

A novel proxy to assess the environmental fate and impact of complex (bio)pesticides: methodological development and challenges

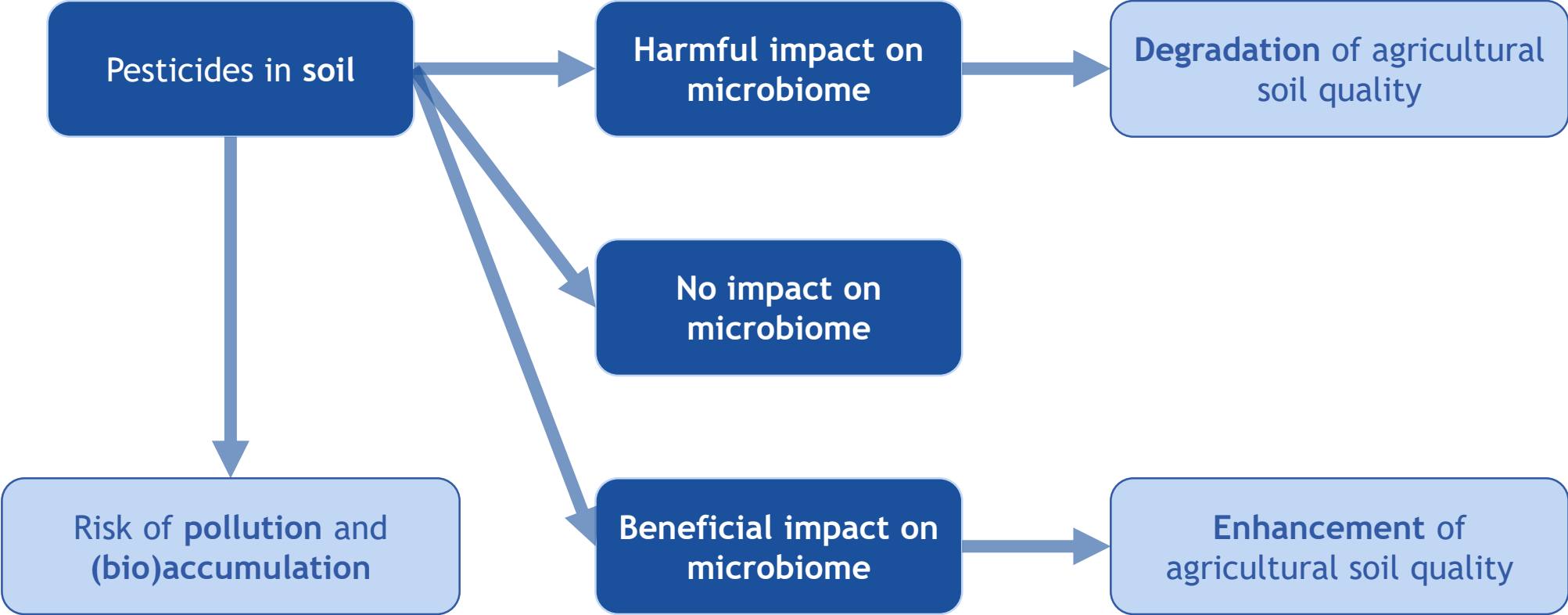
Hikmat Ghosson^{1,2}, Amani Ben Jrad^{1,2}, Chandrashekhar Patil¹, Yannick Brunato^{1,2},
Delphine Raviglione^{1,2}, Marie-Virginie Salvia^{1,2}, Cédric Bertrand^{1,2,3}

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2: UFR Sciences Exactes et Expérimentales, Université de Perpignan Via Domitia, 52 Avenue Paul Alduy, 66860 Perpignan Cedex, France

3: S.A.S. AkiNaO, Université de Perpignan, 52 Avenue Paul Alduy, 66860 Perpignan Cedex, France

Environmental risks of pesticides and/or biopesticides



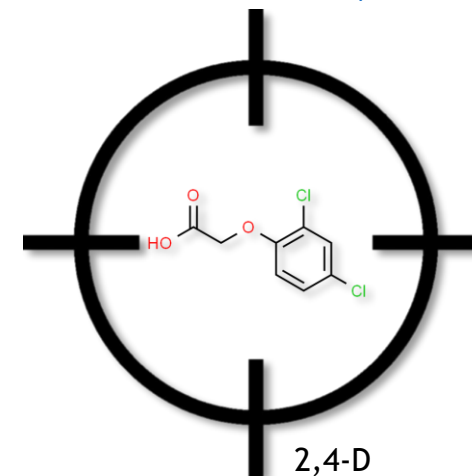
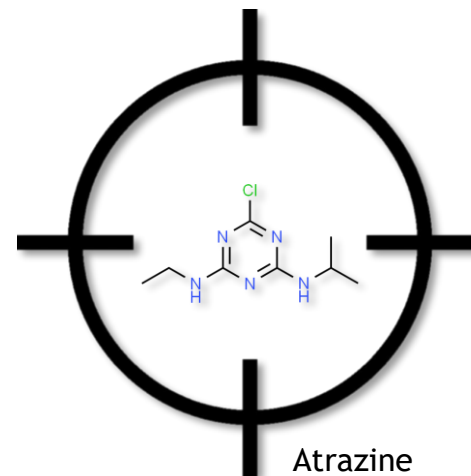
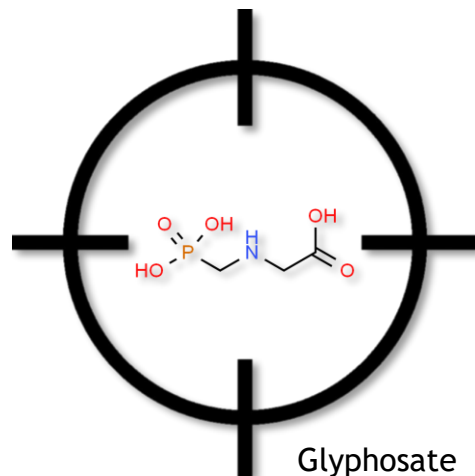
Stanley & Preetha, Springer, Dordrecht, 2016

Classic protocols for fate assessment

Half-life (DT50) approach:

- Studies the **persistence** of **known compounds**
- It is the time taken for a molecule to have **50% of its initial quantity** disappeared
- Tracks **targeted compounds'** transformation through the time
- Applicable for the **active substance** of **synthetic pesticides**

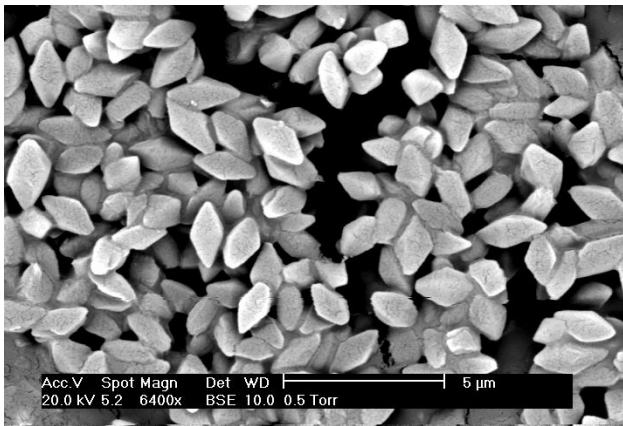
<https://ec.europa.eu/>, October 12, 2020



Classic protocols for fate assessment: *limitations for biopesticides studies*

- **Half-life:**
 - × Not applicable for **biopesticides** with **partially-unknown** compositions
 - × Not applicable for **microorganisms based-biopesticides**
 - × Cannot reveal **novel transformation by-products**
 - × No information about the **environmental impact**

<https://commons.wikimedia.org/>, November 20, 2020

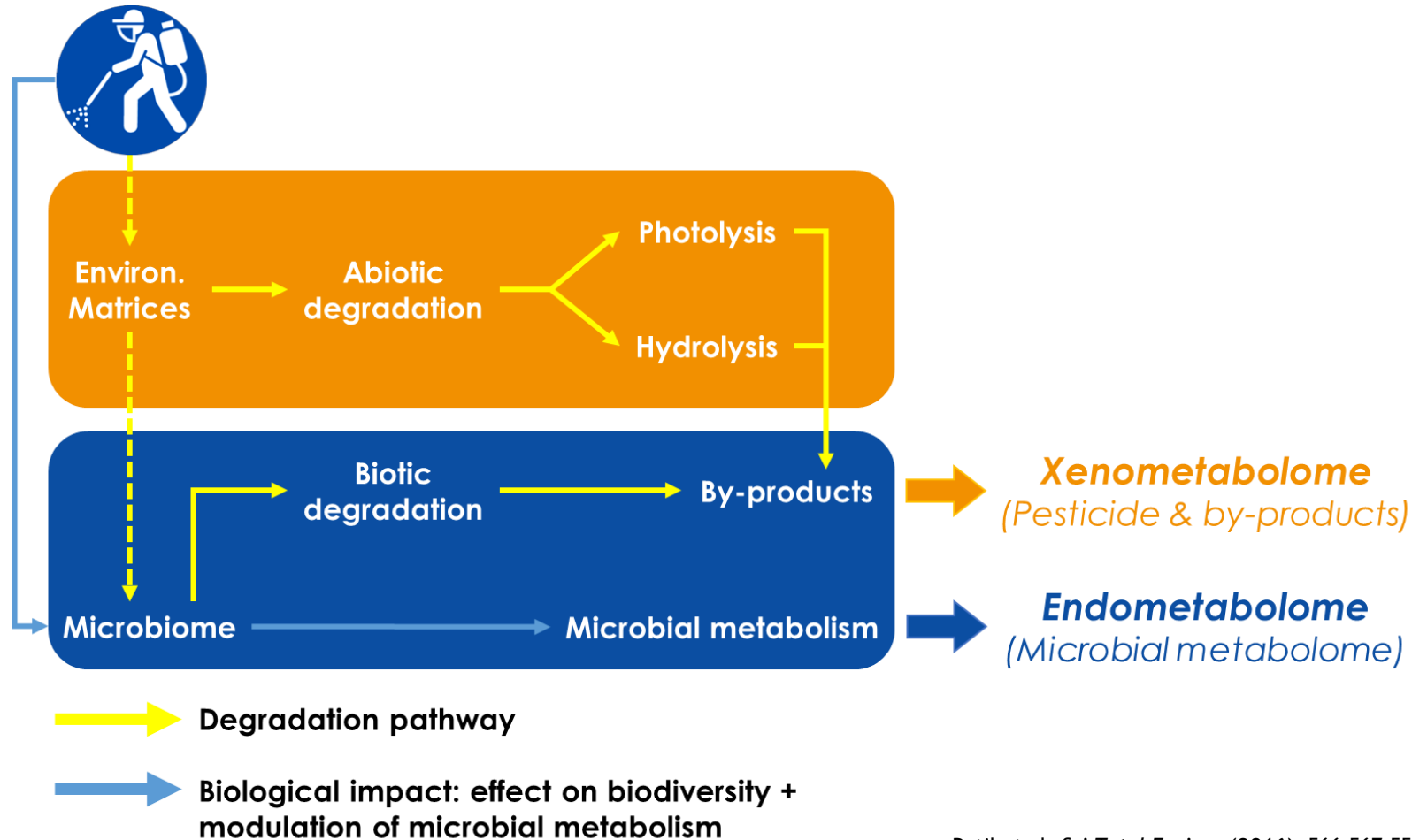


Bacillus thuringiensis morrisoni toxin crystals

*For such complex products..
How could it be done?*



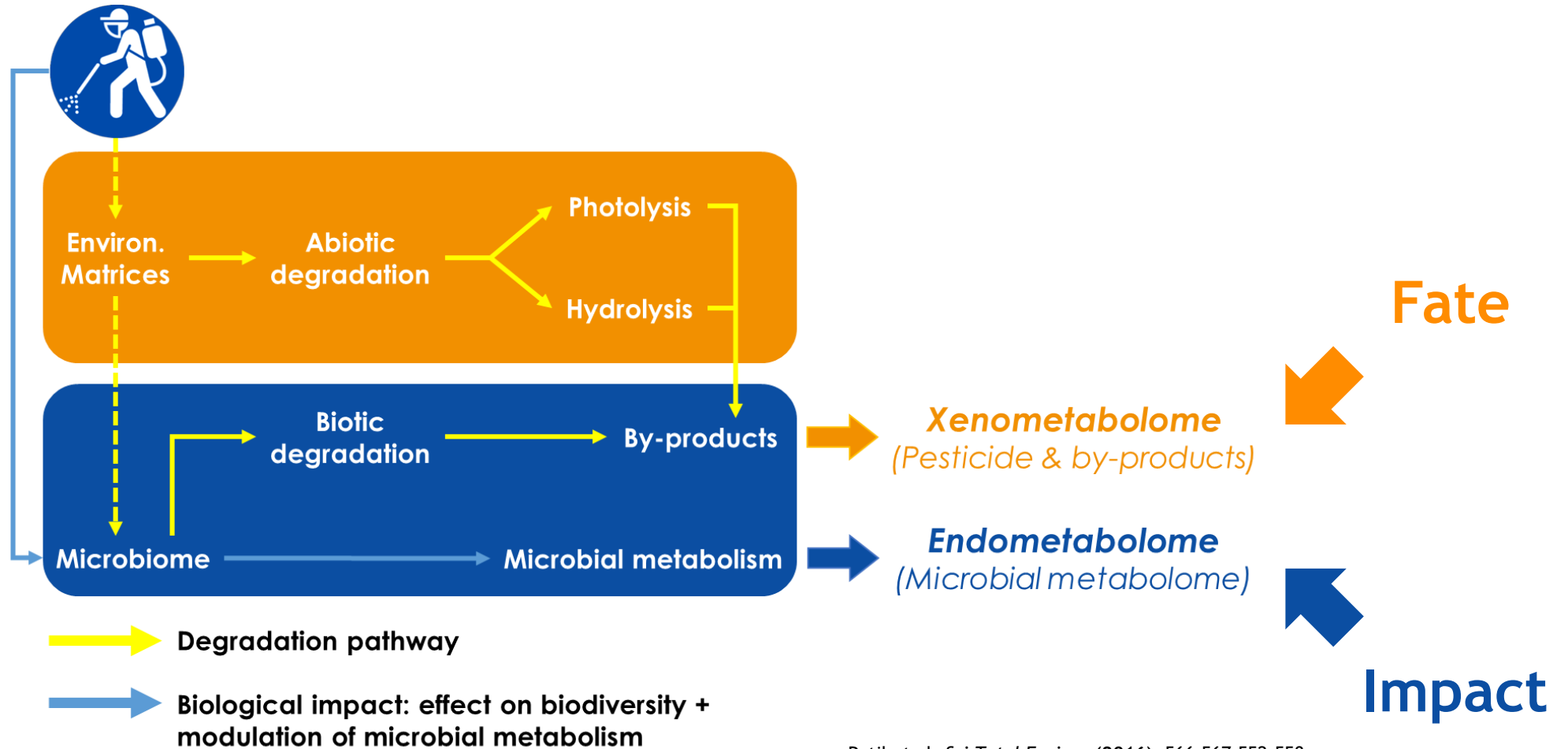
“Environmental Metabolic Footprinting” as an alternative proxy



