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developed at

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by

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Study of polluted oysters from Arcachon Bay by chemical and biomolecular speciation methods: organotin compounds

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LIST OF ABREVIATIONS

ANR: Agence Nationale de la Recherche

BCF: Bioconcentration factor

DBT: Dibutyltin

DisP: Dissolved pathway

GC- ICP MS: Gas Chromatography – Inductively Coupled Plasma Mass Spectrometry

HPLC-ICP MS: High Performance Liquid Chromatography – Inductively Coupled Plasma Mass Spectrometry

IMO: International Maritime Organization

IPREM: Institut Pluridisciplinaire de Recherche en Environnement et Matèriaux

IRSTEA: National research institute in environmental sciences and technologies

iSn: Inorganic tin

LCABIE: Laboratory of Bioinorganic Analytical and Environmental Chemistry

LPTC: Laboratoire de Physico- et Toxico-Chimie de l'Environnement

MBT: Monobutyltin

MQ: Milli-Q water

MW: Microwave

PFA: Perfluoroalkoxy

Phto: Phytoplankton

RIPOST: Interdisciplinary Research of the oysters problematic in the Arcachon Bay; in situ and experimental approaches

SEC: Size Exclusion Chromatography

SPC: Self-polishing copolymers

TBT: Tributyltin

TMAH: Tetramethylammonium hydroxide

TroP: Trophic pathway

ABTRACT

The use of a spike of ^{117}TBT as a tracer has assisted in the investigation of butyltin contamination in oysters (*C. Gigas*). The analysis of incubated oyster organ tissues by gas chromatography-ICP MS has revealed the organotin cytosolic and non-cytosolic distribution and they have been related to the results of the elucidation of contamination pathways of oyster organ tissues. Furthermore, bioconcentration and degradation in the oyster has been studied revealing a high bioconcentration for TBT and DBT, and moderate bioconcentration for MBT whereas the contribution of degradation processes is very low.

The analysis of the water-soluble fraction by size exclusion chromatography-ICP MS revealed the metabolization of a minor part of butyltin compounds through the association of Sn species with biomolecules in a molecular weight range between $3{\cdot}10^3$ and $6{\cdot}10^5$ Da, biomolecules which sheltered affinity for other metals (Se, Hg, Cd, Cu and Zn).

1. INTRODUCTION

In the 1960s, organotin compounds were introduced into many industrial products. Being established the toxicity levels of trisubstituded species they began to be used mainly for their biocide efficiency in industries of wood, textile, paper and leather, industrial cooling systems, domestic detergents, agriculture and above all, in antifouling paints [1].

In anti-fouling paints, the most successful of the trisubstituted organotin compounds was tributyltin (TBT), (see Fig.1).

Fig.1 Tributyltin molecule

Firstly used in 1947 in anti-fouling paints, by the start of the 1960s 140000 tons of TBT were consumed each year in boating and the merchant marine in the United States. It was estimated that in the meanwhile 70% of the global fleet was covered by this kind of paints. [1]

This compound in the composition of paints provides high efficacy and 4-7 years of lifetime if its used next to self-polishing copolymers (SPC). With time, TBT copolymer leaches to the seawater by hydrolysation, leaving the surface exposed to the action of the erosion with a fresh new paint layer. [2] (see Fig.2)

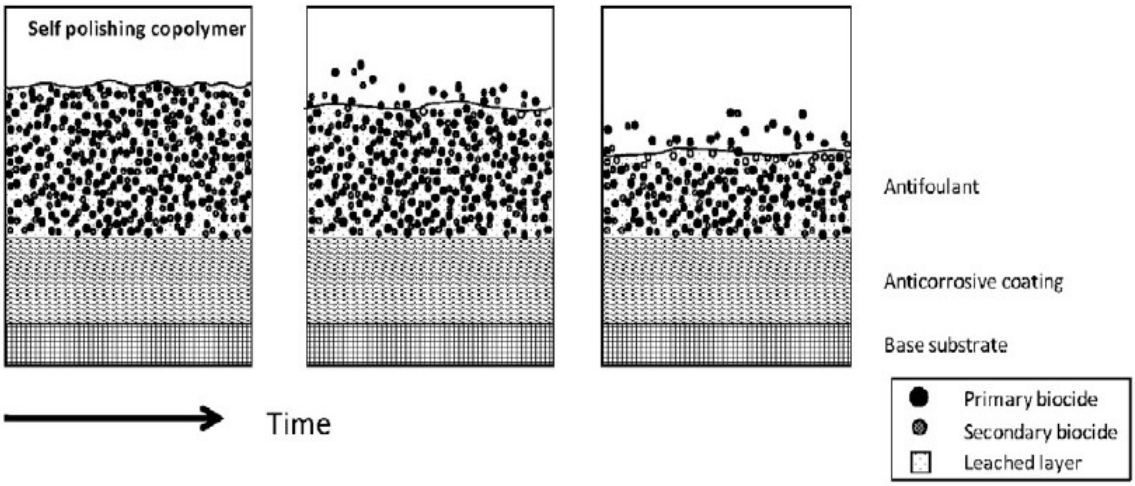

Fig.2 Self-polishing copolymer [3]

When TBT began to be used, there was little knowledge about the effects of releasing TBT to seawater. Nowadays, the behaviour and persistence of organotin compounds in the environment is being studied and it is known that organotin compounds environmental impact is mainly due to the use of anti-fouling paints.

The fate of organotin compounds attends to not only hydrodynamic but also biogeochemical conditions, and they regulate their dispersion and transformation in the environment.
TBT is hydrophobic, as the majority of trisubstituted organotin compounds, which makes it able to be linked to dynamics of particulates in the aquatic environment. This leads to storage of this compound at long-term level in sediments by sorption, but, depending on the conditions, organotin compounds can be desorbed.
Anyway, some of TBT remains out of the sediments as it can be found in the surface microlayer at the air-water surface. Photochemical reactions (UV radiation, chemical cleavage) contribute to a rapid degradation of organotin species (successive de-alkylations or de-arylations), (see Fig.3), resulting in less toxic compounds, in the case of TBT, resulting into dibutyltin (DBT) and monobutyltin (MBT) [1] (see Fig.4)

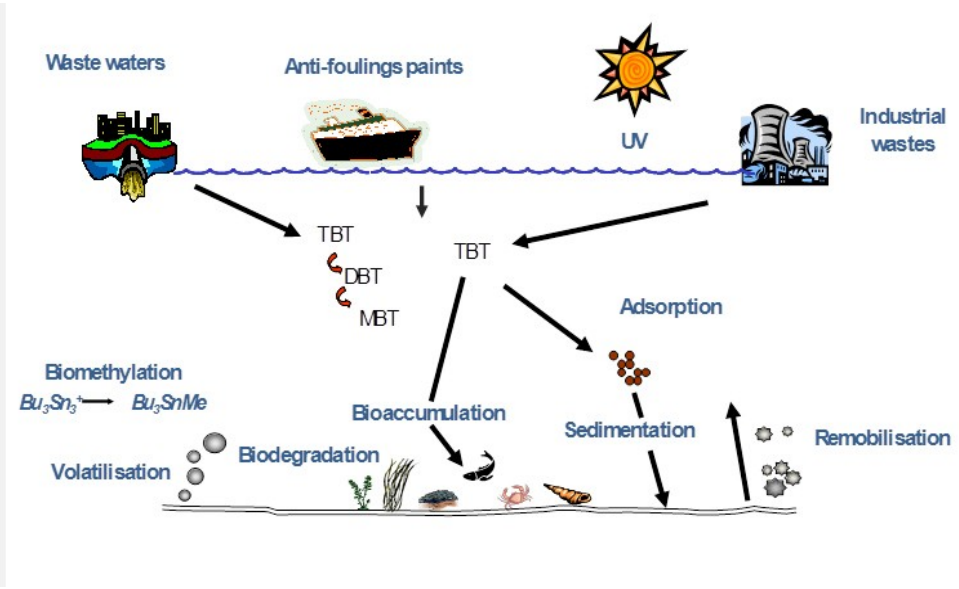

Fig.3 Sources and fates of TBT into biodegradation, bioaccumulation and storage in aquatic systems

Biological degradation by phytoplankton and bacteria can also precipitate the degradation of TBT in these appointed less toxic compounds, DBT and MBT or even to harmless specie, inorganic tin (iSn). [1]

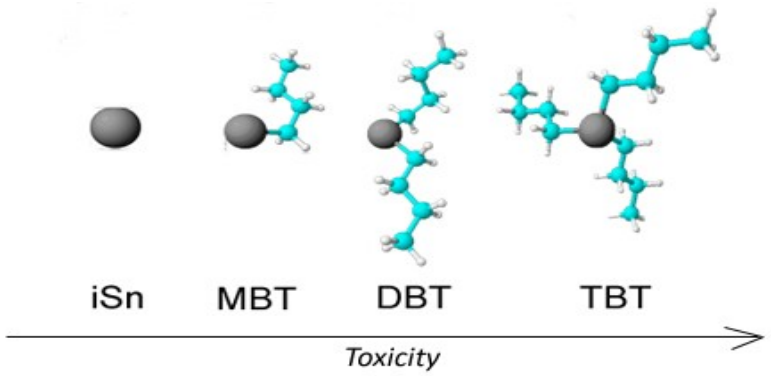

Fig.4 Toxicity scale of several alkyltin compounds and inorganic tin (iSn)

In the 1980s decade, French authorities became aware of TBT-associated problems in farms of oysters (*C. Gigas*) it Arcachon Bay (see Fig.5). Some of the detected issues were the oyster spawning reduction, anomalies in larval development and shell-malformation affecting 80-100% of oyster population. [1],[3] without forgetting the human risk consumption [4],[10].

Fig.5 Arcachon Bay (*Bassin d'Arcachon*) location in map

TBT was consistently forbidden on vessels of less than 25 m length and consequently this action triggered a TBT decrease in coastal waters [1]

Since the record of imposex (male characteristics development on female individuals) in gastropods, restrictions have been spread to other countries: UK (1987), USA (1989), Australia (1989) and the EU (1989) [6] Only 2ng·L^{-1} of TBT is needed to appear this effect.

From 1990s bioaccumulation in sea mammals has been also reported, like in steller sea lion [7], and cetaceans in Korean coasts [13] (see Fig. 8)

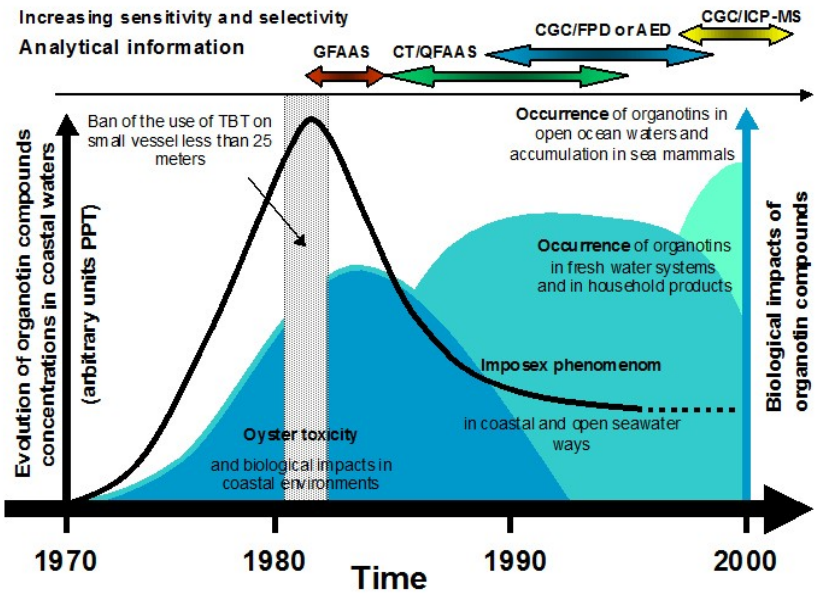

Fig.6 Evolution of concentration of organotin compounds and biological impacts. Biological and instrumental answers (GFAAS Graphite Furnace Atomic Absorption Spectrometry. CT/QFAAS Cryo Trapping Quartz Furnace Atomic Absorption Spectrometry, CGC/FPD Capillary Gas Chromatography Flame Photometric Detection, AED Atomic Emission Detection, CGC/ICPMS Capillary Gas Chromatography Inductive Coupled Plasma Mass Spectrometry [1]

In November 2001, the International Maritime Organisation (IMO) adopted the intention of banning the application of TBT on all vessels after January 1th, 2003 and absence of active coating requirement on all vessels after January 1th, 2008. [8] Only 25% of the raw tonnage of the world's merchant shipping ratified this agreement

In 2010 a fundamental research project funded by the French national research agency ANR (Agence Nationale de la Recherche) called RIPOST (Interdisciplinary Research of the oysters problematic in the Arcachon Bay; in situ and experimental approaches) was established. [9]. The purpose of the project is the characterization of the main sources of contamination in the Arcachon Bay , and the goals are:

- To study the impact of the contaminants presented in the water column and its spatial and temporal variability in oysters from the Arcachon Bay
- To define the toxic impacts of pollutants on organisms and to understand the toxic impacts in oyster populations through molecular, cellular and physiological responses.

The research groups involved and functions are:

- EPOC (Oceanic and Continental Environments and Paleoenvironments), University of Bordeaux with the LPTC team (research in analytical chemistry of organic pollutants) and the EA team (Aquatic ecotoxicology, biochemistry and genetic research), IPREM (Institut Pluridisciplinaire de Recherche en Environnement et Matériaux, University of Pau et les Pays de L'Adour with the LCABIE team (Laboratory of Bioinorganic Analytical and Environmental Chemistry) (research of chemistry of metallic and organometallic pollutants)
- IRSTEA (National research institute in environmental sciences and technologies), Bordeaux, with the REBX team (Networks, sewage and water quality) (research of chemistry of organic pollutants in freshwaters and peripherical communities)

The tasks of the project to complete are the experimental approach about the distribution, fate and impacts of major environmental pollutants in the oyster (work supervised by supervisors D. Amouroux (LCABIE) and P. Gonzalez (EA)), the characterisation of the source of contamination of the Arcachon Bay and reactivity of its pollutants (task leaded by H. Budzinski (LPTC)), making an i*n situ* approach of toxic impact of pollutants in oysters (supervised by M.Baudrimont (EA) and F. Delmas (REBX)), and finally, a methodological transfer of methodologies developed in the Arcachon Bay to other French oyster sites (P. Gonzalez (EA) is in charge).

