Section 3

Assessment of the Eco-Toxicological Effects of Disinfection Processes on the Seine River

Chapter 1

Description of the biological models used to monitor water quality

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1.1 DESCRIPTION OF THE BIOLOGICAL PANELS USED TO ASSESS GENERAL TOXICITY

The biological models used in this project to assess the general toxicity of Seine River water under the influence of chemical disinfection have been grouped in Table 20.

doi: 10.2166/9781789062106_0093

The description of each model and the representation of results obtained will be described in this section. The following proprietary conditions reflect the need for specific modifications to common microbiology protocols and thus to the experimental setting, in order to test the various water samples by directly using a 'Whole Effluent Testing' (WET) approach.

1.1.1 General toxicity in the bacterial model

E. coli bacteria are maintained in culture at 4°C in Petri dishes on a solid agar LB medium and regularly transplanted, as required, onto identical dishes. The day before the experiment, a sample is extracted and the two strains of differential sensitivities are exposed to a culture step in a liquid LB medium under gentle agitation at 37°C (12 h). The next day, the bacteria are resuspended at an optical density at 600 nm (OD600 – Victor 3 Perkin Elmer Reader) of 0.2 in a mixture containing 90% test water and 10% of a proprietary concentrated bacterial culture medium. The bacteria are incubated at 37°C under gentle agitation, and the OD600 corresponding to their multiplication is measured continuously every 40 min for 7 h. In parallel, the same experiment is performed for each strain, with a negative control consisting of double-distilled irrigation water and an ampicillin toxicity control. The response curve profiles are integrated by means of a mathematical operation that takes the entire response into account on all experimental data points, in comparing the sample curves to the controls.

1.1.2 General toxicity in the yeast model

S. cerevisiae yeast is maintained in culture at 4°C in Petri dishes on a solid agar Sabouraud medium and regularly transplanted, as required, onto identical dishes. The day before the experiment, a sample is extracted and the two strains of yeast with differential sensitivities are exposed to a culture step – 12 h for non-susceptible yeasts and 36 h for susceptible yeasts – in a liquid YPD medium under stirring at 25°C in a moisture-saturated atmosphere. On the day of the experiment, the non-susceptible yeasts are collected at a concentration of 100,000 cells/mL in a mixture containing 90% test water and 10% of a proprietary concentrated yeast culture medium; the susceptible yeasts are then repeated at 500,000 cells/mL in a proprietary medium containing 90% test water. The yeasts are incubated at 37°C under gentle agitation in a moisture-saturated atmosphere, and the OD600 corresponding to their multiplication is measured continuously (Victor 3 Perkin Elmer Reader) every hour for 7 h. In parallel, the same experiment is carried out for each strain on a negative control consisting of double-distilled irrigation water and a cadmium toxicity control. The response curve profiles are integrated by means of a mathematical operation that takes the

entire response into account on all experimental data points, when comparing the sample curves to the controls.

1.1.3 General toxicity in the fungal model

The *S. tritici* strain is cultured at room temperature in Petri dishes on a solid agar PDA medium and regularly transplanted, as required, onto identical dishes. On the day of the experiment, a sample is scraped from the spore-forming areas on the jelly, and the spores are returned to suspension at 1000 cells/mL in a proprietary nutrient broth mixture containing 90% test water. Incubation is performed at room temperature (21°C). The OD600, proportional to the number of cells in solution, is measured (Victor 3 Perkin Elmer Drive) at a set time every day for 7 days. In parallel, the same experiment is performed for each strain on a negative control consisting of micropurified water and a cadmium toxicity control (3 mg/L). The response curve profiles are integrated by means of a mathematical operation that takes the entire response into account on all experimental data points, when comparing the sample curves to the controls.

1.1.4 Statement of results

The levels of proliferation or toxicity effects obtained are illustrated by a color code: green for no significant effect, red for a strong toxic effect, and dark blue for a strong proliferation effect. Each biological model has different sensitivity limits, which are taken into account in the graphic representation (Table 21). For the bacteria and yeast models, two distinct strains are used: a so-called sensitive strain, devoid of its defense systems against environmental toxins; and a natural strain (called wild-type), which does not exhibit any of these deficiencies. The sensitive model is unable to survive in the environment because it lacks all capacity to adapt and resistance to toxic stress. These conditions attest, at a very low sensitivity level, to the presence of a substance affecting living organisms. The wild-type model, commonly found in the environment, places the presence and impact of pollutants into perspective by measuring their impacts on an organism able to adapt and resist. The combination of these two information elements makes it possible to weight the interpretation of the measured signal.

Each test is based on measuring the growth of the target population in the presence of the sample to be tested. This measured growth is then compared to the reference (control) growth obtained under ideal conditions (devoid of any toxic stress). The comparison is carried out, after integrating the signal measured as a function of time, on both the growth kinetics and the final growth rate. Results are expressed as a percentage increase in the population, corresponding to the ratio of development acceleration between the stress-free control conditions and the stress test conditions.

