BIOSENSOR BASED TOXICITY DISSECTION OF TANNERY AND ASSOCIATED ENVIRONMENTAL SAMPLES

by

MWINYIKIONE MWINYIHIJA, NORVAL J. C. STRACHAN, ANDY MEHARG AND KEN KILLHAM

School of Biological Sciences, Plant and Soil Sciences,

University of Aberdeen

ST. MACHAR DRIVE, CRUICKSHANK BUILDING

ABERDEEN, AB24 3UU, U.K

ABSTRACT

Kenyan tannery and associated environmental samples were selected for ecotoxicological assessment. A tool-kit of techniques was developed, including whole-cell biosensor and chemical assays. A luminescence based bacterial biosensor (*Escherichia coli* HB101 pUCD607) (via a multi-copy plasmid) was used for toxicity assessment. Samples were manipulated prior to biosensor interrogation to identify the nature of the toxic contaminants. Untreated samples (before any manipulations) showed a strong toxic effect at the discharge point in comparison to other sampling points. Sparging was used to identify toxicity associated with volatile organics. The toxicity of contaminants, removed by treatment with activated charcoal was identified for all the sampling points except for those upstream of effluent discharges. Filtration identified toxicity associated with suspended solids. Changes in availability of toxic contaminants due to pH adjustment of most samples from the tannery effluent treatment pits were also associated with the extreme pH values (4.0 and 8.0). The approach used has highlighted the complexicity of toxic pollutants in effluent from the tanning industry and the dissection of toxicity points to possible remediation strategies for effluents from the tanning industry.

ABSTRACTO

Muestras tomadas de una curtimbre y sus entornos localizada en Kenia fueron seleccionadas para un aforo eco-toxicológico. Una caja de herramientas técnicas fue desarrollada, incluyendo un biodetector a nivel celular y agentes aforadores para químicos. Un biodetector bacterial basado en luminiscencia (*Escherichia coli* HB 101 p UCD607) (por medio de una multicopia plasmídica) fue empleado para la evaluación de toxicidad. Las muestras fueron manipuladas anterior al examen por biodetector para averiguar la naturaleza de los contaminantes tóxicos. Muestras vírgenes (antes de la manipulación) indicaron un fuerte efecto tóxico en el sitio de descarga comparado con los otros sitios de muestreo. Dispersión fue utilizada para identificar la toxicidad asociada con sustancias orgánicas volátiles. La toxicidad de contaminantes removidos por tratamiento con carbón activado fue identificada para todos los puntos de muestreo con excepción de aquellos localizados río-arriba de los sitios de descarga de efluentes. Filtración identificó la toxicidad asociada con los sólidos en suspensión. Cambios en la disponibilidad de contaminantes tóxicos debido a ajustes de pH de la mayoría de los fosos de tratamientos fueron también relacionados con los valores extremos del pH (4.0 y 8.0). Este enfoque empleado enfatiza la complejidad de la contaminación tóxica de la industria curtidora y la identificación de la toxicidad puntual podrá ocasionar estrategias para sanear los efluentes de la industria curtidora.

INTRODUCTION

Tanning is a major polluter worldwide and tannery wastewater, in particular, is a potential environmental pollutant.[1] It can cause a serious environmental impact on water systems with its high oxygen demand, discoloration and toxic chemical constituents.[2]

Tannery waste characteristically contains a complex mixture of both organic and inorganic pollutants. For

Corresponding author: Mwinyikione Mwinyihija, Tel.: +44 1224 272260 Fax: +44 1224 272703
E-mail: m.mwinyihija@abdn.ac.uk
Manuscript received November 1, 2004, accepted for publication January 20, 2005
This paper is not a duplicate of the paper published on pages 380ff in the October 2005 issue of *JALCA*.

example, chlorinated phenols and chromium are closely associated with the waste.[3] Chromium is a transition metal and exists in several oxidation states, with Cr(III) and Cr(VI) species being the most common forms.[4] These two species are known to differ from each other in chemical properties.[5] Chlorinated phenols (e.g. 3,5-dichlorophenol) are highly toxic and affect the cellular compounds of organisms.[6]

Because tannery wastewater is a complex mixture of pollutants including chromium and chlorinated phenols, it is vital to dissect the toxic nature of such wastewater both to understand its environmental impacts and identify potential remediation strategies. Furthermore, there are strict regulations imposed for the environmental control of pollutants such as heavy metals and persistent organic pollutants.[7] Tannery wastewater is generally treated by various physico-chemical and biological methods and by a combination of both.[3] Physical and chemical processes are frequently employed to treat contaminated sites, but often do not destroy contaminants.[8] Although chemical analysis of environmental samples can produce a measure of the total concentration of the chemical present in the sample, it will not indicate what is bioavailable in the sample[9] and hence what the likely toxic impact will be. Bioavailability is important for many organic compounds, particularly when they are associated with humic and other natural complexing agents.[10] Bioremediation is practiced by optimising environmental conditions to stimulate the degradation of the pollutants by naturally occurring microorganisms. However, bioremediation is limited in the materials that it can treat and by conditions at the treatment site.[11]

There is an increasing trend towards the use of biological techniques for monitoring the hazards associated with environmental pollution by industry and regulators alike. This is particularly applicable to components of tannery waste. Chromium toxicity has been investigated using biological techniques involving microorganism[12,13,14,15,16] and plants.[17] The toxicity of chlorinated phenols has also been assessed by bioluminescence-based ecotoxicity tests.[18]

Lux (reporter genes encoding marine bacterial luminescence (i.e. light production)) bacterial biosensor technology has been used to measure the toxicity of heavy metals in a number of matrices ranging from aqueous solutions of single compounds to industrial effluents.[19] The light emission intensity is proportional to the concentration of the toxic analyte over a certain concentration range, allowing one to perform a quantitative analysis. For example, Paton *et al.*,[20] used the luminescence response of a chromosomally *lux*-marked bacterium, *P. fluorescens*, to assess the toxicity of metal salts. Chaudri *et al.*[21] used the luminescence response of *lux*-marked bacteria to assess the toxicity of zinc in pore water in a long-term sewage sludge field. Galli *et al.*,[22] used naturally luminescent marine bacteria (*Microtox*) to test the toxicity of soil from a site contaminated with various pesticides, dyes and other chemicals.