As part of the first of these tasks, on November 2010, H. Bijoux and S. Bouchet (LCABIE) and on March 2011, Ph.D. Romain Bridou (LCABIE) having avoided aquarium walls adsorptions studied the potential of transformation and bioaccumulation in two oysters' organs: digestive gland and gills and a bulk of the residual tissues and organs (the rest) and the biodegradation pathways of TBT at environmental level. For this purpose, Ph. D. Romain Bridou made an experiment with aquarium oysters and studied the behaviour of a spike of ^{117}TBT

This project has tackled the distribution of organotin species in cytosolic and non-cytosolic fractions in order to identify the partition of the biodegradation and bioaccumulation processes in oyster gills and digestive gland and in the bulk of the residual tissues and organs left. Also, it has been interesting knowing in which grade these compounds are associated to proteins. Since

literature has collected a previous study of organomercury binding to metallothioneins in dolphin liver [15], the approach of this project seems to be feasible.

2. EXPERIMENTAL PART

2.1 Materials and methods

2.1.1. SAMPLES

AQUARIUM SET UP:

Five aquariums were used (see Fig. 11) for simulating two conditions of oyster contamination. Phytoplankton presence will give information about trophic pathway and no presence of phytoplankton, information about dissolved pathway.

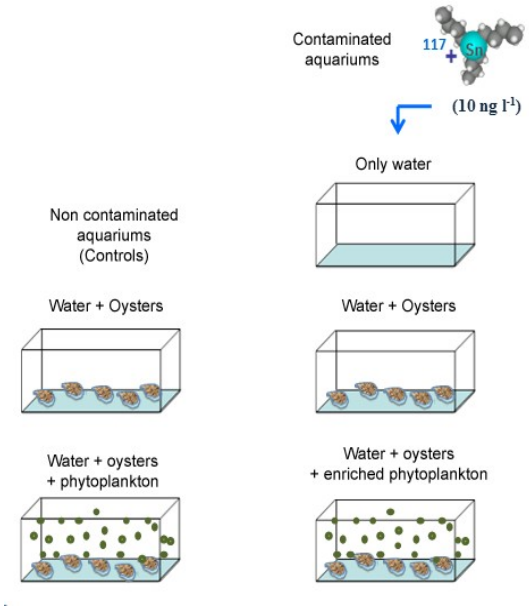

Fig. 11. Aquarium set up

OYSTER INCUBATION:

Raised oysters in non-contaminated environment were placed in several aquariums following these conditions (see Table 1)

Aquarium coating	**PFA (perfluoroalkoxy)**
Aquarium water volume	50 L
Aereation	By bubbling (filtered air)
Circadiam rythm	12 hours: 12 hours
Oyster density	1,26 individuals · L^{-1}
Organotin spike	^{117}TBT (10 ng · L^{-1})
Algae	$35 \cdot 10^5$ cells · $oysters^{-1} \cdot L^{-1}$

Table 1. Oyster incubation conditions

SPIKE ADDITION AND OYSTER SAMPLING:

A first spike of 10 ppb of ^{117}TBT was made and compensatory spikes of 3 ppb ^{117}TBT were introduced as Fig. 8 shows. As losses of the spiked 117TBT may occur during the incubation (due to adsportion or abiotic degradation), compensatory spike was performed in order to prevent this loss. This loss could not be avoided (see Annexes A.1.1). Six samples, one per day, were taken from each aquarium according the schedule in Fig. 8

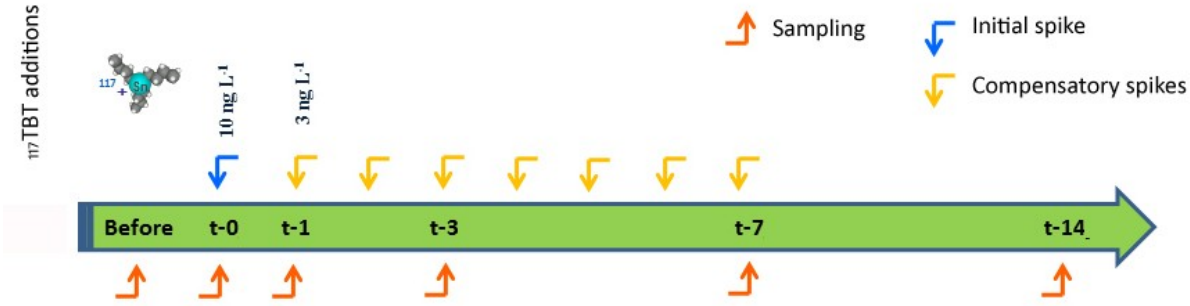

Fig. 12. Sampling schedule

Later on, each oyster was split into gills, digestive gland (digestive tract) and the rest, and they were frozen until procedure.

CONCENTRATIONS:

Tributyltin concentrations in water and oyster (see Annexes 2.1.1.A) (see Table 2), were obtained by Ph.D. Romain Bridou (LCABIE) using ID-GC-ICPMS (Isotope Dilution-Gas Chromatography-Inductively Coupled Plasma-Mass Spectrometry)

ID-GC-ICPMS relies on the intentional alteration of the isotope abundances of an endogenous element in a given sample by addition of a known amount of an enriched

isotope of the same element (spike), which in this case is ^{117}Sn of tributyltin (^{117}TBT). The element to be analysed must have, then, at least two stable or long-lived radiactive isotopes (in our case ^{117}TBT and ^{120}TBT) able to be measured in a mass spectrometer free of spectral interferences. This is illustrated in Fig. 13. As it can be observed, the isotope A is the most abundant in the sample meanwhile the spike is sotopically enriched in the isotope B. It is clear that the abundance of the two isotopes and, therefore, the isotope ratio in the mixture will be intermediate between those in the sample and the spike and it will depend both on the amount of spike added and on the initial amount of the element in the sample. Those relationships can be expressed mathematically using the isotope dilution equation, Equation 1. In this equation, the concentration of the element in the sample Cs (see Equation 1) is determined just by measuring Rm by mass spectrometry as all other parameters in the equation are known.[11]

TroP		ppb			**DisP**		ppb		
		MBT	DBT	TBT			MBT	DBT	TBT
OYSTER, t-0	Gills	0,02	0,04	0,61	OYSTER, t-0	Gills	0,03	0,05	0,84
	Digestive gland	0,03	0,38	11,03		Digestive gland	0,01	0,02	0,54
	Rest	0,01	0,03	0,87		Rest	0,02	0,01	0,11
OYSTER, t-7	Gills	0,04	0,06	0,66	OYSTER, t-7	Gills	0,17	0,94	10,53
	Digestive gland	0,06	0,74	16,52		Digestive gland	0,06	0,29	3,1
	Rest	0,03	0,25	2,95		Rest	0,02	0,11	1,9
WATER, t-0		0,0004	0,0011	0,0075	WATER, t-0		0,0002	0,0007	0,0036
WATER, t-7		0,00025	0,0007	0,0005	WATER, t-7		0,0001	0,00025	0,0002

Table 2. Oyster and water TBT, DBT and MBT concentrations determined by Ph.D. Romain Bridou

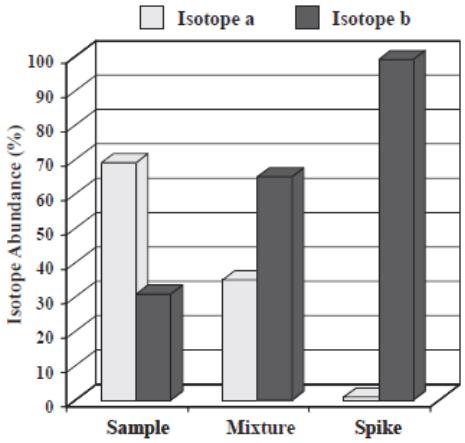

Fig 3. Illustration of the principle of isotope dilution analysis for an element combining two different isotopes A and B [11]

$$c_S = c_{Sp}\frac{m_{Sp}}{m_S}\frac{M_S}{M_{Sp}}\frac{A^b_{Sp}}{A^a_S}\left(\frac{R_m - R_{Sp}}{1 - R_m \cdot R_S}\right)$$

- *C_s and c_{sp} are the concentrations of the element in the sample and the spike, respectively*
- *m_s and m_{sp} the mass taken from sample and spike in the mixture, repectively*
- *Ms and Msp the elemental atomic weights of the element in the sample and the spike, respectively*
- *$Rs=As^b/As^a$ as is isotope ratio (b/a) in the sample*
- *$Rsp=Asp^a/Asp^b$ the isotope ratio (a/b) in the spike)*

Equation 1. Final expression for isotope dilution analysis equation [11]

2.1.1. ANALYTICAL BALANCE

Sartorius (Goettingen, Germany) model BP211D. Precision of 10^{-5} g (see Fig. 13)

Fig. 13. Analytical balance used at the IPREM

2.1.2. DISPERSER

An IKA (Germany/Deutschland) model T 18 basic ULTRA-TURRAX® disperser was used for triturating the oyster samples (see Fig. 14)

Fig. 14. Disperser IKA model T18 basic ULTRA-TURRAX

2.1.3. REAGENTS

- Ultrapure water obtained from a Milli-Q system (Quantum, EX, Milipore, USA)
- Analytical reagent-grade hexane and TMAH (tetramethylammonium hydroxide) Sigma-Aldrich (Belgium)
- Buffer solution 0,1M sodium acetate pH=4,9 prepared by dissolving sodium acetate in MQ water and adjusting pH with glacial acetic acid.
- Derivatizating solution 2% (w/w) sodium tetraethylborate ($NaBEt_4$), purity > 99%, purchased from GALAB (Germany). Every $NaBEt_4$ water solution was prepared every 6 hours and stored in the fridge.
- NH_4Ac mobile phase 100mM of pH=7,5

2.1.4. ANALYTICAL MICROWAVE DIGESTION SYSTEM

CEM discover SP-D system coupled to an autosampler Explorer 4872 96 (USA), (see Fig. 15)

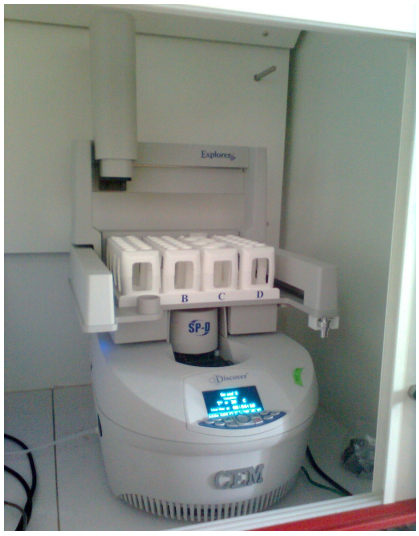

Fig. 15. Analytical MW Digestion system used at the IPREM

2.1.5. ULTRASONICATOR

Bioblock Scientific Secteur Vibra Cell 75115 (France), (see Fig. 16)

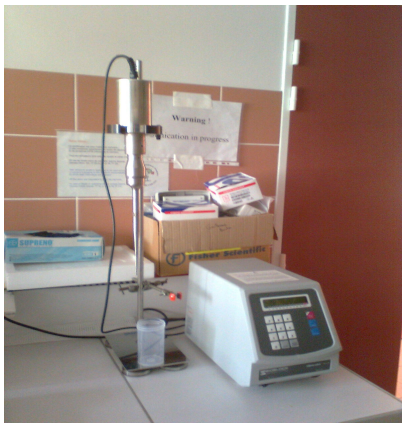

Fig. 16. Ultrasonicator used at the IPREM

2.1.6. CENTRIFUGATOR

Rotofix 32 A Hettich Zentrifugen (Germany), (see Fig. 17)

Fig. 17. Centrifugator used at the IPREM

2.1.7. GC-ICP MS INSTRUMENTATION

Thermo Electron GC (Focus) equiped with a capilar column is coupled to a Thermo Electron ICP-MS (X7 series) (USA) (see Fig. 18, 19) was used following the conditions shown on Annexes 2.1.8.A

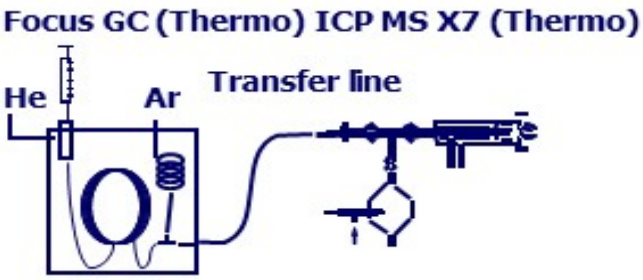

Fig 18. Scheme of the GC-ICP MS instrumentation

Fig. 19. GC-ICP MS used at the IPREM

2.1.8. HPLC-ICP MS INSTRUMENTATION

Agilent 1100 liquid chromatograph (Agilent, Wilmington, DE, USA) equipped with a binary HPLC pump, an autosampler and a diode array detector. An Agilent ICP MS 7500ce (Yokogawa Analytical Systems, Tokyo, Japan) was used as a liquid chromatographic detector and a Superdex 200 chromatographic column was utilized (see Fig. 20). Conditions in Annexes 2.1.9.A.

