The application of an Ames fluctuation assay to determine the mutagenicity of freshwater sediment in Minnesota

Are sediment samples from various bodies of freshwater around the state of Minnesota mutagenic?

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CONTENTS

INTRODUCTION

Known colloquially as "The Land of 10,000 Lakes," Minnesota is widely renowned for its abundance of lakes, rivers, and streams. Residents take advantage of these natural resources in a variety of ways, including recreational swimming and subsistence fishing. Pollution control is necessary to maintain safe human activity in and around Minnesota fresh water and to preserve the health of aquatic ecosystems. As such, the Minnesota Pollution Control Agency measures and observes many indicators of surface water quality, but as of yet does not perform even irregular mutagenicity tests of aquatic environments.[1,2]

Mutagenicity is the ability for a substance to cause mutations in the genomes of living organisms. These substances are known as mutagens. Numerous classes of chemicals are known to be mutagenic, including aromatic amines, polycyclic aromatic hydrocarbons and heavy metals.[3] Organic mutagens are organic molecules that have a mutagenic capability. While mutagenicity does not always correspond to carcinogenicity, it can, and mutagenic compounds play a role in the development of cancer.[4] Mutagens are also genotoxic, as mutations are a type of genetic harm.

Due to the nature of organic mutagens and their use in modern society, they are common in limnic ecosystems. For example, a majority of pesticides useful in agriculture are mutagenic in some capacity: 59% of common compounds are active in gene mutation and 71% are active in DNA damage across a variety of endpoints.[5] Cropland runoff is a major source of pollution of Minnesota surface water, and these pesticides can be transported by precipitation into rivers, lakes, and streams.[6,7] In addition, industrial effluents and wastes discharged from factories and manufacturing facilities into surface water are often heavily contaminated with organic mutagens.[8,9] These bodies of water are often used for recreation or as drinking water. In the case of the Mississippi River, a two thousand mile long river that begins in Minnesota and ultimately ends in the Gulf of Mexico, amounts of lead, uranium, and cadmium have been found dissolved in water and settled in both organic and inorganic forms in sediment.[10] Other non-industrial facilities may also release mutagenic wastewaters into the aquatic environment, such as hospitals[11] or wastewater treatment plants.[12] Finally, another primary source of surface water toxicity and possible mutagenicity is urban runoff, which is shown to contribute known mutagens (polycyclic aromatic hydrocarbons) to nearby bodies of water.[13]

When mutagenic material is released into freshwater, it accumulates in sediment deposits where it can cause harm to the ecosystem and remain for many years.[3,14–17] Specifically, organic molecules easily ab-

sorb onto particulates accumulating into sediments and continually affect the health of the ecosystem.[18] Mutagenic water conditions negatively impact the fitness of species present in aquatic ecosystems, causing a condition—termed mutational meltdown—where population bottlenecks and increased mutation rates lead to eventual extinction.[19,20] Moreover, mutagenic compounds extracted from sediment have been shown to induce DNA adducts in mammalian cells and carcinogenic activity in fish tissues, indicating possible human carcinogenicity and danger to aquatic life.[14,17] Thus, tests of sediment mutagenicity are important in determining the overall quality of freshwater ecosystems, as they can indicate unsafe and unhealthy conditions for life.

Aim and intention

This test is conducted to analyze the possibility of detrimental effects as a result of mutagenicity in some limnic sediments in Minnesota and to provide insight into any necessity for further research. In order to do so, we use an Ames fluctuation assay, an ex situ bacterial bioassay employing mutant strains of *Salmonella typhimurium* to assess sample mutagenicity. However, this assay is limited, and so are the aims of this study: the carcinogenic potential of sediment will not be analyzed, although previous studies link mutagenicity with carcinogenicity;[4] and chemical analysis of sediment samples to determine individual mutagenic compounds will not be carried out.

The scope of this study is intentionally clearly defined, and a limited scope does not imply uselessness. No measurements of the mutagenicity of Minnesota's surface water sediment exist currently for public consumption. This study will aim to fill that void.

