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Dr. Markus Brinkmann

Salish Sea Science Roundtable

Innovation in Ecotoxicology – Integrating Next-Generation Chemical and Biological Tools

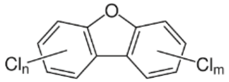
Overview

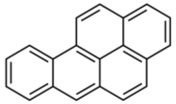
- **Status quo in environmental monitoring and risk assessment**
  – Are current approaches still relevant today?
- **Our changing chemical landscape**
  – How modern chemical and analytical tools help us advance environmental assessments
- **Case study 1**
  – Integrating chemical analysis and transcriptomics to assess contaminants in water, sediment, and fish
- **Case study 2**
  – Characterizing biotransformation products of recently discovered chemicals

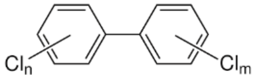
Chemical pollution is one of the greatest threats globally

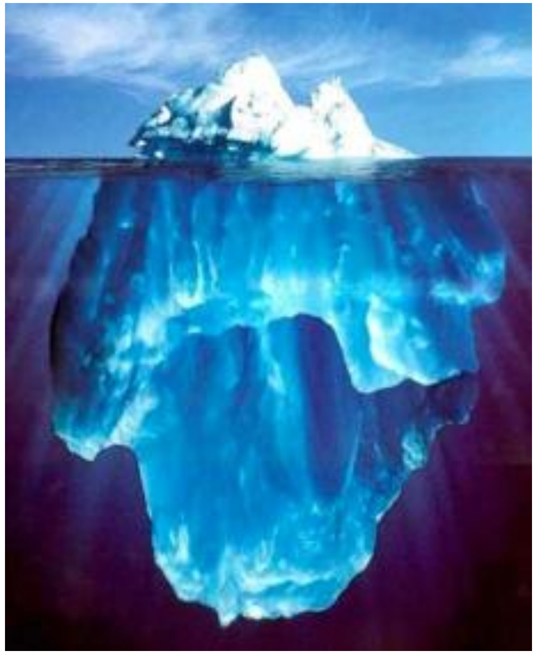
Status quo in environmental monitoring and risk assessment

- In addition to prospective risk assessment, retrospective monitoring efforts are designed to observe and assess the contamination state of and potential impacts on the environment
- These efforts are often based on priority lists of chemicals of concern in various environmental media, which are mandated to be monitored using chemical analysis in various jurisdictions
- Prominent examples include polycyclic aromatic hydrocarbons, dioxin-like chemicals, such as dioxins, furans, and polychlorinated biphenyls, as well as a variety of legacy pesticides









Our chemical society

- These strategies have been developed based on a relatively simple chemical space, and are based on chemical-by-chemical assessments
- We now use an ever-increasing number of chemicals in our everyday lives, from pharmaceuticals and personal care products to household and construction chemicals, plant protection products, and many more
- This leads to the presence of complex mixtures of emerging chemicals in our environment, the risks of which are often relatively poorly understood

Known chemical space versus available safety information

- The Chemical Abstract Service (CAS) database, a registry of known chemical substances lists >219,000,000 chemical substances (Dec 2024), with 15,000 added daily
- Safety information is available for only about 10,000 chemicals
- Priority lists cover a few dozen

Non-target methods

- This clearly shows the limitations of monitoring using targeted methods
- Traditional chemical analytical methods are designed to find the proverbial needle in the haystack
- However, to keep up with the pace of chemical development, we need methods that are suitable for characterizing the entire haystack
- Advancements in chemical analytical techniques, in particular soft ionization techniques and high-resolution mass spectrometry, have enabled novel approaches in non-target chemical analysis that promise just that

The benefits of non-target methods

Say we are attempting to learn more about this environment

Please don’t judge me for the firearm analogy!

The benefits of non-target methods

Conventional targeted methods are similar to a sniper rifle…

The benefits of non-target methods

…while non-target methods are more like a shotgun.

The benefits of non-target methods

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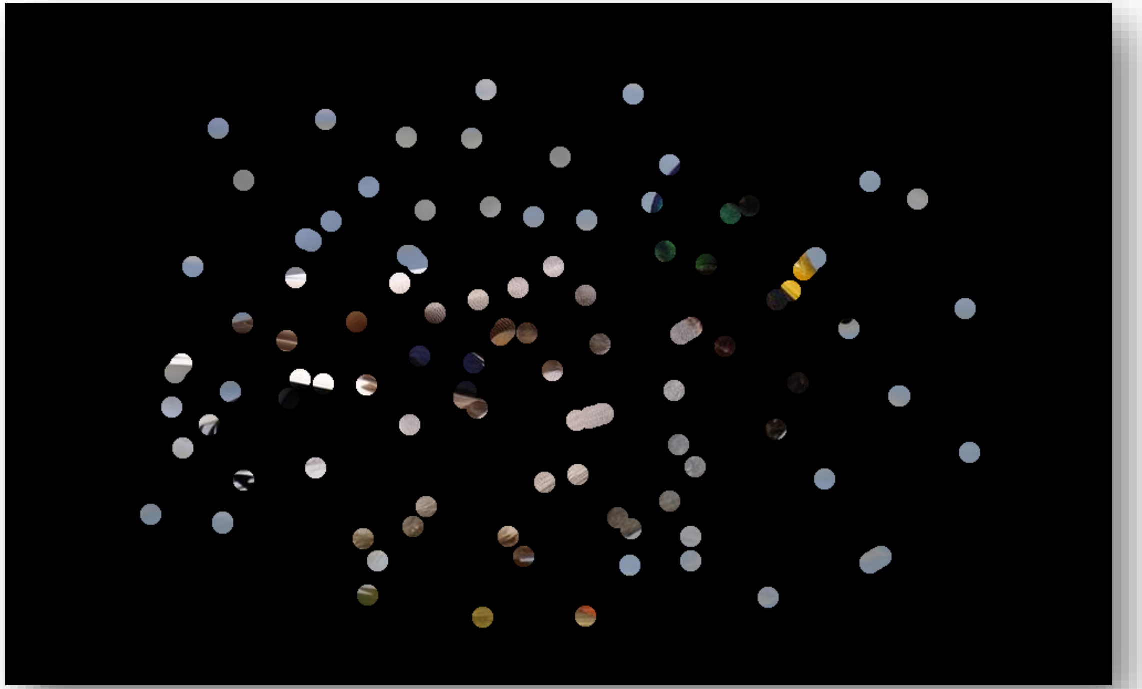
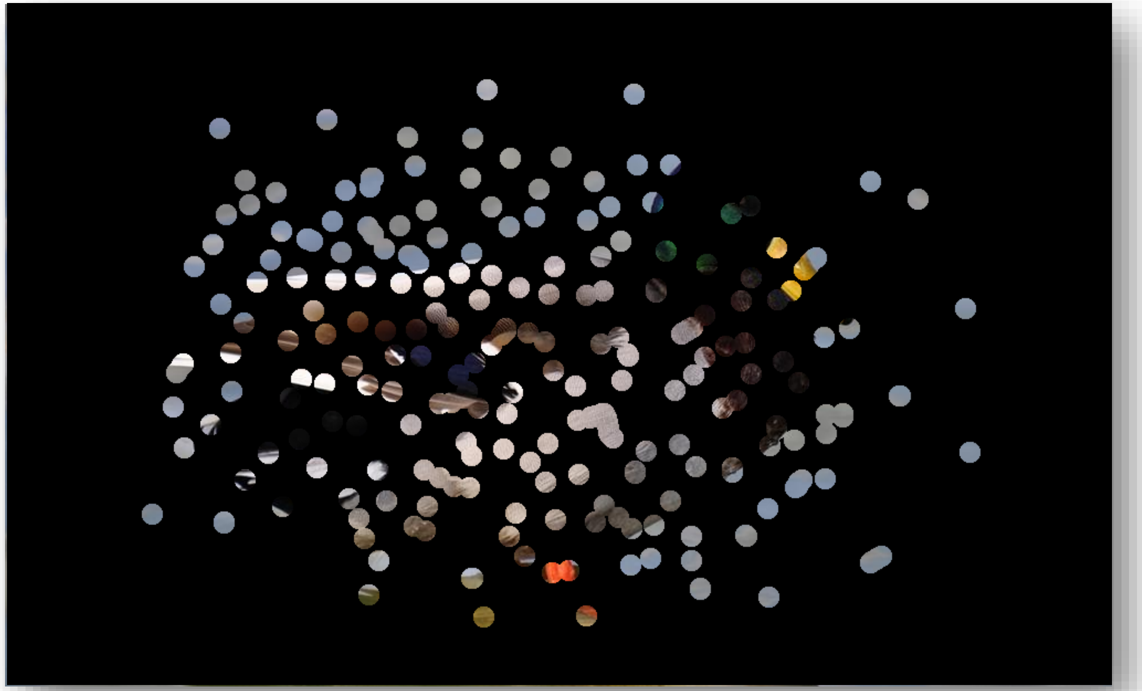


The benefits of non-target methods

…while non-target methods are more like a shotgun.