1.2 DESCRIPTION OF THE BIOLOGICAL PANELS USED TO ASSESS ENDOCRINE DISRUPTION

1.2.1 Description of biological panels

The biological models used in this project to assess endocrine disruption in Seine River water samples following chemical disinfection have been grouped in Table 22.

Three types of endocrine disruptors are evaluated: thyroid, estrogenic, and androgenic. In order to detect these types, tests using aquatic vertebrate organisms, fish and amphibians representative of animals in the ecosystem have been used (AFNOR T90-716-1 and 2: 'Water Quality – In vivo fluorescence measurement of endocrine disrupting effects in natural water and wastewater – Part 1: Measurement of effects on the thyroid axis of amphibian embryos (Xenopus laevis) – Part 2: Measurement of estrogenic axis effects and activity of the fish embryo aromatase enzyme (*Oryzias latipes*)').

The tests using fish can detect estrogenic and androgenic disruptors, which are known to induce sex reversal in fish. Thyroid disruptors are targeted by a test using tadpoles of amphibians whose metamorphosis is affected when in contact with such compounds. More specifically, an endocrine disruption assessment is based on the use of small aquatic model organisms: tadpoles of the amphibian Xenopus for thyroid disruption, and fry of Medaka fish for estrogenic and androgenic disruption. These organisms harbor genetic markers that allow them to fluoresce when coming into contact with molecules that change how the hormonal axes function. Figure 40 illustrates the increase in fluorescence obtained when a bio-indicator tadpole comes into contact with water polluted by thyroid disruptors.

To detect all possible disruption, the samples are tested with or without co-treatment using a hormone; this step is referred to as 'stimulated' and 'non-stimulated', respectively. The larval stages used do not yet synthesize these hormones, hence this stimulation reveals certain action mechanisms of endocrine-disrupting micropollutants that cannot be detected in the absence of hormones. Tests are carried out in the laboratory as follows: larvae are exposed to plate samples (six wells) during 24 or 96 hours for fry and during 48 hours for tadpoles. The larvae are then transferred to plates (96 wells), and the fluorescence is analyzed using robotic imaging: an image is captured of each larva under specific illumination to reveal the fluorescence, and an image analysis algorithm is run to obtain fluorescence quantification. Statistical analysis is then performed to compare the fluorescence values obtained for the samples with those of the control larvae exposed to Evian water.

1.2.2 Physiological states of applied biological models

Each sample is tested in two physiological states, non-stimulated and stimulated. For the thyroid axis in the non-stimulated state, the larvae are exposed to the

sample, or to the reference molecules diluted in the test medium for the control groups. In this state, it becomes possible to measure an increase in larval fluorescence induced by the test condition; this is a pro-thyroid step that activates the thyroid axis. The physiological threshold for pro-thyroid disruption (100% on the disruption scale) is demonstrated by the positive activation control, indicating the activity of the 3.25 µg/L thyroid hormone T3. This concentration corresponds to the plasma concentration of T3 hormone during tadpole metamorphosis, that is, a physiological reference for thyroid disruption as defined by OECD Test Guideline 231. Once this level of activity has been reached, it is certain that the sample has an endocrine disrupting power with deleterious effects on wildlife. In the stimulated state, the larvae are exposed to the sample or reference molecules (for control), diluted in the test medium and supplemented with T3 thyroid hormone at 3.25 µg/L. In this state, it is possible to measure an increase or decrease in larval fluorescence induced by the test condition, which indicates pro-thyroid or anti-thyroid activity, thereby activating or inhibiting the thyroid axis, respectively:

- Pro-thyroid activity (100% on the disruption scale): the threshold for pro-thyroid disruption is demonstrated by a positive control for enhancement, showing the activity of thyroid hormone T3 in the stimulated state (test medium supplemented with T3 thyroid hormone), in indicating the level of larval fluorescence after exposure to a dose of hormone that saturates the physiological response.
- Anti-thyroid activity (−100% on the disruption scale): the physiological threshold for anti-thyroid disruption is indicated by a negative control in an undisturbed state (test medium only). A tested experimental condition that induces inhibition of larvae fluorescence in a stimulated state (test medium supplemented with thyroid hormone T3) reveals anti-thyroid activity.

