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ORIGINAL PAPER

Development of analytical strategies using U-HPLC-MS/MS and LC-ToF-MS for the quantification of micropollutants in marine organisms

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Abstract Organic micropollutants such as pharmaceuticals, perfluorinated compounds (PFCs), and pesticides, are important environmental contaminants. To obtain more information regarding their presence in marine organisms, an increasing demand exists for reliable analytical methods for quantification of these micropollutants in biotic matrices. Therefore, we developed extraction procedures and new analytical methods for the quantification of 14 pesticides, 10 PFCs, and 11 pharmaceuticals in tissue of marine organisms, namely blue mussels (*Mytilus edulis*). This paper presents these optimized analytical procedures and their application to *M. edulis*, deployed at five stations in the Belgian coastal zone. The methods consisted of a pressurized liquid extraction and solid-phase extraction (SPE) followed by ultra high-performance liquid chromatography coupled to triple quadrupole mass spectrometry for pharmaceuticals and pesticides, and of a liquid extraction using acetonitrile and SPE, followed by liquid chromatography coupled to time-of-flight mass spectrometry for PFCs. The limits of quantification of the three newly optimized analytical procedures in *M. edulis* tissue varied between 0.1 and 10 ng g^{-1}, and satisfactory linearities (≥0.98) and recoveries (90–106%) were obtained. Application of these methods to *M. edulis* revealed the presence of five pharmaceuticals, two PFCs, and seven pesticides at levels up to 490, 5, and 60 ng g^{-1}, respectively. The most prevalent micropollutants were salicylic acid, paracetamol, perfluorooctane sulfonate, chloridazon, and dichlorvos.

Keywords Pharmaceuticals · Perfluorinated compounds · Pesticides · Marine organisms · Liquid chromatography · Mass spectrometry

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Introduction

Due to increasing anthropogenic activities and the release of various types of contaminants, marine ecosystems worldwide are subjected to a continuous pollution pressure [1].

The introduction of the European Reach Legislation has led to the development of less persistent, bioaccumulative, or toxic chemicals [2]. Generally, these newly designed chemicals could be characterized as hydrophilic compounds. As a consequence, the emerging more polar anthropogenic contaminants, such as pharmaceuticals, perfluorinated compounds (PFCs), and pesticides have recently gained more attention. Pharmaceuticals, PFCs, and pesticides are, to a large extent, dissolved in the water column. Consequently, several studies have demonstrated the occurrence of these micropollutants in marine and estuarine waters [3–7]. As such, these hydrophilic micropollutants are directly bioavailable to filter-feeding organisms including mussels and oysters. In light of the possible toxic, genotoxic and/or endocrine disrupting properties of some of these micropollutants, their potential to cause adverse effects in marine organisms should not be neglected [8, 9]. Moreover, the ingestion of contaminated seafood forms a major source of human exposure to micropollutants [10–12]. To study and evaluate the fate, effects, and environmental and human risks posed by these polar micropollutants in aquatic ecosystems, information regarding their presence in marine organisms and more particular in species that are important in terms of human consumption such as mussels is urgently needed.

Biotic samples are complex matrices demanding extensive extraction and clean-up procedures to obtain extracts amenable to analysis. In addition, relatively low concentrations may be expected to occur in these matrices. As a result, the occurrence of the above-mentioned micropollutants in marine organisms has been rarely studied and an increasing demand exists for reliable analytical methods allowing the quantification of these micropollutants in biotic matrices [13]. Analytical methods for the quantification of PFCs in biotic samples have been reported in literature [14–16]. Nevertheless, within this study, the existing method of Powley et al. [17] was adapted and optimized for this application, because of the significantly different sample matrix and detection technique. Also, the study area, which is suspected to be highly polluted with PFCs [18], offered an additional motivation to include the PFCs as a target group of contaminants within this study. Until now, only few studies are available for the analysis of pharmaceuticals in marine organisms. Ramirez et al. [19] reported a screening method for the detection of 23 pharmaceuticals in fish tissue, while Cueva-Mestanza et al. [20] described an analytical method for the detection of six pharmaceuticals in mollusks. With respect to the more polar pesticides, such as atrazine, simazine, chloridazon, Carafa et al. [21] reported an analytical procedure for the detection of 29 pesticides in clams. To the best of our knowledge, little attention has been paid to the prevalence of pesticides in marine organisms—aside from the organochlorine pesticides. Because an in depth evaluation of the presence of a wide range of pharmaceuticals and polar pesticides in this specific matrix was intended, new analytical procedures needed to be developed to enable the quantification of these compounds in a reliable and sensitive manner.

The objective of this study was to develop new extraction procedures and analytical methods for the quantification of 14 of the most intensively applied pesticides in Belgium, and 11 of most frequently used pharmaceuticals in Belgium [6] in blue mussels (*Mytilus edulis*). The existing analytical approach [17] for the detection of the most important PFCs in biotic samples was optimized for this specific biotic matrix as well. The analytical procedure for analysis of pharmaceuticals and pesticides consisted of a pressurized liquid extraction (PLE) and solid-phase extraction (SPE), followed by ultra high-performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS/MS). For the detection of PFCs, liquid extraction, and SPE were applied followed by liquid chromatography coupled to time-of-flight mass spectrometry (LC-ToF-MS).

Material and methods

Study area and sampling

M. edulis was collected in the Eastern Scheldt (ES) from subtidal plots and 50 *M. edulis* organisms were transplanted to cages deployed at different stations in the Belgian coastal zone. Two cage experiments were conducted during 2008. A long-term cage experiment ran from February till July 2008 at five stations: the marinas of Nieuwpoort (NP), Oostende (OO), and Zeebrugge (ZB2), the outport of Zeebrugge (ZB1), and one station situated in open sea at the Nieuwpoortbank (SEA; Fig. 1). *M. edulis* was sampled monthly to determine body concentrations of the target micropollutants. A short-term cage experiment was set up in November 2008. Cages with *M. edulis*, also originating from subtidal plots in the Eastern Scheldt, were deployed at the same stations, but not at the SEA-station, for 6 weeks. All cage-organisms were removed from the shell, homogenized, and these composite biotic samples were freeze-dried and stored at 4 °C prior to analysis.

Reagents and chemicals

The analytical method for pharmaceutical analysis included 11 compounds. Paracetamol (99%), ketoprofen (99%), carbamazepine (>99%), diclofenac (>99%), salicylic acid (>99%), clofibric acid (97%), atenolol (≥98%), trimethoprim (≥98%), and chloramphenicol (≥99%) were purchased from Sigma-Aldrich (St-Louis, MO, USA). Ofloxacin (>99%) was obtained from ICN Biomedicals Inc. (Ohio, USA), while propranolol (>99%) was purchased from Eurogenerics (Brussel, Belgium).

