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# The wave nature of molecular responses in ecotoxicology

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## ABSTRACT

Toxicity is described by classical dose-response (also called concentration-response) relationships that usually follow linear or sigmoidal trends. The toxicity of a compound is interpreted as the increase in intensity of the effect as a function of exposure concentration or time. This classic descriptor of toxicity was and is still successfully used in toxicology and pharmacology to describe various toxic responses (decreased survival) and sub-lethal effects at the organ or systemic levels (e.g., weight loss or decreased growth). The advent of biomarkers at the molecular level enabled a better understanding of the way chemicals negatively act at the fundamental level. In some cases, dose-response curves showed curious non-linear relationships which complicate the prediction of adverse toxic outcomes of biochemical/molecular changes. A close examination of the relationships between the intensity of biochemical markers and concentration suggests that biological effects are oscillatory (cyclic) in nature. Spectral analysis using Fourier transformation can decompose signal intensities as a combination of wave functions to describe the cyclic nature of biochemical changes. We tested this approach with a real case, exposing juvenile rainbow trout to seven rare earths using a gene expression quantitative real-time polymerase chain reactions (qPCR) array composed of 12 genes involved in toxic stress responses and compared those responses with the hepatic somatic index, fish condition and trout mortality endpoints. Multiple regression analysis on the classical endpoints (intensity of the 12 transcripts) showed only a few significant relationships between gene expression changes and toxicity. However, spectral analysis transformation of the gene expression data revealed highly significant relationships ($r > 0.95$) with liver weight data and mortality. This study proposes a data transformation based on the cyclic properties of molecular changes using spectral analysis with Fourier transformation. This novel approach considers the possibility that toxic responses could behave as waves providing a means to explore relationships between the mode of action of chemicals and adverse outcome pathways.

**KEYWORDS:** wave theory, toxic responses, biomarkers, adverse outcome pathways

## INTRODUCTION

The toxicity of chemicals or physical agents is typically described by dose-response relationships, which usually follow a linear or sigmoidal trend. The intensity of effects is plotted against exposure concentration or duration (Figure 1A). The direct interpretation of this relationship is that the intensity of a given effect is directly proportional to the exposure concentration or duration. These relationships are classical textbook descriptors of toxicity [1]. To quote Paracelsus (1493-1541), “All things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison”. It follows that the more intense the response, the more harmful the damage, and that at low doses no toxicity occurs. Thus, the relationships between the intensity of responses and exposure concentration or duration formed one of the fundamental pillars of pharmaceutical and toxicological sciences. Toxic effects at the sub-cellular and molecular levels have gradually emerged in the last 50 years through the use of biomarkers to gain a better understanding of the

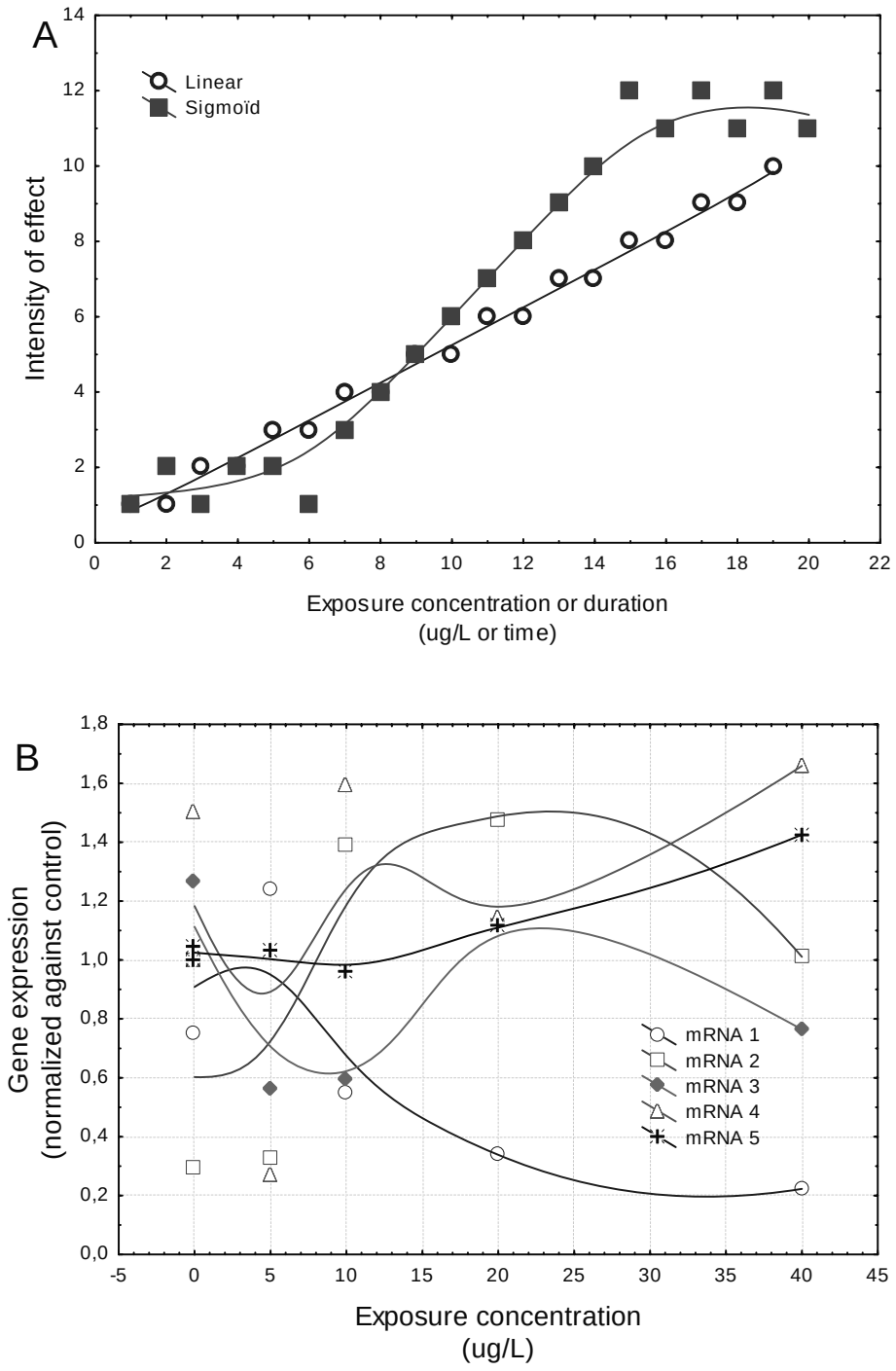


**Figure 1.** Classical dose-response relationships and cyclic behaviour of biochemical markers. Intensity of effects (e.g., number of injured organisms) is plotted against exposure concentration or duration, which could be the internal dose (amount in blood or in tissues), external dose (amount in the water or food) or the time exposed to a given concentration of contaminant (A). A typical example of cyclic behaviour based on real expression of genes with the exposure concentration. The y axis represents the intensity (relative to control cells); the x axis shows the exposure concentration (B). The curves represent the least square fitting of the data points. The curves revealed the periodic or cyclic behaviour in gene expression.

