Deliverable of WG5

Deliverable 19

List of quality criteria concerning ARB&ARGs and biological risks related to contaminants of emerging concern

August 2017

COST is supported by the EU Framework Programme Horizon 2020

This article is based upon work from the COST Action ES1403: New and emerging challenges and opportunities in wastewater reuse (NEREUS), supported by COST (European Cooperation in Science and Technology).

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1. Executive Summary

The aim of this report, based on the current knowledge, is to define a list of quality criteria concerning the contaminants of emerging concern (CECs) and the antibiotic-resistant bacteria and resistance genes (ARB&ARGs) for treated wastewater (TWW). The quality criteria in this deliverable are considered the threshold or limit values. Within this deliverable we use agricultural irrigation and aquifer recharge as two examples of relevant uses, considered also at EU level, and we describe the methodological approach that should be used to derive quality criteria. The quality criteria of the monitoring parameters should protect environment and health. The deliverable takes into account the current work that is currently performed at EU level for the elaboration of the minimum quality requirements for water reuse, for which NEREUS has given a contribution and it takes into account the work performed in the different NEREUS WGs. The deliverable includes the quality criteria for CECs and ARB&ARGs. Furthermore, it is recommended to consider in the evaluation also the use of effect-based methods for the detection of mixtures and effects caused by unknown compounds. A section is related also to the environmental risks taking into account the risks for soil, surface and ground waterbodies that can happen in certain local conditions. In conclusion, a list of CECs based on analysis performed in the context of NEREUS is suggested for the monitoring of TWW.

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Acronyms

ADI	Acceptable Daily Intake
AMR	Antimicrobial Resistance
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistance Genes
CECs	Contaminants of Emerging Concern
CIS	Common Implementation Strategy
COD	Chemical Oxygen Demand
DBPs	Disinfection By-Products
DWD	Drinking Water Directive
EC	European Commission
ECB	European Chemicals Bureau
EDC	Endocrine Disrupting Compound
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EBM	Effect-Based Methods
EQS	Environmental Quality Standards
EQSD	Environmental Quality Standards Directive
ESBL	Extended Spectrum Beta Lactamases
ERA	Environmental Risk Assessement
EU	European Union
FAO	Food and Agriculture Organization
GWD	Groundwater Directive
HACCP	Hazard Analysis and Critical Control Points
IPR	Indirect Potable Reuse

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ISO	International Organization for Standardization
IPR	Indirect Potable Reuse
JECFA	Joint Expert Committee on Food Additives
JRC	Joint Research Center
LOAEL	Lowest observed adverse effect level
MS	Member States
MOAs	Mode of Actions
MTD	Minimum Therapeutic Dose
NOEL	No Observable Effect Level
NOAEL	No observed adverse effects level
PNEC	Predicted no-effect concentration
POD	Point of departure
RfD	Reference dose
TDI	Tolerable Daily Intake
TOC	Total Organic Carbon
TTC	Threshold of Toxicological Concern
TWW	Treated Wastewater
USEPA	United States Environmental Protection Agency
WFD	Water Framework Directive
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

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2. Introduction

The use of treated wastewater (TWW) can cause negative effects on human health and the environment. TWW may contain hazards such as physical, chemical, radiological and microbial agents that constitute risk to human health (WHO, 2006-2015-2016; JRC EU 2017; JRC, 2014). These health concerns have prompted the development of TWW reuse guidelines and regulations in many countries, primarily focused on the use of TWW in agricultural irrigation (Report of WG3 "Wastewater Reuse on an International Scale: Application and Linked Legislation on Quality Requirements"). Guidelines and criteria developed to date follow mainly the classical principles of the risk assessment methodology and recommend the monitoring of a set of regulated traditional parameters (e.g. heavy metals, organic regulated chemicals, macro-descriptors, indicators of faecal pollution) and their comparison to derived values with a variety of quality criteria based mainly on the risk for human health in relation to consumption of drinking water and, in some cases, of crops (NEREUS Deliverable 13; DM, 2003; NP, 2005; CMD, 2011; Jorf, 2014).

