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# Evaluation of surface water quality in the Vuachère watershed using a bioassay battery

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### Photo header: Vincent Gregorio, Ville de Lausanne (site Denantou outlet)

# Summary

*Background*: In this project a bioassay battery was applied to three sampling sites in the Vuachère watershed in the municipality of Lausanne. The applied bioassay battery covers several important pollutant effects and substance groups. The applicability and relevance of the selected bioassays to environmental samples have been shown in various recent international monitoring studies. In the present study, these effect-based methods were applied to enable a comprehensive evaluation of water quality (also for different precipitation conditions) and to perform a risk assessment based on the bioassay results.

*Methods*: Long-term samples (14 days composite samples) at different precipitation conditions as well as rain weather samples were assessed in eleven ecotoxicological bioassays: a panel of six CALUX® assays to assess cytotoxicity, pollutant metabolism, oxidative stress, estrogenic and anti-androgenic activity as well as PAH-like activity. In addition, a combined algae test was applied to assess effects of the water samples on photosynthesis and growth of unicellular green algae (*Raphidocelis subcapitata*). These tests were supplemented by *in vivo* bioassays, performed with selected native samples (long-term dry weather samples and rain weather samples only), evaluating effects on the growth of *R. subcapitata* over 72 h, on the growth *of Lemna minor* over 7 days and on the survival and reproduction of water fleas (*Ceriodaphnia dubia*). Potential effects on early life stages of fish (FET) were evaluated in one sample (dry weather sample from Denantou outlet). The bioassays were performed on six 14d composite samples as well as on three dry weather and two rain weather samples. Samples were evaluated with SPE extracts in the CALUX® panel and the combined algae test, and native in the waterflea reproduction and the FET assays. Bioassay results were compared with effect-based trigger (EBT) values to evaluate a potential risk for aquatic organisms.

*Results and Discussion*: The effect-based risk assessment shows exceedances of EBTs for multiple endpoints at multiple sites and sampling types with different precipitation conditions during sampling. With regard to the different sampling sites, at Denantou outlet and Valmont upstream higher EBT exceedances were detected than at Flon tributary. Fourteen day composite samples from the second sampling event showed the highest number of exceedances and the highest maximum exceedances (up to 14 fold) at all three sites, followed by dry and rain weather samples, whereas the 14d composite samples from the first sampling event showed the lowest number of exceedances. When evaluating the EBT exceedances in number of samples (14d composite and dry weather samples), the bioassays for xenobiotic sensing and oxidative stress (PXR- and Nrf2-CALUX®) and the *Lemna minor* growth inhibition assay (run in screening mode) showed the highest number of exceedances (in 9 of 9 resp. 3 of 3 samples), i.e. they were the most responsive assays. This was followed by the PAH-, the Anti-AR- and the ERα-CALUX®, where 4 resp. 3 of 9 samples exceeded the EBT. EBT values for algae PSII and growth inhibition were exceeded in two samples. No exceedances and/or effects were detected in the algae growth inhibition assay with native samples, the *C. dubia* reproduction assay, the Cytotox-CALUX® and the fish embryo toxicity assay. Values in the Nrf2- and PXR-CALUX® were partly higher than in than in previous studies in Switzerland and The Netherlands, whereas values measured in the anti-AR, the ERα- and the PAH-CALUX® were in the same range or partly lower than those detected in previous studies.

*Conclusions*: The bioassays applied in the present study allowed the evaluation of mixtures of pollutants in surface water samples. Exceedances of thresholds for multiple endpoints and for multiple sites and sampling types with different precipitation conditions during sampling were detected, indicating possible negative impacts on aquatic organisms at the sampled locations. Effect-based risks in samples from Flon tributary were lowest, while highest threshold exceedances were found in the second set of 14d composite samples from Denantou outlet and Valmont upstream. Assays for xenobiotic sensing as well as oxidative stress were most responsive. To draw further conclusions about potentially relevant compounds for the observed effects, a comparison of a risk assessment based on bioassay results with one from chemical analysis would be beneficial.

# Content

# 1 Introduction

Chemical substances such as herbicides, insecticides and pharmaceuticals can affect individual organisms in the short term as well as entire communities in the long term. Chemical investigations enable the measurement of substance concentrations in water bodies and an assessment of the associated risk for impacts on aquatic life (Langer et al., 2017; Wittmer, 2014). Biological investigations allow a statement to be made about the state of the biotic communities of, for example, aquatic plants, aquatic invertebrates and fish (Känel et al., 2018; Schager and Peter, 2004; Stucki, 2010). However, due to the complex composition of surface waters, a chemical-analytical detection of all substances present is not possible. In addition, a potential mixture toxicity of these substances is difficult to assess with chemical analysis only. Ecotoxicological bioassays as screening tools and/or early indicators provide thus an important bridge between measured chemicals, i.e. exposure and associated risk to aquatic life, and effects on organisms in the environment. Bioassays are analytical methods that use living cells, organisms or communities of a defined type and number to measure their response to exposure to contaminants in environmental samples (Fent, 2013). A distinction is made here between bioassays that examine specific effects on individual cells or cell lines (*in vitro* bioassays), tests with whole, multicellular organisms (*in vivo* bioassays) and investigations with whole organisms in the field (*in situ* bioassays) (Connon et al., 2012; Kienle et al., 2015b).

*In vitro* bioassays detect specific effects that can be attributed to a certain group of substances (e.g. estrogenic substances, photosynthesis-inhibiting herbicides, neurotoxic insecticides). All these effects represent processes that take place in cells and organisms. Thus, *in vitro* bioassay assessments can provide clues to possible effects on organisms in the environment. An already very well researched and understood example of such indications/inferences is the effect of estrogenic substances on aquatic organisms. Here we have good indications around which levels measured in the *in vitro* bioassay effects on fish in the water body are to be expected (Arlos et al., 2020; Kidd et al., 2007; Vermeirssen et al., 2005). This understanding is not yet as advanced for other effects, yet they allow cost- and time-efficient screening to assess the risk of certain groups of substances to organisms in the environment and to guide further investigations.

In the past, several studies have shown that the use of ecotoxicological bioassays in environmental monitoring provides valuable information. In the Schussenaktivplus project (Triebskorn et al., 2013), both *in vitro* and *in vivo* bioassays proved suitable for assessing the effects of micropollutants on organisms in water bodies. Hormone-active effects measured in *in vitro* bioassays in stream samples reflected the potential for adverse reproductive and hormone-active effects in snails and fish in the stream (Henneberg et al., 2014). Similar results were observed for genotoxic, dioxin-like and embryotoxic effects measured in stream samples in the laboratory, reflecting the corresponding effects on wild fish (Maier et al., 2015). Studies conducted under NAWA SPEZ in Switzerland revealed high calculated ecotoxicological risks in small and medium-sized streams by chemical measurements (Doppler et al., 2017; Spycher et al., 2018; Wittmer, 2014), which could be confirmed by *in vitro* and *in vivo* bioassays in the laboratory. In addition, in these NAWA SPEZ studies, effects on organisms occurred directly in the field (Junghans et al., 2019; Langer et al., 2017). The results of these studies show that ecotoxicological bioassays can serve as screening tools and/or early indicators of effects in the field.

Since there is no single bioassay that can detect all possible effects on different organisms, it makes sense to combine different *in vitro* and *in vivo* bioassays in a "bioassay battery". Various proposals have been developed for this in recent years (Altenburger et al., 2019; Brack et al., 2019; Brack et al., 2017; De Baat et al., 2019; Di Paolo et al., 2016; Escher et al., 2014; Kienle et al., 2015a; Neale et al., 2017a). These bioassay batteries contain both bioassays that measure the metabolism of pollutants (pollutant metabolism), hormone-active effects (endocrine disruption), oxidative stress, mutagenic effects, effects on photosynthesis and plant growth, and effects on aquatic invertebrates and, in some cases, fish. A comparison with so-called effect-based trigger values has recently made it possible to assess the risk to aquatic organisms (Escher et al., 2018; Kienle et al., 2018; van der Oost et al., 2017). The application of such a bioassay battery

to a large number of stream samples with different pressures in the Netherlands has shown that the bioassay results allow a differentiated picture of the pressures (De Baat et al., 2019).

In the current study, the water quality of three sampling sites in the Vuachère watershed was assessed using a bioassay battery. To enable a comprehensive evaluation of the water quality, the following bioassays were performed for all or a selection of samples:

Bioassays with enriched water samples:

- Cytotox-CALUX® to evaluate damage to cell components such as membranes, cell nucleus and lysosomes (Van der Linden et al., 2008).
- PXR-CALUX® to evaluate xenobiotic metabolism. It measures activation of the Pregnane X receptor (PXR), an important xenobiotic metabolism receptor, which induces various phase I enzymes (CYP) and can act as sensitive indicator of the presence of chemicals. It rather responds to a large number of chemicals, and is thus not specific to a certain group (Alygizakis et al., 2019; Escher et al., 2018).
- Nrf2-CALUX® to evaluate cellular reactions to oxidative stress (Van der Linden et al., 2014). Oxidative stress is induced by reactive oxygen species (ROS), which the cell forms in response to exposure, which can be enhanced by chemical stress. Examples for compound classes, which can elicit oxidative stress are certain radical chemicals (e.g., paraquat) and redox cyclers (e.g., quinones) (Escher et al., 2021). This can impair various cell functions and lead to membrane and DNA damage. The Nrf2 gene is one gene involved in response to oxidative stress. It codes for NF-E2-related factor 2, which regulates cellular defence against oxidative stress by activating detoxification genes and antioxidant genes.
- PAH-CALUX® to evaluate cellular responses to polyaromatic hydrocarbons (Pieterse et al., 2013). In normal cells, PAH activation of the aryl hydrocarbon receptor induces metabolic enzymes to oxidise PAHs. The aryl hydrocarbon receptor thus plays an important role in the metabolism of these pollutants. It mediates the toxic effects associated with PAHs and dioxin-like compounds, such as DNA damage and carcinogenicity (Escher et al., 2021; Fent, 2013).
- ERα-CALUX® (International Organization for Standardization, 2018) and Anti-AR CALUX® (Van der Linden et al., 2008) to evaluate feminising effects, which also imply effects on reproduction and development. While the ERα-CALUX® indicates the presence of compounds acting similar as natural estrogens by binding to the estrogen receptor, the Anti-AR CALUX® indicates the presence of compounds blocking the androgen receptor.
- Combined algae test over 24 h to evaluate effects of photosystem II inhibiting herbicides and compounds affecting algae growth (Escher et al., 2008; Glauch and Escher, 2020).

*In vivo* bioassays with native water samples:

- Algae growth inhibition test over 72 h to evaluate effects of compounds affecting algae growth (International Organization for Standardization, 2012).
- *Lemna minor* growth inhibition test over 7 d to evaluate effects of compounds affecting growth of aquatic plants (International Organization for Standardization, 2005).
- *Ceriodaphnia dubia* reproduction test over 8 d to assess effects on reproduction and mortality of water fleas (International Organization for Standardization, 2008).
- *Fish embryo toxicity test* over 4 d to assess acute toxicity on development and mortality of zebrafish embryos and larvae (OECD, 2013).

Results from bioassays were compared with so-called effect-based trigger (EBT) values. An EBT is defined as a value below which harmful effects on organisms are unlikely (with regard to the observed effect) (Escher et al., 2021). With these EBTs an effect-based risk quotient ($RQ_{bio}$) can be calculated by dividing the measured effect through the EBT. An $RQ_{bio}$ below one indicates no risk, while an $RQ_{bio}$ above one indicates a risk with regard to the observed effect (De Baat et al., 2020).

In the next chapters, the methods are described and the results of the bioassays are reviewed and discussed.

# 2 Material and Methods

## 2.1 Sampling and Transport

To investigate water quality in the Vuachère watershed, three sites were sampled in three long-term campaigns, and during one rain event. Sampling was carried out at times when expected concentrations were high (June - July). For the three long-term campaigns (100 mL/h), two 14 d composite samples and one 7 d composite sample per site were collected during dry weather only. The amount of rain varied for the three samples (0, 23 and 58 mm; Table 1). An additional 4.5 h sample was taken during a rain event (100 mL/15 min). The sampling period included the peak of the rain event. Samples (volume 2.3 to 3 L) were collected in solvent-cleaned glass bottles time-proportionally with automatic samplers.

Tab. 1 gives an overview on the sampling campaigns and provides details about the sampling sites and dates.

***Tab. 1: Overview on sampling campaigns, rivers, sampling sites and dates, sample types and codes and water type.***

| Composite samples | Station | June | | | | | | | | | | | | | | | | | | | | | July | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 02 | 03 | 06 | 07 | 09 | 10 | 13 | 14 | 16 | 17 | 20 | 21 | 23 | 24 | 25 | 27 | 28 | 30 | 01 | 04 | 05 | 11 | 12 | 14 | 15 | 18 | 19 | 21 | 22 | 25 | 26 | 28 | | | | | | | | |
| Long term sample 100ml/h | Denantou | | | | 14days (DO_14_1)* | | | | | | | | 14days (DO_14_2)* | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Flon | | | | 13.5days (FT_14_1)* | | | | | | | | 14days (FT_14_2)* | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Valmont | | | | 14days (VU_14_1)* | | | | | | | | 12.5days (VU_14_2)* | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Dry Weather 100ml/h | Denantou | | | | | | | | 7d(DO_DW)*,**,*** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Flon | | | | | | | | 7d(FT_DW) *,** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Valmont | | | | | | | | 7d(VU_DW) *,** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Rain Weather (flood event) 100ml/15min | Denantou | | | | | | | | | | | | | | | | | | | | | | | | | | | | <4h30(DO_RW)* ** | | | | | | | | | | | |
| | Flon | | | | | | | | | | | | | | | | | | | | | | | | | | | | <4h30(FT_RW)* ** | | | | | | | | | | | |
| | Valmont | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Rainfall [mm] | | | | 23 | | 0 | | 0 | | 0 | | 18 | | 21.3 | | 4 | | 15 | | | | | | | | 4 | | 1 | | | | | | | | | | | | |

**Bioassays performed:** *** CALUX(Cytotox-; ERα-; Anti-AR-; Nrf2-; PXR-; PAH-) + Combined algae test**
**** *Ceriodaphnia dubia* reproduction test + Plant grothw *** Fish embryo acute toxicity (FET) test**

*DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary, DW = Dry weather, RW = Rain weather, FB = field blank*

Fig. 1 shows the Vuachère watershed with the three sampling sites.

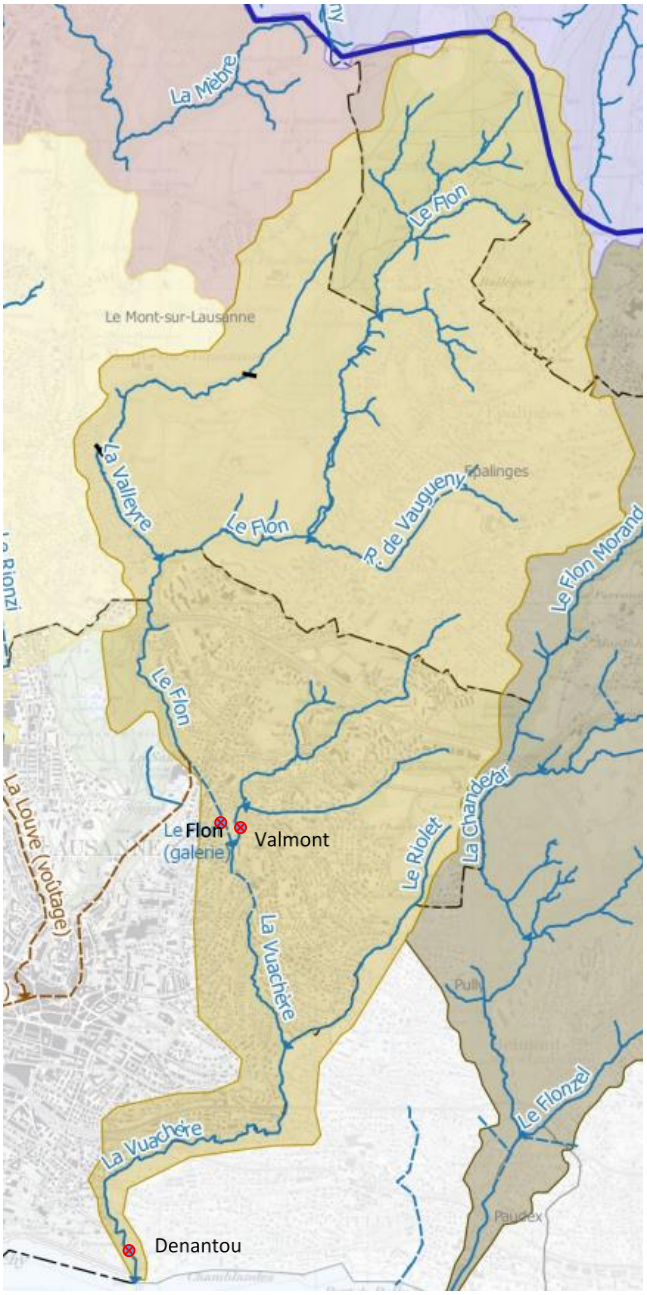


***Fig. 1: Map of the Vuachère watershed with the sampling sites:*** *Flon tributary, Valmont upstream and Denantou outlet*

After completion of each campaign, the samples were transported refrigerated to Soluval Santiago and the Ecotox Centre. *In vivo* bioassays were performed with native water samples, while samples for *in vitro* bioassays were enriched by solid phase extraction (SPE) as described in chapter 2.2.