mechanisms of action of xenobiotics in cells or tissues. In order to understand mechanisms of action, it is fundamental to know how and why a substance can be toxic. Moreover, from the ecological risk perspective, risk assessment based on short-term toxicity tests with classical endpoints at the individual level proved inadequate for prediction and prevention of toxic impacts in organisms. According to the National Academy of Science report *Toxicity Testing in the 21st Century* [2], toxicity tests based on the classical endpoints of survival, growth and reproduction were less able to prevent impacts in ecosystems. It was recognized that early warning systems — based, for example, on molecular or cellular function endpoints — were needed to better understand (and predict) long-term impacts.

Studies involving molecular markers quickly revealed strange dose-response curves that deviated from linearity and were totally unpredictable by classical toxicology [3, 4]. This complicates the prediction of toxic outcomes based on biochemical/molecular changes. For example, endocrine disrupters are a special class of compounds that act at very low concentrations and behave strangely when exposure concentrations are increased progressively. Indeed, the direction of effects could completely reverse as the exposure concentration increases, forming U curves (or inverted U's) and sometimes M-shaped dose-response curves. One noteworthy case of such strange behaviour is the inverted U-shaped concentration-response curve, representing hormesis. Hormesis is the phenomenon in which an agent produces harmful biological effects at moderate to high doses but has beneficial effects at low doses [5]. For example, a low dose of ionizing radiation could stimulate DNA repair activity and protect cells from further damage, thereby improving cell survival. This raised the possibility that chemicals could have opposite effects at lower and higher doses, which could dramatically change the risk-assessment paradigm for environmental pollutants. A close examination of the relationships between the intensity of biochemical markers and concentration suggests that biological effects are cyclic in nature and may behave as waves. Indeed, U-shaped, inverted U-shaped and other non-linear concentration-response relationships suggest that the effects of contaminants are cyclic (periodic) in nature (Figure 1B). In this respect, expressing sub-lethal effects in the traditional way, i.e., intensity of effect as a function of exposure concentration, might not be appropriate and might hide a more fundamental nature of biochemical changes in cells.

Biochemical oscillations or cyclic behaviour are not uncommon in organisms. Glycolytic oscillation of cofactors such as reduced nicotinamide adenine dinucleotide (NADH) was first reported in yeast cells in the late 1950s [6, 7]. Indeed, yeast cell lysates supplemented with trehalose will initiate oscillations in NADH levels for up to 90 min; phosphofructokinase was identified as the target enzyme responsible for these fluctuations. This enzyme is regulated by NAD+ and ADP, which respectively activate and inhibit the conversion of fructose 6-phosphate into fructose-1,6-biphosphate. Another key oscillatory system is the peroxidase–oxidase reaction, which is important during oxidative stress in ecotoxicology [8]. The enzyme catalyzes the oxidation of NADH in the presence of $O_2$. The co-factors 2,4-dichlorophenol/methylene blue are also needed to assist the transfer of electrons. Sustained NADH oscillations are produced when NADH is continuously added or generated by the glucose 6-phosphate/NAD+ dehydrogenase system. This suggests that some enzymes involved in the elimination of oxygen radicals could show oscillatory behaviour. Oscillatory changes are also observed at the gene expression level [9]. Large-amplitude and phase-locked oscillations of gene expression in developing *C. elegans* larvae was produced by periodic transcription. Nearly $1/5^{th}$ of the expressed genes oscillated with an 8 hr period and hundreds of gene changed at > 10-fold. These large-amplitude oscillations in RNA from whole worms indicate synchronization of gene expression pathways across cells and tissues, suggesting that oscillatory changes in transcript constitute a new approach to studying the process of coordinated gene expression. At a more toxicological level, hepatic induction of cytochrome P4501A1 by 2,3,7,8-tetrachloro-p-dioxin showed a biphasic response in rats [10]. The activity of 7-ethoxyresorufin O-deethylase was first maximally induced in mice at 7 days following exposure to 3 µg and 30 µg TCDD/kg. However, at higher doses of TCDD (> 45 µg/kg), the activity was further increased two-fold from the apparent maximal response, resulting in a strange biphasic log dose-response curve.

The cyclic nature of any signal could be analyzed by spectral analysis using Fourier transformation, which decomposes the signal into specific wave functions at different frequencies. Similar to the way white light separates into different colours after passing through a prism, spectral analysis splits a signal into underlying waves at different frequencies (colours). Wave functions at the same frequency and in phase could combine and result in resonance. Resonance is the accumulation of wave amplitudes at the same frequency, which could culminate in drastic effects at higher level of organization (for example, the culmination of acoustic notes at the same frequency that makes a glass vibrate until it shatters). This process could form the basis of emerging properties of complex mixtures. An emergent property arises from the interaction between the elements forming the mixtures and biological macromolecules where each element alone does not produce the observed effects. If toxic responses at the molecular level at a given frequency combine, then the effects at the next level of biological organization could emerge. This study examines the hypothesis that changes at the sub-cellular and molecular levels display a cyclic, wave-like behaviour that is not fully described by classical dose-response based on intensity–exposure concentration relationships.

The purpose of this study was to characterize the cyclic nature of concentration-response (intensity) relationships as another means to describe toxicity and to verify whether changes at the molecular level display wave-like properties. First, spectral analysis using Fourier transformation will be applied to simulated but realistic data in order to gain an understanding of the basic properties of wave-like transformation of biochemical changes. Then, this transformation will be used to predict changes in toxicity, in fish weight changes, and liver index using real examples of trout juveniles exposed to selected lanthanides for 96 h followed by change in the levels of 12 transcripts involved in toxic stress. An attempt will be made to find significant predictions of toxicity using wave descriptors of molecular changes in cells.

