GENETIC TOOLS FOR ASSESSING EXPOSURE TO CONTAMINANTS

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ASSESSMENT OF EXPOSURE TO CONTAMINANTS. Decisions on the identification, assessment, and management of contaminated material are frequently limited by information on how much risk the material poses. A tiered assessment framework has been adopted to determine the suitability of dredged material for disposal (US Environmental Protection Agency and US Army Corps Engineers 1991). According to this framework, if there is reason to believe contamination, sediment samples should be collected, extracted, and analyzed. Chemical analyses are developed to maximize recovery of pre-targeted contaminants of interest. However, a manager must know what chemicals to look for as these tests do not detect non-targeted contaminants or those with poor detection limits. With the exception of the use of theoretical bioaccumulation estimates for nonpolar organics, chemical analyses provide no information on the fraction of the targeted contaminant available (bioavailability) to cause toxicity and subsequent risks. Managers are then required to use lengthy bioaccumulation studies to assess contaminant exposure and bioavailability.

Confounding the remediation or mitigation of contaminated sediment, the causes of toxicity in sediment may never be conclusively identified using standard methods (Larson 2001). As a result, the high cost of sediment analysis constrains effective management decisions. Given the large volumes of material involved in most dredging operations, the heterogeneous distribution of contaminants in sediments, and the frequent need to make decisions during dredging operations the constraints imposed by traditional sampling regimes and analyses can result in overly conservative, and very expensive, decisions. Moreover, it may result in the unnecessary treatment of sediments where contaminants pose no risk. The lack of timely and accurate information can also result in localized hot spots escaping detection, and dispersal of toxic material into aquatic ecosystems. Direct assessment of effects precludes uncertainty due to unknown bioavailability improving the quality of information in terms of environmental risk.

Sediment matrices create difficult challenges for identification of bioavailable contaminants that may cause toxicity. For a contaminant to cause any response, it must be biologically available. While bioavailability can seldom be determined solely from analytical chemistry data, responses in exposed organisms can interpreted and used to identify which chemicals are bioavailable and their potential for causing effects and toxicity due to exposure. Standard EPA tests use ecologically relevant organisms including marine benthic invertebrates, *Neanthes arenaceodentata* and *Leptocheirus plumulosus*, and freshwater benthic invertebrates, *Chironomus tentans* and *Hyalella azteca* (US Environmental Protection Agency and US Army Corps Engineers 1991; US Environmental Protection Agency 2000).

GENETIC MEASURES OF EXPOSURE AND TOXICITY**.** We are applying genetic tools for assessing exposure effects in benthic invertebrates. Genetic tools include assays that rapidly measure the responses of select genes to environmental insult. Introduction of rapid, inexpensive, and high-throughput screening methods, such as that described below, will significantly increase the amount of material that can be assessed, resulting in more informed decision making regarding the disposal of dredged material (Fredrickson et al. 2001).

When invertebrates are exposed to contaminants that are harmful, or biologically available at concentrations that may be harmful, a wide range of responses occurs based on the type of chemical and the level of exposure (Figure 1). The cells of an organism respond to stimuli by synthesizing proteins required for the cell to function (i.e. cell growth, cellular repair, and detoxification). Responses are created by transcribing, or copying many times, genes into messenger RNA (mRNA) that in turn are translated into proteins. At low levels of exposure, organisms generally attempt to eliminate or detoxify the contaminant. For example, mercury exposure results in accumulation of metallothionines to sequester mercury by increasing, or inducing, transcription of genes coding for the protein (Durnam and

Palmiter 1981). Increasingly higher exposures cause cellular damage triggering compensatory pathways and repair mechanisms that mitigate the damage. Eventually, high levels of toxicants can cause organ damage and affect the animal's behavior (e.g. reproductive success and other common endpoints of chronic exposure bioassays). Exposures that overwhelm the cell's defense result in the transcription of a suite of genes that encode enzymes associated with cell death.

Chironomus tentans contains thousands of genes that provide it with many biochemical means to deal with low-level toxicant exposures. Therefore the amount of mRNA that accumulates for specific genes can be used to determine how much of a dose an animal receives. The bioavailability of a chemical will be indicated by changes in the types of mRNA that are produced in an animal as stress responses begin to occur. As a result the profile of genes transcribed that can be toxicant and dosage specific (Burczynski et al. 2000; Waring et al. 2001a,b; Bartosiewicz et al. 2001; Gerhold et al. 2001).

