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Using metabolomics to highlight biomarkers of (non)-exposure of mussels and oysters to phycotoxins

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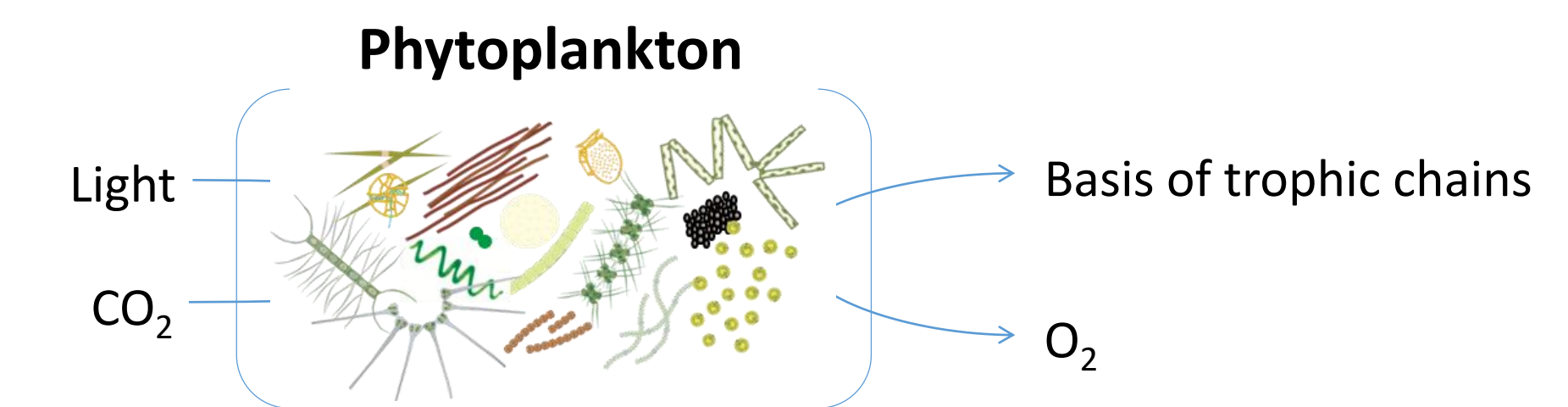
Context

- Some micro-algae species produce toxins that can cause food poisoning in humans through the consumption of contaminated shellfish
- Monitoring programmes have been implemented to protect the consumers → quantification by targeted analyses using LC-MS/MS
- Alternatively, we could try to consider the consequences of a contamination by phycotoxins on the global metabolite expression of bivalves to find biomarkers of exposition or non-exposition → **metabolomics?**

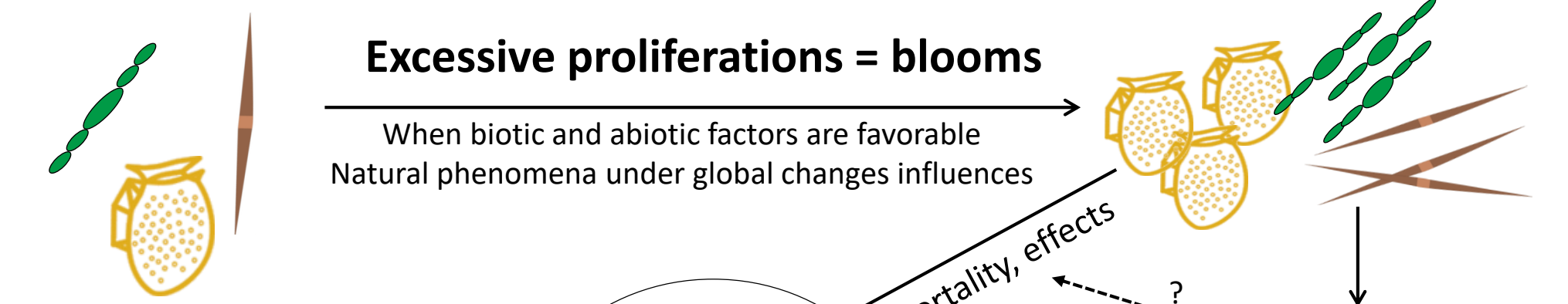
Objectives: Innovate for the assessment of shellfish safety via a double approach: metabolomics + molecular network
Highlight **biomarkers** characteristic of a (non)-exposure of bivalves to phycotoxins

Strategy: Use the latest generation of high resolution mass spectrometers and bioinformatic tools, in collaboration with the metabolomic platform of a leading partner in food chemical safety (LABERCA).

