Endocrinobiotox and Farmacotox

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Rise – 3rd Workshop
Faro 13 de December 2010

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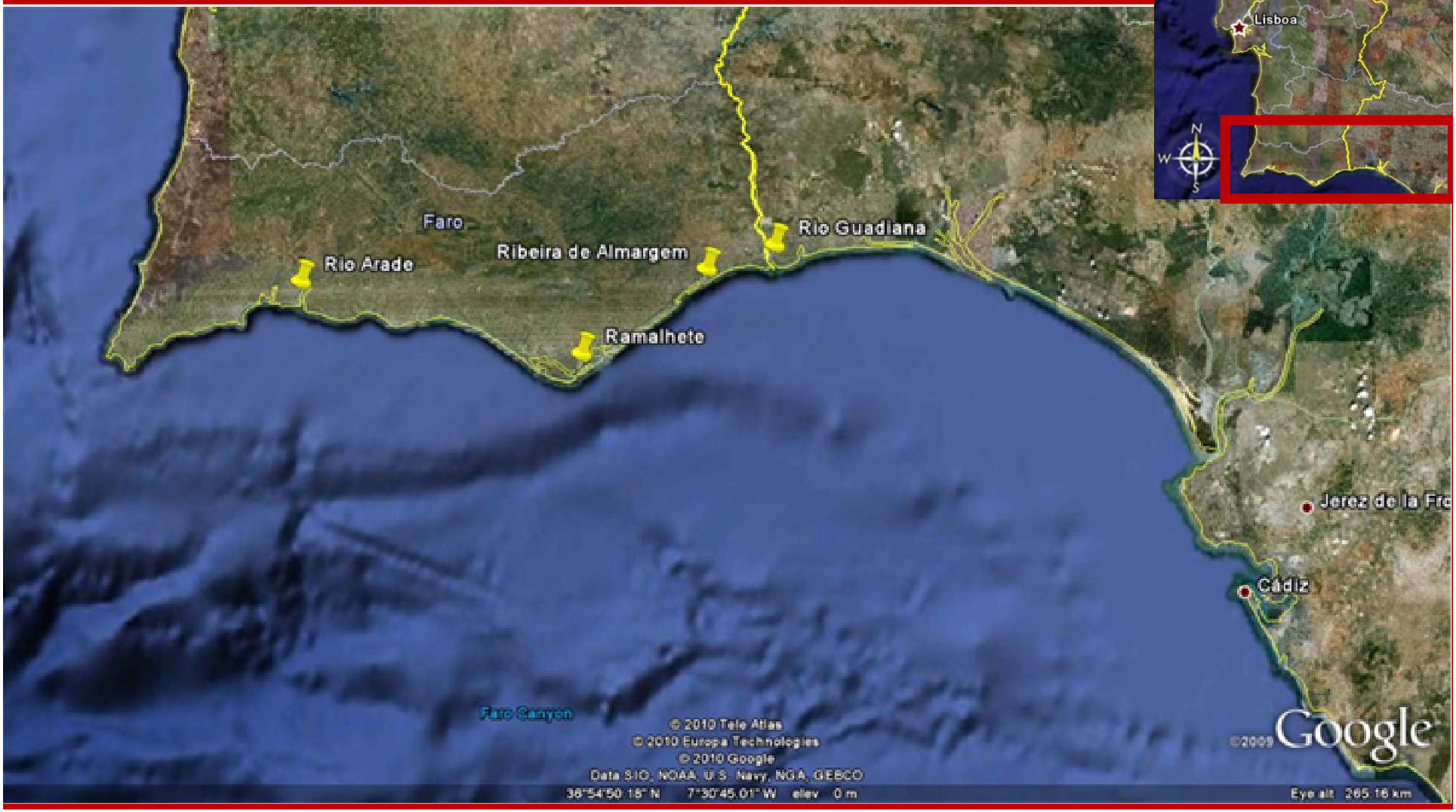
SAMPLING LOCATIONS



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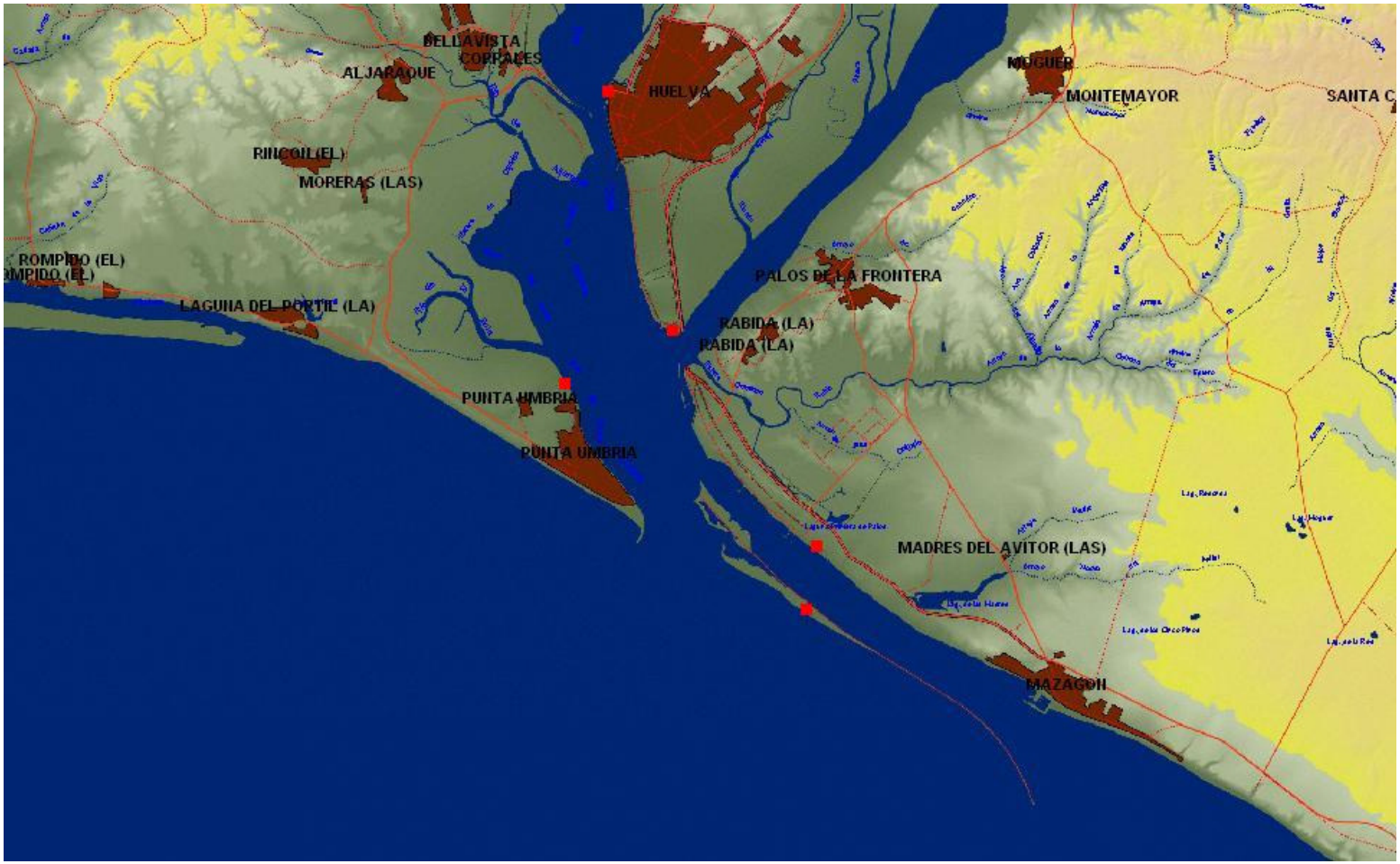
Sampling stations Huelva area



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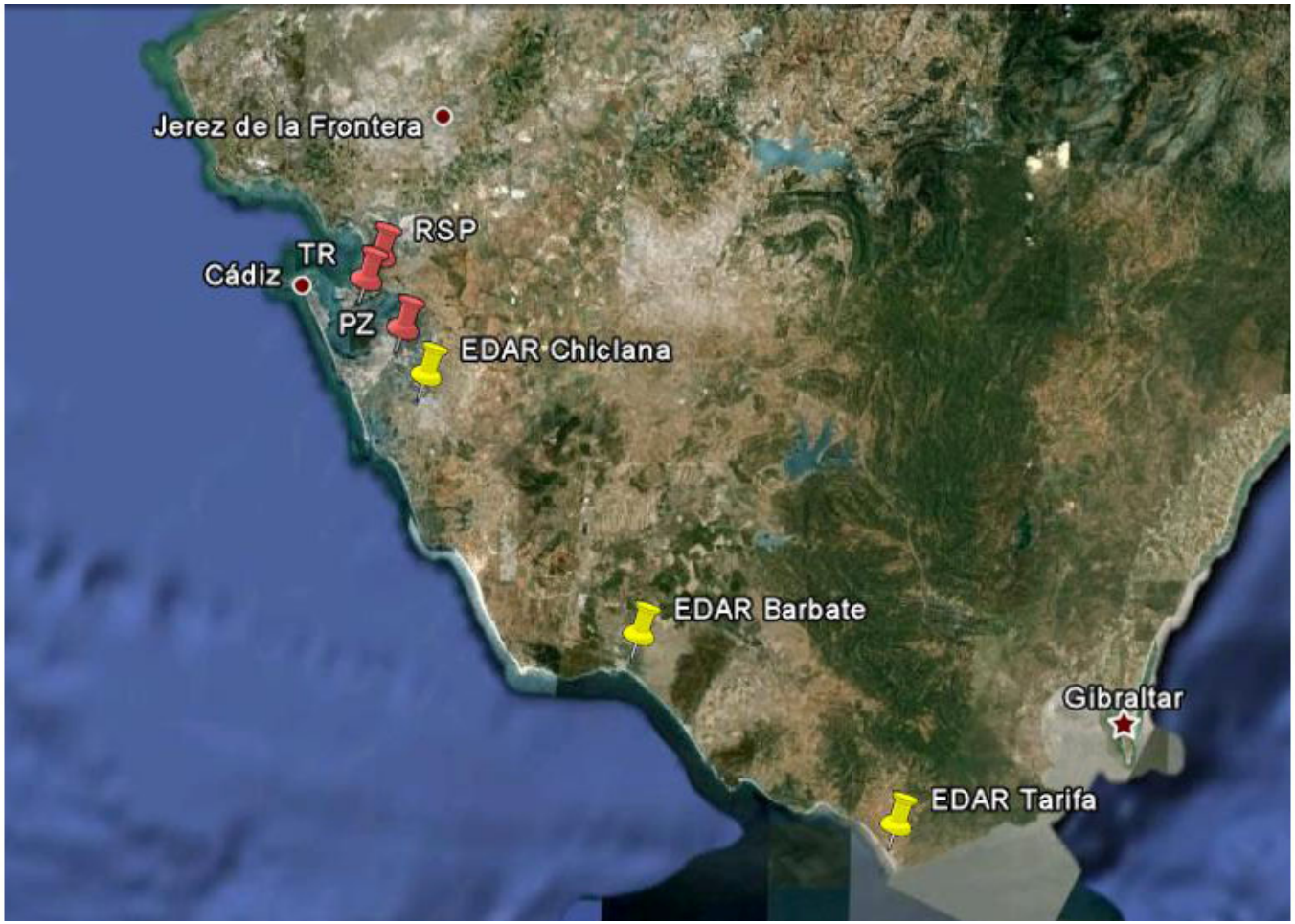
Sampling sites



Sampling

➢ Sediments

Random collection of 5 sediment cores (in duplicate), with 10cm depth (stored at -20ºC)

➢ Species

Estuarine bivalve species, the clam *Scrobicularia plana* (common name "lambujinha")

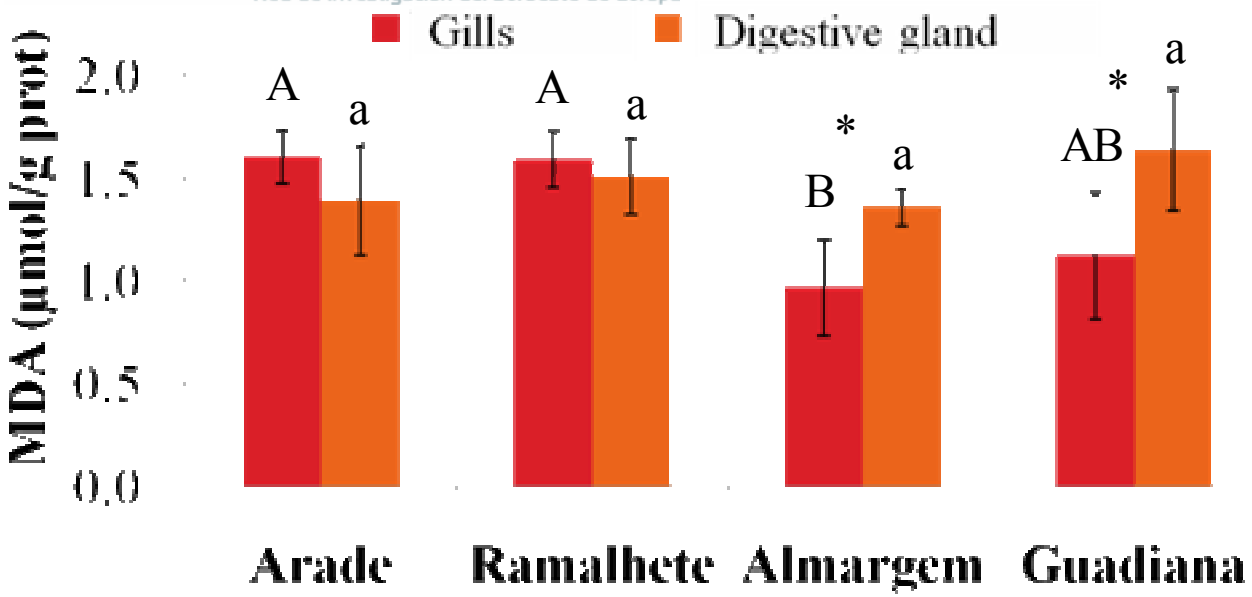
Intertidal polychaete species, *Nereis diversicolor* (common name "minhoca da lama")

