Helping contaminants emerge: The role of non-targeted analysis in monitoring contaminants of emerging concern

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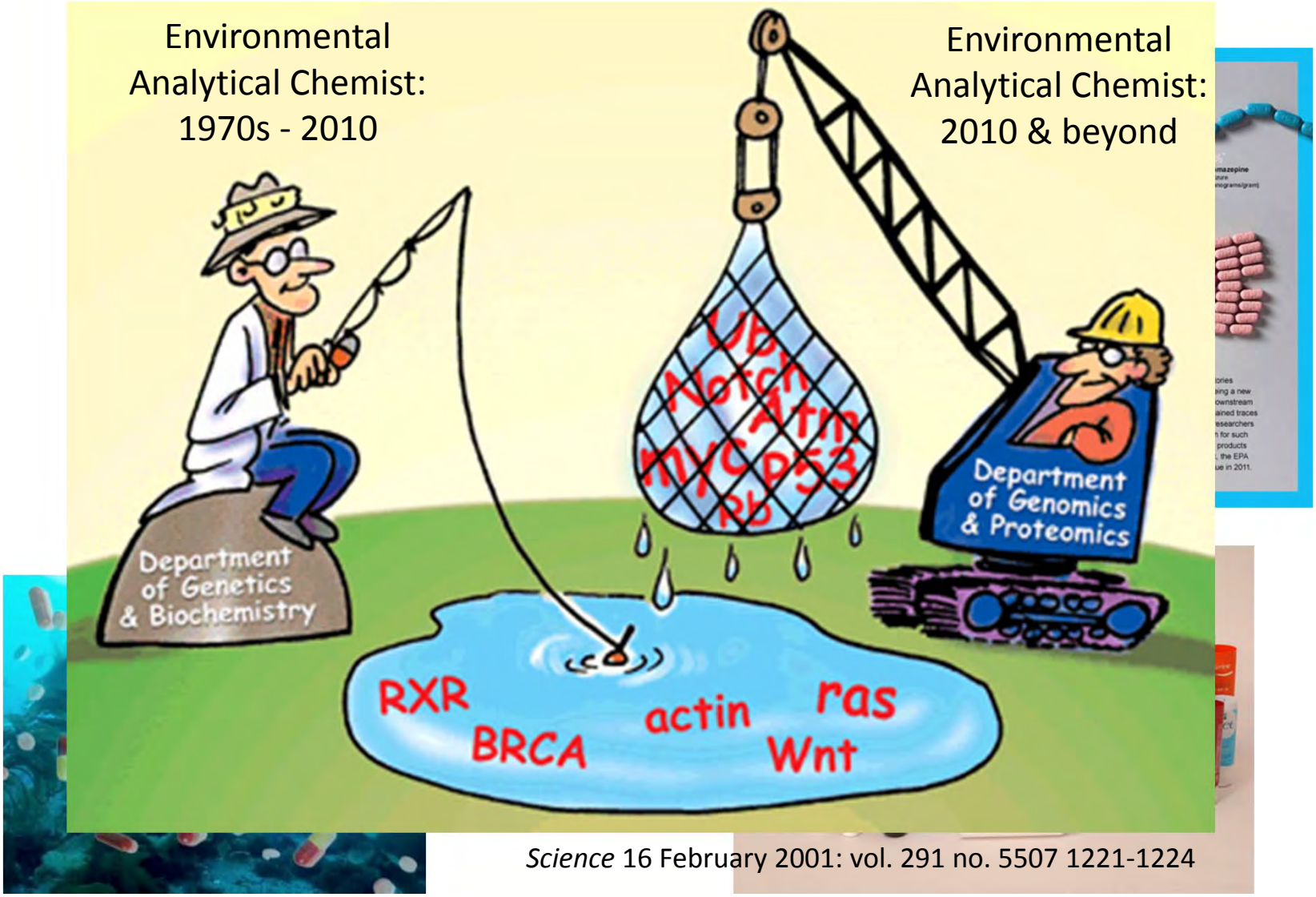
What are the next emerging contaminants and how can we find them in the environment?

LF5



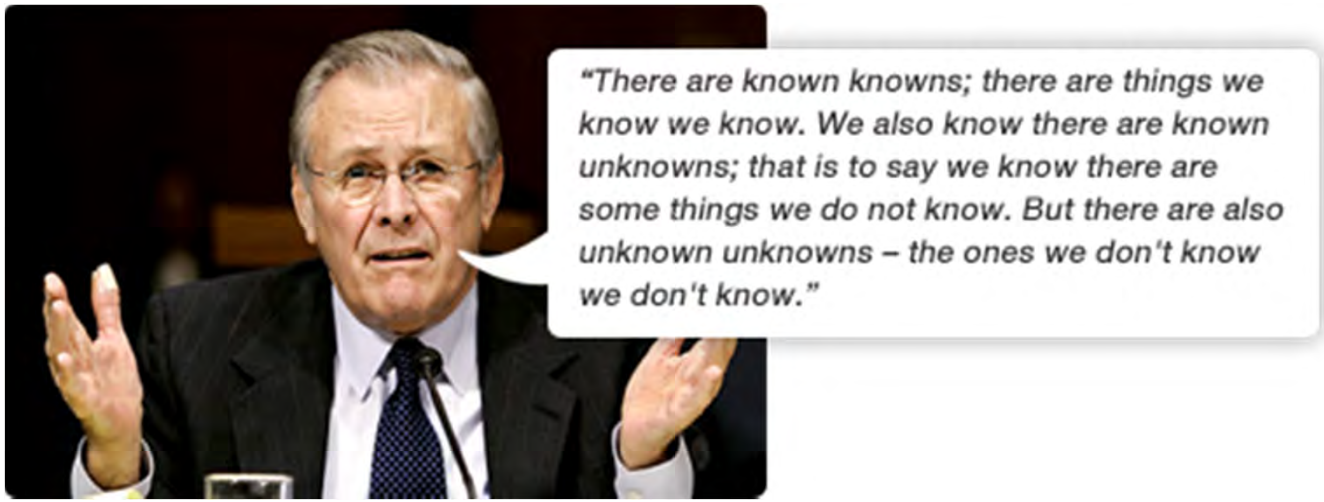
Science 16 February 2001: vol. 291 no. 5507 1221-1224

Slide 2

LF5 Probably expand this a bit to make it more relevant to wastewater… tie to treatment processes, etc. etc.
Lee Ferguson, 6/7/2013

LC-HRMS: An emerging technique for “helping contaminants emerge”

LC-MS strategies for characterization of organic contaminants			
Screening technique:	*Targeted*	*Suspect*	*Non-target*
Question:	*Are compounds x, y, & z present in this sample?*	*Which compounds of a defined list are present in this sample?*	*Which compounds are present in this sample?*
Compound Types:	*Known-knowns*	*Known-unknowns*	*Known-unknowns & unknown-unknowns*



In context of water reuse, it is critical to identify the most relevant CECs for monitoring

- The major challenge in non-target analysis of CECs is compound ***prioritization*** for data reduction.
- CECs may have process-dependent fate within water/wastewater treatment.
- Needed: methods to allow focus on recalcitrant, high-abundance, and/or particularly toxic CECs.

<u>Two Approaches:</u>

- Fate-dependent analysis of CECs in water/wastewater treatment
- Activity-directed analysis of CECs that may have particular biological activities

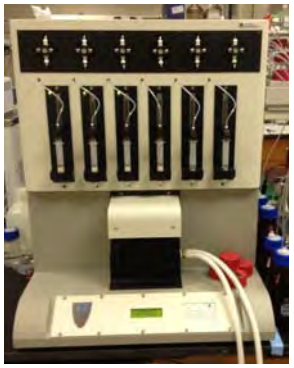
Fate-dependent analysis of CECs in wastewater LF6

Slide 5

LF6 Update and modify to reflect the new study

Lee Ferguson, 6/7/2013

Sample Preparation and Instrumental Analysis



Sampling:

- Daily grab samples (Tue-Fri)
- Triplicate sampling on one day

Sample enrichment:

- 500 mL sample (primary effluent diluted 1:5 (v/v))
- Spiked with stable isotope labeled standards (19)
- Automated SPE, 500 mg Oasis HLB

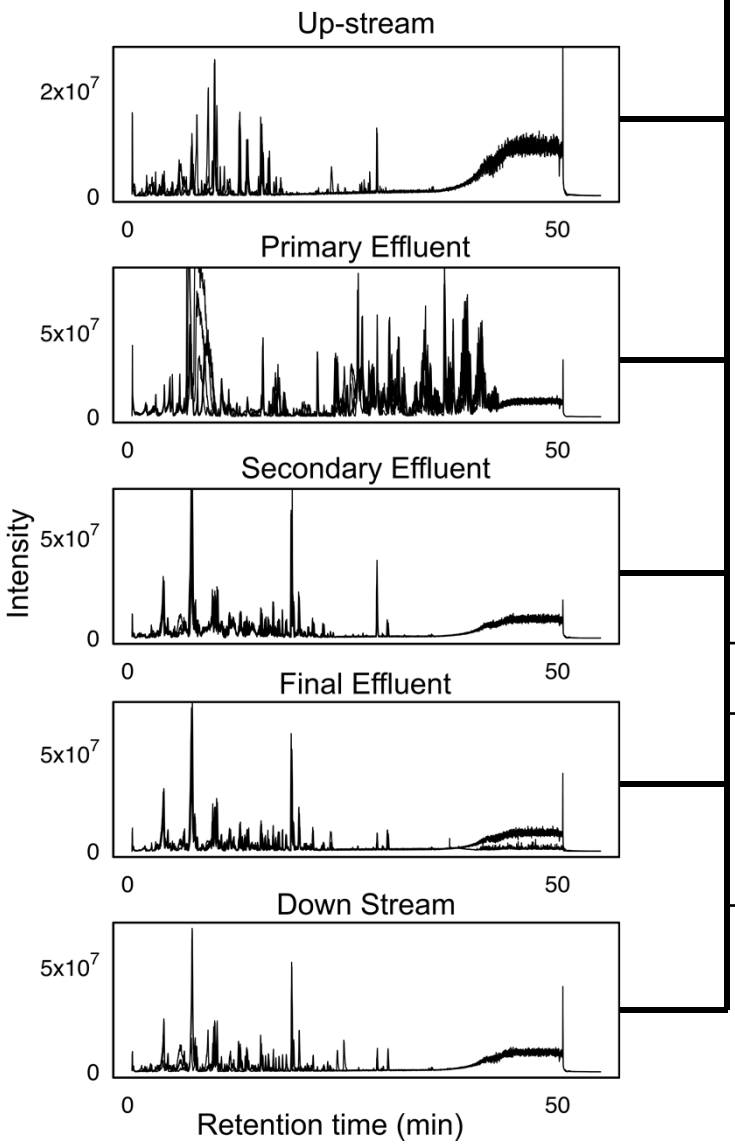
UHPLC

- Dionex Ultimate 3000, 100x2.1 Thermo Hypersil Gold aQ
- H2O/ACN gradient, 95% to 1% H2O in 55 min, 0.5 mL min-1

High-resolution mass spectrometry

- Thermo LTQ Orbitrap Velos, ESI(+)
- Full-scan (m/z 100-2000), accurate mass, R=60k FWHM
- Top-4 data-dependent accurate mass MS^2, R=7500 FWHM

Data analysis workflow



Compound Discover 2.0 (Thermo)

- Retention time alignment
- Peak detection
- De-isotope and de-adduct
- Feature consolidation
- Gap filling