For the estrogenic axis in the non-stimulated state, the larvae are exposed to either the sample or reference molecules diluted in the test medium for the control groups. In this state, it is possible to measure an increase in larval fluorescence induced by the test condition. This pro-estrogenic activity activates the estrogenic axis. The physiological threshold for pro-estrogenic disruption is indicated by the fluorescence level corresponding to the dose of 64 ng/L ethinyl estradiol hormone. This dose is the lowest capable of inducing a physiological effect according to OECD test guideline 230 in Medaka fish. The physiological effect observed at this concentration is the sex reversal of a portion of the population, as identified by the presence of ovarian tissue in the testes of male fish. Once this level of activity has been reached, it is certain that the sample has endocrine disrupting power with deleterious effects on wildlife. In the stimulated state, the larvae are exposed to the sample and reference molecules, diluted in the test medium and supplemented with testosterone at 30 µg/L. In this state, it is possible to measure an increase or decrease in larval fluorescence induced by the

test condition, thus indicating pro-estrogenic or anti-estrogenic activity, which serves to activate or inhibit the thyroid axis, respectively:

- Pro-estrogenic activity (100% on the disruption scale): the threshold for pro-estrogenic disruption is demonstrated by a positive control for enhancement showing the activity of 64 ng/L of stimulated ethinyl estradiol (test medium supplemented with testosterone), in indicating the level of larval fluorescence after exposure to a dose of hormone that saturates the physiological response.
- Anti-estrogenic activity (−100% on the disruption scale): the physiological threshold for anti-estrogenic disruption is demonstrated by a positive control for inhibition by treating larvae with fadrozole at 10 µg/L in a stimulated state (test medium supplemented with testosterone). The observed physiological effect is a change in the gonadosomatic ratio of male fish during the OECD test (Ankley *et al.*, 2002).

For the androgenic axis in the non-stimulated state, the larvae are exposed to the sample or reference molecules diluted in the test medium for the control groups. In this state, it is possible to measure an increase in larval fluorescence induced by the test condition. This pro-androgenic activity activates the androgenic axis. The physiological threshold for pro-androgenic disruption is demonstrated by a positive control for enhancement, in revealing the activity of 5 µg/L of 17α-methyltestosterone. This dose of hormone leads to a decrease in fertility and the presence of male secondary sexual characteristics in females (Pawlowski *et al.*, 2004). In the stimulated state, the larvae are exposed to the sample or reference molecules (for control groups), diluted in the test medium and supplemented with 5 µg/L androgen hormone 17α-methyltestosterone. In this state, it is possible to measure an increase or decrease in larval fluorescence induced by the experimental condition, thus indicating pro-androgenic or anti-androgenic activity, which serves to activate or inhibit the androgenic axis, respectively:

- Pro-androgenic activity (100% on the disruption scale): the threshold for pro-androgenic disruption is demonstrated by a positive control for enhancement, showing the 5 µg/L activity of 17α-methyltestosterone in a stimulated state (test medium supplemented with 17α-methyltestosterone), indicating the level of larval fluorescence after exposure to a dose of hormone that saturates the physiological response.
- Anti-androgenic activity (−100% on the disruption scale): the physiological threshold for anti-androgenic disruption is demonstrated by a positive control for enhancement, in showing 500 µg/L flutamide activity in a stimulated state (test medium supplemented with 17α-methyltestosterone). The physiological effect induced at a flutamide concentration of 500 µg/L is the absence of nest production due to the complete inhibition of Spiggin protein synthesis in males (Sebire *et al.*, 2008), as well as significant inhibition, in both males

and females, of Spiggin protein synthesis induced following pro-androgenic treatment (Katsiadaki *et al.*, 2006). Spiggin protein production is the physiological criterion used in the OECD GD 148 test guideline.

1.2.3 Statement of results

To specify the level of sample contamination, the larval fluorescence induced by the tested water sample is compared with that of negative (mineral water) and positive (water doped with reference hormones) controls. The fluorescence values obtained for the negative controls are consistent with the normal natural biological activity of living organisms and are not consistent with any externally induced hormonal disturbance. The fluorescence values obtained for positive controls serve to define the endocrine disruption threshold. Wherever the fluorescence intensity induced by a sample exceeds this threshold, it is likely that exposure to this sample will result in observable adverse physiological effects (Figure 41). The concentration of triiodothyronine (thyroid hormone T3) used for the positive thyroid test control is 3.25 µg/L. This concentration corresponds to the physiological load found in the plasma of tadpoles during metamorphosis, in their natural state (Leloup & Buscagalia, 1977), and is described in the literature as capable of causing observable adverse physiological effects, that is, an acceleration in the rate of Xenopus metamorphosis, and mortality if tadpoles are exposed at non-metamorphic stages (Shi *et al.*, 1998). For the positive estrogen test control, the ethinyl estradiol concentration used equals 64 ng/L, which is described in the literature as being capable of altering fish reproduction (Seki *et al.*, 2002).

Beyond illustrating results in this qualitative form in order to provide information on the ability of the sample to induce a harmful physiological effect, these results are also expressed in quantitative form. In each experiment, in conjunction with the larvae being exposed to the samples, some larvae are exposed to a range of different reference hormone concentrations that generate a 'standard' curve defining the fluorescence obtained as a function of hormone concentration. Using this 'standard curve', the fluorescence values derived for the samples can be converted into hormonal equivalents and thus establish the hormone concentration that would induce the same fluorescence variation as the sample. Use of these hormone equivalents makes it possible to compare results obtained for samples tested in different experiments.