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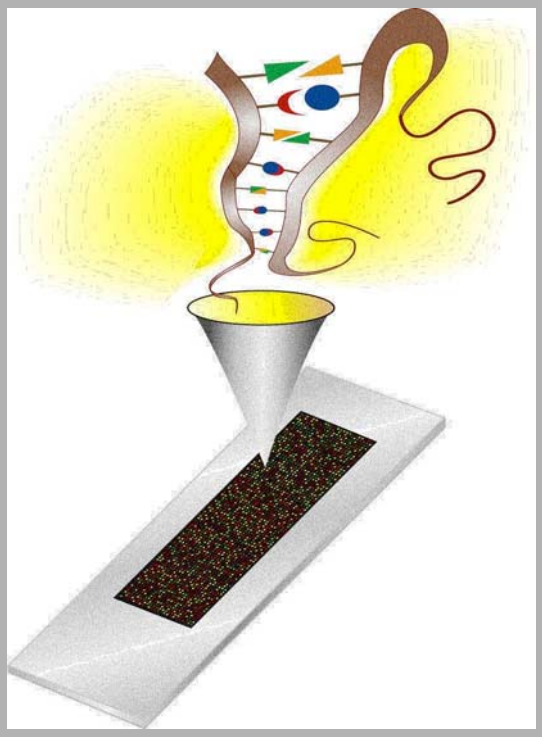

Rapid Environmental Impact Screening For Engineered Nanomaterials:

A Case Study Using Microarray Technology

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Project on
Emerging Nanotechnologies
at the Woodrow Wilson International Center for Scholars

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The **Project on Emerging Nanotechnologies** is an initiative launched by the Wilson Center and The Pew Charitable Trusts in 2005. It is dedicated to helping business, government and the public anticipate and manage possible health and environmental implications of nanotechnology.

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Patrick Larkin, Ph.D. is Vice-President of Research & Development, Chief Scientific Officer, and a co-founder of EcoArray Inc. Dr. Larkin received his Ph.D. from the University of Florida. He has over 10 years experience as a molecular biologist and has considerable expertise in sequence analysis, microarray construction, real time PCR assay development, and analysis and interpretation of microarray data. He has co-authored 9 peer-reviewed paper in the aquatic toxicology field using this technology in the last three years.

John Rogers is President CEO, and a founder of EcoArray, Inc. as well as AquaGene, Inc., a biotechnology company using transgenic fish to produce human therapeutic proteins. He was formerly Vice President Finance and Treasurer of Zytec Corporation. Mr. Rogers is an Advisory Council member of Keck Graduate Institute (Claremont, CA) and past Chairman of BioFlorida, an industry association. He received his AB from Dartmouth College, an MBA in finance from the New York University Graduate School of Business and completed postgraduate studies at the University of Chicago Business School.

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Introduction

Recent public attitude research undertaken by the Project on Emerging Nanotechnologies indicated a desire on the part of the public for (A) more pre- and post-market testing/tracking of nanotechnology-based products and (B) a greater disclosure by firms producing these products of their possible environmental and human health impacts (Macoubrie, 2005). What the public is asking is: "Are nanotechnologies safe?"

In addressing this question, businesses concerned with the potential toxicology of nano-based products face two interrelated challenges regarding testing: speed and cost. Given both domestic and global competition, firms are under pressure to develop and introduce new nanotechnology-based products into the marketplace rapidly or face potential losses in market share, revenues, and strategic position. This means that companies need toxicity screening methods that can fit into product development cycles, which will allow environmental and human health problems to be identified early and hopefully engineered out of products before they are introduced into the marketplace. These screening techniques also have to be affordable. Given the many small businesses and start-ups involved with nanotechnology, financial constraints will limit their options vis-à-vis toxicity testing. Realizing this need, the Project on Emerging Nanotechnologies launched an initiative to work with firms and scientists to develop and apply fast-turnaround toxicity screening methods to emerging nanotech products.

This proof-of-concept study involved the development and application of a genomic-based, ecotoxitity screening method to nano-scale iron particles being used for environmental remediation. Ecotoxicity is an area that has received far less attention and funding than the study of the potential human health impacts of nanomaterials. The screening was completed in less than four months with the complete cooperation of the company and the test showed no significant ecotoxicity effects for two important indicator species. It is important to remember that the findings do not constitute a product endorsement but an additional set of data that the company and consumers can evaluate to make more informed decisions.

