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Background document on environmental monitoring of pharmaceuticals

**Project APRIORA,
GoA 1.1
Background document**

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Scope of this document

This background document was prepared as a part of project APRIORA (Improved risk assessment for strategic water management to reduce micro-pollutant emissions in the Baltic Sea Region), funded by Interreg Baltic Sea Region. The document is accompanied by a set of flowcharts compiled during the project ([link](#)). The goal of these deliverables is to provide regional authorities, wastewater treatment plant operators, and potential other interested stakeholders with a streamlined approach for assessing the risks related to pharmaceuticals in the environment, focusing on discharges from wastewater treatment plants. The risk assessment framework proposed in the flowcharts and this background document can be utilized e.g. when prioritizing wastewater treatment plants for the implementation of advanced treatment methods.

This background document is divided into three modules:

- A. Monitoring pharmaceuticals in the environment,
- B. Modelling pharmaceutical loads and concentrations in the environment,
- C. Assessing the risks related to pharmaceuticals in the environment.

Module A focuses on issues related to producing experimentally quantified concentration data on pharmaceuticals in wastewater matrices and recipient waters, along with occurrence data of antimicrobial resistance in those matrices. This module starts from the selection of target compounds, moving to sampling practices, and eventually analyses and data management.

Module B provides a framework for the computational estimation of pharmaceutical discharges and concentrations in wastewaters and recipient waters. The modelling approach contains a mass-balance model, using pharmaceutical sales statistics as the driving parameter, and a hydrological model, used to estimate recipient flow volumes at different points along the river network.

Module C covers risk assessment from multiple aspects. It relies on measured or predicted environmental pharmaceutical concentrations, and certain compound-specific parameters such as predicted no-effect concentrations. Environmental risks are assessed using a conventional approach of risk quotients. On the other hand, Module C also covers the risk of antimicrobial resistance promotion. This approach is computationally similar to environmental risk assessment, but the threshold values used in the assessment are derived based on the concentration levels at which antimicrobial resistance is promoted. The third risk category considered in this background document covers human exposure via drinking water (incl. involuntary drinking during swimming) and fish consumption. Finally, the three risk categories are merged to create an index-like value, which can be intuitively used to prioritize different sampling sites.

Module A:

Monitoring of pharmaceuticals

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List of abbreviations

Abbreviation	Explanation
AAS	Atomic absorption spectrometry
AMR	Antimicrobial resistance
AMR-B	Antibiotic resistant bacteria
AMR-G	Antibiotic resistance gene
API	Active pharmaceutical ingredient
ATC	Anatomical therapeutic chemical classification system
BOD	Biological oxygen demand
BSR	Baltic Sea Region
CAS	Chemical abstracts service
COD	Chemical oxygen demand
E1	Estrone
E2	17 β -estradiol
EC50	Half maximal effect concentration
EE2	17 α -Ethinylestradiol
EQS	Environmental quality standards
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
HDPE	High-density polyethylene
HLB	Hydrophile lipophile balance
HPLC	High performance Liquid Chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
IS	Internal standard
KM	Kaplan-Meier estimator
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MDL	Method detection limit
MEC	Measured environmental concentration
MLE	Maximum likelihood estimation
ML	Method quantification limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MU	Measurement uncertainty
NOEC	No-observed-effect-concentration
PAH16	16 Polycyclic aromatic hydrocarbons regulated by the USEPA
PC	Process contribution
PE	Population equivalent
PEC	Predicted environmental concentration
PLE	Pressurised liquid extraction
PNEC	Predicted no-effect concentration
POCIS	Polar organic chemical integrative sampler
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
ROS	Regression on Order Statistics
RQ	Risk quotient
SLE	Solid-liquid extraction
SPE	Solid phase extraction
SSD	Species sensitivity distribution
TOC	Total organic carbon
UAE	Ultrasound assisted extraction
UBA	German Environmental Agency
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry
USEPA	U.S. Environmental Protection Agency
UWWTD	Urban wastewater treatment directive
WFD	Water framework directive
WL	Watch list
WWTP	Wastewater treatment plant

Introduction

Environmental monitoring and screening campaigns of hazardous substances in water matrices aim to assess the occurrence, concentration, and distribution of those substances in the environment. Often, the goal is to determine if the contaminants cause adverse effects in the environment or to human health, or if they comply with regulatory requirements.

Sampling campaigns can be divided into two major categories: monitoring and screening. Monitoring refers to systematic, continuous, or recurring sampling campaigns, taking place at the same location, using the same methodologies over an extended period of time. Screening, on the other hand, refers to more sporadic or short-term sampling campaigns. Both types of sampling campaigns provide information on contaminant occurrence in the selected matrix, each with its own advantages and disadvantages. While monitoring campaigns produce directly comparable information e.g., on trends, screening campaigns are often more flexible and useful in preliminary studies for identifying emission sources or contaminants of relevance.

So far, few monitoring campaigns for pharmaceutical residues in the environment have been implemented. The first EU-wide monitoring campaign for pharmaceuticals was initiated in 2015 with the first Water Framework Directive (WFD, 2000/60/EC) watch list (WL, 2015/495). While the watch list mechanism has covered only 20 active pharmaceutical ingredients (APIs) or their metabolites, the PHARMS-UBA database (UBA, 2021) shows that nearly 500 individual compounds have been analysed at least once within the Baltic Sea Region (BSR). Despite the increasing number of distinct pharmaceuticals analysed, most measurements originate from project-based screening campaigns, and long-term time series are lacking.

It is crucial that sampling campaigns produce quantitative, representative, and reliable information on contaminant concentrations. Contaminant loads can be estimated using representative concentration data and flow volume. Especially when assessing contaminant emissions from selected sites, or when tracking contaminant transport through e.g. a river network, care should be taken to ensure the measured concentrations reflect the desired conditions. I.e. sample representativeness is a key factor, whether estimating long term mean concentrations to help assess e.g. annual loads or capturing very short discharge events.

This background document aims to describe steps necessary to

- 1) select a compact and meaningful set of target compounds for environmental monitoring,
- 2) collect and analyse samples, so that the campaign produces useful information for risk assessment,
- 3) select most representative sampling sites and selecting sampling frequencies.

To obtain an overview of current practices in BSR countries, a questionnaire was circulated among relevant stakeholders in Finland, Germany, Latvia, Poland, and Sweden, including regional and/or national authorities in each country. Additionally, the questionnaire was circulated to all wastewater treatment plants (WWTPs) exceeding the PE limit of 10,000 in Finland. The responses to this questionnaire are summarized in this background document, and complemented by the authors' views and expertise.

Whenever carrying out sampling campaigns, their goals and target environments ultimately dictate which approach is the most appropriate. Thus, a universal, step-by-step guideline cannot be given. However, this background document aims to condense the most relevant issues into easily followable flowcharts. Topics highlighted in the flowcharts are discussed more in depth in the following chapters.

I: Selecting target compounds

1. Introduction

Selecting a relevant list of target compounds for environmental monitoring is crucial to ensure meaningful data is being produced, and to help limit the costs of the monitoring campaign. When contracting a commercial laboratory to conduct chemical analyses of pharmaceutical residues in environmental matrices, the price of a single sample may reach thousands of euros. Thus, it is important to target only chemicals considered relevant for the current study. When resources are limited, the target compounds should be prioritized so that the benefit is maximized.

According to Keith (2015), governmental organizations have a tendency to sometimes adopt regulatory substance lists without questioning the background behind the list. This is demonstrated by the list of 16 polycyclic aromatic hydrocarbons (PAH16), shortlisted by the USEPA in 1976. The list was compiled based on previous detections in US environmental samples, information on their production, and availability of analytical standards. The list was compiled as the best available list for the US but it was widely adopted by countries across the world (Keith, 2015). When writing this background document, PAH16 is still included for example in the Finnish Government Decree on the Assessment of Soil Contamination and Remediation Needs (2014/2007). The original PAH16 might not be the most relevant PAH-compounds nowadays, considering especially the huge leaps made in analytical chemistry, and subsequent detections of a wider range of compounds belonging to the group. However, PAH16 still lingers on.

To help avoid potentially redundant "legacy priority lists", it is important that regulatory authorities and legislators have an understanding of target compound selection criteria. This section aims to summarise the target compound lists in use in the project countries, and how those compounds were selected. Furthermore, we aim to present potential selection criteria to be used when selecting target compounds for monitoring campaigns. A proposal for target compounds will be compiled based on these criteria.

2. Current practices

Based on the questionnaire and other relevant information from each country, all countries have some monitoring or screening campaigns in place for hazardous substances. The campaigns reported by authorities were mainly campaigns carried out in surface waters. Moreover, pharmaceuticals and/or hormones are included in at least some of these campaigns in each of these countries. Wastewater sampling campaigns were only reported by Finnish and Latvian authorities, along with Finnish WWTP operators.

On a scale of one ("not important") to five ("very important"), the most important goals for the sampling campaigns were reported as meeting regulatory obligations (mean values of 4.7 and 4.2 for authorities and WWTP operators, respectively), and producing data suitable for risk assessment (mean values of 4.3 and 3.5 for authorities and WWTP operators, respectively). Authorities also considered pinpointing emission sources important, especially outside Finland. Furthermore, Finnish WWTP operators considered assessing substance mass flows equally as important as producing data suitable for risk assessment.

When asked about how the target compounds were selected, risks to the environment, prioritization in regulatory lists, and requirements set in legislation were the three most commonly selected options among the respondents. Prioritization in regulatory lists was clarified to refer to e.g., the WFD watchlist, and national priority lists (VNA 1022/2006).

Other popularly stated selection criteria included hazard properties, and previous detections in environmental samples. High sales or usage of the substance was commonly selected among authorities outside Finland. Risks to human health were the least popular selection criteria. Requirements set in environmental permits were only selected as a selection criterion by some Finnish authorities, and WWTP operators. This might reflect the fact that only some of the responding Finnish authorities are involved in environmental permit supervision, while others focus on surface water quality.

Other selection criteria brought up in the questionnaire responses included HELCOM and NORMAN network databases, presumably indicating that occurrence data is being compiled from international databases, to help select the most relevant target compounds. Furthermore, national statistics on pharmaceutical consumption and production were mentioned as useful resources. The costs of analyses were used as selection criterion by one respondent, presumably indicating that compounds considered less relevant are being excluded due to limited resources.

The questionnaire responses listed numerous APIs that had been included in at least one screening or monitoring campaign. The APIs reported as having been analysed in at least one campaign in at least two partner countries are presented in Table A1. In addition to listing specific APIs, some responses listed substance groups that had been represented in their analyses. Furthermore, respondents occasionally included references to scientific papers or reports, where more information is available.

Table A1. Individual substances reported in the responses been included in monitoring or screening campaigns.

Compound		Country and type of sampling campaign ¹⁾			
Generic name	CAS	DE	FI	LV	SE
Carbamazepine	298-46-4	WW-M, WW-S	RW-M, SW-M, WW-M, WW-S, SW-S	WW-S, SW-M, SW-S	SW-M
Clarithromycin	81103-11-9	WW-M, WW-S	SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
Diclofenac	15307-86-5	WW-M, WW-S	WW-S, SW-M, WW-M, SW-S	SW-M, WW-S, SW-S	SW-M
Sulfamethoxazole	723-46-6	WW-M, WW-S	RW-M, SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
17-α-ethinyl estradiol (EE2)	57-63-6		WW-S, SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
17-β-estradiol (E2)	50-28-2		RW-M, WW-S, SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
Atenolol	29122-68-7		WW-S	WW-S	SW-M
Azithromycin	83905-01-5		WW-S, SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
Ciprofloxacin	85721-33-1		SW-M, SW-S	WW-S, SW-M, SW-S	SW-M
Erythromycin	114-07-8		SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
Ibuprofen	15687-27-1		SW-M, WW-M, WW-S, SW-S	SW-M, WW-S, SW-S	SW-M
Metoprolol	51384-51-1	WW-M, WW-S		WW-S, SW-M, SW-S	SW-M
Trimethoprim	738-70-5		SW-M, SW-S	SW-M, WW-S, SW-S	SW-M
Venlafaxine	93413-69-5		SW-M, SW-S	SW-M, WW-S, SW-S	SW-M

Compound		Country and type of sampling campaign ¹⁾			
Generic name	CAS	DE	FI	LV	SE
Amidotrizoate	117-96-4	WW-M, WW-S	WW-S		
Amoxicillin	26787-78-0		SW-M, WW-S, SW-S	SW-M, WW-S, SW-S	
Ampicillin	69-53-4		WW-S	SW-M, WW-S, SW-S	
Atorvastatin	134523-00-5		WW-S	SW-M, WW-S, SW-S	
Doxycycline	564-25-0		WW-S	SW-M, WW-S, SW-S	
Enrofloxacin	93106-60-6		WW-S	SW-M, WW-S, SW-S	
Estrone (E1)	53-16-7		WW-S, SW-M, SW-S	SW-M, WW-S, SW-S	
Fluconazole	86386-73-4		SW-M, SW-S		SW-M
Losartan	114798-26-4			WW-S	SW-M
Metformin	657-24-9		SW-M, SW-S	SW-M, WW-S, SW-S	
Naproxen	22204-53-1			SW-M, WW-S	SW-M
O-desmethyl-venlafaxine	93413-62-8		SW-M, SW-S	SW-M, WW-S, SW-S	
Paracetamol	103-90-2			SW-M, WW-S, SW-S	SW-M
Propranolol	525-66-6			WW-S	SW-M

¹⁾ WW-M = Wastewater monitoring; WW-S = Wastewater screening; RW-M = Raw water intake monitoring; SW-M = Surface water monitoring; SW-S = Surface water screening

In addition to target compounds, several respondents reported measuring other variables as well. These included i.e. pH, electric conductivity, COD, BOD, nutrients, and heavy metals. Some WWTP-operators also reported monitoring recreational drugs and Covid-19 in wastewaters.

3. Criteria to be considered when selecting target compounds

3.1. Regulatory requirements

Regulatory requirements are commonly used to guide compound selection targets. Including a substance on any regulatory list implies that a significant amount of work has already been carried out, often with resources outside the reach of regional or national authorities. These substance lists usually contain a limited number of well justified compounds, backed by expert knowledge. Some regulatory substance lists, such as the WFD priority substances, are based on the identified risks (e.g. WFD priority substances), while others, such as the WFD watch lists, reflect knowledge gaps. The pharmaceuticals listed in European regulatory lists at the time of writing this background document are presented in Table A2.

Table A2. Pharmaceuticals listed in EU-wide or national regulatory lists

Name of API	CAS No.	UWWTD proposal (EC, 2022a)	EQS proposal (Council of the European Commission, 2024)	GW EQS proposal (EC, 2024)	WL1 (2015/495)	WL2 (2018/840)	WL3 (2020/1161)	WL4 (2022/1307)	Swedish RBSP (HVMFS 2019:25)
17 α -ethinylestradiol (EE2)	57-63-6		X		X	X			X
17 β -estradiol (E2)	50-28-2		X		X	X			X
Amisulpride	71675-85-9	X							
Amoxicillin	26787-78-0					X	X		
Azithromycin	83905-01-5		X		X	X			
Candesartan	139481-59-7	X							
Carbamazepine	298-46-4	X	X	X					
Ciprofloxacin	85721-33-1					X	X		X
Citalopram	59729-33-8	X							
Clarithromycin	81103-11-9	X	X		X	X			
Clindamycin	18323-44-9							X	
Clotrimazole	23593-75-1						X	X	
Diclofenac	15307-86-5	X	X		X				X
Erythromycin	114-07-8		X		X	X			
Estrone (E1)	53-16-7		X		X	X			
Fluconazole	86386-73-4						X	X	
Guanylfurea	141-83-3							X	
Hydrochlorothiazide	58-93-5	X							
Ibuprofen	15687-27-1		X						
Irbesartan	138402-11-6	X							
Metformin	657-24-9							X	
Metoprolol	51384-51-1	X							
Miconazole	22916-47-8						X	X	
O-desmethyl- venlafaxine	93413-62-8						X	X	
Ofloxacin	82419-36-1							X	
Primidone	125-33-7			X					
Sulfamethoxazole	723-46-6			X			X	X	
Triclosan	3380-34-5		X						X
Trimethoprim	738-70-5						X	X	
Venlafaxine	93413-69-5	X					X		

When utilizing regulatory substance lists as selection criteria for target compounds, the nature of the list and the selection criteria used to compile that list should be taken into consideration. Some existing substance lists are outlined in Information box 1. Unless the goals of the sampling campaign demand otherwise, risk-causing substances (e.g. WFD priority substances) should be given highest priority, followed by substances requiring further information (e.g. WFD watch lists). It could be argued that substances required to be monitored as indicator substances for WWTP process compliance assessment (e.g. UWWTD indicator substances) are not relevant for environmental monitoring. However, such substances are likely to have relatively vast datasets of monitoring data in WWTP matrices. Thus, including them into environmental

monitoring can be resource efficient, and may help produce insights on other APIs as well. Considering the compound lists summarised in Information box 1, the substance lists should be prioritized as follows: WFD priority substances > WFD watch lists > UWWTD indicator substance list.

Information box 1. Some regulatory lists present at the EU-level.

EU WFD priority substances

This list contains compounds that have been identified to cause an EU-wide risk to waters. They should have good quality analytical methods available, and reliable threshold values to help assess whether a concentration is high or low. Each priority substance has been given an environmental quality standard, which is a legally binding threshold value that should not be exceeded in surface waters. A compound listed as a WFD priority list can be considered an ecotoxicologically relevant risk substance.

EU WFD watch list substances

The WFD watch list mechanism aims to produce new, high-quality information in an EU-scale on compounds that have been identified as potential risk substances, but for which there is not enough information on occurrence. The watch list is to be updated every two years, and the number of compounds on the list is limited. A compound listed on a WFD watch list has been identified as a substance of potential concern, but for which more information is required to draw more robust conclusions.

UWWTD indicator substance

The indicator substances were introduced into the UWWTD in the 2022 directive proposal (EC, 2022b). They are not compounds identified as posing especially high risks or hazards, but indicator substances used to assess whether the quaternary treatment removal requirements set for micropollutants is achieved. The list of indicator substances is not a compound list tailored for the EU, but a list originally compiled for a similar piece of legislation in Switzerland (e.g. Götz et al, 2015). A compound listed as an UWWTD indicator substance has not been prioritized based on risks, hazards, occurrence, etc., nor has it been identified as a substance requiring further information. Instead, it is an indicator substance to be used to assess overall micropollutant removal efficiency.

Legally binding regulatory requirements, such as the WFD priority substances, often focus on well-known compounds that are already shown to be harmful. This might result in neglecting emerging compounds or compounds for which reliable chemical analytics are not yet available. Moreover, regulatory requirements set in e.g., EU legislation may overlook national characteristics. Thus, if APIs are listed as river basin-specific pollutants (RBSP), those substances should be considered at the same level of urgency as WFD priority substances. At the time of writing this background document, Sweden is the only country in the BSR, with APIs recognized as RBSP. However, Mehtonen et al. (2023) proposed 17 α -ethinylestradiol, estrone, ibuprofen, and diclofenac as Finnish RBSP. Furthermore, Braun et al. (2022) have proposed a minimum list of compounds, including several APIs, that should be included into wastewater monitoring.

Compound lists set in EU and national regulation should be used as the first step for selecting target compounds. If resources allow including a wider set of compounds, further criteria should be considered.

3.2. Risks

In general, contaminants are being monitored to help assess if they cause adverse effects in the environment. Thus, risks should be considered a primary selection criterion for target substances. However, as risks are often already incorporated into selection criteria in regulatory substance lists, those lists can be used as an easily accessible first step.

Chemical risks are commonly defined as the product of exposure-response information acquired from toxicological analysis, and exposure to the chemical. Toxicity of chemicals on different organisms varies. A common approach to assess risk potential is to calculate a risk quotient (RQ) using Eq. 1. RQ exceeding one indicates risk. As the ecotoxicological tests used to derive the PNEC-value may involve different end-points, an increase in RQ should not be interpreted as more severe adverse effects taking place, but a higher likelihood of adverse effects.

Eq. 1
$$RQ = \frac{C}{PNEC}$$

,where
RQ = Risk quotient,
C = Concentration in the relevant compartment (mass/volume),
PNEC = Predicted no-effect concentration in the relevant compartment (mass/volume)

When calculating RQ, predicted (PEC) or measured environmental concentrations (MEC) are commonly used. PECs are often especially useful, when selecting APIs for sampling campaigns (see Chapter 3.3).

If the compound is very persistent, the likelihood of an organism being exposed to it increases. Similarly, if the compound has the capacity to bioaccumulate, there is a risk of secondary poisoning, if a predator consumes lower trophic level organism containing increased levels of the compound. Incorporating persistence and bioaccumulation into the RQ-calculation requires defining a more specific exposure scenario.

3.3. Consumption

APIs are primarily discharged into the environment following their appropriate use. Thus, pharmaceutical sales can help identify APIs of highest concern. Pharmaceutical sales statistics can be useful in identifying risk-causing compounds, or compounds with trends in their consumption. Pharmaceutical sales statistics may be used to estimate API concentrations in wastewaters or the environment. These predicted environmental concentrations (PECs) can be compared to ecotoxicological threshold values (e.g. PNECs) or to regulatory threshold values (e.g. EQS), to help identify compounds of concern. Variations of Eq. 2 have been proposed by e.g. EMA (2006), Äystö et al. (2020), and Kisielius et al. (2024).

Eq. 2
$$PEC = \frac{\left(\frac{M_{pc}}{V}\right)}{D}$$

,where
PEC = Predicted environmental concentration,
 M_{pc} = Per capita API sales [mg/(d*person)],
V = Volume of generated wastewater [L/(d*person)],
D = Dilution factor [-]

When calculating PECs from sales statistics, the chemical form referred in the statistics should be noted. In many cases, the statistics refer to the chemical form present in pharmaceutical preparations (e.g. salt form). For instance, metoprolol sales are commonly reported as metoprolol succinate, while chemical analyses usually target the active substance metoprolol. Thus, M_{pc} in Eq. 2 should be replaced with sales as the active molecule, m_{act} , using Eq. 3, as proposed by Kisielius et al. (2024).

Eq. 3
$$m_{act} = \frac{(n_{act} \times m)_{prep} \times M_{act}}{M_{prep}}$$
, where
 m_{act} = Mass of the active substance ([kg/year],
 n_{act} = Number of active moieties released from the preparation form [-],
 m_{prep} = mass of sold preparation form [kg/year],
 M_{act} = Molar mass of the active form [g/mol],
 M_{prep} = Molar mass of the preparation form [g/mol]

Eq. 2 assumes that all sales eventually end up in the wastewaters or environment. Thus, it is likely to give an overestimate of actual concentrations. Including relevant removal processes (e.g. metabolism and removal in wastewater treatment) (see. Eq. 4) have been shown to improve the correlation between measured and estimated concentrations (e.g. Kisielius et al., 2024).

Eq. 4
$$PEC_{eff-adj} = \frac{M_{pc} \times E \times (1 - RR)}{V}$$
, where
 $PEC_{eff-adj}$ = Adjusted predicted concentration in WWTP effluent
 E = API-specific fraction of used API, excreted as the active form (in ratios, 1 equalling 100%),
 RR = API-specific removal rate at WWTPs (in ratios, 1 equalling 100%)

Pharmaceutical sales statistics are often available on a rough temporal and regional scale, e.g., one year for the whole country. Thus, when PECs are calculated using this data, the resulting values represent annual national averages.

If pharmaceutical sales are not distributed uniformly across time and space, the annual average PEC may be an over- or underestimate. In Finland, weekly sales statistics, divided by hospital district, are available from 2019 onwards (Kainu et al., 2023). The dataset contains information on the monetary value of prescription pharmaceutical sales, along with the number of purchases and the number of persons making the purchases. While this dataset does not allow estimating sold masses or PECs, it gives indication on the regional and temporal distribution of the sales.

Sales data can be aggregated using e.g. the Anatomical Therapeutic Chemical Classification System (ATC). The World Health Organization has assigned ATC codes for each API (WHO, 2024). The ATC system is structured hierarchically into five levels: the first level denotes the main anatomical group (e.g., cardiovascular or respiratory systems), and subsequent levels specify therapeutic subgroups, chemical properties, and the substance itself. When aggregating the sales data on a rough scale (ATC level 2), some groups are highlighted. These include e.g. antihistamines for systemic use (R06). These sales are presented in Figure A1. Based on weekly information on the value of sales in Finland, more than 50 % of the costs related to ATC class R06 purchases are generated between March and June. Assuming that the pharmaceuticals are consumed around the same time, the emissions into wastewaters and the environment can reasonably be estimated to be 1.5-fold compared to the annual average PEC, calculated using only annual sales statistics.

A similar pattern is exhibited by e.g., pharmaceuticals falling into ATC class S01GX ("Other antiallergics"). The pattern is especially clear for Olopatadine (S01GX09) (see Figure A2). The compound is on the Finnish market only as a prescription product. Thus, the dataset can be considered to give a reliable estimate of the overall temporal distribution of the sales of the API in Finland. The concentrations in wastewaters and the environment can thus be assumed considerably higher in spring/summer months than in the fall and winter.

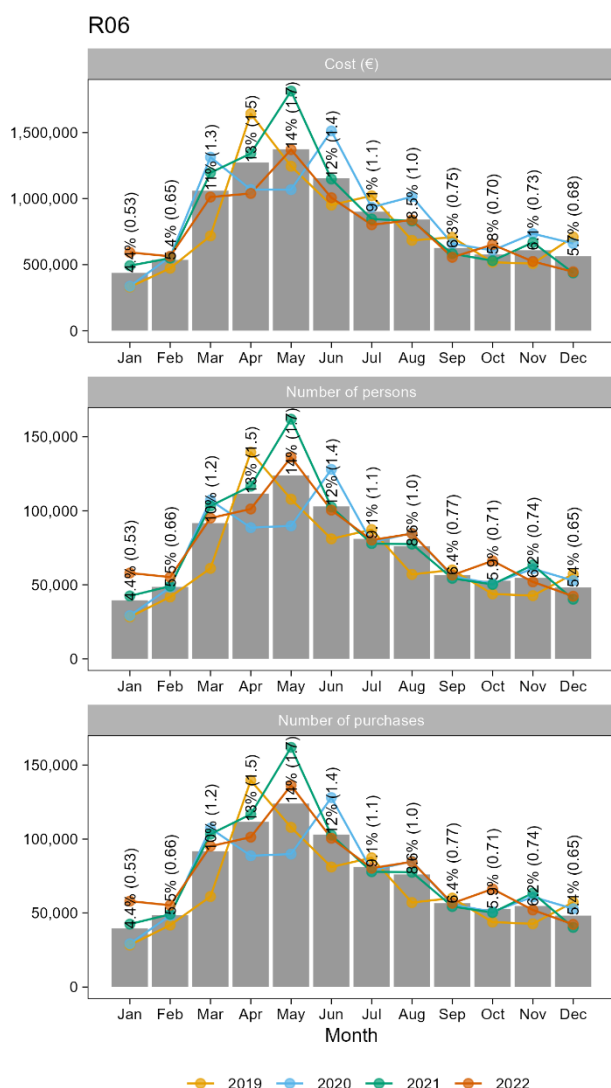


Figure A1. Sales for ATC group R06 (Antihistamines for systemic use) for the years 2019–2022. The bars show mean monthly sales for the four years. The labels in the columns shows the percentage of annual sales, and the value in parentheses the theoretical multiplier for the annual average PEC value.

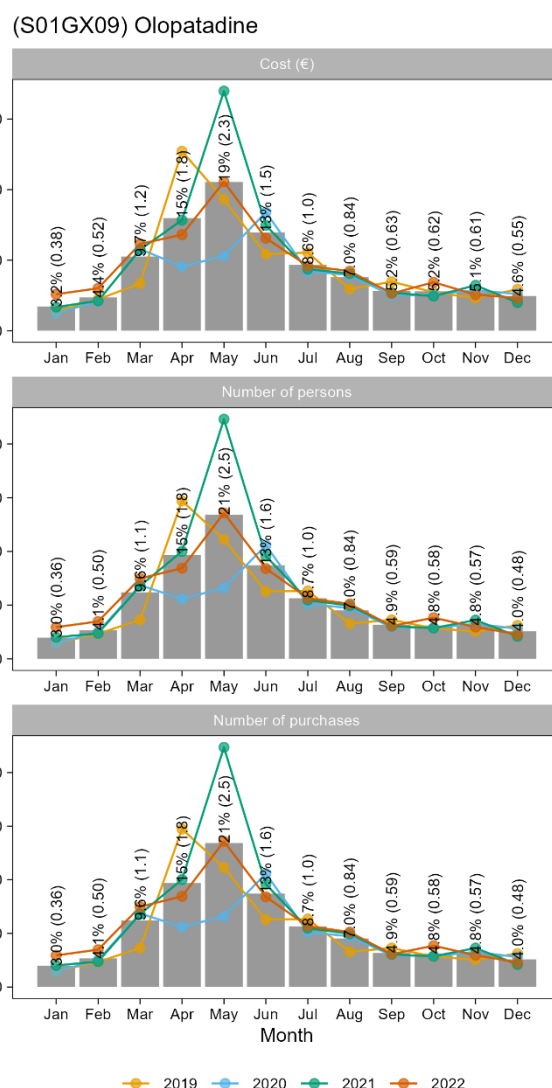


Figure A2. Sales for olopatadine for the years 2019–2022. The bars show mean monthly sales for the four years. The labels in the columns shows the percentage of annual sales, and the value in parentheses the theoretical multiplier for the annual average PEC value.

Seasonal trends are clear for only a limited number of APIs. For instance, the sales value of ATC class J01 (antibacterials for systemic use) does not show any clear seasonal pattern. While seasonal trends are present for only some APIs, sales statistics with higher temporal resolution also make it easier to detect long-term trends. For instance, the monetary value of sales of ATC class J06BA01 (immunoglobulins) exhibits a clear increasing trend in Finland (see Figure A3). Similarly, cefuroxime (J01DC02) shows a clear decreasing trend through the 4-year time frame (Figure A4).

While increased temporal resolution would allow for more refined PECs, such information might have its caveats. For instance, the Finnish weekly dataset only covers prescription medicines. Furthermore, the statistics are only available as monetary value and in the numbers of purchases and people, limiting their utility. Perhaps most importantly, the dataset does not cover all preparations. For instance, diclofenac is only reported for ATC codes M01AB05 and M01AB55, i.e., excluding topical preparations (M02AA15), which has been reported to account for over two thirds of diclofenac sales in Finland and Denmark (Kisieliu et al. 2024).

Whenever using pharmaceutical sales statistics for planning environmental monitoring programs, special considerations must be given to the coverage and reliability of the dataset. Previous studies (e.g. Kisieliu et al. 2024) have shown annual average PECs to correlate reasonably well with MECs when relevant removal processes are taken into consideration. Whenever sales statistics are available in high temporal or spatial resolution, they should be looked into for refining PEC. This should be done especially whenever PEC and MEC show a discrepancy that might be explained by temporal variation in sales. However, it should be considered that pharmaceutical consumption might be spread across a long time after the sales. I.e., all pharmaceuticals sold in April are not used in April. Sales statistics with high temporal resolution could be utilized in prioritizing compounds. A clear increasing trend in a potentially hazardous API should be considered a basis for considering its inclusion to environmental monitoring programs.

3.4. Analytical capabilities

For any compound to be included into monitoring programs, it must have reliable analytical methods available. If the limit of quantification (LOQ) is higher than the relevant ecotoxicological or regulatory threshold values, or if the LOQ is higher than actual environmental levels, the analytical results may give very little insight. Similarly, if the measurement uncertainty is too high, results cannot be used reliably.

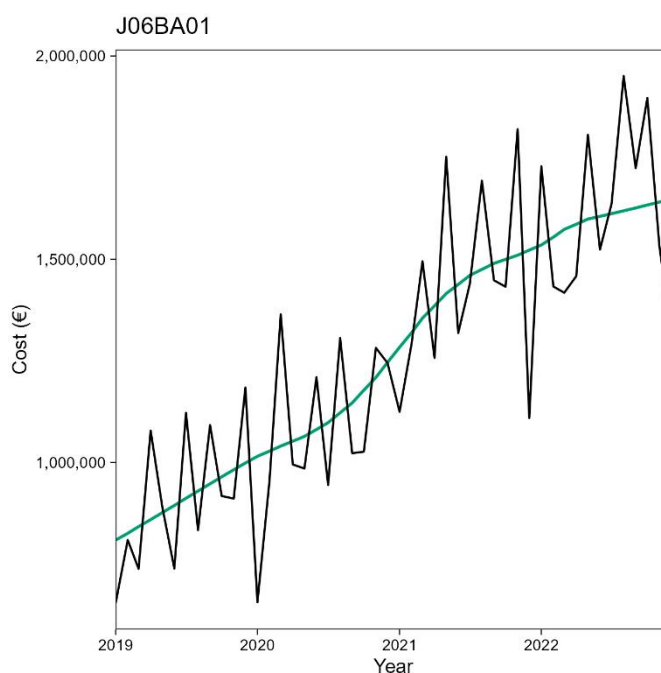


Figure A3. Sales of J06BA01 in Finland in 2019–2022. The green line presents the trend.

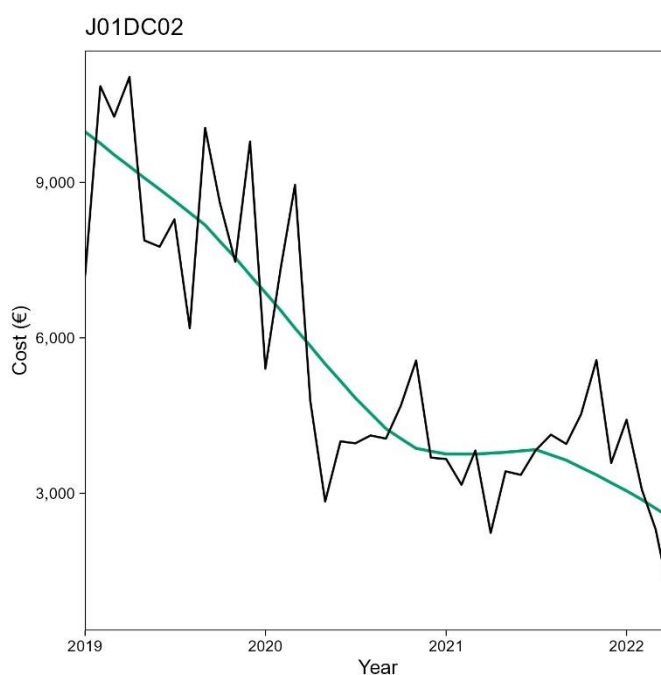


Figure A4. The value of sales of cefuroxime in Finland in 2019–2022. The green line presents the trend.

Measurement uncertainties, as reported by commercial laboratories for pharmaceutical concentrations in surface waters or wastewaters is often around 40%.

Commission directive 2009/90/EC laying down, pursuant to Directive 2000/60/EC of the European Parliament and of the Council, technical specifications for chemical analysis and monitoring of water status (31.07.2009) determines the requirements for the performance parameters of the analytical methods used for chemical analyses. Member States shall ensure that the minimum performance criteria for all methods of analysis applied are based on an uncertainty of measurement of 50 % or below ($k = 2$) estimated at the level of relevant EQS and a limit of quantification equal or below a value of 30 % of the relevant EQS. In the absence of relevant EQS for a given parameter, or in the absence of method of analysis meeting the minimum performance criteria, Member States shall ensure that monitoring is carried out using best available techniques not entailing excessive costs.

While including compounds for which adequately low LOQs are not yet available might not be good use of resources, it is important to highlight the relevance of these compounds to laboratories. This may help commercial operators develop their analytical methods towards a direction that helps produce the most benefit for future monitoring activities.

3.5. Previous results

When selecting target compounds, previous results are useful from at least three perspectives:

- a) They help identify compounds that are most commonly present in the matrix in question,
- b) They help assess if existing analytical methods have adequately low LOQs,
- c) When paired with ecotoxicological or regulatory threshold values, they help identify the compounds of highest concern.

Contaminants present in the environment in concentrations exceeding respective LOQs, should be considered for inclusion into monitoring. Furthermore, compounds for which the LOQs are not sufficiently low for assessing risks, should be considered as well.

Previously, schemes for selecting substances for WWTP monitoring, relying heavily on existing data, have been proposed by e.g. Brückner et al. (2020). The approach suggested by the authors requires a pre-study being carried out, based on which each analyte is categorized and possibly selected as indicator substance. The framework primarily aims to select substances for assessing the impact of individual WWTPs to their recipient water bodies.

The approach proposed by Brückner et al. (2020) does not rely only on the analyte being detected but requires the mean concentration to exceed the LOQ threefold. This is because concentrations very close to the LOQ are comparatively uncertain. Thus, for instance, when comparing such concentrations to ecotoxicological threshold values, there is a higher risk of a false risk signal. However, as authorities carrying out environmental monitoring rarely have the luxury of carrying out site-specific pre-studies, we propose excluding this filtering step. If there is a significant number of previous results from the monitoring area, including this filter should be considered.

When using previous results as a selection criterion, it is beneficial to utilize databases on chemical occurrence. National databases should be given highest priority if they exist. However, international databases, such as the PHARMS-UBA database (UBA, 2021) can be considered to give a wider overview. At the time of writing this background document, the database contains measured concentrations from Baltic

Sea coastal countries for nearly 500 individual APIs. Figure A5 shows the number of datapoints for all APIs with at least 100 datapoints from the Baltic Sea coastal countries.

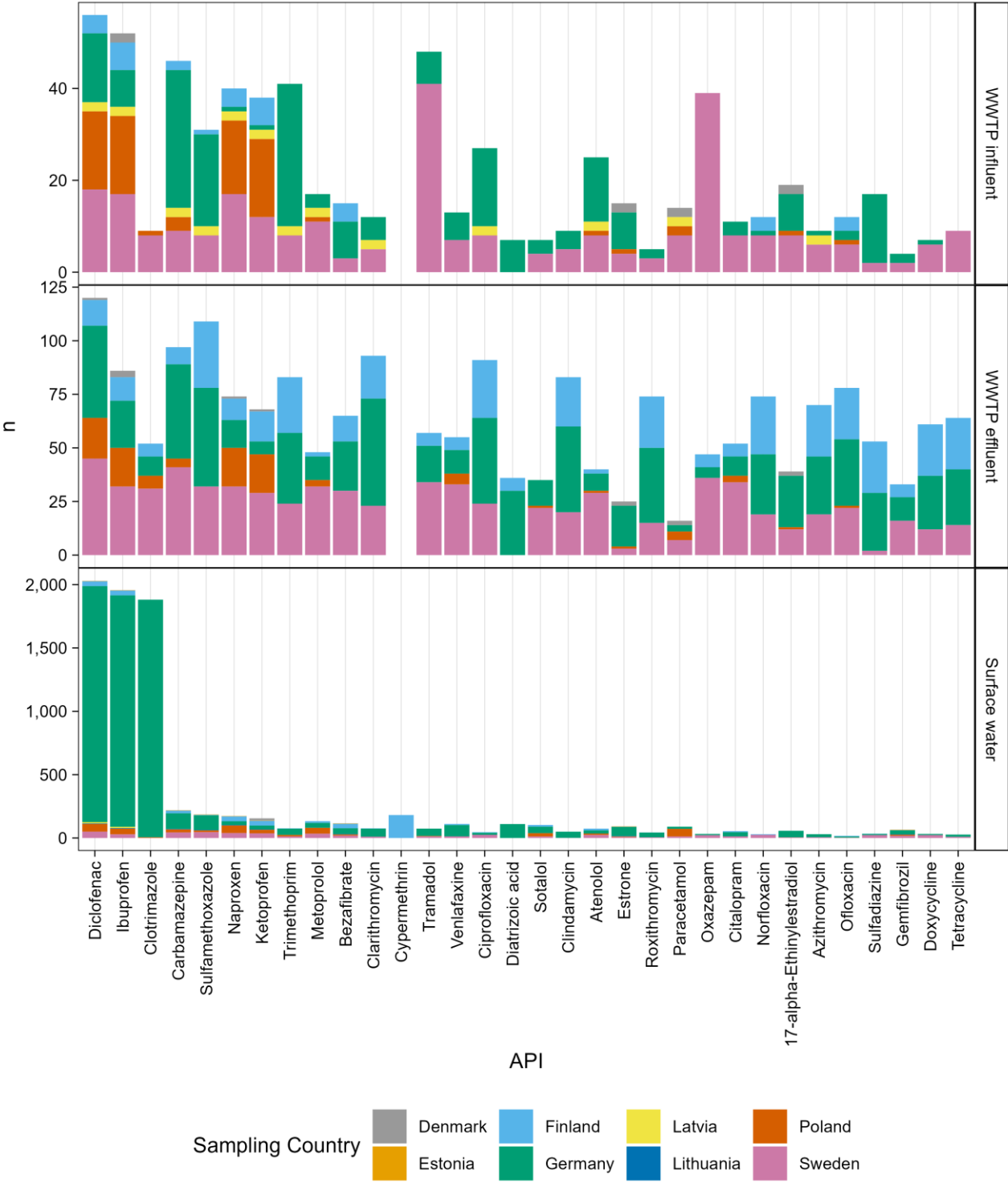
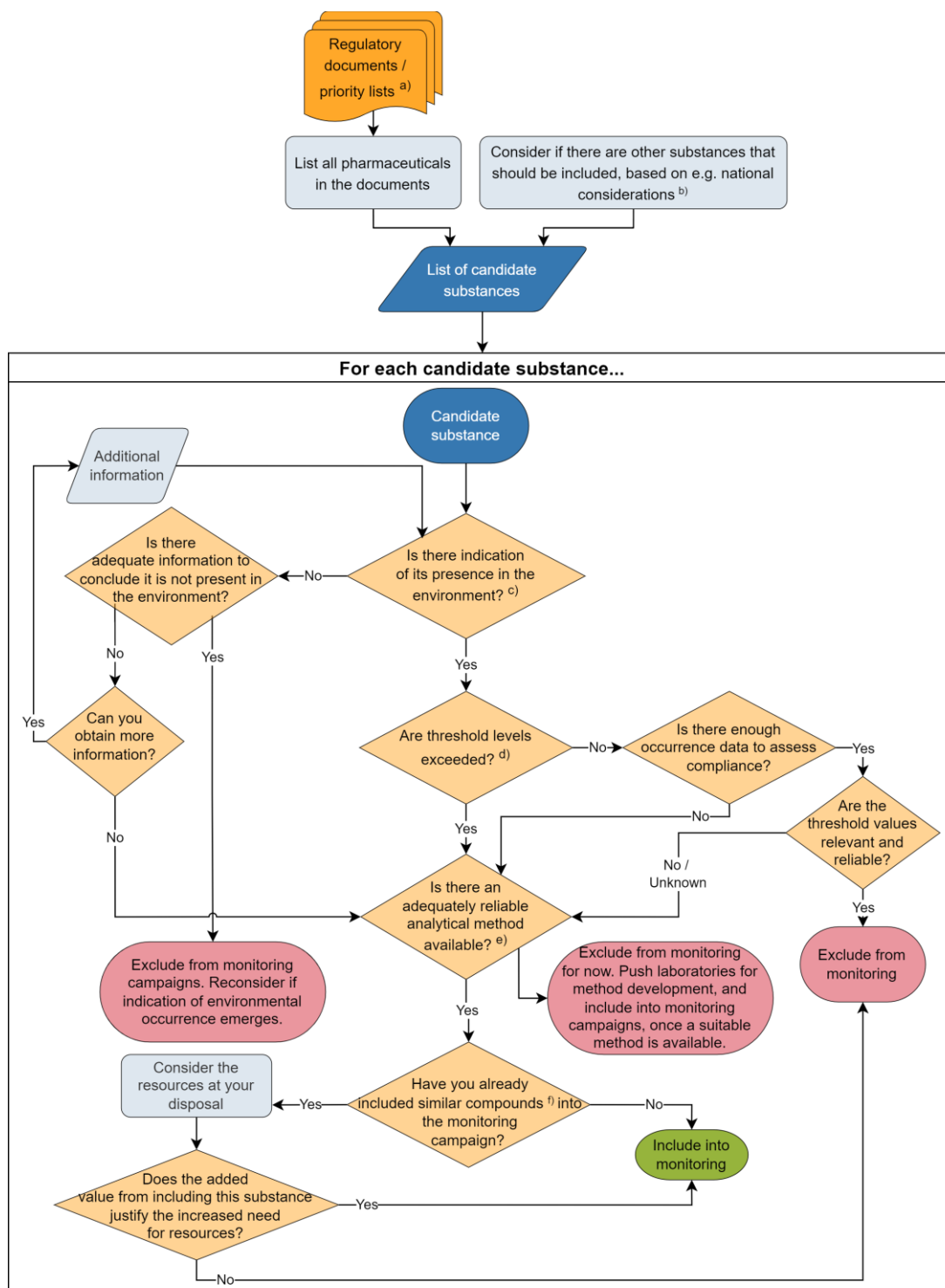


Figure A5. The number of datapoints in the PHARMS-UBA database, per API and matrix.

