# Venezia2021

# Programma di ricerca scientifica per una laguna “regolata”

**Linea 2.3**

***Contaminanti emergenti in laguna, esposizione ed effetti***

## D2.3.2.2 *Versione 2.0*

## *Messa a punto di metodi analitici per inquinanti emergenti*

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31/10/2019

**Sommario**

# 1 Introduction

Contaminants of Emerging Concern (CECs) are defined as any synthetic or naturally occurring chemical that is not commonly monitored in the environment, though having the potential to enter soil and aquatic ecosystems and cause adverse effects in humans, wildlife, and the environment. CECs include synthesized and commercialized chemicals that have just gained entry into the environment and a range of chemicals that have been produced and released into the environment for long, for which new concerns (occurrence, fate, adverse effects on human health and the environment) have recently raised.

They include herbicides, pesticides (i.e. glyphosate, piretroids, neonicotinoids), pharmaceuticals and cosmetics, perfluorinated alkylated substances (PFAS), bisphenol A, and microplastics. The contribution to the environmental status of water, sediments and biota of some classes of these contaminants could be even higher with respect to the contribution of priority pollutants. Recent Italian regulations added some emerging contaminants to the list of the priority pollutants (D. Lgs. 172/2015), such as some flame-retardants and pesticides.

The line 2.3 has the specific objectives to deepen the knowledge regarding the contamination of water, sediments and biota in the Venice lagoon, due to the presence of emerging contaminants and to investigate the distribution and fate of these contaminants in the water environment of the lagoon, experimentally and using models. With this aim, the classes of substance that should be included in future monitoring activities will be identified by the assessment of the ecotoxicological effects and environmental risk associated with these contaminants.

Two approaches are considered: target quantification of environmental contaminants reported in the EU Watch List (2015/495) and untargeted characterization of the environmental matrices.

The present document, on the basis of the scrupulous literature research previously carried out to identify the classes of emerging pollutants to be investigated, their main sources, the diffusion pathways and targets (refer to D2.3.1), deals with the validated analytical methods for the analysis of all contaminants classes.

The report, following the setup of the D2.3.1, is subdivided into chapters, one chapter for each class of contaminants: Pharmaceuticals, Plant Protection Products, Neonicotinoid insecticides, Industrial chemicals, Fragrances, PFASs, and Microplastics. In each chapter the analytical methods' information are described with all the validation parameters carried out in our laboratories: the details regarding the used reagents and standards, sample preparation and the instruments' conditions.

# 2 Pharmaceuticals

Pharmaceutical compounds cover all classes of chemicals used primarily to prevent or treat human and animal diseases. The main representative therapeutic groups are antibiotics, analgesics and painkillers, cardiovascular drugs and blood lipid regulators and antidepressants. Pharmaceuticals are generally excreted and emitted into the sewerage system following use and can then be released into surface water bodies or enter terrestrial systems. Moreover, this chemical class includes veterinary pharmaceuticals, used in intensive farming and aquaculture to increase livestock production. They are often released directly in surface waters or indirectly during the land application of manure and slurry from livestock facilities.

The capability of pharmaceutical ingredients to be absorbed and to interact with living organisms makes them a potential hazard for the whole ecosystem. Pharmaceuticals are specifically designed to be biologically active substances (also at low doses) and to target certain metabolic, enzymatic or cell-signaling mechanisms. Thus, when released into the environment, their biological activity may interact with non-target organisms and impair the ecosystem health. Highly lipid-soluble medicinal products may also have the ability to accumulate in the fat tissues of animals and can be thus introduced into the food chain.

A major concern raised by the presence of pharmaceuticals in the environment is their ability to act as endocrine disruptors, i.e. interfere with the endocrine system to produce undesired effects/disruption of homeostasis. This applies for example to hormones.

Among other pharmaceuticals, the presence of antibiotics (antimicrobial compounds specifically targeting bacteria or fungi in human and animal hosts) in the environment causes significant concern due to the possibility of increasing multi-resistant bacteria.

In literature several papers report methods for the analysis of pharmaceuticals in natural waters (surface water, marine water, waste water); among these documents, the JRC report on “Analytical method for the determination of compounds selected for the first Surface water watch list” proposes a well-established study on analytical methods for most of the selected contaminants.

According to the information reported in the JRC report (Tavazzi et al., 2016), a SPE-LC-MS/MS multi-compound method has been developed for the quantitative determination of environmental contaminants selected in the watch list for surface water monitoring.

These methods are based on SPE-LC-MS/MS analysis, using OASIS HLB as sorbent material for the extraction of 1 liter of marine water sample and quantitative determination. Neither pH modification nor any other sample’s pre-treatment will be performed in order to allow the extraction of all the selected compounds.

## 2.1 Materials and methods

### *2.1.1 Reagents*

- Methanol, Hypergrade for LC-MS LiChrosolv, (Merck)
- Acetonitrile, Hypergrade for LC-MS LiChrosolv, (Merck)
- Ammonium acetate, for LC-MS LiChromapure (Merck)
- Ammonium hydroxide solution 25 %, for LC-MS, (Merck)
- MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA)
- OASIS HLB cartridges 6CC (0.2g), Waters, Milford, MA, (USA)

Table 1. Analytical standards.

| Analyte | Supplier code | CAS | Grade | Supplier |
|---|---|---|---|---|
| 17α-Ethynylestradiol | 46263-250MG | 57-63-6 | Analytical Standard | Sigma Aldrich (Merck) |
| β-Estradiol | E1132-1VL | 50-28-2 | Analytical Standard | Sigma Aldrich (Merck) |
| Estrone | 46573-250MG | 53-16-7 | Analytical Standard | Sigma Aldrich (Merck) |
| Diclofenac | 93484-100MG | 15307-79-6 | Analytical Standard | Sigma Aldrich (Merck) |
| Erythromycin A | BP794 | 114-07-8 | British Pharmacopea Reference Standard | Sigma Aldrich (Merck) |
| Clarithromycin | A3487-100MG | 81103-11-9 | ≥98 % HPLC | Sigma Aldrich (Merck) |
| Azithromycin | 75199-25MG-F | 83905-01-5 | Analytical Standard | Sigma Aldrich (Merck) |
| Amoxicillin | 31586-250MG | 61336-70-7 | Analytical Standard | Sigma Aldrich (Merck) |
| Ciprofloxacin | 33434-100MG-R | 85721-33-1 | Analytical Standard | Sigma Aldrich (Merck) |

Labeled Internal Standard in the next table.

Table 2. Labelled analytical standards.

| Analyte | Supplier code | CAS | Grade | Supplier |
|---|---|---|---|---|
| Ciprofloxacin-d8 | 32982-10MG | 1216659-54-9 | Analytical Standard | Sigma Aldrich (Merck) |
| Diclofenac-(acetophenyl ring-13C6) | 35361-10MG | 1261393-73-0 | Analytical Standard | Sigma Aldrich (Merck) |
| Estrone-2,3,4-13C3 | 802921-1ML | 1241684-29-6 | Analytical Standard | Sigma Aldrich (Merck) |
| 17β-Estradiol-D5 | E-061-1ML | 221093-45-4 | Certified Reference Material | Sigma Aldrich (Merck) |

### *2.1.2 Standard preparation*

For chemical standards purchased as solid, stock standard solutions are prepared in methanol, except for amoxicillin, that has to be dissolved in ultrapure water because it is not stable in methanol.

For labeled standards, stock standard solutions are prepared in methanol, reaching a final concentration of 100 ng/ml of each.

### 2.1.3 Sample preparation

Whole marine water has been filtered at 0.7 µm and frozen for storing in glass bottle. SPE OASIS HLB cartridges are conditioned with 10 ml methanol followed by 10 ml water. One liter of water sample, spiked with 10 µl of internal standard, is loaded at 5 ml/min and successively the cartridges are cleaned with 5 ml of ultrapure water and dried under a stream of nitrogen for 30 minutes. The sorbent is eluted with 10 ml of methanol (3 ml/minute) and the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 0.5 mL of water and acetonitrile (9:1, v/v) and analyzed.

Dry sediment is rehydrated with 3.5 % of NaCl in ultrapure water. 10 grams of this sediment are placed in a 50 ml PP tubes, spiked with both internal and analyte standard, and homogenized by vortex. Each sample is extracted by adding 10 ml of AcCN to the tubes containing the sediment, and sonicated for 15 min. At the end of the sonication, the solution is centrifuged at 3000 rpm and the supernatant is evaporated to dryness by means Turbovap. The solids centrifuged are extracted two times more, with 10 ml of MeOH, repeating the extraction procedure described above. The evaporated extracts are collected and diluted with 400 ml of ultrapure water and concentrate by means SPE OASIS HLB cartridges, loading the liquid at 5 ml/min. The sorbent is eluted with 10 ml of methanol (3 ml/minute) and the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 1 mL of water and acetonitrile (9:1, v/v), filtered at 0.45 µm and analyzed.

### 2.1.4 Instrument conditions

According to the JRC Report (Tavazzi et al., 2016), two different LC-MS/MS methods have been developed and optimized for the quantification of selected chemicals.

Method 1 “Pharmaceuticals”

Method “Pharmaceuticals” will be used for the quantification of diclofenac, azithromycin, clarithromycin, erythromycin, amoxicillin and ciprofloxacin. Chromatography is performed in gradient mode by using ammonium acetate and methanol as mobile phase.

Table 3. Gradient scheme for “Pharmaceuticals” HPLC separation.

| Time | A: $CH_3COONH_4$ (10 mM, pH3) | B: MeOH | Flow (ml/min) |
|---|---|---|---|
| 0 | 90 | 10 | 0.4 |
| 0,2 | 90 | 10 | 0.4 |
| 10 | 10 | 90 | 0.4 |
| 10,2 | 90 | 10 | 0.4 |
| 16 | 90 | 10 | 0.4 |

Table 4. Analytical results for the “Pharmaceuticals” separation.

| | Exact mass measured | Ionization Polarity | RT (min) | Mass transition | Calibration range water (ng/L) | R2 | Calibration range sediment (µg/Kg d.w.) | R2 |
|---|---|---|---|---|---|---|---|---|
| Diclofenac | 294,0094 | negative | 9,7 | 294>250, 214 | 64-1035 | 0.993 | 0.65-10.3 | 0.991 |
| Azithromycin | 749,5158 | positive | 9,2 | 749>591, 573 | 81-1310 | 0.963 | 0.81-13.1 | 0.999 |
| Clarithromycin | 748,4841 | positive | 9,2 | 748>590, 558 | 71-1140 | 0.989 | 0.71-11.4 | 0.999 |
| Ciprofloxacin | 332,1449 | positive | 5,5 | 332>288, 231 | 81-1310 | 0.973 | 0.81-13.1 | 0.968 |
| Amoxicillin | 366,1112 | positive | 1,7 | 366>349 | 131-2100 | 0.987 | 1.31-21 | 0.998 |
| Erythromycin | *In progress* | | | | | | | |

Method 2 “Hormones”

Method “Hormones” will be used for the quantification of EE2, E2, and E1 in negative polarity. Chromatography is performed in gradient mode by using ammonia solution and acetonitrile as mobile phase.

Table 5. Gradient scheme for “Hormones” HPLC separation.

| Time | A: 0.1% $NH_4OH$ | B: AcCN | Flow (ml/min) |
|---|---|---|---|
| 0 | 90 | 10 | 0.4 |
| 1 | 80 | 20 | 0.4 |
| 2 | 60 | 40 | 0.4 |
| 10 | 10 | 90 | 0.4 |
| 12 | 10 | 90 | 0.4 |
| 13 | 90 | 10 | 0.4 |
| 24 | 90 | 10 | 0.4 |

Table 6. Analytical results for the “Hormones” separation.

| | Exact mass mesured | Ionization Polarity | RT (min) | Mass transition | Calibration range water (ng/L) | R2 | Calibration range sediment (µg/Kg d.w.) | R2 |
|---|---|---|---|---|---|---|---|---|
| EE2 | 295,1703 | negative | 4.9 | 295> 67, 145 | 2.42-38.8 | 0.998 | 0.02-0.39 | 0.997 |
| E2 | 271,1703 | negative | 4.6 | 271>145, 143 | 1.6-25.6 | 0.999 | 0.01-0.25 | 0.969 |
| E1 | 269,1547 | negative | 5.1 | 269>145 | 1.92-30.8 | 0.999 | 0.02-0.31 | 0.94 |

#### *2.1.4.1 Mass Spectrometer settings*

The mass spectrometer Compact QTOF (Bruker) is calibrated before each analysis, and the peak identification is carried out by means a double acquisition experiments, a full-scan TOF-MS and a TOF MS/MS analysis. The ionization of the substances is conducted in positive polarity for azithromycin, clarithromycin, erythromycin, amoxicillin and ciprofloxacin, and in negative polarity for the Diclofenac, EE2, E2 and E1.

All calculation are based on the ratio between the chromatographic peak area obtained by MS/MS TOF for the analyte and those for the IS.

## 2.2 17-alpha-ethinylestradiol (EE2)

17-alpha-ethinylestradiol (EE2) is a synthetic feminine sexual hormone, metabolite of mestranol, the estrogen used in contraceptives (mestranol is the prodrug that is activated in the body to improve ADME) (Human metabolome HBMD, n.d.). Several analytical methods for the detection and quantification of EE2 in natural water are reported In literature (Pojana et al., 2004, 2007; Tavazzi et al., 2016; Loos et al., 2018; Sousa et al., 2019), reaching a limit of detection (LOD) from 0.01 to 40 ng/L. The analytical protocol described in the JRC document reports LOD of 0.01 ng/L and 0.03 ng/L in ultrapure water and surface water, respectively (Tavazzi et al., 2016). With our equipment LOD of 0.14 ng/L in ultrapure water and 0.001 µg/Kg (d.w.) for sediments, are achieved.

## 2.3 17-beta-estradiol (E2)

17-beta- estradiol (E2) is a natural feminine sexual hormone. It is produced especially during the fertile period, and its production decreases at low levels during menopause. It is present also in men at a lower extent and also in other animal species. It is also used as an estrogen, especially in the menopausal hormone therapy. For this compound, available analytical methods report a LOD between 0.04 and 40 ng/L. (Pojana et al., 2004, 2007; Tavazzi et al., 2016; Loos et al., 2018; Sousa et al., 2019). The analytical method described in the JRC report ensures a LOD of 0.04 ng/L and 0.05 ng/L in ultrapure water and surface water, respectively (Tavazzi et al., 2016). With our equipment LOD of 0.18 ng/L in ultrapure water and 0.039 µg/Kg (d.w.) for sediments, are achieved.