Research question

Are sediment samples from various bodies of freshwater around the state of Minnesota mutagenic?

Hypotheses

H_0: None of the sediment samples will show significant mutagenicity.

This result may indicate that at the concentrations tested, the samples are not mutagenic. However, it does not rule out mutagenicity, as explained in the Results and discussion.

This hypothesis is supported if a sample induces no more mutations than the negative control.

H_1: Some or all of the sediment samples will show significant mutagenicity.

This result indicates that the sediments of the positive bodies of water are mutagenic. It does not, however, indicate human carcinogenicity, or provide insight into environmental impacts.

	Coordinates			
Body of water	Latitude	Longitude	Date	Body characteristics
Lake Calhoun	N44°56′9″	W93°19′7″	2017-11	Urban lake; 25 m deep; warm-water recreational; good clarity; low algae levels
Mississippi River	N44°45′45″	W92°52′24″	2015-06	Urban and rural river; used for drinking water; collects agricultural runoff; high sediment turbidity
Leech Lake	N47°9′55″	W94°37′8″	2016-08	Rural lake; 45 m deep; warm-water recreational; bad clarity
Tanner Lake	N44°57′10″	W92°58′44″	2017-07	Urban lake; 21 m deep; warm-water recreational; good clarity; low algae levels
Lake Winona	N45°52′30″	W95°24′14″	2014-02	Rural lake; 3 m deep; developed surrounding area with agriculture and homes; bad clarity; high algae levels

Table 1. List and description of samples taken.

This hypothesis is supported if a sample induces more mutations than the negative control.

EXPERIMENTAL

The experiment consisted of four parts: sample collection, extraction, the Ames fluctuation assay, and statistical analysis.

Sampling

Five separate samples were extracted and analyzed, each from a different body of water Sample locations, dataes, and characteristics of each body are given in Table 1. Samples of sediment were taken from the bottom of the body of water and stored frozen in a sterile test tube until needed. The date and coordinates of each sample were recorded.

The Mississippi River, Leech Lake, Tanner Lake, and Lake Winona samples were obtained from previous studies; only the Lake Calhoun sample was collected as a part of this experiment.

Extraction

Excess water was decanted off the sediment samples and between 0.4 g to 1.4 g of relatively dry mass was scooped into separate 50 mL plastic test tubes. Using a micro-pipette, 2 mL of methanol and 2 mL of distilled water were added to the test tube (only 1 mL of each if the sample mass was below 0.7 g). Methanol and water are polar solvents and work to extract a wide range of polar genotoxins expected to be present in sediment possibly contaminated with municipal pollution.[18] The extraction was then sonicated for at least 5 min to dissolve particle-sorbed mutagens into the solvent. Next, samples were centrifuged at 1000 rpm for 2 min

to separate any solids.

Using a micro-pipette, any liquid free of solids was extracted to a labeled glass test tube. 4 mL of methanol was added and the extraction was performed twice again exactly as above with only methanol (only 2 mL was used for samples below 750 mg). The liquid drawn off in each extraction became more and more transparent, as fewer polar solutes remained in the sediment sample. After the third dissolution, each sample's extract was evaporated to near dryness under a N_2 stream in a water bath at 47 °C. Some solid precipitation was observed in the Lake Tanner and Leech Lake samples during evaporation. When sufficiently dry, each sample was redissolved in 1 mL of dimethyl sulfoxide (DMSO) and transferred to a small vial. If any solid phase material settled out, the extract was centrifuged and re-extracted into another small vial. This process continued until there was no sign of any solid material in any of the extract vials. Lastly, each extract was stored in a freezer below 0 °C.