4. Proposal for a parameter list

4.1. Rationale for proposed parameters

The aim of the proposed parameter list is to present a compact and meaningful list of target parameters. The selection approach followed the rationale and considerations presented in Chapter 3. Considering that regulatory requirements are usually based on criteria such as risks, consumption statistics and previous results, it should be the first criteria to be considered when selecting substances for monitoring campaigns. However, when resources are limiting, the list can be condensed by selecting only the substances that meet the criteria presented in Figure A6. Furthermore, it should be considered, if regional or national characteristics give grounds for including additional substances.



a) E.g. EU regulatory lists (e.g. WFD priority substances), or HELCOM priority substances, etc.

b) These considerations could be e.g. national/regional consumption patterns.

c) Indication could be e.g. previous detections, or occurrence predicted using sales statistics.

d) Threshold levels could be e.g. ecotoxicological thresholds, such as predicted no-effect concentrations (PNEC), or regulatory guidance values such as environmental quality standards (EQS). Ecotoxicological threshold values are available e.g. via the NORMAN network. EQS values should be used, whenever they are established.

e) The analytical method should have a limit of quantification (LOQ) lower than relevant threshold values, and lower than expected concentrations in the matrix to be monitored. Furthermore, the method should produce reliable and robust results. However, often even less robust analytical results will help make further assessments.

f) The similarity could be expressed e.g. as substances falling within the same usage category (e.g. antibiotic), or chemical properties. It may be reasonable to avoid selecting multiple similar compounds, that can be assumed to be discharged in similar quantities, and to behave in similar ways, especially if resources are limited. Whenever there are multiple similar compounds you have to choose from, the choices should be justified. For instance, you could select the substance exhibiting the highest toxicity.

Figure A6. Rationale for selecting the proposed target compounds.

4.2. Selection process

4.2.1. Regulatory documents

The pharmaceuticals listed in relevant EU legislation, along with national legislation and other documents were compiled. The EU legislation covered the UWWTD proposal from October 2022 (EC, 2022a), WFD EQS proposal (Council of the European Commission, 2024), groundwater EQS proposal (Council of the European Commission, 2024), and WFD watchlists 1–4 (2015/495, 2018/840, 2020/1161, 2022/1307). Only one project member state was identified to have binding legislation related to pharmaceuticals in the environment, namely Sweden with HVMFS 2019:25. Furthermore, the Finnish proposal for river-basin specific pollutants (Mehtonen et al. 2023) and the German proposal for micropollutants to be monitored in wastewater effluents (Braun et al., 2022) were considered relevant documents. Additionally, two German documents were taken into consideration, focusing on pharmaceutical occurrence in river waters in relation to drinking water quality standards (ERM 2020), and whether or not drinking water quality standards were set. This resulted in altogether 72 pharmaceuticals. However, as the goal was to compile a condensed list of target compounds, only the 30 compounds identified in any of the EU documents were selected for further processing.

4.2.2. Indication of presence in the environment

To assess whether there is indication of the presence of the 30 APIs in the BSR environment, measurements reported in the PHARMS-UBA database were compiled. Only the datapoints originating from Baltic Sea coastal countries were selected, and grouped into three broad matrices: surface waters, WWTP effluents, and WWTP influents. The occurrence data is presented in Table A3.

For each of the 30 APIs, there is at least some indication of their presence in the environment. Amisulpride was the only API for which no data was reported for surface waters, while all other compounds were detected in at least one sample. Furthermore, most APIs were also detected in WWTP influent and effluent, highlighting their inputs into the environment. Thus, all 30 substances can be assumed to be present in the BSR environment.

Table A3. Concentration data available for the APIs listed in EU regulatory documents, as reported in PHARMS-UBA database. Mean values were calculated using the approaches presented in Section III, Chapter 3.4. Censoring frequency presents the frequency of non-detects in the dataset.

API	CAS number	Surface water			WWTP effluent			WWTP influent		
		n	Censoring frequency [%]	Mean (SD) [µg/l]	n	Censoring frequency [%]	Mean (SD) [µg/l]	n	Censoring frequency [%]	Mean (SD) [µg/l]
17α-ethinylestradiol (EE2)	57-63-6	57	33.3%	0.0081 (0.018) ¹⁾	39	46.2%	0.034 (0.097) ¹⁾	19	42.1%	0.15 (0.43) ¹⁾
17β-estradiol (E2)	50-28-2	56	83.9%	- (-) ²⁾	33	48.5%	0.0061 (0.013) ¹⁾	18	22.2%	0.047 (0.099) ¹⁾
Amisulpride	71675-85-9	0	-	- (-)	2	0.0%	0.16 (0.078) ³⁾	0	-	- (-)
Amoxicillin	26787-78-0	17	82.4%	- (-) ⁴⁾	53	98.1%	- (-) ⁵⁾	1	0.0%	1.3 (-) ⁶⁾
Azithromycin	83905-01-5	30	13.3%	0.073 (0.11) ¹⁾	70	25.7%	0.13 (0.18) ¹⁾	9	11.1%	0.14 (0.31) ⁷⁾
Candesartan	139481-59-7	1	0.0%	1.1 (-) ⁶⁾	2	0.0%	1.4 (0.82) ³⁾	2	0.0%	1.5 (0.74) ³⁾
Carbamazepine	298-46-4	220	12.3%	0.35 (1.6) ¹⁾	97	4.1%	1.4 (3.0) ¹⁾	46	32.6%	1.1 (2.0) ⁷⁾
Ciprofloxacin	85721-33-1	46	73.9%	0.034 (0.070) ¹⁾	91	26.4%	0.35 (1.0) ¹⁾	27	0.0%	0.80 (0.91) ³⁾
Citalopram	59729-33-8	54	9.3%	0.025 (0.052) ¹⁾	52	0.0%	0.10 (0.13) ³⁾	11	0.0%	0.18 (0.30) ³⁾
Clarithromycin	81103-11-9	76	11.8%	0.13 (0.66) ¹⁾	93	34.4%	0.18 (0.62) ¹⁾	12	8.3%	0.21 (0.44) ⁷⁾
Clindamycin	18323-44-9	50	20.0%	0.051 (0.065) ¹⁾	83	14.5%	0.094 (0.14) ¹⁾	9	11.1%	0.067 (0.087) ⁷⁾
Clotrimazole	23593-75-1	1881	98.4%	- (-) ⁸⁾	52	42.3%	0.011 (0.028) ¹⁾	9	66.7%	0.0096 (0.021) ⁷⁾
Diclofenac	15307-86-5	2030	28.7%	0.20 (2.5) ¹⁾	120	12.5%	1.6 (3.3) ¹⁾	56	7.1%	2.1 (4.9) ⁷⁾
Erythromycin	114-07-8	46	19.6%	0.021 (0.025) ¹⁾	12	0.0%	0.20 (0.20) ³⁾	4	0.0%	0.56 (1.0) ³⁾
Estrone (E1)	53-16-7	93	57.0%	0.00097 (0.0028) ¹⁾	25	36.0%	0.026 (0.050) ¹⁾	15	13.3%	0.33 (0.86) ¹⁾
Fluconazole	86386-73-4	14	85.7%	- (-) ⁹⁾	50	8.0%	0.12 (0.18) ¹⁾	13	30.8%	0.22 (0.57) ⁷⁾
Guanyurea	141-83-3	18	0.0%	2.3 (1.4) ³⁾	10	0.0%	52 (53) ³⁾	0	-	- (-)
Hydrochlorothiazide	58-93-5	31	25.8%	0.29 (0.51) ¹⁾	14	7.1%	1.7 (1.9) ⁷⁾	7	0.0%	2.1 (2.6) ³⁾
Ibuprofen	15687-27-1	1957	36.6%	0.35 (8.3) ¹⁾	86	31.4%	1.9 (6.6) ¹⁾	52	1.9%	14 (18) ⁷⁾
Irbesartan	138402-11-6	15	46.7%	0.29 (0.18) ⁷⁾	44	0.0%	0.30 (0.57) ³⁾	7	0.0%	1.3 (1.8) ³⁾
Metformin	657-24-9	42	2.4%	0.54 (0.44) ⁷⁾	16	0.0%	3.0 (5.5) ³⁾	18	0.0%	150 (81) ³⁾
Metoprolol	37350-58-6	133	30.1%	0.16 (0.37) ¹⁾	48	2.1%	1.4 (2.1) ⁷⁾	17	5.9%	1.8 (2.6) ⁷⁾
Miconazole	22916-47-8	1	0.0%	0.019 (-) ⁶⁾	39	66.7%	0.011 (0.017) ¹⁾	7	85.7%	- (-) ¹⁰⁾

API	CAS number	Surface water			WWTP effluent			WWTP influent		
		n	Censoring frequency [%]	Mean (SD) [µg/l]	n	Censoring frequency [%]	Mean (SD) [µg/l]	n	Censoring frequency [%]	Mean (SD) [µg/l]
O-desmethylvenlafaxine	93413-62-8	79	16.5%	0.11 (0.16) ¹⁾	7	28.6%	0.58 (0.16) ⁷⁾	7	28.6%	0.50 (0.20) ⁷⁾
Ofloxacin	82419-36-1	17	88.2%	- (-) ¹¹⁾	78	57.7%	0.036 (0.085) ¹⁾	12	0.0%	0.15 (0.27) ³⁾
Primidone	125-33-7	20	10.0%	0.20 (0.25) ⁷⁾	10	0.0%	1.7 (2.9) ³⁾	1	0.0%	0.96 (-) ⁶⁾
Sulfamethoxazole	723-46-6	186	31.7%	0.054 (0.10) ¹⁾	109	40.4%	0.25 (0.90) ¹⁾	31	41.9%	0.53 (1.5) ⁷⁾
Triclosan	3380-34-5	21	23.8%	2.7 (7.7) ¹⁾	42	52.4%	0.15 (0.29) ¹⁾	26	19.2%	1.6 (2.1) ¹⁾
Trimethoprim	738-70-5	76	48.7%	0.033 (0.061) ¹⁾	83	12.0%	0.15 (0.17) ¹⁾	41	34.1%	0.13 (0.23) ⁷⁾
Venlafaxine	93413-69-5	111	27.9%	0.034 (0.064) ¹⁾	55	0.0%	0.20 (0.15) ³⁾	13	0.0%	0.32 (0.60) ³⁾

Data source: PHARMS-UBA database (UBA, 2021)

¹⁾ Mean type: Kaplan-Meier (KM)

²⁾ Only 9 samples (16% of samples) quantified (range of detections: 0.0001–0.0036 µg/l, LOQ range for non-detects: 0.00005–0.075 µg/l)

³⁾ Mean type: Arithmetic mean

⁴⁾ Only 3 samples (18% of samples) quantified (range of detections: 0.09 µg/l, LOQ range for non-detects: 0.0025–0.01 µg/l)

⁵⁾ Only 1 sample (1.9%) quantified (0.19 µg/l, LOQ range for non-detects: 0.0038–0.02 µg/l)

⁶⁾ Mean type: Single value

⁷⁾ Mean type: Regression on order statistics (ROS)

⁸⁾ Only 31 samples (1.6% of samples) quantified (range of detections: 0.0031–0.34 µg/l, LOQ range for non-detects: 0.002–0.005 µg/l)

⁹⁾ Only 2 samples (14% of samples) quantified (range of detections: 0.06–0.29 µg/l, LOQ for non-detects: 0.014 µg/l)

¹⁰⁾ Only 1 sample (14% of samples) quantified (0.048 µg/l, LOQ for non-detects: 0.1 µg/l)

¹¹⁾ Only 2 samples (12% of samples) quantified (range of detections: 0.0061–0.061 µg/l, LOQ range for non-detects: 0.00056–0.023 µg/l)

4.2.3. Are threshold levels exceeded?

For most of the APIs, reference concentrations are available through regulatory documents, such as EQS proposals or WFD Watch lists. For the rest of the compounds, PNECs are available in databases and literature. One of the most comprehensive compilations is the one made available by Giunchi & Poluzzi (2023). They compiled PNEC values from various databases, presenting reference concentrations for over 90,000 pharmaceuticals or their metabolites. Additional PNECs have been collected from literature by e.g. Zhou et al. (2019) and Äystö et al. (2023). Furthermore, species-sensitivity distributions (SSD) developed by Posthuma et al. (2019) could be utilized to derive PNECs. Table A4 shows reference values for the 30 substances, and a comparison between the threshold and concentrations reported in the PHARMS-UBA database.

Altogether 21 of the APIs have exceeded their reference values in surface values. The concentrations reported for four substances never exceeded their respective reference values in any of the matrices. These substances were amisulpride, hydrochlorothiazide, irbesartan, and miconazole. While no exceedances were reported for amisulpride, it should be noted that there were very few datapoints for the substance in the PHARMS-UBA dataset. Thus, the dataset cannot be considered extensive enough to allow for a robust assessment as to whether the reference concentration is exceeded.

For hydrochlorothiazide and irbesartan, no reference values derived as part of EU regulatory frameworks were available. Thus, the reference values for those compounds could be less robust than for many others. For instance, the SSD developed by Posthuma et al. (2019) for hydrochlorothiazide, would result in a somewhat lower PNEC (9.02 µg/l) than the one reported by Zhou et al. (2019), 56.2 µg/l. On the other hand, the PNEC reported by Giunchi & Poluzzi (2023) is considerably higher (1,000 µg/l). Despite the variability in the PNEC values, none of the presented values were exceeded in the UBA-PHARMS dataset. Thus, it can be considered unlikely that hydrochlorothiazide causes risk in the BSR region. The PNEC Giunchi & Poluzzi (2023) presented for irbesartan originates from the NORMAN network database, where it was considered of the highest credibility. Thus, the PNEC could be considered relatively robust, and existing data does not indicate a risk in the BSR.

According to PHARMS-UBA database, miconazole has not exceeded its reference value in any of the three matrices within the BSR. The PNEC can be considered relatively robust, as it has been derived in a WFD WL context. While there is little information in surface waters and in WWTP influents, the available data on WWTP effluents indicates that the concentrations in WWTP effluents are well below the reference value.

Based on these considerations, it would be reasonable to exclude miconazole from monitoring programs. Furthermore, excluding hydrochlorothiazide and irbesartan would be justified, as their PNECs are not exceeded. However, as there is a limited number of measurements especially for these substances, they should be included if resources allow.

Table A4. Concentrations reported in the PHARMS-UBA databases compared to reference values for each API. The APIs exceeding the threshold in at least one matrix are highlighted with red.

API	CAS number	Number of reference concentration exceedances			Reference conc. [µg/l]	Type
		Surface water	WWTP effluent	WWTP influent		
17α-ethinylestradiol (EE2)	57-63-6	38	21	11	0.0000170 ¹⁾	AA-EQS proposal
17β-estradiol (E2)	50-28-2	7	16	14	0.000180 ¹⁾	AA-EQS proposal
Amisulpride	71675-85-9	0	0	0	140 ²⁾	PNEC
Amoxicillin	26787-78-0	3	1	1	0.0780 ³⁾	PNEC (max. MDL/MQL)
Azithromycin	83905-01-5	24	45	4	0.0190 ¹⁾	AA-EQS proposal
Candesartan	139481-59-7	0	1	1	1.20 ⁴⁾	PNEC
Carbamazepine	298-46-4	2	10	7	2.50 ¹⁾	AA-EQS proposal
Ciprofloxacin	85721-33-1	7	25	25	0.0890 ³⁾	PNEC (max. MDL/MQL)
Citalopram	59729-33-8	26	47	7	0.00635 ⁵⁾	PNEC
Clarithromycin	81103-11-9	6	30	3	0.130 ¹⁾	MAC-EQS proposal
Clindamycin	18323-44-9	17	55	4	0.0440 ⁶⁾	PNEC (max. MDL/MQL)
Clotrimazole	23593-75-1	12	8	2	0.0200 ⁶⁾	PNEC (max. MDL/MQL)
Diclofenac	15307-86-5	870	89	46	0.0400 ¹⁾	AA-EQS proposal
Erythromycin	114-07-8	0	2	1	0.500 ¹⁾	AA-EQS proposal
Estrone (E1)	53-16-7	21	14	13	0.000360 ¹⁾	AA-EQS proposal
Fluconazole	86386-73-4	1	7	2	0.250 ⁶⁾	PNEC (max. MDL/MQL)
Gualynurea	141-83-3	0	3	0	100 ⁶⁾	PNEC (max. MDL/MQL)
Hydrochlorothiazide	58-93-5	0	0	0	56.2 ⁴⁾	PNEC
Ibuprofen	15687-27-1	138	23	45	0.140 ¹⁾	AA-EQS proposal
Irbesartan	138402-11-6	0	0	0	700 ²⁾	PNEC
Metformin	657-24-9	0	0	9	156 ⁶⁾	PNEC (max. MDL/MQL)
Metoprolol	37350-58-6	38	41	11	0.100 ⁵⁾	PNEC
Miconazole	22916-47-8	0	0	0	0.200 ⁶⁾	PNEC (max. MDL/MQL)
O-desmethylvenlafaxine	93413-62-8	28	5	5	0.0600 ⁶⁾	PNEC (max. MDL/MQL)
Ofloxacin	82419-36-1	1	24	7	0.0260 ⁶⁾	PNEC (max. MDL/MQL)
Primidone	125-33-7	0	1	0	9.11 ²⁾	PNEC
Sulfamethoxazole	723-46-6	21	27	9	0.100 ⁶⁾	PNEC (max. MDL/MQL)
Triclosan	3380-34-5	5	16	21	0.0200 ¹⁾	MAC-EQS proposal
Trimethoprim	738-70-5	4	35	18	0.100 ⁶⁾	PNEC (max. MDL/MQL)
Venlafaxine	93413-69-5	15	48	9	0.0600 ⁶⁾	PNEC (max. MDL/MQL)

¹⁾ EC, 2022a

²⁾ Giunchi & Poluzzi, 2023

³⁾ WL3, 2020/1161

⁴⁾ Zhou et al., 2019

⁵⁾ Äystö et al., 2023

⁶⁾ WL4, 2022/1307

4.2.4. Analytical capabilities

Each of the 30 compounds have been analysed in at least some Baltic Sea coastal countries, according to the PHARMS-UBA database. Thus, there are analytical methods suitable for them. However, reliability of the

methods, and their availability in individual countries or regions, where the monitoring program is to be implemented, should be looked into whenever selecting compounds for monitoring activities.

According to the WFD, LOQ should be at most one third of the EQS. However, for substances on the watchlist, an LOQ equal to the threshold value is considered adequate. Considering these requirements, at the time of writing this background document, there are 21 APIs with adequate analytics commercially available in Finland (see Table A5).

Table A5. Commercial availability of chemical analyses.

API	CAS number	Analytics with sufficiently low LOQ available? ¹⁾
17α-ethinylestradiol (EE2)	57-63-6	No
17β-estradiol (E2)	50-28-2	No
Amisulpride	71675-85-9	Yes
Amoxicillin	26787-78-0	No
Azithromycin	83905-01-5	No
Candesartan	139481-59-7	Yes
Carbamazepine	298-46-4	Yes
Ciprofloxacin	85721-33-1	Yes
Citalopram	59729-33-8	No
Clarithromycin	81103-11-9	Yes
Clindamycin	18323-44-9	Yes
Clotrimazole	23593-75-1	Yes
Diclofenac	15307-86-5	Yes
Erythromycin	114-07-8	No
Estrone (E1)	53-16-7	No
Fluconazole	86386-73-4	Yes
Guanylurea	141-83-3	Yes
Hydrochlorothiazide	58-93-5	Yes
Ibuprofen	15687-27-1	No
Irbesartan	138402-11-6	Yes
Metformin	657-24-9	Yes
Metoprolol	37350-58-6	Yes
Miconazole	22916-47-8	Yes
O-desmethylvenlafaxine	93413-62-8	Yes
Ofloxacin	82419-36-1	No
Primidone	125-33-7	Yes
Sulfamethoxazole	723-46-6	Yes
Triclosan	3380-34-5	Yes
Trimethoprim	738-70-5	Yes
Venlafaxine	93413-69-5	Yes

¹⁾Indicative of the situation in Finland, at the time of writing this background document

4.3. Detailed parameter list

A detailed proposal parameter list for monitoring campaigns is presented in Table A6. For the piloting activities within the APRIORA project, a subselection of ten compounds was made based on analytical capabilities within the project consortium.

Table A6. Proposed list of target compounds for monitoring campaigns. Compounds selected for piloting activities are marked with an asterisk (*).

API	CAS number	Class	ATC (level2)	Regulatory basis	Indication of presence in the environment	Indication of risk to the environment	Analytics with sufficiently low LOQ available? ¹⁾
17α-ethinylestradiol (EE2)	57-63-6	Hormonal contraceptive	G03	2), 3), 4)	Yes	Yes	No
17β-estradiol (E2)	50-28-2	Hormonal therapy	G03	2), 3), 4)	Yes	Yes	No
Amisulpride	71675-85-9	Antipsychotic	N05	5)	Yes	Cannot be excluded	Yes
Amoxicillin	26787-78-0	Antibiotic	J01	4), 6)	Yes	Yes	No
Azithromycin	83905-01-5	Antibiotic	J01	2), 3), 4)	Yes	Yes	No
Candesartan	139481-59-7	Angiotensin II receptor antagonist (ARB)	C09	5)	Yes	Yes	Yes
Carbamazepine*	298-46-4	Anticonvulsant	N03	2), 3), 5)	Yes	Yes	Yes
Ciprofloxacin	85721-33-1	Antibiotic	J01	4), 6)	Yes	Yes	Yes
Citalopram	59729-33-8	Selective serotonin reuptake inhibitor (SSRI) antidepressant	N06	5)	Yes	Yes	No
Clarithromycin*	81103-11-9	Antibiotic	J01	2), 3), 4), 5)	Yes	Yes	Yes
Clindamycin	18323-44-9	Antibiotic	J01	7)	Yes	Yes	Yes
Clotrimazole	23593-75-1	Antifungal	D01	6), 7)	Yes	Yes	Yes
Diclofenac*	15307-86-5	Nonsteroidal anti-inflammatory drug (NSAID)	M01	2), 3), 4)	Yes	Yes	Yes
Erythromycin	114-07-8	Antibiotic	J01	2), 3), 4)	Yes	Yes	No
Estrone (E1)*	53-16-7	Hormone replacement therapy (HRT)	G03	2), 3), 4)	Yes	Yes	No
Fluconazole*	86386-73-4	Antifungal	J02	6), 7)	Yes	Yes	Yes
Guanyurea	141-83-3	Antidiabetic (metabolite of metformin)	A10	7)	Yes	Yes	Yes
Hydrochlorothiazide	58-93-5	Diuretic	C03	5)	Yes	Unlikely	Yes

API	CAS number	Class	ATC (level2)	Regulatory basis	Indication of presence in the environment	Indication of risk to the environment	Analytics with sufficiently low LOQ available? ¹⁾
Ibuprofen	15687-27-1	Nonsteroidal anti-inflammatory drug (NSAID)	M01	2)	Yes	Yes	No
Irbesartan	138402-11-6	Angiotensin II receptor antagonist (ARB)	C09	5)	Yes	Unlikely	Yes
Metformin	657-24-9	Antidiabetic	A10	7)	Yes	Yes	Yes
Metoprolol*	51384-51-1	Beta blocker	C07	5)	Yes	Yes	Yes
Miconazole	22916-47-8	Antifungal	D01	6), 7)	Yes	No	Yes
O-desmethyl-venlafaxine	93413-62-8	SNRI (metabolite of venlafaxine)	-	6), 7)	Yes	Yes	Yes
Ofloxacin	82419-36-1	Antibiotic	J01	7)	Yes	Yes	No
Primidone*	125-33-7	Anticonvulsant	N03	8)	Yes	Yes	Yes
Sulfamethoxazole*	723-46-6	Antibiotic	J01	6), 7), 8)	Yes	Yes	Yes
Triclosan	3380-34-5	Antimicrobial	D08	2)	Yes	Yes	Yes
Trimethoprim*	738-70-5	Antibiotic	J01	6), 7)	Yes	Yes	Yes
Venlafaxine*	93413-69-5	Serotonin-norepinephrine reuptake inhibitor (SNRI) antidepressant	N06	5), 6)	Yes	Yes	Yes

- 1) Indicative of the situation in Finland, at the time of writing this background document
- 2) EQS proposal (Council of the European Commission, 2024)
- 3) 1st WFD Watch list (2015/495)
- 4) 2nd WFD Watch list (2018/840)
- 5) Proposed UWWTD indicator substance (EC, 2022a)
- 6) 3rd Watch list (2020/1161)
- 7) 4th Watch list (2022/1307)
- 8) GW EQS proposal (EC, 2022b)

5. Complementary parameters

Complementary parameters, such as chemical oxygen demand (COD), total organic carbon (TOC), pH, and conductivity are useful when monitoring pharmaceutical occurrence in wastewaters and surface waters, as they provide a more comprehensive understanding of the water's chemical and physical properties. TOC and COD, along with nutrient concentrations, are especially useful for assessing wastewater treatment efficiency. If these parameters indicate poor performance, then pharmaceutical concentrations may be elevated as well. Furthermore, as there is little historical monitoring for pharmaceuticals, but basic complementary parameters have been monitored in several locations for decades, it may be possible to find correlations between pharmaceutical occurrence and these complementary parameters.

Complementary parameters may also be important for chemical analyses. Water quality and composition can vary considerably, depending on where or when the sample is taken. Particles, salts, and other impurities in the sample can have a significant effect, for example, on the yield of the sample analysis. Sample loss and other matrix effects are controlled by adding mass labelled internal standards to the samples and with proper cleaning of the samples (see Section III, Chapter 3.2). However, monitoring other variables may be useful in mapping the factors that interfere with the analysis and in choosing the right analysis method for the micropollutants.

In addition to chemical parameters, physical parameters, such as water flow and temperature should also be noted down during sampling. Information on flow is especially important for assessing substance flows.

II: Selecting sampling sites and sampling frequency

1. Introduction

Sampling sites and timing of the sampling occasions must be selected based on the objectives of the sampling campaign. Location, distribution, number of sampling sites and sampling frequency can affect the quality and applicability of the resulting data. In most water bodies, a single sampling site or point is not adequate to describe the physical properties and the distribution and abundance of chemical substances in the aquatic environment in question. Especially in standing water, potential stratification should be considered as well when choosing sampling points. Similarly, a single grab sample cannot be considered to describe temporal variation. However, often the resources of the screening and monitoring campaigns are quite limited, and it is not possible to take as many water samples as would be optimal in terms of research objectives.

It is very important to have sufficient background information from the research area so that the limited resources can be allocated correctly. For example, physical characteristics of the area, such as size and shape, land use, tributary and run-off characteristics, geology, point and non-point sources of contamination, hydraulic conditions, climate, water depth, and sediment characteristics are things to consider when selecting sampling sites and frequency. It is important to consider the study objectives, types and quality of data needed, sampling methods, and equipment requirements.

If the sampling is carried out in WWTPs, the logical sampling points may be readily apparent. However, special attention should be paid to sampling frequency. The sampling frequency required to obtain representative samples will increase with the increased temporal variability of the matrix.

2. Current practices

2.1. Selecting sampling sites

2.1.1. Wastewaters

Samples from wastewaters are taken from influent and effluent discharged into the recipient water body, and in some cases also from wastewater sludge. Usually, the sampling sites are specified in the environmental permits of the WWTPs. The samples from wastewater effluent and influent are taken mainly quarter annually, seasonally or biannually. But in some cases, even weekly or daily. From wastewater sludge the samples are taken less frequently, usually quarter annually, biannually or annually but in some cases monthly or weekly.

In Latvia, during the MEDWATER project, wastewater samples were taken from the influent and effluent of WWTPs. **In Poland**, the sampling at a WWTP takes place over the inflow and outflow at a location determined by the WWTP. The Environmental Protection law sets the minimum of 12 samples per year and if the wastewater meets the required conditions four samples in the following year.

The current practises may be useful in evaluating the emission sites. However, this will be a rough indicator due to the limited number of measurement and control points. The methods mentioned in the questionnaire responses can be used to evaluate the load of rivers to the Baltic Sea and the impact of point sources on the quality of water bodies. The current practices can partly allow mass-balancing, but flow data is not

accompanied regularly. In screening projects, when samples are taken in influent, effluent and sludge, mass balancing is possible, assuming that sample representativeness is ensured.

2.1.2. Surface waters

In Finland and Sweden, the surface water samples are taken from recipient waters downstream and upstream from an emission point source to assess the impact of discharge on water quality. Samples are also taken at the discharge location point. In Finland, the sampling sites are usually selected from the biggest rivers so that riverine loading to the Baltic Sea can be calculated. Authorities have specified the sampling points and sites of the surface water samples in the environmental permits of WWTPs. The accessibility of the site throughout the year is taken into consideration when selecting the sampling points.

In Poland, monitoring is carried out at a measurement and control point representative of the assessed water body. Surveys within the framework of research monitoring and protected area monitoring are conducted at a site depending on the occurrence of the phenomenon/event/contamination under study and the location of the protected area. The General Inspectorate for Environmental Protection designates measurement and control points in surface water bodies.

In Latvia, monitoring stations are situated downstream from potential emission sources. API sampling campaigns target locations where pollutants are most likely to be found, primarily near small rivers downstream from major cities. Screening project sample locations are chosen based on the criteria from European Commission (2010). For wastewater outlet samples, downstream sites are selected in relation to the receiving water body's width, according to Eq. 5, with a maximum distance of 1,000 m for large rivers. According to EU guidelines (European Commission, 2010), recipient water must meet the EQS criteria mixing zone edge (i.e. distance L_{mix} from the discharge point). Latvian national monitoring stations in marine environments avoid river discharge impacts. Screening project sampling sites are often targeted to fill data gaps.

$$\text{Eq. 5} \quad L_{mix} = 10 \times w_{recipient} \quad , \text{ where}$$

L_{mix} = Length of the mixing zone [m],
 $w_{recipient}$ = Width of the recipient [m]

2.2. Selecting sampling frequency

According to questionnaire responses, there is a great deal of variation in timing when samples are taken. From rivers and lakes, they are usually taken quarter annually, seasonally, or monthly, but also annually or biannually sampling is used. In some research projects surface and wastewater samples are taken weekly.

The frequency of sampling in surface waters depends on many factors. For example, nature conservation, water use and ecological status of waters and risk assessments. Sampling frequency is often selected based on the guidance given by an authority or WFD requirements. The financial aspects and limitations also must be considered.

Usually, the samples are timed so that different hydrological seasons are covered. For example, **in Finland** more intense monitoring is done during spring high flow and in summer when ecological processes are most active. However, since the purpose of watch list surveys is to investigate the occurrence of concentrations harmful to the environment, sampling has also been attempted to be carried out during low-flow periods, when contaminant concentrations in surface waters are assumed to be at their highest.

In Sweden, regional monitoring is run in a six-year programs. Each year, a different problem/topic is surveyed. So, each problem/topic repeats every sixth year. On each year four samples are taken to represent high and low flow in the rivers. **In Latvia**, the samples in screening projects have been taken during summer and winter, to detect the most important seasonal variations (for example, antibiotics are in higher concentrations in winter and anti-allergy medications in summer).

3. Factors influencing site selection

Goal of the monitoring campaign usually defines boundary conditions for site selection. Depending on the objectives, the sampling site can be anything from a single sampling point to a river catchment. However, certain general criteria should be considered when selecting specific sampling sites. Representativeness is a key consideration whenever selecting sampling sites for any environmental monitoring. In this context, representativeness refers to the selected site being characteristic of the matrix or waterbody being sampled. When selecting sampling sites, it is important to take into account all relevant data from previous or ongoing programmes in the area or similar locations. Figure A7 shows a suggestion for the process of selecting sampling sites and points.

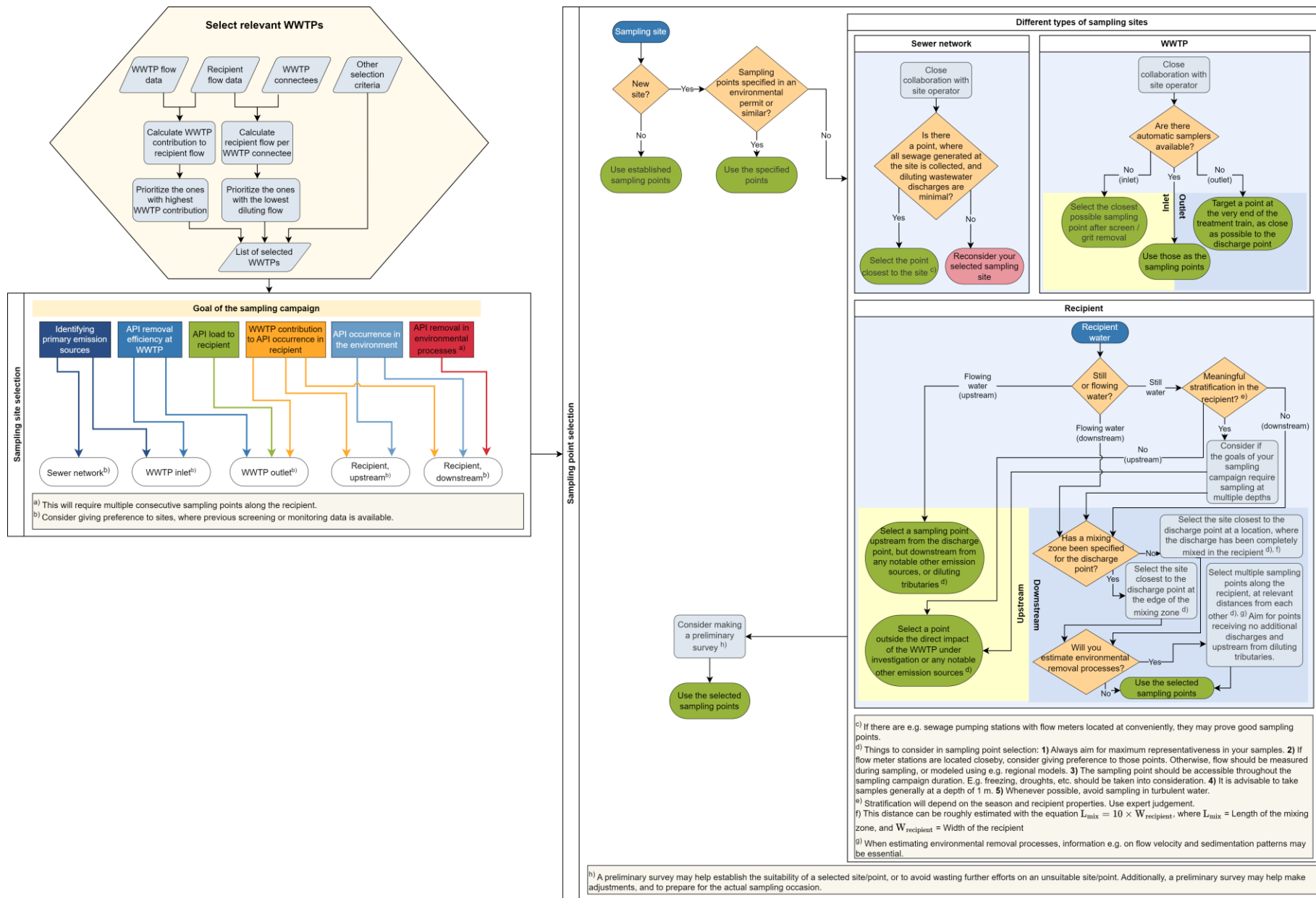


Figure A7. Suggested process for selecting sampling sites and points.

3.1. Wastewaters

Sampling point selection for wastewater monitoring depends on the campaign's goals. If the goal is to estimate current emissions in wastewater and their presence in recipient waters, sampling can be limited to WWTP effluent and relevant recipient sites. However, if the goal is to provide data for setting up, validating, or calibrating predictive models, additional samples from WWTP influents are advisable. These samples help estimate the load discharged by society before WWTP removal processes. Furthermore, sampling both influent and effluent allows for estimates of the removal efficiency of target compounds. For identifying primary emission sources, expanding the sampling campaign into the sewer network may be necessary.

When deciding which WWTPs to include in the sampling campaign, available resources and WWTP-specific details should be considered. If available resources do not allow all WWTPs in the catchment to be sampled, prioritising WWTPs and their recipients estimated to discharge and receive the highest pharmaceutical loads is reasonable. In this prioritization exercise, pharmaceutical consumption within WWTP sewer network coverage is a key factor. While high-resolution spatial consumption data is usually unavailable, assuming it to be uniformly distributed, and focusing on the number of connected persons is practical. Whenever possible, target the actual number of connected persons, because the population equivalent (PE) can give a distorted impression due to factors such as industrial activities within the WWTP sewer network. Another, ideally complementary, approach for prioritizing WWTPs is to compare the sewage flow to recipient flow. If the WWTP discharge accounts for a considerable portion of recipient flow, it should be considered a basis for including the WWTP in the sampling campaign.

When prioritizing WWTPs based on the number of connected persons, or sewage flow, cumulative loads should be taken into consideration. I.e. if there are multiple WWTPs discharging into the same river, the loads from consecutive WWTPs could be added up. This would be especially important for persistent APIs.

Figure A8 demonstrates the above-mentioned considerations for an anonymised pilot region. In this case, WWTP E would be a reasonable selection, considering its relatively high contribution to the recipient flow. On the other hand, WWTP F would be a justified option, based on the high cumulative number of connected persons, along with the relatively low recipient flow per WWTP connectees. Furthermore, WWTP F is the last WWTP before the domestic water intake. The remaining WWTPs could be prioritized e.g. based on increasing cumulative wastewater contribution, or on decreasing recipient flow per cumulative WWTP connectees. Using these criteria, WWTPs B and D would be the next ones to select.

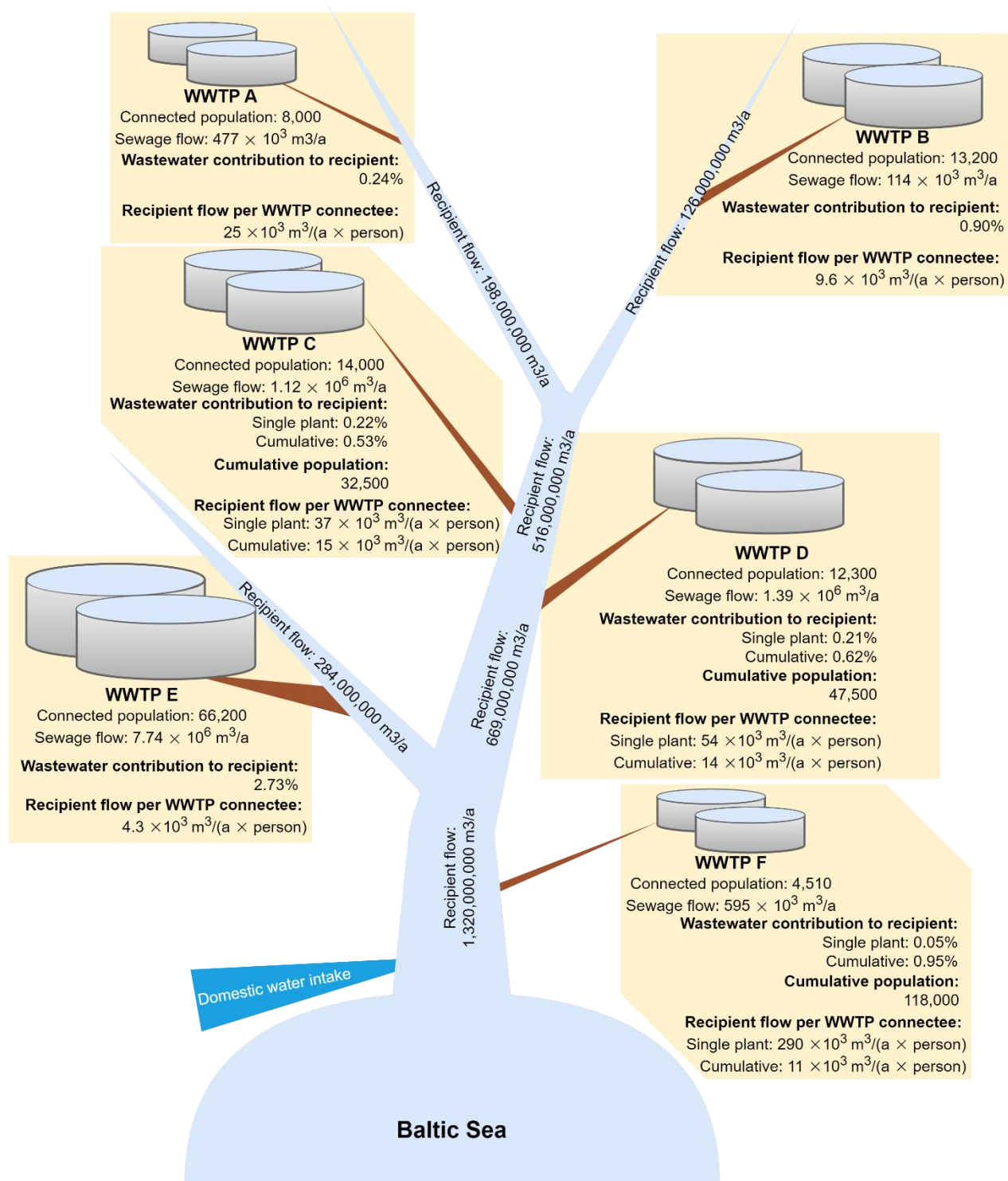


Figure A8. Some parameters to be taken into consideration when selecting WWTPs for monitoring.

3.1.1. Representative sampling sites

Sampling points should ideally be selected so that they best represent the wastewater flow at the WWTP at each timepoint. However, in practice, WWTPs often have automatic sampling equipment available at specific points of the treatment train, usually in the inlet and outlet. For practicality, these are the points where the samples should be taken within the WWTP. The relevance of possible bypass flow should also be evaluated. Sampling of such flows may also be needed for the sampling to be representative for the overall effluent.

In case of manual sampling, the samples should be taken in the middle of the flow in a well-mixed area. The sampling point should be off the bottom and walls of the flow channel to avoid sample contamination by deposits or biofilms. Generally, a sampling point between one third and one half of the effluent water depth below the surface of the water is recommended (SFS-ISO 5667-10).

When sampling waste waters, caution should be exercised to overcome or minimize the substantial heterogeneity caused by suspended solids. Also, thermal stratification may have to be considered when sampling effluents or discharges from industries. Measures have to be taken to promote the mixing of such streams before sampling.

Preliminary investigations are recommended when selecting sampling locations from the sewer network. Possible sampling sites can be identified by studying the drawings of the sewer system, but a site inspection and tracer studies may be necessary to ensure that the path of the waste stream corresponds to the drawings, and to make sure that the selected location is representative for the sampling purpose. The sampling intake point should always be located downstream from a flow restricting barrier. As a general rule, it should be located at least three times the pipe diameter, or width of the channel, downstream of the restriction (SFS-ISO 5667-10).

3.1.2. Assumed emission hot-spots

Potential emission hotspots for APIs include pharmaceutical industries and healthcare facilities. If the goal is to identify primary sources of contaminants, these sites should be considered for sampling. For instance, Scott et al. (2018) found higher API concentrations at WWTPs receiving wastewater from the pharmaceutical industry compared to those that do not. On the other hand, Äystö et al. (2023) found that hospitals could account for up to 78% of the load for specific APIs detected at WWTPs. However, this fraction varied significantly between APIs, and hospitals generally accounted for less than 3% of the total API load. Thus, while API concentrations and per capita loads are often higher in hospital settings than at WWTPs, the overall impact on WWTPs and recipient waters might not be as significant.

Whenever sampling at primary emission sources, the sampling point should ideally be located at the highest possible point upstream, where all the sewage generated at the site, and subsequently directed to the WWTP, is collected. In practice, finding such sampling points requires close collaboration with the sampling site, and might not exist at all, as documented by e.g. Äystö et al. (2023) and Azuma et al. (2019).

Furthermore, due to the lack of representative sampling points, and the space limitations set for sampling equipment, sample representativeness and load estimates must be given special consideration.

3.1.3. Accessibility and safety

Sampling at WWTPs and assumed emission hotspots requires close collaboration with site operators. WWTPs are commonly required to carry out extensive monitoring programs as part of their permit conditions. Thus, they not only have the best knowledge of their WWTP, but also extensive expertise in sampling procedures and equipment. Thus, seeking fruitful collaboration with WWTP operators is advisable. Similarly, successful sampling at industrial settings or healthcare facilities also relies on collaboration.

3.2. Surface waters

Some considerations relevant for sampling site selection in surface waters are presented in the following subsections.

3.2.1. Hydrological Conditions

Choose a sampling site with moving, well mixed water, i.e. not in an eddy or backwater where the flow is not typical of the main water body water. The sampling depth should be 1 m if possible. When sampling in standing water, the monitoring goals should be considered, to establish if samples are required from multiple depths. Furthermore, sampling sites located at estuaries or coastal waters will have increased salinity, which may affect chemical analyses. Thus, whenever selecting sampling sites, it is essential that all relevant background information is recorded and forwarded to the analysing laboratory.

To accurately assess contaminant loads, sampling sites should ideally be located at or near stream gauging stations where reliable water discharge data is available. Flow information should be collected at the time of sampling. If stream gauging stations are unavailable, use regional models or measure flow during sampling. Regardless of the flow estimation method, avoid sampling during unusually heavy rains or droughts to ensure representativeness of the samples.

According to USGS (2006), sampling sites should be selected from straight river sections, with uniform flow. If there are eddies or turbulent flow at the selected sampling site, sampling should be aimed at sections with unidirectional flow. However, turbulent flow at any section of the sampling site may increase the difficulty of measuring water flow. Furthermore, the bottom should be stable at the sampling site. Uniform flow and bottom stability help avoid e.g. sediment resuspension, that might complicate result interpretation and usage. These criteria help improve the representativeness of the samples and make the results more readily comparable with other sampling sites.

If the monitoring campaign aims to assess water quality in the waterbody in question, sampling sites should be located far enough downstream or upstream from tributary confluences. The downstream sampling site should be located an adequate distance downstream from the confluence, to allow for thorough mixing of the two water streams. However, whenever the tributaries are suspected to increase the contaminant load in the mainstream, sampling should be carried out in the mainstream, both upstream and downstream from the confluence, as well as upstream from the confluence in the tributary.

