



Characterisation of wastewater effluents in the Danube River Basin with target and non-target chemical screening techniques, *in vitro* bioassays and antibiotic resistant genes analysis

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- **Sampling campaign of effluent wastewater in Danube River Catchment**
- **Chemical characterization using wide-scope screening methods**
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- **Application of storage of chromatograms for future use by regulatory authorities; Case of Bis(4-chlorophenyl) sulphone**
- **Use of NTS data for prioritisation of the Danube RBSPs**

Location of WWTPs



List of WWTPs in the Danube River Basin selected for effluent monitoring

Country	Town	PE	Treatment type
Romania	Bucharest	1,327,995	tertiary
Romania	Cluj-Napoca	382,031	tertiary
Serbia	Šabac	84,000	tertiary
Croatia	Varaždin	97,450	secondary
Croatia	Zagreb	842,425	secondary
Slovenia	Ljubljana	537,712	secondary
Slovenia	Vipap	152,487	tertiary
Hungary	Budapest	1,174,643	tertiary
Slovak Republic	Žilina	139,934	tertiary
Czech Republic	Brno-Modřice	397,945	tertiary
Austria	Amstetten	150,000	tertiary
Germany	Augsburg	659,387	tertiary

WWTP samples – SOLUTIONS parameters



x12



Chemical Analysis

Highly sensitive determination of **antibiotics and their TPs**

Target screening of **2248 compounds**

Suspect screening (>40,000 substances)

Non-target screening

Upload of the chromatograms to the DSFP



BioDetection Systems



Bioassays

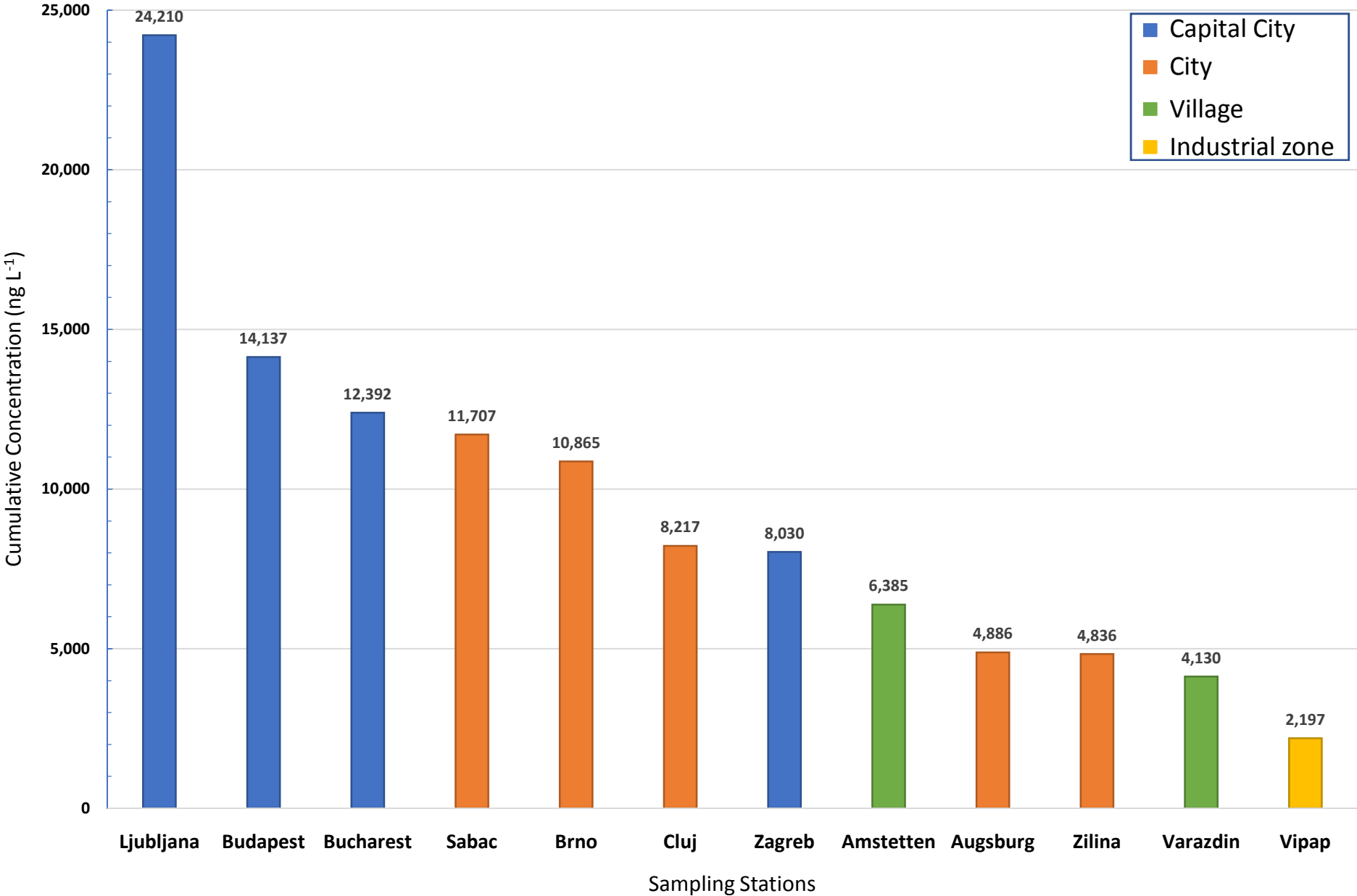
CALUX ER α
CALUX anti-AR
CALUX anti-PR
CALUX GR
CALUX PPAR α 2
CALUX PPAR γ 2
CALUX PAH
CALUX PXR
CALUX Nrf2
CALUX Cytotox



