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# Treball Final de Grau

**Non-target analysis of organic contaminants mixtures in mussel from Ebro Delta**

**Anàlisi non-target de mescles de contaminants orgànics en musclos del Delta de l'Ebre**

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*Reserve your right to think, for even to think wrongly is better than not to think at all*

Hypatia

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# REPORT

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# 1. SUMMARY

Nowadays, the levels of chemical contamination present in the aquatic environment are of high relevance and pose a potential risk for the environment and also for human health. Marine organisms suffer alterations in metabolic pathways as a direct consequence from anthropogenic activities, which are considered one of the main sources of chemical and environmental pollution. Besides, the accumulation of contaminants in edible species may have negative implications in human health due to the ingestion of contaminated seafood.

In this context, the present study arose, as part of the XENOMETABOLOMIC project, with the main goal of assessing environmental pollution present in Ebro delta through the identification of priority mixtures of contaminants bioaccumulated in wild mussels (*Mytilus galloprovincialis*), a high consumed seafood type. Besides, this work is focused on the characterization of contamination patterns present in the two bays located at Ebro Delta (Alfacs and Fangar), and scientific evidence regarding the identity of the pollutants bioaccumulated in mussels from each bay is presented.

For this purpose, a non-target approach was applied based on ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS). The analysis of priority mixtures of contaminants was performed in two different biological matrices: haemolymph and soft tissue.

The non-target analysis reveals the presence of 17 and 25 organic contaminants in mussel's haemolymph serum and soft tissue, respectively, and demonstrate that both pesticides and pharmaceutically active compounds might cause a major environmental impact in the Ebro Delta area among other contaminants.

**Keywords**: priority contaminant mixtures, non-target analysis, bioaccumulation, environmental impact, chemical pollution, mussels, UHPLC-HRMS

# 2. RESUM

Actualment els nivells de contaminació química presents al medi aquàtic són tant significatius que suposen un risc potencial tant pel medi ambient com per la salut humana. Els organismes marins pateixen greus alteracions metabòliques com a conseqüència directa de les activitats antropogèniques que són considerades una de les principals fonts de contaminació química i ambiental. A més, l'acumulació de contaminants en espècies comestibles pot tenir implicacions negatives en la salut humana degut a la ingesta d'aquests aliments.

En aquest context va sorgir aquest estudi, com a part del projecte XENOMETABOLOMIC, que té com principal objectiu avaluar la contaminació ambiental present al Delta de l'Ebre a partir de la identificació de les mescles prioritàries de contaminants acumulades en musclos silvestres *Mytilus galloprovincialis*, un tipus de marisc molt consumit. A més, aquest treball es centra en la caracterització dels patrons de contaminació existents a les dues badies situades al Delta de l'Ebre (Alfacs i Fangar) i alhora presenta evidència científica sobre la identitat dels contaminants bioaccumulats en musclos cultivats a cadascuna de les badies.

Amb aquests objectiu, s'ha utilitzat un mètode non-target basat en cromatografía de líquids d'alta ressolució (UHPLC) acoblada a espectrometria de masses d'alta ressolució (HRMS). L'anàlisi de les mescles prioritàries de contaminants s'ha dut a terme en dos tipus de matriu: teixit i hemolinfa.

L'anàlisi non-target revela la presencia de 17 i 25 contaminants orgànics en les matrius d'hemolinfa i organisme sencer, respectivament, i manifesta que tant els pesticides com els compostos farmacològicament actius poden causar un major impacte ambiental a la zona del Delta de l'Ebre, entre altres contaminants.

**Paraules clau**: mescles prioritàries de contaminants, anàlisi non-target, bioaccumulació, impacte ambiental, contaminació química, musclos, UHPLC-HRMS

# 3. INTRODUCTION

A wide variety of organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), pharmaceutically active compounds (PHACs), Personal Care Products (PCPs) and organochlorine pesticides (OCPs) among others, enter the aquatic environment as a consequence of several anthropogenic activities [1]. These include urban and industrial wastewater discharges, recreational activities, Waste Water Treatment Plants effluents (WWTPs), agricultural runoff and aquaculture facilities [2]. Therefore, coastal areas are suffering constantly the impact from all these sources.

Chemicals, which are widely distributed in the environment and bioaccumulated by aquatic organisms, can damage considerably marine wildlife itself and cause toxicological implications to humans (through the consumption of contaminated seafood) even at low concentrations.

Despite regulatory institutions have established maximum residue limits (MRLs) for a large variety of chemical substances in daily food products of animal origin [2], more action is required to solve the current emerging situation we are living in. In fact, those compounds that regulatory and enforcement agencies classify as priority pollutants only represent a minor fraction of thousands of known and yet-to-be identified chemicals [3]. In this context, more efforts are needed from the scientific community in order to identify priority contaminants present in the environment. New monitoring studies of persistent and emerging pollutants are required and the application of non-target analysis seems to be the way forward. Besides, governments should be aware of this environmental problem and adopt new measures in order to protect consumer's health and to ensure the wellbeing of ecosystems.

## 3.1. TARGET VERSUS NON-TARGET ANALYSIS: THE XENOMETABOLOMIC APPROACH

To date, the analytical approach widely recognized to address monitoring programmes with the main purpose of identification and quantification of pollutants in the aquatic system has been target analysis.

Although target analysis is considered a powerful tool, offering good sensitivity and reliable identification of substances, it presents a clear limitation: this technique is focused on those

compounds included in the method and misses the rest, which might act as potential chemical stressors as well. Under these circumstances, new analytical strategies, such as non-target analysis, are needed to overcome these drawbacks occurring in the assessment of environmental pollutant mixtures. Indeed, target-based environmental monitoring combined with non-target strategies is chosen in order to achieve more representative and accurate results nowadays [3], [4]. According to this, untargeted analytical approaches are characterized by presenting numerous advantages in field-based investigations; provide more relevant information on chemical mixtures accumulating in marine species (the exposome), and simultaneously investigate for any associated disruption of metabolite profiles (the metabolome) [5]. Notice that the pre-selection step is not included in non-target studies, since no particular chemical is in mind prior to analysis.

The current non-target research is part of the XENOMETABOLOMIC project, which adopts the xenometabolomic approach with the main purpose of establishing priority mixtures of contaminants and detecting alterations in metabolic pathways for further regulations. Therefore, it combines both the profiling of the xenometabolome (or exposome) and the metabolome. This approach arises from the duty to establish modes of action of chemical mixtures in marine organisms that are constantly exposed to an immense amount of anthropogenic sources of pollution (large variety of chemicals and its structures). Hence, the use of “omics” (specifically xenometabolomics) seems to be appropriate to assess this problem of high complexity. In fact, metabolomics is gaining ground in characterizing biological responses to environmental stressors in a variety of aquatic species [6], [7], [8].

## 3.2. STUDY SITE: THE EBRO DELTA

The present study was conducted in the Ebro Delta, a natural wetland hugely recognised for its birdlife preservation in the western Mediterranean [9].

The Ebro Delta comprises 320km$^2$ area characterised mainly by two coastal shallow bays, Alfacs and Fangar, which extend along both sides of the Ebro River mouth. [10] Surprisingly, the simple transportation of alluvial sediments, together with natural phenomena, has originated this dynamic and particular shape. Nevertheless, the Ebro Delta is not only committed to nature conservation [11].

Both bays play such an important role in deltaic economy due to the development of agricultural activities and mariculture production in the area. Intensive agriculture basically depends on rice production, assigning the 80% of the land to its cultivation. For this reason, agriculture has a great impact on deltaic income, being considered the main economic activity in the Ebro Delta. Despite the fact that shellfish aquaculture has reached a production of 1,575,000 kg/year [12], this activity would be placed in the second position in economic terms. Moreover, only mussels farming, which represents the 95% of total shellfish production, provides great benefits through 166 fixed culture rafts spread between Alfacs and Fangar bays [9].

Under this scenario, there's no doubt that either estuarine or marine waters are directly affected by the exposure to pollutants associated to agriculture (generally chemicals for pests treatment). Concretely, pesticides might be the ones that present the major ecological impact since these substances, among others, are collected by drainage channels and released into the bays during rice cultivation season. Previous studies have reported that rice farming uses around 20,000 t/year of agrochemicals for the maintenance of the rice crops [13].

In addition, other anthropogenic sources of contamination in the area, such as urban and industrial inputs, are increasingly threatening marine ecosystems as well.

All these evidences suggest the necessity of effecting monitoring studies in order to assess exposure of pollutants in marine species cultured at Ebro Delta and destined for human consumption as mussels. Thus, this area was selected for the present untargeted investigation.

### 3.2.1. Previous studies in deltaic area

Previous target studies undertaken at Ebro Delta have provided relevant information about the existing contamination in the area.

During the nineties, several investigations were focused on the determination of organoghlorinated compounds like polychlorinated biphenils (PCBs), polychlorinated terphenyls (PCTs) and dichlorodiphenyltrichloroethane (DDT) that are mainly used as pesticides. For instance, high levels of PCBs and DDTs were found in red mullet showing the evidence of industrial and agricultural inputs in the Ebro Delta. Actually, some of these pollutants were bioaccumulated or even subject to metabolic transformations in benthic fish and mussel [12],

[14], [15]. Nevertheless, restrictions adopted by regulatory institutions along the eighties cause a notable decrease in concentrations of organochlorinated pesticides and PCBs.

Since the beginning of the 21st century, monitoring programmes included organophosphorous pesticides (OPs) and polycyclic aromatic hydrocarbons (PAHs) for its identification and quantification in bivalves cultured in Ebro Delta. Particularly, fenitrothion, an organophosphorous insecticide widely used in deltaic crops, presented high concentration (exactly 5 ng/g wet weight) in comparison with other OPs in mussels. However, some previous studies concluded that levels of organic pollutants (OPs, OCIs and PAHs) present in edible bivalves were minimal, far below MRLs, and thus these chemicals are not hazardous for either wildlife or human health [16].

