evaluated through RQ_{STU} (Table 21).

Table 21: Sum of the toxic units per trophic level (RQ_{STU}) of each continent (with available data) and worldwide (data grouped), organized by pesticide category.

	RQ_{STU}					
	Africa	Asia	Europe	Oceania	South America	World
Algae						
Fungicides	0.01	0.00	**0.03**	0.00	0.00	0.03
Herbicides	0.00	0.00	**1.91**	0.90	0.01	2.28
Insecticides	0.01	0.01	**0.14**	0.00	0.00	0.14
TOTAL	0.02	0.01	**2.08**	0.90	0.01	2.45
Crustacean						
Fungicides	0.00	0.00	**0.01**	0.00	0.00	0.01
Herbicides	0.00	0.00	**0.02**	0.02	0.00	0.03
Insecticides	0.80	9.35	**19.50**	0.00	0.08	26.50
TOTAL	0.80	9.35	**19.52**	0.02	0.08	26.53
Fish						
Fungicides	0.00	0.00	**0.01**	0.00	0.00	0.01
Herbicides	0.00	0.00	**0.02**	0.00	0.00	0.02
Insecticides	0.85	0.72	**33.21**	0.00	0.04	33.35
TOTAL	0.85	0.72	**33.24**	0.00	0.04	33.37

When compared to other continents, the highest RQ_{STU} ratios were attained in Europe, for all trophic levels (represented in bold; Table 21) resulting from higher concentrations and/or number of available data; herein, fish presented a higher RQ_{STU} value (33), compared to crustacean (20) and algae (4), indicating a higher sensitivity of vertebrates to pesticides (mainly insecticides). Among the other continents, Africa had similar RQ_{STU} values for crustacean and fish (0.8), being both represented by insecticides. In Asia, the most sensitive group was the crustacean (9.35), once again represented by the insecticide category; the same was observed in South America (0.08). Oceania, on the contrary, presented the highest RQ_{STU} for algae, represented by the herbicides (Table 21).

The worldwide results reflect mainly the European data, proving fish (33) and crustacean (27) to be the most sensitive groups to insecticides (Table 21).

Independently from the continent, $RQ_{(MEC/PNEC)}$ and RQ_{STU} demonstrate that one or more biotest organism are sensitive to the concentrations on that continent. In accordance, a second-tier was calculated through the ratio RQ_{STU}/highest RQ_{TU}, applying the highest sum among trophic levels (Table 22).

Table 22: Second-tier, using RQ_{STU} and the highest RQ_{TU} per trophic level and continent.

Continent	No. of compounds (toxic/total)	RQ_{TU}	ΣRQ_{STU}			RQ_{STU}/RQ_{TU}
			algae	crustacean	fish	
Africa	9/14	0.27			0.85	3.11
Asia	11/20	5.22		9.35		1.79
Europe	42/102	20.11			33.24	1.65
Oceania	7/8	0.79	0.90			1.14
South America	3/17	0.06		0.08		1.33

For each of these scenarios, the maximal possible ratio RQ_{STU}/RQ_{TU} was lower than the value given by the number of mixture of toxic components, suggesting that the possible observed toxicity is due to a low number of pesticides. However, in South America and Africa the number of toxic compounds are still significant when compared to the RQ_{STU}/RQ_{TU} values obtained for each continent (Table 22).

3. Global perspective

Between individual and predictive pesticide mixture effects, results (discussed on point 1. and 2.) support that fish and crustacean are affected by the concentrations reported worldwide and compiled and analysed herein; these results are a "window view" to the effects of pesticides in aquatic systems.

In this review the collected biological data grouped according to Metazoan lineages reached a clear biomagnification pattern (Figure 9).

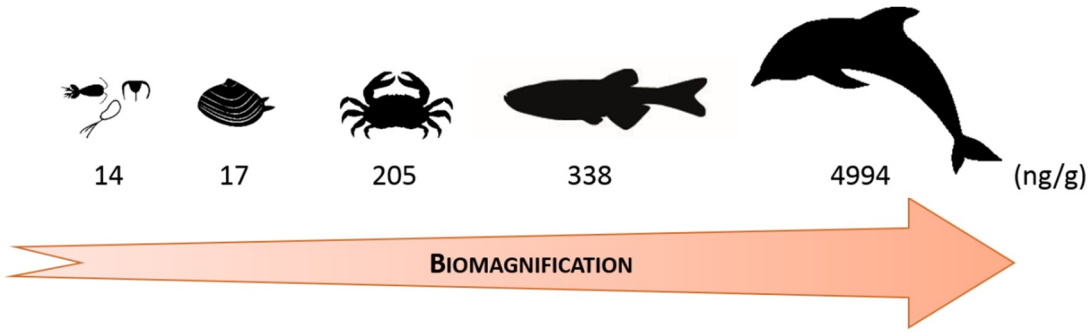

Figure 9: Biomagnification diagram considering different trophic levels ad-dressed and the estimated average concentration of pesticides.

To avoid escalation processes, as the one pictured in this work, more systematic preventive monitoring programs should be implemented, involving the target species. Bivalves, as sentinel and bottom food-chain species, are ideal for these programs. Besides, bivalves are important as a human food resource, and as such, the same programs may (and more cost effectively) help to define quality control standards for consumers.

From all continents (discussed on IV, point 3.), Europe registered the lowest average concentrations of the analyzed organisms. However, many aquatic species are migratory and therefore subjected to diverse levels of pollutions through the surrounding environment and/or feed.

As persistent compounds, pesticides should be treated not only locally (national/governmental institutions) but also on a global scale; further

international discussions and pacts, like the Stockholm Convention, should exist to alert mankind, to broadly regulate usages and monitorings, and, whenever justified, to ban the most hazardous pesticides.

VI. Pesticide effects (metabolic level)

As demonstrated in this work, numerous aquatic systems worldwide are contaminated by pesticides, at several trophic levels Yet, the first big wakeup call about negative secondary effects of pesticides dates back to the early 1960's, with the publication of the Silent Spring [15]. From that time onwards, the bulk of available information about the hazard of pesticides to wildlife has been based on their environmental fate, persistence, application rate, and toxicity [179]; the latter usually assessed through laboratory experiments, reaching the LC_{50} and/or LD_{50} in different trophic levels; usually fish, crustacean, and algae [6]. On the other hand, such studies did not provide significant insights into metabolic and genomic alterations that may occur.

Biocide effects on biota depend on different factors: the presence of a compound in the surrounding environment, its bioavailability to that organism, and the capacity to reach specific target receptors [180]. Physical and physiological characteristics such as shape, respiratory systems, feeding selectivity, and metabolic rate, can all interfere on the rate of a chemical's absorption [181]. Besides these factors, some pesticides are generally known by their specific action mode:

a) Insecticides, mostly organochlorines and organophosphates, affect the nervous system at specific target sites, blocking the transport of sodium, potassium, calcium, and chlorine ions, inhibiting the release of neurotransmitters [180]. Pesticides, as DDT, endrin, lindane, malathion, and parathion, are known for blocking the γ-aminobutyric acid (GABA) receptors and acetylcholinesterase enzyme (AChE) [180];

b) Herbicides are recognized by their ability to affect diverse mechanisms, such as photosynthesis, electron transport, growth, cell and nucleus division, and synthesis of proteins, carotenoids or lipids. Inhibition

of plant enzymes, as 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), blocks protein synthesis, essential for plant growth and photosynthesis [182]; compounds like 2,4-D, 2,4,5-T, and MCPA are known for these effects;

c) Fungicides take part in the breakdown of organic molecules that provide energy, affecting spore germination and inhibiting several enzymes involved in respiratory processes and electron transport. Amongst them are fenpiclonil, iprodionel, and dichlobenil [180].

However, in non-target organisms the same compounds may cause endocrine disruption, carcinogenesis, and immunotoxicity; later is characterized by the inhibition of serine hydrolases esterases, oxidative damage, and modulation of signal transduction pathways [6].

In all cases, serial pathways are activated to metabolize (phase-I and -II) and excretion (phase-III) these compounds [183]. As phase-I, enzymatic oxidation and hydrolysis metabolic reactions occur, producing metabolites with diverse functional groups (-OH, -COOH and -NH2, -SH). The oxidation reactions are mainly characterized by the catalytic function of cytochrome P450 (CYP) enzymes, which can be found in diverse organisms from bacteria to vertebrates [184]. The metabolites are subject to conjugation (phase-II) of more polar functional groups such as carbohydrates, glutathione, sulfate, and amino acids [185], allowing the detoxification process of xenobiotics. Subsequently, the metabolites are eliminated through the membrane, completing the phase-III process [186]. Nuclear receptors (NRs) up or down-regulate the transcription of enzymes and transporters (target genes), which play a critical role in the detoxification pathway capable to alter normal homeostasis [186]. Among these xenosensors, the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have received much attention (Figure 10).

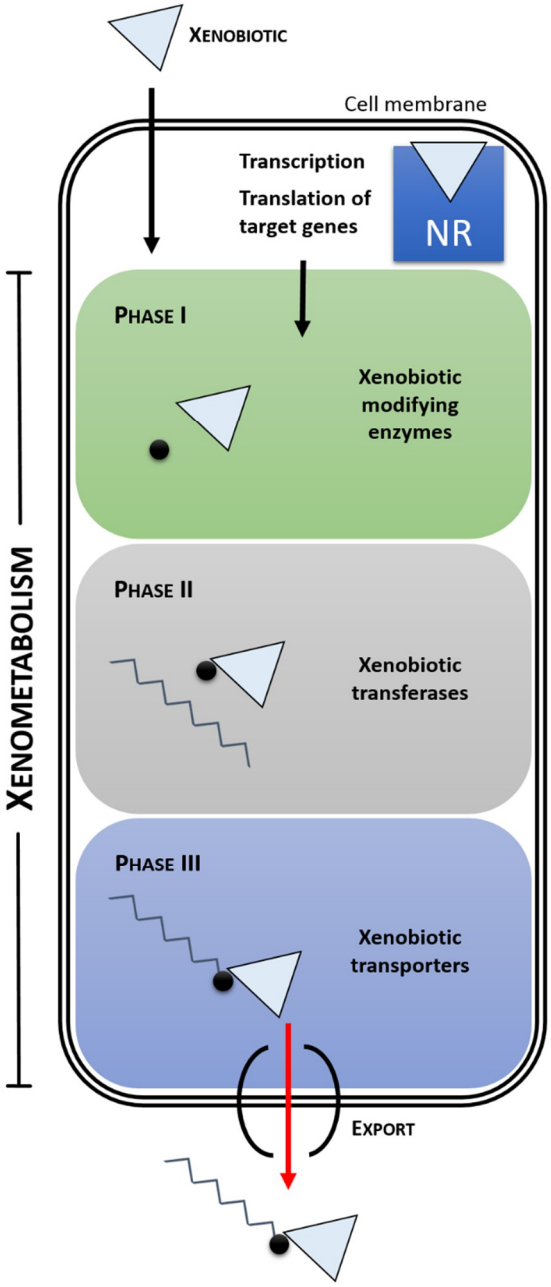

Figure 10: Metabolic pathway diagram: biochemical modification of xenobiotics by living organisms.

Oxidative stress, micronuclei and nuclear abnormalities, DNA damage, and mortality are associated to pesticide exposure. Through them we can evaluate the damage caused by these xenobiotics, but the results do not enlighten us on the biological processes involved; molecular techniques, like gene expression by quantitative real-time polymerase chain reaction (qPCR), ligand binding and transactivation assays, can provide a better

perception of the entire process [187-192]. While PCR characterizes the impact of a xenobiotic at the genesis of a metabolic pathway (up/down regulation of target gene expression), the cell-based transactivation assays with NRs provide insight into the impact of xenobiotic mimicking capacities and their impact into endocrine and metabolic functions [193, 194]. The later system uses two vectors, one reporter containing a firefly luciferase gene with Gal4 binding sites upstream, and the second containing our protein of interest fused to a GAL4 DNA-binding domain. The association of the GAL4 fused protein with the GAL4 binding sites in the reporter vector induces the activation of the luciferase reporter gene. Transcription levels of the reporter gene will vary according to conformational changes in the target nuclear receptor upon binding of the test compound (Figure 11) [195].

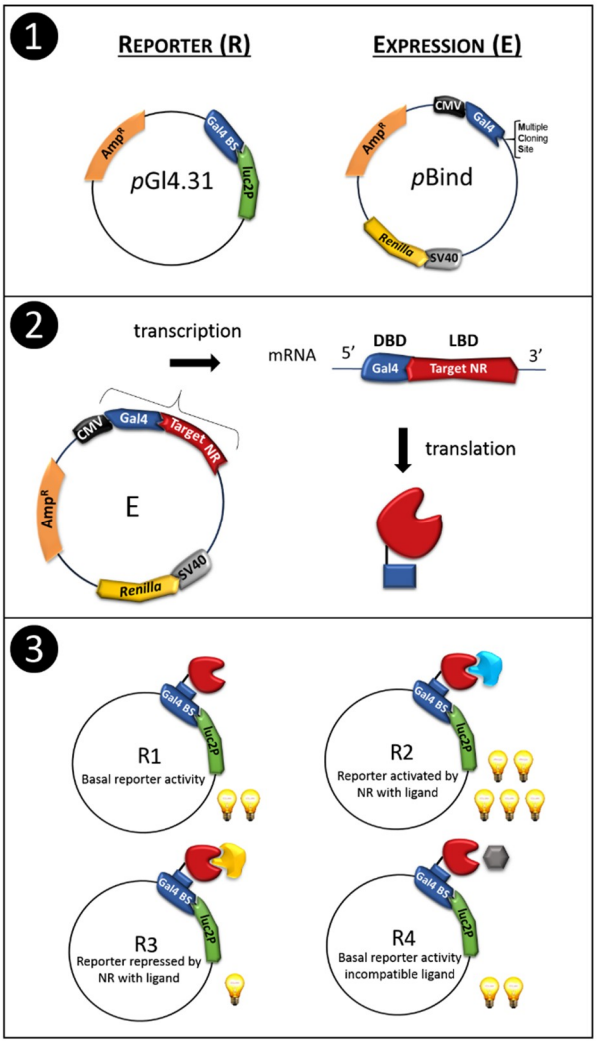

Figure 11: Transactivation assays. Step 1: Reporter plasmid (R): designed for transcriptional activation of the synthetic firefly luciferase reporter gene (green) by association of the GAL4 DNA-binding bound (dark blue) upstream of the luciferase gene; Expression plasmid (E): the early enhancer promoter (black), originated from the human cytomegalovirus (CMV), triggers the transcription machinery of the host mammalian cell line. The yeast Gal4 gene (dark blue) upstream of the Multiple Cloning Site (MCS) will serve as a DNA binding domain for the target protein inserted in the MCS. This plasmid also has a *Renilla reniformis* luciferase gene (yellow) preceded by the SV40 early promoter (grey); both plasmids codes for ampicillin resistance (AmpR) for propagation in *Escherichia coli* (orange). **Step 2**: Both plasmids (E&R) are simultaneously transfected into a mammalian cell line; Vector E will be expressed by the transfected cells producing a functional fusion protein; the LBD can be activated by diverse compounds. The vector R only triggers the expression of the gene upon binding of the fusion protein GAL4-NR upstream of the luciferase gene. **Step 3:** R1 and R4: Basal luciferase expression upon the binding of the fusion protein GAL4-NR LBD in absence/incompatible ligands. R2 and R3: reported gene expression enhancement (R2) or repression (R3) if there is a compound linked to the target NR-LBD R4.

As it was said before, PXR/CAR/VDR gene family (NR1I/J) have been linked to pesticide exposure [196-198]. However, few studies have been carried out in invertebrates to prove the impact of these pollutants in the nuclear receptor (NR1I/J/K) class [199, 200].

As surface deposit and/or filter-feeders, bivalves uptake the contaminants present in the surrounding environment. Anthropogenic substances that are released in the aquatic environment are thus prone to being uptaken and impact on the animal's normal homeostasis via NR binding. Therefore an enormous potential for exploring the yet poorly known pesticides disrupting effects via NR exists.

Given the above scenario, we considered that the NR1I/J nuclear receptor family as an important target for understanding the mechanistic actions of specific pesticides and for predicting their effects in the homeostasis of bivalves (e.g. *Scrobicularia plana*).

VII. Objectives

1. Brief rationale

The main purpose of this Thesis was to establish a bridge between the aquatic systems reality and the actual scientific knowledge. To link both worlds, the goal was to establish analytical methods able to infer spatio-temporal occurrence of pesticides in different matrices, and select significant and representative aquatic systems to evaluate the environmental pressure caused by these biocides. To obtain a clear picture of pesticides' impact in estuaries, three fractions were considered:

- ✓ Dissolved water fraction, as the first contact with these compounds (first matrix);
- ✓ Suspended particulate matter, as part of the water fraction but with a higher appetence to absorb hydrophobic molecules (second matrix);

- ✓ *Scrobicularia plana* soft tissue, as sessile animals and surface deposit and suspension feeders, are ideal to evaluate possible bioaccumulation processes. Besides, it is a commercial species in the Iberian Peninsula, known as lambujinha (third matrix).

A total of 56 pesticides, belonging to three different categories (insecticides, herbicides and fungicides), were selected based on national and European databases.

Thinking further, it is also important to understand the pesticide interactions with organisms. These type of xenobiotics play important roles from mechanistic aspects; however few is known, namely in fine molecular processes underlying their ability to influence homeostasis. Nuclear receptors (NR) are a recognised group of transcription factors involved in important physiological processes, including reproduction and energy-status. The yet poorly known disrupting effects of pesticides via NR brings an enormous potential to explore their capacity to be ligand-activated, vital to endocrine disruption processes. Here, we challenge ourselves to isolate and characterize the nuclear receptor orthologue of PXR/CAR/VDR class in *S. plana* and its experimental and environmental modulation by pesticides.

2. Specific aims

In resume, the specific objectives of this Thesis were:

To optimize a solid-phase extraction method capable to pre-concentrate several pesticides (from different categories) and validate the analytical method by gas chromatography coupled with mass spectrometry (GC-MS) for the identification and quantification of these compounds from surface water samples (first matrix)(data presented in Chapter 2);

To apply the validated method and quantify the samples, collected between 2010 and 2011 from Ria Formosa Lagoon, Tagus and Mondego River that were previously selected from nine aquatic systems. Use this information to evaluate possible local and seasonal pesticide fluctuations, defining them as models for further multi-matrix studies (data presented in Chapter 2, 4 and 5);

To develop a method for the identification and quantification of the selected pesticides, from suspended particulate matter collected from surface water samples (second matrix); apply the same for soft bivalve tissue (third matrix) (data presented in Chapter 3 and 6);

Gathered the above conditions, to perform a one-year sampling campaign in the three selected aquatic systems (2012-2013), englobing all year seasons, and collect the selected matrices at three strategical sites (defined *a priori*). With these data, identify and quantify all target compounds and evaluate the spatio-temporal distribution. Additionally characterize the predominant pesticide category, evaluate the average concentrations according to European directives, determine theoretically the hazardous effects of the quantified pesticides (individual or as a mixture), and estimate the potential hazardous effects to human health through the consumption of wild lambujinha (data presented in Chapter 6 and 7);

Finally, to isolate and characterize the NR orthologue of the PXR/CAR/VDR class in *S. plana*, and to study the agonistic/antagonistic activity of this NR when exposed to target compounds (selected pesticides and reference natural toxins), via cell-based transactivation assays (data presented in Chapter 8).

VIII. References

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Chapter 2

Uncovering seasonal patterns of 56 pesticides in surface coastal waters of the Ria Formosa lagoon (Portugal), using a GC-MS method

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Uncovering seasonal patterns of 56 pesticides in surface coastal waters of the Ria Formosa lagoon (Portugal), using a GC-MS method

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ABSTRACT

This study describes the simultaneous quantification of 56 pesticides in surface coastal water, supported by the development and validation of a gas chromatography (GC)–ion trap (IT) mass spectrometry (MS) method. Samples (500 mL) were pre-concentrated 2500 times by solid phase extraction (OASIS^{TM} HLB). The compounds were identified and quantified, within 35 minutes, by GC tandem mass spectrometry (GC-MS/MS) and GC-MS, respectively. The methodology proved to be highly specific for all target pesticides, with an average linearity of 0.99. Detection limits and recovery rates ranged from 0.4 to 1.3 ng L^{-1} and 71% to 120%, respectively. The performance of the method was checked using water samples collected from nine sampling sites along the Ria Formosa Lagoon Natural Park (south of Portugal, n = 54) in each season (2010). The total annual concentrations of all pesticides in each category (fungicides, herbicides and insecticides) were 1.4, 0.6 and 9.0 µg L^{-1}, respectively. Moreover, 89% of the pesticides tested for were detected, 84% could be quantified and 25% had concentrations above the European recommended levels (2013/39/EU). The highest total loads of pesticides were found in the spring, which is in agreement with their seasonal application. Physicochemical parameters such as, nitrites, nitrates, ammonia and phosphates, also indicate poor water quality, supporting the fact that the Ria Formosa lagoon actually needs an effective monitoring programme for effective preservation of its natural reserve status.

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1. Introduction

Pesticides are chemicals used to enhance agricultural productivity, but due to their physicochemical properties and chemical structure, some of them are listed as persistent organic pollutants (POPs) [1], toxic for the biota and prone to bioaccumulation [2,3]. The variety and extensive usage of these compounds has increased their environmental pollution levels. Only in Europe, 449 pesticides are classified as approved for use by the European Communities (EC) regulation No. 1107/2009 [4], from a total of 1297 active substances. Besides, it was estimated that only a minimum percentage (0.1%) of the total quantity of used pesticides reach the application target, whereas the other 99.9% are a surplus that have greater potential to affect different environmental systems [5]. Therefore, non-conscious utilisation may lead to overuse of these compounds, which may reach hazardous concentrations in soil, crops and, eventually, in water [6,7]. Moreover, the fact

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that some of these pesticides are biodegradation-resistant leads to environmental accumulation and bio-amplification of properties through the food web [8]. Therefore, international initiatives have been taken in order to promote effective regulation and management of these compounds. In this sense, the European Union (EU) directive 98/83/EC established strict maximum levels for water and human consumption [9]. Due to the inherent toxic characteristics of pesticides, a more effective and specific regulation (directive 2008/105/EC) has been used on the basis of environmental quality standards [1]. Presently, the EU directive 2013/39/EU shows the importance of strict control of pesticides in soils and biota, which should be put into practice between 2015 and 2021 [10]. Therefore, the pesticides investigated herein were selected after detailed research on most frequently detected pesticides in Europe, between 2000 and 2010, using official databases, such as the Portuguese Regional Directorate of Agriculture and Fisheries (DRAP) and the European Commission database Regulation (EC No 1107/2009). They were used to cover a wide range of authorised, unauthorised and banned compounds [11,12]. The extraction method, based on a previous study [13], together with the development and validation of an analytical protocol, allows evaluation of the amounts of 56 pesticides (fungicides, herbicides and insecticides) in coastal matrices – lagoons and estuarine environments – by gas chromatography coupled to ion trap mass detector (GC-MS and GC-MS/MS).

Ria Formosa lagoon, located on the south Portuguese coast, is recognised internationally for its natural reserve and touristic interest. It also holds a vast area for agriculture, where high amounts of citrics, almonds, carob, wine and cork are produced [14], along with bivalve and fish aquaculture farms [15]. Despite this, several studies have proved high anthropogenic activity [16–18] and even occurrence of endocrine disruptive conditions in the area [19,20]. Because there are no data on the presence of pesticides in the Ria Formosa lagoon, a first diagnostic study is necessary in order to know the loads of these pesticides and to conclude the eventual need of a monitoring programme.

To sum up, the objectives of this work were to provide: a) a validation of a robust analytic protocol to evaluate 56 pesticides in surface coastal waters; b) results of an annual monitoring survey in the Ria Formosa lagoon superficial waters and c) a correlation between the pesticide load and the values of physicochemical water quality parameters.

2. Experimental

2.1. Chemicals and materials

The analytical grade solvents methanol (MeOH), ethyl acetate (EtOAc) and n-hexane were purchased from Romil (Cambridge, England). Ultrapure water was obtained from a Milli-Q water system (conductivity = 0.054 μS cm^{-1}, at 25°C). The solid-phase extraction (SPE) cartridges, 200 mg OasisTM HLB (Hydrophilic-Lipophilic Balance), 6 mL, were acquired from Waters Corporation (Milford, USA).

2.2. Reference standards

All pesticide standards were supplied by Sigma-Aldrich (Steinheim, Germany); with the exception of Mix A (EPA 505/525, 500 mg L^{-1}) and Mix B (EPA 505/525, 500 mg L^{-1}), all other pesticides were purchased individually. The 4,4′-DDT-d_8 ($C_{14}HCl_5D_8$) and atrazine-d_5 ($C_8H_9D_5ClN_5$) were both used as surrogates. All standard solutions were individually prepared in MeOH to produce a final stock solution of 1000 mg L^{-1} and kept in the dark at −20°C. From the stock solution, eight nominal calibration standard mixtures, prepared in MeOH, were spiked, before the beginning of the extraction procedure in clean water from the headspring of the Febros river (41°01′58.0′ N, 8°33′11.1′ W), with added sodium chloride (99.8%; EMSURE® Merck, Germany) to obtain an average salinity of 23 (w/v) in order to simulate both estuarine and lagoon coastal water conditions. This matrix was used as a calibration standard (blank) and to validate the method, as it was not possible to find estuarine water free of pesticides. The final range of concentrations, in spiked water samples, were 10–400 ng L^{-1} for all 56 pesticides and 160 ng L^{-1} for atrazine-d_5 and 4,4′-DDT-d_8. All pesticides which RT ranged from 7.16 to 14.81 min, and those from 15.05 to 32.22 min were used as surrogates for atrazine-d_5 and 4,4′-DDT-d_8, respectively.

2.3. Sample collection and preparation

Ria Formosa lagoon is a mesotidal system located on the south of Portugal. Due to its extension (approx. 60 km), nine sampling stations (S1–S9, Figure 1A) were selected along the coast covering several urban centres and the natural park protected area. Thus, S1–S3 (Zone I) encompass the cities of Faro and Olhão, and S4–S9 (Zone II) comprises the wildest/major fraction of the Ria Formosa natural park [21]. The selected region presents 28 waste-water treatment plants (WWTPs), where 12 of them are located at the coastline [17]. Water samples were collected at the shore (50 cm depth) during ebb tide, between February and December of 2010 (n = 54 samples, *i.e.*, 9 sites × 6 surveys), into 2.5-L pre-rinsed amber glass bottles until completely full and then kept at 4 ± 1ºC during transport and until sample preparation.

2.4. Water quality measurement

Physicochemical parameters such as temperature (°C), dissolved oxygen (DO; mg L^{-1}), salinity and conductivity (mS cm^{-1}) were evaluated, *in situ*, using the portable meters OXi 330i/ Set WTW and LF 330/ Set WTW, respectively. Other parameters, such as pH (Hech HQ40d), nitrites (mg L^{-1}), nitrates (mg L^{-1}), ammonium (mg L^{-1}) and phosphates (mg L^{-1}) were measured using the Palintest Photometer 700 interface, at the laboratory.

2.5. Sample preparation

Water samples (1 L) were immediately filtrated, to eliminate particulate matter and other suspended solids, through a 0.45-µm glass fibre filter (Munktell, Germany). The filtrates were acidified with H_2SO_4 to pH 7 and, then, 500 mL was subjected to SPE within a maximum period of 24 h – during this phase, all samples were maintained in the

fridge at ±4°C in the dark until extraction, as already described in Rocha et al. (2012) [13].

The compounds were extracted, based on previous works [13,22], using the HLB™ cartridges adapted to an off-line SPE vacuum extraction device (Waters). Briefly, the cartridges were conditioned sequentially with 5 mL of EtOAc, followed by 5 mL of MeOH and 2.5 mL of ultrapure water, at a flow rate of 1–2 mL min^{-1}. Water samples (500 mL) were loaded into SPE cartridges at a constant flow-rate of 5 mL min^{-1}. Cartridges were dried under vacuum for 1 h, to avoid residual water in the final extract, and then eluted with 6 mL of EtOAc, at 1 mL min^{-1}. The extracts were evaporated to dryness under a gentle N_2 (99.9997%) stream and then reconstituted with 200 µL of n-hexane and kept in vials at −80°C until analysis.

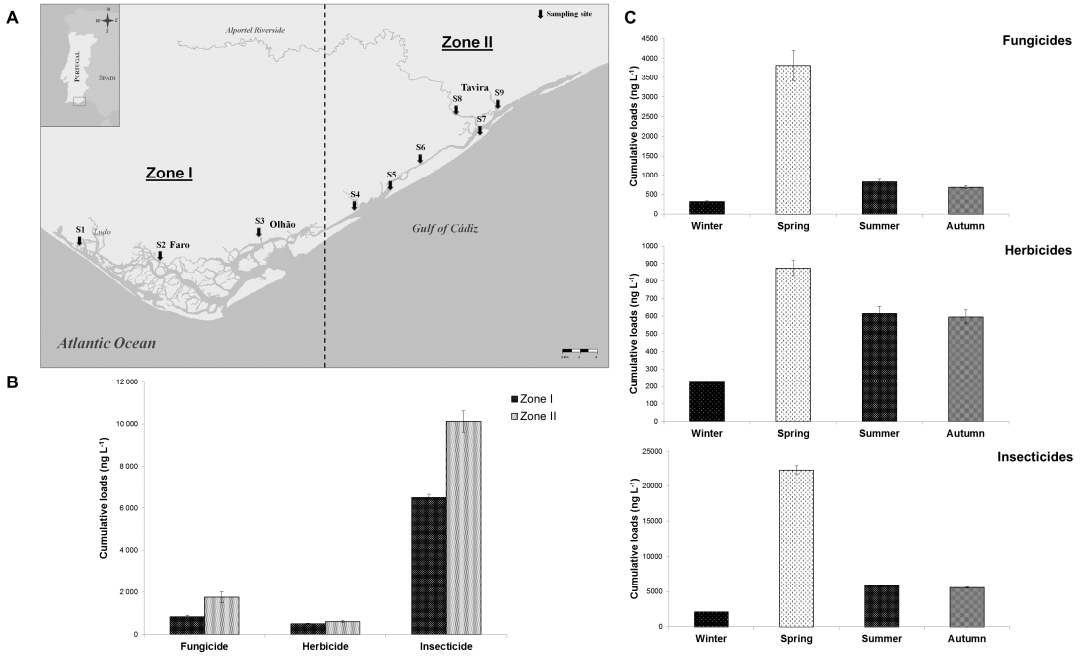


Figure 1. Studied area, amount of pesticides and physicochemical data: A) Location of sampling sites within the Ria Formosa Lagoon (S1-S9), Portugal (adapted from Microsoft MapPoint, 2010); B) Pesticide concentrations (∑ ng L^{-1}) by categories per zone (I and II) and C) per season; Data is expressed as cumulative loads ± SE (n = number of pesticides per zone and number of pesticides per season).

2.6. Gas chromatography–ion trap mass spectrometry

Analyses were carried out using a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation), coupled with an ion trap mass spectrometer detector Thermo Scientific ITQ™ 1100 GC-MS^n), an autosampler (Thermo Scientific TriPlus™) and a Trace GOLD column (TG-5SILMS, 30 m × 0.25 mm × 0.25 µm). Column oven temperatures were programmed for a 35-min period using several ramps: a) from 65°C with an initial equilibrium time of 2 min to b) 180°C at 20°C min^{-1} until c) 280°C at 5°C min^{-1}, where the temperature was maintained for 7 min. A solvent delay time of 5 min was used to protect the MS ion multiplier from saturation. The injector port temperature

was set to 250°C, and both ion source and MS transfer line were at 280°C. Helium (99.9999% purity) was used as carrier gas and was maintained at a constant flow rate of 1 mL min^{-1}.

Sample injection (2 µL) was in the splitless mode (3-mm straight liner), using a 50-mm long needle. The product ions were compared with previously published methods [13,23–25] and supported by the NIST Mass Spectral Search Program (version 2.0, 2005) library to create a selected ion-monitoring mode (SIM) for quantification purposes. MS/MS conditions were optimised for pesticide identification. The software Xcalibur (version 2.0.7, 2007, Thermo Scientific), together with the Mass Frontier (version 1.0, 1998) and the NIST library, were used to evaluate the ion products. The MS/MS transitions were optimised for each pesticide (supplementary data – Table 1).

2.7. Validation studies and matrix effect

The validation procedure followed the European guidance document on pesticide residue analytical methods [26] that includes internationally accepted criteria from the International Conference on Harmonization (ICH) and International Union of Pure and Applied Chemistry (IUPAC) [27,28]. This process includes the evaluation of linearity, accuracy, precision, method detection limits (MDLs) and method quantification limits (MQLs), calculated using the ratio between the spiked pesticide area by the spiked surrogates area. Both MDLs and MQLs were calculated, based on three calibration curves (10–400 ng L^{-1}) of each pesticide as follows: MDL = 3.3 α/S and MQL = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves. The calibration curves were prepared by spiking both pesticide standards and surrogates in 500 mL of headspring water samples, as described earlier. In order to avoid interferences derived from the matrix (headspring water), the fortified samples were subtracted from a non-fortified sample (blanks).The recoveries, accuracy and precision (intra- and inter-batch) were evaluated by analysing, on different consecutive days, three replicates of each quality control samples (QCs) at three levels of concentration (low, medium and high) calculated accordingly to the Brazilian Health Surveillance Agency (ANVISA) guidelines [29], *i.e.*, QC_{low} = 3 × MQL (14 ng L^{-1}), QC_{medium} = average value of QC_{low} and QChigh (180 ng L^{-1}) and QC_{high} = 75–90% of the highest standard used for each pesticide (330 ng L^{-1}). All quantifications were done by comparing the ratio areas of standards spiked in real samples with those of fortified matrices in the SIM mode (Figure 2A). An extra injection in the MS/MS mode (Figure 2B) was done for all the analysed samples to ensure unequivocal identification of the analysed pesticides; all samples were injected in triplicate.

During all processes, solvent (n-hexane) and matrix blanks (estuarine waters) were systematically analysed to prevent occurrence of potential contaminations.

2.8. Statistical analyses

Data analysis was done by considering the average values of all replicates (n = 3). For seasonal and geographical analysis, the sampling site data were grouped (average of means) to calculate the mean values ± standard error (SE) (Figures 1B to 1 C). In other instances, data are presented as mean values ± standard deviation (SD). Statistical tests were performed by STATISTICA 8 (StatSoft 2007). Data normality and homogeneity of variances were evaluated by the Shapiro–Wilk W-test and Levene's test, respectively. Comparisons between levels, seasons and categorical groups were achieved by one-way analysis of variance (ANOVA) using Tukey's post- hoc test. A non-parametric test (Kruskal–Wallis ANOVA) was also applied when data transformation failed the normalisation attempt; results were considered statistically significant when $p < 0.05$.

Table 1. Environmental levels of all pesticides measured at the Ria Formosa lagoon, during 2010, per season. Data is presented as mean ± SD (n = 6/site).

						Frequency	MDL	MQL	Environmental levels (ng L^{-1})			
Pesticides	Class	License[#]	log Kow	log Koc	GUS index	%	% above		Winter	Spring	Summer	Autumn
Fungicides												
Azoxystrobin	Antibiotic fungicide	A	2.5	2.8	2.6	94.4	100	100	22.1 ± 0.01	2148.3 ± 0.80	349.1 ± 0.19	86.4 ± 0.04
Difenoconazol	Conazole fungicides	A	4.4	3.6	0.9	94.4	100	100	39.5 ± 0.04	1018.8 ± 0.90	178.9 ± 0.17	244.7 ± 0.31
HCB	Organochlorines	B	3.9	4.7	-2.3	100	2.8	-	-	-	-	-
PCB	Aromatic fungicide	NA	4.8-5.2	4.5	–1.2	100	100	100	34.7 ± 0.01	49.9 ± 0.02	29.2 ± 0.00	27.0 ± 0.00
Procymidone	Conazole fungicides	NA	3.3	2.6	1.2	97.2	80	77.1	52.0 ± 0.25	28.6 ± 0.19	18.6 ± 0.14	88.8 ± 0.55
Tebuconazole	Conazole fungicides	A	3.7	3	2	97.2	97.1	97.1	168.3 ± 0.05	564.5 ± 0.11	252.4 ± 0.07	237.2 ± 0.09
Herbicides												
Alachlor	Organochlorines	NA	3.7	2.5	0.8	91.7	100	100	10.7 ± 0.00	10.5 ± 0.00	11.0 ± 0.00	10.3 ± 0.00
Atrazine	Triazine	NA	2.7	2	3.3	100	16.7	11.1	-	2.3 ± 0.00	2.8 ± 0.00	2.1 ± 0.00
Atrazine-desethyl	Triazine	NA	2.7	1.9	3.5	100	100	100	10.1 ± 0.00	10.1 ± 0.00	10.5 ± 0.00	10.3 ± 0.00
Cyanazine	Triazine	NA	2.1	2.3	2.1	94.4	100	100	9.6 ± 0.00	9.9 ± 0.00	10.3 ± 0.00	8.9 ± 0.00
Cyhalofop-butyl	Phenoxy herbicides	A	6	3.7	-0.2	94.4	5.9	5.9	-	3.2 ± 0.05	-	-
Metolachlor	Amide herbicides	NA	3.4	2.1	3.5	72.2	-	-	-	-	-	-
Metribuzin	Triazinone herbicides	A	1.7	1.8	2.6	50	5.6	-	-	-	-	-
Pendimethalin	Dinitroaniline herbicides	A	5.2	4.4	-0.4	100	100	97.2	64.1 ± 0.01	493.9 ± 0.03	468.9 ± 0.04	443.8 ± 0.03
Propazine	Triazine	NA	4	2.2	3.8	13.9	-	-	-	-	-	-
Propyzamide	Amide herbicides	A	3.3	2.9	1.8	94.4	88.2	85.3	52.6 ± 0.04	84.4 ± 0.04	25.7 ± 0.02	28.7 ± 0.02
Simazine	Triazine	NA	2.3	2.1	2	100	100	100	15.6 ± 0.00	12.7 ± 0.00	13.1 ± 0.00	13.4 ± 0.00
Simetryn	Triazine	NA	2.8	2.3	3	91.7	100	100	4.6 ± 0.00	5.7 ± 0.01	5.8 ± 0.01	4.7 ± 0.00
Terbuthylazine	Triazine	A	3.4	2.3	3.1	100	100	100	37.9 ± 0.01	219.1 ± 0.08	45.7 ± 0.02	51.5 ± 0.03
Terbutryn	Triazine	NA	3.7	3.4	2.4	77.8	100	100	12.8 ± 0.01	16.1 ± 0.02	13.0 ± 0.02	14.8 ± 0.05
Trifluralin	Carbamate insecticide	NA	5.3	4.2	0.1	94.4	100	100	6.7 ± 0.00	6.5 ± 0.00	6.6 ± 0.00	6.5 ± 0.00
Insecticides												
Aldrin	Organochlorines	B	6.5	4.2	-0.4	88.9	3.1	3.1	-	-	15.1 ± 0.01	-
Azinphos-methyl	Organothiophosphate insecticides	NA	3	3	1	100	86.1	86.1	86.3 ± 0.06	45.6 ± 0.04	78.1 ± 0.05	51.2 ± 0.04
Lindane	Organochlorines	NA	3.7	3.1	4	100	100	100	9.4 ± 0.01	11.2 ± 0.01	16.3 ± 0.01	7.7 ± 0.01
Chlordane (gamma)	Organochlorines	B	2.8	4.3	-0.8	80.6	-	-	-	-	-	-
Chlorfenvinphos Z	Organophosphorus	NA	3.8	2.8	1.9	100	41.7	41.7	8.2 ± 0.12	16.9 ± 0.13	15.9 ± 0.08	-
Chlorpyriphos	Organophosphorus	A	4.7	3.9	0.2	63.9	100	100	23.0 ± 0.02	25.8 ± 0.03	26.7 ± 0.03	22.7 ± 0.02

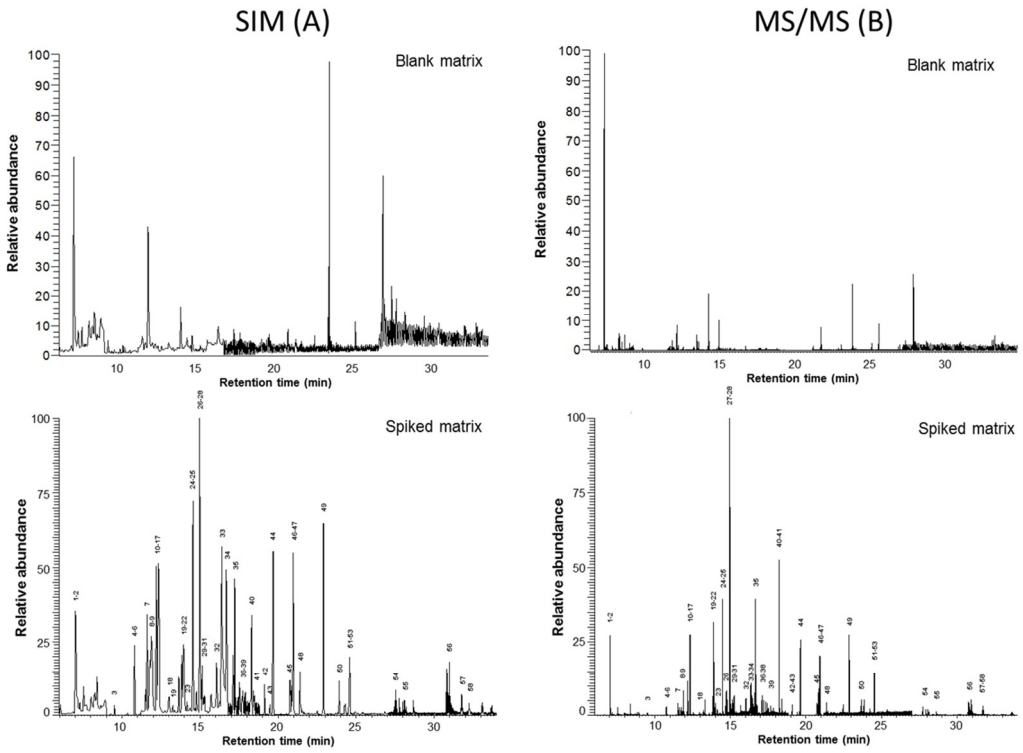
(Continued)

Table 1. (Continued).

Pesticides	Class	License[#]	log Kow	log Koc	GUS index	Frequency	MDL	MQL	Environmental levels (ng L^{-1})			
						%	% above		Winter	Spring	Summer	Autumn
Cyfluthrin (beta)	Pyrethroid	A	5.6	4.8	–1.7	88.9	100	100	121.7 ± 0.11	110.6 ± 0.13	154.6 ± 0.16	161.1 ± 0.13
Cyhalothrin (lambda)	Pyrethroid	A	6.8	5.2	–2.1	97.2	97.1	97.1	90.6 ± 0.12	2997.9 ± 3.44	1580.7 ± 1.62	1910.8 ± 2.32
Cypermethrin (alpha)	Pyrethroid	A	6.9	4.4	–2.1	100	100	100	291.4 ± 0.17	397.0 ± 0.31	453.0 ± 0.14	449.1 ± 0.13
4,4´-DDD	Organochlorines	B	6.9	4.7	–0.9	94.4	-	-	-	-	-	-
4,4´-DDT	Organochlorines	B	6.9	5.9	-4.5	100	100	100	171.7 ± 0.66	370.6 ± 1.00	162.7 ± 0.43	156.5 ± 0.20
4,4´-DDE	Organochlorines	B	6.9	4.9	-2	97.2	-	-	-	-	-	-
Deltamethrin	Pyrethroid	A	4.6	7	-3.4	94.4	100	100	64.8 ± 0.04	16615.4 ± 4.54	2181.5 ± 0.39	2051.2 ± 0.21
Diazinon	Organophosphorus	NA	3.7	2.8	1.1	100	100	100	73.8 ± 0.00	148.9 ± 0.01	123.0 ± 0.00	84.3 ± 0.01
Dichlorvos	Organophosphorus	NA	1.9	1.7	0.7	100	25	25	106.2 ± 0.00	-	-	-
Dieldrin	Organochlorines	B	3.7	4.4	-0.3	100	100	100	129.9 ± 0.15	183.6 ± 0.44	155.2 ± 0.38	143.7 ± 0.38
Dimethoate	Organophosphorus	A	0.7	1	1.1	100	100	100	30.7 ± 0.02	61.7 ± 0.02	39.4 ± 0.01	46.5 ± 0.02
Endosulfan (alpha)	Organochlorines	NA	4.7	4.1	-0.1	80.6	100	100	105.8 ± 0.02	72.3 ± 0.04	66.0 ± 0.03	65.1 ± 0.04
Endosulfan (beta)	Organochlorines	NA	4.8	4.3	-0.1	63.9	100	100	15.5 ± 0.02	13.3 ± 0.02	14.3 ± 0.03	8.5 ± 0.02
Endosulfan sulfate	Organochlorines	NA	3.7	3.7	0.5	97.2	20	20	-	21.3 ± 0.05	-	-
Endrin	Organochlorines	NA	3.2	4	0	16.7	100	100	-	26.0 ± 0.03	17.4 ± 0.01	23.6 ± 0.01
Fenamiphos	Organophosphorus	A	3.3	2	-0.1	97.2	100	100	123.7 ± 0.04	175.2 ± 0.06	29.1 ± 0.02	24.7 ± 0.01
Fenitrothion	Organophosphorus	NA	3.3	3.3	0.5	66.7	8.3	8.3	-	27.6	1.6 ± 0.00	-
Fonofos	Organophosphorus	NA	3.9	2.9	2.1	91.7	100	100	5.9 ± 0.01	7.7 ± 0.01	9.2 ± 0.01	6.7 ± 0.01
Heptachlor	Organochlorines	B	5.4	4.4	-0.9	75	100	100	10.8 ± 0.00	9.5 ± 0.00	11.2 ± 0.00	9.0 ± 0.00
Heptachlor epoxide	Organochlorines	NA	4.4-5.5	4.3	-1.1	100	-	-	-	-	-	-
HCCP	Organochlorines	*	4	3.6	0.4	100	33.3	30.6	31.5 ± 0.05	68.0 ± 0.09	-	-
Malathion	Organophosphorus	A	2.8	3.3	-1.3	41.7	40	40	-	5.6 ± 0.00	1.3 ± 0.00	-
Methoxychlor	Organochlorines	NA	3.8	4.9	-1.9	100	72.2	72.2	3.0 ± 0.02	161.7 ± 0.41	39.6 ± 0.08	48.6 ± 0.06
Mirex	Organochlorines	B	5.3	3.8	0.6	100	66.7	61.1	2.9 ± 0.02	4.6 ± 1.78	3.1 ± 0.42	2.2 ± 1.97
Parathion-ethyl	Organophosphorus	NA	3.8	3.9	2.1	88.9	100	100	63.0 ± 0.02	104.5 ± 0.07	38.0 ± 0.03	43.0 ± 0.05
Parathion-methyl	Organophosphorus	NA	3	2.4	1.5	88.9	37.5	37.5	228.0 ± 0.02	242.0 ± 0.05	288.2 ± 0.02	-
Phosmet	Organothiophosphate insecticides	A	3	3.6	0.2	100	100	100	214.6 ± 0.01	368.0 ± 0.02	301.0 ± 0.01	202.8 ± 0.01
Pirimicarb	Dinitroaniline herbicides	A	1.7	2.6	2.7	100	-	-	-	-	-	-
Tetrachlorvinphos	Organophosphorus	NA	3.5	3	0.3	94.4	100	100	96.8 ± 0.27	38.0 ± 0.09	43.0 ± 0.08	38.8 ± 0.10

[#]NA- Not authorized; A- Authorized; B- Banned; according to the EU Pesticides Database; GUS index (groundwater ubiquity score; GUS = log10 (half life-days) X [4-log10 (Koc)]); * Information not found; MDL: method detection limit; MQL: method quantification limit.

Figure 2. Chromatograms represented in SIM (A) and MS/MS (B) mode of the blank and spiked matrix (target pesticides [400 ng L^{-1}] and the internal standards [160 ng L^{-1}]); * represent the surrogates.



1 - Dichlorvos	**11** - Atrazine	**21** - Alachlor	**31** - Parathion-ethyl	**41** - Dieldrin	**51** - Cyhalofop-butyl
2 - HCCP	**12** - Propazine	**22** - Simetryn	**32** - Pendimethalin	**42** - Endrin	**52** - Cyhalothrin (lambda)
3 - PCB	**13** - Lindane	**23** - Heptachlor	**33** - Chlorfenvinphos Z	**43** - Endosulfan (alpha)	**53** - Mirex
4 - Phosmet	**14** - Terbuthylazine	**24** - Terbutryn	**34** - Heptachlor epoxide	**44** - 4,4´-DDD	**54** - Cyfluthrine (beta)
5 - Atrazine-desethyl	**15** - Propyzamide	**25** - Fenitrothion	**35** - Procymidone	**45** - Endosulfan sulfate	**55** - Cypermethrin (alpha)
6 - Trifluralin	**16** - Fonofos	**26** - Malathion	**36** - Chlordane (gamma)	**46** - 4,4´-DDT-d_8*	**56** - Difenoconazol
7 - HCB	**17** - Diazinon	**27** - Metolachlor	**37** - Tetrachlorvinphos	**47** - 4,4´-DDT	**57** - Deltamethrin
8 - Dimethoate	**18** - Pirimicarb	**28** - Chlorpyriphos	**38** - Endosulfan (beta)	**48** - Tebuconazole	**58** - Azoxystrobin
9 - Simazine	**19** - Metribuzin	**29** - Cyanazine	**39** - Fenamiphos	**49** - Methoxychlor	
10 - Atrazine-d_5*	**20** - Parathion-methyl	**30** - Aldrin	**40** - 4,4'-DDE	**50** - Azinphos-methyl	

3. Results

3.1. Solid-phase extraction and GC-MS instrumental data

Sample pre-treatment was successfully optimised for simultaneous extraction of 56 pesticides as the recovery rates ranged from 71% to 120%, demonstrating the SPE feasibility for the extraction of the selected compounds (supplementary data – Table 2).

GC separation was achieved by evaluation of different ranges of temperatures and injection conditions, initially using full-scan mass spectra of individual pesticides. The SIM segments were established, containing for each compound the specific ion mass-to-charge ratio (m/z).

Table 2. Physicochemical data evaluated per zone (I and II); Data is expressed as mean ± SD (n = 18/Zone I and 36/Zone II).

Physicochemical parameters	Zone I	Zone II
Dissolved O_2 (mg L^{-1})	8.65 ± 1.32	9.28 ± 2.28
Temperature (°C)	20.41 ± 4.61	20.02 ± 4.55
pH	8.35 ± 0.25	8.32 ± 0.22
Salinity	35.53 ± 1.48	28.95 ± 9.25
Conductivity (mS cm^{-1})	48.91 ± 11.38	47.58 ± 12.20
Nitrites (mg L^{-1})	0.01 ± 0.01	0.02 ± 0.02
Nitrates (mg L^{-1})	0.22 ± 0.18	0.49 ± 0.64
Ammonia (mg L^{-1})	0.52 ± 0.50	0.99 ± 1.67
Phosphates (mg L^{-1})	0.60 ± 0.69	0.69 ± 0.93

3.2. Validation data

Retention times and mass spectra were similar between standards and fortified matrices (%RSD < 5), proving that this chromatographic procedure is a selective method for the quantification of all pesticides because it was able to select, with high precision, the pesticides at different concentrations. Precision, expressed in terms of relative standard deviation (%RSD), and accuracy, calculated as a percentage of agreement between the results and the nominal concentrations, were determined based on intra- and inter-day assays (a total of nine replicates per quality control); the mean ± SD values were 9.2% ± 4.2 (for precision) and 99.9% ± 9.4 (for accuracy) (supplementary data – Table 2).

When comparing a solvent sample (400 ng L^{-1}) with a spiked sample at the same concentration, the retention times and the ion presence of the target pesticides were not affected (Figures 2A and 2B), which is in accordance with the 2002/657/EC directive [30], *i.e.*, the tolerances were ±10% for ions with a relative intensity above 50% of the base peak, ±15% for ions with a relative intensity between 20% and 50%, ±20% for ions with a relative intensity of 10–20% and ±50% for ions with a relative intensity lower than 10%. However, a signal enhancement in the matrix (pesticide area in a spiked matrix/pesticide area in solvent) was observed, which ranged from 1- to 66-fold for all the analysed compounds, indicating a matrix effect and, therefore, the need of matrix-matched calibration standards.

The stability of the pesticides in water samples was evaluated by comparing the initial results of the QCs with those obtained after a period of 24 and 48 h, kept at −20°C, and no degradation (%RSD < 20) was observed [29].

3.3. Pesticides in water samples from Ria Formosa lagoon

Of the 3024 measurements made (54 samples × 56 compounds), 89% of the pesticides were identified using the MS/MS mode, and 84% were quantified using a GC-MS SIM method. Nine of the 56 target pesticides were below the average MDL of the method. Table 1 shows the average concentrations ± SD (ng L^{-1}) of each pesticide per season, and on Figure 1C data are assembled by categories of pesticides (fungicides, herbicides and

insecticides), where it is possible to find an increasing trend that reaches maximum total amounts in the
spring, mainly for fungicides and insecticides. During the monitoring of the Ria Formosa lagoon samples, blanks and controls (QC_{medium}) were systematically injected to ensure the reliability of the results.

3.3.1 Fungicides

From six fungicides, only the hexachlorobenzene (HCB) showed concentrations below its MQL. On average, the annual summed concentration of all fungicides was $\Sigma \approx 1.4$ µg L^{-1} and their frequency in samples was close to 97%. Analysing the current data by season, the lowest total average amounts were measured in winter ($\Sigma_{Fungicides} \approx 317$ ng L^{-1}) and the highest were found in the spring ($\Sigma_{Fungicides} \approx 3.8$ µg L^{-1}), representing a 12-fold increase. The highest individual mean value was found for azoxystrobin (≈ 2 µg L^{-1}), difenoconazol (≈ 1 µg L^{-1}) and tebuconazole (≈ 0.6 µg L^{-1}), during spring, presenting significant differences ($p < 0.05$) when compared with the other seasons. The less abundant fungicide was procymidone (≈ 19 ng L^{-1}), observed during summer.

3.3.2 Herbicides

Twelve out of 15 herbicides were detected and quantified, with their annual loads and frequency in samples being $\Sigma \approx 576$ ng L^{-1} and 85%, respectively. Amongst seasons, the lowest amounts were measured in winter ($\Sigma_{Herbicides} \approx 225$ ng L^{-1}), being similar in the other seasons ($\Sigma_{Herbicides} \approx 694$ ng L^{-1}). Individually, pendimethalin (≈ 494 ng L^{-1}) and terbuthylazine (≈ 219 ng L^{-1}) were the most abundant pesticides and their highest amounts occurred in spring. In winter, the levels of pendimethalin decreased significantly (64 ng L^{-1}; $p < 0.05$).

3.3.3. Insecticides

This category represents 62.5% of all pesticides that were intended to be analysed. From 35 pesticides, only five (4,4′-DDD, 4,4′-DDE, chlordane [gamma], heptachlor epoxide and pirimicarb) were not detected and, therefore, were not quantified. The total annual average concentration and frequency were $\Sigma \approx 9.0$ µg L^{-1} and 89%, respectively. Insecticide concentrations were lower in winter ($\Sigma_{Insecticides} \approx 2.1$ µg L^{-1}) and higher in spring ($\Sigma_{Insecticides} \approx 22.3$ µg L^{-1}). Individually, insecticides that showed higher concentrations were cyhalothrin (lambda) (≈ 3 µg L^{-1}, in spring), cypermethrin (alpha) (453 ng L^{-1}, in summer) and deltamethrin (17 µg L^{-1}, in spring). Both 4,4′-DDT and fenamiphos showed higher levels in spring than in the other seasons ($p < 0.05$).

3.4. Physicochemical parameters

In parallel, several physicochemical parameters were measured and they were grouped as it is indicated on Table 2. The annual average levels of temperature (≈ 20°C), salinity (≈31), pH (≈8) and DO (≈9 mg L^{-1}) were similar amongst sampling sites. Similar occurrence was measured for nitrites (≈ 0.02 mg L^{-1}) and phosphates (≈ 0.7 mg L^{-1}). The nitrates (≈ 0.22 mg L^{-1}) and ammonia (≈ 0.52 mg L^{-1}) were 2-fold higher in Zone II than

in Zone I, although no significant differences were observed.

4. Discussion

Validation and optimisation of the SPE followed by GC-MS and the GC-MS/MS method allowed quantification and identification of 56 pesticides (more 17 compounds than the original method) from 14 different chemical classes in coastal matrices. Its low MQLs (ng L^{-1} levels) associated with its speed (10 minutes lesser than the original method) and moderate costs make it excellent for analysing complex coastal matrices. Another advantage of this method, comparatively to others [31,32], is its feasibility for analysing the most currently used pesticides in the EU. The applicability of the method was tested in a seasonal monitoring study, done in nine sampling sites of Ria Formosa lagoon, where 84% of the assayed pesticides were measured. The maximum values of all pesticides were attained in spring and Zone II (Figure 1C and 1B, respectively) where agricultural activities seem to be more intense [33].

4.1 Fungicides

The directive 2008/105/EC established individual maximum levels for some fungicides. In this vein, it is important to mention that pentachlorobenzene (PCB) concentration is 5-fold above the permitted level for inland surface waters (7 ng L^{-1}) and 50-fold higher than the maximum level for other surface waters (0.7 ng L^{-1}) [1,34].

Three fungicides (azoxystrobin, difenoconazol and tebuconazole) showed concentrations above 100 ng L^{-1} (Table 1), which is the maximum level established by the directive 98/83/EC [9]. According to the Groundwater Ubiquity Score (GUS) [35], the cited fungicides show low to moderate leachable levels, as their GUS ranged from 2–3 for azoxystrobin and tebuconazole and 0.1–1.0 for difenoconazol, suggesting that these pesticides are being overused, by themselves or by being the main compound in commercial mixtures, leading to high amounts in water.

Similar amounts were also found in the Save river (France) for tebuconazole (≈255 ng L^{-1}) and in the US streams for azoxystrobin (163–1130 ng L^{-1}), while higher quantities were observed in Alava (Spain) for difenoconazol (970–1440 ng L^{-1}) [7,36,37].

Annually, the total average loads of fungicides (1.4 µg L^{-1}) were approximately 3-fold higher than the maximum allowed (0.5 µg L^{-1}) by the directive 98/83/EC [9].

4.2 Herbicides

The levels of herbicides were 2.4-fold lower than reported for fungicides. Nonetheless, pendimethalin (≈376 ng L^{-1}) surpassed the 100 ng L^{-1}, established by the directive 98/83/EC [9]. As it has an extremely low GUS (−0.4), the presented amounts suggest an overuse of this pesticide in this area.

Some of these compounds were already found in other Portuguese water systems, such as Póvoa do Varzim ($\Sigma_{\text{terbuthylazine, propyzamide and pendimethalin}}$ ≈3.7 µg L^{-1}) [38] and the Douro river ($\Sigma_{\text{simazine, metribuzin, simetryn and atrazine-desethyl}}$ ≈288 ng L^{-1}) [13]. The total annual average loads of herbicides (0.6 µg L^{-1}) were close to the maximum levels (0.5 µg L^{-1}) specified by the directive 98/83/EC [9].

4.3 Insecticides

In this group, 29% of insecticides were measured in amounts higher than 100 ng L^{-1}. Both, cyhalothrin (lambda) (1.6 µg L^{-1}) and deltamethrin (5.5 µg L^{-1}) presented at 16- and 52-fold over that level ($p < 0.05$). Compared to other studies conducted in other Portuguese water sources, these values were the highest [13,39]. 4,4′-DDT residues were measured in concentrations 21.5-fold above the maximum (10 ng L^{-1}) level acceptable for inland and surface waters [1]. As the usage of DDT was banned in Portugal during the 1990s, the recorded levels can be a consequence of misuse from that time, together with its possible illegal usage [13,31]. Besides, the half-life time of this compound, in the aquatic environment, is over 100 years [40], which may explain the existing values. 4,4′-DDT residues were also measured in France (≈144 ng L^{-1}) and Spain (≈39 ng L^{-1}), supporting both the hypotheses referred to above [41,42]. The presence of HCCP, for which average values were ≈48 ng L^{-1}, may be due to its role as a precursor of other pesticides and its usage in the production of flame-retardant, plastic additives among others [43]. As successors/degradation products, endosulfan (alpha) (≈77 ng L^{-1}) and dieldrin (≈153 ng L^{-1}) were measured with levels above those observed for the HCCP. Additionally, the $\Sigma_{\text{aldrin, endrin, dieldrin}}$ and $\Sigma_{\alpha\text{- and }\beta\text{-endosulfan}}$ attained average concentrations of ≈174 ng L^{-1} and ≈90 ng L^{-1}, respectively, representing ≈17-fold higher than the annual average levels referred for inland waters (10 ng L^{-1} and 5 ng L^{-1}, respectively) [10]. All referred insecticides have GUS score that ranged from 0 to 4.5, which means that their presence in surface water continues to indicate their extreme and/or indiscriminate usage.

Similarly with the fungicides, the total annual average loads of insecticides (9.0 µg L^{-1}) were 18-fold higher than the levels established by the European legislation (directive 98/83/EC) [9].

4.4. Physicochemical data

Physicochemical data (Figure 1D) support the present environmental monitoring study. The DO levels were always greater than 8 mg L^{-1} and no signs of hypoxia (below 2 mg L^{-1}) were observed. The total annual average level of phosphorous was 0.05 mg L^{-1}, which is 20-fold lower than the maximum established value of 1 mg L^{-1} for surface waters (Directive 236/98) [44]; however, the values attained in summer (0.1 mg L^{-1}) represent the maximum acceptable to avoid accelerated eutrophication, established by the Water Quality Criteria [45]. Their likely origins are effluents from WWTPs and the usage of organophosphorus pesticides. In agreement with this hypothesis, Zone II – mainly agricultural [33] – had higher concentrations of nitrites, nitrates, ammonia and phosphates than Zone I. In addition, un-ionised ammonia (0.09 mg L^{-1}) and total nitrogen (2.3 mg L^{-1}) had, in Zone II, greater concentrations than the recommended levels of 0.06 and 1.0 mg L^{-1}, respectively [44,46]. These data also support the role of WWTPs and agricultural activities, as described earlier.

5. Concluding remarks

The current method allowed the quantification (GC-MS) and unequivocal identification (GC-MS/MS) of 56 pesticides in environmental coastal water matrices within a 35-min chromatographic run. The applicability of this method to Ria Formosa lagoon samples showed that 84% of the analysed compounds were above the MQLs, 25% were above the recommended levels of 100 ng L^{-1} and 20% had higher concentrations than the maximum established by directive 2013/39/EU [10]. Additionally, considering that directive 236/98 specifies 2.5 µg L^{-1} as the maximum acceptable concentration for pesticides in surface waters, the present data revealed an average value of ≈11 µg L^{-1}, which is 5-fold higher than the recommended value. Moreover, a seasonal pattern of pesticides loads was observed that were higher during spring, probably related to seasonal application in the fields.

Because there are aquacultures in the studied area, and as some pesticides are considered as POPs, bioaccumulation may occur in animals intended for human consumption. A non-negligible risk for human health may, thus, exist in view of the amounts found and because these compounds affect the whole food chain [3,47]. Eventual negative impacts may well occur together with other anthropogenic pollutants measured in the Ria Formosa lagoon [17,33]. Given that the cumulative loads of the selected pesticides were above the legal limits in all seasons, it is clear that this area is under an anthropogenic impact and, therefore, regular surveys should be undertaken to help local and governmental departments minimise the impact of pesticides usage in the Ria Formosa lagoon.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

Table S1. Quantification and diagnostic ions used in GC-MS and GC-MS/MS analysis. The relative abundance of ions (m/z) for each target pesticide is indicated inside brackets.

Pesticides	Molecular mass g mol^{-1}	RT (min)	GC-MS/SIM Target ion (t)	Q1 (%Q1/t)	Q2 (%Q2/t)	Q3 (%Q3/t)	Q 4 (%Q4/t)	GC-MS/MS Percursor		Products			CE	Ranges
Alachlor [a]	269.8	13.84	**188**	160 (86.8)	146 (58.8)			**160**	→	*132*	*130*		1.10	116-161
Aldrin [a]	364.9	15.21	**263**	261 (92.7)	265 (65.6)	66 (57.2)		**263**	→	*193*	*191*	*227*	1.60	190-264
Atrazine [b]	215.7	11.85	**200**	215 (56.8)	173 (36.9)			**200**	→	*122*	*132*	164	158	1.25	121-201
Atrazine-d_5	220.7	11.80	**205**	220 (43.0)	178 (41.7)			***205***	→	*127*	*137*	*105*		1.25	104-206
Atrazine-desethyl	187.6	10.80	**172**	68 (32.2)	174 (29.2)			**172**	→	*130*	*145*	*152*		1.15	104-173
Azinphos-methyl	317.3	23.91	**77**	132 (88.0)	104 (43.4)	160 (32.9)		**77**	→	*51*	*50*			1.30	49-78
Azoxystrobin	403.4	32.22	**344**	388 (41.6)	345 (32.7)			**344**	→	*329*	*328*			1.72	325-345
BHC (gamma) (Lindane) [a]	290.8	12.18	**181**	183 (76.6)	219 (69.3)			**181**	→	*145*	*146*			1.20	108-184
Chlordane (gamma)	338.9	17.14	**375**	373 (93.8)	377 (59.9)			**373**	→	*266*	*264*			1.20	263-374
Chlorfenvinphos Z	359.6	16.38	**267**	269 (52.9)	323 (51.5)			**267**	→	*159*	*203*			1.50	158-268
Chlorpyriphos	350.6	15.05	**314**	316 (72.3)	258 (67.3)	199 (46.8)	197 (41.4)	**314**	→	*258*	*286*			0.90	257-315
Cyanazine	240.7	15.09	**225**	212 (59.4)	198 (35.2)	68 (32.8)		**225**	→	*189*	*172*	*198*		1.28	171-226
Cyfluthrine (beta) *	434.3	27.45	**206**	199 (76.9)	91 (70.9)	226 (55.0)	227 (42.2)	**199**	→	*193*	*191*	163		1.80	190-200
Cyhalofop-butyl	357.4	24.28	**256**	357 (72.6)	229 (41.1)	120 (31.0)		**256**	→	*228*	*200*			1.13	199-257
Cyhalothrin (lambda) *	449.9	24.55	**181**	141 (45.8)	197 (42.1)			**181**	→	*152*	*151*			1.50	120-182
Cypermethrin (alpha) *	416.3	27.78	**181**	91 (76.3)	163 (75.0)	165 (47.3)		**181**	→	*152*	*151*			1.70	150-153/179-182
4,4´-DDD	320.0	19.67	**235**	237 (64.2)	165 (61.7)			**235**	→	*165*	*199*			1.15	162-236
4,4´-DDT	354.5	20.96	**235**	237 (64.2)	212 (59.0)	165 (44.0)		**235**	→	*165*	*199*			1.15	117-236
4,4´-DDT-d_8	362.5	20.88	**220**	243 (62.6)	280 (57.8)			***243***	→	*173*	*206*			1.15	172-244
4,4'-DDE	318.0	18.26	**246**	248 (58.6)	318 (31.9)	316 (29.3)		**246**	→	*176*	*175*			1.70	174-247
Deltamethrin *	505.2	31.75	**181**	207 (61.4)	253 (58.7)			**181**	→	*152*	*151*			1.70	150-153/179-182
Diazinon	304.4	12.36	**137**	179 (44.7)	304 (10.2)			**179**	→	*121*	*163*	137	122	1.35	110-180
Dichlorvos	221.0	7.16	**109**	185 (91.2)	79 (46.7)			**109**	→	*79*	*93*			0.98	66-83/90-110
Dieldrin [a]	380.9	18.44	**79**	263 (93.1)	237 (43.5)			**79**	→	*51*	*50*			1.10	49-80
Difenoconazol	406.3	30.95	**265**	267 (89.9)	323 (68.5)	325 (62.2)		**323**	→	*265*	*249*			1.35	245-266/321-324
Dimethoate	229.3	11.59	**87**	93 (52.5)	125 (44.3)			**87**	→	*86*	*59*			1.10	53-88
Endosulfan (alpha)	406.9	19.46	**241**	195 (78.2)	237 (70.8)	243 (65.5)		**241**	→	*206*	*205*			1.45	165-242
Endosulfan (beta)	406.9	17.59	**241**	195 (72.7)	243 (71.2)	207 (54.0)		**241**	→	*206*	*204*	*170*		1.45	165-242
Endosulfan sulfate	422.9	20.76	**272**	237 (68.0)	274 (60.5)	387 (47.9)		**272**	→	*237*	*235*			1.10	234-273
Endrin [b]	380.9	19.10	**243**	263 (99.0)	281 (68.4)	81 (47.4)		**243**	→	*207*	*173*			1.15	172-244
Fenamiphos	303.4	17.70	**303**	243 (62.4)	217 (54.9)	288 (42.9)	154 (40.6)	**303**	→	*268*	*266*			1.10	175-304
Fenitrothion	277.2	14.55	**260**	109 (83.4)	125 (77.4)	277 (41.8)		**260**	→	*228*	*217*	*232*		1.20	160-261
Fonofos	246.3	12.36	**137**	109 (70.7)	246 (40.0)			**137**	→	*109*	*81*			0.85	80-138
Heptachlor [b]	373.3	14.19	**272**	274 (73.9)	270 (63.3)	100 (43.9)		**272**	→	*237*	*235*			1.05	236-275
Heptachlor epoxide [b]	389.3	16.38	**353**	355 (66.0)	351 (44.4)	81 (25.7)		**353**	→	*263*	*282*			1.10	262-354
Hexachlorobenzene (HCB)[b]	284.8	11.49	**284**	282 (46.4)	249 (41.2)			**284**	→	*214*	*249*			1.50	211-285
Hexachlorocyclopentadiene (HCCP) [b]	272.3	7.93	**237**	239 (54.6)	235 (48.1)	272 (14.2)		**237**	→	*143*	*141*	*203*		2.05	140-145/200-238
Malathion	330.4	14.81	**125**	127 (90.9)	99 (73.2)	173 (40.2)		**173**	→	*99*	*117*	145		0.80	92-173
Methoxychlor [b]	345.7	22.86	**227**	228 (16.5)	274 (15.4)			**227**	→	*169*	*181*			*1.30*	*140-228*
Metolachlor	283.8	15.02	**162**	238 (36.7)	163 (15.1)			**162**	→	*132*	*133*			1.15	115-163
Metribuzin	214.3	13.63	**198**	199 (29.8)				**198**	→	*150*	*110*			1.10	109-199
Mirex	545.5	24.58	**272**	274 (73.3)	237 (62.7)			**272**	→	*237*	*235*			1.13	234-273
Parathion-ethyl	291.3	15.25	**291**	109 (79.0)	263 (61.0)	97 (57.2)	141 (47.0)	**109**	→	*81*	*91*			0.99	60-110
Parathion-methyl	263.2	13.84	**263**	109 (67.9)	79 (44.7)	246 (43.5)		**263**	→	*246*	*153*			1.31	150-264
Pendimethalin	281.3	16.08	**252**	162 (61.3)	191 (28.9)			**252**	→	*162*	*191*			1.00	160-253
Pentachlorobenzene (PCB)	250.3	9.53	**250**	248 (65.1)	252 (66.1)			**250**	→	*215*	*144*			2.01	143-251
Phosmet	317.3	10.31	**160**	161 (86.2)	133 (40.7)			**160**	→	*130*	*140*			1.15	104-161
Pirimicarb	238.4	13.02	**166**	238 (29.0)	72 (18.8)			**166**	→	*96*	*137*	*121*		1.35	95-167
Procymidone	284.1	16.67	**96**	283 (85.3)	285 (29.0)			**96**	→	*67*	*68*			1.00	64-97
Propazine	229.7	11.96	**214**	172 (70.6)	187 (38.0)			**214**	→	*200*	*172*	*138*		1.20	137-215
Propyzamide	256.1	12.31	**173**	175 (41.2)	254 (35.6)			**173**	→	*138*	*145*			1.10	130-174
Simazine [a]	201.6	11.75	**201**	186 (67.1)	173 (51.9)	44 (37.6)		**201**	→	*186*	*174*	*138*		1.20	135-201
Simetryn	213.3	13.94	**213**	170 (22.9)	155 (13.4)			**213**	→	*170*	*185*			1.10	151-214
Tebuconazole	307.8	21.40	**250**	125 (80.6)	163 (44.4)			**125**	→	*89*	*99*			1.60	62-126
Terbuthylazine	229.7	12.22	**214**	173 (71.8)	138 (27.3)	229 (24.3)		**214**	→	*173*	*132*			1.40	131-215
Terbutryn	241.4	14.50	**185**	226 (68.0)	170 (45.2)			**185**	→	*170*	*128*			0.90	127-186
Tetrachlorvinphos	366.0	17.25	**329**	331 (90.4)	109 (51.3)	333 (32.8)		**329**	→	*314*	*278*			1.30	219-330
Trifluralin	335.3	10.80	**264**	306 (44.9)	206 (27.2)			**264**	→	*206*	*160*	*188*	*171*	1.05	159-265

Internal standards; [a] Compounds present on the mix A (EPA 505/525); [b] Compounds present on the mix B (EPA 505/525); * Contain several diastereoisomers
RT: retention time; **CE:** collision energy (V); **Q:** qualifier ion; **%Qn/T:** percent qualifier-to-target ratio where n = 1, 2, 3, and 4;

Table S2. Average values from the intra- and inter-day absolute recovery, precision and accuracy, method detection and quantification limits (MDLs and MQLs) of 53 pesticides in three different QCs. All the calculations were performed in agreement with both ICH and ANVISA guidelines.