➢ Abiotic Parameters

Temperature, salinity, pH and dissolved oxygen, measures *in situ* with a multiparametric probe

LIPID PEROXIDATION (LPO)

Scrobicularia plana

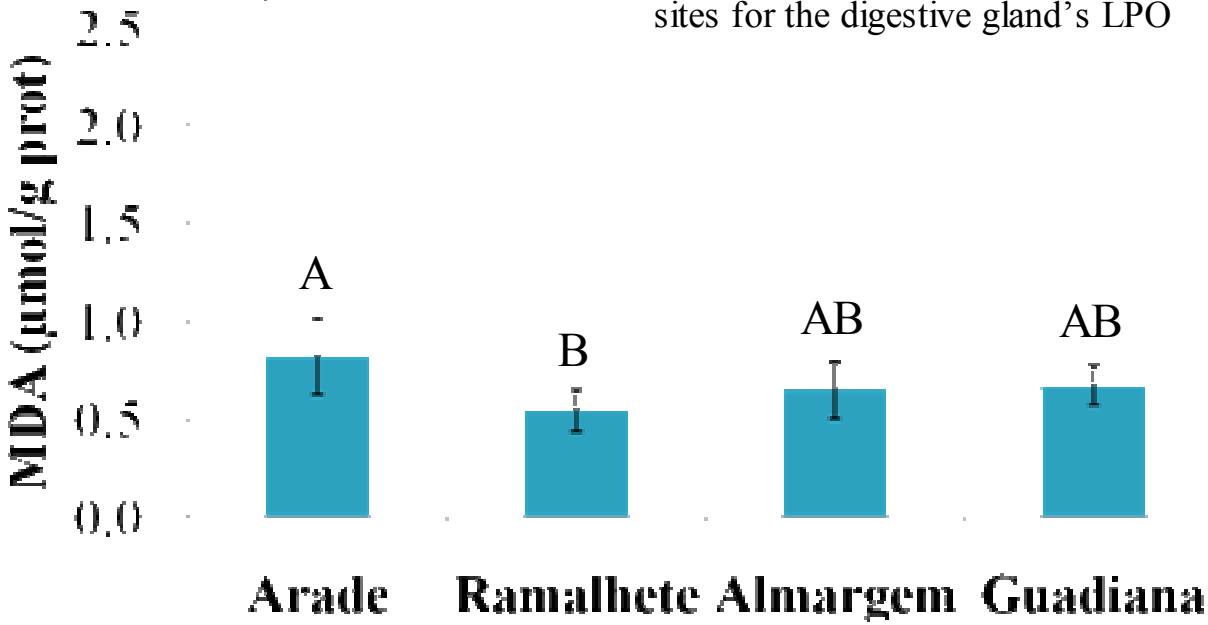


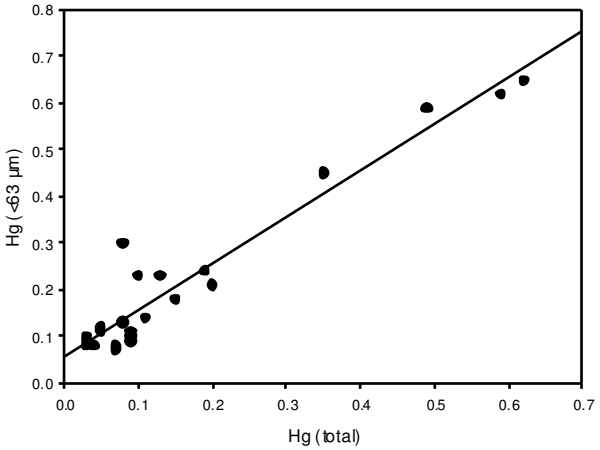
*: differences between tissues

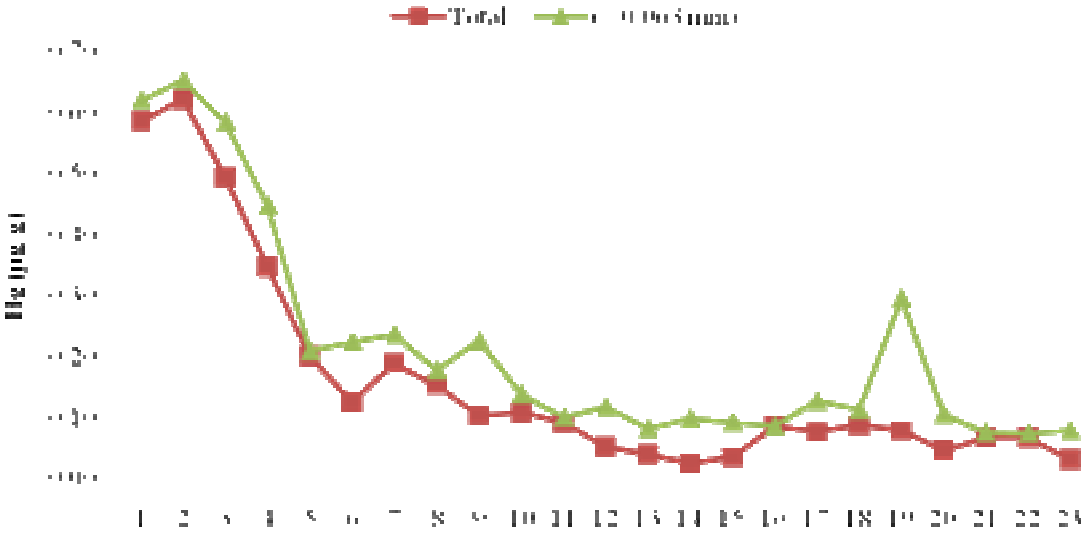
Capital letters: differences between sites for the gill's LPO

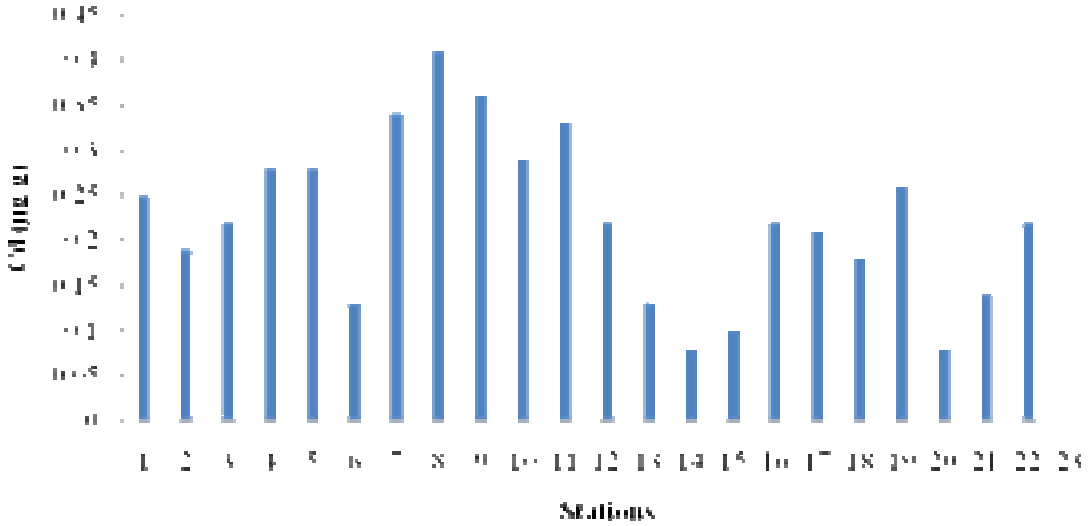
Low case letters: differences between sites for the digestive gland's LPO

Nereis diversicolor




Hg (<63 µm)
Hg (total)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8


Total


Stations

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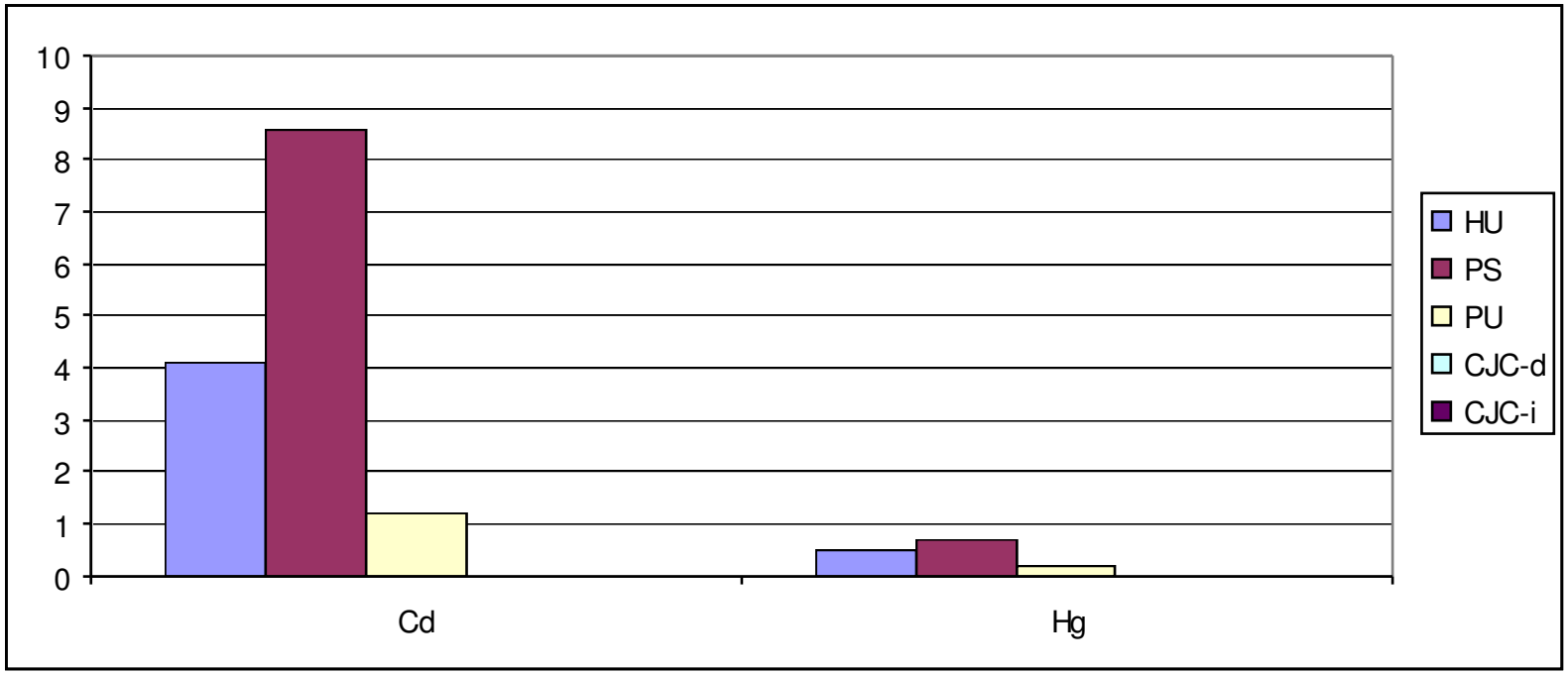
- **Presencia de Cd y Hg (µg g-1, base seca) en sedimentos Ría de Huelva**



