nereus COST ACTION ES1403

# Deliverable of WG3

## Deliverable 14

**Bioassays able to serve as routine tools for analysis and evaluation of the efficiency of the various treatment technologies to remove toxicological hazards and evaluate the quality of the wastewater to be reused (relevant to the reuse practice)**

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This deliverable 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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## Acronyms

| | |
|---|---|
| ADI | Acceptable Daily Intake |
| AR | Androgenic |
| CEC | Contaminants of Emerging Concern |
| DEX | Dexamethasone |
| DHT | Dihydrotestosterone |
| EBTs | Effect-based trigger values |
| EC | European Commission |
| EDA | Effect Directed Analysis |
| EDC | Endocrine Disrupting Compound |
| E1 | Estrone |
| E2 | 17β-estradiol |
| EE2 | 17α-ethinylestradiol |
| ELV | Emission Limit Value |
| ERα | Estrogenic |
| EQSD | Environmental Quality Standards Directive, 2008/105/EC and its update 2013/39/EU |
| GR | Glucocorticoid |
| GWD | Ground Water Directive, 2006/118/EC |
| HRGA | Human *in vitro* Reporter Gene Assays |
| HRL | Human health relevant level |
| JECFA | Joint Expert Committee on Food Additives |
| JRC | DG Joint Research Centre of the European Commission |
| LOAEL | Lowest-observed-adverse-effect-level |
| MEC | Measured Environmental Concentration |
| MS | EU Member State |
| msPFA | Multiple Substances Potentially affected Fraction of species |
| NOAEL | No-Observed-Adverse-Effect-Level |
| PCBs | Polychlorinated biphenyls |



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| | |
|---|---|
| PNEC | Predicted no-effect concentration |
| PR | Progestagenic |
| RBMP | River Basin Management Plan |
| RBSPs | River Basin Specific Pollutants |
| RfD | Reference Dose |
| SAT | Soil-Aquifer Treatment |
| tba | to be added |
| TDI | Tolerable Daily Intake |
| TEQ | Toxic Equivalent |
| TIE | Toxicity Identification & Evaluation |
| TOC | Total Organic Carbon |
| TTL | 'Toxicity Traffic Light' system |
| US EPA | United States Environmental Protection Agency |
| UWWTP | Urban Wastewater Treatment Plant |
| WFD | Water Framework Directive, 2000/60/EC |
| WHO | World Health Organisation |



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## 1. Executive summary

Two sets of bioassays, one addressing the human health-based effects (related to treated wastewater reuse for aquifer recharge) and the other addressing ecological adverse effects (related to river water impacted with treated wastewater or treated wastewater used for irrigation), are proposed here as routine tools for analysis and evaluation of the efficiency of various wastewater treatment technologies. A set of bioassays selection criteria was designed including the practicality of use, robustness, costs and market availability of the bioassays. Effect-based trigger values (EBTs) and clearly described methodology for their derivation are available for each of the selected bioassays. EBTs allow for the use of bioassays in a regulatory context and this report presents proposals of response plans when the EBTs are exceeded. A smart monitoring combining the use of cost-effective bioassays for screening with analysis of a basic set of technology related indicator chemicals is proposed. The approach was included in the proposal for the Water Reuse Policy Instrument prepared by DG Joint Research Centre (JRC) of the European Commission and commented by EU Member States (MS) in 2017.

## 2. Background

As analytical techniques have improved, a number of chemical compounds that are not commonly regulated have been detected in drinking water, wastewater, or the aquatic environment, generally at very low levels. This broad group of chemicals is termed Contaminants of Emerging Concern (CECs). They are sometimes grouped by their environmental- and human-health effects (e.g., hormonally active agents or endocrine disrupting compounds [EDCs]), or by the type of compound (e.g. antibiotics) and may include transformation products resulting from various biotic and abiotic processes.

Frequent monitoring for every potential chemical substance is neither feasible nor plausible. Research is focusing on the development of a science-based framework to guide the identification of CECs that should be monitored or otherwise regulated, including the context of treated wastewater use, especially for direct potable use (Drewes



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et al., 2013). A sound selection framework is needed that can provide a short list of meaningful indicator measurements that can address both human health or ecological relevance and assurance of proper performance of water treatment processes in addition to routine monitoring for compliance with guidelines and/or regulations.

The EU Technical Report on aquatic effect-based monitoring tools (Wernersson et al., 2015) presents, in the context of the EU Water Framework Directive (WFD), a range of effect-based tools (e.g. biomarkers, bioassays) that could be used in the context of different monitoring programmes, and that might be able to take account of the presence of several known and unknown compounds with similar effects.

Effect-based bioassays could be used as a screening and prioritisation tool for subsequent chemical analysis (NRMMC, 2008). WHO report (2016) states that there is still significant uncertainty regarding the role of effect-based tools in a regulatory context and developments in bioanalytical science should be examined to identify validated bioassay candidates. However, in our opinion latest developments in the field of bioassays standardisation and applicability show their readiness to be applied in a regulatory context.

Bioassays are already used routinely for analysis of dioxins and dioxin-like polychlorinated biphenyls (PCBs) in foodstuffs since 2002 (Commission Directive, 2002) and the same limit value (sum PCDD+PCDF+PCB-DL - 0.0065 µg/kg Toxic Equivalent [TEQ]) appeared in the most recent update of the EU Environmental Quality Standards Directive (EQSD, 2013/39/EU) for analysis of biota samples.

Three steroidal estrogens, 17α-ethinylestradiol (EE2), 17β-estradiol (E2) and estrone (E1), are currently included in the so-called “watch list” of the WFD (Commission Implementing Decision, 2015) aiming at standardised monitoring data for European water bodies. This obligation may be difficult to meet because the detection limits of most existing routine analytical methods are above the biological effect concentrations, and high-end analytical methods are costly. An innovative monitoring approach which combines effect-based with chemical analysis was tested in a collaborative project



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(demEAUmed, FP7-ENV-2013-WATER-INNO-DEMO), in which seven specific effect-based methods were compared with three highly sensitive chemical analyses for 35 surface water and wastewater samples. The activity involved approximately 20 institutions from 10 European countries. The project was based on the Science-to-Policy-Interface and "Chemical Monitoring of Emerging Pollutants" activities under the Common Implementation Strategy for the EU WFD, in which the Working Group Chemicals identified a strong need for research on effect-based monitoring tools in support of implementing the relevant legislation. The results clearly indicate that these bioassays are ready for routine use in support of the current EU water legislation (Kase et al., 2018).

A big step towards further standardisation of the bioassays is the availability of ISO standard for the human *in vitro* reporter gene assays (HRGA) for determining estrogenic water and wastewater (ISO/DIS 19040-3, 2017), published in 2018 (ISO, 2018).

One should be aware that the limit of detection in an *in vitro* bioassay has no bearing on the adversity of effect related to a given assay and in many cases there is no direct relationship between the effected biological quality element or human health and the degree of adversity of *in vivo* effects. Rather, *in vitro* bioassays are used as analytical tools to quantify mixtures of chemicals and to differentiate between acceptable and poor water quality with respect to the organic micropollutants, with further testing recommended if a water sample exceeds an effect-based trigger value (EBT).

