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Research Article

Fourier Transformation Analysis of Stress Biomarkers in Mussels Exposed to Lethal Municipal Effluents.

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Abstract

The release of municipal waste waters into the environment could threaten the health and maintenance of populations of organisms living in the vicinity of urban areas. The purpose of this study was to examine the toxicity of municipal waste waters to fresh water mussels based on the biomarker responses involved in oxidative stress and genotoxicity. *Elliptio complanata* mussels were placed in cages and immersed in two different terminal aeration ponds treating domestic waste waters and at one river reference site for up to 48 days. Mussels were collected at different times (0, 12, 24 and 48 days) and analyzed for the following biomarkers: metallothioneins (MT), total peroxidase (Perox) activity, lipid peroxidation (LPO) and DNA damage (DD). The waste water from the first aeration pond was lethally toxic, causing 45% mortality, and the second waste water was less toxic, causing 20% mortality, after 48 days. The data revealed that most of the above biomarkers were initially induced at 12 or 24 days but the response declined afterwards as mortality was manifested in mussels exposed to the waste waters. These cyclic changes were analyzed by Fourier transform which showed that MT, LPO, Perox biomarkers produced amplitude changes at frequencies higher than those observed for reference mussels. Phase analysis revealed that these frequencies were not in phase with those of the reference mussels, which suggests heterogeneous responses or the appearance of toxicity-related frequencies that seemingly did not occur in control mussels. The number of out of phased frequencies was also higher in mussels exposed to the most toxic (lethal) waste waters compared to the least toxic waste water. Moreover, some of these frequencies occurred at similar frequencies with MT, Perox and LPO in the most toxic waste waters, suggesting that resonant signals could contribute to toxicity in fresh water mussels. In conclusion, the cyclic properties of biomarkers could provide new insights into the adverse effects on mussel health and survival.

Keywords: DNA damage; Fourier transformation; municipal wastewaters, mussels, oxidative stress, wave behavior,

Introduction

Municipal waste waters (MW) are well known sources of contamination in aquatic habitats which can threaten local populations [1]. Indeed, they are reported to have various deleterious effects such as endocrine disruption of reproduction, oxidative stress, activation of xenobiotic biotransformation and genotoxicity [2-4]. MW also contains a cocktail of pharmaceuticals and personal care products such as ibuprofen, antibiotics, cholesterol regulator drugs, and neuroactive drugs [5]. These could also contribute to the long-term toxicity of MW to aquatic organisms, given the regular and continuous release of these effluents into aquatic ecosystems. Mussels are particularly at risk from urban pollution because they are sessile and they feed on suspended material in the water column. Union mussels are endobenthic, i.e., they live at the sediment/water interface and are exposed to virtually all hydrophilic and hydrophobic contaminants. Among the effects observed in mussels exposed to municipal effluents, noteworthy ones include an increase in lipid peroxidation (LPO) as evidenced by increased metabolic respiration, infection/inflammation associated with microorganisms, biotransformation of xenobiotic leading to the production of reactive oxygen species [6]. Oxidative stress may be modulated by various antioxidants such as thiol-rich metal binding proteins called metallothioneins (MT), peroxidase (Per-

ox) and other enzymatic systems (superoxide dismutase, catalase, etc.). If their production is uncontrolled, reactive oxygen species can react with proteins, unsaturated lipids, leading to LPO and to DNA damage resulting in oxidative-mediated DNA breakage and repair activity. However, municipal effluents also contain many direct DNA-damaging and repair inhibitors, which could lead to decreased DNA strand, break formation and sustained DNA damage [3].

Perox is an enzyme involved in the sequestration of reactive oxygen species and has several specific properties besides its capacity to degrade H_2O_2 in the presence of an electrophonic substrate. In some conditions, Perox shows oscillatory non-linear behavior in its activity depending on the concentrations of H_2O_2 or the presence of O_2 and NADH. Perox can oxidize NADH in the presence of dissolved O2 ($NADH + O_2 \rightarrow NAD+ + H_2O$) in a periodic/oscillatory manner called the peroxidase-oxidize reaction [7,8] It was also reported that cyclic changes in mitochondria respiration rates were modulated by the release and elimination of reactive oxygen species, suggesting that oxidative stress could result from altered oscillatory/periodic changes in cellular respiration rates [9] There is a growing awareness that biochemical changes follow cyclic or wave-like behavior as suggested above. For example, biomarkers based on molecular endpoints often follow U-shaped dose-response patterns and may even represent thormesis [10, 11].

