PTDC/SAU-ESA/100107/2008 HERA - Environmental Risk Assessment of a contaminated estuarine environment: A case study

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Aims

The main aim of this research project was to develop and apply an innovative methodology to evaluate the environmental risk, including ecologic and human health, of a contaminated sedimentary estuarine environment: the sado estuary, namely through food intake. A control population away from this estuary was used (in Vila Nova de Mil Fontes). An integrated approach was used including evaluation of difference lines of evidence:

i) epidemiology characterization of target population, according to the developed study design, allowing to evaluate the human contamination pathways and human health effects (chronic disease, reproductive health, health care) of the population of Carrasqueira (task 1);
ii) Characterization of metal contamination in the local farming foodstuff (in the vegetables more consumed by the local population, according to the food frequency questionnaire) as well as soil and water holes (task 2);
iii) Quality assessment of sediments and estuarine species (in the species more consumed by the local population, according to the food frequency questionnaire) (task 3)
iv) Characterization of human genotoxic of the estuarine sediments (task 4)
v) Integrated assessment of human health and ecological risk assessment of the estuarine sediment environment (task 5)
vi) Organization of two seminars, one for the local population and other more broader for local stakeholders and also scientific community and public in general, to disseminate the project results and methodologies used (task 6).

The project aimed to developed innovative lines of evidence and integrated methodologies, in a qualitative and quantitative way, for a weight of evidence assessment of human health and ecological risk. Based on project results, recommendations are proposed to minimize, manage and improve the environmental quality of the estuary. Future developments as a way forward are also highlighted.

Task 1
Epidemiological characterization of the target population of the village of Carrasqueira
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Abstract

Sado River estuary is located in the west coast of Portugal. Previous environmental studies identified industrial contamination, non-point anthropogenic sources and contamination coming from the river, all promoting accumulation of polluted sediments with known impacts on the ecological system. Surrounding human populations have intense economic fishery activities. Together with agriculture, estuary fishing products are available to local residents. Food usage previously characterized through ethnographic studies suggests exposure to estuarine products, farming products, and water in daily activities, as potential routes of contamination. It is well established that long term exposure to heavy metals are associated with renal and neurological diseases, most heavy metals are classified as carcinogenic and teratogenic.

Although considerable investigation has been done on environmental contamination assessment and its effects to ecological systems, in this particular geographical area no study relating the health effects due to exposure to heavy metals was ever conducted. In order to overcome this lack of knowledge, an integrated/multidisciplinary research was implemented so to evaluate the environmental risk, including ecologic and human health, of Sado estuary.

HERA project (Environmental Risk Assessment of a contaminated estuarine environment) is financed by the National Science Foundation (FCT- PTDC/SAU-ESA/100107/2008) and includes an epidemiological study aiming to characterize exposure pathways to estuarine products and its potential health effects.

A cross-sectional comparative study of residents in Carrasqueira, a small riverside village in the south channel of the Sado Estuary, and residents in a second different population (Vila Nova de Mil Fontes-VNMF) 200 Km off Carrasqueira, selected as the non-exposed population. VNMF sits near another river estuary with similar fishing and agricultural activities but no known industrial or other contamination exposures.

Simple random samples of individuals were selected in each study population from the National Health Service Lists. Questionnaire data collected at home (31 questions) intends to characterize: 1)Health effects: morbidity (diagnosed illnesses, medication), use of health services, reproductive history (pregnancies, abortions, congenital anomalies); 2) potential routes of exposure: socio-demographic, occupational (fishing and farming related occupations), leisure habits and hobbies (including recreational fishing), lifestyles (tobacco, alcohol); 3) Potential routes of human contamination from the estuary (including use of water, subsistence fishing and farming). Questionnaires were applied at home by trained interviewers by face to face interviews of selected individuals (June and July 2011) using computed assisted personal interview (CAPI). All participants were included after a written informed consent.

Statistical analysis was performed using descriptive analysis and associations were tested using Chi-squared test. The odds ratio of having an adverse outcome within Exposed and Non-Exposed groups was adjusted for possible confounders. Adjustment was performed using the non conditional logistic regression. Potential confounders were investigated and included if they changed crude OR estimate in at least 10% after adjustment by the Mantel-Haenszel method. It was considered a 5% significance level to reject the null hypothesis of the tests.

Data was collected from a total of 202 participants from all ages. Participation rates were 62.5% in Carrasqueira and 48.3% in VNMF. Of selected Carrasqueira participants 58.3% were male (population=55.3%) and 60% were 20-59 in age. In VNMF, 44.2% were male (population=48.4%) the majority in the 20-59 age group (58.3%). Professions like fishers and agricultures are more likely to have higher risks of exposure to estuary river contaminants (directly or indirectly) and were more common in Carrasqueira. Past and actual leisure activities with higher probability of exposure were also more frequent in the estuary exposed village of Carrasqueira. The participants of Carrasqueira reported a significant higher proportion of tasks inherent to their actual job, promoting direct (48.8%) and/or indirect (30.0%) exposure to contaminants ($p<0.001$ and $p=0.006$ respectively). There were no differences in the other routes of contamination ($p=0.445$). On the opposite VNMF participants were not exposed to any contaminant route. Similar results were found in the analysis of tasks in previous professions. Also the period of time that was spent in the fishers and agricultures activities in Carrasqueira was higher than in VNMF (about 90% of individuals had more than 8 years of exposure, $p<0.001$).

Overall analysis of health data, showed a higher proportion of respondents that declared to have a chronic disease, confirmed by a medical doctor, in Carrasqueira than in VNMF location. In the reproductive history, a higher proportion of Miscarriages (<20 Weeks), Fetal Deaths and pregnancies with abnormal outcomes was found in the exposed village of Carrasqueira. No significant differences were found in the rest of the health indicators.

Considering multivariate analysis, a higher odd of having chronic heavy metal related morbidity (OR= 1.91; CI95%: 1.01-3.64), and congenital anomalies (OR= 1.53; CI95%: 0.47- 4.92) were observed in Carrasqueira. Only age and years living in the local was retained as confounder in logistic regression, resulting in a 2,1 higher risk of having at least one of the diseases in Carrasqueira compared to VNMF (CI95%: 1.02-4.69).

Introduction

The Sado estuary is located in the West Coast of Portugal, with an area of approximately 240 km2, and consists of a large basin of great socio-economical importance. Although a large part of the estuary is classified as a natural reserve, the area is very important for tourism, local fisheries, aquaculture, maritime transport and upstream agriculture. The estuary is generally threatened by many sources of anthropogenic pressure: urban pollution (from the city of Setúbal), industrial pollution (from heavy-industry belt that includes chemical plants, a thermoelectrical unit, shipyards, ore deployment facilities and others), and from runoffs from the agriculture grounds (Costa et al. 2012).

The presence of these potential pollution sources has originated a moderately contaminated estuary, particularly in areas near industrial areas and the lower estuary were levels of concern for many contaminants, both organic and inorganic, with adverse toxicological consequences to biota have been found in recent studies (Neuparth et al. 2005; Lobo et al. 2008; Caeiro et al. 2009; Ribeiro et al. 2009).

Contamination of estuarine waters and sediments with xenobiotics metals (e.g. Cd and Ni), pesticides, PAHs and other persistent organic pollutants, known to be mutagenic or carcinogenic to humans has been widely reported (Chen and White 2004). Some metals, such as copper (Cu) and zinc (Zn), are essential for life and play important roles in the functioning of critical enzyme systems. Other metals and organic compounds, like lead (Pb), are nonessential with no useful role in human physiology and are toxic even at trace levels of exposure (Serafim et al. 2012).

These compounds can be accumulated in the edible parts of fish, mollusks and other aquatic species that enter the human food chain (van der Oost, Beyer, and Vermeulen 2003). In fact consumption of contaminated marine food and drinking water and beverages has been reported as an important route of human exposure (Chien et al. 2002; Forsyth and Jay 1997.; Azenha and Vasconcelos 2002; Antizar-Ladislao 2008). Food consumption has been identified as the major pathway of human exposure to xenobiotics, namely metals, making the assessment risks of these elements to human via dietary intake important (Urban et al. 2009; Wang et al. 2005; Zheng et al. 2007).

The mere presence of contaminants in and of itself does not pose a human direct health risk, thus a complete exposure pathway needs to be identified and characterized for justifying that individuals are potentially at risk. Characterization of specific leisure and occupational activities of populations potentially exposed to estuarine contaminants is crucial to accurate health risk assessment. Population questionnaire surveys are the main approach to describe exposure pathways including those related with leisure and occupational activities, and with consumption habits (Urban et al. 2009; Moe et al. 2001; Roseman 1998).

The small fishermen community of the Carrasqueira Village is located on the south margin of the Sado Estuary and has an estimated population of approximately 350 residents (Martins and Souto 2000). Carrasqueira fishermen trawl the area for estuarine species that are associated with the sedimentary environment and are important natural resources for human food intake like: the flatfish Solea spp., the cuttlefish Sepia officianalis, the crab Carcinus maenas and the cockle Cerastoderma edule. Food usage among Carrasqueira residents has been previously characterized through ethnographic studies which suggest exposure to estuarine products, farming products, and water in daily activities (Martins and Souto 2000).

Considerable investigation has been done on environmental contamination assessment and its effects to ecological systems, in this particular geographical area no study relating the health effects due to exposure to heavy metals was ever conducted. In order to overcome this lack of knowledge, the main goal of the present work is to characterize human exposure pathways of the Carrasqueira village population in the contaminated estuarine environment of the Sado river. This study is part of HERA project (Environmental Risk Assessment of a contaminated estuarine environment) financed by the National Science Foundation (FCT- PTDC/SAU-ESA/100107/2008).

Materials and Methods

Study design

A cross-sectional comparative study of residents in Carrasqueira, a small riverside village in the south channel of the Sado Estuary, and residents in a second different population (Vila Nova de Mil Fontes-VNMF) 200 Km off Carrasqueira, selected as the non-exposed population. VNMF sits near another river estuary with similar fishing and agricultural activities but no known industrial or other contamination exposures. Survey data was collected through an epidemiological structured questionnaire (with close ended response choices) in both populations.

Recruitment process

Simple random samples of individuals were selected in each study population from the National Health Service Lists. A total of 140 individuals were selected in Carrasqueira and 219 in VNMF (Figure 1). This study has an exploratory nature, so sample size calculation depended largely on available resources and aimed at contributing identification and characterization of contamination routes of exposure (Matias Dias, 2011).

One week before the scheduled day for data collection, a letter was mailed to each selected registered user, explaining the purpose of the study and asking for participation. Leaflets with the research outcomes were distributed and posted to the local community to increase awareness and participation.

Questionnaire

A first draft questionnaire was prepared and discussed with a groups of experts working in the project from different expertise's (namely from human and ecology toxicology, environmental contamination, public health, nutrition and epidemiology). The questionnaire was designed specifically for this study, and included questions already used in others questionnaires namely the National Health Survey (INE 2007).

This draft was later on submitted to a pilot test applied to 12 individuals (in VNMF). This pilot test was conducted to assess the overall quality of the draft questionnaire, which was especially designed for questionnaire clarity, comprehensiveness and acceptability, as proposed by Rea and Parker (1997). This way, the necessary adjustments to poorly understood questions were identified.

Questionnaire included 57 questions and intends to characterize: 1)Health effects: morbidity (diagnosed illnesses, medication), use of health services, reproductive history (pregnancies, abortions, congenital anomalies); 2) potential routes of exposure: socio-demographic, occupational (fishing and farming related occupations), leisure habits and hobbies (including recreational fishing), lifestyles (tobacco, alcohol); 3) Potential routes of human contamination from the estuary (including use of water, subsistence fishing and farming) (Table 1).

Table 1. Summary description of dimensions and variables included in the epidemiological questionnaire.

Dimension	Variables	Comments
Socio-demografic	Sex Age Educational level Marital status Labour status Residence in the location (Nr. of years)	
Occupation	Main profession (or last applied for unemployed or retired individuals) Duration of main profession (years) Average number of weekly hours spent in the main profession Tasks inherent to main profession Occupational history (Past professions and respective duration and tasks)	
Leisure and hobbies	Fishing Agriculture Painting/ Joinery/Carpentry Domestic activities (include gardening) Other	Hobbies and leisure activities were selected considering the potential exposure effect to contaminants
Food intake	Individual food frequency consumption for the 12 months previous to the interview was collected using a food frequency questionnaire (FFQ) with the 32 food/food items most consumed in the population of Carrasqueira with seven possible responses ranging from never to two or three per day (Willett 1998).	The choice of relevant species items to be included in the FFQ was based on exploratory local interviews, in order to determine which foods were most consumed and to avoid the loss of information.
Water use and consumption of farming products	Farming Products Consumption Consumption of water from well and hole Utilization of water from well and hole to cook Utilization of water in farm from the well, hole and channel river	
Health effects	Health status Reproductive history Health determinants (including tobacco and alcohol) Use of health care services	

Data Collection

Questionnaires were applied at home by trained interviewers by face to face interviews of selected individuals during June and July 2011 using Computed Assisted Personal Interview (CAPI).

Interviewers were health professionals and had a one day training course. The training team included core team members of the HERA project (2 from the epidemiology area and 2 from the nutritional area). The methodology used included the theoretical description of the questionnaire content (including occupational exposure and food frequency parts) and details of the CAPI application. For instruction purposes a training manual containing project and field work goals, interviewers' behavior component and specific formulation of the questions was prepared and provided to the interviewer's team.

Ethical procedures

Study protocol and questionnaire were submitted and approved by the Ethics Commission of the Portuguese National Health Institute and by the National Data Protection Commission. Participants were included in the study only after agreement and written informed consent. All participant information was coded to maintain confidentiality.

Statistical analysis

Statistical analysis was performed using descriptive analysis and associations were tested using Chi-squared test and Fisher Exact Test. The odds ratio of having an adverse outcome within Exposed and Non-Exposed groups was adjusted for possible confounders. Adjustment was performed using the non conditional logistic regression. Potential confounders were investigated and included if they changed crude OR estimate in at least 10% after adjustment by the Mantel-Haenszel method. It was considered a 5% significance level to reject the null hypothesis of the tests.

Results

Sociodemographic characterization

Data was collected from a total of 202 participants from all ages. Participation rates were 81.6% in Carrasqueira and 69.0% in VNMF (Figure 1). Of selected Carrasqueira participants 57.8% were male (population in the=55.3%) and 44.2% were male in VNMF (population=48.4%) (Figure 1 and Table 2.).

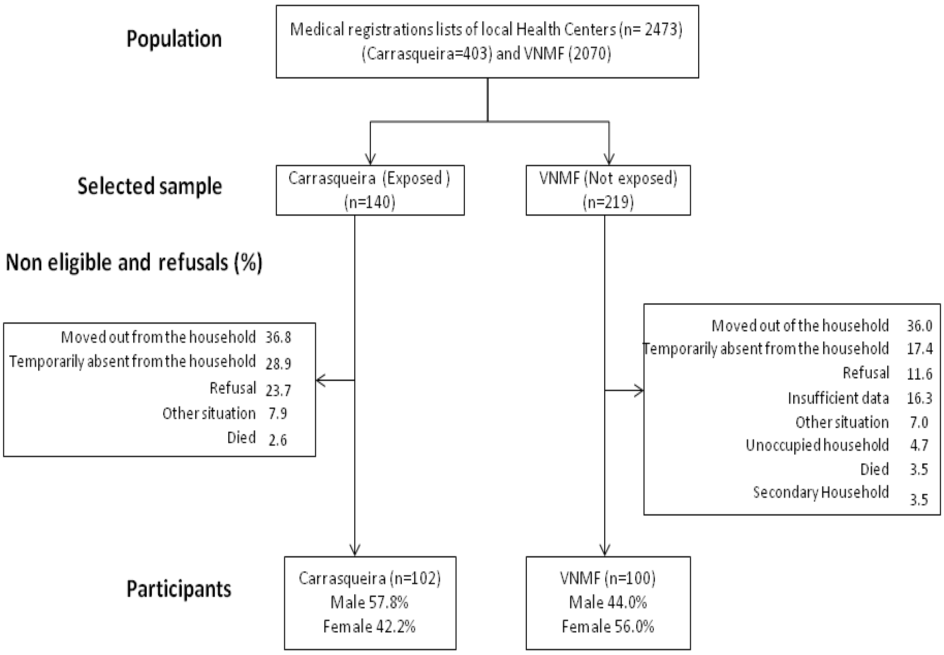

Figure 1. Study Implementation

No significant differences were observed in the age distribution between both samples but there were significant differences in the sex, educational level and occupation (Table 2.). Respondents in VNMF had a higher education level than the Carrasqueira population. The Carrasqueira sample revealed a higher proportion of self employed respondents (38.8%), while higher proportions of employees (34.0%), retired (25.5%) and unemployed or student (11.7%) were observed in VNMF.

Table 2. Socio-Demographic characteristics of studied populations

	Location Carrasqueira (exposed)		VNMF (unexposed)		p-value
	n	%	n	%	
Sex	**102**		**100**		0.049
Male		57.8		44.0	
Female		42.2		56.0	
Age group (years)	**102**		**100**		0.267
≤17		14.7		14.0	
18- 44		29.4		26.0	
45-64		40.2		33.0	
65- 74		9.8		12.0	
≥75		5.9		15.0	
Education level	**99**		**94**		**<0.001**
No formal education		14.1		11.7	
Primary and lower secondary education		69.7		42.6	

	Location Carrasqueira (exposed)		VNMF (unexposed)		p-value
	n	%	n	%	
Upper secondary education		10.1		21.3	
Post - secondary or Higher education		6.1		24.5	
Labour status	**98**		**94**		**<0.001**
Self employed		38.8		11.7	
Employee		23.5		34.0	
Fulfilling domestic tasks		5.1		5.3	
Retired		14.3		25.5	
Unemployed		4.1		11.7	
Student		14.3		11.7	

n-valid cases; %-Proportion of respondents

Occupational Exposure

In what regards the occupational exposure, participants were asked about their main profession or the last before retiring/ unemployment. Table 3 shows the distribution of participants by the main classification groups of the Portuguese classification of professions (CPP), according to National Classification of Occupations (National Institute of Statistics, 2011) and their comparison between the two study groups.

The results revealed statistical significant differences between both populations. The majority of the Carrasqueira participants reported an occupation included in the "farmers and skilled workers of agriculture and fisheries" group, while in VNMF the most referred professions were included in the "technical Professions and professionals of intermediate level, ...". In detail (2 digit CPP codification), it turns out that 46.3% of the participants of the exposed population were "skilled workers in the forest, fishing and hunting" while in VNMF only 1.2% of the participants reported this category of profession. For study purposes professions included in this group, such as fishermen and farmers, are considered more likely to have greater exposure to contaminants from the river estuary (directly or indirectly). Not only the exposed population revealed a higher proportion of participants in this group of occupation, but also the period of time that was spent in the fishery and agriculture activities in Carrasqueira was higher than in VNMF (about 90% of individuals had more than 8 years of exposure, $p<0.001$) (Table 4).

Professions like fishers and agricultures are more likely to have higher risks of exposure to estuary river contaminants (directly or indirectly) and were more common in Carrasqueira.

Table 3. Occupational exposure: distribution of both participants according to main groups of the Portuguese classification of occupations.

Exposure Factors	**Location** *Carrasqueira (exposed)* *n*	*%*	*VNMF (unexposed)* *n*	*%*	***p-value***
Profession	80		84		<0.001
Managers, Professionals and Armed forces occupations		8.8		10.4	
Technicians and associate professionals		12.5		32.9	
Skilled agricultural, forestry and fishery workers; craft and related trades workers; plant and machine operators, and assemblers		67.5		44.5	
Skilled agricultural and animal producers workers		7.5		6.1	
Skilled workers in the forest, fishing and hunting		46.3		1.2	
Elementary occupations		11.3		12.2	

n-valid cases; %-Proportion of respondents

Table 4. Occupational exposure: distribution of both participants according to main groups of the PCO and correspondent working period.

		Location ***Carrasqueira (exposed)*** n	%	***VNMF (unexposed)*** n	%	p-value
						<0.001
Agriculture and fishing		**80**	**67.5**	**84**	**22.6**	
	0-7 yrs	54	9.3	19	15.8	
	8-20 yrs	54	20.4	19	15.8	
	21-35 yrs	54	25.9	19	21.1	
	36 yrs	54	44.4	19	47.4	
Other profissions		**80**	**32.5**	**84**	**77.4**	
	0-7 yrs	26	42.3	63	34.9	
	8-20 yrs	26	19.2	63	38.1	
	21-35 yrs s	26	26.9	63	20.6	
	36 yrs	26	11.5	63	6.3	

n-valid cases; %-Proportion of respondents

Considering the specific tasks inherent to the main occupation (Table 5), participants of Carrasqueira reported a significantly higher proportion of tasks, promoting direct (48.8%) and/or indirect (30.0%) exposure to contaminants ($p<0.001$ and $p=0.004$ respectively). There were no differences in the other routes of contamination ($p=0.445$). On the other hand, VNMF participants were not exposed to any contaminant route.

Table 5. Occupational exposure: distribution of both participants by route of exposure (direct, indirect, another via or without relevant exhibition) related to the main occupation.

	Location				
	Carrasqueira (exposed)		***VNMF (unexposed)***		p-value
	n	%	n	%	
Direct exposure	80	48.8	84	1.2	<0.001
Indirect exposure	80	30.0	84	11.9	0.004
Other routes of contamination	80	31.3	84	36.9	0.445
No relevant exposure	80	17.5	84	53.6	<0.001

n-valid cases; %-Proportion of respondents

Considering the potential exposure routes through hobbies or leisure activities (Table 6), fishing and agriculture as past or actual leisure activities or hobbies were the main factors of exposure that showed a statistical significant difference between the two samples and were more frequent in the exposed population (41.4 % and 59.6%, respectively, in Carrasqueira compared to 18.1% and 19.1% in VNMF).

Table 6. Exposure through hobbies or leisure activities (past or actual): comparison of both participants.

Past or Actual Leisure Activities	**Location** ***Carrasqueira (exposed)***		***VNMF (unexposed)***		p-value
	n	%	n	%	
Fishing	99	41.4	94	18.1	0.001
Agriculture	99	59.6	94	19.1	<0.001
Painting/ Joinery/Carpentry	102	6.9	100	5.0	0.768
Domestic activities (include gardening)	102	26.5	100	14.0	0.052
Others activities	102	18.6	100	25.0	0.234

n-valid cases; %-Proportion of respondents

Water and seafood consumption

Water consumption differed significantly between the two samples (Table 7). Consumption of farming products, as well as water use for drinking, cooking and farming coming from water wells, water holes or river channels were higher in Carrasqueira than in VNMF (99%, 62.6%, 81.4%, 94.9%, respectively, in Carrasqueira compared to 88%, 1%, 2% and 80% in VNMF). Consumption and use of water for food preparation is very small in VNMF, compared to higher values in Carrasqueira, and so is an important source of exposure to be considered in the latter population.

Table 7. Food and water use

	Location				
	Carrasqueira (exposed)		***VNMF (unexposed)***		***p-value***
	n	*%*	*n*	*%*	
Fish consumption from estuary or fish market	102	98.0	100	77.0	<0.001
Farming Products Consumption from Own/familiar/friends farms	102	99.0	100	88.0	0.001
Consumption of water from well and hole	99	62.6	100	1.0	<0.001
Utilization of water from well and hole to cook	102	81.4	99	2.0	<0.001
Utilization of water in farm from the well, hole and channel river	102	94.9	99	80.0	0.004

n-valid cases; %-Proportion of respondents

The food frequency questionnaire (Table 8) showed significant higher consumption of seafood in Carrasqueira namely cuttlefish (p=0.007, 23.5 % reported to be consumed often in Carrasqueira compared to 9.0% in VNMF), sole (p<0.001, 22.5 % consumed often in Carrasqueira compared to 4% in VNMF) and clam (*R. decussatus*) (p=0.004, 18.6 % consumed often in Carrasqueira compared to 5% in VNMF). Mackerel was the unique fish that showed significant higher frequency of consumption in VNMF (p=0.003, 19.6% often consumed in Carrasqueira compared to 39.0% in VNMF).

Table 8. Frequency of food intake in both locations: Seafood

	Carrasqueira (exposed) n	%	*VNMF (unexposed)* n	%	*p-value*
	120		100		
Cuttlefish					0.007
Occasionally	78	76.5	91	91.0	
Often	24	23.5	9	9.0	
Octupus					0.535
Occasionally	98	96.1	94	94.0	
Often	4	3.9	6	6.0	
Sole					<0.001
Occasionally	79	77.5	96	96.0	
Often	23	22.5	4	4.0	
Gilt head (*Sparus aurata*)					0.569
Occasionally	77	81.9	85	85.0	
Often	17	18.1	15	15.0	
Seebass					0.141
Occasionally	85	83.3	91	91.0	
Often	17	16.7	9	9.0	
Clam (*Ruditapes decussatus*)					0.004
Grooved carpet shell clam					
Occasionally	81	79.4	98	95.0	
Often	19	18.6	5	5.0	
Very Frequently	2	2.0	0	0.0	
Clam (*Venerupis pullastra*)					0.128
Pullet carpet shell clam					
Occasionally	92	90.2	97	97.0	
Often	9	8.8	3	3.0	
Stakefish					0.333
Occasionally	95	93.1	89	89.0	
Often	7	6.9	11	11.0.	
Sardine					0.521
Occasionally	78	76.5	72	72.0	
Often	24	23.5	28	28.0	
Mackerel					0.003
Occasionally	82	80.4	61	61.0	
Often	20	19.6	39	39.0	

n-valid cases; %-Proportion of respondents

Regarding the origin of the seafood consumed (Table 9), we found significant higher estuarine fish frequency consumption in Carrasqueira (70%) compared to 1% in VNMF (p<0.001). In contrast, consumption of fish from sea was significantly more common in VNMF (11% in Carrasqueira compared to 25% in VNMF, p<0.001) as well as fish from local fish market (1%

in Carrasqueira compared to 49% in VNMF, p<0.001). No significant differences were found concerning fish bought in stores.