## METHODS

### Simulated concentration-response curves

Concentration-response data were simulated to generate typical relationships (linear increases and decreases, inverted U-shaped dose-response curves). In addition, non-significant baseline (noise) responses that do not differ from controls were also prepared. Curve fitting of intensity of the signal (hypothetical biomarker response) was done using the least square difference method. These relationships were then analyzed using spectral analysis and Fourier transformation as described below.

### Case study of rainbow trout exposed to rare earth elements

A real case study is presented to examine changes at gene expression level and whether they can predict impacts on hepatic somatic index (organ level), fish condition (fish weight/length ratio) and lethal concentration (mortality). Rainbow trout juveniles (1- to 2-cm fork length) were exposed to increasing concentrations of selected rare earth elements (Ce, Er, Sm, Nd, Y, Gd and La) for 96 h at 15 °C. The exposure concentrations were 40, 8, 1.6, 0.32 and 0.064 mg/L for each element cited above. The fish were continuously aerated to reach 98% saturation or better and were subjected to an 8/16 hr dark/light cycle. Fish were monitored three times daily. Any fish showing signs of distress or found belly-up were euthanized in 50 mg/L Tricaine in dechlorinated tap water. At the end of the exposure period, the surviving fish were placed in 50 mg/L Tricaine for 2 min, then placed on ice and weighed. The livers were excised, washed in ice-cold phosphate buffered saline (145 mM NaCl, 5 mM $KH_2PO_4$, pH 7.4, containing 5 mM ethylenediamine tetraacetate (EDTA)), weighed (after blotting on filter paper) and immediately transferred to RNALater$^R$ solution (Thermo Fisher Scientific, Ontario, Canada) to ensure total RNA integrity against RNases. The livers were then stored at -85 °C until the gene expression assessment stage.

Changes in gene expression were determined using real-time quantitative qPCR methodology [11]. Total RNA levels were extracted using a commercial extraction kit (Quiagen Inc, Ontario, Canada) and RNA concentration/purity was determined using a Nanodrop 1000 ultraviolet-visible spectrometer (Thermo Fisher Scientific, On, Canada). RNA integrity was confirmed using a microfluidic-based electrophoresis system (Experion Automated Electrophoresis System; BioRAD, Ontario, Canada). Genomic DNA was completely removed and cDNA was produced from extracted RNA samples using a commercial kit (QuantiTect reverse transcription

kit; Quiagen Inc., Ontario, Canada). The incubation temperature was 42 °C for 15 min followed by 95 °C for 3 min. The newly produced cDNA was then stored at -85 °C. The genes used in this study are listed in table 1. For each gene, at least two primer pairs were evaluated and were synthesized from integrated DNA technologies (Coralville, IA, USA). Quantitative real-time polymerase chain reactions (qPCR) were performed with the iQ SYBR Green Supermix (Bio-Rad, Ontario, Canada) using a Mastercycler ep realplex2 thermocycler (Eppendorf, USA). For each primer pair, a calibration curve (starting cDNA concentration 20 ng, 8 serial dilutions in five-fold increments) was produced with amplification efficiencies between 90% and 110%. Each reaction was run in duplicate and was composed as follows: 5 µL of cDNA (20 ng), 12.5 µL of iQ SYBR Green Supermix, 0.2 mM of each dNTP (dATP, dTTP, dCTP, and dGTP), 25 U/mL iTag DNA polymerase, 3 mM $MgCl_2$ and 10 nM SYBR Green 1. Primer concentrations were 300 nM, and diethylpyrocarbonate-treated bidistilled water (Ambion, USA) was added to reach 25 µL total volume. Temperature cycles were as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 70 °C for 15 s. Amplification specificity was verified using melting curve analysis: 95 °C for 15 s and 57 °C to 95 °C gradient steps in 10 min total time. Controls consisted of the reaction mix without addition of template cDNA. The following genes were used as reference for normalization: β-tubuline and Elongator factor 1α (EL1α).

### Data analysis, spectral analysis and Fourier transformation

The data were first analyzed the traditional way by the mean and standard deviation from N = 8 replicates in each treatment. The normality of the data was checked using the Shapiro–Wilk tests before analysis of variance (ANOVA). Analysis of variance and critical difference between treatments were appraised with the least square difference test. Significance was set at $\alpha < 0.05$. Spectral analysis using Fourier transformation was performed on the gene expression data normalized against the reference gene EL1α. Each measurement (8 replicates per treatment) was randomly placed in series of 8 replicates starting with the controls, the lowest concentration treatment etc. Hence, the first 8 observations were from the controls, the next 8 observations were from the lowest exposure concentration, the next 8 observations were from the next-highest exposure concentration, and so forth. Fourier analysis could be performed on space or time series where the exposure concentration range is the progression in "space". Spectral analysis using Fourier transformation will transform any series of data into a 2D plot with the frequency of the signal on the *x* axis and the periodogram (Pk) value, which represents the signal "variance", on the *y* axis. Fourier transformation seeks wave functions that best describe the data at various frequencies: $F(x,t) = a_0 + \sum [A_k*\sin(f_k*n\pi(x/L)) + B_k*\cos(f_k*n\pi(x/L))]$ with k = 1 to q observations, and A and B are the coefficients related to the amplitude of the wave function. The value *x* corresponds to a given exposure concentration, L is the exposure concentration range, and n is the harmonic value (n = 1, 2, 3…). The Pk value is mathematically defined as the sum of squared values of the sinus and cosinus coefficients A and B of the wave function: $Pk = \sum(A\ \sin_k)^2 + (B\ \cos in_k)^2 * N/2$. The Pk could be interpreted as the sum square (variance) of the data at each frequency with k = 1 to q observations (here, 3 treatments × 8 replicates = 24 observations). All statistical analyses were performed using the Statistica software package (Version 8, France).