## 2.4 Estrone (E1)

Estrone (E1) is a natural feminine sexual hormone; it is less active compared to EE2 and it can be converted into estradiol. It is also used as an estrogen, especially in the menopausal hormone therapy. This compound is well studied in natural water and the LOD reported are between 0.01 and 40 ng/L. (Pojana et al., 2004, 2007; Tavazzi et al., 2016; Loos et al., 2018; Sousa et al., 2019). Among these methods, the JRC document reports a LOD of 0.01 ng/L and 0.09 ng/L in ultrapure water and surface water, respectively. (Tavazzi et al., 2016). With our equipment LOD of 0.11 ng/L in ultrapure water and 0.015 µg/Kg (d.w.) for sediments, are achieved.

## 2.5 Diclofenac

Diclofenac is a nonsteroidal anti-inflammatory drug used to treat pain and inflammatory diseases. At the national level it ranks second after azelastine, an antihistaminic, with regard to national spending on pharmaceuticals for self-medication, and it shows an increasing trend compared to 2016 (AIFA, 2018).

Most of the analytical methods reported in literature provide SPE preconcentration and LC-MS/MS analysis, reaching LOD between 0.9 and 3 ng/L in natural waters (Tavazzi et al., 2016; Seo et al., 2019; Sousa et al., 2019). The method proposed in the JRC report reaches a LOD of 0.47 ng/L and 1 ng/L in ultrapure water and surface water, respectively (Tavazzi et al., 2016). With our equipment LOD of 0.66 ng/L in ultrapure water and 0.008 µg/Kg (d.w.) for sediments, are achieved.

## 2.6 Amoxicillin

The antimicrobial pharmaceutical amoxicillin ranks first in terms of national expenditure in the category of antibiotics of systemic application (AIFA, 2018). Due to its instability in natural water, this compound is not well studied, in fact a wide range of LOD is reported, namely 100-0.2 ng/L (Hu et al., 2018; Loos et al., 2018). No information is reported for the analysis of this compound in the JRC report (Tavazzi et al., 2016). With our equipment LOD of 106.7 ng/L in ultrapure water and 1.02 µg/Kg (d.w.) for sediments, are achieved.

## 2.7 Ciprofloxacin

Ciprofloxacin is an antibiotic. It is at the third place in terms of antimicrobial agents consumption after amoxicillin and ceftriaxone at the national level, and the trend in consumption has been slightly decreasing in the period 2016-2017 (-3.9%) (AIFA, 2018). The LOD reported for this compound are between 200 and 2 µg/L for natural water (Mirzaei et al., 2017; Loos et al., 2018; Hernández et al., 2019). No information is

reported in the JRC report for this analyte. With our equipment LOD of 164 ng/L in ultrapure water and 4.16 µg/Kg (d.w.) for sediments, are achieved.

## 2.8 Erythromycin

Erythromycin is a macrolide antibiotic and is commercialized in Italy, but it is not present in the main ranking on pharmaceuticals consumption and expenditure described in the national report from AIFA (AIFA, 2018).

All the methods reported in literature apply an SPE concentration and analysis by means of LC-MS. Better results are reported for direct analysis or SPE-online. The LOD reported in literature range from 5 to 0.5 µg/L (Boix et al., 2015; Tavazzi et al., 2016; Mirzaei et al., 2017; Hernández et al., 2019; Sousa et al., 2019).

## 2.9 Clarithromycin

Clarithromycin is a macrolide antibiotic and is prescribed in Italy, although it is not the most widespread. A decrease of 5.6%, in 2017 compared to 2016, in medical prescriptions and consumption, is reported (AIFA, 2018). The analytical methods found in literature report LOD between 0.13 and 0.03 ng/L. The analytical method applied in the JRC report reaches LOD of 0.13 ng/L and 2.1 ng/L in ultrapure water and surface water respectively. With our equipment LOD of 84.26 ng/L in ultrapure water and 0.6 µg/Kg (d.w.) for sediments, are achieved.

## 2.10 Azithromycin

Azithromycin is an antibiotic and, according to AIFA (AIFA, 2018), it is more prescribed than clarithromycin at national level. The LOD reported in literature for this compound are between 90 and 0.3 ng/L (Tavazzi et al., 2016; Mirzaei et al., 2017; Loos et al., 2018; Hernández et al., 2019; Sousa et al., 2019). The method used in the JRC study reaches LOD of 2.6 ng/L (Tavazzi et al., 2016). With our equipment LOD of 70.7 ng/L in ultrapure water and 0.57 µg/Kg (d.w.) for sediments, are achieved.

# 3 Plant Protection Products

Plant protection products (PPP) are pesticides that are mainly used to keep crops (or other useful or desirable plants) healthy and prevent them and their products from being destroyed by disease and infestation. They include herbicides, fungicides, insecticides, acaricides, molluscicides, plant growth regulators and repellents (while "pesticides" are a wider class which includes also biocides, intended for non-plant use) (EC, 2019). They are primarily used in the agricultural sector, but also in forestry, horticulture, amenity areas, and in home gardens. Each formulation consists of one or more active substances, responsible for the properties of the plant protection product, and substances called co-formulants.

A large body of EU legislation regulates the marketing and use of PPPs and companies have to produce a large dossier of information to the regulatory authorities before a PPP can be placed on the market. A dual system is in place, under which the European Food Safety Agency (EFSA) evaluates active substances used in PPPs and Member States evaluate and authorise the products at national level. Plant protection products are principally regulated by framework Regulation (EC) No 1107/2009.

## 3.1 Methiocarb

Methiocarb is used as an insecticide and bird repellent for maize crops. EFSA re-evaluated the risk assessment in 2018 (Arena et al., 2018). From the analytical point of view, the best LOD reached for this compound are between 0.01 and 40 ng/L (Tavazzi et al., 2016; Loos et al., 2018; Sousa et al., 2019). The SPE-LC-MS method proposed by JRC reaches LOD of 0.07 ng/L and 0.01 ng/L in ultrapure water and surface water, respectively (Tavazzi et al., 2016). With our equipment LOD of 0.59 ng/L in ultrapure water and 0.21 µg/Kg (d.w.) for sediments, are achieved.

### *3.1.1 Materials and methods*

#### *3.1.1.1 Reagents*

- Methanol, Hypergrade for LC-MS LiChrosolv, (Merck)
- Acetonitrile, Hypergrade for LC-MS LiChrosolv, (Merck)
- Ammonium acetate, for LC-MS LiChromapure (Merck)
- MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA)
- OASIS HLB cartridges 6CC (0.2g), Waters, Milford, MA, (USA).

Table 7. Analytical standard.

| Analyte | Supplier code | CAS | Grade | Supplier |
|---|---|---|---|---|
| Methiocarb | 36152-100MG | 2032-65-7 | Analytical Standard | Sigma Aldrich (Merck) |

Labeled Internal Standard in next table.

Table 8. Labelled analytical standard.

| Analyte | Supplier code | Grade | Supplier |
|---|---|---|---|
| Thiacloprid-(thiazolidin ring-d4) | 30673-10MG | Analytical Standard | Sigma Aldrich (Merck) |

#### *3.1.1.2 Standard preparation*

Methiocarb stock standard solution is prepared in methanol.

A stock standard solution of Thiacloprid-(thiazolidin ring-$d_4$) is prepared in methanol, reaching a final concentration of 100 ng/ml.

#### *3.1.1.3 Sample preparation*

Whole marine water has been filtered at 0.7 µm and frozen for storing in glass bottle. SPE OASIS HLB cartridges are conditioned with 10 ml methanol followed by 10 ml water. One liter of water sample, spiked with 10 µl of internal standard, is loaded at 5 ml/min and successively the cartridges are cleaned with 5 ml of ultrapure water and dried under a stream of nitrogen for 30 minutes. The sorbent is eluted with 10 ml of methanol (3 ml/minute), the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 0.5 mL of water and acetonitrile (9:1, v/v) and analyzed.

Dry sediment is rehydrated with 3.5 % of NaCl in ultrapure water. 10 grams of dried sediment are placed in a 50 ml PP tubes, spiked with both internal and analyte standard, and homogenized by vortex. Each sample is extracted by adding 10 ml of AcCN to the tubes containing the sediment, and sonicated for 15 min. At the end of the sonication, the solution is centrifuged at 3000 rpm and the supernatant is evaporated to dryness by means Turbovap. The solids centrifuged are extracted two times more, with 10 ml of MeOH, repeating the extraction procedure described above. The evaporated extracts are collected, diluted with 400 ml of ultrapure water and concentrate by means SPE OASIS HLB cartridges, loading the liquid at 5 ml/min. The sorbent is eluted with 10 ml of methanol (3 ml/minute) and the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 1 mL of water and acetonitrile (9:1, v/v), filtered at 0.45 µm, and analyzed.

#### *3.1.1.4 Instrument conditions*

According to the JRC Report the chromatographic separation is performed in gradient mode by using ammonium acetate and methanol as mobile phase, as described in the method “Pharmaceutics” (section 2.1.4). The ionization is conducted in positive polarity.

Table 9. Analytical results for the methiocarb analysis.

| | Exact mass mesured | Ionization Polarity | RT (min) | Mass transition | Calibration range water (ng/L) | R2 | Calibration range sediment (µg/Kg d.w.) | R2 |
|---|---|---|---|---|---|---|---|---|
| Methiocarb | 226.0896 | positive | 8.8 | 226>169, 121 | 44-705 | 0.976 | 0.44-7.0 | 0.997 |

## 3.2 Oxadiazon

Oxadiazon is an herbicide. Few analytical methods are reported in literature, reaching LOD between 52 and 1 ng/L (Tavazzi et al., 2016; Sousa et al., 2019). The best LOD reported by JRC are reached by means of SPE-LC-MS/MS (0.2 ng/L in ultrapure water and 0.4 ng/L in surface water) (Tavazzi et al., 2016). With our equipment LOD of 1.92 ng/L in ultrapure water and 0.84 µg/Kg (d.w.) for sediments, are achieved.

### 3.2.1 Materials and methods

#### 3.2.1.1 Reagents

- Methanol, Hypergrade for LC-MS LiChrosolv, (Merck)
- Acetonitrile, Hypergrade for LC-MS LiChrosolv, (Merck)
- Ammonium acetate, for LC-MS LiChromapure (Merck)
- MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA)
- OASIS HLB cartridges 6CC (0.2g), Waters, Milford, MA, (USA).

Table 10. Analytical standard.

| Analyte | Supplier code | CAS | Grade | Supplier |
|---|---|---|---|---|
| Oxadiazon | 33382-100MG | 19666-30-9 | Analytical Standard | Sigma Aldrich (Merck) |

Labeled Internal Standard in next table.

Table 11. Labelled analytical standard.

| Analyte | Supplier code | Grade | Supplier |
|---|---|---|---|
| Thiacloprid-(thiazolidin ring-d4) | 30673-10MG | Analytical Standard | Sigma Aldrich (Merck) |

#### 3.2.1.2 Standard preparation

Oxadiazon stock standard solution is prepared in methanol.

Stock standard solutions of Thiacloprid-(thiazolidin ring-$d_4$) is prepared in methanol, reaching a final concentration of 100 ng/ml.

#### 3.2.1.3 Sample preparation

Whole marine water has been filtered at 0.7 µm and frozen for storing in glass bottle. SPE OASIS HLB cartridges are conditioned with 10 ml methanol followed by 10 ml water. One liter of water sample, spiked with 10 µl of internal standard, is loaded at 5 ml/min and successively the cartridges are cleaned with 5 ml of ultrapure water and dried under nitrogen for 30 minutes. The sorbent is eluted with 10 ml of methanol (3 ml/minute), the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 0.5 mL of water and acetonitrile (9:1, v/v) and analyzed.

Dry sediment is rehydrated with 3.5 % of NaCl in ultrapure water. 10 grams of this sediment are placed in a 50 ml PP tubes, spiked with both internal and analyte standard, and homogenized by vortex. Each sample is extracted by adding 10 ml of AcCN to the tubes containing the sediment, and sonicated for 15 min. At the end of the sonication, the solution is centrifuged at 3000 rpm and the supernatant is evaporated to dryness by means Turbovap. The solids centrifuged are extracted two times more, with 10 ml of MeOH, repeating the extraction procedure described above. The evaporated extracts are collected and diluted with 400 ml of ultrapure water and concentrated by means SPE OASIS HLB cartridges, loading the liquid at 5 ml/min. The sorbent is eluted with 10 ml of methanol (3 ml/minute) and the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 1 mL of water and acetonitrile (9:1, v/v), filtered at 0.45 µm, and analyzed.

#### *3.2.1.4 Instrument conditions*

According to the JRC Report, the chromatographic separation is performed in gradient mode by using ammonium acetate and methanol as mobile phase as described in the method "Pharmaceutics" (section 2.1.4). The ionization is conducted in positive polarity.

Table 12. Analytical results for the oxadiazon analysis.

| | Exact mass mesured | Ionization Polarity | RT (min) | Mass transition | Calibration range water (ng/L) | R2 | Calibration range sediment (µg/Kg d.w.) | R2 |
|---|---|---|---|---|---|---|---|---|
| Oxadiazon | 345.0767 | positive | 10.8 | 345>220, 303 | 83-1330 | 0.992 | 0.8-13.3 | 0.998 |

## 3.3 Triallate

Triallate (S-2,3,3-trichloroallyl di-isopropyl thiocarbamate) is a carbamothioate herbicide widely used to control annual and perennial grasses in wheat, barley, legumes and a number of other crops. Its use, in the last decades, has exceeded 500 tons per year in some European countries (Barbosa et al., 2016). Triallate is highly hydrophobic; therefore it adsorbs to loam and clay soils and is not readily dissolved in water, suggesting that this herbicide is not likely to move through the soil, even though it has a long soil half-life (82 days). Nevertheless, it may be desorbed if there is significant moisture and/or low levels of organic matter in the soil. Leaching and consequent groundwater contamination would be possible in such situations (Barbosa et al., 2016). A lack of knowledge exists about its occurrence and removal in the aquatic environment due to its chemical nature.

For information about the analytical method, please refer to paragraph 6.

## 3.4 Metaflumizone

Metaflumizone is an active substance applied as an insecticide; EFSA reviewed this PPP for risk assessment under request by BASF in 2013. Few methods are reported in literature for the analysis of metaflumizone, obtaining LOD from 0.05 to 0.025 µg/L (EFSA, 2013; Loos et al., 2018). These methods must be upgraded and adapted to be applied by using instruments available in our laboratory. With our equipment LOD of 23.2 ng/L in ultrapure water and 0.106 µg/Kg (d.w.) for sediments, are achieved.

#### *3.4.1 Materials and methods*

#### *3.4.1.1 Reagents*

- Methanol, Hypergrade for LC-MS LiChrosolv, (Merck)
- Acetonitrile, Hypergrade for LC-MS LiChrosolv, (Merck)
- MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA)
- OASIS HLB cartridges 6CC (0.2g), Waters, Milford, MA, (USA).