Table 9. Frequency of consumption of seafood regarding reported sources

	Location				*p-value*
	Carrasqueira (exposed)		***VNMF (unexposed)***		
	n	%	n	%	
	120		100		
Estuary origin					**<0.001**
Usually/nearly always	70	68.6	1	1.0	
Sometimes/scarcely	30	29.4	5	5.0	
Never	2	2.0	94	94.0	
Sea origin					**<0.001**
Usually/nearly always	11	10.8	25	25.0	
Sometimes/scarcely	37	36.3	13	13.0	
Never	54	52.9	62	62.0	
Fish market origin					**<0.001**
Usually/nearly always	1	1.0	49	49.0	
Sometimes/scarcely	3	2.9	23	23.0	
Never	98	96.1	26	26.0	
Store origin					0.423
Usually/nearly always	5	4.9	7	7.0	
Sometimes/scarcely	16	15.7	10	10.0	
Never	81	79.4	83	83.0	

n-valid cases; %-Proportion of respondents

Farming and consumption of farming products

Table 10 shows the habits of farming and consumption of farming products produced by family and friends. The differences found between localities are significant in relation to the question of habit of farming, but the consumption of products produced by family and friends don't show differences statistically significant.

The participants from both locations declared the consumption of the products from their farming.

Table 10. Farming and consumption of farming products

	Location				
	Carrasqueira (exposed)		*VNMF (unexposed)*		*p-value*
	n	%	n	%	
Farming	102	54.90	100	20.00	***<0.001***
Consumption of farming products produced by family and friends	102	82.35	100	83.00	0.903

n-valid cases; %-Proportion of respondents

Considering the products from farming, there are no differences statistically significant between the two locations for the majority of the analyzed groups, only the group of "Cucumber, zucchini and eggplant" shows significant differences (table 11).

Table 11. Frequency of consumption of farming products

	Location				
	Carrasqueira (exposed)		*VNMF (unexposed)*		*p-value*
	n	%	n	%	
Fruits	56	67,9	20	65,0	0,815
Vegetables	56	42,9	20	45,0	0,868
Pumpkin and Carrots	56	26,8	20	40,0	0,269
Lettuce and Watercress	56	32,1	20	25,0	0,551
Tubers	56	89,3	20	75,0	0,145
Green Vegetables	56	71,4	20	55,0	0,179
Aromatic Herbs	56	37,5	20	30,0	0,547
Cucumber, Zucchini and Eggplant	56	60,7	20	20,0	***0,002***
Peppers and Tomatoes	56	83,9	20	75,0	0,502

n-valid cases; %-Proportion of respondents

Health Status

Overall analysis of health data, showed a higher proportion of respondents that declared to have a chronic disease, confirmed by a medical doctor, in Carrasqueira than in VNMF location (Table 10). A higher prevalence of Malignant Tumor, Renal Failure and Stroke was observed in VNMF. Prevalence of Kidney Diseases was significantly higher in Carrasqueira, while Malignant Tumors were significantly higher in VNMF.

Table 12. Proportion of chronic diseases in participants by location

Chronic diseases	Location				
	Carrasqueira (exposed)		*VNMF (unexposed)*		*p-value*
	n	*%*	*n*	*%*	
Osteoarticular Disease	99	39.0	100	28.0	0.089
Hypertension	100	32.0	100	25.0	0.273
Kidney Diseases	99	14.0	100	3.0	***0.005***
Liver Disease	100	9.0	99	5.0	0.276
Ischaemic Heart Disease	102	9.0	100	3.0	0.080
Neurological Disease	101	9.0	100	3.0	0.134
Asthma	102	7.8	100	3.0	0.129
Skin Diseases	102	7.0	100	4.0	0.370
Diabetes	100	6.0	100	5.0	0.756
Stroke	102	2.0	100	4.0	0.393
Renal Failure	100	1.0	100	2.0	0.561
Malignant Tumor	102	0.0	98	6.0	***0.011***

n-valid cases; %-Proportion of respondents

Considering the diseases whose etiology was related to exposure to heavy metals and other contaminants existing in the Sado estuary, particularly diseases of kidneys, liver disease, neurological diseases, skin disease, renal failure and malignant tumor, was created a new variable that measures the existence of at least one of these diseases. The existence of co-morbidity (the presence of two or more diseases considered relevant) was also evaluated.

The results obtained are shown in Table 11. As observed for each chronic disease individually, the participants of Carrasqueira showed a higher proportion of diseases related to exposure in study. The difference is significant, but borderline. The participants of Carrasqueira also showed a higher proportion of disease considering the co-morbidities, however these difference was not statistically significant.

Table 13. Proportion of disease in participants by location

Disease	Location				
	Carrasqueira (exposed)		*VNMF (unexposed)*		*p-value*
	n	*%*	*n*	*%*	
At least 1 disease	102	32.4	100	20.0	***0.046***
Number of Chronic diseases	102		100		
1 disease		16.7		14.0	***0.069***
2 diseases		11.8		6.0	
3 or more diseases		3.9		0.0	

n-valid cases; %-Proportion of respondents

Reproductive history

No significant differences were observed in variables of reproductive history (Table 11). However, respondents in the exposed village of Carrasqueira declared a higher proportion of Miscarriages (<20 Weeks), Fetal Death and pregnancies with Physical Malformation/ Mental Disease/ Metabolic Malfunctions of organs/ Genetic Disease/Syndrome as outcomes.

Table 14. Reproductive history of participants by location

Reproductive indicators		Location				
		Carrasqueira (exposed)		***VNMF (unexposed)***		***p-value***
	Nr.of pregnancies	***n***	***%***	***n***	***%***	
Miscarriage (<20 Weeks)/ Fetal Death		**74**		**69**		
	0	61	82.4	52	75.4	0.124
	1	6	8.1	14	20.3	
	2	6	8.1	3	4.3	
	5	1	1.4	0	0.0	
Physical Malformation/ Menthal Disease/ Metabolic Malfunctions of organs/ Genetic Disease/Syndrome		**74**		**69**		
	0	66	89.2	63	92.6	0.268
	1	5	6.8	1	1.5	
	2	3	4.1	4	5.9	

n-valid cases; %-Proportion of respondents

Health determinants and use of health care services

No significant differences were found in the rest of the studied health indicators (including medical appointment, hospitalizations, tobacco and alcohol consumption).

In the use of medical care during the previous three months, the mean of medical appointment was one in both locations (p >0.05), being the main reason referenced "because they felt sick". Hospitalizations in the previous 12 months were declared by 10% of both samples with no statistical significant differences. The main reason of hospitalization was programed surgery with a prevalence equal or superior a 50% in Carrasqueira and in VNMF, but was not found differences statistically significant in the reasons for hospitalization reported by the participant.

The majority of participants were nonsmokers, however the prevalence of daily tobacco consumption was higher in Carrasqueira (26.45) than in VNMF (21.1%). Also the majority of participants referred that alcohol consumption was made occasionally (never up to 3 times per month) and like the consumption of tobacco the differences between localities were not significant.

Multivariate analysis

Considering multivariate analysis, a higher *odd* of having chronic heavy metal related morbidity (OR= 1.91; CI95%: 1.01-3.64), and congenital anomalies (OR= 1.53; CI95%: 0.47- 4.92) were observed in Carrasqueira. Only age and years living in the local was retained as confounder in logistic regression, resulting in a 2,1 higher risk of having at least one of the diseases in Carrasqueira compared to VNMF (CI95%: 1.02-4.69).

Table 15. Estimates of the Odds Ratio of having at least a disease associated with exposure to heavy metals, pregnancy with adverse outcome

At least one…	**Comparison between Carrasqueira and VNMF**			
	OR_{Crude}	IC95	$OR_{Adjusted*}$	95% IC
Chronic heavy metal related disease	1,91	(1,00;3,64)	2,10	(1,02;4,30)
Pregnancy with Miscarriages (<20 Weeks) or Fetal Deaths	0,65	(0,29;1,47)	---	---
Pregnancy with Abnormal outcomes	1,53	(0,47;4,92)	---	---

* Adjusted OR for age groups and years of residence in the locality

Discussion

Exposure pathways

This study aimed to identify and characterize exposure pathways in a contaminated estuary environment. The evaluation of exposure pathways is essential to identify specific exposure situations, the potential population at risk and in what conditions this exposure occurs (ATSDR 2005).

Results show that, compared to VNMF, the exposed population of Carrasqueira had a higher probability of exposure to the river estuary due to occupational characteristics, leisure activities, and food and water consumption behaviors. Differences in occupational exposure were evident both in terms of the tasks and period of exposure. Participants from Carrasqueira revealed an increased frequency of tasks that included direct or indirect exposure to the estuary (mainly through fishing activities), and these activities were reported to have been performed for long periods of time.

It was also demonstrated that consumption of water placed the population of Carrasqueira in higher exposure to contaminants from the Sado estuary land area. Preliminary results on the frequency of consumption of vegetables grown in the surrounding lands (Vaz-Fernandes et al. 2011), revealed that the frequency was significantly higher in Carrasqueira than in VNMF.

Regarding estuarine species frequency of consumption indicates that the exposed population, Carrasqueira, had a higher probability of exposure to Sado estuary due to higher frequency consumption of estuarine species like cuttlefish, sole and clams. In fact, these species have an important weight on the dietary patterns of the Carrasqueira population compared to the comparison population. These species are benthic and are collected near sediment areas that are impacted by different stressors and have the potential to cause adverse effects to the biota (Costa et al. 2008; Costa et al. 2009). Also, recent studies (within this research project) shown that these species bioaccumulated xenobiotics and that the estuarine environment have caused damage and responses, evaluated through biomarkers, with possible consequences to humans that exploit them (Carreira et al submitted and Costa et al. submitted), it is possible that the exploitations and consumption of these species may be responsible for transferring estuarine toxicants to populations.

Intake of a single heavy metal through consumption of either contaminated vegetables or fish only, were found to indicate relative absence of health risks. However, consumption of both vegetables and fish could lead to potential health risks especially for children (Wang et al. 2005). Adding to these the combination of moderate levels of contamination with toxicant mixtures, existent in the Sado estuary (Carreira et al submitted) enhance the potential human health risk.

Study design

The characterization of human exposure pathways in a contaminated Portuguese estuarine environment was evaluated. We used a cross-sectional comparative design where survey data from a sample of residents in an area exposed to a contaminated estuarine environment (Carrasqueira) was compared with data from a sample of residents in an non-contaminated estuarine environment (Vila Nova de Mil Fontes - VNMF). This epidemiological design has also been used by other authors to assess human exposure to estuarine environments outside Portugal. Swinker *et al.* (2001) conducted a cross sectional comparative study to access the denominated estuary-associated syndrome due to occupational exposure. Lepesteur *et al.* (2006) conducted a social survey to study recreational water environment exposure. Harris *et al.* (2009) estimated fish and shellfish consumption by fishermen using a food consumption survey aiming to investigate the risk of eating contaminated fish in an occupational setting.

The present study design has some limitations that should be accounted for, namely its cross sectional nature that only allows selecting and obtaining information on individuals that were alive and living in the location at the time of the survey. If there are differences in exposure factors or exposure intensity between both populations, and if those factors are associated with lower survivals one of the study populations, comparison of exposed and unexposed populations may be biased (Roseman 1998; Gibb 2002).

Another point for discussion is the potential bias due to non-response (Stang 2008). According to the study implementation results refusal rates were low and similar in the two samples (7.2% in Carrasqueira vs 6.9% in VNMF), making it unlikely that bias has occurred (Matias Dias, 2011).

Information on exposure was obtained through direct interview collecting self reported information or proxy information in the case of children under 14 years of age, but recall bias may influence retrospective information collected using this approach. However, the survey questionnaire was applied using the same protocol in both populations thus minimizing differential bias. Data values for “don’t remember” were also similar in both groups (Matias Dias, 2011). Eventual effects of under or over reporting of exposure due to recall bias could only be assessed by validation of self-reports which is very difficult concerning occupational and leisure routes of exposure such as the ones that have been studied.

Another important limitation is on data collected for food frequency of consumption. It would have been better to collect information on the intake and the quantity consumed on each occasion, preferably in a recent past or with direct observation, that would permit to infer on contaminants in each food item. Nevertheless some authors state that it may be advantageous to sacrifice precise intake measurements obtainable on one or a few days in exchange for more crude information relating to an extended period of time (Willett, 1998).

Comparison group

Evidence on contamination routes leading to human exposure was assessed and enhanced through comparison of the exposed population with a similar population, living near an estuarine environment, but with no evidence of environmental contamination (VNMF). This contrast is often used for etiological purposes in case-control or cohort studies (Rothman, Greenland, and Lash 2008) and specifically in environmental risk assessment evaluations (Roseman 1998). In the present study however, the objective was not etiological but to identify and assess eventual exposure routes for contaminants from the Sado estuary to humans, including occupational, leisure and food related routes of exposure. Also, the future development of the study is to assess health effects due to exposure and the existence of a comparison group will robust the analysis and respective results.

The choice of VNMF as comparison group was due to the fact that this is also a population resident next to another river estuary with similar fishing and agriculture activities. The results contradict initial assumptions, since the VNMF population revealed habits and behaviors very divergent of the exposed population: less occupational and leisure exposure, with a lower consumption of fish/agricultural products and use of the water from hole/channel.

Differences observed between the two populations suggest that the potential routes for contamination transfer are more frequent in Carrasqueira, putting added risk to existing estuarine contamination differences.

The study shows that demographic characteristics (gender and age), are similar in both populations, except for educational level. On the other hand occupational factors associated with exposure were different between the two populations. Comparison between both population lead to evidence that the population exposed to the contaminated estuarine environment has different and more frequent potential routes of exposure that the other population living near a non-contaminated estuary.

Taking in consideration the points discussed above, the population of VNMF was a good option, as it differs from the Carrasqueira only on exposure pathways to contaminants of the estuary, and the other factors that eventually could be linked to potential exposure/health effects were equally distributed in both populations. Further analysis of the impact of this difference will have to include biological markers of exposure.

Conclusions

In Portugal, HERA project is one of the first environmental investigations to include an descriptive observational epidemiological study to assess possible routes of exposure and risks of health effects due to exposure to Sado contaminated estuary and products.

Planning and implementation of epidemiological studies on exposure, contamination routes and health effects of estuarine pollution should involve local Public Health professionals since early stages of planning and study design. Data collection and field work had no major drawbacks with a good response rate in the exposed population but lower in the comparison population. With this exploratory study we conclude that this approach is feasible and allows the characterization of exposure pathways in a contaminated estuary environment.

The study revealed that the population (living nearby and commercially and food depended of an estuarine contaminated area) had several exposure routes to contaminants through occupation, hobbies and leisure activities, and water and seafood consumption, and that this was statistically different from the unexposed population, which revealed habits and behaviors that do not provide significant exposure to the uncontaminated estuary. So, the results obtained showed that VNMF population was a good selection since they differ from Carrasqueira in the exposure indicators. Overall results indicate fragile but possible health effects of exposure to Sado estuary.

Overall results indicate fragile but possible health effects of exposure to Sado estuary. Nevertheless, these results should be analyzed carefully since the outcomes in study are not specific and are based in small samples. Further studies should use bioindicators of exposure and outcome.

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Task 2

Characterization of the contamination of local farming food

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Aims

The previous epidemiological study (task 1) confirmed several human exposure routes to Sado estuary contaminants namely trough occupation, water and food consumption. The aim of this task was to evaluate the contamination and mineral profile from local farming as an important food intake of two estuarine populations: the Carrasqueira community and Vila Nova de Mil Fontes - VNMF (used in this project as control population). To determinate the contamination of metals from local farming products, samples of vegetables, soils and water holes were collected.

Methods

The Food Frequency Questionnaire launched in 2011 (see task 1) allowed to select the vegetables more consumed by the local population. Due to budget constraints only the four more consumed were selected: tomato, lettuce, cabbage and potatoes. To determinate the contamination of metals from local farming products, samples of those vegetables, soils and water holes were collected. Water holes analysis were added since in accordance with the questionnaire of Task 1, the population of Carrasqueira drink and use this water for cooking and agriculture.

For the implementation of the field work sampling, 3 places (local farming) in Carrasqueira and 3 places (local farming) in Vila Nova de Mil Fontes were selected (see figures 1 and 2). It was implemented a convenience sample strategy (participants from Carrasqueira agreed on the sampling from their farms (3 places), and participants from Vila Nova de Mil Fontes selling their own vegetables at local marked and produced them in their local farm in Vila Nova de Mil Fontes area (3 places). Four visits were conducted to Carrasqueira and two visits to VNMF, because early results from the evaluation of contamination of lettuces, soil and water shown the need to repeat sampling and, therefore, the respective analyzes. Thus, soil analysis were carried out at different depths and roots and leaves of lettuce were collected also. Also because of the need to collect species available in different times of the year there was the need to do the sampling more than once.

[1] In collaboration with the researchers Paula Vaz Fernandes and Ana Paula Martinho from Universidade Aberta

Figure 1 – Local farms used in the task 2 in Carrasqueira. Image from google.

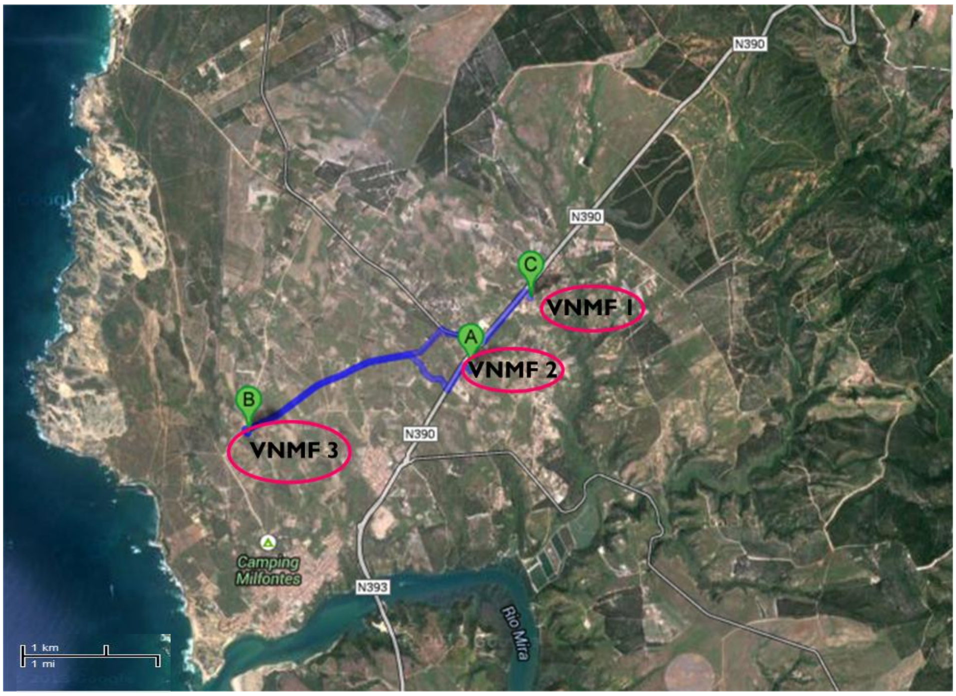

Figure 2 – Local farms used in the task 2 in Vila Nova de Mil Fontes. Image from google.

Table 1 presents the quantity of vegetables and water and soil samples that were analyzed in this task. The quantities were also related with available products in the farms at the time. Products were storage and refrigerated until laboratory procedure.

Table 1. Products and quantities analyzed in the task 2.

Products analyzed
20 potatoes
5 cabbages
5 lettuces
20 tomatoes
Water hole(1 L)
Soil (compost sample of about 500 gr)

Edible parts of the samples were separated, crushed, grinded and frozen at -20ºC. Afterwards samples were freeze–dried for a minimum of 48 hours. The dried samples were stored in a chamber with controlled humidity and temperature until analysis. Then they were analysed by ICP-MS and ICP-OES preceded by assisted microwave digestion to determine inorganic contents. Composition in chromium, manganese, nickel, copper, zinc, arsenic, selenium, cadmium, lead, were evaluated. In the case of the water hole samples, nitrate and nitrites were also analyse to outwit possible organic contamination. Due to INSA laboratory constraints and standardize procedures it was not possible to measure pesticides on vegetables, soils and water samples. Organic matter was also measured in the soils, by organic carbon loss-on-ignition at 500 ± 50 ºC. For the risk assessment of the foodstuff the methodology explained in figure 3 was used, based on: i) the quantification of each metal concentration in the vegetables; ii) the frequency of consumption of the vegetables according to the questionnaire developed in task 1; iii) the use of a worst scenario based on estimation of food daily intake.

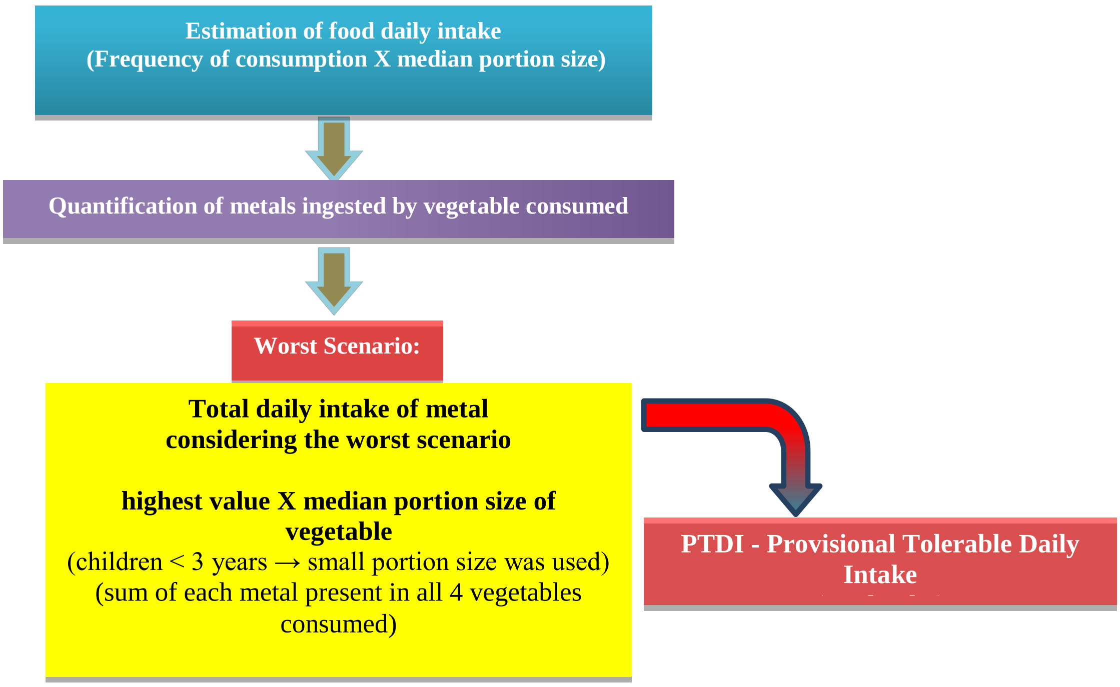

Figure 3. Methodologies to estimate the risk of local farm food intake.

Results and discussion

Composition in chromium, manganese, nickel, copper, zinc, arsenic, selenium, cadmium, lead, were evaluated in soil, water hole and in the vegetables tomato, lettuce, cabbage and potatoes (Table 2 and 3; Figures 4 to 7).

High content of cobalt (Co) was found in the Carrasqueira agriculture soils, although below Canadian Soil Quality Guidelines, also this element was not found in a high content in the analyzed foods. According to the Commission Regulation (EC) No 1881/2006 of 19 December 2006, that set maximum levels for certain contaminants in foodstuffs, the maximum limit for Pb should 100 µg/kg and Cd - 100 µg/kg. All the concentrations found in the vegetables are below that limit for that metals.

The results obtained under appropriated quality control were analyzed for risk assessment according with EFSA guidelines and the methodology earlier explained for estimation of food daily intake (see Table 4 to 6). In the worst Scenario 5 persons (3 children's and 2 adults) from Carrasqueira exceeded the value of EFSA (2009) for As (90% of inorganic As - 0.3 to 8 g / day / kg), above that value there is a potential to cause teratogenic effects. Also 3 person (3 children´s) from VNMF are near the limit value but from Cd (25 µg/kg bw/month according to the JECFA 2011 guidelines) (guidelines available at table 7). The others metals and metalloids concentrations were below values that can cause effects on human health. These results are of concern in particular for children since only 4 vegetables (potatoes , lettuce, tomato and cabbages) were analyzed and already exceeded the amount of daily intake established by EFSA.

The high content of As found in potatoes suggest that further work is necessary to clarify the potential health risks of As to young children of the Carrasqueira. Also higher Cd concentrations were found in Vila Nova de Mil Fontes, who are already close to the maximum allowed for this metal, so the farms of this village should not be considered as a reference location, in terms of this line of evidence.

Table 2. Concentration of metals and metalloids in soils from Carrasqueira and Vila Nova de Mil Fontes. Values in grey rectangle are above the limit guidelines.

