UNIVERSITA' CATTOLICA DEL SACRO CUORE
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Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXIII

S.S.D: AGR/13

ASSESSING EFFECTS TO AQUATIC ORGANISMS OF CONTAMINANTS EXPOSURE ACROSS LEVELS OF BIOLOGICAL ORGANISATION, IN THE FRAME OF THE WFD 2000/60/EC

Candidate: Angela Sacchi
Matr. n.: 3611485

Academic Year 2009/2010

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AIM OF THE THESIS

Aquatic ecosystems are exposed to chronic releases of pollutants coming from different anthropic sources, with increasingly threats for their ability to provide services (i.e. quality and water availability) and of risk to human health (MEA, 2005). Most of the freshwater and coastal organisms are exposed, and their physiology may be affected by toxic chemicals (i.e. tributyltin causing imposex in mollusks; Matthiessen and Gibbs, 1998) with a cascade event on population dynamics.

Developments in biological assessment techniques have given rise to the concept of ecological indicators and biomarkers techniques. Their primary role is to provide measures of responses to anthropogenic disturbance and assessing for ecological integrity of these systems (Niemi et al., 2004). Nowadays there is an increasing need for an appropriate biomonitoring tool in aquatic environments, which allows a diagnosis of ecosystem, which identifies causes of biological impairment and contributes to the ecological risk assessment.

Currently legislations are under development and worldwide revision e.g. Clean Water Act in US, Water Framework Directive (EC, 2000) or Marine Strategy Framework Directive (EC, 2008), National Water Act in South Africa, to address the ecological quality of estuarine and coastal systems. Numbers of studies are ongoing to the development of tools for different physiochemical or biological parameters of the ecosystems, trying to integrate all the elements into a unique evaluation of the status of a water body.

Throughout this thesis, the emphasis has been focused on the study of physiological changes and response of aquatic organisms at different levels of biological organisation to assess the influence of xenobiotics. The thesis is structured in three different chapters were bioindicators and multibiomarker approaches were tested in order to gain an overview of monitoring methodologies as representative picture of ecotoxicological tools useful to investigate the biological effects of xenobiotics in aquatic environments. This study has an insight on the application of the requested methodologies in the application of the WFD (EC, 2000), and application of indicators at different levels of organisation provides different types of information necessary for ecological risk assessment procedures. Selected aquatic species models were invertebrates: as bivalve mollusks and freshwater macroinvertebrates. Clams were selected for their sedentary nature, filter-feeding behaviour of large water-mass volumes, ability to integrate exposure over time and to bioaccumulate pollutants (i.e. International Mussel Watch Project). Freshwater macroinvertebrate communities were selected in the biological and

ecological traits perspective, looking at species and their composition as the results of traits tolerant of altered environmental conditions.

In this general introduction the concept of bioindicators and biomarkers at the sub-individual level is defined. Main focus of biologically based approaches will be on the aquatic environment since most of the present chemicals in widespread use, an high portion potentially reach aquatic environment and are known as potential contaminants for freshwater resources. Additionally an highlight legislation overview for community actions in the field of water policy is underlined, European Framework Directive (WFD, EC 2000).

- In Chapter 1 the research is focused on diagnostic approach at the community composition level with trait-based SPEAR (SPEcies At Risk) indicator of pesticide contamination; developed by Von der Ohe and Liess (2004) and Liess and Von der Ohe, 2005. Based on the well developed SPEAR indicator on macroinvertebrate species-level data, we performed a comparison between the SPEAR indices based on species and family levels of taxonomic resolution using data sets for European regions (Finland, France, and Germany). An assessment of whether the family-level taxonomical resolution remains sensitive enough to indicate pesticide exposure.
- In Chapter 2 changes in biomarkers responses, will be investigated in laboratory experiment, as indicators for the possible effects of toxic chemicals on organisms. The herbicide, based on chropophenoxy pesticides, with an increasing temperature factor were tested for sub-organismal level effect in clams *Mya arenaria*, with a physiological response in aerobic capacity and oxidative stress.
- In Chapter 3 physiological essays were tested in a field study to investigate the health status of *Tapes philippinarum*, a commercial species farmed in one of the most important aquaculture site in Italy, the estuarine costal ecosystem of the lagoon of Sacca di Goro, Nord East Adriatic coast. Were aquatic organisms are exposed simultaneously to different classes of chemicals in complex mixtures, interacting with metabolic pathways or act directly as toxicants. Analytical chemistry will be used to monitoring the sediment compartment and concentration levels detected were correlated with biological responses.

Data collected concern species abundance, trait based approach, activity of different enzymes involved in the aerobic (CCO) and antioxidant responses, (SOD, and CAT), behavioural endpoints, and genotoxicity biomarker associated with micronuclei frequency. This bioindicators and biomarker techniques are promising tools in environmental status diagnosis,

which should not be considered as an absolute numerical data, by looking at multiple indicators simultaneously, we can better understand how organism response changes as environmental conditions vary (Maycock et al., 2003).

1. GENERAL INTRODUCTION

1.1. BIOMARKERS AND BIOINDICATORS

Biomarkers are a biologically-based approach in the detection of early biological effects to exposure of contaminants at low environmental concentrations which may result in long-term physiological disturbances. Biomarkers are stressor-induced variations in cellular or biochemical, structures, or functions that are measurable in a biological system or sample, such as variation in tissues or organs within an organism; enzymatic responses or production of metabolites are examples of biomarkers, which can be related to toxic effects of environment. Biomarkers can be defined as biological responses to an environmental chemical at either the individual or cellular level, which indicate a departure from the "normal-physiological" status (Figure 1). A battery of biomarkers may be useful to evaluate the various responses to a mixture of pollutants in organisms under stress (Aarab et al., 2004).

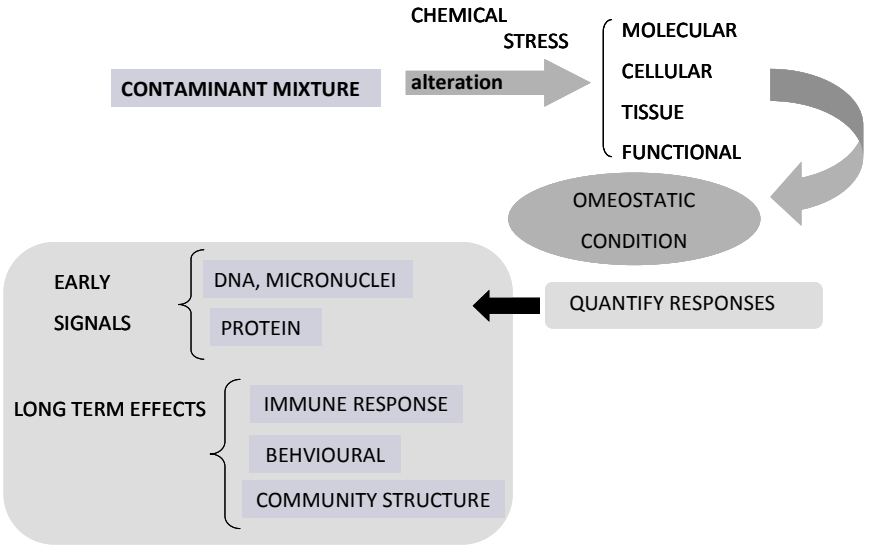

Figure 1. Schematic representation the hierarchical relationship between ecotoxicological responses measured at different levels of biological organization.

The use of biomarkers does not replace chemical monitoring, but it integrates them providing a contribution in determining the toxicity of pollutants, even when they are present at low, sub-lethal concentrations. As a result chemical monitoring, principally evaluates the presence of pollutants in cells and tissues using chemical analyses, while biomonitoring methods evaluate the response of the organisms to these pollutants.

Molecular effects of toxicants may propagate to higher levels of biological organisation. At the individual level, potential effects include increased mortality of invertebrates as well as sublethal endpoints like reduced growth or fecundity but also behavioural alterations (Amirard-Triquet, 2009). These toxicant-induced alterations may affect the performance of populations (i.e. population growth rate) and can propagate from population to community level. As a result, toxicant stress may change the species composition in communities (Liess et al., 2008).

Therefore changes in communities composition of freshwater organisms can be considered as bioindicators (Liess and Von der Ohe, 2005).

Bioindicators are considered as responses to environmental effects that occur at higher levels of biological organization than sub-organism (Adams et al., 2001), and they can be measured at individual, population (reproductive success, mortality, size distribution, reduction in abundance and biomass), community (primary production, disruption of the nutrient cycle) or ecosystem levels. Endpoints at higher levels of biological complexity have high ecological relevance (Forbes et al., 2006) even if these studies conducted at the population and community level often lack the early warning diagnostic potential provided by more sensitive and rapid responding endpoints at lower levels of organization such as biochemical endpoints (biomarkers). A major challenge in conducting ecosystem level studies, therefore, is to bridge this gap between endpoints across the various levels of biological organization (Sibley et al., 2000).

Biomarkers and bioindicators are widely used to determine the response of organisms to stressing agents, and often a set of biological endpoints are required, as additionally what types of questions are to be answered by applying a biological monitoring.

1.2. AQUATIC ENVIRONMENT

Coastal and estuarine zones are amongst the most productive ecosystems of the world. These systems are complex and undergoing rapid changes, both by natural dynamic processes and also threatened by direct anthropogenic influences and climate change. The changes are of different origins, such as dredging and pollution, derived from land and freshwater use in the watersheds through changes in hydrology, deforestation and associated land erosion inducing sediment transport, and agricultural related pollution (Dynesius and Nilsson, 1994; Sumpter, 2009). Nutrient enrichment and related eutrophication is expected to become more widespread, with greater incidences of hypoxia and anoxia (Diaz and Rosemberg, 2008), particularly in shallow coastal lagoons with limited circulation. Many chemical contaminants (especially persistent organic pollutants), as pesticides and fertilizers originating from agriculture, as well as sewage inputs from industrial and urban areas, cause changes in the structure and function of biotic communities (Cannicci et al, 2009).

Changing water flows and regimes in rivers (Dynesius and Nilsson, 1994) will affect salinity intrusion, sediment, and nutrient loads (Syvistki et al., 2005) and these hydrological modifications have downstream impacts on ecosystems, as the influence on distribution of estuarine wetland plant communities, which typically follow an ecological succession from the marine to the terrestrial environments (IPCC, 2007). A trend underlined for climate change model is the intensification of extreme-event risk, such as floods, which are expected to increase in frequency and intensity (Milly et al., 2002; IPCC, 2007). Changes reported as primary threats in relation to impacts, alterations and degradation, of estuarine environments.

As result alteration in estuarine environments can occurs as changes in physical characteristics caused by changes in freshwater quality runoff (Scavia et al., 2002) with effects on shallow near-shore marine environments like productivity within phytoplankton growth rates (Justic et al., 2005).

The productive estuarine systems and their associated resources are highly vulnerable to this increasing pressure and are of primary importance for populations and economies, a generalized reduction of ecosystem goods and services is reported with threats on human health and ecosystem functioning system (Costanza et al., 1987).

1.3. LEGISLATION CONTEXT IN EU

Over the last 60 years, the application of risk assessment to protect human health has increased and during last years the ecological risk assessment has become more widely used (Solomon and Sibly, 2002). The aim of the WFD Water Frame directive (2000/60/EC) is to contribute to the protection, prevention of deterioration and improvement of all water bodies across the European Union. EU waters, throughout member states policies, have to achieve a good quality status for all surface, ground and coastal waters by 2015. Objectives that complement a number of exisiting legislation Drinking (98/83/EC) Water Directive, as well as those based on specific substances or sources of pollution i.e. Directives on Dangerous Substances (76/464/EC), Groundwater (80/68/EEC), Nitrate (91/676/EEC) and Pesticide (91/414/EEC and Regulation (EC) No 1107/2009). Some key aspects of the WFD include participative river basins coordination and cost effective management, protecting ground and surface waters, reducing pollutants, putting common standards, addressing monitoring programmes. The implementation of WFD requires an approach were the ecological status of a body of water is determined according to the a) biological, with an estimation of risks that arise for aquatic communities, exposed to pollutants b) physical and chemical, and c) hydrological and morphological quality standards, and is categorized in classes as excellent- good-moderate-poor or bad.

The WFD does not mandate the use of a particular set of methods, but aims to ensure the establishment of adequate monitoring program and intercalibration at EU level. The WFD also implements the development of relevant bioindicators and European-wide index for the assessment of the ecological quality status of EU waters. It is suggested to use bioindicators able to detect impacts on structure and relative functions of aquatic ecosystems as well as index based on multibiomarker approach, capable to provide an integrated relative measure of the general status of ecosystems (Hagger et al., 2008). Advances in molecular biology and genetics make possible to extend the biomarker concept to other methods for the ecological risk assessment like quantify the expression of specific proteins or examine of the up-regulation and down-regulation of many different genes, a new research area known as ecotoxicogenomics (Snape et al., 2004).

The WFD has a clear objective of reducing pollutants in EU's waters, EQSs (Environmental Quality Values) have been set for 33 priority substances. The list includes selected existing chemicals, plant protection products, biocides, metals and other groups like Polyaromatic Hydrocarbons (PAHs) and Polybrominated Biphenylethers (PBDE). More specifically,

endocrine disruptors such as nonylphenol, di-2-ethylhexylphthalate (DEHP) and polybrominated diphenyl ethers (PBDEs) are considered in the WFD as high priority hazardous substances to be monitored for their toxicity, persistence, tendency to bio-accumulate and having the potential to interfere and disrupt hormonal systems of humans and wildlife (Brack et al., 2007).

According to Fuerhacker (2009), the detection of substances and risk assessment for regulatory decisions needs to be harmonised among different texts as the Urban Wastewater Treatment Directive (EC, 1991) the WFD and the global scale document from United Nations Environmental Programme (UNEP), the Stockholm Convention to reduce POPs (2004).

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2. BIOLOGICAL INDICATOR OF FRESHWATER CONTAMINATION BY PESTICIDES

2.1. INTRODUCTION

Freshwater is one of the most valuable resources on earth its protection and conservation for future generations is a great challenge, since freshwater ecosystem delivers various goods and services for human societies (i.g water purification and pollination; MEA, 2005; EFSA, 2010). At present however, around 100,000 chemicals are in widespread use and many of them are potential contaminants for our freshwater resources. In this context, pesticides, deliberately deployed into the environment, are a hazard to non-target organisms; effects reported for all taxonomic groups of aquatic organisms from microorganisms in DeLorenzo et al., 2001 to aquatic communities as reported by Relyea, 2005. They are often persistent and become toxic at certain thresholds, they can influence structure and functional parameters of biological communities (Liess and Von der Ohe, 2005; Schäfer et al., 2007; Liess et al., 2008). Infact this toxicants induced alterations in the structure and functional parameters of biological macroinvertebrate communities (Liess and Von der Ohe, 2005; Schafer et al., 2007), as the results of the propagation of effects from suborganismal level (Duquesne, 2006) to higher level of biological organisation. Thus, there is a strong need to assess the risks that may originate from these compounds.

The EU Regulation 1107/2009 concerning the placing of plant protection products on the market and repealing 79/117/EEC and 91/414/EEC require as in the Uniform Principles for the assessment of pesticides (Annex VI, Directive 91/414/EEC) that no unacceptable impacts on the viability of exposed organisms occur under field conditions. The Water Framework Directive of the European Community (European Commission, 2000) aims to achieve a good biological quality of the aquatic ecosystems by 2015. This means that for streams, there is a call of actions of the respective governments to achieve a good ecological quality status of streams until 2015. For the description and monitoring of the present status of streams, the assessment will be based on both chemical and ecological aspects, whereas the analysis of the ecological data is an important component.

The distribution of macroinvertebrate taxa and densities in agricultural streams is influenced by many factors such as organic pollution (Whitehurst, 1991) habitat degradation (Hilsenhoff, 1977) and pesticides (Relyea, 2006). As many invertebrate species have low dispersal abilities

and constantly populate streams, macroinvertebrates may serve as valuable indicators of the degradation of streams. So far, many conventional ecological assessments in freshwater indices are based on taxonomic composition and abundance parameters of stream invertebrates as Saprobic Index (Friedrich, 1990) EPT – sum of Ephemeroptera, Plecoptera and Trichoptera species richnesses (Lenat, 1998) Biotic Index (EBI) (Ghetti, 1997). Their variability, espressed by the biological communities inhabiting freshwaters, is affected by numbers of factors including biotic and abiotic parameters. These bioassessment methods shows a low power of resolution to detect effects of pesticides, since they were not designed to detect effects of pesticide specific stressor and vary significantly with environmental variables such as pH, current velocity, and temperature (Liess et al., 2008). Additionally the approach is based on systematic and is restricted to taxonomic identity liming the effectiveness of ecoregions comparisons of ecosystems (i.e. applicableness across different biogeographical regions in Europe).

An alternative approach for assessing the effects of human impacts at large spatial scales is the use of ecological functions of several species (Gayraud et al., 2003). Ecological functions are summarised in the biological traits that reflect the adaptation of species to environmental parameters and habitats status. Environment act in the "selection" of communities species structures, therefore presence of a species should be related with its traits as life-history specialization, body mass and reproductive rate (Larsen and Ormerod, 2010). Species are not equally at risk but species specific ecological traits determine how well the species is able to withstand threats or environmental changes to which it is exposed to. Biological traits assessment could offer a biomonitoring tool more oriented to conservation management and to a deeper knowledge of the species at risk.

Biomonitoring generally identifies macroinvertebrates to the family level. However, previous studies with the SPEAR bioindicator have been based to a great extent on species-level data (Liess and von der Ohe, 2005; Schäfer et al., 2007). SPEAR system is based on biological traits of stream invertebrates and define species as SPEcies At Risk (SPEAR) and SPEcies not At Risk (SPEnotAR), based on their biological traits and not on taxonomic composition or abundance parameters. According to this concept, a species is classified as being at risk of being affected by pesticides if it matches following criteria: physiological sensitivity to organic toxicants including pesticides compare to the sensitivity of *Daphnia magna* ($S_{organic}$ value > -0.36), numbers of generations per year (≥0.5 year), it is fully aquatic unable to avoid exposure

during intensive pesticide usage during their adult life stages or does not emerge before the main period of agrochemical application in a particular study area (i.e. before May), and its migration ability is low. If at least one of the criteria is not met, it is assumed that the species can tolerate exposure, can avoid exposure due to early emergence or short-time migration, or can quickly reproduce after exposure. The response of aquatic communities to toxicant exposure is strongly influenced by the physiological sensitivity that members of these communities show to toxic compounds. However, life-history traits also determine how single species, and communities as a consequence, respond to toxicant exposure. A firm link has been established between the abundance of SPEAR in relation to the overall abundance per site (%SPEAR abundance) and measured pesticide levels (insecticides and fungicides) in three field studies that were conducted in Finland, France and Germany (Liess and von der Ohe, 2005; Schäfer et al., 2007). Measured maximum pesticide concentrations that were expressed in Toxic Units (i.e. concentration of a compound divided by the related LC50 for *D. magna*) best described the observed variance in %SPEAR abundance. Other parameters that contributed slightly to the variability in %SPEAR abundance were length of forested stream sections, type of stream bed substrate, and cover of submerged plants. An analysis of the pooled data from Finland, France and Germany showed that a significant change in community structure occurred at sites characterised by pesticide contamination at a concentration range as lower than the acute 48h-LC50 of *D. magna*. Further, a significant decrease in SPEAR from the pre- to the main agrochemical application period was observed, while no indication was found in the investigations that parameters other than pesticides (e.g. hydrodynamic stress, water quality parameters, etc.) might be responsible for the observed short-term reduction of sensitive species. This suggested that the short-term changes in SPEAR from pre to main application period are best attributed to pesticides.

Validation of the SPEAR system has shown that it is (i) exceptionally sensitive to pesticide contamination (ii) relatively independent of abiotic environmental factors other than pesticides, and (iii) applicable across different biogeographical regions in Europe (Liess and Von der Ohe, 2005; Schäfer et al., 2007; Schriever et al., 2007; Von der Ohe et al., 2007).

The aim of this study was to find patterns in the family composition of aquatic invertebrate communities that are related to the indirect effects of pesticides. To this end, measured pesticide concentrations were linked to the structure of respective invertebrate assemblages. To reduce the site-specific variation of community descriptors due to environmental factors other than

pesticides, species and families were grouped according to their vulnerability to pesticides. The pesticide-specific bioindicator system SPEAR was applied (Liess and Von der Ohe, 2005) to link pesticide exposure and effects.

Therefore, prior to validation and possible routine use of SPEAR indices with family as taxonomic category, it was necessary to compare the SPEAR indices based on family- and species-level data, to establish whether family-level information could be used without significant loss of diagnostic capability. Consequently propose the pesticide-specific SPEAR index based on family level dataset, in order to detect related pesticide effects in European streams as promising candidate to be included in the biomonitoring programmes according to the EU Water Framework Directive (EU, 2000), indicator applicable in different countries.

