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Application and Relevance of Biosensors in The Tanning Industry

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ABSTRACT: The tanning industry has for a long time been associated as the primal source of pollution to various ecosystems wherever they are sited. Effluent spewed by these leather industries including particulate matter has remained very complex requiring more advanced techniques to examine. One such avenue is the use of ecotoxicological diagnosis of the tanning sector to comprehend the underlying environmental impact. Thus recent advances in biotechnology brought to the fore the relevancy and appropriateness of biosensors in determining pollution load and associated impact to the atmospheric, terrestrial and aquatic ecosystems. Indeed other investigations on Lead, Arsenic and complex anthropogenic and xenobiotics have spurred the development of specific genetically modified organism to facilitate in such investigation. Thus in this paper review the relevancy and advances of the application of biosensors is addressed for purposes of recording, some of the achievements so far attained towards the tanning industry are discussed. However it should be realized that the application of biosensors in evaluating tanning industry effluent is very recent but very opportune area that requires an in-depth ecotoxicological diagnosis through of established biotechnological advances.

Key words: Biological tools, Bioluminescence, Biotechnology, Ecotoxicology,Tanning

INTRODUCTION

A great variety of bioassays have been run to determine the levels of ecotoxicity all around the world (Gomes *et al.,* 2011; Ekmekyapar *et al.,* 2011; Frahadi *et al.,* 2011; Moliterni *et al.,* 2012; Lalevic *et al.,* 2012; Kim *et al.,* 2012; Krika *et al.,* 2012). Mwinyihija (2010) indicates that application of biosensors as a bioassay for tanning based samples is quick, cheap and reliable for toxicity testing of tannery effluents. As such Manly (2000), defines ecotoxicology as the study of toxic effects of substances on the biotic and abiotic components of the biosphere, especially on populations and communities within defined ecosystems. While environmental toxicology in general, tests individual species rather than combined species. Mwinyihija et al. (2009) suggest that it is imperative to understand that ecotoxicology is a discipline within a wider field of environmental toxicology. Thus there is preference to develop bioassays that integrate toxicity to examine the effects or impacts of chemicals in a broad range of ecosystems (Trogl and Benediktova, 2011; Gousterova *et al.,* 2011; Khurram, 2011; Castro-Gutierrez *et al.,* 2012). Bioassays are described as tests determining or estimating the effects of biologically active substances under standardized and reproducible conditions. Mwinyihija (2009, 2010) applied this novel technique and used biosensors to intensively determine the toxicity levels emanating from the tanning industry. Biosensor as an emerging novel technique for ecotoxicological analysis has been used in various fields recently. A successful analytical work on biosensors was reported by Mwinyihija (2010), who began with tannery dust produced from various tanning areas, effluents, soil supernatants and finally riverine sediments. The application of biosensor (scientific basis of its mechanism will be discussed immediately hereafter) was mainly based from genetically modified microorganism. Initially the use of whole cell biosensor applying microorganism was prominent. To-date we have moved to microbiological level exploring fully on the genetic makeup of the microorganism. A good example expected properties of a biosensor is narrated from Schwedt *et al.,* 1997 who indicated that efficiency, accuracy, rapidity, convenience and on-line monitoring are some of the advantages conferred by the use of biosensors over other forms of biomonitoring. On the other hand Vo-Dinh and Cullum (2000) defined biosensors as a combination of a bioreceptor, biological component, and a transducer as the detector. They further alluded that the interaction of the analyte with the bioreceptor has the functional characteristic of producing an effect measured by a transducer. Finally it was subscribed that the transducer then converts the information into measurable effect such as an electrical or optical signal also referred to as bioluminescence.

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Areas that have come to be of use by applying the biosensor, include acute and chronic physiological toxicity, genotoxicity, immunotoxicity and endocrine toxicity. In reference to the report by Mwinyihija (2009) he enumerates that the concentration of certain specific analytes are difficult to detect and are important contaminants of water, waste, soil or air (e.g. surfactants, chlorinated hydrocarbons, sulphophenyls carboxylates, sulphonated dyes, fluorescent whitening agents, naphthalensulphonates, carboxylic acids, dioxins, pesticides and metabolites). Indeed the measurability of biosensors is based mainly through luminescence or bioluminescence.

MATERIALS & METHODS

Escherichia coli HB101 (pUCD607) is lux-marked (and derived from E. coli K12 strain) using plasmid pUCD607 (Rattray *et al.,* 1990, Paton *et al.,* 1997) (with a lux CDABE cassette from V. fischeri, Shaw & Kado, 1986) using calcium chloride transformation. The plasmid is under the control of a constitutive promoter of a tetracycline resistance gene (Close *et al.,* 1984). Cells of *E. coli* HB101 are grown on Luria Bertani Glucose (LBG) agar plates containing 50 µg/L of kanamycin for 2 days in a 25 °C incubator. An overnight culture of bacteria is then inoculated with 10 mL of LBG medium containing 50 µg/L of kanamycin with a single colony from the agar plate. The cultures are grown overnight on an orbital shaker incubator at 25 °C, 200 rpm. Aliquots (500 µL) of these bacterial cultures are used to inoculate 2 conical flasks each containing 500 mL of LBG medium amended with 50 µg/L of kanamycin. These flask cultures are incubated on an orbital shaker at 25 °C, 200 rpm until an OD_{550} of 0.6, with relative luminescence units of 1×10^6, is reached. The cultures are then harvested by centrifugation in a MSE Coolspin 2 at 2000 g, 4 °C for 40 min. Thereafter the supernatant is discarded and the cells are resuspended in 130 mL of sterile *Mist desiccans* (100 mL of horse serum, 30 mL of LBG medium and 10 g of glucose). Aliquots (1 mL) of resuspended cultures are pipetted into 5 mL sterile freeze-drying vials with rubber stoppers. The vials are then placed in liquid nitrogen for at least 90 min and transferred to a Modulyo Edwards freeze drier for 16-24 h at –80 °C under vacuum. Freeze dried cultures are finally stored at –20 °C. Growth rates could then be obtained by plotting plate counts in colony forming units (c.f.u/mL) performed by log transforming and fitting a linear regression ($R^2 \geq 95\%$) to the points (Fig. 1).

Determination of toxicity is based on the bioluminescence response of the *lux*-modified biosensor, *E. coli* HB101 pUCD607, which had previously been, marked with the *lux* CDABE genes, (isolated from *Vibrio fischeri)* using the multi-copy plasmid pUCD607 (Amin-Hanjani *et al.,* 1993). The biosensor is stored at -20°C and resuscitated from freeze dried cultures prior to bioassay. Freeze dried cultures of *Escherichia coli* HB101 pUCD607 is resuscitated in 10 mL of sterile 0.1 M KCl (contained in a Universal). 1mL of KCl is then added and the culture resuspended by mixing (drawing up and down 5 times into a PI000 Gilson pipette). The resuspended culture is eventually transferred back to the universal and the culture placed in a shaking (200 rpm) incubator (25° C) for 1 h. One hundred µL of the resuscitated biosensor suspension is added to the samples at 15 s intervals, accurately timed for measurement in the Bio Orbit 1253 luminometer (Labtech International, Uckfield, U.K). Each sample is exposed to the sensor for exactly the same time. Samples are thereafter incubated for 15 min before light output measurements are carried out at 15 s intervals. This ensures the same exposure time to the potentially toxic elements for cells in each of the cuvettes (Fig. 2).