Table 13. Analytical standard.

| Analyte | Supplier code | CAS | Grade | Supplier |
|---|---|---|---|---|
| Metaflumizone | 32966-100MG | 139968-49-3 | Analytical Standard | Sigma Aldrich (Merck) |

Labelled Internal Standard in next table.

Table 14. Labelled analytical standard.

| Analyte | Supplier code | CAS | Grade | Supplier |
|---|---|---|---|---|
| 17β-Estradiol-D5 | E-061-1ML | 221093-45-4 | Certified Reference Material | Sigma Aldrich (Merck) |

#### *3.4.1.2 Standard preparation*

Metaflumizone stock standard solution is prepared in methanol.

A stock standard solution of 17β-Estradiol-D5 is prepared in methanol, reaching a final concentration of 100 ng/ml.

#### *3.4.1.3 Sample preparation*

Whole marine water has been filtered at 0.7 µm and frozen for storing in glass bottle. SPE OASIS HLB cartridges are conditioned with 10 ml methanol followed by 10 ml water. One liter of water sample, spiked with 10 µl of internal standard, is loaded at 5 ml/min and successively the cartridges are cleaned with 5 ml of ultrapure water and dried under nitrogen for 30 minutes. The sorbent is eluted with 10 ml of methanol (3 ml/minute) and the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 0.5 mL of water and acetonitrile (9:1, v/v) and analyzed.

Dry sediment is rehydrated with 3.5 % of NaCl in ultrapure water. 10 grams of this sediment are placed in a 50 ml PP tubes, spiked with both internal and analyte standard, and homogenized by vortex. Each sample is extracted by adding 10 ml of AcCN to the tubes containing the sediment, and sonicated for 15 min. At the end of the sonication, the solution is centrifuged at 3000 rpm and the supernatant is evaporated to dryness by means Turbovap. The solids centrifuged are extracted two times more, with 10 ml of MeOH, repeating the extraction procedure described above. The evaporated extracts are collected and diluted with 400 ml of ultrapure water and concentrate by means SPE OASIS HLB cartridges, loading the liquid at 5 ml/min. The sorbent is eluted with 10 ml of methanol (3 ml/minute) and the eluent is evaporated to dryness under a gentle stream of nitrogen at 40°C by using a TurboVap system. Samples are then reconstituted with 1 mL of water and acetonitrile (9:1, v/v), filtered at 0.45 µm, and analyzed.

#### *3.4.1.4 Instrument conditions*

As reported in the JRC Report, metaflumizone is instable at low pH and, for this reason, the chromatographic separation is performed in gradient mode by using ammonia solution (2.5%) and acetonitrile as mobile phase, as described in the method “Hormones” (section 2.1.4). The ionization is conducted in negative polarity.

Table 15. Analytical results for the metaflumizone analysis.

| | Exact mass mesured | Ionization Polarity | RT (min) | Mass transition | Calibration range water (ng/L) | R2 | Calibration range sediment (µg/Kg d.w.) | R2 |
|---|---|---|---|---|---|---|---|---|
| Metaflumizone | 505.1104 | negative | 8.6 | 505>302, 116 | 40-640 | 0.999 | 0.4-6.4 | 0.998 |

## 3.5 Glyphosate and AMPA

Glyphosate is a non-selective and broad-spectrum herbicide and is the most widely used worldwide. Glyphosate-based pesticides – i.e. formulations containing glyphosate and other chemicals – are used as agriculture and horticulture primarily to combat weeds that compete with cultivated crops. They are typically applied before crops, are sown to control weeds and their root systems and therefore facilitate better growth of crops. To a lesser extent glyphosate is also used as a pre-harvest or desiccating treatment, accelerating and evening the ripening process. In March 2019 the European Commission announced a plan to set up a group of Member States to act as co-rapporteurs for the next assessment of glyphosate. If the plan will be approved, the Assessment Group on Glyphosate (AGG) will assess the application dossier and prepare a draft renewal assessment report to be reviewed by EFSA in 2021. In the degradation pathway of glyphosate, the first step is essentially the cleavage to glyoxylate and amino-methylphosphonic acid (AMPA).

Degradation of AMPA is generally slower than that of glyphosate possibly because AMPA may adsorb onto soil particles more strongly than glyphosate and/or because it may be less likely to permeate the cell walls or membranes of soil microorganisms. AMPA is not ecotoxicologically relevant for water, sediment and groundwater compartments. For precautionary reasons AMPA is proposed as relevant residue due to the frequent detections in surface waters and groundwater and the widespread intended uses of glyphosate in almost all crops. (EFSA, 2015).

Glufosinate is a very polar compound and its structural formula is similar to that of glyphosate. These three compounds are usually determined using a single analysis for both research and regulatory purposes (Stalikas and Konidari, 2001). It is a challenge to detect glyphosate residue due to its poor solubility in common organic solvents, difficult evaporation, high polarity, and absences of chromophores and fluorophores. In the most recent years, several methods were developed to detect glyphosate residues including Gas Chromatography-Mass Spectrometry (GC/MS), Liquid Chromatography-Mass Spectrometry (LC/MS), Ion Chromatography (IC), and Capillary Electrophoresis (CE) (Ding et al., 2015). Unlike GC, HPLC is a common method for separate the glyphosate residue, but pre-column or post-column derivatization procedure needs to be conducted due to the absences of fluorophore and chromophore.

### *3.5.1 Methods*

#### *3.5.1.1 Instrumental method*

We developed an innovative method to separate glyphosate, AMPA and glufosinate using high-pressure anion exchange chromatography coupled with mass spectrometry (HPAEC-MS), without derivatization procedure and through direct injection. To improve the sensitivity of instrumental method, an online-solid phase extraction (online-SPE) was optimized. The three residues were separated using an anion exchange column (Dionex Ion Pac AS 11 2× 250 mm) with a guard column (Dionex Ion Pac AG11 2 × 50 mm). The gradient of sodium hydroxide (NaOH) was produced by an eluent generator (Dionex ICS 5000EG, Thermo Scientific), with a 0.25 mL/min as flow rate. Using the preconcentration cartridge IonPac® UTAC-LP1 Ultratrace Anion (5x23 mm, DionexTM), 250 µL of sample was injected. The MS was operating with an electro-spray ionization (ESI) interface in negative mode with a temperature of 400 °C and a needle voltage of 2.5 kV. Selected ion monitoring (SIM) was used for detection (table 16). The chromatographic separation is reported in figure 1.

Table 16. Optimized mass spectrometric parameters of AMPA, glyphosate (Gly) and Gluphosinate with the relative labelled standards.

| Analyte | Mass | Span | Range(min) | Dwell Time (s) | Polarity | Cone (Volt) |
|---|---|---|---|---|---|---|
| Cl | 35 | 1 | 1-12 | 0.05 | - | 90 |
| AMPA | 110 | 1 | 1-12 | 0.05 | - | 50 |
| AMPA$^{13}C^{15}N$ | 112 | 1 | 1-12 | 0.05 | - | 50 |
| Gly | 168 | 1 | 1-12 | 0.05 | - | 50 |
| Gly$^{13}C^{15}N$ | 171 | 1 | 1-12 | 0.05 | - | 50 |
| Glu | 180 | 1 | 1-12 | 0.05 | - | 50 |

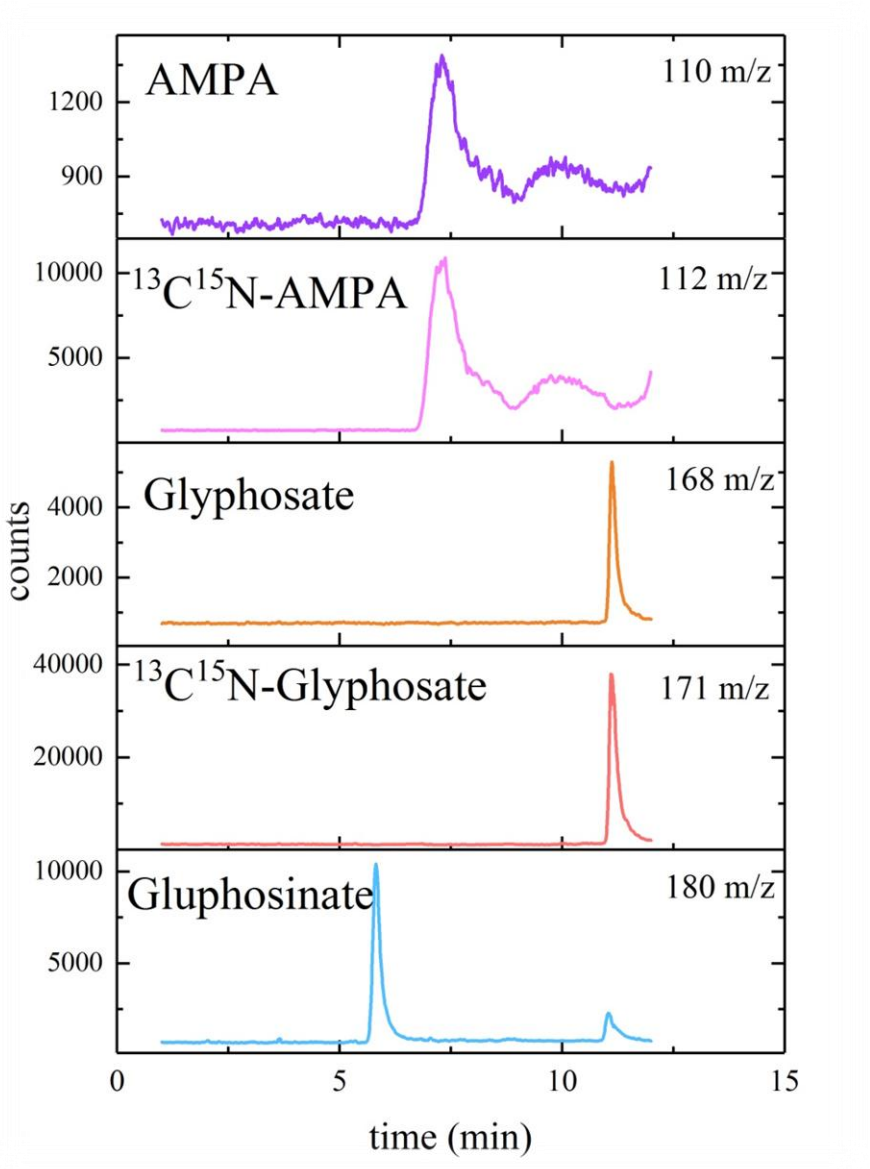


Figure 1. Chromatographic separation of AMPA, glyphosate and gluphosinate.

### *3.5.1.2 Instrumental method performance*

A series of standard solutions was prepared in ultrapure water and in tap water at average concentrations of 0.1, 1, 2.5, 5, 10, 20, 50, 100, and 500 ng/mL, with labelled internal standards at a constant

concentration of 100 ng/mL. Considering the ratio between the concentration of residues and internal standard and the ratio between the relative peak areas, we obtained R2 value > 0.99 for each compound for the solutions prepared in ultrapure water and in tap water. Instrumental precision was tested by a threefold analysis of each standard solution of each compound, resulting in a relative standard deviation (RSD) below 10%. The instrumental detection limits were 2 ng/mL, 0.2 ng/mL and 0.3 ng/mL for AMPA, glyphosate and glufosinate, respectively.

### *3.5.2 Critical points*

Considering that the European concentration limits of glyphosate and AMPA are 0.1 µg L-1, as all the pesticides in drinking water (Directive 1998/83/EC), our method for glyphosate and AMPA will be improved to reduce the limits of detection. Although the European limits referred only to drinking water and no legislative limits are defined for sea water and sediment, we are trying to reduce the sensitivity of our method.

# 4 Neonicotinoid insecticides

In July 2009, a group of entomologists met at Notre Dame de Londres (France), following an international survey which revealed the dramatic decrease in insects and arthropods throughout Europe since the 1950s (van Lexmond et al., 2015) Among the various causes of the problem they identified the intensive agriculture and the use of pesticides and herbicides. They also noticed an abrupt decline of insects in the decade 1990-2000, with consequent decline of insectivorous bird species as well. On the basis of the available studies, the investigations in the field and overwhelming evidence suggested the hypothesis that the new generation of systemic neonicotinoid pesticides, characterized by persistence and neurotoxicity together with fipronil, another insecticide, was responsible at least in part for this decline (van Lexmond et al., 2015).

Neonicotinoid insecticides (NeoNs) were discovered in the late 1980s and include: imidacloprid (IMI), thiamethoxam (TMX), clothianidin (CLO), acetamiprid (ACE), and thiacloprid (TCLO). They are currently the most widely used insecticides in the world. It is estimated that together with fipronil they represent one third of the world market of insecticides and that in 2010, at the global level, imidacloprid, the most representative compound, was produced in about 20,000 tons (Simon-Delso et al., 2015). They are used on a large scale for the protection of plants (crops, vegetables, fruit), as veterinary products, biocides to invertebrate pest control in fish farming. Neonicotinoid compounds act as neurotoxins as they destroy the neuronal transmission of the nervous system of invertebrates. In fact, they mimic the action of neurotransmitters and by continuously stimulating neurons lead to the death of target organisms. However, they can have a lethal or sub-lethal impact even on non-target organisms such as predator insects and vertebrate animals. Considering their wide commercial expansion, mode of action, the systemic properties in plants, persistence, and environmental fate, coupled with limited information about the toxicity profiles of these compounds and their metabolites, NeoNs may entail significant risks to the environment (Simon-Delso et al., 2015).

Environmental contamination occurs via a number of routes including dust generated during drilling of dressed seeds, contamination and accumulation in arable soils and soil water, runoff into waterways, and uptake of pesticides by no-target plants via their roots or dust deposition on leaves. Persistence in soils, waterways, and no-target plants is variable but can be prolonged; for example, the half-lives of NeoNs in soils can exceed 1,000 days, so they can accumulate when used repeatedly. Similarly, they can persist in woody plants for periods exceeding 1 year (Bonmatin et al., 2015).

A range of concerns has emerged about the impacts of NeoNs on the environment: other than soil persistence and accumulation, their impact on soil invertebrates. Being water soluble, NeoNs leach into ponds, ditches and streams and contaminate groundwater. Contamination of marine environments has been observed but as yet has not been monitored systematically (van Lexmond et al., 2015). Dust created during drilling of treated seeds is lethal to flying insects and has caused large-scale acute losses of honeybee colonies. Although vertebrates are less susceptible than arthropods, consumption of small numbers of dressed seeds offers a potential route for direct mortality in granivorous birds and mammals, for such birds need to eat only a few spilt seeds to receive a lethal dose (van Lexmond et al., 2015). Breakdown results in toxic metabolites that can themselves be toxic (Simon-Delso et al., 2015), though concentrations of these in the environment are rarely measured (Bonmatin et al., 2015).

