Biomarkers of oxidatively induced DNA damage in dreissenid mussels: A genotoxicity assessment tool for the Laurentian Great Lakes

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Abstract

Activities of fast growing human population are altering freshwater ecosystems, endangering their inhabitants and public health. Organic and trace compounds have a high potential for adverse impacts on aquatic organisms in some Great Lakes tributaries. Toxic compounds in tissues of organisms living in contaminated environments change their metabolism and alter cellular components. We measured oxidatively induced DNA damage in the soft tissues of dreissenid mussels to check on the possible contaminant-induced impact on their DNA.

The animals were obtained from archived samples of the National Oceanic and Atmospheric Administration (NOAA) Mussel Watch Program (MWP). Mussels were collected from the harbor of Ashtabula River in Ohio, and a reference area located at the Lake Erie shore. Using gas chromatography-tandem mass spectrometry with isotope dilution, we identified and quantified numerous oxidatively modified DNA bases and 8,5′-cyclopurine-2′-deoxynucleosides. We found significant differences in the concentrations of these potentially mutagenic and/or lethal lesions in the DNA of mussels from the harbor as compared to the animals collected at the reference site.

These results align NOAA’s data showing that elevated concentrations of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and heavy metals were found in mussels within the harbor as compared to mussels collected in the reference site.

The measured DNA lesions can be used as biomarkers for identifying DNA damage in mussels from polluted and reference sites. Such biomarkers are needed to identify the bioeffects of contaminants in affected organisms, as well as whether remedial actions have proven successful in reducing observed toxic effects.

Keywords: Mussel Watch Program, dreissenid mussels, Great Lakes, oxidatively induced DNA damage, PAHs, PCBs, trace metals, GC-MS/MS.
Introduction

The Laurentian Great Lakes region, with its wealth of natural resources and maritime and railroad transportation systems, underwent rapid industrialization and ultimately a legacy of chemical contamination in many of its rivers and harbors.

The National Oceanic and Atmospheric Administration (NOAA), National Centers for Coastal Ocean Science, Mussel Watch Program (MWP), uses dreissenid mussels to monitor chemical contamination in the Great Lakes. In recognizing the need for both chemical and biological monitoring information, the MWP partnered with the National Institute of Standards and Technology (NIST), to conduct a pilot project to implement alternative biomonitoring techniques that complement chemicals concentrations measurements in environmental media (water, sediment, biota). This paper describes an oxidatively induced DNA damage monitoring tool applied to the dreissenid mussel species to distinguish reference site from impacted sites in the Ashtabula River harbor and nearby Lake Erie.

After entering an aquatic system, contaminants can move between media, for example through accumulation in the food chain or through deposition in sediments. Contaminants can cause alterations at different levels of the hierarchy of biological organizations. Among numerous contaminants, legacy and of emerging concern (CEC), found in the Ashtabula River harbor, PAHs, PCBs and trace elements are of particular concern, with most of them being toxic even at low concentrations. Usually, when high levels of PAHs are present in aquatic systems, abnormal growth of tissue (neoplasms) is observed in fish and other inhabiting animals. For years, PAHs have been known to be strong inducers of cytochrome P450 (CYP450) activity in fish and other vertebrates, and are metabolized in living organisms to trigger reactions resulting in oxidatively induced DNA damage. In some circumstances, when parallel presence of non-dioxin-like PCBs and their hydroxyl metabolites occur in the environment, they can induce
DNA damage signaling and enhance the DNA damaging effect of benzo[a]pyrene. Metabolites of PAHS and PCBs are known to generate oxygen-derived species such as hydroxyl radical (OH), superoxide radical (O_2^{*−}) and hydrogen peroxide (H_2O_2). Among invertebrates the ability to metabolize PAHs differs significantly due to variable levels and activities of the cytochrome P450 dependent oxidases. Mollusks are species with a wide range of metabolic capability but clearly showing presence of PAHs metabolites generated by reactions involving oxidative and genotoxic effects. Reactions of OH cause damage to biological molecules including DNA, proteins and lipids, and thus can lead to increased genetic instability, inflammation, proliferation, reduction of antioxidants, cell death, apoptosis and angiogenesis. If not repaired, DNA damage may lead to harmful biological effects in living organisms, including mutations, disease and death.