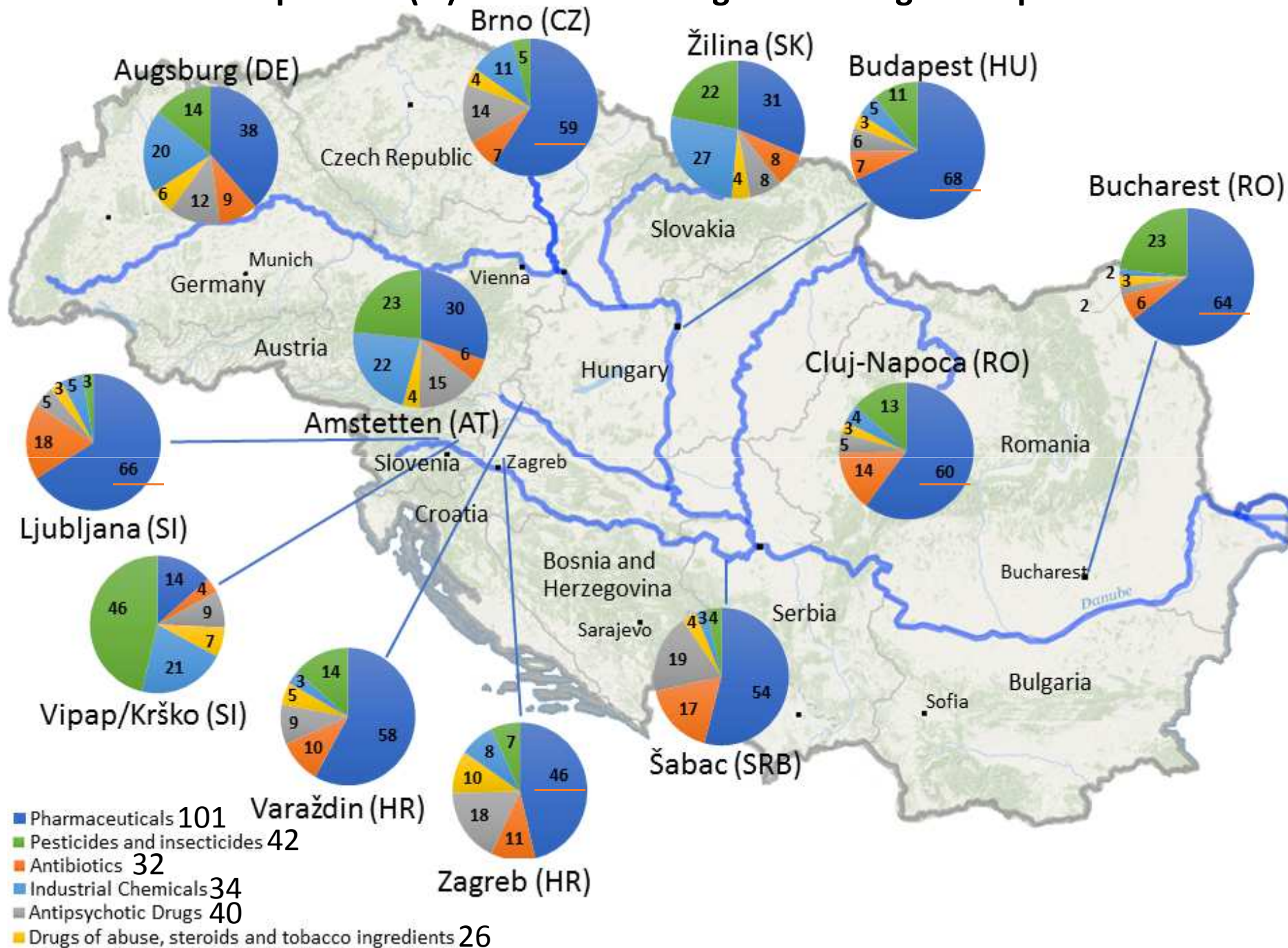
Genomics

Determination of **ARGs** by **multiplex-PCR**

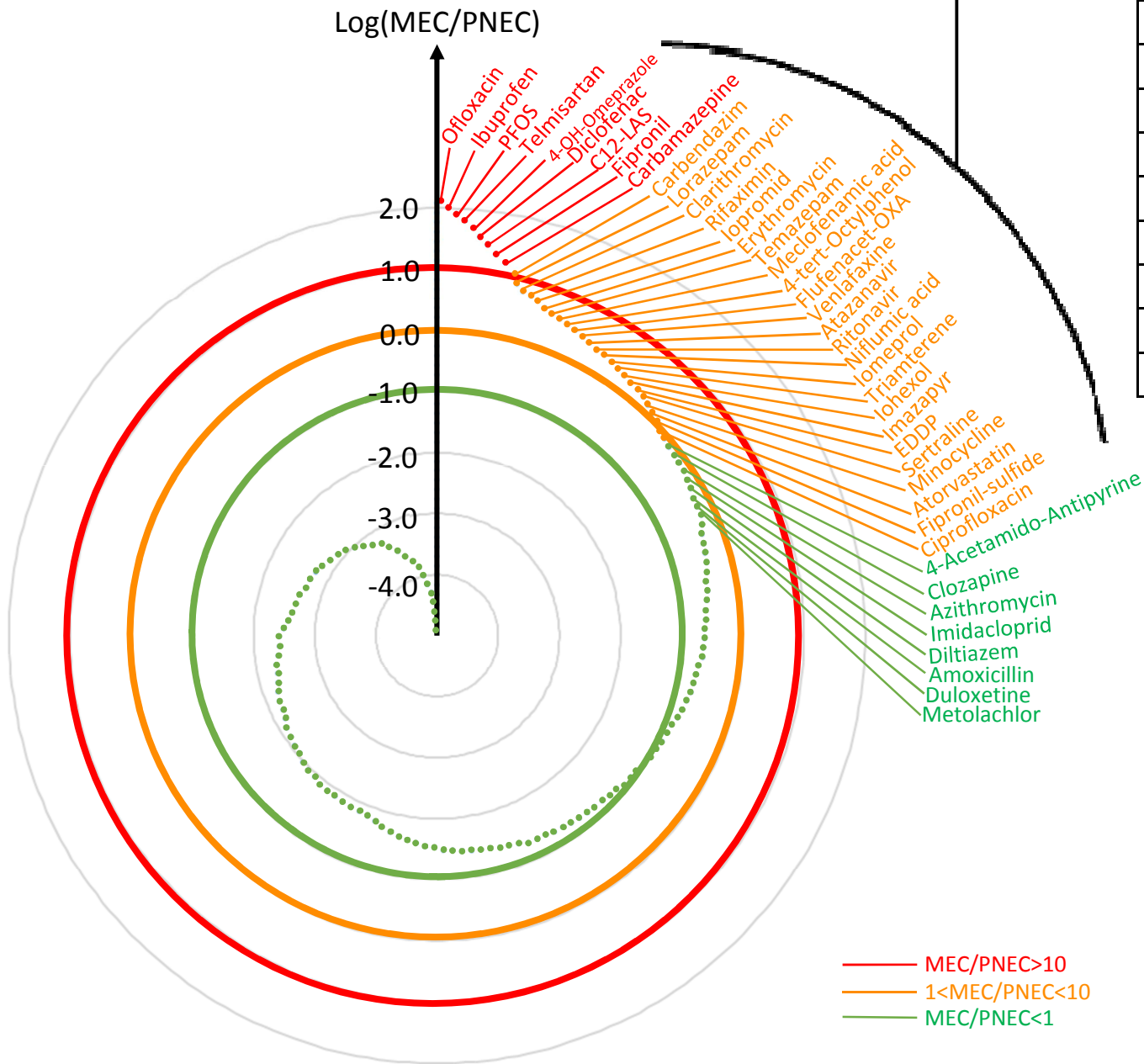
Cumulative concentration of detected targets in the sampling stations



Composition (%) of detected categories of target compounds



Frequency of Exceedance (0-1)
 Extent of Exceedance (0-1)
 Frequency of Exceedance (0-1)



Bioassay results expressed as fold-induction relative to the LOQ of the respective bioassay

	Cytotox CALUX	anti-AR CALUX	ERa CALUX	GR CALUX	anti-PR CALUX	PPARa2 CALUX	PPARG2 CALUX	PAH CALUX	PXR CALUX	Nrf2 CALUX
Varazdin	4.5	0.5	49	0.5	4.9	2.3	0.6	40	2.3	1.2
Amstetten	0.5	1.9	10	0.5	5.3	0.5	0.5	68	3.6	1.4
Cluj	2.3	2.7	0.5	0.7	12	0.5	0.5	28	2.3	0.5
Augsburg	0.5	0.9	8.8	1.5	1.7	0.5	0.5	38	3.6	1.4
Vipap	0.8	2.7	5.6	0.5	7.1	0.5	0.5	159	9.2	2.1
Budapest	0.5	1.0	5.3	0.5	3.1	0.5	0.5	46	3.0	1.5
Ljubljana	0.5	0.7	60	3.8	3.6	0.5	0.5	17	2.7	1.5
Bucharest	2.0	0.5	69	1.3	6.6	0.5	0.5	22	2.9	3.9
Zilina	0.5	0.8	20	1.0	0.5	0.5	0.5	57	1.1	1.8
Sabac	0.5	1.2	9.5	0.5	0.5	0.5	0.5	57	0.8	1.4
Brno	0.5	1.1	10	1.1	0.9	1.2	0.5	80	1.7	2.4
Zagreb	0.5	0.5	15	0.5	0.5	0.5	0.5	34	1.6	0.5