Consolidated Peak Table

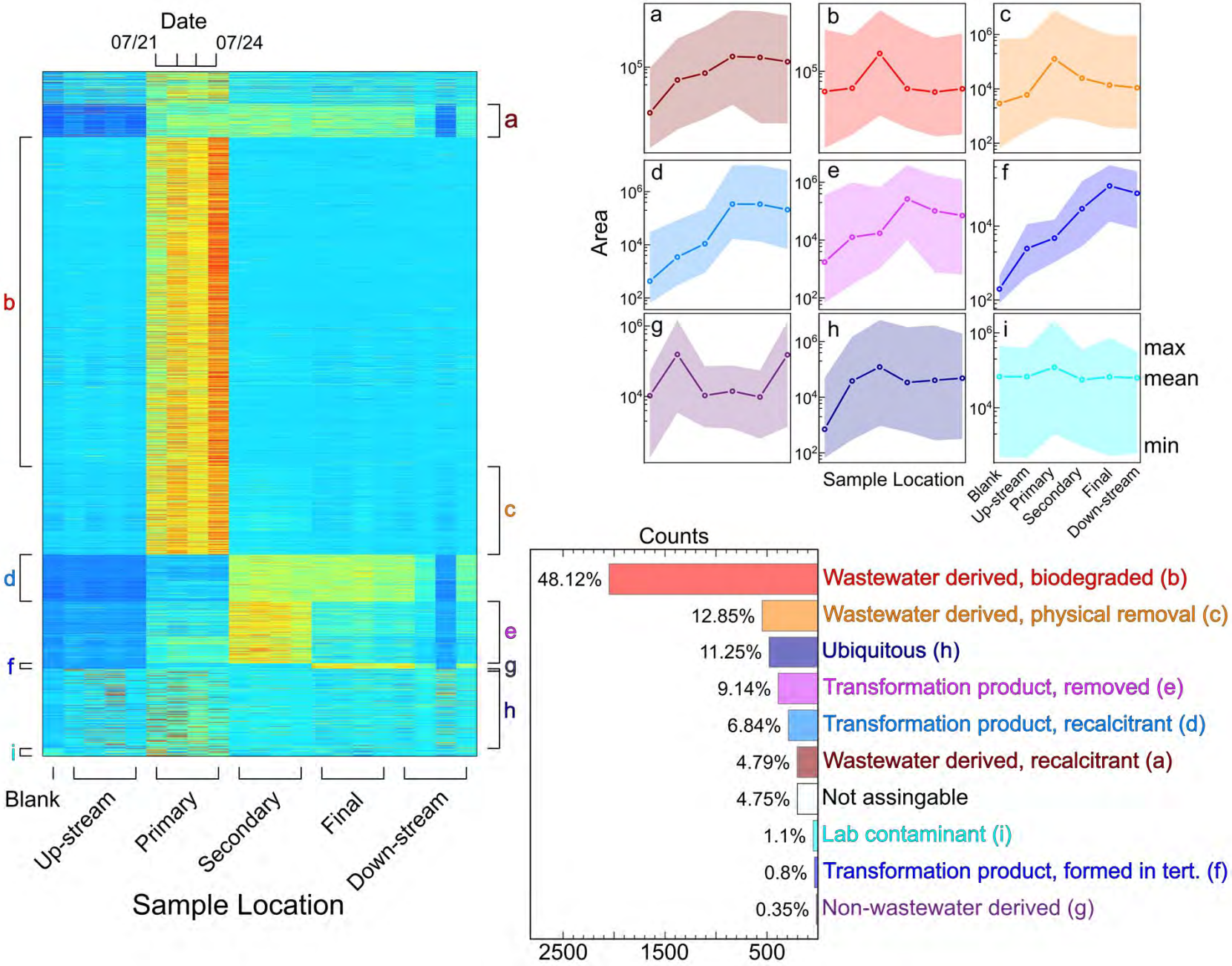
Mol. wt.	RT	Measured Area			
		Samp. 1	Samp. 2	Samp. 3	Samp n
x_1	y_1	$z_{1,1}$	$z_{1,2}$	$z_{1,3}$	$z_{i,1}$
⋮	⋮	⋮	⋮	⋮	⋮
x_i	y_i	$z_{i,1}$	$z_{i,2}$	$z_{i,3}$	$z_{i,n}$

Differential analysis of non-target compounds in wastewater to reveal emerging contaminants

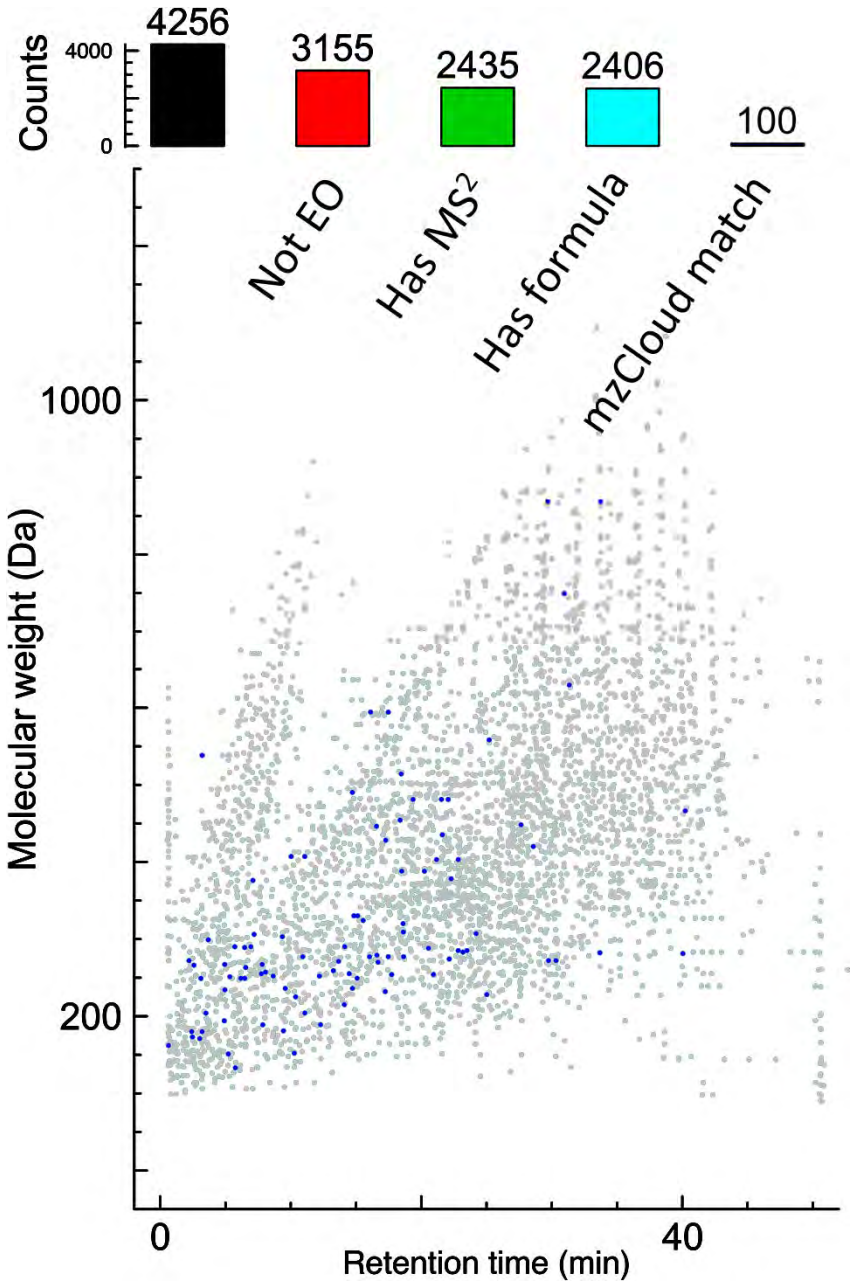
- Screening results were used to generate target lists for differential screening
- ID in screening = mass error <+/-2.5 ppm and ΔRT< 0.5min

Category	Blank	Upstream	Primary eff.	Secondary eff.	Final eff.	Downstream
Wastewater-derived, biodegradable	o	o	↑	↓	→	→
Wastewater-derived, physical removal	o	o	↑	↘	↘	→
Wastewater-derived, recalcitrant	o	o	↑	→	→	→
Transformation product, removed	o	o	o	↑	↓	→
Transformation product, recalcitrant	o	o	o	↑	→	→
Transformation product, produced in tertiary treatment	o	o	o	↗	↑	→
Non wastewater-derived	o	↑	o	o	o	↑
Ubiquitous	o	→	→	→	→	→
Laboratory contaminant	→	→	→	→	→	→