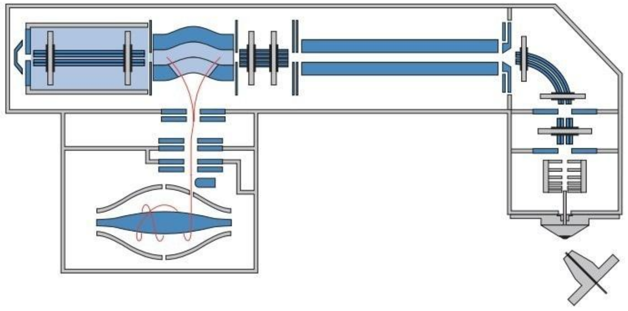
The benefits of non-target methods

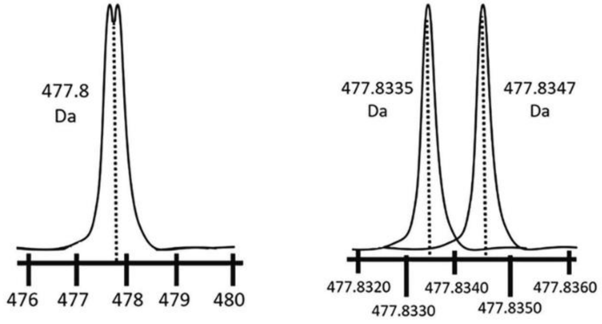
…while non-target methods are more like a shotgun.

High-resolution mass spectrometry (HRMS)

- High-resolution mass spectrometry (HRMS, such as Orbitrap-based systems) offers greater non-target capabilities due to its improved mass resolution
- The improved mass resolution enables the simultaneous detection of tens of thousands of chemicals in complex samples and has revolutionized environmental monitoring

Schematic of the
Thermo Scientific Q Exactive Benchtop LC-MS/MS



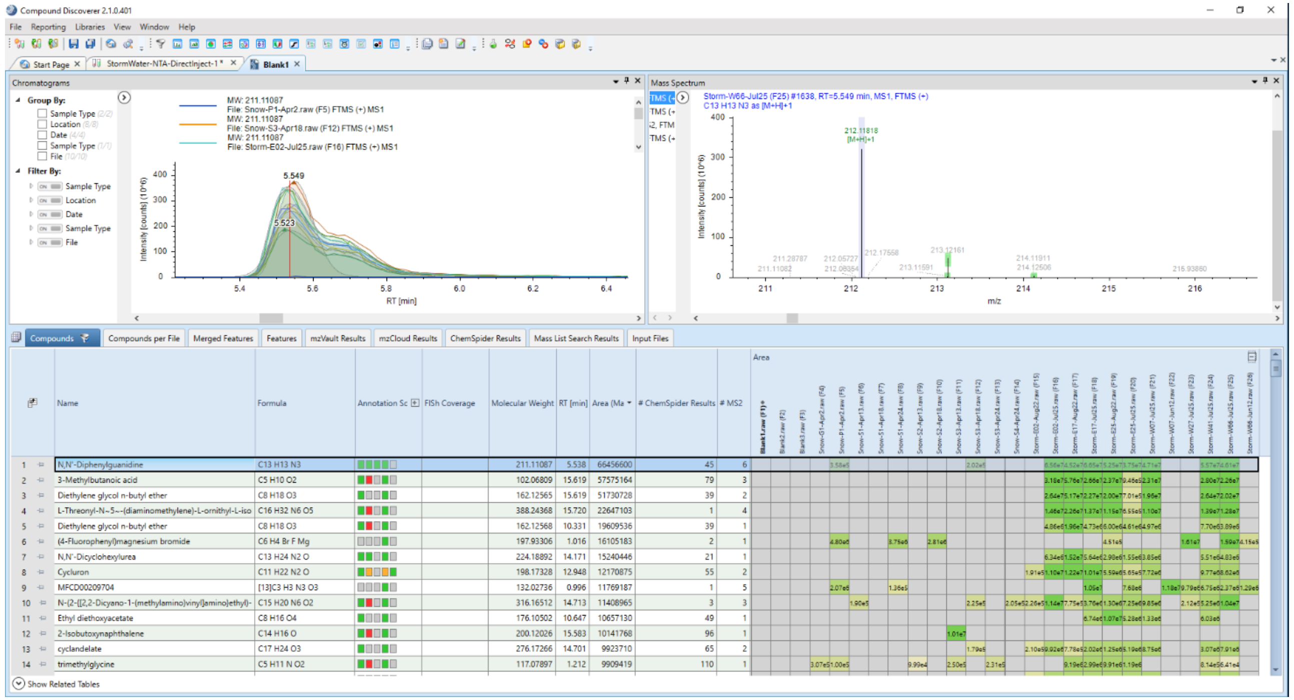


m/z Using Low-Resolution Mass Spectrometry

m/z Using High-Resolution Mass Spectrometry

High-resolution mass spectrometry (HRMS)

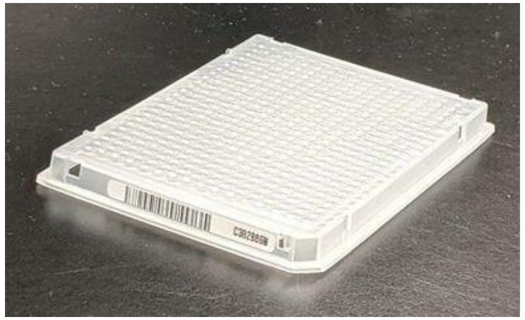
- Resulting data files are huge (several TB per experiment)
- Complex data processing and analysis needs arise from this complexity
- Commercial software is only able to support part of that process, and custom cheminformatics software code is often required, representing a qualification bottleneck

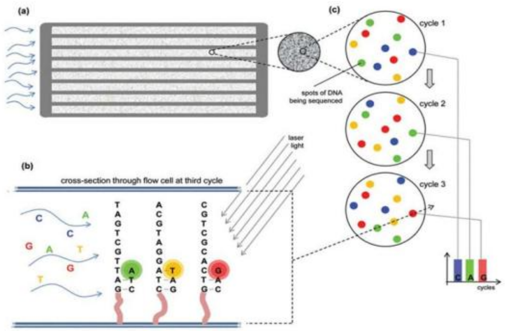


Molecular toxicology methods

- Similarly, advanced methods in molecular toxicology, such as next-generation sequencing and high-throughput techniques (e.g., qPCR arrays such as the EcoToxChip) have revolutionized biological impact assessments
- Similarly, datasets are large, and specialized skills are needed to analyze them

www.ecotoxchip.ca
info@ecotoxchip.ca





This raises many conceptual and practical questions

- What fraction of the chemical space can we detect?
- What fraction of the detectable chemical space is toxicologically relevant?
- If both chemical analytical and effect datasets become ever more complex, how do we bring them together?
- Can we use these methods, in combination or along, to inform prioritization efforts?

Case study 1 – Background

- MSc research of former student Ana Cardenas
- Studied Wascana Creek near Regina, Saskatchewan, Canada, which is a heavily polluted, wastewater-dominated stream
- Previously shown to contain high concentrations of endocrine disruptors
- Efficacy of recent upgrades of the wastewater treatment plant was unknown
- Information on other chemicals was unavailable

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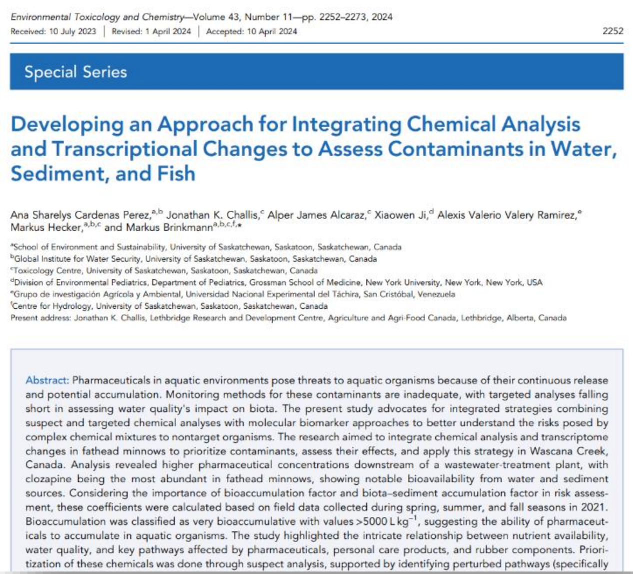
Case study 1 – Methods

- Water, sediment, and fish (fathead minnow) samples were collected in spring, summer, and fall of 2021, upstream and downstream of the Regina municipal wastewater treatment plant
- In addition to duplicate grab samples (water and sediment) and ten replicate fish, we also used six replicate passive samplers (diffusive gradients in thin films, DGTs) to measure time-weighted average concentrations of analytes
- Both targeted and non-target chemical analysis using a data-dependent MS2 non-target workflow
- Brain samples of fish (5-8 replicates) were collected for EcoToxChip analysis



Environmental Toxicology and Chemistry—Volume 43, Number 11—pp. 2252–2273, 2024
Received: 10 July 2023 | Revised: 1 April 2024 | Accepted: 10 April 2024 2252

Special Series

Developing an Approach for Integrating Chemical Analysis and Transcriptional Changes to Assess Contaminants in Water, Sediment, and Fish