The advantages associated with the use of genetically modified bacterial biosensors over other forms of ecotoxicity testing are that they are rapid, sensitive, easy to culture and maintain, flexible in terms of selecting for environmental relevance, and reliable tools that integrate the many factors contributing to environmental toxicity.[23]

Lux bacterial biosensor assays of toxicity can be linked to sample manipulation to assess the scope and the nature of possible remediation strategies. Sousa *et al.*[11] used sample manipulation (coupled to bioassay with *lux*-marked bacteria) to examine the toxicity of a site contaminated with BTEX (benzene, toluene, ethylbenzene, xylene) compounds. The use of biosensors enabled reporting on site toxicity characteristics and contaminant bioavailability. The manipulations included sparging, filtration, muffle furnace and pH adjustment of the sediment samples. This enabled any constraints to bioremediation (such as adverse pH, heavy metals or volatile organics) to be identified.[19] In the case of environmental constraints, success is only likely to be attained if these constraints are identified and means are devised to alleviate them to an extent where bioremediation can effectively proceed.[24] The use of the *lux* biosensor *E. coli* HB101 pUCD607 in relation to sample manipulation, allowing dissection and classification of sample toxicity, has not previously been applied to tannery waste.

The aim of this study was to use a biosensor-based approach to dissect the toxic nature of effluent and environmental samples (river sediments and water) from the tannery industry and to use sample manipulation coupled to biosensor toxicity assay to identify possible remediation strategies for future environmental protection.

EXPERIMENTAL

All chemicals used were of analytical-reagent grade and all test solutions were prepared using double deionised water. The method of sample manipulation (Fig. 1) to examine the toxicity of the samples was modified from that reported by Sousa *et al.*[11] Determination of toxicity was based on the bioluminescence response of the *lux*-modified biosensor, *E. coli* HB101 pUCD607 which was marked with the *lux* CDABE genes (*lux* A and B are structural genes for the luciferase enzyme involved in light production; *lux* C,, E and D are the genes involved in syntheis and recycling of the fatty acid substrate for the enzyme), isolated from *Vibrio fischeri* using the multi-copy plasmid pUCD60725.[27] The biosensor was stored at -20°C and resuscitated from freeze dried prior to bioassay.

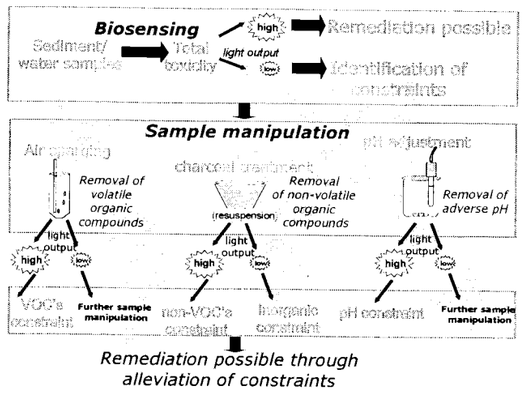

Figure 1. - Schematic representation of toxicity dissection in identifying VOC's (volatile organic compounds), non-VOC's, inorganic, and pH constraints in Kenyan tannery effluents and riverine samples

Samples were collected from a Kenyan riverine site and tannery effluent pits and lagoons. Sediments and effluent samples were collected in 20 ml super polyethylene vials. Bottles were filled to the brim and carefully capped without air bubbles. During storage and transportation from the field, they were stored in the dark at 4°C and tested within four weeks of sampling. Triplicate 900 µl aliquots of each sample were taken using a Gilson pipette for biosensor test immediately after opening the bottles and the bioassay carried out immediately. Additional aliquots were taken for pH measurement and to be submitted subsequently to a series of protocols in order to identify underlying toxic constraints to remediation and assess the scope for their alleviation.

Preparation of sediment/effluent samples

Sediment extraction during this study, was performed using a method adapted from that described by Matthews.[26] Samples (10 g) of the Kenyan riverine sediment were weighed into a centrifuge tube and 20 ml of deionised water (Millipore-Q, Quantum™ EX ultra pure organex cartridge) added (in triplicate). The pH of the samples was measured using a standard pH electrode (Hanna HI 8424, Norlab Instrument Ltd., Aberdeen, U.K). The samples were placed in an end-over-end shaker for 30 minutes (did not affect subsequent biosensor response) and then centrifuged at 1100 g for 15 minutes. Parallel extractions for toxicity dissection were carried out and triplicate samples taken from the supernatants immediately prior to the bioassay.

Toxicity dissection

i.) Sparging

Aliquots (900 µl) of effluent and sediment extract were pipetted into luminometer cuvettes and sparged with air (N_2) for 10 minutes (at 1650 ml/min). The high flow of air allowed for rapid removal of volatiles present.

ii.) Activated charcoal treatment

Charcoal was first conditioned by placing 100 g of charcoal in a Duran bottle filled with double deionised water and allowed to stand for 48 hours, prior to multiple (x10) deionised water rinsing and recovery by filtration.