## RESULTS

In order to understand the cyclic nature of concentration-response relationships using Fourier transformation, the analysis was performed first on simulated but realistic concentration-response curves (Figures 2A to 2F). In the first example, there was no significant effect, as confirmed by ANOVA (Figure 3A). Thus, the data represents the natural or "normal" variability of the biomarker response. Spectral analysis of the data revealed a series of frequencies (0.07, 0.22, 0.24 and 0.47) with small non-significant Pk values (0.024, 0.031, 0.032 and 0.06 respectively) (Figure 2B). The Pk value is a measure of the natural variance of the hypothetical biomarker. These frequencies correspond to the $14^{th}$, $5^{th}$, $4^{th}$ and $2^{nd}$ observations, indicating small, non-significant fluctuations of the biomarker. The lowest frequency (0.47) with the highest Pk value (0.06) is considered the inter-individual or replicate variability, which is the fundamental

**Table 1.** Gene expression targets.

| SYMBOL | GENE | BIOLOGICAL PROCESS | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|---|
| CAT | Catalase | Oxidative/metallic stress | TGATGTCACACAGGTGCGTA | GTGGGCTCAGTGTTGTTGAG |
| CYP1A | Cytochrome P450 1A | Xenobiotic biotransformation | GATGTCAGTGGCAGCTTTGA | TCCTGGTCATCATGGCTGTA |
| GADD45 | growth arrest and DNA-damage-inducible, alpha | DNA repair/growth arrest/senescence regulation | GACTTTTGAGGAAACCAGCGG | GGCACAACACCACATTATCCG |
| GLUD | Glutamate deshydrogenase | Ammonia metabolism | TCGGAGGGGCTAAAGCTGGTGT | TGTCACACATGCGTGGGCGTT |
| GST | Glutathion-S-Transferase-P | Xenobiotic biotransformation | ATTTTGGGACGGGCTGACA | CCTGGTGCTCTGCTCCAGTT |
| HSP70 | heat shock cognate 70-kDa protein | Protein folding | ACCACACCCAGTTATGTCGCCT | CTTCCGCCCTATCAGCCGC |
| MT | Metallothionein | Oxidative/metallic stress | GCTCTAAAACTGGCTCTTGC | GTCTAGGCTCAAGATGGTAC |
| PCNA | proliferating cell nuclear antigen | DNA repair/cell growth/DNA replication | CAGAGGACAACGCAGACACA | CACGGCAGATACGGGCAAAC |
| SOD | Superoxyde dismutase (Cu/Zn cytosolic) | Oxidative/metallic stress | TGGTCCTGTGAAGCTGATTG | TTGTCAGCTCCTGCAGTCAC |
| SPARC | secreted acidic cysteine rich glycoprotein | Calcium binding | TCACCCTGTACGAGCGCGATGA | AGCTGACCGAACTGCCAGTGGA |
| Reference genes | | | | |
| SYMBOL | GENE | BIOLOGICAL PROCESS | FORWARD PRIMER | REVERSE PRIMER |
| EF1a | Elongation factor I α | Protein synthesis | GAATCGGCTATGCCTGGTGAC | GGATGATGACCTGAGCGGTG |
| HPRT | Hypoxanthine phosphoribosyl transferase I | Protein conformation | CCGCCTCAAGAGCTACTGTAAT | GTCTGGAACCTCAAACCCTATG |
| PPIA | Prolylpeptidyl isomerase I | Protein synthesis | CATCCCAGGTTTCATGTGC | CCGTTCAGCCAGTCAGTGTT |

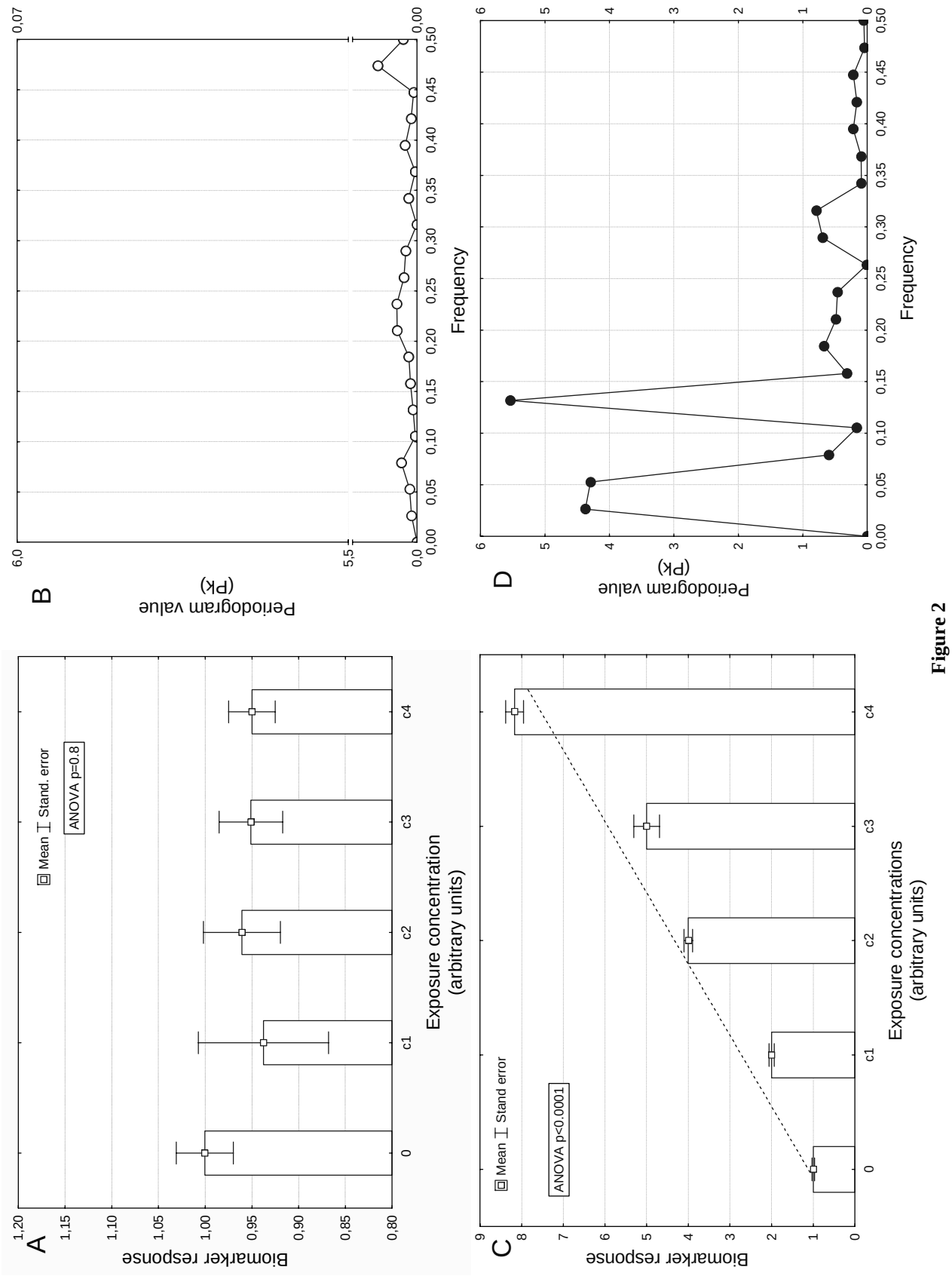
A
Biomarker response
Mean ⌶ Stand. error
ANOVA p=0.8
Exposure concentration (arbitrary units)
0
c1
c2
c3
c4
B
Periodogram value (PK)
Frequency
C
Biomarker response
Mean ⌶ Stand error
ANOVA p<0.0001
Exposure concentrations (arbitrary units)
D
Periodogram value (PK)
Frequency

**Figure 2**

Figure 2 continued..

