Genotoxicity of the Musi River (Hyderabad, India) Investigated with the VITOTOX® Test

(Vitotox test / genotoxicity / toxicity / chemical analyses / Musi river / Hyderabad)

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Abstract. The bacterial VITOTOX® genotoxicity test was used to screen water samples collected from three different stations along the banks of the river Musi, in Hyderabad, India. Water was collected at three stations that differed from each other in the nature of the surrounding industrial and other activities. A number of different pollutants were also measured in water, soil and air samples. The three stations were found highly polluted and different with regard to the genotoxicity and toxicity of their samples. These results demonstrate the need for further biological studies in this area to generate valuable data on genomic instability, risk assessment of cancer, and to provide avenues for risk management.

As environmental consciousness increased, in the 60's and 70's concern arose about the effect of the open dumps on air pollution and especially on both surface and ground-water contamination (Delong, 1993). Since then studies on complex mixtures and their effects on living systems have gained a lot of significance. Genotoxic health effects, such as cancers, birth defects and reproductive anomalies, have been cited among persons living near a sanitary landfill site in Montreal (Al-Homsi, 1993), eventually drawing the attention of researchers to the field of environmental biomonitoring and environmental protection.

Because of the need for early detection of the environmental presence of genotoxic chemicals researchers have been working on developing several short-term genotoxicity assays that combine speed with low costs and accuracy. One of such tests based on bioluminescence was developed by the Flemish Institute for Technological Research at Mol, Belgium. This test, referred to as the VITOTOX® test, was shown to be simple, quick and cost-effective (Van der Lelie et al., 1997; Verschaeve et al., 1999). It requires only minimal amounts of a test compound and is therefore at present essentially used for product testing in the discovery phase of a new chemical. However, it may also be applied for other purposes, e.g., in research on eco(geno)toxicology (Brits et al., 2003; Verschaeve, 2002, 2005;).

A report of a study where the VITOTOX® test was performed on newly synthesized pharmaceutical compounds, or intermediate products in the synthesis of drug candidates, demonstrated that the test gives identical results when performed independently in two different laboratories and that it correlates well with either the Ames test or SOS-chromotest (Verschaeve et al., 1999; Van Gompel, 2001). According to the results of an international technical workshop the test was also considered as one of the most efficient tests among a battery of many tests (Corbisier et al., 2001). A Japanese study concluded that the VITOTOX® test could be used as a high-throughput genotoxicity assay (Muto et al., 2003).

The Musi river, which flows through Hyderabad city in South India, was once a source of drinking water to that city. It has now, over the years, become a dumping ground for wastes from sewage canals, slaughterhouses, hospitals and industrial effluents especially from textile and dye industries along with oil wastes from oil refineries. Approximately 120 million gallons of sewage are being discharged into the Musi river every day. The effluents are a complex mixture of hazardous wastes, such as mutagenic and carcinogenic heavy metals and polyaromatic hydrocarbons (PAHs), which may potentiate a major biological hazard (Rao, 1995). Hence, the present study was undertaken to screen for toxicity and genotoxicity of water samples collected from different stations along this highly polluted Musi river (Fig. 1). For this purpose the above-mentioned VITOTOX® test was used. As this test is performed with *Salmonella typhimurium* bacteria that lack the necessary oxidative enzyme systems for metabolizing for-

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Abbreviations: PAHs – polyaromatic hydrocarbons, S9 – post-mitochondrial supernatant, S/N – signal-to-noise ratio(s).

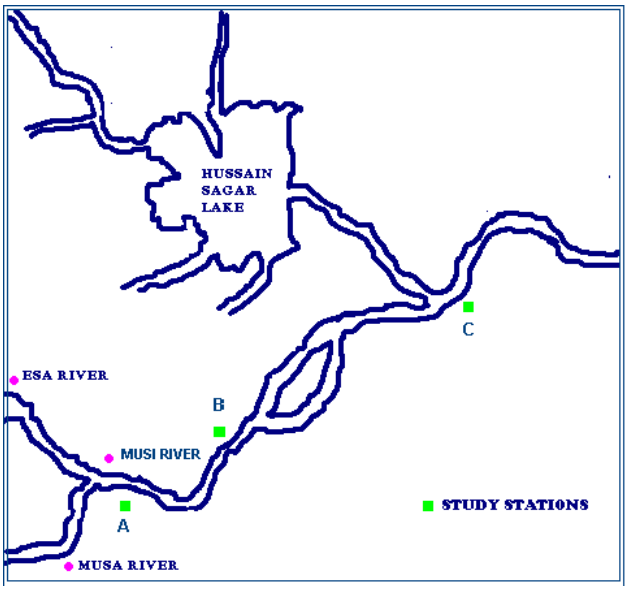


Fig. 1. Different sampling stations considered for the study located on the banks of river Musi.