RAPID TOXICITY ASSAYS. Gene based assays offer a great potential for rapid high throughput analysis of effects of chemical contaminants on test organisms. We have developed several assays using genes isolated from *C. tentans* and *L. plumulosus*. These assays use a technique, real time Polymerase Chain Reaction (real time PCR), that amplifies cDNA targets a million-fold and monitors target accumulation using dyes that fluoresce when bound to DNA. (for review see Bustin 2000 and Mackay et al. 2002). Real time PCR is sensitive enough that just a single organism can be analyzed. Many different approaches have been developed to monitor real time PCR (e.g. Taqman assays, molecular beacons, hybridization probes.), the simplest approach uses the dye SYBR Green I^{tm} whose fluorescence is 100 times greater when bound to double stranded DNA (Figure 2).

We have used this approach to monitor effects of the explosive compound 2,4,6-trinitrotoluene (TNT) on *C. tentans*. Histone genes encode proteins essential for the integrity of chromosome structure and are expressed during cell division and organism growth. Expression of the histone 1a gene is dramatically effected at toxic concentrations of TNT (Figure 3).

IDENTIFICATION OF SOURCES OF TOXICITY. To identify the causative agent of toxicity in an environmental sample using mRNA profiling, test organisms are exposed for a short period to the sediment to be analyzed and surviving animals are recovered. RNA is extracted from exposed organisms, providing a "snapshot" of the proteins the organism is currently synthesizing. Since a cell makes mRNA so that it can make more enzymes or other proteins, the types and levels of gene transcripts in the extract provide a snapshot of the cell's status (Figure 4).

Under controlled test conditions toxicants having similar modes of action will have generally similar patterns of *C. tentans* gene expression. Experiments with rats and rat hepatocyte cell lines exposed to 15 known liver toxins demonstrate that mRNA profiles can be clustered into compounds with similar toxic mechanisms (Waring et al. 2001a, b). However, the complete gene expression profile is unlikely to be the same for any two toxicants. That is, while rat liver toxicants with known and related modes of action produced generally similar expression profiles, each exposure resulted in a unique, reproducible gene expression profile. mRNA profile patterns in rats also correlated with cellular histopathology and biochemical changes in rat physiology directly linking changes in transcription profiles with whole organism toxicity.

Viewed from a different perspective, under controlled test conditions gene expression profiles can be used to identify which contaminants *C. tentans* is sensing and, possibly, also the dosage of the toxicant. However, before this approach can be practically applied a knowledge base of *C. tentans* mRNA profiles in response to specific toxicants must be compiled.

***CHIRONOMUS TENTANS* CONTAMINANT mRNA PROFILES.** Although this approach can be used for any organisms and stressor we chose to develop it for the transcriptional responses of *Chironomus tentans* to common sedimentary chemical contaminants. *C. tentans* can be used for both sediment and water testing, and there are standard EPA and ASTM protocols for its use in sediment testing (USEPA 2000). Chironomids are important prey for many fish including juvenile endangered salmon

species. In studies of the diet of Chinook fry in the Sacramento River, California, chironomids comprised 44% of the stomach contents of salmon fry, outnumbering any other item in the diet (Moore, 1997).

This approach is demonstrated by mRNA profiling of *C. tentans* larvae exposed to six common sedimentary contaminants. Midge larvae were exposed for 12 hours to water containing dosages approximating one-tenth published LD_{50}'s; phenanthrene (25 ug/L) and fluoranthene (30 ug/L), DDT (0.2 ug/L), zinc chloride (125 ug/L), cupric chloride (54 ug/L), and cadmium chloride (100 ug/L). Total RNA was isolated from larvae in each exposure and analyzed using the method outlined in Figure 3. Different sets of adaptors can be selected so that the total population of mRNA-derived cDNA can be comprehensively interrogated by iteratively amplifying different subsets of the cDNA population. The adaptors are used to detect different cDNAs and hence different genes being expressed.

Each exposure produced a unique and reproducible gene expression profile (Figure 5). Toxicants with similar modes of action (e.g. phenanthrene and fluoranthene) produced expression profiles that were more similar to each other than to those of other toxicants (e.g., metals or DDT).

We will continue the development of this technology using *C. tentans* and the saltwater bioassay species *Leptochierus plumulosus*. Additional work is required in the design of exposures that control gene expression, and to focus analytical methods to minimize the reporting of gene expression that is not related to toxicity. This streamlining will reduce costs and potentially confounding results, and assists in the transitioning of this technology to the private sector.