In the last decade, pesticides continued being under investigation in field-based programmes since shellfish mortality episodes occurred yearly in this area at spring time [9]. Moreover, endocrine disruptor compounds (EDCs) and pharmaceuticals (including some major metabolites) were explored in bivalves, fish and microalgae organisms from Ebro Delta. Those studies paid special attention to venlafaxine and azithromycin drugs, which were found in relatively high concentrations in bivalves (2.7 ng/g and 3 ng/g dry weight (dw), respectively) [2], [17].

Recent studies reported EDCs as one of the groups of contaminants with notable influence in deltaic environment. Concretely, five different EDCs were identified in this area, reaching a maximum concentration of 19.25 ng/g dw for methylparaben in wild mussels [18]. Besides, the most occurring EDCs in bivalve samples from Ebro Delta were the organophosphorus flame retardant tris(2-butoxyethyl)phosphate (TBEP), triclosan, 1H-benzotriazole and parabens [19]. Taking into consideration levels of bentazone and (4-chloro-2-methylphenoxy)acetic acid (MCPA) bioaccumulated by mussels, herbicides may be considered pollutants of emerging concern in this area as well [18].

Recently, scientific researches have been focused on the determination of musk fragrances and UV-filters due to their persistency in the environment and their potential toxicity. In fact, their co-occurrence was verified in marine organisms in Ebro Delta, in which galaxolide (HHCB) presented the highest levels (33.10 ng/g dw) [20].

A wide range of chemicals causing negative effects in both marine wildlife and environment has been presented. Therefore, new monitoring programmes based on the identification of priority mixtures of contaminants need to be developed.

### 3.3. MUSSELS AS BIOMONITORING ORGANISMS

Mediterranean native mussels, *Mytilus galloprovincialis*, were selected as biomonitoring organisms due to their wide use for research purposes in environmental framework [21]. For instance, some projects focused on the dentification of priority mixtures of contaminants chose mussels as biomonitoring tools:

- The United Nation's sponsored "Earthwatch" approach applied worldwide that assess environmental quality through bivalve species.
- The United States Environmental Protection Agency established "Mussel Watch" in order to control anthropogenic activities in oceanic ecosystem [21].

Generally, bivalves are considered sentinel organisms for environmental pollution in numerous monitoring programs. Its nature, characterised by filter-feeding and low metabolic capacity, allows them to act as biological indicators of current contamination in aquatic systems [16]. Large amounts of pollutants present in the surrounding waters or sediments can be retained in their tissues, making them capable of bioaccumulating or even metabolizing hazardous substances [15].

Besides, bivalve molluscs are food commodities highly consumed worldwide, mainly in Italy, France and Spain, due to high quality of proteins and the numerous benefits they provide. Yet, consumption levels of mussels are very different depending on the country. Whereas some countries reach a mussel consumption per capita of 3kg per year, others don't even introduce these kind of shellfish in their daily diet. However, the European market for mussels is estimated to be slightly below 600.000 tones in equivalent live animal weight [22]. Mussel business is of high importance in European countries, especially its market is concentrated in five countries: Spain, the United Kingdom, France, Italy and Greece, and Spain leads the sector with 32% of the total jobs [23].

# 4. OBJECTIVES

The main goal of the present research was to apply a novel non-target approach to wild mussels from Ebro Delta in order to identify priority mixtures of contaminants bioaccumulated that may be of potential risk to wildlife and human health. In addition, other specific objectives established were:

- To determine the contaminant mixtures present in Alfacs and Fangar bays.

- To discern if there was a different contamination pattern between bays and if it was related to different contamination sources.

- To find out whether mussel's haemolymph or soft tissue was more suitable for the application of the non-target analysis (xenometabolomic approach).

# 5. MATERIALS AND METHODS

## 5.1. STANDARDS AND REAGENTS

All isotopically labelled standards employed in our analytical method as internal standards were of high purity grade (>90%) and they were purchased from Sigma Aldrich with the exception of the following ones:

**TABLE 1**
Isotopically labelled standards used in the analytical procedure together with the supplier company.

| Supplier Company | IS |
|---|---|
| Dr. Ehrenstorfer | Metolachlor-d6, Thiabendazole-c13, Malathion-d7 and Triclosan-d3 |
| Toronto Research Chemicals | Propanil-d5 and Sulfamethoxazole-d4 |
| CDN Isotopes | Caffeine-d3 and Bisphenol A d-4 |

On the one hand, all required reagents to prepare the internal standard mixture for the analysis of mussel's soft tissue (whole organism) are summarised in Appendix 1. The selection of these compounds was done according to Álvarez-Muñoz et al. 2019 and Terrado et al. 2018 studies [18], [24]. On the other hand, the number of isotopically labelled standards considered for haemolymph study was limited to: venlafaxine-d6, caffeine-d3 and benzotriazole-d4 for positive ionisation mode and bentazone-d4, triclosan-d3 and ethylparaben-c13 for the negative one. Isotopically labelled standards were prepared in methanol at a concentration of 10 µg/mL. Working standard solutions of 1 µg/mL, containing isotopically labelled internal standards were prepared in 100% acetonitrile (ACN) before each analytical run.

Both QuEChERS BEKOlut Citrat-Kit-01 and QuEChERS BEKOlut PSA-Kit-04A were kindly supplied by BEKOlut (Barcelona, Spain). The Ostro™ 96-Well Plate was purchased from Waters (Barcelona, Spain). The HPLC grade water and ACN were supplied by Merk (Darmstadt, Germany).

## 5.2. FIELD EXPERIMENT AND SAMPLING

Considering previous target studies in Ebro Delta [18], the selection of five sampling points was carried out including both bays, Alfacs and Fangar (specific locations are shown in Fig. 4 and Fig. 5 in Appendix 2). As a consequence of the different dimensions of the two bays, 3 sampling sites were located in Alfacs bay (BAP1, BAP2 and BAP3), whereas only 2 sampling sites were allocated in Fangar bay (BFP1 and BFP2). This sampling plan will allow to have an overview of the pollution pattern present in each bay. BAP1 was considered as "clean site" due to its external location, outside of Alfacs bay, and the results obtained in previous research [18].The other sampling sites were treated as "exposed" sampling points, because initially they were considered to be potentially affected by a higher number of contaminant sources.

A rope of approximately 3 m of length was fixed in BAP1 with Mediterranean mussels *Mytilus galloprovincialis.* They were maintained there until May 2017 when the experiment started. At this moment, nets containing 100 specimens each were deployed at each sampling site, and the molluscs were freely exposed to natural Delta waters. They were maintained there during one month (May 25$^{th}$ – June 28$^{th}$). The exposure period was slightly shorter for BAP3 bivalves as a preventive measure because high mortality episodes were previously reported in this sampling point. The explanation to this lies in the fact that urban untreated wastewater inputs occur frequently near the shallow area of study, where pollutants and bacteria are easily retained by mussels. For this reason, the exposition for BAP3 shellfish only lasted 1 week.

By the end of June, a total of 50 molluscs, which were of similar size (shell length between 5-7 cm) and satisfied the legal requirements of harvestable size for human consumption, were collected from each sampling point. All specimens were transported carefully to the laboratory, at IDAEA-CSIC, under refrigerated conditions. Then, 40 specimens from each sampling site were used for the study of exogenous pollutants accumulated in mussel's soft tissue, whereas only 10 of them were used for the analysis of haemolymph.

## 5.3. ANALYTICAL PROCEDURE

### 5.3.1. Mussel's soft tissue

A complete description of analytical protocol for mussel's soft tissue is given elsewhere [24]. Briefly, the shell was removed and the edible content, including all tissue and intervalvar liquid, was added to a pool. Then, each pool was grinded, homogenised, freeze-dried and kept at -20 ºC until its analysis.

To start with, each lyophilised sample was grounded with a mortar and three replicates were prepared (1g each) and analysed by using QuEChERS (quick, easy, cheap, effective, rugged and safe) methodology. Prior to the extraction, the internal standards mixture was added (see detailed information in Appendix 1), vortexed and left to equilibrate overnight under refrigerated conditions (12h, 4ºC). Then, the extraction process involved 2 main steps: liquid-liquid extraction (LLE) and dispersive solid phase extraction for purification of the extracts (dSPE). Firstly, during the LLE step, both 10ml of ACN and 5ml of HPLC water were added to homogenized samples together with a mixture of MgSO4, NaCl and buffering salts (QuEChERS BEKOlut Citrat-Kit-01). The resulting composite sample was vortexed (2500 rpm, 1 min) and centrifuged (4000 rpm, 15ºC, 5 min) to separate the phases. Next, 6ml of the supernatant were transferred to a centrifuge tube and cleaned using the dSPE technique. This step consisted basically of adding bulk drying salts and SPE sorbent (QuEChERS BEKOlut PSA-Kit-04A), vortexing and centrifuging in the same conditions specified earlier. Doing so, the removal of water excess and undesired co-extractives from the extracts was guaranteed. The cleaned extracts were transferred to a glass tube, where they were subjected to evaporation under a gentle stream of nitrogen until complete dryness and reconstituted in 1ml of ACN. Last but not least, a filtration step was required through a phospholipids removal plate (Ostro™ 96-Well Plate) to ensure a successful analysis by ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS).

### 5.3.2. Mussel's haemolymph

Prior to chemical analysis, 1 ml of haemolymph fluid from bivalve molluscs was extracted and collected. After the centrifugation (800 rpm, 10 ºC, 15 min), the supernatant was transferred to a cryogenic vial and kept under refrigerated conditions, -80 ºC.

Haemolymph extracts were defrosted for 45 min at room temperature before starting the sample preparation process. To start with, each replicate was agitated vigorously (2500 rpm, 1 min) and both ACN and the internal standard mixture (500 µg and 50 µg respectively) were added. Some lipid content and proteins that remained in solution precipitated as a result of the polarity difference between the solvent and these biological components. As these compounds could act as interferences in the analytical determination, the filtration using a phospholipid removal plate (Ostro™ 96-Well Plate) was executed. Ultimately, the filtered solution was transferred to a vial and it was analysed by UHPLC-HRMS.