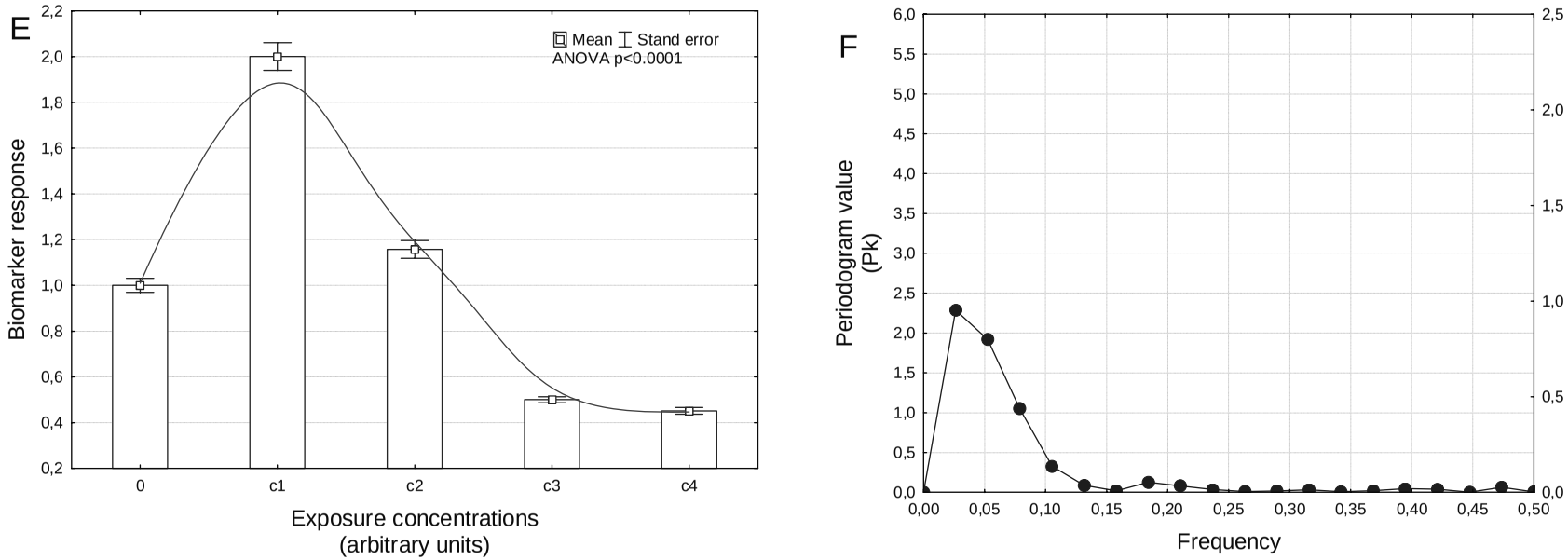


**Figure 2.** Representative concentration-response curves usually observed with molecular biomarkers.
Typical and representative concentration-response curves are shown. Absence of effects, showing only the natural variation of the biomarker data (A and B), positive linear concentration responses case (C and D) and inverted U trend (E and F).

frequency of the biomarker response. In the next example, a classical linear concentration-response curve is provided (Figure 2C). With respect to the controls, the response factor reaches 9-fold; such high intensity is not unusual with gene expression data. Spectral analysis of the data revealed two important signals at frequencies 0.07-0.09 and 0.13 (Figure 2D). The analysis shows that a strong concentration-response trend is transformed into two signals that differ from the fundamental frequency (natural variability) of the biomarker. The frequencies of the signal correspond to the $8^{th}$ and $12^{th}$ observations, which correspond to the inter-treatment replicate number (8) and the next level of treatment (12). This indicates that most of the variance or change in the biomarker occurs after 8 observations and is much higher (Pk values of 4.3 and 5.6) than the Pk values of the fundamental frequencies (0.33 and 0.45). We repeated the analysis with downregulation (negative response compared to controls), and the results were similar. The occurrence of down-expressed responses does not influence the Pk in the same manner (the sums of squares are always positive). The analysis was also performed on inverted U (hormetic) concentration-response curves (Figures 2E and 2F). The analysis revealed that the major frequency occurred at 0.1 with a Pk value of 2.35, which corresponds to a cycle of 10 observations for the maximum variance in the data. Based on these simulated but realistic data trends, the induced effects often occur at a lower frequency than the fundamental normal frequency (variation) of the biomarker, which permits to separate the chemically (treatment-) induced effects from the natural variation of the biomarker.

In the real case, we evaluated the acute toxicity and mode of action of selected rare earth elements (REs) in juvenile rainbow trout. The gene expression data, together with the lethal concentration that kills 20% of the fish, fish condition and hepatic somatic index (HSI), are reported in table 2. The 96 h lethal concentrations ranged from 0.7 mg/L for Y to > 40 mg/L for Nd and Ce, in the following order: Y < Sm < Gd = Er < Nd = Ce. All the tested REs induced the expression of HSP70, PCNA, GADD45 and SOD, while MT, CYP1A1 and GST were influenced by at least 5 out of 7 of the REs. This suggests that exposures to low concentrations of REs lead to changes in protein conformation (denaturation), cell proliferation, cell growth-arrested DNA repair and oxidative stress (SOD). Moreover, genes involved in xenobiotic biotransformation were often influenced by most REs (70%).

