nereus COST ACTION ES1403

# Deliverable of WG3

## Deliverable 15

# Harmonized protocols for effect-based *in vitro* biotests (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

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## Contents





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## Executive summary

Deliverable 15 *"Harmonized protocols for effect-based in vitro biotests (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"* is a compilation of discussions and results derived in WG3 *"Effect-based bioassays required for wastewater reuse schemes"* within the framework of the NEREUS COST Action ES1403 *"New and emerging challenges and opportunities in wastewater reuse"*.

During the course of the Action, members of WG3 got involved in the elaboration of the ISO international standard ISO 19040: *Water quality -- Determination of the estrogenic potential of water and wastewater -- Part 1 to 3*. Despite the fact that ISO 19040 refers to estrogenicity as biological endpoint for the specific *in vitro* biotests addressed in the standard, core aspects are applicable on a broad scale for other *in vitro* assays using different biological endpoints. It is therefore recommended, that aspects as e.g. sample preparation, as well as reporting of results should be done following ISO 19040. Recommendations refer to routine application of *in vitro* biotests in the framework of water quality assessment as in a monitoring for meeting guideline values or quality standards. For scientific investigations basic standard rules of course should be followed too, but especially sample preparation may significantly deviate from standard procedures if e.g. chemical fractioning of samples by different extraction methods or other specific questions are of interest.

In particular, recommendations refer to the following contents:

- Terms and definitions
- Sampling and samples
- Data analysis (analogous approach)
- Validation criteria (analogous approach)
- Test report
- Plate setup
- Test set up for chemicals and extracts
- Calculation of reference substance equivalents
- Measurement of the lowest ineffective dilution (LID) of wastewater



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Besides following the protocols and procedures mentioned above, it is recommended to report results including the following information:

- Equivalent concentrations referring to reference chemical (EQ)
- Lowest ineffective dilution (LID)
- Effect of undiluted sample
- Effect of sample diluted 1:10



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## 1. Aim of the Deliverable

The benefits of complementing the physicochemical evaluation of wastewater with a biological one are demonstrated in an increasing number of studies (e.g. Vasquez and Fatta-Kassinos, 2013; Neale et al., 2017; Välitalo et al., 2017). The toxicity profiling should be considered as an effective tool for screening the hazard of complex environmental mixtures with known and unknown toxicologically active constituents. Biological-based assays can analyse mixture effects providing data from molecular up to organism level. Treatment technologies need to be evaluated in respect to their capacity not only to remove organic microcontaminants, but also potential biological effects. Toxicity (i.e. acute and chronic, estrogenicity, mutagenicity, cytotoxicity, and phytotoxicity) are mere examples of potential effects. The variety of applicable bioassays, the variety of protocols and the way of implementation, the variety in the endpoints that may be considered, significantly increase the complexity of the issue. Additionally, the relevance of the endpoint of a specific test on the stability of the systems exposed to the reused water and the fitness of organisms is a central aspect to be considered.

Whereas conventional ecotoxicological test procedures like acute and chronic tests with organisms of various tropic levels or even advanced toxicological tests applied in REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) for the assessment of chemicals are well established, addressed in international norms and applied in routine, biological in vitro tests targeting a broad variety of physiological endpoints are rather new. In addition to chemical methods which are intended to detect individual compounds, those *in vitro* bioassays are 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; Escher and Leusch, 2012; Leusch et al., 2015; Leusch and Snyder, 2015; Prasse et al., 2015; Wernersson et al., 2015).

As already indicated by the name of NEREUS WG3 “Effect-based bioassays required for wastewater reuse schemes”, the prime focus was set on those emerging *in vitro* biotests during the whole Action. Development of those tests during the course of the implementation of the Action occurred in a tremendously fast way as a lot of international



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research was conducted and results were published. In contrast to only few years ago, nowadays a broad variety of biological endpoints can be assessed and in-depth research on methodology, interpretation and assessment of environmental matrices has been conducted. In 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)",* those new approaches that allow easy implementation in routine laboratories (even when only specialized in chemical analysis) and high throughput of samples at affordable prices are discussed in order to determine the most appropriate and relevant ones to be applied for wastewater quality evaluation.

As more and more *in vitro* biotests are proposed and published over time, it was considered essential to propose harmonized protocols of the procedures used for the individual tests. This should guarantee a comparability between tests and avoid systematic errors when using results for further risk assessment studies. As a consequence, Deliverable D15 was intended to specify recommendations for *in vitro* biotests considered useful, meaningful and suitable for the assessment of water quality for wastewater reuse.

## 2. Status of topic related

In the course of the Action, WG3 members got involved in the elaboration of an ISO international standard addressing the very same aspect as the intended deliverable. It was agreed at an early stage to not work in parallel to the international standardisation procedure, but refer to the standards and amend – if necessary – missing information in Deliverable 14.

Work on the relevant ISO document lasted until spring 2017 and the standard document ISO 19040 was finally published in August 2018. The standard consists of three parts that all deal with the determination of estrogenic potential of water and wastewater, but focuses on three individual biotests for that purpose. The parts are:

- **ISO 19040-1**: Water quality -- Determination of the estrogenic potential of water and wastewater -- Part 1: Yeast estrogen screen (*Saccharomyces cerevisiae*) ISO 19040-1: 2018 08 15 (https://www.iso.org/standard/64450.html)



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- **ISO 19040-2**: Water quality -- Determination of the estrogenic potential of water and wastewater -- Part 2: Yeast estrogen screen (A-YES, *Arxula adeninivorans*) ISO 19040-2: 2018 08 15 (https://www.iso.org/standard/64451.html)
- **ISO 19040-3**: Water quality -- Determination of the estrogenic potential of water and wastewater -- Part 3: *In vitro* human cell-based reporter gene assay ISO 19040-3: 2018 08 15 (https://www.iso.org/standard/66297.html)

A broad part of ISO 19040 contents is homologous as they on the one hand refer to general procedures and approaches valid for *in vitro* biotests in general and sample preparation as well as reporting of results on the other hand. The main difference between the three parts is on the procedure conducting the bioassays with different organisms that require an organism-specific handling and setup.