Ames fluctuation assay

The Ames test, also known as the *Salmonella*/microsome test, is a bioassay designed to detect mutagenicity.[21] It is the most commonly used ex situ assay for this task and has been standardized and tested well.[3,18]

Without histidine, an essential amino acid, bacteria cannot grow or form colonies. Mutant strains of *Salmonella typhimurium* lack the ability to synthesize histidine on their own and are histidine dependent. Without reversion to the wild type or exposure to histidine, they cannot grow. Different varieties of these strains have different mutations in the operon responsible for histidine production: in this experiment, *S. typhimurium* TA100 was used, which is sensitive to base-pair substitutions. Other strains, such as TA98 or variations on TA100, are used to detect frame-shift mutations.[3] In this case, TA100 was chosen due to unavailability of other options from suppliers.

When histidine dependent (his^-) bacteria are exposed to mutagenic material, they can revert to the wild type genome and are then able to synthesize histidine and grow (his^+). This growth is thus indicative of mutagenicity, and can be observed to determine if a sample is mutagenic. This can be done in two main ways: the plate incorporation test first described by Ames and his colleagues and the newer Ames fluctuation assay. In the plate incorporation test, agar plates are cultured with bacteria and samples are placed directly on the plates. After incubation, the number of bacterial colonies that grew are counted to give a measurement of mutagenic activity. However, this procedure is extremely time-intensive and can be inaccurate and prone to mistakes. These issues are

resolved in the other form of the Ames test.

In the fluctuation assay, a plastic plate with a number of small wells is used to hold the growth media, bacteria, and sample during incubation. Each well is independent and constitutes a separate trial. Also added to the wells is a purple indicator that will turn yellow in the presence of bacterial growth. If any growth occurs, which is only possible after a mutation due to the his^- genotype, the well will turn yellow and is counted as revertant.

While some strains of *S. typhimurium* can be dangerous, the strains used in this experiment are not known pathogens. The experimental procedure described here minimizes danger through the use of fume hoods, gloves, and specially indicated biohazard waste containment. At all times, care was taken to ensure both the sterility of the experiment and the safety of the surrounding area and people.

The rest of the fluctuation assay procedure follows the instructions from Environmental Bio-Detection Products Inc., which sells the reagents used in this study. The night before the procedure was undertaken, lyophilized *S. typhimurium* bacteria were rehydrated in growth medium and placed in an incubator set at 37 °C. After 16 to 18 hours, the bacteria were removed from the incubator and all of the sample extracts from the five bodies of water were removed from the freezer and brought to room temperature. Interestingly, only one of the vials froze while the others stayed liquid. After checking the bacteria for turbidity, a volume of distilled water was autoclaved and vacuum filtered through a 22 mm membrane filter to ensure sterility. Next, four dilutions of each extract were prepared according to Table 2. The Mississippi River extract only had enough material for three dilutions, and so only the first three were produced. The reaction mixture was prepared for all of the trials in two batches according to the following ratios:

- 21.62 mL Davis-Mingoli salt solution
- 4.75 mL D-glucose solution
- 2.38 mL bromocresol purple solution
- 1.19 mL D-biotin solution
- 0.06 mL L-histidine solution

Each dilution was measured out into a number of sterile 50 mL plastic test tubes 17.5 mL of water was measured into a test tube as a negative control. Another 17.5 mL of water was measured out into a test tube and 110 µL of sodium azide (NaN_3) was added as a positive control. The proper amount of reaction mix (see Table 2) was then added to each tube along with 5 µL of the *S. typhimurium* culture broth (2500 µL of reaction mix was added the controls, since they used 96 wells).

	Treatment portions (µL)		
Dilution (% volume)	Extract	Water	Reaction mix
100	250	8500	1250
50	125	8625	1250
25	63	8687	1250
12.5	32	8718	1250

Table 2. Amount of reagent corresponding with dilution of sample extract.

The microplates were filled in halves except for the controls, which were given whole plates. One at a time, each tube was poured into a sterile plastic reagent boat and an eight-channel micropipette was used to place 200 µL aliquots of the mixture into the designated wells. When one plate was filled, it was covered and placed to the side. When all of the plates were full, they were sealed in sterile plastic bags to prevent evaporation and incubated for five days at 37 °C. The positive control was stored separately to prevent the volatile sodium azide from interfering with the other trials.