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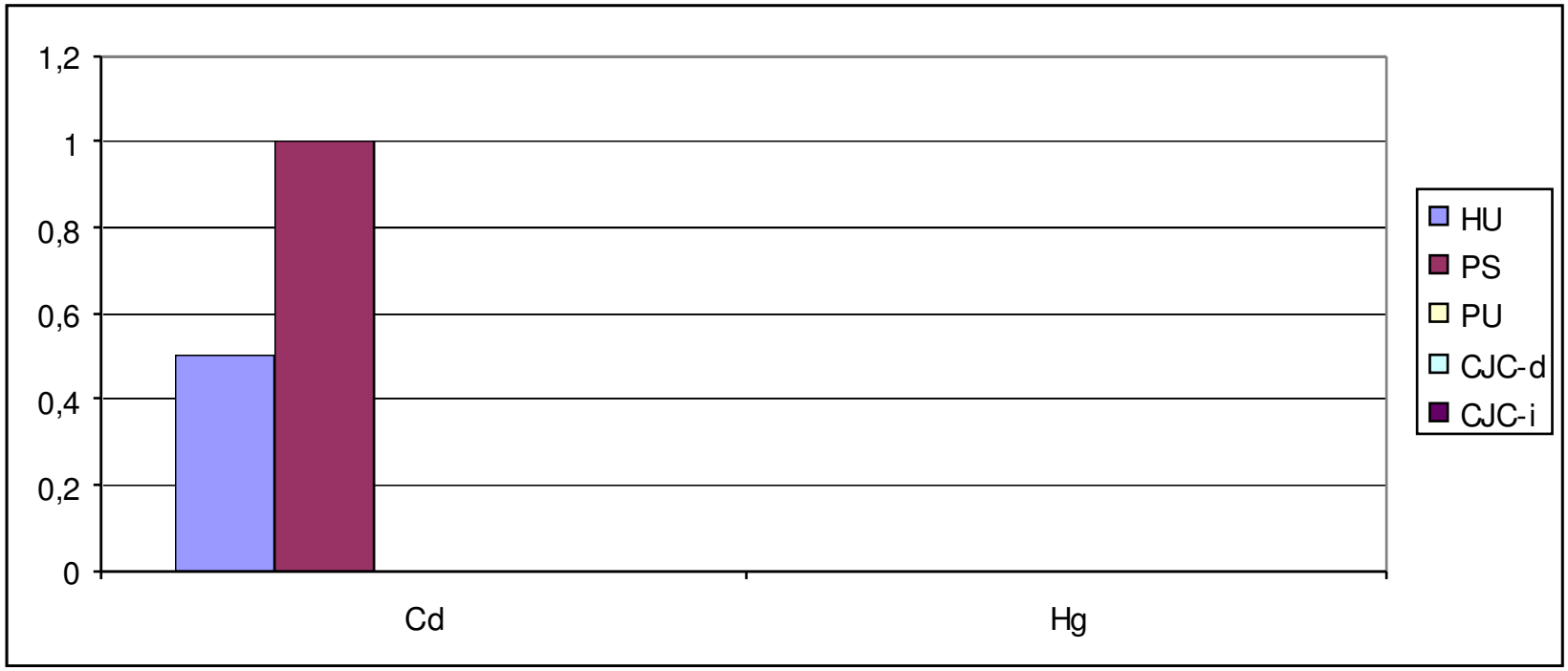
- **Presencia de CD y Hg en *Chamaelea gallina* (µg g-1, peso seco)**



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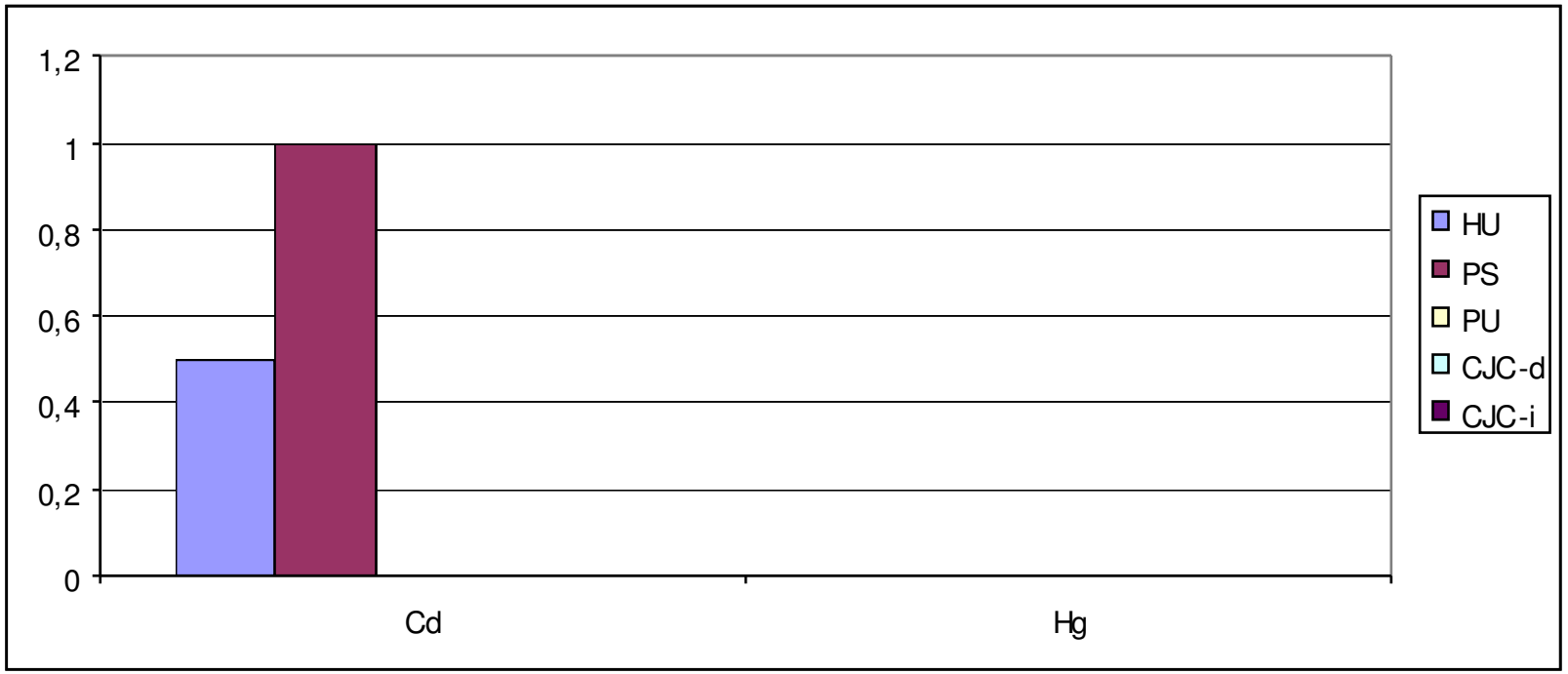
- **Presencia de Cd y Hg en *Nereis diversicolor* (µg g-1, peso seco)**



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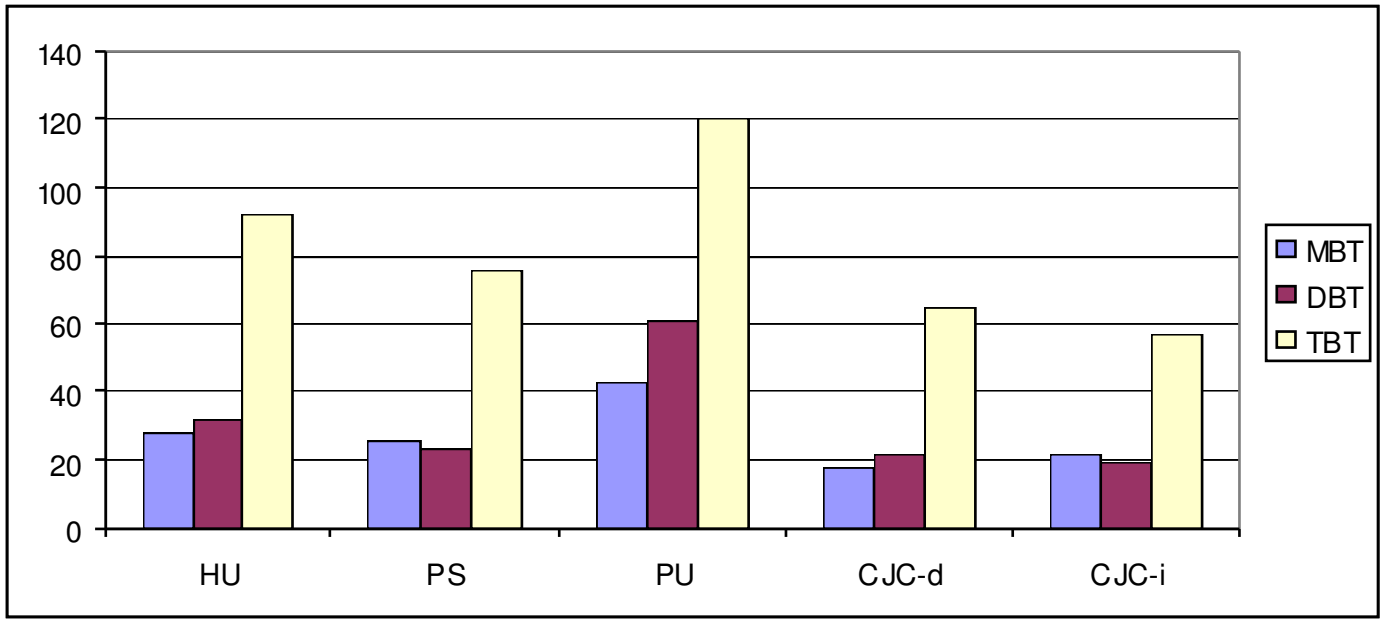
- **Presencia de especies de estaño en muestras de Chamaelea gallina (ng g-1 de Sn, base húmeda)**



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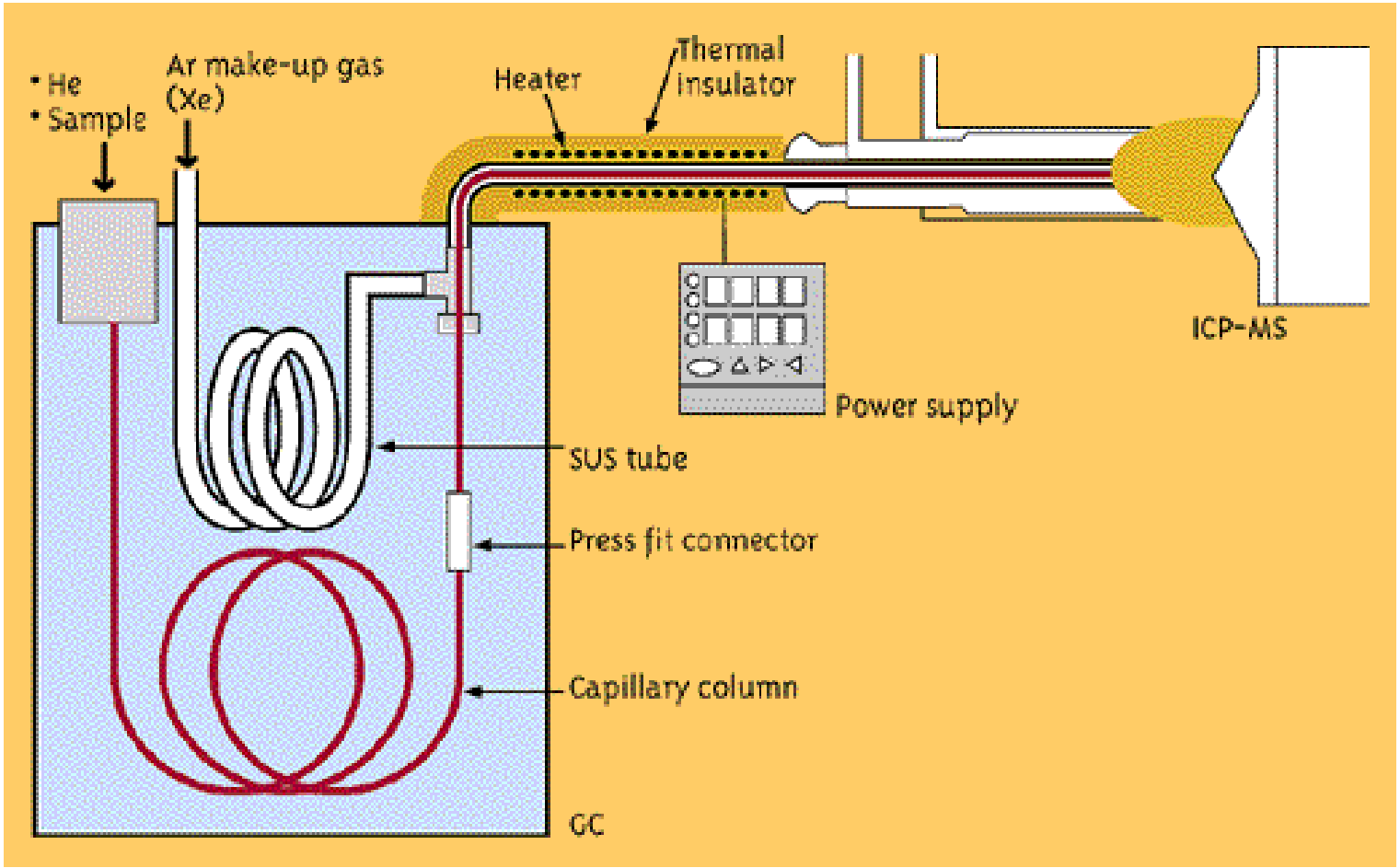
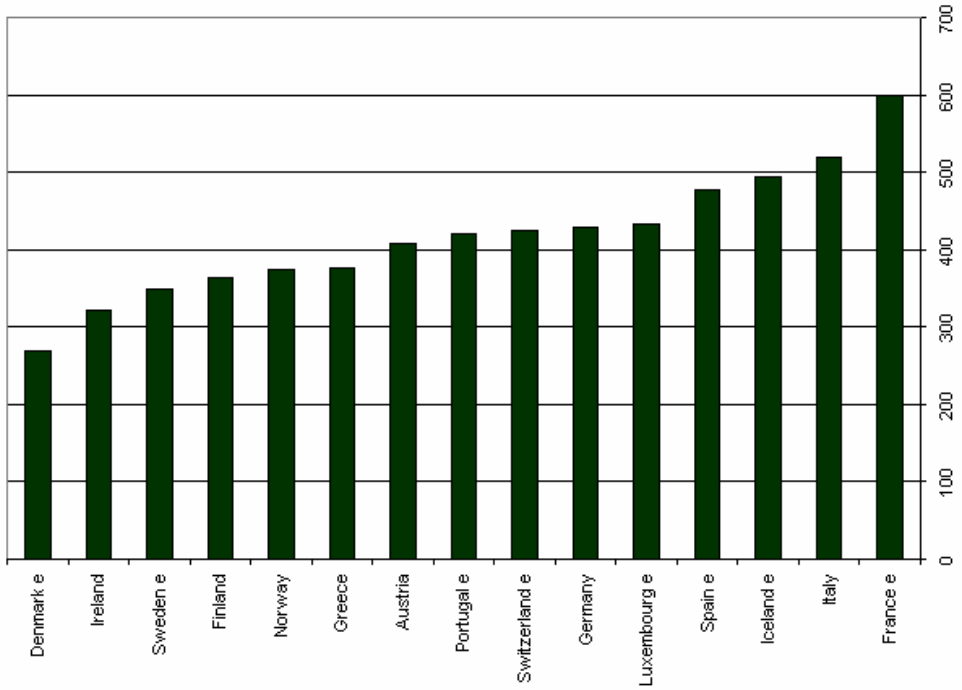
Methodological development: TBT speciation