10 ml of the effluent or sediment extract was placed in a centrifuge tube. Charcoal (0.1g) was added and shaken for 30 minutes. Samples were centrifuged (3000 g) for 20 minutes and toxicity of the supernatant tested by transferring 900 µl of the sample into a luminometer cuvette (Clinicon, Petworth, West Sussex, U.K.) and adding 100 µl of resuscitated biosensor suspension.

iii.) Filtration

Aliquots of water samples and sediment extracts (50 ml) were filtered through a cellulose acetate membrane filter (0.22 µm pore diameter) in order to determine toxicity related to suspended solids. Triplicates of each sample were submitted to the bioassay immediately after filtration.

iv.) pH adjustment

Aliquots of the water samples (pH 5.5) and effluent/sediment extracts were adjusted to pH 4.0, 6.0 and 8.0 with 0.1M sodium hydroxide and hydrochloric acid. Triplicates of the samples were submitted to the bioassay immediately after pH adjustment.

Resuscitation of freeze dried cultures

Freeze-dried cultures of *Escherichia coli* HB101 pUCD607 were resuscitated in 10 ml of sterile 0.1 M KCl (contained in a Universal bottle). A 1 ml aliquot of KCl was added to the culture, which was resuspended by mixing (drawing up and down 5 times into a PI000 Gilson pipette). The resuspended culture was transferred back to the universal and the culture placed in a shaking (200 rpm) incubator (25° C) for an hour.

Sample addition and luminometry measurements

After incubation of the resuspended culture, 100 µl of the resuscitated biosensor was added to the samples at 15 seconds intervals, accurately timed for measurement in the Bio Orbit 1253 luminometer (Labtech International, Uckfield, U.K). Each riverine and tannery sample was exposed to the sensor for exactly the same time. Samples (exposed to the resuscitated biosensor) were further incubated for 15 minutes before light output measurements were carried out at 15 seconds intervals. This ensured the same exposure time to the potentially toxic elements for cells in each of the cuvettes.

Chemical analysis

Aliquots (2.5 ml) of concentrated HNO_3 (69% Analar grade) were added to 200 mg dry riverine sediments from a

Kenyan site and weighed into 75 ml digestion tubes and allowed to stand overnight at 15°C. The following day, the digestion tubes were placed on a heating block and the temperature was gradually raised to 100°C for 8 h. The samples were allowed to digest for three hours, after which, the volume was reduced to 3-4 ml. The digest was cooled at room temperature, and diluted to 10 mL with double deionised water in graduated tubes. Total concentrations of Pb, Cu, Zn, Fe, Ni, Cd, and Cr were determined using atomic absorption spectrometry (Perkin Elmer Analyst 100). Total phenols were determined by gas chromatography.

Statistical analysis and results calculation

The output from the Bio Orbit 1253 luminometer produced after each assay carried out was recorded in relative light units (RLU's) (luminometer readings correspond to mV/10 s/ml).[27] The light output was then converted to percentage maximum bioluminescence. This was calculated against a blank of double deionised water adjusted to pH 5.5, the optimum pH for bioluminescence.[20]

$$\% \text{ maximum bioluminescence} = I_S/I_{C*100} \qquad (1)$$

where

I_S = RLU's emitted by the cells exposed to the sample

I_C = RLU's emitted by the cells exposed to the control

The percentage maximum bioluminescence was determined for the three sample replicates. A mean of this determination was then calculated. The assay performance was monitored by the response to the control, the reproducibility of the response to the three replicates, and the response to a standard of trichlorophenol (TCP).

The experimental data were analysed by a one-way analysis of variance (ANOVA) and regression analysis using Minitab.

RESULTS

A toxic substance generates a reduction in light output proportional to the concentration present. In this study, the percentage maximum bioluminescence results for all the treatments (sparging, activated charcoal, filtration and pH adjustment) were calculated against a blank of double deionised water at pH 5.5. The response to sparging suggests that toxicity could be due to VOC's (volatile organic compounds), activated charcoal to certain inorganics and organics (particularly chlorinated hydrocarbons), and filtration to particulate and colloidal fractions.

Ecotoxicity assessment

Generally, before any manipulations were carried out (untreated samples), high toxicity ($p \leq 0.05$) was observed for the samples obtained from the effluent treatment pits (Table I). Tannery samples from the beam house, chrome stripping and general sedimentation pits showed extreme toxicity. Results from the different effluent treatment pits were all significantly different ($p \leq 0.05$). This observation was similar for the analysed samples obtained from the anaerobic lagoons (except for lagoon 3) (Table II). However, the riverine samples showed higher % bioluminescence values ($p \leq 0.05$), with the exception of the discharge point (64%) (Table III).

TABLE I

Percentage Maximum Bioluminescence of Effluent Treatment Pits Untreated and after Treating with Sparging, Activated Charcoal, Filtration and pH Adjustment Calculated against a Blank of Double-deionised Water. Figures in Parentheses are Standard Errors of the Mean (n=9).

Samples	No Treatment means	N_2 Sparged means	Activated charcoal means	Filtration means	pH adjustment 4.00	6.00	8.00
Beam house	0.06	101.82	125.14	63.15	4.32	83.42	0.04
	(0.04)	(8.2)	(1.06)	(0.83)	(0.72)	(1.68)	(0.04)
General Sedimentation	2.40	141.21	167.53	105.56	4.72	202.00	2.40
	(1.02)	(2.40)	(2.20)	(1.61)	(0.81)	(23.37)	(1.02)
Strip Chrome Tank	0.004	84.67	65.26	0.01	1.83	43.29	0.00
	(0.004)	(1.40)	(0.90)	(0.003)	(1.05)	(2.20)	(0.001)
Chrome sedimentation	26.26	120.84	34.36	0.43	169.62	284.10	26.26
	(12.70)	(8.3)	(1.50)	(0.02)	(33.7)	(8.10)	(12.67)
Equalisation. Tank	6.83	46.36	39.14	5.88	0.03	36.77	6.83
	(2.60)	(46.4)	(0.9)	(0.34)	(0.02)	(1.81)	(2.56)
Reference (Ddw)	104.85	94.75	101	99.97	102.87	107.29	114.24
	(5.36)	(0.49)	(1.61)	(1.13)	(3.82)	(4.06)	(2.70)
LSD (5%)	18.27	18.23	4.39	2.61	42.71	31.80	16.66

Ddw - Double deionised water

TABLE II

Percentage Maximum Bioluminescence of Anaerobic Effluent Treatment Lagoons Untreated and After Treating by Sparging, Filtration and pH Adjustment.