These approaches have some limitations. Firstly, they do not consider the possible risks deriving from contaminants of emerging concern (CECs) that are present in wastewaters (Loos, 2013). To ensure that risks to human health and environmental health are minimized, quality criteria for identified CECs are strongly needed (Fatta-Kassinos 2016; SCHEER 2017) in addition to the routine parameters that are monitored for compliance with guidelines and/or regulations. Another limitation in existing guidelines is that the classical guidelines and criteria do not take into consideration the risks for the aquatic and terrestrial ecosystems deriving from practices such as irrigation and aquifer recharge; for example for the use of pesticides, EFSA (European Food Safety Authority) has produced several guidelines and recommendations about ERA (Environmental Risk Assessment) in order to protect terrestrial and aquatic organisms (https://www.efsa.europa.eu/en/topics/topic/environmental-risk-assessment), and avoid also bioaccumulation of the compounds in target and non-target species. Furthermore, another limitation to the current guidelines is that the risks caused by the mixtures of chemical compounds are not taken into considerations (EC, 2014; Wernersson, 2015). Other key concerns are related to the spread of antibiotic resistance genes in the environment and for this reason there is a strong need of quality criteria also for antibiotic-resistant bacteria and resistance genes (ARB&ARGs) (EFSA Opinion 2017; WG1 Deliverables). The quality criteria are key elements in risk assessment in order to define the appropriate measures to apply for the protection of environment and human health; an

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exceedance of quality criteria for a single chemical compound for example could potentially represent a risk and a risk assessment procedure should be elaborated to evaluate the magnitude of the risk, the duration and the typology of adverse effects (acute or chronic).

3. Contaminants of Emerging Concern (Quality Criteria for Aquifer recharge and Irrigation)

CECs include many groups of chemical compounds which can be categorized by end use (e.g. pharmaceuticals, non-prescription drugs, personal care products, household chemicals, food additives, flame retardants, plasticizers, disinfection by-products, and biocides), by environmental and/or human health effects (e.g. hormonally active agents, endocrine disrupting compounds), as well as transformation products resulting from various biotic and abiotic processes, both as single substance and mixtures of chemicals (Ternes, 1998; Zuccato, 2000; Laws, 2011; WHO, 2011; USEPA, 2012). The possibility of these substances having an impact on the environment and human health due to their reported carcinogenic, teratogenic, mutagenic, neurotoxic and other adverse effects cannot be neglected and quality criteria are needed.

3.1 Quality Criteria for Aquifer Recharge

The main categories of use defined for aquifer recharge are the following:

- Aquifer recharge by surface spreading for non-drinking water purposes at the present and medium future
- Aquifer recharge by direct injection for non-drinking water purposes at the present and medium future
- Aquifer recharge by surface spreading for drinking water purposes or intended for such future use (indirect potable reuse)
- Aquifer recharge by direct injection for drinking water purposes or intended for such future use (indirect potable reuse)

Safe quality criteria for aquifer recharge may potentially be derived on the basis of the same toxicological considerations used for the establishment of current EU Drinking Water Directive (DWD) threshold values (Directive 98/83/EC) and WHO guidelines (Directive, 1998; WHO, 2011).

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In the context of the risk assessment, the dose-response is the establishment of the relationship between the dose of the hazard and the incidence or likelihood of illness. For chemical compounds, it is necessary to define the NOAEL (*No Observed Adverse Effect Level*), LOAEL (*Lowest Observed Adverse Effect Level*) and RfD (*Reference Dose*) according to scientific knowledge.

The POD (point of departure) for determining an ADI (or total daily intake; TDI) is commonly the “no observed adverse effects level” (NOAEL) for a given toxic end point. However, for many ADIs, the POD is the lowest dose resulting in an observable effect level (LOEL). In response to uncertainties associated with calculating the level of risk posed by these compounds in indirect potable reuse (IPR) applications, some scientists and regulators support the adoption of treatment technologies (technologically based-values), based on the precautionary principle, to minimize exposure of humans and aquatic ecosystems (e.g. for reservoir augmentation) until more data on potential risks are collected.

For example, acceptable daily intakes (ADIs) have been developed for 26 active pharmaceutical ingredients on the basis of various end points (USEPA, 2012) from which an appropriate *point of departure* (*POD*) was determined for the ADI calculation.