eign compounds to electrophilic metabolites capable of reacting with DNA, the bacteria are, as for most other *in vitro* assays, treated with the test sample in the presence and absence of a post-mitochondrial supernatant ('S9' or 'microsome') fraction. This is prepared from the livers of aroclor-treated rats (purchased from Moltox®, Boone, NC).

Material and Methods

Sampling

The VITOTOX® test was used to assess the toxicity and genotoxicity of water samples collected randomly from three different stations (A–C) along the bank of the river Musi. The choice of the stations for this study was made based on the data gathered from a previous study, which suggested increased levels of air, water and soil pollution in these areas. Chemical analysis done at these stations showed that PAHs, Pb, Cr, H_2S, SO_2 and NO_X were present in rather high concentrations (Rao, 1995). Station "A" is located at the beginning of the river's path of flow into the city, where its banks are populated with small-scale industries such as textile and dye industries, and oil refineries whose wastes are dumped into the river. Station "B" is located near launderettes, slaughterhouses and a hospital which dump their wastes into the river directly. Station "C" is at an area populated with people of low income who lack proper drainage facilities and thus release domestic and sewage wastes into the river Musi. The river is now also known to carry industrial effluents, which may be partially processed before being released into the river.

The water samples from the above stations were collected, micro-filtered and processed for genotoxicity testing. Due to the expected high pollution load and the demonstrated sensitivity of the VITOTOX® test (Van der Lelie et al., 1997) we did not concentrate the pollutants in the water samples using XAD-resins or other concentration tools as is usually done. On the contrary, a number of dilutions of the raw sample were tested.

VITOTOX® test

The VITOTOX® test is a bacterial luminescence test aimed at detecting genotoxic and toxic chemicals or genotoxicity and toxicity of environmental samples (water/soil/air).

Principle. Ames test bacteria were transformed with a plasmid containing a "lux operon" that was placed under the control of the bacterial SOS system (TA104, recN2-4). This gene is normally not transcribed (no light production), but linked to the *recN* gene it will be "switched on" when the bacteria are exposed to a genotoxic compound (mutagen or SOS-inducing substance) (Van der Lelie et al., 1997).

As it was realised that some compounds may act directly on the light production or enhance the metabolism of the bacteria, creating false-positive results, a constitutive light-producing strain with a *lux* operon under the control of a strong SOS-independent promoter (*pr1)* was also constructed. This is used as an internal control system. For example, increased light production in TA104 (recN2-4) will only be considered indicative of genotoxicity if it is not accompanied by comparable increased light production in the pr1 strain (Verschaeve et al., 1999; Verschaeve, 2005).

Cultures. Bacteria were incubated overnight on a rotative shaker at 37 °C in a normal bacterial growth medium supplemented with extra $CaCl_2$ to allow optimal bacterial growth. The next morning the bacterial suspension was diluted 10 times in medium and 50 µl of the suspension was then inoculated in 2.5 ml medium and incubated for one more hour at 37 °C on a rotative shaker (170 rpm).

Preparation of the 96-well plates. Ninety-six-well plates were prepared so as to contain 10 µl of different dilutions (1/10,1/20,1/40) of Musi river water samples for genotoxicity testing. A single well was used per dilution or replicate. The samples were tested in the presence and absence of a metabolizing S9mix fraction.

For tests with S9 mix, 140 µl of the bacteria (recN2-4 or pr1) were added to 860 µl of medium and 400 µl of S9 mix. From the above mixture, 90 µl were then added to the 10 µl solution of the test sample with different concentrations already present in the wells. Without S9 mix 1,260 µl of growth medium were added to 140 µl of the bacterial suspension (recN2-4 or pr1) and 90 µl of the mixture were then transferred to wells containing 10 µl of the test sample with different concentrations.