Due to the chemical and toxicological characteristics, the spread use in Europe, the possibility of contamination of water, these substances are suspected of posing a significant risk to, or via, the aquatic environment. So there is reliable evidence of hazard and of a possible exposure to aquatic organisms and mammals (JRC, technical report). For these reasons neonicotinoid insecticides (imidacloprid, thiamethoxam, clothianidin, acetamiprid and thiacloprid) were inserted in a watch list of substances (DECISION (EU), 2015). The monitoring data are to be gathered for the purpose of supporting future prioritisation exercises.

## 4.1 Measured concentration in environmental matrices

Neonicotinoid insecticides are usually determined in surface water and in groundwater. The European legislation (Directive 2008/105/EC) sets the Environmental Quality Standard (EQS) for a limited number of priority substances (including few pesticides) in surface waters. Moreover the Italian legislation (D.lgs. 172/2015) sets EQS for some other pesticides and fixes for all the other pesticides (including metabolites), not explicitly regulated, the limit of 100 ng/L and, for the sum of pesticides, the limit of 1000 ng/L. The Directive 2006/118/EC on the protection of groundwater sets standards of environmental quality, defined as the concentrations that should not be exceeded in order to protect the human health and the environment. In particular, for the pesticides and their degradation products, the limits are equal to those for drinking water, equal to 100 ng/L and 500 ng/L, respectively for the single substance and for the sum of the substances (ISPRA, Rapporti 282/2018).

Regarding NeoNs in the Veneto Region (Italy), to the best of our knowledge, there is only a publication (De Liguoro et al., 2014), reporting levels of contamination in waters used for field irrigation and livestock watering. Among this class of compounds Imidacloprid was in livestock watering ranging from 3 to 14 ng/L. Clothianidin and acetamiprid were only in one sample both at a concentration of 3 ng/L. The regional agency Arpav performs a regular monitoring of pesticides in surficial and ground waters (Arpav 2017a, 2017b). Imidacloprid is included in the list of pesticides analysed. In surficial waters imidacloprid was observed sporadically at levels above the quantification limit in a range from 10-250 ng/L. In ground waters imidacloprid was quantified at concentrations of < 50 ng/L in eleven stations mainly in the Treviso province.

Recently ISPRA (ISPRA, 289/2018) published the results of a pesticide monitoring performed in 2016 in Italy. Regarding the NeoNs, the document reported that the occurrence of the insecticide imidacloprid both in surface and groundwater is relatively recent. Imidacloprid is the insecticide most frequently found in groundwater. The rate of investigation is significantly increasing over the years, but it is still largely incomplete, considering that the substance is used throughout Italy and determines the highest number of exceedances of the EQSs. In Italy, the highest imidacloprid concentrations measured in 2016 were of 690 ng/L in surface water and 1670 ng/L in groundwater.

NeoNs are compounds used all around the world. The most frequently observed in surficial and ground waters and in river sediments are imidacloprid and thiamethoxam. Imidacloprid, thiamethoxam, and clothianidin have in general higher concentration, especially in surface waters: they have been quantified in concentration from BDL to hundreds ng/L (see corresponding tables). The highest concentration of imidacloprid (range from below detection limit, BDL to 480 ng/L) and chlotinanidin (BDL-159 ng/L) in surface water were registered in Portugal (Sousa et al, 2019). Acetamiprid and thiacloprid were in lower concentration, both were observed in Pearl River, China: acetamiprid at a mean concentration of 52.2±32.5 ng/L in Pearl River Guangzhou, China (Xiong et al., 2019) and thiacloprid in a range 0.40-0.90 ng/L (Zhang et al., 2019). In river sediment NeoNs were quantified in concentration in general below 1 ng/g dw.

## 4.2 Analytical methods employed

The revision of literature available shows that the majority of studies were performed in surface and ground waters. Only one study deals with the quantification of thiamethoxam in marine coastal waters of China (Xie et al., 2019). The analytical technique mostly employed is LC-MS/MS, which guarantees sensitivity and selectivity, in order to obtain the low limit of detection required of 9 ng/L (DECISION EU 2015/495). It is a choice of the author of the paper to express the sensitivity of an analytical method as instrumental detection limit (LOD), instrumental quantification limit (LOQ), or method detection limit (MDL), or quantification limit (MQL), or a combination of these parameters. In the following tables we report the available information. In general published methods report LOQ or MDL lower than 9 ng/L. Neonicotinoid compounds are generally extracted from water samples (volumes from 100mL to 1L) using SPE Oasis HLB cartridges. The analytes are eluted with methanol or mixture of methanol and

dichloromethane or methanol and acetone. The extract is then concentrated to dryness and reconstituted with a solvent compatible with those used during HPLC analysis. Chromatographic separation is performed using reverse phase C18 stationary phase, using a mobile phase of water and methanol of water acetonitrile. Sometimes modifiers such as formic acid or acetic acid are employed. Due to the availability in our laboratory of a liquid chromatograph coupled to a triple quadrupole MS we chose as analytical technique the HPLC-MS/MS for the quantification of NeoNs. The sample preparation procedure reported in a great number of publications seems well established and we will extract target compound using the methodology previously described.

Different is the case of marine sediment of biota (mussels). Publications on these topics to the best of our knowledge are lacking. Some papers treat the analysis of these compounds in soil, river sediment, sludge, insects, and fish. For the analysis of these matrices frequently a QuEChERS approach is chosen. The extract is then analysed using HPLC-MS/MS. Because this approach is very often used for solid matrixes we will employ it for the sample preparation procedure.

## 4.3 Materials and methods

### *4.3.1 Reagents*

Formic acid (98%) was obtained by Fluka (Sigma Aldrich, Buchs, Switzerland). Acetamiprid (ACE) (≥98%), imidacloprid (IMI) (≥98%), clothianidin (CLO) (≥ 98%), thiacloprid (TCLO) (≥98%), thiamethoxam (TMX) (≥98%), acetamiprid-d3 (ACE*) (98%), clothianidin-d3 (CLO*) (≥97%), imidacloprid-d4 (IMI*) (≥98%), thiamethoxam-d3 (TMX*) (≥98%), sodium chloride (≥99%), sodium citrate tribasic dehydrate (≥99%), Sodium citrate dibasic sesquihydrate (≥99%), magnesium sulfate (≥99.5 %), sodium chloride (≥99%), ammonium hydroxide solution in water (> 25%), formic acid eluent additive for LC-MS, Supelclean™ PSA SPE, Discovery® DSC-18 SPE, HPLC/MS-grade methanol (MeOH), were purchased from Sigma Aldrich. HPLC/MS-grade acetonitrile (ACN) and ultrapure water were purchased from VWR International. The ultrapure water (18.2 MΩ cm, 0.01 TOC) was produced by a Purelab Flex II system (Elga, High Wycombe, U.K.). Oasis HLB, 6mL, 500 mg SPE cartridge were obtained from Waters (Milford Massachusetts, USA).

### *4.3.2 Sample preparation for waters analysis*

In a volumetric flask 10 ng (absolute amount) of labeled neonicotinoids internal standard: acetamiprid-d3 (ACE*), clothianidin-d3 (CLO*), imidacloprid-d4 (IMI*), thiamethoxam-d3 (TMX*), were spiked to 1000 mL of filtered (GF/F , porosity 0.7um, diameter 47mm) sea water samples previously acidified with formic acid (1%, v/v). The clean-up and pre-concentration of samples, was performed using the OASIS HLB SPE cartridge (6cc, 500 mg sorbent per cartridge, Waters). The cartridge was conditioned under vacuum with methanol (10 mL), and equilibrated with formic acid (1%) in water (10 mL). Then, the sample was loaded on the cartridge. To eliminate sea salt remaining in the cartridge, a clean-up step was added to the procedure using 10 mL of formic acid (1%) in water. The SPE cartridge was dried under vacuum for 5 minutes and NeoNs were eluted at atmospheric pressure in a 15 mL vial with 10 mL of methanol. The sample was evaporated under a stream of nitrogen at 45°C in a Zymark Turbovap evaporator (Hopkinton, MA, USA) at a volume of 100 µL, then reconstituted with 800 µL of water. The sample was filtered through a 0.45 µm PTFE filter into the autosampler vial before the analysis.

### *4.3.3 Sample preparation for sediment analysis*

Sediments were dried at 105° C for 48 h in order to obtain the dried weight.

The determination of neonicotinoids insecticides in sediments was performed using the QuEchERS method. 1 g of wet homogenised sediment was weighted in a 50 mL centrifuge tube. Then 7 mL of ultrapure water and 10 mL of ACN and 1 ng absolute amount of labelled internal standard acetamiprid-d3 (ACE*), clothianidin-d3 (CLO*), imidacloprid-d4 (IMI*), thiamethoxam-d3 (TMX*)were added and shaken with a vortex for 1 min; MgSO4 (6 g), NaCl (1.5 g), tri-sodium citrate dehydrate[Na3C6H5O7·2H2O (1.5 g) and disodium hydrogen citrate sesqui-hydrate [HOC(COOH)(CH2COONa)2·1.5H2O] (0.75 g) were used for phase-separation adjustment, vortexed for 1 min and centrifuged for 5 min at 3000 rpm. The extract was transferred in a 50 mL centrifuge tube for a clean-up step was performed using PSA (825 mg), MgSO4 (2.5 g) and C18 (825 mg). The sample was vortexed for 1min and centrifuged for 5 min at 3000 rpm. The sample was evaporated at 100 µL under a stream of nitrogen at 45°C in a Zymark Turbovap, reconstituted with 800 µL of water and filtered through a 0.45 µm PTFE filter before the HPLC-MS/MS analysis.

### *4.3.4 Instrumental conditions*

For the determination of neonicotinoid insecticides was developed an HPLC/(+)ESI-MS/MS instrumental method. Analysis were performed using an Agilent 1100 Series HPLC system (Agilent, Waldbronn, Germany). The chromatographic separation was obtained using a Synergy Hydro (50 mm × 4.6 mm, 4 µm, Phenomenex) column, a mobile phase constituted by a 0.1% formic acid water-based solution (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with an elution flow of 500 µL min−1. The binary elution program was as follows: 0−1 min 100% A, 1-15 min gradient to 30% of A, 15-16 min gradient to 0% of A, 16−21 min washing, from 21 to 22 gradient to 100% of A and equilibration. The run lasted 30 min. Chromatographic separation is shown in figure 2. Injection volume was of 100 µL of sample.

The mass spectrometric analysis was performed employing an API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Ontario, Canada) equipped with a Turbo V source operating in positive polarity with source parameters of: Source Temperature 600 °C, Nebulizer gas 40.00 psi, Auxiliary gas 60.00 psi, Curtain gas 20 psi, Collision gas 6.00 psi, Ionization voltage 5500 V. Data acquisition was obtained in multiple reaction monitoring with a 50 ms dwell time/transition. Precursors and fragment ions, declustering potential, entrance potential, collision energy, and collision cell exit potential monitored are reported in table 17.

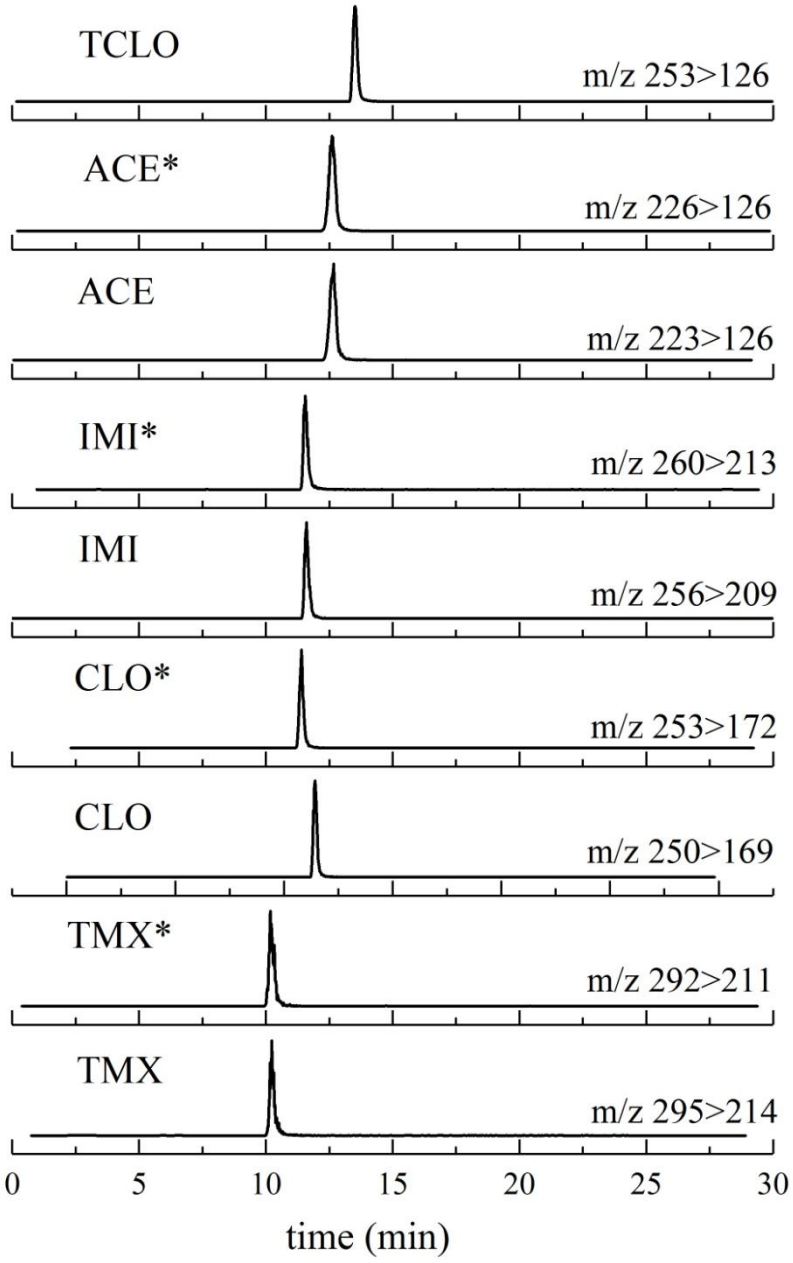


Figure 2. Chromatographic separation of neonicotinoid insecticides.