Samples	No Treatment	N_2 Sparged	Activated charcoal	Filtration	pH adjustment 4.00	6.00	8.00
Lagoon1	27.86	90.89	70.92	149.32	2.10	4.86	115.11
	(7.0)	(9.93)	(3.60)	(1.70)	(0.78)	(0.11)	(21.24)
Lagoon2	34.07	87.07	122.43	139.58	1.40	56.00	116.49
	(21.2)	(8.00)	(22.55)	(6.64)	(0.36)	(1.45)	(23.14)
Lagoon3	77.08	89.39	154.07	145.62	1.02	4.89	91.76
	(23.1)	(13.5)	(3.44)	(1.67)	(0.35)	(0.49)	(0.88)
Lagoon4	8.39	100.54	104.92	94.56	15.17	182.65	138.23
	(0.90)	(7.70)	(20.30)	(1.50)	(0.91)	(0.70)	(0.50)
Lagoon5	1.83	95.59	69.88	104.77	0.62	3.16	110.74
	(0.50)	(11.70)	(10)	(1.22)	(0.62)	(0.70)	(0.39)
Reference (Ddw)	104.85	94.75	101	99.97	102.87	107.29	114.24
	(5.36)	(0.49)	(1.61)	(1.13)	(3.82)	(4.06)	(2.70)
LSD (5%)	45.37	32.72	45.52	10.29	1.85	2.43	44.29

Ddw = Double deionized water

Figures in Parentheses are Standard Errors of the Mean (n=9).

TABLE III

Percentage Maximum Bioluminescence of Riverine Sediments Untreated and after Treating by Sparging, Activated Charcoal, and Ph Adjustment Calculated against a Blank of Double-Deionized Water.

Samples	No Treatment	N_2 Sparged	Activated charcoal	Filtration	pH adjustment 4.00	6.00	8.00
200 m upstream	97.13	96.33	82.54	100.67	86.38	71.83	49.80
	(2.5)	(7.4)	(15.0)	(4.04)	(1.4)	(3.1)	(11.1)
100 m upstream	87.40	85.86	46.80	99.26	85.84	74.37	35.75
	(9.3)	(1.0)	(3.0)	(3.02)	(4.6)	(8.1)	(2.6)
0 m discharge point	64.01	57.06	85.30	115.68	102.16	74.26	1.15
	(6.0)	(7.3)	(8.6)	(1.81)	(3.2)	(3.9)	(0.2)
100 m downstream	90.86	88.93	65.56	105.07	68.65	72.73	24.28
	(3.8)	(1.92)	(22.3)	(2.10)	(1.7)	(12.3)	(1.8)
200 m downstream	78.49	81.50	74.57	104.83	53.36	71.03	10.85
	(3.6)	(4.6)	(9.0)	(8.3)	(1.3)	(1.7)	(0.4)
400 m downstream	69.91	87.25	100.13	103.17	68.67	68.60	26.17
	(3.9)	(3.6)	(4.0)	(2.70)	(2.8)	(19.4)	(1.8)
800 m downstream	87.21	83.51	103.09	100.14	73.95	78.66	30.66
	(9.0)	(2.7)	(6.1)	(0.70)	(2.6)	(12.9)	(2.7)
Reference (Ddw)	104.85	94.75	101	100.80	92.25	104.85	80.58
	(5.36)	(0.49)	(1.61)	(4.40)	(1.97)	(1.79)	(3.14)
LSD (5%)	18.51	14.23	35.08	9.47	7.41	32.20	12.26

Ddw - Double deionized water

Figures in Parentheses are Standard Error of the Means (N=9)

The use of N_2 sparging significantly ($p \leq 0.001$) increased the luminescence for all the effluent treatment samples (Table I). The general sedimentation (141.2%) and beam house samples (101.82%) showed the highest increase, with a highly significant difference observed for all of the effluent treatment pits. The lowest % bioluminescence was observed for the sparged tannery equalisation pit samples (46.4%) (Table I). This trend was not observed when N_2 sparging was applied to the anaerobic lagoons (Table II). The riverine sediment samples generally either showed no effect of sparging, on luminescence or a slight decrease (64 to 57%) (Table III).

Manipulation of the effluent treatment-pit samples using activated charcoal showed a significant ($p \leq 0.001$) overall increase in % bioluminescence in all the samples with the exception of a minimal increase (34.4%) observed for chrome sedimentation ($p \leq 0.05$) (Table I). The anaerobic lagoons showed a significant increase in bioluminescence ($p<0.05$) when activated charcoal was used. Stimulation (light output above that of the control) was noted for samples from Lagoon 3 (154.1%) when subjected to the same treatment, showing a significant difference from lagoons 2 ($p \leq 0.05$), 3 and 5 ($p \leq 0.01$) (Table II). The effect of activated charcoal on riverine sediments was most significantly ($p \leq 0.05$) pronounced for samples at the discharge point with an increased luminescence due to charcoal treatment from 69% to 93% (Table III).