A large number of bioassays indicative of different endpoints have been developed over recent decades. Their strength is that they account for mixtures of chemicals acting together - all chemicals in the case of apical endpoints and groups of chemicals with the same mode of action (MOA) for reporter gene assays. By applying a panel of cellular and small-scale whole-organism assays it is possible to obtain a more holistic profile of the effects of all chemicals present in a water sample without identifying the causative compounds individually. Test batteries should ideally include bioassays indicative of different stages of the cellular toxicity pathway, including induction of xenobiotic metabolism, receptor mediated effects, reactive mode of action, adaptive stress responses and cell viability, as well as apical effects in whole organisms. These may



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include algal growth inhibition, daphnia immobilization or fish embryotoxicity (Escher et al., 2018). *In vivo* bioassays were not included in this study since only high-throughput assays with established EBTs were considered as candidates for immediate regulatory action, providing a possibility to trace back the substance(s) causing the effect.

## 3. Bioassays and assessment of risk of mixtures of pollutants in treated wastewater for aquifer recharge

To address the issue of mixtures of pollutants, a paradigm shift is suggested; to employ low cost bioassays in combination with target analysis of the minimum list of pollutants. This allows to combine a higher level of protection with decreased economic burden caused by monitoring. The use of bioassays has reached sufficient level of maturity and accessibility on the market to include them now in the legislation.

In addition to chemical methods which are intended to detect individual compounds, *in vitro* bioassays (also called bioanalytical tools or effect-based assays) are now recognized as sensitive monitoring tools to screen for contaminants based on 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, *in vitro* bioassays are highly suitable tools to examine the presence of specific acting chemicals in complex mixtures (Van der Linden et al., 2008; Leusch et al., 2010; Escher and Leusch, 2012; Leusch and Snyder, 2015; Prasse et al., 2015; Wernersson et al., 2015).

At present, the WHO, the US Environmental Protection Agency (US EPA) and health agencies in Europe have derived approximately 125 statutory guideline values for drinking water (US EPA, 2004, 2006; WHO, 2011; Schriks et al., 2010; Umweltbundesamt, 2003a, 2003b). The Australian drinking water guidelines contain 181 organic chemicals (NHMRC, 2011), most of which are a subset of the chemicals regulated in recycled water for indirect potable reuse (NRMMC, 2008). The scope of such evaluation is rather limited since many compounds that are present in the aquatic



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environment are not analysed and for the compounds that are analysed, toxicological information is often lacking or insufficient for hazard identification purposes.

In addition, while drinking water sources (including potable groundwater) contain complex mixtures of chemicals, analytical chemistry does not account for combined effects. Smart combinations of chemical- and biological analytics therefore lead to reduced uncertainty in safety assessments at lower costs.

To put bioassays into a regulatory context, a list of robust bioassays and bioassay threshold values (effect-based trigger values (EBTs)) are required. A list of criteria was developed in the DEMEAU project (DEMEAU Deliverable D41.1., 2015) to assess the suitability of effect-based assays to detect activity towards selected endpoints in drinking water (Table 1). Applying the criteria, a list of ready-to-use bioassays available on the market has been compiled (Table 2; DEMEAU WA4 Deliverable D42.2., 2015).

**Table 1** - Example of a set of selected criteria to evaluate bioassays (scoring matrix).

| Primary criteria | Sub-criteria | Max Points | Assay X/Y/Z |
|---|---|---|---|
| Assay applicability and ease of use (max 21 points) | Applied to environmental samples | 3 | |
| | Validated to water samples | 3 | |
| | Standardised protocol available | 3 | |
| | Service and support available | 3 | |
| | Costs | 3 | |
| | Ease of use (maximum 6 points based on criteria as indicated below) | 6 | |
| | • Non-GMO[1] | 1 | |
| | • No specialised equipment/skills required | 1 | |
| | • Automation possible | 1 | |
| | • Non-licensed (cell) in vitro model | 1 | |
| | • Kit available | 1 | |
| | • Training available | 1 | |
| | Total score | 21 | |
| Assay performance (max 33 points) | Selectivity | 3 | |
| | Accuracy | 3 | |
| | Reproducibility | 3 | |



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| | | | |
|---|---|---|---|
| | Robustness | 3 | |
| | Sensitivity | 3 | |
| | Specificity | 3 | |
| | Limit of Detection (LOD) | 3 | |
| | Cytotoxicity control | 3 | |
| | Quick | 3 | |
| | Clear/Straightforward read-out | 3 | |
| | High-throughput capacity | 3 | |
| | Total score | 33 | |
| | Total maximum score | 54 | |

[1] Non-Genetically Modified Organisms

**Table 2** - Overview of recommended *in vitro* bioassays to detect activity towards selected endpoints relevant for drinking water.

| Toxicity endpoints relevant for monitoring of reclaimed water quality | Specific pathway | Most promising bioassay(s) |
|---|---|---|
| **Xenobiotic metabolism** | PXR receptor agonists | HG5LN PXR assay, PXR HepG2 assay |
| | AhR receptor agonists | DR CALUX, AhR-CALUX |
| **Hormone-mediated mode of action** | (anti)estrogenic activity | ERα CALUX, YES assay, ERα Geneblazer, AR CALUX, AR-MDA-kb2, AR Geneblazer, GR CALUX, GR-MDA-kb2, GR-Geneblazer |
| | (anti)androgenic activity | |
| | (anti)glucocorticoid activity | |
| **Reactive mode of action** | Gene mutations | Ames fluctuation assay, ToxTracker |
| | Chromosomal mutations | Micronucleus assay, ToxTracker |
| | DNA damage response | UMUc assay, Vitotox, p53 CALUX, p53-Geneblazer, BlueScreen |
| **Adaptive stress response** | Oxidative stress pathway | Nrf2 CALUX, AREc32 assay, ARE-Geneblazer |
| **Developmental toxicity** | Focus point endocrine disruption | Various nuclear receptor activation assays, H295R assay |

A detailed definition of criteria for validation of the performance of the bioassays and validation of their fitness for purpose should still be a subject of further refinement.



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### 3.1 Effect-based trigger values (EBTs)

For monitoring of treated wastewater for groundwater recharge (often used as a source for production of drinking water), a list of suitable bioassays covering four different toxicity endpoints with specific EBTs is proposed ((equivalents (eq)/L) for 17β-estradiol (E2), dihydrotestosterone (DHT), 16α-ethyl-21-hydroxy-19-norprogesteron (Org2058) and dexamethasone (DEX)) (Table 3)). Recognised (i.e., defined by the FAO/WHO Joint Expert Committee on Food Additives (JECFA)) Acceptable Daily Intake (ADI) values of specific reference compounds were chosen as point of departure for the derivation of the proposed EBTs. It is expected that a mechanism for regular revision/update of the list of bioassays and their trigger values each five years might be considered by the European Commission (EC).

**Table 3** - Effect-based trigger values for a battery of bioassays indicating estrogenic (ERα), androgenic (AR), progestagenic (PR), and glucocorticoid (GR) activities in treated wastewater used for recharge of potable aquifers.

| Activity | EBT[a] |
|---|---|
| **Estrogenic (ERα)** | 3.8 ng E2-eq/L |
| **Androgenic (AR)** | 11 ng DHT-eq/L |
| **Progestagenic (PR)** | 333 ng Org2058-eq/L |
| **Glucocorticoid (GR)** | 21 ng DEX-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 (Brand et al., 2013).

The EBTs are based on 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.