	Soil (mg/kg) – soluble fraction							Limits of agriculture soil	Intervention Values - standard soil (10% organic matter and 25% clay)
	Carrasqueira 1	Carrasqueira 2	Carrasqueira 3	VNMF 1	VNMF 2	VNMF 3	LQ	Canadian Soil Quality Guidelines	Holland Soil Remediation Circular 2009
	µ ± sd	µ ± sd	µ ± sd	µ ± sd	µ ± sd	µ ± sd		(mg/kg)	(mg/kg d.s.)
Cr	4,27±0,076	3,628±0,075	3,434±0,304	6,587±0,853	2,144±0,159	2,249±0,212	0,96	64 (total Cr)	180 (Cr III); 78 (Cr VI)
Mn	0,483±0,004	0,483±0,004	0,561±0,067	88,460±2,855	68,414±0,138	34,501±1,347	0,48		
Co	48,849±4,645	25,041±2,297	45,429±3,248	0,763±0,00002	<LQ(0,500)	<LQ(0,500)	0,48	40	190
Ni	1,186±0,107	1,186±0,107	1,492±0,063	2,759±0,071	<LQ (1000)	<LQ (1000)	0,96	50	100
Cu	4,536±0,157	5,59±0,349	4,352±0,231	4,937±0,116	1,917±0,022	2,044±0,185	0,48	63	190
Zn	15,243±0,754	10,08±0,036	<LQ(10,755)	3,077±1,868	<LQ (20,000)	<LQ (20,000)	9,6	200	720
As	1,136±0,125	1,096±0,17	1,331±0,329	2,304±0,194	<LQ (0,500)	0,725±0,073	0,48	12	76
Se	<LQ(0,976)	<LQ(0,976)	<LQ(1,076)	<LQ (1,000	1,208±0,041	1,036±0,042	0,96	1	100
Sr	15,419±1,197	3,448±0,118	6,351±0,58	12,125±0,11	5,775±0,069	5,523±0,623	0,96		
Cd	<LQ(0,483)	<LQ(0,483)	<LQ(0,505)	<LQ(500)	<LQ(500)	<LQ(500)	0,48	1,4	13
Pb	4,757±0,308	10,229±0,444	23,602±0,482	6,474±0,922	2,117±0,034	2,682±0,022	0,96	70	530
MO (%)	39,74	30,35	31,39	26,44	54,77	38,76			

Table 3. Metals and metalloids concentrations in water holes from Carrasqueira and Vila Nova de Mil Fontes, and comparison with national guidelines. In Carrasqueira a forth water hole was analyzed (of a fisherman house).

	water holes (µg/kg)								Water law **D.L.306/2007** water quality to human consumption **Maximum limit**
	Carrasqueira 1 µ ± sd	**Carrasqueira 2** µ ± sd	**Carrasqueira 3** µ ± sd	**Carrasqueira 4 (Sr. Joaquim)** µ ± sd	**VNMF 1** µ ± sd	**VNMF 1** µ ± sd	**VNMF 1** µ ± sd	**LQ**	
Cr	<LQ (0,5)	<LQ (0,5)	<LQ (0,5)	<LQ (0,5)	1,09±0,08	0,73±0,05	0,77±0,04	0,5	50
Mn	0,6±0,02	0,3±0,00	2,0±0,08	0,5±0,00				0,25	50
Co	<LQ (0,25)	<LQ (0,25)	<LQ (0,25)	<LQ (0,25)	0,64±0,00	0,40±0,01	0,51±0,00	0,5	
Ni	0,5±0,00	<LQ (0,5)	<LQ (0,5)	<LQ (0,5)	6,82±0,01	1,61±0,05	1,69±0,01	0,5	20
Cu	3,1±0,05	2,6±0,03	5,8±0,22	4,4±0,03	9,64±0,22		1,07±0,00	0,25	2 (mg/l)
Zn	6,9±0,07	5,4±0,02	5,8±0,18	29,9±0,17				5	
As	0,6±0,02	<LQ (0,25)	<LQ (0,25)	<LQ (0,25)	0,80±0,05	0,77±0,02	0,63±0,02	0,25	10 (µg/kg)
Se	0,6±0,05	0,5±0,01	0,9±0,04	<LQ (0,5)	2,61±0,09	1,30±0,05	1,63±0,19	0,5	10 (µg/kg)
Sr	1,9±0,03	4,6±0,01	3,5±0,03	20,5±0,13				0,5	
Cd	<LQ (0,25)	<LQ (0,25)	<LQ (0,25)	<LQ (0,25)	0,26±0,00	<LQ (0,25)	<LQ (0,25)	0,25	5 (µg/kg)
Pb	0,9±0,01	<LQ (0,5)	<LQ (0,5)	<LQ (0,5)	4,13±0,04	1,01±0,03	<LQ (0,5)	0,5	25 (µg/kg)

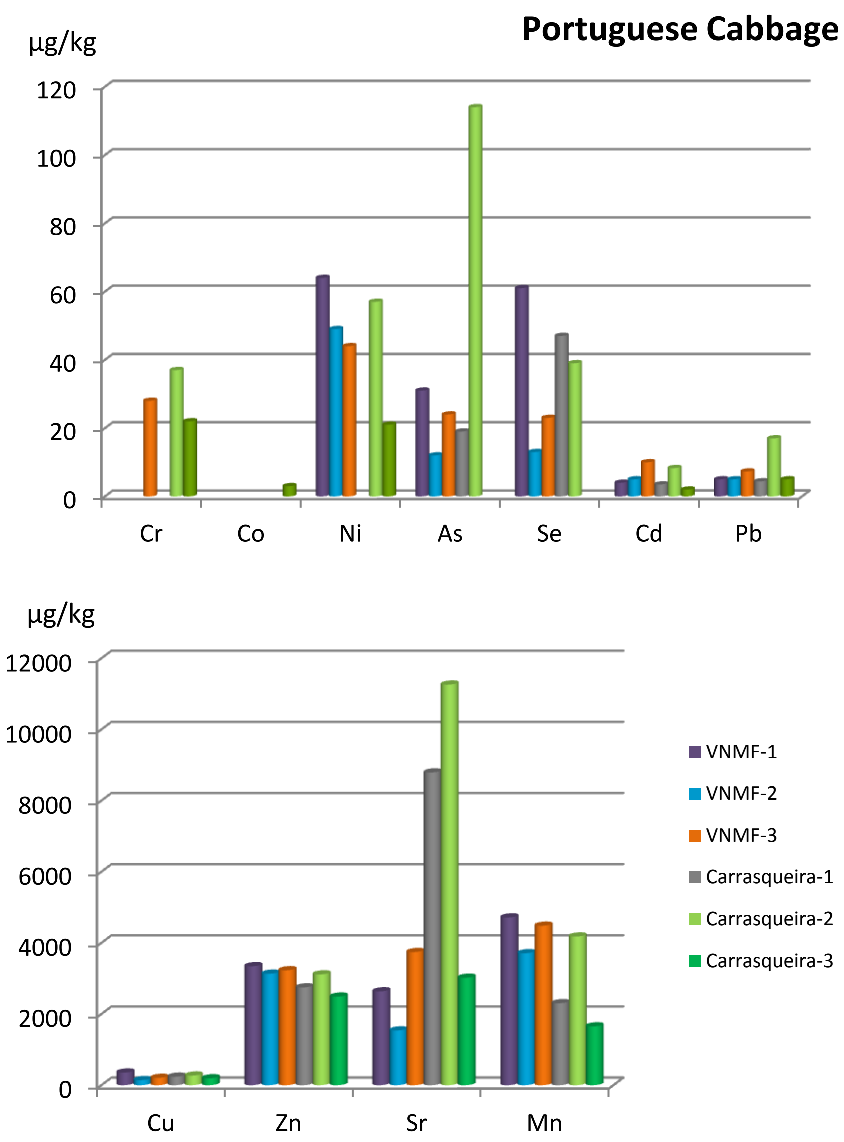

Figure 4. Concentrations of metals and metalloids in Portuguese Cabbage, from Carrasqueira and Vila Nova de Mil Fontes

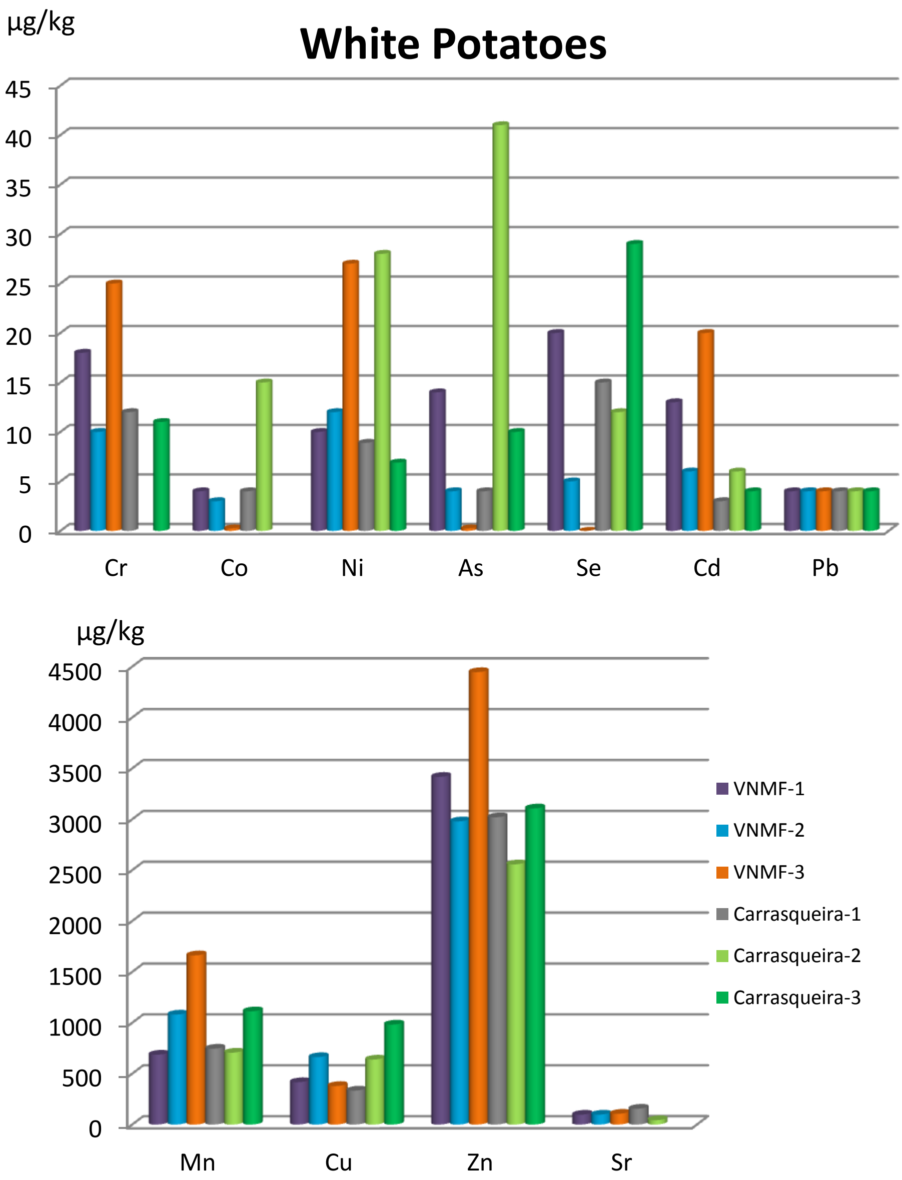

Figure 5. Concentration of metals and metalloids in white potatoes, from Carrasqueira and Vila Nova de Mil Fontes.

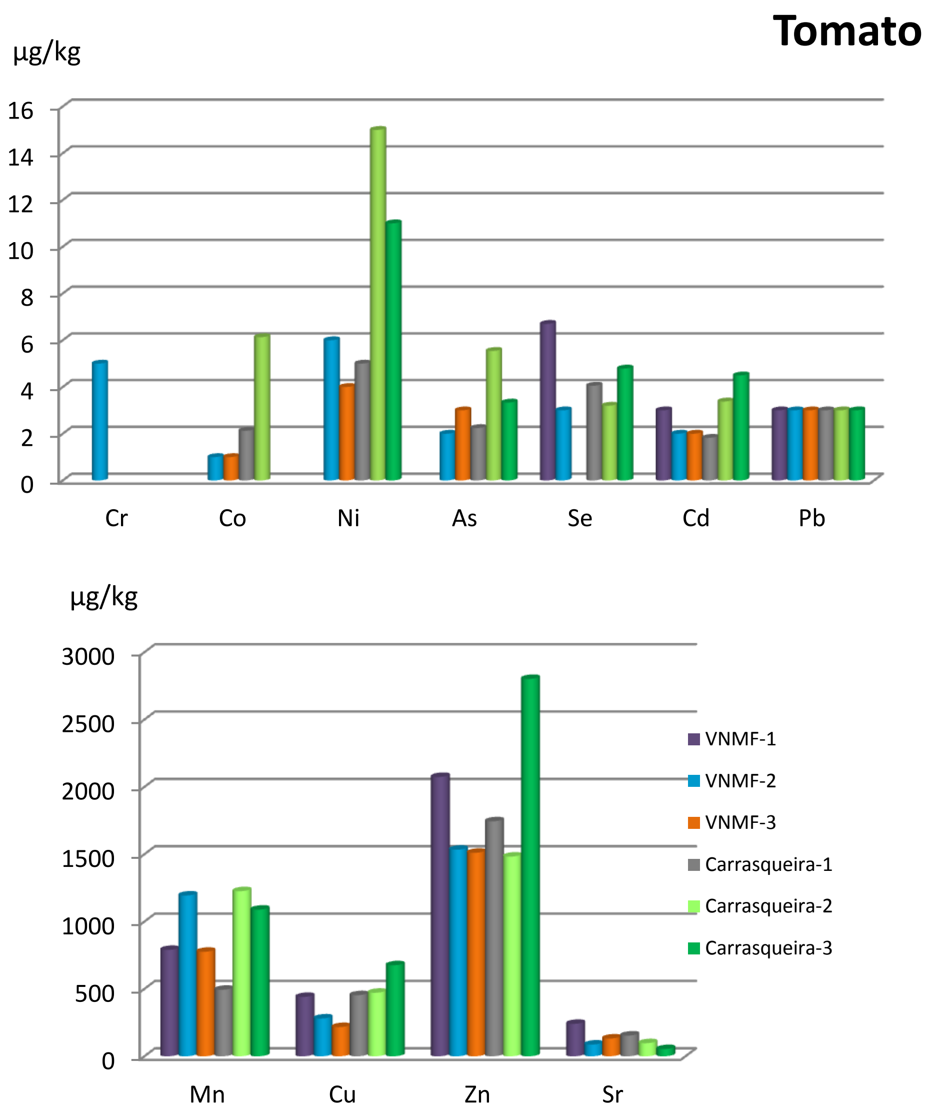

Figure 6. Concentrations of metals and metalloids in Tomato, from Carrasqueira and Vila Nova de Mil Fontes.

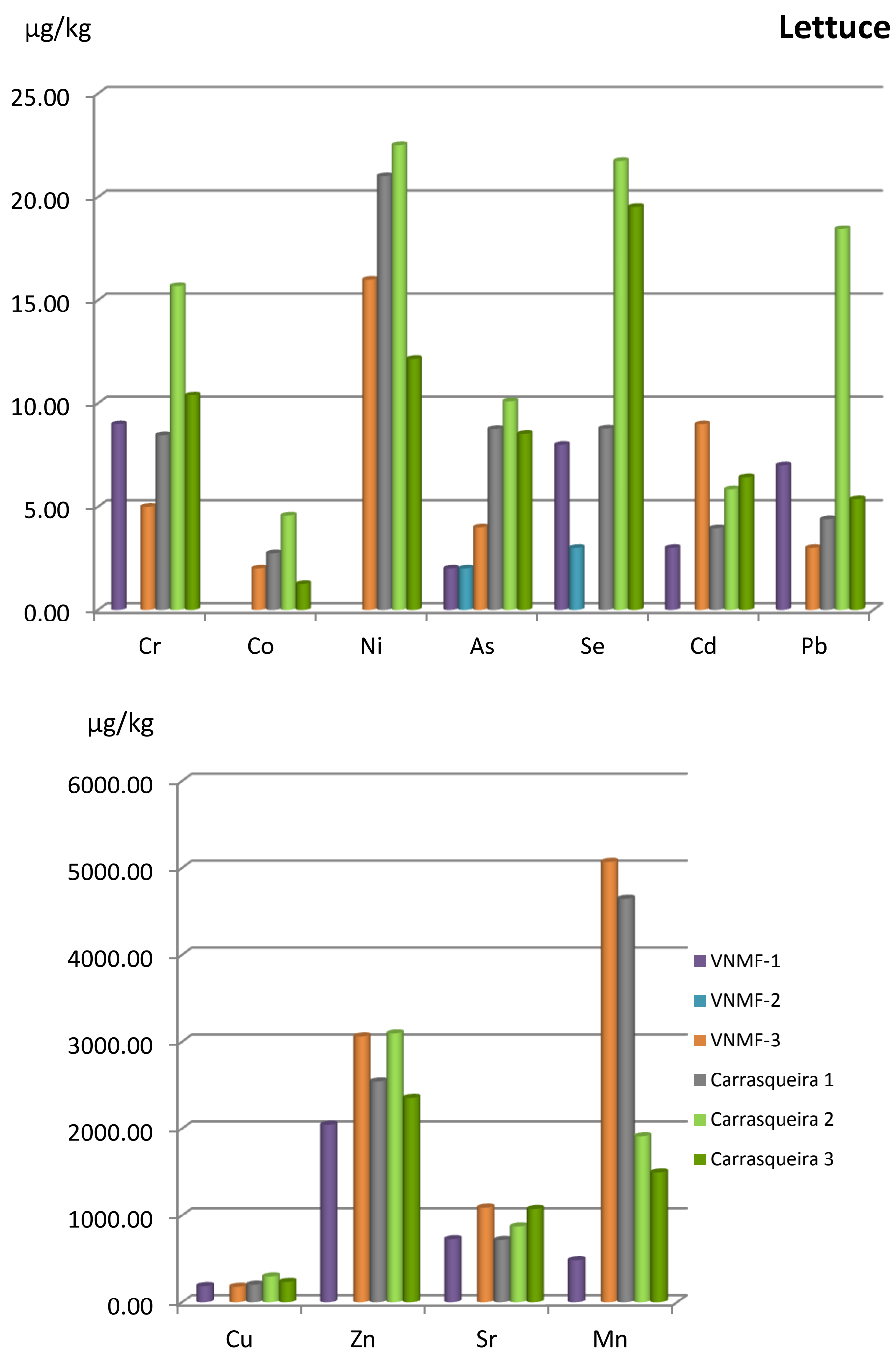

Figure 7. Concentrations of metals and metalloids in Lettuce, from Carrasqueira and Vila Nova de Mil Fontes.

Table 4. Determination of Provisional Tolerable Daily Intake, in population of Carrasqueira (who participate in food frequency questionnaire), using the worst scenario (highest levels of contaminants). Shadow rectangles shown values above guidelines.

PDI (provisional tolerable daily intake) worst scenario									
As	**Cr**	**Ni**	**Cu**	**Pb**	**Cd**	**Zn**	**Co**	Year of birth	
0,037	0,014	0,029	0,991	0,006	0,007	3,502	0,015	1998	children
0,055	0,023	0,055	1,872	0,014	0,014	7,629	0,024	1999	children
0,270	0,101	0,228	6,588	0,056	0,051	27,237	0,086	2000	children
0,102	0,043	0,068	0,905	0,024	0,013	6,384	0,012	2001	children
0,191	0,067	0,140	4,240	0,026	0,029	15,032	0,064	2001	children
0,137	0,060	0,136	4,078	0,042	0,033	18,844	0,049	2003	children
0,123	0,044	0,068	0,325	0,020	0,010	3,737	0,004	2003	children
0,155	0,074	0,178	5,677	0,059	0,046	26,900	0,066	2003	children
0,326	0,110	0,240	7,568	0,040	0,050	25,468	0,115	2004	children
0,140	0,056	0,130	4,119	0,034	0,031	17,169	0,054	2005	children
0,607	0,228	0,513	14,823	0,127	0,114	61,283	0,194	2006	children
0,423	0,153	0,314	9,291	0,065	0,067	34,369	0,139	2009	children – small dose
0,291	0,114	0,208	3,463	0,064	0,040	19,995	0,040	2009	children – small dose
0,102	0,043	0,088	2,662	0,024	0,021	11,291	0,037		
0,094	0,035	0,081	2,663	0,018	0,019	10,152	0,037		
0,055	0,022	0,051	1,634	0,013	0,012	6,808	0,021		
0,044	0,023	0,037	0,909	0,014	0,009	5,124	0,012		
0,048	0,017	0,041	1,238	0,009	0,009	4,710	0,016		
0,177	0,060	0,124	3,357	0,024	0,024	12,167	0,050		
0,147	0,049	0,114	3,987	0,017	0,025	12,888	0,060		
						…			
0,061	0,028	0,050	1,116	0,017	0,011	6,121	0,014		
0,281	0,097	0,214	6,680	0,039	0,045	23,469	0,098		
0,086	0,029	0,070	2,239	0,013	0,015	8,000	0,031		
0,109	0,037	0,083	2,631	0,015	0,018	9,098	0,039		
0,019	0,013	0,027	0,765	0,012	0,008	4,682	0,007		
0,084	0,030	0,070	2,349	0,015	0,016	8,618	0,033		
0,141	0,048	0,106	2,969	0,021	0,021	10,983	0,042		
0,008	0,003	0,005	0,035	0,002	0,001	0,437	0,000		
0,088	0,034	0,070	2,087	0,016	0,015	8,158	0,030		
0,105	0,035	0,083	2,315	0,017	0,017	8,872	0,031		
0,067	0,025	0,058	1,902	0,013	0,013	7,251	0,026		
0,093	0,030	0,082	2,806	0,015	0,018	9,869	0,038		
0,343	0,131	0,207	1,991	0,064	0,034	14,986	0,026		
					…	**n = 102**			

Table 5. Determination of Provisional Tolerable Daily Intake, in population of Vila Nova de Mil Fontes (who participate in food frequency questionnaire), using the worst scenario (highest levels of contaminants). Shadow rectangles shown values above guidelines. Shadow rectangles shown values above guidelines.

PDI (provisional tolerable daily intake) worst case scenario										
As	**Cr**	**Ni**	**Cu**	**Pb**	**Cd**	**Zn**	**Co**	Cd_PI_month	Year of birth	
0,305	0,604	0,653	16,023	0,097	0,483	107,445	0,097	14,50	2010	< children 2 years old - small dose
0,329	0,628	0,708	16,330	0,103	0,492	110,344	0,102	14,76	2009	< children 2 years old - small dose
0,333	0,668	0,718	17,541	0,108	0,530	118,745	0,106	15,91	2008	children
0,175	0,336	0,383	8,414	0,057	0,255	58,423	0,054	7,64	2008	children
0,179	0,341	0,389	8,825	0,064	0,258	60,329	0,055	7,73	2008	children
0,014	0,031	0,032	0,763	0,005	0,023	5,511	0,005	0,70	2005	children
0,102	0,203	0,225	5,330	0,045	0,147	36,842	0,032	4,42	2004	children
0,072	0,132	0,156	3,341	0,022	0,101	22,789	0,022	3,02	2003	children
0,070	0,127	0,157	2,953	0,024	0,089	21,590	0,021	2,66	2002	children
0,049	0,096	0,104	2,555	0,015	0,077	17,133	0,015	2,31	2001	children
0,058	0,109	0,132	2,738	0,031	0,069	19,680	0,017	2,08		
0,094	0,190	0,205	4,901	0,032	0,148	33,764	0,030	4,43		
0,037	0,074	0,079	1,958	0,013	0,058	13,180	0,012	1,75		
0,031	0,067	0,068	1,831	0,017	0,048	12,686	0,010	1,43		
0,046	0,083	0,102	2,073	0,019	0,057	14,593	0,014	1,72		
0,016	0,036	0,038	0,786	0,007	0,025	6,177	0,005	0,74		
0,041	0,084	0,090	2,251	0,019	0,062	15,476	0,013	1,87		
0,005	0,005	0,011	0,063	0,001	0,002	0,593	0,001	0,05		
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0		
0,035	0,065	0,075	1,684	0,011	0,050	11,469	0,011	1,50		
0,008	0,017	0,018	0,463	0,007	0,010	3,389	0,002	0,29		
0,031	0,070	0,070	1,927	0,020	0,048	13,459	0,010	1,45		
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0		
0,026	0,061	0,060	1,688	0,023	0,036	12,177	0,008	1,09		
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0		
0,018	0,038	0,041	0,909	0,011	0,024	7,004	0,006	0,71		
0,005	0,014	0,015	0,202	0,004	0,007	2,312	0,002	0,21		
0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0		
0,018	0,034	0,038	1,044	0,009	0,026	6,565	0,006	0,79		

… n = 100

Table 6. Limit values used in the Provisional Tolerable Daily Intake (bw - body weight).