These changes also occur in time as many gene transcripts "oscillate" in time. For example, this is observed during the process of cell differentiation and growth in embryos of the nematode *C. elegans* [12] The authors found that nearly 1/5 of expressed transcripts oscillated (cyclic increase and decrease in transcripts) with an 8 h period and hundreds of genes changed >10-fold. These periodic mRNA concentrations led to rhythmic translation, thereby maintaining and/or transiently increasing protein and metabolite levels. The periodic changes of NADH levels during glycolysis were first observed in yeast cells during anaerobic glycolysis [13]. These periodic changes are thought to form the basis of biological clocks/rhythms and pace making activity (i.e., periodic contraction/relaxation of blood vessels) at the corresponding tissues. It follows that conventional reporting of data using the mean with a variability measure (e.g., confidence intervals) for intensity responses may not be entirely suitable for understanding these types of behavior in cells [2] It has been proposed that molecular biomarkers behave non-linearly as wave-like signals which can be analyzed by Fourier transformation analysis. Fourier transformation is a mathematical procedure that transforms the cyclic behavior of signal changes usually in time (but not restricted to) in the frequency domain. The effects of toxic substances on the periodic properties of biomarkers are poorly understood [14].

Indeed, anaerobic glycolysis of yeast populations was shown to oscillate in time for NADH and this process was disrupted in the presence of selected toxicants such as copper sulfate, gadolinium chloride and silver nanoparticles. Moreover, these compounds disrupted NADH levels at specific frequencies outside the normal range of frequencies. The purpose of this study was therefore to examine the changes in toxic stress biomarkers in caged mussels exposed to 2 chronically lethal municipal waste waters using Fourier transform. Changes in MT, LPO, Perox and DNA strand breaks were followed after 1, 12, 24, 36 and 48 days, in mussels immersed into 2 municipal waste waters. Fourier transform was performed on the data to highlight changes in the above biomarkers in the frequency domain. An attempt was made to relate the frequency descriptors of the biomarkers in relation to the toxic properties of the waste waters.

Materials and Methods

Mussel collection and Handling

Elliptio complanata mussels were collected by hand in the Richelieu River, Quebec, Canada, in June 2011. They were maintained in 300-L aerated tanks for no longer than 10 days at 15°C and fed with commercial algal preparations (Coral reef mix). Mussels (N=12) were then placed in cylinder nets (24 cm diameter x 48 cm height) at 4 nets were used per site. The first site consisted of a terminal aeration pond (municipal wastewater 1 or MW_1) treating essentially domestic and industrial wastewaters from a municipality of circa 25,000 inhabitants. The other site consisted of a terminal aeration pond (MW_2) treating domestic and industrial waste waters from a municipality of 15,000 inhabitants. The other consisted of the reference site which was in the Richelieu River at 3 km upstream the City of Chambly (Québec, Canada). The mussel cages were immersed at depths between 2-3 meters with the help of buoys and exposed as such for 1, 12, 24, 36 and 48 days. One cage was retrieved every12 days and the cages were placed overnight in dechlorinated, charcoal-treated water at 15oC to allow depuration. Water temperature in the river and aeration pond was monitored daily with temperature recorders.

The mussels were then measured, weighted and tissues (gills, digestive gland and gonad) weighted and dissected out and placed on ice. The tissues were homogenized with a Teflon pestle tissue grinder in 10 mM Hepes-NaOH, pH 7.4, containing 100 mM NaCl, 1 mM EDTA and 1 mM β-mercapto ethanol and 0.1 µg/mL apoprotin in at a ratio of 20 % weight/volume ratio. The homogenate was centrifuged first at 1,500 ×g for 10 min at 2°C, the supernatant removed and centrifuged at 15,000 ×g for 30 min at 2oC. The supernatant (S15fraction) was removed and stored at -85oC. The total protein content in the homogenate andS15fractions was determined using the principle of protein-Coomassie brilliant blue binding [15] Calibration was achieved using serum bovine albumin as standards.

