Monitoring toxicity of industrial wastewater and specific chemicals to a green alga, nitrifying bacteria and an aquatic bacterium

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Abstract Treatment plants may be exposed to a whole range of toxic organic and inorganic compounds that may inhibit the removal of organic matter and nitrogen. In order to secure maximum treatment efficiency, the plant manager has to monitor the toxicity of the influent sewage. With regard to the receiving water the manager also has to make sure that toxicity in the influent is significantly reduced during treatment. Because a whole range of chemicals may be present, chemical analysis may be insufficient and expensive as a control instrument. Instead, direct toxicity measurements are preferable to capture the complexity of the wastewater. The monitoring methods have to be relevant and sensitive for the processes in the treatment plant, i.e. removal of organic matter and nutrients. The methods also have to be simple and inexpensive. The paper reports on recent results from the application of nitrification, algae and Biotox tests, and summarises the experience with monitoring of toxicity. Although the sensitivity of the tests varies with respect to individual chemicals or group of chemicals, the application of a combination of the tests gives a high likelihood of detecting toxic impacts on treatment plants and receiving waters.

Keywords Algae test; benzothiophene; Biotox test; industrial wastewater; naphthalene; nitrification test

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Introduction

In industrialised areas with petrochemical plants, oil industry, creosote-, paint- or varnish producing companies, etc., sewage treatment plants may receive wastewater containing substantial concentrations of toxic organic compounds like aromatic hydrocarbons, phenols, heterocyclic aromatic compounds, chlorinated hydrocarbons, aldehydes, ketones and many other organic compounds. Complex samples can be characterized by laborious and expensive chemical analysis, which can only provide limited information for evaluation of possible toxic effects on biological processes at treatment plants and in recipients. The chemical analysis cannot provide information on the combined inhibitory effect of all chemicals present, nor can the bio-availability be assessed directly. In general, only a fraction of an experienced inhibition can be explained by the compounds identified by a chemical analysis. Simple toxicity tests, on the other hand, can provide direct measures of the actual environmental toxicity of polluted waters.

For large chemical industries that produce a vast number of products, chemical analysis of the wastewater for specific compounds is not a feasible option. At the chemical industries Perstorp AB, Sweden, inhibition of the activated sludge processes by components in the wastewater can occur several times a year. Here bioassays as the simple toxicity tests could be an option for monitoring the potential inhibiting effects of the wastewater. In regard to specific chemical compounds and their effect on treatment plants, ground water and surface water, the simple toxicity tests may provide a direct quantitative measure of the actual toxicity related to the compound. In relation to a mixture of aromatic organic compounds the usefulness of the test has been demonstrated by Dyreborg and Arvin (1995). They showed how creosote contaminated water was toxic to nitrifying bacteria, and that benzene, toluene, o-xylene, phenol, and o-cresol were the most important contributors to

the toxicity. Dyreborg *et al.* (1995) showed that when heterocyclic aromatic compounds like pyrrole, 1-methylpyrrole, thiophene and benzofurane were present, they were strong inhibitors of nitrification. Even the otherwise easily biodegradable compound toluene was slowly removed when the heterocyclic compounds were present.

The aim of the present study was to investigate the potential of three different methods for assessing toxicity of complex mixtures and simple binary mixtures, to compare the methods with respect to sensitivity and their ability to complement each other, and finally to discuss which of the tests can be recommended for different monitoring purposes. The three toxicity tests use the aquatic bacterium *Vibrio fischeri*, mixed nitrifying bacteria from wastewater treatment plants and the fresh water green alga *Pseudokirchneriella subcapitata*. The two tests with bacteria are frequently used as screening tests for acute toxicity of chemicals and water samples, whereas the test with the green alga measures inhibition of growth and therefore reflects subchronic effects. The bacteria test and the algae test represent tests for effect on bacteria and primary producers, respectively. In order to evaluate how the tests assess the toxicity of a given sample, the testing of complex samples of wastewater from a chemical industry will be evaluated first. Secondly the testing of simple samples of binary mixtures of the chemicals naphthalene and benzothiophene will be evaluated. Here the information on the bio-toxicity of the single compounds is already available, and it will be assessed whether the results of the tests with the binary mixtures can be explained based on the knowledge regarding the single compounds.

