Chapter 2

Biological models applied to the case of chemical disinfection using PFA

© SIAAP

2.1 TOXICITY ASSESSMENT AT THE LABORATORY SCALE

2.1.1 Description of the experimental approach

The general toxicity tests for PFA were carried out using spot samples of the Seine Valenton WWTP (SEV) discharge and Seine-Choisy water upstream of the SEV plant. In order to assess the impact of the disinfected PFA discharge from the

doi: 10.2166/9781789062106_0100

SEV plant on the natural environment, two key points were taken into account. The first concerns the preparation of samples to be tested, which entails reconstituting a representative sample of the natural medium in the immediate vicinity of the station discharge (Figure 42).

To this end, 'unfavorable' conditions were adopted, corresponding to a proportion (10% by volume) of water being discharged into Seine River water; this proportion was calculated based on a high summer discharge rate (90th percentile over the period 2016–2017) and a low Seine flow upstream of SEV (10th percentile over the summer period 2016–2017). For the disinfection of discharge water, a shock concentration of 2 ppm PFA was applied. Two types of samples were obtained: Seine River water containing discharge water before disinfection, and Seine water containing disinfected discharge water. For the latter, the discharge water was obviously disinfected before coming into contact with the Seine water. The second point pertains to the instability of the PFA disinfectant. It was decided to prepare the disinfectant just prior to launching the various bioassays.

2.1.2 Estimation of general toxicity

The general toxicity to bacterial, yeast and fungal models applied during the three laboratory-scale tests is depicted in Figure 43. These bioassays were conducted on two types of samples: Seine water samples containing non-disinfected discharge water (indicated in the figure as 'samples without disinfection'), and Seine water samples containing the same discharge water disinfected at a 2 ppm PFA concentration (indicated 'samples with disinfection').

The levels of proliferation or toxicity effects obtained are color-coded, ranging from green for no effect to either red for a strong toxicity effect or blue for a strong proliferation effect. Results are expressed as a percentage of population increase, corresponding to the ratio of development acceleration between stress-free control conditions and test conditions. In the remainder of this section, each test will be discussed separately before concluding on the overall toxicity.

2.1.2.1 General toxicity in the bacterial model

Results obtained from samples tested at the laboratory scale show an absence of general toxicity to bacterial models, for samples containing discharge water either before or after disinfection (Figure 43a and b). The two models tested, both wild-type and susceptible, display values of growth difference from control ranging from −12 to 7% and from −1.5 to −19.2%, respectively. As indicated in Figure 43a, these values are no different from the reference value (green color code). The bacteria do not appear to be impacted by the samples tested, even on the susceptible model without its defense systems. The presence of disinfected discharge water at the shock concentration (2 ppm PFA) is not, under these conditions, toxic to the bacterial model.

2.1.2.2 General toxicity in the yeast model

Results obtained from samples tested at the laboratory scale show a lack of general toxicity to the yeast model for the wild-type strain (Figure 43c and d). The values of growth difference from control, which range from −1 to 13.7%, reflect a similarity to the baseline state. On the other hand, for the susceptible model, a slight toxic effect is revealed in the test samples: for this strain, a growth differential of −12.7 and −32.1% with respect to the control is obtained for samples containing the disinfected discharge water. These values correspond to a zero effect for two of the three samples, but the most impactful value (−32.1%) indicates an effect described as medium. On samples containing disinfected discharge water, toxic effects are identified by a growth reduction ranging from −18.9 to −27.3% compared to the control. These toxic effects occur only on the sensitive model and remain moderate, hence qualifying on the impact scale as a 'weak but significant effect' according to the classification code. Yeasts without defense systems appear to be systematically impacted by the presence of disinfected discharge water, compared to the same samples without prior disinfection. The presence of disinfected discharge water at a shock concentration (2 ppm PFA) has no effect on the wild-type yeast model, though it does exert a small yet statistically significant impact on the susceptible model.

2.1.2.3 General toxicity in the fungal model

The results obtained on samples tested at the laboratory scale show a slight proliferation of the cell population (Figure 43e). Samples containing previously disinfected discharge water do not appear to differ in this respect from samples containing discharge water prior to disinfection. Values range from −3.8 to 101.2% for samples before the disinfection of discharge water and from 4.1 to 92.44% for samples containing disinfected discharge water. These values indicate the presence of moderate to strong proliferation effects, likely reflecting the presence of organic matter in the medium coupled with an absence of specific stressors in the test fungi physiology. These effects are observed for both types of samples regardless of the presence of disinfectant.