<LOQ marked as 0.5

Comparison of bioactivities with EBTs

Sampling stations	Escher et al. (2018)								van der Oost et al. (2017)							
	ERa CALUX	anti-AR CALUX	GR CALUX	anti-PR CALUX	PPARg CALUX	PAH CALUX	Nrf2 CALUX	PXR CALUX	ERa CALUX	anti-AR CALUX	GR CALUX	anti-PR CALUX	PPARg CALUX	PAH CALUX	Nrf2 CALUX	PXR CALUX
Varazdin	Orange	Green	a	Orange	a	Orange	Orange	Green	X	Orange	Green	a	Orange	Green	Orange	Orange
Amstetten	Orange	Orange	a	Orange	a	Orange	Orange	Orange	X	Orange	Green	a	b	Green	Orange	Orange
Cluj	Green	Orange	a	Orange	a	Orange	b	Green	X	Green	Orange	a	b	Green	b	Orange
Augsburg	Orange	Green	a	Orange	a	Orange	Orange	Orange	X	Orange	Green	a	b	Green	Orange	Orange
Vipap	Orange	Orange	a	Orange	a	Orange	Orange	Orange	X	Orange	Orange	a	b	Orange	Orange	Orange
Budapest	Orange	Green	a	Orange	a	Orange	Orange	Orange	X	Orange	Green	a	b	Green	Orange	Orange
Ljubljana	Orange	Green	a	Orange	a	Orange	Orange	Green	X	Orange	Green	a	b	Green	Orange	Orange
Bucharest	Orange	Green	a	Orange	a	Orange	Orange	Green	X	Orange	Green	a	b	Green	Orange	Orange
Zilina	Orange	Green	a	Green	a	Orange	Orange	Green	X	Orange	Green	a	b	Green	Orange	Orange
Sabac	Orange	Green	a	Green	a	Orange	Orange	Green	X	Orange	Green	a	b	Green	Orange	Orange
Brno	Orange	Green	a	Orange	a	Orange	Orange	Orange	X	Orange	Green	a	b	Green	Orange	Orange
Zagreb	Orange	Green	a	Green	a	Orange	b	Orange	X	Orange	Green	a	b	Green	b	Orange

a: no trigger value available; b: LOQ of bioassay exceeding EBT

Putative action plan based on *in vitro* bioassays results

1 < EBT

- No further action required

1 < EBT < 3

- Quality check of data
- Monitor every three months for 1 year until EBT < 1

3 < EBT < 10

- All actions of above category
- Immediate re-sampling and re-analysis to confirm EBT exceedance
- Quantify drivers of toxicity

10 < EBT < 100

- All actions of above category
- Enhance **source identification program**
- Monitor in the **distribution system** closer to the point of exposure to confirm attenuation of CEC is occurring and to confirm the magnitude of assumed safety factors associated with removal efficiency, dilution and post-treatment

EBT > 100

- All actions of above category
- Immediately confer with the **local environmental authorities** to determine the required response action
- Confirm plant corrective actions through **additional monitoring** that indicates the CEC levels are below at least an EBT of 100

Application of putative action plan based on *in vitro* bioassays results

	ER α CALUX	anti-AR CALUX	GR CALUX	PPARY CALUX	PAH CALUX	Nrf2 CALUX
Varazdin	5	5.7	<19	640	72	41
Amstetten	1.1	22	<20	<520	122	46
Cluj	<0,06	31	34	<420	52	<50
Augsburg	1	10	72	<410	72	46
Vipap	0.65	32	<25	<460	242	74
Budapest	0.56	11	<23	<430	62	46
Ljubljana	6.6	8.4	120	<350	62	50
Bucharest	7.4	5.7	38	<340	82	130
Zilina	2.2	8.9	78	<480	72	60
Sabac	1.1	14	<41	<490	72	46
Brno	0.54	13	47	<1100	122	80
Zagreb	0.8	6	<42	<1100	52	<17

1<EBT<3

- Quality check of data
- Monitor every three months for 1 year until EBT < 1

Application of putative action plan based on *in vitro* bioassays results

	ER α CALUX	anti-AR CALUX	GR CALUX	PPARY CALUX	PAH CALUX	Nrf2 CALUX
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3<EBT<10

- Quality check of data
- Monitor every three months for 1 year until EBT < 1
- Immediate **re-sampling and re-analysis** to confirm EBT exceedance
- Quantify drivers of toxicity**

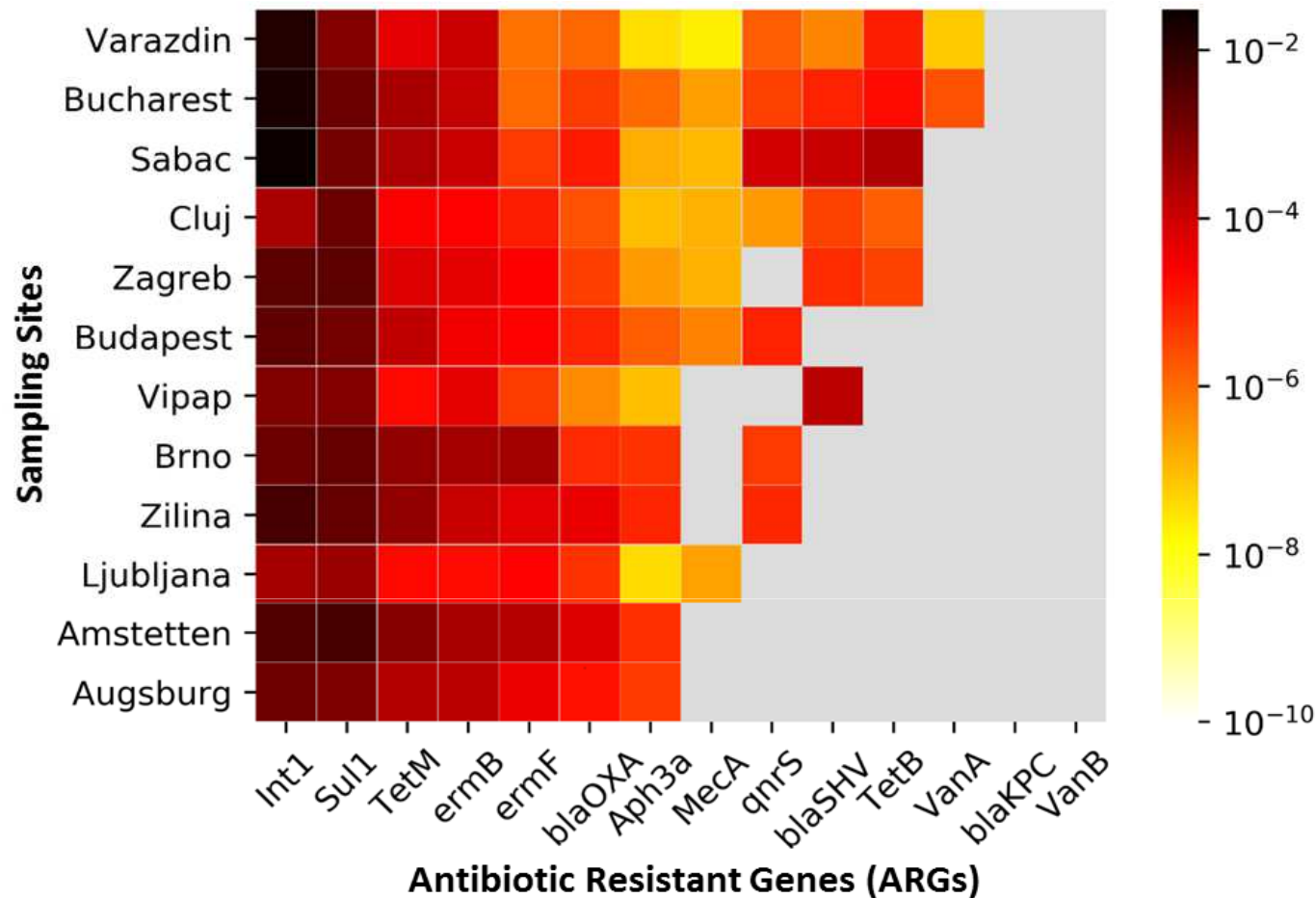
Application of putative action plan based on *in vitro* bioassays results

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- Enhance **source identification program**
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10<EBT<100

Concentration of ARGs [Gene copies/16S rRNA]



