Non-targeted investigation of benthic invertebrates exposed to wastewater treatment plant effluents using NanoLC-HRMS

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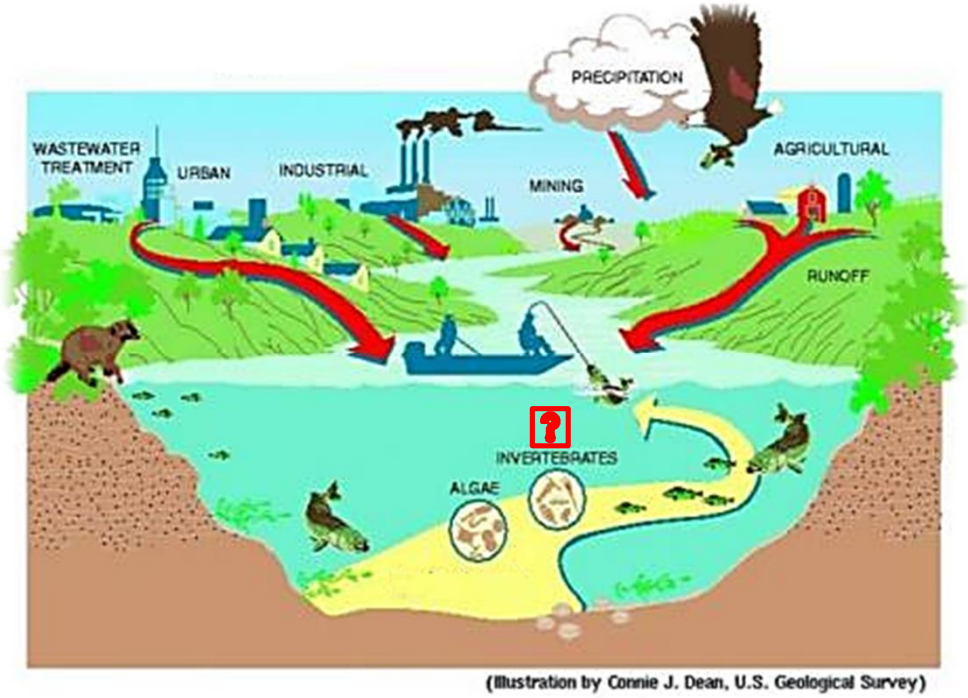
INTRODUCTION

- Higher consumption of chemical products in everyday life

  ↳ Pollution of aquatic ecosystems



➡ Non-targeted approach, a valuable tool to assess the impact of anthropic pressures on benthic invertebrates?

OBJECTIVES

- Based on a non-targeted fingerprinting (metabolomics) strategy:

  - Assess the impact of WWTP effluents on the metabolome of 3 benthic invertebrates
  - Evaluate the influence of exposure conditions and seasonal variations
  - Highlight the inter-species diversity

The development of analytical tools for holistic analysis of biota matrices are required