To specify *de minimis* levels for the CECs for aquifer recharge, any of the following can be adopted and usually modified by a relative source contribution (e.g., 0.2) and used as a point of departure (POD) for estimating risks for carcinogens and non-carcinogens by applying appropriate uncertainty factors (Schwab et al., 2005; Snyder et al., 2008; Bull et al., 2011;):

- Acceptable daily intake (ADI).
- Reference dose (RfD) which is derived from a no-observed-adverse-effect-level (NOAEL) or lowest-observed-adverse-effect-level (LOAEL) and applying several uncertainty factors depending upon the nature of the toxicological data.
- Predicted no-effect concentration (PNEC) that expresses the toxicological potency of health-based contaminants.

Analytical methods used for monitoring must comply with the ‘Technical specifications for chemical analysis and monitoring of water status’ (Commission Directive 2009/90/EC of 31 July 2009), which establishes minimum performance criteria for the analytical methods used in monitoring water status. Those criteria ensure meaningful and relevant monitoring information by requiring the use of analytical methods that are sensitive enough to ensure that any exceedance of a threshold value can be reliably detected and measured. EU

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Member countries should be permitted to monitor in TWW only if the analytical method used meets the minimum performance criteria set out in Article 4 of Directive 2009/90/EC.

It is recommended that the agency responsible for the recharge project confer with the local health regulator to develop a response plan with specific actions to be implemented by the recharge agency as part of interpreting appropriate responses to the monitoring results.

In Table 1 there is an example of a proposed list of initial CECs (Krzeminski et al, 2018) and human health relevant level to be included in monitoring programs of indirect potable reuse projects leading to aquifer recharge.

Table 1 - List of CECs to be included in monitoring programmes for aquifer recharge (the selection criteria are provided and explained in NEREUS Deliverable 14).

Indicator chemical	Human health relevant level (HRL) (ng/L)	Frequency
Biodegradable[1]		
Diclofenac	100	Every 6 months
Gabapentin	1,000	Every 6 months
Sulfamethoxazole	150	Every 6 months
Valsartanic acid	300	Every 6 months
Not biodegradable, but oxidizable[2]		
Carbamazepine	500	Every 6 months
Difficult to degrade biologically; not amendable to chemical oxidation[3]		
Sucralose	tba	Every 6 months

[1] Biodegradable during biofiltration or soil-aquifer treatment.
[2] Not degradable during conventional activated sludge treatment, biofiltration or soil-aquifer treatment, but amendable to chemical oxidation.
[3] Not degradable during conventional activated sludge treatment, biofiltration or soil-aquifer treatment, not amendable to chemical oxidation.
tba: to be added

As soil – aquifer treatment has limited capabilities in removing some of the CECs, aquifer recharge by surface spreading or by direct injection for drinking water purposes or intended for such future use (indirect potable reuse), should be allowed only after applying sufficient treatment technologies, with proven removal capabilities (i.e. AOPs, activated carbon, membranes, etc.).

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3.2 Quality Criteria for Agricultural Irrigation

There are different approaches for the estimation of the health risks as a consequence of dietary exposure. Both probabilistic and deterministic risk assessment have been used to evaluate the risk of contaminants in crops and they have been recently reviewed (Prosser and Sibley, 2015). Some of the most common approaches are reported in the NEREUS Deliverable 10 and are briefly summarized below.

Estimated daily intake vs accepted daily intake

The risk can be calculated from the comparison of the **Estimated Daily Intake** (EDI) of a chemical to the **Accepted Daily Intake** (ADI) levels. The ADI value of a substance is the amount of a contaminant that can be consumed daily over a person's lifespan without evocating adverse effects. The ADI values for pharmaceuticals used for treatment of humans are determined by dividing the lowest daily therapeutic dose for an adult (mg/d) by a safety factor of 1000 and a body weight of 70 kg (WHO, 2011). The **estimated daily intake (EDI)** for each of the pharmaceuticals and plant species under the investigation in a particular study can be calculated as follows:

$$EDI = C_{food} \times IR/M$$

Where:

- C_{food} is the concentration of the pharmaceutical in the analyzed plant tissue (ng/gfw) in fresh weight,
- *IR* is intake rate of vegetables (g/d),
- *M* is body weight, usually averaged for the population group under investigation (e.g. infant, toddler, adult).