Background: Based on preliminary results suggesting metabolites characteristic of non-exposure in mussel (Mondeguer et al., 2012, Ifremer-LABERCA collaboration) and on the implementation of a monitoring method based on biomarkers obtained from untargeted metabolomics by LABERCA



Huge biodiversity including harmful dinoflagellates/diatoms/cyanobacteria
> 100 species (e.g. *Dinophysis*, *Ostreopsis*, *Alexandrium*, *Gambierdiscus*) > 29 species (e.g. *Pseudo-nitzschia*) > 35 species (e.g. *Microcystis*, *Planktothrix*, *Dolichospermum*)



Sanitary and economic impacts
Intoxications, bans on sale and consumption, beach closure

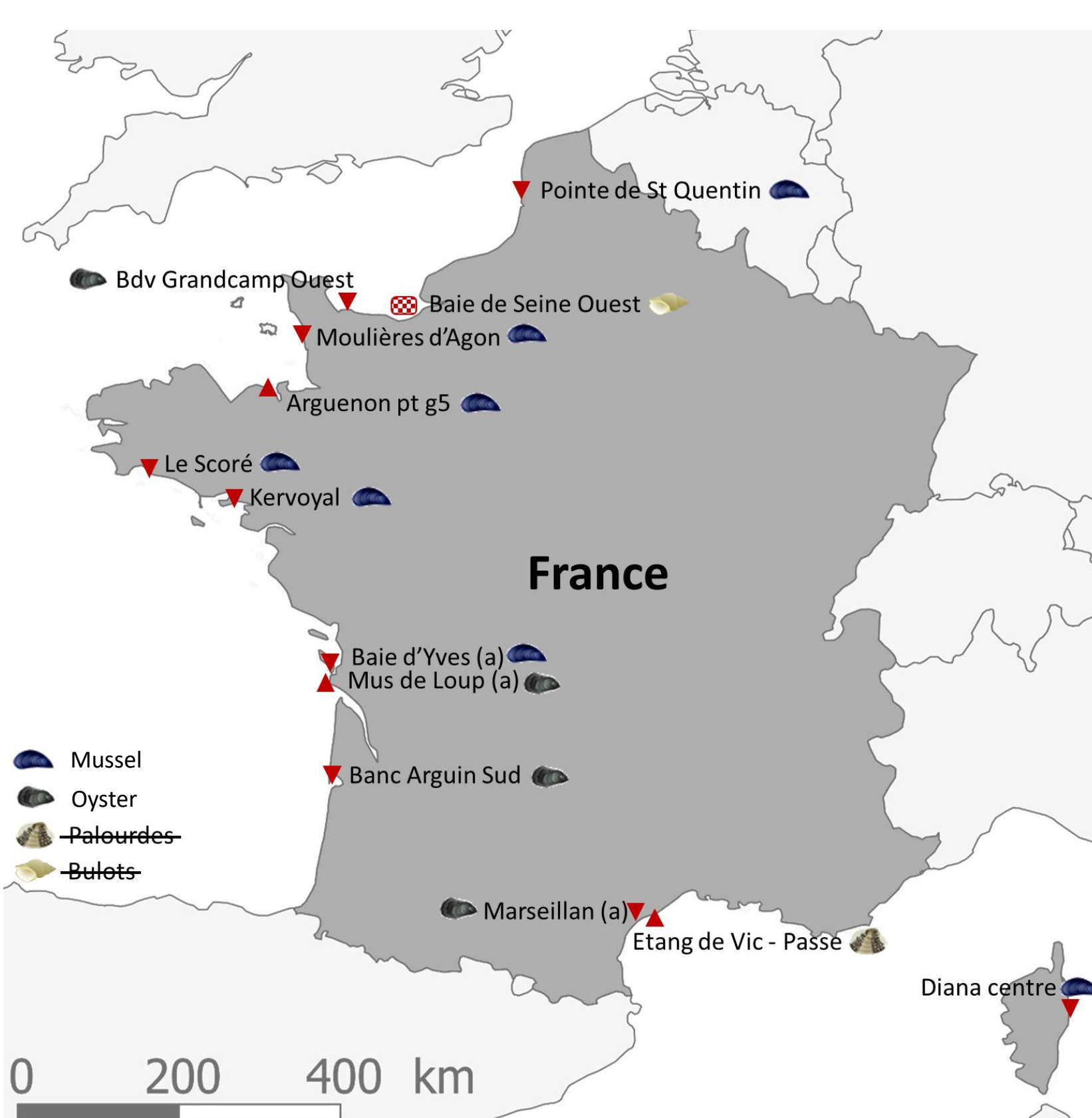
Environmental and animal health impacts
Behavioral changes (e.g. feeding), toxicities (e.g. cytotoxicity, reprotoxicity) up to mass mortalities