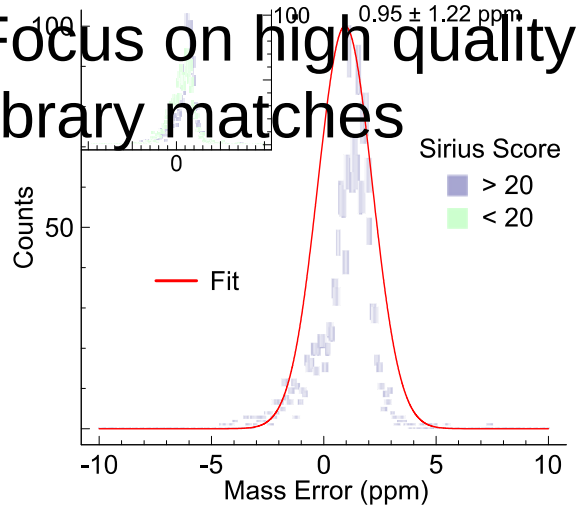
Fate-dependent feature prioritization



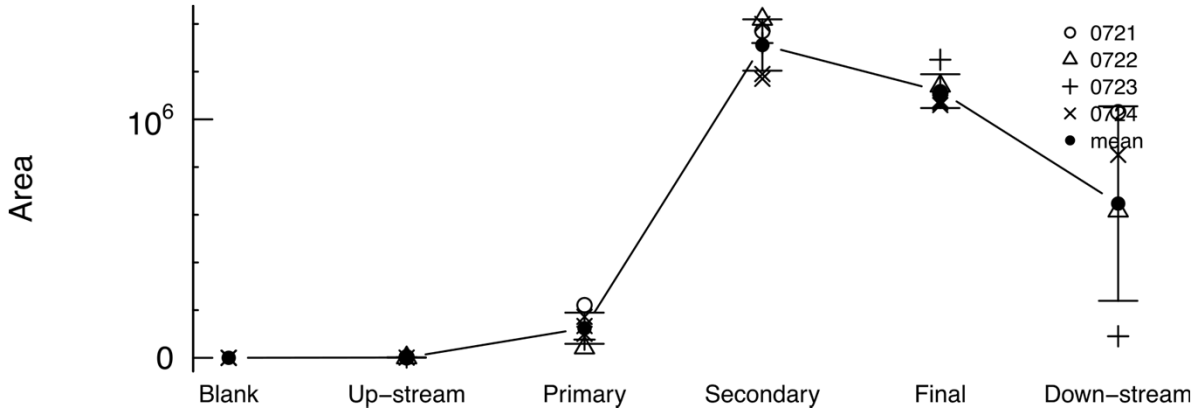
Data filtering

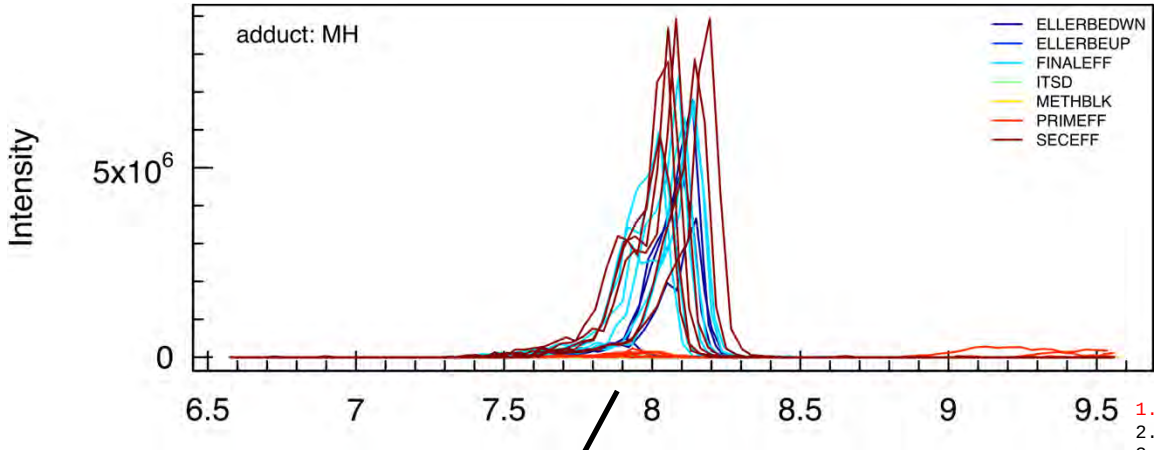


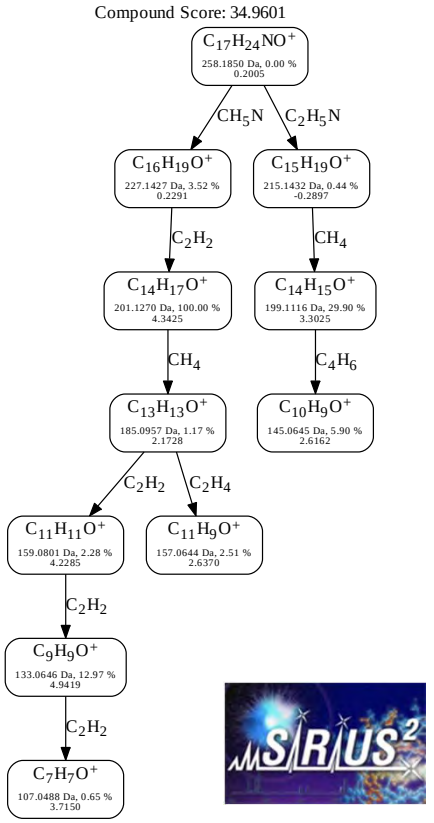
- Remove features corresponding to ethoxylated oligomers
- Filter based on MS^2 spectra
- Retain features with molecular formula assignment
- Prioritize based on differential category
- Focus on high quality mzCloud library matches

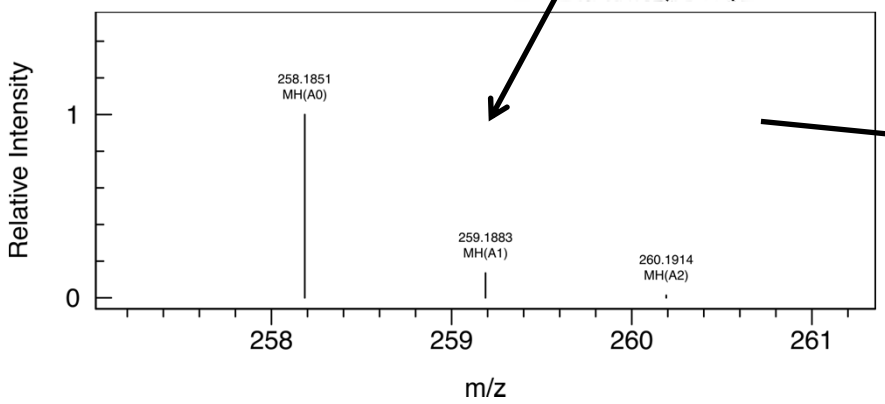


257.1777 Da feature follows a recalcitrant transformation product profile

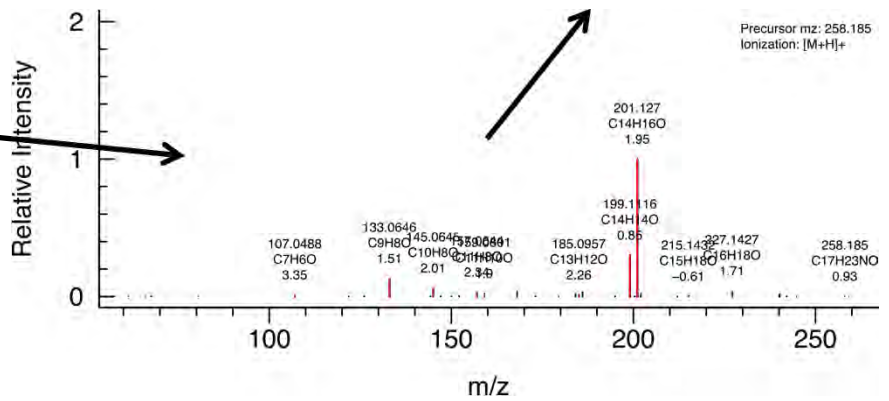








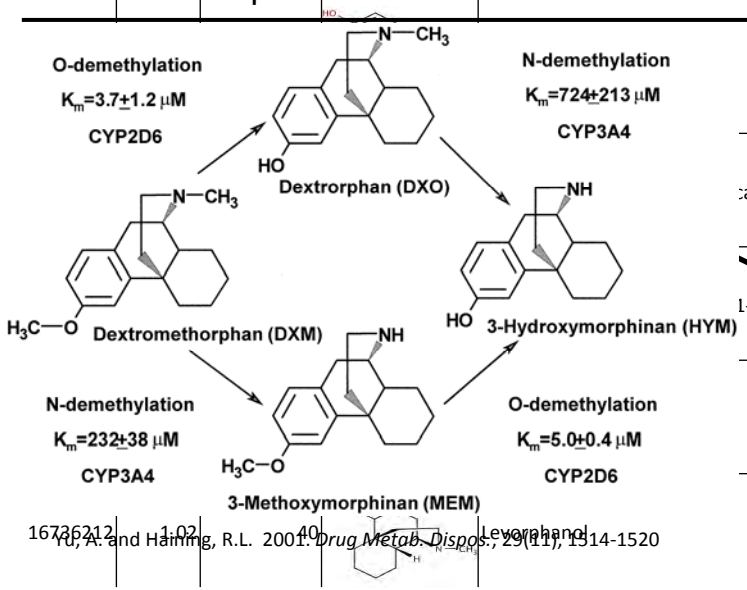
1.) C17H23NO score: 34.96 tree: +28.10 iso: 6.86 peaks: 11 91.33 %
2.) C13H26N2OP score: 23.35 tree: +15.03 iso: 8.33 peaks: 13 93.83 %
3.) C12H22FN4O score: 21.51 tree: +12.90 iso: 8.61 peaks: 11 88.13 %
4.) C7H21F2N7O score: 6.13 tree: +6.13 iso: 0.00 peaks: 11 91.26 %



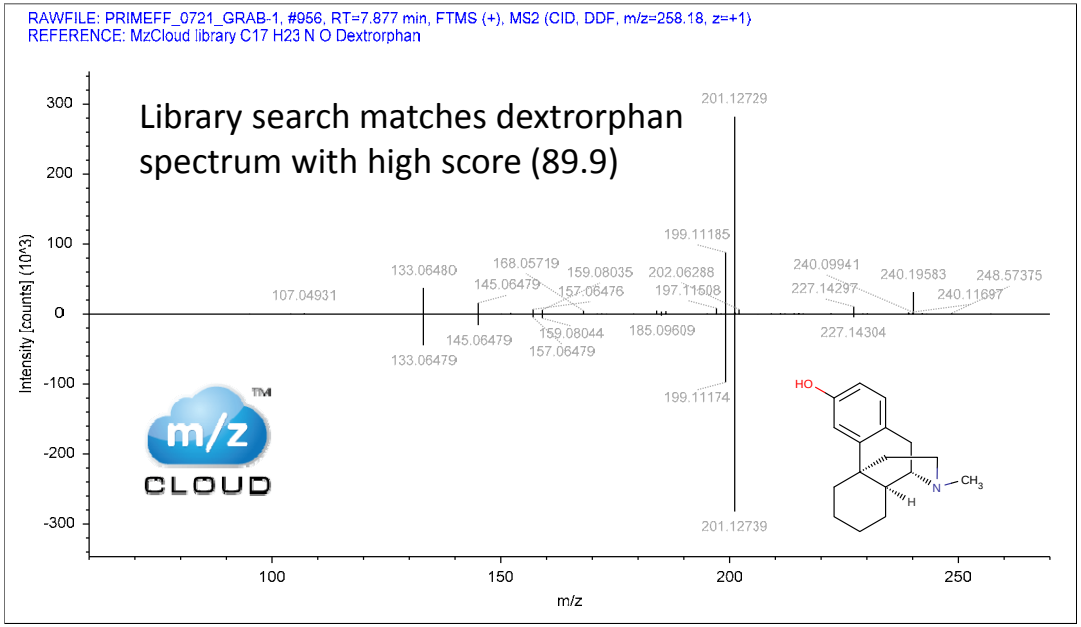
Dextrorphan tentatively identified by library match and *in silico* fragments

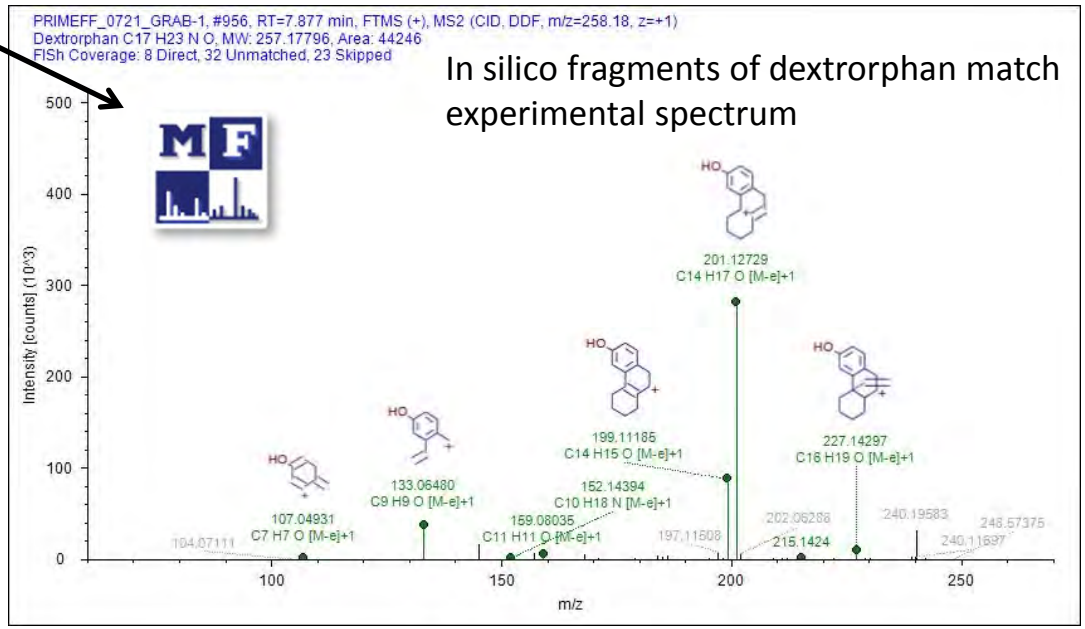
Chemspider matches for $C_{17}H_{23}NO$

Dextrorphan is a known, activated human metabolite of the cough suppressant dextromethorphan