The comparison between EDI and ADI supports an assessment of whether, for a specific population group, a specified consumption rate of food products will lead to an intake of the investigated contaminant at an amount exceeding the ADI and thus pose a health risk.

In cases where an ADI is not available, the **Minimum Therapeutic Dose** (MTD) in (mg/d) that induces the desired therapeutic effect among target populations may be used or a **No Observable Effect Level** (NOEL) where available. In either case, the MTD or NOEL is divided by an uncertainty factor (e.g. 1000 for most of compounds) and the body weight. Full details on the process to develop the ADI is described in NEREUS Deliverable 5.

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The Hazard Quotient (HQ) approach

The hazard quotient can be estimated from *EDI* and *ADI* as follows:

HQ = EDI / ADI

Where:

- *EDI* is the estimated daily intake
- *ADI* the accepted daily intake.

In the case of pharmaceuticals, taking into account the precautionary principle, a HQ value of ≥ 0.1 has been selected as the value which indicates a risk to human health, since many exposure sources of pharmaceuticals are expected.

In the case of genotoxic or potentially genotoxic compounds, the ADI approach needs to include an additional safety factor taking into account chronic exposure to chemical contaminants. According with the Cramer's classification (Cramer et al., 1978) some pharmaceuticals have been already described as genotoxic or potentially genotoxic.

Threshold of toxicological concern

The **Threshold of Toxicological Concern** (TTC) provides a preliminary risk assessment evaluation for a large number of chemical contaminants occurring in food and environmental matrices . It provides a risk estimate of microcontaminants, even in cases of no available toxicity data. The threshold of toxicological concentration (TTC) approach has been developed to assess the risk of low-level substances present in the diet. It can be used for an initial screening assessment of a substance to determine whether a comprehensive risk assessment is required. A TTC value is estimated based on an assessment of the structural properties of chemicals and available toxicological data according with the Cramer's et al. (1978) classification tree (Toxtree) into three categories (I, II and III) by increasing the suspected toxicity. The Toxtree software has been encoded specifically commissioned by the European Chemicals Bureau (ECB). This software determines the TTC values and the compound classification.

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4. Effect-Based Methods

This Section includes a possible list of effect-based methods that could be used to evaluate the quality of TWW for different types of reuses, mainly aquifer recharge and agriculture.

The bioassays should guarantee the protection of human health and environment covering most of the possible MOAs (Mode of Actions) of the chemical substances present in the environment and the risk caused by complex mixtures. The bioassays should allow for a better assessment and monitoring of the overall characteristics of wastewater based on the biological effects caused by the effluent.In the EU Technical report on Aquatic Effect Based Tools of the WFD several Bioassays and approaches have been proposed (Wernersson et al., 2015).

The Bioassays should be used as screening methods and as early warning systems on the basis of the different monitoring programmes foreseen. The use of a battery of bioassays *in vitro* and *in vivo* is the best way to evaluate the quality of TWW in support to the chemical analysis. The bioassays should be used as screening methods and as early-warning systems on the basis of the different monitoring programmes foreseen.

To address the problem of mixtures caused by groups of substance with specific MOAs the work undertaken within WG3 (see NEREUS Deliverable 14) proposes to employ low-cost bioassays in combination with targeted analysis of a short list of pollutants. This is proposed as an approach which combines a higher level of protection with a decreased economic burden caused by monitoring.

In the following Sections some effect-based methods (EBM) that could be used to evaluate the TWW for different reuses are described

In vitro assays

In addition to chemical methods which detect individual compounds, *in vitro* bioassays are increasingly promoted as sensitive monitoring tools to screen waters from a variety of sources based on an assessment of their specific biological action (e.g. mutagenic and genotoxic effects). As the chemical composition of a sample is often unknown and mixture effects cannot be detected or predicted based on the results of the chemical analysis alone, *in vitro* bioassays are considered as suitable complimentary tools to examine the impact of specific acting chemicals in complex mixtures (;;; Leusch and Snyder, 2015; Prasse et al., 2015; Wernersson et al., 2015).

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To put bioassays into a regulatory context, a list of robust bioassays and bioassay threshold values (trigger values) are required. A list of criteria was developed in the DEMEAU project (DEMEAU Deliverable D41.1., 2015) to assess the suitability of a range of effect-based methods to detect activity towards selected endpoints in drinking water.