Genotoxicity and toxicity measurements. A detailed description of the VITOTOX® test procedure is given

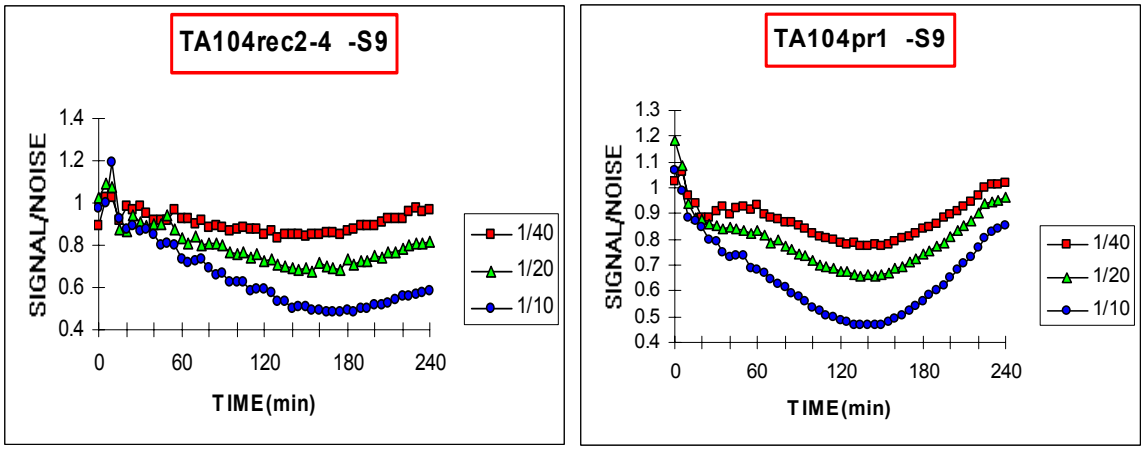
elsewhere (Verschaeve et al., 1999). In short, a 96-well microplate luminometer (Microlumat LB96P from EG and G Berthold, Vilvoorde, Belgium) was used for measurements of light production following exposure to the test samples. Light emission from each of the wells was measured every 5 min during a period of 4 h (30 °C, 1 sec/well, 60 cycles of 300 s each). After completing the measurements, the data were transferred into an Excel (Microsoft, Redmond, WA) macrosheet and the signal-to-noise ratios (S/N) were plotted as a function of the incubation time. A genotoxic effect is obtained when the ratio of the maximum S/N of the recN strain versus the maximum S/N of the pr1 strain (rec/pr1) reaches levels larger than 1.5 and when a clear dose-dependent relationship is observed. In the case of toxic effects, the maximum S/N for the pr1 strain becomes considerably smaller than 0.8.

Results

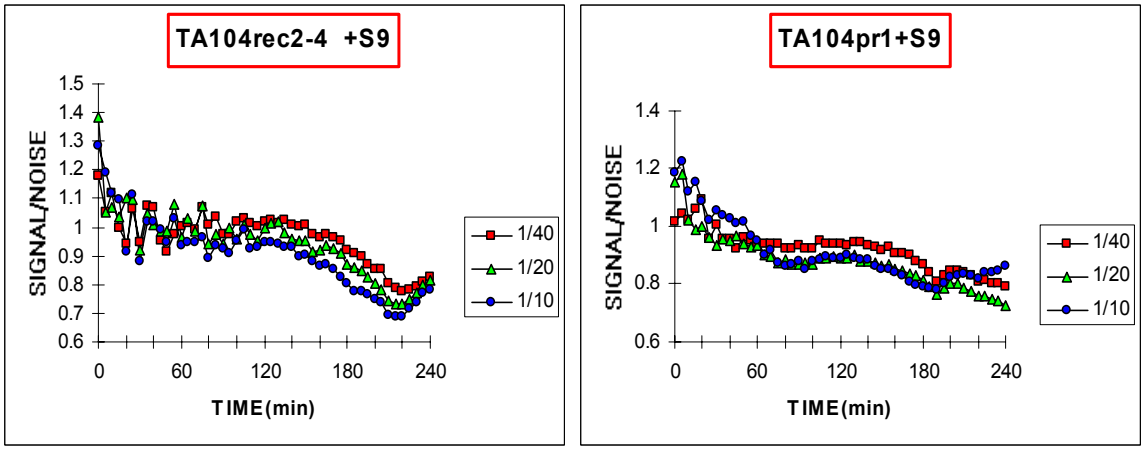
Samples from station A tested without S9 mix showed no genotoxicity, but clearly indicated toxicity as higher doses gave lower responses in both the pr1 and recN2-4 strains and as S/N ratios decreased to approximately 0.5 (Figs. 2, 3). Also with S9 mix, toxicity but no genotoxicity was detected (Figs. 4, 5).