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Chemical (compound-directed) analysis provides a method of absolute quantification of certain compounds in water samples, but the toxicological properties of these compounds are not always known (Schriks et al., 2010a). Bioassays do not discriminate between different specific compounds but detect the total specific biological activity, and so the concentration of specific biological activity is expressed as an equivalent (eq) to a potent reference compound, e.g. 17β-estradiol (E2) for ERα-, dihydrotestosterone (DHT) for AR-, dexamethasone (DEX) for GR-, and Org2058 for PR-mediated activity (Sonneveld et al., 2005). By using *in vitro* bioassays, the combined biological activities of the mixture can be quantified and expressed as ng eq of a reference compound per L. This enables an assessment of the effect of unknown compounds by their activity and provides toxicological relevance of this mixture (i.e., specific endocrine activity). Given the sensitivity and robustness, these properties make the use of *in vitro* bioassays suitable as a screening tool for endocrine activity in water samples (Escher and Leusch, 2012; Schriks et al., 2010b; Van der Linden et al., 2008). A list of compounds with their relative potencies (to be compared to the trigger values) can be established for each bioassay (for an example, see Table 4). It is expected that the relative potency (Benchmark Quotient) will be determined for a large number of new substances identified as relevant in treated wastewater allowing for risk assessment of mixtures of substances (Escher at al., 2014, 2015).

The EBTs for the selected bioassays for agonistic and antiagonistic hormonal activities in the treated wastewater used to recharge potable aquifers define the level above which human health risk cannot be waived *a priori* and additional examination of specific endocrine activity may be warranted (Brand et al., 2013).

EBTs have already been proposed for drinking water for the CALUX series of bioassays (Brand et al., 2013). The derivation of safe level was based on acceptable effect levels *in vivo* translated to *in vitro* by extrapolation methods that took into account toxicokinetics.

Another approach was used to proposed EBTs for treated wastewater by reading across from existing single chemical guideline values of the Australian Guidelines for Water



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Recycling and applying mixture toxicity considerations (Escher at al., 2015, 2013, Tang et al. 2013). The trigger values derived by these various approaches do not differ significantly and the methodology proposed by Brand et al. (2013) is followed in this document.

**Table 4** - Relative potencies (Benchmark Quotients) of a selection of compounds measured using the ERα, AR, GR, and PR CALUX bioassays.

| Compound | Relative potency[a] |
|---|---|
| Estrogens | |
| 17β-estradiol (E2) | 1.00 |
| 17α-ethinylestradiol (EE2) | 1.86 |
| Estrone (E1) | 0.02 |
| Estriol | 0.04 |
| Bisphenol A | $2.5 \times 10^{-5}$ |
| Nonylphenol | $4.6 \times 10^{-5}$ |
| Genistein | $5.0 \times 10^{-5}$ |
| ... | |
| Androgens | |
| Dihydrotestosterone (DHT) | 1.00 |
| Testosterone | 0.15 |
| Trenbolone | 0.94 |
| Androstenedione | 0.06 |
| Androstanediol | 0.01 |
| Androstenediol | 0.02 |
| Androsterone | 0.01 |
| ... | |
| Glucocorticoids | |
| Dexamethasone (DEX) | 1.00 |
| Cortisol | 0.07 |
| Cortinose | 0.00 |
| Prednisolone | 0.09 |
| Prednisone | 0.00 |
| 6α-methylprednisolone | 0.4 |
| Triamcinolone acetonide | 2.3 |
| ... | |
| Progestagens | |
| Org2058 | 1.00 |



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| | |
|---|---|
| Progsterone (P4) | 0.07 |
| Levonorgestrel | 0.46 |
| Norethisterone | 0.08 |
| Medroxyprogesterone Acetate | 0.59 |

[a] For a list of references, see DEMEAU Deliverable D41.2., 2014.

The application of EBTs can help to deciding 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. As limit values are aimed at the protection of human health in the finally produced drinking water, such limits should be sufficiently conservative to serve as a warning signal. On the other hand, such limits should not be too conservative, to avoid unnecessary and costly additional protection measures.

The sample location and frequency for these bioassays should be linked to specified monitoring requirements for CECs (*i.e.,* minimum frequency of six months with an option to monitor more frequently to reflect seasonal variation of occurrence of CECs and their increased concentrations during the droughts, e.g. in the Mediterranean area; collected at the point of compliance - cf. text below). Due to the limited sensitivity of the bioassays it is recommended to enrich the water via solid phase extraction prior to testing using appropriate solid phase materials.

An exceedance of the EBTs listed in Table should initiate the following actions:

- If the measured value/EBT < 1: no further action required.
- If 1 < measured value/EBT < 3: quality check data, continue to monitor every three months, until 1 year and until the EBT < 1.
- If 3 < measured value/EBT < 10: data check, immediate re-sampling and quantify specific target compounds which are known to cause the effects observed in the respective bioassay (toxicity drivers). Continue to monitor every three months, until 1 year and the EBT < 1.



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- If 10 < measured value/EBT < 100: all of the above plus enhance source identification program. Also monitoring of influent waste water to confirm the magnitude of assumed safety factors associated with removal efficiency by the available WWT technology and dilution in the receiving water body.
- If measured value/EBT > 100: all of the above plus immediately confer with the local public health authority to determine the required response action. Confirm WWTP corrective actions through additional monitoring that indicates the measured value/EBT ratio is below at least 100.

  The ranges of exceedances of limit values (3, 10, 100 etc.) have been selected on the basis of previous long-term studies and expert considerations in , e.g., California (https://watereuse.org/wp-content/uploads/2015/01/WRCA-Bioassay.pdf). An approach taken by the California water authorities, suggesting that three- to five-year data collection period to justify response actions, is recommended here as well.

## 4. Bioassays and assessment of risk of mixtures of pollutants in wastewater released into recipient (and potentially used for irrigation)

In the recent publications by van der Oost et al. (2017a) and Escher et al. (2018) a selection was made of market-ready relevant and cost-effective bioanalytical endpoints to cover a wide spectrum of micropollutant modes of action. Specific endpoints may indicate which classes of chemicals might cause adverse effects. EBTs were derived for these bioassays to indicate potential ecological risks (Table 5). Comparison of EBT with bioassay responses should discriminate sites exhibiting different chemical hazards. In addition, a model was designed to estimate the overall risks for aquatic ecosystems. The associated follow-up for risk management was proposed as a 'Toxicity Traffic Light' (TTL) system (Figure 1): green, low hazard (no action required); orange, potential risk (further research needed) and red, high risk (mitigation measures). The strategy is already being applied in the Netherlands and its potential to become the first bioanalytical tool to be



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applied in regular water quality monitoring programmes has been successfully tested (van der Oost, 2017b).

**Table 5 -** Effect-based trigger values (EBTs) for a battery of bioassays indicating potential ecological risk.

| Activity | EBT[a] | EBT[b] |
|---|---|---|
| **Estrogenic (ERα)[c]** | 0.5 ng E2-eq/L | 0.1 ng E2-eq/L |
| **Anti-androgenic (anti-AR)** | 25 µg Flu-eq/L | **14** µg Flu-eq/L |
| **Glucocorticoid (GR)** | **100** ng DEX-eq/L | - |
| **Dioxin-like (DR)** | **50** pg TD-eq/L | 50 pg TD-eq/L |
| **PPARγ receptor (PPARγ)** | **10** ng Ros-eq/L | - |
| **Toxic PAHs (PAH)** | 150 ng BaP-eq/L | **6,2** ng BaP-eq/L |
| **Oxidative stress (Nrf2)** | **10** µg Cur-eq/L | 21 µg Cur-eq/L |
| **Pregnane X receptor (PXR)** | **3** µg Nic-eq/L | 54 µg Nic-eq/L |

[a] Expressed as equivalents of the reference compounds E2 = 17β-estradiol; eq = equivalent; Flu = flutamide; DEX = dexamethasone; T = 2378-TCDD; Ros = rosiglitazone; BaP = benzo[a]pyrene; Cur = curcumine; Nic = nicardipine; van der Oost et al., 2017.
[b] As above, Escher et al., 2018.
[c] EBT of 0.4 ng E2-eq/L reported in the paper by Kase et al., 2018 is proposed to be used.