For monitoring of TWW for groundwater recharge, a list of suitable bioassays covering four different toxicity endpoints with specific effect-based trigger values is proposed (equivalents (eq)/L) for 17β-estradiol (E2), dihydrotestosterone (DHT), dexamethasone (DEX) and Org2058). Recognised (i.e. defined by the FAO/WHO Joint Expert Committee on Food Additives (JECFA)) ADI values of specific reference compounds were chosen as point of departure for the derivation of the proposed trigger values). The developed trigger values are based on a combination of 1) acceptable or tolerable daily intake (ADI/TDI) values of specific compounds, 2) pharmacokinetic factors defining their bioavailability, 3) estimations of the bioavailability of unknown compounds with equivalent hormonal activity, 4) relative endocrine potencies, and 5) physiological, and drinking water allocation factors.

For full details on how trigger values were developed please refer to NEREUS Deliverable 14.

The application of effect-based trigger values can help to decide whether further examination of specific endocrine activity followed by a subsequent safety evaluation may be warranted, or whether concentrations of such activity are of low priority with respect to health concerns in the human population. An exceedance of the trigger values listed in Table 2 should initiate a series of actions.

Table 2 - Examples of trigger values for estrogenic (ERα), androgenic (AR), progestagenic (PR), and glucocorticoid (GR) activities in reclaimed water used for recharge of potable aquifers.

Activity	Trigger value[a]
ERα	3.8 ng E2-eq/L
AR	11 ng DHT-eq/L
GR	21 ng DEX-eq/L
PR	333 ng Org2058-eq/L

[a]Based on Acceptable Daily Intake (ADI) values reported by the JECFA (FAO/WHO, 1995, 2000). E2 = 17β-estradiol; eq = equivalent; DHT = dihydrotestosterone; DEX = dexamethasone; Org2058 = 16a-ethyl-21-hydroxy-19nor-4-pregnene-3,20-dione.

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Mutagenicity/Genotoxicity (e.g. Ames)

Mutagenicity tests are rapid, relatively cheap, and predictive of integral mutagenic/carcinogenic activity, and can evaluate the combined action of potentially hazardous compounds present in drinking water as complex mixtures and not only a specific compound. They are able to take into consideration the bioavailability of (geno)toxic compounds, their synergism, additivity or even antagonism.

Ames

The *Salmonella typhimurium*/mammalian microsome assay (Ames) is the most widely used short-term test to identify genetic damage. This is used to assess the mutagenic and antimutagenic potential of compounds and mixtures. The Ames test uses several strains of bacteria (*Salmonella*, *E. coli*) that carry mutation. This assay uses histidine-dependent strains to detect mutations, e.g., substitutions, additions, or deletions of one or several DNA nucleotides reverting originally changed gene sequence of the tester strains. This test is based on the principle of reverse mutation or back mutation. So, the test is also known as bacterial reverse mutation assay. if there is a positive result a monitoring of possible genotoxic compounds should be performed. This test is also recommended by the EU Project SOLUTIONS (https://www.solutions-project.eu/) and the EU technical report on aquatic effect based tools (A.S. Wernersson et al, 2015).

Whole Effluent Assessment

WEA (Whole Effluent Assessment) is an example of approach used in the last decade. WEA is carried out through the use of aquatic test organisms to wastewater samples determining effects on biological parameters (e.g., survival, growth, mobility or reproduction, embryos development). The use of a battery of bioassays *in vitro* and *in vivo* is the best way to evaluate the quality of TWW in support to the chemical analysis (A.S. Wernersson et al, 2015)). Existing databases about traditional biological models (e.g. daphnids, microalgae, vertebrate and invertebrate embryotoxicity) have been used for years for evaluation of water/wastewater quality monitoring. Moreover, most EU projects used batteries of toxicity tests at various levels of complexity.