Additionally, it was necessary to evaluate working conditions state and the separation efficiency during the analytical run. Hence, a quality control composite sample (QC) was prepared considering different extracts from the control group (BAP1 sampling site). Concretely, 100 µl of each BAP1 replicate were added together with 1ml of ACN and 50 µl of IS mixture to a clean vial. The QC sample was also filtrated through Ostro™ 96-Well Plate.

## 5.4. ANALYSIS BY UHPLC-HRMS

Both mussel's soft tissue and haemolymph samples were analysed by ultra-high performance liquid chromatography coupled to orbitrap Q-exactive high resolution mass spectrometry. The chromatographic separation was performed by an Acquity Ultra-Performance™ Water liquid chromatograph system (Milford, MA, USA), equipped with two binary pumps systems using a Purospher STAR RP-18 end-capped column (specific dimensions: 150 mm x 2.1 mm, particle size: 2 µm).

The mobile phase was a binary mixture of solvents A (acetonitrile 100%) and B (HPLC water). The run (flow rate = 0.2 ml/min) started with 10% A (2.5 min), which was then gradually increased to 100% (at 13.5 min) and remained constant for 3.5 min (Table 2). The initial conditions were reached at 17 min, with an equilibration time of 8 minutes. The injection volume was 20 µl.

A Q-exactive orbitrap mass spectrometer (Q exactive™ Thermofisher Scientific, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in the positive and negative ionisation modes was used to detect all organic contaminants. The mussel extracts were run twice in positive and negative modes. Full scan data (m/z 70-1000) were acquired at a resolving power of 70,000 FWHM.

The extracts were randomly placed in the sample queue. The quality control composite sample was injected along the sample list and in the different runs in order to monitor Orbitrap performance during batch analysis. Besides, a chromatographic blank (100% ACN) was run every 5 samples to detect any possible carryover effect.

**TABLE 2**
Elution gradient where mobile phase A = ACN and phase B = $H_2O$.

| Time (min) | % A | % B |
|---|---|---|
| **0** | 10 | 90 |
| **2.5** | 50 | 50 |
| **12.5** | 80 | 20 |
| **13.5** | 100 | - |
| **16** | 100 | - |
| **17** | 10 | 90 |
| **25** | 10 | 90 |

## 5.5. DATA PROCESSING AND POLLUTANTS IDENTIFICATION

For data analysis the mass spectra generated in the Orbitrap™ were analysed by using Compound Discoverer 2.0 software from Thermo Fisher Scientific (v. 2.0; Thermo Scientific, Fremont, CA, USA). For the non-target analysis (xenometabolomics approach of both mussel's soft tissue and haemolymph) the spectra corresponding to mussels from the control site (BAP1) was compared with the mussel's spectra from the exposed sampling points (BAP2, BAP3, BFP1 and BFP2). Compound discoverer workflow (Fig. 1) was established for background subtraction, spectra alignment, differential analysis, component detection, grouping, composition prediction and chemical identification. Some statistical tools were also included in the workflow in order to guarantee the validity of the results: t-test, p-value ($p < 0.05$) and ratios. For tentative identification of the compounds four databases were used: mzCloud, Chemspider, Mass Lists and mzVault. The putative identity of discriminatory compounds was proposed from their accurate mass composition using elemental with an error below 5 ppm. In addition, well-defined chromatographic peaks (Gaussian profile and high resolution) was a requirement to suggest the presence of a specific pollutant.

The identity of the discriminatory compounds was double checked by using their accurate mass composition and the elemental composition tool from Thermo Xcalibur 3.1 software.

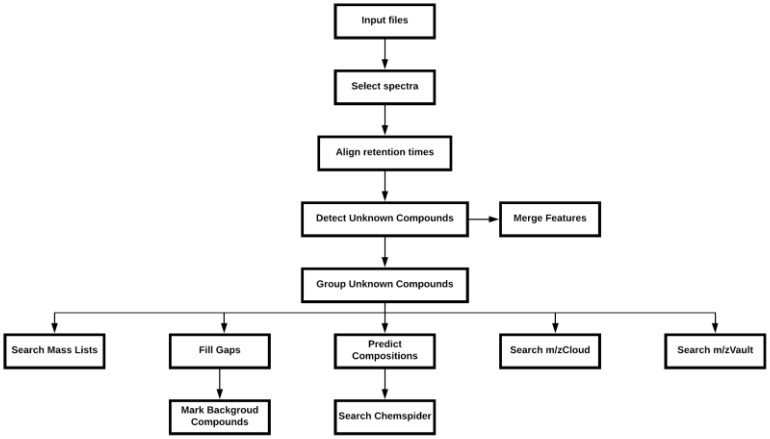


**Fig. 1** Custom workflow in Compound Discoverer software.

It deserves to be mentioned that chemometrics analysis based on mass spectrometry associated with Principal Component Analysis (PCA) and Partial Least Discriminant Square Analysis (PLS-DA) are needed in order to ensure significant differences between sampling sites in each bay. Indeed, PCA has been developed with mussel's haemolymph samples due to its higher number of experimental replicates in each sampling site (n = 10).

Multivariate data analysis was performed using Solo (v. 8.7; Eigenvector Research, Inc., Manson, WA, USA). Autoscale pre-processing was applied in order to unit variance (differences in scale amplitude and magnitude were deleted). Principal component analysis (PCA) model was constructed with exogenous contaminants data (compiled from Xcalibur) to identify similarities and differences between exposed and control groups of samples.

# 6. RESULTS AND DISCUSSION

Initially, between 10 and 13 thousands of biomarkers were detected as potential contaminants present in soft tissue samples from each sampling site (Table 3). However, the identification strategy was limited to those organic compounds that presented significant differences between control and exposed specimens (exclusive condition defined by p value < 0.05). Taking into consideration these significant compounds, a second selection was done following three specific requirements (well-defined chromatographic peak, p value < 0.05 and error < 5 ppm) to suggest the putative identity of potential contaminants. Although Compound Discoverer software proposed both exogenous and endogenous metabolites to describe potential mixtures of contaminants present in the samples, only the exogenous ones were selected in this field-based research to evaluate environmental pollution in wild mussels (the xenometabolomic approach).

Table 3 summarises the screening method followed in the determination of priority mixtures of contaminants in soft tissue matrix from *Mytilus galloprovincialis* specimens. Same information for mussel's haemolymph samples is given in Appendix 3 and similar results were obtained.

**TABLE 3**
Screening method employed for the identification of xenobiotics in mussel's soft tissue.

| Sampling site | ESI | All markers[1] | Significant markers[2] | Markers with chemical formula[3] | Exogenous markers[4] | Tentative identified markers[5] |
|---|---|---|---|---|---|---|
| **BAP2** | + | 10.690 | 1.695 | 62 | 15 | 5 |
| | - | 12.015 | 1.397 | 43 | 5 | 3 |
| **BAP3** | + | 10.698 | 1.399 | 60 | 6 | 3 |
| | - | 12.070 | 663 | 27 | 4 | 1 |
| **BFP1** | + | 11.207 | 3.019 | 75 | 9 | 8 |
| | - | 12.630 | 1.943 | 55 | 8 | 4 |
| **BFP2** | + | 11.678 | 3447 | 83 | 15 | 7 |
| | - | 13.027 | 2.140 | 70 | 3 | 3 |

(1) All markers suggested by Compound Discoverer software.
(2) Significant markers with p value < 0.05.
(3) Markers with well-defined chromatographic peak, p value < 0.05 and error < 5ppm.
(4) Markers considered exogenous contaminants (endogenous metabolites excluded).
(5) Potential identities of markers double-checked using Xcalibur software (elemental composition tool).

The results of the current non-target study for two biological matrices, mussel's soft tissue and haemolymph are presented in Tables 4 and 5.