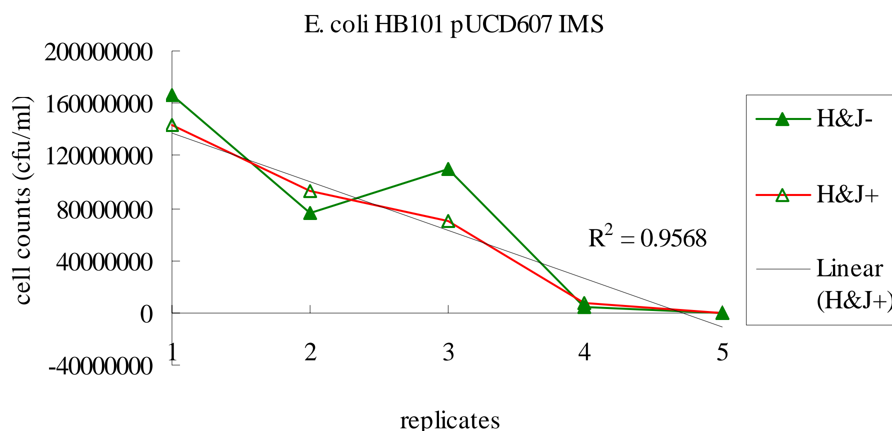


Fig. 1. Comparison of two trial test of *E. coli* HB101 pUCD607 IMS by plotting plate counts in colony forming units (c.f.u/mL) and fitting linear regression to the points

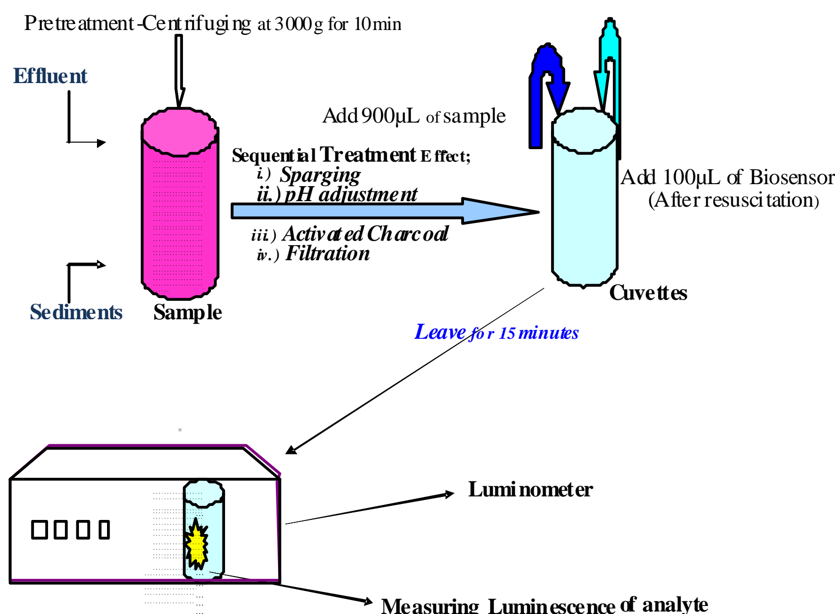


Fig. 2. Bioassay protocol measuring sequential treatment effect during the dissection of the toxic nature of the sediment and effluent

The output from the luminometer resulting from each assay carried out is recorded in relative light units (RLU's) (equating to mV/10 s/mL). The light output is then converted to percentage maximum bioluminescence. This is then calculated against a blank of double deionised water adjusted to pH 5.5, the optimum pH for bioluminescence.

% maximum bioluminescence = I_S/I_{C*100} (2)

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 is determined at the minimum for three sample replicates. A mean of this determination is then calculated. The assay performance continues to be monitored by reference to the response to the control, the reproducibility of the response to the three replicates and the response to a standard of trichlorophenol (TCP) (Fig. 3). Effect of exposure time on toxicity to a range of standard solutions of Zn and Cu (Fig. 4) were prepared by dilution with double deionised water at pH 5.5.

Freeze dried cultures of *Ps. flourescens* 10586s pUCD607 are resuscitated in 10mL autoclaved Luria-Bertani (LB) broth. This is carried out by adding 1mL of autoclaved LB, taken from 10mL LB (autoclaved in a 30mL universal bottle) to the dried culture, mixing with the Gibson pipette tip and returning the LB plus cells to the 9mL of LB. The culture is then incubated with shaking (180 rpm) for up to 2h at 25°C. After 1h the bioluminescence of the culture is checked using the Bio-Orbit 1251 luminometer. After 2h, 1mL aliquots are removed and placed in 1.5mL micro centrifuge tubes. The tubes are then centrifuged for 1min in a Micro Centaur MSE centrifuge at 7550 *g*. The pellet is then resuspended and centrifuged as before. The supernatant is again discarded and the pellet resuspended in 1mL of 0.1M KCl. All the cells suspensions are then placed together in a sterile 5mL bijou bottle. *Ps. flourescens* is resuscitated in LB and then resuspended in 0.1M KCl as described. Cells must be clean in order to be responsive to sample substrate

RESULTS & DISCUSSION

According to Meighen, 1992 bioluminescence refers to visible light emission in living organisms that accompanies the oxidation of organic compounds (luciferins) mediated by an enzyme catalyst (luciferase). There are many organisms that have been reported through various studies known to naturally luminesce. For example some species of earth worms (e.g. *Diplocardia longa*), snails, beetles, limpets, seafirs, star fish, shrimps, fireflies (e.g. *Photinus pyralis*) and bacteria found in the marine environment (e.g. *Vibrio, Photobacterium, Alteromonas* and *Xenorhabdus* i.e. the four major genera of luminescent bacteria) (Campbell, 1989, Lampinen *et al.,* 1995, Meighen, 1988). Hastings & Nealson, 1977 indicated that luminescent