Zebrafish Embryo Toxicity Test

Effect-based methods should be used also to evaluate if there is a presence of a complex mixture effect and in this case it can be useful to also apply an "*in vivo*" assay such as the

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use of the embryos of Zebrafish, in particular the test FET (Fish embryo toxicity test) OECD 236 (OECD, 2013) that can detect the risk of acute toxicity caused by particularly toxic mixtures present in wastewaters; alongside with the lethality evaluation foreseen by the Zebrafish embryo test, also the presence of sub-lethal effects (Di Paolo, 2016) should be carefully evaluated to understand also the possible presence of teratogenic, neurotoxic or embryotoxic compounds. The use of an "*in vivo*" bioassay, that is in compliance with the animal lab ethical regulation, it is recommended also for a confirmation of the results of the "*in vitro* assays". The ZF FET has been applied in the context of the EU JPI Water Project FRAME (A novel framework to assess contaminantss of emerging concern in indirect potable reuse) http://www.frame-project.eu/ and in the mentioned EU SOLUTIONS Project. In the case of *in vivo* bioassays (FET), the compliance should be based on the observations of the Lethal Effects and Sublethal Effects. For example, ıf there is acute toxicity detected (lethality) with results above 20% lethality, it means that there is a possible acute risk caused by the mixtures of pollutants or other stressors. If there is not acute toxicity, but sub-lethal effects are detected a supplement of chemical monitoring or additional measures should be foreseen to the reuse project also on the basis of the sublethal effects. ZF embryo test can be integrated also with different endpoints (for example mutagenicity using comet assays or neurotoxicity/cardiotoxicity with the use of specific softwares.

5. ARB&ARGs (Quality Criteria for Aquifer Recharge and Irrigation)

Considering the threat posed by the spread of antibiotic-resistant bacteria (ARB) and the multiple evidences that domestic wastewater treatment plants are major environmental reservoirs, the issue of antimicrobial resistance (AMR) has to be addressed and not only in TWW use but in a general context of wastewater sanitation (NEREUS Deliverable 4; Manaia et al., 2012; Manaia, 2017; Rizzo, 2013). As a viable compromise for the application of monitoring programmes it is suggested that *E. coli* resistant to cefotaxime (a third generation cephalosporin antibiotic used to treat a range of bacterial infections) is used as a surrogate of a wider range of clinically-relevant ARB (see NEREUS Deliverable 4). *E. coli* is an indicator of faecal contamination and a common host of acquired antibiotic resistance genes, supporting its use as an adequate monitoring tool. Cefotaxime resistance, is associated with extended spectrum beta-lactamases (ESBL) and broader multidrug resistance, and is therefore a good indicator for human sources of antibiotic resistance. ESBL-producing *E. coli* are widespread in the community and a potential source of to human infections.

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As the contribution of TWW irrigation to antibiotic resistance infections has yet to be determined, monitoring of cefotaxime-resistant *E. coli* in effluent used for irrigation should be compulsory. For irrigation, minimum values for *E. coli*, BOD_5, TSS and turbidity are included in the JRC proposal (add ref) according to four reclaimed water quality classes, namely: class A (Secondary treatment, filtration, and disinfection (advanced water treatments), class B (Secondary treatment, and disinfection), class C (Secondary treatment, and disinfection), class D (Secondary treatment, and storage, stabilization ponds or constructed wetlands). In particular, *E. coli* minimum values (CFU/100 mL) were set as _10 or below detection limit, _100, _1000 and _10,000 for class A, B, C and D, respectively.

Minimum values for *E. coli*, TSS and turbidity as well as "Indicative technology target" (Secondary treatment, filtration, and disinfection (advanced water treatments); Secondary treatment, and disinfection) were proposed for two "managed aquifer recharge categories" (direct injection vs surface spreading).

The SCHEER and NEREUS are of the opinion that the JRC document should recommend that disinfection and advanced treatments be selected and operated to address the corresponding limits of *E. coli* set in Table 1 of the JRC report, as well as to minimise the release of antibiotic resistant *E. coli*, while complying with disinfection by-products (DBPs) concentration and toxicity requirements. Additionally, as antibiotic resistance spread is of concern a realistic first step to control this threat would be to incorporate the measurement of antibiotic-resistant *E. coli* when measuring "total" *E. coli* in WWTP's effluents.

In particular, cefotaxime (a third generation cephalosporin that is on the WHO essential list of medicines) resistance is also a good indicator for human sources of antibiotic resistance. Accordingly, Table 1 of the JRC document may be revised by adding to *E. coli* values_1 (or below detection limit), 10, 100 and 1000 CFU/ 100 mL cefotaxime resistant E. coli for A, B, C and D reclaimed water quality classes, respectively. These values correspond to 10% of resistance prevalence which is a compromise between adequacy to monitor wastewater resistance levels and the feasibility of analyses.

