

## Bioassay Kits for Ecotoxicological Testing of Wastewaters with Nanoparticles

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Evaluation of complex wastewaters should include acute/chronic ecotoxicological tests [1] and the potential for endocrine disruption [2,3] to complement the physicochemical characterization. The advantages of bioassay (with a bacterium, alga or crustacean) are simplicity, speed and low cost for monitoring toxicity over time. Its strongest attribute is usefulness as a primary screening test for a broad spectrum of toxicants. Several bioassay kits are commercially available, including the Lux-fluoro, Polytox, Microtox and Thamnotox kits that require no specialized equipment. A combined approach using instrumental methods for chemical analysis and bioassays for ecotoxicological testing would be extremely important to hazard/risk assessment of wastewater treatment plant discharges.

The Lux-fluoro test was developed for the rapid detection and quantification of environmental pollutants with genotoxic and/or cytotoxic potential [4]. This bacterial test system uses two different reporter genes whose products and reactions can be measured easily and simultaneously by optical methods. Genotoxicity is measured by the increase of bioluminescence in genetically modified bacteria which carry a plasmid with a complete lux operon for the enzyme luciferase from the marine photobacterium *P. leiognathi*. If the deoxyribonucleic acid (DNA) in these bacteria is damaged by a genotoxic chemical, the SOS promoter is turned on and the lux operon is expressed. Other genetically modified bacteria carry the gene for the green fluorescent protein from the jellyfish *Aequora victoria* downstream from a constitutively expressed promoter. These bacteria are fluorescent under common growth conditions. If their cellular metabolism is disturbed by the action of cytotoxic chemicals, the fluorescence decreases in a dose-proportional manner. A temperature-controlled microplate reader capable of sequential reading of luminescence and fluorescence can be programmed for repeating the measurement cycle at 30°C from 10 min up to 8 hours of continuous incubation [5].

The Microtox assay is based on the luminescent mechanism of freeze dried *Photobacterium phosphoreum* or *Vibrio fischeri* [6]. If metabolic processes are changed upon cell damage by a toxic substance, a reduction in light output can be detected within 5 to 30 minutes. After correcting the effects of color and turbidity on bacterial light output measurements [7], an EC50 concentration can be calculated as a toxicity value for comparison against a control substance to evaluate relative toxicity [8]. Microtox is probably the most sensitive test that can be used in a screening phase. The test has recently been used to quantify the impact of storage time on toxic compounds formed in the electrochemical treatment of phenol. Chlorinated phenols exhibited little toxicity change while highly toxic benzoquinone exhibited 92% loss of its initial toxicity over 18 days [9].

Polytox is a 30-minute rapid biological test for measuring the toxicity of raw water or wastewater to biological organisms. This blend of specialized microbial cultures, free of nitrifying microorganisms, provides a simple and economical test that eliminates the need for expensive instrumentation [10]. It is a cost-effective and non-pathogenic product that runs with a standard dissolved oxygen meter. Reliable, consistent results can be obtained in 30 minutes. The shelf life is, however, only 3 months from the date of product activation upon order.

Thamnotox bioassay with the crustacean *Thamnocephalus platyurus* [11] is cost-effective and culture maintenance-free. Test organisms are included in the kit as dormant eggs (or cysts) which can be hatched on demand, in 24 hours, to supply the biota. The assay is based on mortality of the test organisms, with calculation of the 24hLC50. A quality control test with a reference chemical provides for accuracy and reproducibility check. Minimal equipment is needed for test performance: dissecting microscope, small incubator and conventional laboratory glassware. The shelf-life of cysts is guaranteed for several months when stored properly, thereby reducing test scheduling constraints. Total performance time of the assay is approximately 1 hour. The Thamnotox kit is used in many laboratories worldwide for routine screening of chemicals. It is specifically sensitive to biotoxins produced by blue-green algae in environmental samples. Test results have shown significant correlation with organic load parameters and total suspended solids.