Statistical analysis

After five days of incubation, the plates were removed from heat and photographed. Each purple well was counted as negative and each yellow or partly yellow well was counted as positive, as color only changed in the presence of growth and therefore mutation.

As mutations in bacterial genomes occur spontaneously without the presence of a mutagen, the negative control provided a measurement of the background mutation and reversion rate. In order to determine if a sample is mutagenic, its treatment's level of reversions must be compared to this background reversion level. In the study of mutagenicity, the proper way to make this comparison is not agreed upon. Originally, the preferred method was the so-called 'twofold test': determining statistical significance by checking if the number of reversions of the treatment was at least twice that of the negative control. However, such a simple test is not a thorough statistical analysis, and so more statistically-proven techniques were also explored.

In particular, we use a logistic regression to compare a proportion (the count of revertant wells per total treatment wells) with a continuous variable (the concentration of the treatment calculated from the original mass of the sample that was then extracted and diluted with DMSO, water, and reaction mix). While this technique does not tell us if a sample was statistically significantly mutagenic, it can show a general trend by creating a dose-response curve. If the curve has a positive slope, it can be assumed that the extract concentration is causally responsible for an increase in mutagenicity and that the sample is mutagenic. However, this method must be employed with

another test of significance, as otherwise it can give false positives and negatives. For example, even if all of the dilutions report insignificant reversion rates on their own, the slope of the logistic regression can still be positive. At the same time, a negative slope does not mean that a sample is not mutagenic, as cytotoxicity can inhibit the ability of an Ames test to correctly report mutagenicity at higher concentrations.

For these reasons, this study further employed a modified chi-square (χ^2) test of significance with $\alpha = 0.05$ according to the methods explained by Gilbert.[22] This method is specifically designed to work on fluctuation tests. As the negative control was performed on 96 wells and each sample was only performed on 48 wells, the observations obtained for the negative control were halved. This preserved the resolution of the 96 wells while allowing the computation of the χ^2. The exact equation used to compute the distribution is shown in Equation 1.

$$z^2 = \frac{(n-1)(n_{21} - n_{11})^2}{n_{.1} n_{.2}} \tag{1}$$

In this equation, z^2 corresponds to the χ^2 distribution with one degree of freedom. The other parameters correspond to the chi-square in Table 3, reproduced from the original.[22]

	+	−	Total
Neg. control	n_{11}	n_{12}	$n_{1.}$
Treated	n_{21}	n_{22}	$n_{2.}$
Total	$n_{.1}$	$n_{.2}$	n

Table 3. A chi-square.

RESULTS AND DISCUSSION

The data obtained from the Ames fluctuation assay is given in Table 4, as well as statistical analysis for significance by the chi-square and twofold methods. This data is plotted by sample location in Figure 1 and a logistic regression was performed on all trials for each sample to provide a dose-response curve. These three statistical methods provide a view of the data that can answer the research question posed earlier.

Both of the controls upheld the invariants of the Ames fluctuation assay. All of the wells in the NaN_3-spiked positive control reverted, affirming that bacteria were successfully able to mutate and revert and that the experimental procedure could reliably express revertants. 13 out of the 96 wells in the untreated negative control reverted, a relatively low number as was expected. This control ensured sterility (if the test was contaminated, many more wells would turn yellow than what was observed) and that it was possible to observe unreverted wells.