To identify key adverse pathways, we compared gene expression results with acute toxicity, change in condition factor and hepatic somatic index (HSI) using multiple regression analysis (Table 3). An example of gene expression data for Ce(III) is shown for GADD45 and MT genes in juvenile fish exposed to Ce(III) for 96 h at 15 °C (Figure 3). The first graph (A) shows the classical representation of transcript levels (mean and standard deviation), and the second (B) shows spectral analysis using Fourier transformation. The transformation revealed that changes in transcript levels were found at two frequencies (0.05 and 0.25), which were lower than the fundamental frequency of GADD45 and MT transcripts (0.4 and 0.45). The analysis was conducted using groupings based on the influence of REs on the expression of genes, as described above. Hence, group 1 genes are those that were influenced by all REs (HSP70, PCNA, GADD45 and SOD), group 2 genes were affected by at least 5 out of 7 of the tested REs (CYP1A1, GST and MT), and those in group 3 were less influenced by the REs. Interestingly, group 2 comprises genes involved in xenobiotic biotransformation. Factorial and discriminant function analysis confirmed that the above genes were significant factors in the total variance of the data (data not shown). The analysis revealed that group 1 genes (HSP70, GADD45, PCNA and SOD) and group 2 genes (GST, MT and CYP1A1) had high factorial weights which explained 67% of the total variance. The predictions were first calculated based on the response factor (normalized against E1α) (the classical method of analyzing this type of data) and then using the Pk values (derived from gene expression data normalized against the Elα reference gene) to integrate the “amplitude” of the signal at given frequencies. Fourier transformation of the gene expression data revealed that genes often expressed important changes at frequencies between 0.05 and 0.2 which could bring about resonance. In this approach, the Pk values were added to determine whether this metric was also predictive to RE toxicity. Resonance could form the basis of emergent properties of chemical mixtures where

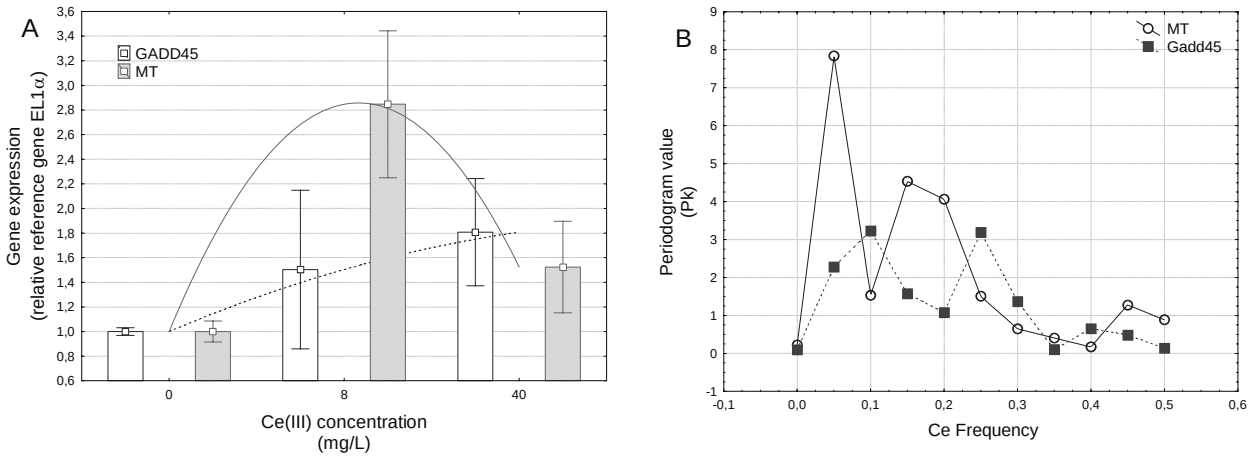


**Figure 3.** Spectral analysis of gene expression data in rainbow trout exposed to cerium(III).
A representative example of gene expression data in rainbow trout exposed to Ce. Classical representation of data with curve fitting using the least square difference (A) and following Fourier transformation (B).

the components do not produce change individually, but they produce toxicity effects when combined. The resonance values at frequency 0.1 are also reported in table 3, since the other frequencies had no predictive value for REs' toxic properties, HSI or fish condition (fish weight/head-to-fork length). Fish mortality was best predicted by group 3 genes after Fourier transformation of the gene expression data (Table 3). Group 2 genes also had good predictive value at r = 0.7. It is noteworthy that a prediction of r = 0.98 was obtained with four genes, GLUD, SOD, CAT and CYP, which are involved in ammonia metabolism, oxidative stress and phase 1 biotransformation of planar organic hydrocarbons. The resonance signal at 0.1 was able to predict fish mortality concentrations (r = 0.63; p = 0.02). The HSI was best predicted by group 2 genes, which are involved in the hepatic metabolism of xenobiotic with r = 0.96. Group 1 genes after Fourier transformation were also good predictors of changes in HSI at r = 0.91. The resonance value (sum of Pk values) at frequency 0.1 was also able to predict changes in HSI at r = 0.82 ($p < 0.01$). Fish condition (weight/head-to-fork length) was also best predicted by group 2 genes (r = 0.96); for group 1 genes, r = 0.90. The resonance value at frequency 0.1 was also predictive of changes in fish condition with r = 0.8. On the whole, the non-transformed database on intensity thresholds had consistently lower predictive values for RE lethal concentration, HSI and fish condition, compared with Fourier-transformed Pk values at low frequencies (0.05-0.2).

## DISCUSSION

Fourier transformation analysis sheds light on the cyclic/oscillatory behaviour of molecular responses (here, gene expression data) and the discussion is developed based on the concept of the cyclic properties of gene expression changes (Pk and frequency values). The following elements were toxic to Rainbow trout juveniles: Y, Sm, Er and Gd. The toxicity of La and Ce was not detected at concentrations ≤ 40 mg/L after 96 h at 15 °C. In another study, Yb was shown to cause much more alteration in the development of zebrafish embryos than did similar concentrations of La [12]. The 40-day toxicity of Yb in the goldfish *Carassius auratus* was reported at concentrations of about 0.1 mg/L [13]. The activity of glutamate-pyruvate transaminase was decreased at concentrations of 0.05 mg/L and was inhibited at higher concentrations, indicating perturbations in gluconeogenesis and ammonia metabolism. This was corroborated by our results on increased expression of GLUD, another enzyme involved in ammonia metabolism, which was one of the genes that provided the best predictions of toxicity (Table 3). It was also reported that SOD activity was significantly induced but CAT activity was inhibited, suggesting that oxidative stress also occurred [13]. Gene expression analysis of SOD and CAT revealed that SOD was decreased by Y, while