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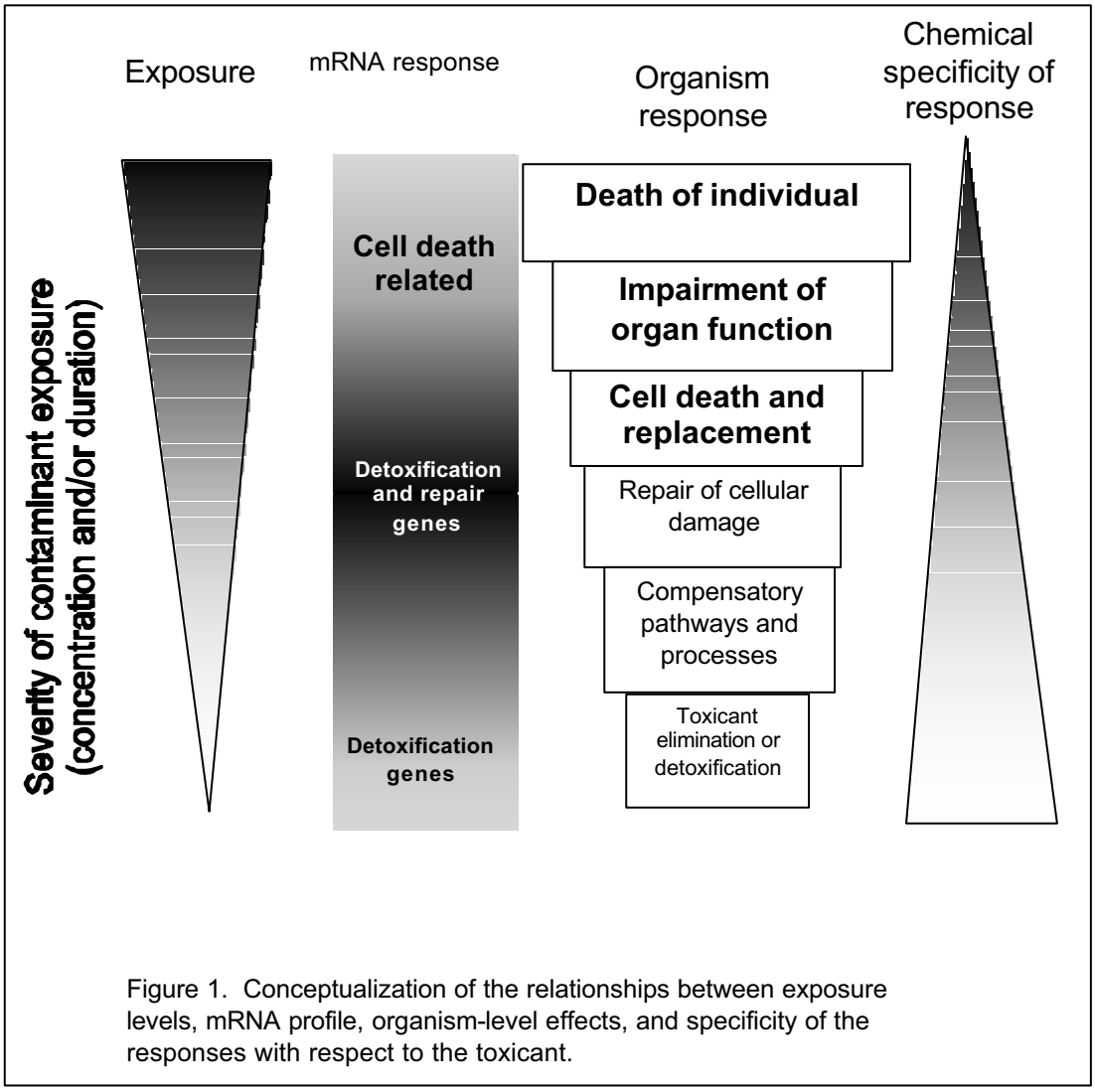

Figure 1. Conceptualization of the relationships between exposure levels, mRNA profile, organism-level effects, and specificity of the responses with respect to the toxicant.