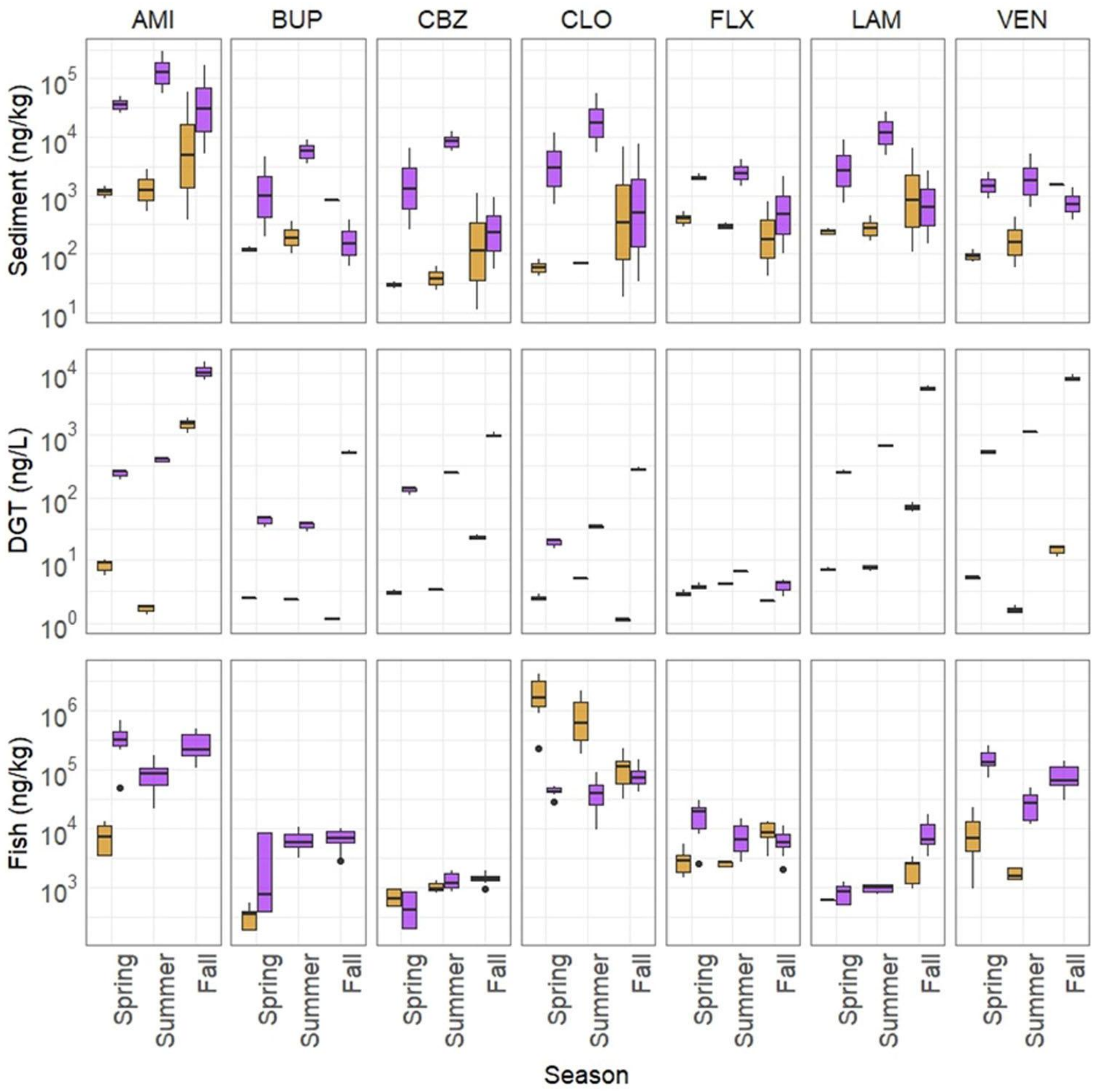
Ana Sharelys Cardenas Perez,[a,b] Jonathan K. Challis,[c] Alper James Alcaraz,[c] Xiaowen Ji,[d] Alexis Valerio Valery Ramirez,[e] Markus Hecker,[a,b,c] and Markus Brinkmann[a,b,c,f,*]

[a]School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[b]Global Institute for Water Security, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[c]Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[d]Division of Environmental Pediatrics, Department of Pediatrics, Grossman School of Medicine, New York University, New York, New York, USA
[e]Grupo de investigación Agrícola y Ambiental, Universidad Nacional Experimental del Táchira, San Cristóbal, Venezuela
[f]Centre for Hydrology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
Present address: Jonathan K. Challis, Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada

Abstract: Pharmaceuticals in aquatic environments pose threats to aquatic organisms because of their continuous release and potential accumulation. Monitoring methods for these contaminants are inadequate, with targeted analyses falling short in assessing water quality's impact on biota. The present study advocates for integrated strategies combining suspect and targeted chemical analyses with molecular biomarker approaches to better understand the risks posed by complex chemical mixtures to nontarget organisms. The research aimed to integrate chemical analysis and transcriptome changes in fathead minnows to prioritize contaminants, assess their effects, and apply this strategy in Wascana Creek, Canada. Analysis revealed higher pharmaceutical concentrations downstream of a wastewater-treatment plant, with clozapine being the most abundant in fathead minnows, showing notable bioavailability from water and sediment sources. Considering the importance of bioaccumulation factor and biota–sediment accumulation factor in risk assessment, these coefficients were calculated based on field data collected during spring, summer, and fall seasons in 2021. Bioaccumulation was classified as very bioaccumulative with values >5000 L kg^{-1}, suggesting the ability of pharmaceuticals to accumulate in aquatic organisms. The study highlighted the intricate relationship between nutrient availability, water quality, and key pathways affected by pharmaceuticals, personal care products, and rubber components. Prioritization of these chemicals was done through suspect analysis, supported by identifying perturbed pathways (specifically

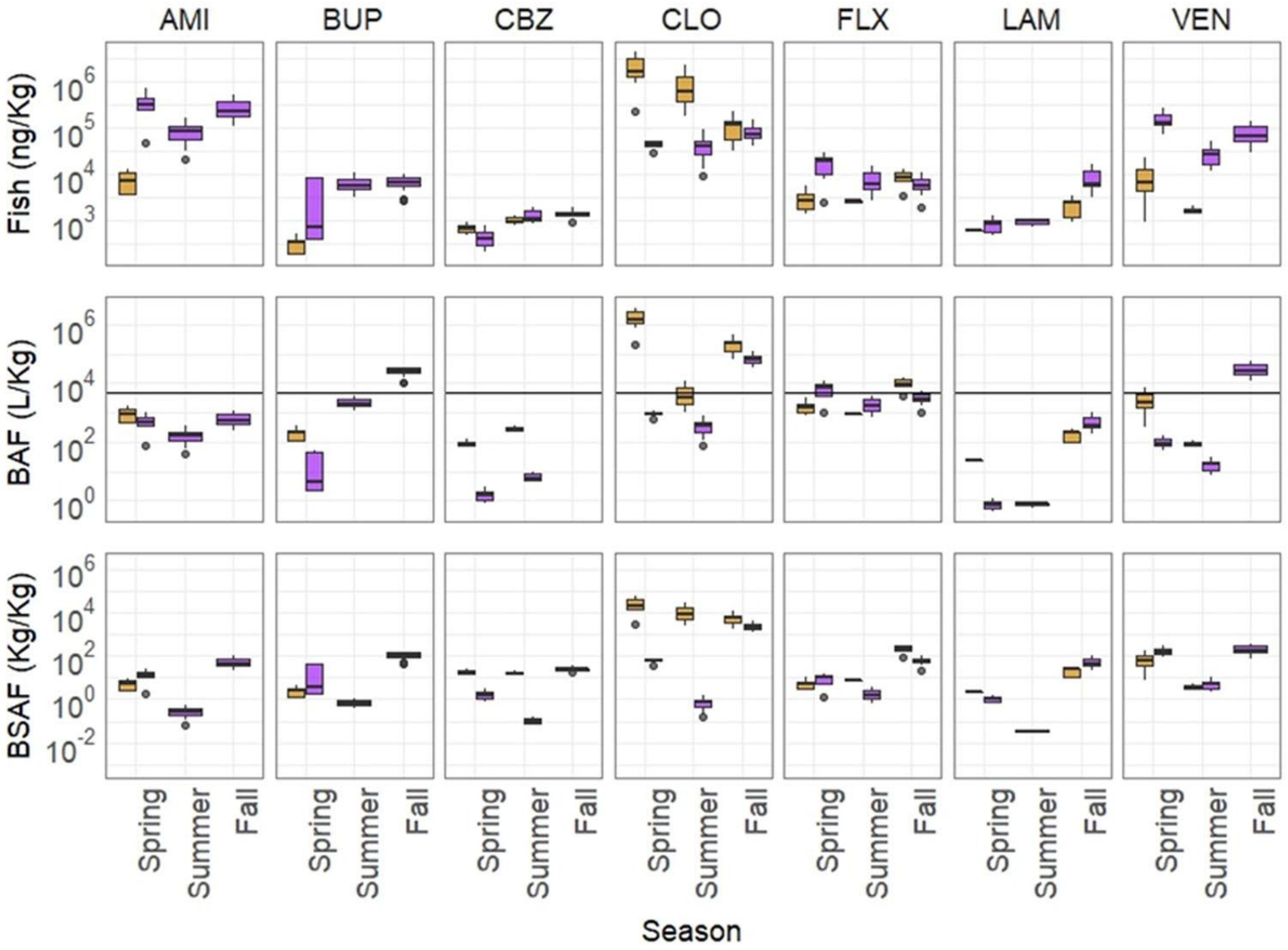
Case study 1 – Targeted analysis

- Upstream, downstream
- Eight targeted analytes, all of which were neuroactive drugs
- AMI = amitriptyline
  BUP = bupropion
  CBZ = carbamazepine
  CLO = clozapine
  FLX = fluoxetine
  LAM = lamotrigine
  VEN = venlafaxine