Material and methods

Chemicals: The chemicals naphthalene and benzothiophene were purchased from Fluka, Germany.

Wastewater samples: Untreated chemical industrial wastewater from Perstorp AB, Perstorp in Sweden, was collected either as grab or flow proportional samples at the inlet to the buffer tank at the wastewater treatment plant of the company. Prior to testing, the samples were adjusted to pH 7. In the buffer tank at Perstorp AB's treatment plant pH is adjusted to between 4 and 9.

Algae test: The freshwater green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*) was used as a test organism (Halling-Sørensen *et al.*, 1996). The growth rate reduction during 72 h of incubation was used as the toxicity endpoint. The toxicity was determined from the reduction in growth rate in dose–response experiments, and was quantified by measuring the fluorescence of solvent-extracted algal pigments. Concentration–response curves were fitted according to the Weibull model (Christensen and Nyholm, 1984), and EC values and corresponding 95% confidence intervals were estimated (Andersen *et al.*, 1998).

Nitrification test: A mixed culture from a municipal treatment plant with nitrogen removal was used (Lundtofte wastewater treatment plant, Lyngby, Denmark) (Arvin *et al.*, 1994). The toxicity was determined from the reduction in ammonia-oxidation (nitrification) rate in dose–response experiments. For the test on the wastewater samples from Perstorp AB, the nitrification test was modified by the addition of 12 mmol l^{-1} phosphate buffer in order to keep pH constant at 7.5, and measurements of nitrite and nitrate were performed instead of ammonia (Eilersen *et al.*, 2003). Concentration–response curves and EC values were estimated as described for the algae test.

Biotox test: A light emission test using the aquatic bacterium *Vibrio fischeri* was carried out (Biotox™ Kit, Bio-Orbit OY, Turku, Finland). The test is equivalent to

the Microtox test. Reduction in luminescence after 15 min was used as endpoint. Concentration–response curves and EC values were estimated as described for the algae test.

Results and discussion

Toxicity of industrial chemical wastewater

Direct toxicity measurements were performed on wastewater from the chemical industry Perstorp AB in Sweden. pH, alkalinity and conductivity varied significantly between samples. pH varied from 2 to 13.4, alkalinity from –2 to 108 meq/l, while conductivity fell within the range of 3 to 24 mS/cm. The average concentration of COD total was 3,000 mg/l, 90–97% of which is soluble. Nitrification tests were performed with activated sludge from Perstorp AB's own treatment plant and from Lundtofte municipal treatment plant. When the EC values for the three toxicity tests are compared, see Table 1, it appears that the EC10, EC20 and EC50 for all three tests are in congruence, while the EC90 values for the Biotox test are significantly higher than the results for the Nitrification test and the Algae test.

The Nitrification test is the most sensitive test in defining the EC10, EC20 and EC50 values. For determination of the EC90 values, the Algae test is most sensitive. The least sensitive test in defining EC10 and EC20 is the Algae test. The inhibition of the algae

Table 1 Effective concentration (EC) values and 95% confidence interval for the Nitrification test, Algae test and the Biotox test. Measurements given as % of wastewater in the sample