Pesticides	QC	Recovery		RSD		Accuracy		MDLs	MQLs
	µg L^{-1}	(%)	SD	(%)	SD	(%)	SD	ng L^{-1}	ng L^{-1}
Alachlor	0.01	118.0	4.7	9.0	3.9	104.8	8.4		
	0.18	98.2	5.4	7.5	3.1	104.6	16.4	0.167	0.505
	0.33	92.0	11.7	7.0	2.5	103.1	8.3		
Aldrin	0.01	99.3	14.0	12.4	2.8	107.3	13.1		
	0.18	75.1	8.4	7.8	3.8	93.6	14.9	0.989	2.996
	0.33	90.4	12.5	4.8	3.7	97.6	16.5		
Atrazine	0.01	93.3	8.1	10.7	8.6	107.5	6.8		
	0.18	104.5	6.0	6.7	1.7	111.7	6.6	0.173	0.523
	0.33	90.4	10.5	6.1	3.6	112.9	11.9		
Atrazine-desethyl	0.01	111.2	6.3	8.8	5.2	116.8	7.4		
	0.18	99.8	7.2	8.8	4.8	112.2	10.2	0.128	0.388
	0.33	119.0	13.0	5.6	2.9	93.8	11.5		
Azinphos-methyl	0.01	110.0	12.5	9.1	5.3	100.9	11.1		
	0.18	112.0	17.1	8.9	4.6	89.3	3.6	0.470	1.424
	0.33	108.1	7.9	9.8	4.9	80.3	3.1		
Azoxystrobin	0.01	113.7	15.3	13.3	9.3	110.9	13.2		
	0.18	97.2	13.2	18.1	13.3	101.1	9.3	1.225	3.713
	0.33	87.5	10.2	5.3	4.8	84.0	12.6		
Chlordane (gamma)	0.01	84.8	18.2	5.3	3.2	79.7	5.0		
	0.18	81.7	1.8	2.5	0.6	119.4	2.1	0.456	1.382
	0.33	82.1	16.4	11.8	7.6	101.9	10.3		
Chlorfenvinphos Z	0.01	110.0	9.0	10.2	2.8	118.6	4.9		
	0.18	111.2	2.8	4.6	2.4	94.1	9.0	0.362	1.098
	0.33	105.4	9.9	7.3	4.1	92.3	8.1		
Chlorpyriphos	0.01	113.3	7.7	16.5	7.7	110.9	10.1		
	0.18	87.9	6.2	8.5	2.3	113.6	7.8	0.878	2.659
	0.33	92.2	12.0	20.5	16.0	100.4	9.0		
Cyanazine	0.01	92.2	13.7	14.2	9.2	111.9	5.1		
	0.18	78.2	18.6	9.5	4.6	86.2	1.8	0.481	1.459
	0.33	102.1	14.4	8.4	2.9	80.0	8.1		
Cyfluthrine (beta)	0.01	97.1	13.8	9.1	4.5	96.4	19.4		
	0.18	112.0	7.3	7.7	1.8	94.1	6.6	0.858	2.600
	0.33	92.8	10.2	8.9	3.2	106.4	13.6		
Cyhalofop-butyl	0.01	94.4	17.9	12.4	2.0	109.0	2.2		
	0.18	94.6	17.1	6.1	4.3	105.2	13.6	0.502	1.521
	0.33	117.3	3.0	3.3	1.3	89.6	2.7		
Cyhalothrin (lamdba)	0.01	98.9	9.9	9.1	3.6	87.9	10.4		
	0.18	98.3	11.8	7.8	3.8	111.4	5.7	0.495	1.500
	0.33	104.7	9.4	9.2	3.3	101.7	10.7		
Cypermethrin (alpha)	0.01	107.0	15.6	9.2	5.5	95.8	7.7		
	0.18	101.8	15.9	7.8	2.2	111.7	4.0	0.305	0.924
	0.33	91.5	9.3	7.8	1.8	90.6	11.2		
4,4´-DDD	0.01	98.5	13.0	9.6	6.2	108.8	11.8		
	0.18	86.7	10.7	5.3	2.3	103.7	11.3	0.486	1.473
	0.33	83.2	8.8	10.9	4.4	111.1	4.2		
4,4´-DDE	0.01	95.2	15.8	6.9	3.6	84.1	9.0		
	0.18	88.5	10.4	5.1	2.4	90.8	12.4	0.312	0.945
	0.33	95.7	11.7	8.6	4.1	98.0	13.5		
4,4´- DDT	0.01	88.9	2.7	9.0	4.5	110.9	3.2		
	0.18	84.8	11.5	5.7	2.9	105.0	19.0	0.934	2.830
	0.33	104.3	6.6	9.7	3.3	91.2	6.7		
Deltamethrin	0.01	100.4	9.9	7.5	4.6	108.0	9.7		
	0.18	99.3	11.8	7.2	3.4	86.8	12.2	0.690	2.092
	0.33	96.8	4.5	12.8	3.1	104.8	14.2		
Diazinon	0.01	96.8	3.3	9.8	3.1	109.8	7.2		
	0.18	113.3	9.7	7.8	3.8	90.2	9.5	0.001	0.003
	0.33	102.3	6.9	8.8	2.5	106.6	9.0		
Dichlorvos	0.01	106.0	8.8	10.8	6.1	89.2	9.0		
	0.18	89.9	17.1	5.8	1.6	96.2	4.9	0.256	0.775
	0.33	98.5	12.6	6.5	2.8	105.9	8.8		
Dieldrin	0.01	104.1	14.2	7.0	2.2	101.2	15.6		
	0.18	89.0	10.8	10.5	2.6	100.7	18.0	0.516	1.564
	0.33	82.7	6.3	6.8	3.1	92.1	4.6		
Difenoconazol	0.01	101.6	8.6	7.0	6.0	101.3	7.3		
	0.18	105.1	12.2	9.0	2.1	100.5	7.2	0.252	0.765
	0.33	105.1	17.6	10.4	2.5	94.0	13.9		

(continued)	QC	Recovery		RSD		Accuracy		MDLs	MQLs
	µg L^{-1}	(%)	SD	(%)	SD	(%)	SD	ng L^{-1}	ng L^{-1}
Dimethoate	0.01	93.4	19.7	15.4	10.3	116.0	6.9		
	0.18	109.0	11.1	4.6	1.9	87.0	11.3	0.749	2.270
	0.33	80.3	8.0	7.0	3.8	84.3	8.1		
Endosulfan (alfa)	0.01	93.7	8.1	12.0	2.7	107.0	13.2		
	0.18	93.6	12.0	8.9	4.6	101.7	9.7	0.031	0.093
	0.33	89.6	8.6	10.7	4.2	84.0	6.2		
Endosulfan (beta)	0.01	90.7	6.5	9.0	3.0	102.4	9.9		
	0.18	101.8	17.4	7.7	4.5	102.7	11.9	0.694	2.103
	0.33	97.0	11.7	9.1	2.3	105.7	9.6		
Endosulfan sulfate	0.01	83.0	8.7	14.0	2.0	111.9	12.1		
	0.18	101.2	11.2	5.9	3.9	113.2	12.2	0.641	1.941
	0.33	93.9	9.4	8.2	3.8	104.0	11.0		
Endrin	0.01	78.5	6.1	7.2	6.0	114.7	6.7		
	0.18	88.4	10.7	12.6	7.7	115.5	8.5	0.455	1.378
	0.33	100.7	11.7	5.4	4.1	95.1	12.7		
Fenamiphos	0.01	93.4	7.3	12.5	0.9	86.5	3.7		
	0.18	89.9	9.8	5.4	4.6	93.2	10.4	0.863	2.616
	0.33	85.4	10.5	7.4	5.2	87.7	7.6		
Fenitrothion	0.01	117.5	13.8	9.8	5.3	86.9	7.6		
	0.18	120.7	4.5	7.3	2.7	102.8	10.6	0.163	0.495
	0.33	80.8	7.0	9.1	4.6	90.9	8.2		
Fonofos	0.01	112.3	4.0	12.7	9.7	112.3	6.7		
	0.18	97.6	9.4	8.7	4.6	103.1	11.5	0.058	0.176
	0.33	92.1	5.8	9.2	3.6	106.4	14.8		
HCB	0.01	97.3	17.4	13.8	11.5	81.3	10.2		
	0.18	89.6	15.7	8.3	4.2	84.9	12.7	0.172	0.521
	0.33	93.4	10.7	8.2	2.0	110.9	11.7		
HCCP	0.01	101.4	16.7	8.6	2.7	78.7	1.4		
	0.18	104.9	12.8	11.6	3.8	85.7	2.7	0.855	2.592
	0.33	118.8	13.5	15.0	0.0	103.3	0.0		
Heptachlor	0.01	98.1	10.3	12.1	10.6	117.2	0.8		
	0.18	83.9	12.0	9.3	3.2	120.2	8.7	0.453	1.374
	0.33	82.2	14.4	18.9	17.9	103.7	18.1		
Heptachlor epoxide	0.01	88.3	16.6	11.7	8.0	118.9	1.8		
	0.18	102.1	8.8	4.7	1.9	84.8	10.8	0.393	1.190
	0.33	102.2	8.8	5.6	2.1	95.1	4.1		
Lindane	0.01	108.7	4.8	9.4	6.9	87.5	3.8		
	0.18	119.0	3.9	9.9	1.0	85.3	1.3	0.720	2.181
	0.33	117.8	19.1	8.9	1.4	96.2	16.8		
Malathion	0.01	99.5	18.5	12.2	1.0	101.2	18.2		
	0.18	95.7	23.4	8.0	3.0	99.3	21.0	1.530	4.636
	0.33	96.6	14.5	10.0	4.4	101.6	9.8		
Methoxychlor	0.01	114.2	6.6	9.5	4.0	101.8	6.0		
	0.18	98.3	8.6	7.6	3.5	106.7	9.7	0.109	0.330
	0.33	81.8	5.5	8.1	3.0	88.3	8.0		
Metolachlor	0.01	108.6	10.5	5.2	1.8	101.6	20.7		
	0.18	91.1	6.0	6.9	2.1	111.7	10.7	0.367	1.113
	0.33	86.1	9.5	11.8	9.0	103.9	11.4		
Metribuzin	0.01	101.0	15.8	10.0	3.8	84.4	1.4		
	0.18	102.1	9.0	10.2	1.5	93.1	7.7	0.095	0.288
	0.33	99.7	11.8	11.9	4.8	103.7	13.9		
Mirex	0.01	71.4	0.4	7.8	2.5	104.9	4.8		
	0.18	92.4	13.4	7.2	3.5	103.5	10.3	0.342	1.037
	0.33	77.9	6.2	6.7	1.4	108.2	8.6		
Parathion-ethyl	0.01	94.1	11.8	14.0	12.5	86.0	5.8		
	0.18	119.1	2.7	8.7	1.2	104.9	13.8	0.931	2.821
	0.33	100.1	11.3	11.3	3.3	99.2	17.8		
Parathion-methyl	0.01	93.4	9.9	18.3	11.4	101.3	14.1		
	0.18	107.9	4.9	11.4	3.1	90.3	6.1	0.305	0.925
	0.33	111.1	6.5	10.8	2.3	99.6	6.0		
PCB	0.01	111.7	11.0	10.3	3.4	81.5	7.0		
	0.18	101.0	12.9	11.9	2.9	74.4	13.6	1.096	3.323
	0.33	86.7	5.0	6.1	3.2	113.9	4.9		
Pendimethalin	0.01	98.8	10.3	10.4	3.1	100.6	10.7		
	0.18	99.9	6.2	7.2	3.1	94.2	12.8	0.151	0.457
	0.33	91.1	3.7	9.1	3.3	108.5	12.4		

(continued)	QC	Recovery		RSD		Accuracy		MDLs	MQLs
	µg L^{-1}	(%)	SD	(%)	SD	(%)	SD	ng L^{-1}	ng L^{-1}
Phosmet	0.01	87.8	11.7	7.3	4.1	87.2	6.7		
	0.18	97.1	11.1	8.9	3.8	87.3	10.4	0.283	0.857
	0.33	90.1	3.2	8.0	3.3	101.2	20.6		
Pirimicarb	0.01	118.4	11.0	12.9	8.8	93.5	2.5		
	0.18	101.1	18.6	9.3	1.9	100.8	17.0	0.032	0.098
	0.33	106.2	13.5	10.9	4.2	88.7	5.3		
Procymidone	0.01	108.4	11.8	8.2	3.6	108.5	9.0		
	0.18	103.4	8.7	4.7	4.7	103.8	13.0	0.527	1.597
	0.33	88.7	10.2	7.2	1.5	97.7	13.5		
Propazine	0.01	99.5	13.9	8.8	3.2	100.4	18.6		
	0.18	93.5	5.8	9.5	2.8	94.7	12.1	0.048	0.144
	0.33	97.9	10.2	7.1	3.0	95.0	6.5		
Propyzamide	0.01	102.8	7.7	8.7	7.4	96.5	7.8		
	0.18	103.1	11.2	5.3	4.9	115.2	9.8	0.344	1.042
	0.33	106.9	9.9	3.7	2.8	119.2	8.1		
Simazine	0.01	98.6	8.5	11.1	7.4	111.6	6.0		
	0.18	109.4	5.0	8.1	3.5	102.7	2.8	0.890	2.696
	0.33	100.8	10.3	8.2	3.3	101.4	11.5		
Simetryn	0.01	118.8	1.7	7.3	5.5	110.1	12.8		
	0.18	78.4	5.9	11.5	0.9	113.9	5.0	0.077	0.235
	0.33	88.6	16.0	8.4	2.9	106.1	12.0		
Tebuconazole	0.01	105.1	6.5	9.9	4.0	111.6	5.7		
	0.18	110.3	7.8	7.6	3.3	106.1	12.5	0.146	0.442
	0.33	119.2	0.5	6.3	3.4	95.0	10.7		
Terbuthylazine	0.01	111.8	6.4	7.8	5.0	112.3	8.7		
	0.18	96.8	5.9	8.7	2.8	95.6	6.4	0.059	0.179
	0.33	97.5	6.7	6.3	3.9	100.6	4.5		
Terbutryn	0.01	95.1	13.4	12.4	7.4	89.7	6.9		
	0.18	104.7	13.4	6.2	3.2	85.1	7.3	0.106	0.320
	0.33	116.2	5.3	8.3	3.0	85.1	2.2		
Tetrachlorvinphos	0.01	96.8	14.5	9.2	5.4	87.8	10.3		
	0.18	110.5	10.0	7.1	2.5	114.4	4.2	0.024	0.072
	0.33	100.3	14.2	8.8	4.0	103.2	12.0		
Trifluralin	0.01	95.3	15.0	9.0	5.4	83.1	20.2		
	0.18	81.4	13.5	8.2	4.0	106.2	9.3	0.180	0.547
	0.33	100.6	5.8	9.7	4.0	105.3	11.4		

Recoveries (%)- obtained for the 3 quality controls (QCs) for 3 independent replicates and days;

Precision (relative standard deviation- **RSD**); **Accuracy** (%); **SD**- standard deviation between replicates (3)

Chapter 3

Occurrence and seasonal loads of pesticides in surface water and suspended particulate matter from a wetland of worldwide interest—the Ria Formosa Lagoon, Portugal

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Occurrence and seasonal loads of pesticides in surface water and suspended particulate matter from a wetland of worldwide interest—the Ria Formosa Lagoon, Portugal

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Abstract Two novel methods were developed to extract and quantify 56 pesticides in surface waters, considering their content in both dissolved aqueous phase (DAP) and suspended particulate matter (SPM) fractions. These procedures were applied to coastal samples taken seasonally during 2012–2013, from three strategic sampling sites along the Ria Formosa Lagoon (south of Portugal). Briefly, 500 mL of water samples were filtrated, separating both fractions. The DAP fraction was extracted and pre-concentrated by solid-phase extraction (SPE), while the SPM was extracted using ultrasonic extraction technique (USE). Both fractions were then analyzed, and the pesticides were quantified and identified, within 35 min, by gas chromatography (GC) coupled to mass spectrometry (GC-MS and GC-MS/MS), respectively. The extraction of pesticides from the SPM fraction showed average recoveries of 102 %, detection limits below 2.2 ng/L, and quantification limits ranging from 0.3 to 6.6 ng/L. Considering the real water samples, 73 % of the selected pesticides were quantified in both DAP and SPM fractions ($\sum_{DAP+SPM}$ 2.3 μg/L) and their maximum levels were measured in autumn and winter. By category, the global loads of fungicides, herbicides, and insecticides were ≈407, ≈323, and ≈1.6 μg/L, respectively. Thirty-one percent of the quantified pesticides exceeded the European directives levels (2008/105/ EC and 98/83/EC). From the total loads, the SPM fraction contribution was 32 %, showing the importance of measuring pesticides in that fraction. The water physicochemical parameters revealed that the total nitrogen amounts were very high relatively to the legal required values, mainly close to the city of Faro (2.6 mg/L). In light of the above, measures are in need to meet European directives and protect both fauna and humans that use this area for leisure.

Keywords Dissolved aqueous phase · Environmental monitoring · GC-MS/MS Lagoon · Pesticides · Surface waters · Suspended particulate matter · Ultrasonic extraction

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Introduction

Several pesticides are commonly toxic presenting slow degradation rates being prone to bioaccumulation (Ritter et al. 1995; Vallack et al. 1998) where their chemical characteristics make them highly able to clutch with water suspended particulate matter (SPM), sediments, and soils (Tang et al. 2008). Environmental pollution by pesticides has become of great concern not only due to immediate effects but also by their potential to produce global and severe ecologic injuries. This type of contamination, currently disseminated in both waters and soils, has become so daunting that restrictive European legislation has been implemented (2008/105/EC and 2013/39/EU) in order to control and limit the usage of these chemicals, being stressed the importance of management and control of pesticides in different matrices (EU 2008a, 2013). New laws have been enhancing the control and management of pesticide usage, while restricting as much as possible the mobilization of valuable economic and technical resources for the eradication of several compounds considered hazardous for the biota and humans.

Among the most toxic pesticides are the organochlorine pesticides, recognized to have potential to induce hormonal disruption and cancer (Ehrlich et al. 2011; Mearns et al. 2012; Vallack et al. 1998), mainly due to their extreme persistency, bioaccumulating and/or rippling through the food chain (Chopra et al. 2011; Katagi 2010). So, the abusive use of pesticides and inefficient treatment of residues increase their probability of reaching estuarine and marine environments affecting fish nurseries as well as the benthic and pelagic communities (Liu et al. 2008; McMillin and Means 1996). The Ria Formosa Lagoon, located on the south Portuguese coast, is an aquatic system recognized at national level, as a natural reserve (Ministério do Ambiente 2004) as well as, a wetland of worldwide interest, defined by the intergovernmental treaty "Convention on Wetlands" (Ramsar 2014). Besides, this area is also known by the bivalve and aquaculture production (Cachola and Campos 2006). However, it is still vulnerable to human activity (Cravo et al. 2009; Ferreira et al. 2003; Vasconcelos et al. 2010), where pesticides may contribute to this state.

Algarve is a well-known region for the citrus production, reaching annual values of 224,000 t that are equivalent to 17,749 ha of production (Ministério da Agricultura 2007). Additional crops, such as, corn, almond, and red fruits (hydroponic production), are also produced in this region (Vaz et al. 2013), contributing for the increase of pesticide usage.

Taking into account the above concerns, after a detailed research through Portuguese and European databases (DRAP 2014; EU 2008b), 56 pesticides were selected to be quantified in surface waters of the Ria Formosa Lagoon considering both, dissolved in the aqueous phase (DAP) and SPM fractions; the selection of these compounds was based on pesticide application lists (DRAP lists) complemented with the quantified levels, registered in other scientific publications, inside of European Union.

This approach will allow a more realistic picture about pesticide contamination on this vulnerable estuarine milieu. Nonetheless, the complexity of these environmental matrices, allied with the rigorous European law, implies developing highly sensitive and accurate analytical methods to monitor the pesticide levels.

Taking into account these concerns, one objective of this study was to optimize and validate an analytical GC-MS/MS method to analyze pesticides in SPM of coastal water samples using a simple and low-cost sample preparation—an ultrasonic extraction (USE) method. Further on, surface water samples were seasonally collected from the Ria Formosa Lagoon and analyzed (both DAP and SPM water fractions), allowing the quantification of different categories of pesticides (fungicides, herbicides, and insecticides). Physicochemical quality parameters—linked with the presence of fecal contamination and eutrophication—such as dissolved oxygen, pH, nitrates, nitritites, ammonia, un-ionized ammonia, and phosphates were measured, complementing the information about the estuary status. The data are relevant for filling

in the case gaps of information of the southwestern Europe and is far from being of interest only locally, namely in view that the Formosa is a well-recognized nursery ground for several (highly prized) oceanic species that spend their early stages of life there and considering the major importance of the area for intercontinental routes of the migrating birds.

Materials and methods

Sample collection and preparation

The Ria Formosa Lagoon is a mesotidal system situated on the south of Portugal, characterized by many small islands protected with dunes, which make it a perfect environment for fish nurseries and bivalve colonies (Ribeiro et al. 2006). Along its extension (60 km), three strategic sampling sites (S1 to S3, Fig. 1) were selected. S1 holds the city of Faro, S2 comprises the fraction of the Ria Formosa Lagoon Natural Park, and S3, the Tavira city and the Gilão River. Water samples were acquired during six sampling collections (December (2012), January, February, May, June, and October 2013) at the shore (50-cm depth) during ebb tide; the samples were collected into 2.5 L amber glass bottles and kept refrigerated (±4 °C) during sampling, until further extraction.

Physicochemical parameters

As a complement, temperature (°C), dissolved oxygen (DO; mg/L), salinity, and conductivity (mS/cm) were evaluated, in situ, using the portable meters OXi 330i/Set WTW and LF 330/Set WTW, respectively. Other parameters, such as pH (Hech HQ40d), nitrites (mg/L), nitrates (mg/L), ammonium (mg/L), and phosphates (mg/L) were measured at the laboratory, using the Palintest Photometer 700 interface.

Reagents and standard solutions

All organic solvents such as methanol (MeOH), ethyl acetate (EtOAc), and hexane were purchased from Romil (Cambridge, England). Ultrapure water was purified through a Milli-Q water system (conductivity = 0.054 μS cm/L, at 25 °C). The cartridges, 200 mg Oasis hydrophilic-lipophilic balance (HLB), 6 cc, were acquired from Waters Corporation (Milford, MA, USA), and the glass fiber filters (0.45 μm) were purchased from Munktell (Bärenstein, Germany). All pesticides were supplied by Sigma-Aldrich (Steinheim, Germany), individually, with the exception of Mix A (EPA 505/525, 500 mg/L) and Mix B (EPA 505/525, 500 mg/L). The 4,4′-DDT-d_8 (DDT-d_8) and atrazine-d_5 (ATZ-d_5) were used as both surrogates and internal standards (IS). All reference standards were above 98 % of purity.

The standard solutions were individually prepared in MeOH (1000 mg/L) and kept in the dark at −20 °C to avoid potential decay. From the stock solutions, eight nominal calibration standard mixtures prepared and spiked in both matrices.

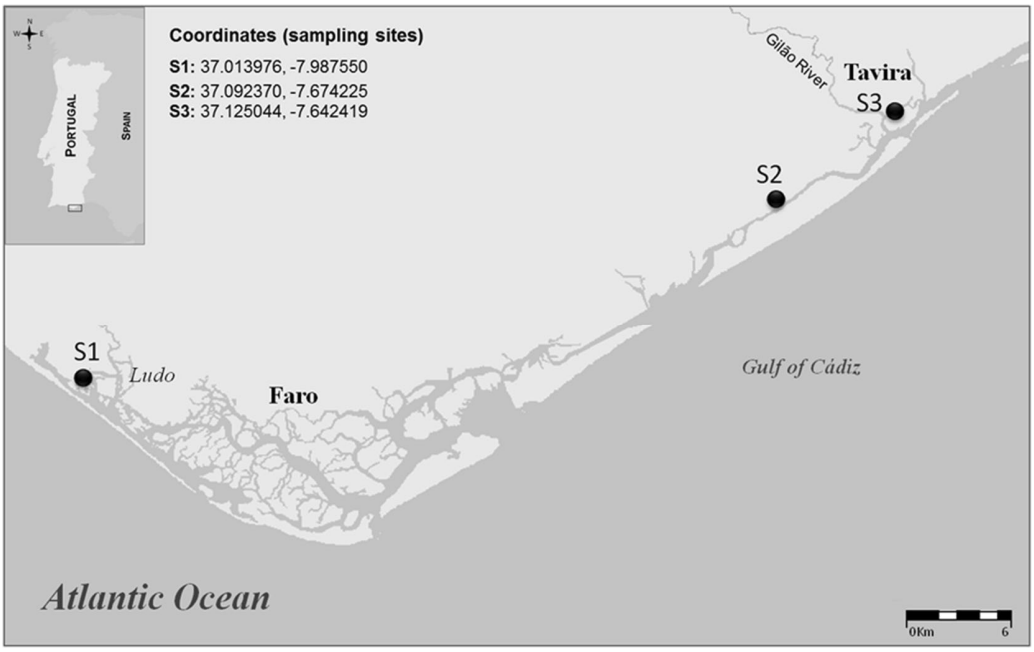

Fig. 1 Location of sampling sites within the Ria Formosa Lagoon (S1 to S3), Portugal (adapted from Microsoft MapPoint, 2010)

Sample preparation

The water samples (500 mL) were filtrated, within 24 h after collection, dividing both fractions for further extraction procedures (Fig. 2).

The protocol used for the extraction of pesticides from DAP fraction, followed a previous solid-phase extraction (SPE; Rocha et al. 2011) and a GC-MS/ MS analytical protocol recently amplified, for the analysis of 56 pesticides (Cruzeiro et al. 2015). The recovery rates were always above 71 %, and limits ranged from 0.4 to 1.3 ng/L. Briefly, water samples (500 mL) were subjected to SPE using OASIS HLB cartridges adapted in an off-line SPE vacuum extraction device (Waters). Firstly, the cartridges were conditioned with 5 mL of EtOAc and MeOH and then 2.5 mL of ultrapure water at a flow rate of 1–2 mL/ min. Thereafter, the water samples were loaded in to the SPE cartridges at a constant flow rate of 5 mL/ min, which were later dried under vacuum for 1 h and then eluted with 6 mL of EtOAc, at 1 mL/min. The extracts were concentrated into 200 μL of hexane and kept in vials at −80 °C until analysis. The pesticides extracted from the SPM fraction followed a USE method previously developed for pesticide extraction from soils matrices (Gonçalves and Alpendurada 2005). The resultant glass fiber filters were soaked in 3 mL of EtOAc for 8 min in an ultrasonic bath (Axtor-Lovango, model CD-4820, 170 W). This procedure was done twice, and cooling devices were used to avoid the increase of temperature during this process.

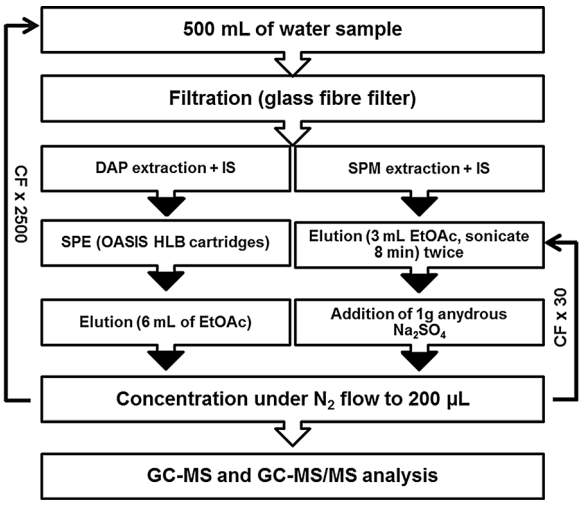

Fig. 2 Diagram of the extraction procedures of both fractions (DAP and SPM)

Then, 1 g of anhydrous Na_2SO_4 was added to the extract (6 mL) to eliminate possible water residues, which was further on evaporated to

dryness under a gentle N_2 (99.9997 %) stream. Similarly to the DAP fraction, the extracts were reconstituted with hexane (200 μL) and kept at −80 °C until analysis.

Gas chromatography-ion trap mass spectrometry

Analyzes were carried out using a gas chromatograph Trace GC ultra (Thermo Finnigan Electron Corporation), coupled with an ion trap mass spectrometer detector Thermo Scientific ITQ™ 1100 GC-MS^n) that operates at 70 eV of electron impact (EI) and an autosampler Thermo Scientific TriPlus™. For the analysis of both DAP and SPM fraction was used a Trace GOLD column (TG-5SILMS, 30 m × 0.25 mm × 0.25 μm) and a 2 μL injection volume sample in splitless mode using a 50-mm length needle. Column oven temperatures were programmed as 65 °C (initial hold time of 2 min) to 180 °C at 20 °C/min until 280 °C at 5 °C/min (hold time of 7 min). The injector port temperature was set to 250 °C, and both ion source and MS transfer line were at 280 °C. The helium (99.9999 % purity) was used as gas carrier, and it was maintained at a constant flow rate of 1 mL/min. The target and qualifier ions (MS) were determined by injection of individual pesticide standards, using full-scan mode (40–650 *m/z*). The product ions were compared with other methods (Lian et al. 2010; Rocha et al. 2011; Wong et al. 2010; X. Yang et al. 2011) and supported by NIST Mass Spectral Search Program (version 2.0, 2005) library to create a selected ion monitoring (SIM; Table 1). The MS/MS conditions were optimized for the identification of each pesticide. The software Xcalibur (version 2.0.7, 2007, Thermo Scientific), together with Mass Frontier (version 1.0, 1998) and NIST library, were used to evaluate the ion products. The MS/MS transitions were optimized for each pesticide (Table 1); the excitation energy ranged from 0.8 to 2.05 eV.

Validation studies and matrix effect in the SPM fraction

The validation procedure for the quantification of pesticides in the SPM fraction followed the European guidance document on pesticide residue analytical methods (EU 2010), which enfolds the international accepted criterions (ICH 2006; Thompson et al. 2002). For validation purposes, an average amount (45.5 ± 0.1 mg) of the standard reference material (SRM 1941b; Organics in Marine Sediments), certified by the National Institute of Standards and Technology (NIST, Maryland), was spiked with the pesticide standards and the respective IS, at eight nominal concentrations (ranging from 10 to 297 ng/L) to evaluate the linearity.

The method detection limits (MDLs) and method quantification limits (MQLs) were calculated, based on the calibration curves of each pesticide, as follows: MDL = 3.3 α/S and MQL = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves (Table 2).

The precision, accuracy, and recoveries were evaluated by analyzing at three consecutive days, independent triplicates of each quality control (QC) concentration level: QC_{low} (29 ng/L), QC_{medium} (143 ng/L), and QC_{high} (267 ng/L), for all pesticides and IS (158 ng/L; Table 3; ANVISA 2003).

Table 1 Quantification and diagnostic ions used in GC-MS and GC-MS/MS analysis for both DAP and SPM fraction

Pesticides	GC-MS							GC-MS/MS			
	Molecular mass	RT	Target ion	Q1	Q2	Q3	Q4	Precursor	Products	EV	Ranges
Pentachlorobenzene (PCB)	250.3	9.55	250	248 (65.1)	252 (66.1)			250 → 215	144	2.01	143–251
Trifluralin	335.3	10.90	264	306 (44.9)	206 (27.2)			264 → 206	160 188 171	1.05	159–265
Atrazine-desethyl	187.6	10.93	172	68 (32.2)	174 (29.2)			172 → 130	145 152	1.15	104–173
Propazine	229.7	11.66	214	172 (70.6)	187 (38.0)			214 → 200	172 138	1.20	137–215
Hexachlorobenzene (HCB)[b]	284.8	11.68	284	282 (46.4)	249 (41.2)			284 → 214	249	1.50	211–285
Dimethoate	229.3	11.77	87	93 (52.5)	125 (44.3)			87 → 86	59	1.10	53–88
Simazine[a]	201.6	11.88	201	186 (67.1)	173 (51.9)	44 (37.6)		201 → 186	174 138	1.20	135–201
ATZ-d_5	220.7	11.95	205	220 (43.0)	178 (41.7)			205 → 127	137 105	1.25	104–206
Atrazine[b]	215.7	12.00	200	215 (56.8)	173 (36.9)			200 → 122	132 164 158	1.25	121–201
BHC (gamma) (Lindane)[a]	290.8	12.33	181	183 (76.6)	219 (69.3)			181 → 145	146	1.20	108–184
Terbuthylazine	229.7	12.41	214	173 (71.8)	138 (27.3)	229 (24.3)		214 → 173	132	1.40	131–215
Propyzamide	256.1	12.45	173	175 (41.2)	254 (35.6)			173 → 138	145	1.10	130–174
Diazinon	304.4	12.46	137	179 (44.7)	304 (10.2)			179 → 121	163 137 122	1.35	110–180
Fonofos	246.3	12.51	137	109 (70.7)	246 (40.0)			137 → 109	81	0.85	80–138
Pirimicar[b]	238.4	13.10	166	238 (29.0)	72 (18.8)			166 → 96	137 121	1.35	95–167
Parathion-methyl	263.2	13.98	263	109 (67.9)	79 (44.7)	246 (43.5)		263 → 246	153	1.31	150–264
Alachlor[a]	269.8	14.01	188	160 (86.8)	146 (58.8)			160 → 132	130	1.10	116–161
Simetryn	213.3	14.10	213	170 (22.9)	155 (13.4)			213 → 170	185	1.10	151–214
Heptachlor[b]	373.3	14.25	272	274 (73.9)	270 (63.3)	100 (43.9)		272 → 237	235	1.05	236–275
Metribuzin	214.3	14.43	198	199 (29.8)				198 → 150	110	1.10	109–199
Terbutryn	241.4	14.67	185	226 (68.0)	170 (45.2)			185 → 170	128	0.90	127–186
Fenitrothion	277.2	14.71	260	109 (83.4)	125 (77.4)	277 (41.8)		260 → 228	217 232	1.20	160–261
Malathion	330.4	14.95	125	127 (90.9)	99 (73.2)	173 (40.2)		173 → 99	117 145	0.80	92–173
Metolachlor	283.8	15.12	162	238 (36.7)	163 (15.1)			162 → 132	133	1.15	115–163
Chlorpyriphos	350.6	15.19	314	316 (72.3)	258 (67.3)	199 (46.8)	197 (41.4)	314 → 258	286	0.90	257–315
Cyanazine	240.7	15.26	225	212 (59.4)	198 (35.2)	68 (32.8)		225 → 189	172 198	1.28	171–226
Aldrin[a]	364.9	15.35	263	261 (92.7)	265 (65.6)	66 (57.2)		263 → 193	191 227	1.60	190–264
Parathion-ethyl	291.3	15.38	291	109 (79.0)	263 (61.0)	97 (57.2)	141 (47.0)	109 → 81	91	0.99	60–110
Pendimethalin	281.3	16.27	252	162 (61.3)	191 (28.9)			252 → 162	191	1.00	160–253
Chlorfenvinphos Z	359.6	16.53	267	269 (52.9)	323 (51.5)			267 → 159	203	1.50	158–268
Heptachlor epoxide[b]	389.3	16.53	353	355 (66.0)	351 (44.4)	81 (25.7)		353 → 263	282	1.10	262–354
Procymidone	284.1	16.86	96	283 (85.3)	285 (29.0)			96 → 67	68	1.00	64–97
Chlordane (gamma)	338.9	17.28	375	373 (93.8)	377 (59.9)			373 → 266	264	1.20	263–374
Tetrachlorvinphos	366.0	17.40	329	331 (90.4)	109 (51.3)	333 (32.8)		329 → 314	278	1.30	219–330
Endosulfan (beta)	406.9	17.70	241	195 (72.7)	243 (71.2)	207 (54.0)		241 → 206	204 170	1.45	165–242
Fenamiphos	303.4	17.87	303	243 (62.4)	217 (54.9)	288 (42.9)	154 (40.6)	303 → 268	266	1.10	175–304
4,4′-DDE	318.0	18.40	246	248 (58.6)	318 (31.9)	316 (29.3)		246 → 176	175	1.70	174–247
Dieldrin[a]	380.9	18.60	79	263 (93.1)	237 (43.5)			79 → 51	50	1.10	49–80
Endosulfan (alpha)	406.9	18.60	241	195 (78.2)	237 (70.8)	243 (65.5)		241 → 206	205	1.45	165–242
Endrin[b]	380.9	18.61	243	263 (99.0)	281 (68.4)	81 (47.4)		243 → 207	173	1.15	172–244
4,4′-DDD	320.0	19.84	235	237 (64.2)	165 (61.7)			235 → 165	199	1.15	162–236
Endosulfan sulfate	422.9	20.95	272	237 (68.0)	274 (60.5)	387 (47.9)		272 → 237	235	1.10	234–273
DDT-d_8	362.5	21.03	220	243 (62.6)	280 (57.8)			243 → 173	206	1.15	172–244
4,4′-DDT	354.5	21.11	235	237 (64.2)	212 (59.0)	165 (44.0)		235 → 165	199	1.15	117–236
Methoxychlor[b]	345.7	23.08	227	228 (16.5)	274 (15.4)			227 → 169	181	1.30	140–228
Azinphos-methyl	317.3	23.85	77	132 (88.0)	104 (43.4)	160 (32.9)		77 → 51	50	1.30	49–78
Tebuconazole	307.8	24.44	250	125 (80.6)	163 (44.4)			125 → 89	99	1.60	62–126
Cyhalofop-butyl	357.4	24.44	256	357 (72.6)	229 (41.1)	120 (31.0)		256 → 228	200	1.13	199–257
Mirex	545.5	24.71	272	274 (73.3)	237 (62.7)			272 → 237	235	1.13	234–273
Cyhalothrin (lambda)[c]	449.9	24.73	181	141 (45.8)	197 (42.1)			181 → 152	151	1.50	120–182
Cyfluthrine (beta)[c]	434.3	27.71	206	199 (76.9)	91 (70.9)	226 (55.0)	227 (42.2)	199 → 193	191 163	1.80	190–200
Cypermethrin (alpha)*	416.3	28.24	181	91 (76.3)	163 (75.0)	165 (47.3)		181 → 152	151	1.70	150–153/179–182
Difenoconazol	406.3	31.25	265	267 (89.9)	323 (68.5)	325 (62.2)		323 → 265	249	1.35	245–266/321–324
Deltamethrinc	505.2	32.00	181	207 (61.4)	253 (58.7)			181 → 152	151	1.70	150–153/179–182

The relative abundance of ions (m/z) for each target pesticide is indicated between brackets

Internal standards; [a] compounds present in the mix A (EPA 505/525); [b] compounds present in the mix B (EPA 505/525); [c] contain several diastereoisomers

Data analyses

Analyses were done considering the average values of all replicates (n = 3) per compound. Data are given as mean values (standard error (SE)), on Table 4, and as total loads (Σ) of pesticides/category (SE), on Fig. 3. The last approach was done to characterize the impact of each category (fungicides, herbicides, and insecticides) in the estuary.

Statistics were performed with STATISTICA 8 (StatSoft 2007). All data was tested for normality and homogeneity of variances using the Shapiro–Wilk's W test and Levine's tests, respectively. Independent comparisons, between sites, seasons, and categorical groups were achieved by one-way analysis of variance (ANOVA), using the post hoc Tukey's test. Non-parametric test (Kruskal–Wallis ANOVA) was also applied when data normalization failed. To reject the null hypothesis, we adopted the standard significance level of 5 %.

It was also calculated the groundwater ubiquity score (GUS) index (Table 2—supplementary data), as an indicator of the potential pollution by pesticides, considering their persistence and binding ability to soil particles: GUS = log10 (half-life days) × [4- log10 (K_{oc})]). The GUS score range from extremely low (<0.1) to very high (>4.0), rating the leaching potential of pesticides of moving toward to groundwaters (Gustafson 1989) as it was used on Claver et al. (2006).

Additionally, a way to infer the impact of these compounds into the aquatic environment is to analyze data according to the distribution of the pesticide between the suspended solids and water fraction (K_d, sorption coefficient; Dueri et al. 2008). This ratio can give us a rough perception of what can directly affect the benthic communities, since the particulate matter works as a contaminant transport to the aquatic environment (Bilotta et al. 2012). Thus, all available data from other aquatic systems was transformed (K_d ratio) and compared with the Ria Formosa Lagoon results (2012/2013; Table 6).

Finally, it was calculated the SPM data expressed in μg/g, dividing by the particulate matter (g) weighted in each sample (156.9 mg ± 0.1 (SE); supplementary material).

Table 2 Calibration parameters of the SPM method, including the calibration curves equation, the correlation coefficients (r), and the method detection and quantification limits (MDLs and MQLs)

Pesticide	Linearity parameters			MDLs ng/L	MQLs ng/L
	Regression equation		*r*		
PCB	Y=0.0120X	−0.73	0.98	1.04	3.14
Trifluralin	Y=0.0210X	−0.14	0.99	2.19	6.63
Atrazine-desethyl	Y=0.0019X	−0.09	0.99	0.85	2.56
Propazine	Y=0.0019X	−0.05	0.99	0.32	0.96
HCB	Y=0.0027X	−0.06	0.99	0.83	2.52
Dimethoate	Y=0.0014X	+0.11	0.99	0.68	2.06
Simazine	Y=0.0011X	−0.04	0.99	0.87	2.65
Atrazine	Y=0.0021X	−0.11	0.99	0.63	1.89
Lindane	Y=0.0021X	+0.07	0.99	0.90	2.72
Terbuthylazine	Y=0.0018X	+0.06	0.99	1.81	5.49
Propyzamide	Y=0.0045X	−0.15	0.99	0.91	2.76
Diazinon	Y=0.0025X	+0.06	0.99	0.55	1.68
Fonofos	Y=0.0069X	−0.00	0.99	0.60	1.83
Pirimicarb	Y=0.0064X	−0.36	0.99	0.69	2.08
Parathion-methyl	Y=0.0032X	+0.09	0.99	0.71	2.14
Alachlor	Y=0.0019X	+0.08	0.99	0.85	2.56
Simetryn	Y=0.0011X	−0.00	0.99	0.94	2.83
Heptachlor	Y=0.0001X	+0.01	0.98	1.25	3.80
Metribuzin	Y=0.0023X	−0.10	0.99	1.17	3.55
Terbutryn	Y=0.0032X	−0.01	0.99	0.42	1.26
Fenitrothion	Y=0.0050X	+1.49	1.00	0.69	2.08
Malathion	Y=0.0008X	+0.01	0.98	0.78	2.37
Metolachlor	Y=0.0299X	−0.84	0.99	0.66	1.99
Chlorpyriphos	Y=0.0235X	−0.44	0.99	0.55	1.67
Cyanazine	Y=0.0017X	−0.07	0.99	0.56	1.70
Aldrin	Y=0.0015X	+0.05	0.99	0.40	1.22
Parathion-ethyl	Y=0.0046X	−0.26	0.99	0.73	2.21
Pendimethalin	Y=0.0030X	+0.28	0.99	0.60	1.82
Chlorfenvinphos Z	Y=0.0188X	+0.07	0.99	0.80	2.43
Heptachlor epoxide	Y=0.0075X	+0.17	0.99	1.37	4.15
Procymidone	Y=0.2624X	−3.85	1.00	2.06	6.25
Chlordane (gamma)	Y=0.0229X	+0.04	1.00	1.68	5.10
Tetrachlorvinphos	Y=0.0113X	+0.74	0.99	0.56	1.70
Endosulfan (beta)	Y=0.0024X	−0.03	0.99	0.42	1.26
Fenamiphos	Y=0.0063X	−0.15	0.99	1.87	5.67
4,4′-DDE	Y=0.1133X	−0.24	0.99	0.93	2.82
Dieldrin	Y=0.0294X	+4.23	0.98	1.05	3.18
Endosulfan (alpha)	Y=0.0015X	+0.03	0.99	0.47	1.42
Endrin	Y=0.0013X	+0.07	0.99	0.21	0.63
4,4′-DDD	Y=0.0534X	+2.37	0.99	0.83	2.52
Endosulfan sulfate	Y=0.0081X	−0.38	0.99	0.85	2.58
4,4′-DDT	Y=0.1243X	−1.36	1.00	1.53	4.65
Methoxychlor	Y = 0.0037X	+ 1.69	1.00	0.99	3.00
Azinphos-methyl	Y = 0.0029X	+ 0.06	0.99	0.11	0.33
Tebuconazole	Y = 0.0076X	+ 0.07	0.99	0.62	1.89
Cyhalofop-butyl	Y = 0.0005X	+ 0.07	0.98	0.19	0.58
Mirex	Y = 0.0125X	− 0.05	0.99	0.50	1.51
Cyhalothrin (lambda)	Y = 0.0306X	− 0.17	0.99	0.76	2.29
Cyfluthrine (beta)	Y = 0.0165X	− 0.03	1.00	0.70	2.11
Cypermethrin (alpha)	Y = 0.0256X	+ 0.76	1.00	0.77	2.32
Difenoconazol	Y = 0.0110X	+ 0.43	0.99	0.46	1.39
Deltamethrin	Y = 0.0076X	+ 0.37	0.99	0.32	0.98

r correlation coefficients, **MDL** method detection limits, **MQL** method quantification limits

Results

Extraction and validation of the analytical method (USE-GC-MS) for SPM samples

Fifty-two pesticides were successfully extracted (Table 3), and only four compounds did not accomplish the linearity (hexachlorocyclopentadiene and dichlorvos), accuracy, and precision (phosmet and azoxystrobin) minimum requirements of the European guidance document on pesticide residue analytical methods.

The selectivity of the optimized GC-MS/MS method was shown by comparing of the retention times and mass spectra of the selected pesticides, at QC concentration levels, in solvent standards and in spiked matrices (estuarine SPM and SRM 1941b; RSD <5 %). All pesticides presented linear calibration curves for the established concentration ranges (10 to 297 ng/L), attaining correlation coefficients above 0.98 %. The MDL values were lower than 2.2 ng/L and MQL ranged from 0.3 to 6.6 ng/L (Table 2).

Precision (% RSD) and accuracy (intra-batch and interbatch) were calculated for the three QC levels that ranged from 2.8 to 15.0 % and 86.7 to 113.9 %, respectively (Table 3).

To evaluate the stability, the QC samples were stored at −20 °C for 24 and 48 h; it was not observed any significant effects on the quantification of the selected pesticides (RSD <10 %).

Pesticides in water samples from Ria Formosa Lagoon

All the results (3 sites × 6 DAP fraction and 6 SPM fraction) are shown on Table 4, organized by seasons, grouped according to the pesticide categories (fungicides, herbicides, and insecticides) and characterized by the European legislation as approved, not approved, and banned pesticides. The total loads (sum of all pesticides/category/season) are represented on Fig. 3, for both fractions.

Evaluation of pesticides in the DAP fraction

In this fraction, 85.7 % of the analyzed pesticides were detected and quantified, being their annual average total loads (Σ of all compounds) of ≈1.8 μg/L, ranging from 2.4 μg/L (autumn) to 1.1 μg/L (spring; Fig. 3a).

Considering the fungicides, their annual average total loads reached ≈ 321 ng/L and only procymidone was not detected. Individually, pentachlorobenzene (PCB, ≈301 ng/L) stand out by having higher amounts in autumn and winter than in the other seasons ($p < 0.05$).

All herbicides (14 compounds) were detected and quantified; summed, their annual average total loads reached ≈133 ng/L, and no significant differences were observed between seasons. For cyhalofop-butyl (≈82 ng/L; $p < 0.05$) and pendimethalin (≈20 ng/L), the highest values were obtained in winter.

From a total of 35 insecticides, only six (chlorpyriphos, cyfluthrin (beta), cypermethrin (alpha), fenamiphos, heptachlor, and lindane) were not detected. Moreover, the concentrations of 4,4′-DDT residues (≈116 ng/L) and hexachlorocyclopentadiene (HCCP; ≈305 ng/L) were higher in winter than in all other seasons ($p < 0.05$).

Evaluation of pesticides in the SPM fraction

A total of 31 pesticides were quantified in the SPM fraction. The annual average total loads for fungicides, herbicides, and insecticides were ≈87, ≈190, and ≈292 ng/L, respectively.

Table 3 Average recovery, precision (RSD), and accuracy data for the 52 pesticides assayed of the SPM fraction at three QC levels, for three independent analyses

Pesticides	QC	Recovery		RSD		Accuracy	
	µg/L	(%)	SD	(%)	SD	(%)	SD
PCB	0.03	102.1	± 18.9	7.5	± 4.7	104.5	± 5.9
	0.14	103.6	± 14.1	7.9	± 1.0	106.0	± 11.4
	0.27	94.5	± 5.2	5.4	± 2.9	97.0	± 6.8
Trifluralin	0.03	90.3	± 16.3	10.2	± 5.7	94.2	± 12.4
	0.14	94.1	± 14.9	15.0	± 7.7	96.8	± 8.8
	0.27	100.4	± 14.8	14.2	± 16.5	101.9	± 16.0
Atrazine-desethyl	0.03	110.3	± 7.4	9.1	± 3.8	103.8	± 9.9
	0.14	112.5	± 10.8	9.4	± 4.5	93.6	± 8.8
	0.27	105.8	± 13.5	8.2	± 3.2	98.8	± 12.8
Propazine	0.03	95.1	± 9.5	7.1	± 6.3	110.3	± 12.0
	0.14	91.1	± 12.2	9.3	± 3.9	110.1	± 13.8
	0.27	102.6	± 9.1	8.6	± 4.0	106.6	± 13.6
HCB	0.03	103.0	± 11.1	9.2	± 5.2	107.3	± 8.8
	0.14	88.7	± 12.5	10.6	± 10.4	102.1	± 13.2
	0.27	98.4	± 9.1	6.3	± 3.9	100.6	± 12.6
Dimethoate	0.03	101.8	± 9.7	8.8	± 3.6	109.0	± 10.0
	0.14	98.1	± 16.9	7.0	± 2.7	96.5	± 15.6
	0.27	91.1	± 5.3	7.5	± 3.6	95.0	± 9.2
Simazine	0.03	99.1	± 14.3	7.5	± 2.9	102.3	± 13.0
	0.14	104.9	± 14.5	7.3	± 5.0	101.7	± 12.3
	0.27	104.0	± 7.9	5.6	± 4.2	92.6	± 6.6
Atrazine	0.03	96.8	± 7.6	5.0	± 3.4	95.6	± 9.9
	0.14	110.6	± 8.8	7.2	± 4.6	110.8	± 11.3
	0.27	99.4	± 8.2	8.3	± 4.6	100.1	± 11.2
Lindane	0.03	106.0	± 16.9	8.3	± 3.3	105.5	± 17.0
	0.14	102.3	± 14.7	6.0	± 2.6	109.1	± 10.6
	0.27	114.7	± 6.2	4.9	± 3.5	100.0	± 14.0
Terbuthylazine	0.03	102.2	± 15.7	10.4	± 3.0	99.5	± 15.5
	0.14	105.8	± 11.3	5.7	± 3.5	102.1	± 12.0
	0.27	95.1	± 19.6	5.8	± 5.3	94.8	± 16.5
Propyzamide	0.03	111.9	± 7.1	10.2	± 3.0	106.7	± 13.8
	0.14	92.4	± 13.8	7.0	± 3.7	102.4	± 11.5
	0.27	104.2	± 11.9	7.8	± 4.8	103.2	± 15.5
Diazinon	0.03	112.0	± 10.4	4.7	± 3.7	101.8	± 13.3
	0.14	98.4	± 11.1	7.4	± 3.9	98.3	± 16.5
	0.27	96.7	± 9.8	6.3	± 5.2	98.0	± 11.1
Fonofos	0.03	103.5	± 14.4	5.7	± 5.2	100.0	± 9.5
	0.14	94.6	± 8.2	9.8	± 3.4	98.4	± 9.8
	0.27	104.8	± 13.5	7.3	± 4.8	92.1	± 7.7
Pirimicarb	0.03	102.1	± 7.9	9.1	± 4.4	100.9	± 9.1
	0.14	98.4	± 15.5	7.9	± 4.4	101.6	± 14.2
	0.27	92.4	± 8.8	8.2	± 3.4	94.5	± 6.2
Parathion-methyl	0.03	104.0	± 9.7	6.0	± 5.0	107.6	± 8.3
	0.14	90.7	± 9.0	8.8	± 3.7	86.7	± 21.2
	0.27	105.6	± 4.6	9.2	± 4.8	99.7	± 12.0
Alachlor	0.03	93.9	± 9.7	4.7	± 3.2	106.6	± 17.3
	0.14	100.2	± 12.4	6.3	± 4.0	108.5	± 16.7
	0.27	101.0	± 9.8	6.2	± 4.6	95.3	± 11.6
Simetryn	0.03	101.7	± 14.2	6.4	± 5.7	100.1	± 11.5
	0.14	106.0	± 22.3	7.8	± 4.5	97.4	± 12.6
	0.27	110.1	± 7.5	7.7	± 4.2	104.4	± 14.4
Heptachlor	0.03	103.6	± 13.1	11.1	± 3.5	102.8	± 15.3
	0.14	104.9	± 10.6	7.9	± 3.6	98.2	± 12.9
Metribuzin	0.03	100.4	± 10.5	6.1	± 4.3	104.8	± 17.0
	0.14	98.4	± 12.8	8.7	± 1.4	107.6	± 14.8
	0.27	87.7	± 7.4	6.2	± 3.2	97.1	± 12.2

Table 3 (continued)

Pesticides	QC µg/L	Recovery (%)	Recovery SD	RSD (%)	RSD SD	Accuracy (%)	Accuracy SD
Terbutryn	0.03	94.7	7.0	10.6	4.5	101.4	13.8
	0.14	95.4	6.9	7.4	3.6	99.7	14.1
	0.27	108.9	13.5	7.5	3.7	98.6	9.8
Fenitrothion	0.03	100.8	3.8	6.6	4.2	107.2	11.1
	0.14	99.7	13.2	10.6	2.8	101.6	15.6
	0.27	102.8	10.2	8.5	4.9	102.7	7.3
Malathion	0.03	102.3	10.1	7.1	3.7	107.5	15.2
	0.14	93.9	8.0	5.7	4.5	92.0	11.8
	0.27	96.7	10.7	9.4	5.1	98.8	9.1
Metolachlor	0.03	105.5	6.2	3.9	3.1	113.1	3.9
	0.14	93.2	13.2	7.3	6.3	99.5	14.6
	0.27	85.4	11.9	8.2	5.1	102.8	9.1
Chlorpyriphos	0.03	104.3	7.6	6.5	3.8	108.9	7.6
	0.14	99.0	10.4	10.7	6.3	99.7	10.6
	0.27	108.3	7.7	10.0	3.5	94.5	8.6
Cyanazine	0.03	101.4	9.1	7.6	4.7	107.3	13.3
	0.14	100.4	13.2	8.6	7.2	101.4	13.6
	0.27	95.3	12.2	7.5	4.2	103.9	13.7
Aldrin	0.03	116.0	4.3	9.1	4.0	101.7	15.0
	0.14	87.1	11.3	8.6	5.7	99.2	12.2
	0.27	112.2	6.7	9.9	4.6	97.3	7.2
Parathion-ethyl	0.03	106.4	6.3	2.8	2.1	100.2	10.4
	0.14	97.7	14.1	7.3	2.5	103.5	11.2
	0.27	100.6	12.3	9.4	2.7	95.1	16.8
Pendimethalin	0.03	109.1	16.0	4.1	1.9	113.9	3.3
	0.14	107.6	11.0	5.4	4.0	104.9	18.0
	0.27	104.7	9.8	7.9	6.9	103.3	17.9
Chlorfenvinphos Z	0.03	110.2	11.7	7.6	4.1	101.0	12.3
	0.14	93.9	17.2	9.4	3.5	103.8	10.4
	0.27	107.8	7.7	5.4	5.3	90.6	6.1
Heptachlor epoxide	0.03	102.7	17.4	10.0	4.3	102.9	13.6
	0.14	99.7	16.1	7.2	5.0	104.5	12.0
	0.27	112.2	7.9	8.3	4.9	96.1	6.2
Procymidone	0.03	89.2	12.1	5.3	4.1	99.4	5.7
	0.14	103.6	11.8	6.3	4.5	102.9	9.7
	0.27	103.3	12.7	5.2	3.9	99.6	14.2
Chlordane (gamma)	0.03	100.1	6.8	6.4	2.2	102.0	13.3
	0.14	112.1	9.7	8.0	3.1	103.2	12.0
	0.27	101.4	12.7	7.1	3.2	95.7	12.2
Tetrachlorvinphos	0.03	103.9	6.6	11.0	2.5	109.4	6.8
	0.14	93.3	15.2	7.6	3.9	103.0	12.6
	0.27	102.8	16.2	9.4	5.2	98.8	14.7
Endosulfan (beta)	0.03	91.9	6.3	5.3	4.1	106.6	10.8
	0.14	99.6	12.6	8.1	5.4	109.6	8.6
	0.27	98.6	13.6	9.1	5.8	98.3	12.1
Fenamiphos	0.03	103.5	8.0	8.0	3.0	103.0	12.2
	0.14	101.4	12.0	8.0	3.8	98.4	9.0
	0.27	101.1	13.4	6.8	4.3	98.6	15.3
4,4′-DDE	0.03	105.3	17.5	5.0	4.0	100.4	3.9
	0.14	101.3	13.3	8.1	2.6	101.4	14.9
	0.27	99.9	10.5	6.9	5.0	103.5	7.5
Dieldrin	0.03	93.0	3.1	6.7	6.1	100.4	9.9
	0.14	103.8	10.8	8.8	3.0	109.9	20.8
	0.27	107.9	9.8	10.8	3.0	104.0	13.0
Endosulfan (alfa)	0.03	97.4	7.3	10.3	3.8	99.6	6.3
	0.14	113.7	12.1	5.3	3.6	94.0	7.2
	0.27	96.9	11.1	8.4	4.3	93.4	10.1
Endrin	0.03	109.2	13.6	10.9	4.3	93.1	10.6
	0.14	108.8	10.1	7.1	4.8	99.1	7.6
	0.27	99.5	13.5	5.7	3.5	92.7	11.2

Table 3 (continued)

Pesticides	QC µg/L	Recovery (%)	SD	RSD (%)	SD	Accuracy (%)	SD
4,4´-DDD	0.03	95.7	14.8	6.7	4.4	98.4	12.3
	0.14	105.3	12.5	9.9	3.7	105.9	11.7
	0.27	99.6	11.3	9.7	3.6	87.4	16.9
Endosulfan sulfate	0.03	102.8	5.1	9.4	4.3	102.7	12.9
	0.14	112.5	8.1	7.4	3.5	102.6	10.7
	0.27	89.8	6.9	9.6	3.0	97.2	8.3
4,4´- DDT	0.03	104.4	14.6	7.2	3.6	109.6	14.7
	0.14	105.2	6.0	6.5	3.1	97.9	7.7
	0.27	98.3	6.8	9.0	5.1	99.8	15.1
Methoxychlor	0.03	95.1	9.4	8.0	2.6	99.0	6.4
	0.14	102.2	10.8	7.9	4.5	92.1	4.0
	0.27	105.4	10.6	7.3	3.0	107.9	6.5
Azinphos-methyl	0.03	106.8	9.6	3.3	2.8	99.6	11.3
	0.14	100.8	11.3	9.4	4.8	92.4	9.1
	0.27	108.6	5.4	7.2	4.5	98.7	8.0
Tebuconazole	0.03	95.1	9.4	8.0	2.6	99.0	6.4
	0.14	102.2	10.8	7.9	4.5	92.1	4.0
	0.27	105.4	10.6	7.3	3.0	107.9	6.5
Cyhalofop-butyl	0.03	108.9	9.5	13.3	1.7	107.6	6.9
	0.14	99.6	12.6	7.8	4.4	106.8	11.9
	0.27	84.7	8.7	6.8	3.8	106.2	8.1
Mirex	0.03	96.3	11.0	8.2	4.5	101.3	14.9
	0.14	110.6	7.6	8.2	3.2	101.5	13.5
	0.27	108.8	11.7	6.6	5.4	100.2	11.9
Cyhalothrin (lamdba)	0.03	109.2	9.1	7.5	6.9	103.3	15.5
	0.14	106.7	8.0	6.3	3.6	105.5	11.8
	0.27	102.8	7.9	10.4	4.8	102.1	14.3
Cyfluthrine (beta)	0.03	101.4	9.2	10.4	4.1	111.5	8.7
	0.14	105.1	11.6	6.0	2.0	93.6	11.3
	0.27	107.4	6.7	6.6	4.4	98.5	10.6
Cypermethrin (alpha)	0.03	105.0	7.1	11.0	6.4	103.4	9.8
	0.14	100.4	9.8	6.6	4.3	97.3	10.5
	0.27	100.2	9.9	5.5	3.4	96.3	10.7
Difenoconazol	0.03	111.6	4.6	5.2	2.4	107.8	9.1
	0.14	99.5	9.8	7.6	4.9	103.2	11.2
	0.27	112.8	7.4	7.0	3.3	102.3	8.6
Deltamethrin	0.03	91.5	8.5	8.8	2.7	103.1	15.7
	0.14	105.8	7.1	8.5	6.5	105.4	6.7
	0.27	98.3	7.9	9.4	2.7	93.9	14.6

Recoveries (%) obtained for the three quality controls (QCs) for three independent replicates and days; **precision**: relative standard deviation (RSD); **accuracy** (%); **SD** standard deviation between replicates (3)

Table 4 Environmental levels (ng/L) of the selected pesticides, for both fractions (DAP and SPM) in the Ria Formosa Lagoon, per season

Pesticides	Frequency	MDL	MQL	DAP (ng/L)				Frequency	MDL	MQL	SPM (ng/L)			
	%	% above		autumn	winter	spring	summer	%	% above		autumn	winter	spring	summer
Fungicide														
Azoxystrobin	100	100	100	154.6 ± 0.1	49.4 ± 0.20	46 ± 0.00	49.5 ± 0.00	X	-	-	-	-	-	-
Difenoconazol	100	100	100	93.2 ± 0.1	90.7 ± 0.10	67 ± 0.00	58.7 ± 0.10	100	100	100	37.7 ± 16.3	41.7 ± 23.4	14.3 ± 4.8	11.3 ± 2.7
HCB	25	100	100	6.1 ± 0	-	-	-	*ND*	-	-	-	-	-	-
PCB	92	100	100	186.8 ± 0	301 ± 0.00	11.4 ± 0.00	10.9 ± 0.00	100	100	100	8.3 ± 0.5	8.4 ± 0.2	12 ± 1.8	7.3 ± 0.6
Procymidone	*ND*	-	-	-	-	-	-	17	100	50	9.4 ± 0	-	-	-
Tebuconazole	100	100	100	30.9 ± 0	64.2 ± 0.00	46.6 ± 0.00	15.6 ± 0.00	92	100	100	57.8 ± 10.7	39 ± 15.8	72.5 ± 10.6	27.4 ± 0.4
Herbicide														
Alachlor	58	100	100	7.4 ± 0.1	-	12.3 ± 0.00	9.7 ± 0.00	58	100	100	51.6 ± 18.4	42.9 ± 14.6	21.6 ± 3.6	11.7 ± 0
Atrazine	50	100	83	3.7 ± 0	1.7 ± 0.00	-	-	17	100	100	-		7.9 ± 0	-
Atrazine-desethyl	100	100	100	9.8 ± 0	8.3 ± 0.00	12.5 ± 0.00	10.5 ± 0.00	*100*	8	0	-	-	-	-
Cyanazine	92	100	100	19.5	6.8 ± 0.00	9 ± 0.00	8.8 ± 0.00	*ND*	-	-	-	-	-	-
Cyhalofop-butyl	100	100	100	51.9 ± 0.7	81.6 ± 0.70	10 ± 0.00	9.4 ± 0.00	100	100	100	2.7 ± 0.8	3.5 ± 0.6	4.4 ± 0.7	2.7 ± 0.8
Metolachlor	25	100	33	-	1.3	-	-	33	100	100	12.4 ± 1.3	16.5	10.6	-
Metribuzin	8	100	100	-	1.4	-	-	17	100	50	-	-	8.7 ± 0	-
Pendimethalin	100	100	100	16.3 ± 0	20.4 ± 0.00	9.3 ± 0.00	8.4 ± 0.00	25	100	100	40.4	76.7	32.7	-
Propyzamide	67	100	100	7.7 ± 0	9 ± 0.00	-	6.5 ± 0.00	*ND*	-	-	-	-	-	-
Simazine	100	100	100	12.1 ± 0	11.1 ± 0.00	12.2 ± 0.00	13.3 ± 0.00	100	100	100	14 ± 2.5	13.5 ± 1.5	25 ± 4.5	14.4 ± 0.1
Simetryn	100	100	100	7.7 ± 0	3.8 ± 0.00	6.7 ± 0.00	6.3 ± 0.00	*ND*	-	-	-	-	-	-
Terbuthylazine	83	100	100	5.5 ± 0	2.4 ± 0.00	8.4 ± 0.00	22.1 ± 0.00	*ND*	-	-	-	-	-	-
Terbutryn	92	100	100	12.5 ± 0	5.9 ± 0.00	12.4 ± 0.00	11.7 ± 0.00	*ND*	-	-	-	-	-	-
Trifluralin	75	100	100	1.7 ± 0	9.3 ± 0.00	6.4 ± 0.00	6.6 ± 0.00	100	100	100	117 ± 33.9	97.7 ± 20.6	105.2 ± 13.5	27.9 ± 5.6
Insecticide														
Azinphos-methyl	92	100	100	59.5 ± 0	24 ± 0.00	75.5 ± 0.00	70.7 ± 0.00	67	100	100	127.3 ± 29.3	53.1 ± 25.4	62.9 ± 6.1	52.1 ± 0
Chlorfenvinphos Z	100	100	100	47.2 ± 0.1	97.5 ± 0.10	29.7 ± 0.10	10.9 ± 0.00	100	100	100	20.1 ± 2.4	56.1 ± 32.2	19.1 ± 1.9	13.4 ± 0.4
Chlorpyriphos	67	100	100	15.6 ± 0	-	22 ± 0.00	19.9 ± 0.00	*ND*	-	-	-	-	-	-
Cyfluthrin (beta)	75	100	78	13.8 ± 0	24.6 ± 0.10	11.4 ± 0.00	5.9	*ND*	-	-	-	-	-	-
Cyhalothrin (lambda)	100	100	92	78.9 ± 0.1	31 ± 0.10	2.6 ± 0.00	7.2 ± 0.00	50	100	100	21.2 ± 0.1	9.3 ± 1.6	10.9 ± 0	3.3 ± 0
Cypermethrin (alpha)	100	100	100	174.2 ± 0	152.3 ± 0.20	126.5 ± 0.00	87 ± 0.00	*ND*	-	-	-	-	-	-
4,4´-DDD	100	100	100	4.5 ± 0	5.5 ± 0.00	3.5 ± 0.00	3.3 ± 0.00	67	100	63	5.4 ± 2.1	2.6 ± 0	4.7 ± 0	-
4,4'-DDE	*ND*							*ND*	-	-	-	-	-	-

Table 4 (continued)

Pesticides	Frequency	MDL	MQL	DAP (ng/L)												Frequency	MDL	MQL	SPM (ng/L)											
	%	% above		autumn			winter			spring			summer			%	% above		autumn			winter			spring			summer		
4,4'-DDT	100	100	100	51.3	±	0.10	116.3	±	0.10	48.6	±	0.10	48	±	0.10	8	100	100		34.8			-			-			-		
Deltamethrin	100	100	100	392.2	±	0.10	222.5	±	0.10	128.8	±	0.00	67.2	±	0.00	58	100	100	59.4	±	5.2	39.5	±	5.9		15.4			-		
Diazinon	100	100	100	139.2	±	0.00	26.8	±	0.00	116.2	±	0.00	97.5	±	0.00	92	100	100	17.5	±	6.1	20.2	±	14.2	19.1	±	7.2	16.3	±	3.9
Dichlorvos	50	100	100		-		29.5	±	0.00	13.1	±	0.00	21.6	±	0.00	X	-	-		-			-			-			-		
Dieldrin	100	100	100	120.1	±	0.00	163.7	±	0.00	14.5	±	0.00	20.2	±	0.00	67	100	100	28.9	±	29.2	52.3	±	36.8	4.9	±	0.9	44.4		
Dimethoate	100	100	100	115.8	±	0.00	7.2	±	0.00	33.1	±	0.00	202.1	±	0.00	92	100	100	10.7	±	1	11.4	±	3.3	15.9	±	9.2	19.2	±	11.1
Endosulfan (alpha)	83	100	100	27.9	±	0.00	27.4	±	0.00	37.4	±	0.00	36.1	±	0.00	8	100	100	7.9	±	0		-			-			-		
Endosulfan (beta)	67	100	88	28.3	±	0.00		-		10.3	±	0.00	10.9	±	0.00	25	100	100	24.2	±	9.7	4.1		-			-			
Endosulfan sulfate	42	100	100	90.5	±	0.10	39.1	±	0.00		-			-		75	100	89	41.5	±	24.4	78.4	±	35.6	8.9	±	0	13.5	±	0.7
Fenamiphos	75	100	100	14.5	±	0.00		-		28.9	±	0.00	22.2	±	0.00	*ND*	-	-		-			-			-			-		
Fenitrothion	8	100	100		-		5.3	±	0.00		-			-		8	100	100		-			-		3	±	0		-	
Fonofos	50	100	100	2.4	±	0.00	3.8	±	0.00	4.6	±	0.00	5.3	±	0.00	100	100	100	2.6	±	0.2	2.9	±	0.2	3	±	0.5	2.4	±	0.2
HCCP	67	100	100	201.4	±	0.00	304.7	±	0.00	42.8	±	0.00		-		X	-	-		-			-			-			-		
Heptachlor	100	100	100	15.6	±	0.00	20.3	±	0.00	16.6	±	0.00	11.2	±	0.00	*ND*	-	-		-			-			-			-		
Lindane	83	100	90	34.2	±	0.00	42.3	±	0.00	16.9	±	0.00	11.8	±	0.00	*ND*	-	-		-			-			-			-		
Malathion	33	100	100	13.1	±	0.00	6	±	0.00		-			-		8	100	100		-		14.2	±	0		-			-		
Mirex	25	100	67		-			-		1.5	±	0.00	1.5	±	0.00	25	100	33		-		4	±	0		-			-		
Parathion-ethyl	100	100	100	60	±	0.00	37.7	±	0.00	11.6	±	0.00	17.4	±	0.00	58	100	100	12.2	±	0.3	14	±	1.1	11.8	±	0		-	
Parathion-methyl	83	100	100	33.5	±	0.00	189.4	±	0.10	33.1	±	0.00	62.9	±	0.00	50	100	50		-		7.4	±	2	13.7	±	0		-	
Phosmet	83	100	100	106.3	±	0.00	40.3	±	0.00	8.3	±	0.00	135.4	±	0.00	X	-	-		-			-			-			-		
Pirimicarb	25	100	100		-		4.2	±	0.00		-			-		*ND*	-	-		-			-			-			-		
Tetrachlorvinphos	67	100	100	16.4	±	0.00	10	±	0.00	13.4	±	0.00	10.7	±	0.00	17	100	100		11.7			14.9			-			-		

Data are presented as mean (SE) (n=3 sites)

ND not detected, **X** pesticide that did not accomplish the requirements during method validation for SPM matrix, **MDL** method detection limit, **MQL** method quantification limit

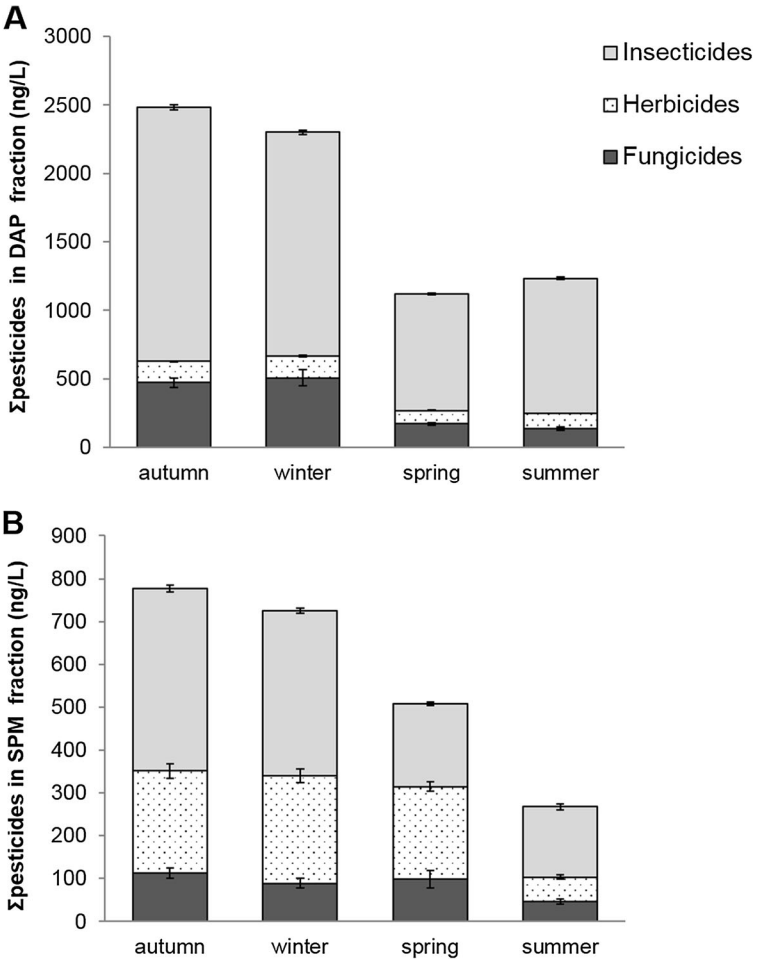

Fig. 3 Total loads of pesticides (Σ ng/L) from DAP (A) and SPM (B) fractions, by categories per season; data is expressed as mean (SE) (n = number of pesticides/ season)

As the Σ of all compounds per season, the SPM had the same pattern as the DAP fraction, where the maximum loads were attained in autumn (≈777 ng/L) and the minimum in summer (≈267 ng/L) (Fig. 3b). Individually, the highest values were found for tebuconazole (≈49.2 ng/L), Trifluralin (≈87.0 ng/L), and for azinphos-methyl (≈74 ng/L).