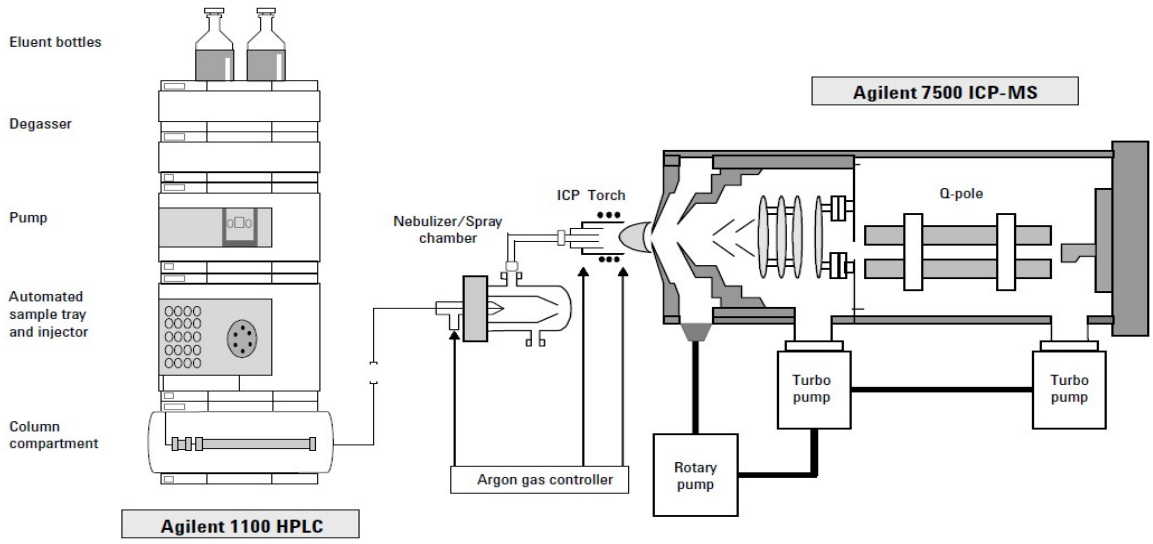

Fig 20. Schematic of the HPLC-ICPMS instrumentation

2.1.9. ANALYTICAL PROCEDURE

A flowchart of the analytical procedure followed is shown in Fig. 22.

See Annexes 2.1.10.A for table of weights.

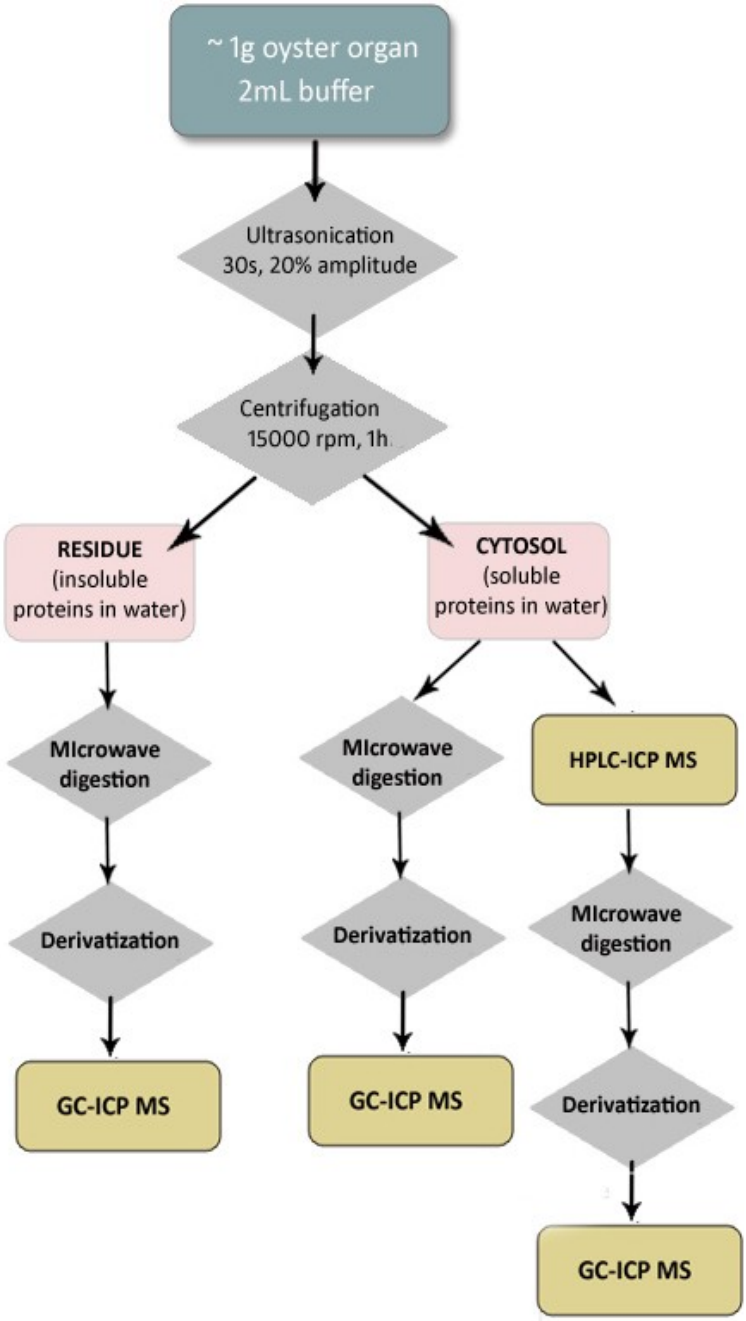

Fig 22. Scheme of the analytical procedure used in this study

ORGANOTIN COMPOUNDS DISTRIBUTION IN CYTOSOLIC AND NON-CYTOSOLIC FRACTIONS:

1) Each oysters samples were weighed on an analytical balance and transferred to vials and then 2 mL of buffer solution 0,1M sodium acetate pH=4,9 was added.
2) 30s - 20% amplitude sonication was performed in pulse mode and immersing the vial in ice to avoid overheating. Ultrasound probe was immersed 1cm in each vial. Later, the obtained extracts were centrifugated at least 1h at 15000 rpm for separating the solid residue and the liquid cytosol.
3) Each phase was trasferred to MW vials, leaving ~0,5 mL of liquid phase in 1,5 mL HPLC flacons. 2 mL TMAH were weighed and dispensed to the MW phials and magnetic stirrers were inserted. A MW method optimized for Hg and Sn samples of 75 W of maximum power over 4 min was set.
4) Aliquots from the digested extracts were derivatized with 1 mL of derivatizating solution 2% (w/w) $NaBEt_4$, vials were rapidly closed for avoiding hexane releasing to the air and shaken for 5 min.
5) Once the derivatization stage is finished, organic phase was analyzed by GC-ICPMS

ORGANOTIN COMPOUNDS BINDING PROTEINS DISTRIBUTION

1) For fractionation of the cytosolic fraction, 150 µL were directly doubled injected in the Superdex 200 chromatographic column coupled to ICP-MS.
2) The different SEC fractions containing Sn (between 9-13 min and 26-30 min for gills and 9-15 min, 26-30 min and 30-34,5 min for digestive) were individually collected after double injection of 150 µL of protein extracts. The collected fractions were digested following the same procedure previously described and after derivatization analyzed by GC-ICP-MS.

EXTERNAL CALIBRATION METHOD

An external calibration curve (see Annexes 2.1.10.C) was made from a 100 ppb mix solution (see Annexes 2.1.11.B).

3. RESULTS AND DISCUSSION

3.1 Bioconcentration and degradation of butyltin species

3.1.1. BIOCONCENTRATION OF ^{117}TBT WITHIN OYSTER ORGAN TISSUES

Concentrations for butyltin compounds in oyster and water are represented in Table 23. Comparing 7th with 0 day in oysters, when ^{117}TBT spike is performed in water, TBT is highly concentrated in gills, whilst when the spike is added in phytoplankton highest TBT concentration is found in the digestive tract. Thus, these results are coherent with the fact that the gills are more in contact with contaminated water than the digestive tract and this last, more in contact with contaminated phytoplankton than the gills. Therefore, it is coherent that water contamination contribution in gills is higher than in digestive gland and phytoplankton pollution contribution is higher in digestive gland than in gills. Bioconcentration factor results are in good agreetment with this coherence.

117TBT dissolved in phytoplankton		ppb			**117TBT dissolved in water**		ppb		
		MBT	DBT	TBT			MBT	DBT	TBT
OYSTER, t-0	Gills	0,02	0,04	0,61	OYSTER, t-0	Gills	0,03	0,05	0,84
	Digestive gland	0,03	0,38	11,03		Digestive gland	0,01	0,02	0,54
	Rest	0,01	0,03	0,87		Rest	0,02	0,01	0,11
OYSTER, t-7	Gills	0,04	0,06	0,66	OYSTER, t-7	Gills	0,17	0,94	10,53
	Digestive gland	0,06	0,74	16,52		Digestive gland	0,06	0,29	3,1
	Rest	0,03	0,25	2,95		Rest	0,02	0,11	1,9
WATER, t-0		0,0004	0,0011	0,0075	WATER, t-0		0,0002	0,0007	0,0036
WATER, t-7		0,00025	0,0007	0,0005	WATER, t-7		0,0001	0,00025	0,0002
		Dimentionless units					Dimentionless units		
BCF (t-7)	Digestive gland	240	2960	66080	**BCF (t-7)**	Digestive gland	240	1160	12400
	Gills	160	240	2640		Gills	680	3760	42120
	Rest	120	357	5900		Rest	200	440	9500

Table 23. Concentration of butyltin compounds in oyster and water on 0 and 7th day

BCF is a value that points how much of a chemical is in a tissue relative to how much of that chemical exists in the environment. It can be calculated with Equation 2:

$$BCF = \frac{Concentration\ in\ organism}{Concentration\ of\ surrounding\ environment\ of\ the\ organism}\ (dimentionless\ units)$$

Equation 2. Bioconcentration Factor expression

When BCF > 1000 bioconcentration is high, when BCF= 1000 – 150 it is moderate and a BCF < 150 is considered low

As hydrophobicity rises from MBT < DBT < TBT (as number of alkylitic groups), and organs and tissue should tend to bioconcentrate hydrophobic species, this tendency should be higher from TBT > DBT > MBT. BCF was calculated for t-7 of incubation for ^{117}Sn spike (see Table 23).

Attending to BCF values, high bioconcentration is manifested for TBT and DBT (TBT > DBT), while MBT moderate bioconcentrated. This is in agreetment with theorical tendency.

It is published that BCF for TBT reached over four orders of magnitude in fishes [17] and shrimp [18], [19], and from four to six orders of magnitude in case of TBT in mussels [20]

3.1.1. DEGRADATION OF ^{117}TBT DURING THE EXPERIMENTATION

Distribution of compounds in organs tissue provide an idea about degradation because degradation takes place through butyltin conversions, therefore, if there is degradation, this distribution should change with time (see Table 24).

TroP		% in organ		
		MBT	DBT	TBT
OYSTER, t-0	Gills	2,99	5,97	91,04
	Digestive gland	0,26	3,32	96,42
	Rest	1,1	3,3	95,6
OYSTER, t-7	Gills	5,26	7,89	86,84
	Digestive gland	0,35	4,27	95,38
	Rest	0,96	4,79	94,25
DisP		% in organ		
		MBT	DBT	TBT
OYSTER, t-0	Gills	3,26	5,43	91,3
	Digestive gland	1,75	3,51	94,74
	Rest	14,29	7,14	78,57
OYSTER, t-7	Gills	1,46	8,08	90,46
	Digestive gland	1,74	8,41	89,86
	Rest	0,99	5,42	93,6

Table 24. Distribution of butyltin compounds in organs and tissue on 0 and 7th day of incubation when

Comparing results from t-0 to t-7 per organ/tissue and pathway, we can see that there is a slight decrease and increase of %TBT and %DBT respectively in all cases, point to a little degradation of TBT leading to DBT transformation. This TBT degradation scopes MBT formation when ^{117}TBT is spiked in phytoplankton ('TroP' in Table 24), but %MBT decreases in the seventh day if spike is performed in water ('DisP' in Table 24) which indicates that TBT degradation to MBT in DisP is performed in a lower extent than TroP. In both cases of ^{117}TBT spike in water and phytoplankton, biodegradation may be perform by abiotic action in water, and also, in case of TroP, this biodegradation might be perform by phytoplankton of the aquarium. This explains why TBT degradation into DBT is lower when ^{117}TBT is spiked in water than when it is spiked in phytoplankton.

Anyway, these degradations represent only 1% of ^{117}TBT spike, so this manifest the low occurrence of degradation of TBT in oysters.

3.2 Butyltin compounds distribution in cytosolic and non-cytosolic fractions

The distribution of butyltin compounds in cytosolic and non-cytosolic fractions per sample for natural ^{120}Sn and spike ^{117}Sn is shown in Fig. 25. If we follow 117Sn tendency, we can observe that TBT and MBT is present in all samples, with more occurrency in solid (there is more MBT in non-cytosolic fraction than TBT). There is also some MBT in cytosol when 117TBT contaminates the water and digestive gland is analyzed (see Annexes 3.1.A):

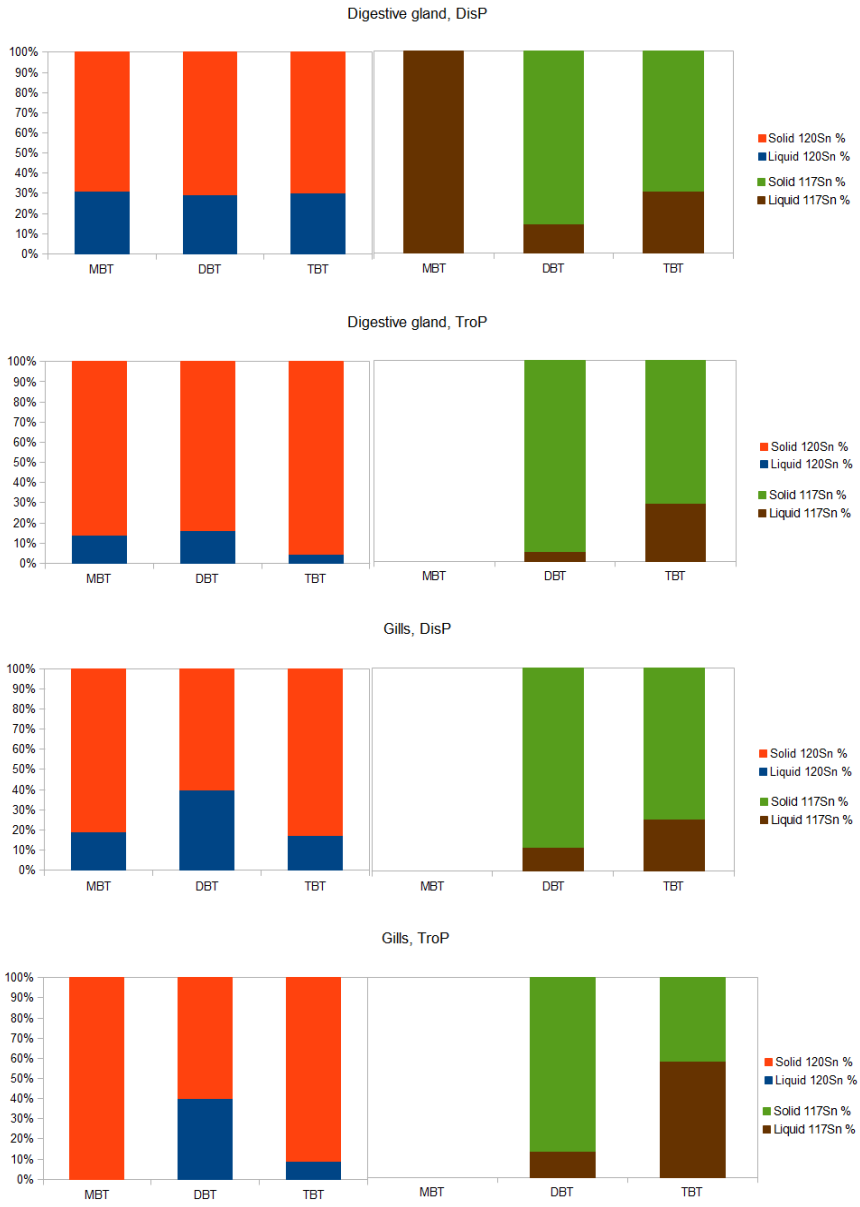

Fig. 25. Distribution of butyltin compounds on 7th day of incubation

Here total concentrations are graphicated (bars) with its liquid and solid fraction distributions (circles) (see Fig.26):