Sample	EC10				EC20			
	Nitrif Perstorp	Nitrif Lundtofte	Algae	Biotox	Nitrif Perstorp	Nitrif Lundtofte	Algae	Biotox
Flow proportional								
1	0.12 [0.08–0.2]	0.79 [0.48–1.3]	1.4 [1.1–1.8]	1.1 [1.0–1.4]	0.14 [0.08–0.3]	1.3 [0.80–1.9]	1.7 [1.4–2.1]	1.3 [1.1–1.7]
2	1.7 [0.02–140]	0.85 [0.61–1.2]	2.5 [1.9–3.3]	1.7 [1.4–2.3]	2.0 [0.02–210]	1.3 [0.96–1.7]	2.7 [2.2–3.4]	2.4 [1.8–3.2]
Grab								
3	0.88 [0.06–12.7]	1.3 [0.60–2.8]	1.2 [1.1–1.4]	1.0 [1.0–1.2]	1.4 [0.13–14.6]	2.1 [1.10–4.1]	1.5 [1.3–1.6]	1.1 [1.0–1.3]
4	0.36 [0.14–0.9]	1.4 [0.72–2.8]	4.5 [4.0–5.0]	1.4 [1.2–2.0]	0.63 [0.23–1.7]	2.2 [1.26–3.9]	5.0 [4.5–5.4]	1.9 [1.5–2.7]
5	0.41 [0.19–0.9]	0.66 [0.29–1.5]	4.1 [3.8–4.5]	3.7 [3.2–4.5]	0.59 [0.30–1.2]	1.1 [0.52–2.3]	4.8 [4.5–5.1]	6.1 [5.2–7.1]
6	0.38 [0.21–0.7]	0.68 [0.36–1.3]	1.5 [1.1–1.9]	1.3 [1.1–1.8]	0.64 [0.35–1.2]	1.1 [0.61–1.9]	1.7 [1.4–2.1]	1.6 [1.3–2.2]

	EC50				EC90			
	Nitrif Perstorp	Nitrif Lundtofte	Algae	Biotox	Nitrif Perstorp	Nitrif Lundtofte	Algae	Biotox
Flow proportional								
1	0.29 [0.12–0.7]	3.0 [2.3–4.1]	2.4 [2.1–2.6]	2.7 [1.9–3.8]	3.5 [0.64–19]	11 [6.9–18]	3.4 [2.7–4.4]	47 [13–425]
2	2.6 [0.02–410]	2.7 [2.2–3.2]	3.2 [2.8–3.7]	7.1 [5.4–9.4]	3.5 [0.01–880]	7.6 [5.6–10]	4.1 [3.4–5.0]	> 100 [35–428]
Grab								
3	3.2 [0.65–16]	5.3 [3.6–7.9]	2.1 [2.0–2.2]	1.8 [1.3–2.4]	11 [0.93–130]	19 [11–35]	3.7 [3.4–4.0]	30 [7.8–364]
4	2.5 [1.0–6.1]	5.2 [3.6–7.4]	6.1 [5.8–6.3]	5.2 [3.6–7.4]	35 [10–120]	16 [9.5–28]	8.3 [7.8–8.9]	79 [22–45]
5	1.2 [0.79–1.9]	3.1 [1.9–5.1]	6.1 [6.0–6.3]	19 [17–21]	3.6 [1.8–7.3]	16 [7.1–33]	8.1 [7.7–8.5]	> 100 [96–192]
6	2.1 [1.29–3.5]	2.5 [1.75–3.6]	2.5 [2.2–2.8]	3.5 [2.4–4.8]	18 [8.87–36]	8.9 [4.87–16.1]	4.2 [3.5–5.0]	31 [12–127]

increases from 10 to 90% within a very narrow concentration range, on average from 2.5 to 5.3% wastewater.

For the Nitrification and the Biotox test, this concentration interval is wider, especially for the Biotox test where the inhibition of the bacteria changes from 10% to 90% within an average concentration interval from 1.7 to 48% wastewater. The inhibition of the nitrifying bacteria increases from 10 to 90% with an increase in % wastewater from 0.8 to 13%. The sensitivity of the Nitrification test using sludge from Perstorp AB might be good, but the accuracy is low. This is due to the rather low nitrifying activity of the sludge, since the treatment plant at Perstorp AB is not constructed for nitrification. The accuracy of the Nitrification test using sludge from a municipal treatment plant is similar to that of the Algae test and the Biotox test.

In an investigation on inhibition of nitrification by industrial wastewater (Naturvårdsverket, 1996) 47 different samples from 9 Swedish industries were tested by the Nitrification test and the Biotox test. When the results of the Nitrification test and Biotox test were compared, it was clear that there was no correlation between the results of the tests. The deviation in the results could not be explained by the type of wastewater. It was concluded that the Biotox test could not be expected to describe the inhibiting effect of a given wastewater sample on nitrification.

