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Environmental characterization of leachates from waste and soil using reproductive and toxicological gene expression in *Daphnia magna*

Caractérisation environnementale des lixiviats de déchets et de sols à l'aide de l'expression génétique reproductive et toxicologique chez *Daphnia magna*

Umwelttechnische Charakterisierung von Sickerwässern aus Abfall und Boden mittels reproduktiver und toxikologischer Genexpression in *Daphnia magna*

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European foreword

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Introduction

The aim of this document is to describe the procedure used to set up and perform quantitative PCR to quantify effects of leachates from waste and soil on reproductive and toxicological endpoints in *Daphnia magna*. The presented method allows for rapid, robust and sensitive detection of molecular responses and can be used to analyse the toxic effects of water leachates from soil and waste as well as the recipient waters.

The study of messenger RNA (mRNA) from different living organisms, using different molecular approaches, can be used to identify the responses of organisms to exposure to toxic substances. Messenger RNA (mRNA) transfers information from the DNA, which stores all the necessary information needed for life, to the cellular machinery that synthesizes proteins. Proteins are the working units in the cell and their abundance is highly dependent on the RNA levels in the cell as all proteins are translation products of mRNA. As mRNA is the first step in the response to toxic substances it is also a quick, precise and sensitive biomarker of exposure that gives information on the mechanisms responsible for the responses. The relationship between physiological impact and mechanistic resolution is shown in Figure 1.

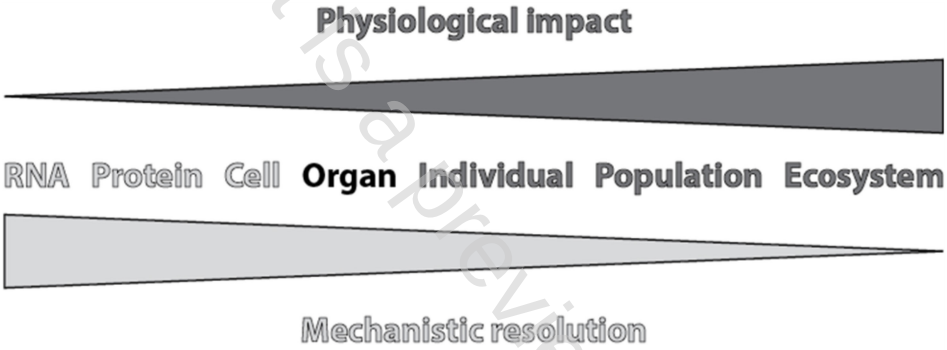

Figure 1 — Differences in physiological impact and mechanistic resolution at different organizational levels

There is an important difference between determining an effect of a toxic substance and understanding what it is that cause the effect. Effects that cause harm to entire ecosystems are usually identified by changes in populations. Effects at the organism/individual level are easiest observed by changes in the physiology, morphology or behaviour of an organism. However, as the environment is highly complex and highly variable, the measurement of changes in ecosystems, population or individuals has very low resolution when it comes to identifying the cause of any observed effect. To avoid "guilt by association" it is important to identify the connection between an exposure and the observed effect. The most sensitive methods for this are at the level of RNA and protein regulation at the cellular level. RNA forms the link between proteins and chromosomal DNA (genes). The chromosomally located genes can be regulated in several different ways, but independent of the regulatory mechanism, exposure to toxic substances will result in a change in active RNA and thereby in the corresponding protein.

A toxic response is initiated by a molecular initiating event (MIE). This can be the exposure to an inorganic or organic compound as well as to radioactivity. The MIE is followed by the molecular interaction between the compound initiating the MIE and a molecule (receptor activation, protein binding, DNA binding) that results in alterations in a set of key events (KE) starting with changes in gene expression leading to changes in protein production. This in turn leads to an altered signal cascade that can, if it overrides homoeostatic control, result in altered functions of cells, tissues and organs. These alterations in function can then lead to adverse outcomes (AO) such as a malformation, organ dysfunction and eventually lethality. This results in an AO of regulatory relevance for risk assessment that represents overt adversity at either organism or population level (Figure 2).