## 2.2 Sample pre-treatment

For the *in vitro* bioassays (see chapter 2.4), the samples were enriched at the Ecotox Centre by means of SPE (see Tab. 10 in Appendix 1). For this purpose, they were filtered through a glass fibre filter (2.7 µm, type APFD 09050, Millipore, Billerica, MA, USA) upon arrival in the laboratory, and the pH was adjusted to 7.2 with HCl (1 M). Sample enrichment was performed as follows: 1.5 L of each sample was extracted using Strata XL cartridges (Phenomenex). One and a half litres of phosphate buffered Millipore water (pH 7.2) served as a blank sample (Blank). The sample was eluted from the cartridges with 2 mL acetone, 2 mL methanol and 3 mL acetone and the 7 mL solvent was concentrated under vacuum to 0.5 - 0.8 mL using an Eppendorf concentrator (V-AL, 51 min, 30°C). Then, ethanol was added to reach a final volume of 1.5 mL. Final extracts were stored at -20°C and a 1 mL aliquot sent refrigerated to Biodetection Systems (BDS) to perform the CALUX panel. The combined algae test was performed with the remaining aliquot.

An algae growth inhibition test as well as tests with *Lemna minor*, water fleas and fish embryos were performed with native samples (see chapter 2.5). In each case, tests were started on the day the samples were delivered, i.e. on the day after the sampling period ended.

## 2.3 Overview on bioassays and effect-based trigger values for water quality evaluation

Fig. 2 provides an overview on sample distribution, pre-treatment and the bioassays performed with either native or enriched samples.

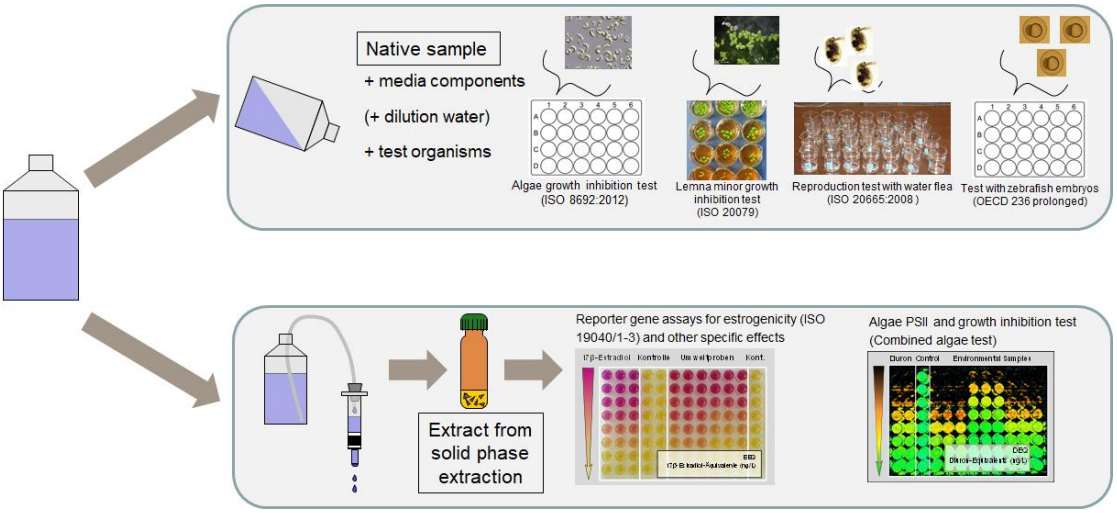


***Fig. 2: Overview on the procedure for the bioassays***

Tab. 2 gives an overview on the selected bioassays for the evaluation of the samples.

***Tab. 2: Overview on the applied in vitro and in vivo bioassays.***

| Effect | Mechanism (organism group) | Test |
|---|---|---|
| **Cell toxicity** | damage to cell components such as membranes, cell nucleus and lysosomes | Cytotox-CALUX® (human cell line) |
| **Oxidative stress** | Cellular reaction to oxidative stress | Nrf2-CALUX® |
| **Pollutant metabolism** | Activation of:<br>• Cell response to aromatic hydrocarbons<br>• Detection and detoxification of xenobiotics | PAH-CALUX®<br>PXR-CALUX® (Pregnane X receptor) |
| **Endocrine disruption** | Estrogenicity<br>Anti-androgenicity | ERα-CALUX® (ISO 19040)<br>Anti-AR-CALUX® |
| **Plant photosynthesis and growth** | Herbicidal effects<br>Growth inhibition | Combined algae test<br>Algae growth inhibition test<br>*Lemna minor* growth inhibition test |
| **Mortality, reproduction** | Non-specific (zooplankton) | Water flea reproduction test (*Ceriodaphnia dubia*, ISO 20665) |
| **Early life stage development, mortality** | Non-specific (fish) | Fish embryo acute toxicity (FET) test (OECD 236 prolonged to 120 h) |

Tab. 3 lists the corresponding effect-based trigger values.

***Tab. 3: Effect-based thresholds for the selected bioassays***

| Effect | Bioassay | Effect-based trigger value | Reference compound |
|---|---|---|---|
| **Bioassays with enriched samples** | | | |
| Cell toxicity | Cytotox-CALUX® | | Tributyltin acetate |
| Pollutant metabolism | PAH-CALUX® | 6.21, 62.1, 150 ng BaPEQ/L [2,1,3] | Benzo(a)pyren |
| | PXR-CALUX® | 3, 5.4, 54 µg NicEQ/L [1,2,3] | Nicardipine |
| Oxidative stress | Nrf2-CALUX® | 10 µg/L CurEQ/L [1] | Curcumine |
| Endocrine disruption | ERα-CALUX® | 0.1, 0.4, 0.5 ng EEQ/L [2,4,1] | 17β-Estradiol |
| | Anti-AR-CALUX® | 14.4, 25 µg FluEQ/L [2,1] | Flutamide |
| Photosynthesis and plant growth | Combined algae test | 70 ng DEQ/L (PSII inhibition) [2]<br>130 ng DEQ/L (growth inhibition) [2] | Diuron |
| **Bioassays with native samples** | | | |
| Plant growth | Algae growth inhibition test | ≤ 75% growth [5,6] | |
| | *Lemna minor* growth inhibition test | ≤ 75% growth [5,6] | |
| Effects on aquatic invertebrates | *Ceriodaphnia dubia* reproduction test | ≤ 80% survival [5,6]<br>≤ 70% reproduction [5,6] | |
| Effects on aquatic vertebrates | *Fish embryo acute toxicity (FET) test* | ≥ 30% sublethal effects [7]<br>≤ 80% survival/hatching success [7] | |

[1](van der Oost et al., 2017); [2](Escher et al., 2018); [3](De Baat et al., 2020), [4](Kienle et al., 2018; Kunz et al., 2015), [5]ISO 17616:2019 (International Organization for Standardization, 2019), [6](Ferrari et al., 2017), [7](Kienle et al., 2023)

## 2.4 Bioassays with enriched water samples

### 2.4.1 CALUX panel for the detection of cell toxicity and specific modes of action

CALUX® assays are carried out with mammalian cell lines. They are receptor activation tests for the detection of hormone-active and other toxic substances. A variant of this test, the ERα-CALUX®, is a sensitive and established method for the detection of oestrogenic activity in environmental samples, which is ISO certified (ISO 19040-3). The reporter gene cells used for the tests are derived from human cells and are applied to assess water extracts for various hormonal activities (Van der Linden et al., 2008).

#### 2.4.1.1 Test organism

Most CALUX® assays are carried out with the genetically modified human osteosarcom cell line U2OS. In addition to the gene for a specific hormone receptor, e.g. the human oestrogen receptor, the human androgen receptor, etc., the used cells contain a luciferase gene which is also read when the hormone receptor gene is read. The cells are cultivated and distributed by the company Biodetection Systems (BDS) in the Netherlands.

#### 2.4.1.2 Test principle and performance

The test was carried out by BDS in 96-well microtitre plates according to the method of Van der Linden et al. (2008) and ISO (International Organization for Standardization, 2018). Positive controls were: tributyltin acetate (Cytotox-CALUX®), 17β-estradiol (ERα-CALUX®), flutamide (Anti-AR-CALUX®), curcurmine (Nrf2-CALUX®), nicardipine (PXR-CALUX®), and benzo[a]pyrene (PAH-CALUX®; using a rat cell line). Pure growth medium (DF medium) with 0.1 % of the solvent DMSO served as solvent control. The positive control and the extracts of the environmental samples were tested in triplicates.

For this purpose, the sample extracts were transferred into DMSO and further concentrated by a factor of 10 (new: 10,000-fold). From this sample extract, dilutions of 1:3, 1:10, 1:30 and 1:100 in DMSO were prepared. The undiluted sample extract and the dilutions were mixed 1:1000 with test medium before transfer to the test plate. Thus, the maximum enrichment factor for the environmental samples in these bioassays was 10.

The day before testing, 96-well plates were seeded with cells and DF medium. After 24 h incubation (37 °C, 5% $CO_2$), the medium was replaced by sample medium containing the sample extracts to be tested (0.1% DMSO). After a further 24 h incubation (37 °C, 5% $CO_2$) the cells were checked microscopically for cytotoxic effects (visible morphological changes of the cells, reduced cell density or cell death). Sample dilutions showing such effects were excluded from the evaluation. The medium was subsequently removed and the cells lysed in 30 µL Triton lysis buffer. The activity of the enzyme luciferase, which converts the protein luciferin by generating light, was measured using a luminometer (e.g. Lucy 2, Anthos, Austria) and reported in relative light units (RLU).

### 2.4.2 Combined algae test to assess photosystem II and growth inhibition

#### 2.4.2.1 Test organism

The test was carried out with the unicellular green alga *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*). The algae were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany).

#### 2.4.2.2 Test principle and performance

The test was performed in 96-well microtitre plates as described in Escher et al. (2008). The herbicide diuron served as reference substance and ethanol as negative control. Diuron and the environmental samples were tested in duplicate in a 1:3 dilution series over eight wells (80 µL/well). The initial concentration of diuron in the test was $1.3 \times 10^{-6}$ M or 310 µg/L. 80 µL of sample extract was pipetted into each well. After complete evaporation of the solvent, reference and samples were re-dissolved in 150 µL of medium and 150 µL of the algal culture was added to each well. The maximum enrichment factor for the environmental samples in the algae test was thus 267.

Photosynthetic inhibition was measured by effective quantum yield (Y) using a maxi-imaging PAM device (Walz, Germany) after 2 h (see also Escher et al. (2008) and (Schreiber et al., 2007)). Algal growth was recorded by measuring absorbance at 685 nm in a microplate photometer (Synergy 4, Biotek, USA) after 0, 2, 24 h and two time points between 2 and 24 h. Algae density was measured by a microplate photometer (Synergy 4, Biotek, USA) to determine growth rates. The absorption of light at 685 nm is proportional to the chlorophyll A content of the algae and thus proportional to the cell number in the medium.

### 2.4.3 Data evaluation

Bioanalytical equivalent concentrations (BEQ) were calculated to quantify toxicity. The BEQ is defined as the concentration of a reference substance that has the same effect as the environmental sample (International Organization for Standardization, 2022). The reference substances vary depending on the specific endpoint measured. Thus, a toxic potency (or toxicity quantity) of a mixture can be expressed as a concentration of a reference substance. The higher the BEQ value, the more toxic the sample under investigation.

For example, RLU raw data from the ERα-CALUX® assay was normalised: 0% corresponds to the solvent control activity and 100% to the highest 17β-estradiol (E2) activity. From the E2 concentration-effect curve, the 10% effect level ($PC_{10}$) of each sample was interpolated and E2 equivalent concentrations (ng EEQ/L) were derived considering the tested sample dilutions.

In the same way, BEQs were determined for further assays with the “B” in BEQ reflecting tributyltin acetate (Cytotox-CALUX®), flutamide (Anti-AR-CALUX®), curcurmine (Nrf2-CALUX®), nicardipine (PXR-CALUX®), benzo[a]pyrene (PAH-CALUX®), and diuron (algae test).

Data evaluation was performed using Excel and the statistical programme GraphPad Prism (Graph-Pad Prism 5 Software, La Jolla California USA) by determining a concentration-effect relationship for the reference substance and the environmental samples.

## 2.5 Bioassays with native water samples

### 2.5.1 Algae growth inhibition test

This assay was conducted complementary to the originally planned bioassay battery with rain and dry weather samples. The assay was performed in screening mode with a reduced number of concentrations and replicates.

#### 2.5.1.1 Test organism

The test was carried out with the unicellular green alga *Raphidocelis subcapitata (strain from UTEX 1648, obtained via Institut F.-A. Forel, University of Geneva).*

#### 2.5.1.2 Test principle and performance

The test was performed according to AFNOR T90-375 in 24 well microtitre plates with a volume of 2 mL per well. Samples were assessed in three to four dilution levels (between 85.9% and 47.7 % of sample) in two replicates per dilution level. Algae were cultivated at 23±2 °C and 5'000 lux.

Growth of the algae at the end of the test (72 h) was determined by optical density at 680 nm (OD680) in each well.

### 2.5.2 *Lemna minor* growth inhibition test

This assay was conducted complementary to the originally planned bioassay battery with rain and dry weather samples. The assay was performed in screening mode with a reduced number of concentrations and replicates.

#### 2.5.2.1 Test organism

The test was carried out with the aquatic plant *Lemna minor*. The culture was obtained from the Ecotoxicological institute, Stuttgart via Institut F.-A. Forel, University of Geneva.

#### 2.5.2.2 Test principle and performance

The test was performed in 80 mL glass beakers. Samples were assessed in two dilution levels (88.7 and 69% of sample) in one replicate per dilution level. The test organisms were cultivated at 24±2 °C and 5'000 lux. The samples were diluted with OECD medium (OECD2 221; modified SIS medium).

Duckweed growth at 7 days was determined by counting number of fronds.

### 2.5.3 *Ceriodaphnia dubia* reproduction test

This assay was conducted with rain and dry weather samples only.

#### 2.5.3.1 Test organism

Effects of the samples on the water flea *Ceriodaphnia dubia* were determined in a chronic toxicity test over 8 days (inhibition of reproduction according to ISO/CD 20665 (International Organization for Standardization, 2008) and AFNOR T90-376 (AFNOR, 2000)).

#### 2.5.3.2 Media

The test was performed with a slight modification of the standards: The control or dilution medium comprised a mixture of ¼ Evian mineral water, ¼ Elendt M4 medium (Elendt and Bias, 1990) and ½ deionized water corresponded to a moderately hard water supplemented with selenium and

vitamin B12. A mixture of yeast, a digested suspension of fish flakes (TetraMin®) and green algae (*Raphidocelis subcapitata* and *Chlorella* sp.) served as feed.