**Table 2.** Gene expression data of livers in rainbow trout exposed to lanthanides.

| | Ce | Er | Sm | La | Y | Nd | Gd |
|---|---|---|---|---|---|---|---|
| CAT | 0.6 (8 mg/L)[1] | 1.2 (0.32 mg/L) | 0.7 (0.06 mg/L) | ns | ns | ns | ns |
| CYP1A | 0.23 (8 mg/L) | 1.9 (0.32 mg/L) | 2 (0.06 mg/L) | ns | ns | 0.6 (1.6 mg/L) | 0.4 (1.6 mg/L) |
| GADD45 | 1.8 (40 mg/L) | 11 (1.6 mg/L) | 1.7 (0.32 mg/L) | 2.9 (8 mg/L) | 0.7 (0.06 mg/L) | 2.6 (8 mg/L) | 2.6 (1.6 mg/L) |
| GLUD | 1.2 (40 mg/L) | 2.3 (0.32 mg/L) | 0.75 (0.32 mg/L) | ns | 1.5 (0.32 mg/L) | ns | ns |
| GST | 0.5 (40 mg/L) | ns | 2.3 (0.32 mg/L) | ns | 1.5 (0.32 mg/L) | 1.3 (8 mg/L) | 1.4 (0.32 mg/L) |
| HSP70 | 3 (8 mg/L) | 2.6 (1.6 mg/L) | 2 (0.32 mg/L) | 1.7 (8 mg/L) | 1.7 (0.32 mg/L) | 1.5 (1.6 mg/L) | 1.9 (1.6 mg/L) |
| MT | 2.5 (8mg/L) | 1.5 (1.6 mg/L) | 2.6 (0.32 mg/L) | 0.7 (40 mg/L) | ns | ns | 2.3 (1.6 mg/L) |
| PCNA | 1.6 (8 mg/L) | 1.2 (0.32 mg/L) | 1.23 (0.32 mg/L) | 1.4 (8 mg/L) | 1.5 (0.32 mg/L) | 1.3 (1.6 mg/L) | 1.5 (0.32 mg/L) |
| SOD | 0.7 (8 mg/L) | 3.6 (1.6 mg/L) | 0.6 (0.32 mg/L) | 0.7 (8 mg/L) | 0.7 (0.06 mg/L) | 1.3 (8 mg/L) | 1.3 (0.32 mg/L) |
| SPARC | ns | ns | 1.3 (0.32 mg/L) | ns | 0.75 (0.06 mg/L) | 1.4 (8 mg/L) | 1.3 (1.6 mg/L) |
| HSI | 0.85 (8 mg/L) | ns | 0.75 (0.32 mg/L) | ns | ns | ns | ns |
| CF | 1.2 (40 mg/L) | 1.15 (1.6 mg/L) | 1.2 (0.06 mg/L) | 1.2 (40 mg/L) | ns | 1.2 (8 mg/L) | ns |
| Trout mortality[2] | > 40 mg/L | 8 mg/L | 1.6 mg/L | > 40 mg/L | 0.7 mg/L | 40 mg/L | 8 mg/L |

[1]: The data represent the fold induction/repression at the lowest exposure concentration (mg/L); ns: not significant. Response factors between 0.85 and 1.15 were usually not statistically significant.
[2]: Concentration at which at least 20% mortality was observed.

CAT gene expression was not significantly affected. This suggests that SOD was downregulated to protect against oxidative stress, since CAT would be inhibited. Moreover, SOD and CAT genes were also included with GLUD in group 4, the highly predictive group for fish mortality (r = 0.98). CAT gene expression levels were also included in the group 3 genes, which were highly predictive of fish mortality (r = 0.95). Lastly, GST activity was found to be induced at 0.05 mg/L, while inhibited at higher concentrations in the goldfish study [13]. GST gene expression was significantly induced in fish exposed to Y in the present study and GST was one of the group 2 genes, which were highly predictive of fish condition and HSI. These relationships were revealed after the cyclic nature of their expression was considered, i.e., after spectral analysis using Fourier transformation.

It is noteworthy that, most of the time, CYP1A1 gene expression was inhibited by the REs, especially with Gd, Nd and Ce, suggesting impairment of xenobiotic phase 1 biotransformation. CYP1A1 was one of the three genes involved in xenobiotic detoxification (Group 2 genes), which were highly predictive of fish condition and HSI. Studies on the effects of REs on aquatic organisms are lacking at present. When female Wistar rats were injected with praseiodynium nitrate, they developed fatty livers, and their cytochrome P450 and b5 levels

**Table 3.** Multiple regression analysis of gene expression and toxicity effects.

| Endpoints | Gene expression (response factor) | Gene expression (Fourier transformed) | Resonance at frequency 0.1 |
|---|---|---|---|
| Fish mortality (LC20)[1] | Group 1 (GADD, HSP70, PCNA, SOD): r = 0.72; p = 0.04<br>Group 2 (MT, GST, CYP): r = 0.7; p = 0.02<br>Group 3 (GLUD, SPARC, CAT): r = 0.45; p = 0.34<br>Group 4 (CYP, GLUD, SOD, CAT): r = 0.54; p = 0.12 | Group 1 (GADD, HSP70, PCNA, SOD): r = 0.5; p > 0.1<br>Group 2 (MT, GST, CYP): r = 0.7; p = 0.02<br>**Group 3 (GLUD, SPARC, CAT): r = 0.95; p < 0.0001**<br>**Group 4 Best prediction (CYP, GLUD, SOD, CAT): r = 0.98; p < 0.0001** | r = 0.63 p = 0.02 |
| Hepatic somatic index | Group 1 (GADD, HSP70, PCNA, SOD): r = 0.87; p = 0.001<br>Group 2 (MT, GST, CYP): r = 0.85; p < 0.001<br>Group 3 (GLUD, SPARC, CAT): r = 0.58; p < 0.01 | Group 1 (GADD, HSP70, PCNA, SOD): r = 0.91; p = 0.001<br>**Group 2 (MT, GST, CYP): r = 0.96; p < 0.0001**<br>Group 3 (GLUD, SPARC, CAT): r = 0.52; p < 0.01 | r = 0.82 p < 0.01 |
| Fish weight/shell length | Group 1 (GADD, HSP70, PCNA, SOD): r = 0.79; p < 0.001<br>Group 2 (MT, GST, CYP): r = 0.44; p = 0.36<br>Group 3 (GLUD, SPARC, CAT): r = 0.66; p < 0.001 | Group 1 (GADD, HSP70, PCNA, SOD): r = 0.90 p < 0.001<br>**Group 2 (MT, GST, CYP): r = 0.96 p < 0.0001**<br>Group 3 (GLUD, SPARC, CAT): r = 0.5; p < 0.01 | r = 0.8 p < 0.01 |

