https://doi.org/10.1007/s11356-023-31698-9

Occurrence and environmental risk assessment of 4 estrogenic compounds in surface water in Belgiumin the frame of the EU Watch List

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https://doi.org/10.1007/s11356-023-31698-9

Abstract

The presence of natural estrogens estrone (E1), 17β-estradiol (E2), estriol (E3) and synthetic estrogen 17α-ethynylestradiol (EE2) in the aquatic environment has raised concerns because of their high potency as endocrine disrupting chemicals. The European Commission (EC) established a Watch List of contaminants of emerging concerns including E1, E2 and EE2. The proposed environmental quality standards (EQSs) are 3.6, 0.4, 0.035 ng/L, for E1, E2, EE2, respectively. A thorough evaluation of analytical procedures developed by several studies aiming to perform sampling campaigns in different European countries highlighted that the required limits of quantification in surface water were not reached, especially for EE2 and to a lesser extent for E2. Moreover, data regarding the occurrence of these contaminants in Belgian surface water are very limited. A sampling campaign was therefore performed on a wide range of rivers in Belgium (accounting for a total of 63 samples). The detection frequencies of E1, E2, E3 and EE2 were 100, 98, 86 and 48 %, respectively. E1 showed the highest mean concentration (=4.433 ng/L). In contrast, the mean concentration of EE2 was 0.042 ng/L. The risk quotients (RQs) were calculated based on the respective EQS of each analyte. The frequency of exceedance of the EQS was 31.7 % for E1, EE2, while it increased to 44.4 % for E2. The extent of exceedance of the EQS, represented by the 95^{th} percentile of the RQ dataset, was higher than 1 for E1, E2, EE2. The use of a confusion matrix was investigated to try to predict the risk posed by E2, EE2, based on the concentration of E1.

Keywords: EU Watch List, estrogenic compounds, Belgian river waters, occurrence data, environmental risk assessment, risk prediction,

https://doi.org/10.1007/s11356-023-31698-9

1 Introduction

The occurrence of estrogens in surface water has been reported throughout the world (Du et al., 2020; Yu et al., 2019; Zhang et al., 2016). These contaminants have attracted scientific attention due to their endocrine-disrupting effects on aquatic organisms, even at extremely low concentrations (down to sub ng/L level) (Caldwell et al., 2012; Mills and Chichester, 2005). This group of contaminants includes natural and synthetic estrogens. Natural estrogens, namely estrone (E1), 17β-estradiol (E2), and estriol (E3) are steroid hormones involved in the functioning of the reproductive system in vertebrates (Amenyogbe et al., 2020; Johnson et al., 2013; Mills and Chichester, 2005). The natural estrogen E2 is further used in human medicine for hormone replacement therapy (HRT), along with a synthetic estrogen, i.e., 17α-ethynylestradiol (EE2), which is mainly used in the formulation of oral contraceptives (Barreiros et al., 2016; Du et al., 2020; Johnson et al., 2013). These compounds are widely present in the environment mainly due to their excretion through the urine and feces of humans and animals from intensive animal husbandry. Estrogens are excreted mainly as conjugated forms (glucuronide/sulfate) and to a lesser extent as free forms (Hamid and Eskicioglu, 2012; Ting and Praveena, 2017; Yu et al., 2019). The wastewater treatment plants (WWTPs) are not designed to completely remove these contaminants (Barreiros et al., 2016; Du et al., 2020; Ting and Praveena, 2017). Moreover, conjugated estrogens can undergo a deconjugation process due to microbial activity in the sewage pipes and also in WWTPs, therefore releasing free and active forms (Hamid and Eskicioglu, 2012; Liu et al., 2015). Surface water, receiving continuously effluents from WWTPs in addition to untreated wastewater, can therefore be highly contaminated (Zhang et al., 2016). Moreover, the application of manure/effluent from intensive animal husbandry to agricultural lands can also contribute to the contamination of surface water following the runoff process (Du et al., 2020; Yu et al., 2019; Zhao et al., 2019).

The European Commission (EC) highlighted the issue posed by these contaminants in surface water by including E1, E2, EE2 in the first Watch List (EC Decision 2015/495, 2015). The Watch List procedure was implemented under the Water Framework Directive (WFD) (Directive 2000/60/EC, 2000) and aims to obtain monitoring data on a list of emerging contaminants in surface water at the European Union (EU) scale (Kase et al., 2018; Könemann et al., 2018; Loos et al., 2018). The proposed annual average environmental quality standards (AA-EQSs) by the EC are 3.6, 0.4 and 0.035 ng/L, respectively for E1, E2 and EE2 (Kase et al., 2018; Loos et al.,

2018). According to the Joint Research Center (JRC) technical report (Loos et al., 2018), analytical techniques used by Member States should be characterized by a limit of quantification (LOQ) equal to or below the EQS of each analyte. The Watch List (EC Decision 2015/495, 2015) provided guidance concerning the analytical procedure that may be used to achieve suitable performance, indicating solid-phase extraction (SPE) and analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the best choice. Nevertheless, as underlined by the JRC technical report (Loos et al., 2018), the analytical procedures applied by several Member States were not able to attain the LOQs equal to the proposed EQSs, especially for EE2 (16 out of 24 Member States). Only a limited number of studies from the literature have so far developed an analytical procedure achieving an LOQ close/equal to the EQS of the most challenging analyte, i.e., EE2. All of them used LC-MS/MS determination but differed in the sample preparation procedure, as well as in the chromatographic conditions, both impacting significantly the results. Celic et al. and Lardy-Fontan et al. (Čelić et al., 2017; Lardy-Fontan et al., 2018) developed analytical methods that reached LOQs (=0.1 ng/L) just above the EQS of EE2. The sample preparation in Celic et al. (Čelić et al., 2017) consisted of an online SPE with an extraction volume of 5 mL. Celic et al. (Čelić et al., 2017) reported that adjusting the pH of samples to a basic value (pH=11) improved sensitivity of their procedure. An extraction of 1000 mL water sample on a H_2O-Philic DVB SPE disk (offline) followed by a purification on an NH_2 SPE cartridge (offline) was performed by Lardy-Fontan et al. (Lardy-Fontan et al., 2018), followed by derivatization with dansyl chloride. In the study by Konemann et al. (2018) (Könemann et al., 2018), three laboratories used the same sample preparation consisting of extraction of 1000 mL of water sample on a C18 SPE cartridge (offline) followed by purification on a SiOH SPE cartridge (offline), but differed in the LC-MS/MS method applied (mobile phases, additives, columns). The LOQs achieved were different between the three laboratories and changed according to the samples analyzed. The LOQ required for EE2 was not achieved in 9 surface water samples (out of 16) by any of the three laboratories. Barreca et al. (Barreca et al., 2019) attained the target LOQ for EE2 (=0.035 ng/L) using an Oasis HLB SPE (offline) followed by an online C18 SPE. In our previous work, we developed and thoroughly validated a method consisting of an extraction of a 250 mL water sample on a C18 SPE disk (offline) followed by a purification on a Florisil SPE cartridge (offline) (Glineur et al., 2020). The derivatization of analytes was performed with pyridine-3-sulfonyl chloride (Glineur

et al., 2020) prior to injection into an LC-MS/MS reaching LOQs below EQSs for E1, E2, EE2 in surface water matrix.

Few surveys in Europe relied on methods attaining LOQs close/equal to the EQSs (mainly, regarding EE2, E2); hence, there is a lack of monitoring data of sufficient quality for surface water within the EU. Concerning the previously listed analytical procedures, sampling campaigns were performed in France (Lardy-Fontan et al., 2018), Serbia (Čelić et al., 2017), Italy (Barreca et al., 2019), and throughout different EU countries (Könemann et al., 2018). To the best of the authors' knowledge, few studies have investigated the presence of E1, E2, E3, EE2 at ultra-trace levels in a wide array of rivers in Belgium. In the study of Konemann et al. (Könemann et al., 2018), 2 surface water samples from Belgium were present. Huysman et al. (Huysman et al., 2017) used high-resolution Q-Orbitrap mass spectrometry (MS) for the quantification of a vast array of steroidal endocrine-disrupting compounds in seawater but on a limited number of samples, i.e., 8 samples. Sghaier et al. (Sghaier et al., 2017) examined the presence of E1, E2, E3, and EE2 in 3 samples from river Escaut (Belgium).