The sample analysed from station B without S9 mix showed a clear dose-response relationship in the recN2-4 strain with a S/N ratio of > 10.4 after 120 min in a dilution of 1/10, and S/N ratio of > 6.4 and 4.4 in dilutions of respectively 1/20 and 1/40 (Fig. 6). The pr1 strain indicated a maximal S/N ratio of > 1.6 for sample dilution 1/10 and 1/20 and about 1.4 for the 1/40 dilution (Fig. 7). This sample thus gave in the recN2-4 strain an S/N ratio much higher than in the constitutive strain pr1, which indicates a clear genotoxic dose response, although a somewhat increased light production is also found in the pr1 strain where light induction is independent of SOS induction. Besides genotoxicity the highest concentration (lowest dilution) also showed a somewhat toxic response (pr1: S/N < 0.8) (Fig. 7).

The sample tested with S9 mix exhibited an S/N ratio greater than 6 at 120 min (Fig. 8). The effects are thus less important (but yet substantial) than without S9 mix, which is indicative of reduced genotoxicity due to gradual detoxification at all concentrations. Yet, dilution 1/10 still showed severe toxicity (pr1: S/N < 0.8) (Fig. 9).



Figs. 2, 3. VITOTOX® results for the strains TA104 rec2-4 and TA104 pr1 without addition of S9 mix at station "A".



Figs. 4, 5. VITOTOX® results for the strains TA104 rec2-4 and TA104 pr1 with the addition of S9 mix at station "A".

Samples from station C tested without S9 mix indicated no genotoxic effect as the maximal S/N ratios of recN2-4 strain never reached values above 1.5 (Fig. 10). The pr1 strain showed S/N ratios similar to the recN-2-4 strain. Dilution 1/10 showed a mild toxic response as S/N in pr1 reached values lower than 0.8 (Fig. 11).

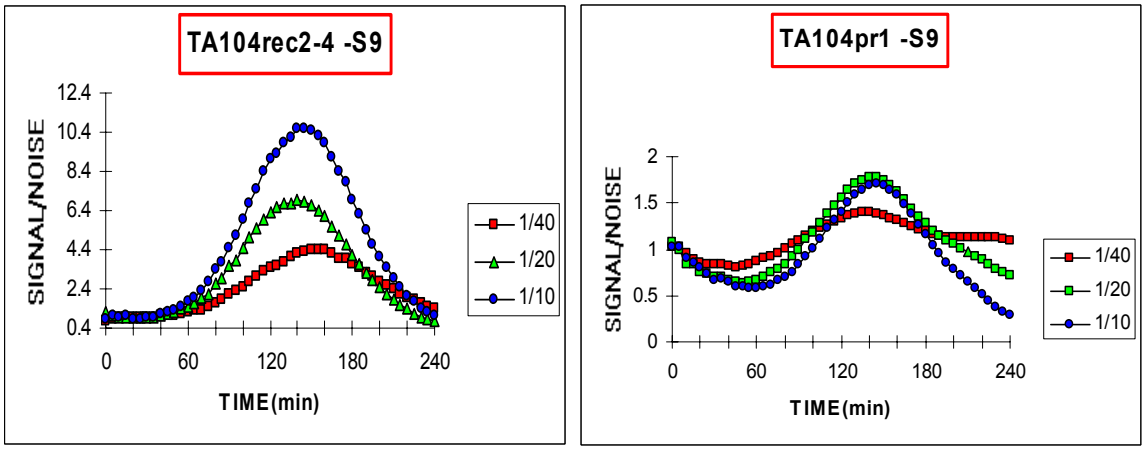
Dilutions of 1/10 and 1/20 showed a genotoxic response in the presence of S9 mix, as the S/N in recN2-4 reached values up to 2.4 and 1.5, respectively. The 1/40 dilution was not genotoxic as almost no increased light production was found over the 240 min period (Fig. 12). In the pr1 strain, S/N fluctuated around 1, indicating no toxicity or false-positive genetic effect. Samples from station C are thus non genotoxic without S9, but appeared genotoxic in the presence of the metabolic enzyme fraction (Fig. 13).