MATERIELS & METHODS

Mollusc

Potamopyrgus antipodarum

Crustacean

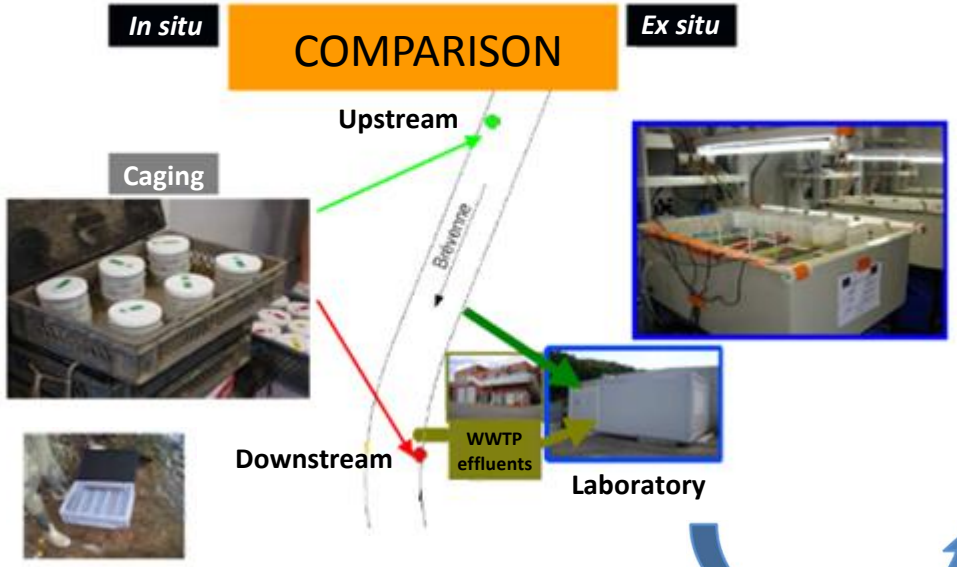
Gammarus fossarum

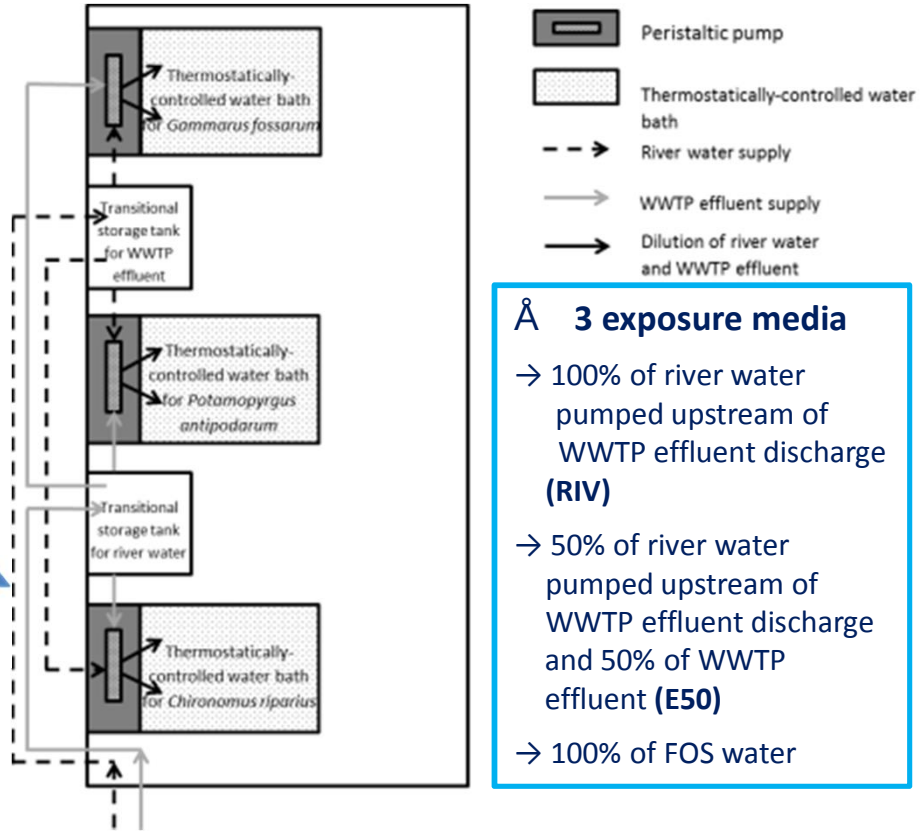
Insect larvae

Chironomus riparius

- High capability to accumulate pollutants
- Commonly used in biomonitoring programs
- Major food source for macro-invertebrates and vertebrates
- Easy to transport and cultivate

MATERIELS & METHODS





Å **2 exposure sites**

→ Upstream and downstream from WWTP effluent discharge

Å **3 exposure media**

→ 100% of river water pumped upstream of WWTP effluent discharge **(RIV)**

→ 50% of river water pumped upstream of WWTP effluent discharge and 50% of WWTP effluent **(E50)**

→ 100% of FOS water

Simultaneous exposure experiments (7 days)
2 exposure campaigns: autumn and summer

MATERIELS & METHODS

- Sample preparation using MicroQuEChERS extraction

One freeze-dried organism for mollusc and crustacean and a pool of 4 organisms for insect larvae

500 µl H20
500 µl ACN
200 µl Hexane

500,5 mg of citrate buffer

Grinding and agitation (2min)

Centrifugation

Elimination of hexane (green) and recovery of ACN (yellow)

- Analysis by NanoLC-QqToF

Nanochromatography
(Ultimate 3000, Thermofisher®)

↳ **Separation of compounds**

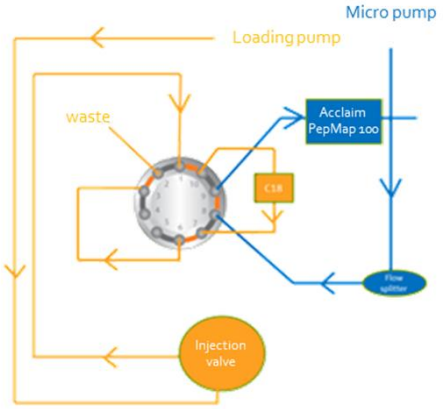
Hybrid High Resolution Mass Spectrometer
(MicrOTOF-QII, Bruker Daltonics®)

↳ **Combination of mass accuracy, high resolution and True Isotopic Pattern (TIP) for precursor and fragment ions**

MATERIELS & METHODS

- Use of small quantities of sample
- Improvement of the sensitivity of analysis
- Only suitable for small injection volumes

Step 1: On-line preconcentration (trapping step)



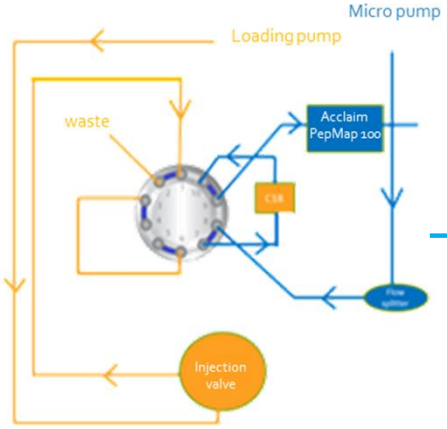
Injection volume: 1µL

Heavily dependent on:

→ loading solvent
→ loading flow
→ loading ?me

Optimizations are required

Step 2: Elution



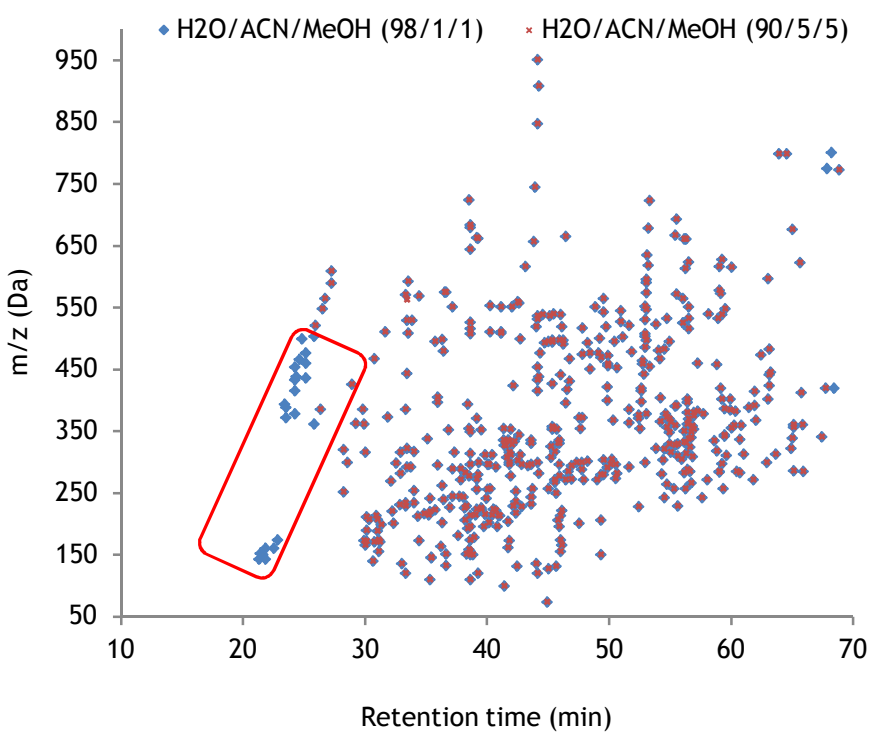
Step 3: Detection by HRMS and HRMS/MS

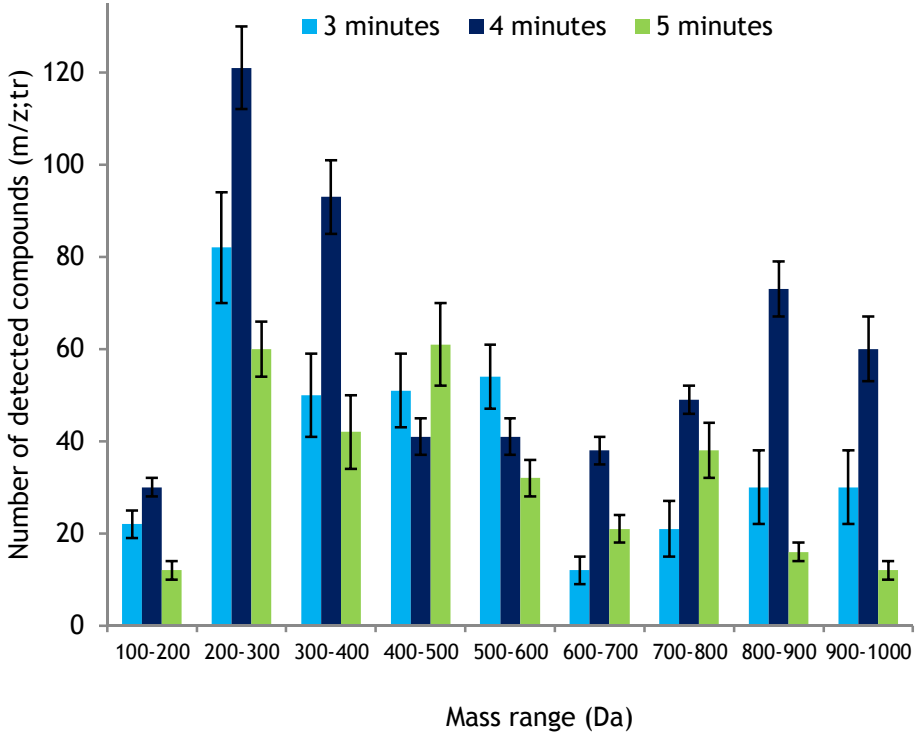
→ First injec?on by full scan (50-1000 Da)
→ Second injec?on using Auto MS/MS (collision energy: 25 and 45 eV)