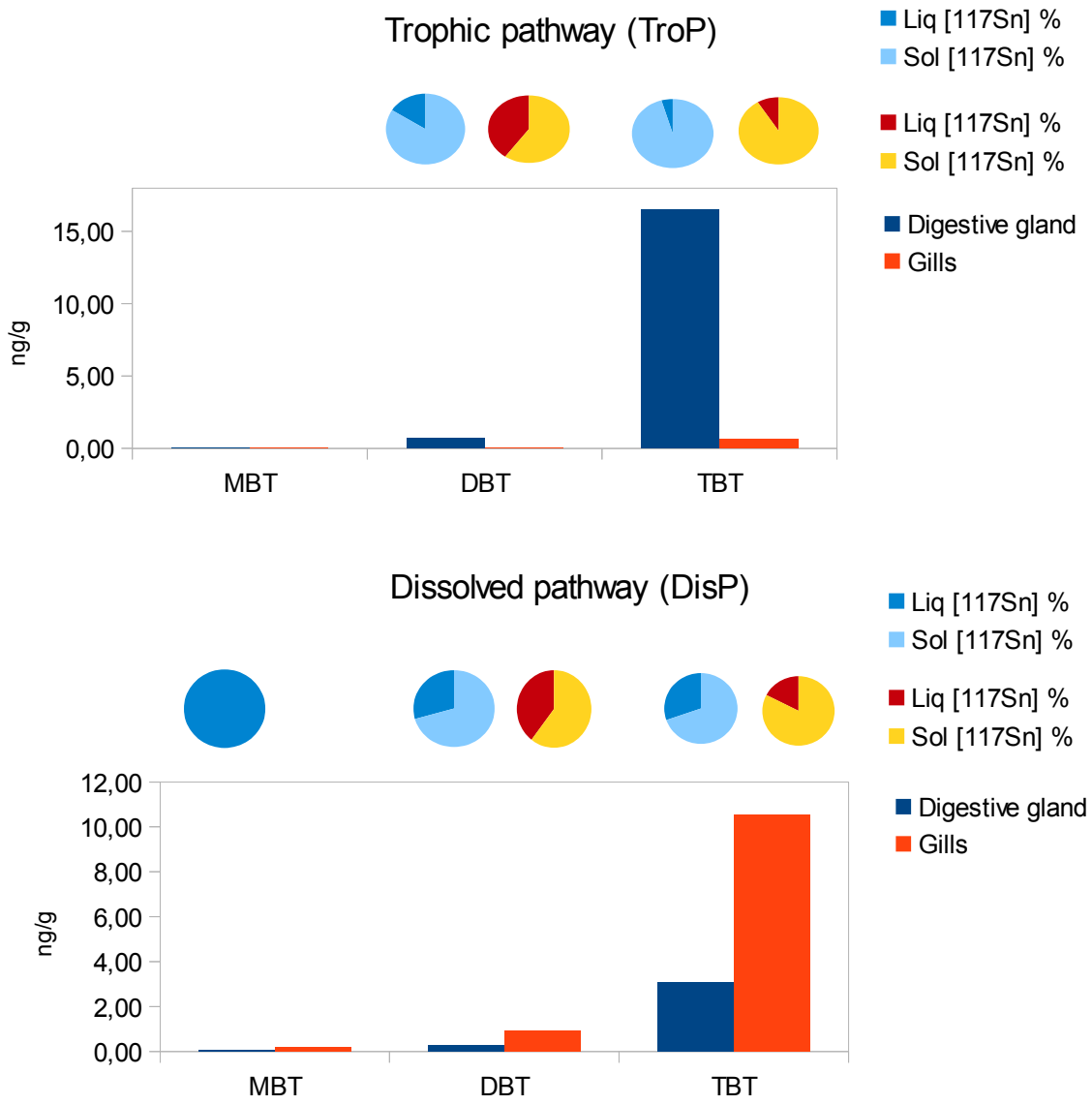

Fig. 26. Total concentrations and solid and liquid distributions on 7th day of oyster incubation

In all cases we can see that $^{117}Sn_{spike}$ use to be stored more in solid fraction than in liquid one. This means that the majority of $^{117}Sn_{spike}$ that arrives to oyster organs, it is not metabolized. $^{117}Sn_{spike}$ solid fraction is slightly higher when trophic pathway is followed.

So then, we could conclude that TBT contamination is worst when it is consumed trophically.

3.3 Butyltin compounds binding proteins distribution

3.3.1. ORGANOTIN BINDING PROTEINS

In first place, Sn containing biomolecules were screened in the water soluble extract fractionated in a size exclusion chromatographic column (Superdex 200) with separation range from 3000 to 600000 Da of molecular weight. In Fig. 14 can be seen that Sn is distributed in two main peaks: one between 9-13 min and the other one at approx. 30 min. The coelution of Sn binding groups of proteins with other elements (Se, Hg, Cd, Cu and Zn) was observed too (see Fig. 27)

Observing Se, Hg, Cd, Cu and Zn chromatograms not all of the manifestate the same profile in the contrary as Sn does. In Fig. 27, the Se chromatograms (a) and (b) have some reseamblance, Se chromatograms (c) and (d) have profiles almost alike, whereas (a) and (b) does not match with (c) and (d). This tendency is followed by Hg, Cd, Cu and Zn, which manifestate that the binding proteins present in digestive tract and gills are different.

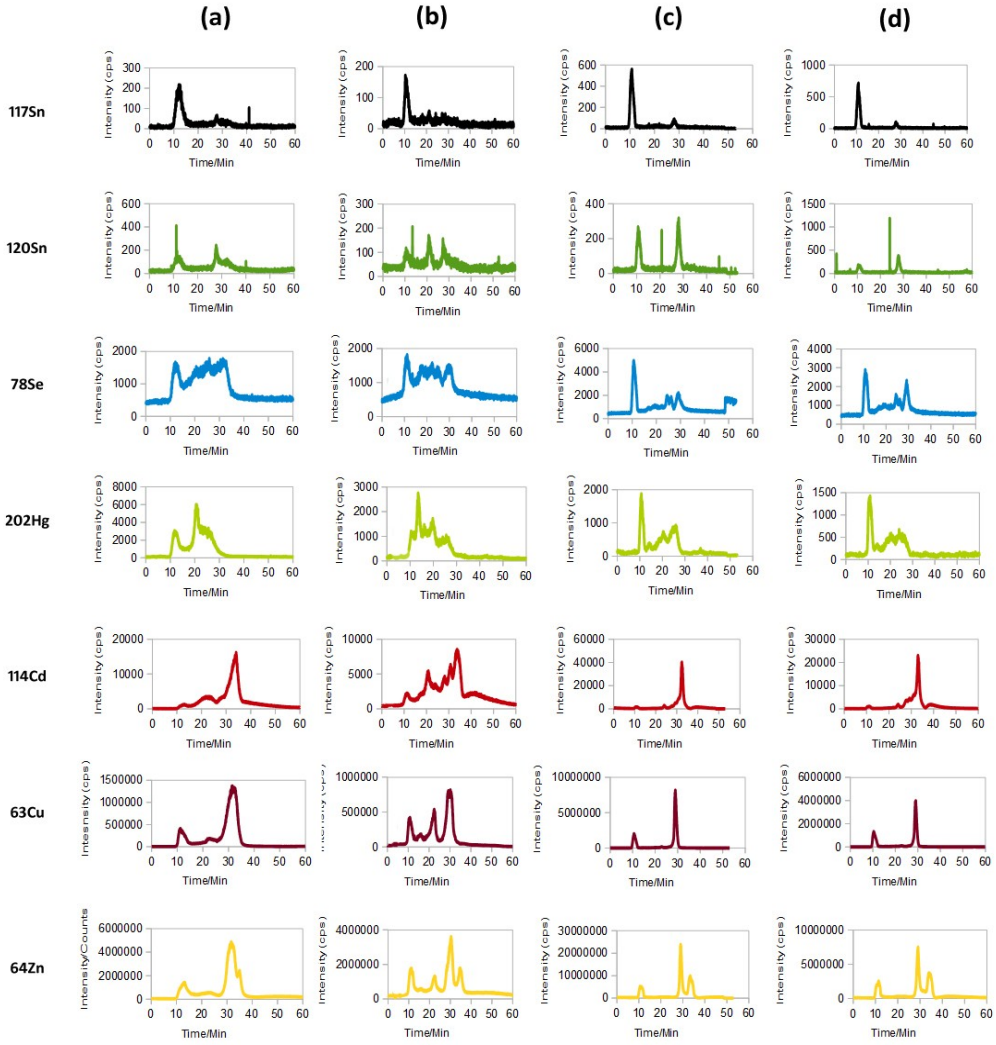

Fig. 27. Tin, cadmium, copper, zinc, selenium and mercury chromatograms. (a) Digestive tract, ^{117}TBT dissolved in phytoplankton, (b) Digestive tract, ^{117}TBT dissolved in water, (c) Gills, ^{117}TBT dissolved in water, (d) Gills, ^{117}TBT dissolved in phytoplankton

As expected, Sn containing fractions observed in the control samples correspond to Sn natural composition (Fig. 28). We check the absence of $^{117}Sn_{spike}$ compairing the Sn profile of the samples with the controls (see Fig. 28). As we can see in controls (Fig.28 , final row of chromatograms), ^{117}Sn and ^{120}Sn have the same profile, with more intensity in ^{120}Sn because it is the more abundant isotope. Compairing controls with samples (Fig 28., two first rows of chromatograms), profiles for ^{120}Sn are coincident. In the contrary, the profiles for ^{117}Sn in samples and controls mismatch because abundance of ^{117}Sn in samples, there are some ^{117}Sn peaks higher than in controls due to presence of ^{117}Sn coming from the spike of ^{117}TBT. If we also check intensities for controls and samples, we notice of lower values for sample ^{120}Sn. This ID-GC-ICP MS consequence is illustrated in Fig. 3: the abundances of our contaminated samples, must be for isotopes ^{120}Sn and ^{117}Sn, lower and higher, respectively, than its respective control. In Fig. 28, the control for digestive tract without ^{117}TBT in water does not satisfy this because there was a mistake in previous labelling in experimental process and both controls for digestive tract were mixed. Even so, controls for digestive tract were represented twice for each ^{117}TBT spiking way because although the control is a mixture, it still have ^{120}Sn profile resemblance with both digestive tract samples.

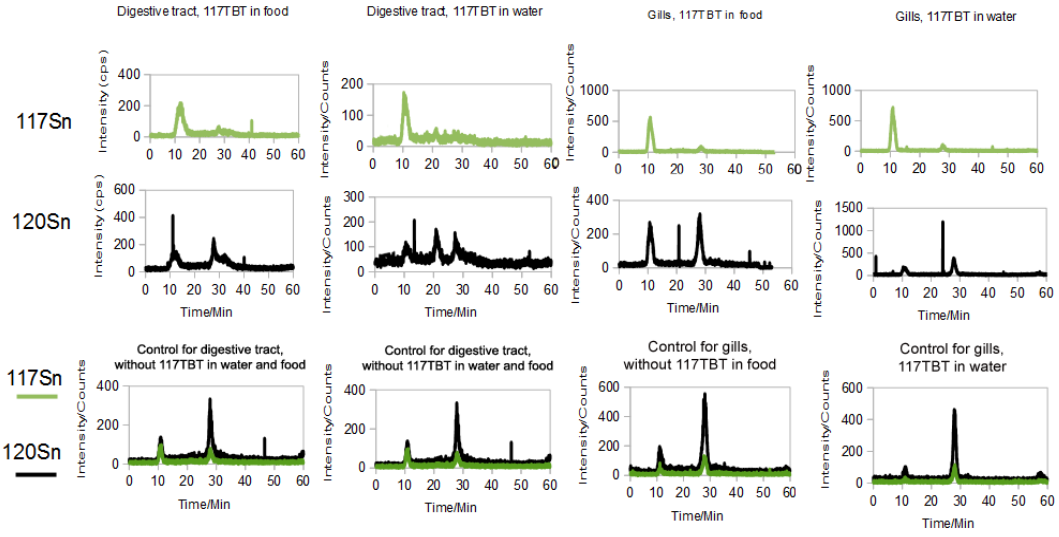

Fig. 28 Chromatograms of samples and its controls

We confirmed $^{117}Sn_{spike}$ presence in HPLC fractions analysis plotting ^{120}Sn/^{117}Sn ratio. If ^{117}Sn is naturally abundant, ratio with natural ^{120}Sn must be constant (over a 4,2461 value). If ratio is lower than 4,2461 is due to the incorporation of ^{117}Sn .