David Rejeski
Director, Project on Emerging Nanotechnologies
Washington, DC
December 2005

Executive Summary

The increasingly rapid introduction of nano-based substances into the marketplace will require new methods to assess both short and long-term environmental impacts. This project explores the application of genomics technologies to nanotechnology, to provide faster, cheaper, and more sophisticated ecotoxicity testing. Microarrays – a relatively new technology capable of measuring subtle changes in DNA in response to exposure to toxins -- were used in conjunction with more traditional methods already approved by the EPA to assess ecotoxicity.

The goal was to demonstrate the potential of this approach for ecotoxicity screening by collaborating directly with a company that had developed an engineered nanomaterial. The company, Toda, manufactures Reactive Nano-Iron Particles (RNIP), which are currently being used to remediate toxic waste sites.

The project combined both a standard EPA-approved ecotoxicology test using daphnia (a water flea) with assays using a newly developed, 2000-gene DNA array for the fathead minnow, an important indicator species that EPA uses for freshwater testing. Three primary advantages emerged:

- Speed: All testing was completed within 4 months. This fast turnaround provides important advantages to businesses seeking to screen out potential environmental risks early in the product development cycle, before products enter the market.

- Sensitivity: The genetic based screens allow the examination of a variety of toxicity endpoints and sub-lethal effects that standard ecotoxicology testing often does often not address.

- Cost: This approach presently costs in the range of $20,000 – 40,000 and the costs can be expected to drop given the rapid cost decreases in DNA arrays.

The testing of the Reative Nano-Iron Particles revealed no significant toxicity issues for the material, though the tests are not all-inclusive since only two species were studied, and only 2000 genes are printed on the DNA array. However, data of this type can serve to quickly highlight toxic materials and can serve as a basis for more detailed mechanistic studies.

Importantly, the testing design described in this report can serve as a model for other companies that are currently developing nanotechnologies. Having these types of rapid screening tests available allows for quick screening, data analysis, and data dissemination, which ultimately will build public trust and help ensure eco-safe nano-products.

I. Background

In August 2004, the Woodrow Wilson International Center for Scholars hosted a meeting in Washington, DC, to explore both the applications and implications of nanotechnology. At that meeting questions were raised about the current state-of-the-science of the ecotoxicology of engineered nanoparticles. Until that point, 'eco-nano' research had focused primarily on applications such as remediation and green manufacturing (see Appendix, Table 2), not on nanoparticles themselves as potential toxicants in the environment. Although the US EPA initiated funding to study the possible environmental implications (versus applications) of nanotechnology in 2004 However, there is a lengthy process of writing grants, peer review, and approval of funding before research can begin. It is evident that information on the impact of NP on the environment will lag years behind the commercialization and use of such particles for environmental applications.

The Project on Emerging Nanotechnologies at the Wilson Center saw a need to fill this information gap and began an initiative to help validate new techniques for the rapid testing of nanoparticles to determine their effects on species that are good indicators of ecosystem health. The ultimate goal is to establish simple, cost-effective methods that can be used to screen nano-based substances and products for eco-toxicity **before** they are commercialized and in the marketplace. The establishment and validation of new techniques could also increase public trust in both government and industry and raise consumer confidence in emerging products.

Existing techniques for testing compounds

To measure the toxicity of a compound, short (acute) and longer (chronic) term exposures are run using one or more environmental "indicator species". The standard 'value' that is used for comparing toxicity of chemicals is the 48-hour LC_{50}, which is the amount of chemical needed to kill 50% of the animals in 48 hours (LC = Lethal Concentration). Other parameters that are measured in these bioassays include measuring fertility, fecundity, and egg hatchability of animals and survival of young. While these endpoints have high ecological value, they often lack information about the compound's mechanism of action and are typically not sensitive

enough to measure low dose effects of a compound. Finally, these biological endpoints are expensive to acquire.