#### 2.5.3.3 Exposure of the test organisms

Test organisms were from a laboratory culture (Soluval Santiago, Couvet, CH). Juveniles (less than 24 h old and all within 8 h of the same age at the start of the test) were exposed to the different samples for up to 8 d in a static system with regular water changes. For each sampling campaign, a control preparation was assessed with 24 replicates. Samples were tested at one concentration (90%). All tests were performed at 25 ± 1°C in a climate chamber with an illumination intensity of 300 to 500 lux and a 16:8 h light:dark rhythm.

#### 2.5.3.4 Endpoints/observations

Survival of mothers and number of offspring were determined daily, each time at water change. Physicochemical characteristics of the samples (pH, dissolved oxygen [mg/L], and electrical conductivity [µS/cm]) were measured upon arrival of the samples at the laboratory, at 4-5 time points during the test, and at the end of the test.

#### 2.5.3.5 Statistical evaluation

Statistical analysis was performed using the GraphPad Prism® statistical program (version 9.4.1). The data were first tested for normal distribution (Shapiro-Wilk test). Since all data sets were normally distributed, the data for the individual treatment levels were analyzed per measurement campaign using an analysis of variance (one-way ANOVA). If this was significant, it was tested whether the number of offspring in the water samples was significantly different from the number of offspring in the respective control (Dunnett's multiple comparisons test).

### 2.5.4 Fish embryo acute toxicity (FET) test

#### 2.5.4.1 Test organism

Effects of the samples on embryos and larvae of zebrafish (*Danio rerio*) were determined in an acute toxicity test over 4 d according to OECD guideline 236 (OECD, 2013). The test was performed with freshly spawned embryos from a wild-type strain of *Danio rerio* (Eawag WM-Strain), kept in-house, age of 14 months.

#### 2.5.4.2 Test principle and performance

The purpose of this test was to determine the acute and sub-lethal toxicity of the environmental water sample on embryonic stages of zebrafish. Newly fertilized zebrafish embryos were exposed to sample for a period of 120 h. Every 24 h, up to five apical observations were recorded as indicators of lethality (see Table 2). At the end of the exposure period, acute toxicity was determined based on a positive outcome in any of the four lethal apical observations recorded, and the $LC_{50}$ value was calculated. Additionally, at each observation, sub-lethal endpoints were recorded (see Tab. 4). If one or more sub-lethal endpoints were observed in an embryo, it was declared affected. The percentage of sub-lethal effects ($EC_{50}$) was calculated on the basis of surviving embryos, which was set to 100%.

*Tab. 4: Lethal and sub-lethal end-points of the FET-Test*

| Exposure Time | 24 hpf | 48 hpf | 72 hpf | 96 hpf | 120 hpf |
|---|---|---|---|---|---|
| n= No lethal endpoints observed | x | x | x | x | x |
| HA = Hatch | x | x | x | x | x |
| NA= Not available (e.g. lost embryo) | x | x | x | x | x |
| **Lethal endpoints / macroscopic observation** | | | | | |
| C= Coagulation | x | x | x | x | x |
| S= No somite formation | x | x | x | x | x |
| T= Tail not detached | x | x | x | x | x |
| H= No heart beat | | x | x | x | x |
| LH= Lack of hatching** | | | | | x |
| **Sub-lethal endpoints / macroscopic observation** | | | | | |
| D= General delay of development / growth | x | x | x | x | x |
| MH= Malformation head | x | x | x | x | x |
| MT= Malformation tail | x | x | x | x | x |
| EY= Modified eye development | x | x | x | x | x |
| A= Modified axis structure | x | x | x | x | x |
| Y= Yolk deformations | x | x | x | x | x |
| HE= Heart edema | | x | x | x | x |
| HY= Yolk edema | x | x | x | x | x |
| M= uncontrolled movements/ trembling | x | x | x | x | x |
| P= No pigmentation | | | | | x |
| NR = no reaction after trigger | x | x | x | x | x |

**Copied from OECD 236 guideline: *"Hatching rates of all treatment and control groups should be recorded from 48 hrs onwards and reported. Although hatching is not an endpoint used for the calculation of the LC50, hatching ensures exposure of the embryo without a potential barrier function of the chorion, and as such may help data interpretation." The exposure up to 120 h is not according to the OECD guideline, in which the test duration is set to 96 h. For 120 h exposure, it is suggested to count the lack of hatching as lethal endpoint. Therefore, lack of hatching is included in the 120 hpf LC50 calculation.*

For water samples, also the lowest-ineffective-dilution (LID) (dilution which has no significant difference to the negative control) was calculated for lethal and sub-lethal endpoints.

Controls: 4 mg/L 3,4-Dichloroaniline exposure was used as positive control and exposure with dilution water as negative control.

#### 2.5.4.3 Exposure of the test organisms

*Test concentrations*: Five dilutions containing 100, 80, 60, 40 and 20% of the water sample, respectively and a control (dilution water only) were used for testing and prepared as presented in Tab. 5.

*Tab. 5: Preparation of dilution series of the water sample.*

| Conc. Nr.: | Water sample concentration [%] | Water sample added [mL] | Dilution water [mL] |
|---|---|---|---|
| 1 | 100 | 100 | 0 |
| 2 | 80 | 40 | 10 |
| 3 | 60 | 30 | 20 |
| 4 | 40 | 20 | 30 |
| 5 | 20 | 10 | 40 |

*Pre-exposure of the embryos*: Temperature of the test item dilutions was adapted to the test temperature of 26 ± 1 °C. Afterwards, 5 mL of all test concentrations were transferred to a petri dish for pre-exposure of the embryos. Fertilized embryos were then transferred from the pre-exposure petri dishes into the 24-well plate and 2 mL of the respective exposure solution was added per well.

*Positive control 3,4-dichloroaniline*: A stock solution of 1.5 mg 3,4-dichloroaniline with 15 mL embryo dilution water was prepared in a 20 mL glass vial on the day before the test. The final stock concentration obtained was of 100 mg/L. At the test day a 4 mg/L dilution was prepared by mixing 4 mL stock with 96 mL dilution water. This dilution was used for both the pre-exposure and final FET-Test performance.

### 2.5.5 Interpretation of results

No recommendations for interpreting the results are given in the ISO/CD 20665 guideline (International Organization for Standardization, 2008) (reproduction assay with water flea) or in OECD guideline 236 (OECD, 2013) (fish embryo toxicity assay). Therefore, the results were compared with effect-based thresholds. These make it possible to classify toxicity according to the parameters studied (CIPEL, 2017). On this basis, the following toxicity classes were applied (see **Error! Reference source not found.** and 7).

***Tab. 6: Toxicity thresholds for biological effects in in vivo tests (ISO 17616) and differentiated classification of effects (adapted from Ferrari et al. (2017))***

EBT = effect-based trigger value, in % compared to control

| | | **Species** | | | |
|---|---|---|---|---|---|
| | | ***Raphidocelis subcapitata*** | ***Lemna minor*** | ***Ceriodaphnia dubia*** | |
| **Category** | **Toxicity** | **Growth** | **Growth** | **Survival** | **Reproduction** |
| 1 | Not significant (< EBT) | >75% | >75% | <80% | <70% |
| 2 | Slight | 50-75% | 50-75% | 60-80% | 50-70% |
| 3 | Moderate | 25-50% | 25-50% | 20-60% | 25-50% |
| 4 | Strong | <25% | <25% | <20% | <25% |

***Tab. 7 Fish embryo toxicity test - Toxicity thresholds and differentiated classification of effects (Kienle et al., 2023).***

EBT = effect-based threshold value, in % of control

| **Category** | **Toxicity** | **sublethal effects (%)** | **Hatching failure / Mortality (%)** |
|---|---|---|---|
| 1 | None or very low (< EBT) | < 30 | < 20 |
| 2 | Slight | 30 - 50 | 20 - 30 |
| 3 | Moderate | 50 - 70 | 30 - 50 |
| 4 | Severe | > 70 | > 50 |

## 2.6 Effect-based risk assessment - Comparison of bioassay results with effects-based trigger values

To compare the results of all the bioassays, an effect-based risk assessment was carried out. For this purpose, effect-based risk quotients ($RQ_{bio}$) were calculated as the ratio of the value measured in the bioassay to the effect-based trigger value (toxicity threshold) (Escher et al., 2021).

The $RQ_{bio}$ was calculated according to equation (2):

(2) $$RQ_{bio} = \frac{measured\ effect}{toxicity\ threshold}$$

Thus, both *in vitro* bioassays with enriched samples, where bioanalytical equivalent (BEQ) concentrations (ng/L) are calculated, and *in vivo* bioassays with native samples, where effect concentrations (% native sample) are determined, can be included in an overall assessment. However, it should be noted that the maximum $RQ_{bio}$ for *in vivo* bioassays is 4 to 5, depending on the effect-based threshold values (see Tab. 3), while that for *in vitro* bioassays with BEQ values may be higher.

To obtain an overall impression of the $RQ_{bio}$ of all bioassays and to assess whether there are differences between site types, the sum of the $RQ_{bio}$ per site was calculated by summing the risk quotients of the individual bioassays (De Baat et al., 2020). This was performed separately for bioassays with enriched samples and for bioassays with native samples.

To identify the bioassays that showed the most diverse effects in the water samples, the proportion of effects-based threshold exceedances was also determined and compared.

# 3 Results

## 3.1 Overview on bioassay results – Effect-based risk assessment

Tab. 8 provides an overview of the results for effect-based risk assessment in the applied bioassays.

***Tab. 8: Overview on effect-based risk assessment results for the bioassays.***

*Numbers show effect-based risk quotients ($RQ_{bio}$) with cells marked in a 2-color-scale (blue = $RQ_{bio}$ < 1; red = $RQ_{bio}$ ≥ 1). White cells indicate that the respective bioassay was not applied at this site. 14 = 14 days composite sample, DW = dry weather sample, RW = rain weather sample. * For calculating $\sum RQ_{bio}$ negative values were set to zero.*

| | | Denantou (D) | | | | Valmont (V) | | | Flon (F) | | | | Field |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample Code | DO_14_1 | DO_14_2 | DO_DW | DO_RW | VU_14_1 | VU_14_2 | VU_DW | FT_14_1 | FT_14_2 | FT_DW | FT_RW | Blank |
| | Sample Type | Outlet (O) | | | | Upstream (U) | | | Tributary (T) | | | | FB |
| ***Bioassays with enriched samples*** | ***Effect*** $\sum RQ_{bio}$ | 15.8 | 20.9 | 11.4 | 23.1 | 17.6 | 23.2 | 12.6 | 10.1 | 13.3 | 9.2 | 10.8 | 0.0 |
| Cytotox CALUX® | Cytotoxicity | 0.0 | 0.4 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| ER-CALUX® | Estrogenic activity | 0.6 | 0.8 | 1.6 | 1.3 | 1.0 | 0.5 | 1.8 | 0.9 | 0.5 | 1.5 | 1.5 | 0.0 |
| Anti-AR-CALUX® | Anti-androgenic activity | 1.3 | 1.5 | 0.7 | 1.0 | 1.0 | 1.2 | 1.2 | 0.0 | 0.8 | 0.9 | 1.0 | 0.0 |
| Nrf2-CALUX® | Oxidative stress | 3.2 | 4.1 | 3.0 | 8.7 | 2.9 | 4.1 | 3.3 | 3.8 | 6.2 | 2.4 | 4.6 | 0.0 |
| PXR-CALUX® | Pollutant metabolism | 9.1 | 10.6 | 5.2 | 10.0 | 10.2 | 13.7 | 5.2 | 4.6 | 3.9 | 3.9 | 3.0 | 0.0 |
| PAH-CALUX® | | 1.0 | 1.1 | 0.5 | 0.8 | 1.0 | 1.1 | 0.4 | 0.6 | 1.0 | 0.4 | 0.6 | 0.0 |
| Combined algae assay | PSII inhibition | 0.4 | 1.1 | 0.2 | 0.4 | 0.9 | 1.6 | 0.4 | 0.2 | 0.6 | 0.1 | 0.1 | 0.0 |
| | Growth inhibition | 0.3 | 1.3 | 0.2 | 0.5 | 0.7 | 1.0 | 0.3 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 |
| ***Bioassays with native samples*** | $\sum RQ_{bio}$ | 0.0 | 0.0 | 1.5 | 2.3 | 0.0 | 0.0 | 1.8 | 0.0 | 0.0 | 1.7 | 2.3 | 0.0 |
| Algae growth inhibition assay | Growth inhibition | | | -0.7 | -0.7 | | | -0.5 | | | -0.6 | -0.3 | |
| Lemna growth inhibition assay | Growth inhibition | | | 1.1 | 1.8 | | | 1.3 | | | 1.7 | 1.9 | |
| Ceriodaphnia reproduction assay | Reproduction | | | 0.2 | 0.5 | | | 0.5 | | | -0.1 | 0.4 | |
| | Mortality | | | 0.0 | 0.0 | | | 0.0 | | | 0.0 | 0.0 | |
| Fish embryo toxicity test | Mortality | | | 0.0 | | | | | | | | | |
| | Hatching | | | 0.0 | | | | | | | | | |
| | Sublethal effects | | | 0.2 | | | | | | | | | |

The effect-based risk assessment shows exceedances of risk quotients for multiple endpoints and for multiple sites and sampling types with different precipitation conditions during sampling. With regard to the different sampling sites, no clear distinction can be seen. With regard to precipitation conditions during sampling, the 14d composite samples from the second sampling event (14_2) showed the highest number of exceedances at all three sites (3-6 per site), followed by dry and rain weather samples, which were relatively similar (4-5 per site). The 14d composite samples from the first sampling event showed the lowest number of exceedances (2-3 per site). Tab. 11 in Appendix 2 shows the results in a 3-color-scale.

When looking at the effects in number of all samples (Fig. 3), eight endpoints from seven different bioassays showed exceedances of EBTs.

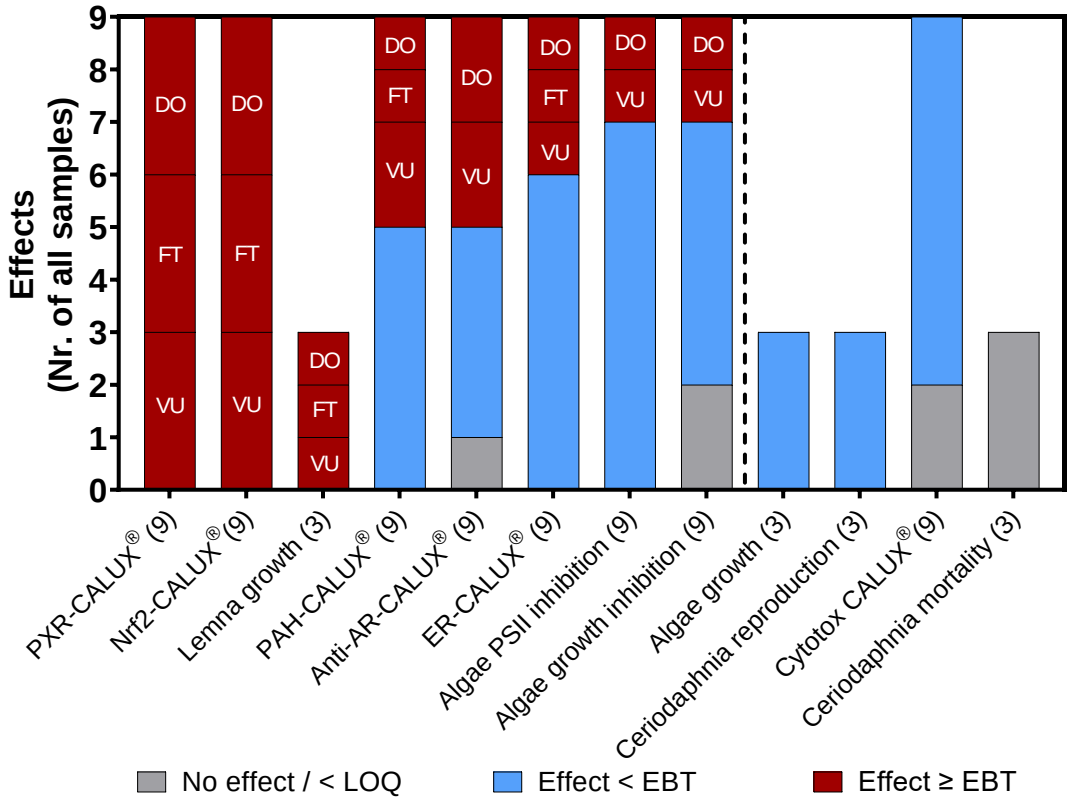


***Fig. 3: Number of samples, which showed an effect in the bioassays, combined with the information on effect-based trigger (EBT) value exceedances.***

*Grey = no effect / effect < LOQ, blue = effect < EBT, red = effect ≥ EBT (DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary). The number of samples is provided in brackets after each test. Only 14d composite and dry weather samples were included in this evaluation. Rain weather samples were excluded as they were not collected at all sites. In addition, the fish embryo toxicity test was excluded, as it was only conducted with one sample.*

The highest number of EBT exceedances (in 9 of 9 resp. 3 of 3 samples) were detected in the PXR-CALUX®, the Nrf2-CALUX® and the *Lemna minor* growth inhibition test. This was followed by the PAH-, the Anti-AR-, and the ERα-CALUX®, where 4 resp. 3 of 9 samples exceeded the EBT. EBT values for algae PSII and growth inhibition were exceeded in two samples. No exceedances and/or effects were detected in the algae growth inhibition assay with native samples, the *C. dubia* reproduction assay, and the Cytotox-CALUX®.