The sample location and frequency for these bioassays should be linked to specified monitoring requirements for CECs (i.e., frequency of six months; collected at the point of compliance, which is in this case urban wastewater treatment plant (UWWTP) effluent). Due to the limited sensitivity of the bioassays it is recommended to enrich the water via solid phase extraction prior to testing using appropriate solid phase materials.

A proposal discussed during the Action would be that the lowest (cf. Table 6, EBTs in bold letters) or most reliable (cf. Table 6; footnote to Estrogenic (ERα)) EBT is taken for the comparison with the signal obtained by any of the used bioassay with the same Mode of Action (Table 6). An exceedance of the EBTs listed in Table 6 would initiate the following actions:



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The sample location and frequency (proposed once in six months) for these bioassays should be linked to specified monitoring requirements in the WWTPs. An exceedance of the above proposed trigger values should initiate the following actions:

- If the measured value/EBT < 1: no further action required.
- If 1 < measured value/EBT < 3: quality check data, continue to monitor every three months, until 1 year and until the EBT < 1.
- If 3 < measured value/EBT < 10: data check, immediate re-sampling and quantify specific target compounds which are known to cause the effects observed in the respective bioassay (toxicity drivers). Continue to monitor every three months, until 1 year and the EBT < 1.
- If 10 < measured value/EBT < 100: all of the above plus enhance source identification program. Also monitoring of influent waste water to confirm the magnitude of assumed safety factors associated with removal efficiency by the available WWT technology and dilution in the receiving water body.
- If measured value/EBT > 100: all of the above plus immediately confer with the local environmental authority to determine the required response action. Confirm WWTP corrective actions through additional monitoring that indicates the measured value/EBT ratio is below at least 100.



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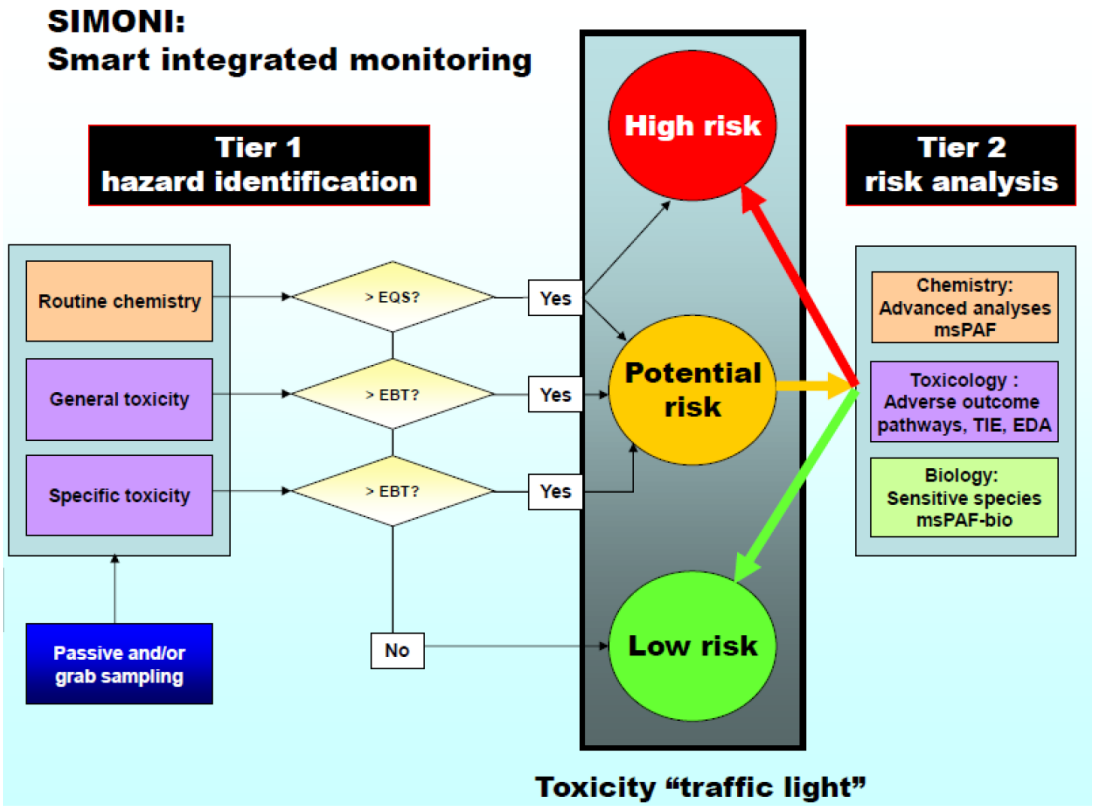


**Figure 1** - Schematic presentation of the SIMONI (Smart Integrated Monitoring; van der Oost et al., 2017a) effect-based monitoring strategy; EQS = environmental quality standard; EBT = effect-based trigger value; msPAF = multiple substances potentially affected fraction of species; TIE = toxicity identification & evaluation; EDA = effect directed analysis.

This would mean that proposed ranges of EBTs for the here discussed bioassays and Modes of Actions would be as in Table 6.



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**Table 6 -** List of applied bioassays, with their mode of action, reference compounds, proposed effect-based trigger values (EBTs) and ranges of exceedance of EBTs indicating a need for different response actions by WWTP operators (labelled with different font/colour styles; for more details see text).

| Mode of Action | Reference compound | Cell TA assay e.g. | EBT | 1 to 3-times EBT level *(italic)* | 3- to 10-times EBT level (underlinded) | 10- to 100-times EBT level **(bold)** | Above 100-times EBT level ***(italic bold underlined)*** |
|---|---|---|---|---|---|---|---|
| **Estrogenicity (ER)[a]** | ng eq E2/l | Human or Yeast | 0.4[d] | *0.4-1.2* | 1.2-4.0 | **4.0-40** | ***>40*** |
| **Inhibition Androgenicity(anti-AR)[b]** | µg eq Flutamide/l | Human or Yeast | 3.3[e] | *3.3-9.9* | 9.9-33 | **33-330** | ***>330*** |
| **Glucocorticoid receptor activation (GR)** | ng eq Dexamethasone /l | Human | 100[e] | *100-300* | 300-1000 | **1000-10000** | ***<10000*** |
| **Activation of peroxisome proliferator-activated receptor (PPAR)** | ng eq Roziglitazone/l | Human | 36[e] | *36-108* | 108-360 | **360-3600** | ***>3600*** |
| **AhR receptor activation (PAH)[c]** | ng eq B(a)P/l | Rat | 6.2[e] | *6.2-18.6* | 18.6-62 | **62-620** | ***>620*** |
| **Adaptive Stress (Nrf2)** | µg eq Dichlorvos/l | Human | 26[e] | *26-78* | 78-260 | **260-2600** | ***>2600*** |
| **Activation pregnane x receptor (PXR)** | µg eq DEHP/l | Human | 272[e] | *272-816* | 816-2720 | **2720-27200** | ***>27200*** |

[a]: Estrogenicity testing according to ISO19040 and OECD TG455.
[b]: Anti-androgenicity testing according to OECD TG458.
[c]: AhR receptor activation testing according to ISO standards (in progress).
[d]; Kase et al., 2018, Sci. Tot. Environ., 628-629, 748-765.
[e]; Escher at al., 2018., Trends in Analytical Chemistry, 102, 343-358.
[f]; Van der Oost et al., 2017, Environ. Toxicol. Chem 36, 2385-2399.