Filtration had a significant ($p \leq 0.001$) effect on treatment effluent samples, showing increases in bioluminescence except for chrome sedimentation and the equalisation tank (Table I). Filtered samples from the treatment effluent were significantly different ($p \leq 0.001$) in all the pits except between chromium sedimentation and the chromium stripping tank. However, the effect of filtration was less ($p \leq 0.05$) for the anaerobic lagoons, with the only significant difference being observed between lagoons 2 and 3 ($p \leq 0.05$), 4 and 5 ($p \leq 0.01$) (Table II). Filtration significantly ($p \leq 0.05$) increased luminescence for all the riverine sampling points. The discharge point demonstrated a strong difference ($p \leq 0.01$) from the reference material (filtered double deionised water), upstream points (100 m and 200 m) and at 800 m downstream in comparison to the lower areas ($p \leq 0.05$) of the river (100 m, 200 m and 400 m).

Metal toxicity (and any other pH dependent toxicity) within the effluent treatment pits was identified by pH adjustment treatment to pH 4.0, 6.0 and 8.0, which generally showed significant ($p<0.001$) effects on the samples (Tables I, II and III). All samples from the effluent treatment pits and lagoons showed an increase in bioluminescence with adjustment from pH 4.0 to pH 6). Furthermore, pH adjustment caused significant differences ($p<0.001$) between all riverine sampling points (Table III), with the discharge point demonstrating the greatest difference ($p<0.001$).

Chemical analysis

Total phenols were present at elevated concentrations within most of the effluent treatment pits, lagoons and at the discharge point. However, a gradual decrease in phenol concentration was observed downstream (Table IV). The samples with the highest total phenols were the general sedimentation, chrome sedimentation, equalisation tank, lagoons 1 and 3, and the discharge point at the river (> 30 mg l^{-1}). The presence of high total phenols within the sampled areas was demonstrated by increased (except lagoon 3) bioluminescence (Table I, II) after sparging, and treatment with activated charcoal. Increase in bioluminescence is related to the uncoupling of the proton gradient (release of the fatty acid substrate for the luciferase enzyme) by chlorinated phenols, causing an increase in electron transfer rate and therefore an increase in the respiration rate, resulting in an increase in light output.

The metals chromium and iron showed high concentrations (Table IV). Chromium stripping (22.58 mg l^{-1}) and chrome sedimentation (191.47 mg l^{-1}) samples were, not surprisingly, associated with high chromium concentrations, hence linked to decreased luminescence of the sensor (Table I). High iron concentrations were observed in the riverine samples (but this is largely attributed to soil erosion, which is common in the Tropics).

Discussion

The *lux*-marked biosensor *E. coli* HB101 pUCD607 was used to dissect the toxic nature of the effluent from the tanning industry and the riverine sediments. Biosensors have been used to monitor environmental contamination[9,28] by heavy metals[20] and organic contaminants, as well as toxicity in soils and water contaminated by industrial effluents,[29] BTEX (benzene, toluene, ethlybenzene, xylene) compounds[11] and chlorinated aromatics.[30] Toxicity is assessed by examining a decrease in light output from the bacterium when it is exposed to environmental samples or chemicals.[9] The reduction (due to a toxic substance) in light output is proportional to the bioavailable concentration present.[11] Therefore, maximum % bioluminescence for all the treatment (sparging, activated charcoal, filtration and pH adjustment results), were calculated against a blank of double deionised water at pH 5.5.

The choice of the *lux*-marked biosensor in this work offered great environmental relevance in dissecting and categorising into broad groups the toxic nature of the effluent from the Kenyan tanning industry. The result in this study compared well with studies by Sousa *et al.*,[11] with differences between sparged and untreated samples providing the contribution to total toxicity of VOC's (Volatile organic compounds). Samples from the effluent treatment pits (Table I) and anaerobic lagoons (lagoons 1,2,4 and 5) (Table II) were associated with this type of toxicity.

The response of N_2 sparged samples reflected the toxicity of the samples once volatile organics had been removed (untreated samples showing total toxicity). This residual toxicity would be caused by inorganic and/or non-volatile

organics in the sample. However, the increase of luminescence in this study in all the effluent treatment pits and anaerobic lagoons suggested that considerable toxicity was caused by volatile organics. The river samples showed no difference between untreated and sparged (N_2), demonstrating the "self sparging effect" inherent in high flow, active rivers. Alleviation of toxicity in sparged, effluent treatment pit and anaerobic lagoon tannery samples highlights sparging as a potential remediative technique for tannery effluent, which would be based on proven technology.[33]

Activated charcoal treatment to the effluent treatment pit samples showed toxicity associated with organics after the removal of certain inorganics and organics (particularly chlorinated hydrocarbons). Samples from the beam house, general sedimentation and the anaerobic lagoon responded by showing an increased % luminescence (stimulation). This observation was probably related to the charcoal-mediated removal of the high total phenols load (Table IV) in the samples. Toxicity from chlorinated phenolics has been reported by Sinclair *et al.*,[19] and most chlorinated and non chlorinated phenolics are considered to be narcotics.[31] Phenolics compounds are known to be toxic through a protonophoric mechanism by acting as uncouplers, and/or inhibiting electron flow in the electron transfer chain. Phenols can accumulate in the membrane and disturb membrane function, causing narcotic effects.[32] The observed impact of charcoal filtration on the toxicity of key tannery and associated environmental samples suggests that it may provide an important remediative step, exploiting established and cost-effective technologies.