In summary, the aim of this study was to compare the SPEAR indices based on species and family levels of taxonomic identification using available data sets for European regions (Finland, France, and Germany).

2.2. MATERIALS AND METHODS

2.2.1. DATA SET $SPEAR_{pesticides}$-INDEX CALCULATION AND ENDPOINTS

The SPEcies At Risk (SPEAR) concept (Liess and von der Ohe, 2005) combines information on physiological sensitivity to organic compounds according to Wogram and Liess (2001) and Von der Ohe and Liess (2004) with information on life-history traits to identify species that are at risk from being affected in particular by one group of organic compounds, pesticides.

Currently SPEAR-bioindicator was developed for other stressor-specific, investigations in Western Siberia demonstrated the applicability of a modified SPEAR approach for organic toxicants such as petrochemicals and synthetic surfactants in habitats of this region. The index $SPEAR_{organic}$ was developed for these contaminants and applied across a large gradient of longitudinal environmental factors (Beketov and Liess, 2008).

$SPEAR_{pesticide}$ was introduced in the present study instead of the index name %SPEAR(abundance) reported in numbers of previous studies (Liess and Von der Ohe, 2005; Schäfer et al., 2007; Schriever et al., 2007; Von der Ohe et al., 2007).

Since the aim of this study was to verify the power of resolution of SPEAR index based on family level classification of data, was necessary to differentiate $SPEAR(sp)_{pesticide}$ from $SPEAR(fm)_{pesticide}$, for the indices based on species and family levels, two different taxonomic identifications.

This trait-based SPEAR was developed on binary classification of species (or other taxonomic categories) into ‘‘species at risk’’ and ‘‘species not at risk’’ according to the following biological traits: (i) physiological sensitivity to organic toxicant ($S_{organic}$, Wogram and Liess, 2001; Von der Ohe and Liess, 2004), (ii) generation time (Beketov et al., 2009), (iii) presence of aquatic stages in water during the maximum pesticide usage period, and (iv) migration abilities (information on taxon-specific traits is available in the SPEAR database Liess et al., 2008).

A taxon is classified as a ‘‘species at risk’’ only if it has: (i) $S_{organic}$ value > –0.36, (ii) generation time ≥ 0.5 year (iii) aquatic stages (eggs, larvae, pupae) during the periods of intensive pesticide usage, and (iv) low migration abilities. Classification of species at risk (1) and not at risk (0) was performed automatically according to an algorithm included in the table. Since information regarding species (where species is at risk or not) are freely available on the database (http://www.systemecology.eu/SPEAR/Start.html), we needed to adapt SPEAR concept on family level taxonomic resolution and derive the binary classification for families, define for each family whether family is at risk or not at risk. These definitions were calculated according to the majority of the species comprising the family (≥ 50%). After defining the “species and families at risk” (can be any taxonomic category), the relative abundance of these taxa was computed for each site and date as follows:

$$SPEAR_{pesticides} = \frac{\sum_{i=1}^{n} \log (x_i + 1) \bullet y}{\sum_{i=1}^{n} \log (x_i + 1)} \bullet 100$$

where n is the number of taxa, x_i is the abundance of the taxon i and y is one if taxon i is classified as SPEAR, otherwise zero. These calculations where performed for the lowest possible identified taxonomic levels (down to species level) to define $SPEAR(sp)_{pesticides}$ and for the families to define $SPEAR(fm)_{pesticides.}$ The ranges of $SPEAR(sp)_{pesticides}$ and $SPEAR(fm)_{pesticides}$ were 2.14-69.41 and 4.86–69.71 respectively. For calculation no particular software is required, because data management and calculation of SPEAR values can be done with standard database software such as Microsoft Excel or Microsoft Access. Currently the program SPEAR Calculator (UFZ, Leipzig, Germany) that is freely available on the internet (http://www.systemecology.eu/SPEAR/Start.html), and was recently developed to automate

computation of the $SPEAR_{pesticides}$ indices. This program derives site- and date-specific values of both the species- and family-level indices automatically using information on all the relevant traits accumulated in the SPEAR database (Liess et al., 2008).

In order to compare the family- and species-level SPEAR indices, biomonitoring data sets from Germany (Liess and von der Ohe, 2005), France, and Finland (Schäfer et al., 2007) were combined and analysed. These data sets included the sampling station, sampling month, results of extensive pesticide measurements expressed in TU, information on macroinvertebrate community composition (abundance of species) and relative SPEAR abundance, SPEAR ratio useful for the algorithm calculation and relevant landscape characteristics (presence of undisturbed upstream reaches) summarised in a binary classification as recovery present (1) and recovery area absent (0) and sets of basic water quality parameters (for details see Liess and von der Ohe, 2005; Schäfer et al., 2007). In total, the data sets comprise information on 48 sampling sites. The samples were collected during the periods of intensive pesticide usage two times (Finland – July and August, France – April and May, Germany – May and June) with a Surber sampler (area 0.062 m^2, four replicate samples per each site/date). The sites present pollution coming from agricultural sources, upstream no other source of contamination as waste-water treatment plants has to be present.

2.2.2. INDEX OF PESTICIDE TOXICITY

To compare the toxicity of pesticide concentrations measured in the different sites, toxic units (TU) were calculated from the maximum peak water concentrations measured at each site according to Liess and von der Ohe (2005).

The TU values for each compound were based on the acute (48-h) LC_{50} of *D. magna:*

$$TU(D.\ magna) = \max^{n}_{i=1}(\log (C_i / LC50_i))$$

where TU(*D. magna*) is the maximum number of toxic units of the *n* pesticides detected at the considered site, C_i is the concentration (μg/L) of pesticide *i* and $LC50_i$ is the 48- hour LC50 of pesticide *i* for *D. magna* (μg/L) as given in Tomlin (2001). This method is an estimation of water toxicity, measuring maximum pesticide concentrations that were expressed in Toxic Units (i.e. concentration of a compound divided by the related LC50 for *D. magna*). Measured residues are inputs of pesticide due to runoff-induced inputs via non point sources. Therefore an underestimation of pesticide concentrations could be detected since runoff event decrease strongly during 24 hours, and therefore 48-h LC50s of *D. magna* were used and only the

maximum toxic unit was considered instead of the sum toxicity of the pesticides detected at the respective site. These exposure-response relationship was applied for diagnose pesticide contamination from biological community data, expected to be reflected by SPEAR (Liess and von der Ohe 2005; Schäfer et al., 2007)

2.2.3. DATA ANALYSIS

The relationship between the SPEAR indices and water toxicity was analysed by linear regression. Analysis of covariance (ANCOVA) was applied to check for significant differences in slope and intercept between the models for $SPEAR(sp)_{pesticides}$ and $SPEAR(fm)_{pesticides}$. A paired t-test was used to check for significant differences in values of these two indices, with the data points paired for each observation site.

To test for significant differences between groups of sites among studied countries, the sites were grouped according to TU as: characterised by low (TU < -4: uncontaminated/reference), medium (-4 < TU < -2: slightly contaminated) and high contamination level (TU > -2). To compare values of $SPEAR(fm)_{pesticides}$ for the study sites in Finland, France, and Germany, two-way analyses of variance (ANOVA) were applied with the factors ‘‘region’’ and ‘‘TU’’. Currently the database comprises information for the following regions: Central Europe, Finland, UK, and Western Siberia. For computation of the $SPEAR_{pesticides}$ indices for Germany and France the information from Central Europe was used, and respectively, such information from Finland was used to derive the indices for sampling sites in this region. In this analysis sites with and without recovery areas were analysed together due to limited amount of uncontaminated and heavily contaminated sites without recovery areas in France. Levene’s test was used to test for homogeneity of variances. As variances were not homogeneous in all cases, Games-Howell post-hoc test (robust with respect to the potential deviations from normality or variance homogeneity) was used to compare the site groups with different contamination levels. Prior to analysis, the average values for the two sampling dates were calculated for all variables that were measured twice at each site, in order to avoid temporal pseudoreplication. The data set for the streams having upstream undisturbed reaches (recovery areas) was analysed separately from the set for streams without undisturbed reaches, as it had previously been shown that the presence of such reaches significantly influenced the correlation between pesticide exposure and observed effect (Liess and von der Ohe 2005; Schäfer et al. 2007; Schriever et al., 2007). The analyses were performed using STATISTICA® 7.1 for Windows (StatSoft, Tulsa, OK, USA).

2.3. RESULTS

All the correlations between the SPEAR indices and water toxicity were statistically significant ($p < 0.001$), with higher values of Spearman's r^2 for the sites without upstream recovery areas. Comparison of the correlations found for the species- and family-level SPEAR indices showed that these indices similarly correlate with water pesticide toxicity (Figure 1). No significant differences in slope were found between the linear regressions for $SPEAR(sp)_{pesticides}$ and $SPEAR(fm)_{pesticides}$ for sites both with and without upstream recovery areas ($p > 0.05$, ANCOVA), although in sites without recovery areas the slope of $SPEAR(sp)_{pesticides}$ was slightly steeper than that of and $SPEAR(fm)_{pesticides}$. The r^2 values were only slightly higher for $SPEAR(sp)_{pesticides}$ than for $SPEAR(fm)_{pesticides}$ in sites both with and without recovery areas. Comparison of $SPEAR(sp)_{pesticides}$ and $SPEAR(fm)_{pesticides}$ values by paired *t*-test showed statistically significant differences between them for streams both with and without upstream recovery areas ($p < 0.01$, paired t-test), with $SPEAR(fm)_{pesticides}$ values being higher for the same sample. These results indicate that correlation patterns (slopes) derived for the family- and species-level SPEAR indices are similar, although the actual values of the family-level index are relatively higher than those of the species-level index.

Two-way ANOVA for the site groups with low, medium, and high levels of contamination with factors "taxonomic level" and "TU" showed insignificant effect of the former factor ($p > 0.05$), but significant effect of the latter ($p < 0.001$) in sites both with and without recovery areas. As the level of taxonomic resolution caused no significant effect, the following one-way ANOVA was performed with "TU" as the only factor. This statistical technique, followed by post-hoc Games-howell test, showed significant differences between sites of low contamination and those with both medium and high levels of pesticide contamination, in sites both with and without recovery areas ($p < 0.004$). However, no significant difference was found between the slightly and highly contaminated sites ($p > 0.05$).

Comparison of the family-level $SPEAR(fm)_{pesticides}$ values for the study sites in Finland, France, and Germany performed with two-way ANOVA showed that effect of the factor ''region'' is insignificant ($p > 0.05$).

The presence of upstream undisturbed reaches (recovery areas) has been shown to significantly affect correlations between pesticide exposure and SPEAR indices (Liess and von der Ohe 2005; Schäfer et al. 2007; Schriever et al., 2007). The mechanisms underlying this effect are thought to be due to downstream drift of sensitive aquatic taxa from undisturbed upstream

reaches to the contaminated stream sections. Downstream drift is well known for many stream invertebrates and be initiated by natural and anthropogenic factors (Brittain and Eikeland 1988; Beketov and Liess 2008). Previous studies have shown significant effects of upstream recovery areas on SPEAR indices based on the species level of taxonomic resolution. Similar effects were expected for family-level indices, but had not previously been investigated. In order to evaluate the effect of upstream recovery areas on the $SPEAR(fm)_{pesticides}$ index, we compared linear regressions for this index and water toxicity computed separately for the sites with and without recovery areas using ANCOVA. The same comparison was performed for the species-level index $SPEAR(sp)_{pesticides}$ values. Comparisons of the correlations derived for the sites with and without upstream recovery areas showed that both $SPEAR(fm)_{pesticides}$ and $SPEAR(sp)_{pesticides}$ intercepts were significantly different ($p < 0.05$, ANCOVA) intercepts at sites with upstream recovery area were significantly higher than at sites without, but the slopes were not significantly different between these two types of sites ($p > 0.05$, ANCOVA).This suggests that although the presence of recovery areas significantly increases the values of both $SPEAR(fm)_{pesticides}$ and $SPEAR(sp)_{pesticides}$ compared to sites with the same TU but without upstream recovery areas, it does not influence the correlation patterns between the indices and water toxicity imposed by pesticides (Figure 1).

Consequently, the effect of the recovery areas on the family-level index was similar to the effect on the species-level index. All this suggests that for both the species- and family-level SPEAR indices, the presence of upstream recovery areas should be taken into account in monitoring programmes. Pesticide effects in streams with and without such recovery areas should be analysed separately.

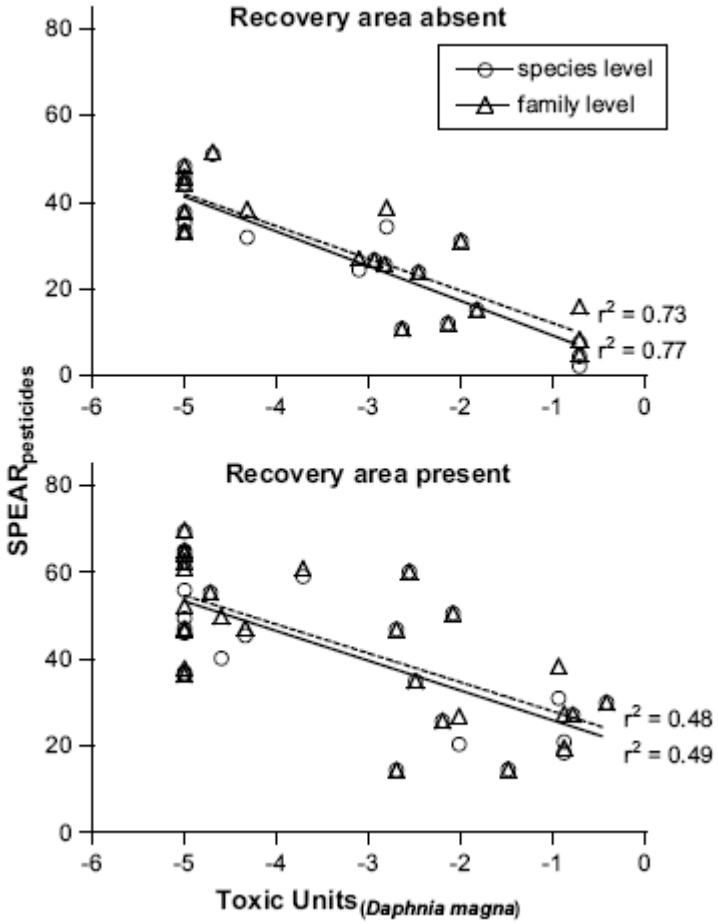

Figure 1. Graphical resolution of up-stream recovery areas on the species- and family-level SPEAR indices. Sites without upstream recovery areas linear regressions for $SPEAR_{pesticide}$ indices based on family ($r^2 = 0.73$, $p < 0.001$) and species ($r^2 = 0.77$, $p < 0.001$) levels of taxonomic resolution and water toxicity expressed as Toxic Units (*D. magna*). For sites with upstream recovery areas linear regressions for SPEAR indices based on family ($r^2 = 0.48$, $p < 0.001$) and species ($r^2 = 0.49$, $p < 0.001$) levels of taxonomic resolution and water toxicity expressed as Toxic Units (*D. magna*). The intercepts and slopes are not significantly different ($p > 0.05$, ANCOVA) with and without recovery areas.

2.4. DISCUSSION

Comparison of the pesticide-specific SPEAR indices based on family and species levels of taxonomic resolution ($SPEAR(fm)_{pesticides}$ and $SPEAR(sp)_{pesticides}$ respectively) for the site groups Finland, France, and Germany has shown that the family-level index can be used to detect pesticide contamination in streams over these different biogeographical regions. These results are in accordance with Schäfer et al., (2007) shows that SPEAR approach is able to discriminate uncontaminated versus contaminated across different biogeographical regions, as powerful tool in biomonitoring programme over large spatial scales.

Results of the present study shown that the $SPEAR(fm)_{pesticides}$ index can be effectively use for detection of pesticide contamination in stream. As shown by Figure 1 the explanatory power of the index and the efficiency is only slightly and non-significantly lower than that of the species-level index, that exhibited no significant differences concerning the slopes and intercepts in the regression models. However, both descriptors are greatly increased when undisturbed stream sections are present in upstream reaches. The levels of biological impairment observed at sites with high pesticide contamination and good habitat quality in the upstream reaches were similar to those at sites with low pesticide contamination and poor habitat quality in the upstream reaches. These results suggest that the geographical unit of the risk assessment of streams should be extended to include the recovery potential of the landscape associated with undisturbed stream sections.

Hoekstra et al. (1994), examining the literature regarding 26 chemicals, demonstrated that the variation of sensitivity between species within a family is usually less than the variation between families, concluded that the sensitivities of species depend on their taxonomic position since taxonomy is a reflection of the phylogenetic relationships between the species, and closely related species may share many characteristics relevant to their sensitivity to chemicals through descent from common ancestors. This is a possible reason of the observed uniformity between the species- and family-level $SPEAR_{pesticides}$ indices that within-family variability of selected traits for SPEAR classification is lower than the variability between families. Additional reason is that information reported for single species are extrapolated from information on higher taxonomic level, as a consequence high resemblance between species and family levels could be an artefact due to extrapolation of information; from family to species.

Taking into account the time-consuming nature, cost and difficulties of species-level identification, the $SPEAR(fm)_{pesticides}$ index is a promising and cost-effective bioindicator tool for detecting pesticide contamination in streams.

Use of the SPEAR approach at the family level of taxonomic resolution and its validation across numbers of countries suggest that this bioindicator can be easily included in biomonitoring programmes in different countries, assuming that exposure-effect relationships in streams do not differ greatly to other investigated areas in Europe (Finland, France and Germany).

2.5. CONCLUSIONS

In conclusion, the present study showed that the index $SPEAR(fm)_{pesticides}$ is potentially applicable across different types of watercourses as well as across different biogeographical regions in Europe with similar boundaries of ecological status classes (in WFD as excellent-good-moderate-poor or bad). Stability at the large spatial scale indicates that this index is a promising bioindicator of organic toxicants for large territories such as entire EU within the biomonitoring programs according the Water Framework Directive (EU, 2000; see also Beketov et al., 2009). The SPEAR-based indicator is not implemented in current Environmental programme procedures, but is a transparent and simple indicator of pesticide contamination. A possible application in a combine approach could be with molecular methods where SPEAR-based indicator is used to diagnose the magnitude of contamination and biomarkers are applied subsequently to identify the type of contaminant i.e. the Acetylcholinesterase (AChE) assay, because this technique has the potential to identify or exclude organophosphate or carbamates insecticides as contaminants responsible for observed biological impairment.

This statement is in accordance with what is reported in Schriever et al., (2008) were the review of current biological indicators shows the within the group of community-based approaches, SPEAR is one of the most promising bioindicator method to detect pesticide contamination in ecosystems.

2.6. PAPER

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3. BIOMARKER RESPONSES OF TEMPERATURE AND POLLUTION ON SOFT-SHELL CLAM *Mya Arenaria*

3.1. INTRODUCTION

Human activities as gas emissions have altered the Earth's atmosphere and sufficient evidence on direct consequences at global scale are reported on increasing in global temperature, rising sea levels, and changes in nutrient loads (Micheli, 1999; Dynesius and Nilsson, 1994; Gillett et al., 2003; Brierley and Kingsford, 2009; IPCC 2002; IPCC 2007). Mathematical models estimated the rise of temperature at global scale around 1.8 - 4°C by 2100. (Lanning et al., 2008; IPCC, 2007). The consequences of global warming affect biological process in marine and terrestrial ecosystems from individual organisms to population structure, geographical distribution and species composition of communities.

The knowledge about effects of temperature changes and chemical toxicity to aquatic organisms, has so far been lacking (Cairns et al., 1975; Portner, 2010), but is needed to understand and predict future global changes and possible risks. Physiological studies on temperature and pollution interactions in ectothermic species, can address susceptibility of aquatic organisms to the toxic effects of contaminants as consequence of temperature fluctutions. High temperature can cause critical reduction in tissue oxygenation, mitochondrial function and energy production (concept of thermal tolerance Portner, 2002). Thermal stress, beyond low or high critical temperatures, was shown to increase oxidative stress (Abele et al., 1998; Heise et al., 2003; Keller et al., 2004). Additionally also studies on pesticide effects indicated a dose-dependent response in the rate of superoxide anion increase and increase on energy demand in clams i.e. effect of paraquat on *Geukensia demissa* and *Rangia cuneata* (Wenning and Di Giulio, 1988) or stimulation of metabolic heat rates in *Elliptio complanata* (Cheney et al., 1997). In other studies, a positive correlation between xenobiotics concentration presents in ecosystem and increase in defense mechanisms of molluscs has been reported (Fisher et al., 2000; Gagnè et al., 2007). Electron transport in mitochondria is closely correlated with oxygen demand and consumption rate (Pörtner and Knust, 2007), that may be accelerated by xenobiotics in Lab experiments (De Coen and Janssen, 2003; Smolders et al., 2004).

This defense system is the lowest level of response a subcellular level, as interference with metabolic pathways, mechanism involved in a cascade event with effects on a higher level of organisation such constraint in behavioural responses, growth and reproduction, and possible long term effects on population size and structure.