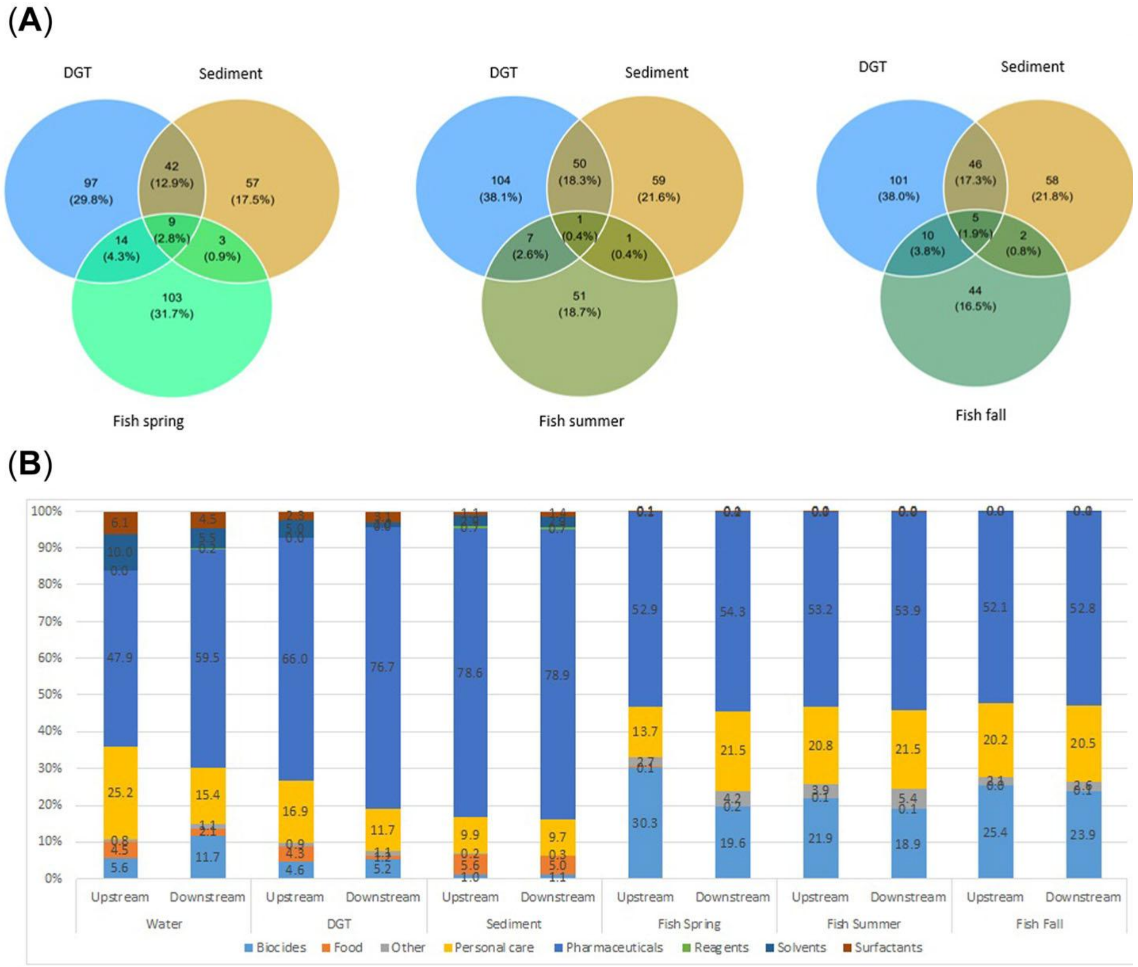


Case study 1 – Targeted analysis

- Upstream, downstream
- Field bioaccumulation factors (BAFs) for some chemicals (BUP, CLO, FLX, and VEN) exceeded 5,000 L/kg in some instances, indicating potential for bioaccumulation

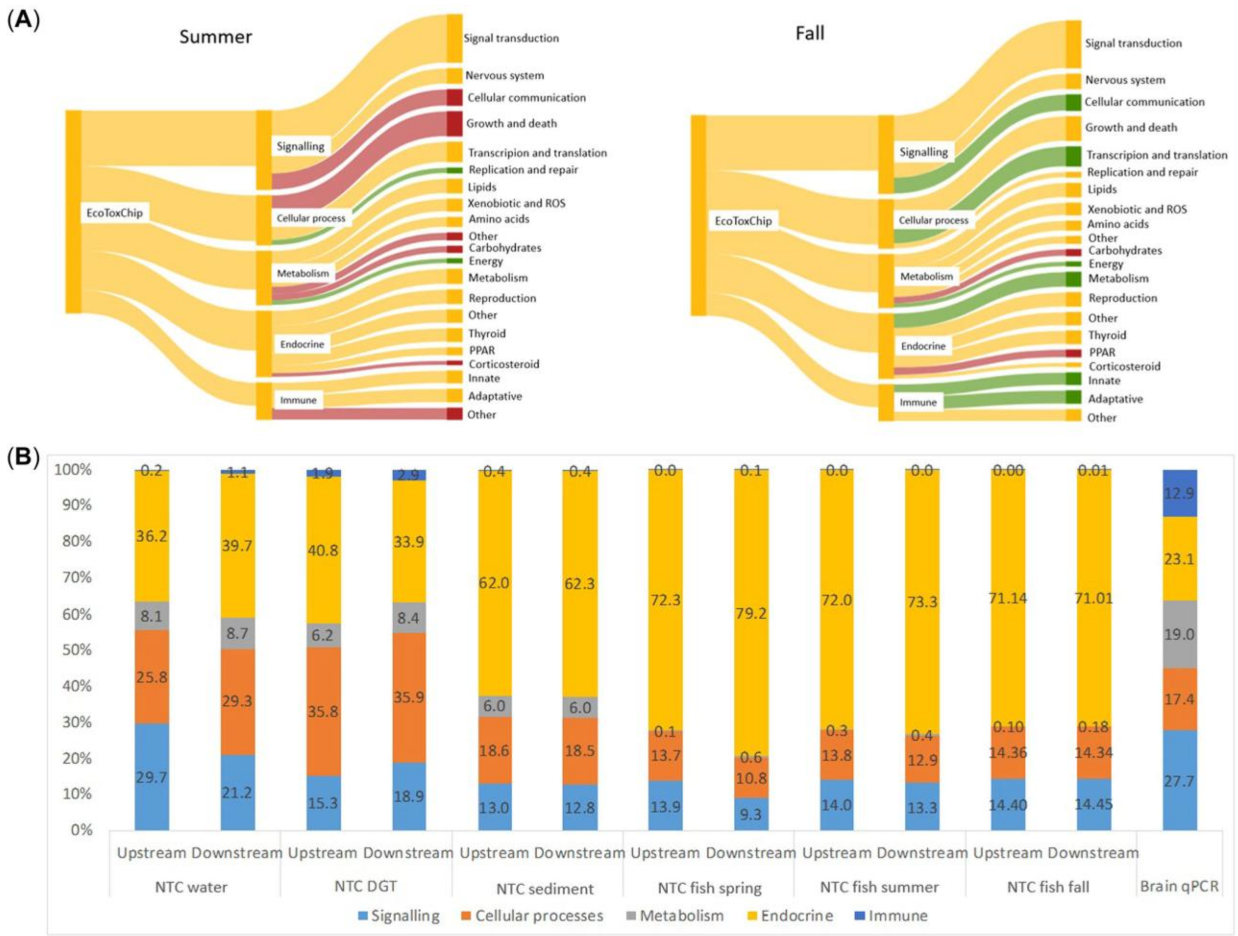


Case study 1 – Non-target analysis



- Following extensive filtering and QA/QC in Thermo Compound Discoverer software, non-target analysis revealed several hundred distinct chemicals in DGT extracts, sediments, and fish
- Only a small fraction of these were present in all environmental matrices
- Chemical use classes were derived using the CompTox Chemicals Dashboard
- Across all matrices and sites, pharmaceuticals dominated the chemical use class (48 to 79%), followed by personal care products (10 to 25%)

Case study 1 – EcoToxChip analysis



- Sankey plots indicate which biological processes and functions were perturbed (yellow = log FC >1.5, red = log FC >2.0 when comparing upstream vs. downstream samples) in fathead minnows from Wascana Creek in summer and fall (A)
- Screening the list of chemicals identified through non-target analysis for their toxicity profiles (CompTox Chemicals Dashboard) revealed a remarkable similarity between EcoToxChip results and their predicted effects profile (B), indicating promising congruency and possibly predictivity

Case study 1 – Prioritization

Based on both presence in the various sample matrices and co-occurrence with biological perturbation of molecular processes in fathead minnows, ten chemical compounds have been identified as potential drivers of biological impacts:

TABLE 3: Chemical compounds prioritized from relationships between time and sampling matrices including water (conventional grab and DGT), sediments, and fish samples using Venn diagrams

Name	CAS No.	Chemical use	Water–Sediment–Fish	DGT–Sediment–Fish
2-Propyl-[1]H-benzimidazole	5465-29-2	Pharmaceutical (various)	C1	C1d
3,5-Dimethyl-1-phenylpyrazole	1131-16-4	Pharmaceutical (anticancer)	C2	C2d
5,6-Dimethylbenzimidazole	582-60-5	Pharmaceutical (component of vitamin B_{12})	C3	C3d
Choline	62-49-7	Natural nutrient	C4	C4d
Clozapine	5786-21-0	Pharmaceutical (antipsychotic)	C5	C5d
Desmethylcitalopram	62498-67-3	Pharmaceutical (antidepressant)	C6	C6d
Galaxolidone	507442-49-1	PPCPs (various)	C7	C7d
Lidocaine	137-58-6	Pharmaceutical (anesthetic)	C8	C8d
N,N′-Diphenylguanidine	20277-92-3	Rubber industry (accelerator)	C9	C9d
Propranolol	525-66-6	Pharmaceutical (hypertension)	C10	

CAS = Chemical Abstracts Service; DGT = diffusive gradients in thin films; PPCPs = pharmaceuticals and personal care products.