Biomarker assessments

The levels of metallothioneins (MT) in gills were determined using the spectrophotometric assay [16] The S15 fraction was first

reduced with one volume of 50 mM di thiothreitol for 15 min prior to the fractionation procedure with ethanol and chloroform. Calibration was achieved using standard solutions of reduced glutathione and data were expressed as µg thiol/mg proteins. Single- and double-stranded DNA were determined using the alkaline precipitation assay [17] Briefly, the procedure separates genomic DNA from DNA strands which remain in solution and the concentrations determined by fluorescence spectrometry in the presence of high pH and salt content to prevent interference by trace amounts of detergent (sodium Dodecyl sulfate) [18].

Calibration was performed using salmon sperm DNA, and fluorescence readings were taken at 485 nm excitation and 520 nm emission using the SYBR Green dye (In vitro gen, USA). Total peroxidase activity was determined in the post-mitochondrial fraction (S15 fraction) of the homogenate as described elsewhere [4] The S15 fraction was first treated with 2 volumes of 0.1 M sodium acetate (pH5) containing 0.1% Triton X-100 for 15 min on ice. Peroxidase activity was then determined by adding 10 mM H_2O_2 and 10mMbenzidine, followed by incubation for 30 min at 22°C. Absorbance was then measured at 650 nm. The data were expressed as absorbance change ($A_{30min} - A_{0\text{-}1min}$)/min/mg protein. The levels of lipid peroxidation were determined in the homogenate fraction using the thiobarbituric acid reactants (TBARS) methodology [19]. The levels of TBARS were determined by fluorescence at 520 nm excitation and 590 nm emission (micro plate reader, Bioscan, USA). Standard solutions of tetramethoxypropane were used for calibration and the data were expressed as □g TBARS/mg proteins.

Data Analysis

Biomarkers were determined in N=12 mussels at each sampling time and samples were collected 5 times (at day 1, 12, 24, 36 and 48). The data were first expressed as the mean and standard error at each exposure time. The normality and homogeneity of variance were determined using Shapiro-Wilk and Bartlett tests. Heterogeneous or non-normal data were log transformed. The data were subjected to analysis of variance (ANOVA), and critical differences between treatments were determined with Fisher's least significant difference test. Correlation analysis was performed using the Pearson-moment procedure. Significance was set at $p<0.05$.

The non-linear cyclic changes in biomarkers were analyzed using spectral analysis and Fourier transformation analysis (Statistical software package, version 8, France). In the present study, measurements collected from 12 mussels at each sampling time were randomly selected (the 10 replicates were randomly combined) and gradually compiled from day 1 to day 48 to form a data series (5 sampling times *12 mussels biomarker level=60 points). The Fourier transform procedure models the periodic nature of the data by fitting sine and cosine functions at different frequencies. In essence, the procedure transforms any functions f (x,t) into functions of frequencies g (k). More precisely, the Fourier series is defined as $g(k) = a_0 + \sum [Ak^*\cos(2\pi(kn/N) + Bk^*\sin(2\pi(kn/N)]$, where n = 1 to *N* observations (time in the present case).

The variable n represents the individual observations of the series expressed in time (min or h) or space (x) and k is the frequency. The constants Ak and Bk are used to calculate the period gram (Pg) value, which is related to the amplitude variance of the sine and cosine functions at each frequency k: Pg = (Ak2 + Bk2)*N/2, where N is the total number of observations. The Pg value is thus related to the variance of the function at a given frequency. The significance of each Pg value was tested and compared to random "noise" using the Kolmogorov–Smirnov test (exponential adjustment). Phase analysis (two-dimensional Fourier transform) comparing signals from mussels exposed to municipal waste water (MW) and those from the reference group were performed to verify whether signals originated from the normal frequencies (reference mussels), i.e., were in phase. Significance was set at $p < 0.05$ in all cases.

Results

Mussels were exposed to 2 municipal waste waters (MW_1 and MW_2) for 1, 12, 24, 36 and 48 days. MW_1 came from the last aeration pond serving a city of 25,000 in habitants and had a pH 6.1-7.1, conductivity 400-800 µS*cm-1 and total coli form counts of 500-1,000 coli forms/100 mL.MW2, which was collected from the last aeration pond serving a city of 15,000 in habitants, had a pH of 7-7.5, conductivity of 500-800 µS*cm-1 and total coli form counts of 400-800 coli forms/100 mL

The river water had a pH level of 8.0-8.2, conductivity of 200 µS*cm-1and total coli forms levels <20 counts. Mussels exposed to the river water showed no significant changes in mortality. In mussels exposed to MW1, mortality appeared after 24 days and reached 50% at the end of the exposure period (48 days).In mussels exposed to MW_2, mortality appeared after 24 days, reaching 20% at the end of the exposure period (48 days). The MT levels were determined in mussels exposed to MW_1, MW_2 and the reference river water (Figure 1). Gill MT levels were significantly increased at day 12 compared to day 1 in mussels exposed to MW1 as well as in the reference mussels at day 12(Figure 1A).