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All chromatograms show the same: when $^{117}Sn_{spike}$ to the organs, only one binding groups of proteins elutes with it in 9-15 min fraction (see Fig.29 for ^{120}Sn and ^{117}Sn chromatograms vs $^{120}Sn/^{117}Sn$ for digestive gland with ^{117}TBT water addition, the rest are in Annexes 3.3.1.A)

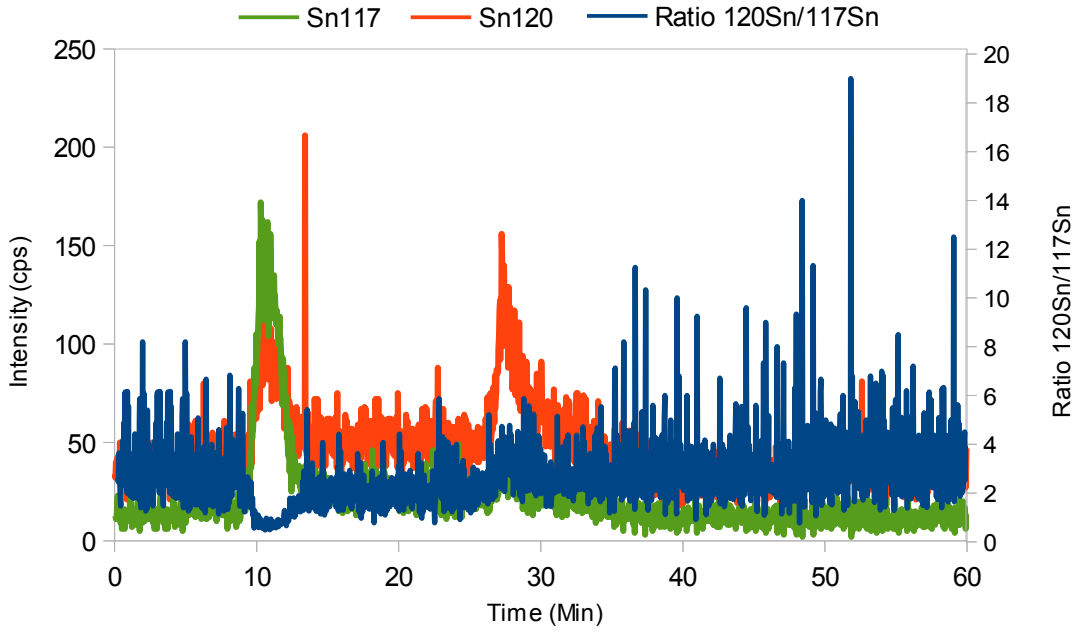


Fig. 29. ^{120}Sn and ^{117}Sn chromatograms vs $^{120}Sn/^{117}Sn$ for digestive gland with ^{117}TBT water addition

3.3.2. IDENTIFICATION OF ORGANOTIN SPECIES BINDING PROTEINS

A screening of Sn groups of proteins in the water soluble extract was made and it was fractionated in a size exclusion chromatographic column (Superdex 200) with separation range from 3000 to 600000 Da of molecular weight. In Fig. 28 we saw that detected Sn is associated with groups of proteins in the molecular weight range selected. Then, each collected fraction was analyzed with GC-ICP MS in order to know the organotin specie associated to its respective Sn binding protein.

Only 9 – 15 min fraction the sample of digestive glands with ^{117}TBT enriched phytoplankton is available, so we are not able to compare with other samples. A binding to TBT, DBT, MMT and DMT is appreciated.

Comparing 26 – 30 min fractions, the case of gills (Fig. 31, 32), we can appreciate a binding to DBT, DMT and MMT. In Fig. 33 corresponding to digestive glands with ^{117}TBT enriched phytoplankton, there is only MMT binding to proteins.

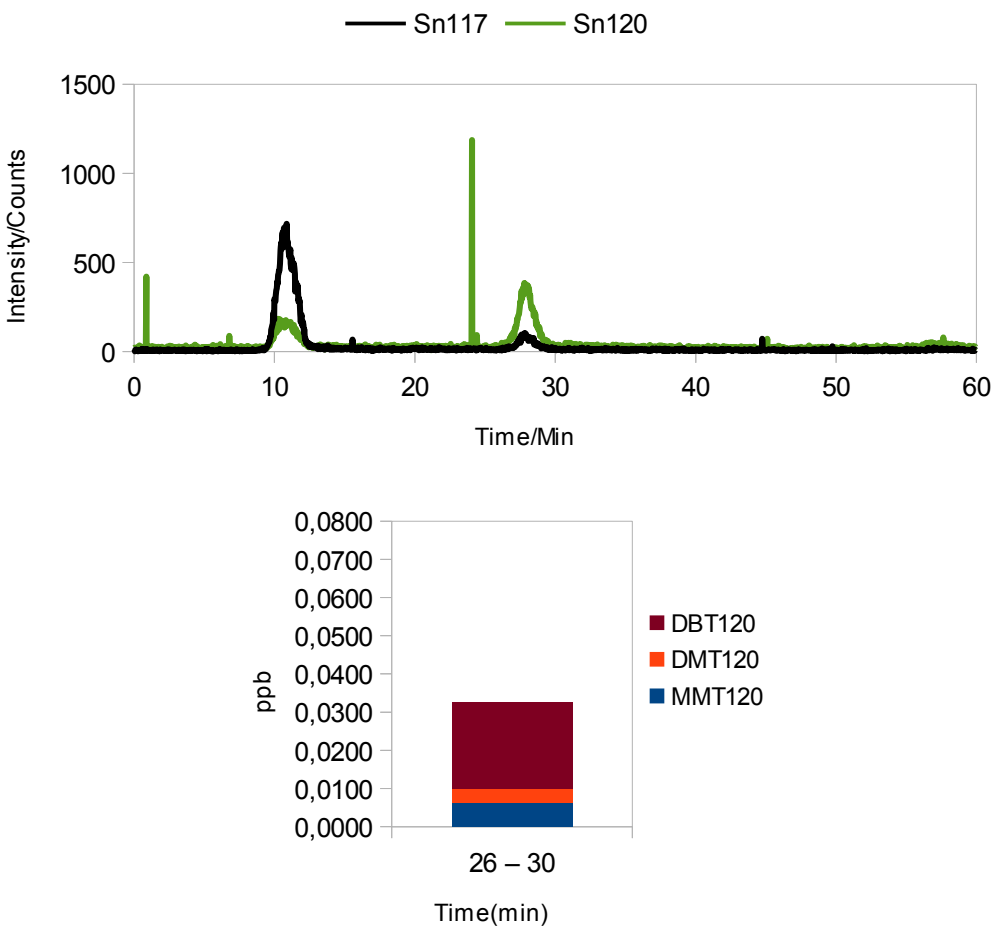
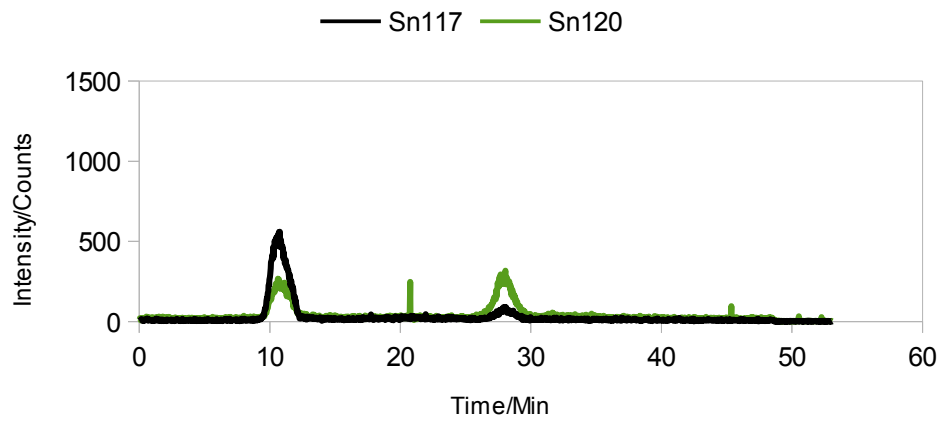


Fig. 31. Gills with ^{117}TBT pollution coming water



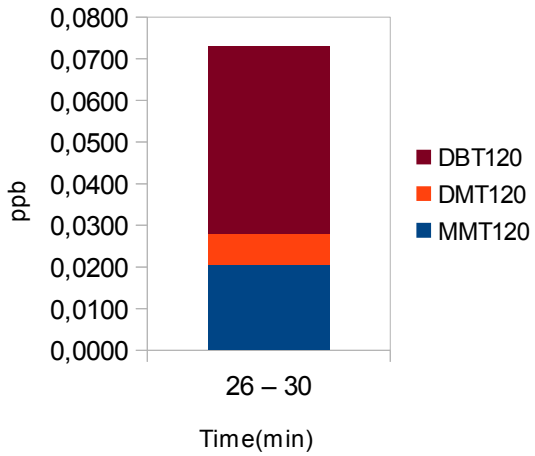
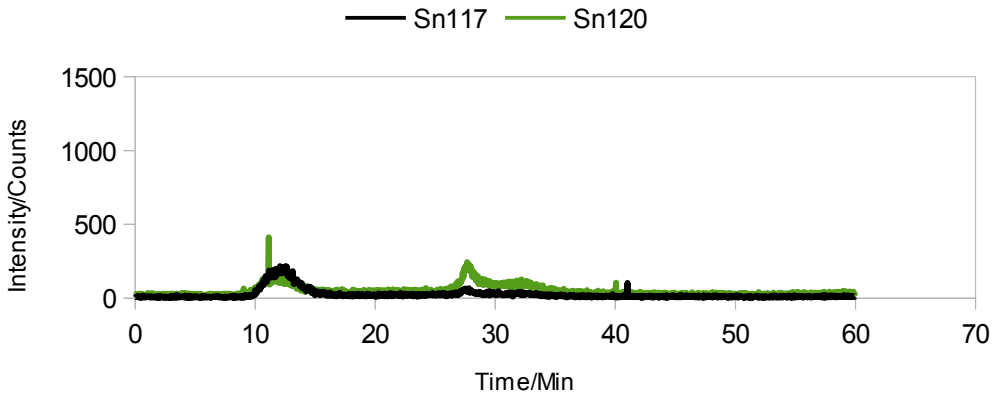


Fig. 32. Gills with ^{117}TBT pollution from enriched phytoplankton



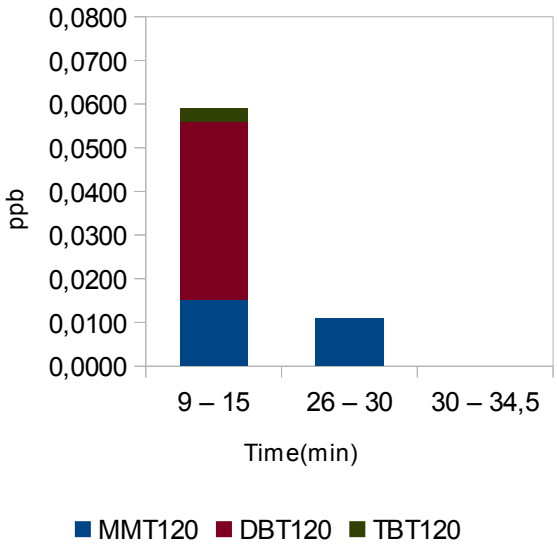


Fig. 33. Digestive tract with ^{117}TBT contamination coming from enriched phytoplankton

There were not any results for the sample of digestive gland when ^{117}TBT is added in water because it was used for plotting the first chromatogram which helped as reference for fraction collecting. 9-15 min fractions of the samples of gills when ^{117}TBT is dissolved in water and spiked in phytoplankton were evaporated after first hexane dilution.Therefore, we only have one fraction that may elucidate what butyltin specie coming from contamination with ^{117}TBT is binding cytosolic proteins. It is the case digestive gland sample with enrichment of phytoplankton as ^{117}TBT contamination source (see Fig. 30) (see Annexes 3.3.2.A)

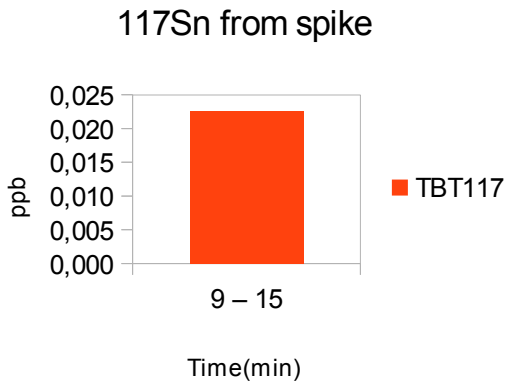


Fig. 30. ^{117}TBT coming from ^{117}TBT enrichment in phytoplankton that was binded by cytosolic Sn binding groups of proteins in digestive gland.

CONCLUSION

This study has manifested that organotin compounds consumption by oysters may lead not only to a bioaccumulation in organ tissues but also a metabolization with proteins.

Attending bioconcentration factor values, high bioconcentration in TBT and DBT is manifested whereas MBT presents a moderate value. In spite of great bioaccumulation of butyltin compounds, there is little degradation occurrence of TBT into DBT and in lower extent to MBT when butyltin compounds follow the dissolved pathway. This is explained by the degradation factor of each case: in the dissolved pathway only abiotic action is performed to degradate TBT, but in the trophic pathway, appart of abiotic action there is also the phytoplankton which can degradate organotin matter. This degradations represent only 1% of the original ^{117}TBT spike, so we can conclude that butyltin compounds are mainly bioaccumulated in oyster organ tissues.

Bioaccumulation was also studied through partition of the sample into cytosolic and non-cytosolic extract. We observed that Sn use to be more stored in non-cytosolic fraction than in cytosol, being slightly higher when the route continued by tin is trophic pathway. This means that the most of arriving Sn to the oyster it is not metabolized.

In addition, metabolization was studied screening Sn binding proteins of the cytosolic part of oyster organ tissues. Beyond the observation of coelution of Sn binding groups of proteins with same affinity for other elements (Se, Hg, Cd, Cu and Zn), it was found that from the linear molecular weight range between $3{\cdot}10^3$ and $6{\cdot}10^5$ Da, there is a kind of Sn binding protein groups which binds, at least in the case of the gills, TBT from contaminated phytoplankton. Natural Sn bindings to organotin compounds were also reported.