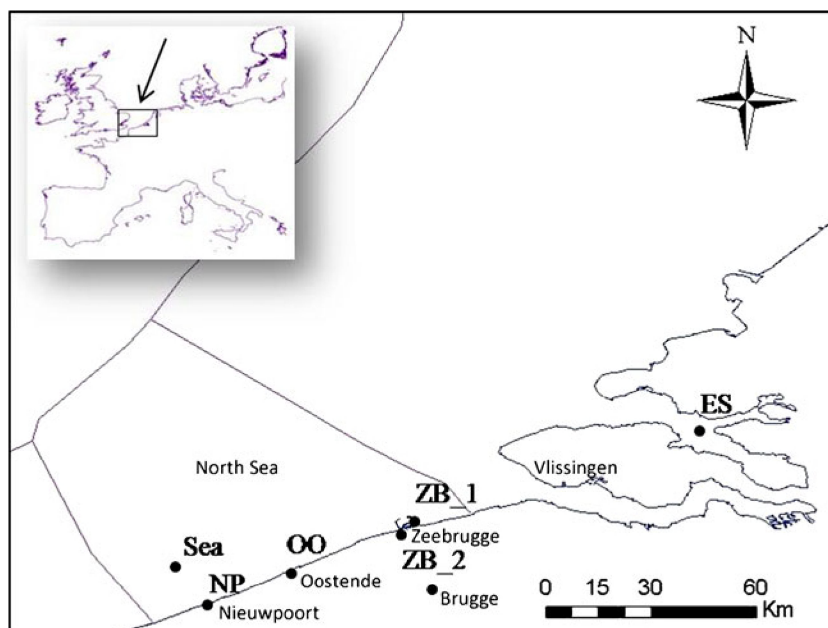

Fig. 1 Study area of the *Mytilus edulis* cage experiments in the Belgian coastal zone

The synthetic isobutcar 61 (4-3(isobutylamino-2-hydroxy-propoxy)carbazole) and two deuterated pharmaceuticals, atenolol-d_7 (≥95%) and salicylic acid-d_4 (≥98%) from Toronto Research Chemicals Inc. (North York, ON, Canada), were used as internal standard.

Fourteen pesticides were included in this study. Dichlorvos (>98%), dimethoate (>99%), diazinon (>98%), pirimicarb (≥99%), linuron (>99%), metolachlor (≥98%), chloridazon (≥99%), chlorpyriphos (>99%), simazine (>99%), isoproturon (>99%), terbutylazine (>98%), and diuron (>99%) were obtained from Sigma-Aldrich (St-Louis, MO, USA), while atrazine (>99%) and kepone (≥98%) were purchased from Chem Service (West Hester, PA, USA). Isoproturon-d_6 (>99%) and atrazine-d_5 (>99%) from Sigma-Aldrich (St-Louis, MO, USA) were used as internal standards.

Ten PFCs were examined in this study: four perfluorosulfonates (potassium perfluoro-1-butane sulfonate (PFBS), sodium perfluoro-1-hexane sulfonate (PFHxS), sodium perfluoro-1-octane sulfonate (PFOS), and sodium perfluoro-1-decane sulfonate (PFDS)) and six perfluorocarboxylates (perfluoro-*n*-heptanoic acid or PFHpA, perfluoro-*n*-octanoic acid or PFOA, perfluoro-*n*-nonanoic acid or PFNA, perfluoro-*n*-decanoic acid or PFDA, perfluoro-*n*-undecanoic acid or PFUnA, and perfluoro-*n*-dodecanoic acid or PFDoA). All analytical PFC-standards were purchased from Wellington Laboratories (Guelph, Ontario, Canada) with chemical purities of more than 98%. Five ^{13}C-labelled internal standards were used as well: sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octane sulfonate, perfluoro-*n*-[1,2,3,4-$^{13}C_4$] octanoic acid, perfluoro-*n*-[1,2,3,4,5-$^{13}C_5$]nonanoic acid, perfluoro-*n*-[1,2-$^{13}C_2$]decanoic acid, and perfluoro-*n*-[1,2-$^{13}C_2$]dodecanoic acid.

Analytical grade reagents were used for extraction and purification purposes, and Optima® LC-MS grade for U-HPLC-MS/MS analysis. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively. For LC-ToF-MS analysis, methanol was purchased from Rathburn Chemicals (LTd Walkerburn, Scotland), while HPLC-grade water was obtained from Biosolve (Biosolve Chemicals, The Netherlands). Ammonium acetate (2.5 mM) in water was obtained through dilution of LC-MS Chromasolv® water containing 0.1% ammonium acetate (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany). Aqueous ammonium carbonate (Merck, Darmstadt, Germany; 2 mM) and aqueous formic acid (Merck, Darmstadt, Germany; 0.08%) were prepared by appropriate dissolution or dilution in ultra-pure water (Arium 611 UV system, Sartorius Stedim Biotech, Aubagne, France).

Primary stock solutions of the pharmaceuticals and pesticides were prepared in ethanol at a concentration of 1 mg mL^{-1}, while methanol was used for the PFCs. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in ethanol and methanol, respectively. All solutions were stored at −20 °C in the dark.

Extraction and clean-up

The sample preparation for pharmaceuticals consisted of a PLE, which was performed on a Dionex ASE® 350 Accelerated Extractor with Solvent Controller (Dionex Corp., Sunnyvale, CA, USA). A cellulose filter (27 mm, Dionex Corp.) was placed on the bottom of a 22-mL stainless steel extraction cell. Each cell was filled with 9.5 g of aluminum

oxide 90 aktiv neutral (Dionex Corp.). A mixture of 1 g of freeze-dried biotic sample with 1.5 g of diatomaceous earth (DE, ASE® Prep Diatomaceous Earth, Dionex Corp.) was placed on top of the aluminum oxide. The internal standards were added prior to extraction to a final concentration of 200 ng g^{-1}. A combination of acetonitrile/water (3/1) with 1% formic acid was used as the extraction solvent. Extraction was carried out at 100 °C for three cycles of each 10 min. The extract (±45 mL) obtained by accelerated solvent extraction (ASE) was evaporated under nitrogen at 55 °C to a final volume of 5 mL and further diluted to 100 mL with ultra-pure water. Next, SPE was carried out using Strata-X cartridges (6 mL, 200 mg, Phenomenex B.V., Utrecht, Netherlands). The cartridges were preconditioned with 5 mL of methanol and 5 mL of ultra-pure water. After loading, the cartridges were rinsed with 5 mL of ultra-pure water. Elution was performed using 2×3 mL of methanol. Finally, this eluate was evaporated under nitrogen at 55 °C and reconstituted in 50 µL of acetonitrile with formic acid (0.08%) and 250 µL aqueous formic acid (0.08%). Prior to U-HPLC-MS analysis, the extracts were centrifuged at 9,000 rpm for 10 min at 4 °C.

A similar combination of PLE and SPE was optimized for pesticide extraction and clean-up from biotic samples, with the following differences: the internal standards were spiked at a concentration of 100 ng g^{-1}, and each extraction cell was filled with 0.25 g of sample, 2 g of aluminum oxide, and 4.5 g of diatomaceous earth. Extraction was performed using 1:1 acetonitrile/methanol, at 100 °C for three cycle times of 3 min. The ASE-extract obtained was evaporated to 0.5 mL before dissolving it in 10 mL of ultra-pure water. SPE was carried out using Isolute ENV + cartridges (10 mL, 200 mg, Biotage, Uppsala, Sweden). The cartridges were preconditioned with 5 mL of methanol and 5 mL of ultra-pure water with methanol (5%). Elution was performed using 5 mL of methanol and acetonitrile. Next, the eluate was evaporated under nitrogen at 55 °C to dryness and reconstituted in 50 µL methanol and 150 µL of 2 mM aqueous ammonium carbonate. After centrifugation, the eluate was filtered using a 0.22-µm Syringe-Driven Filter Unit (Millipore, Carritwohill, Cork, Ireland).