Out of the 19 trials conducted, seven reported a sig-

Sample	Conc. ($\pm 0.01\,\mathrm{mg\,mL^{-1}}$)	Revertants	Significance	
			Chi-square	Twofold
Positive control[a]	N/A	96	N/A	N/A
Negative control[a]	N/A	13	N/A	N/A
Lake Calhoun	12.93	9	0.476	No
Lake Calhoun	6.46	6	0.023	No
Lake Calhoun	3.23	11	1.400	No
Lake Calhoun	1.62	14	3.453	**Yes**
Mississippi River	11.73	17	**6.147**	**Yes**
Mississippi River	5.86	15	**4.285**	**Yes**
Mississippi River	1.47	11	1.400	No
Leech Lake	32.40	27	**19.068**	**Yes**
Leech Lake	16.20	25	**16.003**	**Yes**
Leech Lake	8.10	14	3.453	**Yes**
Leech Lake	4.05	10	0.887	No
Tanner Lake	28.68	19	**8.257**	**Yes**
Tanner Lake	14.34	12	2.004	No
Tanner Lake	7.17	16	**5.184**	**Yes**
Tanner Lake	3.58	14	3.453	**Yes**
Lake Winona	10.93	11	1.400	No
Lake Winona	5.46	18	**7.172**	**Yes**
Lake Winona	2.73	14	3.453	**Yes**
Lake Winona	1.37	13	2.691	**Yes**

[a]This trial was conducted on a plate of 96 wells.

Table 4. Results of the Ames fluctuation analysis. Bold values signify that a sample was considered statistically significant by the respective column.

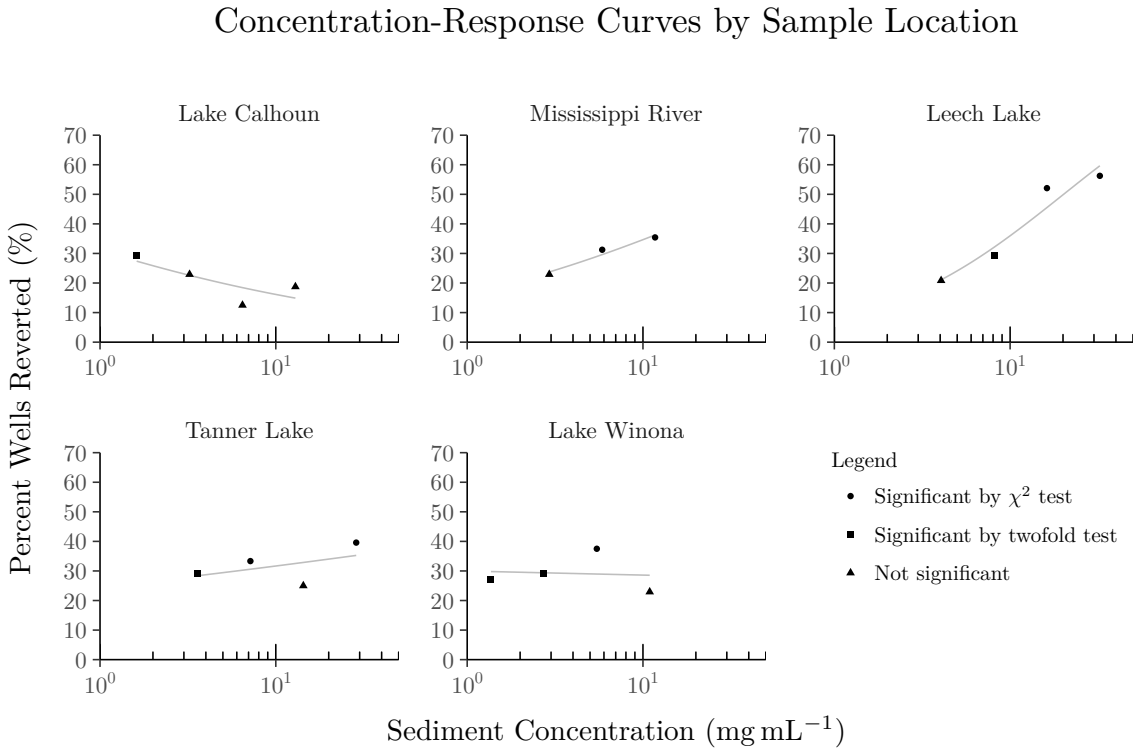

Figure 1. Sample dilutions plotted by body of water and concentration. Logistic regressions are plotted in grey. Shape corresponds to sample significance.

nificantly increased number of revertants from the negative control by the chi-square test (Mississippi River at 100 % and 50 %, Leech Lake at 100 % and 50 %, Tanner Lake at 100 % and 25 %, and Lake Winona at 50 %). In order to be significant, a trial must have had at least 15 revertant wells. As the twofold test was strictly less restrictive, a further five trials were calculated to be significantly different by that method (Lake Calhoun at 12.5 %, Leech Lake at 25 %, Tanner Lake at 12.5 %, and Lake Winona at 25 % and 12.5 %). Only at least 13 wells were required to be revertant by the twofold test to show significance. In the evaluation, however, only the trials that showed a significant amount of mutagenicity by the χ^2 test are considered to be mutagenic, for reasons explained in the Statistical analysis section.