MT levels gradually declined as of day 24, with at three-fold reduction observed at day 48. In mussels exposed to MW_2, MT levels dropped after day 1 and remained at the same level until the end of the exposure period. At day 24, the levels were significantly lower in the MW_2 exposed mussels than in those from the reference site. In mussels exposed to control (river) water, the levels were not significantly influenced by the exposure period, except at 48 days, when the MT levels were significantly lower. The MT data were analyzed by Fourier transformation, given the cyclic and non-linear changes in time (Figure 1B). In mussels exposed to the less toxic effluent, MW_2, signals at higher frequencies were similarly observed but to a lesser extent than for MW_1. The major fre-

quencies were (in decreasing order) 0.02, 0.06, 0.1, 0.14 0.42 and 0.36, corresponding to signals with periods of 50, 17, 10, 7, 2 and 3 observations. This suggests that more changes in the biomarker responses were observed after 17 observations, which is indicative of a more general (decreasing) temporal pattern of MT levels in mussels compared to the results for the more toxic aeration pond effluents (MW1). The data were also analyzed using two-dimensional Fourier transform with phase spectral analysis (Figure 1C).

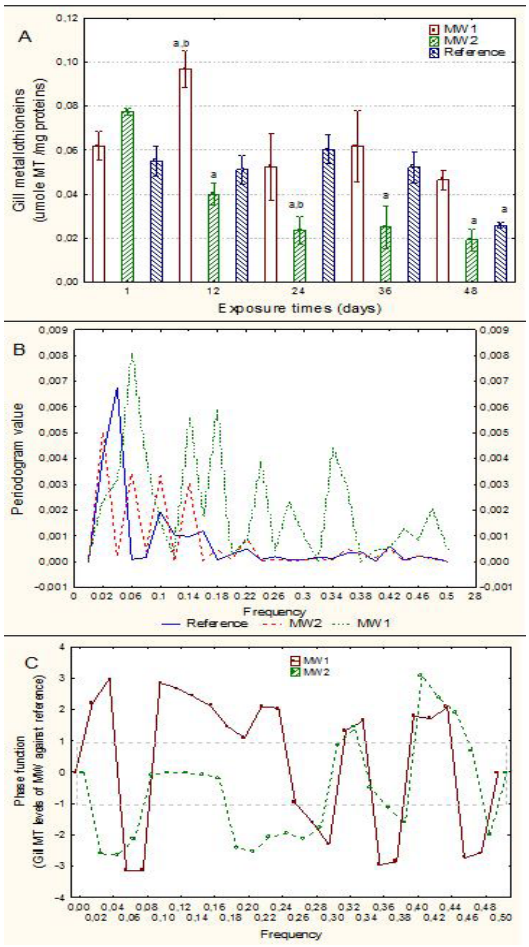


Figure1: The figure shows the signal intensities (Pg) as a function of frequencies on the x axis. The frequency is defined by 1/period which is the number of observations to complete one cycle (for example, a frequency of 0.2 corresponds to 5 observations). In mussels exposed to river water only, we found 3 major frequencies at 0.04, 0.1 and 0.16, which corresponds to periods of 25, 10 and 6 observations. The 10 and 6 observations are within the number of 10 mussels per time, which corresponds to the inter-individual variations, while the 25 observations suggest a periodic response that encompasses day 1 to day 24. In the case of mussels exposed to MW1 (mortality beginning at >24 days and 7 5,3, 4, and 2 observations. This indicates that mussels exposed to MW tend to show stronger inter-individual changes in MT, i.e., more variability between individuals is observed.