Case study 1 – Summary

- Non-target chemical analysis of water, sediment, and fish (fathead minnow) from Wascana Creek facilitated the prioritization of 10 chemicals for further study that are suspected to drive biological effects in the system
- EcoToxChip analysis and predicted toxicity profiles showed remarkable similarities
- While the study was limited in terms of scope, the approaches and methods described here have great potential for solving some pressing environmental issues in other ecosystems and watersheds

Case study 2 – Background

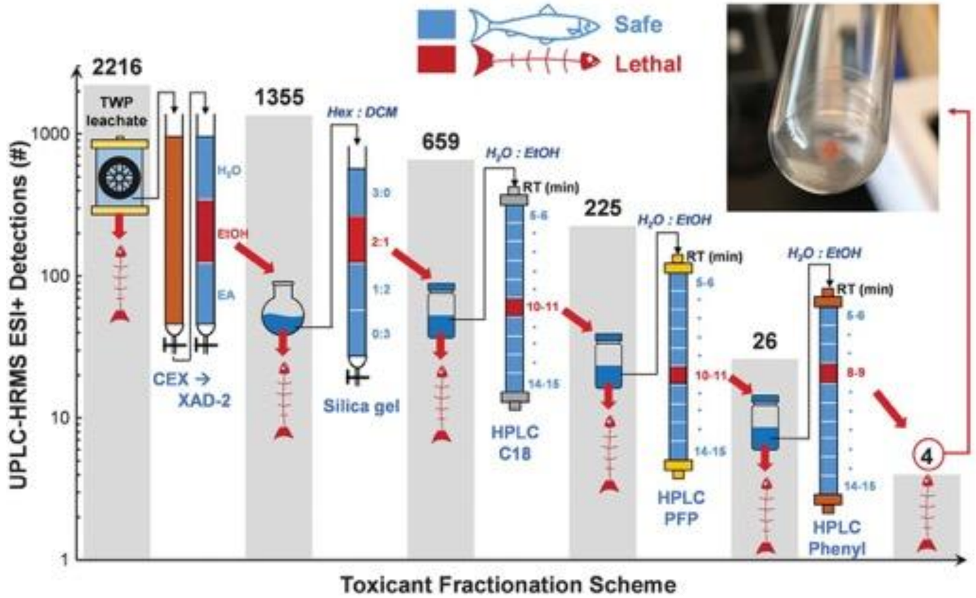
- Since the 1990s, coho salmon in the Pacific Northwest began gasping and spiralling immediately following runoff events in urban streams, leading to death
- These mass die-offs have been dubbed urban runoff mortality syndrome (URMS)

Photo by University of Washington, USA

Photo by Tiffany Linbo, NOAA Fisheries, USA

Case study 2 – Background

In 2021, N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-Q) has been identified as chemical responsible for URMS



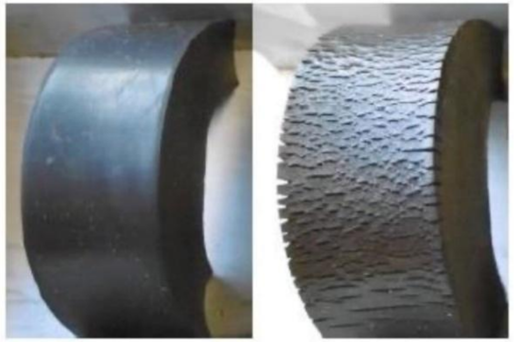
RESEARCH

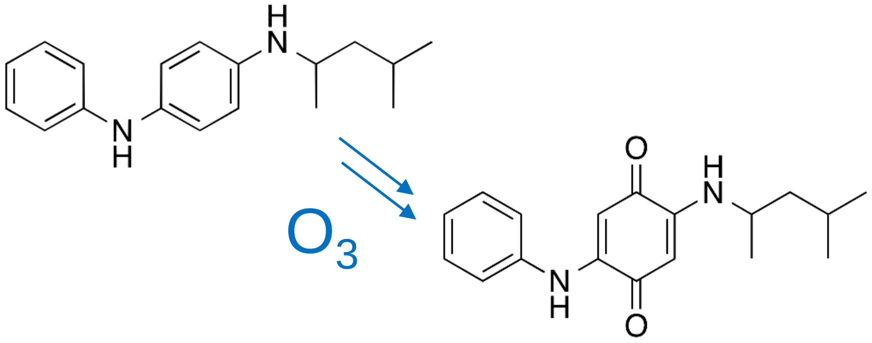
ECOTOXICOLOGY

A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon

Zhenyu Tian[1,2], Haoqi Zhao[3], Katherine T. Peter[1,2], Melissa Gonzalez[1,2], Jill Wetzel[4], Christopher Wu[1,2],

With 6PPD Without 6PPD





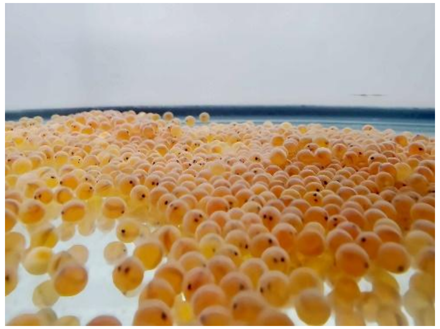
6PPD-Q is formed in the environment from the tire antidegradant 6PPD

Case study 2 – Background

- Coho salmon are exceptionally sensitive to 6PPD-Q (LC_{50} = 95 ng/L) (Tian et al. 2022)
- This places 6PPD-Q among the most toxic environmental chemicals known to date
- Except for coho salmon, other fishes, as well as invertebrates, tested at the time were insensitive
- Environmental concentrations of 6PPD-Q in stormwater frequently exceed the toxicity threshold for coho salmon after runoff events
- Need to understand how widespread sensitivity to 6PPD-Q is across fishes and what drives potential differences

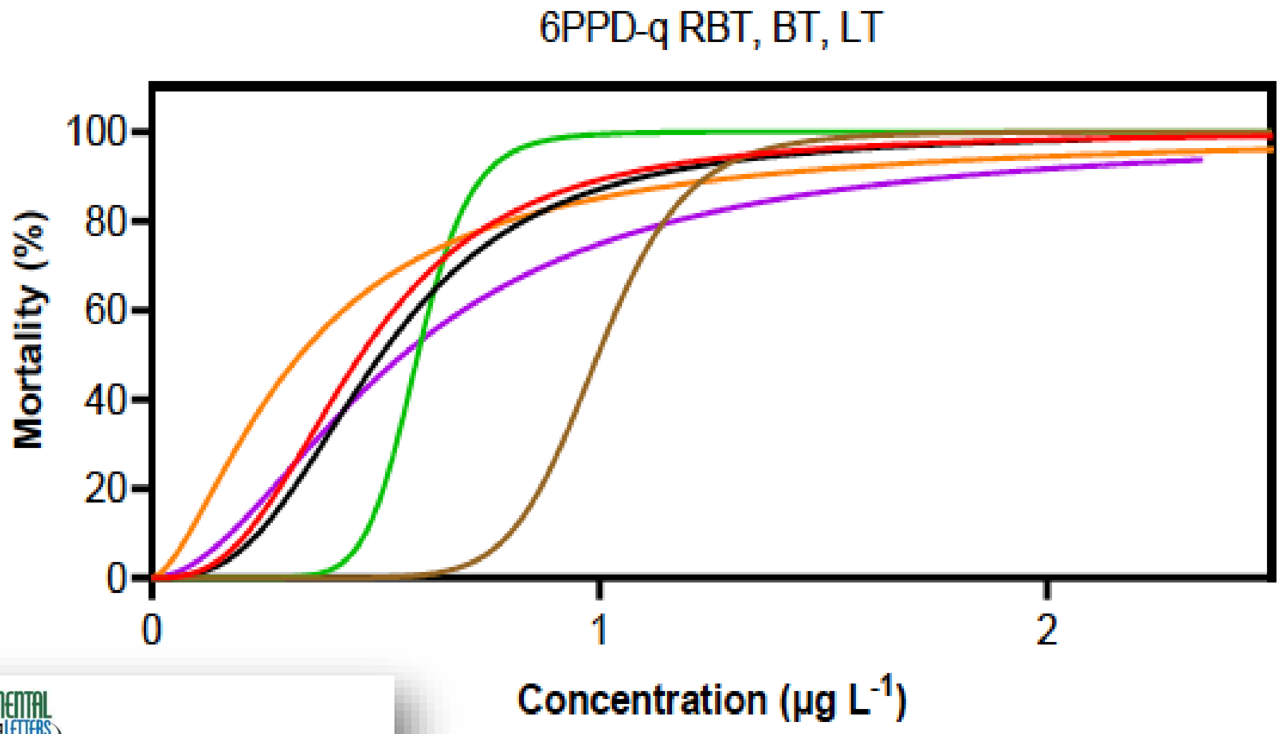
Case study 2 – Acute lethality

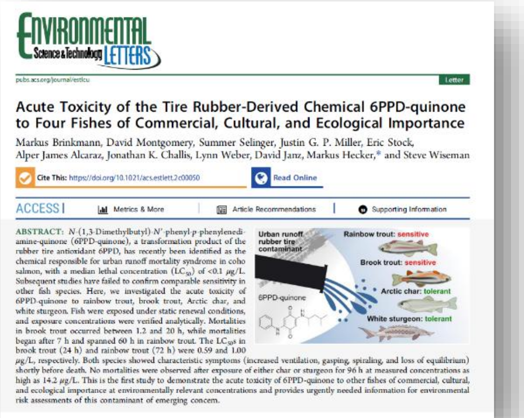
- We conducted laboratory experiments to assess the acute and sub-chronic toxicity of 6PPD-Q to various fishes of commercial, cultural, and ecological importance
- 12 to 96-hour acute toxicity tests with sub-adult and juvenile life stages
- 28- and 45-day sub-chronic tests with rainbow trout and lake trout
- Static renewal; ~70% water change every 24 hours
- Exposure concentrations were confirmed with LC-HRMS