Limit values used in the Provisional Tolerable Daily Intake.								
EFSA's (2009)	**(OEHHA 2010)**	**(AFSSA 2008; WHO 2005)**	**(SCF 2006)**	**EFSA's (2010)**		**(JECFA 2011)**	**(SCF 2006)**	**(AFSSA 2010)**
As	**Cr**	**Ni**	**Cu**		**Pb**	**Cd**	**Zn**	**Co**
0.3 a 8 µg/kg bw/day	0.5 (mg/kg bw/day)-1	22 µg/kg bw/day	5 mg/day	1.5	cardiovascular	25 µg/kg bw/month	25 mg/day	1.6 and 8 µg/kg bw/day
				0.63	neprotoxic			
				0.5	neurotoxic			

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Task 3

Sample collection and laboratorial quantification of estuarine species bioaccumulation and health biomarkers

IMAR

Background and objectives

Coastal areas have long endured strong anthropogenic pressures. Since the dawn of the Industrial revolution human activities have been responsible for the dumping onto these areas large amounts of potential pollutants, either from point or diffuse sources, many of which tend to become trapped and stored in sediments, depending on their chemical nature. For such reason, monitoring sediment contamination has become a paradigm for the determination of environmental quality in estuarine sediments. However, estuaries are very complex ecosystems and measuring pollution, i.e., when contamination is building up to the level of causing deleterious effects to the biota, remains challenging (see Chapman et al., 2013, for a review).

In the past decades, a considerable effort has been put into motion to monitor the ecological status of estuarine environments, ultimately to provide knowledge-sustained tools for effective environmental management plans. The European Union's (EU) Water Framework Directive (updated through the Directive 2008/56/EC) and the Marine Strategy Framework Directive (Directive 2008/56/EC) contain the first EU-wide conceptual and practical guidelines to develop efficient monitoring programmes for coastal environments and adjacent waterbodies (see Borja et al., 2010; Lyons et al. 2010).

The Sado estuary is a large estuarine basin (≈ 180 km2) located in SW Portugal, characterized by its high biogeographical diversity and high ecological and socio-economic importance. The area comprises the city of Setúbal and its adjacent urban surroundings, deep-water harbours and a large heavy-industry belt. The estuary is important for tourism, fisheries, aquaculture and agriculture and part is classified as a national reserve. Previous studies revealed the existence of contamination levels capable of inducing adverse effects to the biota, albeit the estuary being globally judged as moderately contaminated (Caeiro et al., 2009; Costa et al., 2012a). Nevertheless, the full ecotoxicological evaluation of the estuary is yet incipient (no permanent monitoring programmes have been developed) and no comparisons exist between the northern and southern areas, enduring urban/industrial and riverine/rural stressors, respectively. Furthermore, no attempts have yet been made to link environmental contamination and human health in the area, which constitutes per se a worldwide novelty of the HERA project.

The present task of the project HERA aimed essentially at producing a thorough ecotoxicological appraisal of the Sado Estuary. It was aimed also at establishing also a bridge between environmental and human health through a biomonitoring approach focused on commercial species of high value in the area, namely a flatfish, a clam and the cuttlefish, all prized resources for the inhabitants of the Carrasqueira village and other local populations.

Methods and Materials

Sediment sampling and characterization

During the research period, as stipulated, sediment samples were collected from several sites of the Sado Estuary and from the reference area, specifically, the Mira Estuary, located in the same biogeographical area, albeit considered one of the least impacted coastal areas in Portugal. Two potentially distinct areas were identified within the Sado Estuary, the "North" holding urban and industrial influence and the "South" potentially impacted by rural activities and direct river inputs (Fig. 1).

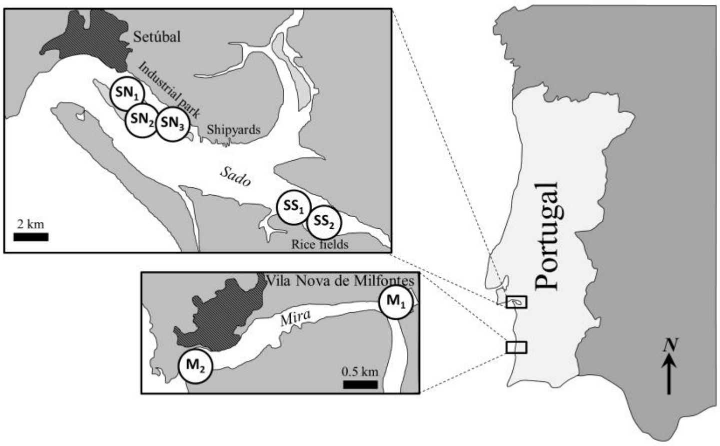

Figure 1. Map of the study area indicating the sediment collection sites, namely Sado "North" (SN_1–SN_3), Sado "South" (SS_1–SS_2) and Mira (M_1–M_2), the latter considered the reference area.

Sediment inorganic toxicants (metals and metalloids) were surveyed by ICP–MS after acid digestion. Organic toxicants (PAHs and organochlorines, including pesticides) were determined by gas-chromatography techniques following extraction with specific solvents, depending on class of toxicants. The sediment contamination profiles are given in Table 1. Contaminant levels were contrasted to available sediment quality guidelines for coastal waters, namely the threshold effects level (TEL) and the probable effects level (PEL) guidelines (MacDonald et al. 1996). Risk (i.e. the potential to cause adverse biological effects) was determined through the estimation of Sediment Quality Guideline Quotients (SQG–Qs), as described by Long and MacDonald (1998):

$$SQG - Q = \frac{\sum_{i=1}^{n} PEL - Q_i}{n} \qquad [1]$$

Where the PEL quotient (PEL–Q) for the i^{th} group of n contaminants is calculated by:

$$PEL - Q_i = \frac{C_i}{PEL_i} \qquad [2]$$

Being C_i the concentration of the i^{th} contaminant and PEL_i its respective PEL guideline. A classification of potential impact to cause adverse effects was performed according to (MacDonald et al. 2004), according to which the sediments are ranked as unimpacted (SQG–Q < 0.1); moderately impacted (0.1 to 1) and strongly impacted (SQG–Q > 1).

Biomarker approach

In accordance with the sediment contamination profiles, animals collected from the Sado estuary yielded differential responses according to the sampling area, i.e., "north" and "south". Multiple endpoints have been analysed in the three target species abovementioned (a flatfish, clam and the cuttlefish), chosen for their important commercial value, which allows a plausible link between environmental and human health.

Several organs were analysed in each species, however, the liver and its invertebrate analogue, the digestive gland, were the main targets. The biomarker approach included the determination of lipid peroxides through the TBARS (thiobarbituric acid-reactive substances) method as a measure of oxidative damage; total and oxidized glutathione, the activity of glutathione S-transferase (GST), metallothionein (MT) and other responses. Endocrine disruption was also determined in fish though histopathology and vitellogenin (VTG) induction in the liver of soles, accompanied by the analysis if cytochrome P450 (CYP) 1A induction and ethoxyresorufin–*O*–deethylase (EROD) activity, two acknowledged biomarkers of exposure to organic toxicants such as PAHs, since most known endocrine disruptors are, in fact, organic pollutants (Gonçalves et al., *in press*). A strong histopathological component was included, for all species, following specially-developed quantitative/semi–quantitative indice-based approaches (Costa et al., 2013a, 2013b, Gonçalves et al, 2013).

The semi–quantitative histopathological condition indices (I_h) constituted a keystone approach throughout the project. The indices being estimated for each individual based on the weighted indices approach proposed by Bernet et al. (1999), with modifications, according to the concepts of the differential biological significance of each surveyed alteration (weight) and its degree of dissemination (score). The weight is a value that ranges between 1 and 3 (maximum severity) and the score a value between 0 (feature/alteration not observed) and 6 (diffuse). The histopathological condition indices were estimated according to the simple formula:

$$I_h = \frac{\sum_{1}^{j} w_j a_{jh}}{\sum_{1}^{j} M_j} \qquad [3]$$

Where I_h is the histopathological condition indice for the individual h; w_j the weight of the j^{th} histopathological alteration; a_{jh} the score attributed to the h^{th} individual for the j^{th} alteration and M_j the maximum attributable value for the j^{th} alteration, i.e., weight × maximum score. The equation's denominator normalizes I_h to a value between 0 and 1, thus permitting comparisons between distinct situations (such as different organs).

Other important technical achievements were accomplished through the use and development of molecular, biochemical and histopathological indicators of exposure to environmental stressors, from metals to parasites in the surveyed species (Costa et al., 2013a, 2012b). Bioaccumulation of relevant toxicants was also determined in the edible portion of surveyed animals as a complement to the biomarker analysis and to provide an insight on risk to human consumption.

Statistics and integration of data

The data from the multiple lines-of-evidence for the Sado Estuary's contamination profiles has been processed statistically using a wide–range of computational techniques, from simple univariate statistics to advanced modelling, such as principal component analyses and discriminant analyses. The process of data integration is continuous and critical for the preparation of manuscripts, proceeding according to the achievements of each component of the research. Other integrative approaches, such as the Integrated Biomarker Response were also endeavoured.

The IBR indice was computed to integrate all biomarker responses determined in both organs, according to the method described by Beliaeff and Burgeot (2002). In brief: the score (*S*) for each biomarker was calculated as:

$$S = Z + |Min| \qquad [4]$$

Where $S \geq 0$, since $|Min|$ is the absolute minimum value obtained for the biomarker and:

$$Z = \pm \frac{X - m}{s} \qquad [5]$$

Being *Z* either positive or negative, depending on the activation or inhibition of the biological effect, respectively. The standardized values *Z* were estimated through the mean value for each biomarker in each site (*X*), the mean value for each biomarker (*m*) and the standard deviation of *X(s)*. The area (*A*) connecting two consecutive coordinates is calculated for each biomarker result as if in star plots, being S_i and S_{i+1} two consecutive scores and *n* the number of biomarkers under analysis:

$$A_i = \frac{S_i}{2} \sin\beta \left(S_i \cos\beta + S_{i+1} \sin\beta \right) \qquad [6]$$

Where:

$$\beta = \arctan\left(\frac{S_{i+1} \sin\alpha}{S_i - S_{i+1} \cos\alpha} \right); \; \alpha = \frac{2\pi}{n} \qquad [7]$$

The final IBR indices are then calculated through the sum of all the areas (*A*).

Results and Discussion

The sediment contamination profiles revealed a distinction between the northern (urban/industrial) and southern (rural/riverine) areas of the estuary (Table 1). Although the overall levels of metallic contaminants are similar between the two areas, there is a clear difference between the patterns of organic toxicants, more concentration (especially PAHs) in the northern areas. Sediment SN_3, collected from a site just off the city's heavy–industry belt was the overall most contaminated. Still in the northern area of the estuary, sediments SN_1 and SN_2 were found to be essentially uncontaminated, however, these samples were collected from a high–hydrodynamics sandbank that is an important commercial shellfish bed, therefore very distinct from the deep–water channel facing the industrial zone where the muddy SN_3 sediment was collected from. The toxicants of most concern in the estuary were As, Cr, Ni, Cu and Zn, all of which presented values above TEL (the lowest guideline) in sediments SN_3, SS_1 and SS_2. Lead was found above the TEL threshold in sediments SN_3 and SS_2. The PEL guideline was surpassed for the metals Cu (S1 and S3) and Zn (SN_3 and SS_2). Only sediment sample SN_3 (northern Sado) yielded organic toxicant levels above TEL, namely for a few 3- to 5-ring PAHs. Total PAH levels in sediments from the industrial area of the estuary attained ten-fold those from the rural area. The levels of organochlorines were globally low.

Table 1. Summary of the sediment characterization for Sado "North" (samples SN_1–SN_2), Sado South (SS_1–SS_2) and the reference estuary, Mira (M_1–M_2). Contamination data (per sediment dry weight) is contrasted to available sediment quality guidelines, namely TEL (threshold effects level) and PEL (probable effects level), retrieved from MacDonald et al. (1996). Ranges indicate standard deviation. Adapted from Carreira et al. (2013).

	Site								
	SN_1	**SN_2**	**SN_3**	**SS_1**	**SS_2**	**M_1**	**M_2**		
Parameter									
TOM (%)	0.8 ± 0.1	0.9 ± 0.1	10.4 ± 0.0	6.9 ± 0.1	8.8 ± 0.0	7.9 ± 0.3	0.7 ± 0.0		
FF (%)	2.5	3.5	52.9	63.7	74.3	72.5	0.8		
								SQGs	
Contaminant								TEL	PEL
Inorganic ($\mu g.g^{-1}$)									
Non–metal									
As	3.50 ± 1.00	0.34 ± 0.26	19.7 ± 5.21^{a}	26.44 ± 2.68^{a}	25.02 ± 8.84^{a}	20.21 ± 3.49^{a}	0.88 ± 0.43	7.24	41.6
Se	0.63 ± 0.24	1.84 ± 0.84	1.92 ± 1.45	0.59 ± 0.21	0.72 ± 0.08	2.12 ± 0.32	0.43 ± 0.39	n/a	n/a
Metal									
Cr	2.30 ± 0.33	2.36 ± 0.36	77.67 ± 4.57^{a}	62.22 ± 4.45^{a}	87.61 ± 2.97^{a}	120.76 ± 2.36^{a}	1.81 ± 0.12	52.3	160
Ni	1.71 ± 1.02	4.10 ± 1.66	16.67 ± 1.1^{a}	17.15 ± 1.21^{a}	22.79 ± 9.47^{a}	40.80 ± 3.09^{a}	3.04 ± 0.65	15.9	42.8
Cu	4.04 ± 0.14	4.51 ± 1.05	178.64 ± 7.01^{b}	74.15 ± 13.16^{a}	92.3 ± 5.63^{a}	62.22 ± 1.44^{a}	2.31 ± 0.36	18.7	108
Zn	14.51 ± 3.76	13.10 ± 1.51	327.51 ± 1.16^{b}	269.79 ± 7.81^{a}	385.11 ± 35.69^{b}	97.90 ± 2.42	1.04 ± 0.51	124	271
Cd	0.13 ± 0.10	0.03 ± 0.02	0.27 ± 0.03	0.33 ± 0.13	0.43 ± 0.19	0.23 ± 0.06	0.1 ± 0.05	0.68	4.21
Pb	5.73 ± 0.60	3.50 ± 0.48	56.45 ± 3.1^{a}	25.3 ± 0.91	32.7 ± 1.21^{a}	29.25 ± 0.52	1.48 ± 1.64	30.2	112
$SQG–Q_{inorganic}$	**0.0445**	**0.0352**	**0.6825**	**0.6213**	**0.4871**	**0.4918**	**0.0236**		
Organic ($ng.g^{-1}$)									
SPAH	23.88 ± 4.06	19.60 ± 3.33	1.076.98 ± 183.09	215.03 ± 36.55	82.47 ± 14.02	322.28 ± 54.79	6.72 ± 1.14	1684	16770
$SQG–Q_{PAH}$	**0.0032**	**0.0029**	**0.0937**	**0.0206**	**0.0089**	**0.0239**	**0.0012**		
Pesticides									
tDDT	0.02 ± 0.00	0.02 ± 0.00	1.22 ± 0.21	0.21 ± 0.04	0.13 ± 0.02	0.14 ± 0.02	0.00 ± 0.00	3.89	51.7
HCB	0.02 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	20	480
$SQG–Q_{pesticide}$	**0.0003**	**0.0001**	**0.0525**	**0.0039**	**0.0006**	**0.0014**	**0.0000**		
SPCB	0.18 ± 0.03	0.05 ± 0.01	5.37 ± 0.91	0.26 ± 0.04	0.27 ± 0.05	0.24 ± 0.04	0.02 ± 0.00	34.1	277
$SQG–Q_{PCB}$	**0.0006**	**0.0002**	**0.0194**	**0.0009**	**0.001**	**0.0009**	**0.0001**		
$SQG–Q_{organic}$	**0.0014**	**0.001**	**0.0552**	**0.0085**	**0.0035**	**0.0087**	**0.0004**		
$SQG–Q_{total}$	**0.0229**	**0.0181**	**0.3688**	**0.3149**	**0.2453**	**0.2503**	**0.0120**		

< d.l., below detection limit; n/a, no guideline available; a), values above TEL; b), values above PEL; SQG–Q, Sediment Quality Guideline Quotients for each contaminant class and total.

Altogether, the SQG–Q approach revealed that sediments from the Sado Estuary are moderately impacted. Whereas sediment M_2 was found to be clean, sediment M_1, also from the Mira estuary, contained significant levels of contaminants, especially metallic. Nevertheless, this sediment was collected from an aquaculture inlet channel (the only industrial facility in the area), which should be responsible for the relatively high levels of toxicants there trapped. In general, the contamination profiles for the Sado estuary are in accordance with those obtained from preliminary studies (Costa et al., 2012a).

The results confirmed the division of the Sado Estuary between a northern area, impacted by urban and industrial stressors and a southern area, of rural and riverine influence. This division is translated into different sets of biomarker responses for all three species, as exemplified in Fig. 2, which shows the results for the hepatic biomarkers for *S. senegalensis*, including metallothionein induction, catalase and GST activities, lipid peroxidation and the histopathological condition indice (I_h). In general, the biomarkers approach revealed a similar trend between fish and cuttlefish, meaning higher responses in animals collected from Sado 1, which is in accordance with the overall sediment contamination profiles and higher SQG–Q score (for sediment sample SN_3, collected from this area). Still, enzyme activity (GST and catalase was lowered in fish from Sado 1, which may reflect some degree of metabolic impairment (see Gonçalves et al., 2013). On its turn, the cuttlefish yielded a promising debut in ecotoxicological studies (while contributing to the understanding of its physiology and anatomy), which has already been translated into successful publications (Costa et al., *in press*; Rodrigo et al., 2013).

Clams from Sado 2 were clearly more affected than those from Sado 1, with especial respect to oxidative stress biomarkers, such as lipid peroxidation (Fig. 3). This divergence from fish and cuttlefish is probably caused by the clams' sedentary disposition, in parallel with the low contamination measured in sediments SN_1 (from the location where the animals were collected), which is a clean sediment that results from higher hydrodynamics and strong oceanic influence (see Carreira et al., 2013). Cuttlefish and soles, although the former a territorial predator and the second a predator exhibiting high fidelity for its nursery habitats (all fish were juveniles), tend to reflect a wider biogeographical area than clams, meaning more exposed to environmental contamination of the area between the sandbanks and Setúbal's industry belt (see Fig. 1). The findings also suggest that bivalve molluscs may be more sensitive to agriculture runoffs (impacting Sado 2) than fish and cuttlefish. The degree of impact to estuarine contamination is visible in histopathological lesions and alterations that attain higher degree of severity and dissemination in clams collected from Sado 2, comparatively to clams from Sado 1. The semi–quantitative histopathological analyses were performed through a weighted indices approach (Bernet et al., 1999), developed specifically for the species during the current research, as exemplified in Fig. 3 (Costa et al., 2013b).

Interestingly, clams revealed a link between parasites/commensals and environmental stressors, while no significant signs of parasites were found in fish of cuttlefish. Clams from the reference location (Ria Formosa, Algarve) presented more and more diverse parasites than their counterparts from Sado, an issue that was explored by combining microscopical and molecular tools (Costa et al., 2012b, 2013b). The study brought to light, inclusively, novel species of prokaryote parasites (Fig. 5).

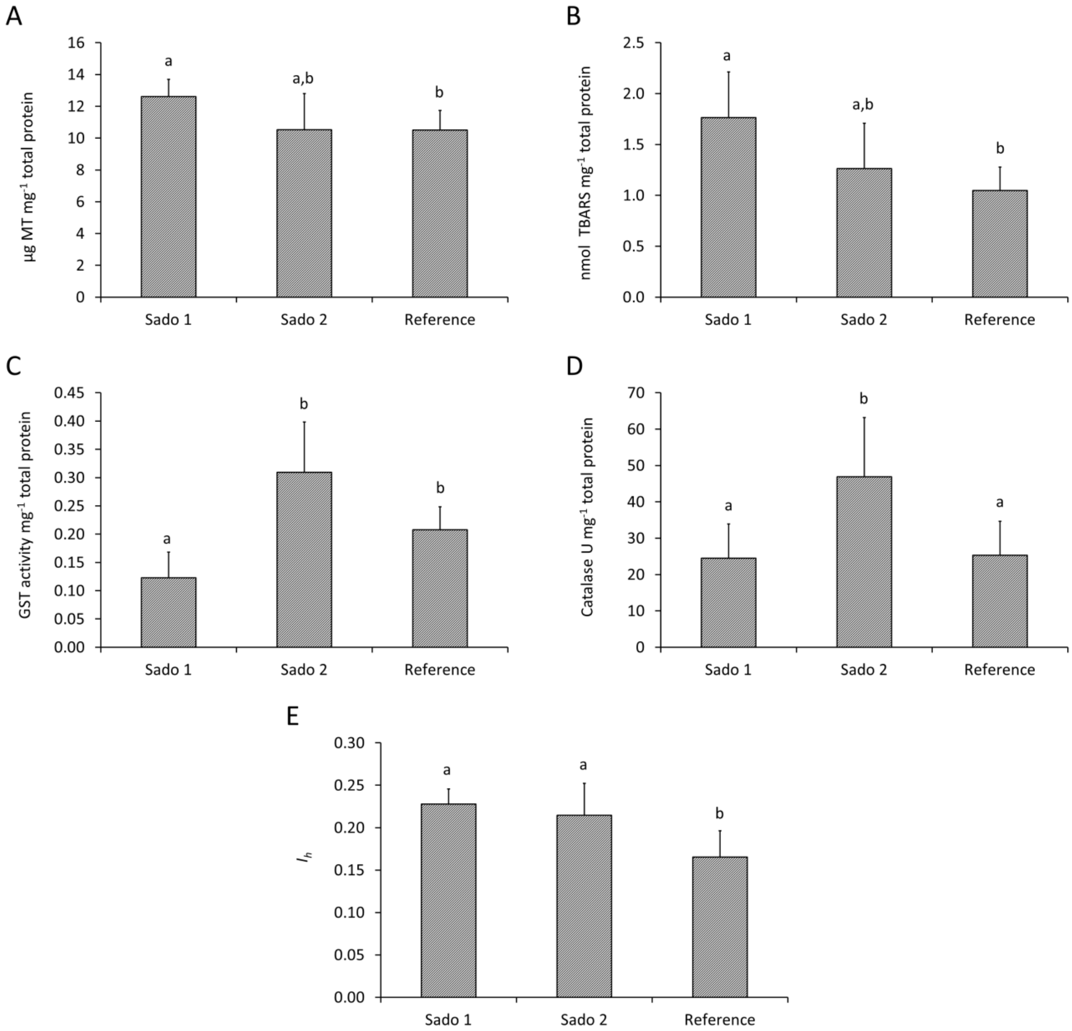

Figure 3. Mean biomarker response results ± 95% confidence intervals in the livers of Senegalese sole collected from the three areas, Sado 1 (northern estuary), Sado 2 (southern estuary) and Reference (off the Mira Estuary). A) Metallothionein-like protein (MT). B) Lipid peroxides (given by TBARS). C) Glutathione S-transferase activity (as mol conjugated CDNB min-1). D) Catalase activity. E) Hepatic histopathological condition indice (I_h). Different letters indicate significant differences (Mann-Whitney U, p < 0.05). From Gonçalves et al. (2013).

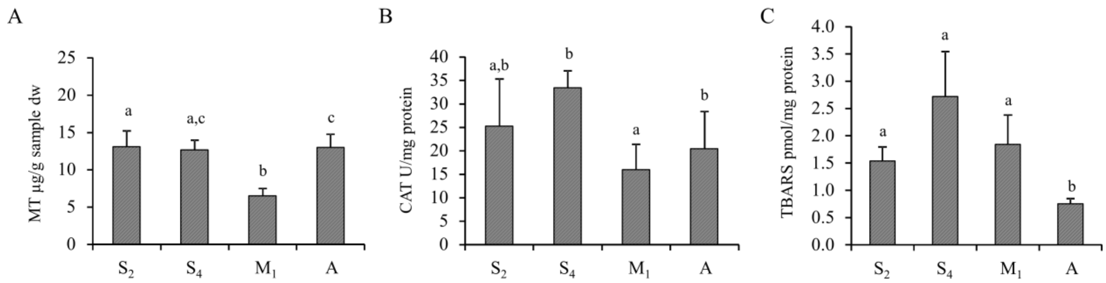

Figure 3. Mean biomarker responses (± 95% confidence intervals) in the clams' digestive glands for the Sado (sites S_2 – Sado 1 and S_4 – Sado 2) and Mira (M_1) plus Ria Formosa (A), the latter constituting external reference areas. A) metallothionein–like protein (MT) concentration; B) catalase (CAT) activity and C) lipid peroxidation, inferred from the thiobarbituric acid reactive species (TBARS) assay. Different letters indicate significant differences (Wald-Wolfowitz runs test, $p <$ 0.05). From Carreira et al. (2013).