The removal of particulate matter and colloidal materials through filtration was critical for samples from the beam house, general sedimentation and all the anaerobic lagoons. In relation to this observation, studies by Thanikaivelan *et al.*[35] reported that activities such as soaking, liming, reliming (including fleshing) and deliming (beam house activities) account for 15 - 20% total solids containing lime sludge, fleshing and hair. Chrome sedimentation, chrome stripping and the equalisation tank showed the lowest response to filtration, suggesting that toxicity was not bound within the particulate and colloidal content of the samples. However, as the effluent flows towards the general sedimentation tank, an effect of filtration was observed, suggesting aggregation of the effluent contents to particulate matter. Coagulation and flocculation are envisaged to be the main activities in sedimentation tanks.[36] Filtering of chrome sedimentation samples was associated with a slight increase in bioluminescence (not necessarily indicating a decrease of toxicity) in comparison to other samples within the effluent treatment pits. This

TABLE IV

Total Phenols, pH, and Metal Concentrations of Tannery Effluent Treatment Pits (mg l^{-1}), Anaerobic Lagoons (mg l^{-1}) and Riverine Sediments (mg kg^{-1}).

Samples	pH	Cr ppm	Pb ppm	Fe ppm	Cu ppm	Cd ppm	Zn ppm	Ni ppm	Total Phenol, ppm
Effluent treatment pits (mg l^{-1})									
beam house	12	0.07	0	1.75	0.02	0.01	0.07	0.05	ND
general sedimentation	8.34	0.31	0	0.15	0.01	0.01	0..07	0	72
chrome stripping	9.6	22.58	0.06	1.39	0.03	0.01	0	0.15	ND
chrome sedimentation	8.25	191.47	0	7.42	0.06	0.01	0.71	0.53	52.9
equalization tank	8.05	0.3	0	0.5	0	0.01	0	0.02	36.8
Anaerobic lagoons (mg l^{-1})									
lagoon 1	7.8	0.1	0	0.17	0	0.01	0.02	0.03	30
lagoon 2	7.92	0.06	0	0.09	0.01	0.01	0	0.04	NA
lagoon 3	8.3	0.07	0.01	0.03	0	0.01	0	0.04	48.1
lagoon 4	7.82	0.13	0.04	0.06	0.01	0.01	0.05	0.01	24.5
lagoon 5	8.40	0.03	0	0	0	0.01	0.01	0.06	16.6
Riverine samples (mg kg^{-1})									
200m upstream	7.02	0.91	0.55	1139	0.51	0.04	1.94	0.75	ND
100m upstream	7.06	1.14	0.27	772	0.16	0.02	0.79	0.43	ND
0 m discharge point	8.01	1.41	0.39	4237	0.57	0.02	1.63	0.72	30
100m downstream	7.31	1.31	0.36	1048	0.44	0.03	1.14	0.62	17.7
200m downstream	7.30	1.65	0.58	1362	0.69	0.03	1.15	1.01	11.4
400m downstream	7.4	1.76	0.58	1349	0.52	0.04	1.37	1.02	5.5
800m downstream	7.2	1.22	0.41	1234	0.48	0.03	1.34	0.71	ND

phenomenon was also observed when the river samples were filtered, with the discharge point indicating stimulation. Along with the results from charcoal treatment, the effects of filtration on sample toxicity also highlighted this treatment as likely to have an important role in the remediation of tannery effluents, again using proven technologies.

Available metals are generally in the form of soluble cations and their tendency to be present in ionic form increases with increasing acidity.[37] Sarin[38] reported the toxicity response of lux-marked *E. coli* HB101 to a range of metals. In this study, metal toxicity and bioavailability patterns were identified through pH adjustment (4.0, 6.0 and 8.0) in the tannery effluent (Table I), anaerobic lagoons (Table II) and riverine sampling points (Table III). The increase in % luminescence (>80%) on adjustment from pH 4.0 to pH 6.0 was demonstrated for all of the tannery related samples tested (Table I and II). This suggested the presence of metal toxicity and the response to pH variation on bioavailability, which is imparted by changes in speciation and portioning effects of the metals.[39,40,41,42] The tannery treatment effluent showed increased % luminescence at pH 6.0 (representing typical environmental conditions) where the majority of the metals are limited in their bioavailability.[43] Because of the alleviation in toxicity of all tannery samples through adjustment to pH 6, pH treatment (along with charcoal treatment and filtration) offers a potentially useful remediative option for tannery effluents.

Toxicity in samples such as treatment effluents, anaerobic lagoons and downstream riverine sampling points (Table I, II and III) was attributed to high concentrations of chromium and phenols (Table IV). For example although in tannery waste water Cr(III) is the most expected Cr form, the redox reactions occurring in the sludge can increase the concentration of the hexavalent form.[4]. Most metals show increased solubility with decreased pH,[45] indicating increased bioavailability (chemical assimilation and possible toxicity) of organic/inorganic compounds.[9,10,46] Under slightly acidic or neutral pH conditions in this type of wastewater, the poorly soluble $Cr(OH)_3$ aq. should be the preferred form, but a high content of organic matter originating from the hide material processing is effective in forming soluble organic Cr(III) complexes.[47,48,49]. Samples from the discharge points showed higher toxicity when the samples were adjusted from pH 6.0 to pH 8.0. Other related studies investigating the fractionation of chromium toxicity in water using *E. coli* HB101 pUCD607 showed that speciation of chromium at different pH levels and a synergistic effect with other metals (e.g. copper and zinc) contributed to its toxicity.[44] This observation suggested that chromium is frequently a constraint to bioremediation in contaminated environments.*

The choice of the organism was suitable for the study as it is found in a variety of habitats including sewage sludge and contaminated river water, and it functions over a wide pH range. However, the future use of indigenous organisms as biosensors should complement this study and enable exploration of adaptability and acquired pollutant resistance over time to be addressed.