Four of the bodies of water tested exhibited at least one trial with significant amounts of mutagenicity: Mississippi River, Leech Lake, Lake Tanner, and Lake Winona. Lake Calhoun's trials reported low revertants,

with the highest count at the 12.5 % dilution with 14 in a 48 well plate. In fact, this is opposite of what is expected. Higher concentrations of sample sediment should cause more wells to revert, due to an increased potency of any mutagenicity. In addition, in the Lake Calhoun samples, the dose-response curve has a negative slope; as concentration increases, revertant wells decrease. While this behavior is unexpected, it can be explained.

One possible cause of this behavior is acute cytotoxicity of the sample at higher concentrations.[3] At high concentrations, like those tested at the first three dilutions, some chemicals can kill microbes, including the *S. typhimurium* used in the Ames test. Since the fluctuation assay counts any lack of bacterial growth as an unreverted well (a negative result), any phenomena that kills bacteria will interfere with the measurements. Before any mutagens in the sample were able to affect the bacterial genome and revert bacteria to the his^+ phenotype, the well may have been sterilized. At lower concentrations, the cytotoxic capabilities of some chemicals lose potency, and where before the well may have been sterilized it is not, allowing mutagens to affect the trial.

In this way, cytotoxity can 'shadow' the mutagenicity of samples when analyzed by the Ames fluctuation test. While there is no evidence obtained in this study to definitively support that this is the correct explanation for the observed data in the case of Lake Calhoun, it is plausible and has been observed elsewhere where the Ames assay is used.[citation needed] A negative slope in the dose-response curve of the Lake Winona samples is also observed, though less severe. However, the Lake Winona sediment sample is also significantly mutagenic in the 50 % dilution, and so this study is able to report conclusively on Lake Winona while it cannot on Lake Calhoun.

In all other sediment samples, the dose-response curve is positive and at least one of the dilutions is mutagenic when compared to the negative control. The positive dose-response curves suggest a relationship between the sample concentrations and the fraction of revertant wells. However, none of the curves calculated in this experiment have $p < 0.05$, and they cannot be evaluated as statistically significant, including the two with negative slopes.

The highest number of revertant wells was observed in the 100 % dilution of the Leech Lake sample, with a count of 27. In fact, the Leech Lake sediment sample also had the second highest number of revertant wells, 25, at the 50 % dilution. The Lake Calhoun sample had the lowest number of revertant wells: 6 at the 50 % dilution. Thus, the revertants observed ranged from 6 to 27 per 48, with an average of 14.

CONCLUSION

The evidence from the Ames fluctuation assay supports H_0, as mutagenicity was indeed found in four of the five samples. While not every dilution was found to be mutagenic, one or two of the concentrations for each of the four mutagenic samples had a statistically significantly higher number of revertants than the negative control, which provided a reading on the background mutation rate. Only Lake Calhoun, the most urban of the location tested, had no significantly mutagenic dilutions. Overall, these findings suggest that more research should be performed into the mutagenicity of Minnesota surface water.