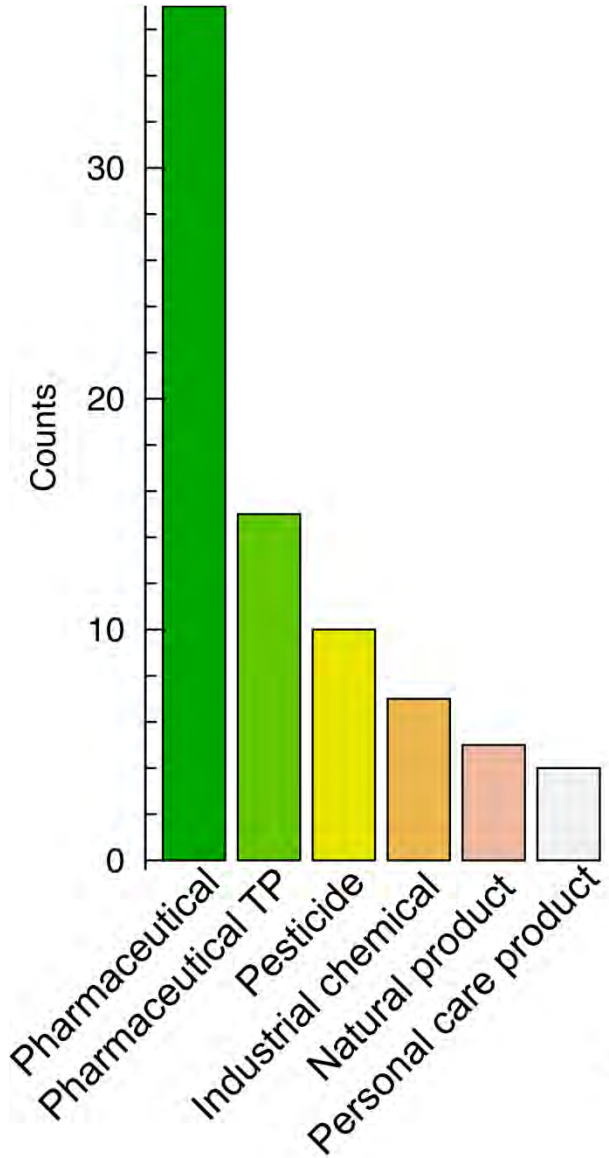
CSID	Δmass [ppm]	# References	Structure	Name
16736212	1.02	40		Levorphanol

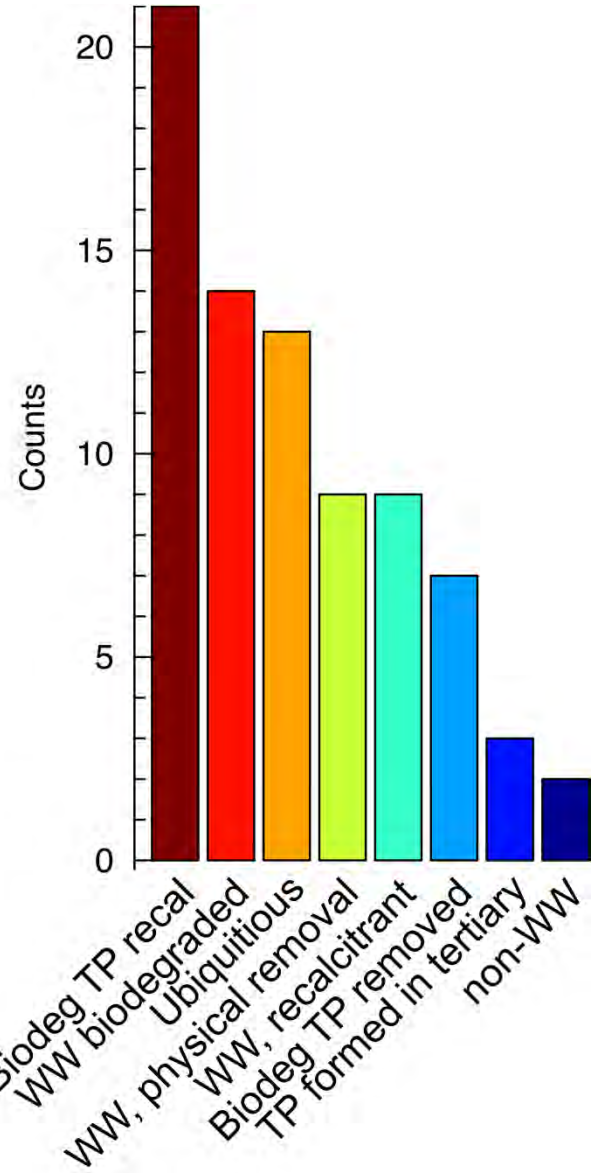
Yu, A. and Haining, R.L. 2001. *Drug Metab. Dispos.*, 29(11), 1514-1520





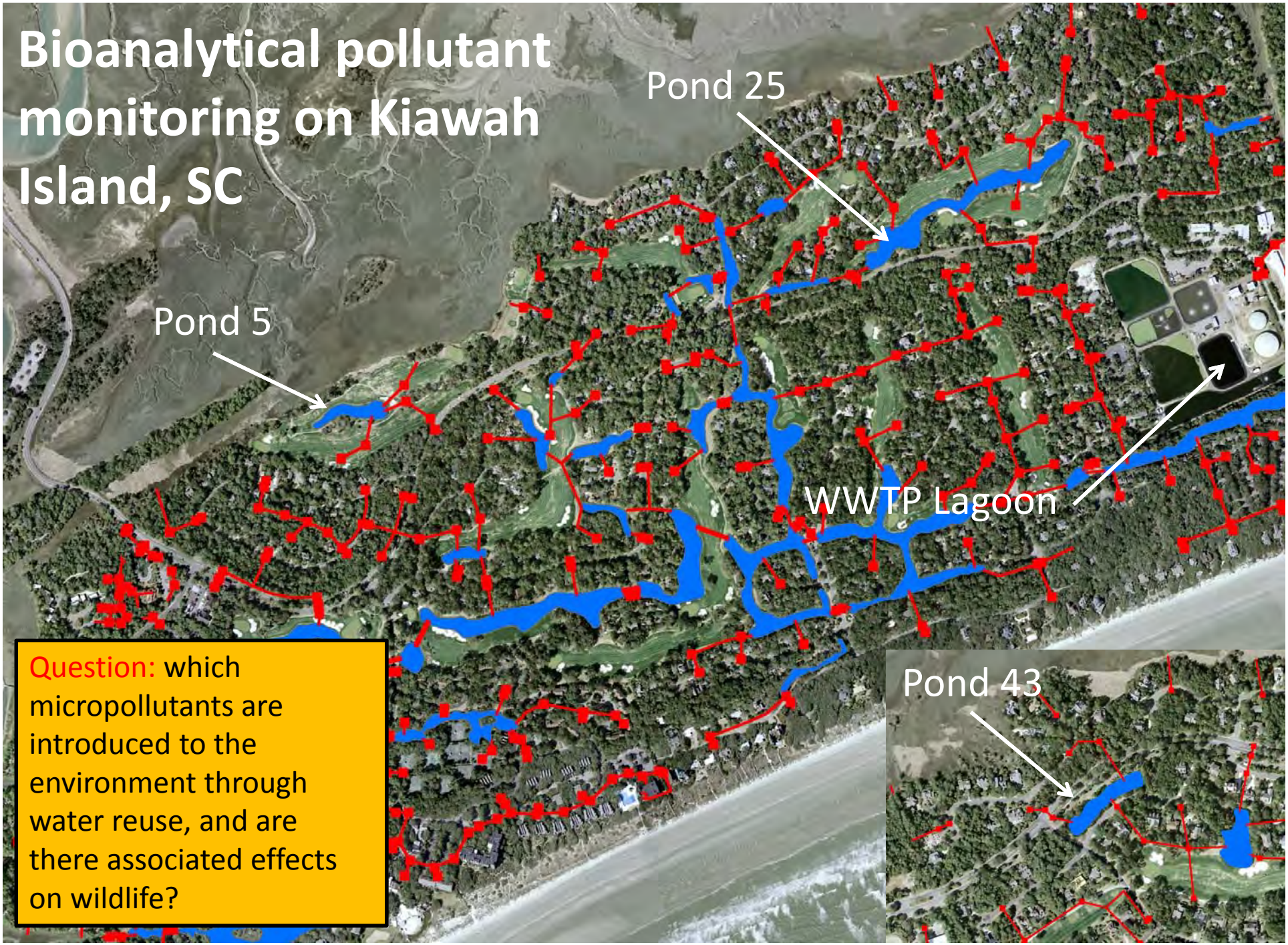
Results: Suspect compounds tentatively identified



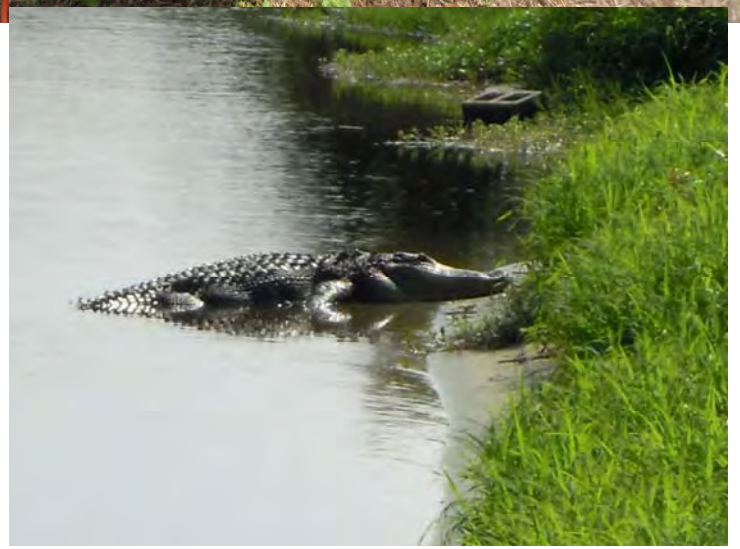


- 78 compounds from six classes were tentatively identified in wastewater/surface water
- 20 of these were confirmed with standards (100% correct assignment)
- A further 1,101 features were annotated as polyethoxylated surfactants (comprising 39% of features identified as wastewater-derived, biodegradable)
- The largest fraction of ID'd compounds was classified as recalcitrant transformation products.

Finding the “causative stressors” within a water reuse scenario using non-targeted analysis coupled with bioanalytical tools

