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Summary recommendations on “Analytical methods for substances in the Watch List under the Water Framework Directive”

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Abstract

The Watch List (WL) is a monitoring program under the European Water Framework Directive (WFD) to obtain high-quality Union-wide monitoring data on potential water pollutants for which scarce monitoring data or data of insufficient quality are available. The main purpose of the WL data collection is to determine if the substances pose a risk to the aquatic environment at EU level and subsequently to decide whether a threshold, the Environmental Quality Standards (EQS) should be set for them and, potentially to be listed as priority substance in the WFD. The first WL was established in 2015 and contained 10 individual or groups of substances while the 4th WL was launched in 2022. The results of monitoring the substances of the first WL showed that some countries had difficulties to reach an analytical Limit of Quantification (LOQ) below or equal to the Predicted No-Effect Concentrations (PNEC) or EQS. The Joint Research Centre (JRC) of the European Commission (EC) organised a series of workshops to support the EU Member States (MS) and their activities under the WFD. Sharing the knowledge among the Member States on the analytical methods is important to deliver good data quality. The outcome and the discussion engaged with the experts are described in this paper, and in addition a literature review of the most important publications on the analysis of 17-alpha-ethinylestradiol (EE2), amoxicillin, ciprofloxacin, metaflumizone, fipronil, metformin, and guanylurea from the last years is presented.

Keywords: Limit of Quantification (LOQ); water sample; analytical method; technical guidance; partitioning

1. Introduction

The Watch List (WL) under the European Water Framework Directive (WFD) is a mechanism for obtaining high-quality Union-wide monitoring data on potential water pollutants for which scarce monitoring data or data of insufficient quality are available. The main purpose of the WL data collection is to determine the risk which the substances pose to the aquatic environment at EU level and to decide whether Environmental Quality Standards (EQS) should be set for them (EU, 2013). Therefore, high quality measurements are expected from the Member States (MS) meaning that the monitoring methods shall be validated and reach the stated method quantification limits.

The first WL was established by Commission Decision 2015/495 (EU, 2015) and contained 10 individual or groups of substances. The results of monitoring the substances of the first WL (Loos et al., 2018a) showed that some countries had difficulties to reach an analytical Limit of Quantification (LOQ) below or equal to the Predicted No-Effect Concentrations (PNEC) or EQS for 5 out of the 17 WL substances (17-alpha-ethinylestradiol, 17-beta-estradiol, azithromycin, imidacloprid and methiocarb).

For this reason, the Joint Research Centre (JRC) of the European Commission (EC) created a knowledge platform around the experts to provide support to the EU Member States and their activities under the WFD. To do so, the JRC organised a series of workshops and their follow up focused on the substances listed in the WL and their measurements. MS experts came together to share their experience and knowledge on analytical methods for the existing problematic and new WL substances, to identify the obstacles in developing methods that meet the required PNEC or EQS, and to collaborate in method development by sharing technical details and information on method validation. In addition, the JRC technical workshops facilitated MS experts to openly discuss and exchange opinions about good analytical practices through which actions for improvement by learning from each other were proposed. Laboratory experts also discussed the possibility to facilitate the analysis of the WL substances for other MS.

Before the 1st workshop in 2018, the JRC distributed a questionnaire to the participating laboratories in order to gather information about the analytical methods applied and the LOQ achieved for these substances. The answers to the questionnaire and a full detailed workshop report are published separately (Loos et al., 2018b).

The workshop in 2018 was attended by 35 experts from 21 countries (EU Member States and Iceland, Switzerland and Turkey; the participants list is provided in the SI). During this first workshop, the discussion focused mainly on the analytical methods for 5 substances from the 1st WL (see above), which were already identified with difficulties to reach the relevant LOQ (Loos et al., 2018a), as well as, on the methods for 3 new WL substances (amoxicillin, ciprofloxacin and metaflumizone), which were expected to show an EU-wide concern. Indeed, the latter have been included in the second WL (EU, 2018; Loos et al., 2018a), since the WL is being revised every two years in accordance with the EQS Directive (EU, 2013).

Moreover, in 2022, two additional online workshops were organised. During these workshops “Sharing knowledge on analytical methods for substances in the 4th Watch List under the Water Framework Directive” held in September and October 2022, a specific focus was put on the analysis of fipronil,

metformin and its transformation product guanyurea, which were included in the 4th WL (EU, 2022a). The participants shared their knowledge regarding the methods used to monitor them in water.

The workshop in 2022 was attended by 65 experts from 20 countries (EU Member States, Switzerland, North Macedonia and Turkey; participants list in the SI).

The outcome and discussion points of these workshops are described in the following sections.