First, a valuable extension is to continue to perform more fluctuation assays on samples from Lake Calhoun with lower concentrations than used here to test the possibility of cytotoxicity shadowing mutagenicity. In fact, more concentrations should be tested across all of these samples in order to fully flesh out dose-response curves, which can provide insight into the potency of a mutagen. The polarity of the solvent used for sample extraction should also be varied to ensure that all mutagens present in the sediment are present in the test dilution. As this study only used two polar solvents, only the presence of polar mutagens was informed by the evidence. Extracting only polar solutes can lower observed reversion rates due to less mutagenic material in each dilution. It can also lower revertant counts further by reducing the synergistic effects present in interactions between chemicals.[3]

Secondly, testing more locations along the Mississippi River is important to determining both possible sources of mutagenicity and to fully understanding the nature of the river's mutagenic sediment. This study only tested one location on the river, leaving the vast majority of the river's path in both the United States and Minnesota untested.

Thirdly, a greater number of test strains should be used, and a mammalian liver extract should be added to the reagent mix. More test strains would provide insight into different classes of mutagenicity, as some chemicals only cause certain types of mutations and each test strain is sensitive to different types of mutation. Moreover, TA100 has been found to be less sensitive than TA98, a widely used alternate test strain.[3,23] The use of a mammalian liver extract such as S9 would provide greater insight into human genotoxicity. As the body of an organism is complex, some innocuous chemicals can be metabolized into mutagens by the natural processes of life.[12] Liver extract contains many of the enzymes in mammalian metabolic pathways that can act to form mutagens.

Lastly, further genotoxin analysis of limnic sediment

in Minnesota must be performed. Now that the presence of mutagens in the sediment of these bodies of water is confirmed, it is now important to understand why these samples are mutagenic and how much of an effect on aquatic ecosystems and human life this can have. With the use of gas spectrometry or a chemical fractionation procedure, it is possible to find classes of chemicals and individual compounds responsible for sample mutagenicity.[3,24] Other tests for mutagenicity, such as the comet assay, which tests the effect of a sample on the human genome, can confirm these results with a different procedure.[25]

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REFERENCES

1. Anderson, P. & Martin, I. *Standard Operating Procedures: Lake Water Quality Sampling* Mar. 2015. https://www.pca.state.mn.us/sites/default/files/wq-s1-16.pdf (2017).
2. Team, S. I. L. *Stressors to Biological Communities in Minnesota's Rivers and Streams* Jan. 2017. https://www.pca.state.mn.us/sites/default/files/wq-ws1-27.pdf (2017).
3. Reifferscheid, G., Buchinger, S., Cao, Z. & Claus, E. Identification of mutagens in freshwater sediments by the Ames-fluctuation assay using nitroreductase and acetyltransferase overproducing test strains. *Environmental and Molecular Mutagenesis* **52,** 397–408. ISSN: 08936692 (June 2011).
4. Mohn, G. R. in *Genetic Origins of Tumor Cells* (eds Cleton, F. J. & Simons, J. W. I. M.) DOI: 10.1007/978-94-009-8823-1_2, 11–24 (Springer Netherlands, Dordrecht, 1980). ISBN: 978-94-009-8825-5 978-94-009-8823-1. http://www.springerlink.com/index/10.1007/978-94-009-8823-1_2 (2018).
5. Bolognesi, C. & Morasso, G. Genotoxicity of pesticides. *Trends in Food Science & Technology* **11,** 182–187. ISSN: 09242244 (Apr. 2000).
6. Wall, D. *et al. Nitrogen in Minnesota Surface Waters* (Minnesota Pollution Control Agency, 2013). https://www.pca.state.mn.us/sites/default/files/wq-s6-26a.pdf (2017).
7. *Pesticides in Surface Waters: Fact Sheet FS-039-97* Mar. 4, 2014. https://water.usgs.gov/nawqa/pnsp/pubs/fs97039/sw4.html (2017).
8. Houk, V. S. The genotoxicity of industrial wastes and effluents. *Mutation Research/Reviews in Genetic Toxicology* **277,** 91–138. ISSN: 01651110 (Aug. 1992).
9. Cerná, M. *et al.* Genotoxicity of industrial effluents, river waters, and their fractions using the Ames test and in vitro cytogenetic assay. *Toxicology Letters* **88,** 191–197. ISSN: 0378-4274 (Nov. 1996).
10. Garbarino, J. *et al.* Heavy Metals in the Mississippi River. *U.S. Geological Survey Circular*. https://pubs.usgs.gov/circ/circ1133/heavy-metals.html (2017) (1995).
11. Jolibois, B., Guerbet, M. & Vassal, S. Detection of hospital wastewater genotoxicity with the SOS chromotest and Ames fluctuation test. *Chemosphere* **51,** 539–543. ISSN: 00456535 (May 2003).
12. Pérez, S., Reifferscheid, G., Eichhorn, P. & Barceló, D. Assessment of the mutagenic potency of sewage sludges contaminated with polycyclic aromatic hydrocarbons by an Ames fluctuation assay. *Environmental Toxicology and Chemistry* **22,** 2576. ISSN: 0730-7268, 1552-8618 (2003).