## 3.2 Bioassays with enriched water samples

### 3.2.1 CALUX panel

#### 3.2.1.1 Validity of the tests

The measurement uncertainty for the CALUX method is typically below 30%. The ERα-CALUX® is accredited by ISO17025 (RvA L401). The reference compounds showed the expected effects and no effects were measured in any CALUX® assay in the blanks.

#### 3.2.1.2 Evaluation of samples

Fig. 4 provides an overview about results for the CALUX® panel.

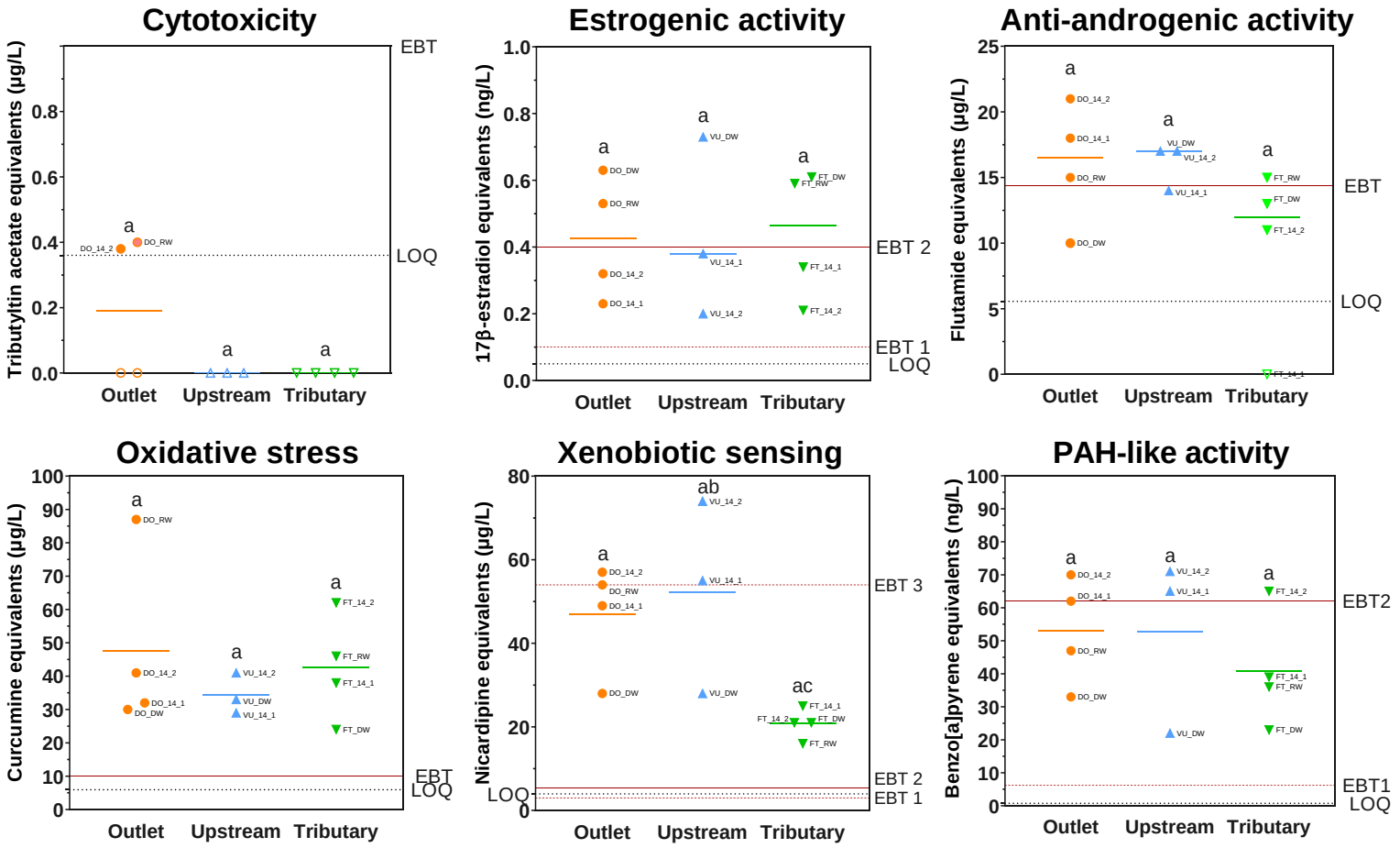


***Fig. 4: CALUX® panel: Overview of the results of reporter gene assays for cytotoxicity (cytotoxicity-CALUX®), estrogenic activity (ERα-CALUX®), anti-androgenic activity (Anti-AR-CALUX®), oxidative stress (Nrf2-CALUX®), xenobiotic sensing (PXR-CALUX®) and PAH-like activity (PAH-CALUX®)***

*Scatter dot plot: the line represents the mean, each symbol represents the result for one sample, and empty symbols indicate values below the limit of quantification (LOQ). 3-4 samples per sample type. EBT = Effect-based trigger value. Different letters indicate significant difference between sample types. Sampling site and sample type: DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary. Sampling type: 14 = 14d composite sample, DW = Dry weather sample, RW = Rain weather sample.*

No EBT exceedances for cytotoxicity were observed and only two outlet samples were slightly above LOQ. EBTs for estrogenic and anti-androgenic activity (0.4 ng EEQ/L and 14.4 µg FEQ/L, respectively) were exceeded in 5 to 6 of 11 samples. For estrogenic activity dry weather samples showed the highest values (0.6 - 0.7 ng EEQ/L), followed by rain weather samples (0.5 - 0.6 ng EEQ/L) and 14d composite samples (0.2 - 0.4 ng EEQ/L). For anti-androgenic activity, the EBT was exceeded in the outlet in 14d composite samples (18 and 21 µg FEQ/L, respectively), as well as in one 14d composite sample from the upstream site (14 µg FEQ/L). In addition, EBT exceedance was measured in the rain weather samples from outlet and tributary (15 µg FEQ/L, respectively) (Fig. 4 first row).

The EBTs for oxidative stress and xenobiotic sensing (10 µg CEQ/L and 5.4 µg NEQ/L) were exceeded in all samples (in one sample up to 9fold) and the EBT for PAH-like activity (62.1 µg BaP EQ/L) in 5 of 11 samples. For oxidative stress, highest values were measured in the second set of 14d composite samples (41 - 62 µg CEQ/L) (precipitation: 58 mm, compared to 23 mm in the first set of 14d composite samples) as well as in rain weather samples (46 and 87 µg CEQ/L). Highest EBT exceedances for xenobiotic sensing (PXR-CALUX®) were measured in the outlet rain weather sample (54 µg NEQ/L) as well as in the second 14d composite sample for outlet and upstream sites (57 and 74 µg CEQ/L, respectively). With regard to PAH-like activity, 14d composite samples exhibited the highest values (39 - 71 µg BaP EQ/L), followed by rain and dry weather samples (Fig. 4 second row).

Further details on the results can be found in Appendix 3.

### 3.2.2 Combined algae test

#### 3.2.2.1 Validity of the test

Negative controls in the assay met the validity criteria, no growth or PSII inhibition was detected and algae growth was good. A positive control was assessed on each plate and $EC_{50}$ values were within the validity range. In addition, neither the blank nor the field blank exhibited PSII or growth inhibition.

#### 3.2.2.2 Evaluation of samples

Fig. 5 provides an overview about results for the combined algae test.

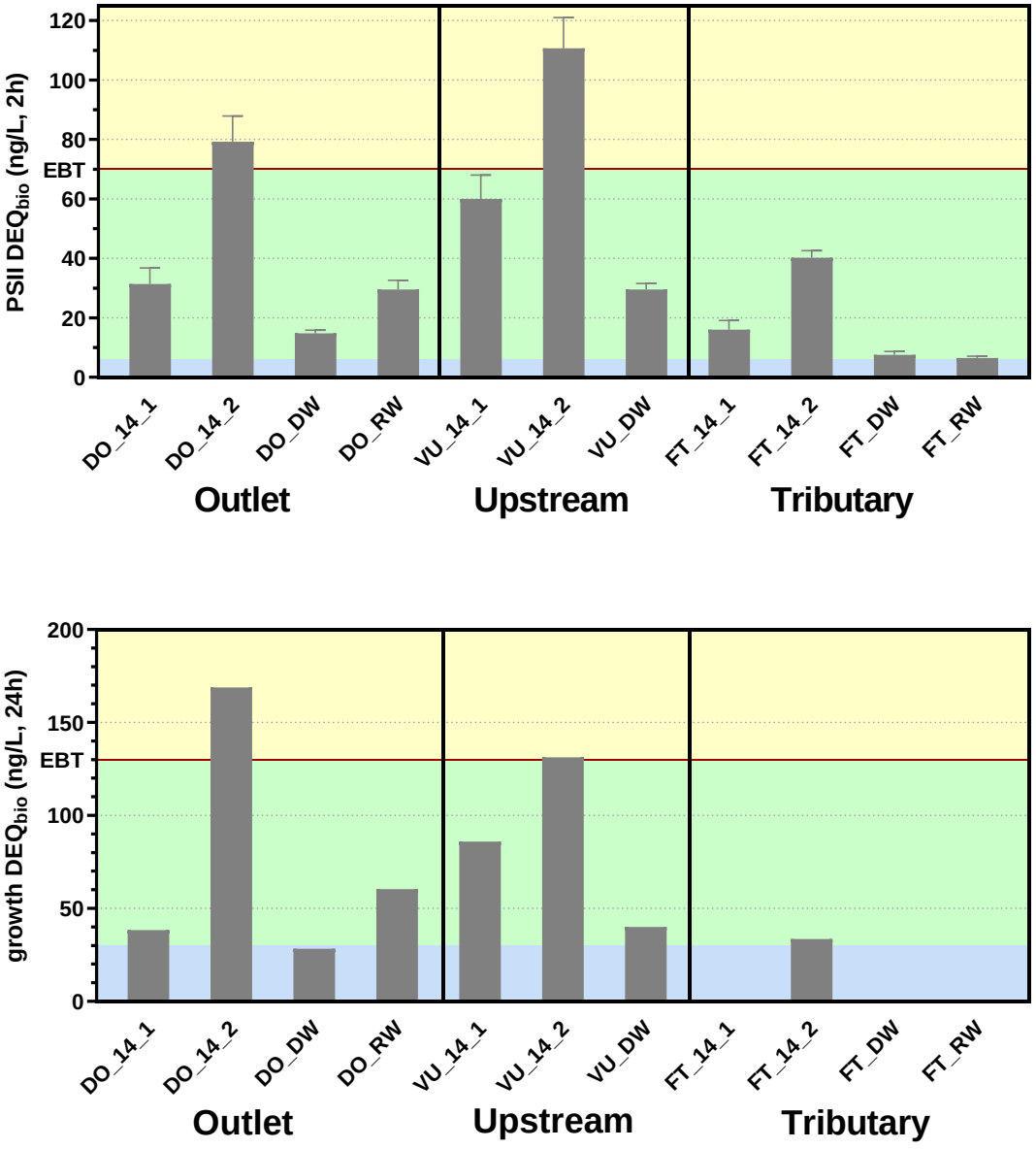


***Fig. 5: Combined algae test with Raphidocelis subcapitata: diuron equivalent concentrations (DEQ, ng/L) for photosystem II (PSII) inhibition (above) and growth inhibition (below).***

*Bar plot: the bar represents the mean and the error bars the 95% confidence limit (available for PSII inhibition only). The shaded areas indicate different water quality levels (blue = very good, green = good, yellow = moderate, orange = insufficient). Sampling site and sample type: DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary. Sampling type: 14 = 14d composite sample, DW = Dry weather sample, RW = Rain weather sample.*

*Algae PSII inhibition*: The EBT for PSII inhibition (70 ng PSII-DEQ/L) was exceeded in two samples (one 14 d composite sample from Denantou and one from Valmont) (79 and 111 ng PSII-DEQ/L, respectively). All other samples were below the EBT (range: 6.5 - 60 ng PSII-DEQ/L). Lowest values were measured in the dry and rain weather samples from Flon tributary (7.6 and 6.5 ng PSII-DEQ/L). Overall, this indicates very good to good water quality with regard to PSII inhibition in all Flon tributary samples and several Denantou and Valmont samples and moderate water quality at one Denantou and one Valmont sample from July 2022 (Fig. 5 top). Chemical

analysis showed a clear correlation between diuron concentration in samples and algae PSII inhibition (V. Gregorio, personal communication).

*Algae growth inhibition*: The EBT for growth inhibition (130 ng growth-DEQ/L) was exceeded in the second 14d composite sample of the Denantou outlet and Valmont upstream sites (169 and 131 ng growth-DEQ/L). In several samples from the Flon tributary site, growth inhibition was below the LOQ. This indicates very good to good water quality with regard to algae growth inhibition in the majority of samples and a moderate water quality in one outlet and one upstream sample (Fig. 5 bottom).

To find out whether more growth-inhibiting substances are present in the water samples than PSII inhibitors or vice versa, the $EC_{50}$ values (in relative enrichment factors (REF)) of PSII inhibition and growth inhibition can be compared (see also (Kienle et al., 2019; Tang et al., 2013)). Fig. 6 shows the results of this evaluation.

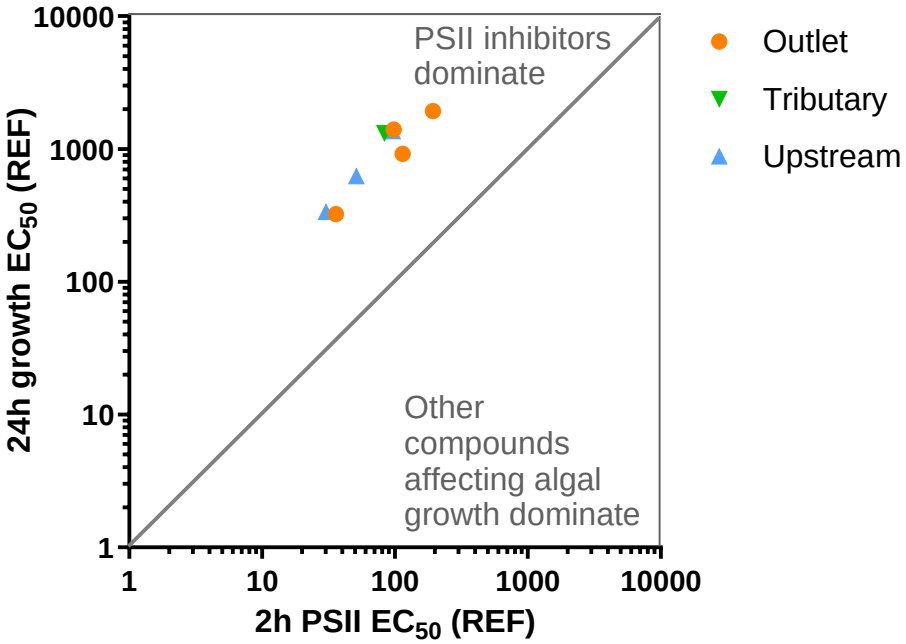


***Fig. 6: Combined algae test: correlation of both endpoints measured in the test: Relationship between $EC_{50}$ values for growth inhibition after 24 h and photosystem II (PSII) inhibition after 2 h in the water samples from the Vuachère watershed.***

*The grey line marks the 1:1 line. REF = relative enrichment factor, $EC_{50}$ (REF) = relative enrichment factor at which a 50 % inhibition of photosystem II or algal growth occurred.*

Results show that PSII inhibitors were more relevant in the samples than other substances affecting algal growth: All samples are clearly above the 1:1 line, i.e. PSII inhibitors dominate in these samples.