The present work aimed to perform a sampling campaign on a wide range of rivers in Belgium using the previously validated method (Glineur et al., 2020). Performances of the method were systematically verified while carrying out the sampling campaign by analyzing quality control samples.

2 Material and method

2.1 Chemicals and reagents

Standards in neat form (powder) were purchased from MilliporeSigma, Overijse, Belgium (E2, EE2) and from LGC, Köln, Germany (E1, E3). Stable isotope labeled internal standards (SIL-ISs) were purchased from Alsachim (Shimadzu group company, Illkirch-Graffenstaden, France): E1-d4, E2-d5, E3-d3, EE2-d4. A multi-analyte stock solution was prepared by weighing and dissolving in acetone all 4 analytes (100 mg/L for E2, E3, EE2, and 400 mg/L for E1). A stock solution containing all 4 SIL-ISs was also prepared in acetone. Stock solutions were stored in amber glass tubes at 4 °C.

Acetone was purchased from VWR chemicals (Leuven, Belgium). Water ULC/MS grade used for mobile phase preparation was bought from Biosolve (Dieuze, France). Methanol, acetonitrile, dichloromethane, n-heptane were from Biosolve. Pyridine-3-sulfonyl chloride,

sodium hydrogencarbonate, formic acid, and ammonium formate were purchased from MilliporeSigma (Germany).

2.2 Sampling campaign: sampling sites selection, sampling procedure, samples handling

Two parameters were considered and varied to select the sampling points: 1) WWTP located nearby the sampling site and its capacity in population equivalent (see supplementary data, Table S1); 2) rivers median flow rate (see supplementary data, Table S2). Summary information on the sampled rivers is presented in Table 1. Fifteen flow rate monitoring stations were selected accordingly to estimate the flow rate of the rivers at several sampling sites. Information on the flow rates (one-hour step, over a five-year period, from 2016 to 2021) is provided by the "Service Public de Wallonie" (Belgium). Daily mean flow rates were calculated and represent the data set used in further calculations. The mean, median (F_m), 95th percentile flow rate (F_{95}) of a river were calculated for the five-year period (see supplementary data, Table S2, Figure S1). Unfortunately, for some sampling locations, no flow rate monitoring station was located nearby to collect this information. The number at the end of each sampling site code corresponds to the location on the river (from upstream to downstream on a river). More details about sampling sites, WWTPs and river flow rates are reported in supplementary data, Tables S1 and S2. Sampling sites belonging to the same watershed were regrouped to locate them from a hydrological perspective (as for the website Aqualim, https://hydrometrie.wallonie.be/home/observations/debit.html) (Aqualim). As an example of all the maps, Figure 1 shows a map of the Haine watershed and indicates sampling sites and WWTPs locations. Maps of other investigated watersheds in Belgium (mainly in Wallonia) are presented in supplementary Figure S2.

<insert Table 1>

<insert Figure 1>

Sixty-three samples of surface water were collected between 10 June 2020 and 10 May 2021. All samples were grabbed samples and collected in the center of the river, where the stream of water was visible (Baker and Kasprzyk-Hordern, 2013). Stainless steel bucket and funnel, amber glass bottles used for the sampling were pre-washed with milli-Q water in the

laboratory. At the sampling site, the sampling material was rinsed first with the water sample (Sousa et al., 2019). Amber glass bottles were kept in a container with several cold accumulators before carrying them to the laboratory. Once they arrived in the laboratory, the bottles were refrigerated at 4°C. Samples were extracted within 24 hours after sampling to minimize the storage time and hence, the biodegradation (Gabet et al., 2007; Loos, 2015; Miège et al., 2009).

2.3 Analytical procedure

The analytical procedure for the quantification of E1, E2, E3, and EE2 in surface water was developed and published in a previous study (Glineur et al., 2020). The main steps are briefly described hereafter. The surface water sample (250 mL) was spiked with SIL-ISs and was extracted on an SPE disk based on a reverse phase-type sorbent (C18). SPE disk allows for the filtration of the water sample as well as the extraction of the analytes, thus avoiding an additional filtration step and complying with the EU Watch List (EC Decision 2015/495, 2015) that requires the analysis of whole water samples (including suspended particulate matter). Analytes were eluted with 3 aliquots of 10 mL of acetonitrile. The extract was evaporated to dryness under a flow of nitrogen at 50 °C (TurboVap II from Zymark) and reconstituted with 6 mL of n-heptane/dichloromethane (1:1, v/v). The extract was further purified on a Florisil® SPE cartridge, which consisted of a highly polar sorbent (magnesium silicate). E1, E2, and EE2 were first eluted by 3 aliquots of 2 mL of acetone/dichloromethane (1:9, v/v). Secondly, E3 was eluted by 3 aliquots of 2 mL of methanol/acetone (5:95, v/v). Purified extracts were evaporated to dryness, followed by a derivatization reaction: sodium hydrogencarbonate (0.1 mol/L in water, pH adjusted to 10.5) and pyridine-3-sulfonyl chloride (1 mg/mL in acetone) were added to the dry residue.

The high-performance liquid chromatography instrument was an Agilent 1260 Infinity. The analytical LC separation was achieved on a Kinetex biphenyl column (50 × 2.1 mm, 2.6 µm) using a gradient, with solvent A: water and solvent B: methanol. Both solvents A and B contained 0.1 % (v/v) formic acid and 0.6 g/L of ammonium formate as additives. More details on LC separation are described elsewhere (Glineur et al., 2018). The detector was an Agilent 6490 triple quadrupole with iFunnel technology equipped with an electrospray ionization source (ESI) and positive mode was used for the detection of the derivatized analytes. Further

information on ion source and MS/MS parameters are presented in Glineur et al. 2018 (Glineur et al., 2018).

2.4 Quality control

The analytical procedure was validated according to the standard NF T90-210 as previously published (Glineur et al., 2020). During each batch of samples, a procedural blank (following the whole analytical procedure) and a quality control (QC) spiked at 0.1 ng/L for E2, E3, and EE2; at 0.4 ng/L for E1 were analyzed. These concentrations correspond to the low spiking level (low-level QC) from the validation procedure (Glineur et al., 2020). The matrix was mineral water with no detectable analytes. During the whole sampling campaign, two performance characteristics were checked by the analysis of the QC: precision and trueness, through the calculation of the relative standard deviation (RSD, expressed as %) and the relative bias (expressed as %), respectively. Each water sample was analyzed in duplicate and the relative difference between individual results and the mean was calculated (EC Sante/11813/2017, 2017). Identification of the analytes in a sample was confirmed following the same criteria selected during the validation procedure (Glineur et al., 2020).

Besides, two complex surface water samples, namely Sambre (5) and Haine (4) were selected for spiking experiments on different no-consecutive days (n=5) close to the estimated LOQ for EE2 (at 0.035 ng/L, equal to the EQS), to challenge the analytical procedure. River Sambre is characterized by a higher median flow rate (= 12.5 m^3/s) compared to river Haine (= 1.4 m^3/s). Both rivers flow through highly populated areas.

2.5 Data treatment

When the concentration of an analyte was below the limit of quantification/limit of detection, <LOQ/<LOD was reported, respectively. For statistical analysis, when the result of a sample was below LOQ (<LOQ) or LOD (<LOD), concentration values were set to LOQ/2 or LOD/2, respectively (Gusmaroli et al., 2019; Kase et al., 2018; Loos et al., 2018). For some samples, as concentration values were higher than the highest level of the calibration curve, those were set at 20 ng/L for E1; 5 ng/L for E2 and E3, corresponding to the last calibration point. Data treatment was performed on Microsoft Excel (Microsoft Office, version 2016).

223

224 **2.6 Risk quotient (RQ)**

225 The assessment of the risk posed by estrogenic compounds (E1, E2, EE2, E3) to aquatic
226 organisms was performed through the calculation of the risk quotients (RQs). The RQ for an
227 individual chemical *i* is defined as the ratio between the measured environmental
228 concentration (MEC) and the predicted no-effect concentration (PNEC) (Equation 1) (Sousa et
229 al., 2019). In this study, EQSs for surface water proposed by the EC were used as PNEC for E1,
230 E2, EE2 (Kase et al., 2018; Loos et al., 2018). For E3, PNEC from Caldwell et al. (Caldwell et al.,
231 2012) was used for the assessment of the risk.