**TABLE 4**
Priority mixtures of contaminants identified in mussel's soft tissue.

| Sampling site | ESI | Experimental m/z | RT (min) | Theorical m/z | Δ ppm | Experimental molecular formula of ion[a] | Tentative identification[b] | p value | Levels[e] |
|---|---|---|---|---|---|---|---|---|---|
| BAP2 | + | 291.1458 | 2.21 | 290.1379 | -1.97 | $C_{14}H_{19}N_4O_3$ | Trimethoprim* | 7.50E-03 | ↓ |
| | | 279.1963 | 10.73 | 278.1882 | -2.59 | $C_{17}H_{27}O_3$ | Irganox degradate* | 8.40E-03 | ≈ |
| | | 229.1437 | 5.89 | 228.1362 | -1.79 | $C_{12}H_{20}O_4$ | Dibutyl maleate* | 1.00E-02 | ↓ |
| | | 149.0603 | 11.23 | 148.0524 | -3.57 | $C_9H_9O_2$ | 1-Phenylpropane-1,2-dione | 1.50E-02 | |
| | | 219.1961 | 9.18 | 218.1882 | -2.71 | $C_{12}H_{27}O_3$ | Diethylene Glycol Dibutyl Ether* | 3.90E-02 | ↑ |
| | - | 193.0710 | 1.95 | 194.0787 | 1.87 | $C_7H_{13}O_6$ | (-)-Quebrachitol* | 4.20E-03 | ↓ |
| | | 233.1182 | 7.17 | 234.1254 | 1.01 | $C_{14}H_{17}O_3$ | Stiripentol* | 5.80E-03 | ≈ |
| | | 180.0328 | 2.22 | 181.0409 | 3.93 | $C_5H_{10}NO_4S$ | Acamprosate* | 1.00E-02 | ↓ |
| BAP3 | + | 259.1310 | 8.79 | 236.1412 | 2.24 | $C_{14}H_{20}O_3Na$[b] | Heptylparaben* | 3.30E-05 | ≈ |
| | | 228.2329 | 13.16 | 227.2249 | -3.15 | $C_{14}H_{30}NO$ | N-Dimethyldodecanamide* | 7.50E-03 | ≈ |
| | | 297.0588 | 12.36 | 296.0509 | -2.77 | $C_{10}H_{21}N_2S_4$ | Tetraethylthiuram Disulfide* | 3.40E-02 | ↑ |
| | - | 253.0926 | 1.95 | 194.0790 | 1.87 | $C_9H_{17}O_8$[c] | (-)-Quebrachitol* | 1.30E-02 | ↓ |
| BFP1 | + | 228.2328 | 13.20 | 227.2249 | -2.66 | $C_{14}H_{30}NO$ | N-Dimethyldodecanamide | 1.20E-06 | |
| | | 223.1445 | 2.09 | 222.1368 | -1.58 | $C_{12}H_{19}N_2O_2$ | Isophorone Diisocyanate* | 6.40E-05 | ↓ |
| | | 247.1056 | 5.39 | 224.1161 | -1.37 | $C_{11}H_{16}N_2O_3Na$[b] | Butalbital | 9.00E-05 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 230.2485 | 15.99 | 229.2406 | -3 | $C_{14}H_{32}NO$ | N,N-Dimethyldodecylamine N-oxide | 5.90E-04 | |
| | | 108.0428 | 1.86 | 107.0351 | -2.8 | $C_7H_{12}N_4O_4$[c] | Etanidazole* | 7.30E-04 | ↑ |
| | | 429.3196 | 15.99 | 428.3138 | 3.53 | $C_{24}H_{45}O_6$ | Glycerol triheptanoate* | 1.30E-03 | ≈ |
| | | 291.1458 | 2.16 | 290.1379 | -1.99 | $C_{14}H_{19}N_4O_3$ | Trimethoprim* | 2.10E-02 | ↓ |
| | | 253.0927 | 2.01 | 194.0790 | 1.85 | $C_9H_{17}O_8$[c] | (-)-Quebrachitol* | 1.00E-04 | ↓ |
| | | 247.1338 | 15.04 | 248.1412 | 0.32 | $C_{15}H_{19}O_3$ | Amiloxate* | 3.10E-04 | ↓ |
| | - | 209.0846 | 4.32 | 210.0920 | 2.93 | $C_8H_{17}O_4S$ | 2-Ethylhexyl hydrogen sulfate* | 2.10E-03 | ≈ |
| | | 172.0064 | 2.01 | 173.0147 | 4.81 | $C_6H_6NO_3S$ | Sulfanilic acid* | 6.60E-03 | ≈ |
| | | 216.1598 | 15.21 | 215.1521 | -2.69 | $C_{11}H_{22}NO_3$ | Hexaminolevulinate | 3.50E-05 | |
| | | 230.1756 | 15.09 | 229.1678 | -2.62 | $C_{12}H_{24}NO_3$ | Icaridin | 2.40E-04 | |
| | | 279.1497 | 6.74 | 278.1419 | -1.36 | $C_{18}H_{19}N_2O$ | Demexiptiline | 4.30E-04 | |
| | + | 223.1445 | 2.06 | 222.1368 | -1.59 | $C_{12}H_{19}N_2O_2$ | Isophorone Diisocyanate* | 2.70E-03 | ↓ |
| | | 112.8965 | 1.63 | 111.8886 | -4.57 | HSSe | Selsun | 3.60E-03 | |
| **BFP2** | | 195.1386 | 15.09 | 194.1307 | -3.09 | $C_{12}H_{19}O_2$ | Hexylresorcinol | 1.60E-02 | |
| | | 291.1458 | 2.14 | 290.1379 | -1.96 | $C_{14}H_{19}N_4O_3$ | Trimethoprim* | 2.10E-02 | ≈ |
| | | 209.0846 | 4.25 | 210.0926 | 2.84 | $C_8H_{17}O_4S$ | 2-Ethylhexyl hydrogen sulfate* | 1.50E-03 | ≈ |
| | - | 293.1766 | 10.98 | 294.1831 | -1.82 | $C_{17}H_{25}O_4$ | [6]-Gingerol* | 1.80E-03 | ≈ |
| | | 193.0710 | 1.94 | 194.0790 | 1.85 | $C_7H_{13}O_6$ | (-)-Quebrachitol* | 2.10E-03 | ↓ |

([a]) Marker ions for +ESI and -ESI respectively: $[M+H]^+$ and $[M-H]^-$.
([b]) Marker ion = $[M+Na]^{+1}$

(c) Marker ion = $[M-H+HAc]^{+1}$
(d) Marker ion = $[M+2H]^{+2}$
(e) Levels of contaminants in exposed samples taking compared to BAP1 (↑ higher levels, ↓ lower levels, ≈ similar levels).
(*) Xenobiotics present in control group BAP1.

**TABLE 5**
Priority mixtures of contaminants identified in mussel's haemolymph.

| Sampling site | ESI | Experimental m/z | RT (min) | Theorical m/z | Δ ppm | Experimental molecular formula of ion[a] | Tentative identification | p value | Levels[b] |
|---|---|---|---|---|---|---|---|---|---|
| BAP2 | + | 146.0612 | 4.99 | 146.0606 | -1.11 | $C_9H_8NO$ | 1 (2H) - Isoquinolinone | 1.10E-06 | |
| | | 120.0810 | 3.12 | 120.0813 | -2.94 | $C_8H_{10}N$ | 3-Vinylaniline | 4.40E-02 | |
| | - | 235.0999 | 4.54 | 235.1002 | 2.59 | $C_9H_{19}O_5Si$ | Silquest A-187 | 3.40E-03 | |
| | | 350.1635 | 4.12 | 350.1617 | -3.34 | $C_{19}H_{20}N_5O_2$ | Pirenzepine | 9.90E-03 | |
| | | 209.0644 | 4.37 | 209.0636 | -0.89 | $C_{11}H_{13}O_2S$ | Thiolactomycin | 3.80E-02 | |
| BAP3 | + | 164.1073 | 4.82 | 164.1076 | 1.46 | $C_{10}H_{14}NO$ | Methcathionone | 1.40E-03 | |
| | | 277.1797 | 13.70 | 277.1804 | 1.26 | $C_{17}H_{25}O_3$ | Cyclandelate* | 4.50E-02 | ≈ |
| | - | 218.9611 | 3.69 | 218.9616 | 3.89 | $C_8H_5Cl_2O_3$ | 2,4-Dichlorophenoxyacetic acid | 2.90E-10 | |
| | | 195.0687 | 3.96 | 195.0689 | 4.11 | $C_{10}H_{12}ClN_2$ | meta-Chlorophenylpiperazine | 1.80E-04 | |
| BFP1 | + | 146.0602 | 4.97 | 146.0606 | -1 | $C_9H_8NO$ | 1 (2H)-Isoquinolinone | 1.30E-03 | |
| | | 128.1072 | 4.25 | 128.1075 | -2.13 | $C_7H_{14}NO$ | N-Methylcaprolactam* | 6.00E-03 | ≈ |
| | | 204.1384 | 12.73 | 204.1388 | -0.06 | $C_{13}H_{18}NO$ | 2,6-Diisopropylphenyl-isocyanate* | 1.40E-02 | ≈ |
| | | 172.1696 | 6.70 | 172.1701 | -0.66 | $C_{10}H_{22}NO$ | N,N-Dibutylacetamide* | 2.00E-02 | ≈ |
| | | 109.0653 | 6.96 | 109.0653 | -4.75 | $C_7H_9O$ | Anisole* | 2.50E-02 | ≈ |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 183.0785 | 5.00 | 183.0786 | -1.35 | $C_6H_{16}O_4P$ | Diisopropylphosphate* | 4.30E-02 | ↓ |
| | | 192.1384 | 7.15 | 192.1388 | -0.54 | $C_{12}H_{18}NO$ | Diethyl-2-phenylacetamide* | 4.50E-02 | ≈ |
| | - | 235.1001 | 4.49 | 235.1002 | 2.69 | $C_9H_{19}O_5Si$ | Silquest A-187 | 1.10E-02 | |
| | | 128.1072 | 4.25 | 128.1075 | 2.11 | $C_7H_{14}NO$ | N-Methylcaprolactam* | 2.30E-04 | ↓ |
| | | 109.0653 | 6.96 | 109.0653 | -4.75 | $C_7H_9O$ | Anisole* | 7.70E-03 | ≈ |
| | | 172.1696 | 6.70 | 172.1701 | -0.65 | $C_{10}H_{22}NO$ | N,N-dibutylacetamide* | 7.80E-03 | ↓ |
| **BFP2** | + | 192.1384 | 7.15 | 192.1388 | -0.45 | $C_{12}H_{18}NO$ | Diethyl-2-phenylacetamide*<br>Phendimetrazine* | 9.30E-03 | ↓ |
| | | 204.1384 | 12.73 | 204.1388 | -0.06 | $C_{13}H_{18}NO$ | 2,6-Diisopropylphenyl-isocyanate* | 1.60E-02 | ≈ |
| | | 158.1541 | 7.71 | 158.1545 | -0.95 | $C_9H_{20}NO$ | Tetramethyl piperidylol* | 2.50E-02 | ≈ |

([a]) Marker ions for +ESI and -ESI respectively: $[M+H]^+$ and $[M-H]^-$.
([b]) Levels of contaminants in exposed samples taking compared to BAP1 (↑ higher levels , ↓ lower levels, ≈ similar levels).
(*) Xenobiotics present in control group BAP1.

## 6.1. CONTAMINANTS MIXTURES IN EBRO DELTA

The potential components of the xenobiotic's "cocktail" present in mussels from Ebro Delta (both in soft tissue and haemolymph serum samples) are shown in Tables 4 and 5, respectively.