The presence of disinfected discharge water at a high concentration (2 ppm PFA) does not appear to alter the propensity to induce cell proliferation, as observed for Seine water samples containing discharge water prior to disinfection. The observed effects are due to the presence of factors conducive to fungal cell multiplication.

2.1.3 Estimation of endocrine disruption

2.1.3.1 Thyroid disruption

The results obtained for the three campaigns show, for all samples, no effect on the thyroid axis. Whether the Seine water is supplemented with discharge water before

disinfection or supplemented with disinfected discharge water, the values lie below the thyroid test detection limit.

2.1.3.2 Estrogenic disruption

An evaluation of estrogenic disruption of the three samples tested is shown in Figure 44.

These results are expressed in both hormone equivalents and physiological effects in each state, that is, non-stimulated and stimulated. The physiological threshold (as represented by the red zone) is established by a dose of endocrine-disrupting reference hormone or molecule that induces an adverse effect at the organism level.

It should be noted that in stimulated mode, a co-treatment with 10 ng/L of testosterone is applied to the test sample (reference level in stimulated state). Treatment with 64 ng/L ethinyl estradiol (EE2) is then performed to identify the physiological threshold of pro-estrogenic disruption (positive activation control in the stimulated state) or 10 µg/L fadrozole to identify the physiological level of anti-estrogenic disruption (positive control for inhibition in a stimulated state). Testosterone treatment allows embryos to be placed under conditions where the estrogen axis is activated. Under these conditions, the test in fact also takes into consideration the effects on the aromatase enzyme. Aromatase is an essential enzyme for the endogenous production of estradiol from testosterone; it controls the fine balance between estrogen and testosterone in the body and thus participates in the sexual identity of individuals. Aromatase disruption is one of the OECD estrogenic disruption criteria (OECD Guidelines 229 and 230). The stimulated mode allows detecting compounds that alter the aromatase function (inhibition of protein synthesis, expression activator or inhibitor) as well as receptor antagonists. Results are expressed in terms of an ethinyl estradiol equivalent. This analysis indicates which concentration of ethinyl estradiol hormone yields the same hormonal potential as the sample. To complete this analysis, a dose response range of ethinyl estradiol is conducted in parallel with the test in order to model a standard curve. The fluorescence value obtained for the sample is then plotted on this curve and converted into an ethinyl estradiol equivalent. On the disruption scale, 0% denotes the total absence of endocrine activity and 100% refers to endocrine activity that reaches the physiological threshold.

These results assessed the absence of any estrogenic activity detectable in the non-stimulated mode, for all three tests. Let us note that the Seine water samples with disinfected discharge water do not activate the estrogen axis and moreover that adding disinfected discharge water does not activate it either. On the other hand, when in the stimulated mode by activating the estrogenic axis, an anti-estrogenic potential is detected. For 'control' samples, Seine water with disinfected discharge water has an estrogenic potential equivalent to −34, −46

and −86 ng EE2/L for the three tests, reaching 39, 38 and 72%, respectively, of the physiological estrogen axis inhibition threshold. These results, although quantifiable (by their difference with respect to controls), lie below the levels producing an adverse effect on development of the aquatic organisms under study. As for the Seine water samples supplemented with disinfected discharge water, no effect on the estrogenic axis is found in the stimulated mode for the first two tests, and a potential of −62 ng EE2/L exists for the third test.

When comparing samples with and without disinfected discharge water, differences appear between all three tests. For the first two tests, a reduction in the anti-estrogenic effect is observed after adding disinfected discharge water. This minimal effect on the estrogen axis after contact with disinfected discharge water means that the molecules in the medium no longer exhibit estrogenic activity, which suggests a phenomenon of cancelling or limiting the anti-estrogenic effect due to the disinfected discharge water. This cancellation/limitation action could have taken place at three levels: hormonal conversion, protein expression, or receptor level. For the third test however, a decrease in endocrine potential is recorded: −86 to −62 ng EE2/L equivalent and 72 to 52% physiological effect impairment, respectively, between the two samples. This campaign has confirmed the presence of pro-estrogenic molecules or molecules that extinguish the anti-estrogenic signal.