Further details on the results can be found in Appendix 3.

## 3.3 Bioassays with native water samples

### 3.3.1 Algae growth inhibition test

#### 3.3.1.1 Validity of the test

Negative controls in the assay met the validity criteria, thus all assays were valid. At test end, the cell number in the controls had increased by more than 16fold compared to the test start. pH in the samples did not vary by more than 1.5 throughout the test and the $EC_{50}$ for the positive control (potassium dichromate) was between 0.25 and 0.8 mg/L (0.73 mg/L tested in April 2022).

#### 3.3.1.2 Evaluation of samples

Fig. 7 provides an overview about results for the algae growth inhibition test.

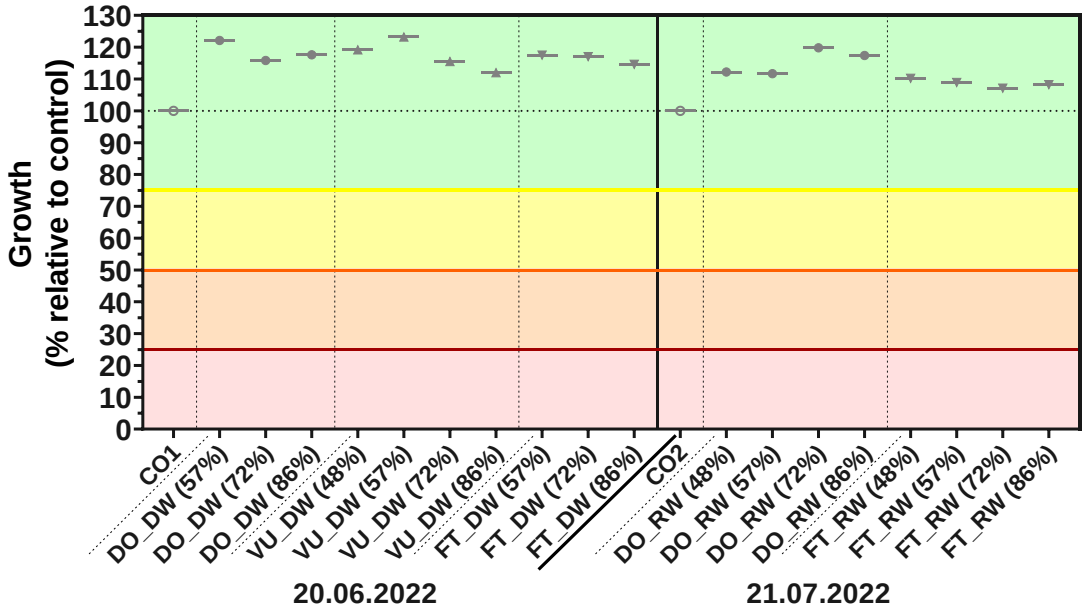


***Fig. 7: Algae growth inhibition test with Raphidocelis subcapitata: growth inhibition after 72 h of exposure to the different samples (shown in % relative to the respective control = CO).***

*Symbols with lines represent the mean growth of R. subcapitata from 3 technical replicates (for CO1 and CO2) and from 2 technical replicates for each sample. Samples were tested in three to four concentrations between 48 to 86%. The shaded areas indicate different toxicity levels (green = not significant, yellow = slight, orange = medium, red = strong). Sampling site and sample type: DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary. Sampling type: DW = Dry weather sample, RW = Rain weather sample.*

All tested samples and sample dilutions induced growth enhancement in the algae. Values ranged from 112 to 123% and no clear decrease of growth enhancement was observed with increasing sample concentration. It has to be noted that the two samples, which showed EBT exceedances in the combined algae test (DO_14_2 and VU_14_2) were not assessed in the algae growth inhibition test.

Further details on the results can be found in Appendix 4.

### 3.3.2 *Lemna minor* growth inhibition test

#### 3.3.2.1 Validity of the test

Negative controls in the assay met the validity criteria, thus all assays were valid. At test end, the frond number in the controls had increased by more than 7fold compared to the test start. pH in the samples did not vary by more than 1.5 throughout the test.

#### 3.3.2.2 Evaluation of samples

Fig. 8 provides an overview about results for the growth inhibition test with *Lemna minor*.

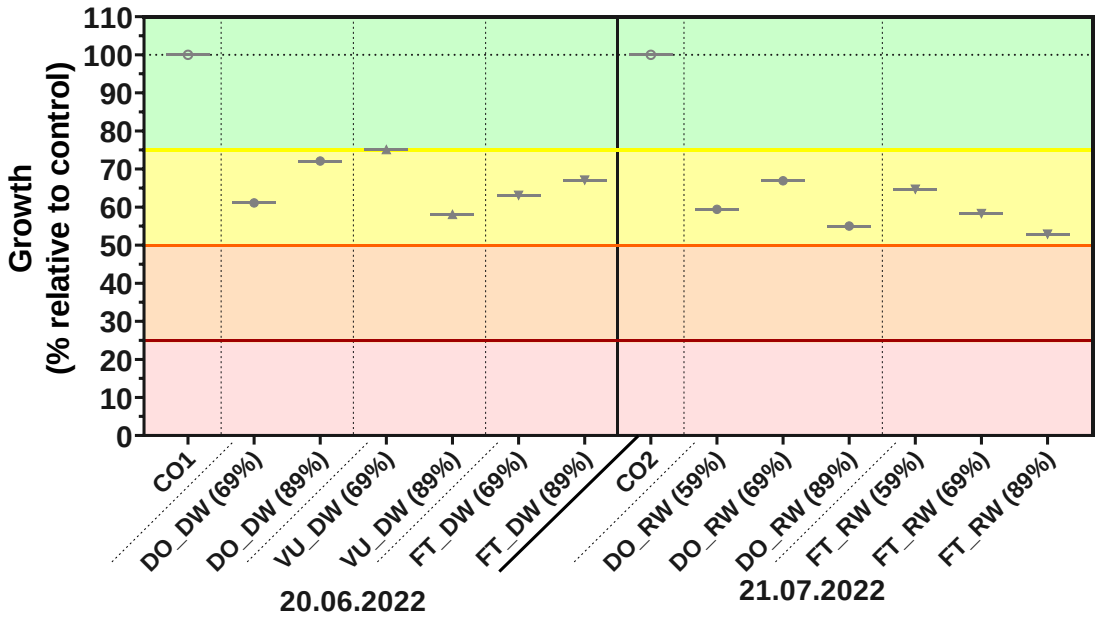


***Fig. 8: Lemna minor growth inhibition test: growth inhibition after 7 d of exposure to the different samples (shown in % relative to the respective control = CO).***

*Symbols with lines represent the mean growth of L. minor from 6 technical replicates (for CO1 and CO2) and the measured growth from 1 technical replicate for each sample. Samples were tested in two to three concentrations (i.e. 59, 69 and 89%). The shaded areas indicate different toxicity levels (green = not significant, yellow = slight, orange = medium, red = strong). Sampling site and sample type: DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary. Sampling type: DW = Dry weather sample, RW = Rain weather sample.*

All tested samples and sample dilutions induced an inhibition of growth in *Lemna minor*. Growth ranged from 53 to 75% in the samples relative to controls (i.e. 100%). Strongest effects were detected in the rain weather samples, where both, the Denantou outlet sample and the Flon tributary sample, inhibited growth by more than 45% in the highest concentration (89%). For the latter one a slight increase in inhibition with increasing sample concentration was observed. The dry weather samples inhibited *Lemna* growth by 28, 33 and 42% in the highest concentration (samples DO_DW, FT_DW and VU_DW, respectively) (see Fig. 8). For VU_DW a clear increase in inhibition with increasing sample concentration could be observed.

Further details on the results can be found in Appendix 5.

### 3.3.3 *Ceriodaphnia dubia* reproduction test

#### 3.3.3.1 Validity of the test

Negative controls met the validity criteria, thus both test series were valid: On day 7, maternal mortality was ≤ 20% and the proportion of males was ≤ 20%; at least 60% of live mothers had produced a minimum of three broods, and the mean number of offspring per live mother was ≥15.

#### 3.3.3.2 Evaluation of samples

Samples did not have a negative effect on maternal survival. Fig. 9 shows the results for reproduction of the tests with 90% sample.

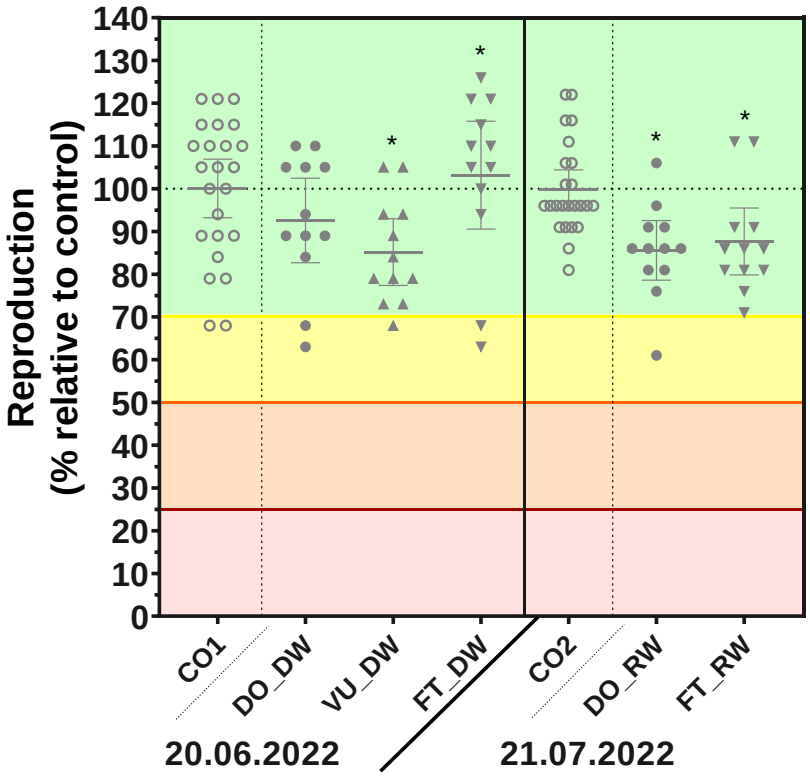


***Fig. 9: Reproduction test with Ceriodaphnia dubia: reproduction after 8 days of exposure to the different samples (shown in % relative to the respective control = CO).***

*Scatter dot plot: the line represents the mean and the error bars the 95% confidence limit. n = 12 replicates of 1 water flea per sample and 24 replicates of 1 water flea per control. *: significant difference to the respective control (one-way ANOVA with Dunnett's multiple comparison test). The shaded areas indicated different toxicity levels (green = not significant, yellow = slight, orange = medium, red = strong). Sampling site and sample type: DO = Denantou outlet, VU = Valmont upstream, FT = Flon tributary. Sampling type: DW = Dry weather sample, RW = Rain weather sample.*

The reproduction of each measurement campaign was compared with the reproduction in the respective control. A significant increase of reproduction could be observed in one sample (Flon tributary, dry weather, FT_DW). This might be caused by an additional availability of nutrients and thus algae as food for the water flea in this sample in comparison to the control. Reproduction was significantly decreased in three samples: Valmont upstream (dry weather, VU_DW), Denantou outlet (rain weather, DO_RW), and Flon tributary (rain weather, FT_RW). No exceedance of thresholds was detected in any sample. Thus, the water samples do not indicate high toxicity for water flea.
Further details on the results can be found in Appendix 6.

### 3.3.4 Fish embryo toxicity test

#### 3.3.4.1 Validity of the test

Two percent mortality was observed in the dilution water control. This can be evaluated as natural background mortality. The positive control exposure to 4 mg/L 3,4-Dichloroaniline showed 100 % mortality after 120 h. In addition, all other validity criteria indicated in OECD 236 were fulfilled (see Tab. 9).

*Tab. 9: FET test acceptance criteria according to OECD 236*

| Test conditions | 0 h -120 h test duration | | Acceptance criteria |
|---|---|---|---|
| Parents fertility rate | 90 | % | [>70 %] |
| pH test chambers | 7.62 – 8.36 | | [6.5-8.5] |
| Oxygen level test chambers | 99.8 – 100 | % | [≥80 %] |
| Temperature test chambers | 25.8 – 26.9 | °C | [26 ±1 °C] |
| Temperature incubator | 26 | °C | [26 ±1 °C] |
| Photoperiod incubator | 14 | h light /day | [12-16h light / day] |
| Survival rate negative control (dilution water) | 98 | % | [>90 %] |
| Mortality rate positive control (3,4-Dichloroaniline 4 mg/L) | 100 | % | [>30 %] |
| Hatching rate negative control (dilution water) | 98 | % | [>80 %] |

#### 3.3.4.2 Evaluation of samples

The tested sample did not have a negative effect on fish embryo development, hatching or survival. All measured values were below the level for significant effects (<10%).

*Mortality:* During the 120 h test, no concentration dependent mortality was observed (0% mortality in 100% water sample at 120 h). The calculated $LC_{50}$ value for 120 h was >100% water sample and the calculated LID value at 120 h for lethal effects was >100% water sample.

*Sub-lethal effects*: The water sample exposure showed no concentration dependent effect on the hatching rate of embryos (delayed hatching or non-hatching). In addition, no concentration dependent sub-lethal effects were observed for the water sample (5% effect in 100% water sample at 120 h). The $EC_{50}$ value at 120 h was > 100% water sample. For the tested water sample, the calculated LID value at 120 h for sub-lethal effects was >100% water sample.

Further details on the results can be found in the test report (aQuaTox-Solutions, 2022).

# 4 Discussion

## 4.1 Effect-based risk assessment using the bioassay results

In the present study, the effect-based risk assessment showed exceedances of EBTs for multiple endpoints, sites and sampling types (Tab. 8). Exceedances were observed in bioassays with both enriched and native water samples. In total, exceedances of the EBTs were detected for eight endpoints from seven different bioassays (Fig. 3). No EBT exceedances were detected for the Cytotox-CALUX®, *Ceriodaphnia* reproduction and mortality and the fish embryo toxicity test. It has to be noted that bioassays with enriched samples were conducted on 11 samples while only five samples (resp. one in the FET assay) were assessed in the bioassays with native samples.

These differences in bioassay responsiveness highlight differences in the pollution profile of the different sites and sampling types. A comparison with risk assessment based on chemical analyses could allow further conclusions to be drawn about compounds potentially responsible for the effects measured in the bioassays. Mixture risk assessment based on data from chemical analysis for plants, invertebrates and vertebrates could be compared with the risk assessment based on bioassay results for the respective organism groups, as was done in Kienle et al. (2023).

Results from CALUX (and algae) may best be compared to two recent studies:

- Kienle et al. (2023) evaluated 15 sites in Switzerland with different land uses (extensive, agricultural, and agricultural-urban). PXR-CALUX® exceeded its respective EBT in all samples from agricultural-urban sites, which corresponds well to the results in the present study. In total, the EBT for this assay was exceeded in 12 of 15 samples. However, Kienle et al. (2023) found fewer EBT exceedances for Nrf2-CALUX® (6 of 15 samples), ER-CALUX® (one of 15 samples) and anti-AR-CALUX® (none of 15 samples). Thus, these three CALUX assays showed less EBT exceedances than in the present study. The EBT for algal growth inhibition was exceeded in seven of 15 samples, whereas the EBT for algae PSII inhibition was exceeded only in two of 15 samples, similar to the present study.
- De Baat et al. (2019) investigated 45 sites with different land use in the Netherlands (reference, urban, WWTP, horticulture, agri mix and complex) using passive sampler extracts, and found that the PAH-, the PXR- and the Nrf2-CALUX® had effects above the LOQ at all sites. The highest number of EBT exceedances (at 70 resp. 65 % of all sites) was found for the ERα- and the PXR- CALUX®. The PAH-CALUX® had exceedances at all 45 sites; however, for this assay a 10times lower EBT was used for assessment than in the present study, so the results are not comparable. If the same EBT had been used as in the current study, exceedances would have occurred at 11 of 45 sites (24 %). This is a lower proportion than in the current study, where the EBT was exceeded in four of 11 samples (36 %). The Cytotox-CALUX® showed effects above the LOQ, but below the EBT in the majority of cases. The same was true for the Nrf2-CALUX®. Overall, the values for this assay were much lower than in the present study. In the follow-up study at 15 sites, including reference sites, horticultural sites and sites influenced by WWTP (De Baat et al., 2020), the ERα-CALUX® and the anti-AR-CALUX® exposed to polar extracts exceeded their respective EBT values at >75% of the sites, and the PXR-CALUX® at >70% of locations. This corresponds well to the results of the present study. The PAH-CALUX®, assessed this time with the same EBT as in the present study, exceeded its EBT in <10% of the samples.