The results obtained have shown that a different number of exogenous compounds are part of the mixture of contaminants accumulated in haemolymph and soft tissue samples: 17 and 25 hazardous substances identified, respectively. A higher number of xenobiotics that were identified in soft tissue may be related to the greater bioaccumulation capacity of certain chemicals; such as those ones of non-polar nature and higher octanol-water partition coefficient (LogKow). No coincidence is observed between those compounds identified in the two mussel matrices. Nonetheless, similarities in their chemical structures are noticed since most of them contain an aromatic benzene ring (concretely 11/17 in haemolymph serum and 10/25 in mussel tissue). Besides, heteroatoms such as sulphur have been scarcely found in molluscs specimens according to the results presented. Notice that only haemolymph matrix reveals the presence of chlorinated and phosphorus compounds (2,4-dichlorophenoxyacetic acid, meta-chlorophenylpiperazine and diisopropylphosphate).

Overall, identified compounds in haemolymph samples were characterized by compact structures and polar functional groups, whereas the ones found in soft tissue presented bigger dimensions with large aliphatic chains, which reduce polarity and increase LogKow favouring accumulation.

Almost all xenobiotic compounds identified are commonly found in our daily life, including plasticizers (e.g., N,N-dibutylacetamide and N-dimethyldodecanamide), pharmaceuticals (e.g., thiolactomycin and trimethoprim) and personal care products (e.g. diethyl-2-phenylacetamide and heptylparaben). In fact, chemicals identified cover a wide range of applications. Hence, a simple classification based on their use has been done in order to identify the contamination sources at Ebro Delta:

- Pharmaceuticals: medical and recreational uses (addictive drugs)
- Pesticides
- Industrial uses, including plasticizers, surfactants and coating materials
- Personal care products (e.g., shampoo and sunscreen)
- Natural products

## 6.2. CONTAMINATION DIFFERENCES BETWEEN BAYS

### 6.2.1. Mussel's soft tissue

Exogenous compounds identified in soft tissue confirm the contamination pattern detected in Ebro Delta by previous studies [18] : Fangar bay may be more affected by anthropogenic inputs.

Findings in mussel's soft tissue referring to contaminant mixtures in southern bay (Alfacs) reveal that 11 different compounds have a predominant impact on these specimens. Concretely, the mixture of xenobiotics predominant in this bay was formed by trimethoprim, irganox degradate, dibutyl maleate, 1-phenylpropane-1,2-dione, diethylene glycol dibutyl ether, (-)-quebrachitol, stiripentol, acamprosate, heptylparaben, N-dimethyldodecanamide and tetraethylthiuram disulphide (details presented in Table 4).

First, trimethoprim, stiripentol and acamprosate are classified as pharmaceuticals, 1-phenylpropane-1, 2-dione as a flavouring ingredient and heptylparaben as a personal care product. All these compounds could come either from wastewater discharges or municipal wastewater effluents since sampling points in Alfacs bay are close to these contamination sources.

Second, diethylene glycol dibutyl ether, N-dimethyldodecanamide and tetraethylthiuram disulphide are commonly applied in commercial products for plants protection. According to previous studies in Ebro Delta, agricultural runoff is highlighted as the main source of pesticides and herbicides contamination in this area [9], [12], [15].

Lastly, both irganox degradate and dibutyl maleate are linked to plastics industry, since they are involved in polymerization processes. Consequently, inputs from industrial activities might be contributing to their presence in mussel's soft tissue. Besides, dibutyl maleate also acts as a comonomer in paints and adhesives production and thus might be employed in boats coating. So, another source of contamination of this compound may be maritime traffic.

Regarding contamination patterns in Fangar bay, 17 xenobiotics were found in mussel's soft tissue and were classified as follows:

- Pharmaceuticals: butalbital, etanidazole, sulfanilic acid, hexaminolevulinate, demexiptiline and trimethoprim.
- Pesticides: N-dimethyldodecanamide and glycerol triheptanoate

- Personal care products: N,N-dimethyldodecylamine N-oxide, amiloxate, 2-ethylhexyl hydrogen sulphate, icaridin, selsun and hexylsorcitol.
- Industrial uses: isophorone diisocyanate.
- Natural products: (-)-quebrachitol and [6]-gingerol.

According to this classification, pharmaceuticals, natural and personal care products may be related to industrial and urban inputs. Interestingly, previous studies reported that (-)-quebrachitol was found in black rice [25], [26]. Therefore, this compound is related to agricultural activities in rice fields located in Fangar bay. Pesticides bioaccumulated by mussels are bounded to agrochemical runoff as previously stated for Alfacs bay.

On the other hand, isophorone diisocyanate is used in coating dyes due to its abrasion resistance. Although the maritime traffic is less notorious in Fangar bay, the presence of isophorone diisocyanate in mussel's soft tissue may suggest the growth of tourism and recreational activities in the area.

It deserves to be mentioned that (-)-quebrachitol, N-dimethyldodecanamide and trimethoprim have been detected in both bays. Therefore, the presence of (-)-quebrachitol and N-dimethyldodecanamide in both bays verifies the environmental impact caused by agricultural sector.

Regarding the complexity of the mixture and number of contaminants identified in each bay, the results obtained here are in agreement with previous target studies where higher complexity of the mixture of pollutants was found in northern bay (Fangar). Terrado et al. (2018) detected between 1 and 5 different contaminants accumulated in mussels tissue's from Alfacs bay, while Fangar's samples presented 8 or 9 different compounds in the mixture [18]. In the present research 11 different contaminants have been identified in Alfacs while 17 in Fangar supporting the same hypothesis. These observations are also supported by the smaller dimensions of Fangar bay compared to Alfacs bay. Thus, the movement of marine waters is limited (lower water exchanged with open sea) and the accumulation of pollutants and residues is promoted.

### 6.2.2. Haemolymph matrix

Significant different patterns of contamination have been also found in Alfacs and Fangar bays, according to the analysis of the mussel's haemolymph (Table 5).

On the one hand, 9 compounds were identified in Alfacs bay: 1 (2H)-isoquinolinone, 3-vinylalanine, silquest A-187, pirenzepine, thiolactomycin, methcathionone, cyclandelate, meta-chlorophenylpiperazine (mCPP) and 2,4-dichlorophenoxyacetic acid (2,4-D). These xenobiotics detected in bivalve's haemolymph serum can be divided in four categories:

- Pharmaceuticals: 1 (2H)-isoquinolinone, pirenzepine, thiolactomycin, cyclandelate and mCPP.
- Stimulant drugs: methcathionone.
- Herbicides: 2,4-D.
- Industrial uses: 3-vinylalanine and silquest A-187.

Substances such as silquest A-187 can be present in paints employed in boat surfaces as coating for chemical and corrosion resistance. The occurrence of this xenobiotic in haemolymph serum can be related to marine traffic in the area that is certainly active in both Alfacs harbour (located in Sant Carles de la Ràpita) and Alcanar pier (close to BAP1 sampling site). 2,4-D is commonly found in pesticides, which would explain the environmental impact of agricultural runoff from rice crops in this area. Nonetheless, pharmaceuticals and recreational drugs is the group with more representative compounds in this bay. The occurrence of this group of chemicals can be related to inputs from urban and recreational activities.

On the other hand, 10 exogenous compounds were identified in Fangar bay: 1 (2H)-isoquinolinone, N-methylcaprolactam, 2,6-diisopropylphenyl-isocyanate, N,N-dibutylacetamide, anisole, diisopropylphosphate, diethyl-2-phenylacetamide, silquest A-187, phendimetrazine and tetramethyl piperidylol. These substances present multiple applications as well:

- Pharmaceuticals: 1 (2H)-isoquinolinone, phendimetrazine, diisopropylphosphate and tetramethyl piperidylol.
- Pesticides: anisole.
- Industrial uses: N-methylcaprolactam, 2,6-diisopropylphenyl-isocyanate, N,N-dibutylacetamide and silquest A-187.
- Personal care products: diethyl-2-phenylacetamide.

Almost all compounds presented might be bounded to industrial (highlighting polymer production) and medical uses. This phenomenon agrees with the fact that sampling sites selected are placed near wastewater discharges from industrial and urban activities. Both anisole and silquest A-187 might be an exception to this trend. Anisole presence in mussel's

haemolymph could be explained by the fact that agricultural waste from the rice crops treated with pesticides is released into Ebro Delta bays. Marine traffic may explain silquest A-187 occurrence as it has been previously commented.

Finally, both bays presented 2 contaminants in common: 1 (2H)-isoquinolinone, natural product used to synthetize drugs, and silquest A-187, organic compound previously found in adhesives and coating materials.

## 6.3. CONTAMINATION DIFFERENCES BETWEEN SAMPLING POINTS IN THE SAME BAY

### 6.3.1. Mussel's soft tissue

- **Alfacs bay**

Considering our findings (Table 4), as well as the results reported Terrado et al. [18], notable differences appeared between sampling points located in the same bay.

Firstly, the xenobiotics presenting a major accumulation in soft tissue of wild mussels in BAP2 site were: trimethoprim, irganox degradate, dibutyl maleate, 1-phenylpropane-1,2-dione, diethylene glycol dibutyl ether, (-)-quebrachitol, stiripentol and acamprosate.

According to the classification presented before (section 6.2.1), pharmaceuticals, pesticides, natural products, flavouring agents and compounds used in plastics production were the families of contaminants accumulated in BAP2. Concretely, pharmaceuticals were the main pollution source in BAP2 sampling point (3/8 drugs found).

Secondly, heptylparaben (personal care product), (-)-quebrachitol (natural product found in rice) together with N-dimethyldodecanamide and tetraethylthiuram disulfide (pesticides) form the priority mixtures of contaminants detected in BAP3 sampling point.