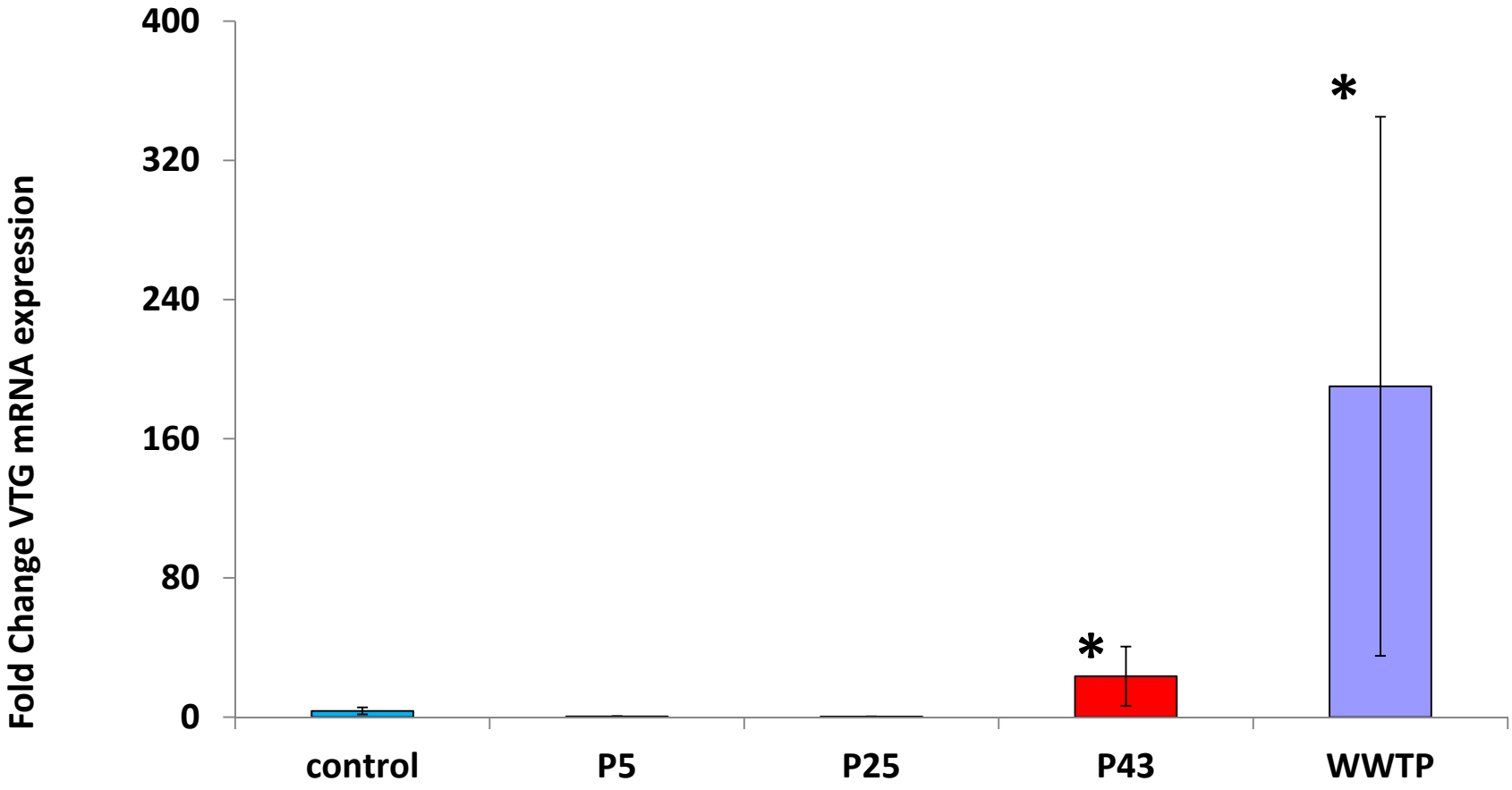

Bioanalytical pollutant monitoring on Kiawah Island, SC
Pond 25
Pond 5
WWTP Lagoon
Pond 43
Question: which micropollutants are introduced to the environment through water reuse, and are there associated effects on wildlife?

Male Fathead Minnow Exposures



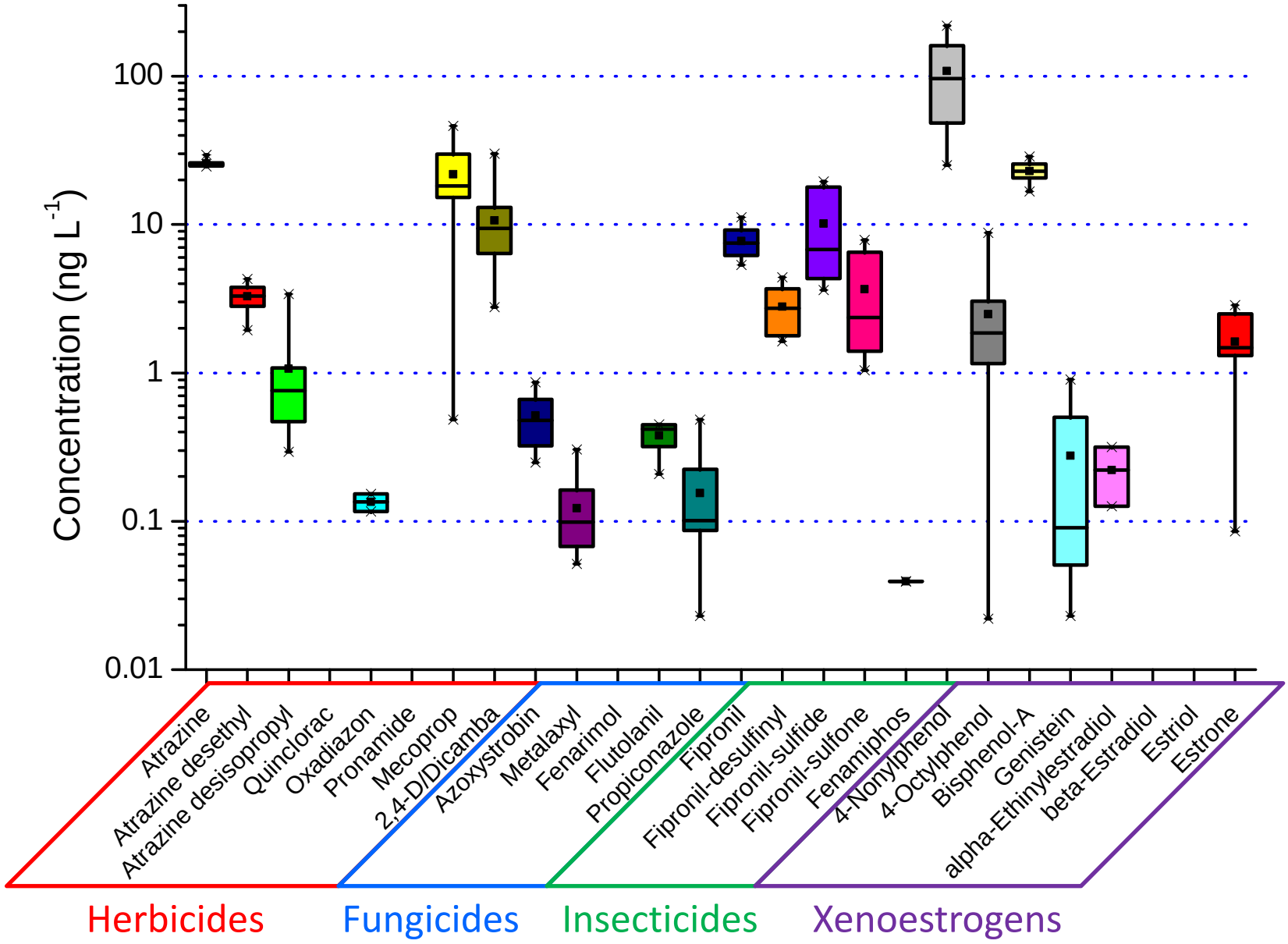
Mini Mobile Units for “safer” in-situ fish exposure (Alan Kolok)

Hepatic Vitellogenin mRNA Expression in Male Fathead Minnows After 1 Week Exposure



* Indicate statistically significant values compared to control using one-way ANOVA ($p < 0.05$)

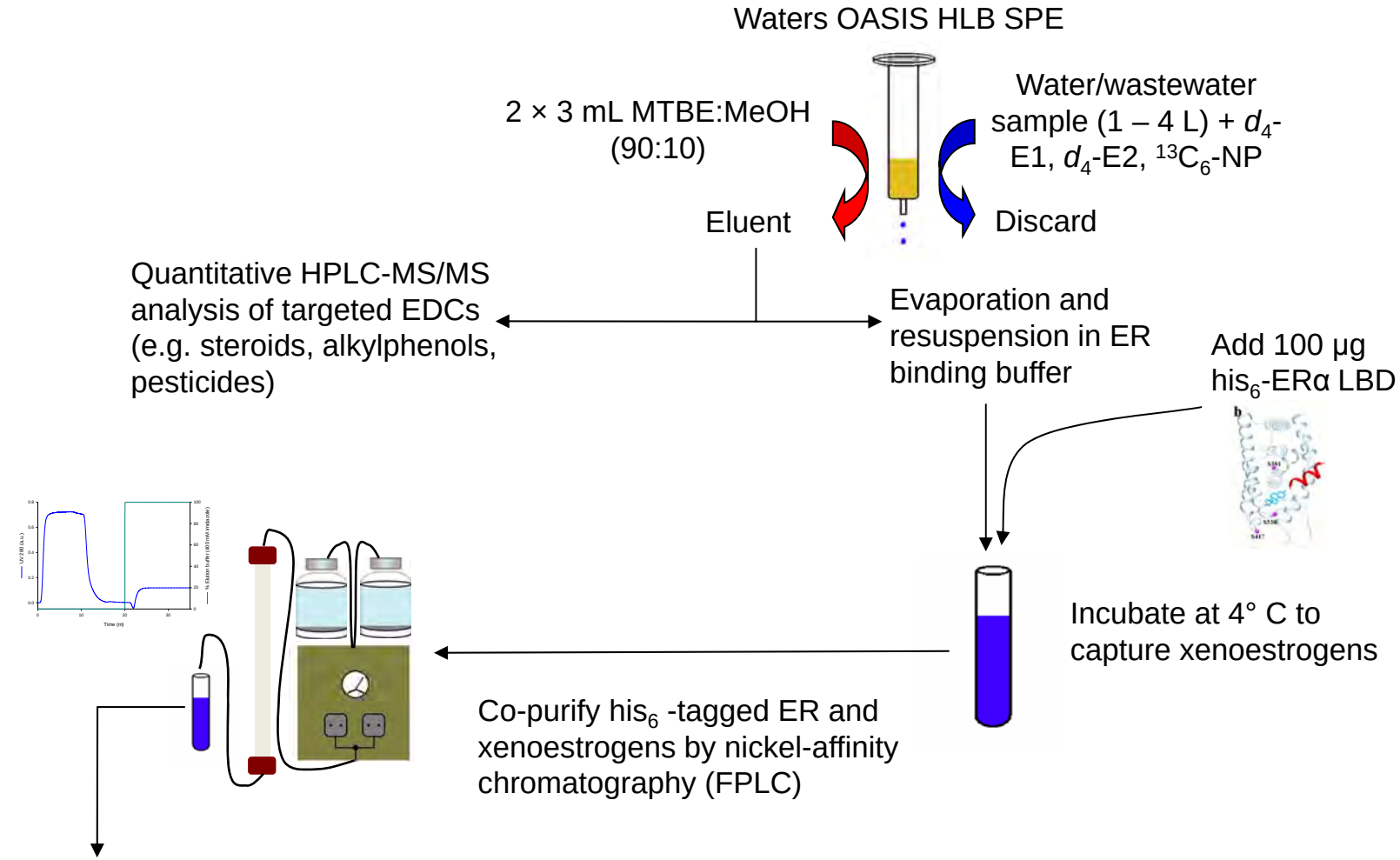
Target Micropollutants in WWTP Lagoon



Yes, but what ELSE might be contributing to estrogenicity in the reclaimed water??