Pharmaceuticals: "new" environmental contaminants

- Included in priority list of persistent and emerging contaminants (EU)

Consumption/ capita [€] in Western European countries (2004)



Source: Espicom Business Intelligence management report: Major Pharmaceutical Markets in Europe

→ Introduction into environment:

- Human and animal excretion (urine, faeces)
- Disposal expired products
- Pharmaceutical industry

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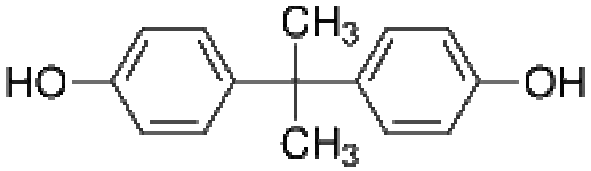
Endocrine disruptor and pharmaceuticals compounds:

Based matrix analysis and new methodological approach (liquid-phase-microextraction with porous hollow fiber)*



Ibuprofeno
Diclofenac
Carbamezapine

*Ramos-Payán, MD., Fernández-Torres, R. Bello-López, MA., Gomez-Ariza, JL., Callejóm-Mochón, M. (2010) Talanta, 81 (3), pp. 871-880

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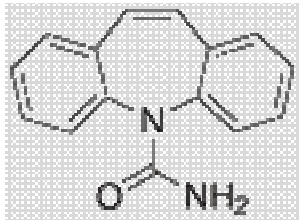
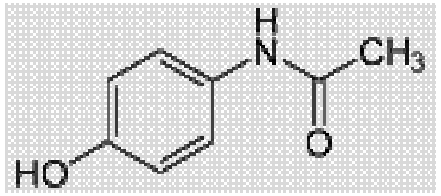
Carbamazepine (CA):

anticonvulsant ➔ epilepsy and bipolar disorders



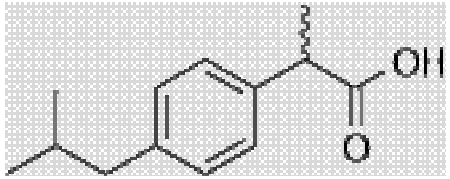
Acetaminophen (AC):

Analgesic and antipyretic ➔ pain relieve and reduction of fever .

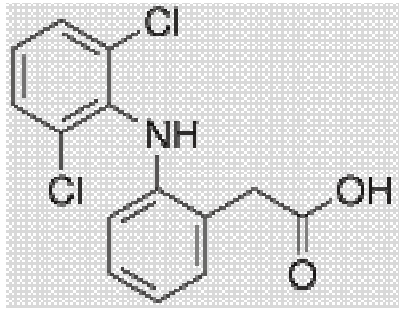


Ibuprofen (IB):

non-steroidal anti-inflammatory drugs (NSAID) ➔ reduction of inflammation and pain



Diclofenac (DF):



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Test organism: *Tisbe battagliai*

- Marine copepod *Tisbe battagliai* (Crustacea, Copepoda, Harpacticoida)
- Distribution: shallow waters of coastal regions of Europe and US Atlantic coast.
- Feeding: epiphytic micro-algae, detritus and bacteria
- Development:

Adult female carrying ova

Second stage copepodid

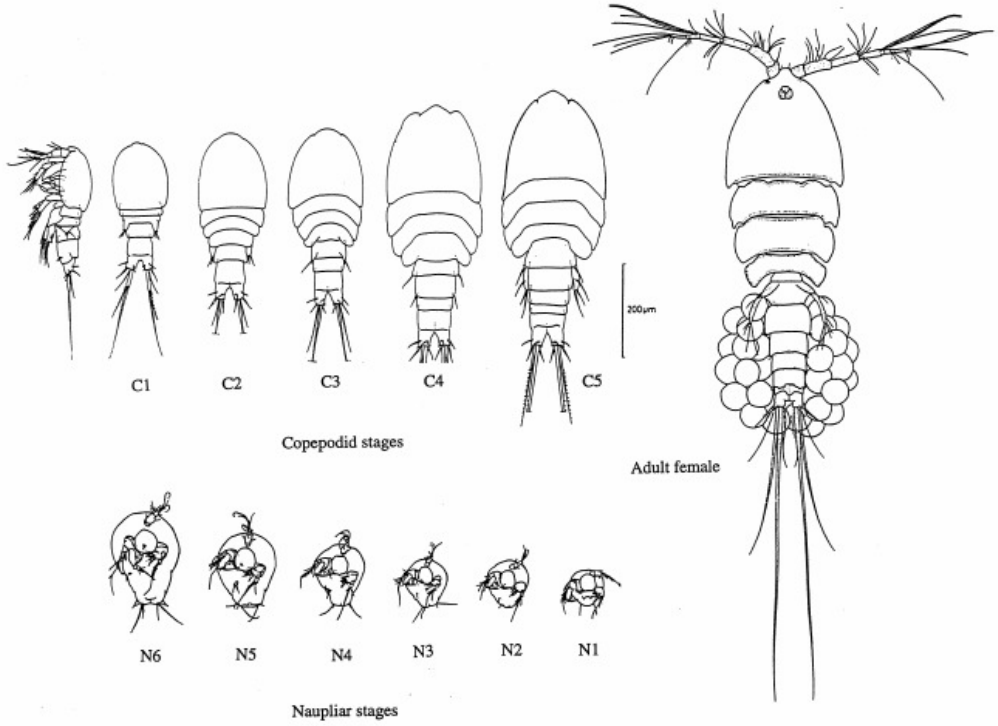

Fig. 2. Summary of developmental stages of *Tisbe battagliai* (Crustacea: Copepoda), adapted from Volkmann-Rocco (1972). Scale bar represents 200 µm.

T.H. Hutchinson et al. The Science of the Total Environment 233 (1999) 167-179

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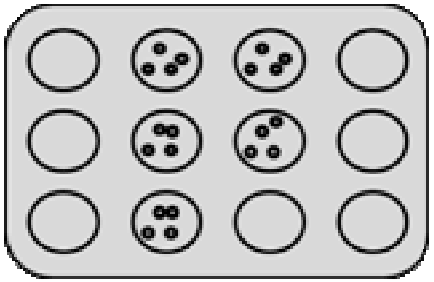
Test protocol: Diz et al., 2009

<24h –old nauplii, 48h, 16h light: 8h darkness, 21ºC

Exposure system: 12 well plates (5mL); 4 nauplii/well; 5 replicates/concentration



Control
Solvent Control (DMSO)
Increasing concentrations (from STOCKs in DMSO)

Nominal concentrations: 1 and 150 $mg \cdot L^{-1}$

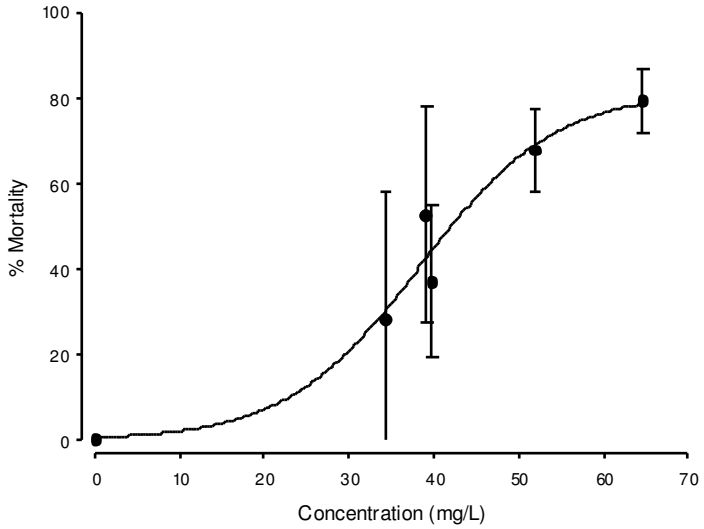
Every 24h: mortality control and renewal of 80% of exposure media

Mortality data: generalized linear models (GLMstat)

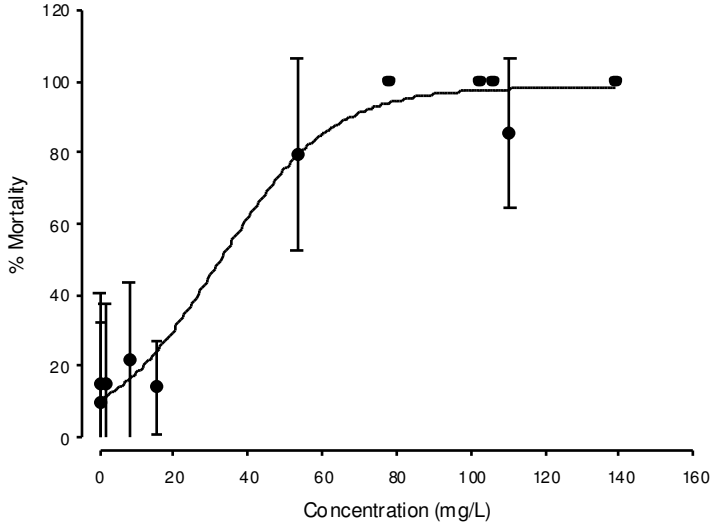
➔ LC50, LC30, LC20 and LC10: Kerr y Meador (1996)

1. Single compounds

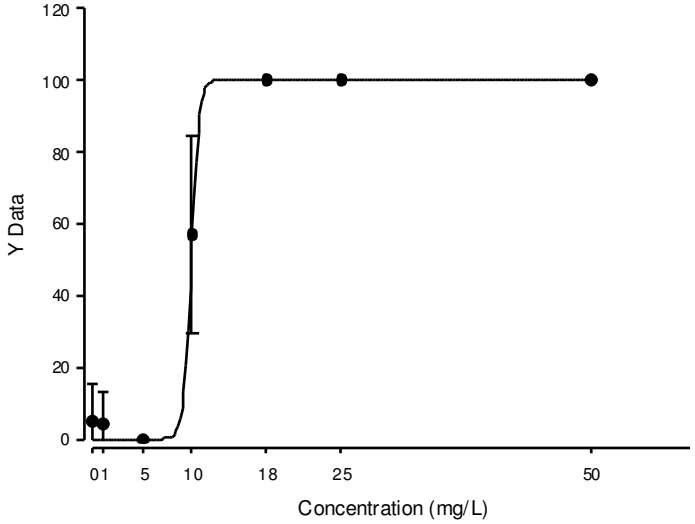
Acetaminophen



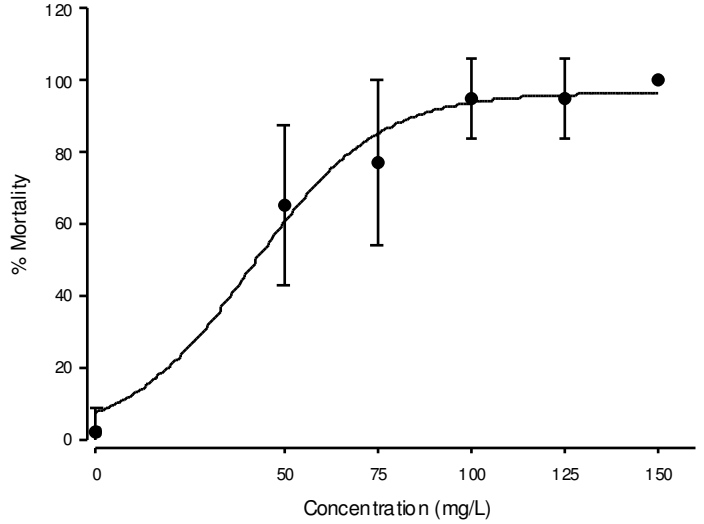
Carbamazepine



Diclofenac



Ibuprofen



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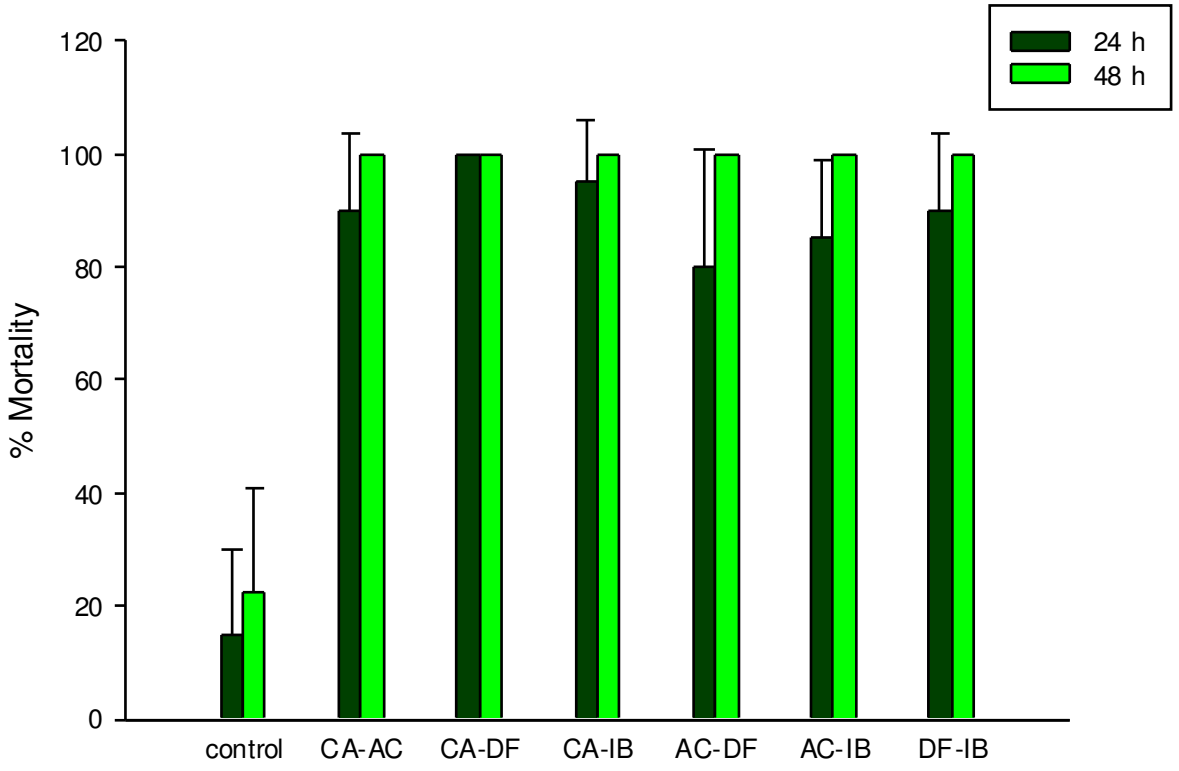
2. Binary mixtures