Patil et al. *Sci Total Environ* (2016), 566-567:552-558
Salvia et al. *Environ Sci Pollut Res Int* (2017), 25(30):29841-29847

Context

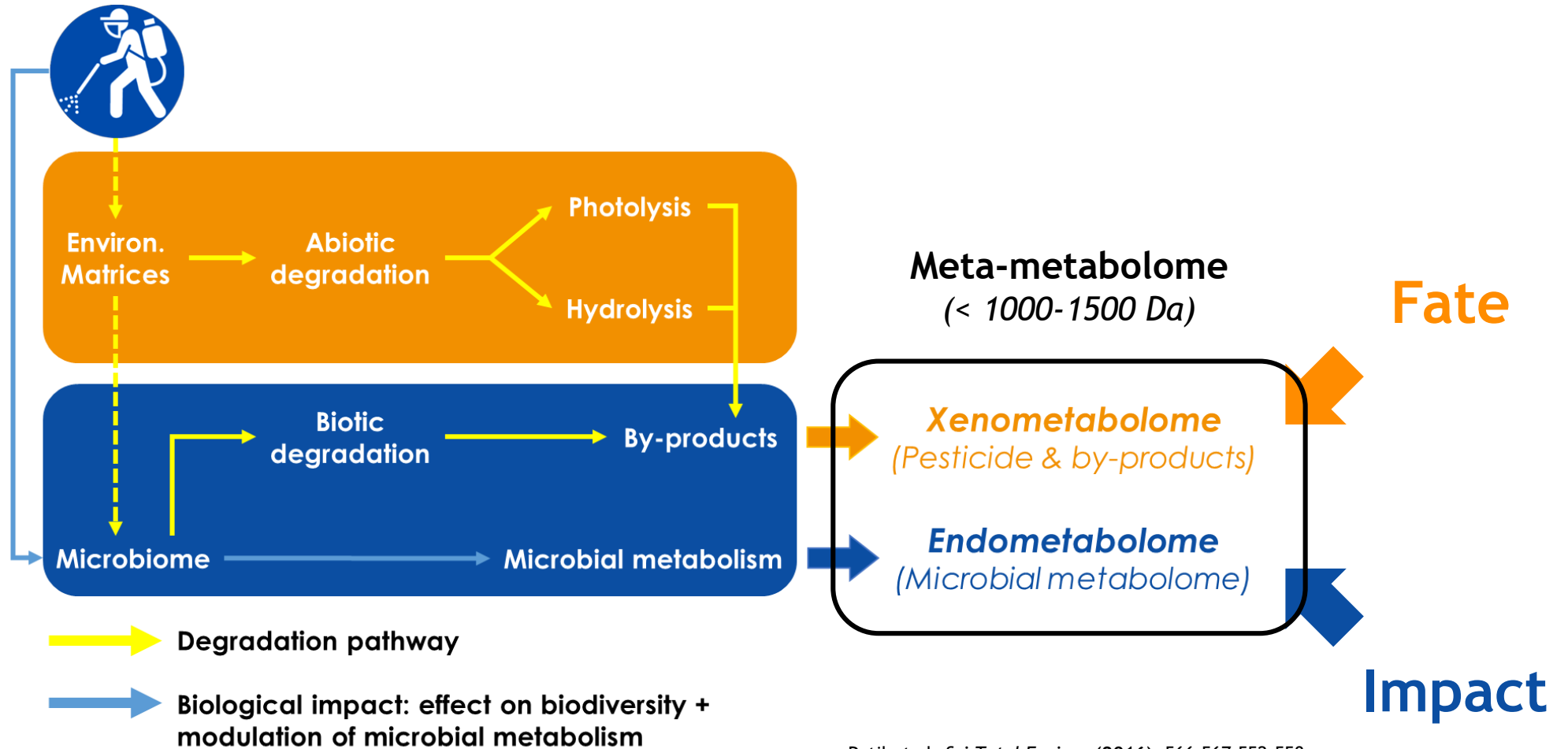
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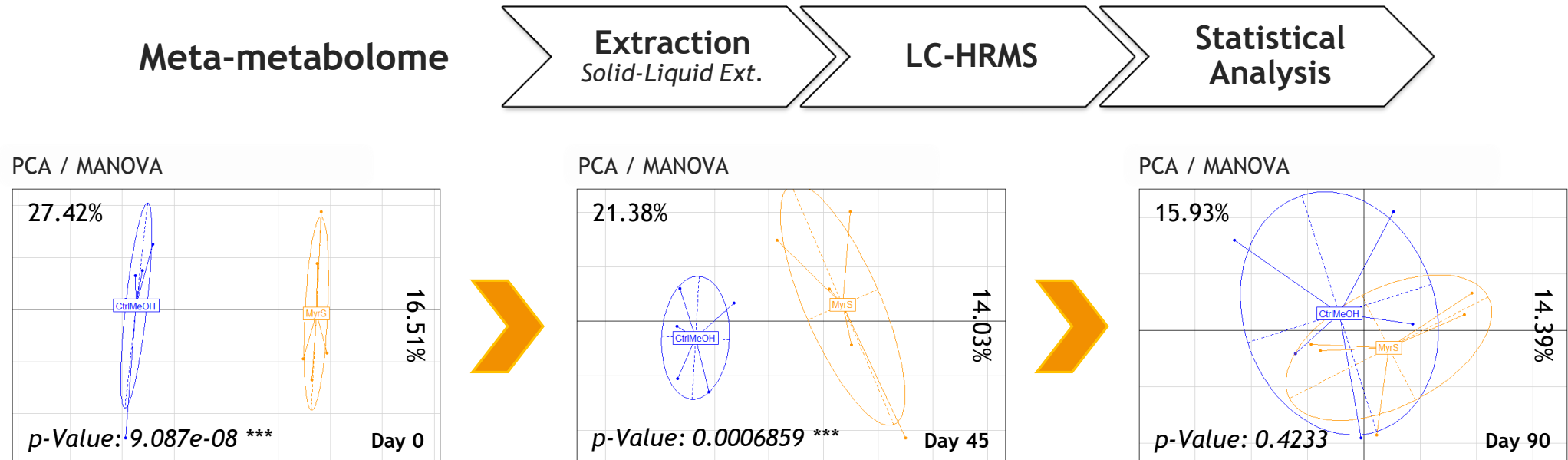
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“Environmental Metabolic Footprinting” as an alternative proxy

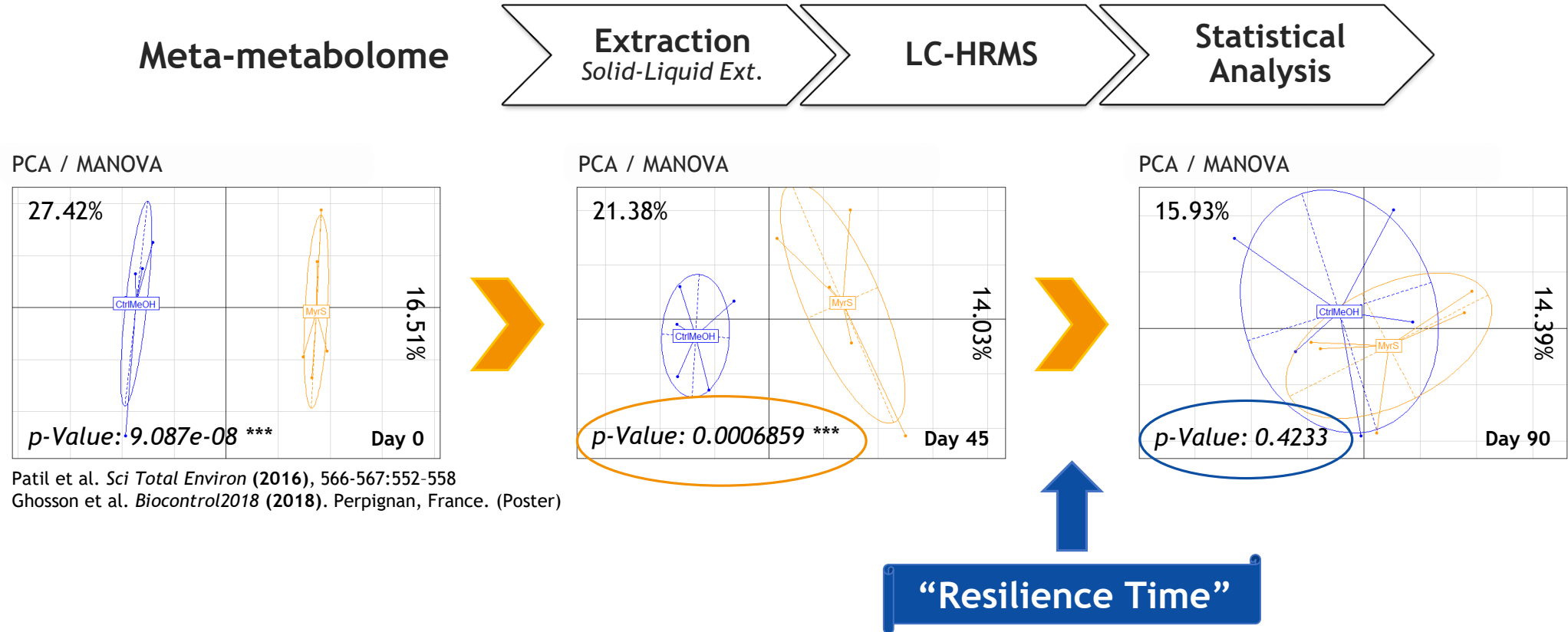


Patil et al. *Sci Total Environ* (2016), 566-567:552-558

Ghosson et al. *Biocontrol2018* (2018). Perpignan, France. (Poster)

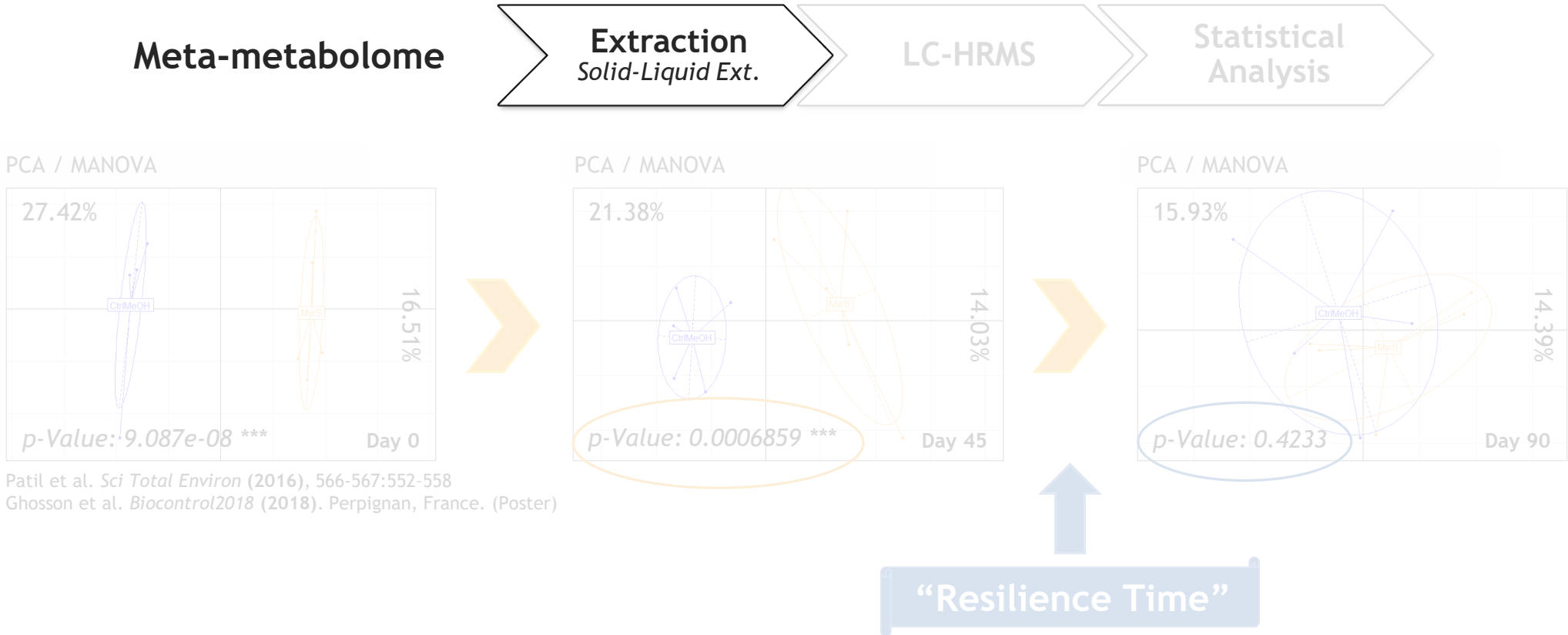
Comparison of Meta-metabolomes of Control and Spiked Samples