Phase analysis of signals at each corresponding frequency from mussels exposed to the MW compared to the reference mussels was performed to see whether the observed frequencies in mussels exposed to MW1 or MW2 originate (are in phase) from existing signals of frequencies for reference mussels. Phase signals between -1 and 1 are considered not significant and can be treated as in phase with each other (MW and river water/reference). Conversely, signals outside this interval were significant and considered out of phase which one another, meaning that the signal frequency of the MW group either differs or drastically out of phase from the reference background signal.

The phase signals of mussels exposed to MW_1 when compared with the reference signals revealed out-of-phase signals throughout the frequency domain (x-axis). Indeed, over 88% of the frequencies were out of phase with those of the control mussels. In the case of MW_2, which was about 2.5 times less toxic, there were more frequencies in phase with the reference signals (MW_2 signals were closer/in phase with those of the reference signals) and 61% of frequencies were out of phase with reference mussels. Correlation analysis performed between the phase function of MW_2 and MW_1 revealed no significant trend ($r=0.13$; $p>0.1$). Hence, an increase in the number of out-of-phase frequencies over60% of the frequency spectrum is associated with the appearance of mortality events (20% mortality) and when the number reaches 90%, mortality is increased to 50%.The levels of LPO in gills were also determined in mussels exposed to the MW (Figure 2).

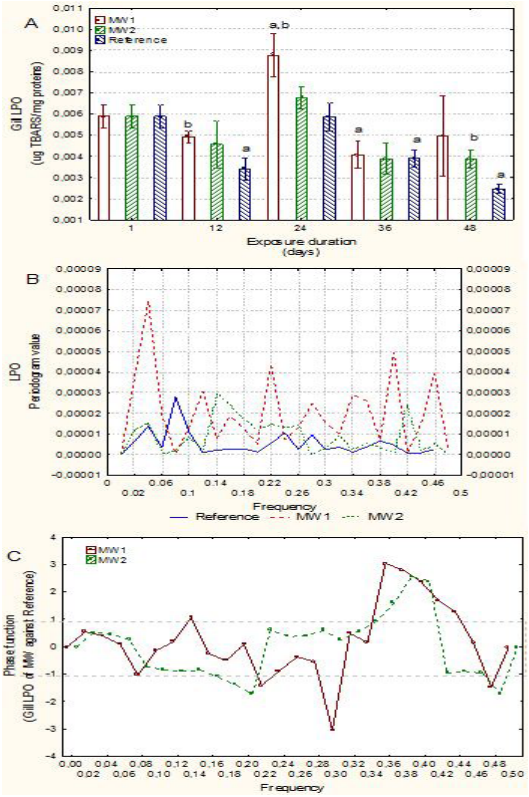


Figure 2: Variation in gill LPO in mussels exposed to toxic municipal wastewaters.

Levels of LPO in gills were determined in mussels at different exposure times to the municipal wastewaters. The data are conventionally reported as the mean with standard error (A). The letter indicates a significant difference compared to day 1 and the letter b indicates significance from reference mussels at the same time. Fourier transformation of data showing the period gram (Pg) values as a function of frequency is shown in B. In C, 2D Fourier transform analysis of the biomarker data obtained for MW compared with those obtained for the reference site. Frequencies that fall within the dotted lines were not significantly different (in phase). Conversely, signals outside the dotted lines (> +1 or <-1) are significantly different (i.e., the frequency of the MW differs out of phase from reference signal) at $p<0.05$ which suggests the appearance of "toxic" frequency In mussels exposed to the reference water, LPO levels fluctuated with a small but significant decrease in LPO at days 12, 36 and 48 relative compared to day 1 mussels. In mussels exposed to MW1, LPO levels increased at day 12, followed by a significant decrease at day 24 relative to day 1. Gill LPO levels, were increased at day12 and day 24 in relation to reference mussels and returned to their values afterwards. In mussels exposed to MW2, gill LPO levels were not significantly affected (ANOVA $p>0.1$).Gill LPO levels were only increased at day 48 relative to the control mussels group on the same day. Gill LPO levels were then analyzed using Fourier transformation (Figure 2B).