Regarding inhibition of nitrification it can be seen from Table 2 that there is no significant difference between inhibition by flow proportional and grab samples, just as no significant difference between the usage of sludge from Perstorp AB and Lundtofte could be observed from the average EC values. The tendency of the Perstorp AB sludge to be more sensitive to inhibition by the wastewater than sludge from Lundtofte that can be seen in Table 2 is confirmed in Table 1, which shows the EC values from the single experiments. Here, in 7 out of 24 cases, the EC values for the Perstorp AB sludge are significantly lower than for the Lundtofte sludge.

For the Algae and the Biotox tests there is no significant difference between inhibition by flow proportional and grab samples, see Table 3 and 4. The grab samples could be expected to be more toxic than the flow proportional, since the extreme values for some parameters will be reduced in the flow proportional samples. Contrary to expectation, all three tests show a tendency for the flow proportional samples to be more toxic than the grab samples.

Experiments with binary mixtures of naphthalene and benzothiophene

Biodegradation experiments were performed with naphthalene and benzothiophene, each at concentrations of 4–6 mg/L (Dyreborg *et al.*, 1997; Dyreborg and Arvin, 1997). The compounds were degraded in batch systems as single compounds as well as a binary mixture with a culture previously exposed to creosote compounds. Toxicity was determined by the Nitrification test (Arvin *et al.*, 1994) and the Algae test (Halling-Sørensen *et al.*, 1996). Naphthalene was completely biodegraded within one week. No metabolites could be

Table 2 Average effective concentration (EC) values and their standard deviation for the Nitrification test. Measurements given as % of wastewater in the sample

Sample	EC10	EC20	EC50	EC90
All flow proportional	0.88 ± 0.66	1.18 ± 0.78	2.16 ± 1.26	6.40 ± 3.63
All grab	0.76 ± 0.41	1.22 ± 0.65	3.15 ± 1.44	15.9 ± 9.3
All (flow proportional and grab) using Perstorp AB sludge	0.65 ± 0.59	0.90 ± 0.69	2.00 ± 1.07	12.4 ± 12.5
All (flow proportional and grab) using Lundtofte sludge	0.95 ± 0.32	1.50 ± 0.52	3.64 ± 1.27	13.1 ± 4.61

Table 3 Average effective concentration (EC) values and their standard deviation for the Algae test. Measurements given as % of wastewater in the sample

Sample	EC10	EC20	EC50	EC90
All flow proportional	1.96 ± 0.79	2.22 ± 0.74	2.78 ± 0.61	3.75 ± 0.50
All grab	2.82 ± 1.71	3.25 ± 1.90	4.19 ± 2.20	6.06 ± 2.47

Table 4 Average effective concentration (EC) values and their standard deviation for the Biotox test. Measurements given as % of wastewater in the sample

Sample	EC10	EC20	EC50	EC90
All flow proportional	1.40 ± 0.37	1.85 ± 0.75	4.88 ± 3.08	74.6 ± 38.8
All grab	1.48 ± 1.08	2.22 ± 1.95	7.29 ± 7.76	68.5 ± 49.3

detected by GC-analysis. Naphthalene was not toxic to algae and nitrifying bacteria at the initial concentration and by the end of the experiments. Benzothiophene could not be biodegraded as a single compound and it was highly toxic to both nitrifying bacteria and algae. However, benzothiophene could be biodegraded co-metabolically in the binary mixture with naphthalene as the primary substrate. Both naphthalene and benzothiophene were degraded effectively from the initial concentrations of 4–6 mg/L to 0.02–0.04 mg/L. No metabolites could be detected by GC-analysis. During this process, the inhibition to nitrifying bacteria disappeared, whereas the toxicity to algae was only reduced from 90% to 40–60%. The strong residual toxicity to algae might be due to accumulation of one or more metabolites and it was actually observed that the concentration of non-volatile organic compounds (NVOC) increased from 0.8 to 4.5 mg/L during degradation in the binary mixture. One likely metabolite from benzothiophene is benzothiophenesulfone.