“Environmental Metabolic Footprinting” as an alternative proxy



Extraction development

“Environmental Metabolic Footprinting”: *development and challenges*

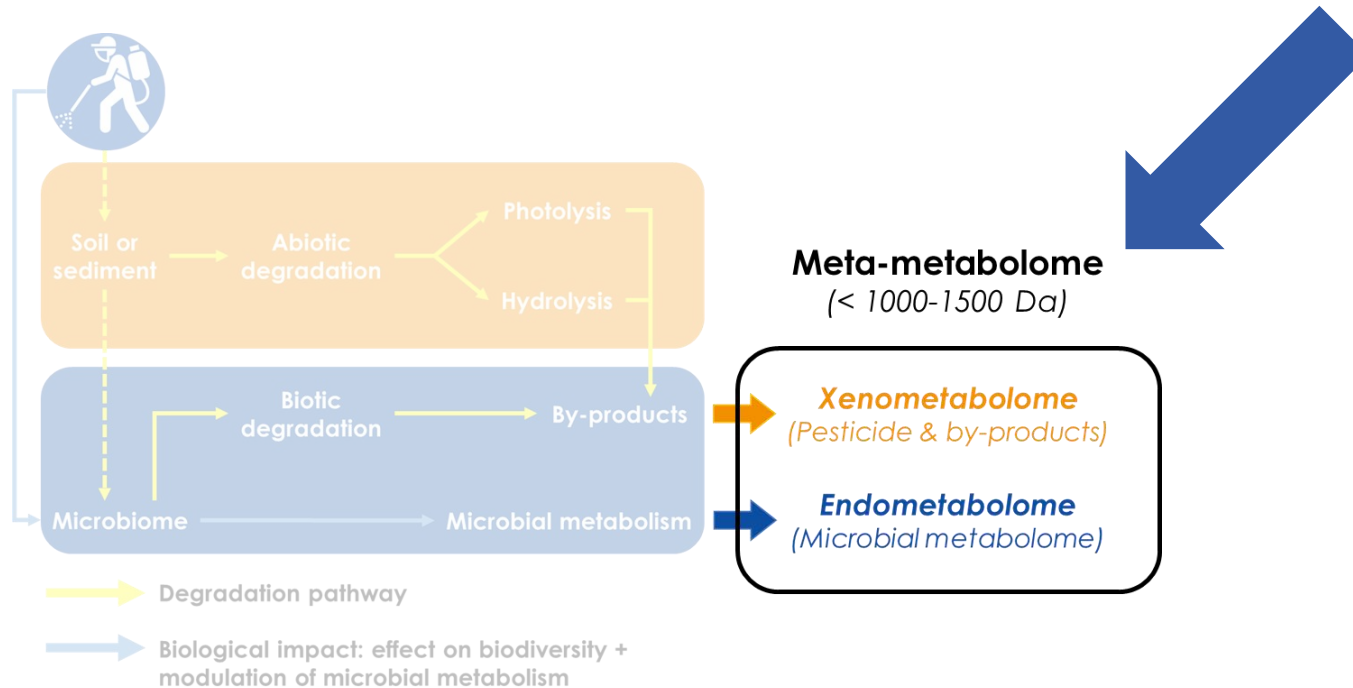


Extraction development

Why the extraction?

Meta-metabolome is the information source for the EMF:

- ✓ Xenometabolome: information about the **fate**
- ✓ Endometabolome: information about the **impact**

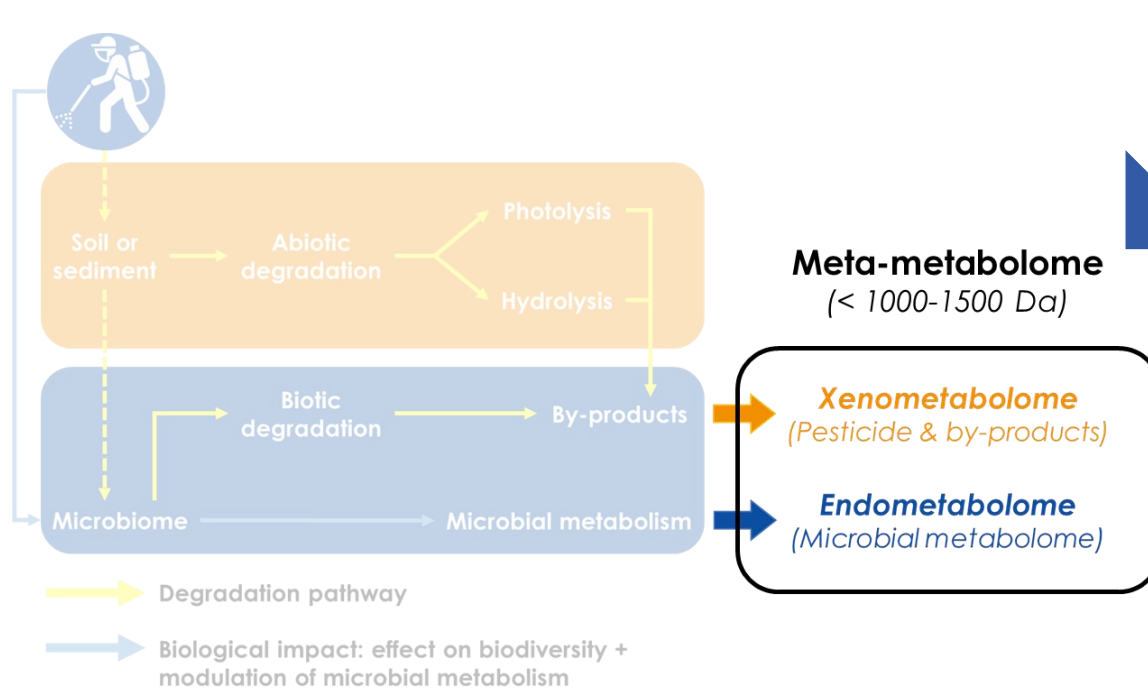


Extraction development

Why the extraction?

Meta-metabolome is the information source for the EMF:

- ✓ Xenometabolome: information about the **fate**
- ✓ Endometabolome: information about the **impact**



To collect this information:
“Optimal” meta-metabolome extraction is the key-step



Extraction development

What are the requirements?

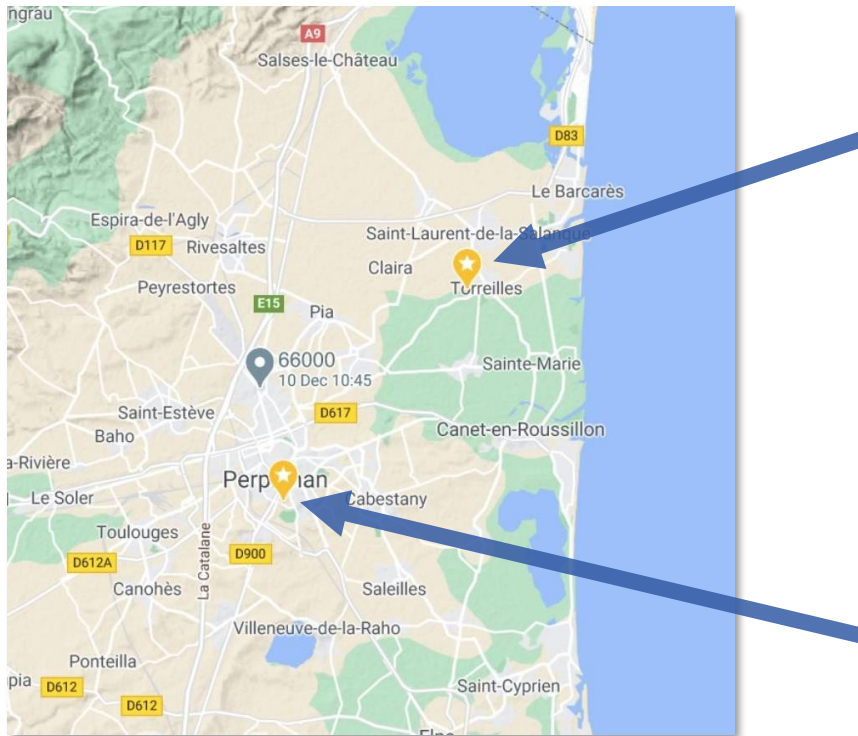
To maximize the meta-metabolic information:

- ✓ Extract a maximum number of both **xeno** and **endometabolites**
- ✓ Cover a **wide range of polarity** → various families of metabolites
- ✓ Reasonable balance between **wide polarity coverage** & **extraction yield**

Aims:

- ✓ Enhancing the detection of **pesticide** components and their TPs (**fate**)
- ✓ Increasing the **probability** to detect environmental **biomarkers** (**impact**)

Experimental design



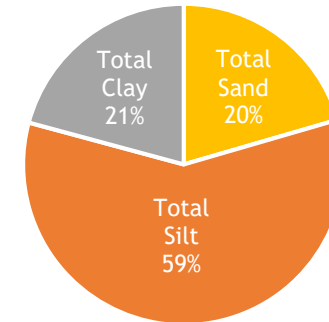
© Google

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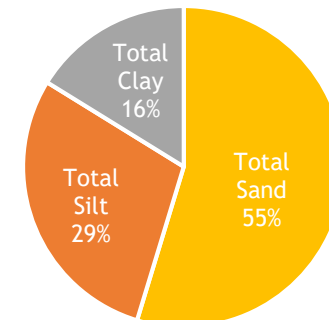
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Torreilles (ST)



Silt Loam soil

Perpignan (SP)



Sandy Loam soil

Experimental design

Laboratory soil microcosms for the 2 different soils (*SP* & *ST*):

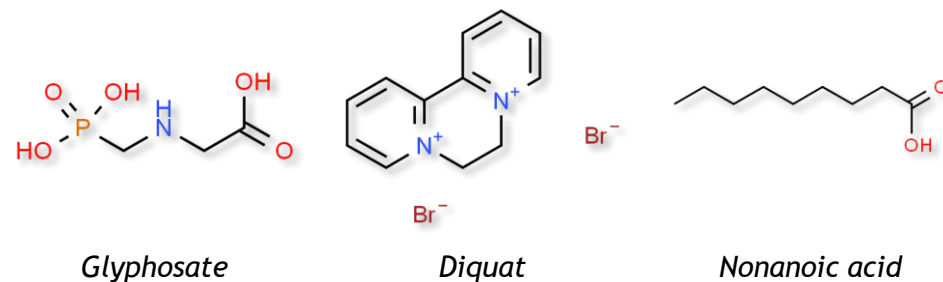
➤ 3 environmental modalities with 5 replicates for each:

- ✓ Unpolluted Control soil (*Ctr*)
- ✓ Soil polluted with Tartan Super 360™: Formulated Glyphosate/Diquat (*Gly*)
- ✓ Soil polluted with Beloukha®: Formulated Nonanoic acid (*Bel*)*
- ✓ To compare two different soil treatments with different natures & polarities

Spiking:
1× the field
dose



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Experimental design

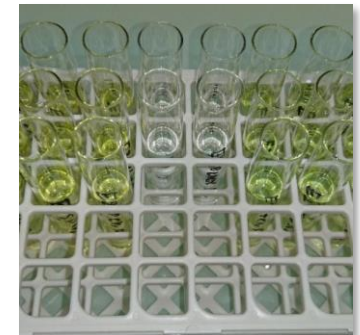
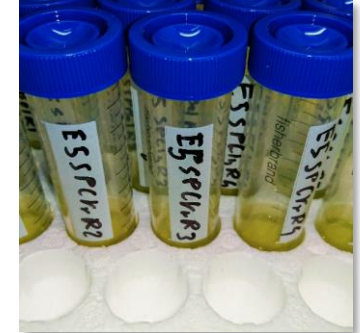
5 different protocols with different solvents and reagents were applied:

- ✓ **E1: Simple extraction based on acidified (HCl) Ethyl Acetate** (*Romdhane et al.*)
- ✓ **E2: Simple extraction based on Methanol** (*Anastassiades et al.*)
- ✓ **E3: Simple extraction based on Methanol + 1% FA (v/v)** (*Anastassiades et al.*)
- ✓ **E4: Two-steps extraction based on miscible mixtures of solvents** (*Ghosson 2020*)
- ✓ **E5: Two-steps extraction based on miscible mixtures of acidified solvents** (*Ghosson 2020*)