232 The mean of two analyses performed on each sample was used as MEC.

233 $$Equation\ 1: RQ = \frac{MEC}{PNEC} \qquad \text{(Eq. 1)}$$

234 RQ≥1 denotes a high risk for aquatic organisms (Kase et al., 2018).

235

236 **3 Results and discussion**

237 **3.1 Insight into the method's performance**

238

239 As emphasized by the Watch List procedure, high-quality monitoring data are required for
240 performing a robust risk assessment. Careful validation of the analytical procedure should
241 therefore be performed. Table 2 reports a list of studies from literature which have dealt with
242 the determination of estrogenic compounds in surface water, along with the information
243 regarding the procedure followed to validate the method (LOQ, precision and trueness).

244 According to Loos et al. (2018) (Loos et al., 2018), in order to assess the risk posed by E1, E2,
245 EE2, analytical methods applied by Member States should attain LOQs ≤ their respective EQS.
246 As displayed in Table 2, this requirement is not fulfilled in many of the listed articles for EE2
247 and in about half of them for E2. The difficulty of reaching such low LOQs was previously
248 underlined by Tiedeken et al. (2017) (Tiedeken et al., 2017). This observation is particularly
249 relevant as several samples in the listed papers were reported as <LOQ or <LOD. In this case,
250 if a sample is reported as <LOQ/<LOD and the applied method is characterized by a LOQ>EQS

(or LOD>(3/10)*EQS, based on signal-to-noise approach), this method is not suitable for the purpose of evaluating the risk (Loos et al., 2018).

<Insert Table 2>

The method used in this paper followed the most severe guidance for validation in order to ensure the reliability of the data reported and that when <LOQ was reported no exceedance of the EQS was present. Trueness and precision were assessed at three different spiking levels (LOQ, 5*LOQ, 20*LOQ), to cover the calibration range. Seven different surface water samples were spiked at the previously mentioned spiking levels, and analyses were carried out on 7 no-consecutive days (over a period of about one month, n=7). The LOQ for EE2 was first estimated by spiking/analyzing surface water samples with a concentration giving a signal-to-noise ratio close to 10. The estimated LOQ (≈ 0.029 ng/L) was then verified by spiking experiments in order to assess precision and trueness.

Moreover, the validated method was systematically verified throughout the sampling period from 2020 to 2021. The procedural blank was tested at every batch, always obtaining levels <LOD for all analytes. The inter-day RSDs (n=16 over a period of roughly one year, June 2020-May 2021) at the low-level QC were in the 2.8 – 8.4 % Range. Inter-day relative biases (n=16) ranged from -4.4 % to 6.1 %. The relative differences between the measurements of a sample (duplicate) and the mean were included in the range -10 and 10 %. The spiking experiments for EE2 in complex surface water samples led to the following results: inter-day RSD (n=5) equal to 8.4 % and inter-day relative bias (n=5) equal to -1.3 %.

3.2 Occurrence data

This sampling campaign aimed to obtain high-quality monitoring data on the concentrations of E1, E2, EE2, E3 in Belgian surface water. Data on these estrogenic compounds at ultra-trace levels in Belgian surface water are lacking. The sampling campaign was carried out on a wide array of rivers (26 in total) to draw a preliminary map of the levels of contamination. The presence of WWTPs is a determining factor as WWTPs effluents are considered an important source of estrogenic compounds that are released in receiving rivers (Adeel et al., 2017; Ting and Praveena, 2017). Consequently, in the different watersheds investigated, sampling sites

were selected nearby WWTPs of various capacities (see supplementary data, Table S1, Figure S2). The contamination of receiving rivers is also dependent on their flow rate, as a higher flow rate could result in a higher dilution of WWTP effluent/untreated sewage and inversely (López-Serna et al., 2012; Riva et al., 2019). Considering this factor, rivers with various median flow rates were selected (Table S2). Raw data concentrations are presented in supplementary Table S3 for the 63 samples analyzed. For each sample, analyses were performed in duplicate and the mean value was reported.

The results showed general widespread contamination of the Belgian surface water. The detection frequency (DF in %), which represents the ratio between the number of samples for which either a concentration value or <LOQ is reported, and the total number of samples, was rather substantial for all the 4 target analytes. Concerning natural estrogens, the DF of E1 was 100 %, followed closely by E2 (DF = 98 %) and E3 (DF =86 %). The synthetic estrogen EE2 showed a lower DF (48 %). Natural estrogens DFs were higher compared to the DF of the synthetic estrogen EE2, which is in accordance with several studies/reviews ((Du et al., 2020), Table 2). As can be observed in Table 2, the DF of E1 is typically higher than all other natural estrogens, attesting to its ubiquity in surface water.

<Insert Figure 2>

The mean concentration observed for E1 (4.433 ng/L) was the highest compared to other estrogens (Figure 2). These observations for E1 can be explained by different factors such as: high biotransformation rate of E2 into E1, lower removal efficiency for E1 in WWTP, and high excretion rate of E1 from livestock and poultry (Liu et al., 2015; Yu et al., 2019). In contrast, the mean concentration of synthetic estrogen EE2 was the lowest value (0.042 ng/L), roughly 2 orders of magnitude lower than the mean concentration of E1. The mean concentration of E3 is positioned between those of E1 and E2. The average concentrations of E1 and E2 are close to those calculated from several studies from literature (Gusmaroli et al., 2019; Könemann et al., 2018; Lardy-Fontan et al., 2018; Riva et al., 2019). Most of the concentration data ranged from 0.215 to 18.983 ng/L for E1 and from <LOD to 3.832 ng/L for E2. Only 8 and 6 % of the data exceeded the highest level of the calibration curve for E1 and E2, respectively. For EE2, the concentration data ranged from <LOD to 0.198 ng/L and there was no exceedance of the highest level of the calibration curve. The concentration range for EE2 is noticeably close

311 to the ones determined by two recent studies: Lardy-Fontan et al. and Konemman et al.
312 (Könemann et al., 2018; Lardy-Fontan et al., 2018) (Table 2).

313 The sum of the 4 estrogenic compounds was calculated and the summed values were ≥ 10
314 ng/L for eight rivers, at least at one sampling site: Sambre, Ligne, Senne, Hain, Thines, Samme,
315 Sennette, Haine, highlighting an important level of contamination (Figure 3). Rivers Senne and
316 Haine displayed multiple sampling sites with a sum ≥ 10 ng/L. River Haine is the only river for
317 which concentrations of E1, E2 exceeded the highest calibration level at multiple sampling
318 sites. For sampling sites Haine (1) and Haine (2), the extremely high concentrations of
319 estrogenic compounds would not be explained solely by the contribution of upstream WWTP
320 of moderate capacity. The release of untreated wastewater in river Haine could be the main
321 contributor to the contamination. Besides, it is worth noting that the sum of the 4 estrogenic
322 compounds did not exceed 5 ng/L in all sampling sites on river Meuse, characterized by the
323 highest median flow rate among investigated rivers. The mean % of each analyte, relative to
324 the sum of the 4 estrogenic compounds, was in descending order: 68.5, 17.4, 12.8, 1.2, for E1,
325 E3, E2, EE2, respectively. E1 is the prevailing contaminant when considering the mean % of all
326 4 estrogenic compounds.

327 *<Insert Figure 3>*

328 Different factors affect environmental concentrations of pollutants in rivers, for example
329 biotrasformation, vertical transport process, hydrology (including dilution effect) and in-
330 stream attenuation processes (biodegradation, sorption to sediments) (Burns et al., 2018;
331 Kasprzyk-Hordern et al., 2008). In here, we examined the dilution effect due to the river flow
332 at the sampling time. As can be seen in Figure S1, histogram charts representing the
333 distribution of the daily mean flow rates are rather asymmetric, with a median lower than the
334 mean for all rivers. Most of the studied rivers were sampled on a day characterized by a flow
335 rate localized on the left part of the histogram, for which a high frequency was observed. For
336 rivers By (1), Orneau (1) on day 04/02/2021, flow rates were noticeably high (> 95^{th}
337 percentile), as heavy rainfalls preceded the sampling (Table S2). For these rivers (By and
338 Orneau), a second sampling was carried out on a day for which the flow rate was significantly
339 lower and within the main flow distribution. Only for one river Ligne (1), flow rate was above
340 the 95^{th} percentile on day 12/01/2021 and it was not possible to repeat the sampling.
341 Exceptionally high flow rates may lead to uncommon concentration values as observed by

Kasprzyk-Hordern et al. (Kasprzyk-Hordern et al., 2008). They studied the concentrations of an array of pharmaceuticals in rivers in the South Wales (UK), which is characterized by a similar climate as Belgium, including precipitation patterns. The authors observed that concentrations of pharmaceuticals were lower after heavy rainfalls, arising from the larger dilution factor (Kasprzyk-Hordern et al., 2008).