Use of microarray technology for testing nanoparticles

Microarrays (also called gene chips) are tools that are made by spotting or synthesizing hundreds to thousands of genes specific to an organism onto a solid support matrix (Brown and Botstein, 1999; Brazma and Vilo, 2000; Burgess, 2001; Churchill, 2002). Microarrays detect changes in messenger RNA (mRNA) within an animal. By measuring mRNA, one can detect subtle responses in an animal upon exposure to a compound. Microarrays offer the advantage of being able to provide biologically relevant, mechanistically based data compared to existing assays that are currently used for compound screening. Microarrays can also be used to detect adverse responses of animals to toxicants earlier than existing assays, which often measure various physiological endpoints. This "early detection" is because changes in the normal physiology of an animal due to exposure to compounds in the environment are ultimately a result of initial changes at the molecular and cellular levels. In addition, microarrays can be used to identify dose response relationships for compounds (Larkin et al., 2003) and therefore could be used to identify levels of exposure of animals in a laboratory or field setting.

Animal models

Fathead minnows (*Pimephales promelas*) were chosen as a model species in this study for several reasons. First and most important, they have been used as a standard test species for aquatic toxicology since the 1960s (Mayer and Ellersieck 1986) and are widely used in eco-toxicology. There are over 9,000 records for fathead minnows in the ECOTOX database alone. Second, their reproductive physiology is well known (Jensen et al., 2001), and they can be propagated easily in the laboratory. Third, there is a 2,000 gene microarray available for this species. While there are larger, whole genome microarrays available in other aquatic species like zebrafish, for this project we wanted to use a sentinel species that is found in the United States and is commonly used as a standard species for eco-toxicology.

Water fleas (*Daphnia magna*) were also used to examine the toxicity of RNIP. *Daphnia* are small crustaceans that live in fresh water such as ponds and lakes. They are an important source of food for fish and other aquatic organisms. Like the fathead minnow, *Daphnia* are

commonly used as a bioindicator species by various governmental agencies, including the US EPA. This species is also easily grown and maintained in a laboratory setting.

Industry Partnership

For the first phase of this project, we chose to work with a company that had already introduced a product into the market. Several nanoparticle products are currently in use

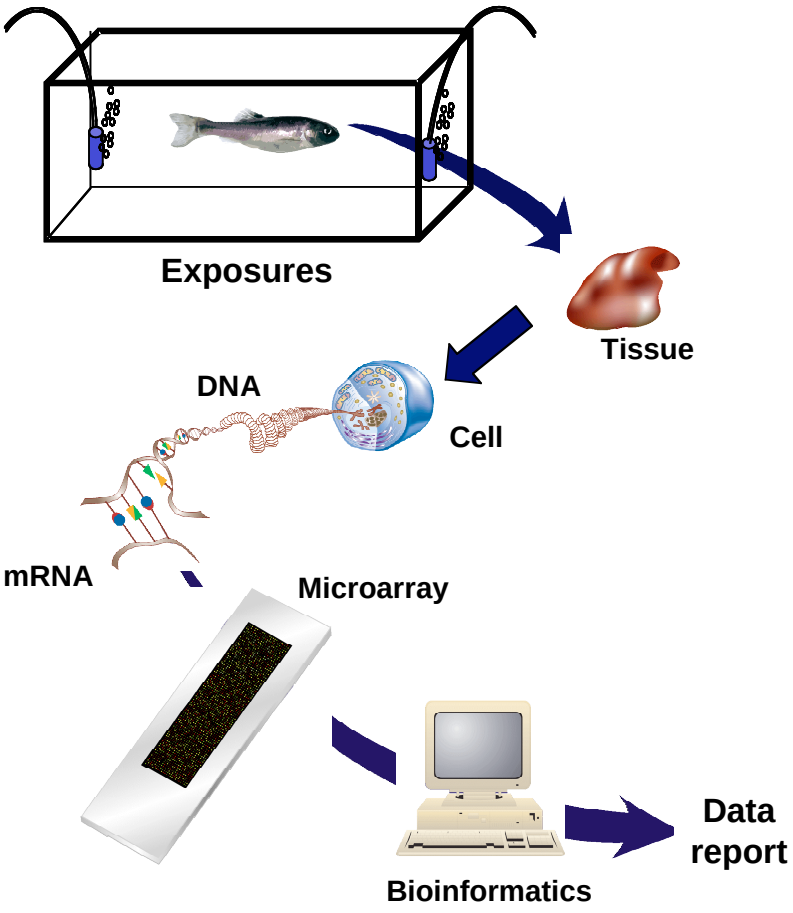

Figure 1: Rapid gene expression screening using a fathead minnow microarray.