Total number of samples: $2 \times 3 \times 5 \times 5 = 150$

Romdhane et al. *Front Microbiol* (2016), 7:775
Anastassiades et al. *EURL-SRM* (2016), V9.1
Ghosson, *Ph.D. Thesis* (2020)

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Chemical analyses

LC: Vanquish UHPLC⁺ Focused System (Thermo Fisher Scientific)

- ▶ Modified C18 UHPLC Column (polar analytes retention enhanced)
- ▶ 20 min/run

HRMS: maXis ESI-Q/ToF System (Bruker)

- ▶ Scan range: m/z 90-1000
- ▶ Resolution (at FWHM):
 - ▶ 23000-26500 at m/z 90.9766-974.8133
 - ▶ Max Res. of 35500 at m/z 226.9516 (S/N: 10800.3)



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Raw data

Codes:

E1: EtOAc + HCl

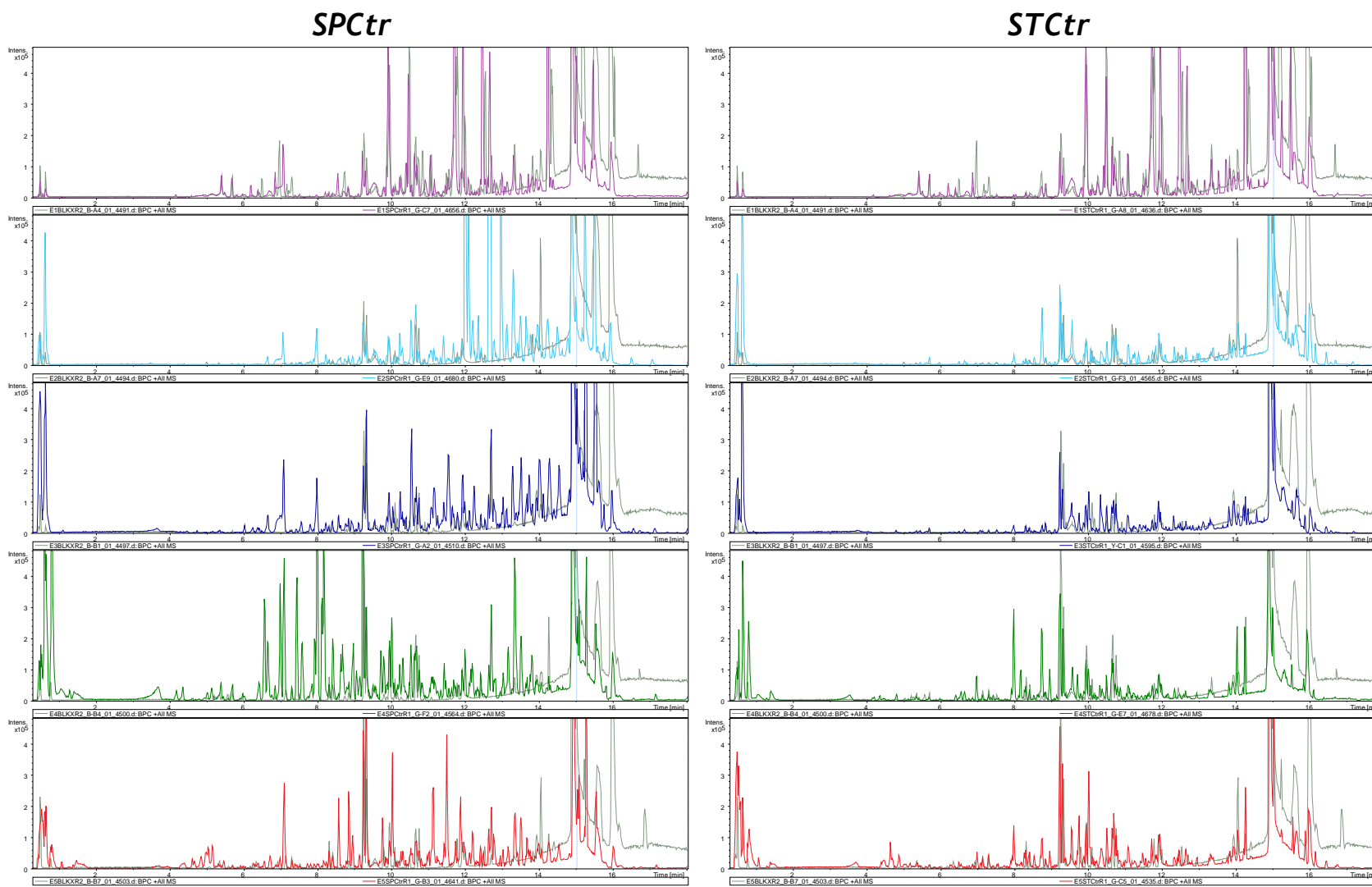
E2: MeOH

E3: MeOH + FA

E4: DE-Mix

E5: DE-Mix Acid

Blank Extraction



BPCs: m/z 90-1000, Intensity fixed at 4.8E5
Grey background: Blank Extraction



Raw data

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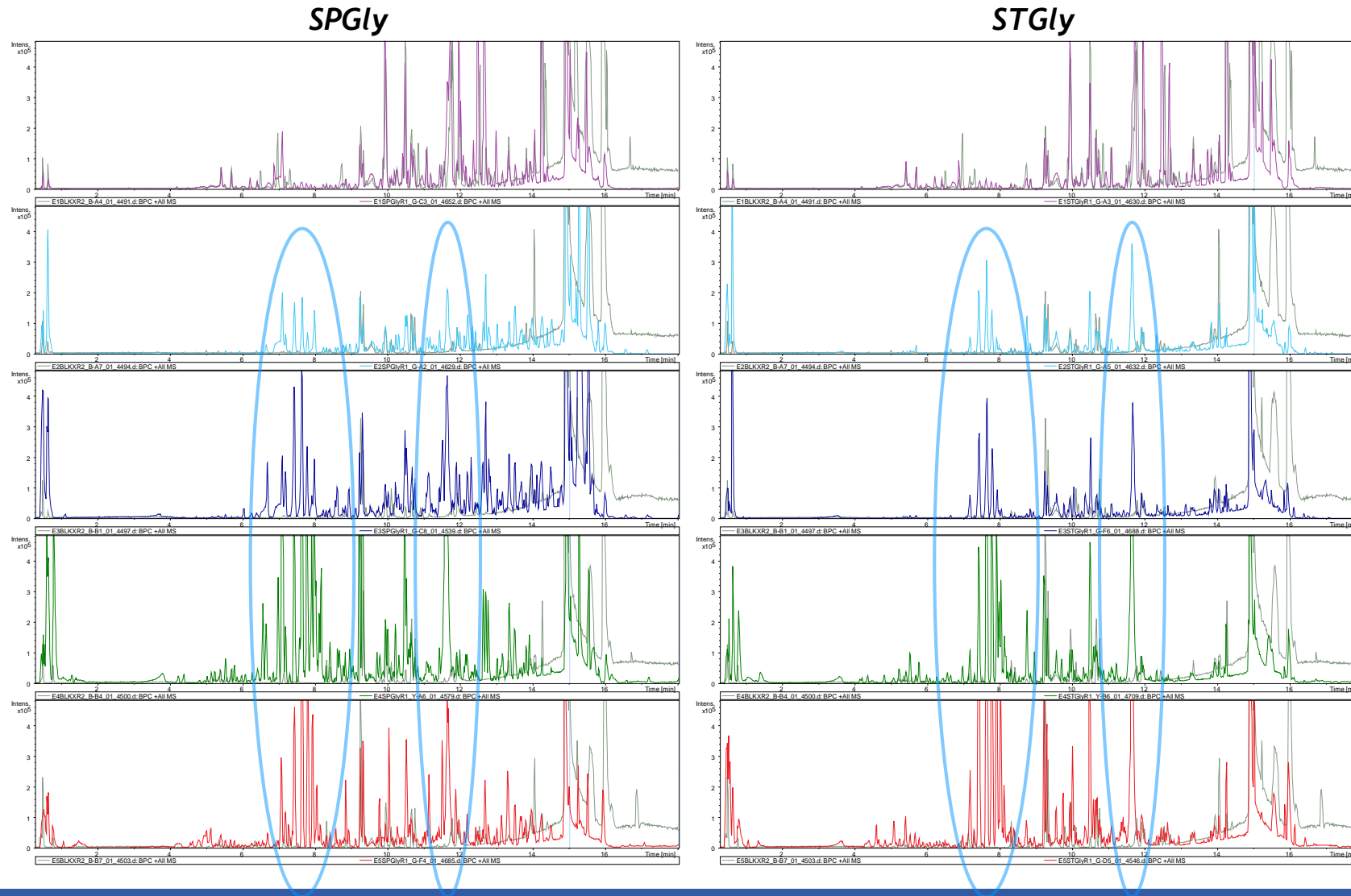
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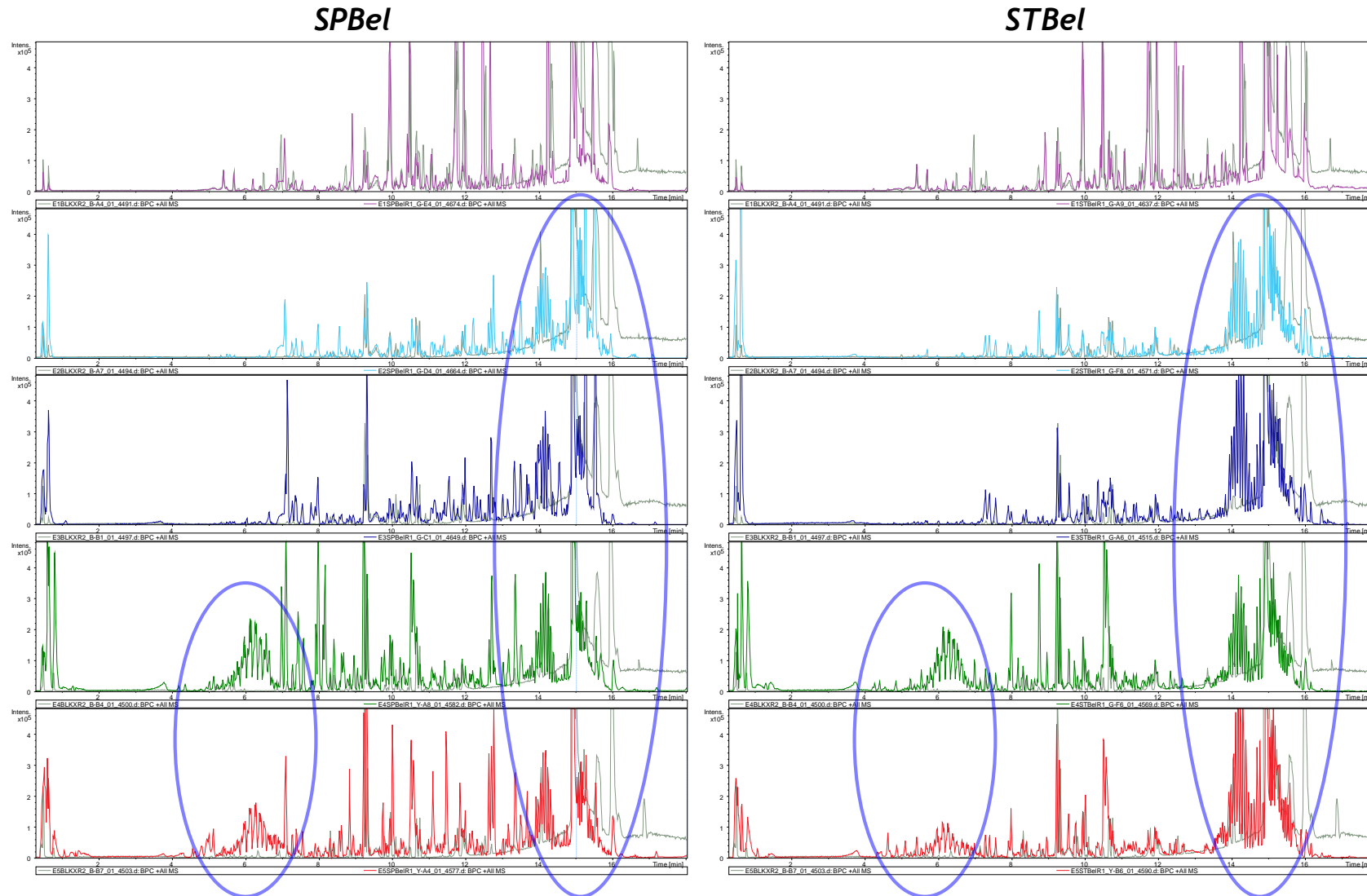
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Blank Extraction



BPCs: m/z 90-1000, Intensity fixed at 4.8E5
Grey background: Blank Extraction



Optimal extraction..?

LC-HRMS data are more complex than they appear...

- ▶ Assessing Chromatograms: is it sufficient to define the “optimal” extraction?
- ▶ How to judge the “optimum” for a “without *a priori*” untargeted approach?
- ▶ Which criteria and parameters to assess?



Optimal extraction..?