In aquatic systems, bivalves are recognized as important biomonitoring organisms because of their ubiquity, abundance, easy sampling and filter-feeding lifestyle. Dreissenid mussels are useful monitoring organisms because they usually dwell in large numbers and are well adapted to naturally stressful environments. Dreissenid mussels in the Great Lakes have been used to monitor chemical contaminant levels for over two decades by the NOAA Mussel Watch Program and in recent years for mussel health assessment. Here, we report on oxidatively induced DNA damage in dreissenid mussels (Dreissena polymorpha; zebra) collected from the harbor of the Ashtabula River and compare with mussels collected from a reference location in Lake Erie. We identified and quantified numerous DNA lesions including 8,5′-cyclopurine-2′-deoxynucleosides in DNA of dreissenid mussels as potential bioindicators for environmental genotoxicity.
Materials and Methods

Study Area and Sample Collection

The Ashtabula River flows into Lake Erie and like many rivers of the Great Lakes it received industrial pollution in the years prior to environmental laws and regulations. Chemical contamination from PCBs, PAHs, and heavy metals, led to restrictions on fish and wildlife consumption, degradation of fish and wildlife populations, and loss of fish and wildlife habitat.

In 2014 all management actions to clean up the Ashtabula River had been completed. Samples were collected from two sites (LEAR-1 and LEAR-3) outer Ashtabula harbor and from a reference site (LEAB) in Lake Erie approximately 6.5 km east of the Ashtabula River mouth, in 10 m of water and 230 m off-shore (Figure 1) in September 2014. The reference site (LEAB) was established by the NOAA Mussel Watch Program in 1992 and sampled every two years for analysis of over 100 chemical contaminants. Scuba divers removed dreissenid mussels from rock substrate using stainless steel scrapers, placed them in a nylon mesh bag, and upon surfacing transferred them to coolers containing site water.

Within one hour of collection, the mussels were rinsed free of debris with site water, placed in 5 Ziploc bags in composites of approximately 20 to 30 mussels each. The bagged mussels were then placed in a cooler of dry ice and shipped blind coded to the NIST laboratory, where they were transferred to a freezer at -80 °C until they were thawed for further processing and analyses.

In addition, mussels were sent to a separate laboratory for analysis of chemical contaminants in the soft tissue according to NOAA protocols for sample collection, preparation, and analysis.\textsuperscript{33-35}
Methods of PAHs, chlorinated hydrocarbons including PCBs, and trace elements (Cu, Ni, Cr, Fe) analyses are summarized in Supporting Information section, including links and citations for detailed information.

**DNA isolation**

After removal from the Dewar container, mussels were thawed on ice, then washed with ice-cold deionized water. To minimize the influence of body mass and age of studied animals on the results of measurements of markers of oxidative DNA damage, similar size (~2 cm) mussels have been selected for each group. Mussel tissues (~100 mg) separated from shells with a scalpel were processed according to the product manual of E.Z.N.A. Mollusc DNA Kit, Omega Bio-tek (Norcross, GA) with modification involving homogenization with Bullet Blender Storm 24 high-throughput bead-mill homogenizer (Next Advance, Averill Park, NY). Tissues were placed in the 1.5 mL Rhino Screw cap tubes (Next Advance, Averill Park, NY) kept on ice, containing 350 µL of ML1 Buffer from the kit and three 2 mm zirconium oxide beads. Tubes were transferred into the Bullet Blender kept in the refrigerator at 4 °C and processed 2 x 30 s at speed 12 with 30 s break between runs. Subsequently 25 µL of Proteinase K from the kit was added and samples were incubated for 2 h at 60 °C. Then, subsequent steps of the Mollusc DNA Kit protocol were applied. For the final DNA elution, two portions of 100 µL of sterile high-performance liquid chromatography grade water (Sigma-Aldrich, St. Louis, MO) warmed to 70 °C were used. The UV absorbance spectrum of each DNA sample was recorded by absorption spectrophotometry between the wavelengths of 200 nm and 350 nm to ascertain the quality of DNA and to measure the DNA concentration at 260 nm (absorbance of 1 = 50 µg of DNA per milliliter). Aliquots (50 µg) of DNA samples were dried in 1.5 mL deoxyribonuclease-free Eppendorf tubes in a SpeedVac under vacuum and then kept at −80 °C for further analysis.
Gas chromatography-tandem mass spectrometry