2. Analytical Methods

2.1. 17-alpha-Ethinylestradiol (EE2)

For the first WL, the most challenging substance to analyse was 17-alpha-ethinylestradiol (EE2) with its very low EQS value of 0.035 ng/L. In the new WFD Commission revision proposal, this EQS has been lowered to 0.017 ng/L (EU, 2022b). Five countries have reported for EE2 $LOQ \leq EQS$ (0.035 ng/L), and other four countries a close LOQ (0.05 - 0.1 ng/L) (Loos et al., 2018a). However, the workshop participants stressed that the analysis of EE2 at ultra-trace levels is complicated and work intensive because an efficient clean-up procedure (silica, florisil, aminopropyl, or gel permeation chromatography, GPC) and a very clean and sensitive liquid chromatography/triple quadrupole mass spectrometry (LC-MS/MS) instrument are obligatory. Selective extraction, additional clean-up and efficient LC separation are important for the analysis of estrogens, because matrix effects can cause a loss in sensitivity. Overall, many different analytical strategies, including sample extraction, clean-up and determination are applied in the countries. Analytical techniques employed for EE2 are mainly based on solid-phase extraction (SPE) (with online- or off-line SPE cartridges or off-line SPE disks) followed by LC-MS/MS, but also liquid-liquid-extraction (LLE) and GC-MS/MS (gas chromatography) after derivatization are used. Reviews on analytical methods have been published by Tomsikova et al. (2012) and Loos (2015), and on the occurrence in environmental samples by Du et al. (2020). Table 1 gives a summary on selected analytical methods for EE2 including extraction, clean-up, detection, and LOD(Q). The optimization of the sample preparation is important for reducing matrix interferences and plays a significant role in enhancing sensitivity of analytical procedures for the determination of steroid hormones in environmental matrices. In several studies, derivatization improved the sensitivity of LC-MS detection because steroid estrogens are weak acids and their ionization on ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) are not very efficient compared with other more polar chemicals. Chemical derivatization can add on moieties improving ionization and enhance signals. Disadvantageous is the higher work intensiveness. On-line SPE is more straight forward, less sample volume is needed, but off-line SPE is more robust. The lowest LOQ reported in the literature for EE2 is 0.035 ng/L (equal to the annual average EQS) (Barreca et al., 2019; Mirmont et al., 2021). Barreca et al. (2019) have developed a two-step off-line-SPE (water volume: 1L) followed by an on-line SPE LC-MS-MS method, and Mirmont et al. (2021) have applied C18 SPE disk extraction of 1 L water, LC-NH₂ SPE (500 mg) clean-up, and dansyl derivatization LC-MS-MS analysis. Dansylated derivatives are analysed in positive ionization mode which increases sensitivity by 1-2 orders of magnitude.

The workshop participants from Austria (AT) and Sweden (SE) reported that in order to reduce the matrix effect it is better to inject a very small volume of sample extract (AT: 2 μ L; SE: 10 μ L). In addition, it was reported by the Czech Republic (CZ) that the less intensive, but more specific MS/MS transition (295.2 > 267.0) is better for real water samples because of less co-elution from matrix substances. The

usage of an isotopically labelled internal standard added before extraction has significant positive effect on precision and accuracy. No agreement on the use of ammonia in the HPLC eluent (used by AT and CZ) could be made, because SE has found a better ionization without ammonia (Svahn and Björklund, 2016). It was reported by Latvia that by applying SPE sample preparation and detection with GC-MS/MS it is possible to reach LOQ < EQS, although a laborious derivatization procedure should be applied before the instrumental analysis.

Lardy-Fontan et al. (2018) developed and validated in real matrix (with high suspended particulate matter (SPM) content) a two-step solid-phase extraction (aminopropyl SPE clean-up) LC-MS/MS method for estrone (E1), 17-beta-estradiol (E2), and EE2. LOQ of the method were 0.4 ng/L for E1 and E2 and 0.1 ng/L for EE2. The developed method was successfully implemented to monitor French inland surface waters contamination by E1, E2 and EE2, and the survey revealed a chronic state of contamination by E1 and significant one by E2 and EE2 (15 % quantification rate). Maximum measured concentrations were above PNEC concentrations (0.23 ng/L for EE2), indicating potential risk toward the environment.

Deich et al. (2021) analysed surface water samples from the South China Sea near the Pearl River Estuary by SPE-LC-MS/MS (1L water; C18ec cartridge; LOQ 0.6 ng/L). The maximum EE2 concentration in proximity to the coast was 0.6 ng/L.

Solaun et al. (2021) analysed emerging WL substances in waste water treatment plant effluents and receiving transitional and coastal water bodies of the Basque coast (N-Spain) by on-line SPE-LC-MS/MS (water sample volume 50 mL) and achieved for EE2 a LOQ of 0.116 ng/L. EE2 was not detected.

Mirmont et al. (2021) developed a multi-residue analytical procedure for the quantification of several natural and synthetic steroid hormones in whole surface water samples. Extraction of 1L water is performed with a C18 SPE disk followed by clean-up of the extracts by NH₂-SPE (500 mg, 6 mL) and dansylation derivatization before LC-MS/MS analysis. The reported LOQ for EE2 is 0.035 ng/L. The method was applied to real samples of the Rivers Meuse, Ourthe, Lesse, and Sambre in Belgium, and EE2 was not detected, but E1, E2, and E3 (estriol) at < 1 ng/L.

Borrull et al. (2020) included EE2 in a multi-compound direct injection (500 µL filtered water sample) on-line SPE LC-MS/MS analytical method for 34 priority and emerging pollutants in water, and obtained for EE2 a LOQ of 5 ng/L.

Lu et al. (2023) conducted a pharmaceuticals monitoring program in the Jiaozhou Bay (Qingdao, China). Analysis was performed by SPE-LC-MS/MS (1L water; Oasis HLB). The LOQ for EE2 is not given, but it was detected in river, bay and coastal water samples with maximum concentrations of 11 ng/L.