The hypothesis of this study was to assess the combine and cumulative effects of temperature and pesticide exposure on soft-shell clam physiology. Different biomarkers were selected in order to measure an early response, an initial changes caused by toxicological interactions between the chemical and the (biological) receptor site. Specimens of temperate molluscs *Mya arenaria* inhabiting mudflat of St Laurence estuary, is already selected species as marine bioindicator organism in numbers of studies (Fournier et al., 2002; Frouin et al., 2007; Gagnè et al., 2007). Clams were acclimated to two different temperatures: the basic Atlantic seawater temperature (7°C) and an elevated temperature (18°C) and were exposed to a commonly used herbicide, (registered trademark Weedout®) based on three chlorophenoxy pesticides: Dichlorophenoxyacetic acid (2,4-D), 2-(2-methyl-4-chlorophenoxy) propionic acid (mecoprop) and 3,6-dichloro-2-methoxybenzoic acid (dicamba) for 28 days. Chlorophenoxy herbicides with a half-life range in the water of 13 to 50 d are of high solubility and persistence in water. 2,4-D, mecoprop, and dicamba are active ingredients reported in 2002 (OMAFRA, 2002) as most frequently used by professional lawn-care applicators in Ontario province and discharged directly through overland runoff to rivers such as the Don and Humber Rivers (rivers that flows into lake Ontario and downstream to St Lawrence river). These herbicides were applied on soils, and adjacent water bodies which are usually the ultimate recipient for pesticide residues. Therefore, non-target organisms belonging to sediment and water compartments, as the infaunal bivalve *M. arenaria* are of primary interest when addressing the potential adverse effects of pesticides. 2,4-D and 3,6-dichloro-2-methoxy-benzoic acid (dicamba) are considered to be of low-to-moderate mammalian toxicity and have a limited persistence in the environment (Bradberry et al., 2000). Chlorophenoxy herbicides have been shown to form analogues of acetyl-CoA *in vitro* with disrupting potential in the cellular metabolic pathways that involve acetyl-CoA (Sastry et al., 1997). Alteration of energy metabolism in rat by uncoupling oxidative phosphorylation maybe correlated with the disruption of the phospholipids bilayer of mitochondrial membranes and ion channels transport mechanism (Peixoto et al., 2003) which can promote inhibition of respiratory complex. Additionally some evidence are reported for pesticide like 2,4-Dichlorophenoxyacetic acid (2,4-D) and its metabolites which, act as neurotoxic agent and possible endocrine disruptors (Colborn, 2006; Cheney et al., 1997). Studies realized with Pacific oyster *Crassostea gigas* exposure to pesticide, showed that 2,4-Dichlorophenoxyacetic acid (2,4D), significantly increased ROS-positive cells and cell mortality at 450 µmol/l after a 4 h incubation period (Gagnaire et al., 2006).

Biochemical analysis were tested on gills, this tissue was selected since is the major respiratory organ and major site of uptake of xenobiotics chemicals in this filter-feeding infaunal clams. Additionally antioxidant enzyme activities (AOX) tend to be higher in metabolically active tissues like gills compared to muscle tissue in fish (Lemaire et al., 1993).

On this target tissue we carried out enzyme activity measurement of cytochrome C oxidase (CCO) and the antioxidant enzyme superoxide dismutase (SOD) used as biomarker of oxidative damage (Regoli and Principato, 1995). The CCO enzyme located in the inner side of the mitochondrial membrane is recognized as a good indicator of aerobic metabolism in numbers of studies (Couture P and Pyle G, 2008; Pelletier et al., 1993). Moreover, is the last enzyme in the respiratory electron transport chain in mitochondria, and participates in the establishment of the transmembrane proton gradient required for the ATP synthesiation. SOD is the enzyme involved in the cellular defense system against toxicity from ROS, and metalloenzyme, that catalyse the dismutation of the superoxide radical to hydrogen peroxide (H_2O_2) and oxygen (O_2). However, H_2O_2, can also be converted via the Fenton reaction to the highly reactive hydroxyl radical (OH), although the catalase and glutathione peroxidase systems can neutralise the hydrogen peroxide by converting it to H_2O and O_2. Several studies in aquatic organisms demonstrated the importance of enzymatic antioxidant defenses in protecting cellular systems from oxidative stress induced by xenobiotics (Gagné et al., 2006; Livingstone, 2001).

Biomarker responses of superoxide dismutase (SOD) and the metabolic enzyme cytochrome C oxidase (CCO), presented here were part of a wider project were additional studies were carried out at different levels of biological organisation (biochemical, metabolic and cellular) as: immune parameters (phagocytosis activity and efficiency), biomarkers of oxidative stress (catalase (CAT) and activities and malondialdehyde (MDA) content), a biomarker of pesticide exposure (acetylcholinesterase (AchE) activity) and the activity of an enzyme related to gametogenetic activity (aspartate transcarbamylase (ATC)). Additionally status of the reproductive cycle, gonado-somatic index, condition factor and sex were also assessed. Results of this mulibiomarker approach, using *M. arenaria* as test species in laboratory experiment to evaluate biomarker responses to organic toxic agent was published on 2010.

3.2. MATERIALS AND METHODS

3.2.1. EXPOSURE PROTOCOL AND SAMPLING

Clams with a mean shell length 57±6 mm (n = 390), *M. arenaria*, were collected in summer 2006 in south shore of the estuary of St Lawrence at Metis beach (N 48° 40', W 68° 80'), Québec Canada. Clams were placed in 10 aquaria (volume 50 L) with a constant filtered seawater flow rate of 150 ml/min and 10 cm of clean sediment. The study consist of 28 day-long experiment with 5 treatments: two control aquaria at 7°C (T_0); 4 aquaria kept at 7°C, two with pesticide and two without pesticide; 4 aquaria kept at 18°C 2 with and 2 without pesticide. Forty-five clams (except for control aquaria with 15 clams) were transferred and acclimated (24 h) in each aquarium, and within the treatment of 18°C temperature increase from 7 to 18°C within 8 days, with a daily increase of 1-2°C. Clams were exposed to a constant concentration of Weedout, from a stock solution obtained by diluting 1ml Weedout (containing 3 g/L 2,4-D; 3 g/L mecoprop; 0.3 g/L dicamba) in 3 L of seawater, corresponding to a nominal water concentrations of 0.01 mg/L 2,4-D, 0.01 mg/L mecoprop, and 0.001 mg/L dicamba. Herbicide concentrations were selected on the basis of expected environmental concentrations (Syracuse, 2004; US Forest Service, 2006). Since the aquaria are open seawater system, seawater is drawn from estuarine and seston was available during the exposure experiment. Sampling were performed at T_0, T_7, T_{14}, and T_{28}, at each sampling time 15 clams were randomly selected and transported in coolers to Laboratory of Institut des Sciences de la Mer of the Université du Québec à Rimouski for processing samples.

3.2.2. BIOCHEMICAL ANALYSIS

Specimens of *M. arenaria* were collected at T_0, T_7, T_{14}, and T_{28} (sampling were performed on day 0 and after 7, 14, 28 days of exposure) and gills rapidly dissected for the biochemical analyses frozen in liquid nitrogen and stored at -80 °C. Analysis carried out in March-April 2008 at Laboratoires of Institut National de la Recherche Scientifique–Centre Eau Terre Environnement (INRS-ETE), Quebec, Canada. Gills tissues were homogenized in ice-cold buffer for three times of 20s using an Ultra Turrax T25 tissue homogeniser, in five volumes of ice-cold homogenization buffer 20 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), pH 7.2, containing 1 mM ethylenediaminetetra-acetic acid (EDTA), 1% Triton X-100, 210 mM mannitol, and 70 mM sucrose.

Enzyme activities were measured on a UV-VIS Varian Cary 100 Bio spectrophotometer using a 6x6 multi cell block equipped with a thermostated cell holder and circulating refrigerated water bath to adjust the temperature analysis to the temperature used in the experimental design. Reactions were measured for 5 min, and the last 3 min were used to calculate reaction rates. Protein concentrations were determined using the bicinchoninic acid method according to Smith et al., (1985).

Cytochrome c oxidase activity was measured (Gauthier et al., 2008; Pelletier et al., 1994) in the homogenate, spectrophotometrically at 550 nm against blank containing 100 mM phosphate buffer, 0.07 mM cytochrome c, and 0.5 mM KFeCN (ferricyanide). For the samples, 10 ul of homogenate substitute KFeCN. Dithionite (sodium hydrosulphite) was added at the solution for the reduction of cytochrome c and aerated for 5 minutes to eliminate the exceeding reducing agent. Results were expressed as mU/mg protein.

The protocol for the SOD measurements was based on Paoletti et al. (1986) and Paoletti and Mocali (1990) adjusted on our case of study. This method is based on the inhibition of the NADH oxidation reaction, which rely upon the superoxide. The SOD activity is measured by spectrophotometry. A chain reaction involving thiol oxidation and O_2 reduction occurs in presence of sufficient concentrations of EDTA, Mn^{2+} and mercaptoethanol. The addition of SOD in the solution causes a decrease of the NADH oxidation rate that is proportionate to the quantity of enzyme added. The homogenate was centrifuged for 5 min at 1500 rpm at 4°C. The resulting supernatant was transferred in an eppendorf tube, dialyzed for 20 min in ice-cold buffer over a magnetic bar, and diluted (1/100) with buffer (20 mM HEPES (4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid), 1 mM ethylenediaminetetra-acetic acid (EDTA), 210 mM mannitol, and 70 mM sucrose, pH 7.2). In the dialysed supernatant SOD activity was determined spectrophotometrically at 340 nm. Results were expressed as U/mg protein.

3.2.3. STATISTICAL ANALYSIS

Results of all parameters measured were expressed as mean ± standard deviation (SD). Normality and homogeneity of variances were verified by Shapiro-Wilk and Cochran test, respectively (PAST software, Oslo University, Oslo, NO). Due to the non parametric distribution of all variables studied, a PERMANOVA (PERmutational Multivariate ANalysis Of VAriance) analysis was performed (Anderson et al., 2001). Significance and high significance were set at $p < 0.05$ and at $p < 0.001$, respectively.

To test for differences in temperature and pesticide effects at each sampling time, a first pair-wise one-way PERMANOVA analysis was conducted with factor: treatment (temperature and pesticide exposure) (fixed, orthogonal, four levels 7°C, pesticide exposed clams 7°C, 18°C and 18°C pesticide exposed clams).

To test for differences among all sampling times, a second pair-wise two-way PERMANOVA analysis was conducted with factors: time (fixed, orthogonal, four levels, day 0 control, day 7, day 14, day 28) and treatment (temperature and pesticide exposure) (fixed, orthogonal, four levels 7°C, pesticide exposed clams 7°C, 18°C and 18°C pesticide exposed clams). Additionally, to test for differences between day 0 control *versus* all treatments, a pair-wise one-way PERMANOVA analysis was conducted with factor: interaction between time and treatment (temperature and pesticide exposure) (Fixed, orthogonal, 13 levels, day 0 control and 7°C, pesticide exposed clams 7°C, 18°C, and 18°C pesticide exposed clams at day 7, 14, and 28). The software used for analysis of variance was PERMANOVA + for PRIMER 6 (PRIMER-E Ltd, Plymouth, UK; Anderson et al., 2008). No statistical analysis was carried out on histological data of gonad development.

3.3. RESULTS

3.3.1. OXIDATIVE STRESS

After 28 days a significantly high increase in CCO activity was observed in all treatments compare to the control (Figure 1). Moreover a significant increase was detected also between day 7 and 28 for each treatment at temperature 7°C exposed and not exposed to pesticide and at temperature 18°C with and without pesticide. Except for 18°C without pesticide treatment were the highest value (3.75 ±0.58 mU/mg protein) was detected on T_7 (Figure 1), and a following decrease of CCO activity was observed after 14 (2.15±0.45 mU/mg protein) and 28 days (3.09±0.59 mU/mg protein). After 7 and 28 d, the exposure to pesticide enhanced CCO activity in samples kept at 7°C ($p < 0.05$). In samples kept at 18°C pesticide effects was detected after 14 days as well as after 28 days with a significantly higher increase in the CCO activity ($p < 0.001$). At all the sampling times, CCO activity was significantly increased by the effects of the higher temperature in pesticide-exposed and not-exposed clams (Table 1).

A decrease in SOD activity between day 7 and 28 was observed only in clams kept at 18°C with pesticide (T_7 633±262; T_{14} 568±183 and T_{28} 567±140) not evidenced by PERMANOVA results were *p* value result > 0.05. Within the treatment 18°C at T_{14} SOD activity was significantly enhanced (Figure 1), while exposure to pesticide in samples kept at 18°C significantly inhibited the activity of this enzyme (Table 1; $p < 0.05$). After 28 d, pesticide exposure in samples kept at 7 and 18°C significantly inhibited SOD activity, whereas increased temperature in pesticide exposed clams had the opposite significant effect on the same enzyme (Table 1). At T_{28} we register a significantly inhibition of SOD activity compare to T_0.

Table 1. Level of significance (*p* values) of the effects (increase (+) or decrease (-) of CCO and SOD observed activities). Pesticide effect at 7° C (7°C vs 7°C pest); pesticide effect at 18°C (18°C vs 18°C pest); effects of increasing temperature in not exposed samples (7°C vs 18°C) and exposed to pesticide (7°C pest vs 18°C pest). ns stand for not significant effect ($p > 0.05$).

		***p* values**			
	Days	**Pesticide effect at 7°C**	**Pesticide effect at 18°C**	**Increasing temperature effect**	**Increasing temperature effect in pesticide exposed clams**
CCO					
mU/mg protein	7	**< 0.05 (+)**	ns	**< 0.001 (+)**	**< 0.001 (+)**
	14	ns	**< 0.001 (+)**	**< 0.001 (+)**	**< 0.001 (+)**
	28	**< 0.05 (+)**	**< 0.001 (+)**	**< 0.001 (+)**	**< 0.001 (+)**
SOD					
U/mg protein	7	ns	ns	ns	ns
	14	ns	**< 0.05 (-)**	**< 0.05 (+)**	ns
	28	**< 0.001 (-)**	**< 0.05 (-)**	ns	**< 0.001 (+)**

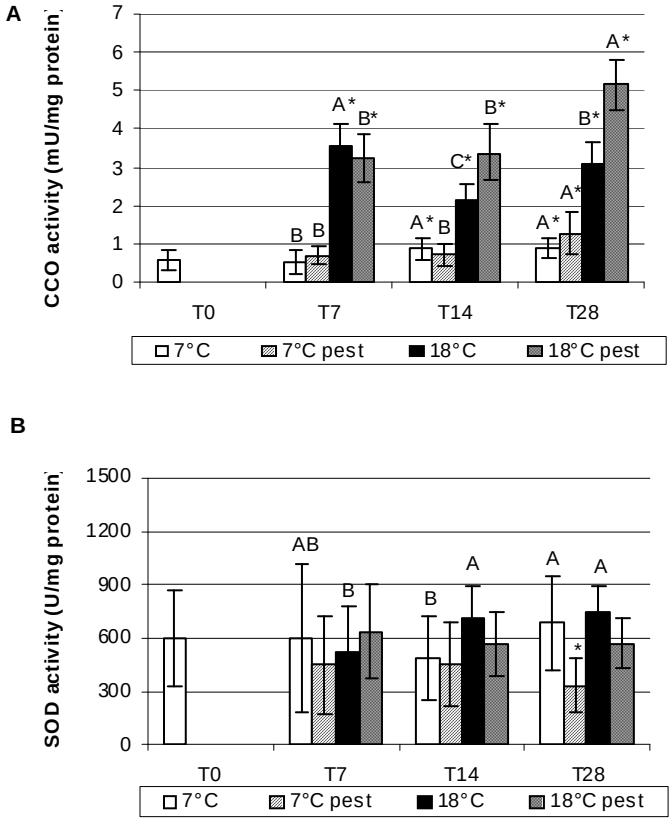

Figure 1. Data are shown as mean and ±SD in A) CCO activity (mU/mg protein) and in B) SOD activity (U/mg protein). Significant statistical differences for each parameter and treatment among sampling times (7°C T_7 vs 7°C T_{14}) are represented by different uppercase letters ($p < 0.05$). Values marked with an asterisk differ significantly from controls (T_0).

3.4. DISCUSSION

Bivalves are ectothermic species, where their metabolism has to cope with the environmental variables of their habitats (e.g. temperature, salinity, pH, dissolved oxygen and rate of sediment deposition) in order to maintain homeostasis for reproduction, growth and survival. This ability depends on functional capacity of ventilation or circulation confined in their thermal tolerance window, which allow a tissue oxygenation and mitochondrial functionality. *M. arenaria* is exposed to variable environmental conditions of a tidal ecosystem, showing a specific geographical distribution according to their thermal window, which spans from 4° C in winter to 18°C during low tide on hot summer days (Heise et al., 2003). Therefore in a possible scenario of a climate change, additional stressors like variation in physical chemical parameters (i.e. increasing temperature and pollution) could add additional stressors to ectotherms populations.

From our results a tight linkage between temperature and physiological response in clams was underline, since increasing temperature has an effect on CCO, aerobic capacity indicator, with a consistently increase in clams after only 7d after exposure ($p < 0.001$). These observation of a reduction in aerobic capacity suggest that animals are close to what is define as critical temperature (Tc) by Pörtner (2002) the highest border of their thermal tolerance window. This reduction is not caused by falling levels of PO_2 but to the limited capacity of the clams to cope with the animal oxygen demand by ventilation and circulation. The main observed effects were that pesticide and temperature have an effect on aerobic capacity and energy demand in clams. In agreement with Gagné et al. (2007) in *M. arenaria*, Mitochondrial Electron Transport (MET) or cellular energy consumption is a temperature-dependent and readily accelerated by different xenobiotics for maintaining cost of homeostasis. Therefore at higher critical temperature constraints occurs with a reflection on tissue oxygenation mitochondrial function and energy production (Frederich and Pörtner, 2000), and a consequent increased energy demand to maintain homeostasis.

Additionally pesticide exposed clams showed to be affected in their aerobic capacity after 7d exposure ($p < 0.05$) as well as exacerbated effects due to the additional temperature after 14 d ($p < 0.001$; effect of combine effects of pesticide and temperature). This statement is in agreement with the effects reported by Cheney et al. (1997) in *Elliptio complanata* used to test effects of herbicide on energy metabolism, short-term exposure to low levels of 2,4-D that enhanced mitochondrial respiration rates, probably due to alteration in membranes. In our research a significantly increase in effect of pesticide was found after 7 days at 7°C ($p < 0.05$) and after 14

days at 18° C ($p < 0.001$). Therefore it is likely that xenobiotics exposition accelerate cellular energy consumption, according to what is shown by Lanning et al. (2008) in *Crassostrea virginica* and Gagné et al., (2006) in *Elliptio complanata* mussels.

With increasing temperature and pesticide exposure after 28 d SOD activity was increased ($p < 0.001$), these results support the hypothesis of hierarchy of effects from aerobic capacity to molecular protection by antioxidative defense. Results are in agreement with what was observed by Abele et al. (2002) where mitochondrial activity and production of ROS in *M. arenaria*, has been described as temperature-dependent response. In this study at higher temperatures (15 to 25°C), mitochondrial release of ROS increase, contributing significantly to oxidative stress in mitochondria. Even if the experiment reported that temperature induced system of ROS formation by increasing in AOX activity, was not found under temperature close to habitat (20°C) indicating that the antioxidant system in thermal window are able to prevent ROS damage, therefore animals have no need to invest into AOX synthesis.

It is unclear however, the reduction in SOD activity after 28 d in clams exposed to pesticide (both temperature), may other mechanisms than oxidative stress should be detected. In the case of higher temperature this factor could induce protein unfolding or reflect a general metabolic disturbance affecting the synthesis of relevant part in the antioxidant defense system (Portner, 2002). This statement is reported in an *in vitro* experiment for *M. arenaria* were SOD activity shown a temperature dependency, with a maximum catalytic activity at 18-22°C and fast denaturation of the enzyme above the maximal habitat temperature with an activity loss by 40% (25°C) and 70% at 30°C (Able et al., 2002). Additionally the drop in the of SOD activity registered after 28 d in the treatment of 7°C with pesticide indicated that compare to control may a decrease in the equilibrium occurs and should be interesting to further investigate for recovery patterns after 24 h from this event to look on this impairment induced by long exposure to pesticide at low temperature. Since studies with fish (Otto and Moon, 1996) in activities of cytosolic superoxide dismutase (SOD) observed that, in response to toxicant-induced inflammation by ROS, the concentrations of certain antioxidant enzymes are increased, but under high levels of pollution the antioxidant defenses are reduced (inhibition weakened antioxidant status).