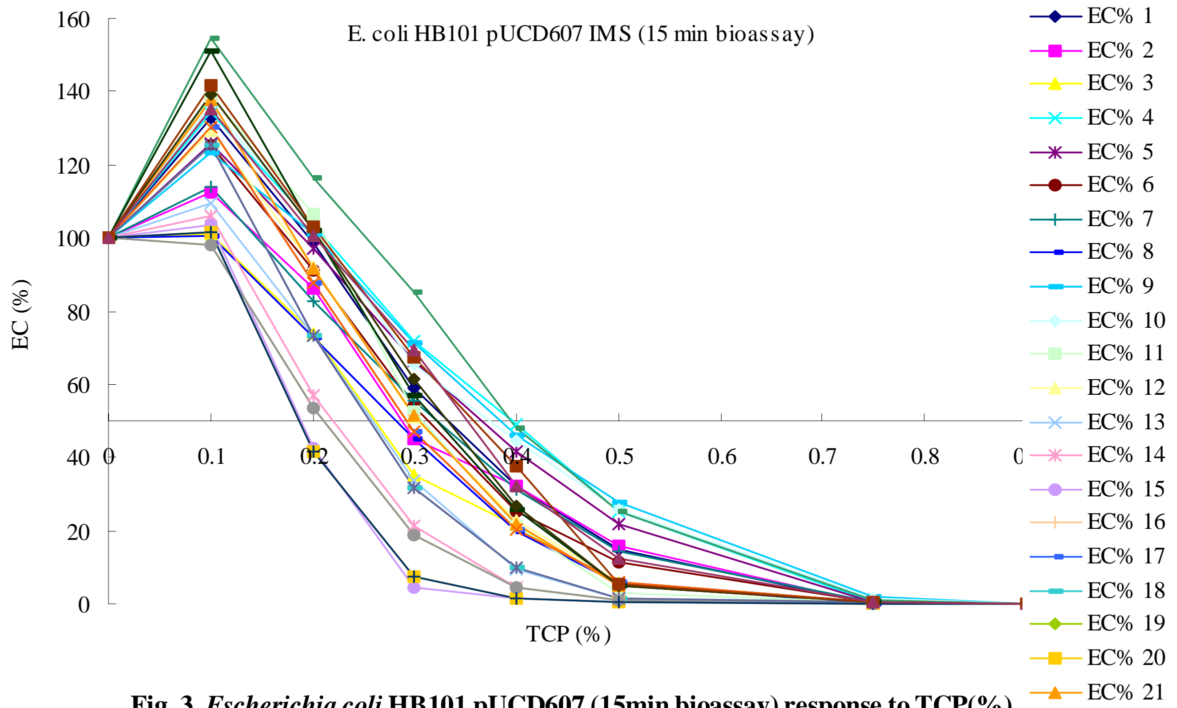


Fig. 3. ***Escherichia coli*** **HB101 pUCD607 (15min bioassay) response to TCP(%)**

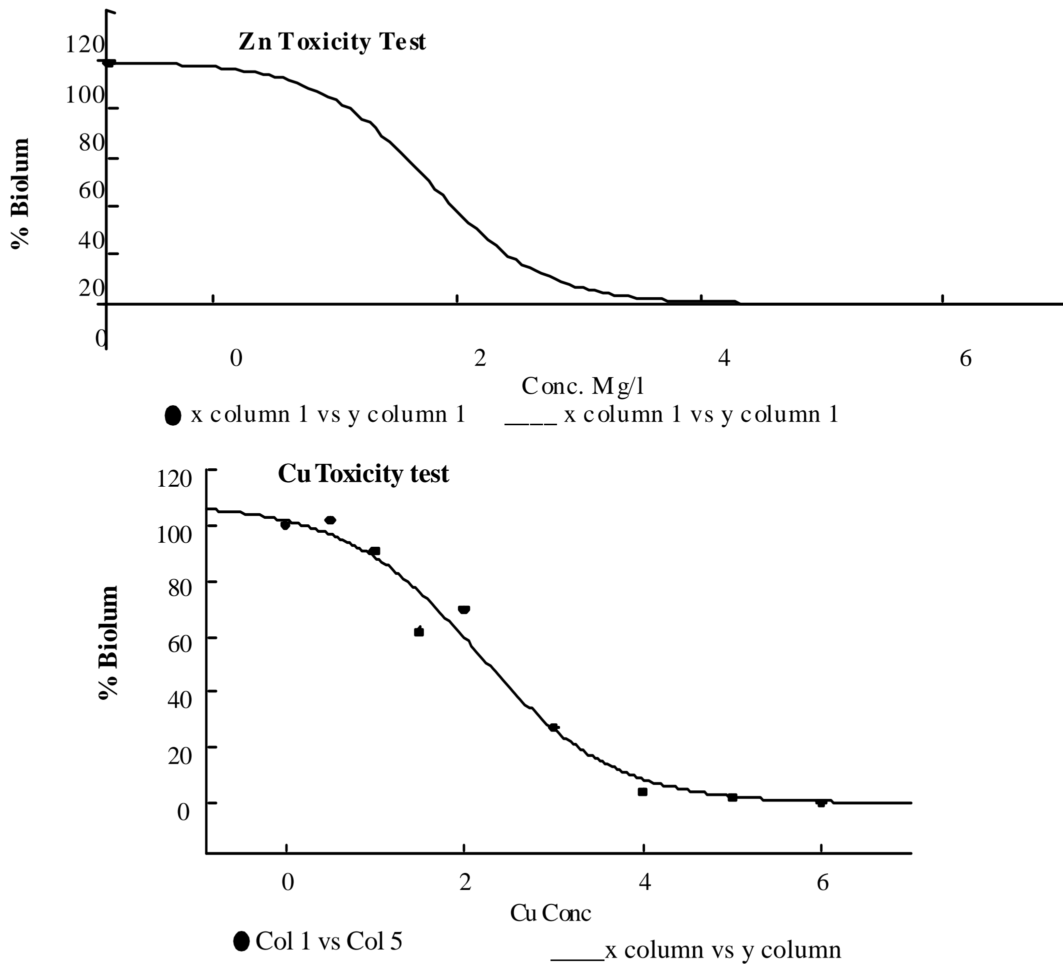
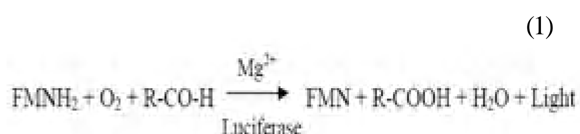


Fig. 4. Bioluminescence % indicating the effect of exposure time (15s) on toxicity to a range of standard solutions of Zn and Cu

bacteria all share a common morphology as they are Gram-negative, motile rods, facultative anaerobes and produce extracellular chitanase. Moreover Belas *et al.,* 1982, reported that luminescent bacteria found in the sea exist as free-living planktonic forms or in symbiosis with fish, squid and other organisms, usually in the gut or on special light organs. Campbell, 1989 alludes that symbiotic luminous bacteria are responsible for luminescence in approximately one third of the 190 fish genera. For example Hastings & Nealson, 1977 reported that *Vibrio fischeri* is found as a symbiont in *Monocentris japonica.* However it is important to note that to date the only known non-marine luminescent bacteria is *Vibrio cholerae* var. *albensis* (i.e. Fresh water bacterium) which was isolated from the Elbe river in Germany (Hastings & Nealson, 1977). Bioluminescence is one of the products of reaction that involves enzyme luciferase, oxidation of reduced flavin mononucleotide ($FMNH_2$), and a long chain aliphatic aldehyde. Most of the microorganism lack the 'genetic blue print' for luciferase and reductase but can supply $FMNH_2$. To convert bacteria into bioluminescent organism a genetic transfer is required of genes for luciferase and reductase with aldehyde added exogenously. Indeed bioluminescence reaction also involves the reduction of Aldehyde to the corresponding fatty acid with the production of light where;-

(1)