Some hydrological considerations may be possible to be accounted for only at the sampling site. For instance, turbulent flows during high-flow season, or drought during dry-season might not have been identified during sampling site selection. Thus, the sampling personnel must be able to adjust their sampling procedure accordingly. For instance, if there are turbulent flows, sampling should be targeted towards river sections with unidirectional flow. On the other hand, if the stream is too dry for appropriate sampling, it may be most resource-efficient to not to take samples at that time.

3.2.2. Land use patterns

In addition to wastewaters, pharmaceutical residues can be released into the environment from veterinary medication, soil application of sewage sludge or manure, and e.g. property-specific wastewater treatment. Thus, land use within the drainage basin may have an impact on the concentrations and loads of pharmaceutical residues.

Sewage sludge-based fertilizers are commonly utilized as growing media e.g. in landscaping, but also as fertilizers in agriculture. Thus, urban green areas and arable land may leach some pharmaceuticals in the environment. On the other hand, veterinary medication of companion animals may cause dispersed pharmaceutical emissions. However, veterinary medication of cattle whose manure is subsequently utilized as fertilizer is likely to cause more centralized emissions.

These issues should be taken into consideration when selecting sampling sites, especially those representing areas with no direct wastewater discharges (i.e. background sites). Furthermore, land use patterns might help explain unexpected results from chemical analyses. For instance, if pharmaceutical residues are detected in river stretches upstream from all known wastewater discharge points, the explanation might be e.g. veterinary medication.

3.2.3. Source Proximity

In recipient water bodies, sampling sites should be selected so that they reliably reflect the conditions upstream and downstream from the discharge point. The upstream sampling sites are especially important to help pinpoint the emission source, and to help estimate its effect on the contaminant load.

Furthermore, if environmental removal processes, such as degradation or sedimentation are relevant, it is recommended to take samples at several distances downstream from the discharge point. This is important especially when setting up, calibrating, or validating any calculation models for concentration or load estimation.

Any samples taken downstream from the discharge point must be taken at a location where the discharged wastewater can be expected to be completely mixed with the recipient water, but where the environmental processes have not yet had a drastic impact on the contaminant load. API concentrations are higher in WWTP effluents than in recipient waters, and threshold value exceedances more common (see Section I, Chapter 4.2.2). Due to the higher concentration in WWTP effluents, the concentration directly at the discharge point is unrepresentative of the recipient waterbody. Thus, in many parts of the world, EU included, environmental regulation gives the option of designating so called mixing zones (see e.g. Campos et al., 2022). These are the zones where the discharge is diluted into the recipient water, and within which water quality criteria, such as the WFD EQSs, are allowed to be exceeded.

The extent of a mixing zone is affected by e.g. discharge volume and quality, and by discharge outlet type. Furthermore, the type of recipient, its water quality, flow, and potential seasonal changes are important in determining a mixing zone (Campos et al., 2022). The definition of a mixing zone within the WFD is presented in Information box 2. If a mixing zone has been derived for the WWTP in question according to requirements set in the WFD (see Information box 2), it is prudent to take the downstream samples at the downstream edge of the mixing zone.

According to 2008/105/EC, Article 4:

- Mixing zones are areas adjacent to points of discharge, where the concentration of one or more substances may exceed the EQS given in Part A of Annex I of that directive.
- The Mixing zones must not affect the compliance of other parts of the recipient waterbody in relation to the EQSs.
- Mixing zones are restricted to the proximity of the point of discharge.

Concentration at the sampling point downstream from the discharge point could be estimated computationally, by utilizing information on representative concentration in the discharged water, along with its volume, and flow volume of the recipient water. The concentration calculated using this information would be free of the risk of taking unrepresentative samples at the recipient. However, it is advisable not to omit downstream sampling sites at least before a match between measured and predicted concentrations has been established. Furthermore, requirements set in national regulation should be taken into account.

According to the technical guidance on mixing zones (EC, 2010), mixing zones should be determined using a tiered approach. The first step is to evaluate, if discharge concentration of the target compound exceeds surface waters EQS. If it does, then a rough evaluation should be made to identify if the discharge is clearly acceptable or unacceptable. This is done by checking the percentage concentration increase relative to the EQS, caused by the discharge, and comparing this value to proposed allowable increase thresholds. The third step of mixing zone derivation relies on estimating the dimensions of the mixing zone computationally. Relatively simple models to do this are available as excel spreadsheets, but more complex ones are available as well. All these models require extensive knowledge of the recipient system. If modelling is unfeasible, or input required for model parametrization is not available, rough estimates have been proposed. For example, in Latvia, sampling points have been selected based on the width of the recipient water (see Eq. 5). Further details on mixing zone derivation are presented in Annex A1.

3.2.4. Other considerations

Sites, such as domestic water intakes, bathing waters, or nature reserves can justifiably be considered of special concern. Whenever relevant, and if in line with goals of the monitoring campaign, such sites should be considered as sampling sites.

Logistics of the sampling procedure, sampling methods and the equipment needs often play an important role in site selection. For instance, in larger rivers, sampling at the cross-section mid-point is seldom possible without the use of e.g. a bridge or a boat. However, according to USGS (2006), sampling sites should be selected upstream from bridges and other artificial structures, to ensure the structures, road surface, or e.g. surface run-off do not cause contamination. However, as these sources should not be significant for APIs, bridges can be considered useful platforms from where to take samples. Boats, on the other hand, are more invasive. Whenever taking samples from a boat, special consideration should be given for e.g. sediment resuspension. Approach the sampling location from downstream if possible, and avoid anchoring during sampling especially in shallow water.

Sampling sites should be accessible, and their characteristics should remain mainly unchanged throughout the planned monitoring campaign. i.e. sampling sites at or directly downstream from water construction or e.g. dredging operations should be considered less than optimal.

Sampling sites where other parameters are being monitored, and/or where there is historical data available should be given preference. Monitoring other parameters, while not necessarily being indispensable for assessing the occurrence and risks of pharmaceutical residues, may help give context to the results and benefit result interpretation.

4. Factors influencing sampling frequency

The selection of sampling frequency is typically driven by the need to obtain as accurate and comprehensive data on concentrations as possible within the budgetary constraints set for the campaign. This leads to the necessity of scheduling a limited number of samples at times that are most relevant for covering the variability in the occurrence of substances. When selecting sampling timing and frequency, it is important to consider objectives of the monitoring as well as type and intensity of changes in both natural and anthropogenic conditions of the monitored water body. Examples of these changes include seasonal variations in temperature and light, changes in flow conditions, changes in amount and type of pollution from societies, and alterations in the biota present in the water body. These conditions affect the levels at which pharmaceuticals appear in water bodies, what are their concentrations, how long they persist, and which organisms can be impacted. National and international legislation may also set requirements on sampling frequency. Figure A9 shows a framework for selecting sampling times and frequencies.

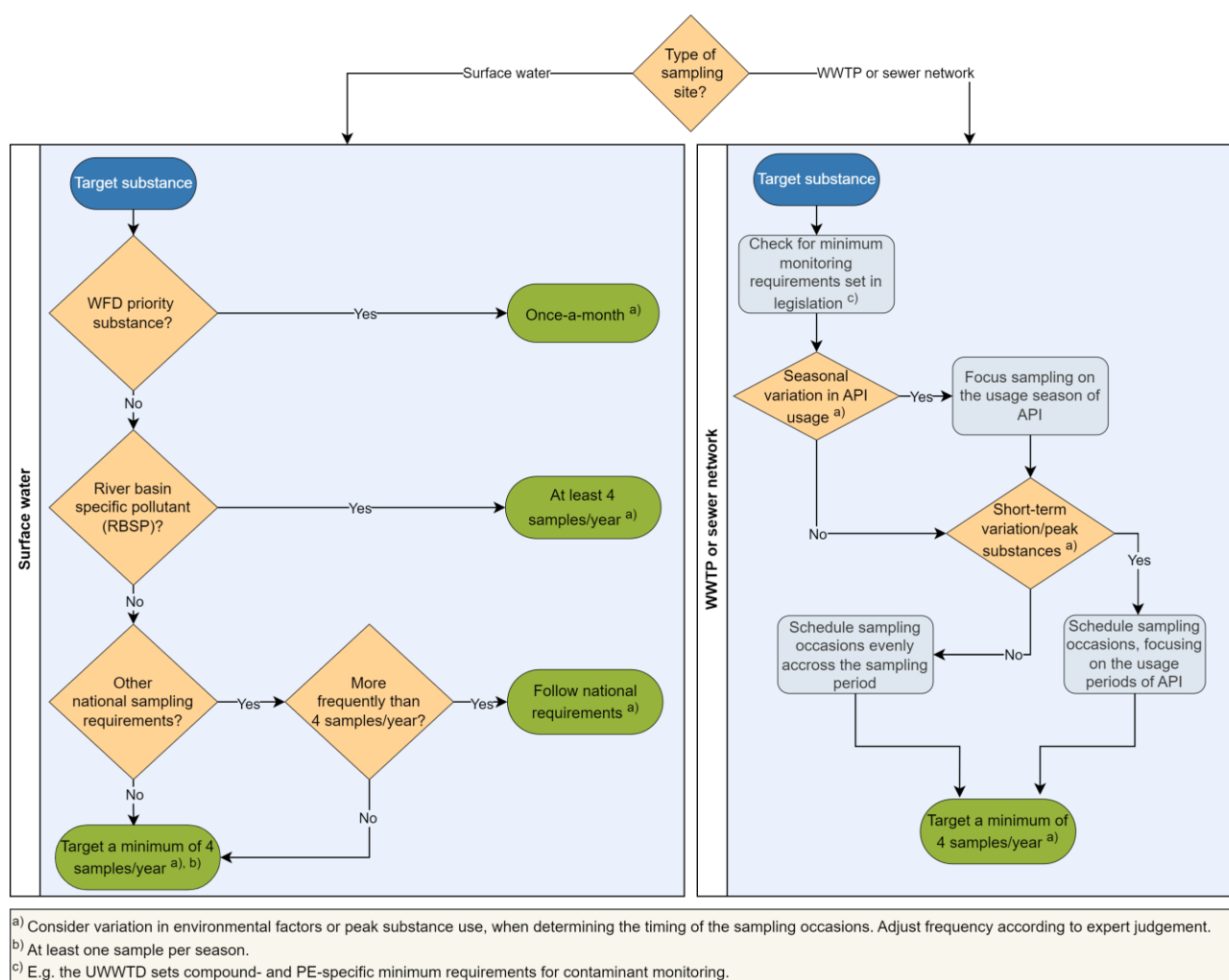


Figure A9. Guidance on choosing appropriate sampling times and frequencies.

4.1. Wastewaters

4.1.1. Temporal variation in target compound load

Consumption of certain pharmaceutical groups varies over time, which in turn affects their environmental load (e.g. Sui et al. 2011). Consumption levels naturally impact wastewater concentrations. An example of such group is the seasonal variation of allergy medications, which are used more in spring compared to winter (see e.g. Figure A1). Similarly, flu seasons can result in higher usage of antibiotics. Such seasonal variation can be expected to be lower for pharmaceuticals used as maintenance medication, such as compounds used to treat high blood pressure or other chronic conditions.

Similarly to seasonal variation, some compounds may exhibit more short-term temporal patterns. For instance, Kisielius et al. (2024) observed higher concentrations of the contrast media iohexol during weekdays than during weekends. The authors hypothesized, that this could be due to contrast imaging taking place primarily during weekdays, with only the most urgent examinations taking place during weekends.

It should be noted, that when the number of connected persons is very low, pharmaceutical discharges may be sporadic. In extreme cases, pharmaceutical usage of a single medicated person can increase concentrations in the samples. These situations should be avoided by appropriate selection of sampling sites, as the results are not representative and are likely not applicable to a wider setting.

In addition to usage patterns, weather conditions may affect pharmaceutical loads from WWTPs. For instance, in cold conditions the biological degradation rates of pharmaceuticals in activated sludge can be reduced, leading to higher concentration levels in effluents (Sun et al. 2014).

Whatever the source of variation is, its impact on the sampling campaign should be considered before sampling. If the goal of the sampling campaign is to produce an estimate of the annual average concentration, temporally distributed sampling, covering seasonal variation, is necessary. On the other hand, it may be reasonable to target the seasons when the target compounds are most likely to be detected. In these cases, the periods of assumed peak discharges must be identified.

In addition to sampling timing and frequency, subsample frequency is an important factor, when taking composite samples. According to Ort et al. (2010), wastewater samples should preferably be taken as flow-proportional continuous composite samples. However, if continuous sampling is not possible, the samples should be taken at a high sampling frequency. A high sampling frequency can mean e.g. <15 min to 2 hour subsample intervals.

Determining a high enough subsample frequency requires some prior knowledge. Pharmaceuticals are only excreted by the people using them. Thus, pharmaceutical emissions received at WWTPs can be considered as “packets” from those people. Ort et al. (2010) suggested Eq. 6 for estimating the number of pulses containing a specific API received at a WWTP.

$$\text{Eq. 6} \quad N = \frac{Cons_{nat}}{365} \times \frac{Pop_{WWTP}}{Pop_{nat}} \times \frac{1}{dose_{day}} \times n_T$$

, where
 N = Number of pulses containing substance of interest [-],
 $Cons_{nat}$ = Total annual pharmaceutical consumption [kg/a],
 Pop_{WWTP} = Population connected to the WWTP,
 Pop_{nat} = Total population,
 $dose_{day}$ = Typical dose per patient [kg/d],
 n_T = Number of times a person goes to the toilet per day (default = 5)

According to Ort et al. (2010), whenever the number of pulses containing the API under investigation is in thousands or tens of thousands, increasing the frequency from 2 hours to 15 minutes gives little added value. However, whenever the number of pulses is unknown or smaller than 1,000, a minimum frequency of 15 minutes should be used.

4.1.2. Wastewater flow dynamics

Similar to natural waters, the quantities of wastewaters also exhibit seasonal variations. Wastewater flow dynamics vary depending on how much wastewater is generated, and on weather conditions. In general, the more people and industrial and other activities are connected to the WWTP, the higher the volume of generated wastewater. However, wastewater volume may vary depending on the time of day or season. Typically, household wastewater is generated in highest quantities during daytime, peaking in the morning and evening, and falling to the lowest levels in the nighttime (e.g. Weiner et al., 2003). Furthermore, holiday periods can affect the amount of wastewater generated by households, industry, and other societal activities. Weather conditions may increase the amount of wastewater received at the WWTP. Combined sewers may result in increased inflow during periods of rainfall, snowmelt, and other wet seasons. Similarly, leaking sewer lines may result in infiltration. Inflow and infiltration will dilute the pharmaceutical discharges. Additionally,

peak flows can reduce wastewater treatment efficiency due to reduced hydraulic retention times, or result in bypass events.

When aiming to estimate representative concentrations, 24-hour composite samples are often considered appropriate. Challenges caused by temporal variations in wastewater quality can be addressed by taking these samples flow-proportionally, either continuously or sufficiently frequently. In this sampling mode, the sample volume (e.g. each subsample) is adjusted to the current flow. This way the resulting composite sample will give the most representative results possible.

Volume- and time-proportional sampling modes have their respective caveats. Volume-proportional sampling takes subsamples of constant volume at a frequency determined by the wastewater flow rate. On the other hand, when taking samples using the time-proportional mode, subsamples of constant volume are taken at a constant time frequency. In this sampling mode, night-time hours may be overrepresented. Whatever sampling mode and frequency are selected, it should be thoroughly documented.

4.2. Surface waters

Pharmaceuticals enter waterbodies primarily via WWTPs. However, emissions from agricultural and urban areas, as well as grazing land, can also occur through leaching or surface run-off. Discharges from land areas may be relevant wherever sewage sludge has been used as a fertilizer or in landscaping, and where veterinary medicines are excreted. Surface run-off and soil leaching increase during heavy rainfall which potentially raises pharmaceutical emissions. High surface run-off can also cause leaks in sewage systems and bypasses at WWTPs, increasing the contaminant load to surface waters. On the other hand, during high flow periods, a significant amount of water can dilute wastewater discharges. In dry periods, a significant portion of the water in the water body may consist of treated wastewater.

Temperature and light conditions can affect the persistence of pharmaceuticals in water bodies. Warm and bright conditions can improve the degradation of organic compounds. Conversely, cold and dark condition can slow down the degradation and prolong the presence of these compounds in the environment.

The adverse effects caused by pharmaceuticals vary between species. In water bodies with strong seasonal variations in environmental conditions, there are also changes in the organisms present in the ecosystem. Thus, the risks caused by pharmaceuticals may also vary seasonally. For instance, invertebrates and algae might spend the winter in dormant forms which reduces their exposure to pollutants. Some species migrate to different areas depending on the season, what naturally affects to their exposure.

The sampling frequency must be planned in such a way that the impact of seasonal variation on the results is minimized and sampling objectives are accomplished, taking into account the aforementioned factors affecting on the occurrence of substances. As a bare minimum, the number of samples should match the number of seasons in the region, meaning at least four samples per year. However, the environmental conditions within each season can vary so much, that obtaining comprehensive picture of the occurrence of pharmaceuticals, and risks they cause, may require more frequent and in some cases unevenly distributed sampling. Similar to wastewaters, the timing of sampling can also be influenced by variation and peaks in substance use as described in Section II, Chapter 4.1.1. When monitoring WFD priority substances or other pollutants for the purpose of determination of the chemical status of a waterbody, the requirement to take samples in once-a-month for priority substances and once-per-three-months for other pollutants, unless greater intervals are justified on the basis of technical knowledge and expert judgment (2000/60/EC, Annex V).

III: Procedures for sampling and analytics

1. Introduction

Taking samples is the first concrete step towards producing new information on chemical occurrence in selected matrix and location. It is a crucial step, and errors made in this step cannot be remedied later. Samples must be representative. I.e. they need to represent the characteristics of the sampling site in an unbiased and accurate way. Poorly taken, unrepresentative samples will not help produce meaningful results, and may give false results when utilized in e.g. risk assessment. Consequently, the state-of-the-art work carried out when analysing trace levels of pharmaceuticals, may be wasted if errors are made during the sampling stage.

Organic trace analysis usually requires sample preparation once the sample is collected. The samples are hereby concentrated, and purified from background substances, that might have so called matrix effects, by various techniques. More information on matrix effect is provided in Section III, Chapter 3.2. Most common technique for the sample preparation in the analysis of micropollutants, including pharmaceutical residues, is Solid Phase Extraction (SPE). In SPE the compounds of interest in the sample are retained on a solid phase material housing within an SPE column, which can have different chemical composition depending on the nature of the micropollutants. The most common choice of SPE in environmental analysis is some sort of HLB-material, which is composed of a hydrophilic (H), lipophilic (L) balanced (B) polymer. These HLB materials are brand specific.

Sample preparation preferably begins with the addition of isotopically labelled internal standards (IS) to the sample. The sample is then transferred to the SPE column that separates the analytes from the liquid phase and reduces the content of unwanted background substances (matrix). In the final step, the SPE column is eluted with an organic solvent. The solvent is evaporated, and the analytes are dissolved in a smaller liquid volume and transferred to a special sample vial. The sample is now concentrated and ready for final analysis in HPLC-MS/MS (more detailed in Section III, Chapter 3.2).

Mass spectrometry, more specifically tandem mass spectrometry (MS/MS), in combination with liquid chromatography (HPLC) has become the standard for analysing these types of micropollutants. The three techniques are combined with associated methods into a complete analysis chain, which is abbreviated SPE-HPLC-MS/MS. The analysis chain is recommended by the EU for the analysis of most substances on the EU's Watchlists 1, 2, 3 and 4 (2015/495, 2018/840, 2020/1161, 2022/1307).

2. Current practices

2.1. Sampling, storage and transportation

Previously presented sampling procedures varied among countries. According to German responses, samples are taken as grab samples or manual composite samples. In Latvia, grab samples are employed for surface waters, and passive samplers like POCIS may be used in research projects. Automatic 24-hour flow- or time-weighted composite samples are utilized in Latvian WWTPs. In Finland, various methods including grab samples, manual and automatic composite samples, continuous online monitoring, and passive samplers are used for surface waters, while WWTPs use time- or flow-weighted composite samples.

There is also difference in the responses concerning the water flow measurements. Latvian responses indicate that surface water flow measurements are not done except in some research projects whereas in

Germany the flow is not measured during sampling but can be retrieved from databases. In Finland, some respondents measure surface water flow during sampling while others collect flow data retrospectively from data bases. Wastewater treatment operators in Finland monitor the flow of both influent and effluent.

In Germany and Finland, the riverine mass balancing can be calculated at least on some occasions by combining analysis results to the flow rate data collected from databases. According to Latvian responses, mass balance can be calculated for treated wastewater because flow rates are monitored at treatment plants. Population biomarkers and other parameters have also been used in research projects to normalize loads. Polish responses indicate that mass balancing is not feasible due to a lack of legislation requiring flow monitoring.

A common practice is to deliver samples to an analysing laboratory in a cooler, or to store them in a freezer.

2.2. Chemical analysis

German and Polish responses had no information available regarding pretreatment steps. In Latvia, water samples undergo solid-phase extraction (SPE), while solid samples are treated with methods like QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), SLE (Solid-Liquid Extraction), UAE (Ultrasound Assisted Extraction), or PLE (Pressurised Liquid Extraction). In Finland wastewater samples may be filtered or unfiltered. Addition to this SPE is used for non-filtered samples as well as other waters samples as a pretreatment method.

Analytical methods vary depending on analyte, although responses do not indicate which methods are used for which compound groups. Mentioned instrumental setups are LC-HRMS, GC-MS, GC-MS/MS, LC-MS/MS, UHPLC-MS/MS, ICP-MS, AAS, gravimetric filtration and spectrophotometer. All respondents are not aware of the specific methods used by commercial laboratories, but they trust in their adherence to standards.

The survey did not provide information on the quality control methods used in the analyses.

3. Best Practices

3.1. Sampling

Below is some practical, simple advice on sampling and sample handling, which form an important part of the analysis chain to ensure good analysis results. Figure A10 shows a suggestion for sampling, transport, and related preparations. Whenever the abundance of antibiotic resistant bacteria, or resistance genes is to be analysed, special care should be given to avoiding potential contamination. Sterile sample containers should be used, and analyses should commence within six hours from sampling.

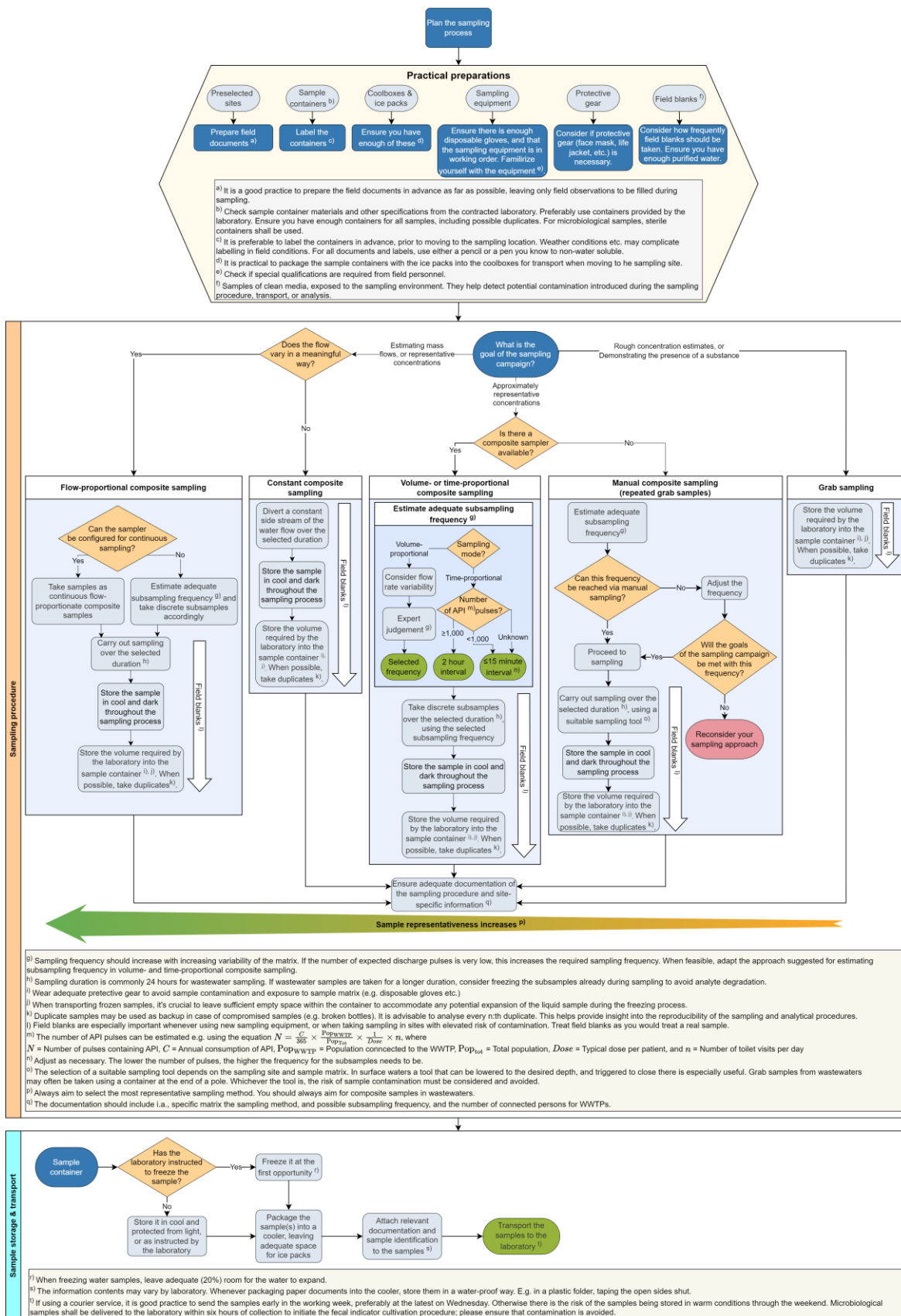


Figure A10. Procedures for sampling and storage.

3.1.1. Sampling advice

Only use the sample bottles specified, or preferably provided by the contracted laboratory. When analysing API residues, there are generally two options; high-density polyethylene (HDPE) bottles or glass bottles specifically designated by the laboratory. When the correct sampling vessel is used, losses of the studied compounds caused by binding (adsorption) to the material of the sampling vessel are avoided. Consequently, the LOQ and the measurement uncertainty defined by the laboratory are also valid.

Be careful when transferring samples and handling vessels to avoid sample contamination. Consider good hand hygiene, use gloves if possible and avoid touching the mouth of the sample bottle. When taking water samples, the sampling equipment is to be rinsed with the sample water at each sampling point. To further help avoid contamination, it is preferable to take samples starting from the site where the concentrations of the target compound are assumed to be lowest, moving towards assumedly higher concentrations, whenever feasible. Avoid using cosmetics and, if possible, non-essential medications on the sampling day.

Do not overfill the bottle so that it does not break in the cold. This is especially important when freezing the samples. If the sample bottles are frozen during any stage of the process, leave circa 20% of the container empty. This allows the water to expand during freezing.

Label sample bottles with a designated label that does not come off or use a permanent marker pen. Carry out labelling and fill in the delivery note according to the instructions of the contracted laboratory. In general, it is preferable to label the sample containers in advance, before moving out to the sampling location. Take the cooler with you to the sampling location so that you can immediately put the sample bottles in a cold and dark.

Waste water samples

Composite samples are collected over time, by either continuous sampling or by mixing discrete samples. Flow proportional sampling is preferred, if possible. These samples are collected either by diverting a constant sidestream, proportional to the flow, into the sample container, or by taking discrete subsamples at constant time intervals while the volume of each individual aliquot is kept proportional to the flow.

In cases of composite sampling (24-hour or weekly sampling) cooling and keeping samples in the dark is important. When weekly samples are taken; if possible, remember to freeze the partial samples. Conveniently, these smaller sample amounts can be added to a larger sample vial, which is kept frozen.

Grab samples consist of either a single discrete sample or individual samples collected over a period not to exceeding 15 minutes. Grab samples are often used at smaller sewage treatment plants where equipment for collective sampling might be missing, and in surface waters. However, the grab samples can only be considered representative if the quality of the water being sampled is constant. Furthermore, mass loads calculated from grab samples may contain drastic errors.

Note the daily flow if available, together with the sampling date. For weekly samples, the average flow from the current week can be used.

Recipient sample

Samples are most commonly taken as grab samples, and occasionally as composite samples. The recipient sampling, which applies to watercourses, lakes and seas, is designed and adapted to the character of the recipient, available resources, and the project's objectives.

If a watercourse is the recipient; take at least one upstream sample during the sampling series. If aiming to estimate background conditions (i.e. unpolluted site), the upstream sampling point should be located upstream from any relevant discharge points. Take the downstream samples at a point where sufficient mixing of the wastewater plume has taken place – as ballpark a figure, 10 times the width of the recipient downstream from the outlet, in the middle of the stream and 1 m below the surface. Ensure that downstream samples are taken in different seasons (if possible; spring, summer, autumn and winter; four samples in one year) so that flow variations are reflected in the sampling series. This ensures that pollution load at low water flow is included.

Flow measurements

When sampling in WWTP settings, wastewater flow is commonly logged continuously. This information should be used for load calculations. In recipients, flow data can be measured during sampling or compiled for the sampling time if the sampling site is located at a river gauging station, or if there are established flow models for the region. When measuring the flow during sampling, the methods and possible uncertainties related to the process should be documented.

There are several ways for measuring the flow volume over a cross section of a river. Some of them rely on highly sophisticated equipment, such as acoustic doppler current profilers. On the other hand, perhaps the simplest method is to measure the time it takes a floating object to travel from point A to point B. This information then must be accompanied by information on river morphology to estimate the flow volume. When using different types of current meters, commonly multiple measuring points across the river cross-section are required. Therefore, when selecting the method, sampling site characteristics such as width and depth should be taken into consideration.

3.1.2. Sample storage and transportation

If analysis does not take place immediately after sampling; freeze the sample and store the samples at minimum -18°C until transport. During sampling, possible subsamples should be stored in cold and protected from light to prevent decomposition of the analytes. After taking the final subsample, the sample should be frozen as soon as possible. Transport of frozen samples must take place with the time aspect in mind; the shorter the better. Always use insulating packaging and ice packs to avoid thawing. If samples are sent with a transport company, the shipping is planned so that the samples do not have a risk of being left lying around over the weekend. I.e. It is preferable to ship samples early in the week (e.g. Monday or Tuesday).

Wastewater samples are more biologically active than natural waters, with influent samples being more active than effluent samples. Consequently, the micropollutant content in these samples is more susceptible to biodegradation. Therefore, it is especially important to freeze the samples as quickly as possible.

Current methods of sampling and transport are resource intensive, as water samples of varying sizes are transported between regions and countries to specialized laboratories for analysis. These transports not only cause greenhouse gas emissions, but also risks the quality of subsequent analyses. In the laboratory, the water samples are processed using SPE. With a technique (see Figure A11) invented and currently developed by Ola Svahn (Kristianstad University) the sample can instead be transferred immediately to the SPE sample cartridge in a robust, simple and reliable way, also minimizing the risk of contamination. When applying this technique, the first essential step in the analytical chain can be performed at the sample location and by the person taking the sample. When widely applied, this method will improve environmental monitoring and the evaluation of advanced micropollutant treatment. The technique lowers the cost of analysis and may increase the quality of analysis.

Sampling procedures will be followed by analyses. Figure A12 shows a basic flow of the analytical processes for chemical and microbiological analyses.

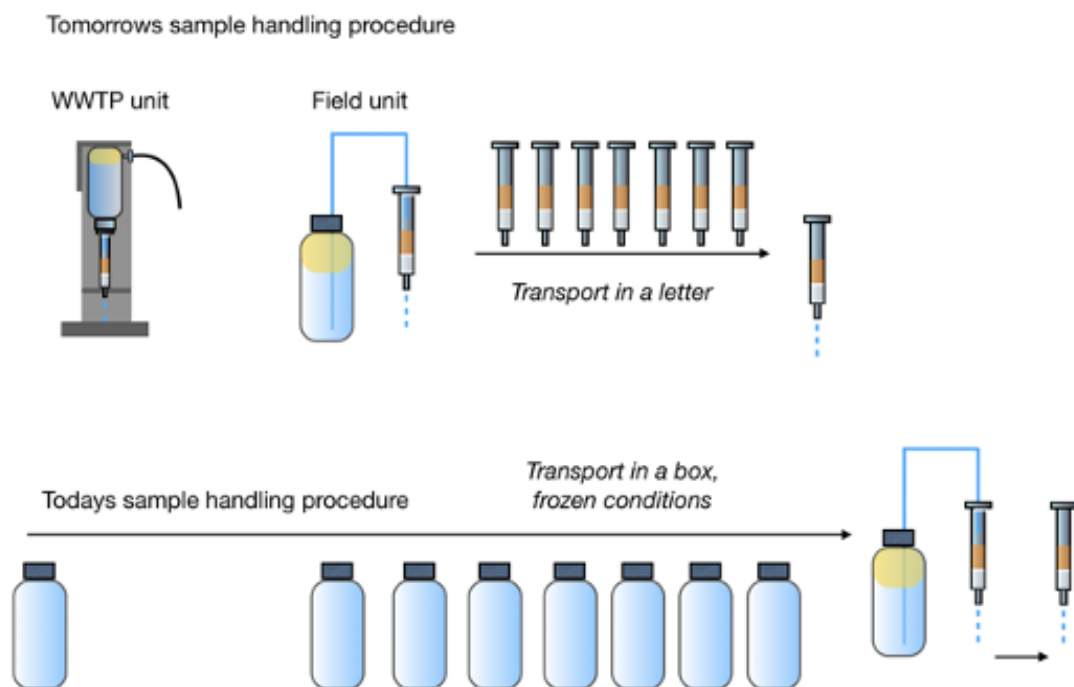


Figure A11. Sample pre-treatment in field conditions, using a method developed at the Kristianstad University.

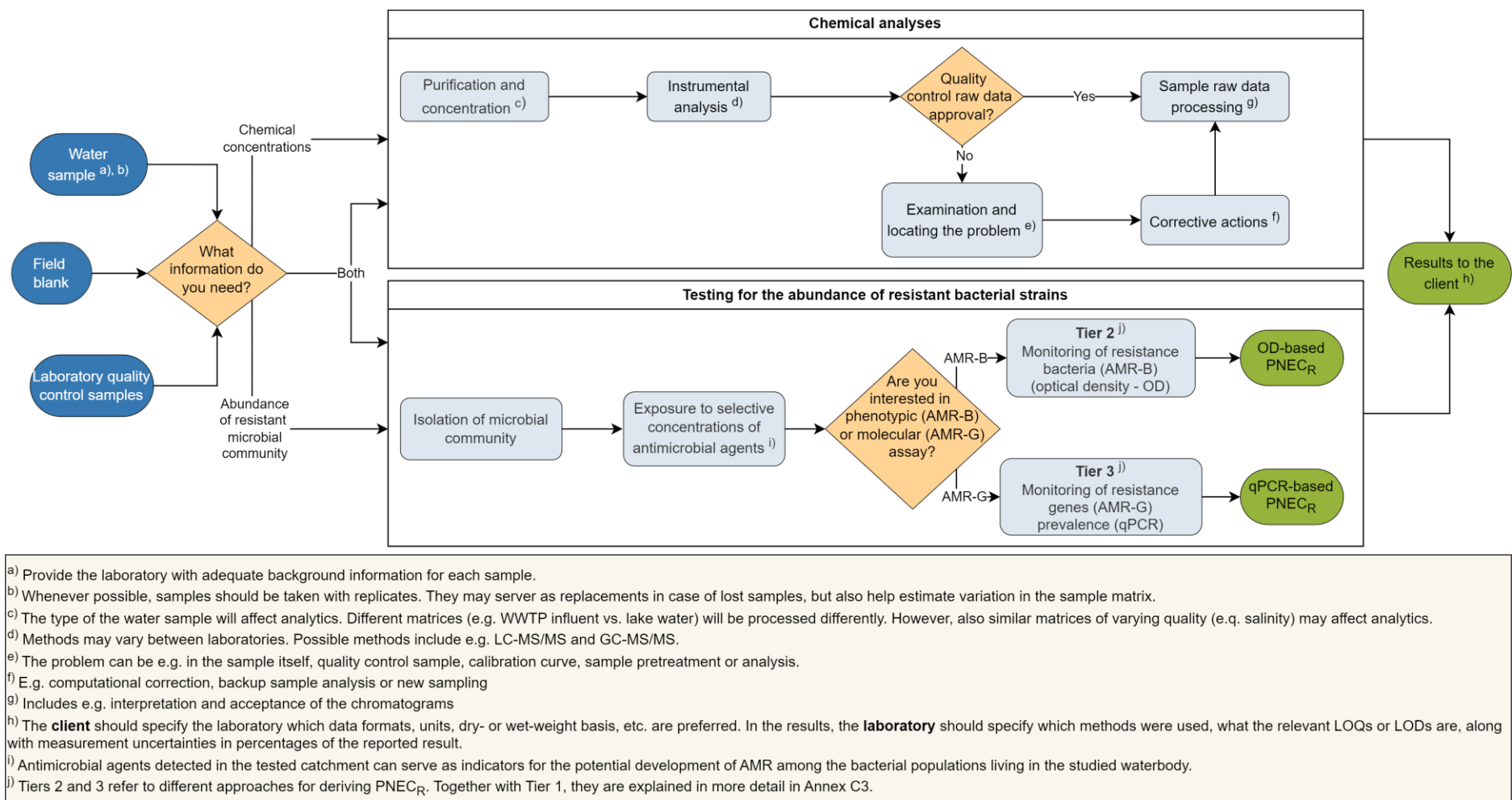


Figure A12. The process of sample analysis. Sample purification and analysis methods may vary depending on the laboratory.

3.2. Chemical analysis

3.2.1. Selecting the analytical methods

The laboratory analysing the samples needs information about the sample type (for example surface water, groundwater, wastewater), the compounds to be analysed and the desired level of sensitivity. Furthermore, sample properties such as salinity may affect analytics. Thus, all relevant background information should be delivered to the laboratory, for example, the customer's contact information, name of the sampler, sampling location, coordinates or customer's sample ID, collection date and time, sampling depth, water temperature, odor, if the sample contains a lot of humic substances, unusual appearance of the water and other environmental conditions that may affect the representativeness of the sample. A laboratory may have several types of methods for the same compounds. Based on information provided to the laboratory, a suitable method can be chosen. Figure A12 shows a basic flow of the chemical analytical process.

There are differences between laboratories, and, for example, the reported LOQ levels of the methods may vary. This is due to the challenge of analysing environmental samples but also the different equipment's of the laboratories. If possible, you should ask for offers from several laboratories. It is also good to be aware that the more compounds you want to analyse, the more likely the measurement uncertainty of the result will increase. Analytical devices have a certain capacity, how many compounds can be analysed with one measurement. The more compounds to be measured, more samples and more measurements might be needed, which naturally increases the price of the analysis.

When analysing micropollutants from an environmental sample, one of the biggest challenges is getting the sample cleaned well enough that the matrix does not significantly affect the analysis's sensitivity. In general, the matrix effect is caused by matrix components co-eluting with the target analytes, which change the chromatographic response of the analytes in the analytical column and the ionization in the mass spectrometer, leading to a decrease or increase in the response. By adding a mass-labelled IS appropriate for each analyte, interference from the matrix can be controlled up to a certain point. However, IS does not eliminate the need for careful SPE purification suitable for the target analytes.

In SPE, target analytes are isolated from a liquid sample by extraction, partitioning, or adsorption onto the solid phase of an SPE column. Unwanted particles and chemical compounds are washed away, after which the analytes are extracted from the solid phase with suitable solvents. Purified and concentrated sample is injected first into the liquid chromatography, then led to the mass spectrometer.

In the chromatographic column of the liquid chromatograph, the compounds of the sample mixture are separated from each other by means of a suitable solvent mixture. A mass spectrometer measures the mass-to-charge ratio of the ions. In the device's ion source, the liquid molecules of the sample are vaporized, and with the help of suitable voltage, the ionized analytes are directed forward to the analyser. There are several types of ion sources and analysers on the market, and the selection of a suitable combination depends on the composition of the sample and the chemical properties of the compounds to be analysed. In the triple quadrupole devices, which are commonly used in micropollutant analysis, the ionised molecules are filtered in the quadrupoles and fragmented in the collision cell. When operating on a multiple reaction monitoring (MRM) mode, triple quadrupole analyser becomes even more sensitive and selective. Depending on the structure of the molecule, it can be fragmented into one or several so-called product ions. The signal ratio of the product ions to the precursor ion is used to identify the molecule and the quality assurance of the result.

At the end of the device is a detector, which measures the strength of the physical signal produced by the ion. A detector leads the signal to a computer which draws the graph, i.e. mass spectrum.

In quantitative analytics, reference substances are also needed to identify compounds and determine their concentration. The calibration curve is used to verify the accuracy of the concentrations.

In the measurement result obtained from the measuring device, the sample volume and possible concentration coefficients must also be considered. Most measurement software calculates these automatically if the figures are entered into the software correctly. The micropollutant results from water samples are usually reported in ng/L or µg/L.

3.2.2. Quality control procedures

The most important thing is that the chosen method must be able to reliably determine the concentrations of the selected compounds. The analysing laboratory may not have an accreditation to the method in question, but it must be able to demonstrate the quality control it performs and the trueness of the results in other ways. The laboratory can also partially or fully comply with a suitable ISO standard, if one exists. The accuracy and the functionality of the method are monitored with different quality control and blank samples. Accredited laboratories are also required to participate regularly in various comparison tests to ensure the quality of their methods. Reliable storage and traceability of raw data of analysis results are also an important part of quality controlling.

The result report usually states at least the measurement uncertainty (MU) and LOQ of the results. In organic chemistry residue analysis, measurement uncertainty is generally greater than, for example, in inorganic chemistry measurements. This is mainly due to more challenging compounds, more complex analyses and often technically more challenging equipment.

3.2.3. Instrument calibration and maintenance

Regardless of the method used, equipment must be regularly maintained. Quality systems require the laboratory to keep records of device maintenance, potential problems, and solutions to problems. The equipment operator's professional competence includes identifying and reacting to various analytical and equipment problems which will inevitably arise.

3.3. AMR-analyses

There are multiple possible methods for determining antimicrobial resistance in a sample. One of the most important distinctions between these methods is whether they target the abundance of antibiotic resistance genes (AMR-Gs) or the abundance of antibiotic-resistant bacteria (AMR-B). It is also important to note that exposure to non-antibiotic pharmaceuticals, heavy metals, and other biocides may co-select for antimicrobial resistance.

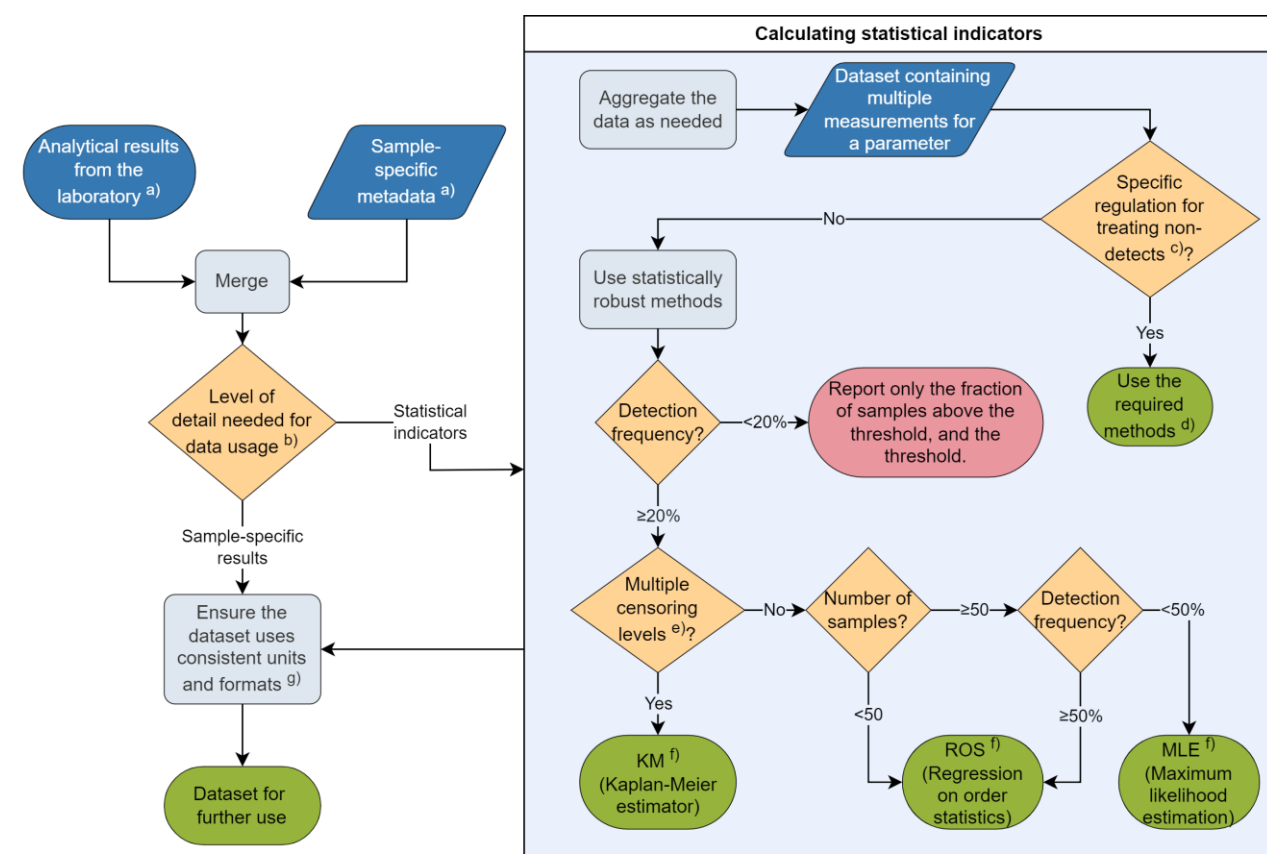
Methods targeting AMR-Gs provide information on the abundance of genes with the potential to confer resistance to specific antibiotics. These methods rely on DNA extracted from the sample, after which selected AMR-Gs are quantified. In addition to these targeted approaches, metagenomic sequencing may be used to provide a broader overview of the resistome present in the sample. However, such methods do not distinguish between gene expression, therefore, they reflect resistance potential rather than confirmed phenotypic resistance.