2.1. Combinations $LC50_i$ + $LC50_j$ ➔ 100% mortality after 24h in all mixtures

2.2. Combinations $LC50_i/2$ + $LC50_j/2$



Carbamazepine (CA):
Acetaminophen (AC):
Ibuprofen (IB):
Diclofenac (DF):

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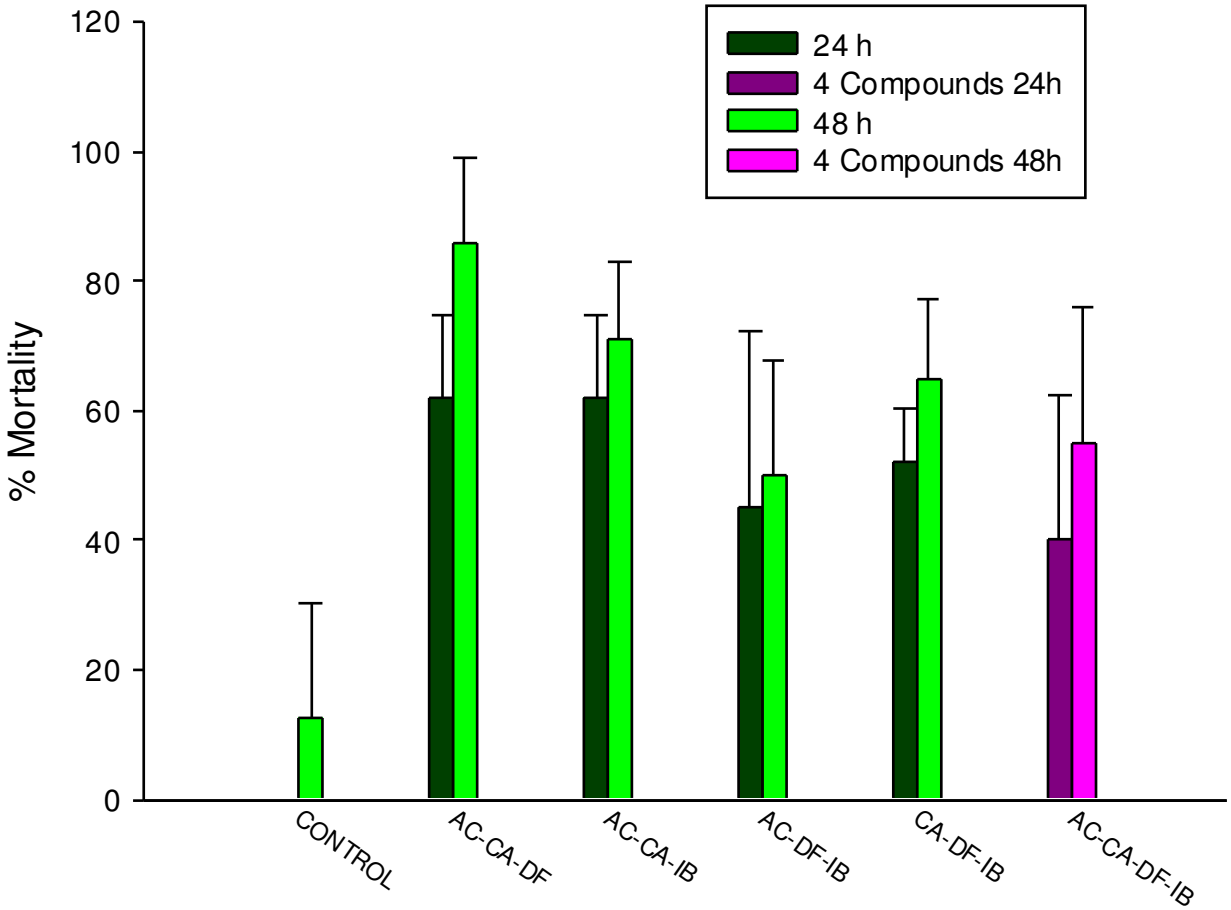
3. Tertiary mixtures

$LC50_i/3 + LC50_j/3 + LC50_k/3$

4. Cuarternary mixtures

$LC50_i/4 + LC50_j/4 + LC50_k/4 + LC50_l/4$



Carbamazepine (CA)
Acetaminophen (AC)
Ibuprofen (IB)
Diclofenac (DF)

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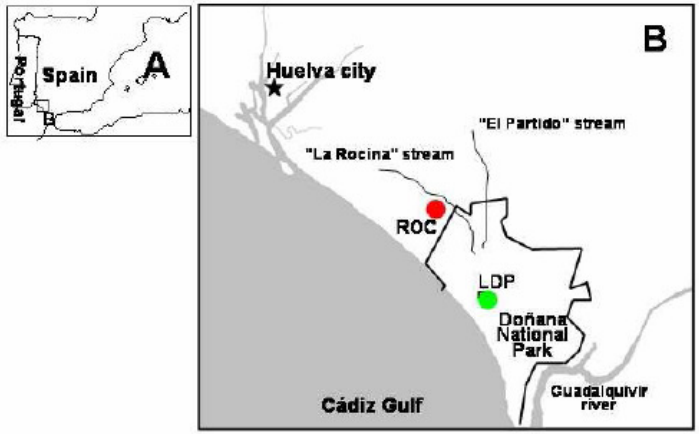
Mus musculus and Mus spretus



Ruiz-Laguna, J., Abril, N., Garcia-Barrera, T., Gomez-Ariza, J.L. Lopez-Barea J, Pueyo C., Environ. Sci. Technol. (2006) 40: 3646

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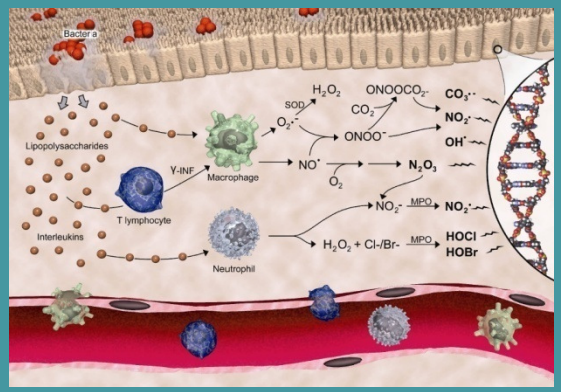
Metal-binding molecules importance in environmental pollution assessment

Metal +Proteins → **Metallomolecules** → Metabolic processes

- Enzyme cofactors
- Structural components of proteins

Deficiency or excess



- Toxics effects of pollutants (Cd, Pb, As)
- Oxidative stress
- Damage of biomolecules
- Alteration of glutathione status
- Induction of antioxidative enzymes
- Homeostasis failure

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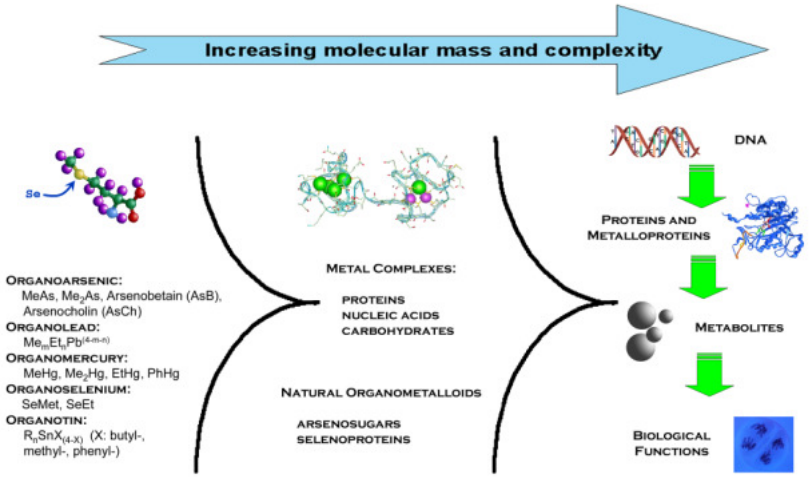
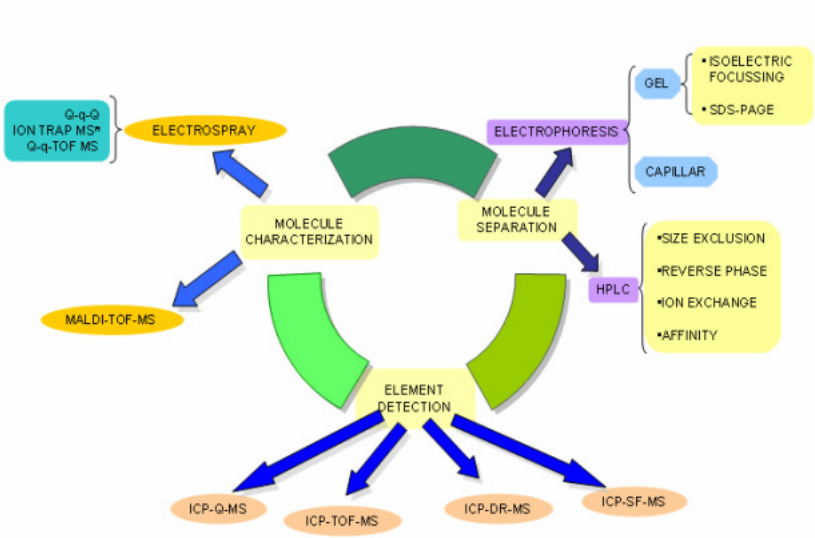
Metallomics Approach

Metallome:The entirety of metal and metalloid species present in a cell or tissue type, their identity, quantity and localization





S. Monicou, J. Szpunar, R. Lobinski, *Chem. Soc. Rev.*, 38, 1119-138 (2009)

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Sample preparation

1. Entire liver and brain from 5 different animals were pooled.
2. **Pool was disaggregated with cryogenic homogenization (1min to rate 15) cryogenic** SPEX SamplePrep (Freezer/Mills 6770)
3. **After that was homogenized (1:3, w/v) with 25 mM Tris-HCl (pH 7.2) buffer containing 1 mM GSH and 1 mM PMSF.** Using a glass/teflon tissue homogenizer.
4. **Benzonase was added (50 U mL^{-1}) to the extracts which were incubated for 30 min at room temperature**
5. **Finally, centrifuged at 45,000 rpm for 1 h to 4ºC.** Beckman model L9-90K equipped with a rotor 70.1 Ti. Polycarbonate bottles with cap assembly (Beckman Coulter)
6. **Extracts were stored at -80 °C until analysis.**

No use metal – complexing reagents

EDTA

Avoid to the interaction with metal bound biomolecules.