MATERIELS & METHODS

- Optimisation of trapping step

  ↳ Influence of the eluting strength of loading solvent



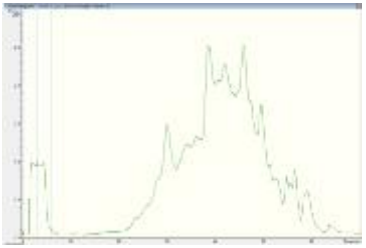


Loading solvent: H_2O/ACN/MeOH (98/1/1)
Loading time: 4 minutes

MATERIELS & METHODS

Data analysis strategy

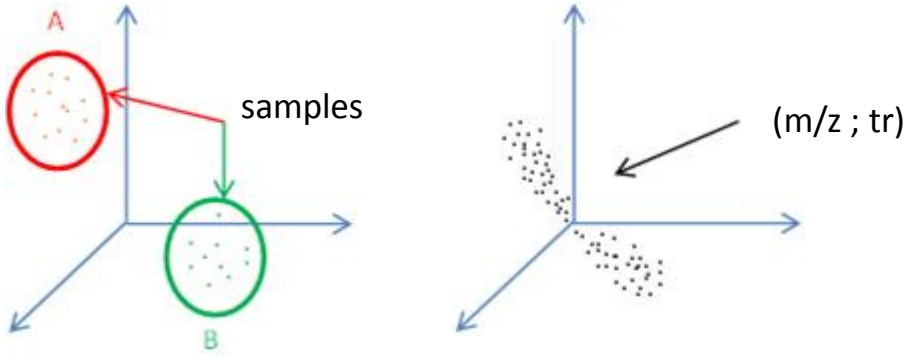
Step 1: Calibration, peak alignment, bucketing



Step 2: Data Matrix creation

	Group	$(m/z, tr)_1$	$(m/z, tr)_2$	...	$(m/z, tr)_p$
Sample 1	A	I_{11}	I_{12}	...	I_{1p}
Sample 2	B	I_{21}	I_{22}	...	I_{2p}
...	...	...	...	...	...
Sample m	A	I_{m1}	I_{m2}	...	I_{mp}

Step 3: Statistical analysis (PCA)

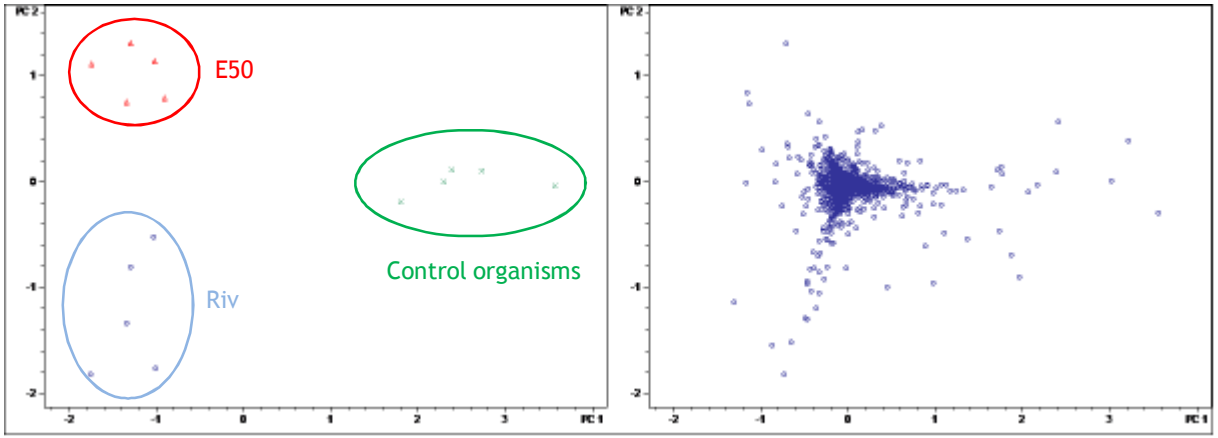


Reducing the data complexity to facilitate result interpretation

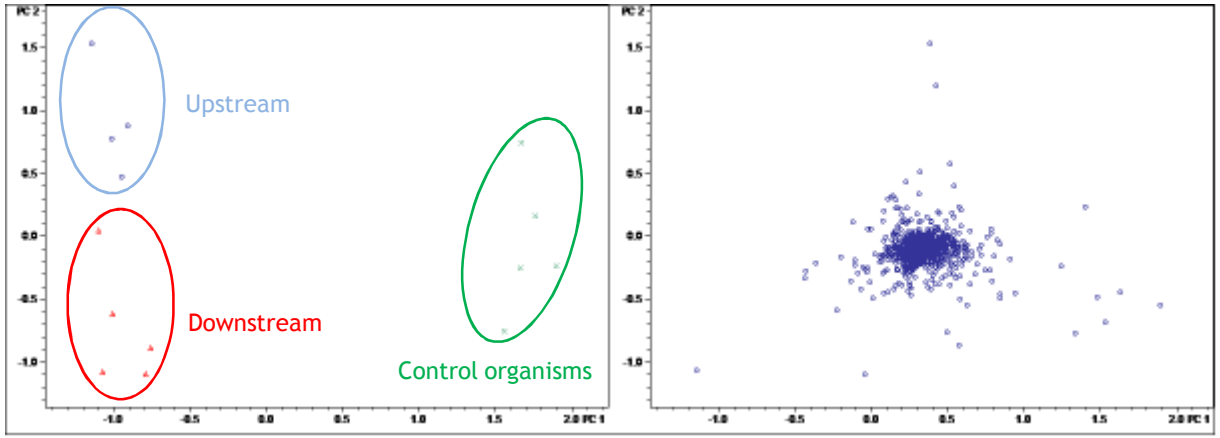
RESULTS & DISCUSSIONS

- Example of *Chironomus riparius*

Laboratory exposure experiments (*ex situ* approach)