Int1: Measure of mobile resistant elements present

Sul1: ARG against **sulfonamides**

tetM, tetB: ARG against **tetracycline**

ermB, ermF: ARG against **macrolides and lincosamide**

blaOXA, blaSHV, blaKPC, MecA: ARG against **beta-lactamase**

Aph3a: ARG against **aminoglycosides**

qnrS: ARG against **fluoroquinolones**

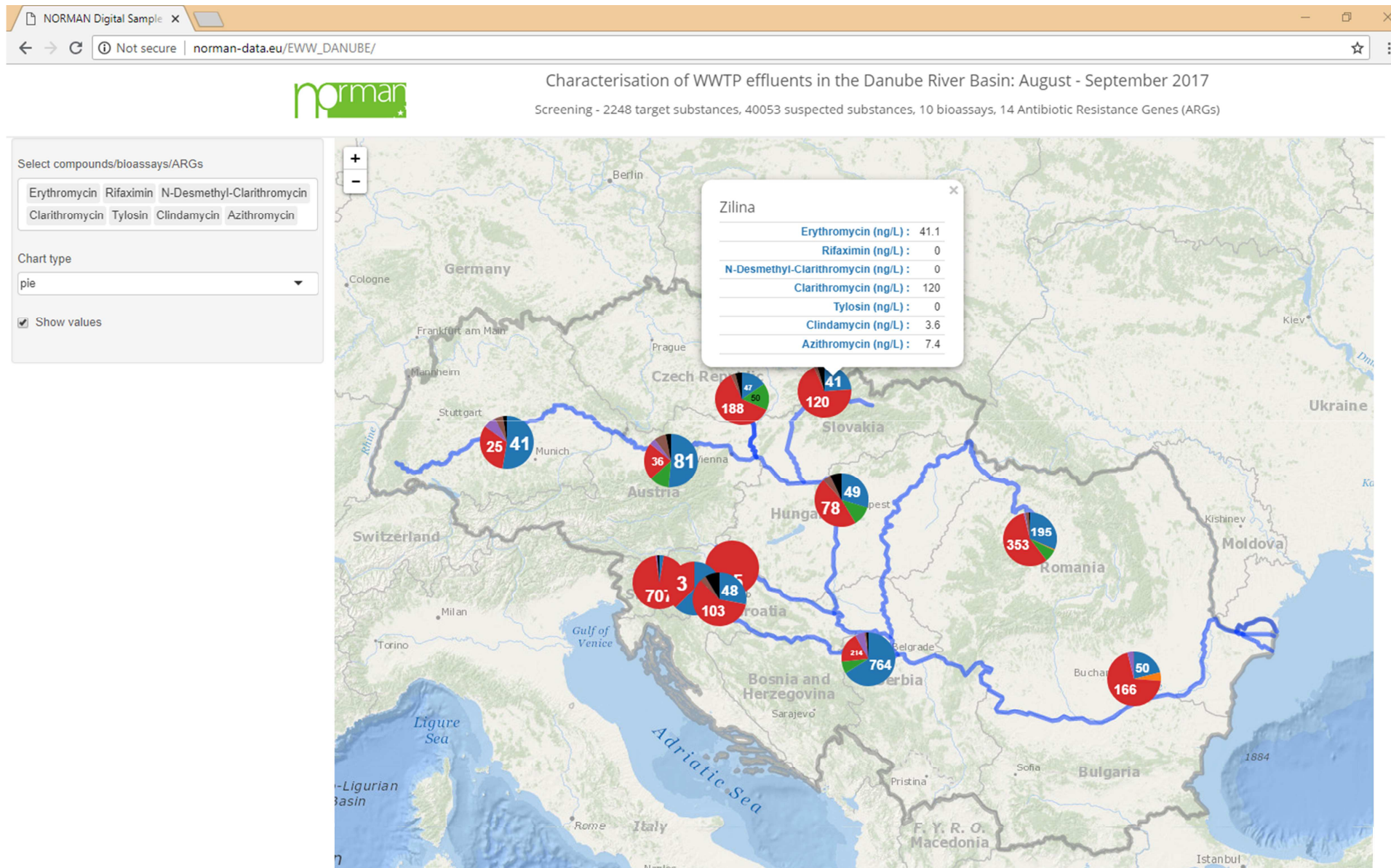
VanA, VanB: ARG against **vancomycin**

High correlation factors between A&ARGs

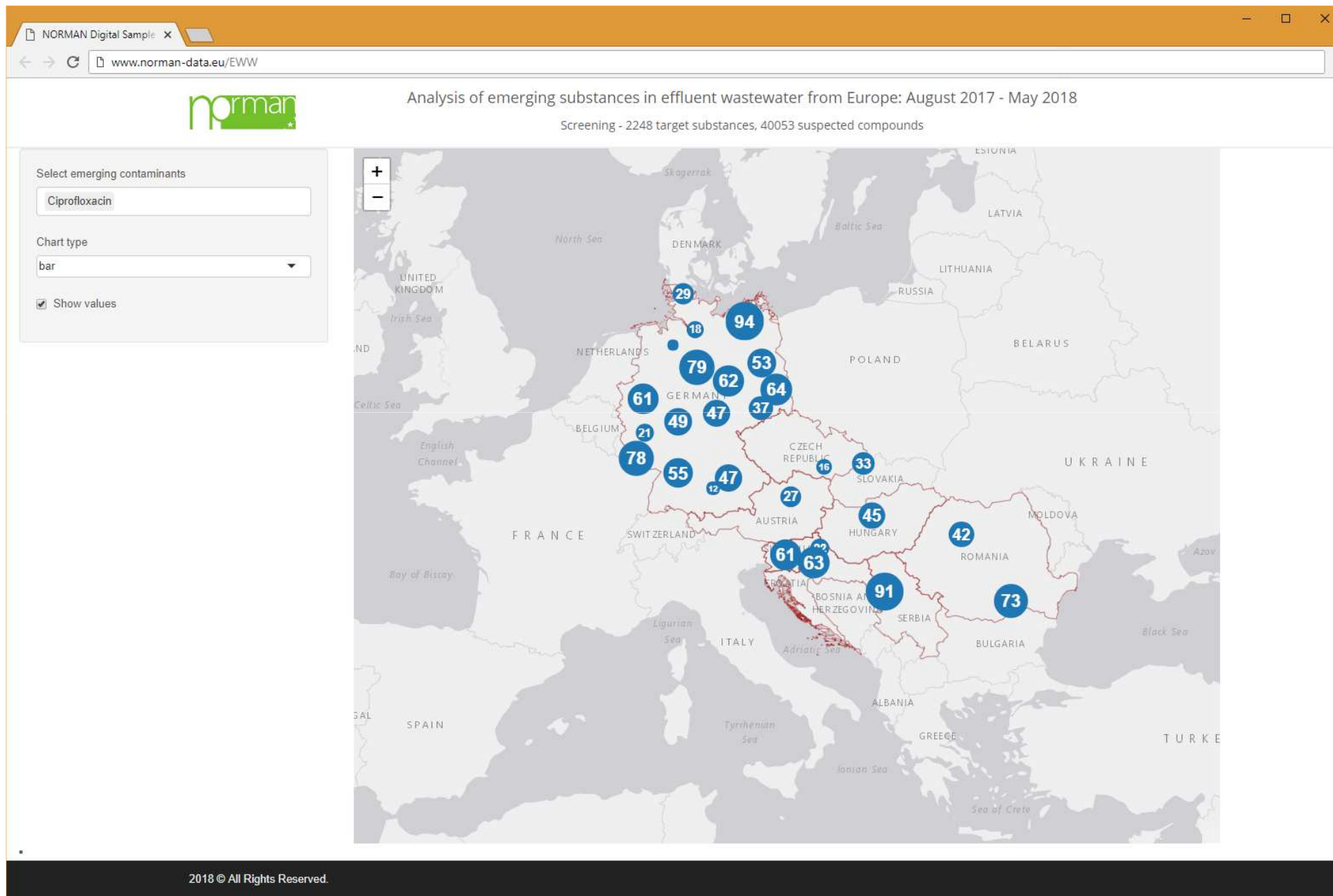
in some cases; $r \approx 0.77$ for qnrS and sum of Oxolinic Acid, Marbofloxacin, Enrofloxacin, Ofloxacin, Norfloxacin, Ciprofloxacin, Flumequine

Online interface for results of all analysis [Chemical/Effects/Genes]