- **Receptor affinity extraction:** Similar in concept to immunoaffinity chromatography – relies on high specificity/selectivity molecular interaction to isolate target analytes from a mixture prior to analysis
- Recombinant protein engineering:
  - ERα ligand binding domain triple Cys → Ser mutant
  - Fusion of thioredoxin to ER enhances solubility
  - His_6 tag allows subsequent purification
- Proteins are cloned, expressed in bacterial vectors, and purified chromatographically

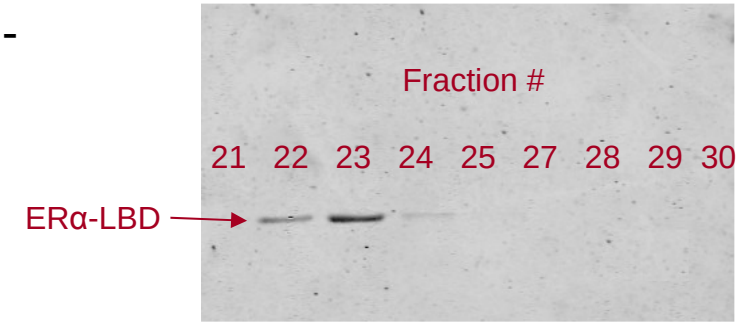
Estrogen receptor-affinity isolation for activity-directed analysis

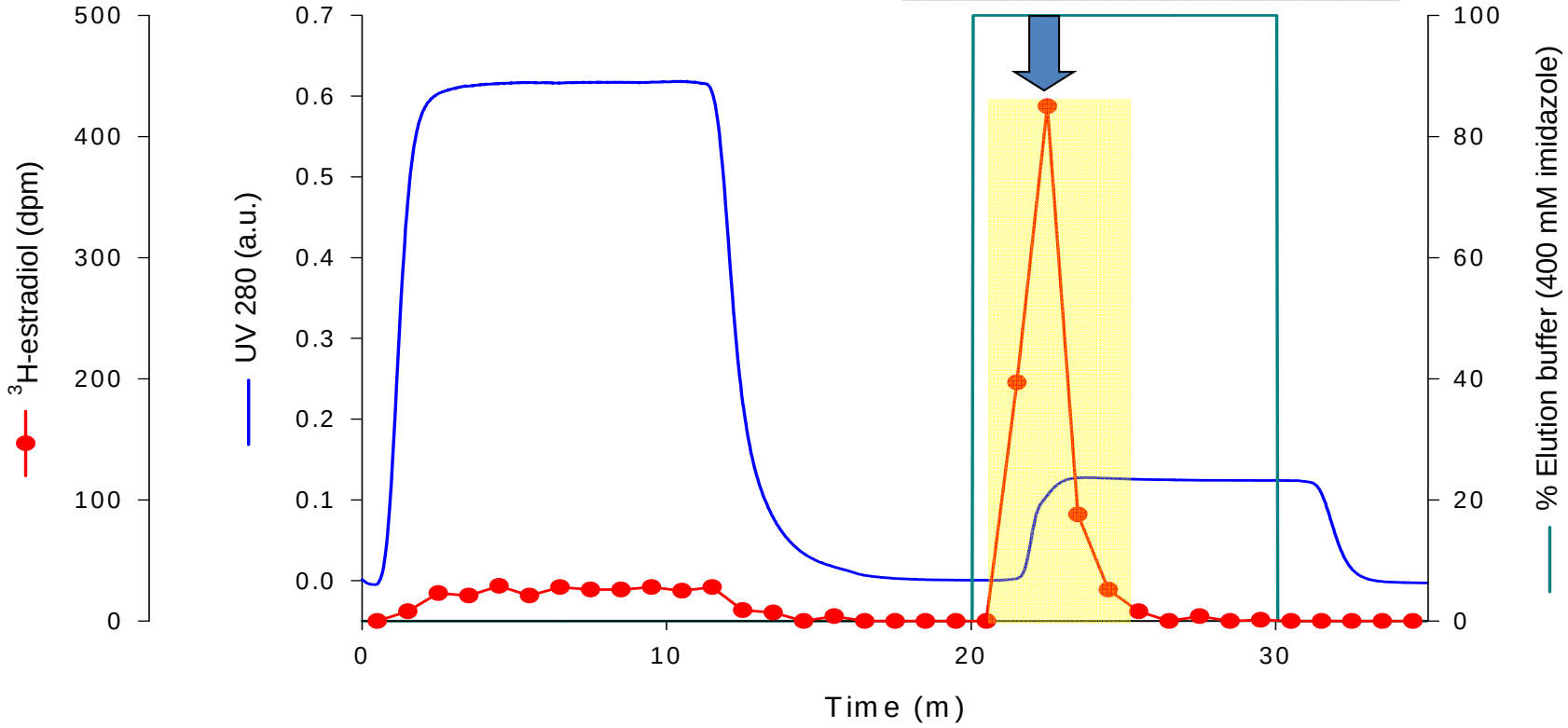


Analysis of receptor-active EDCs in eluent by UHPLC-Orbitrap MS/MS or triple-quadrupole MS

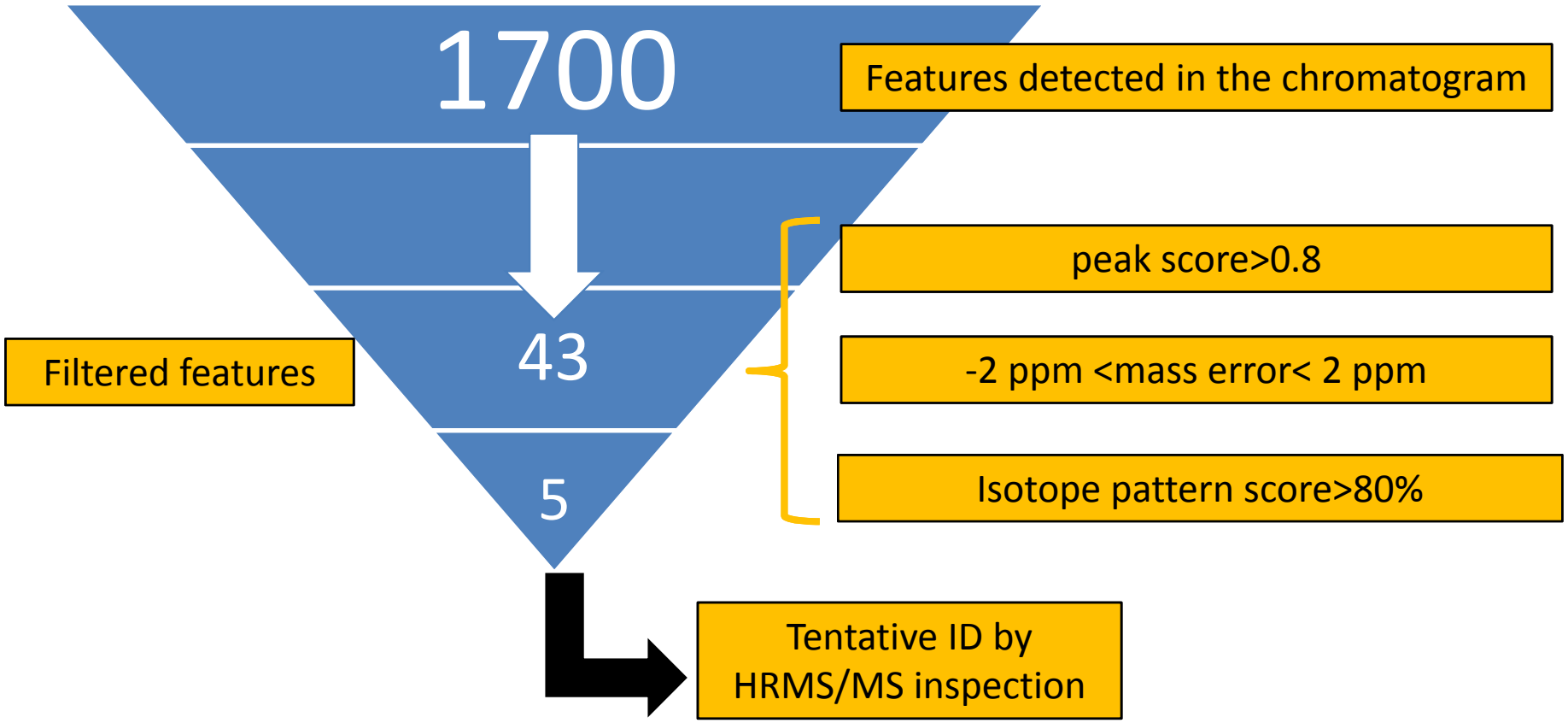
Purification of ER-bound xenoestrogens from wastewater

ERα-LBD and radioligand tracer (^{3}H-17β-estradiol) are selectively retained from wastewater extract by Ni^{2+} agarose, and can be co-eluted with 400 mM imidazole





Data filtering and tentative compound identification



Xenoestrog… tor affinity
extracts … screening

Name	Class	… Pattern ore	Confirmed & Quantified?
17β-estradiol	Endoge… steroid	00	0.70 ng/L
Estrone	Endoge… steroid	95	11.5 ng/L
Celecoxib	**NSAID Pharma**	**00**	**no**
PFOA	Fluorina… surfacta…	00	no
Efavirens	**Antivira… pharma…**	**00**	**no**

DOI: 10.1111/j.1468-1293.2010.00831.x
HIV Medicine (2010), 11, 603–607

SHORT COMMUNICATION

Efavirenz directly modulates the oestrogen receptor and induces breast cancer cell growth

MJ Sikora,[1] JM Rae,[1,2] MD Johnson[3] and Z Desta[4]

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Objectives
Efavirenz-based HIV therapy is associated with breast hypertrophy and gynaecomastia. Here, we tested the hypothesis that efavirenz induces gynaecomastia through direct binding and modulation of the oestrogen receptor (ER).

Methods
To determine the effect of efavirenz on growth, the oestrogen-dependent, ER-positive breast cancer cell lines MCF-7, T47D and ZR-75-1 were treated with efavirenz under oestrogen-free conditions in the presence or absence of the anti-oestrogen ICI 182,780. Cells treated with 17β-oestradiol in the absence or presence of ICI 182,780 served as positive and negative controls, respectively. Cellular growth was assayed using the crystal violet staining method and an *in vitro* receptor binding assay was used to measure the ER binding affinity of efavirenz.

Results
Efavirenz induced growth in MCF-7 cells with an estimated effective concentration for half-maximal growth (EC_{50}) of 15.7 μM. This growth was reversed by ICI 182,780. Further, efavirenz binds directly to the ER [inhibitory concentration for half maximal binding (IC_{50}) of ~ 52 μM] at a roughly 1000-fold higher concentration than observed with 17β-oestradiol.

Conclusions
Our data suggest that efavirenz-induced gynaecomastia may be caused, at least in part, by drug-induced ER activation in breast tissues.

Keywords: efavirenz, gynaecomastia, highly active antiretroviral therapy, oestrogen receptor, oestrogens

Accepted 12 January 2010

Introduction

The introduction of highly active antiretroviral therapy (HAART) multi-drug combination regimens has considerably improved the prognosis of patients infected with HIV by reducing AIDS-related morbidity and mortality [1]. However, chronic treatment with these regimens is associated with multiple adverse effects, nonadherence and eventually therapy failure [2]. Treatment regimens containing the nonnucleoside reverse transcriptase inhibitor efavirenz are preferred in treatment-naïve patients and are widely used in other settings [3]. While efavirenz is generally well tolerated, concentration-dependent side effects that impact drug adherence and promote resistance have been documented [4]. Common adverse effects of efavirenz include central nervous system symptoms, occurring in up to 50% of patients [5], but other less common adverse effects have also been reported. An increasing number of reports suggest that the use of HAART, in particular efavirenz-based therapy, is associated with breast hypertrophy or gynaecomastia [6–11]. While mechanisms underlying efavirenz-induced gynaecomastia are not well understood, a number of hypotheses exist, including a direct oestrogenic effect, induction of an immune response, or altered steroid hormone metabolism by cytochrome P450 enzymes. To our knowledge, none of