Table 17. Optimized mass spectrometric parameters for neonicotinoids analysis. DP: Declustering. Potential, EP: Entrance Potential, CE: Collision Energy, CXP: Cell Exit Potential. Transitions used for quantification are in bold. For IMI 256.1/209.2 was used for quantification in waters, while 256.1/175.1 transition in sediments.

| **Precursor ion** | **Q1 (m/z)** | **Q3 (m/z)** | **DP (V)** | **EP (V)** | **CE (V)** | **CXP (V)** |
|---|---|---|---|---|---|---|
| $[ACE+H]^+$ | 223.1 | **126.1** | 59 | 10 | 29 | 8 |
| | | 90.2 | 59 | 10 | 47 | 10 |

| Precursor ion | Q1 (m/z) | Q3 (m/z) | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| $[IMI+H]^+$ | 256.1 | **209.2** | 56 | 12 | 21 | 5 |
| | | **175.1** | 56 | 12 | 23 | 18 |
| $[CLO+H]^+$ | 250.0 | **169.0** | 51 | 10 | 19 | 11 |
| | | 132.0 | 51 | 10 | 21 | 9 |
| $[TCLO+H]^+$ | 253.1 | **126.1** | 70 | 9 | 31 | 8 |
| | | 90.0 | 70 | 9 | 50 | 6 |
| $[TMX+H]^+$ | 292.2 | **211.1** | 56 | 11 | 17 | 5 |
| | | 181.2 | 56 | 11 | 32 | 4 |
| $[ACE*+H]^+$ | 226.0 | **126.0** | 68 | 11 | 27 | 8 |
| | | 90.0 | 68 | 11 | 46 | 6 |
| $[IMI*+H]^+$ | 260.0 | **213.0** | 61 | 11 | 19 | 15 |
| | | 179.0 | 61 | 11 | 24 | 12 |
| $[CLO*+H]^+$ | 253.0 | **172.0** | 57 | 15 | 17 | 12 |
| | | 132.0 | 57 | 15 | 21 | 9 |
| $[TMX*+H]^+$ | 295.0 | **214.0** | 61 | 11 | 16 | 15 |
| | | 184.0 | 61 | 11 | 30 | 13 |

## 4.4 Method validation for neonicotinoids in sea waters

Quantification was performed using internal standard. ACE*, IMI*, CLO* and TMX* were used for the determination of ACE, IMI, CLO, TCLO, TMX. To quantify the concentration the peaks area of native compounds were compared with those of labelled internal standard. Results were corrected for the instrumental response factors. During the method validation linear instrumental response for NeoNs were between 5 pg/mL and 1200 ng/mL; NeoN* were at a fixed concentration of 10 ng/mL. Good linearity was obtained for all compounds and the R2 values were generally above 0.99. Instrumental limit of detection (LOD) for NeoNs ranged from 2 pg/mL to 18 pg/mL, while instrumental limit of quantification ranged from 5 pg/mL to 60 pg/mL. Details are in table 18.

Table 18. Linear instrumental response, instrumental limit of detection (LOD), limit of quantification (LOQ) and $R^2$ for ACE, IMI, CLO, TCLO, TMX.

| | Internal standard | Linear range | LOD pg/mL | LOQ pg/mL | R2 |
|---|---|---|---|---|---|
| ACE | ACE* | 8 pg/mL - 1000 ng/mL | 2 | 8 | 0,9997 |
| IMI | IMI* | 22 pg/mL - 1100 ng/mL | 6 | 22 | 0,9991 |
| CLO | CLO* | 49 pg/mL - 600 ng/mL | 15 | 49 | 0,9857 |
| TCLO | ACE* | 5 pg/mL - 1050 ng/mL | 2 | 5 | 0,9935 |
| TMX | TMX* | 61 pg/mL - 1200 ng/mL | 18 | 61 | 0,9965 |

The analytical method was validated using waters collected in the Venice lagoon and the validated method will be applied to the determination of target compounds in the sea water.

For the validation of analytical method for sea waters, 10 ng (absolute amount, abs) of native standard of NeoNs, and 10 ng abs of ACE*, IMI*, CLO* and TMX* were added to filtered Venice lagoon water. The water lagoon without the addiction of native spikes was considered as blank for validation. Quantitation for IMI was obtained using the 256.1/209.2 transition. The reproducibility (expressed as CV%) and trueness (as percentual error) were evaluated preparing 5 replicate samples and resulted in CV% and percent error in general less than 10%. Validation data and the internal standard employed for the quantification are reported in table 19.

Table 19. Summary of trueness and reproducibility expressed as a relative standard deviation (CV%) for neonicotinoids determined in seawater. For each compound, the internal standard used is reported. Blank refers to Venice lagoon water. Based on the reported results the method resulted validated for the quantification of NeoNs in seawater.

| | Internal standard | Blank (ng abs) | Std dev (ng abs) | MDL (ng abs) | MQL (ng abs) | CV% | Trueness |
|---|---|---|---|---|---|---|---|
| ACE | ACE* | 0.52 | 0.05 | 0,18 | 0.46 | 7 | 4 |
| IMI | IMI* | 0.36 | 0.03 | 0.10 | 0.35 | 9 | -2 |
| CLO | CLO* | 0.55 | 0.06 | 0.19 | 0.64 | 9 | -3 |
| TCLO | ACE* | 0.17 | 0.04 | 0.11 | 0.38 | 1 | 7 |
| TMX | TMX* | 0.13 | 0.05 | 0.14 | 0.47 | 5 | 6 |

## 4.5 Method validation for Neonicotinoids in sediments.

Similarly for seawaters, quantification of NeoNs was performed in sediments using internal standards: ACE*, IMI*, CLO* and TMX*. Quantification was performed comparing areas of native compounds with those of internal standard, with the correction of result for the instrumental response factor.

Linear instrumental response for NeoNs is reported in validation method for the sea water section.

The method was validated using a sediment collected in the Venice Lagoon at a depth of 10 m dated back to Pleistocene age (Teatini et al. 2017) and for these reason judged free from modern contaminant compounds.

Validation of method for sediments was performed at two levels of concentration: spiking 1 ng (absolute amount, abs) and 10 ng abs of native standard of NeoNs, and in both 1 ng abs of ACE*, IMI*, CLO* and TMX* were added to 1 g of the Pleistocene sediment. The Pleistocene sediment without the addiction of native spikes was considered as blank for validation. Quantitation for IMI was obtained using the 256.1/175.1 transition. The reproducibility (expressed as CV%) and trueness (as percentual error) were evaluated preparing 5 replicate samples and resulted in CV% and percent error in general less than 10%. Validation data for both concentration levels and the internal standard employed for the quantification are reported in table 20.

Table 20. Summary of trueness and reproducibility expressed as a relative standard deviation (CV%) for neonicotinoids determined in sediments. For each compound, the internal standard used is reported. Blank refers to the Pleistocene sediment. Based on the reported results the method resulted validated for the quantification of NeoNs in sediment

| | **Internal standard** | **Blank (pg/g)** | **Std dev (pg/g)** | **MDL (pg/g)** | **MQL (pg/g)** | **CV% (10ng/g)** | **Trueness (10ng/g)** | **CV% (1ng/g)** | **Trueness (1ng/g)** |
|---|---|---|---|---|---|---|---|---|---|
| ACE | ACE* | 8 | 1 | 4 | 12 | 8 | 9 | 5 | -8 |
| IMI | IMI* | 199 | 38 | 114 | 381 | 6 | -1 | 4 | -10 |
| CLO | CLO* | 19 | 2 | 6 | 20 | 11 | 4 | 3 | 12 |
| TCLO | ACE* | 1 | 1 | 4 | 14 | 9 | 5 | 6 | -9 |
| TMX | TMX* | 26 | 3 | 9 | 30 | 10 | -1 | 11 | -1 |

# 5 Industrial chemicals

## 5.1 2,6-Di-tert-butyl-4-methylphenol (BHT)

2,6-di-tert-butyl-4-methylphenol (BHT) is an anti-oxidant commonly used since the 1950s to preserve and stabilize the freshness, nutritive value, flavour and colour of food and animal feed products. BHT is also used to improve the stability of pharmaceuticals and cosmetics and increase the durability of rubber and plastics (Barbosa et al., 2016). The use of BHT as a food additive is generally not considered to pose a public health risk and it has been detected in different aquatic environments (Barbosa et al., 2016). However, in the natural environment, BHT is degraded biologically to 3,5-di-tert-butyl-4-hydroxybenzaldehyde (BHT-CHO), which is reported to generate peroxides in mice and rats and induce cellular DNA damage (Barbosa et al., 2016). Additional data are needed to understand the human health and environmental risks associated with the exposure to this compound.

For information about the analytical method, please refer to paragraph 6.

## 5.2 EHMC

2-ethylhexyl-4-methoxycinnamate (EHMC) is an organic UV filter used in many personal care products. Its occurrence in the environment has been described in several papers that have been given a great attention to the aqueous matrices, entering in the environment by wash off from skin or through wastewater or swimming pool water and finally reaching the sediments ant the biota (Barbosa et al., 2016).

Organic UV filters have known estrogenic effects on biota and humans with recognized in vivo and in vitro estrogenic activity to fish and mammals, but also other non- estrogenic hormonal targets in such organisms. Little is known about the removal of EHMC in the aquatic environment, while in WWTPs it resulted refractory to ozonation, but could be removed by UV treatment (Barbosa et al., 2016).

For information about the analytical method, please refer to paragraph 6.

## 5.3 Bisphenol A

Bisphenol A (BPA) is a contaminant of emerging concern that has been already measured in many European environmental samples and belong to different classes of widely used emerging substances (plasticizers, surfactants, personal care products, and industrial chemicals). Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) is an organic compound composed of two phenol molecules bonded by a methyl bridge and two methyl groups.

BPA is used as an intermediate (binding, plasticizing, and hardening) in plastics, paints/lacquers, binding materials, and filling materials. Furthermore, it is used as an additive for flame-retardants, brake fluids, and thermal papers. About 95 % of BPA produced in industry is used to make plastics, in particular polycarbonate resins (71 %) and epoxy resins (29 %). Due to the increasing demand for polycarbonates and epoxy resins, BPA production has constantly grown in the last years: the global demand was 3.2, 3.9, and 5.0 million tons in 2003, 2006, and 2010, respectively.

### 5.3.1 Materials and methods

#### 5.3.1.1 Reagents

BpA was determined after extracting the sample or the blank in 0.5 ml of methanol (LC-MS ChromaSolv® grade, 99.9% purity, Fluka, Sigma-Aldrich, Milan, Italy) for 10 min in an ultrasonic bath (SONICA®, Soltec, Milan, Italy). The extract was filtered by a 0.45 µm pore size PTFE filter, in order to remove any particles before the instrumental analysis was carried out. All samples and blanks were spiked with labelled BpA13C12 (10 ng/ml; ChemService, West Chester, PA, USA) as internal standard.

Biphenol A(≥99%), sodium chloride (≥99%), sodium citrate tribasic dehydrate (≥99%), sodium citrate dibasic sesquihydrate (≥99%), magnesium sulphate (≥99.5 %), sodium chloride (≥99%), ammonium hydroxide solution in water (> 25%), formic acid eluent additive for LC-MS, Supelclean™ PSA SPE, Discovery® DSC-18 SPE, HPLC/MS-grade methanol (MeOH), were purchased from Sigma Aldrich. Labeled BPA$^{13}C_{12}$ (BPA*, 10 ng/ml), used as internal standard, was purchased from ChemService, West (Chester, PA, USA).

HPLC/MS-grade methanol (ACN) and ultrapure water were purchased from VWR International. The ultrapure water (18.2 MΩ cm, 0.01 TOC) was produced by a Purelab Flex II system (Elga, High Wycombe, U.K.). Oasis HLB, 6mL, 500 mg SPE cartridge were obtained from Waters (Milford Massachusetts, USA).

#### 5.3.1.2 Instrumental method

An Agilent 1100 Series HPLC Systems (Waldbronn, Germany; with a binary pump, vacuum degasser, auto-sampler) was coupled with an API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystem/MSD SCIEX, Concord, Ontario, Canada) using a TurboV electrospray source that operated in positive mode by multiple reaction monitoring (MRM).

Chromatographic separation was performed using a C18 Synergi Hydro-RP (50 mm length, 4.6 mm i.d., 4 µm particle size; Phenomenex, Torrance, CA) with elution flow at 500 µl/min of a mobile phase constituted by water (solvent A) and ultrapure methanol (solvent B). The BPA's retention time of BPA was 4.53 min, and the run lasted 15 min. The binary elution program was as follows: at 0 min 50% B, 0-2 min gradient to 100% B, 2-7 min isocratic elution of B, 7-9 min to 50% B and equilibration at 50% B from 9 to 15 min. The injection volume was 100 µL.

The mass spectrometer source revealed the formation of $[M-H]^-$ precursor ions at m/z 227.00 for BPA and at m/z 239.00 for $^{13}C_{12}$BPA. Data were collected in negative ion mode by multiple reaction monitoring (MRM) with a 100 ms dwell time/transition.

The following transitions were monitored for BPA: 227.00/211.90 m/z (Declustering Potential, DP, -71.00 V, Collision Energy, CE, -25.20 V, Collision Cell exit Potential, CXP, -5.00 V), 227.00/210.80 m/z (DP -71.00 V, CE -28.00 V, CXP -6.00 V), 227.00/132.80 m/z (DP -71.00 V, CE -36.00 V, CXP -10.00 V), whereas for BPA*: 239.00/224.10 m/z (DP -74.00 V, CE -25.00 V, CXP -4.30 V), 239.00/222.90 m/z (DP -74.00 V, CE -35.00 V, CXP -5.00 V), 239.00/138.80 m/z (DP -74.00 V, CE -35.00 V, CXP -10.00 V). Instrumental conditions for the electrospray ionization source were: source temperature 550°C, nebulizer gas 30.00 psi, auxiliary gas 50.00 psi, curtain gas 25 psi, collision gas 4.00 psi, -54.00 V, entrance potential -10.00 V, ionization voltage -4450.00 V. The transitions 227.00/132.80 m/z for BPA, and 239.00/138.80 m/z for $^{13}C_{12}$BPA, were used for sample quantification.

#### 5.3.1.3 Instrumental method performance

The isotope dilution mass spectrometry method was used to quantify BPA, by comparing the native compound peak area with that of BPA*. The results were corrected for the instrumental response factor, which was evaluated by analysing a solution containing BPA at a concentration of 30 pg/µl and BPA at 25

pg/µl in ultrapure water. A series of external standards were used to calibrate the linearity of the instrument response. Standard solutions containing BPA at the concentrations between 0.015 and 150 pg/µl were prepared. In each standard solution, BPA* was added at a concentration of 25 pg/µl as internal standard and the analyses were performed by HPLC/MS/MS. The ratios between the unlabelled and labelled BPA areas were plotted against the ratio of unlabelled to labelled BPA concentrations. Good linearity was obtained, with an $R^2$ value of 0.998. The instrumental precision, obtained by three determinations of each of the standard solutions, and expressed as the relative standard deviation (RSD%), was always <10%. Instrumental limit of detection (LOD) and of quantification (LOQ), as three and ten times the signal-to-noise ratio of the known absolute amounts of the analysed target compound in a standard solution, was 3 e 10 pg/mL.