The generation of fatty acids is catalysed by acyl-transferase which reacts with various esters such as acyl-CoA, acyl-ACP and acyl-p-nitrophenol (Meighen, 1988). The acyl group from the ester is transferred to the enzymes and then to the water to produce fatty acid. Acyl-ACP, has fourteen carbon atoms and is therefore the preferred substrate of luminescence bacteria (Meighen, 1988). Essentially it is suggested that luminescence may be part of the electron transport system where electrons are transferred to oxygen by two flavin enzymes i.e. flavin mononucleotide reductase and luciferase (Prosser *et al.,* 1994). Bioluminescence reactions have important applications in bioanalytical chemistry. Many different luciferins and mutant luciferase have been investigated to optimise the reaction performance. For example the North American firefly *Photinus pyralis* uses luciferase which catalyses the oxidation of D-luciferin in the presence of ATP to produce light (Lampinen *et al.,* 1995). The genes responsible for the occurrence of bioluminescence in bacteria are present on two operons. Operon R contains the *lux* ICDABEGH genes and operon L contains the *lux* R gene (Meikle *et al.,* 1992). Environmental toxicity tests are normally measured by exposing test organisms such as fish, insects and plants to environmental samples (Steinberg *et al.,* 1995). Endpoints such as survival have been used but bacterial toxicity tests can measure a variety of different endpoints such as mutagenicity, population, carbon dioxide production, enzymes biosynthesis, glucose mineralization and inhibition of bioluminescence (Layton *et al.,* 1999). Biosensor can be classified as specific or general toxicity sensors. Luminescent toxicity sensors generally occur naturally from *Vibrio fischeri* a marine bacterium. This was introduced in 1981 as a toxicity testing procedure called Microtox™ (Steinberg *et al.,* 1995) and is now widely accepted as an international toxicity test (Paton *et al.,* 1995b). The toxicity is assessed by examining the decrease in light output from the bacterium when it is exposed to environmental samples or chemicals (Steinberg *et al.,* 1995). Toxicity is measured as EC_{50} which is the concentration of the aqueous sample required to reduce light output by the bacterium by 50% (Ribo & Rogers, 1990). The EC_{50} is dependent on test temperature and exposure time to the samples (Ribo & Rogers, 1990). Microtox has been used to test the toxicity of a wide range of compounds including pesticides, trinitrotoluene, tributyltin compounds, tributenyltin compounds, river water, sediment interstitial pore waters and aquatic toxicants (Steinberg *et al.,* 1995). Paton *et al.,* 1995b demonstrated that Microtox toxicity data correlate well with other aquatic bioassays. The test has been adopted for water quality screening in various industrialized country such as Canada, France, Germany, Spain and Sweden (Brown *et al.,* 1996). However certain limitation had been observed by Paton *et al.,* 1995a where the marine organism has a limited pH range and the sample to be tested requires to be buffered with 22% sodium chloride to condition the samples for determination. Examples of other *lux*-marked terrestrial bacteria used for general toxicity testing include; *Pseudomonas fluorescens* 10586s pUCD607 (Paton *et al.,* 1995a, 1995b, Brown *et al.,* 1996, Sousa *et al.,* 1998) *Rhizobium leguminosarum* bv. *Trifolii* F6 pUCD607 (Paton *et al.,* 1997, Palmer *et al.,* 1998, Reid *et al.,* 1998), Pseudomonas putida F1 (Reid *et al.,* 1998), Escherichia coli HB101 (Reid *et al.,* 1998, Chaudri *et al.,* 1999). In comparison to the general sensors, specific pollutant sensors are for example those that emanate from specific reporter genes such as *lux*. These are fundamentally activated when the bacteria comes into contact with an activator such as specific xenobiotics (Barkay *et al.,* 1995). The reporter gene is placed under the expression control of the promoter where the former (i.e. reporter gene) is then

expressed as an indirect measure of the activity of the gene of interest (Burlage *et al.,* 1995). This therefore enables the specific bacteria (e.g. genetically modified) with degradative properties to be used as biosensors for particular xenobiotics. Thus as bioluminescence forms an integral part of the pollutant determination process, Steinberg *et al.,* 1995 demonstrated that luminescence systems activates due to the presences of the operon for bioluminescence where the specific bacteria emits light as it degrades the xenobiotic. The principle behind bioluminescence is attained when the regulatory gene or operon which codes for the induction of enzymes for the degradation of specific xenobiotic can be fused with the operon that code for bioluminescence. Some examples of pollutant specific *lux*-marked biosensors include; *Alcaligenes eutrophus* AE1239 and *Alcaligenes eutrophus* AE1433 (pollutant specific to Copper, Zinc, Cadmium, Cobalt and lead) (Corbisier et. al., 1996); *Pseudomonas flourescens* HK44 (pollutant specific to hydrocarbons) (Heitzer *et al.,* 1998); *Pseudomonas flourescens* pUTK21 (e.g. Naphthalene) (King *et al.,* 1990); *Escherichia coli* JM109(pSB450) (e.g. Nitrate) (Prest *et al.,* 1997); *Pseudomonas putida* B2 (e.g. Toulene, Naphthalene) (Applegate *et al.,* 1997). Fundamental advantages of biosensor technology in ecotoxicology testing could also be attributed to the fact the technology is non-invasive, non-destructive and highly sensitive in real time analysis. Environmental diagnostics and the need to ascertain pollution levels have led to the rapid development of genetically modified bacteria in the field of ecotoxicology. Thus advancement in genetic engineering has allowed the introduction of the *lux* operon into indigenous bacteria and more environmentally relevant bacteria in pursuance to such diagnostics demand to the environment. The applications of *lux*-marked bacteria to assess toxicity in both aquatic and terrestrial systems have been used by several experts worldwide in both anthropogenic and natural ecosystem. In addition the same type of biosensor has been used in a cost effective manner to screen both metal organic pollutants (Boyd *et al.,* 1997). In recent times some of the experts who have used *lux*-marked biosensor as a primary diagnostic tool include; Tiensing *et al.,* 2001, 2002 (assessing bioavailability of pollutants in soils); Wararatananurak, 2000 (Fractionation of Chromium toxicity in water); Sinclair, 1999 (soil toxicity assessment of 2,4-DCP), Mwinyihija, 2005 (Ecotoxicological impact of the tanning industry), Edmond, 2006a (Evaluation of ameliorants for interception of lead leaching from soil) etc. The application of biosensors has both its advantages and disadvantages although when compared with other types of bioassays the advantages associated with biosensors outweigh them all. According to various reported works for example by Burlage *et al.,* 1992, Cronin and Schultz, 1997, Paton *et al.,* 1997 and Mwinyihija *et al.,* 2005, 2006 they all indicated several advantages accredited to the use of biosensors; that bioluminescence are rare in nature and so elicits no background effects; light measurement is fast and sensitive; equipment is easy to use; technique is inexpensive; assays are quick and simple; bacterial are integral part of ecosystems; bacteria are cheap to grow and can be freeze dried; bacteria responds to toxins; Can select environmentally relevant bacteria for a given purpose; biosensors can indicate bioavailability; can detect specific compounds; could be used for both solid and liquid state matrices (e.g. sediments, dust etc). However a few disadvantages have been cited by Bulage *et al.,* 1992 where they indicated that molecular oxygen is required and that bacteria must produce the appropriated Aldehyde substrate for optimal results to be attained. In addition to some of the disadvantage cited, Mwinyihija *et al.,* 2005, Benton *et al.,* 1995 and Brower *et al.,* 1990 reported that the use of biosensor in toxicity assessment of dust, there is extreme variability of bacterial cells to the dust particles depending on the degree of adsorption. This impairs luminescence measurement due to the inherent colour of the solution and presence of suspended particles. In addition the application of biosensors has successfully been used in tannery effluents, tannery dust, river sediments (Mwinyihija *et al.,* 2005a, 2005b, 2006), lead contaminated soils (Edmond *et al.,* 2006, 2007a, 2007b), water, soils contaminated with organics (e.g. phenolics, BETEX (benzene, toluene, ethylbenzene, xylene) etc.) (Sousa *et al.,* 1998, Sinclair, 2000, Tiensing *et al.,* 2002) and wastes (Sarin, 2000, Chaudri *et al.,* 2000). Others include detection of heavy metals such as Hg^{2+} (Selifonova *et al.,* 1993), Zn^{2+} (Erbe *et al.,* 1996), chromate-copper-arsenate (Cai and DuBow, 1997), Ni^{2+} and chromate (Peitzsch *et al.,* 1998), and Cd^{2+} and Pb^{2+} (Tauriainen *et al.,* 1998). Moreover Heitzer *et al.,* 1998 reported on the bioavailability assessment of naphthalene and salicylate using *Pseudomonas fluorescens,* which carried a transcriptional *nah-lux* fusion for naphthalene and salicylate catabolism. Burlage *et al.,* 1994 used luciferase genes as a bioreporter for toluene catabolism by bio-engineered microbes. Due to the various types of biosensors available for analysis of environmental samples worldwide as discussed earlier, it is imperative to select the most ideal and ecologically relevant biosensor of choice. Therefore the type of biosensors that has had prominent interest in most recent studies was the *lux*-marked *Escherichia coli* HB101 pUCD607 and *Pseudomonas fluorescens* 10586s pUCD607. The basis of the choice on *E. coli* is due its abundance in natural habitats and certain anthropogenic sources e.g.