2.2. Amoxicillin and ciprofloxacin

The antibiotics amoxicillin and ciprofloxacin are usually analysed in multi-compound analytical methods based on off-line SPE followed by LC-MS/MS (Arun et al., 2022; Ben et al., 2020; Biel-Maeso et al., 2018; Boger et al., 2021; Cui et al., 2018; Fonseca et al., 2020; Fu et al., 2022; Gros et al., 2013; Jiang et al., 2023; Kairigo et al., 2020; Li et al., 2018; Mirzaei et al., 2017; Wang et al., 2023; Zhou et al., 2022). However, amoxicillin is less frequently included in these multi-residue analytical methods (Biel-Maeso et al., 2018; Boger et al., 2021; Gros et al., 2013; Ismail et al., 2019; Kairigo et al., 2020; Li et al., 2018; Mirzaei et al., 2017). One reason for this is that it is, due to its polar nature, less well extracted with conventional SPE absorber materials, resulting in low recoveries (Cha et al., 2006; Fabregat-Safont et al.,

2021). The achievement of the respective PNEC of 0.078 $\mu\text{g/L}$ for amoxicillin and 0.089 $\mu\text{g/L}$ for ciprofloxacin is usually not a problem, with the exception of the work of Boger et al. (2021) for amoxicillin (LOQ: 200 ng/L). Cha et al. (2006) developed a SPE LC-MS/MS method for β -lactam antibiotics in water (200 mL water; EDTA addition) but observed recovery problems for amoxicillin.

Also four on-line SPE LC-MS/MS methods for antibiotics are reported in the literature, with LOQ of 2 ng/L for ciprofloxacin (injection volume: 5 mL) (Shen et al., 2022) (LOD: 2.3 ng/L; injection volume: 9.8 mL; Pozzo et al. (2006)), and 3.3 and 39 ng/L for ciprofloxacin and amoxicillin, respectively (injection volume: 900 μL) (Tuc Dinh et al., 2011), and LOQ of 5 and 2.5 ng/L for ciprofloxacin and amoxicillin (injection volume: 500 μL), respectively (Borrull et al., 2020).

Boix et al. (2014) presented a rapid analytical method for 40 drugs (amoxicillin and ciprofloxacin not included) based on direct sample injection of water samples (100 μL) with LC-MS/MS; reported LOQ are around 3 ng/L.

Fabregat-Safont et al. (2021) developed a rapid and robust direct injection LC-MS/MS method for amoxicillin in surface water (together with other six related penicillin antibiotics) and achieved an LOD of 9 ng/L (injection volume: 100 μL).

Rico et al. (2019) developed a SPE QTOF (quadrupole time-of-flight) multi-residue method (sample volume: 200 mL) with LOQ of 60 and 500 ng/L for amoxicillin and ciprofloxacin, respectively. TOF MS is less sensitive.

Goessens et al. (2020) developed a multi-class analysis of 46 antimicrobial drug residues in pond water using SPE LC-Orbitrap-HRMS; an LOQ of 0.05 ng/L was achieved for ciprofloxacin (sample volume: 500 mL).

Due to the relatively fast degradation or removal of amoxicillin (Braschi et al., 2013; Ecke et al., 2023; Elizalde-Velázquez et al., 2016) and ciprofloxacin (Cardoza et al., 2005; Lam et al., 2003; Van Doorslaer et al., 2014) in water, the samples should be analysed as fast as possible, preferably within one week. The water samples can be preserved in the laboratory in the freezer at -20°C (use of glass or HDPE bottles), but the stability and recovery should be tested for amoxicillin and ciprofloxacin. The water samples can also be stored after SPE extraction by freezing the SPE cartridges or disks (Llorca et al., 2014). Another conservation method is the addition of ascorbic acid and sodium azide and storage in the fridge at 4°C (Vanderford et al., 2011). In addition, the stock solutions have to be stored in the freezer and working standards should be prepared freshly and, due to its instability when prepared in methanol, amoxicillin standard has to be dissolved in Milli-Q water (Gros et al., 2013; Kantiani et al., 2009; Mirzaei et al., 2017). After addition of the internal standard, the use of a chelating agent such as Na_2EDTA (ethylene diamine tetra acetic acid) is recommended because it improves the extraction efficiency for many antibiotics by lowering their complexation with metals or multivalent cations (Arun et al., 2022; Ben et al., 2020; Cui et al., 2018; Gros et al., 2013; Ismail et al., 2019; Kazprzyk-Horden et al., 2007; Li et al., 2018; Mirzaei et al., 2017; Shen et al., 2022; Zhou et al., 2022). The sample pH should be adjusted to ca. 2-3 with hydrochloric acid (HCl) and the SPE cartridges or disks (Oasis HLB or similar) conditioned with methanol and water (pH 2-3; using a pH buffer such as formic acid). Elution should be performed with methanol (despite its reported instability in methanol) and evaporation to dryness, followed by reconstitution in the HPLC solvent and LC-MS/MS analysis. Multiple reaction monitoring (MRM)

transitions after positive ionisation are 366 > 349, 114, 160, 208 for amoxicillin and 332 > 288, 231, 245 (m/z) for ciprofloxacin.