Case study 2 – Acute lethality

- No mortality occurred for Arctic char, brown trout, bull trout, westslope cutthroat trout, or white sturgeon even at unrealistically high concentrations (>13 µg/L)
- Brook (BT), rainbow (RBT), and lake (LT) trout showed significant mortality with LC50 values between 0.33 – 1.0 µg/L (on the right)




Environmental Science & Technology Letters

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Acute Toxicity of the Tire Rubber-Derived Chemical 6PPD-quinone to Four Fishes of Commercial, Cultural, and Ecological Importance

Markus Brinkmann, David Montgomery, Summer Selinger, Justin G. P. Miller, Eric Stock, Alper James Alcaraz, Jonathan K. Challis, Lynn Weber, David Janz, Markus Hecker,* and Steve Wiseman

Cite This: https://doi.org/10.1021/acs.estlett.2c00050 Read Online

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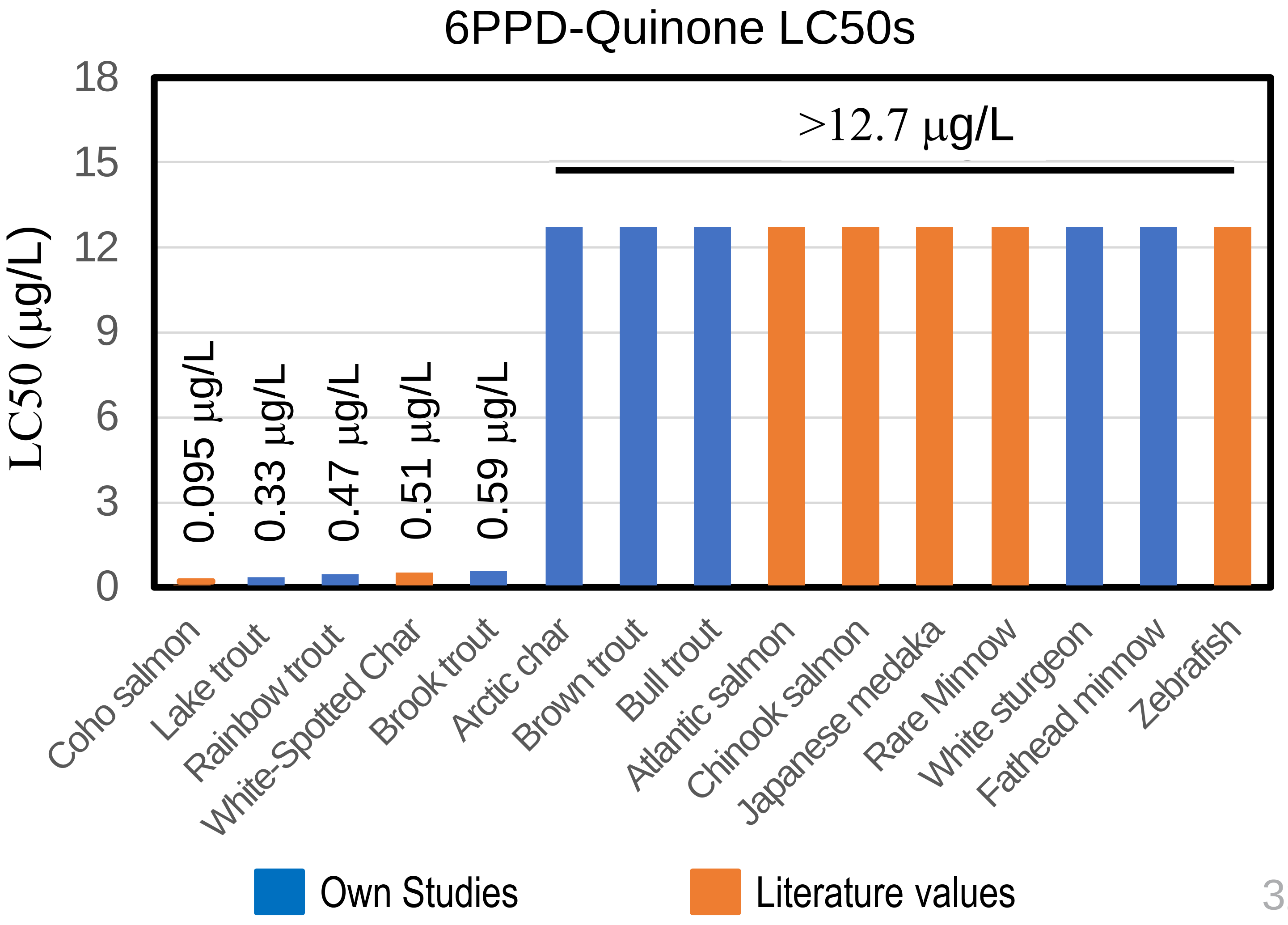
ABSTRACT: N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-quinone), a transformation product of the rubber tire antioxidant 6PPD, has recently been identified as the chemical responsible for urban runoff mortality syndrome in coho salmon, with a median lethal concentration (LC_{50}) of <0.1 µg/L. Subsequent studies have failed to confirm comparable sensitivity in other fish species. Here, we investigated the acute toxicity of 6PPD-quinone to rainbow trout, brook trout, Arctic char, and white sturgeon. Fish were exposed under static renewal conditions, and exposure concentrations were verified analytically. Mortalities in brook trout occurred between 1.2 and 20 h, while mortalities began after 7 h and spanned 60 h in rainbow trout. The LC_{50}s in brook trout (24 h) and rainbow trout (72 h) were 0.59 and 1.00 µg/L, respectively. Both species showed characteristic symptoms (increased ventilation, gasping, spiraling, and loss of equilibrium) shortly before death. No mortalities were observed after exposure of either char or sturgeon for 96 h at measured concentrations as high as 14.2 µg/L. This is the first study to demonstrate the acute toxicity of 6PPD-quinone to other fishes of commercial, cultural, and ecological importance at environmentally relevant concentrations and provides urgently needed information for environmental risk assessments of this contaminant of emerging concern.

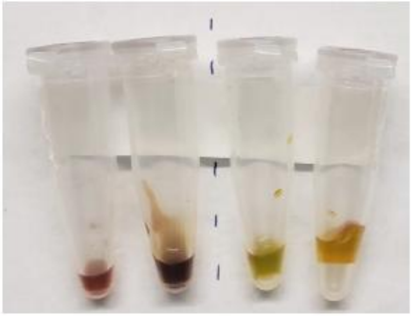
Case study 2 – Acute lethality

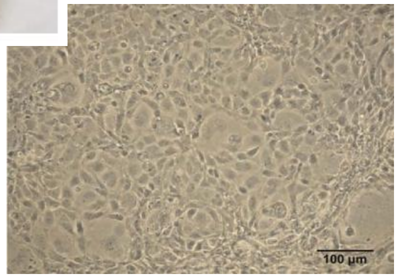
- Significant differences in sensitivity among fishes
- Either very sensitive or completely insensitive at environmental levels
- To date, only salmonids are shown to be sensitive to 6PPD-Q exposure
- Otherwise, phylogeny is a poor predictor of sensitivity
- How can we explain these differences?



Case study 2 – Insights into biotransformation

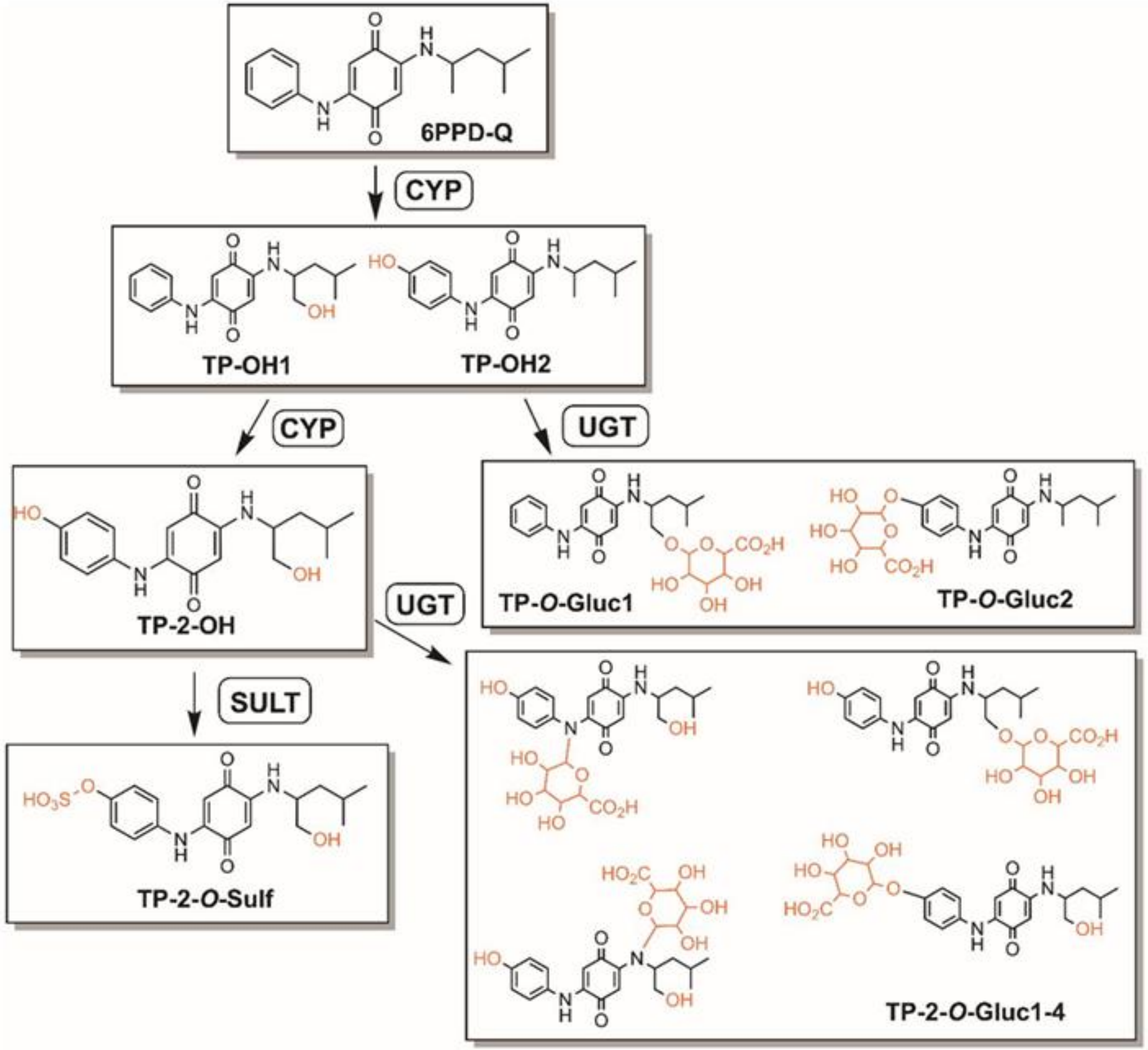
- We suspected differences in the ability of species to biotransform 6PPD-Q as a potential driver of species differences in sensitivity – i.e., differences in detoxification or bioactivation
- We collected gall bladder bile and whole-body samples of sub-adults and juveniles, respectively
- We also conducted incubations of rainbow trout cell lines and hepatocytes with 6PPD-Q to identify the responsible enzymes
- Samples were diluted and analyzed using a data-dependent MS2 non-target workflow to screen for transformation products using LC-Orbitrap HRMS