Overall, the presence of disinfected discharge water at a shock concentration of 2 ppm PFA and under unfavorable hydrological conditions (i.e., high proportion of disinfected discharge water in the natural environment) does not exert a significant impact on the estrogenic axis. The values quantified at the laboratory scale never exceed the thresholds for physiological effects A potential anti-estrogenic effect limitation has been identified in the presence of disinfected discharge water.

2.1.3.3 Androgenic disruption

The assessment of androgenic disruption on the three samples tested is shown in Figure 45.

Results are obtained by comparing the sample to the physiological threshold in each physiological state, that is, non-stimulated and stimulated. This comparison is expressed as both a hormonal equivalent (Flutamide equivalent) and a percentage of the physiological effect threshold. This threshold is established by a dose of endocrine-disrupting hormone or reference molecule inducing an adverse effect at the body level.

It should be noted that in stimulated mode, a co-treatment with 5 µg/L of pro-androgenic hormone 17α-Methyltestosterone (17MT) is applied to the test sample (positive control of activation in the stimulated state). This threshold corresponds to the one induced by both a decrease in fertility and the presence of male secondary sexual characteristics in female medaka fish (Pawlowski *et al.*,

2004). A treatment with 500 µg/L of the anti-androgenic hormone, flutamide (in addition to 17α-Methyltestosterone), is then added. A threshold has thus been defined, as corresponding to the extinction of the pro-androgenic signal, beyond which an anti-androgenic disruption can be identified. This threshold induces an absence of nest production due to complete inhibition of Spiggin protein synthesis in male stickleback (Sebire *et al.*, 2008), as well as a significant inhibition of Spiggin protein synthesis induced in females and males following pro-androgenic treatment (Katsiadaki *et al.*, 2006). This flutamide concentration also leads to decreased fish fecundity along with histological alterations of ovaries and testis (Jensen *et al.*, 2004). Results are expressed as a hormonal equivalent (17MT or flutamide). This analysis indicates which concentration of the anti-androgen flutamide yields the same hormonal potential as the sample. To complete this analysis, a flutamide dose response range is conducted in parallel with the test in order to model a standard curve. The fluorescence value obtained for the sample is then plotted on this curve and converted into a hormonal equivalent. On the disruption scale, 0% denotes the total absence of endocrine activity while 100% refers to endocrine activity that reaches the physiological threshold.

These results allow us to assess the absence of any androgenic activity, in undiagnosed mode, for all three tests. Let us note that the Seine water samples with disinfected discharge water do not activate the androgenic axis and moreover that adding disinfected discharge water does not appear to activate it either. When in the stimulated mode by activating the androgenic axis, an anti-androgenic potential is detected for two of the three tests. The initial detection, which is very weak, was observed during the first test on the 'control' sample, corresponding to Seine water supplemented with disinfected discharge water at a potential of 7.1 µg/L flutamide equivalent, 1% physiological effect threshold and no disruption in the presence of disinfected discharge water. This difference in response can be attributed to the proximity of the values to the detection limits; moreover, the absence of an anti-androgenic effect due to the disinfected discharge water is to be considered. The second and higher detection level was obtained on the Seine water sample supplemented with disinfected discharge water; an anti-androgenic potential of 300 µg/L flutamide equivalent and 60% physiological threshold was achieved. A potential anti-androgenic effect was found in the presence of disinfected discharge water. For the third test, no disruptions were identified. Overall, in all three tests, no pro-androgenic activity could be identified, when in the non-stimulated mode. On the other hand, when in the stimulated mode, three distinct behaviors were observed for samples containing disinfected discharge water: total absence of effect, disappearance of an existing effect, and a significant occurrence of effect, in relation to the sample containing disinfected discharge water. In light of this erratic behavior, no systematic trend can be advanced concerning a potential anti-androgenic disruption due to the presence of the disinfectant. The differences in more or less

quantified responses seem to depend on the seemingly evolving nature of the molecules present in the medium. Some molecules will tend to interact positively, negatively or inertly with the disinfected discharge water (or disinfection byproducts) with respect to the androgenic axis. Even though a 2 ppm PFA shock concentration was applied to the disinfected discharge water, the values quantified under these controlled laboratory-scale conditions have never exceeded the physiological effect thresholds.