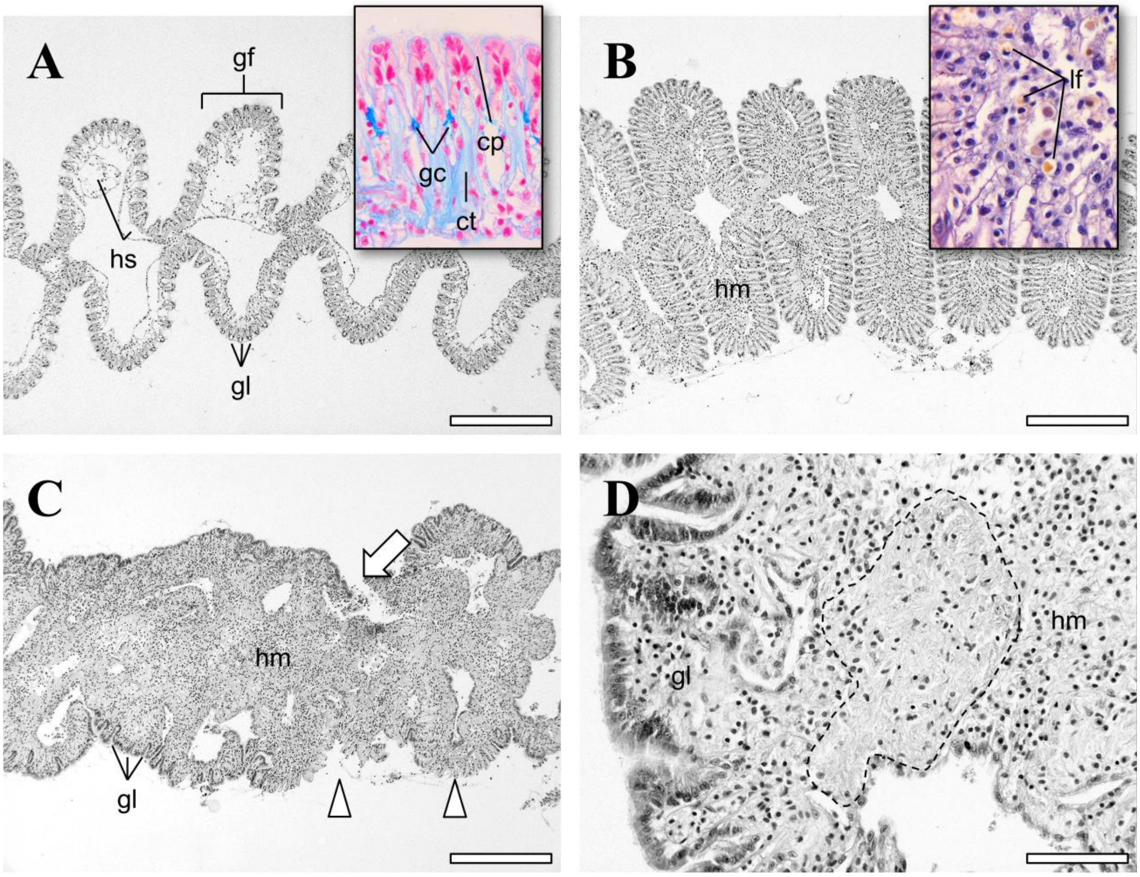

Figure 4. Example micrographs of histopathological lesions and alterations in the gill ctenidia of *R. decussatus*. A) normal structure of clam gills (of an individual collected from site A) showing the regular arrangement of filaments (gf) and lamellae (gl). The haemolymphatic sinuses (hs) contain few haemocytes. HE. Inset: detail of gill lamellae stained with Alcian Blue and Nuclear Fast Red. Lamellae are comprised of ciliated epithelial cells (afrontal region) that form the ciliary plates (cp) between lamellae. Goblet (mucous–secreting) cells (gc) are present at each side of the exposed. The structure is supported by a delicate cartilage, stained blue (ct). B) diffuse haemocytic infiltration in the gills of a clam from site M (hm). The overall microstructure of the organ is seemingly unaffected and without signs of any parasitosis. Inset: detail of the haemocytes (likely granulocytes), revealing lipofuscin–containing bodies inside (lp). HE. C) severely affected gills of a clam from site M, without signs of parasites. Besides diffuse haemocytic infiltration (hm), indicating inflammation, apparently responsible for the engorgement of ctenidium (including lamellae – gl), there are necrotic foci (arrow), loss of the lamellar epithelium (arrowheads), exposing the supportive cartilage of the structures. HE. D) a developing fibroma in a clam also from site M (inside dashed line). The heavy haemocytic infiltration caused swelling and subsequent deformation of lamellae. HE. Scale bars: A–C, 500 µm; D, 50 µm. Retrieved from Costa et al. (2013b).

The very first attempt to detect potential endocrine disrupting effects in the Sado Estuary was also endeavoured; in the flatfish *S. senegalensis* (see Gonçalves et al., *in press*). Hepatic vitellogenin (VTG) concentrations in males from the industrial area were higher than those from the Reference area, which may indicate an oestrogenic effect resulting from the complex contaminant mixture. Higher levels of cytochrome P450 (CYP1A) induction and ethoxyresorufin-O-deethylase (EROD) activity were also found in those animals as well as in females, which is consistent with contamination by organic substances, such PAHs, which are the most significant organic toxicants measured the sediments from the Estuary, in particular from Sado 1 (urban and industrial). This endocrine disrupting effects was not observed in animals from Sado 2.

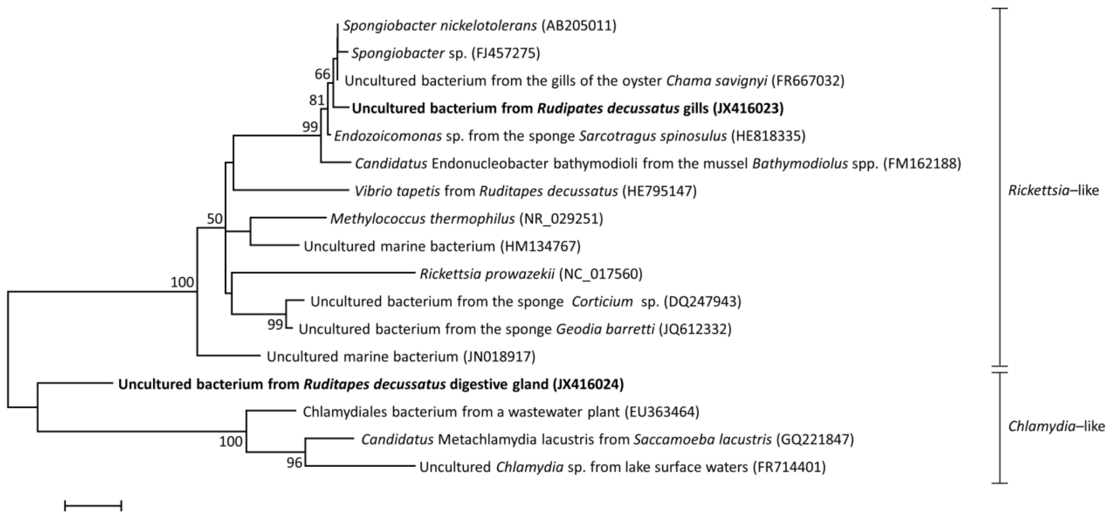

Figure 5. Consensus phylogenetic tree showing the evolutionary position of the two bacterial sequences obtained from the gills and digestive glands of *R. decussatus* (boldface text) relatively to other 16S sequences of aquatic, parasitic or free–living, *Rickettsia/Chlamydia*–like bacteria. *Rickettsia prowazekii* (etiological agent of human epidemic typhus) was included as outgroup. The accession number of each sequence is indicated. The tree was built using the Maximum likelihood method (Tamura and Nei, 1993), based on 5 000 bootstrap replicates (internode bootstrap values below 50% are not shown). The scale bar indicates 2% sequence divergence. From Costa et al. (2012b).

The Integrated Biomarker Approach (Table 2) confirmed the differences between species and sites by combining biomarker responses in liver (fish) or digestive glands (molluscs), showing that clams from Sado 2 were the most affected organisms, followed by cuttlefish from Sado 1 and fish from both sites. These results indicate that each species is sensitive to a specific, site–dependent, set of stressors, according to its basic physiology and behaviour. In comparison, the flatfish yielded a more integrative image of the overall status of the estuary by reflecting the potential adverse effects impacting both areas, Sado 1 and Sado 2, with very distinct characteristics. Nevertheless, it was the combination of the three species provided the most complete and insightful appraisal of the ecotoxicological status of the estuary, which confirms the importance of integrative, multi–indicator approaches in environmental monitoring.

Toxicant analyses in edible tissues of surveyed animals (exemplified in Fig. 6) revealed a trend towards elevated bioaccumulation in organisms from Sado 1, in accordance with biomarker results. Bioaccumulation of toxicants was, in general, low and, with the exception for animals, especially clams, from Sado 1, should not inspire great caution with respect to risk to human consumption. Nonetheless, the results indicate that animals from this location should undergo further analyses and continuous surveillance, due to their importance as estuarine resources.

Table 2. Comparative Integrated Biomarker Response (IBR) scores for clams (*R. decussatus*), soles (*S. senegalensis*) and cuttlefish (*S. officinalis*).

	Ruditapes decussatus[1,2]		*Solea senegalensis*[3]		*Sepia officinalis*[4]	
Site	Biomarkers	IBR	Biomarkers	IBR	Biomarkers	IBR
Sado 1	MT, CAT, LPO, Ih	0.16	MT,CAT, GST, LPO, Ih	1.03	MT, GST, GSH, rGSH, LPO	2.28
Sado 2	MT, CAT, LPO, Ih	2.61	MT,CAT, GST, LPO, Ih	1.01	MT, GST, GSH, rGSH, LPO	0.23
Reference	MT, CAT, LPO, Ih	0.00	MT,CAT, GST, LPO, Ih	0.01	MT, GST, GSH, rGSH, LPO	0.00

[1]Carreira et al. (2013)

[2]Costa et al. (2013b)

[3]Gonçalves et al. (2013)

[4]Rodrigo et al. (2013)

CAT, catalase activity; GSH, total glutathione; GST, glutathione S–transferase activity; Ih, histopathological condition indice; LPO, lipid peroxidation; MT, metallothionein induction, rGSH, reduced/oxidized glutathione ratio.

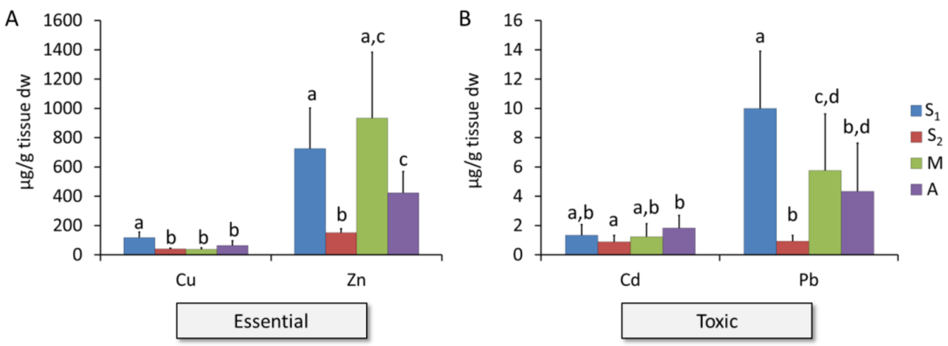

Figure 6. Average metal concentrations in the digestive gland ± 95% confidence intervals, divided by essential (A) and non–essential, i.e. "toxic" metals (B). Different letters indicate differences between sites (Mann–Whitney U, $p < 0.05$).

Concluding remarks

The objectives of the task were completed in full, through the delivery of the most complete, to date, ecotoxicological appraisal for the Sado Estuary. Deviations from the original plan were essentially additive, meaning that more techniques were applied and mote productivity indicators were produced than the originally expected (see Annex 1). The current findings indicate that the Sado Estuary can be globally regarded as a moderately impacted ecosystem, however in need of developing full biomonitoring programmes to establish time– and season–dependent trends on the effects of pollutants (refer, for instance, to the criteria proposed by Chapman and Anderson, 2005).

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Task 4

Characterization of the genotoxic potential of sediments collected in the areas of local fishing activities

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Abstract

In this task the cytotoxic, genotoxic and endocrine disruption effects of sediment samples collected at five fishing sites of the Sado river estuary were characterized in a human liver-derived cell line (HepG2 cell line), comparatively to those of a reference sample from the Mira river estuary. Furthermore, HepG2 cells were subjected to bioassays with sediment extracts obtained from the several sediment samples with a series of progressively polar solvents. Overall, the results showed a close association between the level and nature of contaminants presents in each sediment sample and its cyto and genotoxic effects in HepG2 cells. More mechanistic studies were also performed, comprising the induction oxidative stress and interference with the DNA repair machinery. From those data a synthetic risk indicator for environmental and human risk assessment based on the *in vitro* genotoxicity of sediments was derived, which constituted a line of evidence for the overall environmental and human risk assessment of the Sado estuary. Finally, the genotoxic effects of the most contaminated sample was tested in a transgenic mouse model by application of an integrated approach, providing information on induction of DNA damage in several organs/tissues as well as on mutations in somatic cells, clastogenic and aneugenic effects.

Objectives

The general objective of this task was to contribute to evaluate the risk to human health, associated with a multi-purpose contaminated estuary. Because the exposure of the Carrasqueira village population to the estuary contaminants - during their fishing activities and through the consumption of potentially contaminated estuarine species - had not been addressed prior to this project (tasks 1 and 2), this work was focused on hazard characterization of the complex mixture of contaminants present in the estuarine sediments collected from the fishing sites of the Sado Estuary; the Mira river estuary was used as the presumably uncontaminated reference area. The adverse effects of those contaminants were characterized through the analysis of the potential cytotoxic, genotoxic and endocrine disrupting effects of contaminants extracted from sediments (total mixture and fractions) in human cell lines and the investigation of their mutagenic effect in a transgenic mouse model.

Methodologies

Extracts preparation

Sediment samples were collected (task 3) from four distinct fishing sites of the Sado Estuary (samples N_2 and R, in the northern area; and S_1 and S_2 from the southern area) from October 2010 through June 2011, along with two samples from the Mira Estuary (samples ME and MW). Another sample from the Sado Estuary, collected from a contaminated area in 2007 (N_1, northern area; Costa et al., 2011) was added as a positive control. Total contaminants were extracted from each sediment samples with a mixture of methanol:dichloromethane (Met:DCM, 1:2). Fractionated extracts were also prepared from samples N_2, S_1, S_2, and R from the Sado Estuary, and from samples ME and MW of the Mira Estuary. For this, three distinct extractions were performed, using solvents of different polarities: n-hexane (n-hex), dichloromethane and methanol.

In vitro bioassays

Characterization of the cytotoxic and genotoxic effects of the total and fractioned extracts

Neutral red assay

The human hepatoma-derived HepG2 cells in exponential growth phase were exposed to serial dilutions of the 7 total extract samples, and to dimethyl sulfoxide (DMSO, solvent control) for 48h. Cytotoxicity was evaluated by the Neutral Red (NR) assay as described Repetto et al. (2008). In order to determine cell survival after exposure, the mean value of cell viability was calculated, with 3 independent experiments, each with 3 replicate treatment conditions.

Comet assay

To evaluate the level of DNA damage induced by several dilutions of the total extracts, the single cell gel electrophoresis, or Comet assay, was performed as described by Collins et al., (2008). After exposure, HepG2 cells were immobilized in an agarose gel, over a microscope slide, and subjected to lysis. An alkaline incubation was performed for DNA unwinding, allowing after electrophoresis the detection of DNA strand breaks. An additional enzymatic treatment step was introduced, with the DNA repair endonuclease FPG, to permit the detection of oxidative DNA damage. Cells were also exposed to solvent and positive controls (DMSO and H_2O_2, respectively). One hundred nucleoids were analyzed for each treatment condition and 3 independent experiments were performed. The mean percentage of DNA in tail was determined, as well as the % of FPG sensitive sites.

Micronucleus assay

The level of chromosomal damage, induced by several dilutions of the total extracts, was evaluated by the micronucleus assay as described by Fenech (2007). After exposure, HepG2 cells were incubated with cytochalasin-B, to inhibit cytokinesis, and were collected after 28h, in order to obtain a population of binucleated cells in a microscope slide. After staining, 2000 binucleated cells were analyzed per treatment condition, in duplicate cultures, and the frequency of micronucleus, nucleoplasmic bridges, nuclear buds were calculated, along with the replication and cytokinesis-block proliferation indices.

Evaluation of oxidative DNA damage

To confirm the presence of oxidative DNA damage revealed in the Comet assay by the FPG enzyme, HepG2 cells were co-exposed to two concentration of extracts N_2, R, S_1 and S_2 (that revealed oxidative DNA damage) and an known antioxidant (quercetin) for 48h. Cytotoxicity was also determined for quercetin after 48h exposure. Three independent experiments were performed for each treatment condition. The difference in FPG sensitive sites between cells exposed and non-exposed to quercetin indicates that quercetin was able to revert the observed oxidative DNA damage.

Another approach to the characterization of oxidative DNA damage consisted of inhibiting the glutathione synthesis with buthionine-sulfoximine (BSO) prior to a short (3h) HepG2 cells exposure to sediment extracts. Higher cytotoxicity or DNA damage (specially revealed by FPG) in cells pre-exposed to BSO, suggests the presence of oxidative stress induced by the contaminants present in the sediment extracts.

Evaluation of the potential interference with DNA repair mechanisms

The Comet assay was adapted to evaluate the potential interference of the total sediment extracts with DNA repair mechanisms. HepG2 cells were exposed to an alkylating agent (Ethyl methanesulfonate, EMS), for 1h at different concentrations, in order to induce DNA strand breakage. Following this exposure, cells were allowed to recover, in order to repair DNA damage, for 24 or 48h. Simultaneously, during this recovery period, cells were exposed to a non-genotoxic concentration of extracts N_1 ,N_2, R, S_1, S_2 and MW. The presence of a higher level of DNA strand breaks after the recovery period, in the presence of sediment extracts in comparison with its absence, suggests a potential interference with DNA repair mechanisms. Three independent experiments were performed for every treatment condition. For each sample and recovery period, the percentage of unrepaired damage and the percentage of relative recovery were calculated.

Evaluation of endocrine disrupting effects

To evaluate endocrine disrupting effects, the human mammary-derived MCF-7 cells were exposed for 5 days to different dilution of the total extracts, 2 positive controls (β-estradiol and bisphenol-A), and a solvent control (DMSO). The E-screen assay was then performed as described by Soto et al., (1995). After treatment, cells were stained and proliferation rates were calculated.

In vivo assays

Mice exposure to sediment extract

Mice maintenance, reproduction, treatment and sacrifice were performed according to the European Union and the Instituto Nacional de Saúde, Dr. Ricardo Jorge, I.P., Lisbon, guidelines. Three groups of six mice each were exposed orally (through drinking water) to two different concentrations of sediment extract N_2 (final doses of 73.7 and 147.3 g SEQ/day/Kg body weight; dose 1 and dose 2, respectively) and to the solvent control (DMSO, 3.33 %), during a 28 day period.

Characterization of the in vivo mutagenic effects of sediment N_2

The micronucleus assay in peripheral blood reticulocytes (Hayashi et al., 2000) was performed at 5 different time-points (i.e., before exposure and at 7 days intervals). DNA damage, as well as FPG and ENDO III sensitive-sites, were evaluated in peripheral leukocytes at the same time-points, using the *in vivo* Comet assay (Hartmann et al., 2003). At the end of the exposure period, the Comet assay was carried out with liver, spleen and kidney cells as putative target organs of the extract contaminants.

The mutant frequency was determined in liver, spleen and kidney tissues after the recovery of the *LacZ* transgene as described by Louro et al., (2002). Briefly, after genomic DNA extraction, the transgene was immunomagnetically separated and electrotransformed into E.coli ΔLacZ bacteria. After growth, cell colonies were counted in X-gal (blue colonies, e.g. non-mutated plasmid with *LacZ* gene) and P-gal mediums (white colonies, e.g. mutated plasmid with *LacZ* gene). Mutant frequency was then determined by the ratio between the number of mutant colonies and the total number of colonies.

Histopathology evaluation

After sacrifice, liver, spleen, kidney, stomach and testicle samples were collected for histopathological analysis, namely inflammation and necrosis evaluation.

Statistical analyses

Statistical analysis of data was performed using the SPSS Statistics 20.0 software. Data normality and homogeneity of variances were tested through the Shapiro-Wilk's and Levene's tests, respectively. Cytotoxicity data were analyzed by the non-parametric Mann-Whitney *U* test and the Spearman's correlation *R* statistics. Regression analysis was applied to search for dose-effect relationships and to determine the mathematical function that best describes that relationship. Comet assay results were analyzed through the 2-tailed Student's *t*-test for inter-sample comparisons following log-transformation of data to achieve a normal distribution and to comply with the homogeneity of variances. Comparison of the micronucleus assay data between exposed and control cultures was performed by the two-tailed Fisher's Exact Test. The potential interference with DNA repair mechanisms was analyzed after application of a regression analysis to search for dose-effect. Statistical significance was assumed for $p \leq 0.05$.

Results and Discussion

In vitro bioassays

Evaluation of the cytotoxic and genotoxic effects of the total extracts

The cytotoxic and genotoxic effects of total extracts obtained from sediment samples collected in several sites of the Sado and Mira Estuaries were characterized in HepG2 cells. Following a 48h exposure period, extracts of sediments from both impacted areas, the industrial/urban area (N1 and N2) and the riverine/agricultural area (S1 and S2,) produced deleterious effects in a dose-response manner as compared either to a sample from a clean area of the Sado Estuary (R) or to one sample from the Mira Estuary (MW). Furthermore, the other sample (ME) from the latter estuary was also cytotoxic and genotoxic, which might be due to the presence of unsurveyed contaminants from a nearby aquaculture facility (see Pinto et al., 2013a for details).

However, the sediment extracts from the industrial area of the Sado estuary caused a higher cytotoxicity and genotoxicity (concerning total DNA strand breakage and clastogenesis). Conversely, sediment extracts from the rural area of the estuary displayed the highest potential to induce oxidative DNA damage, as revealed by the FPG-modified Comet assay. Although the estuary has been globally classified as moderately contaminated, the results suggest that the sediments from the industrial area are significantly genotoxic and, furthermore, elicit permanent chromosome breakage as assessed by the micronucleus assay, thus being potentially more hazardous than those from the rural area. The results are consistent with contamination by pro-mutagens like polycyclic aromatic hydrocarbons (PAHs), potentiated by metals. On the other hand, the sediments from the agriculture-influenced area likely owe their genotoxic effects to metals and other toxicants, probably pesticides and fertilizers, able to induce reactive oxygen species without the formation of DNA strand breaks (see Pinto et al., 2013b for details).

The study of the potential interference of the contaminants mixture with the DNA repair mechanisms of HepG2 cells yielded positive results, suggesting that the mixture of contaminants might impact on the cells capacity to deal with genotoxic stress, possibly leading to accumulation of mutations. This effect was more accentuated in the presence of extracts from the northern industrial/urban area when compared with those from the southern riverine/agricultural area.

Regarding the endocrine disrupting effect, it was not possible to obtain reproducible results due to technical problems, related to the lack of sensitivity of the commercially available cell line (MCF-7 cells) for the performance of the E-screen assay (reported on the MSc thesis of Ana Vicente, 2012). The suitable cell line was developed from MCF-7 cells and is available from Tufts University, EUA, but its transport in adequate conditions has not been possible so far.

In conclusion, these findings suggest that the mixtures of contaminants present in the assayed sediments are genotoxic to HepG2 cells, ultimately providing a useful approach to hazard identification and being an effective line-of-evidence for environmental monitoring of anthropogenically-impacted coastal ecosystems (Pinto et al., 2013b).

Evaluation of oxidative DNA damage

Since oxidative DNA damage was observed by the Comet assay following 48h exposure to sediment extracts, a shorter exposure period, was then used in order to further investigate that effect. Interestingly, the level of extracts-induced oxidative DNA damage, as assessed by the FPG-modified comet assay, was increased and was even amplified when cells were pre-exposed to an inhibitor of glutathione synthesis, BSO. These data confirm that the induction of oxidative damage is a relevant mode of action of the mixture of contaminants extracted from the sediments, particularly from those of the southern area.

Evaluation of the cytotoxic and genotoxic effects of the fractioned extracts

The results described above together with the identification of the contaminants present in each sediment sample (task 3) suggest that the presence of metals, PAHs and other organic contaminants are responsible for the observed effects, either by inducing genotoxic effects alone or as co-mutagens in a mixture.

The fractioning with solvents of different polarities was expected to further allow the establishment of an association between a set of contaminants and its particular biological effects. DCM and *n*-hex (non-polar solvents) should be able to extract many organic compounds, mainly PAHs, which is compatible with the low levels of cytotoxicity observed. Nevertheless, only extract N_{2hex} revealed genotoxicity, which could reflect that the levels of PAHs present did not induce detectable genotoxic effects at the tested concentration range. Genotoxicity (particularly oxidative DNA damage) was observed with the methanol extraction which, along with the contamination data, could suggest that these extracts contain, predominantly, metals. Overall, the data obtained for the different extracts from each sediment sample indicate that the mixture of Met:DCM is the most appropriate to determine the overall toxic effects of a complex environmental sample. In fact, possible interactions between contaminants might be responsible for the effects detected in Met:DCM extracts, which were lost after fractioning.

Results for sediments samples from the Mira Estuary are discussed in Pinto et al., (2013a). Briefly, all extracts from sediment sample MW failed to induce cytotoxicity and genotoxicity. Nevertheless, the most polar extracts of ME yielded the highest cytotoxicity while the highest genotoxicity (including oxidative damage) was induced by the non-polar extracts. While the former caused effects similar to those expected from biocides, the latter triggered effects compatible with known pro-mutagens like PAHs, even though the overall levels of toxicants were considered of low risk.

The current results show that different extraction methods may aid at differentiating the genotoxic effects of certain sets of contaminants among complex mixtures of environmental contaminants, contributing to the establishment of a unique signature for a given anthropogenic-impacted area. Furthermore, this approach may add relevant information on the contaminants responsible for biological effects that may be subsequently used for more focused contaminant analyses

Characterization of the in vivo mutagenic effects of sediment N_2

Along the exposure period, an induction of MN was observed in mouse peripheral blood reticulocytes from the two groups of mice exposed to sediment extract N_2, as compared with the control group. Also, the level of MN at the end of the exposure period was significantly higher than at the day 7 of exposure. Nevertheless, no genotoxicity was observed by the comet assay (with or without FPG and ENDO III treatments), either in leukocytes or in liver, spleen and kidney (reported in the MSc thesis of Joana Sacadura, 2013). Results of the histopathology analysis and mutant frequencies are not presented because these two parameters are still currently being analyzed. This delay was due to the time consumption of the microscopic analysis and to a technical constrain in the recovery of the transgene DNA that make it impossible to finish this complex analysis within the duration of the project.