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## 5. Monitoring requirements for CECs in aquifer recharge

In addition to meeting core requirements of the Ground Water Directive (GWD, 2006/118/EC) and to protect human health from CECs, monitoring of a selection of health-based indicator CECs and performance-based indicator chemicals and appropriate surrogates (such as bulk parameters) should be regulated at the EU level for monitoring of planned treated wastewater reuse activities leading to aquifer recharge. For the determination of health-based indicator chemicals with available toxicological information, a *de minimis* risk approach can be used. To specify *de minimis* levels for these health-based contaminants, 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; WHO, 2010; Bull et al., 2011; Khan, 2013):

- 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.

Performance validation and verification of wastewater treatment trains can be obtained through direct measurements of certain performance-based indicator contaminants that frequently occur in treated wastewater and correlate with core removal mechanisms (i.e., biotransformation, adsorption, size exclusion, chemical oxidation) of individual treatment processes (Drewes et al., 2008; Dickenson et al., 2009, 2011). The following factors must be considered for the selection of performance-based indicator contaminants to assess the treatment efficacy of potable reuse schemes (Drewes and Horstmeyer, 2016):



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- Target substances chosen to assess treatment performance must permanently occur at concentrations significantly above their analytical method detection limit (preferably, the ratio between the measured environmental concentration and the method detection limit should exceed at least 10).
- Appropriate and commercially available analytical methods must exist to quantify the target contaminants in treated wastewater.
- Performance-based indicator chemicals used for monitoring should broadly represent the range of physico-chemical and biological properties affecting their removals by the various treatment processes within an indirect potable reuse treatment train.
- Substances with a high potential to contaminate groundwater and drinking water.
- Substances with toxicological relevance.

Monitoring of health-based and performance-based indicator chemicals provides an additional safeguard for proper removal of CECs. However, the reliability of the treatment processes in properly removing microbial and chemical contaminants is assured by measuring bulk parameters ("surrogate parameters") that correlate with the removal of CECs (Drewes et al., 2008; Dickenson et al., 2009). Examples of specific performance-based bulk measurements that can be monitored continuously include turbidity, electrical conductivity, UV absorbance, or total organic carbon (TOC). Such surrogate parameters can also indicate out-of-specification performance or treatment process failure. Thus, the monitoring frequency of indicator chemicals can be much longer (e.g., semi-annual) and provides an additional check on proper operation of the overall treatment train in addition to continuous monitoring of surrogates. In principle, this concept is well established in conventional drinking water treatment to manage the risk from microbial contaminants, where treatment efficiency is assessed by measuring turbidity and residual chlorine levels online while monitoring indicator organisms occurs less frequently. The choice of appropriate surrogate parameter will depend on the treatment processes selected, which might differ from project to project. Thus, the selection of surrogate parameters and their



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expected removal efficiency (expressed as percent removal) should be specified during the start-up and validation monitoring phase of a treatment train (Drewes et al., 2011).

Meeting the removal criteria for both the selected indicator chemicals and surrogate parameters can be accomplished by establishing multiple barriers within the overall treated wastewater reuse project. Such barriers can be obtained through the use of several different treatment processes. Treatment processes should be operated in series to provide redundancy and robustness in the removal of both pathogens and unwanted chemicals, and to ensure the failure of a single process does not render the system vulnerable to penetration by microbial or chemical contaminants that pose a significant risk to public health or the environment.

While the requirements to meeting these standards for the two groundwater recharge practices (a. surface spreading; b. direct injection) are the same, the point of compliance differs since removal credit can be given to soil-aquifer treatment (SAT) resulting in additional attenuation of contaminants during vadose zone treatment (Figure 2). For surface spreading operations, the point of compliance is the uppermost groundwater downstream of the recharge facility, which can be monitored by lysimeters or groundwater monitoring wells (Figure 2a). For direct injection projects, all standards have to be met prior to injection (Figure 2b).

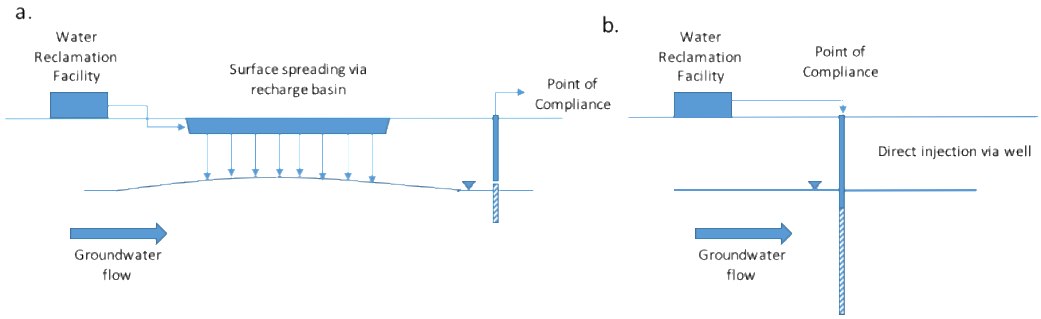


**Figure 2** - A typical conceptual schematic of groundwater recharge practices and point of compliance (a. surface spreading; b. direct injection).



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Where groundwater aquifers are already severely compromised due to local environmental conditions (e.g., brackish groundwater; seawater intrusion; geologically-occurring arsenic), Member States (MS) might define these aquifers as non-potable and specify less stringent water quality requirements (GWD).

A minimum list of chemicals and appropriate surrogate parameters in treated wastewater used for groundwater recharge to potable aquifers and frequency of their monitoring should be established by the Commission. The Commission should be responsible for defining threshold values for the health-based indicator chemicals at the EU level, whereas Member States (MS) could define more stringent threshold values at a national, river basin or other level addressing regional or local conditions. It is recommended that an initial list should contain approximately 10 substances and it should indicate the possible methods of analysis not entailing excessive costs for each substance. A mechanism for regular revision/update of the list of CECs each five years should be proposed by the Commission. A list of initial CECs is provided in Table 7. The chemicals were proposed based on the experience and European data available to the NEREUS experts with reference to Australian and Californian water reuse legislation (NRMMC & EPHC & NHMRC, 2008; Drewes, et al., 2013). For the general criteria used for the selection of the substances, kindly see the footnote of Table 7 and Chapter 6 below.

Analytical methods used for monitoring must comply with the QA/QC Directive (Commission Directive, 2009). 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. Member States should be permitted to monitor in wastewater only if the analytical method used meets the minimum performance criteria set out in Article 4 of the Directive 2009/90/EC.