According to the present results, BAP3 sampling site is more affected by pollutants that come directly from the drainage of rice crops. These observations have general similarities to previous target study performed in XENOMETABOLOMIC project that detected and quantified pesticides and herbicides in both sampling sites, BAP2 and BAP3 (for instance, bentazone showed appreciable levels in bivalves) [18]. Our results also agree with this study that demonstrated wide variety of contaminants in BAP2 in comparison to BAP3. Levels of contaminants cannot be mentioned in the present study due to the lack of quantification process.

Finally, almost all of the xenobiotics found in exposed groups (BAP2 and BAP3) are also present in the sampling point considered as a reference (BAP1) (Table 4). Whereas some xenobiotics such as diethylene glycol dibutyl ether and tetraethylthiuram disulphide have been detected at higher levels in exposed samples (and thus lower levels in control), some others like trimethoprim, dibutyl maleate, (-)-quebrachitol and acamprosate have been notably found in control samples. In contrast, irganox degradate, stiripentol, heptylparaben and N-dimethyldodecamide presented similar levels in both exposed and control groups. Previous detection of methylparaben, another compound from parabens family, in fish and bivalves support our results regarding heptylparaben [18], [19], [27].

Among others, trimethoprim would cause controversial effects, like the development of bacterial resistance, in marine ecosystem [28]. Additionally, the occurrence of this contaminant in wild mussels has been reported in Atlantic Northeast and Estern-Central Pacific by McEneff et al. and Klosterhaus et al. [29], [30].

- **Fangar bay**

Taking into consideration the results in soft tissue analysis (Table 4) certain xenobiotics would be damaging both sampling sites located in Fangar bay. However, the similarity in contamination patters is not quite clear.

First of all, xenobiotics identified in BFP1 presented wide variety of applications: medical uses (butalbital, etanidazole and trimethoprim), products used for rice crops maintenance (N-dimethyldodecanamide and glycerol triheptanoate), personal care products (N,N,dimethyldodecylamine N-oxide, amiloxate and 2-ethylhehyl hydrogen sulphate), coating paints for boats (isophorone diisocyanate) and natural products ((-)-quebrachitol). Products used to treat pests and personal care products would be the ones causing a bigger impact in mussels in this sampling site, though. Concretely, a substance with surfactant and carcirogenic properties that may threat wild organisms safety has been identified: 2-ethylhexyl hydrogen sulphate. For this reason, future monitoring programmes are required urgently in order to check its presence and evaluate its potential risk to animals and human health.

Second, BFP2 samples presented chemical contaminants from different families as well, but the most remarkable ones might be personal care products and pharmaceuticals. Specifically, 4/10 substances identified were classified as personal care products (icaridin, selsun,

hexylresorcinol and 2-ethylhexyl hydrogen sulphate), including shampoos, insect repellents and sunscreen agents, whereas 3/10 compounds were described as drugs (hexaminolevulinate, demexiptiline and trimethoprim). The three compounds remaining were considered either natural products ([6]-gingerol and (-)-quebrachitol) or synthetic materials (isophorone diisocyanate mainly used in coating paints).

Notice that both sampling sites in Fangar bay presented 4 contaminants in common: (-)-quebrachitol, isophorone diisocyanate, trimethoprim and 2-ethylhexyl hydrogen sulphate. This fact may reflect the environmental impact in coastal areas promoted by urban and agricultural inputs.

According to Table 4 (soft tissue), the following contaminants are also present in the control group: isophorone diisocyanate, etanidazole, glycerol triheptanoate, trimethoprim, (-)-quebrachitol, amiloxate, 2-ethylhexyl hydrogen sulphate, sulfanilic acid, selsun, hexylsorcitol and [6]-gingerol. The levels of the respective xenobiotics are detailed in Table 4 as well.

Lastly, the presence of demexiptiline in BFP2 samples requires special attention since a investigation demonstrate that tricyclic antidepressants induce therapeutic adverse effects in fish [31].

### 6.3.2. Haemolymph matrix

- **Alfacs bay**

Regarding contamination patterns in Alfacs bay, notable differences have been found between two sampling sites selected, BAP2 and BAP3 (Table 5).

On the one hand, priority mixtures of pollutants present in BAP2 include both pharmaceuticals (1 (2H)-isoquinolinone, pirenzepine and thiolactomycin) and products with industrial applications (silquest A-187 and 3-vinylaniline).

On the other hand, two classes of xenobiotics were detected in BAP3 sampling point too: drugs (methcathionone, cyclandelate and mCPP) and herbicides (2,4-D).

Despite our study is mainly focused on the determination of xenobiotics in biota samples, it seems appropriate to mention the presence of mCPP in BAP5 (metabolite of antidepressant drug known by its commercial name Trazodona). This finding is consistent with a previous study

carried out with mussels from Ebro Delta, where venlafaxine (antidepressant drug) and its metabolite were detected [19].

Although almost all compounds are classified as pharmaceuticals (derived from WWTPs efllluents) in Alfacs, their chemical structures and properties are quite different. Further multivariate data analysis (section 6.4.) will elucidate these observations.

Note that the vasodilator known by its chemical name "cyclandelate" has been identified in both exposed and control mussel's haemolymph (BAP3 and BAP1 sampling sites). Similar bioaccumulation levels of this contaminant have been detected in these two sampling points.

- **Fangar bay**

Our results show that the potential mixtures of chemicals to which marine bivalves are exposed to in both sampling points located in the northern bay are quite similar (Table 5).

Contaminant mixtures present in BFP1 are basically composed by pharmaceuticals (1 (2H)-isoquinolinone and diisopropylphosphate), manufacturing facilities (N-methylcaprolactam, 2,6-diisopropylphenyl-isocyanate, silquest A-187 and N,N-dibutylacetamide), pesticides (anisole) and personal care products (diethyl-2-phenylacetamide).

Compounds identified in BFP3 are classified according to their uses in a similar way to the ones found in BFP1: medical uses (phendimetrazine and tetramethyl piperidylol), industrial applications (N-methylcaprolactam, 2,6-diisopropylphenyl-isocyanate and N,N-dibutylacetamide), pesticides (anisole) and personal care products (diethyl-2-phenylacetamide).

In contrast to the contamination pattern observed in Alfacs, where difference in contamination among sampling sites were clear, Fangar bay presents a uniform behaviour in chemical pollution terms. Indeed, most exogenous compounds are detected in both sampling points, BFP1 and BFP2 (concretely, 5 substances in common). The explanation to this fact lies in the reduced dimensions of the northern bay, as it has been mentioned before. This approach will be confirmed further in data statistical analysis section (following section 6.4.).

Interestingly, diethyl-2-phenylacetamide, an insect repellent whose presence in bivalves has not been reported previously in Ebro Delta, was detected in both sampling sites of Fangar bay. Nonetheless, the tentative identification proposed by Compound Discovered seems to be reasonable since Ebro Delta is characterised by warm weather and high humidity levels. Consequently, among other insects, mosquitoes are frequently found in this area [32].

Last but not least, chemicals such as N-methylcaprolactam, 2,6-diisopropylphenyl-isocyanate, N,N-dibutylacetamide, anisole, diisopropylphosphate, diethyl-2-phenylacetamide, phendimetrazine and tetramethyl piperidylol have been found at similar or higher levels in mussel's haemolymph collected from the control sampling site (Table 5).

## 6.4. STATISTICAL ANALYSIS

PCA performed with all the replicates and classes including control and exposed groups from both bays showed 4 outliers, 2 from BAP2 sampling site (evaluation though Hotelling $T^2$ test at the 95% confidence) and 2 from BAP1 (Appendix 5). After excluding the 2 outliers from BAP2 a similar separation of the groups was obtained (Fig. 2) with 2 samples from BAP1 remaining as extreme values but not considered as anomalous. Therefore, it was decided to keep them in the model.

Significant class separations in PCA scores plot were found applying a model with 2 principal components which explained a total of 56.70% data variability. Concretely, PC1 axis explains 41.4% of data variability while PC2 only explains 15.3%. PC1 axis elucidates clear separation between exposed and control samples and, at the same time, different contamination patterns between each bay and the control site. Complementary, PC2 axis allows to distinguish differences in the contaminants mixtures between sampling sites in the same bay. The figure shows a clear separation between BAP2 and BAP3 indicating different contamination pattern, while BFP1 and BFP2 cluster together with some overlapping, which indicates similar contamination pattern in both sampling points as previously explained.

The scores plot (Fig. 2) shows a clear separation between control and exposed samples. Hence, the hypothesis established in this work of considering BAP1 as control site because there wasn't "a priori" relevant contaminants inputs was appropriate to study pollution patterns of other specific areas in Ebro Delta.

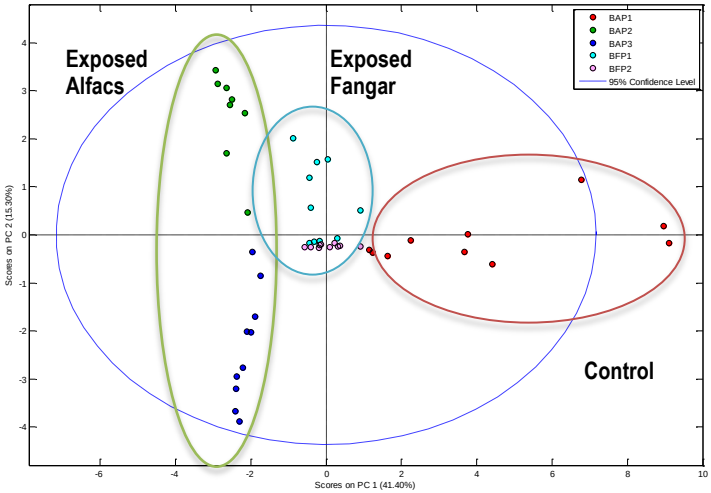


**Fig. 2** Principal component analysis (PCA) scores plot, illustrating the different contamination profiles of the sampling sites selected in Ebro Delta (Alfacs bay: BAP1, BAP2, BAP3 and Fangar bay: BFP1 and BFP2). 3 groups detected: exposed mussel's from Alfacs bay (green), exposed mussel's from Fangar bay (blue) and control specimens from Alfacs bay (red).