Conclusions

This study successfully demonstrated the potential of *lux*-marked biosensors to determine toxicity of tannery effluent. Furthermore, through toxicity dissection involving sample manipulation coupled to biosensor assay, potential remediative strategies have been identified. Where appropriate, chemical analysis was used to further confirm the biosensor-based identification of the cause of toxicity and potential constraint to remediation. In combination with focused analysis, therefore, *lux*-marked biosensors offer a powerful tool for the rapid, toxicity based assessment of tannery effluents and associated environmental samples.

Acknowledgement

The authors thank the Kenyan Government and The University of Aberdeen for financial support and thank Chandrika Nanayakkara for her assistance.

References

1. Ros, M. and Ganter, A.; Possibilities of reduction of recipient loading of tannery waste Slovenia. *Water Sci. Technol.* **37**, 145-152, 1998
2. Song, Z., Williams, C. J., and Edyvean, R. J.; Sedimentation of tannery wastewater. *Water Res.* **34**, 2171-2176, 2000
3. Reemste, T. and Jekel, M.; organics in tannery wastewaters and their alteration by a combined anaerobic and aerobic treatment. *Water Res.* **31**, 1035-1046, 1997
4. Kota, J. and Stascika, Z.; Chromium occurrence in the environment and method of its speciation. *Environ. Pollut.* **107**, 263-285, 2000
5. Andersen, J. E. T.; Introduction of hydrogen peroxide as an oxidant in flow injection analysis: speciation of Cr(III) and Cr(VI). *Anal. Chim. Acta* **361**, 125-131, 1998
6. Pasco, N., Hay, J., and Webber, J.; Biosensors: MICREDOX -a new biosensor technique for rapid measurement of BOD and toxicity. *Biomarkers* **6**, 83-89, 2000
7. Tunay, O., Orhon, D., and Kabdasli, I.; requirements for leather tanning industry wastewaters. *Water Sci. Technol.* **29(9)**, 121 - 128, 1994

*Killham, K., (2004). Personal Communication. University of Aberdeen, Scotland

8. Bouwer, E., Durant, N., Wilson, L., Zhang, W., and Cunningham, A.; Degradation of xenobiotic compounds in situ: Capabilities and limits. *FEMS Microbiol. Rev.* **15**, 307-317, 1994
9. Steinberg, S. M., Poziomek, E. J., Engelman, W. H., and Rogers, K. R.; A review of environmental applications of bioluminescence measurements. *Chemosphere* **30**, 2155-2195, 1995
10. Shaw, L. J., Y., Glover, L. A., Killham, K., Osborn, D., and Meharg, A. A.; Bioavailability of 2,4-dichlorophenol associated with soil water-soluble humic material. *Environ. Sci. Technol.* **34**, 4721-4726, 2000
11. Sousa, S., Duffy, C., Weitz, H., Glover, A. L., Bar, E., Henkler, R., and Killham, K.; of a *lux*-modified bacterial biosensor to identify constraints to bioremediation of BTEX-contaminated sites. *Environ. Toxicol. Chem.* **17(6)**, 1039-1045, 1998
12. Cervantes, C., Campos-Garcia, J., Devars, S., Guitierrez-Corona, F., Loza-Tavera, H., Torres-Guzman, J. C., and Moreno-Sanchez, R.; Interactions of the chromium with microorganisms and plants. *FEMS Microbiol. Rev.* **25**, 335-347, 2000
13. Saxena, D., Levin, R., and Firer, M. A.; Removal of chromate from industrial effluent by a new isolate *Staphylococcus chonii. Water Sci.Technol.* **42**, 93-98, 2000
14. Chen, J. M., Hao, O. J.; Microbial chromium (VI) reduction. *Critical Reviews in Environ. Sci. Technol.* **28**, 219-251, 1998
15. Chirwa, E. M. N. and Wang, Y. T.; Chromium(VI) reduction by *Pseudomonas fluorescens* LB 300 in fixed-film bioreactor. *J. Environ. Eng.* **123**, 760-766, 1997
16. Shen, H. and Wang, Y. T.; Characterisation of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. *Appl. Environ. Microbiol.* **59**, 3771 - 3777, 1993
17. Prasard, B. G. S., Thyagarajan, G., and Nayudamma, Y.; Utilisation of Water Hyacinth in the treatment and disposal of tannery wastewater. *JSLTC* **75**, 56-57, 1991
18. Lagido, C., Pettit, J., Porter, A. J. R., Paton, G. I. and Glover, L. A.; Development and application of bioluminescent *Caenorhabditis elegans* as multicellular eukaryotic biosensors. *FEMS Microbiol. Lett.* **493**, 95-107, 2001
19. Sinclair, M. G.; Soil toxicity assessment of 2,4-DCP using lux microbial biosensors. PhD thesis, University of Aberdeen, U.K., 1999
20. Paton, G. I, Campbell, C. D., Glover, L. A., and Killham, K. Assessment of bioavailability of heavy metals using *lux* modified constructs of Pseudomonas fluorescens. *Lett. Appl. Microbiol.* **20**, 52-56, 1995
21. Chaudri, A. M., Knight, B. P., Barbosa-Jefferson, V. L., Preston, S., Paton, G. I., Killham, K., Coad, N., Nicholas, F. A., Chambers, B. J., and McGrath, S. P.; Determination of acute Zn toxicity in pore water from soils previously treated with sewage sludge using bioluminescence assays. *Environ. Sci. Technol.* **33**, 1880-1885, 1999
22. Galli, R., Munz, C. D., and Scholtz, R.; Evaluation and application of aquatic toxicity tests: use of the Microtox test for the prediction of toxicity based upon concentrations of contaminants in the soil. *Hydrobiologia* **273**, 179-189, 1994
23. Wild, S. R., Harrad, S. J., and Jones, K.; Chlorophenols in digested UK sewage sludges. *Water Res.* **27**, 1527-1534, 1993
24. Allan-King R. M., Baker J. F., Gillham R. W., and Jensen, B. K. Substrate and nutrient limited toluene biotransformation in sandy soil. *Environ. Toxicol. Chem.* **13**, 693 - 705, 1994
25. Amin-Hanjani, S., Meikle, A., Glover, L. A., Prosser, J. I., and Killham, K.; Plasmid and chromosomally encoded luminescence marker systems for detection of *Pseudomonas fluorescens* in soil. *Mol. Ecol.* **2**, 47 - 54, 1993
26. Matthews, J. E.; Evaluation of toxicity test procedure for screening treatability potential of waste in soil. *Toxic. Assess.* **2**, 265 - 281, 1987
27. Rattray, E. A. S., Prosser, J. I., Killham, K., and Glover, L.A.; Luminescence-based non extractive techniques for in situ detection of *Escherichia coli* in soil. *Appl. Environ. Microbiol.* **56**, 3368-3374, 1990
28. Van de Lelie, D., Corbisier, P., Baeyens, W., Wuertz, S., Diels, L., and Mergeay, M.; The use of biosensors for environmental monitoring. *Res. Microbiol.* **145**, 67 - 74, 1994
29. Brown, J. S., Rattray, E. A. S., Paton, G. I., Reid, G., Caffoor, I., and Killham, K.; Comparative assessment of the toxicity of a paper mill effluent by respirometry and luminescence-based bacterial assay. *Chemosphere* **32**, 1553-1561, 1996
30. Steiert, J. G. and Crawford, R. L.; Microbial degradation of chlorinated phenols. *Trends Biotechnol.* **3**, 300 - 305, 1985
31. Cronin, M. T. D., Schultz, T. W.; Validation of Vibrio fisheri acute toxicity data: mechanism of action-based QSARs for non-polar narcotics and polar narcotic phenols. *Sci. Total Environ.* **204**, 75-88, 1997
32. Escher, B. I., Snozzi, M., and Schwarzenbach, R. P.; Uptake, speciation and uncoupling activity of substituted phenols in energy transducing membranes. *Environ. Sci. Technol.* **30**, 3071 - 3079, 1996
33. Boyd, E. M., Meharg, A. A., Wright, J., and Killham, K.; Toxicity of chlorobenzene to a lux-marked terrestrial bacterium, *Pseudomonas flourescens*. *Environ. Toxicol. Chem.* **16**, 849-856, 1998