3.3 Risk assessment

As previously mentioned, the RQ is defined as the ratio between the MEC (calculated using the mean of two analyses on the same sample) and the EQS/PNEC. If the RQ is ≥1, it means that the MEC exceeds the EQS/PNEC for a given analyte and implies that the MEC might pose a risk for aquatic organisms. On the contrary, if the RQ is <1, it means that the MEC is below the EQS/PNEC and implies that the MEC can be considered safe for aquatic organisms (Sousa et al., 2019; Tousova et al., 2017). The frequency of exceedance of EQS/PNEC is calculated as % of all samples with RQ ≥1. RQs for E1, EE2 were ≥1 for 31.7 % of all samples analyzed, whereas this % rose to 44.4 % for E2. For some samples (8 %, 6% for E1, E2, respectively), concentrations were higher than the highest calibration level, and it was decided to set them as equal to this level. This choice did not impact the conclusion on the frequency of exceedance of the EQS/PNEC, because these values were already substantially above the ecotoxicological threshold either for E1 or E2 (see Table S3 with EQS/PNEC values for all target analytes). For E3, in 84 % of the samples, RQs were <1, signifying low risk associated with this pollutant. However, 16 % of the samples exceeded the highest level of the calibration curve for E3 and concentrations were set as equal to this level (= 5 ng/L). Therefore, it was not possible to conclude if the PNEC for E3 was exceeded for these samples. In the worst-case scenario 16 % of the samples would exceed the PNEC. This % is still considerably lower than % of samples with RQs≥1 observed for E1, E2 and EE2. Moreover, if all target estrogenic compounds are considered for the assessment of the chemical status, these same samples can be classified as “high risk” because RQ was ≥1 for at least one of the following analytes: E1, E2, EE2. The extent of exceedance was obtained by calculating the 95^{th} percentile of the RQ dataset including all samples (n=63) (Tousova et al., 2017). The extent of exceedance is ranked in descending order for each analyte: 12.2, 5.6, 4.3 for E2, E1 and EE2, respectively.

Samples were first arranged by rivers, to identify rivers for which E1, E2, EE2 may pose a risk. On several rivers, the number of samples was >1 and therefore it allowed to study if a RQ ≥1 was observed multiple times on the same river for a given analyte. N1 was designated as the number of samples on a given river for which RQ was ≥1 for an analyte, so meaning a high risk related to this analyte. N1≥1 was selected as a criterion, meaning that at least one sample on a given river exhibited a RQ≥1 (cells in orange color in Table 3). N1≥1 for E2 was observed for 14 rivers, while N1≥1 for E1 or EE2 was observed for 11 rivers, out of the total number of investigated rivers (=26). 8 rivers displayed N1≥1 for E1 and E2 and EE2. In contrast, 9 rivers displayed N1=0 for E1 and E2 and EE2. The sum of N1 for all sampled rivers belonging to the same watershed was determined. Three watersheds appeared to be particularly exposed to a risk for aquatic life with sum ≥10 and were classified hereafter by descending order: Dyle-Senne; Senne and Haine with equal value. The RQs for all analytes for each sample and arranged by river and by watershed are displayed in Figure 4.

<Insert Table 3>

<Insert Figure 4a, 4b, 4c>

3.4 Risk prediction: confusion matrix

E1 concentration was used to predict the risk linked to the two other analytes E2 and EE2. The ultimate idea is to evaluate the possibility of screening only for E1, the most abundant estrogenic compound and thus easier to analyze and to predict the related risk. Whether necessary, only selected samples can undergo a more demanding analysis to characterize the concentrations of the other compounds.

E1 concentration value was used as the predicted class and the principle was to determine a concentration threshold ([E1] > x or [E1] < x), in order to predict the risk (high risk/no risk, respectively). The actual class corresponded to the risk level for a sample (high risk/no risk) related to the contaminants E2 and EE2. If a sample was at high risk, it meant that either E2 or EE2 showed a RQ≥1. The samples (n=63) were arranged by building a confusion matrix composed of 4 classes (Figure 5). Class A corresponds to the case where the sample is predicted to be at high risk, and it is (True Positive, TP). Class B: the sample is predicted to be at high risk, and it is not (false positive, FP). Class C: the sample is predicted to be at no risk, and it is not (false negative, FN). Class D: the sample is predicted to be at no risk, and it is (True

Negative, TN). Ideally, the number of samples in class C (FN) needs to be maintained at 0, and in class B (FP) minimized in order to avoid more expensive analysis that would not be needed. E3 was not considered in this approach because the risk associated to this compound is considered to be lower compared to E1, E2 and EE2. For 84 % of the samples, RQs were <1.

<Insert Figure 5>

Using a threshold value of 1.1 ng/L for E1 allowed to obtain 0 samples in class C (FN) and only 9 samples in class B (FP) out of the total 63 samples. These results provided sensitivity and negative predicted values of 1, as desired. Regarding precision and specificity, their values need to be as close as possible to 1, in this case they were 0.78 and 0.71, respectively.

The outcomes of this prediction study suggest that E1 can be used as a marker of the risk posed by estrogenic compounds (E2, EE2). This means that the concentration of E1 may be first determined in surface water samples by using a multi-analyte analytical procedure less time-consuming in terms of sample preparation. However, this procedure needs to achieve reliably a LOQ lower than the threshold of 1.1 ng/L, required for the risk prediction. Worth to note that a LOQ <1.1 ng/L was attained by several studies using multi-analyte analytical procedure (Table 2) (Gorga et al., 2015; Gusmaroli et al., 2019; Rubirola et al., 2017). If the concentration of E1 in the sample is below the threshold (1.1 ng/L), there is no need to investigate for E2, EE2 using the more complex procedure. Conversely, if the concentration of E1 is above the threshold, E2, EE2 have to be analyzed. Considering the data set in the present study, this would enable to avoid the analysis of 22 samples out of the 63 (about 35%) with a significant saving of time and money.

4 Conclusion

An analytical method using solid phase extraction and liquid chromatography hyphenated with tandem mass spectrometry was applied to the analysis of surface water samples collected from a wide range of rivers in Belgium to obtain an overview of contamination levels. During the risk assessment procedure, the frequency of exceedance (31.7-44.4 %) and the extent of exceedance (4.3-12.2) of the EQS were calculated and indicated that E1, E2, EE2 might pose a risk for aquatic organisms. Furthermore, for some rivers, estrogenic compounds

(E1, E2, EE2) may pose a serious threat to aquatic organisms. Indeed, 8 rivers presented a high risk considering E1, E2 and EE2. Three watersheds showed substantial risk: Dyle-Senne, Senne and Haine. The contamination of these rivers by estrogenic compounds can be linked to different factors. As these pollutants are only partially eliminated in WWTPs, trace levels were observed in effluents, eventually discharged in rivers. Some estrogens are more recalcitrant to degradation in WWTPs, like the synthetic estrogen EE2 (Ting & Praveena). In rivers with medium to low flow rates receiving effluents, the dilution factor may not be sufficient and concentrations above EQS will occur. Lastly, some rivers likely receive untreated wastewater, thus further contributing to the contamination.

A confusion matrix was built to predict the risk posed by E2 and EE2 based on E1 concentration, showing that a first analysis using a simpler multi-analytes method can reliably provide information regarding the related risk. If the concentration of E1 is below the determined threshold (i.e., 1.1 ng/L) in the selected samples, their analysis for searching E2 and EE2 can be avoided. This could be advantageous in future sampling campaigns in order to decrease the number of analyses.