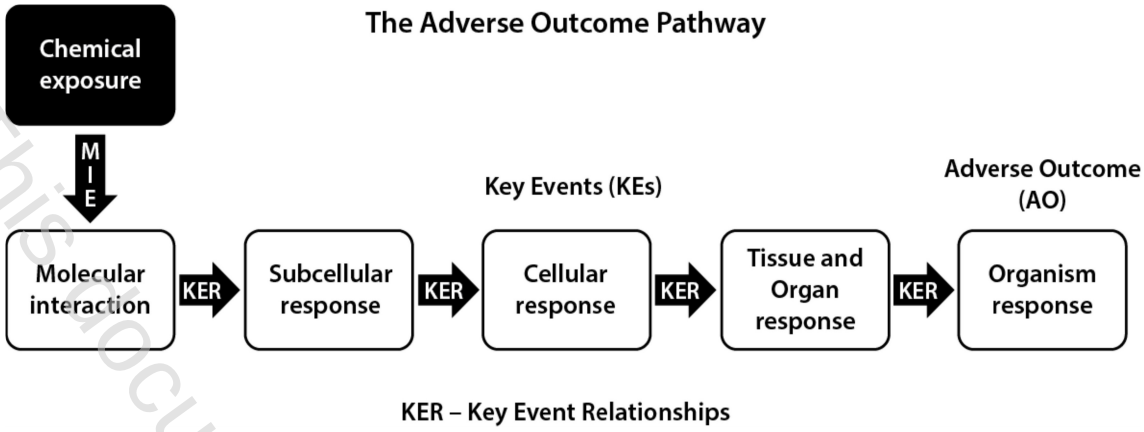

Figure 2 — The Adverse Outcome Pathway

An AOP (Adverse Outcome Pathway), starts with a molecular interaction between a chemical and a molecule. This step is called the Molecular Initiating Event (MIE). This is followed by a set of Key Events (KE) propagating the signal to higher levels of organization. This leads to an outcome on the physiology of an organism and if this outcome is deleterious it is called and Adverse Outcome (AO). The complete chain of events is therefore called AO Pathway (AOP) and connects the initial exposure, through a molecular interaction to a physiological change in the organism. At sufficient concentrations of the chemical and durations of exposure, a KE can increase to a level where it will trigger another KE to shut down, overcoming cell defence mechanisms and adaptation processes.

Thus, in order for an AO to occur, there has to be an initial molecular interaction leading to alterations in gene activity. While the AO is the measure of the damage/toxicity of the exposure, the initial changes in gene activity is the direct response to the exposure.

With the toxicogenomic qPCR method it is possible to directly measure the first KE in the cascade leading from MIE to AO. This can be done by analysis of genes belonging to selected AOP such as metal toxicity, xenobiotic toxicity, stress response, reproduction, metabolism, respiration, cell cycle (cancer and apoptosis). By determining the correlation between significant changes in gene expression and specific AOPs it is thereby possible to identify the causative factor. Lack of correlation between an AOP and its MIE indicates that there is no effect caused by the exposure on that AOP. An advantage with this method is that it allows for identification of the cause of the AO as each gene is regulated by a specific set of receptors and transcription factors.

Analysis of RNA levels is therefore the most robust and sensitive way to determine the mechanism leading to the effects observed following an exposure. For comparison, reproduction in *Daphnia magna* can be analysed by measuring the number of offspring in a 21-day assay. If there is a reduction, or increase, in offspring this indicates that the exposure affects reproduction. At the same time, a short exposure (24 h to 96 h) followed by analysis of RNA levels will be able to show if there is a change in the machinery necessary for reproduction.

One advantage of analysing the regulation of genes is that the induction or reduction in RNA is directly correlated to specific compounds that can regulate the gene expression. Thus, by combining the analysis of reproductive RNA with the analysis of RNA measuring toxicity it is possible to determine what type of toxic effect is caused by the compounds that the organism is exposed to. By analysing the RNA regulation, it is therefore possible to determine effects related to both HP10 (Toxic for reproduction) and HP14 (Ecotoxic, i.e. causing toxicity to organisms in the environment).

While young daphnids (24 h to 48 h old) can be used to investigate the expression of genes related to biological pathways activated during early development of the animal (i.e.; metal response, oxidative stress), older daphnids (>72 h old) are required to analyse effects of the tested compound on genes related to pathways that are functional in later life stages, such as reproduction. To cover genes involved

in both development, toxicity, and reproduction it is therefore important to expose the animals from hatching/birth to 96 h.

The analyses can be designed based on previous chemical data indicating what type of exposure that the *Daphnia magna* will be experiencing. The resolution of the assay can be increased by selecting specific genes that are known to respond to specific insults. In addition, by using controlled exposures to specific compounds it is possible to identify if these are contributing to a measured biological effect see Figure 3. Thus, it is possible to determine if the effect is caused by a compound of concern or by normally occurring abiotic properties of the exposure solution.