Gas chromatography-tandem mass spectrometry (GC-MS/MS) with isotope-dilution was used to identify and quantify modified DNA bases and 8,5′-cyclopurine-2′-deoxynucleosides. A set of DNA samples was used to identify and quantify oxidatively induced DNA base lesions 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), thymine glycol (ThyGly), 5,6-dihydroxyuracil (5,6-diOH-Ura), 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-hydroxyguanine (8-OH-Gua). DNA samples (50 μg each) were supplemented with the aliquots of the stable isotope-labeled analogs of these compounds, i.e., 5-OH-5-MeHyd-\(^{13}\text{C},^{15}\text{N}_2\), ThyGly-\(^{d}_4\), 5,6-diOH-Ura-\(^{13}\text{C},^{15}\text{N}_2\), FapyAde-\(^{13}\text{C},^{15}\text{N}_2\), FapyGua-\(^{13}\text{C},^{15}\text{N}_2\) and 8-OH-Gua-\(^{15}\text{N}_5\) as internal standards, which are a part of the NIST Standard Reference Material 2396 Oxidative DNA Damage Mass Spectrometry Standards (for details see http://www.nist.gov/srm/index.cfm and https://www-s.nist.gov/srmors/view_detail.cfm?srn=2396). The samples were dried in a SpeedVac under vacuum, and then dissolved in 50 μL of an incubation buffer consisting of 50 mmol/L phosphate buffer (pH 7.4), 100 mmol/L potassium chloride (KCl), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.1 mmol/L dithiothreitol. Subsequently, they were incubated with 2 μg of \(E.\ coli\) formamidopyrimidine-DNA glycosylase (Fpg) and 2 μg of \(E.\ coli\) endonuclease III (Nth) at 37 °C for 1 h to release the modified DNA bases from DNA. An aliquot of 100 μL ethanol was added to precipitate DNA and to stop the reaction. After centrifugation, the supernatant fractions were separated, lyophilized and trimethylsilylated as described\(^{36}\). Derivatized samples were analyzed by GC-MS/MS using multiple reaction monitoring (MRM) as described previously\(^{36}\, 37\). The mass transitions used for this purpose were: \(\text{m/z } 331 \rightarrow \text{m/z } 316\) and \(\text{m/z } 334 \rightarrow \text{m/z } 319\) for 5-OH-5-MeHyd and 5-OH-5-MeHyd-\(^{13}\text{C},^{15}\text{N}_2\), respectively; \(\text{m/z } 432 \rightarrow \text{m/z } 417\) and \(435 \rightarrow \text{m/z } 420\) for 5,6-diOH-Ura and 5,6-diOH-Ura-\(^{13}\text{C},^{15}\text{N}_2\), respectively; \(\text{m/z } 448 \rightarrow \text{m/z } 259\) and \(\text{m/z }
452 → m/z 262 for ThyGly and ThyGly-d₄, respectively; m/z 369 → m/z 368 and m/z 372 → m/z 371 for FapyAde and FapyAde-¹³C,¹⁵N₂, respectively; m/z 457 → m/z 368 and m/z 460 → m/z 371 for FapyGua and FapyGua-¹³C,¹⁵N₂, respectively; m/z 455 → m/z 440 and m/z 460 → m/z 371 for 8-OH-Gua and 8-OH-Gua-¹⁵N₅, respectively. These transitions are based on the known mass spectra of the trimethylsilyl derivatives of modified DNA bases and their fragmentation patterns, which were reported previously (for a review see38). 

*E. coli* Nth was obtained from Dr. Susan Wallace at the University of Vermont. *E. coli* Fpg was prepared by Dr. Prasad Reddy at NIST39. 

Another set of DNA samples (50 μg each) was used for the measurement of 8,5′-cyclopurine-2′-deoxynucleosides, i.e., (5′S)-8,5′-cyclo-2′-deoxyadenosine (S-cdA), (5′R)-8,5′-cyclo-2′-deoxyguanosine (R-cdG) and (5′S)-cyclo-2′-deoxyguanosine (S-cdG). DNA samples were supplemented with the aliquots of the stable isotope-labeled analogs of these compounds. The stable isotope-labeled internal standards for modified 2′-deoxynucleosides, i.e., (5′S)-8,5′-cyclo-2′-deoxyguanosine-¹⁵N₅ (S-cdG-¹⁵N₅), (5′R)-8,5′-cyclo-2′-deoxyguanosine-¹⁵N₅ (R-cdG-¹⁵N₅), (5′S)-8,5′-cyclo-2′-deoxyadenosine-¹⁵N₅ (S-cdA-¹⁵N₅) and (5′R)-8,5′-cyclo-2′-deoxyadenosine-¹⁵N₅ (S-cdA-¹⁵N₅) were synthesized and isolated as described40, 41. The samples were dried in SpeedVac and then dissolved in 50 μL of 10 mmol/L tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) solution (pH 7.5) containing 45 mmol/L zinc chloride (ZnCl₂), supplemented with 2.5 μL of 1 mol/L sodium acetate (final pH 6.0). Aliquots of nuclease P1 (2 U), snake venom phosphodiesterase (0.004 U) and alkaline phosphatase (16 U) were added and the samples were incubated at 37 °C for 24 h. After hydrolysis, the samples were filtered using ultrafiltration membranes with a molecular mass cutoff of 3 kDa by centrifugation at 12000 g for 30 min. Filtered samples were lyophilized and then trimethylsilylated as described37. MRM scans were performed with mass transitions m/z...
465 → m/z 309 for S-cdA, m/z 470 → m/z 314 for S-dA$^{15}$N$_5$, m/z 553 → m/z 397 for R-cdG and 
S-cdG, and m/z 558 → m/z 402 for R-cdG$^{15}$N$_5$ and S-cdG$^{15}$N$_5$. These transitions are based on 
the known mass spectra of the trimethylsilyl derivatives of 8,5′-cyclopurine-2′-deoxynucleosides 
and their fragmentation patterns, which were reported previously$^{38,41-44}$. The optimal (maximum) 
collision energies of the trimethylsilyl derivatives of S-cdA, R-cdG and S-cdG were determined 
by varying the collision energy between 5 V and 35 V with 5 V increments. The maximum 
collision energy for each of these compounds was found to be 15 V, and this was used for the 
measurements.