The loadings plot (Fig. 3) has been also evaluated to determine which xenobiotics contributed to the separation of contaminant-exposed and control classes in the PCA scores plot. Parameters that present a larger vector module are considered essential for the separation between the controls and the exposed groups.

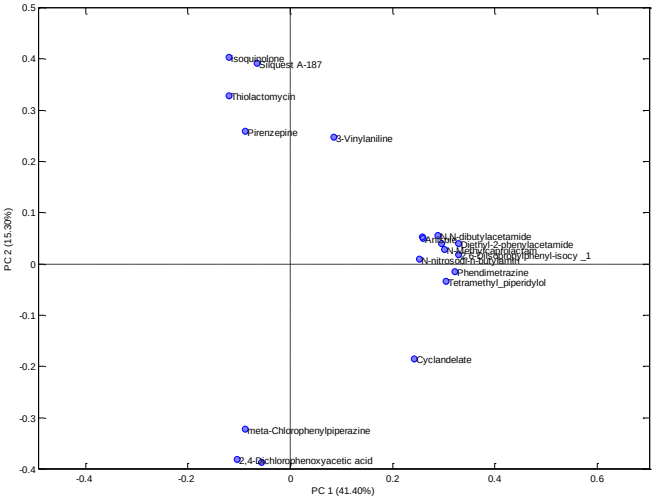


**Fig. 3.** Principal components analysis (PCA) loadings plot. The percentage of explained variation of the first two components considered in the model is displayed on the relative axes.

Therefore, loadings representation support what has been detailed in previous sections for haemolymph analysis:

- BAP2 is highly influenced by pharmaceuticals (thiolactomycin, pirenzepine and isoquinolone).
- BAP3 by organochlorine contaminants such as 2,4-dichlorophenoxyacetic acid and m-chlorophenylpiperazine which are proposed as the main responsible of contamination in this sampling site inside Alfacs bay.
- BFP1, BFP2 and BAP1 present similar contamination profiles including compounds such as: N,N-dibutylacetamide, diethyl-2-phenylacetamide, anisole, N-methylcaprolactam, N-nitrosodi-n-butylamine, 2,6-diisopropylphenyl-isocyanate, phendimetrazine, tetramethyl-piperidylol, isophorone and diisopropylphosphate.

Finally, taking into consideration the existing correlations shown in loadings plot, we conclude that there's no relationship between Alfacs and Fangar contamination patters (the angle described by the vectors is approximately 90º). Besides, BAP2 and BAP3 are indirectly correlated.

# 7. CONCLUSIONS

Priority mixtures of organic contaminants accumulated in mussels from Ebro Delta have been identified in the present research by using non-target analysis. Overall the results obtained demonstrate the bioaccumulation of different mixtures of xenobiotics in wild mussels collected from polluted and initially considered "control" sites. Besides, this research has allowed the detection of 17 and 25 contaminants in mussel haemolymph and soft tissue, respectively, collected from two bays located in Ebro Delta (Alfacs and Fangar). Details about the identity of the mixtures of contaminants found are shown in Tables 4 and 5.

According to results obtained, pesticides and herbicides, which are used for rice crops maintenance, together with pharmaceutically active compounds were considered the main contaminants groups accounting for the mixtures of pollutants present in study area. Furthermore, clear trends were observed in contamination patterns between Alfacs and Fangar bays using principal components analysis (PCA). While southern bay (Alfacs) seems to be most vulnerable to waste water effluents, which provide wide variety of pharmaceuticals, the northern one (Fangar) does not present a specific pattern of contamination. In contrast, Fangar bay presents a complex pollution profile since it is potentially affected by wastewater discharges and inputs from industrial activities. In addition, both bays present a common source of pollution: draining channels discharging the output water from the rice fields.

Taking into consideration the simplicity of the sample treatment and that it is a non-invasive extraction technique (the animal doesn't need to be sacrificed for the analysis), haemolymph matrix would considered as adequate in order to provide qualitative information about contaminants mixtures present in marine ecosystem. However, bioaccumulation of certain substances cannot be reflected in the analysis of this matrix (particularly less polar ones). For that reason, a complementary study in soft tissue is highly recommended to elucidate different types of contaminants present in the environment that are bioaccumulated in bivalves. Thus,

information collected from both matrices provides more wide and representative information about contaminants mixtures present in marine bivalves and their sources.

Our findings demonstrate the usefulness of non-target analysis to explain the complexity of anthropogenic mixtures present in biota samples. However, the combination of target and non-target studies is essential to assess stressors of potential concern in aquatic ecosystems, since uncontrolled ecological parameters may hinder their investigation. Additionally, laboratory experiments are suitable to study carefully the exposure of potential mixtures of contaminants and their effects in target organisms under controlled conditions.

Future research needs to be conducted in order to further confirm the identities of the contaminants tentatively identified here. This can be done either by comparison with authentic standards or by collision induced dissociation (CID). Besides, the study of alterations in metabolic pathways that occur in natural ecosystems might be required to evaluate toxicity and negative effects of xenobiotics in both marine wildlife and human health.

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# 9. ACRONYMS

| | | | |
|---|---|---|---|
| **2,4-D** | 2,4-Dichlorophenoxyacetic acid | **MRL** | Maximum residue limit |
| **ACN** | Acetonitrile | **MS** | Mass spectrometry |
| **BAPx** | Alfacs bay sampling point | **OCIs** | Organochlorine compounds |
| **BFPx** | Fangar bay sampling point | **OCPs** | Organochlorine pesticides |
| **DDT** | Dichlorodiphenyltrichloroethane | **OPs** | Organophosphorous pesticides |
| **dSPE** | Dispersive solid phase extraction | **PAHs** | Polycyclic aromatic hydrocarbons |
| **DW** | Dry weight | **PCA** | Principal component analysis |
| **EDC** | Endocrine disruptor compound | **PCBs** | Polychlorinated biphenils |
| **ESI** | Electrospray ionization | **PCPs** | Personal Care Products |
| **HHCB** | Galaxolide | **PCTs** | Polychlorinated terphenyls |
| **HRMS** | High resolution mass spectrometry | **PHACs** | Pharmaceutically active compounds |
| **HPLC** | High performance liquid chromatography | **PLS-DA** | Partial Least Discriminant Square Analysis |
| **IS** | Internal standard | **QC** | Quality control |
| **LLE** | Liquid-liquid extraction | **TBEP** | Tris(2-butoxyethyl)phosphate |
| **MCPA** | (4-chloro-2-methylphenoxy)acetic acid | **UHPLC** | Ultra-high performance liquid chromatography |
| **mCPP** | meta-chlorophenylpiperazine | **WWTPs** | Waste Water Treatment Plants |

# APPENDICES

# APPENDIX 1: UHPLC-HRMS INFORMATION

**TABLE 6**
Isotopically labelled standards used in mussel's soft tissue analysis, organized by their family, ionization mode, precursor ions and retention time.

| Chemical family | ESI | Internal Standard | m/z | RT (min) |
|---|---|---|---|---|
| Organonitrogen pesticides | + | Atrazine-d5 | 221.1324 | 7.09 |
| | | Atrazine-d5 | 221.1324 | 7.07 |
| | | Metoalachlor-d6 | 290.1789 | 10.66 |
| | | Simazine-d10 | 212.1481 | 5.87 |
| Organophosphorus pesticides | + | Diazinon-d10 | 315.1710 | 12.82 |
| | | Malathion-d7 | 338.0875 | 10.31 |
| | | Thiabendazole-C13 | 208.0636 | 5.1 |
| Herbicides | - | Bentazon-d7 | 246.0926 | 2.48 |
| | | MCPA-d3 | 202.0342 | 4.04 |
| | | Propanil-d5 | 223.0261 | 8.39 |
| Insecticides | + | Acetamiprid-d3 | 226.0935 | 4.69 |
| | | Imidacloprid-d4 | 260.0850 | 4.6 |
| EDCs | + | Benzotriazole-d4 | 124.0814 | 4.15 |
| | - | Bisphenol A d-4 | 231.1325 | 6.45 |
| | + | Caffeine-d3 | 198.1064 | 3.59 |
| | | Ethylparaben-C13 | 171.0743 | 5.9 |
| | | Ethylparaben-C13 | 171.0743 | 5.9 |
| | - | Ethylparaben-C13 | 171.0743 | 5.9 |
| | | Triclosan-d3 | 289.9622 | 12.92 |
| | | Triclosan-d3 | 289.9622 | 12.92 |
| PhACs | + | Carbamazepine-d10 | 247.1653 | 5.46 |
| | | Sulfamethoxazole-d4 | 258.0844 | 4.83 |
| | | Venlafaxine-d6 | 284.2491 | 9.97 |

# APPENDIX 2: SAMPLING POINTS IN EBRO DELTA


**Fig. 4** Map of the Alfacs bay indicating the location of the sampling points (19/05/2019 via Google Earth).

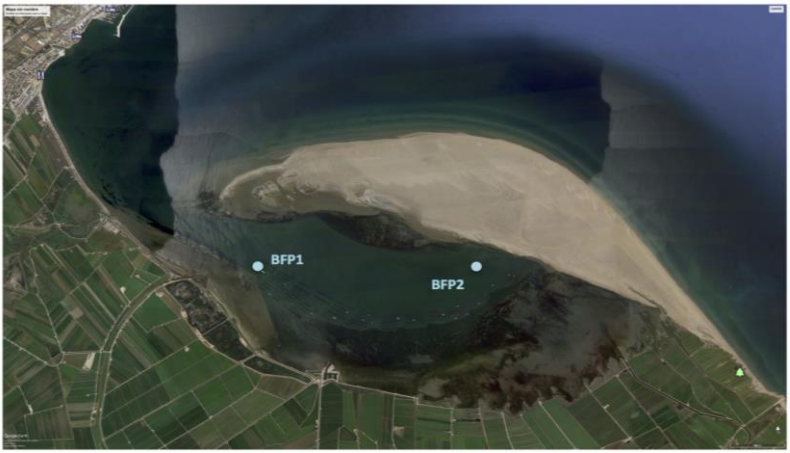


**Fig. 5** Map of the Fangar bay indicating the location of the sampling points (19/05/2019 via Google Earth).

# APPENDIX 3: DATA-PROCESSING INFORMATION

**TABLE 7**

Screening method employed for the identification of xenobiotics in mussel's haemolymph serum.