In contrast, methods targeting AMR-B quantify the number of organisms that are actively resistant and therefore provide information on expressed resistance in the sample. These methods may rely, for example, on culture-based approaches, in which bacteria from the sample are cultured and exposed to antibiotics. Figure A12 shows the basic procedures used in culture-based AMR-B analyses. AMR-B-based methods may also rely on determining the minimum inhibitory concentration (MIC) for specific antibiotics, i.e. the lowest concentration at which an antibiotic inhibits the growth of an isolated microbial strain.

3.4. Data management

3.4.1. Metadata

When producing contaminant occurrence data, it is especially important to keep comprehensive records of all relevant metadata, i.e. information which enables its more detailed analysis, tracking the chain of custody, etc. This information should be incorporated into a sampling protocol, that is filled for each sampling occasion and point. Annex A2 presents an example template for such a sampling protocol. Furthermore, Annex A3 and Annex A4 show suggestions for templates for monitoring data. Figure A13 shows a flowchart for data management. Annex A5 presents an R script for applying the framework presented in Figure A13.



- a) It is essential to have common sample identifiers for the laboratory results and metadata.
- b) Statistical indicators may be more robust e.g. for risk assessment. However, when checking for compliance with regulatory threshold values, individual datapoints may be required. When in doubt, it is advisable to forward the complete dataset.
- c) i.e. results below the limit of detection or limit of quantification.
- d) If utilizing the data e.g. for modelling purposes, or carrying out statistical analyses for the data, it is advisable to avoid using substitution methods, but to utilize more robust methods, such as KM or ROS.
- e) The term 'Censoring level' refers to e.g. the limit of quantification (LOQ).
- f) Accessible via e.g. the R package NADA.
- g) To avoid conversion errors, it is advisable to use standardised units for results (e.g. 'ng/l'), and to report metadata consistently. It is advisable to report coordinates using e.g. the widely used WGS84 decimal format. Especially for solid samples, it is essential to specify clearly whether the results are expressed per dry weight (e.g. 'µg/kg dw') or wet weight ('µg/kg ww') basis.

Figure A13. Flowchart for management of data produced in environmental monitoring campaigns.

3.4.2. Data integration

The data should ideally use a structure that can be directly applied in subsequent applications. I.e. it is advisable to request the analysing laboratory to report the data using a pre-delivered data template. The monitoring campaign organizer should issue every individual sample a unique identifier. It should be delivered to the analysing laboratory (e.g. a sample bottle code) and be requested to be linked with the results when reporting. The identifier should then be used to merge the analysis results with the sample-specific meta data.

The data formats and units should be unified. I.e. sampling site coordinates should be given in a consistent coordinate reference system (e.g. WGS84, decimal format). Similarly, concentration data should be reported using a consistent format (e.g. µg/l), to avoid conversion errors.

Whenever it is not possible to use the standard units, it is important to state clearly which units are being used. For instance, the number of people connected to WWTPs is commonly expressed in population equivalents (PE). While PE often provides a good estimate of the actual number of people and may better

reflect seasonal changes (e.g., holiday seasons) compared to occasional population counts, it can significantly overestimate the actual population e.g. in regions with extensive food industry operations.

3.4.3. Data analysis & censored data

Analysing compounds present at trace levels in complex environmental matrices is a challenging task. It is common to encounter data points that fall below the LOQ or the limit of detection (LOD). A common way to handle these data points is to use substitution methods, i.e., substituting the censored value with an arbitrary numerical value.

The use of substitution methods is often encouraged. For instance, the Finnish Government Decree 868/2010 states that when calculating means, individual results below the LOQ should be substituted with half the LOQ. If the resulting mean is below the LOQ, it should be reported as below LOQ. On the other hand, according to decree, when calculating a sum parameter, individual constituents below the LOQ should be treated as zero.

While substitution methods offer quick and easy solutions, they may lead to incorrect statistical conclusions. More robust, scientifically justified, and statistically valid approaches are extensively described in sources such as Helsel et al. (2020) and Helsel (2012). These documents outline methods such as Kaplan-Meier (KM), regression on order statistics (ROS), and maximum likelihood estimation (MLE), all of which are suitable for estimating mean values and standard deviations from datasets containing data points below the LOQ. These methods are accessible through the NADA package in statistical computing software R (Lee, 2020). Each of these approaches has its own strengths and should be applied according to the specific situation. The flowchart presented in Figure A13 shows some guidance for selecting the appropriate approach. For reference, the approach presented in Figure A13 was applied in Table A3.

4. What to consider when contracting a lab?

When contracting a laboratory there are some things to reflect upon as a customer. First and foremost, it's important to consider if the project needs can be met by the laboratory, not the other way around. Only you as a customer can ultimately set these criteria. However, discussions are of course important to find a common ground, and compromises are probably bound to be made, from both sides. Some issues to be discussed and agreed upon are presented in Information box 3.

Information box 3. Things to consider when contracting a laboratory.

- Timeline:
 - How long will the analyses take?
 - How quickly can the laboratory deliver the results?
- Analytical capacity:
 - What is included in the analyses?
 - Ensure LOQs meet project requirements.
 - Ensure analytical precision (MU) meets project requirements.
- Transport logistics
 - Will the laboratory send the bottles?
 - Commercial laboratories may have contract couriers available, or they may be able to recommend a company.
 - Ensure you know the exact address and contact person for sample reception.
- Reporting results
 - In which format will the laboratory deliver the results? Can they use a format predefined by the project?
 - Ensure the laboratory knows which identifier is to be used for sample identification.
- Support
 - Ensure that the laboratory will provide support in interpreting the results, if necessary.

Challenges and solutions

- **Legacy lists.** Target compounds should always be selected based on current information needs. E.g. consumption patterns will change, resulting in changes in environmental risks, and new compounds may arise.
- **Lack of historical monitoring data.** Until now, APIs have not been under widescale monitoring. Thus, it is important to utilize existing data, while keeping in mind the potential problems caused by multiple data sources and methodologies.
- **Resource limitations.** Monitoring campaigns are commonly carried out under budgetary restraints. This makes it especially important, that the monitoring focuses on relevant parameters and that adequate efforts are directed into sampling procedures.
- **Temporal variation.** Not accounting for seasonal variation e.g. in API sales may result in misleading results. Always aim to identify potential variation e.g. in API sales in advance, and adjust the sampling campaign according to its goals.
- **Uncertainties.** Aim to identify and record the sources of uncertainties during the monitoring program. This can be helpful in interpreting results or possible inconsistencies later on. Qualitative description or estimation of uncertainty may include writing down e.g., challenges in selecting sampling frequency, sampling site selection, or any inherent variation involved in the process, imprecision, or lack in understanding phenomena or situation. Furthermore, any deviations from standard procedures are to be noted.
- **Errors in sampling cannot be remedied later on.** Always avoid sample contamination, and ensure adequate cooling for frozen samples. Analysing compromised samples may be a waste of resources.

Summary

- **Regulatory requirements.** If a substance has been identified on a regulatory substance list, it indicates that a substantial amount of work has already been carried out on the compound. Thus, it is likely that relevant background data and established analytical methods are readily available. Furthermore, monitoring may have to meet legislative obligations, which often focus on substances identified in regulatory lists.
- **Environmental risks.** Focusing on APIs known to cause environmental risks is reasonable use of resources.
- **Analytical capabilities.** Target compounds should ideally have robust and reliable analytical methods available. However, especially for risk-posing compounds, avoid justifying exclusion from monitoring due to a lack of analytical capabilities.
- **Representativeness and the goals of the campaign dictate sampling sites.** In general, aim for sampling sites where the discharge can be assumed completely mixed.
- **Seasonal variation** (both natural and anthropogenic) should be considered when timing the sampling occasions.
- **Plan ahead.** Sampling campaigns should be planned well in advance, considering all relevant variables. Obtaining samples ex tempore is not always possible.
- **Prepare for adjustments.** Field work does not always go according to plan. Be prepared to adjust the plans if e.g. field conditions demand it.

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Annexes

Annex A1 - Mixing zones approach according to technical guidelines, 2010

1st step - evaluate, if concentration in discharge exceeds surface waters EQS;

2nd step – rough evaluation, if wastewater discharge is acceptable.

The criterion is defined as the proposed allowable increase in concentration after complete mixing due to the discharge, expressed as a percentage of EQS. In order to check if this criterion is fulfilled one has to calculate the so-called process contribution (PC). This is defined as:

$$\frac{C_{effl.} \times Q_{effl.}}{Q_{river} + Q_{effl.}} = PC$$

In the next step, the PC as a percentage of the EQS needs to be checked with the proposed allowable increase:

$$\frac{PC}{AAC\ EQS} \times 100\% = \text{concentration increase, \%}$$

If the increase is below the significance criterion as shown in Table SA 1.1 of the report, this discharge is deselected if there are no sensitivities present in the vicinity of the point of discharge.

Table SA 1.1. Proposed allowable increase in concentration after complete mixing for different water types, which can meet criteria for MAC- and EQS mixing zone (EC, Technical guidelines, 2010)

Size of river	River flow, Q_{90}^1 , m^3/s	Proposed allowable increase in concentration after complete mixing as % EQS
Small	≤ 100	4
Medium	$100 < Q_{90} < 300$	1
Big	> 300	0,5

1 The flow which is exceeded during 90 % of the time in a year. The third step of mixing zone derivation relies on estimating the dimensions of the mixing zone computationally. Relatively simple models to do this are available as excel spreadsheets, but more complex ones are available as well. All of these models require extensive knowledge of the recipient system. If modelling is unfeasible, or input required for model parametrization is not available, rough estimates have been proposed. For example, in the Netherlands the length (L) of the mixing zone for chemical substances is proportionate to the width of the water body and equals $10 \times W$ (width) with a maximum of 1000 m.

3rd step: the dimensions of the mixing zone must be estimated to judge whether a discharge is acceptable. This requires an assessment using simple computation of the size of the mixing zone.

A simple approach for dilution as a function of the distance from the point of discharge can be derived from the Fischer equations, the most important of which are given below. An important limiting condition is that the flow of the effluent is negligible to the flow of the water body.

$$\varphi(x, y) = \frac{W}{a \times u \times \sqrt{\pi \times K_y \times \frac{x}{u}}} \times \sum_{n=-\infty}^{n=\infty} e^{-\frac{(y-2 \times n \times B)^2}{4 \times K_y \times \frac{x}{u}}}$$

, where

W = emitted load of a substance [g/s];

a = depth of the receiving surface water [m];

u = flow rate of the surface water [m/s];

B = width of the receiving surface water [m];

K_y = transversal dispersion coefficient in y direction

X = distance to the point of discharge (maximum L) [m];

$\varphi(x, y)$ = concentration at a distance x from the point of discharge and distance y from the bank of the receiving surface water.

An Excel tool has been developed for the calculations of mixing zones size according to these Technical guidelines.

In some EU member states the extent of the allowable mixing zone is proportionate to the width of the waterbody and limited to a chosen fixed maximum value. For example, in the Netherlands the length (L) of the mixing zone for chemical substances is proportionate to the width of the water body and equals 10*W (width) with a maximum of 1000 m. This is also the criterion of the Technical guidelines: in order to ensure that EQS-exceedence does not impair the quality of the overall water body, it is therefore proposed that at Tier 2 the extent of EQS exceedence should be restricted to the lesser of 10*W (river width) or 1 kilometre provided that this does not exceed 10% of water body length overall.

The main criterion for the length of the mixing zone to achieve MAC EQS: mixing zone length maximum length: 0.25*river width at outlet (maximum 25 m). In this criterion there has been taken into account situations where the receiving water body has a narrow channel or may be subject to low flows there is a risk that the mixing zone will occupy a major part of the cross-section which can have adverse consequences for the passage of migratory species.

In other steps in case of mixing zones exceedences more scientific models should be used for deeper research.

Annex A2 - Example sampling protocol

Sampling protocol Id	Unique identifier, e.g. "FI-001"
----------------------	----------------------------------

Client	
Project	Reference to relevant project or similar
Date	Date, using a consistent format, e.g. 2024-08-22
Person taking the samples	Firstname, Lastname, Affiliation, email, phone number

Site information	
Site type	E.g. River, Lake, Estuary, WWTP, etc.
Site specification	Fill in if relevant (e.g. "WWTP effluent, discharge pipe")
Name of site	Regular name for the site, e.g. "WWTP x"
Site ID	If sampling at an established site, please fill in any relevant identifiers. E.g., for WWTPs a relevant ID could be the WWTP-specific UWWTD identifier.
Coordinates	Latitude, e.g. 60.2297 Longitude, e.g. 25.0199
Site information confidential (if yes, please indicate which information)	☐ Yes ☐ No
Site contact person	Firstname, Lastname, Affiliation, email, phone number

Site specifics
Please document all relevant information, e.g. the following: - Extreme weather events - Sampling site type, E.g. "Downstream from WWTP" - Deviations in WWTP processes - Connected persons at the WWTP - Closest discharge point - Sludge retention time - References to any previous monitoring - ...

Sampling procedure

Detailed information on sampling.

- *Sampling method (grab/composite/passive/etc.) + specification if relevant*
- *Sampling mode (if taking composite samples)*
- *Sampling duration (relevant for composite and passive samples)*
- *Sample volume*
- *Number of subsamples (relevant when taking composite samples)*
- *Subsample frequency (relevant when taking composite samples)*
- ...

Sample storage & logistics

Please indicate the storage conditions for the sample(s), and who carried out the transport.

Identified sources of uncertainty

Please indicate any deviation from prior plans, along with any problems or errors occurred in the field work.

Results

Delivery format of results:

- ☐ Text report (pdf)
- ☐ Text file (txt)
- ☐ Excel file (xlsx)
- ☐ Format specified by the client (template to be delivered)

Annex A3 - Example result template for the laboratory

[illegible]

Annex A4 - Example structure for monitoring data

The produced data should be structured so that it contains all relevant background data, and identifiers allowing it to be linked with sampling protocols. The information to be included in the monitoring data structure includes, but might not be limited to the following:

- Sample identification
 - o Sample ID
 - o Sampling protocol Id
- Site identification
 - o Sampling site type
 - o Sampling site type, specification
 - o Name of site
 - o Site ID
 - o Lat WGS84 (Decimal Degrees)
 - o Lon WGS84 (Decimal Degrees)
 - o Additional information
- Matrix
 - o Matrix
 - o Matrix, specification
- Analyte
 - o Generic name
 - o Analyte CAS No.
- Results
 - o Result
 - o Flag
 - o Unit
- Analytical method
 - o Analytical method
 - o Limit of detection (LOD)
 - o Limit of quantification (LOQ)
 - o Measurement uncertainty (%)
- Sampling procedure
 - o Date
 - o Time of day
 - o Sampling method
 - o Sampling method, specification
 - o Composite sample, sampling mode
 - o Sampling duration
- General
 - o Notes

A proposal for a data template is attached as an MS Excel file.



APRIORA_Monitoring
data template.xlsx

Annex A5 - Data management demonstration

1. Introduction

This annex aims to present the commands required for processing environmental monitoring data, utilizing the approach suggested in Section III, Chapter 3.4.

2. Libraries

Libraries in R are collections of functions and code, that extend the functionality of base R. Libraries such as dplyr and tidyr, included in wider tidyverse library, make data manipulation drastically easier and more intuitive than the commands available in base R. On the other hand, the library NADA contains useful functions for dealing with non-detects in environmental data.

2.1. Installing libraries

If you run R for the first time, you first need to install the libraries we will be using. To install libraries, run the following code.

```
install.packages('tidyverse')
```

2.2. Loading libraries

To be able to use the functions and commands in the libraries, you need to load them. To do this, use the library() command.

```
library(tidyverse)
```

```
## — Attaching core tidyverse packages ————— tidyverse 2.0.0 —
## ✓ dplyr      1.1.4      ✓ readr      2.1.5
## ✓ forcats    1.0.0      ✓ stringr   1.5.1
## ✓ ggplot2    3.5.1      ✓ tibble    3.2.1
## ✓ lubridate  1.9.3      ✓ tidyr     1.3.1
## ✓ purrr      1.0.2
## — Conflicts ————— tidyverse_conflicts() —
## ✗ dplyr::filter() masks stats::filter()
## ✗ dplyr::lag()     masks stats::lag()
## ⓘ Use the conflicted package (<http://conflicted.r-lib.org/>) to force all conflicts to become errors
```

3. Generating data

Then let's generate the data for our demonstration. If you are not interested in the process of generating random data, you can simply copy the commands in this section (Section 3), and skip to Section 4.

3.1. Concentration data

```
data <- expand.grid(Sampling_protocol_ID = c('FI-001','FI-002','FI-003',
                                           'FI-004'),
                  Sample_No = sprintf('%03d',seq(1,15)),
                  Generic_name = paste0('Compound ',seq(1:5)),
                  stringsAsFactors = FALSE)

set.seed(123) #Helps ensure reproducibility

randomCompound <- sample(unique(data$Generic_name),size = 2,replace = FALSE) #Selects random compounds, for which we'll set multiple censoring levels

data <- data %>%
  select(Sampling_protocol_ID,Sample_No) %>%
  distinct() %>%
  arrange(Sampling_protocol_ID) %>%
  mutate(Sample_ID=paste0('W-',sprintf('%03d',row_number())) %>%
  right_join(data,by=c('Sampling_protocol_ID','Sample_No')) %>%
```

```

select(-Sample_No)

set.seed(123) #Helps ensure reproducibility

data <- data %>%
  left_join(data.frame(Generic_name=data %>%
    pull(Generic_name) %>%
    unique(),
    LOD=round(runif(n=data %>%
      pull(Generic_name) %>%
      unique() %>%
      length(),
      min = 1,10)/10,1)) %>% #Generates random numbers for our
LODs (with the 'set.seed(123)' command we ensure that the same "random numbers" are being
generated)
    rowwise() %>%
    mutate(LOQ=round(runif(1,1,3)*LOD,1)), #Once again, random LOQs
    by='Generic_name') %>%
  mutate(LOQ2=case_when(Generic_name%in%randomCompound&Sampling_protocol_ID%in%
    c('FI-003','FI-004')~LOQ*sample(x=seq(0.8,1.2,0.05),
    size=1,replace=FALSE), #Generates a
second LOQ, which is a multiplication of the original LOQ
    .default=LOQ),
    LOQ=ifelse(LOQ2<=LOD,LOQ,LOQ2)) %>%
  select(-LOQ2)%>%
  mutate(Unit='ng/l')

set.seed(123) #Helps ensure reproducibility

generate_concentration <- function(LOQ, LOD, decPoints, nonDetectProb = 0.15) {
  if (runif(1) < nonDetectProb) {
    return(paste0('<',LOQ))
  } else {
    return(round(runif(1, min = LOQ, max = LOQ*abs(rnorm(n=1,mean=50,sd=25))),
      decPoints))
  }
}
}#This command creates a custom function, that will help us generate random concentrations that
are higher than the LOQ

set.seed(123) #Helps ensure reproducibility
non_detect_generic_names <- sample(unique(data$Generic_name),size = 2) #Selects random compounds
for which we'll set a lower detection frequency

data <-
data %>%
  group_by(Generic_name) %>%
  mutate(Concentration = sapply(1:n(), function(x) {
    if (Generic_name[x] %in% non_detect_generic_names) {
      generate_concentration(LOQ[x], LOD[x], 2, nonDetectProb = 0.85)
    } else {
      generate_concentration(LOQ[x], LOD[x], 2, nonDetectProb = 0.15)
    }
  }
),
  Concentration=ifelse(Concentration=='NaN',as.character(LOQ),Concentration)) %>%
  ungroup()

```

Let's check what our data looks like.

```
print(slice_sample(data,n=10))
```

```
## # A tibble: 10 × 7
##   Sampling_protocol_ID Sample_ID Generic_name   LOD   LOQ Unit Concentration
##   <chr>                <chr>    <chr>    <dbl> <dbl> <chr> <chr>
## 1 FI-004              W-053    Compound 4    0.9   1.9  ng/l   76.61
## 2 FI-001              W-014    Compound 2    0.8   1.6  ng/l    <1.6
## 3 FI-004              W-048    Compound 3    0.5   1.33 ng/l   60.53
```

## 4	FI-004	W-054	Compound 5	0.9	1.7	ng/l	33.82
## 5	FI-002	W-027	Compound 4	0.9	1.9	ng/l	22.86
## 6	FI-002	W-028	Compound 2	0.8	1.6	ng/l	<1.6
## 7	FI-003	W-033	Compound 1	0.4	0.4	ng/l	8.89
## 8	FI-002	W-024	Compound 1	0.4	0.4	ng/l	<0.4
## 9	FI-001	W-010	Compound 1	0.4	0.4	ng/l	<0.4
## 10	FI-001	W-013	Compound 4	0.9	1.9	ng/l	<1.9

3.2. Sample-specifics

Now let's generate some arbitrary background information for our concentrations. In reality this dataset would originate from your field notes, site specific background data, etc. But for demonstration purposes, let's keep it simple for now.

```
sampleData <- data.frame(Sample_ID=data %>% pull(Sample_ID) %>% unique(),
  Site=expand.grid(paste0('Site ',LETTERS[1:5]),paste0('Point ',1:3)),
  Matrix='Surface water') %>% rename('Site'=2,
  'Point'=3)
```

3.3. Merging the data with our imaginary site-specifics

Next, we'll combine our two datasets (concentrations and background data).

```
data <- data %>%
  left_join(sampleData,by='Sample_ID')
```

4. Getting to know the data

Before doing any analyses, you should get to know the structure of your dataset. By using the `glimpse()` command, we can see the first values for each column, along with the datatype for each column.

```
glimpse(data)

## Rows: 300
## Columns: 10
## $ Sampling_protocol_ID <chr> "FI-001", "FI-001", "FI-001", "FI-001", "FI-001",...
## $ Sample_ID            <chr> "W-001", "W-001", "W-001", "W-001", "W-001", "W-0...
## $ Generic_name         <chr> "Compound 1", "Compound 2", "Compound 3", "Compou...
## $ LOD                  <dbl> 0.4, 0.8, 0.5, 0.9, 0.9, 0.4, 0.8, 0.5, 0.9, 0.9,...
## $ LOQ                  <dbl> 0.4, 1.6, 1.4, 1.9, 1.7, 0.4, 1.6, 1.4, 1.9, 1.7,...
## $ Unit                 <chr> "ng/l", "ng/l", "ng/l", "ng/l", "ng/l", "ng/l", "ng/l",...
## $ Concentration        <chr> "2.81", "<1.6", "<1.4", "<1.9", "99.84", "8.79", "...
## $ Site                 <fct> Site A, Site A, Site A, Site A, Site A, Site B, S...
## $ Point                <fct> Point 1, Point 1, Point 1, Point 1, Point 1, Poin...
## $ Matrix               <chr> "Surface water", "Surface water", "Surface water"...
```

- Our dataset contains concentrations for five compounds.
- The concentration column ('Concentration') contains character strings (''). To do any kind of calculation with it, we first need to convert it into a numeric format.
- The concentration column also contains flags for non-detects ('<1.6', etc.). We need to move these flags into another column to help us do the calculations.

Let's look at how often each compound has been detected.

```
data %>% group_by(Generic_name) %>%
  summarise(n=n(),
    n_det=sum(!is.na(as.numeric(Concentration))),
    detection_frequency=n_det/n*100)

## # A tibble: 5 × 4
##   Generic_name      n n_det detection_frequency
##   <chr>          <int> <int>          <dbl>
## 1 Compound 1      60    52           86.7
## 2 Compound 2      60     8           13.3
```

## 3 Compound 3	60	11	18.3
## 4 Compound 4	60	49	81.7
## 5 Compound 5	60	53	88.3

So, the detection frequencies in our dataset vary from 13.3% for Compound 2 to 88.3% for Compound 5.

5. Summarising the data

If we wanted to calculate compound-specific statistical indicators, we need to find a way to deal with non-detects, and to identify relevant ways of aggregating our data. But for the sake of demonstration, let's calculate the indicators using two levels of aggregation: a) by compound and site, b) by compound.

If the concentration data is not already in a numeric format, you should convert it into such. Our 'Concentration' column contains character strings, so it needs to be converted into numeric format. The following approach assumes, that periods ('.') are used as the decimal separator. If your dataset uses e.g. comma (',') as the decimal separator, you should take it into account (e.g. replace commas with periods using the gsub() command).

```
data <- data %>%
  mutate(censored=grepl('<',Concentration), #This command checks if the concentration contains
any '<' signs. If it does, the row is flagged as TRUE into the new column 'censored'.
         Concentration=as.numeric(gsub('^0-9.','',Concentration))) #This replaces all symbols
with blanks, except numbers and periods ('.'). It is assumed, that the concentration data uses
period as the decimal separator. If it uses commas (','), it is advisable to adjust the data.

#Then Let's check our data once more
glimpse(data)

## Rows: 300
## Columns: 11
## $ Sampling_protocol_ID <chr> "FI-001", "FI-001", "FI-001", "FI-001", "FI-001",...
## $ Sample_ID            <chr> "W-001", "W-001", "W-001", "W-001", "W-001", "W-0...
## $ Generic_name         <chr> "Compound 1", "Compound 2", "Compound 3", "Compou...
## $ LOD                  <dbl> 0.4, 0.8, 0.5, 0.9, 0.9, 0.4, 0.8, 0.5, 0.9, 0.9,...
## $ LOQ                  <dbl> 0.4, 1.6, 1.4, 1.9, 1.7, 0.4, 1.6, 1.4, 1.9, 1.7,...
## $ Unit                 <chr> "ng/l", "ng/l", "ng/l", "ng/l", "ng/l", "ng/l", "...
## $ Concentration        <dbl> 2.81, 1.60, 1.40, 1.90, 99.84, 8.79, 7.03, 1.40, ...
## $ Site                 <fct> Site A, Site A, Site A, Site A, Site A, Site B, S...
## $ Point                <fct> Point 1, Point 1, Point 1, Point 1, Point 1, Poin...
## $ Matrix               <chr> "Surface water", "Surface water", "Surface water"...
## $ censored             <lgl> FALSE, TRUE, TRUE, TRUE, FALSE, FALSE, FALSE, TRU...
```

Now we see, that our concentration data is in numerical format (""), and the non-detects are flagged in the column 'censored'.

5.1. Substitution methods

Let's assume that regulation requires us to use substitution methods. When using substitution methods, non-detects are commonly reported as medium bound (MB), i.e. LOQ/2. You could calculate the statistical indicators using the approaches presented below.

#Let's calculate the medium bound values for non-detects, and assign them into a new column called 'Conc_MB'

```
data <- data %>%
  mutate(Conc_MB=ifelse(censored==TRUE,Concentration/2,Concentration))
```

5.1.1. By compound and site

If we wanted to calculate the statistical indicators dividing by compound and site, we could do it e.g. in the following way:

```

substitution_summary_byCompoundSite <- data %>%
  group_by(Generic_name, Site) %>%
  summarise(n_obs=n(), #Number of datapoints per combination
            n_det=sum(!censored), #Counts the number of positive detections
            mean=mean(Conc_MB), #Calculates the mean concentration from the medium bound values
            sd=sd(Conc_MB)) #Calculates the standard deviation from the medium bound values

## `summarise()` has grouped output by 'Generic_name'. You can override using the
## `.groups` argument.

print(substitution_summary_byCompoundSite)

## # A tibble: 25 × 6
## # Groups:   Generic_name [5]
##   Generic_name Site   n_obs n_det  mean    sd
##   <chr>         <fct> <int> <int> <dbl> <dbl>
## 1 Compound 1   Site A     12    12  7.05  5.24
## 2 Compound 1   Site B     12     9  8.60  6.81
## 3 Compound 1   Site C     12   11 12.6   7.57
## 4 Compound 1   Site D     12   10  8.21  6.97
## 5 Compound 1   Site E     12   10 15.1   11.7
## 6 Compound 2   Site A     12     2  8.41 25.3
## 7 Compound 2   Site B     12     4 11.0   18.0
## 8 Compound 2   Site C     12     1  9.08 28.7
## 9 Compound 2   Site D     12     1  4.70 13.6
## 10 Compound 2  Site E     12     0  0.78  0.0209
## # i 15 more rows

```

5.1.2. By compound

If we are only interested in e.g. the mean concentration for individual compounds, we could do it as follows:

```

substitution_summary_byCompound <- data %>%
  group_by(Generic_name) %>%
  summarise(n_obs=n(), #Number of datapoints per combination
            n_det=sum(!censored), #Counts the number of positive detections
            mean=mean(Conc_MB), #Calculates the mean concentration from the medium bound values
            sd=sd(Conc_MB)) #Calculates the standard deviation from the medium bound values

print(substitution_summary_byCompound)

## # A tibble: 5 × 5
##   Generic_name n_obs n_det  mean    sd
##   <chr>        <int> <int> <dbl> <dbl>
## 1 Compound 1     60    52 10.3   8.28
## 2 Compound 2     60     8  6.79 19.5
## 3 Compound 3     60    11  6.12 13.4
## 4 Compound 4     60    49 44.1  45.1
## 5 Compound 5     60    53 37.2  33.7

```

5.2. Using more robust methods

If we are not required to use substitution methods, we should use the methods available in the library NADA.

```

install.packages('NADA')

library(NADA)

## Loading required package: survival

##
## Attaching package: 'NADA'

## The following object is masked from 'package:stats':
##
##   cor

```

As we already prepared the data into a compatible format (concentrations as numerical, censored datapoints as logical), we can skip that phase and move straight to calculating the statistical indicators.

To use the framework in Section III, Chapter 3.4, we need to

- 1) calculate the detection frequency (or censoring frequency),
- 2) Check if the dataset contains multiple LOQs (i.e. censoring levels),
- 3) Check the number of samples
- 4) Calculate relevant indicators.

5.2.1. By compound and site

```
data_byCompoundSite <- data %>%
  left_join(data %>%
    group_by(Generic_name,Site) %>%
    summarise(n_obs=n(),
              n_det=sum(!censored),
              detFreq=n_det/n_obs, #calculates detection frequency as a percentage
              n_LOQ=n_distinct(LOQ)), #Counts the number of distinct censoring levels)
  by=c('Generic_name','Site'))

## `summarise()` has grouped output by 'Generic_name'. You can override using the
## `.groups` argument.

head(data_byCompoundSite)

## # A tibble: 6 × 16
##   Sampling_protocol_ID Sample_ID Generic_name   LOD   LOQ Unit Concentration
##   <chr>                <chr>    <chr>   <dbl> <dbl> <chr>      <dbl>
## 1 FI-001              W-001    Compound 1   0.4   0.4 ng/l      2.81
## 2 FI-001              W-001    Compound 2   0.8   1.6 ng/l      1.6
## 3 FI-001              W-001    Compound 3   0.5   1.4 ng/l      1.4
## 4 FI-001              W-001    Compound 4   0.9   1.9 ng/l      1.9
## 5 FI-001              W-001    Compound 5   0.9   1.7 ng/l     99.8
## 6 FI-001              W-002    Compound 1   0.4   0.4 ng/l      8.79
## # i 9 more variables: Site <fct>, Point <fct>, Matrix <chr>, censored <lgl>,
## #   Conc_MB <dbl>, n_obs <int>, n_det <int>, detFreq <dbl>, n_LOQ <int>

data_summary_byCompoundSite <- data_byCompoundSite %>%
  group_by(Generic_name,Site) %>%
  mutate(stat_type=case_when(detFreq<0.2~NA,

                             detFreq==1~'Arithmetic mean',

                             n_LOQ>1~'KM',

                             n_obs<50~'ROS',

                             (n_obs>=50&detFreq>=0.5)~'ROS',

                             detFreq<0.5~'MLE',
                             .default=NA),

  mean=case_when(is.na(stat_type)~NA,

                 stat_type=='Arithmetic mean'~mean(Concentration),

                 stat_type=='KM'~tryCatch({
   cen_data <- with(cur_data(),cenfit(Concentration,censored))
   mean <- as.numeric(mean(cen_data)['mean'])
   ifelse(length(mean)>0,mean,NA)}, error=function(e) {return(NA)}),

                 stat_type=='ROS'~tryCatch({
   cen_data <- with(cur_data(),ros(Concentration,censored))
```

```

      mean <- mean(cen_data)
      ifelse(length(mean)>0,mean,NA)}, error =function(e) {return(NA)}},

stat_type=='MLE'~tryCatch({
  cen_data <- with(cur_data(),cenmle(Concentration,censored))
  mean <- as.numeric(mean(cen_data)['mean'])
  ifelse(length(mean)>0,mean,NA)}, error=function(e){return(NA)})

, .default=NA),

sd=case_when(is.na(stat_type)~NA,

stat_type=='Arithmetic mean'~sd(Concentration),

stat_type=='KM'~tryCatch({
  cen_data <- with(cur_data(),cenfit(Concentration,censored))
  sd <- as.numeric(sd(cen_data))
  ifelse(length(sd)>0,sd,NA)}, error=function(e) {return(NA)}},

stat_type=='ROS'~tryCatch({
  cen_data <- with(cur_data(),ros(Concentration,censored))
  sd <- sd(cen_data)
  ifelse(length(sd)>0,sd,NA)}, error =function(e) {return(NA)}},

stat_type=='MLE'~tryCatch({
  cen_data <- with(cur_data(),cenmle(Concentration,censored))
  sd <- sd(cen_data)
  ifelse(length(sd)>0,sd,NA)}, error=function(e){return(NA)}},

. default=NA)) %>%
relocate(stat_type,.after=sd) %>%
mutate(Notes=case_when(detFreq==0~paste0('All observations <',max(LQ),',' ,Unit),
  detFreq<0.2~paste0(round(sum(Concentration>max(LQ))/n_obs*100,1),'% of
observations \nhigher than ',max(LQ),',' ,Unit),
. default=NA)) %>%
distinct(Generic_name,Site,n_obs,n_det,mean,sd,stat_type,Notes)

print(data_summary_byCompoundSite)

## # A tibble: 25 × 8
## # Groups:   Generic_name, Site [25]
##   Generic_name Site   n_obs n_det mean    sd stat_type      Notes
##   <chr>         <fct> <int> <int> <dbl> <dbl> <chr>         <chr>
## 1 Compound 1   Site A    12    12  7.05  5.24 Arithmetic mean <NA>
## 2 Compound 2   Site A    12     2  NA    NA    <NA>         16.7% of observa...
## 3 Compound 3   Site A    12     3 20.9  11.0 KM          <NA>
## 4 Compound 4   Site A    12    11 42.0  38.9 ROS        <NA>
## 5 Compound 5   Site A    12    12 46.3  31.0 Arithmetic mean <NA>
## 6 Compound 1   Site B    12     9  9.51  5.71 ROS        <NA>
## 7 Compound 2   Site B    12     4 15.1  17.2 KM        <NA>
## 8 Compound 3   Site B    12     3 28.2   3.86 KM        <NA>
## 9 Compound 4   Site B    12     8 27.5  43.7 ROS        <NA>
## 10 Compound 5  Site B    12    10 24.5  25.9 ROS        <NA>
## # i 15 more rows

```

5.2.2. By compound

```

data_byCompound <- data %>%
  left_join(data %>%
    group_by(Generic_name) %>%
    summarise(n_obs=n(),
              n_det=sum(!censored),
              detFreq=n_det/n_obs, #calculates detection frequency as a percentage
              n_LOQ=n_distinct(LQ)), #Counts the number of distinct censoring levels)
  by=c('Generic_name'))

head(data_byCompound)

```

```
## # A tibble: 6 × 16
##   Sampling_protocol_ID Sample_ID Generic_name   LOD   LOQ Unit Concentration
##   <chr>                <chr>    <chr>    <dbl> <dbl> <chr>    <dbl>
## 1 FI-001              W-001    Compound 1    0.4   0.4 ng/l      2.81
## 2 FI-001              W-001    Compound 2    0.8   1.6 ng/l      1.6
## 3 FI-001              W-001    Compound 3    0.5   1.4 ng/l      1.4
## 4 FI-001              W-001    Compound 4    0.9   1.9 ng/l      1.9
## 5 FI-001              W-001    Compound 5    0.9   1.7 ng/l     99.8
## 6 FI-001              W-002    Compound 1    0.4   0.4 ng/l      8.79
## # i 9 more variables: Site <fct>, Point <fct>, Matrix <chr>, censored <lgl>,
## #   Conc_MB <dbl>, n_obs <int>, n_det <int>, detFreq <dbl>, n_LOQ <int>

data_summary_byCompound <- data_byCompound %>%
  group_by(Generic_name) %>%
  mutate(stat_type=case_when(detFreq<0.2~NA,

                             detFreq==1~'Arithmetic mean',

                             n_LOQ>1~'KM',

                             n_obs<50~'ROS',

                             (n_obs>=50&detFreq>=0.5)~'ROS',

                             detFreq<0.5~'MLE',
                             .default=NA),

         mean=case_when(is.na(stat_type)~NA,

                         stat_type=='Arithmetic mean'~mean(Concentration),

                         stat_type=='KM'~tryCatch({
                           cen_data <- with(cur_data(),cenfit(Concentration,censored))
                           mean <- as.numeric(mean(cen_data)['mean'])
                           ifelse(length(mean)>0,mean,NA)}, error=function(e) {return(NA)}),

                         stat_type=='ROS'~tryCatch({
                           cen_data <- with(cur_data(),ros(Concentration,censored))
                           mean <- mean(cen_data)
                           ifelse(length(mean)>0,mean,NA)}, error =function(e) {return(NA)}),

                         stat_type=='MLE'~tryCatch({
                           cen_data <- with(cur_data(),cenmle(Concentration,censored))
                           mean <- as.numeric(mean(cen_data)['mean'])
                           ifelse(length(mean)>0,mean,NA)}, error=function(e){return(NA)})
                         ,.default=NA),

         sd=case_when(is.na(stat_type)~NA,

                       stat_type=='Arithmetic mean'~sd(Concentration),

                       stat_type=='KM'~tryCatch({
                         cen_data <- with(cur_data(),cenfit(Concentration,censored))
                         sd <- as.numeric(sd(cen_data))
                         ifelse(length(sd)>0,sd,NA)}, error=function(e) {return(NA)}),

                       stat_type=='ROS'~tryCatch({
                         cen_data <- with(cur_data(),ros(Concentration,censored))
                         sd <- sd(cen_data)
                         ifelse(length(sd)>0,sd,NA)}, error =function(e) {return(NA)}),

                       stat_type=='MLE'~tryCatch({
                         cen_data <- with(cur_data(),cenmle(Concentration,censored))
                         sd <- sd(cen_data)
                         ifelse(length(sd)>0,sd,NA)}, error=function(e){return(NA)}),
```

```

      .default=NA)) %>%
relocate(stat_type, .after=sd) %>%
mutate(Notes=case_when(detFreq==0~paste0('All observations <',max(LOQ),' ',Unit),
                        detFreq<0.2~paste0(round(sum(Concentration>max(LOQ))/n_obs*100,1),'% of
observations \nhigher than ',max(LOQ),' ',Unit),
                        .default=NA)) %>%
  distinct(Generic_name,n_obs,n_det,mean,sd,stat_type,Notes)

## Warning: There were 4 warnings in `mutate()`.
## The first warning was:
## i In argument: `mean = case_when(...)` .
## i In group 2: `Generic_name = "Compound 2"` .
## Caused by warning in `ros()` :
## ! Input > 80% censored -- Results are tenuous.
## i Run `dplyr::last_dplyr_warnings()` to see the 3 remaining warnings.

print(data_summary_byCompound)

## # A tibble: 5 × 7
## # Groups:   Generic_name [5]
##   Generic_name n_obs n_det mean    sd stat_type Notes
##   <chr>         <int> <int> <dbl> <dbl> <chr>    <chr>
## 1 Compound 1      60    52  10.5  8.00 ROS      <NA>
## 2 Compound 2      60     8   NA    NA   <NA>    13.3% of observations higher t...
## 3 Compound 3      60    11   NA    NA   <NA>    18.3% of observations higher t...
## 4 Compound 4      60    49  44.6  44.7 ROS      <NA>
## 5 Compound 5      60    53  37.5  33.5 ROS      <NA>

```

6. Comparing the results

Let's see how our means and standard deviations compare with the substitution methods and the ones available in the NADA library.

```

substitution_summary_byCompound %>%
  left_join(data_summary_byCompound %>%
    select(-contains('n_')) %>%
    rename('mean_NADA'=mean,
           'sd_NADA'=sd), by='Generic_name')

## # A tibble: 5 × 9
##   Generic_name n_obs n_det mean    sd mean_NADA sd_NADA stat_type Notes
##   <chr>         <int> <int> <dbl> <dbl> <dbl>    <dbl> <chr>    <chr>
## 1 Compound 1      60    52  10.3  8.28  10.5    8.00 ROS      <NA>
## 2 Compound 2      60     8   6.79 19.5   NA      NA   <NA>    13.3% of obs...
## 3 Compound 3      60    11   6.12 13.4   NA      NA   <NA>    18.3% of obs...
## 4 Compound 4      60    49  44.1  45.1  44.6    44.7 ROS      <NA>
## 5 Compound 5      60    53  37.2  33.7  37.5    33.5 ROS      <NA>

```

When comparing by compound, there are not drastic discrepancies between the results in our dataset. However, the differences are sometimes more drastic. The concentrations divided by compound and site contain more significant discrepancies. Below is a plot, where selected compounds and sites are presented as a bar chart.

```

#First we need to merge our datasets
p_data <- data_summary_byCompoundSite %>%
  left_join(substitution_summary_byCompoundSite %>%
    select(-contains('n_')),
    by=c('Generic_name', 'Site'),
    suffix=c('_NADA', '_Substitution')) %>%
  relocate(contains('mean')|contains('sd_'), .after='n_det') %>%
  mutate(df=n_det/n_obs) %>%
  select(-c(n_obs,n_det)) %>%
  filter(Generic_name%in%c('Compound 1','Compound 2','Compound 3'),
         Site%in%c('Site A','Site B','Site E'))

```

```

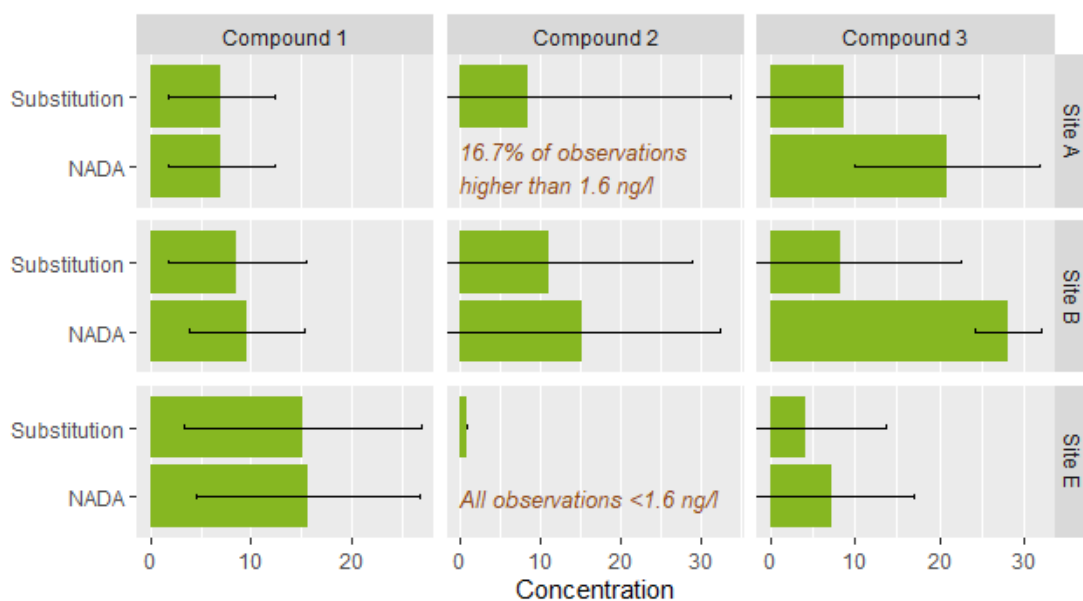
#...and flip into long format
p_data_long <- p_data %>%
  pivot_longer(cols=contains('mean'),names_to = 'type',values_to='mean',names_prefix =
'mean_') %>%
  select(-contains('sd_')) %>%
  left_join(p_data %>%
    pivot_longer(cols=contains('sd_'),
      names_to='type',
      values_to='sd',
      names_prefix='sd_') %>%
    select(-contains('mean_')),
    by=c('Generic_name','Site','stat_type','Notes','df','type'))

#Then, we'll plot the data using the library ggplot2. Once again, you need to install the package
if you haven't already
install.packages('ggplot2')
library(ggplot2)

p_data_long %>%
  ggplot(aes(y=type,x=mean))+
  geom_col(fill='#86B722')+
  facet_grid(cols=vars(Generic_name),
    rows=vars(Site),
    scales = 'free_x')+
  geom_errorbar(aes(xmin=mean-sd,
    xmax=mean+sd),
    width=0.2/.pt)+
  geom_text(data=p_data_long %>%

filter(!is.na(Notes),type=='NADA'),aes(y=type,x=0,label=Notes),hjust=0,size=10,size.unit='pt',col
or='chocolate4',fontface='italic')+
  coord_cartesian(xlim=c(0,NA))+
  theme(panel.grid.major.y = element_blank())+
  labs(x='Concentration',
    y=element_blank(),
    caption='Mean concentrations for our demonstration data, divided by compound and site.
\nThe bars show the mean concentration and the whiskers the standard deviation.')