While molecular methods such as qPCR and shotgun metagenomics provide a holistic evaluation of ARG in municipal wastewater effluents and TWW (these have been comprehensively discussed in the NEREUS WG1 report), the requirement for expensive equipment and robust analyses make them unsuitable for routine monitoring and evaluation at this time. One possible solution, would be to be to screen the cefotaxime-resistant isolates

for ESBL genes, using a rapid multiplex PCR approach such as that suggested by Roschanski et. al. (2014).

6. Environmental Risks (surface and groundwater, soils)

The use of TWW in applications such as agricultural irrigation and aquifer recharge must not have adverse effects on environmental matrices (e.g. soil, crops, surface waters and associated biota) and should be in compliance with the related EU directives for environmental protection. TWW applied for groundwater recharge can affect for example surface water quality and related ecosystems due to the interactions surface/groundwaters. Regulatory requirements of related EU Directives for environmental protection have to be always fulfilled. Authorities should ensure that the introduction of TWW use schemes do not compromise the objectives specified by the related EU Directives, such as the Directive 2000/60/EC (Water Framework Directive (WFD), the Directive 2008/105/EC (Environmental Quality Standards Directive (EQSD)) (amended by Directive 2013/39/EU), the Directive 2006/118/EC (Groundwater Directive (GWD)), the Directive 91/676/EEC (Nitrates Directive), the Directive 91/2717EEC and other related EU Directives that may apply. It is necessary also to perform an environmental risk assessment to protect soils, and dependent ecosystems, including crops to be irrigated, on a case-by-case basis according to site specific conditions. The Directive 86/78/EEC (Sludge Directive) defines limit values for heavy metals concentration in soils where sludge is applied, which may be an indication for establishing limit values in soils irrigated with TWW. The Sludge Directive is now under a revision process and any update should be considered accordingly Furthermore, soil quality criteria (residential, agricultural and industrial use) are usually applied in the context of remediation monitoring programmes; they can be applied and checked in order to evaluate the possible risks deriving from irrigation practices. A verification monitoring programme must be established for all environmental matrices that, based on the evaluation of the risks according to site-specific conditions, are considered at risk due to the use of TWW for e.g. irrigation. Analytical methods used for monitoring shall comply with the requirements included in the related Directives to conform to the quality control principles, including, if relevant, ISO/CEN or national standardized methods, to ensure the provision of data of an equivalent scientific quality and comparability. If the monitoring results indicate that the use of TWW for irrigation may affect the content of any of these parameters in the related environmental matrices, either the water should not be used, the practices for using it should be changed, or additional treatment should be undertaken beforehand (JRC, 2018).

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7. Proposed list of CECs for Risk Assessment Scenarios

In the context of NEREUS an effort has been made to define a list of CECs for the monitoring of TWW (please refer to the supporting excel file available at http://www.nereus-cost.eu/mycloud/index.php/s/2nysrIHOapolQfz). The substances analysed are those that have been studied during the activity of NEREUS, the CECs considered are in total 41 (see tables below) and the following properties and parameters have been considered:

- Baseline Toxicity (e.g. cytotoxicity, lethality)
- Genotoxicity/Mutagenicity
- Hormone-related toxicity endpoints (e.g. estrogenicity)
- Human Health Risk assessment values (e.g. ADI)
- PNEC (predicted no effect concentration)
- Persistence
- Bioavailability
- Bioaccumulation
- Presence of active by products
- Frequency of occurrence in wastewaters
- Abundance
- Log K_{ow}

It is important to remark that the table is not exhaustive, but it represents an example of a "work in progress" document that has been elaborated during the NEREUS activity by the Blue Circle Society; the information related to many CECs is still scarce and some gaps remain.

In general, it is recommended to perform a risk assessment when the substances exceed available quality criteria. The toxicological approaches that should be used to derive quality criteria are those suggested in this Deliverable.