For analysis of PFCs in biotic samples, 1 g of freeze-dried sample, spiked with the ^{13}C-labelled internal standards at a concentration of 50 ng g^{-1}, was extracted with 10 mL of acetonitrile by homogenization with an Ultra-Turrax dispersing unit (Ika, Staufen, Germany). After centrifugation at 5,000 rpm for 20 min at 4 °C, the supernatant was reduced to 5 mL by evaporation under nitrogen at 55 °C and subsequently diluted to 100 mL with ultra-pure water. Next, SPE was carried out using OASIS HLB cartridges (6 mL, 200 mg, Waters, Milford, MA). The cartridges were preconditioned with 2 mL of methanol and 2 mL of ultra-pure water. After loading, the cartridges were rinsed with 5 mL of ultra-pure water. Elution was performed using 3×2 mL of methanol and the eluates were centrifuged at 9,000 rpm for 10 min at 4 °C. Next, the eluates were evaporated to dryness under nitrogen at 55 °C. Finally, 0.25 mL of methanol and 0.25 mL of 2.5 mM aqueous ammonium acetate were added before transfer to LC-MS vials. Samples were stored at −20 °C before analysis.

Chromatography

For both the pesticides and pharmaceuticals, chromatographic separation was carried out using ultra high-performance liquid chromatography (U-HPLC). The apparatus comprised of an Accela™ High Speed LC and an Accela™ Autosampler (Thermo Scientific, San Jose, CA, USA). Chromatographic separation was achieved using a Nucleodur C18 Pyramid U-HPLC column (1.8 µm, 100×2 mm, Macherey-Nagel, Düren, Germany). For the pharmaceuticals, the mobile phase constituted of 0.08% aqueous formic acid (A), 0.08% formic acid in acetonitrile (B), and isopropanol (C). A linear gradient was used starting from 98% A and 2% B, which was held for 0.8 min. The percentage of acetonitrile was increased to 65% B in 30 s, and further to 100% B in 1 min and held for 4 min. Next, 90% B and 10% C were applied to the column for 2 min, before equilibration at initial conditions for 2 min. Pesticide separation was achieved using methanol (D) and aqueous ammonium carbonate (2 mM; E). The linear gradient started with a mixture of 98% E and 2% D for 1 min. The methanol percentage increased to 90% in 30 s, and further to 100% in 3 min. Between samples, the column was allowed to equilibrate at initial conditions for 1 min.

For PFC-analysis, the LC-apparatus comprised of a 1,200 series binary gradient pump and a 1,100 series autosampler (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using a Luna® C18 (2) HPLC column (5 µm particle size, 250×2.0 mm; Phenomenex Inc., Utrecht). The mobile phase consisted of a mixture of (F) 2.5 mM aqueous ammonium acetate and (G) methanol. A linear gradient of 0.3 mL min^{-1} was used starting with a mixture of 50% F and 50% G, increasing to 90% G in 10 min. This ratio was kept for 6 min before reversion to the initial conditions.

Mass spectrometric detection

Detection of pharmaceuticals and pesticides was carried out using a TSQ Vantage Triple-Stage Quadrupole Mass Spectrometer (Thermo Electron) equipped with a heated electrospray ionization probe (HESI-II). The parameters as presented in Table 1 were found to be the optimal ionization source working parameters for the respective analytes. The mass resolution at the first (Q1) and third (Q3) quadrupole was set to 0.7 Da at full width at half maximum. The cycle time was

Table 1 HESI-II working parameters for ionization of the selected pharmaceuticals and pesticides

	Pharmaceuticals	Pesticides
Spray voltage (V)	3,500	4,000
Capillary temperature (°C)	270	315
Sheath gas pressure (arbitrary units, au)	25	25
Auxiliary gas pressure (au)	5	5
Ion sweep gas pressure (au)	2	2
Vaporizer temperature (°C)	25	35

adjusted to 0.5 and 0.9 s for pharmaceutical and pesticide analysis, respectively. Argon was used as collision gas, the collision gas pressure was set at 1.5 mTorr and the chrom filter peak width at 10 s.

Perfluorinated compounds were detected with a time-of-flight mass spectrometer equipped with a dual electrospray ionization interface (ESI MSD TOF, Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer was operated in the negative ion mode. Instrument parameters were: drying gas temperature of 325 °C, drying gas flow of 5 L min^{-1}, nebuliser pressure of 20 psi, capillary voltage of 3,500 V, and chamber voltage of 3,000 V. Before analyzing a series of samples, the ToF-MS apparatus was tuned and calibrated using the ESI Tuning Mix (Agilent Technologies, Santa Clara, CA, USA). During analysis, a reference solution was pumped into the MS-system at a rate of 50 μL min^{-1} using a separate sprayer connected to a 1100 series pump (Hewlett Packard, Palo Alto, CA, USA). This reference solution consisted of purine with a *m/z* ratio of 119.0363 and HP-0921 (hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) with a *m/z* ratio of 980.0164 in ACN/ H_20 (95/5; Agilent Technologies, Santa Clara, CA, USA). Accurate mass measurements could only be achieved if these reference masses were clearly present. Chromatograms and spectra were recorded and processed using Agilent MassHunter Quantitative Analysis® software (Agilent Technologies, Santa Clara, CA, USA).

Identification and quantification

The target analytes were identified based on their retention time relative to that of the internal standards. For pharmaceutical and pesticide analysis, using U-HPLC-QqQ-MS/MS in the selected reaction monitoring mode (SRM), at least two transitions were monitored. The relative abundances of these specific transitions were compared with those of the standards and both product ions were used for quantification purposes. Identification of the PFCs, using LC-ToF-MS, was performed on the basis of their accurate mass. Within this study, a maximum mass error of 10 ppm was allowed [7].

Upon identification, area ratios were determined by integration of the area of an analyte under the obtained chromatograms in reference to the integrated area of the internal standard. The analyte concentrations were calculated by fitting their area ratios in a seven-point calibration curve in tissue matrix. To this end, freeze-dried *M. edulis* samples were spiked with a standard mixture obtaining seven final concentrations in the range of 0.1 to 250 ng g^{-1} and with the appropriate concentrations of the respective internal standard mixtures.

Quality assurance

Before and after analysis of a series of samples, a standard mixture (0.1 ng on column) of the targeted analytes and the internal standards was injected to check the instrument performance of the LC-ToF-MS and U-HPLC-QqQ-MS/MS systems. Quality control of the method was performed by analysis of a blank sample, together with linear calibration curves constructed using matrix samples spiked with standard solutions at seven concentration levels ranging from 0.1 to 250 ng g^{-1}. This was performed for every series of samples at least in duplicate.

Results and discussion

Sample preparation

Pharmaceuticals

Many studies describe analytical methods for the detection of pharmaceuticals in water. For marine organisms, however, only few studies are available [19, 20]. Extraction of environmental matrices such as biotic tissue and sediment, is conventionally performed by means of Soxhlet extraction or sonication, demanding long extraction times and large solvent volumes [22]. In recent years, techniques such as microwave-assisted extraction (MAE) and PLE are gaining in popularity [20]. The latter extraction technique has been reported in several recent studies about the detection of pharmaceuticals in soil, sediment, and sewage sludge [23–25]. Since preliminary experiments using classical solid/liquid extraction versus PLE and MAE provided higher extraction recoveries for the target pharmaceuticals in case of PLE application, this technique was selected and further optimized for pharmaceutical extraction from biotic tissue.