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Acknowledgments

This work was carried out in the framework of the DIADEM project (Développement d'une Approche Intégrée pour le Diagnostic de la qualité des Eaux de la Meuse) and was supported by the Interreg France-Wallonia-Vlaanderen Programme (European Regional Development Fund) and by the Public Service of Wallonia (SPW).

The authors would like to thank the "Service Public de Wallonie, mobilité et infrastructures, département expertises hydraulique et environnement, direction de la gestion hydrologique" for the flow rate data.

Statements and Declarations

Funding

This work was financed by the fund Ernest du Bois managed by the "Roi Baudouin foundation" (2018-F2812650-211494).

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

The authors have declared that no ethical issues exist.

Consent to participate

Not applicable.

Consent to publish

All authors agreed with the content and all gave explicit consent to submit.

Authors contributions

https://doi.org/10.1007/s11356-023-31698-9

652 **Alex Glineur, Giorgia Purcaro, Katherine Nott, Thomas Pollet**: Conceptualization,
653 Methodology, Visualization; **Alex Glineur**: Validation, Data curation, Writing- Original draft
654 preparation, Investigation; **Giorgia Purcaro, Katherine Nott, Philippe Carbonnelle, Sébastien**
655 **Ronkart**: Supervision, Writing- Reviewing and Editing, Project administration; **Alex Glineur,**
656 **Giorgia Purcaro, Katherine Nott, Sébastien Ronkart**: Fund raising for the research.

657 **Availability of data and materials**

658 Available on request, most raw data is provided in supplementary materials.

https://doi.org/10.1007/s11356-023-31698-9

659 **Figure and Table Captions**

660 **Table 1.** Summary information on the sampled rivers: number of samples, WWTP(s)
661 discharging effluent into the river and median flow rate at one/several sampling site(s)
662 investigated.

663 **Table 2.** Studies on the determination of estrogenic compounds (E1, E2, EE2, E3) in surface
664 water from different European countries. Method performance characteristics,
665 concentration range and detection frequency are summarized in the table. A note on this
666 table is included in the supplementary data.

667 **Table 3.** Name of the 26 rivers sampled, number of samples collected on each river. N1
668 corresponds to the number of samples for which a RQ ≥ 1 was observed on a river for a
669 contaminant, signifying a high risk related to the contaminant. Cells in orange corresponds to
670 N1 ≥ 1, signifying that at least one sample on a determined river exhibits a RQ ≥ 1.

671

672 **Figure 1.** Location of sampling sites and nearby wastewater treatment plants in the Haine
673 watershed.

674 **Figure 2.** Box plot of the concentration data for the 4 target analytes: E1, E2, EE2, E3 (ng/L, log
675 scale), triangle represents the mean concentration. If <LOQ, = LOQ/2; if <LOD, = LOD/2.

676 **Figure 3.** Cumulative concentrations (ng/L) of the 4 target analytes. The number at the end of
677 each river name corresponds to the location on the river (from upstream to downstream on
678 the river).

679 **Figure 4a.** Risk quotient (RQ) calculated for all samples. The number at the end of each river
680 name corresponds to the location on the river (from upstream to downstream on the river).
681 Rivers are arranged by watershed. RQ ≥ 1 indicates a high risk related to the contaminant (E1).

682 **Figure 4b.** Risk quotient (RQ) calculated for all samples. The number at the end of each river
683 name corresponds to the location on the river (from upstream to downstream on the river).
684 Rivers are arranged by watershed. RQ ≥ 1 indicates a high risk related to the contaminant
685 (E2).

686 **Figure 4c.** Risk quotient (RQ) calculated for all samples. The number at the end of each river
687 name corresponds to the location on the river (from upstream to downstream on the river).
688 Rivers are arranged by watershed. RQ ≥ 1 indicates a high risk related to the contaminant
689 (EE2).

690 **Figure 5.** Confusion matrix. The predicted class corresponds to E1 concentration (ng/L) based
691 on a threshold value. The actual class corresponds to the risk posed by E2 and EE2 in each
692 sample (n=63).

693

694 **Supplementary Figures and Tables captions**

695 **Table S4.** Location of sampling sites and nearby wastewater treatment plant. The number of
696 each sampling site code corresponds to the location on the river (from upstream to
697 downstream on a river).

698 **Table S5.** Sampling date and hour, sampling site code, river flow rate on the day of sampling
699 (Fi). Median, mean and 95th percentile of the daily mean flow rates, over a five-year period
700 (from 2016 to 2021).

701 **Table S6.** Sampling date and hour, sampling site code, raw data concentration (based on the
702 mean of two analysis of the same sample), measured environmental concentration (ng/L)
703 and risk quotient.

704 **Figure S1.** Histogram charts of daily mean flow rates over a five-year period for different rivers
705 in Belgium

706 **Figure S2.** Location of sampling points and nearby wastewater treatment plants in several of
707 the watersheds investigated (a: Senne, b: Meuse aval, c: Sambre, d: Meuse amont, e: Dyle-
708 Gette, f: Lesse)

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714 ***Table 7. Summary information on the sampled rivers: number of samples, WWTP(s) discharging effluent into the river***
715 ***and median flow rate at one/several sampling site(s) investigated.***

Watershed	River/water resource	Wastewater treatment plant (WWTP)	Wastewater treatment plant capacity (Population Equivalent, PE)	Median flow rate, m^3/s
Sambre	Sambre (n=6)	Montignies-sur-Sambre	200,000	9.45-12.45
		Mornimont	45,000	
		Floreffe	20,700	
	Orneau (n=3)	Corroy-Le-Château	19,800	0.66
	Ligne (n=1)	Wanfercee-Baulet	10,800	0.14
Meuse amont	Meuse (n=3)	Godinne	9,800	/
		Pont de Wepion	13,450	
	Bocq (n=1)	/	/	/
Meuse aval	Meuse (n=5)	Namur-Brumagne	93,100	94.11-102.78
		Amay+Engis	76,400	
		Liège Sclessin	135,000	
		Liège Oupeye	401,850	
	Mehaigne (n=2)	Eghezée+Wasseiges	5,400	/
	Hoyoux (n=1)	Marchin	2,250	0.97
Lesse	Lesse (n=2)	Resteigne+Belvaux	2,070	2.29
Dyle-Gette	Argentine (n=1)	/	/	/
	Grande Gette (n=1)	/	/	/
Dyle-Senne	Lasne (n=2)	Rosières	100,000	0.93
	Dyle (n=3)	Grez-Doiceau	20,000	/
	Senne (n=6)	Bruxelles-Nord	1,400,000	6.7
	Senne former path (n=1)	/	/	/
Senne	Hain (n=2)	Vallée Du Hain (L'orchis)	92,000	0.24
	Thines (n=1)	Nivelles	44,450	0.12
	Samme (n=1)	Seneffe (Soudromont)	58,500	/
	Sennette (n=1)	Ecaussinnes	5,000	0.08
Nete	Nete (n=1)	Lier	34,000	/
	De Wimp (n=1)	Morkhoven	48,000	/
	Grote Nete (n=1)	Westerlo	34,000	/
Escaut aval	Rupel (n=1)	/	/	/
Haine	Haine (n=5)	Morlanwelz	18,000	1.35
		Trivières	19,000	
		Wasmuel	250,000	
	By (n=2)	Frameries	18,000	0.09
	Trouille (n=1)	/	/	/
Ourthe	Eau de Somme (n=2)	/	/	/
	Ourthe (n=6)	Embourg	24,300	/
		Liège (Grosses Battes)	53,137	/

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https://doi.org/10.1007/s11356-023-31698-9

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718 ***Table 2. Studies on the determination of estrogenic compounds (E1, E2, EE2, E3) in surface water from different European countries. Method performance characteristics, concentration***
719 ***range and detection frequency are summarized in the table. A note on this table is included in the supplementary data.***