Table 5 Physicochemical data evaluated per site (S1 to S3)

Physicochemical parameters	Site 1	Site 2	Site 3
Dissolved O_2 (mg L^{-1})	10.92 ± 0.6	10.27 ± 0.4	10.56 ± 1.4
Temperature (°C)	21.12 ± 2.7	16.87 ± 2.0	17.73 ± 2.1
pH	8.50 ± 0.1	8.18 ± 0.0	8.27 ± 0.2
Salinity	27.88 ± 5.8	33.52 ± 0.6	29.43 ± 2.8
Conductivity (mS cm^{-1})	39.87 ± 9.1	43.87 ± 2.5	39.37 ± 3.1
Nitrites (mg L^{-1})	0.04 ± 0.0	0.02 ± 0.0	0.03 ± 0.0
Nitrates (mg L^{-1})	0.56 ± 0.1	0.94 ± 0.1	1.05 ± 0.3
Ammonia (mg L^{-1})	4.07 ± 2.1	0.23 ± 0.0	0.15 ± 0.0
Un-ionized ammonia (mg L^{-1})	0.33 ± 0.1	0.03 ± 0.0	0.01 ± 0.0
Phosphates (mg L^{-1})	0.33 ± 0.1	0.22 ± 0.1	0.18 ± 0.1

Data is expressed as mean (SE) (n =6/site)

Table 6 K_d index values from Ria Formosa Lagoon and other aquatic environments

Pesticides	K_d values	
	Formosa Lagoon	Other aquatic systems
Fungicides		
Difenoconazol	0.34	
HCB		0.62[e]
PCB	0.07	0.71[e]
Tebuconazole	1.25	
Herbicides		
Alachlor	3.26	0.06[a]
Atrazine	2.95	0.63[a]
Cyanazine		1.77[a]
Cyhalofop-butyl	0.09	
Metolachlor	10.18	1.44[a]
Metribuzin	6.11	
Pendimethalin	3.68	
Simazine	1.37	1.32a
Trifluralin	14.53	3.56[a]
Insecticides		
4,4'-DDD	1.00	6.80[c]
4,4'-DDE		3.86[a]/3.87[d]/6.00[c]
4,4'-DDT	0.53	6.37d / 6.75c
Azinphos-methyl	1.29	
Chlorfenvinphos Z	0.59	
Cyhalothrin (lambda)	0.37	
Cypermethrin (alpha)		
Deltamethrin	0.19	
Diazinon	0.19	
Dieldrin	0.41	0.30[c]/1.73[a]
Dimethoate	0.16	
Endosulfan (alpha)	0.24	9.71[c]
Endosulfan (beta)	0.86	1.24[c]
Endosulfan sulfate	0.55	
Fenitrothion	0.57	0.71[f]
Fonofos	0.68	
Heptachlor		0.11[c]
Lindane		0.20[c] / 0.45[d]
Malathion	1.49	1.24[b]
Mirex	2.70	
Parathion-ethyl	0.40	
Parathion-methyl	0.13	
Tetrachlorvinphos	1.06	

[a] Gulf of Mexico (McMillin and Means 1996)
[b] Laguna de Bay (Varca 2012)
[c] Pearl River (Luo et al. 2004)
[d] Quanzhou Bay (Yang et al. 2013)
[e] Yangtse River (Jiang et al. 2000)
[f] Takahamairi Bay (Inoue et al. 2002)

Table 7 Comparison between the total annual values (DAP and SPM fraction; ng/L) obtained, for some pesticides, at Ria Formosa Lagoon with the maximum annual average values (ng/L) established by the European legislations (98/83/EC and 2013/39/ EU)

Annual average value Legislation		This study ng/L	Directive
98/83/EC	PCB	137	100
	HCCP	183	100
	Endosulfan sulfate	100	100
	Dimethoate	104	100
	Difenoconazol	104	100
	Dieldrin	112	100
	Diazinon	113	100
	Deltamethrin	241	100
	Cypermethrin (alpha)	135	100
	Azinphos-methyl	131	100
	4,4'-DDT	101	100
	Σdieldrin and heptachlor*,1	128	50
2013/39/EU	Dieldrin*,2	112	10
	Dichlorvos	21	0.6
	4,4'-DDT	101	10
	Endosulfan (alpha + beta)	71	5
	Heptachlor*,3	16	0.0002
	Trifluralin	93	30
	PCB	137	7

* the sum of the detected pesticides from the following directives:
98/83/EC [1]Σaldrin, dieldrin, heptachlor and heptachlor epoxide
2013/39/EU [2]Σaldrin, dieldrin, endrin and isodrin
[3]Σheptachlor and heptachlor epoxide

Physicochemical parameters

The average annual values of the physicochemical parameters were grouped per sampling site (Tables 5 and 6). With the exception of the levels of ammonia (≈4 mg/L; $p < 0.001$) measured at S1, all other parameters were similar among sampling sites. As to seasonality, only the phosphates showed significant differences between seasons ($p < 0.05$), being higher in spring (0.7 mg/L).

Discussion

From previous studies, it was concluded that the current SPE technique followed by GC-MS analysis was considered suitable for environmental analysis of pesticides in the DAP fraction (Rocha et al. 2011). Also, the presently validated USE method, followed by GC-MS/MS, proved to be an ideal protocol to analyze pesticides from the SPM fraction, following all the validation parameters of the European guidelines (EU 2010). Herein, it is stressed that this procedure presented extremely low MQL levels (below 0.22 ng/L). Besides, some methods for the evaluation of pesticides in the SPM fraction have yet been published, and none of them quantifies such a considerable number of pesticides (Luo et al. 2004; Varca 2012; Yang et al. 2013). In addition, throughout the validation protocol, and later on, during the everyday analysis, real water samples and standard reference material (SRM) were spiked with QC levels, to confirm the robustness of this method by the direct use of complex matrices. The main input of pesticides in the Ria Formosa Lagoon occurred in autumn and winter, in both DAP and SPM fractions (Fig. 3), matching with the raining seasons; considering both fractions as a whole, the cumulative values from all pesticides (Σ3.1 μg/L) surpass the maximum levels (2.5 and 0.5 μg/L) established by the 236/98 Portuguese and the 98/83/EC European directives, respectively (Ministério do Ambiente 1998; EU 1998).

Fungicides

Considering the DAP fraction, the obtained results from 2012/2013 samples were 2-fold lower (Σ≈2.8 μg/L) than those measured in 2010 (Σ≈6.6 μg/L) at the same region (Cruzeiro et al. 2015), probably due to the policies enforced in this region, aiming the reduction of pesticides in aquatic systems (ICN 2005). In spite of this, in 2012/2013, the fungicide PCB was 3.6-fold more concentrated in the DAP fraction (≈128 ng/L) than those measured in 2010. This observation reveals that the banned pesticide was being used, as an active intermediate of other manufacturing pesticides, or as a flame retardant, as suggested by Cabeza et al. (2012). However, few data had been reported regarding the presence of PCB in other aquatic systems (Estévez et al. 2012; Robles-Molina et al. 2014; Wang et al. 2009). Considering the SPM fraction, the values herein obtained are higher than those already published, for the Yangtze River (Jiang et al. 2000). As a whole, the annual concentrations of fungicides were 4-fold lower in 2013 than in 2010 (Cruzeiro et al. 2015). In respect to K_d ratio, comparable average values (0.6) were obtained to what was found in the literature (0.7; Yangtse River; Jiang et al. 2000), indicating similar contamination potency in organisms for both systems—this is quite worrying in view of Formosa ecological grandness.

Herbicides

With respect to the DAP fraction results from the herbicide category, the concentration of cyhalofop-butyl (≈42 ng/L) was noticeably higher than those amounts measured (3.2 ng/L) in 2010 in this aquatic system (Cruzeiro et al. 2015) but lower than the measured amounts in the Rhône River (France; ≈60 ng/L; Comoretto et al. 2007). Since this compound is quickly hydrolyzable (less than 8 days; Comoretto et al. 2007) and has low GUS index (Gustafson 1989) and high log K_{ow}, its high environmental amounts in the DAP fraction may be due to its active use. Pendimethalin was also found in great amounts in both DAP (≈14 ng/L) and SPM (≈50 ng/L) fractions; however, the values are lower than those previously referred in the north of Portugal (≈100–710 ng/L; Gonçalves et al. 2007; Rocha et al. 2011), at Ria Formosa in 2010 (≈370 ng/L; Cruzeiro et al. 2015), at Santa Catarina rivers (Brazil, ≈50–250 ng/L; Freitas et al. 2011) and at Thames River estuary (England, ≈40 ng/L; St. George et al. 2011) for the DAP fraction. As a whole, the data from 2012/2013 was 1.8-fold lower than that from 2010 (Cruzeiro et al. 2015), suggesting that herbicide contamination is decreasing, but more time series are needed. Despite the eventual trend, the herbicides presented the

highest K_d ratio values (5.3) when compared to the other categories; this fact corroborates with their chemical properties (log K_{ow} (3.5) and low GUS index (2.0)). A higher ratio (3.6-fold higher) was also found when comparing to data available elsewhere, *viz.* the Gulf of Mexico (average K_d 1.5; Table 7; McMillin and Means 1996).

Insecticides

Considering the HCCP in DAP fraction, it was observed that its levels (≈183 ng/L) were higher than their precursors ($\Sigma_{\text{dieldrin, endosulfan (alpha and beta) and mirex}} \approx 125$ ng/L). Because HCCP has a rapid hydrolysis process (less than 6 days; EPA 2014) and has low GUS index (0.1), it is hypothesized that this compound was being used in this lagoon as an industrial product (flame retardants, resins, and other; EU 2007). Moreover, 4,4′-DDT residue was measured in both DAP and SPM fractions, in amounts higher than its metabolites, showing a possible current use of this compound. In spite of the fact that DDT was banned from Europe in the 1970s (Barriada Pereira et al. 2005), their metabolites were found recently in Portuguese aquatic systems (Gonçalves et al. 2007; Rocha et al. 2011), in line with the amounts obtained here for 2012/2013.

Considering the SPM fraction of the insecticides, the azinphos-methyl presented great amounts (1.7 μg/kg); however, much higher values (53.2 μg/kg) were found in the Lourens River (Western Cape, Schulz et al. 2001). In opposite, and for endosulfan sulfate, lower levels (0.03 ng/L) were found in Macau (Luo et al. 2004) than in this lagoon (35.6 ng/L). For that insecticide, the registered levels might be explained by the degradation of endosulfan alpha and beta (7.9 and 14.2 ng/L) into their metabolite (endosulfan sulfate); the same pattern was verified for the DAP fraction.

Likewise for the other pesticide categories, the accumulative values were lower in 2012/2013 (about 5-fold) than in 2010 (Cruzeiro et al. 2015), suggesting an improving trend that should be monitored.

Considering the K_d ratio of this category, higher values (3.3) were found in other aquatic systems than in this lagoon (0.7). This demonstrates that other ecosystems, such as Takahamairi Bay, Pearl River, Gulf of Mexico, Laguna de Bay, and Quanzhou bay, have been presenting higher availabilities to contaminate the aquatic biota (Inoue et al. 2002; Luo et al. 2004; McMillin and Means 1996; Varca 2012; Yang et al. 2013).

Legislative limits

Considering the directives 98/83/EC (water intended for human consumption) and 2013/39/EU (annual values for inland and surface waters), some pesticides were above the maximum allowed values (EU 1998, 2013; Table 7). The most concerning pesticides were dichlorvos and heptachlor, which were 35- and 80,000-fold higher than the maximum levels defined by the 2013/39/EU directive, *viz*. 0.7 and 0.3, respectively (EU 2013). Taking into account both directives, most of the pesticides that are above the limits were insecticides (79 %), followed by fungicides (17 %), and then herbicides (5 %), evidencing an abusive use of insecticides above the other categories. Annually and considering both fractions, the quantified loads (2.3 μg/L) were 11-fold higher that the maximum amount (0.5 μg/L) set by the 98/83/EC directive; the same was also verified by season, varying from 3.3 μg/L in autumn to 1.5 μg/L in summer.

Physicochemical data

Normal oxygen levels (above 2 mg/L), pH (between 5 and 9), and total phosphorous (until 1 mg/L) were registered as established by the directive 236/98. Only the total amounts of nitrogen were above the maximum levels established for superficial waters (1 mg/L; Ministério do Ambiente 1998), being 5-fold higher at S1 (close to city of Faro).

The same was verified for the un-ionized ammonia levels (maximum toxic level for fish 0.06 mg/L; Durborow et al. 1997). These levels may be related to the regional agriculture (mainly orchards) and/or leisure sports (*viz*. golf) activities (Postigo et al. 2010), allied with insufficient/inefficient waste water treatment plants (WWTPs; Ferreira et al. 2003).

Conclusions

Both extraction methods (for DAP and SPM fraction) presented suitability for coastal water samples, allowing the quantification (GC-MS) of 86 and 60 % of the pesticides, respectively and the identification by GC- MS/MS. As total loads ($\Sigma_{DAP+SPM}$), the maximum values were obtained during the raining seasons (autumn and winter), which were above the maximum amounts established by the 236/98 and 98/83/EC directives. These higher levels may be correlated with the application of pesticides and/or their leachable properties (low K_{ow} and high GUS index). From 48 quantified pesticides, 31 % (mostly insecticides) exceeded the European directive levels. Some chemicals overcame the limits because of the added SPM fraction; this contributed to an additional 32 % for the total load of pesticides. The attained levels may be explained by the nature of the estuarine samples, presenting high SPM weights (min-max; 38 mg/L–3.6 g/L). In view of the K_d ratio, herbicides were the most dangerous for the local aquatic fauna. At last, we found that both total nitrogen and un-ionized ammonia were at critical levels, in line with the pesticide pollution, backing an ecologically relevant anthropogenic impact in the area, mainly at S1. This study calls for actions from the Ria Formosa regulators, coupled to fine chemical and biological monitoring.

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Supplementary data

Table 1 Environmental levels (μg/g) of the selected pesticides, for SPM fraction at the Ria Formosa Lagoon, per season. Data is presented as mean (SE) (n = 3 sites)

Pesticides	Environmental levels (μg/g)			
	autunm	winter	spring	summer
Fungicides				
Difenoconazol	0.8 ± 0.0	0.9 ± 0.5	0.3 ± 0.5	0.3 ± 0.2
PCB	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.2
Procymidone	0.1 ± 0.0	-	-	-
Tebuconazole	2.0 ± 0.0	0.6 ± 1.2	2.0 ± 0.4	0.5 ± 1.1
Herbicides				
Alachlor	1.6 ± 0.0	0.8 ± 0.9	0.5 ± 0.5	0.3 ± 0.3
Atrazine	-	0.2 ± 0.0	0.1 ± 0.1	-
Cyhalofop-butyl	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Metolachlor	0.2 ± 0.0	0.0 ± 0.1	0.4 ± 0.0	-
Metribuzin	-	-	0.1 ± 0.0	-
Pendimethalin	2.0	1.6	1.3	-
Simazine	0.5 ± 0.0	0.3 ± 0.3	0.7 ± 0.1	0.3 ± 0.4
Trifluralin	3.0 ± 0.0	2.0 ± 1.8	2.6 ± 1.1	0.6 ± 1.5
Insecticides				
Azinphos-methyl	3.0 ± 0.0	1.1 ± 0.7	1.3 ± 0.6	1.4
Chlorfenvinphos Z	0.6 ± 0.0	1.2 ± 0.4	0.5 ± 0.7	0.3 ± 0.3
Cyhalothrin (lambda)	0.6 ± 0.0	0.3 ± 0.3	0.4 ± 0.2	0.1 ± 0.2
4,4'-DDD	0.2 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	-
4,4'-DDT	1.4	-	-	-
Deltamethrin	1.5 ± 0.4	1.9 ± 1.5	0.9 ± 0.3	0.6
Diazinon	0.6 ± 0.1	0.5 ± 0.3	0.4 ± 0.2	0.3 ± 0.0
Dieldrin	0.6 ± 0.0	1.0 ± 0.3	0.1 ± 0.6	0.4 ± 0.1
Dimethoate	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.2
Endosulfan (alpha)	0.3 ± 0.0	-	-	-
Endosulfan (beta)	0.3 ± 0.0	0.1 ± 0.2	-	-
Endosulfan sulfate	0.8 ± 0.0	1.5 ± 0.5	0.3 ± 0.9	0.2 ± 0.2
Fenitrothion	-	-	0.0 ± 0.0	-
Fonofos	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Malathion	-	0.4 ± 0.0	-	-
Mirex	-	0.1 ± 0.0	-	-
Parathion-ethyl	0.4 ± 0.0	0.3 ± 0.2	0.4 ± 0.2	-
Parathion-methyl	-	0.2 ± 0.0	0.4 ± 0.1	-
Tetrachlorvinphos	0.2 ± 0.0	0.1 ± 0.1	-	-

Table 2 Chemical characteristics (class, log Kow, log Koc and GUS index) and license category (according to European pesticides database) of the selected pesticides.

Pesticides	Class	License[#]	log K_{ow}	log K_{oc}	GUS index
Fungicide					
Azoxystrobin	Antibiotic fungicide	A	2.5	2.8	2.6
Difenoconazol	Conazole fungicides	A	4.4	3.6	0.9
HCB	Organochlorines	B	3.9	4.7	-2.3
PCB	Aromatic fungicide	NA	4.8-5.2	4.5	-1.2
Procymidone	Conazole fungicides	NA	3.3	2.6	1.2
Tebuconazole	Conazole fungicides	A	3.7	3.0	2.0
Herbicide					
Alachlor	Organochlorines	NA	3.7	2.5	0.8
Atrazine	Triazine	NA	2.7	2.0	3.3
Atrazine-desethyl	Triazine	NA	2.7	1.9	3.5
Cyanazine	Triazine	NA	2.1	2.3	2.1
Cyhalofop-butyl	Phenoxy herbicides	A	6.0	3.7	-0.2
Metolachlor	Amide herbicides	NA	3.4	2.1	3.5
Metribuzin	Triazinone herbicides	A	1.7	1.8	2.6
Pendimethalin	Dinitroaniline herbicides	A	5.2	4.4	-0.4
Propyzamide	Amide herbicides	A	3.3	2.9	1.8
Simazine	Triazine	NA	2.3	2.1	2.0
Simetryn	Triazine	NA	2.8	2.3	3.0
Terbuthylazine	Triazine	A	3.4	2.3	3.1
Terbutryn	Triazine	NA	3.7	3.4	2.4
Trifluralin	Carbamate insecticide	NA	5.3	4.2	0.1
Insecticide					
Azinphos-methyl	Organothiophosphate insecticides	NA	3.0	3.0	1.0
Chlorfenvinphos Z	Organophosphorus	NA	3.8	2.8	1.9
Chlorpyriphos	Organophosphorus	A	4.7	3.9	0.2
Cyfluthrin (beta)	Pyrethroid	A	5.6	4.8	-1.7
Cyhalothrin (lambda)	Pyrethroid	A	6.8	5.2	-2.1
Cypermethrin (alpha)	Pyrethroid	A	6.9	4.4	-2.1
4,4'-DDD	Organochlorines	B	6.9	4.7	-0.9
4,4'-DDE	Organochlorines	B	6.9	4.9	-2.0
4,4'-DDT	Organochlorines	B	6.9	5.9	-4.5
Deltamethrin	Pyrethroid	A	4.6	7.0	-3.4
Diazinon	Organophosphorus	NA	3.7	2.8	1.1
Dichlorvos	Organophosphorus	NA	1.9	1.7	0.7
Dieldrin	Organochlorines	B	3.7	4.4	-0.3
Dimethoate	Organophosphorus	A	0.7	1.0	1.1
Endosulfan (alpha)	Organochlorines	NA	4.7	4.1	-0.1
Endosulfan (beta)	Organochlorines	NA	4.8	4.3	-0.1
Endosulfan sulfate	Organochlorines	NA	3.7	3.7	0.5
Fenamiphos	Organophosphorus	A	3.3	2.0	-0.1
Fenitrothion	Organophosphorus	NA	3.3	3.3	0.5
Fonofos	Organophosphorus	NA	3.9	2.9	2.1
HCCP	Organochlorines	*	4.0	3.6	0.4
Heptachlor	Organochlorines	B	5.4	4.4	-0.9
Lindane	Organochlorines	NA	3.7	3.1	4.0
Malathion	Organophosphorus	A	2.8	3.3	-1.3
Mirex	Organochlorines	B	5.3	3.8	0.6
Parathion-ethyl	Organophosphorus	NA	3.8	3.9	2.1
Parathion-methyl	Organophosphorus	NA	3.0	2.4	1.5
Phosmet	Organothiophosphate insecticides	A	3.0	3.6	0.2
Pirimicarb	Dinitroaniline herbicides	A	1.7	2.6	2.7
Tetrachlorvinphos	Organophosphorus	NA	3.5	3.0	0.3

[#]**NA**- Not authorized; **A**- Authorized; **B**- Banned; according to the EU Pesticides Database * Information not found
GUS index (groundwater ubiquity score; GUS= log10 (half life-days) X [4-log10 (Koc)])

Chapter 4

Environmental assessment of pesticides in the Mondego River Estuary (Portugal)

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Environmental assessment of pesticides in the Mondego River Estuary (Portugal)

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ABSTRACT

The Mondego River estuary, located on the North Atlantic Ocean Ecoregion, is a basin affected by agricultural run-off with increasing signs of eutrophication. We evaluated the amounts and distribution of 56 priority pesticides belonging to distinct categories (insecticides, herbicides and fungicides). Temporal trends were considered and a total of 42 surface water samples were collected between 2010 and 2011. More than 55% of the GC–MS/MS- quantified pesticides were above the maximum amounts established by the European Directives (98/83/EC and 2013/39/EU). Based on the concentration addition (CA) and independent action (IA) models, we used a two-tiered approach to assess the hazard of the pesticide mixture, at the maximum concentration found, reflecting a potential risk. Short-term exposure using *Artemia salina* indicated a significant toxic effect where the locomotion of the animals was clearly affected.

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Introduction

The Mondego River estuary (Fig. 1), located on the North Atlantic Ocean Ecoregion (Flindt et al., 1997), is a basin intensely affected by agricultural run-off, caused by corn and rice fields located upstream (Lillebø et al., 2012). Despite the resulting eutrophication of the last decades (Lillebø et al., 2005, 2012), there have been few studies on the quantification and monitoring of pesticides in this area (Andrade and Stigter, 2009; Silva and Cerejeira, 2014; Villaverde et al., 2008).

The European Directive 2013/39/EU promotes the control of pesticides in inland waters and biota (European Union, 2013). Seven sampling sites in the Mondego River estuary were used to study the presence of 56 selected pesticides, over a year, in surface waters.

Brine shrimp larvae (*Artemia salina*) were used in laboratory toxicity tests using the maximum environmental concentrations found in the estuary. In parallel, a two-tiered approach, based on concentration addition (CA) and independent action (IA), was conducted to evaluate the environmental hazard of chemical mixtures at concentrations found in the same area (Backhaus and Faust, 2012; Silva and Cerejeira, 2014).

The specific aims of this study were to: (1) quantify the selected pesticides from surface waters collected in the Mondego River estuary over a year; (2) characterize the estuary status according to the concentration of the predictive ecotoxicologic risk assessment of the pesticide mixtures; (3) use brine shrimp larvae for acute toxicity (24 h) assessment of water quality for the highest measured concentrations; and (4) provide an overall hazard assessment.

2. Materials and methods

2.1. Study area

Mondego River flows along 227 km from the mountain Serra da Estrela to the Atlantic Ocean, in the city of Figueira da Foz. It receives waters from the tributaries Dão, Ançã, and Foja on the northern side and from Alva, Ceira, Cernache, Ega, Arunca, and Pranto on the southern side (Ferreira dos Santos and Freitas, 2012). During its course, the Mondego River passes through agriculture fields (12286 ha, mainly of rice and corn), industrial areas (mostly factories of cellulose and paper), many salt-works and aquaculture systems (semi-intensive farming—ponds) (Ferreira dos Santos and Freitas, 2012; Marques et al., 1993). The Mondego River basin (40°07′59.8″ N, 8°49′57.9″ W; Fig. 1) is an estuary that covers 3.4 km^2 and has a drainage area of 6670 km^2 which is divided by the Murraceira Island into two branches. These branches diverge 7.5 km upstream, presenting different hydrologic characteristics (Ribeiro et al., 2009).

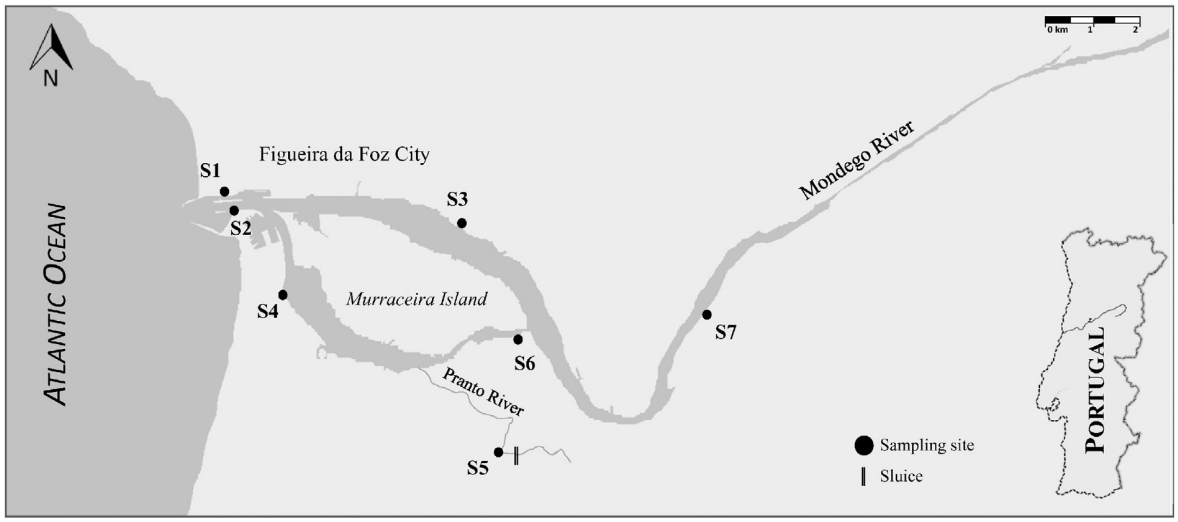

Fig. 1. Map of the studied area showing the location of the sampling sites at Mondego River estuary, Portugal.

The northern arm receives most of the freshwater, being the deepest one and strongly influenced by seasonal water fluctuations, while the southern arm depends on the tides and on freshwater input from the Pranto River (small tributary) for water circulation (Chainho et al., 2006; Marques et al., 1993).

Seven sampling sites were selected to assess the input of pesticides and characterize the most affected areas (Fig. 1). On the north margin, while S_1 represents Figueira da Foz, S_3 characterizes the harbour area. On the south margin, S_2 is parallel to S_1 at the mouth of the river, S_4 characterizes the exposed intertidal areas during low tide, S_5 matches the Pranto River stream, S_6 is located on the silted area of the southern arm and S_7 represents the river before the branch division.

2.2. Water collection and quality measurement

Surface water samples (2.5 L) were collected at ebb tide, from 2010 (in January, March, May, July, September, and November) to January 2011, once per month, into pre-rinsed amber glass bottles. In the laboratory, the samples (1 L) were immediately filtered (0.45 μm glass fibre filter), pH = 7 adjusted with H_2SO_4 and then stored at 4 °C in the dark, for a maximum period of 24 h.

Physicochemical parameters, temperature (°C), dissolved oxygen (DO; mg/L), salinity and conductivity (mS/cm), were measured in situ, using portable meters OXi 330i/Set WTW and LF 330/Set WTW, respectively. Other parameters, such as pH (Sartorius PB-11), nitrites, nitrates, ammonium and phosphates (mg/L) were measured in the laboratory, using a photometer device from Palintest (Gateshead, UK).

2.3. Chemical analyses

2.3.1. Water sample pre-concentration

The selected pesticides were extracted using solid-phase extraction (SPE) cartridges following a published protocol (Cruzeiro et al., 2015). The cartridges were conditioned sequentially with 5 mL of ethyl acetate, followed by 5 mL of methanol and 2.5 mL of ultrapure water (Milli-Q water), at a flow rate of 1–2 mL/min.

The water samples (500 mL), spiked with internal standards (IS), were loaded into SPE cartridges at a constant flow-rate of 5 mL/min and allowed to dry. Subsequently, the samples were eluted with 6 mL of ethyl acetate, at a rate of 1 mL/min. The extracts were then concentrated into 200 μL of hexane, under N_2 stream (99.9997%), and kept in vials at –80 °C until further analysis.

2.3.2. Quantification of pesticides

GC–MS/MS analyses were carried out using a Trace GC ultra (Thermo Finnigan Electron Corporation) and the software Xcalibur (version 2.0.7, 2007, Thermo Scientific) (Supplementary information, Table SI1). The selected ion monitoring mode (SIM) for pesticide quantification was optimized with the acquired standards, which matched the NIST Mass Spectral Search Program (version 2.0, 2005) library and other published methods (Lian et al., 2010; Rocha et al., 2012; Wong et al., 2010; Yang et al., 2011). To get the best parent/daughters ratio, optimized energies were applied when identifying the compounds by MS/MS (Supplementary information, Table SI 2).

The validation procedure followed the European guidance document on pesticide residue analytical methods (European Commission Directorate General Health and Consumer Protection, 2010). The limits of detection (LOD) and quantification (LOQ) were determined by the ratio (spiked pesticide area/spiked surrogates area). Both LODs and LOQs were calculated based on three calibration curves (10–400 ng/L) of each pesticide as follows: LOD = 3.3 α/S and LOQ = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves. Recoveries, accuracy and precision were evaluated by analysing three independent replicates of each quality control samples (QCs) at three levels of concentration (low, medium and high) calculated according to the Brazilian Health Surveillance Agency (ANVISA) guidelines (ANVISA, 2003). Eight nominal working concentrations were prepared by spiking clean estuarine water samples. This matrix was used as calibration standard with a range of concentrations of 10–400 ng/L for all 56 pesticides and 160 ng/L for IS. Blanks and an intermediate concentration (160 ng/L) were used as quality control. During all processes, solvent (n-hexane) and matrix blanks (estuarine waters) were systematically analysed to prevent potential contamination.

2.4. Hazard analyses

Pesticides may reach the aquatic environments in different mixtures and quantities, making difficult the toxicological assessment for all cases. Two reference models—concentration addition (CA) and independent action (IA)—allow to calculate the expected risk of a mixture toxicity (ECHA, 2012). The two-tiered approach suggested by Backhaus and Faust (2012) was followed to predict environmental hazard and risk evaluation (Fig. 2).

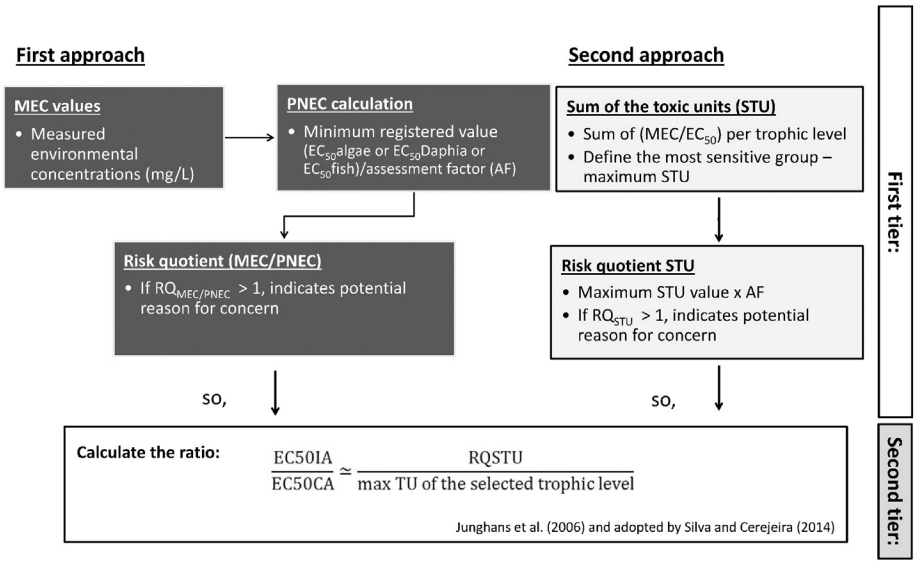

Fig. 2. Illustration of the two-tiered approach to predict the environmental risk caused by a pesticide mixture.

Classical CA (first tier) is replaced by the sum of the measured environmental concentrations (MEC) divided by the predicted no effect (PNEC) ratio and/or by the risk quotient of sum of toxic units (RQ_{STU}). In turn, PNEC was calculated based on the minimum EC_{50}, of one trophic level, divided by an assessment factor of 100. If both approaches fail (*i.e.*, $RQ_{MEC/PNEC}$ and $RQ_{STU} > 1$) additional IA considerations should be done (Backhaus and Faust, 2012). According to Junghans et al. (2006), the ratio STU/maxSU can be used to predict the second tier (CA and IA classical application), giving us the maximum value from which CA may predict a higher toxicity than IA, as described by Silva and Cerejeira (2014). The AF = 100 was adopted as the maximum acceptable concentration-quality standards (MAC-QS) in order to assess the short term effects (European Communities, 2011).

The average effective concentration (EC_{50}) values for each trophic level algae, crustaceans and fish, were obtained from FOOTPRINT pesticide and PubChem chemistry databases (National Center for Biotechnology Information, 2015; PPDB, 2013); no or limited data were accessible for pentachlorobenzene (PeCB), atrazine-desethyl, endosulfan sulphate and hexachlorocyclopentadiene (HCCP).

2.5. Toxicity test with A. salina

A. salina is a primitive aquatic arthropod (about 100 million years) that belongs to the phylum Arthropoda, class Crustacea and the Artemiidae family. It is a very adaptable larva that can survive a wide range of salinities (5–250 g/L) and temperatures (6–35 °C). Its life cycle begins by the hatching of dormant cysts (0.2–0.3 mm) into free-swimming naupliae (0.45 mm; instar I/II), in a period of 24–36 h, reaching their adult life in 3 to 5 weeks' time, passing through 15 moulting stages (Van Stappen, 1996; Lu et al., 2012).

The hatching and the experimental design followed the "Artemia Reference Center-test" protocol (Vanhaecke and Persoone, 1981, 1984)

where 50 mg of dry cysts (Ocean Nutrition, batch number: 0N13280) were incubated in a well-aerated bottle with 200 mL of artificial salty water (35 g/L of Tropic Marin Pro Reef Sea Salt) at 25 °C and 14:10 h photoperiod (light:dark). Thirty-six hours later, groups of 10 free-swimming nauplii (between instar I and II) were randomly transferred into 2 mL glass beakers and placed in a 24-multiwall plate.

Three plates were used per exposure (24 h maintained in the dark) at 25 °C. The treatment groups (control, solvent control (0.01% of EtOH), pesticides mixture (maximum concentration found) and the reference toxicant ($K_2Cr_2O_7$; 50 mg/L) were distributed by line per plate, with randomization using tools offered at www.random.org. The procedure was repeated on three different days.

Toxicity was analysed by counting the dead nauplii (no movement in 10 s of observation), using a binocular microscope. The plate results were valid if mortality of the control group was below 10%.

2.6. Data presentation and statistical analyses

For each pesticide, the analytical data were displayed as average values of the sampled sites (n = 7) per season, followed by the 95% confidence interval (CI). The same data were also grouped by the total average (TA) loads of the compounds and displayed by category (fungicides, herbicides and insecticides), per season (spring, summer, autumn and winter). The physicochemical parameters are grouped by season and presented as average (CI) loads of all sampling sites.

Descriptive and inferential statistics were made with the PAST 3.06 software (Hammer et al., 2001). After checking the assumptions of normality (Shapiro–Wilk W-test) and homogeneity of variances (Levine's test), independent comparisons between seasons and categorical groups were analysed by one-way analysis of variance (ANOVA), and post-hoc comparisons were made using the Tukey's test. When the cited parametric assumptions were not valid, and data transformation was ineffective, the non-parametric Kruskal–Wallis ANOVA was used, followed by the Mann–Whitney U test, with a sequential Bonferroni correction; the significance level was set at the conventional 5%.

The GUS index (groundwater ubiquity score) was calculated (Table SI 3) as an indicator of potential pollution based on an empiric approach that allows the classification of pesticides into leachable (GUS > 2.8), non-leachable (GUS < 1.8) and transition (1.8 < GUS > 2.8), considering their persistence and ability to bind to soil particles: GUS = log10 (half-life days) × [4 – log10 (Koc)]). The GUS score ranges from extremely low (< 0.1) to very high (> 4.0), rating the potential of pesticides leaching into groundwater (Gustafson, 1989).

3. Results

3.1. Pesticide concentrations in the Mondego estuary surface waters

Because no significant differences were observed between sites, data were grouped by season. The average concentrations (ng/L) and the percentage of samples above the detection and quantification limits (LOD and LOQ,

respectively), for each pesticide, are provided in the supplementary information (Table SI 4). The total cumulative (TA) values represented on Fig. 3 demonstrate similar concentrations throughout the year, ranging from ∑≈ 5000 to 7000 ng/L. The values further show that there were higher cumulative loads for insecticides (about 74%, $p < 0.05$) when compared to herbicides and fungicides. From all of the selected pesticides, only simetryn was below the LOD; both procymidone and chlorpyriphos were below the LOQ.

The highest fungicide TA values were quantified in spring ($\sum_{spring} \approx 900$ ng/L), being statistically different ($p < 0.05$) from autumn ($\sum_{autumn} \approx 100$ ng/L) but not from summer and winter ($\sum_{summer\ and\ winter} \approx 700$ ng/L). As for specific pesticides, the average annual values of difenoconazol (≈ 380 ng/L) stand out from the other fungicides (≈ 51 ng/L) but with no significant differences (Table SI 4).

With regard to the herbicides, 93% of the selected herbicides were quantified, registering average annual loads of ≈ 1000 ng/L, with no significant differences between seasons (Table SI 4); the same pattern was observed per compound.

With regard to the insecticides, the annual average loads of this category was $\sum_{annual} \approx 4100$ ng/L and, like in the herbicides category, no seasonal statistical differences were registered, but higher TA amounts were quantified in winter ($\sum_{winter} \approx 5000$ ng/L) than for other seasons ($\sum_{spring,\ summer,\ autumn} \approx 3800$ ng/L) (Fig. 3). Individually, only deltamethrin presented significant differences between spring ($\sum_{spring} \approx 800$ ng/L) and summer or winter ($\sum_{summer\ and\ winter} \approx 300$ ng/L) (Table SI 4).

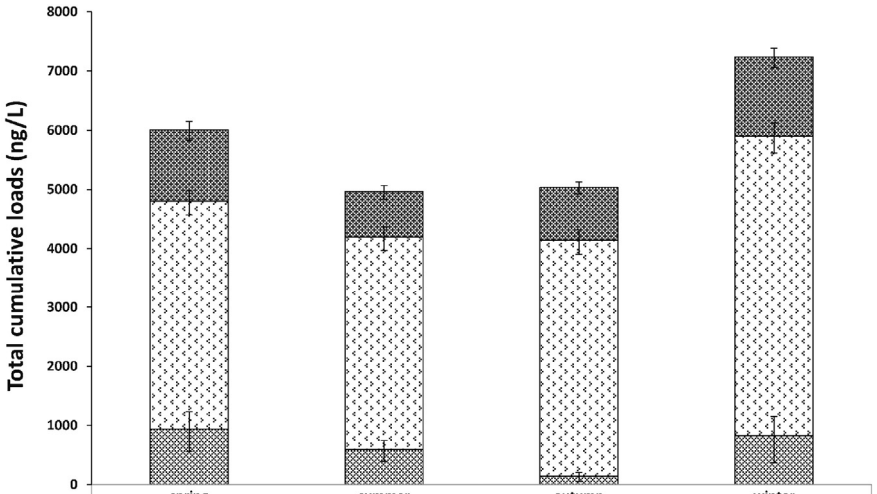

Fig. 3. Seasonal fluctuation of the pesticide concentrations (∑ ng/L) grouped by category (fungicides, herbicides and insecticides). Data are expressed as total average (TA) loads per pesticide category (CI).

3.2. Physicochemical parameters

The physicochemical parameters were grouped by season (Fig. 4). Significant seasonal fluctuations ($p < 0.05$) were observed for phosphates, ammonia and nitrites but with no common pattern between them. Other parameters, such as temperature ≈ 16.5 °C, salinity ≈ 13.1, pH ≈ 8.1, DO ≈ 8.1 mg/L and nitrates ≈ 2.1 mg/L, were also registered, but no differences were observed among seasons.

3.3. Values above the limits permitted by European legislation

Table 1 shows all the pesticides measured in this study and which concentrations are above the maximum values set by both EU Directives (98/83/EC and 2013/39/EU) (European Union, 1998, 2013). Several herbicides and insecticides were at or up to 6.1-fold higher than the concentrations established for water intended for human consumption; the highest average annual value was obtained for endosulfan (alpha) (≈ 615 ng/L). As a total sum per season, all values were at least 10 times above the allowed maximum value of 500 ng/L (European Union, 1998), attaining the highest amounts in winter ≈ 7200 ng/L.

According to Directive 2013/39/EU, several insecticides registered concentrations between 6 and 421155-fold higher than the allowed average annual amounts set for surface waters. The same occurred for dichlorvos, $\sum_{\text{endosulfan (alpha + beta)}}$, and $\sum_{\text{heptachlor, heptachlor epoxide}}$, attaining amounts above the maximum allowable concentrations. Both the fungicides hexachlorobenzene (HCB) and PeCB, and the herbicide trifluralin were above the admissible amounts (5.3 and 3.8-fold, respectively).

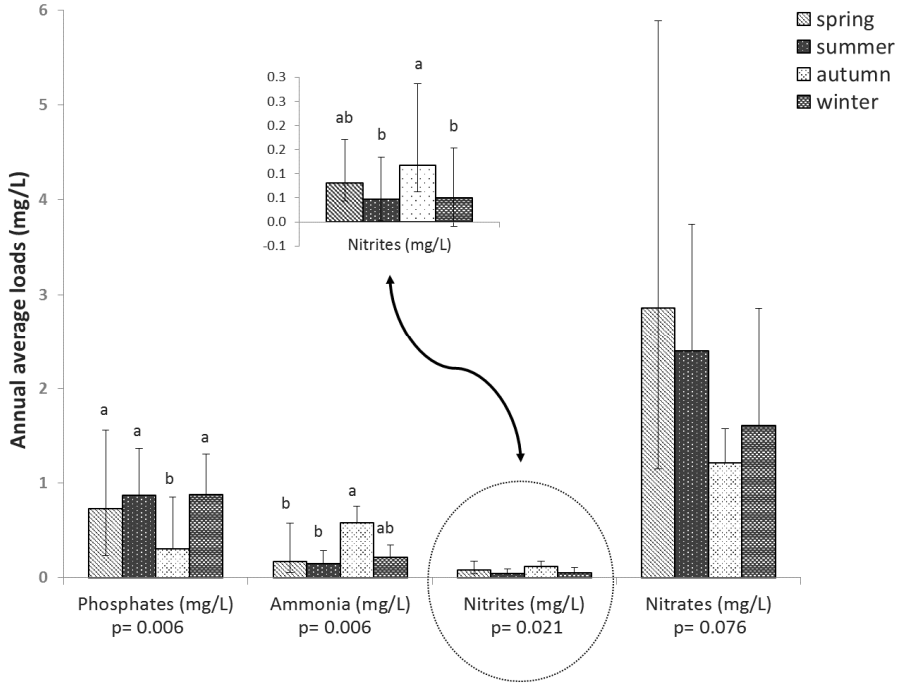

Fig. 4. Physicochemical parameter results of the collected samples, from 2010 until 2011, grouped by season. Data are shown as mean values (CI); n = 14 in spring and summer and n = 7 in autumn and winter.

3.4. Evaluation of the aquatic hazard of pesticide mixtures

The two-tiered approach to predict an environmental risk caused by a pesticide mixture is summarized in Fig. 2. A database of 52 pesticides was used for the evaluation, considering the maximum concentrations found in the Mondego estuary water—all the calculations are given in the Supplementary information (Table SI 5). As a first approach to the first tier, more than 50% of the compounds presented an individual MEC/PNEC ratio above 1, indicating a potential risk. A second approach was done to define the most sensitive (maximum sum of toxic units, STU) trophic group (algae, invertebrates or fish) to this environmental mixture, where the highest value was obtained for fish (STU = 14.27); this value was 48 and 1.5-fold higher than for algae and the invertebrate groups, respectively. Afterwards, the risk quotient was calculated (RQ_{STU}) achieving a value of 1427. Both approaches indicate a potential risk, which leads us to the second tier. The most sensitive group is taken into account, obtaining a ratio of 1.188 that indicates an estimative of the maximal value by which CA may predict a higher toxicity than IA.

Table 1
Evaluation between the average annual values obtained, for certain pesticides, at Mondego River estuary, with the maximum annual average values (ng/L) established by the European legislations (98/83/EC and 2013/39/EU).

Legislation		Mondego estuary	Directive
		ng/L	
98/83/EC	Alachlor	100.3	100
	Atrazine-desethyl	100.9	100
	Cyanazine	154.1	100
	Cyfluthrin (beta)	262.4	100
	Cyhalothrin (lambda)	203.3	100
	Cypermethrin (alpha)	124.7	100
	Deltamethrin	499.4	100
	Diazinon	530.8	100
	Difenoconazol	379.8	100
	Dimethoate	161.4	100
	Endosulfan (alpha)	614.9	100
	Endrin	148.4	100
	Fenitrothion	202.8	100
	HCCP	141.9	100
	Lindane	468.1	100
	Methoxychlor	123.7	100
	Metolachlor	145.5	100
	Mirex	138.7	100
	Phosmet	233.4	100
	Pirimicarb	154.6	100
	Propazine	196.1	100
	Trifluralin	114.4	100
	Σaldrin, dieldrin, heptachlor and heptachlor epoxide [*,1]	166.0	50
2013/39/EU	Dichlorvos	22.6	0.6
	4,4'-DDT	59.3	10
	Endosulfan (alpha + beta)	625.1	5
	Heptachlor[*,3]	84.2	0.0002
	HCB	52.7	10.0
	PeCB	37.4	7
	Trifluralin	114.4	30
	Σaldrin, dieldrin, endrin [*,2]	230.3	10

* the sum of the detected pesticides from the following directives:

98/83/EC [1]Σaldrin, dieldrin, heptachlor and heptachlor epoxide

2013/39/EU [2]Σaldrin, dieldrin, endrin and isodrin

[3]Σheptachlor and heptachlor epoxide

3.5. Acute exposure

After 24-hour exposure of all replicates (individual wells) were counted, giving a total of 54 samples per treatment (control, positive control, dichromate and solvent control). The positive control attained 50% mortality at 33 mg/L $K_2Cr_2O_7$, which is in accordance with the ARC-test conditions (to obtain 50% of mortality between 25 and 50 mg/L $K_2Cr_2O_7$) and was statistically different ($p < 0.001$) from the other groups (Fig. 5). While both solvent and control groups had an average mortality of 2%, the pesticide mixture group reached a mortality of 4%; with significant differences between them ($p < 0.05$). The pesticide mixture group also presented perceivable alterations in the animals' mobility when compared to the animals of control group, affecting both swim speed and coordination of movements (Supplementary information: video data).

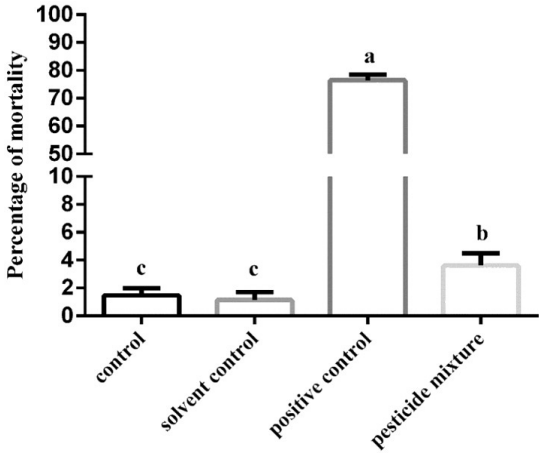

Fig. 5. Artemia assay results, expressed as percentage of mortality from all exposed groups.

4. Discussion

The concentrations of the pesticides quantified herein reflect an estuary severely affected by the agriculture carried on in the area (12300 ha) (Ferreira dos Santos and Freitas, 2012). Indeed, the cumulative values were always above the limits established by the Directive 98/83/EC (European Union, 1998).

The fungicides difenoconazol (≈ 380 ng/L), PeCB (≈ 37 ng/L) and HCB (≈ 53 ng/L) presented concentrations 4 to 7 times higher than the legal ones. Since their chemical characteristics (GUS index of − 0.9 and a log Kow of 4.4) demonstrate a higher craving for organic matter, it is assumed that their presence in surface waters only occurs when they are applied in excessive amounts (Gustafson, 1989).

Comparing present data with other aquatic systems, lower amounts were quantified in the Netherlands (120 ng/L) for the difenoconazol, and higher concentration were observed at the Douro river estuary (65 ng/L) for HCB.

The average measured concentrations (100 ng/L) of the herbicides alachlor, atrazine-desethyl, cyanazine, propazine and trifluralin stand out from the others, which may indicate a run-off of these compounds into the estuary. These compounds have a high leaching potential (average GUS index of 2.1 and an average log K_{ow} of 3.6), which makes them prone to contaminate surface and groundwaters (Gustafson, 1989).

The same compounds were quantified in other aquatic European systems (Ave River (Portugal), Kalamas River (Greece), Elbe River (Germany) and Llobregat (Spain), reaching levels 40 times higher for trifluralin (Lambropoulou et al., 2002)

and also alachlor and atrazine- desethyl (Gonçalves et al., 2007), and 17 to 36 times lower levels for cyanazine and propazine (Koal et al., 2003; Köck-Schulmeyer et al., 2012).

This study revealed that 45% of the selected compounds (most of them being insecticides) were above concentrations of possible concern (European Union, 1998, 2013). Some of them are directly associated with rice production done at the lower Mondego River valley (Marques, 2009), namely lindane, malathion, endosulfan, deltamethrin, dimethoate and parathion, with their individual annual average values ranging from 99 to 615 ng/L (Direção Regional de Agricultura e Pescas (DRAP), 2014; Faria et al., 2007; Jørgensen et al., 1997). Considering Directive 2013/39/EU, dichlorvos, endosulfan and heptachlor reached levels 38 to 421,155-fold higher than the maximum annual amounts set for inland and surface waters (European Union, 2013).

Some banned pesticides (4,4′-DDT and heptachlor) and their metabolites (4,4′-DDD, 4,4′-DDD and heptachlor epoxide, respectively) were quantified. The ratio precursor/metabolites for DDT/$\sum_{(DDE,\ DDD)}$ (1.8), heptachlor/heptachlor epoxide (7.9) were above 1, suggesting a regular usage of these compounds (Marques et al., 2003). The same active response was observed in the North of Portugal, surveying the Douro River estuary (Rocha et al., 2012), which presented ratios of 1.3 and 2.9, respectively. Like for the fungicides, the chemical properties of insecticides (average GUS index of –0.1 and an average log K_{ow} of 4.2) indicate a higher tendency to be adsorbed to organic matter. This shows that their presence in surface waters only occurs when they are applied in excessive amounts, suggesting that pesticides are being overused or used/treated improperly. This practice and consequent chronic pesticide loads may impose direct (pesticide application and contaminated water) and/or indirect potential risks (eutrophication and contaminated food) to the local and migratory animals (Marques, 2009). Compared with our study, higher amounts were found in 2004 for alachlor, aldrin and drieldrin, which were 45, 113 and 167-fold higher (Abrantes et al., 2010) respectively, suggesting a decrease in the usage of these pesticides.

Along with the quantification of pesticides, physicochemical parameters linked to water quality were measured. Parameters, such as DO and pH were considered normal (*i.e.*, DO above 2 mg/L and pH between 5 and 9) even in summer, when water temperatures were above 20 °C (Ministério do Ambiente, 1998). Nitrites (0.07 mg/L), nitrates (2.02 mg/L) and un-ionized ammonia (0.01 mg/L) presented average amounts (Fig. 4) below the maximum recommended values defined by the Directive 236/98, which are 0.1, 50.0, and 0.06 mg/L, respectively (Ministério do Ambiente, 1998). However, ammonia presented values 5.6-fold higher than recommended for surface waters (0.05 mg/L) (Ministério do Ambiente, 1998), which is reflected in the total amounts of nitrogen (1.02 mg/L) measured. The total average amounts of phosphates (0.05 mg/L) were above the range (0.01–0.03 mg/L) set for an uncontaminated environment and in between the range (0.025–0.1 mg/L) where plants' growth is stimulated (USEPA, 1986). The same range of values, for nitrites, nitrates, ammonia and phosphates, were earlier measured in this estuary, associated at the time to agricultural fertilization and

demonstrating the impact of this activity in the basin (Marques et al., 2003).

This aquatic system is being impacted by human activities, such as agriculture and waste water treatment plants (WWTPs) discharges, which may cause a wide range of adverse effects in the biota. In accordance with this hazard, some studies reported signs of endocrine disruption occurring in the grey mullet fish (Carrola et al., 2014), in isopods (Lemos et al., 2010) and in the harlequin fly (Faria et al., 2007).

Comparing the obtained values with the International Union of Pure and Applied Chemistry (IUPAC) database, some pesticides (cyfluthrin, cyhalothrin, deltamethrin, fonofos and methoxychlor) were present at concentrations able to cause a 50% mortality of the exposed population (LC_{50}) of fish (*Oncorhynchus mykiss*), invertebrate (*Daphnia magna*) and crustacean (*Americamysis bahia*) (PPDB, 2013).

We cannot extrapolate these results to an environmental mixture. Therefore, the maximum concentrations found for each pesticide were used to simulate the worst scenario in the estuary, using a concentration addition-based approach (theoretical) and a short-term exposure (48 h) founded on the ARC-test assay (practical). The results of the two-tiered approach indicate that this mixture has a potential risk to fish, being determined by the deltamethrin LC_{50} concentration (260 ng/L; Table SI 5), which is also supported by the low IA/CA ratio (1.188). Silva and Cerejeira (2014) made comparable predictions for the same basin (1.003) and for the Tagus River (1.025).

The *Artemia* mortality results presented significant differences between the controls (solvent and media) and the exposed group (maximum pesticide concentrations found), where a behaviour alteration was visible (in swim speed and motor coordination), indicating a toxic effect of the pesticides in this crustacean. The visible alterations may be linked to the physiological effects of the insecticides, since they affect the nervous system, interfering with the membrane transport ions, enzyme activities inhibition and/or release of chemical transmitters at nerve ending (Åkerblom, 2004; Fulton and Key, 2001).

4.1. Concluding remarks

Fifty three pesticides from three different categories were quantified in the Mondego River estuary from 2010 to 2011. Several pesticides were above the maximum amounts established by the European Directives (98/83/EC and 2013/39/EU), some of them able to cause mortality (LC_{50}) in some animals (fish, invertebrates and crustaceans). Seasonally, the average total concentrations were always above the legal limits. Additionally, some physicochemical parameters measured corroborate earlier published data and indicate that the area is impacted by human activities.

The two-tiered approach used to assess the pesticide mixture, at the maximum concentration, reflected a potential risk, mainly for fish, while the short-term exposure, which used *Artemia* as a biological model, indicated a potential toxic risk of (at least) affecting the animal's locomotion. Our data backs the view that the pesticide usage in the Mondego Basin should be reduced.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2015.12. 013.

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Supplementary information

Table SI1 – GC-MS and GC-MS/MS characteristics and conditions for the analysis of the selected pesticides in surface coastal waters samples.

GC-MS conditions			
Gas chromatograph:	Trace GC ultra, Thermo Finnigan Electron Corporation		
Detector:	Ion trap mass spectrometer (Thermo Scientific ITQ™ 1100 GC-MS^n)		
Autosampler:	Thermo Scientific TriPlus™		
Injector:	SSL (3 mm straight liner)		
Mode	splitless mode		
Temperature (°C)	250		
Volume (μL)	2uL (50 mm lenght needle)		
Carrier Gas:	Helium (99.9999%); constant flow 1 mL/min		
Column:	TG-5SilMS (30m x 0.25 mm x 0.25 μm)		
Program:	**temperature (° C)**	**hold time (min)**	**Rate °C/min**
1st ramp	65	2	-
2nd ramp	180	-	20
3rd ramp	280	7	5
Solvent delay:	5min		
Transfer line (°C):	280		
Ion source (°C):	280		

Table SI2 – Quantification and diagnostic ions used in GC-MS and GC-MS/MS analysis. The relative abundance of ions (*m/z*) for each target pesticide is indicated between brackets.

Pesticides	RT (min)	GC-MS/SIM					GC-MS/MS						
		Target ion (t)	Q1 (%Q1/t)	Q2 (%Q2/t)	Q3 (%Q3/t)	Q 4 (%Q4/t)	Percursor	Products				EV	Ranges
Hexachlorocyclopentadiene (HCCP) [b]	7.20	**237**	239 (54.6)	235 (48.1)	272 (14.2)		**237**	→ *143*	*141*	*203*		2.05	140-145/200-238
Pentachlorobenzene (PeCB)	9.00	**250**	248 (65.1)	252 (66.1)			**250**	→ *215*	*144*			2.01	143-251
Phosmet	9.80	**160**	161 (86.2)	133 (40.7)			**160**	→ *130*	*140*			1.15	104-161
Trifluralin	10.30	**264**	306 (44.9)	206 (27.2)			**264**	→ *206*	*160*	*188*	*171*	1.05	159-265
Atrazine-desethyl	10.30	**172**	68 (32.2)	174 (29.2)			**172**	→ *130*	*145*	*152*		1.15	104-173
Hexachlorobenzene (HCB)[b]	10.90	**284**	282 (46.4)	249 (41.2)			**284**	→ *214*	*249*			1.50	211-285
Dimethoate	10.96	**87**	93 (52.5)	125 (44.3)			**87**	→ *86*	*59*			1.10	53-88
Simazine [a]	11.20	**201**	186 (67.1)	173 (51.9)	44 (37.6)		**201**	→ *186*	*174*	*138*		1.20	135-201
Atrazine-d_5	11.21	**205**	220 (43.0)	178 (41.7)			***205***	→ *127*	*137*	*105*		1.25	104-206
Atrazine [b]	11.27	**200**	215 (56.8)	173 (36.9)			**200**	→ *122*	*132*	164	158	1.25	121-201
Propazine	11.30	**214**	172 (70.6)	187 (38.0)			**214**	→ *200*	*172*	*138*		1.20	137-215
BHC (gamma) (Lindane) [a]	11.50	**181**	183 (76.6)	219 (69.3)			**181**	→ *145*	*146*			1.20	108-184
Terbuthylazine	11.60	**214**	173 (71.8)	138 (27.3)	229 (24.3)		**214**	→ *173*	*132*			1.40	131-215
Diazinon	11.70	**137**	179 (44.7)	304 (10.2)			**179**	→ *121*	*163*	137	122	1.35	110-180
Fonofos	11.74	**137**	109 (70.7)	246 (40.0)			**137**	→ *109*	*81*			0.85	80-138
Propyzamide	11.80	**173**	175 (41.2)	254 (35.6)			**173**	→ *138*	*145*			1.10	130-174
Pirimicarb	12.37	**166**	238 (29.0)	72 (18.8)			**166**	→ *96*	*137*	*121*		1.35	95-167
Metribuzin	12.93	**198**	199 (29.8)				**198**	→ *150*	*110*			1.10	109-199
Alachlor [a]	13.20	**188**	160 (86.8)	146 (58.8)			**160**	→ *132*	*130*			1.10	116-161
Parathion-methyl	13.33	**263**	109 (67.9)	79 (44.7)	246 (43.5)		**263**	→ *246*	*153*			1.31	150-264
Simetryn	13.33	**213**	170 (22.9)	155 (13.4)			**213**	→ *170*	*185*			1.10	151-214
Heptachlor [b]	13.38	**272**	274 (73.9)	270 (63.3)	100 (43.9)		**272**	→ *237*	*235*			1.05	236-275
Terbutryn	13.79	**185**	226 (68.0)	170 (45.2)			**185**	→ *170*	*128*			0.90	127-186
Fenitrothion	13.82	**260**	109 (83.4)	125 (77.4)	277 (41.8)		**260**	→ *228*	*217*	*232*		1.20	160-261
Malathion	14.04	**125**	127 (90.9)	99 (73.2)	173 (40.2)		**173**	→ *99*	*117*	145		0.80	92-173
Chlorpyriphos	14.20	**314**	316 (72.3)	258 (67.3)	199 (46.8)	197 (41.4)	**314**	→ *258*	*286*			0.90	257-315
Metolachlor	14.27	**162**	238 (36.7)	163 (15.1)			**162**	→ *132*	*133*			1.15	115-163
Cyanazine	14.35	**225**	212 (59.4)	198 (35.2)	68 (32.8)		**225**	→ *189*	*172*	*198*		1.28	171-226
Aldrin [a]	14.41	**263**	261 (92.7)	265 (65.6)	66 (57.2)		**263**	→ *193*	*191*	*227*		1.60	190-264
Parathion-ethyl	14.45	**291**	109 (79.0)	263 (61.0)	97 (57.2)	141 (47.0)	**109**	→ *81*	*91*			0.99	60-110
Pendimethalin	15.28	**252**	162 (61.3)	191 (28.9)			**252**	→ *162*	*191*			1.00	160-253
Heptachlor epoxide [b]	15.57	**353**	355 (66.0)	351 (44.4)	81 (25.7)		**353**	→ *263*	*282*			1.10	262-354
Chlorfenvinphos Z	15.61	**267**	269 (52.9)	323 (51.5)			**267**	→ *159*	*203*			1.50	158-268
Procymidone	15.90	**96**	283 (85.3)	285 (29.0)			**96**	→ *67*	*68*			1.00	64-97
Chlordane (gamma)	16.26	**375**	373 (93.8)	377 (59.9)			**373**	→ *266*	*264*			1.20	263-374
Tetrachlorvinphos	16.45	**329**	331 (90.4)	109 (51.3)	333 (32.8)		**329**	→ *314*	*278*			1.30	219-330
Endosulfan (beta)	16.70	**241**	195 (72.7)	243 (71.2)	207 (54.0)		**241**	→ *206*	*204*	*170*		1.45	165-242
Fenamiphos	16.96	**303**	243 (62.4)	217 (54.9)	288 (42.9)	154 (40.6)	**303**	→ *268*	*266*			1.10	175-304
4,4'-DDE	17.45	**246**	248 (58.6)	318 (31.9)	316 (29.3)		**246**	→ *176*	*175*			1.70	174-247
Dieldrin [a]	17.56	**79**	263 (93.1)	237 (43.5)			**79**	→ *51*	*50*			1.10	49-80
Endrin [b]	18.24	**243**	263 (99.0)	281 (68.4)	81 (47.4)		**243**	→ *207*	*173*			1.15	172-244
Endosulfan (alpha)	18.55	**241**	195 (78.2)	237 (70.8)	243 (65.5)		**241**	→ *206*	*205*			1.45	165-242
4,4´-DDD	18.78	**235**	237 (64.2)	165 (61.7)			**235**	→ *165*	*199*			1.15	162-236
Endosulfan sulfate	19.84	**272**	237 (68.0)	274 (60.5)	387 (47.9)		**272**	→ *237*	*235*			1.10	234-273
4,4'-DDT-d_8	20.01	**220**	243 (62.6)	280 (57.8)			***243***	→ *173*	*206*			1.15	172-244
4,4´-DDT	20.04	**235**	237 (64.2)	212 (59.0)	165 (44.0)		**235**	→ *165*	*199*			1.15	117-236
Tebuconazole	20.55	**250**	125 (80.6)	163 (44.4)			**125**	→ *89*	*99*			1.60	62-126
Methoxychlor [b]	22.00	**227**	228 (16.5)	274 (15.4)			**227**	→ *169*	*181*			*1.30*	*140-228*
Azinphos-methyl	22.96	**77**	132 (88.0)	104 (43.4)	160 (32.9)		**77**	→ *51*	*50*			1.30	49-78
Mirex	23.60	**272**	274 (73.3)	237 (62.7)			**272**	→ *237*	*235*			1.13	234-273
Cyhalothrin (lambda) *	23.70	**181**	141 (45.8)	197 (42.1)			**181**	→ *152*	*151*			1.50	120-182
Cyhalofop-butyl	23.96	**256**	357 (72.6)	229 (41.1)	120 (31.0)		**256**	→ *228*	*200*			1.13	199-257
Cyfluthrine (beta) *	26.64	**206**	199 (76.9)	91 (70.9)	226 (55.0)	227 (42.2)	**199**	→ *193*	*191*	163		1.80	190-200
Cypermethrin (alpha) *	27.18	**181**	91 (76.3)	163 (75.0)	165 (47.3)		**181**	→ *152*	*151*			1.70	150-153/179-182
Difenoconazol	29.76	**265**	267 (89.9)	323 (68.5)	325 (62.2)		**323**	→ *265*	*249*			1.35	245-266/321-324
Deltamethrin *	30.44	**181**	207 (61.4)	253 (58.7)			**181**	→ *152*	*151*			1.70	150-153/179-182
Azoxystrobin	30.89	**344**	388 (41.6)	345 (32.7)			**344**	→ *329*	*328*			1.72	325-345

Internal standards ; [a] Compounds present on the mix A (EPA 505/525); [b] Compounds present on the mix B (EPA 505/525); * Contain several diastereoisomers

Table SI3 – Chemical characteristics (class, log K_{ow}, log K_{oc} and GUS index) and license category (according to European pesticides database) of the selected pesticides.