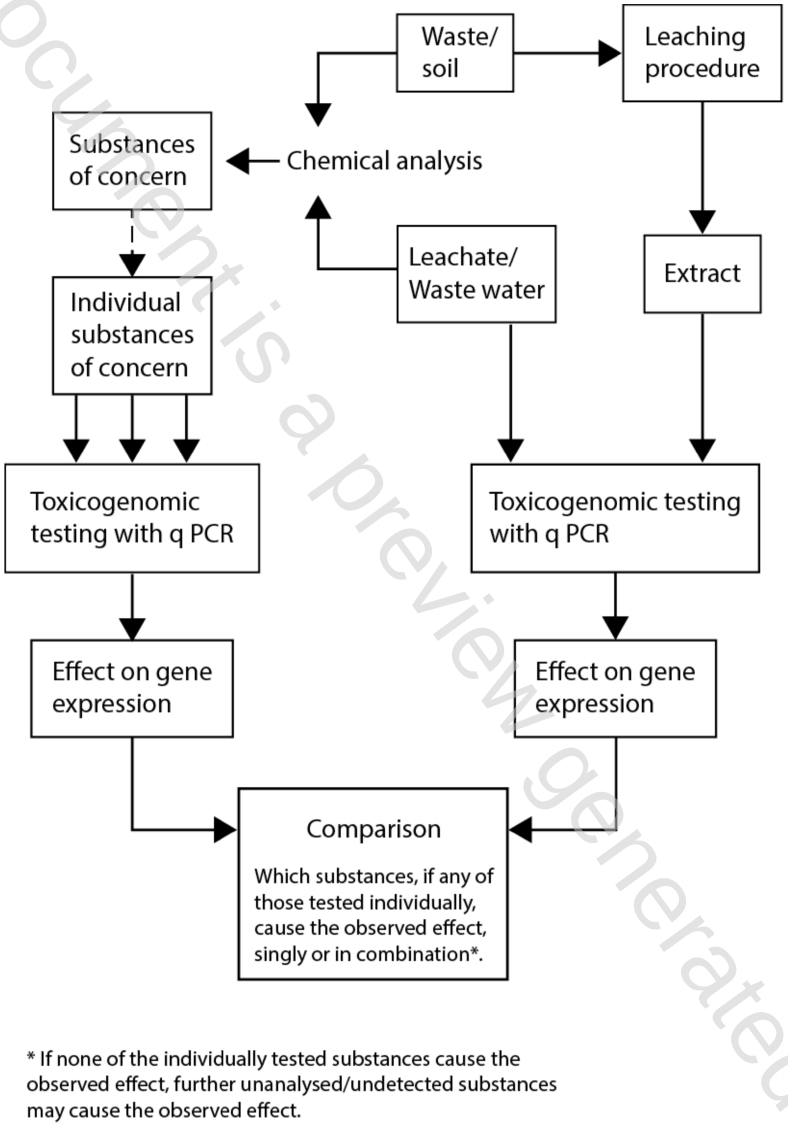

Figure 3 — Testing the effect of identified contaminants in waste, soils or water

The rational for choosing *Daphnia magna* for toxicological analyses is that it is a sensitive organism. *Daphnia magna* is considered a keystone species for the analysis of effects caused by complex environmental exposure [1]. It has successfully been applied as a sensitive model system for studies of effects caused by building material and bottom-ash [2,3].

The advantages of the method can be summarized as:

1. Robust: Analysis at the RNA level using qPCR is a robust method. An exposure that affects an animal has to involve bioavailable compounds that can activate or inhibit the conversion of DNA information into RNA. This gives information on the responses to the exposure.

2. Quantitative: Analysis at the RNA level is quantifiable. As the amount of RNA is directly proportional to the activation of genes and occurs rapidly following an exposure this is an easily quantifiable method.

3. Mechanistic: The resolution of qPCR arrays (including many genes) allows for identification of the cause leading to the effect. This cannot be achieved by traditional methods at the individual or higher organizational level.

4. Sensitive: Alterations at the RNA level is more sensitive than analysis of physiological, morphological or behavioural effects at the level of individual organisms or higher. This is due to alterations in RNA being the first step in the organismal responses to exposure.

5. Complex mixtures: Using the biological qPCR approach allows for the detection of effects, even if they are caused by complex mixtures, where several compounds in low doses are functioning in an additive, synergistic or antagonistic manner.

6. Other advantages: The method is less prone to false positives (or negatives). The method is fast. The method is relatively cheaper than corresponding physiological assays that require longer time and thus more man hours to perform.

1 Scope

This document specifies the crucial steps of a quantitative real-time polymerase chain reaction (qPCR) method to quantify the abundance of specific mRNA molecules extracted from *Daphnia magna*.

The method allows the identification of molecular responses to exposures for potentially toxic substances through the analysis of the abundance of specific mRNA molecules. In this document, the central genes involved in reproductive and toxic responses are included.

NOTE The selection of genes can be adapted to specific exposure conditions, for example, exposure to known toxic substances, by adding genes known to respond to a specific insult.

The present method allows for rapid, robust and sensitive detection of molecular responses and can be used to analyse the toxic effects of water leachates from soil and waste. The method gives information of the concentration of a substance or test-liquid at which toxic effects begin to occur prior to observations of reproductive or toxic effects at higher levels of organization, which reduces the need for the use of safety factors in toxicity assessment.

The method is useful in several types of risk assessment. In this document, the genes studied are appropriate for the assessment of the risks when recycling materials and for the classification of waste, but the method can be adapted to other types of risk assessment by including other genes.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12457-2, *Characterization of waste — Leaching — Compliance test for leaching of granular waste materials and sludges — Part 2: One stage batch test at a liquid to solid ratio of 10 l/kg for materials with particle size below 4 mm (without or with size reduction)*

EN ISO 21268-2, *Soil quality — Leaching procedures for subsequent chemical and ecotoxicological testing of soil and soil-like material — Part 2: Batch test using a liquid to solid ratio of 10 l/kg dry matter (ISO 21268-2)*

ISO 20395, *Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org

3.1
cDNA
conversion product from mRNA

3.2
cycle threshold
Ct
number of cycles required for the signal to cross the threshold above the base line