**Statistical analysis**

Six independently prepared DNA samples from 6 different mussels from each collection site 
were used to identify and quantify the levels of oxidatively induced DNA base lesions and 8,5′-
cyclopurine-2′-deoxynucleosides. Statistical analyses of the data were performed using the 
GraphPad Prism 7.01 software (La Jolla, CA, USA) and nonparametric Kruskal-Wallis and 
Dunn’s multiple comparisons tests. A $p$-value < 0.05 was assumed to correspond to statistically 
significant difference between medians.

**Results**

We identified and quantified six DNA base lesions and three 8,5′-cyclopurine-2′- 
deoxynucleosides in DNA of zebra mussels from the Ashtabula River harbor (LEAR-1 and 
LEAR-3) and the reference site (LEAB) using GC-MS/MS with isotope-dilution. The identified 
DNA lesions and their measured concentrations in DNA of the mussels are shown in Figure 2
and Figure 3, respectively. The results show that the mussels from LEAR-1 had significantly
greater levels of oxidatively induced DNA bases, except for 5,6-diOH-Ura \( (p=0.7873) \), and 8,5'-
cyclopurine-2'-deoxynucleosides with the \( p \)-values varying from \( p<0.0001 \) to 0.0488 than those
from LEAB. The greater level of FapyGua in mussels’ DNA than that of 8-OH-Gua suggests
possible low level of \( O_2 \) in their cells/tissues (perhaps reflecting low oxygen level in the
river/lake waters). Such phenomenon is known to generate suitable environment for the one-
electron reduction of the guanine ring-opened C8-OH-adduct radical, which yields FapyGua at a
greater level than 8-OH-Gua\(^45\).

To the best of our knowledge, our work is the first to present application of quantitative mass
spectrometric assessment of oxidatively induced DNA base damage in dreissenid mussels from a
historically polluted harbor and a corresponding reference area. Previously, NOAA researchers
had found elevated concentrations of PAHs and PCBs, including several of the approximately
40000 organic compounds identified as contaminants of emerging

concern\(^46,47\), in the outer harbor of Ashtabula River compared to the concentrations observed at
relatively clean areas\(^33\). Figure 4 shows the representative concentrations of fourteen PAHs
measured in tissues of mussels in three sampling points LEAB, LEAR-1 and LEAR-3. In all
cases, the levels of PAHs in LEAR-1 were greater than those in LEAB.
Fig. 4. Representative concentrations of fourteen PAHs measured in tissues of mussels in sampling points LEAB, LEAR-1 and LEAR-3.