In relation to control mussels, the Pg intensities were generally higher than reference mussels and the following 5 major frequencies were observed in decreasing order of intensity: 0.08, 0.04, 0.24, 0.28, 0.38, corresponding to periods of 12, 25, 4 (2 times) and 3 observations, respectively. In the case of mussels exposed to MW_1, the intensities (Pg) were generally higher than those of the control mussels with the following 5 major frequencies: 0.04, 0.4, 0.22, 0.46, 0. 34, corresponding to periods of 25, 2 (twice), 4 and 3 observations. This suggests that mussels exposed to the most toxic MW_1 (50% mortality) show stronger and higher frequencies than mussels exposed to the river water only. In mussels exposed to MW_2 (less toxic than MW_1), the 5 major frequency signals were (in decreasing order) 0.16, 0.42, 0.04, 0.22 and 0.26, corresponding to periods of 6, 2, 25 and 4 (2 times). The intensities were generally lower than for the more toxic MW_1 but some higher and stronger frequencies were observed compared to reference mussels. Phase analysis of mussels exposed to either MW1 or MW_2 compared to control mussels also revealed out-of-phase signals at higher frequencies (Figure 2C). The proportion of out-of-phase frequencies was 38% and 23% for MW_1 and MW_2, respectively. The out-of-phase frequencies occurred mostly at higher frequencies ($f>0.28$) and the most toxic MW1 contained more of out-of-phase and higher frequencies compared to the less toxic one (MW_2). Total Perox activity was determined in the digestive gland of mussels exposed to the MW (Figure 3A).

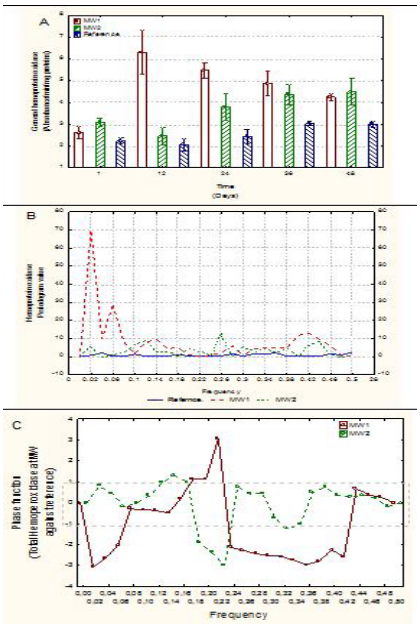


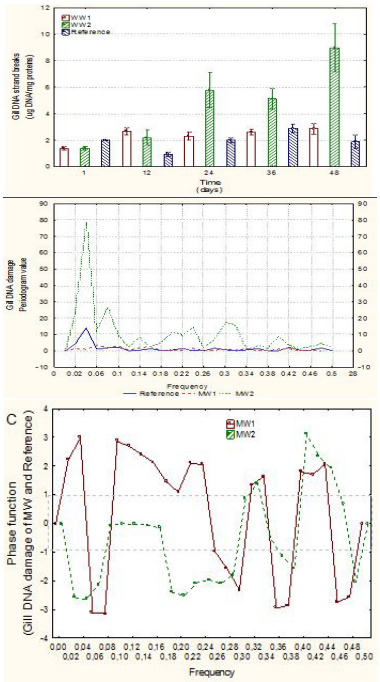
Figure 3: Total hemoprotein oxidase activity in the digestive gland mussels exposed to municipal wastewaters

Total hemo protein oxidase activity was determined in the digestive gland of mussels exposed to the municipal waste waters. The data are conventionally reported as the mean with standard error (A). The letter indicates significant difference compared to day 1 and letter b indicates significance from reference mussels at the same time. Fourier transformation of data showing the period gram (Pg) values as a function of frequency is shown in B. In C, 2D Fourier analysis of the biomarker data obtained in MW compared with those obtained in the reference. Frequencies that fall within the dotted lines were not significantly different (in phase). Conversely signals outside the dotted lines (> +1 or <-1) are significantly different (i.e., the frequency of the MW differs out of phase from reference signal) at $p<0.05$ which suggests the appearance of "toxic" frequencies.

In mussels exposed to MW_1, activity was significantly increased at day 12 and thereafter in relation to either day 1 or the reference group at the corresponding time. In the case of MW_2, a significant increase was observed at day 36 and thereafter. The activity of Perox, a marker enzyme for oxidative stress and the presence of hydroxyl radicals, was mildly but significantly increased at 36 and 48 days in the control mussels. Fourier transformation analysis was also performed on the Perox activity (Figure 3B).