This project might be an starting point to identify the oyster binding proteins deepening in organotin species metabolization of oysters and finding the connection between cause-effects of organotin pollution seen in oysters and beyond, in other aquatic animal species.

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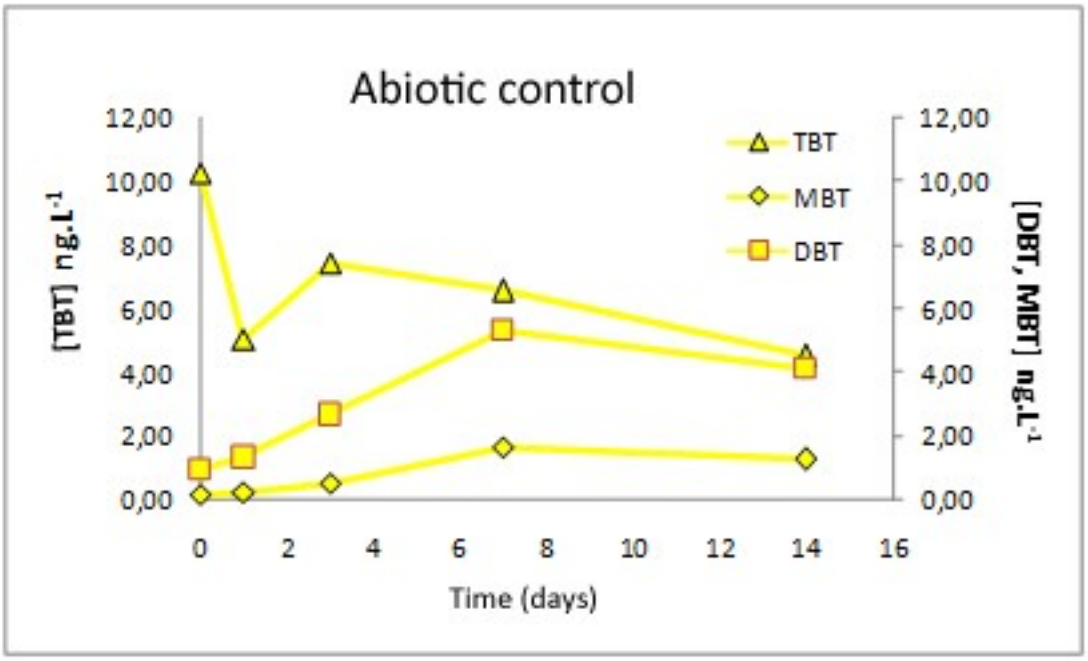
Annexes

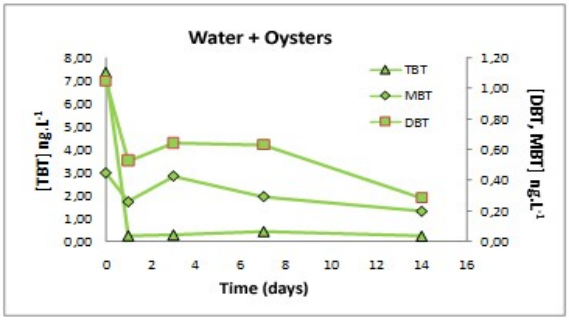
2) Experimental part

2. 1) Materials and methods

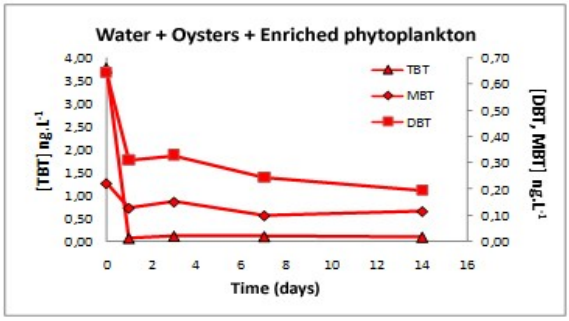
2.1.1.A



Dissolved pathway



Trophic pathway



2.1.8.A CG-ICPMS parameters for separation of tin

GC Parameters	
Column	MXT Silicosteel 30 m, i.d. 0,53 mm, df 1 μm
Injection port	Splitness
Injection port temperature	200ºC
Injection volume	2 μL
Carrier gas flow	He 25 mL/min
Make up gas flow	Ar 300 mL/min
Oven program	
Initial temperature	60ºC
Initial time	0 min
Ramp time	60ºC/min
Final temmperature	250ºC
Number of inyections	2
Transfer line	
Lenght	1 m
Inner	Silicosteel, i.d. 0,28 mm, o.d. 0,53 mm
Outer	Silicosteel, i.d. 0,10 mm, o.d. $^{1}/_{16}$ inch
ICP-MS parameters	
RF power	1250 W
Gas flow	
Plasma	15 L/min
Auxiliary	0,9 L/min
Nebulizer	0,6 L/min
Isotopes/dwell time	Hg: 202, 201: 30 ms Sn: 117, 120; 30 ms Tl: 203, 205; 5 ms Sb: 121, 123; 5 ms

2.1.9.A HPLC-ICPMS parameters for separation of tin

HPLC parameters

Column	
Model	Superdex 200 10/300 GL 10 0071
Chromatographic method	Size exclusion
Separation range	M_r 3000 - 600000 (globular proteins)
Matrix	Aragose and dextran
Volume	24 mL
Columne lenght	30 cm
Maximun / Limit	260 psi
Mobile phase	
Composition	NH_4Ac 100mM of pH=7,5
Flow	0,7 mL/min
Analyte	
Inyection volume	150 µL
Number of inyections	2

2.1.10.A Table of reagent weights

ORGANOTIN COMPOUNDS DISTRIBUTION IN CYTOSOLIC ('SOLID') AND NON-CYTOSOLIC ('LIQUID') FRACTIONS

SAMPLE	OYSTER weight/g	SOLID		Extract		Hexane	
		Solid weight/g	m TMAH/g	V ext/µL	m ext/g	V hex/µL	m hex/g
Digestive gland, DisP	**1,5745**	1,1738	1,9924	500	0,4806	300	0,1881
Digestive gland, TroP	**1,3337**	0,5936	1,0060	400	0,3561	400	0,3561
Control digestive gland, Disp&TroP	**1,9588**	0,7514	1,2895	400	0,3914	300	0,1917
Gills, DisP	**2,5380**	0,9900	1,6651	500	0,4361	300	0,1892
Control gills, DisP	**0,9777**	0,4048	0,8670	500	0,4591	300	0,1887
Gills, TroP	**2,3700**	1,0482	2,0935	350	0,3286	300	0,1676
Control gills, TroP	**1,2162**	0,7310	1,2232	400	0,3596	300	0,1841

SAMPLE	OYSTER weight/g	LIQUID		Extract		Hexane	
		Liquid weight/g	m TMAH/g	V ext/µL	m ext/g	V hex/µL	m hex/g
Digestive gland, DisP	**1,5745**	0,5777	0,9792	400	0,3765	300	0,1873
Digestive gland, TroP	**1,3337**	0,4561	0,7662	400	0,3182	300	0,1883
Control digestive gland, Disp&TroP	**1,9588**	2,4542	4,0998	400	0,3929	300	0,1934
Gills, DisP	**2,5380**	0,4451	0,7761	500	0,4394	300	0,1828
Control gills, DisP	**0,9777**	1,3739	2,3011	500	0,469	300	0,1887
Gills, TroP	**2,3700**	0,9444	1,6397	300	0,2899	400	0,2546
Control gills, TroP	**1,2162**	1,5866	2,6857	400	0,3447	300	0,1882

Controls of DisP and TroP of digestive gland were mixed to a mistake in labelling.

ORGANOTIN COMPOUNDS BINDING PROTEINS DISTRIBUTION

SAMPLE	OYSTER weight/g	LIQUID		HPLC				Concentrating
		Liquid weight/g	m TMAH/g	Fraction	m extract/g	m hex/g	m aliquot/g	m hex/g
Gills, DisP	**2,5380**	1,0777	0,7761	# 1	7,5705	0,3038	-	-
				#2	7,8429	0,2904	0,0052	0,0675
Gills, TroP	**2,3700**	1,4444	1,6397	#1	8,0583	0,2964	-	-
				#2	8,2985	0,3019	0,0057	0,0668
Digestive gland, TroP	**0,5936**	0,9561	0,7662	#1	6,9791	0,2947	0,0053	0,0669
				#2	8,4289	0,2679	0,0043	0,3039
					6,9375	0,2774		
				#3	6,2582	0,2986	0,0051	0,2977
					5,9518	0,3037		

No results for 'Digestive gland, DisP' because it was used for plotting first chromatogram which helped as reference for fraction collecting. First fractions of 'Gills, DisP' and 'Gills DisP' were evaporated after first first hexane dilution.

2.1.10.B Mix solution

A mix solution was utilized for performing standards of the external calibration curve. This solution of 1 ppb was made from an original 100ppb solution:

$Ci \cdot Vi = Cf \cdot Vf \rightarrow 100\ ppb \cdot Vi = 1ppb \cdot 2mL \rightarrow Vi$=0,02 mL of 100 ppb solution was taken and raising until 2mL with water.

100ppb solution was made as follows from standards (std) prepared by Ph.D. Joana Cavalheiro (UPPA)

Compound (Concentration of std)	Compound weight (~100 µL)/g	Water weight (~8,8g)/g	HCl weight (~100 µL)/g	Concentration of specie in 100 ppb mix solution
MMT (9,633ppm)	0,1085	8,8028	0,1208	104,3428
DMT (8,287 ppm)	0,0994			82,2346
TMT (9,309 ppm)	0,1024			95,1643
MBT (8,911 ppm)	0,0958			92,4599
DBT (9,499 ppm)	0,0975			93,4672
TBT (9,505 ppm)	0,0985			100,4672
MphT (10,033 ppm)	0,1000			96,1321
DphT (9,591 ppm)	0,1004			86,8166
TphT (9,021 ppm)	0,0964			99,8323
MeHg (10 ppm)	0,1000			99,8323
IHg (10 ppm)	0,0943			94,1418

Mercury concentrations were added as this original project was going to study also mercurial but later it was rejected due to lack of time.

2.1.10.C External Calibration Curve

Three inyections of each calibration standard were made.

Specie	STD	**Mean Real [std]/ppb**	Mean Area ^{120}Sn	**Net Mean Area ^{120}Sn**	Mean Area ^{117}Sn	**Net Mean Area ^{117}Sn**
MMT	BL-0	0,0000	0	0	513147	0
	S1	0,4273	101698388	101698388	29389499	28876352
	S2	1,1734	373951665	373951665	77396417	76883270
	S3	1,3926	321693057	321693057	97142736	96629589
DMT	BL-0	0,0000	0	0	0	0
	S1	0,3367	112502134	112502134	26401682	26401682
	S2	0,9248	307066102	307066102	70428740	70428740
	S3	1,0976	369169116	369169116	84554772	84554772
TMT	BL-0	0,0000	0	0	0	0
	S1	0,3897	134273865	134273865	25358557	25358557
	S2	1,0702	268635347	268635347	68616063	68616063
	S3	1,2701	424906618	424906618	73326096	73326096
MBT	BL-0	0,0000	0	0	668051	0
	S1	0,3490	89680098	89680098	21765056	21097004
	S2	0,9584	264956449	264956449	61314553	60646502
	S3	1,1375	317036005	317036005	73043835	72375784
DBT	BL-0	0,0000	16423479	0	3644822	0
	S1	0,3786	91551882	75128403	21471269	17826447
	S2	1,0398	231190567	214767088	51584971	47940149
	S3	1,2340	250029293	233605814	56883216	53238394
TBT	BL-0	0,0000	0	0	0	0
	S1	0,3827	48654007	48654007	12767821	12767821
	S2	1,0511	155456041	155456041	33002212	33002212
	S3	1,2475	185795557	185795557	41676029	41676029
MphT	BL-0	0,0000	0	0	0	0
	S1	0,4102	34527689	34527689	8345802	8345802
	S2	1,1264	109410938	109410938	24077209	24077209
	S3	1,3368	91585289	91585289	23039122	23039122
DphT	BL-0	0,0000	0	0	0	0
	S1	0,3937	74450529	74450529	17304522	17304522
	S2	1,0811	231853621	231853621	45522533	45522533
	S3	1,2830	273819484	273819484	59978301	59978301
TphT	BL-0	0,0000	0	0	0	0
	S1	0,3555	20174530	20174530	3548396	3548396
	S2	0,9763	51564580	51564580	12705477	12705477
	S3	1,1587	64067833	64067833	13605161	13605161

	^{120}Sn			^{117}Sn			^{120}Sn	^{117}Sn
Specie	**Slope**	**Intercept**	r	**Slope**	**Intercept**	r	**[BL-0]**	**[BL-0]**
MMT	264978019	1044903	0,9660	68051588	-327710	0,9990	0,0000	0,0048
DMT	334892263	-326267	0,9999	76540810	204552	0,9999	0,0010	0,0000
TMT	300352457	1962503	0,9748	59460972	1242887	0,9962	0,0000	0,0000
MBT	280194457	-3340732	0,9997	63836525	-487977	0,9999	0,0119	0,0076
DBT	195142655	1474725	0,9975	43886907	649501	0,9984	0,0766	0,0000
TBT	150902684	-3678492	0,9990	32616985	-2693	0,9987	0,0244	0,0001
MphT	78143607	2746997	0,9602	18535827	550435	0,9846	0,0000	0,0000
DphT	216477324	-4218148	0,9994	45212893	-470432	0,9967	0,0195	0,0104
TphT	54152243	234672	0,9992	12437489	-279254	0,9948	0,0000	0,0225

Blanks concentration were recalculated from calibration curve.