To obtain the optimal extraction parameters, subsequent experiments were performed using 1 g of freeze-dried biotic tissue spiked at 250 ng g^{-1}. Selection of the optimal parameters was based on the resulting peak area, signal-to-

noise ratio and peak shape of each analyte upon U-HPLC-MS/MS analysis, but also on visual characteristics of the extract such as colour and turbidity.

First, different extraction solvents were tested (acetone, methanol, acetone/methanol (1:1), *n*-hexane, acetone:ethyl acetate (1:1), acetonitrile+1% formic acid, acetonitrile: water (3:1)+1% formic acid). This is of crucial importance, since all pharmaceuticals of interest should be simultaneously extracted, irrespective of their chemical structure or physico-chemical properties. A mixture of acetonitrile/ water (3:1) with 1% formic acid provided the best results. Second, the optimal temperature (60–100–140–180 °C), static time (3–5–10–15 min), and number of extraction cycles (1–2–3) were investigated. Three cycles of 10 min were found to be optimal for the extraction of the target pharmaceuticals. Moreover, it was found that 100 °C resulted in slightly higher recoveries compared to 60 °C or 140 °C. The flush volume, which is the amount of solvent flushed through the sample cell after extraction, was evaluated as well. Since previous studies at our laboratory showed slightly better recoveries when using a flush volume of 60%, as proposed by the manufacturer, this flush volume was further applied during this study as well [26]. Next, the addition of Al_2O_3 to the extraction cell was evaluated. Since Al_2O_3 is known to inhibit the co-extraction of lipids and other hydrophobic matrix constituents, addition of different quantities (0–4–6–9.5 g) of Al_2O_3 to the PLE cells was tested. Cleaner extracts were obtained by inserting 9.5 g of Al_2O_3 into the PLE cells. For fine powdery samples, such as freeze-dried biotic tissue, it is recommended to mix the sample with diatomaceous earth to inhibit the aggregation of the sample and to improve the solvent–matrix interactions. Therefore, 1.5 g diatomaceous earth was inserted into the extraction cell as well. The sample mass was tested by analysis of 1, 3 or 5 g of freeze-dried *M. edulis* tissue spiked at 250 ng g^{-1}. It was found that increasing the sample mass to 3 or 5 g, resulted in lower extraction efficiencies and turbid extracts. Therefore, further experiments were conducted using 1 g of tissue.

Due to the complexity of biotic samples, further clean-up was required following PLE to allow sufficiently high *S/N* ratios and peak resolution upon U-HPLC-MS/MS analyses. Several techniques have been described in literature for the clean-up of pharmaceuticals from complex matrices: gel permeation chromatography [27, 28], solid-phase microextraction [29], and solid-phase extraction [23–25, 30, 31]. In this study, we applied the most adequate technique, namely SPE. Indeed, SPE has shown good performance in extraction of pharmaceuticals from various aqueous matrices and simultaneously allows the concentration of the sample. Several SPE-cartridges were tested: Strata-X, Chromabond HR-X (6 mL, 200 mg, Marchery-Nagel, Düren, Germany), and Oasis HLB (6 mL, 200 mg, Waters, Milford, MA). Based on recoveries obtained (peak area and *S/N* ratio) and the clarity of the final extract, it was decided to retain the Strata-X columns for this application.

Pesticides

Pang et al. [32] determined more than 400 pesticides in grain by accelerated solvent extraction using acetonitrile as extraction solvent, followed by SPE. Besides, Carafa et al. [21] described an analytical procedure for 29 pesticides in clams. This method included extraction with ultrasonication using a mixture of methanol and water (4:1) followed by SPE using Oasis HLB cartridges. In this study, the combination of PLE and SPE was again selected, because of its excellent performance in extraction of pharmaceuticals from biotic tissue, and since PLE proved more efficient in extraction of our selected pesticides.

First, the optimal PLE-parameters were determined by analysis of a 1 g freeze-dried *M. edulis* sample spiked at 100 ng g^{-1} by using a similar approach to that described above. Some significant differences resulting in better analytical results upon U-HPLC-MS/MS with the application of the pharmaceuticals were identified. A sample mass of 0.25 g was found to be sufficient. Two and 4.5 g Al_2O_3 and diatomaceous earth, respectively, were inserted in the cell. A mixture of methanol and acetonitrile (1:1) was found to be the optimal extraction solvent for extraction of the target pesticides at a temperature of 100 °C. An extraction time above 10 min did not significantly increase the extraction efficiency of the analytes. Three cycles of 3 min were found to be sufficient. Further clean-up was again performed by SPE. The choice of the SPE-sorbent was determined based on the recovery rates (*S/N* ratio and peak area) obtained and clarity of the extract. The Isolute ENV + cartridges provided the best results over Strata-X, Chromabond HR-X, and Chromabond Easy (6 mL, 200 mg, Macherey-Nagel, Düren, Germany).

Perfluorinated compounds

Our analytical procedure is based on the study of Powley et al. [17] on quantification of PFCs in biological samples, which is commonly used in this field [33]. The use of acetonitrile as extraction solvent was adapted from Powley et al. [17], while for clean-up and concentration of the extracts, SPE with Oasis HLB cartridges was performed. Oasis HLB cartridges have been repeatedly applied for PFC-extraction from aqueous samples [7, 34, 35] and their superiority above other SPE sorbents has been clearly demonstrated [7]. After SPE, clear extracts were obtained by centrifugation of the eluates.

Chromatography and mass spectrometric detection

Pharmaceuticals and pesticides

According to literature [36, 37], LC-MS/MS is the best tool for sensitive detection of pharmaceuticals from different therapeutic classes as well as for multi-residue pesticide analysis in complex environmental matrices. Therefore, new U-HPLC-MS/MS methods were developed allowing unequivocal confirmation and quantification of the targeted pharmaceuticals and pesticides. For both groups of micropollutants, rapid chromatographic separation was achieved using a Nucleodur C18 Pyramid U-HPLC column. Based on peak intensities, areas, *S*/*N* ratios, and peak resolution of the individual analytes, this column provided better results than the Hypersil Gold (1.9 µm, 50 and 100×2.1 mm, Thermo Electron) and Acquity HSS T3 or HSS C18 (1.8 µm, 50 and 100×2.1 mm, Waters, Milford, USA) U-HPLC columns. The Nucleodur C18 Pyramid also exerted a better retention for the fast-eluting pharmaceutical atenolol. Too early elution of compounds should be avoided, in order to prevent interference with the solvent peak. To this end, the mobile phase started with a gradient of 98% 0.08% aqueous formic acid and 2% 0.08% formic acid in acetonitrile. For separation of pharmaceutical compounds using liquid chromatography, water and acetonitrile are commonly used solvents [36]. In addition, a higher ionization rate in positive ion mode may be obtained by

Table 2 SRM transitions, MS parameters, recoveries, and limits of quantification (LOQs) of the targeted pharmaceuticals and pesticides in *Mytilus edulis* extracts (n=21)