and commercially available. These products had been approved for use by the US EPA and presented a baseline of 'eco-nano-technologies'. With input from US EPA, we requested the collaboration of one of the largest companies, Toda America, which is presently manufacturing Reactive Nano-Iron Particles (RNIP) for use in superfund site remediation (Appendix 1). Toda

America donated 1 kg (250 g RNIP in 750 mL water, as a slurry) for toxicity testing using standard aquatic species.

Compound used

The reactive Nano Iron Particles (RNIP) that were used in this study are iron solids with an average particle size of 70 nanometers, composed of an iron oxide shell and an elemental iron core. In comparison, a typical bacterial cell is approximately 1000 nanometers in diameter. One property that characterizes these particles from their micro-scale counterparts is a large reactive surface area of over 9,000 square feet per ounce (28.27 square meter per mL) of material. The ability of iron to rust, or oxidize, gives it the ability to treat hazardous substances and chemicals. The particles can be injected directly into groundwater or used to detoxify contaminated water in above-ground tanks. The technology has been shown to be very effective in treating chlorinated organic solvents, organochlorine pesticides, and polychlorinated biphenyls (PCBs) (Zhang, 2003). Though the use of iron for remediation has been generally accepted as an environmentally safe practice, applications so far have not made use of particles in the nanoscale range. Two important questions raised are whether our understanding of iron at a bulk or micro scale translates to the nano-scale and whether the large increases in oxidative ability also poses additional threats to the environment.

II. Overview of Project

The goal of the project was to develop a quick turnaround genomic based ecotoxicity screen for companies developing and commercializing new nanotechnologies which provides results in 3-4 months. Based on these efforts, we hope to encourage industries that are developing nanotechnologies to test their materials using genomic tools.

Study Design

The project utilized both a standard EPA-approved ecotoxicology test using daphnia with assays using a newly developed, 2000-gene DNA microaarray for the fathead minnow, an important indicator species that EPA uses for freshwater testing From the initial assays to the final report, the process took approximately 4 months (May-August 2005).

Step 1: Range-finding assay using water fleas (*Daphnia magna*)

Step 2: Exposure studies using fathead minnows (*Pimephales promelas*) exposed to a high, but tolerable dose of RNIP for 5 days

Step 3: Gene expression study to examine changes in liver and gill of fish using DNA microarrays

III. Daphnia study

Initial range find-studies were carried out with a common aquatic zooplankton, *Daphnia magna*, the water flea (picture at right). Daphnia are the basis of many aquatic food chains since they filter-feed on phytoplankton (microscopic algae), and are in turn eaten by fish. Daphnia are also used by the US EPA as a standard organism for testing the toxicity of various chemicals. Therefore a large database is available to compare the toxicity of new chemicals to those that are already in use.

The 48-hour LC_{50} of RNIP was found experimentally to be ~55 parts per million (ppm), which is approximately the same as that for bulk iron. Figure 2 below shows mortality after exposures to various concentrations of RNIP.

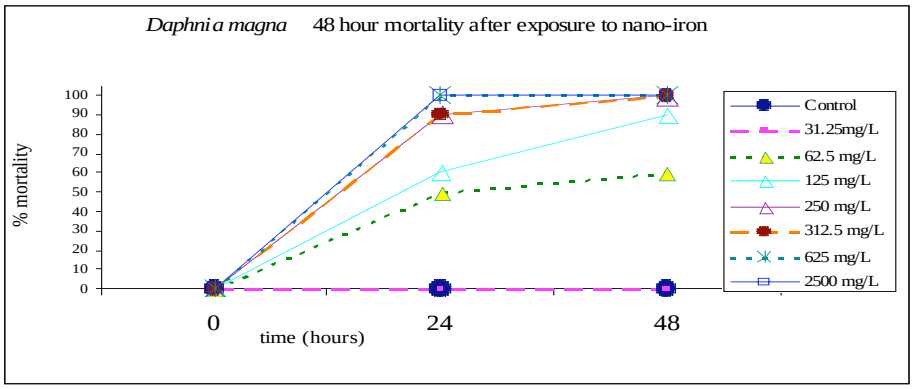

Figure 2: Daphnia mortality curve.