Table 1 presents a summary report on genotoxicity and toxicity evaluation together with concentrations of a number of pollutants that were measured in randomly collected water, soil and/or air samples. It appears that overall the lowest concentrations of pollutants were found at station A (which showed toxicity but no genotoxicity). The pollution level at stations B and C was comparable and considerably higher than at station A. Several measurements exceeded international standard levels.

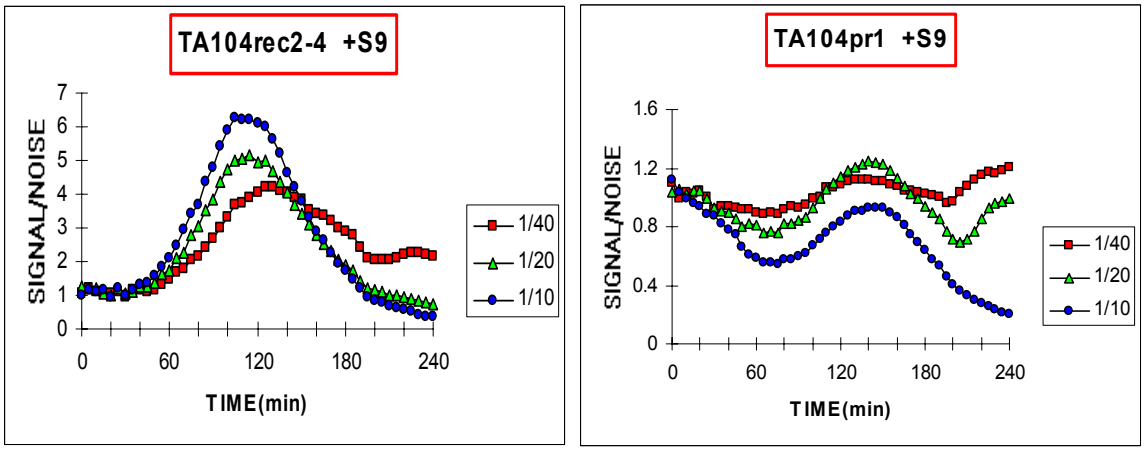
Disscussion

The Musi river was a source of drinking water until the 1950s. However, over this period, many factors like rapid urbanization have reduced this river into a stream of filth. The river became heavily contaminated with domestic sewage and industrial effluents besides air pollution caused by industrial smoke, automobile exhaust and fowl smell from the river itself. Water of the Musi river is highly discoloured by the presence of inorganic metals from the industrial effluents, which are directly released into the river. Effluents from Kattedean, Jeedimetla and Nacharam industrial estates are dumped into the river either in an untreated or partially treated form. These areas are abounding in textile, dye industries and oil refineries.

It is well known that many hazardous industrial pollutants cause genetic damage, while the genetic damage due to domestic pollutants is less well studied. Since sewage consists essentially of organic material and nutrients, along with potentially toxic components such as metals



Figs. 6, 7. VITOTOX® results for the strains TA104 rec2-4 and TA104 pr1 without addition of S9 mix at station "B".



Figs. 8, 9. VITOTOX® results for the strains TA104 rec2-4 and TA104 pr1 with the addition of S9 mix at station "B".