Pesticides	Class	Molecular mass (g/mol)	License[#]	log K_{ow}	log K_{oc}	GUS index
Fungicides						
Azoxystrobin	Antibiotic fungicide	403.4	A	2.5	2.8	2.6
Difenoconazol	Conazole fungicides	406.3	A	4.4	3.6	0.9
HCB	Organochlorines	284.8	B	3.9	4.7	-2.3
PeCB	Aromatic fungicide	250.3	NA	4.8-5.2	4.5	-1.2
Procymidone	Conazole fungicides	284.1	NA	3.3	2.6	1.2
Tebuconazole	Conazole fungicides	307.8	A	3.7	3.0	2.0
Herbicides						
Alachlor	Organochlorines	269.8	NA	3.7	2.5	0.8
Atrazine	Triazine	215.7	NA	2.7	2.0	3.3
Atrazine-desethyl	Triazine	187.6	NA	2.7	1.9	3.5
Cyanazine	Triazine	240.7	NA	2.1	2.3	2.1
Cyhalofop-butyl	Phenoxy herbicides	357.4	A	6.0	3.7	-0.2
Metolachlor	Amide herbicides	283.8	NA	3.4	2.1	3.5
Metribuzin	Triazinone herbicides	214.3	A	1.7	1.8	2.6
Pendimethalin	Dinitroaniline herbicides	281.3	A	5.2	4.4	-0.4
Propazine	Triazine	229.7	NA	4.0	2.2	3.8
Propyzamide	Amide herbicides	256.1	A	3.3	2.9	1.8
Simazine	Triazine	201.6	NA	2.3	2.1	2.0
Simetryn	Triazine	213.3	NA	2.8	2.3	3.0
Terbuthylazine	Triazine	229.7	A	3.4	2.3	3.1
Terbutryn	Triazine	241.4	NA	3.7	3.4	2.4
Trifluralin	Carbamate insecticide	335.3	NA	5.3	4.2	0.1
Insecticides						
Aldrin	Organochlorines	364.9	B	6.5	4.2	-0.4
Azinphos-methyl	Organothiophosphate insecticides	317.3	NA	3.0	3.0	1.0
Chlordane-gamma	Organochlorines	338.9	B	2.8	4.3	-0.8
Chlorfenvinphos Z	Organophosphorus	359.6	NA	3.8	2.8	1.9
Chlorpyriphos	Organophosphorus	350.6	A	4.7	3.9	0.2
Cyfluthrin (beta)	Pyrethroid	434.3	A	5.6	4.8	-1.7
Cyhalothrin (lambda)	Pyrethroid	449.9	A	6.8	5.2	-2.1
Cypermethrin (alpha)	Pyrethroid	416.3	A	6.9	4.4	-2.1
4,4´-DDD	Organochlorines	320.0	B	6.9	4.7	-0.9
4,4'-DDE	Organochlorines	354.5	B	6.9	4.9	-2.0
4,4'-DDT	Organochlorines	318.0	B	6.9	5.9	-4.5
Deltamethrin	Pyrethroid	505.2	A	4.6	7.0	-3.4
Diazinon	Organophosphorus	304.4	NA	3.7	2.8	1.1
Dichlorvos	Organophosphorus	221.0	NA	1.9	1.7	0.7
Dieldrin	Organochlorines	380.9	B	3.7	4.4	-0.3
Dimethoate	Organophosphorus	229.3	A	0.7	1.0	1.1
Endosulfan (alpha)	Organochlorines	406.9	NA	4.7	4.1	-0.1
Endosulfan (beta)	Organochlorines	406.9	NA	4.8	4.3	-0.1
Endosulfan sulfate	Organochlorines	422.9	NA	3.7	3.7	0.5
Endrin	Organochlorines	380.9	NA	3.2	4.0	0.0
Fenamiphos	Organophosphorus	303.4	A	3.3	2.0	-0.1
Fenitrothion	Organophosphorus	277.2	NA	3.3	3.3	0.5
Fonofos	Organophosphorus	246.3	NA	3.9	2.9	2.1
HCCP	Organochlorines	272.3	*	4.0	3.6	0.4
Heptachlor	Organochlorines	373.3	B	5.4	4.4	-0.9
Heptachlor epoxide	Organochlorines	389.3	NA	4.4-5.5	4.3	-1.1
Lindane	Organochlorines	290.8	NA	3.7	3.1	4.0
Malathion	Organophosphorus	330.4	A	2.8	3.3	-1.3
Methoxychlor	Organochlorines	345.7	NA	3.8	4.9	-1.9
Mirex	Organochlorines	545.5	B	5.3	3.8	0.6
Parathion-ethyl	Organophosphorus	291.3	NA	3.8	3.9	2.1
Parathion-methyl	Organophosphorus	263.2	NA	3.0	2.4	1.5
Phosmet	Organothiophosphate insecticides	317.3	A	3.0	3.6	0.2
Pirimicarb	Dinitroaniline herbicides	238.4	A	1.7	2.6	2.7
Tetrachlorvinphos	Organophosphorus	366.0	NA	3.5	3.0	0.3

[#]**NA**- Not authorized; **A**- Authorized; **B**- Banned; according to the EU Pesticides Database **ND**- Not detected;
GUS index (groundwater ubiquity score; GUS= log10 (half life-days) X [4-log10 (Koc)])

Table SI4 – Environmental levels (ng/L) of the selected pesticides, from the surface waters of the Mondego River estuary, per season. Data is presented as mean (CI); n = 7 sites.

Pesticides	Frequency	MDL	MQL	Environmental levels (ng/L)			
	%	% above		spring	summer	autumn	winter
Fungicide							
Azoxystrobin	57	71	94	31.31 (-67.8 - 50.6)	38.72 (-69.8 - 53.4)	2.20 (-37.0 - 29.2)	26.70 (-49.8 - 38.7)
Difenoconazol	79	73	100	687.40 (-1032.8 - 1336.4)	173.18 (-365.8 - 275.0)	4.00 (-3.8 - 4.0)	654.74 (-1714.2 - 1208.8)
HCB	64	78	86	152.27 (-38.8 - 152.3)	36.18 (-51.7 - 36.2)	15.53 (-32.5 - 15.5)	6.75 (-15.3 - 17.7)
PeCB	100	86	100	24.60 (-45.2 - 35.4)	14.73 (-22.0 - 18.7)	53.22 (-187.0 - 122.5)	57.20 (-167.3 - 114.4)
Procymidone	5	100	0	-	238.28 (-55.2 - 379.3)	-	-
Tebuconazole	98	85	100	44.28 (-38.8 - 152.3)	92.52 (-194.7 - 146.5)	69.91 (-183.8 - 129.4)	84.21 (-198.1 - 144.0)
Herbicide							
Alachlor	12	60	100	264.76 (-244.0 - 264.8)	44.87 (-43.1 - 44.9)	78.79 (-230.3 - 157.7)	12.84 (-111.7 - 80.4)
Atrazine	90	71	81	47.18 (-63.3 - 49.9)	38.31 (-26.9 - 59.0)	11.51 (-15.2 - 34.0)	27.95 (-70.2 - 50.1)
Atrazine-desethyl	40	82	93	25.93 (-99.2 - 74.9)	9.41 (-30.8 - 20.5)	165.58 (-263.0 - 174.9)	202.68 (-212.8 - 212.0)
Cyanazine	43	67	100	209.17 (-441.3- 341.5)	149.82 (-248.0 - 203.0)	59.71 (-123.1 - 93.3)	197.82 (-299.0 - 253.5)
Cyhalofop-butyl	83	74	77	43.02 (-67.8 - 52.5)	28.09 (-105.6- 74.9)	20.02 (-36.1 - 28.7)	51.25 (-134.0 - 94.5)
Metolachlor	33	79	91	266.43 (-512.5 - 397.4)	157.25 (-337.8- 295.2)	12.91 (-24.3 - 17.1)	-
Metribuzin	2	100	100	48.72 (-46.8 - 48.7)	-	-	-
Pendimethalin	100	86	100	65.12 (-211.2- 103.7)	53.63 (-43.4 - 64.7)	77.09 (-180.93 - 131.7)	93.10 (-227.7 - 163.7)
Propazine	69	79	100	86.65 (-213.6 - 173.7)	142.63 (-212.4 - 181.1)	214.60 (-282.7 - 253.7)	340.55 (-428.9 - 377.7)
Propyzamide	5	100	100	-	-	100.06 (-96.1 - 100.1)	38.37 (-36.8 - 38.4)
Simazine	48	80	88	23.92 (-35.9 - 27.7)	7.14 (-29.0 - 18.5)	39.92 (-59.4 - 61.9)	64.01 (-84.2 - 75.6)
Simetryn	0	-	-	-	-	-	-
Terbuthylazine	2	100	100	-	-	-	88.44 (-84.9 - 88.4)
Terbutryn	100	86	100	41.88 (-97.3 - 72.6)	40.88 (-88.7 - 66.1)	27.34 (-77.2- 53.3)	24.69 (-74.0 - 50.4)
Trifluralin	74	81	92	86.41 (-180.8 - 136.3)	94.61 (-264.2 - 187.4)	79.66 (-164.9 - 124.8)	196.95 (-274.0 - 240.3)
Insecticide							
Aldrin	67	79	100	14.87 (-55.2 - 43.8)	35.76 (-98.9 - 72.8)	23.85 (-31.3 - 26.8)	41.56 (-78.2 - 61.1)
Azinphos-methyl	21	78	100	28.56 (-55.9 - 43.1)	18.12 (-32.1 - 25.6)	5.86	5.78
Chlordane-gamma	64	89	100	6.53 (-12.3 - 10.1)	5.45 (-7.7 - 6.8)	4.82 (-5.4 - 5.4)	8.43 (-7.1 - 12.3)
Chlorfenvinphos Z	50	90	84	11.03 (-12.6 - 16.2)	16.58 (-25.8 - 19.6)	6.01 (-27.9 - 10.0)	9.02 (-18.3 - 14.0)
Chlorpyriphos	2	100	0	7.72	-	-	-
Cyfluthrin (beta)	67	79	100	16.22 (-26.1 - 21.6)	236.18 (-747.4 - 501.8)	252.88 (-700.6 - 486.5)	544.16 (-1677.3 - 1133.4)
Cyhalothrin (lambda)	43	72	100	328.32 (-427.1 - 414.8)	123.80 (-244.0 - 151.5)	66.13 (-182.4 - 117.3)	295.14 (-485.3 - 369.6)
Cypermethrin (alpha)	19	138	100		121.77 (-342.7-258.2)	125.20 (-267.5 - 200.4)	127.05 (-269.2 - 218.0)
4,4´-DDD	43	67	100	6.94 (-21.6 - 16.8)	9.67 (-15.9 - 13.1)	10.04 (-15.9 - 15.0)	20.04 (-30.1 - 25.6)
4,4'-DDE	98	85	100	38.77 (-74.0 - 56.1)	18.39 (-46.5 - 33.1)	13.78 (-34.6 - 24.7)	13.88 (-23.8 - 19.2)
4,4'-DDT	100	86	100	79.02 (-248.7 - 177.5)	64.55 (-73.6 - 122.7)	46.32 (-88.7 - 68.9)	47.17 (-141.3 - 80.1)
Deltamethrin	88	84	100	806.91 (-1866.1 - 1363.8)	319.12 (-725.2 - 532.8)	372.22 (-1089.2 - 745.6)	-
Diazinon	21	100	100	389.42 (-601.8 - 489.6)	-	-	672.17 (-574.2 - 672.2)
Dichlorvos	98	85	100	21.14 (-44.5 - 37.4)	19.87 (-25.4 - 21.6)	20.99 (-30.1 - 25.1)	28.29 (-35.6 - 29.4)
Dieldrin	76	81	100	27.54 (-252.0 - 63.8)	85.09 (-99.2 - 168.3)	66.45 (-23.3 - 131.8)	32.24 (-68.8 - 54.6)
Dimethoate	100	86	100	188.96 (-431.6 - 317.4)	182.97 (-360.6 - 276.7)	179.90 (-380.1 - 278.4)	93.66 (-250.6 - 182.7)
Endosulfan (alpha)	52	82	100	151.38 (-255.8 - 207.8)	603.46 (-1355.8 - 871.15)	1199.1 (-2422.5 - 1847.8)	505.79 (-1139.3 - 839.3)
Endosulfan (beta)	79	82	100	8.29 (-41.0 - 12.4)	17.57 (-34.3 - 33.0)	8.39 (-6.9 - 10.9)	6.57 (-17.6 - 13.1)
Endosulfan sulfate	55	74	100	67.01 (-115.1 - 92.9)	38.03 (-85.8 - 63.2)	26.75 (-60.2 - 26.75)	19.01 (-53.9 - 37.2)
Endrin	31	62	100	28.15 (-45.7 - 37.7)	57.33 (-73.4 - 62.4)	175.84 (-494.5 - 342.0)	332.40 (-1029.0 - 694.6)
Fenamiphos	29	67	100	20.07 (-25.4 - 24.9)	26.48 (-45.1 - 43.3)	15.41 (-31.5 - 23.9)	35.13 (-34.0 - 35.3)
Fenitrothion	100	86	100	143.85 (-489.2 - 369.8)	172.16 (-332.3- 246.1)	285.14 (-549.3 - 429.1)	210.09 (-557.9 - 391.8)
Fonofos	100	86	100	66.08 (-222.6 - 175.6)	29.13 (-77.0 - 54.1)	21.63 (-26.1 - 38.0)	17.36 (-36.0 - 27.3)
Heptachlor	55	74	100	146.56 (-283.6 - 208.1)	93.54 (-246.9 - 173.7)	14.04 (-13.5 - 14.0)	45.02 (-69.4 - 52.9)
Heptachlor epoxide	100	86	100	9.31 (-17.5 - 14.7)	10.35 (-14.3 - 11.9)	8.12 (-8.2 - 8.2)	9.98 (-16.5 - 13.0)
HCCP	71	97	100	153.73 (-372.4 - 293.2)	89.43 (-186.4 - 140.7)	146.36 (-282.6 - 201.6)	178.09 (-550.5 - 371.7)
Lindane	40	100	100	365.44 (-607.9 - 477.9)	144.19 (-116.3 - 202.7)	419.05 (-341.2 - 652.4)	943.76 (-906.0 - 943.8)
Malathion	83	83	100	129.62 (-336.0 - 237.5)	159.35 (-367.0 - 268.6)	56.10 (-85.4 - 72.2)	50.66 (-89.3 - 75.0)
Methoxychlor	60	76	100	256.17 (-558.2 - 415.5)	217.02 (-406.0 - 317.9)	5.22	16.36
Mirex	19	88	100	75.72 (-145.5 - 112.9)	338.00 (-765.4 - 563.0)	2.43	-
Parathion-ethyl	19	75	100	19.27 (-101.7 - 32.7)	63.79 (-151.8- 110.0)	-	20.35 (-32.0 - 26.7)
Phosmet	93	74	97	66.60 (-195.4 - 139.2)	174.76 (-391.6 - 289.0)	267.65 (-534.7 - 463.9)	424.72 (-879.9 - 665.6)
Pirimicarb	5	100	100	111.38 (-106.9 - 111.4)	-	-	197.74 (-189.8 - 197.7)
Parathion-methyl	86	83	100	48.54 (-56.2 - 74.1)	89.31 (-244.6 - 170.3)	135.13 (-309.9 - 225.1)	98.73 (-157.2 - 103.5)
Tetrachlorvinphos	88	86	100	20.12 (-33.8 - 24.5)	23.09 (-27.3 - 27.5)	21.78 (-35.4 - 31.7)	21.91 (-26.8 -29.8)

Table SI5 – The maximum measured environmental concentrations (MEC) along with the median lethal (effective) concentrations (LC_{50} and EC_{50}) for three distinct groups (algae, invertebrate and fish) used to calculate the predicted no effect concentration (PNEC), the individual MEC/PNEC and the sum of the toxic units (STU) per group.

	Pesticides	MEC (mg/L)	Algae 72h EC_{50} growth (mg/L)	Invertebrate 48h EC_{50} (mg/L)	Fish 96h LC_{50} (mg/L)	PNEC (mg/L)	Individual MEC/PNEC	Individual STU (algae)	Individual STU (invertebrate)	Individual STU (fish)	
FUNGICIDES	Azoxystrobin	9E-05	4E-01	2E-01	5E-01	2E-03	4E-02	3E-04	4E-04	2E-04	
	Difenoconazol	2E-03	3E-02	8E-01	1E+00	3E-04	6E+00	6E-02	3E-03	2E-03	
	HCB	6E-04	1E-02	5E-01	3E-02	1E-04	6E+00	6E-02	1E-03	2E-02	
	PeCB	6E-05	1E+01	-	3E-01	3E-03	2E-02	5E-06	-	2E-04	
	Procymidone	2E-06	3E+00	2E+00	7E+00	2E-02	9E-05	6E-07	9E-07	2E-07	
	Tebuconazole	2E-04	2E+00	3E+00	4E+00	2E-02	1E-02	1E-04	7E-05	5E-05	
HERBICIDES	Alachlor	3E-04	1E+00	1E+01	2E+00	1E-02	3E-02	3E-04	3E-05	1E-04	
	Atrazine	1E-04	6E-02	9E+01	5E+00	6E-04	2E-01	2E-03	1E-06	3E-05	
	Atrazine-desethyl	5E-05	1E-01	-	-	1E-03	5E-02	5E-04	-	-	
	Cyanazine	6E-04	2E-01	5E+01	1E+01	2E-03	3E-01	3E-03	1E-05	6E-05	
	Cyhalofop-butyl	1E-04	1E+00	3E+00	8E-01	8E-03	1E-02	1E-04	4E-05	1E-04	
	Metolachlor	6E-04	6E+01	2E+01	4E+00	4E-02	2E-02	1E-05	3E-05	2E-04	
	Metribuzin	5E-05	2E-02	5E+01	7E+01	2E-04	2E-01	2E-03	1E-06	7E-07	
	Pendimethalin	3E-04	6E-03	3E-01	1E-01	6E-05	5E+00	5E-02	1E-03	2E-03	
	Propazine	2E-04	2E-01	2E+01	2E+01	2E-03	1E-01	1E-03	1E-05	1E-05	
	Propyzamide	7E-07	3E+00	6E+00	5E+00	3E-02	3E-05	3E-07	1E-07	2E-07	
	Simazine	5E-05	4E-02	1E+00	9E+01	4E-04	1E-01	1E-03	5E-05	6E-07	
	Simetryn	4E-07	1E-02	-	7E+00	1E-04	4E-03	4E-05	-	6E-08	
	Terbuthylazine	5E-07	1E-02	2E+01	2E+00	1E-04	4E-03	4E-05	2E-08	2E-07	
	Terbutryn	1E-04	2E-03	3E+00	1E+00	2E-05	6E+00	6E-02	5E-05	1E-04	
	Trifluralin	3E-04	1E-02	2E-01	9E-02	1E-04	2E+00	2E-02	1E-03	3E-03	
INSECTICIDES	Aldrin	3E-05	-	3E-02	5E-03	5E-05	6E-01	-	1E-03	6E-03	
	Azinphos-methyl	6E-05	7E+00	1E-03	2E-02	1E-05	6E+00	9E-06	6E-02	3E-03	
	Chlordane-gamma	1E-05	-	6E-01	9E-02	9E-04	1E-02	-	2E-05	1E-04	
	Chlorpyriphos	8E-06	5E-01	4E-05	1E-03	4E-07	2E+01	2E-05	2E-01	6E-03	
	Chlorfenvinphos Z	2E-05	1E+00	3E-04	1E+00	3E-06	9E+00	2E-05	9E-02	2E-05	
	Cyfluthrin (beta)	3E-05	1E+01	2E-04	5E-04	2E-06	2E+01	3E-06	2E-01	7E-02	
	Cyhalothrin (lambda)	6E-04	-	4E-01	5E-04	5E-06	1E+02	-	2E-03	1E+00	
	Cypermethrin (alpha)	1E-06	1E-01	3E-04	3E-03	3E-06	4E-01	1E-05	4E-03	4E-04	
	4,4´-Σ DDD,DDE,DDT	6E-04	-	5E-03	7E+00	5E-05	1E+01	-	1E-01	9E-05	
	Deltamethrin	3E-03	9E+00	6E-04	3E-04	3E-06	1E+03	3E-04	6E+00	**1E+01**	
	Diazinon	5E-04	6E+00	1E-03	3E+00	1E-05	5E+01	9E-05	5E-01	2E-04	
	Dichlorvos	4E-05	5E+01	2E-04	6E-01	2E-06	2E+01	7E-07	2E-01	7E-05	
	Dieldrin	3E-04	1E-01	3E-01	1E-03	1E-05	2E+01	3E-03	1E-03	2E-01	
	Dimethoate	5E-04	9E+01	2E+00	3E+01	2E-02	2E-02	5E-06	2E-04	2E-05	
	Endosulfan (alpha+beta)	3E-04	2E+00	4E-01	2E-03	2E-05	1E+01	1E-04	7E-04	1E-01	
	Endosulfan sulfate	2E-04	-	-	-	-	-	-	-	-	
	Endrin	5E-05	-	4E-03	7E-04	7E-06	7E+00	-	1E-02	7E-02	
	Fenamiphos	1E-04	4E+00	2E-03	9E-03	2E-05	6E+00	3E-05	6E-02	1E-02	
	Fenitrothion	7E-04	1E+00	9E-03	1E+00	9E-05	8E+00	5E-04	8E-02	5E-04	
	Fonofos	5E-04	2E+00	2E-03	3E-02	2E-05	2E+01	3E-04	2E-01	2E-02	
	Heptachlor	4E-04	3E-02	4E-02	7E-03	7E-05	6E+00	2E-02	1E-02	6E-02	
	Heptachlor epoxide	2E-05	2E+02	2E-01	2E-02	2E-04	1E-01	1E-07	9E-05	1E-03	
	HCCP	5E-04	-	5E-02	2E+00	5E-04	1E+00	-	1E-02	2E-04	
	Lindane	9E-04	3E+00	2E+00	3E-03	3E-05	3E+01	4E-04	6E-04	3E-01	
	Malathion	6E-04	1E+01	7E-04	2E-02	7E-06	8E+01	4E-05	8E-01	3E-02	
	Methoxychlor	8E-04	6E-01	8E-04	5E-02	8E-06	1E+02	1E-03	1E+00	2E-02	
	Mirex	2E-04	1E-01	1E-01	1E+02	1E-03	2E-01	2E-03	2E-03	2E-06	
	Parathion-ethyl	1E-04	5E-01	3E-03	2E+00	3E-05	4E+00	2E-04	4E-02	7E-05	
	Phosmet	2E-04	7E-02	2E-03	2E-01	2E-05	1E+01	3E-03	1E-01	9E-04	
	Pirimicarb	1E-04	1E+02	2E-02	1E+02	2E-04	7E-01	8E-07	7E-03	1E-06	
	Parathion-methyl	2E-04	3E+00	7E-03	3E+00	7E-05	2E+00	5E-05	2E-02	6E-05	
	Tetrachlorvinphos	4E-05	-	2E-03	4E-01	2E-05	2E+00	-	2E-02	1E-04	
						$RQ_{MEC/PNEC}$	1816	0.30	9.41	**14.27**	SUM
						$RQ_{STU(100)}$	1427				
						IA/CA	1.188				

Chapter 5

Seasonal-spatial survey of pesticides in the most relevant estuary of the Iberian Peninsula — The Tagus River

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Seasonal-spatial survey of pesticides in the most significant estuary of the Iberian Peninsula—The Tagus River Estuary

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Abstract

Estuarine environments are being constantly pressured by new sources of pollution (e.g. new pesticides) derived from the activities of industry and intensive agriculture. The present study aim at quantify pesticides of three different categories (fungicides, herbicides and insecticides) in the Tagus River basin. Seasonal transition water samples were collected during 2010 and 2011, from seven sites distributed on both margins (north and south) of this estuary. Pesticides were subjected to solid phase extraction and then analyzed using gas chromatography coupled to tandem mass spectroscopy. Data showed that 95% of the analyzed pesticides were measurable in all samples, some being above the 2013/39/EU Directive levels (total loads ranged from 1.4 μg/L, in winter, up to 5.5 μg/L, in spring). Additionally, a panel of selected physicochemical parameters—linked with water quality standards, such as total phosphorous and total nitrogen—revealed high amounts close to the Trancão River mouth. Centered on the concentration addition and independent action models, a two-tiered approach was used to assess the risk of the pesticide mixture, at the maximum concentration found. This reflected the potential risk, mainly to fish. From the results, we conclude that legal quality standards are not being followed in this emblematic ecosystem and so there are risks to the biota. Hence, refined studies are required to identify both sources and impacts of the pesticides, and also to monitor preventive and depollution measures.

1. Introduction

Estuarine and coastal environments play a vital role as fish nurseries and habitat for benthic communities and local fauna. In spite of their importance, these aquatic environments are being heavily impacted by human activities through pollution (Barbier et al., 2010). Pesticides are compounds intentionally used mainly for agricultural purposes, with biodegradation-resistance able to affect aquatic habitats directly (through water/sediments) and/or indirectly (bio-amplification)(Katagi, 2010). Negative effects, such as slow growth rate, larval deficiencies, and mortality have already been described for bivalves, crustaceans, fishes and birds (Köhler and Triebskorn, 2013). Therefore, international initiatives have been taken to promote an effective regulation and management of pesticides, alerting already for the present levels in soils and biota, through Directive 2013/39/EU (EU, 2013). Significant total loads of pesticides were registered in several Portuguese estuarine systems, like the Douro River (3000 ng/L; Rocha et al., 2012), Mondego River (6000 ng/L; Cruzeiro et al., 2016), and the Ria Formosa Lagoon (1800 ng/L; Cruzeiro et al., 2015) attaining individual concentrations above the Environmental Quality Standards set by 2013/39/EU Directive.

The Tagus River estuary holds one of the most important natural reserves of Europe, because it contains the primary wetlands used by birds migrating between northern Europe and Africa (Catry et al., 2011). It is characterized by an extensive surface of estuarine waters, vast fields intersected by creeks, marshes, salt flats, and alluvial agricultural land (marshlands), and more than 250 species of birds can be found there (Instituto da Conservação da Natureza e da Biodiversidade, 2007). Along its 1038 km path, which makes it the longest river in Iberian Peninsula, the Tagus is subjected to anthropogenic impacts from urban, agricultural, and industrial areas, mainly near the cities of Madrid and Lisbon (Ferreira et al., 2003). In spite of this, few surveys have been done of this aquatic system (Batista et al., 2002; Sanches et al., 2012; Silva et al., 2015; Silva et al., 2012b).

In the current study, we evaluated the annual/seasonal patterns of fluctuation for a wide panel of pesticides (insecticides, herbicides and fungicides)

using seven sampling sites in the Tagus River estuary. Official databases, such as European Commission database Regulation (EC No 1107/2009) and Portuguese Regional Directorate of Agriculture and Fisheries (DRAP) were consulted in order to select a representative panel of the authorized, unauthorized, and banned pesticides in use (DRAP, 2014; EU, 2009). The fifty-six pesticides were analyzed using gas chromatography coupled with mass detection (GC-MS/MS). Physicochemical water-quality parameters linked with anthropogenic contamination (dissolved oxygen (DO), pH, nitrates, nitrites, ammonia and phosphates) were also measured (Pesce et al., 2008). These, and a two-tiered approach based on concentration addition and independent action, was used to predict the environmental risk of chemical mixtures at the concentrations found in this estuary (Backhaus and Faust, 2012).

The present research aims to: (1) provide seasonal data on the concentrations of fifty-six pesticides in surface transitional water-samples collected from seven sites in the Tagus River estuary; (2) determine which pesticides were above the European directive limits; (3) discover if banned pesticides were being used in this habitat; (4) link the physicochemical water-quality parameters with pesticide concentrations; (5) prepare an ecotoxicological risk assessment of the pesticide mixtures found at maximum concentrations.

2. Materials and methods

2.1. Tagus River estuary

The Tagus River extends 1038 km, from Spain to Portugal, debouching into the Atlantic Ocean near the Portuguese capital, Lisbon. The Tagus Estuary is approximately 30 km long (320 km^2), representing the second largest watershed in Portuguese territory (Ferreira et al., 2003). As a mesotidal estuary formed by mudflats, the Tagus tidal amplitude ranges from 1 to 4 m and, creating an intertidal area that makes up 20-40% of the total area. The fresh water flows into the estuary, are affected by seasonal variation and human interventions upstream (Costa et al., 2007).

For this study, seven intertidal sampling sites were selected along the estuary (Fig. 1). At the north margin, there were four sampling stations. Two were near Lisbon (S_1 - 38°42'16.1" N, 9°09'21.4" W and S_2 - 38°46'02.0" N 9°05'31.8" W), while the other two were near the cities of Sacavém (S_3 - 38°47'47.6", N 9°05'46.1" W), and Vila Franca de Xira (S_4 - 38°55'30.1"N, 9°00'22.2"W). The one near Sacavém is proximal to the entrance of the Trancão River tributary into the Tagus Estuary, whereas, the one near Vila France de Xira, is where the Tagus River enters in its estuary. Three other sampling sites occurred on the south margin located near the cities of Alcochete (S_5 - 38°45'20.9" N, 8°57'50.6" W), Montijo (S_6 - 38°41'58.7" N, 8°58'02.9" W), and Seixal (S_7 - 38°38'42.0" N, 9°06'13.6" W).

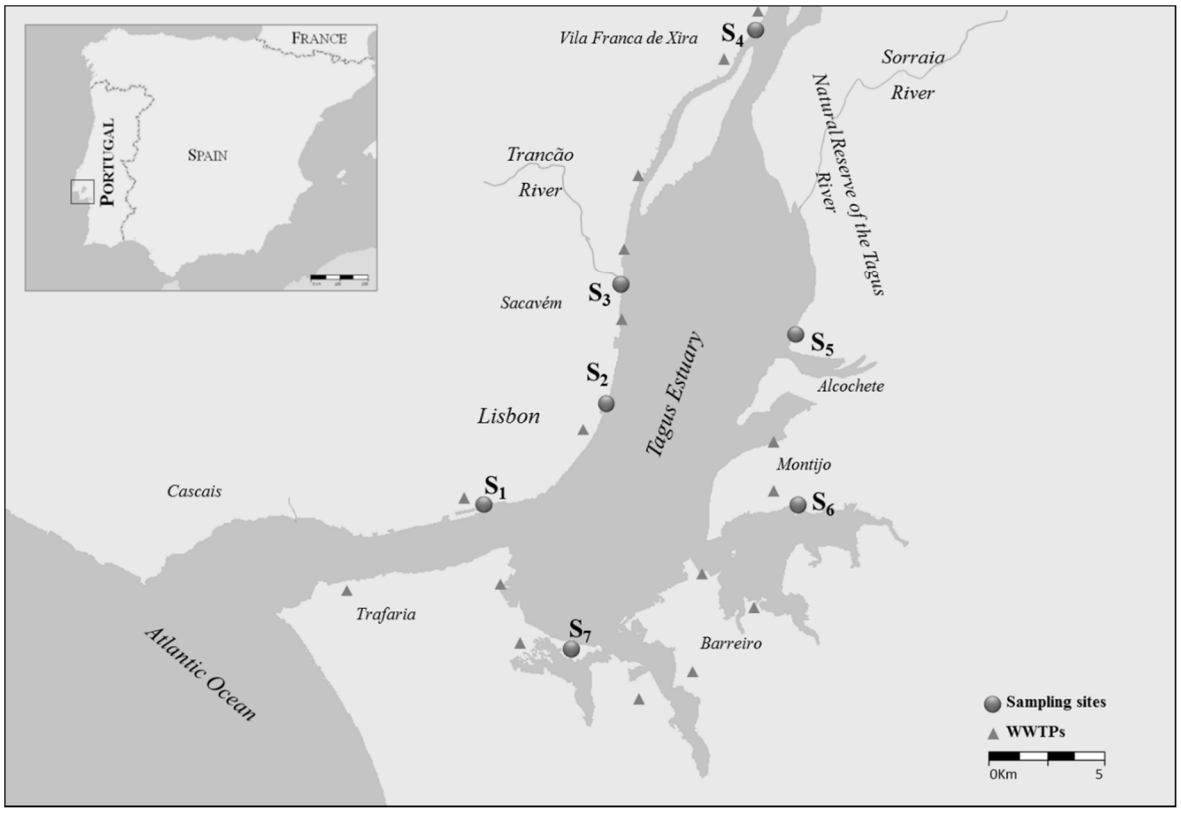

Fig. 1 – Location of the sampling sites in the Tagus River estuary (S_1 to S_7), Portugal, as well as the main cities and wastewater treatment plants (WWTPs) (adapted from Microsoft MapPoint, 2010).

2.2. Chemicals and materials

The analytical grade solvents methanol (MeOH), ethyl acetate (EtOAc) and hexane were purchased from Romil (Cambridge, England). The pesticide standards were supplied by Sigma-Aldrich (Steinheim, Germany). With the exception of Mix A (EPA 505/525, 500 mg/L) and Mix B (EPA 505/525, 500 mg/L), all other pesticides were purchased individually. The 4,4'-DDT-d_8 and atrazine-d_5 were used as both surrogates and internal standards (IS). Individual pesticides were prepared in MeOH at 1000 mg/L and kept in the dark at -20 ºC. Ultrapure water was obtained from a Milli-Q water system (conductivity = 0.054 µS cm/L, at 25 ºC). The solid-phase extraction (SPE) cartridges, 200 mg Oasis HLB (Hydrophilic-Lipophilic Balance), 6 cc, were acquired from Waters Corporation (Milford, USA).

2.3. Water collection and quality measurement

Transitional water samples (2.5 L) were collected at ebb tide, at half meter depth, from 2010 (at April, June, September, October and December) until February 2011, into amber glass bottles, pre-rinsed with local water. In the laboratory, samples (1 L) were immediately filtrated through a 0.45 µm glass fiber filter (Munktell, Germany). The filtrates were acidified with H_2SO_4 to pH 7, and then 500 mL was subjected to SPE, within 24 h. From transport until extraction, samples were kept at ~ 4 °C. The temperature (ºC), DO (mg/L), salinity, and conductivity (mS/cm) were measured *in situ*, using portable meters (OXi 330i/ Set WTW and LF 330/ Set WTW). The pH (portable Hech HQ40d), as well as nitrites, nitrates, ammonium and phosphates (Palintest Photometer 7000) were measured in the laboratory

2.4. Sample preparation

All pesticides were extracted by solid phase extraction (SPE, OASIS HLB) following the published protocol (Cruzeiro et al., 2015). Briefly, cartridges were conditioned sequentially with 5 mL of ethyl acetate, followed by 5 mL of methanol and 2.5 mL of ultrapure water, at a flow rate of 1-2 mL/min.

The water samples (500 mL), added with the IS, were loaded in to SPE cartridges at a constant flow-rate of 5 mL/min. The cartridges were dried and then eluted with 6 mL of ethyl acetate, at 1 mL/min. The extracts were concentrated into 200 μL of hexane and kept in vials at -80 ºC.

2.5. Quantitative analyses

The analyses were carried out using GC-MS/MS, with the software Xcalibur (version 2.0.7, 2007, Thermo Scientific) (see supplementary material Table SM1 and SM2). The final chromatogram (full scan) was then analyzed using the NIST Mass Spectral Search Program library (version 2.0, 2005) to create a selected-ion-monitoring mode (SIM) for pesticide quantification (Table SM2). For the identification of the compounds by MS/MS, optimized energies were applied to get the best parent/daughters ratio (Table SM2).

Eight nominal working concentrations were prepared by spiking clean water samples from a headspring of the Febros River (41°01'58.0" N, 8°33'11.1" W), to which was added sodium chloride (99.8%; EMSURE® Merck, Germany) to obtain an average salinity of 23 (w/v). This matrix was used as a calibration standard and to validate the method. The final range of concentrations was 10 – 400 ng/L for all pesticides and 160 ng/L for the IS. Blanks at an intermediate concentration (160 ng/L) were used to ensure both the absence of contamination and the existence of a quality control.

2.6. Data presentation and statistical analyses

Analytical data for each pesticide are displayed as the average values of the instrumental replicates (n = 3) per season, followed by the 95% confidence interval (CI). For having comparable estimates of the total average (TA) loads of the compounds (*i.e.*, total sums) the data are organized by chemical, grouped by category (fungicides, herbicides and insecticides) and displayed by the season (spring, summer, autumn and winter). Physicochemical parameters are presented by site as average (CI) loads.

Descriptive and inferential statistics were primed with PAST 3.06 software (Hammer et al., 2001). First, the assumptions of normality and homogeneity

of variances were checked using the Shapiro-Wilk W-test and Levine's test, respectively. Then, independent comparisons between seasons and categorical groups were analyzed using one-way analysis of variance (ANOVA), and post-hoc comparisons were made using Tukey's test. When the cited parametric assumptions were not valid, and data transformation was ineffective, the Kruskal-Wallis non-parametric ANOVA test was used. In the latter case, post-hoc comparisons were made using the Mann-Whitney U test, with sequential Bonferroni correction. The usual standard level of significance (α = 0.05) was seen as the threshold for a greater strength of evidence.

The correlation analysis was achieved with GraphPad Software (Prism 6) using the Pearson's correlation test to evaluate statistical differences (Muzyka et al., 2012).

The GUS index (groundwater ubiquity score; supplementary material Table SM3) was also calculated as an indicator of the potential pollution by pesticides, considering their persistence and binding ability to soil particles: GUS= log10 (half-life days) X [4-log10 (Koc)]). The GUS score ranges from extremely low (< 0.1) to very high (> 4.0), and is used to rate the leaching potential of pesticides for moving towards groundwater (Gustafson, 1989), as it was used by Claver et al. (2006).

Based on the two reference models, concentration addition (CA) and independent action (IA), a two-tiered approach was suggested by Backhaus & Faust (2012) to predict environmental hazard and risks evaluation of several pesticides. In accordance with the last authors, the classical CA (first tier) was herein calculated using the sum of the maximum Measured Environmental Concentrations (MEC) of pesticides in the Tagus River estuary. This was followed by the ratio between MEC and their Predicted No Effect (PNEC) and/or the ratio between MEC and the biological end point per trophic level, defined as the toxic units risk quotient (RQ_{TU}). If both CA and IA approaches failed (*i.e.*, risk quotient (RQ) > 1), additional IA considerations must be evaluated (Backhaus and Faust, 2012). According to Junghans et al. (2006) a new ratio should be calculated, considering STU and $maxRQ_{TU}$, to predict the second tier. This new ratio gives the maximum value by which CA may predict a higher toxicity than IA (Silva and Cerejeira, 2014). To proceed with the first

tier outline, the average effective and/or lethal concentration (EC_{50}/ LC_{50}) values, for each trophic level algae, crustaceans and fish), were obtained from the FOOTPRINT pesticide and PubChem chemistry databases (Agriculture & Environment Research Unit (AERU), 2013; National Center for Biotechnology Information (NCBI), 2015); no data were available for pentachlorobenzene (PeCB), atrazine-desethyl, endosulfan sulphate and hexachlorocyclopentadiene (HCCP).

3. Results

3.1. Pesticide levels in the Tagus estuary surface waters

Table 1 shows pesticide concentrations per season, as well as, the percentage of samples above the detection (MDL) and quantification limits (MQL). Based on 2352 determinations, the total annual (TA) loads of pesticides were grouped per category (insecticides, fungicides and herbicides) and per season (Fig. 2A). The highest concentrations were measured in spring, for insecticides ($p < 0.05$) and fungicides ($p = 0.071$), while no suggestive seasonal fluctuations were found for herbicides ($p = 0.957$). S_3 presented the highest average annual concentration of pesticides ($p < 0.05$; Fig. 2B).

Table 1 – Percentage of samples above the detection (MDL) and quantification limits (MQL), as well as the environmental levels (ng/L) of the selected pesticides, for coastal surface waters in the Tagus River estuary, per season; data are presented as mean (confidence interval).

Pesticides	Frequency	MDL	MQL	Environmental levels (ng/L)			
	%	% above		spring	summer	autumn	winter
Fungicides (total amounts)				**184.74**	**123.27**	**99.63**	**86.08**
Azoxystrobin	90	26.3	18.4	9.85 (9.77-9.92)	9.08 (9.06-9.11)	11.1 (11.1-11.1)	6.20 (6.1-6.3)
Difenoconazol	100	90.5	90.5	104.4 (104.3-104.6)	72.6 (72.4-72.7)	36.6 (36.4-36.7)	23.1 (23.10-23.19)
HCB	100	83.3	76.2	5.38 (5.36-5.39)	2.85 (2.84-2.86)	4.37 (4.36-4.38)	4.49 (4.48-4.50)
PeCB	100	90.5	90.5	20.8 (20.75-20.77)	16.9 (16.88-16.89)	16.9 (16.947-16.950)	17.2 (17.176-17.184)
Procymidone	100	88.1	88.1	15.6 (15.59-15.71)	13.5 (13.48-13.55)	22.8 (22.77-22.90)	24.0 (23.9-24.1)
Tebuconazole	100	16.7	16.7	28.7 (28.60-28.73)	8.35 (8.29-8.41)	7.81 (7.7-7.9)	11.1 (10.9-11.3)
Herbicides (total amounts)				**1640.49**	**509.92**	**553.89**	**574.46**
Alachlor	100	16.7	16.7	6.16 (6.14-6.17)	13.8 (13.82-13.87)	5.65 (5.64-5.66)	4.39 (4.38-4.40)
Atrazine	98	90.2	90.2	40.1 (40.05-40.10)	20.1 (20.13-20.14)	20.4 (20.44-20.45)	25.4 (25.38-25.40)
Atrazine-desethyl	100	90.5	90.5	11.8 (11.80-11.81)	11.1 (11.12-11.13)	11.3 (11.34-11.35)	10.8 (10.84-10.85)
Cyanazine	100	90.5	90.5	523.0 (522.96-522.99)	21.2 (21.23-21.24)	30.0 (29.97-29.98)	44.4 (44.35-44.37)
Cyhalofop-butyl	100	88.1	88.1	295.8 (293.1-298.5)	59.8 (59.3-60.3)	98.7 (98.71-98.74)	356.9 (356.7-357.1)
Metolachlor	86	88.9	88.9	5.04 (5.01-5.06)	3.99 (3.98-4.00)	4.31 (4.29-4.32)	5.65 (5.59-5.71)
Metribuzin	52	81.8	81.8	21.0 (20.98-21.00)	18.1 (18.12-18.13)	15.2 (15.22-15.23)	17.5 (17.50-17.51)
Pendimethalin	100	66.7	66.7	167.1 (167.06-167.22)	175.1 (175.0-175.2)	176.4 (176.31-176.44)	15.3 (15.29-15.30)
Propazine	100	0.0	0.0	-	-	-	-
Propyzamide	100	90.5	90.5	16.9 (16.85-16.88)	18.3 (18.33-18.35)	15.6 (15.62-15.64)	15.2 (15.20-15.23)
Simazine	100	90.5	73.8	66.8 (66.78-66.81)	24.4 (24.40-24.42)	10.8 (10.78-10.80)	29.8 (29.75-29.77)
Simetryn	52	81.8	81.8	23.2 (23.21-23.22)	22.5 (22.51-22.52)	19.8 (19.84-19.85)	19.5 (19.54-19.55)
Terbuthylazine	100	11.9	11.9	326.5 (326.3-326.6)	49.3 (49.30-49.39)	93.5 (93.49-93.52)	7.13 (7.10-7.17)
Terbutryn	100	83.3	83.3	137.2 (137.1-137.3)	71.9 (71.87-72.01)	52.1 (51.9-52.2)	22.5 (22.42-22.50)
Trifluralin	100	0.0	0.0	-	-	-	-
Insecticides (total amounts)				**3658.81**	**2200.65**	**975.97**	**731.54**
Aldrin	29	66.7	66.7	20.1 (20.08-20.13)	21.5 (21.44-21.52)	21.4 (21.32-21.46)	27.1 (26.9-27.4)
Azinphos-methyl	100	90.5	90.5	179.8 (179.6-180.0)	59.2 (59.08-59.23)	53.3 (53.18-53.32)	46.3 (46.1-46.6)
Chlordane-gamma	83	88.6	88.6	2.95 (2.95-2.96)	2.89 (2.89-2.90)	2.92 (2.92-2.93)	2.92 (2.92-2.92)
Chlorfenvinphos Z	100	7.1	4.8	1.62 (1.55-1.69)	-	-	2.81 (2.5-3.1)
Chlorpyriphos	74	87.1	87.1	19.2 (18.9-19.6)	10.2 (10.1-10.3)	10.5 (10.4-10.6)	19.6 (19.4-19.8)
Cyfluthrin (beta)	86	88.9	88.9	43.0 (42.99-43.09)	115.3 (115.20-115.31)	62.9 (62.87-62.94)	32.0 (31.92-32.00)
Cyhalothrin (lambda)	100	81.0	78.6	388.9 (387.9-389.9)	372.9 (372.0-373.7)	73.2 (73.0-73.5)	39.3 (39.2-39.5)

(continued)

Pesticides	Frequency	MDL	MQL	Environmental levels (ng/L)			
	%	% above		spring	summer	autumn	winter
Insecticides (total amounts)				**3967.61**	**1977.03**	**739.84**	**676.77**
Cypermethrin (alpha)	100	11.9	11.9	108.6 (108.47-108.64)	42.9 (42.873-42.874)	42.0 (41.8-42.1)	-
4,4'-DDD	76	87.5	87.5	3.16 (3.157-3.170)	3.1 (3.091-3.099)	3.04 (3.040-3.046)	3.26 (3.24-3.27)
4,4'-DDE	100	90.5	90.5	13.9 (13.87-13.91)	13.5 (13.51-13.55)	13.3 (13.24-13.27)	13.4 (13.35-13.43)
4,4'-DDT	100	83.3	83.3	49.7 (49.4-50.1)	52.5 (52.1-52.9)	38.4 (38.1-38.6)	73.3 (73.0-73.6)
Deltamethrin	90	86.8	86.8	1145 (1144.1-1145.3)	878.6 (878.2-879.0)	187.9 (187.8-188.0)	30.1 (30.10-30.17)
Diazinon	98	90.2	90.2	55.3 (55.24-55.28)	37.1 (37.11-37.19)	20.8 (20.82-20.85)	18.1 (18.04-18.10)
Dichlorvos	100	90.5	90.5	19.7 (19.66-19.68)	16.3 (16.29-16.30)	15.6 (15.57-15.58)	15.8 (15.77-15.79)
Dieldrin	100	45.2	40.5	18.3 (18.1-18.5)	12.7 (12.4-13.0)	14.5 (14.1-14.8)	40.9 (40.7-41.0)
Dimethoate	100	78.6	76.2	145.5 (145.4-145.6)	99.2 (99.1-99.3)	42.8 (42.77-42.86)	15.0 (14.9-15.1)
Endosulfan (alpha)	100	90.5	90.5	90.5 (90.45-90.51)	90.5 (90.44-90.51)	84.1 (84.04-84.09)	64.1 (63.7-64.5)
Endosulfan (beta)	76	6.3	6.3	3.02 (3.00-3.04)	-	-	15.9 (15.89-15.95)
Endosulfan sulfate	100	90.5	90.5	26.8 (26.72-26.84)	17.3 (17.32-17.35)	13.3 (13.27-13.31)	16.1 (16.08-16.13)
Endrin	71	0.0	0.0	-	-	-	-
Fenamiphos	88	89.2	89.2	13.1 (13.12-13.15)	11.3 (11.25-11.27)	12.4 (12.35-12.37)	11.6 (11.59-11.61)
Fenitrothion	100	83.3	83.3	109.1 (109.0-109.3)	46.6 (46.5-46.7)	33.5 (33.38-33.53)	35.5 (35.4-35.6)
Fonofos	100	90.5	90.5	22.5 (22.43-22.52)	18.8 (18.81-18.87)	16.8 (16.79-16.85)	16.8 (16.77-16.86)
Heptachlor	71	86.7	86.7	25.3 (25.250-25.255)	23.5 (23.48-23.49)	23.4 (23.445-23.447)	22.8 (22.83-22.84)
Heptachlor epoxide	100	23.8	2.4	-	-	-	2.33 (2.32-2.34)
HCCP	100	90.5	90.5	559.7 (559.5-560.0)	42.5 (42.46-42.49)	35.8 (35.80-35.82)	22.7 (22.670-22.674)
Lindane	100	85.7	73.8	14.2 (14.13-14.23)	11.5 (11.50-11.55)	7.09 (7.07-7.11)	5.29 (5.27-5.31)
Malathion	76	87.5	87.5	52.0 (51.98-52.05)	41.5 (41.51-41.57)	31.7 (31.63-31.68)	33.8 (33.80-33.86)
Methoxychlor	98	90.2	90.2	21.9 (21.8-22.0)	25.7 (25.6-25.8)	7.31 (7.28-7.33)	4.60 (4.59-4.61)
Mirex	100	90.5	90.5	92.0 (91.8-92.1)	11.8 (11.78-11.89)	6.02 (5.99-6.05)	8.05 (8.01-8.08)
Parathion-ethyl	98	56.1	43.9	15.0 (15.02-15.03)	8.89 (8.89-8.90)	6.91 (6.907-6.911)	6.28 (6.281-6.282)
Phosmet	100	28.6	28.6	306.4 (306.37-306.44)	30.0 (29.95-29.97)	30.7 (30.71-30.73)	-
Pirimicarb	98	90.2	90.2	26.4 (26.40-26.45)	25.3 (25.31-25.35)	26.7 (26.70-26.75)	25.3 (25.33-25.35)
Parathion-methyl	95	72.5	70.0	52.1 (52.06-52.15)	43.7 (43.58-43.73)	21.8 (21.70-21.82)	51.3 (51.25-51.41)
Tetrachlorvinphos	100	90.5	90.5	14.4 (14.3-14.5)	14.0 (13.9-14.0)	15.1 (15.0-15.2)	11.3 (11.2-11.5)

MDL- method detection limit; **MQL**- method quantification limit

Fungicides: All the selected fungicides were quantified (Table 1) but no seasonal differences were observed. However the highest TA amounts were measured in spring (Σ_{spring} ≈ 185 ng/L (40.0–200.9, CI)) and the lower ones in winter (Σ_{winter} ≈ 86 ng/L (57.5–88.3, CI)), representing a 2-fold decrease between these two seasons. Within this category, only the levels of difenoconazol suggestively increased (≈ 104 ng/L) in spring ($p = 0.055$).

Herbicides: Thirteen of the fifteen screened herbicides were quantified, registering annual average loads and frequencies of ≈ 820 ng/L and 87%, respectively (Table 1). Like the fungicides, no seasonal differences were observed for herbicides. In spring, the TA amounted ≈ 1.6 µg/L and in the other seasons ≈ 546 ng/L. Individually, constant concentrations were observed throughout the year for atrazine-desethyl (≈ 11 ng/L), metolachlor (≈ 5 ng/L), metribuzin (≈ 18 ng/L), propyzamide (≈ 17 ng/L) and simetryn (≈ 21 ng/L). Both propazine and trifluralin were below the MDLs.

Insecticides: A pool of thirty-five insecticides was selected and only endrin was below the MDL (Table 1). The total annual average concentration of this category was Σ ≈ 1.9 µg/L, presenting higher values in spring (Σ_{spring} ≈ 3.7 µg/L (694.1–4973.9, CI)) than in all other seasons. In winter, the levels of these compounds were 5-fold lower than in spring ($p < 0.05$). Following this pattern, the insecticides azinphos-methyl (≈180 ng/L), cyhalothrin (lambda) (≈ 389 ng/L), cypermethrin (alpha) (≈ 109 ng/L), deltamethrin (≈1.1 µg/L), dimethoate (≈ 146 ng/L), fenitrothion (≈ 109 ng/L), HCCP (≈ 560 ng/L) and phosmet (≈ 306 ng/L), also show higher concentrations ($p < 0.05$) in spring than in winter, when their levels decreased 3- to 38-fold.

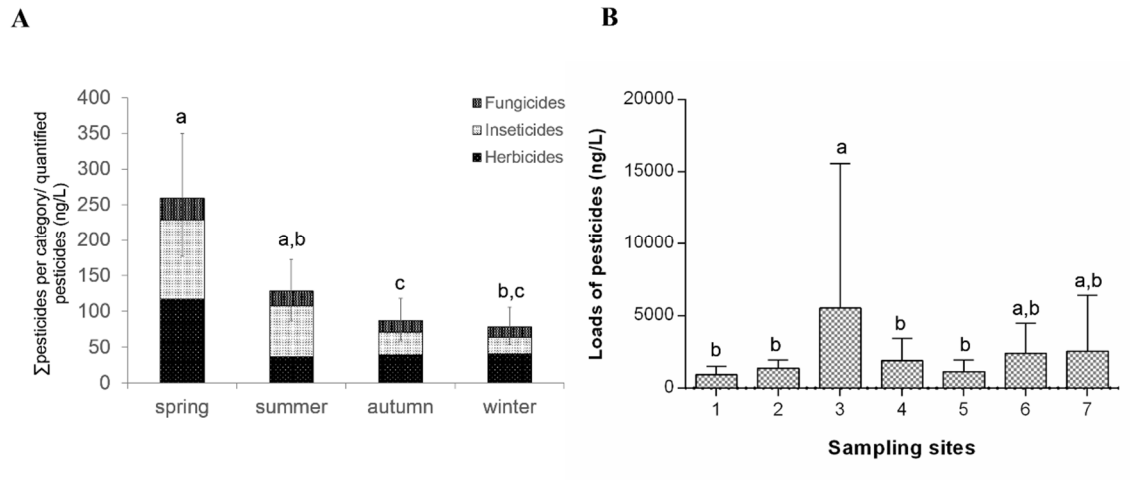

Fig. 2 – Pesticide concentrations (Σ ng/L): (A) per season and normalized by the number of quantified pesticides; (B) per sampled site; data are expressed as total average (TA) loads per pesticide category (CI, confidence interval).

3.2. Physicochemical parameters

Physicochemical parameters are organized by margin, site, and season (Table 2). Annually, the temperature fluctuated from 12 ºC (winter) to 25 ºC (summer), while salinity, conductivity, and pH were constant between seasons (Table 2). According to the two margins, there was a trend of higher amounts of nitrates, nitrites, and phosphates on the north margin, where the levels of both ammonia and un-ionized ammonia were significantly higher ($p < 0.003$ and $p < 0.018$, respectively), than those measured on the south margin. Among the sampling sites, S_3 presented the highest and most significant amounts of nitrites ($p = 0.003$), ammonia ($p = 0.010$), and phosphates ($p = 0.025$, Table 2). Pearson's correlations (r) were also done between TA loads of compounds and physicochemical parameters. TA loads of pesticides presented significant correlations ($p < 0.05$) between salinity ($r = -0.37$), temperature ($r = 0.50$), nitrites ($r = 0.49$) and phosphates ($r = 0.62$), mainly due to data of the north margin in spring and autumn (Table 3).

Table 2 – Physicochemical parameter data by site, margin and season.

Season	Margin	Site	Dissolved O_2		Temperature	Salinity	Condutivity	pH	Nitrites	Nitrates	Ammonia	Un-ionized ammonia	Phosphates
			mg/L	%	ºC		mS/cm				mg/L		
Spring	North	S_1	8.65	95.5	17.50	25.20	38.30	8.05	0.05	0.20	0.57	0.02	0.22
		S_2	8.10	92.5	18.00	21.70	33.65	7.98	0.15	0.58	0.90	0.03	0.17
		S_3	4.95	57.0	20.55	5.00	8.73	8.05	0.84*	0.40	7.25*	0.24*	3.30*
		S_4	8.30	94.5	18.50	21.15	29.20	7.98	0.07	0.14	0.72	0.02	0.79
	South	S_5	8.60	95.5	19.45	25.30	40.20	8.03	0.08	0.20	0.60	0.02	1.35
		S_6	9.20	101.0	17.55	0.10	0.70	8.09	0.05	0.74	0.11	0.00	1.80
		S_7	9.20	103.5	20.25	15.65	33.25	8.02	0.24	0.14	0.85	0.03	0.90
Summer	North	S_1	8.60	113.0	23.60	27.30	42.60	8.30	0.07	0.25	2.44	0.12	1.15
		S_2	7.10	93.0	24.50	21.40	43.20	8.06	0.50	0.10	0.57	0.03	1.40
		S_3	4.70	63.0	26.10	14.90	24.40	8.10	0.93*	0.90	3.54*	0.18*	3.4*
		S_4	6.90	91.0	24.60	24.60	38.70	8.07	0.16	0.27	0.84	0.04	1.10
	South	S_5	7.40	98.0	25.10	30.80	47.40	8.12	0.23	0.15	0.81	0.04	0.90
		S_6	7.70	103.0	25.30	0.30	1.05	8.31	0.01	0.85	0.01	0.00	2.50
		S_7	6.90	92.0	25.50	30.60	37.20	8.09	0.29	0.24	1.71	0.09	1.10
Autumn	North	S_1	7.90	89.0	17.65	27.85	43.60	8.38	0.04	0.58	1.05	0.03	0.19
		S_2	6.05	72.5	17.50	21.00	33.80	8.52	0.09	0.61	3.21	0.11	0.52
		S_3	4.45	49.5	18.80	7.70	12.83	8.26	0.32*	1.85	5.65*	0.19	0.98*
		S_4	6.55	79.5	19.70	18.50	30.25	8.54	0.04	0.67	1.44	0.05	0.39
	South	S_5	6.35	75.0	18.35	28.70	44.80	8.41	0.04	0.66	1.09	0.04	0.19
		S_6	6.33	71.5	14.00	0.35	1.08	8.63	0.06	1.28	0.80	0.03	0.49
		S_7	6.70	78.0	18.40	30.40	47.10	8.35	0.20	1.06	1.12	0.04	0.40
Winter	North	S_1	8.70	83.0	13.50	18.90	31.00	7.83	0.09	2.20	0.19	0.00	0.86
		S_2	7.80	74.0	13.80	9.70	16.70	7.33	0.39	8.42	2.29	0.05	1.65
		S_3	4.20	40.0	13.30	11.70	3.51	8.20	0.54	8.33	6.54*	0.14	3.10*
		S_4	9.30	84.0	11.60	11.80	20.80	7.85	0.04	2.60	0.16	0.00	1.05
	South	S_5	8.60	78.0	11.80	17.00	28.30	7.63	0.06	1.68	1.26	0.03	1.80
		S_6	10.20	99.0	11.80	0.00	0.20	8.24	0.06	3.90	0.31	0.01	1.45
		S_7	7.10	85.0	12.10	16.90	28.00	7.64	0.19	2.60	2.13	0.05	1.20

*S_3 with significant differences

Table 3 – Pearson's correlation between TA loads (ng/L) and the physicochemical parameters.

Season	Margin	Dissolved O_2 (mg/L)	Temperature (°C)	Salinity	Condutivity (mS/cm)	pH	Nitrites	Nitrates	Ammonia	Un-ionized ammonia	Phosphates
Spring	North	0.99*	0.92*	0.97*	0.93*	0.31	0.99*	0.05	0.99*	0.99*	0.97*
	South	0.76	0.08	0.15	0.03	0.02	0.60	0.01	0.10	0.92	0.24
Summer	North	0.83	0.81	0.55	0.75	0.36	0.42	0.60	0.13	0.13	0.51
	South	0.22	0.16	0.84	0.96	0.73	0.66	0.91	0.32	0.29	0.91
Autumn	North	0.78	0.00	0.67	0.70	0.54	0.95*	0.83	0.97*	0.95*	0.89*
	South	0.42	0.98	0.99	0.99	0.99	0.26	0.46	0.99	0.98	0.40
Winter	North	0.00	0.62	0.47	0.23	0.05	0.00	0.01	0.02	0.02	0.05
	South	0.53	0.07	0.94	0.94	0.94	0.07	0.97	0.53	0.51	0.11

*Pearson correlations with significant differences ($p \leq 0.05$)

3.3 European limits legislation

Figures 3A and 3B show, respectively, the number and the percentage of water samples, per season, in which the concentrations of pesticides were above the reference values established in the 2013/39/EU directive. Most the samples collected (83%) approximated the established levels (2013/39/EU) for dichlorvos, 4,4'-DDT, endosulfan (alpha + beta), heptachlor + heptachlor epoxide, and PeCB (Fig. 3A). This pattern was expressed more in spring (30%) and autumn (35%; Fig. 3B) than in the other seasons.

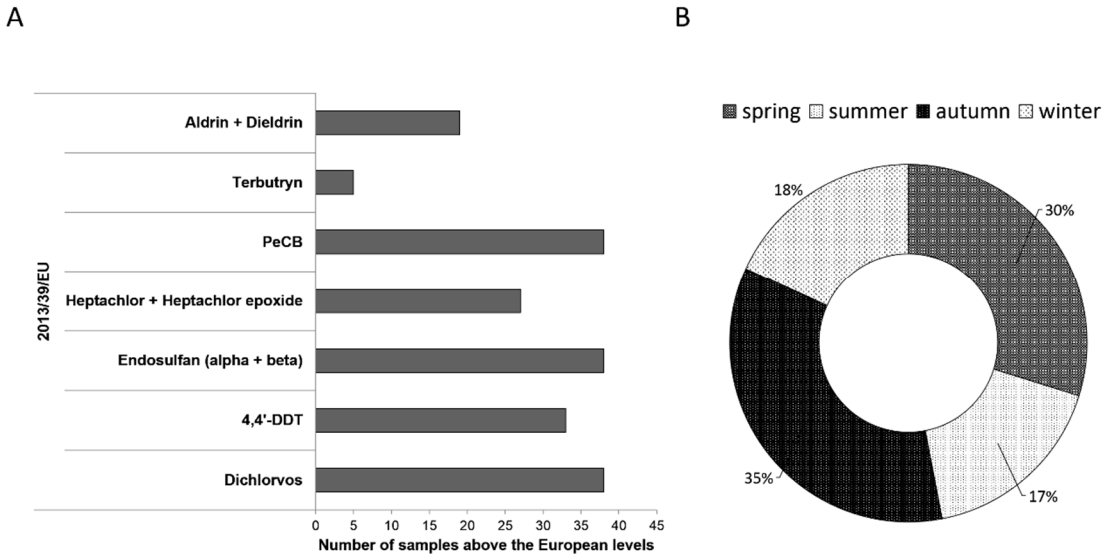

Fig. 3 – Pesticides exceeding the European directive levels: (A) Number of pesticides above legislation and (B) Percentage of pesticides that exceed the limits, by season.

3.4 Aquatic risk of pesticide mixture

Table 4 shows the two-tiered approach, based on classical models IA and CA. Here, the maximum amount of each pesticide was used to calculate the MEC that ranged from 2.3E-06 to 7.1E-03 mg/L. The assessment factor (AF) of 100 was adopted as a Maximum Acceptable Concentration- Quality Standard (MAC-QS) to estimate the short term effects of pesticides in this estuarine environment (European Communities, 2011). As a first approach, 57% of the compounds presented an individual MEC/PNEC ratio above '1'. This,

indicated a potential environmental risk. Consequently, the second approach was done to define the most sensitive trophic group (algae, invertebrates, or fish). This approach showed that the highest value of STU was obtained for fish (25.60).

Table 4 – Maximum measured environmental concentrations (MEC) along with the median lethal (effective) concentrations (LC_{50} and EC_{50}) for three distinct groups (algae, invertebrate and fish) used to calculate the predicted no effect concentration (PNEC), the individual MEC/PNEC and the toxic units (TU) per group.

	Pesticides	MEC (mg/L)	Algae 72h EC_{50} growth (mg/L)	Invertebrate 48h EC_{50} (mg/L)	Fish 96h LC_{50} (mg/L)	PNEC (mg/L)	Individual MEC/PNEC	RQ_{TU} (algae)	RQ_{TU} (invertebrate)	RQ_{TU} (fish)
FUNGICIDES	Azoxystrobin	2.0E-05	3.6E-01	2.3E-01	4.7E-01	2.3E-03	8.7E-03	5.5E-05	8.7E-05	4.2E-05
	Difenoconazol	4.0E-04	3.2E-02	7.7E-01	1.1E+00	3.2E-04	**1.2E+00**	1.2E-02	5.2E-04	3.6E-04
	HCB	1.4E-05	1.0E-02	5.0E-01	3.0E-02	1.0E-04	1.4E-01	1.4E-03	2.9E-05	4.8E-04
	PeCB	3.9E-05	1.3E+01	nf	2.5E-01	2.5E-03	1.6E-02	3.0E-06	-	1.6E-04
	Procymidone	5.1E-05	2.6E+00	1.8E+00	7.2E+00	1.8E-02	2.9E-03	2.0E-05	2.9E-05	7.1E-06
	Tebuconazole	8.9E-05	2.0E+00	2.8E+00	4.4E+00	2.0E-02	4.5E-03	4.5E-05	3.2E-05	2.0E-05
HERBICIDES	Alachlor	1.5E-05	9.7E-01	1.0E+01	1.8E+00	9.7E-03	1.5E-03	1.5E-05	1.5E-06	8.2E-06
	Atrazine	2.8E-04	5.9E-02	8.5E+01	4.5E+00	5.9E-04	4.7E-01	4.7E-03	3.3E-06	6.2E-05
	Atrazine-desethyl	2.0E-05	1.0E-01	nf	nf	1.0E-03	2.0E-02	2.0E-04	-	-
	Cyanazine	7.1E-03	2.0E-01	4.9E+01	1.0E+01	2.0E-03	**3.5E+00**	3.5E-02	1.4E-04	7.1E-04
	Cyhalofop-butyl	1.6E-03	9.6E-01	2.7E+00	7.9E-01	7.9E-03	2.1E-01	1.7E-03	6.1E-04	2.1E-03
	Metolachlor	1.3E-05	5.7E+01	2.4E+01	3.9E+00	3.9E-02	3.3E-04	2.2E-07	5.4E-07	3.3E-06
	Metribuzin	4.1E-05	2.0E-02	4.9E+01	7.5E+01	2.0E-04	2.1E-01	2.1E-03	8.4E-07	5.5E-07
	Pendimethalin	2.9E-04	6.0E-03	2.8E-01	1.4E-01	6.0E-05	**4.8E+00**	4.8E-02	1.0E-03	2.1E-03
	Propyzamide	4.2E-05	2.8E+00	5.6E+00	4.7E+00	2.8E-02	1.5E-03	1.5E-05	7.5E-06	8.9E-06
	Simazine	5.5E-04	4.0E-02	1.1E+00	9.0E+01	4.0E-04	**1.4E+00**	1.4E-02	5.0E-04	6.1E-06
	Simetryn	3.5E-05	9.8E-03	nf	7.0E+00	9.8E-05	3.5E-01	3.5E-03	-	5.0E-06
	Terbuthylazine	6.2E-04	1.2E-02	2.1E+01	2.2E+00	1.2E-04	**5.1E+00**	5.1E-02	2.9E-05	2.8E-04
	Terbutryn	1.6E-03	2.4E-03	2.7E+00	1.1E+00	2.4E-05	**6.5E+01**	6.5E-01	5.8E-04	1.4E-03

(continued)

	Pesticides	MEC (mg/L)	Algae 72h EC_{50} growth (mg/L)	Invertebrate 48h EC_{50} (mg/L)	Fish 96h LC_{50} (mg/L)	PNEC (mg/L)	Individual MEC/PNEC	RQ_{TU} (algae)	RQ_{TU} (invertebrate)	RQ_{TU} (fish)
INSECTICIDES	Aldrin	2.7E-05	nf	2.8E-02	4.6E-03	4.6E-05	5.9E-01	-	9.7E-04	5.9E-03
	Azinphos-methyl	1.2E-03	7.2E+00	1.1E-03	2.0E-02	1.1E-05	**1.1E+02**	1.6E-04	1.1E+00	5.8E-02
	Chlordane-gamma	3.3E-06	nf	5.9E-01	9.0E-02	9.0E-04	3.7E-03	-	5.6E-06	3.7E-05
	Chlorpyriphos	7.4E-05	4.8E-01	4.0E-05	1.3E-03	4.0E-07	**1.9E+02**	1.6E-04	1.9E+00	5.7E-02
	Chlorfenvinphos Z	2.8E-06	1.4E+00	2.5E-04	1.1E+00	2.5E-06	1.1E+00	2.1E-06	1.1E-02	2.6E-06
	Cyfluthrin (beta)	3.1E-04	1.0E+01	1.6E-04	4.7E-04	1.6E-06	**2.0E+02**	3.1E-05	2.0E+00	6.7E-01
	Cyhalothrin (lambda)	1.8E-03	nf	3.8E-01	4.6E-04	4.6E-06	**3.9E+02**	-	4.7E-03	3.9E+00
	Cypermethrin (alpha)	1.9E-04	1.0E-01	3.0E-04	2.8E-03	3.0E-06	**6.2E+01**	1.9E-03	6.2E-01	6.7E-02
	4,4'-ΣDDD,DDE,DDT	2.7E-04	nf	5.0E-03	7.0E+00	5.0E-05	**5.4E+00**	-	5.4E-02	3.8E-05
	Deltamethrin	5.3E-03	9.1E+00	5.6E-04	2.6E-04	2.6E-06	**2.0E+03**	5.8E-04	9.5E+00	2.0E+01
	Diazinon	2.7E-04	6.4E+00	1.0E-03	3.1E+00	1.0E-05	**2.7E+01**	4.3E-05	2.7E-01	8.8E-05
	Dichlorvos	3.9E-05	5.3E+01	1.9E-04	5.5E-01	1.9E-06	**2.0E+01**	7.4E-07	2.0E-01	7.1E-05
	Dieldrin	8.5E-05	1.0E-01	2.5E-01	1.2E-03	1.2E-05	**7.1E+00**	8.5E-04	3.4E-04	7.1E-02
	Dimethoate	6.4E-04	9.0E+01	2.0E+00	3.0E+01	2.0E-02	3.2E-02	7.1E-06	3.2E-04	2.1E-05
	Endosulfan (alpha+beta)	7.4E-04	2.2E+00	4.4E-01	2.0E-03	2.0E-05	**3.7E+01**	3.4E-04	1.7E-03	3.7E-01

(continued)

	Pesticides	MEC (mg/L)	Algae 72h EC_{50} growth (mg/L)	Invertebrate 48h EC_{50} (mg/L)	Fish 96h LC_{50} (mg/L)	PNEC (mg/L)	Individual MEC/PNEC	RQ_{TU} (algae)	RQ_{TU} (invertebrate)	RQ_{TU} (fish)
INSECTICIDES	Fenamiphos	3.0E-05	3.8E+00	1.9E-03	9.3E-03	1.9E-05	**1.6E+00**	7.8E-06	1.6E-02	3.2E-03
	Fenitrothion	3.9E-04	1.3E+00	8.6E-03	1.3E+00	8.6E-05	**4.5E+00**	3.0E-04	4.5E-02	3.0E-04
	Fonofos	7.7E-05	1.5E+00	2.3E-03	2.8E-02	2.3E-05	3.4E+00	5.2E-05	3.4E-02	2.8E-03
	Heptachlor	3.4E-05	2.7E-02	4.2E-02	7.0E-03	7.0E-05	4.8E-01	1.2E-03	8.0E-04	4.8E-03
	Heptachlor epoxide	2.3E-06	2.0E+02	2.4E-01	2.0E-02	2.0E-04	1.2E-02	1.2E-08	9.7E-06	1.2E-04
	HCCP	2.0E-03	nf	5.2E-02	2.4E+00	5.2E-04	**3.9E+00**	-	3.9E-02	8.5E-04
	Lindane	4.4E-05	2.5E+00	1.6E+00	2.9E-03	2.9E-05	**1.5E+00**	1.8E-05	2.7E-05	1.5E-02
	Malathion	2.2E-04	1.3E+01	7.0E-04	1.8E-02	7.0E-06	**3.2E+01**	1.7E-05	3.2E-01	1.2E-02
	Methoxychlor	8.6E-05	6.0E-01	7.8E-04	5.2E-02	7.8E-06	**1.1E+01**	1.4E-04	1.1E-01	1.7E-03
	Mirex	1.0E-03	1.0E-01	1.0E-01	1.0E+02	1.0E-03	**1.0E+00**	1.0E-02	1.0E-02	1.0E-05
	Parathion-ethyl	3.9E-05	5.0E-01	2.5E-03	1.5E+00	2.5E-05	**1.6E+00**	7.8E-05	1.6E-02	2.6E-05
	Phosmet	1.2E-03	7.0E-02	2.0E-03	2.3E-01	2.0E-05	**5.9E+01**	1.7E-02	5.9E-01	5.1E-03
	Pirimicarb	5.4E-05	1.4E+02	1.7E-02	1.0E+02	1.7E-04	3.2E-01	3.9E-07	3.2E-03	5.4E-07
	Parathion-methyl	3.1E-04	3.0E+00	7.3E-03	2.7E+00	7.3E-05	**4.2E+00**	1.0E-04	4.2E-02	1.1E-04
	Tetrachlorvinphos	3.1E-05	nf	2.0E-03	4.3E-01	2.0E-05	**1.6E+00**	-	1.6E-02	7.2E-05
						$RQ_{MEC/PNEC}$	3283.06	0.86	16.76	**25.60**
						$RQ_{STU(100)}$	2559.92			
						STU/max RQ_{TU}	1.257			

nf: information not found; **MEC** (mg/L): maximum measured environmental concentration; **PNEC** (mg/L): predicted no effect concentration; **EC_{50}** (mg/L): half maximal effective concentration; **LC_{50}** (mg/L): median lethal dose; **TU**: toxic units; **STU**: sum of the toxic units;

4. Discussion

The present study reveals that the Tagus River estuary is intensely impacted by the ubiquitous presence of pesticides. The concentrations of these surpass in some circumstances, or even all year round, those limited by EU Directive for transitional surface waters (2013/39/EU). The origin of these compounds is mainly agricultural activity, which is intensive and involves a significant area (39 3624 ha) of the Tagus River basin (DRAP LVT, 2012a, b). This observation is supported by the seasonal fluctuation of several pesticides of which greater concentrations were measured in spring, when they are applied to the fields (Mendes et al., 2008). However, there are also other possible sources of local contamination such as industries involved in food processing and chemical production (Peneda and Frazão, 1995; Picado et al., 2008). The industrial impact of pesticides in the aquatic environment is clearly observed at the sampling site located close to Trancão River mouth (S_3). At this sampling site, denoted as a "hot spot" of pollution (van den Berg et al., 2007), the concentrations of almost all pesticides were ca. 3-fold higher than those, measured at the other sampling sites. The steady occurrence of PeCB, used as a precursor of other pesticides and/or as a flame retardant, is also coincident with the presence of chemical industries near S_3 (Cabeza et al., 2012).