```



Mean concentrations for our demonstration data, divided by compound and site.
The bars show the mean concentration and the whiskers the standard deviation.

Here, there are obvious differences between the derivation methods. In general, the discrepancies are higher when the detection frequency is lower. The detection frequencies are shown below.

```
p_data %>%
  select(Generic_name, Site, df) %>%
  pivot_wider(id_cols = Generic_name, names_from=Site, values_from=df) %>%
  print()

## # A tibble: 3 × 4
## # Groups:   Generic_name [3]
##   Generic_name `Site A` `Site B` `Site E`
##   <chr>         <dbl>    <dbl>    <dbl>
## 1 Compound 1      1      0.75    0.833
## 2 Compound 2    0.167    0.333     0
## 3 Compound 3    0.25     0.25    0.25
```

- For Compound 1 at Site A detection frequency is 100%, leading to the results being identical
- For Compound 2 at Sites A and E, the results are very different. When using substitution methods, the mean is reported as 8.4 ng/l and 0.78 ng/l at sites A and E, respectively. However, in both sites, over 80% of samples were below the LOQ of 1.6 ng/l.
- For compound 3, the statistical indicators could be calculated using both approaches. However, the values vary between approaches. This could affect the conclusions made based on the data.

Module B:

Modelling pharmaceutical concentrations in rivers

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List of abbreviations

Abbreviation	Explanation
API	Active pharmaceutical ingredient
EPA	Environmental protection agencies
PiE	Pharmaceuticals in the environment
DDD	Defined daily dose
ATC	Anatomical therapeutic chemical
WWTP	Wastewater treatment plant
PEC	Predicted environmental concentration, estimates
WFD	Water framework directive
UWWTD	Urban Wasterwater Treatment Directive

I: Modelling approach and flow scheme

In a perfect world, environmental data is completely available for all compartments, all times and all potential polluting substances posing a risk. During the last decades, monitoring capabilities and analytical methods have been largely improved. At the same time, the risk awareness for anthropogenic micropollutants, including pharmaceuticals, has increased. At the time of writing this document, this increased awareness is being introduced into EU legislation. The European Urban Wastewater Treatment Directive (UWWTD) is proposed to include requirements for the removal of pharmaceuticals and other micropollutants from wastewaters. Furthermore, several pharmaceuticals have been proposed as priority substances under the Water Framework Directive (WFD).

Until now, pharmaceuticals have rarely been included into monitoring programs. Thus, data is commonly available from screening studies carried out in individual projects (compare Module A). Therefore pharmaceutical concentration in the environment (PIE) data is sparsely available especially in rural areas. Modelling is a common tool to fill the gaps with estimations since we need complete environmental data for a meaningful risk assessment and prioritising actions. Environmental agencies in charge of this task mostly work at local or regional level. Therefore, they need a model which is spatially as accurate as possible as well as easy to implement in their daily working practice. There exist various modelling approaches with different complexity and resolution. However, there is an obvious gap due to practical boundary conditions: limited expert knowledge at end-user side (EPA-staff), required spatial data for risk assessment and prioritisation of measures, availability of input data (mostly only statistical data with low time resolution). Considering that produced model estimates cannot be more exact than applied datasets, the APRIORA approach uses the best available spatial resolution of geodata but avoids numeric modelling since the required input data are often not available. Furthermore, a model producing daily estimates would give the impression of high accuracy but is based on yearly input data. Here, the future application of end-users/ target groups has to be considered to avoid misinterpretation.

The APRIORA model is based on pharmaceutical pathways into the environment and its implementation follows a deterministic approach, using pharmaceutical sales statistics as the driving parameter. Figure B1 gives an overview of the workflow and defines the structure of this background document.

1. Pharmaceutical pollution pathways into the environment

Compared to other substances and micropollutant groups, the sources of pharmaceuticals are distinct: consumption in human and veterinary medicine. While distribution of veterinary application is usually not possible to classify in time and place, the

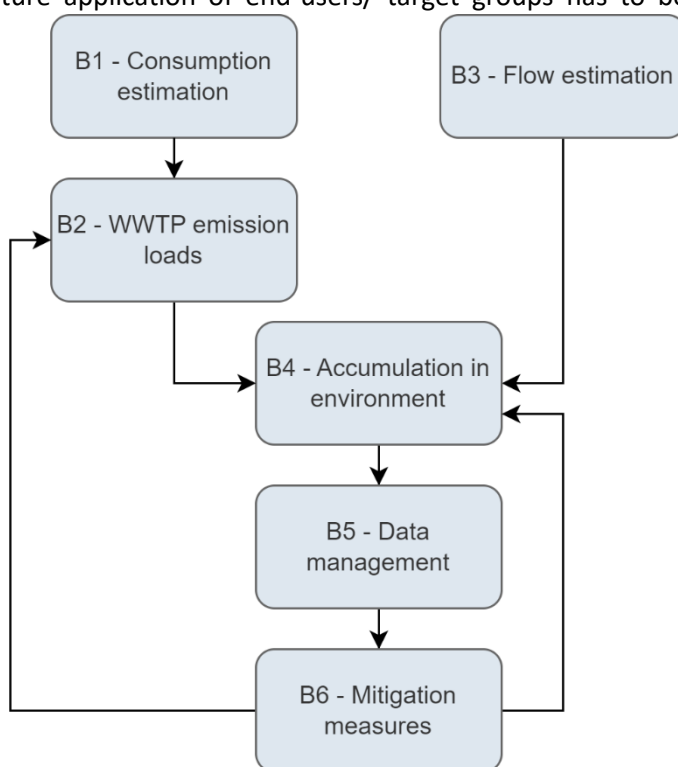


Figure B1. Flow chart of Module B: Modelling.

consumption of human pharmaceuticals is widely known and usually published in yearly statistics. Related uncertainties are discussed in Module A.

Figure B2 shows the main pathways of pharmaceutical pollutions into the environment. Depending on substance characteristics and application form, the intake dose of APIs is only partly metabolized and excreted as metabolites, with some proportions remaining as the parent compound. The APIs excreted by each inhabitant are collected in municipal wastewater, transported via local sewer systems to wastewater treatment plants (WWTPs). Additionally, improper disposal of unused pharmaceuticals into the toilet or sinks flushes API into the sewer system. In the Baltic Sea region, usually between 70 and 95% of the population is connected to a sewer and WWTP infrastructure (REF-A). While the wastewaters

are widely transported to WWTPs, the APIs they contain are removed to a variable degree in conventional wastewater treatment processes. For instance, in a study carried out in Finland, the removal efficiencies of 17 analysed pharmaceuticals ranged between -6% and over 99% (Vieno & Arjonen, 2021). WWTPs then discharge treated wastewater into receiving water bodies and with this, APIs reach the environment. Accumulated within a catchment, the transported APIs reach the Baltic Sea. In the environment, all media constantly interact with each other. Therefore, an exchange with soil and/or groundwater layers is inevitable and will vary by season and catchments. The complex interactive transport and transformation processes cause potential increases or decreases in PiE.

During wastewater treatment at WWTPs, the majority of organic matter is transformed into biomass or together with inorganic particulate directly incorporated into sludge. Depending on the process, chemical precipitation may be used to bind excess phosphorus in sludge as well. A variety of chemical contaminants, including APIs have been shown to accumulate in sludge. While nutrients and organic matter make sludge utilization an attractive prospect, attached contaminants may cause environmental risks, if not managed properly. However, estimating the API load discharged from land application of sewage sludge requires at least information on soil sludge treatment methods, application methods, soil properties, local weather and further knowledge about soil passages in highly varying soil conditions. This is a broad field of current research. Thus, discharges from sludge application are outside the scope of the APRIORA model.

While human pharmaceutical sources are easy to localise by WWTP representing point sources, veterinary applications differ in their environmental paths. API discharges from veterinary medication depend not only on animals' intake and excretion rates, but also on manure treatment and storage conditions, techniques and timing of land-application, and local drainage systems. As a consequence, date, amount, location and specific way of manure application on agricultural soils is widely uncertain, so is the potential emission of veterinary API to the water environment. However, this path may increase the occurrence of certain PiE

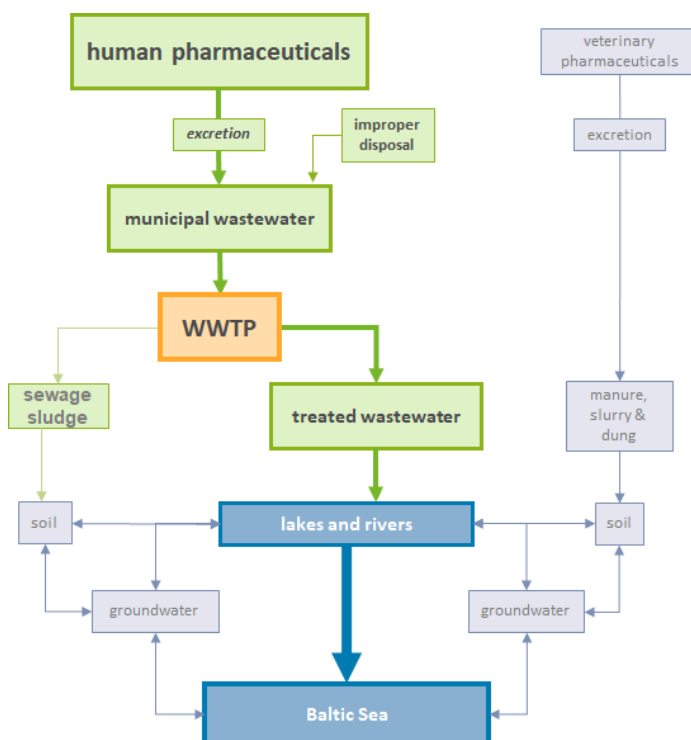


Figure B2. Emission pathways of pharmaceuticals into the environment, according to Ebert et al. 2014)

parallel to human use, e.g. the well investigated antibiotic sulfamethoxazole. Mitigation options implemented at WWTPs may not lead to sufficient reductions of PiE in these cases.

In summary, WWTPs represent the main source for pharmaceutical pollution. Other anthropogenic sources are collected via sewer systems and are most likely covered via this pathway. Diffuse sources such as sludge and manure in agriculture are known but not yet quantified sufficiently.

2. APRIORA model approach in a flow scheme

The APRIORA model approach is based on main API pathways into the environment. By combining key information of regional population and their pharmaceutical consumption with catchment and known flow characteristics, so-called predicted environmental concentrations (PECs) are estimated. The general idea is that the end-user is able to implement as much own data as possible but can make use of the model with a basic set, too. The more regional expertise is used, the better the results.

The main input data types can be allocated to two different branches (see Figure B2): On the left side, the environment and on the right side, human's API emissions via wastewater. While estimations of API consumption and pathways require knowledge in the fields of medicine, pharmacology, chemistry and wastewater treatment, regional hydrologists are the experts for geographical and flow related data. Due to this highly interdisciplinary work to be performed in an estimation model, APRIORA supports the end-user with pre-processed data pools available for several modelling steps. This shall compensate eventually missing expertise for producing PEC estimates. Another advantage of data pools is the flexibility to implement further aspects, this could be for example related to other substances, own monitoring data, (yearly) updates, other Baltic Sea regions, etc. In a data pool, the user may also have an insight into the data ranges and can compare or classify own regional and applied data.

API consumption is always considered as substance related and specific to a certain region. Based on statistical data, most often yearly loads (kg/a) can be calculated. Since each statistic is related to a different region or country with corresponding population, an average value per inhabitant shows the best option to compare and observe trends or changes of these input data. To allocate consumption to the emission path via WWTPs, the connected inhabitants of each plant serves as transfer information. By integrating two data pools, one for average inhabitant's consumption and one for WWTP removal rates, the total emission load discharged by WWTPs can be used as intermediate input data points. Once an emission load is calculated, it is projected geographically into the river catchment and is used to estimate regional and substance specific PEC in WWTP recipients.

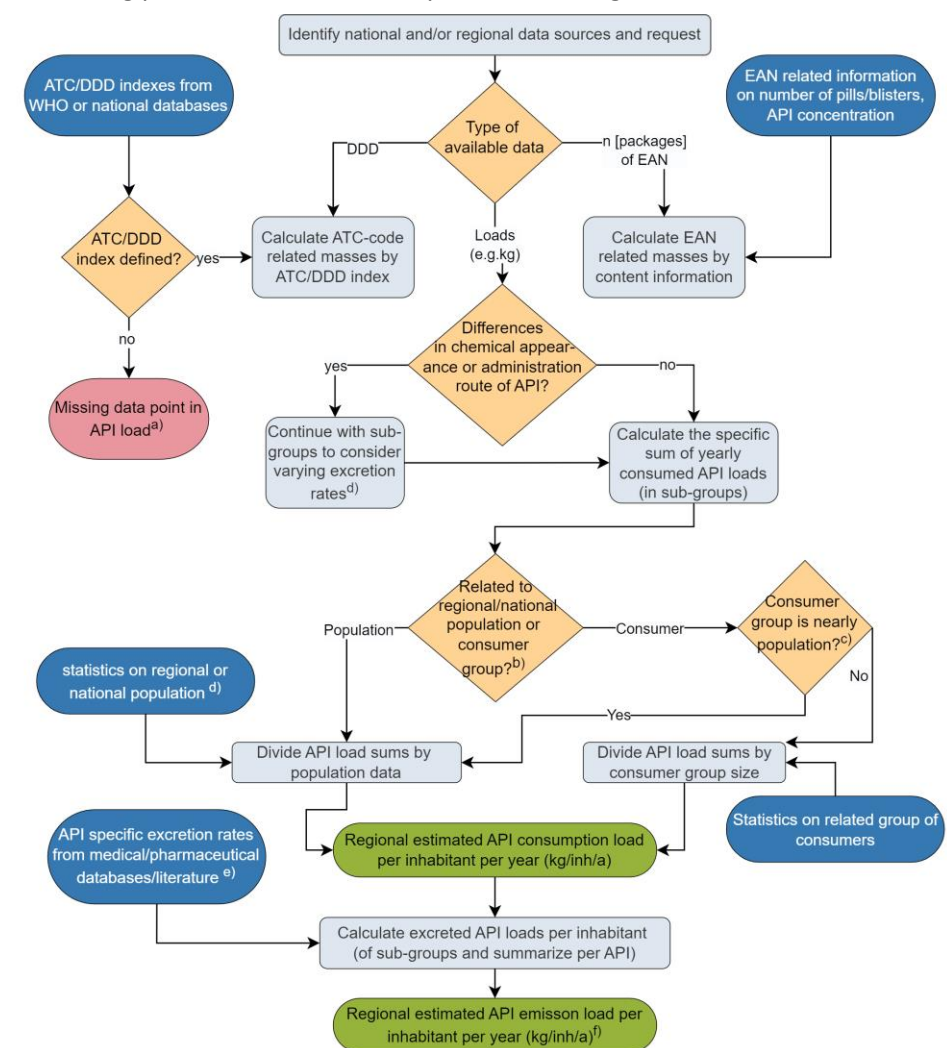
River catchments are highly complex environments which are usually observed in gauging stations regarding flow. Flow varies in seasons, depending on weather conditions, river types, geomorphology and anthropogenic modifications, such as dams, hydropower plants or river regulations. Statistical mean values like yearly average flow and so-called mean low flow are hydrological key numbers we apply for yearly PECs. With standard hydrological methods, it is common to regionalize the flow into subcatchments. The model user is given the option of implementing data produced by e.g. national or regional hydrological models, or using a method incorporated in the APRIORA model, based on a selected range of geofactors such a regionalisation of the flow. The better the resolution of subcatchment flows, the more accurate the model can estimate yearly PECs.

The two outputs of subcatchment flow and WWTP discharge load are combined via accumulation alongside the catchment flow direction towards the Baltic Sea. Accumulation in this context is the transport and

combination of several discharge loads within a river course from upstream to downstream until reaching the Baltic Sea. As a result, API PECs can now be calculated for regional river sections and used for further risk assessment (Module C) and following prioritization of mitigation options.

II: Estimation of discharged pharmaceutical loads

The model estimates pharmaceutical load discharges into the aquatic environment completely based on human consumption statistics and potential removal at WWTPs. In this part II, the whole emission pathway and underlying data is described in detail. Firstly, background information of consumption data and load calculations are provided. A guideline to prepare data calls for potential upgrades and extensions of the data pool aims to improve the outcome and quality of API consumption data sets. Secondly, WWTP characteristics and standard data to be inquired are listed. Within these two chapters, both related data pools of the model are explained and (intermediate) output values are described in detail. Figure B3 shows the procedure for estimating pharmaceutical consumption for the region to be modelled.



^{a)} E.g. for ATC codes representing combinations of at least two APIs there are no ATC-Indexes defined. This might lead to missing a data point showing considerable consumptions.

^{b)} Based on information of data providers, it needs to be clarified to which and how many people these statistics belong to. Usually, national or regional data (e.g. voivodships, federal states) are consolidated. It might occur that consumption is allocated to a specific consumer group, defined by insurance status (e.g. in Germany: legally insured inhabitants appr. 90% of population) or those who are able to receive reimbursement for selected APIs.

^{c)} In some cases, the consumer group is nearly covering the whole population (national or regional). Here, the data acquire can be simplified by using the population statistics as substitute number.

^{d)} Regional and national population statistics are published yearly. Ensure to use either the most recent or the one where consumption data was requested for.

^{e)} excretion rates vary regarding API and also depends on the administration route or chemical appearance of API; e.g. diclofenac consumed as pill is excreted by approximately 2 to 5%, whereas dermal use as cream results in an excretion higher than 90%.

^{f)} The output data for each region/nation calculated in this project will be provided in a data pool as input for further modelling.

Figure B3. Procedure for estimating pharmaceutical consumption.

1. Human consumption to quantify pharmaceutical input

In general, consumption data is usually available either in standard pharmaceutical statistic units or in sales data. Depending on the data source and regarded API, consumption data sets may cover only certain fractions of the total consumption in the countries or regions. Medical agency data, when accessible, is often complete if over-the-counter products are included, whereas public sales reports in DDDs are more or less complete for many APIs except topical applied forms. With the aim of the completest data sets to be processed in the model, extrapolations and interpolations are partly applied. However, to develop a solid consumption load with known potential variances, the meta data is crucial to assess and evaluate. Furthermore, it is important to highlight that each data set is related to a specific consumer group or respective population.

1.1. Classification system, chemical characteristics

In Europe's highly developed healthcare, thousands of pharmaceuticals are available with varying active ingredients and related mechanisms. Already in 1969, the European Market Research Association (EPHRA) developed a classification system for pharmaceuticals. The World Health Organization (WHO) – Collaborating Centre for Drug Statistics Methodology (WHOCC) adapted the EPHRA-classification at international level towards the current anatomical-therapeutic-chemical classification (ATC). Herein, pharmaceuticals are classified in several levels according to their characteristics and application areas:

- Level 1: Main Anatomic group (LETTER)
- Level 2: therapeutic/pharmacologic sub-group (TWO DIGITS)
- Level 3: therapeutic/pharmacologic sub-group (LETTER)
- Level 4: chemical/therapeutic/pharmacologic sub-group (LETTER)
- Level 5: Sub-group of chemical substance (TWO DIGITS)

As a result of this classification, each active pharmaceutical ingredient (API) can be coded by numerous ATCs if it is applied for different purposes in human health care.

In combination with the ATC-codes, a so-called DDD (defined daily dose) value is defined. Only one DDD is assigned to each individual route of administration within an ATC-code. "The DDD is the assumed average maintenance dose per day for a drug used for its main indication in adults." (WHOCC 2018) which is commonly used for pharmaceutical consumption statistics. The DDD is a theoretical value and not necessarily the same as recommended daily dose or prescribed dose. The relation of DDD-consumption numbers and resulting loads can be calculated by the use of the ATC/DDD-Index which provides the conversion factor from DDD to an actual load (unit: g). The ATC/DDD classification system is slightly modified according to different national markets (DIMDI 2017, REF-A).

Based on experiences from previous projects (REF-A), publicly available data in this classification format is generally limited. APIs used in combination products may fall within multiple ATC codes, where the DDD value refers only to one of the components, if available. Thus, combination products cannot be completely covered using statistics in DDDs. A sub-division into different chemical forms of the APIs are not included which have a direct impact on API emissions. For multiple APIs, the chemical form used in pharmaceutical preparations is not the free API molecule. These include e.g. tramadol, which is present in pharmaceutical preparations as tramadol hydrochloride. Some APIs may be on the market in several different chemical

forms. These include e.g. diclofenac (present in preparations as diclofenac sodium, diclofenac potassium, and diclofenac diethylamine) and metoprolol (present in preparations as metoprolol succinate and metoprolol tartrate). Different chemical forms of APIs have different molecular masses. While it varies whether the sales statistics refer to the free molecule or the so-called preparation form of the API, chemical analyses carried out as part of environmental monitoring routinely focus on the free API. Kisielius, Äystö et al. (2024) found that for a set of 35 APIs, the discrepancy in chemical forms may result in an up to 38% error in the load calculated using sales statistics. Thus, whenever possible, it is advisable to gather data on the consumption, distinguishing between different chemical forms.

Another important aspect for load estimation is the route of administration. APIs administered via different routes (e.g. oral, topical, ophthalmological) are absorbed to a varying degree in the human organism. Once absorbed, the human metabolism is responsible for the further API emission pathway. Depending on the design of the API and its route of administration since APIs are provided as pills, injection, topical creams etc, a fraction of the compound is transformed into metabolites. The remaining fraction is excreted as so-called parent-compound. Topical application is perhaps the most notable administration route affecting absorption. For instance, only 6% of topically applied diclofenac has been reported to be absorbed (REF-A). In medical databases (e.g. <https://go.drugbank.com/>), API specific excretion rates are listed as pharmacokinetic characteristic. Additionally, these databases provide information on chemical structures which lead to conclusions regarding environmental behaviours, such as transformation, degradation, potential re-formation of metabolites.

1.2. Preparation of regional consumption data calls

Consumption statistics are the core input data of our model. In some BSR countries, such as Denmark, Sweden and Finland, pharmaceutical consumption data is provided freely in the desired format for environmental discharge estimations, namely in masses per year (e.g. kg/a). These data bases (see Table B1) are usually covering national data.

Table B1. Data sources for pharmaceutical sales in the project countries.

Country	Data source	Is the data public/private?	Data format	Data coverage			
				Time	Area	ATC groups	Route of distribution
Germany	WIdO	public	DDD	Yearly	National	Several	Prescription
Germany	WIdO	public	DDD, kg	Yearly	Federal state	plus combined	Prescription
Germany	IQVIA	private	kg				Prescription+ hospital+OTC
Sweden	Swedish National Board of Health	public	DDD	Yearly	National	several	Prescription
Sweden	Region Skåne	public	DDD	Yearly	Regional	several	Prescription+hospital+OTC

Country	Data source	Is the data public/private?	Data format	Data coverage			
				Time	Area	ATC groups	Route of distribution
Sweden	E-hälsomyndigheten	public	kg	Yearly	National	?	Prescription-OTC
Poland	National Health Fund	public	Number of reimbursed packages	Monthly	Regional	plus combined	Reimburse
Finland	Finnish Medicines Agency	public	DDD	Yearly	National, regional data on request	all with defined DDD	Prescription+hospital+OTC
Latvia	State Agency of Medicines Republic of Latvia	public	DDD	Yearly	National	?	Prescription + OTC
Denmark	The Danish Health Data Authority	public	DDD, Number of sold packages	Yearly	National + regional	?	All

In general, the more regional and distinctive the data points are, the more reliable can the model estimate discharged loads. Despite known limitations of the common ATC classification system, it is very helpful to prepare a good and comprehensive call for consumption statistic data which is not freely available. It emphasizes the potential extent of single data points when APIs are represented in several application classes, and is used to ensure data sets as complete as available. Even in countries with free accessible sources, internal or private regional data sets could improve the input into our model.

Based on different health care systems, the available data sources and their extents vary in each BSR country. Table B1 provides an overview of the five countries in APRIORA and related data sources including meta data. Not only data format is listed but also coverage of data sets. Mainly yearly data is provided, monthly for Poland. National/regional/local data vary as much as the ATC group coverage with defined ATC/DDD-Index or more. The distribution via prescription, reimburse system, hospitals or as so-called over the counter sales (OTC) are key meta data to evaluate the potential completeness of substance related data points.

For data call preparation, this guideline helps to receive meaningful API raw data to be further processed and managed:

1. Select a well-manageable list of API substances (Module A).
2. Use the list in spreadsheet software and attach all ATC-codes to each substance using the WHO data base (https://atcddd.fhi.no/atc_ddd_index/). Here, also ATC/DDD indexes for later transfer into loads can be found for most of the substances.

3. Ask for data of the last completed year.
 - a. Ask for the most regional data covering at least the pilot region completely. For complementary data sets to compensate e.g. the distribution paths (hospital, OTC and so on), also national data sets are valuable.
 - b. Add the following requests about the data set if not known yet:
 - Administration routes (oral, renal, parenteral), alternatively info about pills, injection, cream, etc.
 - data coverage: reimbursed, only prescription, hospital, OTC (free purchase), etc.
 - Group of related inhabitants, national/regional groups, e.g. Voivodship population in the year of investigation
 - c. For some substances, the chemical form should preferably be reported separately, examples from the list of substances considered in the piloting phase of project APRIORA:
 - diclofenac: diclofenac, diclofenac sodium, diclofenac potassium, diclofenac ethylamine
 - estrone: sodium estrone sulphate.
 - metoprolol: metoprolol, metoprolol succinate and metoprolol tartrate.
 - venlafaxine: venlafaxine and venlafaxine hydrochloride

In APRIORA, we requested consumption data in all piloting countries for the year 2023. Our national contact partners reached out to consumption data providers and gathered information for all 10 substances on the APRIORA piloting list (Module A) if available. The selected substances were accompanied with in total 46 ATC-codes. In Finland, the technical system for consumption data statistics is currently under construction (02.09.2023), so a preliminary data set from 2020 is gathered. As soon as the system is running, data will be updated.

The received data is based on fundamentally different health care systems and resulting statistic formats are either in DDD units or counted sold/refunded packages. Equation (a) and (b) show the universal approach for unit's conversion into loads:

$$\text{API (x,y) intake} * \text{ATC/DDD Index} / 1000 = \text{API load (x,y)} \quad (\text{a})$$

$$\text{API (x,z) intake} * \text{doses/package} * \text{packages sold} = \text{API load (x,z)} \quad (\text{b})$$

- X Specific API
- Y ATC-class
- Z EAN product code

Each calculated API consumption load represents a fraction of the total API consumption. If literature provides excretion rates distinguished according to their administration routes (pills, injection, topical) or their chemical form, each API load fraction is multiplied with its related excretion rate and excreted API loads

summarized afterwards. Otherwise, an average excretion rate is used with the total consumed API load. Divided by the number of inhabitants of the data set related population, the yearly excreted API load per inhabitant is calculated (see equation (d)).

$$\text{SUM} (\text{API}_{\text{load}} (x, y_n) * e_x (y_n) = \text{Tot}_{\text{API,load}}(\text{ex}) \quad (\text{c})$$

$$\text{Total}_{\text{API,load}} (\text{ex}) / \text{inhabitants}_{\text{regional}} = \text{API}_{\text{in}} \quad (\text{d})$$

E_x	Excretion rate, API and application specific [%]
$\text{Tot}_{\text{API,load}} (\text{ex})$	Total API load excreted [g/a]
API_{in}	yearly consumed and excreted API load per inhabitant as model input

In case, more than one data set is available for the same year covering overlapping but not identical groups of consumers/ regions, the data then is used to interpolate or extrapolate. As example, a national data set including all distribution paths (legal and private insurance, hospitals and OTC sales) is applied to extrapolate a regional data set related to consumption financed by legal insurances. As a first step, the national data set is transferred into percentage shares of legal insurance data points, see equation (e). Assuming that national numbers are similar to regional distributions, API specific numbers are incorporated in the regional API load sum equation (d), see equation (f).

$$\text{API}_{\text{intake, nat}} (\text{legal insurance}) / \text{API}_{\text{intake, nat}} (\text{all distributions}) = \text{API}_{\text{intake, nat, \%}} \quad (\text{e})$$

$$(\text{Total}_{\text{API,load}} (\text{ex}) / \text{API}_{\text{intake, nat, \%}}) / \text{inhabitants}_{\text{regional}} = \text{API}_{\text{in}} \quad (\text{f})$$

$\text{API}_{\text{intake, nat}}$ (distribution)	API intake based on national data set according to specific distribution path(s)
$\text{API}_{\text{intake, nat, \%}}$	API intake based on national data set according to specific distribution path(s) [%]

1.3. Optional input extensions for API sources or consumer groups

One big advantage of simple model approaches is the low hurdle to expand, update and adapt according to (new) boundary conditions in the case study areas. We are aware that average consumption data represents the main API input path but not always the single one. However, there are multiple ways to integrate further relevant API sources and their pathways with either slight adaptations of our calculations or substituting numbers as add-on loads. Here, we describe four optional input extensions related to further API sources or consumer groups.

Disposed unused APIs: Consumption statistics assume that each API handed out is consumed completely. In many previous research projects, it was shown that varying amounts of APIs was disposed irregularly into the sink/toilet and reached the sewer system (REF-A). Even though campaigns raise awareness and unused pharmaceuticals can be returned in pharmacies, there is still a fraction disposed unused. If knowledge about API specific disposal compared to consumption is available, this fraction is easily added up to the total API load without any excretion rate (= set to 100%) in equation (c). Since the API does not pass the human organism, no metabolism can reduce the API and it reaches completely the sewer system. This approach was

previously implemented by Äystö et al. (2020). According to their modelling exercise, improper waste management can be especially relevant for extensively metabolized APIs, such as ibuprofen and carbamazepine. According to the data source, a dependency on inhabitant characteristics may lead to a finer subdivision of unused API loads. This API path is most relevant for liquid substances, e.g. injections or cough syrups (REF-A). For the APIs selected in APRIORA, most of them do have a commonly used liquid formulation meant to be used by non-healthcare professionals in the Baltic area, estrone, metoprolol, sulfamethoxazole and venlafaxin being the notable exceptions. It is considered that liquid formulations meant to be administered by healthcare professionals (e.g. solution for infusion) would generally be properly disposed of.

Tourists: Tourism may influence the domestic wastewater composition and quantity seasonally. Usually, the tourism sector publishes regularly regional statistics about overnight-stays, day-tourism and other details. Evenly distributed or logically allocated, extra demands for wastewater treatment can be estimated. With the assumption that all tourisms are well represented by a regional average inhabitant consumer, we can transfer this information. This tourism data is implementable in the model as temporal “additional inhabitants” to extrapolate previously calculated API inputs according to (d). The model could run once without and once with tourism integration. Seasonal wastewater volume fluctuations at specific WWTP are later used for a plausibility check.

Hospitals: It is well known that in medical care, larger and different amounts of APIs are consumed. In case no consumption data is available for this distribution path, hospital’s additional API input can be estimated using statistical standard data of medical care in regions of interests. In yearly reports, the general capacity, utilization of beds and ambulant treatments are allocated to specific hospital operators. Combined with their localization, either hospital consumption data or estimates can be allocated directly to a specific WWTP (see 2.2).

Hormones: Estradiol (E2) is a common contraceptive API, used by females in the fertile years, which is in part metabolised into estrone (E1) and subsequently excreted as estrone. Since it is most often not possible to receive related consumption data via statistics described in this chapter, regional demography data and average consumption assumptions can be used to estimate hormone consumption in catchment regions.

1.4. Intermediate output: Data pool with regional average consumptions per inhabitant

The API input data pool shows categorized yearly average API consumption loads in the unit kg/a/inh. (=kilogram/year/inhabitant). The following categories are noted and will help to select the most appropriate load for the region of interest:

- API, pharmaceutical substance
- year of consumption statistic
- country
- region, e.g. Federal State, Voivodship, Province
- source
- estimated coverage of population

The API input data pool can be updated with the years, extended by polluting substances or countries/regions in the BSR and corrected quite easily. This flexibility requires expert’s maintenance but offers further

application options of the model itself. With a selected API input load in kg/a/inh., the model returns to the catchment level and allocates loads to WWTPs.

2. Pharmaceutical point sources: WWTPs discharge API loads

In general, sewer systems collect and transport domestic wastewater for treatment in small to large WWTPs nearby. The degree of connectivity is usually higher in rural areas than in urban areas. The remaining wastewaters are either collected in smaller ponds or private septic tanks and regularly transported to WWTPs by tank trucks or discharged treated directly into the environment. This supports the APRIORA approach to consider WWTPs as main emission points which will be quantified. The average consumption loads can now be directly linked to local WWTPs with specific characteristics to be collected and classified.

2.1. WWTP data requirements

According to EU reporting requirements in the UWWTD, many key facts of WWTPs are collected and visualized in the UWWTD map, latest published in November 2021 (see <https://www.eea.europa.eu/themes/water/european-waters/water-use-and-environmental-pressures/uwwtd/interactive-maps/urban-waste-water-treatment-maps-3>). This is only the case for WWTPs larger than 2,000 p.e. meaning that information of smaller plants is missing. In many cases, the local/regional EPA in charge has main data in place of all WWTP size classes related to further national or regional reporting duties. For additional information, the operators or associations can support with their expertise.

Table B2 summarizes the WWTP data requirements sub-divided into mandatory key facts and optional information accompanied with formatting examples. Yearly averages can always be supported with seasonal subdivision if relevant. For monitoring campaign preparation, required records during sampling campaigns are noted to implement monitored API concentrations into the model. This listed data is further used to estimate inflow and outflow loads of WWTPs. Potentially relevant additional emission sources or groups and related data are described in 2.3.

Table B2. WWTP data

Parameter	unit	description	example (year 2022)	sampling records	Mandatory?
WWTP_name	-	usual name for identification/location	KA Schwaan		Yes
ID	-	ID according to EU, national and or regional standards	GID: 691		Yes
Location, alternatively:	Lat./ Long .	geographical location of plant, define CRS, (e.g. WGS84 or ETRS89)	5981822,33310302		Yes
Location_dis.	Lat./ Long .	geographical location of discharge point into receiving waters	5981771,33310353		Yes
inh._conn.	-	real connected inhabitants, either via sewer system or manually collected wastewater	7011 inh.		Yes
cap.	p.e.	treatment capacity	13000 p.e.		Yes

Parameter	unit	description	example (year 2022)	sampling records	Mandatory?
utilization	p.e.	actually used capacity of plant; yearly average	10.767 p.e.		Yes
tech-class	1-5	degree of technical treatment of plants, classified according to UWWTD Article 2 (primary, secondary, tertiary/appropriate)	1 – primary 2 – secondary 3 – appropriate/tertiary		Yes
Q_WWTP	m ³ /d	daily treated wastewater volume; yearly average	314.485 m ³ /a	yes	Yes
SRT	days	sludge age, sludge retention time	n.a.		No
T	°C	Temperature	n.a.	yes	No
pH	-	how acidic/basic is the sample	n.a.	yes	No
COD	mg/L	chemical oxygen demand, yearly average	inflow: 1.393,4 mg/L outflow: 32.46 mg/L	yes	No
P_tot	mg/L	total phosphorus concentration; yearly average	inflow: 22.95 mg/L outflow: 0.24 mg/L	yes	No
TOC	mg/L	total organic carbon concentration; yearly average	n.a.	yes	No
NH4	mg/L	ammonium concentration; yearly average	inflow: 50.94 mg/L outflow: 0.997 mg/L	yes	No

According to usual wastewater inflow volume variations hourly, daily and seasonally, also concentrations of pharmaceuticals vary. API input is mostly limited to yearly data applied in the model so that these variations cannot be shown reasonably. Additionally, average API consumption data is provided in loads (kg/a). Therefore, we follow the API path from sewer systems towards the environment consistently in loads, too. To estimate incoming and discharged API loads, WWTP's characteristic data are determining.

In case, API monitoring data is available for selected WWTPs in the investigated river catchment, experts can replace classified estimations (see 2.3) with actual WWTP's data. Preferably converted into loads using the monitoring flow volume at the sampling day, the API concentration data in the inflow and outflow can be implemented using equation (g).

$$API_{WWTP, inflow} \text{ concentration } (d_s) * Q_{WWTP, inflow} (d_s) = API_{WWTP, inflow} \text{ load} \quad (g)$$

$API_{WWTP, inflow}$ API concentration [g/L] or load [g/d] monitored at the WWTP inflow of a specific monitoring day d_m

$Q_{WWTP, inflow}$ WWTP inflow [L/d] of a specific monitoring day d_m

2.2. Determination of API inflow loads

Generally, WWTPs receive wastewater, surface run-off and/or mixed water depending on the sewer system structure. Besides households, also industries discharge wastewater with highly diverse characteristics. All these components are represented in the yearly reported standard flow volume and load in the unit p.e.. Also given in p.e., the WWTP capacity was estimated for construction planning and discharge permission processes. With regard to API inflow load calculations, both standard data points would lead to an overestimation. Assuming that additional loads according to chapter 1.3 are quantified extra, the real connected inhabitants are the crucial data points to link API input with individual WWTPs. The API inflow loads is therefore determined by the following equation (h):

$$(h) \quad API_{inflow}(WWTP) = \sum \left(\frac{API_{input} \times Inh_{con}(WWTP)}{extr_{to} + extr_{tr} + \dots} \right) \quad , \text{ where}$$

API_{input} = yearly consumed and excreted API load per inhabitant as model input [g/inh/a],

$Inh_{con}(WWTP)$ = Connected inhabitant of WWTP,

$extr_{to}$ = Extra load based on tourism [kg/a],

$extr_{tr}$ = Extra load based on additional wastewater deliveries/transport [kg/a],

$API_{inflow}(WWTP)$ = Total yearly WWTP API inflow load [kg/a]

In some cases, local expertise might improve the estimation by better information about incoming wastewaters and should be checked by WWTP operators.

2.3. Determination of API outflow loads

The potential reduction of API pollution loads at WWTPs is represented in the so-called removal rate. Given in % or decimal, this value shows the ability of an individual WWTP to remove the API from the aqueous phase and resulting lower concentrations and loads in the discharged treated wastewater. Since individual WWTP removal data are sparse, API specific removal rates are collected from literature. Furthermore, concentrations measured during the APRIORA piloting phase will be utilized to derive additional removal rates. Research projects, feasibility studies and case studies often provide a removal potential of existing technology to be compared with upgrading technologies for micropollutant removals, such as ozonation and activated carbon (REF-A). These removal rates vary considerably, even for single APIs. Therefore, a subdivision into technical classes (compare Table B2) categorize collected removal rates. This is the basis for linking WWTP to API removal rates of our provided data pool.

The API removal rates data pool shows categorized and API specific removal rates in a decimal number. The technical categories are supported by the following information if available and will help to select the most appropriate rates for the WWTP of interest:

- Range of rates
- Number of data points
- API, pharmaceutical substance

- Technical classification of sampled WWTP(s)
- country
- related project
- source

If the WWTPs are categorized as suggested, removal rates for selected APIs are directly suggested. We aim to keep this link manually adjustable so that experts can test mitigation options (see 2.4) or insert own, individual WWTP monitoring data, pre-calculated removal rates explicitly here. After selection of a removal rate, the discharged API load by individual WWTPs is calculated as shown in equation (i):

$$(i) \quad API_{dis}(WWTP) = API_{inflow}(WWTP) \times (1 - RR_{API}(WWTP)) \quad , \text{ where}$$

$API_{dis}(WWTP)$ = yearly WWTP API discharge load [kg/a],

$RR_{API}(WWTP)$ = Technique-specific API removal rate [-],

$API_{inflow}(WWTP)$ = Total yearly WWTP API inflow load [kg/a]

2.4. Mitigation options at WWTPs

The model approach aims not only to show the current status of pharmaceutical pollution in the catchments but also potential mitigation scenarios I, II and III. Besides source-oriented reduction of API consumption, end-of-pipe options can be implemented mostly at WWTPs to directly reduce the emission loads into the aquatic environment. Once mitigation options are identified, their effect on risk assessment should be a main prioritization criteria. Therefore, this model is applicable to test these scenarios and directly affects the WWTP's API discharge load as output.

In close collaboration with associated partners, namely WWTP operators in the piloting areas, realistic mitigation options were discussed. Upgrading a WWTP with a fourth treatment step to remove API and other micropollutants is widely recommended by the two technologies ozonation and activated carbon. For these techniques, numerous published studies listed API specific removal rates at upgraded WWTPs. Studies and databases were scanned for removal rates for the 10 APIs included into APRIORA piloting phase. These rates can be selected in our API removal rates data pool and attached to relevant WWTPs for running the model with this upgrading mitigation scenario (Option I).

Besides upgrading techniques, the model can also show mitigation by changes in the infrastructure. Potential options discussed with operators are either wastewater re-allocation to other larger WWTPs with higher removal rates (Option II) or redirect the discharge point of a WWTP to a larger or less-sensitive receiving water body (Option III).

In the first case, the total WWTP discharge loads into the catchment are likely reduced. Even though the risk is reduced within the previous receiving water body, it may occur that the risk is increased for the larger WWTP treating higher pollution loads. In the second case, the total WWTP discharge load is not decreased but moved to water bodies are less sensitive due to higher flow volume, no relevant usage risks or else. In the model itself, the applicant can change manually the WWTP discharge loads, redirect WWTP input and

discharge location data to test these two infrastructure mitigation options. After these changes, the model shows the effects in the risk assessment (Module C).

However, the operator needs to evaluate the potential mitigation options if they are appropriate and integrable into current treatment techniques including local specifications. Only after operator's feedback, a model mitigation scenario is reasonable to support prioritization processes.

3. Output: WWTP discharge loads of API

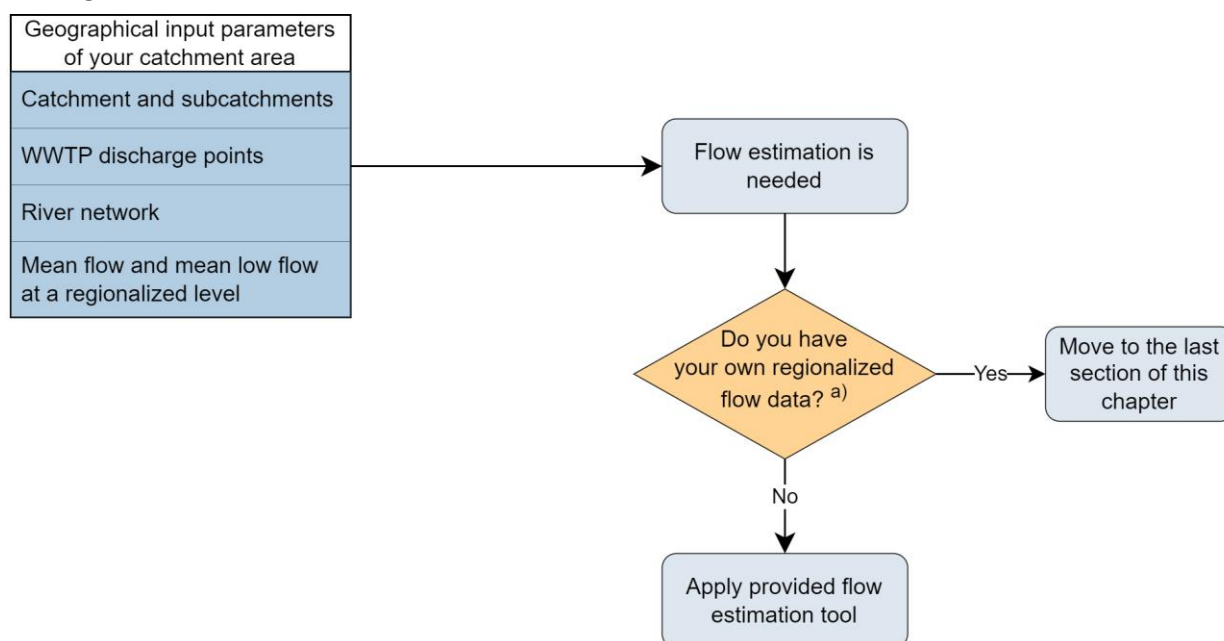
Based on statistical data, interdisciplinary background knowledge regarding pharmaceuticals in pharmacology, medicine and wastewater treatment, the emission pathway via WWTPs was tracked and quantified with the best available input. As a result, each WWTP located in the catchment area and discharging treated wastewater into the water bodies is accompanied with the key information of pharmaceutical pollution, the yearly API discharge load calculated according to equation (f), in chapter 2.3. This so-called attribute contains the following information:

- API name, abbreviation
- Local discharge load in kg/a
- Scenario: current status or mitigation option number
- Comment field

The API discharge load is calculated for 3-5 APIs in parallel or 3-5 mitigation scenarios, respectively. In this way, the results, especially regarding risk assessment are directly comparable and can be checked for plausibility. This geographically referenced output of API discharge loads represents the pollution quantity and links human input with the aquatic environment.