In the pharmaceuticals monitoring study by Lu et al. (2023) (see above), amoxicillin was identified as the dominant antibiotic, with maximum concentrations of 1334 ng/L in river and 93 ng/L in bay sea water samples, respectively. The maximum ciprofloxacin concentration in the river was 60 ng/L.

Li et al. (2018) investigated the occurrence, spatiotemporal distribution and ecological risks of 27 antibiotics in water and sediments from the Pearl and Yangtze Rivers estuary, S-China. Maximum river water concentrations of ciprofloxacin and amoxicillin were 459 and 125 n/L, respectively.

Biel-Maeso et al. (2018) investigated the occurrence and distribution of 78 pharmaceuticals in different aquatic marine environments from the Gulf of Cadiz (SW-Spain); ciprofloxacin was found in coastal waters with a maximum concentration of 212 ng/L (detection frequency 43%); amoxicillin was not detected.

2.3. Metaflumizone

Very few publications on the analysis of metaflumizone in environmental water exist. Metaflumizone is a hydrophobic compound ($\log K_{ow}$ 6.9) (Jurado et al., 2022) and should be extracted from whole water samples by LLE or SPE disk or cartridge, followed by LC-MS/MS analysis (MRM transitions: 507 > 178, 287 (m/z)) (Loos et al., 2018a).

Metaflumizone was included in the watch list monitoring study of Solaun et al. (2021) in the Basque coast (N-Spain) (see above). Using on-line SPE-LC-MS/MS (water sample volume 50 mL) an LOQ of 3.3 ng/L was achieved, but metaflumizone was not detected in any of the wastewater or receiving waters investigated.

Lopez et al. (2022) investigated several pharmaceuticals and pesticides in the influents and effluents of a wastewater treatment plant in Madrid (Spain) and the Manzanares River. The pesticides, including metaflumizone, were analysed by SPE (50 mL water) LC-MS/MS, and an LOQ of 0.2 ng/L was achieved for metaflumizone, but it was not detected in the real water samples.

Metaflumizone was also included in the direct injection on-line SPE LC-MS/MS analytical method of Borrull et al. (2020) (see above); the LOQ was 10 ng/L.

It can therefore be concluded that the requested maximum acceptable method detection limit of 65 ng/L for metaflumizone of the second WL (EU, 2018) is well achievable.

2.4. Fipronil

Fipronil is a nonpolar lipophilic compound ($\log K_{ow}$ = 4.0) which (bio-)accumulates in sediment, SPM (suspended particulate matter) and fish (mainly its metabolites f. desulfinyl and f. sulphide) (Deiu et al. 2021). It is therefore mainly analysed in solid matrices such as sediment, biota, or food (Gonçalves et al., 2022; Pasupuleti et al., 2023), but has recently also been analysed in water samples by SPE and LC-MS/MS (He et al., 2021; Labad et al., 2023; Li et al., 2019; Michel et al., 2016; Montiel-León et al., 2018; Onduka et al., 2022; Santos et al., 2022; Shi et al., 2020) or direct injection (DI) and LC-MS/MS (Halbach et al., 2021).

Its maximum acceptable quantification limit of Decision 2022/1307 (EU, 2022) is 0.77 ng/L, which is difficult to reach with DI-LC-MS/MS systems, which are the current tendency nowadays. That is why some laboratories (Italy, Croatia, German State Saxony and Swiss Cantons) analyse this compound with Liquid-Liquid Extraction (LLE) and GC-MS/MS systems (unpublished methods). Using LLE allows concentrating the sample and reducing the possible matrix effects. Several organic solvent can be used (hexan, cyclohexane, heptane, etc.). The use of dispersive liquid-liquid microextraction (DLLME), as presented by Croatia, can reduce the amount of solvent used. Compared to DI, LLE allows the analysis of the whole water fraction (see sample preparation discussion below). Finally, the sensitivity of the methods will depend on the instrument, especially on the ionization source (NCI, APCI, ESI).

Different Swiss cantonal water protection laboratories developed LLE-GC-MS/MS methods for the analysis of pyrethroid and organophosphate insecticides in water samples (Moschet et al., 2019), based on Rösch et al. (2019)). Aside from those compounds, fipronil is analysed using the same methods since 2021. LOQ are in the range 0.025-0.5 ng/L and the first results from Swiss river samples indicate concentrations between 0.05 and 8 ng/L, and high detection frequency (100%) in small stream with high wastewater content (unpublished data).

In Germany, the Saxony State report a LLE-GC-MS/MS method with NCI ionization source with an LOQ of 0.02 ng/L. In Italy and Croatia, LOQ of 0.2 and 0.05 ng/L, respectively, are reported (unpublished method, Personal Communication).

Labad et al. (2023) conducted a multi-compound river and groundwater monitoring program in the Besòs River delta (Spain). Analysis of 500 mL water samples was performed by SPE LC-MS/MS. SPE was performed with manually prepared cartridges. The reported LOQ for fipronil is 0.34 ng/L, and the maximum concentration of fipronil in river water was 16 ng/L (average 13 ng/L).