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**Table 7** - Proposed list of initial CECs to be included in monitoring programmes of indirect potable reuse projects leading to aquifer recharge.

| Indicator chemical | Human health relevant level (HRL) (ng/L) | Frequency[4] | References - analytical method |
|---|---|---|---|
| **Biodegradable[1]** | | | |
| Benzotriazole | tba | Every 6 months | Loos et al., 2013 |
| Diclofenac | 100 | Every 6 months | Loos et al., 2013 |
| Gabapentin | 1,000 | Every 6 months | Kasprzyk-Horderna et al., 2008 |
| Trimethoprim | tba | Every 6 months | Kostich et al., 2014 |
| Sulfamethoxazole | 150 | Every 6 months | |
| Valsartanic acid | 300 | Every 6 months | Schultz et al., 2010 |
| Oxypurinol | tba | Every 6 months | Funke et al., 2015 |
| **Not biodegradable, but oxidizable[2]** | | | |
| Acesulfam | tba | Every 6 months | Loos et al., 2013 |
| Carbamazepine | 500 | Every 6 months | Loos et al., 2013 |
| **Difficult to degrade biologically; not amendable to chemical oxidation[3]** | | | |
| Tris (2-carboxyethyl)phosphine (TCEP) | tba | Every 6 months | Loos et al., 2013 |
| Sucralose | tba | Every 6 months | Loos et al., 2013 |

[1] Biodegradable during conventional activated sludge treatment, 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.

[4] One of the measurements to be carried out in the summer period.



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An exceedance of any of the threshold values at the point of compliance (measured environmental concentration, MEC) and the recommended associated follow-up action is described in the following section. This guidance on thresholds for each of these tiers is based on conservative values because of the limited toxicological information available and the fact that the suggested point of compliance does not represent the point of exposure to public health. In indirect potable reuse projects, additional attenuation in the aquifer, dilution with native groundwater or other source waters after abstraction, as well as post-treatment, will result in further reductions of contaminant concentrations. 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.

- If 1 < MEC/HRL < 10: quality check data, continue to monitor every three months, until 1 year and the MEC/HRL < 1 and preferably is consistently less than 5 times the ratio of MEC/HRL.
- If 10 < MEC /HRL < 100: data check, immediate re-sampling and analysis to confirm MEC, also monitor at the point of abstraction. Continue to monitor every three months, until 1 year and the MEC/HRL<1 and preferably is consistently less than 5 times the ratio of MEC/HRL.
- If 100 < MEC/HRL < 1,000: all of the above plus enhance source identification programme. Also monitoring at a point of abstraction and in the distribution system closer to the point of exposure to confirm attenuation of CEC is occurring and to confirm the magnitude of assumed safety factors associated with removal efficiency, dilution and post-treatment.
- MEC/HRL > 1,000: all of the above plus immediately confer with the local health regulator to determine the required response action. Confirm plant corrective actions through additional monitoring that indicates the CEC levels are below at least an MEC/HRL of 100.



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## 6. Emission Limit Values (ELV) and monitoring requirements for CECs in treated wastewater released into recipient or used for irrigation

The same list of indicator chemicals (Table 7) as for aquifer recharge is proposed with limit values related to the ecological risk (ecological PNEC-based values). In addition, each WWTP operator is requested to include into the list of monitored compounds also River Basin Specific Substances (RBSPs) taken from the latest formally adopted version of the River Basin Management Plan (RBMP) for the basin where WWTP is located. Top ten RBSPs (Table 8) should be chosen according to the following criteria:

- Target substances chosen to assess treatment performance must permanently occur at concentrations significantly above their analytical method detection limit (preferably, the ratio between the measured environmental concentration and the method detection limit should exceed at least 10).
- Appropriate and commercially available analytical methods must exist to quantify the target contaminants in recycled water.
- Performance-based indicator chemicals used for monitoring should broadly represent the range of physico-chemical and biological properties affecting their removals by the various treatment processes.
- Substances with toxicological relevance.
- Cost of analysis should be minimal.

The limit values should be related to the EQS set for each RBSP at the national level using an appropriate dilution factor used at the definition of 'mixing zones' according to the EQS Directive (2008/105/EC). As the river water impacted by the wastewater is most commonly used for irrigation (in some cases even before the pollution plume reaches the end of mixing zone), taking into account precautionary principle, these values should apply also for any treated wastewater used for irrigation.

In case of exceedance of ELVs following actions are proposed:



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- If 1 < MEC/ELV < 10: quality check data, continue to monitor every three months, until 1 year and the MEC/ELV < 1 and preferably is consistently less than 5 times the ratio of MEC/ELV.
- If 10 < MEC/ELV < 100: data check, immediate re-sampling and analysis to confirm MEC. Continue to monitor every three months, until 1 year and the MEC/ELV < 1 and preferably is consistently less than 5 times the ratio of MEC/ELV.
- If 100 < MEC/ELV < 1,000: all of the above plus enhance source identification programme. Monitoring to confirm the magnitude of assumed safety factors associated with the accumulative contributions by upstream WWTPs, removal efficiency, dilution and post-treatment. It should be noted that dilution is not always a refinement measure in Mediterranean rivers or streams, which run dry in summertime.
- MEC/ELV > 1,000: all of the above plus immediately confer with the local regulator to determine the required response action. Confirm plant corrective actions through additional monitoring that indicates the CEC levels are below at least an MEC/ELV of 100.

**Table 8** - Example of proposed RBSPs to be included in monitoring programmes of treated wastewater released into the recipient or used for irrigation.

| Indicator chemical | ELV= PNEC*dilution factor (ng/L) | Frequency[4] | LOQ (ng/L) | References - analytical method |
|---|---|---|---|---|
| **Biodegradable[1]** | | | | |
| **RBSP1** | tba | Every 6 months | tba | |
| **RBSP2** | tba | Every 6 months | tba | |
| **...** | tba | Every 6 months | tba | |
| **Not biodegradable, but oxidizable[2]** | | | | |
| **RBSP5** | tba | Every 6 months | tba | |
| **RBSP6** | tba | Every 6 months | tba | |



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| | | | | |
|---|---|---|---|---|
| **...** | tba | Every months | 6 | tba |
| **Difficult to degrade biologically; not amendable to chemical oxidation[3]** | | | | |
| **...** | tba | Every months | 6 | tba |
| **RBSP9** | tba | Every months | 6 | tba |
| **RBSP10** | tba | Every months | 6 | tba |

[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.
[4] One of the measurements to be carried out in the summer period.

## 7. Conclusions and future perspectives

Two batteries of bioassays were identified as being able to serve as routine tools for analysis and evaluation of the efficiency of the various treatment technologies to remove toxicological hazards and evaluate the quality of the treated wastewater to be reused. The first battery for monitoring of treated wastewater used for aquifer recharge consisted of four bioassays (ERα, AR, PR, GR) for detecting endocrine disruptive effects and relevant human health effect-based trigger values (EBTs) were defined for each bioassay. The second battery designed to monitor ecological EBTs (treated wastewater released into recipient or used for irrigation) contained eight bioassays (ERα, anti-AR, GR, DR, PPARγ, PAH, Nrf2, PRX).

The bioassays are already used in the European context for a long time as an alternative to chemical measurements for dioxins and dioxin-like compounds (Commission Directive, 2002). Results of a successful pan-European study on the use of bioassays for estrogenic compounds as a viable alternative to chemical measurements were presented (Kase et al., 2018). The use of bioassays for this group of compounds is acknowledged also in the JRC Technical Reports (JRC, 2013). A new ISO/DIS standard 19040-3 (ISO, 2018) was released in 2018, which makes the development of bioassays equal to development of any chemical analytical technique.



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This report foresees the use of above battery of bioassays as early-warning indicators of the toxicological adverse effects observed in treated wastewater used for aquifer recharge or being released into the recipient or used for irrigation. A sequence of actions was defined to be taken in cases of exceedance of the EBTs at various levels. A smart monitoring is proposed in which samples are pre-screened with bioassays and only those showing an effect with any of the bioassays are subjected to more expensive chemical target analysis.