III: Estimation of regionalized average river flows

Emission data of API are in most cases only available as yearly average, a higher time resolution for flow is not required. However, we need PEC as a mean yearly concentration and for ecologically relevant low flow conditions. Accordingly, flow estimation aims at estimating the key numbers mean flow and mean low flow at a sufficient spatial resolution. The aim of this chapter is to describe how to develop a geographical layout of a catchment, which includes the estimation of river flows at a regionalized level. By regionalized, we mean splitting the catchment into smaller, manageable subcatchments. This allows us to estimate river flow at these subcatchment levels, taking into account the spatial variability of factors such as precipitation, land use and topography. Figure B4 shows a flowchart describing the entire process. It begins with a box listing the basic datasets required (in detail described in Section 1) to create the geographical layout. Most of the data listed here should be easy to obtain, but one potential challenge is getting the mean flow and mean low flow at a regionalized level.



^{a)} By "regionalized flow data", we refer to estimated values for both the mean flow and mean low flow at a subcatchment level.

Figure B4. Flowchart illustrating the process of developing the geographical layout of the catchment and flow estimation.

There are two possibilities for estimating the flow: if the user have access to regionalized flow statistics as geographical data (e.g. shapefile), they can use this information directly and move to the last section of this chapter. If not, a standard approach is provided based on data from gauging stations and so-called geofactors to estimate the flow. The expected output is a shapefile showing average yearly flow and average yearly low flow for each subcatchment within the catchment. This is a one-time task and once the user has created the shapefile, they will not need to repeat the process.

1. Boundary conditions

In order to develop a comprehensive understanding of catchment characteristics for modelling the emission pathway, a complete set of spatial and hydrological data is required. The key data categories include: catchment and subcatchment boundaries, WWTP discharge location, river network structure, mean flow and

mean low flow at a regionalized subcatchment level. In this chapter it is described what it is needed for this process before moving onto the next part of the document.

1.1. Catchment, river network and WWTP

One of the key pieces of information required for the model application process is the catchment area and its subdivision into smaller subcatchments. This data should be provided as a vector file containing polygons that represent both the overall catchment area and its subdivided units. Each subcatchment works as a standard unit for localized analysis and modelling of water flow and pollutant loads.

In addition to the catchment data, it is necessary to include the location of the waste water treatment plant (WWTP) discharge points. These points should be provided as point vector data, indicating the precise location of WWTP discharge sites. Mapping the discharge sites is fundamental for assessing the impact of API on the receiving water bodies. By analysing these points, it is possible to better understand how the treated waste water interacts with the river system and to simulate downstream effects on water quality.

Since the focus is on water bodies and how pollutants move, it is crucial to have detailed information about the river network. The river network constitutes the primary pathway through which water and pollutants flow. Understanding the geometry and connectivity of this network is key to simulating hydrological processes and predict how APIs may be distributed along the system. This data should be provided as a polyline vector file.

In Figure B5 it is illustrated an example from the Warnow catchment (test case of Germany) on how the data should look like.

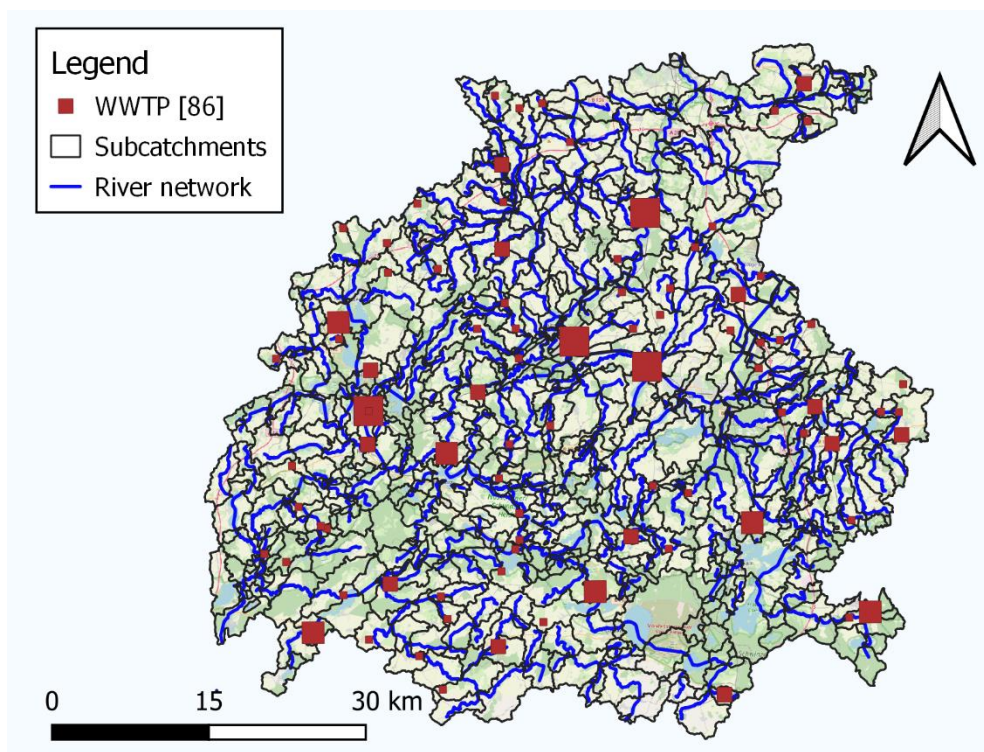


Figure B5. Warnow catchment and its subcatchments, river network and WWTP within the basin.

1.2. Statistical flow data: mean flow, mean low flow at a regionalized level

When developing a geographical layout of a catchment, accurate flow data is crucial. For this approach, data related to mean flow and mean low flow of each subcatchment will be necessary. Our model offers a method for estimating both of the requirements, but it is recommended to use regionalized data provided by local experts whenever possible. The regional experts possess a deeper knowledge of the specific catchment characteristics and typically offer more reliable estimations. It can happen that the flow data produced by the local experts might require further subdivision into smaller subcatchments to better allocate the discharge point from WWTPs into the river system. However, this is manageable and the model is capable of handling this type of processing. The required data format is a vector file containing mean flow and mean low flow values for each subcatchment. While Section 2 will discuss our approach to flow estimation, if you already possess the regionalized flow data you can skip ahead to Section 3.

2. Flow regionalization with biota approach – how it works

Regionalization is a method used to predict hydrological behaviour in ungauged catchments by transferring model parameters from gauged catchments to similar ungauged areas. This approach is mainly used in areas where there is a lack of direct measurements.

In this study, for flow regionalization, it was decided to adopt a methodology developed by Biota, an environmental institute based in Bützow, Germany (REF-A). This approach has been successfully tested in the Mecklenburg – Vorpommern region and it consists of a statistical model that correlates subcatchments with similar characteristics to predict similar flow patterns. The estimation process involves a non linear regression equation that uses several geofactors calculated for each subcatchment as input. The model's output is a vector file containing the estimated mean flow and mean low flow values for each subcatchment. To guarantee accurate predictions, water level data from gauging stations within the basin are essential for both model calibration and validation. This method comes up with robust estimation of flows, even in areas where direct measurements may be limited.

2.1. Input of the model

For the flow regionalization, the input data is divided into two categories: required data that the user must provide and optional data that the user can choose to provide. If the optional data is not given, the model will automatically source the necessary information from existing accessible datasets. The general advice is to use the user's data (if they are available) in order to obtain better performances of the model. Table B3 shows a list of the required and optional input data.

Table B3. List of required and optional input data.

Input data	Format	Data requirement
Catchment and subcatchments	Vector	Required
River network	Vector	Required
Water level data at gauging stations	Vector	Required
Digital Elevation Model	Raster	Optional
Land use/land cover map	Raster/vector	Optional

Soil permeability	Raster	Optional
Precipitation	Raster	Optional

Water level data from gauging stations is necessary for the calibration and validation of the model, while the other datasets are essential for calculating the geofactors. These geofactors are required as input of the non linear regression equation, as mentioned earlier in Section 2. In case, no regional flow data is available, Annex B1 provides an overview of relevant open-source databases with suitable input data applicable for flow estimation. However, it should be noticed that regional data is preferred.

2.1.1. Catchment and subcatchments

The catchment features were already introduced in Section 1.1 but it is necessary to understand their importance in the flow regionalization model as well. The vector file of the catchment, along with its subdivision into smaller subcatchments, is not only essential for developing a geographical layout of the catchment but also plays a key role in the flow estimation process. These subdivisions allow to calculate specific “geofactors”, which work as inputs to the model and represent important characteristics of the catchment.

Some of these geofactors include perimeter, area and form factor. While the perimeter and area are straightforward, the form factor requires a deeper explanation. It is calculated using the following formula:

$$k = \frac{P^2}{2 * \pi * A}$$

Where k is the form factor, P the perimeter of the catchment and A the area of the catchment. This parameter gives an indication of the catchment’s shape, which can influence flow characteristics.

Moreover, the catchment and subcatchments are critical in subsequent steps because they define the spatial boundaries for other processes. For instance, when working with a raster file covering all of Europe, having a catchment vector file allows for targeted analysis within the area of interest.

2.1.2. River network

Like catchment and subcatchments in Section 2.1.1, the features of the river network were already discussed in Section 1.1, but it’s important to highlight their role in the flow regionalization model as well. The vector file of the river network allow to calculate the geofactor “water body density”, which is defined as the total length of all watercourses relative to the catchment area. This parameter is measured in km/km² and provides insight on the density of the river network within a catchment.

Additionally, the river network is needed for modelling how the flow is distributed within the basin. Together with the WWTPs discharge points, it helps split API concentrations across different part of the river system. This guarantees a more realistic representation of how pollutants disperse, with a weighted average calculation that reflects the actual conditions in different segments of the river.

2.1.3. Water level data at gauging stations

The model requires mean low flow and mean flow data from the gauging stations within the catchment. This data should be provided in a shapefile that includes the locations of the gauging stations along with the

calculated mean low flow and mean flow values for each station. These average values should be calculated between 1991 and 2020 (period selected for this study). A higher number or density of gauging stations within the catchment improves the performance of the model and leads to more accurate output estimations. Below there is an example of the data format, including a map showing gauging stations across the Warnow catchment (Figure B6) and a sample of flow data from six gauging stations (Table B4).

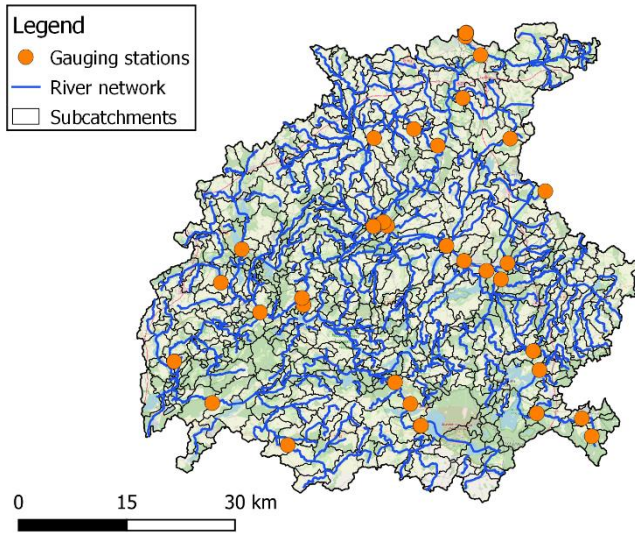


Figure B6. Location of the gauging stations across the Warnow catchment.

Table B4. Sample of water level data from six gauging stations within the Warnow catchment.

Station name	MQ [m ³ /s]	MQ _{Aug90} [m ³ /s]
Schwaan Beke	1.80	0.21
Wolken	4.30	0.83
Kessin	0.50	0.05
Reez	0.21	0.02
Bützow Gesamt	7.17	1.33
Rostock-Geinitzbrücke	14.56	2.40

The water level data converted into flow data from the gauging stations is essential for calibrating and validating the model. In most cases, the mean flow (MQ) and mean low flow (MQ_{Aug90}) values are not directly available and require additional calculations. The data from the gauging stations is typically available in tabular format, representing daily values. Therefore, further processing is necessary to calculate mean flow and mean low flow from the raw data. In the following section, it is explained an example from Germany of how the tabular data is processed to quantify the mean flow (MQ) and the mean low flow (MQ_{Aug90}) values.

a) Mean flow (MQ)

The mean flow is calculated as the average of the daily flow values from the entire time series (1991 - 2020). It is defined by the formula:

$$MQ = \frac{\sum Q_d}{n}$$

With MQ as mean flow, Q_d as daily flow values from the gauging station and n as the total number of daily values.

b) Mean low flow (MQ_{Aug90})

The mean low flow in the German example is determined by calculating the 90% exceedance probability of the mean August flow (MQ_{Aug}) over the study period (1991 - 2020). The steps are as follows:

1. Calculate the average flow in August for each year within the study period.

- Sort the 30 MQ_{Aug} values for each year in descending order.
- Fit a regression line $MQ_{Augp} = a \cdot p + b$ for the lowest 7 MQ_{Aug} values, corresponding to ranks 23 - 29 (Figure B7). To avoid possible outlier effects, the lowest ranked value is not taken into account. The probability of exceedance p is defined as follow:

$$p = \frac{m - 0.4}{n + 0.2}$$

Where m is the rank of the respective value and n is the total number of values.

- Determine the MQ_{Aug90} value for each gauge with $p = 0.9$ using the regression line MQ_{Augp} .

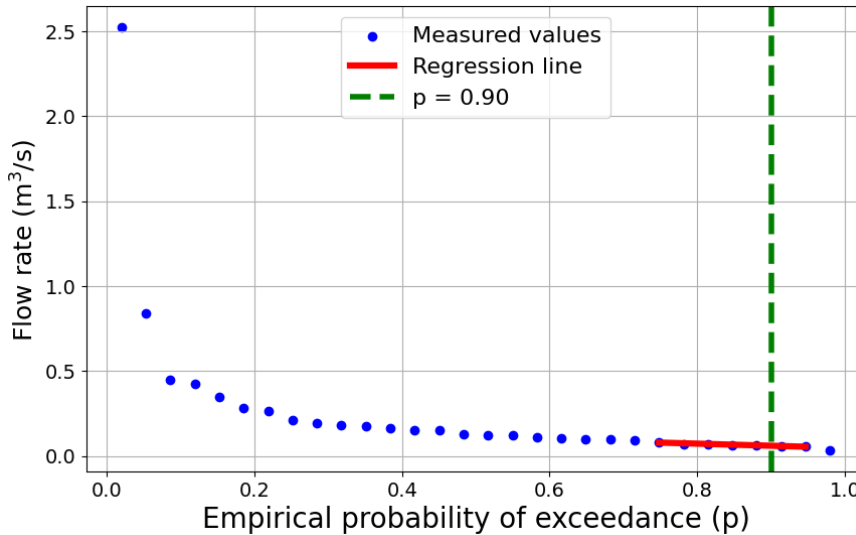


Figure B7. Determination of the discharge value with a 90% of exceedance probability (MQ_{Aug90}) using the example of the Kessin/Kösterbeck gauging station.

2.1.4. Digital elevation model

Another important dataset for the approach is the Digital Elevation Model (DEM) of the catchment. This data should be provided as a raster file and is essential for calculating several geofactors: average height, minimum height, standard deviation of height, average slope and standard deviation of slope. These features describe the catchment's topography and act as key inputs during the flow estimation process.

2.1.5. Land use/land cover map

The land use and land cover map is a database that classifies the landscape into different categories such as forests, settlements and water bodies. This data is useful for understanding how different land cover types can impact hydrological processes within the catchment. It should be provided as either a vector or raster file and it is necessary for calculating geofactors such as forest share, settlement share and the proportion of water area. These features are determined by comparing their extent to the total catchment area and are expressed as percentages.

2.1.6. Soil permeability

Soil permeability is a dataset that contains information on clay content at various depths. This data should be given as a raster file and is used to calculate the percentage of permeable soil within the catchment. This

geofactor significantly impacts flow estimation, as areas with higher soil permeability allow more infiltration, influencing both surface run-off and groundwater recharge.

2.1.7. Precipitation

A key dataset is the one related to precipitation. This data should be provided as a raster file and should cover the period from 1991 to 2020. It is needed to calculate the average annual precipitation and the average August precipitation within the catchment.

2.2. Intermediate output: list of geofactors

Table B5 summarises all the geofactors explained in the previous paragraphs with the unit and the database source.

Table B5. List of geofactors necessary for the model. () Temporal extent 1991 – 2020.*

Geofactors	Unit	Data source
Perimeter	km	Catchment geometry (2.1.1)
Area	km ²	Catchment geometry (2.1.1)
Form factor	—	Catchment geometry (2.1.1)
Water body density	km/km ²	River network (2.1.2)
Mean flow and mean low flow*	m ³ /s	Gauging stations (2.1.3)
Average height	m	DEM (2.1.4)
Minimum height	m	DEM (2.1.4)
Standard deviation of height	m	DEM (2.1.4)
Average slope	%	DEM (2.1.4)
Standard deviation of slope	%	DEM (2.1.4)
Forest share	%	LULC (2.1.5)
Settlement share	%	LULC (2.1.5)
Proportion of water area	%	LULC (2.1.5)
Percentage of permeable soil	%	Soil permeability (2.1.6)
Average annual precipitation*	mm	Precipitation (2.1.7)
Average August precipitation*	mm	Precipitation (2.1.7)

2.3. Non-linear multiple regression model

In this approach, a nonlinear multiple regression equation (Eq. 1) is developed using a stepwise regression with forward selection approach. The forward selection process starts with an empty model and sequentially adds explanatory geofactors (listed in Table B5) based on their statistical significance. In each iteration, the geofactor with the highest correlation to the remaining unexplained variability (residual) is incorporated into the model. This guarantees that each added geofactor is the one that most improves the model's ability to estimate the target variable.

Eq. 1
$$MQ(catch) = a_0 + a_1 * f_{1a}(GF_{1a}(catch)) + \dots + a_n * f_{na}(GF_{na}(catch)),$$
 , where
or
$$MQ_{Aug90}(catch) = b_0 + b_1 * f_{1b}(GF_{1b}(catch)) + \dots + b_n * f_{nb}(GF_{nb}(catch))$$
 $MQ(catch)$ = Mean flow of the observation period in the unobserved catchment area,
 $MQ_{Aug90}(catch)$ = Mean low flow of the observation period in the unobserved catchment area,
 a_x, b_x = model parameters,
 $f_x(x)$ = non-linear variable transformation,
 $GF_x(catch)$ = geofactor of a catchment area as an explanatory variable of the model

2.4. Calibration and validation

Calibration and validation are critical steps in ensuring the accuracy and reliability of the flow estimation model. Calibration involves identifying a unique set of model parameters that accurately reflect the model's behaviour, and it can be achieved by comparing model predictions with actual measurements. In this scenario, it is used water level data from the gauging stations described in section 2.1.3.

Validation, on the other hand, is the process of testing the calibrated model with independent data that were not used during the calibration phase. Typically, the dataset is split into a training set (around 70 - 80% of the data) for calibration and a test set (20 - 30%) for validation. During both calibration and validation, performance metrics such as R^2 (coefficient of determination) and NSE (Nash – Sutcliffe efficiency) are used to evaluate the model's accuracy.

3. Output of the model

The main outputs of the flow regionalization model are the mean low flow and mean flow values for each subcatchment. These outputs are crucial for the next step in the process that is calculating the concentration of API in the rivers. The results are provided in vector file format and expressed in m^3/s . Figure B8 shows an example where the data is displayed as a mean cumulative discharge (REF-A). A cumulative value that represents the mean discharge from all contributing upstream subcatchments is assigned to each subcatchment.

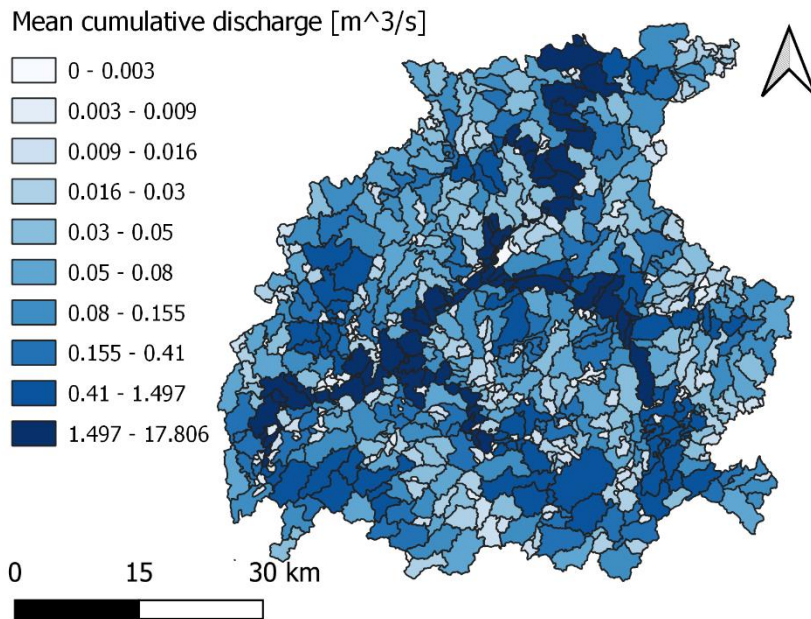


Figure B8. Mean cumulative discharge of every subcatchments within the Warnow area.

When a wastewater treatment plant (WWTP) is located within a subcatchment, a further subdivision of the subcatchment may be necessary. In this scenario, the river is segmented at the WWTP's discharge point to better localize the emission source and assess the corresponding risk. To estimate the flow at the WWTP's discharge point within the subcatchment, the following approach is proposed: since the flow values at the upstream and downstream boundaries of the subcatchment are known, a weighted average can be applied. This weighted average would be based on the relative area of the upstream portion of the subcatchment, providing an estimate of the flow at the discharge point.

To summarize, the model output is a shapefile containing the mean low flow and mean flow values for each subcatchment, expressed in m^3/s . In special cases, such as when a WWTP is located within the subcatchment, a further subdivision is applied. Using a weighted average based on the area, the mean flow and mean low flow can also be estimated at the WWTP's discharge location.

IV: Pollution loads and concentrations in the environment

In the environment, pollution is caused by either constant release of low to medium loads of hazardous substances or accidents with very high loads. Related to APIs, the constant discharge of pollution loads by local emission points of API is the main source. It is important to consider that API emissions also have a downstream effect and accumulate. At the same time, they are further diluted by the flow in the receiving river water bodies. If not completely persistent, the API loads are also reduced by biodegradation processes. This results in API concentrations, diverse in different river sections, regionally different. This process of estimating PECs (predicted environmental concentrations), if not monitored, is further described by combining output from parts II and III. Additionally, one of the considered mitigation options in our approach is linked to the WWTP discharge locations.

1. River flow accumulation of upstream contributing subcatchments

Once regional flow of subcatchments are estimated either for gauged or ungauged catchments, the river network of the complete catchment becomes relevant again for accumulation purposes. It is crucial to define the flow direction meaning that for each river section in a subcatchment source and discharge ends are known. In other words, information about upstream and downstream subcatchments are required and need to be provided as attribute attached to the subcatchment area or the river itself. For example in the German data set of the river network, this information is embedded in a specific code indentifying upstream sections. QGIS and similar programmes also contain tools for this so-called “routing” process in case flow direction is missing or identifiers/attributes are not feasible to be further interpreted. According to flow direction, the upstream flow for each subcatchment including own contributing flow can be calculated and attached as information to the regional discharge point.

With the information of accumulated regional flow, the environmental conditions are described with a high spatial resolution. However, this model approach simplifies temporal variations and applies standard flow characteristics on an annual basis, namely yearly flow and yearly low flow to indicate seasonal variation.

If flow is monitored during seasonal monitoring campaigns, measured flow data can be compared to modelled accumulated flow data at the same location. Considering the monitoring season, either mean flow or mean low flow (MQ/MNQ) should be selected to check for plausibility. If all measurements indicate deviating ranges of flow, the check reveals a need for adjustment in the model for this specific pilot.

2. Merging environmental conditions with distinct emissions

Finally merging two different input data types, namely spatial and point emission data, the model profits from a sufficient regional resolution of the environmental flow conditions. Technically, the point information of WWTPs is projected onto the river catchment by the location described in coordinates. Depending on the accurateness of the data set, the closest vicinity of WWTPs to water bodies are applied. Here, it makes sense to re-ensure the correct discharge location with local WWTP operators. By this projection, the WWTP specific informations such as yearly API emission loads (kg/a) are transferred to the environment. One of the described mitigation options (OPTION III, see II.2.4) interferes directly at this point: If considered as reasonable and realistic by the WWTP operators, the discharge location may change due to infrastructure changes. For example, if an alternative recipient in reachable distance has higher dilution capacities, a potential bypass can be used as a mitigation scenario in the model.

As explained in III.3, at least upstream and downstream of our point emission sources WWTPs, a catchment or river is subdivided. This enables the model to insert the WWTP emissions into the beginning of receiving river sections. As a consequence, we are able to model downstream effects of the API emission loads in the whole subcatchment and avoid to estimate unlikely/non-occurring upstream load effects.

With a worst-case assumption that no reduction of API loads flowing downstream occurs in the aquatic environment, also emission loads can now be accumulated alongside the river network. At each discharge point of a subcatchment and river section, an accumulated yearly API load, flow and low flow are now determined. By dividing API load with flow, the predicted environmental concentration is calculated (=PEC).

3. Options to include degradation, transformation, and sedimentation into the model

For many API substances, it is known that natural processes in the aquatic environment occur and have an impact on API concentrations. Conceptionally, the complex processes can be split into photo-degradation and various biological transformation processes. These very complex processes are mainly dependent on residence time in the medium or transport distance and can be expressed in a simplified way by first order degradation kinetics. We aim to apply the CWPharma approach, combining two exponential degradation terms as function of residence time. The residence time needs to be estimated from the regionalised flow data, river section length and estimate of the average cross section. Further details are provided in CWPharma (2020). In the model, these reductions of API loads or concentrations are integrated as alternative route to the worst-case assumption for selected APIs.

To this stage of the work, there is no concept yet if and how to include sedimentation as a complex process into the model approach. It will be further evaluated if the general concept and resulting estimates is feasible to show reasonable numbers with sedimentation processes included.

4. Output: API concentrations

At each discharge point of a subcatchment and river section, the predicted environmental concentration of specific API is calculated (=PEC). Hence, the final output is a geographical dataset (shapefile) containing information both about the environmental flow characteristics, WWTP locations and estimated API concentrations:

- Accumulated flow, namely MQ/MNQ in m³/s
- Locations of emitting WWTPs
- API_A concentration (PEC_A) in ng/L
- API_B concentration (PEC_B) in ng/L
- API_B_MitigationOII (PEC_B_MO_II) in ng/L

Most likely, it is possible to model either several API substances or mitigation options at the same time. In the latter case, the ID name of calculated new PEC should contain the mitigation option number I, II or III. The regional API concentrations in the aquatic environment are further used in the risk assessment (Module C).

We have average regional flow of rivers and local emission points of API (=WWTP discharge locations). It is important to consider that API emissions also have a downstream effect and accumulate. At the same time, they are further diluted. This results in API concentrations, diverse in different river sections, regionally different. Mitigation options affect WWTP discharge loads as input and accumulation can be repeated as often/for many substances

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Annexes

Annex B1 – Open-source databases as potential flow model input

Example of database	Useful for...	Reference in the text	Coverage	Link
Coperincus	Digital Elevation Model	Chapter III, 2.1.4	Global	click
Corine Landcover	Land use/land cover	Chapter III, 2.1.5	Europe	click
LUNG	Catchment, subcatchments, river network	Chapter III, 2.1.1 & 2.1.2	Mecklenburg-Vorpommern (Germany)	click
ISRIC	Soil permeability	Chapter III, 2.1.6	World	click
Deutschen Wetterdienstes (DWD)	Precipitation	Chapter III, 2.1.7	Germany	click
Polish national weather service (IMGW)	Water level data at gauging stations	Chapter III, 2.1.3	Poland	click
Urban Waste Water Treatment Directive (UWWTD)	WWTP discharge points	Chapter III, 1.1	Europe	click

Module C:

Risk assessment of pharmaceuticals

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List of abbreviations

Abbreviation	Explanation
AA-EQS	EQS parameter expressed as an annual average value
ACRS	Aggregated component risk score
AF	Assessment factor
AMR	Antimicrobial resistance
AMR-B	Antimicrobial resistant bacteria
AMR-G	Antimicrobial resistance gene
AMR-RA	Antimicrobial resistance risk assessment
ASRIT	Activated sludge respiration inhibition test
API	Active pharmaceutical ingredient
ATC	Anatomical therapeutic chemical classification system
BCF	Bioconcentration factor
CAMRI	Comparative AMR Risk Index
CCRI	Component cumulative risk index
CFU	Colony forming unit
CRA	Cumulative risk assessment
EMA	European medicines agency
EQS	Environmental quality standards
ERA	Environmental risk assessment
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HHRA	Human health risks assessment
HQ	Hazard quotient
ISD	Internal safe dose
LOEC _R	Lowest observed effect concentration for resistance
LOQ	Limit of quantification
MAR	Multiple antibiotic resistance
MEC	Measured environmental concentration
MIC	Minimal inhibitory concentrations
NOEC	No observed effect concentration
NOEC _R	No observed effect concentration for resistance
NSAID	Nonsteroidal anti-inflammatory drugs
OD	Optical density
PBT	Persistence, bioaccumulation, and toxicity
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
PNECs	Predicted no effect concentrations
PNEC _{RS}	Predicted no effect concentrations for resistance
QSAR	Quantitative structure-activity relationship
RfD	Reference dose
RQ	Risk quotient
SNRI	Serotonin-norepinephrine reuptake inhibitor
U.S.EPA	United States environmental pollution agency
WWTP	Wastewater treatment plant

Introduction

In the present day, people often encounter a great number of stressors in the environment, some of which are chemical and others are non-chemical in nature. These stressors are not always singular in nature as their multiplicity can cause a synergistic effect of negative health outcome greater than that of any of the stressors taken in isolation. To assess negative impacts on the environment, it is important to recognize and measure the cumulative nature of these effects on top of generalizing the risk in question.

Water contamination exposes the environment and humans to chemical stressors. Urban wastewater has been found to be the main channel for active pharmaceutical ingredients (APIs) to aquatic systems (aus der Beek et al., 2016; Boxall, 2004). When non-target species are exposed to APIs, the effects may range from behavioral changes to alterations in the gender distribution, all the way to lethal toxicity. Furthermore, certain APIs, such as antibiotics, disinfectants and antimycotics, may promote antimicrobial resistance (AMR). Unfortunately, conventional wastewater treatments can not treat a large percentage of these pollutants and they are released into the environment after treatment (Michael et al., 2013). This constant addition results in disruption of the ecosystem and thus presents a serious threat to the environment including the health of the recipient biota.

This report aims to provide regional authorities with a framework of assessing the risks linked to the presence of pharmaceutical substances in water bodies. The scope of this framework covers not only conventional environmental risk assessment (ERA), but also the assessment of human health risks (HHRA) caused by exposure to pharmaceutical residues via the environment and risks of AMR (Figure C1).

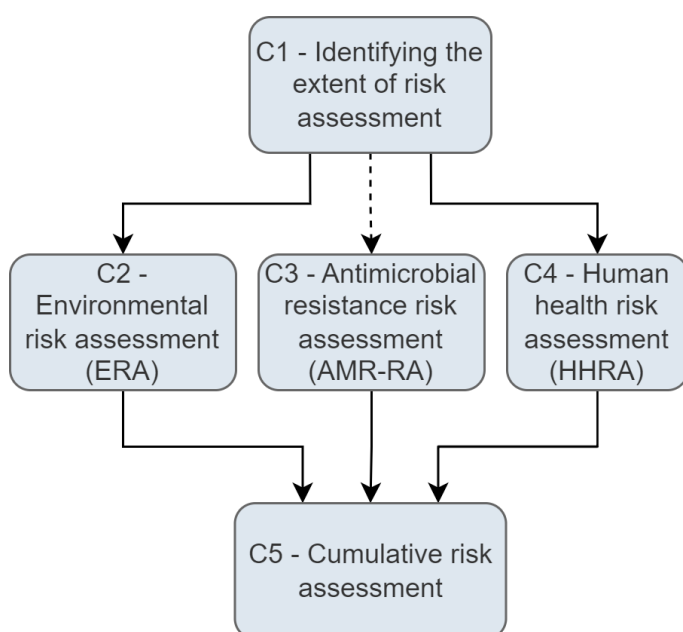


Figure C1. Framework for assessing Risk assessment of pharmaceutical substances in water bodies.

ERA is based on the risk assessment framework utilizing Predicted No Effect Concentrations (PNECs) and measured or predicted environmental concentrations (MEC or PEC, respectively). By examining PNEC data from scientific sources and ecotoxicity databases the report aims to clarify potential impacts and support well informed decision making.

Moreover, the report delves into the interactions and combined effects of pharmaceutical substances, known as "cocktail effects" by establishing an accumulative risk quotient. This method enhances our understanding of the risks arising from pollutants being present simultaneously.

To address the growing challenge of AMR, this report introduces a comprehensive AMR Risk Assessment (AMR-RA) framework. This framework utilizes PNECs tailored to selection and dissemination of both resistant bacteria (AMR-B) and resistant genes (AMR-Gs) in environmental contexts (PNEC_{RS} - predicted no effect concentrations for resistance). It is important to highlight that, to date, there is no standardized method for determining PNEC_R values. Therefore, a tiered strategy was proposed within the APRIORA project. As the first step, the assessment should begin with the theoretical approach (MIC-based PNEC_R), proposed by Bengtsson-Palme and Larsson (2016). They provided minimal selection concentration boundaries and PNEC_R for 111 antibiotics and 11 antibiotics combinations. If this approach is not appropriate—e.g., because no robust compound-specific threshold is available, the existing value is not sufficiently representative, or additional site-specific validation is required—a second-tier phenotypic community assay, based on optical density (OD), can be applied (OD-based PNEC_R). This assay is based on measurements of bacterial growth across a gradient of increasing antibiotic concentrations. If phenotypic assay does not provide a clear and reproducible threshold, the assessment should proceed to a third-tier molecular assay (qPCR-based PNEC_R) of the same bacterial community, which offers greater analytical sensitivity and mechanistic resolution but is also more methodologically demanding. This sequence is intended to balance scientific robustness, practical feasibility, and regulatory implementability. All approaches are described below and PNEC_R values provided by literature can be found in Annex C2.

Human health implications are further explored through a dedicated Human Health Risk Assessment (HHRA). This assessment applies health-oriented guidance values to examine human populations from environmental exposure to pharmaceutical contaminants, emphasizing the broader impact of these substances on public health.

Cumulative risk assessment examines the combined effects of multiple chemicals, but the approach can also be extended to include mixtures and other stressors. These scenarios in cumulative risk assessments will require various types of data, many of which are not typically used in current risk assessments and often in large quantities. Until this data becomes usable, these assessments may highlight key information gaps and research needs.

The purpose of the background document and accompanying flowcharts is to offer regional authorities a risk assessment framework that identifies WWTPs or river sections with the highest risk from pharmaceutical discharges. This targeted approach is invaluable for determining where to prioritize and implement advanced treatment processes or other mitigation measures. Figure C2 shows a proposal for a framework for choosing the scope of risk assessment relevant for APIs in the environment.

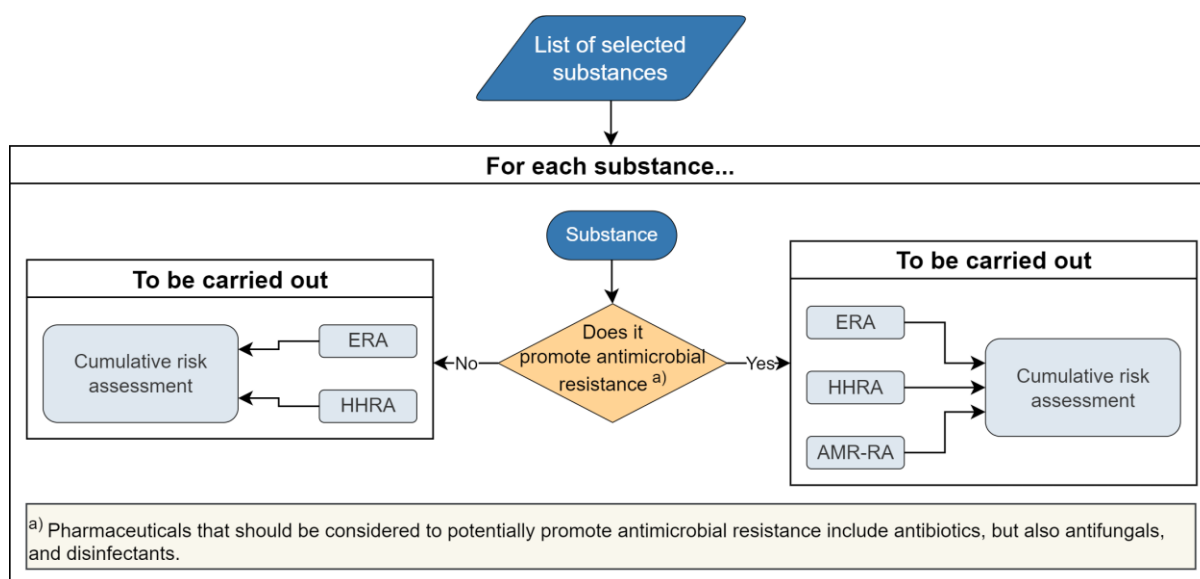


Figure C2. Flowchart for determining the scope of risk assessment according to the APRIORA risk assessment framework.

I: Environmental risk assessment

In the EU, assessing environmental risks related to pharmaceutical products has been a mandatory part of applying for marketing authorization since 2005. APIs that are naturally occurring substances, such as vitamins and electrolytes are exempted from this requirement. In 2006, the European Medicines Agency published a guideline on how to carry out the risk assessment (EMA 2006). This guideline was updated in 2024 (EMA 2024).

The risk assessment framework proposed by EMA is divided into two phases, complemented by an assessment of the compound's PBT properties (persistence, bioaccumulation, and toxicity). In general, Phase I risk assessment focuses on predicting an environmental concentration and comparing it to a cut-off value of 0.1 µg/l. As the predicted environmental concentration (PEC) is primarily calculated for substances that are not yet on the market, parameters such as market penetration factor will affect the outcome. If the PEC exceeds the cut-off value, the compound will require Phase II risk assessment. Furthermore, if the API has a specific mode of action that makes using the cut-off value unsuitable (e.g. antibiotics and hormones), the API will either require a tailored risk assessment or Phase II risk assessment.

Phase II risk assessment will require determining physico-chemical properties, environmental fate, and toxicity applying the methods presented in the risk assessment guideline (EMA 2024). The results from these tests will be used to determine if specific trigger values for groundwater, soil, and secondary poisoning risk assessments are exceeded. Risk assessments for surface water, sediment and WWTPs are required for all APIs entering Phase II.

While the current practices help ensure environmental risks related to new pharmaceuticals are acknowledged, the results of the assessment do not affect the outcome of the marketing authorisation application of human pharmaceuticals. I.e. identified environmental risks cannot result in denying the marketing authorisation. Furthermore, current practices are applied only to new compounds, not so-called legacy-APIs, that were introduced to the market before 2005. Most of the APIs monitored or screened in the

environment were introduced to the market well before 2005. For instance, in Finland, the APIs proposed as monitoring candidates in (Table A6) were first approved for market between 1964 and 1997.

The ERA concept is based on predicted no effect concentrations, or PNECs. We have compiled the most recent PNEC data from scientific literature and ecotoxicity databases. Table C1 presents the PNEC and proposed regulatory threshold values for the ten APIs included in the APRIORA project monitoring campaigns, while more APIs are listed in Annex C1. Annex C1 Table SC2 provide an overview of physical and chemical properties, uses, and potential environmental fate of the selected pharmaceuticals for APRIORA pilot. The substances discussed include carbamazepine, clarithromycin, diclofenac, metoprolol, sulfamethoxazole, trimethoprim, venlafaxine, estrone (E1), fluconazole, and primidone (see Module A, Section I, Chapter 4). Each of these substances belongs to a specific pharmaceutical class, including anticonvulsants, antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), beta blockers, serotonin-norepinephrine reuptake inhibitors (SNRIs), hormone replacement therapy (HRT), and antifungals.

Table C1. PNEC and proposed regulatory threshold value in proposed list of API for APRIORA project monitoring campaigns.

Name of API	CAS No.	ATC (level2)	Proposed regulatory threshold (Inland surface waters) µg/L	Proposed regulatory threshold (Other surface waters) µg/L	*PNEC (NORMAN lowest value freshwaters µg/L)	*PNEC (NORMAN lowest value marine waters µg/L)
Carbamazepine	298-46-4	N03	0.006 ^{a)} 2.5 ^{b)}	0.006 ^{a)} 0.25 ^{b)}	2	0.25
Clarithromycin	81103-11-9	J01	0.13 ^{b)}	0.013 ^{b)}	0.12	0.013
Diclofenac	15307-86-5	M01	0.04 ^{b)}	0.004 ^{b)}	0.04	0.004
Metoprolol	51384-51-1	C07			8.6	0.86
Sulfamethoxazole	723-46-6	J01	0.1 ^{c)} 0.6 ^{e)}	0.06 ^{e)}	0.6	0.06
Trimethoprim	738-70-5	J01	0.15 ^{c)} 0.1 ^{c)}		0.5	10
Venlafaxine	93413-69-5	N06	0.0061 ^{c)}		0.88	0.088
Estrone (E1)	53-16-7	G03	0.00036 ^{b)}	0.000018 ^{b)}	0.00036	0.000018
Fluconazole	86386-73-4	J02	0.25 ^{c)}		1.04	0.1
Primidone	125-33-7	N03	2.5 ^{d)}		9.11	0.91

* NORMAN Substance Database –

<https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php>).

a) AA-EQS as derived in Carbamazepine draft EQS dossier (2021), <https://circabc.europa.eu/ui/group/9ab5926d-bed4-4322-9aa7-9964bbe8312d/library/ad07a599-cd2e-49c3-9fa0-d0203495a5e1/details>

b) AA-EQS as proposed in EC 2024 Document P9_TA(2024)0358, [https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=EP:P9_TA\(2024\)0358](https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=EP:P9_TA(2024)0358)

c) Maximum acceptable method detection or quantification limit as presented in COMMISSION IMPLEMENTING DECISION (EU) 2022/1307,

https://www.stradalex.eu/en/se_src_publ_leg_eur_jo/toc/leg_eur_jo_3_20220726_197/doc/ojeu_2022.197.01.0117.01

d) Groundwater quality standard (annual average) as proposed in EC 2024, chrome-extension://efaidnbmninnibpcapjpcglclefindmkaj/https://health.ec.europa.eu/document/download/506c48f1-02a9-4e8d-a5cc-ff8855b917fc_en?filename=scheer_o_035.pdf

e) Nendza, M. (2014): EQS DATASHEET Environmental Quality Standard Sulfamethoxazol, <https://webetox.uba.de/webETOX/public/basics/literatur.do?id=24270>

The ten compounds selected for the APRIORA piloting phase are presented in Table C1, along with relevant regulatory threshold values and PNEC values extracted from the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (NORMAN) database. NORMAN organises the development and maintenance of various web-based databases for the collection & evaluation of data / information on emerging substances in the environment. The Ecotoxicology Database contains – for substances listed in the NORMAN Substance Database – experimental endpoints from ecotox tests as well as quality targets from different regulatory contexts (<https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php>).

If the measured environmental concentrations (MEC) in the NORMAN Substance Database exceed the lowest PNEC which is not robust, a review is required to determine whether a regulatory concern exists. The lowest PNECs are preferably based on experimental ecotoxicity data.

It should be noted that PNECs and regulatory thresholds are estimates based on incomplete datasets. Optimally, the threshold values would be derived from a dataset covering all relevant species in all relevant conditions. However, ecotoxicological tests are commonly carried out using only certain species and conditions. For instance, according to EU guidance documents (EC, 2018), ecotoxicity data such as NOEC (no observed effect concentration) or EC10 (concentration where 10% of the organisms are affected) is used to derive the PNEC by dividing it with the assessment factor (AF). AF may commonly range from 10 to 1,000, depending on the level of information. Furthermore, PNECs may be derived using the so-called species sensitivity distribution (SSD) method. In this approach, an ecotoxicity dataset covering multiple species from different taxa is compiled and used to fit a distribution function. A percentile is then selected from this distribution (commonly the 5th percentile). This approach produces the concentration where the selected percentage of species is affected (e.g. HC5). A PNEC is then derived from the resulting concentration by dividing it with the AF, which for SSDs commonly ranges between 1–5.

Thus, the threshold values may change as more information becomes available. Furthermore, some species may not be directly relevant for certain environments. For instance, the commonly used fish species zebrafish (*Danio rerio*) naturally occurs in South Asia. Thus, it could be argued that PNECs derived utilizing zebrafish are not relevant for the BSR. However, as the goal of the PNEC approach is to predict a concentration below which adverse effects should not be observed, even data on exotic species is useful and relevant. In the rare situation where there is an abundance of ecotoxicity data on native species, it may be justified to focus on it.

In case no PNEC datasets are available, it may be necessary to produce the required data to allow for an ERA. PNEC derivation in general requires ecotoxicity data, preferably from multiple trophic levels. If you do not have the capacity to carry out ecotoxicity testing, rough estimates can technically be derived using readily available QSAR (Quantitative Structure-Activity Relationship) tools, such as ECOSAR (Wright et al., 2022) or VegaHub (Benfenati et al., 2013). The tools estimate toxicity based on the chemical structure and known data from similar substances. However, APIs often fall outside the domain of applicability for existing tools. Thus, it is advisable to use QSAR tools primarily as a screening level tool.