Except for acenaphthylene and fluorene, LEAR-3 also had elevated levels of PAHs when compared to LEAB. LEAR-3 site situated behind breakwater wall and closer to open lake waters, thus not directly exposed as LEAR-1 to sediments transported by the river stream, ship waste and substances eluted by rainwater from tons of coal and minerals stored on harbor banks. Moreover, prevailing winds and lake current move lake water into the harbor inlet and out through the southeast hole in the breakwater. Therefore, significant differences were observed between the total concentration of PAHs found in the sediments collected near sites LEAR-1 and LEAR-3, 4201 µg/kg and 2603 µg/kg, respectively. Some individual examples of analyzed PAHs show even greater differences, e.g., benzo[a]pyrene, 267.4 µg/kg dry vs. 92 µg/kg dry; pyrene, 646.1 µg/kg dry vs. 235.0 µg/kg dry, and fluorene, 62.3 µg/kg vs. 26.3 µg/kg dry. The same NOAA report also shows striking differences in the results of the toxicity tests for Ashtabula River and harbor stations, conducted near site LEAR-1 and LEAR-3, e.g., *Hyalella azteca* mean survival (% ±SD), 2.50%±0.46% vs. 43.75%±4.07%, respectively. Average concentrations of PCBs in the sediments where site LEAR-1 were located were also higher than at site LEAR-3: 168 µg/kg dry mass and 58.7 µg/kg dry mass, respectively. Data showing PAHs concentrations in mussels’ tissues and sediments together with the observed lower level of oxidatively induced DNA damage in animals from LEAR-3 vs. LEAR-1 site suggest possible higher impact on DNA damage of concentration of contaminants absorbed and metabolized through mussels’ digestive and respiratory structures than already accumulated in the tissues. Those findings point also to the sensitivity of the employed technique, which can distinguish between organisms exposed to different concentrations of pollutants; one should notice that the lower concentration of some pollutants has been also found in tissues of mussels at LEAR-3, e.g., acenaphtylene, fluorene, anthracene, phenanthrene and benz[a]anthracene (Fig. 4), which suggests a possible significant
impact of those compounds on the concentrations of the markers of oxidatively induced DNA
damage. The concentrations of PCBs are shown in Figure 5A as the sums of the concentrations
of 39 compounds measured in tissues of mussels in three sampling points.

Fig. 5. (A) PCBs concentrations shown as the sums of the concentrations of 39 compounds
measured in tissues of mussels in sampling points; (B) concentrations of copper, nickel,
chromium and iron at the sampling areas.

In this case, too, at least 50 % higher levels of PCBs were observed in LEAR-1 and LEAR-3
than those in LEAB. Figure 5B shows the concentrations of four metals measured at three
sampling areas (data presented as from last NOAA report of concentration of those metals in
2011; concentrations of PAHs and PCBs are from 2014, NOAA personal communication:
https://coastalscience.noaa.gov/projects/detail?key=179). Only the concentration of Cu was
greater in LEAR-1 and LEAR-3 than in LEAB. The concentration of Fe was found to be higher
in LEAR-3 than in LEAR-1 and LEAB.

Discussion
Reactions of the highly reactive •OH with DNA constituents generate a plethora of products
from all four heterocyclic bases and the sugar moiety of DNA (reviewed in45). Among these
products, 8,5'-cyclopurine-2'-deoxynucleosides are unique tandem lesions in that they are formed
by abstraction of an H atom by •OH from the C-5' of 2'-deoxyribose of the nucleoside, followed
by C5'–C8-intramolecular cyclization and oxidation. Both (5'-R)- and (5'-S)-diastereomers of
8,5'-cyclopurine-2'-deoxynucleosides are formed (reviewed in45). Many of these DNA lesions
have been identified and quantified in DNA in vitro, in cultured mammalian cells, and in human
and animal tissues in vivo (reviewed in45, 49-51). Early studies discovered that PAHs and PCBs
cause hepatocellular carcinoma of fish from polluted sites and in rodents, and to other
detrimental biological effects such as alterations in gene expression and immune response\textsuperscript{52-62}. These findings drew considerable attention to the health of aquatic animals in such environments that may have the potential to affect human health as well. Subsequent studies have suggested that oxidatively induced DNA lesions can be used as highly promising biomarkers for identifying contaminant-induced genomic changes in fish at polluted sites compared to reference sites, and also for determining whether remedial actions were successful in reducing toxic effects\textsuperscript{3, 63}. Thus, a number of DNA lesions have been identified in English sole in a highly industrialized river site at greater concentrations than in a reference site\textsuperscript{3}. In the present work, we hypothesized that other aquatic animals such as dreissenid mussels in the impacted sites around large lakes with expected pollution and near human populations may accumulate oxidatively induced DNA lesions, which may be used as early warning biomarkers for pollution and determining the efficacy of remedial actions.