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Task 5

Integrated assessment of environmental risk assessment of Sado estuary sediments

Part I - Qualitative assessment

IMAR and INSA

Introduction

The integration of different Lines of Evidence (LOE), through Weight of Evidence (WOE) approaches to provide the best information for informed decision-making, is essential given the natural complexity of transitional waters. Sediment management in transitional waters needs to be sustainable, including processes at local scales using different LOE appropriately and incorporating them into integrative assessments for management decision making. Although scientific knowledge has improved, and will continue to improve, management decisions, particularly in transitional waters, must still be made without complete information and will require triage, which means a process of prioritization based upon clear goals and allocation of resources hence the importance of screening and integrative assessments (Chapman et al., 2013).

WOE is a way of synthesis and integration, as recommended for estuaries and can incorporate relative risk modeling (Chen et al., 2012). It is defined according to Burton et al. (2002), by a process used to evaluate multiple lines-of-evidence concerning ecological condition. Presently, in the absence of mechanistic stressor-response linkages, WOE relies more on Best Professional Judgment (BPJ, according to Burton et al., 2002 and Chapman and Anderson, 2005). Best Professional Judgment, which means expert opinion and judgment, can be used to address the limitation of field and laboratory investigations (Chapman et al., 2002, Bay et al, 2007). Usually these WOE and BPJ methods are mainly used for ecological risk assessment (with usual LOE for chemical assessments, toxicity and biological surveys). However, human health assessment can also be added by considering epidemiological data, often based on questionnaire surveys.

The final aim of this task was to develop an integrative methodology to evaluate the adverse effects of contaminated estuarine sediments to human and ecosystem health, based on the different lines of evidence obtained on the other tasks of the project. The integrative qualitative assessment herein presented was based on a best professional expert judgment.

Methods

Considering the data provided by the earlier tasks (1 to 4) the following lines-of-evidence were evaluated:

i) Human contamination pathways (task 1)

ii) Human health effects: chronic disease (task 1)

iii) Human health effects: reproductive health (task 1)

iv) Human health effects: health care (task 1)

v) Human health effects of local farming products consumption (tomato, lettuce, cabbage and potatoes) (task 2)

vi) Contamination of water holes and agriculture soils (task 2)

vii) Contamination of estuarine sedimentary environment (task 3)

viii) Effects on benthic organism with commercial value (sole, clam and cuttlefish) (task 3)

ix) Genotoxic potential of sediments (task 4)

A WOE approach according to a BPJ was performed based on Chapman et al. (2002) and Bay et al. (2007) procedure as explained in the next paragraphs. Each of the lines of evidence were ordinal ranked from 1 to 3 level of ecological or human health risk assessment as indicative of high, moderate, or negligible/low risk, according to a tabular decision matrix (see table 1). For each of the two surveyed areas of Sado estuary (Fig. 1), the score was ranked, comparatively to the reference location, with also an explanation of the given classification.

Table 1. Ordinal ranking scheme applied for weight of evidence categorization for each line-of-evidence.

Line of evidence	Low risk(1)	Moderate risk(2)	High Risk(3)	Explanation of the score
• Human contamination pathways • Human health effects: chronic disease • Human health effects: reproductive health • Human health effects: health care	• No evidence of epidemiologic risk (measures of comparative risk - OR- between the two study populations was not statistically significant for a significance level of 5%).	• Low evidence of epidemiologic risk (measures of comparative risk - OR- between the two study populations was not statistically significant for a significance level of 5% or potential biases identified as the study design, sample size or the absence of biological markers).	• High evidence of epidemiologic risk (measures of comparative risk - OR- between the two study populations was statistically significant for a significance level of 5%; high epidemiological plausibility of the identified associations).	• The epidemiological score was defined by qualitative integration of the following parameters: a) magnitude measurements calculated statistics (OR different from the null value of "1"; b) statistical significance (p <0.05); c) the number and quality of bias identified; d) epidemiological plausibility of the observed differences in the comparison between the two populations).
• Human health effects of local farming products consumption	• Without human health risk (according to EFSA guidelines)	• With low human health risk (according to EFSA guidelines)	• With high human health risk (according to EFSA guidelines)	• Score levels were determined based on the values guide EFSA (2009) and based on food average dose, frequency of consumption and actual weight in individual population value of Carrasqueira (obtained by food frequency questionnaire - see task 1). Analyzed on the 4 vegetables (potatoes, lettuce, tomato and cabbage), which according to the food frequency questionnaire, are the vegetables most consumed by the population of Carrasqueira. The contaminants analyzed were metals and metalloids, As, Pb, Cd, Cr, Cu, Zn and Ni. It were considered the maximum values found in samples collected in the farms. It were not considered metal concentrations in estuarine food, since bioaccumulation was only measured in target organs for ecotoxicology purpose.

Line of evidence	Low risk(1)	Moderate risk(2)	High Risk(3)	Explanation of the score
• Contamination of water hole and soil	• Without human health risk (according to national and international threshold limits)	• With low human health risk (according to national and international threshold limits)	• With high human health risk (according to national and international threshold limits)	• The water samples were analyzed in the same water hole (inside the farms) where we sampled the horticultural food, as well soil samples. The water hole is used for irrigation and also for human consumption. The contaminants analyzed were metals and metalloids (As, Pb, Cd, Cr, Cu, Zn, Ni and in water also nitrates and nitrites). Values were compare with water national law (for water DL 306/2007 de 27 de Agosto) and soil quality Canadian guidelines (CCME, 2007).
• Contamination of estuarine sedimentary environment	• Sediments without risk of causing adverse effects in estuarine organisms: Sediment Quality Guideline-Quotient <0.1 (SQG-Q)	• Sediments wit low risk of causing adverse effects in estuarine organisms: Sediment Quality Guideline-Quotient 0.1<SQG_Q < 1	• Sediments with high risk of causing adverse effects in estuarine organisms: Sediment Quality Guideline-Quotient > 1.	• The levels of this score are defined by MacDonald et al, 2004 and calculated according to Long and MacDonald, 1998 through the overall average ratio for all contaminants analyzed (SQG-Q). Concentration of each contaminant (those who were measured: metals, PAHs, pesticides (DDT and metabolites) and PCBs), divided by the value guide defined by Long and MacDonald (1998). These values measure the potential to cause adverse effects on estuarine organisms. It was considered the maximum value of SQG-Q for each station in each area.
• Effects on benthic organism with commercial value	• Without risk of causing adverse effects in estuarine organisms (based on IBR)	• With low risk of causing adverse effects in estuarine organisms (based on IBR)	• With high risk of causing adverse effects in estuarine organisms (based on IBR)	• The levels of this score were determined based on an aggregation of integrated response index (IBR - see task 3) to several biomarkers in the analyzed species (cuttlefish, clams and sole - according to the food frequency questionnaire, are the estuarine foods most consumed by the population of Carrasqueira): The biomarkers analyzed were: i) the induction of methalothionines (biomarker that evaluates exposure of the metal bodies), ii) antioxidant enzymes such as catalase and glutathione S-transferase (the activity of these enzymes is a biomarker of exposure given that these enzymes are induced in response) , iii) the histopathological condition weighted indices (these ratios determined in Costa et al. , 2013) is mostly determined by biomarkers of effect , evaluating the changes and lesions in tissues of target organs of the body such as the liver or digestive gland), iv) lipid peroxidation, which is a biomarker for assessing the biochemical effect lesions caused by reactive oxygen species. Biomarkers of effect, although less specific (hindering the cause and effect relationship), are standardized, and their interpretation is made as a single index.
• Genotoxic potential of sediments	• Absence of a genotoxic potential (GPI < 10; No cytotoxic, genotoxic, oxidative stress or DNA repair interference were observed).	• Evidence of low genotoxic potential (10 ≥ IPG > 50; Cytotoxic or genotoxic effects were observed in 10 to 50 % of the parameters considered for the calculation of the GPI).	• Evidence of moderate genotoxic potential (IPG ≥ 50; Cytotoxic or genotoxic effects were observed in more than 50 % of the parameters).	• The Genotoxic Potential Index (GPI) was derived following data obtained for different endpoints from the in vitro assays in HepG2 cells (see task 4), namely: DNA strand breakage (Comet assay); oxidative DNA damage (Comet assay with FPG); chromosomal instability (micronucleus assay); replication index (micronucleus assay); cytotoxicity (NR assay) and interference with DNA repair (Comet assay following a genotoxic stimulus). The index was calculated by attributing a value of 0 (negative) or 1 (positive)

Line of evidence	**Low risk(1)**	**Moderate risk(2)**	**High Risk(3)**	**Explanation of the score**
				to the following analyzed parameters. p value: Positive when $p < 0.05$. Relative to statistical significance when comparing the observed effect in a cell culture exposed to a given concentration of a sediment extract with that observed for a non-exposed culture (negative control). Induction factor: Positive when ≥ 2 (e.g. higher than twice the effect observed for the negative control). Given by the ratio between the value obtained for the observed effect in a cell culture exposed to a given concentration of a sediment extract with that observed for a non-exposed culture (negative control). Dose response: Positive when $R^2 \geq 0.8$ and slope p value < 0.05. Refers to mathematical model that best describe the dose-response relationship(linear regression). Interference with the DNA repair: positive when more than 25 % of the gentoxin-induced DNA damage remained unrepaired in the presence of a sediment extract. For each sediment sample, the GPI was then calculated as the ratio between the sum of the obtained values and the total number of observations (x100).

Fifteen experts elected from the team members from different scientific backgrounds (epidemiology, nutrition, ecology, human health and ecotoxicology), scored the two areas of Sado estuary (Fig. 1 and see task 3), compared to the control population and estuarine area. In the case of the epidemiological LOE, only one global estuarine area was considered. Experts were asked to rank each of the two Sado areas in terms of human health and ecological risk from best to worst assigning each site to 1 to 14 absolute condition categories (see table 2), according to their best expert professional judgment. The categories were adapted from Bay et al., 2007, Chapman and Anderson, 2005 and Bay and Weisberg, 2012, although those authors only considered the ecological risk. Besides the rank, experts were also called to justify their qualitative scoring .

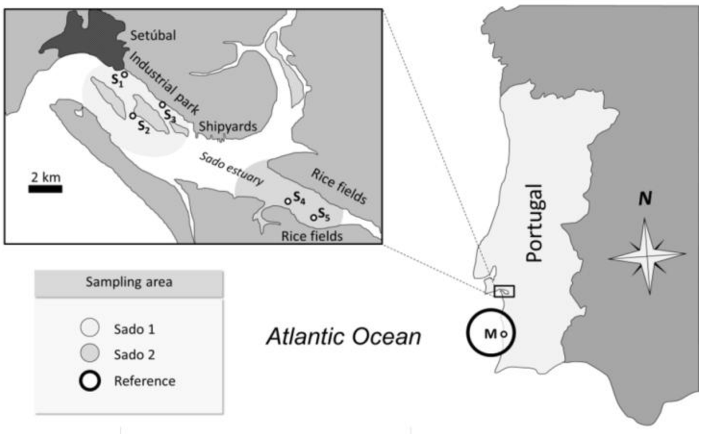


Figure 1. Location of Sado estuary areas and reference location (the Mira estuary and Vila Nova de Mil Fontes, VNMF).

The expert scores were analyzed in terms of median and relative frequencies and overall disagreement. For the overall disagreement, first, for each expert, we identified the total number of categories for which the expert's categorical assessment of a site different from the median categorical assessment of all other experts for that site. The number of differences was then summed for all sites to indicate the overall rate of disagreement. A content analysis was conducted to each expert explanation of their rank. Categories and units of analysis were coded and their frequencies' rate calculated, according to Bryman (2012).

Table 2. Absolute condition categories for ecological and human health risk assessment. EE, estuarine ecosystem. HH, human health.

Grade	Designation	Impact likelihood	Disagreement among LOEs	Impact target	Action
1	Unimpacted	Negligible	No	EE + HH	None
2	Likely ecologically unimpacted	Likely unimpacted	Yes	EE	More ecological studies, understanding of reasons of alterations
3	Likely human health unimpacted	Likely unimpacted	Yes	HH	More epidemiological studies understanding of reasons of alterations
4	Likely environmentally unimpacted	Likely unimpacted	Yes	EE + HH	More epidemiological understanding of ecological studies and reasons of alterations
5	Possibly ecologically impacted	Possible	Yes	EE	As above and monitor EE
6	Possibly human health impacted	Possible	Yes	HH	As above and monitor HH
7	Possibly environmentally impacted	Possible	Yes	EE + HH	As above and monitor EE + HH
8	Likely ecologically Impacted	Persuasive	Yes	EE	As above and EE alert/response system
9	Likely human health Impacted	Persuasive	Yes	HH	As above and HH alert/response system
10	Likely environmentally Impacted	Persuasive	Yes	EE + HH	As above and EE + HH alert/response system
11	Clearly ecologically Impacted	Certain (severe adverse impact)	No	EE	As above and EE remediation, management, regulation
12	Clearly huamn health Impacted	Certain (severe adverse impact)	No	HH	As above and HH remediation, management, regulation
13	Clearly environmentally Impacted	Certain (severe adverse impact)	No	EE + HH	As above and EE + HH remediation, management, regulation
14	Inconclusive	Unknown	Yes	EE + HH	Review data, gather additional information

Results and discussion

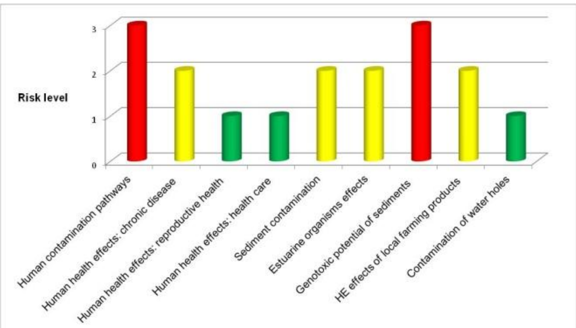
Prior to expert classification, each LOE were scored from 1 to 3 for the Sado 1 and 2 areas, and an explanation given (developed by the team member of each task and corresponding lines of evidence) (see table 3).

Both Sado areas had the same scores with exception for the LOE of genotoxicity in sediments. Nevertheless, the explanation of the scores showed differences in both stations in terms of exposure and effects (see Figure 2 and Table 3).

Table 3. Scores for each Sado 1 and 2 areas, according to the results of each line of evidence, obtained in earlier tasks.

Line of evidence	Sado 1	Sado 2	Explanation of the score
• Human contamination pathways	3	3	• Participants of Carrasqueira reported higher percentage of tasks that promote occupational exposure to direct and / or indirect contaminants, with statistically significant differences ($p < 0.001$ direct exposure and $p < 0.004$ indirect exposure). The ratio of the percentage of individuals Carrasqueira vs VNMF with direct or indirect exposure is 40.6 and 2.5, respectively. There were no differences in other pathways and participants of VNMF still arise more frequently related activities without important exposure. As for leisure, related to fishing and farming activities, participants Carrasqueira also revealed a higher frequency of such activities (statistically significant difference, $p < 0.001$), a ratio of 2.3 to 3.1 in agriculture and fishing. Same classification in Sado 1 and 2, since this line of evidence was measured in population of Carrasqueira.
• Human health effects: chronic disease	2	2	• The participants of Carrasqueira revealed a higher proportion of diseases related to exposure in the study, including kidney disease, liver disease, neurological diseases, skin diseases, renal failure and malignancy. The ability to have at least one of these chronic disease in Carrasqueira was 2.1 times higher when compared with the same possibility in VNMF (association measured by Odd ratio adjusted for age and time of residence in the town, 95% CI: 1.02, 4.30). This risk was statistically significant, however cannot be classified as level 3 due to: i) reduced sample size; ii) the study design is not appropriate to draw conclusions about the causality of exposure and the appropriate effect; iii) information corresponds to self-reported data, which may introduce information bias. Same classification in Sado 1 and 2, since this line of evidence was measured in population of Carrasqueira.
• Human health effects: reproductive health	1	1	• About the adverse pregnancy effects, outputs estimates indicate that the possibility of at least one pregnancy which resulted in a miscarriage or stillborn baby is lower than in the Carrasqueira compared to VNMF (OR = 0.65, 95% CI: 0.29, 1.47). When the effect in question is a pregnancy end in a child / malformed fetus, or syndrome or genetic disorder, mental illness or the possibility of metabolic disease, occurrence is 1.5 times higher in exposed than in unexposed (95% CI of OR = 0.47, 4.92). Nevertheless none of these OR are statistically significant. Given the dispersion of the results, the small sample size, the study design not be appropriate and the potential bias of information by data being self-reported, there is no evidence of effects on reproductive health. Same classification in Sado 1 and 2, since this line of evidence was measured in population of Carrasqueira.
• Human health effects: health care	1	1	• The multiple indicators of health care surveyed did not indicate differences between the two samples. Notably, there is no significant differences in the number of medical appointments, and most participants were not subject to any hospitalization. Participants of VNMF had a higher percentage of being hospitalized at least once in the last 12 months and the use of other health professionals mostly revealed no statistically significant differences. Thus, compared to VNMF there is no evidence of increased use of health care of the population of Carrasqueira. Same classification in Sado 1 and 2, since this line of evidence was measured in population of Carrasqueira.
• Human health effects of local farming products consumption	2	2	• The score of this classification is due to the concentration found for arsenic, that have the highest value of all analyzed metals and metalloids (As, Pb, Cd, Cr, Cu, Zn, Ni); in the worst case an intake of 0.8 g / day / kg of As was found (body weight). This value was found in the group of children. For its calculation it was considered: i) the maximum concentration of arsenic and that is 100 % inorganic (according to literature inorganic arsenic in vegetables is more than 90 % of the total, Nortan , et al., 2013); ii) the food average dose; iii) frequency of consumption and individual weight of the population of Carrasqueira. The obtained value was compared with the guideline of the EFSA (2009) - 0.3 to 8 g / day / kg a threshold of having potential to cause teratogenic effects. The others metals and metalloids concentrations were below values that can cause effects on human health. These results are of concern in particular for children since only 4 vegetables (potatoes , lettuce, tomato and cabbages) were analyzed and already exceeded the amount of daily intake established by EFSA. The study of the speciation of arsenic in these

Line of evidence	Sado 1	Sado 2	Explanation of the score
			vegetables should be conducted to confirm the levels of inorganic arsenic. Same classification in Sado 1 and 2, since this line of evidence was measured in the farms of the population of Carrasqueira.
• Contamination of water hole and soil	1	1	• Water and soil contaminants concentrations were all below recommended values for the legislation of drinking water and soil quality for all elements analyzed. Same classification in Sado 1 and 2, since this line of evidence was measured in water holes of the farms of the population of Carrasqueira.
• Contamination of estuarine sedimentary environment	2	2	• .1 <SQG_Q <1 in both areas. In sediments located in **Sado 1** area, S3 (see task 3) is the most contaminated station (the other stations are fundamentally clean sand with little organic content). The Sado 1 is one of the areas where fishermen caught and harvest bivalve. At station S3 (see task 3) metallic contaminants with higher concentration and that makes sado 1 rated risk level as 2 are the concentrations of Zinc and Copper (with the upper limit of likely adverse effect on organisms), followed by at lower concentrations Cr, and Pb (values above the lower limit of possible adverse effects on estuarine organisms), some PAHS (acenaphthylene, acenaphthene, fluoranthene, pyrene, anthracene dibenzo) also have values above the lower limit of possible adverse effects on estuarine organisms. Both S4 and S5 sediments stations located in **Sado 2** (see task 3) have moderate levels of risk of causing adverse effects in organisms. The Sado 2 represents one of the other areas where fishermen caught and harvest bivalve. The metal contaminants with higher concentrations in this area and that makes Sado 2 rated with risk level 2 are Zinc (with the upper limit of likely adverse effect on organisms values), followed in smaller concentrations by, As, Cr, Cu and Pb (values above the lower limit of possible adverse effects on estuarine organisms).
• Effects on benthic organism with commercial value	2	2	• **Sado 1**: The index of response biomarkers was similar to **Sado 2**, although showing a distinct pattern of response. It revealed a number of mixed responses and apparently related with heterogeneous mixture of contaminants found in this area. The organisms of this area of the estuary have significantly different responses (compared to the reference station): i) induction of metalotieninas in sole and cuttlefish, ii) the activity of enzymes related to oxidative stress, particularly in cuttlefish, iii) the lipid peroxidation (indicating stress oxidative) in all species, especially fish and cuttlefish. The presence of pathological liver injury in the sole is consistent with moderate to prolonged stress (being absent neoplastic and neoplastic lesions). Fish and cuttlefish were potentially most affected organisms in Sado 1. **Sado 2:** The index of response biomarkers was broadly similar to Sado 1, but also revealing a set of mixed responses probably related to mixing and heterogeneity of contaminants found in this area. The organism in this area of the estuary have significantly different responses (compared to the reference station) for: i) induction of methalothionines but only in clams, ii) the activity of enzymes related to oxidative stress in all species analyzed, iii) the lipid peroxidation (indicating oxidative stress) in clams and cuttlefish and iv) moderate histopathological changes in the liver of soles and more severe in the digestive gland of clams (including necrotic lesions and acute inflammation not motivated by infectious agents). Bivalves showed more significant responses in Sado 2.
• Genotoxic potential of sediments	3	2	• **Sado 1**: IPG ≥ 50.It was observed cytotoxic or genotoxic effects in more than 50% of parameters considered for calculating the IPG (endpoint for each considered value of p statistical comparison between treated and negative control cells, the existence of a dose-response relationship, and increment the value obtained for treated cells compared to negative control). **Sado 2:** 10 <IPG <50. It was observed cytotoxic or genotoxic effects in 10 to 50% of parameters considered for calculating the IPG.

a) 

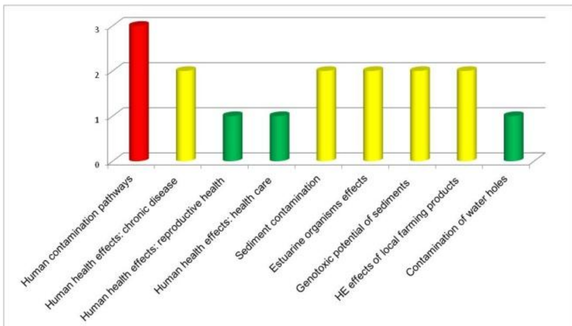
b) 

Figure 2. Risk level of each LOE for a) Sado 1; b Sado 2.

Based on the data from Table 2, the fifteen experts categorized, in terms of ecological and human health risk assessment the two locations, Sado 1 and 2 (see table 3), and justified their choices. Results of the content analysis of the explanations/justifications given by the experts for their classification are shown in Table 4. Sado 1 was classified has a likely ecologically impacted compared to Sado 2 that was classified as a possibly environmentally impacted location, although a much higher disagreement rate was observed in Sado 1 compared to station 2 (73 % compared to 13 %, respectively).

Table 3. Results of overall classification by the 15 experts for Sado 1 and 2.

Category	Designation	Number of votes Sado 1	Sado 2
7	Possibly environmentally impacted	6	13
8	Likely ecologically Impacted	4	1
9	Likely human health Impacted	0	0
10	Likely environmentally Impacted	5	1
	Median	**8**	**7**
	Disagreement Rate	**73 %**	**13 %**

Ecological risk in terms of effects on estuarine species and sediments contamination, both in Sado 1 and 2, is a concern pointed out by the majority of experts (see table 4). According to Caeiro et al. (2009), in recent years, the reduction of direct industrial and urban wastewater discharges into the Sado estuary reduced the pollution in the estuary, but "hotspots" in confined sedimentary areas still persist, which is in accordance with these results. The main differences between Sado 1 and 2 are related with different contaminants of concern (Zn, Cu, PAH in Sado 1 compared to only Zn in Sado 2). Also, the ecological effects occurred differently in both areas, with more significant effects being shown in species with more mobility in Sado 1, like the cuttlefish and sole, and higher in burrowing species, namely clams, in Sado 2. Genotoxic (and potentially carcinogenic) effects in sediments and human exposure from agriculture and fisheries activities are issues that were also pointed out by half of the experts, in terms of human health risk, in both locations. Genotoxic effects were higher in Sado 1 (see Table 3 and figure 2), an area in the northern part of the estuary near the industrial belt and point pollution sources. In terms of agriculture exposure the concerns are related with the observed relatively high concentration of As in potatoes (see table e and task 2).

The need for more studies, pointed out by half of experts (53 and 54 % for Sado 1 and 2 - table 4) confirmed the need to find better cause-effects associations that link the anthropogenic pressures in the estuary with the ecological and human health effects. 20 % of the experts stressed that there is no clear evidence of effects on human health. Although integrative WOE assessments are considered the most appropriate approach, establishing causation requires knowledge of all stressors, receptors, and potential activities that could affect both in any transitional water body and adjoining terrestrial environments (Chapman et al., 2013). More detailed epidemiological studies, determination of other persistent organic pollutants in sediments and biomarkers in humans are suggestions given by the experts as the more studies needed. The requirement of monitoring programs in the estuary was also highlighted by the experts as future recommendation (27 and 20 % for Sado 1 and 2 - table 4).

Table 4. Content analysis of the explanations/justifications given by the experts for their scores.