## 4.2 Bioassays with enriched water samples

### 4.2.1 Specific effects measured in CALUX® assays

In the present study two CALUX® assay, the Nrf2-CALUX®, indicating oxidative stress, and the PXR-CALUX®, indicating xenobiotic sensing, exceeded their respective EBTs (10 µg CEQ/L and 5.4 µg NEQ/L) in all samples (Fig. 4):

- Values for Nrf2-CALUX® did not differ significantly between the three sites and ranged from 24 to 87 µg CEQ/L, with the highest value measured in the rain weather sample from Denantou outlet (Tab. 12). These values were higher than those measured by Kienle et al. (2023) and De Baat et al. (2019), where values ranged from 3.4 to 36 µg CEQ/L and 2.5 to 15 µg CEQ/L, respectively. Even lower values were measured in the second study from the Netherlands (De Baat et al., 2020) (<LOQ - 10.1 µg CEQ/L), where the EBT was exceeded at only one of 14 sites.
- In the PXR-CALUX® significantly lower values were measured in the Flon tributary samples than in the Valmont upstream samples (Fig. 4). Also in this assay, the values were higher than in previous studies in Switzerland (Kienle et al., 2023) and in the Netherlands (De Baat et al., 2020) (16 - 74 µg NEQ/L compared to 0.9 – 15.3 µg NEQ/L and 2.4 - 24 µg NEQ/L). The reasons for these differences are not easily explained, as the receptor activated in the PXR-CALUX® is involved in the recognition of xenobiotics and is thus activated by a wider group of chemicals and not only those indicating a specific mode-of-action.

EBT exceedances were also measured in the anti-AR-CALUX® and the ERα-CALUX® (14.4 µg FEQ/L / 0.4 ng EEQ/L), both indicating feminising effects. No significant differences were found between the three sites in either assay (Fig. 4):

- Values in the anti-AR-CALUX® ranged from <LOQ - 21 µg FEQ/L and were in a similar range as in the previous Swiss study (<LOQ - 13 µg FEQ/L (Kienle et al., 2023)), but lower than at several sites in the Netherlands (maximum values: 139 and 252 µg FEQ/L) (De Baat et al., 2019; De Baat et al., 2020). While values in Kienle et al. (2023) remained below the EBT (14.4 µg FEQ/L), the EBT in the current study was exceeding this value in 6 of 11 samples. In addition, it has to be kept in mind that in the current study only one river was assessed compared to several rivers in the other studies.
- Values in the ERα-CALUX® ranged from 0.2 - 0.73 ng EEQ/L, with the highest values measured in dry weather conditions. Values in this range were also measured in samples from 12 Swiss rivers downstream of WWTPs (0.1 - 0.84 ng EEQ/L) (Kienle et al., 2019) and in samples from sites with agricultural-urban impact (0.2 - 0.44 ng EEQ/L) (Kienle et al., 2023). However, they were considerably lower than those measured at several sites in The Netherlands (De Baat et al., 2019; De Baat et al., 2020) (maximum values: 1.59 and 4.92 ng EEQ/L, respectively).

The EBT for the PAH-CALUX® (62.1 ng BaP EQ/L), which indicates effects of polycyclic aromatic hydrocarbons, was exceeded in about 40 % of the samples (four of 11). Again, no significant differences could be found between the three sites (Fig. 4). The measured values were in a similar range as in a previous study in Switzerland (Kienle et al., 2023) (22 - 71 ng BaP EQ/L compared to <LOQ - 72 ng BaP EQ/L). However, in this study only one of 15 samples exceeded the respective EBT (7 %). Partly higher values were measured in the Netherlands (maximum values: 395 ng BaP EQ/L (24 % exceedance, i.e. 11 of 45 samples) (De Baat et al., 2019) and 1430 ng BaP EQ/L (De Baat et al., 2020) (14 % exceedance, i.e. 2 of 14 samples).

### 4.2.2 Algae photosystem II and growth inhibition

EBTs for algae PSII and growth inhibition (70 and 130 ng DEQ/L, respectively) were exceeded at two sites (Denantou outlet and Valmont upstream). Values ranged from 6.5 to 111 ng PSII-DEQ/L and from <LOQ - 169 ng growth-DEQ/L, and are similar or lower than in previous studies in Switzerland:

- *PSII inhibition*: Values at five sites with agricultural-urban land use from 2021 ranged between 15 and 91 ng PSII-DEQ/L, with EBT exceedances measured at two sites (Kienle et al., 2023), which is a similar range as in the present study. In another study (SPEZ 2015 and 2017), where the focus was on the assessment of pesticide impact on five small streams in Switzerland, maximum values ranged from 69 to 272 ng PSII-DEQ/L (Langer et al., 2017) and from 51 to 507 ng PSII-DEQ/L (Junghans et al., 2019). These values were thus at least partly higher than in the present study; however, the different focus of the study should be kept in mind.
- *Growth inhibition*: In the 2021 measurements at five sites with agricultural-urban land use (Kienle et al., 2023), values ranged from 41 to 515 ng growth-DEQ/L. The EBT of 130 ng growth-DEQ/L was exceeded at 7 out of 15 sites (five with extensive, five with agricultural and five with agricultural-urban land use). These values are partly higher than in the present study. Maximum values in the SPEZ 2017 study (Junghans et al., 2019), ranged from 187 to 1721 ng growth-DEQ/L. In general, it has to be kept in mind that this EBT is not as well founded as the EBT for PSII inhibition. It was derived by Escher et al. (2018) based on available environmental quality criteria and effect data from bioassays. The effect of various algal toxicants on the growth of algae was included and the effect values of these substances were integrated into the calculation of the threshold value, taking into account their respective relative potencies in the bioassay (i.e. their effect compared to the reference substance diuron). It should be noted, however, that only data for primarily photosynthesis-inhibiting substances were taken into account and not substances that only inhibit growth and not photosynthesis. Therefore, this value is to be regarded as provisional.

In the past, PSII-DEQ values correlated very well with calculated DEQ values based on the results of chemical analysis of PSII inhibitors (Junghans et al., 2019; Kienle et al., 2019; Langer et al., 2017; Vermeirssen et al., 2010). In the present study, a comparison of the bioassay results with the results of the chemical analysis could also provide information on the compounds responsible for the observed effects, similar to what was done in Neale et al. (2017b) and Kienle et al. (2019).

## 4.3 Bioassays with native water samples

### 4.3.1 Algae growth inhibition

The algal growth inhibition test, conducted as a screening test with a reduced number of concentrations and replicates, showed an increase in growth in all samples and dilutions tested (Fig. 7). This is often observed when testing native samples and can be explained by the additional abundance of nutrients in the samples, which at the same time can also mask potential toxicity of the samples (e.g. (Altenburger et al., 2010)). However, it must be taken into account that a thorough evaluation of the samples would require a larger number of replicates and sample dilutions to allow for a robust statistical evaluation.

### 4.3.2 *Lemna minor* growth inhibition

The test with *Lemna minor*, also performed as a screening test with one replicate and a reduced number of concentrations, showed slight toxicity for all samples with remarkably even results for all samples (Fig. 8). Similar results were observed in other studies with surface water samples in Switzerland (S. Santiago, personal communication and Ferrari et al. (2017)) and Croatia (Radić et al., 2011), while a study with surface water samples in Poland (Kaza et al., 2007)

measured lower inhibition and partial growth promotion. Also for this test, the results provide a first indication of possible effects, but more replicates would have to be considered for a thorough evaluation of the samples. To draw conclusions on compounds that may be relevant for the observed effects, the measured concentrations of metals and pesticides should be taken into account.

### 4.3.3 *Ceriodaphnia dubia* reproduction inhibition

This test led to an increase in reproduction in one sample and to a significant decrease in reproduction in three samples (Fig. 9). However, an exceedance of the toxicity thresholds was not detected in any sample. These results are in line with previous studies in Swiss surface waters (Kienle et al. (2023), Ferrari et al. (2017), Junghans, Langer et al. (unpublished)). In some cases, exceedances of threshold values were found (S. Santiago, personal communication). One reason for the low number of threshold exceedances could be the relatively high threshold for reproductive inhibition of 30 %, which is currently applied following International Organization for Standardization (2019) and Ferrari et al. (2017). It is currently under discussion whether this threshold could and should be reduced to a reproduction inhibition of 20%, as the "minimum statistical difference" when comparing results in samples with reproduction in controls is ≤ 15% in most cases. Furthermore, the coefficient of variation for controls is usually between 8 and 15% (S. Santiago, personal communication). Lowering the toxicity threshold could allow better differentiation between different sites and pollution levels. However, also with a lower i.e. 20% effect threshold, none of the samples in the present study would have exceeded this lower value.

### 4.3.4 Fish embryo toxicity assay

In the present study, no effects were observed in the FET assay for the one evaluated sample.

To date, there is limited experience with this assay and the evaluation of surface water samples. Kienle et al. (2023) found effects in a considerable number of samples with different land use: survival was impaired at three sites with extensive land use, four sites with agricultural land use, and five sites with agricultural-urban land use. Effects on hatching and the development of fish embryos were also found.

# 5 Conclusions

In the present study, exceedances of risk quotients for multiple endpoints and for multiple sites and sampling types with different precipitation conditions during sampling were detected.

- Effect-based risks in sites from Flon tributary were lowest, while highest summed risks were found in the second set of 14d composite samples from Denantou outlet and Valmont upstream.
- Assays for xenobiotic sensing as well as oxidative stress were most responsive.

Bioassays allowed the evaluation of mixtures of pollutants in surface water samples. This is highly relevant as not all substances present can be measured (e.g. commercial products, wastewater with unknown composition). Thus, bioassays provide evidence for toxic effects, both *in vitro* and *in vivo*. Bioassay batteries enable the assessment of water quality. However, some bioassays and most effect-based trigger values still need further validation.

In the present study, the most responsive assays (i.e. those with the highest number of EBT exceedances) were the PXR-CALUX®, the Nrf2-CALUX® and the *Lemna* growth inhibition assay, followed by the PAH-CALUX®, the ERα-CALUX® and the PSII inhibition endpoint of the combined algae assay. Toxicity measured in the Vuachère delta was partly higher than in previous studies (Nrf2 and PXR-CALUX®). For anti-AR, ERα- and PAH-CALUX®, the values were similar to other Swiss rivers. Rivers in the Netherlands showed partly higher values. With regard to algal PSII inhibition, the values in the present study were in a similar range or partly lower than in previous studies in Switzerland. For the parameter algae growth inhibition, the values measured in the present study were also partly lower than in previous Swiss studies.

Overall, the risk assessment based on bioassay results from the present study can provide relevant additional information to a risk assessment based on chemical analysis. To draw further conclusions about potentially relevant compounds for the observed effects, a comparison of risk assessment based on bioassay results with the one from chemical analysis would be beneficial.

The applied bioassay battery could serve as a tool to assess a future improvement of the water quality. For this assessment the following assays with enriched samples can be recommended: PXR-, Nrf2-, PAH-, and ERα-CALUX®, as well as the combined algae assay. In addition, to take into account *in vivo* effects on aquatic organisms, the *Lemna minor* growth inhibition assay and the *Ceriodaphnia dubia* reproduction assay could be included.

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# 7 Glossary

| | |
|---|---|
| CEQ | Curcumine equivalent |
| BaP EQ | Benzo[a]pyrene equivalent |
| DEQ | Diuron equivalent |
| DO | Denantou outlet |
| DW | Dry weather |
| EEQ | 17β-estradiol equivalent |
| FT | Flon tributary |
| FEQ | Flutamide equivalent |
| ISO | International Organisation for Standardisation |
| LOQ | Limit of quantification |
| NEQ | Nicardipine equivalent |
| PSII | Photosystem II |
| PXR | Pregnane X receptor |
| RW | Rain weather |
| RQ | Risk quotient |
| TEQ | Tributyltin acetate equivalent |
| VU | Valmont upstream |

# 8 Indices

## 8.1 List of Figures



## 8.2 List of Tables

# Appendix 1 Background information on sample preparation

***Tab. 10: Solid phase extraction for bioassays.***

| | |
|---|---|
| ***General information*** | |
| *Sample type* | *Water samples* |
| *Sample volume* | *1500 mL surface water* |
| *Blank sample* | *1500 mL ultrapure water* |
| ***Sample preparation*** | |
| *Filtration* | *Glas fiber filter type APFD 09050 (2.7 µm) (Millipore)* |
| *Acidification* | *With HCl to pH 7.2* |
| ***Sample preparation*** | |
| *Enrichment* | *Solid Phase Extraction (SPE)* |
| *SPE cartridges* | *Strata-XL (100 µm Polymeric Reversed Phase, 500 mg / 6 mL) (Phenomenex: 8B-S043-HCH)* |
| *Conditioning* | *5 mL acetone*<br>*5 mL methanol*<br>*5 mL ultrapure water*<br>*5 mL ultrapure water* |
| *Elution* | *2 mL acetone*<br>*2 mL methanol*<br>*3 mL acetone* |
| *Concentration* | *Under vacuum to approx. 500 – 800 µL, then adding up to 1000 µL with ethanol* |
| *Enrichment factor* | *1500-fold* |
| *Storage* | *In the dark, at -20°C* |

# Appendix 2 Effect-based risk quotients – 3 color scale

***Tab. 11: Overview on effect-based risk assessment results for all bioassays.***

Numbers show effect-based risk quotients marked in a 3-color scale from blue (0.0001) over yellow (1) to red (≥ 10). White cells indicate that the respective bioassay was not applied at this site. 14 = 14 days composite sample, DW = dry weather sample, RW = rain weather sample. * For calculating $\sum RQ_{bio}$ negative values were set to zero.

| | Sampling site | Denantou (D) | | | | Valmont (V) | | | Flon (F) | | | | Field |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample Code | DO_14_1 | DO_14_2 | DO_DW | DO_RW | VU_14_1 | VU_14_2 | VU_DW | FT_14_1 | FT_14_2 | FT_DW | FT_RW | blank |
| | Type | Outlet (O) | | | | Upstream (U) | | | Tributary (T) | | | | FB |
| ***Bioassays with enriched samples*** | ***Effect*** $\sum RQ_{bio}$ | 15.8 | 20.9 | 11.4 | 23.1 | 17.6 | 23.2 | 12.6 | 10.1 | 13.3 | 9.2 | 10.8 | 0.0 |
| Cytotox CALUX® | Cytotoxicity | 0.0 | 0.4 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| ER-CALUX® | Estrogenic activity | 0.6 | 0.8 | 1.6 | 1.3 | 1.0 | 0.5 | 1.8 | 0.9 | 0.5 | 1.5 | 1.5 | 0.0 |
| Anti-AR-CALUX® | Anti-androgenic activity | 1.3 | 1.5 | 0.7 | 1.0 | 1.0 | 1.2 | 1.2 | 0.0 | 0.8 | 0.9 | 1.0 | 0.0 |
| Nrf2-CALUX® | Oxidative stress | 3.2 | 4.1 | 3.0 | 8.7 | 2.9 | 4.1 | 3.3 | 3.8 | 6.2 | 2.4 | 4.6 | 0.0 |
| PXR-CALUX® | Pollutant metabolism | 9.1 | 10.6 | 5.2 | 10.0 | 10.2 | 13.7 | 5.2 | 4.6 | 3.9 | 3.9 | 3.0 | 0.0 |
| PAH-CALUX® | | 1.0 | 1.1 | 0.5 | 0.8 | 1.0 | 1.1 | 0.4 | 0.6 | 1.0 | 0.4 | 0.6 | 0.0 |
| Combined algae assay | PSII inhibition | 0.4 | 1.1 | 0.2 | 0.4 | 0.9 | 1.6 | 0.4 | 0.2 | 0.6 | 0.1 | 0.1 | 0.0 |
| | Growth inhibition | 0.3 | 1.3 | 0.2 | 0.5 | 0.7 | 1.0 | 0.3 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 |
| ***Bioassays with native samples*** | $\sum RQ_{bio}$ | 0.0 | 0.0 | 1.5 | 2.3 | 0.0 | 0.0 | 1.8 | 0.0 | 0.0 | 1.7 | 2.3 | 0.0 |
| Algae growth inhibition assay | Growth inhibition | | | -0.7 | -0.7 | | | -0.5 | | | -0.6 | -0.3 | |
| Lemna growth inhibition assay | Growth inhibition | | | 1.1 | 1.8 | | | 1.3 | | | 1.7 | 1.9 | |
| Ceriodaphnia reproduction assay | Reproduction | | | 0.2 | 0.5 | | | 0.5 | | | -0.1 | 0.4 | |
| | Mortality | | | 0.0 | 0.0 | | | 0.0 | | | 0.0 | 0.0 | |
| Fish embryo toxicity test | Mortality | | | 0.0 | | | | | | | | | |
| | Hatching | | | 0.0 | | | | | | | | | |
| | Sublethal effects | | | 0.2 | | | | | | | | | |