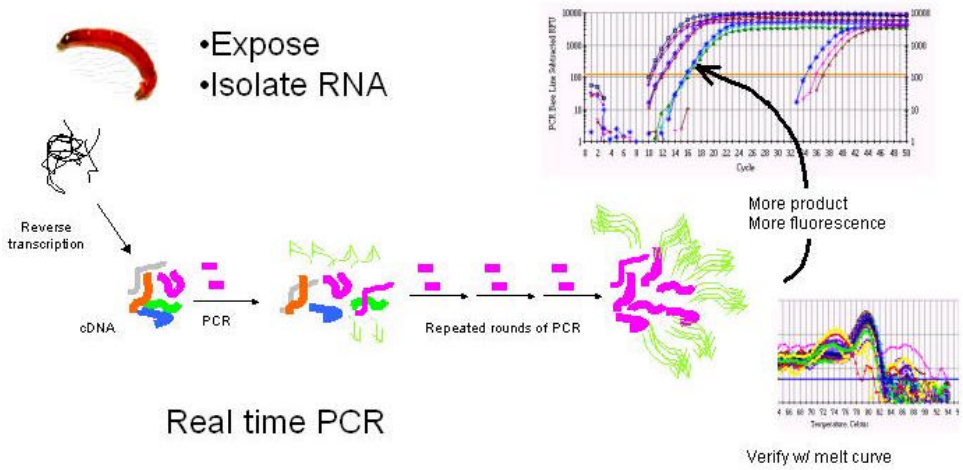

Figure 2. Outline of real time, reverse transcriptase PCR. RNA is isolated from an exposed and a control organism. Total RNA is converted to DNA using reverse transcriptase to create a cDNA (complementary DNA) pool of all mRNAs present in the organism when it was harvested. Repeated rounds of amplification by PCR determine the level of expression for a particular gene. A double strand DNA binding dye, SYBR Green I™, is used to detect PCR. As double stranded PCR product accumulates in the reaction tube, more SYBR Green I™ is bound, resulting in successively higher levels of fluorescence. The concentration of the target cDNA (representing mRNA isolated from the test organism) can then be determined from the point at which fluorescence emerges from background. The target gene is then quantified by comparison to a control or standards of known concentration. The identity of the PCR product can be confirmed using melt curve analysis. In melt curve analysis, the completed real-time PCR reactions are slowly ramped from a temperature of 55^{o}C to 95^{o}C. As the temperature increases, double stranded PCR fragments reach the temperature at which the strands separate, or melt (T_m). As a result of duplex DNA melting, fluorescence is decreased because SYBR Green I does not bind single strand DNA. Since the T_m of a DNA fragment is length and sequence dependent, the melt curve can be used to discriminate between primer dimers and successful amplification of the target product.