3) Results and conclusion

3. 1) Organotin compounds distribution in cytosolic and non-cytosolic fractions

3.1.A List of concentrations and RSD% of samples

(See next page for concentration values)

%RSD – 120 Sn – CYTOSOLIC FRACTIONS

Samples	MMT	DMT	TMT	MBT	DBT	MphT	TBT	DphT	TphT
Digestive gland, 117TBT dissolved in water	21	8	0	5	15	0	10	0	0
Digestive gland, 117TBT dissolved in phto.	14	5	0	20	2	0	5	0	0
Control of digestive gland, 117TBT dissolved in water+phto.	13	1	0	6	9	0	10	0	0
Gills, 117TBT dissolved in water	0	13	0	14	12	0	7	0	0
Control of gills, 117TBT dissolved in water	34	1	0	13	2	0	24	0	0
Gills, 117TBT dissolved in phto.	6	1	0	6	6	0	11	0	0
Control of gills, 117TBT dissolved in phto.	11	13	0	7	5	0	6	0	0

%RSD – 120 Sn – NON-CYTOSOLIC FRACTIONS

Samples	MMT	DMT	TMT	MBT	DBT	MphT	TBT	DphT	TphT
Digestive gland, 117TBT dissolved in water	3	11	0	10	12	0	7	0	0
Digestive gland, 117TBT dissolved in phto.	5	21	0	21	14	0	7	0	0
Control of digestive gland, 117TBT dissolved in water+phto.	2	16	0	4	2	0	4	0	0
Gills, 117TBT dissolved in water	2	5	0	53	19	0	3	0	0
Control of gills, 117TBT dissolved in water	3	9	0	3	12	0	5	0	0
Gills, 117TBT dissolved in phto.	11	4	0	11	18	0	11	0	0
Control of gills, 117TBT dissolved in phto.	6	6	0	6	7	0	24	0	0

%RSD – total 117Sn - CYTOSOLIC FRACTIONS

Samples	MMT	DMT	TMT	MBT	DBT	MphT	TBT	DphT	TphT
Digestive gland, 117TBT dissolved in water	21	6	0	20	26	0	19	0	0
Digestive gland, 117TBT dissolved in phto.	9	6	0	8	7	0	6	0	0
Control of digestive gland, 117TBT dissolved in water+phto.	5	0	0	6	10	0	7	0	0
Gills, 117TBT dissolved in water	6	11	0	17	11	0	10	0	0
Control of gills, 117TBT dissolved in water	22	2	0	16	2	0	16	0	0
Gills, 117TBT dissolved in phto.	0	1	0	0	7	0	12	0	0
Control of gills, 117TBT dissolved in phto.	6	11	0	7	5	0	1	0	0

%RSD –total 117Sn - NON-CYTOSOLIC FRACTIONS

Samples	MMT	DMT	TMT	MBT	DBT	MphT	TBT	DphT	TphT
Digestive gland, 117TBT dissolved in water	3	11	0	10	12	0	7	0	0
Digestive gland, 117TBT dissolved in phto.	5	21	0	21	14	0	7	0	0
Control of digestive gland, 117TBT dissolved in water+phto.	2	16	0	4	2	0	4	0	0
Gills, 117TBT dissolved in water	2	5	0	53	19	0	3	0	0
Control of gills, 117TBT dissolved in water	3	9	0	3	12	0	5	0	0
Gills, 117TBT dissolved in phto.	11	4	0	11	18	0	11	0	0
Control of gills, 117TBT dissolved in phto.	6	6	0	6	7	0	24	0	0

%RSD – spike117Sn - CYTOSOLIC FRACTIONS

Samples	MMT	DMT	TMT	MBT	DBT	MphT	TBT	DphT	TphT
Digestive gland, 117TBT dissolved in water	6	17	0	21	29	0	12	0	0
Digestive gland, 117TBT dissolved in phto.	27	10	0	15	9	0	8	0	0
Control of digestive gland, 117TBT dissolved in water+phto.	8	12	0	1	19	0	24	0	0
Gills, 117TBT dissolved in water	38	8	0	22	11	0	1	0	0
Control of gills, 117TBT dissolved in water	20	12	0	24	13	0	62	0	0
Gills, 117TBT dissolved in phto.	36	15	0	22	17	0	11	0	0
Control of gills, 117TBT dissolved in phto.	8	16	0	8	35	0	53	0	0

%RSD – spike 117Sn - NON-CYTOSOLIC FRACTIONS

Samples	MMT	DMT	TMT	MBT	DBT	MphT	TBT	DphT	TphT
Digestive gland, 117TBT dissolved in water	13	30	0	2	13	0	12	0	0
Digestive gland, 117TBT dissolved in phto.	11	15	0	16	15	0	8	0	0
Control of digestive gland, 117TBT dissolved in water+phto.	19	3	0	22	27	0	9	0	0
Gills, 117TBT dissolved in water	26	2	0	7	10	0	1	0	0
Control of gills, 117TBT dissolved in water	26	23	0	8	6	0	8	0	0
Gills, 117TBT dissolved in phto.	19	25	0	14	12	0	11	0	0
Control of gills, 117TBT dissolved in phto.	34	3	0	7	20	0	11	0	0

[120Sn]/ppb in sample – CYTOSOLIC FRACTIONS

Samples	Liq [MMT 120Sn]/ppb	Liq [DMT 120Sn]/ppb	Liq [TMT 120Sn]/ppb	Liq [MBT 120Sn]/ppb	Liq [DBT 120Sn]/ppb	Liq [MphT 120Sn]/ppb	Liq [TBT 120Sn]/ppb	Liq [DphT 120Sn]/ppb	Sol [TphT 120Sn]/ppb
Digestive gland, 117TBT dissolved in water	0,1307	0,0309	0,0000	0,0547	0,1802	0,0000	0,3084	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	0,0574	0,0227	0,0000	0,0330	0,1297	0,0000	0,0476	0,0000	0,0000
Control of digestive gland, 117TBT dissolved in water+phto.	0,0370	0,0504	0,0000	0,0616	0,2341	0,0000	0,1330	0,0000	0,0000
Gills, 117TBT dissolved in water	0,0429	0,0095	0,0000	0,0224	0,0748	0,0000	0,0568	0,0000	0,0000
Control of gills, 117TBT dissolved in water	0,0455	0,0246	0,0000	0,0451	0,0976	0,0000	0,0369	0,0000	0,0000
Gills, 117TBT dissolved in phto.	0,0326	0,0275	0,0000	0,0188	0,1324	0,0000	0,0272	0,0000	0,0000
Control of gills, 117TBT dissolved in phto.	0,0664	0,0558	0,0000	0,1319	0,2225	0,0000	0,2043	0,0000	0,0000

[120Sn]/ppb in sample – NON-CYTOSOLIC FRACTIONS

Samples	Sol [MMT 120Sn]/ppb	Sol [DMT 120Sn]/ppb	Sol [TMT 120Sn]/ppb	Sol [MBT 120Sn]/ppb	Sol [DBT 120Sn]/ppb	Sol [MphT 120Sn]/ppb	Sol [TBT 120Sn]/ppb	Sol [DphT 120Sn]/ppb	Sol [TphT 120Sn]/ppb
Digestive gland, 117TBT dissolved in water	0,0505	0,0249	0,0000	0,0601	0,2132	0,0000	0,3495	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	0,1429	0,0482	0,0000	0,1569	0,5217	0,0000	0,7777	0,0000	0,0000
Control of digestive gland, 117TBT dissolved in water+phto.	0,0373	0,0190	0,0000	0,0730	0,2356	0,0000	0,2616	0,0000	0,0000
Gills, 117TBT dissolved in water	0,0629	0,0043	0,0000	0,0425	0,0513	0,0000	0,1213	0,0000	0,0000
Control of gills, 117TBT dissolved in water	0,0458	0,0055	0,0000	0,0155	0,0650	0,0000	0,1463	0,0000	0,0000
Gills, 117TBT dissolved in phto.	0,0751	0,0413	0,0000	0,0655	0,1778	0,0000	0,2543	0,0000	0,0000
Control of gills, 117TBT dissolved in phto.	0,0759	0,0299	0,0000	0,0399	0,1755	0,0000	0,2175	0,0000	0,0000

[total 117Sn]/ppb in sample – CYTOSOLIC FRACTIONS

Samples	Liq [MMT 117Sn]/ppb	Liq [DMT Area 117Sn]/ppb	Liq [TMT 117Sn]/ppb	Liq [MBT 117Sn]/ppb	Liq [DBT 117Sn]/ppb	Liq [MphT 117Sn]/ppb	Liq [TBT 117Sn]/ppb	Liq [DphT 117Sn]/ppb	Liq [TphT 117Sn]/ppb
Digestive gland, 117TBT dissolved in water	0,0311	0,0073	0,0000	0,1851	0,2089	0,0000	0,6171	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	0,0142	0,0054	0,0000	0,0044	0,0681	0,0000	0,4601	0,0000	0,0000
Control of digestive gland, 117TBT dissolved in water+phto.	0,0116	0,0112	0,0000	0,0011	0,0000	0,0000	0,0100	0,0000	0,0000
Gills, 117TBT dissolved in water	0,0131	0,0030	0,0000	0,0047	0,1735	0,0000	1,7458	0,0000	0,0000
Control of gills, 117TBT dissolved in water	0,0111	0,0056	0,0000	0,0035	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT dissolved in phto.	0,0090	0,0062	0,0000	0,0000	0,0238	0,0000	0,3128	0,0000	0,0000
Control of gills, 117TBT dissolved in phto.	0,0177	0,0129	0,0000	0,0159	0,0000	0,0000	0,0254	0,0000	0,0000

[total 117Sn]/ppb in sample – NON CYTOSOLIC FRACTIONS

Samples	Sol [MMT 117Sn]/ppb	Sol [DMT Area 117Sn]/ppb	Sol [TMT 117Sn]/ppb	Sol [MBT 117Sn]/ppb	Sol [DBT 117Sn]/ppb	Sol [MphT 117Sn]/ppb	Sol [TBT 117Sn]/ppb	Sol [DphT 117Sn]/ppb	Sol [TphT 117Sn]/ppb
Digestive gland, 117TBT dissolved in water	0,0135	0,0057	0,0000	0,0441	0,6177	0,0000	3,3636	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	0,0359	0,0148	0,0000	0,1150	1,3615	0,0000	6,0491	0,0000	0,0000
Control of digestive gland, 117TBT dissolved in water+phto.	0,0126	0,0045	0,0000	0,0110	0,0107	0,0000	0,0564	0,0000	0,0000
Gills, 117TBT dissolved in water	0,0161	0,0000	0,0000	0,0119	0,5987	0,0000	3,0142	0,0000	0,0000
Control of gills, 117TBT dissolved in water	0,0113	0,0000	0,0000	0,0000	0,0000	0,0000	0,0239	0,0000	0,0000
Gills, 117TBT dissolved in phto.	0,0186	0,0103	0,0000	0,0167	0,4466	0,0000	1,6472	0,0000	0,0000
Control of gills, 117TBT dissolved in phto.	0,0192	0,0084	0,0000	0,0004	0,0000	0,0000	0,0428	0,0000	0,0000

[spike 117Sn]/ppb in sample – CYTOSOLIC FRACTIONS

Samples	Liq [MMT 117Sn]/ppb	Liq [DMT Area 117Sn]/ppb	Liq [TMT 117Sn]/ppb	Liq [MBT 117Sn]/ppb	Liq [DBT 117Sn]/ppb	Liq [MphT 117Sn]/ppb	Liq [TBT 117Sn]/ppb	Liq [DphT 117Sn]/ppb	Liq [TphT 117Sn]/ppb
Digestive gland, 117TBT dissolved in water	0,0000	0,0000	0,0000	0,1608	0,1915	0,0000	2,7001	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	0,0006	0,0000	0,0000	0,0000	0,0820	0,0000	2,8186	0,0000	0,0000
Control of digestive gland, 117TBT dissolved in water+phto.	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT dissolved in water	0,0000	0,0000	0,0000	0,0000	0,1684	0,0000	2,0403	0,0000	0,0000
Control of gills, 117TBT dissolved in water	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0114	0,0000	0,0000
Gills, 117TBT dissolved in phto.	0,0000	0,0000	0,0000	0,0000	0,0702	0,0000	2,2332	0,0000	0,0000
Control of gills, 117TBT dissolved in phto.	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000

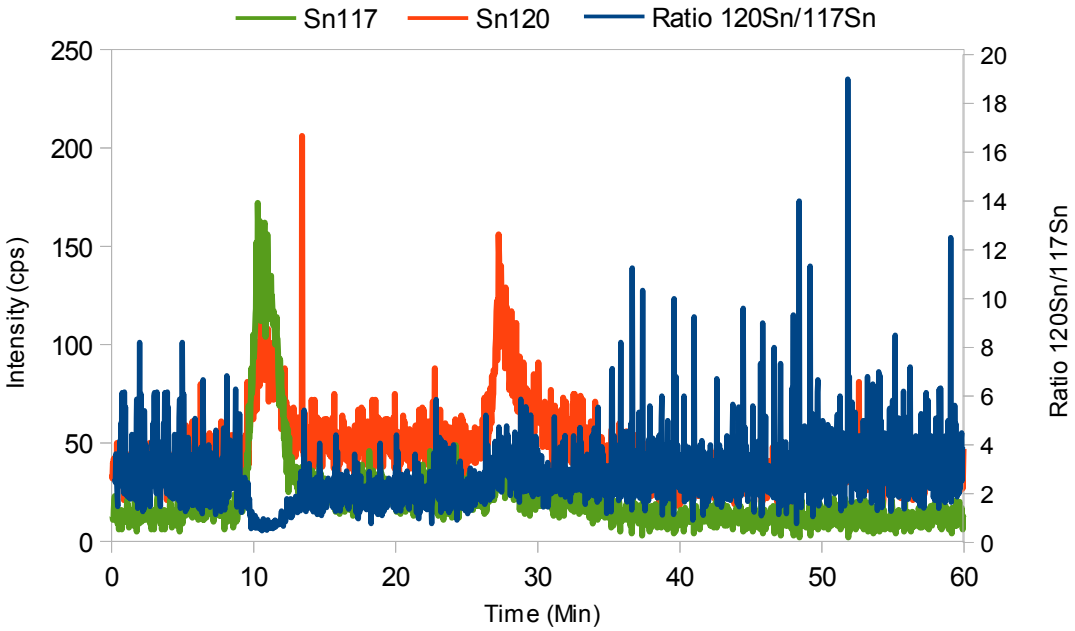
[spike 117Sn]/ppb in sample – NON CYTOSOLIC FRACTIONS