► Criteria to judge the **optimal extraction**:

1. The ability to cover the **largest range of polarity** (qualitative)
2. The best **extraction yield** (quantitative)
3. The best **reproducibility**
4. The **performance in discrimination** between polluted and unpolluted groups (EMF objective)

*Untargeted metabolomics preprocessing & statistical tools:
To be exploited...*



Chong et al. *Nucleic Acids Res* (2018), 46:486-494
Giacomini et al. *Bioinformatics* (2015), 31(9):1493-1495
Guillon et al. *Int J Biochem Cell Biol* (2017), 93:89-101

Data transformation

- Transformation of raw LC-HRMS data to processed data matrix (3D: Molecular traces; Samples; Intensities)
- Data matrix allows simplifying results visualization and revealing minor molecular information

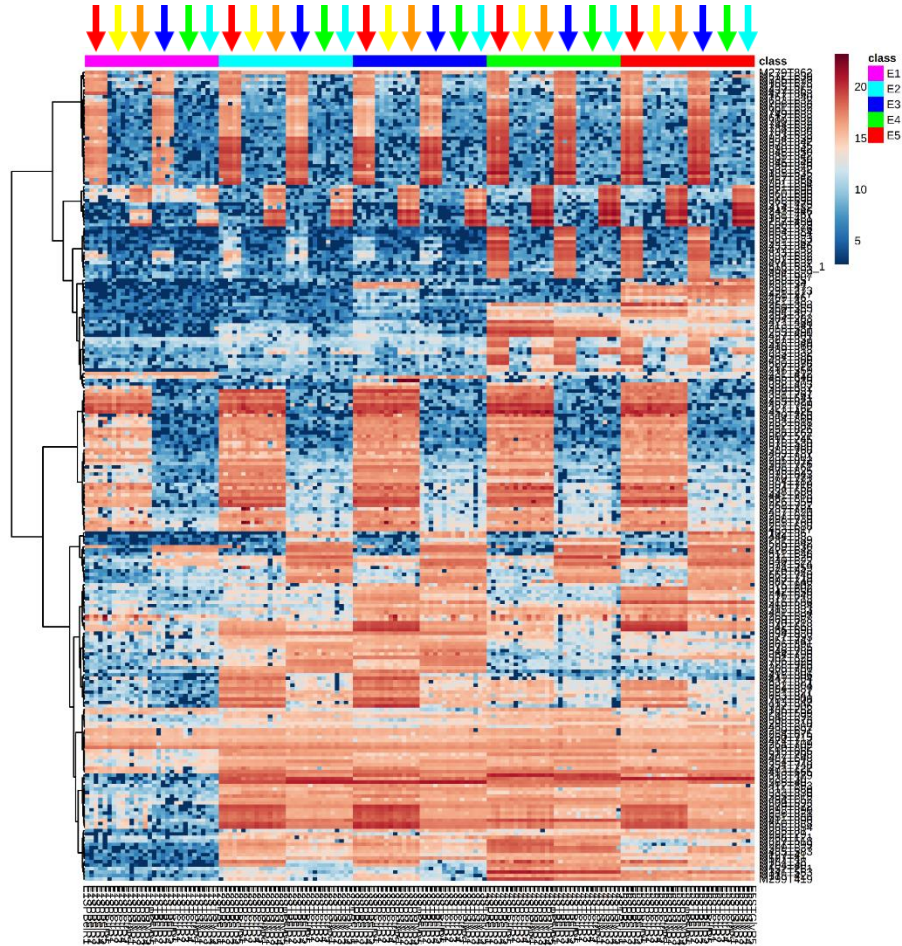
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
1	name	class	M102T36	M110T30	M118T42	M124T36	M126T30	M127T41	M128T30	M132T302	M136T252	M145T40	M146T29	M152T261	M159T628	M16
2	E1SPBelR1	E1SPBel	0	421.597023	365.317627	170.743897	101.557362	0	0	1191.28903	36452.148	0	431.186667	1490.38642	121680.727	
3	E1SPBelR2	E1SPBel	0	263.351494	35.7762427	0	0	0	2779.43706	132.359432	47210.4294	156.292991	5971.61095	2969.81111	100528.495	
4	E1SPBelR3	E1SPBel	0	588.222848	380.906838	0	479.166098	131.2858	3138.76532	470.678896	41217.6004	337.068521	6199.54535	835.925315	87386.2799	
5	E1SPBelR4	E1SPBel	0	0	269.030884	42.2166575	1696.70944	161.11947	6662.83953	215.724985	2194.4825	205.648673	6131.41189	205.845912	80994.9128	
6	E1SPBelR5	E1SPBel	0	832.644334	419.956272	26.7372164	826.602037	226.342006	6398.3848	221.801745	47029.9277	0	10033.9178	239.273881	72083.9325	
7	E1SPCtrR1	E1SPCtr	0	0	250.042449	152.904983	923.248743	0	2612.93396	1147.38893	59658.7317	61.9549691	3516.64536	4013.03648	0	135
8	E1SPCtrR2	E1SPCtr	0	512.959875	160.649321	53.4744329	1293.29601	472.479969	5234.074	0	41675.3539	227.036134	4122.86665	1581.67073	213.509742	
9	E1SPCtrR3	E1SPCtr	0	320.73092	140.705949	0	1148.93728	110.51533	3517.78049	195.310869	41775.6675	0	4616.39992	2945.17997	1441.19076	161
10	E1SPCtrR4	E1SPCtr	73.6578638	601.100663	209.663468	206.861622	23.730202	202.06454	2768.78787	115.458443	54678.7543	626.817154	7507.91822	1301.93141	532.126903	
11	E1SPCtrR5	E1SPCtr	0	776.769558	151.516619	0	916.579052	207.200542	5171.95376	453.573175	42729.731	298.601873	7616.52302	393.511701	1293.24959	
12	E1SPGlyR1	E1SPGly	0	395.27005	38.1504254	186.034396	412.384439	238.279816	1908.64242	1087.52516	22355.6657	0	4400.22459	0	659.341119	
13	E1SPGlyR2	E1SPGly	0	668.654936	126.652293	33.773326	0	74.1693184	1920.40287	237.278494	20903.0946	0	5953.04581	758.287077	773.972814	
14	E1SPGlyR3	E1SPGly	0	35.5120822	190.958912	38.2262457	249.277161	151.688411	3818.07289	828.115492	56169.3357	83.7229312	1859.05071	713.957564	0	
15	E1SPGlyR4	E1SPGly	0	901.42961	589.267577	0	0	0	918.818859	799.306052	42794.2826	0	2019.86213	992.599336	524.349113	
16	E1SPGlyR5	E1SPGly	0	734.616914	42.6436173	0	961.073181	366.173461	3347.39354	536.274083	42823.3866	0	12282.716	978.207923	624.219454	175
17	E1STBelR1	E1STBel	0	68.8695424	0	0	0	0	3522.78423	1454.68962	58875.1043	75.3506381	7192.33498	5949.64637	116899.368	
18	E1STBelR2	E1STBel	0	769.26052	451.972695	140.162901	1246.3858	194.449746	965.425381	654.510558	52717.4817	0	31.8088525	2204.34398	95208.2932	
19	E1STBelR3	E1STBel	0	510.254502	0	0	3506.13735	52.4959437	4119.45939	337.355137	44547.7237	0	10431.7024	3004.99844	69045.9475	
20	E1STBelR4	E1STBel	0	36.0771304	286.132935	52.0672109	1303.18359	76.6362364	3342.06895	100.266542	6000.78549	0	7120.39626	235.755147	71149.8337	
21	E1STBelR5	E1STBel	170.576106	460.040319	0	24.1238043	503.673538	391.458847	3214.27874	127.517014	39864.5468	229.832956	5099.67488	1203.11364	70249.9475	
22	E1STCtrR1	E1STCtr	0	66.6060154	308.551265	66.2588258	1643.38276	0	68.7922451	1077.54787	64852.4601	115.537645	1933.27137	2617.8444	1827.41244	
23	E1STCtrR2	E1STCtr	0	108.204713	260.022411	28.1444383	0	0	386.920357	342.197556	39643.9503	262.407706	5061.63818	62.897362	0	

Extraction efficiency

► Criteria to judge the optimal extraction:

1. The ability to cover the **largest range of polarity** (qualitative)
2. The best **extraction yield** (quantitative)
3. The best reproducibility
4. The performance in discrimination between polluted and unpolluted groups (EMF objective)

Extraction efficiency

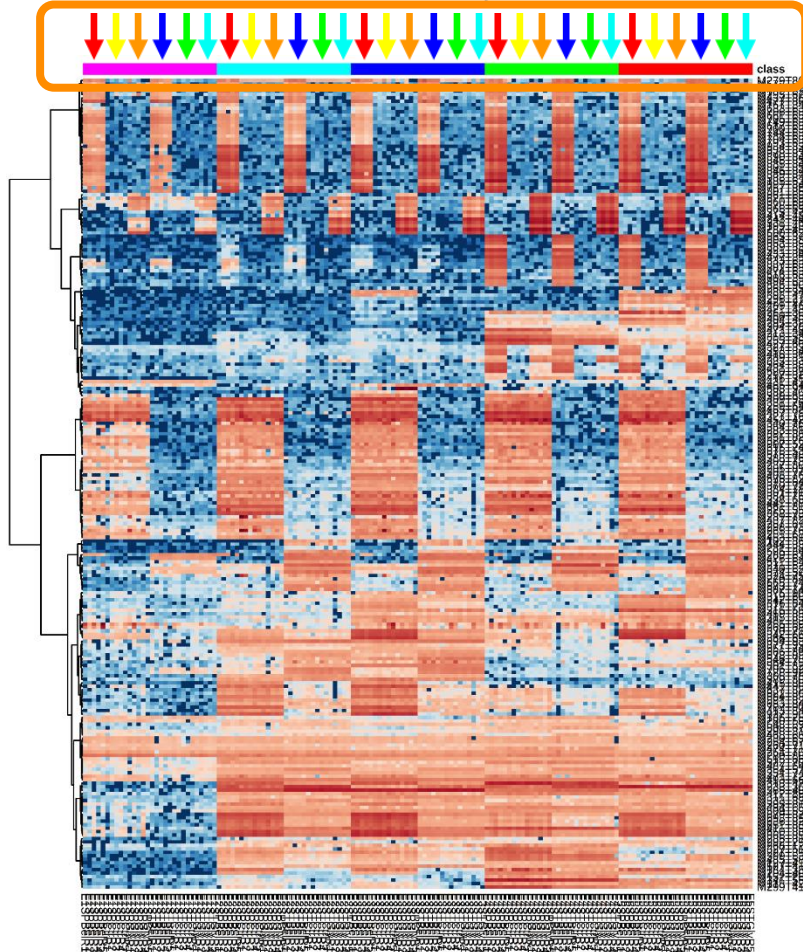


*Heatmap is a powerful tool
to visualize the whole data matrix*

Wilkinson & Friendly. *Am Stat* (2009), 63(2):179-184

- ▶ The 3D (Molecular traces; Samples; Intensities) are projected in a colored figure

Extraction efficiency



- class
- E1: EtOAc + HCl
 - E2: MeOH
 - E3: MeOH + FA
 - E4: DE-Mix
 - E5: DE-Mix Acid

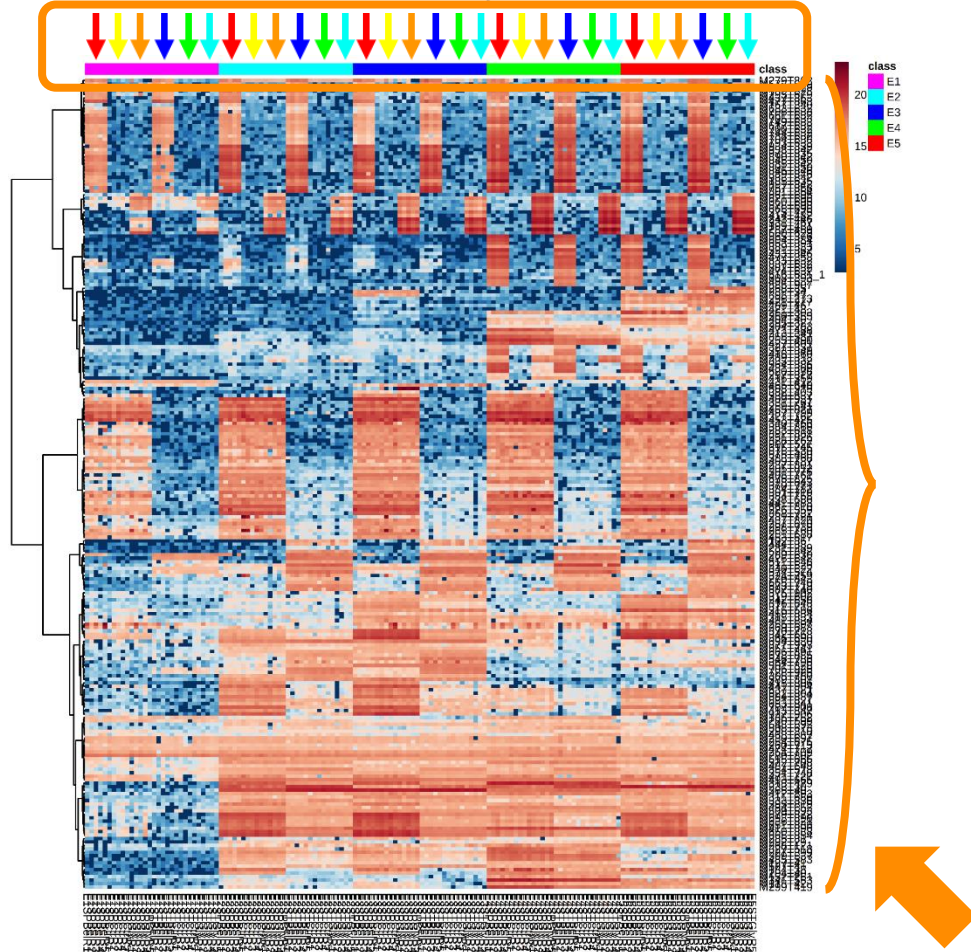
- Soil Perpignan - Bel
- Soil Perpignan - Ctr
- Soil Perpignan - Gly
- Soil Torreilles - Bel
- Soil Torreilles - Ctr
- Soil Torreilles - Gly