Based on a toxicity rating scale that is used by toxicologists to classify compounds for aquatic toxicity, RNIP would be considered slightly toxic (see Table 1). In addition, 55 ppm of RNIP is an extremely high dose, and would likely not be seen in the watershed after remediation is completed.

Toxicity Category	LC50 (ppm)
Very highly toxic	<0.1
Highly toxic	0.1-1.0
Moderately toxic	1.0-10
Slightly toxic	10-100
Practically nontoxic	>100

TABLE 1: Toxicity scales as defined in: M. A. Kamrin, *Pesticide Profiles: Toxicity, Environmental Impact, and Fate,* Lewis Publishers (Boca Raton, FL, 1997), p. 8

By comparison, chemicals such as benzo[a]pyrene have an LC_{50} of less than 0.1 ppm (Govers *et al.*, 1984), and are considered to be very highly toxic.

An interesting observation from the exposure study was that the Daphnia ingested RNIP and this NP also coated their carapace (outer shell), including filtering apparatus and appendages (Figure 3). Even though the daphnids were coated with RNIP, they were able to survive and were able to feed and reproduce over a 21-day life-cycle test. The significance of this observation is currently not known.

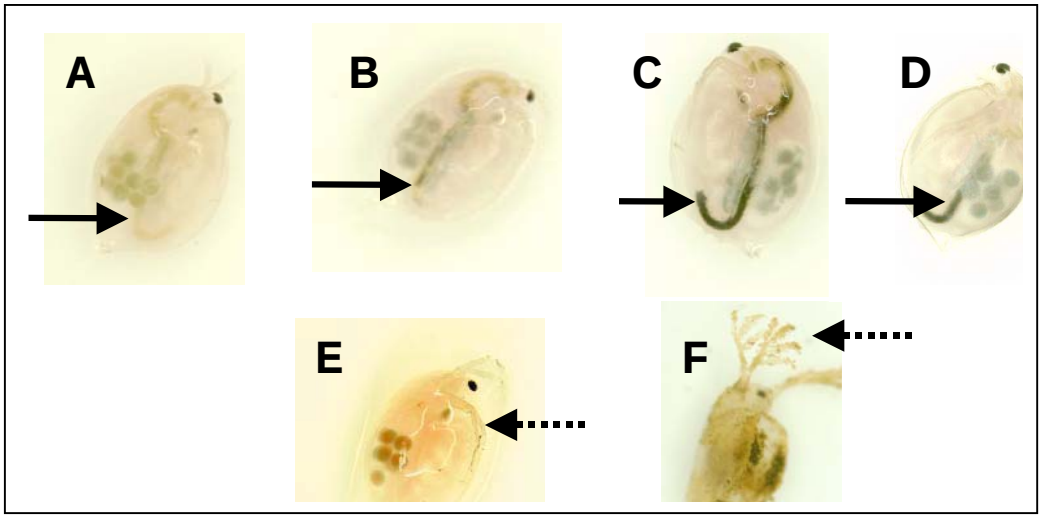

Figure 3: Daphnia exposed to various concentrations of RNIP used in remediation. A = control; B = 3 mg/L; C = 7.5 mg/L; D = 15 mg/L; E = 30 mg/L; F = 125 mg/L (dead daphnid). All daphnids shown are 21 days old and eggs are visible in their brood pouches (small green circles). Note the darkening of the digestive tract from A (normal greenish color) to D with increased ingestion of RNIP particles (solid arrows). Antennae become clogged with nano-iron in E and F (dashed arrows).