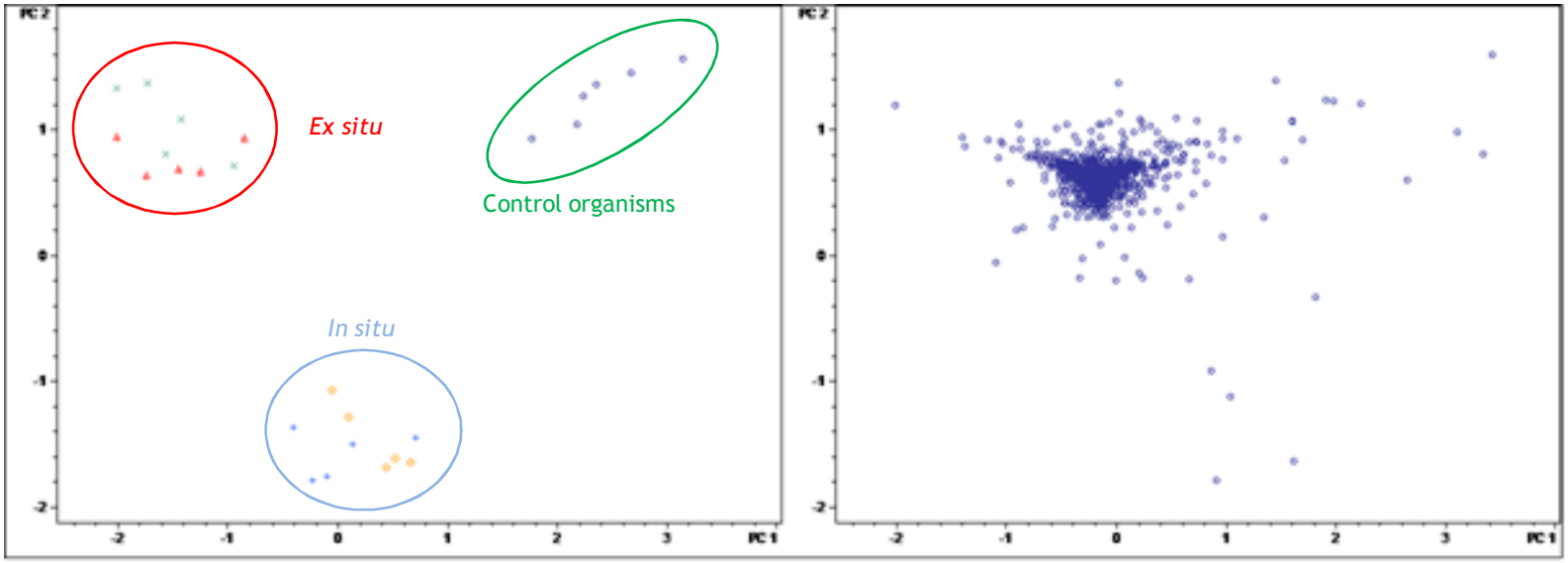
Caging experiments (*in situ* approach)



WWTP effluents modify the metabolome of insect larvae

RESULTS & DISCUSSIONS

- Example of *Chironomus riparius*



The metabolome is also influenced by the exposure conditions

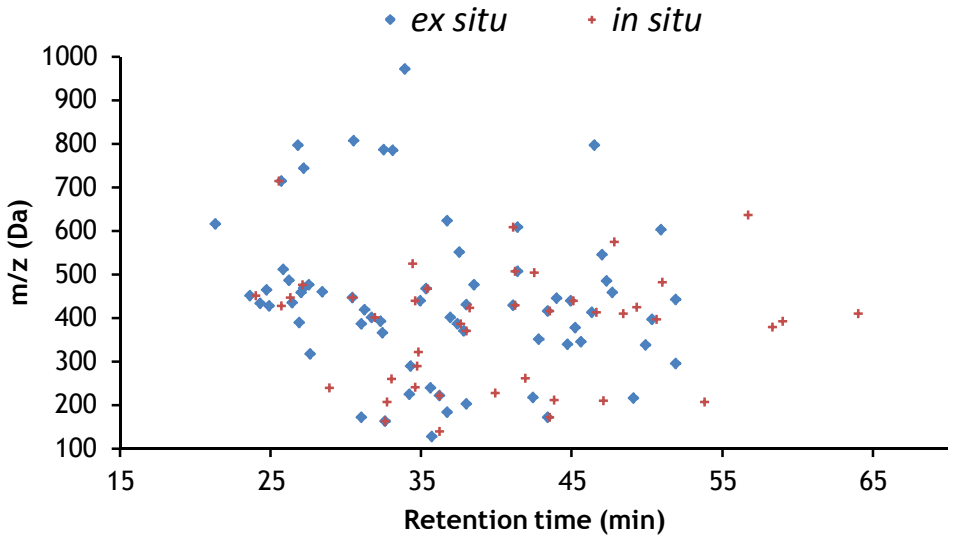
RESULTS & DISCUSSIONS

- Example of *Chironomus riparius*

  Study of discriminating signals (m/z ; tr) based on:

  - ✓ ANOVA with FDR correction results (p<0,05)
  - ✓ Study of PCA loading-plot
  - ✓ Evaluation of intensity profiles

Number of discriminating signals (m/z ; tr)			
Autumn		**Summer**	
Ex situ	*In situ*	*Ex situ*	*In situ*
61	34	24	122



RESULTS & DISCUSSIONS

- Strategy of discriminating signals identification

Step 1

Annotation of discriminating signals by interrogation of databases (HMDB and DrugBank) using MetaboTrack®

→ Generation of a short list of potential candidates

Step 2

Verification of the correlation of isotope patterns according to putative atomic compositions

→ Reduction of the list of putative biomarkers

Step 3

Investigation of MS/MS data using web-based spectral DB MassBank and/or *in silico* fragmentation tool MetFrag

→ Obtaining structural information

Step 4

Analysis of standards is the only means of reaching unambiguous identification

→ Retention time, full scan and product ions spectra should be compared

RESULTS & DISCUSSIONS

- 1st example of discriminating signal identification

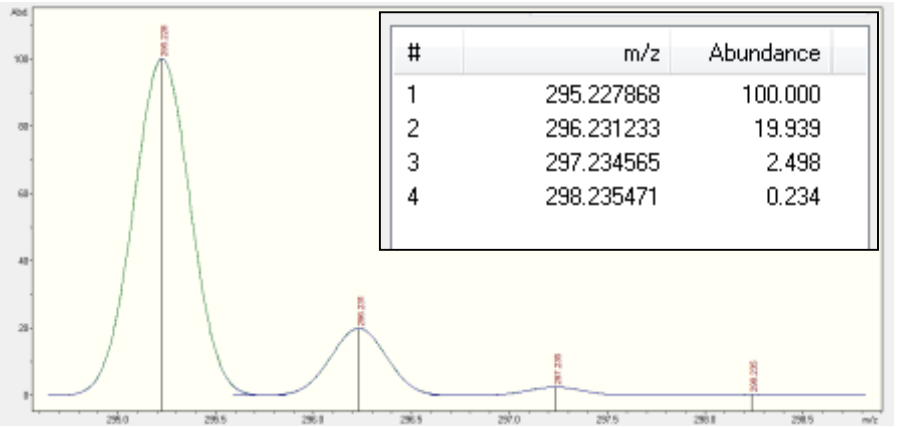
Step 1

Annotation of discriminating signal corresponding to **(m/z = 295.2293 Da ; tr = 51,4 min)** by interrogation of HMDB database using MetaboTrack

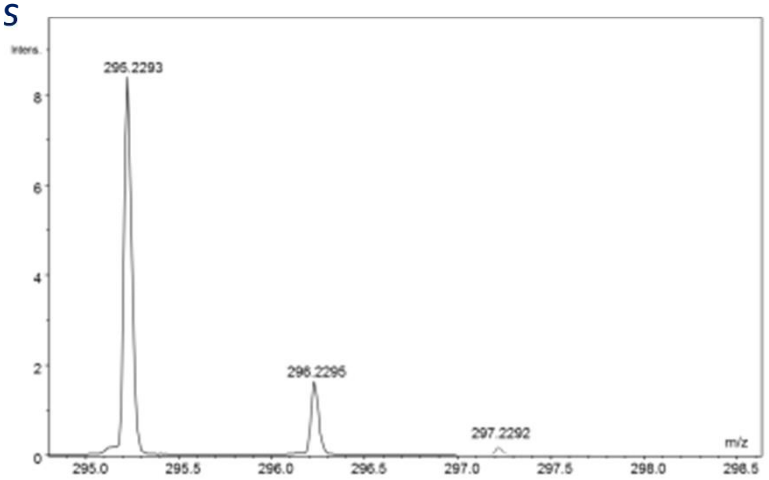
→ 5 candidates ($C_{18}H_{32}O_3$)

Step 2

Verification of the correlation of isotope patterns according to putative atomic compositions