The present data revealed that fungicides are widely spread not only in this estuary but also in other aquatic systems from Portugal (≈ 65 ng/L for hexachlorbenzene)(Rocha et al., 2012) to other European countries, such as the Netherlands (≈ 120 ng/L for azoxystrobin and difenoconazol)(S. Wuijts et al., 2008) and France (≈ 60 ng/L for procymidone and 255 ng/l for tebuconazole)(Pesce et al., 2008; Taghavi et al., 2011). Herein, their unwavering presence in considerable concentrations may be linked to large amounts of sediment and/or particulate matter, because all samples were collected at the shore.

The herbicides—*viz.* cyanazine, terbuthylazine, and terbutryn—were measured in extremely high concentrations and demonstrated that these are likely being overused in this basin. Due to their high leaching potential

(2.5 GUS index value and 3.1 log K_{ow}), they easily contaminate surface and groundwater (Gustafson, 1989). Comparing with others, it is observed that a similar panorama is also occurring in other aquatic systems across Europe (Portugal, Spain, and Germany), as exemplified for terbuthylazine (≈ 250 ng/L) and terbutryn (≈ 303 ng/L) (Köck et al., 2010; Quednow and Püttmann, 2007; Rocha et al., 2012). Thus, comparable fates and impacts of herbicides are occurring within Europe.

Among insecticides, five cases (aldrin+dieldrin, heptachlor+heptachlor epoxide, endosulfan, 4,4'-DDT, and dichlorvos), were above the 2013/39/EU limits established for transitional waters in almost all water samples analyzed. The concentration of banned insecticides, such as 4,4'-DDT and heptachlor, revealed recent illegal use of these pesticides. Proportional ratios between their metabolites, (DDD+DDE) and heptachlor epoxide, 3.2 and 10.3, respectively, indicate an active presence of these banned compounds ((NPIC), 1999). Similar results were also registered in other Portuguese water systems, such as Douro, Sado, Ave, and Minho River estuaries (Carvalho et al., 2009; Rocha et al., 2012), and at the natural park in Doñana Ana, Spain (Fernández et al., 2000).

The present study demonstrated that, compared to previous studies done in the Tagus River estuary from 2004 to 2006 (Silva et al., 2012a; Silva et al., 2012b), the amounts of some herbicides (metolachlor, alachlor, atrazine, atrazine-desethyl, metribuzin, and simazine) and insecticides (chlorpyrifos, endosulfan and lindane) were, respectively, 1000 to 42 fold lower than as reported earlier. This may be the result of decontamination efforts done since 2000 (van den Berg et al., 2007). Thus, it seems that there is an increasing interest in accomplishing the EU directive for transitional surface waters (2013/39/EU).

To estimate possible impacts of the studied pesticides on aquatic life, the highest concentration of each pesticide measured, in the Tagus River estuary was used, as well as the toxic levels listed in IUPAC databases (Table 4; (University of Hertfordshire, 2006-2013)). It was concluded that some insecticides, like azinphos-methyl, chlorpyriphos, cyhalothrin-lambda, and deltamethrin, attained environmental concentrations able to cause mortality to

50% (LC_{50}) of the exposed population of fish (*Oncorhynchus mykiss*), and invertebrates (crustaceans: the water flea *Daphnia magna* and the mysid shrimp *Americamysis bahia*) (Agriculture & Environment Research Unit (AERU), 2013). However, a worse scenario may exist as these compounds are present, not isolated but in environmental mixtures. A two-tiered approach was used to evaluate the impact of our environmental pesticide mixture on three trophic levels (fish, invertebrates and algae). Using the current data, the first tier demonstrated a potential risk mainly for fish. This exhibited the highest total sum of the toxic units STU (25.60), and was determined by the deltamethrin LC_{50} concentration, which presents the highest toxicity (260 ng/L; Table 4). The second tier represents the maximal value by which CA may predict toxicity higher than IA (by the IA/CA ratio) in this case, the value was 1.257, demonstrating that this toxicity is controlled by a small number of compounds. Similar predictions had already been calculated for fish (1.025 and 1.005) in the Tagus and Mondego basins, respectively; and attaining even higher average values for algae (2.318 and 2.163) in the same basins (Silva and Cerejeira, 2014).

Because several physicochemical parameters can be linked with pollution, this aspect was also taken into consideration. Despite both pH and DO values being within acceptable ranges (as defined by Portuguese and EU legislation, (Ministério do Ambiente, 1998)), the levels for total nitrogen surpassed the hazardous amount of 1 mg/L (World Health Organization-WHO, 2011), particularly in autumn and winter. Moreover, the phosphates at S_3—usually originating from WWTPs and excess organophosphorus pesticides—exceeded the recommended value of 0.1 mg/L defined as a limit to prevent eutrophication in flowing streams (Environmental Protection Agency (EPA), 1986). The WWTPs output near S_3 and S_6 explain also the very low salinity values at these sites. Significant positive correlations were calculated between the TA loads of pesticides vs. nitrites and phosphates, supporting the present data. This corroborates the pesticide data, because it also highlights the S_3 sampling site as pollution hotspot within the Tagus River estuary.

Conclusions

For the first time, a broad range of pesticides (from different categories) were assessed in the Tagus River estuary, where fifty-three of them were quantified. The measured concentrations, mainly from insecticides, were above the European Directive concentrations (2013/39/EU) set for transitional waters. Concerning the nature of the assayed pesticides, fifty percent of them were not approved, and sixteen percent are currently banned by European legislation, indicating that their use is abusive and illegal

Site S_3, near the Trancão River was the most polluted area. Physicochemical data, mainly total nitrogen and phosphates, corroborate the presence/loads of pesticides. The other sites studied were similarly impacted by pesticides, the source of which seems to be agricultural run-off.

A two-tiered approach was used to assess the hazard of pesticide mixtures, at maximum concentration, which reflected a potential risk in this basin mainly for fish. This study highlights the need for monitoring studies, as well as for the implementation of more green technologies and policies, to decrease the impacts of pesticides. This is in line with European legislation (e.g., Water Framework Directive), which has strict parameters for prevention, protection and improvement of environmental quality and human health as well as for the rational use of natural resources.

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Supplementary material

Table SM1 – GC-MS characteristics and conditions for the analysis of selected pesticides in transitional waters samples.

GC-MS conditions			
Gas chromatograph:	Trace GC ultra, Thermo Finnigan Electron Corporation		
Detector:	Ion trap mass spectrometer (Thermo Scientific ITQ™ 1100 GC-MS^n)		
Autosampler:	Thermo Scientific TriPlus™		
Injector:	SSL (3 mm straight liner)		
Mode	splitless mode		
Temperature (ºC)	250		
Volume (µL)	2 mL (50 mm lenght needle)		
Carrier Gas:	Helium (99.9999%); constant flow 1 mL/min		
Column:	TG-5SilMS (30m x 0.25 mm x 0.25 µm)		
Program:	temperature (º C)	hold time (min)	Rate ºC/min
1st ramp	65	2	-
2nd ramp	180	-	20
3rd ramp	280	7	5
Solvent delay:	5 min		
Transfer line (ºC):	280		
Ion source (ºC):	280		

Table SM2 – Quantification and diagnostic ions used in GC-MS and GC-MS/MS analyses. The relative abundance of ions (*m/z*) for each target pesticide is indicated between brackets.

Pesticides	Molecular mass	RT	GC-MS/SIM					GC-MS/MS						
			Target ion	Q1	Q2	Q3	Q 4	Percursor		Products			EV	Ranges
	g/mol	(min)	(t)	(%Q1/t)	(%Q2/t)	(%Q3/t)	(%Q4/t)							
PeCB	250.3	9.55	**250**	248 (65.1)	252 (66.1)			**250**	→	215	144		2.01	143-251
Trifluralin	335.3	10.90	**264**	306 (44.9)	206 (27.2)			**264**	→	206	160	188 171	1.05	159-265
Atrazine-desethyl	187.6	10.93	**172**	68 (32.2)	174 (29.2)			**172**	→	130	145	152	1.15	104-173
Propazine	229.7	11.66	**214**	172 (70.6)	187 (38.0)			**214**	→	200	172	138	1.20	137-215
HCB [b]	284.8	11.68	**284**	282 (46.4)	249 (41.2)			**284**	→	214	249		1.50	211-285
Dimethoate	229.3	11.77	**87**	93 (52.5)	125 (44.3)			**87**	→	86	59		1.10	53-88
Simazine [a]	201.6	11.88	**201**	186 (67.1)	173 (51.9)	44 (37.6)		**201**	→	186	174	138	1.20	135-201
ATZ-d_5	220.7	11.95	**205**	220 (43.0)	178 (41.7)			***205***	→	127	137	105	1.25	104-206
Atrazine [b]	215.7	12.00	**200**	215 (56.8)	173 (36.9)			**200**	→	122	132	164 158	1.25	121-201
Lindane [a]	290.8	12.33	**181**	183 (76.6)	219 (69.3)			**181**	→	145	146		1.20	108-184
Terbuthylazine	229.7	12.41	**214**	173 (71.8)	138 (27.3)	229 (24.3)		**214**	→	173	132		1.40	131-215
Propyzamide	256.1	12.45	**173**	175 (41.2)	254 (35.6)			**173**	→	138	145		1.10	130-174
Diazinon	304.4	12.46	**137**	179 (44.7)	304 (10.2)			**179**	→	121	163	137 122	1.35	110-180
Fonofos	246.3	12.51	**137**	109 (70.7)	246 (40.0)			**137**	→	109	81		0.85	80-138
Pirimicarb	238.4	13.10	**166**	238 (29.0)	72 (18.8)			**166**	→	96	137	121	1.35	95-167
Parathion-methyl	263.2	13.98	**263**	109 (67.9)	79 (44.7)	246 (43.5)		**263**	→	246	153		1.31	150-264
Alachlor [a]	269.8	14.01	**188**	160 (86.8)	146 (58.8)			**160**	→	132	130		1.10	116-161
Simetryn	213.3	14.10	**213**	170 (22.9)	155 (13.4)			**213**	→	170	185		1.10	151-214
Heptachlor [b]	373.3	14.25	**272**	274 (73.9)	270 (63.3)	100 (43.9)		**272**	→	237	235		1.05	236-275
Metribuzin	214.3	14.43	**198**	199 (29.8)				**198**	→	150	110		1.10	109-199
Terbutryn	241.4	14.67	**185**	226 (68.0)	170 (45.2)			**185**	→	170	128		0.90	127-186
Fenitrothion	277.2	14.71	**260**	109 (83.4)	125 (77.4)	277 (41.8)		**260**	→	228	217	232	1.20	160-261
Malathion	330.4	14.95	**125**	127 (90.9)	99 (73.2)	173 (40.2)		**173**	→	99	117	145	0.80	92-173
Metolachlor	283.8	15.12	**162**	238 (36.7)	163 (15.1)			**162**	→	132	133		1.15	115-163
Chlorpyriphos	350.6	15.19	**314**	316 (72.3)	258 (67.3)	199 (46.8)	197 (41.4)	**314**	→	258	286		0.90	257-315
Cyanazine	240.7	15.26	**225**	212 (59.4)	198 (35.2)	68 (32.8)		**225**	→	189	172	198	1.28	171-226
Aldrin [a]	364.9	15.35	**263**	261 (92.7)	265 (65.6)	66 (57.2)		**263**	→	193	191	227	1.60	190-264

(*continued*)

Pesticides	Molecular mass g/mol	RT (min)	GC-MS/SIM Target ion (t)	Q1 (%Q1/t)	Q2 (%Q2/t)	Q3 (%Q3/t)	Q 4 (%Q4/t)	GC-MS/MS Percursor		Products			EV	Ranges
Parathion-ethyl	291.3	15.38	**291**	109 (79.0)	263 (61.0)	97 (57.2)	141 (47.0)	**109**	→	81	91		0.99	60-110
Pendimethalin	281.3	16.27	**252**	162 (61.3)	191 (28.9)			**252**	→	162	191		1.00	160-253
Chlorfenvinphos Z	359.6	16.53	**267**	269 (52.9)	323 (51.5)			**267**	→	159	203		1.50	158-268
Heptachlor epoxide [b]	389.3	16.53	**353**	355 (66.0)	351 (44.4)	81 (25.7)		**353**	→	263	282		1.10	262-354
Procymidone	284.1	16.86	**96**	283 (85.3)	285 (29.0)			**96**	→	67	68		1.00	64-97
Chlordane (gamma)	338.9	17.28	**375**	373 (93.8)	377 (59.9)			**373**	→	266	264		1.20	263-374
Tetrachlorvinphos	366.0	17.40	**329**	331 (90.4)	109 (51.3)	333 (32.8)		**329**	→	314	278		1.30	219-330
Endosulfan (beta)	406.9	17.70	**241**	195 (72.7)	243 (71.2)	207 (54.0)		**241**	→	206	204	170	1.45	165-242
Fenamiphos	303.4	17.87	**303**	243 (62.4)	217 (54.9)	288 (42.9)	154 (40.6)	**303**	→	268	266		1.10	175-304
4,4'-DDE	318.0	18.40	**246**	248 (58.6)	318 (31.9)	316 (29.3)		**246**	→	176	175		1.70	174-247
Dieldrin [a]	380.9	18.60	**79**	263 (93.1)	237 (43.5)			**79**	→	51	50		1.10	49-80
Endosulfan (alpha)	406.9	18.60	**241**	195 (78.2)	237 (70.8)	243 (65.5)		**241**	→	206	205		1.45	165-242
Endrin [b]	380.9	18.61	**243**	263 (99.0)	281 (68.4)	81 (47.4)		**243**	→	207	173		1.15	172-244
4,4´-DDD	320.0	19.84	**235**	237 (64.2)	165 (61.7)			**235**	→	165	199		1.15	162-236
Endosulfan sulfate	422.9	20.95	**272**	237 (68.0)	274 (60.5)	387 (47.9)		**272**	→	237	235		1.10	234-273
DDT-d_8	362.5	21.03	**220**	243 (62.6)	280 (57.8)			***243***	→	173	206		1.15	172-244
4,4´-DDT	354.5	21.11	**235**	237 (64.2)	212 (59.0)	165 (44.0)		**235**	→	165	199		1.15	117-236
Methoxychlor [b]	345.7	23.08	**227**	228 (16.5)	274 (15.4)			**227**	→	169	181		*1.30*	*140-228*
Azinphos-methyl	317.3	23.85	**77**	132 (88.0)	104 (43.4)	160 (32.9)		**77**	→	51	50		1.30	49-78
Tebuconazole	307.8	24.44	**250**	125 (80.6)	163 (44.4)			**125**	→	89	99		1.60	62-126
Cyhalofop-butyl	357.4	24.44	**256**	357 (72.6)	229 (41.1)	120 (31.0)		**256**	→	228	200		1.13	199-257
Mirex	545.5	24.71	**272**	274 (73.3)	237 (62.7)			**272**	→	237	235		1.13	234-273
Cyhalothrin (lambda)	449.9	24.73	**181**	141 (45.8)	197 (42.1)			**181**	→	152	151		1.50	120-182
Cyfluthrine (beta) *	434.3	27.71	**206**	199 (76.9)	91 (70.9)	226 (55.0)	227 (42.2)	**199**	→	193	191	163	1.80	190-200
Cypermethrin (alpha)	416.3	28.24	**181**	91 (76.3)	163 (75.0)	165 (47.3)		**181**	→	152	151		1.70	150-153/179-182
Difenoconazol	406.3	31.25	**265**	267 (89.9)	323 (68.5)	325 (62.2)		**323**	→	265	249		1.35	245-266/321-324
Deltamethrin *	505.2	32.00	**181**	207 (61.4)	253 (58.7)			**181**	→	152	151		1.70	150-153/179-182

Internal standards ; [a] Compounds present on the mix A (EPA 505/525); [b] Compounds present on the mix B (EPA 505/525); * Contain several diastereoisomers

Table SM3 - Chemical characteristics (class, log K_{ow}, log K_{oc} and GUS index) and license category (according to European pesticides database) of the selected pesticides.

Pesticides	Class	License[#]	log K_{ow}	log K_{oc}	GUS index
Fungicides					
Azoxystrobin	Antibiotic fungicide	A	2.5	2.8	2.6
Difenoconazol	Conazole fungicides	A	4.4	3.6	0.9
HCB	Organochlorines	B	3.9	4.7	-2.3
PeCB	Aromatic fungicide	NA	4.8-5.2	4.5	-1.2
Procymidone	Conazole fungicides	NA	3.3	2.6	1.2
Tebuconazole	Conazole fungicides	A	3.7	3.0	2.0
Herbicides					
Alachlor	Organochlorines	NA	3.7	2.5	0.8
Atrazine	Triazine	NA	2.7	2.0	3.3
Atrazine-desethyl	Triazine	NA	2.7	1.9	3.5
Cyanazine	Triazine	NA	2.1	2.3	2.1
Cyhalofop-butyl	Phenoxy herbicides	A	6.0	3.7	-0.2
Metolachlor	Amide herbicides	NA	3.4	2.1	3.5
Metribuzin	Triazinone herbicides	A	1.7	1.8	2.6
Pendimethalin	Dinitroaniline herbicides	A	5.2	4.4	-0.4
Propazine	Triazine	NA	4.0	2.2	3.8
Propyzamide	Amide herbicides	A	3.3	2.9	1.8
Simazine	Triazine	NA	2.3	2.1	2.0
Simetryn	Triazine	NA	2.8	2.3	3.0
Terbuthylazine	Triazine	A	3.4	2.3	3.1
Terbutryn	Triazine	NA	3.7	3.4	2.4
Trifluralin	Carbamate insecticide	NA	5.3	4.2	0.1
Insecticides					
Aldrin	Organochlorines	B	6.5	4.2	-0.4
Azinphos-methyl	Organothiophosphate insecticides	NA	3.0	3.0	1.0
Chlordane-gamma	Organochlorines	B	2.8	4.3	-0.8
Chlorfenvinphos Z	Organophosphorus	NA	3.8	2.8	1.9
Chlorpyriphos	Organophosphorus	A	4.7	3.9	0.2
Cyfluthrin (beta)	Pyrethroid	A	5.6	4.8	-1.7
Cyhalothrin (lambda)	Pyrethroid	A	6.8	5.2	-2.1
Cypermethrin (alpha)	Pyrethroid	A	6.9	4.4	-2.1
4,4'-DDD	Organochlorines	B	6.9	4.7	-0.9
4,4'-DDE	Organochlorines	B	6.9	4.9	-2.0
4,4'-DDT	Organochlorines	B	6.9	5.9	-4.5
Deltamethrin	Pyrethroid	A	4.6	7.0	-3.4
Diazinon	Organophosphorus	NA	3.7	2.8	1.1
Dichlorvos	Organophosphorus	NA	1.9	1.7	0.7
Dieldrin	Organochlorines	B	3.7	4.4	-0.3
Dimethoate	Organophosphorus	A	0.7	1.0	1.1
Endosulfan (alpha)	Organochlorines	NA	4.7	4.1	-0.1
Endosulfan (beta)	Organochlorines	NA	4.8	4.3	-0.1
Endosulfan sulfate	Organochlorines	NA	3.7	3.7	0.5
Endrin	Organochlorines	NA	3.2	4.0	0.0
Fenamiphos	Organophosphorus	A	3.3	2.0	-0.1
Fenitrothion	Organophosphorus	NA	3.3	3.3	0.5
Fonofos	Organophosphorus	NA	3.9	2.9	2.1
Heptachlor	Organochlorines	B	5.4	4.4	-0.9
Heptachlor epoxide	Organochlorines	NA	4.4-5.5	4.3	-1.1
HCCP	Organochlorines	*	4.0	3.6	0.4
Lindane	Organochlorines	NA	3.7	3.1	4.0
Malathion	Organophosphorus	A	2.8	3.3	-1.3
Methoxychlor	Organochlorines	NA	3.8	4.9	-1.9
Mirex	Organochlorines	B	5.3	3.8	0.6
Parathion-ethyl	Organophosphorus	NA	3.8	3.9	2.1
Phosmet	Organothiophosphate insecticides	A	3.0	3.6	0.2
Pirimicarb	Dinitroaniline herbicides	A	1.7	2.6	2.7
Parathion-methyl	Organophosphorus	NA	3.0	2.4	1.5
Tetrachlorvinphos	Organophosphorus	NA	3.5	3.0	0.3

[#]**NA**- Not authorized; **A**- Authorized; **B**- Banned; according to the EU Pesticides D * Information not found
GUS index (groundwater ubiquity score; GUS= log10 (half life-days) X [4-log10 (Koc)])

Chapter 6

Development and application of a QuEChERS-based extraction method for the analysis of 55 pesticides in the bivalve *Scrobicularia plana* by GC-MS/MS

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Abstract

A method for quantitative determination of 55 pesticides in a bivalve matrix was established, based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and using gas chromatography (GC)-ion trap (IT) mass spectrometry (MS/MS). Accomplishing the European SANCO guidelines, this method was validated using 5 g of homogenized soft tissue, allowing the quantification of pesticides at ng/g of wet weight (ww). Quantification limits and recovery rates ranged from 0.33 to 10.3 µg/L and from 78 to 119%, respectively. As an important mollusc, not only from an ecological perspective but also for food consumption, the peppery furrow shell (*Scrobicularia plana*) was sampled at three strategical sites (Ria Formosa Lagoon, in the south of Portugal) during 2012-2013, over 6 campaigns. A total of 2160 animals were pooled by place and sex. No statistical differences were found among sites or between sexes. Forty percent of the sampled pools were above quantification limits, reaching total annual average concentrations of Σ800 ng/g ww. Additionally, 83% of the selected compounds showed concentrations above the legal limits set by the European Directive 2013/39/EU. In conclusion, the applied method was successful and proved that bivalves were contaminated by the selected pesticides. In future work, this methodology can be used to monitor body burdens and obtain data for predicting impacts in shellfish consumers.

1. Introduction

Anthropogenic activities such as industry and agriculture are distressing to the aquatic ecosystems through atmospheric pollution, effluent discharges and land use [1, 2]. As a consequence of intensive agriculture activity, millions of tons of fertilizers and pesticides are applied yearly, contaminating surface, waste and ground waters [3], normally at low ranges of concentrations (ng to µg/L) [4-6]. Habitats such as estuarine and coastal environments are highly impacted by the presence of pesticides [7-9], bringing harmful effects to biota [10-12], and affecting different trophic levels through bio-accumulation and bio-magnification mechanisms [13].

Bivalves are close the base of the food-chain. These are sessile filter-feeders and/or surface deposit feeders, able to accumulate pesticide residues in higher concentrations than the surrounding habitat [14]. This class of organisms can be used as time-integrated indicators of environmental contamination, allowing the identification of potential sources of pollution [15], as well as a reliable source of information about human exposure to contaminated seafood [16]. In Portugal, an Atlantic country famous for having one of the highest world rates of seafood consumption per capita, more than 3 000 tons of molluscs and crustaceans were sold in 2010, two-thirds of which were bivalves [17]. This represents a consumption of 3.94 kg/capita/2011, which is higher than the average European and Worldwide consumptions, with 1.89 and 2.44 kg/capita/2011, respectively [18]. Due to the importance of bivalve as human food, this study selected the benthic peppery furrow shell (*Scrobicularia plana*) as model species. This bivalve plays an important role in estuarine ecosystems and has a wide geographic distribution (from Senegal to the Norwegian Sea) [19] and is a commercial species exploited in the Iberian Peninsula [20, 21]. Since it is known that the ubiquitous presence of pesticides in aquatic environments gives rise to their bioaccumulation in bivalves, some studies quantified those compounds in consumed species (mussels, clams, oysters and scallops), thus demonstrating the need of food control for human health safety [22-27]. Analytically,

whole-body bivalves are complex matrices. These normally require time-consuming sample preparation, mainly during the extraction procedure [28-30]. Due to its rapid protocol, reliable results and affordable costs, the extraction technique QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) became a preferable alternative to other methods, such as ultrasonication, rotary extraction, microwave extraction, pressurized-liquid extraction (PLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME) [31-33]. However, up to now the adaptation of QuEChERS to extract pesticides from bivalve matrices was never attempted for more than 26 pesticides [22-27]. Multiresidue approaches, such as chromatography coupled with mass spectrometry (MS), are reliable sensitive techniques to trace pollutants [34]. In particular, GC-tandem mass spectrometry is valuable to minimize matrix effects, particularly when determining pesticides at ng/kg or ng/L ranges, in food and environmental matrices [34]. The quantification of such low levels is vital, namely in view of requirements by European directives [35-37]. Supplemental features and procedures, such as matrix normalization and analyte protectants' addition, can further contribute to more accurate results [38, 29].

The aim of this study was to develop and implement a QuEChERS method together with a multiresidue analytical method (GC-MS/MS) to extract and quantify 56 pesticides from the whole-body *S. plana* matrix. The applicability and performance of the combined methods were demonstrated by the first seasonal study (2012–2103) on the pesticide burden in animals collected from the Ria Formosa Lagoon (south of Portugal).

2. Materials and methods

2.1. Chemicals and materials

Reagents: LC/GC grade methanol (MeOH) and acetonitrile, anhydrous magnesium sulfate ($MgSO_4$), sodium acetate, and Supelclean™ PSA SPE Bulk Packing (polymerically bonded, ethylenediamine-*N*-propyl phase that contains both primary and secondary amines), were acquired from Sigma-Al-

drich (Steinheim, Germany); $MgSO_4$ was pre-heated (5 h at 500 ºC) to eliminate residual water and phthalates. Dimethyldichlorosilane solution (DMDCS, 99.5%, used as per the producer's protocol), was obtained from Sigma-Aldrich (China).

Pesticide standards: reference standards (98–99%) were acquired from Sigma-Aldrich (Steinheim, Germany). With exception of Mix A (EPA 505/525, 500 mg/L) and Mix B (EPA 505/525, 500 mg/L), all other pesticides were purchased individually. All standard solutions were individually prepared in MeOH to produce a final stock solution of 10 000 µg/L and kept in dark at –20 ºC to avoid possible decay.

Internal standards: atrazine-d_5 ($C_8H_9D_5ClN_5$) and 4,4´-DDT-d_8 ($C_{14}HCl_5D_8$) were used both as surrogates and internal standards, at a final concentration of 10 µg/L in the matrix. Due to their different molecular structure (one or two benzene rings) and weight, they could cover all the target pesticides; retention time intervals, 6.55-13.57 and 13.58-29.55 min, used atrazine-d_5 and 4,4'-DDT-d_8, respectively.

Analyte protectants: the compounds used as protectants had a purity above 98% and were acquired from Sigma-Aldrich (Saint Louis, USA). Stock solutions of 3-ethoxy-1,2-propanediol (400 mg/mL) and D-sorbitol (182 mg/mL) were prepared in acetonitrile and in 7 water : 3 acetonitrile, respectively.

2.2. Sampling area, bivalve collection and maintenance

Ria Formosa Lagoon is a mesotidal system located in the south of Portugal, providing a perfect environment for fish nurseries and bivalve stable populations [39]. Along its extension (60 km), three strategic sampling sites (S_1 to S_3) were selected. S_1 corresponds to the city of Faro (37º00'50.3'' N, 7º59'15.2'' W), S_2 comprises the fraction of Ria Formosa Lagoon Natural Park (37º05'32.5'' N, 7º40'27.2'' W), and S_3 marks the city of Tavira and the Gilão River (37º07'30.2'' N, 7º38'32.7'' W) (see Electronic Supplementary Material Fig. S1). The animals were collected manually at the shore (±20 cm depth) during ebb tide, in December 2012 and in January, February, May, June and

October 2013. Specimens were transported with local sediment to in-house facilities. There they were kept in water for 24 hours, in constant salinity, temperature and oxygenation. Afterwards, animals were drained and left for at least one hour in an anesthetic solution of $MgCl_2$ (Sigma-Aldrich; Saint Louis, USA) at 60 g/L until muscle relaxation [40]. Biometric parameters were registered (length, width, and height, plus total and soft tissue weights) and the condition index (CI) computed according to $\mathrm{CI} = \left[\left(\frac{\text{fresh weight}}{\text{shell weight}}\right) \mathrm{x}\ 100\right]$ as complementary information about the health status of the organisms [41] and for statistical correlations. Before storage at –80 ºC, a small fragment of gonad was collected in each specimen, smashed between two histological glass slides, and dried for 24 h. The slides were then subjected to Diff-Quick staining and the sex of the animals was determined.

2.3. QuEChERS extraction procedure

The extraction protocol was based on Anastassiades et al. [42] and AOAC 2007.01 [43] methodologies to create a more suitable extraction procedure (Fig.1). Different quantities and salts are used in these two methods; the main difference is on the extraction processes where in the AOAC method the acetonitrile is acidified with acetic acid (1%) and uses the sodium acetate instead of NaCl.

During the validation process, 10 g of homogenate tissue were primarily used, then being optimized to 5 g. To this end, live animals captured at Ria Formosa Lagoon and marketed by an authorized retailer were used. Bivalve samples were thawed, chopped and then grinded (IKA T10 basic Ultra-Turrax®, Lille, France), then 5 g of this homogenate were transferred into a 50 mL Teflon centrifuge tube (Nalgene, Rochester, USA). For validation purposes, samples were spiked with 10 µL of both surrogates (10 µg/L) and with 50 µL of each calibration curve concentration and let stand for one hour. Briefly, the matrix was vortexed (VX-200 Vortex Mixer, Labnet International Inc.) with 5 mL of acetonitrile , followed by the addition of partition salts that were mixed and centrifuged (Sigma 2-16K centrifuge; extraction phase). Subsequently, 2.5 mL of the acetonitrile layer were transferred into

a tube containing the clean-up salts to collect the extract a posteriori and store it at -20 ºC for further GC-MS/MS analysis. Before injection, the protectants 3-ethoxy-1,2-propanediol (5 mg/mL) and D-sorbitol (0.5 mg/mL) were added to 100 µL of sample (more detailed information on Fig.1)

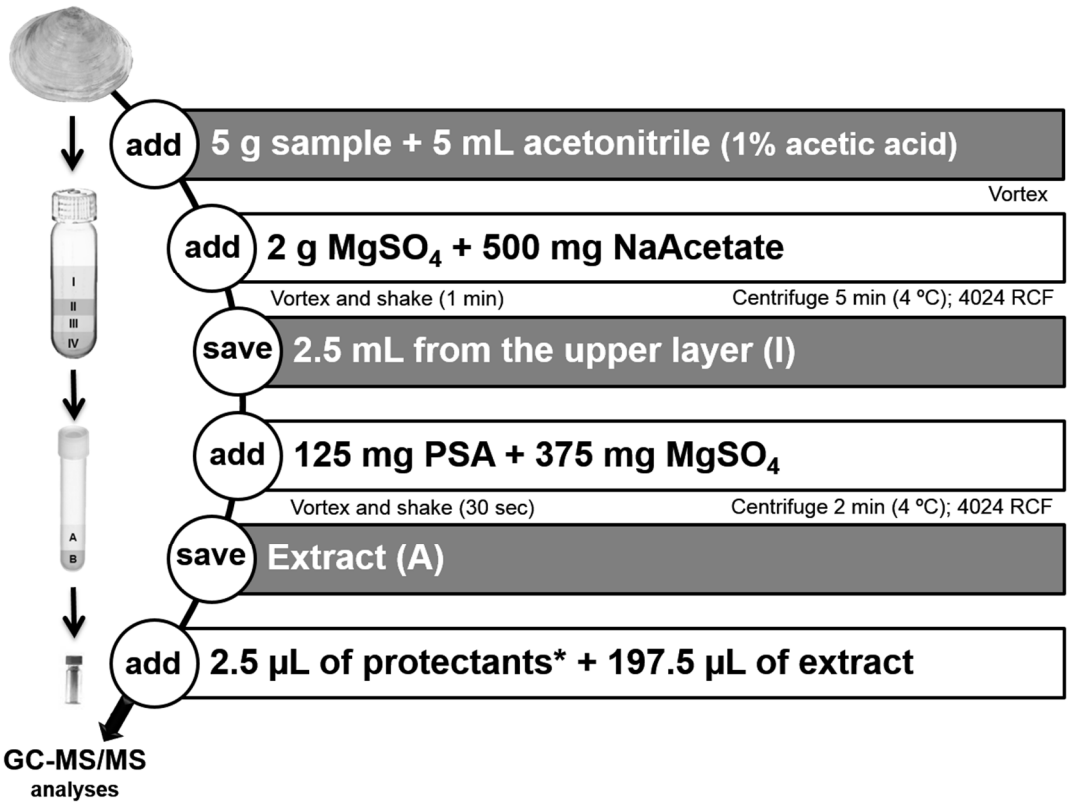

Fig. 1 QuEChERS extraction diagram adopted for the extraction of 55 pesticides from S. plana matrix; I- organic phase, II- organic matter, III- water phase, IV- salts; A- organic phase, B- salts; * 3-ethoxy-1,2-propanediol (5 mg/mL) + D-sorbitol (0.5 mg/mL)

2.4. Gas chromatography-ion trap mass spectrometry

Analyses were performed on a chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation), coupled with an ion trap mass spectrometer detector Thermo Scientific ITQ™ 1100 GC-MS^n), an autosampler (Thermo Scientific TriPlus™) and a Trace GOLD column (TG-5SILMS, 30 m × 0.25 mm × 0.25 µm). Column oven temperatures were programmed (35 min) using several ramps: i) from 65 °C with an initial equilibrium time of 2 min to ii) 180 ºC at 20 ºC/min until iii) 280 ºC at 5 ºC/min, where the temperature was

maintained for 7 min. A solvent delay of 5 min was used to protect, the MS ion multiplier from saturation. The injector port temperature was set to 250 ºC and both ion source and MS transfer line were at 280 ºC. Helium (99.99997% purity) was used as carrier gas at a constant flow rate of 1 mL/min. Sample injection (1 µL) was in splitless mode (3 mm ID, 105 mm, highly deactivated borosilicate glass liner), using a 50 mm length needle. GC separation was achieved by the evaluation of different ranges of temperatures and injection conditions, using initially full-scan mass spectra (65–550 *m/z*) of individual pesticides. SIM segments were established containing a specific ion mass-to-charge ratio (*m/z*) for each compound, followed by the MS/MS characterization. Precursor ions were then subjected to different collision energy voltages (between 0.8 and 2.05) to generate the subsequent product ions (Table 1). The software Xcalibur (version 2.0.7, 2007, Thermo Scientific) together with the Mass Frontier (version 1.0, 1998) and the NIST library were used to evaluate the ion products; the same instrumental protocol was used in other works [44, 45].

Table 1 Molecular mass (g/mol), retention time (min), quantification and diagnostic ions used in GC-MS/MS analysis

Pesticides	Molecular mass	RT	GC-MS/MS							
			Percursor		Products				CE	Ranges
	g/mol	(min)								
Alachlor [a]	269.8	12.50	**160**	→	132	130			1.05	116-161
Aldrin [a]	364.9	13.63	**263**	→	193	191	227		1.60	190-264
Atrazine [b]	215.7	10.83	**200**	→	122	132	164	158	1.50	121-201
Atrazine-d_5	220.7	10.96	**205**	→	127	137	105		1.35	104-206
Atrazine-desethyl	187.6	9.98	**172**	→	130	145	152		1.05	104-173
Azinphos-methyl	317.3	21.58	**132**	→	117	104	114		1.10	100-133
Azoxystrobin	403.4	29.49	**344**	→	329	328			2.00	325-345
BHC (gamma) (Lindane) [a]	290.8	10.94	**181**	→	145	146			1.50	108-184
Chlordane (gamma)	338.9	15.44	**373**	→	266	264			1.10	263-374
Chlorpyriphos	350.6	13.57	**314**	→	258	286			0.90	257-315
Cyanazine	240.7	15.56	**212**	→	209	197	176	171	1.15	170-226
Cyfluthrine (beta) *	434.3	26.53	**199**	→	193	191	163		1.20	190-200
Cyhalofop-butyl	357.4	22.58	**357**	→	342	287			1.50	280-358
Cyhalothrin (lambda) *	449.9	22.83	**181**	→	152	151			1.30	150-182
Cypermethrin (alpha) *	416.3	26.54	**181**	→	152	151			1.30	150-182
4,4´-DDD	320.0	17.97	**235**	→	165	199			1.20	162-236
4,4´-DDT	354.5	17.96	**235**	→	165	199			1.20	117-236
4,4´-DDT-d_8	362.5	17.90	**243**	→	173	206			1.20	172-244
4,4'-DDE	318.0	16.58	**246**	→	176	175			1.70	174-247
Deltamethrin *	505.2	29.54	**181**	→	152	151			1.30	150-182
Diazinon	304.4	12.58	**179**	→	121	163	137	122	1.20	110-180
Dichlorvos	221.0	6.59	**109**	→	79	93			1.10	66-83/90-110
Dieldrin [a]	380.9	17.85	**79**	→	51	50			1.05	49-80
Difenoconazol	406.3	28.60	**323**	→	265	249			1.30	245-324
Dimethoate	229.3	10.39	**87**	→	86	59			1.15	55-88
Endosulfan (alpha)	406.9	18.02	**241**	→	206	205			1.35	165-242
Endosulfan (beta)	406.9	16.75	**241**	→	206	204	170		1.35	165-242
Endosulfan sulfate	422.9	18.96	**272**	→	237	235			0.90	234-273
Endrin [b]	380.9	17.33	**243**	→	207	173			1.20	172-244
Fenamiphos	303.4	15.45	**303**	→	268	266			1.20	265-304
Fenitrothion	277.2	13.53	**260**	→	228	217	232		1.10	190-261
Fonofos	246.3	11.15	**137**	→	109	81			0.85	80-138

(continued)

Pesticides	Molecular mass g/mol	RT (min)	GC-MS/MS Percursor		Products				CE	Ranges
Fonofos	246.3	11.15	**137**	→	109	81			0.85	80-138
Heptachlor [b]	373.3	12.70	**272**	→	237	235			1.05	236-275
Heptachlor epoxide [b]	389.3	14.75	**353**	→	263	282			1.05	262-354
Hexachlorobenzene (HCB)[b]	284.8	10.38	**284**	→	214	249			1.50	211-285
Hexachlorocyclopentadiene (HCCP) [b]	272.3	6.88	**237**	→	143	141	203		1.60	140-145/200-238
Malathion	330.4	13.62	**173**	→	99	117	145		0.90	95-173
Methoxychlor [b]	345.7	19.70	**227**	→	169	181			1.30	165-228
Metamidophos	141.1	8.29	**141**	→	113	115			1.50	110-142
Metolachlor	283.8	13.50	**162**	→	132	133			1.05	130-163
Metribuzin	214.3	12.59	**198**	→	150	110			1.35	109-199
Mirex	545.5	22.58	**272**	→	237	235			1.00	234-273
Parathion-ethyl	291.3	13.65	**291**	→	186	220	255	256	1.15	185-292
Parathion-methyl	263.2	12.51	**263**	→	246	153			1.40	150-264
Pendimethalin	281.3	14.99	**252**	→	162	191			1.25	160-253
Pentachlorobenzene (PeCB)	250.3	8.66	**250**	→	215	144			2.01	143-251
Phosmet	317.3	8.64	**160**	→	130	140			1.15	125-161
Pirimicarb	238.4	11.78	**166**	→	96	137	121		1.20	95-167
Procymidone	284.1	15.39	**96**	→	67	68			0.90	64-97
Propazine	229.7	10.87	**214**	→	200	172	138		1.00	137-215
Propyzamide	256.1	11.17	**173**	→	138	145			1.05	135-174
Simazine [a]	201.6	10.93	**201**	→	186	174	138		1.45	135-201
Simetryn	213.3	12.71	**213**	→	170	185			1.25	169-214
Tebuconazole	307.8	19.68	**125**	→	89	99			1.40	87-126
Terbuthylazine	229.7	11.08	**214**	→	173	132			1.00	131-215
Terbutryn	241.4	13.15	**185**	→	170	128			1.10	127-186
Tetrachlorvinphos	366.0	14.96	**329**	→	314	278			1.20	219-330
Trifluralin	335.3	9.76	**264**	→	206	160	188	171	1.00	159-265

Internal standards; [a] Compounds present on the mix A (EPA 505/525); [b] Compounds present on the mix B (EPA 505/525); [*] Contain several diastereoisomers; **RT:** retention time; **CE:** collision energy (V)

2.5. Validation studies

The validation process followed the European SANCO guidelines for pesticide residue analytical methods [37]. Linearity was evaluated using three independent calibration curves, each with eight nominal calibration standard mixtures (ranging from 0.5 to 100 µg/L) spiked (50 µL) into pre-homogenized bivalve pools with 56 pesticides and both IS (each at 10 µg/L). The curves were plotted using the ratio between pesticide and surrogate area. Eight-point levels were established based on the quantified concentrations found in the literature for aquatic organisms (see Electronic Supplementary Material Fig. S2).

The limits of detection (LODs) and quantification (LOQs) were calculated with the same curves, using the following formulas: LOD = 3.3 α/S and LOQ = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves. Afterwards, the eight-point calibration curve was adjusted to five concentration levels taking into consideration the LOD and LOQ concentrations, for each pesticide and the best linearity response (r^2). Two calibration curves in matrix extract (4, 8, 16, 32 and 64 µg/L), with a minimum of 5 different analyses at the limit of quantification (LOQ) and at two multiples of the LOQ (2LOQ and 10LOQ), were used for validation.

To evaluate the recoveries, precision, accuracy and stability of the method, three different pools of bivalve homogenate were fortified at three concentrations (LOQ, 2LOQ and 10LOQ) as quality controls (QCs). The extracts were injected in triplicate and analysed for each QC in three different days. Recoveries were determined comparing the peak area ratio of each pesticide in spiked bivalve matrix with the peak area ratio of the same concentration in a matrix blank spiked after extraction. Precision was expressed as the relative standard deviation (%RSD) of the replicate measurements, and the accuracy of the method was evaluated as the percentage of agreement between the method results and the nominal amount of added compound. Finally, the stability of pesticides in the extracts was analyzed immediately after preparation and after 24, 48, and 96 hours at –20 ºC.

New deactivated liners were used every 100 injections; when dirty, the glassware was cleaned with 5% DMDCS in toluene solution. Pesticide concentrations were validated against a set of quality control parameters, such as laboratory (solvent) and commercial animal blanks, matrix spikes, and triplicate samples that were used in every day injection.

2.6. Data analyses

A total of 90 pools of *S. plana* were analyzed per compound and expressed as ng/g of wet weight (ww). As a first approach for handling the data, pesticides were examined summed and grouped per category, that is, fungicides, herbicides, insecticides. Additionally, data were also organized by total average loads (TAL) as the sum (Σ) of the average concentrations found for each pesticide. All of these categories were analyzed against factors such as site, season and sex.

Descriptive and inferential statistics were made with the PAST 3.06 software [46]. Since data did not accomplish the parametric assumptions (normality and/or homogeneity of variances) and data transformation was ineffective, the non-parametric Kruskal-Wallis ANOVA was used, followed by Mann-Whitney U test, with a sequential Bonferroni correction, considering a significant level of 5%.

The analyses investigating correlations between CI and other selected parameters, as well as the F-test for linear regression (concerning linearity evaluation as required in the validation process), were done using the software GraphPad Prism [47].

3. Results and discussion

3.1. QuEChERS extraction

The QuEChERS extraction protocols of Anastassiades et al. [42] and AOAC 2007.01 [43] were tested to find which method would yield the best results in terms of recoveries and lower matrix interference. For each one, four pools of bivalves (10 g of homogenized soft tissue) were spiked with 10 µg/L of the selected pesticides and surrogates. Between repetitions, the second extraction procedure revealed to be more stable, with fewer pesticides (34%) with recoveries below 100% when compared to the first method (55%). This fact may be related to pH reduction, by addition of acid acetic and the anhydrous sodium acetate, which buffers the extract [30].

For both methods, the extracts were also evaporated and reconstituted in n-hexane (99%, from Sigma-Aldrich, Deisenhofen, Germany), but none of them presented good reproducibility and recovery results. Consequently, the final extracts were kept in acetonitrile.

The final protocol was then reduced to half of the quantities (homogenate tissue, solvent and salts) since there were no significant differences (p = 0.123) between the percentage of the recoveries when compared to the original one, saving expenses and allowing the duplication of injected samples.

3.2. Method validation

The validation protocol followed the criteria established by SANCO/825/00 rev 8.1 [37].

Retention times and mass spectra were similar between standards and fortified matrices (RSD < 5%), thus proving that this chromatographic procedure is a selective method for the quantification of all pesticides. The parent-daughters ions, resulted from specific individual collision energies, had the same proportions between spiked and wild samples, demonstrating the robustness of the method. As a result, the method was able to select the pesticides with a high precision at different concentrations.

The five-point calibration curves proved to have good fits, with r^2 ranging from 0.986 to 0.999. Additionally, the F-test was always significant (p < 0.001), pointing to good linearity responses.

LOD values ranged from 0.22 µg/L to 3.4 µg/L and LOQ values ranged from 0.33 µg/L to 10.3 µg/L, where the last values accomplished the limits established (0.01 mg/kg) by the annex IIA point 4.2.1 of Directive 91/414/EEC described by the European Commission Directorate General Health and Consumer Protection [37].

Recovery, precision, accuracy and stability were studied for three independent replicates in three different days for each spiked concentration; the related data are summarized in Table 2. The tested concentrations were suitable for the determination of recoveries according to SANCO/825/00 rev 8.1 (concentration range from 30% of the LOQ to 20% above the highest value) [37]. The successfully optimized extraction procedure, obtained recovery rates ranging from 78 to 119%, demonstrating the feasibility of this QuEChERS protocol for the extraction of 55 pesticides from bivalve matrix. The obtained values are in accordance with the range of mean recoveries (70–120%) established by the protocol cited above. The recoveries success of this protocol were also likely favored by the matrix composition, since bivalves have a high water content (>79%) and low lipid profile (<1.5%) [48]. Comparable average recoveries were obtained for comparable matrices, such as fish (70–115% for 22 pesticides) [49], mussels (90–106% for 14 pesticides) [22] and shrimps (90–105% for 9 pesticides) [50].

Precision (%RSD) results varied from 2 to 27%, where the highest values were attained for the lowest concentrations (LOQ and 2LOQ). In spite of this, the precision was never above the maximum established (30%) by SANCO/825/00 rev 8.1 protocol, for concentrations > 1 µg/kg ≤ 0.01 mg/kg. Accuracy ranged from 76 to 120% demonstrating high stability for 55 pesticides, with the exception of chlorfenvinphos Z that was posteriorly excluded from this method validation.

Table 2 Average recovery, precision (RSD), accuracy, LODs and LOQs data for the 55 selected pesticides assayed at three quality control (QC) levels, for three independent analyses

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Alachlor	2.82		95.4	13.6	12.2	10.0	100.6	9.7		
	5.64	< 0.0001	90.1	9.0	14.9	8.6	88.1	17.4	1.24	3.76
	28.20		101.9	9.1	10.4	6.7	91.7	8.3		
Aldrin	2.82		93.1	13.7	18.3	5.3	87.7	6.9		
	5.64	< 0.0001	92.6	11.7	11.6	3.5	90.5	16.0	0.85	2.57
	28.20		97.2	16.0	8.9	5.0	102.9	12.3		
Atrazine	3.26		104.7	10.1	12.1	7.4	99.4	7.6		
	6.52	< 0.0001	85.9	7.4	8.5	5.0	83.4	9.3	1.20	3.63
	32.60		90.1	14.6	9.4	5.4	84.5	16.7		
Atrazine-desethyl	4.53		102.4	14.9	9.3	8.4	99.3	14.4		
	9.06	< 0.0001	96.0	10.3	9.6	5.0	95.4	7.9	1.49	4.53
	45.30		100.3	11.1	15.1	3.7	78.1	41.2		
Azinphos-methyl	2.11		99.6	1.2	20.2	0.0	88.5	3.1		
	4.22	0.0008	94.8	13.4	22.9	9.0	119.7	0.6	0.70	2.11
	21.10		101.1	15.5	14.4	7.3	86.4	32.6		
Azoxystrobin	1.23		109.8	17.0	16.2	7.6	93.2	13.2		
	2.46	< 0.0001	104.2	11.3	12.9	7.1	98.0	7.0	0.41	1.23
	12.30		84.0	6.8	12.8	8.1	92.4	18.9		
Chlordane (gamma)	4.69		96.7	10.1	13.6	8.9	103.9	9.2		
	9.38	< 0.0001	91.6	11.0	14.4	9.2	92.0	10.6	1.55	4.69
	46.90		86.4	13.8	11.7	5.8	88.1	12.1		
Chlorpyriphos	3.27		107.6	18.4	12.3	9.9	103.1	14.4		
	6.54	< 0.0001	102.0	8.1	11.1	5.9	97.8	7.3	1.08	3.27
	32.70		101.8	12.0	7.4	5.2	101.3	11.1		

(continued)

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Cyanazine	3.38		85.2	6.9	21.1	6.1	90.8	11.6		
	6.76	< 0.0001	78.7	13.2	23.5	2.1	81.2	6.5	1.12	3.38
	33.80		90.6	15.0	12.8	6.6	85.7	10.1		
Cyfluthrine (beta)	1.2		93.6	8.6	21.6	5.9	98.1	0.1		
	2.40	< 0.0001	117.3	4.8	26.5	28.8	112.6	2.7	0.39	1.20
	12.00		104.8	2.6	11.3	4.5	111.8	12.1		
Cyhalofop-butyl	4.28		94.4	16.5	7.4	5.0	100.8	15.2		
	8.56	< 0.0001	95.5	5.3	10.6	6.5	91.3	20.7	1.41	4.28
	42.80		94.0	12.1	13.5	5.0	107.4	7.7		
Cyhalothrin (lamdba)	2.97		113.9	5.2	10.1	7.0	99.0	4.9		
	5.94	< 0.0001	101.8	8.1	8.4	3.9	101.2	9.7	0.98	2.97
	29.70		98.1	11.6	9.8	6.5	103.7	10.1		
Cypermethrin (alpha)	10.3		91.9	5.7	15.2	8.2	96.1	7.6		
	20.60	0.0006	100.5	4.0	1.9	1.2	103.9	12.5	3.40	10.30
	103.0		100.9	11.5	12.4	4.3	90.4	7.7		
4,4´-DDD	3.56		101.3	17.5	6.9	5.2	92.3	15.6		
	7.12	< 0.0001	100.7	8.3	15.8	7.0	107.8	3.2	1.17	3.56
	35.60		81.1	7.3	12.3	5.8	94.1	6.1		
4,4´-DDE	3.85		112.9	5.6	25.2	5.1	90.1	5.0		
	7.70	< 0.0001	119.3	12.1	13.9	5.7	90.3	13.6	1.27	3.85
	38.50		91.7	10.2	13.8	4.3	87.1	14.5		
4,4´-DDT	3.51		102.1	12.6	13.6	9.9	98.5	13.7		
	7.02	< 0.0001	88.3	5.9	18.8	5.0	92.9	11.3	1.16	3.51
	35.10		86.6	8.9	8.8	5.2	86.3	10.4		

(continued)

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Deltamethrin	1.43		102.5	8.8	13.9	8.9	92.6	10.1		
	2.86	< 0.0001	108.6	12.2	16.3	4.4	115.6	10.8	0.47	1.43
	14.30		97.8	0.4	5.8	0.8	97.2	1.9		
Diazinon	4.53		110.0	7.2	13.4	9.2	107.3	33.9		
	9.06	0.0024	96.7	7.9	11.6	8.1	89.6	16.8	1.49	4.53
	45.30		98.8	7.5	9.3	5.2	98.7	15.5		
Dichlorvos	1.82		98.1	11.9	14.5	8.4	99.4	10.7		
	3.64	< 0.0001	105.4	6.0	8.0	4.6	100.5	11.3	0.60	1.82
	18.20		89.1	12.8	5.7	3.3	76.1	27.0		
Dieldrin	2.82		103.4	4.4	15.6	4.0	107.8	5.5		
	5.64	< 0.0001	89.1	4.3	6.2	2.9	97.0	15.6	0.22	0.66
	28.20		96.1	12.1	9.5	3.6	103.8	14.6		
Difenoconazol	2.77		109.2	22.0	15.6	10.0	111.4	18.5		
	5.54	< 0.0001	103.4	10.8	7.7	5.0	100.7	6.5	0.92	2.77
	27.70		78.2	12.4	6.7	5.6	84.7	9.6		
Dimethoate	3.87		101.9	5.7	7.8	4.5	96.9	15.2		
	7.74	< 0.0001	93.8	8.2	11.7	5.9	77.6	2.8	1.28	3.87
	38.70		101.1	9.4	7.1	5.2	91.8	10.6		
Endosulfan (alfa)	3.53		107.5	11.3	17.4	7.3	103.6	12.3		
	7.06	< 0.0001	107.7	11.2	21.0	4.2	99.1	12.0	1.17	3.53
	35.30		86.6	13.4	10.1	4.6	88.9	14.7		
Endosulfan (beta)	3.53		115.5	3.9	8.1	2.5	96.0	17.5		
	7.06	< 0.0001	111.5	5.3	17.1	4.0	109.5	6.8	1.14	3.44
	35.30		98.9	9.1	12.7	6.5	93.9	13.5		

(continued)

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Endosulfan sulfate	4.7		106.7	10.5	11.4	7.0	90.2	17.5		
	9.40	< 0.0001	98.6	4.0	16.7	7.9	86.2	8.8	1.55	4.70
	47.00		100.5	14.7	14.1	7.9	76.6	11.6		
Endrin	3.26		106.2	7.1	15.8	12.6	97.6	12.0		
	6.52	< 0.0001	98.3	2.4	11.0	10.7	92.4	12.4	1.09	3.30
	32.60		99.5	9.9	7.9	5.4	96.5	13.4		
Fenamiphos	4.27		109.9	18.5	21.6	7.7	105.5	18.7		
	8.54	< 0.0001	93.5	9.7	7.9	5.8	83.2	12.3	1.41	4.27
	42.70		110.1	19.7	9.9	4.7	100.4	13.3		
Fenitrothion	3.87		100.7	4.1	5.2	3.0	90.4	5.5		
	7.74	< 0.0001	94.7	4.6	15.9	9.8	89.5	7.2	1.28	3.87
	38.70		107.1	14.9	9.9	5.5	95.9	13.1		
Fonofos	2.26		83.0	6.9	13.4	8.8	91.1	7.0		
	4.52	< 0.0001	102.1	4.7	14.1	10.3	102.2	3.0	0.75	2.26
	22.60		92.4	10.1	9.6	4.1	99.1	9.5		
HCB	3.26		100.1	13.4	12.4	7.8	98.1	9.7		
	6.52	< 0.0001	97.1	9.6	13.2	8.1	91.0	10.5	0.72	2.18
	32.60		100.8	10.7	9.6	5.1	88.1	26.3		
HCCP	3.26		102.8	4.5	6.7	4.6	115.4	29.4		
	6.52	0.0055	103.2	11.2	11.9	6.5	99.8	28.4	2.12	6.42
	32.60		87.5	10.6	8.8	4.4	82.1	32.0		
Heptachlor	3.26		102.8	4.5	6.7	4.6	115.4	29.4		
	6.52	< 0.0001	103.2	11.2	11.9	6.5	99.8	28.4	1.55	4.70
	32.60		87.5	10.6	8.8	4.4	82.1	32.0		

(continued)

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Heptachlor epoxide	3.26		107.4	11.8	8.4	6.0	107.3	8.7		
	6.52	< 0.0001	94.8	15.3	9.9	7.9	91.2	15.4	0.75	2.27
	32.60		105.9	10.3	9.5	6.4	107.0	10.7		
Lindane	2.82		95.7	7.5	8.9	6.0	88.4	8.2		
	5.64	< 0.0001	107.1	9.5	12.4	6.5	102.6	6.0	0.91	2.76
	28.20		91.7	15.6	11.2	4.3	98.1	12.0		
Malathion	4.17		104.1	15.2	7.1	2.5	93.1	18.9		
	8.34	< 0.0001	102.9	10.9	12.0	10.1	93.5	17.6	1.38	4.17
	41.70		110.1	12.5	10.9	6.7	92.8	8.6		
Metamidophos	3.41		103.5	10.7	9.5	2.5	96.5	18.3		
	6.8	< 0.0001	99.4	4.2	5.0	2.0	99.0	11.8	1.1	3.41
	34.1		102.2	5.5	10.9	2.7	90.6	29.0		
Methoxychlor	3.26		119.0	3.2	15.6	9.9	102.5	10.7		
	6.52	< 0.0001	77.7	20.0	15.3	3.0	84.9	25.8	1.1	3.45
	32.60		105.2	8.8	12.4	11.3	101.2	9.3		
Metolachlor	4.41		91.7	20.7	11.7	6.2	94.0	13.6		
	8.82	< 0.0001	99.7	9.6	9.5	6.1	98.6	7.5	1.46	4.41
	44.10		90.4	6.3	7.8	3.6	89.9	5.3		
Metribuzin	3.52		97.4	6.4	14.1	12.3	86.4	14.1		
	7.04	< 0.0001	107.1	18.5	12.9	7.0	94.9	13.0	1.16	3.52
	35.20		90.9	13.0	8.8	3.9	93.9	14.9		
Mirex	4.14		100.1	8.7	12.0	7.1	92.1	13.0		
	8.28	< 0.0001	80.9	23.6	16.4	3.9	85.2	7.9	1.37	4.14
	41.40		80.2	10.7	7.5	4.2	84.1	11.0		

(continued)

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Parathion-ethyl	3.12		99.8	3.2	18.2	6.3	113.7	5.2		
	6.24	< 0.0001	103.7	11.1	13.7	5.8	92.5	20.2	1.03	3.12
	31.20		98.4	10.4	11.0	5.8	101.8	9.5		
Parathion-methyl	3.75		101.7	3.8	11.8	7.1	92.3	10.9		
	7.50	< 0.0001	94.6	8.4	18.6	18.5	95.1	13.7	1.24	3.75
	37.50		113.8	3.4	4.3	3.4	114.0	3.6		
PeCB	3.63		106.9	8.0	10.2	5.8	107.4	12.4		
	7.26	< 0.0001	99.2	10.8	12.6	8.3	90.6	13.0	1.20	3.63
	36.30		92.4	12.4	15.1	4.5	80.2	26.4		
Pendimethalin	2.6		103.0	11.9	7.5	5.9	95.2	21.3		
	5.20	< 0.0001	94.5	7.6	7.2	3.9	95.2	19.9	0.86	2.60
	26.00		99.9	9.6	12.6	3.4	95.7	18.7		
Phosmet	4.4		97.0	4.0	7.5	2.4	96.5	6.1		
	8.80	< 0.0001	118.2	20.4	8.9	8.5	96.2	7.7	1.45	4.40
	44.00		89.0	10.1	8.0	4.1	76.5	28.4		
Pirimicarb	3.02		93.6	12.0	13.2	7.3	99.0	16.4		
	6.04	< 0.0001	99.9	5.8	8.2	6.4	88.8	12.5	1.00	3.02
	30.20		94.4	8.7	7.8	5.4	97.8	11.4		
Procymidone	3.84		90.7	6.3	15.3	2.9	80.5	1.0		
	7.68	< 0.0001	99.2	6.4	14.4	7.1	96.1	11.4	1.27	3.84
	38.40		100.7	8.8	12.4	6.5	101.2	2.7		
Propazine	3.37		98.8	6.6	4.7	3.6	92.2	9.6		
	6.74	< 0.0001	103.2	11.7	13.5	7.8	106.1	14.3	1.11	3.37
	33.70		105.8	14.0	8.7	1.7	101.1	12.7		

(continued)

Pesticides	QC	F ratio test	Recovery		RSD		Accuracy		LODs	LOQs
	µg/L	p value	(%)	SD	(%)	SD	(%)	SD	µg/L	µg/L
Propyzamide	2.46		89.4	9.4	14.3	8.5	88.7	8.0		
	4.92	< 0.0001	79.7	17.6	12.1	8.5	77.4	18.7	0.81	2.46
	24.60		94.8	13.4	9.7	6.5	88.4	12.9		
Simazine	2.82		97.5	15.6	20.1	7.2	92.5	10.0		
	5.64	< 0.0001	94.0	10.7	10.9	3.7	95.0	15.5	0.72	2.17
	28.20		103.4	9.4	10.4	6.4	83.9	29.8		
Simetryn	4.49		94.0	19.1	16.6	8.6	98.5	15.6		
	8.98	< 0.0001	86.8	11.0	13.9	10.3	88.5	15.1	1.48	4.49
	44.90		95.2	5.7	11.9	4.8	93.1	6.3		
Tebuconazole	2.78		98.5	12.2	17.9	2.3	94.9	13.6		
	5.56	< 0.0001	85.5	9.7	12.2	4.9	92.9	15.1	0.92	2.78
	27.80		98.8	12.4	10.9	5.5	95.1	13.9		
Terbuthylazine	1.83		93.6	9.0	11.3	2.7	98.7	14.1		
	3.66	< 0.0001	105.6	8.3	14.0	7.5	104.1	7.1	0.61	1.83
	18.30		97.9	13.5	18.8	13.9	91.6	13.7		
Terbutryn	2.35		98.3	9.1	15.2	10.2	99.9	13.2		
	4.70	< 0.0001	101.9	9.8	17.2	11.8	105.8	11.4	0.78	2.35
	23.50		96.7	10.4	9.1	6.1	90.9	11.4		
Tetrachlorvinphos	2.53		80.5	2.3	22.7	10.7	94.7	11.9		
	5.06	< 0.0001	101.2	7.9	7.0	3.5	102.7	11.8	0.84	2.53
	25.30		90.7	9.1	10.3	7.0	90.8	9.0		
Trifluralin	1.39		96.9	5.5	18.9	4.3	91.7	13.0		
	2.78	< 0.0001	101.5	13.7	9.4	5.1	97.9	13.4	0.46	1.39
	13.90		99.0	10.7	9.8	4.6	83.6	23.2		

Recoveries (%)- obtained for the 3 quality controls (LOQ, 2LOQ and 10LOQ) for 3 independent replicates and days; **Precision** (relative standard deviation- RSD); **Accuracy** (%); **SD**- standard deviation between independent replicates (min. 5)

The stability of the extracted pesticides from bivalve matrix was evaluated by comparing the initial results of the QCs with those obtained after a period of 24, 48, and 96 h, kept at −20 ºC (Fig. 2). Higher standard deviations were observed for the lowest concentrations (LOQ and 2LOQ), as well as a decrease of the average percentages of extraction after 48 h, presenting significant differences between time periods for two categories of pesticides (fungicides and herbicides) at the highest concentration (10LOQ). Therefore, due to the low stability of the samples, the extracts should always be analyzed within a maximum period of 24 h.

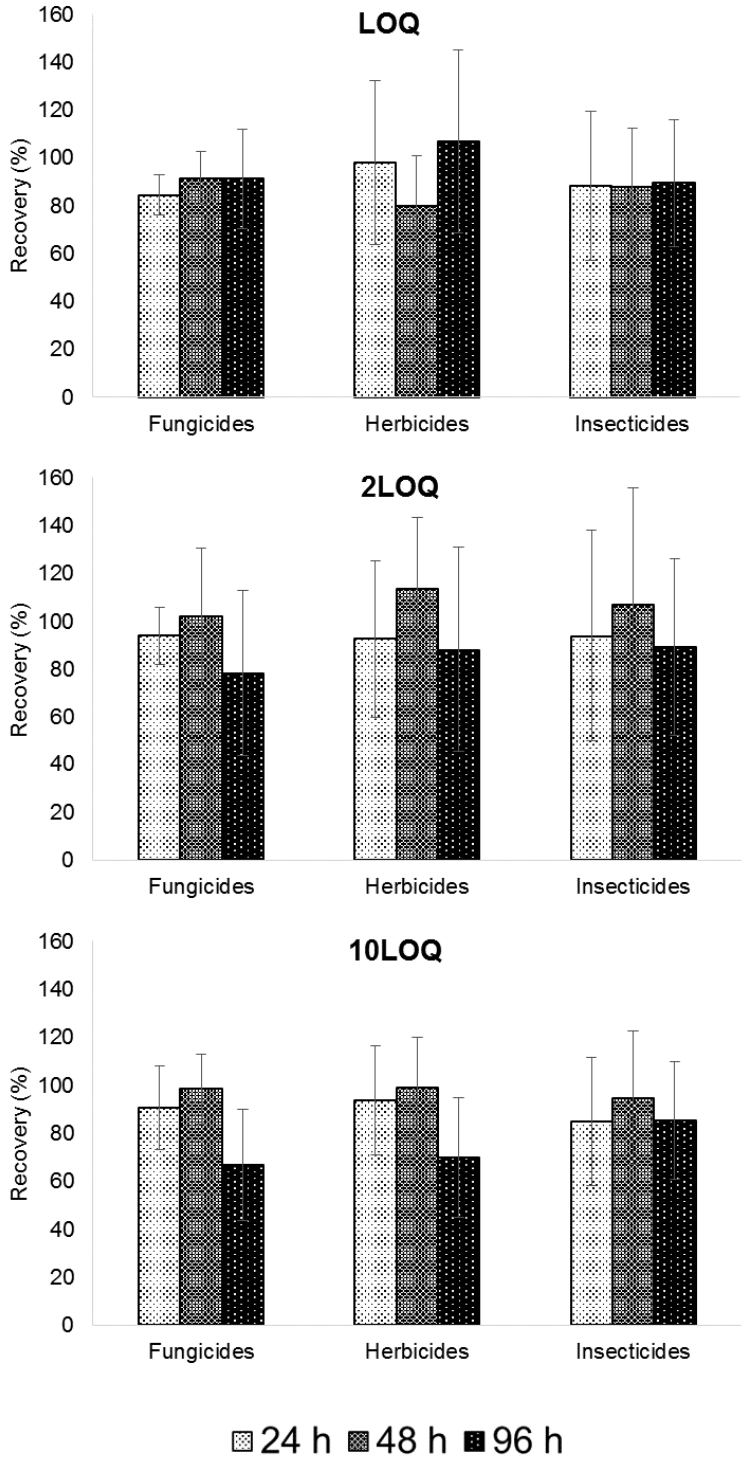

Fig. 2 Stability of the extracted pesticides, at three different concentrations (LOQ, 2LOQ and 10LOQ) from bivalve matrix after 24, 48 and 96 hours; the results are expressed as average percentages (%) and the bars standard deviation

3.2.1 Matrix effects

The quantitative determination of pesticide residues from food extracts can be challenging because of the matrix co-extractives, which may vary with food type [51]. The most common problem is the enhancement and/or suppression of the signal when compared with the standards. Different approaches have been established to minimize these effects, such as the use of different type of injectors, changing sorbents, the use of isotopically labelled internal standards, the use of a matrix-matched calibration and analyte protectants [52, 53].

Here a deactivated glass liner was adopted, which was cleaned with DMDCS every 100 injections to avoid interactions with silanol groups, as suggested by Čajka et al. [52] Two surrogates were used to express data as area ratio where their RSD injection were below 20%.

The protectants were also evaluated considering type, number and concentration used to obtain the best results [52, 29, 38]. The 3-ethoxy-1,2-propanediol and D-sorbitol were chosen and tested at two concentrations (1 and 10 mg/mL). Both proved to enhance the signal by more than 50% for all the selected compounds (see Electronic Supplementary Material Fig. S3); the highest concentration of protectants provided better results. The data prove that protectants strongly interact with active sites in the GC equipment (injector and column) and therefore decrease the adsorption of the target analytes. As a compromise between reliable results and equipment stability, we decided to use half of the concentration used by Maštovská et al. [29] and to wash the syringe with acetone:water (1:1) mixture, followed by acetonitrile between injections.

The matrix effect (ME) was evaluated at the lowest concentration (LOQ), where eight samples were spiked after extraction ($A_{\text{standard in matrix}}$) and compared to those of injected standards ($A_{\text{standards}}$), as indicated on the following equation:

$$\mathrm{ME} = -\left[\frac{A_{\text{standards}} - A_{\text{standards in matrix}}}{A_{\text{standards}}}\right] \times 100$$

If ME results equal to zero no matrix effect is presented, while ME above or below zero represents a signal enhancement/suppression, respectively. The matrix effects involving the selected compounds are shown in Fig.3, where most (80%) presented a signal suppression with average values of –83.9%. Signal enhancement was verified for one fungicide (procymidone) and one herbicide (metribuzin), with the others being observed among insecticides; the highest average value (4821%) was due to dieldrin and azinphos-methyl areas. The atrazine-d_5 and 4,4´-DDT-d_8 presented –10.4% and 1289.8%, respectively as ME. Thus, to compensate this matrix effect and avoid under or over estimation, a matrix-matched calibration was used.