## 3. Suggested approach and recommendations

The suggested approach for harmonising protocols for effect based *in vitro* biotests is based on regulations stated in ISO 19040 - Water quality -- Determination of the estrogenic potential of water and wastewater Part 1 to 3. Despite the fact, that ISO 19040 - due to the nature of the standardized biotests using estrogenicity as biological endpoint – only refers to estrogenic potential, the procedures and approaches can – and should – be used for other biological endpoints or cellular toxicity pathways respectively too. Typically, the same type of organisms – but with various other as e.g. androgenic, glucocorticoid, progestagenic, oxidative stress related endpoints are available, so test organism-specific procedures, as well as sample preparation would be independent from the endpoint assay used.

### 3.1 Basic approach and procedures

Based on the TOC of ISO 19040 Part 1 to 3, contents summarized in Table 1 were identified as generally applicable for a broader variety of *in vitro* biotests (especially those mentioned in Deliverable D14 as is the core target of D15) and therefore are recommended to follow.



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If a direct approach is not possible due to test-specific circumstances, reasoning for deviations should be given and the standards be adopted as needed without neglecting the basic idea behind it.

### 3.2 Sample preparation

Water and wastewater samples can be tested directly or after concentration (retention) of the mixed chemicals on suitable columns and subsequent extraction with a lower amount of solvent compared to the volume of sample assessed. The concentration / extraction step is used to significantly increase the sensitivity (LOQ) of the tests and is necessary to derive equivalent concentrations (EC) referring to the reference substance used for the test. The procedure applied in general follows concentration / extraction used in chemical analysis as liquid-liquid extraction (LLE) or solid phase extraction (SPE) with suitable columns and solvents.

Multistep fractionation procedures applying a subsequent series of different solvents for obtaining e.g. acidic, basic and neutral fraction usually are not applied nor recommended for monitoring in order to access compliance of tested waters with quality standards. Nevertheless, they may be of interest for the isolation and identification of individual toxicants even in very complex mixtures or assessment of bioavailability of chemicals in complex mixtures.

**Table 1** - Headlines from ISO 19040 relevant for *in vitro* biotests in general.

| **Main content** | |
|---|---|
| **Terms and definitions** | |
| **Sampling and samples** | |
| | General |
| | Bottles and material for sampling |
| | Bottles and material pre-cleaning |
| | Sampling procedure |
| | Transport of samples |
| | Pre-treatment of sample |
| | Storage of samples |
| **Data analysis (semi test specific!)** | |
| **Validation criteria** | |
| **Assessment criteria** | |
| **Test report** | |



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| | |
|---|---|
| | |
| **Informative Annexes** | |
| **Plate setup** | |
| **Test set up for chemicals and extracts** | |
| | General |
| | Extraction of water samples |
| | Test with organic solutions or extracts |
| **Calculation of reference substance equivalents** | |
| | General |
| | Modelling of dose response relationship |
| | Calculation of reference substance equivalents for test samples |
| | Reporting of reference substance equivalents for test samples |
| **Measurement of the lowest ineffective dilution (LID) of wastewater - A simplified evaluation for testing of wastewater** | |
| | General |
| | Principle |
| | Preparation of dilutions for LID assessment |
| | Test for LID |
| | Assessment of results — LID value. effluents |
| | Reporting of results |

### 3.3 Reporting of results

The way results are reported usually depends on the questions to be answered. Usually two reasons for performing *in vitro* bioassays are of given:

1. In a whole effluent assessment; what is the observed effect of a water sample for the biological endpoint investigated?

   Quantification can be done by comparing the mixed toxicity effects by comparing the response in the sample to a calibration curve for a reference substance. For the reference substance a dose-response relationship is modelled to correlate the effect measured by the assay and a series of given reference concentrations. The resulting (usually sigmoid shaped) calibration function can be used to recalculate an equivalent concentration for the water sample. This translates to the information, that the sum effect of all substances in the water samples show the same effect as that specific concentration of the reference substance. Due to sample concentration, very low limits of quantification, frequently surpassing chemical analysis can be achieved and the compliance with trigger values or environmental quality standards can be assessed.



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As a result, the EQ (equivalent) concentration is reported together with the type of the reference substance.

2. The second question of interest is, if the native sample triggers a measurable effect or what dilution of the sample would be needed to show no effect in the test applied. In that case, defined dilutions of the sample are tested and the most concentrated dilution reported, at which no significant response in the test system is observed. The result could be that even the undiluted sample shows no effect. Whereas for the assessment of the equivalent concentration a concentration step has to be performed (see previous bullet point), in this case dilutions of the sample are assessed. As for practical application it is of interest, if the undiluted 1:1 sample and the 1:10 diluted sample show any significant effect in the test, these two variations should be tested at least. Dilution levels higher than 1,000 are not required for routine assessments.

It is recommended, that both numeric results, the

- Equivalent concentration - EQ and the
- Lowest ineffective dilution - LID

are reported together with the qualitative information, if the undiluted simple and the 1:10 diluted simple show any significant effect.

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