34. Heitzer, A., Appegate, B., Kehrmeyer, S., Pinkart, H., Webb, O. F., Phelps, T. J., White, D. C., and Sayler, G S.; Physiological considerations of environmental applications of lux reporter fusions. *J. Microbiol. Meth.* **33**, 45-57. 1998
35. Thanikaivelan, P., Rao, R. J., Nair, B. U., and Ramasami, T.; Approach towards zero discharge tanning: role of concentration on the development of eco-friendly liming-reliming processes. *J. Clean. Prod.* **11**, 79-90, 2003
36. UNEP IE/PAC, (2nd Print 1994). Tanneries and the Environment - A Technical Guide, Technical Report Series No 4, ISBN 92 807 1276 4.
37. Sposito, G; The Chemistry of Soils. Oxford University Press, London, 1989
38. Sarin, C.; A lux-based bioassay of heavy metal contamination of wastes. PhD Thesis, University of Aberdeen, U.K, 2000
39. Ritchie, J. M., Cresser, M., and Cotter-Howells; Toxicological response of a bioluminescence microbial assay to Zn, Pb and Cu in artificial soil solution: relationship with total metal concentrations and free ion activities. *Environ. Pollut.* **144**, 129-136, 2001
40. Van Leeuwen, H. P.; Metal speciation dynamics and bioavailability: inert and labile complexes. *Environ. Sci.Technol.* **33**, 3734-3748, 1999
41. McGrath, S. P., Knight, B., Killham, K., Preston, S., and Paton, G I.; Assessment of the toxicity of metals in soils amended with sewage sludge using a chemical speciation technique and a *lux*-based biosensor. *Environ. Toxicol. Chem.* **18**, 659-663, 1999
42. Knight, B. P. and McGrath, S. P.; A method to buffer the concentrations of free Zn and Cd ions using a cation exchange resin in bacterial toxicity studies. *Environ. Toxicol. Chem.* **14(12)**, 2033 - 2039, 1995
43. Gadd, G M.; Metal tolerance. In: Edwards, C., Eds, Microbiology of Extreme Environments, Open University Press, Milton Keynes, U.K., pp 178 - 210, 1990
44. Wararatananurak, P.; Fractionation of chromium toxicity in water. PhD Thesis, University of Aberdeen, U.K, 2000
45. Artiola, J. F.; Waste disposal, chapter 10, pp 142, In: Pepper, L.I., Gerba, P.C., Brusseau, M.L Pollution Science, Academic Press (Elsevier Science, USA), San Diego, California, USA, 1996
46. Alexander, M.; Ageing, bioavailability, and overestimation of risk from environmental pollutants: critical review. *Environ. Toxicol. Chem.* **34**, 4259-4265, 2000
47. Stein, K. and Schwedt, G; Chromium speciation in the wastewater from a tannery. *Fresenius J. Analyt. Chem.* **350**, 38-41, 1994
48. Walsh, A. R. and O'Halloran, J.; Chromium speciation in tannery effluent-I. An assessment of techniques and role of organic Cr(III) complexes. *Water Res.* 30, 2393 - 2400, 1996
49. Walsh, A. R. and O'Halloran, J.; Chromium speciation in tannery effluent-II. Speciation in effluent and in a receiving estuary. *Water Res.* 30, 2401 - 2412, 1996