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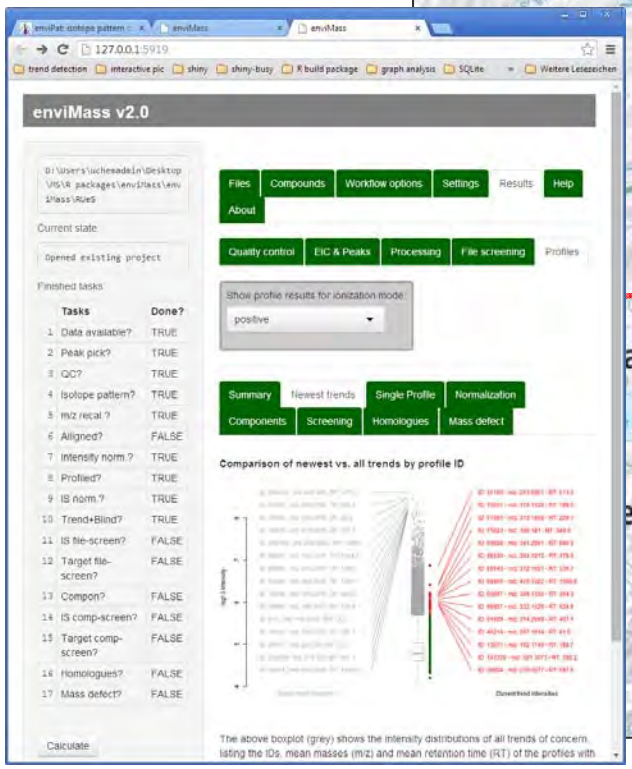
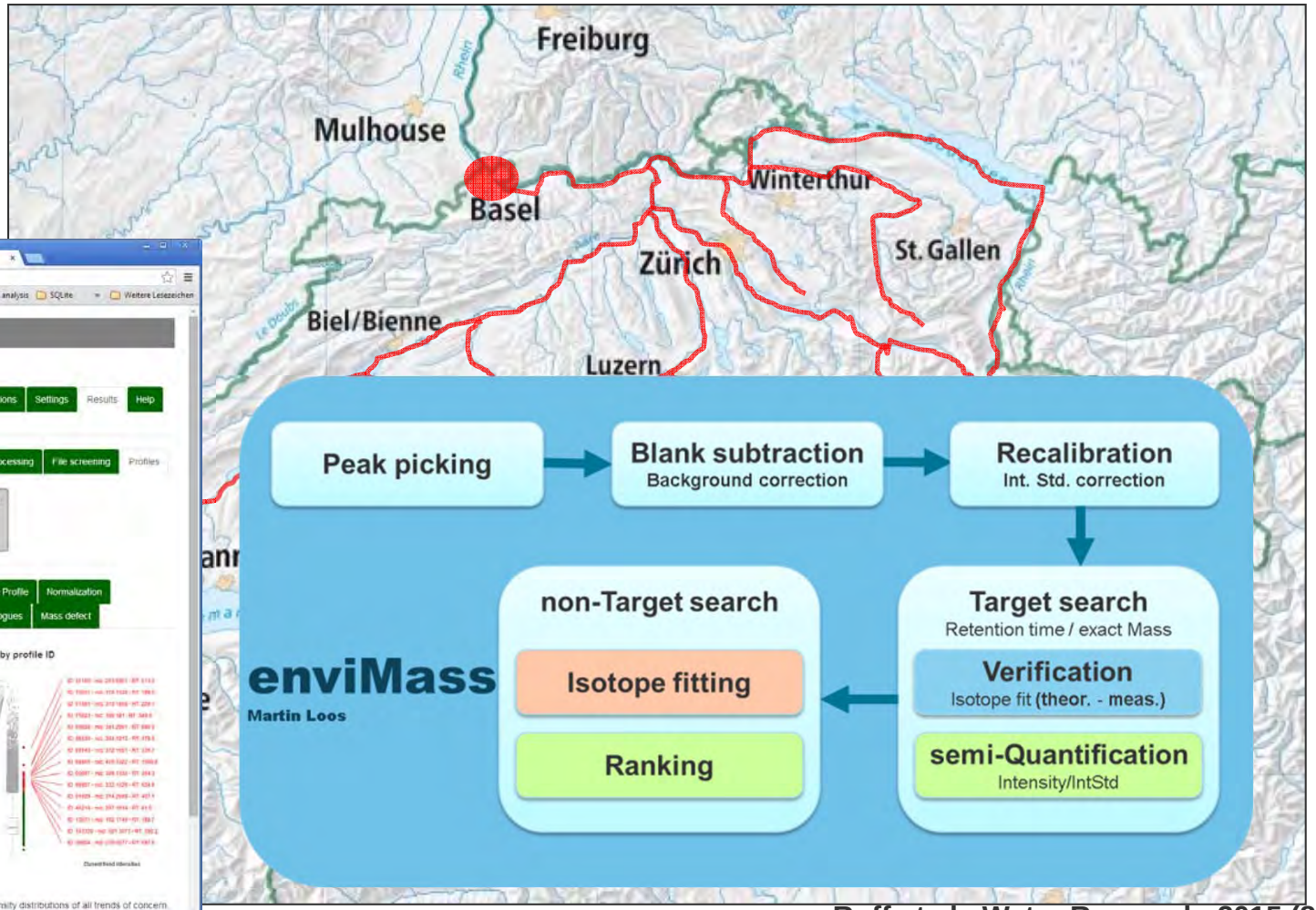
Case study: Daily routine monitoring of CECs in the Rhine river at the Swiss-German border

(Matthias Ruff, Heinz Singer, and Juliane Hollender)

enviMass





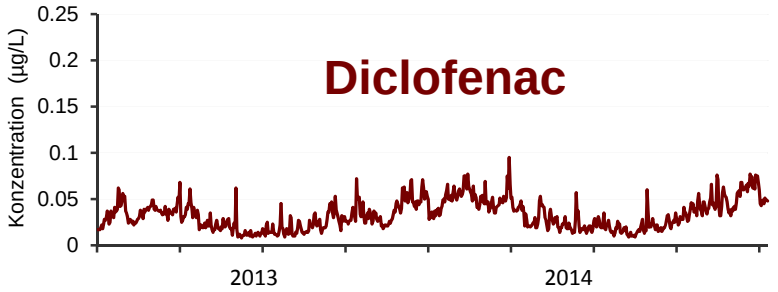
Ruff et al., Water Research, 2015 (87)

Target/non-target CEC monitoring in the Rhine

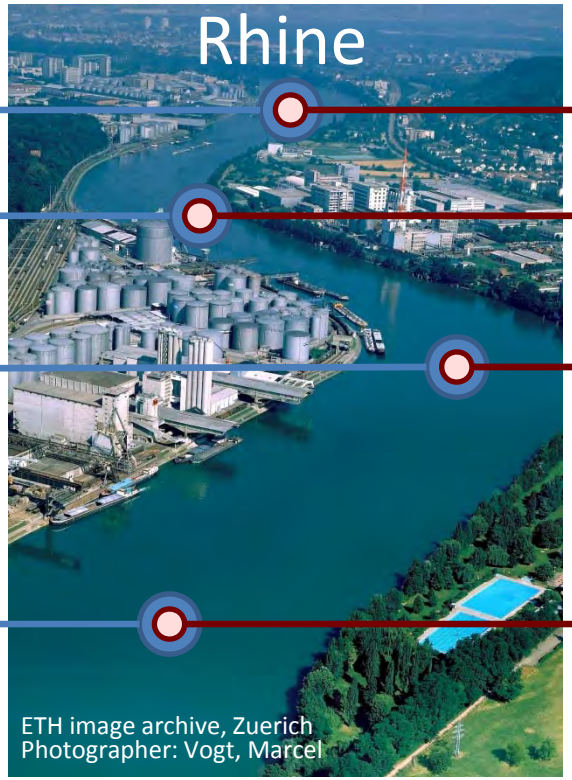
eawag aquatic research

spill

trend



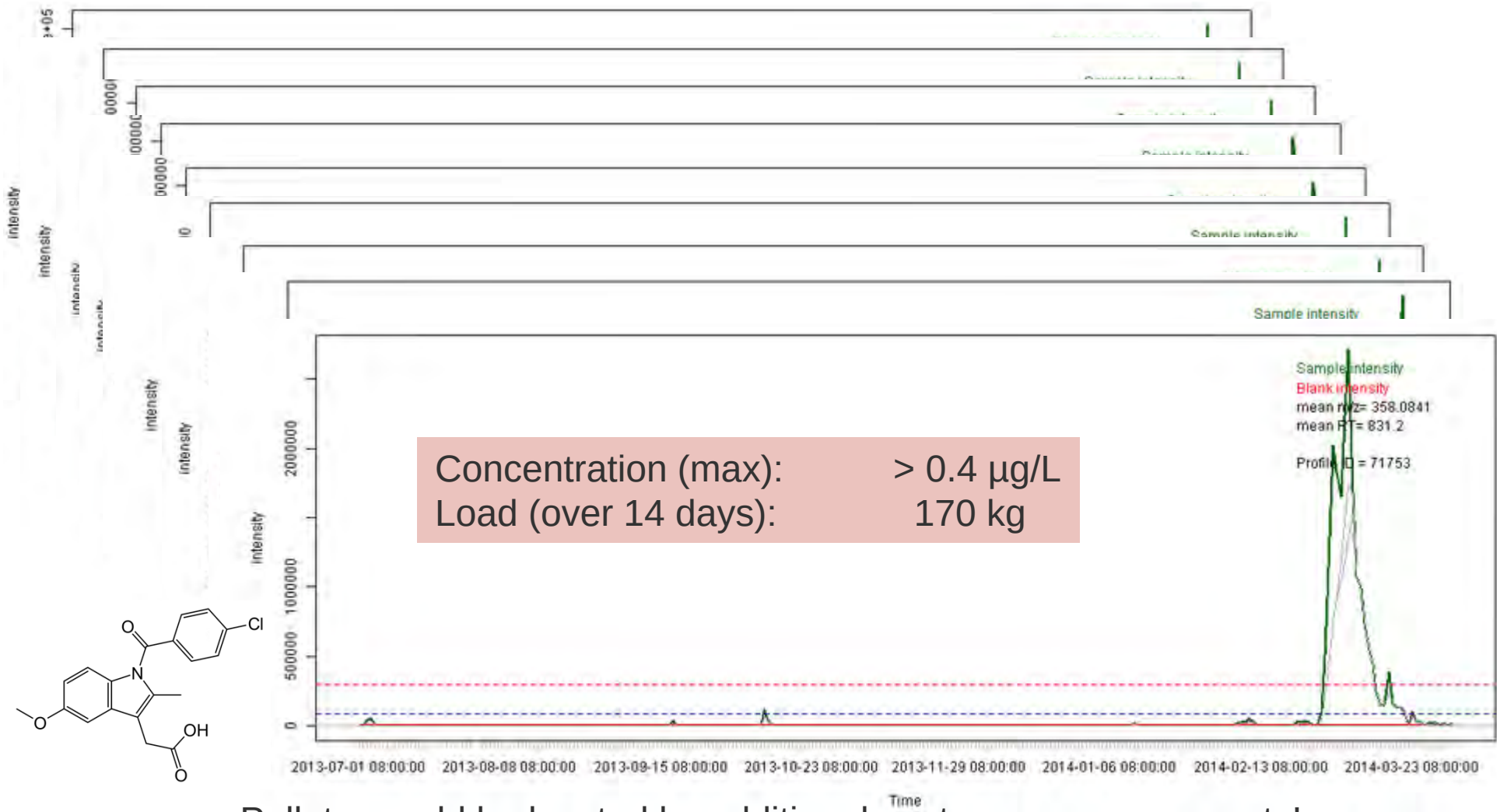
Industry



Households

Ruff et al., Water Research, 2015 (87)

Indomethacin spill



Polluter could be located by additional upstream measurements!