Case study 2 – Tentative biotransformation pathway

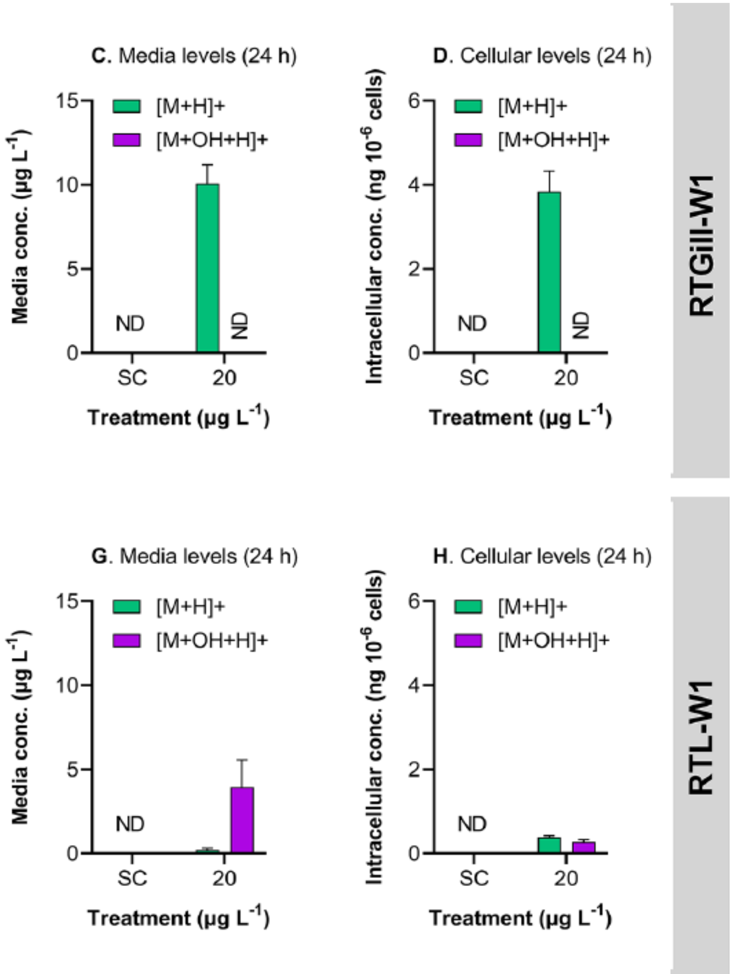
- We detected and quantified a variety of singly and double-hydroxylated metabolites and confirmed their identity with custom standards (Cayman)
- We also detected the associated glucuronides and sulfates (no standards)
- TP-OH2 was dominant and present in all species
- TP-OH1 was present in lake and rainbow trout, but not brown trout



From: Ankley et al. (2024) *https://doi.org/10.1101/2024.06.12.598661*

Case study 2 – Difference across cell lines

- In gill cells, only the parent compound was found in the media and cells
- The predominant compound(s) in the media of liver cells were mono-hydroxylated metabolites
- This was accompanied by an increased oxygen consumption rate (indicative of interferences with mitochondrial respiration) in gill cells, but not in liver cells



Environmental Science & Technology Letters

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Exposure to the Tire Rubber-Derived Contaminant 6PPD-Quinone Causes Mitochondrial Dysfunction *In Vitro*

Hannah Mahoney, Francisco C. da Silva Junior, Catherine Roberts, Matthew Schultz, Xiaowen Ji, Alper James Alcaraz, David Montgomery, Summer Selinger, Jonathan K. Challis, John P. Giesy, Lynn Weber, David Janz, Steve Wiseman, Markus Hecker, and Markus Brinkmann*

Cite This: https://doi.org/10.1021/acs.estlett.2c00431 Read Online

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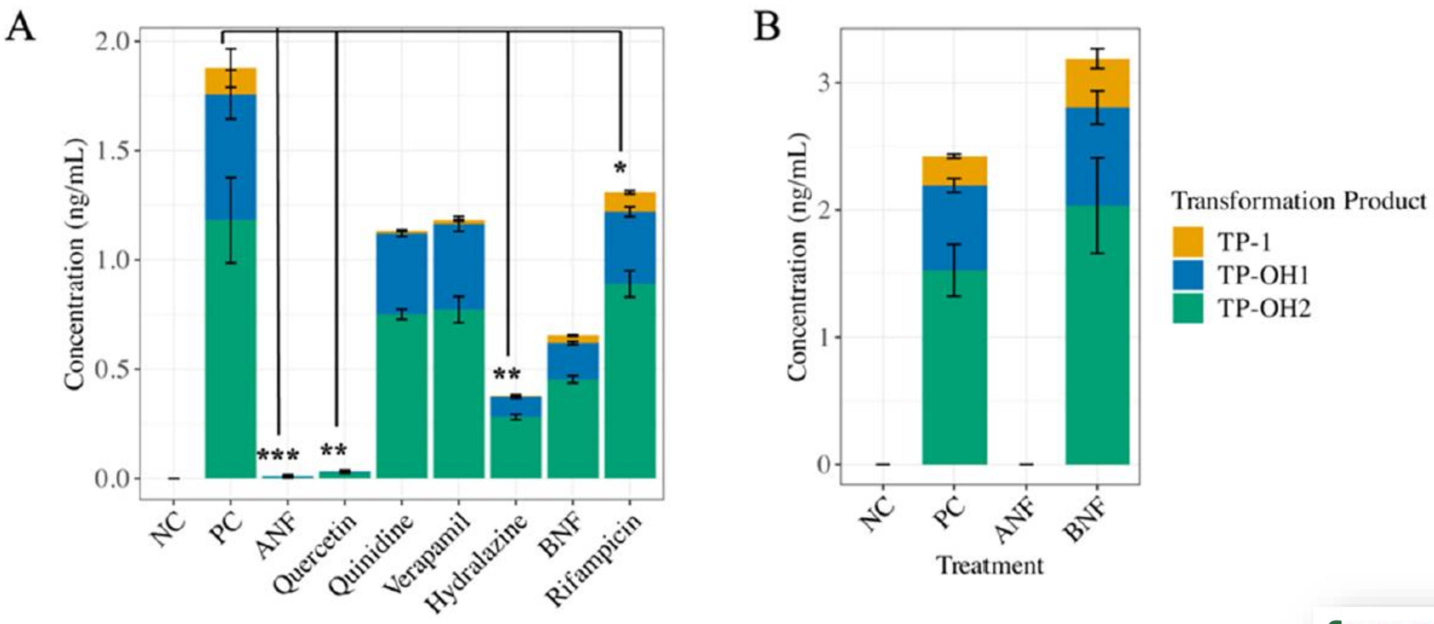
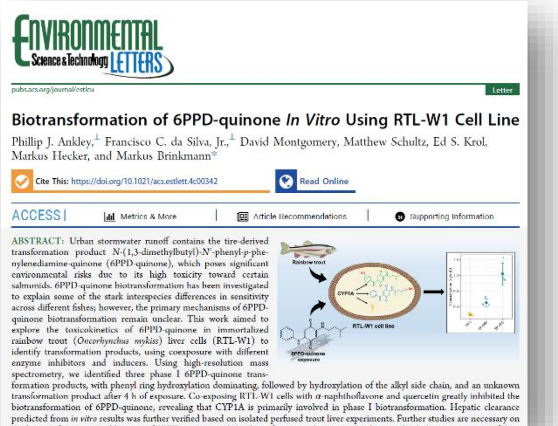
ABSTRACT: N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-quinone), a rubber tire oxidation product found in road runoff, is highly and acutely toxic to selected salmonids including coho salmon, brook trout, and rainbow trout but not other fish species and invertebrates studied to date. Sensitive species displayed increased ventilation and gasping, suggesting a possible impact on respiration. Here, adherent cell lines RTL-W1 and RTgill-W1 were exposed to 5–80 µg/L 6PPD-quinone, and cytotoxicity, oxygen consumption rate (OCR), and biotransformation of 6PPD-quinone were measured to assess the ability of 6PPD-quinone to uncouple mitochondrial respiration *in vitro*. RTL-W1 cells were not sensitive to 6PPD-quinone, and exposure did not result in significant impacts on cytotoxicity or OCR. In contrast, RTgill-W1 cells demonstrated decreased cell viability at 80 µg/L and a 2-fold increase in OCR at 20 µg/L. Effects appear to be partly driven by toxicokinetic differences where incubation of RTL-W1 cells with 6PPD-quinone led to almost quantitative conversion of 6PPD-quinone into a suspected hydroxy-metabolite, which was not observed in RTgill-W1 cells. Exposure studies with primary cultures of rainbow trout gill cells indicated that 6PPD-quinone increased OCR by uncoupling the mitochondrial electron transport chain. Together, these findings