# Appendix 3 Test results for the CALUX® panel and the combined algae test

***Tab. 12: Results of the CALUX® panel.***

*TEQ = Tributyltin acetate equivalent, EEQ = 17β-estradiol equivalent, FEQ = flutamide equivalent, CEQ = curcumine equivalent, NEQ = nicardipine equivalent, BaP EQ = Benzo[a]pyrene equivalent, LOQ = limit of quantification, in green: values < effect-based trigger value, in red: values ≥ effect-based trigger value*

| | | | | | | Cytotox-CALUX® | | ERα-CALUX® | | Anti-AR-CALUX® | | Nrf2-CALUX® | | PXR-CALUX® | | PAH-CALUX® | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cluster** | **Sampling site** | **Sample type** | **Sampling type** | **Sample Code** | **Sampling Date** | **TEQ (µg/L)** | **LOQ** | **EEQ (ng/L)** | **LOQ** | **FEQ (µg/L)** | **LOQ** | **CEQ (µg/L)** | **LOQ** | **NEQ (µg/L)** | **LOQ** | **BaP EQ (ng/L)** | **LOQ** |
| 1 | Denantou | outlet | 14d | DO_14_1 | 20.06.2022 | < LOQ | 0.38 | 0.23 | 0.056 | 18 | 5.9 | 32 | 5.93 | 49 | 4.2 | 62 | 0.82 |
| 1 | Valmont | upstream | 14d | VU_14_1 | 20.06.2022 | < LOQ | 0.35 | 0.38 | 0.063 | 14 | 5.7 | 29 | 5.93 | 55 | 3.6 | 65 | 0.68 |
| 1 | Flon | tributary | 14d | FT_14_1 | 20.06.2022 | < LOQ | 0.35 | 0.34 | 0.062 | < LOQ | 5.8 | 38 | 5.93 | 25 | 3.6 | 39 | 0.68 |
| 1 | Denantou | outlet | DW | DO_DW | 20.06.2022 | < LOQ | 0.33 | 0.63 | 0.043 | 10 | 4.5 | 30 | 5.93 | 28 | 4.1 | 33 | 0.65 |
| 1 | Valmont | upstream | DW | VU_DW | 20.06.2022 | < LOQ | 0.33 | 0.73 | 0.043 | 17 | 4.6 | 33 | 5.93 | 28 | 4 | 22 | 0.63 |
| 1 | Flon | tributary | DW | FT_DW | 20.06.2022 | < LOQ | 0.37 | 0.61 | 0.05 | 13 | 7.1 | 24 | 5.93 | 21 | 4.1 | 23 | 0.59 |
| 2 | Denantou | outlet | 14d | DO_14_2 | 05.07.2022 | 0.38 | 0.37 | 0.32 | 0.049 | 21 | 6.7 | 41 | 17.8 | 57 | 4.2 | 70 | 0.59 |
| 2 | Valmont | upstream | 14d | VU_14_2 | 05.07.2022 | < LOQ | 0.36 | 0.2 | 0.048 | 17 | 4.7 | 41 | 5.93 | 74 | 4.1 | 71 | 0.69 |
| 2 | Flon | tributary | 14d | FT_14_2 | 05.07.2022 | < LOQ | 0.36 | 0.21 | 0.048 | 11 | 4.6 | 62 | 5.93 | 21 | 4.1 | 65 | 0.7 |
| 3 | Denantou | outlet | RW | DO_RW | 21.07.2022 | 0.4 | 0.36 | 0.53 | 0.05 | 15 | 5.9 | 87 | 17.8 | 54 | 3.9 | 47 | 1.1 |
| 3 | Flon | tributary | RW | FT_RW | 21.07.2022 | < LOQ | 0.36 | 0.59 | 0.051 | 15 | 5.6 | 46 | 5.93 | 16 | 3.9 | 36 | 1.1 |
| 2 | Field Blank | Blank | FB | FB | 05.07.2022 | < LOQ | 0.35 | < LOQ | 0.049 | < LOQ | 5.6 | < LOQ | 5.93 | < LOQ | 3.8 | < LOQ | 0.99 |
| 1,2,3 | | SPE blanc | | SPE blanc_ges | | < LOQ | 0.25 | < LOQ | 0.037 | < LOQ | 4 | < LOQ | 4 | < LOQ | 2.8 | < LOQ | 0.54 |
| EBT | | | | | | | | 0.4 | | 14.4 | | 10 | | 5.4 | | 62.1 | |

***Tab. 13: Results of the combined algae test***

*PSII-DEQ = Diuron equivalent concentration for PSII inhibition, Growth-DEQ = Diuron equivalent concentration for growth inhibition, LOQ = limit of quantification, $EC_{50}$ REF = $EC_{50}$ value in relative enrichment factors*

| Cluster | Sampling site | Sample type | Sampling type | Sample Code | Sampling Date | 2h PSII-$DEQ_{bio}$ (ng/L) | LOQ | $EC_{50}$ REF | 24h Growth-$DEQ_{bio}$ (ng/L) | LOQ | $EC_{50}$ REF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Denantou | outlet | 14d | DO_14_1 | 20.06.2022 | 31.4 | 1.0 | 98.0 | 38.3 | 22.3 | 1397.7 |
| 1 | Valmont | upstream | 14d | VU_14_1 | 20.06.2022 | 60.0 | 1.0 | 51.3 | 86.0 | 22.3 | 623.3 |
| 1 | Flon | tributary | 14d | FT_14_1 | 20.06.2022 | 16.1 | 1.0 | 191.5 | < LOQ | 22.3 | |
| 1 | Denantou | outlet | DW | DO_DW | 20.06.2022 | 14.8 | 1.3 | 192.7 | 28.3 | 22.7 | 1928.6 |
| 1 | Valmont | upstream | DW | VU_DW | 20.06.2022 | 29.5 | 1.3 | 96.3 | 40.0 | 22.7 | 1361.3 |
| 1 | Flon | tributary | DW | FT_DW | 20.06.2022 | 7.6 | 1.3 | 376.1 | < LOQ | 22.7 | |
| 2 | Denantou | outlet | 14d | DO_14_2 | 05.07.2022 | 79.2 | 1.3 | 35.9 | 168.9 | 22.7 | 322.7 |
| 2 | Valmont | upstream | 14d | VU_14_2 | 05.07.2022 | 110.7 | 1.4 | 30.3 | 131.3 | 18.4 | 335.8 |
| 2 | Flon | tributary | 14d | FT_14_2 | 05.07.2022 | 40.2 | 1.4 | 83.5 | 33.5 | 18.4 | 1317.0 |
| 3 | Denantou | outlet | RW | DO_RW | 21.07.2022 | 29.5 | 1.3 | 114.3 | 60.4 | 23.2 | 921.4 |
| 3 | Flon | tributary | RW | FT_RW | 21.07.2022 | 6.5 | 1.3 | 516.7 | < LOQ | 23.2 | |
| 2 | Field Blank | Blank | FB | FB | 05.07.2022 | < LOQ | 1.4 | | < LOQ | 18.4 | |
| 1 | | SPE blanc | | SPE blanc 1 | | < LOQ | 1.0 | | < LOQ | 22.3 | |
| 2 | | SPE blanc | | SPE blanc 2 | | < LOQ | 1.4 | | < LOQ | 18.4 | |
| 3 | | SPE blanc | | SPE blanc 3 | | < LOQ | 1.3 | | < LOQ | 23.2 | |

# Appendix 4 Test reports for algae growth inhibition test

**Soluval Santiago**
*Analyses environnementales*
Rue Edouard-Dubied 2 Tél: 032 863 43 60
CH - 2108 COUVET e-mail: ssantiago@bluewin.ch

***Bioessais de toxicité***
***Récapitulation des résultats***

**Identification**

Origine : *Eau Service de Ville de Lausanne (VD)*
Type d'échantillon : *Eaux de surface ; La Vuachère*
Echantillonnage : ☐ *instantané* ☑ *composite*
*1ère campagne* *2ème camp.*
Dates : *20 - 06 - 2022*
Echantillons n$^{os}$ : *A. 1.2 DEN*
*B. 2.2 FLO*
*C. 3.2 VAL*

*Remarques :*

Destinataire : *M. Vincent GREGORIO*
Société : *Service de l'Eau - Lausanne*
Addresse : *CH - 1095 Lutry*
Plan d'analyse(s) : *Ceriodaphnia ;*
*(complément Algues vertes; Lemna minor)*
Dates de réception : *21-06-2022 /*
Enregistrements n° : *8860- 01 et xx*
Responsable : *S. Santiago*

***Algues vertes***
*Raphidocelis subcapitata*
(selon AFNOR T90-375)

Conduite de l'essai
Organisme : *R. subcapitata (S. capricornutum)* UTEX1648 Date : 30-06-2022
Microplaque (2ml); 2 répliques; 23±2°C; 5 Klux; 0 t/m
Dilution : milieu AAP (*USEPA*); Densité optique à 680 nm Effectué par : SS

Densité cellulaire initiale = 1.00E+04

| Echantillon n° / Concentration | | Croissance des algues – Densité optique à 72 h. ($DO_{680}$) Moyenne | coef. var.% | Croissance (%) | Densité optique à 96 h. ($DO_{680}$) Moyenne | Ecart-type | Croissance (%) |
|---|---|---|---|---|---|---|---|
| Contrôles (moyenne; n= 8) | | 0.267 | 5.1% | **100%** | | | |
| *A. 1.2 DEN* *20-06-2022* | 85.9% | 0.314 | 9.0% | **117.6%** | | | |
| | 71.6% | 0.309 | 5.4% | **115.8%** | | | |
| | 57.3% | 0.326 | 4.2% | **122.1%** | | | |
| *B. 2.2 FLO* *20-06-2022* | 85.9% | 0.306 | 14.7% | **114.6%** | | | |
| | 71.6% | 0.312 | 2.9% | **117.0%** | | | |
| | 57.3% | 0.313 | 4.7% | **117.5%** | | | |
| *C. 3.2 VAL* *20-06-2022* | 85.9% | 0.299 | 2.9% | **112.0%** | | | |
| | 71.6% | 0.308 | 0.3% | **115.5%** | | | |
| | 57.3% | 0.329 | 4.2% | **123.2%** | | | |
| | 47.7% | 0.318 | 3.2% | **119.2%** | | | |

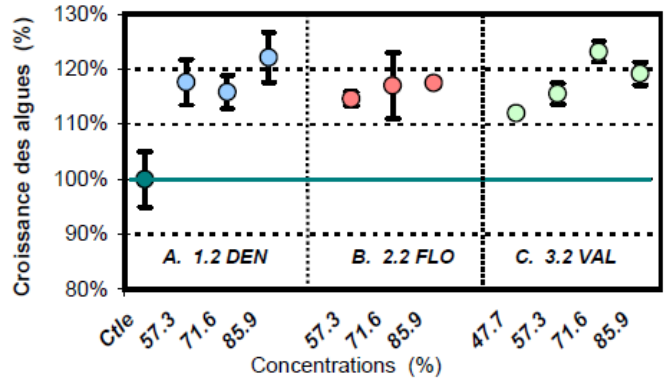
Remarques : *avec ajout de nutriments = P, N, oligoéléments + EDTA (concentrations identiques aux contrôles - milieu USEPA)*

**Conclusions - Commentaires**

*A. 1.2 DEN (22-06-22)* ☛ *Non toxique*
*B. 2.2 FLO (22-06-22) :* ☛ *Non toxique*
*C. 3.2 VAL (22-06-22) :* ☛ *Non toxique*

Essai valide ☑ oui - ☐ non
Contrôle : $N_{fin} \geq 16 \times N_{init.}$ ☑
Variation pH ≤ 1,5 ☑

Réf. $K_2Cr_2O_7$ [0,25 - 0,80 mg/l]
Date : 07-04-2022 ☑
$CE_{50b}$-72h. = 0,73 mg/l [0,67 - 0,77]




Couvet, 08-07-2022

S. Santiago

2 /

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## *Bioessais de toxicité*
### *Récapitulation des résultats*

**Identification**

Origine : ***Eau Service de Ville de Lausanne (VD)***
Type d'échantillon : ***Eaux de surface ; La Vuachère***
Echantillonnage ❑ *instantané* ☑ *composite*

| | *1ère campagne* | *2ème campagne* |
|---|---|---|
| Dates : | ***20 - 06 - 2022*** | ***21 - 07 - 2022*** |
| Echantillons n$^{os}$ | ***A. 1.2 DEN*** | ***D. 1.4 DEN*** |
| | ***B. 2.2 FLO*** | ***E. 2.4 FLO*** |
| | ***C. 3.2 VAL*** | |

Destinataire : ***M. Vincent GREGORIO***
Société : ***Service de l'Eau - Lausanne***
Addresse : ***CH - 1095 Lutry***
Plan d'analyse(s) : ***Ceriodaphnia ;***
*(complément Algues vertes; Lemna minor)*
Dates de réception : ***21-06 / 21-07-2022***
Enregistrements n° : ***8860-01 et -02***
Responsable : ***S. Santiago***

*Remarques :*

***Algues vertes***
*Raphidocelis subcapitata*
(selon AFNOR T90-375)

Conduite de l'essai
Organisme : *R. subcapitata (S. capricornutum)* UTEX1648 Date : 02-08-2022
Microplaque (2ml); 2 répliques; 23±2°C; 5 Klux; 0 t/m
Dilution : milieu AAP (*USEPA);* Densité optique à 680 nm Effectué par : SS

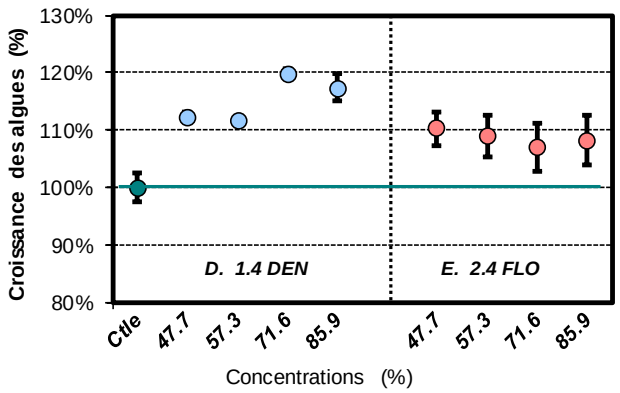
Croissance des algues — Densité cellulaire initiale = 1.00E+04

| Echantillon n° / Concentration | Densité optique à 72 h. ($DO_{680}$) Moyenne | coef. var.% | **Croissance (%)** | Densité optique à 96 h. ($DO_{680}$) Moyenne | Ecart-type | Croissance (%) |
|---|---|---|---|---|---|---|
| Contrôles (moyenne; n= 8) | 0.279 | 2.4% | **100%** | | | |
| ***D. 1.4 DEN*** *21-07-2022* 85.9% | 0.328 | 2.5% | **117.4%** | | | |
| 71.6% | 0.335 | 0.8% | **119.8%** | | | |
| 57.3% | 0.312 | 0.3% | **111.7%** | | | |
| 47.7% | 0.314 | 0.8% | **112.2%** | | | |
| ***E. 2.4 FLO*** *21-07-2022* 85.9% | 0.302 | 4.3% | **108.2%** | | | |
| 71.6% | 0.299 | 4.2% | **107.1%** | | | |
| 57.3% | 0.304 | 3.6% | **108.9%** | | | |
| 47.7% | 0.308 | 2.8% | **110.2%** | | | |
| | | | | | | |