Shi et al. (2023) conducted a monitoring and risk assessment study for fipronil and its metabolites in the Yangtze River (China). Analysis was performed by SPE of filtered water samples (500 mL) followed by LC-MS/MS (LOQ: 0.01-0.02 ng/L). High detection frequencies of fipronil and its transformation products were observed. The median fipronil concentration was 0.13 ng/L (below the PNEC of 0.77 ng/L).

Halbach et al. (2021) performed a pesticide-monitoring program in small agricultural streams in Germany during dry weather and rainfall. The water samples were filtered with a disposable syringe filter and 1 mL of the filtered sample was spiked with five isotope-labeled internal standards of the pesticides. Fipronil was quantified using its $^{13}\text{C}_4$ isotope-labeled internal standard. Analyses were carried out with an LC-MS/MS system by direct injection of the aqueous samples and multiple-reaction-monitoring (LOQ: 0.7 ng/L). The insecticides thiacloprid, clothianidin and fipronil were the three compounds with the most frequent regulatory acceptable concentrations (RAC; 0.77 ng/L for fipronil) exceedances during dry weather (7 % of the samples with exceedances) as well as during rainfall events (13 % of the samples with exceedances).

He et al. (2021) analysed neonicotinoids, fipronil and its metabolites in a semi-closed bay in China by SPE disk followed by ion-trap LC-MS (LOQ 0.05 ng/L). The maximum concentration of fipronil in river water was 10 ng/L and in surface seawater 1.4 ng/L.

Wan et al. (2021) analysed neonicotinoids, fipronil, and other pesticides in surface water and drinking water from northern Vietnam by SPE LC-MS/MS (sample volume: 1L; LOQ: 0.01 ng/L). The median fipronil plus degradates concentration was 3.8 ng/L.

Shi et al. (2020) analysed fipronil and its metabolites in Chinese tap and ground water by SPE (500 mL) LC-MS/MS (LOQ: 0.02 ng/L); the maximum sum concentration detected was 5 ng/L.

Li et al. (2019) performed a simultaneous determination of seven neonicotinoids and fipronil and its metabolites in seawater and river water using disk-based SPE (water volume: 2L) followed by LC-MS/MS and achieved an LOQ of 0.05 ng/L.

Montiel-León et al. (2018) developed a fast automated on-line enrichment (direct injection of 2 mL water) coupled to LC-MS/MS for neonicotinoids and achieved for fipronil an LOD of 0.1 ng/L for tap and 0.5 ng/L for surface water.

Michel et al. (2016) analysed fipronil and its metabolites in water and sediment from the River Elbe (Germany) by SPE (200 mL) LC-MS/MS (LOQ: 0.08 ng/L). Total concentrations in the water samples were 0.5-1.6 ng/L.

2.5. Metformin and Guanylurea

The antidiabetic drug metformin is the pharmaceutical with the highest concentration in wastewater (up to 100 µg/L) but it is removed with > 98% in sewage treatment plants (STP, Oosterhuis et al.; 2013). The specified LOQ of Decision 2022/1307 (EU, 2022) is 156 µg/L while for guanylurea, biodegradation product of metformin, it is 100 µg/L. While these LOQ are rather high, the high polarity of the compounds requires a specific chromatographic separation. Moreover, the necessary LOQ for metformin probably needs to be reduced in the upcoming years, since there is evidence that this substance has reproductive effects in vertebrates (Crago et al., 2016; Niemuth et al., 2015; Niemuth and Klaper, 2015), which could lower the EQS by two orders of magnitude. For this reason, it is important to have validated sensitive analytical methods with lower LOQ to monitor this substance.

So far, several of the available analytical methods use hydrophilic interaction liquid chromatography (HILIC)-tandem mass spectrometry (HILIC-MS/MS) for the quantitative analysis of metformin and guanylurea (e.g. Boulard et al., 2018; Oertel et al., 2018; Scheurer et al., 2012).

The method described by Boulard et al. (2018) is based on a non-specific sample preparation using freeze-drying followed by detection with large injection (70 µL) zwitterionic HILIC-ESI-MS/MS reaching LOQ of 5 and 20 ng/L for metformin and guanylurea, respectively.

Scheurer et al. (2012) extracted metformin and guanylurea by cation-exchange SPE (10 mL water sample) followed by HILIC-MS/MS. Metformin surface water concentrations in German rivers and creeks were between 0.22 and 3.1 µg/L, and between 0.66 and 28 µg/L for guanylurea.

Oosterhuis et al. (2013) reported the presence of metformin in the Netherlands with a concentration of 1800–3900 ng/L in surface water. Guanylurea was detected in STP effluents and surface waters at concentrations of 39–56 µg/L and 1.8–3.9 µg/L, respectively.

Blair et al. (2013) studied the presence of 54 pharmaceuticals and personal care products in Lake Michigan (USA), and the most widely detected pharmaceutical was metformin (median: > 100 ng/L; max. 3.8 µg/L).

Posselt et al. (2018) investigated polar organic micropollutants in water samples from River Erpe (Germany) and reported a maximum concentration of 222 µg/L guanylurea and 1.66 µg/L metformin. Analysis was performed by direct injection LC-MS/MS (LOQ for guanylurea 213 ng/L, and for metformin 364 ng/L).