Toxins & other metabolites
Significant chemodiversity >20 families and hundreds of analogs
4 major syndromes
In France, prevalence of okadaic acid & derivatives, yessotoxins, pectenotoxins, spirolides, domoic acid

Overview of the problems related to harmful algal blooms (HABs)

Metabolomic approach

1) Samples and experimental design



Sampling sites for EMERGTOX (monthly sampling for the monitoring of regulated and emerging toxins in mollusks)

Samples available from:

- EMERGTOX monitoring programme:
- 459 bivalves harvested between 2018 and 2021 (73%)
- Metadata: quantification of 44 phycotoxins
- Organisms maintained in controlled conditions at the experimental platform of Bouin (= "healthy")

Experimental design:

Healthy vs. contaminated (all toxins considered)

- ≥ 1000 µg/kg for
- ≥ 200 µg/kg for
- Characteristic of natural contaminations
- Trying to consider the *in situ* physiological status (resting, sexual maturation, spawning, fattening)

Selection of 50 and 45 (Filtered MeOH extracts of digestive glands)

2) LC-HRMS/MS

UPLC
Kinetex® C18 (100 x 2,1 mm, 1,7 µm)
Gradient: ACN/water/2 mM ammonium formate + 50 mM formic acid

Comparison/complementarity Q-TOF & Q-Exactive

High Resolution Mass spectrometry

Electrospray (+ and -MS)
DDA (5 precursors/scan, 3 collision energies)