IV. Fish study

For the fish experiments, we exposed fathead minnows, a standard EPA test species, for 5-days to 50 ppm of RNIP. In the absence of formal standards yet to be established by the EPA for using microarray data in exposure studies, we based our fish exposure design on the Daphnia range-finding studies and a report in the literature that examined gene expression profiles in the sheepshead minnow, which were exposed 4-5 days to a number of different compounds that mimic estrogens (Larkin et al., 2003). The Daphnia range-finding study served as a cost-effective, quick screen to identify a dose of RNIP that was subsequently used for the fish exposures. Because of the longer exposure time in these studies, we used a slightly lower dose of RNIP than the dose used for the Daphnia studies.

Fish were exposed as groups of 3 in 10 liter aquaria (Figure 4), with 5 aquaria serving as controls and 5 aquaria containing RNIP (30 fish total). A 50% water change was done at 24 and 72 hours, and water quality and temperature were monitored and were at all times in normal ranges.

Figure 4: Control (Left) and 50-ppm RNIP (Right) exposure aquaria, each containing three male adult fathead minnows. Note that the tanks with RNIP had a dark color to the water.

During the 5-day exposure the fish were monitored daily for overt physical changes (such as lesions). The concentration used did not cause any mortality of the fish. Mortality is an endpoint that is currently used in acute toxicity tests. In addition to measuring this traditional endpoint, we also measured gene expression patterns in the fish using a fathead minnow 2000 gene microarray that was developed by EcoArray and the US EPA and implemented on the Agilent® Technologies platform. The genes on the fathead minnow microarray were obtained by sequencing clones obtained from cDNA and subtraction libraries that were constructed from different tissues (brain, liver, gonad, gill, and others) in male and female fathead minnows. The 2,000 genes encode proteins that are part of a variety of diverse biological pathways in fathead minnows. Because of the broad representation of genes on the microarray, these tools can be used to examine the toxicity of nanoparticles.

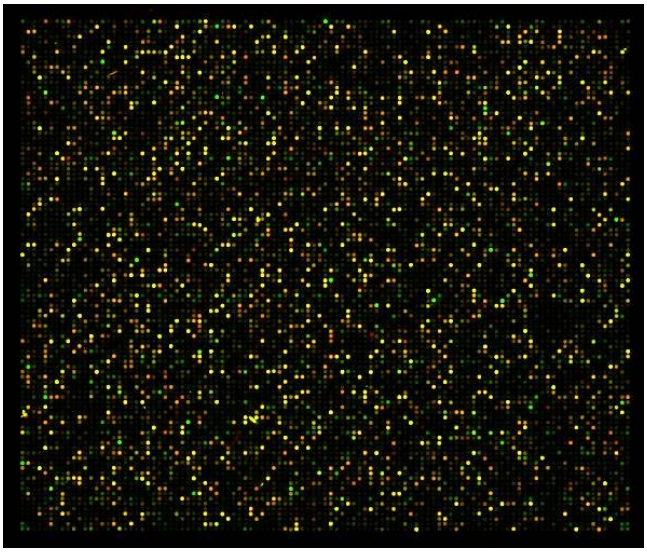

Figure 4: Picture of fathead minnow microarray.

To carry out the microarray experiments, preserved tissue samples were shipped to EcoArray for analysis. Twenty microarrays were used to examine gene expression patterns in liver and gill tissue of fathead minnows exposed to RNIP or vehicle control.

The microarray results revealed that very few genes were robustly changed in the RNIP exposed animals compared to controls. Some genes were differentially regulated based on standard statistical measures (t-Tests, $P<0.01$). These included genes that encode proteins involved in tissue repair, inflammation (the first line of defense against any foreign chemical or organism), and anti-oxidant defenses. See Appendix, Table 3 for a summary of some of the genes that were changed.

V. Conclusions and Long-Term View

The project demonstrated that a new technology can be brought to bear to the issue of screening NP quickly and cost-efficiently. While there is additional work to be done to develop robust databases that can be used to further examine and document the microarray data, microarrays show substantial promise of being useful in screening NP products. Working with a new technology in a new area, we came to meaningful conclusions in four months, and at a cost that would compare very favorably to current tests. While these studies measured gene expression profiles in fathead minnows exposed to RNIP at a specific dose, additional exposures of fathead minnows to RNIP need to be conducted to identify Lowest-observed-effect (LOEL) and No observable effect (NOEL) levels. Furthermore, additional studies that examine other endpoints like histology and reproduction could be conducted in the future as well as studies that characterize the gene expression profiles in *Daphnia* that are exposed to RNIP.