Malnes et al. (2022) extracted metformin by using Oasis HLB-cartridges (6 mL, 200 mg; 500 mL water sample; recovery 1-4 %; LOQ 0.016-0.021 ng/L) followed by LC-MS/MS. Mean, median, and maximum concentrations of metformin in Swedish rivers and lakes samples were 11, 2.6 and 120 ng/L and 1.6, 1.1 and 11 ng/L, respectively.

Ogunbanwo et al. (2020) analysed metformin within a multi-compound direct injection (100 µL) C18 chromatography column LC-MS/MS method (LOQ: 8.4 ng/L), and found very high metformin concentration in surface waters from Nigeria (mean: 10 µg/L; max: 2 µg/L).

Balakrishnan et al. (2022) published a review on the occurrence, analysis and treatment methods of metformin.

3. Determination of the Limit of Quantification (LOQ)

Some Member States (MS) asked during the workshop in 2018 to specify in detail the different methods for the determination or calculation of the Limit of Quantification (LOQ) including method validation and uncertainty estimations, because the available general guidelines (DIN 32645, 2008; EN ISO 17025, 2005; Eurachem Guide, 2014; IUPAC, 2002) are not sufficient for a uniform approach. It remained unclear whether the LOQ of the presented methods are derived from appropriate real surface water samples with or without standard addition, or from calibration curves. A comparison of method performances and of LOQ of different methods are almost impossible since their evaluation is rarely described (matrix used, statistical approach chosen, etc.). For a better harmonisation of the methods, a consensus on the determination of the LOQ is necessary, which needs a thorough discussion on the minimum requirements for sample preparation and analysis, i.e. representative surface water matrix, standard addition, recovery, reproducibility, uncertainty and multiplication factor. In general, the LOQ can be derived from a calibration curve, the analysis of blank water samples, or the analysis of (real) water samples spiked at 2–5 times the pre-estimated limit of quantification and then using the signal-to-noise ratio ≥ 10 (≥ 3 for LOD). The latter approach appears to be the most reliable. The discussion held during the workshops should provide technical guidelines on monitoring strategies and analytical methods for substances in order to facilitate the implementation for all MS.

4. Which fraction analysing: Whole water or dissolved water phase?

The workshop showed that nowadays many MS are using Direct Injection (DI) techniques with LC-MS/MS. DI-LC-MS/MS should however only be performed for water soluble substances for which

partitioning to suspended particulate matter (SPM) can be neglected (low K_{OW} and $K_{d(sed)}$ values). The question is whether filtration or centrifugation of the water sample is allowed when DI is used. According to the EQS Directive (EU, 2013), “whole water” analysis, including the particle bound fraction, should be performed for the organic priority substances. Total concentration should be obtained by direct analysis of the whole water sample (without filtration) or by a separate determinations on filterable (dissolved) and solid phases (particulate) (Lardy-Fontan et al., 2018).

General principles of pollutant partitioning can be found in the literature (e.g., Vignati et al. (2009) and references therein). The Expert Group on Analysis and Monitoring of Priority Substances (AMPS) concluded in 2005 that no specific requirements could be made regarding which matrix (whole water, liquid or particulate phase) should be analyzed. Indeed, variations in hydrological and environmental circumstances, which are reflected in the quantity and the quality of SPM, preclude rigid categorization and make it difficult to establish general rules for the choice of the appropriate matrix (Ademollo et al., 2012). However, the first and simplest approach is to consider the hydrophobicity of the analytes, as recommended in the WFD CIS Guidance document No. 25 on sediment and biota monitoring (2010). The proposed rule of thumb is that compounds with $\log K_{ow} > 5$ should preferably be measured in sediments or in SPM, while compounds with a $\log K_{ow} < 3$ should preferably be measured in water (Ademollo et al., 2012). The WFD CIS Technical guidance document No. 27 for deriving Environmental Quality Standards (2011) however states in section 3.8 that “discrepancies between total and dissolved concentrations may only become evident for very hydrophobic substances [...] with a $\log K_{ow}$ above 6”. Since the EQS are usually derived in standard laboratory toxicity and bioconcentration tests with low levels of total organic carbon, the EQS refer to the dissolved (bioavailable) concentrations.

It has been shown for lipophilic compounds (polycyclic aromatic hydrocarbons, PAH) (Chen et al., 2023; Wang et al., 2023), and brominated diphenyl ethers (BDE)) that the measurement of the dissolved fraction underestimates the total concentrations in water (Ademollo et al., 2012). For nonylphenol, a compound of medium lipophilicity ($\log K_{ow}$ 4.5), up to 50 % of the substance has been found adsorbed on the particles (Patrolecco et al., 2006; Rusconi et al., 2015). The European Committee for Standardization (CEN) has already developed three standard methods for whole water analysis of hydrophobic substances (PAH, BDE, and organochlorine pesticides) in water with SPM content up to 500 mg/L and the use of SPE disks (EN 16691, EN 16694, EN 16693; 2015).