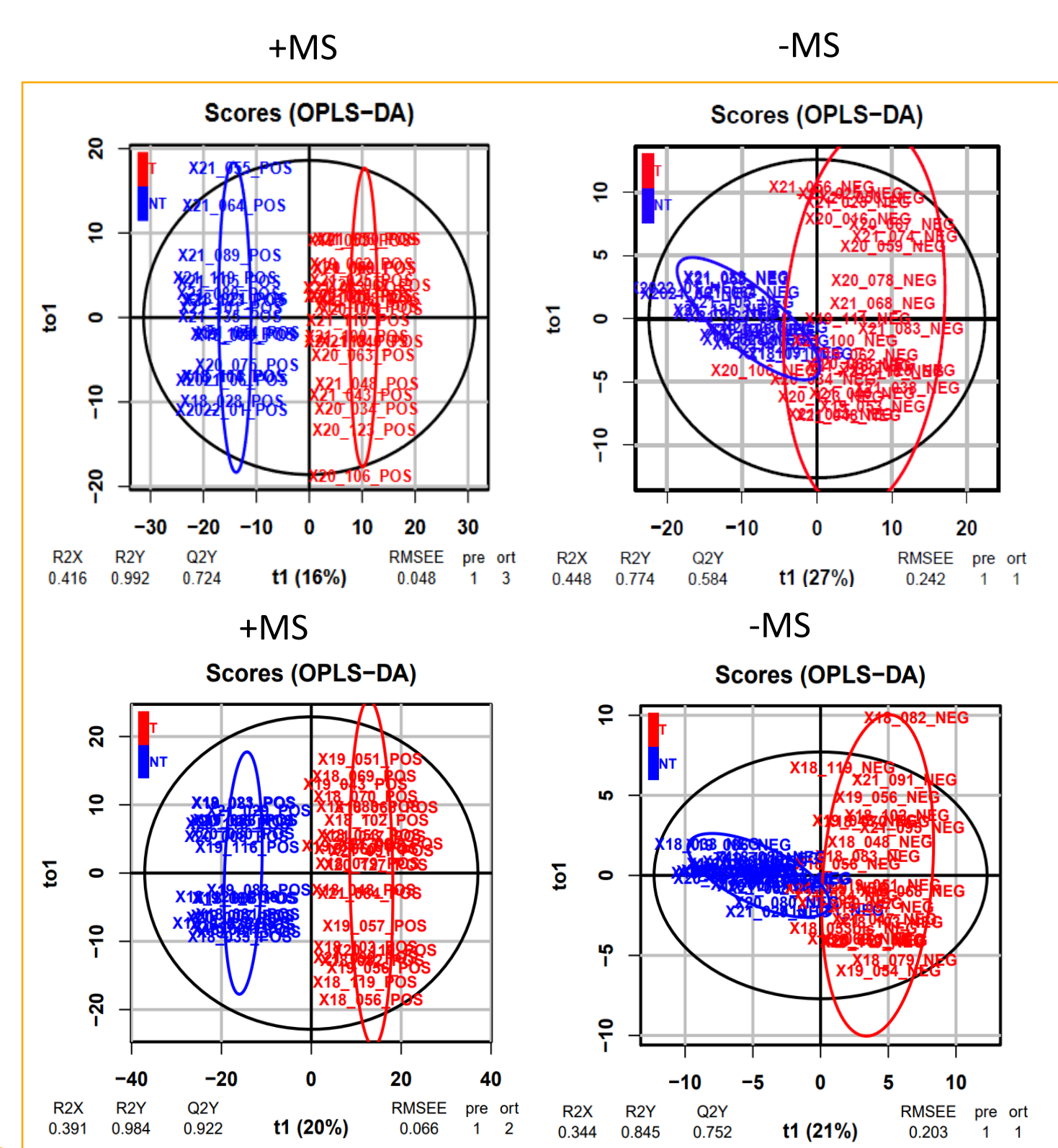
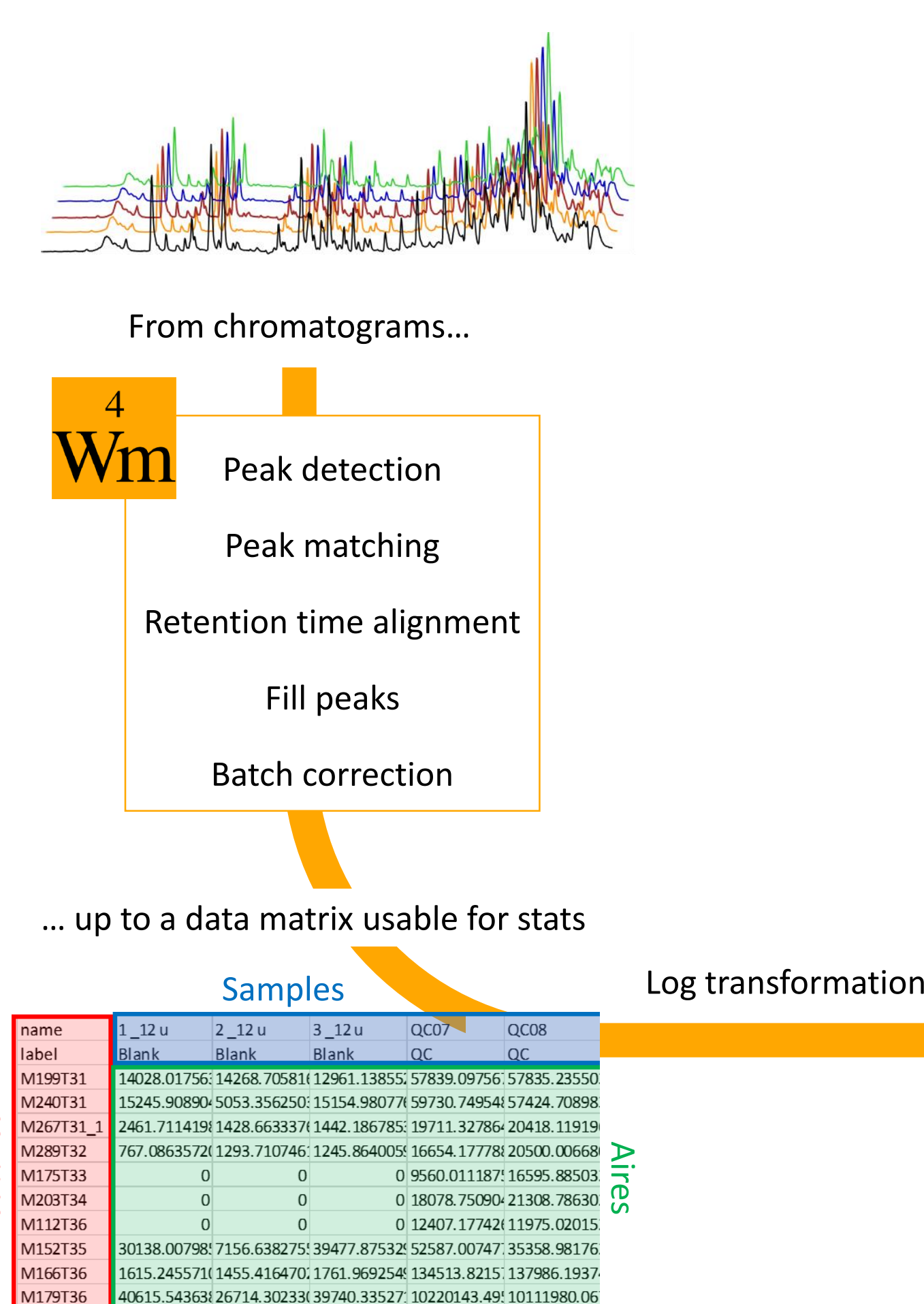
Untargeted metabolomics (following the guidelines of Broadhurst et al., (2018))