2.2 TOXICITY ASSESSMENT AT THE INDUSTRIAL SCALE

General toxicity and endocrine disruption were measured on samples collected from the Seine River both upstream and downstream of the WWTP discharge point in order to evaluate the impact of WWTP discharge on the river with and without PFA injection. These samples were collected at two sampling points of the MeSeine platform, which oversees Seine River quality monitoring within the SIAAP Authority: Choisy-le-Roi (PK 622.440) (3) and Port à L'anglais (PK 626.152) (4). The Choisy-le-Roi sampling point is located approximately 1,500 m upstream of the WWTP discharge point, on the first bridge accessible to pedestrians. Sampling was performed once a week (on Wednesdays). It must be kept in mind that depending on weather conditions, single rainfall sewer overflows can occur between these two Seine River sampling spots, thus significantly impacting water quality. More information was given in a previous section dedicated to the sampling description (Section 2, Chapter 1).

2.2.1 Estimation of general toxicity in the Seine River

The monitoring of general toxicity in the Seine River during industrial-scale testing entailed tests on bacteria, yeasts and fungi; these were carried out both upstream and downstream of the SEV station outfall. During these tests, periods without chemical disinfection were also monitored in order to establish a baseline (prior to the start of testing) and assess any potential aquatic system disruptions during the post-disinfection period. Results obtained on general toxicity during the 10-week follow-up phase (between weeks 35 and 44) are illustrated in Figure 46.

As described above, for bacterial tests as well as yeast tests, two variants have been mobilized: a susceptible strain lacking certain defense mechanisms, and a wild-type strain without such defects. Results are expressed as a percentage of population growth, corresponding to the ratio of development acceleration between stress-free control and test conditions. A color code indicating the level of toxicity obtained is given in each figure, representing increased toxic effects from green to red.

2.2.1.1 Bacterial tests

Results obtained in the Seine River during the disinfection tests show an overall absence of general toxicity to the wild-type bacterial model, with negative responses in about 90% of cases, either upstream or downstream of the SEV station. The values range from −16 to 15% growth for the Seine upstream of SEV and from −5 to +17% growth alteration for downstream of SEV (Figure 46a), for the most part similar to the reference state. A small, but significant, effect was observed downstream of SEV during the first season, with a slightly more pronounced impact than that upstream. These effects are likely due to the rainy conditions of that particular day. In the other case, again a small, but significant, effect was observed in the Seine upstream of SEV that was no longer found downstream. Regarding the susceptible bacterial model, no significant effects were observed in the Seine upstream of SEV. On the other hand, downstream of SEV, a small significant effect was detected in half the cases (Figure 46b). These weak effects, comprising between −2 and −29% growth modulation for the Seine upstream of SEV and between −6 and −52% for the Seine downstream of SEV, do not appear to coincide with any particular periods of disinfection. We can in fact observe that the remainder of the time (50% of occurrences), no significant effects were detected between upstream and downstream of SEV. It should be noted that the observed toxic effects, though moderate, systematically correspond to rainy episodes. The well-known quality degradations associated with these contexts are visibly expressed by effluent inputs causing physiological stress on the susceptible strain. Laboratory-scale results on reconstituted samples of Seine water supplemented with 2 ppm PFA disinfected discharge water did not reveal a significant toxic effect of chemical disinfection on both models, that is, wild-type and susceptible, of bacteria. This finding corresponds perfectly with the results observed during the industrial-scale tests and would instead tend to support the external effluent input track (thunderstorm weirs, soil leaching, etc.) or a change in the discharge water matrix prior to disinfection, as observed by Ragazzo *et al.* (2017).