Remarques : *avec ajout de nutriments = P, N, oligoéléments + EDTA (concentrations identiques aux contrôles - milieu USEPA)*

**Conclusions - Commentaires**

***D. 1.4 DEN*** *(21-07-22)* ☛ ***Non toxique***
***E. 2.4 FLO*** *(21-07-22)* ☛ ***Non toxique***




Essai valide ☑ oui - ❑ non
Contrôle : $N_{fin} \geq 16 \times N_{init.}$ ☑
Variation pH ≤ 1,5 ☑

Réf. $K_2Cr_2O_7$ [0,25 - 0,80 mg/l]
Date : 07-04-2022 ☑
$CE_{50b}$-72h. = 0,73 mg/l [0,67 - 0,77]

Couvet, 06-08-2022

S. Santiago

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# Appendix 5 Test reports for *Lemna minor* growth inhibition test

**Soluval Santiago**
Analyses environnementales
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***Bioessais de toxicité***
***Récapitulation des résultats***

**Identification**

Origine : *Eau Service de Ville de Lausanne (VD)*
Type d'échantillon : *Eaux de surface ; La Vuachère*
Echantillonnage : ☐ *instantané* ☑ *composite*
*1ère campagne* *2ème camp.*
Dates : *20 - 06 - 2022*
Echantillons n°s : *A. 1.2 DEN*
*B. 2.2 FLO*
*C. 3.2 VAL*

Destinataire : *M. Vincent GREGORIO*
Société : *Service de l'Eau - Lausanne*
Addresse : *CH - 1095 Lutry*
Plan d'analyse(s) : *Ceriodaphnia ;*
*(complément Algues vertes; Lemna minor)*
Dates de réception : *21-06-2022 /*
Enregistrements n°: *8860- 01 et xx*
Responsable : *S. Santiago*

*Remarques : Mode screening (nombre réduit de concentrations testées et de réplques)*

***Macrophytes***
*Lemna minor*
(selon OCDE 221; ISO 20079)

Conduite de l'essai
Organisme : *Lemna minor* (Ökotox. Inst., Stuttgart; via *IFAF*) Date : 01-07-2022
Bécher (80 ml); 1 réplique; 24±2°C; 5 Klux;
Dilution : OCDE 221 (milieu *SIS* modifié) Effectué par : SS

Croissance des macrophytes

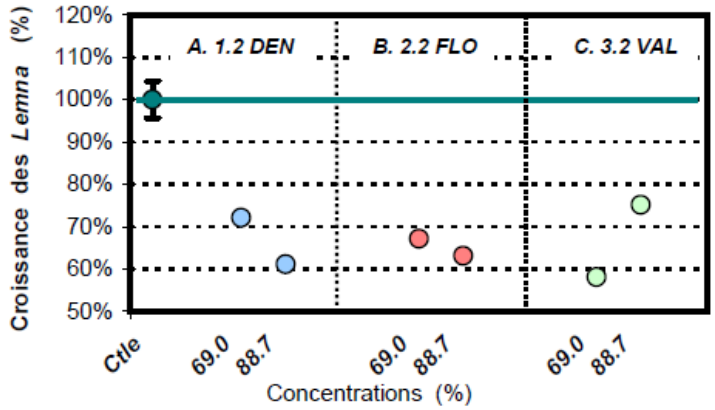
| Echantillon n° / Concentration | | Nombre de fronds Répl.a | b | c | d | e | f | Moyenne [ - $N_{init.}$] | N initial = 12 coef. var.% | Croissance (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Contrôles | | 114 | 111 | 110 | 106 | 119 | 111 | 99.8 | 4.4% | **100%** |
| ***A. 1.2 DEN*** | 88.7% | 84 | | | | | | 72.0 | | **72.1%** |
| *20-06-2022* | 69.0% | 73 | | | | | | 61.0 | | **61.1%** |
| ***B. 2.2 FLO*** | 88.7% | 79 | | | | | | 67.0 | | **67.1%** |
| *20-06-2022* | 69.0% | 75 | | | | | | 63.0 | | **63.1%** |
| ***C. 3.2 VAL*** | 88.7% | 70 | | | | | | 58.0 | | **58.1%** |
| *20-06-2022* | 69.0% | 87 | | | | | | 75.0 | | **75.1%** |

Remarque : *avec ajout de nutriments = P, N, oligoéléments + EDTA (concentrations identiques au contrôle - milieu SIS modifié)*

**Conclusions - Commentaires**

*A. 1.2 DEN (22-06-22) :* *Peu toxique* ($CE_{20}$ < 70%)
*B. 2.2 FLO (22-06-22) :* *Peu toxique* ($CE_{20}$ < 70%)
*C. 3.2 VAL (22-06-22) :* *Peu toxique* ($CE_{20}$ < 70%)

Essai valide ☑ oui - ☐ non
Contrôle : $N_{final} \geq 7 \times N_{initial}$ ☑
Variation pH ≤ 1,5 ☑




Couvet, 08-07-2022
S. Santiago
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**Soluval Santiago**
*Analyses environnementales*
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## *Bioessais de toxicité*
### *Récapitulation des résultats*

**Identification**

Origine : ***Eau Service de Ville de Lausanne (VD)***
Type d'échantillon : ***Eaux de surface ; La Vuachère***
Echantillonnage ❑ *instantané* ☑ *composite*

| | *1ère campagne* | *2ème campagne* |
|---|---|---|
| Dates : | ***20 - 06 - 2022*** | ***21 - 07 - 2022*** |
| Echantillons n$^{os}$ | ***A. 1.2 DEN*** | ***D. 1.4 DEN*** |
| | ***B. 2.2 FLO*** | ***E. 2.4 FLO*** |
| | ***C. 3.2 VAL*** | |

Destinataire : ***M. Vincent GREGORIO***
Société : ***Service de l'Eau - Lausanne***
Addresse : ***CH - 1095 Lutry***
Plan d'analyse(s) : ***Ceriodaphnia ;***
*(complément Algues vertes; Lemna minor)*
Dates de réception : ***21-06 / 21-07-2022***
Enregistrements n° : ***8860-01 et -02***
Responsable : ***S. Santiago***

*Remarques : Mode screening (nombre réduit de concentrations testées et de réplques)*

***Macrophytes***
*Lemna minor*
(selon OCDE 221; ISO 20079)

Conduite de l'essai
Organisme : *Lemna minor* (Ökotox. Inst., Stuttgart; via *IFAF*) Date : 03-08-2022
Bécher (80 ml); 1 réplique; 24 ± 2°C; 5 Klux;
Dilution : OCDE 221 (milieu *SIS* modifié) Effectué par : SS

Croissance des macrophytes

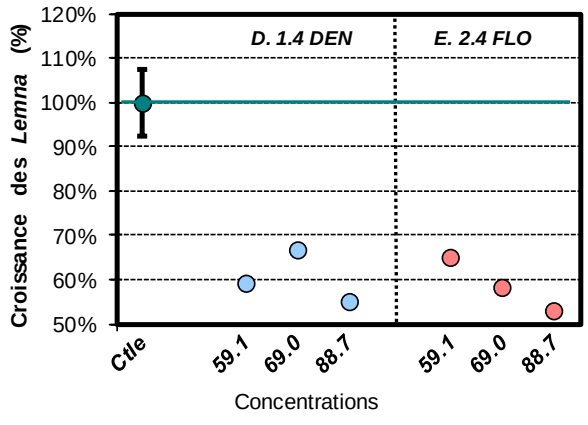
| Echantillon n° / Concentration | | Nombre de fronds Répl.a | b | c | d | e | f | Moyenne [ - $N_{init.}$] | N initial = 12 coef. var.% | **Croissance (%)** |
|---|---|---|---|---|---|---|---|---|---|---|
| Contrôles | | 114 | 94 | 106 | 113 | 102 | 99 | 92.7 | 7.5% | **100%** |
| ***D. 1.4 DEN*** | 88.7% | 63 | | | | | | 51.0 | | **55.0%** |
| *21-07-2022* | 69.0% | 74 | | | | | | 62.0 | | **66.9%** |
| | 59.1% | 67 | | | | | | 55.0 | | **59.4%** |
| ***E. 2.4 FLO*** | 88.7% | 61 | | | | | | 49.0 | | **52.9%** |
| *21-07-2022* | 69.0% | 66 | | | | | | 54.0 | | **58.3%** |
| | 59.1% | 72 | | | | | | 60.0 | | **64.7%** |
| | | | | | | | | | | |

Remarque : *avec ajout de nutriments = P, N, oligoéléments + EDTA (concentrations identiques au contrôle - milieu SIS modifié)*

**Conclusions - Commentaires**

***D. 1.4 DEN*** *(21-07-22)* **:** ☛ ***Peu toxique*** ($CE_{20}$ < 50%)
***E. 2.4 FLO*** *(21-07-22)* **:** ☛ ***Peu toxique*** ($CE_{20}$ < 50%; $CE_{50}$ ≈ 100% )

Essai valide ☑ oui - ❑ non
Contrôle : $N_{final} \geq 7 \times N_{initial}$ ☑
Variation pH ≤ 1,5 ☑




Couvet, 11-08-2022

S. Santiago

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# Appendix 6 Test reports for reproduction test with *Ceriodaphnia dubia*

**Soluval Santiago**
*Analyses environnementales*
Rue Edouard-Dubied 2 Tél: 032 863 43 60
CH - 2108 COUVET e-mail: ssantiago@bluewin.ch

***Bioassays of toxicity***
***Summary of results***

**Identification**

Origine : *Eau Service de Ville de Lausanne (VD)*
Type d'échantillon : *Eaux de surface ; La Vuachère*
Echantillonnage : ❑ *instantané* ☑ *composite*

| | 1ère campagne | 2ème campagne |
|---|---|---|
| Dates : | *20 - 06 - 2022* | *21 - 07 - 2022* |
| Echantillons n°s : | *A. 1.2 DEN* | *D. 1.4 DEN* |
| | *B. 2.2 FLO* | *E. 2.4 FLO* |
| | *C. 3.2 VAL* | |

*Remarques : Mode screening (nombre réduit de concentrations testées)*

Destinataire : *M. Vincent GREGORIO*
Société : *Service de l'Eau - Lausanne*
Addresse : *CH - 1095 Lutry*
Plan d'analyse(s) : *Ceriodaphnia ; (complément Algues vertes; Lemna minor)*
Dates de réception : *21-06 / 21-07-2022*
Enregistrements n° : *8860- 01 et -02*
Responsable : *S. Santiago*

***Ceriodaphnia dubia***
(ISO 20665 ; AFNOR T90-376 ; Environnement Canada SPE 1/RM/21)

Organisme : *Ceriodaphnia dubia (IFAF-Cemagref)*
PP béchers (25 ml); 25±1°C; 0,4±0,1 Klux (photopér.16h:8h)
Dilution : milieu AFNOR T90-376 modifié; nour. *A, Y, xt.T*

Dates : 21-06 / 22-07-22
Effectué par : SS
Contrôlé par :

Toxicité chronique : inhibition de la croissance de population à 7 jours

| Echantillon n° / Concentration | | Mortalité à 7 jours | Nombre de néonés ; ♀ = mère morte ; ⊙ = + œuf non éclos — Réplicats | | | | Σ néon. | Moyenne | Ec.-type | Croissance (%) | Inhibition (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Contrôles 01 (milieu synthétique = milieu de dilution) 21-06-2022 | | 0 / 24 = 0 % | 16 21 19 20 13 22 | 20 21 17 20 23 18 | 19 15 23 17 21 13 | 22 17 21 23 15 22 | *458* | **19.1** | 3.1 = 16.2% | **100%** | **0%** |
| *A. 1.2 DEN* *20-06-2022* | 90.0% | 0 / 12 = 0 % | 21 16 12 | 17 20 18 | 20 17 21 | 13 20 17 | *212* | **17.7** | 3.0 | **92.6%** | **7.4%** |
| *B. 2.2 FLO* *20-06-2022* | 90.0% | 0 / 12 = 0 % | 18 13 23 | 20 24 19 | 12 21 20 | 22 21 23 | *236* | **19.7** | 3.8 | **103.1%** | **-3.1%** |
| *C. 3.2 VAL* *20-06-2022* | 90.0% | 0 / 12 = 0 % | 14 17 18 | 15 20 13 | 18 16 15 | 20 15 14 | *195* | **16.3** | 2.3 | **85.2%** | **14.8%** |
| Contrôles 02 (milieu synthétique = milieu de dilution) 22-07-2022 | | 0 / 24 = 0 % | 18 24 19 16 21 19 | 17 19 22 18 21 19 | 23 19 18 19 23 18 | 20 19 24 19 20 19 | *474* | **19.8** | 2.1 = 10.8% | **100%** | **0%** |
| *D. 1.4 DEN* *21-07-2022* | 90.0% | 0 / 12 = 0 % | 21 18 17 | 15 17 16 | 18 12 17 | 17 19 16 | *203* | **16.9** | 2.2 | **88.6%** | **11.4%** |
| *E. 2.4 FLO* *21-07-2022* | 90.0% | 0 / 12 = 0 % | 16 22 17 | 14 18 16 | 22 17 18 | 15 17 16 | *208* | **17.3** | 2.5 | **90.8%** | **9.2%** |

Remarks : *pH : contrôles* = 7,5 ; *A* = 7,7 ; *B* = 7,8 ; *C* = 7,8 ; D = 7,6 ; E = 7,8.
conductivité électrique [µS/cm] : *contrôle* = 305 ; *A* = 505 ; *B* = 475 ; *C* = 535 ; D = 560 ; E = 545 µS/cm.

**Conclusions - Commentaires**

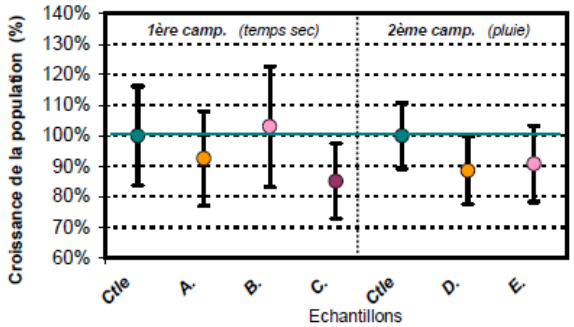
*1ère campagne (temps sec; 20-06-2022) :*
*A. 1.2 DEN (20-06-22) :* ☛ *Non toxique*
*Aucune mortalité ; pas d'inhibition significative de la reproduction*
*B. 2.2 FLO (20-06-22) :* ☛ *Non toxique*
*Aucune mortalité ; aucune inhibition de la reproduction à 7 jours*
*C. 3.2 VAL (20-06-22) :* ☛ *Peu toxique*
*Aucune mortalité ; inhibition de la reproduction statistiquement significative*

*2ème campagne (pluie; 21-07-2022) :*
*D. 1.4 DEN (21-07-22) :* ☛ *Peu toxique*
*Aucune mortalité ; inhibition de la reproduction statistiquement significative*
*E. 2.4 FLO (21-07-22) :* ☛ *Peu toxique*
*Aucune mortalité ; inhibition de la reproduction statistiquement significative*

*MSD = Minimum statistical difference*
*(% d'inhibition par rapport au contrôle) :*
*1ère camp.* = 12,5 % ; 2ème camp. = 8,1 %

Essais valides ☑ oui - ❑ non

Contrôles (à 7 jours) :
Mortalité des mères ≤ 20% ☑
Proportion de mâles ≤ 20% ☑
Min. 3 portées pour ≥ 60% de mères survivantes ☑
Moyenne de néonés par mère survivante ≥ 15 ☑




S. Santiago

Couvet, 04-08-2022

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