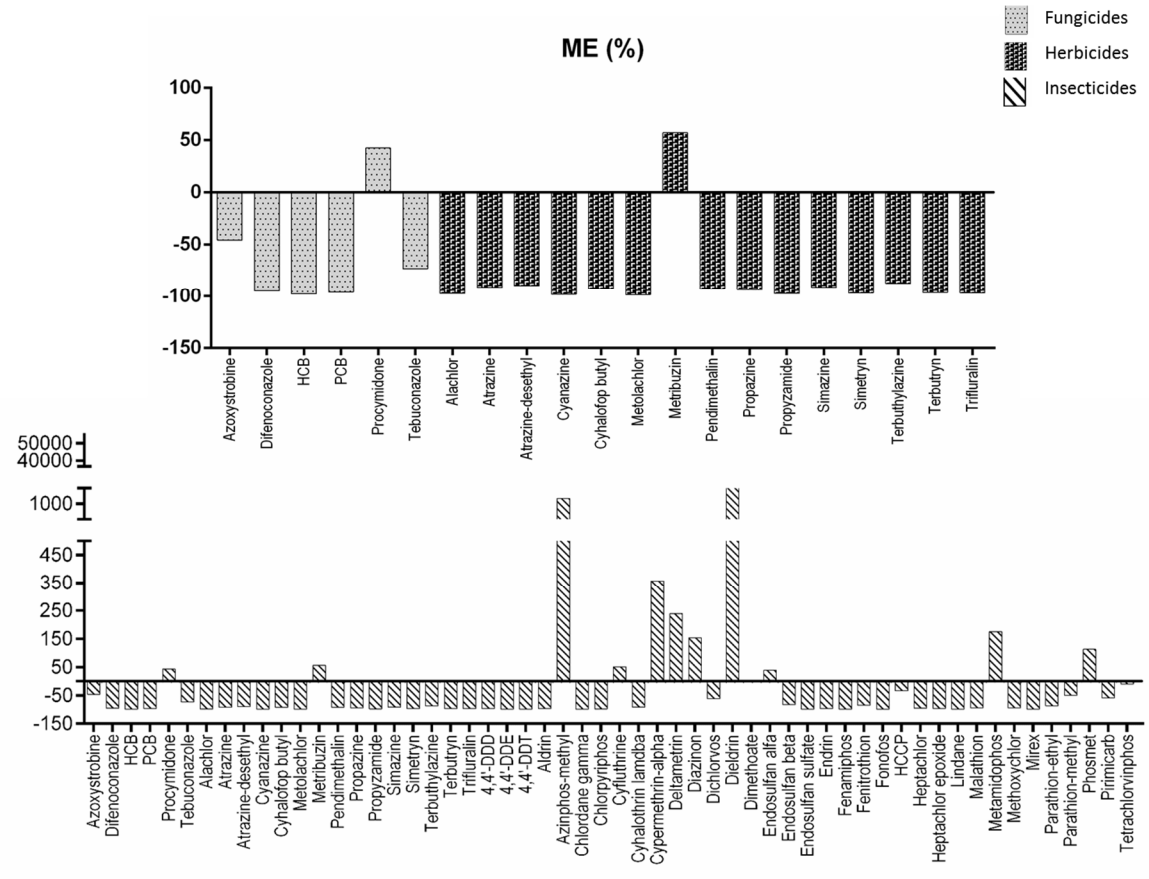

Fig. 3 Evaluation of ME at LOQ concentration for all 55 selected pesticides; the results are expressed as percentage (%)

3.3. Pesticide levels in bivalve samples from Ria Formosa Lagoon

From all selected pesticides only metolachlor and 4,4´-DDE were not detected in the analyzed bivalves. A total of 76% of the samples presented levels of pesticides above LOD and 40% above LOQ values, with nominal concentrations ranging from 1.7 to 53.6 ng/g ww. Similar ranges of concentrations were reported by others in both blue and Mediterranean mussels (1–60 ng/g ww and 2–246 ng/g ww, respectively) [22, 24, 27], in scallops (0.008–4.2 ng/g ww) [26], and in undifferentiated shellfish (1.4–22.5 ng/g ww) [25]. Here no statistical differences were found among sites and sexes (undefined, female, male). Thus, data were grouped and displayed by season (Table 3). The animals collected during autumn presented significantly higher TAL of pesticides (Σ930 ng/g ww, $p < 0.05$) than those collected in summer (Σ500 ng/g ww; Fig. 4A). Perfectly in line with this finding, an identical seasonal fluctuation was observed in surface water samples (dissolved and particulate matter) collected at the same time in this basin [44].

Table 3 Pesticides concentrations (ng/g ww) in soft tissue of *S. plana* collected from Ria Formosa Lagoon, and displayed by season, as well as the percentage of samples that were above the LOD and LOQ values per compounds; data are presented as minimum–maximum (average; standard deviation)

	Pesticides	LOD	LOQ	*S. plana* soft tissue (ng/g ww)			
		% above		Autumn	Winter	Spring	Summer
Fungicides	Azoxystrobine	70.67	47.35	12.76 - 61.11 (28.51; 7.5)	1.70 - 38.24 (13.39; 5.9)	3.40 - 44.63 (17.17; 5.7)	36.50 - 52.35 (44.42; 3.3)
	Difenoconazole	97.33	67.70	4.42 - 27.31 (15.01; 4.3)	3.46 - 9.06 (13.76; 1.5)	3.37 - 8.55 (5.69; 1.2)	3.84 - 13.32 (6.91; 1.4)
	HCB	46.67	19.03	3.51 - 3.51 (3.51; 0.3)	2.61 - 2.81 (13.71; 0.1)	-	-
	PeCB	72.00	20.35	4.59 - 6.21 (5.40; 0.2)	3.68 - 5.80 (13.47; 0.4)	4.37 - 6.07 (5.22; 0.7)	-
	Procymidone	22.67	11.50	18.09 - 18.09 (18.09; -)	7.43 - 18.88 (13.70; 4.2)	-	-
	Tebuconazole	92.00	58.41	5.19 - 49.07 (15.96; 4.8)	3.20 - 12.55 (13.74; 2.3)	6.06 - 6.06 (6.06; 0.5)	4.31 - 15.11 (9.71; 1.7)
Herbicides	Alachlor	97.33	41.59	4.55 - 5.59 (4.95; 1.0)	3.90 - 6.59 (13.05; 0.7)	4.18 - 4.60 (4.32; 0.5)	4.59 - 4.59 (4.59; 0.6)
	Atrazine	94.67	58.41	7.08 - 56.88 (24.59; 7.1)	4.52 - 24.03 (13.64; 2.7)	9.13 - 37.13 (21.27; 6.7)	5.90 - 6.15 (6.02; 2.4)
	Atrazine-desethyl	81.33	35.84	6.55 - 9.52 (7.91; 3.2)	4.98 - 12.63 (13.65; 2.5)	7.53 - 30.30 (16.44; 4.4)	6.12 - 6.12 (6.12; 1.6)
	Cyanazine	94.67	55.75	6.53 - 33.85 (18.71; 9.5)	3.34 - 29.60 (13.12; 4.3)	7.75 - 14.44 (10.43; 4.1)	9.43 - 13.38 (11.23; 4.5)
	Cyhalofop-butyl	73.33	27.88	-	4.95 - 24.89 (13.71; 3.7)	7.21 - 13.83 (11.21; 1.9)	6.29 - 7.99 (6.96; 2.0)
	Metribuzin	94.67	50.44	4.52 - 28.58 (15.32; 6.4)	4.41 - 17.46 (13.21; 4.0)	10.36 - 24.97 (15.62; 4.6)	6.25 - 13.43 (9.69; 2.5)
	Pendimethalin	93.33	55.31	3.47 - 26.09 (11.29; 2.2)	5.07 - 73.94 (13.70; 4.6)	3.61 - 34.48 (17.82; 5.2)	5.51 - 16.27 (10.89; 3.0)
	Propazine	98.67	65.49	5.16 - 25.37 (12.96; 2.4)	4.24 - 21.90 (13.33; 2.2)	5.51 - 10.25 (6.98; 1.8)	6.11 - 8.81 (7.11; 1.9)
	Propyzamide	72.00	38.05	2.58 - 3.81 (3.24; 0.4)	2.50 - 7.58 (13.14; 0.9)	3.20 - 4.47 (3.67; 0.7)	4.00 - 4.96 (4.34; 0.3)
	Simazine	64.00	27.88	10.28 - 72.21 (29.80; 14)	11.86 - 32.96 (13.25; 6.3)	12.49 - 13.11 (12.80; 6.9)	13.38 - 38.24 (25.81; 0.9)
	Simetryn	98.67	42.48	6.48 - 12.93 (9.16; 2.1)	5.77 - 16.92 (13.05; 2.6)	4.65 - 7.16 (5.87; 1.2)	8.25 - 8.96 (8.60; 1.7)
	Terbuthylazine	77.33	39.82	3.97 - 32.58 (11.50; 3.9)	6.61 - 39.01 (13.28; 4.3)	2.24 - 25.31 (13.98; 6.9)	14.54 - 20.57 (16.90; 4.6)
	Terbutryn	93.33	61.95	3.08 - 17.14 (7.20; 1.8)	3.02 - 7.17 (13.86; 1.6)	4.29 - 9.42 (6.19; 1.3)	3.42 - 10.88 (5.57; 1.5)
	Trifluralin	65.33	25.66	9.63 - 46.78 (22.22; 4.9)	1.49 - 3.70 (13.61; 0.8)	2.04 - 2.48 (2.26; 0.6)	-

(continued)

	Pesticides	LOD	LOQ	*S. plana* soft tissue (ng/g ww)			
		% above		Autumn	Winter	Spring	Summer
Insecticides	Aldrin	36.00	10.18	22.82 - 22.82 (22.82; 0.0)	4.82 - 7.01 (13.86; 1.7)	-	-
	Azinphos-methyl	88.00	49.12	22.67 - 90.61 (50.62; 8.4)	5.69 - 36.42 (13.00; 5.8)	3.05 - 32.30 (13.88; 4.4)	2.66 - 11.09 (6.87; 0.9)
	Chlordane (gamma)	18.67	2.65	8.84 - 8.84 (8.84; 5.0)	-	-	-
	Chlorpyriphos	96.00	17.26	4.20 - 4.20 (4.20; 1.2)	3.30 - 3.83 (13.59; 0.3)	4.44 - 4.44 (4.44; 0.6)	4.26 - 4.26 (4.26; 0.2)
	Cyfluthrine	57.33	42.04	14.18 - 138.0 (46.70; 18)	5.33 - 86.39 (13.25; 7.2)	14.63 - 63.60 (42.09; 6.7)	-
	Cyhalothrin (lamdba)	65.33	20.35	4.51 - 21.70 (12.19; 2.3)	3.07 - 6.35 (13.95; 2.0)	-	3.74 - 3.74 (3.74; 0.5)
	Cypermethrin (alpha)	49.33	34.07	24.69 - 77.51 (55.39; 22)	13.81 - 66.82 (13.03; 7.9)	31.23 - 43.04 (36.24; 8.9)	-
	4,4´-DDD	34.67	7.96	4.04 - 17.46 (8.57; 2.6)	-	-	5.17 - 5.17 (5.17; 0.5)
	4,4´-DDT	100.00	62.39	3.97 - 18.16 (9.12; 1.6)	3.76 - 8.34 (13.91; 0.6)	4.71 - 7.66 (6.04; 1.1)	4.43 - 12.42 (7.94; 0.9)
	Deltametrin	77.33	50.44	4.71 100.0 (46.17; 9.2)	2.52 12.21 (13.69; 2.0)	2.41 11.80 (6.10; 1.6)	3.54 3.99 (3.77; 0.5)
	Diazinon	90.67	42.92	5.88 - 13.68 (8.24; 2.0)	5.70 - 32.71 (13.10; 4.5)	15.35 - 49.86 (28.72; 9.9)	15.15 - 26.12 (20.63; 6.5)
	Dichlorvos	97.33	62.83	3.84 - 90.35 (31.78; 8.6)	3.93 - 55.90 (13.65; 8.8)	3.48 - 58.01 (30.39; 7.2)	17.12 - 35.47 (23.51; 5.1)
	Dieldrin	14.67	8.41	-	-	1.23 - 2.48 (1.72; 0.4)	-
	Dimethoate	9.33	3.54	-	-	37.36 - 69.86 (53.61; 18)	-
	Endosulfan (alfa)	77.33	36.73	13.55 - 60.75 (39.46; 18)	4.83 - 33.96 (13.50; 6.3)	10.80 - 49.88 (23.87; 8.0)	9.14 - 13.30 (11.22; 2.9)
	Endosulfan (beta)	86.67	49.12	6.39 - 51.93 (24.55; 9.9)	5.58 - 22.21 (13.43; 2.5)	14.21 - 58.63 (30.81; 2.1)	5.30 - 14.55 (8.63; 1.6)
	Endosulfan sulfate	73.33	30.53	5.77 - 26.36 (12.01; 1.2)	7.21 - 16.08 (13.25; 2.7)	6.33 - 10.50 (7.94; 1.6)	4.88 - 9.31 (7.35; 1.9)
	Endrin	65.33	24.34	11.32 - 11.32 (11.32; 1.0)	3.71 - 9.48 (13.68; 1.0)	5.21 - 6.21 (5.56; 1.7)	5.68 - 5.68 (5.68; 1.0)
	Fenamiphos	68.00	20.80	5.42 - 52.21 (21.70; 7.5)	8.79 - 14.81 (13.88; 2.0)	7.82 - 7.82 (7.82; 0.1)	5.88 - 9.34 (7.61; 1.9)
	Fenitrothion	77.33	27.88	6.95 - 7.47 (7.21; 2.7)	4.88 - 17.74 (13.23; 2.1)	-	4.70 - 4.70 (4.70; 0.1)
	Fonofos	88.00	54.42	3.21 - 11.34 (6.36; 3.1)	2.54 - 8.43 (13.05; 0.9)	2.46 - 6.08 (4.57; 1.1)	3.13 - 10.22 (5.53; 0.8)
	HCCP	86.67	56.64	11.66 - 89.24 (40.89; 6.2)	11.36 - 71.94 (13.47; 9.0)	14.32 - 117.5 (51.56; 11)	38.90 - 48.82 (43.86; 13)
	Heptachlor	85.33	41.59	7.14 - 15.40 (10.68; 3.4)	4.96 - 11.29 (13.11; 1.3)	8.11 - 11.50 (9.81; 1.5)	6.57 - 8.41 (7.49; 1.0)

(continued)

	Pesticides	LOD	LOQ	*S. plana* soft tissue (ng/g ww)			
		% above		Autumn	Winter	Spring	Summer
Insecticides	Heptachlor epoxide	90.67	60.18	4.96 - 18.45 (8.82; 2.8)	4.68 - 6.47 (13.45; 0.8)	2.91 - 3.86 (3.42; 0.9)	2.88 - 3.90 (3.44; 0.8)
	Lindane	81.33	32.30	3.72 - 16.04 (7.16; 3.2)	4.07 - 8.55 (13.95; 2.8)	6.15 - 12.67 (9.41; 4.5)	8.47 - 8.47 (8.47; 4.1)
	Malathion	81.33	43.36	4.65 - 32.42 (15.44; 5.3)	5.06 - 52.68 (13.22; 4.1)	9.66 - 15.58 (12.62; 1.4)	5.87 - 5.87 (5.87; 1.2)
	Metamidophos	68.00	38.50	8.45 - 43.50 (25.50; 7.5)	6.56 - 34.91 (13.53; 4.3)	10.01 - 78.57 (38.05; 12)	20.86 - 20.86 (20.86; 11)
	Methoxychlor	100.00	51.77	3.83 - 18.67 (10.15; 1.9)	3.45 - 7.73 (13.12; 0.7)	3.81 - 5.48 (4.64; 0.8)	3.56 - 6.45 (5.01; 0.3)
	Mirex	97.33	60.18	4.45 - 34.27 (13.04; 2.7)	4.79 - 19.65 (13.55; 1.8)	4.96 - 15.38 (9.31; 3.8)	7.51 - 13.72 (11.05; 2.9)
	Parathion-ethyl	85.33	50.00	4.21 - 28.37 (11.54; 4.1)	4.56 - 12.64 (13.79; 1.7)	6.38 - 12.46 (8.52; 2.2)	3.82 - 6.09 (4.92; 1.6)
	Parathion-methyl	84.00	47.35	7.54 - 33.32 (15.73; 6.6)	11.82 - 122.8 (13.36; 19)	14.92 - 100.1 (42.32; 8.1)	6.50 - 9.89 (8.19; 0.8)
	Phosmet	78.67	47.35	5.68 - 45.46 (18.91; 5.7)	7.04 - 45.01 (13.21; 7.6)	7.16 - 51.61 (26.23; 3.8)	5.70 - 12.92 (9.51; 2.2)
	Pirimicarb	81.33	50.44	8.46 - 95.20 (21.97; 5.7)	3.80 - 39.44 (13.35; 3.4)	6.56 - 53.89 (24.76; 9.1)	3.94 - 35.82 (16.57; 1.4)
	Tetrachlorvinphos	97.33	63.27	4.64 - 153.1 (37.47; 9.4)	6.93 - 109.3 (13.23; 12)	10.06 - 46.96 (23.86; 7.1)	8.45 - 52.19 (31.49; 14)

The observed seasonal fluctuation in the TAL of pesticides is somewhat inversely proportional to both water temperature and CI of animals (Fig.4B), presenting a significant (despite weak) negative linear correlation with the last one ($r = -0.38$; $p < 0.0007$). These data suggest that depleted animals present higher amounts of pesticides per body weight than well-fed bivalves. The same pattern was already observed in accumulated polychlorinated biphenyls (PCBs) compounds in mussels [54, 55]. However, both results contradict the expectations. Indeed, these compounds are classified as lipophilic substances and so higher pesticide concentrations were expected in animals with greater CI [55, 41, 56]. Therefore, at least in our study, the fluctuations in the environmental concentrations of pesticides seem to largely govern the *S. plana* burden because, as cited, there is agreement of seasonal patterns [52].

The gonad differentiation occurred along with the temperature and CI increase, reaching the maximum of sexual maturation in spring, decreasing abruptly in summer (Fig. 4C). This pattern represents the typical spawning period (March and September) and gonad repose (October to January) of this species settled in the Mediterranean area [57, 20]. In spite of no visual gonad maturation alterations, studies should be done to evaluate the anthropogenic impacts on the fertilization rate and/or the viability of the first life-stages.

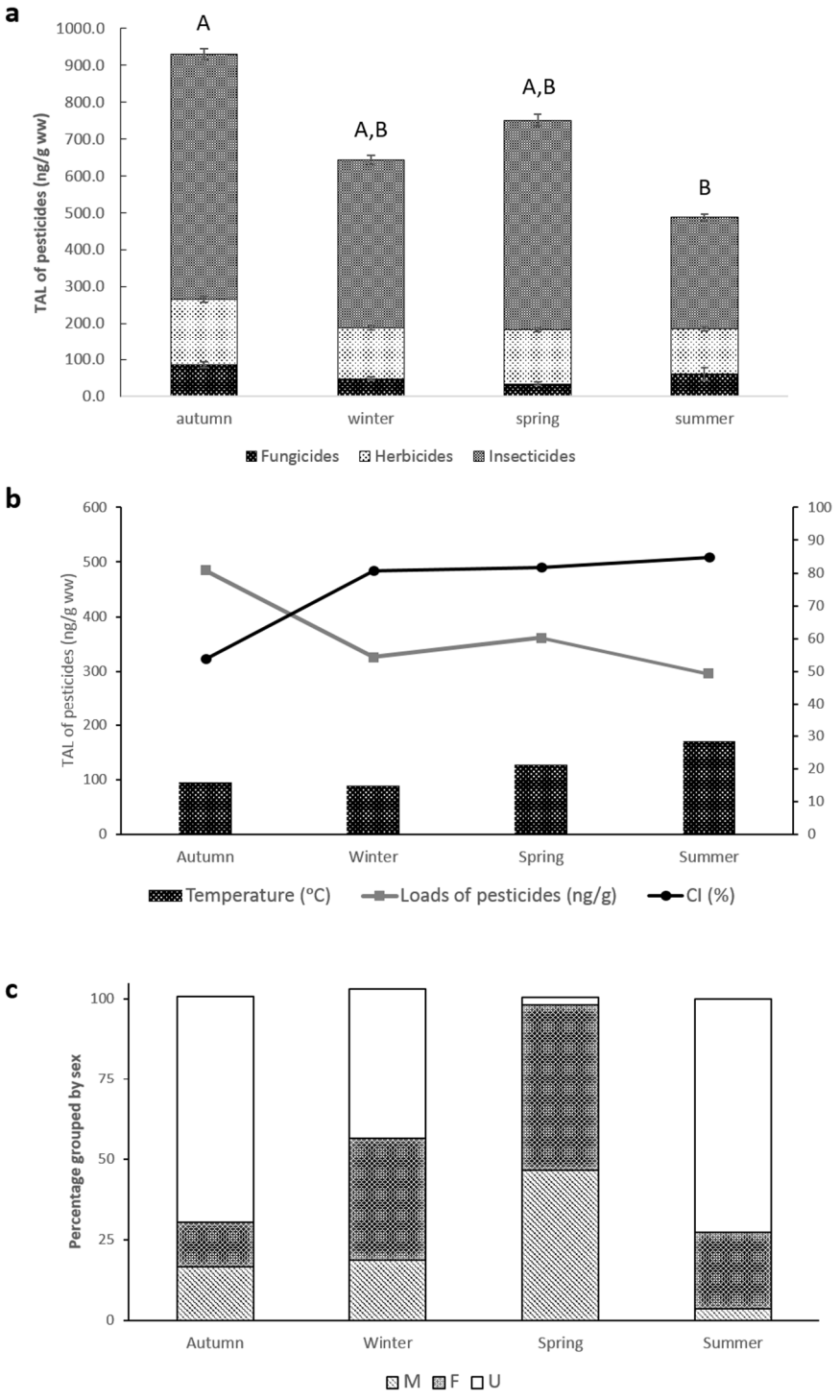

Fig. 4 a TAL of pesticides (ng/g ww) grouped by categories (fungicides, herbicides and insecticides) and represented by season; bars represent standard deviation and capital letters represent the significant differences; **b** interaction between TAL of pesticides (ng/g ww), condition index (CI;%) and temperature (ºC) displayed by seasons; **c** percentage of males (M), females (F) and undefined animals (U) grouped by season

3.4 Bioaccumulation versus European water framework directive

The application of the Directive 2013/39/EU denotes a need for measuring priority substances in matrices such as sediment and biota, along with water, setting strict levels for each one (environmental quality standards, EQS). In this directive, the sum of heptachlor and heptachlor epoxide ($\Sigma_{\text{heptachlor and heptachlor epoxide}}$) and the sum of all other individual pesticides are settled for a maximum amount of 0.0067 and 10 ng/g of ww, respectively. Herein, most of the selected pesticides (83%) were present, at least once, above the legal limits, where the insecticides, tetrachlorvinphos, $\Sigma_{\text{heptachlor and heptachlor epoxide}}$, HCCP, and dichlorvos were in more than 50% of the collected samples (Fig. 5).

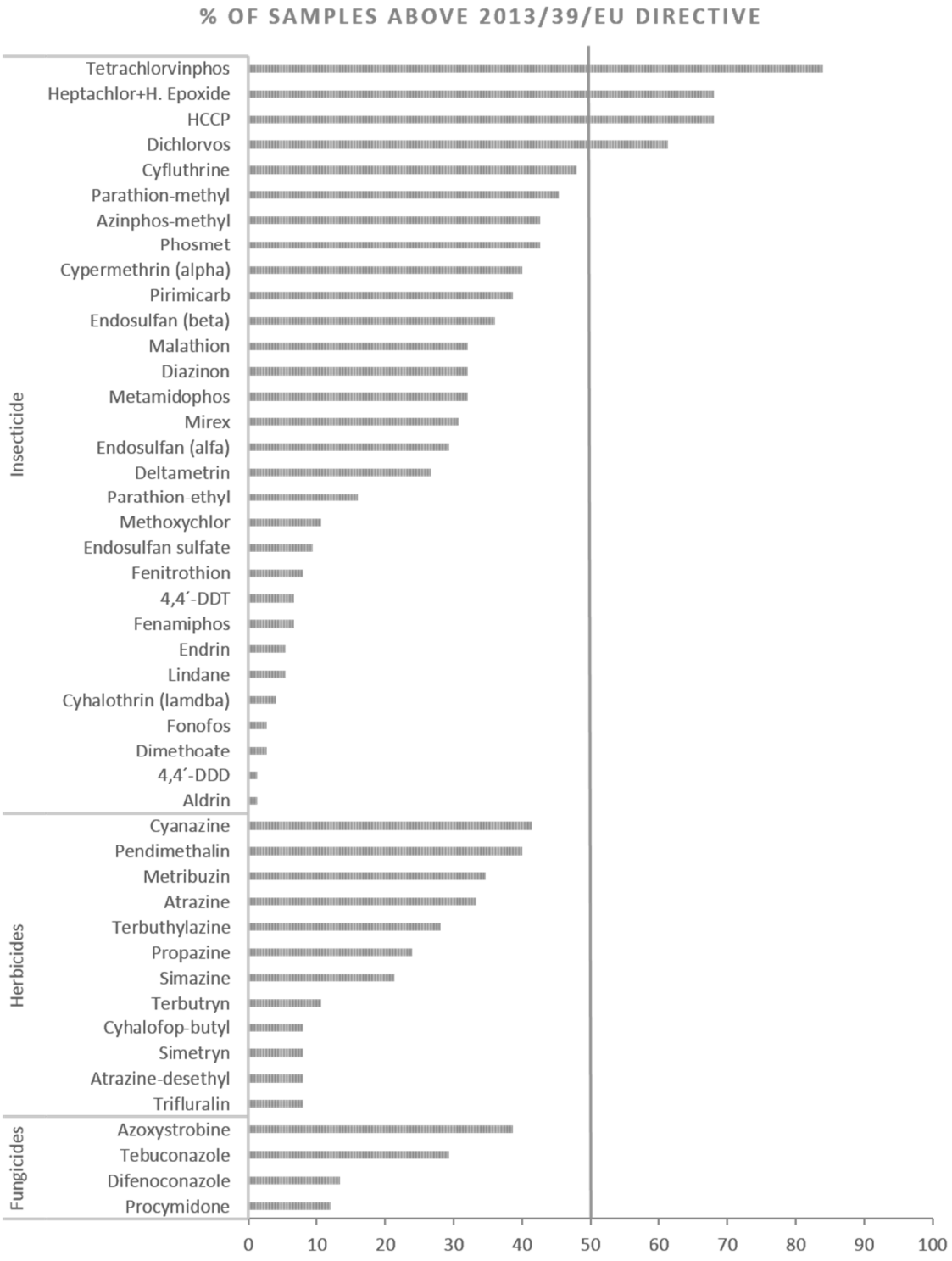

Fig. 5 Representation of the percentage (%) of collected samples above the 2013/39/EU Directive limits

The present data are a picture of a waterbody very impacted by the presence of pesticides, where bivalves can be affected—from endocrine disruption and reproduction disturbances to the viability of the species [58-60]—by one or more pesticides that are above the EQS limits. Moreover, as a suspensive filter-feeder and a bottom-chain animal, the bivalve *S. plana* is prone to bioaccumulating pesticides and other contaminants passible of causing disturbance, not only to the ecosystems but also to humans, via biomagnification processes and/or direct food consumption [61-63]; the latter is common in the sampled areas, in line with the fact that the south touristic coastal region (Algarve) sums ≈ 20% of all Portuguese fisheries [64].

4. Concluding remarks

The development and validation of a QuEChERS-based extraction method for the analysis of 55 pesticides in the bivalve *S. plana* by GC-MS/MS was successfully made, including a large monitoring application to specimens captured from the Ria Formosa Lagoon. In the process, all the requirements set by SANCO protocol SANCO/825/00 rev 8.1 for the extraction of pesticides from plants, plant products, foodstuffs and feeding stuffs were accomplished, increasing the reliability of our data. Due to its simplicity and implementation speediness—and also perceived lower-price when compared to other more complex technical solutions—the new methodology seems excellent, not only for future monitoring campaigns in ecotoxicological contexts, but also for seafood quality control and assessment of risks to human health.

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Compliance with ethics standards including statements on:

Conflict of interest: The authors declare that they have no competing interests.

Ethical approval: All the animals received human care and all experimental protocols were performed in accordance with the Portuguese Animal Welfare Law (Decreto-Lei n°.113/2013, 7 de Agosto D.R. n°. 151, Série I) and animal protocols approved by CIIMAR/UP and DGAV (Direcção-Geral de Alimentação e Veterinária, the Portuguese National Authority for Animal Health).

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Electronic supplementary materials:

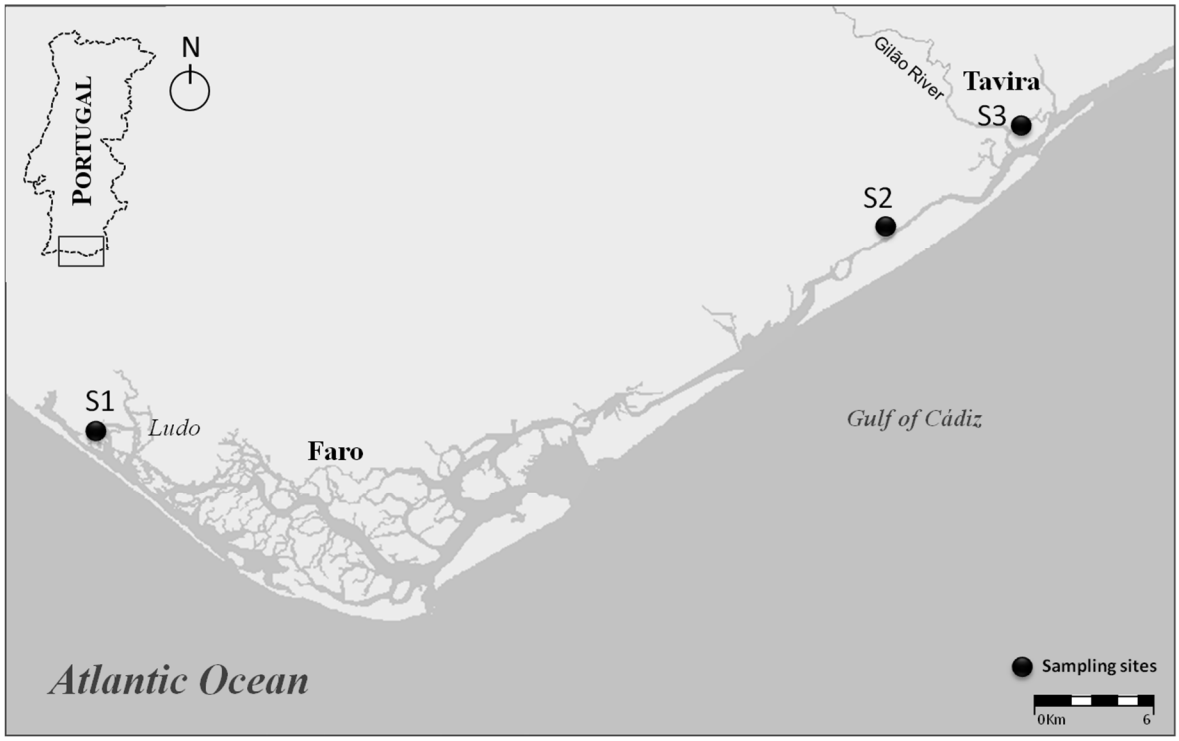

Fig. S1 Illustration map of the sampled area (Ria Formosa Lagoon, Portugal)

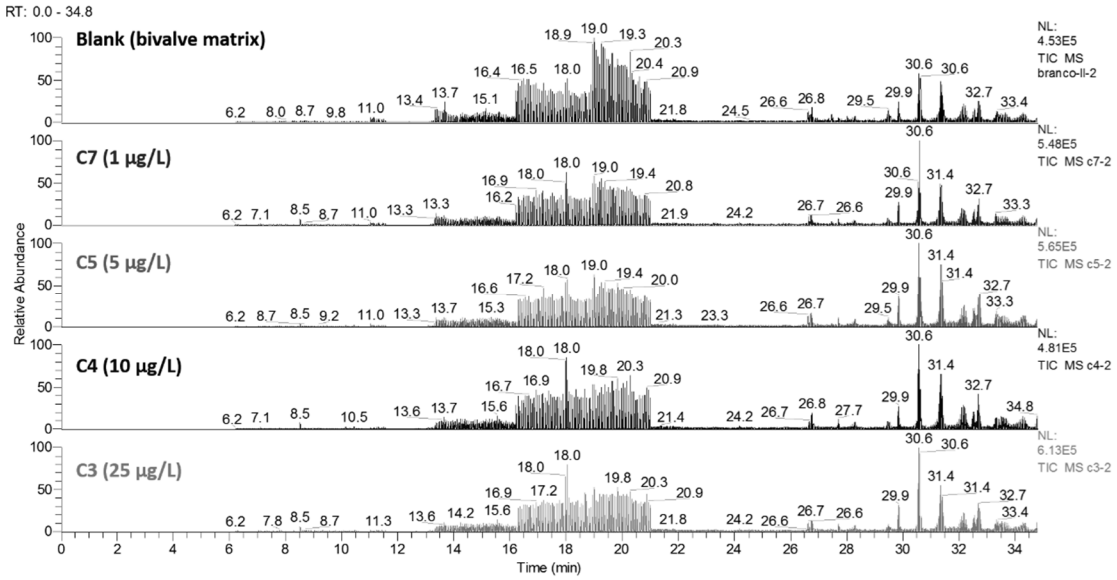

Fig. S2 Chromatograms represented in MS/MS mode of the blank (black) and spiked matrix (colored; target pesticides 1, 5, 10, and 25 µg/L and the surrogates 10 µg/L)

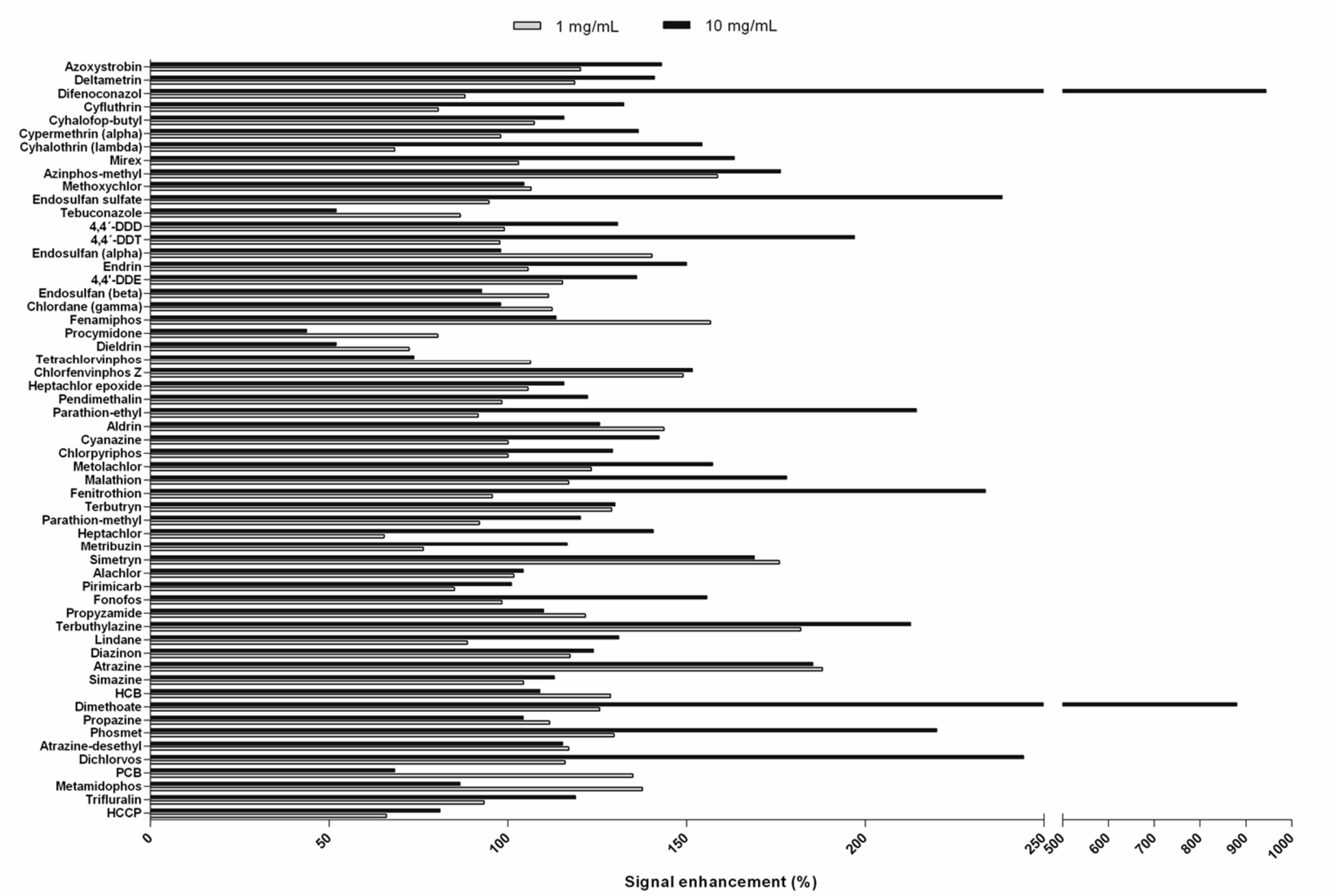

Fig. S3 Representation of the signal enhancement (%) at two concentration (1 and 10 mg/L) for each selected pesticide

Chapter 7

Three-tiered matrix quantification (in aqueous phase, suspended particulate matter, and in bivalve soft tissues) of pesticides in the estuary of the Iberian Peninsula longest river—The Tagus

Catarina Cruzeiro, Eduardo Rocha, Miguel Ângelo Pardal & Maria João Rocha

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Environmental Pollution

Three-tiered matrix quantification (in aqueous phase, suspended particulate matter, and in bivalve soft tissues) of pesticides in the estuary of the Iberian Peninsula longest river—The Tagus

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Keywords: estimated daily intake, MRLs, *Scrobicularia plana,* seafood, 2013/39/EU

Abstract

The distribution of pesticides in dissolved aqueous phase (DAP), suspended particulate matter (SPM) and *Scrobicularia plana* soft tissues from the Tagus River estuary was determined to evaluate the chemicals pollution status and their hazard potential in this area. Samples were collected in 6 campaigns (from December 2012 to October 2013), from 3 strategical sites, and analysed via different extraction procedures followed by gas chromatography tandem mass spectrometry (GC-MS/MS) determination. The contamination profile among matrices (DAP, SPM, and soft tissue from bivalves (STB)) was marked by average concentrations of 345 ng/L, 0.51 mg/kg, and 0.02 mg/kg, respectively, with several samples above the 2013/39/EU Directive of environmental quality standards (EQS). A wider range of pesticides was present in STB (n = 53) than in SPM (n = 36) and DAP (n = 19) matrices. Sediment–water partition coefficient (log K_d), bioaccumulation factor (BAF) in both DAP and SPM fraction were estimated ranging between 2.5-4.4 and 0.008-2799, respectively. The spatial distribution of most pesticides and physicochemical parameters were consistent, indicating a pollution pattern primarily near the Trancão River mouth (site S_3). Due to the presence of the target compounds, calculated risk quotients pointed out potential hazards for aquatic organisms, mainly to invertebrates. The estimated average daily intake (EADI), theoretical maximum daily intake (TMDI), and hazard quotient (HQ) of the studied pesticides–via bivalve ingestion–indicated no risk for human health, although it is important to note possible biomagnification processes that may happen along the estuarine food-chain.

1. Introduction

Contamination of aquatic environments is a worldwide problem, affecting, directly or indirectly, both human and wildlife (Serrano et al., 2012). Anthropogenic activities, such as industry and agriculture, distress the aquatic ecosystems via atmospheric pollution, effluent discharges and land use (Falconer, 2006; US Environmental Protection Agency (EPA), 2002). As a consequence of the agriculture activity, several million tons of fertilizers and pesticides are applied each year, contaminating both surface and groundwater (Schwarzenbach et al., 2006). By providing habitat for local and migratory fauna, the estuarine and coastal environments are highly impacted by the presence of these compounds (Barbier et al., 2010; Katagi, 2010; Pitarch et al., 2007), bringing deleterious effects to non-target organisms, such as birds, fish, aquatic invertebrates and plants (Köhler and Triebskorn, 2013; Osterberg et al., 2012; Scholz et al., 2012; Slaninova et al., 2009).

The Tagus River, chosen as case study, is one of the major freshwater sources of Europe and the longest river (1038 km) of the Iberian Peninsula (Ferreira et al., 2003). Due to an extensive surface of estuarine waters and vast mud and sandflats, and saltmarshes, it provides an ideal habitat for local and migratory waders (Catry et al., 2011; Instituto da Conservação da Natureza e da Biodiversidade, 2007). As a unique habitat, that includes a natural reserve of international relevance, it offers conditions for over 250 bird species and numerous benthic communities that include bivalves, crustaceans, and fishes, being of crucial interest to maintain the good quality of this ecosystem (Instituto da Conservação da Natureza e da Biodiversidade, 2007).

Due to the ubiquitous presence of pesticides, monitoring studies involving different contamination layers are essential to enforce regulatory limits and warn for possible negligent agriculture and waste water treatment practices. This work monitored 56 pesticides in samples of water (DAP), of suspended particulate matter (SPM), and of an edible bivalve (STB), collected in 6 campaigns (from December 2012 until October 2013). To create a representative panel of authorized, unauthorized, and banned pesticides (insec-

ticides, herbicides and fungicides) currently in use in Portugal, it was consulted the European Commission database Regulation (EC No 1107/2009) (EU, 2009) and the Portuguese Regional Directorate of Agriculture and Fisheries (DRAP). The biologic model used herein to study the bioaccumulation of pesticides, was the peppery furrow shell (*Scrobicularia plana*) which, beyond its commercial interest for human consumption, it is also a crucial prey to higher trophic levels (Grilo et al., 2013).

In this vein, the main objectives of this work were: i) to evaluate the residual concentrations of 56 pesticides in DAP, SPM, and STB matrices collected in the Tagus River estuary during four seasons; ii) to identify possible ecological risks upon exposure to the maximum concentrations found in ΣDAP, SPM fractions; iii) to infer about human health risks after consumption of local bivalves; iv) to evaluate the registered levels according to Directive 2013/39/EU; and v) to link the physicochemical water-quality parameters with pesticide concentrations found in the aqueous matrices.

2. Material and methods

2.1 Study area and sample collection

The Tagus is the largest river of Iberian Peninsula, ending in a large tidal estuary covering an area of 320 km^2. The estuary is located close to the Portuguese capital Lisbon, being formed by several channels, small islands, and mudflats, which provide optimum conditions for benthic communities that constitute an important source of food for higher trophic levels, such as crabs, fish, birds (local and migratory waders), and for humans too (Ferreira et al., 2003).

In this study three sampling sites were chosen, considering several factors, such as the margin side, the incidence of *S. plana* specimens, the degree of pollution previously identified by others (Rocha et al., 2015; Silva et al., 2012a; Silva et al., 2012b) and the location of the international Tagus natural park (Figure 1). Thus, two selected sampling stations were on the south margin, close to the cities of Moita (S_1 - 38°39'14.8" N, 8°59'48.5" W) and Alcochete (S_2 - 38°45'11.2" N, 8°57'57.2" W), and the other one was on

the north margin, near the Sacavém city (S_3 - 38°47'47.6" N, 9°05'46.1" W) and close to the entrance of the Trancão River tributary into the Tagus River estuary (Figure 1).

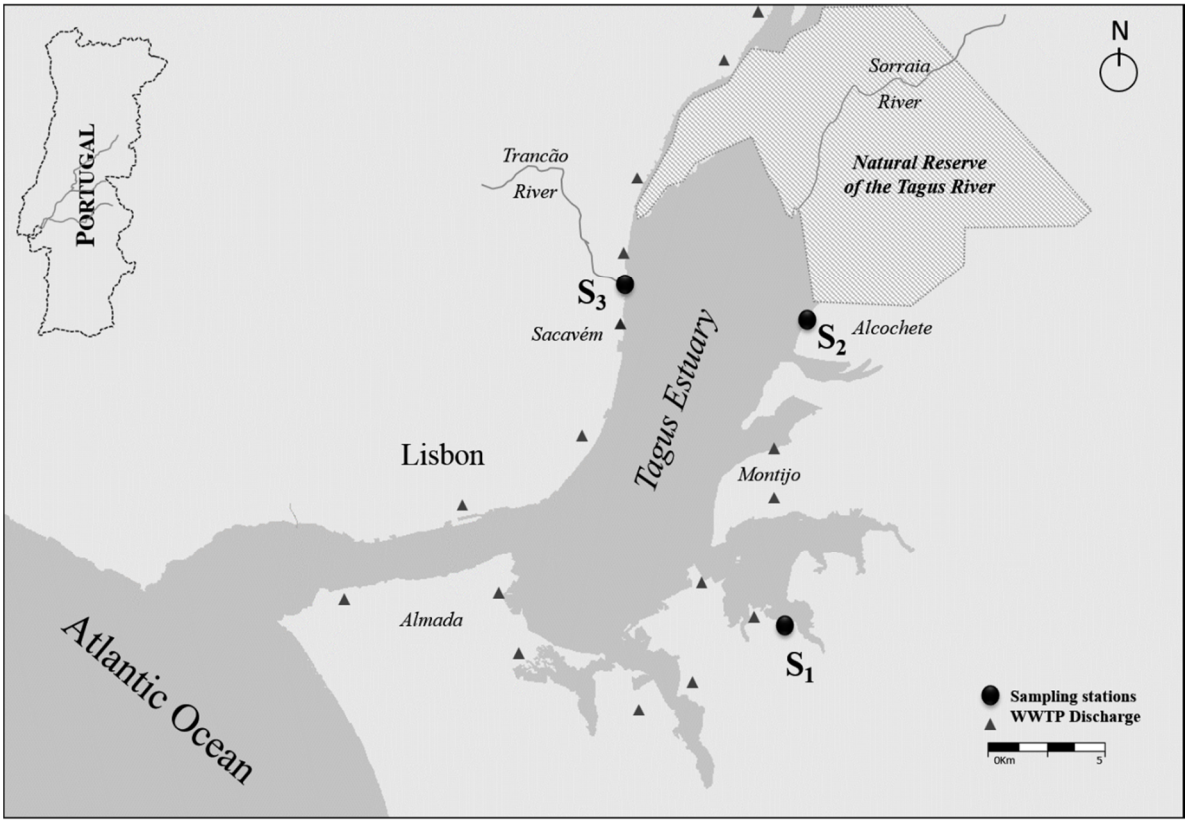


Figure 1- Map of the Tagus River Estuary tagged with the sampled locations (S_1 to S_3). The natural reserve is represented by the dashed area.

On each sampling site, the water samples were collected at half meter depth, into pre-rinsed amber bottles, and kept refrigerated (~ 5ºC) during their transport to the laboratory. The animals (120 organisms *per* sampling site) were collected manually on the shore, at *ca*. 20 cm depth, and transported to in their sediment. A total of six campaigns were completed, from December 2012 to October 2013, scheduled to involve all four seasons.

2.2 Chemicals and reagents

Reagents: methanol (MeOH), acetonitrile (MeCN), ethyl acetate (EtOAc) and hexane were purchased from Romil (Cambridge, England), with LC/GC grade, while the anhydrous magnesium sulfate ($MgSO_4$), sodium acetate (NaAcetate) and the Supelclean™ PSA SPE Bulk Packing were obtained from Sigma-Aldrich (Steinheim, Germany). The $MgSO_4$ was pre-heated (5 h/500 ºC) to eliminate residual water and phthalates.

Pesticide standards and GC-MS/MS protectants: the pesticide reference standards, all with 98-99% of purity, were acquired from Sigma-Aldrich (Steinheim, Germany). With exception of Mix A (EPA 505/525, 500 mg/L) and Mix B (EPA 505/525, 500 mg/L), all other pesticides were purchased individually. All standard solutions were prepared individually in MeOH, to produce a final stock solution of 10 000 µg/L, and kept in dark at - 20ºC, to avoid possible decay. The deuterated internal standards (IS) 4,4´-DDT-d_8 and atrazine-d_5 were used herein as surrogates. The GC-MS/MS protectants, 3-ethoxy-1,2-propanediol and D-sorbitol were acquired from Sigma-Aldrich (Saint Louis, USA).

2.3 Sample preparation and pesticides extraction

2.3.1 Water samples: dissolved aqueous phase (DAP) and suspended particulate matter (SPM)

Within 24 hours, the water samples (500 mL) were filtrated through a 0.45 µm glass fibre filter (Munktell, Germany), and the fractions DAP and SPM followed independent extraction procedures (Cruzeiro et al., 2015a; Cruzeiro et al., 2015b). The pH of DAP was adjusted to ~7, and the pesticides dissolved in this fraction were extracted by solid phase extraction (SPE) using pre-conditioned OASIS HLB cartridges (Waters Corporation, Milford, MA, USA). Briefly, the cartridges were pre-conditioned with 5 mL of EtOAc, 5 mL of MeOH and 2.5 mL of ultrapure water (Cruzeiro et al., 2015b). The final extracts were eluted with 6 mL of EtOAc (Cruzeiro et al., 2015b). The pesticides adsorbed to the particulate matter, which were retained by the above referred glass fibre filters, were soaked in 3 mL of EtOAc for 8 min in an

ultrasonic bath (Axtor- Lovango, model CD-4820, 170 W); this procedure was done twice with the application of cooling devices to avoid temperature increase (Cruzeiro et al., 2015a).

The final DAP and SPM extracts were evaporated to dryness under a gentle N_2 (99.9997 %) stream, reconstituted in hexane (200 µL) and kept at - 80°C until GC-MS/MS analysis.

2.3.2 Soft tissues from bivalve (STB)

The animals were left for depuration during 24 hours in clean water, at constant salinity, temperature, and oxygenation. Before dissection, they were placed into an anaesthetic solution of magnesium chloride hexahydrate ($MgCl_2$) at 60 g/L (Butt et al., 2008), until muscle relaxation. Biometric parameters such as length, width, height, total and soft tissue weight were noted for further condition index (CI) calculation. Based on sex (females, males and undefined *S. plana*) and taking in consideration the proportion sex-ratio per sampling site, five independent pools were extracted in order to have a representative sample. Then, the bivalve soft tissues were chopped and grinded (IKA T10 basic Ultra-Turrax®, Lille, France), and 5 g of the STB homogenate was transferred into 50 mL Teflon centrifuge tube (Nalgene, Rochester, USA). In this study two protocols, used for pesticide extraction, were merged in order to adapt them to STB (Cruzeiro et al., 2016). Shortly, 5 mL of MeCN with 1% of acetic acid was added and mixed by vortex (VX-200 Vortex Mixer, Labnet International Inc.). As a next step, 2 g of anhydrous $MgSO_4$ and 0.5 g of NaAcetate were added and mixed together during 1 min. The extracts were centrifuged (Sigma 2-16K centrifuge) for 5 min at 4024 RCF, at 4°C. Subsequently, 2.5 mL of the MeCN layer was transferred into a tube, containing 375 mg $MgSO_4$ and 125 mg PSA, and mixed by vortex for 30 s. After centrifugation (2 min, 4024 RCF), the extracts were collected and immediately analysed. Before injection, the protectants 3-ethoxy-1,2-propanediol (5 mg/mL) and D-sorbitol (0.5 mg/mL) were added to 100 µL of sample (Maštovská et al., 2005).

2.4 Histocytological analyses of *S. plana* gonads

The sex and the maturation status of the sampled animals (120 bivalves × 3 sites × 6 campaigns) were evaluated, using the squash technique. A parallel study (using ten animals/site/campaign) was done to compare both histological and cytological approaches (Figure SM1). From each animal two gonadal fragments were collected where one followed the squash technique and the other went for routine histological procedures. Briefly, the fragment was fixed in 10% buffered formalin (24 h) and subsequently infiltrated with paraffin following a routine program in an automatic tissue processor (Leica TP1020, Germany). Each fragment was embedded in paraffin, sectioned (4 µm thickness) on a motorized rotary microtome (Leica RM2155, Germany), and then stained with hematoxylin-eosin for microscopic evaluation. Thus, before individual storage of the bivalves (-80ºC), a small fragment of the gonad was collected, smashed between two histological slides and left to dry for 24 hours. Both slides were then subjected to Diff-Quik coloration for gonadal assessment and the animals were classified as males (M), females (F) or undifferentiated (U). The U classification included animals with empty and undifferentiated gonads.

2.5 Instrumental and analytical methods

DAP, SPM and STB extracts were analysed in a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation), coupled with an ion trap mass spectrometer detector Thermo Scientific ITQ™ 1100 GC-MS^n), an autosampler (Thermo Scientific TriPlus™), and equipped with a Trace GOLD column (TG-5SILMS, 30 m x 0.25 mm x 0.25 µm). Column oven temperatures were programmed according to previous published methods (Cruzeiro et al., 2015a; Cruzeiro et al., 2015b). Briefly, the injector port temperature was set to 250ºC and both ion source and MS transfer line were at 280ºC. Helium (99.99999 % purity) was present as carrier gas, at a constant flow rate of 1 mL/min. The sample injection (1 µL), in splitless mode, required a 50 mm length needle. GC separation was initially achieved by the evaluation of different ranges of temperatures and injection conditions, using full-scan

mass spectra (65–550 *m/z*) of individual pesticides. As a next step, SIM segments were established, containing the specific ion mass-to-charge ratio (*m/z*) for each compound, followed by the MS/MS characterization which was refined, using specific collision energy voltages (between 0.8 and 2.05) to generate the subsequent product ions (see supplementary material document SM1). The ion products were evaluated using Software Xcalibur (version 2.0.7, 2007, Thermo Scientific) together with Mass Frontier (version 1.0, 1998) and NIST library.

2.6 Quality assurance (QA) and quality control (QC) procedures

The performance of the methods were daily checked using method blanks (solvent controls), quality controls (two-fold higher than the limit of quantification), fortified samples spiked with both surrogates, and using, weekly, new calibration curves. The limits of detection (LODs) and quantification (LOQs) for each pesticide in DAP, SPM and STB was defined as LOD = $3.3\frac{\alpha}{S}$ and $LOQ = 10\frac{\alpha}{S}$ (Table 1); here, α is the standard deviation of the response, and S is the average slope of the calibration curves. The LOQs ranged from 0.01 to 11.6 µg/L in DAP, from 0.01 to 12.2 µg/L in SPM, and from 0.33 to 10.3 µg/L in STB. Among matrices, the fortified samples presented average recovery rates varying from 71% and 120%. Precision, accuracy and recoveries were evaluated following the criteria established by SANCO/825/00 rev 8.1 (European Commission Directorate General Health and Consumer Protection, 2010) (see supplementary material, Document SM1).

2.7 Mathematical analysis of data

2.7.1 Bioaccumulation accumulation factors

The extent of pesticides bioaccumulation in *S. plana* can be promoted by their concentrations dissolved in water (C_{DAP}, mg/L) and sorbed to particulate matter (C_{SPM}, mg/kg). In this context the bioaccumulation factor was calcu-

lated considering the ratio of the average chemical concentration in the organism (C_{STB}, mg/kg ww) to that in DAP $\left(BAF_{DAP} = \frac{C_{STB}}{C_{DAP}}\right)$ and SPM$\left(BAF_{SPM} = \frac{C_{STB}}{C_{SPM}}\right)$ (Arnot and Gobas, 2006; Mirsadeghi et al., 2013).

2.7.2 Partition coefficient (K_d)

The relationships between pesticides and suspended sediments have been highlighted in several studies (Boithias et al., 2014; Dueri et al., 2008; Taghavi et al., 2010; Turner and Millward, 2002). Here, to appreciate the distribution of pesticides in between both DAP and SPM the partition coefficient K_d (L/kg) of each molecule was calculated as follows $\left(K_d = \frac{C_{SPM}}{C_{DAP}}\right)$, where C_{SPM} (mg/kg) and C_{DAP} (mg/L) are the observed pesticide concentrations in sorbed and dissolved phases respectively.

2.7.3 Evaluation of potential risks for aquatic life

The calculation of risk quotients (RQ) involves the evaluation of measured environmental concentrations MEC (mg/L) of the chemical (pesticide) and the predicted no-effect of the same compound (PNEC, mg/L) in species from different trophic levels $\left(RQ = \frac{MEC}{PNEC}\right)$. When RQ is higher than 1, usually reflects a potential risk for aquatic organisms (Backhaus and Faust, 2012). Here, the PNECs were calculated considering documented values of $L(E)C_{50}$ (mg/L) for algae, crustaceans and fish, and their respective assessment factors (AF) (Backhaus and Faust, 2012); that is the maximum acceptable concentrations - quality standards (MAC-QS) used to assess short term effects for each assayed pesticide $\left(PNEC = \frac{L(E)C_{50}}{AF}\right)$ (Agriculture & Environment Research Unit (AERU), 2013; European Communities, 2011; National Center for Biotechnology Information (NCBI), 2015). So, when RQ was higher than 1 a second mathematic approach was considered to define which trophic level was the most sensitive to the environmental concentrations of pesticides. This second approach involves the calculation of the toxic units $\left(RQ_{TU} = \frac{MEC}{L(E)C_{50}}\right)$ *per* trophic level and their respective sum, denoted as STU (RQ_{STU}). Then, the highest RQ_{STU} value indicate the most affected group of organisms (Backhaus and Faust, 2012). If $RQ_{(MEC/PNEC)}$ and RQ_{STU} is higher than

1, additional considerations are required (Backhaus and Faust, 2012). Based on the two reference models–concentration addition (CA) and independent action (IA)–the RQ_{STU}/max_{TU} can be used to predict the second-tier, resulting in the maximum value from which CA may display higher toxicity values than IA (Silva and Cerejeira, 2014).

2.7.4 Human health risk assessment by food intake of pesticide residues in bivalves

To estimate the dietary pesticide risks, it is crucial to compare the estimated pesticide exposure with that established by health criteria, defined by USEPA (US Environmental Protection Agency (EPA), 1998), FAO (Food and Agriculture Organization of the United Nations, 2011), and European Union Directives (European Union, 2008). For this purpose, several indices can be used to predict effects caused by the intake of pesticide residues. One of these indices is the theoretical maximum daily intake (TMDI) that uses the multiplication of the consumption rate (kg/capita/day) by the maximum residue limits (MRLs), which represent the maximum concentration of a pesticide residue (mg/kg) legally permitted in food (European Union, 2008); when no specific MRL is published a 0.01 mg/kg value is applied (The European Parliament and the Council of the European Union, 2005).

Other index is the evaluation of the acceptable daily intake (ADI), which is the estimated amount of a substance in food (expressed on a body-weight basis) that can be ingested daily over a lifetime without appreciable health risk to the consumer (European Union, 2008). Accordingly to U.S. Environmental Protection Agency (1998), the estimated average daily intake (EADI = pesticide residue intake / standard body weight) of pesticide residues should be less than the established ADI values. Both values, EADI and ADI can be used to predict the hazard quotients (HQ), for developing non-carcinogenic health effects. So, herein HQ was calculated as HQ = EADI / acute reference dose (ARfD) or HQ = EADI / ADI, to estimate the hazard index based on long intake periods (European Union, 2008; Ogbeide et al., 2015).

2.7.5 Data organization

A total of 18 DAP (ng/L), 18 SPM (mg/kg), and 90 STB samples were evaluated per pesticide. Data was grouped per categories *i.e.*, fungicides, herbicides, and insecticides and displayed as minimum and maximum concentrations, average concentrations and frequency of quantification (%) (Table 1). Pesticide average concentrations were summed (Σaverage concentrations) per matrix, and grouped by category and season (Figures 2 and 3). The health status of organisms was assessed through the individual condition index CI = $\left[\left(\frac{\text{fresh weight}}{\text{shell weight}}\right) \text{x } 100\right]$ (Mouneyrac et al., 2008) and further correlated with Σaverage concentrations and temperature (Figure 4).

2.7.6 Statistical analyses

Since data did not accomplish the parametric assumptions (normality and/or homogeneity of variances) and data transformation was ineffective, paired comparisons were made with non-parametric Kruskal-Wallis ANOVA followed by the Mann-Whitney U test (with a sequential Bonferroni correction), for a significant level of 5%; analyses were performed using the software PAST 3.06 (Hammer et al., 2001). Correlation analyses were done using the software GraphPad Prism (Muzyka et al., 2012).

2.8 Physicochemical parameters

Temperature (°C), dissolved oxygen (DO; mg/L), salinity, conductivity (mS/cm), and pH were measured *in situ*, using a multi-parameter Hech HQ40d. Other parameters, such as nitrites (mg/L, NO_2), nitrates (mg/L, NO_3), ammonium (mg/L, NH_4) and phosphates (mg/L, PO_4), were measured in the laboratory with a photometer (Palintest 7000, Gateshead, UK). Dissolved inorganic nitrogen (DIN) was determined by the sum of nitrogen from NO_2, NO_3, and NH_4. Total inorganic phosphorous (TIP) was calculated multiplying PO_4 by the constant multiplication 0.075 (Kwok et al., 2013). Additionally, the un-ionized ammonia was derived from the multiplication of the un-ionized ammonia form (set for a specific pH and temperature) by the nitrogen calculated from NH_4 (Durborow et al., 1997).

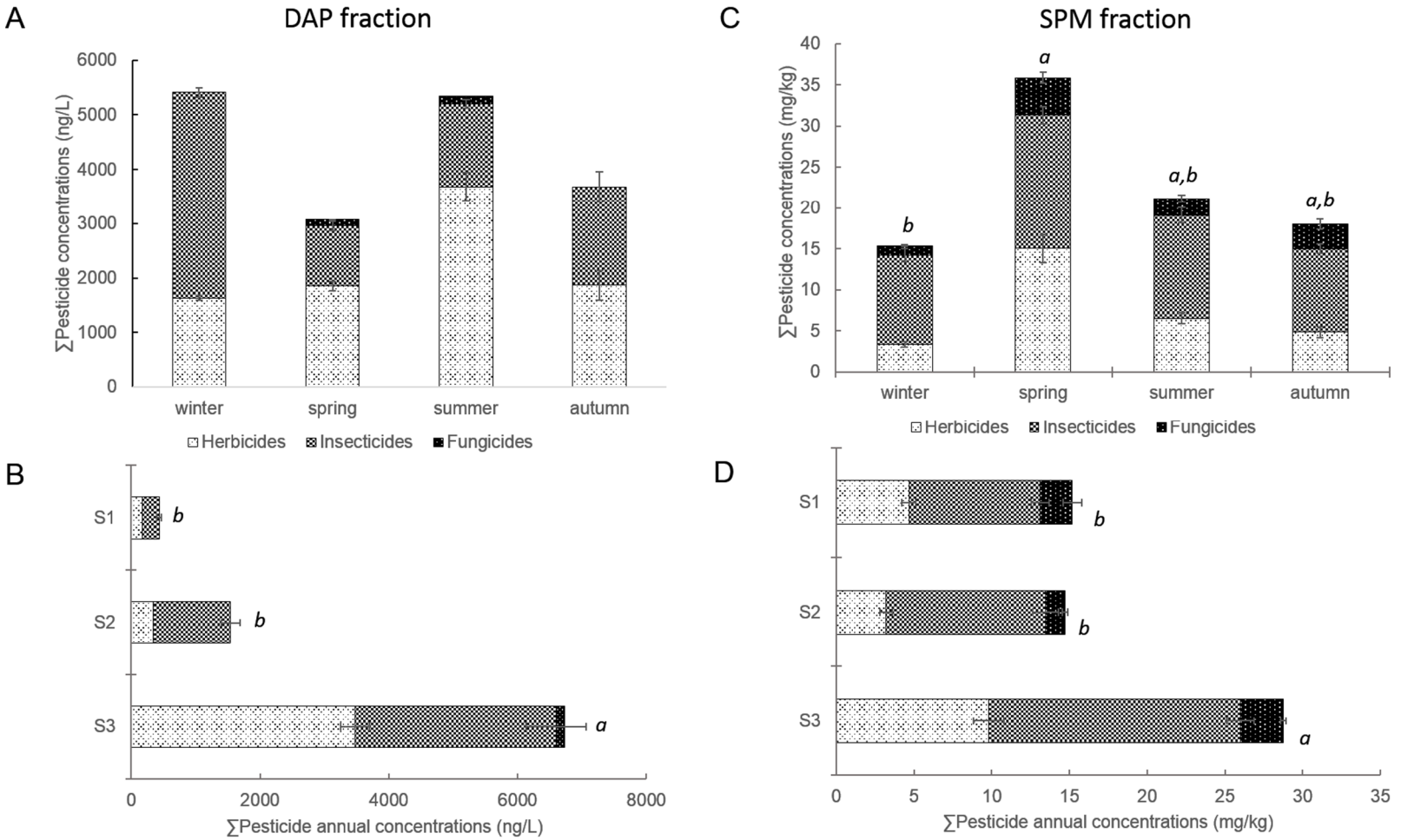
3. Results and discussion

Pesticide residues were detected in all selected matrices (Table 1). Data are represented by minimum, maximum, and average values, together with the frequency of detection.

3.1 Pesticides dissolved in water (DAP fraction)

From the 56 pesticides proposed in this study, 19 were quantified during the 2012-2013 campaign, in one or more samples. In average, their concentrations ranged from ~211 ng/L to ~548 ng/L. Regarding their frequency of occurrence, only pendimethalin and diazinon were quantified in more than 80% of the collected samples, indicating a constant output from local and adjacent land use. The same profile was already reported in 2011 for diazinon, when high and constant concentrations were found in the Swiss Plateau due to the abusive application of agricultural and urban pesticides (Wittmer et al. 2011). Considering the pesticides *per* category, both herbicides and insecticides, which annual average concentrations ranged respectively, from ~1600 ng/L to ~4400 ng/L and from ~1100 ng/L to ~3700 ng/L — we can thus conclude that the amounts of the two categories are similar in the Tagus River estuary. Within the selected fungicides, only tebuconazole (fungicide) was measured indicating a minor use of these pesticides in this area (Figure 2A). Besides that, this fungicide was only measured between spring and summer (~ 130 ng/L, Figure 2A) suggesting a temporal application in the crops. Seasonally, no statistical differences were verified.

Figure 2- Sum of the average concentrations of pesticides in DAP (ng/L) and SPM (mg/kg) fractions, expressed by category and season; n = 18 and bars represent SD.



Considering data *per* sampling site (Figure 2B), it is shown that S_3 registered the highest annual average concentrations (~361 ng/L, p = 0.014), the highest sum of all pesticide average concentrations (~6700 ng/L), and the highest frequency of pesticides dissolved in water (19%). This sampling site matches with Trancão River mouth, which is the receiver of neighbouring industries involved in food processing and chemical production (Peneda and Frazão, 1995; Picado et al., 2008). Denoted in the past as a "hot spot" of pollution (Rocha et al., 2015; van den Berg et al., 2007), this river still continues to receive a continuous rate of pesticides.

3.2 Pesticides in suspended particulate matter (SPM fraction)

A total of 36 pesticides were quantified in SPM (Table 1), presenting annual average values of 0.51 mg/kg. Comparatively to DAP, this fraction had a higher frequency of pesticides, above 80% (Table 1), namely atrazine, cyhalofop-butyl, simazine, chlorfenvinphos Z, diazinon, dimethoate, endosulfan sulfate, malathion, parathion-ethyl, hexachlorobenzene (HCB), and pentachlorobenzene (PeCB). This observation may derive from the pesticide chemical properties, such as octanol-water values (log K_{OW}), which in this study were in average ~4, a fact that points to a higher affinity of these chemicals to SPM rather than DAP.

In general, the pesticide annual average levels in SPM ranged from ~0.015 mg/kg to ~1.7 mg/kg. Considering data *per* season, and summing their average concentrations it is observed a significant increase of their amounts in spring (~ 34.2 mg/kg; p = 0.026), comparatively to the other seasons (~ 15.0 mg/kg; Figure 2C). These changes may be related to lower river flow and the increment of suspended particulate matter during warmer seasons, as previously referred in another study performed for the Tagus River (Zell et al., 2014) and for another Iberian river, the Guadalquivir (Masiá et al., 2013). As observed in the DAP fraction, both alachlor and diazinon registered high concentrations in the SPM fraction (~2 mg/kg), demonstrating an abusive application of these pesticides in the Tagus region (Table 1). Considering the average sum of all pesticides in SPM, this study reveals that pesticide concentrations in the Tagus River estuary are in the same order of

magnitude of others measured worldwide (Darwano et al., 2014; Liu et al., 2008; Schulz et al., 2001; Smalling et al., 2013).

Considering data *per* sampling site, significant differences ($p = 0.003$) occurred for S_3, that showed always higher concentrations of pesticides (~ 29.0 mg/kg) than the other sampling sites (Figure 2D). To assess the ability of the assayed pesticides to be sorbed by the particulate matter, the log K_d show values that ranged from ~ 2.5 to ~ 4.4, which is in accordance with the existence of conditions for the accumulation of the studied compounds in SPM. Similar log K_d ranges were observed in other aquatic environments suggesting that this fraction is an excellent repository of this type of compounds (Cruzeiro et al., 2015a; Darwano et al., 2014; Domagalski and Kuivila, 1993; Magnusson et al., 2013; Schulz et al., 2001; Tang et al., 2008) (Table SM1).

Table 1- Environmental concentration of pesticides in surface waters (DAP and SPM fractions) and in bivalves collected from the Tagus River estuary (STB fraction).