Stations	Categories	Units of analysis (percentage of frequency)
Sado 1	Ecological risk	• Effects on estuarine species cuttlefish and sole (87 %) • Sediments contamination (Zn, Cu, PAH) (47 %)
	Human health risk	• Genotoxic effects in sediments (potentially carcinogenic) (47%) • Human exposure (agriculture and fisheries) (20%) • Association with health effects: chronic diseases (53%) • Contamination of food (although only low and for As) (20%)
	Recommendations/gaps	• More studies (epidemiological, persistent organic pollutants (POP), biomarkers in humans, bioaccumulation) (53%) • Disagreement in the LOE (33%) • No clear evidence of effects on human health (20%) • Alert / monitoring systems (27%)
Sado 2	Ecological risk	• Effects of estuarine species but less than 1 Sado (clams) (87%) • Sediment contamination but smaller than 1 Sado (Zn) (53%)
	Human health risk	• Genotoxic effects in sediment but smaller than Sado 1(53%) • Human exposure (agriculture and fisheries) (33%) • Association with health effects: chronic diseases (53%) • Contamination of foods (although only low and for As) (27%)
	Recommendations/gaps	• More studies (epidemiological, POP, human biomarkers bioaccumulation) (47%) • Disagreement in LOE (27%) • No clear evidence of human health effects (20%) • Alert / monitoring systems (20%)

Considering the ranked condition categories for risk assessment in the locations of fishing areas of Sado 1 and 2 and besides the uncertainties the BPJ highlighted, management options should be advised. As stressed by Chapman et al. (2013), we need to begin intentional interventions where necessary and appropriate to maintain essential ecosystem services associated with sediments in transitional water bodies. At the environmental level, several local interventions could be conducted, specifically:

- adopt, through the appropriate state agencies, control and environmental requalification measures;

- define and implement a monitoring plan for the estuary (including ecological risk and human health)

In the field of health effects in the exposed populations, the following issues should be considered:

- local populations should reduce direct contact with the sediment, particularly when harvesting clams and other shellfish.

- regarding the cuttlefish, in particular, it is advisable to consume without the ink sac and viscera.

- propose to conduct further epidemiological studies with more representative samples - including blood analysis in sampled population for analysis of contaminants and biomarkers, allowing to detect early biological effects before the onset of illness.

Conclusions

A best professional judgment for a weight of evidence approach based on eight different lines of evidence was used to assess not only the usual ecological risk assessment but also the risk for human health, allowing an overall environmental assessment.

The estuary should not be regarded as impacted by a specific toxicant, but by a complex mixture of contaminants (both organic and inorganic). According to the WOE approach, there is a likely impact on estuarine ecosystems, since there are proven adverse effects on species with commercial value. Although there is no clear evidence of adverse health effects in the local population, it is possible that exposure to estuarine contaminants during their lifetime constitutes a risk factor for the development of chronic-degenerative diseases that have a long latency period before being symptomatic . Public health awareness campaigns will be conducted by the local health professionals.

As futures developments, more deep epidemiological studies should be conducted including blood analyses in the sample population for analysis of contaminants and biomarkers of effects and exposure. Analysis of more persistent organic pollutants in sediments and bioaccumulation in estuarine species should be also conducted. Analysis of organic contaminants in vegetable, water and soil should also be investigated, as well as arsenic speciation. Quantitative methods for environmental risk assessment using multivariate statistical of integrated assessment or risk assessment per toxic substance (e.g. modeling of chemical equilibrium/partition, probabilistic estimation and / or methods of quotients of toxicity and index of epidermal risk), should also be performed and compared with BPJ following Bay and Weisberg, 2010. Nevertheless the qualitative methodology used could be applied to other estuaries in Portugal to also evaluate and compare their environment risk assessment associated with the estuarine ecosystem.

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Task 5

Integrated assessment of environmental risk assessment for Sado estuary

Part II - Quantitative assessment

Universidade do Algarve

Objective

According to the working plan for the extended period of the project (from May to October of 2013) a collaboration with Universidade do Algarve, researcher Luis Nunes, resulted in a first draft calculations for the probabilistic estimation of the risk due to ingestion of contaminated food, through the use of Monte Carlo simulation. The present section makes the human health risk assessment for the Carrasqueira population due to the ingestion of contaminated food, as proposed in Task 5. In the present text only the ingestion of clams from the Sado estuary is considered.

Methods

To determine individual exposure due to the ingestion of clams contaminated with heavy metals, the estimated daily intake of a metal i, is obtained by multiplying daily consumption rate, *IR* (kg/day), by the concentration of the metal, C_i (mg/kg). This result is designated the average daily intake, ADI_i (mg/day):

$$ADI_i = IR \cdot C_i$$

The individual risk for carcinogenic substances is determined by the "incremental individual lifetime cancer risk" is given by:

$$ILCR_i = \frac{ADI_i \cdot ED \cdot EF}{BW \cdot LE} \cdot CSF$$

Where ED is the exposure duration (year); EF is the exposure frequency (day/year); BW is the body weight (kg); LE is the life expectancy of the exposed person (day); CSF (cancer slope factor) is the oral cancer slope factor for the heavy metal (per mg/kg/day).

Total carcinogenic risk due to ingestion of contaminated food is obtained by adding the ILTR calculated for each metal.

For non-carcinogenic substances the hazard quotient is used instead:

$$HQ_i = \frac{ADI_i}{RfD_i}$$

Where RfD is the reference dose (mg/kg/day). Total non-carcinogenic hazard due to ingestion of contaminated food is obtained by adding the HQ calculated for each metal.

Values of CSF and RfD are shown in Table 1.

Table 1. Cancer slope factor and reference dose.

Heavy metal	RfD (mg/kg/d)*	CSF $(mg/kd/d)^{-1}$ **
As		1.5
Cr		0.5
Pb		No value yet established
Cd	1.0x10-3	
Cu	1.5x10-1	
Ni	2.0x10-2	
Se	5.0x10-3	
Zn	2.0x10-1	

*: from: http://rais.ornl.gov/tox/profiles/copper_ragsa.html;
**: http://www.deq.virginia.gov/Portals/0/DEQ/Land/ Remediation Programs/VRPRisk/RagTables

Data and assumptions

The present risk estimates are based on the field data available at the moment, which is limited to concentrations of the different heavy metals in the digestive tract of clams. The data is presented in Table 2. The remaining model parameters were obtained from bibliography, as presented in Table 3.

Table 2. Metal content in the digestive tract of clams collected in the Sado estuary.

	Heavy metal (μg/g dw)							
Site	**Cr**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Cd**	**Pb**
Sado 1	5.46	12.89	45.07	564.85	50.02	7.38	0.61	2.53
Sado 1	2.09	11.58	68.53	226.03	46.67	8.44	4.61	7.94
Sado 1	16.70	30.72	84.95	638.96	27.58	6.33	2.53	9.79
Sado 1	0.80	1.84	14.36	57.71	7.00	1.56	0.08	1.09
Sado 1	14.82	21.75	144.87	338.00	49.11	11.74	0.50	10.19
Sado 1	5.33	8.27	69.57	282.62	24.59	6.07	1.28	7.90
Sado 1	6.12	9.48	325.43	447.69	6.68	3.79	0.43	19.68
Sado 1	7.87	2.32	52.81	622.21	52.47	78.61	0.85	3.21
Sado 1	12.30	17.49	104.29	928.51	33.59	10.04	1.26	5.83
Sado 1	8.63	4.06	66.87	686.77	11.30	4.09	3.10	8.25
Sado 1	7.56	7.89	158.72	442.72	36.55	6.05	0.05	3.06
Sado 1	13.61	5.81	129.46	1451.17	26.68	7.41		18.39
Sado 1	21.80	19.77	349.26	2913.15	18.84	6.28	44.25	205.21
Sado 1	13.27	7.61	127.40	674.69	31.90	8.67	4.30	31.86
Sado 1	9.51	2.26	70.31	453.27	33.86	8.10	0.79	9.53
Sado 1	6.34	4.41	98.02	684.11	19.12	5.72	0.89	24.62
Sado 1	3.72	2.98	131.81	493.13	37.73	8.79	0.10	0.23
Sado 1	11.22	5.22	83.77	1021.80	31.98	10.55	0.04	9.54
Sado 1	15.25	8.15	106.00	851.57	41.96	8.29		6.29
Sado 2	8.18	5.06	57.92	113.15	22.23	4.91	0.35	-
Sado 2	9.09	10.53	46.83	147.48	18.20	3.49	0.07	
Sado 2	6.28	4.89	34.63	85.61	31.50	5.73		
Sado 2	1.70	3.76	31.85	91.76	26.78	3.10	0.27	
Sado 2	11.71	4.75	28.27	75.15	14.60	2.05		
Sado 2	13.17	6.10	51.11	132.30	18.91	3.09		
Sado 2	18.98	7.58	35.48	144.83	19.97	1.90	3.02	
Sado 2	2.53	3.69	39.54	113.92	30.39	3.53	0.41	
Sado 2	13.34	5.26	37.24	104.03	27.57	5.96	0.37	
Sado 2	6.42	3.24	27.68	155.01	17.95	2.77		
Sado 2	14.22	20.36	43.78	191.48	26.96	4.21	1.90	
Sado 2	4.01	6.42	34.35	80.49	24.09	4.68	0.35	
Sado 2	3.85	2.10	25.11	87.58	16.21	3.42	0.22	

	Heavy metal (µg/g dw)							
Site	**Cr**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Cd**	**Pb**
Sado 2	47.57	14.37	30.91	106.25	25.55	0.00	1.94	
Sado 2	13.20	5.17	20.99	149.81	22.52	2.03	1.33	
Sado 2	7.55	10.50	50.64	187.50	31.18	3.53		
Sado 2	10.06	5.64	47.75	198.04	19.03	5.39	2.79	
Sado 2	20.11	8.41	71.43	333.73	21.62	3.20	1.53	
Sado 2	9.29	5.59	37.08	99.19	23.60	4.01	0.02	
Sado 2	5.67	5.22	46.25	155.38	22.19	4.91	0.10	1.44
Sado 2	22.86	8.39	42.97	344.98	15.29	4.83	0.42	0.23
Sado 2	10.88	6.51	44.63	183.77	25.74	5.31	0.26	1.25
Sado 2	7.59	4.90	56.52	146.02	23.62	3.71		0.59
Sado 2	5.38	3.20	38.28	230.52	17.18	2.82	0.57	1.43
Sado 2	7.18	3.13	33.44	124.09	20.03	4.73		0.59

Table 3. Assumptions

Parameter	Value	unit	Observation	Source
Ingestion rate (IR)	3.0859×10^{-3}	kg/day		This study
Exposure duration (ED)	70	year		
Exposure frequency (EF)	365	day/year		
Life expectancy (LE)	70 x 365	day		
Body weight (BW)	70	kg	Average of male and female	ISHST (2006)
Conversion factor from dw to ww (clams)	Divide by 4			This study
Concentration of metals in clam tissues	Multiply by 0.1		Digestive tract is about 10% of total weight	This study

Results and discussion

The data available for the estimation of the risks are affected by many sources of variability, many of which are not intrinsic to the phenomenon in study, as for instance due to incorrect choice of sampling locations, sample handling, chemical determination. Therefore generating a large number of concentration values and passing them with random combinations into the models, i.e., a probabilistic analysis may provide a better representation of the phenomenon and allow the estimation of posterior statistical density functions. An effective way to achieve this is by identifying probability density functions (pdf) for each variable in the model and then to compare them to theoretical distributions. Probabilistic analysis uses the Monte Carlo method by developing a large number of realizations of heavy metal concentrations using the adjusted pdf. Monte Carlo simulation produces pdf of possible outcome values, instead of single values, allowing a richer analysis than that of a deterministic approach. Probabilistic risk estimation was implemented in Model RisK® (Oracle). Several statistical distributions were adjusted to concentration data, after adjusting for wet weight, i.e., dividing by 4, using the Anderson-Darling test. Lognormal distribution was the one that best fitted to all heavy metal experimental pdf. These results are shown in Table 4.

Table 4. Anderson-Darling test results for lognormal distribution

Heavy metal	A-D value	*p*	Average	Stand. deviation
As	0.320	0.384	6.54	2.65
Cr	0.223	0.719	2.63	1.82
Pb	0.358	0.534	4	13.9
Cd	0.262	0.734	0.52	1.79
Cu	0.421	0.214	18.2	15.5

Ni	0.229	0.719	2.02	1.72
Se	0.689	0.032	1.59	1.34
Zn	0.296	0.558	106.1	181.5

Monte Carlo simulations were made using the models for ILCR and HQ using the pdf presented in Table 4 for heavy metal concentrations in clam digestive tract. Concentrations in the edible part was obtained by multiplying the concentration in the digestive tract by the fraction of its weight on the mass of the organism, i.e., 0.1. The value of the remaining parameters was considered constant, as presented in Table 3. One thousand iterations with Latin Hypercube sampling were made to assure convergence, measured by the stabilization of the estimated percentiles. The following estimates were obtained: $ILCR_i$; HQ_i; total ILCR; total HQ. Results are shown in Table 5. Histograms for both data and estimates pdf are provided in Annex A.

Table 5. Percentiles of health risk estimates **-** probabilistic assessment.

Heavy metal	**ILCR**			**HQ**		
	P10	**Median**	**P90**	**P10**	**Median**	**P90**
As	2.26x10^{-5}	4.13x10^{-5}	6.64x10^{-5}			
Cr	1.83x10^{-6}	4.89x10^{-6}	1.08x10^{-5}			
Pb	-	-	-			
Cd				8.35x10^{-5}	6.45x10^{-4}	4.99x10^{-3}
Cu				1.85x10^{-4}	4.01x10^{-4}	1.02x10^{-3}
Ni				1.55x10^{-4}	3.33x10^{-4}	8.47x10^{-4}
Se				5.21x10^{-4}	1.05x10^{-3}	2.63x10^{-3}
Zn				4.70x10^{-4}	1.22x10^{-3}	4.96x10^{-3}
TOTAL	2.78 x10^{-5}	4.71 x10^{-5}	7.28 x10^{-5}	2.72x10^{-3}	5.03 x10^{-3}	1.23 x10^{-2}

Percentile 90, commonly used as the conservative health risk estimate, indicates a ILCR above the 1x10^{-6} threshold, i.e., of a relevant risk, for both As (6.64x10^{-5}) and Cr (1.08x10^{-5}), and in consequence also for Total ILCR (7.28 x10^{-5}). Non-carcinogenic risks are all well below the 1.0 threshold, indicating a large margin of safety.

These results are still preliminary in terms of the probabilistic analysis, as the pdf for a large number of parameters remains to be assessed. This is the case of the population weight, age and sex distribution, and ingestion rates. Other sources of incorporation must also be considered, namely due to the ingestion of water, soil, ingestion of other foodstuff, and dermic contact with contaminated sediment.

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ANNEX A – Probability density functions

Table A1. Assumption pdf

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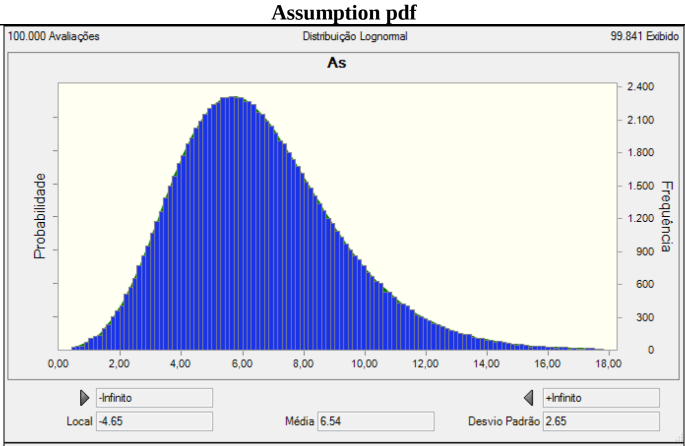
<tr><td>As</td><td>
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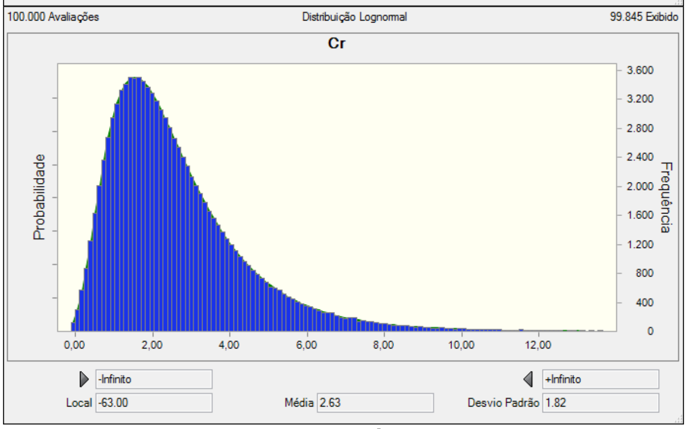
<tr><td>Cr</td><td>
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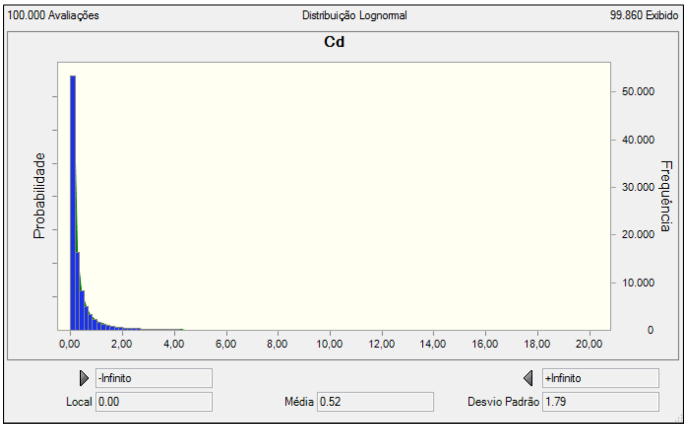
<tr><td>Cd</td><td>
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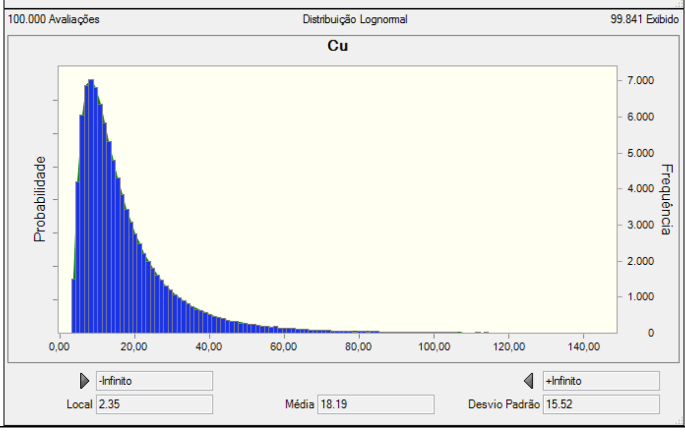
<tr><td>Cu</td><td>
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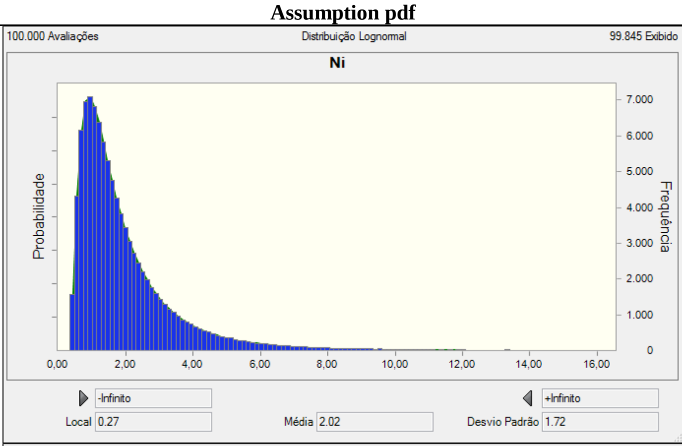
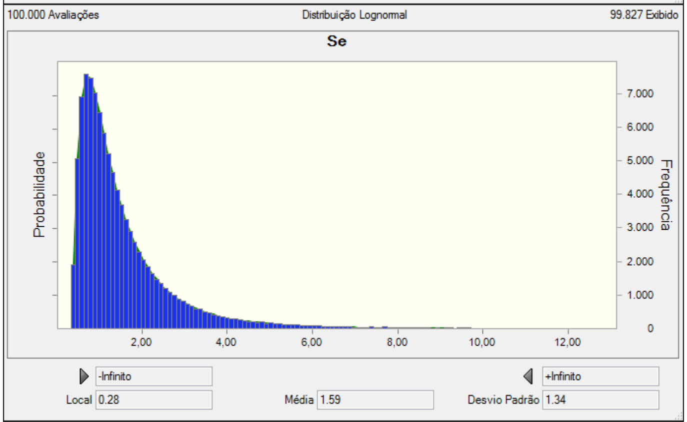
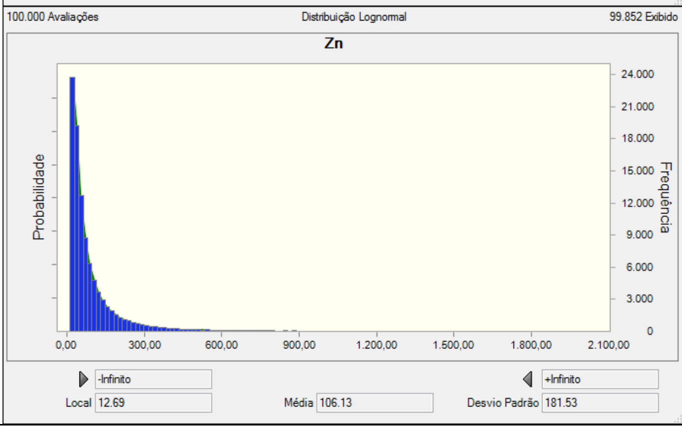
Heavy metal	Assumption pdf
Ni	
Se	
Zn	