1. Method description of ERA

The environmental risk assessment (ERA) relies on the calculation of the risk quotient (RQ). RQ is a key metric for evaluating the potential ecological impact of a substance by comparing its concentration in the environment to its toxicological effects. This ratio helps determine the potential harm to non-target

organisms within an ecosystem. The risk quotient (RQ) for each substance shows the potential risk caused by the API to the environment and is calculated by formula below:

$$RQ = \frac{C}{PNEC}, \text{ where } C = \text{Environmental concentration, should be given either as measured or predicted environmental concentration (MEC or PEC, respectively),}$$
$$PNEC = \text{Predicted no-effect concentration in the relevant compartment (mass/volume)}$$

Result interpretation:

RQ values exceeding one indicate risk. If a substance is not detected but the LOQ is higher than the PNEC, RQ should be calculated using LOQ as the numerator. In these cases the RQ is conservative, and presents the upper bound of possible actual RQ values.

Key considerations from the RQ interpretation:

- 1) Risk Threshold ($RQ > 1$): An RQ value greater than 1 indicates potential environmental risk.
- 2) Binary Nature of RQ: When $RQ > 1$, a higher value does not necessarily mean greater severity of risk because the interpretation depends on variability in ecotoxicological tests and the assessment factors (AFs) used in deriving PNEC. The RQ primarily serves as a binary risk indicator.
- 3) Summing Up RQs: Individual RQs are only summed at the end as part of the Cumulative Risk Assessment (CRA).
- 4) Severity of Potential Risk: An alternative approach presented in chapter IV considers the magnitude of potential risk by evaluating how close compound values are to exceeding the RQ/HQ threshold. This method captures the degree of risk presence, even when thresholds are not directly surpassed.

Not all APIs represent the same level of risk, and their concentrations are therefore important to monitor. Some of them, because of their persistence in the environment, their potential to bioaccumulate in living organisms, or inherent toxicity, may be harmful even at a very low concentration. By focusing on the compounds most likely to impact aquatic ecosystems, regional authorities and researchers could prioritize their efforts and allocate resources more effectively.

The flowchart of exposure and risk assessment used in APRIORA is described in Figure C3. This flowchart outlines the process of obtaining a reference value and assessing the RQ. The ERA detection process starts with either a measured or predicted concentration of a substance and focuses on identifying a relevant reference or threshold value. Figure C3 presents a series of decision points to guide this process.

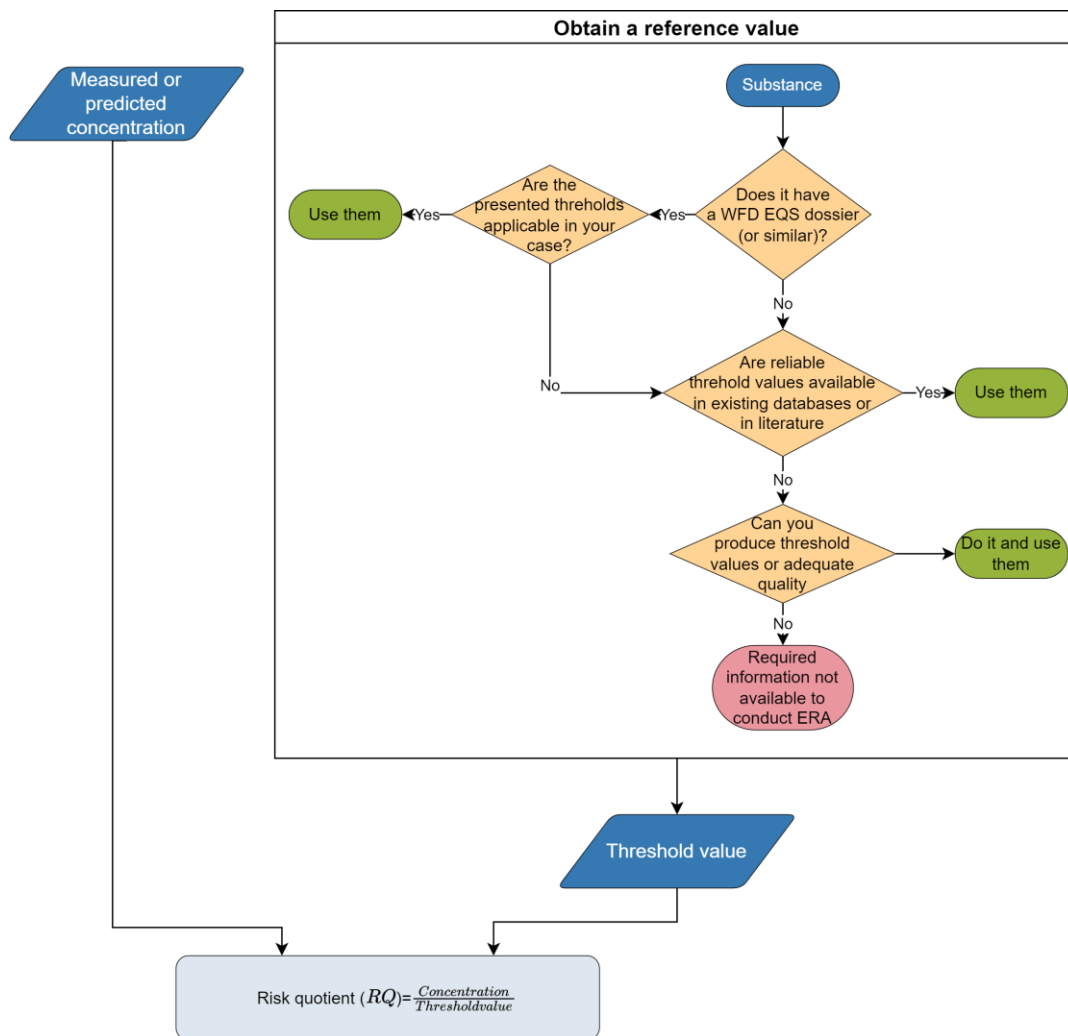


Figure C3. Flowchart of ERA

II: AMR risk assessment (AMR-RA)

Environmental concentrations of antimicrobial agents (referred to as measured or predicted environmental concentrations; MECs/PECs), are significantly lower than therapeutic levels. Thus, AMR development outside clinical niches has long been overlooked and underestimated. However, increasing evidence indicates that even low environmental concentrations may contribute to the selection and maintenance of antibiotic-resistant bacteria (AMR-B) and antibiotic resistance genes (AMR-Gs). Co-selection that can protect bacteria occurs through three processes: co-resistance (resistance genes are genetically linked, on e.g. a conjugative plasmid), cross-resistance (a single resistance mechanism in bacteria confers resistance to multiple antimicrobial agents, e.g. multi-drug efflux pump) and co-regulation (coordinated control of multiple genes or pathways by common regulatory mechanisms).

Within current pharmaceutical environmental risk assessment, the activated sludge respiration inhibition test (ASRIT; OECD 209) remains the main bacterial ecotoxicity test used to assess effects on wastewater-treatment microorganisms. In this assay, activated sludge is exposed to a test substance and changes in respiration are measured (EMA 2024, OECD 2010). However, ASRIT and conventional ecotoxicity endpoints were not developed to identify concentrations that specifically select for AMR, and their adequacy for this purpose has been widely questioned.

A range of experimental and literature-based approaches has been proposed to derive thresholds relevant to AMR selection and maintenance. Experimental studies usually report thresholds: commonly expressed as minimal inhibitory concentrations (MICs), and effect-based thresholds analogous to the lowest observed effect concentration for resistance ($LOEC_R$) and the no observed effect concentration for resistance ($NOEC_R$), from which a predicted no-effect concentration for resistance selection ($PNEC_R$) can subsequently be derived. A key distinction between approaches is whether they target AMR-Gs or AMR-B (Figure A12).

Methods targeting AMR-Gs (molecular approach) provide quantitative information on the abundance of genes that may confer resistance to specific antibiotics. These methods typically rely on the extraction of DNA from environmental samples, such as water, biofilms, or sediments, followed by qPCR to quantify selected AMR-Gs with high analytical sensitivity and specificity. Through qPCR, gene abundance is measured by amplifying targeted resistance-associated DNA sequences and monitoring amplification in real time, enabling precise quantification of the selected genes. However, qPCR is limited to predefined targets included in the assay design. Integrins can also serve as useful proxies for resistance determinants because they are associated with the capture, accumulation, and horizontal transfer of AMR-Gs; accordingly, detection of integron-integrase genes such as *intI1* by qPCR is commonly used as an indirect indicator of resistance potential in a studied microbial community.

In addition to these targeted approaches, metagenomic sequencing can provide a broader overview of the resistome, encompassing all AMR-Gs present in a sample and enabling the identification of genes that were not initially targeted. Although valuable for comprehensive screening, metagenomics is more complex and resource-intensive than targeted qPCR-based approaches. Moreover, neither DNA-based qPCR nor metagenomics can, on their own, distinguish between live and dead cells or confirm whether the detected genes are actively expressed. These methods therefore characterise resistance potential rather than direct phenotypic activity, unless combined with RNA-based or viability-targeted approaches.

Methods quantifying the number of AMR-B (phenotypic approach) commonly rely on culture-based assays in which isolates or microbial communities are exposed to increasing antibiotic concentrations and their growth response is measured. Although such methods confirm expressed resistance, they also have

important limitations and may underestimate the overall risk. Not all bacteria grow equally well under laboratory conditions, which may result in underrepresentation of slow-growing or fastidious organisms. In mixed communities, differences in growth dynamics among taxa can strongly influence the apparent selection threshold, and multiple breakpoints may be observed rather than a single clear threshold. Moreover, only the culturable fraction of the microbial community is captured, potentially excluding a substantial proportion of microorganisms present in the sample. Because these approaches assess phenotypic resistance only, they may also fail to reflect resistance genes that are present but not expressed under the test conditions. Consequently, AMR-B-based methods provide only a partial picture of the overall resistance potential of environmental samples.

It should also be emphasised that in AMR-B growth response approach the minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial that prevents visible growth under test conditions. But in contrast to ecotoxicological effect thresholds, such as EC10, which are expected to be relatively stable when derived for the same chemical and species under comparable conditions, MIC values may vary substantially depending on the characteristics and exposure history of the tested microbial population. For example, microbial populations originating from environments with frequent or high antibiotic exposure, such as clinical settings, are more likely to contain resistant bacteria and may therefore require higher concentrations to inhibit growth. In contrast, populations from less impacted environments are less likely to have been subjected to selection for resistance and may respond to much lower antibiotic concentrations. MIC should therefore be interpreted as a population-specific susceptibility parameter rather than a universal chemical-specific threshold.

However, no standardised experimental method is currently available for routine determination of the lowest concentrations that select for AMR in complex microbial populations, and there is still ongoing debate about the most appropriate way to derive $PNEC_R$ values. Although studies using complex communities are generally regarded as more environmentally relevant than single-strain systems, the available methods differ in microbial community composition, endpoint selection, analytical workflow, and interpretation. Bengtsson-Palme and Larsson (2016) therefore proposed **MIC-based $PNEC_R$** values as a pragmatic screening tool, based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC data and an assessment factor of 10.

Figure C4 outlines the proposed workflow for AMR risk assessment. Annex C2 summarises the experimental and estimated $PNEC_R$ values currently available in the literature that can be used for risk quotient (RQ) calculations. Where literature-derived thresholds appear not to be applicable to the study region, site-specific experimental studies may be undertaken to derive threshold values that better reflect the characteristics of the investigated catchment (Figure A12).

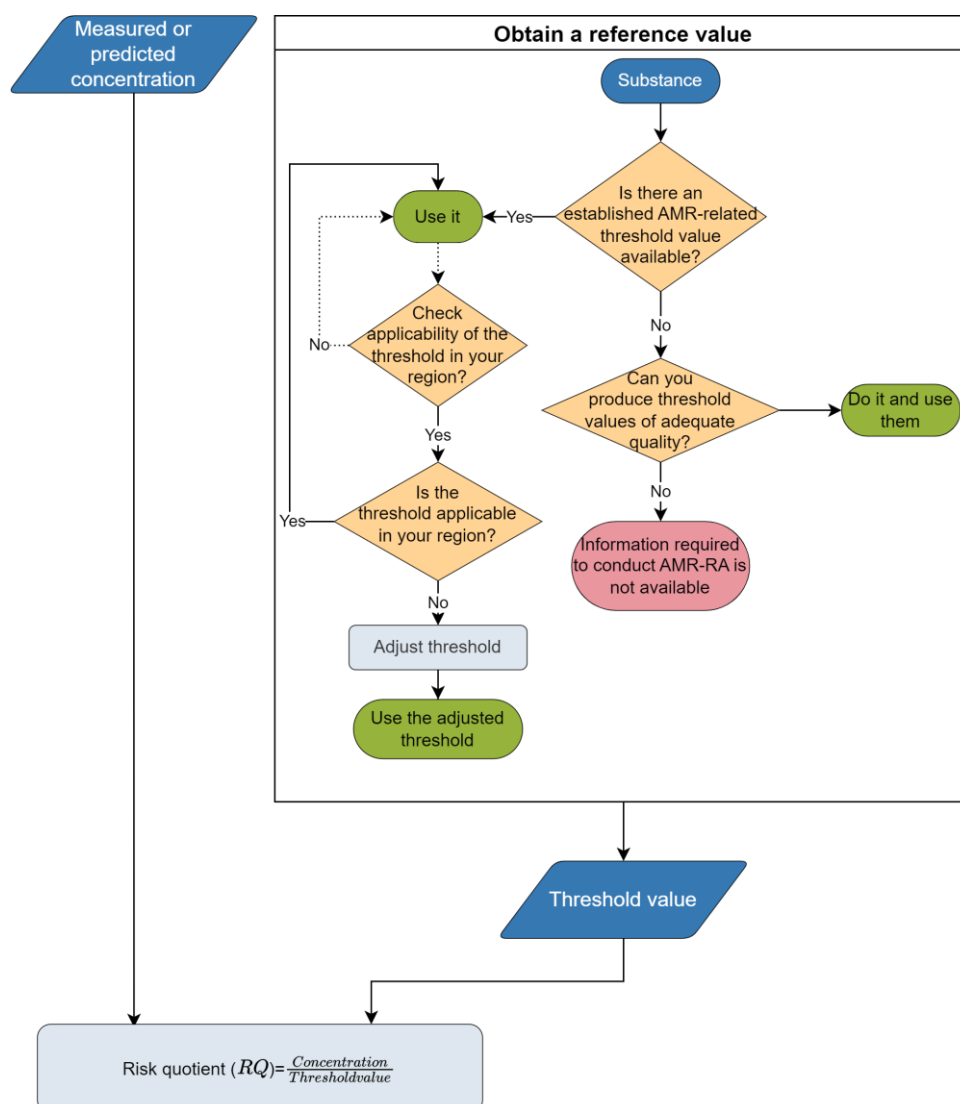


Figure C4. Flowchart of AMR-RA. For details, see Annex 3, Figure SC3.1.

1. Identification of APIs for AMR detection

The risk assessment related to AMR (AMR-RA) will be limited to APIs, which act either bactericidal or bacteriostatic, and to which microorganisms can develop resistance. These include antibiotics and other antimicrobial agents.

Among the substances selected for piloting during the APRIORA project, three are regarded as antimicrobial agents:

- Clarithromycin: bacteriostatic; is a semisynthetic macrolide antibiotic that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, thereby preventing bacterial growth
- Trimethoprim: bacteriostatic; inhibits the bacterial enzyme dihydrofolate reductase, which is involved in folic acid synthesis, a crucial process for DNA replication and cell division
- Sulfamethoxazole: bacteriostatic; inhibits bacterial folic acid synthesis by competing with para-aminobenzoic acid (PABA), which bacteria need for DNA synthesis

Trimethoprim and sulfamethoxazole are often used in combination (as in the drug - co-trimoxazole), and together they have a bactericidal effect due to the synergistic inhibition of the bacterial folate synthesis pathway at two different points.

2. Approaches proposed for generation of Risk Quotients (RQs)

As discussed on page 124, up to now there is a lack of standardized experimental method for routinely determining the lowest antibiotic concentration that selects for AMR in microbial populations.

Thus a tiered strategy was proposed. As a first-line option, assessment should begin with the **theoretical approach (e.g. MIC-based PNEC_R)**. Where this approach is not appropriate—for example, because no robust compound-specific threshold is available, the available value is considered insufficiently representative for the assessment objective, or additional site-specific validation is required—a second-tier **phenotypic community assay (OD-based PNEC_R)** may be applied. In this assay local microbial communities are exposed to increasing antibiotic concentrations and their growth response is measured by optical density (OD). If this assay does not provide a clear and reproducible threshold, the assessment should proceed to a third-tier **molecular assay (qPCR-based PNEC_R)** of resistance genes abundance in mentioned above bacterial community. This assay offers greater analytical sensitivity and mechanistic resolution but is also more methodologically demanding. This sequence is intended to balance scientific robustness, practical feasibility, and regulatory implementability.

The prioritisation of the theoretical approach is justified by the current state of knowledge. Although a range of experimental and data-driven approaches has been proposed for deriving PNEC_R or related selection thresholds, recent reviews conclude that there is still minimal standardisation of existing approaches and no consensus on the relative merits of different methods.

Table C2 summarises experimental and estimated PNEC_R values, which can be used for generating Risk Quotients (RQs) for antibiotics of APRIORA concerns (clarithromycin, trimethoprim, sulfamethoxazole). PNEC_R values for other APIs determined to date are presented in Annex C2.

Table C2. Predicted no effect concentrations for resistance (PNEC_R) determined to date for antibiotics of APRIORA concerns (adapted from Bengtsson-Palme & Larsson, 2016 and Murray et al., 2021); theoretical approach of MIC-derived PNEC_R is highlighted in bold

Antibiotic	PNEC _R (µg/L)	Experimental system*	Method	Method endpoint	Reference
Clarithromycin	250	Microcosm wastewater influent	qPCR statistic	<i>ermF</i>	Stanton et al., 2020
	50	Microcosm wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020
	0.25	MIC-derived PNEC_R - MICs dataset for clinical strains	theoretical approach (extrapolation)	-	Bengtsson-Palme & Larsson, 2016
Sulfamethoxazole	16	MIC-derived PNEC_R - MICs dataset for clinical strains	theoretical approach (extrapolation)	-	Bengtsson-Palme & Larsson, 2016
Trimethoprim	42.7	Isogenic competition (<i>E. coli</i>)	Ratio MSC	<i>dfrA12</i>	Kraupner 2020
	33	Isogenic competition (<i>E. coli</i>)	Ratio MSC	<i>dhfr</i>	Gullberg 2014
	3.13	Microcosm wastewater influent	qPCR statistic	<i>intI1</i>	Murray et al., 2020
	1.56	SELECT wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020

Antibiotic	PNEC _R (µg/L)	Experimental system*	Method	Method endpoint	Reference
	1	Microcosm wastewater effluent	CFU statistics	% resistant <i>E. coli</i>	Kraupner 2020
	1	Microcosm <i>E. coli</i> , mixed population	CFU statistics	% resistant <i>E. coli</i>	Kraupner 2020
	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)	-	Bengtsson-Palme & Larsson, 2016

*MIC - minimum inhibitory concentration data for various antimicrobial agents against a wide range of bacterial species provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST); MICs represents the lowest concentration of an antimicrobial that inhibits visible bacterial growth - database used to establish clinical breakpoints; Complex bacterial community (of wastewater origin) capture some intra- and interspecies competition expected in natural environments. Metagenomics recommended to determine changes of representative resistance genes and community composition providing information on co-selection.

In this context, the **theoretical approach of MIC-derived PNEC_R**, proposed by Bengtsson-Palme and Larsson (2016) remains the most suitable screening-level option which provides minimal selection concentration boundaries and PNEC_R for 111 antibiotics and 11 antibiotics combinations. This method uses EUCAST MIC data and an assessment factor of 10 to derive compound-specific thresholds intended to represent upper protective boundaries for resistance selection. While MIC-based approaches do not fully capture community interactions, mixture effects, or site-specific environmental conditions, they are currently the most practical, rapid, and reproducible option where harmonised experimental data are lacking. Importantly, Environment Agency (2024) found that MIC-based PNEC_R values were often lower than experimental PNEC_R values for the antibiotics that could be compared, indicating that MIC-based values may provide a more conservative, and therefore more protective, basis for screening. Until a harmonised experimental method has been validated across laboratories and environmental matrices, MIC-derived PNEC_R values should therefore be regarded as the most defensible first-tier basis for WWTP-related AMR risk screening.

The **phenotypic community assay (OD-based PNEC_R)** nevertheless remains a useful second-tier option when a more site-specific experimental assessment is needed. In this approach (described in detail in Annex C3), growth of the AMR-B among an isolated microbial community is monitored in 96-well plates under increasing antibiotic concentrations using OD as the readout. This format is relatively simple, rapid, and comparatively cost-effective, and similar plate-based community growth assays have been proposed in the literature as potentially practical tools for AMR-related environmental screening. However, its implementation still requires basic microbiological expertise in microbial community isolation and cultivation, sterile handling, and access to standard laboratory infrastructure, including incubators, a microplate reader, and facilities for routine culture work, which may not be available in all wastewater laboratories. Additionally phenotypic assay cannot yield a clear and reproducible threshold for resistance selection. This outcome can be biologically plausible because selection in mixed microbial communities is strongly influenced by community composition and ecological interactions. Suppression of one fraction of the microbial community may create ecological space for other organisms, and community-level responses may therefore obscure a single, clearly defined breakpoint (see Annex C3).

The **molecular community assay (qPCR-based PNEC_R)** should therefore be considered a higher-tier option when the phenotypic assay is inconclusive. In this approach (described in detail in Annex C3), isolated microbial community response is assessed by quantifying resistance genes (AMR-Gs) associated with the applied antibiotic, providing a more sensitive and biologically informative signal than simple growth inhibition alone. Its use, however, requires specialised molecular biology expertise and more advanced infrastructure, including validated DNA extraction procedures, dedicated clean areas for PCR workflow, a

real-time PCR platform, appropriate standards and controls, and personnel experienced in primer selection, assay validation, and data normalisation, which substantially limits its applicability in routine laboratories. This approach can be more informative than OD-based growth monitoring, but it still may not yield a single unambiguous threshold suitable for routine implementation. The main limitations are methodological as well as analytical: qPCR is inherently restricted to predefined targets, does not by itself confirm gene expression, and results depend strongly on target-gene selection, primer design, normalisation strategy, and endpoint definition. Importantly, qPCR can only quantify genes that are already known and intentionally included in the assay design. This is a major limitation because resistance to a given antimicrobial may be mediated by multiple genes and mechanisms that vary across taxa and environmental settings. In the case of macrolides such as clarithromycin, for example, resistance may involve ribosomal methylases encoded by *erm* genes, efflux- or protection-related determinants such as *mef* and *msr* genes, and in some organisms also target-site alterations, including mutations in 23S rRNA. A fully comprehensive qPCR panel would therefore need to cover numerous potential determinants, which is analytically demanding and often too costly for routine use. A similar issue applies to other antibiotic classes. Because it is practically difficult to quantify all possible determinants, qPCR-based environmental studies usually select a limited number of representative or indicator genes that are considered most relevant for the antibiotic class of interest. In Table C3, the most relevant indicator genes for clarithromycin, trimethoprim, and sulfamethoxazole (antimicrobials of APRIORA concern) are presented and may be used as qPCR targets in environmental analyses. This improves feasibility and comparability, but it is also an inherent weakness of the method, because important untargeted determinants may be missed and the resulting signal may not fully reflect the total resistance potential of the studied microbial community.

In Table C3 the most important genes driving AMR to clarithromycin, trimethoprim, sulfamethoxazole are presented. These genes can be used as resistance indicators in qPCR analyses.

Table C3. Main resistance determinants relevant to the antibiotics of APRIORA concern and suggested qPCR indicator targets for routine environmental screening*

Antibiotic	Main genes (major documented resistance determinants; not exhaustive)	Recommended qPCR indicators (pragmatic panel)
Clarithromycin	<i>erm</i> family: especially <i>ermA</i> , <i>ermB</i> , <i>ermC</i> , <i>ermF</i> ; <i>ermT</i> , <i>ermX</i> , <i>mef</i> family: <i>mefA</i> , <i>mefE</i> , <i>msr</i> family: <i>msrD</i> , <i>msrA</i> ; and <i>mphA</i> ;	Core: <i>ermB</i> , <i>ermF</i> Extended: <i>mefA</i> , <i>mefE</i> , <i>msrD</i> Optional: <i>mphA</i>
Trimethoprim	<i>dfrA</i> family (e.g. <i>dfrA1</i> , <i>dfrA12</i> , <i>dfrA14</i> , <i>dfrA17</i>), <i>dfrB</i> family;	Core: <i>dfrA1</i> Extended: <i>dfrA12</i> or <i>dfrA17</i> Optional: selected <i>dfrB</i> target(s)
Sulfamethoxazole	<i>sul1</i> , <i>sul2</i> , <i>sul3</i> , <i>sul4</i>	Core: <i>sul1</i> , <i>sul2</i> Extended: <i>sul3</i>

*As a complementary, non-antibiotic-specific marker of anthropogenic AMR pressure and horizontal gene mobility, the class 1 integron-integrase gene *int11* may also be included in the qPCR panel; however, it should be interpreted as a general AMR-associated marker rather than as an antibiotic-specific determinant

The broader literature also shows that molecular methods require further standardisation if results are to be comparable across studies and laboratories. In addition, in their review the Environment Agency (2024) notes that single-target qPCR may overestimate $PNEC_R$ if the most conservative gene target is not selected. For these reasons, qPCR-based assays are best regarded as a higher-resolution but more resource-intensive tool for resolving cases in which lower-tier approaches do not provide sufficient confidence

Overall, a tiered strategy that starts with MIC-derived $PNEC_R$ values and proceeds to phenotypic and molecular assays only when needed provides the most scientifically defensible and practically implementable framework for screening WWTP-related AMR selection pressure under current methodological and laboratory constraints.

In the AMR-RA, similar to the ERA, the RQ for resistance (RQ_R) is calculated using the following formula:

$$RQ_R = \frac{C_R}{PNEC_R}, \text{ where}$$

C_R = measured or predicted environmental concentration (MEC or PEC, respectively) of antimicrobial agent.
 $PNEC_R$ = Predicted no-effect concentration for resistance

Result interpretation:

As described above in the ERA section (p. 121), the conventional environmental risk assessment typically focuses on exposure in environmental matrices within receiving ecosystems, and $PNEC$ values are generally interpreted in relation to compartments such as freshwater or marine waters. In the case of antimicrobial resistance, however, it is also informative to assess wastewater itself, including WWTP influent and effluent, because sewer networks and treatment systems constitute densely populated microbial environments in which antimicrobial residues may already exert selection pressure before discharge to the receiving water. Although such matrices are not receiving environments in the classical sense, their inclusion in AMR-oriented risk screening can provide important information on microbial exposure along the entire urban wastewater pathway and improve understanding of where conditions favourable to resistance selection and dissemination may occur.

The interpretation of RQ_R may therefore be extended as follows:

- (a) **$RQ_R > 1$ in WWTP influent** suggests that selective pressure may already be present within the sewer network, indicating that conditions favourable to the selection of antibiotic-resistant bacteria (AMR-B) and the dissemination of antibiotic resistance genes (AMR-Gs) may occur before treatment begins.
- (b) **$RQ_R > 1$ in WWTP effluent** indicates that wastewater treatment did not reduce concentrations of the tested antimicrobial agents below the $PNEC_R$ threshold; therefore, residual concentrations discharged with the effluent may continue to sustain selection pressure and may co-occur with the release of AMR-B and AMR-Gs into the receiving environment.
- (c) **$RQ_R > 1$ in receiving surface waters** suggests that selection pressure may extend into the aquatic environment. If exceedances are observed mainly downstream rather than upstream of the discharge point, this pattern is consistent with a substantial influence of WWTP effluent on local selection pressure and on the potential dissemination of resistance determinants within aquatic microbial communities. In such cases, mitigation measures should consider both reduction of antimicrobial loads and limitation of AMR-B/AMR-G release.
- d) **$RQ_R < 1$ regardless the sampling points**, indicates a low likelihood of resistance selection

When interpreting RQ_R values, data quality should also be considered. If there is reason to question the quality of either the concentration data or the threshold value, these should be verified or refined before drawing conclusions. Additionally, the RQ_R approach is substance-specific, as each risk quotient is calculated

for a single antimicrobial by comparing its measured or predicted concentration with the corresponding PNEC_R value. As such, a single RQ_R does not capture the combined selective pressure exerted by mixtures of antimicrobial agents occurring simultaneously in wastewater or receiving waters. In reality, however, microorganisms are exposed to complex antimicrobial cocktails, which may promote co-selection, cross-resistance and, consequently, the emergence and maintenance of multidrug-resistant bacteria. For this reason, complementary indicators such as the Multiple Antibiotic Resistance (MAR) Index and the Comparative AMR Risk Index (CAMRI) have also been proposed (Goh et al. 2024). The MAR Index reflects the extent of resistance of bacterial isolates to multiple antibiotics, whereas CAMRI is intended to compare AMR risk across samples and identify potential AMR hotspots. These indices may therefore complement compound-specific RQ_R values, providing a broader perspective on mixture-related and cumulative AMR risk.

III: Human health risk assessment (HHRA)

Human health risks can be assessed in many ways. For example, by calculating the number of estimated health effects for each health end point due to exposure to a substance. This approach, however, requires epidemiological or toxicological exposure-response information, specific data on exposures, and statistics on diseases. Such information is often readily unavailable and other means of estimating the risk are more plausible, such as the use of reference doses (RfD) or tolerable daily intakes (TDI). These are more straightforward to use e.g., in regulatory risk assessments, and the process is similar to the commonly used PNEC approach in environmental risk assessment. Importantly, this approach produces similar format of risk estimate and enables the results to be utilized in the cumulative risk assessment approach, described in the next section.

API contaminated water human health risk assessment usually considers exposures acquired via drinking water, swimming (ingestion of water during swimming, and dermal exposure) and fish consumption. Dermal exposure, however, is considered out of scope in APRIORA which leaves three exposure routes; drinking water, ingestion during swimming, and fish consumption. If local data on fish and drinking water is unavailable, approximations can be used, e.g. API concentration in fish can be approximated using bioconcentration factors (BCF) and surface water API concentration.

The flowchart of exposure assessment and risk assessment used in APRIORA is described in Figure C5. Human risk assessment in APRIORA is performed according to the approach described by Duarte et al., 2022. The authors estimate human risk from multiple exposure routes, in five different age groups, and in three different exposure scenarios for 15 APIs in the Vecht River (in Netherlands and Germany). For the purpose of this project and simplicity, only the age group 18-80 (adults) and average exposure, is considered. Combined effects of APIs will not be estimated in this model. Further, in the piloting phase, HHRA will be performed for only three APIs: carbamazepine, metoprolol and diclofenac for which sufficient data is available. This framework is suitable for other APIs as well, and they can be incorporated into the framework as the input parameters needed in the calculations become available. Measured or modelled concentration of the APIs in the studied pilot river catchment area (and drinking water if data available) will be used for estimating the total uptake of pharmaceuticals in a lifetime.

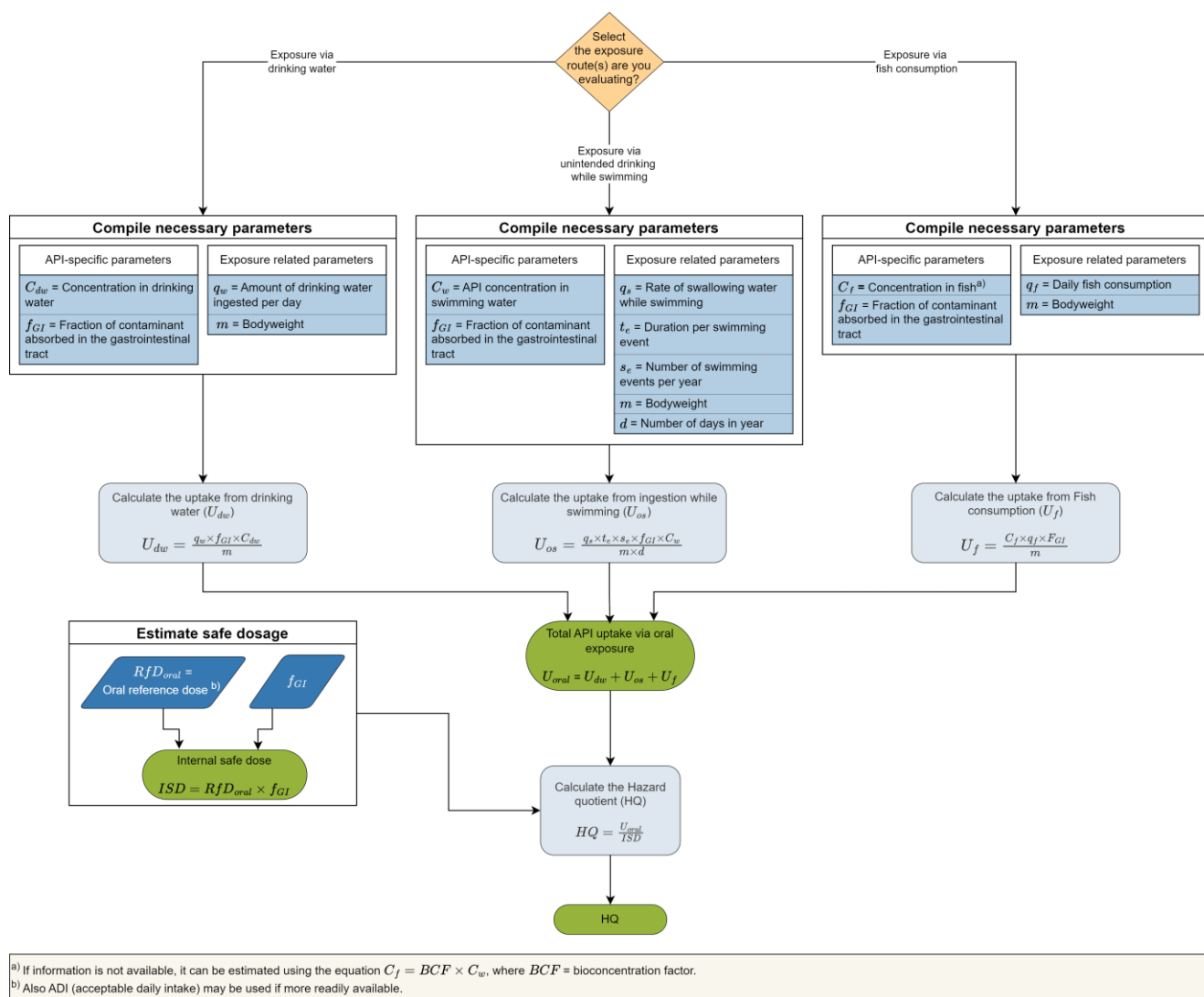


Figure C5. HHRA flowchart with calculation details.

The end result, human risk assessment hazard quotient (HQ) is acquired by dividing the calculated total lifetime uptake of the API with the Internal safety dose (ISD/ mg/kg/day). The total lifetime uptake only takes into consideration the intake from the environment, and not through pharmaceutical treatment. The ISD is calculated by multiplying the oral absorption fraction of an API with its safe dose for oral exposure, for threshold substances the Acceptable Daily Intake.

List of variables for all three oral exposure routes is provided in Table C4, and API-specific calculation parameter values in Table C5. The values listed in the tables are taken from Duarte et al., 2022. The calculation parameters contain many sources of uncertainties and the result of the human health risk provides an indication, rather than an accurate prediction. If local data is available e.g. on fish consumption, drinking water consumption, or other behavioural parameters, their use is encouraged. There are some useful literature sources for conducting more refined exposure assessments. For example, U.S.EPA's Exposure Factors Handbook provides risk assessors the latest information and various useful parameter values to be used in different phases of exposure assessments.

All calculations can be performed using the Excel model provided in the **Virhe. Viitteen lähde ei löytynyt..** Simply, in Excel worksheet "Inputs and results" type in each API concentration (average) in surface

water using the correct units (mg/ml), and the model calculates the hazard quotients (HQ). All other parameter values needed are provided in the model on tab “Calculation sheet” where the user can assign values if more specific information is available.

Table C4. List of variables and parameters of three oral exposure routes and their values used in the APRIORA HHRA.

Symbol	Name	Unit and/or value	Description
c_f	Pharmaceutical concentration in fish tissue	mg/mg	$C_f = C_w \times BSC$
c_w	Pharmaceutical concentration in surface water	mg/mL	Measured or modelled (input values)
BCF	Pharmaceutical-specific bioconcentration factor	mL/mg	API-specific values (see Table C5)
U_f	Pharmaceutical uptake after ingestion of fish	mg/kg/day	$U_f = q_f \times f_{GI} \times c_f / m$
q_f	Daily fish consumption	108,969 mg/day	
f_{GI}	Gastrointestinal absorption fraction	-	API-specific values (see Table C5)
m	Human body weight	70 kg	
U_{dw}	Pharmaceutical uptake after ingestion of drinking water	mg/kg/day	$U_{dw} = q_w \times f_{GI} \times c_{dw} / m$
q_w	Amount of drinking water ingested per day	1,757 mL/day	
c_{dw}	Concentration in drinking water	mg/mL	Measured or modelled
U_{os}	Pharmaceutical uptake via water ingestion during recreational swimming	mg/kg/day	$U_{os} = q_s \times t_e \times s_e \times f_{GI} \times c_w / d / m$
q_s	Rate of water swallowing while swimming	0,4 mL/min	
t_e	Duration per swimming event	54 min/event	
s_e	Number of swimming events per year	7 events/year	
d	Number of days in a year	365	
U_{oral}	Total pharmaceutical uptake via oral exposure	mg/kg/day	$U_{oral} = U_{os} + U_{dw} + U_f$
ISD	Internal reference dose	mg/kg/day	$ISD = RfD_{oral} \times f_{GI}$
RfD_{oral}	Oral reference dose or ADI (of threshold or nonthreshold compounds)	mg/kg/day	API-specific values (see Table C5)
HQ	Hazard quotient of a pharmaceutical		$HQ = U_{oral} / ISD$

Table C5. API-specific parameter information

Calculation parameter	Parameter description	Unit	Carbamazepine	Diclofenac	Metoprolol
BCF	Bioconcentration factor	mL/mg	22,39	275,42	1,35
f_{GI}	Gastrointestinal absorption factor	-	1	0,97	0,96
RfD_{oral}	Reference dose	mg/kg/day	0,0675	0,0042	0,0075

Result interpretation:

The utility of the presented simple exposure model relies on the data quality and availability, especially API concentration in the surface water. Parameter values used in the model provide a general picture to be drawn, locally adjusted values would improve the reliability of the results.

If HQ turns out to be close to one or greater, considerations of the parameter values used is suggested and adjustments done if needed. In the end, HQ greater than one can be interpreted as increased risk. Frequent monitoring, more elaborated risk assessment, and mitigation measures should be considered.

IV: Cumulative Risk Assessment (CRA)

The presence of mixture of pharmaceuticals in aquatic environments can lead to complex interactions that affect both ecosystem and human health. Each drug targets different biological systems: antibiotics disrupt microbial communities, anti-inflammatory medications and beta-blockers alter physiology and behaviour, anticonvulsants influence neurological functions, and hormones interfere with endocrine systems. When these substances co-occur, their combined effects may amplify their impacts, creating multifaceted risks. Research has shown that mixtures of antibiotics and estrogen-like compounds in aquatic environments disrupt fish reproductive health and affect microbial diversity by reducing nutrient cycling. For example, in Swedish waters, low-level exposure to antibiotics and endocrine disruptors significantly altered the behaviour of fish and microbial communities, preventing essential organic matter decomposition processes from occurring (Brodin et al., 2013). Additionally, findings from Rosi-Marshall et al. (2013) revealed that pharmaceutical mixtures in rivers suppress algal growth, reduce microbial respiration, and alter bacterial communities in stream biofilms. These biofilms are foundational to aquatic food webs, supporting nutrient cycling and energy flow. Such interactions may lead to unforeseen consequences, including disruptions in food webs, changes in nutrient cycling, and potential risks to human health, particularly through water contamination.

Due to these complex interactions between pharmaceuticals, traditional risk assessments that focus on single substances or isolated pathways fall short in capturing the full scope of ecological and health risks. To address this, cumulative risk assessments (CRAs) are crucial, as they offer a more comprehensive view by considering the combined impact of multiple stressors, along with covering multiple risk categories such as environmental, antimicrobial, and HHRA.

However, conducting a CRA poses challenges. It requires the development of new methodologies that go beyond the standard approaches. This often involves applying mathematical models and functions that have not been used before in environmental studies. APRIORA provides tools bringing together different types of data and assesses how pharmaceutical mixtures interact on a broader scope. While building and refining these mathematical frameworks is necessary for accurately evaluating cumulative impacts, it also demands significant innovation, since current methods are limited in addressing the complexities involved with mixtures of contaminants.

1. Component Cumulative Risk Index (CCRI)

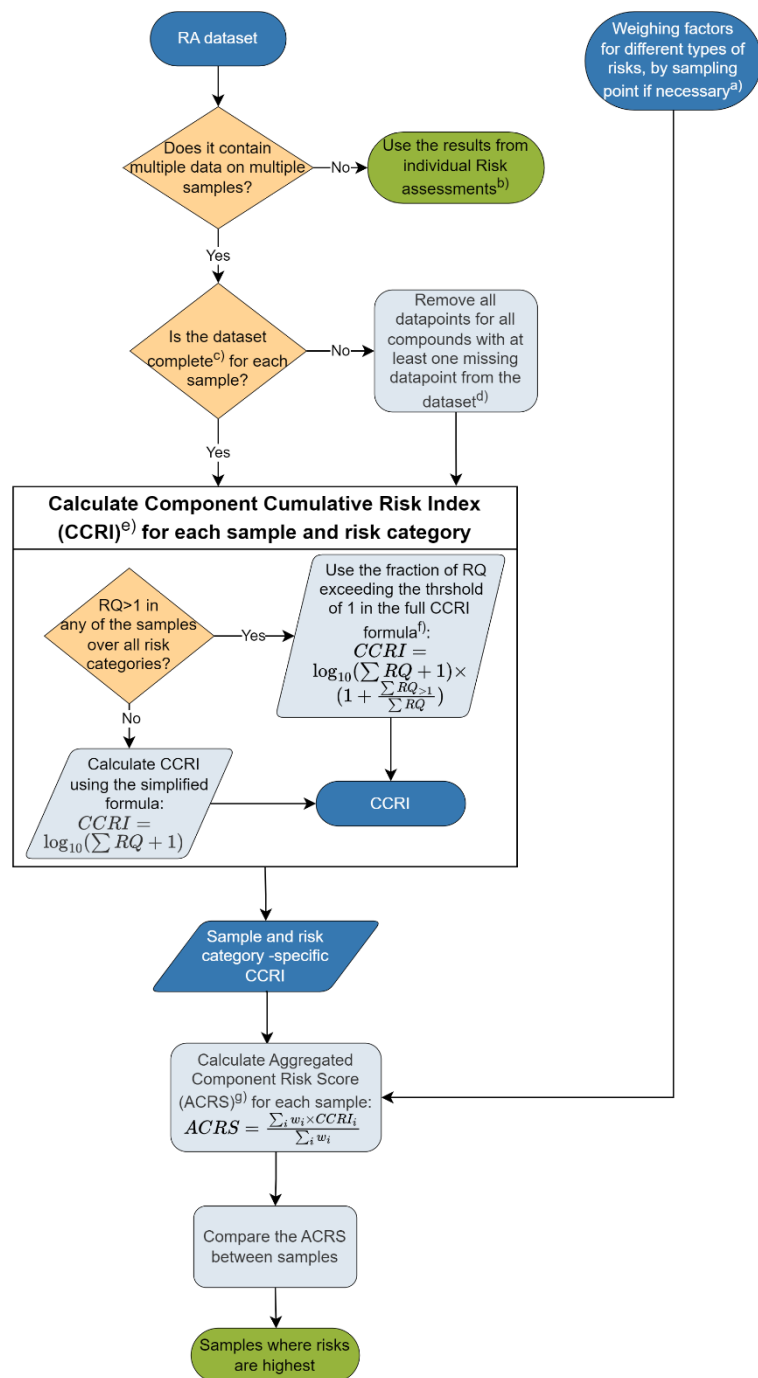
The first step in calculating an overall cumulative risk is to determine the risk index for each component—environmental, antimicrobial, and human health. This is achieved by calculating the **Component Cumulative Risk Index (CCRI)** for each category. The CCRI allows for the aggregation of risks from multiple compounds within each component by using an adjusted logistic function.

$$CCRI = \log(\sum RQ + 1) \times \left(1 + \frac{\sum RQ_{>1}}{\sum RQ}\right) \quad , \text{ where}$$

$\sum RQ$ = total risk of the analysed APIs,
 $\sum RQ_{>1}$ = sum of the RQ's that exceed the threshold of 1

Before calculating the CCRI (Figure C6), it is essential to analyze the risk assessment datasets for completeness and applicability. First, determine whether the dataset contains multiple samples from different sources or only single sample results for each compound. If only individual risk assessments are available, proceed directly with these results. However, if the dataset includes multiple compounds and samples, conduct a preliminary review to check for missing values. In this case, any compound with at least one missing value across samples should be removed from the dataset to ensure only complete data points are used for subsequent CCRI calculations.

Using the APRIORA suggested approaches, the CCRI provides a consistent and comparable measure of risk for each component, enabling a more accurate assessment of the individual risks component before they are combined into an overall component risk score. This method simplifies the process of managing complex datasets and also facilitates clarity in evaluating how each component contributes to the broader risk landscape.