Heatmap is a powerful tool
to visualize the whole data matrix

Wilkinson & Friendly. *Am Stat* (2009), 63(2):179-184

- The 3D (Molecular traces; Samples; Intensities) are projected in a colored figure
- Samples can be sorted following the examined factors

Extraction efficiency

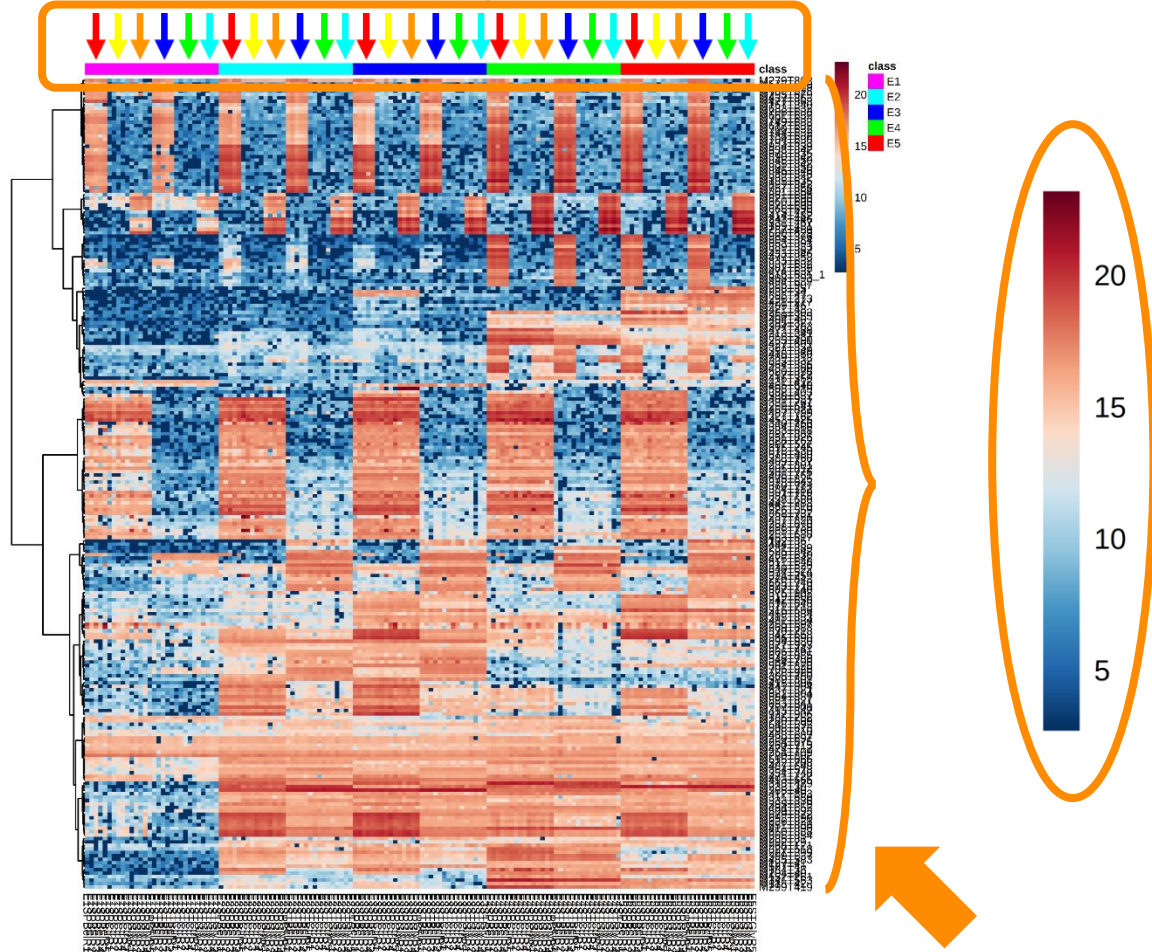


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- ▶ The 3D (Molecular traces; Samples; Intensities) are projected in a colored figure
- ▶ **Samples** can be sorted following the **examined factors**
- ▶ **Molecular traces** are sorted following their **behavior through samples**

Extraction efficiency



*Heatmap is a powerful tool
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Wilkinson & Friendly. *Am Stat* (2009), 63(2):179-184

- ▶ The 3D (Molecular traces; Samples; Intensities) are projected in a colored figure
- ▶ **Samples** can be sorted following the **examined factors**
- ▶ **Molecular traces** are sorted following their **behavior** through samples
- ▶ **Intensities** are represented by **color gradient**

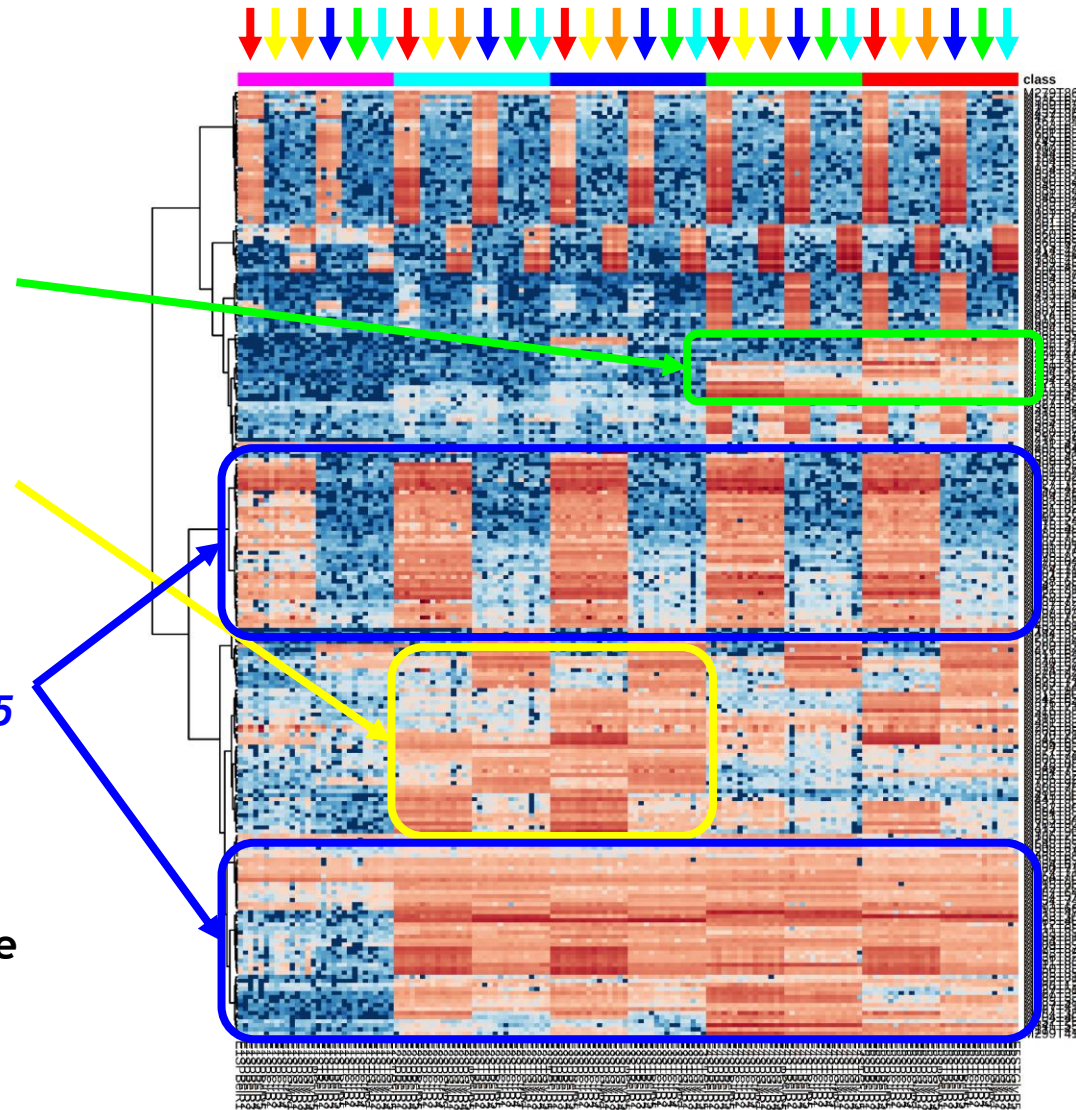
Extraction efficiency

Endometabolites more detected in E4 and E5

Endometabolites more detected in E2 and E3

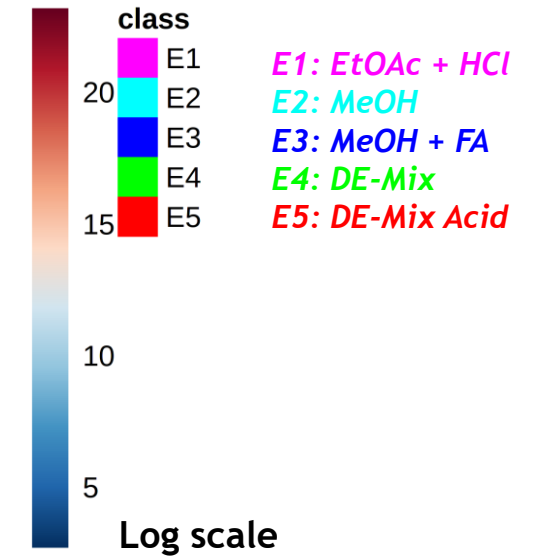
Endometabolites more detected in E2, E3, E4 and E5

- ✓ Compromises to be done
- ✓ Other criteria to be decisive



Condition indicators:

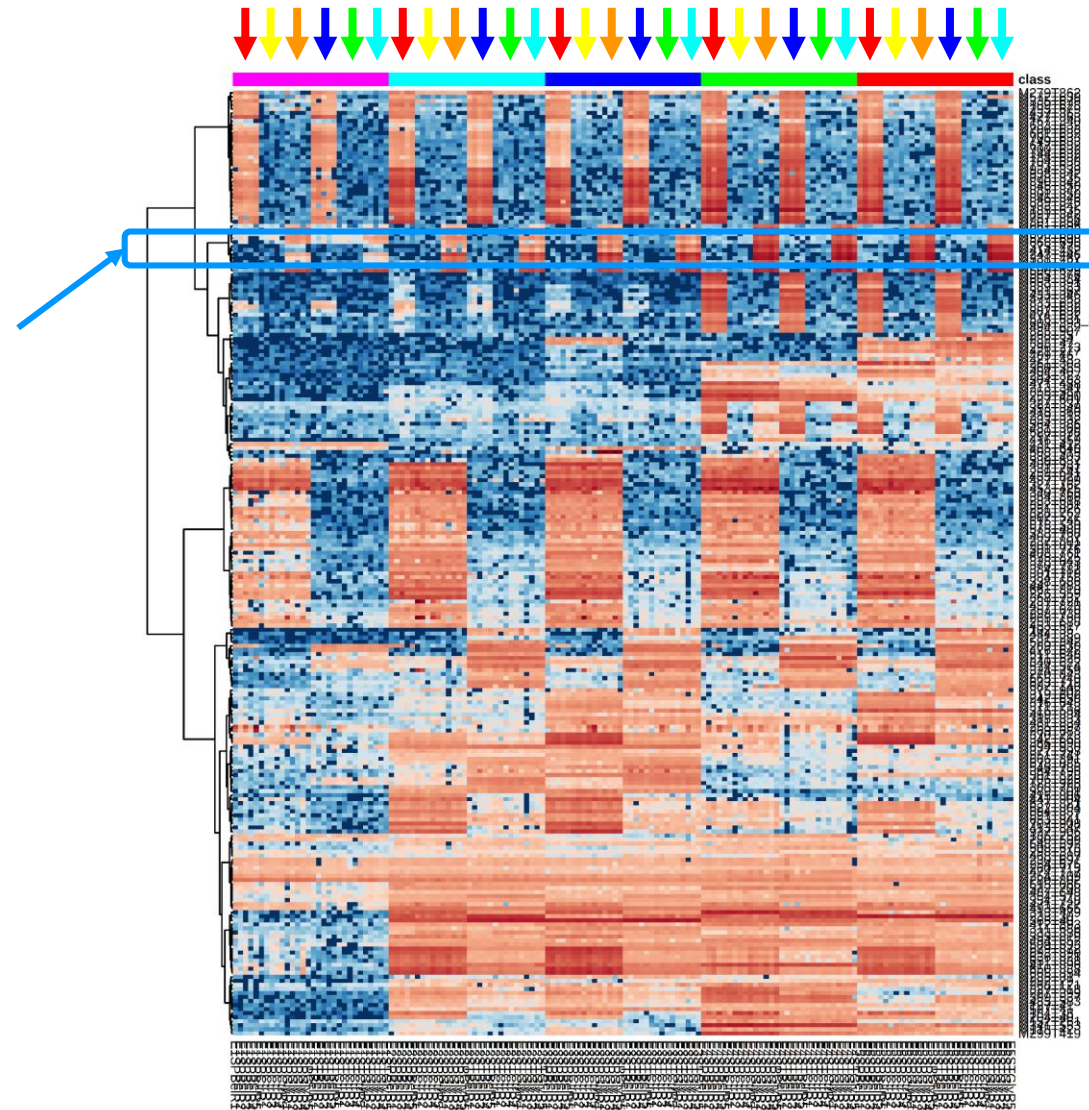
- : Soil Perpignan - Bel
- : Soil Perpignan - Ctr
- : Soil Perpignan - Gly
- : Soil Torreilles - Bel
- : Soil Torreilles - Ctr
- : Soil Torreilles - Gly