[1]: For fish mortality data, the concentration that killed at least 20% of the fish was reported.
In the case for toxicity at concentrations > 40 mg/L, a default concentration of 200 mg/L was used for regression analysis. The strongest multiple regression coefficients are highlighted in bold.

were drastically reduced in the rough endoplasmic reticulum [14]. In another study, a single dose of a light RE, praseodymium (5 mg/kg), reduced the cytochrome P450-mediated metabolism of hexobarbital and zoxazolamine in rats [15]. However, Ce was shown to increase the activity of coumarin 7-hydroxylase at lower doses (0.5 and 1 mg/kg) in DBA/2 mice, and at higher concentrations morphological changes occurred in the liver which were associated with decreased coumarin 7-hydroxylase activity [16]. Lastly, similar effects were observed in rats exposed to Gd: total cytochrome P450 levels were significantly reduced [17]. Interestingly, pretreatment of rats with Gd(III) reduced the toxicity of $CCl_4$, which requires activation by cytochrome P450. However, pretreatment with Gd(III) had no effect on the toxicity of $CdCl_2$. These studies revealed that cytochrome P450 activity could be either increased or decreased depending on the exposure and duration of concentration and the type of RE. Spectral analysis seems to cope more easily with these "opposite" effects, as it highlights the oscillatory nature of the responses rather than the intensity of either the increase or the decrease of the biomarker that could occur depending on exposure time and concentration. It is not surprising that hepatic detoxification gene transcripts (CYP1A1, GST and MT) were predictive of changes in the HSI, since inducers of cytochrome P450 and phase GST were recognized to increase the HSI in some cases [18].

Gene expression of GADD45, which is involved in cell growth-arrested DNA repair activity, was overexpressed for nearly all the tested REs. The exception was Y, which downregulated GADD45. Y is genotoxic in human lymphocytes, as determined by the micronucleus test [19], and it triggers DNA breaks, leading to S phase arrest and apoptosis [20]. La(III) and Ce(IV) ions were shown to mediate DNA hydrolysis, making them efficient artificial nucleases [21]. GADD45 expression was included in group 1 genes, which were very good predictors of HSI and fish condition. GADD45 gene expression data for Ce (Figure 3) revealed that gene expression was induced at two frequencies, 0.1 and 0.25, and that the frequency 0.1 contributed to the resonance value, which was significantly associated with fish mortality concentrations, HSI and fish condition.

Spectral analysis using Fourier transformation represents another data transformation approach based on the principle that molecular responses behave as waves in space and time, space being the exposure concentration range in the present context. It is reasonable to assume that a biochemical/ physiological response could not always respond proportionally to the exposure concentration in a given timeframe, because, as the exposure concentration increases, the biochemical response could manifest earlier, making it possible to find non-significant or even decreased changes compared to the control at higher concentrations. Moreover, although biochemical pathways are statistically analyzed as independent variables, biochemical changes are expected to have many inter-dependencies at the physiological level (glycolysis is influenced by gluconeogenesis; cellular respiration could be impacted by oxidative stress or xenobiotic metabolism, etc.). The mechanism of action of xenobiotics is not always specific (i.e., receptor-mediated), and many pleiotropic interactions could occur, leading to unsuspected effects in biochemical changes. This highlights the possibility that when some xenobiotics are present alone they do not show toxicity, but when they are present in mixtures, toxicity is observed even if the concentration of the xenobiotic in the mixture is lower than that of the xenobiotic alone. One such example was observed in hydra exposed to a mixture of eleven pharmaceuticals usually found in municipal wastewater [22]. The combined toxicity of the eleven pharmaceutical mixtures was compared with the individual toxicity of each pharmaceutical compound. The concentrations that produced toxicity of the pharmaceuticals in the mixture were 2 to 3 orders of magnitude lower than the concentrations of the individual pharmaceutical compounds administered alone to the hydra. By considering the wave-like nature of molecular responses (biomarkers), we can quantify effects based on resonance and/or emergence. Resonance occurs when signals are at the same frequency (i.e., synchronized): the individual signals are integrated and enhance the net amplitude of the biochemical changes to the point that they cause damage to the system. This allows for emergence, where an effect could be observed with a mixture of xenobiotics but not with the individual components. In general, the toxicity of individual compounds is additive, but spectral analysis introduces the notion that the biochemical changes caused by the individual components of the mixture must occur at the same frequency in order to make it possible to observe additivity or integration of “non-detectable” effects. Moreover, the frequency at which the effects are observed was always lower than the natural/biological variation (the fundamental frequency) of the biomarker. In addition, the wave-like properties of biochemical changes are also suitable for modelling the phenomena of circadian rhythms and synchronization in biochemical changes. Synchronization is the process in which biochemical changes occur in phase with other biochemical changes. It was observed that cells in population have the ability to synchronize their metabolic pathways (e.g., glycolysis) with each other, enabling the global functioning of population (yeast cells) or tissues such as the heart [23]. The intermediary metabolite acetaldehyde has been suggested as synchronization agent for glycolysis in dense populations of cells [24]. However, more research will be needed to determine whether these wave-like properties could be generalized further in molecular toxicology. The present study revealed that the Pk metric (an indicator of the variance of the changes) identified genes or pathways that were corroborated by other studies and highly predictive ($r > 0.90$) of effects at higher levels of biological organization such as mortality, HSI and fish condition. The resonance metric (sum of Pk values of signals occurring at

the same frequency) was also significantly correlated with the above endpoints, suggesting that some effects arise from the process of resonance. Again, more research will be required to further validate this statement.

## CONCLUSION

In conclusion, toxic responses at the molecular level appeared cyclic in nature and transformation of the responses based on Fourier analysis could provide further insights on the toxic properties of biochemical changes in cells.

## CONFLICT OF INTEREST STATEMENT

The author of this article declares no conflict of interest.

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