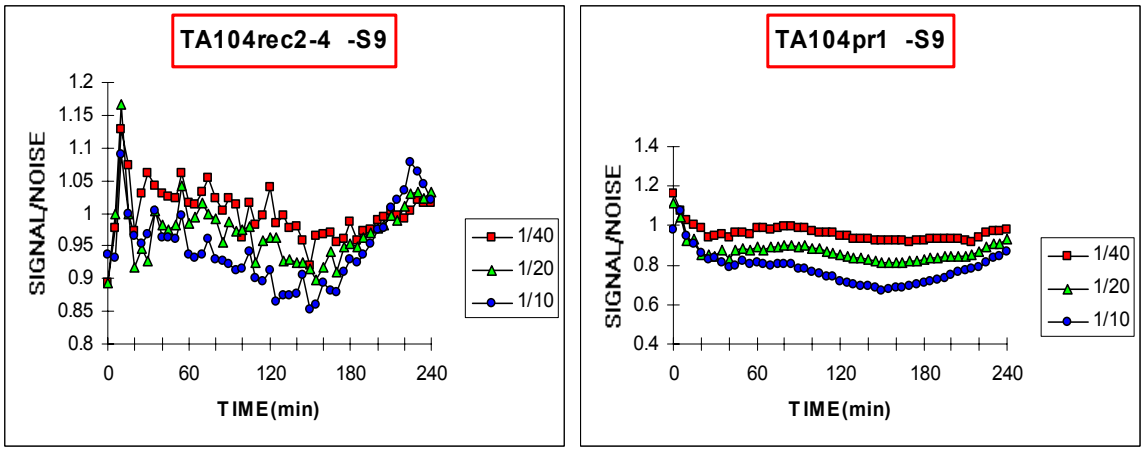
and organohalogens, eutrophication and toxic effects might be expected (Reddy et al., 2002). This assertion reflects a growing concern, mainly over the pollution caused by toxic chemicals in sewage and sludge. Studies by Hopke (Hopke et al., 1982, 1984) have shown that the municipal sewage sludge from Chicago was mutagenic in the bacterial Ames test. The herbage grown on the soil treated with sludge indicated a significant increase in the levels of heavy metals (copper, lead, nickel, zinc and cadmium) as compared to the herbage grown on the control soil. Crop plants grown on sludge-treated soil can bioconcentrate these heavy metals.

In the present study, water samples were collected from three different stations (A, B and C) located along the Musi river, which is highly polluted with complex mixtures of sewage and industrial wastes (Table 1).

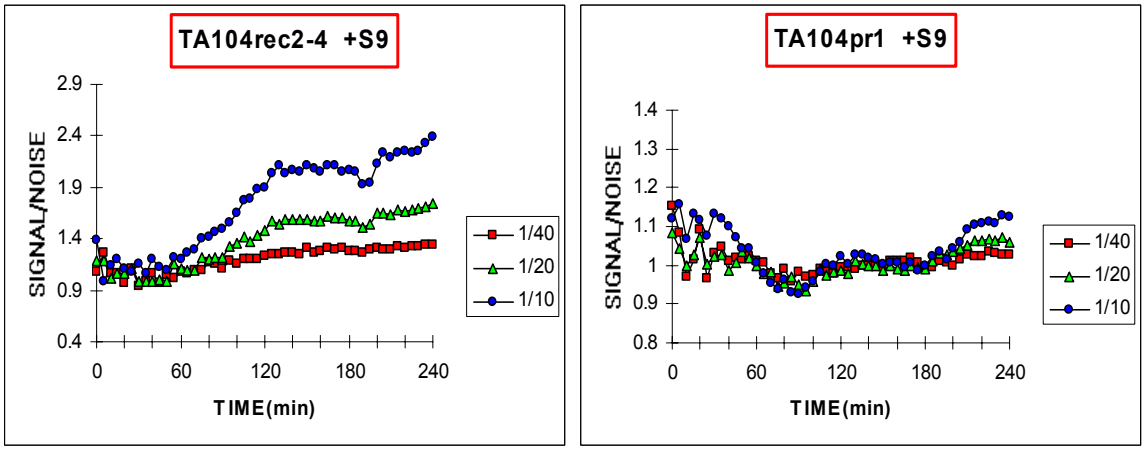
Among the three stations (A, B and C) studied for toxicity and genotoxicity using the VITOTOX® test, the results indicated that water samples collected from station A (oil refineries and textile and dye industries) were highly toxic both with and without S9 mix. Therefore, genotoxicity cannot be completely excluded although none was found (a genotoxic response can be 'masked' by toxicity). Station B (launderettes, slaughterhouses and hospital) only indicated genotoxicity directly without addition of S9 mix, and station C (domestic sewage) showed no genotoxicity without S9 mix but expressed its genotoxic nature after addition of S9 mix.