Case study 2 – Inhibition studies in liver cell lines



- Through co-exposure (A) and pre-exposure (B) experiments with inhibitors, we could demonstrate that CYP1A appears to be responsible for catalyzing these hydroxylation reactions


Environmental Science & Technology LETTERS

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Biotransformation of 6PPD-quinone *In Vitro* Using RTL-W1 Cell Line

Phillip J. Ankley,[⊥] Francisco C. da Silva, Jr.,[⊥] David Montgomery, Matthew Schultz, Ed S. Krol, Markus Hecker, and Markus Brinkmann*

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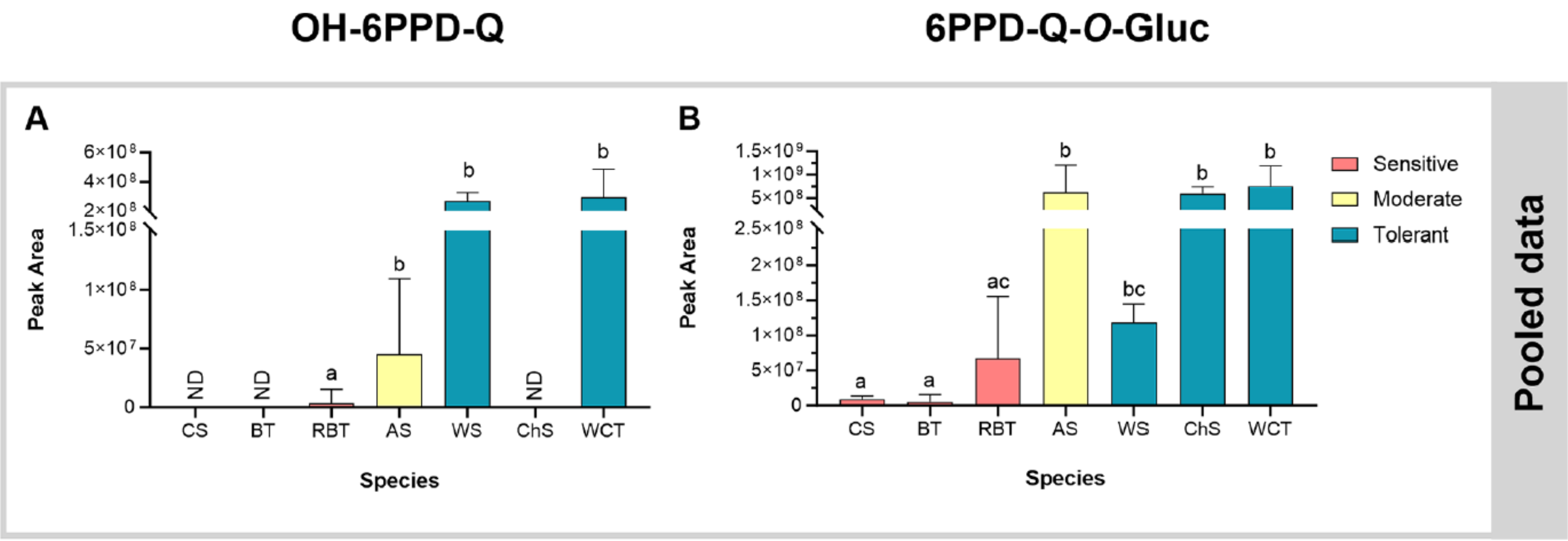
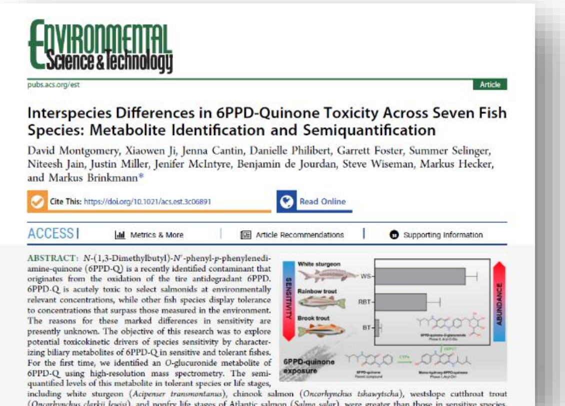
ABSTRACT: Urban stormwater runoff contains the tire-derived transformation product N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-quinone), which poses significant environmental risks due to its high toxicity toward certain salmonids. 6PPD-quinone biotransformation has been investigated to explain some of the stark interspecies differences in sensitivity across different fishes; however, the primary mechanisms of 6PPD-quinone biotransformation remain unclear. This work aimed to explore the toxicokinetics of 6PPD-quinone in immortalized rainbow trout (*Oncorhynchus mykiss*) liver cells (RTL-W1) to identify transformation products, using coexposure with different enzyme inhibitors and inducers. Using high-resolution mass spectrometry, we identified three phase I 6PPD-quinone transformation products, with phenyl ring hydroxylation dominating, followed by hydroxylation of the alkyl side chain, and an unknown transformation product after 4 h of exposure. Co-exposing RTL-W1 cells with α-naphthoflavone and quercetin greatly inhibited the biotransformation of 6PPD-quinone, revealing that CYP1A is primarily involved in phase I biotransformation. Hepatic clearance predicted from *in vitro* results was further verified based on isolated perfused trout liver experiments. Further studies are necessary on

Case study 2 – Differences across fish species



- In fish bile, we found OH-6PPD-Q and its glucuronide
- Their overall abundance increased with decreasing sensitivity
- This may suggest that sensitive species cannot detoxify 6PPD-Q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Interspecies Differences in 6PPD-Quinone Toxicity Across Seven Fish Species: Metabolite Identification and Semiquantification

David Montgomery, Xiaowen Ji, Jenna Cantin, Danielle Philibert, Garrett Foster, Summer Selinger, Niteesh Jain, Justin Miller, Jenifer McIntyre, Benjamin de Jourdan, Steve Wiseman, Markus Hecker, and Markus Brinkmann*

Cite This: https://doi.org/10.1021/acs.est.3c06891
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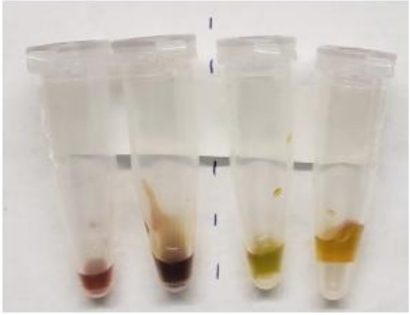
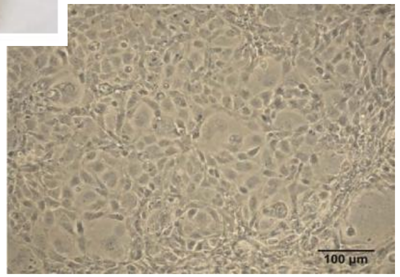
ABSTRACT: N-(1,3-Dimethylbutyl)-N′-phenyl-p-phenylenediamine-quinone (6PPD-Q) is a recently identified contaminant that originates from the oxidation of the tire antidegradant 6PPD. 6PPD-Q is acutely toxic to select salmonids at environmentally relevant concentrations, while other fish species display tolerance to concentrations that surpass those measured in the environment. The reasons for these marked differences in sensitivity are presently unknown. The objective of this research was to explore potential toxicokinetic drivers of species sensitivity by characterizing biliary metabolites of 6PPD-Q in sensitive and tolerant fishes. For the first time, we identified an O-glucuronide metabolite of 6PPD-Q using high-resolution mass spectrometry. The semi-quantified levels of this metabolite in tolerant species or life stages, including white sturgeon (*Acipenser transmontanus*), chinook salmon (*Oncorhynchus tshawytscha*), westslope cutthroat trout

Case study 2 – Conclusion

- Differences in the ability of various species of fishes to biotransform 6PPD-Q are likely contributing to species differences in sensitivity
- The jury is still out on whether these are differences in detoxification or bioactivation
- We are in the process of conducting acute and sub-chronic toxicity tests, exposing early-life stage and juvenile rainbow trout to both 6PPD-Q, as well as TP-OH1 and TP-OH2
- The results of these experiments should answer the remaining open questions related to the detoxification versus bioactivation question





Overall take-home messages

- Novel non-target chemical analytical methods based on high-resolution mass spectrometry (HRMS) have the potential to revolutionize environmental monitoring and laboratory-based assessments
- In combination with non-biased molecular tools, such as transcriptomics, HRMS methods can provide deep mechanistic insights for causal analysis and prioritization
- These findings can guide decision makers and risk assessors in developing refined monitoring efforts and environmental assessments
- Presently, these methods are subject to ongoing research and development, and more work is needed before they can be broadly applied in monitoring and risk assessment

Thank you for your attention!

Dr. Markus Brinkmann
University of Saskatchewan
Saskatoon, SK S7N 5B3
Tel: (306) 966 – 1204
markus.brinkmann@usask.ca