sewage sludge. Moreover it has been demonstrated through many related studies that the organism operates in a wide pH range (3.0 - 10) with very satisfactory results. This type of *lux*-marked biosensor has been found to be ideal as general toxicity bioassay when compared to other ecological niche based species such as *Bacillus* and Pseudomonads e.g. *Rhizobium* sp. (Prosser 1994). In addition it is worth noting that *lux*-marked biosensors such as *Pseudomonas fluorescens* (a ubiquitous soil bacteria and important rhizobacterium) and *Rhizobium trifolii* (an associative N_2 fixer) can be used to detect the bioavailability of specific potentially toxic elements (PTE's) or to indicate general metabolic status.

Growth and bioluminescence characteristics could be assessed to evaluate the optimal phase of growth and expression of bioluminescence. Cell growth and bioluminescence of *Pseudomonas fluorescens* 10586's pUCD607 can then be studied by taking a single colony of cells from a clean plate and shaking in 10mL LB containing 10 µg of Kanamycin antibiotic and incubating overnight at 25°C. After 16h, the culture is checked for bioluminescence and, if loading on the luminometer, a sub culture of 1 mL is aliquoted in each of three 250 mL Erlenmeyer flask containing 100mL LB and µl Kanamycin. The triplicate cultures are then incubated with shaking of the samples at 25°C. A 1 mL sample is removed every 60min for the measurement of optical density (OD 550) on a Cecil CE373 spectrophotometer and a separate 1mL sample removed to measure bioluminescence on the Bio-Orbit 1251 luminometer. During the initial stages of growth (lag phase), no dilution of the culture is required but, after 5h, dilution is necessary. As the culture enters the exponential phase, serial dilutions in 9mL of 25mL of 25% Ringers are made (x10, and x100). As growth declines RLU and OD are again read undiluted. Environmental studies as discussed earlier have taken new dimension especially with the application of biosensor in determining toxicity levels in various contaminated ecosystems. In review of recent works (between the period of 2005 to 2010) it is conspicuous that biosensors have continued to be used in both spiked and unspiked (including industrial effluents and sewage sludge) environmental samples. A specific study by Edmond (2006b) investigated the toxicity levels of lead spiked soils with or without ameliorants using a biosensor. The choice of the biosensor was appropriate due to its robust, wide pH ranges (3.0 – 10.00) and sensitivity in measuring the toxicity of the final leachate collected (Palmer *et al.*, 1998). To ascertain that the application of the ameliorants to the lead spiked soils had a toxicity reduction effect on the soil sample leachate, a toxicity test based on a *lux*-biosensor was used (Fig. 5).The results compared the toxicity effect between several lead sources with the application of individual ameliorants.

For example when soil samples spiked with various lead compounds ($PbNO_3$, $PbCO_3$, $PbSO_4$ and PbS) were treated to Red earth a significant effect was observed ($p<0.05$) (with the exception of $PbCO_3$) (Fig. 5). The highest difference was observed with PbS where on application of red earth significantly increased percentage bioluminescence from 63% ±7 to 100.3±2.9. When the differences in percentage bioluminescence between samples treated with/ without Red earth were further analyzed the following trends were due to the application of the mentioned

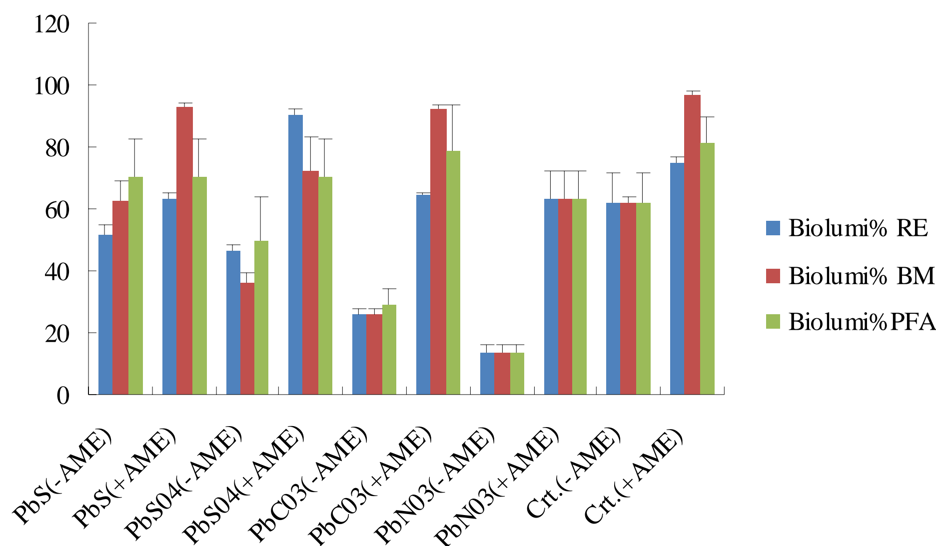


Fig. 5. Effect of ameliorant on Pb bioavailability in soils spiked with various lead compounds

bioassay was observed; PbS>$PbNO_3$> $PbSO_4$. The application of bone meal as an ameliorant had a significant ($p<0.05$) effect on lead spiked soils as demonstrated with the % bioluminescence results. However, the effect of samples treated with bone meal demonstrated only a difference in toxicity levels between $PbNO_3$ and its control ($p<0.05$). In another related study by Edmond *et al.,* (2006b) the application of PFA (Pulverized Fly Ash) treated lead spiked samples showed a significant increase ($p<0.001$) in luminescence in all the treatments. However, for leachates from samples treated with PFA, the highest luminescence result was shown with $PbNO_3$ (74%±1.24), while the lowest was with $PbSO_4$ (35.05%± 2.60). The highest difference was observed with $PbNO_3$ (23.60%) and $PbSO_4$ (22.05%), with the lowest luminescence noted with PbS (2.24%) (Fig. 5).