A set of evaluation criteria to justify the 'market-readiness' of a bioassay had been established including parameters such as costs, robustness, training available, automation and high throughput analysis possible, etc.

The cost of the four bioassays analyses used for water quality measurements in aquifer recharge installations is currently less than 500 EUR, which means that the monitoring requirements for one treated wastewater reuse installation would not exceed 1000 EUR/year considering the proposed 6 months monitoring interval. It is expected that with a widespread use of bioassays the cost of analyses will rapidly fall.

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## 9. References

Australian Guidelines for Water Recycling: Managing Health and Environmental Risks (Phase 1), 2006. Natural Resource Management Ministerial Council Environment Protection and Heritage Council Australian Health Ministers Conference. Web copy: ISBN 1 921173 06 8; Print copy: ISBN 1 921173 07 6, November 2006.

Brand, W., De Jongh, C.M., van Linden, S.C., Mennes, W., Puijker, L.M., van Leeuwen, C., van Wezel, A., Schriks, M. and Heringa, M., 2013. Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. Environment International, 55: 109-118.

Bull, R., J. Crook, M. Whittaker, and J. Cotruvo, 2011. “Therapeutic dose as the point of departure in assessing potential health hazards from drugs in recycled municipal wastewater.” Regulatory Toxicology and Pharmacology, 60(1): 1-19.

Commission Directive 2002/69/EC of 26 July 2002 laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBsin foodstuffs, Official Journal of the European Communities, L 209/5, 6.8.2002.

Commission Directive 2009/90/EC of 31 July 2009 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, which establishes minimum performance criteria for the analytical methods used in monitoring water status.



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Commission Implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council,

Official Journal of the European Union, L 78/40, 24.3.2015.

DEMEAU - Demonstration of promising technologies to address emerging pollutants in water and waste water, European Union Seventh Framework Programme (FP7/2007-2013) under Grant Agreement no. 308339; www.demeau-fp7.eu.

DEMEAU WA4 Deliverable D41.1., 2015 - Selection criteria to select in vitro bioassays for implementation and use, October 2015.

DEMEAU WA4 Deliverable D41.2., 2014 - Establishment of trigger values for effect-based water quality assessment, May 2014.

DEMEAU WA4 Deliverable D42.2., 2015 - Report on robustness of novel water treatment technologies as determined with bioassays in a novel testing framework, October 2015.

Directive 2002/69/EC, 2002. Commission Directive laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in foodstaffs, Official Journal of the European Communities, L 209/5, 6.8.2002.

Dickenson, E.R.V., Drewes, J.E., Sedlak, D.L., Wert, E.C. and Snyder, S.A., 2009. Applying Surrogates and Indicators to Assess Removal Efficiency of Trace Organic Chemicals during Chemical Oxidation of Wastewaters. Environmental Science & Technology, 43(16): 6242-6247.

Dickenson, E.R.V., Snyder, S.A., Sedlak, D.L. and Drewes, J.E., 2011. Indicator compounds for assessment of wastewater effluent contributions to flow and water quality. Water Research, 45(3): 1199-1212.



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COST is supported by the EU Framework Programme Horizon 2020

This deliverable 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).

Drewes, J.E., Sedlak, D., Snyder, S. and Dickenson, E., 2008. Development of indicators and surrogates for chemical contaminant removal during wastewater treatment and reclamation., Alexandria, VA, USA.

Drewes, J.E. and Khan, S., 2011. Water Reuse for Drinking Water Augmentation. J. Edzwald (ed.) Water Quality and Treatment, 6th Edition. 16.1-16.48. American Water Works Association. Denver, Colorado.

Drewes, J.E., Anderson, P., Denslow, N., Olivieri, A., Schlenk, D., Snyder, S.A. and Maruya, K.A., 2013. Designing monitoring programs for chemicals of emerging concern in potable reuse - what to include and what not to include? Water Science and Technology, 67(2): 433-439.

Drewes, J.E. & Horstmeyer, N., 2016. Recent Developments in Potable Reuse. D. Fatta-Kassinos et al. (eds.), Advanced Treatment Technologies for Urban Wastewater Reuse, Hdb Env Chem 45: 269–290.

Escher B. I., Ait-Aissa S., Behnisch P. A., Brack W., Brion F., Brouwer A., Buchinger S., Crawford S. E., Du Pasquier D., Hamers T., Hettwer K., Hilscherova K., Hollert H., Kase R., Kienle C., Tindall A. J., Tuerk J., van der Oost R., Vermeirssen E., Neale P. A., 2018. Effect-based trigger values for in vitro and in vivo bioassays performed on surface water extracts supporting the environmental quality standards (EQS) of the European Water Framework Directive. Science of The Total Environment 628-629, 748-765, doi:https://doi.org/10.1016/j.scitotenv.2018.01.340.

Escher B, Leusch F. Bioanalytical tools in water quality assessment, 2012. With contributions by Heather Chapman and Anita Poulson. IWA Publishing, London, UK, 2012.

Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R., 2013. Most oxidative stress response in water samples comes from unknown chemicals: the need for effect-based water quality trigger values. Environmental Science & Technology, 47(13): 7002-7011.



cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

COST is supported by the EU Framework Programme Horizon 2020

This deliverable 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).

Escher B.I. et al., 2014. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays. Environ Sci Technol 48(3):1940-56.

Escher, B.I., Neale, P.A. and Leusch, F.D.L., 2015. Effect-based trigger values for in vitro bioassays: Reading across from existing water quality guideline values. Water Research, 81(0): 137-148.

FAO/WHO. Evaluation of certain veterinary drug residues in food (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series 851, 1995.

F.AO/WHO, 2000. Evaluation of certain veterinary drug residues in food (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series 893, 2000.

Funke, J.; Prasse, C.; Lütke Eversloh, C.; Ternes, T. A. Oxypurinol – A novel marker for wastewater contamination of the aquatic environment. Water Res. 2015, 74, 257–265.

ISO 19040-3:2018: Water quality — Determination of the estrogenic potential of water and waste water — Part 3: In vitro human cell-based reporter gene assay, https://www.iso.org/obp/ui/#iso:std:iso:19040:-3:ed-1:v1:en.

JRC Technical Reports, 2013. Analytical methods for new Priority Substances of the European Water Framework Directive.

Kase R., Werner I., Hollert H., Vermeirssen E., Buchinger S., Behnisch P., Jarosova B., Mariani G., Gawlik B., Clayton H., Perceval O., Ait-Aissa S., Creusot N., Reifferscheid G., Ternes T., Heiss Ch., Di Paolo C., Seiler T.-B., Kunz P., Kienle C., Dulio V., Valsecchi S., Carere M., 2015. Effect-based and chemical analytical monitoring for the steroidal estrogens: An international project to cope with a monitoring challenge SETAC Barcelona.

Kasprzyk-Horderna, B., Dinsdaleb, R.M., Guwyb. A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. 42 (13), 3498–3518.



cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

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Khan S.J., 2013. Drinking water through recycling. A report of a study by the Australian Academy of Technological Sciences and Engineering (ATSE). Melbourne, Australia.

Kostich, M. S.; Batt, A. L.; Lazorchak, J. M., 2014. Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation. Environ. Pollut., 184, 354–359.