2.2.1.2 Yeast tests

Results obtained in the Seine during disinfection testing systematically showed no general toxicity to wild-type yeasts upstream of the SEV station, as well as downstream. The values ranged from −3 to +28% growth modulation for the upstream Seine SEV and between −6 and +37% for the downstream SEV (Figure 46c); these values are broadly similar to the baseline, except for two points (weeks 39 and 43). For the latter, a proliferation-stimulating effect, described as 'weak but significant', was observed in the Seine downstream of SEV, indicating the probable presence of organic matter conducive to

developing yeasts. As regards the sensitive yeast model, a significant absence of effect was observed in the Seine upstream of SEV 70% of the time, compared to 80% of the time downstream of SEV (Figure 46d). These values ranged from +3 to −31% change in growth for the Seine upstream of SEV vs. between −28 and +11% downstream. Overall, during the 10 follow-up campaigns, only one showed stronger pressure downstream of SEV than upstream. This day was marked by especially high rainfall, which may explain the difference. Results observed during industrial-scale tests were consistent with the laboratory-scale tests for the wild-type strain (reconstituted samples from Seine water supplemented with discharge water disinfected at 2 ppm PFA), for which a lack of disinfection effect was noted. For the susceptible strain, a lack of industrial-scale effects was observed, in contrast with the laboratory tests, which revealed a slight, but systematic, effect after the disinfection step. This difference in response could be attributed to the more adverse laboratory test conditions (characterized by a larger proportion of disinfected discharge water in the reaction volume).

2.2.1.3 Fungal tests

Results obtained in the Seine during the disinfection tests show no general toxicity in the fungal model 60% of the time downstream of SEV vs. 40% of the time upstream. These values ranged from 3 to −55% growth modulation for the upstream Seine SEV and between −2 and −58% downstream (Figure 46e). Instances of proven impact are systematically assigned to the 'weak but significant' category and appear, interestingly, more frequently upstream than downstream of SEV. With the exception of week 37, for which an impact was observed downstream of SEV and not upstream, the minimal effects observed downstream of SEV were generally identical to those observed upstream. In other cases, either no effect between upstream and downstream or even a slight improvement over the upstream could be observed. The observed effects, though limited, thus seem to correspond not to the periods of disinfection, but rather to wider variations in Seine River quality and/or SEV discharge water. This conclusion becomes even more pronounced when taking into account various rainy events during the industrial-scale tests. Laboratory-scale fungal model testing on reconstituted samples of Seine water and discharge water, both before and after disinfection, showed no impact of disinfection in two of the three tests, with a stimulated response in the post-disinfection case. The only significant difference between industrial and laboratory tests stems from toxicity observations in the natural environment (overall negative values), whereas a tendency to activate proliferation is observed in the laboratory, due in all likelihood to the better controlled laboratory conditions, hence allowing for more efficient removal of external inputs influencing the biological test response.

2.2.2 Estimation of endocrine disruption in the Seine River

The monitoring of endocrine disruption in the Seine during the industrial-scale trials was focused on thyroid and estrogenic disruption; it was carried out upstream and downstream of the SEV station outfall. Estrogenic disruption results over the 10 weeks of follow-up (between weeks 35 and 44) are shown in Figure 47. As described above, two physiological states of the biological model were applied: the non-stimulated mode (Figure 47a and b), and the stimulated mode (Figure 47c and d). For each physiological condition, results were expressed in hormonal equivalents. A color code for the level of disruption found is indicated on the results, which are expressed as a percentage of physiological threshold attainment.

2.2.2.1 *Thyroid disruption*

The first thing to note from these results is the absence of thyroid disruption in the Seine during the entire monitoring period, whether upstream or downstream of SEV. Chemical disinfection applied during this period did not appear to disturb the natural environment as regards thyroid disruption. These observations are consistent with laboratory-scale results. In fact, the laboratory tests were carried out on a matrix of Seine water supplemented with SEV discharge water disinfected at 2 ppm PFA, in simulating the receiving environment immediately downstream of the station outfall under 'unfavorable' conditions. Of the three tests conducted with a 1-week interval, no disruption was quantified. These results are also consistent with studies carried out on the discharge water of the same station, in which the authors found no thyroid disruption (Du Pasquier *et al.*, 2018). According to the monitoring performed within the framework of *Meseine Innovation* (the R&D component of the Seine River quality monitoring network sponsored by the SIAAP), among the 13 sampling campaigns run between 2016 and 2017, effects ranged from 11 to 56% for both non-stimulated and stimulated modes, and moreover no effect was detected during the summer period (unpublished data).