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Table 3 - Oestrogenic substances

CECs Long-List	Nature of CECs
17alpha-Ethinylestradiol	Synthetic, steroidal oestrogen
17-Beta-estradiol	Oestrogenic steroid
Estrone	Natural oestrogen

Table 4 - Artificial sweeteners

CECs Long-List	Nature of CECs
Acesulfame	Synthetic sweetener (K salt; calorie free)
Sucralose	Artificial sweetener

Table 5 - Insecticides, pesticides and herbicides

CECs Long-List	Nature of CECs
Acetamiprid	Neonicotinoid insecticide
Clothianidin	Neonicotinoid insecticide
Imidacloprid	Neonicotinoid insecticide
Thiacloprid	Neonicotinoid insecticide
Thiamethoxam	Neonicotinoid insecticide
Methiocarb	Carbamate pesticide
Mecoprop	Herbicide
Oxadiazon	Herbicide
Triallate	Selective pre-emergent herbicide

Table 6 - Antibiotics

CECs Long-List	Nature of CECs
Azithromycin	Antibiotic
Ciprofloxacin	Antibiotic (fluoroquinoline)
Clarithromycin	Antibiotic

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Enrofloxacin	Antibiotic (fluoroquinoline)
Erythromycin	Antibiotic
Trimethoprim	Antibiotic
Sulfamethoxazole	Sulphonamide antibiotic

Table 7 - Flame retardants

CECs Long-List	Nature of CECs
Hexabromocyclododecane (HBCD)	Brominated flame retardant
Tetrabromobisphenol A (TBBPA)	Brominated flame retardant
Tris(2-carboxyethyl)phosphine) (TCEP)	Fire retardant

Table 8 - Other Pharmaceuticals

CECs Long-List	Nature of CECs
Carbamazepine	Anti-epileptic and anti-depressant
Diclofenac	Nonsteroidal anti-inflammatory
Triclosan	Antibacterial and antifungal agent
Gabapentin	Drug for epilepsy and neuropathic pain
Lamotrigine	Anti-convulsant drug
Metformin	Treatment of type 2 diabetes,
Oxypurinol	Xanthine oxidase inhibitor; heart failure treatment
Valsartanic acid	Treatment of high blood pressure

Table 9 - Other uses

CECs Long-List	Nature of CECs
Benzotriazole	Corrosion inhibitor
2,6-Ditert-butyl-4-methylphenol (BHT)	Phenolic antioxidant used as food additive
2-Ethylhexyl 4-methoxycinnamate (EHMC)	Sunscreen and lip balm
N-Nitrosodimethylamine (NDMA)	By-product of several industrial processes; present at very low levels in certain foodstuffs (carcinogen)
Perfluorobutanoic acid	Used in household and industrial products such as: stain repellents, lubricants, fire retardants and

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	suppressants, pesticides, surfactants, and emulsifiers
Perfluorohexanoic acid	Used in household and industrial products such as: stain repellents, lubricants, fire retardants and suppressants, pesticides, surfactants, and emulsifiers
Perfluoropentanoic acid	Used in household and industrial products such as: stain repellents, lubricants, fire retardants and suppressants, pesticides, surfactants, and emulsifiers
Perfluorooctanoic acid (PFOA)	A fluorosurfactant e.g. used in the emulsion polymerization of fluoropolymers.
Perfluorooctanesulphonic acid (PFOS)	A fluorosurfactant e.g. used in the emulsion polymerization of fluoropolymers.

From this long-list of CECs it is possible to recommend case-specific short priority lists of substances that have the potential to be present widely in wastewater, have relevant toxicological properties, can bioaccumulate in plants, are persistent and can have ecotoxicological effects on aquatic ecosystems and soil.

8. Acknowledgments

Mario Carere, Italian Institute of Health, Italy
Lian Lundy, Middlesex University, UK
Celia Manaia, Catholic University of Portugal, Portugal
Despo Fatta-Kassinos, Nireas-IWRC, University of Cyprus, Cyprus
Marlen Vazquez, Cyprus University of Technology, Cyprus
Sarah Koenemann, Eawag, Switzerland
David Weinberg, Ministry of Health, Israel
Sara Rodriguez, ICRA, Spain
Irene Michael-Kordatou, Nireas-IWRC, University of Cyprus, Cyprus
Heidemarie Paula Schaar, TU, Wien, Institute of Water Quality, resource and management, Austria
European Chemicals Bureau (ECB) Members

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