13. Maltby, L., Boxall, A. B., Forrow, D. M., Calow, P. & Betton, C. I. The effects of motorway runoff on freshwater ecosystems: 2. Identifying major toxicants. *Environmental Toxicology and Chemistry* **14,** 1093–1101. ISSN: 07307268, 15528618 (June 1995).

14. Metcalfe, C., Balch, G., Cairns, V., Fitzsimons, J. & Dunn, B. Carcinogenic and genotoxic activity of extracts from contaminated sediments in Western Lake Ontario. *Science of The Total Environment* **94,** 125–141. ISSN: 00489697 (May 1990).

15. Hollert, H., Dürr, M., Erdinger, L. & Braunbeck, T. Cytotoxicity of settling particulate matter and sediments of the Neckar River (Germany) during a winter flood. *Environmental Toxicology and Chemistry* **19,** 528–534. ISSN: 07307268, 15528618 (Mar. 2000).

16. Keiter, S. *et al.* Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches (12 pp). *Environmental Science and Pollution Research - International* **13,** 308–319. ISSN: 0944-1344, 1614-7499 (Sept. 2006).

17. Balch, G., Metcalfe, C. & Huestis, S. Identification of potential fish carcinogens in sediment from Hamilton Harbour, Ontario, Canada. *Environmental Toxicology and Chemistry* **14,** 79–91. ISSN: 07307268, 15528618 (Jan. 1995).

18. Chen, G. & White, P. A. The mutagenic hazards of aquatic sediments: a review. *Mutation Research/Reviews in Mutation Research* **567,** 151–225. ISSN: 13835742 (Nov. 2004).

19. Lynch, M., Conery, J. & Burger, R. Mutational Meltdowns in Sexual Populations. *Evolution* **49,** 1067. ISSN: 00143820 (Dec. 1995).

20. Bickham, J. Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. *Mutation Research/Reviews in Mutation Research* **463,** 33–51. ISSN: 13835742 (June 2000).

21. Mortelmans, K. & Zeiger, E. The Ames Salmonella/microsome mutagenicity assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **455,** 29–60. ISSN: 00275107 (Nov. 2000).

22. Gilbert, R. The analysis of fluctuation tests. *Mutation Research/Environmental Mutagenesis and Related Subjects* **74,** 283–289. ISSN: 01651161 (Aug. 1980).

23. Kosmehl, T. *et al.* Comparative genotoxicity testing of rhine river sediment extracts using the comet assay with permanent fish cell lines (rtg-2 and rtl-w1) and the ames test*. *Journal of Soils and Sediments* **4,** 84. ISSN: 1614-7480 (June 1, 2004).

24. Samoiloff, M. R. *et al.* Combined bioassay-chemical fractionation scheme for the determination and ranking of toxic chemicals in sediments. *Environmental Science & Technology* **17,** 329–334. ISSN: 0013-936X, 1520-5851 (June 1983).

25. Ubomba-Jaswa, E., Fernández-Ibáñez, P. & McGuigan, K. G. A preliminary Ames fluctuation assay assessment of the genotoxicity of drinking water that has been solar disinfected in polyethylene terephthalate (PET) bottles. *Journal of Water and Health* **8,** 712. ISSN: 1477-8920 (Dec. 2010).