- ^{a)} Weighing factors should be selected based on expert judgement before looking further into the sampling point specific risk assessments. It should be considered if e.g. HHRA-RA should be given higher priority in specific locations, e.g. close to recipient sections used for water intake or bathing.
- ^{b)} If the dataset only contains risk assessment results for a single sampling point, there is not need to combine the results from different risk categories, or to compare the aggregated risks between sampling points.
- ^{c)} Here a complete dataset refers to whether the dataset contains missing information due to e.g. unsuccessful analyses or missing information resulting in RA not being carried out.
- ^{d)} Removing compounds with missing data is important for keeping the CCRI comparable between sampling points. If the number of analytes increases, CCRI has a tendency to increase.
- ^{e)} The goal of the CCRI is to help normalise the risks at each sampling point within each risk category.
- ^{f)} In the equations, RQ = Risk quotient, $RQ_{>1}$ = Risk quotients exceeding the threshold of 1
- ^{g)} The goal of the ACRS is to help identify the samples with the highest normalised risks. I.e., it should be used for risk-based prioritisation of e.g. sampling locations. Please note that ACRS's are not comparable if the analyte list varies between studies. In the equation, $i \in \{ERA, AMR, HHRA\}$, w_i = weighing factors for each risk category, $CCRI_i$ = CCRI's calculated for each risk category.

Figure C6. Flowchart of APRIORA cumulative risk assessment.

2. Aggregated Component Risk Score (ACRS) as an overall cumulative risk measure

Evaluating cumulative risk across different categories presents challenges due to the varying structures of individual risk assessments. To address this, the **Aggregated Component Risk Score (ACRS)** is proposed, which calculates weight sum of risks from each CCRI. This approach enables end users to assign weights based on the significance of each risk type, ensuring that the contribution of each category is adjusted to reflect the specific environmental and health conditions at the sampled site. This allows for a more accurate assessment of cumulative risk based on the unique characteristics and risks present in each case. It also mitigates the potential for results to be skewed by the number of substances sampled or by extreme values encountered within a category.

$$ACRS = \frac{w_{ERA}CCRI_{ERA} + w_{ARA}CCRI_{ARA} + w_{HHRA}CCRI_{HHRA}}{(w_{ERA} + w)_{ARA} + w)_{HHRA}}, \text{ where}$$

w = weights assigned to each risk category ranging from 0 to 1, with a total sum of 1

Weights assigned to each risk category should ideally be adjusted by the end-user based on the specific impact of each risk type at each sampling point. If no adjustments are made, the default approach is to assign equal weight to each risk category. This flexibility allows for targeted assessments where site-specific factors, such as relevance to drinking water and recreational use, are accurately reflected. Implementing the ACRS in this way supports a more nuanced understanding of cumulative risks, aiding informed decision-making and optimizing resource allocation in public health and environmental protection.

3. Challenges in assessing risk accumulation in the Baltic Sea

Due to the restricted water exchange of the Baltic Sea, the cumulative presence of pharmaceuticals amplifies their ecological impact. Advancing CRA methodologies—such as the APRIORA-provided approach—will be crucial for prioritizing potential polluting areas. Tools like the CCRI and the ACRS offer a structured means to assess and compare risks across environmental, antimicrobial, and human health impacts. However, transitioning from cumulative risk assessment to accumulation risk assessment necessitates additional steps. This process requires longitudinal data to evaluate how risks change over time, as well as an analysis of the persistence and degradation rates of contaminants to understand their long-term impacts. Identifying spatial hotspots where pollutants are more likely to accumulate is the first step provided by APRIORA; the next step involves assessing the effects of bioaccumulation within organisms and biomagnification in food webs. Moreover, adjusted thresholds for chronic exposure need to be established, as long-term, low-dose exposure can differ significantly from acute exposure scenarios. It is also important for the Baltic Sea accumulation risk to use the specific PNEC values for marine water (as provided in Table C1), when assessing ERA, rather than general PNEC values.

Summary

This report presents a comprehensive framework designed to assist regional authorities in evaluating the risks associated with pharmaceutical contaminants in water and can be used for identification of places with increased risks and optimisation of monitoring in the end. It integrates traditional environmental risk assessments (ERA), which compare predicted environmental concentrations (PEC) to predicted no-effect concentrations (PNECs), with a targeted antimicrobial resistance risk assessment (AMR-RA). Additionally, it includes HHRA to analyze both direct and indirect risks to human populations.

Cumulative risk assessments (CRAs) provide an effective means of understanding the combined effects of multiple environmental stressors. Although single-substance assessments yield important insights, they often overlook the complex interactions between various pollutants that can intensify risks. CRAs are essential for pinpointing areas in aquatic environments where contaminants, especially pharmaceuticals, pose the highest risks, making them crucial for prioritizing targeted ecological and health risk mitigation efforts.

To implement CRAs effectively, innovative methodologies are necessary. The APRIORA framework offers tools that integrate diverse data sources and advanced mathematical models to evaluate the cumulative impacts of pharmaceutical mixtures. It calculates a Component Cumulative Risk Index (CCRI) for each risk category—environmental, antimicrobial resistance, and human health—allowing for a clearer understanding of the risks posed by multiple stressors. The final step involves determining an Aggregated Component Risk Score (ACRS), which consolidates these individual risk indices into a comprehensive measure. This enables regional authorities to make more informed decisions regarding resource allocation and public health interventions.

In summary, the rising presence of pharmaceutical contaminants in water underscores the limitations of traditional risk assessment methods. The CRA approach outlined in this framework offers a more thorough and realistic evaluation of the cumulative risks associated with pharmaceutical pollutants, serving as a critical tool for protecting both environmental and human health.

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Annexes

Annex C1 - Summary of proposed list of API for monitoring campaigns

Table S1. Summary of PNEC and EQS value for different API

Name of API	CAS No.	Class	ATC (level2)	Proposed regulatory threshold (Inland surface waters) µg/L		Proposed regulatory threshold (Other surface waters) µg/L	*PNEC (NORMAN lowest value freshwaters µg/L)	*PNEC (NORMAN lowest value marine waters µg/L)
Carbamazepine	298-46-4	Anticonvulsant	N03	0.006 ^{a)} 2.5 ^{b)}		0.006 ^{a)} 0.25 ^{b)}	2	0.25
Clarithromycin	81103-11-9	Antibiotic	J01	0.13 ^{b)}		0.013 ^{b)}	0.12	0.013
Diclofenac	15307-86-5	Nonsteroidal anti-inflammatory drug (NSAID)	M01	0.041 ^{b)}		0.004 ^{b)}	0.04	0.004
Metoprolol	51384-51-1	Beta blocker	C07				8.6	0.86
Sulfamethoxazole	723-46-6	Antibiotic	J01	0.1 ^{c)}	0.6 ^{e)}	0.06 ^{e)}	0.6	0.06
Trimethoprim	738-70-5	Antibiotic	J01	0.15 ^{c)} 0.1 ^{c)}			0.5	10
Venlafaxine	93413-69-5	Serotonin-norepinephrine reuptake inhibitor (SNRI) antidepressant	N06	0.0061 ^{c)}			0.88	0.088
Estrone (E1)	53-16-7	Hormone replacement therapy (HRT)	G03	0.00036 ^{b)}		0.000018 ^{b)}	0.00036	0.000018
Fluconazole	86386-73-4	Antifungal	J02	0.25 ^{c)}			1.04	0.1
Primidone	125-33-7	Anticonvulsant	N03	2.5 ^{d)}			9.11	0.91
Ciprofloxacin	85721-33-1	Antibiotic	J01				0.064	0.0064
17Alpha-Ethinylestradiol (EE2)	57-63-6	Hormonal contraceptive	G03				0.000037	0.0000016
Citalopram	59729-33-8	Selective serotonin reuptake inhibitor (SSRI) antidepressant	N06				16	1.6
17Beta-Estradiol (E2)	50-28-2	Hormonal therapy	G03				0.0004	0.000009
Ibuprofen	58560-75-1	Nonsteroidal anti-inflammatory drug (NSAID)	M01	0.22 ^{b)}		0.022 ^{b)}	0.011	0.0011

Name of API	CAS No.	Class	ATC (level2)	Proposed regulatory threshold (Inland surface waters) µg/L	Proposed regulatory threshold (Other surface waters) µg/L	*PNEC (NORMAN lowest value freshwaters µg/L)	*PNEC (NORMAN lowest value marine waters µg/L)
Azithromycin	83905-01-5	Antibiotic	J01	0.019 ^{b)}	0.0019 ^{b)}	0.019	0.0019
Triclosan	3380-34-5	Antimicrobial	D08	0.02 ^{b)}	0.002 ^{b)}	0.02	0.002
Erythromycin	114-07-8	Antibiotic	J01	0.5 ^{b)}	0.05 ^{b)}	0.3	0.05
Irbesartan	138402-11-6	Angiotensin II receptor antagonist (ARB)	C09			700	70
Candesartan	139481-59-7	Angiotensin II receptor antagonist (ARB)	C09			100	10
Amoxicillin	26787-78-0	Antibiotic	J01			0.078	0.078
Clotrimazole	23593-75-1	Antifungal	D01			0.03	0.003
Hydrochlorothiazide	58-93-5	Diuretic	C03			100	10
Guanylurea	141-83-3	Antidiabetic (metabolite of metformin)	A10			100	10
Amisulpride	71675-85-9	Antipsychotic	N05			140	14
Metformin	657-24-9	Antidiabetic	A10			160	16
Miconazole	22916-47-8	Antifungal	D01			0.025	0.0025
O-desmethylenlafaxine	93413-62-8	SNRI (metabolite of venlafaxine)	-			0.88	0.088
Clindamycin	18323-44-9	Antibiotic	J01			0.044	0.0044
Ofloxacin	82419-36-1	Antibiotic	J01			0.5	0.005

* NORMAN Substance Database –

<https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php>).

a) AA-EQS as derived in Carbamazepine draft EQS dossier, 2021 (<https://circabc.europa.eu/ui/group/9ab5926d-bed4-4322-9aa7-9964bbe8312d/library/ad07a599-cd2e-49c3-9fa0-d0203495a5e1/details>)

b) AA-EQS as proposed in EC 2024 Document P9_TA(2024)0358 ([https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=EP:P9_TA\(2024\)0358](https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=EP:P9_TA(2024)0358))

c) Maximum acceptable method detection or quantification limit as presented in COMMISSION IMPLEMENTING DECISION (EU) 2022/1307 (https://www.stradalex.eu/en/se_src_publ_leg_eur_jo/toc/leg_eur_jo_3_20220726_197/doc/ojeu_2022.197.01.0117.01)

d) Groundwater quality standard (annual average) as proposed in EC 2024 (chrome-extension://efaidnbmninnibpcapjpcglclefindmkaj/https://health.ec.europa.eu/document/download/506c48f1-02a9-4e8d-a5cc-ff8855b917fc_en?filename=scheer_o_035.pdf)

e) Nendza, M. (2014): EQS DATASHEET Environmental Quality Standard Sulfamethoxazol, <https://webetox.uba.de/webETOX/public/basics/literatur.do?id=24270>

Table S2. Description of proposed list of API for monitoring campaigns.

API name	Description	Reference
Carbamazepine	White, crystalline substance with limited solubility in water but good solubility in organic solvents such as ethanol. Widely used as an anticonvulsant to treat epilepsy and bipolar disorder, carbamazepine is also prescribed for neuropathic pain. Due to	Baali, H., & Cosio, C. (2022).

API name	Description	Reference
	its extensive use, it is commonly found in aquatic environments. Carbamazepine has resistance to biodegradation that leads to its persistence in water bodies, where it may accumulate in sediments and potentially affect aquatic life.	Canadian Council of Ministers of the Environment (CCME). (2018).
Clarithromycin	An antibiotic moderately soluble in water and primarily used to treat various bacterial infections, including respiratory tract infections, skin infections, and <i>Helicobacter pylori</i> infection. In the environment, clarithromycin can persist due to its stability and may contribute to the development of antibiotic resistance in microbial communities.	Fouz, N. et. al. (2020)
Diclofenac	Nonsteroidal anti-inflammatory drug that appears as a white to slightly yellow crystalline powder. It is sparingly soluble in water and is commonly used to reduce inflammation and as an analgesic in conditions like arthritis. Environmentally, diclofenac is known for its poor biodegradability, leading to its frequent detection in water bodies. It has been associated with adverse effects on aquatic organisms due to its toxic effects at environmentally relevant concentrations.	Bonnefille, B. et. al., (2017).
Metoprolol	Selective beta-1 receptor blocker. Highly soluble in water and is used primarily for managing hypertension, angina, and heart failure. Metoprolol's environmental impact indicates that it is not readily biodegradable and has been detected in treated wastewater and surface waters in Stockholm. There are concerns regarding its effects on aquatic species due to its mode of action as a beta-blocker.	Janusinfo. (2021).
Sulfamethoxazole	A sulfonamide antibiotic, exhibiting moderate solubility in water. It is often used in combination with trimethoprim to treat bacterial infections, including urinary tract infections. Its environmental presence raises concerns about the promotion of antibiotic resistance and its potential toxic effects on aquatic organisms.	Rauseo, J., et. al., (2021).
Trimethoprim	Dihydrofolate reductase inhibitor. It has moderate water solubility and is commonly used in combination with sulfamethoxazole for treating bacterial infections. Trimethoprim is frequently detected in aquatic environments due to its widespread use and resistance to biodegradation. Its presence in the environment can contribute to the development of antibiotic-resistant bacteria.	Straub, J. O. (2013).
Venlafaxine	Antidepressant with a good solubility in water. It is primarily prescribed for the treatment of depression, anxiety, and panic disorders. In the environment, venlafaxine is persistent and often detected in wastewater effluents and surface waters. Its presence in aquatic environments may affect the behaviour and physiology of aquatic organisms due to its influence on neurotransmitter systems.	Mehdi, H., et. al., (2019).
Estrone	Naturally occurring estrogen. It is slightly soluble in water and is commonly used in hormone replacement therapy. Estrone can enter the environment through wastewater effluents and is often found in surface waters. It is of concern due to its endocrine-disrupting potential, which can adversely affect the reproductive systems of aquatic organisms even at low concentrations.	Pironti, C., et. al (2021).
Fluconazole	Antifungal agent. It is highly soluble in water and is used to treat fungal infections, including candidiasis. Its presence may affect microbial communities and contribute to the development of antifungal resistance.	Assress, H., et. al., (2021).
Primidone	Anticonvulsant with moderate solubility in water. It is used primarily to manage seizures and essential tremors. If primidone's environmental behaviour resembles that of carbamazepine, it raises significant concerns about its potential impacts on aquatic life.	Khan, A. H. A., & Barros, R. (2023).

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Annex C2 - Summary of PNEC_R values for different API

Antibiotic	PNEC _R (µg/L)	Experimental system	Method	Method endpoint	Reference
Amikacin	16	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Amoxicillin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Amphotericin B	0.016	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ampicillin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Anidulafungin	0.016	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Avilamycin	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Azithromycin	50	Microcosm wastewater influent	qPCR statistic	AMR-G (ermF)	Stanton et al., 2020
Azithromycin	50	SELECT wastewater influent	SELECT statistic	Growth (OD)	Murray et al., 2020
Azithromycin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Aztreonam	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Bacitracin	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Benzylpenicillin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Capreomycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefaclor	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefadroxil	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefalexin	4	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefaloridine	4	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefalothin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefazolin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefdinir	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefepime	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefixime	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefoperazone	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefotaxime	4	Microcosm wastewater influent	qPCR MSC	CTX-M	Murray et al., 2018

Antibiotic	PNEC _R (µg/L)	Experimental system	Method	Method endpoint	Reference
Cefotaxime	0.78	Microcosm wastewater influent	SELECT statistic	Growth (OD)	Murray et al., 2020
Cefotaxime	6.25	Microcosm wastewater influent	qPCR statistic	CTX-M & <i>int1</i>	Murray et al., 2020
Cefotaxime	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefoxitin	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefpirome	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefpodoxime	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ceftaroline	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ceftazidime	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ceftibuten	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ceftiofur	0.064	MIC-derived PNEC _R - MICs dataset for clinical strain	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ceftobiprole	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ceftriaxone	0.032	MIC-derived PNEC _R - MICs dataset for clinical strain	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cefuroxime	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Chloramphenicol	12.5	SELECT wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020
Chloramphenicol	25	Microcosm wastewater influent	qPCR statistic	<i>int1</i>	Murray et al., 2020
Chloramphenicol	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ciprofloxacin	0.1	Isogenic competition (E. coli)	Ratio MSC	<i>gyrA1</i>	Gullberg et al. 2011
Ciprofloxacin	0.05	SELECT wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020
Ciprofloxacin	0.01	Wastewater Biofilm microcosm	Metagenome statistics	<i>qnrD</i> , <i>qnrS</i> , <i>qnrB</i>	Kraupner et al., 2018
Ciprofloxacin	10.77	Microcosm wastewater influent	qPCR MSC	<i>int1</i>	Stanton et al., 2020
Ciprofloxacin	4	Isogenic competition (E. coli)	Ratio MSC	Unknown mutant	Vos et al., 2020
Ciprofloxacin	0.78	Microcosm wastewater influent	qPCR statistics	<i>int1</i>	Stanton et al., 2020
Ciprofloxacin	0.064	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Clarithromycin	250	Microcosm wastewater influent	qPCR statistic	<i>ermF</i>	Stanton et al., 2020
Clarithromycin	50	Microcosm wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020

Antibiotic	PNEC _R (µg/L)	Experimental system	Method	Method endpoint	Reference
Clarithromycin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Clavulanic acid	64	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Clinafloxacin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Clindamycin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Cloxacillin	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Colistin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Daptomycin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Doripenem	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Doxycycline	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Enrofloxacin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ertapenem	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Erythromycin	514.1	Microcosm wastewater influent	qPCR statistics	<i>ermF</i>	Stanton et al., 2020
Erythromycin	50	Microcosm wastewater influent	qPCR statistics	<i>ermF</i>	Stanton et al., 2020
Erythromycin	200	Isogenic competition (E. coli)	Ratio MSC	<i>mph</i>	Gullberg et al. 2011
Erythromycin	1250	SELECT wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020
Erythromycin	1	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ethambutol	2	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Faropenem	0.016	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Fidaxomicin	0.016	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Florfenicol	2	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Fluconazole	0.25	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Flumequine	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Fosfomycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Fusidic acid	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Gatifloxacin	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016

Antibiotic	PNEC _R (µg/L)	Experimental system	Method	Method endpoint	Reference
Gemifloxacin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Gentamycin	12.5	SELECT wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020
Gentamycin	12.5	SELECT wastewater influent	qPCR statistics	<i>int1</i>	Murray et al., 2020
Gentamycin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Imipenem	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Isoniazid	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Itraconazole	0.008	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Kanamycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Levofloxacin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Lincomycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Linezolid	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Loracarbef	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Mecillinam	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Meropenem	0.064	MICs dataset for individual clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Metronidazole	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Minocycline	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Moxifloxacin	0.125	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Mupirocin	0.25	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Nalidixic acid	16	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Narasin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Neomycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Netilmicin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Nitrofurantoin	64	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Norfloxacin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ofloxacin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016

Antibiotic	PNEC _R (µg/L)	Experimental system	Method	Method endpoint	Reference
Oxacillin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Oxytetracycline	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Pefloxacin	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Phenoxymethylpenicillin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Piperacillin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Quinupristin–dalfopristin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Retapamulin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Rifampicin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Roxithromycin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Secnidazole	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Sparfloxacin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Spectinomycin	32	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Spiramycin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Streptomycin	16	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Sulbactam	16	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Sulfamethoxazole	16	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Teicoplanin	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Telithromycin	0.064	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Tetracycline	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Thiamphenicol	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Tiamulin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Ticarcillin	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Tigecycline	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Tilmicosin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Tobramycin	1	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016

Antibiotic	PNEC _R (µg/L)	Experimental system	Method	Method endpoint	Reference
Trimethoprim	42.7	Isogenic competition (E. coli)	Ratio MSC	<i>dfrA12</i>	Kraupner 2020
Trimethoprim	33	Isogenic competition (E. coli)	Ratio MSC	<i>dhfr</i>	Gullberg 2014
Trimethoprim	3.13	Microcosm wastewater influent	qPCR statistic	<i>int1</i>	Murray et al., 2020
Trimethoprim	1.56	SELECT wastewater influent	SELECT statistics	Growth (OD)	Murray et al., 2020
Trimethoprim	1	Microcosm wastewater effluent	CFU statistics	% resistant E. coli	Kraupner 2020
Trimethoprim	1	Microcosm E. coli, mixed population	CFU statistics	% resistant E. coli	Kraupner 2020
Trimethoprim	0.5	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Trovafloracin	0.032	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Tylosin	4	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Vancomycin	8	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Viomycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016
Virginiamycin	2	MIC-derived PNEC _R - MICs dataset for clinical strains	theoretical approach (extrapolation)		Bengtsson-Palme & Larsson, 2016

Annex C3 - APRIORA stepwise strategy to derive PNEC_R values for AMR-RA (procedures)

1. Introduction

In the AMR-RA, the (RQ_R) is calculated using the following formula:

$$RQ_R = \frac{C_R}{PNEC_R},$$

where

- C_R is the measured environmental concentration (MEC) or the predicted environmental concentration (PEC) of the antimicrobial API
- PNEC_R is the predicted no-effect concentration for resistance selection

In APRIORA project, a stepwise strategy is applied to select or derive PNEC_R values (see GoA 1.1, Module C for details).

Tier 1 – Theoretical assay (MIC-based PNEC_R)

Use MIC-derived PNEC_R values compiled by Bengtsson-Palme and Larsson (2016), which provide minimal selection concentration boundaries and PNEC_R for 111 antibiotics and 11 antibiotic combinations (for details see Anex 2).

Tier 2 – Phenotypic assay (OD-based PNEC_R)

If no robust compound-specific PNEC_R is available, the available value is not sufficiently representative, or additional site-specific validation is required, expose a microbial community to a concentration gradient of antimicrobial agent and apply an OD600-based community growth assay to derive a phenotypic threshold of PNEC_R (for details see below Section 2 and 3).

Tier 3 – Molecular assay (qPCR-based PNEC_R)

If Tier 2 is inconclusive or higher specificity is needed, expose the microbial community to a concentration gradient of the antimicrobial and quantify selected antibiotic resistance genes (AMR-G) by qPCR to derive a gene-based PNEC_R threshold. This approach is more sensitive and informative but more resource-demanding (for details see Section 2 and 4).

The APRIORA stepwise strategy for assessing PNEC_R values is presented in Figure SC3.1.

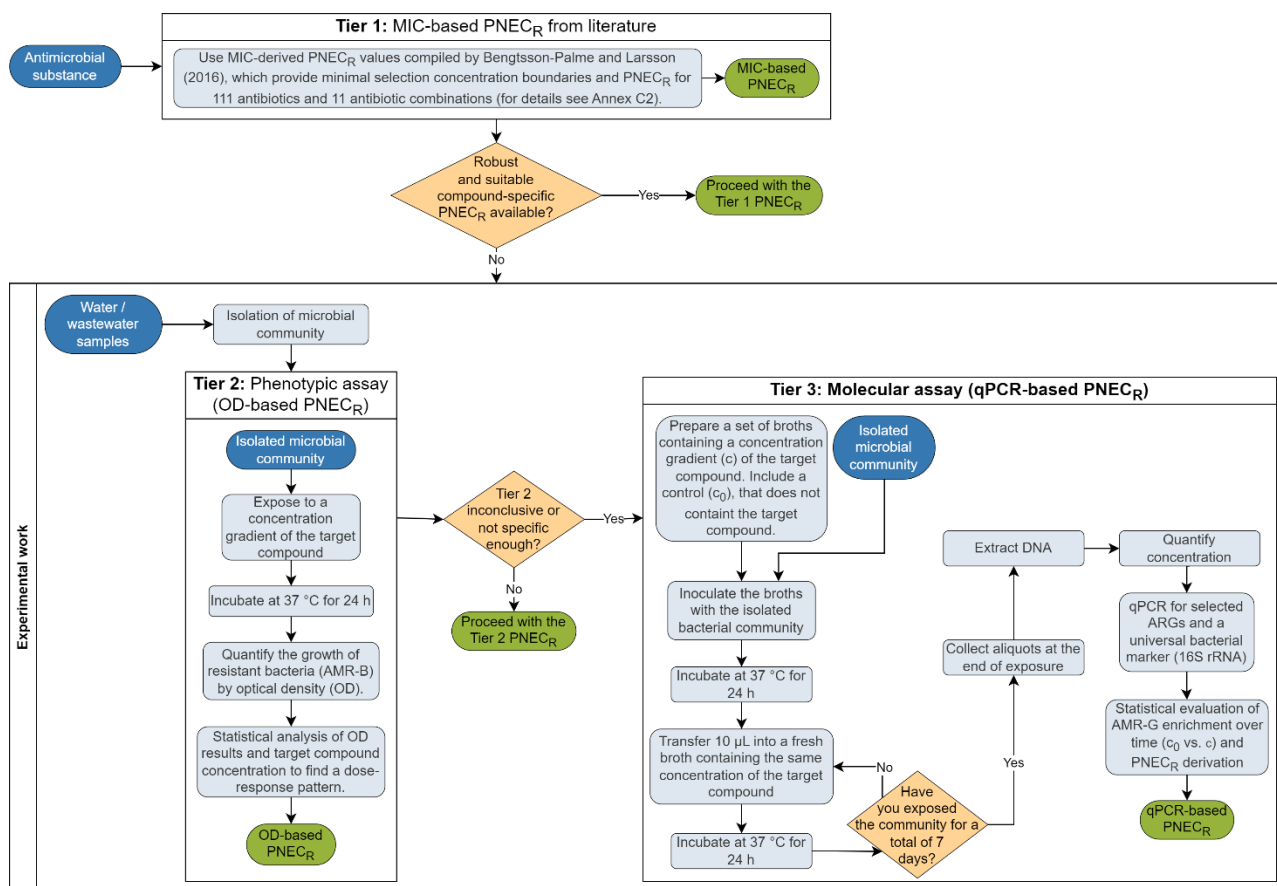


Figure SC3.1. The APRIORA stepwise strategy for assessing PNEC_R values

The methodology of phenotypic assay (OD-based) and molecular assay (qPCR-based) in details is presented in Figure SC3.2 and described below. Both methods are based on microbial community isolated from the tested environment.

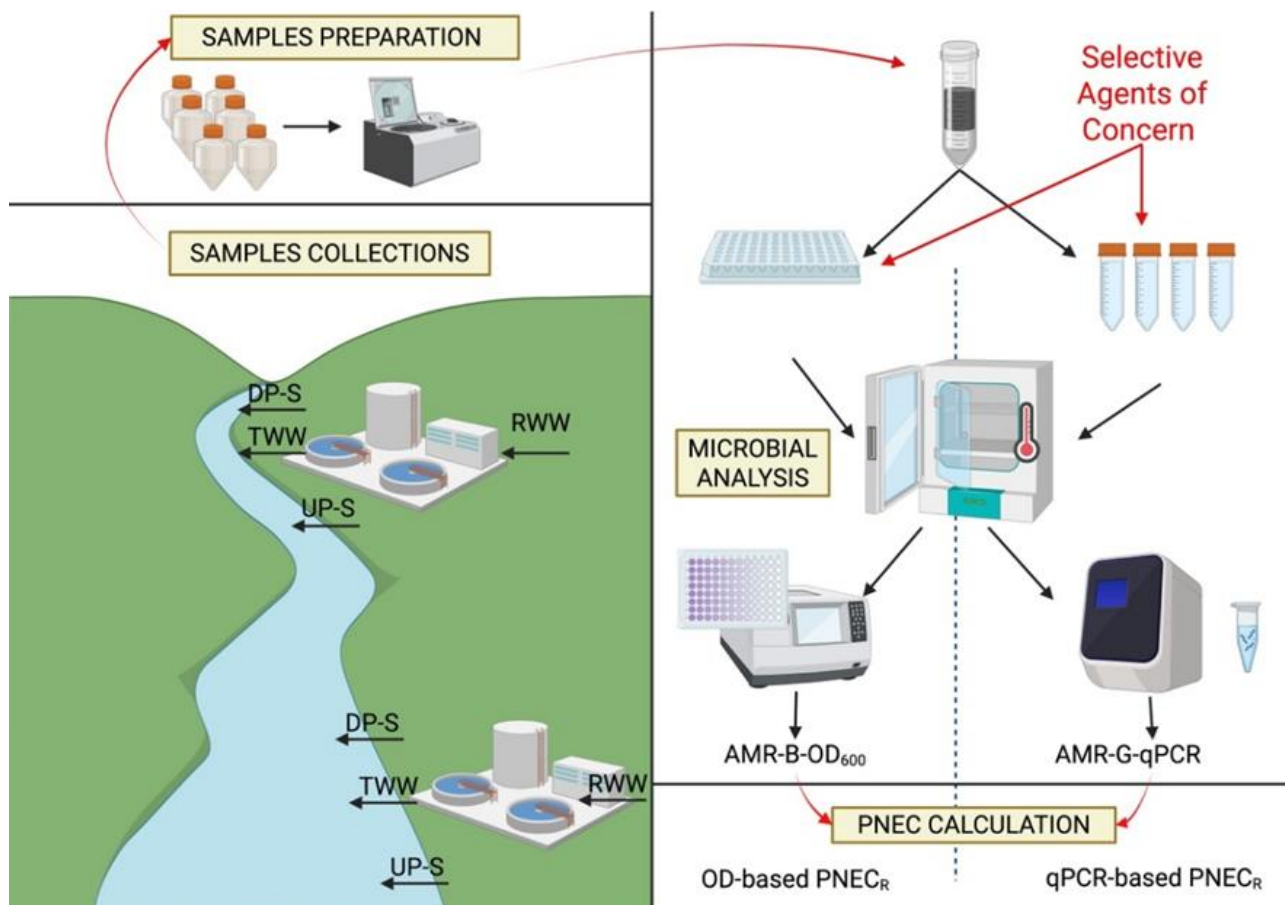


Figure SC3.2 Schematic visualization of phenotypic assay (OD-based $PNEC_R$) and molecular assay (qPCR-based $PNEC_R$)

2. Microbial community isolation

The aim of this step is to recover the environmental bacterial community from the tested sample (water/wastewater) and to obtain the sufficient amount for further phenotypic and molecular assay. The community is collected by membrane filtration. Microorganisms retained on the filter are transferred into sterile growth medium and enriched under controlled laboratory conditions

2.1 Equipment and materials required for microbial community isolation step

- a sterile filtration set (e.g. funnel, flask, filter holder)
- a vacuum pump
- sterile membrane filters (MCE nitrocellulose), 0.45 μm pore size
- sterile forceps
- Iso-Sensitest™ broth (Oxoid; CM0473)
- sterile culture vessels (e.g., sterile 50 mL conical tubes)
- incubator set to 37°C.

2.2 Procedure

Filter environmental samples within 4h after collection. Recommended volumes are ~50 mL for raw/treated wastewater and ≥100 mL for upstream/downstream receiving-water samples (adjust as needed to avoid filter clogging).

After filtration, aseptically transfer the membrane filter into a sterile vessel containing Iso-Sensitest™ broth and incubate for 24 h at 37°C.

The enriched culture can be used directly for Tier 2 - OD-based PNEC_R (Section 3) and Tier 3 - qPCR-based PNEC_R (Section 4). If storage is required, use short-term refrigeration (4°C) and prepare glycerol stocks for long-term storage (–80°C), as appropriate for the project's biosafety procedures.

Before testing, bacteria stored in the freezer should be revived by incubating them for 24 hours at 37°C in sterile Iso-Sensitest™ broth.

3. Phenotypic assay (OD-based PNEC_R)

This assay measures microbial community growth across a gradient of antibiotic concentrations to generate a phenotypic concentration–response. Growth of antibiotic resistant bacteria (AMR-B) is quantified by optical density at 600 nm (OD600) (Figure SC3.3).

Note: Enriched microbial communities comprise many taxa with different antibiotic sensitivities. Inhibition of susceptible bacteria can release resources and ecological niches, allowing more tolerant populations to proliferate; therefore, the overall OD600 signal may not decrease markedly even at higher antibiotic concentrations. Consequently, OD-based endpoints are community-level indicators and the results may be difficult to interpret unambiguously with respect to resistance selection. If OD-based results are inconclusive, qPCR-based assays targeting defined antibiotic resistance genes (AMR-G) should be applied (see Section 4).

3.1 Equipment and materials required for phenotypic assay:

- enriched bacterial culture (prepared in Section 2)
- Iso-Sensitest™ broth
- stock solutions of the tested antibiotics
- sterile 96-well microtiter plates
- pipettes and sterile tips
- an incubator set to 37°C
- a UV/Vis microplate reader capable of measuring absorbance at 600 nm.

3.2 Procedure

Prepare a series of increasing antibiotic concentrations in Iso-Sensitest™ broth in a 96-well plate. The highest tested concentration should be several times higher than the corresponding MIC-derived PNEC_R value (Annex 2). The procedure should then be carried out according to the scheme presented in Figure SC3.1. In addition, a visual representation of the experimental workflow is provided in the video material attached to the document (Walkthrough video 1. OD-based PNEC_R, [Link](#)). Depending on the experimental design, the isolated community can be tested against several antibiotics, each analyzed separately in rows A to F, as shown in Figure SC3.2.

Incubate the prepared plates for 24 h at 37 °C, then immediately measure absorbance at 600 nm (OD600) using a microplate reader

3.3 Interpretation of OD600 results

- High OD600 indicates strong community growth.
- Decreasing OD600 with increasing antibiotic concentration indicates growth inhibition.

- A comparatively weak decrease in OD600 at higher concentrations may indicate a higher tolerance of the community to the tested antibiotic.

Interpretation should consider that OD-based endpoints are community-level indicators, which may make interpretation of OD-based dose–response patterns less straightforward (as discussed above).

3.3 Estimation of OD-based $PNEC_R$

The assay endpoint is $PNEC_R$ derived from the growth response. OD600 values were blank-corrected and normalised to the no-antibiotic growth control. Then, the relationship between OD600 and antibiotic concentration may be analysed statistically in order to identify the concentration range showing the clearest dose–response pattern. The appropriate toxicological endpoint (TE) should be selected according to the type of test.

- Pearson’s correlation coefficient may be used for normally distributed data,
- Spearman’s rank correlation coefficient may be used for non-normally distributed data.
- if the result cannot be determined unambiguously by correlation analysis, the OD600 results are normalised to the highest value obtained for a given antibiotic in a given sample. TE is then estimated by interpolation from the obtained dataset.

Finally, the OD-based $PNEC_R$ can be calculated using the following equation.

$$PNEC_R = \frac{TE}{AF} \quad (1)$$

Where,

- TE - toxicological endpoint appropriate for a used ecotoxicological test
- AF - assessment factor; the AF = 10 acc. to the recommendation of European Medicines Agency (EMA, 2022)

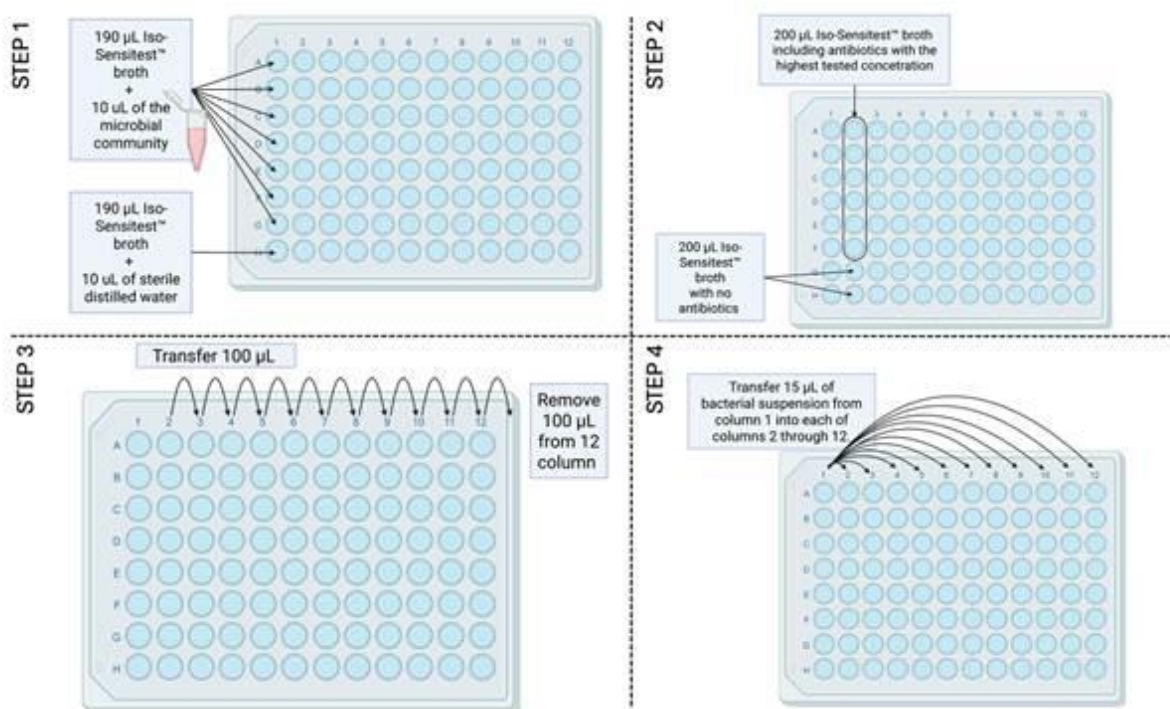


Figure SC3.3. Procedure for preparing a 96-well plate for the phenotypic assay (OD-based $PNEC_R$)

4. Molecular assay (qPCR-based PNEC_R)

This assay evaluates whether exposure of microbial communities to a gradient of increasing antibiotic concentrations results in a concentration-dependent increase of relevant resistance genes (AMR-Gs). For tested antimicrobial agent the highest concentration should be several times higher than the corresponding PNEC_R value given in Annex 2. The method is based on serial passaging microbial suspensions in liquid medium containing defined concentrations of antibiotics, followed by qPCR quantification of target AMR-Gs normalized to 16S rRNA (universal bacterial marker).

Changes in AMR-Gs relative abundance between Day 0 and Day 7 are used to determine selection endpoints (LOEC_R/NOEC_R) and to derive qPCR-based PNEC_R. Schematic visualization of molecular assay (qPCR-based PNEC_R) is presented in Figure SC3.4.

4.1 Equipment and materials required for molecular biofilm assay:

- enriched bacterial culture (prepared in section 2)
- Iso-Sensitest™ broth (Oxoid; CM0473)
- stock solutions of the tested antibiotics
- sterile microcentrifuge tubes (1.5 – 2.0 mL)
- pipettes and sterile tips
- incubator set to 37°C
- freezer (–20°C or below) for sample storage,
- DNA isolation kit suitable for mixed bacterial communities
- instrument for DNA quantification (e.g., Qubit® or NanoDrop)
- qPCR thermocycler and compatible consumables (e.g., sterile 96-well plates, adhesive seals)
- gene-specific flanking primers (for the selected AMR-Gs and for 16S rRNA)

4.2. Procedure

Prepare sterile tubes containing 990 µL Iso-Sensitest™ broth with the specified antibiotic concentration and inoculate with 10 µL of the bacterial community. Incubate for 24 h at 37°C. After each 24 h incubation, transfer 10 µL into 990 µL of fresh broth containing the same antibiotic concentration and incubate for a further 24 h. Repeat this transfer six times (total exposure period: 7 days), as illustrated in Figure SC3.4. In addition, a visual representation of the experimental workflow is provided in the video material attached to the document (Walkthrough video 2. qPCR-based PNEC_R, [Link](#)).

Collect aliquots for DNA extraction on Day 0 (before exposure) and Day 7 (end of exposure). Extract DNA, quantify concentration, and perform qPCR for the selected AMR-Gs and the 16S rRNA reference gene.

4.3 Interpretation of qPCR results

qPCR yields Ct (cycle threshold) values for each targeted gene in a given sample. Ct is the PCR cycle number at which fluorescence exceeds the detection threshold and becomes detectable; therefore, a lower Ct indicates a higher initial abundance of the target sequence. To account for differences in total bacterial biomass and DNA yield between samples, AMR-G signals are normalized to the 16S rRNA reference gene (universal bacterial marker). In environmental samples, the Ct value of the 16S rRNA reference gene is often lower than that of the targeted AMR-Gs, as the reference gene is typically more abundant. For each sample, time point, and antibiotic concentration the relative abundance of the target gene (q) is described by the equations from 2 to 4.

$$Ct = \Delta Ct_g - Ct_{ref}$$

$$q = 2^{(\Delta t_{ref} - Ct_g)} \quad (2)$$

$$(3)$$

where:

- q is the relative abundance (normalized copy number) of the AMR-G
- Ct_{ref} is Ct value for the reference gene (16S rRNA),
- Ct_g is Ct value for the target resistance gene (AMR-G)

To evaluate whether antibiotic exposure is associated with an enrichment of AMR-Gs over time (consistent with selection), compute the change relative to baseline (Day 0):

$$\Delta\Delta Ct = \Delta Ct_{Day7} - \Delta Ct_{Day0} \quad (4)$$

$$FC = 2^{-\Delta\Delta Ct} = \frac{q_{Day7}}{q_{Day0}} \quad (5)$$

where

- FC is the fold change in AMR-G relative abundance (q) between Day 7 and Day 0 for a given antibiotic concentration.

Interpretation logic

- $FC > 1$ indicates an increase in the relative abundance of the targeted AMR-G
- $FC \approx 1$ indicates no change
- $FC < 1$ indicates a decrease in relative abundance

Note: environmental samples may contain numerous compounds and selective agents that contribute to the co-selection of antibiotic resistance. Therefore, the initial abundance of resistance genes may be influenced by factors other than the presence of the tested antibiotic in the environment.

2.4 Estimation of qPCR-based $PNEC_R$

The main endpoint of this assay is the qPCR-based $PNEC_R$ value, derived from changes (between Day 0 and Day 7) in the abundance of the gene conferring resistance to the tested antibiotic. This approach allows the determination of the relationship between the $LOEC_R$ (the lowest concentration at which a statistically significant increase is detected) and $NOEC_R$ (the highest concentration below $LOEC_R$ with no significant increase), as defined by Murray et al. (2020). The qPCR-based $LOEC_R$ for AMR-Gs is determined using Dunn's test

Subsequently, the qPCR-based $PNEC_R$ value was calculated according to Equation (4).

$$PNEC_R = \frac{NOEC_R}{AF} \quad (4)$$

Where:

- $NOEC_R$ – the highest tested concentration at which no effect on resistance was observed.
- AF - assessment factor; the $AF = 10$ acc. to the recommendation of European Medicines Agency (EMA, 2022)

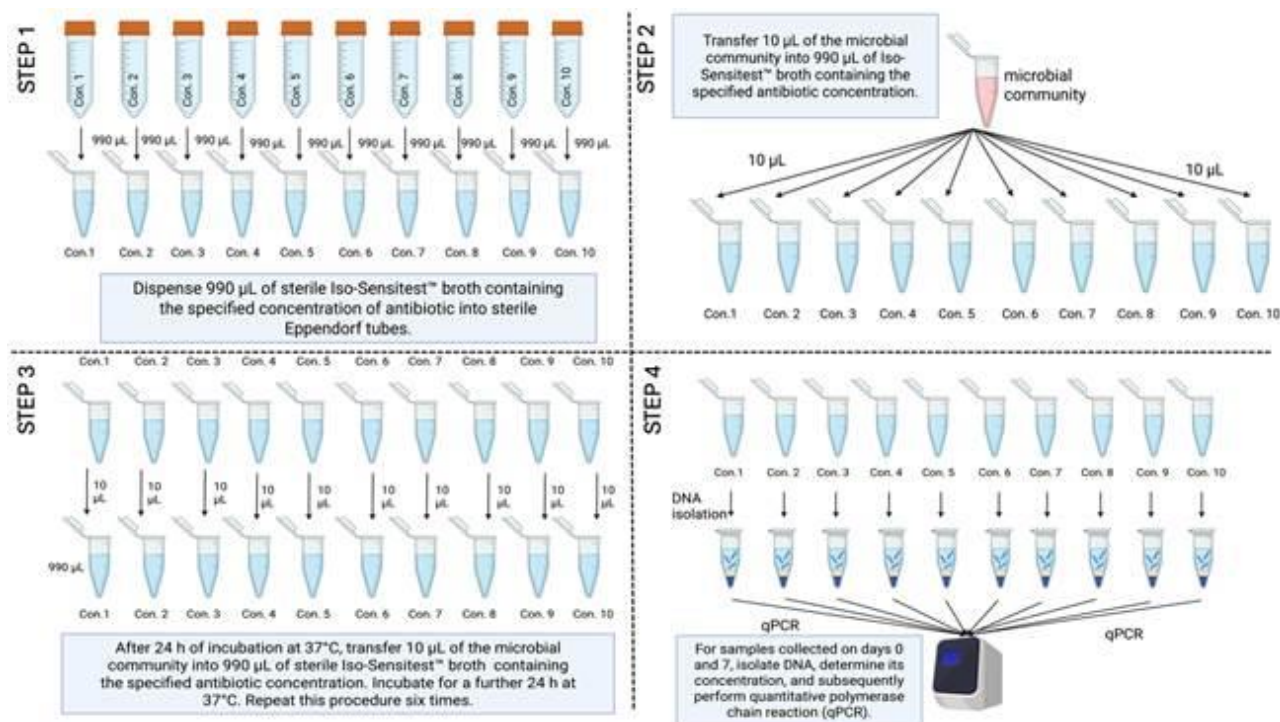


Figure SC3.4. Schematic visualization of molecular assay (qPCR-based PNEC_R)