Enhanced SOD activities were related with the increased temperature after 14 d, and after 28 days in clams exposed to both factors temperature and pesticide. These finding of a significative increasing of effects in SOD activity after 14 d between clams exposed to 7°C to 18°C could be

related with the fact that under thermal stress, next to critical temperature, increasing demand of oxygen and consequently increase of ROS species (Abele et al., 2002) and activate antioxidative defense system. This event may be correlated with the fact that often in marine invertebrate from low oxygen sedimentary habitats hypoxic episode occurs in relation to the request ventilation non able to cover the oxygen demand from tissue, and then oxygen radicals are released during re-oxygenation receive elevated ROS during a recovery from hypoxia episodes (event resembled to ischemia/reperfusion in mammalian tissue; Chi et al., 2004; Heise et al., 2003). Therefore warmer temperature could be characterised also by hypoxic anaerobiosis and uncoupled mitochondrial activity due to membrane damage by ROS cellular oxidative stress. Additionally regarding SOD activity Huang et al. (2000) shown that inhibition should causes accumulation of O_2^- in cells and free radical damage to mitochondrial membranes and apoptosis.

Even if results are more readable on aerobic capacity than in oxidative stress, we could hypothesis that at higher critically temperature of thermal tolerance window the respiratory chain will be seriously affected, and then a reduction in ATP turnover and antioxidant defenses are overcome by cellular production of ROS, resulting in tissue damages which implies i.e. DNA damage and lipid peroxidation.

Our results could show that the presence of pollution combined with increased temperature could accentuate electron transport activity in mitochondria activity (CCO) and leads to increased oxidative stress. Studies on ROS related process in marine ectotherms are still scarce, and the basal dynamics of oxidative stress not well understood (Abele and Puntarulo, 2004). More studies are needed since bivalves are used as sentinel species for monitoring pollution in costal environments.

3.5. CONCLUSIONS

In the present research, both temperature and pesticide had different and measurable effects on the physiology of the test organism, and these effects were more important on aerobic capacity than on oxidative stress. Our study also indicates that the effects of the two stressors combined are to some extent additive. Overall this study suggest that higher temperatures could rise to an enhancement of pesticide toxicity, emphasizes that clams populations subjected to temperature rise due to seasonal warming or global climate change may become more susceptible to pesticide pollution and vice versa. This suggests how temperature is important when studying toxicity in ectotherms, and to understand environmental effects of warmer temperature in the prospective of global climate change in estuaries. In the present research increasing temperatures altered the biochemical and physiological ability of the sentinel species *M. arenaria* to respond to pesticide exposures. Additionally exposure scenario such as those caused by pollutants run-off in estuarine coastal areas coupled with various aspects of global change, able to promote pro-oxidant processes, could seriously imperil population dynamics and life in intertidal habitats. Indeed impacts on *M. arenaria* population could have direct effects on food webs and trophic structure leading to a progressive decline of the ecosystem. Since the massive presence of a species like *M. arenaria* in Saint Lawrence emphasizes intertidal environments in terms of food web dynamics in this ecosystem. Further work, focusing on the relevance of ecotoxicological significance of the observed effects with studies on clam population that include also provisional model approach on the whole Saint Lawrence River ecosystem.

3.6. PAPER

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4. BIOMONITORING STUDY OF ESTUARINE COSTAL ECOSYSTEM: SACCA DI GORO LAGOON USING *Tapes Philippinarum* (MOLLUSCA: BIVALVIA)

4.1. INTRODUCTION

At present, around 100 000 chemicals are in widespread use and since an high proportion of these chemicals probably reach aquatic environment many of them are potential contaminants for freshwater resources with complex interactions and effects of these compounds in the environment (Schwarzenbach et al., 2006; Sumpter, 2009).

Anthropogenic release of chemical contaminants, coming from agricultural watersheds and industrial areas, may significantly affect complex and labile ecosystems having an impact on coastal lagoon, through riverine inputs. For example, pesticides, heavy metals, polycyclic aromatic hydrocarbons (PAHs) end up to the natural water bodies from municipal and industrial waste water treatment plants and from agriculture practices. In water environment, lipophilicity of these compounds causes their adsorption to organic material and sediment, but can be remobilized by resuspension and thus lead to accumulation of filter-feeding organisms which may result in accumulation of the compounds in food chain.

The Po river flow through one of the most densely populated and productive agricultural and industrial regions of the country, by influencing the inputs of freshwater quality flowing to aquatic ecosystems of deltaic costal lagoons. The catchment of Po river, with a basin area of over 70 000 km^2 is heavily exploited for agriculture, and flows through one of the most productive agricultural (36% Total production) area of the country and carry towards the sea large amount of industrial and domestic waste water (Camusso et al., 2002) decreasing progressively further downstream.

Along the East-Adriatic coast Sacca di Goro is an estuarine costal ecosystem of the Po River Delta. Sacca di Goro lagoon is situated in the proximity of the Regional natural park of the Po River Delta and under protection of Ramsar convention, a highly productive aquatic lagoon and a nursery area for many aquatic species. This lagoon is considered extremely relevant for its ecological and socio-economic value and considered as case of study for highly impacted costal environments in numbers of scientific projects (Zaldivar et al., 2003).

Sacca di Goro lagoon is affected by contamination of heavy metals (Locatelli and Torsi, 2001; Viaroli et al., 2006), by nutrient pollution (Bartoli et al., 2001) and pesticide (Carafa et al., 2007) . According to Giordani et al., (2009) who developed index TWQI for water quality i.e.

considering parameters as dissolved oxygen DO and inorganic nitrogen DIN related to eutrophication and phytoplankton blooms during summer, considered a very low water quality.

Sacca di Goro has knowledge on PPP historical contamination episodes as the result of leaching of cultivated inlands (Carafa et al., 2007; Baldi et al., 1994) and considered heavily impacted by PPP used in agriculture (Viaroli et al., 2006). Actually around 80% of the watershed is dedicated to extensive agriculture with different crop typologies as corn, wheat, rice, sugar beets, and soybean. Experimental measurements of selected herbicides in different environmental compartments: water column, sediment and clams reported high concentration of s-triazine – terbuthylazine – urea herbicides – diuron – and alachlor (Carafa et al., 2007) shown that pesticides delivered in the Sacca di Goro (with clear seasonal pattern and spring peaks as main period of agrochemical application) may have effect on coastal lagoon ecosystems. In 1989 and 1990 studies (Baldi et al., 1994) shown the presence of atrazine, molinate and DDT and metabolites as significant concentrations during a monitoring programme that include herbicide (molinate, atrazine, simazine and other s-triazines) and insecticides (DDT, aldrin dieldrin, endrin and endosulfan). Recent studies carried out by ARPA Emilia Romagna (unpublished data, 2007) found that simazine and terbutilazine are still detectable in the sediment even if their use was banned under the Directive 91/414.

Sacca di Goro receive freshwater inputs, rich in organic and mineral nutrients derived from urban, agricultural and/or industrial effluents and domestic sewage, as a consequence the lagoon experienced an abnormal proliferation of macroalgae (*Ulva rigida*), as an increase of eutrophication of this ecosystem (Viaroli et al., 2006). During summer decomposition of significative biomass of this macroalgae bring to severe anoxic event (in 1997 reported a biomass estimation of 100-150 000 tons). Overall these chemicals can cause various types of impacts: toxicity of the water for human's consumption or aquatic life, or contributing towards the eutrophication of water bodies (Viaroli et al., 2005).

Three sampling stations differing along a spatial pollution gradient were selected according to reported source of contamination. Following sampling stations: G site in proximity to port of Goro and P site next to the reported freshwater input from watershed of agricultural origin (Carafa et al., 2007) and with approximately 67 000 inhabitants basin; N site (Nursery) was selected as reference area since is the sampling station near to the southern sand barrier close to the sea mouth (less continental), with a high hydrodynamism and salinity close to the once of

the sea, factors that provide to N site the characteristics to be selected by clams veliger larvae as natural collector site (Carafa et al., 2006).

In the presented field study, intend to investigate the potential of biomarkers and biomonitors in aquatic invertebrates to predict risk of toxicity in the context of current or emerging EU regulation and guidelines. An approach to marine and costal pollution monitoring was proposed in order to assess effects at low concentration in complex mixtures and the ecological status of an ecosystem (Viarengo et al., 2007) combining the traditional chemical analyses with laboratory and field-based biological endpoints. The combination of analytical chemistry and biomarkers (Binelli et al., 2010; Morales-Caselles et al., 2009) is an approach that aids the identification of the impact of chemical contamination on different levels of biological organisation (Galloway et al. 2004), were chemical analyses are able to measure the contaminants present in terms of concentrations values detected, but do not necessarily reveal potential biological effects. Most of the studies report the use of sentinel species (Da Ros and Nesto, 2005; Magni et al., 2006), allowing the assessment of the status of organisms under chronic stressful environmental conditions (Moore et al., 2004). Bivalves have been used in many parts of the world as biomonitors because of wide geographical distribution, filter-feeding behaviour, sedentary nature, comparatively low Cytochrome P450 activities and their ability to sequester some metals and lipophilic contaminants such as petroleum hydrocarbons (PHCs), chlorinated pesticides (CPs), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). Manila clam, *Tapes (Ruditapes) philippinarum* (Adams and Reeve, 1850) is a soft sediment dwelling bivalve chosen as test organism and not so widely used in biomonitoring studies. Although this species is recognized as useful sentinel bioindicator for indirect description of habitat quality in costal marine environment (Solè et al., 1994; Nasci et al., 2000; Moraga et al., 2002; Irato et al., 2007). Crucial in this case is the direct contact with sediments, which gather a number of contaminants and consequently, benthic infaunal organisms are particularly exposed to chemical stress.

In the present study we select this species for the commercial importance, the scientific concern regarding the development of a useful tool able to detect the healthy status of an ecosystem. Additionally a growing concern is focus on controlling biological and chemical hazards associated with aquaculture products (EFSA, 2009) and consequent consumer health i.e. quantitative analysis of the main environmental xenobiotics (Binelli and Provini, 2003). In this

framework studies on selected biomarker could have some implications on human consumption and safety assessment.

Biomarkers related with an higher level of biological organisation (i.e. behaviour) are more able to detect long term effects as the environment of estuarine costal lagoons subjected to a complex mixture of contaminants (Dourou et al., 2007). Behavioural measures have been advocated as a suitable approach to potentially link physiological function of the individual (internal biological processes) with important ecological processes. Behavioural endpoints studies are reported in different taxonomic groups of aquatic organisms: clams *Scrobicularia plana* (Bonnard et al., 2009) as well as in *Tapes philippinarum* (Matozzo et al., 2006; Moschino et al., 2010), infaunal polychaete, *Nereis diversicolor* (Mouneyrac et al., 2010), crabs (Dissanayake et al., 2010) as well in fish (Scott and Sloman, 2004).

One of the most investigated pathways is the exposure of organisms to xenobiotics that could induce oxidative stress in organisms, through the formation of reactive oxygen species (ROS). Organisms, in order to protect themselves against free radicals generated by oxidative pollutants, activate their defense system with antioxidant enzymes such as catalase, superoxido dismutase and glutathione peroxidises (Orbea et al., 2002; Pellerin, 1994). Catalase is often one of the earliest anti-oxidant enzymes, to be induced with non-specificity for a class of contaminants, with increasing levels of catalase following the exposure to xenobiotics (Van der Oost et al., 2003; Tlili et al., 2010).

Different contaminants and their interactions are responsible of ROS formation that could be act as an additional co-inducer in oxidative stress and mutagenic actions (Wiseman and Halliwell 1996) as DNA and chromosomal damage. Carcinogen and teratogen activity of organic contaminants such as PAHs is well establish, and among PAHs benzo[a]pyrene is well known to induce genotoxicity, and additionally research underline its additive or synergistic interactions with Cd or As (Maier et al., 2002; Van den Hurk et al., 2000). Bivalves are one of the taxonomic groups able to transform contaminants in active metabolites and bioaccumulate toxicants in their tissues, even if in the environment chemical analyses are able to detect lower levels of concentrations.

Additional factors that should be taken into consideration, is the cumulative and opposite effects of contaminant interaction in an estuarine environment, where bioavailability and toxic effects could significantly be affected form environmental parameters like temperature and salinity. For these reasons, is feasible to be concerned about the exposure to active substances capable to act

as mutagens and carcinogens since bivalves chosen in this monitoring are reared for human consumption.

A genotoxicity biomarker such as the micronucleus test was selected as index of cumulative exposure, as a sensitive model to evaluate genotoxic compounds where MN, once formed stays present in the cell until the end of her life-span (Bolognesi et al., 2006). Additionally since nowadays genotoxicity biomarkers are considered a validated instrument that should be integral part in the battery of biomarker as long term exposure to genotoxic compounds (Viarengo et al., 2007; Magni et al., 2006). In this study genotoxicity test was evaluated in association with the evaluation of a number of enzymatic activities, as an index of antioxidant defense of the organism (CAT SOD). Among biomonitoring methods for the detection of genotoxic effects, the micronuclei (MN) frequency is a well known test able to detect genetic alteration as chromosomal damage, in wild and caged marine invertebrates (Bolognesi et al., 2004; Izquierdo et al., 2003). MN are small DNA bodies localisated in the cytoplasm, as a resulting process of cells division and their frequency is considered a result of chromosomal and genomic damage caused by genotoxic compound. Numbers of studies showed how MN frequencies are able to correlate with pollution load and showing a clear dose and time-dependent response (Bolognesi et al., 1996; Jha et al., 2005). Micronuclei assay could be applied on different tissues, frequently in bivalves studies MN results are obtained from haemocytes and gills, the latter present a higher sensitivity to genotoxic agents (Bolognesi and Hayashi, 2010).

Therefore aim of this research is to evaluate the response of important commercial species clams *T. philippinarum* (Mollusca: Bivalvia) to a gradient of a costal-estuarine environment quality in transitional waters of estuarine costal ecosystem of Sacca di Goro lagoon, Delta Po river. According to the current European legislation, coastal lagoons are classified as transitional waters and fall under the European Water Framework Directive (EC, 2000).

The evaluation of clams status and of the environment subjected to an anthropogenic impact will be evaluated using a multi-biomarker approach (catalase, superoxide dismutase, and micronuclei frequency). The challenge is to integrate individual biomarker responses into a set of tools and indices capable of detecting and monitoring the degradation in health of selected sentinel organism. Gills, the principal respiratory organ, were selected for measurement of MN determinations. Digestive glands were analysed for catalase (CAT), and superoxido dismutase (SOD) antioxidant enzymes indicative of oxidative stress. Additionally behavioural kinetics will

be performed since higher level endpoints are relatively sensitive, showing disturbances at concentrations far below those inducing mortality (reported in *T. philippinarum* Matozzo et al., 2006; in bivalve *Scrobicularia plana* Bonnard et al., 2009). Analytical chemistry monitoring is restricted to key class of contaminants (metals, PAHs), since a general approach in numbers of studies is based on priority pollutants or key priority contaminants (2000/60, WFD). Additionally sediment matrix characterisation will be performed in order to correlate concentrations with response in biomarkers.

4.2. MATERIALS AND METHODS

4.2.1. STUDY AREA

Sacca di Goro with a surface area of 26 km^2 and an average depth of 1.5 m and a highly variable salinity (10-35%) Sacca is Goro is connected with the open sea, and the western area is influenced by freshwater inflow from the Po di Volano (~350 x $10^6 m^3 yr^{-1}$).

Additional freshwater inputs are Romanina, Canal Bianco and Giralda which the same discharge rates (20–55 x $10^6 m^3$ yr^{-1}) (Viaroli et al., 2006). Sampling stations (Figure 1) localisated in the intensive farming licensed area, were selected along a spatial pollution gradient: G site in proximity to port of Goro and P site (P) next to the freshwater input from watershed of agricultural origin and with approximately 67 000 inhabitants basin.

Third sampling site Nursery site (N) is the less "continental" location near to the southern sand barrier close to the sea mouth, with a high hydrodynamism and a salinity close to the one of Adriatic sea (Carafa et al., 2006). N site is a natural collector of *T. philippinarum* larvae, not exploited since all the cooperatives of fishermans (around 150) collect and sown in licensed area seeds collected in the Nursery area (N). In the present study N site is reported as a "reference" site. Sampling sites location was shown in Figure 1.

The lagoon is one of the most important aquacultural systems in Italy for clams and mussel production.

About 10 km^2 of the aquatic surface are exploited for farming of the Manila clam, *T. philippinarum*, with a mean density of 500 individuals m^2 (up to 2000–2500 adult individuals m^2; Bartoli et al. 2001), with a production of about 15 000 tons/year. Italy is the first producer in EU, and second at global scale (following China), with a high annual production and with revenue oscillating during the last few years around 50 to 100 million Euros.

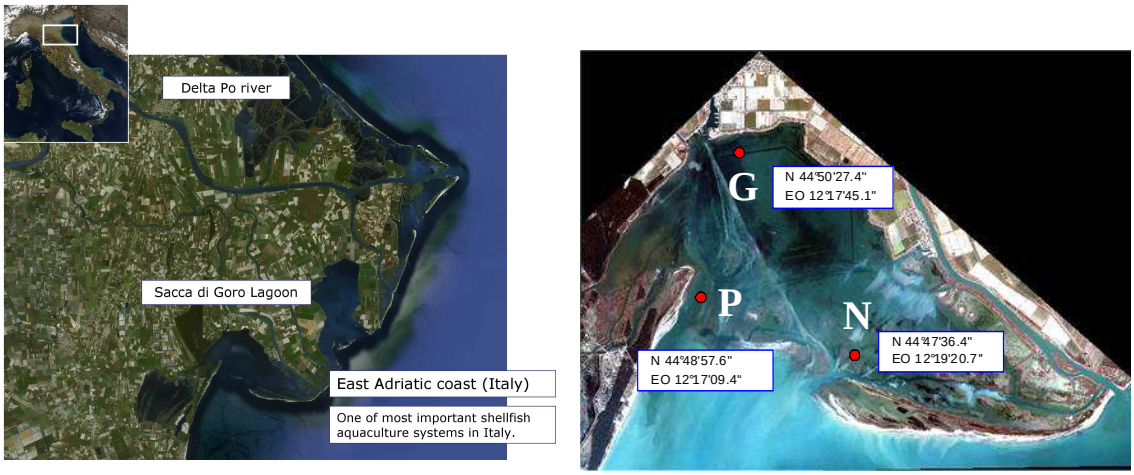


Figure1. Sampling sites located along the Sacca di Goro lagoon, Delta Po River. N nursery area (N 44°47'36.4" EO 12°19'20.7"), P in the plume area that receive fluxes from watershed of Po di Volano (N 44°48'57.6"EO 12°17'09.4''), G close to port of Goro (N 44°50'27.4''EO 12°17'45.1").

4.2.2. SAMPLING

a) *Clams*: two sampling surveys on seasonal scale were carried out, in May and December 2009. Individuals of *T. philippinarum* homogeneous size (long 25-30 mm; $p > 0.05$) were collected from G-N-P sites (Figure 1), and stored immediately in a cooler box and transported to the CRIM Lab facilities in Goro within 2 h, were size was measured using a calliper. A sub-sample of twenty individuals per sample were placed in aquaria containing reference sediment – and behavioural kinetics were performed. Therefore animals were transported under refrigerated condition (+4°C) to Genoa at the National Research Institute of Cancer Research, Environmental Cancerogenesis Unit, were six to ten animals for each sampling site were processed for biomarker analysis. Biological parameters were assessed in two sampling time, May and December 2009, taking to consideration that gametogenesis began in January and is clearly evident until May and spawning mainly occurred in June, continuing during summer until September (Meneghetti et al., 2004).

b) *Sediments* Monitoring comprises four sediment sampling campaigns during the year 2009. The monitoring includes three sites with five randomly selected replicas, for a total of fifteen samples covering the lagoon of Sacca di Goro, each seasonal campaign. Sediment samples were collected in February, May, August and December 2009 their specific locations are shown in Figure 1. On the coastal lagoon, sediment core samples were collected at each sampling site (G, N, and P) using Eijkelcamp multisampler. Upper layer surface sediment (0-10 cm) was

collected in glass jars stored immediately in a portable freezer and then stored at -20° until analysis. Sampling at such depth allows collecting sediment representative of the present status of the sediment compartment in the Sacca di Goro lagoon. At each station, a sediment sample was obtained to determine, organic matter content (TOC g Kg^{-1}), sediment granulometry, TN (% N), pH, metals and As, and PAHs concentrations.

c) *Physical – chemical water characteristics* were determinated at a depth around 50 to 100 cm in each of three sampling sites in February, May, August and December 2009. pH, temperature, salinity and dissolved oxygen (DO) were determined on-site between 8 am and 2 pm, using a multiparameter ion specific meter (Hanna instruments, version HI9828), field equipment checked and calibrated according to manufactures specifications.

4.2.3. BIOCHEMICAL ANALYSIS

Specimens of *T. philippinarum* were collected on a seasonal basis and digestive glands rapidly dissected for the biochemical analyses; for each sampling period, 9 samples were prepared (each constituted by tissues of three up to five specimens, total of 3 g), three pools per site frozen in liquid nitrogen and stored at -80 °C. Hepatopancreatic tissues were homogenized with a Potter-Elvehjem tissue grinder in the appropriate ice-cold buffer, 10 mM of Tris–HCl buffer (pH 7.6) containing 150 mM of KCl and 0,5 M of sucrose. Sub-cellular fractionation, cytosolic and mitochondrial matrix, was conducted in order to localizing antioxidant enzymes activities (Orbea et al., 2002). The homogenate was centrifuged for 15 min at 1480 rpm and at 4 °C. After centrifugation, the supernatant was transferred in an ultracentrifuge system (Beckman, Model SW 41 centrifuge) for 45 min at 9 800 rpm at 4°C. The mitochondrial pellet was resuspended in 3 mL homogenise-buffer. The supernatant collected in two centrifugation steps were centrifuge for 90 min at 28000 rpm at 4°C, and the resulting supernatant was considered as the cytosolic fraction. Aliquots were stored at -80° C until analysis. Total protein contents of all fractions were determined spectrophotometrically using a UV–VIS reader (Beckman DU 6400, USA) a wavelength of 595 nm was used according to the method of Lowry et al. (1951) using bovine serum albumin (BSA, fraction V) as standard. Enzymes were measured on a Beckman DU 6400 UV-VIS reader spectrophotometer, maintained at room temperature. Reactions were measured for 120 seconds and a linear portion was used to calculate reaction rates.

Reaction conditions for selected antioxidant enzyme activity assays were as follows:

Catalase activity (CAT; EC 1.11.1.6) were performed in the cytosolic and mitochondrial fractions and was measured spectrophotometrically at 240 nm using a specific absorption

coefficient at 43.59 cm^{-1} M^{-1} H_2O_2 3 mL of substrate solution made up of 150 mM H_2O_2 in a phosphate buffer 80 mM at pH 7 and 20 µL of homogenate against blank. Results of CAT activity were calculated as mM H_2O_2 degraded, expressed as mmol/min/mg protein. Superoxido dismutase (SOD; EC 1.15.1.1) activities were performed in the homogenate cytosolic fraction of digestive glands. The enzyme activity was indirectly assayed by measuring cytochrome c reduction in potassium phosphate buffer 50 mM, EDTA 0.1 mM, pH 7.8 containing 0.3 mM cytochrome c, hypoxantine 1.5 mM, and xanthine oxidase 56 mUnit/ml (ΔA_{550} = 0.025 min^{-1}) was followed spectrophotometrically at 550 nm. Results were expressed as SOD units U per mg/protein (1 U = amount which cause 50% inhibition of the initial rate of cytochrome c reduction by common substrate $O_2^{\bullet -}$ radicals produced by the enzymatic activity of xanthine oxidase on hypoxantine).

4.2.4. BIOMARKER OF GENOTOXICITY: MICRONUCLEUS TEST

Micronucleus (MN) frequency assay were performed as described by Bolognesi et al. (1999) Schmid, 1976). We analyzed MN in cells from gill tissue, for each site (N-G-P) and sampling time (May and December 2009) we remove gill pairs from specimens of *T. philippinarum*. Preparation of gill cells for micronucleus test consist on an enzymatic digestion with a solution of 0.1 mg/ml dispase I (neutral protease, grade I, Boehringer, Mannheim, Germany) in a modified (20 per thousand) Hank's balanced salt solution, for 7 minutes at 37° C. Cells suspension obtained by filtration on 80 µm filters (Millipore Corporation Bedford, MA, USA) were centrifuge for 5 minutes at 1000 rpm. Cellular gills pellet obtained were fixed in methanol: acetic acid (3:1) for 20 min and then centrifuged at 1000 rpm for 10 min. The resuspended cells were spread on slides, air dried and stained with 3% Giemsa for 7 minutes. One-thousand cells with cytoplasm and cytoplasmic boundaries preserved were scored under 1000 X magnification (oil immersion) in order to perform the assay according to basic criteria for cell scoring defined by Countryman and Heddle (1976). Frequencies of MN were expressed as numbers of micronuclei on thousand of cells scored (MN/1000); additionally granular cells were microscopically examine and counted.

4.2.5. BEHAVIOURAL KINETICS

Experiments were performed in May and December in the CRIM Lab facilities in Goro. A sub-sample of twenty individuals per site, with 3 replica each, 180 animals (25-30 mm; $p > 0.05$) were placed in 3 aquaria containing reference sediment – obtained collecting natural beach sand

at a reference clean costal site in Baratti Gulf (Fratini et al., 2008). Animals were placed in plastic units of 60 L filled with 10 cm of clean sediment, clams were maintained in a flow-through seawater open system and test duration was set of 2h observations. The time organism was totally burrowed in the sediment was considered in the analysis of results and Ln of percentage of unburrowed clams+1 settled for calculation. The reburrowing rate test evaluate the ability of clams to dig into sediment (Byrne and O' Halloran, 2001; Matozzo et al., 2004), as ultimate ending in an alteration of their ecological role The observation of behaviour was set one every 5 minutes for a period of 2 h. The reburrowing rate tests evaluate the ability of clams to dig into sediment and consequently as an indirect measure of the physiological status of clams and a potentially link between effect at a lower level of biological organisation (e.g. biochemical and cellular) and long term ecological process ongoing.

4.2.6. SEDIMENTS SAMPLES ANALYSIS

Samples were unfrozen in order to perform polycyclic aromatic hydrocarbons (PAHs) (µg kg^{-1} dw), metals and As concentration (mg kg^{-1}), TOC (total organic carbon content expressed g kg^{-1}; Walkley-Black method), TN (% N; Kjeldahl method), pH, and sediment granulometry (pipette method). The percentages of sand, silt and clay were calculated as: from 2 mm fraction to 50 µm, 50 µm -2 µm and less than 2 µm, respectively.

Sample digestion *aqua regia* procedure and analysis

For concentration of trace element we performed *acqua regia* digestion method, samples were completely dried at room temperature, passed through a 2 mm mesh inox sieve to eliminate coarse material. A representative aliquot of these samples was then ground to a fine powder (less than 200 mm Φ) in a planetary mill (Pulverisette 7 FRITSCH, Oberstein, Germany) with agate grinding bowls and balls. Grounded to a fine powder (200 µm Φ) and sediment samples of around 0.25 g dry weight were pose to contact to 2.5 mL of HNO_3 (nitric acid 65% m/v) and 7.5 mL of HCl (Hydrochloric acid 37% m/v) reagents (Merck, Darmstadt, FRG) purified using a sub-boiling distillation system (Milestone mod. subPUR, Shelton, CT, USA). Digestion treatments were carried out in an automated system (Digiprep Jr model, SCP science, Baie D'Urfé, Quebec, Canada) for 2 h at 95°C. Digested samples were diluted to volume of 50 mL with Milli-Q water with a resistivity of 18 MΩ cm produced with a Milli-Q system (Millipore, MA, USA), and filtered by 0.45 µm Teflon filter membrane (DigiFilter, SCP science) in

vacuum assisted sample filtration (VAF) in order to obtain a faster and more complete filtration with lower sample preparation time and sample manipulation.

Standard reference materials supplied by Community Bureau of Reference Sample (BCR): Estuarine Sediment CRCRM 277, River Sediment BC-CRM 320 and light sandy soil BCR 142 R were used. Trace elements concentrations were determinated by inductively coupled plasma optical emission spectrometry ICP-OES a Perkin-Elmer Optima 2100 DV spectrometry (Massachusetts, USA) and with an inductively coupled plasma mass spectrometry ICP-MS (Agilent Technologies mod. 7500ce with Octapole Reaction System — OCR, Santa Clara, USA). For Hg analysis we used an Automatic solid Hg analyzer AMA 254 (ALTEC Ltd. Khodlova 1297 19300, PRAHA 9 Czech Republic).

PAHs investigated: sample extraction

The 12 PAHs selected for this study are include as priority substances in 2000/60/EC (Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(ghi)perylene, Indeno(1,2,3-cd)pyrene (44 priority substances have been identified). 15 samples for each campaign were analysed for PAHs content: Fluorene (F), Phenanthrene (Ph), Antracene (A), Fluoranthene (Fl), Pyrene (P), Benzo(a)antracene (B(a)A), Chrysene (Chr), Benzo(bjk)fluoranthene (B(bjk)F), Benzo(a)pyrene (B(a)P), Dibenzo (ah) antracene (D(ah)A), Indeno(1,2,3-cd)pyrene (IP), Benzo(ghi)perylene. The samples were prepared by mixing together anhydrous sodium and sediment (20g ww). The mixture was Soxhlet extracted by mean of hexane (80%) and acetone (20%). The extract was concentrated to 10 ml using a Buchi B-811 Rotavapor. A 5 ml aliquot of the extract was evaporated under gentle flow of nitrogen, redissolved with 500 µl of hexane, centrifuged and analyzed by mean of an gas chromatograph (GC) Agilent Technologies 6890 series equipped with a mass spectrometer (MS) Agilent Technologies 5973 series and the data analysis station ChemStation. The column used for analysis was a Zebron ZB-5ms type (5% Phenyl-Arylene–95% dimethylpolysiloxane; 30 m×0.25 mm internal diameter ×0.25 µm film thickness). As carrier gas, high grade of purity helium was used at a constant flow rate of 1.0 ml min^{-1}. Injection temperature was 250°C, with a 1 µl as injection volume and 0.5 min as purge time. The GC-MS oven temperature was maintained at 140°C, and then increased at a rate of 5°C min^{-1} until 200°C. This temperature was maintained for 5 min and finally increased at a rate of 10°C min^{-1} until 280°C and held for 15 min. The detector temperatures were 150°C (MS quadrupole) and 230°C (MS source). Data were acquired in full scan mode and extracting the m/q from the full scan chromatogram for

each of the analytes. Quantification, for resolved compounds of 12 individual PAHs was performed by comparison with external standard (range) purchased from Dr Ehrenstorfer (Augsburg, Germany), PAH mixtures were prepared from stock solution in hexane. Recoveries calculated by comparing the peak areas of the internal standards with the reference solution, were always better than 88%. As internal standard we use a mix of two PAHs d10 anthracene and perylene d12. Since we could not be able to separate the three benzofluoranthene isomers, the results were expressed as the sum of benzo(b)floranthene, benzo(j)fluoranthene, and benzo(k)fluoranthene. In this work the three isomers were considered as a single PAH indicated as benzo(bjk)fluoranthene.

4.2.7. STATISTICS

Non parametric statistical test was used for comparing results concerning MN frequency in gill cells, differences among sampling and sites were evaluated by Mann-Whitney U-test.

Enzyme activities measured were expressed as mean ± SD; differences in sample enzymatic activity according to site and season were examined using permutational analysis of variance (PERMANOVA) (Anderson, 2001). Significance and highly significance were set at $p < 0.05$ and at $p < 0.001$, respectively. To test for differences in enzyme activity at each sampling time, a pair-wise one-way PERMANOVA was conducted with factor: site (fixed, orthogonal, 3 levels N,G,P) and factor season (random, 2 levels Dec Feb). In order to investigate the relationship between the biological and chemical endpoints a Euclidean dissimilarity matrices were performed, regarding variables as PAHs, metals, CAT, SOD and frequency % MN explored using distance based linear models (DistLM). Distance-based Redundancy Analysis (dbRDA) was used to plot the result of DistLM. PERMANOVA, DistLM and Principal Component Analysis (PCA) were performed with software PERMANOVA + for PRIMER 6 routines (PRIMER-E), (Andersonet al., 2008). Correlation between chemical concentrations in sediments of Cr/Ni, Flu/Pyr, Phe/Ant was carried out using a Spearman's correlation analysis. Wilcoxon test was performed to detect differences in reburrowing behaviour of the bivalves coming from different sites. Metals data were all normalized before the statistical analysis.

4.3. RESULTS

4.3.1. ABIOTIC PARAMETERS

As showed in table 1, the punctual measurements for pH values carried out in February, May, August and December remained relatively constant throughout the sampling period and ranged from 5.29 to 8.5, with the lowest values during the December, winter period in station G. Dissolved oxygen levels (% of saturation) in all stations considered, varied between a minimal value of 80.19% in August station P and a maximum of 120.2% in February at station N. Salinity varied both spatially and temporally from 13.26 to 22.42 P.S.U. The greatest variability in salinity was recorded at the station N. Water temperature at the three stations exhibited comparable seasonal fluctuations from 9.72° C to 29.61° C.

Table 1. Physical-chemical characteristics of the three sampling sites during 4 sampling seasons.

Parameters	Sampling time		N Mean ±SD	G Mean ±SD	P Mean ±SD
T	February	Winter	7.74± 0.01	8.32± 0.02	8.39± 0.01
	May	Spring	18.63± 0.58	17.99± 0.08	16.69± 0.77
	August	Summer	28.1± 0.02	29.61± 0.01	28.64± 0.01
	December	Autumn	9.57± 0.02	9.72± 0.11	9.44± 0.02
pH	February	Winter	7.64± 0.1	7.47± 0.17	7.35± 0.21
	May	Spring	8.5± 0.1	8.43± 0.09	7.87± 0.08
	August	Summer	6.77± 0	6.87± 0	6.82± 0
	December	Autumn	3.91± 0.92	3.65± 0.81	0.24± 0.36
Dissolved oxygen (% of saturation)	February	Winter	120.2± 1.1	74.5± 0.55	85± 0.07
	May	Spring	84.7± 0.12	82.1± 0.12	95.5± 0.07
	August	Summer	82.68± 0.89	91.25± 0.32	80.19± 0.99
	December	Autumn	98.4± 2.64	84.74± 0.55	80.36± 0.54
COND µS/cm	February	Winter	33740± 46.12	26930± 178.46	26580± 241.37
	May	Spring	23678± 117.8	21456± 83.4	2761± 95.3
	August	Summer	22198.06± 62.69	22168.28± 3.84	23070.67± 156.56
	December	Autumn	35626.51± 266.74	29734.59± 531.66	23463.53± 1348.76
TDS ppm	February	Winter	16870± 8.5	13470± 45.79	13290± 32.58
	May	Spring	12639± 128.46	14267± 127.43	15937± 7.27
	August	Summer	11098.39± 32.36	11082.07± 4.12	11536± 78.5
	December	Autumn	17813.36± 133.21	14867.41± 265.81	11731.57± 674.08
PSU	February	Winter	21.05± 0	16.48± 0.35	16.24± 0.15
	May	Spring	18.48± 0.03	17.8± 0.05	19.18± 0.07
	August	Summer	13.3± 0.04	13.26± 0	13.86± 0.1
	December	Autumn	22.42± 0.18	18.4± 0.36	14.21± 0.89

4.3.2. SEDIMENT CHARACTERISATION

Mean clay contents (fraction < 2 µm) varied from 23.1 to 16.6% at Po di Volano (P station), 16.8 to 22.1% G and 16.3 to 10.8% in N site for two sampling season (December and May 2009). A total average of N 0.05%, pH of 7.96 and TOC of 1.54±0.38 (G site) 1.94±0.28 (N site) 2.08±0.30 (P site).

According to the classification proposed by Baumard et al. (1998), PAHs contamination is low in all the studied sites in Sacca di Goro lagoon. Pollution level of sediment expressed as average ∑PAH sediment (ppb), according to each site and season, are all <100, so overall our results indicate a low contamination. If we consider single sample sediment we had the highest contamination levels in the sites consider more "continental", site P next to Po di Volano plume and G site nearby the port of Goro in the sampling campaign of December (PAH: 246 and 115 µg/kg dw, respectively, mainly due to Fluoranthene, Pyrene and Phenanthrene concentrations). On the other hand, sediment from the N site in February present the lowest concentrations of PAHs revealed (1.25 µg/kg dw), whereas these chemicals were not detected in the sampling site located in the Sacca di Goro lagoon.

The studied PAHs ranged from the triaromatics (fluorene, phenanthrene, anthracene) to the hexaaromatics (e.g. benzo[ghi]perylene). Total PAH in sediment presented in Table 2 is the sum of the 12 PAHs analysed as individual concentrations (mean of 5 samples each site and season) and expressed on a dry weight. The compound distributions in Sacca di Goro were dominated by fluoranthene, pyrene and phenantrene in average of all sites, respectively of 26-23 and 20% (Figure 2).

Consequently (Figure 3) sediments present dominance in 3-and 4-rings PAHs. The slight predominance of fluoranthene over pyrene ratio values of specific PAH compounds such fluoranthene/fluoranthene+pyrene ratio indicates the potential pyrolitic (fuel combustion) and urban activities source. In the present study a linear relationship were found also in the case of the Flu/Pyr (n = 60, Spearman's correlation r = 0.99), values fluoranthene/fluoranthene+pyrene ratio higher than 0.1 (Yunker et al., 2002).As well a low phenanthrene/anthracene (Phe/Ant) ratio values (4 to 10) according to Yang et al. (1991) indicate that the major PAH input was from combustion of fossil fuels. In our case we had evidence of anthracene presence in few samples, but all of them with a ratio > 4 (Spearman's R correlation r = 0.97, n = 6). Strong differences among seasons were evidenced (Table 2 and Figure 4), the higher levels were found in Po di Volano and Goro port (66.52 and 36.76 µg kg^{-1} dw) in December, with a high

differences between seasons (p = 0.0001). No any differences were revealed according to sites (p = 0.468).

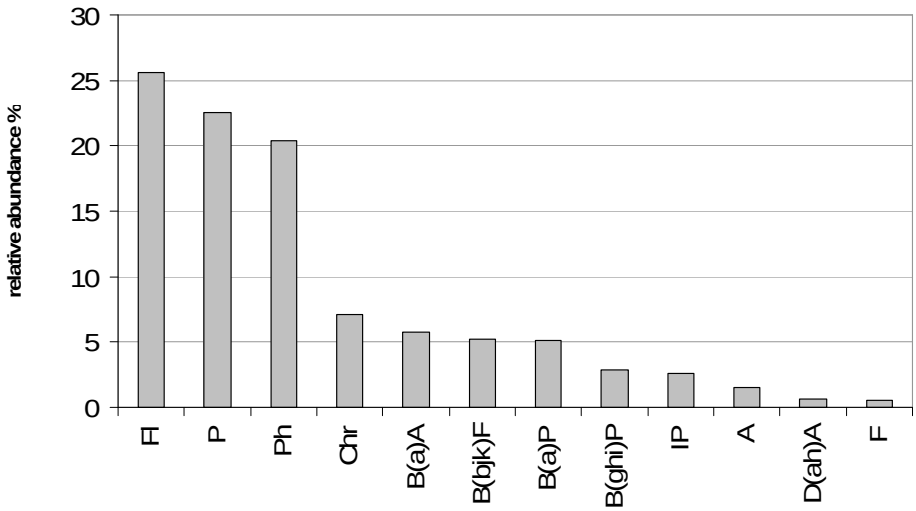


Figure 2. Average relative abundance (%) of PAHs congeners in all the three sites in all year considered (2009). For PAHs acronyms, see text.

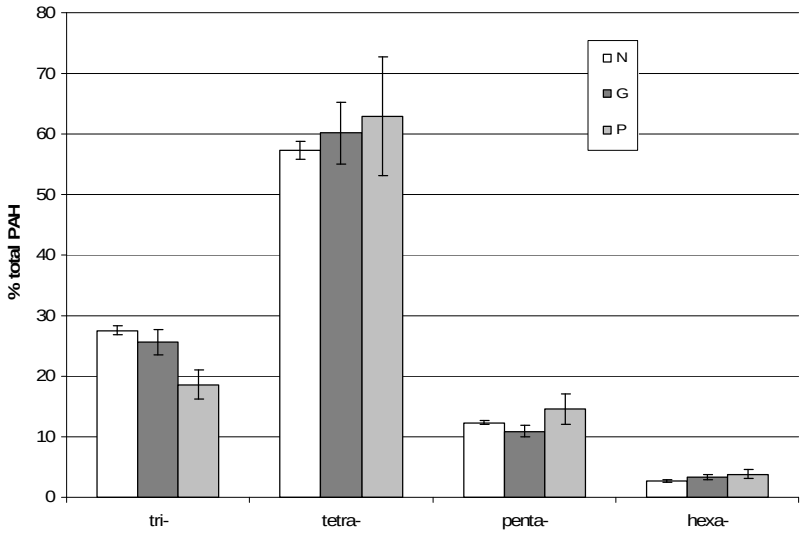


Figure 3. Relative distribution of polycyclic aromatic hydrocarbons (PAHs) per number of rings (tri-, tetra-, penta-, hexa-) in three different sites (N-G-P). Data are expressed as percentage of total PAH and relative ±SD.

Arsenic and heavy metals concentrations were selected and evaluated for characterise the sediment compartment were *T. philippinarum* living in contact with. Concentrations of Cr, Zn, Cd, Pb, V, Ni, Pb, Cu and As, expressed on a dry weight basis, are listed in Table 3 and Table 3A for standard reference materials, summarised variability in sediment samples about the spatial (different sampling sites) and temporal (in different sampling times) distribution in the sediment compartment (Figure 5).

PCA (Figure 6, A and B) was performed in order to have a synthetic overview on how discriminate the intersite variations of the studied parameters and investigate on which of the selected parameters are able to describe better the variance among sites and seasons.

Results shown as PC1 is able to explain 83% of variance and Ni with PC1 coefficient of -0.665, Cr (PC1 = -0.585) and Zn (PC1 = -0.389) are the variables that contribute to data clustering. To summarize we could underline that distribution of data in the principal component analyses evidence how N sites located in the positive part of PC1 are negatively correlated with Ni concentrations and less present in sites among season. Our results are in accordance with the statement that for the Po costal plain is present a geochemical baseline that is not an expression o any anthropogenic influence.

In our results Cr and Ni (Spearman's R correlation 0.88; $p < 0.001$) present high concentration with values that exceed the national standard limits settled for Cr at 50 ppm and for Ni at 30 ppm (Directive 2000/60/EC, 2008/105/EC; in response Italian legislation DL n.152 3/04/2006, n.4 16/01/2008 and DL n.56 14/04/2009). Our results as a general trend, observed a highly differences in significance between sampling campaign (PERMANOVA; $p = 0.0001$), sites shown a significantly difference in the concentrations among seasons. A difference between sites were also evidenced (PERMANOVA; $p = 0.0147$), significantly differences were detected between sites in each sampling campaign (Table 3).

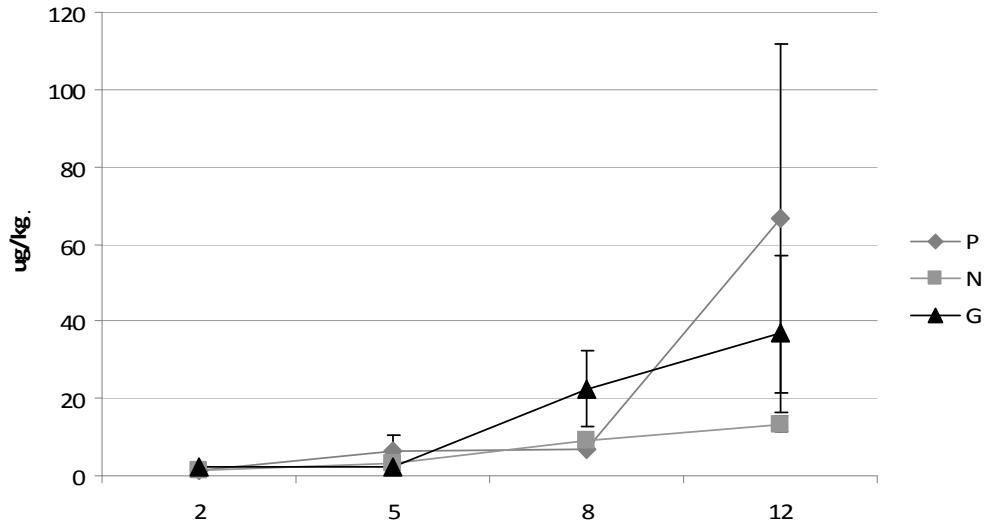
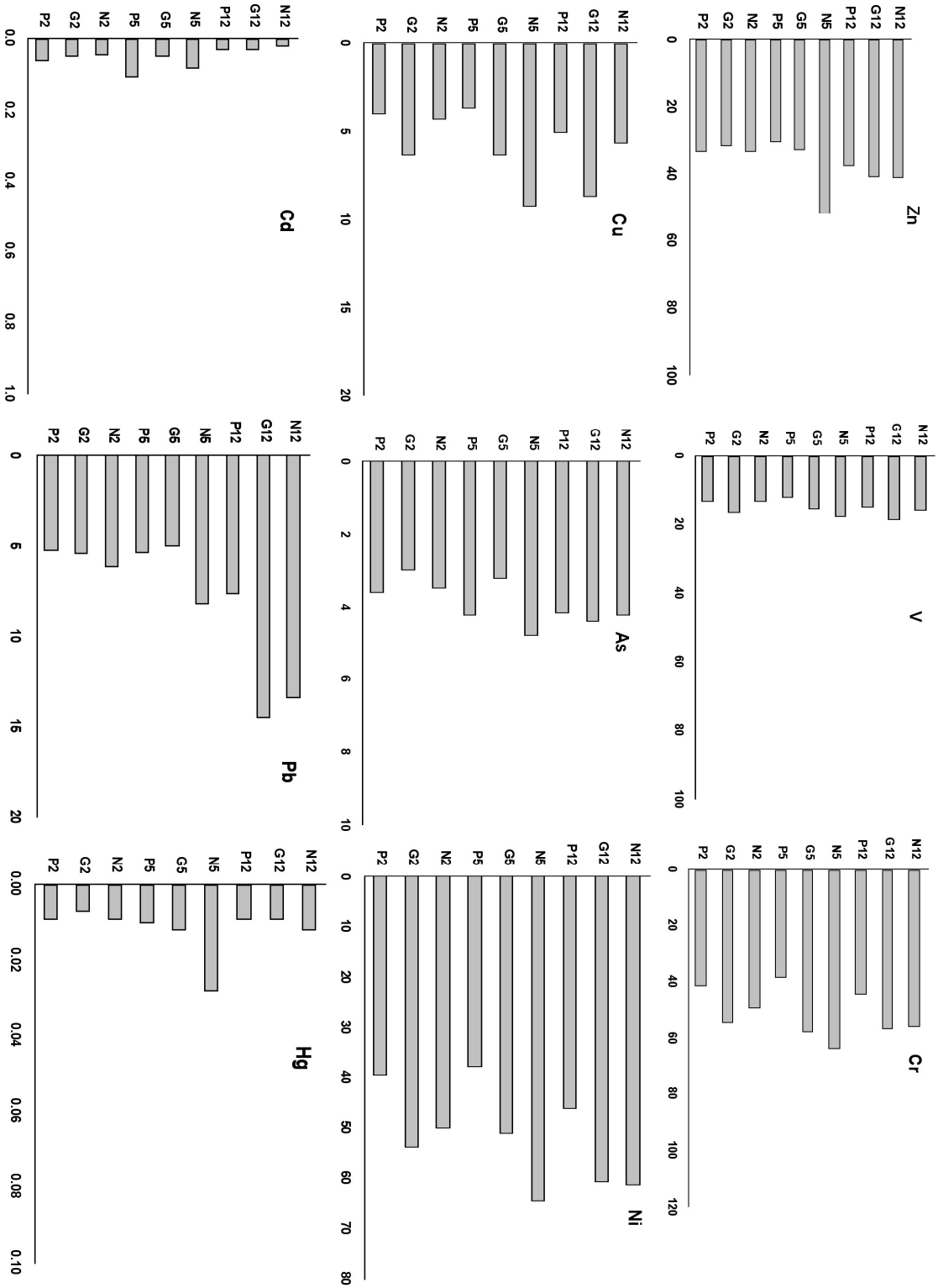


Figure 4. ∑PAHs (ug kg^{-1} dw) ±SD according to sampling time (February: 2, May: 5, August: 8, December: 12) in sediment from study sites in Sacca di Goro, concentrations are expressed as mean values for five replicates for each site.

Figure 5. Chemicals concentration in sediments A) As and metals (mg kg^{-1} dw) sites are reported according to sampling month (i.e. G2: sampling in February on site G, N12 sampling on December in N site)



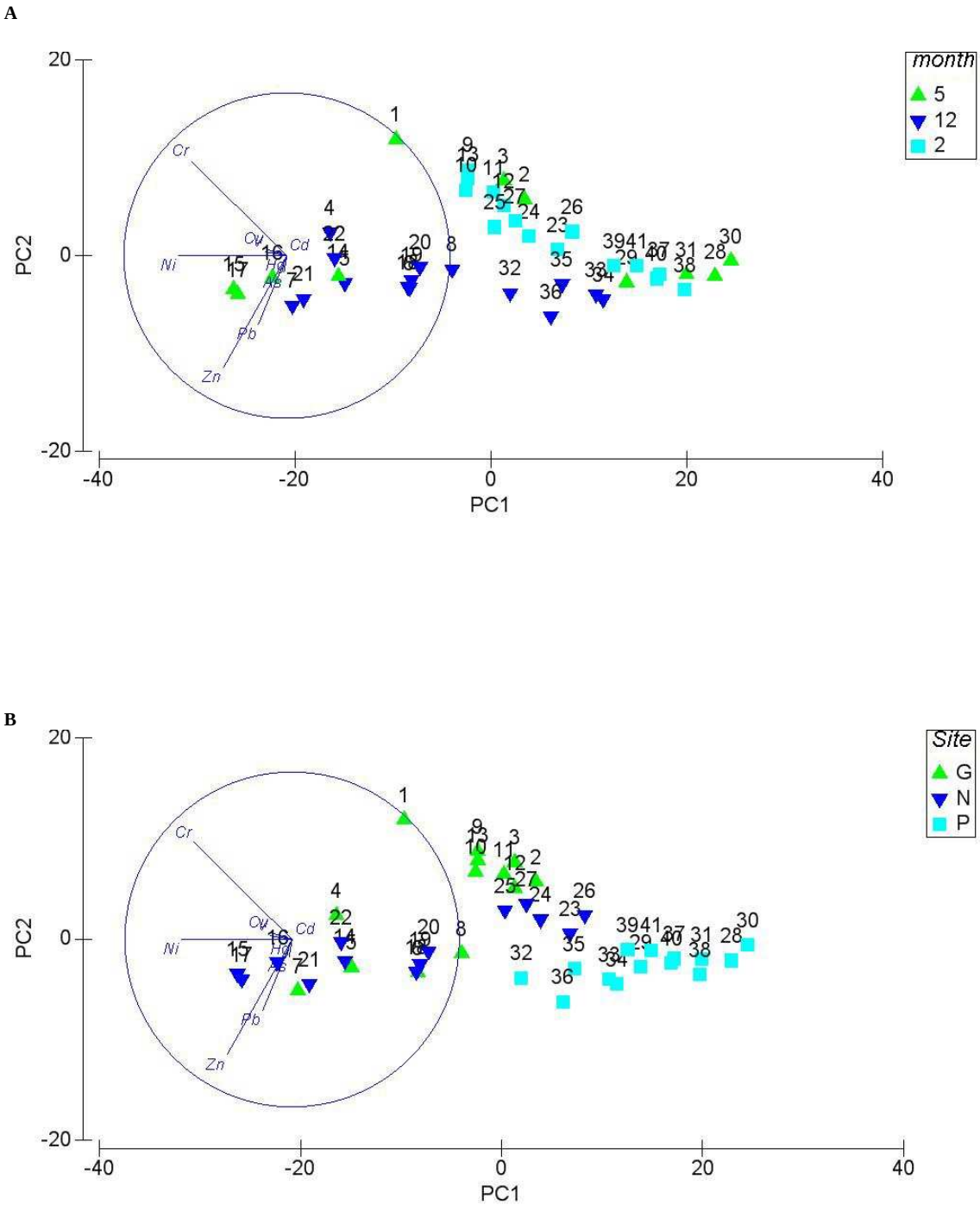


Figure 6. Principal component analysis of sediment metals content measured at the three sampling sites (B) in 3 sampling seasons (A). Vectors of the linear correlations with individual variables are superimposed on the graph.

Table 2. Results of the chemical analysis of PAHs (ug kg^{-1} dw) in surface sediment upper layer (0-10 cm) in Sacca di Goro lagoon. Four sampling campaign from February to December 2009 in three selected sites.

	February			**May**			**August**			**December**		
	N	**G**	**G**	**N**	**G**	**P**	**N**	**G**	**P**	**N**	**G**	**P**
Fluorene	ND	ND	ND	N	ND	ND	ND	0.25	ND	0.08	0.36	0.24
Phenanthrene	0.50	0.57	0.54	0.82	0.63	1.45	2.60	5.19	2.38	3.38	7.81	9.35
Antracene	ND	ND	ND	ND	ND	ND	ND	0.69	ND	0.06	0.66	1.22
Fluoranthene	0.27	0.51	0.48	0.82	0.44	1.87	1.88	5.68	1.34	3.36	9.46	17.91
Pyrene	0.59	0.62	0.55	0.70	0.44	1.68	1.76	4.64	1.25	2.52	8.41	15.49
Benzo (a)anthracene	ND	ND	ND	0.31	0.10	0.37	0.65	1.51	0.54	0.53	1.75	4.10
Chrysene	ND	ND	ND	0.32	ND	0.38	0.87	1.76	0.71	0.95	2.57	4.62
Benzo(b,j,k)fluoranthene	ND	ND	ND	0.26	ND	0.27	0.58	0.89	0.54	0.61	1.13	4.63
Benzo(a)pyrene	ND	0.41	ND	0.07	ND	0.21	0.66	1.14	0.13	0.46	1.78	3.84
Dibenzo (a,h) anthracene	ND	ND	ND	ND	ND	0.12	ND	0.33	ND	0.71	1.17	2.15
Indeno (*1,2,3NDcd*)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.27	0.80
Benzo(ghi)perylene	0.09	ND	ND	ND	ND	0.14	ND	0.44	ND	0.63	1.38	2.16
∑ PAHs (ug kg^{-1} dw)	**1.45 a,b**	**2.10 a**	**1.56 a**	**3.30 b**	**1.60 b, d**	**6.50 a**	**8.99 c**	**22.54 c**	**6.89 b**	**13.30 d, c**	**36.76 d,c**	**66.52 a**

N: Nursery, G: Goro port, P: Po di volano, ND: not detected. Four sampling campaign from February to December 2009, different letters indicate significant differences between seasonal samplings (p = 0.0001) in total PAH within same sites (i.e. N5 vs N12). No any differences between sites (p = 0.469) among each season.

Table 3. The concentrations are the mean value (±SD) of five independent measurements (for each three sites), minimum and maximum values for three sampling periods (February, May and December). The EU set concentration protection limits for sediment matrix (Directive 2000/60/EC, 2008/105/EC; in response Italian legislation DL n.152 3/04/2006, n.4 16/01/2008 and DL n.56 14/04/2009), therefore values underlined are elements which shown exceeding reference quality standard values, for transitional waters. Reference quality standards for Cr and Ni, total Cr: 50 mg kg^{-1} dw and Ni 30 mg kg^{-1} dw. Upper case letters detect differences between sites within same samples campaign ($p < 0.05$); differences between sampling campaign are evidenced smaller case letters.

Sampling	site		Cr	Cu	Zn	V	Cd	Ni	Pb	As	Hg	
Feb 09	G	MEDIA	54.27±2.44	6.29±0.39	31.47±0.58	16.1±0.44	0.05±0	53.77±0.79	5.39±0.32	2.97±0.07	0.007±0.001	A, a
		max	57.03	6.92	32.40	16.72	0.05	54.92	5.91	3.04	0.01	
		min	51.08	5.93	30.82	15.67	0.05	52.87	5.09	2.87	0.01	
Feb 09	N	MEDIA	48.94±3.01	4.3±0.35	33.11±1.33	13.1±0.87	0.04±0.01	49.98±1.84	6.1±0.17	3.46±0.19	0.009±0.001	B, a
		max	52.07	4.71	34.67	14.47	0.05	52.32	6.28	3.74	0.01	
		min	45.37	3.88	31.10	12.33	0.04	47.44	5.91	3.25	0.01	
Feb 09	P	MEDIA	40.93±2.74	3.97±0.24	33.1±0.84	13.1±0.39	0.06±0.01	39.43±1.55	5.2±0.23	3.59±0.16	0.009±0.0005	C,a
		max	43.73	4.24	34.10	13.50	0.08	42.05	5.54	3.86	0.01	
		min	37.08	3.71	32.02	12.55	0.05	38.03	4.96	3.45	0.01	
May 09	G	MEDIA	57.45±5	6.29±2.02	32.53±0.6	15.32±0.93	0.05±0.01	51.09±3.46	4.99±0.47	3.19±0.15	0.012±0.002	A, b
		max	64.57	9.28	33.19	16.67	0.05	55.86	5.39	3.36	0.014	
		min	52.91	4.91	32.00	14.59	0.03	48.12	4.43	3.00	0.01	
May 09	N	MEDIA	63.35±2.53	9.2±1.02	51.7±2.81	17.32±0.6	0.08±0.01	64.47±3.33	8.18±0.6	4.77±0.18	0.028±0.004	B, b
		max	65.34	10.40	53.99	17.81	0.09	67.50	8.78	4.89	0.03	
		min	59.65	8.06	47.77	16.50	0.08	60.18	7.41	4.51	0.02	
May 09	P	MEDIA	38.11±2.08	3.68±0.42	30.33±2.49	11.88±0.83	0.11±0.04	37.7±3.5	5.34±0.65	4.21±0.46	0.01±0.001	C,b
		max	40.84	4.23	33.62	12.95	0.17	42.61	5.93	4.61	0.01	
		min	36.19	3.23	27.71	11.03	0.08	34.42	4.72	3.68	0.01	
Dec 09	G	MEDIA	56.43±4.73	8.66±2.24	40.78±3.47	18.221.21	0.03±0.01	60.59±4.44	14.48±4.15	4.38±0.35	0.009±0.003	A, c
		max	63.13	12.50	43.84	19.38	0.04	67.25	20.08	4.77	0.01	
		min	51.22	6.79	35.80	16.31	0.01	55.73	8.59	4.01	0.01	
Dec 09	N	MEDIA	55.54±4.97	5.61±0.4	40.83±2.59	15.530.59	0.02±0.01	61.26±2.78	13.41±4.52	4.2±0.17	0.012±0.002	B, c
		max	61.33	5.92	44.03	16.19	0.03	64.68	19.38	4.42	0.01	
		min	49.61	5.00	38.26	14.88	0.01	58.00	8.50	3.97	0.01	

Sampling	site		Cr	Cu	Zn	V	Cd	Ni	Pb	As	Hg	
Dec 09	P	MEDIA	43.9±2.71	5.04±0.38	37.49±2.09	14.590.49	0.03±0.01	46.27±2.12	7.61±0.83	4.15±0.44	0.009±0.002	C,c
		max	47.94	5.60	39.97	15.33	0.04	49.32	8.87	4.41	0.01	
		min	41.16	4.57	35.63	14.08	0.02	43.82	6.82	3.37	0.01	

Table 3A In order to confirm and verify the applicability of the analytical procedure, concentrations of standard reference materials (Estuarine Sediment BCR-CRM 277, BCR 320 and BCR 142 R), are the mean value of three independent measurements for three sampling periods (February, May and December).

	BCR 320			BCR 277			BCR 142 R
	I st Sampling campaign	II nd Sampling	III nd Sampling	I st Sampling campaign	II nd Sampling	III nd Sampling	I st Sampling campaign
	Feb 09	May 09	Dec 09	Feb 09	May 09	Dec 09	Feb 09
Cr	47.98±1.85	49.2±1.78	52.67±1.2	131.71±3.31	118.94±0.32	126.24±0.26	
Cu	45.12±2	44.72±1.92	46.81±0.02	106.94±1.28	96.36±0.61	111.01±0.12	
Zn	110.17±4.75	113.21±0.68	125.72±0.95	516.87±13.48	480.25±5.23	559.11±12.03	
V	36.94±1.14	36.41±0.4	43.150.86	49.5±1	47.37±0.19	49.473.49	
Cd	0.47±0.03	0.49±0.02	0.42±0.01	12.19±0.07	11.78±0.15	9.53±0.4	
Ni	51.11±2.53	50.67±1	54.13±0.21	40.56±4.74	31.9±0.18	36.74±0.7	
Pb	25.72±0.86	25.92±0.16	30.35±0.38	147.41±12.17	122.33±0.27	145.73±2.96	
As	71.04±3.39	87.44±2	81.21±1.05	43.59±0.44	50.74±0.85	41.9±0.96	
Hg							0.07±0.003

4.3.3. MICRONUCLEUS TEST

Gills were selected as target organ for their function in gas exchange and for their filtering behaviour of the water containing food particles and potentially toxic chemicals. Additionally cells proliferation in gill tissue had shown a higher sensitivity to detect effect of genotoxic compound exposition.

In the examined cells, we found two main types of abnormalities: MN (Figure 7 B) and granules accumulated in lysosomes. Sporadic cytoplasmic granules observed, were not considered in our analyses for their very low frequency. In Figure 8 and Table 4 are reported results for mean MN frequency observed in gills cells isolated from clams sampled in the three sites in two different sampling seasons. Micronuclei averages ranged from 16% (site G in winter) to 4% (site N).

Significant differences among sampling sites for the winter sampling (Mann-Withney-U-test) N vs G ($p < 0.001$) and G vs P locations ($p < 0.05$). In spring significantly differences among sites G site vs N and N vs P ($p < 0.05$). Significant differences were observed between G sample between spring and winter campaign ($p = 0.004$). Clams in G site expressed more MN than other clams collected in the other sites with respect to MN average, infact we reported the highest level (16%) during winter sampling in G site. In May samples collected in site N present a significant difference with other sites ($p < 0.05$), with the lower frequency observed (4%). N site represent farest site in the lagoon from watershed of Po di volano and Goro port. Revealing a significant decrease in MN frequency (site N) in relation to the distance from the more "continental" sites G and P, with winter level that approximately double in site G. Since G site was the highest level of MN frequency we calculated the ratio between G and the other two locations (G vs P 1.04 G vs N 1.36 in May; G vs P 1.34 and G vs N 1.83).

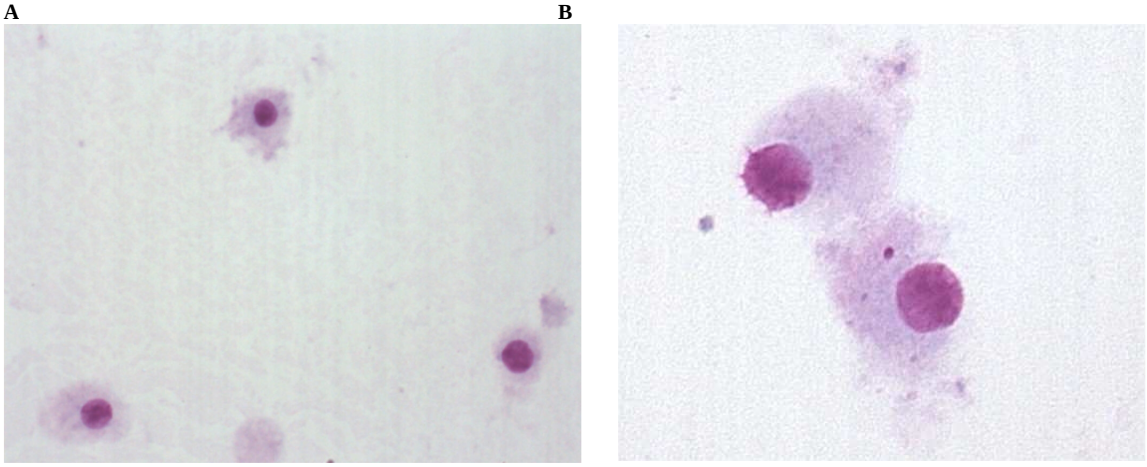


Figure 7 Micronucleus assay image (1000X) of gill cells from *T.philippinarum*, A) normal aspect and B) micronucleated agranular gill cells.

A **B**

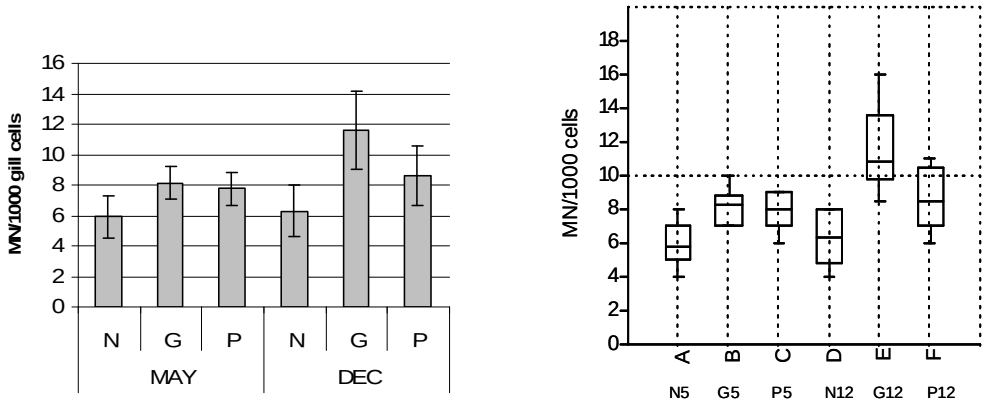


Figure 8. A) Frequency of micronucleated cells (x 1000 cells) in gill cells of *T. philippinarum* (n = 8) from three sites (G,N,P) and two sampling season (May and December). Results are given as mean and ±SD, significant differences are reported. B) Box plot for micronuclei frequency (MN/1000 cells) in gills cells of clams collected in May (N5,G5, P5) and December (N12, G12, P12). Box plots show the median, 25–75% percentiles and range.

Table 4. Mean and standard deviation ±SD are presented for micronuclei frequency (MN/1000 cells). Values marked with an asterisk differ significantly ($p < 0.05$), double asterisk mean a significant difference of $p < 0.001$, within site among different sampling time. Different letters represent significant differences between sites within factor month (Mann-Witney-U-test).

		MN/1000 cells			
Site	**Date**	**n**	**Mean**	**SD**	
N	May	8	5.94	±1.37	a
G	May	8	8.13	±1.09	b*
P	May	8	7.79	±1.07	b
N	December	8	6.28	±1.70	A
G	December	8	11.56	±2.54	B*
P	December	8	8.63	±1.94	A

4.3.4. BIOCHEMICAL ANALYSIS

Mean values of oxidative stress biomarkers SOD and CAT were determined in clams obtained in two sampling campaign and are summarized graphically in Figure 9 and for statistical output on Table 5. The digestive gland was selected since is the major site of uptake of organic xenobiotics (Livingstone et al., 1990) and could reflect chronic pollutants effects. Results obtained showed that enzyme activities and protein levels varied during the experimental campaign. Selected biomarkers of oxidative stress examined in *T. philippinarum* showed significant differences as a function of sampling period, with p value for CAT < 0.001 and for

SOD $p < 0.05$ according to the two seasonal campaigns. CAT activities were measured in the mitochondrial and cytosolic fraction and a total CAT activity (sum of the 2 fractions) was considered (Table 5). An evidence of a higher activity of CAT in the cytosolic fraction was statistically confirmed for samples collected in May ($p < 0.05$). CAT was predominantly present in the cytosolic fraction > 50% in all site with maximum value of 64% in May, while in winter slightly higher average level was found in mitochondrial fraction. CAT activity is higher in samples collected in December than in those collected in spring site N present a significative higher ($p < 0.001$) activity within different season indicating a season-dependent relation, while for seasonal variation P site present an slightly higher activity in December ($p < 0.05$).

SOD shown similar activity in the three different sites, significantly higher activity was found in clams collected in May compare to those collected in December ($p < 0.05$).

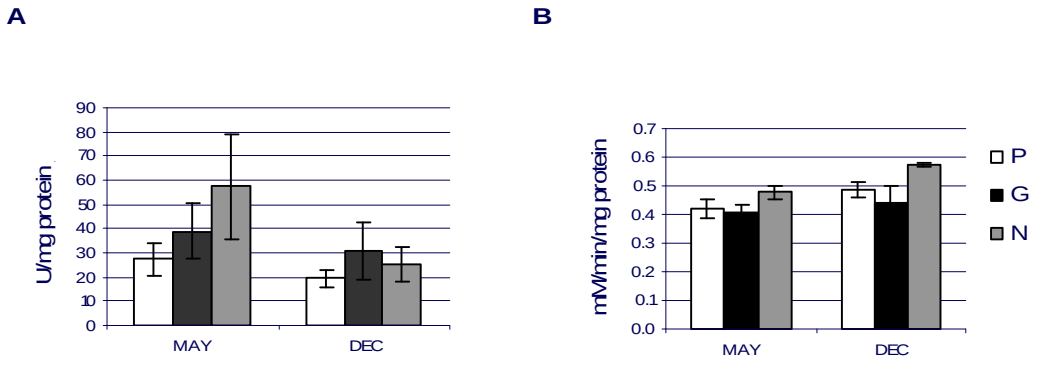


Figure 9. Activity of the antioxidant enzyme A) superoxide dismutase (SOD) expressed in (U/mg/prot) and in B) catalase (CAT) in digestive gland of clams (mM/min/mg prot) collected in different site of the Sacca di Goro Lagoon in May and December, results are shown as means ±SD. Catalase activity was detected in the mitochondrial and cytosolic fraction; total CAT activity was calculated as the sum of the activity of two fractions.

Table 5 Activities of antioxidant enzymes in the digestive gland of clams collected in two sampling seasons and 3 stations. Data are expressed as mean and ±SD, values marked with an asterisk differ significantly ($p < 0.05$) within site among different sampling time, while double asterisk mean a significant difference of $p < 0.001$. Different letters represent significant differences sites among sampling times. Significance was set at $p < 0.05$.

	MAY			DEC		
	P	G	N	P	G	N
SOD	57.44	38.97	27.43	25.28	30.86	19.44
(U/mg prot)	±21.78	±11.48	±6.69	±7.22	±11.56	±3.52
Cyto CAT	0.29	0.26	0.29	0.26	0.19	0.26
mol / min / mg prot)	±0.03 A	±0.02 A*	±0.02 A	±0.02 a	±0.02 b*	±0.02 ca
Mito CAT	0.13	0.15	0.19	0.22	0.25	0.32
(mol / min/ mg prot)	±0.01 A**	±0.04 A*	±0.01 B**	±0.04 a**	±0.04 a*	±0.03 b**
Tot CAT	0.42	0.41	0.48	0.49	0.44	0.57
(mol / min / mg prot)	±0.02 A*	±0.03 A	±0.03 B**	±0.06 a*	±0.03 a	±0.01 b**

4.3.5. BEHAVIOURAL RESPONSES

With regard to reburrowing behaviour, P collected clams had significantly slower reburrowing times compared to all other groups in May (P vs G and P vs N with a $p < 0.0001$; Figure 10). Significant differences were observed between experimental groups, also in December (Figure 11) with a highest reburrowing kinetics in clams collected in G site ($p < 0.0001$ G vs N and G vs P; while P vs N a p value of 0.002). All the values were ln transformed.

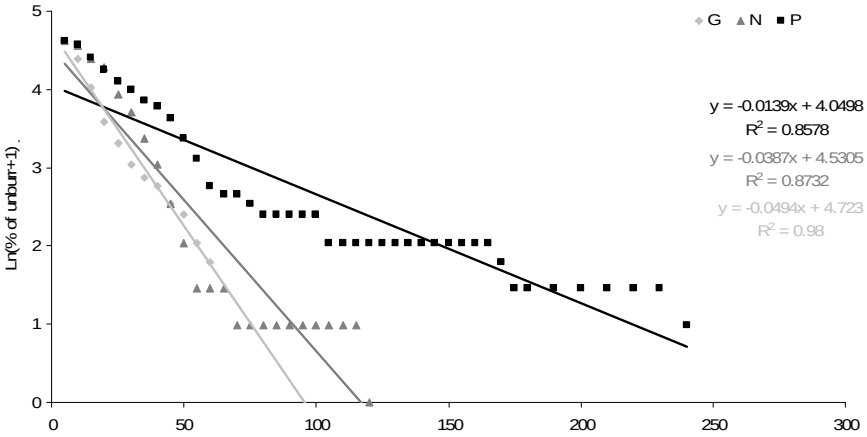


Figure 10. Reburrowing kinetics in May X axis represent time in minutes and Y axis ln(%un-burrowed bivalves +1). Significantly differences ($p < 0.0001$) in the reduction of burrowing behaviour exclusively in: P site (Po plume) vs G (Goro harbour) and vs N (Nursery) p value < 0.0001. No significant differences between site N vs G.

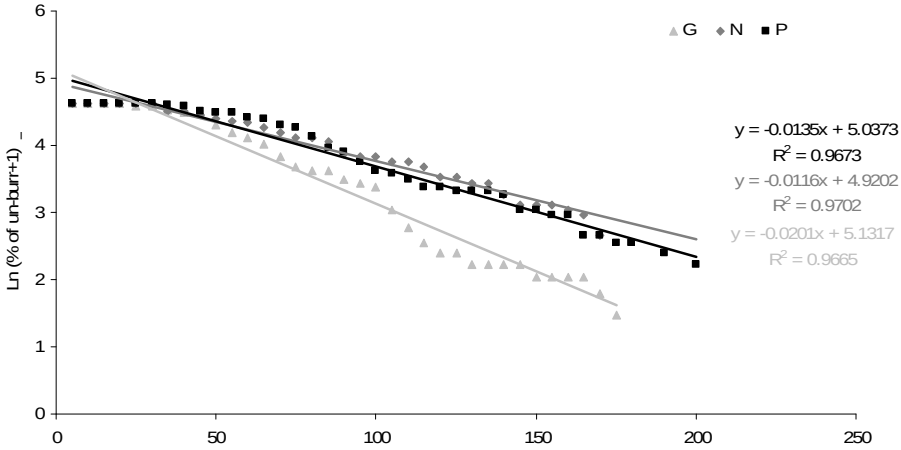
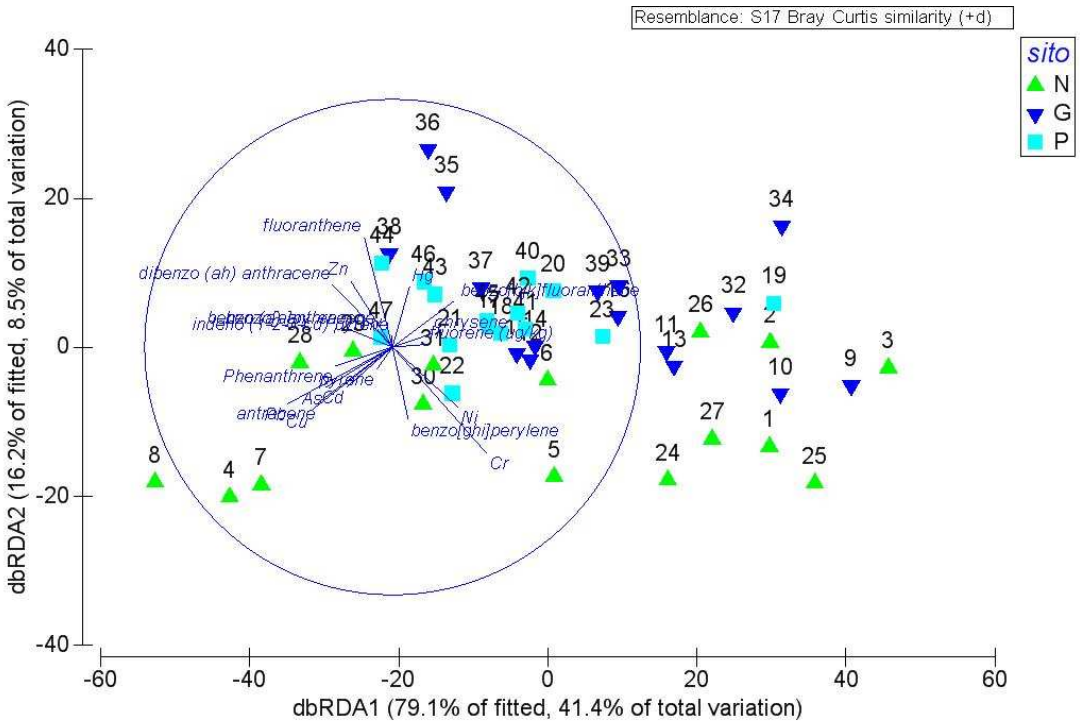


Figure 11. Reburrowing kinetics in December. X axis represent time in minutes and Y axis ln (%un-burrowed bivalves +1). Significantly differences ($p < 0.0001$) in the reduction of burrowing behaviour in December in G site vs N ($p < 0.001$) and G vs P site ($p < 0.001$), and a slight lower difference between P vs N ($p < 0.002$).

4.3.6. LINKING CHEMICALS TO BIOMARKERS

DistLM was utilized as useful method to study the complex relationships between chemical values and biomarker results. DistLM has a similarity matrix, based on biomarker response CAT, SOD, and MN frequency detected in clams using Bray-Curtis index, with a matrix of prediction variables like our selected class of chemicals (PAHs, As and metals). Table 6 shows the results of the regression multivariate analysis performed by the DISTLM forward test. The test suggested a weak or null relationship relationship between most of the observed sediment characteristics and biomarker response. There was a no evidence of relationship between biomarker and contaminant characteristics when analyzed through season (Figure 12). None of the selected chemical variables are able to explain the biological responses in biomarkers.

A



B

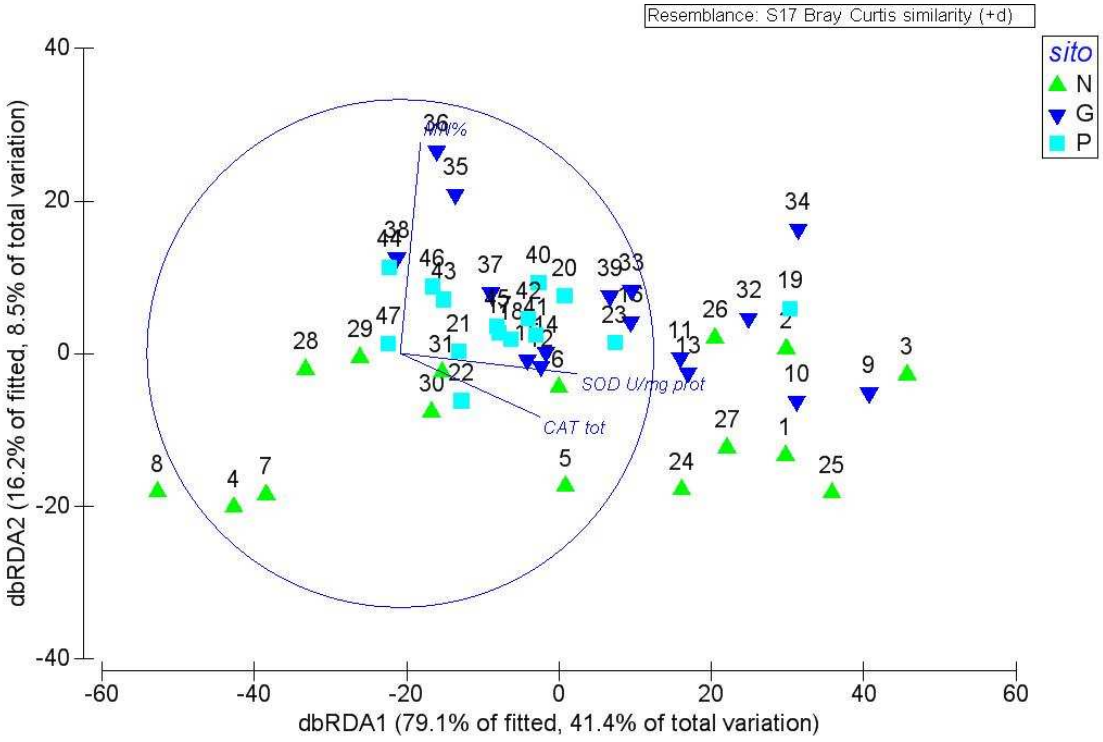


Figure 12 dbRDA models the linear relationship between selected chemical versus biomarker response in *T.philippinarum.* Within the centre vectors of predictor variables which are indicated intensity and direction. Resolution A) according to sampling seasons and B) sites. MN% = frequency of micronucleated cells; CAT = catalase; SOD = superoxide-dismutase.

Table 6 DISTLM of the chemical parameters in relation to biomarker responses in *T. philippinarum.* For each variables are indicated in columns R^2, coefficient of regression, sum of square SS, the value of pseudo F and its significativity P, percentage of variance explained by each single variable Prop, and the cumulative percentage. As variables As, metals and PAHs were selected.

Variable	R^2	SS(trace)	Pseudo-F	P	Prop.	Cumul.
Phenanthrene	4.84E-02	2775	2.2886	0.1104	4.84E-02	4.84E-02
Cr	8.98E-02	2371.3	1.9992	0.15	4.14E-02	8.98E-02
As	0.16002	4028.8	3.597	0.0485	7.03E-02	0.16002
Cu	0.23042	4036.9	3.8424	0.041	7.04E-02	0.23042
Pb	0.27807	2732.1	2.7061	0.0875	4.76E-02	0.27807
Antracene	0.31662	2210	2.2561	0.1209	3.85E-02	0.31662
Zn	0.35518	2210.9	2.3321	0.1125	3.86E-02	0.35518
Cd	0.37138	928.81	0.97922	0.366	1.62E-02	0.37138
Hg	0.38896	1008.4	1.0649	0.3054	1.76E-02	0.38896
V	0.40684	1025	1.0849	0.3127	1.79E-02	0.40684
Benzo[ghi]perylene	0.41652	555.18	0.58081	0.4946	9.68E-03	0.41652
Indeno (1-2-3-cd) pyrene	0.4288	704.17	0.73102	0.4256	1.23E-02	0.4288
Benzo (a) anthracene	0.45434	1464	1.5442	0.2112	2.55E-02	0.45434
Dibenzo (ah) anthracene	0.46586	660.64	0.69027	0.4503	1.15E-02	0.46586
Fluoranthene	0.47528	540.31	0.55672	0.518	9.42E-03	0.47528

Fluorene (ug/kg)	0.487	672.05	0.68544	0.4434	1.17E-02	0.487
Pyrene	0.49727	588.89	0.59246	0.4909	1.03E-02	0.49727
Chrysene	0.50185	262.31	0.25714	0.7153	4.57E-03	0.50185
Benzo[bjk]fluoranthene	0.50818	362.85	0.34741	0.6383	6.33E-03	0.50818
Benzo[a]pyrene	0.5194	643.49	0.60715	0.4736	1.12E-02	0.5194
Ni	0.52344	231.52	0.21182	0.7491	4.04E-03	0.52344

Relationships between dbRDA coordinate axes and orthonormal X variables (multiple partial correlations) that participate in the dbRDA (ordination method of redundancy analysis) shown in Figure 12.

Variable	dbRDA1	dbRDA2
Phenanthrene	-0.23	-0.076
Cr	0.377	-0.427
As	-0.27	-0.164
Cu	-0.33	-0.258
Pb	-0.422	-0.228
antracene	-0.302	-0.227
Zn	-0.167	0.265
Cd	-0.183	-0.16
Hg	0.068	0.243
V	-0.034	0.04
benzo[ghi]perylene	0.064	-0.293

Variable	dbRDA1	dbRDA2
indeno (1-2-3-cd) pyrene	-0.003	0.045
benzo (a) anthracene	-0.059	0.07
dibenzo (ah) anthracene	-0.241	0.251
fluoranthene	-0.113	0.441
fluorene (ug/kg)	0.139	0.011
pyrene	-0.062	-0.087
chrysene	0.157	0.054
benzo[bjk]fluoranthene	0.244	0.182
benzo[a]pyrene	-0.175	0.071
Ni	0.265	-0.244

4.4. DISCUSSION AND CONCLUSIONS

An integration of chemical data and biological responses is strongly recommended in monitoring programmes of ecological status (Allan et al., 2006). These results propose how a combination of biological responses in clams can be combined with analytical chemistry to investigate the status of the marine-costal areas. To allow this the sediments monitoring was performed in order to detect several priority chemicals (e.g. metals and polycyclic aromatic hydrocarbons (PAHs)) since this matrix represents a compartment of primary importance for infaunal organisms and additionally characterisation of sediment quality is essential to understand the *status* of the aquatic environments. Moreover sediments were investigated for polycyclic aromatic hydrocarbons, since PAHs are hydrophobic compounds that rapidly absorb on suspended material in the water column, and sediment is considered as pollution reservoir. Our results shown how sediments from both sites were low polluted compared to other sites such as the western Mediterranean Sea (Baumard et al., 1998), with values around 20 440 (μg kg^{-1} dw), in a heavily polluted area (Ajaccio harbour). In Sacca di Goro the ∑PAH concentrations range from 1.56 to 66 (μg kg^{-1} dw), which are typically low contents for remote locations far from point sources. Ratio values of specific PAH compound such as phenanthrene/anthracene (> 4) and fluoranthene/fluoranthene+pyrene (> 0.1), were calculated to evaluate possible source of PAHs concentrations, suggesting that sediments might have a pattern of pyrolytic inputs of PAHs. Temporal variations of the sediment PAH contents could be related to concentration of dissolved organic carbon that is known to be important in complexing hydrophobic toxic pollutants (Evans et al., 1990). In this process salinity is also known to play an important role for the influence in DOM removal from water to sediment compartment. Therefore salinity is a key factor in transitional water chemistry, mainly for the partitioning of contaminants between sediments and overlying waters and bioavailability (Chapman and Wang, 2001). Phenanthrene, fluorene and pyrene are the predominant PAH in sediment form Sacca di Goro, these compounds are considered priority contaminants from EPA on the basis of their toxicity and frequency that could alter the cell membrane and lead to the formation of ROS with high relationship with biological effects in exposed organisms (Di Giulio et al., 1989). Total PAHs concentration and seasonal variation are in good agreement with the study of Liang et al. (2008), where higher PAH concentrations were found in wet season, and lower concentration in dry season. Even if Dongjiang River, is subjected to subtropical condition we could expect a same trend in total PAHs concentrations. However, at

so low concentrations exposure assessment and establishing relationships with biological effects may be difficult to interpret.

Arsenic and metal concentrations measured in sediments are low and are in substantial disagreement with study conducted in Sacca di Goro of Locatelli and Torso (2001) a monitoring on the heavy metals with voltammetric analytical procedure, since they found an anthropogenic contamination especially of copper, lead, cadmium and zinc. Therefore in our study no special pattern was detected regarding to the concentration of metals due to an anthropogenic impact in different study sites. The relatively elevated concentrations of Ni and Cr detected in our samples are related to the fact that Po River drainage system, reflect a significant contribution of ultra basic rocks, i.e. ophiolites (Amorosi, 2002; Amorosi and Sammartino, 2002) from geological formations present upstream of deltaic zone of Po river. This rocks composition influence the deposition of sediment drained to the Po delta with important consequences as to background levels of some elements such as Cr and Ni. Chemical characterisation of sediments in terms of traces elements and PAH did not reveal evidence of exceeding standard levels for sediment of transitional and marine-costal waters (Decr. Min. 367/03), with the exception of Cr and Ni related to natural composition of sediment deposit of Po plain.

Results on sediment analysis had shown a low contamination with PAH and trace metals. Even if concentrations in the environment are found of low magnitude, is well known that many of bivalves species, have the capacity to accumulate toxicants as well as transform these agents to active metabolites (Sumpter, 1995), and a great concern is focus on this specie since is directly linked with human food consumption and safety issues. Although this study highlighted that the overall results on sediment compartment, could describe the environment in which the clams were raised.

The biomarkers selected in this study are the techniques proposed to monitor effects of pollutant in aquatic ecosystems, and shown showed limited evidence of contaminant effects, which is consistent with the detected low contamination.

The increased activity of antioxidant enzyme is known to serve as a protective response to eliminate reactive free radicals whose production is usually stimulated by exposure to oxidative stress. Therefore abiotic factors as oxygen availability, temperature (Pellerin-Massicotte, 1997; Abele et al., 1998) and sulphide conditions could enhance ROS production (Abele-Oeschger and Oeschger, 1995) and even if the selected specie of the present study is known for a high adaptation to restricted oxygen availability, enzymes activities could be related to seasonal factors. In Sacca di Goro anoxic conditions and sulphide diffusion, related to an abundance high nitrate input and related macro algal blooms are dystrophic events that occurs in this eutrophic

lagoon (Naldi and Viaroli, 2002; Bartoli et al., 2001). Events due to seasonal growth of *Ulva rigida,* since an early spring its biomass decomposition interfere with oxygen availability and sulphide production, this induces the oxygen radical formation as potential cause of enzymatic impairments with effects on benthic fauna. Therefore frequent algal blooms and associated periods of hypoxia due to high levels of nutrients in runoff may determine impairments in their aerobic capacity in the warmer months. Coupled with aerobic capacity mitochondrial activity should be related with antioxidant defense system as evidenced by Abele et al. (2002) where production of ROS in *M. arenaria*, has been described as temperature-dependent response. In addition to this in May, clams maybe at different stage in gonad development and since is well known that between the digestive system and gonad development there is a tight connection this could seriously complicate interpretation of biomonitoring data, and a comparison between animals from different sites.

In the present research, within connected responses in antioxidant enzymes, were found a slightly higher activity of catalase in winter, and evidences that shown differences linked with seasonal factors. In literature enzyme responses in CAT activity shown different results with a general trend in antioxidant enzyme activities, especially catalase, that are generally enhanced in summer (Orbea et al., 2002), Porte et al. (1991) shown a CAT activity elevated in mussels contaminated by PAHs, indicated as well in Tlili (2010) that evidenced an increases in antioxidant activities by exposure to organic xenobiotics (Livingstone et al., 1990).

We evidenced that enzymes, involved in detoxification system as early warning signals are subjected to a biological variation, supported by statistical significance according to season in catalase (PERMANOVA $p = 0.0002$); no significantly relevant differences among sites. A significantly difference ($p < 0.05$) according to season sampling for SOD activity was detected, even if a due to high variability of data statistical difference were not detected within sites among seasons.

Our study showed that the readout of genotoxic end-points are much simpler to interpret than enzymatic responses. Results in the response in enzymes activity not evidenced relevant biological impairments, among the stations and the sampling times, showing that observed genotoxic damage suggesting no or limited involvement of ROS indirect effects of genotoxic damage (results compared with frequencies detected in other studies on other bivalves *Mytilus galloprovincialis*, Bolognesi et al., 1996; Bolognesi et al., 2004; Magni et al., 2006). Results obtained reveal moderate genotoxic damage in clams from G and P sites compare to reference site N. Additionally an increase of genotoxic damage is clear for the winter sampling, mainly in samples from G station, suggesting a difference in the input of contaminants along the year.

Oxidative damage evaluated in the enzymatic response in association with MN frequency often reported as indirect acting agents in the genotoxicity mechanism (Valavanidis et al., 2006). We proposed that the observed effects with an increasing trend in MN frequency in winter are the result of a complex response suggesting a possible seasonal change in chemical mixture characteristics. Additionally since we were not able to detect a clear response in antioxidant defense system we hypothesize that MN impairments are due to a direct-acting genotoxic damage (Mitchelmore and Chipman, 1998), not requiring metabolic activation as reactive oxygen species-mediated. Although this explanation could be associated with seasonal variations and environmental variables our results shown a significant increase in winter samples of gentoxic damage and is feasible to hypotize an involvement of different contaminants from the selected once or a synergic activity of contaminant mixture. Variation in concentration of PAHs and their increase detected in winter should not be addressed as the once mechanism able to drive detected effects. Further studies are needed in order to understand the pollutants involved in the observed differences in genotoxic damage. One of the limitations of in MN frequencies carried out using gills in *T. philippinarum* is mainly the lack of knowledge about baseline genotoxic effects in the selected species. Another limitation may be the lab sensitivity induced against a range of reference concentrations of genotoxic agents. Therefore further studies are needed to define a baseline response in selected specie and explore the bioconcentration of pollutants in the clam tissues.

However since possible other pollutants are responsible of detected genotoxic effects how select the target contaminants? Additionally single species is exposed to a highly variable exposure of chemical mixtures. The issue is beset with other topics like temporal variation in proportions of chemical concentrations in the mixture, persistence, abiotic features of the ecosystem and their interactions especially in estuarine ecosystems.

Such complex mixtures include numbers of industrial well known products as well as, under environmental conditions, numerous unidentified transformation products (Brack et al., 2003; Chiron et al., 2003). Numbers of studies evidence that pollutant concentrations has limits (i.e. detection and identification) and additionally the once selected could be non-target as indicator of toxicity, and might be present at concentrations too low to raise concern. Brian et al., (2005) showed additive and cumulative effects of complex chemical mixture that present no significative response for individual chemicals. Therefore the main difficulties are related to measure environmental alterations and related biological effects due to low concentrations of pollutants, unknown transformations products and contaminants mixture.

A selected response able to detect a higher level of biological organisation impairment, proposed as monitoring tool able to detect long term effects, is the behaviour kinetics (Boldina-Cosqueric et al., 2010). Results shown in the present field monitoring investigation on behavioural response evidenced an impairment in reburrowing rate in May in the site next to the Po di volano watershed ($p < 0.0001$ P vs G and P vs N) and with a higher speed in reburrowing rate for G site in December ($p < 0.0001$). Burrowing behaviour was disturbed in clams from P site in May; the factor avoidance of contaminated sediment is excluded *apriori* for since according to the experimental design the sediment is clean reference sediment. Therefore is impairments of invertebrate responses related to chemical stress, is feasible to correlate the trend in MN frequency detected with the results detected in the reburrowing rate? Linking responses across level of biological organisation, in the cascade event from sub-organism to higher and ecologically relevant biological response is one of the current challenges in the ecological risk assessment (Forbes et al., 2006) and a need in ecological risk assessment is underline for a more scientifically robust methods for extrapolating individual-level effects to the population level.

The model emerged with DistLM analyses underlined that a combination of multibiomarkers with analytical chemistry can be used to investigate influence of chemical variables on biomarker variance according to site and seasons. Methodology employed in this study revealed a non significant relationship between biomarker and selected classes of contaminants, illustrating that other contaminants drive the response detected with DNA damage and impairments in behavioural response detected in clams. This could be related with the fact that other classes of contaminants are not yet accessible to analysis or act with a synergic effects to contaminant mixture in the ecosystem. Explanation could be related to the fact that our analysis doesn't detect an anthropogenic impact that exceeds standard quality reference values, and sites shown a low contamination.

Therefore in order to test biomarkers, a numbers of sites with a marked pollution gradient or a punctual anthropogenic contamination should be well defined in experimental design. Therefore is essential that adequate control samples and an extremely attention be taken in the choice of the experimental design, to reduce factors with a direct interaction with responses in organisms.

This research hypotize that biological impairments evidenced in MN frequencies and behavioural response are due to toxic effects of environmental mixtures caused by other, so far unknown or unexpected contaminants than a priori selected chemicals. This is due to the lack of knowledge about which of the many chemicals present in the environment are responsible for effects at different levels of biological organisation (including mutagenicity and endocrine disruption) and due to the missing knowledge about the mechanisms involved in different

effects following exposure to a combinations of different active substances released in aquatic environments.

Moreover an integrated approach using analytical chemistry combined with biological response at different level of organisation is needed in ecotoxicological risk assessment and each endpoint should be regardered in a holistic approach and not as an absolute value. Each results not interpretable on its own and require substantial additional information to be useful. Consequently multivariate analysis should be considered as the most appropriate and useful tools to cope with data interpretation and variables interactions. The development of such integrated tools to assess ecosystem quality is therefore of high importance, and must take into account the multidisciplinarity of the problems involved, the need for integration of abiotic factors, methods intercalibration and validation, and adequate indicators to follow the evolution of the monitored ecosystems. This study underline the need of an integration of results since alterations at individual level (MN frequency) can provide important insights on the mechanism of toxicity and an indication of exposure, such changes has to be coupled with ecologically relevant indicators at higher level of biological organisation. Despite the difficulty to establish cause-effect relationships due to the co-occurrence of various stressors and their interactions, the adopted integrated monitoring strategy appears to be promising.

This objective in this area of research could be reach with further studies on the incorporation of different enpoints, from molecular to population-level effects with the help of multivariate analysis and predictive models tools to integrate and detect and/or predict adverse chemical impacts on populations, communities, and ecosystems.

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GENERAL CONCLUSIONS

Thesis focussed on suite of techniques of biologically-based approach employed in ecological risk assessments to investigate the healt of aquatic system associated to chemical exposure. Two approach of environmental assessment at different level of biological organisation were considered, bioindicator and biomarkers.

Chapter 1 investigate on how the response of aquatic communities to toxicant exposure is strongly influenced by the physiological sensitivity that members of these communities show to toxic compounds and how life-history traits also determine how single species, and communities as a consequence, respond to toxicant exposure.

Comparison of the pesticide-specific SPEAR indices based on family and species levels of taxonomic resolution ($SPEAR(fm)_{pesticides}$ and $SPEAR(sp)_{pesticides}$ respectively) has shown that the family-level index can be used to detect pesticide contamination in streams. The efficiency of this index is expected to be only slightly lower than that of the species-level index. Taking into account the time-consuming nature, cost and difficulties of species-level identification, the $SPEAR(fm)_{pesticides}$ index is a promising and cost-effective bioindicator tool for detecting pesticide contamination in streams. The study suggest how future biomonitoring programmes may consider applying stressor-specific SPEAR-bioindicator as an effective family taxonomically based approach in biomonitoring programme according to EU Water Framework Directive as a European-wide index.

Chapter 2 physiological responses was investigated in the bivalve *Mya arenaria* of combine factors as increasing temperature and pesticide exposure. Specimens were acclimated to two different temperatures: the basic Atlantic seawater temperature (7°C) and an elevated temperature (18°C) and were exposed to a commonly used herbicide, Weedout® Dichlorophenoxyacetic acid (2,4-D), 2-(2-methyl-4-chlorophenoxy) propionic acid (mecoprop) and 3,6-dichloro-2-methoxybenzoic acid (dicamba) for 28 days.

For biochemical assays, gills were removed at days 7, 14 and 28, and biomarkers involved in aerobic (CCO) and antioxidant resposnses (SOD) were monitored in bivalve tissues.

At all the sampling times, CCO activity was significantly increased ($p < 0.001$) by the effects of higher temperature in pesticide-exposed and not-exposed clams. The activity of CCO in samples kept at 18°C pesticide effects was observed after 14 and 28 days with significantly higher increase ($p < 0.001$). In samples kept at 7°C exposure to pesticide enhanced CCO activity after 7 and 28 days ($p < 0.05$). Moreover a significant increase ($p<0.05$) was detected also between day 7 and 28 for each treatment and temperature.

Exposure to the pesticide inhibited SOD activities at day 28, in *M. arenaria* acclimated to the lower temperature ($p < 0.001$) and 18°C ($p < 0.05$) exposed to pesticide. In pesticide exposed clams the increasing temperature after 28 d exposure had opposite significant effect ($p < 0.001$). At T7 no pesticide effects were observed in clams acclimated at either temperature.

In the present research, both temperature and pesticide had different and measurable effects on the physiology of the test organism, and these effects were more important on aerobic capacity than on oxidative stress. Our study also indicates that the effects of the two stressors combined are to some extent additive. This was done with the aim to have a link with the actual environmental challenges, that climate change will affect contaminant exposure and toxic effects and that both forms of stress impact aquatic ecosystems.

In Chapter 3 the field study presented aimed to evaluate the status of clams collected in three farming sites, with different environmental conditions, in Sacca di Goro Lagoon, Delta Po River. The Manila clams *Tapes philippinarum* have been used as bioindicator organisms, tool for indirect description of their habitat quality within the costal-marine environment.

To assess the quality healt of aquatic system we propose a integrated approach using different chemical and biological tools, genotoxic, enzymatic and behavioral biomarkers, which have been aimed to be indicators of chronic stressful environmental conditions.

Surface sediment sample were collected from sample site in Goro seaport, Po Volano inflow (Po river plume) and a reference site in 4 seasonal sampling campaign. Sediment characterization and chemical analysis for selected contaminant classes (metals, As, Σ PAHs) were performed, since is well established that sediment act as sink for a variety of contaminants and were correlated with biological responses.

Organisms were sampled in May and December for genotoxicity with micronucleus test in gills clams tissue in association with the evaluation of enzymatic activities (CAT and SOD) in digestive gland, as index of the antioxidant defense system of organisms and behavioural endpoints.

Results on sediment analysis showed a low contamination with PAH, As and trace metals. Chemical characterisation of sediments in terms of traces elements and PAH did not reveal evidence of exceeding standard levels for sediment of transitional and marine-costal waters (Decr. Min. 367/03), with the exception of Cr and Ni related to natural composition of sediment deposit of Po plain. The model emerged with DistLM analyses had no significant explanatory power to investigate between the relationship biological variables and analytical chemistry endpoints according to site and seasons, despite the difficulty to establish cause-effect relationships due to the detected lower concentrations, the adopted integrated monitoring

strategy appears to be useful. Biomarker results obtained with micronucleus test reveal a genotoxic damage among sampling sites for the winter sampling campaign mainly in samples from G station ($p<0.001$ N vs G and $p<0.05$ N vs P). Samples collected in N site present the lower frequency observed with a significant difference with other sites in May ($p<0.05$) and $p<0.001$ vs G site in winter (no differences were observed in winter between N and P site). Data on antioxidant defense system suggest a no or limited involvement of ROS species, as indirect driven mechanism of toxicity, in genotoxic damage. Even significant differences among seasons were evidenced ($p = 0.0001$) with the higher levels found in Po di Volano and Goro port (66.52 and 36.76 µg kg^{-1} dw), PAHs concentrations are low according to environmental quality standards. Therefore genotoxic damage is the response on a different contaminat or a low contaminant mixture. Thus, there is a need for a better understanding of genotoxic impairments in clams with futher studies on the baseline MN frequency and bioconcentration of pollutants in clam tissues.

Reburrowing responses reported in May could fit with data of Carafa et al, (2007), where clams from P-site, plume area of Po river, object of seasonal variation of PPP fluxes of pesticides mainly of agricultural origin, shown a significantly slower burrowing speed ($p<0.001$). Observed behavioral effects in bivalves suggest impairment in physiological selected parameters (enzymatic response and genotoxic damage). The integration of different techniques is necessary to give a more reliable picture of the relations between contamination and biological effects.

These studies investigate on the use of biological responses associated with chemical exposure: an approach for the assessment of the quality of a water body. This approach was performed at a different levels of biological organisation specific biomarkers at the cellular and intracellular levels (enzyme activity SOD, CCO, CAT; genotoxicy biomarker ad MN test), whole organism to detect behavioural changes (behaviour kinetics) to an highest level, the measurement of communities forms (SPEAR trait based) as an integral part of ecological status monitoring.

Within the context of theWFD, it is envisaged that biomarkers are proposed efficient tools for investigative and operational monitoring. Biomarkers aim to gain a quick response to a risk of pollution allowing rapid decision making. Their use is one of the challenges in the ecological risk assessment, since needs to be accompanied by an ecological value with consequences at the populations, communities, and ecosystems levels (i.e. reproductive impairments).

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