Tlili et al. (2016) investigated in France the partitioning of 26 pharmaceuticals, including 18 antibiotics, between the dissolved water phase and SPM. Drug residues associated with SPM were extracted from filters by pressurized liquid extraction (PLE) combined with an on-line SPE-LC-MS/MS system. In the investigated French rivers, the drug residues were present mainly in the dissolved phase. The concentrations of ciprofloxacin in the rivers were 6.6 and 6.4 ng/L in the dissolved phase, and 1.2 and 1.4 ng/L on SPM, respectively, which shows that approximately 20 % of ciprofloxacin is present in the particulate water phase. Therefore, analysis of the dissolved water phase by direct LC-MS/MS injection could deliver for this substance acceptable results similar (but a bit lower) to whole water analysis. However, this aspect should be investigated in more detail in order to confirm the results of Tlili et al. (2016). Sediment appears to be a sink for fluoroquinolone antibiotics (Shi et al., 2014).

Lardy-Fontan et al. (2018) give a review on “whole-water analysis” of estradiol hormones. Nie et al. (2014) determined the association of estrogen compounds among different water phases: SPM, filtrate

(dissolved), and colloidal phases. Their results highlighted that, depending on the type of matrix and season, 5-35 % of E2 ($\log K_{ow}$ 4.0) and up to 100 % of EE2 ($\log K_{ow}$ 4.2) were associated with SPM fractions. In 2015, the same authors carried out a study on the occurrence, distribution and risk assessment of six estrogen substances in samples of surface water, SPM, and sediment in the Yangtze Estuary (China) and its coastal areas over four seasons. The SPM phase for total estrogens contributed between 16 to 88 %, however, EE2 was not detected in any of the water samples (Nie et al., 2015). These observations confirmed that colloids and SPM could act as a significant sink for environmental estrogens. Moreover, Neale et al. (2009) showed that ca. 50 % of E2 could be lost by filtration through cellulose acetate filters, and de Graaff et al. (2011) reported up to 64 % adsorption of estrogens (E1, E2, EE2) when filtrated over a glass fiber filter.

Yarahmadi et al. (2018) conducted in Canada extensive environmental monitoring in an urban river impacted by multiple combined sewer overflows and wastewater treatment plant (WWTP) discharge points. Temporal and spatial distributions of dissolved and particulate steroids were investigated in sewage, WWTP effluents, receiving river water and sediments. The water samples were filtered and the SPM was extracted by ultrasonic solvent extraction followed by C18 SPE clean-up. The particle-associated steroids represented 0-82% of their total concentration as some steroids like estrone (E1) and estriol (E3) were detected only in the dissolved phase, while EE2 (>75%) was primarily detected in the particulate phase.

Wilkinson et al. (2017), however, investigated the spatial distribution of some pharmaceuticals (including EE2), illicit drugs, plasticisers, and perfluorinated compounds in three rivers of Southern England bound to SPM and dissolved in water, and the selected analytes were almost exclusively found dissolved in sampled waters with an EE2 mean concentrations of 0.23 ng/L (detection frequency 3%). EE2 was not detected in SPM, but PFOA and PFOS.

Goeury et al. (2022b) investigated the occurrence of steroid hormones in surface waters and suspended sediments in Canada (St. Lawrence River and its major tributaries). The suspended particulate matter (SPM) was sampled on glass fiber filters, which were then extracted with a mixture of methanol and acetone (3:1 v/v; 5 mL) in a 15 mL centrifuge tube by vortex-mixing (10 s) and sonification (20 min), followed by a C18 SPE cartridge clean-up. EE2 detection frequency in the dissolved phase was 1.5 %, and a max. concentration of 2.8 ng/L, and in SPM 2.9 % and 63.9 ng/g, respectively. The most frequent compound in SPM was bisphenol A.

Xu et al. (2024) investigated 31 steroids (E2 and EE2 not included) and metabolites in SPM, water phase and sediments of the Pearl River Estuary (China) during the dry and wet seasons to elucidate their spatiotemporal patterning, partitioning behavior, and environmental fate. The results showed that natural steroids predominated in SPM and sediments while the metabolites predominated in water.

Thus it can be concluded that EE2 and E2 are strongly adsorbed to particulate matter (around 40 % for E2 and 70 % for EE2) and therefore whole water samples have to be extracted and analysed.

Lorre et al. (2023) studied phthalate esters delivery to the largest European lagoon (Curonian Lagoon, south-east Baltic Sea). Di(2-ethylhexyl) phthalate (DEHP) was the most abundant phthalate ester and was mainly found attached to particulate matter, highlighting the importance of this matrix in the

transport of such contaminant. Dibutyl phthalate (DnBP) and diisobutyl phthalate (DiBP) were the other two dominant phthalate esters found in the area, mainly detected in dissolved phase.

The multicompartiment partitioning of per- and polyfluoroalkyl substances (PFAS) was studied by several scientists (Ahrens et al., 2011; Ding et al., 2018; Gao et al., 2020; Li et al., 2020; Liu et al., 2019; Wang et al., 2020; Wang et al., 2022). While perfluorooctanoic acid (PFOA), and perfluorooctansulfonate (PFOS) mainly distribute in the dissolved phase and SPM at low suspended solid concentrations, more diverse compositions were observed in sediment and biota with the increase in contributions of long-chain PFASs.

In conclusion, the participants of the workshop agreed that compounds with a $\log K_{ow} < 3$ should preferably be measured in the dissolved water phase, and compounds with $\log K_{ow} > 5$ in sediment, SPM, or biota. A more precise investigation of the partitioning behavior is necessary for compounds with $\log K_{ow}$ between 3 and 5, such as fipronil. The correction of the discrepancies in the different technical guidance documents is recommended.