| Sampling site | ESI | All markers[1] | Significant markers[2] | Markers with chemical formula [3] | Exogenous markers[4] | Tentative identified markers [5] |
|---|---|---|---|---|---|---|
| **BAP2** | + | 14,225 | 680 | 18 | 5 | 2 |
| | - | 11,060 | 323 | 17 | 4 | 3 |
| **BAP3** | + | 14,118 | 1,617 | 32 | 5 | 2 |
| | - | 10,798 | 291 | 20 | 3 | 2 |
| **BFP1** | + | 14,374 | 1,476 | 42 | 16 | 7 |
| | - | 11,094 | 546 | 24 | 2 | 1 |
| **BFP2** | + | 14,397 | 1,808 | 45 | 18 | 7 |
| | - | 10,819 | 826 | 33 | 4 | 0 |

(1) All markers suggested by Compound Discoverer software.
(2) Significant markers with p value < 0.05.
(3) Markers with well-defined chromatographic peak, p value < 0.05 and error < 5ppm.
(4) Markers considered exogenous contaminants (endogenous metabolites excluded).
(5) Potential identities of markers double-checked using Xcalibur software (elemental composition tool).

# APPENDIX 4: MIXTURES OF CONTAMINANTS IN CONTROL GROUP

**TABLE 8**

Priority mixtures of contaminants identified in mussel's soft tissue collected from BAP1 (reference sampling site).

| Sampling site | ESI | Experimental m/z | RT (min) | Theorical m/z | Δ ppm | Experimental molecular formula of ion | Tentative identification |
|---|---|---|---|---|---|---|---|
| BAP1 | + | 237.1489 | 8.84 | 237.14908 | -2.21 | $C_{14}H_{21}O_3$ | Heptylparaben |
| | | 291.1457 | 2.21 | 291.14571 | -1.96 | $C_{14}H_{19}N_4O_3$ | Trimethoprim |
| | | 279.1963 | 10.73 | 279.19602 | -2.59 | $C_{17}H_{27}O_3$ | Irganox degradate |
| | | 229.1436 | 5.89 | 229.14399 | -1.79 | $C_{12}H_{20}O_4$ | Dibutyl maleate |
| | | 219.1960 | 9.18 | 219.19602 | -2.71 | $C_{12}H_{27}O_3$ | Diethylene Glycol Dibutyl Ether |
| | | 228.2328 | 13.16 | 228.23274 | -3.15 | $C_{14}H_{30}NO$ | N-Dimethyldodecanamide |
| | | 297.0588 | 12.36 | 297.05876 | -2.77 | $C_{10}H_{21}N_2S_4$ | Tetraethylthiuram Disulfide |
| | | 223.1445 | 2.09 | 223.14466 | -1.58 | $C_{12}H_{19}N_2O_2$ | Isophorone Diisocyanate |
| | | 108.042 | 1.86 | 108.042935 | -2.8 | $C_7H_{12}N_4O_4$ | Etanidazole |
| | | 429.3196 | 15.99 | 429.32161 | 3.53 | $C_{24}H_{45}O_6$ | Glycerol triheptanoate |
| | - | 193.0710 | 1.95 | 193.07084 | 1.85 | $C_7H_{15}O_6$ | (-)-Quebrachitol |
| | | 233.1181 | 7.17 | 233.11753 | 1.01 | $C_{14}H_{19}O_3$ | Stiripentol |
| | | 180.0327 | 2.22 | 180.03305 | 3.93 | $C_5H_{10}NO_4S$ | Acamprosate |
| | | 247.1338 | 15.04 | 247.13341 | 0.32 | $C_{15}H_{21}O_3$ | Amiloxate |
| | | 209.0846 | 4.32 | 209.08413 | 2.84 | $C_8H_{17}O_4S$ | 2-Ethylhexyl hydrogen sulfate |
| | | 172.0064 | 2.01 | 172.0068 | 4.81 | $C_6H_6NO_3S$ | sulfanilic acid |
| | | 293.1766 | 10.98 | 293.17528 | -1.82 | $C_{17}H_{25}O_4$ | [6]-Gingerol |

**TABLE 9**
Priority mixtures of contaminants identified in mussel's haemolymph collected from BAP1 (reference sampling site).

| Sampling site | ESI | Experimental m/z | RT (min) | Theorical m/z | Δ ppm | Experimental molecular formula of ion | Tentative identification |
|---|---|---|---|---|---|---|---|
| BAP1 | + | 277.1797 | 13.70 | 277.1804 | 1.26 | $C_{17}H_{25}O_3$ | Cyclandelate |
| | | 128.1072 | 4.25 | 128.1075 | -2.13 | $C_7H_{14}NO$ | N-Methylcaprolactam |
| | | 204.1384 | 12.73 | 204.1388 | -0.06 | $C_{13}H_{18}NO$ | 2,6-Diisopropylphenyl-isocyanate |
| | | 172.1696 | 6.70 | 172.1701 | -0.66 | $C_{10}H_{22}NO$ | N,N-dibutylacetamide |
| | | 109.0653 | 6.96 | 109.0653 | -4.75 | $C_7H_9O$ | Anisole |
| | | 139.1119 | 6.71 | 139.1123 | -0.88 | $C_9H_{15}O$ | Isophorone |
| | | 159.1494 | 9.77 | 159.1497 | -0.97 | $C_8H_{19}N_2O$ | N-nitrosodi-n-butylamin |
| | | 183.0785 | 5.00 | 183.0786 | -1.35 | $C_6H_{16}O_4P$ | Diisopropylphosphate |
| | | 192.1384 | 7.15 | 192.1388 | -0.45 | $C_{12}H_{18}NO$ | Diethyl-2-phenylacetamide<br>Phendimetrazine |
| | | 158.1541 | 7.71 | 158.1545 | -0.95 | $C_9H_{20}NO$ | Tetramethyl piperidylol |

# APPENDIX 5: PRINCIPAL COMPONENTS ANALYSIS INFORMATION

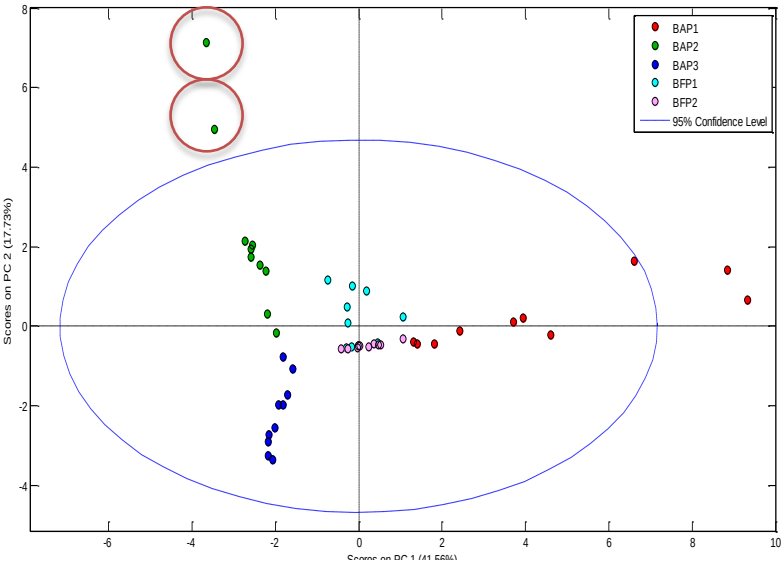


**Fig. 6** Principal component analysis (PCA) scores plot, illustrating the different contamination profiles of the sampling sites selected in Ebro Delta. All replicates from each sampling site are represented (n = 10).

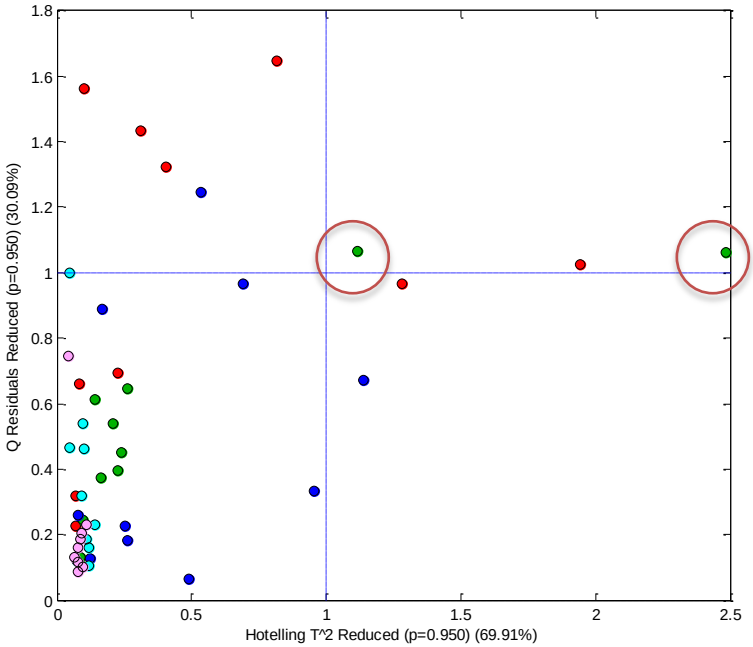


**Fig. 7** Hotelling $T^2$ test at the 95% confidence where 2 samples from BAP2 are presented as outliers.