Analyte(s)	Instrumental technique	Method performance characteristics		Concentration range (…-… ng/L) and (mean)	Detection frequency (%)	Reference
		LOQ (ng/L)	Precision and trueness assessment			
E1,E2,EE2,E3 +27	LC-MS/MS	surface water sample 0.17 (E1) 0.12 (E2) 0.56 (E3) 0.47 (EE2)	Spiking surface water: at 2.5, 25, 250 ng/L One type of surface water (n=6), no information on timespan (only interday is mentioned) Bias (%, based on relative recovery value), 2.5 ng/L; RSD (%), 2.5 ng/L : -7 ; 7.2 (E1) 3 ; 2.7 (E2) -17 ; 11.0 (E3) -15 ; 15.0 (EE2)	<LOD-7.3 (E1) <LOD-7.8 (E2) <LOD-5.7 (E3) <LOD-2.2 (EE2)	43-87 (E1) 0-93 (E2) 0-13 (E3) 0-4.2 (EE2)	(Gorga et al., 2015, 2013)
E1,E2,EE2	LC-MS/MS	surface water sample 0.1 (E1) 0.1 (E2) 0.035 (EE2)	Spiking surface water: at LOQ, 8xLOQ, 16xLOQ (ng/L) Three types of surface water (n=3), only repeatability conditions (same day)	<LOD-4.0 (0.86) (E1) <LOD-1.0 (0.33) (E2) <LOD (EE2)	83 (E1) 36 (E2) 0 (EE2)	(Barreca et al., 2019)
E1, E2, EE2 + 27	LC-MS/MS	Effluent of WWTP 1.5 (E1) 2.6 (E2) 4.6 (EE2)	Instrumental repeatability (n=3)	5.0-20.4 (10.1) (E1) <LOQ-3.9 (2.4) (E2) / (EE2)	100 (E1) / (E2) / (EE2)	(Castiglioni et al., 2005; Riva et al., 2019)
E1,E2,EE2,E3 +130	SFC-MS/MS	not mentioned 0.05 (E1) 0.05 (E2) 0.05 (E3) 0.05 (EE2)	not mentioned	<LOD-10.5 (1.8) (E1) <LOD-19.6 (6.6) (E2) <LOD-0.1 (0.1) (E3) <LOD-2.7 (1.2) (EE2)	95 (E1) 48 (E2) 5 (E3) 18 (EE2)	(Maasz et al., 2019)

E1,E2,EE2	LC-MS/MS	surface water sample 0.4 (E1) 0.4 (E2) 0.1 (EE2)	Spiking surface water: at LOD, 3xLOD (LOQ), 6xLOD (ng/L) one type of surface water (n=6, intermediate precision, no information on timespan, 2 different operators) Bias (%) ; RSD (%): (-21 to + 20%) ; ≈10% (E1,E2) (-33 to +27%) ; <25% (EE2)	<LOQ-7.71 (1.44) (E1) <LOQ-1.3 (0.29) (E2) <LOQ-0.23 (0.07) (EE2)	96 (E1) 15 (E2) 15 (EE2)	(Lardy-Fontan et al., 2018)
E1,E2,EE2 +14	LC-MS/MS	surface water sample 0.32 (E1) 0.51 (E2) 1.6 (EE2)	Spiking surface water: at 5, 100 ng/L (precision); 100 ng/L (trueness) one type of surface water (n=3), 3 consecutive days Bias (%), 100 ng/L ; RSD (%), 100 ng/L : -1.5 ; 4.1 (E1) -3.5 ; 4.2 (E2) -7.9 ; 6.1 (EE2)	<LOD-6.9 (3.11) (E1) <LOD-1.36 (0.99) (E2) <LOD-2.88 (2.29) (EE2)	48.3 (E1) 10.3 (E2) 31 (EE2)	(Gusmaroli et al., 2019, 2018)
E1,E2,EE2 +3	LC-MS/MS	not mentioned 0.52 (E1) 1.5 (E2) 1.88 (EE2)	Instrumental precision (intra-day and inter-day), 1 calibration standard Trueness: spiking deionized water, at 20 ng/L (n=3)	<LOQ-28 (6.4) (E1) <LOQ-39.7 (8.5) (E2) <LOQ (EE2)	48 (E1) 30.5 (E2) 0 (EE2)	(Pignotti et al., 2017)
E1, E2, EE2 +21	LC-MS/MS	surface water sample 0.7 (E1) 1.1 (E2) 0.8 (EE2)	Spiking surface water: at 25 ng/L One type of surface water (n=5), on different days, no information on timespan Bias (%), 25 ng/L ; RSD (%), 25 ng/L : 1.5 ; 3.9 (E1) -0.3 ; 4.4 (E2) 0.9 ; 6.1 (EE2)	<LOD-34 (8.1) (E1) <LOD-30 (E2) <LOD (EE2)	44 (E1) 5 (E2) 0 (EE2)	(Rubirola et al., 2017)

E1,E2,EE2,E3 +6	LC-MS/MS	surface water sample 0.1 (E1) 0.136 (E2) 0.210 (E3) 0.115 (EE2)	Spiking surface water: at 5 ng/L One type of surface water (n=3), no information on timespan Bias (%), 5 ng/L ; RSD (%), 5 ng/L : -0.3 ; 2.2 (E1) -3.4 ; 1 (E2) -15.7 ; 3.1 (E3) 0.2 ; 6.2 (EE2)	<LOD-2.29 (0.90) (E1) <LOD (E2) <LOD-4.24 (2.91) (E3) <LOD (EE2)	26.7 (E1) 0 (E2) 40 (E3) 0 (EE2)	(Čelić et al., 2017)
E1,E2,EE2	LC-MS/MS	several surface water samples (16) 0.023-0.181 (E1) 0.045-0.700 (E2) 0.039-0.397 (EE2)	Spiking surface water: at 0.6, 6 ng/L One type of surface water (n=3), different laboratories Bias (%), 0.6 ng/L ; RSD (%), 0.6 ng/L : 3 ; 17.5 (E2) 28 ; 12.5 (EE2)	0.067-5.561 (1.503) (E1) <LOQ-0.504 (0.231) (E2) <LOD-0.292 (0.154) (EE2)	100 (E1) 100 (E2) 50 (EE2)	(Könemann et al., 2018)
E1,E2,EE2,E3	LC-MS/MS	several surface water samples (7) 0.075 (E1) 0.041 (E2) 0.058 (E3) 0.029 (EE2)	Spiking surface water: at 0.1, 0.5, 2 ng/L for E2, E3, EE2; at 0.4, 2, 8 ng/L for E1 Seven types of surface water (n=7), over a period of one month (intermediate precision) Bias (%), 0.1 ng/L ; RSD (%), 0.1 ng/L : -5.7 ; 14.6 (E2) 8.3 ; 23.3 (E3) 9.7 ; 5.4 (EE2) Bias (%), 0.4 ng/L ; RSD (%), 0.4 ng/L : -31.2 ; 13.8 (E1)	/	/	(Glineur et al., 2020)

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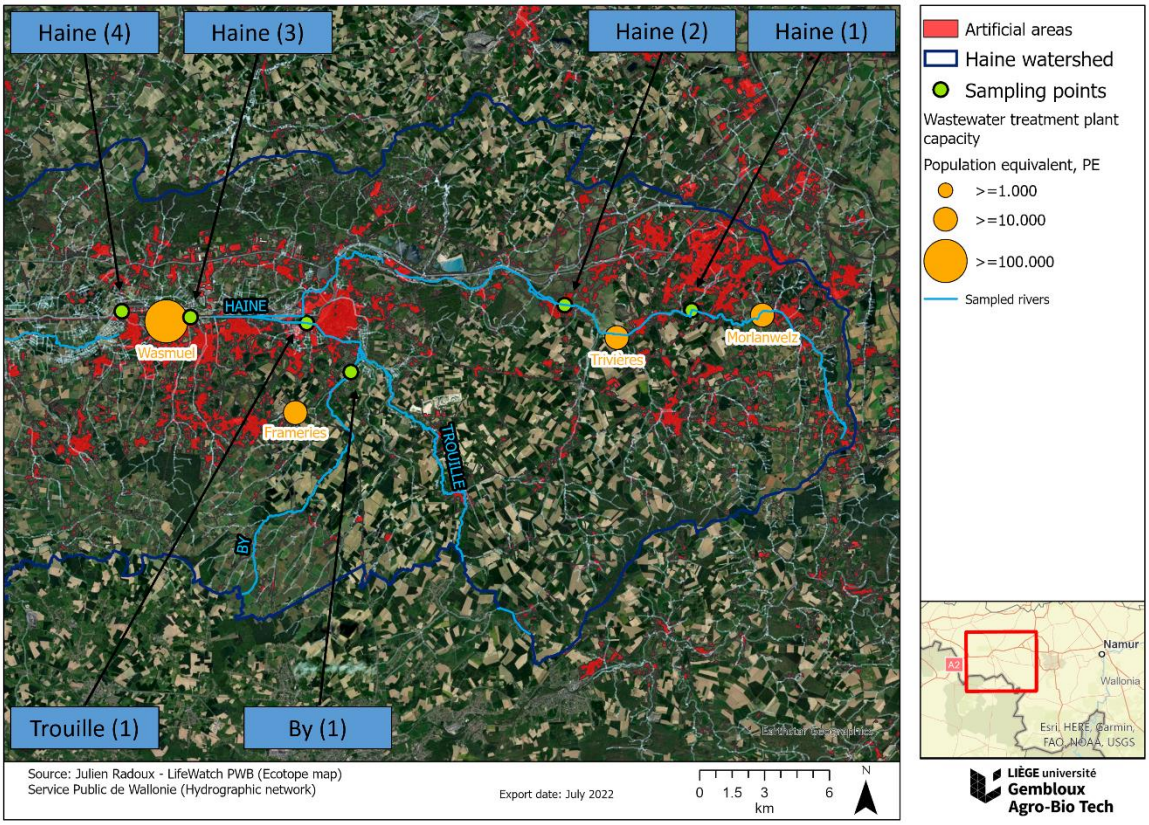
https://doi.org/10.1007/s11356-023-31698-9