March 2014	1	2	3	4	5	6	7	8	……	25

Ruff et al., Water Research, 2015 (87)

International monitoring station - Basel

Results

- Comprehensive and real time trend and spill detection
- Implementation of non-target/suspect screening into routine monitoring

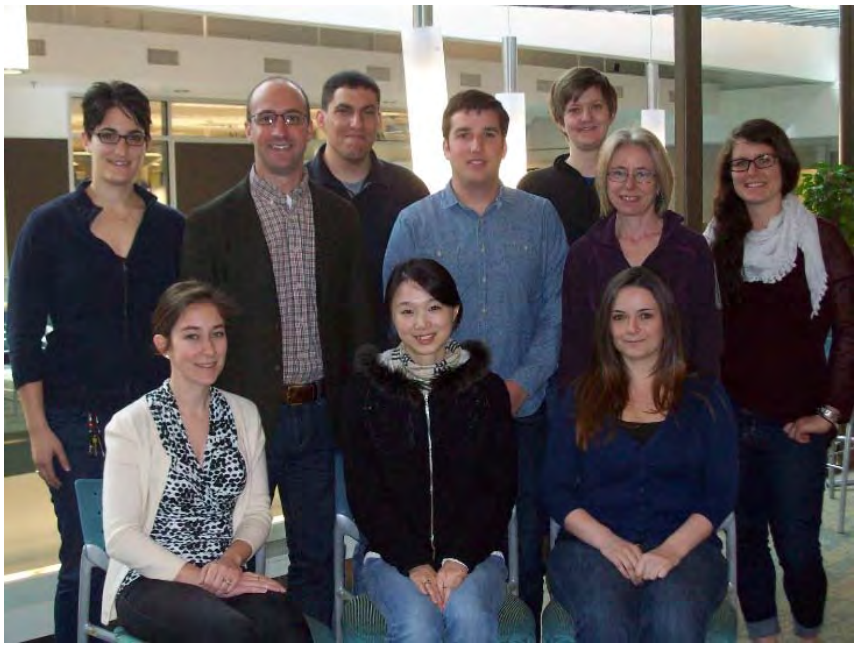
Results:

Conclusions and perspectives for future work

- LC-HRMS coupled with optimized non-targeted screening workflows provide essential tools for conducting “fate-directed analysis” and prioritization of CECs in water/wastewater.
- Coupling non-targeted analysis with mechanism-based bioanalytical tools provides a means of identifying “causative stressors”.
- Non-targeted analysis is complementary to targeted CEC monitoring in recycled water, and may best be applied to supply “leads” for CEC inclusion on monitoring lists.
- Significant challenges remain for robust compound identification tools (chemoinformatics), harmonization of methods, and “routine” application of non-targeted analysis.

Acknowledgement

- **Ferguson Lab Group**
- **Heinz Singer, Juliane Hollender (EAWAG)**



NIH National Institute of Environmental Health Sciences

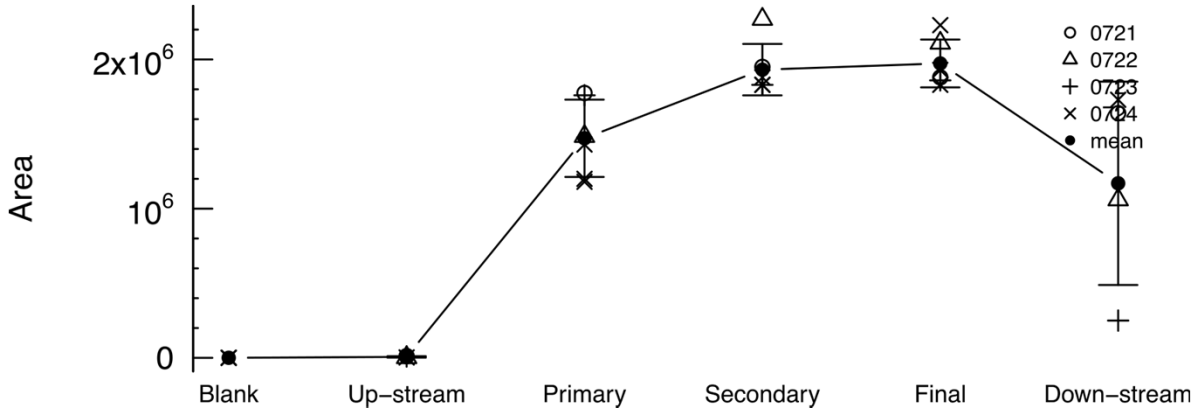
Srp Superfund Research Program

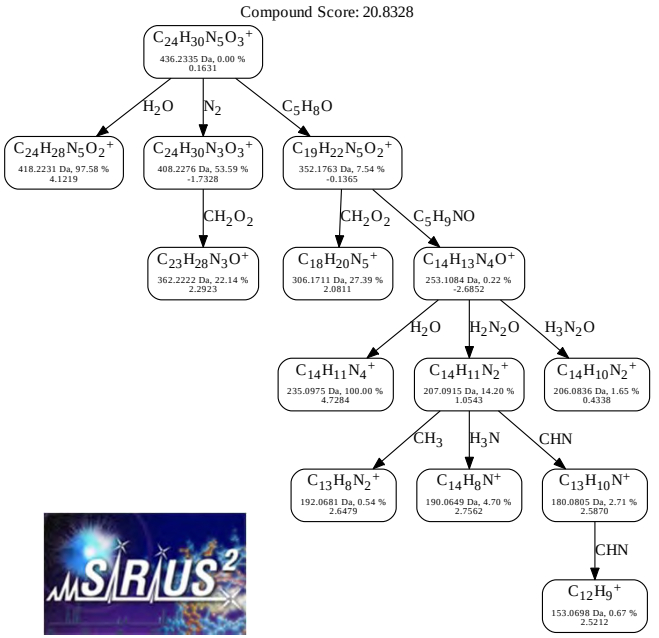
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

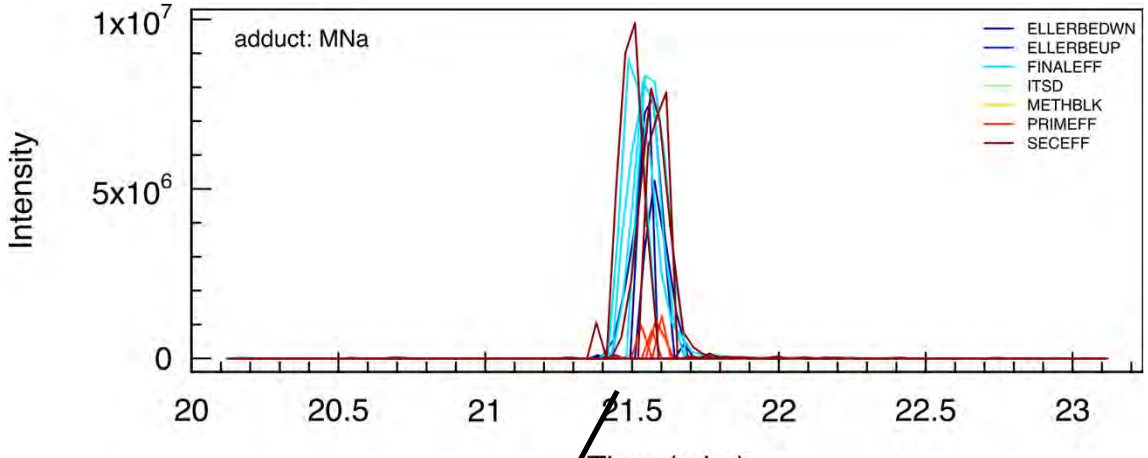
ThermoFisher SCIENTIFIC

Richard Jack and Dipankar Ghosh

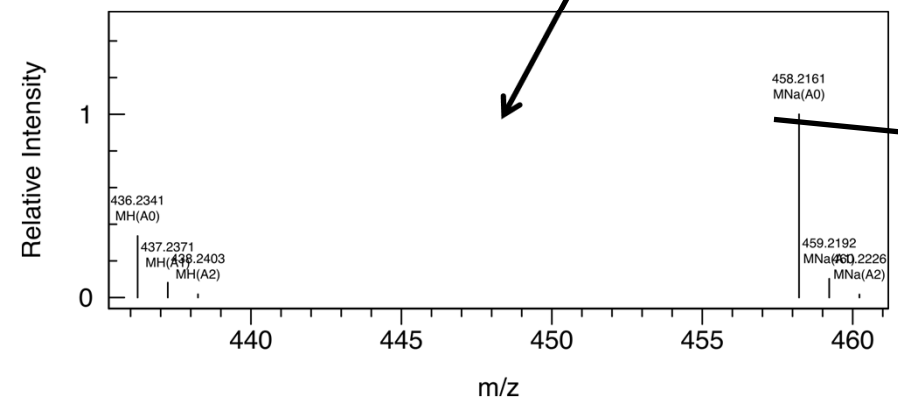
A feature at 435.2244 Da is wastewater-derived and recalcitrant

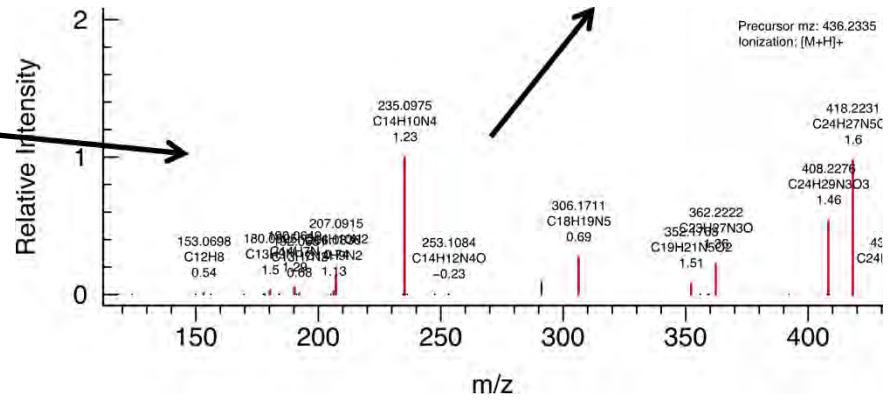






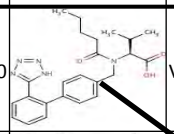
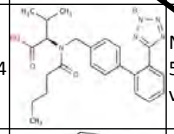
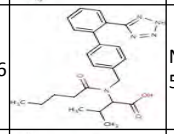
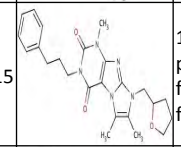
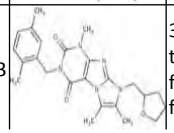
1.) C24H29N5O3 score: 20.83 tree: +20.83 iso: 0.00 peaks: 14 95.67 %
2.) C22H27N8O2 score: 15.72 tree: +15.72 iso: 0.00 peaks: 15 95.87 %
3.) C19H28FN8O3 score: 13.65 tree: +13.65 iso: 0.00 peaks: 14 95.67 %
4.) C16H33N7O5S score: 13.08 tree: +13.08 iso: 0.00 peaks: 12 85.63 %
5.) C19H29F4N5O2 score: 12.88 tree: +12.88 iso: 0.00 peaks: 13 74.11 %
6.) C18H30N9O2P score: 12.26 tree: +12.26 iso: 0.00 peaks: 11 64.07 %
7.) C21H31F4N2O3 score: 12.00 tree: +12.00 iso: 0.00 peaks: 14 95.40 %
8.) C19H30ClN9O score: 11.25 tree: +11.25 iso: 0.00 peaks: 10 63.87 %
9.) C15H33F2N4O8 score: 10.95 tree: +10.95 iso: 0.00 peaks: 14 95.67 %
10.) C15H31FN9O3P score: 9.57 tree: +9.57 iso: 0.00 peaks: 13 80.27 %





Valsartan tentatively identified by library match and *in silico* fragments

Chemspider matches for $C_{24}H_{29}N_5O_3$

CSID	ΔMass [ppm]	# References	Structure	Name
54833	1.4	1050		Valsartan
4447678	1.4	34		N-pentanoyl-N-{[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl}-D-valine
5448	1.4	26		N-Pentanoyl-N-{[2'-(1H-tetrazol-5-yl)-4-biphenylyl]methyl}valine
2414607	1.4	15		1,6,7-Trimethyl-3-(3-phenylpropyl)-8-(tetrahydro-2-furanylmethyl)-1H-imidazo[2,1-f]purine-2,4(3H,8H)-dione
2414601	1.4	13		3-(2,5-Dimethylbenzyl)-1,6,7-trimethyl-8-(tetrahydro-2-furanylmethyl)-1H-imidazo[2,1-f]purine-2,4(3H,8H)-dione

Valsartan is a highly prescribed angiotensin II receptor antagonist and has previously been shown to be recalcitrant to biodegradation in wastewater treatment.

Bergheim, M. et al. 2014. *Environ. Chem.*, 11, 431-444