#	m/z	Abundance
1	295.227868	100.000
2	296.231233	19.939
3	297.234565	2.498
4	298.235471	0.234

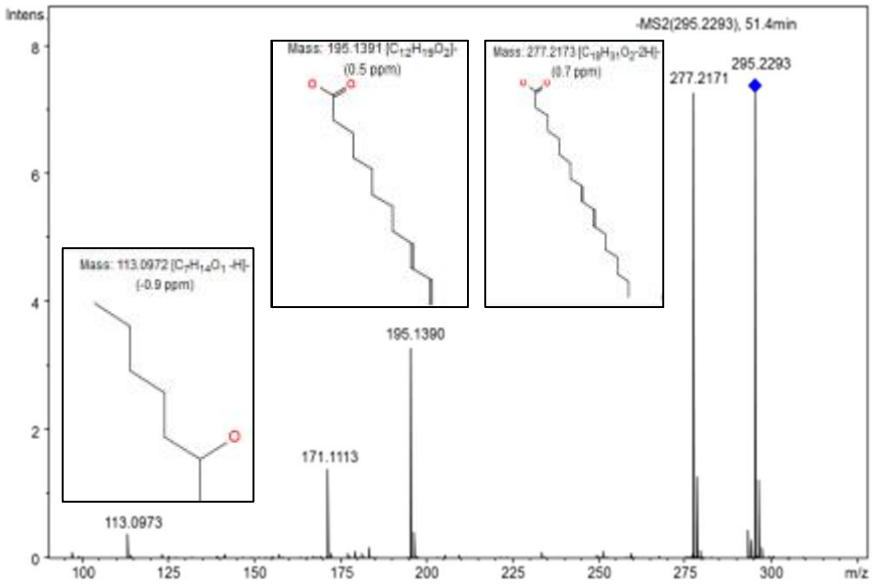


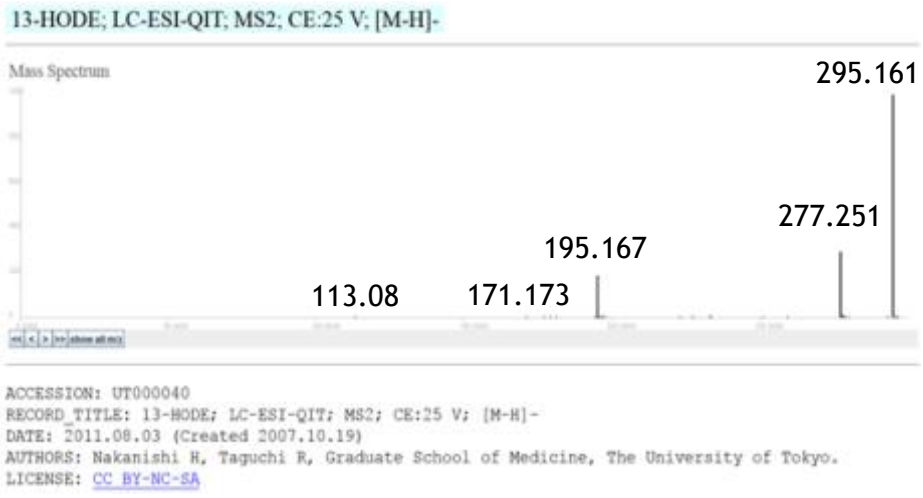
RESULTS & DISCUSSIONS

- 1st example of discriminating signal identification

Step 3

Investigation of MS/MS data using web-based spectral database (MassBank) *in silico* fragmentation tool MetFrag





13-Hydroxyoctadecadienoic acid (13-HODE) is the major lipoxygenation product derived from linoleic acid

RESULTS & DISCUSSIONS

- 2nd example of discriminating signal identification

Step 1

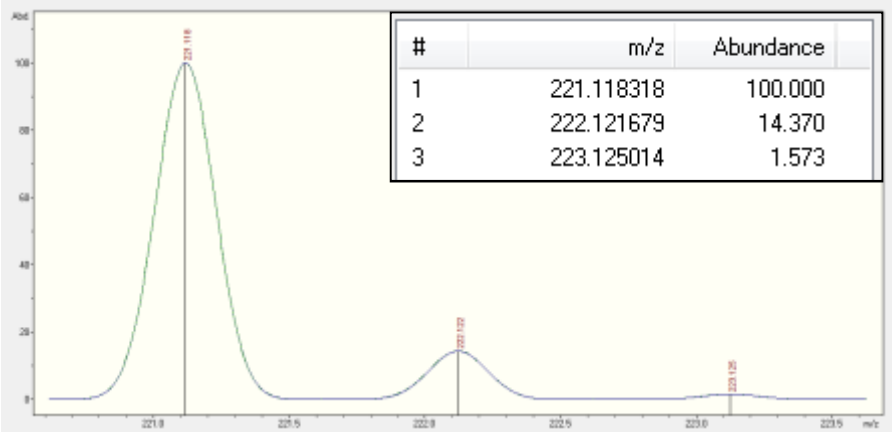
Annotation of discriminant signal corresponding to **(m/z = 221.1170 Da ; tr = 35.9 min)** by interrogation of DrugBank database using MetaboTrack

→ 3 candidates (1-Hydroxyibuprofen, 2-Hydroxyibuprofen and 3-Hydroxyibuprofen)

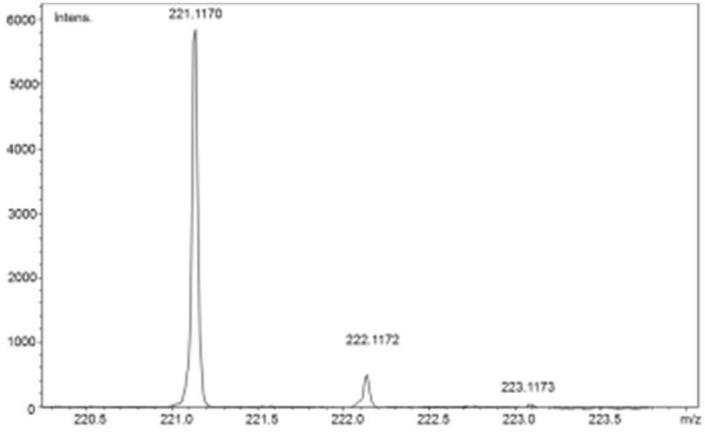
($C_{13}H_{18}O_3$)

Step 2

Verification of the correlation of isotope patterns according to putative atomic compositions