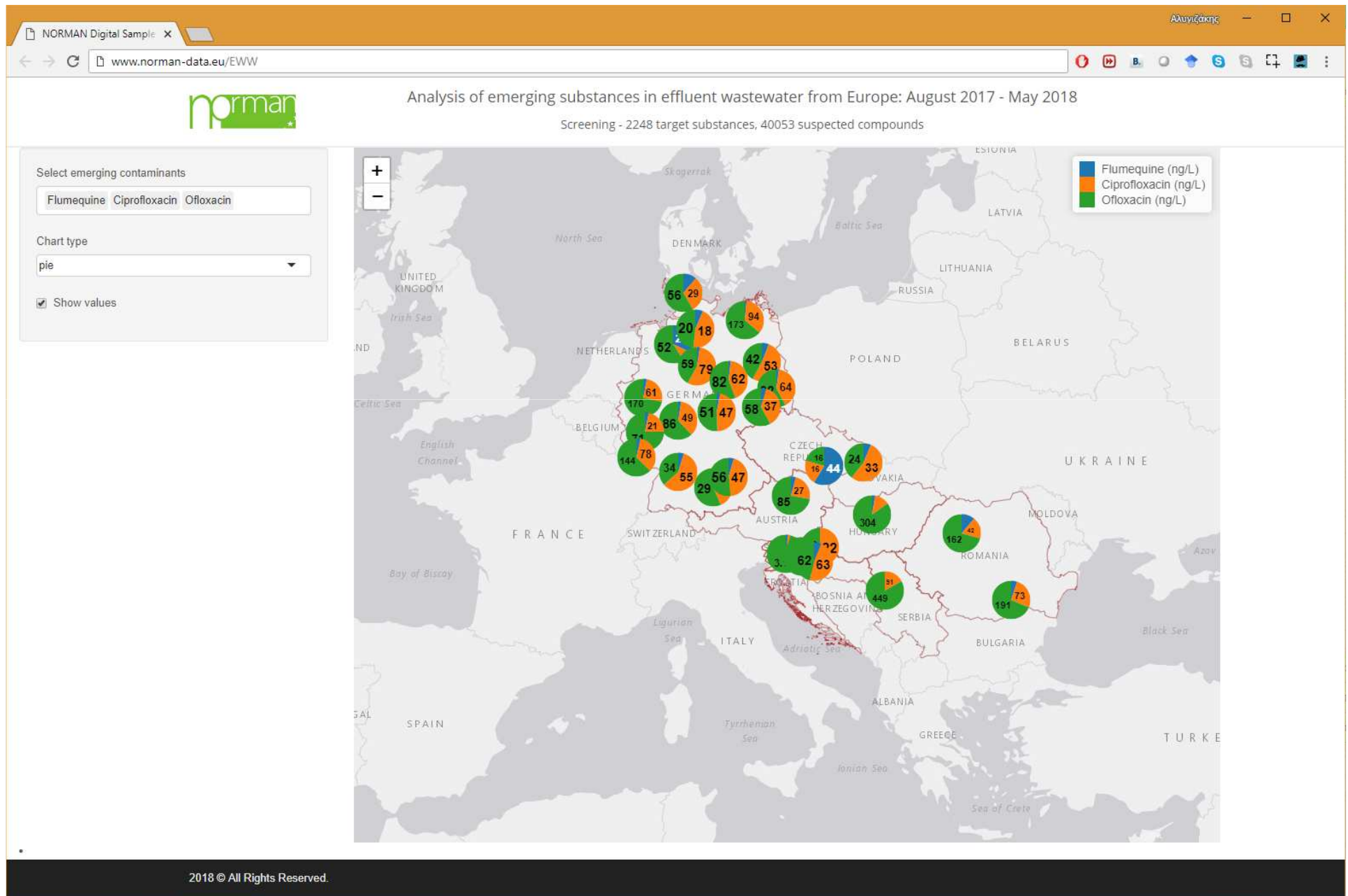
www.norman-data.eu/EWW_DANUBE



Online interface for results of all analysis [Chemical/Effects/Genes]



Online interface for results of all analysis [Chemical/Effects/Genes]



Storage of Chromatograms for future Chemical Screening

www.norman-data.eu

The screenshot displays the NORMAN Digital Sample Platform web interface. The browser address bar shows the URL norman-data.eu/DigitalSFP/. The interface includes a navigation menu with options like 'Main Page', 'Batch mode', 'Contribute', and 'More tools'. A search section on the left allows filtering by 'Substance name or CAS or StdInChIKey' (with a 'Compound' dropdown), 'Precursor m/z' (with the value '254.059389'), 'Mass error in Da' (with a slider set to 0.003), and 'RTI Tolerance (%)' (with a slider set to 20). A 'Submit Job' button is located below these filters. The main content area shows a list of 'Contributed Samples' with columns for 'Country', 'Matrix', and 'Project'. The table lists 10 entries, all from Germany, with various sample names and file formats (xlsx). A pagination bar at the bottom indicates 'Showing 1 to 10 of 306 entries' and includes navigation links for 'Previous', '1', '2', '3', '4', '5', '...', '31', and 'Next'.

	Country	Matrix	Project
543	Germany	Water-Waste water-Municipal	ITN ANSWER
541	Germany	Water-Waste water-Municipal	ITN ANSWER
539	Germany	Water-Waste water-Municipal	ITN ANSWER
535	Germany	Water-Waste water-Municipal	ITN ANSWER
533	Germany	Water-Waste water-Municipal	ITN ANSWER
531	Germany	Water-Waste water-Municipal	ITN ANSWER
529	Germany	Water-Waste water-Municipal	ITN ANSWER
527	Germany	Water-Waste water-Municipal	ITN ANSWER
525	Germany	Water-Waste water-Municipal	ITN ANSWER
523	Germany	Water-Waste water-Municipal	ITN ANSWER



Storage of Chromatograms for future Chemical Screening

Case of Bis(4-chlorophenyl) sulphone



Main Page Batch mode Contribute More tools

Contributed Samples Results Chromatograms Interactive Map Help

Select Chromatogram
UoA_GC-APCI-QTOF_Seawater particulate matter cell 1 from XL-SPE (600 L) Stretch 1_Odesa_Ukraine_30.08.2017_EMBLAS-II (Survey 2017)_27008.xlsx

Data-Independent		Data-Dependent		chromatogram
show	m/z	accuracy_mDa		
<input checked="" type="checkbox"/>	285.9617	0.010000	UoA_POS_4_GC-APCI-QTOF_Seawater particulate matter cell 1 from XL-SPE (600 L) Stretch 1_Odesa_Ukraine_30.08.2017_EMBLAS-II (Survey 2017)_27008.xlsx	
<input checked="" type="checkbox"/>	285.9617	0.010000	UoA_POS_25_GC-APCI-QTOF_Seawater particulate matter cell 1 from XL-SPE (600 L) Stretch 1_Odesa_Ukraine_30.08.2017_EMBLAS-II (Survey 2017)_27008.xlsx	
<input checked="" type="checkbox"/>	285.9617	0.010000	UoA_POS_DataDependent_GC-APCI-QTOF_Seawater particulate matter cell 1 from XL-SPE (600 L) Stretch 1_Odesa_Ukraine_30.08.2017_EMBLAS-II (Survey 2017)_27008.xlsx	

Submit (Press the button everytime changes are done to the table above)

285.9617 ± 0.01

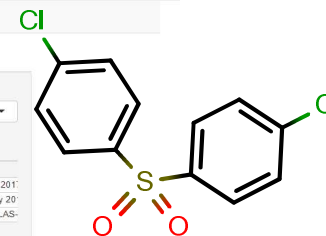
frequency

retention time (min)