The success of the project was also due to Toda Kyogo's willingness to accommodate the project's needs. Without knowing what we would find in the aquatic toxicity tests, Biox cooperated fully with this study. This type of openness and readiness to have their product tested shows not only a high level of faith in their product, but also their willingness to be transparent to the public.

Microarray technology offers several advantages over existing testing methods and may replace them when it is fully developed and better understood by the testing community. The current testing approaches are iterative, approximate and expensive. For example, Donald Versteeg, Ph.D., a senior scientist at Procter & Gamble and advisor to EcoArray, estimates the current cost of screening a product for possible endocrine effects are in the $50,000 to $80,000 range per compound using draft OECD or US EPA methods. With advances in combinatorial chemistry and molecule design, new compounds are being synthesized rapidly and environmental screening costs need to be less than $5,000 per compound to make that new productivity worthwhile. EcoArray estimates that arrays can deliver this kind of cost performance. Environmental remediation assessments would realize similar benefits: the current cost to test a site for toxicants is now $110,000 – 130,000. A microarray-based approach will cost $30,000 – 50,000 using 2006 array costs.

As has happened in other technologies (e.g. computer chips) the cost of gene microarrays is falling over time. Figure 5 shows a projection of the base cost of microarrays for EcoArray's fathead minnow array. The chart shows cost per spot, excluding costs to hybridize, scan and analyze arrays.

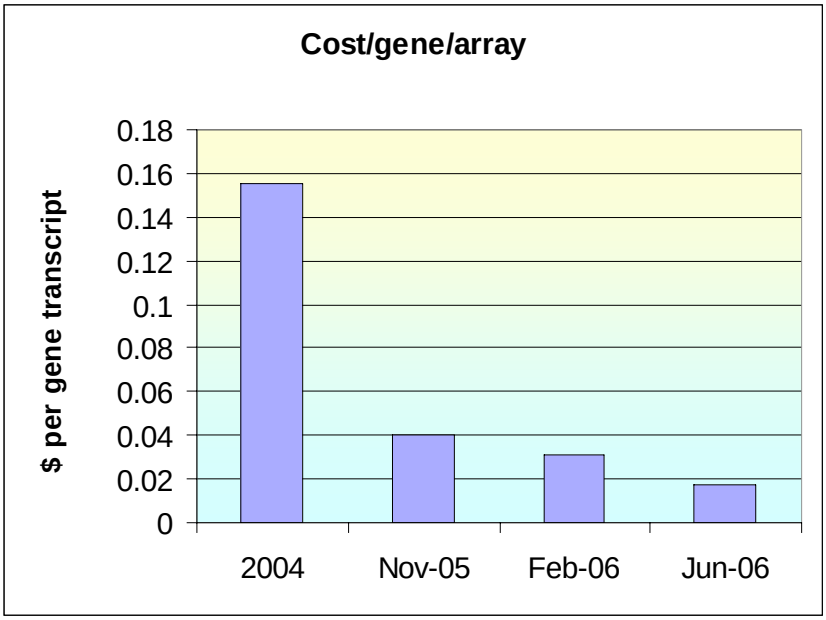

Figure 5: Microarray costs.

For screening of NP, as well as other compounds, the rapidly falling cost per spot on the microarray is important since screens will likely use large microarrays in order to record the expression of as many different genes as possible during testing.

The Future

This study can serve as a model to other companies currently developing nanotechnologies. Having these types of rapid screening tests (3-4 months) available for companies allows for quick screening, data analysis, and data dissemination, and this ultimately will build public trust and ensure eco-safe nano-products.

APPENDIX

Table 2: Some recent funding by the US EPA to develop applications of nanoparticles for use in the Environment (US EPA, 2005).