Table 3. Name of the 26 rivers sampled, number of samples collected on each river. N1 corresponds to the number of samples for which a RQ ≥ 1 was observed on a river for a contaminant, signifying a high risk related to the contaminant. Cells in orange corresponds to N1 ≥ 1, signifying that at least one sample on a determined river exhibits a RQ ≥ 1.

River	Number of samples	N1		
		E1	E2	EE2
Argentine	1	0	0	0
Bocq	1	0	0	0
By	2	0	2	0
De Wimp	1	1	1	1
Dyle	3	0	0	1
Eau de Somme	2	0	1	0
Grande Gette	1	0	0	0
Grote Nete	1	0	0	0
Hain	2	2	2	2
Haine	5	4	5	4
Hoyoux	1	0	0	0
Lasne	2	0	0	2
Lesse	2	0	0	0
Ligne	1	1	1	1
Mehaigne	2	1	1	0
Meuse	8	0	1	0
Nete	1	1	0	1
Orneau	3	0	1	0
Ourthe	6	0	0	0
Ruppel	1	0	0	0
Sambre	6	2	3	0
Samme	1	1	1	1
Senne	7	5	7	5
Sennette	1	1	1	1
Thines	1	1	1	1
Trouille	1	0	0	0

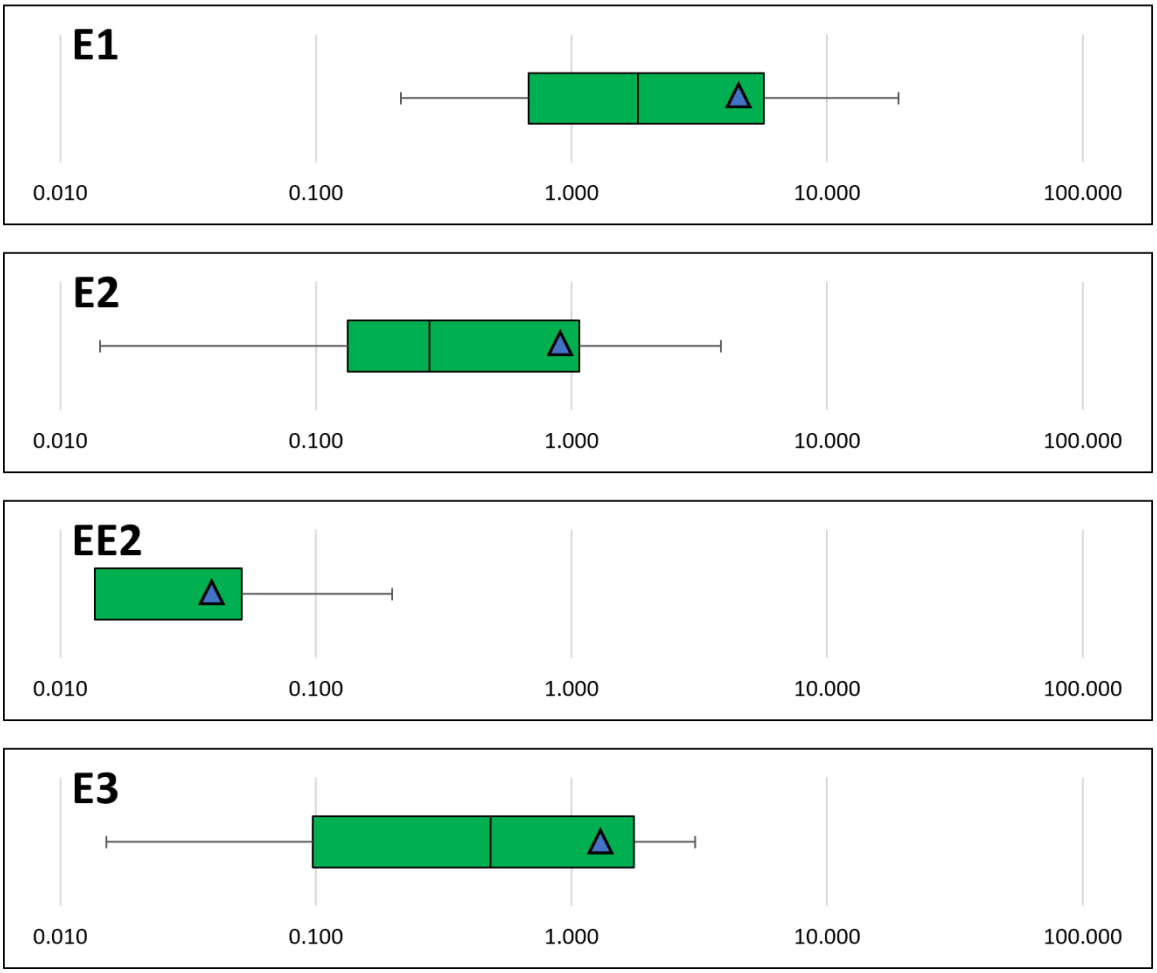
https://doi.org/10.1007/s11356-023-31698-9