V2

frequency

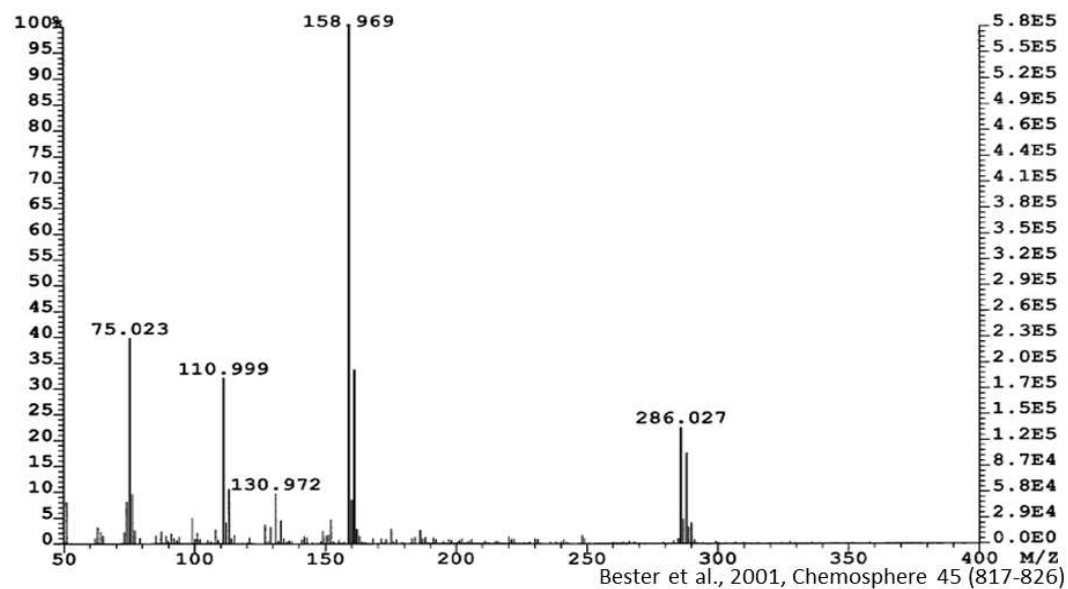
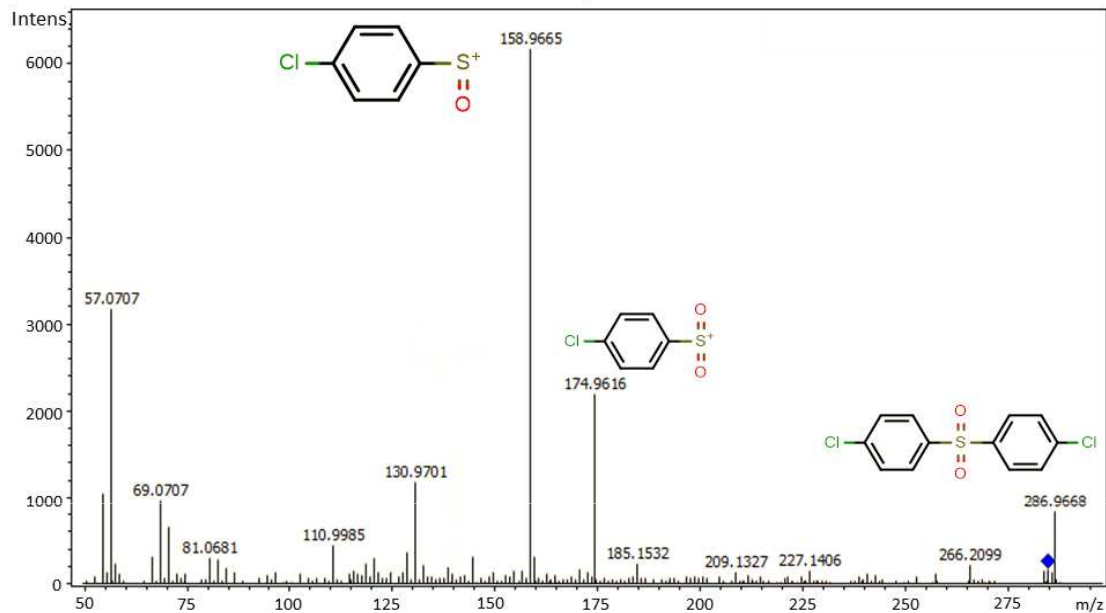
m/z



Storage of Chromatograms for future Chemical Screening

Case of Bis(4-chlorophenyl) sulphone

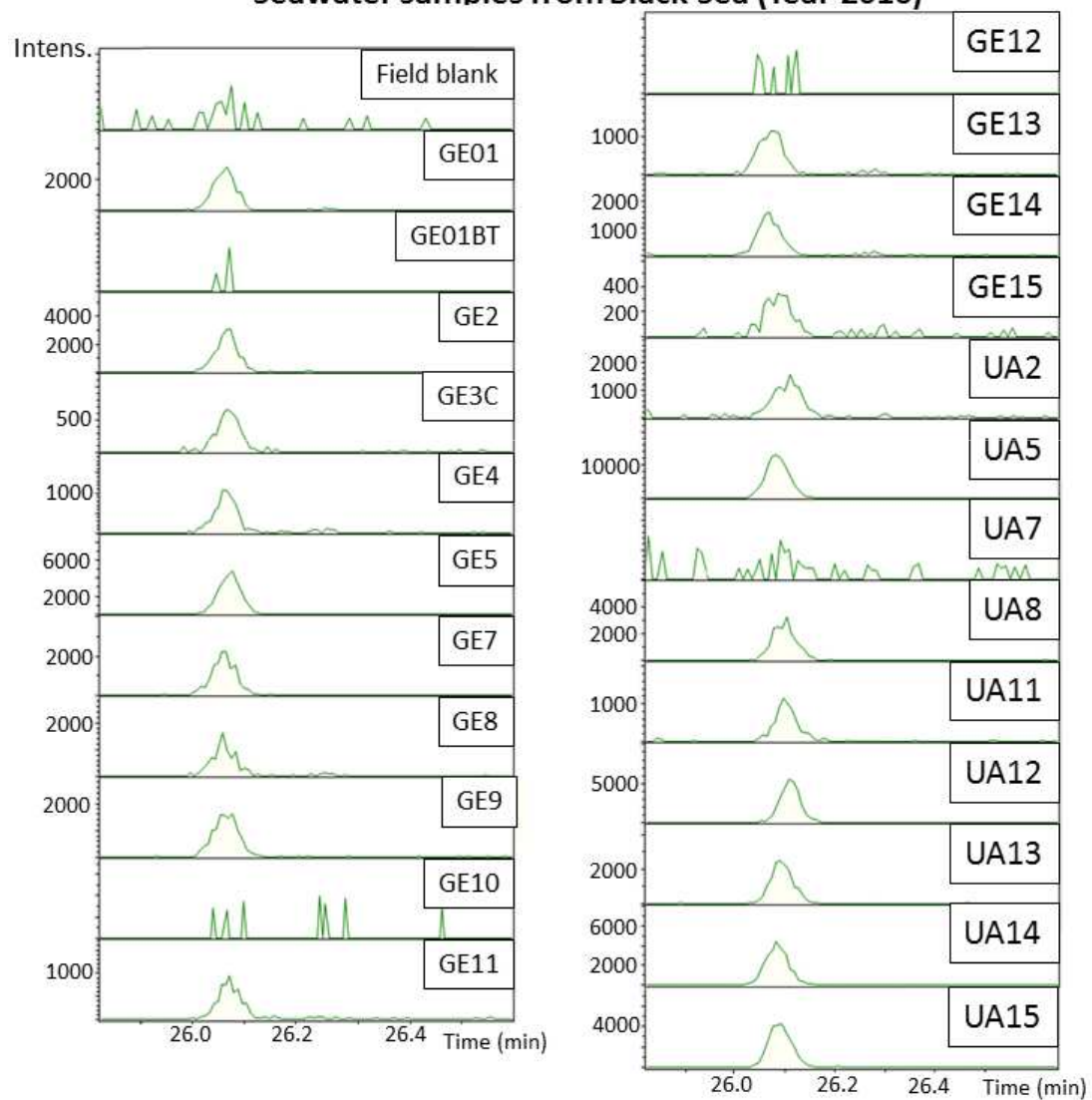
Experimental spectra of Bis-(4-chlorophenyl)-sulphone in GC-APCI(+)-QTOF against literature
GC-EI-MS spectra