#	m/z	Abundance
1	221.118318	100.000
2	222.121679	14.370
3	223.125014	1.573



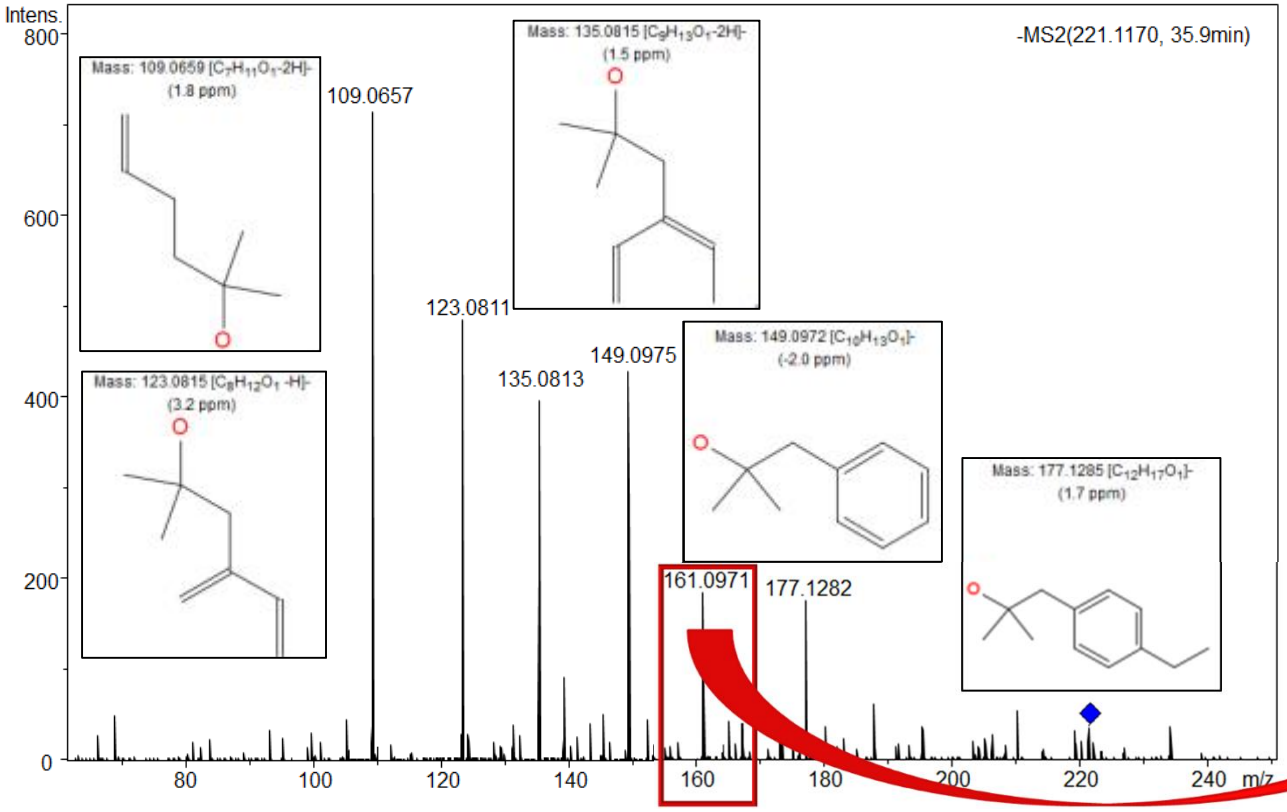
RESULTS & DISCUSSIONS

- 2nd example of discriminating signal identification

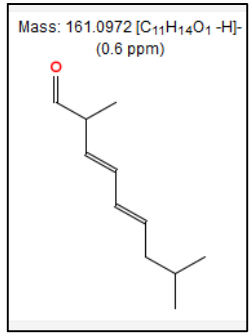
Step 3

Investigation of MS/MS data using *in silico* fragmentation tool MetFrag

→ No MS/MS data are available in MassBank



Characteristic fragment of ibuprofen



RESULTS & DISCUSSIONS

- 2nd example of discriminating signal identification

Step 4

Analysis of authentic standard: 2-hydroxyibuprofen

→ Retention time, full scan and product ions spectra are similar…

BUT, in the absence of the two other analytical standards corresponding to 1-Hydroxyibuprofen and 3-Hydroxyibuprofen, it is not possible to confirm the isomerism of hydroxyibuprofen

↳ This result highlights the bioaccumulation and biotransformation capacities of benthic invertebrate for pharmaceuticals

↳ This result confirms the impact of exposure conditions

→ Hydroxyibuprofen has only been detected during *ex situ* approach

CONCLUSION & OUTLOOK

- This study highlights the usefulness of NanoLC-HRMS for environmental non-targeted approaches

- The use of nanoBooster associated with the Captivespray nanosource can be an appropriate tool to improve the sensitivity of small molecules

- The results confirm the impact of WWTP effluents discharge on the metabolome of benthic invertebrates and show the influence of exposure conditions

- These results could be considered as a first step for the determination of invertebrates 'metabolome but should be investigated by experts with biochemists

ACKNOWLEDGMENTS

Thank you for your attention