In mussels exposed to river water (reference), no specific frequencies were observed with a positive signal. In mussels exposed to the most toxic MW1, we identified the following 5 most important frequency signals in decreasing order of Pg intensities: 0.02, 0.06, 0.42, 0.40, 0.44, corresponding to signals with 50, 17 and 2

(3 times) observations. Hence, exposure to toxic waste waters also leads to Perox changes at higher frequencies than in the control. In mussels exposed to MW_2, the following 5 major frequencies were observed in decreasing order of Pg values: 0.26, 0.12, 0.44, 0.42 and 0.4, corresponding to 4, 8 and 2 (3 times) observations. The intensities (Pg values) were generally lower than for the more toxic MW_1 but higher than for the reference mussels. The data were also analyzed using two-dimensional Fourier transformation for phase analysis (Figure 3C). In mussels exposed to MW_2 compared to reference mussels, 62% of frequencies were out of phase with those of the corresponding frequencies in reference mussels. For the less toxic MW2, only 15% of the frequencies were out of phase with those of reference mussels, suggesting again that mussels exposed to the more toxic MW1 had a greater number of out-of-phase frequencies and that most of them occurred at frequencies > 0.25. The levels of DNA strand breaks in the gills were also analyzed (Figure 4). The levels of DNA strand breaks in mussels exposed to MW_1 were generally increased relative to day 1 but not in comparison with the reference mussels except at day 12 (Figure 4A). In mussels exposed to MW_2, the levels of DNA strand breaks were increased after 12 days relative to day 1 and significantly increased after day 1 when compared with the reference mussels at the corresponding times. In control mussels, DNA strand breaks were significantly lower only at day 12 compared to day 1. The data were subjected to spectral analysis using Fourier transformation (Figure 4B).



The analysis revealed than in mussels exposed to MW_1, low intensity frequencies were observed at 0.06, 0.1, 0.16, 0.24 frequencies, corresponding to periods of 17, 10, 6 and 4 observations. In the case of MW_2, the signals were stronger and the following major frequencies were found: 0.04, 0.08, 0.32, 0.22, 0.2, corresponding to periods of 25, 12, 3 and 5 (2 times) observations. The signals were stronger and some appeared at higher frequencies compared to the case for reference and MW1 mussels. The data were subjected to phase analysis using reference mussels as the independent variable (Figure 4C). In mussels exposed to MW_1, 92% of the 26 frequencies were out of phase with the reference mussels. In the case of MW_2, 62% of the 26 frequencies were out of phase with the reference mussels. This suggests that although lower DNA strands breaks were observed in mussels exposed to the most toxic MW_1, most of the frequencies were still out of phase with those of reference mussels which suggests that decreased DNA levels occurred at higher frequencies which were not in phase with those of the normal (reference) case.

Discussion

This study examined a different approach for analyzing biochemical responses to environmental pollutants. Fourier transformation can be used to analyze biomarker responses not only in time but also in the frequency domain. Mathematically, this transformation is very robust as it can model any signal in time or space into a series of sine and cosine functions at different frequencies. Biologically, it introduces the notion that biological responses can be cyclic in nature and exhibit wave-like behavior. Oscillations at the molecular level are not uncommon in biology, and have been observed at fundamental levels such as glycolysis, oxidative stress, insulin secretion and Ca2+ signaling [20,21] Oscillatory behavior forms the basis of biological rhythms and circadian responses such as insulin secretion during the day [20] A cell-free extract of rat skeletal muscle spontaneously oscillates in terms of glycolysis and ATP/ADP ratio when glucose is supplied continuously. Oscillations in the ATP/ADP ratio are coupled with oscillations in free Ca2+ and insulin secretion. Citrate levels also oscillate in the mitochondria as do NADH and ATP levels [21] The production of citrate depends on the enzyme aconitase derived from isocitrate, which is known to be sensitive to toxic heavy metals such as zinc and cadmium [22,23] Given that metals and oxidative stress can alter critical metabolites involved in cyclic behavior in cells, the role of cyclic changes in eco toxicology needs to be better understood.