5. Conclusions

During the workshops, the current analytical methodologies for the quantification of selected substances from the surface water Watch List and their limits were discussed among experts. It was a good opportunity to share the analytical protocols, experience and eventually to provide analytical service to those countries which could not measure properly some of the existing substances. The discussions revealed some discrepancies between WFD requirements (whole water analysis) and the reality of analytical methods (direct injection after filtration or centrifugation) for several substances. Indeed, the question regarding the particulate vs dissolved content of WL substances (e.g. ciprofloxacin) in the water column was addressed and the latest literature was reviewed for the substances. This question should be addressed for each substance and if necessary, some tests have to be done in order to use the appropriate analytical methods for the appropriate matrix. Moreover, a working group at the EC could make proposals of guidelines on whether and in which cases the dissolved fraction could be a good approximation for the whole water fraction. Some lacks in the calculation and reporting of LOD/LOQ were also clearly identified. Such workshops are really worth as it allows a fruitful exchange between experts from different countries, as well as identifying synergies and discrepancies. They allowed discussions in order to ensure the delivery of good data quality for the WL substances, which will then drive decision-making since it is the prerequisite to assess and evaluate their risk at EU level. However, the discrepancies between the different technical guidance documents and the examination of the partitioning and “whole water” analysis (because the EQS refer to the whole water concentrations) should be addressed for each substance and corrected if necessary. In conclusion, further workshops will be held as a follow-up to the subsequent dynamic list of substances within the WL, with the aim of improving analytical methods for the monitoring strategy that the MS may require.

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Supporting Information

The Supporting Information lists all participants of the workshops.

In addition, it contains statistical analytical results summaries over the years 2015-2021 for the WL substances EE2, amoxicillin, ciprofloxacin, and metaflumizone and information on PNEC exceedances.

Journal Pre-proof

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit author statement

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Table 1: Analytical methods (extraction, clean-up, detection, LOD(Q)) for 17-alpha-ethinylestradiol (EE2). LOD, Limit of Detection.

Extraction, clean-up	Water volume (L)	Detection	LOD(Q) (ng/L)	Reference
LLE; clean-up with alumina/florisil	1	HRGC/HRMS (derivatization)	MDL: 0.1	EPA 1698, 2007
On-line SPE derivatization	1 mL	LC-MS-MS	0.7	Salvador et al., 2007
SPE; clean-up with florisil	0.25	LC-MS-MS (derivatization)	LOQ: 0.22	Matejcek and Kuban, 2008
SPE	1	LC-MS-MS	MDL: 0.2	Vulliet et al., 2008
SPE	2	GC-MS-MS (derivatization) LC-MS-MS	0.3 0.4	Grover et al., 2009
C18 speedisks	50 µL	LC-MS-MS (derivatization)	0.91	Lien et al., 2009
SPE; clean-up with florisil	0.25	LC-MS-MS	LOQ: 1.2	Miège et al., 2009
SPE; clean-up with florisil	2	LC-MS-MS	0.10	Chang et al., 2011
SPE disks (C18 + SDB); silica clean-up	4	GC-MS (derivatization)	1.64	Wang et al., 2012

SPE (SDB); clean-up with GPC	1-2	LC-MS-MS	0.05	Williams et al., 2012
SPE disks (C18); clean-up with florisil	0.5	GC-MS-MS (derivatization)	0.8	Alvarez et al., 2013
SPE (Oasis HLB 200mg); silica gel column clean-up; pH3	2	LC-MS-MS	0.18	Li et al., 2013
On-line SPE	5 mL	LC-MS-MS	0.47	Esteban et al., 2014
On-line SPE	5 mL	LC-MS-MS	0.035; LOQ: 0.115	Čelić et al., 2017
SPE; aminopropyl SPE clean-up	Not given	LC-MS/MS	0.1	Lardy-Fontan et al., 2018
LLE with hexane	0.01	LC-MS/MS	0.4	Chang et al., 2018
SPE	0.5	LC-MS/MS	0.8; LOQ: 1.7	Pignotti and Dinelli, 2018
SPE	0.5	GC-MS (derivatization)	14 ; LOQ: 40	Sousa et al., 2019
On-line SPE	0.01	LC-MS/MS	0.34; LOQ: 1.13	Gusmaroli et al., 2019
On-line SPE	0.01	LC-MS/MS	1.4; LOQ: 4.2	Goeury et al., 2019
SPE and on-line SPE (two-step)	1	LC-MS/MS	LOQ: 0.035	Barreca et al., 2019
SPE	1	LC-MS/MS	0.6	Deich et al., 2021
On-line SPE	0.05	LC-MS/MS	0.116	Solaun et al., 2021
On-line SPE	0.02	LC-MS/MS	0.5-5	Ng et al., 2021
SPE disks (C18); clean-up with LC-NH ₂ SPE (500 mg)	1	LC-MS/MS (derivatization)	LOQ: 0.035	Mirmont et al., 2021
SPE (SolEx HRPHS 200mg); silica SPE clean-up	0.5	LC-MS/MS	0.5; LOQ: 1.5	Goeury et al., 2022a

Graphical abstract





Graphics Abstract