#### *5.3.1.4 Sample preparation for waters analysis*

In a volumetric flask 25 ng (absolute amount) of BPA*, internal standard, were spiked to 1000 mL of filtered sea water samples previously acidified with formic acid (1%, v/v). The clean-up and pre-concentration of samples, was performed using the OASIS HLB SPE cartridge (6cc, 500 mg sorbent per cartridge, Waters). The cartridge was conditioned under vacuum with methanol (10 mL), and equilibrated with formic acid (1%) in water (10 mL). Then, the sample was loaded on the cartridge. To eliminate sea salt remaining in the cartridge, a clean-up step was added to the procedure using 10 mL of formic acid (1%) in water. The SPE cartridge was dried under vacuum for 5 minutes and BPA were eluted at atmospheric pressure in a 15 mL vial with 10 mL of methanol. The sample was evaporated under a stream of nitrogen at 45°C in a Zymark Turbovap evaporator ( Hopkinton, MA, USA) at a volume of 100 µL, then reconstituted with 800 µL of water. The sample was filtered through a 0.45 µm PTFE filter into the auto-sampler vial before the analysis.

#### *5.3.1.5 Critical points*

The procedure checked was exactly the same of Neonicotinoid insecticides in order to simplify the preanalytical preparative. However, we demonstrated that OASIS HLB SPE cartridge (6cc, 500 mg sorbent per cartridge, Waters), used to concentrate and to purify the water samples, released BPA during the elution. We found a mean concentration of 17±6 ng (absolute amount) in five procedural blanks and this concentration is very similar to the amount of BPA used to estimate the trueness and precision (30 ng abs). For these reason, we will use glass SPE cartridge in order to prevent the contamination.

We further worked on the analytical methodology in order to reduce sample contamination analysing different solvent and considering different sample preparation procedure. At the end, we validated the method using this sample preparation protocol: in a volumetric flask 25 ng (absolute amount) of BPA*, used as internal standard, were spiked to 1000 mL of filtered sea water samples. The clean-up and pre-concentration of samples, was performed using the OASIS HLB SPE cartridge (6cc, 200 mg sorbent per cartridge, Waters). The cartridge was conditioned under vacuum with acetone (5mL), methanol (5 mL), and equilibrated with 5 mL of Elga ultrapure water purified using a LC-Pack cartridge (Millipore)(ElgaLCPack). Then, the sample was loaded on the cartridge. To eliminate sea salt remaining in the cartridge, this was washed first with 10 mL of ultrapure water ElgaLCPack and then with 10 mL of ElgaLCPack 5% MeOH. The SPE cartridge was dried under vacuum for 10 minutes and BPA were eluted at atmospheric pressure in a 15 mL vial with 5 mL of acetone. An aliquot of the sample was diluted 1:1 with ultrapure water ElgaLCPack, 10 µL were injected on the column. Procedural blanks (3 replicates) were prepared in the same way analysing ultrapure water ElgaLCPack as blank matrix.

#### *5.3.1.6 Method validation in sea waters.*

Quantification of BSA was performed in sea waters using BPA* as internal standard, by comparing areas of native compound with those of internal standard, with the correction of result for the instrumental response factor. The analytical procedure was validated through an estimation of trueness, precision, and method detection and quantification limits (MDL and MQL).

The analytical method was validated using waters collected in the Venice lagoon. A spike of 30 ng (absolute amount, abs) of BPA and 25 ng abs of BPA* were added to 1 L of sea water sample. The water lagoon without the addiction of native spikes was considered as blank for validation.

The reproducibility (expressed as CV%) and trueness (as percentual error) were evaluated preparing 5 replicate samples and resulted in a CV% of 10% and percent error of 4%. Mean blank was of 5.9 ng (absolute amount, abs), MDL and MQL were of 1.8 ng abs and 9.2 ng abs respectively.

#### *5.3.1.7 Sample preparation for sediment analysis*

Sediments were dried at 105° C for 48 h in order to obtain the dried weight. The determination of BPA in sediments was performed using the QuEchERS method. 1 g of humid homogenised sediment was weighted in a 50 mL centrifuge tube. Then 7 mL of ultrapure water and 10 mL of ACN and 25 ng absolute amount of BPA* was added and shaken with a vortex for 1 min; MgSO4 (6 g), NaCl (1.5 g), tri-sodium citrate dehydrate [Na3C6H5O7•2H2O (1.5 g)] and disodium hydrogen citrate sesqui-hydrate [HOC(COOH)(CH2COONa)2•1.5H2O (0.75 g)] were used for phase-separation adjustment, vortexed for 1 min and centrifuged for 5 min at 3000 rpm. The extract was transferred in a 50 mL centrifuge tube for a clean-up step was performed using PSA (825 mg), MgSO4 (2.5 g) and C18 (825 mg). The sample was vortexed for 1min and centrifuged for 5 min at 3000 rpm. The sample was evaporated at 100 µL under a stream of nitrogen at 45°C in a Zymark Turbovap, reconstituted with 800 µL of water and filtered through a 0.45 µm PTFE filter before the HPLC-MS/MS analysis.

#### *5.3.1.8 Method validation in sediments.*

Quantification of BSA was performed in sediments using BPA* as internal standard, by comparing areas of native compound with those of internal standard, with the correction of result for the instrumental response factor.

The analytical procedure was validated through an estimation of trueness, precision, and method detection and quantification limits (MDL and MQL). The method was validated using a sediment collected in the Venice Lagoon at 10 m depth dated back to Pleistocene age (Teatini et al. 2017) and for these reason judged free from the modern contaminant compounds.

Validation of method for sediments was performed spiking 30 ng (absolute amount, abs) of BPA and 25 ng abs of BPA* were added to the Pleistocene sediment. The Pleistocene sediment without the addiction of native spikes was considered as blank for validation. The reproducibility (expressed as CV%) and trueness (as percentual error), were evaluated preparing 5 replicate samples and resulted in CV% of 7% and percent error of -8%, mean blank was of 7 pg/g, MDL and MQL were of 5 pg/g and 16 pg/g respectively.

# 6 Fragrances

Fragrances are ubiquitous in daily life: the majority of cosmetics, toiletries, and a variety of household and Personal Care Products (PCPs) contain Fragrance Materials (FMs). 17 among the longest-lasting and most stable fragrance ingredients, that are commercially available, were originally selected to study their occurrence and distribution in the environment. The selected fragrances were recently found as contaminants at a global scale, being detected in the Venice Lagoon, Antarctica, Svalbard Islands and open Mediterranean Sea. Local emissions were revealed, together with evidences of long-range atmospheric transport (LRAT), supporting the hypothesis of the environmental persistence of the selected FMs. To fulfil the aims of the line 2.3, the research will focus on the 6 fragrances that in the preliminary studies were detected more frequently and at higher concentrations, namely: Amyl Salicylate, Hexyl Salicylate, Benzyl Salicylate, Oranger Crystals, Ambrofix, and Peonile. In particular, the allergenic and oestrogenic Salicylate compounds are High Production Volume (HPV) chemicals with a large global consumption (>5000 tons/year).

The results of the extensive bibliographical review indicate that the most widely used preanalytical procedures for the determination of **BHT, EHMC, triallate** and the **fragrances** in water samples use SPE extraction with Oasis HLB cartridges. These analytes are also effectively analysed by GC-MS, or GC-MS/MS, generally achieving lower detection limits. These techniques suggest a comprehensive multi-analyte method for their determination in water samples. Similarly, the preparation of samples of solid matrices will be performed following a common procedure for BHT, EHMC, triallate and fragrances using QuEchERS. The analytical method for fragrance analyses was previously validated and published (Vecchiato et al., 2016), both for the preanalytical and the instrumental steps, using a single quadrupole GC-MS (7890A-5975C, Agilent Technologies). However, in order to further improve the instrumental detection limits, a GC-MS/MS method has been developed (Trace 1310 - TSQ 9000 Thermo Fisher) for the selected analytes (fragrances, BHT, EHMC, triallate).

## 6.1 Materials and methods

### *6.1.1 Reagents*

Sodium chloride (≥99%), sodium citrate tribasic dehydrate (≥99%), sodium citrate dibasic sesquihydrate (≥99%), magnesium sulphate (≥99.5 %), sodium chloride (≥99%), Supelclean™ PSA SPE, Discovery® DSC-18 SPE were purchased from Sigma Aldrich. Pesticide-grade solvents, dichloromethane, *n*-hexane, toluene and acetone (Romil Ltd., Cambridge, UK) and HPLC/MS-grade methanol (ACN) (VWR International) were used. The ultrapure water (18.2 MΩ cm, 0.01 TOC) was produced by a Purelab Flex II system (Elga, High Wycombe, U.K.). Oasis HLB, 6mL, 500 mg SPE cartridge were obtained from Waters (Milford Massachusetts, USA).

### *6.1.2 Standard preparation*

$^{13}C$ labelled compounds are used as internal standard (Cambridge Isotope Laboratories Inc., Andover, MA USA), in order to achieve satisfactory accuracy for the quantification.

### 6.1.3 Sample preparation

Water samples (1 L) are spiked with $^{13}C$ internal standards and extracted using SPE cartridges previously conditioned with 10 ml of *n*-hexane, 10 ml of dichloromethane followed by 10 ml of ultrapure water. Cartridges are eluted with 1 mL of toluene, 15 mL of dichloromethane followed by 10 mL of *n*-hexane. Eluates are dried with $Na_2SO_4$ and reduced to 100 µL under a gentle nitrogen flow at 23 °C (Turbovap II®, Caliper Life Science, Hopkinton, MA, USA).

The determination of fragrances, BHT, EHMC and triallate in sediments was performed using the QuEchERS method. 1 g of wet homogenised sediment was weighted in a 50 mL centrifuge tube. Then 7 mL of ultrapure water, 10 mL of ACN and 50 ng absolute amount of labelled internal standard (Phenanthrene $^{13}C$ and triallate $^{13}C$) were added and shaken with a vortex for 1 min; MgSO4 (6 g), NaCl (1.5 g), tri-sodium citrate dehydrate [Na3C6H5O7·2H2O] (1.5 g) and disodium hydrogen citrate sesqui-hydrate [HOC(COOH)(CH2COONa)2·1.5H2O] (0.75 g) were used for phase-separation adjustment, vortexed for 1 min and centrifuged for 5 min at 3000 rpm. The extract was transferred in a 50 mL centrifuge tube for a clean-up step was performed using PSA (825 mg), MgSO4 (2.5 g) and C18 (825 mg). The sample was vortexed for 1min and centrifuged for 5 min at 3000 rpm. The organic phase was evaporated under a gentle nitrogen stream and solvent exchanged with dichloromethane before the instrumental analysis.

### 6.1.4 Instrument conditions

The determination of Fragrances, BHT, EHMC and triallate is conducted on a 60-m HP-5MS column (0.25 mm I.D., 0.25 µm; Agilent Technologies, Avondale, USA) and Multiple Reaction Monitoring (MRM) conditions were optimized (figure 3 and table 21). GC operating conditions are: He flux at 1.2 ml/min with the oven starting at 120°C (1min), increasing to 180°C at 25°C/min, to 250°C at 10°C/min and to 310°C at 20°C/min (1min).

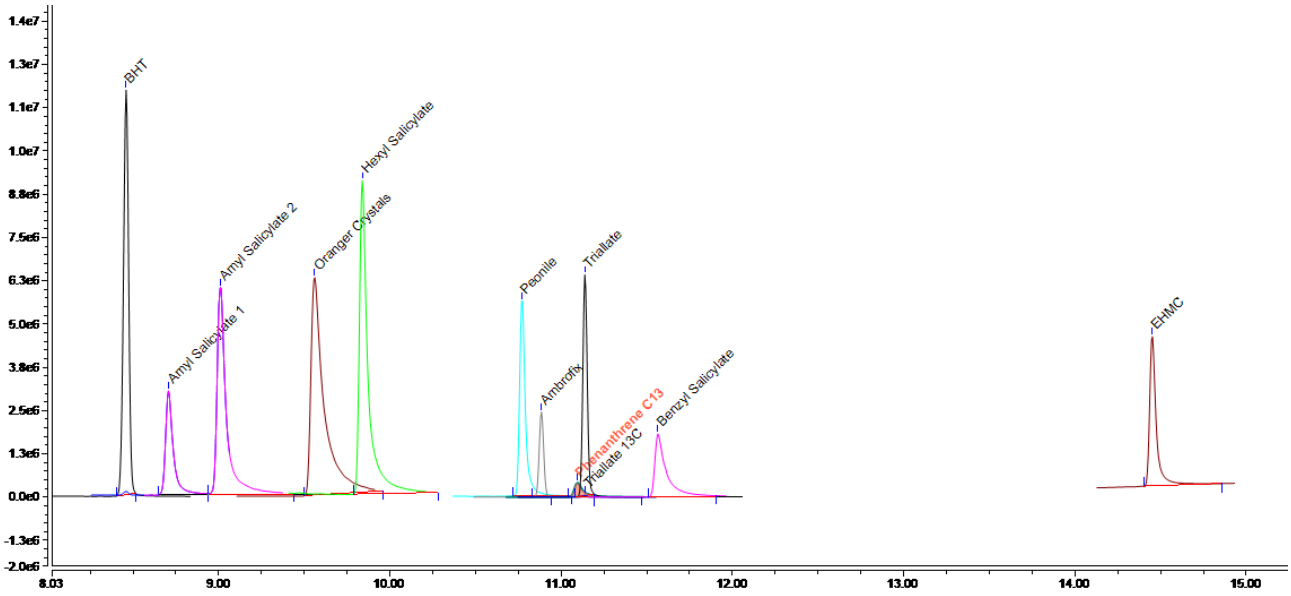


Figure 3. Chromatographic separation of the fragrances, BHT, EHMC and triallate.

Table 21. Optimized mass spectrometric parameters. Transitions used for quantification are in bold. The internal standard used for each compound is reported.

| | Internal standard | Q1 (m/z) | Q3 (m/z) | Collision Energy (V) |
|---|---|---|---|---|
| BHT | Phe. $^{13}C$ | 205.2 | 145.1 | 15 |
| | | 205.2 | 177.1 | 10 |
| | | **220.2** | **205.2** | **10** |
| Amyl Salicylate | Phe. $^{13}C$ | **120** | **92** | **10** |
| | | 138 | 92 | 15 |
| | | 138 | 120 | 5 |
| Oranger Crystals | Phe. $^{13}C$ | **155** | **127** | **15** |
| | | 170.1 | 155 | 10 |
| Hexyl Salicylate | Phe. $^{13}C$ | **120** | **92** | **10** |
| | | 138 | 92 | 15 |
| | | 138 | 120 | 5 |
| Peonile | Phe. $^{13}C$ | **130.1** | **103.1** | **10** |
| | | 197.1 | 168.1 | 10 |
| Ambrofix | Phe. $^{13}C$ | 97.1 | 55 | 10 |
| | | **221.2** | **97** | **10** |
| Triallate | Trial. $^{13}C$ | 86.1 | 43.1 | 5 |
| | | **268** | **184** | **20** |
| | | 268 | 226 | 10 |
| Benzyl Salicylate | Phe. $^{13}C$ | 91.1 | 65 | 15 |
| | | **228.1** | **91** | **10** |
| EHMC | Phe. $^{13}C$ | 161.1 | 133.1 | 10 |
| | | **178.1** | **132.1** | **15** |
| | | 178.1 | 161.1 | 10 |

### *6.1.5 Method validation*

The linearity of the instrumental response was checked in the concentration range 0.5 pg/µl – 25 ng/µl, with values spanning from $R^2$=0.9986 (Triallate) to $R^2$=1.0000 (Amyl Salicylate, Oranger Crystals, Peonile, Ambrofix, EHMC). The remaining compounds fall in the middle. Instrumental detection limits for the selected analytes range from 0.2 to 2 absolute picograms. Validation data for both water and sediment matrices are reported respectively in tables 22 and 23.