- Analytical and extraction process blanks, quality controls
- Standard reference materials (4 IS spiked in all samples + mix of 10 phycotoxins)
- Appropriate order of analysis (randomized for samples)
- Data-dependant analysis

4) Statistical analyses and selection of 'biomarkers'

Supervised orthogonal partial least squares – discriminant analyses (OPLS-DA) were constructed (and validated by permutation tests) using the classes healthy and contaminated.

3) Data processing



Similar trends observed for and :

- Predictive variation (t1) for -MS > +MS (21-27% vs. 16-20% of all variation in the data)
- But in +MS: ↗ discrimination healthy - contaminated (R2Y 0.98-0.99 vs. 0.77-0.84) and ↗ predictive performance (Q2Y 0.72-0.92 vs. 0.58-0.75)
- features discriminating the two groups can be highlighted = putative biomarkers?

+MS				
Feature	m/z	rt (s)	mean area in T	mean area in NT
M3877340	387.2961	339.9139	1.74E+05	4.98E+01
M2767137	276.1262	137.4513	1.31E+06	4.98E+04
M4767251	476.9208	251.2862	3.26E+04	1.46E+01
M3257334	325.2590	323.6006	7.08E+04	1.02E+04
M4757167	474.9731	167.4614	3.61E+04	1.01E+02

Relative abundances of some significant features in contaminated (T) and healthy (NT) organisms

+MS				
Feature	m/z	rt (s)	mean area in T	mean area in NT
M5667313	566.1042	312.6718	1.40E+05	3.55E+02
M191739	191.0741	38.6389	1.79E+05	4.68E+02
M301756	301.1391	55.8024	5.86E+05	4.94E+03
M4407338	440.2787	337.6721	9.28E+05	6.01E+02
M3237345	323.0939	344.8371	1.00E+00	1.54E+04

- Most significant features characteristic of contaminated organisms

- Some contrary examples: M3237345 for M5347675 for

- One feature possibly common between the two bivalves (M6957760-763)

- M4407338 may correspond to lysoPC (12:0) (GNPS annotation)

Results and perspectives

- The untargeted metabolomic workflow allowed for the selection of several putative biomarkers of (non)-exposure to phycotoxins
 - mainly for contaminated bivalves
 - but some could be biomarkers of health status
- Data from the Q-Exactive are being processed
 - Complementarity between the two HRMS will be assessed
- Sensitivity and selectivity should be tested
 - using less contaminated samples
- Annotation has to be improved
 - only few biomarkers present in molecular networks
 - should consider targeted-MS/MS
 - try other annotation tools (e.g. SIRIUS)
- Promising results towards innovating for the assessment of shellfish safety
 - but the list of candidates biomarkers will ultimately have to pass challenge tests