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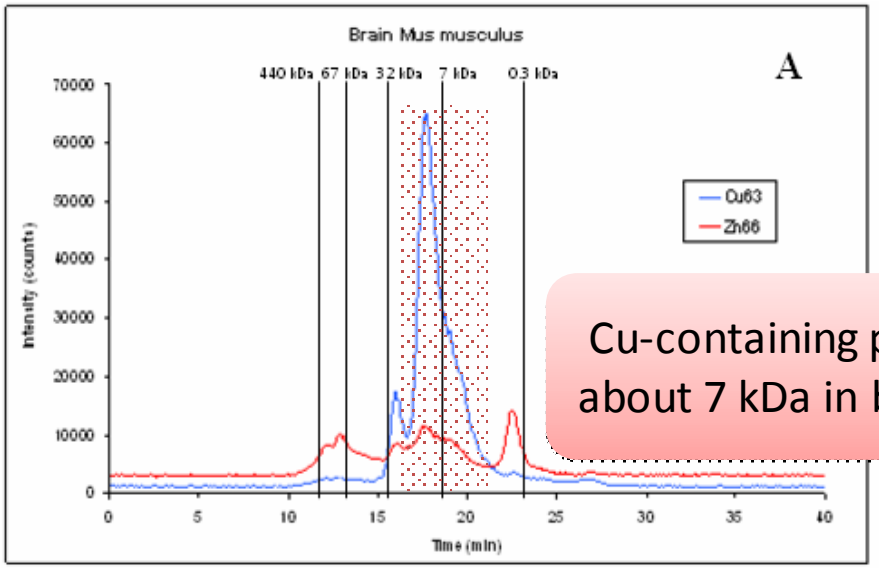
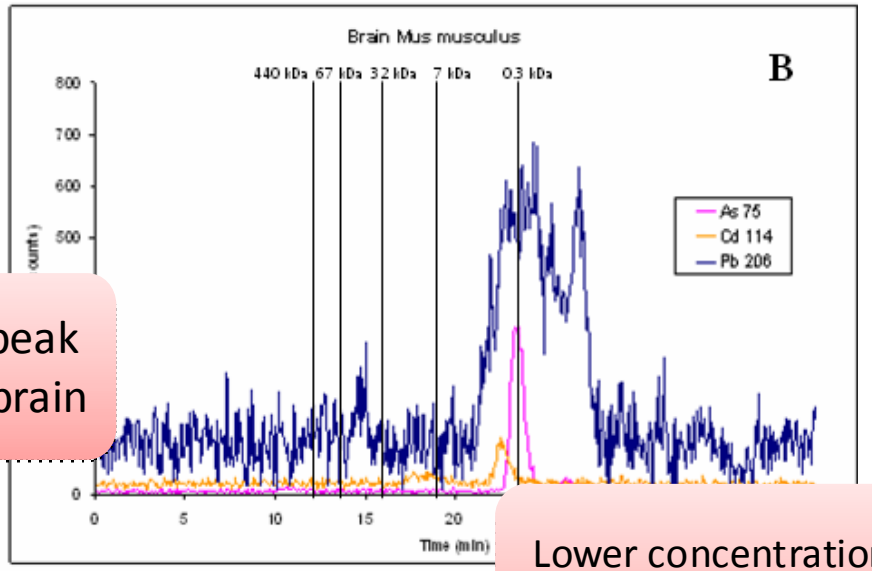
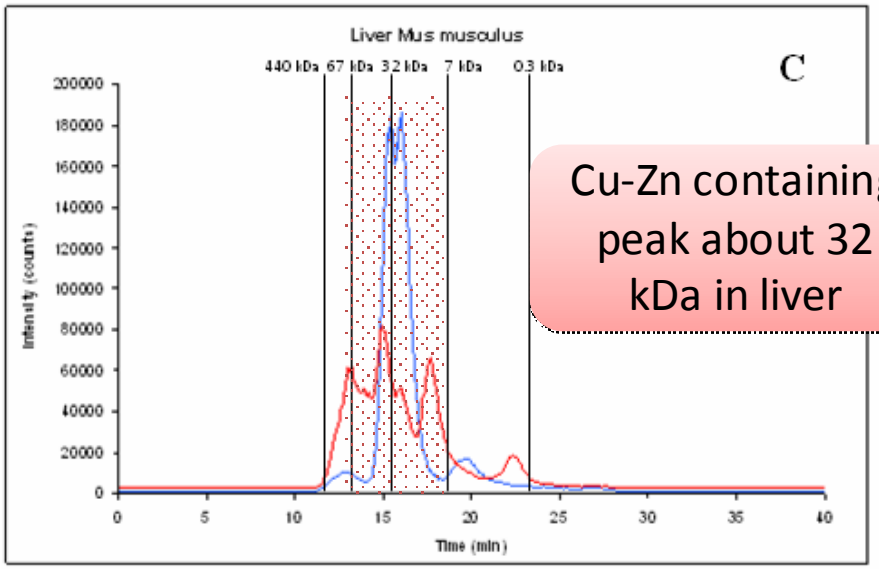
Size characterization of essential and toxic metal species in liver and brain from *Mus musculus* using SEC-ICP-ORC-MS



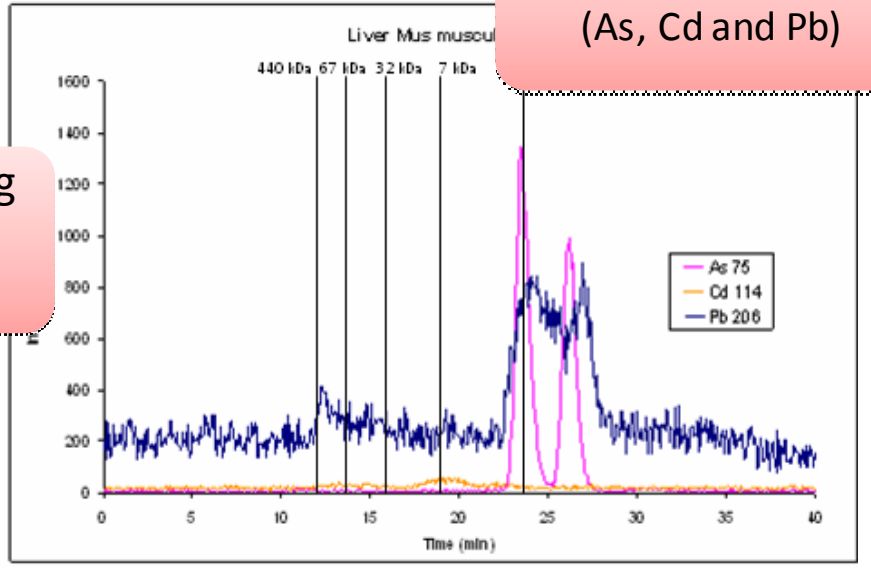


Cu-containing peak about 7 kDa in brain

Lower concentration (As, Cd and Pb)



Cu-Zn containing peak about 32 kDa in liver



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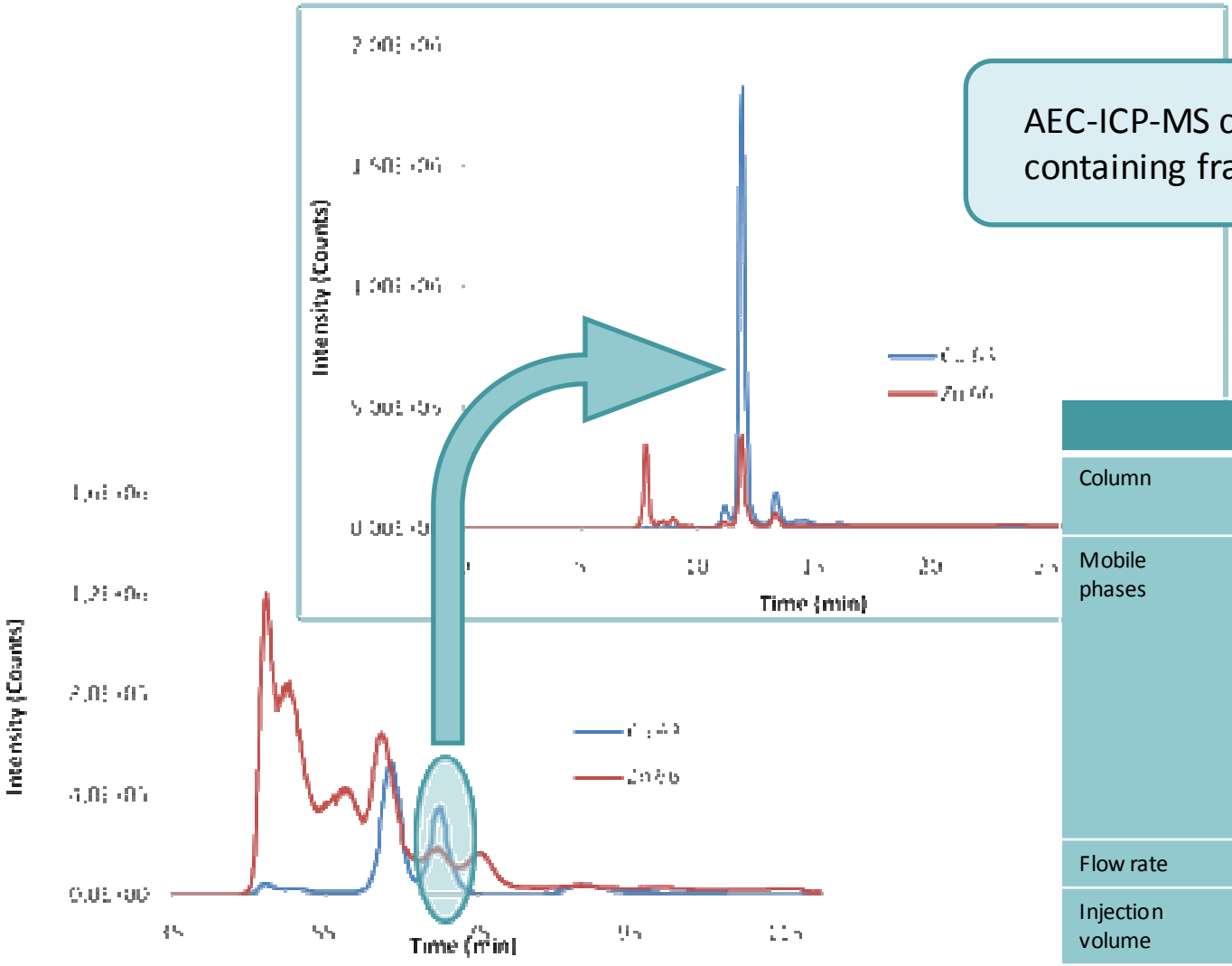
Fractionation of Cu/Zn-molecules in liver from *Mus musculus* using AEC-HPLC-ICP-ORC-MS



AEC-ICP-MS chromatogram of Cu/Zn containing fraction collected by SEC

AEC operating conditions	
Column	Protein Pak DEAE 5PW (Waters) (7.5 x 75 mm)
Mobile phases	A: 2 mM ammonium acetate (pH 7.4) B: 200 mM ammonium acetate (pH 7.4) Time/min — Buffer B (%) 0 — 0 10 — 15 15 — 20 23 — 20 30 — 0
Flow rate	1 mL min^{-1}
Injection volume	50µL
UV-visible wavelength	254 nm

Chromatographic conditions: Hiload 26/60 Superdex 75 Prep, Mr 3-70kDa, 20 mM ammonium acetate (pH 7.4) for isocratic elution

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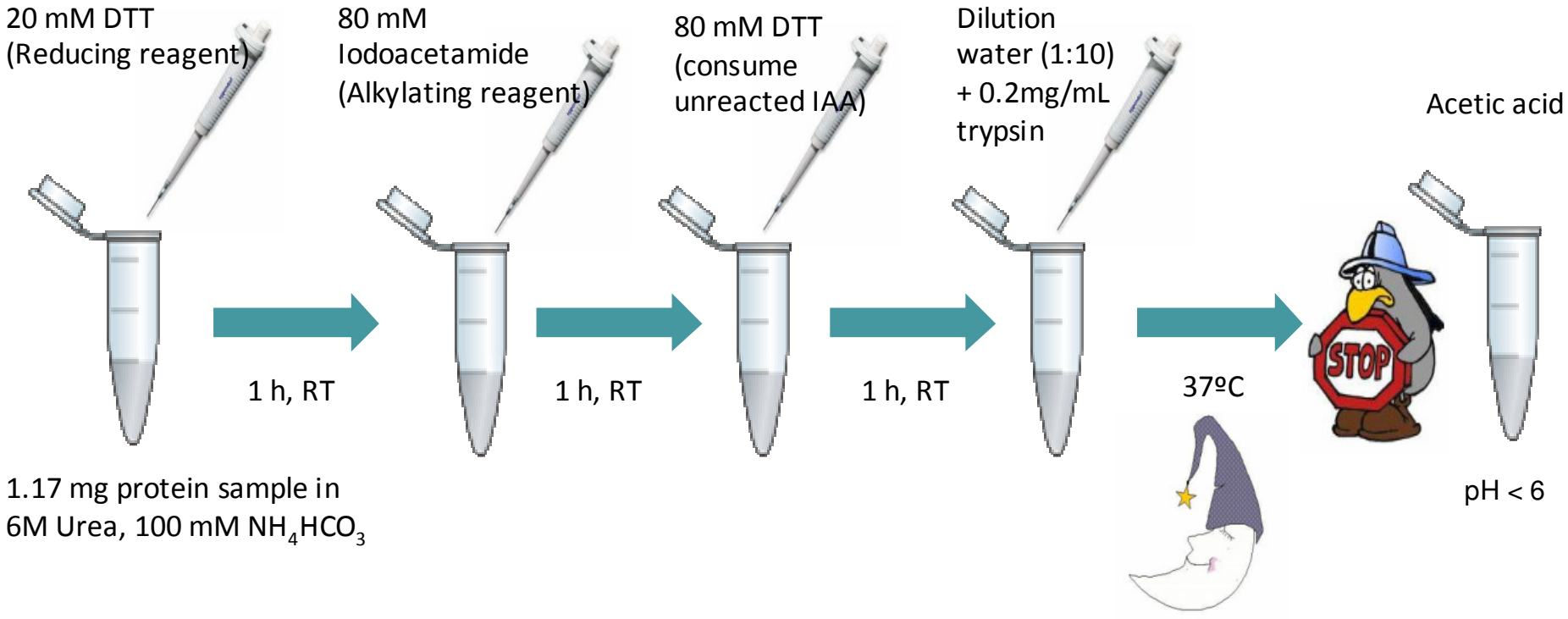
Fragmentation of metallomolecules using trypsin