In order to establish a cause–effect relationship, separate experiments were performed to determine the effect-concentrations of naphthalene, benzothiophene and benzothiophenesulfone (Dyreborg *et al.*, 2004). From Table 5 it can be seen that residual concentrations at 0.02–0.04 mg/L of naphthalene and benzothiophene would not result in any significant toxicity to algae. However, if benzothiophene is transformed into benzothiophenesulfone, this could explain the high residual toxicity of the binary mixture to algae.

The experiment clearly demonstrates that although the traditional GC-analysis shows virtually complete removal of the parent compounds, and no residual toxicity should be expected from the parent compounds, a toxicity test shows that there is a strongly inhibiting metabolite left in the water. According to Table 5, the Biotox test did capture most of the toxicity estimated by the three toxicity tests.

The reported responses of the three toxicity tests to single or binary mixtures of chemical compounds are not well correlated. The different tests may give different responses to the

Table 5 Effective concentration (EC, mg/L) values and 95% confidence interval of naphthalene, benzothiophene and benzothiophenesulfone determined from dose–response experiments

Compound	Algae		Nitrification		Biotox	
	EC20	EC50	EC20	EC50	EC20	EC50
Naphthalene	6.2	9.4 [5.9–17]	4.8	9.0 [7.2–10.8]	0.6	2.3 [1.7–3.1]
Benzothiophene	4	5 [4–7]	0.6	1.4 [0.9–2.1]	0.7	1.8 [1.4–2.3]
Benzothiophenesulfone	2.3	2.9 [2.5–3.5]	50	196 [109–337]	4.7	14 [11–18]

chemicals. This is in accordance with the findings by Jonsson and Baun (2003) who compared the toxicity of mono- and diesters of *o*-phthalic esters assessed by the Biotox-test, the Algae-test and an immobilization test on the crustacean *Daphnia magna*. They found that none of the toxicity tests could be characterized as more sensitive than the others.

Conclusions

All three toxicity tests showed that the complex industrial wastewater was toxic to the nitrifiers, the aquatic bacterium *Vibrio fischeri* and the primary producer the green alga. It can therefore be expected that the wastewater will inhibit the biological processes at the treatment plant. The nitrification process is expected to be inhibited 20% at a dilution of the wastewater of 0.5 to 2%. Although the tests use different test organisms, the responses were surprisingly similar. Therefore, at Perstorp AB the toxicity test that is most easily available and the cheapest can be applied. However, this result is not general. Thus the recommendation for assessment of the toxicity of industrial wastewater, that is to be treated in a nitrogen removal plant, must be that the Nitrification test should be included in the evaluation.

Contrary to the results for the wastewater, the reported responses of the three toxicity tests to single or binary mixtures of chemical compounds were not well correlated. For a simple binary mixture of naphthalene and benzothiophene, it was not possible to establish a simple cause–effect relationship between the chemical measurements and the observed toxicity after degradation. Probably, a metabolite from the degradation of benzothiophene resulted in a residual toxicity although the parent compounds were effectively removed. For the experiment on single solutions of naphthalene, benzothiophene and benzothiophene-sulfone, the Biotox test was the overall most sensitive for the compounds investigated.

It is obvious that most industrial wastewater is too complex to have its toxic effect evaluated by chemical analysis. But here, it was demonstrated that even a simple binary mixture of chemical compounds can be too complex a system to be evaluated for its toxic effects by chemical analysis. On the other hand, the simple toxicity tests presented in this study can provide direct measures of the actual environmental toxicity of the system in question.

This study shows that generally no single toxicity test can be characterized as being more sensitive than the others. In order to capture any inhibition of relevant biological systems, at least two toxicity tests should be applied. Combinations of the Algae test and the Nitrification test or the Algae test and the Biotox test are useful indicators of the toxicity of wastewater. The choice of tests must depend on the system in question. If the effect on nitrogen removal is of interest, the Nitrification test should be one of two choices. If the effect of a treated wastewater on the recipient is of interest, the algae test would be a relevant choice.

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