PAHs and PCBs are known to generate $^\bullet$OH, and other oxygen-derived species such as superoxide radical (O$_2$\textsuperscript{-}) and hydrogen peroxide (H$_2$O$_2$). The latter two can undergo further transformation into $^\bullet$OH in the presence of transition metal ions such as Fe$^{+3}$ or Cu$^{+2}$ ions\textsuperscript{17}. The DNA lesions identified in the present work are typical products of reactions of $^\bullet$OH with the heterocyclic bases and the sugar moiety of DNA\textsuperscript{45}. If not repaired by cellular repair mechanisms prior to replication, such DNA lesions can lead to mutagenicity and/or lethality and, thus cause genetic instability that may lead to disease processes including carcinogenesis\textsuperscript{26, 64-68}. Specifically, both 8-OH-Gua and FapyGua pair with adenine during replication and lead to G $\rightarrow$ T transversions\textsuperscript{69-72}. These mutations are the second most common somatic mutations found in human cancers\textsuperscript{73}. FapyAde causes A $\rightarrow$ T transversions; however, it is weakly mutagenic when compared to 8-OH-Gua and FapyGua\textsuperscript{74}. ThyGly constitutes a strong block to DNA polymerases
and is a lethal lesion and, at best, poorly mutagenic. 5-OH-5-MeHyd derived from thymine acts 
in vitro as a strong block for DNA polymerases and may be a lethal lesion in vivo. Uracil 
derivatives such as uracil glycol, 5-hydroxyuracil and 5,6-diOH-Ura (isodialuric acid) are 
formed in DNA by deamination and dehydration of cytosine-derived lesions. The biological 
effects of 5,6-diOH-Ura are not known; however, uracil glycol and 5-hydroxyuracil are strongly 
mutagenic. In this work, no increase in the level of 5,6-diOH-Ura in the polluted areas was 
oberved when compared to the reference area (Figure 3). Among 8,5'-cyclopurine-2'-
deoxynucleosides, S-cdA blocks transcription and several DNA polymerases, and leads to 
transcriptional mutagenesis and multiple nucleotide deletions. S-cdG blocks replication and 
leads mainly to G → A transitions, and to G → T transversions to a lesser extent. Taken 
together, the DNA lesions identified in this work with greater levels in the polluted area than in 
the reference area are highly mutagenic and may thus contribute to the adverse biological effects 
of PAHs and PCBs.

The increased levels of PAHs and PCBs, and Cu found by NOAA in the two polluted areas when 
compared to the reference area correspond to the increased levels of a variety of DNA lesions in 
mussels in the same polluted areas that we report in this work. This fact links, for the first time, 
the presence of PAHs and PCBs, and metals as possible cause of oxidatively induced DNA 
damage in terms of numerous DNA lesions in dreissenid mussels. The data suggest that the 
known mutagenicity or lethality of the identified DNA lesions are highly likely to contribute to 
carcinogenesis and other disease processes observed in aquatic animals living in polluted areas. 

We propose the use of oxidatively modified DNA bases and 8,5'-cyclopurine-2'-
deoxynucleosides as novel quantitative biomarkers for identifying pollutant-induced changes in 
DNA of aquatic animals. The discovery that numerous mutagenic or lethal DNA lesions
accumulate in mussels in polluted areas adds a novel dimension to previous studies that used a
single DNA lesion only. Further evaluation of this monitoring tool is planned with mussels collected from other Great Lakes harbors in agricultural, and industrial watersheds, for comparison with reference sites as part of a larger strategic plan to identify and assess adverse impacts in Great Lakes tributaries.

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Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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**Figure legends**

Fig. 1. Map of sample collection sites.

Fig. 2. Structures of analyzed modified DNA bases and nucleosides.

Fig. 3. Concentrations of modified DNA bases and nucleosides as measured by GC-MS/MS with isotope dilution; *p values present significant differences of median concentrations of lesions between reference site samples (LARB) and samples from AOC (LEAR-1); no significant difference found for 5,6-diOH-Ura (p=0.7873), Kruskal Wallis Test. Dunns’s multiple comparison test did not demonstrate significant differences between LEAB/LEAR-3 and LEAR-1/LEAR-3 groups. Error bars are standard deviations.

Fig. 4. Representative concentrations of fourteen PAHs measured in tissues of mussels in sampling points LEAB, LEAR-1 and LEAR-3.

Fig. 5. (A) PCBs concentrations shown as the sums of the concentrations of 39 compounds measured in tissues of mussels in sampling points; (B) concentrations of copper, nickel, chromium and iron at the sampling areas.
Figure 1

Figure 2
Figure 3
Figure 4

Figure 5
Supporting Information

Biomarkers of oxidatively induced DNA damage in dreissenid mussels: A genotoxicity assessment tool for the Laurentian Great Lakes

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Biomarkers of oxidatively induced DNA damage in dreissenid mussels
Summary of organic contaminants analysis

The methods described here include polycyclic aromatic hydrocarbons (PAHs), chlorinated hydrocarbons including polychlorinated biphenyls (PCBs) and trace elements (Cu, Ni, Cr, and Fe) analyses. B&B Laboratories, Inc, an affiliate of TDI-Brooks International, Inc. in College Station, Texas under contract to NOAA, used the organic analytical techniques described here. The Trace Element Research Laboratory, Department of Veterinary Integrative Biosciences, Texas A&M University, also an affiliate of TDI-Brooks International, Inc., performed the methods described for trace element analysis.