Table 22. Summary of trueness, recoveries and reproducibility expressed as a relative standard deviation (RSD) for fragrances, BHT, EHMC and triallate determined in water.

| | **Blank (ng abs)** | **Std dev (ng abs)** | **MDL (ng $L^{-1}$)** | **Recovery %** | **RSD** | **Trueness** | **RSD** |
|---|---|---|---|---|---|---|---|
| BHT | 2.11 | 0.48 | 1.44 | 86 | 7 | -15 | 8 |
| Amyl Salicylate 1 | 0.76 | 0.08 | 0.25 | 94 | 1 | -6 | 2 |
| Amyl Salicylate 2 | 0.31 | 0.07 | 0.21 | 94 | 4 | -9 | 3 |
| Oranger Crystals | 0.11 | 0.01 | 0.03 | 92 | 3 | 2 | 8 |
| Hexyl Salicylate | 0.34 | 0.06 | 0.18 | 93 | 6 | -8 | 7 |
| Peonile | 0.20 | 0.07 | 0.20 | 102 | 3 | 9 | 8 |
| Ambrofix | 0.37 | 0.10 | 0.31 | 97 | 3 | 3 | 5 |
| Triallate | 6.13 | 2.37 | 7.10 | 106 | 8 | 2 | 7 |
| Benzyl Salicylate | 0.12 | 0.02 | 0.05 | 94 | 7 | 2 | 8 |
| EHMC | 0.23 | 0.04 | 0.11 | 94 | 4 | 6 | 7 |

Table 23. Summary of trueness, recoveries and reproducibility expressed as a relative standard deviation (RSD) for Fragrances, BHT, EHMC and triallate determined in sediment.

| | **Blank (ng abs)** | **Std dev (ng abs)** | **MDL (ng $L^{-1}$)** | **Recovery %** | **RSD** | **Trueness** | **RSD** |
|---|---|---|---|---|---|---|---|
| BHT | 0.74 | 0.14 | 0.41 | 52 | 3 | 3 | 8 |
| Amyl Salicylate 1 | 0.36 | 0.16 | 0.47 | 60 | 13 | -2 | 2 |
| Amyl Salicylate 2 | 0.71 | 0.25 | 0.76 | 48 | 2 | -7 | 9 |
| Oranger Crystals | 0.51 | 0.20 | 0.59 | 64 | 8 | 8 | 4 |
| Hexyl Salicylate | 0.25 | 0.12 | 0.35 | 52 | 4 | -6 | 10 |
| Peonile | 0.45 | 0.07 | 0.21 | 70 | 8 | 8 | 7 |
| Ambrofix | 0.87 | 0.19 | 0.58 | 51 | 10 | -7 | 7 |
| Triallate | 4.06 | 3.06 | 9.17 | 103 | 7 | 8 | 4 |
| Benzyl Salicylate | 0.15 | 0.06 | 0.19 | 50 | 6 | -5 | 1 |
| EHMC | 0.47 | 0.10 | 0.29 | 64 | 7 | 2 | 8 |

# 7 Perfluoroalkyl substances: PFASs

In recent years perfluoroalkyl substances (PFASs) attracted increasing interest due to their widespread applications, environmental persistence and bioaccumulative potential, becoming a possible threat for human health. PFASs are a large group of anthropogenic organic chemicals widely used for a variety of industrial applications and products, such as surfactants, food packaging, cleaning agents, coating materials and more (Bečanová et al., 2016; Moreta and Tena, 2014), which includes perfluorinated carboxylic acids (PFCAs) and perfluorinated sulfonic acids (PFSAs). Their unique physico-chemical and biological properties make them resistant to hydrolysis, photolysis and biodegradation, as well as to metabolic processes in living organisms. Unlike other persistent organic pollutants (POPs), PFASs are water-soluble, hence easily released into surface water and aquifers, which become the principal medium for their environmental transport (Prevedouros et al., 2006). For this reason, they can enter into the food chain through the direct consumption of this water or its reuse to irrigate crops, intensifying the possible human exposure to PFASs.

In the last few years, a serious PFAS pollution is affecting a large area of the Veneto region, Italy, among the provinces of Vicenza, Verona and Padua (about 1.800.000 inhabitants with 350.000 people involved), caused by industrial discharges that have contaminated the rivers, the aquifers, and the drinking water led to concentrations in serum of the population about forty times higher than the tolerable values (WHO, World Health Organization, 2017).

The liquid chromatography (LC) methods are the most commonly used analytical methods for the determination of PFASs. They can be employed with different detection methods, but determination with mass spectrometry (MS) detection, with different type and configuration of the analyzers, are commonly considered as the reference methods.

Nevertheless, LC tubing and the internal LC parts could be responsible for high PFAS signals in blanks. Therefore, the LC tubing is usually replaced with PEEK (polyether ether ketone) and/or stainless-steel tubing, while solvent inlet filters are replaced with stainless-steel ones. Furthermore, all the Teflon® and other fluoropolymers parts, like vial caps and septa, are replaced with polyethylene ones.

Although analyses for PFASs are now mostly carried out by means of HPLC-MS(-MS) to meet the requirements of high sensitivity and selectivity, many laboratories are not equipped for this method and would prefer readily available, and less expensive, gas chromatography (GC) techniques.

GC is less common than LC methods but is also widely applied in determination of PFASs in different matrices, including environmental ones. If GC offers much larger efficiency of chromatographic separation, its practical limitation is given by the volatility of analytes to be determined, that requires an additional derivatization step to convert the polar functional group to a non-polar derivative (usually ethers) prior to injection in GC columns.

## 7.1 Perfluorinated carboxylic acids (PFCAs)

Among perfluorinated carboxylic acids (PFCAs), Perfluoroheptanoic acid - PFHpA, Perfluorooctanoic acid - PFOA, Perfluorononanoic acid - PFNA, Perfluorodecanoic acid - PFDA, Perfluoroundecanoic acid – PFUnA, and Perfluorododecanoic acid - PFDoA, will be analyzed using a GC/MS instrumentation, adopting a rapid and simple derivatization procedure in acetonitrile proposed by Dufková *et al.* (Dufková et al., 2012, 2009), using isobutyl chloroformate to convert the acids into the more volatile isobutyl esters, under catalysis by pyridine.

## 7.2 Perfluorinated sulfonic acids (PFSAs)

Among perfluorinated sulfonic acids (PFSAs), the Perfluorooctane sulfonic acid - PFOS will be analyzed by means of HPLC-$MS^2$, since GC determination of PFSAs is quite difficult because of the instability of its derivatives, which quickly undergo solvolysis and nucleophilic substitution reactions (Miller, 2010; Teasdale et al., 2010).

## 7.3 Materials and methods

### *7.3.1 Reagents*

All solvents are of pesticide grade. Dichloromethane, *n*-hexane, methanol and acetonitrile are supplied by Romil Ltd. (Cambridge, UK), while ultrapure water (18.2 MΩ, 0.01 Total Organic Carbon, TOC) is produced using a Purelab Ultra System (Elga LabWater Ltd., High Wycombe, UK). Isobutyl chloroformate, isobutyl alcohol, pyridine, sodium acetate, acetic acid, aqueous ammonium hydroxide solution (> 25%, v/v) and formic acid (98%) are purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Nitrogen gas 5.0 (99.999%) is used to concentrate the extracts. Isotope-labeled standard solutions (CLM-8005: $^{13}C_8$ PFOA and CLM-8505: $^{13}C_8$ PFOS) are obtained by CIL Inc. (Cambridge Isotope Laboratories, Tewksbury, MA, USA), while surrogate standard solution (ML163) is purchased from A2S SARL (Analytical Standard Solutions, Saint Jean d'Illac, France). All the tools and glassware are washed with an aqueous (2%, v/v) Contrad®2000 solution (VWR International LLC, Radnor, PA, USA), dried and rinsed three times with dichloromethane, three times with *n*-hexane and one time with methanol.

### *7.3.2 Standard preparation*

All the standard solutions (isotope-labeled and surrogate) are prepared in HPLC-MS grade methanol.

### *7.3.3 Water sample preparation*

Whole marine water is filtered using pre-combusted (400°C for 4 h) glass fiber filters (Whatman®, GF/F, Sigma-Aldrich Co.) at 0.7 µm, and the filtered water is kept frozen at -20°C to await analysis. One liter of water sample, acidified with formic acid (1%, v/v) and spiked with a known amount of $^{13}$C-labeled internal standard, is loaded on an Oasis HLB cartridge (Waters Corp., Milford, MA, USA; Hydrophilic-Lipophilic Balance, 500 mg, 6 mL, 60 µm), previously conditioned by passage of 10 mL of methanol, followed by 10 mL of deionized acidified water at a flow rate of one drop per second. The sample solution is passed through the cartridge under a pressure of 0.3 bar and the cartridge is washed with 10 mL of deionized acidified water to remove all the salts and then dried by passage of the air for 5 min. The analytes are eluted with 10 mL of methanol at a rate of one drop per second and the eluate is divided into two aliquots: one for direct PFOS by HPLC-$MS^2$ analysis and one for PFCAs by GC/MS analysis. The first aliquot is filtered through a 0.45 µm cellulose acetate filter (Vetrotecnica S.r.l., Padova, Italy) into the autosampler vials, while the latter one is evaporated to dryness under a gentle nitrogen flow and reconstituted with 178 µL of acetonitrile. Derivatization is performed by gradually adding 4 µL of pyridine, 8 µL of isobutyl alcohol and 10 µL of isobutyl chloroformate, thus making the reaction mixture overall volume equal to 200 µL. The mixture is stirred for 1 min in an ultrasonic bath and maintained quiet for 8 min. 200 µL of *n*-hexane are then added and the isobutyl esters formed are extracted into them by shaking for 2 min. The upper *n*-hexane layer is collected into vials for the GC/MS analysis.

### *7.3.4 Sediment sample preparation*

Fresh sediment samples are homogenized and kept frozen at -20°C until analysis. The extraction of the analytes from the matrix is performed using an ultrasound-assisted procedure in methanol. Samples are spiked with a known amount of $^{13}$C-labeled internal standard and then ultrasonically extracted three times for 15 min with aliquots of 10 mL each of methanol. The extracts are combined after centrifugation, evaporated under a nitrogen stream to 10 mL and then diluted with 25 mL of deionized water. Clean-up is performed by Solid Phase Extraction (SPE) using Oasis WAX cartridges (Waters Corp.; Weak Anion Exchange, 150 mg, 6 mL, 30 µm) previously conditioned by passage of 4 mL of methanol, followed by 4 mL of deionized water at a flow rate of one drop per second. The sample solution is passed through the cartridge under a pressure of 0.3 bar and the cartridge is dried by passage of the air for 5 min. The interferences are eluted with 4 mL of 25 mM acetate buffer, following by 4 mL of an aqueous 40% methanol solution and by 8 mL of methanol at a rate of one drop per second. The analytes are then collected eluting them with 2 mL of a methanolic 2% solution of ammonium hydroxide and divided into two aliquots: one for direct PFOS by HPLC-MS$^2$ analysis and one for PFCAs by GC/MS analysis, as described above.

### *7.3.5 Instrument conditions*

As previously mentioned, PFCAs are analyzed using a GC/MS instrumentation adopting a simple micro-derivatization procedure, practice that helped to avoid background contamination from internal fluoropolymer parts of LC, while PFOS is analyzed by means of HPLC-MS$^2$ because of the instability of its derivatives.

Quantification is performed using internal standards and the isotopic dilution technique and results are corrected using the instrumental response factors.

## 7.4 Method validation

Since there is no available Standard Reference Materials (SRM) for PFAS concentrations in water and sediment, it is necessary to spike them with a known amount of the surrogate standard solution. Therefore, the validation of the analytical procedure, in terms of precision, expressed as repeatability, accuracy, and recovery is performed on fortified real environmental matrices.

### *7.4.1 Method validation for PFASs in salt waters*

For the validation of analytical method in sea waters, 100 ng (absolute amount, abs) of surrogate standard of PFASs, and 10 ng abs of $^{13}$C-labeled internal standard (PFOA* and PFOS*) are added to filtered Venice lagoon water. The lagoon water without the addiction of the surrogate spikes is considered as blank for validation. Precision, measured as relative standard deviation (RSD) of three replicated analyses of different aliquots of lagoon water, ranged from 0.4 to 3.7% for PFCAs, with an average value of 2.1%, while for PFSAs, RSD for PFOS is 3.8%. The accuracy tests led to good results, with a relative error ($E_R$) in the range of 5-26% for PFCAs and of 2% for PFOS. Procedural average recoveries of the surrogate internal standards are 68±16% for PFCAs, with the lower value of 52% for PFHpA and the higher one for PFDoA (84%), while for PFOS the mean recovery percentage for triplicate analysis is 101%.

### 7.4.2 Method validation for PFASs in lagoon sediments

For the validation of analytical method for lagoon sediments, 100 ng abs of surrogate standard of PFASs and 10 ng abs of $^{13}$C-labeled internal standard (PFOA* and PFOS*) are added to a sediment collected in the Venice lagoon at a depth of 10 m, dated back to Pleistocene age (Teatini et al. 2017) and for these reasons judged free from modern contaminant compounds. As for the lagoon water, the sediment without the addiction of the surrogate spikes is considered as blank for validation.

Generally, the validation results are worse than those obtained for water, with critical points represented by the very low yields of the method and by the lack of accuracy in the quantification process. Precision, measured as RSD of three replicated analyses of different aliquots of lagoon sediment, ranged from 3.1 to 9.7% for PFCAs, with an average value of 5.3%, while for PFSAs, RSD for the PFOS is 3.0%. The accuracy tests led to good results only for those compounds that are quantified with the isotope dilution technique: PFOA* shows a $E_R$ value of 5.7%, while PFOS a value of 2.7%. As for the other PFCA compounds, the $E_R$ is in the range of 41-89%, thus making hard their quantification. Procedural average recoveries of the surrogate internal standards are 23 and 71% for PFUnA and PFDoA, while is always below the 15% for the other PFCA compounds. For PFOS the mean recovery percentage for triplicate analysis is 18%.