Type of NP used	Potential use	Lead PIs and Institutions
	REMEDIATION	
nanoTiO2	photocatalysis of organic contaminants	DD Dionysiou Miami University-Oxford, OH; University of Cincinnati, OH
carbon nanostructures	absorption of organics	MB Tomson Rice University
nano-metal oxides	control NOx production	S Senkan UCLA
nano-iron	degradation of PAH-based contaminants	GV Lowry, SA Majetich, K Matyjaszewski, RD Tilton Carnegie Mellon University
nano-biopolymers	control of heavy metals	W Chen, M Matsumoto, A Mulchandani UC Riverside
bi-metallic nano-Fe/Pd	remediation of inorganics and organics	WX Zhang Lehigh University
nano-crystalline zeolite	NOx, photocatalytic oxidation of organics	SC Larsen, VH Grassian University of Iowa
nano-magnetite	groundwater contamination	M Hull Luna Innovations, Inc.
	FILTRATION	
ferromagnetic particles	using nanocomposites to monitor and filter (smart particles)	WM Sigmund, D Mazyck, CY Wu University of Florida
nano-crystalline catalysts	disinfection by-product control in drinking water	SJ Masten and MJ Baumann Michigan State University
nanostructured electrodes	perchlorate from drinking water	SM Jaffe Material Methods LLC
	SENSORS	
carbon nano-particle based microchip	analytical chemistry of Environmentally Relevant endpoints	J Wang New Mexico State University
nanocrystalline metallic conductors	gas sensor	V Subramanian UC Berkeley
colloidal-metal nanoparticles	monitoring Heavy Metals	O Sadik, J Wang New Mexico State University
polystyrene	detection of aquatic toxins	RE Gawley

beads coated with peptides		University of Miami
fullerene	tracers for water pollution	JB Callegary University of Arizona
	GREEN ENERGY/ MANUFACTURING	
nano-clay	substitute petroleum-based products for nano-composites	LT Drzal, M Misra, AK Mohanty Michigan Sate University
nano-micelles	replacing VOCs with nano-structured microemulsions	DA Sabatini, JH Harwell University of Oklahoma
nano-plastic fibrils and crystals	alternative to Petroleum-based composites	WT Winter SUNY College of Environmental Science and Forestry
nano-TiO2	photocatalyst for solar cells	G Chumanov Clemson University
semi-conducting nanoparticles	catalyst fuel cells	NY Dolney University of Michigan-Ann Arbor

Table 3: Summary of some genes that are differentially regulated (P<0.01) in RNIP exposed animals compared to controls. Many more genes were found to be differentially regulated in the liver compared to the gill.

Gene Hit Definition	Fold Change	Explanation
UNDER-EXPRESSED IN LIVER – MALES EXPOSED TO NANO-IRON		
Complement component C9 precursor	**-1.3**	Plays a key role in innate and adaptive immunity (Boshra *et al.*, 2006)
OVER-EXPRESSED IN LIVER – MALES EXPOSED TO NANO-IRON		
Alpha-2 macroglobulin 2 Alpha-2 macroglobulin 1	**2.0** **1.6**	Act as defense barriers – binding foreign (or host) peptides and particles. (Borth, 1992)
Selenoprotein Pa precursor	**1.8**	An extracelluar glycoprotein; associates with endothelial cells; postulated to protect against oxidative injury and to transport selenium from liver to peripheral tissues. (Burk *et al.*, 2003)
Tubulin, alpha-3	**1.6**	Involved in microtubulin dynamics (growth and shortening of tubules) and possibly motor proteins used for intracellular transport. Targeted by anticancer drugs. (Pellegrini and Budman, 2005)
Ubiquitin	**1.5**	Plays a role in the process of protein degradation. (Walters *et al.*, 2004)
Prothrombin precursor	**1.5**	Thrombin (which has multiple roles) is generated from its inactive precursor prothrombin by factor Xa as part of the prothrombinase complex. (Lane *et al.* epub ahead of print.)
Antithrombin	**1.4**	Mediates the activity of heparin, a major anticoagulant. (Munoz and Linhard, 2004)
Aldolase A fructose-biphosphate	**1.3**	Plays a role in glucose metabolism (Shiokawa *et al.*, 2002)
Hexokinase	**1.2**	Enzyme involved in glycolosis, transcriptional regulation and regulation of apoptosis. (Kim and Dang, 2005)

UNDER-EXPRESSED IN GILL – MALES EXPOSED TO NANOIRON		
Cytosolic alanine aminotransferase (c-AAT)	**-1.2**	Plays a role in glycolosis and energy production (Patel and Olson, 1985)

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