Organic contaminant analysis: Shell length and volume are determined for all bivalves collected at each sampling site. The bivalves are then shucked and the soft tissue homogenized using a stainless steel Waring blender. Homogenized tissue samples are frozen at –20 ºC until extraction. Prior to extraction, tissue samples are thawed and re-homogenized using a stainless steel spatula. A subsample is removed for percent moisture determination. Approximately 15 g of tissue are thoroughly mixed and ground with a sufficient quantity (approximately 40 g) of prepared (combusted) Hydromatrix to “dry” the sample. The tissue samples must be thoroughly dry to optimize the extraction efficiency. Hydromatrix chemically dries samples by binding moisture. The amount of Hydromatrix necessary to dry a sample depends upon the amount of sample and the percent moisture in that sample. Tissues are extracted with dichloromethane using an ASE200. The tissue/Hydromatrix mixture is loaded into 33 mL ASE extraction cells. Appropriate surrogates and spikes are added to the top of the samples. The ASE extractor tubes are sealed and placed in the ASE cell carousel. The ASE conditions are: 100 % dichloromethane as the extraction solvent, 13.789 MPa (2000 psi) solvent pressure, 100 ºC cell temperature, and 2 static cycles for 2 min each. Extracts are collected in 60 mL collection vials. The extracts are
reduced to approximately 10 mL in the 60 mL collection vials in a 55 °C to 60 °C water bath. Extracts are then quantitatively transferred to Kurderna-Danish (K-D) tubes and the volume reduced to 3 mL in a 55 °C to 60 °C water bath. A 100 µL aliquot is removed and weighed to determine lipid content. Quality control samples (e.g., blanks, duplicates, matrix spikes and standard reference materials) are prepared and extracted in the same manner as samples. Extracts are initially purified using alumina/silica gel chromatography columns. Combusted and cooled alumina is deactivated by adding 1% (w/w) HPLC water and tumbled for at least 1 hour using a Lortone rock tumbler. Combusted and cooled silica gel is deactivated by adding 5 % (w/w) HPLC water and tumbling for at least 1 h using a Lortone rock tumbler. Borosilicate glass columns (300 mm x 19 mm) are filled with dichloromethane and packed from the bottom with: glass wool, 1g to 2 g of sodium sulfate, 10 g of deactivated alumina, 20 g of deactivated silica gel, and another 1 g to 2 g of sodium sulfate. The dichloromethane is drained to the top of the column followed by the addition of 50 mL of pentane. The pentane is drained from the top of the upper sodium sulfate layer and discarded. The sample extract (approximately 3 mL) is added to the top of the column and eluted with 200 mL of a 50:50 mixture of pentane and dichloromethane at a flow rate of 1 mL/min. The eluent is collected in a 250 mL flat-bottom flask. The eluent is reduced to approximately 10 mL in a 55 °C to 60 °C water bath. The extract is transferred to 25 mL K-D tubes and reduced to 1 mL to 2 mL. The concentrate is transferred to 4 mL amber HPLC vials and brought up to 4 mL with dichloromethane. The extract is further purified using HPLC. The extract is injected using a Waters, Model 717 Plus autosampler and eluted through one Phenogel 100 Å guard column and two Phenogel 10µm GPC 100 Å size exclusion columns with 100 % dichloromethane at a flow rate of 7 mL per minute. Elution times for compounds of interest are monitored using standards and a UV absorbance detector (254 nm). The appropriate fraction is collected using a Waters Fraction Collector. The sample is
collected in 50 mL Zymark tubes and reduced to 10 mL in a 55 °C to 60 °C water bath. The extract is transferred to K-D tubes and reduced to 1.0 mL. The dichloromethane is exchanged with hexane and reduced to a final volume of 0.5 mL. The concentrate is transferred to 2 mL amber vials and stored at 20 °C until analysis.

PAH analysis: A gas chromatograph/mass spectrometer (GC/MS) in selected ion mode (SIM), coupled to a capillary column, is used to resolve and PAHs in tissues. Samples are injected into a temperature-programmed GC/MS, operated in splitless mode. The capillary column is an HP-5MS (60 m x 0.25 mm ID and 0.25 µm film thickness). The mass spectrometer is capable of scanning from 35 to 500 AMU every second or less and uses 70 electron volts energy in electron impact ionization mode. A data acquisition system continuously acquires and stores all data for quantitation. This method is capable of producing data at parts-per-billion concentrations.