Figure 1



https://doi.org/10.1007/s11356-023-31698-9

Figure 2



https://doi.org/10.1007/s11356-023-31698-9

Figure 3

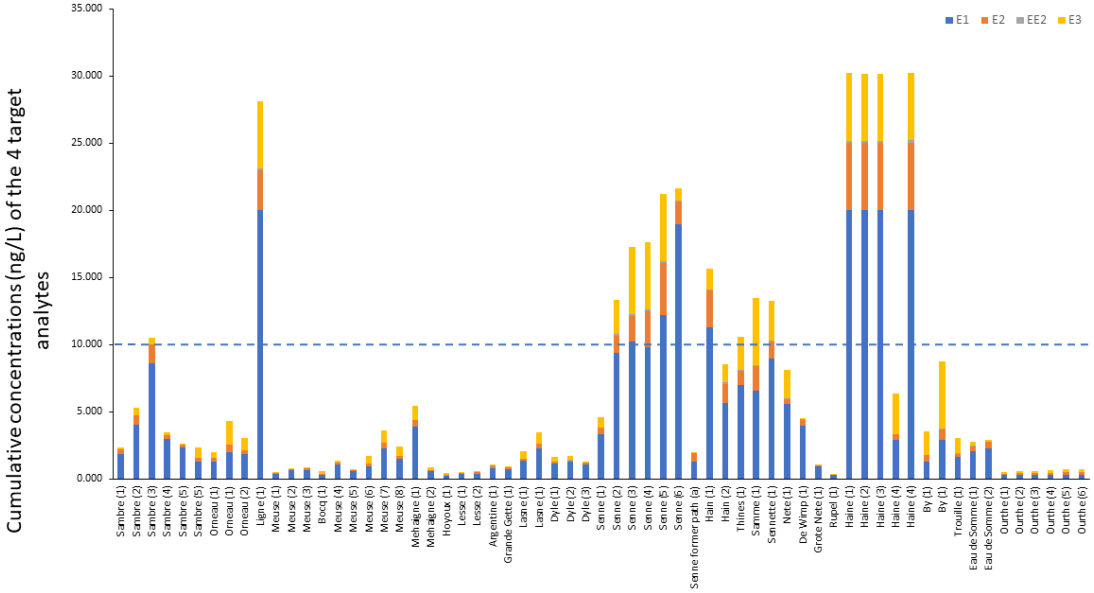
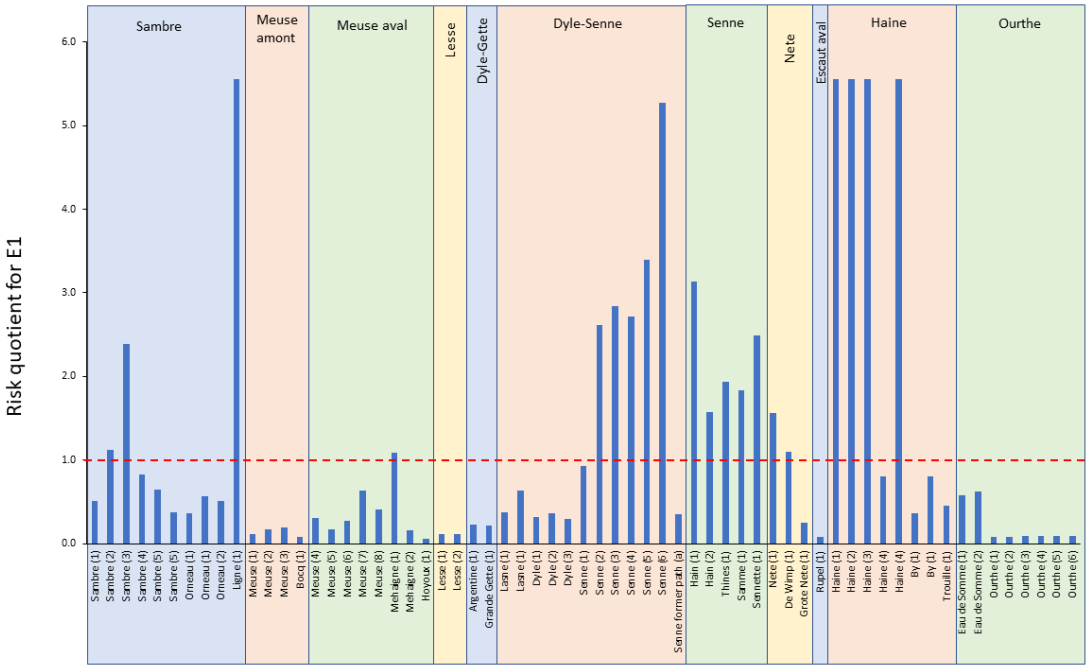


Figure 4a



https://doi.org/10.1007/s11356-023-31698-9

Figure 4b

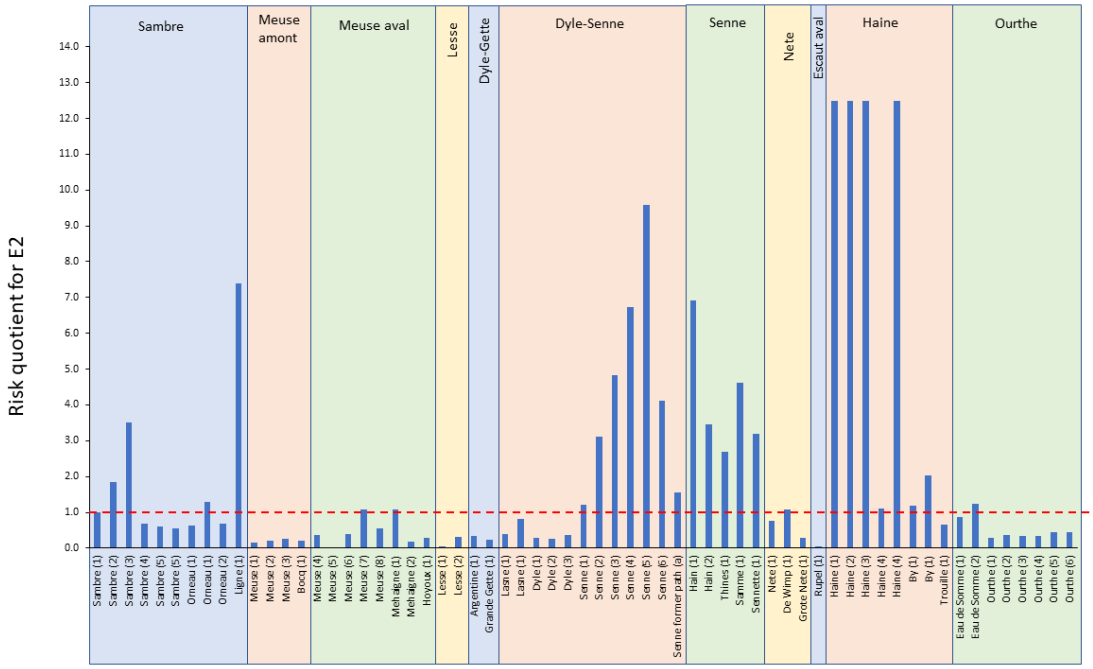


Figure 4c

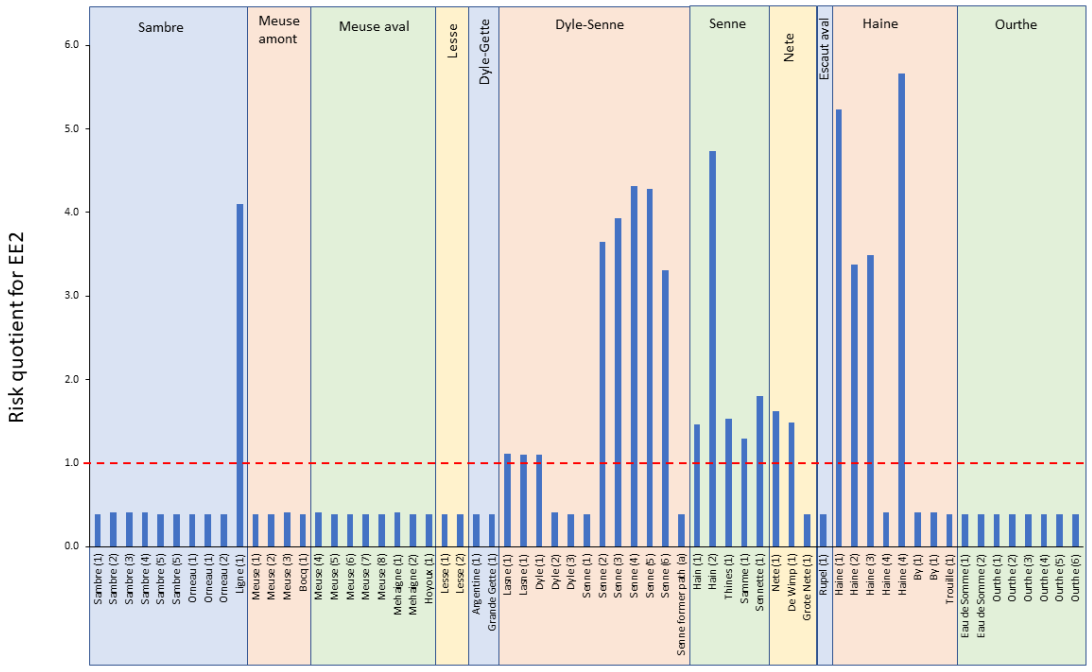


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Figure 5

		Predicted class (E1 concentration, ng/L)			
		Positive	Negative		
Actual class (risk level related to E2 and EE2)	Positive	Class A True positive (TP)	Class C False negative (FN)	**Sensitivity**	TP/(TP+FN)
	Negative	Class B False positive (FP)	Class D True negative (TN)	**Specificity**	TN/(TN+FP)
		Precision	**Negative predicted value**		
		TP/(TP+FP)	TN/(TN+FN)		

		Predicted class (E1 concentration, ng/L)			
		>1.1	**<1.1**		
Actual class (risk level related to E2 and EE2)	High risk	32	0	**Sensitivity**	1
	No risk	9	22	**Specificity**	0.71
		Precision	**Negative predicted value**		
		0.78	1		