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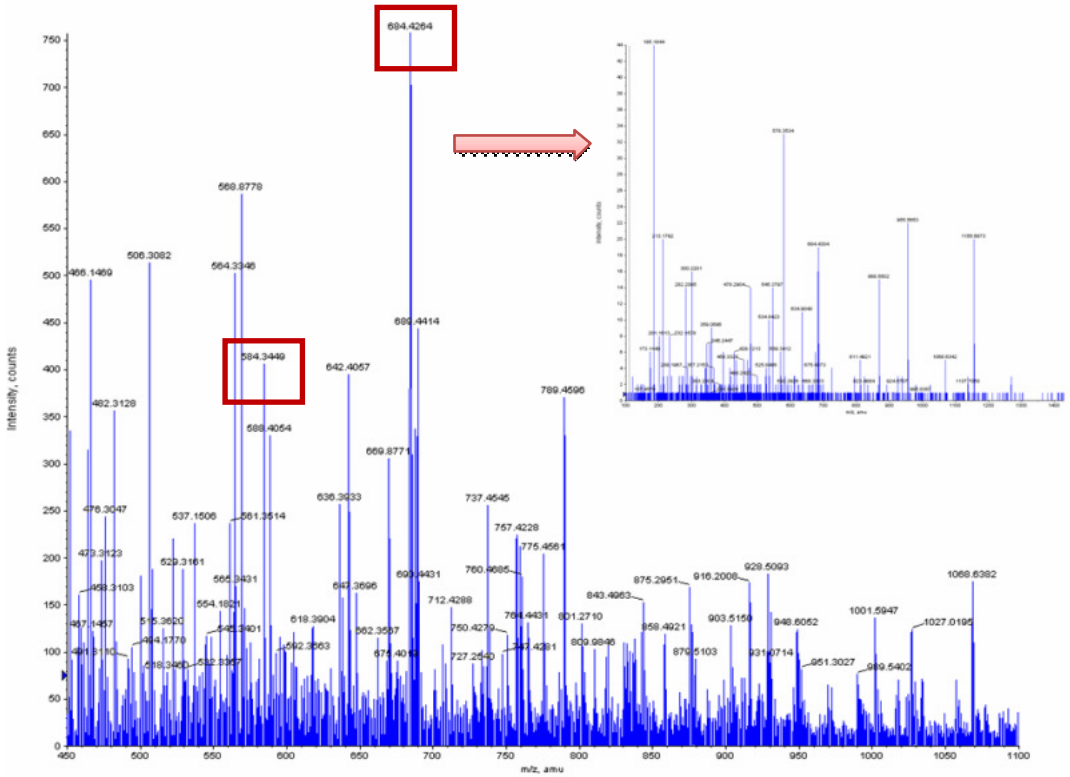
Identification of metal-biomolecules by nESI-QqQ-TOF



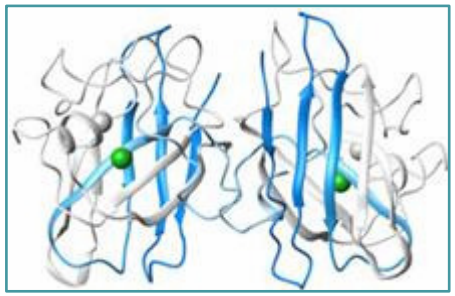
n-ESI-MS spectrum of the tryptic digestion from the unknown peak about 32 kDa

nanoESI-QqQ-TOF parameters	
Ion spray Voltage	1500 V
Curtain gas	20 psi
Declustering potencial	90 V
Electron multiplier voltage	2200 V
Mass range	450- 1500 m/z

Superoxide dismutase (Cu-Zn)

(MASCOT-SwissProt Accesion No. P08228)
Nominal mas: 16104 Da
Subunit structure: Homodimer
Localization subcellular: Cytoplasm

Mass ions	Score	Peptide Sequence
584.3	77	1 MAMKAVCVLK GDGPVQGTIH FEQKASGEPV VLSGQITGLT EGQHGFHVHQ 51 YGDNTQGCTS AGPHFNPHSK KHGGPADEER HVGDLGNVTA GKDGVANVSI 101 EDRVISLSGE HSIIGRTMVV HEKQDDLGKG GNEESTKTGN AGSRLACGVI 151 GIAQ
684.4	88	1 MAMKAVCVLK GDGPVQGTIH FEQKASGEPV VLSGQITGLT EGQHGFHVHQ 51 YGDNTQGCTS AGPHFNPHSK KHGGPADEER HVGDLGNVTA GKDGVANVSI 101 EDRVISLSGE HSIIGRTMVV HEKQDDLGKG GNEESTKTGN AGSRLACGVI 151 GIAQ



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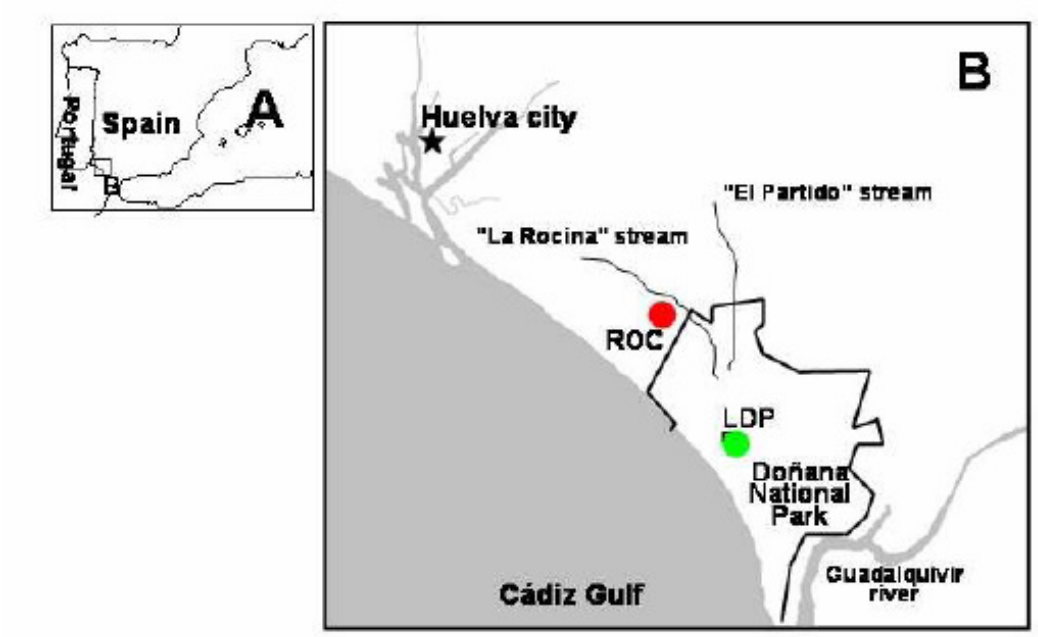
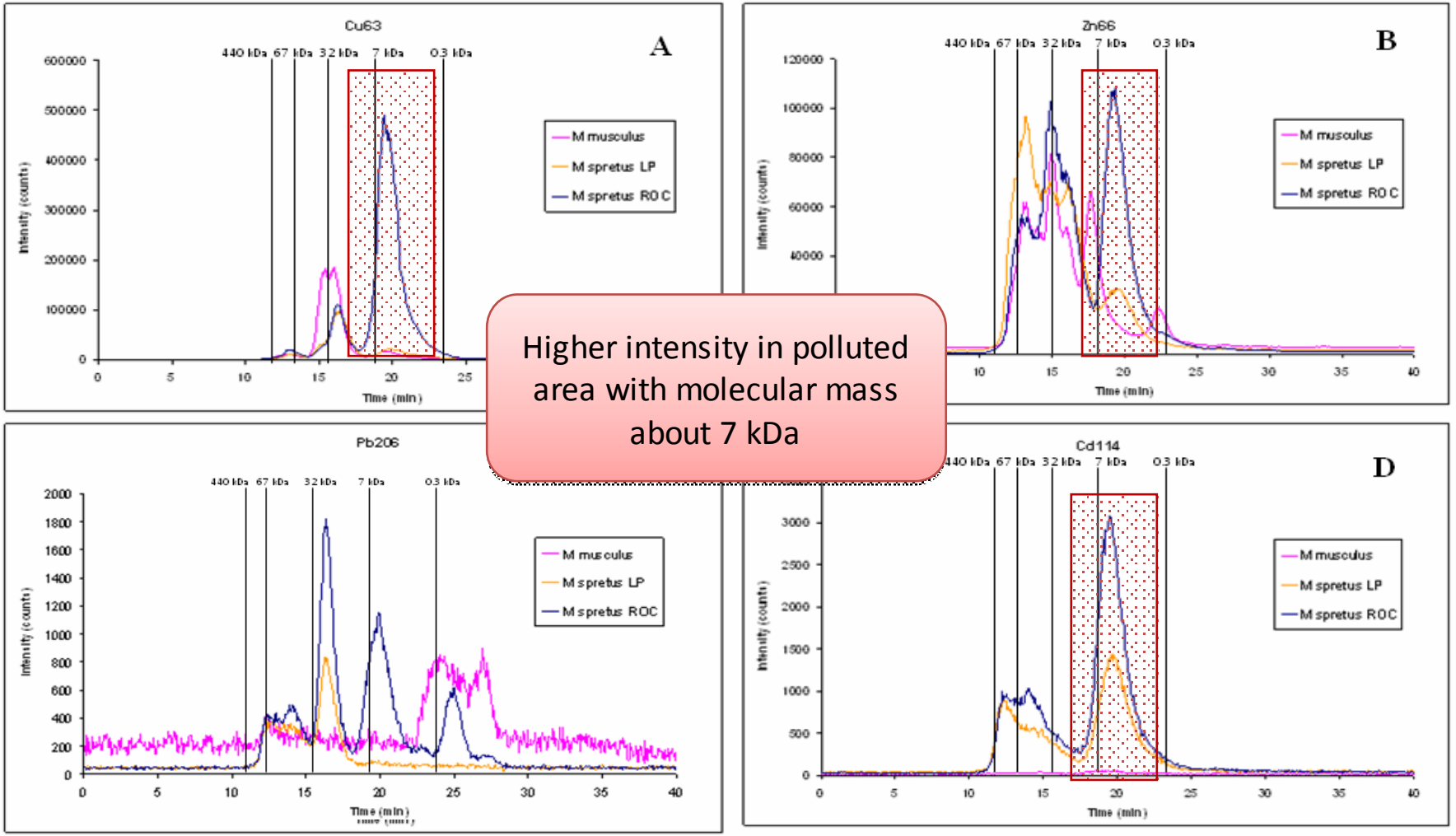
Environmental Metallomics



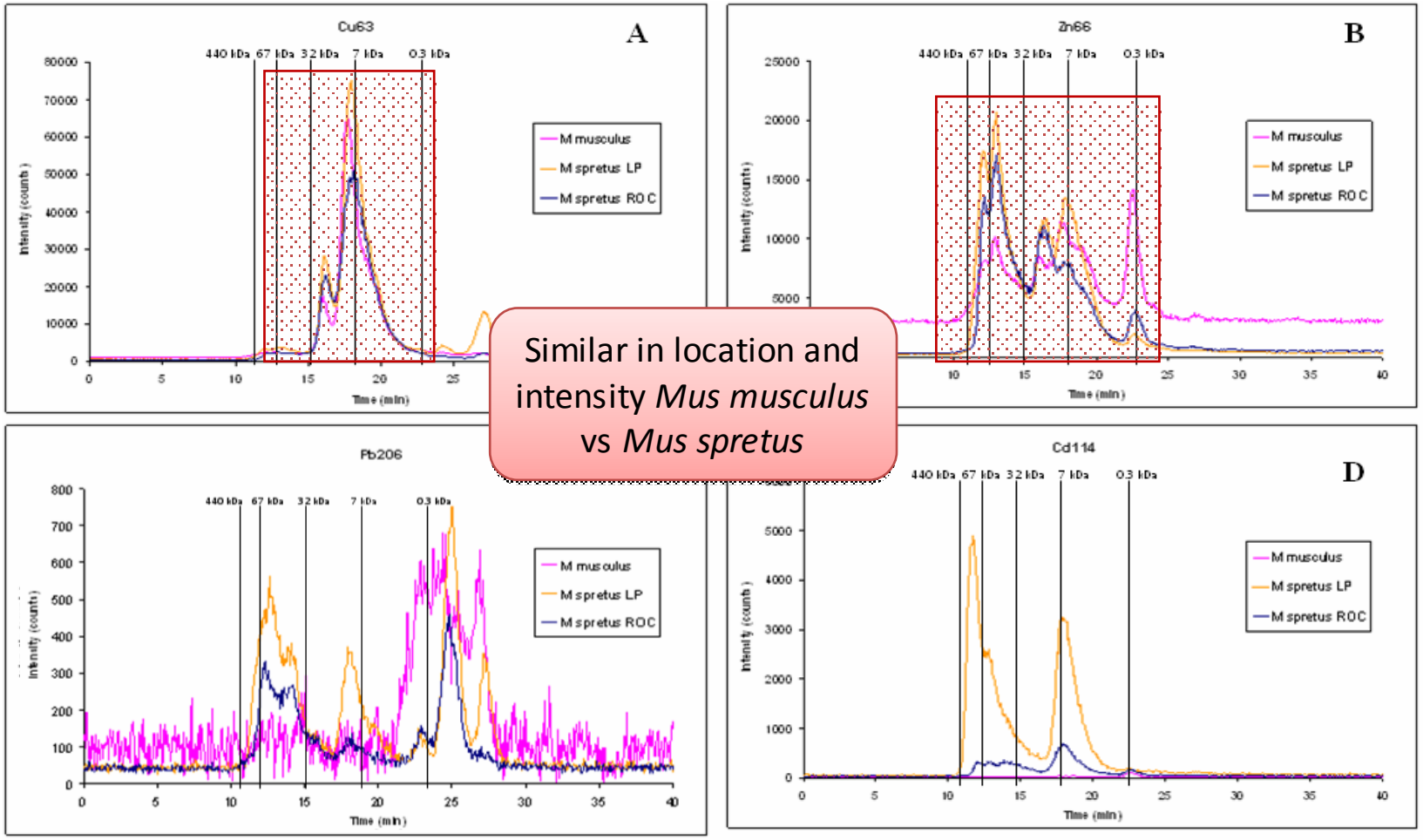
Distribution of metal-biomolecules complexes in liver *Mus musculus* extracts against free-living Mus spretus extracts from non contaminated (LDP) and contaminated (ROC) areas of Doñana Natural Park by SEC-ICP-MS