Alterations in the redox status of cells form the basis of toxicity in many instances. Perturbations in the processes of cellular respiration and oxidative phosphorylation caused by xenobiotic could be a common denominator of toxicity, and hence could underlie many pathophysiological conditions. Mitochondrial oscillators in NADH, membrane potential (electrolytes) and Ca^{2+} are dependent on the release of reactive oxygen species and scavenging by antioxidant mediators such as catalase and Perox [24] When the delicate balance between ROS generation and scavenging is altered (i.e., by environmental toxicants), the mitochondrial network locks onto one main low frequency and high amplitude mode, which could lead to suppression of electrical excitability

and Ca^{2+}handling. This could in turn lead to arrhythmic contractions in muscles, with a potentially fatal outcome in the case of heart muscles. The mitochondria oscillator is dependent on the concentration of reactive oxygen species [9].

The efflux of superoxide anion through the inner membrane anion channel and its scavenging by antioxidant mediators (ascorbate, glutathione, superoxide dismutase, catalase and Perox) were examined in relation to NADH and membrane potential oscillations. The study found that the period of the oscillator could be influenced by altering the concentration of reactive oxygen scavengers, leading to oscillations in reduced glutathione in the cytoplasm as well as in oxidative phosphorylation rates. The enzyme Perox which is involved in the reaction of H_2O_2 with various substrates is also known to oscillate under some conditions [18] For Example, umbelliferone can be oxidized following a sinusoidal pattern by high concentrations of H_2O_2 in the presence of horseradish peroxidase. The oscillatory responses reflected periodic changes in the concentration of Perox active (compounds I and II) and inactive (compound III or $Perox^{+3}$) intermediates. An interesting feature of Perox is that it not only catalyzes the oxidation of substrates by H_2O_2 but can oxidize NADH in the presence of O2 instead of H_2O_2 [25] This is called the Perox-oxidase oscillator, which is associated with periodic (sinus-like) changes in NADH and $Perox^{3+}$ levels. The addition of peroxynitrite, a major reactive oxygen species, can result in a reaction with the Perox-oxidase oscillator leading to a concentration-dependent increase in amplitudes with no change in the frequencies. MT is considered an antioxidant because it sequesters electro Phallic and oxidative compounds such as peroxynitrite and H_2O_2 [26]. In the present study, total Perox activity was significantly correlated with LPO (r=0.43), which suggests that Perox activity was involved in oxidative damage (i.e., could not prevent oxidative damage) in mussels exposed to the most toxic municipal effluent. The frequency profiles of LPO and Perox activity were also significantly correlated (r=0.44). Coherence values of gill LPO as a function of Perox activity (vicariate spectral analysis) revealed a strong value at frequency 0.18 (squared coherence = 0.6) and they were in phase with a significant gain in amplitudes of LPO due to Perox. This suggests that the amplitudes change at given frequencies were in phase and manifested at the same frequency (resonance), leading to increased oxidative damage.

Interestingly, we found the same pattern for LPO in relation to MT levels, suggesting that reactive oxygen species sequestration by Perox and MT were coupled (in phase) with the levels of LPO. It has been shown that MT protect against creatine kinase inhibition by low concentrations of NO and H_2O_2 [27] In the case of mussels exposed to the less toxic effluent (MW2), the analysis revealed that LPO and peroxidase activity were not significantly coherent at the 0.18 frequency and they were out of phase with each other and prevented any gain in the peroxidase signals in relation to the LPO signal. This indicates that the manifestation of changes in oxidative stress mediators (LPO, peroxidase, and MT) at amplitudes in phase at critical (higher) frequencies was associated with the most toxic wastewater, MW_1. This was also shown in telocytes with Ca^{2+} oscillations in the inositol triphosphate/Ca^{2+} signaling pathway [28]. Disruption of these signals could have consequences for the pace making activity of contractions of various systems such as the vascular (valve calcification) and intestinal (inflammation) systems. This highlights the wave-like behavior of biochemical responses which could lead to toxic events in tissues.

Conclusion

Exposure of freshwater mussels to toxic MW led to non-linear changes in antioxidant mediators (MT and Perox), oxidative stress and DNA damage. Analysis of the periodic properties of these responses revealed the appearance of amplitude changes at higher frequencies which were out of phase with the responses obtained in reference mussels, suggesting a heterogeneous response leading to novel frequencies. The amplitudes and frequencies seemed to be associated with the toxic potency of the MW. The analysis supports the hypothesis that contaminants could alter the oscillatory behavior of biomarker responses in mussels. Further research is needed to better understand how the cyclic changes in biochemical responses could lead to adverse effects.

Acknowledgements

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