Storage of Chromatograms for future Chemical Screening

Case of Bis(4-chlorophenyl) sulphone

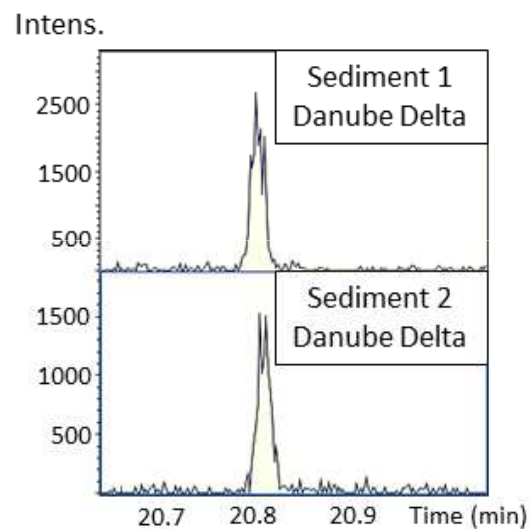
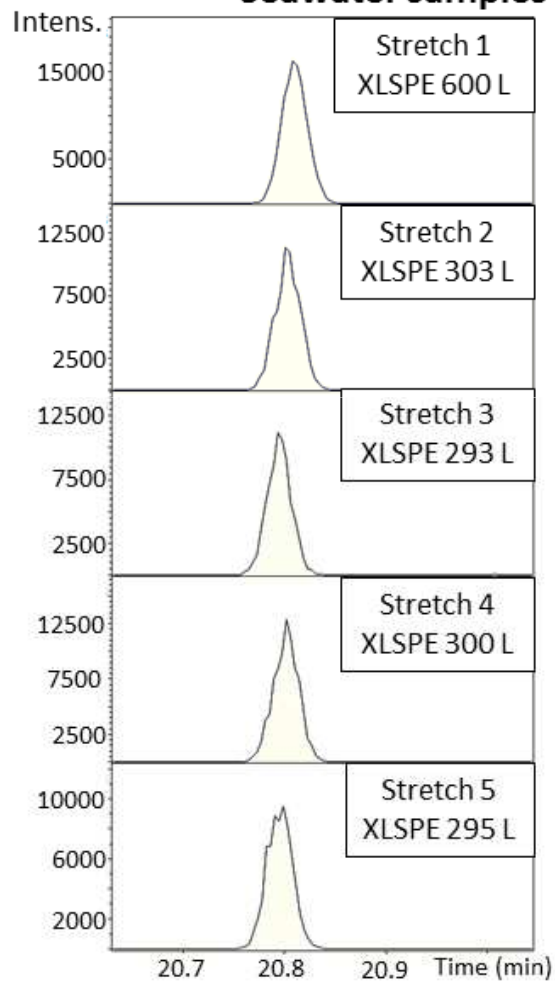
Bis-(4-chlorophenyl)-sulphone in GC-APCI(+)-QTOF ($M^{*+}=285.9622\pm 0.005$)
Seawater samples from Black Sea (Year 2016)