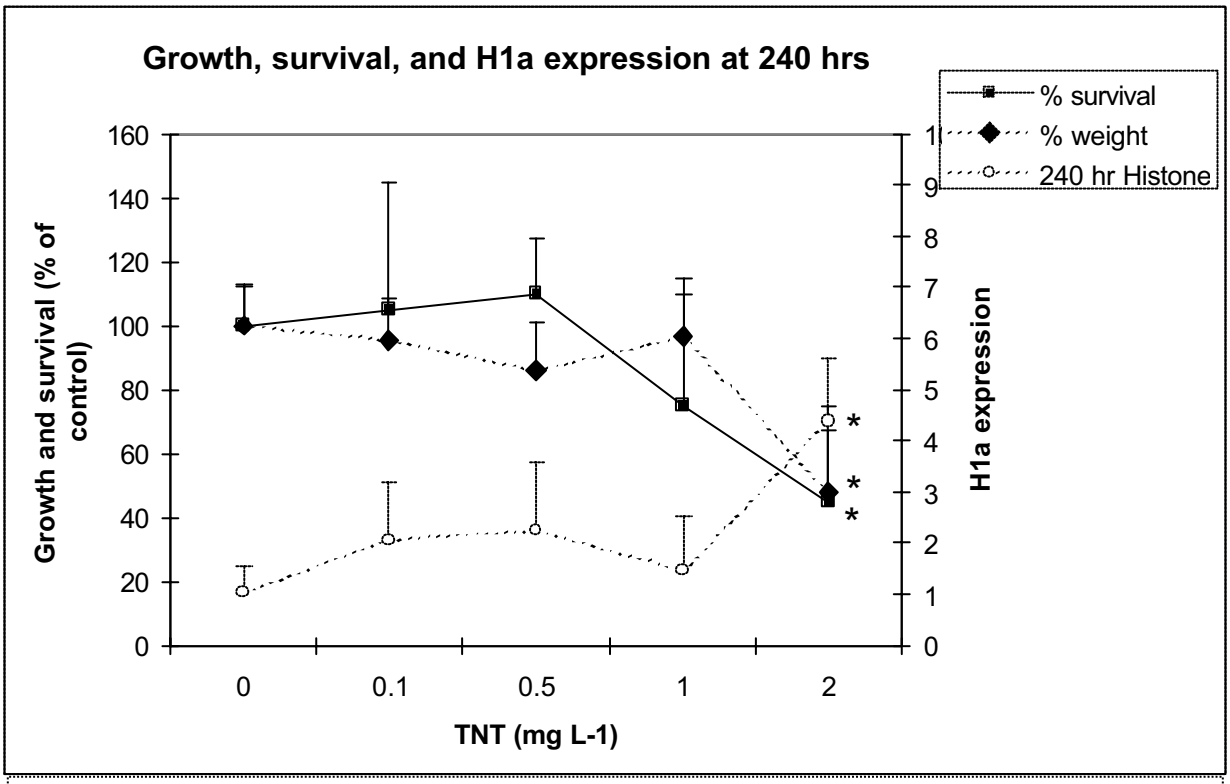

Figure 3. Correlation of histone gene expression with growth and survival at 10 days (240 hrs). Values significantly different from controls are denoted by *.