Samples	Sol [MMT 117Sn]/ppb	Sol [DMT Area 117Sn]/ppb	Sol [TMT 117Sn]/ppb	Sol [MBT 117Sn]/ppb	Sol [DBT 117Sn]/ppb	Sol [MphT 117Sn]/ppb	Sol [TBT 117Sn]/ppb	Sol [DphT 117Sn]/ppb	Sol [TphT 117Sn]/ppb
Digestive gland, 117TBT dissolved in water	0,0000	0,0000	0,0000	0,0000	0,5566	0,0000	2,9671	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	0,0020	0,0000	0,0000	0,0000	1,2055	0,0000	5,3070	0,0000	0,0000
Control of digestive gland, 117TBT dissolved in water+phto.	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT dissolved in water	0,0000	0,0000	0,0000	0,0000	0,5651	0,0000	2,6984	0,0000	0,0000
Control of gills, 117TBT dissolved in water	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT dissolved in phto.	0,0008	0,0000	0,0000	0,0000	0,4139	0,0000	1,4437	0,0000	0,0000
Control of gills, 117TBT dissolved in phto.	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000

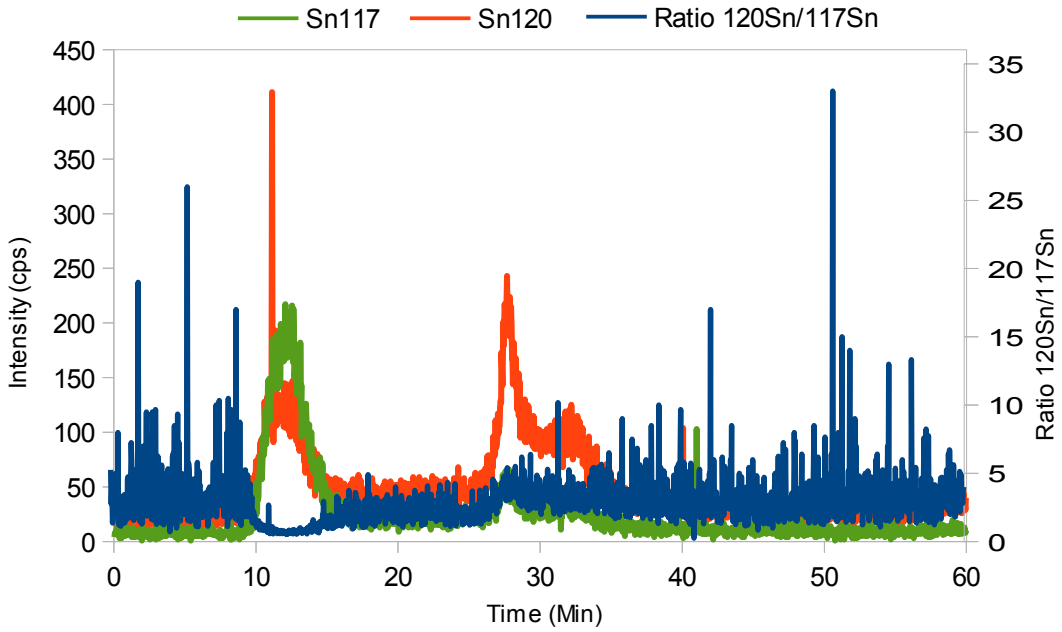
3.3. Butyltin compounds binding proteins distribution

3.3.1. ORGANOTIN BINDING PROTEINS

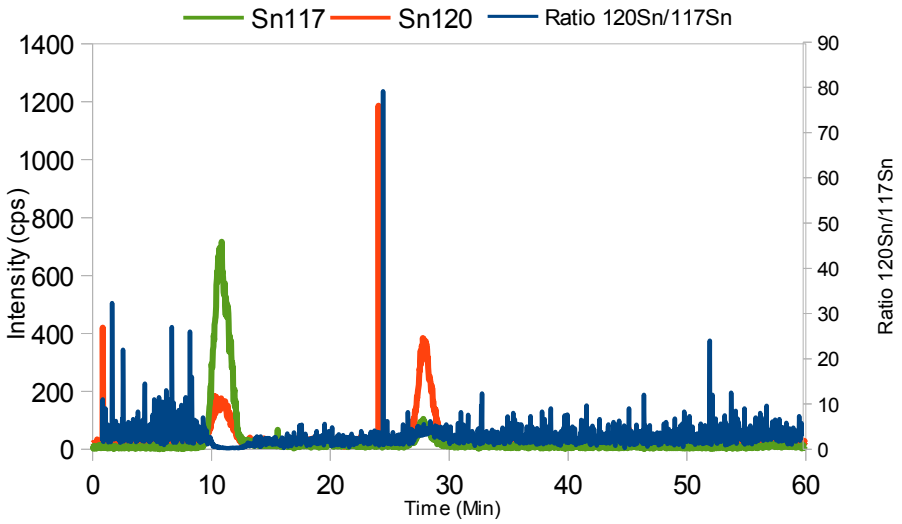
3.3.1.A ^{120}Sn and ^{117}Sn chromatograms vs ^{120}Sn/^{117}Sn



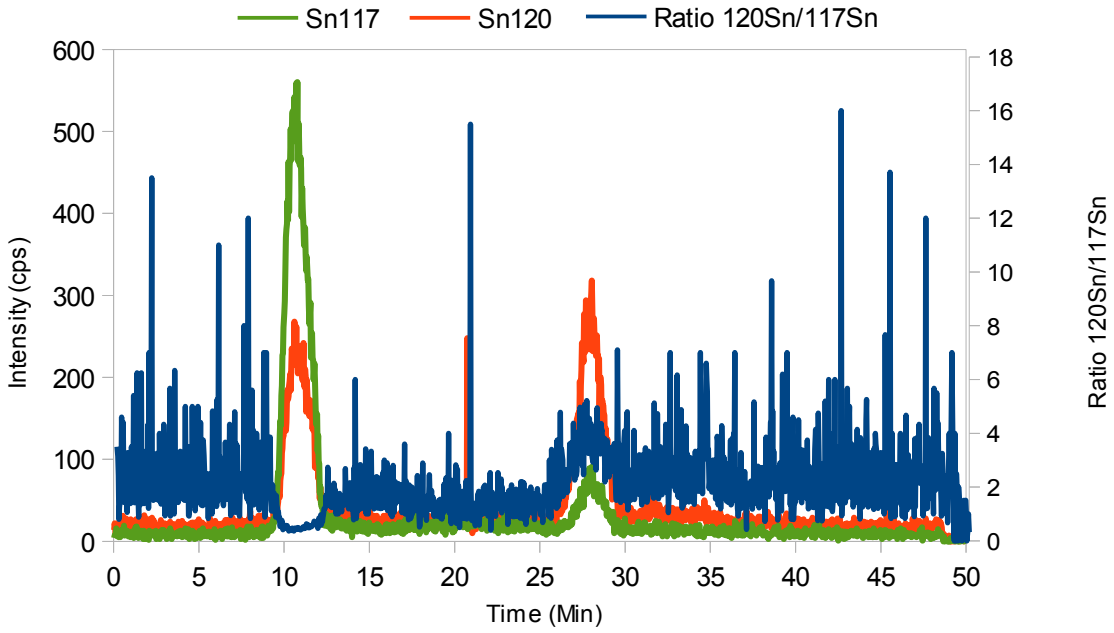
^{120}Sn and ^{117}Sn chromatograms vs ^{120}Sn/^{117}Sn for digestive gland with ^{117}TBT water addition



^{120}Sn and ^{117}Sn chromatograms vs $^{120}Sn/^{117}Sn$ for digestive gland with ^{117}TBT phytoplankton enrichment



^{120}Sn and ^{117}Sn chromatograms vs $^{120}Sn/^{117}Sn$ in the case of gills with ^{117}TBT water addition



^{120}Sn and ^{117}Sn chromatograms vs $^{120}Sn/^{117}Sn$ for gills with ^{117}TBT phytoplankton enrichment

3.3.2. IDENTIFICATION OF ORGANOTIN SPECIES BINDING PROTEINS

3.3.2.A List of concentrations and %RSD of samples

120Sn ng/mL	Time/min	MMT120	DMT120	TMT120	MBT120	DBT120
Gills, 117TBT is dissolved in water	26 – 30	0,0062	0,0038	0,0000	0,0000	0,0228
Gills, 117TBT is dissolved in phto.	26 – 30	0,0206	0,0074	0,0000	0,0000	0,0450
Digestive gland, 117TBT dissolved in phto.	9 – 15	0,0153	0,0000	0,0000	0,0000	0,0408
	26 – 30	0,0109	0,0000	0,0000	0,0000	0,0000
	30 – 34,5	0,0000	0,0000	0,0000	0,0000	0,0000
117Sn(nat+spk) ng/mL	Time/min	MMT117(nat+spk)	DMT117(nat+spk)	TMT117(nat+spk)	MBT117(nat+spk)	DBT117(nat+spk)
Gills, 117TBT is dissolved in water	26 – 30	0,0018	0,0010	0,0000	0,0000	0,0000
Gills, 117TBT is dissolved in phto.	26 – 30	0,0060	0,0017	0,0000	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	9 – 15	0,0051	0,0017	0,0000	0,0000	0,0000
	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
	30 – 34,5	0,0000	0,0000	0,0000	0,0000	0,0000
117Sn (spk) ng/mL	Time/min	MMT117(spk)	DMT(spk)	TMT117(spk)	MBT117(spk)	DBT117(spk)
Gills, 117TBT is dissolved in water	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT is dissolved in phto.	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	9 – 15	0,0000	0,0000	0,0000	0,0000	0,0000
	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
	30 – 34,5	0,0000	0,0000	0,0000	0,0000	0,0000

120Sn ng/mL	Time/min	MphT120	TBT120	DphT120	TphT120	iSn120
Gills, 117TBT is dissolved in water	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT is dissolved in phto.	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	9 – 15	0,0000	0,0030	0,0000	0,0000	0,0000
	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
	30 – 34,5	0,0000	0,0000	0,0000	0,0000	0,0000
117Sn(nat+spk) ng/mL	Time/min	MphT117(nat+spk)	TBT117(nat+spk)	DphT117(nat+spk)	TphT117(nat+spk)	iSn117(nat+spk)
Gills, 117TBT is dissolved in water	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT is dissolved in phto.	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	9 – 15	0,0000	0,0061	0,0000	0,0000	0,0000
	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
	30 – 34,5	0,0000	0,0000	0,0000	0,0000	0,0000
117Sn (spk) ng/mL	Time/min	MphT117(spk)	TBT117(spk)	DphT117(spk)	TphT117(spk)	iSn117(spk)
Gills, 117TBT is dissolved in water	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Gills, 117TBT is dissolved in phto.	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
Digestive gland, 117TBT dissolved in phto.	9 – 15	0,0000	0,0226	0,0000	0,0000	0,0000
	26 – 30	0,0000	0,0000	0,0000	0,0000	0,0000
	30 – 34,5	0,0000	0,0000	0,0000	0,0000	0,0000

120Sn RSD%	Time/min	MMT120	DMT120	TMT120	MBT120	DBT120
Gills, 117TBT is dissolved in water	26 – 30	6	8	0	28	20
Gills, 117TBT is dissolved in phto.	26 – 30	11	4	0	14	18
Digestive gland, 117TBT dissolved in phto.	9 – 15	11	8	0	4	9
	26 – 30	15	0	0	10	33
	30 – 34,5	3	0	0	0	7
117Sn(nat+spk) RSD%	Time/min	MMT117(nat+spk)	DMT117(nat+spk)	TMT117(nat+spk)	MBT117(nat+spk)	DBT117(nat+spk)
Gills, 117TBT is dissolved in water	26 – 30	1	15	0	7	6
Gills, 117TBT is dissolved in phto.	26 – 30	4	1	0	4	22
Digestive gland, 117TBT dissolved in phto.	9 – 15	9	0	0	11	4
	26 – 30	15	0	0	11	25
	30 – 34,5	5	0	0	9	26
117Sn (spk) RSD%	Time/min	MMT117(spk)	DMT(spk)	TMT117(spk)	MBT117(spk)	DBT117(spk)
Gills, 117TBT is dissolved in water	26 – 30	66	52	0	67	91
Gills, 117TBT is dissolved in phto.	26 – 30	18	45	0	59	35
Digestive gland, 117TBT dissolved in phto.	9 – 15	4	49	0	43	59
	26 – 30	28	0	0	16	87
	30 – 34,5	20	0	0	23	44

120Sn RSD%	Time/min	MphT120	TBT120	DphT120	TphT120	iSn120
Gills, 117TBT is dissolved in water	26 – 30	0	0	0	0	0
Gills, 117TBT is dissolved in phto.	26 – 30	0	0	0	0	0
Digestive gland, 117TBT dissolved in phto.	9 – 15	0	0	0	0	0
	26 – 30	0	0	0	0	0
	30 – 34,5	0	0	0	0	0
117Sn(nat+spk) RSD%	Time/min	MphT117(nat+spk)	TBT117(nat+spk)	DphT117(nat+spk)	TphT117(nat+spk)	iSn117(nat+spk)
Gills, 117TBT is dissolved in water	26 – 30	0	0	0	0	0
Gills, 117TBT is dissolved in phto.	26 – 30	0	0	0	0	0
Digestive gland, 117TBT dissolved in phto.	9 – 15	0	4	0	0	0
	26 – 30	0	0	0	0	0
	30 – 34,5	0	0	0	0	0
117Sn (spk) RSD%	Time/min	MphT117(spk)	TBT117(spk)	DphT117(spk)	TphT117(spk)	iSn117(spk)
Gills, 117TBT is dissolved in water	26 – 30	0	0	0	0	0
Gills, 117TBT is dissolved in phto.	26 – 30	0	0	0	0	0
Digestive gland, 117TBT dissolved in phto.	9 – 15	0	5	0	0	0
	26 – 30	0	0	0	0	0
	30 – 34,5	0	0	0	0	0

3.3.2.B Detection limits of compounds

Detection limits were calculated with three replicates.

Specie	120 DL/ng·mL^{-1}	117 DL/ng·mL^{-1}
MMT	**0,003**	**0,007**
DMT	**0,005**	**0**
TMT	**0**	**0**
MBT	**0,01**	**0,02**
DBT	**0,01**	**0,03**
TBT	**0**	**0**
MphT	**0**	**0**
DphT	**0**	**0**
TphT	**0**	**0**
iSn	**4**	**4**