Overall, the use of biosensors brought to the fore application of ameliorants generally appeared to reduce metal release from heavily contaminated soils rendering the final leachate less toxic. Shende *et al.* (1994) for instance, used alkaline Fly Ash to reduce the metal toxicity of contaminated soil through immobilization of heavy metals. The linkage of CEC is closely related to adsorption potential (Fig) where Chien-Jung Lin *et al.* (1999) who investigated the effect of applying Fly Ashes to remove metal ions from wastewater concluded that the cation exchange capacity and specific surface area of Fly Ashes increased with increasing carbon content, and consequently, carbon residual in the Fly Ashes played a much more important role than the mineral content in the removal of metal by the Fly Ashes (Fig. 5). This observation is critical in evaluating the biosensor potential in determining toxicity of complex media especially when ameliorants are introduced to the environmental samples. To complement the results related to the efficacy of the ameliorants based on CEC, the biosensor analysis of leachate toxicity showed that the application of red mud had rendered the leached fraction of the spiked lead less toxic, with an increase in percentage bioluminescence upon its application. The only lead compound which showed a degree of toxicity before the application of Red mud after 193 days was PbS. This could probably be attributed to the low solubility of lead sulphide when spiked into soil samples. Bataillard *et al.* (2003) showed that when lead was added as sulphate, between 10 and 20% of lead particles dissolved, regardless of the soil type with lead sulphide progressively oxidizing over time. This observation was also demonstrated where PbS (as indicated earlier) in comparison to the other lead sources had lower adsorption percentage efficiency in comparison to $PbSO_4$. Therefore application of a biosensor as a diagnostic too indicated that red earth/ mud application as an *in situ* inactivation technique (where an ameliorant was incorporated and mixed with lead contaminated soil). Red earth bound the toxic metals to the additive which essentially reduced their mobility in the soil thus reducing the contaminant leachability and bioavailability (Hartley *et al.*, 2004). Moreover, the advantages of Red mud/earth which relate to factors such as retention of nutrients on infertile sandy soils, reduction of eutrophication of rivers and water ways (Summers *et al.*, 1996a), groundwater recharge areas, improvement of pasture growth (Summers *et al.*, 1996b), plant P uptake (Snars *et al.*, 2004) and water retention in excessively drained soils (Vlahos *et al.*, 1989) outstripped the disadvantages. The disadvantages that were attributed to red earth/mud include high pH values, salinity, and absence of nutrients and organic constituents that could possibly suppress revegetation (Xenidis *et al.*, 2005). However precautionary principle is highly required as a public health concern for wide application of red earth/mud where off-site assessment is conducted before use to avoid recontamination of terrestrial or aquatic systems. Sadeque, 2005 on the other hand used arsenic spiked irrigation water on a selected leguminous plant. The main criterion of the study was based on assessing toxicity (using *lux*-marked biosensor *Escherichia coli* HB101pUCD607). The approach evaluated the effect of arsenic contaminated irrigation water on leguminous plant (*Lens culinaris* L.), plant growth, nutrient uptake and subsequently assesses toxicity. Determination of toxicity was based on the bioluminescence response of the *lux*-modified biosensor, *E. coli* HB101 pUCD607 (Amin-Hanjani *et al.,* 1993). As indicated earlier the biosensor's environmental relevancy and wide pH range (3-10) (Palmer *et al.,* 1998) provided reliable results, which indicated an increase of toxicity on increased arsenic concentration.

Sadeque (2005) reported that evaluation of toxicity effect of Arsenic in irrigation water on plant uptake and bioavailability using a *lux*-marked biosensor was successful. Bioassay is known to provide the bioavailable fraction of the contaminant and readily complements on the chemical analysis which provides the total concentration of the contaminant. Results from such a study showed that the effect of Arsenic on increasing order of sensitivity depicted root length>root dry weight>shoot dry weight>plant height>pod dry weight>flower pod number. This was closely related to Kapustka et al. (1995) who proposed that the sensitivity of vegetative response follows the order: root length>root mass>shoot length>total mass (root+shoot)>shoot mass>germination. The vegetative responses (root length ($r=0.86$), root dry weight ($r=0.83$), shoot dry weight ($r=0.85$), plant height

(r=0.82), pod dry weight (r=0.63) and flower number (r=0.76) had a positive correlation to the bioluminescence. This observation demonstrated that an increase in arsenic toxicity (through irrigation water) resulted to a decrease of all the measured vegetative and reproductive responses of lentil plant indicated above. The bioassay results from the Arsenic related studies further indicated that the effect of filtration was probably attributed to physically held (e.g. arsenic complexes) but not chemically bond arsenic compounds (e.g. arsenite are more soluble in aqueous media) in the soil matrices (Fig. 6).

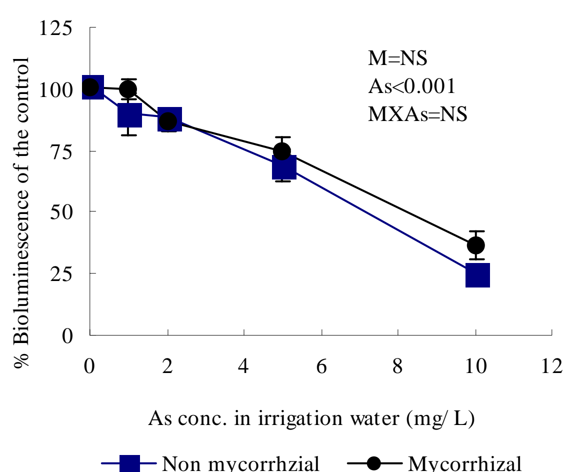


Fig. 6. Percentage bioluminescence effect of arsenic contaminated irrigation water on Mycorrhizal and Non-Mycorrhizal samples