Pesticides	DAP				SPM				STB				BAF_{DAP}	BAF_{SPM}
	Min.	Max.	Average	Freq.	Min.	Max.	Average	Freq.	Min.	Max.	Average	Freq.		
ΣHERBICIDES			**3343.3**				**5.72**				**0.25**		**251.28**	**1.16**
Alachlor	929.17	929.17	929.17	5.88	0.01	6.25	2.08	76.47	0.00	0.02	0.01	61.45	7.42	0.003
Atrazine	382.27	549.75	466.01	11.76	0.00	1.28	0.42	82.35	0.01	0.09	0.03	55.42	64.21	0.072
Atrazine-desethyl	<0.32	<0.32	<0.32		0.00	0.33	0.06	23.53	0.01	0.04	0.01	33.73	-	0.251
Cyanazine	<1.2	<1.2	<1.2		<0.04	<0.04	<0.04		0.01	0.14	0.04	59.04	-	-
Cyhalofop-butyl	<1.25	3.48	<1.25		0.00	0.50	0.16	88.24	0.01	0.06	0.02	20.48	-	0.126
Metolachlor	74.62	170.57	122.60	11.76	<0.07	<0.07	<0.07		0.01	0.01	0.01	1.20	41.83	-
Metribuzin	<0.24	<0.24	<0.24		0.00	1.86	0.44	47.06	0.01	0.08	0.02	69.88	-	0.054
Pendimethalin	161.29	771.54	391.82	82.35	0.00	5.67	1.37	47.06	0.00	0.10	0.02	75.90	46.12	0.013
Propazine	<0.12	<0.12	<0.12		0.00	0.05	0.03	17.65	0.00	0.04	0.01	55.42	-	0.498
Propyzamide	280.51	711.01	514.84	17.65	<0.05	<0.05	<0.05		0.00	0.02	0.01	38.55	22.85	-
Simazine	<2.22	3.37	<2.22		0.01	2.28	0.73	94.12	0.01	0.06	0.02	43.37	-	0.030
Simetryn	<0.19	<0.19	<0.19		0.00	0.42	0.15	58.82	0.00	0.04	0.01	73.49	-	0.085
Terbuthylazine	206.31	870.61	562.66	35.29	<0.03	<0.03	<0.03		0.00	0.12	0.02	67.47	42.17	-
Terbutryn	222.49	515.85	356.20	29.41	0.01	0.81	0.29	11.76	0.00	0.03	0.01	75.90	26.68	0.033
Trifluralin	<0.45	<0.45	<0.45		<0.01	<0.01	<0.01		0.00	0.01	0.01	68.67	-	-
ΣINSECTICIDES			**1513.1**				**10.23**				**0.67**		**4634.0**	**1.4**
Aldrin	<2.47	<2.47	<2.47		0.11	0.70	0.28	17.65	0.00	0.01	0.01	13.25	-	0.024
Azinphos-methyl	<1.18	1.78	<1.18		0.08	0.12	0.10	5.88	0.00	0.03	0.01	37.35	-	0.123
Chlordane (gamma)	<1.14	<1.14	<1.14		0.00	0.22	0.05	52.94	0.01	0.01	0.01	7.23	-	0.134
Chlorfenvinphos Z	<0.91	<0.91	<0.91		0.01	1.39	0.41	94.12			na		-	-

(*continued*)

Pesticides	DAP				SPM				STB				BAF_{DAP}	BAF_{SPM}
	Min.	Max.	Average	Freq.	Min.	Max.	Average	Freq.	Min.	Max.	Average	Freq.		
Chlorpyriphos	96.21	96.21	96.21	5.88	<0.03	<0.03	<0.03		0.00	0.03	0.01	33.73	84.59	-
Cyfluthrine	<2.15	<2.15	<2.15		<0.07	<0.07	<0.07		0.00	0.10	0.02	87.95	-	-
Cyhalothrin (lamdba)	14.68	101.07	42.09	64.71	0.00	1.60	0.30	76.47	0.00	0.02	0.01	46.99	195.32	0.028
Cypermethrin (alpha)	<2.31	<2.31	<2.31		<0.23	<0.23	<0.23		0.01	0.11	0.03	53.01	-	-
4,4´-DDD	<3.68	<3.68	<3.68		0.00	0.41	0.11	64.71	0.00	0.02	0.01	44.58	-	0.065
4,4´-DDE	<2.36	<2.36	<2.36		<0.01	<0.01	<0.01		0.00	0.01	0.01	9.64	-	-
4,4´-DDT	144.84	144.84	144.84	5.88	0.00	3.26	0.85	70.59	0.00	0.03	0.01	68.67	66.15	0.011
Deltametrin	<5.23	<5.23	<5.23		0.03	1.20	0.42	17.65	0.00	0.05	0.02	92.77	-	0.037
Diazinon	16.96	544.62	106.31	100.00	0.03	7.00	2.74	94.12	0.01	0.17	0.03	77.11	286.04	0.011
Dichlorvos	<1.94	<1.94	<1.94				*na*		0.00	0.14	0.03	83.13	-	-
Dieldrin	<1.29	1.48	<1.29		0.00	3.79	0.82	52.94	<0.00	<0.00	<0.00		-	-
Dimethoate	101.78	219.23	155.12	11.76	0.01	1.74	0.50	88.24	0.01	0.06	0.02	66.27	13.05	0.045
Endosulfan (alfa)	104.60	897.53	236.50	70.59	0.02	1.73	0.52	29.41	0.00	0.14	0.03	40.96	127.41	0.058
Endosulfan (beta)	<5.26	<5.26	<5.26		0.00	0.44	0.18	29.41	0.00	0.09	0.02	66.27	-	0.094
Endosulfan sulfate	<4.85	<4.85	<4.85		0.00	1.34	0.45	94.12	0.01	0.05	0.01	27.71	-	0.033
Endrin	<3.44	<3.44	<3.44		0.00	0.38	0.15	11.76	0.00	0.06	0.02	16.87	-	0.118
Fenamiphos	<6.54	<6.54	<6.54		<0.46	<0.46	<0.46		0.01	0.15	0.03	21.69	-	-
Fenitrothion	197.06	308.05	228.05	35.29	<0.02	<0.02	<0.02		0.01	0.08	0.02	53.01	104.18	-
Fonofos	39.39	420.46	115.88	47.06	<0.01	<0.01	<0.01		0.00	0.04	0.01	31.33	70.24	-
HCCP	<6.48	<6.48	<6.48				*na*		0.01	0.20	0.06	78.31	-	-
Heptachlor	<3.43	<3.43	<3.43		0.00	0.30	0.10	76.47	0.01	0.03	0.01	39.76	-	0.138

(*continued*)

Pesticides	DAP				SPM				STB				BAF_{DAP}	BAF_{SPM}
	Min.	Max.	Average	Freq.	Min.	Max.	Average	Freq.	Min.	Max.	Average	Freq.		
Heptachlor epoxide	<2.97	<2.97	<2.97		0.01	1.34	0.33	52.94	0.00	0.02	0.01	10.84	-	0.026
Lindane	<1.8	2.01	<1.8		<0.01	<0.01	<0.01		0.00	0.01	0.01	32.53	-	-
Malathion	<11.59	<11.59	<11.59		0.02	4.75	1.35	82.35	0.01	0.10	0.02	66.27	-	0.015
Metamidophos		na					na		0.00	0.14	0.02	79.52	-	-
Methoxychlor	<0.82	<0.82	<0.82		<18.5	<18.5	<18.5		0.00	0.02	0.01	60.24	-	-
Mirex	<2.59	<2.59	<2.59		0.00	0.08	0.04	35.29	0.00	0.04	0.01	69.88	-	0.316
Parathion-ethyl	<7.05	<7.05	<7.05		0.01	1.43	0.39	88.24	0.00	0.02	0.01	21.69	-	0.024
Parathion-methyl	274.87	362.24	318.56	11.76	0.00	0.35	0.14	47.06	0.01	0.08	0.02	87.95	58.88	0.134
Phosmet	<2.14	<2.14	<2.14				*na*		0.00	0.12	0.04	72.29	-	-
Pirimicarb	38.29	93.17	53.45	70.59	<0.02	<0.02	<0.02		0.01	0.25	0.04	75.90	829.09	-
Tetrachlorvinphos	11.95	27.92	16.14	76.47	<0.01	<0.01	<0.01		0.00	0.17	0.05	67.47	2799.02	-
ΣFUNGICIDES			**128.86**					**258.82**			**0.06**		**91.38**	**0.06**
Azoxystrobin	<9.28	<9.28	<9.28				*na*		0.00	0.22	0.03	73.49	-	-
Difenoconazole	<1.91	<1.91	<1.91		0.01	1.21	0.26	35.29	0.00	0.04	0.01	93.98	-	0.043
HCB	<0.43	0.66	<0.43		0.00	2.57	0.57	82.35	0.00	0.01	0.00	37.35	-	0.008
PeCB	<8.31	<8.31	<8.31		0.01	0.67	0.23	94.12	<0.00	<0.00	<0.00		-	-
Procymidone	<3.99	<3.99	<3.99		0.12	0.93	0.40	11.76	<0.00	<0.00	<0.00		-	-
Tebuconazole	117.66	140.06	128.86	11.76	0.01	2.74	1.04	35.29	0.00	0.06	0.01	92.77	91.38	0.011

DAP (ng/L): dissolved aqueous phase; **SPM** (mg/kg): suspended particulate matter; **STB** (mg/kg ww): soft tissues from bivalve; **BAF_{DAP}**: bioaccumulator factor in DAP fraction; **BAF_{SPM}**: bioaccumulator factor in SPM fraction **<LOQ**: below limits of quantification; **Min**.:minumum value; **Max**.: maximum value; **Freq**. (%): frequencies

3.3 Pesticides in *S. plana* (STB fraction)

The evaluation of pesticides in bivalve soft tissues showed that almost all selected pesticides, with the exception of dieldrin, PeCB and procymidone, were bioaccumulated in *S. plana*. In fact, 54% of the analysed samples were quantified in STB. These showed pesticide concentrations ranging from ~0.005 mg/kg ww to ~0.072 mg/kg ww. Similar values were observed in diverse aquatic animals collected from polluted environments (Galvao et al., 2012; Helaleh et al., 2012; Silva et al., 2008; Wille et al., 2011; Yang et al., 2006). Summing the average concentrations of pesticides, *per* season, it is observed a significant increase of these compounds in *S. plana* in autumn (~ 1.25 mg/kg ww, $p < 0.05$) comparatively to winter (~ 0.89 mg/kg ww) and spring (~ 0.73 mg/kg ww) (Figure 3A).

Observing data per sampling site, results demonstrate that bivalves accumulate similar concentrations, independently from DAP and SPM levels (Figure 3B). However, considering together the surface deposit-feeder nature of these organisms and the chemical properties of the studied pesticides, a positive correlation could be anticipated mainly between the STB fraction and SPM fraction (Bejarano et al., 2003). The opposite results may be linked to biological detoxification processes (metabolization and depuration), once triggered by pesticide concentrations, available in the surrounding environment (Serrano et al.; VanSlooten and Tarradellas, 1994).

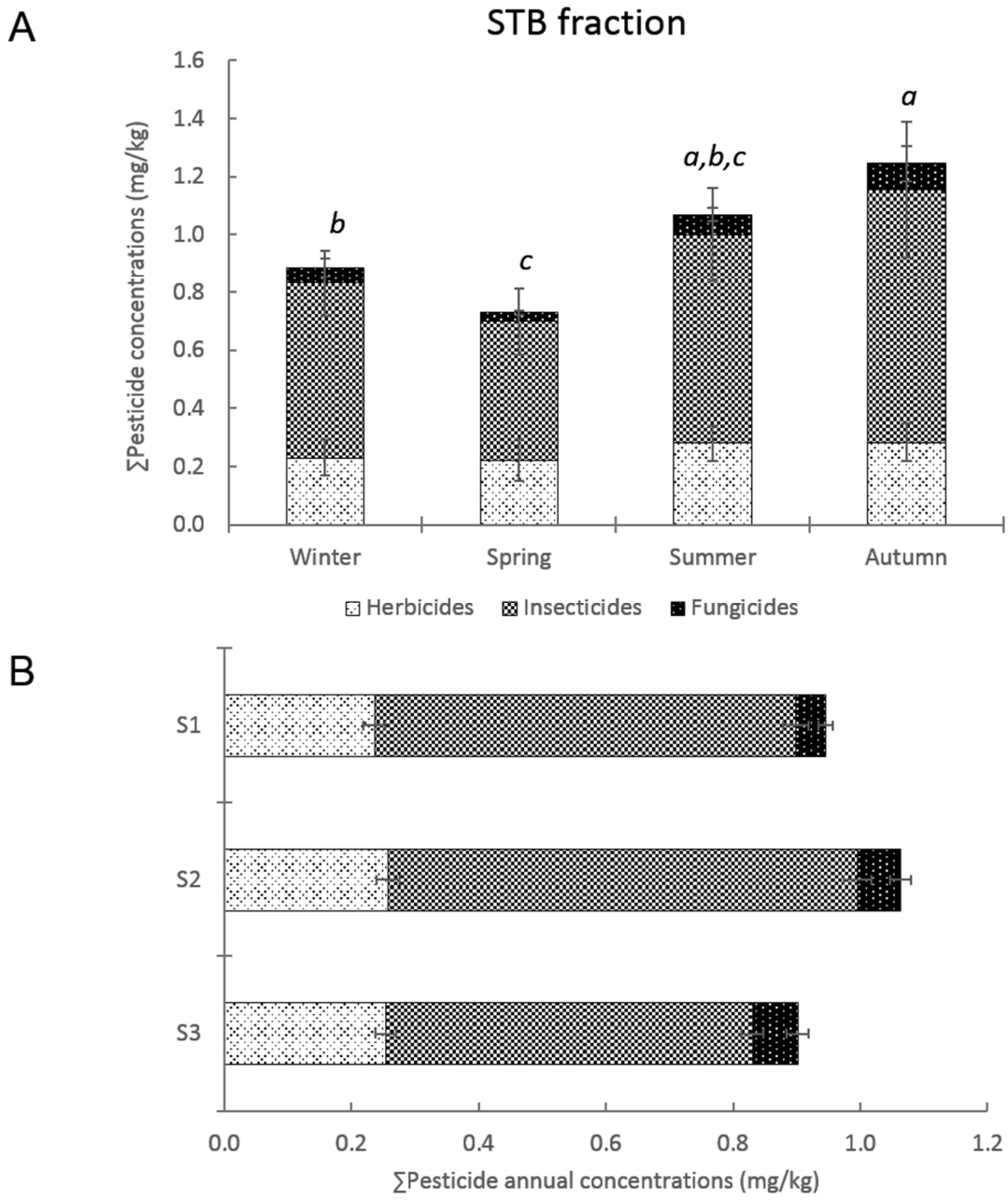


Figure 3- Sum of the average concentration of pesticides in STB (mg/kg) fraction, expressed by category and season; n = 90 and bars represent SD.

Considering the sex and the maturation status of the sampled animals the current histocytological analyses showed that gonadal maturation reaches its maximum in spring (May) and decreases drastically in autumn (October), occasion when 80% of the collected animals were classified as U

(Figure 4). In overall, *S. plana* showed the typical long cycle, expanded from winter to summer, described earlier for southern European populations of this species (Santos et al., 2011). Probably, also due to this, no significant differences were found when analysing the pesticide concentrations in bivalve tissues between seasons. Also, no correlation was observed for *S. plana* between the CI and the Σaverage concentrations of pesticides *per* season, suggesting a pesticide accumulation independent from lipid body content, gonadal ripeness or sex. Similar observations also occurred for other class of lipophilic compounds, such as the polychlorinated biphenyls (PCBs) (Hummel et al., 1990; Suárez et al., 2013), suggesting that further studies about this issue are necessary to fully understand what phenomena are underlying these occurrences.

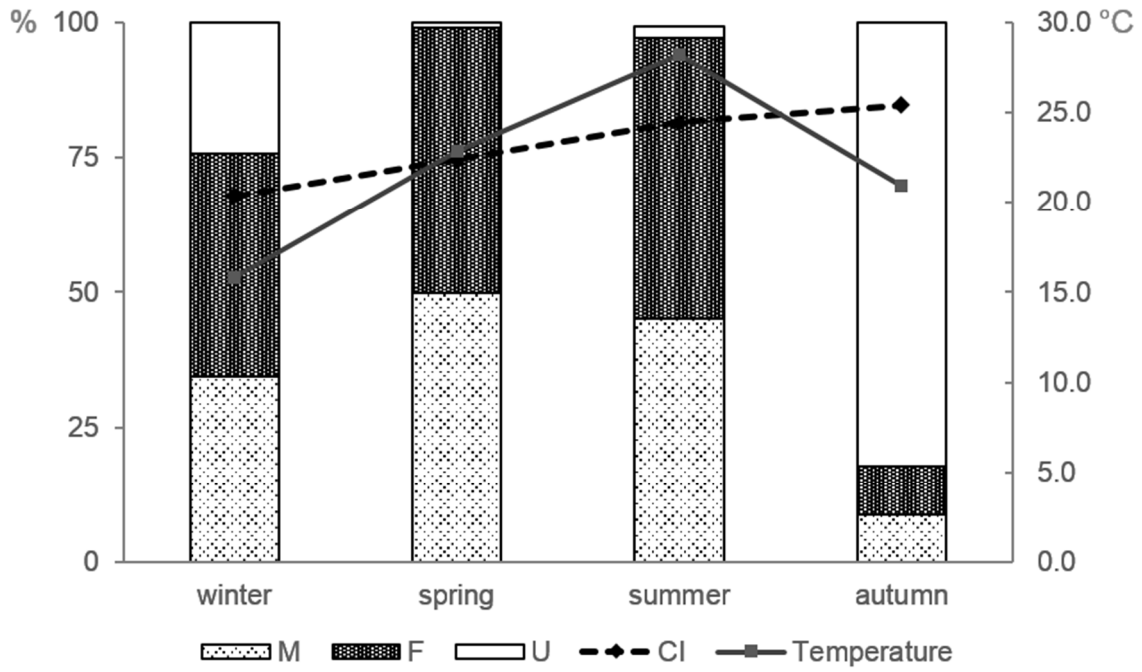


Figure 4- Sex proportion (%, left axis) of males (M), females (F), and undefined animals (U), together with average condition index (CI) (%, left axis and dashed line) and temperature (°C, right axis and continuous line), grouped by season.

Concerning the BAF values, from both DAP and SPM fractions, it is accepted that those above 1000, 2000 or 5000 (depending on the regulatory authority) are considered significantly bioaccumulative (Arnot and Gobas, 2006). In the Tagus River estuary these values ranged from 0.008 to 2799 (Table 1). These data suggest possible bioaccumulation effects and are in accordance with positive correlations found between the concentrations of pesticides in STB fraction and the $BAF_{DAP+SPM}$ values ($r = 0.49$, $p = 0.0001$). These results demonstrate that individual pesticides were below the bioaccumulation level referred above, but as a sum (Σ4979) the scenario changes being classified as "very bioaccumulative". Also, no significant differences were found for BAF_{DAP} and BAF_{SPM} calculated for the different sampled sites.

3.4 Hazard assessment

3.4.1 Biota

The maximum global amounts of pesticides, in both DAP and SPM (ng/L), were used to predict their potential risk for aquatic organisms. Firstly, it was considered the sum of concentrations of all pesticides in both fractions since contaminant exchanges between matrices can occur, and given that both fractions are available for surface deposit–feeder animals. In this sense, the RQ ratio revealed that 4 herbicides and 14 insecticides have the probability to produce noxious effects to different aquatic trophic levels ($RQ \geq 1$). The second approach, through the calculation of the STU values, identified the invertebrates as the most sensitive group living in this estuary (STU = 4.15). Individually, the highest STU values were observed among insecticides, corroborating with the fact that invertebrates are more sensible to this category of pesticides (Åkerblom, 2004; Fulton and Key, 2001). The RQ_{STU}/maxTU ratio of 1.727 was calculated, indicating a toxicity effect led by a small number of pollutants, mainly insecticides (Table SM2).

3.4.2 Human health

Human health hazard assessment was done based on the assumption that humans can ingest contaminants through bivalve consumption (Table SM3).

Based on FAO specifications it was considered the consumption of 15 g/capita/day for the current estimate (Food and Agriculture Organization (FAO) of the United Nations, 2015). Afterwards, and considering an average body weight of 60 kg, it was calculated the EADI and none of the concentrations were above the ADI values and the maximum residue levels (MRLs) set by the European Union (2008). Evaluations were also done between EADI and the theoretical maximum daily intake (TMDI) but, as expected, no chronic or acute health risk was observed, concerning pesticides, for humans consuming *S. plana* from the Tagus River estuary. Similarly, the calculation of HQ confirmed the last assumption (Table SM3). Nonetheless, it is important to stress that bivalves are on the bottom of the food chain and biomagnification events may happen and affect the top-predators, including humans (Guéguen et al., 2011).

3.5 European water framework directive

Data was assessed accordingly with the Directive 2013/39/EU (EU, 2013), set for transitional waters considering the annual average- and the maximum allowable concentration-environmental quality standards (AA-EQS and MAC-EQS, respectively) (Table SM4). As referred previously the concentrations of each pesticide in DAP and SPM fractions were summed (ng/L) to evaluate the percentage of compounds (15 listed pesticides) above legislation. In this situation, a total of 53% and 27% of these contaminants were above AA-EQS and MAC-EQS, respectively. Pesticides, such as Σendosulfan (alpha and beta) and Σheptachlor, heptachlor epoxide (ΣH, He), showed concentrations highly above legislation limits (EU, 2013) (Table SM4). The quantified levels of these last compounds may be derived from SPM fraction, since some of them were not detected in DAP fraction (Table 1).

Among STB samples, 64% of the selected contaminants were above 2013/39/EU limits (0.0067 ng/g for ΣH, He and 10 ng/g for all others) and several samples showed pesticide concentrations above legal limits (EU, 2013) (Figure SM2). These results may indicate that bivalves are subject to a constant exposure to these pollutants and/or they are quite persistent and stable in organisms, making quantification possible during all year round.

3.6 Physicochemical data

Water quality control parameters are resumed in Table 2 grouped by season and represented by site. Temperature (~ 22 ºC) and pH (~ 8.1) were quite stable all year round. Salinity ranged from 2 to 25, due to the variable input of freshwater at each sampled site. Within sampling sites, S_1 and S_3 presented lower DO values (~ 5 mg/L) than S_2 (~ 16 mg/L, $p < 0.05$), which may be linked to local pollution (slaughterhouse near S_1 and Trancão River effluents at S_3). Low DO values are usually associated to high levels of organic matter decomposition, lack of water recirculation, and temperature increase, among other events connected to anthropogenic activities (Best et al., 2007). The increment of nitrogen and phosphate were, in contrast to the DO values, significantly higher at S_1 (0.2 and 1.1 mg/L, respectively) and S_3 (1.2 and 5.6 mg/L, respectively) than at S_2 (0.07 and 1.5 mg/L, respectively). Significant differences were observed for $N\text{-}NH_4$, DIN and $P\text{-}PO_4$ parameters between S_2 and S_3 (Table 2). Moreover, significant negative correlations were found between DO and total nitrogen ($r = -0.74$; $p = 0.0007$), corroborating with these results and denoting an environment highly impacted by anthropogenic compounds. In most cases all significant differences observed were associated with concentration levels above legislation, and/or associated with toxic and/or eutrophication events (Best et al., 2007; Daniel et al., 1998; Environmental Protection Agency (EPA), 1986; Ministério do Ambiente, 1998). No correlations were found between total average concentrations of pesticides and physicochemical parameters, suggesting that the values observed herein may derive from other anthropogenic activities. Altogether, the results obtained herein demonstrate an urgent need for waste water treatment, mainly at S_1 and S_3.

Table 2- Physicochemical parameters, grouped by season and site (S_1 to S_3).

Season	Site	Dissolved O_2	Temperature	Salinity	Condutivity	pH	$N\text{-}NO_2$	$N\text{-}NO_3$	$N\text{-}NH_4$	$N\text{-}NH_3$	DIN	$P\text{-}PO_4$	TIP
		mg/L	℃		mS/cm		mg/L						
Winter	S_1	5.1	15.8	21.0	27.6	7.6	0.35#	2.95	7.55#	0.06	10.85#	1.78#	0.13
	S_2	16.7	16.7	19.2	25.9	8.9	0.05	1.89	0.30	0.11#	2.24#	0.55#	0.04
	S_3	5.8	14.8	2.2	3.3	8.0	0.71#	6.48	4.00#	0.05	9.85#	1.58#	0.12
Spring	S_1	7.6	23.0	24.1	38.0	7.7	0.20#	0.82	1.53#	0.03	2.55#	1.35#	0.10
	S_2	19.1	25.6	18.1	29.2	8.7	0.06	0.90	0.14	0.22#	1.10	0.38#	0.03
	S_3	3.9	19.9	7.6	13.2	7.5	0.86#	2.90	7.09#	0.02	10.85#	2.70#	0.20#
Summer	S_1	5.5	31.0	25.3	44.4	8.0	0.25#	1.34	2.31#	0.13#	3.90#	2.60#	0.20#
	S_2	18.6	27.8	24.2	41.1	9.0	0.04	1.36	0.54	0.19#	1.94#	1.50#	0.11
	S_3	5.8	25.8	15.0	25.0	8.2	2.51#	6.79	4.02#	0.05	13.32#	3.90#	0.29#
Autumn	S_1	6.3	21.0	22.7	33.2	8.0	0.17#	1.68	1.14#	0.11#	2.99#	0.08#	0.01
	S_2	11.3	21.7	21.1	31.5	8.1	0.13#	1.80	0.27	0.35#	2.20#	0.07#	0.01
	S_3	3.2	20.2	10.2	15.6	8.1	0.79#	6.31	4.26#	0.16#	11.36#	0.13#	0.01

DIN: dissolved inorganic nitrogen; **TIP**: total inorganic phosphorous; #:values above legislation limits

4. Conclusions

The present study confirmed the contamination of the Tagus River estuary, by assessing the occurrence and distribution of pesticides in water (DAP + SPM fractions) and bioaccumulated in the soft tissues of *S. plana* (STB fraction). Pesticides were widespread in all the collected matrices, likely as a consequence of a continuous output of pesticides from agricultural and urban land. Notably, many of the concentrations were above the limits established by Directive 2013/39/EU. Moreover, the sampling site located close to the Trancão River mouth was found to be one hotspot of pollution entering the Tagus River estuary. Physicochemical parameters also demonstrate a very poor water quality, which was evident at S_1 and S_3 sampling sites.

Ecological and human health risk assessments were conducted from a theoretically perspective, revealing that there is a possible environmental risk for invertebrates but with no direct consequences for human health. In spite of this, it is important to stress that biomagnification events may happen and may be harmful for top-predators.

The results herein described, mainly concerning the bivalve matrix, should be taken into consideration by the environmental organizations to protect local and migratory fauna that habit the natural reserve, as the wading birds among others.

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Supplementary material

Document SM1- Detailed information concerning the quality assurance (QA) and quality control (QC) procedures.

The validation protocols followed the criteria established by SANCO/825/00 rev 8.1 (European Commission Directorate General Health and Consumer Protection, 2010).

Retention times and mass spectra were similar between standards and fortified matrices (RSD<5%), thus proving that the chromatographic procedures were selective for the quantification of all pesticides. The parent-daughters ions, resulted from specific individual collision energies, had the same proportions between spiked and wild samples, demonstrating the robustness of the method.

The calibration curves proved to have good fits, with r^2 ranging from 0.98 to 0.99.

The protocol also includes the evaluation of detection limits (LOD), quantification limits (LOQ), accuracy, precision, recoveries, and stability of the extracted samples. Recovery, precision, accuracy and stability were studied for three independent replicates in three different days for each spiked concentration.

In DAP fraction, LODs were below 1.3 ng/L, LOQs ranged from 0.01 to 11.6 µg/L, and the average recoveries, precision and accuracy were 99%, 9%, and 100%, respectively (Cruzeiro et al., 2015b).

In SPM fraction, LODs were below 2.2 ng/L, LOQs ranged from 0.01 to 12.2 µg/L, and the average recoveries, precision, and accuracy were 102%, 8%, and 101%, respectively (Cruzeiro et al., 2015a).

In STB fraction, LODs were below 3.4 ng/L, LOQs ranged from 0.33 to 10.3 µg/L, and the average recoveries, precision, and accuracy were 99%, 12%, and 95%, respectively (Cruzeiro et al., 2016).

The stability of the extracted pesticides was evaluated by comparing the initial results of the QCs with those obtained after a period of 24, 48, and 96h, kept at –20 ℃. No alterations were found in all the matrices within a 24h period.

Table 1. Molecular mass (g/mol), quantification and diagnostic ions used in GC-MS/MS analysis.

Pesticides	Molecular mass	GC-MS/MS							
	g/mol	Percursor		Products				CE	Ranges
Alachlor [a]	269.8	**160**	→	132	130			1.05	116-161
Aldrin [a]	364.9	**263**	→	193	191	227		1.60	190-264
Atrazine [b]	215.7	**200**	→	122	132	164	158	1.50	121-201
Atrazine-*d*$_5$	220.7	**205**	→	127	137	105		1.35	104-206
Atrazine-desethyl	187.6	**172**	→	130	145	152		1.05	104-173
Azinphos-methyl	317.3	**132**	→	117	104	114		1.10	100-133
Azoxystrobin	403.4	**344**	→	329	328			2.00	325-345
BHC (gamma) (Lindane) [a]	290.8	**181**	→	145	146			1.50	108-184
Chlordane (gamma)	338.9	**373**	→	266	264			1.10	263-374
Chlorpyriphos	350.6	**314**	→	258	286			0.90	257-315
Cyanazine	240.7	**212**	→	209	197	176	171	1.15	170-226
Cyfluthrine (beta) *	434.3	**199**	→	193	191	163		1.20	190-200
Cyhalofop-butyl	357.4	**357**	→	342	287			1.50	280-358
Cyhalothrin (lambda) *	449.9	**181**	→	152	151			1.30	150-182
Cypermethrin (alpha) *	416.3	**181**	→	152	151			1.30	150-182
4,4´-DDD	320.0	**235**	→	165	199			1.20	162-236
4,4´-DDT	354.5	**235**	→	165	199			1.20	117-236
4,4´-DDT-*d*$_8$	362.5	**243**	→	173	206			1.20	172-244
4,4'-DDE	318.0	**246**	→	176	175			1.70	174-247
Deltamethrin *	505.2	**181**	→	152	151			1.30	150-182
Diazinon	304.4	**179**	→	121	163	137	122	1.20	110-180
Dichlorvos	221.0	**109**	→	79	93			1.10	66-83/90-110
Dieldrin [a]	380.9	**79**	→	51	50			1.05	49-80
Difenoconazol	406.3	**323**	→	265	249			1.30	245-324
Dimethoate	229.3	**87**	→	86	59			1.15	55-88
Endosulfan (alpha)	406.9	**241**	→	206	205			1.35	165-242
Endosulfan (beta)	406.9	**241**	→	206	204	170		1.35	165-242
Endosulfan sulfate	422.9	**272**	→	237	235			0.90	234-273
Endrin [b]	380.9	**243**	→	207	173			1.20	172-244
Fenamiphos	303.4	**303**	→	268	266			1.20	265-304
Fenitrothion	277.2	**260**	→	228	217	232		1.10	190-261
Fonofos	246.3	**137**	→	109	81			0.85	80-138
Heptachlor [b]	373.3	**272**	→	237	235			1.05	236-275
Heptachlor epoxide [b]	389.3	**353**	→	263	282			1.05	262-354
Hexachlorobenzene (HCB)[b]	284.8	**284**	→	214	249			1.50	211-285
Hexachlorocyclopentadiene (HCCP) [b]	272.3	**237**	→	143	141	203		1.60	140-145/200-238
Malathion	330.4	**173**	→	99	117	145		0.90	95-173
Methoxychlor [b]	345.7	**227**	→	169	181			1.30	165-228
Metamidophos	141.1	**141**	→	113	115			1.50	110-142
Metolachlor	283.8	**162**	→	132	133			1.05	130-163
Metribuzin	214.3	**198**	→	150	110			1.35	109-199
Mirex	545.5	**272**	→	237	235			1.00	234-273
Parathion-ethyl	291.3	**291**	→	186	220	255	256	1.15	185-292
Parathion-methyl	263.2	**263**	→	246	153			1.40	150-264
Pendimethalin	281.3	**252**	→	162	191			1.25	160-253
Pentachlorobenzene (PeCB)	250.3	**250**	→	215	144			2.01	143-251
Phosmet	317.3	**160**	→	130	140			1.15	125-161
Pirimicarb	238.4	**166**	→	96	137	121		1.20	95-167
Procymidone	284.1	**96**	→	67	68			0.90	64-97
Propazine	229.7	**214**	→	200	172	138		1.00	137-215
Propyzamide	256.1	**173**	→	138	145			1.05	135-174
Simazine [a]	201.6	**201**	→	186	174	138		1.45	135-201
Simetryn	213.3	**213**	→	170	185			1.25	169-214
Tebuconazole	307.8	**125**	→	89	99			1.40	87-126
Terbuthylazine	229.7	**214**	→	173	132			1.00	131-215
Terbutryn	241.4	**185**	→	170	128			1.10	127-186
Tetrachlorvinphos	366.0	**329**	→	314	278			1.20	219-330
Trifluralin	335.3	**264**	→	206	160	188	171	1.00	159-265

Internal standards; [a] Compounds present on the mix A (EPA 505/525); [b] Compounds present on the mix B (EPA 505/525); * Contain several diastereoisomers; **RT**: retention time; **CE**: collision energy (V)

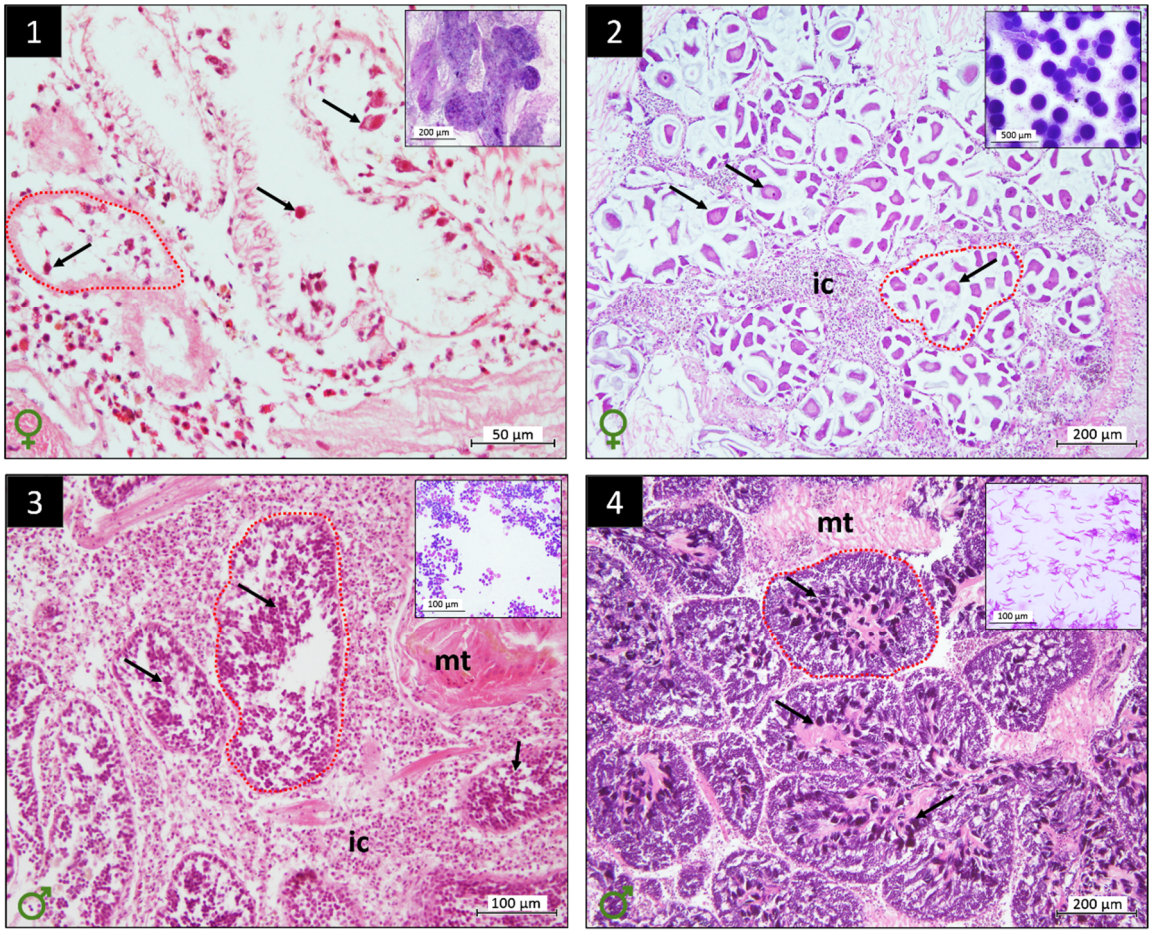

Figure SM1- H&E histology (main pictures) of *S. plana* gonad according to the developing stage and sex (male and female); ic: insterstitial cells; mt: muscle tissue; the arrows (from 1 to 4) represent: growing oocytes, spermatocytes, and sperm; the dashed line contours the acini. The set-in images represent the gonad squash stained with Diff-Quik.

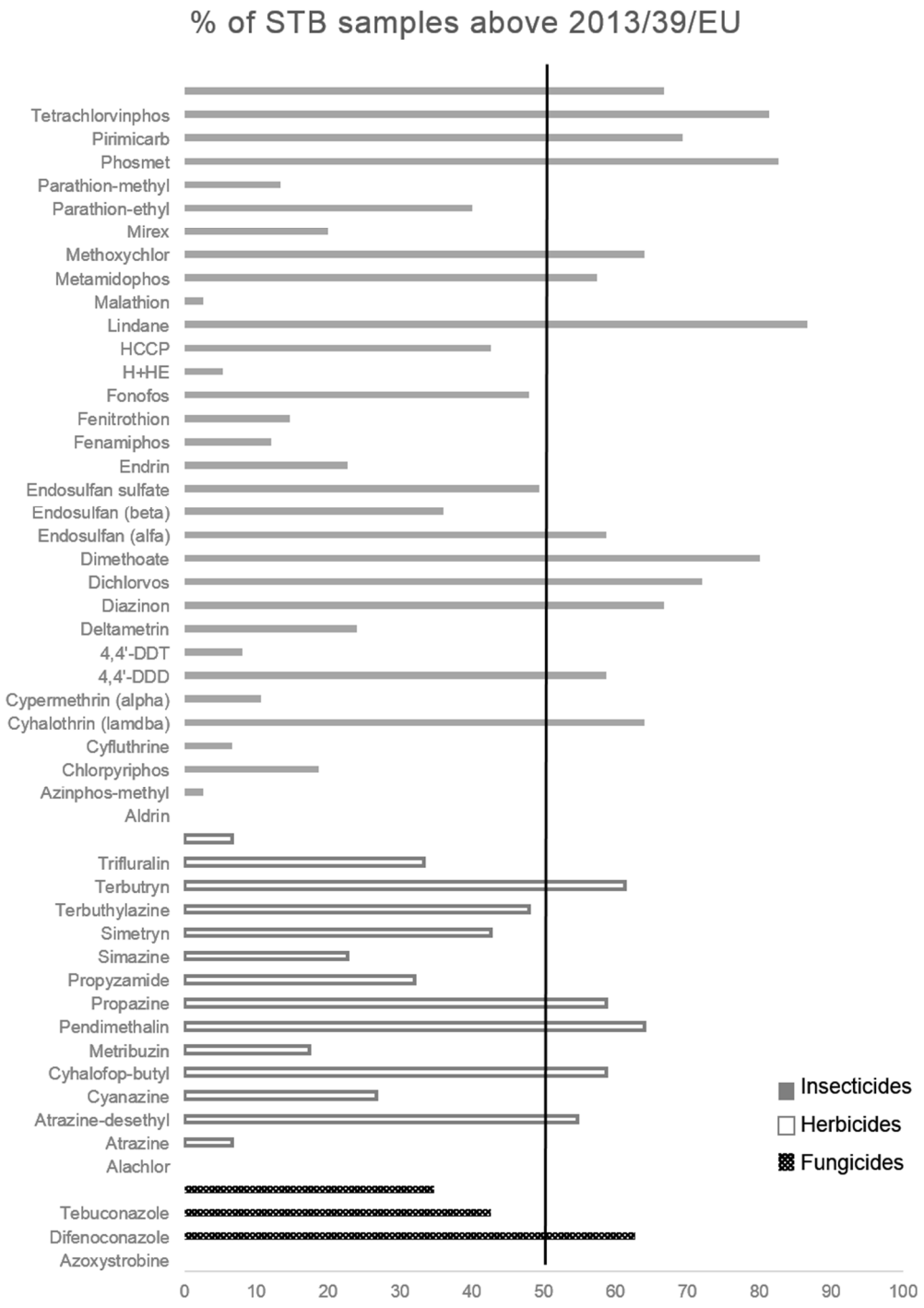

Figure SM2- Percentage of STB samples with concentrations above 2013/39/EU Directive EQS.

Table SM1- Index values of log K_d calculated for this study and reported in other aquatic environments.

Log K_d	This study	San Francisco Bay	Yangtze River	Johnstone River	Ria Formosa Lagoon	Montreal Island	Lourens River
Alachlor	3.35				4.91		
Atrazine	2.95			1.84	4.73	3.84	
Atrazine-desethyl						3.05	
Cyanazine							
Cyhalofop-butyl					3.25		
Metolachlor					5.18		
Metribuzin					4.66		
Pendimethalin	3.54				5.07		
Propazine							
Propyzamide							
Simazine				2.00	4.55		
Simetryn							
Terbuthylazine							
Terbutryn	2.91						
Trifluralin					5.54		
Aldrin							
Azinphos-methyl					4.47		
Chlordane (gamma)							
Chlorfenvinphos Z					4.14		
Chlorpyriphos							
Cyfluthrine						1.25	
Cyhalothrin (lamdba)	3.85				4.06	1.42	
Cypermethrin (alpha)						13.89	
4,4´-DDD		4.09			4.22		
4,4´-DDE		3.36					
4,4´-DDT	3.77	3.81			4.34		
Deltametrin					3.78		2.35
Diazinon	4.41	2.22			3.65		
Dichlorvos							
Dieldrin					3.82		
Dimethoate	2.47				3.56		
Endosulfan (alfa)	3.34				3.96		
Endosulfan (beta)					4.09		
Endosulfan sulfate					4.05		
Endrin							3.18
Fenamiphos							3.20
Fenitrothion					3.63		2.83
Fonofos					4.25		
HCCP							
Heptachlor							
Heptachlor epoxide							
Lindane			8.00				
Malathion					4.63	8.00	
Metamidophos							
Methoxychlor							
Mirex					4.64		
Parathion-ethyl					4.05		
Parathion-methyl	2.64				3.57		
Phosmet							
Pirimicarb							
Tetrachlorvinphos					4.08		
Azoxystrobin							
Difenoconazole					3.87		
HCB							
PeCB					3.23		
Procymidone							
Tebuconazole	3.91				4.52		

Table SM2- Ecological hazard assessment of pesticides in Tagus River estuary waters (DAP + SPM).

	Pesticides	MEC	EC_{50} or LC_{50}			PNEC	MEC/PNEC		RQ_{TU}	
			algae	invertebrates	fish			algae	invertebrates	fish
HERBICIDES	Alachlor	1.2E-03	9.7E-01	1.0E+01	1.8E+00	9.7E-03	1.2E-01	1.2E-03	1.2E-04	6.4E-04
	Atrazine	6.1E-04	5.9E-02	8.5E+01	4.5E+00	5.9E-04	1.0E+00 #	1.0E-02	7.2E-06	1.4E-04
	Atrazine-desethyl	2.4E-06	1.0E-01	nf	nf	1.0E-03	2.4E-03	2.4E-05	-	-
	Cyhalofop-butyl	1.8E-05	9.6E-01	2.7E+00	7.9E-01	7.9E-03	2.3E-03	1.9E-05	6.8E-06	2.3E-05
	Metolachlor	1.7E-04	5.7E+01	2.4E+01	3.9E+00	3.9E-02	4.4E-03	3.0E-06	7.3E-06	4.4E-05
	Metribuzin	6.9E-05	2.0E-02	4.9E+01	7.5E+01	2.0E-04	3.5E-01	3.5E-03	1.4E-06	9.3E-07
	Pendimethalin	9.3E-04	6.0E-03	2.8E-01	1.4E-01	6.0E-05	1.6E+01 #	1.6E-01	3.3E-03	6.8E-03
	Propazine	1.7E-06	1.8E-01	1.8E+01	1.8E+01	1.8E-03	9.7E-04	9.7E-06	9.8E-08	1.0E-07
	Propyzamide	7.1E-04	2.8E+00	5.6E+00	4.7E+00	2.8E-02	2.5E-02	2.5E-04	1.3E-04	1.5E-04
	Simazine	4.7E-05	4.0E-02	1.1E+00	9.0E+01	4.0E-04	1.2E-01	1.2E-03	4.3E-05	5.3E-07
	Simetryn	2.0E-05	9.8E-03	nf	7.0E+00	9.8E-05	2.0E-01	2.0E-03	-	2.8E-06
	Terbuthylazine	8.7E-04	1.2E-02	2.1E+01	2.2E+00	1.2E-04	7.3E+00 #	7.3E-02	4.1E-05	4.0E-04
	Terbutryn	5.3E-04	2.4E-03	2.7E+00	1.1E+00	2.4E-05	2.2E+01 #	2.2E-01	2.0E-04	4.8E-04
INSECTICIDES	Aldrin	8.8E-06	nf	2.8E-02	4.6E-03	4.6E-05	1.9E-01	-	3.1E-04	1.9E-03
	Azinphos-methyl	8.4E-07	7.2E+00	1.1E-03	2.0E-02	1.1E-05	7.7E-02	1.2E-07	7.7E-04	4.2E-05
	Chlordane-gamma	3.4E-06	nf	5.9E-01	9.0E-02	9.0E-04	3.8E-03	-	5.8E-06	3.8E-05
	Chlorpyriphos	9.6E-05	4.8E-01	4.0E-05	1.3E-03	4.0E-07	2.4E+02 #	2.0E-04	2.4E+00 #	7.4E-02
	Chlorfenvinphos Z	2.5E-05	1.4E+00	2.5E-04	1.1E+00	2.5E-06	1.0E+01 #	1.8E-05	1.0E-01	2.3E-05
	Cyhalothrin (lambda)	1.3E-04	nf	3.8E-01	4.6E-04	4.6E-06	2.8E+01 #	-	3.4E-04	2.8E-01
	4,4´-Σ DDD,DDE,DDT	2.6E-04	nf	5.0E-03	7.0E+00	5.0E-05	5.1E+00 #	-	5.1E-02	3.6E-05
	Deltamethrin	1.3E-04	9.1E+00	5.6E-04	2.6E-04	2.6E-06	5.0E+01 #	1.4E-05	2.3E-01	5.0E-01
	Diazinon	8.1E-04	6.4E+00	1.0E-03	3.1E+00	1.0E-05	8.1E+01 #	1.3E-04	8.1E-01	2.6E-04
	Dieldrin	1.4E-04	1.0E-01	2.5E-01	1.2E-03	1.2E-05	1.1E+01 #	1.4E-03	5.4E-04	1.1E-01
	Dimethoate	2.8E-04	9.0E+01	2.0E+00	3.0E+01	2.0E-02	1.4E-02	3.1E-06	1.4E-04	9.2E-06

(*continued*)

	Pesticides	MEC	EC_{50} or LC_{50}			PNEC	MEC/PNEC	RQ_{TU}		
			algae	invertebrates	fish			algae	invertebrates	fish
INSECTICIDES	Σ Endosulfan (alpha, beta)	1.0E-03	2.2E+00	4.4E-01	2.0E-03	2.0E-05	5.0E+01 #	4.7E-04	2.3E-03	5.0E-01
	Endrin	1.2E-05	nf	4.2E-03	7.3E-04	7.3E-06	1.6E+00 #	-	2.9E-03	1.6E-02
	Fenitrothion	3.1E-04	1.3E+00	8.6E-03	1.3E+00	8.6E-05	3.6E+00 #	2.4E-04	3.6E-02	2.4E-04
	Fonofos	4.2E-04	1.5E+00	2.3E-03	2.8E-02	2.3E-05	1.8E+01 #	2.8E-04	1.8E-01	1.5E-02
	Heptachlor	8.8E-06	2.7E-02	4.2E-02	7.0E-03	7.0E-05	1.3E-01	3.2E-04	2.1E-04	1.3E-03
	Heptachlor epoxide	2.0E-05	2.0E+02	2.4E-01	2.0E-02	2.0E-04	1.0E-01	1.0E-07	8.4E-05	1.0E-03
	Malathion	1.7E-04	1.3E+01	7.0E-04	1.8E-02	7.0E-06	2.5E+01 #	1.3E-05	2.5E-01	9.5E-03
	Mirex	4.7E-06	1.0E-01	1.0E-01	1.0E+02	1.0E-03	4.7E-03	4.7E-05	4.7E-05	4.7E-08
	Parathion-ethyl	2.2E-05	5.0E-01	2.5E-03	1.5E+00	2.5E-05	8.6E-01	4.3E-05	8.6E-03	1.4E-05
	Parathion-methyl	3.8E-04	3.0E+00	7.3E-03	2.7E+00	7.3E-05	5.2E+00 #	1.3E-04	5.2E-02	1.4E-04
	Pirimicarb	9.3E-05	1.4E+02	1.7E-02	1.0E+02	1.7E-04	5.5E-01	6.7E-07	5.5E-03	9.3E-07
	Tetrachlorvinphos	2.8E-05	nf	2.0E-03	4.3E-01	2.0E-05	1.4E+00 #	-	1.4E-02	6.5E-05
FUNGICIDES	Difenoconazol	1.4E-05	3.2E-02	7.7E-01	1.1E+00	3.2E-04	4.5E-02	4.5E-04	1.8E-05	1.3E-05
	HCB	3.9E-05	1.0E-02	5.0E-01	3.0E-02	1.0E-04	3.9E-01	3.9E-03	7.7E-05	1.3E-03
	PeCB	1.4E-05	1.3E+01	nf	2.5E-01	2.5E-03	5.8E-03	1.1E-06	-	5.8E-05
	Procymidone	4.9E-05	2.6E+00	1.8E+00	7.2E+00	1.8E-02	2.7E-03	1.9E-05	2.7E-05	6.8E-06
	Tebuconazole	3.2E-04	2.0E+00	2.8E+00	4.4E+00	2.0E-02	1.6E-02	1.6E-04	1.1E-04	7.3E-05
						$RQ_{MEC/PNEC}$	579.6	0.47	4.15	1.52
						$RQ_{STU(100)}$	415.4			
						RQ_{STU}/maxTU	1.727			

nf: information not found; **MEC** (mg/L): maximum measured environmental concentration; **PNEC** (mg/L): predicted no effect concentration; **EC_{50}** (mg/L): half maximal effective concentration; **LC_{50}** (mg/L): median lethal dose; **TU**: toxic units; **STU**: sum of the toxic units; **RQ**: ratio quotient; # values above 1

Table SM3- Human health hazard, associated with *S. plana* consumption captured from the Tagus River estuary.

	MEC	MRL	TMDI	ADI	ARfD	EADI	EADI/ADI	EADI/TMDI	HQ (ARfD)
Alachlor	6.9E-03	1.0E-02	1.5E-04	3.0E-01	4.8E+01	1.7E-06	3.4E-04	1.1E-02	3.6E-08
ΣAldrin, Dieldrin	6.9E-03	6.0E-03	9.0E-05	6.0E-03	nf	1.7E-06	1.7E-02	1.9E-02	-
Atrazine	3.0E-02	1.0E-02	1.5E-04	1.2E+00	6.0E+00	7.6E-06	3.8E-04	5.0E-02	1.3E-06
Atrazine-desethyl	1.4E-02	1.0E-02	1.5E-04	1.3E+00	nf	3.5E-06	1.6E-04	2.3E-02	-
Azinphos-methyl	1.3E-02	1.0E-02	1.5E-04	3.0E-01	6.0E-01	3.4E-06	6.7E-04	2.2E-02	5.6E-06
Azoxystrobin	3.4E-02	1.0E-02	1.5E-04	1.2E+01	1.1E+01	8.5E-06	4.2E-05	5.6E-02	7.8E-07
Chlordane (gamma)	7.3E-03	2.0E-03	3.0E-05	3.0E-02	3.0E-02	1.8E-06	3.6E-03	6.1E-02	6.1E-05
Chlorpyriphos	7.6E-03	1.0E-02	1.5E-04	6.0E-01	6.0E+00	1.9E-06	1.9E-04	1.3E-02	3.2E-07
Cyanazine	3.7E-02	1.0E-02	1.5E-04	1.2E-01	nf	9.3E-06	4.6E-03	6.2E-02	-
Cyfluthrine	2.3E-02	1.0E-02	1.5E-04	1.8E-01	1.2E+00	5.7E-06	1.9E-03	3.8E-02	4.7E-06
Cyhalofop-butyl	2.0E-02	1.0E-02	1.5E-04	1.2E-01	nf	5.0E-06	2.5E-03	3.3E-02	-
Cyhalothrin (lamdba)	8.2E-03	1.0E-02	1.5E-04	3.0E-01	nf	2.0E-06	4.1E-04	1.4E-02	-
Cypermethrin (alpha)	3.1E-02	5.0E-02	7.5E-04	9.0E-01	2.4E+00	7.8E-06	5.2E-04	1.0E-02	3.2E-06
4,4´-ΣDDD, DDT	2.2E-02	1.0E+00	1.5E-02	3.0E-01	nf	5.5E-06	1.1E-03	3.7E-04	-
Deltametrin	1.5E-02	3.0E-02	4.5E-04	6.0E-01	6.0E-01	3.8E-06	3.8E-04	8.5E-03	6.4E-06
Diazinon	3.0E-02	1.0E-02	1.5E-04	1.2E-02	1.5E+00	7.5E-06	3.7E-02	5.0E-02	5.0E-06
Dichlorvos	3.0E-02	1.0E-02	1.5E-04	4.8E-03	1.2E-01	7.4E-06	9.2E-02	4.9E-02	6.2E-05
Difenoconazole	1.1E-02	5.0E-02	7.5E-04	6.0E-01	9.6E+00	2.8E-06	2.8E-04	3.7E-03	2.9E-07
Dimethoate	2.3E-02	1.0E-02	1.5E-04	6.0E-02	6.0E-01	5.6E-06	5.6E-03	3.8E-02	9.4E-06
Σ Endosulfan (a, b, sulfate)	6.2E-02	5.0E-02	7.5E-04	3.6E-01	1.2E+00	1.5E-05	2.6E-03	2.1E-02	1.3E-05
Endrin	1.6E-02	5.0E-02	7.5E-04	1.2E-02	1.8E-02	4.1E-06	2.1E-02	5.5E-03	2.3E-04
Fenamiphos	2.8E-02	5.0E-03	7.5E-05	3.0E-02	1.5E+00	6.9E-06	1.4E-02	9.2E-02	4.6E-06
Fenitrothion	2.4E-02	1.0E-02	1.5E-04	3.0E-01	7.8E-01	5.9E-06	1.2E-03	4.0E-02	7.6E-06
Fonofos	8.0E-03	1.0E-02	1.5E-04	1.2E-01	nf	2.0E-06	9.9E-04	1.3E-02	-
HCB	4.7E-03	1.0E-02	1.5E-04	3.6E-02	4.8E-02	1.2E-06	1.9E-03	7.8E-03	2.4E-05
HCCP	6.2E-02	1.0E-02	1.5E-04	nf	3.6E-01	1.6E-05	-	1.0E-01	4.3E-05
ΣH, HE	2.1E-02	4.0E-03	6.0E-05	6.0E-03	nf	5.3E-06	5.3E-02	8.8E-02	-
Lindane	6.0E-03	1.0E-03	1.5E-05	4.8E-01	1.8E-02	1.5E-06	1.9E-04	1.0E-01	8.3E-05
Malathion	2.0E-02	2.0E-02	3.0E-04	1.2E+00	1.2E+00	5.0E-06	2.5E-04	1.7E-02	4.2E-06

(*continued*)

	MEC	MRL	TMDI	ADI	ARfD	EADI	EADI/ADI	EADI/TMDI	HQ (ARfD)
Metamidophos	2.4E-02	1.0E-02	1.5E-04	6.0E-02	1.8E-01	6.0E-06	6.0E-03	4.0E-02	3.4E-05
Methoxychlor	9.5E-03	1.0E-02	1.5E-04	6.0E+00	3.0E-01	2.4E-06	2.4E-05	1.6E-02	7.9E-06
Metolachlor	5.1E-03	1.0E-02	1.5E-04	6.0E+00	9.0E+00	1.3E-06	1.3E-05	8.5E-03	1.4E-07
Metribuzin	2.4E-02	1.0E-01	1.5E-03	7.8E-01	1.2E+00	5.9E-06	4.6E-04	4.0E-03	4.9E-06
Mirex	1.2E-02	1.0E-02	1.5E-04	nf	1.2E-02	3.0E-06	-	2.0E-02	2.5E-04
Parathion-ethyl	8.6E-03	5.0E-02	7.5E-04	3.6E-02	3.0E-01	2.2E-06	3.6E-03	2.9E-03	7.2E-06
Parathion-methyl	1.9E-02	1.0E-02	1.5E-04	1.8E-01	1.8E+00	4.7E-06	1.6E-03	3.1E-02	2.6E-06
Pendimethalin	1.7E-02	1.0E-02	1.5E-04	7.5E+00	2.4E+00	4.3E-06	3.4E-05	2.9E-02	1.8E-06
Phosmet	4.1E-02	1.0E-01	1.5E-03	6.0E-01	2.7E+00	1.0E-05	1.0E-03	6.9E-03	3.8E-06
Pirimicarb	4.2E-02	5.0E-02	7.5E-04	2.1E+00	6.0E+00	1.0E-05	3.0E-04	1.4E-02	1.7E-06
Propazine	1.4E-02	1.0E-02	1.5E-04	1.2E+00	1.0E+00	3.5E-06	1.7E-04	2.3E-02	3.4E-06
Propyzamide	1.1E-02	1.0E-02	1.5E-04	1.2E+00	4.5E+00	2.8E-06	1.4E-04	1.9E-02	6.3E-07
Simazine	2.1E-02	1.0E-02	1.5E-04	3.0E-01	3.0E-01	5.4E-06	1.1E-03	3.6E-02	1.8E-05
Simetryn	1.2E-02	1.0E-02	1.5E-04	1.5E+00	nf	3.1E-06	1.2E-04	2.1E-02	-
Tebuconazole	1.2E-02	5.0E-02	7.5E-04	1.8E+00	1.8E+00	2.9E-06	9.8E-05	3.9E-03	1.6E-06
Terbuthylazine	2.4E-02	1.0E-02	1.5E-04	2.4E-01	4.8E-01	5.9E-06	1.5E-03	3.9E-02	1.2E-05
Terbutryn	9.3E-03	1.0E-02	1.5E-04	6.0E+00	6.0E-02	2.3E-06	2.3E-05	1.6E-02	3.9E-05
Tetrachlorvinphos	4.6E-02	1.0E-02	1.5E-04	3.0E+00	1.8E+00	1.1E-05	2.3E-04	7.6E-02	6.3E-06
Trifluralin	6.6E-03	1.0E-02	1.5E-04	9.0E-01	9.0E-01	1.6E-06	1.1E-04	1.1E-02	1.8E-06

MRL (mg/kg): maximum residue levels, **MEC** (mg/kg): average measured environmental concentrations, **TMDI** (mg/kg): theoretical maximum daily intake, **ADI** (mg/kg bw/day): acceptable daily intake for 60 kg of bw, **ARfD** (mg/kg bw/day): acute reference dose for 60 kg of bw, **EADI** (mg/kg bw): estimated average daily intake for 60 kg of bw, **HQ**: hazard quotient, **nf**: information not found, **ΣEndosulfan (a, b, sulfate)**: sum of endosulfan alpha, beta and sulfate residues, **ΣH, HE:** sum of heptachlor and heptachlor epoxide residues

Table SM4- Comparison between the annual average and maximum concentrations found (DAP + SPM fractions), at Tagus River Estuary, with AA-EQS and MAC-EQS amounts established by the Directive 2013/39/EU.

Pesticides	**2013/39/EU**		**∑ DAP,SPM**	
	AA-EQS	**MAC-EQS**	**average**	**max**
Alachlor	300	700	1028.5	1153.1
Atrazine	600	2000		
Chlorfenvinphos	100	300		
Chlorpyriphos	30	100	96.2	
∑aldrin, dieldrin, endrin	5	na	62.8	-
4,4´-DDT	10	-	188.3	-
Endosulfan (alpha + beta)	0.5	4	273.4	1002.4
HCB	-	50	-	
PeCB	0.7	-	10.3	-
Simazine	1000	4000		
Trifluralin	30	-	nf	-
Cypermethrin	0.008	0.06	nf	nf
H+He	0.00002	0.03	17.6	29.0
Terbutryn	6.5	34	363.4	530.0
Dichlorvos	0.06	0.07	nf	nf

AA-EQS (ng/L): annual average-environmental quality standards, **MAC-EQS** (ng/L): maximum allowable concentrations-environmental quality standards, **DAP** (ng/L): dissolved aqueous phase, **SPM** (ng/L): suspended particulate matter, **nf**: not found, **na**: not applicable

Chapter 8

A mollusk VDR/PXR/CAR-like (NR1J) nuclear receptor provides insight into ancient detoxification mechanisms

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A mollusk VDR/PXR/CAR-like (NR1J) nuclear receptor provides insight into ancient detoxification mechanisms

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Abstract

The origin and diversification of the metazoan endocrine systems represents a fundamental research issue in biology. Nuclear receptors are critical components of these systems. A particular group named VDR/PXR/CAR (NR1I/J) is central in the mediation of detoxification responses. While orthologues have been thoroughly characterized in vertebrates, a sparse representation is currently available for invertebrates. Here, we provide the first isolation and characterization of a lophotrochozoan protostome VDR/PXR/CAR nuclear receptor (NR1J), in the estuarine bivalve the peppery furrow shell (*Scrobicularia plana*). Using a reporter gene assay, we evaluated the xenobiotic receptor plasticity comparing the human PXR with the *S. plana* NR1Jβ. Our results show that the molluscan receptor responds to a natural toxin (okadaic acid) in a similar fashion to that reported for other invertebrates. In contrast, the pesticide esfenvalerate displayed a unique response, since it down regulated transactivation at higher concentrations, while for triclosan no response was observed. Additionally, we uncovered lineage specific gene duplications and gene loss in this NR gene group in protostomes with likely impacts on the complexity of detoxification mechanisms across different phyla. Our findings pave the way for the development of multi-specific sensor tools to screen xenobiotic compounds acting *via* of the NR1I/J group.

1. Introduction

Metazoans have evolved specific molecular pathways to metabolize and eliminate toxic compounds, both from endogenous or exogenous origins, the so-called "*defensome*" (Goldstone et al., 2006). Their activation provides protection against environmental insults and involves a series of catalytic steps from biotransformation and oxidation to excretion (Bainy et al., 2013). Among the defensome's regulating components, the Nuclear Receptors (NRs) are key since they control a variety of enzymes such as cytochrome P450 (CYP) (*e.g.* CYP2, CYP3, CYP4) implicated in the metabolism and excretion of toxic substances (Zanger and Schwab, 2013). NRs are transcription factors, commonly triggered by ligand binding, that selectively modulate transcription upon recognition of specific DNA responsive elements. Specific ligands include an array of small lipophilic molecules from endogenous or exogenous sources (Nakata et al., 2006). A set of kindred vertebrate NRs has been linked to xenobiotic "*sensing*" (Creusot et al., 2010; Kojima et al., 2011); they comprise members of the NR1I/J gene group (Nuclear Receptors Nomenclature Committee, 1999). Documented ligands of these receptors include endogenously produced molecules (such as steroid hormones, secondary bile acids - including lithocholic acid (LCA) - and dietary fat-soluble vitamins, like vitamin D), along with exogenous compounds, such as xenobiotics (Kojima et al., 2011).

Given that ligand binding typically occurs at rather low concentrations, NRs are prime targets of endocrine disrupting chemicals (EDCs). In fact, the best characterized examples of endocrine disruption, such as feminization of fish (through estrogen receptors) and imposex induction in gastropods (through retinoid X receptor) involve disruption of NR signaling (Santos et al., 2009). VDR/PXR/CAR orthologues have been recognized and characterized in a variety of vertebrate species (Whitfield et al., 2003; Zhao et al., 2015). The Vitamin D receptor (VDR) displays a high affinity mostly to vitamin D (Krasowski et al., 2011), while the Pregnane X receptor (PXR) is a ligand-dependent nuclear receptor activated by a significant number of

compounds with a diverse chemical structure (Kojima et al., 2011). Human PXR studies demonstrate that opposite responses (agonistic and antagonistic) are possible for the same class of compounds like pesticides, polychlorinated biphenyl compounds (PCBs), drugs and others (Jacobs et al., 2005; Kojima et al., 2011). The constitutive androstane receptor (CAR) is also able to bind a broad range of ligands (*e.g.* androstane steroids) partially overlapping with PXR (Krasowski et al., 2011). Some studies have also detailed the diversity and functional features of the NR1I/J gene family in invertebrate phyla. The tunicate *Ciona intestinalis* genome encodes at least 2 VDR/PXR/CAR-like genes, denoted as CiVDR/PXRα and CiVDR/PXRβ (Satou and Satoh, 2003). The CiVDR/PXRα activates transcription in the presence of synthetic compounds such as the pesticide esfenvalerate and natural toxins, such as okadaic acid (OA) and pectenotoxin-2 (Fidler et al., 2012). In the protostome ecdysozoans *Drosophila melanogaster* and *Daphnia pulex* an NR1J orthologue, also named hormone receptor-like 96 (HR96), was also found to be involved in the metabolism of xenobiotics, including pesticides (Karimullina et al., 2012; King-Jones et al., 2006; Thomson et al., 2009).

Biased taxonomic sampling can hamper our ability to understand the molecular specificity of NRs from different species as well as our comprehension of NR-based disruption mechanisms (Castro and Santos, 2014). Metazoan protostome phyla, such as mollusks and annelids (lophotrochozoans), remain uncharacterized with respect to the NR1J gene family (Castro and Santos, 2014; Richter and Fidler, 2014). Mollusks in particular, are fundamental to ecosystem functions and represent the second largest metazoan phylum. Importantly, many are filter-feeders, sedentary or slow-moving species, displaying optimal features to be used as environmental sentinel species (Uno et al., 2001). As a consequence, they are exposed to a wide variety of natural and man-made compounds. Due to their ecological niche locations, invertebrates such as bivalves, are affected/contaminated by diverse persistent compounds, like the organochlorine (OCs) and organophosphorous (OPPs) pesticides resultant from local agriculture activity and run-off of contaminated waters.

The usage of pesticides has increased in the last few decades and with it, the awareness of their harmful effects. Several studies reported on neurotoxicity, endocrine disruption and immunotoxicity from mammals to fish and insects, demonstrating the impact on populations affecting the whole ecosystem (Androutsopoulos et al., 2013; Köhler and Triebskorn, 2013).

Here we provide the first isolation and characterization of a lophotrochozoan protostome VDR/PXR/CAR-like nuclear receptor (NR1J), which we name SplNR1Jβ, in the estuarine bivalve peppery furrow shell (*Scrobicularia plana*), in order to assess the response to a natural toxin (okadaic acid) and pesticides (esfenvalerate and triclosan) at the receptor level. Our findings further provide a conceptual framework to understand xenobiotic responses *via* the NR1I/J group throughout bilaterians.

2. Materials and methods

2.1 Data mining and phylogenetic analysis

Sequences were identified in the National Center for Biotechnology Information (NCBI) database for the following species: *Homo sapiens* (human), *Crassostrea gigas* (pacific oyster), *C. intestinalis* (sea squirt), *D. melanogaster* (fruit fly), *Musca domestica* (fly), *Saccoglossus kowalevskii* (acorn worm), *Apis mellifera* (honey-bee), *D. pulex* (water flea), *Tigriopus japonicas* (copepod) and *Ixodes scapularis* (deer tick). To identify non-annotated genes, Blastp searches were performed at JGI, OMGU, METAZOME and BG genomic databases for *Capitella teleta* (sea worm), *Pinctada fucata* (pearl oyster), *Aplysia californica* (sea hare), *Lottia gigantea* (owl limpet) and *Biomphalaria glabrata* (freshwater snail), while the *Crangon crangon* (brown shrimp) was obtained from Christiaens et al. (2015). The accession numbers are listed in supplementary material Table SI2. The thirty complete sequences—containing the DBD, hinge region and LBD—were aligned (MAFFT, version 7) and were used for subsequent phylogenetic analyses. Sequence blocks, corresponding to the DBD and LBD, were aligned, checked and manually curated to remove the variable hinge region and the

amino- and carboxy-termini (BioEdit, version 7.1.11) (Hall, 1999). The final sequence alignment composed by 30 sequences with 580 amino acids with partial gap positions was submitted to ProtTest 2.4 server to derive the evolutionary model (LG+I+G) (Darriba et al., 2011). Phylogenetic analysis was performed on the online platform PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) and aBayes algorithm was selected to calculate branch support (Guindon et al., 2010).

The final tree was visualized with TreeViewer (version 1.4.2) software (available at http://tree.bio.ed.ac.uk/software/figtree/) and the *C. intestinalis* (EcR-like and FXR-like), *D. melanogaster* (EcR) and *L. gigantea* (EcR_like) sequences were used to root the tree. Additionally, several amino acid sequences were aligned, where *S. plana* was used as the reference sequence, to calculate the percentage of identity using the Sequence Identity And Similarity (SIAS) (Reche, 2008), where the gaps were taken into account and the BLOSUM62 matrix was used to test for global similarity.

2.2 Sampling

S. plana is an estuarine bivalve with a wide geographical distribution (from Norwegian Sea to Senegal including the Mediterranean Sea) (Hughes, 1970) that has also a direct importance for humans, since they are commercially exploited in the Iberian Peninsula (Langston et al., 2007). The animals were collected at Mondego River estuary and the tissues were extracted as it is described in document SI1.

2.3 *S. plana* (SplNR1Jβ) and *H. sapiens* (HsaPXR) gene isolation and cloning into pBIND

Initial gene isolation of *S. plana* gene was achieved by PCR using a set of degenerate primers designed based on two sequences of *L. gigantea* (Bridgham et al., 2010) and one of *C. intestinalis* (Fidler et al., 2012), using a multi-sequence alignment with the CODEHOP software (Rose et al., 1998). Both sequences were classified as part of NR1I/J clade. The amplification product was then purified and further cloned into pGEM-T easy vec-

tor. Rapid Amplification of cDNA Ends (RACE) and PCR reactions were performed according to manufacturer's recommendations and the final products were cloned and sequenced (document SI1 for full detailed description). Gene isolation of *H. sapiens* PXR was achieved using total RNA from liver, obtained from FirstChoice Human Total RNA Survey Panel (Ambion), where cDNA synthesis and full ORF gene amplification followed the same protocol as described above. Full-length of ligand binding domain (LBD) of *S. plana* (AA102-360; KP995063; SplNR1Jβ) and human (AA120-434; NP_003880.3; HsaPXR) were isolated using specific primers incorporated with BamHI and KpnI restriction sites and cloned into the pBIND vector (accession number AF264722), which encodes the yeast GAL4 DBD and expresses the *Renilla reniformis* luciferase under the control of the SV40 promoter. The constructs pBIND-splNR1J and pBIND-HsaPXR, were sent to sequence to ensure integrity and orientation of each sequence in the expression vector (document SI1 for primer and PCR details).

2.4 Cell culture and transactivation assays

Host COS-1 cells were plated in 24-well plate (Orange Scientific) at a density of 2 x 10^5 live cells/well, 1 day before transfection. Transfections were performed using Lipofectamine® 2000 reagent (Invitrogen), 1 µg of pGL4.31 reporter vector (accession number DQ487213) and 0.5 µg of pBIND-SplNR1J or pBIND-HsaPXR and according to manufacturer's recommendations. After 5 h, the transfection medium was removed and cells were exposed to various concentrations of the test compounds (OA, esfenvalerate and triclosan) or to 0.1% DMSO (solvent control) in DMEM without phenol red, containing 10% FBS charcoal stripped and 5% Penicillin/Streptomycin. No toxic effects (cell mortality/viability) were observed for 0.1% DMSO. The test concentrations ranged from 5 nM to 200 nM for the OA, from 100 nM to 100,000 nM for the esfenvalerate, and from 5,000 to 50,000 nM for triclosan. After 24h, the cells were harvested and luciferase activity was quantified using the Promega dual-luciferase reporter assay system and the Synergy HT microplate reader (BioTek), according to manufacturer's recommendations.