A number of important remarks should be made. First, it should be noted that each VITOTOX® experiment comprises the use of a "positive control". In the present investigation this was 50 µg/ml 4-nitroquinoline-oxide (4NQO) (without S9) and 12 µg/ml benzo(α)pyrene (with S9). Typically, these compounds give an S/N in recN2-4 of about 6 and 4, respectively (e.g. Elgorashi et al., 2003). This means that the water samples from Musi river, especially from station B, should be considered extremely polluted by genotoxic compounds as S/N reached higher levels in these samples compared to the positive controls. The high levels of pollutants (Table 1), most of them being recognized mutagens, corroborates this.

It should also be stressed that the water samples were tested in different dilutions and that no concentration procedure was applied. This is however required for most, if not all, currently employed genotoxicity tests.



Figs. 10, 11. VITOTOX® results for the strains TA104 rec2-4 and TA104 pr1 without addition of S9 mix at station "C".



Figs. 12, 13. VITOTOX® results for the strains TA104 rec2-4 and TA104 pr1 with the addition of S9 mix at station "C".

Table 1. Results from chemical analyses along the different stations performed at the time of sampling for genotoxicity and toxicity assessment

	Toxic	Genotoxic	Chromium (ppb)		Lead (ppb)		Air concentrations (µg/m^3)				PAHs (µg/l/kg/m^3)	
			In water	In soil	In water	In soil	H_2S	SO_2	NO_x	PAHs	Water	Soil
Station A	+	-	0.018	0.134	0.003	0.045	1.6	6.0	3.0	0.05	12.13	5.2
Station B	+/-	woS9–/wS9+	0.0059	0.478	0.042	0.33	155.1	9.26	22.83	4.81	157.33	139.4
Station C	-	woS9–/wS9+	0.109	0.348	0.053	0.526	133.73	7.6	19.23	1.43	137.03	127.1

(– non toxic or genotoxic; + toxic or genotoxic; woS9 = without S9; wS9 = with S9).

Surface waters in Europe are for example often concentrated up to 25 000 x times before application of an Ames assay or other genotoxicity test (e.g. Penders and Hoogenboezem, 2001). As it was previously shown that the minimal detectable concentrations of a genotoxic compound can be up to several orders of magnitude lower with the VITOTOX® test compared to other bacterial tests (Van der Lelie et al., 1997) and because of the expected high pollution load, omission of a time-consuming concentration procedure was justified. It was found that such concentration step was indeed not necessary and that even dilutions were needed. The fact that the VITOTOX® test was able to detect genotoxicity in the raw surface water thus underlines both the high pollution level of Musi river and the sensitivity of the test. It may also be interesting to note that the tests were performed twice and gave exactly the same results. As a matter of fact, VITOTOX® test results were shown earlier to be remarkably reproducible (e.g. Verschaeve et al., 1999).

A further remark is that it should be kept in mind that the river Musi contains complex mixtures of chemicals and that these may have different interaction patterns with the VITOTOX® bacteria. Some of them may directly influence the *lux* operon, resulting in a false toxic response. In the past, VITOTOX® tests were performed on many different samples. The test was found very suitable for testing pure compounds (e.g., pharmaceutical compounds and industrial chemicals) and environmental samples, as far as no extraction or concentration procedure was applied (e.g., Verschaeve, 2002, 2005). When extraction and concentration procedures were used, severe toxicity was often found. This may be due to the presence of residues of the solvents that were employed (Verschaeve, 2002, 2005; Elgorashi et al., 2003) or to the complex nature of the sample itself. Maybe this is the reason why the less polluted site (station A) surprisingly showed the highest toxicity. No solvent residues were present as no solvents were employed, but the sample from station A might be so complex that it inhibits the activity of the lux operon, resulting in a "toxic-like" response. At the stations B and C this apparently is not (or much less) the case.

In conclusion, this study confirmed the presence of a complex mixture of toxins and genotoxins in the river water, making it a potential area for further follow-up study. Due to the complex nature of the samples, other genotoxicity tests that are not based on bioluminescence might be necessary to confirm our preliminary results.

Acknowledgement

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