Extraction efficiency

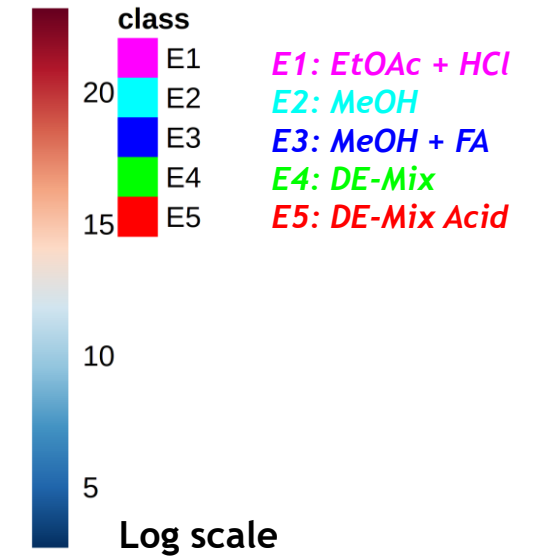
Xenometabolome originating from Tartan Super 360™ herbicide

- ✓ Intensity increasing from E1 to E5
- ✓ Highest intensities in E4



Condition indicators:

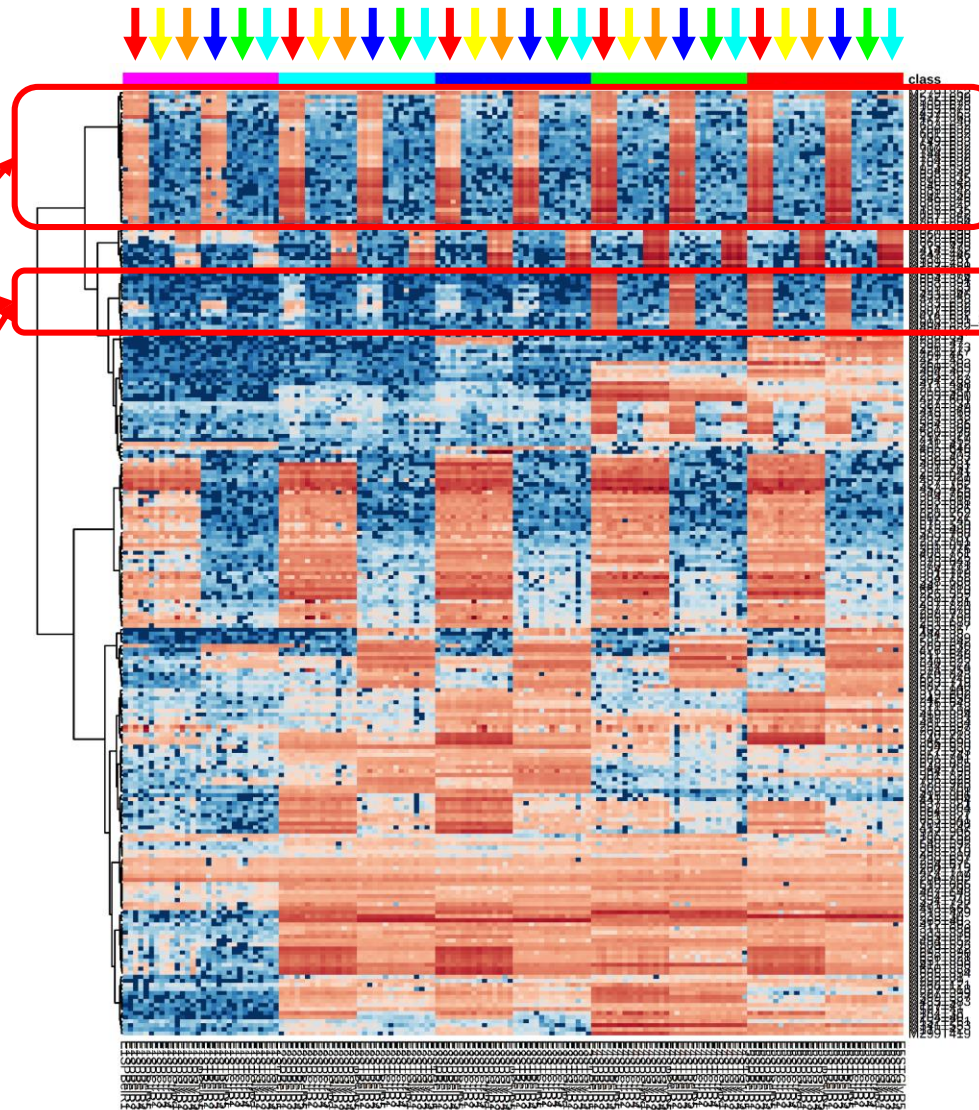
- ➔: Soil Perpignan - Bel
- ➔: Soil Perpignan - Ctr
- ➔: Soil Perpignan - Gly
- ➔: Soil Torreilles - Bel
- ➔: Soil Torreilles - Ctr
- ➔: Soil Torreilles - Gly



Extraction efficiency

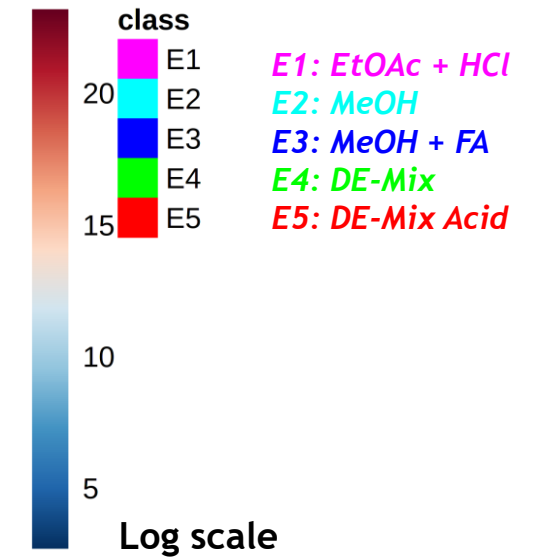
*Xenometabolome
originating from Beloukha®
herbicide*

- ✓ Intensity increasing from E1 to E5
- ✓ Xenometabolites only detected in E4 and E5
- ✓ Highest intensities in E4



Condition indicators:

- ➔: Soil Perpignan - Bel
- : Soil Perpignan - Ctr
- : Soil Perpignan - Gly
- : Soil Torreilles - Bel
- : Soil Torreilles - Ctr
- : Soil Torreilles - Gly



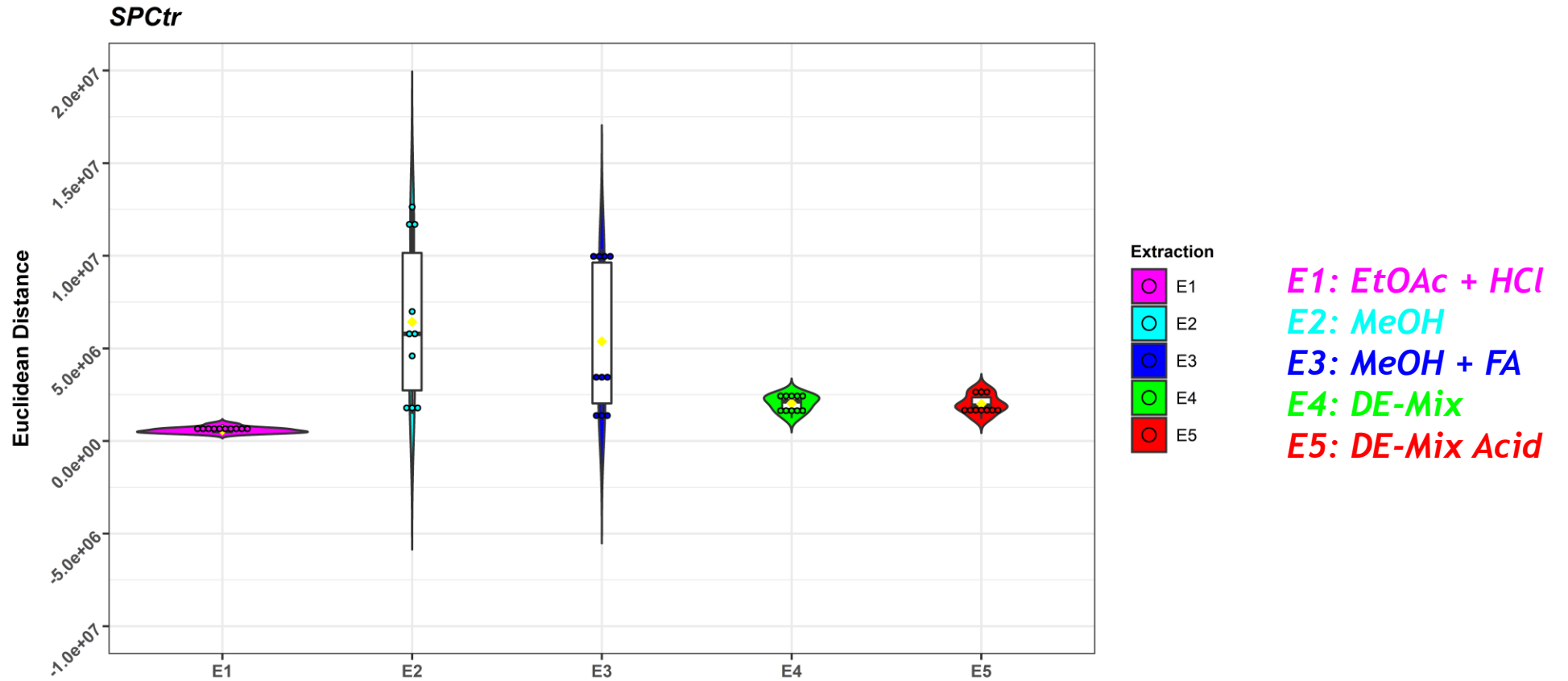
Extraction reproducibility

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2. The best extraction yield (quantitative)
3. **The best reproducibility**
4. The performance in discrimination between polluted and unpolluted groups (EMF objective)

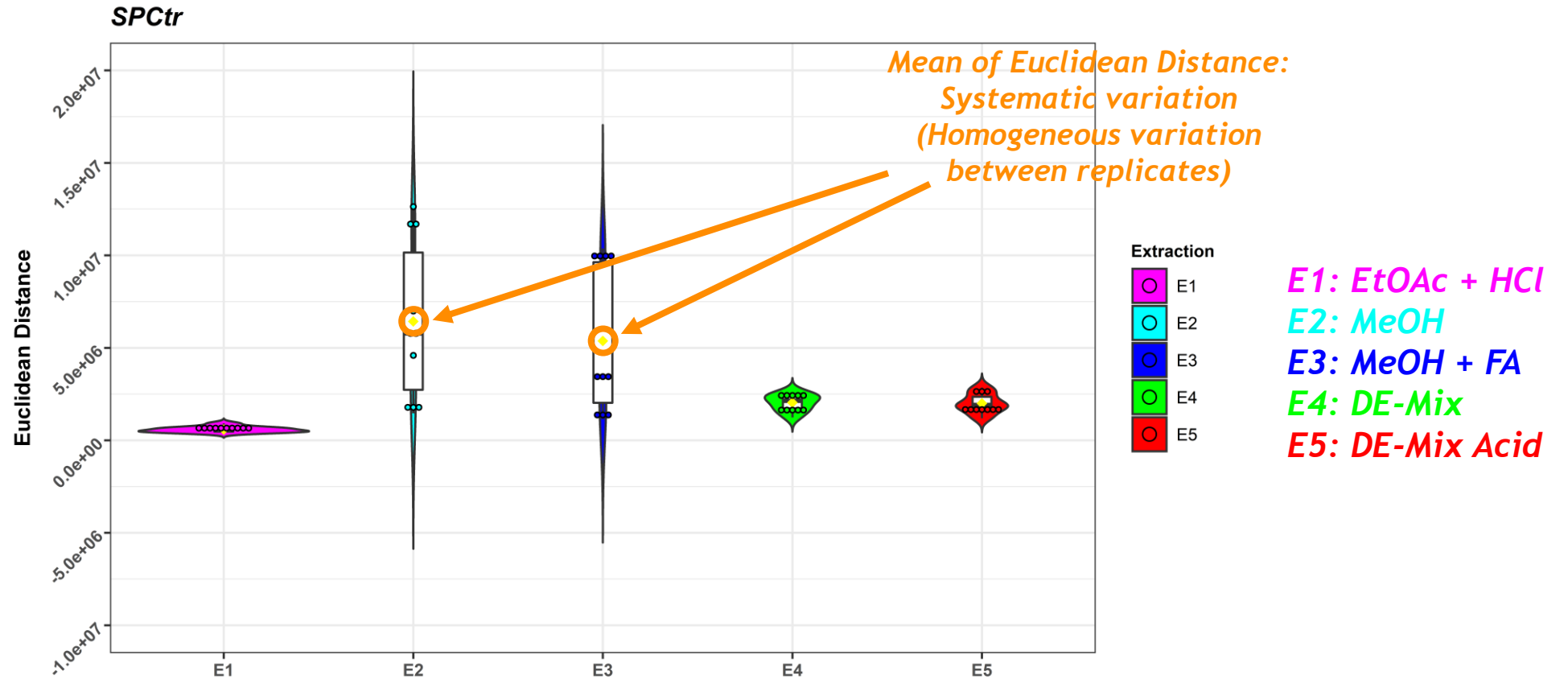
Extraction reproducibility

- ▶ Euclidean Distances calculation: to assess extraction's "inter-replicates" reproducibility