Sewage sludge are complex media that contain a wide range of organic chemicals in varying concentration and structure including volatile organic compounds, polychlorinated, biphenyls, polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and Chlorophenols (Wilson *et al.,* 1997). This complexity of sewage disposed to the terrestrial systems may lead to accumulation of recalcitrant organics in the soil (Wild & Jones, 1992). The source of sewage sludge weather industrial or domestic also attributes to the level or presence of heavy metals, phosphates, sulphates and chlorides. Characteristically anions present in the sludge assists in binding heavy metals cations and precipitate the insoluble metal salts thus reducing the bioavailability of the toxic metals. Therefore a decline in bioluminescence is conspicuous in samples of soils amended with contaminated sewage sludge after an exposure time of 10min to the biosensor (*Ps. flourescens*). Thus in ecotoxicological perspective the presence of increased heavy metals (e.g. Zn and Cu) concentration is known to reduce on bioluminescence. The toxic effect of different heavy metals on the metabolism of the test organism has been reported by Paton *et al.,* 1994 as the main cause of the decline in bioluminescence when exposed to a biosensor. However it is worth noting that biosensor responds differently to various heavy metals. Lewin *et al.,* 1980 observed that the bioavailability of heavy metals is curtailed by their transfer to less labile organic complexes, adsorption complexes and insoluble salts. Sousa *et al.,* 1998 indicated that biosensor technique which assesses heavy metal toxicity is equally suitable to determine the bioavailability of organic xenobiotic by modifying the extraction method. Dust as an environmental concern is becoming critical both as an anthropogenic and natural perspective. Therefore when its effect is previewed and focused in the context of industrial occupational hazard than its impact cannot be underestimated (Mwinyihija *et al.,* 2005). To understand the toxicity of dust and estimating its bioavailability content, an appropriate bioassay technique incorporating a biosensor (*Escherichia coli* HB101pUCD607) was successfully used. To determine inherent toxicity potential it was important first, to agitate the collected dust samples e.g. through centrifugation. Centrifugation enables the solution to be isolated from the matrix (soil/sediment or even dust), allowing rapid and reliable solid phase testing (Vedy and Brucket; 1982; Thibault and Sheppard, 1982; Kittrick, 1983; Elkhatib *et al*., 1986; Elkhatib *et al*., 1987; Ross and Barlett, 1990). Detection of contaminants, sorbed to dust particles, using bacterial biosensors required close contact between the bacterial cells and the particulate matter, while retaining the ability to recover the bacterial cells to measure the toxicity response. Contaminant toxicity in dust will be affected by the type of dust, while quenching of light due to the colour of the sample solution and the presence of suspended particles may affect bioluminescence (Benton *et al.,* 1995; Brower *et al*., 1990). The adsorption will also be dependent on the particle size distribution as observed in soils (Benton *et al*., 1995; Ringwood *et al*., 1997) and dust (Mwinyihija *et al.,* 2006b), affecting the availability of the contaminants and their toxicity (Benton *et al*., 1995, Mwinyihija *et al.,* 2005a, 2006a). Mwinyihija *et al.,* 2006b in related studies carried out a technique where the tubes with dust samples were vortexed and then centrifuged at 7500 *g* for 10 min in a microcentaur centrifuge. Thereafter small filter containers were removed and placed into luminometer cuvettes and bioluminescence measured on a portable luminometer (Jade, Labtech International), at 15 s intervals. The results related to this study showed that biosensors provided a means of assessing bioavailability by measuring the impact of the dust pollutants on the metabolic activity of the

lux-marked micro organism (Fig. 7). Since luminescence is linked to the electron transport chain of the organism, it is, therefore, a measure of the metabolic activity of the cell (Isenberg, 1993).

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.* (1998) used sample manipulation (coupled to bioassay with *lux*- marked bacteria) to examine the toxicity of a site contaminated with BTEX (benzene, toluene, methylbenzene, xylene) compounds. Mwinyihija et al 2005a, also used as a technique, sample manipulation and a biosensor assay to samples from the tannery waste. In these cases the use of biosensors enabled reporting on site toxicity characteristics and contaminant bioavailability. The use of the *lux* biosensor *E. coli* HB101pUCD607, in relation to sample manipulation, allowed dissection and classification of the sample toxicity of tannery waste. Therefore, maximum percentage bioluminescence for all the manipulative treatment (e.g. sparging, activated charcoal, filtration and pH adjustment results) of the samples, were calculated against a blank of double deionised water at pH 5.5. The reduction (due to a toxic substance) in light output is proportional to the bioavailable concentration present (Sousa *et al.*, 1998). The choice of the *lux*-marked biosensor in such an approach offered great environmental relevance in dissecting and categorizing into broad groups (i.e. organic/inorganic, metallic/non-metallic and or acidic/alkalinic manifestation of the samples) the toxic nature of the effluent from the tanning industry. Furthermore, through toxicity dissection involving sample manipulation coupled to biosensor assay, potential remediative strategies could be identified. Where appropriate, chemical analysis was applied to confirm the biosensor-based diagnosis as the cause of toxicity and potential constraint to remediation. Indeed the *lux*-marked biosensors offered a powerful tool for the rapid, toxicity based assessment of tannery effluents and associated remediative potential to the environmental samples. Rivers have the inherent capacity to dilute and detoxify the substances discharged into them and to recover their original nutrient and oxygen levels. An adequate monitoring system should assess both the effects and the distribution of pollutants (Moriarty, 1999). Reliance on measurement of pollutants alone, without also assessing biological effects, ignores potential problems (Price, 1978). The activity of certain enzymes and cofactors such as $F_{420,}$ hydrogenase, dehydrogenase (DHA), and adenosine triphosphate (ATP), may serve as indicators of these biological effects (Nybroe *et al.*, 1992; Le Bihan and Lessard, 1998; Goel *et al.*, 1998). There is also the determination of oxygen level at the surface and river sediment level of the river which is also critical in ascertaining weather the ecosystem is anoxic. Therefore when all the techniques are compared to the use of biosensor in carrying out an evaluation of river sediment health, it was found to be effective, cost effective and providing reproducible results (Mwinyihija *et al.,* 2006a). The recovery of the river is observed when the values of the bioluminescence increases away from the discharge point. Indeed the application of the biosensor further illustrated that the sample value at the end point (800m downstream) is almost equal to the control whilst the discharge point indicates the lowest value compare to the extreme points up and down stream (Fig. 8).