Nanoparticles (NPs) are increasingly applied in a diverse array of commercial, industrial and medical products due to their intrinsic properties. There is a pressing requirement for a comprehensive understanding of their hazard, which is a major research pre-occupation for toxicologists [12]. Since early literature showed that very complex and unexpected interactions could occur between nanomaterials and biological systems, a wide spectrum of NPs varying in charge, composition, shape, size and solubility has been tested of their toxicity. Other than their ability to translocate from the site of deposition, there is no evidence that particles below 100 nm show any step-change in hazard from the conventional particle toxicology. Effort should be focused on measuring their toxicokinetics toward a better understanding of nano-specific risk assessment at the individual organism, tissue, cell and DNA levels. Toxic behavior of zinc oxide NPs at the subcellular level, for instance, has recently been reported [13]. Treatment of isolated rat liver mitochondria with ZnO NPs resulted in collapse of membrane potential, swelling, depression of respiration, increase of inner membrane permeability to H<sup>+</sup> and K<sup>+</sup>, alterations of ultrastructure, release of cytochrome c, and generation of reactive oxygen species. These results suggested a putative mechanism that ZnO NPs can lead to energy dissipation, oxidative stress and even apoptosis.

Dissolution of ZnO NPs plays an important role in the toxicity to aquatic organisms [14]. The effects of water chemistry such as pH, ionic components, and dissolved organic matter (DOM) on the dissolution

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of nano-ZnO and its toxicity to *Escherichia coli* were investigated. The results showed that increasing pH,  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ,  $\text{HPO}_4^{2-}$  and DOM reduced the concentration of free  $\text{Zn}^{2+}$  released and thus lowered the toxicity of ZnO NPs. Suspensions of cerium oxide, iron oxide and titanium oxide nanoparticles were tested for lead removal in water cleaning via adsorption processes [15]. Germination tests with tomato/lettuce/cucumber seeds and the Microtox assay were used to determine the toxicity of synthesized NPs, NPs after lead adsorption, and the supernatant after NPs separation. The  $\text{CeO}_2$  NPs showed a high phytotoxicity while the  $\text{Fe}_3\text{O}_4$  NPs and  $\text{TiO}_2$  NPs did not exhibit any toxicity.

Silica mesoporous nanoparticles are used more and more in oral drug delivery. Their cytotoxicity and genotoxicity on the HT29 human intestine cell line were studied by measuring cell viability, proliferation and global metabolism [16]. Besides conventional sulforhodamine B, flow cytometry, and  $\gamma\text{-H2Ax}$  foci approaches, real-time monitoring of cell proliferation was performed using an impedance-based system to ensure no interference. Experimental results showed that 25-nm and 100-nm  $\text{SiO}_2$  NPs induced a rather limited cytotoxic and genotoxic effects on HT-29 cells after a 24-h exposure. Interestingly, the higher the 100-nm  $\text{SiO}_2$  dose, the lower the cytotoxic/genotoxic effects. These data well illustrated the complexity in understanding the hazards of NPs for human health. Previous work had reported the effect of carboxyl functionalization of graphene in pacifying its strong hydrophobic interaction with cells and associated toxic effects [17]. Pristine graphene (contact angle =  $162^\circ$ ) was found to accumulate on the cell membrane causing high oxidative stress leading to apoptosis, whereas carboxyl functionalized hydrophilic graphene (contact angle =  $30^\circ$ ) was internalized by the cells without causing any toxicity.

Systematic evaluation of NPs toxicology in living systems was based on a determination of the number of NPs and of their toxicity factor [18]. Such a strategy was applied to the ingestion of citrate-capped gold nanoparticles by the model system *Drosophila melanogaster*. Using AuNPs as a reference toxicity standard, different regions in the multiparametric space of toxicity were defined to enable the classification of other nanomaterials, such as quantum dots and pegylated AuNPs. This approach may lead to important developments in risk assessment and regulatory approval of NPs in a wide range of applications.

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