Higher intensity in polluted area with molecular mass about 7 kDa

Chromatographic conditions: Superdex ™ -75 (10x 300x 13 µm), mass molecular 3-70 kDa, 20 mM ammonium acetate (pH 7.4) for isocratic elution

Distribution of metal-biomolecules complexes in brain *Mus spretus* extracts against *Mus musculus* extracts from non contaminated (LDP) and contaminated (ROC) areas of Doñana Natural Park by SEC-ICP-MS



Similar in location and intensity *Mus musculus* vs *Mus spretus*

A lower response of brain to environmental stress than liver which is more active in the metabolism and more sensible to the presence of contaminants

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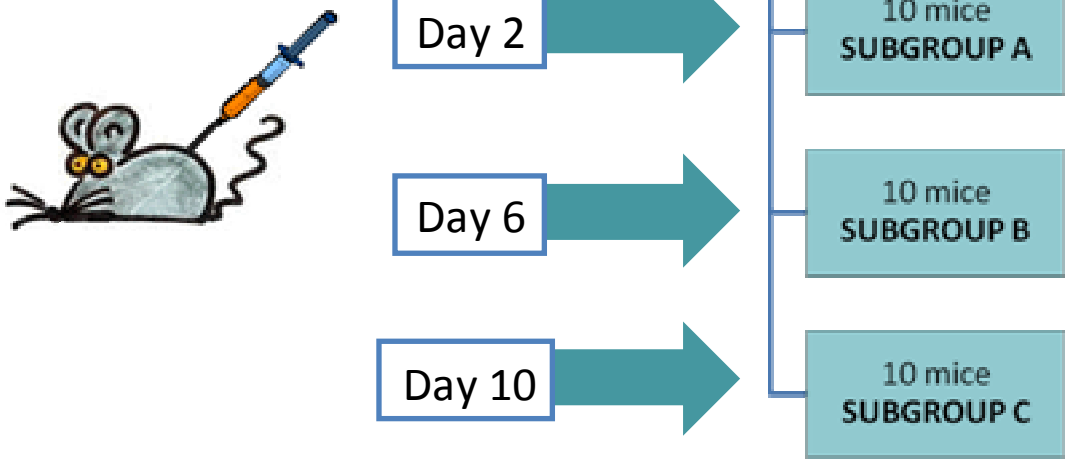
Expossure experiment to Cd of *Mus musculus* in liver extract

Information about the metabolic effects of contaminants in mice.

Subcutaneous injection (100 µL) of a increasing dose from 0.1 to 1 mg Cd (in the form of $CdCl_2$) per kg of body weight per day during a total period of 10 days.

Day	Dosis (mg Cd/Kg weight/day)
1	0,1
2	0,2
3	0,3
4	0,4
5	0,5
6	0,6
7	0,7
8	0,8
9	0,9
10	1



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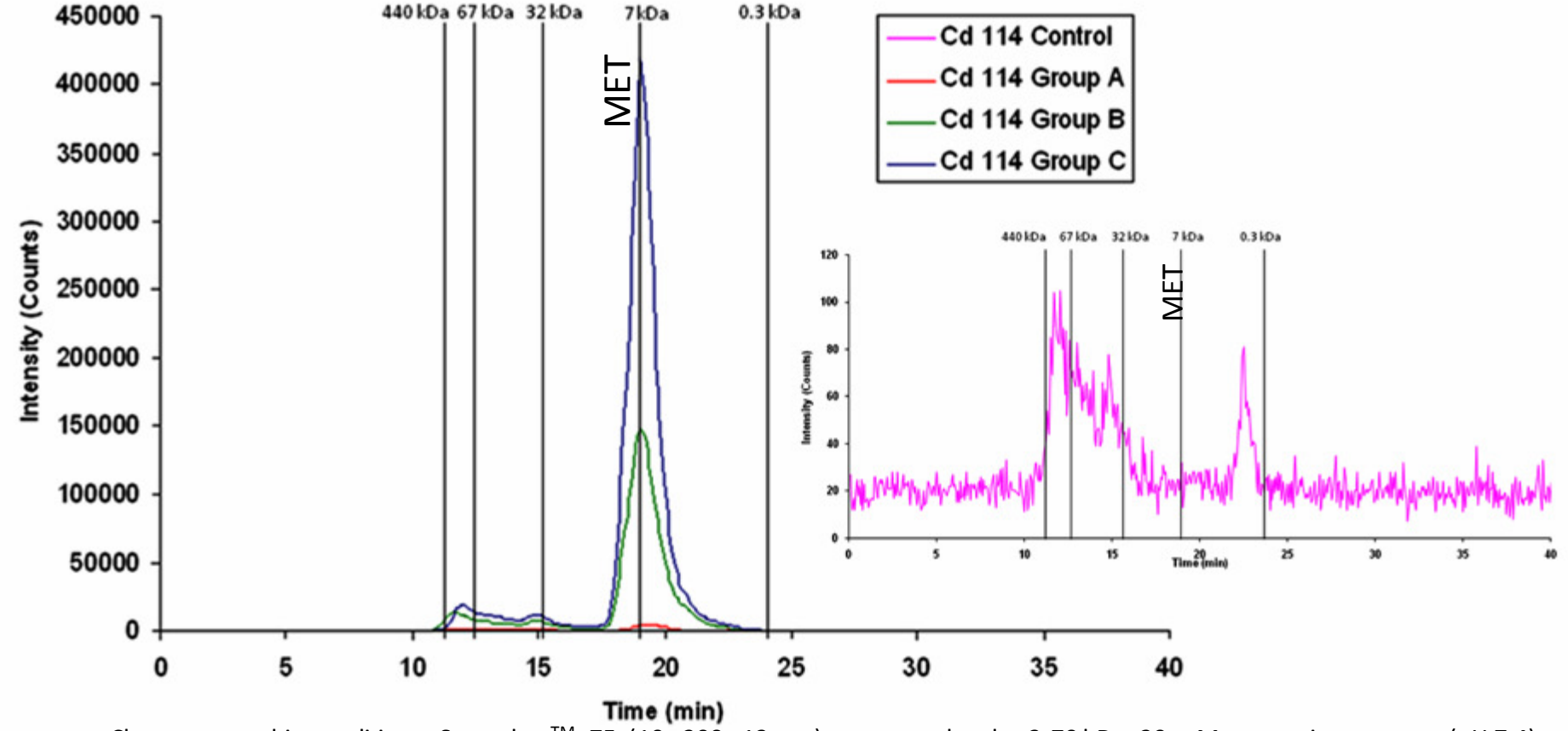
Chromatograms obtained by SEC-ICP-MS of mouse *Mus musculus* in liver cytosolic extracts after subcutaneous administration of Cd aqueous solution

Cd-fraction with molecular mass about 7 kDa increases with the doses, not present in the control group



Chromatographic conditions: Superdex ™ -75 (10x 300x 13 µm), mass molecular 3-70 kDa, 20 mM ammonium acetate (pH 7.4) for isocratic elution. Isotope monitored ^{114}Cd

Conclusions-1

- An assessment about the presence of endocrin disruptors contaminants has been performed in southwest coastal area of Iberian peninsula (ENDOCRINOBIOTOX).

- Presence of emergents contaminants (pharmaceutical drugs) has been studied in the area of study (PHARMACOTOX)

- New analytical approache have been developed for the speciation of Sn, Hg, Se and Cd in sediments and biota.

- Proteomics approaches and biomarkers have been performed for this purpose

Conclusions-2

- Studies of effects of contaminants is being developed on the basis of the followings items:
  - Toxicological test based on larvae marine copepode *Tisbe battagliai* exposed to pharmaceutical drugs and their mixtures
  - Metallomic approach for couple M*us musculus - Mus spretus.*
    - A Cu and Zn-binding protein (32 KDA) was up-regulated in liver of Mus spretus from a contaminated area versus a reference in which contamination is not present.
    - This peak was identified as superoxide dismutase, protein related with the antioxidative defence of the cells.
    - A Cu and Zn-peaks were observed at about 7 KDa in liver of Mus spretus from contaminated areas that can be related to the presence of metallothioneins
    - Contamination affect less significantly to metal-proteins expression in brain ("blood brain barrier").
    - Expossure experiments to Cd provoke an impressive increase of a Cd-peak in liver extract. This fact shows the protective mechanism developed by the organism

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Scientific contributions derived from ENDOCRINOBIOTOX and PHARMACOTOX

Publications in indexed journals

- F. Moreno, T. García-Barrera, J.L. Gómez-Ariza (2010) Analyst, 135 (10), pp. 2700-2705
  (IF: 3.272- Subject: Chemistry (Analytical). Position 11 from 70; Q1)

- M. Gonzalez-Fernández, M. A. García-Sevillano, R. Jara-Biedma, T. García-Barrera, A. Vioque, J. López-Barea, C. Pueyo, J. L. Gómez-Ariza (2011) J. Anal. At. Spectrom, DOI: 10.1039/C0JA00127A
  (IF: 3.435- Subject: Chemistry (Analytical). Position 7 from 70; Q1)

- Ramos-Payán, MD., Fernández-Torres, R. Bello-López, MA., Gomez-Ariza, JL., Callejón-Mochón, M. (2010) Talanta, 81 (3), pp. 871-880
  (IF: 3.757- Subject: Chemiistry (Analytical. Position 5 from 70; Q1)

- A. Mauffret, A. Temara, J. Blasco (2010) Water Res. 44 (9), pp. 2831-2840
  (IF: 4.355- Subject: Environmental Science. Position 9 from 181; Q1)

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Scientific Meeting contributions

- F. Moreno, T. García-Barrera, J. L. Gómez-Ariza, Mulitespeciación quiral de Selenio y Mercurio en muestras biológicas mediante HPLC-ICP-MS. XII GRASEQA. Córdoba. Julio 10-11 de 2010
- M. Contreras-Acuña, T. García-Barrera, J.L. Gómez-Ariza. Desarrollo de un procedimiento rápido para la especiación de arsénico en ortiguillas (anemonia sulcata) mediante el uso de ultrasonidos y microondas. XII GRASEQA. Córdoba. Julio 10-11 de 2010
- M. González-Fernández, M.A. García-Sevillano, R. Jara-Biedma, T. García-Barrera, J.L. Gómez-Ariza, J.López-Barea, C. Pueyo. Metallomic comparison between laboratory mouse (mus musculus) and free-living mouse (Mus spretus). 6th Franco-Spanish Workshop in Bioinorganic Analytical Chemistry, Pau (France, 23-25 September 2010
- M. Contreras-Acuña, T. Garcia-Barrera, J.L. Gómez-Ariza . Speciation of arsenic metabolites in human urine after seafood consumption by elemental and molecular mass spectrometry. 6th Franco-Spanish Workshop in Bioinorganic Analytical Chemistry, Pau (France, 23-25 September 2010
- J.L. Gómez-Ariza, T. García Barrera, M. González-Fernández, M.A. García Sevillano, R. Jara Biedma. Comparative metallomic study of liver and brain cytosolic extracts from laboratory (Mus musculus) and free-living (Mus spretus) mice. 7th Aegean Chemistry Days in Analytical Chemistry (ACDD 2010). Lesvos (Greece), 29 Sept -3 Oct 2010

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Scientific Meeting contributions

- M. González-Fernández, T. García-Barrera, J.L. Gómez-Ariza. Metallomics of laboratory mouse (mus musculus) against the free-living mouse (mus spretus). 11th Rio Symposium on Atomic Spectrometry. Mar del Plata (Argentina), 24-29 October 2010
- M. Contreras-Acuña, T. Garcia-Barrera, J.L. Gómez-Ariza. Speciation of arsenic metabolites in human urine after seafood consumption by elemental and molecular mass spectrometry. 11th Rio Symposium on Atomic Spectrometry. Mar del Plata (Argentina), 24-29 October 2010
- M.A. García-Sevillano, T. García-Barrera, J.L. Gómez-Ariza. Characterization of metal-linking metabolites in laboratory mouse (mus musculus). 11th Rio Symposium on Atomic Spectrometry. Mar del Plata (Argentina), 24-29 October 2010
- R. Jara-Biedma, T. García-Barrera, J.L. Gomez-Ariza. Metallome changes of the mouse mus musculus exposed to toxic species of metals with environmental significance . 11th Rio Symposium on Atomic Spectrometry. Mar del Plata (Argentina), 24-29 October 2010

Acknowledgements

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Thanks all of you for the attention!!!!