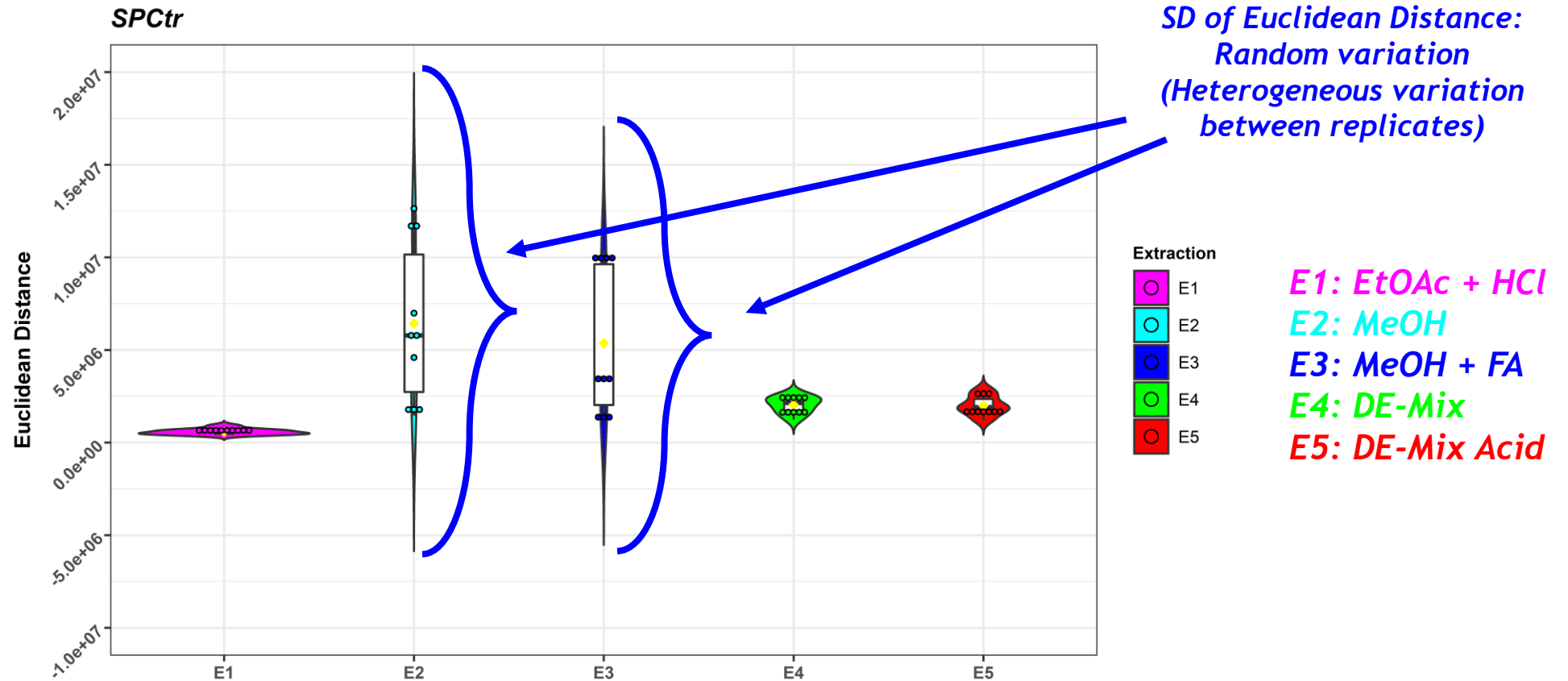
Extraction reproducibility

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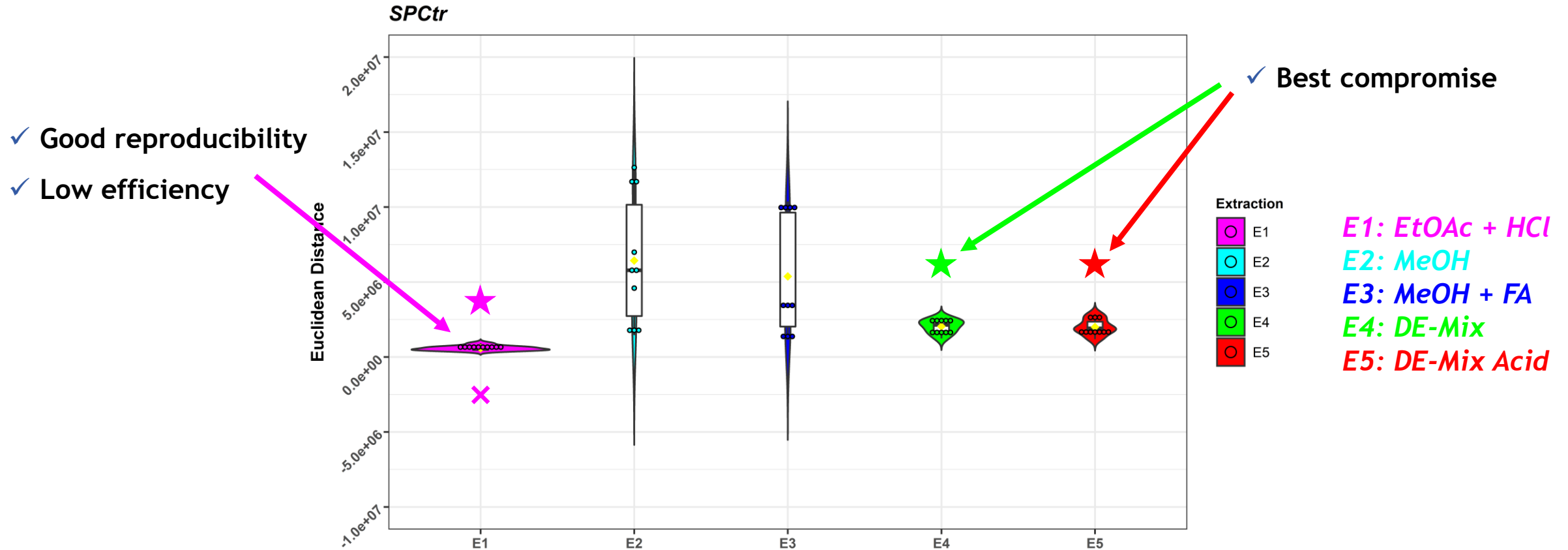
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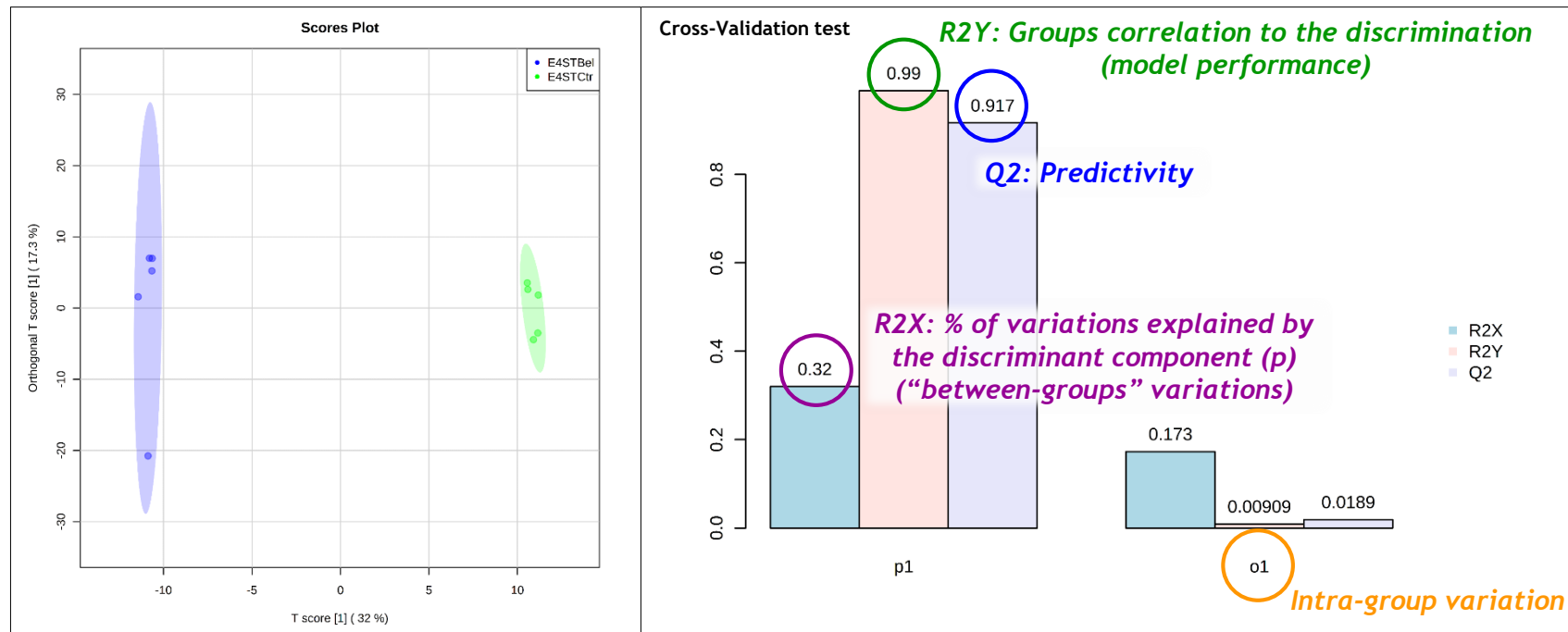
Discrimination ability

► Criteria to judge the optimal extraction:

1. The ability to cover the largest range of polarity (qualitative)
2. The best extraction yield (quantitative)
3. The best reproducibility
4. The **performance in discrimination** between polluted and unpolluted groups (EMF objective)

Discrimination ability

- OPLS-DA is an **explicative, supervised** multivariate statistical analysis
- It allows to assess the **ability** and the **predictivity** of an extraction to **discriminate** between a **control group** of soil samples and a **herbicide-spiked group** of soil samples



Trygg & Wold. *J Chemometrics* (2002), 16:119-128
Boccard & Rutledge. *Anal Chim Acta* (2013), 769:30-39

Discrimination ability

	SP						ST					
	p1			o1			p1			o1		
	R2X	R2Y	Q2	R2X	R2Y	Q2	R2X	R2Y	Q2	R2X	R2Y	Q2
<i>Gly</i>												
E1	17.90	97.40	61.20	14.00	02.51	03.33	16.70	98.50	60.00	10.90	01.51	04.51
E2	19.30	96.40	67.10	12.40	03.21	04.34	17.70	98.10	63.00	10.50	01.74	04.21
E3	16.60	95.80	66.10	15.30	03.56	05.07	18.50	95.60	59.90	13.20	04.19	02.68
E4	24.90	97.70	80.80	10.60	02.14	02.63	20.10	97.80	72.10	13.50	02.06	05.51
E5	20.30	96.40	70.20	10.20	03.37	10.40	18.60	97.00	63.30	13.60	02.83	05.54
<i>Bel</i>												
E1	22.10	99.40	77.50	12.00	00.58	03.46	19.90	96.40	68.60	13.20	03.51	03.53
E2	29.80	98.60	88.40	11.90	01.31	02.56	29.40	96.50	85.60	09.71	03.40	01.34
E3	26.80	97.40	81.60	09.72	02.43	03.46	25.60	96.50	81.00	13.40	03.35	03.30
E4	37.20	98.90	93.40	10.70	01.07	01.39	33.00	98.00	90.80	15.00	01.87	02.53
E5	34.20	96.90	88.40	09.83	02.84	01.17	31.80	98.40	90.00	10.00	01.43	02.42

Extraction development

Summary

- ★ The novel **mix-based extractions** are **promising** for the **EMF** approach
- ➔ Compared to **classic simple extractions**, those protocols can assure:
 - ✓ A highly efficient **xenometabolome** extraction
 - ✓ A **wide coverage** of **endometabolome** in term of polarity
 - ✓ An **acceptable reproducibility**
 - ✓ A **good ability** to **discriminate** between **polluted** and **unpolluted** soils

Summary

- ★ **A novel approach for methodological development based on untargeted metabolomics tools was set-up:**
 - ✓ **Heatmap** → exhaustive **qualitative** and **quantitative** assessment
 - ✓ **Euclidean Distances** → to assess **reproducibility**
 - ✓ **OPLS-DA** → to assess the **ability** to respond to the **environmental objective**
(discrimination between the two conditions)

What is next?

- **Chemometric and statistical workflows** are being in-depth investigated for **kinetics studies** (IMpACT Project)
- Further **laboratory and field** applications: towards assessing the **environmental fate and impact of complex (bio)pesticides**

What is next?

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Stay tuned with the **NEXT PRESENTATION:**

O27 – Application of an innovative method to study the future and the environmental impact of biopesticides: results and prospects

A. Mejait et al.

Acknowledgments



Workflow4metabolomics



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**Thank you for
your attention**