Compound	tR (min)	Precursor ion (m/z)	Product ions (m/z)	S-lens (V)	Collision E (eV)	Recovery (%) (x ± RSD)	LOQ ng g^{-1}
Pharmaceuticals							
Atenolol	0.83	267.1 (+)	190.1, 145.0	102	18, 26	97±13	1
Paracetamol	2.15	152.0 (+)	110.1, 65.1	52	16, 30	97±26	2.5
Trimethoprim	2.25	291.1 (+)	261.1, 230.1	188	25, 23	101±13	1
Propranolol	2.35	260.2 (+)	183.1, 116.1	138	18, 17	98±13	1
Ofloxacin	2.35	362.1 (+)	318.2, 261.1	176	18, 27	102±14	5
Chloramphenicol	2.58	321.0 (−)	257.1, 152.1	104	15, 19	95±15	2.5
Carbamazepine	2.78	237.1 (+)	194.1, 193.1	93	19, 33	100±11	1
Salicylic acid	2.85	137.0 (−)	93.1, 65.1	51	20, 32	103±10	10
Ketoprofen	3.19	255.0 (+)	209.2, 105.0	295	14, 24	100±12	5
Clofibric acid	3.46	213.0 (−)	127.1, 85.1	73	19, 13	100±20	1
Diclofenac	3.55	296.0 (+)	250.1, 214.1	78	13, 34	98±16	2.5
Atenolol-d_7	0.96	274.1 (+)	190.1, 145.0	111	19, 27	–	–
Isobutcar 61	2.36	313.8 (+)	222.1, 130.1	170	19, 20	–	–
Salicylic acid-d_4	2.84	141.1 (−)	97.1, 69.2	52	19, 33	–	–
Pesticides							
Dimethoate	2.87	230.0 (+)	199.0, 79.1	63	10, 34	104±10	10
Chloridazon	2.90	222.0 (+)	104.1, 77.1	95	23, 36	104±9	1
Simazine	3.11	202.1 (+)	132.1, 124.1	77	18, 18	100±8	5
Pirimicarb	3.20	239.1 (+)	182.2, 72.2	74	15, 33	101±10	1
Isoproturon	3.21	207.1 (+)	72.1, 46.2	78	19, 17	102±8	1
Dichlorvos	3.23	221.0 (+)	109.1, 79.1	81	19, 28	100±10	1
Atrazine	3.25	216.1 (+)	174.1, 68.1	83	17, 36	95±19	1
Diuron	3.25	233.0 (+)	72.1, 46.2	71	18, 16	103±8	1
Linuron	3.36	249.0 (+)	182.1, 160.1	83	16, 18	105±11	1
Terbutylazine	3.41	230.2 (+)	174.1, 104.1	70	18, 33	100±8	1
Metolachlor	3.61	284.1 (+)	252.2, 176.2	69	15, 24	101±11	1
Diazinon	3.80	305.1 (+)	169.1, 97.0	87	20, 34	104±17	1
Kepone	4.30	506.6 (−)	426.8, 424.8	157	21, 20	99±19	1
Chlorpyriphos	4.64	349.8 (+)	199.9, 197.9	82	22, 21	98±17	1
Isoproturon-d_6	3.21	213.1 (+)	78.2, 52.2	69	20, 19	–	–
Atrazine-d_5	3.23	221.1 (+)	179.1, 101.1	79	19, 27	–	–

adding formic acid to the mobile phase [36]. For 1 min, isopropanol was added to the mobile phase. The higher elution strength of isopropanol resolved the carry-over problem of ofloxacin and trimethoprim. All pesticides were separated within less than 5 min using a mobile phase consisting of aqueous ammonium carbonate (2 mM) and methanol. Methanol as eluent was preferred over acetonitrile because of its weaker elution strength, thus increasing the retention of the more polar pesticides. In line with Martins-Junior et al. [38] ammonium carbonate was selected as a buffer since it provided better chromatographic elution for polar pesticides than other additives (formic acid, acetic acid, ammonium hydroxide, ammonium acetate).

As mentioned before, we selected triple quadrupole mass spectrometry to allow reliable quantification of the selected pharmaceuticals and pesticides in extracts of tissue. At first, compound-dependent parameters were optimized by direct infusion of individual analytes (10 ng μl^{-1}) into the heated electrospray ionization source (HESI-II). Data acquisition was performed initially in full scan mode to determine an abundant precursor ion. Next, the MS/MS transitions (at least two), S-lens voltages, and collision energies were optimized for each individual compound (Table 2). Finally, the ionization source working parameters were optimized by direct infusion of a standard mixture (10 ng μl^{-1}; Table 1). As such, the use of U-HPLC coupled to triple quadrupole mass spectrometry resulted in a rapid and selective multi-residue analytical method for the detection of 11 pharmaceuticals and 14 pesticides (+internal standards) in biotic extracts. The obtained SRM chromatograms of a *M. edulis* sample fortified with the selected pharmaceuticals at 100 ng g^{-1} are presented in Fig. 2. Some minor matrix components could be noticed at the specific retention times of several pharmaceuticals. This background noise did however not affect quantification or identification, since it was chromatographically resolved from the target compounds. With respect to the targeted pesticides, no interferences were observed at their specific retention time upon analysis of *M. edulis* tissue spiked with 100 ng g^{-1} (Fig. 3).

Perfluorinated compounds

For detection of PFCs in biotic tissue, an analytical methodology was developed using LC-ToF-MS. LC-ToF-MS, which encompasses a high-resolution approach based