Table A1. Estimates pdf

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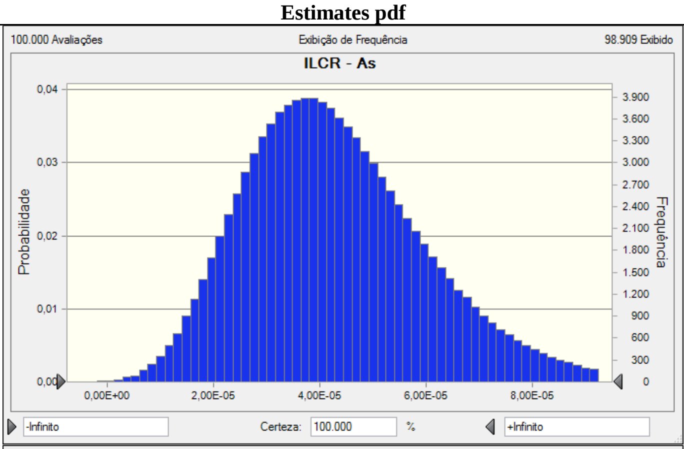
<tr><td>ILCR As</td><td>
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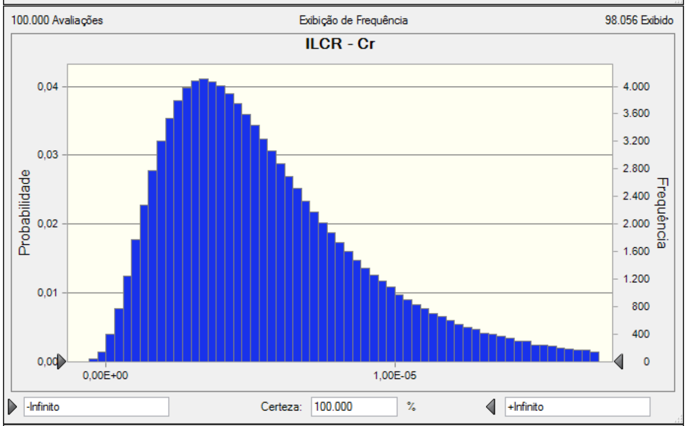
<tr><td>ILCR Cr</td><td>
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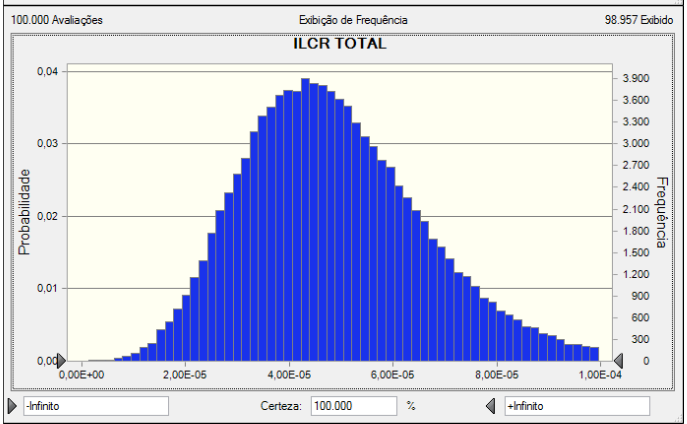
<tr><td>Total ILCR</td><td>
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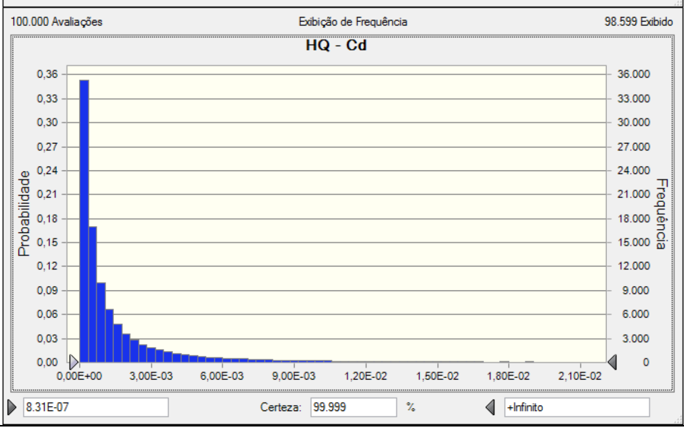
<tr><td>HQ Cd</td><td>
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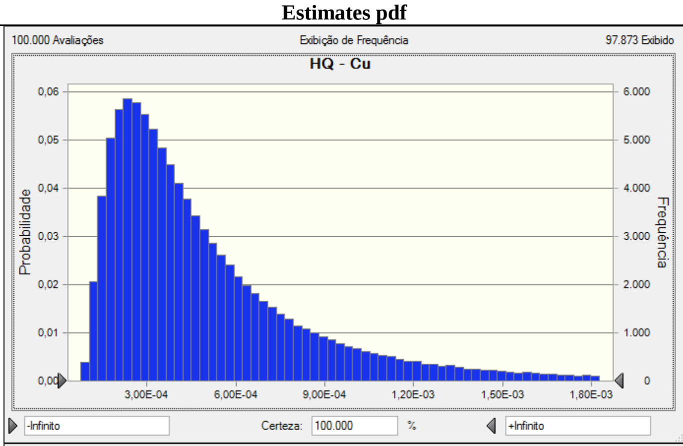
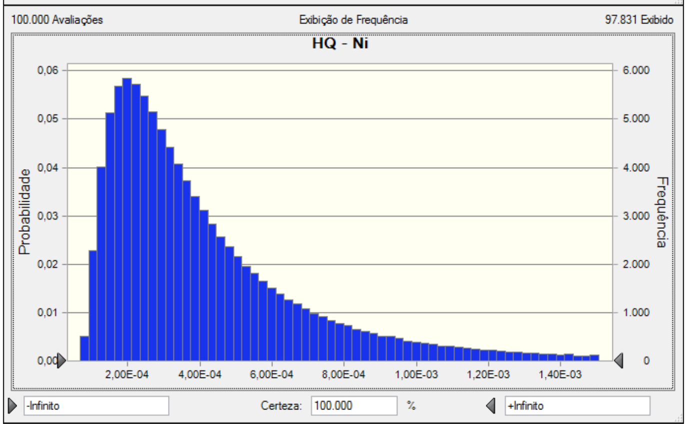
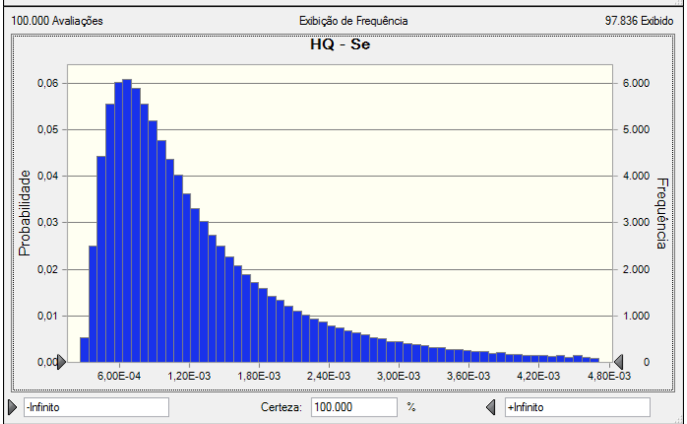
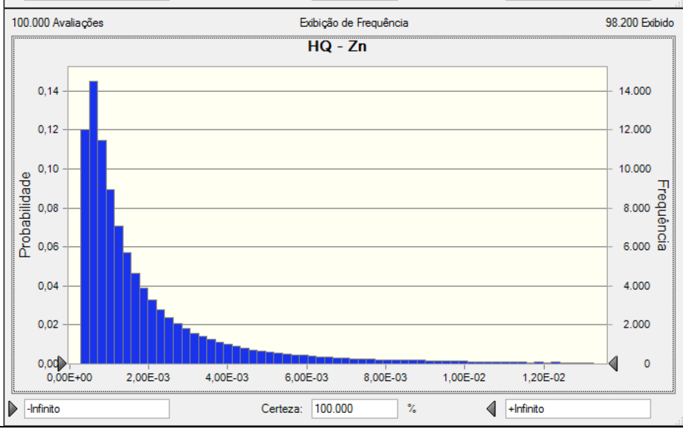
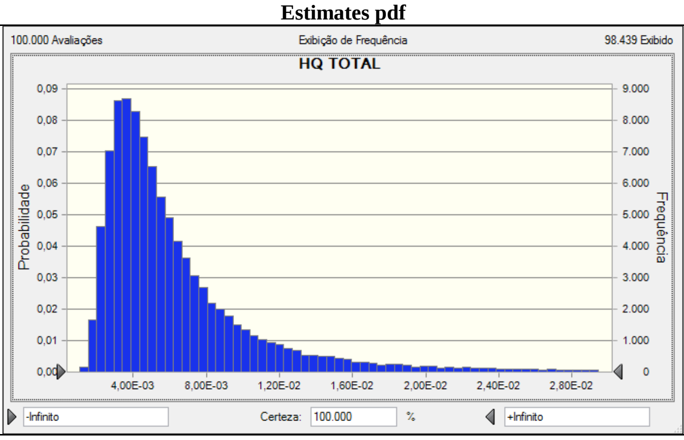
Heavy metal	**Estimates pdf**
HQ Cu	
HQ Ni	
HQ Se	
HQ Zn	

Heavy metal	Estimates pdf
TOTAL HQ	

Task 6
Diffusion of Results
IMAR and INSA

Summary

In task 6 the aims were the diffusion of results produced in each task and overall project results. A initial pamphlet was produced to disseminate the project for local population participation engagement (see Fig 1). Two seminars were organized with different aims. The first one entitled "Environment and food: assessment of the effects on the human health of the population of Alentejo Litoral", was for the local population, for awareness and diffusion of preliminary results that occurred at 19 of February of 2013, at the old primary school of Carrasqueira village. In the seminar about 30 persons participate, like fisherman's, the presidents of Alcacer do Sal municipality and Comporta parish, local regional health administration and population in general (see Fig. 2 with the pamphlet of the seminar).

The other seminar entitled "Environmental risk assessment of a estuarine contaminated: the case of Sado estuary" was conducted near the end of the project (7th of October of 2013) and where main project results were presented (a representative of the team of each task and Line of Evidence present their results) (see fig. 3 with the pamphlet of the seminar). At the beginning of the seminar Prof. Angel Del Valls had a key note lecture about environmental risk assessment methodologies: perspectives and innovation. At the end in a round table it was discussed how to integrate the data and management/recommendation measures. The seminar was held in Fundação Calouste Gulbenkian and had about 60 participants, coming from local municipalities, the natural Sado estuary reserve, general public administration, students and researchers. All the seminar was audio recorded by Fundação Caloust Gulbenkian and a DVD produced. A television program was broadcast in Saturday 9 of November, within the Universidade Aberta (UAb) program at the public and open access channel (RTP2), talking about the seminar and the project (see in http://www.uab.pt/web/guest/uabtv, "emissões na RTP" - "9 de novembro"). A video of 4 min is also available at the website of UAb about the seminar and dissemination of the results (see in http://www.uab.pt/web/guest/uabtv, "seminário/workshop" - "Seminário Sado"). A press release about the seminar and project results was also done by LUSA agency.

The different research activities produced the following indicators, in summary:

i) 9 published papers, 8 in preparation and 2 submitted in scientific international journals,

ii) 18 communications in international meetings (10 orals and 8 posters);

iii) 4 communications in national meetings;

iv) 6 completed MSc thesis and 2 in preparation/finalizing;

v) 4 reports;

vi) 2 awards (for best oral and poster presentations);

vii) 2 organization of seminars. These number are well above the expected output indicators, proving the excellent quality of the work developed.

Figure 1. Pamphlet for local population engagement in the project.

Figure 2. Pamphlet of the Seminar to the local population of Carrasqueira.

Figure 3. Pamphlet of the Gulbenkian Seminar in Lisbon.

Papers in international ISI journals

In preparation

1. CAEIRO, S., COSTA, P. M., SILVA, M. J., LAVINHA, J., PINTO, M., DIAS, C. MACHADO, A., MARTINHO, A. P., FERNANDES, A. P., CASTANHEIRA, I., COSTA, M. H., DELVALLS, A. Environmental risk assessment in a contaminated estuary: A weight of evidence qualitative approach (in prep).

2. CASTANHEIRA, I., GUEIFÃO, S., SARAIVA M., MARTINHO, A. P., VAZ-FERNANDES, P., CAEIRO S. Local farming quality and food composition: The case of Carrasqueira Village, Portugal" (in prep to Journal of Hazardous Materials).

3. COSTA, P.M., GONÇALVES, C., RODRIGO A., CARREIRA, S., COSTA, M.H., & CAEIRO, S. Multi–species monitoring of complex estuarine areas: biological traits and adaptations reflect high ecotoxicological heterogeneity (in prep).

4. GONÇALVES, C., MARTINS, M., DINIZ, M.S., COSTA, M.H., CAEIRO, S. & COSTA, P.M. Can the complex mixture of sediment contaminants cause endocrine disruption on a benthic fish? A case study with Solea senegalensis (in prep).

5. PINTO, M., A.M. VICENTE, J. SACADURA, P.M. COSTA, H. LOURO, J. LAVINHA, S. CAEIRO, M.J. SILVA. Determining cyto- and genotoxicity of sediments from the Sado Estuary using solvent extractions of different polarities (in prep).

6. PINTO, M., H. LOURO, J. LAVINHA, S. CAEIRO, M.J. SILVA. Evaluation of the DNA repair capacity of HepG2 cells after exposure to estuarine sediment contaminants (in prep).

7. PINTO, M., J. SACADURA, H. LOURO, P.M. COSTA, J. LAVINHA, S. CAEIRO, M.J. SILVA. An integrated approach to assess the genotoxicity of an estuarine sediment extract using a transgenic mouse model (in prep).
8. PINTO, M.., J. LAVINHA, H. LOURO, S. CAEIRO, M.J. SILVA Deriving a synthetic risk index for environmental and human risk assessment based on the in vitro genotoxicity of sediments (in prep).

Submitted or other review

1. MACHADO, A., VAZ-FERNANDES, P, PAIXÃO, E, CAEIRO, S., MATIAS DIAS, C. An epidemiological approach to characterize the human exposure pathways in a contaminated estuarine environment. Submitted to Journal of Toxicology and Environmental Safety.
2. PINTO, M., P.M. COSTA, H. LOURO, M.H. COSTA, J. LAVINHA, S. CAEIRO, M.J. SILVA. (2013) Determining oxidative and non-oxidative genotoxic effects driven by estuarine sediment contaminants on a human hepatoma cell line. Science of the Total Environment (under revision).

Published

1. CARREIRA, S., COSTA, P.M., MARTINS, M., LOBO, J., COSTA, M.H. & CAEIRO, S. (2013). Ecotoxicological heterogeneity in transitional coastal habitats assessed through the integration of biomarkers and sediment contamination profiles: a case study with a commercial clam. Archives of Environmental Contamination and Toxicology 64, 97–109. http://dx.doi.org/10.1007/s00244-012-9812-1.
2. COSTA, P.M. & COSTA, M.H. (2012). Development and application of a novel histological multichrome technique on whole–body clam histopathology. Journal of Invertebrate Pathology 110, 411–414. http://dx.doi.org/10.1016/j.jip.2012.04.013.
3. COSTA, P.M., CAEIRO, S. & COSTA, M.H. (2013). Multi–organ histological observations on juvenile Senegalese soles exposed to low concentrations of waterborne cadmium. Fish Physiology and Biochemistry 39, 143–158. http://dx.doi.org/10.1007/s10695-012-9686-1.
4. COSTA, P.M., CARREIRA, S., COSTA, M.H. & CAEIRO, S. (2013). Development of histopathological indices in a commercial marine bivalve (Ruditapes decussatus) to determine environmental quality. Aquatic Toxicology 126, 442–454. http://dx.doi.org/10.1016/j.aquatox.2012.08.013.
5. COSTA, P.M., CARREIRA, S., LOBO, J. & COSTA, M.H. (2012). Molecular detection of prokaryote and protozoan parasites in the commercial bivalve Ruditapes decussatus from southern Portugal. Aquaculture 370/371, 61–67. http://dx.doi.org/10.1016/j.aquaculture.2012.10.006.
6. COSTA, P.M., RODRIGO, A.P. & COSTA, M.H. (2013, in press). Microstructural and histochemical advances on the digestive gland of the common cuttlefish, Sepia officinalis. L. Zoomorphology. http://dx.doi.org/10.1007/s00435-013-0201-8.

7. GONÇALVES, C., MARTINS, M., COSTA, M.H., CAEIRO, S. & COSTA, P.M. (2013). Ecological Risk assessment of impacted estuarine areas: integrating histological and biochemical endpoints in wild Senegalese sole. Ecotoxicology and Environmental Safety 95, 202–211. http://dx.doi.org/10.1016/j.ecoenv.2013.06.004.

8. PINTO, M., COSTA, P.M., LOURO, H., COSTA, M.H., LAVINHA, J., CAEIRO, S., SILVA, M.J. (2014) Human hepatoma cells exposed to sequential estuarine sediment contaminant extracts permitted the differentiation between cytotoxic and pro-mutagenic fractions. Environmental Pollution, 185, 141-148, doi:10.1016/j.envpol.2013.10.03.

9. RODRIGO, A.P., COSTA, P.M., COSTA, M.H. & CAEIRO, S. (2013). Integration of sediment contamination with multi–biomarker responses in a novel potential bioindicator (Sepia officinalis) for risk assessment in impacted estuaries. Ecotoxicology. 22, 1538–1554. http://dx.doi.org/10.1007/s10646-013-1140-3.

Communications in international scientific meetings

Oral

1. PINTO, M., H. LOURO, P.M. COSTA, S. CAEIRO, M.J. SILVA. (2013). Can estuary sediment contaminants interfere with the DNA repair capacity of HepG2 cells? 10th International Comet Assay Workshop (ICAW), 18-20 Setembro 2013, Porto, Portugal.

2. PINTO, M., J. SACADURA, H. LOURO, P.M. COSTA, J. LAVINHA, S. CAEIRO, M.J. SILVA. (2013). DNA and chromosome damaging effects in mice exposed to an estuary sediment extract. International Conference on Occupational & Environmental Toxicology (ICOETox), 16-17 Setembro 2013, Porto, Portugal.

3. PINTO, M, LOURO H, COSTA, PM, COSTA, M.H., CAEIRO, S, LAVINHA, J, SILVA MJ. (2012) Characterization of cytotoxic and genotoxic effects of contaminated sediments from the Sado Estuary and potential human health risk. In Albuquerque, P., Cabo Verde, S., Manteigas, V., Monteiro, A., Ramos, C., Silva, A., Viegas, C., Viegas, S. (ed.). International Congress on Environmental Health (ICEH). 29 Maio - 1 Junho 2012, Lisboa, Portugal. ISBN 978-989-8077-22-6.

4. PAIXÃO, E., A. MACHADO, C. CARVALHO, S. CAEIRO, C. M. DIAS (2012). Potential human exposure pathways in a contaminated estuarine environment. A case study in Sado estuary, Portugal. International Conference on Environmental Pollution and Public Health (EPPH2012), Shanghai, China, Maio de 2012.

5. MACHADO, A., E. PAIXÃO, S. CAEIRO, C. M. DIAS (2012). Risk assessment in an estuarine environment: a case-study in the Sado estuary. 12th World Congress on Environmental Health (IFEH 2012), Vilnius, Lituânia, Maio de 2012.

6. PAIXÃO, E., A. MACHADO, C. CARVALHO, S. CAEIRO, C. M. DIAS (2012). Occupational exposure in a contaminated estuarine environment epidemiological study. International Congress of Environmental Health 2012. ISBN: 978-989-8077-22-6. 29th May-1st June.

7. FERNANDES, A.P.; MARTINHO, A. P.; COSTA, P.M.; CARREIRA, S; COSTA, M.H., CAEIRO, S. (2012). Food frequency consumption in a fishing community from a contaminated estuarine environment. A case study. In Albuquerque, P., Cabo Verde, S., Manteigas, V., Monteiro, A., Ramos, C., Silva, A., Viegas, C., Viegas, S. (ed.). International Congress on Environmental Health (ICEH). ISBN 978-989-8077-22-6. 29th May-1st June.

8. COSTA, P.M., GONÇALVES, C., MARTINS, M., RODRIGO, A., CARREIRA, S., COSTA, M.H. & CAEIRO, S. (2013). Assessment of sediment contamination in an impacted estuary: differential effects and adaptations of sentinel organisms and implications for biomonitoring. SedNet Conference 2013, Lisbon (Portugal), November 2013.

9. COSTA, P.M., CARREIRA, S., RODRIGO, A., GONÇALVES, C., COSTA, M.H., ANTUNES, J., LOBO, J. & CAEIRO, S. (2012). Environmental risk assessment in an estuary affected by anthropogenic contamination: from ecological impacts to risk to human communities. 50th ECSA Symposium, Venice (Italy), June 2012.

10. ANTUNES J., CARREIRA S., COSTA P.M., CAEIRO S., LOBO J., MARTINHO A.P. & COSTA M.H. (2011) Surveying biomarker responses in invertebrates and vertebrates for Ecological Risk Assessment in a Portuguese estuarine area. 5th EUROLAG, Aveiro (Portugal), July 2011.

Posters

1. CASTANHEIRA, I., GUEIFÃO, S., SARAIVA M., MARTINHO, A. P., VAZ-FERNANDES, P.; CAEIRO S., (2013). Local farming quality and food composition: The case of Carrasqueira Village, Portugal,10th International Food Data Conference, Granada, Spain. 12-14 Setembro 2013 .

2. DIAS, C. M., A. MACHADO, E. PAIXÃO, I. MATEUS, J. TORO, S. CAEIRO. (2011). Design and implementation of an epidemiological study for the characterization of potential pathway human exposure in a contaminated estuary environment, Proceedings of the International Conference on Occupational and Environmental Health, Porto, Portugal, 17-19th of October 2011, pp. 99.

3. MACHADO, A., E. PAIXÃO, S. CAEIRO, C. M. DIAS (2012). Risk assessment in an estuarine environment: a case-study in the Sado estuary. IEA-EEF European Congress of Epidemiology, Epidemiology for a Fair and Healthy Society, Porto, Portugal , 6-8 september 2012. URL: http://hdl.handle.net/10400.18/1086.

4. PINTO, M., H. LOURO, P.M. COSTA, M.H. COSTA, S. CAEIRO, J. LAVINHA, M.J. SILVA. (2011). Impact of potentially contaminated sediments from the Sado Estuary in human health: cytotoxic and genotoxic assays in a human cell line. International Conference on Occupational and Environmental Health (ICOEH), 17-19 Outubro 2011, Porto, Portugal.

5. PINTO, M., H. LOURO, P.M. COSTA, M.H. COSTA, S. CAEIRO, J. LAVINHA, M.J. SILVA. (2011). Cytotoxic and genotoxic assessment of potentially contaminated sediments from the Sado Estuary. European Environmental Mutagen Society (EEMS), 4-7 Julho 2011, Barcelona, Espanha.

6. PINTO, M.; H. LOURO; P.M. COSTA; M.H. COSTA; S. CAEIRO; J. LAVINHA; M.J. SILVA. (2013). Cytotoxic and genotoxic potential of sediments from the Portuguese Mira-River Estuary.

Environmental Health 2013 Science and Policy to Protect Future Generations, 3-6 Março 2013, Boston, Massachusetts, EUA.

7. VAZ-FERNANDES, P., GUIOMAR, S., MARTINHO, A. P., CAEIRO, S. (2011) Food frequency pattern in a village exposed to an estuarine contaminated environment - HERA project. Proceedings book International Conference on Occupational and Environmental Health 2011 (ICOEH 2011), Porto, 17th to 19th October.

8. VICENTE, A. M. J. SACADURA, M. PINTO, H. LOURO, P.M. COSTA, J. LAVINHA, S. CAEIRO, M.J. SILVA. (2013). Evaluation of genotoxicity of sediments from the Sado-River Estuary using solvent extractions of different polarities. International Conference on Occupational & Environmental Toxicology (ICOETox), 16-17 Setembro 2013, Porto, Portugal.

Communications in national scientific meetings

Oral

1. GONÇALVES, C., MARTINS, M., DINIZ, M.S., COSTA, M.H., CAEIRO, S. & COSTA, P.M. (2013). Endocrine disruption in soles collected from an estuarine area: comparison with sediment contamination profiles. 10ª Conferência Nacional do Ambiente. 6 e 7 de novembro de 2013, Aveiro, Portugal.

2. PINTO, M. (2013) O projeto HERA: caracterização da toxicidade dos sedimentos do estuário do rio Sado e implementação de um índice de genotoxicidade. Comunicação oral a convite da Prof. Isabel Gaivão, no âmbito do Mestrado em Biologia Clinica e Laboratorial, da Universidade de Trás-os-Montes e Alto Douro. 28 Outubro 2013, Vila Real, Portugal.

3. PINTO, M., J. SACADURA, H. LOURO, P.M. COSTA, M.H. COSTA, S. CAEIRO, J. LAVINHA, M.J. SILVA. (2013). Genotoxicity and oxidative stress induced by sediments from the Sado Estuary and potential antimutagenic effects of quercetin. 3º Encontro de Biologia Molecular em Saúde, 15-16 Março 2013, Lisboa, Portugal.

4. PINTO, M., H. LOURO, S. CAEIRO, P.M. COSTA, M.H. COSTA, J. LAVINHA E M.J. SILVA. (2013). Avaliação do risco ambiental e para a saúde pública de um ambiente estuarino contaminado. Encontro sobre Riscos nas Zonas Costeiras, in: A Ciência na Prevenção e Mitigação dos Riscos em Portugal, 21 Março 2013, Lisboa, Portugal.

Master thesis

1. ANTUNES, J. (2011) *Solea senegalensis* como bioindicador da qualidade sedimentar estuarina. MSc thesis, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Monte de Caparica, 92 pp.

2. CARREIRA, S. (2011). Qualidade de bivalves estuarinos explorados para consumo. Potencial risco para a espécie *Ruditapes decussatus*: um estudo de caso. MSc thesis, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Monte de Caparica, 61 pp.

3. GONÇALVES, C. (2013)Can the complex mixture of sediment contaminants cause endocrine disruption on a benthic fish? A case study with *Solea senegalensis*. MSc thesis, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Monte de Caparica, 32 pp..

4. PINTO, M. (2011). Characterization of cytotoxicity and genotoxicity of sediments from a potentially contaminated estuary. Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa.

5. RODRIGO, A.P.C. (2012). Integration of sediment contamination with multi–biomarker responses in a novel bioindicator candidate (*Sepia officinalis*) for risk assessment in impacted estuaries. MSc thesis, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Monte de Caparica, 38 pp.

6. SACADURA, J.S. (em conclusão) Caracterização do potencial genotóxico de sedimentos estuarinos em sistemas experimentais in vitro e in vivo. Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa (em conclusão).

7. SARAIVA, M. A. (Em preparação). Quantificação de contaminantes químicos em produtos hortícolas por ICP-MS. Mestrado em Tecnologia e Segurança Alimentar. Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa.

8. VICENTE, A. M. (2012). Efeitos adversos de contaminantes estuarinos em células humanas: avaliação da genotoxicidade e desregulação endócrina. Mestrado em Biologia Humana e Ambiente, Faculdade de Ciências, Universidade de Lisboa.

Awards

1. Prémio de Melhor Poster no 3º Encontro de Biologia Molecular em Saúde, 2013, Lisboa, Portugal, atribuído pelo comité cientifico. M. Pinto, J. Sacadura, H. Louro, P.M. Costa, M.H. Costa, S. Caeiro, J. Lavinha, M.J. Silva. Genotoxicity and oxidative stress induced by sediments from the Sado Estuary and potential antimutagenic effects of quercetin.

2. Prémio de Melhor Comunicação Oral no International Congress on Environmental Health (ICEH) 2012, Lisboa, Portugal, atribuído por the Taylor & Francis Group e o comité cientifico. M. Pinto, H. Louro, P.M. Costa, M.H. Costa, S. Caeiro, J. Lavinha, M.J. Silva. Characterization of cytotoxic and genotoxic effects of contaminated sediments from the Sado Estuary and potential human health risk

Reports

1. Caeiro, S. (coord.). (2011). 1º Relatório de progresso do projeto HERA - Avaliação de risco ambiental de um ambiente estuarino contaminado: o estudo de caso do estuário do Sado. PTDC/SAU-ESA/100107/2008.

2. Caeiro, S. (coord.). (2012). 2º Relatório de progresso do projeto HERA - Avaliação de risco ambiental de um ambiente estuarino contaminado: o estudo de caso do estuário do Sado. PTDC/SAU-ESA/100107/2008.

3. Caeiro, S. (coord.). (2013). Final report of HERA - Avaliação de risco ambiental de um ambiente estuarino contaminado: o estudo de caso do estuário do Sado. PTDC/SAU-ESA/100107/2008.

4. Machado, A., E. Paixão, S. Pereira, C. M. Dias. (2013). Projeto HERA Avaliação de risco ambiental de um ambiente estuarino contaminado: Resultados do Estudo Epidemiológico". Lisboa: Instituto Nacional de Saúde Doutor Ricardo Jorge I.P, Departamento de Epidemiologia.