2.2.2.2 *Estrogenic disruption*

For estrogenic disruption, effects were detected periodically throughout the industrial-scale testing campaign (Figure 47).

When in the non-stimulated mode, by placing the sample directly into contact with the biological model, the Seine upstream of SEV exhibited estrogenic activity most of the time and at a frequency equivalent to that downstream of SEV. The estrogenic activity detected in the Seine was in the range of 24–37 ng EE2/L upstream of SEV to 24–31 ng EE2/L downstream (Figure 47a). These effects remain low, reaching 38–58% of the physiological threshold (Figure 47b).

Overall, this low variability is due to measurement uncertainty since the results obtained between upstream and downstream of SEV are statistically equivalent (Mann–Whitney test, $\alpha = 0.05$, p-value $= 0.908$). The chemical disinfection during industrial-scale testing did not disrupt the receiving environment as regards estrogenic disruption in the non-stimulated mode. These observations are consistent with the laboratory results, for which no effects were detected either before or after disinfection.

When in stimulated mode, achieved by adding a hormone activating the estrogenic axis in the sample to be tested, an anti-estrogenic potential was detected 60% of the time in the Seine upstream of the SEV station vs. 20% of the time downstream. When this activity was detected in the Seine, it amounted to between −29 and −53 ng EE2/L for the upstream of SEV and between −25 and −48 ng EE2/L downstream (Figure 47c). The detected effects remain weak overall and range from 23 to 53% of the physiological level of estrogenic axis inhibition (Figure 47d). Results observed between the upstream and downstream of SEV, in contrast with the non-stimulated mode, are statistically significant (Mann–Whitney test, $\alpha = 0.05$, p-value $= 0.066$), thus indicating the presence of an effect due to SEV discharge water. Moreover, over the 10 weeks of follow-up between discharge upstream and downstream of SEV, an anti-estrogenic effect disappeared 50% of the time, broken down as follows: absence of effect 30% of the time, decrease in effect 10% of the time, and appearance of an effect 10% of the time. These erratic trends do not seem to coincide with the applied rates of PFA treatment or even with the periods of chemical disinfection, but rather are correlated with effluent quality (SEV and/or Seine discharge). These observations are consistent with laboratory-scale results; of the three laboratory tests conducted, the anti-estrogenic effect disappeared in two of them and decreased in the other.

In sum, during the industrial-scale disinfection tests, the Seine downstream of the ENS outfall exhibited weak endocrine disruption and equivalent, minimized or cancelled effects relative to those upstream. This finding, which is generally positive, shows the presence of molecules that no longer induce endocrine effects on organisms, either via the cancellation effect (pro- and anti-estrogenic compensation) or via inhibition, which could occur at the level of a hormone conversion process or at the receptor level. The exact action mechanism is normally difficult to precisely identify for endocrine disruptors, since they can carry out several different actions simultaneously (Du Pasquier *et al.*, 2015; Mengeot *et al.*, 2016), hence the value of combining several bioassays. This statement is not surprising, given that endocrine disruption of ETS discharge water has been estimated, based on the work by Du Pasquier *et al.* (2018), at a zero or low level.

Key points

Results obtained from samples tested at the laboratory scale indicate:

- An absence of general toxicity to bacterial models.
- No effect on the wild-type yeast model, although a small but statistically significant impact on the susceptible model.
- No alteration of the cell proliferation of fungi.
- Results obtained for all sampling campaigns showed no effect on the thyroid axis.
- No significant impact on the estrogenic axis, whereas a potential pro-estrogenic or anti-estrogenic effect limitation was identified in the presence of disinfected discharge water.
- No androgenic activity was identified, in either the non-stimulated or stimulated mode, with three distinct behaviors being observed: total absence of effect, disappearance of effect, and significant occurrence of effect, respectively, in relation to the sample containing disinfected discharge water.

Results obtained from samples tested at the industrial scale indicate:

- Regarding general toxicity models (bacteria, yeast, fungi), no significant effects linked to disinfection were observed in the Seine downstream of the SEV outfall.
- Results obtained for all sampling campaigns showed no effect on the thyroid axis.
- Chemical disinfection did not modify the receiving environment in terms of estrogenic disruption.