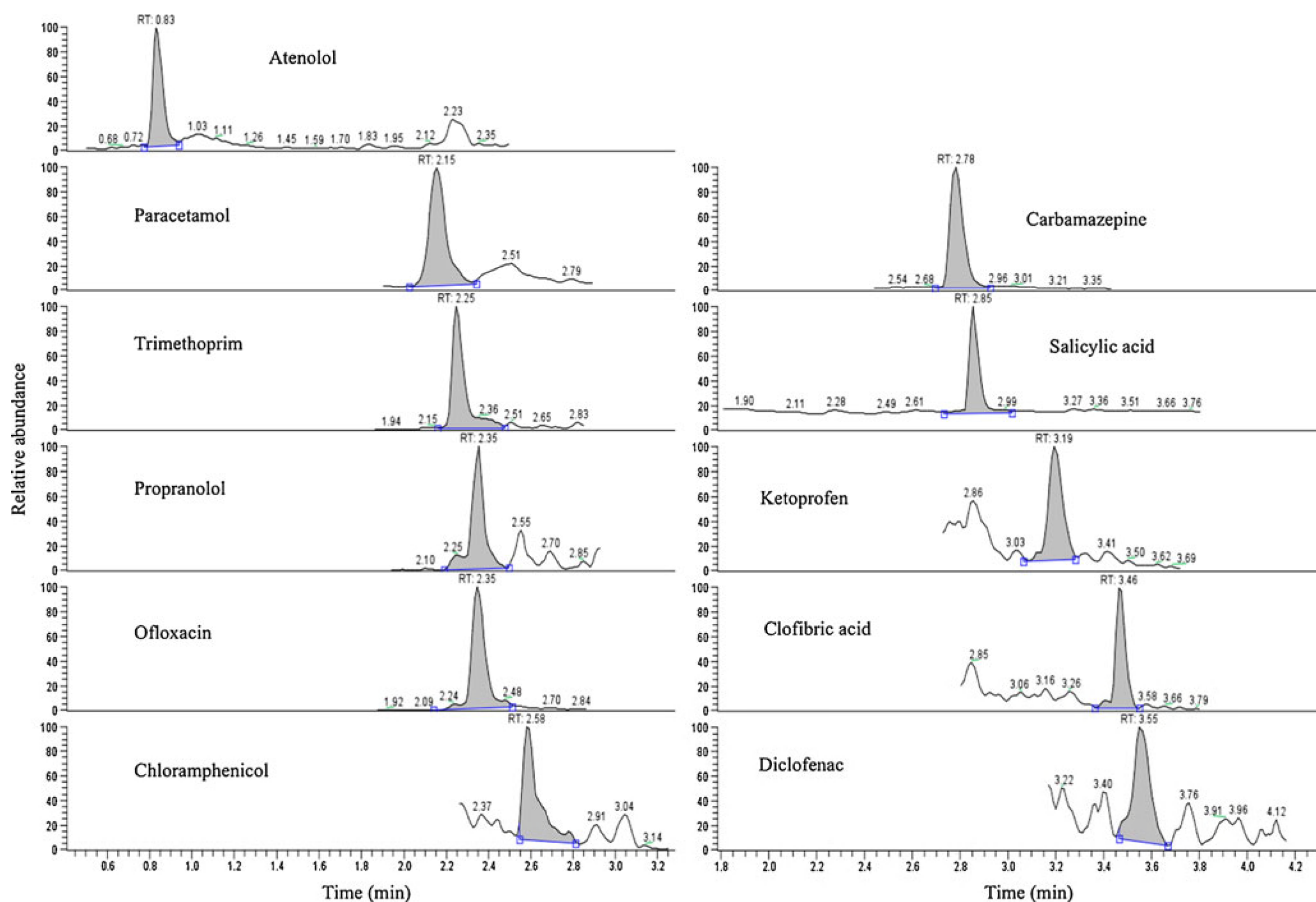

Fig. 2 SRM chromatograms of a *Mytilus edulis* sample fortified with the target pharmaceuticals at 100 ng g^{-1}

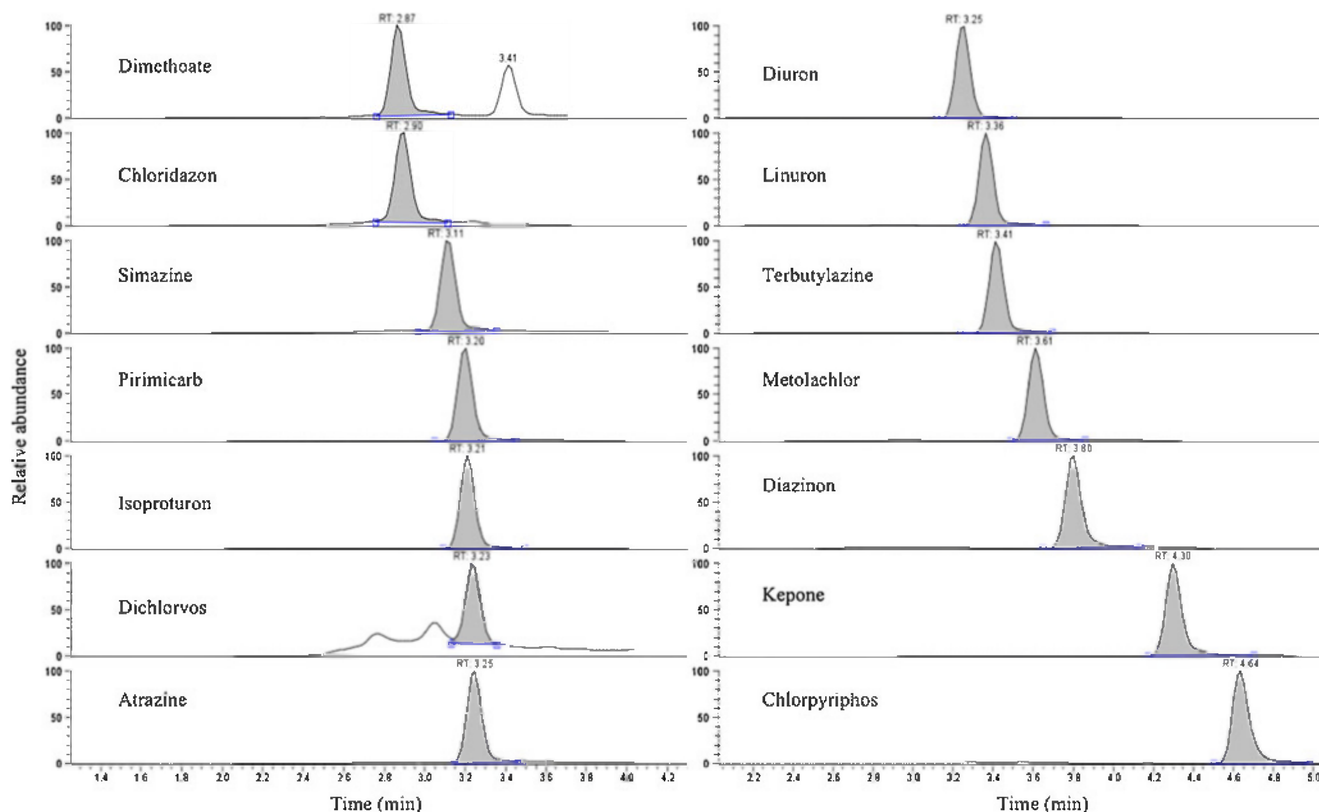

Fig. 3 SRM chromatograms of a *Mytilus edulis* sample fortified with the target pesticides at 100 ng g^{-1}

on accurate mass measurements, has been shown superior for the detection of perfluorosulfonates as compared to tandem MS [39, 40]. This specific class of PFCs, including the major contaminant PFOS, shows a very high stability even at extreme conditions (e.g., high collision energies) which makes the use of tandem MS for the detection of perflurosulfonates very complex and less efficient [39, 40]. Based on our previously developed method in aqueous matrices [7], LC-ToF-MS was found to be a highly selective MS-technique for the detection of PFCs in complex environmental matrices. Also in literature, ToF-MS has proved to be the optimum quantitative method for PFCs [41] and excellent specificity for unequivocal compound identification after a crude sample clean-up is obtained using high-resolution ToF-MS [42].

Good chromatographic separation of all compounds was achieved using the Luna C18 (2) LC-column and a mixture of 2.5 mM ammonium acetate in water and methanol as mobile phase. The detection of the target compounds was obtained via full scan data, from which the calculated theoretical masses of the target PFCs were extracted using very narrow mass tolerance windows. The theoretical masses, the mean measured masses, and the mass errors obtained are presented in Table 3. Except for PFDoA (ppm of 5.5), the obtained mean mass errors were below 5 ppm, resulting in a highly selective MS-technique for the detection of PFCs in complex biotic matrices (Fig. 4).

Method performance

The method performance of each of the three newly optimized analytical procedures was determined by constructing seven-point calibration curves in tissue matrix. To this end, freeze-dried *M. edulis* samples were used. The method proved to be applicable to pacific oysters (*Crassostrea gigas)* and brown shrimps (*Crangon crangon*) as well, and comparable limits of quantification (LOQs) were obtained upon analysis of these biotic organisms. The samples were spiked with a standard mixture at seven final concentrations between 0.1 and 250 ng g^{-1} and with the appropriate concentrations of the respective internal standard mixtures. This was performed in triplicate for each application (n=21). In addition, unspiked *M. edulis* samples (n=3) were also analyzed, to check the occurrence of the target compounds in blank samples. Based on the obtained calibration curves, the LOQ, recovery (trueness), and linearity were assessed.