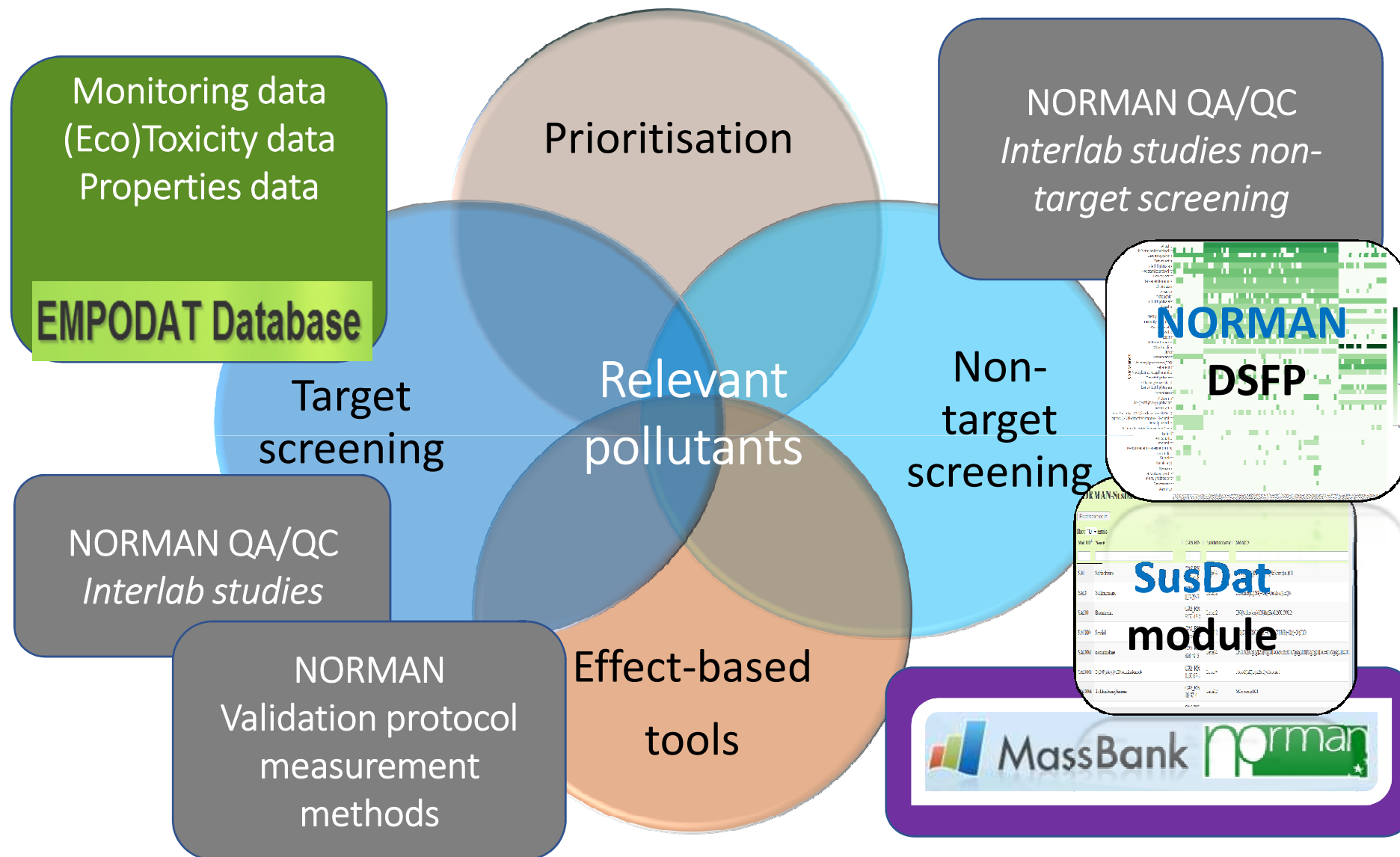
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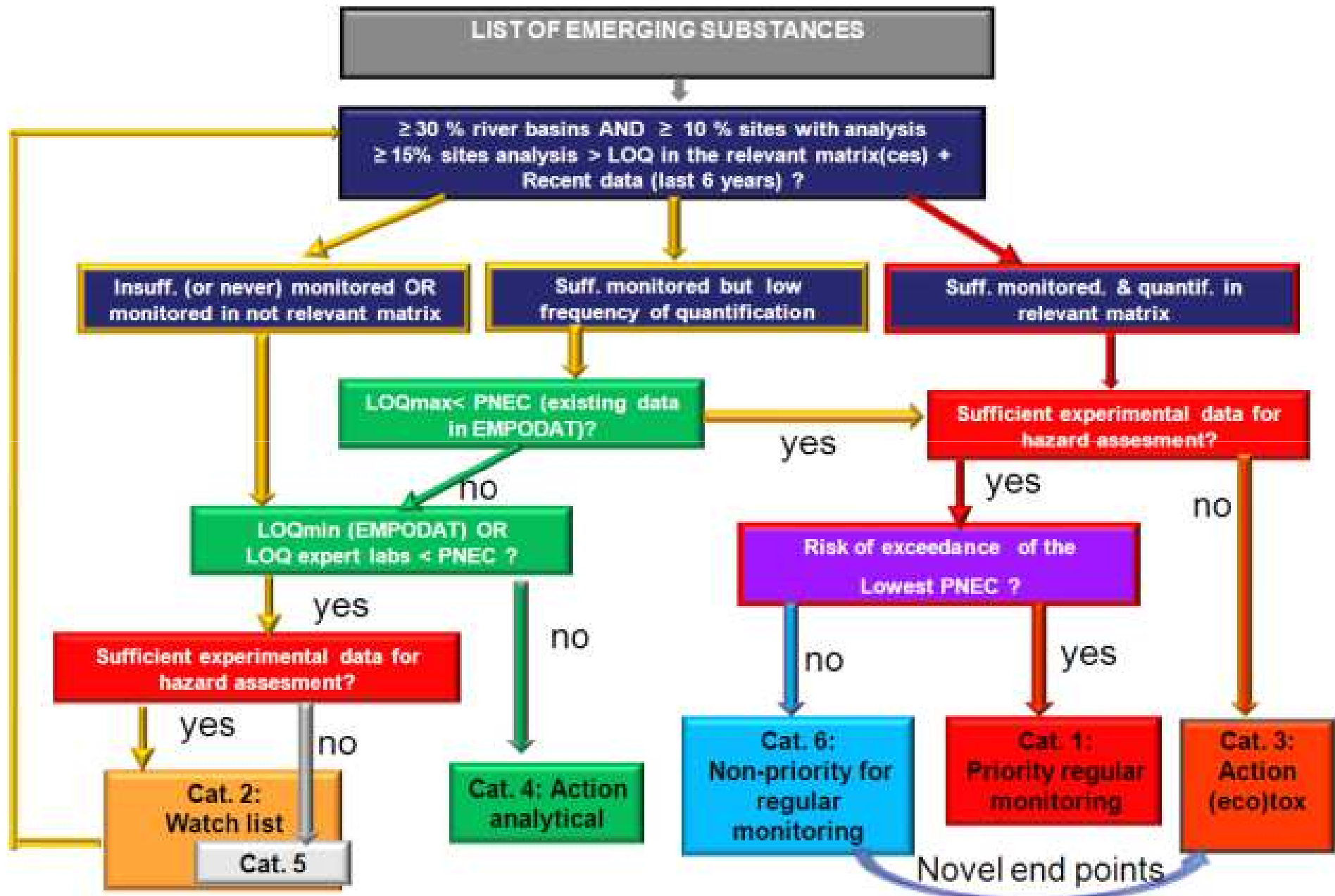
**Bis-(4-chlorophenyl)-sulphone in GC-APCI(+)-QTOF ($M^{*+}=285.9622\pm 0.005$)
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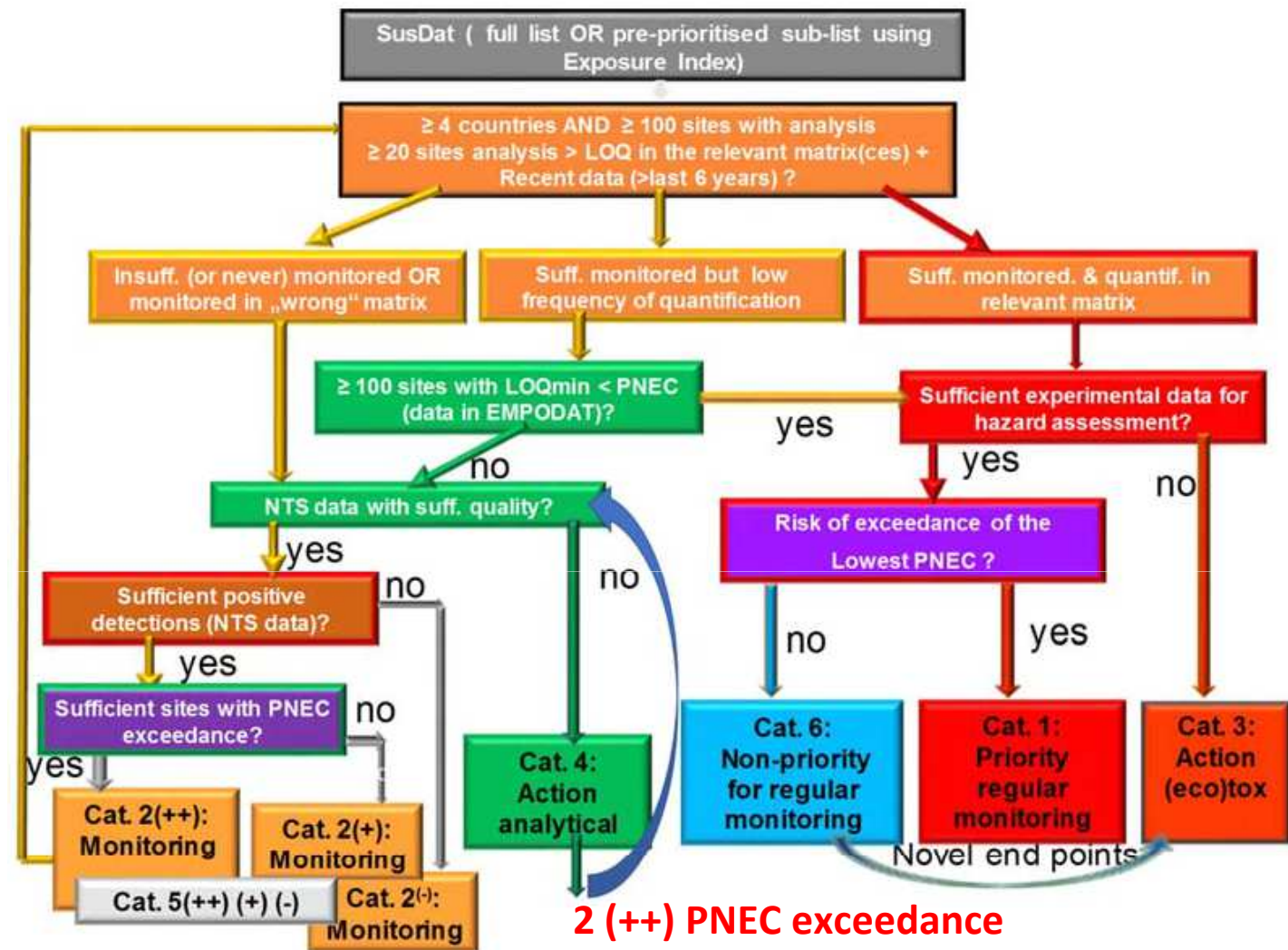
Strategy of NORMAN for the identification of relevant pollutants



NORMAN Prioritisation scheme for emerging substances



Updated NORMAN Prioritisation scheme for emerging substances



2 (++) PNEC exceedance
2(+) No semi-quantification but High frequency of appearance
2 (-) No PNEC exceedance

- 1) Countries with positive detection
- 2) Sites with positive detection
- 3) Frequency of PNEC exceedance
- 4) Extent of exceedance

Conclusions & Proposals

- NTS laboratories to assure application of wide-scope screening LC&GC-HRMS methods to achieve chemical characterization of JDS4 samples
- Chromatograms to be stored in DSFP for future retrospective uses to support regulatory efforts
- **Stored chromatograms can be used to propose river basin specific pollutants though prioritization of NTS data**
- Application of a wide panel of battery of bioassays to detect effects and explanation of effects based on chemicals detected
- Better-understand Antibiotic Resistance at catchment level



EMBLAS
Environmental Monitoring
in the Black sea



solutions



icpdr ikisd
International
Commission
for the Protection
of the Danube River
Internationale
Kommission
zum Schutz
der Donau



nereus
COST ACTION
ES1403



ANSWER

Thanks for your attention!

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Jörg Ahlheim (UFZ)