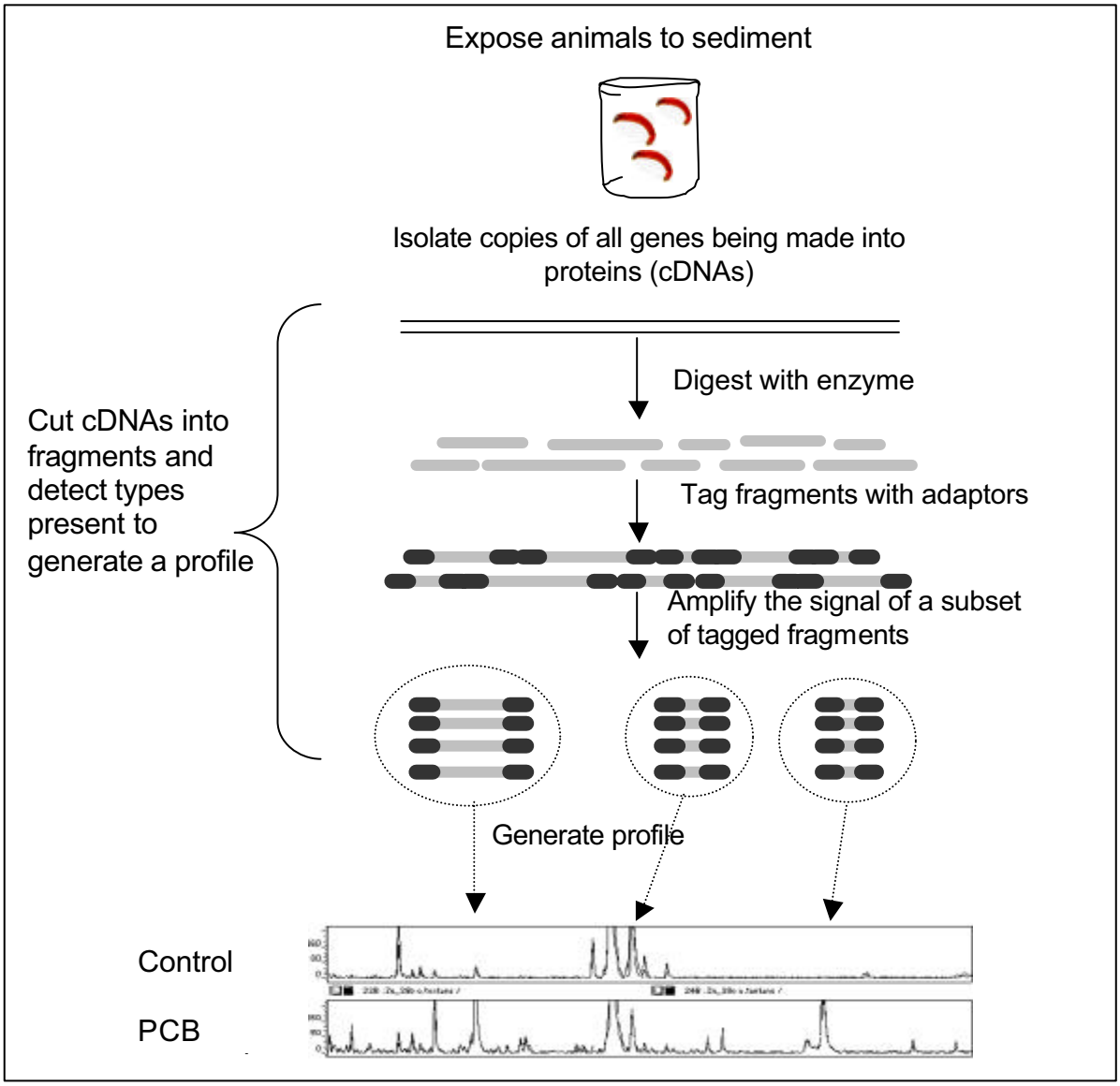

Figure 4. A profile of mRNAs is obtained by extracting and purifying mRNA from exposed and control *Chironomus tentan.* mRNA is reverse transcribed to cDNA, cut with restriction enzymes, and ligated to short pieces of DNA with known sequences (adaptors). Subsets of the adapted cDNA fragments are amplified using PCR. The resulting mixture of cDNA fragments are separated and visualized using an automated DNA sequencer. Each of the resulting peaks represents the expression of a single gene. These mRNA profiles are diagnostic for the type and severity of stress the cell was experiencing at the time of nucleic acid extraction.

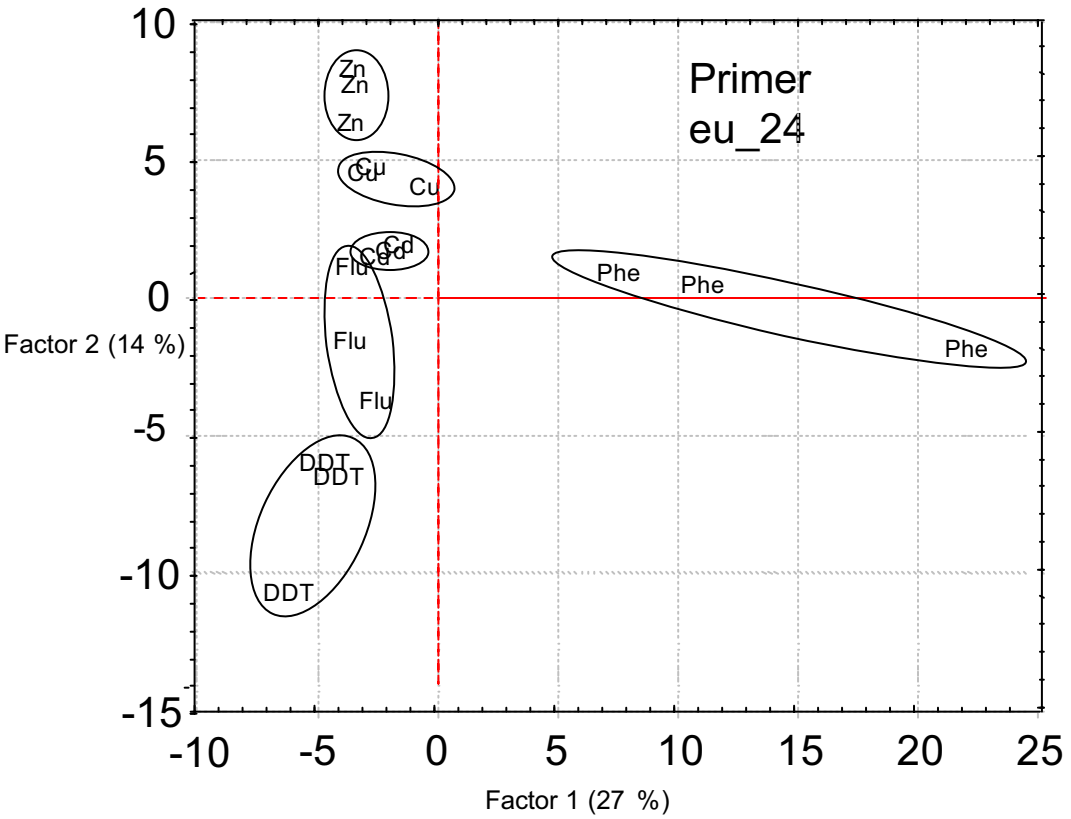

Figure 5. *C. tentans* mRNA profiles revealed using one adaptor (eu_24) were characterized by the gene fragment base pair length and normalized peak height of each peak. The resulting data were subjected to a Principle Component numerical analysis and the sample scores of each profile were plotted on the 2 factors that best differentiated the mRNA profiles. Points representing replicate mRNA profiles from animals exposed to contaminants formed clusters. Phe = phenanthrene exposed; Flu = fluoranthene exposed; DDT = DDT exposed; Cu = copper exposed; Cd = cadmium exposed; Zn = zinc exposed.