Linear regression analysis was carried out by plotting the peak area ratios of the analyte against the I.S. versus the analyte concentrations. Good linearities were obtained for

Table 3 Characteristics of the PFC-analysis using ToF-MS: theoretical masses, mean measured masses, mean mass errors, internal standards, recoveries, and limits of quantification (LOQs)

Compound	Theoretical *m/z* $[M\text{-}H]^-$ ion	Mean measured *m/z*	Mean mass error (ppm)	Internal standard	Recovery (%) (x + RSD)	LOQ ng g^{-1}
PFHpA	362.9696	362.9705	3.2	$^{13}C_4$ PFOA	103±10	2
PFOA	412.9664	412.9680	4.1	$^{13}C_4$ PFOA	100±9	1
PFNA	462.9632	462.9648	4.3	$^{13}C_5$PFNA	101±15	2
PFDA	512.9600	512.9625	4.9	$^{13}C_2$PFDA	105±15	2
PFUnA	562.9563	562.9581	4.9	$^{13}C_2$PFDA	98±15	2
PFDoA	612.9531	612.9565	5.5	$^{13}C_2$PFDoA	90±17	5
PFBS	298.9430	298.9436	2.9	$^{13}C_4$PFOS	94±18	5
PFHxS	398.9366	398.9387	4.9	$^{13}C_4$PFOS	106±23	0.1
PFOS	498.9302	498.9317	3.9	$^{13}C_4$PFOS	100±16	0.1
PFDS	598.9233	598.9250	3.7	$^{13}C_4$PFOS	96±16	0.1

all analytes (regression coefficients ≥0.99), except for chlorpyriphos for which R^2 equalled 0.98. Due to the ubiquitous character of some of the compounds, analysis of unspiked *M. edulis* samples frequently resulted in their detection in the low nanogram per gram range. The calibration curves were corrected for these concentrations.

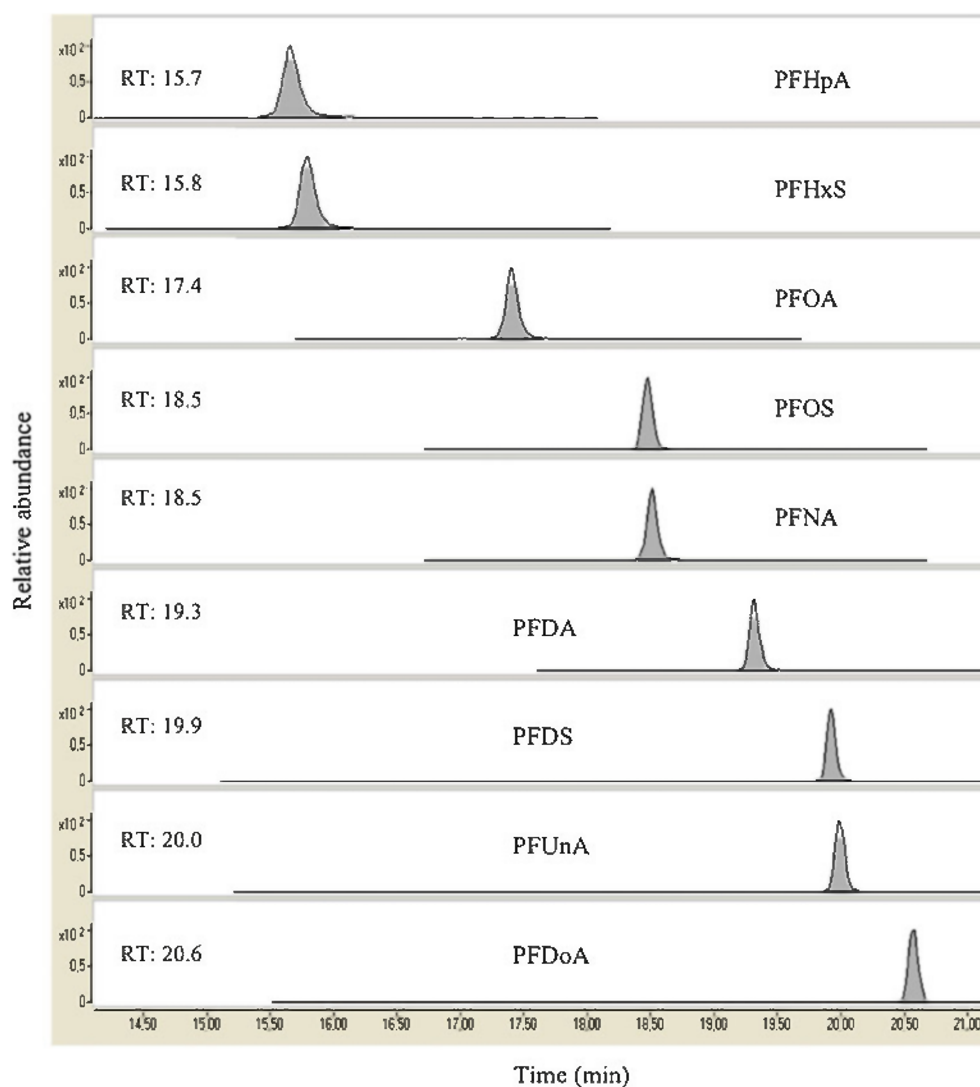

Fig. 4 Chromatograms of a *Mytilus edulis* sample fortified with the target PFCs at 100 ng g^{-1}

Table 4 Detected concentrations (ng g^{-1} on dry weight basis) of the target micropollutants in *Mytilus edulis* deployed in a 6-month cage experiment performed at five stations in the Belgian coastal zone (n.d. = not detected; n.a. = not analyzed)

	Sampling location																		
	ES	ZB1					ZB2					Sea				NP		OO	
	Feb	Mar	Apr	May	Jun	Jul	Mar	Apr	May	Jun	Jul	Apr	May	Jun	Jul	Mar	Apr	Mar	Apr
Pharmaceuticals																			
Ofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	5	n.d.	5	n.d.	n.d.	n.d.	n.d.	8	n.d.	7	n.d.	n.d.	n.d.	65
Propranolol	n.d.	40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	19	n.d.	n.d.	52
Salicylic acid	145	444	208	223	n.d.	172	162	241	339	184	315	264	93	203	119	490	125	41	206
Carbamazepine	1	11	3	1	1	3	n.d.	4	n.d.	3	n.d.	n.d.	n.d.	2	n.d.	n.d.	n.d.	3	n.d.
Paracetamol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	96	n.d.	n.d.	n.d.	n.d.	n.d.	115	65
PFCs																			
PFHxS	n.d.	n.d.	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	1	4	4	4	3	4	2	4	3	1	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	2	3
Pesticides																			
Dichlorvos	21	18	19	5	25	23	20	15	10	19	5	4	n.d.	20	28	5	11	n.a.	14
Diuron	1	1	1	n.d.	1	1	1	1	1	1	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	1
Linuron	n.d.	2	n.d.	n.d.	1	n.d.	n.d.	n.d.	n.d.	1	n.d.	n.d.	n.d.	n.d.	n.d.	1	n.d.	n.a.	n.d.
Chloridazon	n.d.	n.d.	n.d.	n.d.	16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11	n.d.	n.d.	n.a.	n.d.
Isoproturon	n.d. <1 <1	1	n.d. <1 <1	n.d.	n.d. <1 <1	n.d.	n.d. <1 <1	n.d.	n.d. <1 <1	n.d.	n.d. <1 <1	n.d.	n.d. <1 <1	n.d.	n.d.	n.d	n.d.	n.a.	n.d.
Metolachlor	n.d.	n.d.	n.d.	n.d.	n.d.	1	n.d.	1	n.d.	1	1	n.d.	n.d.	n.d.	n.d.	1	1	n.a.	1
Terbutylazine	n.d.	n.d.	n.d.	n.d.	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	n.d.	n.a.	n.d.