2.5 Data analysis and statistics

The concentration-response curves, expressed as fold-induction, were derived for chemicals, using the ratio between luciferase (reporter) and *Renilla* (transfection efficiency control) luminescent activity and then normalized, by dividing by the solvent control (Schagat T. et al., 2007). The results were presented as average of the normalized values (n=6) and the bars, the standard deviations (SD). The Mann–Whitney pairwise ANOVA test (one-way ANOVA on ranks) was used for the comparison of data among concentrations. Sequential Bonferroni significance p-value corrections were done *a posteriori*. A p-value less than 0.05 was chosen as the significant level. The cited analyses were done with the software PAST (Hammer et al., 2001). The half maximal effective concentration (EC_{50}) value and the correlation coefficients (r^2) were determined from the sigmoidal concentration-response curves generated by SigmaPlot 11.

3. Results and discussion

3.1 An orthologue of the NR1J gene family in *S. plana*

We isolated the near-full ORF of a gene encoding a VDR/PXR/CAR-like orthologue from *S. plana* (GenBank accession number KP995063), which we name SplNR1Jβ (see below for details). The predicted protein sequence is characterized by the two typical domains present in NR: the DBD and the LBD (Figure SI1). Additionally, the *S. plana* sequence also displays the well-conserved DBD motif (**ESCKAFFR**), characteristic of the NR1J members (Thomson et al., 2009; Vogeler et al., 2014).

We next analysed the identity levels across DBD and LBD of the *S. plana* sequence compared to a phylogenetic range of NR1I/J sequences, from deuterostome and protostome origin. The DBD from *S. plana* shares between 37% and 87% identity with the selected sequences, with the highest values registered with other NR1J-like sequences from mollusc (Table 1). The identity scores for LBD are slightly lower, ranging from 38% to 84%

(Table 1). As expected, the peppery furrow shell NR1J sequence shares a higher degree of identify with other mollusc sequences, but also with those of annelids and arthropods.

Table 1. Amino acid comparisons of the DBD and LBD from NR1I/J species, using Spl_β as the reference model.

Species	Code	DBD	LBD
S. plana	Spl_β	100.0	100.0
C. gigas	Cgi_β	87.4	82.9
L. gigantea	Lgi_β	81.6	83.8
C. teleta	Cte_β	78.2	79.6
D. pulex	Dpu_HR96	55.2	64.8
L. gigantea	Lgi_α	44.8	64.8
C. gigas	Cgi_α	46.0	63.7
L. gigantea	Lgi_γ	47.1	60.2
C. teleta	Cte_γ	49.4	63.3
C. gigas	Cgi_σ	46.0	50.2
L. gigantea	Lgi_σ	44.8	37.9
C. teleta	Cte_σ	44.8	64.2
H. sapiens	Hsa_VDR	40.2	46.9
H. sapiens	Hsa_PXR	36.8	47.9
H. sapiens	Hsa_CAR	36.8	55.2
C. intestinalis	Cin_α	37.9	46.9
C. intestinalis	Cin_β	42.5	51.2

We next undertook a phylogenetic analysis to address the orthology of the reported sequence. The isolated *S. plana* NR robustly groups within a clade containing other NR1J protostome sequences (Figure 1), and out-grouping the chordate NR1I clade. We were unable to detect NR1I/J-like gene sequences in cephalochordates and echinoderms as previously proposed (Lecroisey et al., 2012). A partial sequence with similarity to VDR/PXR/CAR was also identified in the hemichordate *S. kowalevskii* (XP_002736417) but this was not included in the analysis. The tunicate *C. intestinalis* has two NR1I sequences that group together branching-off the vertebrate clade, thus indicating that they represent lineage specific duplicates (Figure 1). It has recently been noted that molluscs contain between three and four NR1J-like genes in their genome, in contrast to the single copy found in ecdysozoans such as *Drosophila* and *Daphnia* (Kaur et al., 2015; Vogeler et al., 2014). We expanded this analysis by mining the genome of additional species, include-ing the annelid worm, *C. teleta*. We confirm the presence of 4 sequences in other mollusc species (Kaur et al.,

2015; Vogeler et al., 2014), one of which is orthologous of the arthropod HR96 (Figure 1). Interestingly, our findings also indicate that three of the NR1J gene lineages previously identified exclusively in molluscs are also present in annelids (Figure 1). Thus, the previously reported NR1J diversity in molluscs does not correspond to a lineage specific duplication event. The topology and branching pattern of our phylogenetic tree indicates that the NR1J gene family probably diversified early in Protostome evolution to generate 4 distinct gene lineages —α, δ, γ, and β— which includes the *S. plana* sequence we now report (Figure 1). Importantly, all these NR1J lineages have been retained in molluscs and up to a certain point in annelids, with the secondary loss of three lineages in ecdysozoans.

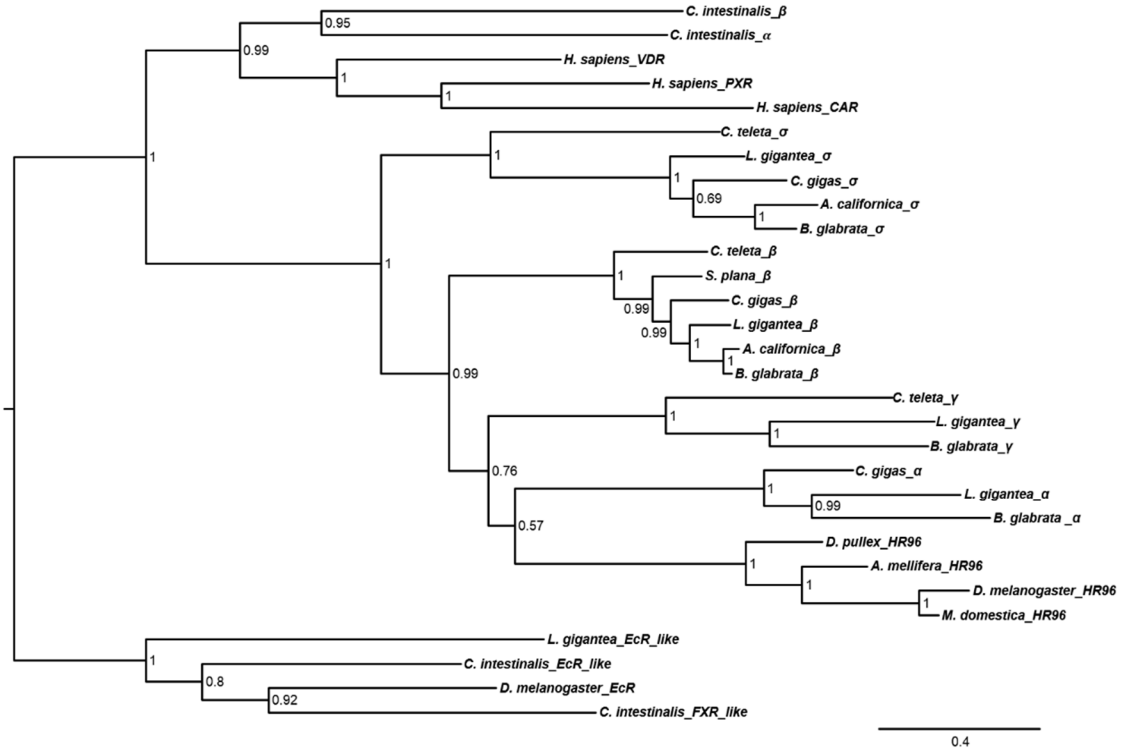

Figure 1. Maximum likelihood phylogenetic tree describing relationships among NR1I/J vertebrates and invertebrates. Node values represent branch support using the aBayes algorithm. Full details of tree construction are given in the material and methods Section 2. Accession numbers for all sequences are provided in the supporting information (Table SI2).

3.2 A natural toxin activates transcription *via* SplNR1Jβ

We next assayed the ability of SplNR1Jβ to activate transcription in the presence of described ligands of this NR gene subfamily. We began by testing a natural toxin from dinoflagellates, okadaic acid (OA), which has been suggested to activate transcription in a ligand-dependent manner in the tunicate *C. intestinalis* (Fidler et al., 2012). Marine algae toxins are responsible for more than 60,000 seafood intoxications every year (Van Dolah, 2000). Since OA is a potent protein phosphatases inhibitor, this effect may be linked to diarrhoea, degenerative changes in absorptive epithelium of the small intestine, and tumor promotion effects (Dominguez et al., 2010). We used the human PXR orthologue (HsaPXR) as positive control. A significant 15-fold induction was observed in HsaPXR at 200 nM (Figure 2A). Regarding the SplNR1Jβ, we observed that OA was able to activate transcription at concentrations between 10 nM and 200 nM, similar to the positive control (Figure 2B). Given that no plateau was reached, it was not possible to accurately compute a complete concentration-response curve to estimate the EC_{50} concentrations. Higher concentrations were not tested, since cell toxicity has been documented above 200 nM (Fidler et al., 2012). Despite similar transactivation responses, our assays yielded overall lower inductions than previously reported for the positive control (Fidler et al., 2012).

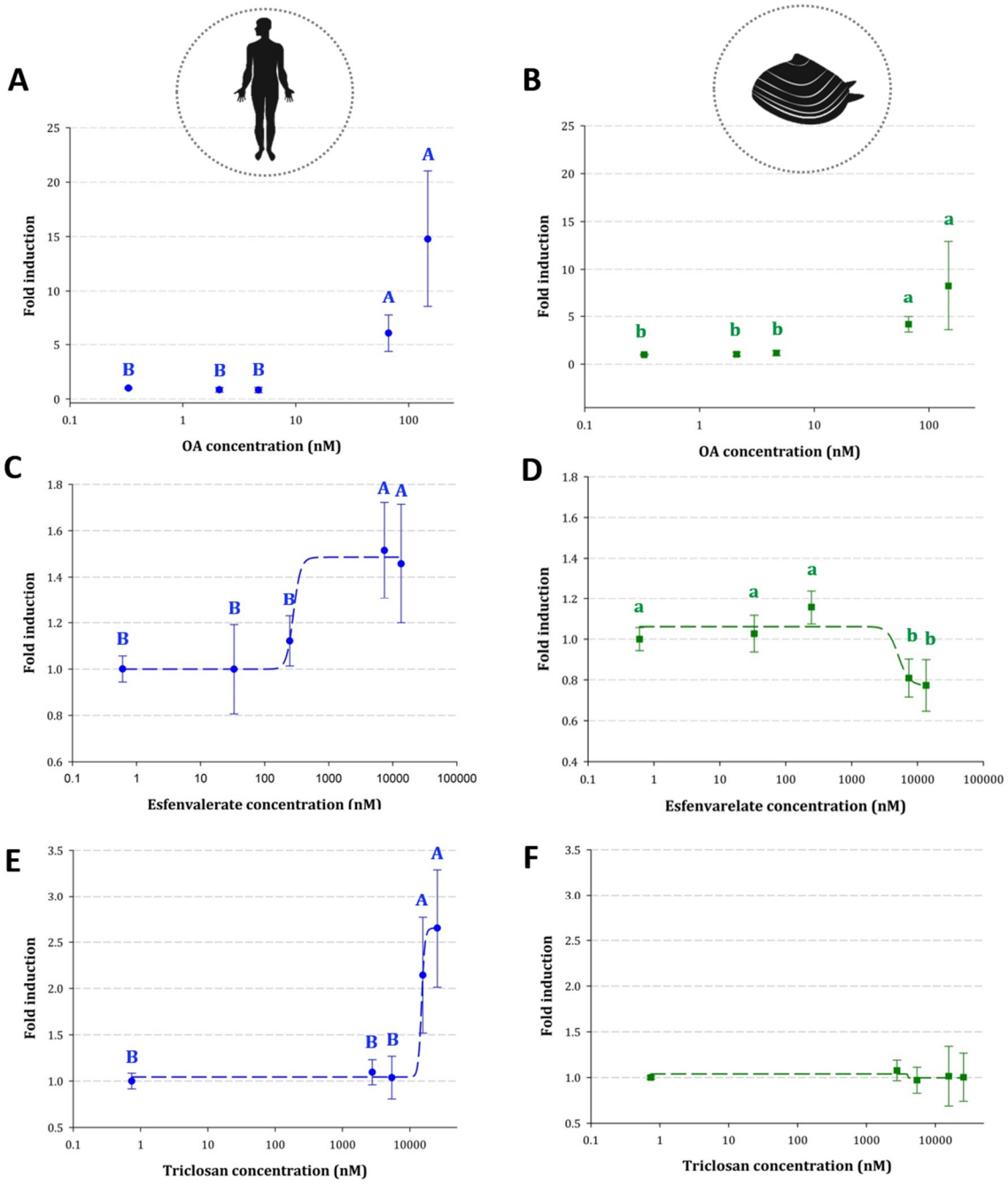

Figure 2. Marine toxin and pesticides concentration-responses curves of luciferase expression by COS-1 cells transfected with GAL4-pBIND-SplVDR/PXR LBD and GAL4-pBIND-HsaPXR LBD fusion genes. The HsaLBD (represented by the letters A, C and E) and the SplLBD (represented by the letters B, D and F) assays treated with OA, esfenvalerate and triclosan, respectively. (Data are represented as the average fold activation normalized to the solvent control group (n = 6) and the bars SD; superscript letters represent significant statistical differences ($p < 0.05$)).

Besides its putative binding affinity toward PXR, OA is also a potent inhibitor of protein phosphatases 1 and 2A, from a family of serine/threonine phosphatases (Bianchini et al., 1991). PXR phosphorylation by protein kinase C was shown to repress transcription in the presence of a steroid ligand, suggesting increased affinity toward co-repressors, and not co-activators (Ding and Staudinger, 2005). Further treatment with OA, leading to phosphatase inhibition and increased phosphorylation, abolished PXR activity in human and rat (Ding and Staudinger, 2005); however this phenomenon is not observed in mouse PXR, suggesting a species-specific profile for the latter (Staudinger et al., 2011). Yet controversial results with OA are increasing, whereas the previous observation, for human and rat PXR, is in sharp contrast with ours and with the previously reported (Fidler et al., 2012), results for human PXR, which displayed OA-induced activation rather than repression. Regardless of the exact regulatory pathway, our results propose a similar mechanism for both human and mollusc receptors, suggesting that the regulatory networks and co-factor interactions might be conserved.

3.3 Two pesticides impact differently the transcription *via* SplNR1Jβ

We then investigated a different category of exogenous ligands, pesticides, which are known activators and repressors of NR1I/J members (Kojima et al., 2011; Richter and Fidler, 2014). Based on previous results published for the tunicate *C. intestinallis* (NR1Jα) (Fidler et al., 2012) and the crustacean *D. pulex* (HR96) (Karimullina et al., 2012), two different pesticides (esfenvalerate and triclosan) were selected. Esfenvalerate is a pyrethroid insecticide, classified by PAN Pesticides Database (PPDB) as highly toxic compound, which presents noxious effects in a wide range of aquatic organisms (from zooplankton to molluscs and fish) (University of Hertfordshire, 2006–2013). Due to its chemical characteristics, is capable of causing toxicity at lower concentrations (100-6,500 ng/L) in species, such as *Oncorhynchus mykiss* and *Daphnia magna* (Lozano et al., 1992). Other studies revealed negative effects on reproduction and larval devel-

opment in bluegill sunfish, amphipod crustacean, harlequin fly, salmon, and rainbow fish (Tanner and Knuth, 1996; *Cold and Forbes*, 2004; Barry et al., 1995; Forbes and Cold, 2005; Moore and Waring, 2001).

Triclosan is an antifungal and antibacterial compound, widely used in several hygienic products in human daily routine (Jacobs et al., 2005). As a highly hydrophobic compound, it can be easily accumulated in fatty tissues; this fact, allied with a constant output to aquatic environments may cause adverse effects on biota (mainly at concentrations above 100 ng/L, defined as long-term predicted no effect concentration) (EPA, 2008; Water Framework Directive-United Kingdom Technical Advisory, 2009). Wide concentrations, were detected worldwide (WWTPs and surface waters), ranging from 23 to 434 ng/L in Australia, until 944 ng/L and 57600 ng/L in India and China, respectively (Ying and Kookana, 2007; Ramaswamy et al., 2011; Zhang et al., 2013). The ubiquitous and persistent existence of triclosan can be detected from lower trophic-level organisms (algae, freshwater snails, and fish) to higher aquatic mammals (dolphins and killer whales) (Coogan et al., 2007; Dann and Hontela, 2011; Adolfsson-Erici et al., 2002; Fair et al., 2009; Dann and Hontela, 2011). It is described as an endocrine disruptor compound (Wang and Tian, 2015), affecting mainly the pregnancy and lactating period (Manservisi et al., 2015); the plasma testosterone and vitellogenin levels in frogs (Matsumura et al., 2005) and clams (Matozzo et al., 2012) are also affected, as well as the modulation of the thyroid hormone metabolism in rats (Crofton et al., 2007) and frogs (Veldhoen et al., 2006).

Due to the "*hate water, love fat*" properties, both pesticides are highly attracted to lipophilic surfaces and thereby, they can easily be incorporated in the filter feeders' diet (Perron et al., 2012). Therefore, the differential ligand potency and efficacy was tested at a concentration range of 0–100,000 nM (for esfenvalerate) and 0–10,000 nM (for triclosan) using *S. plana* NR (SplNR1Jβ**)** and the human PXR (Figure 2C–F). Considering the luciferase activity, significant differences ($p < 0.05$) were obtained above 1000 nM of esfenvalerate and 10,000 nM of triclosan (only for HsaPXR), showing a concentration-dependent luciferase activity.

When exposed to esfenvalerate, the HsaPXR presented a 0.5-fold activation in the two highest concentrations, when compared to the solvent control group (Figure 2C). The opposite was verified for SplNR1Jβ exhibiting a low, yet significant suppression (1.5-fold; Figure 2D). No toxicity was observed since the *renilla* amounts were similar between the control and exposed groups, therefore we speculate that esfenvalerate may behave as an inverse agonist at higher concentrations. Interestingly, the majority of residues involved in ligand binding in human PXR are conserved or replaced by residues with similar biochemical properties in *S. plana* (Figure SI2). Thus, additional experiments will be necessary to confirm this behaviour, such as site directed mutagenesis. Bivalves are described also as relatively insensitive to pyrethroids, being able to bioaccumulate large amounts of these compounds, which are subsequently available for the predators (Werner et al., 2002). This fact may be related to the significant suppression observed herein. The calculated EC_{50} was ~28-fold higher for the human receptor than the bivalve orthologue (Table 2). When comparing with the published data (Fidler et al., 2012), similar EC_{50} values were obtained for human (1,500 nM) and 56-fold lower for tunicate (590 nM), when exposed to the same range of concentrations (0-100,000 nM).

Table 2. EC_{50} and confidence interval (CI) of both chemicals. (DNC: did not compute; EC_{50}: concentration of chemicals that increased/decreased, to one-half of maximum (activators/repressors) activity).

Chemical	CAS number	Organism	EC_{50} (nM)	r^2	Direction
Okadaic acid	78111-17-8	*H. sapiens*	114	DNC	Activator
		S. plana	120	DNC	Activator
Esfenvalerate	66230-04-4	*H. sapiens*	1172	0.993	Activator
		S. plana	33037	0.862	Inverse activator
Triclosan	3380-34-5	*H. sapiens*	28708	0.998	Activator
		S. plana	7244	DNC	No effect

Considering the triclosan exposure, a 2.5-fold induction was observed for the HsaPXR, corresponding to an EC_{50} of 29,000 nM (Figure 2E). Similar fold inductions and EC_{50} values were already published (Jacobs et al., 2005;

Paul et al., 2013) for the same range of concentrations (0–50,000 nM). Regarding SplNR1Jβ, no significant differences were observed in comparison with the control treatment. Our findings in the peppery furrow are in sharp contrast to those reported for the HR96 orthologue from *Daphnia* (Karimullina et al., 2012), yet, the isolated molluscan isoform is most likely a paralogue of NR1Jβ. In *Daphnia* a decrease of the concentration-response was visible for the highest concentrations (10,000–100,000 nM) when compared to the control group (Karimullina et al., 2012). Moreover, these results were expressed in luminescent units (luciferase activity) and therefore not normalized, bringing variation (such as, suboptimal transfection, cytotoxic effects and number of cells) into the results. Additionally, the interactive Chemical Safety for Sustainability (iCSS) dashboard from Environmental Protection Agency (EPA) (Environmental Protection Agency (EPA), 2015) was used to compare their high-throughput chemical screening data with our results. Considering the HsaPXR for the esfenvalerate and triclosan, the database results presented similar EC_{50} (4,560 and 13,900 nM) when compared to ours (1,172 and 28,708 nM), evidencing the reproducibility of this type of chemical screening assay.

3.4 Conserved and derived features of detoxification mechanisms

Animal homeostasis is dependent on the ability to deal with environmental stressors. Physiological systems have evolved a variety of molecular sensors and downstream effector pathways (Phase I, II and III) to engage in detoxification responses. However, it is expected that nuclear receptors (NRs) induce or down-regulate enzymes and transporters (target genes), which are critically involved in the detoxification pathway and are capable to alter normal homeostasis (Nakata et al. 2006). These NRs play important roles in the chemical stress response, by binding to a wide range of structurally unrelated ligands, such as xenobiotics, steroids and natural ligands (di Masi et al., 2009). Yet, distinct activation patterns can be observed between orthologues. For instance, while the human PXR is induced (nM range; EC_{50} 2.3 nM) by hyperforin, the active component of the herbal

antidepressant St. John's wort, the mouse PXR yields an opposite effect (Chang, 2009). Several molecules, classified as endocrine disrupting chemicals (EDCs) and/or endocrine acting chemicals (EACs), are anthropogenic or natural metabolic substances, able to mimic antagonize, alter, or modify hormonal activity through PXR and CAR activation (Kretschmer and Baldwin, 2005; Timsit and Negishi, 2007). Pesticides like alachlor, aldrin, chlordane, DDT, dieldrin and many others are known for their ability to cause disruption of thyroid hormone levels, anti-estrogen and –androgen effects, potential EDC, and synergetic effects with other EDCs (Kojima et al., 2011; Kretschmer and Baldwin, 2005). PXR is a highly promiscuous receptor known for containing a large flexible ligand binding cavity (Watkins et al., 2003) and low cross-species conservation of the ligand binding domain. Additionally it has been suggested that PXR ligand binding selectivity may be linked to selective pressures and environmental features (Krasowski et al., 2011; Richter and Fidler, 2015). For example it was shown that the exposure of marine tunicate PXR/VDR to fresh water toxins rendered no effect (Richter and Fidler, 2015).

While numerous studies have detailed the biological role, ligand specificity and controlled pathways of PXR and CAR in vertebrate species (Jacobs et al., 2005; Xie et al., 2000), a similar approach is largely missing for invertebrate phyla (Richter and Fidler, 2014). NRs related to this group were initially described in *D. melanogaster* (HR96) (King-Jones et al., 2006) and *Caenorhabditis elegans* (DAF-12 and NRH-8) (Lindblom et al., 2001), although they were classified as a different NR group, NR1J (Nuclear Receptors Nomenclature Committee, 1999). In effect, this dual categorization has been misinterpreted occasionally with the proposal that NR1J group genes are absent in vertebrates (Kaur et al., 2015). However, the inclusion of a wider set of bilaterian sequences indicates that NR1I and NR1J are probable sister clades belonging to the same gene lineage, which underwent distinct evolutionary processes in both invertebrates and vertebrates (Figure 3) (Zhao et al., 2015). In this context, we provide the first characterization of a NR1J gene in lophotrochozoans and propose that an ancestral receptor duplicated to yield a minimum of four NR1J genes in

protostomes, although the vast majority was lost in arthropods and nematodes (Figure 3). Why exactly this expansion took place and was subsequently lost is unclear. Lophotrochozoans typically inhabit marine environments, which are brimming with bioactive compounds such as toxins. This environmental setting might have acted as a selective force to drive the expansion of this NR gene subfamily (Richter and Fidler, 2014). In tunicates, a lineage independent duplication has also taken place, although the similarities and differences of both receptors have not been addressed yet (Figure 3) (Richter and Fidler, 2015). The constant exposure of these marine filter-feeding animals to xenobiotics, present in the water and food, was also suggested to promote an adaptive evolution to resistance and/or metabolic responses to such compounds (Fidler et al., 2012). Paradoxically, NR1J are absent from marine phyla such as cephalochordates and echinoderms (Goldstone et al., 2006).

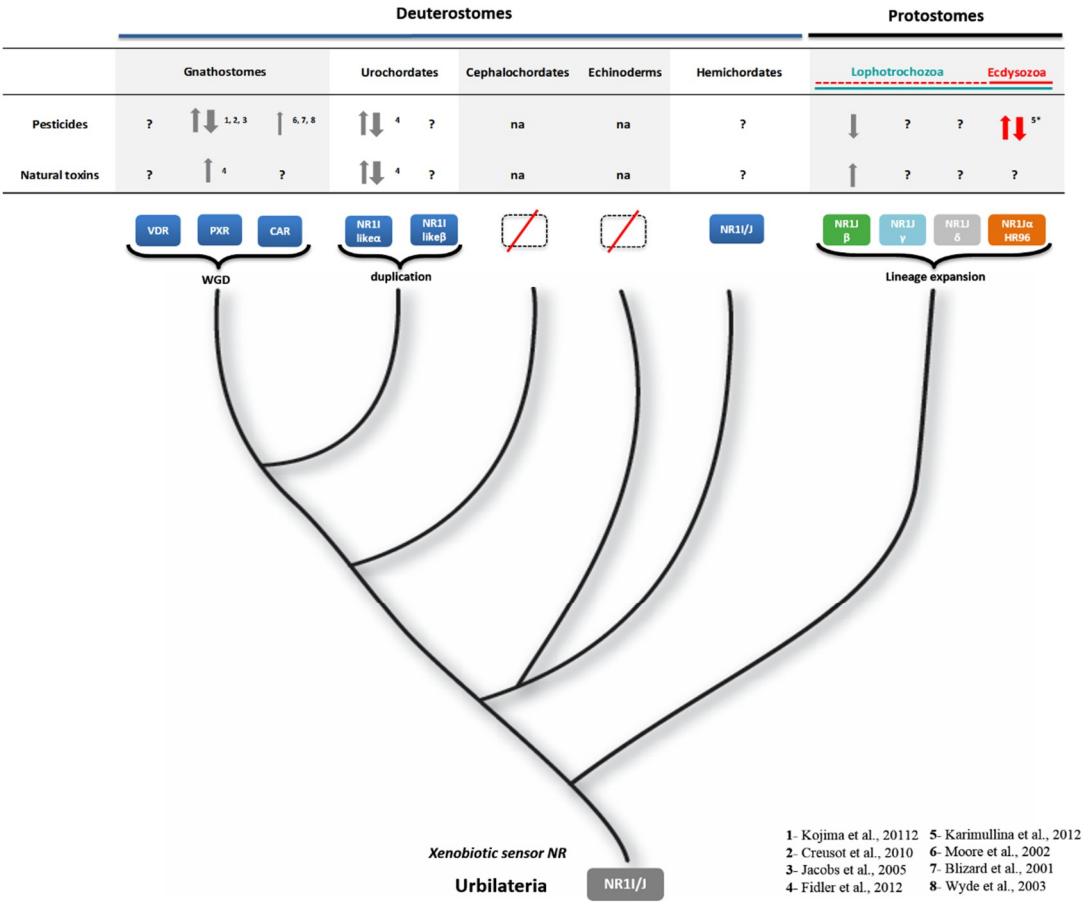

Figure 3. Evolutionary model of NR1I and NR1J in bilaterian phyla together with a table containing the schematics of transactivation responses (Blizard et al., 2001; Creusot et al., 2010; Fidler et al., 2012; Jacobs et al., 2005; Karimullina et al., 2012; Kojima et al., 2011; Moore et al., 2002) (the arrows indicate a positive/negative transactivation response, where the thickness represents roughly the number of compounds capable of causing this effect; *please see discussion; na: not applicable; WGD: whole genome duplication; the red dash line represents gene loss).

The NR repertoire is known to be different between animal lineages both in terms of gene number per *taxon* as well as their precise ligand specificity, a proxy for function (Castro and Santos, 2014). Both these aspects are crucial for the understanding of endocrine systems and their exploitation by xenobiotics. For example, Retinoic Acid receptors (RAR) and Estrogen receptors (ER) albeit absent from ecdysozoans are present in annelids and molluscs with variable ligand specificities (Bridgham et al., 2010; Keay and Thornton, 2009; Thornton et al., 2003). These examples further empha-

size the need to consider an appropriate phylogenetic sampling to recognize NR function in invertebrate phyla (Castro and Santos, 2014).

4. Conclusion

We show that xenobiotic sensing NRs underwent a significant dynamic history in bilateria. Our transactivation studies show that the molluscan NR1Jβ responds to OA similarly to what was previously observed in tunicates and vertebrates, but, in contrast, is apparently repressed by a pesticide. In parallel we identified three other lophotrochozoan NR1J isoforms which future studies should investigate their contribution to detoxification responses. Our study emphasizes the importance of considering a wider taxonomic sampling to address xenobiotic responses at the ecosystem level.

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Supplementary material

Document SI1. Gene cloning and characterization-detailed information.

Sampling

The animals were collected at the Mondego River estuary, during ebb tide, and then maintained in artificial seawater (salinity 30) for 24 hours. Then, animals were drained and left for at least 1 hour in an anaesthetic solution of magnesium chloride ($MgCl_2$) at 60 g/L[35]. After anaesthesia the bivalves were dissected and the kidney, intestine, muscle, gonad, gills and gastric gland were collected and immediately preserved (-80 ºC) in RNA later (R0901, Sigma-Aldrich).

Gene cloning and characterization-detailed information

S. plana softtissues (up to 20 mg of each collected organ) were individually homogenized using a high-performance dispersing T10 basic Ultra-Turrax (Ika) instrument. RNA extraction was performed using illustra RNAspin mini kit (GE Healthcare Life Sciences) and eluted in 30 µl RNase-free water. RNA quantification was performed using a Qubit fluorometer and the Qubit RNA BR assay kit (Q10211; Invitrogen) and RNA integrity was evaluated by loading 1 µL of the RNA in 1% agarose gel. First-strand cDNA synthesis was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad), according to manufacturer's recommendations, using 1 µg of total RNA. Additionally, 5´and 3´ cDNA for Rapid amplification of cDNA ends (RACE) was prepared using RNA pool from the previously sampled tissues, using SMARTer RACE cDNA amplification kit (Clontech) according to manufacturer's guidelines. For an initial isolation of *S. plana* gene, a set of degenerated primers were designed based on two sequences of *Lottia gigantea*[31] and one of *C. intestinalis*[19], using a multi-sequence alignment with the CODEHOP software[32]. PCR was performed with using Phusion® Flash (high-fidelity PCR master mix; Finnzymes), according to manufacturer's guidelines. The amplification reaction was performed with an initial denaturation step of 10 s at 98 ºC, followed by 40 cycles of denaturation at 98 ºC for 1 s, annealing at 53 ºC for 5 s, extension at 72 ºC during 5 s, and a final 60

s extension at 72 ºC during the last cycle. The resulting PCR product was loaded in a 1% agarose gel and separated by electrophoreses at 80 volts. Gel band (≈ 200 bp) was excised and DNA was purified using GRiSP kit according to manufacturer's recommendations. Purified DNA was further cloned into pGEM-T easy vector and transformed into JM109 competent cells, (both from Promega). The plasmid products were sent for sequencing to STABVIDA. After sequence confirmation, gene specific primers for RACE reactions were designed and full-length cDNA sequences were obtained using the polymerase Phusion® Flash and the universal primer SMARTer™ RACE cDNA Amplification (Clontech). The resulting sequence was further used to design gene specific primers for RACE reactions and PCR reactions were performed according to manufacturer's recommendations and the final products were cloned and sent to sequence.

Cell line and cell culture and assay materials

Cell culture reagents, such as Dulbecco's Phosphate-Buffered Saline (DPBS), trypsin 0.25 % in PBS without Ca^{2+} and Mg^{2+}, fetal bovine serum (FBS) and FBS charcoal stripped, penicillin/streptomycin (1:1), Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and L-glutamine and without phenol red and DMEM with 4.5 g/l glucose and L-glutamine and without sodium pyruvate, were purchased from PAN Biotech. The Opti-MEM reduced serum medium was acquired from Gibco Life Technologies and the dimethyl sulfoxide (DMSO; > 99.9 %), from Sigma-Aldrich. The COS-1 simian kidney cells (ATCC number CRL-1650) were cultured in T75 flasks with filter caps (Orange Scientific) using DMEM with phenol red containing 10 % FBS and 5 % Penicillin/Streptomycin, in a 37˚C-5% CO_2 incubator. The esfenvalerate (46277-100 mg) and okadaic acid (O4511-10 µg), were purchased from Sigma-Aldrich, while triclosan (sc-220326) was acquired from Santa Cruz; the compounds were dissolved in DMSO to form a stock solution (1 M, 200 µM and 0.5 M, respectively), with serial dilutions in DMSO in order to obtain a final concentration in the cell culture media of 1 % (v/v).

Transfection and transactivation assays

Full-length of ligand binding domain (LBD) of *S. plana* (SplNR1Jβ) and *H. sapiens* (HsaPXR) were isolated using specific primers incorporated with BamHI and KpnI restriction sites, respectively and cloned into the pBind vector (accession number AF264722). The constructs pBIND-splNR1J or pBIND-hsaPXR, were sent to sequence to ensure integrity and orientation of each sequence in the expression vector.

The host COS-1 cells were plated in 24-well plate (Orange Scientific) at a density of 2 x 10^5 live cells/well, 1 day before transfection. Transfections were performed using Lipofectamine® 2000 reagent (Invitrogen), 1 µg of pGL4.31 reporter vector (accession number DQ487213) and 0.5 µg of pBIND-SplNR1J or pBIND-HsaPXR and according to manufacturer's recommendations. After 5 to 6 hours, the transfection medium was removed and cells were exposed to various concentrations of the test compounds (esfenvalerate and okadaic acid) or to 1 % DMSO (solvent control) in DMEM without phenol red, containing 10 % FBS charcoal stripped and 5 % penicillin/streptomycin. The test concentrations ranged from 100 nM to 100,000 nM for the esfenvalerate, from 5 nM to 200 nM for the okadaic acid and from 5,000 to 50,000 nM for triclosan. After 24 hours, the cells were harvested and luciferase activity was quantified using the Promega dual-luciferase reporter assay system and the Synergy HT microplate reader (BioTek), according to manufactures recommendations.

Table SI1. *S. plana* and *H. sapiens* primers designed to isolate the LBD of each nuclear receptor.

Species	Purpose	Primer F	Primer R	TM (ºC)	Cycles
S. plana	Initial isolation	CAGGGCCATGGGCTACMAYTTYRAYGC	GGTCATGATGCACTCCTTCYKCATNCCDAT	53	40
	5´RACE	UPM	GACGGTTTGAGACGTCCATCTTGC	55	35
	3´RACE	TCTGGCAGTGGATCTGAAAGCTGAC	UPM	65	35
	pBIND cloning	GCTGCTGGATCCTAATGGAGCCGGAGGAGGA	TATAGGTACCATGGCAACGCAGCGTTCCTA-	64	40
H. sapiens	LBD isolation	GGGAGAGCGGCATGAAGAAGGAGA	CTCTGGGTCTGGCTGCCTCTCG	60	30
	pBIND cloning	GTCGTCGGATCCGGGCCTTGATCAAGCGG	TATAGGTACCCCCAAGGGCAGCCGCTCA	67	30

Table SI2. Sequence identification of nuclear receptors from different species and represented by a specific accession number

Species	Name	Accession code	Database
Homo sapiens	HS_VDR	ENST00000395324	Ensembl
Homo sapiens	HS_CAR	ENST00000367979	Ensembl
Homo sapiens	HS_PXR	ENST00000337940	Ensembl
Ciona intestinalis	Cin_α	NP_001071847.1	NCBI
Ciona intestinalis	Cin_β	NP_001037831.1	NCBI
Crassostrea gigas	Cgi_β	EKC36490.1	NCBI
Scrobicularia plana	Spl_β	KP995063	NCBI
Aplysia californica	Acal_β	XP_005106453.1	NCBI
Biomphalaria glabrata	Bgl_β	contig954_2	BG genomic data (4.01)
Lottia gigantea	Lgi_β	63892	JGI
Capitella capitata	Cca_β	KB301483.1	NCBI
Daphnia pullex	Dpu_HR96	EFX89804.1	NCBI
Apis mellifera	Amel_HR96	XP_624213.3	NCBI
Drosophila melanogaster	Dmel_HR96	NP_524493.1	NCBI
Musca domestica	Mdom_HR96	AEC03603.1	NCBI
Crassostrea gigas	Cgi_α	JH816958.1	NCBI
Lottia gigantea	Lgi_α	163618	JGI
Biomphalaria glabrata	Bgl_α	contig48_1	BG genomic data (4.01)
Crassostrea gigas	Cgi_σ	EKC23219.1	NCBI
Lottia gigantea	Lgi_σ	163956	JGI
Aplysia californica	Acal_σ	XP_005113105.1	NCBI
Biomphalaria glabrata	Bgl_σ	contig2148_3	BG genomic data (4.01)
Capitella capitata	Cca_σ	ELT92961.1	NCBI
Capitella capitata	Cca_γ	ELU05478.1	NCBI
Lottia gigantea	Lgi_γ	63892	METAZOME
Biomphalaria glabrata	Bgl_γ	TMP004659_4	BG genomic data (4.01)
Drosophila melanogaster	Dmel_EcR	P34021.1	NCBI
Lottia gigantea	Lgi_EcR	LgGsHFWreduced.11616	METAZOME
Ciona intestinalis	Cin_EcR	287266	JGI
Ciona intestinalis	Cin_FXR	287095	JGI
Others			
Pinctada fucata		5032.1_67145.t1	OMGU
Pinctada fucata		7400.1_52946.t1	OMGU
Pinctada fucata		72427.1_56501.t1	OMGU
Saccoglossus kowalevskii		XP_002736417.1	NCBI
Crassostrea gigas	NR96	EKC26716.1	NCBI

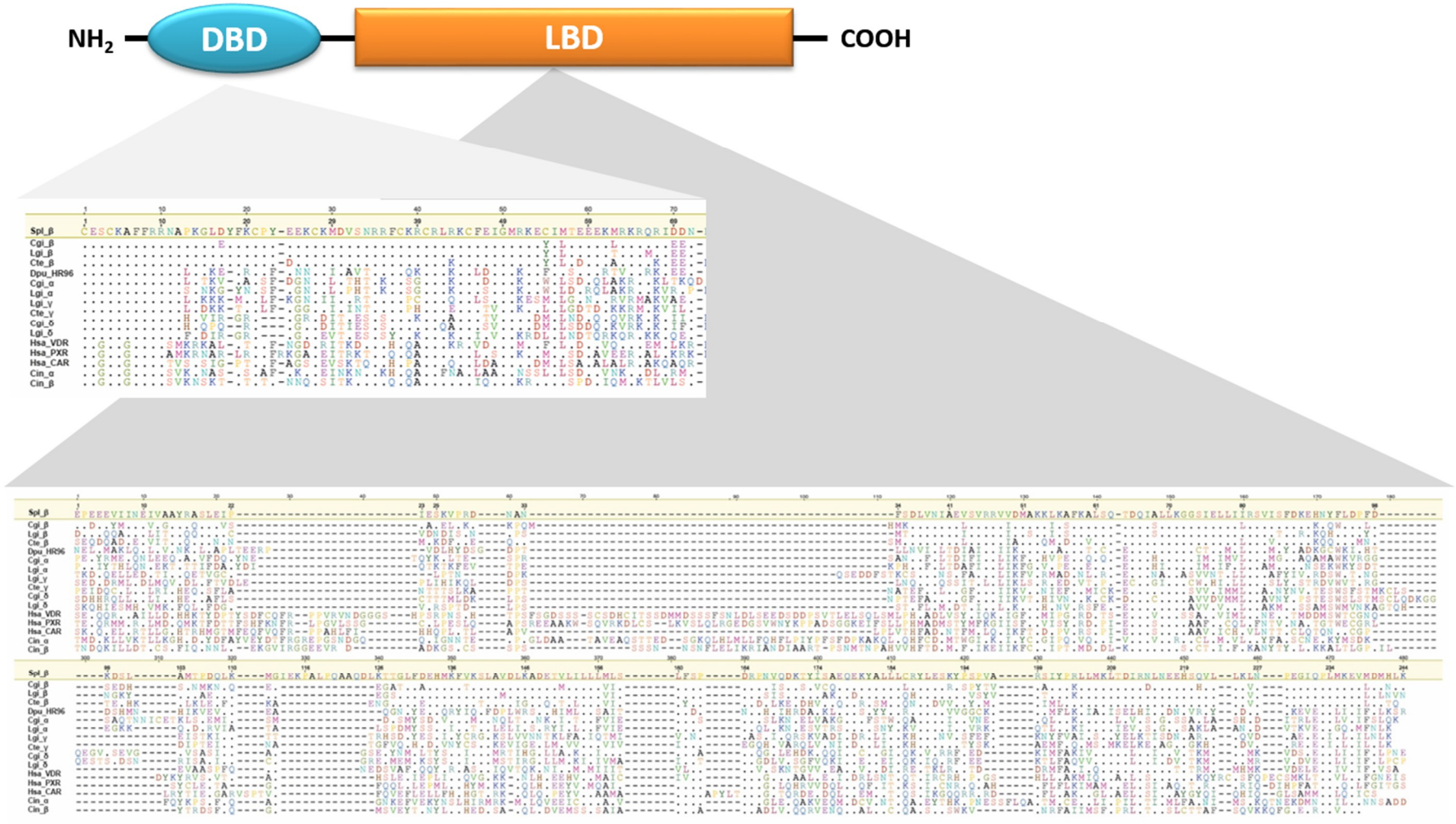

Figure SI1. Alignment of DBDs and LBDs NR1I/J

Figure SI2. Comparative sequence alignment of human PXR and *S. plana* ligand binding domain. Below amino acid replacement score (*) - full conservation of the amino acid; (:) - conservative amino acid replacement; (.)- semi-conservative amino acid replacement; ( ) non conservative amino acid replacement. PDB codes of the several available human PXR crystal structures and binding sites of the corresponding ligand indicated by (+). For 4X1F/4X1G - estrogen 17alpha-ethinylestradiol; 1SKX- rifampicine; 2QNV- colupulone; 4NY9- N-{(2R)-1-[(4S)-4-(4-chlorophenyl)-4-hydroxy- 3,3-dimethylpiperidin-1-yl]-3-methyl-1-oxobutan- 2-yl}-3-hydroxy-3-methylbutanamide; 1M13 – hyperforin.

```
Hs_PXR          SQVRKDLCSLKVSLQLRGEDGSVWNYKPPADSGGKEIFSLLPHMADMSTYMFKGIISFAK
S.plana         NEI---VAAYRASLEIPIES------KVPRDNAN---FSDLVNIAEVSV---RRVVDMAK
Score           .::   :.: :.**::  *.      * * *...   ** * ::*::*.   : ::.:**
4X1F/4X1G            ++                                 +  +   +   +
1SKX                      ++                            +  +       +
2QNV                    +                                   +   +
4NY9                      +                                 ++
1M13                    +                                 +  +   +

Hs_PXR          VISYFRDLPIEDQISLLKGAAFELCQLR-----------FNTVFNAETGTWECGRLSYCL
S.plana         KLKAFKALSQTDQIALLKGGSIELLIIRSVISFDKEHNYFLDPFDKDSLAMTPDQLKMGI
Score            :. *: *.  ***:****.::**  :*           *   *: :: :   .:*.  :
4X1F/4X1G
1SKX                                +  ++                         +        +
2QNV                                   +              +
4NY9                                   + +            +           +      +
1M13                                +  ++             +           +

Hs_PXR          EDTA--GGFQQLLLEPMLKFHYMLKK-----LQLHEEEYVLMQAISLFSPDRPGVLQHRV
S.plana         EKPALPQAAQDLKTTGLFDEHMKFVKSLAVDLKADETVLILLLMLSLFSPDRPNVQDKTY
Score           *..*   . *:*    ::. *  : *     *: .*   :*:  :********.* ::..
4X1F/4X1G                      +
1SKX                           +
2QNV                           +
4NY9
1M13                           +

Hs_PXR          VDQLQEQFAITLKSYIECNRPQPAHRFLFLKIMAMLTELRSINAQHTQRLLRI--QDIHP
S.plana         ISAEQEKYALLLCRYLESKYPSPVARSIYPRLLMKLTDIRNLNEEHSQVLLKLNPEGIQP
Score           :.  **::*: *  *:*.: *.*. * :: :::  **::*.:* :*:* **::  :.*:*
4X1F/4X1G                                                 +  +   +
1SKX                                                      +
2QNV                                                      +  ++  +
4NY9                                                      +
1M13                                                      +      +

Hs_PXR          FATPLMQ----------ELFGITGS
S.plana         LMKEVMDMHLKKDGEDSDSSSVASP
Score           : . :*:          :  .::..
4X1F/4X1G            +
1SKX            +
2QNV
4NY9
1M13            +
```

Chapter 9

Final remarks

Comments, questions and conclusions

The amount of reports on the occurrence of pesticides residues in distinct aquatic compartments are quite significant and has been growing continuously, as demonstrated in Chapter 1. However, few studies have been done involving the Portuguese aquatic systems [1-6]. The latter fact justifies the need to implement studies such as those made in this Thesis.

Do pesticides persist and accumulate in the environment?

As persistent compounds, pesticides have a ubiquitous presence among environmental matrices, with measurable amounts from ng/L to µg/L. Aquatic matrices, like surface and groundwaters, are the primary vehicles of pesticide transportation from the areas of their application into estuarine surfaces.

Which matrices provide the most representative samples to analyze the environmental status of pesticides?

As a first source of information, water samples are ideal to know which pesticides are being used and the total loads that reach into these systems [7]. However, these matrices may not be sufficient to assess the pesticides' impact on ecosystems. The suspended particulate matter (SPM), rich in natural humic substances, is able to clutch hydrophobic compounds, including mostly all pesticides [8]. SPM fraction provides a crucial link for chemical constituents between the dissolved aqueous phase (DAP), bed sediment, and biota [9].

Bivalves, close to the base of the food-chain and as surface deposit and/or filter-feeders can accumulate pesticide residues in higher concentrations than the surrounding habitat [10]. With a strict connection between DAP and SPM fraction, bivalves are the bridge between abiotic and biotic factors.

Which extraction and analytical method is more suitable for the samples?

The variability and complexity of these addressed matrices imply adequate preparation and analytical instrumentation to correctly identify and quantify these organic compounds from environmental samples. Different extraction procedures, like solid-phase extraction (SPM), ultrasonic extraction (USE) and the so called Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method, are reliable procedures that guarantee an efficient isolation of the target compounds as it was referred in this Thesis [11-13]. Associated to the cited processes are the multiresidue methodologies for separation, identification and quantification, such as gas chromatography coupled with mass spectrometry (GC-MS), which is a reliable sensitive technique to trace these compounds [14]. The extraction and analytical methods used in these works, proved to be efficient on the identification and quantification of ~56 compounds belonging to different categories, which includes the need for such precise approaches (Chapter 2, 3, and 6).

Which pesticides should be included in the study?

The pesticides investigated herein were selected, based on national and European databases, with the objective to englobe the most used pesticides between 2000 and 2010, as well as the approved, not approved, and banned pesticides [15, 16].

Is there a pattern of pesticide use practices along the country?

Which aquatic systems may be representative of Portugal?

Does Portugal comply with the European directives' levels?

Between 2010 and 2011, surface water samples from nine Portuguese estuaries were sampled, allowing to determine which aquatic systems would be used in the studies presented in the Thesis. After combining different factors, such as geographical distribution, overall importance, agricultural practices, and sample availability, three systems were selected. Afterwards, a new one yearlong campaign (2012-2013) was done to collect

the selected matrices at Mondego and Tagus rivers, and at Ria Formosa Lagoon.

Ria Formosa Lagoon (Chapter 2, 3, and 6)

Covering a period of three years, both sampling campaigns presented distinctive loads of pesticides (Σ), *i.e.* sum of average values of forty-seven/forty-eight pesticides. While the first campaign (2010-2011) presented annual average concentrations of Σ11000 ng/L, the second one (2012-2013) had concentrations six times lower (Σ1800 ng/L). Moreover, the seasonal fluctuation observed in the first campaign, mainly due to the significantly high concentrations measured in spring (2010-2011; Σ 27000 ng/L), was not so evident in the second one (Figure 1A and 1B). These results strongly suggest a radical change of the application or usage of pesticides in the areas surrounding the Ria Formosa Lagoon. The land abandonment and/or changes in the agriculture practices (e.g., introduction of "biological agriculture") may be partly responsible for this evolution (Chapter 2 and 3). However, as to the priority substances in the field of water policy (Directive 2013/39/EU), in both campaigns the same number of cases (seven) were registered with frequencies above 80% [17]. This suggests a continuous application of these target priority compounds over these three years.

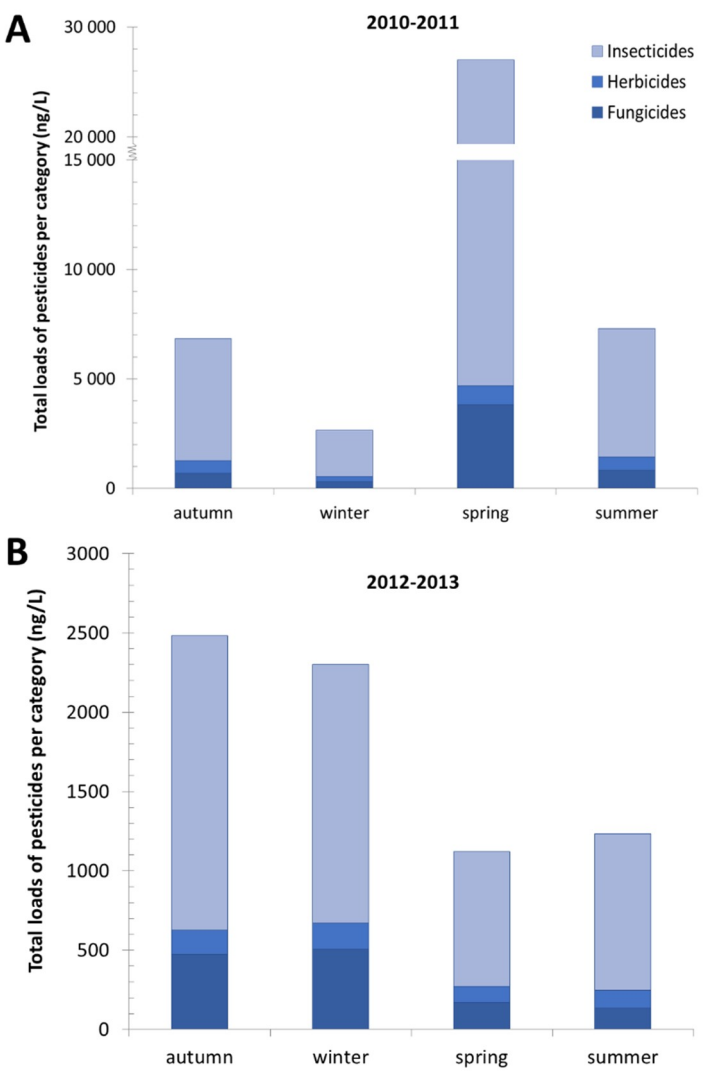

Figure 1: Total average loads of pesticides in water samples (Σng/L) of Ria Formosa Lagoon, per category and displayed by season; A - data from the 2010-2011 campaign; B - data from the 2012-2013 campaign (based on data of Chapter 2 and 3).

During the second campaign, SPM and the bivalve peppery furrow shell (*Scrobicularia plana*) samples were also collected (Chapter 2 and 6). For the first matrix, thirty-one pesticides were quantified, where total average concentrations ranged from Σ6 to 18 mg/kg (in SPM), displaying the same seasonal pattern as the dissolved water phase (DAP) (Figure 2A).

From pooled samples of whole bivalve (n = 90), fifty-three pesticides were quantified with total average concentrations ranging from Σ0.5 to 0.9 mg/kg, presenting once more a comparable seasonal fluctuation as

observed in DAP and SPM fractions (Figure 1B, Figure 2A and 2B). Here, a possible correlation is indicated between matrices. No differences were observed between sexes, suggesting that body composition differences are not determinant for pesticides accumulation, as we initially hypothesized.

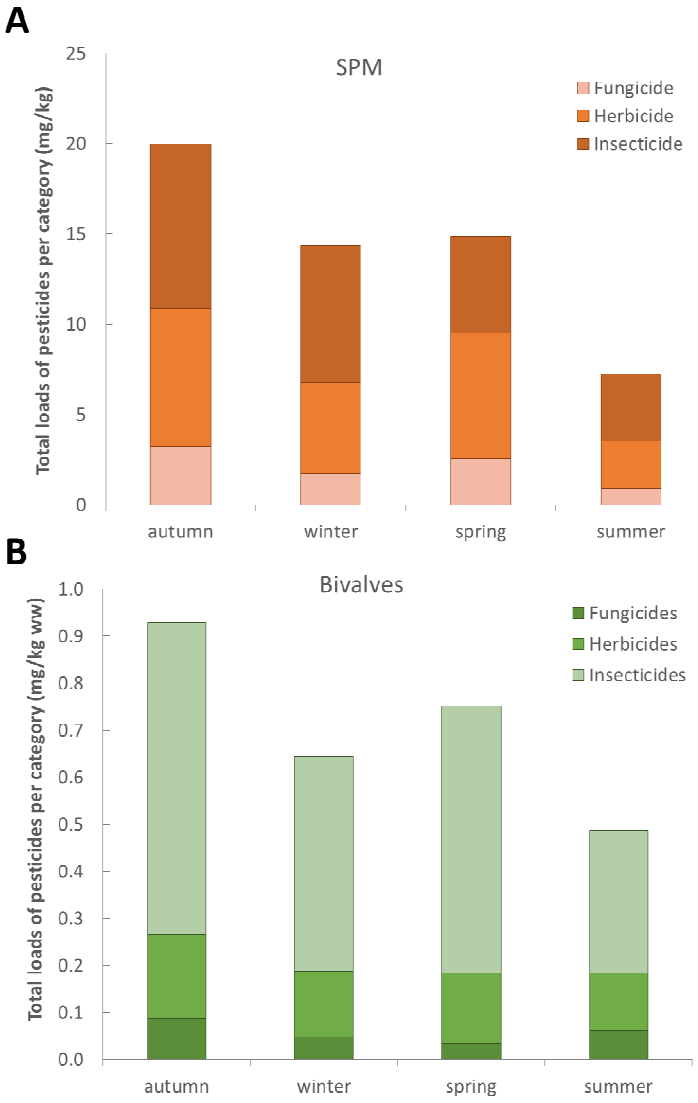

Figure 2: Total average loads of pesticides (Σ) of Ria Formosa Lagoon, represented by categories and displayed by season; A - Suspended particulate matter (SPM; mg/kg); B- Soft *S. plana* tissue (mg/kg ww) (based on data of Chapter 3 and 6).

In spite of average concentrations similar to the values presented in literature [18-22], 83% of the quantified pesticides were present, at least once, above the 2013/39/EU Directive limits set for biota. These results alert for levels that may cause widespread toxicity effects. Indeed, several of the individual maximum levels (between fourteen and seventeen compounds) were toxic mostly for fish and invertebrates, at least at short-time exposures (documented LC_{50} and EC_{50} values). As bivalves are on the base of the food-chain, these results may further escalate through the food-web.

Ria Formosa Lagoon provides ideal conditions for fish and bivalve nurseries [23], which may be compromised not only by the pesticide concentrations herein quantified but also by synergetic effects between them and other anthropogenic compounds.

Mondego River (Chapter 4)

Being under strong influences of the agriculture practices developed along its course, the Mondego River basin is quite affected, mainly by corn and rice fields located upstream [24, 25]. During the first campaign (2010-2011), seven sites were selected covering the main areas of this estuary. A total of forty-two samples were measured, quantifying fifty-six pesticides. Total loads of pesticides demonstrated similar concentrations throughout the year, ranging from Σ5000 to 7000 ng/L. This fact is well compatible with a constant usage/output of these biocides through all seasons (Figure 3).

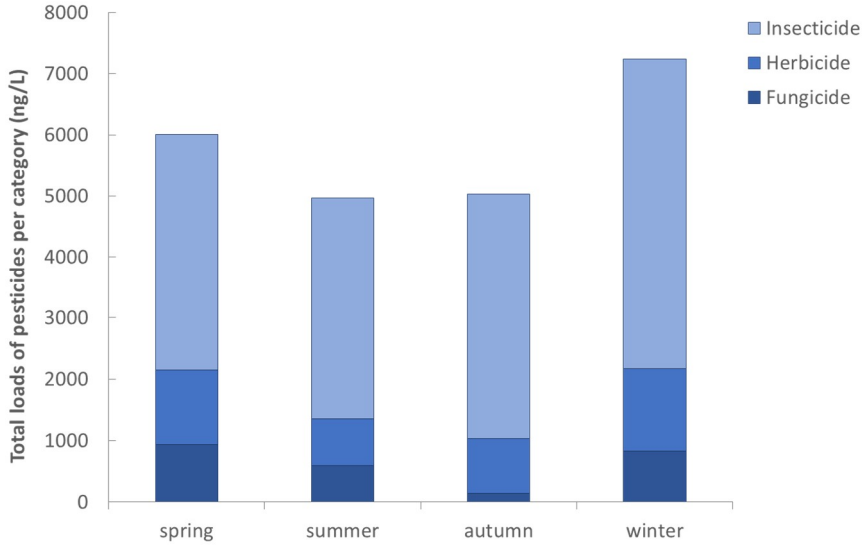

Figure 3: Total average loads of pesticides in water samples (Σng/L) of Mondego River estuary, displayed per category and by season; data are from the 2010-2011 campaign (based on results of Chapter 4).

Eight of the quantified pesticides revealed concentrations above the 2013/39/EU Directive limits [26]. Moreover, deltamethrin, dimethoate, endosulfan, lindane, malathion, and parathion – associated with rice production – registered individual annual average values ranging from 99 to 615 ng/L [27]. Once again, these facts illustrate well the amount of human pressure applied on this ecosystem.

Theoretical and practical approaches, *viz*. environmental hazard analysis and short-term toxicology assays, were also applied to probe the aquatic hazard of pesticide mixture hereby quantified. In both methods, the potential risk to biota was assessed, mainly fish and invertebrates.

Further analyses are under progress to complete the data from the 2012-2013 campaign, and so improving our knowledge of risks and trends.

Tagus River (Chapter 5 and 7)

As the longest river of the Iberian Peninsula, the Tagus is influenced by several anthropogenic features along its course [28]. Being a shelter for several migratory and local birds one of the most important natural reserves of Europe is settled, in its basin [29].

In a first analysis, during the 2010-2011 campaign, fifty-four pesticides were quantified from a total of forty-two samples. Total loads of pesticides ranged between seasons ($p<0.05$), attaining the highest amounts in spring (Σ5500 ng/L) and the lowest in winter (Σ1400 ng/L) (Figure 4A). This profile indicates an overuse of pesticides during the spring season, as observed in Ria Formosa Lagoon during the 2010-2011 campaign. During the 2012-2013 campaign eighteen samples were collected and nineteen pesticides were quantified, presenting total average concentrations ranging from Σ2700 to 5300 ng/L, with no seasonal pattern (Figure 4B). After the evaluation of the Ria Formosa Lagoon, it was expected that the nominal values obtained in the second campaign would decrease. However, this did not occur. As it was referred in Chapter 7, some pesticides are being used for agricultural and urban purposes, increasing their concentrations along the year in the metropolitan area of Lisbon.

Moreover, in both campaigns the selected point near the Trancão River mouth stand out from all sampling sites, with total average loads of Σ5500 ng/L and Σ6700 ng/L in the first and second campaign, respectively (see Chapter 5 and 7). These results demonstrate a very affected water course with increasing pesticide concentrations in the observed period. The industrial and agricultural fields located upstream of Trancão River may be the main cause for these persistent values.

Considering the 2013/39/EU Directive, seven cases were constantly detected (more than 66%) during the 2010-2011 samples (Chapter 5); in the second campaign three cases were registered, where endosulfan had 70% of the quantified values above the optimum levels.

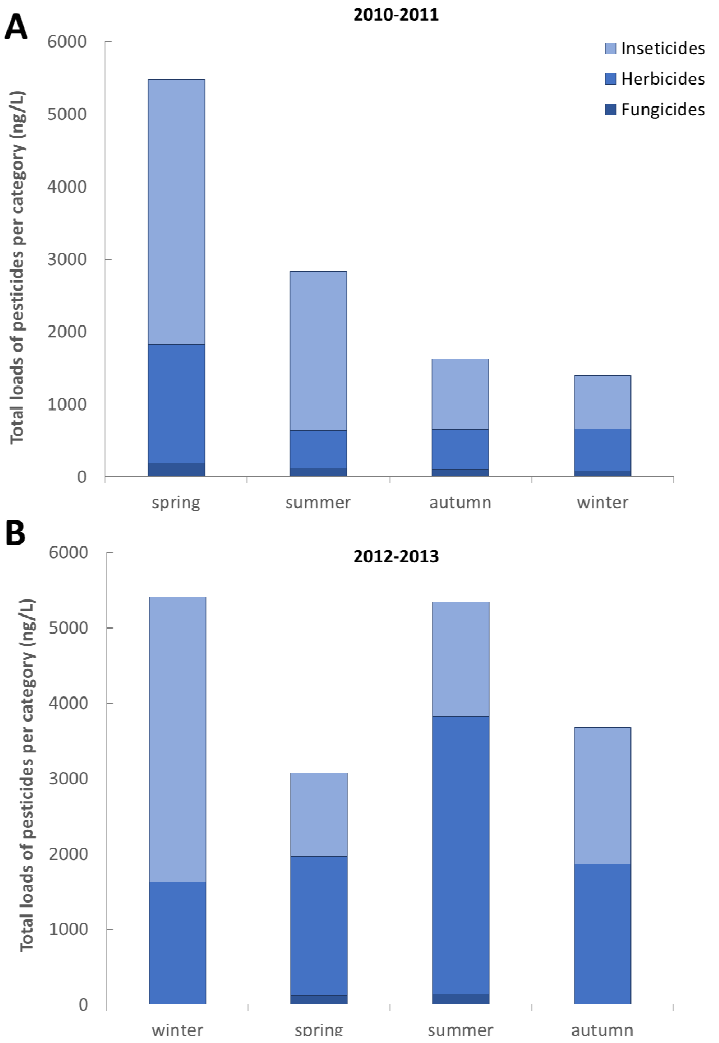

Figure 4: Total average loads of pesticides in water samples (Σng/L) of Tagus River per category and displayed by season; A - data from 2010-2011 campaign; B - data from 2012-2013 campaign (based on results of Chapter 5 and 7).

SPM and bivalve samples were collected during the 2012-2013 campaign. Comparing to DAP fraction the number of pesticides quantified was twice as much in the SPM fraction. The total average SPM values ranged from Σ16 mg/kg (in spring) to Σ7mg/kg (in winter) with significant differences (Figure 5A). The same nominal values were registered at Ria Formosa Lagoon during the 2012-2013 campaign (see Figure 2A), however no seasonal relation was observed with DAP fraction. The site near Trancão

River showed the highest total average amounts (Σ29 mg/kg) corroborating with the DAP results refered above (see Chapter 7).

As to the bivalve matrix, total average concentrations ranged from Σ0.7mg/kg (in spring) to Σ1.2 mg/kg (in autumn), with significant seasonal fluctuations. The same range of concentrations were registered at Ria Formosa Lagoon (Chapter 6), but no seasonal distribution was observed when compared to DAP and SPM fractions; also no differences were observed between samples sites. These results may denote a constant gathering of these organic compounds by the animals; no differences or correlations were established between sexes, achieving the same results as observed for Ria Formosa Lagoon bivalves.

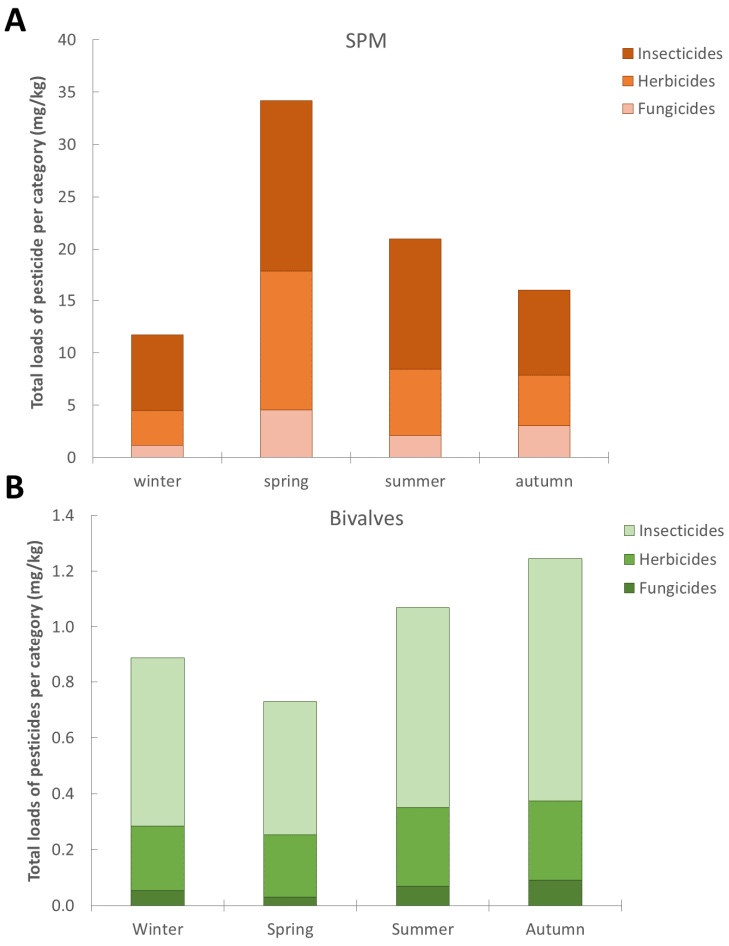

Figure 5: Total average loads of pesticides (Σ) of Tagus River, represented by categories and displayed by season; A - Suspended particulate matter (SPM; mg/kg); B - Soft *S. plana* tissue (mg/kg ww) (based on results of Chapter 5 and 7).

From ninety observations, nineteen compounds (≈ 21%) were above the 2013/39/EU Directive levels in more than 50% of the quantified samples; this, together with the information cited above, indicates that these sessile animals are subject to constant exposure of these pollutants (Chapter 7).

Which nuclear receptor gene family is responsive to xenobiotics?

Are members of this nuclear receptor family expressed in *S. plana*?

Molecular assays (Chapter 8)

Nuclear receptors (NR), as ligand-activated proteins, are the main regulating components of metabolic pathways, implied in the enzymatic oxidation, conjugation, and excretion of toxic compounds [30, 31]. Several works have linked the NR1I/J gene family members, such as PXR/VDR/CAR orthologues, to xenobiotic recognition [32-34].

Bivalves are typically sessile and surface deposit and/or filter-feeders, encompassing a wide range of ecological niche species for studying the effects of xenobiotic compounds in the environment. However, the understanding of NR function and xenobiotic disruption in the phylum is limited. Therefore, our goal was to use *S. plana* as model species to fulfil these gaps of information.

For gene isolation, VDR/PXR/CAR-like genes were used from other aquatic animals (sea squirt and owl limpet) [35, 36], resulting in the isolation of the complete ligand binding domain of the *S. plana* NR, which we named *Spl*NR1Jβ.

Do all xenobiotics have the capacity to activate the studied nuclear receptors?

How does the compounds concentration influence the activation?

To study the xenobiotic receptor plasticity, transactivation assays were used as a model/tool for evaluation and comparison between the human PXR and the *S. plana* NR1Jβ. Based on other works, one natural toxin (okadaic acid) and two pesticides (esfenvarelate and triclosan) were used for a first NR response evaluation [36, 37]. While similar results for okadaic acid were registered, esfenvalerate presented a unique response, since it down regulated transactivation at higher concentrations, and triclosan did not show any response. As first results documented for bivalves, we unveil that different compounds/molecules at different concentrations have distinct ligand potency and efficacy.

In parallel the NR1Jβ orthologue was characterized through phylogenetic analyses, indicating in the NR1J gene family a branching pattern for Protostomes, generating 4 distinct gene lineages — α, β, γ, and δ — where the NR1Jβ is included (Figure 6).

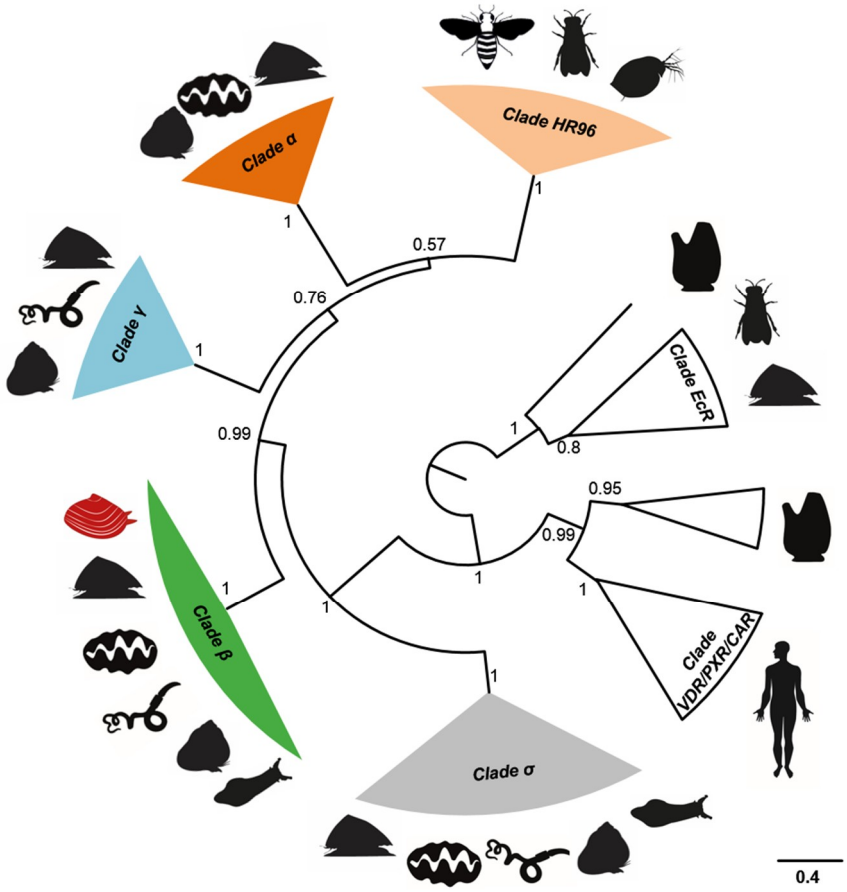

Figure 6: Maximum likelihood phylogenetic tree describing relationships among NR1I/J vertebrates and invertebrates; *S. plana* is represented by the red bivalve illustration (based on Chapter 8).

Further studies are needed to characterize all four isoforms and evaluate their response to xenobiotics. Anyway, our findings demonstrate that transactivation assays are a refined tool that is able to screen xenobiotic compounds acting via NR1I/J group, using human and mollusk models.

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