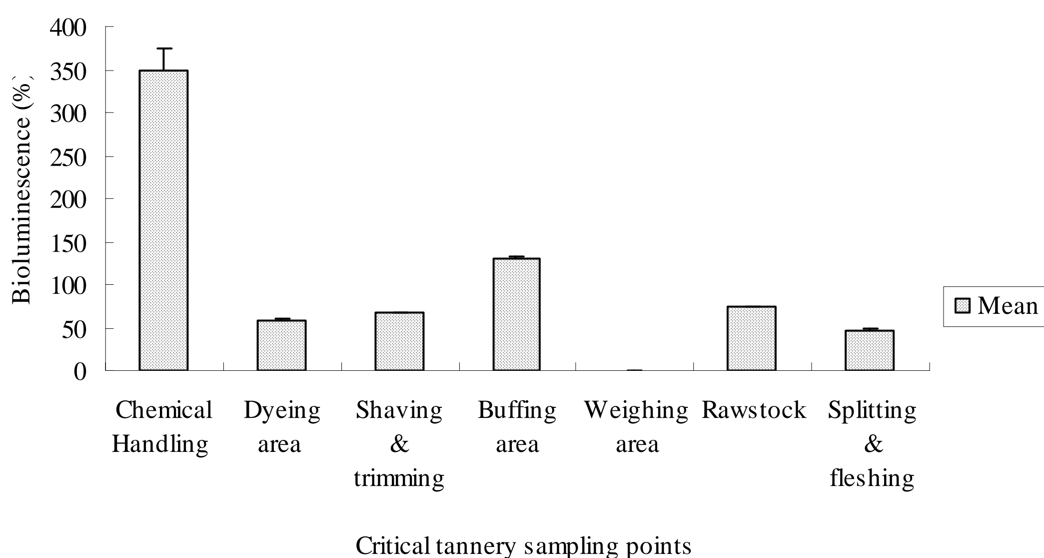


Fig. 7. Percentage bioluminescence on identified tannery dust samples

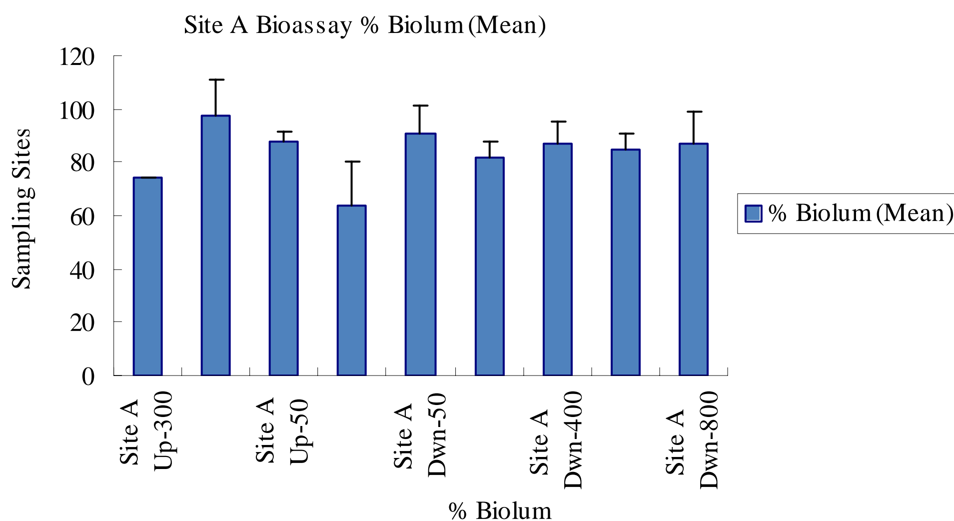


Fig. 8. Bioluminescence (%) response on river samples both up and downstream (three replicates with standard errors indicated by the error bars)

Mwinyihija *et al.,* 2006a determined how a tannery site impacted to adjacent river sediment. The tannery waste after 'treatment' were released or disposed to the river and the fear of contamination was eminent. Therefore *lux*-biosensor, INT-DHA and geochemical methods were used to generate information about the river health at the study site. The study attempted to use a toxicity assay, correlating it with the biomass activity in the sediments and ultimately assessing how the contaminants from tannery effluent impacted on water quality. An approach involving *lux*-based biosensor (reporting on metabolic activity) and DHA (biomass activity) techniques was also found to offer a rapid, reliable and sensitive monitoring procedure. This approach complemented the power of analytical methods, identifying pollutants in the parts per million and parts per billion ranges, the toxic impact of the tannery effluent was therefore assessed by integrating biological sensors. The use of other bioassay techniques positively ascertained and collaborated to the biosensor values depicting in all cases of reducing toxicity away from the discharge point. Ecological risk assessment (ERA) as a tool entails understanding the categories of hazardous waste, its identification, exposure assessment, ecological effects and risk characterization (Mwinyihija *et al.,* 2005b). To identify the toxic nature of the effluent, bioassays (to evaluate responses to stressors) and chemical analysis (to provide information on the concentration and identification of the stressor) were used. ERA is also defined as a process that evaluates the probability that adverse ecological effect will occur as a result of exposure to one or more stressors. Therefore the objective of an ecological risk assessment is to determine and document actual or potential effects of contaminants on ecological receptors and habitats as a basis for evaluating remedial alternatives. Moreover for the purpose of undertaking ERA, a genetically modified biosensor (*E .coli* HB101pUCD607) as a primary decomposer was also used as part of a trophic level representation Mwinyihija *et al.,* 2005b). The major advantage of bioassays is that the total toxicity of wastewater can be assessed by taking into account bioavailability and synergistic or antagonistic effects. Chemical analysis provides information on the concentration of a substance in a sample and may help to identify that substance (Stuhlfauth, 1995). However, this does not give direct information relating to the bioavailability and impact of environmental pollutants. The major advantage of bioassays is that the total toxicity of wastewater can be assessed by taking into account bioavailability and synergistic or antagonistic effects. Also, transforming information on concentration to information on biological response is useful for risk assessment. Chemical analysis provides information on the concentration of a substance in a sample and may help to identify that substance (Stuhlfauth, 1995). However, this does not give direct information relating to the bioavailability and impact of environmental pollutants.The biosensor (*Escherichia coli* HB101 pUCD607) provided acute toxicity data for the stressor during the effluent treatment phase and flow towards the aquatic receptors showing areas that were high in toxicity (Mwinyihija, 2010). The use of bioassays and biomass activity could be complemented with chemical analysis, which quantified the stressors and their effects in the ecosystems. The significant impact of the stressors to

the riverine ecosystem was demonstrated when the upstream, discharge point and downstream sampling points indicated a positive correlation with the other bioassay (i.e. Dehydrogenase and Daphnia) results (Mwinyihija *et al.,* 2006a). Ecological risk assessment (ERA) is a relatively new approach to quantifying the risk of significant harm to organisms and their ecosystems, but it is already a requirement of a number of regulatory regimes worldwide.

CONCLUSION

When using luminescent bacteria for analysis of environmental samples emanating from the tanning industry, toxicity testing could pose certain problems. This can be encountered where light output (luminescence) is affected by coloured supernatant and differing c.f.u/mL numbers in the analyzed sample; loss of bacteria due to adhesion to suspended sediment/dust particles and optical interference of suspended sediment particles. Moreover others could include false positives (due to inducer substrates e.g. salicylate or anthranilate common in the environment) or false negatives (significant quantities of metabolic inhibitors such as naturally occurring compounds including antibiotics or contaminants such as cyanides and chlorinated aromatics) which may be encountered. In addition, the extracts after centrifugation may expose to the biosensor organism, more contaminant than would actually occur in the environment, producing overestimated toxicity values. However due to the main aspect of interest in this paper being the efficacy attributed to the application of selected biosensors to various environmental samples, it was important to minimize the limitations cited by identifying resident organism in areas earmarked for ecotoxicological analysis. The environmental relevance within such identified areas would enhance accuracy and reliability of the data collated. This is because bioavailability (used to describe the biologically active form or fraction of a chemical that is available for uptake or transformation by living organisms) in relation to ecotoxicity assessment of pollutants is species dependent (especially when considering rate of transfer of the compound/xenobiotics from solution, uptake and metabolism). As such use of biosensor is appropriate, relevant, cost effective, easy to adopt and opportune to carry out ecotoxicological diagnosis of the pollutants emanating from the tanning Industry.

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