Kase, R.; Javurkova, B.; Simon, E.; Swart K.; Buchinger. S., Koenemann, S., Escher, B. I.; Carere, M.; Dulio, V.; Ait-Aissa, S. Hollert, H.; Valsecchi, S.; Polesello, S.; Behnisch, P.; di Paolo, C.; Olbrich, D.; Sychrova, E.; Gundlach, M.; Schlichting, R.; Leborgne, L.; Clara, M.; Scheffknecht, Ch.; Marneffe, Y.; Chalon, C.; Tusil, P.; Soldan, P.; von Danwitz, B.; Schwaiger, J.; Moran Palao, A.; Bersani, F.; Perceval, O.; Kienle, C.; Vermeirssen, E.; Hilscherova, K.; Reifferscheid, G.; Werner, I., 2018. Screening and risk management solutions for steroidal estrogens in surface and wastewater. Trends in Anal. Chem. 102, 343-358.

Leusch FDL, de Jager C, Levi Y, Lim R, Puijker L, Sacher F, et al., 2010. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ Sci Technol 2010;44: 3853-60.

Leusch, F.D.L. and Snyder, S.A., 2015. Bioanalytical tools: half a century of application for potable reuse. Environ. Sci.: Water Res. Technol., (1): 606-621.

Loos, R.; Carvalho, R.; António, D. C.; Comero, S.; Locoro, G.; Tavazzi, S.; Paracchini, B.; Ghiani, M.; Lettieri, T.; Blaha, L.; et al., 2013. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water Res., 47 (17), 6475–6487.

NRMMC & EPHC & NHMRC, 2008. Australian guidelines for water recycling: managing health and environmental risks (phase 2). Augmentation of drinking water supplies, National Water Quality Management Strategy (NWQMS), Natural Resource Management Ministerial Council (NRMMC), Environment Protection and Heritage Council (EPHC) and National Health and Medical Research Council (NHRMC),



cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

COST is supported by the EU Framework Programme Horizon 2020

This deliverable 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).

Canberra, Australia. http://www.environment.gov.au/resource/national-water-quality-management-strategy-australian-guidelines-water-recycling-managing.

NHMRC, 2011. Australian Drinking Water Guidelines Paper 6 National Water Quality Management Strategy. www.nhmrc.gov.au/guidelines/publications/eh34, National Health and Medical Research Council (NHMRC) and the Natural Resource Management Ministerial Council, Canberra, Australia.

Prasse, C., Stalter, D., Schulte-Oehlmann, U., Oehlmann, J. Ternes, T.A., 2015. Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. Water Research, 87: 237-270.

Schriks M., van Leerdam J.A., van der Linden S.C., van der Burg B., van Wezel A.P., de Voogt P., 2010a. High-resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in the Netherlands. Environ Sci Technol 2010b;44: 4766-74.

Schriks M., Heringa M.B., van der Kooi M.M.E., de Voogt P., van Wezel A.P., 2010b. Toxicological relevance of emerging contaminants for drinking water quality. Water Res 2010a;44: 461-76.

Schultz, M. M.; Furlong, E. T.; Kolpin, D. W.; Werner, S. L.; Schoenfuss, H. L.; Barber, L. B.; Blazer, V. S.; Norris, D. O.; Vajda, A. M., 2010. Antidepressant pharmaceuticals in two U.S. effluent-impacted streams: Occurrence and fate in water and sediment and selective uptake in fish neural tissue. Environ. Sci. Technol., 44 (6), 1918–1925.

Schwab, B.W., E.P. Hayes, J.M. Fiori, F.J., Mastrocco, N.M. Roden, D., Cragin, R.D. Meyerhoff, V.J. D'Aco, and P.D. Anderson, 2005. “Human pharmaceuticals in US surface waters: A human health risk assessment.” Regul. Toxicol. Pharm., 42(3): 296– 312.

Snyder, S.A., Trenholm R., Snyder E.M., Bruce G.M., Pleus R.C., and Hemming J.D., 2008. Toxicological Relevance of EDCs and Pharmaceuticals in Drinking Water. Awwa Research Foundation Report. Water Research Foundation, Denver, CO.



cost EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

COST is supported by the EU Framework Programme Horizon 2020

This deliverable 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).

Sonneveld E., Jansen H.J., Riteco J.A.C., Brouwer A., van der Burg B., 2005. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci.; 83: 136-48.

Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S. and Escher, B.I., 2013. Mixture effects of organic micropollutants present in water: towards the development of effect-based water quality trigger values for baseline toxicity. Water Research 47(10): 3300-3314.

Umweltbundesamt, 2003a. Bewertung der Anwesenheit teil- oder nicht bewertbarer Stoffe im Trinkwasser aus gesundheitlicher Sicht. Empfehlung des Umweltbundesamtes nach Anhörung der Trinkwasserkommission beim Umweltbundesamt (Evaluation from the health point of view of the presence in drinking water of substances that are not (yet) possible or only partially possible to evaluate. Guidance of the Federal Environment Agency after consultation with the Drinking Water Commission at the Federal Environment Agency). Bundesgesundheitsblatt– Gesundheitsforschung-Gesundheitsschutz 46: 46 249-251.

Umweltbundesamt, 2003b. Maßnahmewerte (MW) für Stoffe im Trinkwasser während befristeter Grenzwert-Überschreitungen gem. § 9 Abs. 6-8 TrinkwV 2001. Empfehlung des Umweltbundesamtes nach Anhörung der Trinkwasserkommission beim Umweltbundesamt (Action values for substances in drinking water during temporary limit value exceedances. Guidance of the Federal Environment Agency after consultation with the Drinking Water Commission at the Federal Environment Agency). Bundesgesundheitsblatt–GesundheitsforschungGesundheitsschutz 46: 46 707-710.

U.S. Environmental Protection Agency, 2004. Guidelines for Water Reuse, EPA/625/R-04/108, September 2004.

US EPA, 2006. Drinking Water Health Advisories 2006 Edition. http://water.epa.gov/action/advisories/drinking/dwstandards.cfm. Accessed September 2015.



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This deliverable 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).

van der Linden S.C., Heringa M.B., Man H-Y., Sonneveld E., Puijker L.M., Brouwer A., van der Burg B., 2008. Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. Environ Sci Technol 2008;42: 5814-20.

van der Oost R., Sileno G., Suárez-Muñoz M., Thao Nguyen M., Besselink H., Brouwer B., 2017a. SIMONI (Smart Integrated MONItoring) as a novel bioanalytical strategy for water quality assessment; Part I: model design and effect-based trigger values, Environ. Toxicol. Chem., Accepted Article, DOI: 10.1002/etc.3836.

van der Oost R., Sileno G., Janse T., Thao Nguyen M., Besselink H., Brouwer A., 2017b. SIMONI (Smart Integrated MONItoring) as a novel bioanalytical strategy for water quality assessment: Part II. Field feasibility survey, Environ. Toxicol. Chem., Accepted Article, DOI: 10.1002/etc.3837.

Wernersson A-S. et al., 2015. The European technical report on aquatic effect-based monitoring tools under the water framework directive, Environmental Sciences Europe, Bridging Science and Regulation at the Regional and European Level, 201527:7, DOI: 10.1186/s12302-015-0039-4.

WHO, 2010. WHO Human Health Risk Assessment Toolkit: Chemical Hazards. World Health Organization, Geneva, Switzerland.

WHO, 2011. Guidelines for drinking-water quality. Ffourth edition. WHO, Geneva, Switzerland.

WHO, 2016. Unpublished draft from the Potable Reuse Guidelines. World Health Organization, Geneva, Switzerland.



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