Since no certified reference material was available, the accuracy in terms of recovery of the methods was assessed using *M. edulis* samples spiked at seven concentration levels between 0.1 and 250 ng g^{-1} (three replicates). According to the guidelines SANCO/10684/2009 [43] on pesticide residues analysis in food and feed, and Commission Decision 2002/657/EC [44] concerning the determination of analytes in products of animal origin, typically a recovery is required within the range of 70–120% and 80–110%, respectively. As can be deduced from Tables 2 and 3, all obtained recoveries were between 90% and 106%, indicating good accuracy for all compounds. LOQs were determined using spiked matrix samples and were defined as the lowest detectable concentrations of the calibration curve with a signal-to-noise of at least 10:1. The LOQs obtained varied between 0.1 and 10 ng g^{-1}. For detection of pharmaceuticals in mussel tissue, the LOQs obtained in this study are an order of magnitude lower than a previous study [20]. For detection in fish muscle tissue, Ramirez et al. [19] determined comparable values for paracetamol, atenolol, and trimethoprim, and lower LOQs for propranolol and carbamazepine. With respect to pesticide analysis, the only analogous study reported limits of detection for simazine, atrazine, and terbutylazine of 0.21, 0.042, and 0.012 ng g^{-1}, respectively [21]. The obtained LOQs for detection of PFCs in mussel tissue are comparable to previous reported values, yet based on wet weight sample volumes [15, 45]. In general, the sensitivity of the reported methodologies is considered acceptable to good.

A well-known interference, which is associated with analysis by LC-MS, is the potential for interaction with matrix co-elutants. Due to the complexity of biotic samples, the number of co-eluting interferences and their interactions with target analytes increase [13]. To anticipate these matrix effects, quantification using matrix-matched calibration curves is suggested in literature. Besides, the use of isotopically labelled internal standards or compounds, which are structurally related with the target analytes, has also been recommended [13, 46]. In this study, both strategies to compensate for matrix effects were applied. The results obtained were thus corrected for possible matrix-induced suppression or enhancement effects, resulting in reliable analytical methods for the detection of the three groups of analytes in biotic matrices.

Application to *M. edulis* samples from the Belgian coastal zone

The developed methods were applied to *M. edulis* samples, derived from two cage experiments (see "Study area and sampling" section). Since freeze-dried samples were analyzed, the obtained results are expressed on dry weight basis in nanograms per gram. As shown in Tables 4 and 5, five different pharmaceuticals were detected in the *M. edulis* samples. The widely used non-steroidal anti-inflammatory drug (NSAID) salicylic acid was found in almost every sample in levels up to 490 ng g^{-1}. A second NSAID, namely paracetamol was detected less frequently at concentrations up to 115 ng g^{-1}. Also the β-blocker propranolol and the antibiotic ofloxacin were measured in some samples: up to 63 and 65 ng g^{-1}, respectively. Finally, carbamazepine was detected in concentrations ≤11 ng g^{-1}. Salicylic acid has been identified by Wille et al. [6] as the most prevalent pharmaceutical in water samples collected in the Belgian coastal waters, which explains the presence of this compound in the *M. edulis* samples observed in this study. No obvious temporal trends could, however, be observed during the cage experiments. Moreover, the measured concentrations of salicylic acid showed large variations over time and location.

Table 5 Detected concentrations (ng g^{-1} on dry weight basis) of the target micropollutants in *Mytilus edulis* deployed in a 6-week cage experiment performed at four stations in the Belgian coastal zone (n.d. = not detected)

	Sampling location				
	ES	ZB1	ZB2	NP	OO
Pharmaceuticals					
Ofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.
Propranolol	n.d.	39	38	30	63
Salicylic acid	33	14	118	288	229
Carbamazepine	n.d.	3	1	4	4
Paracetamol	n.d.	n.d.	n.d.	n.d.	115
PFCs					
PFHxS	n.d.	n.d.	n.d.	n.d.	n.d.
PFOS	n.d.	5	2	n.d.	n.d.
Pesticides					
Dichlorvos	25	7	8	60	8
Diuron	n.d.	1	1	n.d.	n.d.
Linuron	n.d.	n.d.	n.d.	n.d.	n.d.
Chloridazon	13	8	7	6	n.d.
Isoproturon	n.d.	n.d.	n.d.	1	1
Metolachlor	1	n.d.	n.d.	n.d.	1
Terbutylazine	n.d.	n.d.	n.d.	1	1

Seven target pesticides were found in the *M edulis* samples originating from the cage experiments. The concentrations of five pesticides (diuron, linuron, isoproturon, metolachlor, terbutylazine) were close to the limit of quantification, while the detected concentrations of chloridazon and dichlorvos were significantly higher. Chloridazon was observed at up to 16 ng g^{-1} and dichlorvos was found in most samples with a maximum concentration of 60 ng g^{-1}. This implies that the European default maximum pesticide residue level (MRL) in

foodstuffs of 10 ng g^{-1} [47], was exceeded for chloridazon and dichlorvos at several stations. Carafa et al. [21] also reported the exceeding of this MRL in clams, in which up to 73 ng g^{-1} of terbutylazine was retrieved.

Only two PFCs were detected in the *M. edulis* samples; PFHxS was detected only once at a concentrations of 3 ng g^{-1}, while PFOS was found in most samples at levels ≤5 ng g^{-1}. These concentrations were in the same order as those reported by So et al. [48] who found PFHxS and PFOS in mussel samples at levels ≤4 ng g^{-1} in coastal waters of China and Japan. In the study by Van de Vijver et al. [18], much higher concentrations of PFOS were measured in aquatic invertebrates of the southern North Sea, which is the same study area as the present study. PFOS was measured in shrimp (*C. crangon*), crab (*Carcinus maenas*), and starfish (*Asterias rubens*) up to 520, 877, and 176 ng g^{-1}, respectively.

Conclusions

In this study, three separate sensitive, selective, and reliable analytical methods have been developed for the quantification of 11 pharmaceuticals, 14 pesticides, and 10 perfluorinated compounds in tissue from marine organisms. It was shown that these methods exhibited satisfactory linearities and recoveries. The LOQs varied between 0.1 and 10 ng g^{-1} for all target compounds. Application of these analytical procedures to the blue mussel (*M. edulis*) deployed at different stations in the Belgian coastal zone revealed the presence of several of the target micropollutants. Five pharmaceuticals were found in *M. edulis* samples at up to 490 ng g^{-1}, two PFCs were detected at up to 5 ng g^{-1}, and seven pesticides were measured at concentrations up to 60 ng g^{-1}. As a consequence, the present study demonstrates that presence of micropollutants in marine ecosystems clearly affect tissue concentrations in resident marine organisms [3–7]. These findings will contribute to the assessment of the environmental and human health risk of these emerging micropollutants.

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