### 7.4.3 Limits of detection

For the calculation of the method detection limits (MDLs), three times the standard deviation of three replicated procedural blanks is used. Where no detectable peak is found in the blank, the instrumental detection limit (IDL) value is used in the calculation of the limit of detection (LOD). The procedural blanks for the MDL calculation are obtained spiking one liter of deionized water and the fourth aliquot of organic phase used for extraction of the Pleistocene sediment, after the collection of the first three extraction cycles. IDLs are calculated by injecting different standard solutions at decreasing concentration and every solution is injected three times. The calculation of the signal-to-noise ratio (S/N) of each congener is performed for all the replicates. Through linear regression and interpolation, we obtained the values of the IDL by setting a S/N > 3. LOD values are shown in table 24.

Table 24. Limits of detection (LOD) of single PFAS congeners expressed as method detection limits (MDLs) or instrumental detection limit (IDLs).

| PFHpA | PFOA | PFNA | PFDA | PFUnA | PFDoA | PFOS |
|---|---|---|---|---|---|---|
| *Lagoon sediment* | | | | | | |
| 0.10 | 6.7 | 0.22 | 1.18 | 23 | 0.14 | 0.36 |
| MDL (ng abs) | IDL (pg abs) | MDL (ng abs ) | MDL (ng abs) | IDL (pg abs) | MDL (ng abs) | MDL (ng abs) |
| *Salt water* | | | | | | |
| 0.60 | 1.26 | 0.73 | 2.91 | 23 | 0.51 | 0.04 |
| MDL (ng $L^{-1}$) | MDL (ng $L^{-1}$) | MDL (ng $L^{-1}$) | MDL (ng $L^{-1}$) | IDL (pg abs) | MDL (ng $L^{-1}$) | MDL (ng $L^{-1}$) |

### 7.4.4 Critical points

The bad results obtained during the validation of the method for lagoon sediments, require the evaluation of possible solutions. The presence of salts in the matrix, analyzed as fresh sediment and not freeze-dried one, could alter the ionic equilibrium exploited by the SPE cartridges with a WAX stationary phase, used during the purification process. Possible solutions can be the use of cartridges with a HLB stationary phase or the sediment freeze-drying before the analysis. The purification through the use of HLB cartridges is

carried out on three different replicated Pleistocene sediment samples but, at the date of this report, it was not yet possible to analyze them due to instrumental problems. The resolution of this issue is currently ongoing.

# 8 Untargeted characterization of sea waters and sediments from the Venice Lagoon

Has reported in the introduction, two approaches are considered with the aim of characterizing sea waters and sediments of the Venice lagoon in terms of the emerging contaminants: target quantification of environmental contaminants reported in the EU Watch List (2015/495) and untargeted characterization of the environmental matrices. Untargeted characterization supplies additional information performing suspect screening of suspect substances (using their exact mass and isotope pattern) and the non-target screening that involves all remaining components detected in a sample. In this last case, because no structural information are available in advance, a full non-target identification starting from the exact mass, isotope pattern, adduct and fragmentation information needs to be performed.

In order to obtain an untargeted characterization (suspect and non-target) of sample, more analytical methods in HPLC-ESI-HRMS were developed for acquisition of data in positive and negative polarity and also using data dependent acquisition.

## 8.1 Reagents

Acetamiprid-$d_3$ (ACE*) (98%), clothianidin-$d_3$ (CLO*) (≥97%), imidacloprid-$d_4$ (IMI*) (≥98%), thiamethoxam-$d_3$ (TMX*) (≥98%), HPLC/MS-grade methanol (MeOH), ammonium hydroxide solution in water (> 25%), formic acid eluent additive for LC-MS were purchased from Sigma Aldrich. HPLC/MS-grade acetonitrile (ACN) and ultrapure water were purchased from VWR International. The ultrapure water (18.2 MΩ cm, 0.01 TOC) was produced by a Chorus system system (Elga, High Wycombe, U.K.). Oasis HLB, 6mL, 500 mg SPE cartridges and Oasis HLB, 6mL, 200 mg SPE cartridges were obtained from Waters (Milford Massachusetts, USA).

## 8.2 Sample preparation for waters analysis

In a volumetric flask 20 ng (absolute amount) of labeled neonicotinoids internal standard: acetamiprid-$d_3$ (ACE*), clothianidin-$d_3$ (CLO*), imidacloprid-$d_4$ (IMI*), thiamethoxam-$d_3$ (TMX*), and 200 ng of labeled vanillin were spiked to 1000 mL of filtered (GF/F, porosity 0.7um, diameter 47mm) seawater. The sample was of 4L of seawater. The clean-up and pre-concentration of samples, was performed using the OASIS HLB SPE cartridge (6cc, 500 mg sorbent). The cartridge was conditioned under vacuum with methanol (5 mL), and equilibrated with water (5 mL). Then, the 4 L of sample were loaded on the cartridge at a flow of 10 ml/min. The cartridge was washed with 5 mL of water for the purpose of eliminating sea salt. The SPE cartridge was dried under vacuum for 10 minutes. In order to collect the greatest number possible of different molecules the elution was performed as follows: with 7 mL of ultrapure methanol, with 7 mL of formic acid 2% in methanol, with 6 mL of NH4OH 1.25% in methanol. After each elution step the cartridge was dried under vacuum for 10 min. All fractions were collected in a 22 ml amber vial. The sample was evaporated under a stream of nitrogen at 45°C in a Zymark Turbovap evaporator (Hopkinton, MA, USA) at a volume of 600 µL. The sample was filtered through a 0.22 µm mixed cellulose ester membrane. To reducing peak broadening during the chromatographic run, a 200 µL aliquot of the sample was diluted to a final volume of 800 µL with water before analysis. Blanks were prepared treating 4L of ultra-pure water that was produced by a Elga Purelab Chorus 1 system and filtered through a LC pack polisher cartridge (Millipore, Molsheim, France) as described before.

## 8.3 Sample preparation for sediments analysis

Before the pre-treatment, the sample was homogenized by quartering. The samples were dried at 105° C for 48 h in order to determine the dry weight.

An aliquot of 10 g of wet sample in a 15 mL centrifuge tube was spiked with labeled neonicotinoids as internal standard: acetamiprid-d3 (ACE*), clothianidin-d3 (CLO*), imidacloprid-d4 (IMI*), thiamethoxam-d3 (TMX*), and 200 ng of labeled vanillin. In order to extract the greatest number possible of different molecules the sample was sonicated with for 20 minutes in a bath of ice with different solvents: 5 mL of MeOH, 5 mL of MeOH:water 1:1, 5 mL of acetone. After each extraction the sample was centrifuged at 3000 rpm for 10 min. The extracts were collected and were evaporated under a stream of nitrogen at 45°C in a Zymark Turbovap evaporator (Hopkinton, MA, USA) at a volume of 1 mL. Then it was diluted with 200 mL of ultrapure water before the solid phase extraction and clean up step using a the OASIS HLB SPE cartridge (6cc, 200 mg sorbent). The cartridge was conditioned with 3 mL of methanol, equilibrate with with 3 mL of ultrapure water. Sample was loaded at a flow of 10 mL/min and, for the purpose of eliminate the salts, the cartridge was washed with 3 mL of ultrapure water. The cartridge was dried for 10 min before elution that was performed as follows with: 3 mL of methanol, 3 mL of 2 % formic acid in methanol, 3 mL of 1.25% ammonium hydroxide in methanol. After each step the cartridge was dried under vacuum for 10 minutes. The fraction were collected and the sample was evaporated at 45°C in Turbovap at a volume of 600 µL. The sample was filtered through a 0.22 µm mixed cellulose ester membrane, 200 µL were diluted in water at a final volume of 800 µL before analysis. Blanks were prepared treating 10 g of magnesium sulfate previously cleaned using ultrapure methanol as described before for sediments.

## 8.4 Instrumental conditions

Samples were analyzed using a HPLC-ESI-HRMS technique where a HPLC Ultimate 3000 (ThermoFisher Scientific, Germany) was coupled to an LTQ Orbitrap XL (ThermoFisher Scientific, Germany). The ionization was obtained using a HESI ion source (ThermoFisher Scientific, Germany). 19 µL of each sample were injected on a Zorbax SB-Aq (150 mm × I.D. 2.1 mm, 3.5 µm, Agilent) column. Eluent A was 0.01% of formic acid in VWR ultrapure water. Eluent B was 0.01% of formic acid in acetonitrile. Elution was performed at a flow rate of 200 µL/min. Chromatographic conditions were the following: 0-5 min 0% of A, from 5 to 45 min from 1% of A to 100% of B, from 45 to 60 min 100% of B, at 63 min 0% of A and isocratic equilibration until 80 min. HESI ionization was performed in both negative and positive polarity using a voltage of -4 kV and +4kV, Vaporizer Temperature of 300° C, Shealth gas 35 psi, Auxiliary gas 5 psi, capillary voltage +43 V and -43V. HRMS acquisition was in full scan in a 80-600 *m/z* mass range at a resolution of 100,000 at *m/z* 400, using a Tube Lense value of 70V and -70V.

Mass spectrometer calibration was performed every 48 h using sodium dodecil sulfate, sodium taurocolate, MRFA and Ultramark 1621 (Pierce LTQ ESI Negative Ion Calibration Solution, Thermo Scientific) and caffeine, MRFA and Ultramark 1621 (Pierce LTQ ESI Positive Ion Calibration Solution, Thermo Scientific) . In order to ensure mass accuracy lower than 2 ppm, lock masses were employed during data acquisition.

The samples were also analyzed by HPLC-HRMS using the data dependent acquisition consisting of the simultaneous acquisition of HRMS, MS/MS and $MS^n$. The fragmentation of the precursor ions is acquired at a resolution of 30,000 for the first and the second most intense ions observed during the full scan profiling in the selected mass range.

Each sample was injected three times and data were compared with blanks using Sieve 2.0 software (Thermo Scientific) where the detection algorithm was limited to small molecules. Comparing the water and sediment samples versus the blanks as a control group and applying a threshold of 10000 cps for a maximum of 5000 ions, the software calculated a ratio and p-value for each ion detected. The Sieve software was able to distinguish between ions of the same isotopic cluster, where we only considered monoisotopic ($^{12}C$) ions with intensities greater than five times of the blank values (ratio > 5). Molecular

assignments were performed using Xcalibur 2.1.0 software (Thermo Scientific) using guidelines of $^{12}C \leq 100$, $^{1}H \leq 200$, $^{14}N \leq 5$, $^{16}O \leq 50$, $^{32}S \leq 2$. At the moment data acquisition and data analysis are underway.

# 9 Microplastics

The ubiquity of microplastics in the environment has become of increasing concern among scientists, politicians, and the public. Determining the real hazards of microplastics is as essential as the need to develop and implement standardized protocols for sampling, quantification, and polymer identification of microplastics. Furthermore, data treatment and visualization should be standardized to allow for the subsequent comparison between different studies. According to the literature, analyses of microplastics in water and sediments were mainly performed via a microscope (e.g. optical or stereo microscope, SEM, etc.) and then followed by the polymer identification via FTIR. In recent studies microplastic analyses are performed via Micro-FTIR, which allows the quantification of particles and fibers and the identification of polymers. Different methods of separation can be employed to recover microplastics from water and sediments.

## 9.1 Materials and methods

### *9.1.1 Reagents*

For the analysis:

- Hydrogen peroxide 30% Sigma (Merck, Darmstadt, Germany)
- Hexane puriss. ≥99% (GC) Sigma (Merck, Darmstadt Germany)
- Methanol ≥99,9% (HPLC) Sigma (Merck, Darmstadt Germany)
- Ethanol absolute≥99,8% (HPLC) Sigma (Merck, Darmstadt Germany)
- Sunflower Oil (organic, cold pressed, Crudolio, Camisano Vicentino (VI))
- Ultrapure water (Elga Labwater, High Wycombe, UK)

For cleaning and decontamination:

- Contrad 2000 (Deacon Laboratories Limited, Hove, UK)
- Methanol ≥99,9% (HPLC) Sigma (Merck, Darmstadt Germany)
- Ethanol absolute≥99,8% (HPLC) Sigma (Merck, Darmstadt Germany)
- Ultrapure water (Elga Labwater, High Wycombe, UK)

For the filtration

- Whatman® Anodisc inorganic filter membrane 0.2 µm (47 mm diam.)

### *9.1.2 Sample preparation*

Sediment samples were kept in aluminium boxes and covered with aluminium foil and stored at 4 °C until the pre-treatment for the analysis. Water were collected in amber glass bottles, hydrogen peroxide was added to digest organic matter and stored at 4 °C until the pre-treatment for the analysis.

The sample pre-treatment and all the decontamination steps were performed in a plastic free clean room ISO 7. Glassware and steel tweezers, cotton lab coat and nitrile gloves were employed in order to minimize any plastic contamination.

Samples and blanks were tested at least in triplicate. Microplastic particles were extracted from sediments and waters, taking advantage of the oleophilic properties of plastic polymers. Before the extractions sediments were quartered and waters were shaken thoroughly. Separating funnels were employed for the microplastics extraction. On the same aliquot of water or sediments the extraction was repeated twice. Following the phases settling, the oil layer was recovered with hexane and ethanol in a cleaned Erlenmeyer

flask and then filtered on Whatman® Anodisc inorganic filter membrane 0.2 µm (47 mm diam.). Filters were then stored in cleaned glass Petri dishes and they were dried at room temperature in clean room for 72 h, until the analysis with Micro-FTIR.

### *9.1.3 Instrument conditions*

Quantitative analysis and polymer identification were performed with Micro FTIR Nicolet™ iNTM 10 (Thermo Fisher Scientific, Waltham MA, USA), equipped whit an ultra-fast motorized stage and with MCT detector (mercury cadmium telluride detector). Every filter was analysed with Omnic™ Picta™ software. The filters were mounted on the stage with removable tape on the supporting ring of the filter. Assuming that particles are randomly distributed on the surface of the filters, at least 20 areas (2.40 mm2, defined as count fields) were randomly chosen with no overlapping.

### *9.1.4 Method validation*

Blanks were extracted, filtered and analysed following the same procedure employed for sediment and water samples.

Microplastic particles are randomly distributed on the surface of the filter according the Poisson distribution. Standard deviation (σ), relative standard deviation % (CV %) and upper and lower confidence levels (IF) were calculated for each sample.

### *9.1.5 Critical points*

All the samples, especially those of sediments, are very heterogeneous. At least three replicates or more are needed for every sample collected.

Therefore, a certain amount of time is required for pretreatment, blanks, analysis with micro-FTIR and evaluation of results for every replicate and it can vary according to the matrix complexity.

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