Chlorinated hydrocarbons analysis: A gas chromatograph/electron capture detector (GC/ECD), coupled to two capillary columns, is used to resolve and detect chlorinated hydrocarbons (polychlorinated biphenyls and pesticides) in tissues. Samples are injected into a temperature-programmed GC/ECD, operated in splitless mode. The capillary columns are DB-5 (30 m x 0.25 mm ID and 25 µm film thickness) and DB-17HT (30 m x 0.25 mm ID and 0.15 µm film thickness). The DB-17HT column is used for analyte confirmation. A data acquisition system continuously acquires and stores all data for quantitation. This method is capable of producing data at pg/kg to fg/kg concentrations.

Trace element analysis: Whole bivalves were rinsed with distilled water to remove extraneous material and shucked with a stainless steel knife (using care not to touch the tissue). Whole soft tissue was removed with plastic forceps and rinsed with distilled, deionized water to remove
sediment particles from gills and exterior tissue surfaces. Soft parts were transferred to a tared Ziploc polyethylene bag, and the number of individuals shucked and placed in the bag was recorded. When soft tissue from all individuals from a site had been collected, they were weighed on a top loading balance to measure the total sample wet weight. The pooled samples were stored in a freezer. Ziploc bags containing pooled tissue were removed from the freezer and allowed to thaw. The entire pooled sample was transferred to an acid-washed Teflon jar and 3 large Teflon balls were added. The Teflon lids were securely tightened and the jars placed in Ziploc bags and shaken in an industrial paint shaker for 20 min. After the bulk sample was homogenized, an aliquot of the sample was transferred to a clean 40 dram snap vial and frozen. The frozen aliquot from the bulk homogenization step was placed in a freeze drier and allowed to dry for several days, depending upon the total mass of tissue being dried at one time. In some cases it was necessary to remove the samples from the freeze drier and drain accumulated water from the trap before continuing with the drying step. When samples were thoroughly dried, three small Teflon balls were inserted into each snap cap vial, the lids were affixed, and the samples placed in a Spex shaker mill for 1 min. The Teflon balls were then removed, and the samples stored in closed vials until weighing. Approximately 0.2 g samples of dried tissue were weighed to the nearest 0.0001 g and transferred to tared, acid-washed Teflon bombs. A 3 mL aliquot of HNO3 was added and the bombs sealed in a digestion system and cooked according to the appropriate method (Section 3.2.4). The samples were allowed to cool and 1 mL of H2O2 was added to each sample, then heated to promote the reaction. After the samples were allowed to cool, 1 mL of HCl was added to each sample, and then heated gently. The samples were cooled and 15 mL of deionized distilled water was added. The bombs were closed, mixed by shaking, and weighed to 0.01 g to determine the total solution weight. The digest solution was transferred to labeled 1 oz polyethylene bottles. Solution density was determined by weighing known
volumes with calibrated Eppendorf pipettes in order to determine solution volume. For analysis of Hg, tissue samples were digested using a modified version of the Environmental Protection Agency (EPA) method 245.6. Approximately 0.15 g to 0.3 g (dry weight) of sample was weighed into a 70 mL snap cap vial. Concentrated H$_2$SO$_4$ (2.5 mL) and 1.5 mL of concentrated HNO$_3$ were added and the samples heated in a digestion block at 90 °C to 95 °C for 30 min. After cooling, 10 mL of distilled water, 10 mL of 5 % (w/w) KMnO$_4$, and 5 mL of 5 % (w/w) of K$_2$S$_2$O$_8$ were added to each tube, and the samples left overnight without heating. Before analysis, 5 mL of 10 % (w/w) NH$_2$OH x HCl were added to reduce excess KMnO$_4$ and the volume brought to 40 mL with distilled water. Calibration standards were prepared by serial dilution of commercially available standards using calibrated micropipettes, a top loading balance, deionized distilled water and acids to match the matrix of the samples and methods. Concentrations of working standards were verified by comparison with independent standards traceable to the National Institute of Standards and Technology (NIST) Standard Reference Materials. Trace metal concentrations were calculated by comparing analytical signals of unknowns with those of calibration standards, and then multiplying the observed concentration by the instrumental and digestion dilution factors. Inductively coupled plasma-mass spectrometry was used to measure chromium. Inductively coupled plasma-optical emission spectrometry was used to measure copper, iron, and nickel.

Acknowledgements

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or
endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

References


Detailed descriptions of the methods are referenced here:


And available online:
