UNIVERSITÀ CA' FOSCARI DI VENEZIA
DIPARTIMENTO DI SCIENZE AMBIENTALI

DOTTORATO DI RICERCA IN SCIENZE AMBIENTALI
XXII CICLO
(A.A. 2005-2006 – A.A. 2008-2009)

# EFFECTS OF ORGANIC AND METALLORGANIC POLLUTANTS ON EUROPEAN GASTROPOD SPECIES

SETTORE SCIENTIFICO-DISCIPLINARE DI AFFERENZA: CHIM/12

TESI DI DOTTORATO DI **NOVENTA SETA** (MATR. 955343)

COORDINATORE DEL DOTTORATO:
**PROF. PAVONI BRUNO**

TUTOR:
**PROF. PAVONI BRUNO**
(UNIVERSITÀ CA' FOSCARI VENEZIA)

CO-TUTOR:
**PROF.SSA GALLOWAY TAMARA**
(UNIVERSITY OF EXETER. UK)

# CONTENTS

CONTENTS ..... 2
LIST OF ACRONYMS ..... 5
1 INTRODUCTION ..... 7
1.1 RESEARCH AIM ..... 7
1.2 MARINE ORGANIC POLLUTANTS ..... 10
1.2.1 PAH ..... 11
1.2.2 PCB ..... 22
1.2.3 OCP ..... 28
1.2.4 OTC ..... 32
1.3 POLLUTION RELATED STRESSES ..... 37
1.3.1 GENOTOXICITY ..... 38
1.3.2 OXIDATIVE DAMAGE ..... 41
1.3.3 ENDOCRINE DISRUPTION ..... 48
1.3.4 CYTOTOXICITY ..... 55
1.4 BIOLOGICAL TOOLS FOR MARINE POLLUTION ASSESSMENT ..... 58
1.4.1 BIOMARKERS ..... 59
1.5 ASSESSMENT OF POLLUTION RELATED STRESSES IN NATURAL POPULATIONS ..... 64
1.5.1 GENOTOXICITY ASSESSMENT ..... 64
1.5.2 OXIDATIVE STRESS ASSESSMENT ..... 72
1.5.3 ENDOCRINE DISRUPTION ASSESSMENT ..... 76
1.5.4 GENERAL HEALTH STATUS ASSESSMENT ..... 79
1.6 BIOMARKERS APPROACH FOR MARINE POLLUTION ASSESSMENT ..... 83
1.6.1 BIOMARKER RESERCH CHALLENGES ..... 85
1.7 SENTINEL ORGANISMS IN BIOMONITORING PROGRAM ..... 86
1.7.1 MULTI-SPECIES BIOMARKER APPROACH ..... 86
1.7.2 MOLLUSCS ..... 87
1.7.3 GASTROPODS ..... 88
1.8 RESEARCH GOALS AND EXPERIMENTAL DESIGNS ..... 94
1.8.1 RESEARCH LINE 1 ..... 94
1.8.2 RESEARCH LINE 2 ..... 98
2 MATERIALS AND METHODS ..... 100
2.1 RESEARCH LINE 1 ..... 100

2.1.1 SAMPLING SITES ..... 100
2.1.2 SAMPLING AND PRE-TREATMENT ..... 107
**2.2 RESEARCH LINE 2 ..... 109**
2.2.1 TEST-ORGANISMS RECRUITMENT ..... 109
2.2.2 POISONING SYSTEMS ..... 109
2.2.3 TEST CHAMBERS AND EXPERIMENTAL CONDITIONS ..... 111
2.2.4 TESTS SCHEME ..... 111
**2.3 CHEMICAL ANALYSIS ..... 113**
2.3.1 PAH, PCB AND OCP DETERMINATION ..... 113
2.3.2 OTC DETERMINATION ..... 116
**2.4 BIOLOGICAL METHODS ..... 118**
2.4.1 SAMPLE PREPARATION ..... 118
2.4.2 COMET ASSAY ..... 118
2.4.3 MICRONUCLEI ASSAY ..... 119
2.4.4 FRAP ASSAY ..... 120
2.4.5 INTERSEX/IMPOSEX DEVELOPMENT ANALYSIS ..... 120
2.4.6 NEUTRAL RED RETENTION ASSAY ..... 126
**2.5 STATISTICAL ANALYSIS ..... 127**
2.5.1 UNIVARIATE ANALYSES ..... 127
2.5.2 MULTIVARIATE ANALYSIS ..... 127
**3 RESULTS AND DISCUSSION ..... 129**
**3.1 EXPERIMENT 1 ..... 129**
3.1.1 RESULTS ..... 129
3.1.2 DISCUSSION ..... 137
3.1.3 CONCLUSION ..... 140
**3.2 EXPERIMENT 2 ..... 142**
3.2.1 RESULTS ..... 142
3.2.2 DISCUSSION ..... 146
3.2.3 CONCLUSION ..... 150
**3.3 EXPERIMENT 3 ..... 151**
3.3.1 RESULTS ..... 151
3.3.2 DISCUSSION ..... 154
3.3.3 CONCLUSION ..... 157
**4 CONCLUSIONS ..... 158**
**ACKNOWLEDGMENTS ..... 160**
**REFERENCES ..... 161**

**ANNEX I ........................................................................ 178**

# List of acronyms

| | |
|---|---|
| Ah receptor (AhR or AHR) | Aryl hydrocarbon receptor |
| AR | Androgen Receptor |
| BT | Butyltin |
| CAT | Catalase |
| CYP1A1 | P450 isoenzymes 1A2 |
| CYP1A2 | P450 isoenzymes 1A1 |
| CYP1B1 | P450 isoenzymes 1B1 |
| CYT P450 | Cytochrome P450 |
| DL-PCB | Dioxin like-PCB |
| E2 | Estradiol |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ER | Estrogen Receptor |
| FRAP | Ferric Reducing Antioxidant Power Assay |
| GC | Gas Chromatography |
| GPX | Glutathione Peroxidase |
| GR | Glutathione Reductase |
| HPLC | High Performance Liquid Chromatography |
| IARC | International Agency for Research on Cancer |
| ISI | Intersex Index |
| LOD | Limit Of Detection |
| MC- | 3-methylcholantrene- |
| MFO | Mixed Function Oxidase |
| MN | Micronuclei |
| MS | Mass Spectrometry |
| $NAD^+$/ NADH | Nicotinamide adenine dinucleotide (oxidizing/reducing forms) |
| $NADP^+$/ NADPH | Nicotinamide adenine dinucleotide phosphate (oxidizing/reducing forms) |
| NDL-PCB | Non Dioxin Like-PCB |
| NRR | Neutral Red Retention |
| OCP | Organochlorine Pesticides |
| OTC | Organotin Compounds |
| PAH | Polycyclic Aromatic Hydrocarbon |
| PAH | Polycyclic Aromatic Hydrocarbons |
| PB- | Phenobarbital- |
| PCB | Polychlorinated Biphenyls |

| | |
|---|---|
| PCDF | Polychlorinated dibenzofuran |
| POP | Persistent Organic Pollutants |
| RIA | Radio Immuno-Assay |
| ROS | Reactive Oxygen Species |
| RPLI | Relative Penis Length Index |
| SOD | Superoxide Dismutase |
| TCDD | 2,3,7,8-Tetrachlorodibenzodioxin |
| TEF | Toxic Equivalence Factor |
| TEQ | Toxic Equivalent |
| USEPA | United States Environmental Protection Agency |
| VDSI | Vas Deferent Sequence Index |
| WFD | Water Framework Directive (2000/60/EC) |

# 1 INTRODUCTION

## 1.1 RESEARCH AIM

The present PhD project develops two different research lines, as illustrated by the diagram reported in Figure 1.1. The purpose of the first research line is to verify the suitability as *sentinel organisms* of common gastropod species, used until now only in TBT biomonitoring programs, by testing the feasibility of selected biomarkers and their performance in discriminating pollution-related pressures of different nature and intensity.

To achieve this goal two field studies are carried out using different species. More precisely, the two experiences which are reported as *Experiment 1* and *Experiment 2* in the present thesis, consist in:

- *Experiment 1.* Field survey carried out on six *Littorina littorea* populations living along the British south and south-west coast. Because of the feeding habits as detritus-grazer, *L. littorea* health conditions are considered to provide insights about the quality of the sedimentary fraction constituted by organic and inorganic particular matter, on which hydrophobic organic pollutants tend to adsorb.

  Experiment 1 is developed through the partnership and collaboration of Prof. Tamara Galloway, Exeter University (UK). Sampling activities and toxicological investigation are performed with the support of the Invertebrates Toxicology Laboratory, Exeter University, and under the supervision of Prof. Galloway. Differently, the bioaccumulation analyses are carried out in the Environmental Chemistry Laboratory of Ca' Foscari University, Venice (Supervisor Prof. Bruno Pavoni).

  The experimental activities at Exeter University make part of a research program aimed at gaining skills on the use of biomarkers to assess the ecological impact of marine pollution. During the experience at Exeter University as PhD visiting student (from 1st of October 2007 to 23rd of May 2008 - eight months), I joined the Invertebrates Toxicology research group and I worked also on the EC funded 'FACEiT' project, studying the development of rapid biotools for assessing the ecological impact of oil pollution in sediment dwelling invertebrates.

- *Experiment 2.* Field survey which reproduces, in smaller scale (three stations), Experiment 1 design in the area of Venice Lagoon. Among the indigenous gastropod species, *Hexaplex trunculus*, a common mollusc often used in Mediterranean TBT

monitoring programs, is chosen as test-organism. Differently from *L. littorea*, *H. trunculus* is a voracious carnivorous whose contaminants body burden likely mirrors the average contamination in the low part of the trophic pyramid.

The second research line focuses on the possible contribution of organic pollutants, different from TBT, to Imposex development in gastropods. Experimental effort is dedicated to the preliminary phase of a laboratory study aimed at assessing the suspected involvement of PCB in the induction of this sexual malformation, as suggested by Maran et al. (2006) on the basis of field evidences. The laboratory experiment, which is in program in the Environmental Chemistry Laboratory of Ca' Foscari University (Venice), basically consists in a chronic (3 months) dietary exposure of *Nassarius nitidus* specimens to a mixture of PCBs. The preliminary tests included the PhD program are comprehensively referred as Experiment 3 in this manuscript. Basically they consist in:

- *Experiment 3.* Set of "pilot" laboratory studies aimed at 1) assessing the effectiveness of a pre-exposure decontamination period for decreasing the contaminants' background residues in the test-specimens recruited from natural populations (*Decontamination test*) and 2) establishing the efficiency of two poisoning systems via food ingestion, fitting the specific feeding behaviour of the test-organisms (contamination tests: *Recipe A test* and *Recipe B test*). Moreover these preliminary studies aim also at acquiring experience on managing sea-water aquaria and on running long-term exposure experiments.

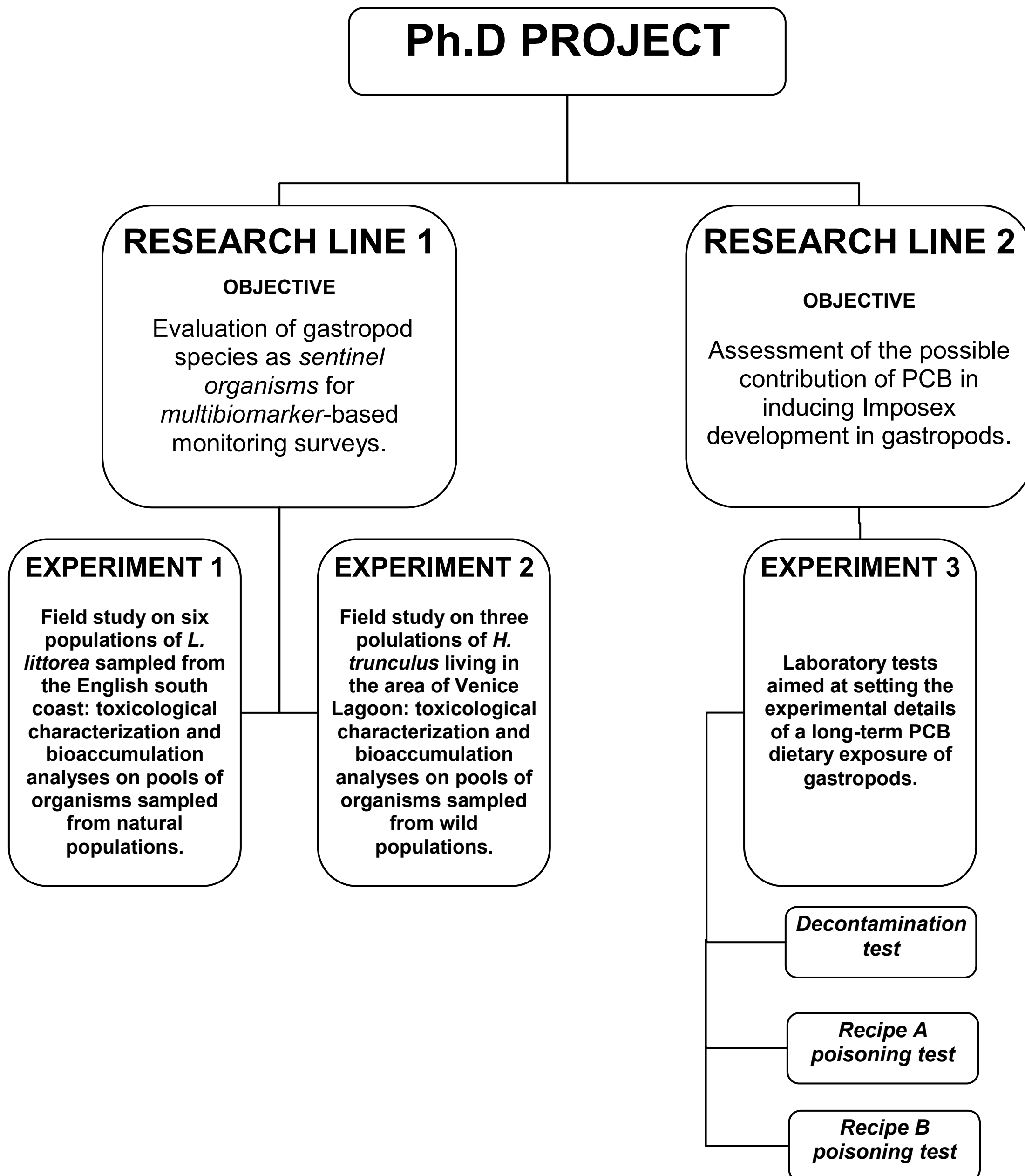



**Figure 1.1 PhD project diagram**

## 1.2 MARINE ORGANIC POLLUTANTS

Industrial, agricultural and urban activities are chiefly responsible of the worldwide presence of chemical pollution. Once released in the environment, pollutants undergo specific physical-chemical processes which determine their different fate and distribution into the geosphere, hydrosphere, atmosphere and biosphere. Several authors refer to the marine environments as the primarily sinks for xenobiotics: in fact, toxicants from the atmosphere, rivers, sewage treatment effluents, as well as those directly produced by maritime activities (fisheries, mariculture, shipping, dredging), reach the sea (Bowen and Depledge, 2006).

Great concern about marine pollution is related to organic contaminants, including organochlorine compounds, hydrocarbons and organometallic compounds (Livingstone, 2001). Many of them, such as organochlorine pesticides (OCP), polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), as well as dioxins and furans, are named *persistent organic pollutants* (POP). POP are semi-volatile and long half-live compounds which are responsible of their global distribution through long-range transport mechanisms (*i.e*. global distillation effects; grasshopper effects; cold trap effect). Residues of POP, in fact, are detected everywhere in the world, including areas absolutely lacking of own contamination sources, such as polar regions (Fernández and Grimalt, 2003). The concern about their environmental presence is mainly due to their tendency to bioaccumulate in the biota and biomagnificate along the food chain inducing harmful toxic effects. Environmental exposure to POP has been associated to detrimental effects on the wildlife and humans, such as carcinogenesis, mutagenesis, endocrine disruption, immunotoxicity etc. Despite national and international measures against organic contaminants production, use and release (*i.e.* Stockholm Convention on POP[1] (2001), Antifouling Systems Convention[2] (2001)), the assessment of organic pollution and risk associated for the biota still remain one of the main purposes of environmental monitoring programs.

---

[1] The full text is available in <http://chm.pops.int/Convention/tabid/54/language/en-US/Default.aspx#convtext>.

[2] International Convention on the Control of Harmful Anti-fouling Systems on Ships, adopted on 5th of October 2001 and entered into force on 17th of September 2008.

### 1.2.1 PAH

Polycyclic aromatic hydrocarbons (PAH) represent a major class of organic compounds (Xue and Warshawsky, 2005). They consist in two or more fused aromatic rings, containing no hetero-atoms.

#### 1.2.1.1 Sources and environmental presence

Even though PAH can be introduced in the environment by natural phenomena, such as volcanic activity and forest fire, generally their environmental presence is related to anthropic activities, in particular production, refining and burning of coal, mineral oil and oil shale (Harvey, 1998). It is worth mentioning that other dangerous pollutants arise from the same processes: PAH are usually produced together with heterocyclic aromatic compounds (HAC), which contain one or more nitrogen, sulphur or oxygen atoms (Xue and Warshawsky, 2005).

According to their origin, PAHs can be classified as (Soclo et al., 2000; Anderson and Lee, 2006; Wenzl et al., 2006):

- *Pyrolitic PAHs*. They are generated by uncompleted combustion at high temperature of organic matter (fossil or recent). The highest production occurs at 500-700°C, the typical temperature of wood fire and cigarettes; lower amounts are produced at higher temperatures (*i.e.* furnaces).
- *Petrogenic PAHs.* They are generated by slow maturation of organic matter under geochemical gradient conditions.

Combustion processes are the major responsible of environmental PAH presence. Once produced, pyrolitic PAHs remain in the atmosphere as gas only temporarily: in fact at environmental temperature they tend to condense and adsorb on atmospheric particles, due to their low vapour tension; thus they return to the geo- and hydrosphere according to the mechanisms of the *long range transport.* Consequently the atmospheric fallout is one of the main responsible of PAH global contamination of geosphere and hydrosphere.

Focusing on aquatic environments, even if PAH sources are represented primarily by oil spills and long-range transport of atmospheric particulates (Francioni et al., 2007), sewage disposal, land run off and industrial wastes represent additional important sources (Wake, 2005; Grundy et al., 1996; Soclo et al., 2000).

Environmental PAH contamination is never constituted by a single compound, but by complex mixtures made up by hundreds of congeners. For practical reasons, PAH environmental monitoring programs do not considered all compounds, but only selected

groups. The 16 PAHs included in the US EPA List of Priority Pollutants (1970) are generally taken as reference in PAH pollution surveys, even if the inclusion of other congeners is specifically recommended for characterizing PAH content in particular matrices (air, food, water). Table 1.1 lists the US EPA 16 priority PAHs, and both JECFA (Joint FAO/WHO Expert Committee on Food Additive) and EFSA-SCF (European Food Safety Authority-Scientific Committee on Food) priority PAHs on food matrices.

**Table 1.1 Priority PAHs according to US EPA, JECFA, EFSA**

| | Abbreviation | US EPA<br>Environmental priority PAHs<br>(1970) | EFSA<br>Priority PAHs on food<br>(2005) | JECFA<br>Priority PAHs on food<br>(2005) |
|---|---|---|---|---|
| Naphthalene | NA | X | | |
| Acenaphthylene | ACL | X | | |
| Acenaphthene | AC | X | | |
| Fluorene | FL | X | | |
| Phenanthrene | PHE | X | | |
| Anthracene | AN | X | | |
| Fluoranthene | FA | X | | |
| Pyrene | PY | X | | |
| Benz[a]anthracene | BaA | X | X | X |
| Chrysene | CHR | X | X | X |
| Benzo[b]fluoranthene | BbFA | X | X | X |
| Benzo[k]fluoranthene | BkFA | X | X | X |
| Benzo[a]pyrene | BaP | X | X | X |
| Indeno[123-cd]pyrene | IP | X | | |
| Dibenzo[a,h]anthracene | DBahA | X | | |
| Benzo[ghi]perylene | BghiP | X | X | |
| Benzo[j]fluoranthene | BjFA | | X | X |
| Cyclopenta[cd]pyrene | CPP | | X | |
| Dibenzo[a,e]pyrene | DBaeP | | X | X |
| Dibenzo[a,h]pyrene | DBahP | | X | X |
| Dibenzo[a,i]pyrene | DBaiP | | X | X |
| Dibenzo[a,l]pyrene | DBalP | | X | X |
| 5-methylchrysene | 5-MCH | | X | X |
| Benzo[c]fluorene | BcFL | | | X |

The specific composition of environmental PAH mixtures depends on physical-chemical properties, degradation processes and on the nature of PAH sources. Through the use of particular diagnostic indexes, it is possible to make hypothesis about the source originating PAH contamination in a studied area. The foundation upon which *molecular fingerprint indexes* are built up is the different molecular structure of petrogenic and pyrogenic PAHs.

Petrogenic PAHs are characterized by lower molecular weight (tri- and tetra-rings PAHs, such as PHE, FL, alkylated naphthalenes); the composition of crude and refined oils differs for the fraction of high molecular weight PAHs, which is greater in refined products due to their origin during catalytic cracking processes. Differently pyrogenic PAHs are high molecular weight compounds (four-six rings, such as BaA, CHR, BaP, P); the specific pattern of pyrogenic mixtures strictly depends on temperature at which pyrolisis occurred, making possible to potentially discriminate among PAHs generated by anaerobic coking processes at high temperature, at moderate temperature and at low temperature (Harvey, 1998; Soclo et al., 2000; Anderson and Lee, 2006).

The available *molecular fingerprint indexes* are generally based on the relative concentrations of diagnostic PAHs (Soclo et al., 2000; Francioni et al., 2007). Even if the use of these tools might be limited by the coexistence of many contamination sources and biotransformation processes in the environment, they are not seldom employed in PAH monitoring programs (Martins et al., 2005).

#### 1.2.1.2 PHYSICAL-CHEMICAL PROPERTIES AND ENVIRONMENTAL BEHAVIOUR

As previously mentioned, PAH are generally featured by low volatility and high tendency to adsorb on particulate matter. In atmosphere, PAH can be in gas or solid phase, depending on the molecular weight: generally naphthalene and three rings PAHs are in vapour phase, four-five rings PAHs can be found both as solid and gas, whereas more heavy PAHs only as solids (Zander, 1980)

In aquatic environments, due to their hydrophobicity, PAH tend to rapidly adsorb on particles, making sediment an important PAH reservoir. In fact the low solubility and structural stability are responsible of their persistence (several years); sediment bioturbation by benthic organisms can influence PAH persistence, by enhancing microbial degradation rate (Palmqvist et al., 2003). Filter-feeders and sediment dwellers are probably the marine organisms most exposed to PAH pollution. PAH biomagnification along the aquatic food chain is partially prevented by the efficient biotransformation systems featuring the organisms at the higher levels of food chain (Galloway at al., 2004).

PAH are quite stable and are not efficiently degraded by hydrolysis; oxidation in presence of light plays an important role as degradation mechanisms in atmosphere, whereas biotransformation by fungi and bacteria is the most important pathway in soil (Scientific Committee on Food, 2002 and references therein). Generally two- and three-ring PAHs are rapidly degraded, whereas the higher molecular weight PAHs are recalcitrant. Evidences about this behaviour are provided by studies on the treatment of soils contaminated by PAH mixtures: in most cases, the highest molecular weight PAHs constitute the residual fraction (Ghoshal et al., 1999).

#### 1.2.1.3 TOXICITY

PAH toxicity, which is highly structure-related, depends on two fundamental PAH properties: 1) the ability to interact with macromolecules, following metabolic activation, and 2) the ability to interact with the Ah receptor. These properties are primarily involved in the induction of PAH genotoxic effects, which can lead to mutagenicity and carcinogenicity, as well as in PAH acute toxicity, immunotoxicity, developmental and reproductive toxicity (Sjögren et al., 1996). These two modes of action are not independent to each other, as suggested by the involvement of Ah receptor binding-dependant mechanisms in the regulation of important enzymes of PAH metabolism/activation.

Because of their ubiquity, PAH can be taken up by the biota through all principal exposure routes, such as oral ingestion, dermal adsorption and inhalation (Lèmiere et al., 2004). Although PAH are distributed in the whole body by the circulatory system, adipose tissues bear the highest PAH contents.

PAH are metabolized through MFO-mediated biotransformation processes primarily in liver. Even though the pathway is specific for each compound, it is possible to outline a general scheme using benzo[a]pyrene as model. As illustrated by the diagram in Figure 1.2, PAH metabolism is a two-phase biotransformation process consisting in:

1) *Phase I: microsomal CYP-dependent mono-oxygenase reactions*.

   CYP1A1, CYP1A2, CYP1B1 and epoxide hydrolase are the enzymes most involved in this phase which consists in two subsequent oxidations:

   a) *Primary oxidation*. The parent compounds are oxidized into epoxides or phenols. Phenols can be generated also through spontaneous epoxide transformation. Epoxide hydrolase catalyzes the subsequent formation of diols (primary metabolites are marked in red in Figure 1.2);

   b) *Second oxidation*. Diols and phenols, produced during the primary oxidation, can further be oxidized into hydroxyepoxides and dihydroxyepoxides (secondary metabolites are marked in green in Figure 1.2).

2) *Phase II: conjunction*.

   It consists in the conjugation of phase I metabolites by glutathione, glucuronic acid and sulphate (conjugate metabolites are marked in yellow in Figure 1.2). The enzymes mainly involved in phase II reactions are glutathione *S*-transferases (GSTs), uridine5′-diphosphate glucuronosyltransferases (UGTs) and sulfotransferases (SULTs).

The soluble conjugates are excreted in urine, bile and faeces.

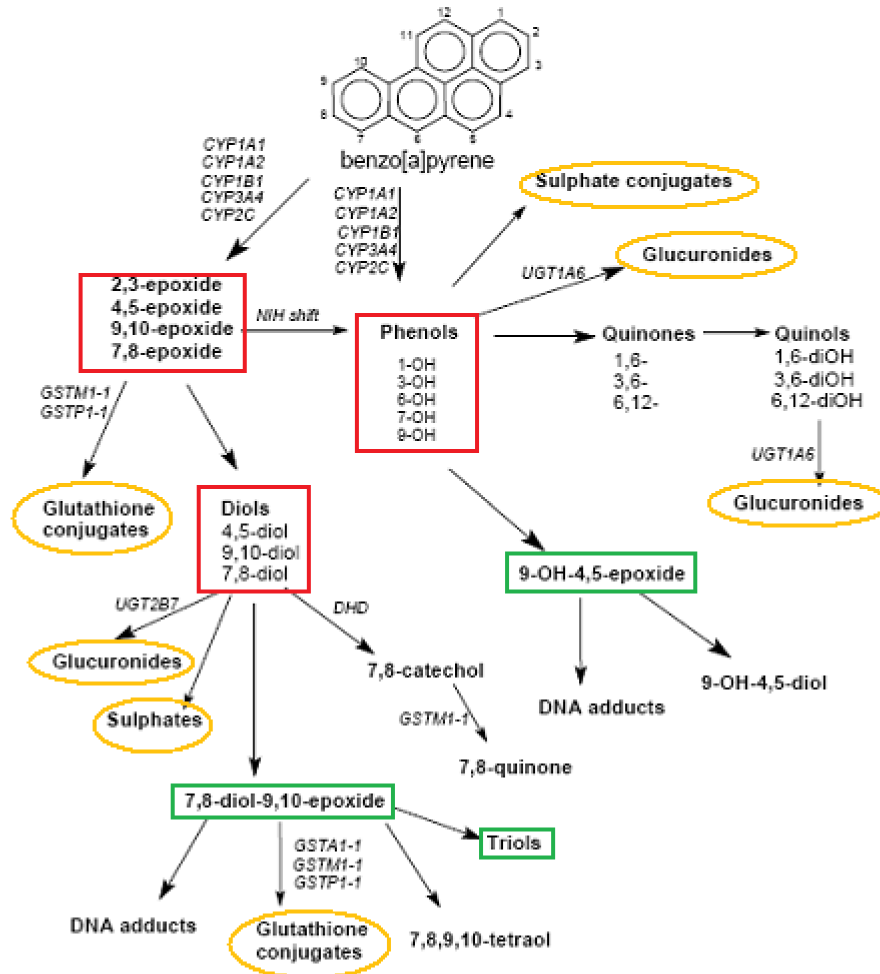



**Figure 1.2 Benzo[a]pyrene metabolic pathway (modified from Scientific Committee on Food, 2002). Phase I primary and secondary metabolites are marked in red and green respectively; phase II conjugation products are marked in yellow.**

PAH constitutes one of the first pollutant classes whose carcinogenic properties have been assessed by laboratory studies. Several research organizations have assessed the potential of individual PAHs for human carcinogenesis. As indicated by PAH classification into different groups of risk (Table 1.2), no all congeners are equally aggressive. This diversity is partially associated to the specific role which individual PAHs can play in the carcinogenesis. In fact, PAH can act as *tumour initiators* and *tumour promoters* (Figure 1.3): as explained below, the mutagenic potency of the former is due to their high tendency to form DNA adducts; differently, the promotion activity of the latter basically consists in the activation of biotransformation enzymes and protein kinase, which respectively enhance the bioavailability of active mutagens and promote the mitosis of initiated cells (Sjögren et al., 1996; Xue and Warshawsky, 2005).

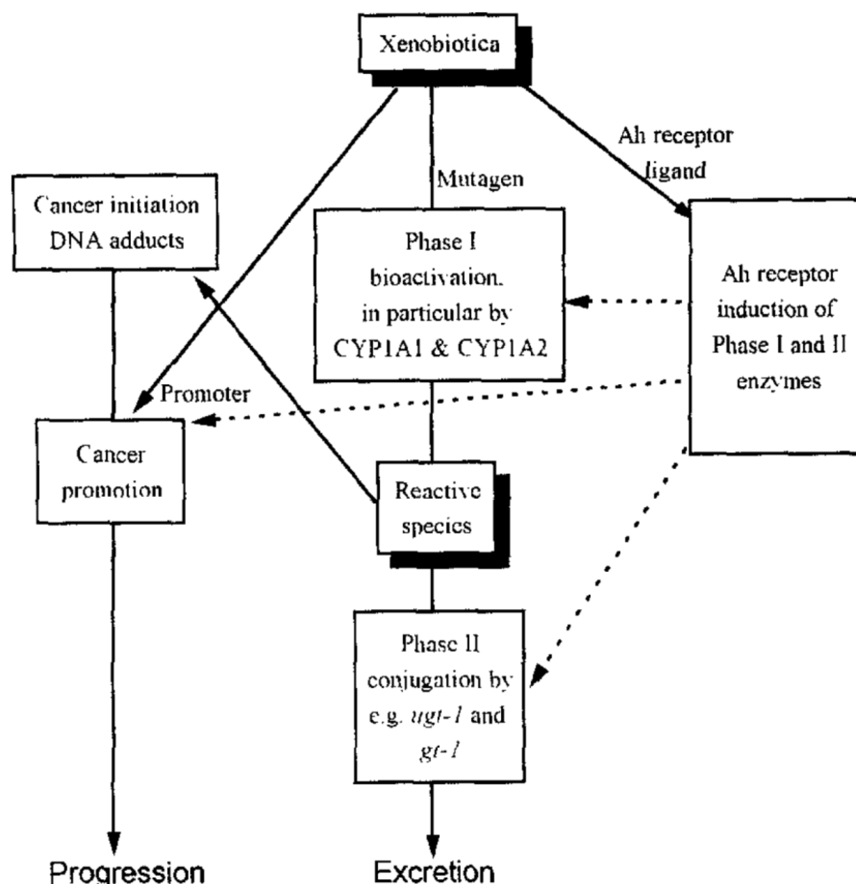



**Figure 1.3 Proposed model for bioactivation, detoxification and cancer initiation and promotion (Sjögren et al., 1996)**

Results from laboratory studies pointed out that some PAHs are *complete carcinogens*, acting both as initiators and promoters in test-animals. In PAH-induced carcinogenesis, the real significance of each mechanism depends on many variables, including the chemical/biological characteristics of individual compounds, test-species, tumour site and level of expression of the enzymes involved in the activation (Xue and Warshawsky, 2005).

To exert their genotoxic properties, PAH must be turned into polar biochemically reactive electrophilic species, able to interact with nucleic acids, as well as cellular proteins. Four metabolic activation pathways have been described, which involve different enzyme-mediated reactions and lead to the following electrophilic metabolites (Xue and Warshawsky, 2005):

1. *Oxide and diol-epoxides*. This pathway, playing an essential role in PAH activation, basically coincides with phase I PAH metabolism. PAH oxides and diolepoxides generated are DNA-reactive metabolites which tend to bind primarily with guanines and adenines, forming stable DNA adducts and both stable and depurinating

adducts respectively. Animal experiments have demonstrated that these genotoxic lesions can induce mutations.

The specific reactivity of PAH metabolites depends on the chemical structure (*i.e.* bay regions, fjord regions, cyclopenta rings) and, in the case of diol epoxides, on the stereochemistry and degree of planarity.

This pathway is responsible for the activation of benzo[a]pyrene, chrysene, 5-methylchrysene, phenanthrene, benzo[c]phenanthrene, benz[a]anthracene, 7,12-dimethylbenz[a]anthracene, dibenzo[a,l]pyrene, as well as several nitrogen heterocyclic aromatic compounds.

2. *Radical cations*. They are formed by removal of one electron from the π electron system of the molecule through electron oxidation catalyzed by P450 peroxidase. The so generated PAH radical cations are extremely reactive and tend to be immediately trapped by nucleophiles present in the system, forming adducts with guanines and adenines. Because of their general instability, spontaneous depurination might occur, generating apurinic sites which can turn into mutations, strongly associated with tumourigenesis.
3. *O-quinones*. They are formed through dihydrodiol dehydrogenases-catalyzed reaction. These enzymes compete with cytochrome P450 to oxidize the non-K-region diols, the carcinogenic metabolites of PAH. This $NADP^+$-dependent oxidation leads to the initial formation of ketols that spontaneously rearranges into catechols. The following autoxidation of the unstable catechols leads to the formation of o-quinones. Besides forming both stable and depurinating DNA adducts, PAH-o-quinones can generate reactive oxygen species (ROS): the reaction reducing PAH-o-quinones into catechols or semiquinones is accomplished through the consumption of molecular oxygen and the production of hydrogen peroxide.
4. *Sulphuric acid esters*. This activation pathway involves mainly methyl-substituted PAHs (*i.e.* 7,12-dimethylbenzo[a]anthracene, 7-methylbenzo[a]anthracene, 6-methyl-benzo[a]pyrene, 5-methyl-chrysene, 9,10-dimethyl-anthracene, 1-methylpyrene). This mechanism basically consists in cytochrome P450s-mediated transformation of methyl-PAHs into hydroxymethyl-PAHs, followed by conversion into reactive sulphate esters forms able to interact with DNA forming adducts. Studies on the role of this mechanism in PAH tumourigenesis are limited.

The assessment of the genotoxic, mutagenic and carcinogenic potential of PAH exposure is one of the priority objectives of several cancer research institutions, primarily IARC (International Agency for Research on Cancer), the intergovernal agency of WHO (World Health Organization). The evaluation of the carcinogenic risk associated to individual compounds and occupational PAH exposures are carried out, respectively, by *in vitro/in vivo*

tests and epidemiological studies. The improvement of the testing systems continuously updates PAH risk characterization. Based on the most recent evidences, individual PAHs and occupational exposures are classified into five groups:

- Class 1: carcinogenic to humans;
- Class 2A: probably carcinogenic to humans;
- Class 2B: possibly carcinogenic to humans;
- Class 3: not classifiable as to their carcinogenicity to humans
- Class 4: probably not carcinogenic to humans.

Table 1.2 reports the classes assigned to each US EPA priority PAHs and to other congeners suspected as carcinogens: it is easy to recognize that there is a considerable overlapping between genotoxic and carcinogenic potential, in agreement with the mechanistic link between DNA adduct formation, mutations and cancer outcomes (Scientific Committee on Food, 2002).

**Table 1.2 Carcinogenic and genotoxic characterization of PAHs (Scientific Committee on Food, 2002)**

| | **US EPA Priority Pollutants (1970)** | **Carcino genicity (IARC, 2008)** | **GENOTOXICITY** | |
|---|---|---|---|---|
| | | | **Evaluation** | **Evidences** |
| Naphthalene | X | | Probably not genotoxic | Mainly negative results *in vitro;* extensive negative data *in vivo* |
| Acenaphthylene | X | | Inadequate data | Mixed results from bacterial studies |
| Acenaphthene | X | | Inadequate data | Mixed results from few *in vitro* studies |
| Fluorene | X | | Inadequate data | Mixed results from *in vitro* studies; no *in vivo* data available |
| Phenanthrene | X | | Equivocal | Mixed results *in vitro ;* negative or borderline positive *in vivo cytogenetics* |
| Anthracene | X | | Non genotoxic | Negative results in the majority of *in vitro* assays and in all *in vivo* tests |
| Fluoranthene | X | | Equivocal | Mixed results from *in vitro* studies; evidences of DNA binding *in vivo* after administration, negative in mutagenicity/genotoxicity tests by oral route |
| Pyrene | X | | Not genotoxic | Mainly negative results *in vitro;* extensive negative data *in vivo* |
| Benz[a]anthracene | X | 2B | Genotoxic | Positive results *in vivo* and *in vitro* for multiple end-points; positive also at germ cell level |
| Chrysene | X | 2B | Genotoxic | Positive results *in vivo* and *in vitro* for multiple end-points; positive also at germ cell level |

| | | | | |
|---|---|---|---|---|
| Benz[b]fluoranthene | X | 2B | Genotoxic | Positive results *in vivo* and *in vitro* for multiple end-points |
| Benz[k]fluoranthene | X | 2B | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |
| Benz[a]pyrene | X | 1 | Genotoxic | Positive results *in vivo* and *in vitro* for multiple end-points; positive also at germ cell level |
| Indeno[1,2,3-cd]pyrene | X | 2B | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |
| Dibenzo[a,h]anthracene | X | 2A | Genotoxic | Positive results *in vivo* and *in vitro* for multiple end-points |
| Benz[ghi]perylene | X | 3 | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |
| Cyclopenta(c,d)pyrene | | 2A | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |
| Dibenzo(a,l)pyrene | | 2A | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |
| Benzo(j)aceantrilene | | 2B | | |
| Benzo(j)fluoranthene | | 2B | | |
| Benzo(c)phenanthrene | | 2B | Limited evidences | Limited evidence of genotoxicity (positive results in assay in vitro; limited evidences for DNA binding in vivo) |
| Dibenzo(a,h)pyrene | | 2B | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |
| Dibenzo(a,i)pyrene | | 2B | | |
| 5-metilcrisene | | 2B | Genotoxic | Positive results in assay *in vitro* and for DNA binding *in vivo* |

The evaluation of the overall risk of environmental PAH exposure for human health is complicated by numerous factors influencing PAH toxicity in environmental mixtures. These modulating factors can be mainly related to (Van Ngan et al., 2007 and references therein):

1. Differential carcinogenic potential of individual PAHs;
2. PAHs interactions in complex environmental mixtures;
3. Environmental factors (*i.e.* light-induced phototoxicity);
4. Differential PAH sensitivity of exposed subjects (associated, for example, to genetic polymorphisms of drug-metabolizing enzymes and differential susceptibility to cancer).

There is no general agreement in using TEF approach (Toxic Equivalent Factor) for the PAH risk assessment as done for other harmful organic pollutants (*i.e.* TCDD, PCDF). This is mainly because the assumptions of homogeneity in the action mechanisms and additivity (dose addition) are not certainly assessed (Scientific Committee on Food, 2002). In fact as regard the genotoxic and mutagenic properties of complex PAH mixtures, the literature is not univocally consistent. White (2002) reviewed 17 studies about the mutagenic behaviour of complex mixtures of PAHs. Summarizing his findings, only five studies indicated that the

mutagenicity of the mixture components is additive; slightly more than half of the studies (9 of 17) described some synergistic interactions, most of the studies (11 of 17) described antagonism between mutagens. Commonly, antagonist effects are explained as a reduction in the conversion of the mutagens into their reactive metabolites, whereas sub-additive effects as a competitive inhibition of enzymes involved in metabolic activation. Similar disagreement about the additive action of PAH mixtures characterizes also the results obtained by considering simple PAH mixtures (*i.e.* binary, tertiary).

About the role of non-mutagenic congeners (*e.g.* anthracene, naphthalene) in the carcinogenesis of complex PAH mixtures, several authors have demonstrated that these compounds can modulate (*e.g.* enhance or diminish) the effects of mutagens (White, 2002).

Consequently alternatives to TEF approach have been studied for different application fields: in food hazard characterization, for example, the single BaP is used as marker of carcinogenic PAHs, and the overall carcinogenic potency of food is calculated as 10 times the carcinogenic potency due to the only presence of BaP (Scientific Committee on Food, 2002).

#### 1.2.1.4 LEGISLATION

Neither Italy nor European Union have adopted comprehensive legislative measures for the management of PAH pollution. Limits on PAH production, emission and exposure can be found in different directives regulating specific activities that contribute to PAH contamination. References about European legislation setting PAH limits in atmosphere, food, water and drinking water are reported below. Because of PAH ubiquity and worldwide production which make difficult to establish safe exposure limits, legislation generally aims at assuring that PAH exposure is *as low as reasonably achievable* (ALARA) (Wenzl et al., 2006).

- *Atmospheric pollution*. The global-scale problem of PAH pollution was firstly debated at international level in 1998, when the Aarhus Protocol on Persistent Organic Pollutants was included in the Convention on Long-range Transboundary Air Pollution (1979)[3]. In UE the protocol came into force in 2003, by the settlement of the Regulation (EC) 850/2004[4]: within this moment four PAHs (BaP, BbF, BkF, IcP) were recommended as PAH pollution indicators. This changed with the Directive 2004/107/CE[5], which took BaP as marker of carcinogenic risk associated to PAH

---

[3] The Protocol to the regional UNECE Convention on Long-Range Transboundary Air Pollution (CLRTAP) on POPs, opened for signatures in June 1998 and entered into force on 23 October 2003. <http://www.unece.org/env/lrtap/full%20text/1998.POPs.e.pdf>.

[4] Regulation (EC) No 850/2004 of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC

[5] Directive 2004/107/EC of the European Parliament and of the Council of 15 December 2004 relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air.

contamination and fixed at 1 ng BaP/$m^3$ air (PM10) the threshold value of annual mean concentration. In Italy the Directive was adopted by the Decreto Legislativo 152/2007[6], which established that this target-concentration has to be achieved by 31/12/2012.

- *Food.* The UE legislation has been recently updated as response to the results emerged from the survey carried out by the UE Scientific Committee on Food (2002). The Commission Regulation (EC) No 208/2005[7] and the Commission Regulation 1881/2006[8] amend Regulation (EC) 466/2001[9] as regards polycyclic aromatic hydrocarbon. Maximum concentrations of BaP and other PAHs were established for different kind of food (*i.e.* high fats and oils containing food, smoking-drying processed food).
- *Water.* PAH are in the list of priority pollutants (Decision 2455/2001/EC[10]) mentioned by the Water Framework Directive (2000/60/EC[11]). According to the WFD prescriptions, five PAHs, selected as representative of the whole class, have to be regularly monitored in surface waters, groundwater and coastal water (BaP, BbF, BkF, IcP, BgP).
- *Drinking water.* The Drinking Water Directive (Council Directive 98/83/EC[12]) fixes the maximum concentration of PAH in drinking water at 0,010 μg/L as BaP and at 0,10 μg/L as sum of BbF, BkF, IcP, BgP.

---

[6] Decreto Legislativo 3 agosto 2007, n. 152, "Attuazione della direttiva 2004/107/CE concernente l'arsenico, il cadmio, il mercurio, il nichel e gli idrocarburi policiclici aromatici nell'aria ambiente ", (*Gazzetta Ufficiale* n. 213 del 13 settembre 2007 - Suppl.Ordinario n. 194).

[7] COMMISSION REGULATION (EC) No 208/2005 of 4 February 2005 amending Regulation (EC) No 466/2001 as regards polycyclic aromatic hydrocarbons.

[8] COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.

[9] COMMISSION REGULATION (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs.

[10] Decision No 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC.

[11] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

[12] Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption.

### 1.2.2 PCB

Polychlorinated biphenyls (PCB) are an important class of persistent halogenated hydrocarbon. They are constituted by 1 to 10 chlorine atoms bound to a biphenyl nucleus. Totally 209 congeners exist, differing from each other for the degree of chlorination and the position of the chloride atoms on the molecule. According to IUPAC nomenclature system, PCBs are identified by a sequence number, attributed according to the number and position of chlorines (from 2-chlorobiphenyl (cb1) to 2,3,4,5,6,2',3',4',5',6'-decachlorobiphenyl (cb209)).

#### 1.2.2.1 SOURCES AND ENVIRONMENTAL PRESENCE

The commercially production of PCB, through catalytic chlorination of biphenyls, raised consistently in the 1970s, thanks to the chemical and physical properties that make them suitable for many industrial purposes (oil in transformers, dielectrics in capacitors, hydraulic fluids, heat exchange liquids and lubricants); thermal stability, chemical inertness, strong resistance to acids, basic hydroxides and corrosive chemicals, non-flammability, high electrical resistivity and dielectric constant are the technical features that mark them out (Fishbein, 1973). PCB commercial products were traded as mixtures containing 60-90 congeners. The commercial names depended on the manufacture brand (Aroclor, Kaneclor, Fenclor, Pyralene, Clophen); commonly the name was followed by a number indicating the chlorine percentage on which technical properties largely depend (Borja et al., 2005).

PCB environmental presence is only partially associated to direct and intentional releases. A large portion arises from waste disposal facilities not perfectly sealed, from which PCB can leak and reach inland and coastal waters through percolation and water transport; thus atmosphere enlarges environmental diffusion. Thanks to their large scale diffusion and stability, PCB are ubiquitous pollutants. Even through restrictions and bans on their production and use have been established almost worldwide since the 1970s, causing a general decrease of levels, residues can be still revealed in any environmental compartments.

PCB occur in the environment as mixture and never as single congeners. The pattern of PCB environmental contamination does not closely resemble the composition of the commercial PCB mixtures; this is progressively more evident at incremental distance from the input areas, which are mainly concentrated in the northern hemisphere. In fact, individual PCBs are featured by specific rates of environmental degradation/transformation and by a peculiar susceptibility to transport processes, explaining their differential behaviours and fates. This implicates that decreases on PCB environmental concentrations might represent

changes in the global picture of PCB contamination, rather than PCB degradation (Fishbein, 1973).

Although the relative abundance of PCB congeners in environmental matrices is strictly linked to the sampling origin, some generalities have been noted, such as the almost worldwide predominance in biological samples of cb138, cb153 and cb180, which are high chlorinated biphenyls, particularly resistant to degradation (Maran et al., 2006; Safe, 1994).

#### 1.2.2.2 PHYSICAL-CHEMICAL PROPERTIES AND ENVIRONMENTAL BEHAVIOUR

As previously mentioned, physical and chemical PCB properties vary according to the degree of chlorination: lower chlorinated congeners (up to 4 Cl) generally appear as colourless oily liquids, whereas medium chlorinated congeners tend to be heavy, honey-like oils; finally high chlorinated PCBs have greases-waxy consistency and dark colour (Borja et al., 2005).

The features responsible of the concern about PCB pollution are the extremely high molecular stability and fat solubility. PCB, in fact, tend to persist in the environment, bioaccumulate in animal tissues and biomagnificate along the food chain (Fishbein, 1973): the highest chlorinated PCBs, which are low soluble and high lipophilic, are generally the most resistant to biotransformation and excretion processes (Borja et al., 2005).

PCB environmental degradation proceeds mainly through microbial decomposition (anaerobic reductive dechlorination and aerobic oxidative degradation). Thanks to these processes, PCB are transformed into less toxic substances and, finally, mineralized. Although PCB biodegradation is generally slow, many factors can influence the degradation rate: probably the most prominent variables are the solubility (low solubility generally prevents degradation) and the chlorination degree. Highly halogenated compounds require, in fact, more energy to break the halogen-C bonds; furthermore the presence of Cl atoms can alter the electron density, deactivating the primary oxidation and influencing the degradation rate. Generally the presence of chlorine atoms in *para* and *meta* positions obstacles the epoxide formation, decreasing the degradation rate (Delm, 2000).

#### 1.2.2.3 TOXICITY

Depending on the organism, PCB up-take may occur through all principal routes of exposure, such as though the skin, lung and gastrointestinal tract. Once inside the body, PCB can be metabolized directly or by CYP P450-mediated reactions. Similarly to PAH (Par. 1.2.1.3), after phase I oxidations, producing mono- and di-hydrodiols via intermediate formation of reactive epoxides, the biotransformation can proceed through several phase II metabolism routes, yielding catechols, phenol conjugates and methyl-, sulfonyl-metabolites (Safe, 1994).

It is interesting to mention that the position of halogen substituents determines the involvement of specific CYP P450 isoenzymes, in particular if typically 3-methylcholantrene-induced monoxigenase or phenobarbital-induced monoxigenase (MC- or PB-). In fact, according to some research results obtained from rat studies (White et al., 1997), MC-inducible P450 isoenzymes (CYP1A1, CYP1A2, CYP2A1) are activated by PCBs with adjacent non-substituted *ortho-meta* carbons, and congeners with at least one chlorine in *para* positions, whereas PB-inducible P450 isoenzymes (CYP2B1, CYP2B2, CYP2A1) are activated by congeners with adjacent non-substituted *para-meta* carbons, and at least one *ortho*-halogen.

PCB toxicity is a function of both number and position of chlorine atoms. Generally the most harmful PCBs are those having 1) high chlorination degree, 2) coplanar structure and 3) absence of chlorines in *ortho* positions (Borja et al., 2005). The congeners that represent the major threat for wildlife and human health are the non *ortho*-coplanar congeners cb77, cb126 and cb169, noted as *dioxin-like* PCBs (DL-PCBs); these molecules present chlorine atoms in both *para* positions, in at least two in *meta* positions, but not in *ortho* positions. This particular structure is considered responsible of their biological activity, which closely resembles that of TCDD (2,3,7,8-tetrachloro-dibenzo-p-dioxin) and MC-like compounds: in fact, dioxin-like PCBs have very high affinity for Ah-receptor, eliciting the biochemical and toxic responses typical of dioxins (*i.e.* induction of CYP1A1 and CYP1A2 gene expression, induction of epoxide hydrolase, wasting syndrome, thymic atrophy, hepatotoxicity, reproductive and developmental toxicity, immunotoxicity, endocrine disruption, tumour promotion). PCB affinity for Ah-receptor diminishes with the occupation of the *ortho* positions by chlorines: monoortho- and diortho- coplanar PCBs still act as Ah-receptor agonist, even if weakly. However, considering the higher proportion of mono- and di*ortho* coplanar PCBs compared to non-*ortho* coplanar congeners in commercial mixtures, it is likely that their contribution in inducing TCDD-like activity is significant (Safe, 1994).

Regarding the carcinogenic potential of PCB, because many *in vivo* and *in vitro* genotoxicity tests are negative, the most likely hypothesis is that PCB are indirect non-genotoxic carcinogens, acting mainly as tumour promoters. In fact even thought unstable arene oxide, able to bind DNA, are generated during PCB oxidative metabolism, experimental evidences seem to suggest that metabolic activation does not play a great role in PCB-induced genotoxicity (Safe, 1994).

Tumour promotion experiments on rats have pointed out that, after initiation with genotoxic carcinogen, both technical PCB mixtures and individual dioxin-like/non-dioxin-like PCBs act as liver tumour promoters: although DL-PCBs are the most potent, a weak carcinogenic potential of individual NDL-congeners cannot be excluded.

The mechanisms proposed to explain PCB role in liver tumour promotion refer to 1) suppression of apoptosis in pre-neoplastic cells, 2) inhibition of intercellular communication and 3) indirect stimulation of cellular proliferation after cells or tissues injury caused by reactive PCB metabolites or ROS (Reactive Oxygen Species).

Because of the similar mode of action of PCB and PCDD/PCCF, explained by the common involvement of Ah-receptor, Safe (1994) proposed to extend TEF approach to the assessment of the carcinogenic risk of PCB mixtures. In fact non-*ortho* and mono-*ortho* coplanar PCBs fulfil the requirements necessary to receive a TEF value, which are 1) to be structurally related to TCDD, 2) to bind the Ah-receptor, 3) to exert dioxin-like biochemical and toxic effects, and 4) to persist in the environment and to accumulate in the biota (Knerr and Schrenk, 2006). PCB carcinogenicity was tested for TEF purposes by several research agency (*i.e.* USEPA, IARC), referring to different Ah-receptor-mediated responses. Table 1.3 reports WHO-TEF values expressing the relative potency of individual congeners compared to that of TCDD (Knerr and Schrenk, 2006).

**Table 1.3 WHO-TEF values of non-ortho coplanar PCBs and mono-ortho coplanar PCBs**

| | **PCB** | **WHO-TEF** |
|---|---|---|
| NON-ORTHO PCBs | cb 77 | 0,0001 |
| | cb 81 | 0,0001 |
| | cb 126 | 0,1 |
| | cb 169 | 0,01 |
| MONO-ORTHO PCBs | cb 105 | 0,0001 |
| | cb 114 | 0,0005 |
| | cb 118 | 0,0001 |
| | cb 123 | 0,0001 |
| | cb 156 | 0,0005 |
| | cb 157 | 0,0005 |
| | cb 167 | 0,00001 |
| | cb 189 | 0,0001 |

Although many studies demonstrated that the carcinogenicity of commercial PCB mixtures is expressed better by TEQ values (Toxic Equivalent) computed by considering only DL-congeners rather than total PCB, the suitability of TEF approach for PCB mixtures recently underwent to further evaluation. The major objections base on the possible non-negligible role of NDL-PCBs as carcinogens, which generally account for more than 90% PCB residues in environmental samples (included food), and on the non-Ah receptor-mediated carcinogenicity of non-planar congeners (Knerr and Schrenk, 2006).

PCB are also assessed as endocrine disrupting agents (ECDs, Endocrine Disrupting Compounds, for details refer to Par. 1.3.3) (Safe, 2001). In particular they are classified among those showing estrogenic activities. In fact, cases of abnormal sexual differentiation and reproductive dysfunction have been recorded in wildlife and in humans. The most evident effects have been observed in adult individuals who have been exposed to PCB during their fetal and neonatal life. The developed abnormalities closely resembled those developed by the male offspring of woman treated with the potent synthetic estrogen diethylstilbestrol (DES) during pregnancy (see Par 1.3.3).

The mechanisms responsible for PCB estrogenic activity are not clear yet, even though there is general agreement in indicating the hydroxylated metabolites as the active agents. Many authors, observing a certain affinity for the estrogen receptor, suggest that PCB-OH are oestrogen receptor agonists; this feature is particularly marked in congeners carrying hydroxyl group in *para* position in only one ring (cb3, cb4). Contrarily Kester et al. (2000), considering that oestrogen receptor affinity is not so pronounced, suggested that PCB-OH and PCB exert their estrogenic activity mainly through indirect mechanisms, for example by enhancing cellular estradiol (E2) level through inhibition of E2 metabolism. These authors found, in fact, that several congeners are potent inhibitors of human estrogen sulfotransferase (EST), the enzyme that catalyzes E2 inhibition by sulfatation. The congeners having OH group in *para* position with two adjacent Cl substituents (*i.e.* 4-OH cb31; 4-OH cb13; 4-OH cb14; 4-OH cb15; 4-OH cb16; 4-OH cb18; 4-OH cb19) seem to have the maximum EST inhibitory potency.

Beside their role in disrupting estrogens homeostasis, PCB, in particular the common congeners cb 28, cb 52, cb 101, cb 138, cb 153, cb 180, which are all *ortho*-substituted nonplanar PCBs, have been associated to thyroid hormones disturbances. Cases of severe PCB poisonings, as those of Yusho's and Yu Chang's accidents (Mukerjee, 1998 and references therein), showed that, beside detrimental effects on the reproductive system, also symptoms typically associated to hypothyroidism were developed by the victims. This was explained by the high affinity of *ortho*-substituted nonplanar PCBs for thyroid hormones' transport proteins: these congeners compete with thyroid hormones (in particular, thyroxine (T4)) to bind Thyroid Binding Globulin (TBG) and transthyretin, resulting in increasing failure of binding the receptor. Furthermore T4 displacement, together with PCB-mediated induction of phase II biotransformation enzymes (particularly UDP-GT), favours T4 metabolism and excretion, leading to a significant decrease of T4 levels. (Vasseur and Cossu-Leguille, 2006) This mechanism is particularly dangerous during the organs growth and development, because it can cause several functional neurological impairments, including effects on brain functions. For this reason big attention has been given to study the exposure *in utero* and via breast milk to PCB, in particular to *ortho*-substituted nonplanar congeners. The alteration of

thyroid hormones homeostasis was found to have a role also in the development of cancers on endocrine glands and on endocrine-dependent organs (Mukerjee, 1998).

### 1.2.3 OCP

The definition *organochlorine pesticides* (OCP) covers a wide range of chemicals used as biocides and featured by the presence of several chlorine atoms and rigid molecular shape (Zitko, 2003). Among OCP, the present study considered hexachlorobenzene (HCB), hexachlorohexane (HCH), DDT (1,1,1-Trichloro-2,2'bis(*p*-chlorophenyl)ethane) and its degradation products DDE (1,1-bis(4-chlorophenyl)-2,2-dichloroethylene) and DDD (1,1-dichloro-2,2,-bis(4-chlorophenyl)ethane), because of their global distribution and harmfulness.

#### 1.2.3.1 Sources and environmental presence

DDT was the first large-scale pesticide used in the world, whose effectiveness was responsible for the elimination of malaria from US and Europe. The molecule was synthesized in 1874, but its properties as insecticide were discovered only in 1939: its first large use was during the Second World War for protecting the soldiers against malaria. Then it was commercially available, remaining the most extensively used pesticide until the 1970s, when its use was banned or restricted in most developed countries (Beard, 2006). Contrarily, DDT use in many developing countries is not dismissed yet, because of its importance for the public health in tropical areas where malaria still exists (Zitko, 2003a). Technical DDT contains about 85% of *p,p'*-DDT, 15% of *o,p'*-DDT and traces of *o,o'*-DDT and impurities.

Similarly HCB was synthesized in the early 1800s, but its use as fungicide and seeds disinfectant started in 1940, reaching the widest diffusion in the 1970s, when it replaced mercury-based fungicides (Zitko, 2003b). As extensively reviewed by Barber et al. (2005), HCB agricultural application started in the 1950/1960s; the peak of HCB production was reached across the 1980s and the 1990s (about 10000 tonnes/year in 1978-1981); then the environmental levels decreased thanks to restrictions/bans come into force in many countries. Nowadays HCB release is low and mainly accidental, mainly as by-product or impurity of several chemical industrial processes (chlorinated solvents, chlorinated aromatics, pesticides). However HCB-contaminated soils and sediments still represent sources of contamination.

HCH is an insecticide used since the 1950s on fruits, vegetables, rice paddies, animals and for seeds treatment. It was used also for medical purposes, in particular for the treatment of lice and scabies. Among the 8 isomers produced via benzene photochlorination, identified by the Greek letters $\alpha$, $\beta$, $\gamma$, $\delta$, $\varepsilon$, $\eta$ ,$\theta$, only $\gamma$-HCH has great potential as pesticide. $\gamma$-HCH was commercially sold both in technical mixtures, containing all isomers in different ratios (60-70% $\alpha$, 5-12% $\beta$, 10-12% $\gamma$, 6-10% $\delta$, and 3-4% $\varepsilon$), and in refined products (pure $\gamma$-HCH), traded under the name of *Lindane* (Walker et al., 1999).

DDT environmental residues still represent sources of exposure: they are particularly high in developing countries, where DDT is still used to subdue malaria (Beard, 2006). Contrarily, HCB presence in the environment is closely related to the level of industrial development, as for the case of PCB; so the highest concentrations are recorded in the northern hemisphere.

With regard to HCH, its employment was particularly encouraged by the low cost of the commercial products, which allowed its use also in developing countries. Lindane, the refined product, was used mainly in the northern hemisphere, because more expensive. Once release in the environment, HCH is subject to spreader diffusion compared to other organochlorine pesticides and POPs in general. In fact, thanks of its medium vapour pressure and long half-live in atmosphere, HCH can be transported across long distances, explaining its presence at relatively high concentrations in Arctic and Antarctic regions. The most common isomers in environmental samples are α– and γ-HCH. In particular the former is predominant in air and oceans. To explain the high α/γ ratio, which is inconsistent with the bulky use of γ-HCH, several hypotheses have been suggested. According to the most supported, during the long range transport HCH isomers undergo global distillation or photoisomerization, explaining the predominance of α-HCH on γ-HCH. Other theories refer to differential biodegradation, bioisomerization in soils and sediments,. Differently β-HCH is generally found in soil and animals' tissues and fluids: in fact it is the most abundant isomer in human tissues thanks to its equatorial configuration, which favours storage and biotransformation resistance (β-HCH bioaccumulation factor is nearly 30 times higher that of γ-HCH). (Walker et al., 1999)

#### 1.2.3.2 PHYSICAL-CHEMICAL PROPERTIES AND ENVIRONMENTAL BEHAVIOUR

The properties that highly influence the environmental behaviour of organochlorine pesticides are the extremely low solubility in water and the low vapour pressure. This yields high values of Henry's law constant, indicating high tendency to wide environmental distribution. Among the pesticides considered in the present studies, HCH is featured by the highest solubility and vapour pressure, explaining its presence both in atmosphere as gas, and in aquatic environments (dissolved rather than adsorbed onto particles) (Walker et al., 1999)

OCP values of octanol/water coefficient clearly indicate high lipophilicity: in aquatic environments these compounds are strongly adsorbed to organic carbon, thus the concentration of dissolved and insoluble organic matter influences respectively the solubility in water column and the accumulation in sediments. The high solubility in lipids is responsible for the bioaccumulation on the biota and the magnification along the food chain. Focusing on marine ecosystems, even if OCP residues are found at all levels of food chain, the highest concentrations occur in aquatic birds and marine mammals, which are at the top

of the food chain and have high lipid content, besides low capability of metabolizing foreign compounds. (Zitko, 2003)

Focusing on HCH, the eight isomers, which differ from each others for the axial and equatorial position of chlorine atoms, have different physical and chemical properties. With regard to the two isomers considered in the present study, γ-HCH and the two enantiomers of α-HCH, it is worth mentioning that γ-HCH is slightly less lipophilic than α-HCH, and its Henry's law constant value is twice as low as that of α-HCH, indicating its minor tendency to both bioaccumulate in tissues and partition to the air (Walker et al., 1999).

Consistently with their success as industrial pesticides, OCP, particularly DDT, are featured by high persistence in the environment, which reduces the need of frequent applications. For example, Mackay et al. (1992) estimated HCB half-lives of 1,9 years in air and 6,3 years in water and sediments (Barber et al., 2005).

DDT degradation proceeds via dehydrochlorination; under methanogenic conditions, the degradation product DDE is hydrogenated to DDD, which has itself insecticide potential (Zitko, 2003a). HCB degradation in the environment proceeds via bacteria reduction to pentachlorobenzene (QCB), which can further decompose into tetra-, tri-, di- and chlorobenzene.

#### 1.2.3.3 TOXICITY

Even if OCP are present in every environmental compartments and the uptake can proceed through all exposure routes, ingestion of contaminated food is the most effective for the organisms at the highest levels of food chain. Being at the top of the ecological pyramid, human beings are exposed to the risk associated to pesticides exposure as well. Beard et al. (2006) reviewed the studies about the epidemiological evidences of possible effects of human exposure to DDT. Even if methodological limits did not allow to establish certain relationships between DDT exposure and several diseases, evidences suggested a possible role of DDT in reproductive disorders (*i.e.* spontaneous abortion, reproductive abnormalities, hypospadias, undescended testes), pancreatic cancer and neuropsychological dysfunctions.
As regard to metabolizing processes, the transformation of DDT to DDE via dehydrochlorination has a key role in DDT detoxification and the efficiency of this reaction is related to the development of resistance in insects. It seems that the reaction rate increases from invertebrate to fish, birds and mammals (Zitko, 2003a). In mammals HCB is metabolized and excreted extremely slowly. The major HCB metabolites are QCB and pentachlorophenol. The pattern of biotransformation consists in a firstly conversion of HCB into glutathione conjugates, and then in cysteine conjugates and mercapturic acids; then these compounds are transformed into pentachlorothiophenol and finally into QCB and pentachlorophenol (Zitko, 2003b).

Pesticides tend to be very reactive compounds, forming covalent bonds with various nucleophilic centres of cellular biomolecules, including DNA. Furthermore it was also showed that several pesticides induce the formation of reactive oxygen species (ROS) which may be involved in the production of DNA-single strand breaks (Poletta et al., 2009). About their carcinogenic potentials, according to US EPA classification system α-HCH and HCH technical grade are located among the *probable human carcinogens* (Group B2), whereas γ-HCH in B2/C (Group C: *possible human carcinogens*). Differently IARC locates α-, β-, γ-HCH in Group 3 (not classifiable as human carcinogens).

Among the OCPs considered in the present study, DDT and the relative degradation products are noted for their endocrine-mediated toxicity. Following the numerous evidences of sexual and reproductive abnormalities in humans and wildlife associated to DDT exposure (*i.e.* cryptorchidism and hypospadias), the properties of DDT and degradation products as endocrine disrupters have been extensively assessed. These studies confirmed their potential as estrogenic chemicals and their involvement in phenotypic alterations observed in both males and females. Mechanistic studies have revealed that the estrogenic potential might result from different modes of action, mediated at the level of both androgen and estrogen receptors. In fact the estrogenic-like effects of the major and persistent DDT metabolite, *p,p'*-DDE, seem to be mediated at the level of androgen receptor rather than estrogen receptor: Kelce et al. (1995) demonstrated that *p,p'*-DDE inhibits androgens from binding androgen receptors, causing a decrease of androgen-induced transcriptional activity and carrying consequences in developing, pubertal and adult male rats. Differently o*,p'*-DDT, which in rat exposure experiment is responsible of estrogenic effects in female specimens (*i.e.* increased uterine weight) acts through ER binding.

### 1.2.4 OTC

Organotin compounds (OTC) are a class of metallorganic molecules featured by the presence of an atom of tin ($Sn^{4+}$) bound to alkyl or aryl groups. They are represented by the general formula $R_{(4-n)}SnX_n$ (R= alkyl/aryl group, X=anion, usually halides, oxide, hydroxyl, acetate). It is a very wide class, made up by more than 800 compounds, whose physical-chemical properties, biological activity and environmental behaviour largely depend on the alkylation/arylation degree (Hock, 2001).

#### 1.2.4.1 SOURCES AND ENVIRONMENTAL PRESENCE

Even thought OTC were synthesized since the beginning 1950s, they found no relevant applications until the 1940s. In this decade, consistently with the growing of plastic industry, there was an incremental employment of mono- and dialkyltin compounds as heat- and light-stabilizers in PVC processing. Since the late 1950s, thanks to the discovery of their biocidal properties, trisubstituted organotins (mainly tributyltin, triphenyltin, tricyclohexyltin) have been used as timber preservatives, fungicides, molluscicides, nematocides, ovicides, rodent repellents and antifouling paints (Hoch, 2001).

The concern over OTC environmental presence almost entirely refers to the congeners employed as biocides, in particular tributyltin (TBT) and triphenyltin (TPhT) (dibutyltin (DBT), monobutyltin (MBT), diphenyltin (DPhT), monophenyltin (MPhT)). Regarding marine environments, since the 1970s coastal areas have been affected by the massive presence of TBT, used in antifouling paints, and its degradation products (DBT and MBT). The new antifouling products, more effective and durable than the previous $Cu_2O$-based, had a worldwide distribution and use. The marine presence of DBT and MBT has been mainly due to TBT–microbial and photochemical degradation. TPhT, in the form of hydroxide and acetate, has been employed as pesticide and fungicide in agriculture since the 1960s, as well as in antifouling paints in combination with TBT (Schulte-Oehlmann et al., 2000).

Because of clear evidences of detrimental effects caused by TBT on marine ecosystems, the production and use of TBT underwent progressively stricter limitations until the global ban in 2008. Consequently environmental levels of butyltin compounds (BT), which peaked between the 1970s and 1980s, started to decrease in the late 1980s and early 1990s leading to clear signs of recovery (decrease of TBT environmental concentrations and improvement of heath conditions in the wildlife). Nowadays TBT environmental residues continue to be high in the areas close to source points (harbours, shipyards, high density shipping places, etc): in fact, due to the low mobility, OTC pollution pattern almost matches the distribution of OTC sources.

#### 1.2.4.2 PHYSICAL-CHEMICAL PROPERTIES AND ENVIRONMENTAL BEHAVIOUR

Because of the strength of the covalent bind Sn-C, TBT and TPhT are rather insensitive to degradation by water, atmospheric oxygen and temperature below 200°C. Degradation proceeds through progressive loss of the alkyl/aryl groups and it is mediated by biological cleavage (by bacteria and microalgae) and UV photolysis.

OTC half-life in marine environments are highly variable, depending on several parameters, as pH, temperature, depth, turbidity, light and concentration of suspended organic materials: the values reported by the literature range between few days and some months for TBT, whereas they are generally lower for TPhT, because of the greater sensitivity to UV radiations (Steward and De Mora, 1990; Watanabe et al., 1992; Hock, 2001).

OTC distribution within the marine compartments is highly influenced by their hydrophobic character, which can be partially influenced by the environmental pH, ionic strength and temperature (Rüdel, 2003). Because the solubility in sea water increases with decreasing alkylation degree, TBT is slightly less soluble than the degradation products (Alzieu, 1998; Rüdel, 2003). Especially in marine environments, where the hydrophobic hydroxyl species are prevalent on the relative cations, TBT tend to form complexes with the carboxyl-, amino- and thiol- groups of the dissolved organic matter and adsorb on suspended particulate matter and sediment (Fent, 2003). Arnold et al. (1998) computed that, depending on the nature and concentration of dissolved organic matter, a fraction, ranging between 10% and 70% of total OTC, is bound to the dissolved organic matter; clay minerals and metal oxides, which are negatively charged, represent efficient adsorbents in sediment.

Once in the sedimentary compartment, the half-life of TBT is in the range of years (1-5 years). The fraction bound to the sediments represents a potential contamination source because of re-suspension and re-mobilization processes (De Mora and Pelletier, 1997; Hoch, 2001).

TBT is highly accumulated by marine organisms; residues of TBT and degradation products are found along the whole food chain. However the big fraction bound to suspended matter and sediment makes filter- and sediment-feeding/dwelling organisms the most exposed. The highest TBT concentrations are found in molluscs; crustaceans and fishes present lower body residues thanks to their active enzymatic degradation system. At the low levels of the marine food chain both diet and general adsorption from the surrounding play similar roles in TBT bioaccumulation: at the highest levels the main route is represented by the ingestion of contaminated food. Differently from POP, TBT does not appear to significantly biomagnify along the food chain (Maguire, 2000)

#### 1.2.4.3 TOXICITY

As for all environmental pollutants, the uptake by marine organisms and the subsequent toxicity is highly dependent on the chemical speciation (Fent, 2003). The predominance of uncharged species (hydroxyl- and chloride forms) in the sea encourages OTC

bioaccumulation because of their greater capability in crossing the biological membranes (Fent and Looser, 1995; Fent, 1996). However OTC bioavailability is influenced by the concentration of dissolved organic matter: it has been observed that, in presence of humic acids, bioaccumulation diminishes because of the formation of chelates whose polarity and large-size prevent the crossing of biological membranes (Fent and Looser, 1995).

TBT is reported as one of the most toxic marine pollutants: chronic and acute toxic effects on the most sensitive marine species (algae, zooplankton, molluscs and larval stage of fishes) are visible at very low concentrations (1-2 ng/L). The diagnostic signs usually warning about TBT presence in coastal ecosystems are: 1) thickening of shell and failure to spat in oysters of the genus *Crassostrea*, and 2) sexual abnormalities in gastropod species. Furthermore growth retardation in mussels and immunological dysfunction on fish have been also reported (Hock, 2001 and references therein).
Even through genotoxic, cytotoxic and immunotoxic properties of different butyltin compounds have been assessed by several authors (Hagger et al., 2002; 2005; 2006; Inadera et al., 2006; Gabbianelli et al., 2006), great concern about OTC pollution is due to their potential as endocrine disrupters.

The ability of TBT to act as endocrine disrupter is worldwide known by the case of Imposex and Intersex. As explained in Par. 1.5.3.1, Imposex and its variant Intersex are sexual malformations developed by female gastropods living in areas affected by TBT. The phenomenon, discovered in 1971 by Smith (Smith, 1971), was associated to leaks of TBT from antifouling paints in the first 1980s. Subsequent field and laboratory researches confirmed the ability of TBT to induce the phenomenon: a strict correlation was assessed between the mean stage of malformation achieved by a population and TBT environmental level (Bauer at al., 1997).

Imposex phenomenon was used to study TBT mode of action as EDC. About the mechanism of Imposex development, different hypotheses have been suggested: the theories, which agree in explaining the phenomenon as indirect endocrine disruption rather than receptor-mediated hormone mimicking action, basically differ for the diverse role given to steroids and peptide hormones in the induction.

According to some authors, TBT acts as androgen-like compound by interfering with the metabolism of testosterone. In particular Spooner et al. (1991) and Stroben et al. (1991) reported that TBT inhibits the ability of cytochrome P450 aromatase to convert androgens into estrogens, enhancing testosterone titres in the tissues; differently Janer et al. (2005) and Ronis and Mason (1996), focusing on the mechanisms that regulate testosterone levels, suggested that TBT interferes with testosterone esterification and sulphur conjugation, preventing testosterone excretion or favouring the predominance of free-forms on conjugate-form. In fact esterification makes hormone apolar, causing its accumulation in adipose

tissues; otherwise TBT seems to block sulphur conjugation of testosterone, preventing excretion.

Completely different is the theory of Féral and LeGrall (1983), which focused on the peptide hormones secreted by pedal ganglia and involved in different physiological and reproductive functions. According to their hypothesis, TBT acts as neurotoxin, inducing the release of a neurohormone, generically indicated as Penis Morphological Factor (PMF), from the pedal ganglia. Once released in the heamolymph, PFM accumulates close to the right tentacle, the site of penis morphogenesis, and it induces the growth of male sexual features. The consequent differentiation of male tissues, in particular prostate and vas deference, is responsible of androgens synthesis, necessary to maintain the "accessory sex organs". Oberdörster and McClellan-Green (2000) identified the APGWamide as PMF: laboratories studies demonstrated that APGWamide, besides accumulating in right haemal cavity, which coincides with PMF concentration site, can induce Imposex as TBT and testosterone.

#### 1.2.4.4 LEGISLATION

Following the increasing evidences of TBT-related detrimental effects on marine wildlife, the production/use of TBT-based antifouling paints underwent to progressively severe restrictions since the mid-1980s. Firstly the prohibition was limited to small boats (less than 25 m in length): France, whose oyster industry suffered heavy economic damages due to TBT pollution, was the first country to adopt legislative measures in 1982. By the end of 1980s, the main industrial countries, such as USA, UK, Australia, New Zealand, Canada, Japan, Hong Kong and most European countries, adopted similar regulations (Antizar-Ladislao, 2008; Champ, 2000). In this time, more precisely in 1988, the environmental quality standard for TBT was fixed at 2 ng TBT/L in UK. Because of the global scale dimension of TBT issue, the International Maritime Organization (IMO) proposed the global phase out of TBT-based antifouling systems starting from the 1$^{st}$ of January 2003, and the total prohibition by the 1st of January 2008[13]. IMO intentions became effective in October 2001, when the International Convention on the control of harmful antifouling systems on ships, noted as AFS Convention (Antifouling System Convention), was adopted. However to come into force, AFS Convention had to be signed by, at least, 25 countries whose total fleet represented 25% of world tonnage. In the mean time Europe, consistently with IMO policy, extended the prohibition of TBT anti-fouling paint to all size boats (Directive 1999/51/CE[14]). In 2003 the

[13] <http://www.imo.org>.

[14] Commission Directive 1999/51/EC of 26 May 1999 adapting to technical progress for the fifth time Annex I to Council Directive 76/769/EEC on the approximations of the laws, regulations, and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations (tin, PCP and cadmium).

European Commission Parliament adopted the Regulation 2003/782/CE[15], which forced EU boats and any boats working under the European authority or landing to a European port/off-shore terminal to respect AFS requirements.

AFS convention was ratified the 17th of September 2007, definitely sanctioning the global ban of TBT antifouling paints.

[15] Regulation (EC) No 782/2003 of the European Parliament and of the Council of 14 April 2003 on the prohibition of organotin compounds on ships.

## 1.3 POLLUTION RELATED STRESSES

The concern about environmental pollution is related to the possible toxic effects that xenobiotics might exert on the wildlife and, ultimately, on humans (Bowen and Depledge, 2006). The mechanisms through which xenobiotics may exert their toxic actions are thousands, and it is beyond the scope of the present thesis to illustrate them. However it is possible to generalize that usually xenobiotics, or their active metabolites, act contemporarily through several pathways; their initial subcellular targets are 1) elements making part of the membranes systems, 2) components of the machineries for the production of energy and synthesis of macromolecules and 3) cellular redox homeostasis system.

The toxic action of many environmental pollutants depends on their non-selective reactivity against cellular structures (*i.e.* electrophilic metabolites against nucleophilic molecules); however it is known that many contaminants can act through specifically binding cellular components, such as enzymes, receptors, etc.

Cellular injuries might be likely prevented by detoxification mechanisms or solved by repairing mechanisms. However, when these systems fail on their functions, cellular lesions may become persistent and cause detrimental consequences at the higher levels of biological organization. In fact, depending on their extent, they can turn into tissues dysfunction and physiological changes, up to population impairments (Vasseur and Cossu-Leguille, 2006; Cajaraville et al., 2000).

Among the toxic mechanisms which have received particular attention from aquatic toxicologists there are genotoxicity, endocrine disruption and oxidative stress. This is likely motivated by two important reasons: 1) the key-role of these mechanisms in the toxicity of the main classes of aquatic pollutants, 2) the high risk that the initial injuries mediated by these mechanisms give rise to effects at the higher organizational levels.

### 1.3.1 GENOTOXICITY

Genotoxic agents can be functionally defined as having the ability to alter DNA replication and genetic transmission. They can be classified into physical and chemical agents, the former including, for example, UV radiation and X-ray, the latter a variety of organic and inorganic compounds. Basically genotoxic lesions consist in DNA structure alterations, which, if not efficiently repaired, can turn into gene damage, chromosome damage, genome damage, causing important consequences up to the highest levels of biological organization (Fairbairn et al., 1995).

#### 1.3.1.1 DNA DAMAGE

Genotoxic lesions can be classified into five typologies: adducts, strand breaks, modified bases, DNA-DNA crosslink, DNA-protein crosslink (Table 1.4). All of them are promutagenic lesions, meaning that, if not correctly repaired before replication, they can turn into mutations (Ross and Nesnow, 1999).

**Table 1.4 DNA structural modifications caused by genotoxicants (modified by Shugart, 2000)**

| | |
|---|---|
| **ADDUCTS** | Structural modification of DNA resulting from the covalent attachment of a chemical, or its active metabolite, usually via a base moiety. |
| **STRAND BREAKS** | Single or double strand breaks occurring when chemical bonds are cleaved along one or both backbones respectively. Besides being associated to physical-chemical agents, strand breaks can be also due to apoptosis, necrosis and excision-repair enzymatic processes (Lee and Steinert, 2003). |
| **MODIFIED BASES/ABASIC SITES** | Lesions usually arising from chemical modifications of existing bases (*i.e.* DNA alkylation and oxidation) and loss of chemically instable adducts or damaged bases. |
| **DNA CROSSLINKS** | DNA–protein crosslinks generated by protein covalent binding to DNA; DNA-DNA cross-link occurring when exogenous or endogenous agents react with two different positions in the DNA (Brendler-Schwaab, et al., 2005). |

A transient DNA population carrying these genetic injuries is regularly present under normal conditions. This background level of DNA damage is efficiently countered by DNA system repair or cellular death. However the concern about the possible transformation of these lesions into irreversible consequences increases when exogenous agents enhance the extent of the phenomenon (Shugart, 2000).

#### 1.3.1.2 GENOTOXIC MECHANISMS

Focusing on chemical genotoxicity, even if genotoxic compounds commonly damage DNA through multiple mechanisms, they can be virtually divided in two groups, those having affinity for direct interaction with DNA and those acting indirectly (Lee and Steinert, 2003).

Basically direct mechanisms are based on the interaction between the active compounds and genetic material. The complete list of direct genotoxicants is reported in Lee and Steinert (2003): briefly it includes alkylating agents, hydrogen peroxide and common herbicides (atrazine, metalochlor, glyphosate, metribuzin, linuron).

Differently, the main indirect mechanisms depend on metabolic activation and ROS formation, or they consist in inhibition of DNA synthesis/repair mechanisms. Metabolic activation processes generally lead to the formation of electrophilic metabolites which can bind to nucleophilic DNA molecules producing a variety of DNA lesions (Lee and Steinert, 2003). As extensively illustrated for PAH, which are usually taken as model for metabolic activated-genotoxic pollutants, several activation pathways can be described, mainly consisting in MFO-mediated reactions and ionization (Xue and Warshawsky, 2005). Ionization processes can be enforced by the presence of light: photoinduced toxic mechanisms basically consist in photoionization yielding cation radicals able to bind DNA and generate ROS (Lee and Steinert, 2003). This mechanism explains the increase of DNA damage extent usually records in presence of light, particularly visible in studies with aquatic organisms. For genotoxicity mediated by ROS formation refer to Par. 1.3.2.

As regard genotoxicity mediated by inhibition of DNA synthesis and repair, the chemicals that have been shown to inhibit DNA synthesis are hydroxyurea, cytosine arabinoside, ethidium bromide, 5-fluorouracil; those able to disrupt DNA repair system (by inhibition of one or more DNA polymerases) are aphidicolin, novobiocin and several heavy metals (cadmium, mercury, arsenic) (Dixon et al., 2002).

#### 1.3.1.3 ADVANCED STEPS OF GENOTOXICITY

As previously mentioned, all forms of DNA damage are promutagenic lesions, meaning that, if not correctly repaired, they can turned into mutations and chromosomal alterations, giving rise to serious consequences, such as tumours and alteration of gene-coded proteins synthesis (Ross and Nesnow, 1999; Poletta et al., 2009; Shugart, 2000). The persistence of DNA damages in cells depends upon different factors, such as the ability of the organisms to repair its damaged DNA, the temporal extent of contact with the genotoxicants (acute or chronic exposure), the dose etc. However, even if pollution-related genotoxic damages are typically *single DNA lesions*, which are easier to repair compared to that induced by physical agents (*i.e.* UV radiation), characterized by both single and double strand breaks in more than one moiety in the DNA, cytogenic alterations have been assessed following exposure to chemicals. The probability that genotoxic alterations will cause aberrant modifications grows

if the exposure occurs during embryogenesis, when rapid organogenesis is in progress (Poletta et al., 2009; Lee and Steinert, 2003). Differently chromosome alterations are more strictly associated to malignancy: mutations and cytogenic anomalies are usually found in tumour cells, and, because of this relationship, specific cytogenic endpoints are usually included in the toxicological evaluation of industrial, pharmaceutical and environmental chemicals (Tucker and Preston, 1996).

### 1.3.2 OXIDATIVE DAMAGE

Oxidative stress is defined as a state of unbalanced tissue oxidation, involving enhanced intra- and extracellular ROS production, typically associated to lipids peroxidation, and oxidation of proteins and DNA. The mechanisms involved in oxidative damages and cellular antioxidant defence are highly conserved, so several aspects of oxidative stress are common to biological systems (Valavanidis et al., 2006; Di Giulio et al., 1995).

#### 1.3.2.1 REACTIVE OXYGEN SPECIES (ROS)

Under the definition of *Reactive Oxygen Species* (ROS), several compounds are included. They can be classified into two different subgroups (Livingstone, 2001):

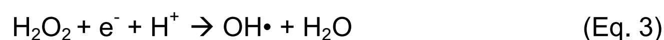
1. Oxygen free radicals:
    - hydroxyl radical (OH•); superoxide anion radical ($O^-_2•$);
    - peroxy radicals (ROO•);
    - nitrogen oxide (NO•);
    - alkoxy radical (RO•);
    - hydroperoxyl radical ($HO_2•$).
2. Nonradical reactive species:
    - hydrogen peroxide ($H_2O_2$);
    - hypochlorous acid (HOCl);
    - singlet oxygen.

A fraction of ROS production is integral part of metabolic processes and is essential for the maintenance of the redox homeostasis. Among the endogenous sources there are (Winston et al., 1998; Di Giulio et al., 1989):

- *Mitochondrial reduction of $O_2$ to $H_2O$*. Animals with aerobic metabolism use molecular oxygen as terminal electron acceptor in mitochondrial respiration (oxidative phosphorylation of ADP to ATP through reduction of $O_2$ to $H_2O$). In the four steps-reduction of $O_2$ to $H_2O$, which consume more than 90% of cellular oxygen, ROS are generated as partially reduced intermediates (Eq. 1, 2, 3, 4, 5).

$O_2 + e^- \rightarrow O^-_2•$ (Eq. 1)

$O^-_2• + e^- + 2H^+ \rightarrow H_2O_2$ (Eq. 2)



$$H_2O_2 + e^- + H^+ \rightarrow OH\bullet + H_2O \qquad \text{(Eq. 3)}$$

$$OH\bullet + e^- + H^+ \rightarrow H_2O \qquad \text{(Eq. 4)}$$

$$\text{Sum} \quad O_2 + 4e^- + 4H^+ \rightarrow 2\ H_2O \qquad \text{(Eq. 5)}$$

It has been calculated by *in vitro* experiments that almost the whole consumed oxygen is regularly reduced to water and about the 1-3% is turned into ROS (Livingstone, 2001; Abele and Puntarulo, 2004). In fact during the mitochondrial transfer of electrons to oxygen, the superoxide anion ($O^-_2\bullet$) can be produced as spin-off and, subsequently, converted into more aggressive oxygen species.

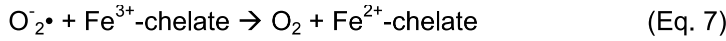
Besides the mitochondrial electron transport, also microsomal and photosynthetic electron transport are sources of ROS.

- *Active phagocytosis.* ROS production by active phagocytosis is particularly visible in leucocytes. It takes place mainly in lysosomes, the organelles involved in cellular digestion. In invertebrates studies, Winston et al. (1996) demonstrated that the lysosomes of mussels' haemocytes actively generate ROS. However, in addition to the fraction actively produced for digestion of endocytosed pathogens, part of lysosomal ROS production has to be associated to the reactivity of redox cycling xenobiotics stored in lysosomal compartment (for more details refer to Par. 1.3.4.1).
- *Activity of several enzymes.* Among the enzymes noted for producing ROS there are xanthine oxidase, tryptophan dioxygenase, glucose oxidase, diamine oxidase and prostaglandin synthase.

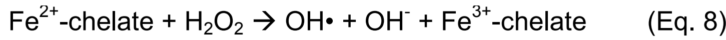
As illustrated in Figure 1.4, a portion of ROS production can be interrelated. In fact $O^-_2\bullet$ can be reduced to $H_2O_2$ both spontaneously and by SOD-mediated reaction. $H_2O_2$ is more mobile in the cell, thanks to its capability to diffuse through the membranes. If not rapidly decomposed, $H_2O_2$ can be converted into OH•, which, despite its short lifetime, is the most harmful free radical because of its higher oxidative potential and indiscriminate reactivity against cellular components (Valavanidis et al., 2006; Abele and Puntarulo, 2004). The generation of OH• from $H_2O_2$ occurs according to following reaction (Eq. 6), noted as Haber-Weiss reaction (Abele and Puntarulo, 2004):

$$O^-_2\bullet + H_2O_2 \rightarrow OH\bullet + O_2 \qquad \text{(Eq. 6)}$$

Being a kinetically discouraged, this reaction proceeds very slowly. However if catalyzed by transitional metals such as metal cations of cell membrane and DNA, Haber-Weiss reaction becomes faster. Equations 7 and 8 represent the iron-catalyzed Haber-Weiss reaction whose sum provides Eq. 6. The second equation (Eq. 8) is noted by the name of Fenton reaction (Valavanidis et al., 2006; Di Giulio et al., 1989).



$O_2^{-}\bullet + Fe^{3+}\text{-chelate} \rightarrow O_2 + Fe^{2+}\text{-chelate}$ (Eq. 7)



$Fe^{2+}\text{-chelate} + H_2O_2 \rightarrow OH\bullet + OH^{-} + Fe^{3+}\text{-chelate}$ (Eq. 8)

### 1.3.2.2 OXIDATIVE DEFENCE

To maintain the redox balance and protect the cell against ROS, a complex defence system was set up during evolution, involving enzymatic and non-enzymatic antioxidant mechanisms (Winston et al., 1998). The antioxidant defence is generally ubiquitous in different tissue-types of aerobic animals. As important adaptation, it is highly responsive to oxidative stress agents, such as redox-active chemicals and particular environmental changes (UV radiation, $O_2$ levels) (Di Giulio et al., 1989; 1995). In accordance with the results of many studies, in aquatic organisms the anti-oxidative defence is made up by (Livingstone, 2001; Winston et al., 1998; Di Giulio et al., 1989; Valavanidis et al., 2006):

- Low molecular weight antioxidants:
    1. water-soluble reductants: ascorbic acid (vitamin C), glutathione (GSH), uric acid;
    2. fat-soluble radical scavengers: α-tocopherol (vitamin E), β-carotene (vitamin A).
- Antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), DT-diaphorase (DTD).

The way through which antioxidant enzymes cooperate in antioxidant defence is illustrated in the following figure (Figure 1.4).

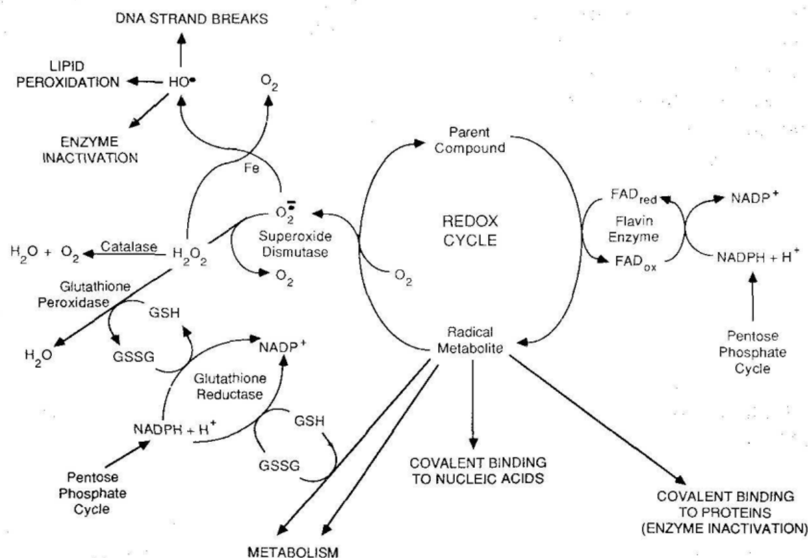
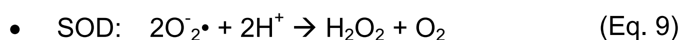
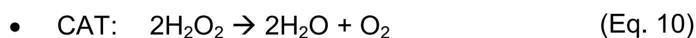



**Figure 1.4 Overviews of redox cycling summarizing free radicals production, antioxidant defence action and toxicological consequences (Di Giulio et al., 1989)**

The specific reactions catalyzed by each antioxidant enzymes are reported below (Di Giulio et al., 1995):



- SOD: $2O^{-}_{2}\bullet + 2H^{+} \rightarrow H_2O_2 + O_2$ (Eq. 9)



- CAT: $2H_2O_2 \rightarrow 2H_2O + O_2$ (Eq. 10)

- GPX: $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ (Eq. 11)

SOD, which is typically found in cytosol, mitochondria and chloroplasts, is considered the most active antioxidant enzymes; it is often induced together with CAT, an enzyme primarily associated to peroxisomes, which removes SOD products.

GPX is a cytosolic enzyme. It catalyzes the reduction of both hydrogen peroxide and organic peroxides (R-OOH) to, respectively, water and the corresponding stable alcohols (R-OH), using cellular glutathione (GSH) as reducing reagent. GPX enzymes exist on two forms: GPX containing a selenocysteine moiety in the active site, and non-selenium containing GPX. Selenocysteine has a direct role in the electron donation to peroxide substrate; then reduced glutathione is used as hydrogen donor to regenerate the oxidized selenocysteine.

Although the enzyme glutathione reductase (GR) does not directly participate in the reduction of reactive oxygen species, it plays an essential role in the antioxidant machinery, maintaining cellular levels of GSH which is involved in both antioxidant and detoxification processes (GSH-dependant peroxidise activity, direct scavenging of radicals, phase II conjugation). In fact GR catalyzes the reduction of GSSG to GSH, regenerating cellular reductants stock.

Finally also the enzymes family glutathione S-transferase (GST) contributes to maintain the redox status, supporting the scavenging activity of GSH: in fact the formation of a thiol group of glutathione to electrophilic xenobiotics is catalyzed by GST.

When this system fails to neutralize ROS overproduction, ROS surplus is responsible for the settlement of particular stress conditions, referred as *oxidative stress* (Winston et al., 1998; Katalinic et al., 2005). Besides the antioxidant defence, cells are supplied of specific systems that can repair and remove damaged proteins, lipids and DNA (Valavanidis et al., 2006). In fact the second tier of defence against ongoing oxidative stress is represented by the cellular lysosomal system: through their phagic activity, lysosomes remove damaged proteins, impaired organelles and portions of nucleus and DNA from the cytoplasm. In this way lysosomes prevent cells from impairments due to malfunctioning proteins and waste accumulation. Furthermore the autophagic activity of lysosomes can provide also a third tier of defence, consisting in cell death, which represents the extreme solution to maintain tissue-organ integrity (Moore et al., 2006).

#### 1.3.2.3 REDOX UNBALANCE: OXIDATIVE STRESS AND OXIDATIVE LESIONS

If the cellular oxidative defence is overwhelmed, ROS accumulate in the cell, representing a threat for organism's health (Valavanidis et al., 2006; Katalinic et al., 2005). Because the reactivity of different ROS varies considerably, it is not possible to conclude that ROS are equally dangerous for cells. The most harmful is OH•, which is featured by instantly and indiscriminately reactivity against almost all organic molecules. However the presence of the other oxygen species is source of concern as well considering their potential to be converted into hydroxyl radicals (Par. 1.3.2.2) (Livingstone, 2001).

The wide range of cellular injuries caused by ROS are comprehensively referred to as *oxidative damage*. In particular the primary biochemical and physiological events are (Winston et al., 1998, 1996; Regoli and Winston, 1999; Abele and Puntarulo, 2004; Di Giulio et al., 1989; 1995; Valavanidis et al., 2006):

- *Lipid peroxidation*. Lipid peroxidation is a chain reaction mainly involving polyunsaturated fatty acids, initiated by hydroxyl radicals and highly favoured by the presence of transition metals. Direct consequence of this process is the loss of permeability and integrity by the membranes, which become rigid. Furthermore many lipids peroxidation products, such as lipid epoxides and lipid alkoxyl radicals, may induce mutagenesis via adducts formation.
- *Protein oxidation.* This process leads to modifications in amino acid side chains, ROS-mediated peptide cleavage, reactions between peptides and oxidation products of lipids and carbohydrates. The phenomenon is related to aging processes.
- *DNA damages*. Superoxid anions and hydroxyl radicals are known to be genotoxic. In particular OH•, once formed, instantly reacts with DNA at the level of metal cations adhering to the polyanionic DNA structure (Fenton reaction, Eq. 8). They preferably attack nucleobases and the sugar-phosphate backbone (the former 5 times faster than the latter). Typical products of nucleobases oxidation are 8-hydroxy-2'-deoxiguanosine (8-OHdG) and its oxidation product 8-oxo-2'-deoxiguanosine (8-oxodG); in the case of radical attack to sugar-phosphate backbone, typical lesions are apurinic sites, single-strand breaks and oxidation of sugar moiety. These lesions might have an important role in mutagenesis and carcinogenesis.
- *Cellular redox unbalance*. Considering that many important cellular processes depend on redox couples (*i.e.* GSH/GSSG, NADH/NAD), the alteration of these ratios can lead to important cytotoxic consequences (*i.e.* GPX activity, responsible of the $H_2O_2$ deactivation, depends on the ratios GSH/GSSG and $NADPH_2/NADP^+$.

- *Enzymes inactivation.* Enzyme inactivation is generally due to attack to thiol groups (-SH).

---

Thus these primary oxidative damages can impair the cellular functioning by disrupting particular mechanisms and constituents. As explained in Par. 1.3.4.11.3.4, lysosomes are one of the most sensitive targets of oxidative stress due to ROS production both inside and outside the membrane (Bocchetti et al., 2002).

Protracted conditions of oxidative stress have been linked to different pathologies in humans, such as reperfusion injuries, inflammation, degenerative diseases, carcinogenesis, heart diseases, rheumatoid arthritis, aging, atherosclerosis, pathogenesis of many reproductive processes (Valavanidis et al., 2006; Winston et al., 1998; Regoli and Winston, 1999; Zhou et al., 2006; Katalinic et al., 2005 and references therein). Regarding aquatic organisms, the literature mostly indicates relationships between oxidative stress and decreased growth, respiratory and acid-base pathophysiology, carcinogenesis (Livingstone, 2001 and references therein).

#### 1.3.2.4 EXOGENOUS ROS SOURCES

Besides the physiological production of ROS, many processes can dangerously enhance ROS supply in the cells. These are mainly related to the exposure to a wide range of xenobiotics and to extreme environmental conditions (UV-radiation, hypoxia, hyperoxia). Many field studies carried out on natural and transplanted populations of *sentinel organisms* have shown that the exposure to poor quality environments leads to a reduced capability of the organisms to deal with free radicals, making them more susceptible to oxidative stress-related diseases (Bacchiocchi et al., 2002). Among the chemical sources of oxidative stress there are (Winston et al., 1996; Livingstone, 2001; Di Giulio et al., 1989):

- Redox cycling compounds (quinones, nitroaromatics, nitroamines, bipyridyl herbicides);
- PAH;
- Halogenated hydrocarbons (bromobenzene, dibromomethane, PCB, lindane);
- Dioxins, pentachlorophenol;
- metal contaminants (Al, Ar, Cd, Cr, Hg, Ni, Va).

These contaminants can stimulate ROS production through different direct and indirect pathways, or they can impair some mechanisms involved in redox balance (Di Giulio et al., 1989). Particularly harmful are the pollutants inducing redox cycles catalyzed by NAD (P)H-dependent reductases. As showed by Figure 1.4**,** in these proceses the xenobiotic is reduced to its corresponding radical metabolite by NAD(P)H-dependent reductases reaction;

the so generated xenobiotic radical donates its unpaired electron to $O_2$, yielding superoxide radical anion and regenerating the parent compound which can start the cycle again.

### 1.3.3 ENDOCRINE DISRUPTION

Endocrine disruption hypothesis was suggested in 1991 by Dr Theo Colburn during Wingspread Conference in Racine (USA). It referred to the capability of several pollutants to interfere with the functions of the endocrine system. Afterwards the thesis was enforced by numerous evidences of developmental and reproductive abnormalities in wildlife, correlating the presence of one or more suspected endocrine active pollutants (Orlando and Guillette, 2002). Among the historical cases of endocrine disruption in wildlife, the most famous refer to 1) feminized male fishes in British rivers which received sewage treatment effluents containing the degradation products of detergents, natural estrogens and residues of oral contraceptive pills; 2) cryptorchidism in Florida panthers, 3) small penis in alligators of Apopka Lake (USA) associated to spills of kelthane, a pesticide formulation containing DDE, 5) sexual malformations in polar bears, and 6) masculinisation of female gastropod molluscs caused by TBT leaking from antifouling paints (Imposex-Intersex development) (Sonnenschein and Soto, 1998 and references therein).

Generally *endocrine disrupters* (*EDs*, or *endocrine-disrupting chemicals*, *EDCs*) are defined as exogenous compounds which elicit effects on organisms, or on their progeny, by interfering with the endocrine system (Gillesby and Zacharewsky, 1998; McKinlay et al., 2008). As shown in Table 1.5, EDCs constitute a structurally heterogeneous group of chemicals having different origin, both natural and anthropogenic; this group can be split into different subclasses (estrogen-like, androgen-like, antiestrogen-like, antiandrogen-like, see Par.1.3.3.2) according to their specific mode of action, in particular to their capability of mimicking/inhibiting the activity of particular hormones.

The scientific and public concern about the wide diffusion of chemicals able to interfere with the endocrine system is easily understandable considering the crucial role of hormones in regulating many body functions (*i.e.* growth, development and reproduction) and the irreversible developmental defects which might arise from the exposure to EDCs during organogenesis (Sonnenschein and Soto, 1998). For humans, the threat associated to the exposure to endocrine active compounds, especially during the *in uterus* development, was worldwide evident from the historical case of DES therapy. The consumption of synthetic estrogen diethylstilbestrol (DES) was prescribed to pregnant women in the 1960s in order to reduce spontaneous abortions. The prenatal exposure to DES yielded transgenerational effects in the offspring: during the puberty females developed high incidence of vaginal cancers, uterine structural malformations and cellular changes in the vagina and fallopian tubes, whereas males abnormal pelvic development, genital tract lesions and gonadal changes (Orlando and Guillette, 2001 and references therein).

The research interest about environmental endocrine disrupters grew in the last decade because of the evidences that many everyday chemicals have hormonal activity. In fact it was suggested that the environmental distribution of these chemicals might be related to increasing incidence of sexual, developmental, reproductive abnormalities in wildlife and humans, such as decreased sperm count, increasing cases of hypospadias, cryptorchidism, testicular cancer earlier age at puberty, increasing incidence of endometriosis and breast cancer.

#### 1.3.3.1 BASICS ON STEROID HORMONE SYSTEM

The research on environmental endocrine disrupters mainly focuses on compounds interfering with steroidal hormones and, secondarily, with thyroidal hormones.

Steroid hormones play the major role in the regulation of development and homeostasis, and stimulate growth and differentiation of their target cells. Their actions are mainly mediated by binding specific hormone-receptors and basically consist in the activation of hormone-related gene transcription.

Steroid hormone receptors belong to a structurally related-nuclear receptor superfamily. They have a modular structure made up by distinct functional domains, including DNA-binding domain and ligand-binding domain. Even thought their structure is highly conserved, explaining the similar modes of action of different hormones, amino acid sequence variations in the ligand-binding domains are responsible for the ligand selectivity and hormone binding affinity (Mueller and Korach, 2001; Kelce and Wilson, 2001).

Basically the mode of action of steroid hormones can be summarized by the following steps (Mueller and Korach, 2001; Kelce and Wilson, 2001):

1) *Hormone transportation to the target cell*. Hormones, produced by specific endocrine glands, are transported to the target-organs by the bloodstream system and enter the cell by passive diffusion through the lipid membrane;
2) *Receptor binding.* Inside the cell, hormones bind specific receptor proteins located within the nucleus (estrogen receptor (ER) and androgen receptor (AR));
3) *Activation of the complex hormone-receptor.* After the hormone–receptor binding, the proteins associated to the receptor, which are responsible of its inactive state (heat shock proteins (Hsp90 and Hsp70) and immunophilins), dissociate, allowing the complex to take its active conformation;
4) *Dimerization*. The activated complex dimerizes forming dimers which seek out specific DNA motifs, called *hormone response elements* which are located upstream of hormone-responsive genes;

5) *DNA binding*. The dimer binds the DNA hormone response element, resulting in chromatin rearrangement which increases the access of the cellular transcriptional machinery to the promoter region of hormone-inducible genes;

6) *Hormone-related gene transcription.*

It is worth mentioning that, besides the chromatin rearrangement, other factors influence mRNA synthesis and the subsequent protein expression. In particular an additional family of transcriptional co-activators interacts with the DNA binding-receptor complex to assist the gene activation. Thus endocrine disruption potential is not merely function of its affinity for hormone receptors, but it depends on the complex interaction of the ligand-receptor with the complete transcription machinery and other signal pathways.

#### 1.3.3.2 EDCS

Endocrine-disrupting chemicals can elicit their effects either directly, by binding the hormone receptors and interfering in the hormone-related gene expression, or indirectly, by disrupting the biochemical processes which regulate the production, availability and metabolism of hormones, or by influencing the hormone receptor levels. Both of these typologies can be further characterized according to the nature of the effects induced. In particular, within the former group it is possible to distinguish *hormone agonists*, which induce hormone-mediated responses (oestrogen EDCs, androgen EDCs), and *hormone antagonists*, which inhibit/prevent the hormone-mediated responses (anti-estrogen EDCs and anti-androgen EDCs); similarly, in the later group EDs can be classified as *estrogen-like compounds* and *androgen-like compounds* (Sonnenschein and Soto, 1998). Because the endocrine disruption potential of xenobiotics is not deducible by their chemical structure, the characterization of endocrine activity is carried out by purposely selected suites of bioassays as recommended by US EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee, US EPA[16]). The potential of environmental EDCs to elicit their toxic effects does not depend only on variables strictly related to their mode of action. In fact it can be influenced by several factors, such as the bioavailability of the compounds, time-window of exposure, metabolic processes and toxicological behaviour of complex mixtures.

#### 1.3.3.3 DIRECT EDCS

In the following table (Table 1.5) direct EDCs, having both pharmaceutical and industrial origin, are grouped according to their affinity for a specific steroid receptor. It is worth highlighting that this classification is not strict because the hormone-mediated action depends not only on the nature of receptor ligand, but also on the cellular and genetic context. In fact some EDs, such as Tamoxifen, are known for their tissue-dependant activity:

[16] <http://epa.gov/endo/pubs/edspoverview/edstac.htm>

Tamoxifen was assessed acting as ER agonist in uterus and bone, whereas as ER antagonist in breast (Mueller and Korach, 2001). Another important feature to take into account is the possible role of metabolism in activating/deactivating ED potential. For example, it is noted that hydroxylation enhances the estrogenic activity of PCBs and bisphenol A (Kester et al., 2000).

Pharmaceutical hormone-like compounds are usually derived from natural hormones and they have high intrinsic activity. Contrarily, environmental EDCs are featured by lower intrinsic hormone activity compared to endogenous hormones: generally they have lower binding affinity for the receptors than the respective endogenous hormones. However the concern about their environmental presence is due to their persistence and lipophilicity, which explain their tendency to bioaccumulate and biomagnify in the trophic pyramid (Porte et al., 2006).

**Table 1.5 Natural steroid hormones and direct EDCs (Metzler and Pfeiffer, 2001)**

| | | | | ESTROGENS | | ANDROGENS | |
|---|---|---|---|---|---|---|---|
| NATURAL | | | MAMMALIAN STEROID HORMONES | 17b-Estradiol (E2)<br>Estrone (E1)<br>Estriol (E3)<br>16a-hydroxyestrone<br>Equin<br>Equilenin | | Testosterone and its potent metabolite 5a-dihydrotestosterone (DHT) | |
| NATURAL | | | PHYTOESTROGEN | Isoflavones<br>Lignans | | | |
| ANTROPOGENIC | PHARMACEUTICAL | CHEMICAL CLASSES | USES | ESTROGENS | ANTI-ESTROGENS | ANDROGENS | ANTI-ANDROGENS |
| ANTROPOGENIC | PHARMACEUTICAL | | | 17b-Ethinylestradiol (EE2)<br>Tamoxifen (SERM)<br>Clomiphene<br>Ramoxifen<br>Stilbene-type agents (*i.e.* DES) | ICI 164,384<br>ICI 182,780<br>Tamoxifen (SERM) | Methyltestosterone<br>17b-trenbolone<br>Methyltrienolone (R1881) | Cyproterone acetate |
| ANTROPOGENIC | INDUSTRIAL | **ALKYLPHENOLS** | ANTIOXIDANT AND SYNTHESIS OF DETERGENTS | Nonylphenol<br>Octylphenol | | | |
| ANTROPOGENIC | INDUSTRIAL | **BISPHENOLS** | PLASTIC SYNTHESIS | Bisphenol A (BPA) | | | |
| ANTROPOGENIC | INDUSTRIAL | **POLYCHLORINATED COMPOUNDS** | INSECTICIDES | p,p'-DDT<br>o,p'-DDT<br>Dieldrin<br>Endosulfan<br>Kepone (Chlordecone) | | | p,p'-DDE |

| | | | | | |
|---|---|---|---|---|---|
| ANTROPOGENIC INDUSTRIAL | | | Toxaphene<br>Methoxychlor | | |
| | POLYCHLORINATED COMPOUNDS | INDUSTRIAL WASTE | PCDD<br>PCDF | | |
| | | INDUSTRIAL PRODUCTS | PCB and hydroxy derivates (PCB-OH) | Hydroxy derivates (PCB-OH) | |
| | PHTHALATES (SUSPECTED EDCs) | PLASTICIZERS | 2-etilexil phthalate (DEHP)<br>butyl benzyl phthalate (BBP) | | |
| | OTHER | FUNGICIDES | Benomyl<br>Mancozeb | | Vinclozolin (in particular its metabolite M1 and M2)<br>Procymidone<br>Linuron |
| | | PESTICIDE | | | Methoxichlor (in particular its metabolite HPTE) |
| | | HERBICIDES | Alachlor<br>Atrazine | | |
| | | METALS | Cadmium<br>Lead<br>Mercury | | |

#### 1.3.3.4 INDIRECT EDCS

TBT is probably the most known environmental pollutant which elicits endocrine disruption by interfering with the endogenous hormones metabolism (Par. 1.2.4.3). Several evidences have suggested that TBT exposure inhibits the activity of P450 aromatase in many species, including molluscs, crustaceans; in other species, such as echinoderms, TBT interferes with testosterone sulphotransferase and palmitoyl-CoA-testosterone transferase, whereas in human and rat tissues it inhibits 5$\alpha$-reductase.

Other environmental pollutants, such as the organophosphorous pesticide malathion, nonylphenol polyethoxylates and pentachlorophenol, have been related to alterations in testosterone metabolism; exposures to PCB (Chlorophen 50) and cadmium have shown to decrease the levels of testosterone and progesterone, probably by disrupting the hormones' synthesis or metabolism (Porte et al., 2006).

### 1.3.4 CYTOTOXICITY

Considering that the primary targets of environmental toxicants are subcellular structures and cellular functions, cellular defence system plays an essential role in preventing initial lesions from yielding critical consequences at the highest organizational levels.

Lysosomes have a primarily role in the cellular defence system. Evolutionary conserved, these organelles take part in cellular defence against environmental stresses and diseases, and in a wide range of normal physiological activities as well. Because of lysosome relevance in the organism's well being, the capability of chemicals to impair their structure integrity and functions might lead to serious pathological alterations (ICES, 2004a).

#### 1.3.4.1 LYSOSOMES

Lysosomes are ubiquitous organelles whose functions are mediated by their phagic capability. Different kind of cellular phagic activity can be observed (Brunk and Terman, 2002):

1. *Macroautophagy* of whole portions of cytoplasm (*i.e.* whole organelles, such as mitochondria). The sequestration proceeds through formation of autophagosomes, which merge into lysosomes, followed by materials degradation by lysosomal enzymes;
2. *Microautophagy* of minor cytoplasmic components, such as macromolecules. They enter through invagination of the lysosomal membrane or through selective (chaperone-mediated) autophagy;
3. *Heterophagy* of material that originates from other cells. Typical degradation process of different cell types, such as macrophages.

Lysosomes contain various hydrolases (more than 60) which are involved in intracellular digestion of endocytosed foods and macromolecules, and in sequestration of xenobiotic compounds and metals. Lysosome compartment have acid pH, maintained by membrane ATPase dependent $H^+$ ion proton pumps (Lowe and Fossato, 2000).

In the cell lysosomes work as (ICES, 2004a; Winston et al., 1996):

- *Waste disposal*. Lysosomes remove metals and organic compounds from the cytosol.
- *Recycling site of cellular components*. Lysosomes are involved in the continuous turnover of intracellular components. Thank to their ability to up-regulate the autophagic processes in time of physiological changes and stress, lysosomes can

produce enhanced supplies of energy and commodities for protein synthesis, breaking down longer-lived proteins or organelles if required (enhanced cellular catabolism).

- *Cellular anti-oxidant defence tier.* Lysosomes can support the anti-oxidant defence system when overloaded, by removing oxidative damaged proteins, impaired organelles and portion of DNA that might constitute a threat for cell health if accumulated in aggregates.
- *Cellular compartment for the digestion of food and ingested microorganisms.*

#### 1.3.4.2 MECHANISMS INVOLVED IN LYSOSOMES IMPAIRMENTS

The capability of lysosomes to carry out their functions can be seriously compromised in presence of particular cytotoxic insults and protracted stressful conditions. Diagnostic signs of the progressively incoming of lysosomal impairments are (Brunk and Terman, 2002; ICES, 2004a):

- *Enhanced autophagy.* Evidences have suggested that an evolutionary conserved response to contaminant stress is the enhancement of lysosomal autophagy. In fact stressful conditions enhance cellular catabolic activity, resulting in the production of a big amount of cellular waste and in the consequent increase of the autophagic rate.
- *Lipofuscinogenesis.* This process, which takes place in postmitotic and slowly dividing cells, is strictly linked to oxidative stress. Lipofuscins are chemically/morphologically polymorphous undegradable waste materials, autofluorescent, constituted by proteins, lipids, carbohydrates and metals, especially iron, that originate from a variety of intracellular structures and that accumulate in the primary cellular waste disposal. Lipofuscins formation and accumulation is a consequence of:
    1. *Decreased activity of lysosomal enzymes, especially some proteases, or decreased recycling rate of proteasomes*.
    2. *Active oxidation inside the lysosomal compartment.* $H_2O_2$ diffuses into lysosomes and, by interacting with iron released by many metalloproteins undergoing degradation, it turns into hydroxyl radical which reacts with autophagocytosed macromolecules producing crosslinks, probably the cause of the undegradability. Autophagocytosed mitochondria give a big contribution to lipofuscins formation because they constitue an important source of ROS.

  At the early stages of lipofuscinogenesis, lysosomes still contain active hydrolases and the proton pumps work. However the progressive increase of lipofuscins

accumulation can lead to detrimental consequences, such as the release of hydrolytic enzymes through impaired lysosomal membranes and consequent damage to cytoplasmatic constituents. Furthermore the attempt to digest undegradable lipofuscins can cause the failure of the primary lysosomal functions, firstly at all phagocytosis. Decreased autophagy can be responsible of impairment of cellular repair machinery and insufficient renewal of cellular components which can ultimately lead to a general vulnerability of cells to stresses (Winston et al., 1996).

#### 1.3.4.3 LYSOSOMAL LESIONS

The protracting of stressful conditions for lysosomes rapidly can turn into pathological alterations to lysosomal structure and functions, which can be responsible of serious consequences at the higher levels of organization. Common lysosomal lesions are (Moore and Lowe, 2004; Lowe and Fossato, 2000):

1. *Changes in lysosomal content, increasing volume of the compartment and cell swelling*. These changes are associated to overload of waste materials.
2. *Activation or inhibition of acid hydrolases activity within the lysosomes.* This phenomenon is due to the toxic action exerted by accumulated pollutants and lipofuscins.
3. *Changes in fusion events* (*failed autophagy*).
4. *Changes in membrane permeability*. This can lead to release of lysosomal content into the cytosol, in particular of hydrolytic enzymes which can cause degradation and damage to other organelles.

It has been demonstrated that recovery of lysosomal integrity can occur by interrupting the exposure to stressors (Lowe et al., 2006).

## 1.4 BIOLOGICAL TOOLS FOR MARINE POLLUTION ASSESSMENT

In recent years the monitoring approach of aquatic environments underwent a drastic revision. All changes have been addressed to guarantee water bodies' quality and integrity, ensuring that all human activities could be carried out in a sustainable way, without impairing water safety, health and productivity. At the base of this comprehensive revision there are few essential concepts (Borja et al., 2008):

- *Ecosystem* is the environmental unit object of all protection and restore actions. Physical-chemical, hydrological and biological criteria contribute to define its *ecological status* in an integrative way;
- All efforts of environmental policy and management have to aim at achieving/maintaining *good ecological status*, resulting from the comprehensive high quality of all constitutive elements (*physical-chemical*, *pollution-related* and *biological* elements);
- The biological quality of aquatic ecosystem depends on the health status of all biological elements (*phytoplankton*, *macroalgae*, *benthos* and *fish*).

The validity of this new approach has been recognized worldwide, thanks to the adoption of regulatory measures, such as Ocean act in USA, Australia and Canada, Water Framework Directive (WFD, 2000/60/EC[17]) and Marine Strategy Directive[18] in Europe, National Water Act in South Africa (Borja et al., 2008).

As a direct consequence, marine pollution monitoring strategies have been reviewed. In fact, under the new point of view, chemical pollution is considered according to its potential to impair the ecological status of water bodies and to affect their biological elements. Consequently primary aim of marine pollution assessment is to evaluate the toxic pressure exerted by pollutants on the ecosystems and to alert about future likely consequences.

It is widely recognized that these goals cannot be achieved by traditional chemical analyses because data on contaminants levels do not provide any information on the effects caused by pollutants on the biota. In fact the threat associated to environmental toxins cannot be inferred from the concentrations, being the toxicity influenced by a multitude of environmental, ecological and biological factors which modulate contaminants behaviour,

[17] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

[18] Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive).

bioavailability, bioaccumulation potency and physiological responses on organisms (Moore et al., 2004; Ruus et al., 2005; Downs et al., 2001; Van den Broeck et al., 2007; Wilson, 2003; Lam and Gray, 2003; Ham et al., 1997; Lowe and Fossato, 2000; Pereira et al., 2007).

On the contrary innovative monitoring tools are regarded with growing interest because of their capability in providing pollution-related information about the actual state of biotic constituents. Among the most promising there are *biomarkers*, measures of pollution-related changes at molecular, cellular, tissue/organs and organism levels (Cajaraville et al., 2000; Francioni et al., 2007; Galloway et al., 2006; Handy et al., 2003; Lam and Gray, 2003; Beliaeff and Burgeot, 2002; Shugart, 2000).

### 1.4.1 BIOMARKERS

Biomarkers have been loaned by the medical sector and tested for ecotoxicological purposes in the mid 1980s (Galloway at al., 2006; Beliaeff and Burgeot, 2002). Even though several definitions have been proposed, the most general defines biomarkers as "quantitative measures of changes in the biological system that response to exposure to (or doses of) xenobiotic substances that lead to biological effects" (Lam and Gray, 2003). Biomarkers are usually grouped into two categories: 1) *biomarkers of exposure*, which are specific indicators of exposure to single classes of contaminants, and 2) *biomarkers of effects*, which are measures of health impairment in the test-organism. The former are generally based on the responses (induction/inhibition) of specific enzymes involved in biotransformation and detoxification processes; consequently they are *early* biomarkers based on endpoints at low levels of biological organization. The latter focus on pathological endpoints, reflecting the overall toxicity induced by toxic exposure, which can be assessed at all levels of biological organization (Broeg et al., 2005). A biomarker can be classified as *exposure and effect biomarker* if its response, besides showing dose-dependent pattern, is involved in biological processes essential for the normal functioning of cells/tissues/organism (Handy et al., 2003).

#### 1.4.1.1 BIOMARKER OF EXPOSURE

Fundamental requirements of exposure biomarkers are (Handy et al., 2003):

1. the response has to be consistent with cell/tissue contaminant concentration;
2. cell/tissue contaminant concentration has to correlate to the respective environmental concentration.

Exposure biomarkers which fulfil these requisites are potent tools for assessing the toxicant's bioavailable fraction, which can be related to potential detrimental effects.

Due to their general inexpensiveness, easiness and rapidity, biomarkers of exposure are considered reliable tools to alert about environmental presence of contaminants belonging to a specific class. Even though the exact chemical characterization is not inferable by their responses, their use can provide some advantages respect to traditional chemical analyses. In fact biomarkers of exposure are usually highly sensitive, able to detect contaminants in trace and, depending on specific methodology, they might result less affected by typical limits of chemical analyses (interferences, sample contamination etc). Finally, depending on the specific pattern of induction-response, they can overcome analytical problems which chemical analyses usually find in detecting intermittent pollution (Galloway et al, 2006; Handy et al., 2003).

Therefore the role suggested for exposure biomarkers in routine monitoring program consists in screening the insurgence/presence of stressors, which would be further characterized by chemical analyses. So they are not suggested as alternatives of chemical analyses in pollution assessment, but rather as complements (Handy et al., 2003; Lam and Gray, 2003; Pereira at al., 2007).

Core examples of endpoints of exposure biomarkers, which have been validated and largely tested in field and laboratory studies, are the up-regulation of CYP1A1 (Anderson and Lee, 2006; Shaw et al., 2002), PAH metabolites concentration in urinary (Fillman et a., 2004), acetylcholinesterase activity (Viarengo et al., 2007 and references therein) and metallothioneins (Viarengo et al., 1997).

#### 1.4.1.2 BIOMARKERS OF EFFECTS

Biomarkers of effects are proposed as reliable tools for monitoring the health status of biotic elements. As previously mentioned, endpoints can be at all levels of biological organization, from subcellular to population/community. Generally the biological level at which the effect is visible suggests the degree of severity of the ongoing toxic process (Figure 1.5). In fact, initially, xenobiotics act at sub-cellular level: if detoxification and repair mechanisms do not act efficiently, the lesions might become persistent and expand toward the higher biological levels.

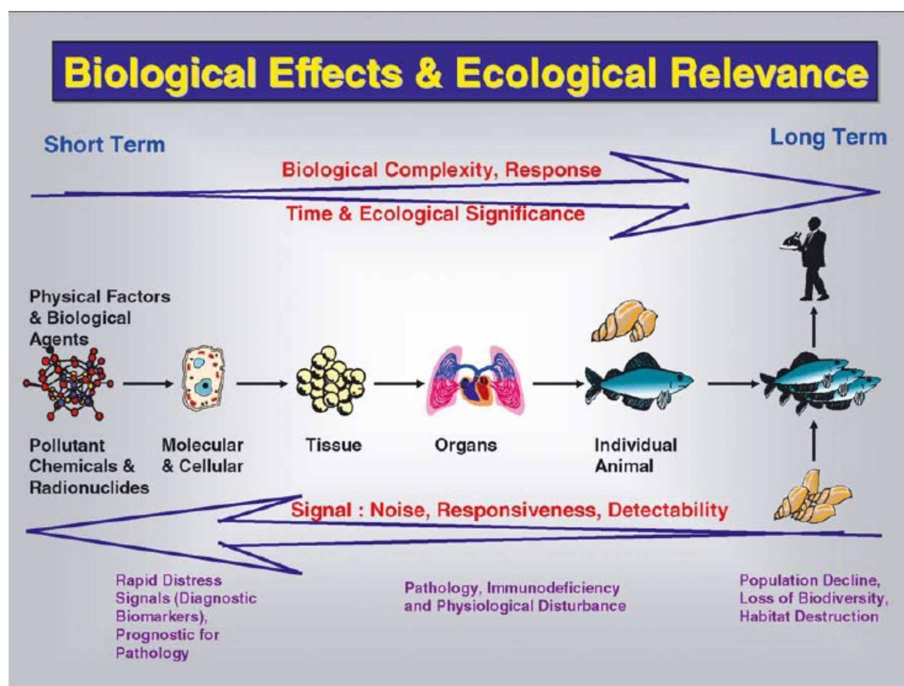



**Figure 1.5 Diagram of the relationship between environmental distress and ecological relevance (Moore et al., 2004)**

A fundamental point of effect biomarkers is the ecological relevance of the endpoint, given by the link with fitness parameters, such as growth rate, reproductive output and viability of the offspring (Depledge and Billinghurst, 1999; Moore at al., 2004). This feature is particularly important for molecular and cellular biomarkers, because at the base of their prognostic use as *early signals* of stress syndromes prior to the occurrence of severe environmental alterations at higher organizational levels (Moore at al., 2004; Vasseur and Cossu-Leguille, 2006; Cajaraville et al., 2000; Broeg et al., 2005 and references therein).

Biomarkers focusing on endpoints at *low levels of biological organization* have been intensively developed in the last years due to the research advances in genomics, proteomics and metabolomics (Lam and Gray, 2003). These tools are considered with great interest because of their potentiality as *short-term indicators of long-term biological effects* (Cajaraville et al., 2000); furthermore, the generic nature of their endpoints makes them easily adaptable to different taxa (Galloway et al., 2004).

Organism-population-community biomarkers are preferentially referred as *bioindicators*. Organism-level biomarkers are fundamental for alerting about the possibility that detrimental changes might occur in a population; anyway without additive information on possible causes, which can be provided by mechanistically-based indicators (*i.e.* biomarkers at molecular and cellular levels, chemical analyses), these tools do not allow an extensive comprehension of the problem and individuation of possible causes (Anderson et al., 2006).

#### 1.4.1.3 LIMITS ON BIOMARKERS USE

A common objection arisen against the use of biochemical/physiological biomarkers as monitoring tools refers to the variability of the response. Biomarkers seasonal fluctuations are usually attributed to both abiotic factors (variation of environmental parameters, such as temperature, salinity, oxygen, etc) and biotic factors (*i.e.* test-specie's age, sex, genotype, nutritional conditions, etc).The supporters of biomarkers' suitability assert that these limits can be easily overcome, or at least countered, by opportunely designing the biomonitoring plan. This implicates a wise choice of reference sites, number of experimental replications and sampling sites, exposure/sampling timing and methods for data elaboration. Anyway further research on non-pathological variations of biomarkers' responses is generally recommended in order to ameliorate the capability to correctly interpret biomarkers' results in field context. In particular, laboratory long-term experiments could be useful to elucidate the pathway of biomarkers response against the time and their sensitivity to short-term changes associated to biotic and abiotic factors (Handy et al., 2003).

#### 1.4.1.4 STATE OF ART

Even through considerable research effort has been invested in biomarkers' development since their discovery in the mid 1980s, the routinely use of biomarkers in environmental management strategies is still seldom. Until now, in fact, the use of biomarkers has been limited to pilot studies, which have been essential to acquire experience about experimental designing criteria and to gain insights about the effectiveness of biomarkers based monitoring strategies (Lam and Gray, 2003; Galloway et al., 2006; 2002; Bloxham et al. 2004; Handy et al., 2003)

However the promotion of biomarkers strategies from the research/development phase to the practical use seems to proceed slowly, as marked by the not still introduction of biomarkers in regulatory procedures. This delay is likely due to the uncertainties about how to design the testing system (*i.e.* which organism to use and how to analyse the dataset) and to the need to further expand the international agreement on biomarker potential and to develop standard operating procedures and inter-laboratory calibration.

To fill these lacks, recently many important initiatives at international level have been carried out (Lam and Gray, 2003). Several important pollution monitoring programmes, incorporating biomarkers among the assessment tools, have been arranged in USA and Europe (*e.g.* NOAA National Status and Trends Program[19]; United Nations Environment Programme for Mediterranean Sea (Med Pol I and II[20])). Particularly relevant was the UE founded BEEP

[19] <http://ccma.nos.noaa.gov/about/coast/nsandt/welcome.html>

[20] <http://www.unepmap.org/index.php?module=content2&catid=001017003>

Program (Biological Effect of Environmental Pollution Program[21]), aimed at evaluating the overall potential of biomarkers. Furthermore practical workshops for comparing different biomarkers methods and protocols have been organized by international institutions, such as International Council for the Exploration of the Sea (ICES[22]) and the Intergovernmental Oceanographic Commission (UNESCO-IOC[23]). Finally working groups, to specifically study biomarker applicability, have been established (OSPARCOM/ICES[24]). All these efforts have led to concrete advancements, such as the availability of standard protocols for the most robust biomarkers and the incorporation of several biomarkers in the Joint Monitoring Programme of the OSPAR convention (Cajaraville et al., 2000).

According to several authors, biomarkers, in particular those focusing on biochemical processes, can play a great role also in the research field of ecological risk assessment. In fact, those whose relationships with responses at population-community levels have been verified and calibrated, can be used as valid biomarkers for predicting detrimental outcomes at higher levels (Broeg et al., 2005).

---

[21] <http://www.beep.u-bordeaux1.fr/>

[22] <http://www.ices.dk/indexfla.asp>

[23] <http://ioc-unesco.org/>

[24] <http://www.ospar.org/>

## 1.5 ASSESSMENT OF POLLUTION RELATED STRESSES IN NATURAL POPULATIONS

### 1.5.1 GENOTOXICITY ASSESSMENT

Consequences of long-term exposure to genotoxic pollutants became of concern in the last decades, because of the increasing related incidence of neoplasia in wildlife and in humans (Di Giulio et al., 1989). However the genotoxic potency of environmental contaminant mixture is not inferable by its chemical composition, because toxicological properties of chemicals and compensatory/repair mechanisms of organisms can highly vary in complex conditions. So biomarkers, measuring the overall genotoxic power associated to environmental pollution exposure, might represent a good alternative approach.

Several biomarkers are currently available to assess genotoxicity *in situ*. Those focusing on structural modifications are considered biomarkers of exposure to genotoxicants, whereas those detecting the presence of abnormal DNA are proposed as biomarkers of effects. The former endpoints are early and transient effects to genotoxicants insults that can be easily repaired without leading to any permanent consequences, and so they are considered only *indicators* of possible mutagenic/carcinogenic outcomes associated to genotoxic exposure; the latter are forms of “expressed genotoxicity” (Fairbairn et al., 1995; Shugart, 2000).

Table 1.6 lists some of the most prominent assays, grouped consistently with the nature of the endpoint (Shugart, 2000; Lee and Steinert, 2003; Albertini et al., 2000; Dixon et al., 2002). Among these, Comet assay and MN assay have been highly tested on aquatic organisms for large scale monitoring investigations (Nigro et al., 2006).

**Table 1.6 List of common methods for measuring biological markers of genotoxic exposure and effects (Shugart, 2000; Tucker and Preston, 1996).**

| ENDPOINTS | BIOMARKERS |
|---|---|
| **DNA structural modifications** | |
| DNA adducts | P-Postlabelling adduct assay; Immunological detection of adducts (RIA, ELISA); HPLC fluorescence spectrophotometry |
| DNA strand breaks | Comet assay; Alkaline Elution and Unwinding assay; Gel Electrophoresis assay (GE) |
| DNA damage capable of interfering with DNA replication (*e.g.* alkylated bases, crosslinks) | Sister-chromatin Exchanges (SCE) |
| Modified bases | Detection of 8OH-dGuo, Fapy-Gua |
| **Abnormal DNA** | |
| Chromosomal aberrations | Chromosomal Aberration analysis (CA); Fluorescence in |

| | |
|---|---|
| (structural and numerical) | situ Hybridization (FISH); Micronuclei assay (MN) |
| Mutations | Hypoxanthine-guanine phosphoribosyltransferase (HPRT) |

### 1.5.1.1 COMET ASSAY

#### 1.5.1.1.1 Method

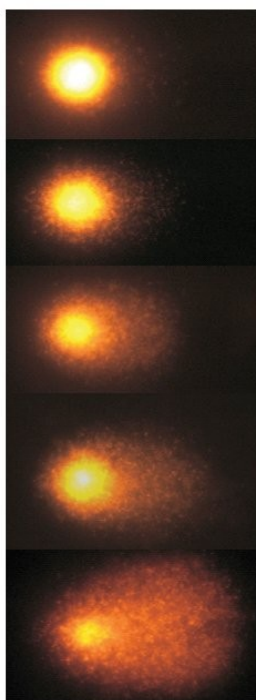


**Figure 1.6 Images of comets: apparence of DNA progressively more damaged. From <http:// egms.de/static/figures/journals/cto/20043/cto000003.f1.png>**

The Comet assay is a test to measure the DNA integrity of single cells (Brendler-Schwaab et al., 2005). Basically single cells, trapped in agarose layered microscope slides, are lysed by immersion in a lysis bath, leading to DNA denaturation. During the following electrophoresis, if present, negatively charged DNA fragments migrate toward the anode. The extent of migration is revealed using a DNA-specific fluorescent dye: under epifluorescent microscope observation, damaged cells look like *comets*, in particular the bulk of undamaged genetic materials constitutes *comets' nucleus*, whereas DNA fragments *comets' tail* (Figure 1.6). Two main principles are believed to determine the ability of DNA to migrate: 1) the size of DNA fragments and 2) the number of broken ends which may be attached to larger pieces of DNA (Fairbairn et al., 1995). The degree of DNA damage is given by the ratio between the extent of comet tail and comet nucleus. This evaluation can be done using a simple ocular micrometer or sophisticated image analysis softwares: the former lets to estimate *tail length* and *length:width ratio*, whereas the latter, measuring fluorescence intensity and DNA distribution, output high sensitive parameters, such as the *percentage of DNA tail intensity* (fluorescence intensity of the tail relative to the head) and the *tail moment* (product of the tail length and the fraction of total DNA in the tail) (pictures of softwares images processing are reported in Figure 1.7. Compared to the tail length, the last two parameters seem to be more consistent with the number of strand breaks. In fact, the tail length, after an initial increase, reaches a maximum dependent on the specific conditions of electrophoresis run (Lee and Steinert, 2003; Fairbairn et al., 1995).

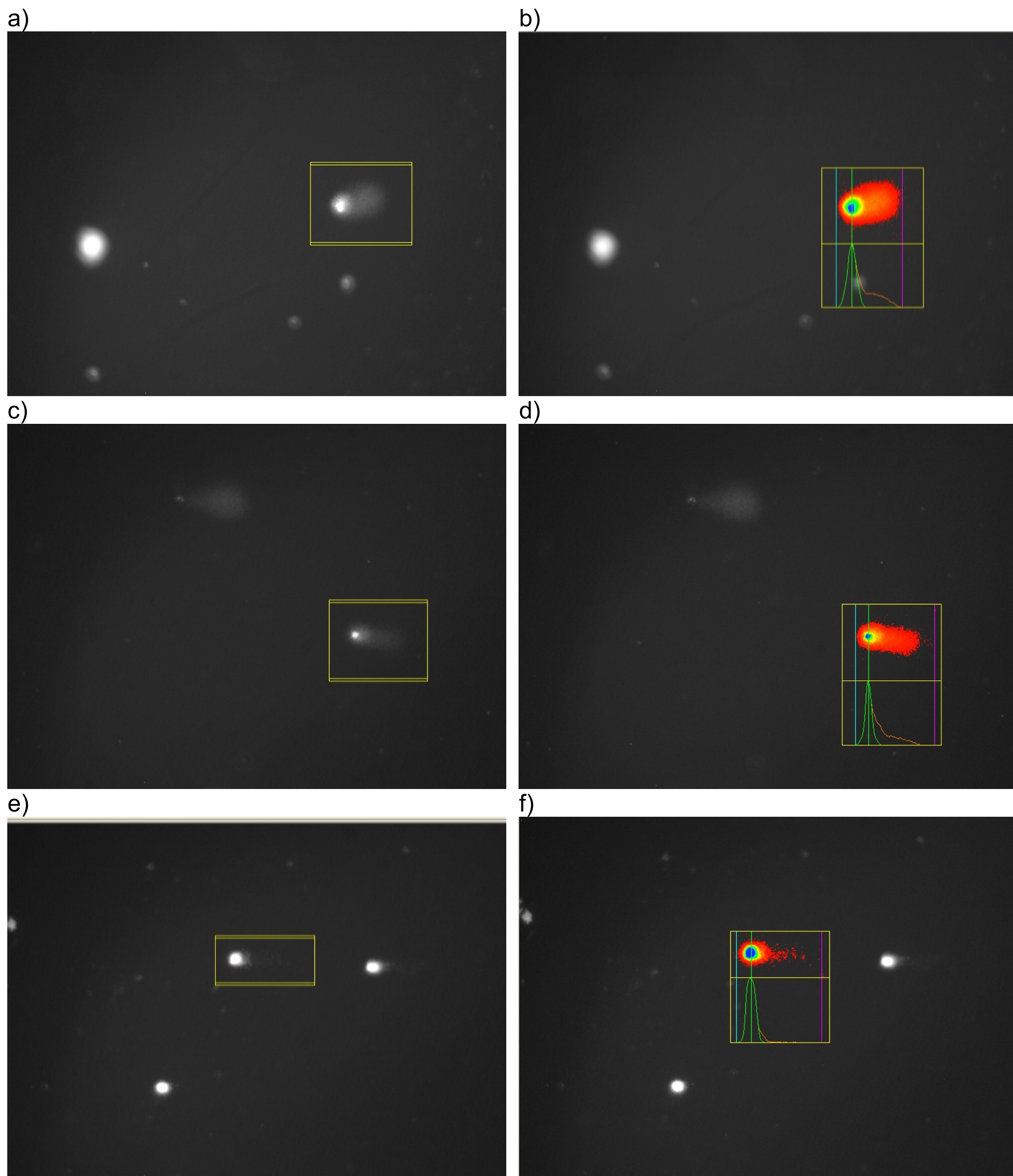



**Figure 1.7 Examples of Comet assay images processing though Kinetic COMET Software. Pictures a and b: comet having 38,66% tail intensity; pictures c and d: comet having 44,51% tail intensity; pictures e and f: comet having 7,50% tail intensity**

Comet assay standardized methods are available (ASTM, 2005). As stressed by OECD guidelines for standards to adopt for *in vivo* genotoxicity tests, the quality of experimental protocol is fundamental for the acceptance of Comet assay results and for comparing data (Brendler-Schwaab et al., 2005; Fairbairn et al., 1995). In fact the method is highly sensitive to small operative variations, such as lysis buffer composition and electrophoresis running

conditions, as well as to the choice of the "tissue-organism" test-system (Dixon et al., 2002; Kim and Hyun, 2006).

#### 1.5.1.1.2 Type of DNA lesions assessed

The test, also known as Single Cell Gel Electrophoresis assay (SGC or SGCE), was initially proposed by Ostling and Johanson (1984). According to the original version, cells lysis and electrophoresis had to be carried out in neutral conditions, making the test sensitive only to double DNA strand breaks, generally caused by radiations and radiomimetic chemicals. The following version, modified by Singh et al. (1988), allowed also the identification of single strand breaks and alkali-labile sites, typical DNA damages caused by common genotoxic agents, such as environmental pollutants. The main change introduced into the protocol was the use of alkaline conditions for the electrophoresis process: at pH value higher that 12,1 (maximum return at pH >13), DNA unwinds into single strands and all kinds of single DNA strand lesions are transformed into breaks, contributing to the formation of the comet tail during the electrophoresis migration (Lee and Steinert, 2003; Albertini et al., 2000). Thus Comet assay-alkaline version provides a measure of the overall DNA damage, including single and double strand breaks, alkali-labile sites, cross-links DNA-DNA and DNA-protein (Taban et al., 2004; Gobbo Braz et al., 2007; Lee and Steinert, 2003; Brendler-Schwaab et al., 2005). Considering that DNA single strands breaks occur 5-200 times more often than DNA double strands, the alkaline version is considered more sensitive (Seo et al., 2008).

The extent of DNA damage measured is related to both the action of extracellular agents, such as genotoxic pollutants and physical agents, and endogenous mechanisms, such as excision-repair enzymes' activity (transient strand breaks) and cellular turnover (apoptosis). It represents the equilibrium between forces acting directly/indirectly on DNA and cellular defence, made up by enzymatic and non-enzymatic antioxidants, DNA repair mechanisms and detoxification processes (Par. 1.3.2.2) (Akcha et al., 2004). Thus Comet assay, which can be carried out on all kind of cells (*i.e.* hemocytes from mammals, fishes, amphibians and birds; lymphocytes from molluscs and crustaceans; coelomocytes from annelids; cellular suspensions obtained from tissues) shows different sensitivity depending on the nature of cells/tissue and test-animal. In fact cells have a specific background of DNA strand breaks, which depends on the efficiency of the own excision-repair and anti-oxidant defence systems and on metabolic processes (Lee and Steinert, 2003).

The performance of Comet assay on tissue's cells suspension is considered more laborious and critical because, during the phase of cells suspension preparation, additional strands breaks and repair processes might occur (Fairbairn et al., 1995; Dixon et al., 2002; Kim and Hyun, 2006).

As previously generalised about results interpretation of tests focusing on early genotoxic endpoints, Comet assay, in particular the alkaline version, is a tool to identify hazard rather

than risk (Albertini et al., 2000). In fact single strand breaks can be quickly repaired, without leading serious consequences for the cell and for the organism (chromosomal and protein aberration, cell death, neoplasia, etc); differently, more serious events could be initiated by double strand breaks, which are more difficult to repair (Dixon et al., 2002).

###### 1.5.1.1.3 Applications

Comet assay is frequently carried out in *in situ* experimental surveys as biomarker of genotoxicants exposure (Dhawan et al., 2009). As regard to laboratory applications, besides its use as *in vivo* test, many authors have used Comet assay as *in vitro* method for mechanistic studies (Dixon et al., 2002). In fact, by introducing some variations on the standard protocol, the method becomes suitable to study singular mechanisms of DNA fragmentation or specific DNA injuries (*i.e.* excisable DNA damage, DNA crosslinks, oxidative stress mediated-DNA damage, apoptosis, oxidized pyrimidines, oxidized purines (Fairbairn et al., 1995; Lee and Steinert, 2003).

###### 1.5.1.1.4 Advantages and limits

The method provides many advantages compared to other common genotoxicity assays. Besides being simple, quick, inexpensive and well-tested (Kim and Hyun, 2006; Seo et al., 2008; Lee and Steinert, 2003; Dixon et al., 2002), it is virtually amenable to all eukaryotic cell types (included those not mitotically active), suitable for both *in vivo* and *in vitro* experiments and without any requirements of cells division (Fairbairn et al., 1995; Dixon et al., 2002). Its applicability on single cells makes it adapted for studying organ-specific genotoxicity. Many evidences have supported its high and reliable sensitivity. To perform the test, only small amounts of sample are required (Seo et al., 2008; Lee and Steinert, 2003; Brendler-Schwaab et al.,2005; Fairbairn et al., 1995; Kim and Hyun, 2006).

Despite these evident advantages, the assay presents some limitations in term of applicability and interpretation. Regarding the use of Comet assay in field surveys, some studies have warned about the possibility that, in populations chronically exposed to pollution, adaptive responses take place in order to prevent genotoxic damages (Shaw et al., 2000; Large et al., 2002); other studies have highlighted that Comet assay response can be affected by a significant level of interindividual and seasonal variability (Dixon et al., 2002; Shaw et al., 2000).

Focusing on interpretative issues, it is important to note that Comet assay does not allow to discriminate among 1) fragmentation due to genotoxic action, 2) fragmentation arising from ongoing repair mechanisms and 3) fragmentation associated to ongoing processes of programmed cell death (apoptosis) (Shugart, 2000). So, as recommended by Gabbianelli et al. (2006), the use of Comet assay as preliminary test should be followed by other investigations able to elucidate the mechanisms involved in the measured DNA damage.

Finally there is not an unanimous agreement about the ecological relevance of Comet assay endpoint for environmental genotoxicity assessment. In fact DNA fragmentation is considered an early stage effect lesions which can be quickly repaired without leading to advanced genotoxic effects (*i.e.* chromosomal aberrations, chromatin exchange and micronuclei formation), protein dysfunctions and tumour formation, which can likely affect organism's health status (Palmqvist et al., 2003).

#### 1.5.1.2 MICRONUCLEUS ASSAY

##### 1.5.1.2.1 Method

Micronucleus assay is a test for measuring chromosome damage. It was developed independently by Schmid and Heddle in the late 1970s. Nowadays it is one of the best tested assay used to quantify *in vivo* chromosome breakage and loss related to exposure to clastogens (chromosome breaking agents, having DNA as target) and aneugens (agents inducing changes in chromosome number, usually by interfering in the spindle formation) (Fenech, 2000; Albertini et al., 2000).

The method consists in estimating micronuclei frequency by visual observation under light microscope. Basically cells suspensions, or cells pellets, are fixed by a fixative solution and spread onto a microscope slide. Then about 1000-2000 cells/individual are scored in order to evaluate micronuclei frequency (Albertini et al., 2000; Venier et al., 1997). The presence of micronuclei must be associated to clastogenic and aneugenic events which took place during the last *in vivo* cell division. Depending on the longevity of the cells used for the study, the test might be suitable for both acute and long-term studies (Albertini et al., 2000).

NM assay can be carried out in dividing eukaryotic cells (Fenech, 2000). In environmental biomonitoring surveys it is usually performed on gill cells or haemocytes of selected test-organisms. Even though gill cells are direct targets of pollutants, haemocytes are generally preferred as matrix for MN assay. In fact the preparation of single gill cells suspension is time consuming and not easy to carry out; furthermore it is a source of cells damage and bias, because usually the suspension obtained is not perfectly pure.

Several authors use the protocol reported in UNEP/RAMOGE (1999) as reference.

##### 1.5.1.2.2 Type of chromosomal lesions assessed

Micronuclei are small intracytoplasmatic masses containing chromatin and surrounded by nuclear membrane that may be present close to the main nucleus (Figure 1.8). They can derive from failures occurring before or during mitosis (Albertini et al., 2000): part of the genome might not be integrated into the daughter nuclei and, during teleophase, it undergoes "regular" nuclear membrane envelopment and uncoiling, gradually assuming the

typical morphology of interphase nucleus, despite the smaller dimension (Fenech, 2000; Nigro et al., 2006; Dolcetti and Venier, 2002).

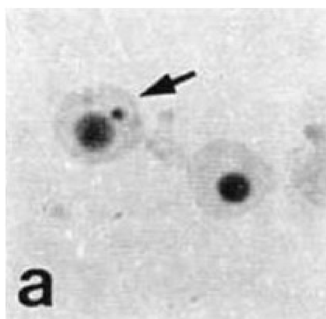



**Figure 1.8 Cell showing micronucleus (arrow) (Dailianis et al., 2003)**

Micronuclei can be classified into two categories, depending on the type of genetic material harboured: 1) micronuclei containing acentric fragments (chromosome breaks lacking centromeres) and 2) micronuclei carrying a whole chromosome. The former result from direct DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis; the latter are associated to defects which block chromosome moving toward the spindle poles. In this second case, the aberration might regard some components of the mitotic apparatus or kinetochore (Albertini et al., 2000).

In order to improve the sensitivity of MN assay, a growing number of authors takes into account the frequency of other nuclear abnormalities (*i.e.* binucleated cells, eight-shaped cells, etc) as additional complementary measure of chromosome rearrangement (Koukouzika and Dimitriadis, 2008 and references therein; Dolcetti and Venier, 2002; Van Ngan et al., 2007).

#### 1.5.1.2.3 Applicability

In environmental surveys, micronuclei frequency is used as index of chromosome loss and chromosome breakage which can turn into long-term consequences, included mutagenesis and carcinogenesis (Venier et al., 1997; Tucker and Preston, 1996; Fenech, 2000). It has been successfully used on fishes and invertebrates to monitor water quality (Kim and Hyun, 2006; Nigro et al., 2006) and in field and laboratory invertebrates studies (Dolcetti and Venier, 2002; Venier et al., 1997). In particular MN test on *Mytilus* spp. has been suggested as tool for large-scale monitoring purposes (Viarengo et al., 2000).

#### 1.5.1.2.4 Advantages and limits

MN assay is very easy to perform. In fact, compared with other techniques for assessing chromosome damage, such as Sister-Chromatid Exchange assay and Chromosomal Aberration test, MN assay is more practical because it does not need cells to be in metaphase (Kim and Hyun, 2006; Venier et al., 1997; Burgeot et al., 1996; Tucker and Preston, 1996). However it results limited by operative aspects, such as the visual micronuclei scoring which makes the method laborious and partially subjective (Burgeot et al., 1996; Fang et al., 2009). Furthermore micronuclei formation in wild organisms seems to be influenced by physiological and environmental factors (Magni et al., 2006): field studies on marine species have shown that MN response may be affected by variability related to both test-organisms and environmental conditions (*i.e.* age, growth moment, viral infections, MFO levels in tissues, mitotic frequency, breeding, bioaccumulation capability, anoxia,

temperature, season, etc). Adaptation mechanisms influencing MN frequency have been suggested to occur and to be dependent on the species, season and site specificities (Pytharopoulou et al., 2006; Burgeot et al., 1996; Sotil et al., 2008; Venier et al., 1997; Dixon et al., 2002).

### 1.5.2 OXIDATIVE STRESS ASSESSMENT

The importance of oxidative stress assessment in aquatic toxicology is underscored by the involvement of oxidative processes in many pollution-related injuries. In fact, biochemical changes associated to oxidative stress make part of the initial reactions induced by many contaminants; from this their relevance as early warning signals of the possible onset of pathologies (Di Giulio et al., 1989; Valavanidis et al., 2006).

ROS are difficult to detect, in particular those extremely reactive and aggressive as hydroxyl radicals (Di Giulio et al., 1989). For this reason researchers have developed several indirect methods for assessing oxidative stress. Table 1.7 reports some of the most recommended biomarkers, grouped according to the nature of the endpoints (Valavanidis et al., 2006; Di Giulio et al., 1989; Viarengo at al., 2007).

**Table 1.7 List of oxidative stress biomarkers**

| **ENDPOINT: OXIDATION PRODUCTS** | **BIOMARKERS** |
|---|---|
| *Lipid peroxidation* | HPLC/GC based methods for the detection of primary/secondary lipid peroxidation products, such as malondialdehyde (MDA) and F2-isoprostane. |
| *DNA damage* | HPLC-based analytical assays for the detection of hydroxylation products of nucleobase guanosine (8-OHdG, 8-oxodG; DNA strand-breaks (for assays list refer to Par. 1.5.1). |
| *Proteins oxidation* | HPLC or MS detection of carbonyl derivates of proteins, such as oxidation products of phenylalanine and tyrosine amino acids; methemoglobidinemia in vertebrate. |
| **ENDPOINT: STATUS OF THE ANTIOXIDANT DEFENSE SYSTEM** | **BIOMARKERS** |
| Levels of non-enzymatic antioxidants | Vitamins C and E; Urate; Retinyl esters; β-carotene; Glutathione (GSH). |
| Activity of enzymes involved in antioxidant machinery | Catalase (CAT); Superoxide dismutase (SOD); Glutathione peroxidase (GPX); Glutathione transferase (GST); Glutathione reductase (GR). |
| **ENDPOINT: CELLULAR LESIONS** | **BIOMARKERS** |
| Lipofuscins accumulation | Lipofuscins accumulation assays. |

The induction of antioxidant enzymes under oxidative stress, that is an important evolutionary adaptation to these conditions, lets the use of this phenomenon as biomarker. In fact, according to Di Giulio at al. (1989), the presence of enzymatic antioxidants (*i.e.* SOD, CAT, GPX) has to be considered one of the most important evidence of ROS activity *in vivo*, because of their primary role in the antioxidant defence. However the authors warned about the difficulties in data interpretation: in fact enzymatic activity, as well as non-enzymatic

scavenging capability, is susceptible to many endogenous and exogenous variables (*i.e.* sex, age, gonadal status, diet, seasons, toxicant bioavailability, etc) that are not easily controllable in the context of environmental survey (Di Giulio et al., 1989; Livingstone, 2001); furthermore enzymes' response trends are featured by specific kinetics (*i.e.* SOD and CAT activities may be elevated by stressors induction and depressed as toxic response to the stressors (Viarengo et al., 2007; Di Giulio et al., 1989) and they vary with the kind of oxidant that attach the system. So activation and inhibition of antioxidant system are not contradictory results because they may represent two different phases of the response against chemical stresses, highly dependent on the intensity and duration of the stress (Pereira et al., 2007 and references therein). The interpretation of biomarkers data based on antioxidant enzymes' activity is even more complicated in the context of environmental mixtures exposure, because induction and inhibition of antioxidant enzymes can be caused simultaneously by different compounds (Di Giulio at al., 1989). For these reasons there is a general agreement about the limited relevance of the information provided individually by single biomarker, considered likelihood of misinterpretation. Thus it is recommended to use selected batteries of oxidative stress biomarkers and to enforce the biochemical based-description through biomarkers focusing on oxidative syndromes at the higher level of biological organization, such as degree of lysosomal membrane integrity and lipofuscins accumulation: these responses may be useful as benchmark against which to evaluate the other oxidative parameters (Morales-Caselles et al., 2008; Katalinic et al., 2005).

More recently, alternative strategies for oxidative stress assessment have been developed. They take rise from the evidence that antioxidant system's components act cooperatively, so methods focusing on single antioxidant agents might not provide a comprehensive picture of the oxidative status of a biological system (Winston et al., 1998; Regoli and Wilson, 1999 and references therein; Livingstone, 2001). These new approaches aim at estimating the overall ability of a test-system to deal with ROS overproduction, by quantifying the comprehensive tissue capability in adsorbing oxyradicals (Winston et al., 1998). Table 1.8 reports a brief description of common assays carried out to estimate the overall oxidative buffering capability of test-systems.

**Table 1.8 Short description of TOSCA, FRAP and TRAP**

| | |
|---|---|
| **TOSCA** **(Total Oxidant Scavenging Capacity Assay)** | The method, described by Winson et al. (1998), is based on the competition between cellular antioxidants and α-keto-γ-methiolbutyric acid (KMBA), a selected substrate purposely introduced into the reaction milieu for reacting with ROS. The reaction between ROS and KMBA produces ethylene, which can be easily measured through GC-MS analysis: low rate of ethylene production means high efficiency of the antioxidant defence to subtract ROS from the system. Generally the total scavenging capability of a systems is assessed separately for several reactive oxygen species which have different reactivity (*i.e.* peroxyl, hydroxyl and alkoxil radicals), in order to evaluate the specific ability of the test-system in countering the threat represented by singular free radicals. |
| **FRAP assay** **(Ferric Reducing Antioxidant Power assay)** | The assay was initially designed by Benzie and Strain (1996) for measuring the antioxidant status of human samples (plasma) and afterward it was adapted for environmental studies. Basically the test measures the global reducing potential of a system provided by non-enzymatic antioxidants, by evaluating their efficiency in reducing Fe(III), the oxidant-model purposely added to the reaction milieu. |
| **TRAP** **(Total Radical-trapping Antioxidant Parameter)** | This technique was developed by Wayner et al. (1985). It is based on the measurement of the time needed by a system (plasma or tissue homogenate) to adsorb ROS. Free radicals are introduced into the system by a free radical generator: TRAP value is calculated comparing the times of induction required to obtaining maximum oxygen consumption in the sample and in the reference system (solution at known concentration of Trolox, a water-soluble vitamin E analogue). |

### 1.5.2.1 FRAP

#### 1.5.2.1.1 Method

As mentioned in Table 1.8, FRAP assay (Ferric Reducing Antioxidant Power assay) is a simple and inexpensive procedure for evaluating the antioxidant level of biological systems. Basically the test measures the global potential of the antioxidant defence (non-enzymatic) to face the oxidative threat represented by an oxidant-model (Fe(III)) (Griffin and Bhagooli, 2004). The foundation upon which the assay is built up is that the inactivation of oxidant species proceeds through a redox reaction where non-enzymatic antioxidants act as reductants; thus the antioxidant power may be referred as *reducing ability*.

Basically the spectrophotometric method proposed by Benzie and Strain (1996) measured the capability of biological samples (tissues homogenate or plasma) to reduce Fe(III)-TPTZ during the reaction time-window.

#### 1.5.2.1.2 Application

In human studies FRAP assay is generally applied to plasma, explaining the original meaning of the acronym FRAP (Ferric Reducing Ability of Plasma). In environmental studies using invertebrates as test-organisms, the assay is usually carried out on tissues homogenates of digestive gland/hepatopancreas, lipids-rich organs which actively biotransform redox-cycling xenobiotics (Winston et al., 1996). It has been successfully performed on tissue homogenates from different organisms, such as corals (Griffin and Bhagooli, 2004), bivalves (Hagger et al., 2005; Morales-Caselles et al., 2008), and crabs (Dissanayake et al., 2008; Morales-Caselles et al., 2008).

#### 1.5.2.1.3 Advantages and limits

FRAP assay is deemed a simple, reproducible and inexpensive method. However it is not strictly selective for biological antioxidants, being sensitive also to chemical reductants which do not make part of the antioxidant defence. Furthermore it does not take into account the role of antioxidants having a SH-group, such as GSH, because of their incapability of reducing Fe(III).

Consistently with general recommendations about the use of biochemical markers in oxidative stress assessment, it would be better to carry out FRAP assay coupled with other available tests in order to enforce FRAP results interpretation (Griffin and Bhagooli, 2004).

### 1.5.3 ENDOCRINE DISRUPTION ASSESSMENT

As reported in Par. 1.3.3, EDCs have been associated to many cases of reproduction and development abnormalities in wildlife, including both vertebrates and invertebrates species (Porte et al., 2006). The assessment of endocrine disruption in marine environments is commonly carried out by the use of biomarkers. The most suitable methods are based on changes in hormone titres (*i.e.* steroid hormones), abnormal gonad development, low gamete viability, alteration of specific enzymes' activity (*i.e.* aromatases) and of protein levels (*i.e.* vitellogenin, zona radiata proteins, spiggin). They are usually performed on fishes, because vertebrate hormonal system is more understood in comparison with invertebrates. However some of them, such as the induction of vitellogenin-like proteins and zona radiata proteins, have been carried out in invertebrate species by analogy with vertebrate systems; in these cases, critical use and further research are highly recommended. Probably one of the most performed endocrine disruption biomarker in invertebrate species is Imposex (or Intersex) development in gastropod molluscs. This sexual malformation, induced by TBT exposure in more than 150 species belonging to Prosobranchia subclass (Oehlmann et al., 2007), is a clear example of *effect* and *exposure biomarker* (Cajaraville et al., 2000): in fact, besides focusing on a deformity which can lead to organism impairment and population instability (Bryan et al., 1986), it shows high correlation with TBT pollution level.

#### 1.5.3.1 IMPOSEX-INTERSEX

##### 1.5.3.1.1 Method

*Imposex* has been defined by Smith (1971) as the superimposition of male sexual organs in female specimens; its variant *Intersex*, developed by Littorinidae species, is a graduated conversion of female pallial oviduct into male morphological structures (Bauer et al., 1995). The virilisation of female gastropods is an irreversible phenomenon which, in the most sensitive species, takes place at TBT water concentrations lower that 1 ng/L (Bryan et al., 1987).

The development of this malformation, despite being species-specific, can be basically described by a 4-6 stages-model: the early stages involve the formation of a vas deference and a small penis, the intermediate stages are characterized by the enlargement of male sexual structures, and the late stages are featured by pallial oviduct blockade or bursa copulatrix/capsule gland split, likely yielding sterilization.

For biomonitoring purposes, indexes expressing the extent and the severity of this TBT-mediated phenomenon have been based on the progressive transformation of female sexual organs. Those showing the best correlation with environmental TBT concentrations are:

- VDSI (Vas Deferent Sequence Index): mean Imposex stage in a population;
- ISI (Intersex Index): mean Intersex stage in a population;
- Mean Female Penis Length;
- FPrl (Mean Female Prostate Length);
- $\text{RPLI (Relative Penis Length Index)} = \frac{\text{Mean Female Penis Length}}{\text{Mean Male Penis Length}} * 100$
- $\text{RPSI (Relative Penis Size Index)} = \frac{(\text{Mean Female Penis Length})^3}{(\text{Mean Male Penis Length})^3} * 100$

Others Imposex/Intersex-related parameters, used to describe the extent of the phenomenon, are:

- Percentage incidence of Imposex/Intersex in the studied population;
- Percentage of sterile females in the studied population.

These indexes provide an estimation of both environmental TBT contamination and reproductive capability of wild gastropod populations. The evident relationship between Imposex/Intersex development and Darwinian fitness parameters is at the base of the high ecological relevance of this biomarker. In fact, the breeding activity of several gastropod species is compromised by the highest stages of malformation. For example, the advanced Intersex stages in the North Atlantic *L. littorea* are featured by ventral splitting and prostate gland formation, which prevent copulation and capsule formation, inhibiting egg-laying. Thus, the most affected *L. littorea* populations usually show few juveniles and low capability to produce planktonic eggs and veliger larvae. However no cases of *L. littorea* populations extinction have been recorded, thanks to the possibility of recruiting planktonic veliger larvae from less impaired areas (Bauer et al., 1995). Differently the late Imposex stages in *N. lapillus* are featured by the maximum extension of vas deference which overgrows the genital papilla, occludes vulva, invades the oviduct and, finally, displaces the capsule gland. Consequently, females are completely sterilized, because of the impossibility to expel the capsules which accumulate within the oviduct, often yielding split and animal death. In the most polluted areas *N. lapillus* populations declined or, even, disappeared (Bryan et al., 1986). In fact, differently from *L. littorea*, the lack of planktonic phase during larval development prevents the recruitment from healthy populations.

#### 1.5.3.1.2 Application

Imposex/Intersex development analysis is highly recommended as TBT biomarker at international level and it has been included in the Joint Assessment and Monitoring Program (JAMP) of the Oslo and Paris Commission (OSPAR).

Imposex/Intersex monitoring programs are designed as spatial surveys aimed at describing the geographical distribution of affected populations by the use of the Imposex/Intersex parameters. Though this approach it is possible to identify TBT point sources, the rate of TBT-dispersion/dilution and temporal changes in pollution pattern (in the case of surveys repeated at regular time intervals) (Davies, 2000).

In Europe, in particular in the Atlantic area, the species recognized as very suitable for large-scale TBT monitoring are *Nucella lapillus* and *Littorina littorea*. The dogwhelk *N. lapillus* is one of the most sensitive species, developing Imposex in presence of TBT concentrations lower that 1 ng/L. It is a really common species in the North Atlantic coast, whose ecology and reproductive biology have been well-characterized (Bryan et al., 1987; Gibbs et al., 1987; Oehlmann et al., 1991). Similarly, the periwinkle *L. littorea*, in spite of its lower sensitivity, is highly recommended as well, in particular for monitoring areas highly affected by TBT pollution (*i.e.* harbours and marinas), where usually *N. lapillus* expires. It is a very common species, present also in some European coastal regions where *N. lapillus* does not occur, such as the Baltic Sea and the southern part of the North Sea. ICES standard protocol n °37 is usually used as reference for carrying out the analysis (ICES, 2004b).

In the Mediterranean area, TBT biomonitoring programs are carried out using different species, such as *Hexaplex trunculus* (Axiak et al., 1995; 2000; Terlizzi et al., 1998; 1999; 2004; Rilov et al., 2000; Pellizzato et al., 2004; Chiavarini et al., 2003; Prime et al., 2006; Garaventa et al., 2007), *Stramonita haemastoma* (Terlizzi et al., 2000; Rilov et al., 2000), *Bolinus brandari*s (Solé et al., 1998; Ramòn and Amor, 2001), *Nassarius* spp (Stroben et al., 1992A; 1992B; Sousa et al., 2005; Pavoni et al., 2007).

###### 1.5.3.1.3 Advantages and limits

Even though some evidences from laboratory and field experiments do not exclude the involvement of other contaminants on gastropod sexual malformations (Oehlmann et al., 2000; Tilmann et al., 2001; Shulte-Oehlmann at al., 2000; Maran et al., 2006), this biomarker has been frequently used as alternative tool to chemical analyses in TBT monitoring programs, thanks to the good correlation between TBT environmental concentrations and Imposex/Intersex indexes. The test, besides providing a measure of TBT pollution levels, gives insights about the reproductive capability of natural gastropod populations living in affected areas (Oehlmann and Schulte-Oehlmann, 2003).

Compared to TBT monitoring approach based on traditional chemical analyses, Imposex/Intersex biomonitoring programs are limited by the availability of the test-organisms in the studied areas. Thus regions not included within the geographical distribution of the test-species or affected by TBT levels higher that its tolerance limit cannot be investigated through this biomarkers.

### 1.5.4 GENERAL HEALTH STATUS ASSESSMENT

To get insights about the general heath conditions of ecosystem's biotic constituents of might be very valuable for environmental quality assessment purposes. However, because of the great complexity of organism functioning, it is not easy to find out single endpoints of general toxic effect in complex systems highly interrelated. However some endpoints, ecologically relevant and primarily involved in the defence against generic insults, are considered fruitful in this context.

Lysosomes stability has shown great potential as non-specific biomarker and as indicator of general heath conditions because their impairment reflects the breakdown of the adaptive capacity to withstand toxic insults. Lysosomal destabilization can be induced by many chemical and non-chemical stressors (metals, organic pollutants, hypoxia, hyperthermia, osmotic shock, food depletion, bacterial infections, etc), acting separately or simultaneously (ICES, 2004a; Hauton et al., 2001); furthermore, due to the essential role of lysosomes in food digestion, proteins metabolism and immunocompetence, it can be linked to detrimental consequences on the growth, reproduction capability and energy balance (Bacchiocchi et al., 2002; Moore et al., 2004; Martins et al., 2005).

To assess lysosomal integrity many tests have been developed. They are based on different mechanisms accounting for lysosomal impairment (*i.e.* membrane instability, lysosomal lipofuscins content, lysosomal neutral lipid accumulation, lysosomal swelling). Lysosomal stability assay and Neutral Red Retention assay, which measure lysosomal membrane integrity, have been well tested and the methodologies standardized by ICES (ICES, 2004a). The use of this endpoint as indicator of general physiological state and early signal of pathologies is based on the critical importance of the intactness of lysosomal membrane in the cells well-being: in fact, leakages of lytic enzymes into the cytosolic compartment can lead to auto-degradation of macromolecules and other cellular constituents, up to severe damages and cellular death (Winston et al., 1996).

#### 1.5.4.1 NEUTRAL RED RETENTION

##### 1.5.4.1.1 Method

Neutral Red Retention assay is a method to measure lysosomal membrane stability in living cells. It is based on the ability of the lipophilic Neutral Red dye to permeate into cell membrane and to be trapped into the lysosomes by protonation. The efficiency of lysosomes in retaining the dye depends on the status of membrane proton pump. Basically the assay measures the time lysosomes last in retaining the dye. According to the method proposed by

Lowe et al. (1992), after incubation in a Neutral Red solution, the cells are observed at regular time intervals under light microscope, in order to catch the start of dye leakage into the cytosol (lysosomal retention time) (Figure 1.9). Retention time values provide a measure of lysosomal tendency to realise the acid hydrolase content, causing cellular impairment up to cell death (Hauton et al., 2001).

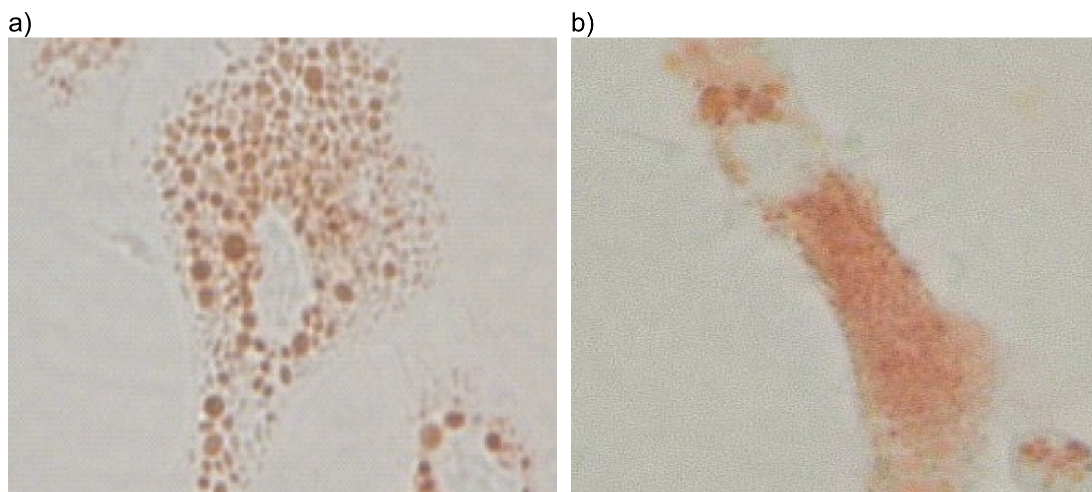



**Figure 1.9 Appearance of cells during NRR assay. Picture a: unstressed cells showing NR dye inside the lysosoms; Picture b: stressed cells with NR dye into the cytosol (ICES, 2004a).**

At the beginning, NRR assay was developed for fish hepatocytes, mussels digestive gland cells and oyster digestive gland cells, requiring the sacrifice of the organism and the digestion of tissue samples. Subsequently the assay was adapted to blood/haemolymph cells, becoming a non-destructive method, feasible for both *in vivo* and *in vitro* studies (ICES, 2004).

The potential of lysosomal membrane stability as non-specific biomarker of xenobiotics exposure has been deeply tested, and now it is generally used as “non-specific index of physiological stress”, showing good relationship with pollution gradient (Da Ros et al., 2002; Castro et al., 2004; Hwang et al., 2002; Fernley et a., 2000; Lowe and Pipe, 1994; ICES, 2004; Mamaca et al., 2005). However, NRR response can provide further information. In fact the possibility to perform the test on different types of cells (*i.e.* digestive gland epithelial cells and haemocytes) makes the assay suitable for assessing the status of specific cells and their efficiency in carrying out their functions. In fact, for example, lysosomal destabilization of molluscs’ haemocytes can be used as endpoint of immune status of test-organism (Hauton et al., 2001; Grundy et al., 1996; Fernley et a., 2000). In fact molluscs’ haemocytes are important components of the internal defence against pathogens (Winston et al., 1996; Grundy et al., 1996; Gorbushin and Iakovleva, 2006; 2007): the level of the lysosomal instability provides insights about the efficiency in removing pathogens through autophagic activity and it is prognostic for pathologies (Galloway et al., 2006; Cajaraville et al., 1996; Martins et al., 2005).

Moreover lysosomal impairment can be linked to the presence of oxidative unbalance, because of lysosomes' role in recycling damaged cellular components and in storing the final products of oxidative processes (*i.e.* lipids and lipofuscins). In fact, inverse correlation is frequently assessed between lysosomal membrane stability and oxidative stress endpoints, such as DNA damage, lipofuscins formation, lysosomal swelling and autophagic accumulation of lipids (Valavanidis et al., 2006; Nigro et al., 2006). From this the use of this biomarker in surveys assessing oxidative stress: this endpoint can give helpful information to correctly interpret the oxidative status description provided by traditional biochemical assay (Par. 1.5.2) (Regoli et al., 2006; Moore et al., 2006).

###### 1.5.4.1.2 Application

The inclusion of NRR assay in biomonitoring strategies as rapid and integrated indicator of non-specific physiological stress is highly recommended (ICES, 2004). In the context of field studies, this test has been successfully performed on fishes, bivalves and, less frequently, on other invertebrate species (Martins et al., 2005; Koukouzika and Dimitriadis, 2008; Hauton et al., 2001; Lowe and Fossato, 2000; Fernley et al., 2000; Da Ros et al., 2002; Castro et al., 2004, Hwang et al., 2002; Reid and MacFarlane, 2003).

NRR assay has been included as core biomarker in many international biomonitoring programs, such as BEEP program (EU VI Framework Program) (Viarengo et al., 2007), GEF International Water Programme (UNESCO-IOC), MEDPOL Mediterranean Action Plan (UNEP). Usually the protocols used as reference are those reported in ICES (2004) and in UNEP/RAMOGE (1999).

Because lysosome membrane stability represents an integrative parameter reflecting the onset and progression of pollution-induced changes, it has been often used as benchmark against which other toxic responses are evaluated (Broeg et al., 2005).

###### 1.5.4.1.3 Advantages and limits

Lysosomal alterations are among the most robust and sensitive effect biomarkers (Cajaraville et al., 2000); its suitability as non-specific physiological stress biomarker has been careful assessed by laboratory and field experiments. The test, which is easy and inexpensive to perform, can be carried out on very small amount of sample.

Lysosome membrane stability seems not to be particularly influenced neither by typical environmental fluctuations (water temperature, salinity, pH, chlorophyll *a*, food, oxygen) nor by test-organisms' features (*i.e.* size) (ICES, 2004; Lowe et al., 2006; Ringhwood et al., 1998; Petrović et al., 2004). However, even thought the endpoint is more sensitive to the direct action of stressors (Lowe and Fossato, 2000) rather than to physiological changes (such as those related to increased metabolic demand during gamete development and spawning activity), a decreased sensitivity of NRR assay during the reproductive period has

been often recorded (Petrović et al., 2004; Kagley et al., 2003; Cho and Jeong, 2005; Pytharopoulou et al., 2006).

## 1.6 BIOMARKERS APPROACH FOR MARINE POLLUTION ASSESSMENT

According to several authors (Hagger et al., 2008; 2009; Borja et al., 2008), Water Framework Directive (2000/60/EC)[25] represents an important opportunity to legally promote biomarkers use in European environmental management strategies. In fact, in order to classify and monitor the ecological quality of water bodies, WFD requires the assessment of the *ecological status* of all *biological elements* constituting the aquatic ecosystem (*i.e.* phytoplankton, macroalgae, benthos, fishes). Consequently, the scientific community is working for developing monitoring tools to evaluate the degree of deviation from the reference status, according with the requirements of WFD.

The ecological status of biotic components is traditionally assessed through biodiversity and community studying approaches. However there is growing awareness that this approach could not be adequate to efficiently protect the biota. In fact, the experience acquired on this field has highlighted that, when detrimental effects are visible at population and community levels, impairing processes may be at their irreversible stage and recovery may be impossible. In this context several authors have suggested that biomarkers can help to cover this lack by warning about the occurrence of stress syndromes at early stages and the possibility of environmental deterioration (Moore et al., 2004; Hagger et at., 2009; Galloway et al., 2006 and references therein).

In the context of biomarker-based environmental quality assessment, the experience has pointed out that only a combination of purposely selected biomarkers can provide relevant information (Broeg et al., 2005; Handy et al., 2003; Viarengo et al., 2000; 2007; Galloway, 2006; Anderson et al., 2006; Cajaraville et al., 2000). In fact field surveys designed to assess pollution-related quality of coastal areas and estuaries have proved that the integration of several responses distinguishes spatial differences in life conditions better than single biomarkers responses. This is because each biomarker reveals only one of the possible biological alterations induced by pollution and it may be sensitive to only one single (or class) of chemical pollutant.

As recommended by several authors (Moore et al., 2004; Adams et al., 1999; Broeg et al., 2005; Viarengo et al., 2007), multi-biomarkers based diagnostic systems should preferentially include both biomarkers of exposure and effect, and they should encompass the whole biological organization hierarchy (from molecular/cellular level up to population/community) and different types of stresses (*e.g.* genotoxicity, immunotoxicity,

---

[25] Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy.

endocrine disruption). This approach could enhance the possibility of obtaining responses reflecting both the nature and the extent of environmental deterioration, finding out relationships between stressors and biological effects and discriminating between natural and anthropogenic stressors (Kagley et al., 2003). Furthermore the effort in integrating the responses of exposure to contaminants has to be included in this approach.

The results of multi-biomarkers data analysis through multivariate statistical methods can provide more powerful and practical information for forming a judgement about the ecological state of water bodies than single biomarkers: they, in fact, can be used to discriminate among sites having different life conditions and to quantify the biological impact of pollution. Furthermore they can help to identify the relationships between different biological effects and to evaluate the relative contribution of each biomarker in discriminating the environmental quality (Galloway et al., 2006).

As regard multi-biomarkers data analysis, several authors have attempted to compute biomarkers-based indexes expressing the overall *health status* of wild populations of species having high ecological relevance. Table 1.9 lists some recent examples of biomarkers-based indexes and field studies using multivariate statistical methods to holistically evaluate the effects of environmental stressors on the health of target species.

**Table 1.9 Example of integrative biomarker-indexes**

| | |
|---|---|
| **BRI (Biomarker Response Index)**<br>(Hagger et al., 2008; 2009) | It is a potential measure of sentinel organism's health. Basically it is computed as sum of biomarkers' scores weighted according to the endpoint relevance. BRI has been successfully tested on different invertebrate species (the crab *Carcinus maenas* and the mussel *Mytilus edulis*). |
| **BAI (Bioeffect Assessment Index)**<br>(Broeg et al., 2005) | This index is a modified version of the Health Assessment Index (HAI) proposed by Adams et al. (1993). It measures the toxicants-induced deviation from the normal condition of the fish physiology by the use of a selected suite of biomarkers of general toxicity. Data integration is performed through cluster analysis. |
| **IBR (Integrated Biomarker Response)**<br>(Beliaeff and Burgeot, 2002) | This index is calculated by exploring the multi-biomarkers dataset through Star Plot visual method. According to this method, the total area of the star plotting biomarkers responses recorded for each population represents the integrated biomarkers response index (IBR). |
| **Adams et al., 1999** | Field study carried out on representative fish species. The description of each population was provided by a |

suite of biomarkers: data were jointly analysed by using a canonical discriminant analysis procedure. The method allowed to identify differences on health status among sites and to recognize the variable that contributed the most in discrimination.

### 1.6.1 BIOMARKER RESERCH CHALLENGES

Considering the prospects that the recent environmental legislation has opened for the effective use of biomarkers as environmental monitoring tools, fundamental goals of the ongoing research on multi-biomarkers strategies are:

1) Creation, validation and implementation of robust, simple, easy to learn and cost-effective standard operating procedures for the identification of early diagnostic changes in the biota, linked to ecologically relevant endpoints (Moore et al., 2004). Acquisition of robust knowledge about the basal values and seasonal fluctuations of biomarkers' responses (Cajaraville et al., 2000);
2) Understanding the interrelations among effects at different levels of biological organization (Moore et al., 2004);
3) Development of procedures for multibiomarker data management (Viarengo et al., 2000; Beliaeff and Burgeot, 2002);
4) Finding efficient statistical methods to holistically interpret the variations among several biomarkers in order to infer robust diagnoses about the physiological status of test-organisms (Beliaeff and Burgeot, 2002; Pampanin et al., 2006; Broeg et al., 2005; Moore et al., 2006B; Anderson et al., 2006);
5) Development of decision support (*expert*) systems linking chemical, biological and ecological data with expert judgement and contextual information (Viarengo et al., 2000; Hagger et al., 2009).

According with Bowen and Depledge (2006), pragmatic and easy to use methodologies which integrate information into models may be very useful tools for guiding management actions and for prioritizing locations. In fact these models, which require extensive validation and continuous refinement, can provide readily interpretable information to the policy makers, available in an understandable form.

## 1.7 SENTINEL ORGANISMS IN BIOMONITORING PROGRAM

In monitoring programs biomarkers are performed on *sentinel organisms*, which are typically mussels and demersal fish, such as flounders (Lam and Gray, 2003). Sentinel organisms may be defined as organisms with high potential in describing environmental quality and in detecting possible human health impacts arising from environmental contamination (Di Giulio et al., 1989).

The efficiency of biomonitoring strategies largely depends on the capacity of sentinel organisms to provide meaningful and accurate information about environmental quality. This largely depends on both biological-ecological features of test-species (*i.e.* geographical distribution, position on the trophic chain, sensitivity) and the possibility to perform a wide set of biomarkers, selected among the most proper for the research purposes (Di Giulio et al., 1989).

From this the permanent effort of ecotoxicologists to test the suitability of new candidates as sentinel organism, by evaluating the relative susceptibility to different environmental stressors and to transfer biomarker methodologies between species (Galloway et al., 2006; Moore et al., 2004).

### 1.7.1 MULTI-SPECIES BIOMARKER APPROACH

A typical objection arisen against biomarkers-based monitoring approach refers to a possible oversimplification in inferring a diagnosis about the state of aquatic systems (status, functions, integrity) from observations referred to few individuals of a single species (Calow and Forbes, 2003; Galloway et al., 2006). In fact, considering that the biotic compartment is featured by complex processes and dynamic interactions, the examination of a small subsystem could be inadequate to understand the overall functioning and to assert about the general state. In fact, the environmental quality characterization obtained by referring to single species is likely strictly related to the biological and ecological features of the selected sentinel species (*i.e.* feeding habits, trophic position). Taking mussels as examples, their physiological status is strictly related to the quality of water column, or the pollutants dissolved and adsorbed to suspended particular matter. From this derives the idea of developing biomonitoring strategies using a suite of organisms belonging to different phyla and occupying various key-positions in the ecosystem (Depledge and Fossi, 1994; Galloway et al., 2004; 2006). According to this approach, an opportune combination of ecotoxicological analyses should be performed in selected sentinel species, having different feeding habits (*i.e.* filter feeder, grazers, omnivores, predators, scavengers), trophic positions and lifestyles.

Within this strategy, invertebrate species are considered of a great interest, thanks to their abundance and biodiversity (Galloway et al., 2006; Depledge and Bullinghurst, 1999).

The literature reports several proposals for "multi-biomarkers – multi-species" monitoring approach and field surveys testing the effectiveness in combining biomarker analyses on different animals to characterize the environmental quality of coastal and estuarine areas (Galloway et al. 2004; 2006; Bloxham et al., 2004; Anderson et al., 2006; Bowen and Depledge, 2006).

### 1.7.2 MOLLUSCS

Molluscs, especially bivalves and gastropods, are powerful invertebrate model-systems for biomonitoring purposes. In fact, several mollusc species are common, highly visible and ecologically important. The sessile/sedentary behaviour and feeding habits, in addition to their fairly resistance to chemical contamination, make them efficient environments "bioaccumulators" (Rittshof and McClellan-Green, 2005).

Nowadays great knowledge exists about mollusc biochemistry, physiology, molecular biology, genetics and neurobiology. The most studied species are the sea hare *Aplysia brasiliansis* and the freshwater pond snail *Lymnaea stagnalis*, about which extensive literature is available (for review article see Rittshof and McClellan-Green, 2005). However, also commercial species, such as abalone, mussels, oyster, scallops, clams and predator molluscs, such as whelk and dogwhelks, have been deeply studied. These studies have highlighted that many of the control pathways for chemical and biological processes in molluscs are highly conserved (Rittshof and McClellan-Green, 2005).

Among mollusc species, at the beginning the attention of environmental scientists was addressed to bivalves, which nowadays are employed in monitoring programs at national and international level (Viarengo et al., 2007). In fact bivalves are excellent bioaccumulators, thanks to their filter-feeding habits and low metabolic transformation rate (Galloway et al., 2006). The analysis of contaminants tissues concentrations allows to reveal the bioavailable fraction of micropollutants which are more difficult to detect in other environmental matrices (*i.e.* water, sediments) (Baumard et al., 1998). Since the mid 1970s, mussel species are used as sentinel organisms in *Mussel Watch Biomonitoring Programs*, a strategy proposed by Dr. Goldberg who, besides considering the advantages related to their wide distribution and sampling easiness, intuited that the assessment of contaminants concentration in their tissues could provide a time-integrated evaluation of water column quality. Afterwards this approach has been enforced by the introduction of biomarkers, rapid tools for revealing contamination-related stresses (Rittshof and McClellan-Green, 2005; Viarengo et al., 2000 and references therein).

## 1.7.3 GASTROPODS

More recently gastropods have attracted the research attention for their potentials as sentinel organisms. This class is constituted by species having different feeding habits (carnivorous, scavengers, herbivorous) and wide geographical distribution. Thanks to Intersex/Intersex development, several species are well known to the ecotoxicologists and they are already included in monitoring programs as bioindicator of TBT pollution (Oehlmann et al., 2007).

### 1.7.3.1 LITTORINA LITTOREA (LINNAEUS, 1758)

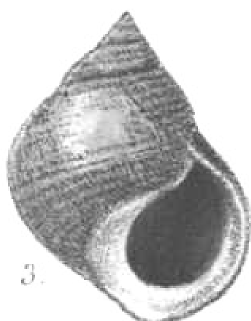


**Figure 1.10** ***L. littorea***
**< www.chestofbooks.com>**

The periwinkle *Littorina littorea* (Gastropoda, Littorinidae) is one of the most common North Atlantic gastropod species, particularly abundant in UK and in the north-western European coast. It lives in almost all kinds of shore (rocky, sandy and muddy), in estuaries and mudflats, occupying the intertidal and subtidal zone (up to 15 m depth). Usually it prefers sheltered areas, like small stones, gullies or surfaces covered by fucoids, where it is bulky found in heaps during the low tide (Lowe et al., 2006; Bauer et al., 1995). *L. littorea* is a quite long-lived species, living for more than 9 years. It reaches the sexual maturity at the age of 12-18 months: females produce about 500 planktonic egg capsules, containing 1-5 eggs each; after 5-6 days eggs hatch as free swimming veliger larvae (Bauer et al., 1995 and references therein).

The feeding habit as detritus grazer makes *L. littorea* very interesting as sentinel organism. In fact, in addition to pollutants uptake from dissolved and re-suspended phases, periwinkles ingest contaminants absorbed to algae, sea-lettuce, detritus and microorganisms (Lowe et al., 2006; Bauer et al., 1995). This is particularly interesting because one of the most toxic matrixes of marine environment is represented by organic and inorganic particulates and colloidal organic carbon on which many pollutants, in particular those highly hydrophobic as the organic compounds, tend to adsorb (Cornelissen et al., 2006; Baumard et al., 1998; Soclo et al., 2000; Moore et al., 2004). Considering that particulate matters tend to sediment, the health status of benthic species living in contact with sediment and having scavenger/detritus-grazer habits is strictly linked with the quality of this matrix (Verrengia Guerrero et al., 2003).

The use of periwinkles as model of grazers in environmental monitoring strategies has been supported by Lowe et al. (2006). In fact, it is positively considered the opportunity of

obtaining a marine quality description which could be complementary to that provided by traditional sentinel species, such as filter-feeding mussels.

The anatomical and biological characterization of common periwinkles has been well assessed by Fretter and Graham (1994) and currently many data exist on physiologically relevant parameters (De Lange and Minnen, 1998; Larade and Storey, 2004; Gorbushin and Iakovleva, 2006). In particular it is relevant the functional characterization of *L. littorea* blood cells taken as models for the whole Prosobranchia subclass (Gorbushin and Iakovleva, 2006; 2007). Haemocytes, in fact, constitute the sample-matrix for many biomarkers proposed as rapid tools in biomonitoring strategies (*i.e.* NRR assay, Comet assay, MN assay, Phagocytosis assay). According to the most recent findings, *L. littorea* haemogram differs from that of common bivalves in the absence of granular haemocytes: in suspensions, haemocytes appear generally spherical, rarely elongate with short flexuous podia. Agranulocytes are reported as hyalinocytes, because of the hyaline-glassy appearance of cytoplasm under light microscope observation. They are subdivided into three morphologically distinct types, differencing mainly for nucleoplasmic ratio, nucleus shape and location, number and size of glycogen deposits. As proposed by Gorbushin and Iakovleva (2006), the three morphotypes correspond to different stages of maturation (*i.e.* juvenile, intermediate and mature):

- *Morphotype I*: juvenile round cells (5-6 mm in diameter) without glycogen aggregates. The central nucleus has regular shape and high nucleo-cytoplasm ratio. The cytoplasm is scarce and contains few organelles (*i.e.* mitochondria, tubules of smooth endoplasmic reticulum, rare vacuoles, few lipid droplets, numerous free ribosomes (giving basophilic properties to the cytoplasm), several lysosomes).
- *Morphotype II*: intermediate cells (about 6 mm). Heterogeneous subpopulation demonstrating a lower nucleoplasmic ratio compared with the morphotype I haemocytes. These cells have a more or less eccentrically located nucleus and several small glycogen aggregates together with numerous single glycogen particles scattered throughout the cytoplasm. They also contain mitochondria with a dense matrix, rough endoplasmic reticulum and developed smooth endoplasmic reticulum similar to that found in haemocytes of the previous type.
- *Morphotype III*: large mature haemocytes (7^8 mm) having a prominent glycogen deposit. These cells, which are asymmetrical because of the eccentrically located nucleus, have a large glycogen deposit and abundant smooth endoplasmic reticulum. Cisternae of rough endoplasmic reticulum are present around the nucleus and near the narrow cell cortex. Generally these mature cells are featured by small nucleoplasmic ratio.

As reviewed by Gorbushin and Iakovleva (2006), glycogen deposits might result from the metabolic conversion of phagocytised material during intracellular digestion: under this hypothesis, the size and form of the glycogen deposits should partially reflect the phagocytic abilities and the degree of maturation of the cell. These glycogen deposits can likely serve as store of energy for possible anoxic periods (low tide) during which glycolysis is the only source of ATP.

It is known that haemogram is featured by seasonal-physiological variations in both cells abundance and morphotypes composition. From a study carried out on *L. littorea* specimens sampled from the White Sea, cell concentration ranges between 700 to 4000 cells per ml (mean values estimated around 2000 cells per ml), reaching two concentration picks during the summer. The proportion of morphotype II and III respectively decreases and increases during the summer, whereas the subpopulation of morphotype I haemocytes seems to be constant (around 15%). Haematopoiesis takes place throughout the blood circulating system, as suggested by the presence of mitotic cells in the haemolymph of *L. littorea* (Gorbushin and Iakovleva, 2006).

From functional studies it is known that haemocytes possess Gorbushin and Iakovleva (2007):

- *Phagocytic capability*. It has been assessed that around 70-85% of haemocytes population showed phagocytic capability, suggesting that the non-phagocytic subpopulation might correspond to the juvenile morphotype.
- *Potent ROS generating system.* The multicomponent enzyme NADPH-oxidase associated to cellular vesicles is the main responsible of superoxide anion formation. Haemocytes can address ROS secretions to both intra- and extracellular targets, depending on the origin of the stimuli.
- *Cytotoxic (haemolytic) activity against non-self material*. The secretion of lytic factors, which are toxic for non self-cells, can be induced in haemocytes. Although it is not certain the exact mechanism through which haemolysis occurs, it is likely that ROS production is involved.

In spite of *L. littorea* relevance in TBT biomonitoring, there is not extensive knowledge about periwinkle sensitivity to the main classes of pollutants; only few ecotoxicological studies, focusing on endpoints different from Intersex development, have been carried out on this species (Downs et al., 2001; Lowe et al., 2006; Morcillo and Porte, 1997; Romeo et al., 2006); differently there is a wide literature about Intersex development on *L. littorea*, consisting in both laboratory and epidemiological studies (Bauer et al., 1995; 1997; Barroso et al., 2000; Davies and Minchin, 2002).

### 1.7.3.2 HEXAPLEX TRUNCULUS (LINNAEUS, 1758)

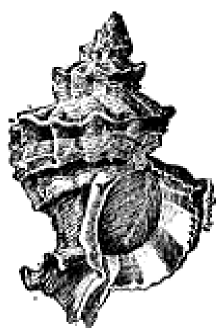


**Figure 1.11 *H. trunculus* < www.gutenberg.org>**

*Hexaplex trunculus* (Gastropoda, Muricidae) is one of the commonest gastropod species in the Mediterranean Sea, also present along the Portuguese coast and in Canarias. It lives in any kinds of substrate (rocky, sandy and muddy), up to 100 m depth, preferably occupying low energy habitats, such as lagoons, small bays and places sheltered by rocks (Terlizzi et al., 1998). The most abundant populations are observed in soft bottom areas, close to farms of oysters and mussels, because it is a voracious predator (Peharda and Morton, 2006; Siboni et al., 2004).

*H. trunculus* has been frequently used in Mediterranean monitoring programs thanks to its high sensitivity to low TBT water concentrations. Good correlation has been assessed between TBT levels and Imposex parameters (Axiak et al., 1995; 2000; 2003, Terlizzi et al., 1998; 1999; 2004; Rilov et al., 2000; Pellizzato et al., 2004; Chiavarini et al., 2003; Prime et al., 2006; Garaventa et al., 2007). As sentinel species for biomonitoring studies *H. trunculus* presents several positive features. In fact the lack of planktonic larval stage and the scarce mobility makes it potentially suitable for assessing small-scale spatial variations of environmental quality. Furthermore, as operative advantage, the big body size and abundance encourage laboratory manipulation and sampling activities (Siboni et al., 2004).

Except its involvement in TBT monitoring studies, *H. trunculus* has not been frequently used as test-organisms in ecotoxicological studies, explaining the scarce knowledge about its sensitivity to common environmental toxicants (Siboni et al., 2004). However, according to Rilov et al. (2004) its use in biomonitoring strategies could provide interesting information thanks to its feeding habits: *H. trunculus* bioaccumulates high concentration of contaminants thanks to its wide diet as carnivorous (eating mainly barnacles and molluscs) and, occasionally, as cannibal and saprophagous.

### 1.7.3.3 NASSARIUS NITIDUS (JEFFREYS, 1867)

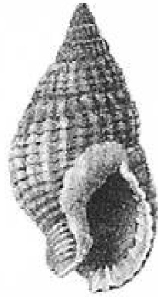


**Figure 1.12 *N. nitidus* <www.wikipedia.org>**

As well explained in Pavoni et al. (2007), *Nassarius nitidus* (Gastropoda, Buccinidae) corresponds to the prosobranch gastropod species generally reported as *N. reticulatus mamillatus* (Risso, 1826), a Mediterranean subspecies of the Atlantic *N. reticulatus*. It probably originated from *N. reticulatus* specimens which entered the Mediterranean Sea from the Strait of Gibraltar, colonizing the Spanish shoreline (Rolán and Luque, 1994). According to the supporters of this theory, the Mediterranean distribution of *N. reticulatus* would be limited to the western coast, whereas *N. nitidus* would be present in the whole Mediterranean area.

Even though there is not unanimous agreement in considering *N. reticulatus* and *N. nitidus* as distinct species, *N. nitidus* has been recently registered as separate species in the European Register of Marine Species (Costello et al., 2004) and in the Checklist of the Species of the Italian Fauna (Minelli et al., 1993).

Considering the high similarity between the two species and the common custom of environmental scientists to generally refer to *N. reticulatus sensu latu*, in the present PhD thesis the bibliographic references about *N. reticulatus* were used to acquire knowledge about *N. nitidus*, consistently with Pavoni et al. (2007). Thus referring to *Nassarius* (*Hinia*) *reticulatus*, this species is commonly found in the Atlantic area, from Azores and Canaries to the Baltic Sea, as well as in the Mediterranean and Black Sea. Its ecology and anatomy are extensively described by Fretter and Graham (1984). It is a borrowing species, living on patches of soft materials along both sandy and rocky shores; even if it usually occupies the intertidal zone (up to 15 m depth), it is found also in off-shore areas (Santos et al., 2004). It is not very sensitive to salinity changes, surviving within the range 20-35‰ (or less), explaining its presence also in areas which receive high inputs of freshwater, such as lagoons and estuaries. *N. reticulatus* is a saprophagous species, eating carrions of *Patella sp.*, *Mytilus sp.* and other invertebrates and fishes. These feeding habits make *N. reticulatus* an interesting species for ecotoxicological research; studies on TBT bioaccumulation pointed out that more than half TBT body burden is due to dietary uptake (Stroben et al., 1992B; Pavoni et al., 2007 and references therein).

*N. reticulatus* has been extensively used as TBT bioindicator, thanks to the advantages provided by its widespread distribution and high colonization capability (Pavoni et al., 2007). In fact the three months-planktonic larval phase (free-swimming veliger larvae) is responsible of its high tendency toward dispersion. Furthermore, because Imposex does not

affect the integrity of ovary, female pallial glands and vaginal opening, the most affected populations do not risk the extinction for sterilization (Stroben et al., 1992A).

# 1.8 RESEARCH GOALS AND EXPERIMENTAL DESIGNS

## 1.8.1 RESEARCH LINE 1

Two field studies are carried out in order to assess the suitability as *sentinel organisms* of common gastropod species, used until now only in TBT biomonitoring programs. The feasibility of selected biomarkers and their performance in discriminating pollution-related pressures of different nature and intensity are tested by matching the toxicological and chemical characterization featuring wild populations living along a pollution gradient.

The gastropod species used as test-organisms are chosen among those commonly used in TBT biomonitoring programs, living also in very poor quality areas. Furthermore the test-species must guarantee sampling easiness and the possibility to extract the heamolymph volume required for performing the bioassays (1 ml).

The selection of the biomarkers suite is done considering the possibility of assessing the presence of stress syndromes of different nature and involving various levels of biological organization. The tests are chosen among those easy and cost-effective to perform, for which the laboratory is supplied by the necessary equipments and experience.

### 1.8.1.1 EXPERIMENT 1

Experiment 1 is an epidemiologic survey carried out thanks to the collaboration and partnership of Prof. Tamara Galloway during the eight month-research experience as PhD visiting student at Exeter University (UK). It aims at characterizing the health conditions of *L. littorea* populations living in areas differently affected by pollution. In particular the research goals are:

- To assess the presence of stress syndromes associated to genotoxicity, oxidative stress, cytotoxicity and endocrine disruption by the use of a selected suite of biomarkers;
- To evaluate possible relationships between biomarkers responses, measured at sub-organism and organism levels, and the exposure to specific environmental mixtures of organic pollutants;
- To enforce the knowledge about the sensitivity of *L. littorea* to environmental pollution.

Briefly, the experimental activities consist in performing a suite of chemical and ecotoxicological analyses on pools of organisms representative of six adult populations of *L. littorea* living along British south and south-west coast. The samples are both chemically and

toxicologically characterized by determining tissues concentrations of the main classes of marine organic pollutants (PAH, OTC, PCB and OCP) and by applying a suite of toxicological tests which can provide insights about the occurrence of impairing processes at different levels of biological organization (Intersex development analysis, Comet assay, NRR assay and FRAP assay). Table 1.10 lists the chemical and ecotoxicological analyses included in the experimental design. Sampling activities and biological tests are performed under the supervision of Prof. T. Galloway and with the support of the Invertebrates Toxicology Laboratory (Exeter University); bioaccumulation analyses are carried out at the Environmental Chemistry Laboratory of Ca' Foscari University (supervisor Prof. B. Pavoni).

Details about the methods used for both kinds of analyses are reported respectively in Par. 2.3 and Par. 2.4.

Thus, biomarkers performance is evaluated in term of 1) capability in discriminating different contamination levels, 2) consistency with the presence of specific pollutants, known for inducing the assessed stress, and 3) correlation with other responses induced by common toxic mechanisms.

**Table 1.10 Experiment 1 schedule: ecotoxicological and chemical analyses**

| **CLASSES OF STRESS ASSESSED** (BIOLOGICAL ASSAYS) | **CLASSES OF ORGANIC CONTAMINANTS** |
|---|---|
| **GENOTOXICITY** | **POLYCYCLIC AROMATIC HYDROCARBONS (PAH)** |
| (COMET ASSAY) | Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene, Benz[k]fluoranthene, Benz[a]pyrene, Dibenzo[a,h]anthracene, Benz[ghi]perylene |
| **OXIDATIVE STRESS** | **POLYCLOROBIPHENILS (PCB)** |
| (FERRIC REDUCING ANTIOXIDANT POWER ASSAY) | PCB 18, PCB 28, PCB 52, PCB 155, PCB 101, PCB 77, PCB 123, PCB 118, PCB 153, PCB 105, PCB 138, PCB 126, PCB 185, PCB 156, PCB 157, PCB 180, PCB 198, PCB 169, PCB 170, PCB 194, PCB 209 |
| **ENDOCRINE DISRUPTION** | **ORGANOCHLORINE PESTICIDES (OCP)** |
| (INTERSEX DEVELOPMENT ANALYSIS) | $\alpha$– HCH, $\gamma$–HCH, HCB, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, p,p'-DDT, o,p'-DDT, |
| **GENERAL PHYSIOLOGICAL STATUS** | **ORGANOTIN COMPOUNDS (OTC)** |
| (NEUTRAL RED RETENTION ASSAY) | TBT, DBT, MBT, TPhT, DPhT, MPhT |

#### 1.8.1.2 EXPERIMENT 2

The main aim of Experiment 2 is to test Experimental 1 design in the Adriatic area, by using a common gastropod, *Hexaplex trunculus*, which seems to have positive features as sentinel organism for the Mediterranean Sea.

More precisely the goals of Experiment 2 are:

- To adapt the protocols of ecotoxicological assays to the specific features of *H. trunculus*;
- To verify the sensitivity of *H. trunculus* along a pollution gradient, by matching biomarker responses to the organic contaminants body burden.

Similarly to Experiment 1, the research consists in a field survey where samples of adult populations of *H. trunculus* are analysed for their organic contaminants content and health impairments.

Due to the preliminary character of this survey, a minor number of wild populations are considered. The three sampling sites are located in areas at increasing distance from contamination sources, both inside and outside Venice Lagoon. The suite of chemical and biological analyses included in Experiment 2 is reported in the following table (Table 1.11).

**Table 1.11 Experiment 2 schedule: ecotoxicological and chemical analyses**

| CLASSES OF STRESS ASSESSED (BIOLOGICAL ASSAYS) | CLASSES OF ORGANIC CONTAMINANTS |
|---|---|
| **GENOTOXICITY** | **POLYCYCLIC AROMATIC HYDROCARBONS (PAH)** |
| (MICRONUCLEI ASSAY) | Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene, Benz[k]fluoranthene, Benz[a]pyrene, Dibenzo[a,h]anthracene, Benz[ghi]perylene |
| **ENDOCRINE DISRUPTION** | **POLYCLOROBIPHENILS (PCB)** |
| (IMPOSEX DEVELOPMENT ANALYSIS) | PCB 18, PCB 54, PCB 28, PCB 155, PCB 101, PCB 77, PCB 123, PCB 118, PCB 153, PCB 105, PCB 138, PCB 126, PCB 185, PCB 156, PCB 157, PCB 180, PCB 198, PCB 169, PCB 170, PCB 194, PCB 209 |
| **GENERAL PHYSIOLOGICAL STATUS** | **ORGANOCHLORINE PESTICIDES (OCP)** |
| (NEUTRAL RED RETENTION ASSAY) | $\alpha$– HCH, $\gamma$–HCH, HCB, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, p,p'-DDT, o,p'-DDT, |
| | **ORGANOTIN COMPOUNDS (OTC)** |
| | TBT, DBT, MBT, TPhT, DPhT, MPhT |

The choice of using a suite of toxicological tests different from that employed in the previous experiment is primarily due to the different equipment available for ecotoxicological

investigation: in fact Experiment 2 is entirely carried out on the laboratories of Environmental Science Department - Ca’ Foscari University (Venice), which are not equipped for performing Comet assay and FRAP assay. For this reason the genotoxic status is assessed by MN assay instead of Comet assay, changing the endpoint from DNA damage to chromosomal damage. Differently FRAP assay is not substituted by other biochemical tests.

### 1.8.2 RESEARCH LINE 2

The final aim of the second research line is to experimentally test the observations pointed out by Maran et al. (2006) about the possible contribution of PCB in the induction of Imposex development. A laboratory experiment is in program in the Environmental Chemistry Laboratory of Ca' Foscari University (Venice) for this porpoise. It is based on the experimental design frequently used by Oehlmann and co-workers to assess the potential of several chemicals as estrogen, androgen and anti-androgen compounds in gastropods (Schulte-Oehlmann et al., 2000; Tillmann et al., 2001; Oehlmann et al., 2000). Basically it consists in a chronic exposure (3 months) of gastropod specimens to environmental concentrations of PCB through the diet. More in detail, the congeners cb138, cb153 and cb180, selected on the basis of their widespread presence in the environment, are taken as PCB-models; the exposure to test-toxicants is carried out in parallel to controls, in particular a negative control (not contaminated food), solvent control (food contaminated with ethanol, the solvent used to dissolve the organic test-toxicants) and positive control (TBTCl contaminated food). The choice of using contaminated food as exposure-mode is due to the proved evidences that dietary accumulation is at least as important as water exposure for many gastropod species (Stroben et al., 1992B, and references therein).

#### 1.8.2.1 EXPERIMENT 3

In the contest preliminary studies are carried out in order to:

- Acquire competence on managing sea-water aquaria and on running long-term exposure experiments;
- Test a poisoning system via food ingestion, fitted for the specific feeding behaviour of the test-organisms, and assess its effectiveness in term of bioaccumulation.

*Nassarius nitidus* is selected as test-organism for research line 2 investigations because of its high suitability for the designed experiment in terms of feeding behaviour, recruitment and laboratory resistance.

A set of three "pilot" laboratory tests, comprehensively referred as Experiment 3, are carried out to specificatelly assess:

- the effectiveness of a pre-exposure decontamination period for decreasing the contaminants' background residues in the test-specimens recruited from natural populations (*Decontamination test*);

- the effectiveness of two different poisoning systems, referred as *Recipe A* and *Recipe B* (*Recipe A test* and *Recipe B test*).

# 2 MATERIALS AND METHODS

## 2.1 RESEARCH LINE 1

### 2.1.1 SAMPLING SITES

#### 2.1.1.1 EXPERIMENT 1

The sampling sites were located in areas involved in different coastal activities, so presumably affected by different patterns and degrees of contamination. As shown by the map in Figure 2.1, two stations were located far away from big urban and industrial settlements (coastal stations), two inside Plymouth Sound, an open bay subject to the presence of many estuaries and relevant anthropic pressures (Plymouth Sound stations) and, finally, two in Southampton Water, a ria with high industrial density, mainly related to petrochemical activities (Southampton Water stations). Details about the sampling areas are reported in the following sub-paragraphs.

**Figure 2.1 South England map. Geographical location of sampling areas**

#### 2.1.1.1.1 Coastal stations

**Figure 2.2 Geographical location of Mothecombe and Brixham sampling sites**

**Figure 2.3 Mothecombe beach <www.members.furtenecity.com/drakehhh/Resources/mothecombe.JPG>**

***MOTHECOMBE station***

*Mothecombe sampling station was selected as reference site. The beach, where the organisms were collected, lies on the Erme estuary, one of the rivers that flow on Dartmoor National Park, Devon* (Figure 2.3). *The estuary is completely surrounded by countryside, far away from any industrial activities, urban settlements and ports.*

**Figure 2.4 Brixham harbour <www.loddiswell.com/BrixhamHarbourBerryheadDSC00606.JPG>**

***BRIXHAM station***

*The samples were collected from a small cove, in proximity of Brixham. Despite the small size of the village, the harbour is quite lively. In fact the marina harbours a large number of boats used for fishing and tourism purposes, the two main economic resources of Brixham* (Figure 2.4).

2.1.1.1.2 Plymouth Sound

**Figure 2.5 Plymouth Sound map: geographical location of Torpoint and Mountbatten sampling sites**

Plymouth Sound (Figure 2.5) is an open bay partially sheltered by an artificial breakwater. The city of Plymouth (250,700 people in 2007[26]) is placed just in the middle of the Sound, behind the breakwater. A big part of its economy is linked to maritime and military activities carried out in Devonport Dockyard and in the close naval base. Devonport Dockyard is a big ship- and boat-building yard, spread along 4 km of shoreline: it was built in the 1960s and continually enlarged during the following years. It was the principal source of TBT in the area until the TBT ban on antifouling paints. Nowadays it is an important source of PAH due to combustions and general activities, together with urban run-off. Many rivers provide freshwater and pollution inputs to the Sound: the rivers Tamar, Tavy and Lynher flow into the inner part of the Sound, called Hamoaze, whereas the estuaries of the rivers Plym and Yealm open into the east part of the Sound, an area less sheltered than the Hamoaze. According to a study carried out by Langston et al. (2003), these rivers, whose catchments pass through areas with long mining history and high agricultural density, provide inputs of metals, pesticides and nutrients.

**Figure 2.6 Mountbatten beach; Plymouth city behind. <www.aphotofauna.com/page37.htlm>**

***MOUNTBATTEN station***

*The sampling site was located to the west of Plymouth (city), on the Plym estuary. This area is devoted to commercial and leisure marine activities* (Figure 2.6).

[26] From <http://www.plymouth.gov.uk/population> in August 2010.

Figure 2.7 View of Devonport Dockyard from Torpoint beach. <www.panoramio.com/photo/original/7688728>

***TORPOINT station***

*The sampling site was located on the mouth of the river Tamar, along the shore of Torpoint town. The estuary separates Torpoint from the city of Plymouth: this stretch of water is called Hamoaze. Just in front to the sampling site, in the opposite bank of the river, there is the Devonport Dockyard* (Figure 2.7).

2.1.1.1.3 Southampton Water

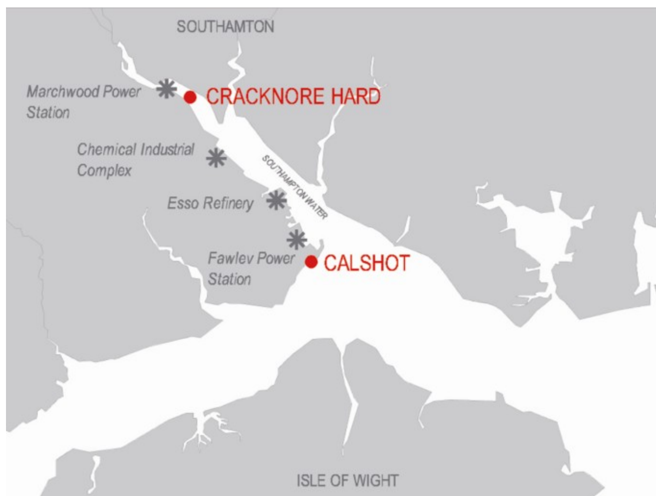



**Figure 2.8 Southampton Water map: geographical location of Cracknore Hard and Calshot sampling sites**

Southampton Water is a ria that opens in front of the Isle of Wight: it is 2 km wide, 10.3 km long and about 12.5 m depth in its central part, thanks to intense dredging activities (Figure 2.8). The substrate is generally mud or sandy mud, rich in organic matter; the western flank is occupied by salt marshes. Water mass circulation is predominantly controlled by fresh water discharges (the rivers Test, Itchen and Hamble), tidal circulation (tidal excursion ranges between 1.5 and 4.5 m) and atmospheric forcing (Levasseur et al., 2007).

Both urban and industrial activities have high density in the ria, influencing the environmental conditions of the area. Southampton, which lies on the east shore, is a medium-size city (228,600 people) whose economy is connected with maritime activities. Southampton's port is considered both the capital of the cruise industry, satisfying the largest share of British cruise passenger market, and the second largest container terminal in UK. The terminal covers a site of over 200 acres, and in 2007 it handled 1.9 million TEU.

The western shore has a high density of industries related to petrochemical activities. The most important plant is represented by Esso oil refinery in Fawley: it is the largest refinery in

UK and one of the most complex in Europe, handling around 2,000 ship movements and 22 million tonnes of crude oil and other products every year. Because of water use in the steam-cracking processes, high amount of hydrocarbon are spilled into the ria together with the effluents: it has been estimated that 300,000 tonnes of sea water are draws over from Southampton Water every day, and most of them return through outfalls. Despite the improvements in the effluents' quality achieved over the last two decades, the plant still remains a source of hydrocarbon pollution in the area (Croudace and Cundy, 1995). Just opposite to Fawley, on the River Hamble, there is the Hamble Oil Terminal, an important hub for oil and refined products.

In the north part of the western shore, there is Marchwood military port which homes army vessels run by the Royal Logistic Corps. Just beside the military port, there is an industrial park equipped with a refuse incinerator, a sewage working plant and a new gas power station that was under construction at the moment of the sampling.

Figure 2.9 Fawley Power Station and Calshot beach

***CALCHOT station***

*This sampling site was placed in the mouth of Southampton Water, at the south of Esso refinery and Fawley Power Station. The samples were collected in the beach used* for leisure water activities (water sports and sailing)*, towards the Solent Sea* (Figure 2.9).

**Figure 2.10 Esso refinery plant in Fawley <www.energyinst.org.uk/education/refineries/fawley.htm>**

***CRACKNORE HARD station***

*One sampling site was placed in Cracknore Hard, more precisely in the muddy beach located between the Marchwood Industrial Park and the Military Port, on the western shore* (Figure 2.10).

### 2.1.1.2 EXPERIMENT 2

The survey considered only three populations living both inside and outside the lagoon because part of the experimental effort was spent on adapting the toxicological methodologies (in particular MN protocol) to the specific features of *H. trunculus*.

**Figure 2.11 Venice Lagoon map. Geographical location of sampling sites**

Venice Lagoon was chosen as sampling area for practical reasons, being the closest suitable environment for recruiting *H. trunculus*. It is a complex lagoon system which receives pollution inputs from Porto Marghera industrial centre (PCB and PAH), from the bordering inland (pesticides and nutrients), from the city of Venice (urban runoff), and from the intense boating and shipping traffic (commercial cargo ships, oil tankers, cruise crafts, urban transport ships, yachts, tourist and fishing vessels).

Sampling sites were placed both inside and outside the lagoon (Figure 2.11): the inner sampling stations (Santa Maria del Mare and Certosa) were necessarily located in the proximity of the lagoon inlets, where the salinity is fairly close to that of open sea, allowing the prosper diffusion of *H. trunculus.* The location of the external sampling site (Adriatic station) was determined by the availability of fishermen who kindly donated the samples.

**Figure 2.12 Jesolo beach <http://www.val-gardena.ws/images/jesolo/.jpg>**

***ADRIATIC SEA station***

*The sampling site extern to Venice Lagoon was located along the coastline of Jesolo, at 3 miles distance from the coast* (Figure 2.12)*. The fishermen who managed the sampling did not provide the exact geographical coordinates of the station.*

Figure 2.13 Malamocco inlet
<http://www.aeroclubpadova.it>

***SANTA MARIA DEL MARE station***

*Santa Maria del Mare station was located closed to Malamocco inlet* (Figure 2.13)*. It was placed along the industrial channel which allows cargo ships and oil tankers to cross the lagoon, connecting the Adriatic Sea with the industrial area of Porto Marghera. Maran et al. (2006) refer to this site as a "low impacted area inside Venice Lagoon", featured by high tidal water exchanges.*

Figure 2.14 Certosa Isle
<http://media.hotelscombined.com/HI6732691.jpg>

***CERTOSA station***

*Certosa sampling station was located in the proximity of Lido inlet, the lagoon access most frequented by tourist and leisure boats, as well as commercial cargo ships* (Figure 2.14).

### 2.1.2 SAMPLING AND PRE-TREATMENT

#### 2.1.2.1 EXPERIMENT 1

*L. littorea* samplings were performed between 28th March and 4th April 2008. About 140 specimens per sampling station were collected by hand during low spring tide and immediately transferred to the laboratory inside a cold box. Once in the lab, 20 specimens devoted to the chemical analyses (reported as group C in Table 2.1) were placed in a separated glass tanks, filled with artificial seawater (T 11°C; 3,5% salinity; constant aeration), and let purge overnight. The day after they were stored at -20°C (until the use). The remaining organisms, devoted to toxicological analyses, were put in another aquaria (artificial seawater at T 11°C, 3,5% salinity, constant aeration), together with some leaves of algae (sea lettuce) collected from the same site. Before performing the bioassays, the specimens were randomly pooled according to the schedule reported in Table 2.1. More precisely 20 gastropods belonging to group A underwent to Comet assay, NRR assay and Intersex development analysis. Another group, counting 20 individuals each, was used for FRAP assay. Finally, in order to obtain reliable Intersex results, 80 more organisms were analysed for Intersex development.

**Table 2.1 *L. littorea* pools devoted to ecotoxicological and chemical analysis**

| COMET ASSAY | NRR ASSAY | INTERSEX | FRAP ASSAY | POP OTC |
|---|---|---|---|---|
| 20 organisms (group A) | | Groups A + 80 org (100 org checked; about 50 females analysed for Intersex development) | 20 organisms (group B) | 20 organisms (group C) |

#### 2.1.2.2 EXPERIMENT 2

*Hexaplex trunculus* were sampled on 13th May, 25th May and 15th June 2008 in S.M. Mare, Certosa and Adriatic stations respectively. About 100 specimens were collected in each sampling station and immediately transferred to the laboratory inside a cold box. In S. Maria del Mare and in Certosa the collection was carried out by hand during low spring tide; in the case of Adriatic station fishermen used a net.

Once in the lab, all specimen were placed in a glass tank filled with artificial seawater (T 13°C; 3,3% salinity, constant aeration), and left purging overnight.

To carry out the analyses, animals were pooled randomly according to the scheme represented in Table 2.2. Three different groups of organisms were used to carry out

separately MN assay, NRR assay and bioaccumulation analyses. Imposex development analysis was performed on all specimens who underwent to toxicological and chemical analyses, and on 60 individuals more.

**Table 2.2 Organisms pools devoted to toxicological and chemical analysis**

| MN ASSAY | NRR ASSAY | INTERSEX | POP<br>OTC |
|---|---|---|---|
| 8 organisms<br>(group A) | 10 organisms<br>(group B) | Groups A, B, C + 60 org<br>(100 org checked; about 50 females analysed for Imposex development) | 20 organisms<br>(group C) |

## 2.2 RESEARCH LINE 2

### 2.2.1 TEST-ORGANISMS RECRUITMENT

About 200 specimens of *Nassarius nitidus* were sampled from Canale Rigà (Lio Piccolo, Venice) on 25$^{th}$ June 2007, using traps (eel basket-like traps) and dead flesh as bait. *N. nitidus* population from Canale Rigà (northern basin of the lagoon) was chosen for recruiting the test-organisms because it is scarcely affected by Imposex. In fact, Pavoni et al. (2007) assessed the presence of normal females and low VDSI value (VDSI=1,2±0,7) during a field survey carried out in 2005.

Once collected, the samples were immediately transported to the laboratory within a cold box and transferred into the aquaria, imitating water temperature and salinity of the collection site (artificial seawater, T 22±1°C, 3,2±1% salinity, aerated conditions).

### 2.2.2 POISONING SYSTEMS

To contaminate the organisms via dietary uptake proved TBT/PCB-free beef heart was used as medium, consistently with the experience reported in Stroben et al. (1992B). The food was provided three times a week (approximately 4 g of flesh per 100 organisms).

Considering the aim of these preliminary experiments, that is to verify the capability of test organisms to bioaccumulate the toxicants via food ingestion during long-term exposure, the food was contaminated simultaneously by TBTCl and PCB mixture.

Two different poisoning systems were tested, respectively referred as *Recipe A* and *Recipe B*:

- *Recipe A*

  Applied concentrations:

  - TBTCl: 65 ng Sn/g heart (ww)
  - cb138: 10 ng/g heart (ww);
  - cb153: 10 ng/g heart (ww);
  - cb180: 6 ng/g heart (ww).

  *Spiking method for 1 g of beef heart*. The flesh was cut in small pieces (approximately 1 g heart split into 10 pieces). 20 µL of *Recipe A* spiking solution were split among the pieces through direct injection (*Recipe A* spiking solution: 9 µg TBTCl/mL, 0,5 µg cb138/mL, 0,5 µg cb153/mL, 0,3 µg cb180/mL in ethanol).

- *Recipe B*

  Applied concentrations:

  - TBTCl: 170 ng Sn/g heart (ww);
  - cb138: 25 ng/g heart (ww);
  - cb153: 25 ng/g heart (ww);
  - cb180: 15 ng/g heart (ww).

  *Spiking method for 1 g of beef heart*. 26 µL of *Recipe B* spiking solution (18 µg TBTCl/mL, 1 µg cb138/mL, 1 µg cb153/mL, 0,6 µg cb180/mL in ethanol) were diluted into 1 mL of ethanol and the heart was hashed. The small pieces of heart were dipped into the contaminant solution and left immersed for 15 minute. Then the solvent was removed by evaporation under feeble nitrogen stream.

The spiking methods were selected among those which minimize the use of the carrier solvent (ethanol), as recommended by Hutchinson et al. (2006). In fact many studies have shown that organic solvents can impact endocrine disruption biomarkers responses (Hutchinson et al., 2006 and references therein).

Test-toxicants concentrations were chosen in order to reproduce the contamination of *N. nitidus* natural food (*i.e.* carrions of invertebrates and fishes (Stroben et al., 1992B)) in medium quality environments. Thus tissues residues of TBT and PCB in clam population (*Tapes spp*) from Venice Lagoon were used as reference. In particular TBT and PCB concentrations reported by Zanon et al. (2009) and Boscolo et al. (2007) were nearly reproduced in *Recipe A*. *Recipe B* concentrations were chosen in order to test greater concentrations: they were calculated as double of *Recipe A* concentrations, plus a 25% due to an expected lower efficiency of the spiking method.

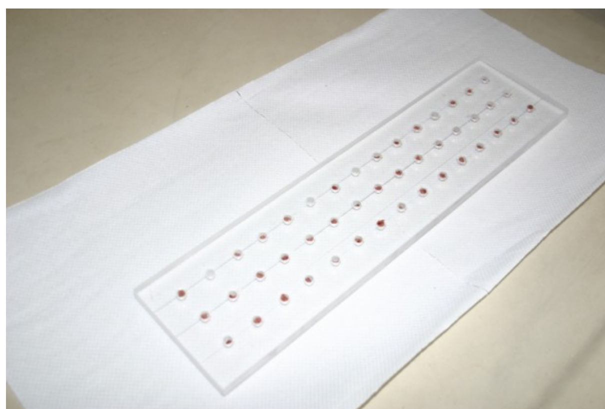


**Figure 2.15 Manger**

In order to encourage a nearly uniform food up-take among the organisms, a special manger was conceived (Figure 2.15), consisting in a Plexiglas board (approximately 20x10 cm) provided with 45 wells on which to insert the small pieces of heart.

### 2.2.3 TEST CHAMBERS AND EXPERIMENTAL CONDITIONS

The experiments were carried out on glass tanks filled with 20 l of artificial seawater (Ocean Fish marine salt, Prodac International, Italy), prepared by dissolving the commercial salt mixture in dechlorinated water. Before its use, the solution was stirred and aerated overnight. The stability of water temperature, oxygen concentration and nutrients levels were respectively guaranteed by a cryostat, a simple air pump and an external filtration apparatus connected to the aquaria. In particular the filtration system was made up by both mechanical and biological filters in order to remove solids from water and encourage the development of bacteria. Before the use, the filtration system was activated for one month at least.

Water change was carried out by renewing daily the 25% of the total volume. During the whole exposure, measurements of temperature, salinity, pH and concentrations of $N\text{-}NH^{+}_{4}$ and $N\text{-}NO^{-}_{2}$ were taken almost regularly (twice at week at least); the concentrations of N-nutrients were measured following the methods reported by Strickland and Parson (1972). All parameters were checked before renewing the water.

### 2.2.4 TESTS SCHEME

Three preliminary laboratory tests, indicated as d*econtamination test*, *Recipe A test* and *Recipe B test*, were carried out on pilot aquaria in order to verify the effectiveness of three weeks decontamination period and two poisoning systems.

The tests were carried out between the 25$^{th}$ June and 6$^{th}$ August 2007; Table 2.3 reports the details about the number of specimens used, the feeding rate and the exposure length for each test.

**Table 2.3 Experiment 3: tests schedule**

| | **DECONTAMINATION TEST** | ***RECIPE A* TEST** | ***RECIPE B* TEST** |
|---|---|---|---|
| N° specimens | 130 | 100 specimens | 70 specimens |
| Food | Beef heart (not contaminated) | Beef heart contaminated following Recipe A poisoning system | Beef heart contaminated following Recipe B poisoning system |
| Feeding frequency | 4 g every 2 days (3 times/week) | 4 g every 2 days (3 times/week) | 3 g every 2 days (3 times/week) |
| Exposure time | 3 weeks | 4 weeks | 3 weeks |

The introduction into the aquaria of a number of organisms significantly greater than the necessary depended on the need of verifying the effectiveness of aquaria running conditions at full capacity (number of specimens required for the definitive experiments).

The real consumption of the whole food-dose administrated was checked every time after 30 min from the introduction of the mangers into the aquaria.

In order to evaluate the effectiveness of decontamination and poisoning processes, at the start and at the end of the exposure period sub-samples of 30 specimens underwent Imposex development characterization and OTC and PCB body burden analyses (for analytical methods refer to Par. 2.3.2 and Par. 2.3.1). The post-exposure samplings were carried out two days after the last feeding.

## 2.3 CHEMICAL ANALYSIS

Chemical analyses were carried out on tissues of pooled organisms (whole body). The samples were previously freeze-dried and homogenated; they were kept at -20°C until the start of the analyses.

Chemical analyses were performed in duplicate: the results were expressed as average of the two replicates, the error as semi-variation interval of the measures. For each compound LOD value (Limit of Detection) was calculated as three times the standard deviation of the concentrations revealed in the controls (blanks).

### 2.3.1 PAH, PCB AND OCP DETERMINATION

Tissue concentrations of PAH, PCB and organochlorine pesticides were quantified using the analytical procedure reported in Raccanelli et al. (1994). The compounds quantified for each category were the following: for PCBs, PCB 18, PCB 28, PCB 52, PCB155, PCB 101, PCB 77, PCB 123, PCB 118, PCB 153, PCB 105, PCB 138, PCB 126, PCB 185, PCB 156, PCB 157, PCB180, PCB 198, PCB 169, PCB 170, PCB 194, and PCB 209; for organochlorine pesticides, α-HCH, γ-HCH, HCB, *p,p*'-DDT, *o,p*'-DDT *p,p*'-DDE, *o,p*'-DDE, *p,p*'-DDD, *o,p*'-DDD; for PAHs, naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[b]fluoranthene, benzo[*k*]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[*a,h*]anthracene and benzo[*ghi*]perylene.

Basically the analytic method consisted in the following 3 steps:

1. *Ultrasonic extraction.*

   Freeze-dried tissue samples (1,000 g) were extracted three times by cycles of two hours extraction with 25 mL of *n*-hexane/dichloromethane (4:1) solution; the whole volume extracted was concentrated at 1 mL under weak nitrogen stream.

2. *Purification and simultaneous separation of chlorinated and non-chlorinated hydrocarbons by column chromatography.*

   Chromatographic columns (length, 45 cm; width, 1 cm) were packed by introducing, in order, silica gel (6 g), alumina (8 g), florisil (1.5 g) and anhydrous sodium sulphate (4,5 g). All adsorbents were previously washed with dichloromethane and activated for 16 h at 250°C; furthermore, just before the start of the extraction, the following analytical components were sonicated for 2 hours: 1) *n*-hexane/dichloromethane (3:2) carrier solution, 2) *n*-hexane/dichloromethane (4:1) solution used to pack the

column 3) silica gel and 4) alumina, previously deactivated with 120 µL of distilled water. After elution of 30 mL of *n*-hexane, sample extracts were introduced into the column and the three classes of compounds were separated through the following elution factions: 20 mL of *n*-hexane to elute alkanes (first fraction), 80 mL of *n*-hexane to elute PCBs and pesticides (second fraction), and 75 mL of *n*-hexane/dichloromethane (3:2) to elute PAH and the remaining pesticides (third fraction). Only the second and third fractions were analyzed. Finally the extracts were concentrated up to 1 mL and let to completely evaporate overnight in order to re-suspend the analytes in 200 µL of iso-octane. Samples were stored at -20°C until instrumental analysis.

3. *Instrumental detection.*

   PCB and OPC (II and III fractions) were detected through GC-ECD (Hewlett-Packard 5890 series II, Wilmington, DE, USA) (operative conditions in Table 2.4, whereas PAH (III fraction) though HRGC-LRMS (Hewlett-Packard 5890 series II/ Hewlett-Packard 5970 B) (operative conditions in Table 2.5).

The quantification was performed with the internal standard method. As internal standards for PCB, PAH and pesticides were used respectively PCB 30, a mixture of deuterated PAHs (naphthalene-d8, phenanthrene-d10, perylene-d12) and pentachloronitrobenzene.

Analytical concentrations were expressed as nanograms of compound per gram of dry tissue (ng/g dw).

Response factors were calculated through a calibration curve drawn using five to seven standard mixtures.

LOD values were lower than 0.1 ng/g for PCB, lower than 0.06 ng/g for pesticides, and lower than 7 ng/g for PAH.

**Table 2.4 GC-ECD analysis of PCB and OCP: operative conditions**

| **GC-ECD ANALYSIS of PCB and OCP** | |
|---|---|
| **Capillary column** | HP-5 (5% phenyl methylsiloxane; inner diameter, 0.20 mm; length, 50 m; film thickness, 0.33 µm) |
| **Injector** | Temperature, 250°C |
| | Splitless injection |
| **Temperature program** | • 110°C for 1 min,<br>• 9°C/min up to 141°C<br>• 3°C/min up to 280°C<br>• Post-run for 5 min at 280°C |
| **Carrier gas** | Helium at 199 kPa head pressure |
| **Detector** | Electron-capture detector |

Temperature, 300°C

**Table 2.5 GC-MS analysis of PAH: operative conditions**

## GC-MS ANALYSIS of PAH

| | | |
|---|---|---|
| **Capillary column** | HP-5 (5% phenyl methylsiloxane; inner diameter, 0.20 mm; length, 50 m; film thickness, 0.33 μm) | |
| **Injector** | Temperature, 300°C<br>Splitless injection | |
| **Temperature program** | • 60°C for 1 min<br>• 18°C/min up to 140°C<br>• 10°C/min up to 252°C<br>• 14°C/min up to 300°C<br>• Post-run for 22 min at 300°C | |
| **Carrier gas** | Helium | |
| **Transfer line** | Temperature, 280°C. | |
| **Detector** | Detection electron impact Ionization (70 ev) in selected-ion monitoring mode (SIM). | |
| **SIM (Single Ion Monitoring)** | Primary ion | Secondary ion |
| **Naphthalene** | **128** | 129 |
| **Acenaphthylene** | **152** | 150 |
| **Acenaphthene** | **153** | 154 |
| **Fluorene** | **166** | 165 |
| **Phenanthrene** | **178** | 176 |
| **Anthracene** | **178** | 176 |
| **Fluoranthene** | **202** | 200 |
| **Pyrene** | **202** | 200 |
| **Benz[a]anthracene** | **228** | 226 |
| **Chrysene** | **228** | 226 |
| **Benz[b]fluoranthene** | **252** | 250 |
| **Benz[k]fluoranthene** | **252** | 250 |
| **Benz[a]pyrene** | **252** | 250 |
| **Indeno[1,2,3-cd]pyrene** | **277** | 276 |
| **Dibenzo[a,h]anthracene** | **278** | 276 |
| **Benz[ghi]perylene** | **276** | 274 |
| **INTERNAL STANDARD** | | |
| ***Naphthalene-d8*** | ***136*** | *108* |
| ***Acenaphthene-d10*** | ***188*** | *187* |
| ***Perilene-d12*** | ***264*** | *260* |

### 2.3.2 OTC DETERMINATION

Organotin compounds (TBT, TPhT, and degradation products DBT, DPhT, MBT and MPhT) were analysed by using the procedure reported in Pellizzato et al. (2004). It is a four steps analysis consisting in:

1. *Extraction with methanol solution of tropolone (0.03%) in acid environment through sonication bath and liquid–liquid separation.*

   Freeze-dried tissues (0,5000 g or 1,000 g) were extracted twice through sonication (15 min) in 13 mL of methanol solution of tropolone (0.03%) and 1 mL of HCl (37%). The extracts, recovered after centrifuging the test tubes for 20 min at 1000 rpm, were poured in separatory funnels: by liquid–liquid partition, which was encouraged by the presence of 100 mL 10% NaCl solution in deionised water into the funnels, analytes were transferred into with 15 mL of $CH_2Cl_2$. The separation step was repeated twice.

2. *OTCs derivatization by Grignard reagent (2M etylmagnesiumchloride in THF)*.

   Before proceeding with derivatization reaction, the organic phase, carefully dewatered by short elution through a volume of anhydrous sodium sulphate, was solvent changed: by evaporation under a gentle stream of nitrogen, analytes passed from $CH_2Cl_2$ to isooctane (1 mL), which was previously pipette into the sample. Then 1 mL of Grignard reagent was added to the sample and pentylation reaction was allowed to occur; the excess of non-reacted reagent was destroyed by carefully adding 2 mL of deionised water. So the pentylated organotins were extracted twice with 2 mL of n-hexane and 5 mL of $H_2SO_4$ 1 M; after concentration to 1 mL, 5 µL of the internal standard (30 ng/ µL of tripropylpentyltin (TPrTPe) in n-hexane) were added to each sample.

3. *Cleanup through chromatographic column packed with florisil and anhydrous sodium sulphate.*

   Samples were eluted with 10 mL of n-hexane–toluene (1:1) in a short column containing 3 g of florisil and 1 spatula of anhydrous sodium sulphate; the column was previously conditioned by elution of 10 mL n-hexane–toluene (1:1) solution. Both adsorbents were activated overnight by heating (250°C). The purified extracts were concentrated at 1 mL.

4. *Instrumental quantification*

   OTC were analysed by HRGC-LRMS (Hewlett-Packard 5890 series II/ Hewlett-Packard 5970B). Operative details are reported in Table 2.6.

OTCs concentrations were expressed as ng Sn/g dry weight tissue (dw).

**Table 2.6 GC-MS analysis of OTC: operative conditions**

| **GC-MS ANALYSIS of OTC** | | |
|---|---|---|
| **Capillary column** | HP-5 (5% phenyl methylsiloxane; inner diameter, 0.20 mm; length, 50 m; film thickness, 0.33 μm) | |
| **Injector** | Temperature, 300°C | |
| | Splitless injection | |
| **Temperature program** | • 80°C for 1 min | |
| | • 20°C/min up to 290°C | |
| | • Post-run for 20 min at 290°C | |
| **Carrier gas** | Helium at 199 kPa head pressure | |
| **Transfer line** | Temperature, 280°C. | |
| **Detector** | Detection electron impact Ionization (70 ev) in selected-ion monitoring mode (SIM). | |
| **SIM (Single Ion Monitoring)** | Primary ion | Secondary ion |
| **TBT** | **305** | 303 |
| **DBT** | **319** | 317 |
| **MBT** | **319** | 317 |
| **MPhT** | **339** | 337 |
| **DPhT** | **345** | 343 |
| **TPhT** | **351** | 349 |
| **INTERNAL STANDARD** | | |
| ***TPrTPe*** | ***277*** | *275* |

In the Environmental Chemistry Laboratory of Venice University the method is regularly tested by analysing mussel reference material certified for butyltin compounds residues (CRM 477); usually the percentage recovery is higher than 80%, 75% and 84% for TBT, DBT and MBT respectively.

# 2.4 BIOLOGICAL METHODS

## 2.4.1 SAMPLE PREPARATION

### 2.4.1.1 HAEMOLYMPH EXTRACTION

Comet assay, NRR assays and MN assay were carried out within 24 hours from the sampling. To perform them, it was necessary to previously extract haemolymph samples. About 100 µL of haemolymph were withdrawn from each specimen by inserting hypodermic syringes (21-gauge hypodermic needle) behind the ocular tentacles. The protrusion of the body (head and foot) from the shell, that is necessary for inserting the needle, was achieved without anaesthetizing the organisms: one by one snails were very gently detached from the inner side of aquaria, trying to avoid the foot to draw back into the shell; in this way, only few minutes were required to achieve the best conditions for the extraction. Afterwards the samples, poured inside Eppendorf tubes filled with 1-2 drops of physiological saline solution (solution obtained by dissolving in 1 L of distilled water 4,77 g of Hepes, 25,48 g of sodium chloride, 13,06 g of magnesium sulphate, 0,75 g of potassium chloride and 1,47 of calcium chloride; pH 7,36), were kept in ice until the start of the assays (no more than 75 minutes). The suitability of samples' cells density was checked by observation under light microscope (20-50 cells per field of view, using x20 objective lens).

### 2.4.1.2 TISSUES HOMOGENIZATION

To perform FRAP assay, specimens were previously homogenized one by one using a hand-held homogenizer. The whole homogenization process was carried out keeping the sample in ice in order to maintain low constant temperature and avoid protein denaturation. The saline solution reported in the previous paragraph was used as homogenate buffer. Homogenate aliquots were stored at -20°C inside Eppendorf tubes until the use.

At the start of FRAP analysis, samples were let thaw out for few minutes and then centrifuged for 30 minutes at 10000g and 4°C.

## 2.4.2 COMET ASSAY

The method used in the present study was a modified version of that described by Singh et al. (1988). Briefly, the cells contained in 0,5 mL of haemolymph samples were concentrated at the bottom of Eppendorf tubes by centrifuging 50 µL at 1000 rpm for 4 minutes and discharging the supernatant fraction. Then they were gently mixed with 1% low melting point agarose (37°C) and smeared onto slides previously coated with 1% normal melting point agarose. The slides were left setting for 10 min at 4°C, applying coverslips on the top. Once

removed the coverslips, the cells underwent to 1 h lysis (lysis solution: 2.5 M NaCl, 100 mM Na2–EDTA, 10 mM Tris, 1%Na sarconisate, pH 10.0, 1% Triton X-100, 10% DMSO), followed by 45 min denaturation process into electrophoresis bath (electrophoresis solution: 0.3M NaOH and 1mM EDTA, pH 13). Then electrophoresis was carried out for 30 min at 25V and 300mA. After a short washing with the neutralising solution (0.4 M Tris, pH 7.5), cells were stained with 20 µL of ethidium bromide solution (5 mg/L) and examined under fluorescence microscope (420–490-nm excitation filter; 520-nm emission filter). 100 cells per haemolymph sample were scored using the Kinetic COMET Software, which outputs the percentage of DNA tail intensity.

For each sampling site the average DNA migration, measured as percentage of DNA tail intensity, and standard deviation were calculated (mean % tail intensity ± std.dev).

### 2.4.3 MICRONUCLEI ASSAY

The MN test was applied according to a modified version of UNEP/RAMOGE (1999). All modifications were aimed at adapting the method to the specific features of the gastropod species used as test-organism. About 1 mL of haemolymph was used to carry out the assay; the presence of a suitable haemocytes density was checked by observation under optical microscope. Eppendorf tubes containing the heamolymph samples were centrifuged for 5 minutes at 1000 rpm in order to concentrate most of the cells at the bottom of the tube; then the supernatant fluid was carefully pipetted off and the cells washed with 1 mL of Hanks' balanced salts solution (136,9 mM NaCl, 5,36 mM KCl, 0,40 mM $MgSO_4*7H_2O$, 1,26 mM $CaCl_2*2H_2O$, 0,337 mM $Na_2HPO_4*2H_2O$, 0,44 mM $KH_2PO_4$, 4,165 mM $NaHCO_3$, 5,045 mM D-Glucose*$H_2O$). Once removed the washing fluid, haemocytes were fixed with Carnoy's solution (methanol:acetic acid, 3:1) for 30 min in ice. Then the cells were concentrated again at the bottom of the tube by short centrifuging (1000 rpm for 5 minutes) and the supernatant fluid was removed; following resuspension in a drop of Carnoy's solution (20 µL), they were spread on glass microscope slides. The slides were let to dry on air and finally stained with a 5% (v/v) Giemsa (Fluka) solution in tap water. Then they were rinsed twice in tap water, clarified by two minutes immersion in Hystolemon solution (Carlo Erba) and then mounted in EUKITT®.

For each sample, 1000 intact haemocytes were scored under light microscope observation (1000x magnification). The following criteria were used to identify the micronuclei (Venier et al., 1997; Baršiene et al., 2006):

- Bodies completely separated from the main nucleus;
- Micronuclei size ≤ 1/3 of the main nucleus size;
- Chromatin structure and colour intensity similar to that of the main nucleus;

- Round or oval shape;
- Location on the same optical plane as the main nucleus;
- Not fragmentation (to exclude small stain particles and apoptotic cells).

Micronuclei frequency was expressed as number of micronuclei per 1000 cells scored (‰). For each sampling site the mean micronuclei frequency and the relative standard deviation was calculated.

### 2.4.4 FRAP ASSAY

The method used to perform FRAP assay was that described by Benzie and Strain (1996).

50 µL-volumes of the following solutions were pipetted into the wells of a 96-wells microtitre plate:

- Samples (for details about FRAP samples preparation refer to Par. 2.4.1.2)
- calibration solutions (six aqueous solutions of $Fe^{2+}$ at the following concentration: 0, 100, 200, 300, 400, 500 µM Fe II);
- Control solutions (homogenate buffer, see Par. 2.4.1.1);

The assay was carried out in three replicates.
Afterward 200 µL of FRAP reagent (mixture containing, in the ratio 10:1:1, the following solutions: 300 mM acetate buffer (pH 3,6); 10 mM TBTZ (2,4,6-tripyridyl-s-triazine) in 40mM HCl; 20 mM $Fe^{3+}$) were added into each well. Then the plate was incubated at 20°C into the spectrophotometric plate reader and the adsorbency at 593 nm was measured twice: the first time just after FRAP reagent addition (time 0), the second time after 10 minutes reaction (time 10). Using the calibration line, the difference in absorbency was transformed into µM $Fe^{2+}$ produced. For each sample the mean FRAP value of three replicates was calculated and the results normalized for protein content (µmol $Fe^{2+}$/g proteins (PRT)); the error was expressed as standard deviation.
Protein concentration was determined following the method of Bradford (1976).

### 2.4.5 INTERSEX/IMPOSEX DEVELOPMENT ANALYSIS

Intersex/Imposex development analysis was usually carried out on frozen or narcotized organisms. The anaesthetization was carried out by 30 minutes immersion into a 7% $MgCl_2$ solution in distilled water.

To sex the specimen and check the presence of Intersex organisms were previously removed from the shell (by cracking the shell with a vice) and the sexual organs exposed by cutting the mantel along the hypobranchial gland.

### 2.4.5.1 INTERSEX ANALYSIS IN *LITTORINA LITTOREA*

Intersex development analysis in *L. littorea* was carried out according to the standardized ICES method No.37 (ICES, 2004).

Following the precise indications of the protocol, the sex was determined considering visceral hump's colour and gonoduct's shape and colour. Table 2.7 summarizes the features that were sex-discriminating at the time of the analyses.

**Table 2.7 Sex discriminating features in *L. littorea***

| | **GONAD** (visceral hump) | **GONODUCT** (columellar side of visceral hump) |
|---|---|---|
| **MALE** | Yellow; smooth texture | Highly coiled; white because of the presence of sperm |
| **FEMALE** | Light red or pale pink; granular texture | Straight; dark colour |

Intersex stage was recognized by observing the pallial oviduct section under dissection microscope. By the support of the "decision tree-diagram" provided by ICES protocol (Figure 2.16), each specimen was attributed to one of the five Intersex stages depicted in Figure 2.17.

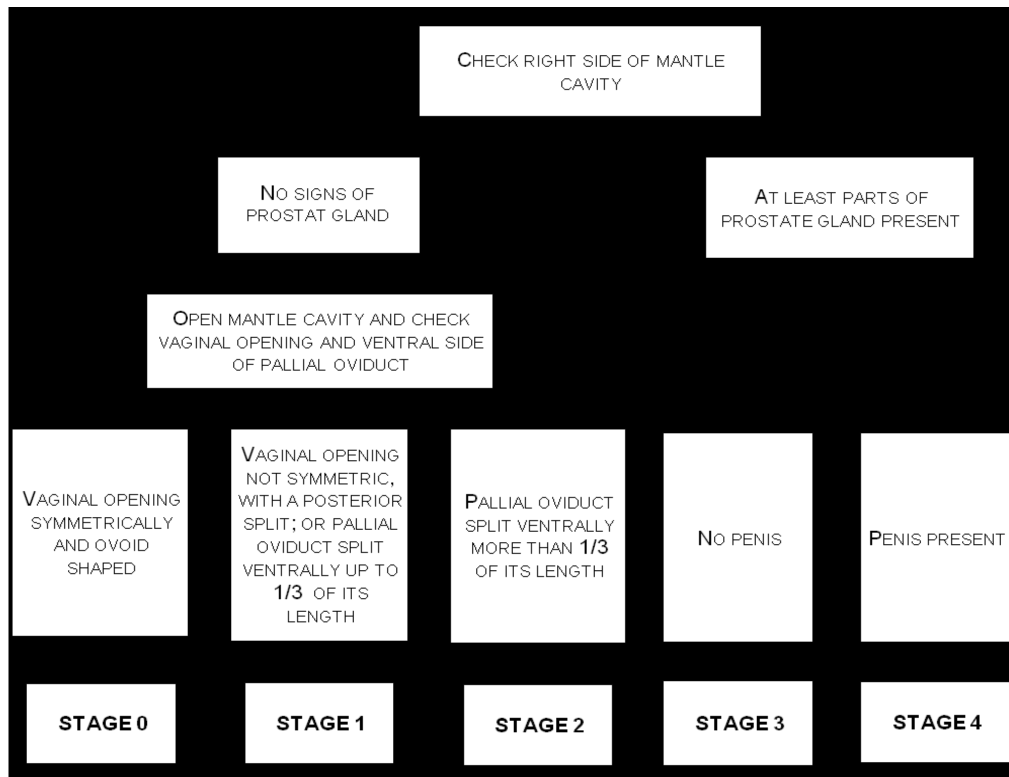



**Figure 2.16 Decision tree for identification of Intersex stages in *Littorina littorea* (ICES, 2004)**

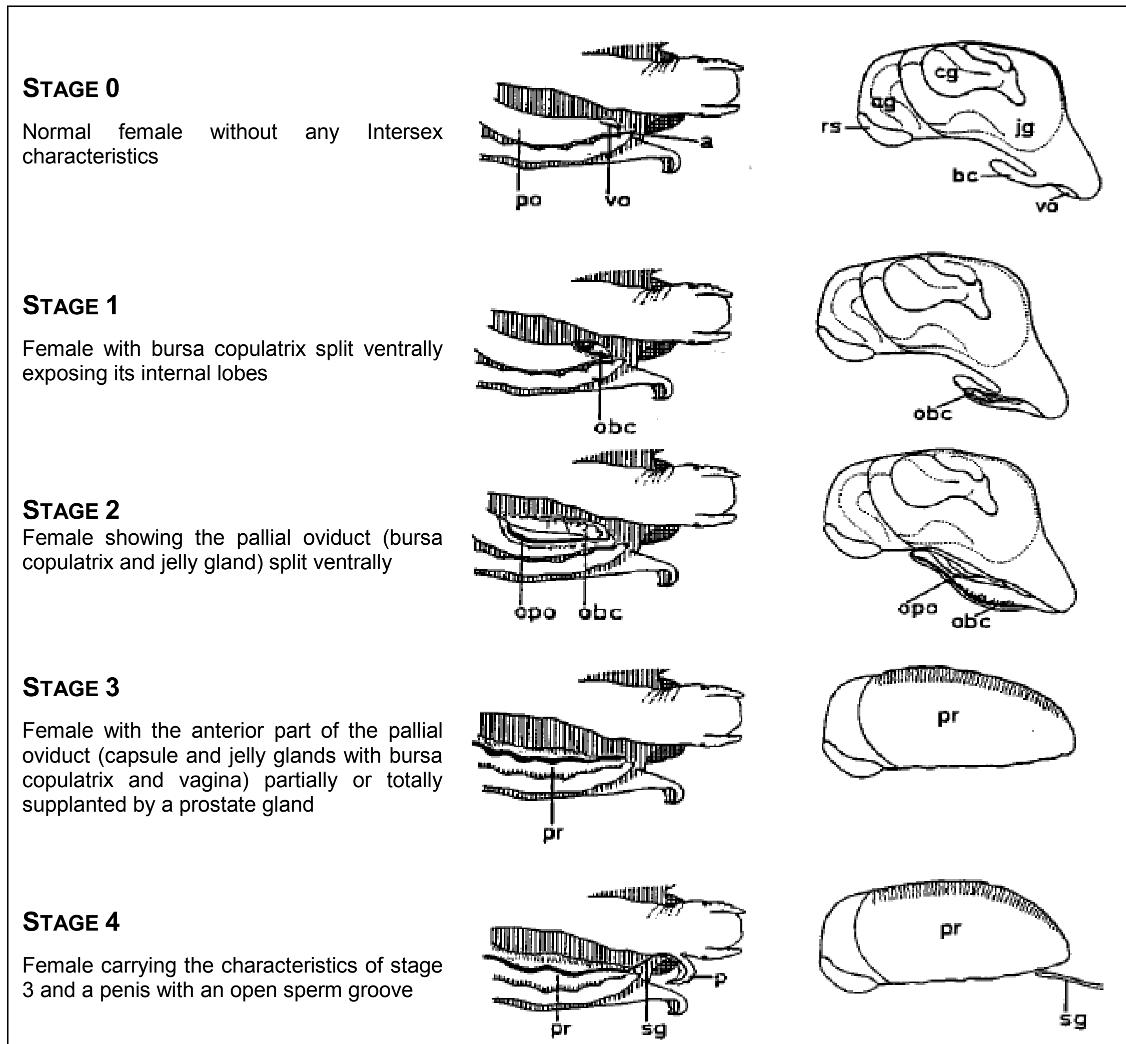



**Figure 2.17 Scheme of *L. littorea* Intersex development: Intersex stages' short description, dorsal view and view of the pallial section of the genital tract (ICES, 2004). Abbreviations: anus (a); albumen gland (ag); bursa copulatrix (bc); capsule gland (cg); jelly gland (jg); open bursa copulatrix (obc); open pallial oviduct (opo); pallial oviduct (po); prostate (pr); sperm groove (sg); receptaculus seminalis (rs); vaginal opening (vo).**

Using the collected data, the Intersex index (ISI) was calculated as mean value of Intersex stages recorded in a population, the corresponding error as standard deviation.

#### 2.4.5.2 IMPOSEX ANALYSIS IN *HEXAPLEX TRUNCULUS*

In *H. trunculus* specimens the sex was identified by observing the genital organs in the mantel cavity. Males were recognized for the absence of capsule gland and the presence of penis and prostate, whereas females for the presence of capsule gland and vagina. Penis length was measured by a Vernier calliper (0,1 mm) in both males and imposexed females.

Imposex stage was attributed according to the scheme proposed by Terlizzi et al. (1999) and Axiak et al. (1995), who adapted the developmental sequence proposed by Stroben et al. (1992A) to the specific features of *H. trunculus*. According to them, each female was associated to one of the stages reported in Figure 2.18.

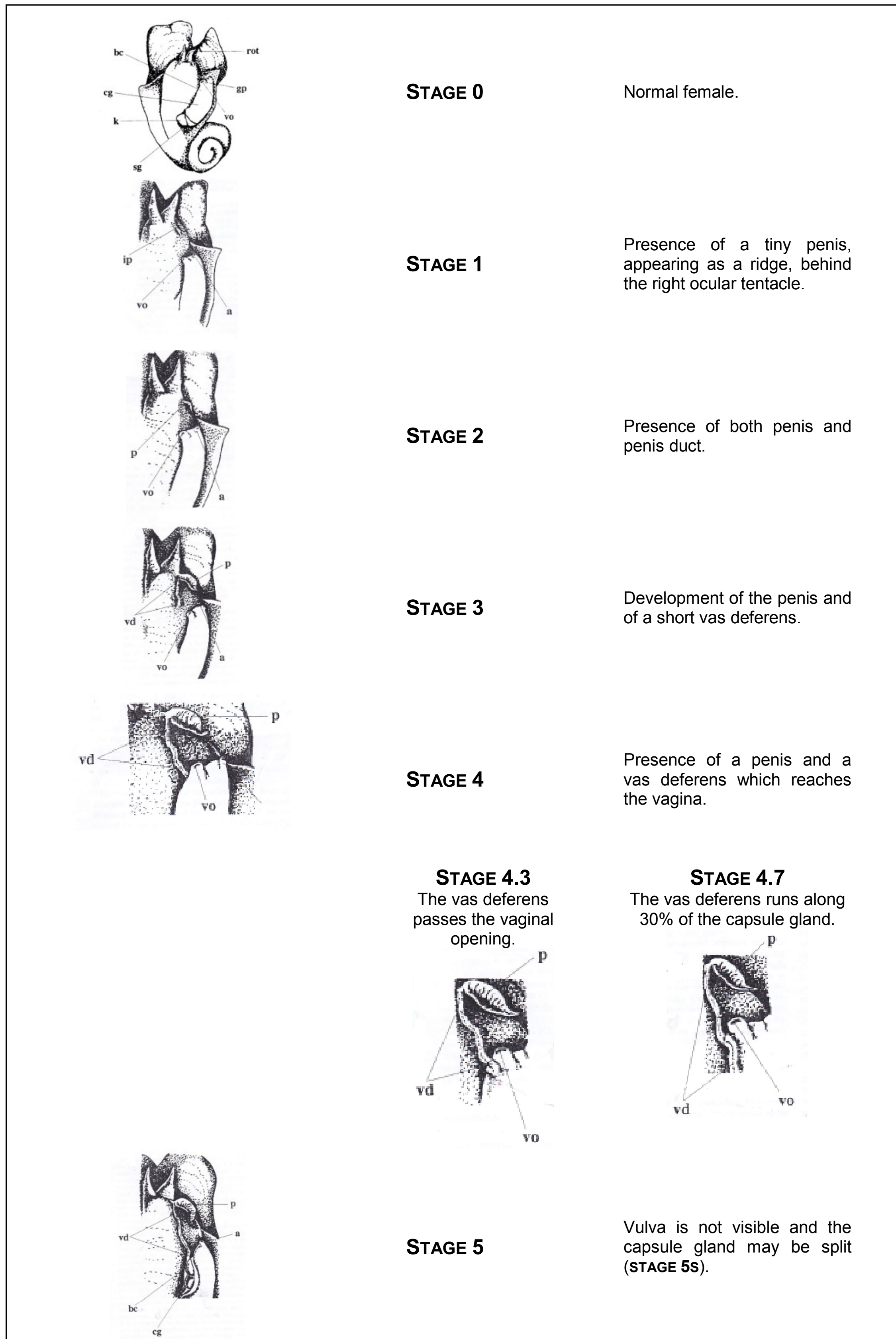



**Figure 2.18 Scheme of H. trunculus Imposex development: Imposex stages' short description and dorsal view (Terlizzi et al., 1999). Abbreviations: anus (a); bursa copulatrix (bc); capsule gland (cg); genital papilla (gp); kidney (k); incipient penis (ip); occlusion of the vulva (ocv); penis (p); sperm ingesting gland (sg);**

**vulva (v); vas deferens (vd); vas deferens tissue (vdt);vaginal opening (vo).**

Data about vas deference development and penis length were used to calculate the following population indexes:

- RPSI = (average of female penis length)$^3$/(average of male penis length)$^3$*100
- VDSI = mean of Imposex stages
- Mean Female Penis Length

### 2.4.5.3 IMPOSEX ANALYSIS IN *NASSARIUS NITIDUS*

Consistently with the assumptions of Par. 1.7.3.3, Imposex development characterization on *N. nitidus* was carried out referring to method reported by Stroben et al. (1992A) for the species *N. reticulatus*.

Snails were sexed by identifying the vulva in females; penis extension was measured at the nearest 0,1 mm in both males and imposexed females.

Imposex development in *N. nitidus* is made up by four main Imposex stages. However two of them, stage 1 and stage 3, can appear in two different versions (morphotype *a* or morphotype *b*), differing from each other for the specific accessory sexual organ developed (respectively penis or vas deferens). The schematic description provided by Stroben et al. (1992A) for each Imposex stage is reported below (Figure 2.19).

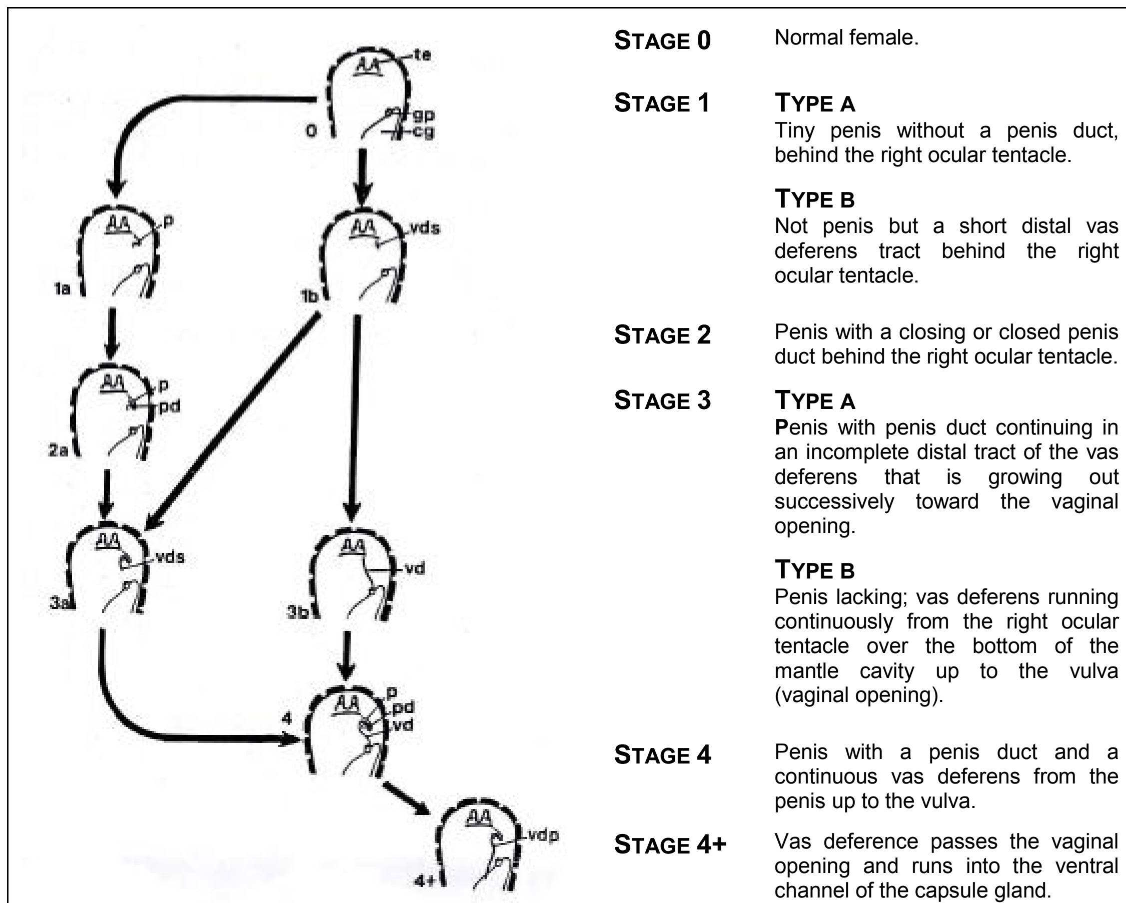



**Figure 2.19 Diagram of Imposex development in *Nassarius nitidus*: graphic scheme and short description of each Imposex stage (modified from Stroben et al. (1992))**

Using the data referring to penis length and Imposex degree, the following three Imposex indexes were calculated for each population:

- VDSI (Vas Deferens Sequence Index) = mean of Imposex degrees in each population;
- RPLI (Relative Penis Length Index) = (Mean Female Penis Length/Mean Male Penis Length) * 100;
- Mean Female Penis Length.

The cubed version of RPLI, called RPSI (Relative Penis Size Index), was not considered in the present study because often it has been rejected and proved as invalid due to its great seasonal variability (Stroben et al., 1992A; 1992B).

### 2.4.6 NEUTRAL RED RETENTION ASSAY

NRR assay was performed using the *in vivo* cytochemical method proposed by Lowe et al. (1992) and reported in ICES method No 36 (ICES, 2004).

Briefly 40 µL of haemolymph sample were smeared on Poly-L-Lysine-coated microscope slides and incubated in a light proof humidity chamber for 30 minutes to allow cells to attach; the excess of heamolymph was removed by carefully tapping off the remaining fluid from the slide. Thus 40 µL of Neutral Red solution (20 mg/mL in DMSO, diluted 50-fold in physiological saline solution at the start of the analysis; see Par. 2.4.1.1 for saline solution composition) was added in each slide and left to penetrate into the cells for 15 min in dark condition. Afterward the progressive dye leaching from lysosomes into the cytoplasm was monitored by observing the cells under light microscope (x20 objective): each slide was systematically examined at 15 minutes intervals for the first hour, and at 30 minutes intervals for the following two hours. The visual estimation of lysosomal conditions was assessed in 1 minute-observation; the endpoint was stated when at least 50% of the cells exhibit either lysosomal leakages (presence of Neutral Red in the cytosol) or lysosomal abnormalities (usually enlargements). The retention time corresponded to the last time period before reaching the endpoint.

For each sampling site, mean retention time value and standard deviation were calculated.

## 2.5 STATISTICAL ANALYSIS

### 2.5.1 UNIVARIATE ANALYSES

Spatial variations of toxicological results obtained in Experiment 1 and 2 were assessed through the analysis of variance. One way ANOVA coupled with Tukey's post-hoc test was carried out on data normally distributed and featured by homogeneous variance; differently Kruskal-Wallis test coupled with Mann Whitney test was used; differences at the <5% level were considered significant. In order to find out possible relationships between bioaccumulation pattern and health impairments, correlation analysis was performed on the database summarizing toxicological and chemical data (Pearson correlation analysis, p-value=0,05; Spearman correlation analysis, p-value=0,05).

The results of biological analyses of Experiment 3 (Female Penis Length and VDS) were tested for differences between pre- and post treatment; because data were not normally distributed, Mann Whitney test was used (p-value=0,05).

The statistical package STATISTICA version 7.1 (StatSoft Italy srl, 2005) was used for univariate analyses.

### 2.5.2 MULTIVARIATE ANALYSIS

In experiment 1, in order to assess the variation pattern between chemical and toxicological description, the whole database was processed by the method of partial least squares (PLS) regression in latent variables (Daren, 2001). PLS is a multivariate regression method based on principal component–like variables. Basically from two blocks of variables, contextually chemical and toxicological groups, the relevant information provided by each variable was extracted by PLS components and then the correlation between these new variables was assessed by linear regression methods. Differently from other multivariate regression methods, the computed latent variables are extracted under the vinculi to maximize the correlation between the two blocks as well as to best fit the point-swarm in each space: so the relevance of the original variables in the new comprehensive description (expressed by the loading values) depends on their contribution in correlating the information between the two blocks.

In the present study PLS analysis was used as tool for assessing variables correlation rather than for prediction purposes. The model was built considering the pollutants concentrations as independent variables (predictor variables, X block), and the toxicological responses as dependant variables (response variables, Y block).

The parameters used to evaluate model performance in fitting were *R2X* and *R2Y,* which indicate the unitary variance of *x* and *y* variables, respectively. The contribution of original variables in the correlation was evaluated considering the loading values, which are the coefficients of the starting variables in the linear combination of principal components; they express the weights of each variable in describing the objects in the PLS space.

The SIMCA-P software (Soft Independent Models of Class Analogy, Ver 8.0; Umetrics AB, Umea°, Sweden) was used for the chemometric processing; it automatically scales and centres the data (means of zero and a variance of one), ensuring similar *a priori* importance to the variables.

# 3 RESULTS AND DISCUSSION

## 3.1 EXPERIMENT 1

### 3.1.1 RESULTS

Chemical results are summarized in Table 3.1 (for the whole dataset see Annex I). They revealed that the six *L. littorea* populations were sampled from areas differently affected by organic chemical pollution: the most polluted station was Cracknore Hard (1131±23 ng/g dw), whereas the least polluted was Mothecombe, the reference station (58±9 ng/g dw).

**Table 3.1 Chemical analyses results: tissue residues of organic pollutants on pools (20 organisms each). The concentration of each pollutant class is the sum of single congener's concentrations; the error was computed through propagation of single compound's error (semi-variation interval of the two replicates analysed). The sign (*) marks the concentrations below the limit of detection (LOD): the values reported were computed as sum of semi-LOD values of single congeners.**

| | **PAH** (ng/g dw) | **OTC** (ng Sn/g dw) | **PCB** (ng/g dw) | **OCP** (ng/g dw) | **ΣORGANIC POLLUTANTs** (ng/g dw) |
|---|---|---|---|---|---|
| **MOTHECOMBE** | 47 ± 9 | 3,73 (*) | 5,9 ± 0,2 | 1,2 ± 0,2 | 58 ± 9 |
| **BRIXHAM** | 35 ± 4 | 45 ± 9 | 4,4 ± 0,5 | 7,4 ± 0,4 | 92 ± 10 |
| **MOUNTBATTEN** | 32 ± 2 | 12 ± 2 | 11,3 ± 0,7 | 4 ± 1 | 59 ± 3 |
| **TORPOINT** | 69 ± 20 | 59 ± 7 | 18,4 ± 0,7 | 7 ± 1 | 154 ± 21 |
| **CALSHOT** | 154 ± 5 | 40 ± 4 | 5 ± 1 | 1,6 ± 0.2 | 200 ± 6 |
| **CRACKNORE HARD** | 812 ± 15 | 266 ± 17 | 48 ± 2 | 6 ± 4 | 1131 ± 23 |

PAH and OTC were the main contaminant classes bioaccumulated by snails. PAH body burden varied between 32±2 ng/g dw, recorded in Mountbatten, and 812±15 ng/g dw, registered in Cracknore Hard, whereas OTC concentrations were from below the LOD (Mothecombe) to 266±17 ng Sn/g dw (Cracknore Hard).

Focusing on the bioaccumulation pattern of each population, it appeared that the chemical pressure in the reference station (Mothecombe) was mainly due to the presence of PAH (Table 3.1): similarly to the other populations living in areas not directly affected by big industrial and urban activities (such as Brixham and Mountbatten), the most abundant congener was phenanthrene, a low molecular weight petrogenetic PAH (12,6±0,9 ng/g dw) (Figure 3.1a).

In Brixham, besides PAH, also a certain amount of OTC was found (45±9 ng Sn/g dw). Considering the predominance of degradation products on the parent compound (Figure 3.1b), OTC pollution might likely represent the residue of the past TBT contamination described by White et al. (1991) and Bryan et al. (1986). In fact, before the ban of TBT-

based antifouling paints, this harbour was one of the most polluted by the leisure-fishing activities of the village.

The most abundant pollutant classes into the Plymouth Sound (Mountbatten and Torpoint) were PAH and OTC (Table 3.1). The concentrations recorded in the two sampling stations reflected their different geographical locations inside the bay. The proximity of Torpoint to the area featured by the highest industrial density and traffic intensity (Hamoaze) was responsible of the greatest contamination level and abundance of high molecular weight PAHs, such as the pyrogenic benzo[b]fluoranthene (32±20 ng/g dw) and benzo[k]fluoranthene (10,9±0,4 ng/g dw) (Figure 3.1a). As explained for Brixham, the residues of OTC in Torpoint (59±7 ng Sn/g dw) might be presumably ascribed to past TBT sources. Similarly, the bioaccumulation patterns of Southampton Water's samples were consistent with both the position of sampling stations inside the ria (external and sea-front for Calshot; internal and sheltered for Cracknore Hard) and the distance to the main contamination sources. Tissues concentrations were definitely the highest in Cracknore Hard. In fact PAH and OTC body loads were respectively 812±15 ng/g dw and 266±17 ng Sn/g dw in Cracknore Hard, whereas 154±5 ng/g dw and 40±4 ng Sn/g dw in Calshot. As suggested by the abundance of the heaviest PAH congeners (benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[a]pyrene, benz[a]anthracene, indeno[1,2,3-cd]pyrene and benzo[ghi]perylene) (Figure 3.1a), the environmental quality of Southampton Water seemed to be highly compromised by the presence of Fawley refinery and Marchwood industries. With respect to OTC, BTs concentrations seemed to indicate the presence of still active TBT sources in the area. In fact, compared to the levels reported for the British south region after TBT ban (Langston et al., 2003), OTC concentrations in Cracknore Hard were higher. Furthermore TBT was approximately as abundant as the degradation products, suggesting recent contamination (86±9 ng Sn/g dw of TBT; 93±12 ng Sn/g dw of DBT; 81±7 ng Sn/g dw of MBT) (Figure 3.1b). This might be explained by the presence of the military port in Marchwood: in fact the European Regulation (EC) No 782/2003, on the prohibition of organotin compounds on ships, allows the use of TBT-based antifouling paints on army ships hulls. Furthermore it was not possible to exclude that the muddy sediment and the sheltered morphology of the area slacken degradation processes. In general PCB concentrations were almost similar in all samples. Slightly higher residues were recorded in Cracknore Hard (48±2 ng/g dw as ΣPCBs), where traces of dioxin-like congeners (2,1±0,2 ng cb169/g dw) were found besides common environmental PCB (cb180, cb153, cb101, cb138, cb123, cb118) (Figure 3.1c). In the other stations PCB body burden ranged between 4,4±0,5 ng/g dw and 18,4±0,7 ng/g dw.

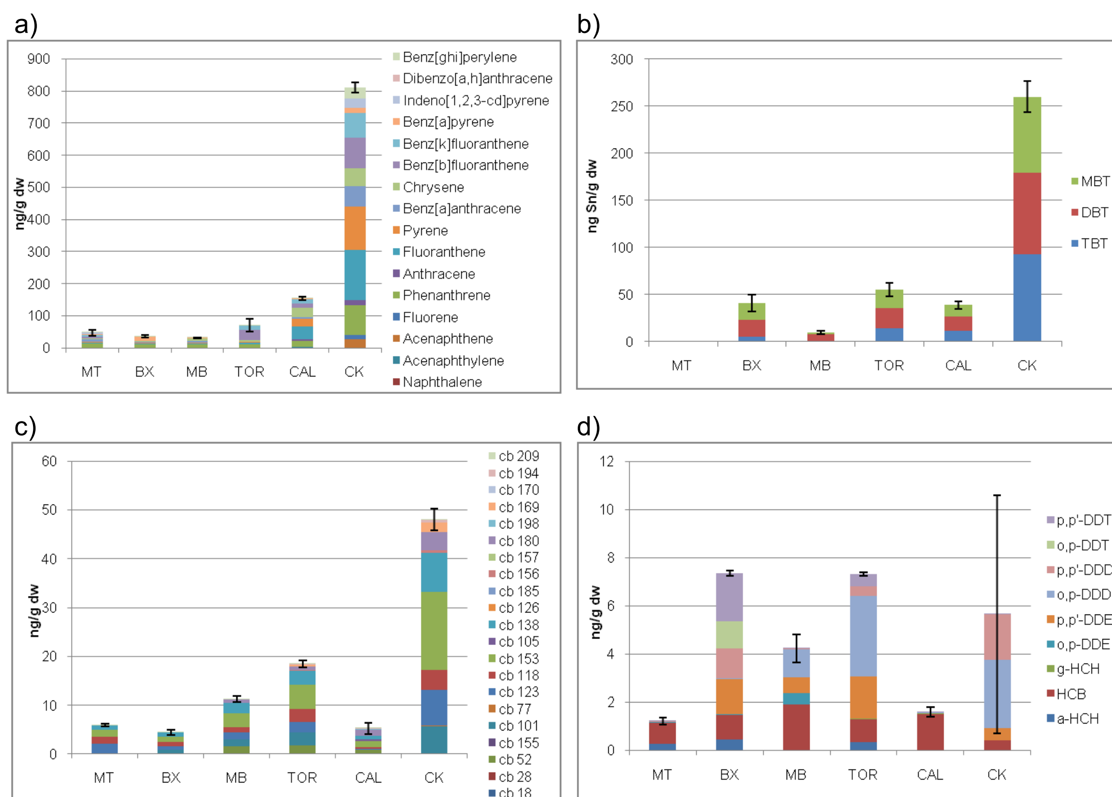



**Figure 3.1 Bioaccumulation patterns of PAHs (graph a), BTs (graph b), PCBs (graph c) and OCPs (graph d) in the six *L. littorea* populations (pools of 20 organisms each); the error bars were computed through propagation of single compound's error (semi-variation interval of the two replicates analysed). Abbreviations: Mothecombe (MT), Brixham (BX), Mountbatten (MB), Torpoint (TOR), Calshot (CAL), Cracknore Hard (CK).**

Focusing on the toxicological results (Table 3.2), Comet assay, NRR assay and Intersex analysis agreed in describing Cracknore Hard as the most impaired population and Mothecombe as the least.

**Table 3.2 Toxicological analyses results.**

| | **COMET ASSAY** | **NRR ASSAY** | **INTERSEX** | **FRAP ASSAY** |
|---|---|---|---|---|
| | **Mean percentage tail intensity (±SD)** (%) | **Mean retention time (±SD)** (minute) | **ISI** | **Mean (±SD)** (µmol $Fe^{+}$/g PRT) |
| **MOTHECOMBE** | 4 ± 2 | 75 ± 25 | 0,11 ± 0,32 | 19 ± 4 |
| **BRIXHAM** | 10 ± 7 | 47 ± 21 | 0,43 ± 0,78 | 28 ± 7 |
| **MOUNTBATTEN** | 10 ± 4 | 18 ± 6 | 0,51 ± 0,52 | 18 ± 7 |
| **TORPOINT** | 7 ± 3 | 14 ± 8 | 0,54 ± 0,50 | 14 ± 3 |
| **CALSHOT** | 17 ± 7 | 13 ± 5 | 0,21 ± 0,41 | 23 ± 6 |
| **CRACKNORE HARD** | 22 ± 7 | 13 ± 10 | 1,13 ± 1,06 | 21 ± 5 |

The analysis of variance of Comet assay results, which are plotted in Figure 3.2a, pointed out that Calshot and Cracknore Hard were similarly affected by genotoxic stresses (ANOVA, p>0,05). In Mothecombe, Brixham, Mountbatten and Torpoint the average of DNA

fragmentation was significantly lesser (ANOVA, p<0,05): these populations did not significantly differ from each other for the degree of DNA damage recorded (p>0,05), except for the pair-case Mothecombe-Brixham (p<0,05). Figure 3.2b plots the distribution of Comet assay results according to the classification system proposed by Giovannelli et al. (2003), who ranked the extension of tail migration in five arbitrary classes (class 1: 0%–5% DNA tail intensity; class 2: 5.1%–17%; class 3: 17.1%–35%; class 4: 35.1%–60%; class 5: 60.1%–100%). According to this classification, 85% of specimens collected in Mothecombe resulted belonging to class 1, whereas the remaining 15% to class 2. Differently, Brixham population had a greater proportion of class 2 organisms, as well as 2 individuals of class 3. The samples from Mountbatten and Torpoint were classified mainly into class 2, which accounted for 90% and 64,3% respectively. The highest degree of tail migration was recorded in gastropods from Southampton Water. Here, in fact, class 1 specimens were not found at all; the most frequent classes were class 2 in Calshot (50%) and class 3 in Cracknore Hard (70%); in both sites only one individual belonging to class 4 was found.

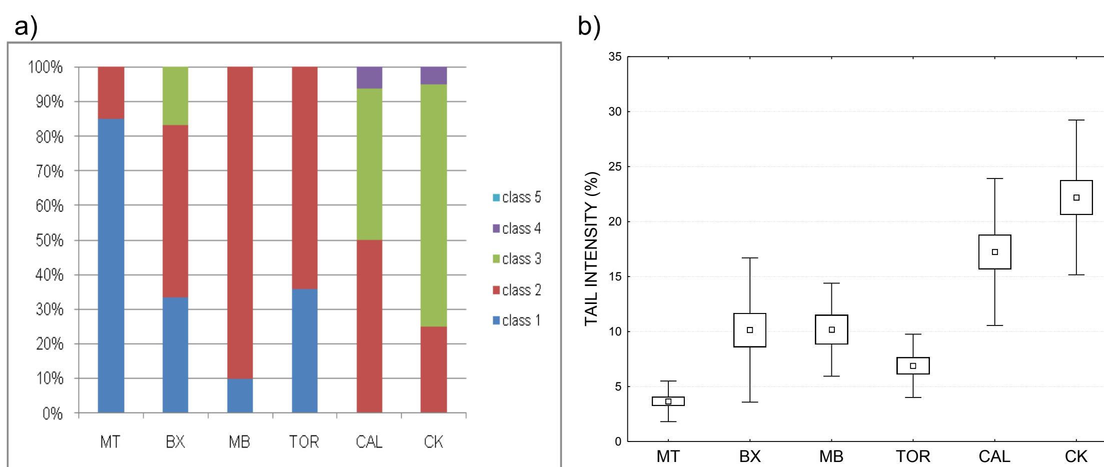



**Figure 3.2 Comet assay results. Graph a: percent frequency of tail migration classes according to Giovannelli et al. (2003). Graph b: box and whisker plot of the average DNA tail intensity. The mean is indicated by the square (□), the standard error by the box and the standard deviation by the whiskers. The results of statistical analyses are deported in the text. Abbreviations: Mothecombe (MT), Brixham (BX), Mountbatten (MB), Torpoint (TOR), Calshot (CAL), Cracknore Hard (CK).**

Considering Neutral Red retention time as indicator of general physiological status (Hwang et al., 2002; Castro et al., 2004), it could be stated that *L. littorea* presented similar health conditions in Mountbatten, Torpoint, Calshot and Cracknore Hard (Kruskal-Wallis, p>0,05): as depicted by the histogram in Figure 3.3a, in Plymouth Sound and in Southampton Water most of the specimens had retention time value of only 15 min (80 % in Mountbatten, 73,7% in Torpoint, 88,9% in Calshot and 68,4% in Cracknore Hard); furthermore, the percentage of organisms leaking Neutral Red dye immediately (retention time 0), was relevant in the last three stations (respectively 1,1% and 21,1%). Opposite conditions were observed in

Mothecombe and Brixham: in the former station most of the specimens recorded values higher than 60 minutes, whereas in the latter around 10% of the sample.

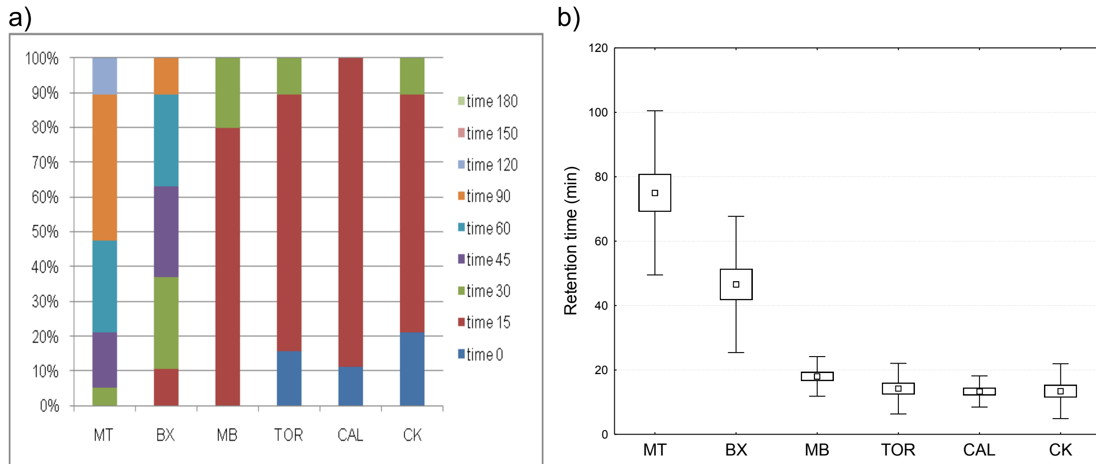



**Figure 3.3 NRR assay results. Graph a: percent distribution of NR retention times. Graph b: box and whisker plot of NR retention times; the mean is indicated by the square (□), the standard error by the box and the standard deviation by the whiskers. The results of statistical analyses are deported in the text. Abbreviations: Mothecombe (MT), Brixham (BX), Mountbatten (MB), Torpoint (TOR), Calshot (CAL), Cracknore Hard (CK).**

Intersex development analyses revealed that all populations were affected by Intersex, even if that from Cracknore Hard resulted the most impaired by the phenomenon (Figure 3.4). Cracknore Hard population recorded the highest ISI value (Kruskal-Wallis, p<0,05) and showed the presence of all Intersex stages. In particular 5 (10,6%), 4 (8,5%) and 2 (4,3%) specimens were counted for the Intersex classes 2, 3, 4 respectively (Figure 3.4a). Differently, the deformation degree reached by the other populations was generally low: only stage 1-females were observed in the other stations excluded Brixham, where two stage 4-females were found. Intersex development in the reference station was the lowest of all populations (Kruskal-Wallis, p<0,05), except Calshot's.

a) b)

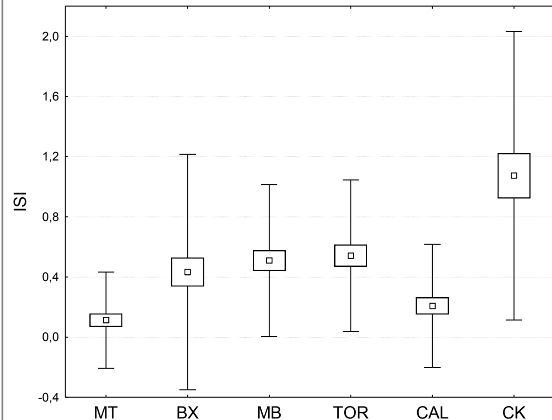



**Figure 3.4 Intersex assay results. Graph a: percent distribution of Intersex stages. Graph b: box and whisker plot of Intersex development results; the mean is indicated by the square (□), the standard error by the box and the standard deviation by the whiskers. The results of statistical analyses are deported in the text. Abbreviations: Mothecombe (MT), Brixham (BX), Mountbatten (MB), Torpoint (TOR), Calshot (CAL), Cracknore Hard (CK).**

The pattern of FRAP results definitely diverged from that featuring the other tests (Figure 3.5). In fact, as indicated in Table 3.3, there was no statistical difference between data obtained in the control station (Mothecombe, 19±4 µmol $Fe^{2+}$/g PRT) and in the most impaired site (Cracknore Hard, 21±5 µmol $Fe^{2+}$/g PRT) (ANOVA, p>0,05). The most dissimilar was Brixham population which recorded the highest antioxidant capability of all stations (ANOVA, p<0,05), except Calshot.

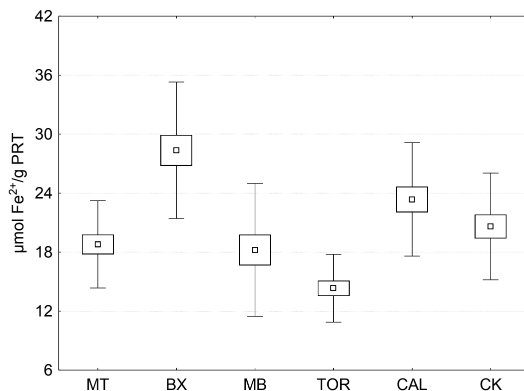



**Figure 3.5 Box and whisker plot of FRAP assay results. The mean is indicated by the square (□), the standard error by the box and the standard deviation by the whiskers. Abbreviations: Mothecombe (MT), Brixham (BX), Mountbatten (MB), Torpoint (TOR), Calshot (CAL), Cracknore Hard (CK).**

**Table 3.3 HSD Tukey test results on FRAP data. Statistical difference at <5% is marked in red. Abbreviations: Mothecombe (MT), Brixham (BX), Mountbatten (MB), Torpoint (TOR), Calshot (CAL), Cracknore Hard (CK).**

| | MT | BX | MB | TOR | CAL | CK |
|---|---|---|---|---|---|---|
| MT | - | 0,0001 | 1,000 | 0,124 | 0,110 | 0,909 |
| BX | | - | 0,0001 | 0,0001 | 0,060 | 0,0005 |
| MB | | | - | 0,269 | 0,060 | 0,776 |
| TOR | | | | - | 0,0001 | 0,007 |
| CAL | | | | | - | 0,629 |
| CK | | | | | | - |

To relate the chemical information to the toxicological description, the database was processed by the multivariate regression method PLS. Because of the limited dimension of the dataset (6 observations), PLS model was used for exploring possible relationships between the way the observations are grouped in the *bioaccumulation space* and in the *health status space*, rather than for predictive purposes. Furthermore it was used as tool to find out the relative relevance of each variable in modelling the correlation.

Table 3.4 shows the report of the linear multivariate model obtained using as descriptors (X-block) the four pollutants classes (PAH, PCB, OCP and OTC) and as responses (Y-block) the toxicological variables (Mean percentage tail intensity, NRR time, ISI, mean FRAP value). Two pairs of latent variables were extracted by the dataset, totally correlating almost the whole bioaccumulation pattern ($R^2X$ cumulative=0,98) to a certain part of the toxicological description ($R^2Y$ cumulative=0,45). Considering the scarce contribution of the second pair of variables in improving the fitting capability, the monodimensional model (marked in bold in Table 3.4) was considered for the discussion. It correlates the 75% of chemical information to the 40% of the toxicological description.

**Table 3.4 PLS analysis summary (6 observations; 8 variables (X=4; Y=4)). $R^2X$ is the fraction of variation in the X block explained by the current component; $R^2X$(cum) is the cumulative variation in the X block explained by all components extracted; Eig is the eigenvalues of the X matrix; $R^2Y$ is the variation in the Y block explained by the current component; $R^2Y$ (cum) is the cumulative variation in the Y block explained by all components extracted; $Q^2$ is the fraction of the total variation in X and Y that can be predicted by the current component; $Q^2$(cum) is the cumulative $Q^2$ for the extracted components; Sign is the significance of the component according to the cross validation rules: according to rules R1 a component is significant when $Q^2$>limit, whereas according to rules $R^2$ a component is significant when at least one Y variable has $Q^2V$>limit ($Q^2V$ is $Q^2$ for variable); Iter is number of iteration till convergence.**

| | $R^2X$ | $R^2X$(cum) | Eig | $R^2Y$ | $R^2Y$(cum) | $Q^2$ | Limit | $Q^2$(cum) | Sign | Iter |
|---|---|---|---|---|---|---|---|---|---|---|
| **1 comp** | **0,754** | **0,754** | **3,014** | **0,407** | **0,407** | **0,239** | **0,097** | **0,239** | **R1** | **7** |
| 2 comp | 0,23 | 0,983 | 0,919 | 0,045 | 0,451 | -0,555 | 0,097 | 0,239 | R2 | 10 |

The choice of using the four contaminant classes, instead of individual congeners, resulted from the optimization of a preliminary PLS model built on the whole dataset (6 observations; 50 variables (X=46; Y=4)). In fact, because no improvement in fitting and predictive capability was observed, this option was discharged.

The plots of Figure 3.6 illustrate the main features of the multivariate analysis results.

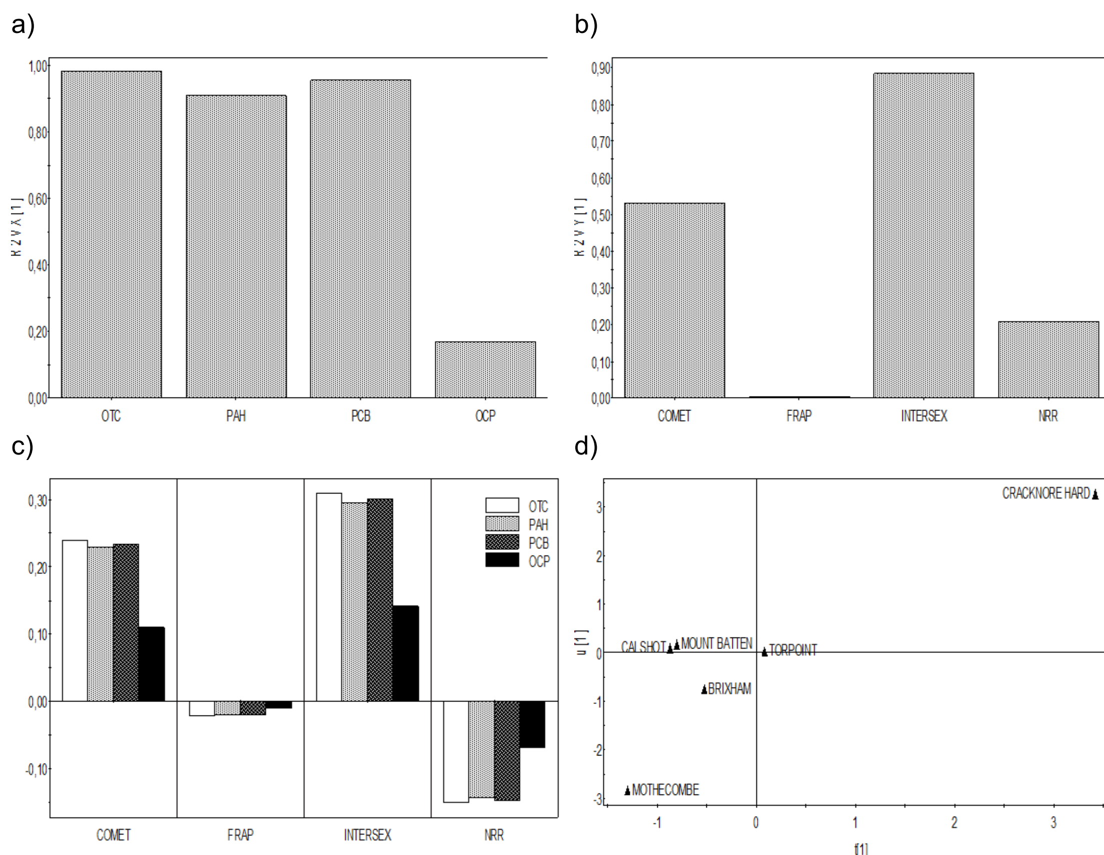



**Figure 3.6 Multivariate analysis overview. Graph a: variance ($R^2$) explained of each predictor (X); graph b: variance explained for each response (Y); graph c: regression coefficients of scaled and centered variables for the four toxicological responses; graph d: t[1]/u[1] score plot; t[1] and u[1] are the latent variables extracted in the X-space and in the Y-space respectively.**

Graphs a and b plot the weights (loadings) of X and Y variables respectively, helping to identify which variables contributed to the correlation and which were not modelled at all. As indicated by the first graph, OTC, PCB and PAH similarly weighted in the toxicological status, whereas the contribution provided by pesticides was definitely less important. As regard to the toxicological variables (graph b), the bar plot revealed that the most modelled biomarker was Intersex development, whereas the lesser was FRAP assay; DNA fragmentation degree and the level of lysosomal integrity had intermediate weight in the correlation.

The coefficients graph (Figure 3.6c) helps to interpret the specific role of each contaminant class in correlating the single toxicological responses. The vectors, in fact, summarizing model information for each toxicological variable, show how the measured variables combined to form the quantitative relation between X and Y. According to the results obtained, the involvement of the four predictors in the toxicological responses had a common pattern: in fact the different biomarkers' responses resulted to be equally caused by OTC, PAH and PCB, whereas OCP had a minor role. The opposite locations of NRR assay and

the other most relevant toxicological variables (Comet assay and Intersex development) were consistent with the inverse relationship existing between lysosomal membrane stability and health impairment-organic chemical bioaccumulation.

Finally, the t/u score plot (graph d) lets to visualize the relation structure between X and Y. The model underscored opposite conditions in terms of bioaccumulation and health status between the reference station (Mothecombe) and Cracknore Hard; intermediate conditions, even if more close to that characterizing Mothecombe population, featured all other stations.

### 3.1.2 DISCUSSION

Comet assay results distinguished different genotoxic pressures on the analysed populations, pointing out the highest extent of DNA fragmentation in Southampton Water, the area particularly affected by PAH pollution because of the high density of oil-related industries. Consistently with the well known genotoxic potential of PAH (see Par. 1.2.1.3), the spatial variation of DNA integrity correlated total PAH residues on the tissues ($n=6$, $r=0,81$, $p<0,05$); focusing on individual PAHs, no significant correlation ($p>0,05$) was observed between Comet results and tissues concentrations of the most genotoxic congeners, such as those forming bay-region diol-epoxides (benzo[a]pyrene, dibenz[a,h]anthracene, benz[a]anthracene, benzo[k]fluoranthene) (Xue and Warshawsky, 2005); a correlation was found only between mean percentage tail intensity and chrysene body burden ($n=6$, $r=0,93$, $p<0,05$).

The relation between DNA damage and PAH exposure was observed in both field and laboratory experiments using different aquatic organisms and genotoxic biomarkers (Taban et al., 2004; Kim and Hyun, 2006; Large et al, 2002; Aas et al., 2000). In the context of epidemiological surveys, Comet assay seems to be able to distinguish between indigenous populations living in pristine and oil-polluted sites, and to discriminate between acutely and chronically exposed samples. In fact Thomas et al. (2007) and Laffon et al. (2006) recorded very high DNA damage in sentinel organisms living in very oiled sites, in particular in those receiving renewing PAH inputs (*i.e.* crude oil spills from oil tankers, fuel discharges and resuspended PAH contaminated-sediments); differently, wild populations chronically exposed to PAH contamination, which is generally intermittent in quantity and patchy in distribution, were featured by levels of DNA damage only slightly higher than those recorded in the relating reference sites. Sometimes differenced in degree of tail migration was not assessed at all, suggesting the presence of adaptation mechanisms (Large et al., 2002; Akcha et al., 2004). Similar results were also obtained by McFarland et al. (1999) who measured the DNA damage through a different genotoxic test, the Alkaline Unwinding assay. The hypothesis about the existence of mechanisms able to prevent genotoxic damage in populations living in poor quality environments have been supported also by other studies

which simply compared reference and urban/industrial sites, not specifically characterized for PAH pollution (Shaw et al., 2002). Thus our findings seemed to agree with the literature: the great incidence of DNA fragmentation in Southampton Water, which receives continuously renewed PAH inputs from Esso refinery of Fawley, and the correlation found between PAH tissue load and average DNA fragmentation confirmed the capability of Comet assay to detect pollution-related genotoxic stress in *L. littorea*. However it was not possible to completely exclude the contribution in the DNA damage of the other pollutant classes whose genotoxic potential has been ascertained (Gabbianelli et al., 2006; Hagger et al., 2005 and references therein).

NRR results pointed out that the six *L. littorea* populations were experiencing environmental pressures of different intensity, stressed by spatial variation of lysosomal integrity. The average retention times were consistent with the pollution gradient, correlating the total organic pollutants body burden (n=6, r=-0,89, p<0,05). A negative correlation between lysosomal integrity and contaminant burden has been assessed through different biomarkers of lysosomal instability during monitoring programs carried out on several sentinel species (Viarengo et al., 2007). The correlation found between Comet assay and NRR assay results (n=70; r=-0,70, p<0,05) can be likely explained by the involvement of oxidative mechanisms in both genotoxic damage and lysosomal membrane impairment (Regoli et al., 2003; 2004; Mitchelmore et al., 1998; Frenzilli et al., 2001). Oxyradicals, in fact, can be generated both inside and outside lysosomes of molluscs' haemocytes, contributing to the fragility of the membranes (Nigro et al., 2006; Winston et al., 1996). The parallel loss of DNA and lysosome integrity in oxidative stressed cells has been observed in different organisms (Frenzilli et al., 2001; Gorbi et al., 2008; Nigro et al., 2006).

The high performance of Intersex analysis on *L. littorea* is widely known and standard protocols are available for its routine use in national and international monitoring programs (ICES, 2004b). In the present study Intersex development correlated total BT concentrations (n=6, r=0,83, p<0,05) rather than TBT body burden (p>0,05). Similar results were observed in other studies carried out on different gastropod species: in Pellizzato et al. (2004) and in Garaventa et al. (2007) the degree of sexual malformation in *Hexaplex trunculus* specimens better correlated the sum of OTCs concentrations than TBT. This might be explained considering that, in the sea, DBT and MBT originate mainly from TBT degradation (considering irrelevant PVC as source), so OTC body burden is almost representative of the initial TBT presence, the effective agent of Intersex induction.

FRAP assay results were more difficult to interpret, consistently with the nature of the endpoint which is not linearly related with the extent of oxidative pressure (Griffin and Bhagooly, 2004). In fact, as indicated by Hagger et al. (2008; 2009) who set a system to rank FRAP results for environmental quality assessment purposes, the presence of oxidative stress may be associated to both enhancement and reduction of antioxidant levels compared

to a reference status. However, differently from what expected, FRAP results did not mark opposite conditions in the reference station, Mothecombe, and in the most impaired one, Cracknore Hard. Contrarily, similar antioxidant capabilities were measured in several populations, as Mothecombe, Mountbatten, Calshot and Cracknore Hard, whereas the highest average was recorded in Brixham. Furthermore the results did not correlate the bioaccumulation pattern (neither single contaminant classes nor individual congeners, $p>0,05$) and they did not show any relationships with the levels of DNA integrity and lysosomal membrane stability, which are endpoints of oxidative unbalance as well ($p>0,05$). These findings are not completely unusual in the literature: fallowing 28-day laboratory exposure to sediment contaminated by environmental mixtures of PAHs, PCBs and heavy metals, FRAP results on exposed clams and crabs did not show any relationship with sediment contamination (Morales-Caselles et al., 2008); moreover the authors observed that, among the biomarkers purposely selected to assess ongoing conditions of oxidative stress, FRAP response was one of the weakest. Similarly, in Dissanayake et al. (2008) and Hagger et al. (2005) FRAP assay provided results less meaningful than those obtained through the other biomarkers making part of the selected battery.

Nevertheless further research is required to test the suitability of FRAP assay in monitoring surveys carried out on *L. littorea.* In our opinion the research has to address to possible spatial-temporal variations of antioxidants basal line and to differences in FRAP performance on single organs. In fact it was not possible to exclude that the low sensitivity of FRAP results might be related to some operative details, such as the choice of performing the assay on whole body homogenate, rather than on single organs homogenate. Future studies must consider with particular attention the antioxidant status of organs primarily involved pollutants biotransformation, such as hepatopancreas.

As previously mentioned, the computed PLS model has to be considered an attempt to explore: 1) the correlation between the comprehensive toxicological description of *L. littorea* populations and the environmental pressure exerted by organic pollutants in natural conditions, 2) the possibility of using a computed variable, expression of the population health status, to discriminate a gradient of environmental contamination, and 3) the contribution of each biomarker in the health status description.

The $R^2Y$ value of the linear model (0,407) confirmed that organic pollution impaired the health conditions of *L. littorea* natural populations, as suggested by the results of the single biomarkers. In fact, it indicated a certain capability of the latent variable extracted in the *bioaccumulation space* to explain the variance in the *health status space*. In this correlation the single contaminant classes did not play specific roles: contrarily PAH, OTC and PCB contributed to the health impairment in similar measure and the general health status of *L. littorea* seemed to be related to the comprehensive pressure exerted by organic contaminants. Regarding OCP, it was not possible to exclude that their contribution was

misunderstood because by the low variance of pesticides concentrations among the samples.

The spatial distribution of the samples in the t[1]/u[1] score graph, locating the reference and most polluted stations at the opposite extremities of the ideal diagonal line and the other samples in the middle, revealed that, according to the model, gastropods health conditions became progressively worse along the pollution gradient. By observing more in detailed the distribution pattern, it was possible to recognize three groups, respectively featured by *very impaired conditions* (Cracknore Hard), *very good conditions* (Mothecombe) and *medium-tending to good conditions* (Brixham, Mountbatten, Torpoint, Calshot). No one sample was placed in the space between Cracknore Hard (bad condition) and the third group (medium-tending to good conditions), meaning that no one population presented intermediate conditions approximately classifiable as *medium-tending to bad*. The capability of the model to potentially distinguish four levels of health impairment, consistently with the environmental quality, was an interesting result. In fact, it supported the idea that a proper integration of biomarkers responses measured at different levels of biological organization might be used as tool to discriminate the ecological status of biological elements, according to the recent requirements of water management policies (Par. 1.6).

Finally the model seemed to support the rationale used by several authors for computing multi-biomarkers based indexes expression of ecological status: similarly to Hagger et al. (2008, 2009), it differently weighted biomarkers responses in accordance with the endpoints' level of biological organization. In fact, as mentioned in Par. 1.6, Hagger et al. (2008, 2009) computed BRI (Biomarker Response Index) by weighting the least molecular-level biomarkers, the most individual-level biomarkers and intermediately cellular-level biomarkers, assuming that alterations at physiological level would have greater impact on the health conditions than changes at molecular level. Similarly, the model assigned the minimum loading value to FRAP, a molecular endpoint weighted 1 in BRI, intermediated relevance to Comet assay and NRR assay, which are measures of cellular impairments (DNA lesions and lysosomal membrane instability) weighted 2 in BRI, and maximum importance to Intersex. Although not mentioned by Hagger et al. (2008, 2009), who used sentinel organisms not developing Intersex, it is likely that Intersex would be maximally weighed by the authors (probably ≥3), being an individual-level biomarker. Thus the highest loading value associated to Intersex by PLS model would be consistent with this choice.

### 3.1.3 CONCLUSION

The present field study revealed positive features of *L. littorea* as sentinel organism for biomonitoring purposes. Firstly, this species showed high resistance to very poor conditions, allowing to sample in areas extremely polluted. At the same time it resulted enough sensitive

to reveal an environmental quality gradient. In fact, the results of biomarkers analyses discriminated environmental pressures of different nature and intensity (genotoxicity, oxidative stress and endocrine disruption), consistently with the presence of specific contaminants which are indicated as causing-agents of the assessed stress by the literature.

Despite the necessity to further develop the multivariate analysis by extending the database, these preliminary results encouraged the use of *L. littorea* in multi-biomarkers based monitoring studies. In particular they suggested positive features of statistical variables, computed as linear combination of biomarkers responses, to discriminate among natural *L. littorea* populations differently stressed by environmental pollution. Furthermore the loading values of the toxicological responses computed by the model supported the biomarkers weighting system establishing the endpoint relevance according to the correspondent level of biological organization.

In order to verify possible improvement of the model, it would be worth including other variables in the future investigations, such as heavy metals and immunotoxic endpoints among, respectively, the chemical and toxicological predictors.

# 3.2 EXPERIMENT 2

## 3.2.1 RESULTS

Table 3.5 summarizes the results of bioaccumulation analyses carried out on the three *H. trunculus* populations. It reports the tissue concentrations of each chemical class analysed and the total organic contaminants body burdens (for the whole dataset see Annex I).

**Table 3.5 Results summary of chemical analyses carried out on on pools of 20 organisms: tissue residues of organic pollutants. Pollutant classes concentrations are expressed as sum of single congeners concentrations; the error was computed through propagation of single compounds' error (semi-variation interval of the two replicates analysed).**

| | **PAH** ng/g dw | **BT** ng Sn/g dw | **PCB** ng/g dw | **OCP** ng/g dw | **ΣORGANIC POLLUTANTS** ng/g dw |
|---|---|---|---|---|---|
| **ADRIATIC** | 13,0 ± 0,4 | 13 ± 2 | 69 ± 4 | 18 ± 1 | 112 ± 5 |
| **S.M. MARE** | 35 ± 3 | 17 ± 4 | 119 ± 15 | 21 ± 2 | 192 ± 16 |
| **CERTOSA** | 42 ± 2 | 42 ± 4 | 199 ± 5 | 40,2 ± 0,9 | 323 ± 7 |

OTC concentrations are reported as sum of butyltin compounds (BT) because TPhT and degradation products were below the limit of detection in all samples.

These data confirmed that the populations selected for the present study were located along a pollution gradient, even if less marked than that observed in Experiment 1. The specimens collected outside Venice Lagoon had the lowest chemical residues in their tissues (112±5 ng/g dw as sum of organic pollutants), representing approximately half and one third of the body burden recorded in S.M. Mare (192±16 ng/g dw) and in Certosa (323±7 ng/g dw) respectively.

Focusing on the specific contamination pattern, it was noticed that PCB was the most abundant class, accounting for nearly the 60% of total body burden in all populations. As illustrated by the histogram in Figure 3.7c, individual PCBs were similarly distributed in all samples. The most abundant were cb153, cb138 and cb180, whose sum accounted for the 62-65% of PCB body burden. The remaining 21-30% was constituted by cb101, cb123, cb118, cb156. Dioxin-like PCBs generally resulted below the LOD, with the exception of cb169, whose concentrations ranged between 4±1 and 6±1 ng/g dw (see Annex I).

Total PAH concentrations in the two stations inside the lagoon were similar (respectively 35±3 ng/g dw and 42±2 ng/g dw in S.M. Mare and in Certosa), whereas they were lower in the sea station (13,0±0,4 ng/g dw). The most abundant congeners in Adriatic station were phenanthrene (5,47±0,02 ng/g dw), pyrene (2,3±0,3 ng/g dw), fluoranthene (1,29±0,07 ng/g dw), which totally accounted for the 70% of PAH body burden (versus the 42% in both S.M. Mare and Certosa). Differently the levels of petrogenic PAHs, such as nathalene and

fluorene, were relatively higher inside the lagoon, totally accounting for 16% in S.M. Mare and 17% in Certosa (versus 7% in Adriatic station). Also the pyrolitic benzo[b]fluoranthene, anthracene and acenaphthene were higher inside the lagoon, globally accounting for 32-34% (vs. 5%).

The concentrations of butyltin compounds were low in all stations. The general decrease of OTC concentrations in the area was proved by comparing the results plotted in Figure 3.7b with those reported for the same *H. trunculus* populations by Pellizzato et al. (2004). In fact, in 2002 S.M. Mare TBT body burden ranged between 50±9 and 64±9 ng Sn/g dw (vs. 11±1 ng Sn/g dw in 2009), DBT concentrations between 46±10 and 65±9 ng Sn/g dw (vs. 4±3 ng Sn/g dw) and MBT concentrations between 43±11 and 53±16 ng Sn/g dw (vs. 2±3 ng Sn/g dw). In the area of S. Nicolò del Lido, which is located close to Certosa sampling site, TBT body burden ranged between 59±2 and 117±49 ng Sn/g dw (vs. 13±3 ng Sn/g dw in 2009), DBT between 91±5 and 151±30 ng Sn/g dw (vs. 24±2 ng Sn/g dw) and MBT between 52±1 and 74± 26 ng Sn/g dw (vs. 5,0±0,6 ng Sn/g dw).

With regard to OCPs distribution pattern, residues of DDT degradation products were found in all samples, whereas HCH and HCB were generally below the LOD. The distribution pattern inside the lagoon slightly differed from that outside, mainly because of the presence of DDT (p,p'-DDT: 1,98±0,10 ng/g dw in S.M Mare and 4,9±0,6 ng/g dw in Certosa).

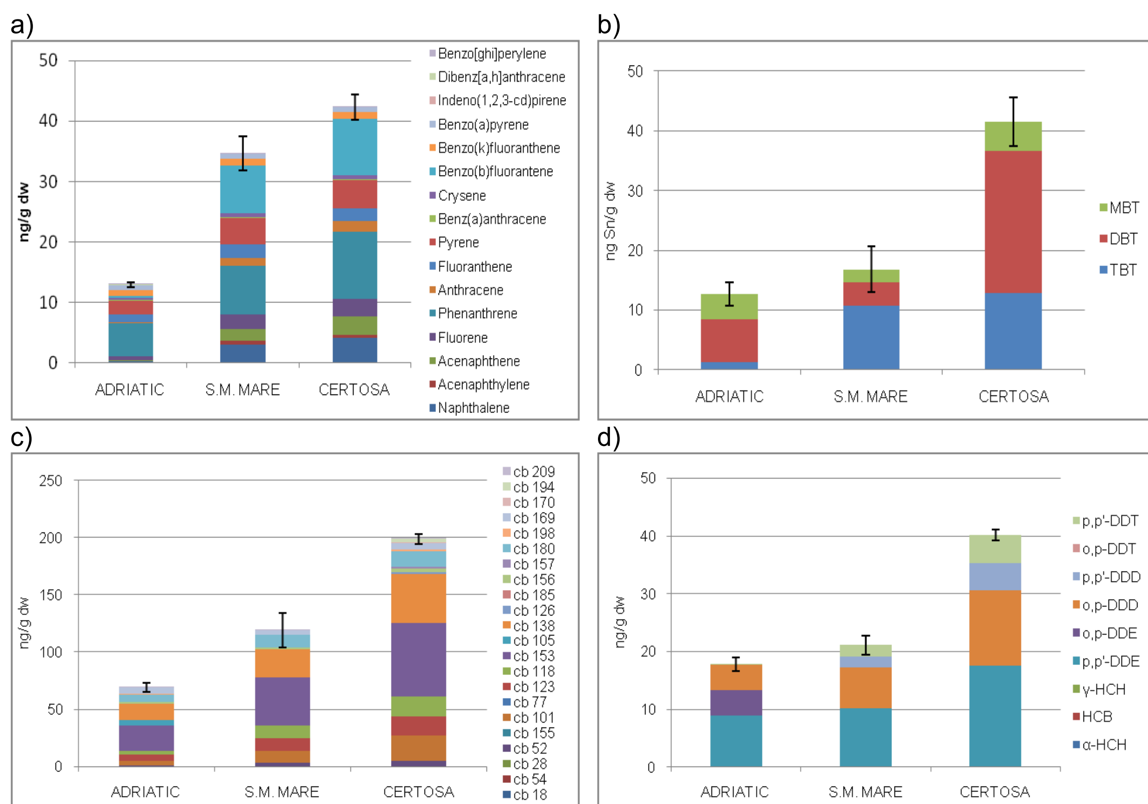



**Figure 3.7 Bioaccumulation patterns of PAHs (graph a), BTs (graph b), PCBs (graph c) and OCPs (graph d) in *H. trunculus* populations; the error bars were computed through propagation of single compound's error**

**(semi-variation interval of the two replicates analysed).**

The results of toxicological assays are summarized in Table 3.6.

**Table 3.6 Summary of results of toxicological assays.**

| | MN ASSAY | NRR ASSAY | IMPOSEX | | |
|---|---|---|---|---|---|
| | Mean micronuclei frequency (±SD) (‰) | Mean NR retention time (±SD) (minute) | Mean Female Penis Length (±SD) (mm) | VDSI | RPSI |
| **ADRIATIC** | 1,7 ± 2,3 | 31,5 ± 42,7 | 1,1 ± 0,9 | 3,7 ± 1,1 | 0,06 ± 0,16 |
| **S.M. MARE** | 3,1 ± 1,6 | 41,5 ± 49,8 | 4 ± 1 | 4,3 ± 0,6 | 4,65 ± 5,01 |
| **CERTOSA** | 1,7 ± 1,8 | 93,0 ± 78,7 | 4 ± 1 | 4,1 ± 0,6 | 5,66 ± 6,15 |

Imposex development analyses pointed out that all populations were affected by the phenomenon. As shown by the graph a in Figure 3.8, all females presented superimposition of sexual male organs, except two cases from Adriatic sampling site. The frequency of the highest Imposex stages was higher inside the lagoon: specimens showing VDS 4,7 and 5 were not found in Adriatic station, whereas they respectively accounted for 19,4% and 12,9% of female sub-population in S.M. Mare, and 10,7% and 3,6% in Certosa. The split of capsule gland was observed in all VDS 5-females.

The Imposex parameters Mean Female Penis Length, VDSI and RPSI were almost consistent in describing the degree of masculinisation reached by *H. trunculus* populations. Especially Mean Female Penis Length and RPSI clearly indicated lower Imposex development outside the lagoon (Figure 3.8c,d). The average extension of female penis was statistically lower in the snails sampled from the sea (ANOVA, $p<0,05$), whereas it was similar in the two populations living inside the lagoon ($p>0,05$). RPSI, expressing the cubed ratio between the mean female penis length and the mean male penis length, further stressed the difference in Imposex development between sea and lagoon populations. Differently, VDS results pointed out a significant difference only between S.M. Mare and Adriatic stations (Kruskal-Wallis, $p<0,05$) (Figure 3.8b). The dominance of stage 4.3-animals in all sampling sites (65,8%, 58,1% and 64,3% respectively in Adriatic, S.M. Mare and Certosa) made VDSI varying within a small range (3,7±1,1 and 4,3±0,6).

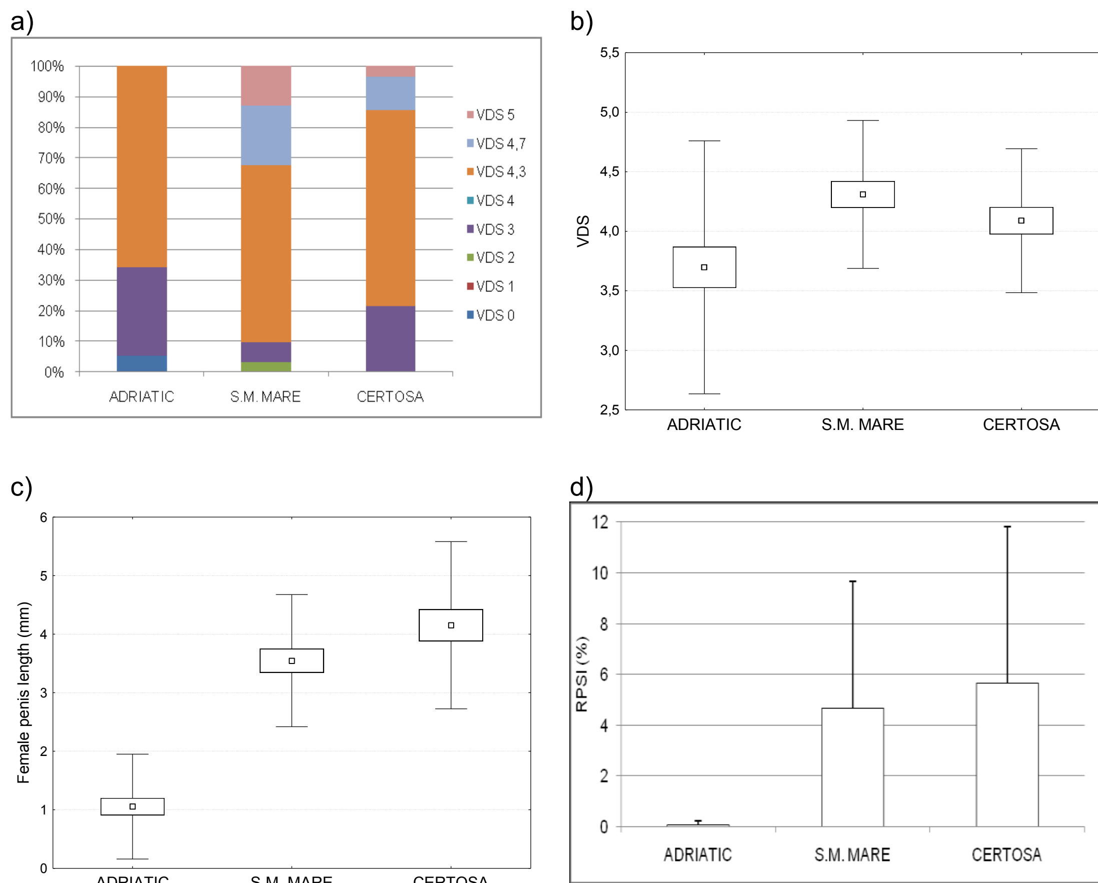



**Figure 3.8 Imposex development analysis results: percent frequency of VDS stages (graph a); box and whisker plots of VDS (graph b) and Mean Female Penis Length (graph c): the mean is indicated by the square (□), the standard error by the box and the standard deviation by the whiskers; graph-bars of RPSI (graph d).**

MN assay results are depicted in Figure 3.9a. The highest mean MN frequency was recorded in S.M. Mare, even if the difference with the other stations did not reach the statistical significance (ANOVA, p>0,05). The analyses pointed out that the incidence of micronucleated cells was highly variable within each population, ranging between 0-6 MN per specimen; thus high values of standard deviation are associated to the mean. No correlation was found between MN results and pollutants body burden (p>0,05).

Figure 3.9b plots the results of NRR assay. NRR analyses did not point out spatial variation in degree of lysosomal integrity (ANOVA, p>0,05); NRR times recorded in the stations were not significantly different from each other. As in the case of MN assay, the results were featured by great variability, as indicated by the high values of standard deviation associated.

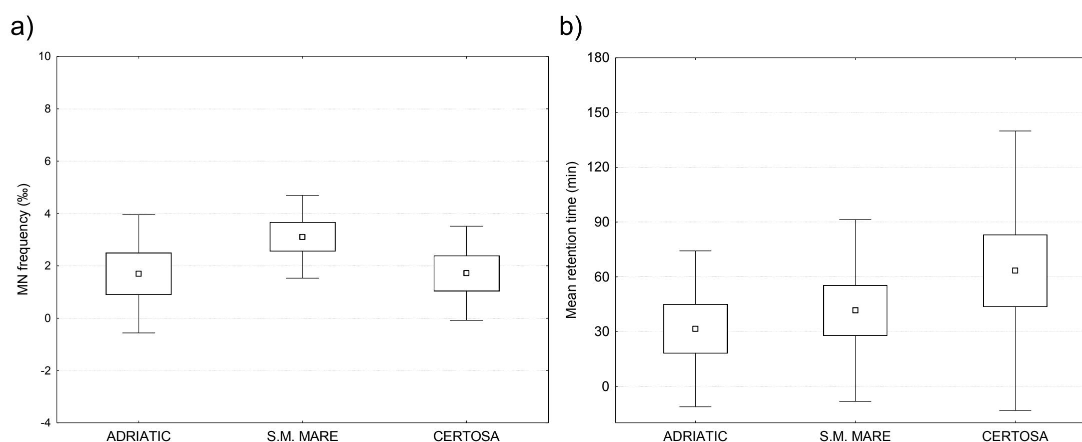



**Figure 3.9 Box and whisker plot of MN assay results (graph a) and NRR assay results (graph b); the mean is indicated by the square (□), the standard error by the box and the standard deviation by the whiskers.**

## 3.2.2 DISCUSSION

Concerning Imposex results, interesting observations were obtained by comparing the present data with those referring to the same *H. trunculus* populations sampled in 2002 by Pellizzato et al. (2004). In fact, although BT tissue concentrations clearly decreased during this period as previously illustrated (Par. 3.2.1), not all Imposex parameters followed the same trend. In particular, comparing S.M. Mare and Certosa results with those reported for S.M. Mare and S. Nicolò del Lido by Pellizzato et al. (2004) (Table 3.7), it was noticed that VDSI values remained quite high, whereas RPSI, Mean Female Penis Length and percentage of split capsule gland dropped.

**Table 3.7 Comparison of Imposex extent in *H. trunculus* populations from S.M. Mare and from the area close to Certosa-S.Nicolò del Lido in 2002 (Pellizzato et al., 2004) and in 2009 (present study)**

| | **VDSI** | | **RPSI** (%) | | **Mean Female Penis Length (±SD)** (mm) | | **Percentage of split capsule gland** (%) | |
|---|---|---|---|---|---|---|---|---|
| | 2002 | 2009 | 2002 | 2009 | 2002 | 2009 | 2002 | 2009 |
| **S.M. MARE area** | 4,1±0,6 | 4,3±0,6 | 8,03 | 4,65 | 8±2 | 4±1 | | |
| **CERTOSA area** | 4,9±0,3 | 4,1±0,6 | 36,2 | 5,66 | 12±2 | 4±1 | >80 | 3,4 |

The great sensitivity of *H. trunculus* RPSI and Mean Female Penis Length to spatial/temporal variations of TBT environmental levels was reported by Garaventa et al. (2007), Pellizzato et al. (2004) and Wilson (2001): generally the degree of Imposex development expressed by these parameters stricter correlated TBT and/or BT body burden than VDSI. Furthermore a specific relationship between OTC and Mean Female Penis Length was found by Pellizato et al. (2004).

Focusing on VDSI data, the presence of medium-high VDS stages in all stations might be explained by the high sensitivity of *H. trunculus* to TBT. Even if the exact quantitative relationship between TBT dose and VDS development is still controversial and evidences of population-related specificity have been observed (Garaventa et al., 2007), it has been estimated that the phenomenon is induced in *H. trunculus* at concentrations lower than 1 ng Sn/g dw, and it rapidly develops following small increases in TBT body load (Axiak et al., 1995; Pellizzato et al., 2004). However also the hypothesis about the role of other factors in *H. trunculus* pseudo-hermaphroditism could not be completely excluded. In fact Garaventa et al. (2006), examining 55 specimens sampled in the Mediterranean Sea between 1845-1930 and conserved in different European museums, found out cases of females with an incipient penis and bifallic males. According to the authors, these sexual abnormalities, developed before the introduction of TBT-based antifouling systems, might be considered evidences that, besides TBT, other factors might contribute to Imposex development in *H. trunculus.* As possible inducing agents, heavy metals, environmental conditions, parasites and other androgenic compounds were suggested (Maran et al., 2006). The same authors, during a laboratory experiment of Imposex induction through direct injection of TBTCl and Aroclor 1260 in *H. trunculus* specimens, observed that not only TBT treated organisms, but also the controls (both organisms simply anesthetized by immersion in 7% $MgCl_2$ water solution and organisms narcotized and injected with ethanol, the solvent used to dissolve the toxicants) developed Imposex (Garaventa et al., 2008). Also in this occasion the authors suggested that Imposex in *H. trunculus* might be a non-specific response to different stimuli. Differently, Axiak et al. (2003) hypothesized that the lowest Imposex stages in *H. trunculus* may be expressed under normal conditions, considering the co-existence of normal and imposexed (pseudoandric) females whose penis and vas deferens are distinguishable only by Scanning Electron Microscopy. Similar findings were found also by Terlizzi et al. (2004).

MN assay and NRR assay failed in discriminating different stress conditions among the populations living inside and outside Venice Lagoon and they generally provided results highly affected by intravariability.

Focusing on MN results, the high interindividual variability and scarce sensitivity has been observed in both laboratory and field studies carried out on several test-organisms (*i.e.* mussels, oysters, fishes etc) and using different cell types (*i.e.* gill cells, haemocytes, erythrocytes, lymphocytes) (Venier et al., 1997; Dailianis et al., 2003; Dolcetti and Venier, 2002). Based on these evidences, the authors have stressed the necessity to further test MN suitability in field applications.

The lack of knowledge about MN basal-line in *H. trunculus* did not allow to establish 1) if the frequency observed in all stations has to be interpreted as natural MN background for this species or 2) if possible differences in genotoxic conditions were masked by other variables (endogenous or exogenous) acting as confounding factors. The first hypothesis would

implicate that the degree of organic pollution in the two stations inside the lagoon was not effective in causing cytogenic damages in *H. trunculus* and so the presence of 0 to 6 micronucleated cells per 1000 haemocytes must be considerate non-pathological; the second hypothesis would suggest that some adaptation mechanisms and/or environmental/physiological changes influenced the frequency of micronucleated cells.

The occurrence of adaptation mechanisms against genotoxic stress in wild populations was suggested by Burgeot et al. (1996), who observed that the MN background was higher in mussels living in uncontaminated areas than in those from polluted sites. These authors hypothesized that the specificity of each site may directly influence the behaviour and physiology of living organisms, inducing adaptation mechanisms. Evidences about the occurrence of adaptation mechanisms against genotoxic stresses in marine organisms have been provided also by Sotil et al. (2008).

With regard to the possible influence of physiological changes in MN results, the role of reproduction-related factors has to be taken particularly into account considering that, unfortunately, the survey shifted into the spawning period of *H. trunculus*, which occurs in the late spring in northern Adriatic Sea. Sexual cycle has been demonstrated to influence MN formation in *Mytilus spp*: field studies on both caged and wild *Mytilus galloprovincialis* specimens showed an increase of MN formation during the breeding season (Magni et al., 2006; Burgeot et al., 1996; Pytharopoulou et al., 2006). According to Burgeot et al. (1996), the breeding period, besides being featured by gametogenesis and punctuated by spawning events, is characterized by an intense activity aimed at reconstructing the reserves and gonad tissues: all these activities, which involve haemocytes as transporters of stock and glycogens, may influence haemopoiesis, MN induction and DNA repair processes. In particular the influence of changes in mitosis frequency must be taken into account because there is no certainty about the fate of MN after more than one nuclear division (Fenech, 2001). As reviewed by Tucker and Preston (1996), the persistence of MN seems not to be indefinitive, and it is not excluded that MN might be lost at mitosis or reincluded in the hypoploid nucleus.

Finally, based on the evidences provided by several authors (Koukouzika and Dimitriadis, 2008; Dolcetti and Venier, 2002, Venier et al., 1997; Van Ngan et al., 2007; Sotil et al., 2008) it was not possible to exclude that the frequency of total nuclear abnormalities (including binucleated cells, incomplete MN, fragmented and abnormally-shaped nuclei), instead of MN only, might have improved the detection of chromosomal damage and results' interpretation. In fact, it is generally observed that the inclusion of these endpoints ameliorates the sensitivity of this biomarker (Koukouzika and Dimitriadis, 2008).

As regard NRR assay, surprisingly the tests underscored very high intra-variability in lysosomal membrane integrity. The results failed to discriminate spatial differences of

general toxic pressure and to underscore the usually observed inverse relationship between the chemical body burden and the lysosomal membrane stability. Even if NRR assay is generally reported as robust biomarker not particularly susceptible to seasonal variations and environmental fluctuations (salinity, food, oxygen) (Castro et al., 2004; ICES, 2004a; Lowe et al., 2006), we might hypothesize that the experimental-timing influenced NRR assay results. In fact the literature is not lacking of laboratories and field studies revealing decreased lysosomal stability during the reproductive period. Cho and Jeong (2005), in a laboratory experiment assessing the impact of spawning on lysosomes of haemocytes of the Pacific oyster *Crassostrea gigas*, observed a significant decrease of average NR retention time in spawned specimens compared to the unspawned. The authors concluded that the spawning involves drastic physiological changes and energetic costs, accounting for lysosomal instability. The influence of reproduction on lysosomal membrane integrity has been assessed also in digestive gland cells using Lysosome Membrane Stability assay (Kagley et al., 2003; Petrović et al., 2004; Pytharopoulou et al., 2006). In a field study aimed at assessing the temporal trend of several biomarkers responses in blue mussels, Kagley et al. (2003) observed that the differences in membrane stability which usually discriminate between reference and contaminated specimens, disappeared during the period of gonadal development and spawning. In agreement with Cho and Jeong (2005), the authors explained that the energy costs of reproduction may cause the impairment of other functions, such as the resistance against pollution. In a similar field survey carried out on *M. galloprovincialis* populations from north Adriatic coast, Petrović et al. (2004) excluded that the general decrease of lysosome membrane stability during the reproductive period may be co-influenced by seasonal fluctuation of environmental conditions (seawater temperature, oxygen saturation, salinity, pH and chlorophyll *a* concentration). Thus under the hypothesis of a possible influence of reproduction changes in NRR assay results, the high variability of lysosomal conditions within each population might be explained as differences in reproductive maturity among individuals. The lack of statistical differences NRR responses between males and females belonging to the same populations (ANOVA two ways, $p>0,05$) induced to think that reproduction-related changes were not sex-dependant and they equally involved males and females.

Finally, in agreement with Fang et al. (2009), the high interindividual variability of MN and NRR results and the failure of these biomarkers in detecting differential spatial distribution of cytogenic and cytotoxic insults might simply be related to insufficient replication. In fact, by re-analysing the statistical power of MN and NRR assays' results recently reported by the literature for wild *Mytilus spp.* populations, Fang et al. (2009) stated that the common use of sampling size of 5-10 organisms does not provide satisfactory statistical power to both MN and NRR data. According to their reviewing analysis, MN frequency seemed to be particularly affected by natural variability, which can be compensated only by the use of a

number of replicates not realistically practicable in the frame of monitoring/screening programs.

### 3.2.3 CONCLUSION

This study provided the first data on *H. trunculus* features *as sentinel organism* for multi-biomarkers based monitoring surveys in Adriatic Sea. Furthermore it allowed to successfully adapt MN assay protocol to the specific features of this species, making it available for future investigations.

Based on the results obtained, it was not possible to conclude the sufficient sensitivity of MN and NRR assay in *H. trunculus* for monitoring purposes. Further data on MN formation and lysosomes stability in haemocytes deserve in view of their possible use in long-term and large-scale monitoring programs, in agreement with what recommended by Dolcetti and Venier (2002), Siu et al., (2008). In particular the research has to address to enhance the knowledge on temporal-spatial variations of micronuclei baseline and membrane stability.

With regard to Imposex analyses results, the great potential of *H. trunculus* RPSI and Mean Female Penis Length compared to VDSI as TBT/OTC pollution indicators was assessed in accordance with the literature. Thanks to the availability of previous data about Imposex development and TBT body burden on the same gastropod populations (Pellizzato et al., 2004), it was ascertained the general decrease of OTC levels in the area of Venice Lagoon, following the ban of TBT based-antifouling paints. However, considering that male features in female gastropods were still pronounced despites the drastic decrease of TBT body load, the collected data raised again some questions about Imposex development in *H. trunculus*. In particular they not seemed to discharge the hypotheses that 1) Imposex in *H. trunculus* is induced by other causing agents different from TBT (Maran et al., 2006) and 2) the low stages of Imposex are naturally expressed in *H. trunculus* (Axiak et al., 2003). However considering the high sensibility of *H. trunculus* to low concentrations of TBT, it was not possible to exclude that the environmental traces of TBT still present in the area accounted for the induction of the phenomenon.

# 3.3 EXPERIMENT 3

## 3.3.1 RESULTS

The chemical and Imposex characterization of *N. nitidus* population used for decontamination and exposure tests is reported in Table 3.8. In the analysed sample (n=30, 21 females and 9 males), 52% of females did not show any Imposex features, 29% had VDS 1, 14% VDS 3, and 5% VDS 4.

**Table 3.8 Characterization of *N. nitidus* population used for decontamination and poisoning tests: BT and PCB tissues residues and imposex development in the tested pool (30 speciemens). The concentrations indicated as ΣBTs, Σ3PCBs and ΣPCBs represent, respectively, the sum of TBT, DBT and MBT, the sum of cb138, cb153, cb180, and the sum of PCB congeners listed in Par. 2.3.1.; the relative error was computed through propagation of single compound's error (semi-variation interval of the two replicates analysed)**

| | TBT | DBT | MBT | ΣBTs | cb153 | cb138 | cb180 | Σ3PCBs | ΣPCBs | Male penis length | Female penis length | VDSI | RPLI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (ng Sn/g dw) | | | | (ng/g dw) | | | | | mm | | % | % |
| mean | 65 | 20,8 | 4 | 90 | 4,2 | 1,9 | 0,54 | 6,6 | 9,9 | 15 | 0,4 | 1 | 2,4 |
| error | 4 | 0,8 | 1 | 4 | 0,7 | 0,4 | 0,08 | 0,8 | 0,9 | 3 | 0,5 | 1 | 3,5 |

Aquaria water temperature, salinity and pH remained almost constant during whole experimental period, respectively 22,7±0,5°C, 32±2‰ and 8,2±0,2, consistently with the values established by the experimental design (Par.1.8.2.1). The graphs in Figure 3.10 plot, separately, the concentrations of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ in the two aquaria. Except for two values (the concentrations of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ recorded respectively on 3rd and 16th of July in aquarium A), $N\text{-}NH_4^+$ level ranged between 0,04 and 0,08 mg/L, whereas $N\text{-}NO_2^-$ level between 0,05 and 0,22 mg/L.

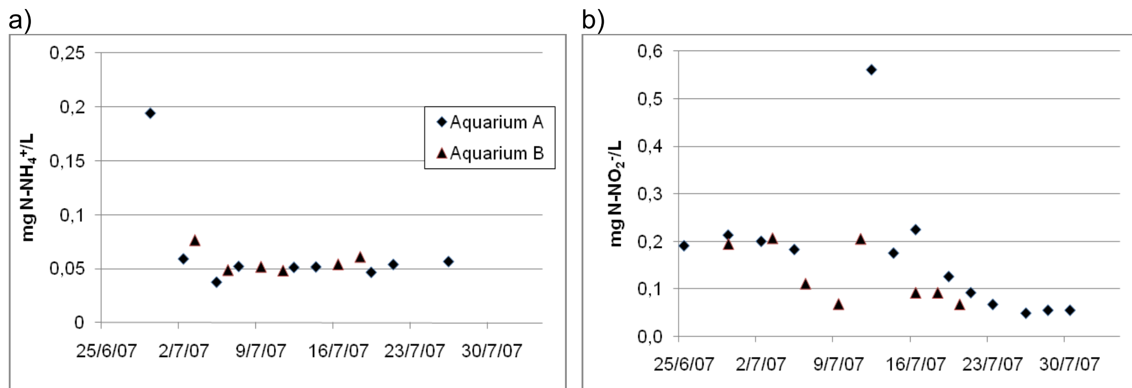



**Figure 3.10. Temporal trend of $N\text{-}NH_4^+$ (graph a) and $N\text{-}NO_2^-$ (graph b) concentrations in the aquaria water**

No organisms died during the tests. Both contaminated and not contaminated food was always completely fed in approximately 30 minutes.

The histograms of Figure 3.11 report the chemical and biological results of each test (for the corresponding tables see Annex I). In particular graphs a, c, e  and graphs b, d, f plot, respectively, the toxicants' tissues concentrations (TBT, cb138, cb153, cb180) and the values of Imposex parameters (Mean Female Penis Length and VDSI) recorded before and after each treatment (decontamination, *Recipe A* dietary poisoning, *Recipe B* dietary poisoning). Considering that part of the ingested TBT could be transformed into degradation products during the experimental period, DBT and MBT tissue concentrations were reported in the same graphs (a, c, e), close to TBT bars; furthermore, in order to make easier the view of bioaccumulation and decontamination efficiencies, the total tissue concentrations of butyltin compounds (ΣBTs,  sum of TBT and degradation products) and of the selected PCB congeners (ΣPCBs, sum of cb138, cb153 and cb180) were plotted as well.

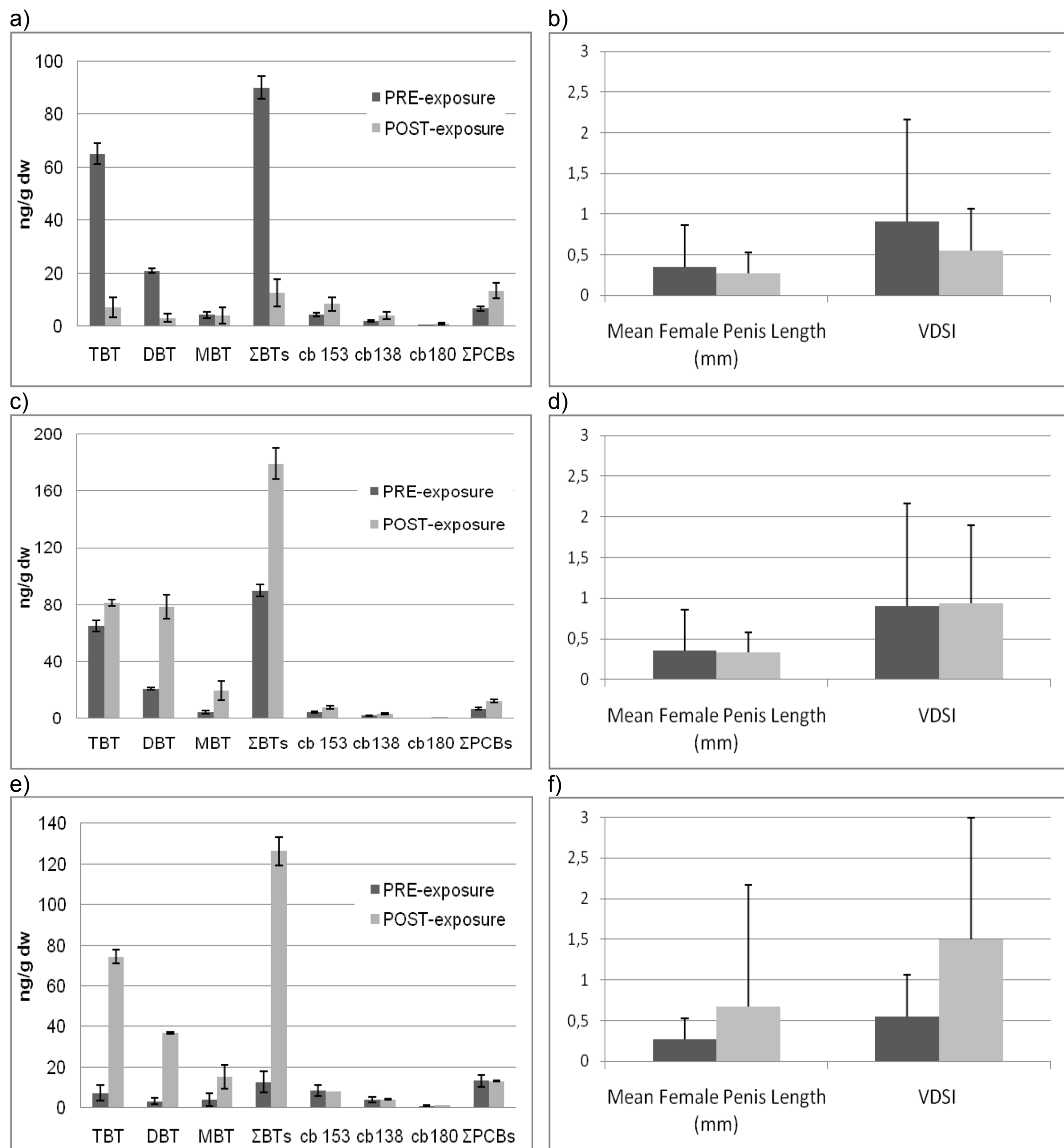



**Figure 3.11 Experiment 3 results summary: Decontamination test results (graph a: chemical analyses; graph b: Imposex analysis), *Recipe A* test results (graph c: chemical analyses; graph d: Imposex analysis), *Recipe B* test results (graph e: chemical analyses; graph f: Imposex analysis). The graphs showing chemical results plot the residues of the analytes (TBT, DBT, MDT, cb153, cb158, cb180) and the sums ΣBTs and ΣPCBs revealed on the tissues of 30 organisms-pools; butyltin compounds' concentrations are expressed as ng Sn/g dw. ΣBTs and ΣPCBs are computed as sum of TBT, DBT, MDT and as sum of cb153, cb158, cb180 respectivelly; the corresponding errors are calculated considering the errors associated to each single compound (semi-variation interval of the two replicates analysed). Imposex analysis results are expressed as Mean Female Penis Lenght and VDSI of pools of 30 organisms each.**

Focusing on the results of decontamination test (Figure 3.11a,b), chemical analyses of tissues residues revealed a significant reduction of TBT body burden after three weeks decontamination (Test t, $p<0,05$); the efficiency of the decontamination period was evident also from the decrease of DBT and total BTs tissues levels ($p<0,05$). Differently the

concentrations of PCB (both individual congeners and total amount) did not change significantly ($p>0,05$). Biological analyses did not demonstrate any significant remission of Imposex features in females, measured as female penis length and VDS stage (Test U Mann-Whitney, $p>0,05$).

Focusing on the two poisoning tests (Figure 3.11c, d, e, f), neither contamination system increased PCB body burden. As regard to TBT bioaccumulation, *Recipe B* food was absolutely the most efficient: in fact three weeks contamination enhanced almost 10 times TBT body load in the test-organisms (74±3 ng Sn/g dw of TBT in the post-exposed subsample vs. 7±4 ng Sn/g dw in the pre-exposed subsample); as consequence, also ΣBTs tissues concentrations significantly increased ($p<0,05$) (ΣBTs: 126±7 vs. 13±5 respectively). Differently, four weeks contamination with *Recipe A* spiked food did not increase significantly TBT body burden ($p>0,05$); however it enhance the general amount of BT in body tissues (ΣBTs) (Test t, $p<0,05$).

As regard to Imposex development analyses, neither female penis length nor vas deference developed significantly in the exposed subsamples (Test U Mann-Whitney, $p>0,05$), despite the increase of BTs body burden in the organisms exposed to both poisoning food. However *Recipe B* seemed to be more effective than *Recipe A* in inducing the endocrine mediated responses, considering the greater increment of VDSI and female penis length (VDSI: from 0,6±0,5 to 2±2 vs. from 1±1 to 0,9±1,0; Mean Female Penis Length: from 0,3±0,3 to 1±2 mm vs. 0,4±0,5 to 0,3±0,2 mm).

### 3.3.2 DISCUSSION

The use of an external water filtration system and the practice of daily water renewings (25% of total volume) seemed to efficiently control N-nutrients concentrations in aquaria water. In fact, the levels of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$, the most toxic N-species for aquatic biota, resembled those usually recorded in Venice Lagoon (Solidoro et al., 2004; Sfriso et al., 1994).

The chemical analyses of decontamination test organisms provided evidences about the effectiveness of a three weeks-period in decreasing TBT body burden, consistently with the experiences reported for other gastropod species (Stroben et al., 1992). However three weeks suspension of PCB dietary uptake was not enough for reducing PCB body burden because of their higher persistence. However the low PCB residues in *N. nitidus* samples from Canale Rigà (ΣPCBs 9,9±0,9 ng/g dw) might likely represent PCB background level in this species which bioaccumulate POP primarily through dietary uptake. As regard to Imposex parameters, the failed remission of male sexual characteristics from imposexed females, following suspension of environmental exposure to TBT and consequent decrease

of TBT body load, was consistent with the irreversibility of this malformation, well assessed by laboratory studies (Stroben et al., 1992B).

*Nassarius nitidus* showed positive features as test-organisms for dietary exposures. In fact, differently from other gastropod species, as the carnivorous *H. trunculus* eating exclusively living preys, its feeding habits as saprophagous allowed the ingestion of artificially poisoned food. During the whole exposure period *N. nitidus* showed good health conditions and constant appetite, as demonstrated by the survival of all specimens and by their immediate reaction to the olfactory stimuli linked to food presence inside the aquaria.

As well documented by the photo-sequence reported in Figure 3.12, within a while from the introduction of the manger into the tank, nearly all specimens went toward the food and took place on the manger almost uniformly (Figure 3.12c). The full consumption of the daily meal was always completed within 30 min; the presence of ethanol traces in the flesh did not seem to discourage food consumption.

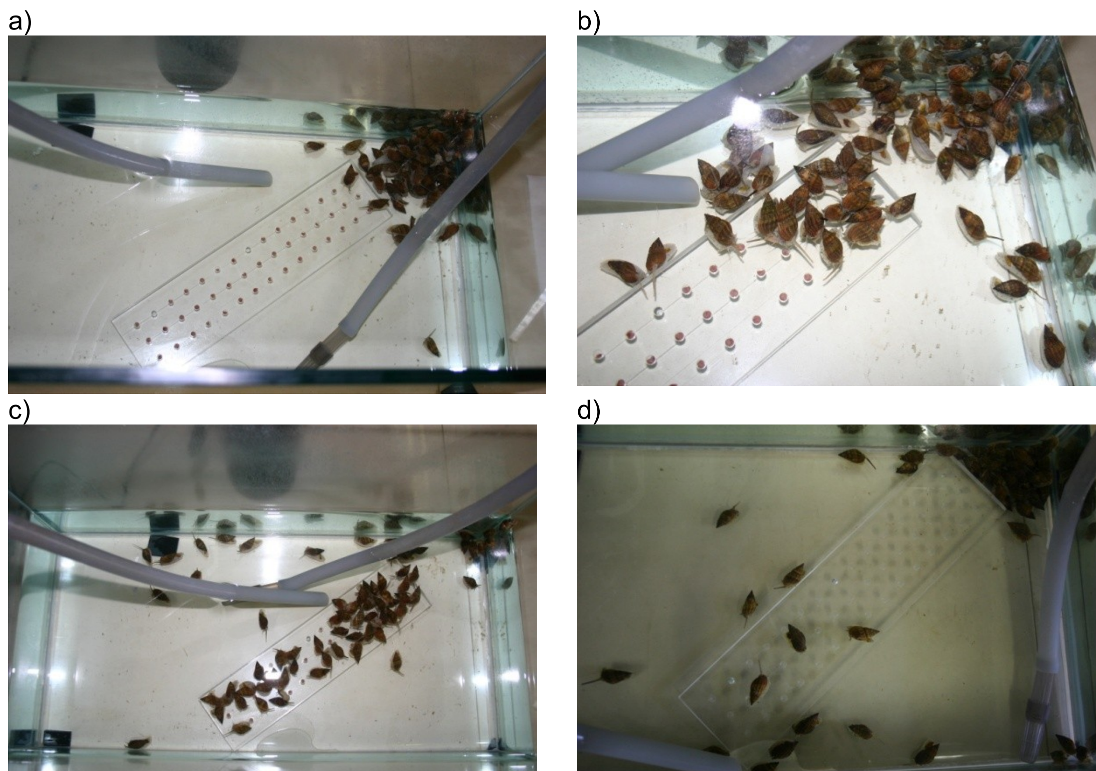



**Figure 3.12 *N. nitidus* feeding sequence (a, b, c, d)**

In fact, as shown by photos reported in Figure 3.13(a,b), generally each specimen attained to one poisoned morsel at least.

a) b)

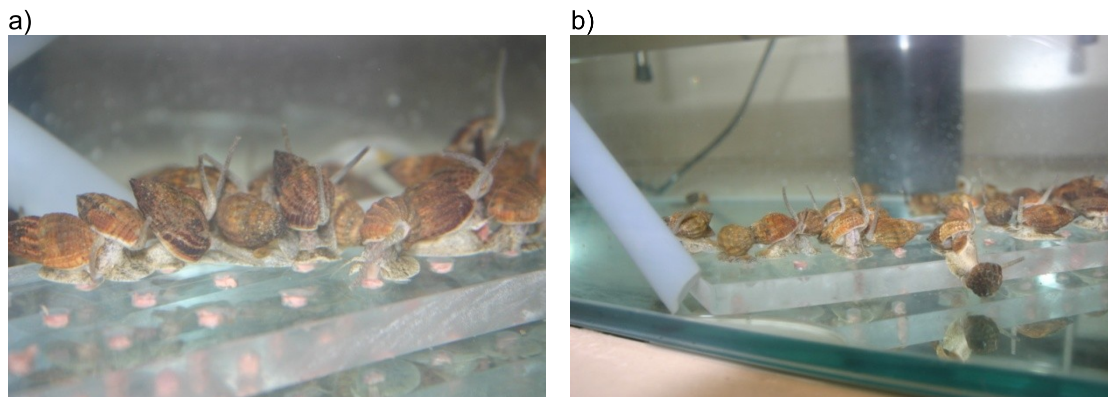


**Figure 3.13 *N. nitidus* during the feeding (a, b)**

With regard to the effectiveness of contamination systems, the lower efficiency of *Recipe A* compared to *Recipe B* in term of TBT bioaccumulation has to be attributed to the lower TBTCl concentration tested rather than to the different spiking method. The failure of both recipes in enhancing PCB body burden was likely due to the excessive low doses supplied. As explained in Par.1.8.2.1, both TBT and PCBs concentrations were chosen in order to mirror the contamination of natural *N. nitidus* food (*i.e.* carrions of invertebrates and fishes), consistently with the final research goal (*i.e.* the assessment of PCB contribution in Imposex induction at realistic environmental concentrations). Thus Venice Lagoon and its clam populations (*Tapes spp.*) were chosen as models of medium quality environment and easily available food respectively: PCBs poisoning concentrations were fixed consistently with the literature (Boscolo et al., 2007; Bayarri et al., 2001). Probably this rationale resulted excessively oversimplified: PCB body burden in *Tapes spp.* might not be a good example for average PCB residues in *N. nitidus* food, considering that this species has a very wide diet as scavenger. PCB load in marine organisms is highly variable, due to PCB biomagnification along the food chain (Perugini et al., 2004). In fact, for example, other bivalve molluscs sharing the habitat with *Tapes spp* inside the lagoon, such as the abundant *Mytilus spp*, usually present higher PCB tissues concentrations than the sympatric clams (Bayarri et al., 2001). Furthermore it was not properly considered that gastropod species bioaccumulating POPs mainly through food ingestion reach relevant PCB body burden by long-term dietary uptake. So the tested PCB concentrations were likely not sufficient for observing an increase of PCB residues after 3-4 weeks exposure.

Even if the pre- and post-exposure subsamples of *Recipe B* test did not statistically differ for Imposex development ($p>0,05$), the greater increase of Imposex parameters (VDSI and Mean Female Penis Length) observed in poisoned organisms was consistent with the major raise of TBT body burden.

### 3.3.3 CONCLUSION

Experiment 3 allowed to settle important details of the experimental design of the long-term laboratory exposure planned to assess the role of PCB in inducing Imposex in gastropod species. In particular it permitted to test the effectiveness of 1) aquaria management mode, 2) decontamination period and 3) two different systems of dietary exposure.

Based on the experimental evidences acquired, three weeks-decontamination was enough to decrease TBT, but not PCB, body burden in *N. nitidus*. However the population from Canale Rigà (Venice), selected for recruiting the test-organisms, provided specimens with low PCB residues in their tissues. Further extension of decontamination period could not be recommendable for the final experiment, considering that it could enhance the risk of affecting test-organisms' health conditions, which, contrarily, have to be preserved for the long dietary exposure (3 months).

The exposure of *N. nitidus* through fortified food seemed to be an effective contamination method. The food was quickly consumed during the whole experimental period; the choice of providing the food through a manger, which maintained a certain distance among the pieces of flesh, resulted to be effective in encouraging an almost uniform repartition of food among the specimens.

TBT doses administered in *Recipe B* experiment resulted more suited for enhancing TBT body burden compared to those used in *Recipe A* experiment; differently both PCB concentrations tested were too lower for the experimental purposes. So the use of higher PCBs doses is recommended for future investigations. Based on the experience acquired, it would be better to enhance PCB concentrations on food rather than to augment the feeding frequency or the size of rations provided: in fact the feeding system experienced (approximately 4 g of beef heart per 100 specimens, three times/week) seemed to guarantee high constant appetite among test-organisms, which is a necessary condition for carrying out long-term dietary exposures. The average PCB concentrations in the biota living in medium-poor quality environments could be taken as reference values for PCB food content. As regard to the spiking methods, the direct immersion of flesh pieces in the contaminant solution, followed by solvent evaporation (method used in *Recipe B* test), seemed to be more suitable because less time-wasting. In the selection of spiking concentrations it would be wise to take into account possible toxicants losses due to the relative efficiency of the method.

The method used for aquaria management seemed to be proper for *N. nitidus* long-term exposure. All monitored physical-chemical parameters resulted nearly stable during the whole experimental period and seemed not to affect organisms' heath conditions.

# 4 CONCLUSIONS

The present PhD project contributed to develop two research lines addressed to the enhancement of the knowledge on the sensitivity of gastropod species toward organic and metallorganic pollutants and their suitability as *sentinel organisms* for biomonitoring purposes.

Focusing on the two field experiences which were carried out in the context of the first research line, Experiment 1 and Experiment 2 provided encouraging evidences for the use of common gastropod species in multi-biomarkers based biomonitoring programs. In particular Experiment 1, which allowed to collect the first data on the sensibility of Comet assay, NRR assay and FRAP assay on the Atlantic species *Littorina littorea*, provided positive evidences about the possibility to discriminate wild gastropod populations experiencing different stress conditions by the use of selected suites of biomarkers. In fact it was observed that stress syndromes were more pronounced in the populations living in areas affected by the presence of specific pollutants classes, in agreement with what reported by the literature for other test-organisms. The multivariate analysis carried out on the whole chemical and toxicological dataset showed positive prospects for the use of multi-biomarkers based indexes computed as linear combination of biomarkers responses: the new variable, comprehensively describing the population *health status,* was able to outline progressive health impairment along the pollution gradient.

Experiment 2, aimed at testing Experiment 1 design in the context of the Mediterranean Sea, allowed to collect the first epidemiological data of MN assay and NRR assay on the species *Hexaplex trunculus*. The results highlighted the necessity to further investigate the possible influence of seasonal variables in biomarkers' responses in gastropod species.

Both field experiences gave the opportunity to adapt the protocols of easy and cost-effective ecotoxicological assays to the features of gastropod species, making them available for future investigations. Furthermore they contributed to update the knowledge on OTC contamination levels in two areas historically affected by TBT pollution: the analyses of Intersex and Imposex development, coupled with the determination of OTC tissues residues in gastropod populations from south-west England and Venice Lagoon, showed a general decrease of TBT levels following to the ban on TBT use in antifouling paints.

The "pilot" tests (Experiment 3) carried out for the second research line allowed to set important details of the laboratory experiment in program in the Environmental Chemistry Laboratory of Ca' Foscari University (Venice), aimed at assessing the possible contribution of PCB in Imposex induction in gastropod species. Through these studies, besides acquiring

the practical skills required for succeeding in long-term exposures of aquatic organisms, important information for the settlement of a powerful dietary poisoning system were obtained. Moreover it was experimentally ascertained the general suitability of *Nassarius nitidus* as test-organism for the designed experiment, the appropriateness of the *N. nitidus* population from Canale Rigà (Northern Venice Lagoon) for test-specimens recruitment and the efficiency of 3 weeks-decontamination period to decrease the pollutants' background load in the test-organisms.

# ACKNOWLEDGMENTS

I would like to thank Prof. Pavoni for the support during the three years research project and Prof. Galloway for the possibility to carry out part of the PhD program in the Invertebrate Ecotoxicology Laboratory (Exeter University). A special thanks to all people that worked with me in the Lab, giving me helps, advices and fun: Elena Centanni (Ca' Foscari University of Venice), Chris Pook, Ceri Lewis, Ida Dolciotti and Katla Jörundsdóttir (Exeter University). I thanks also Sonia Ceoldo, Chiara Facca, Sabrina Manente, Alessio Gomiero and Anna Mietto for their invaluable technical and scientific assistance, and all other people who gave me their advices and concrete helps to overcome difficulties.

A special thank to my family, my friends and Giacomo for encouraging me during the whole PhD project.

# REFERENCES

Aas E, Baussant T, Balk L, Liewenborg B, Andersen OK. 2000. PAHs metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. *Aquatic Toxicology* 51, 241-258

Abele D, Puntarulo S . 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology*, Part A 138, 405– 415

Adams SM, Bevelhimer MS, Greeley MS, Levine DA, Teh SJ. 1999. Ecological risk assessment in a large river–reservoir: 6 bioindicators of fish population health. *Environmental Toxicology and Chemistry* 18(4), 628–640

Adams SM, Brown AM, Goede RW. 1993. A quantitative health assessment index for rapid evaluation of fish condition in the field. *Transactions of the American Fisheries Society* 122, 63–73

Akcha F, Tanguy A, Leday G, Pelluhet L, Budzinski H, Chiffoleau JF. 2004. Measurement of DNA single-strand breaks in gill and hemolymph cells of mussels, *Mytilus* sp., collected on the French Atlantic Coast. *Marine Environmental Research* 58, 753–756

Albertini RJ, Anderson D, Douglas GR, Hagmar L, Hemminki K, Merlo F, Natarajan AT, Norppa H, Shuker DEG, Tice R, Waters MD, Aitio A. 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutation Research* 463, 111–172

Alzieu C. 1998. Tributyltin: case study of a chronic contaminant in the coastal environment. *Ocean & Coastal Management* 40, 23-36

Anderson JW, Lee RF. 2006. Use of biomarkers in oil spill risk assessment in the marine environment. *Human and Ecological Risk Assessment* 12, 1192-1222

Anderson SL, Cherr GN, Morgan SG, Vines CA, Higashi RM, Bennett WA, Rose WL, Brooks AJ, Nisbet RM. 2006. Integrating contaminant responses in indicator saltmarsh species. *Marine Environmental Research* 62, S317–S321

Antizar-Ladislao B. 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. *Environment International* 34, 292-308

Arnold CG, Ciani A, Mùller SR, Amirbahman A, Schwarzenbach RP. 1998. Association of triorganotin compounds with dissolved humic acids. *Environmental Science and Technology* 32, 2976-2983

ASTM, 2005. Standard Guide for determining DNA single-strand damage in eukaryotic cells using the Comet assay, E2186-02a. American Society for Testing and Materials

Axiak V, Micallef D, Muscat J, Vella A, Mintoff B. 2003. Imposex as a biomonitoring tool for marine pollution by tributyltin: some further observations. *Environment International* 28, 743-749

Axiak V, Vella AJ, Agius D, Bonnici P, Cassar G, Cassone R, Chircop P, Micallef D, Mintoff B, Sammut M. 2000. Evaluation of environmental levels and biological impact of TBT in Malta (central Mediterranean). *The Science of Total Environment* 258, 89-97

Axiak V, Vella AJ, Micallef D, Chircop P, Mintoff B. 1995. Imposex in *Hexaplex trunculus* (Gastropoda, Muricidae): first results from biomonitoring of tributyltin contamination in the Mediterranean. *Marine Biology* 121, 685-691

Bacchiocchi S, Gorbi S, Orletti R, Palombo P, Regoli F. 2002. Valutazione rapida della capacità antiossidante totale in *Mytilus galloprovincialis*. *Webzine Sanità Pubblica Veterinaria* 14, 1-9

Barber JL, Sweetman AJ, van Wijk D, Jones KC. 2005. Hexachlorobenzene in the global environment: emissions, levels, distribution trends and processes. *Science of the Total Environment* 349, 1-44

Barker S, Weinfeld M, Murray D. 2005. DNA–protein crosslinks: their induction, repair, and biological consequences. *Mutation Research* 589, 111–135

Barroso CM, Moreira MH, Gibbs PE. 2000. Comparison of imposex and intersex developmentin four prosobranch species for TBT monitoring of a southern European estuarine system (Ria de Aveiro, NW Portugal). *Marine Ecology Progress Series* 201, 221–232

Baršienė J, Lehtonen KK, Koehler A, Broeg K, Vuorinen PJ, Lang T, Pempkowiak J, Syvokiene J, Dedonyte V, Rybakovas A, Repecka R, Vuontisjarvi H, Kopecka J. 2006. Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipeda-Butinge area (Baltic Sea). *Marine Pollution Bulletin* 53, 422–436

Bauer B, Fioroni P, Ide I, Liebe S, Oehlmann J, Stroben E, Watermann B. 1995. TBT effects on the female genital system of *Littorina littorea*: a possible indicator of tributyltin pollution. *Hydrobiologia* 309, 15-27

Bauer B, Fioroni P, Schulte-Oehlmann U, Oehlmann J, Kalbfus W. 1997. The use of *Littorina littorea* for tributyltin (TBT) effect monitoring- results from the german TBT survey 1994/1995 and laboratory experiments. *Environmental Pollution* 96(3), 299-309

Baumard P, Budzinski H, Garrigues P, Sorbe JC, Burgeot T, Bellocq J. 1998. Concentrations of PAHs (Polycyclic Aromatic Hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. *Marine Pollution Bulleti,* 36(12), 951-960

Bayarri S, Baldassarri LT, Iacovella N, Ferrara F, di Domenico A. 2001. PCDDs, PCDFs, PCBs and DDE in edible marine species from the Adriatic Sea. *Chemosphere* 43, 601-610

Beard J. 2006. DDT and human health. *Science of the Total Environment* 355, 78-89

Beliaeff B, Burgeot T. 2002. Integrated biomarker response: a useful tool for ecological risk assessment. *Environmental Toxicology and Chemistry* 21(6), 1316-1322

Benzie IFF, Strain JJ. 1996. The Ferric Reducing Ability of Plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical Biochemistry* 239, 70–76

Borja A, Bricker SB, Dauer DM, Demetriades NT, Ferreira JG, Forbes AT, Hutchings P, Jia X, Kenchington R, Marques JC, Zhu C. 2008. Overview of integrative tools and methods in assessing ecological integrity in estuarine and coastal systems worldwide. *Marine Pollution Bulletin* 56, 1519-1537

Borja J, Taleon DM, Auresenia J, Gallardo S. 2005. Polychlorinated biphenyls and their biodegradation. *Process Biochemistry* 40, 1999-2013

Boscolo R, Cacciatore F, Berto D, Giani M. 2007. Polychlorinated biphenyls in clams *Tapes philippinarum* cultured in the Venice Lagoon (Italy): contamination levels and dietary exposure assessment. *Food and Chemical Toxicology* 45, 1065-1075

Bowen RE, Depledge MH. 2006. Rapid Assessment of Marine Pollution (RAMP). *Marine Pollution Bulletin* 53, 631-639

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254

Brendler-Schwaab S, Hartmann A, Pfuhler S, Speit G. 2005. The *in vivo* comet assay: use and status in genotoxicity testing. *Mutagenesis* 20(4), 245-254

Broeg K, Westernhagen HV, Zander S, Körting W, Koehler A. 2005. The ‘‘bioeffect assessment index’’ (BAI). A concept for the quantification of effects of marine pollution by an integrated biomarker approach. *Marine Pollution Bulletin* 50, 495-503

Brunk UT, Terman A. 2002. Lipofuscin: Mechanisms of age-related accumulation and influence on cell function. *Free Radical Biology & Medicine* 33(5), 611-619

Bryan GW, Gibbs PE, Burt GR, Hummerstone LG. 1987. The effects of tributyltin accumulation on adult dog-welks, *Nucella lapillus*: long term field and laboratory experiments. *Journal of Marine Biological Association of the UK* 66, 524-544

Bryan GW, Gibbs PE, Hummerstone LG, Burt GR. 1986. The decline of the gastropod *Nucella lapillus* around the south-west England: evidence for the effect of tributyltin from antifouling paints. *Journal of the Marine Biological Association of the UK* 66, 611-640

Burgeot T, Woll S, Galgani F. 1996. Evaluation of the micronucleus test on *Mytilus galloprovincialis* for monitoring applications along French coasts. *Marine Pollution Bulletin* 32(1), 39-46

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *The Science of the Total Environment* 247, 295-311

Calow P, Forbes V. 2003. Does ecotoxicology inform ecological risk assessment? *Environmental Toxicology and Chemistry* 37, 146A–151A

Castro M, Santos MM, Monteiro NM, Vieira N. 2004. Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. *Marine Environmental Research* 58, 741–745

Champ MA. 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits. *The Science of the Total Environment* 258, 21-71

Chiavarini S, Massanisso P, Nicolai P, Nobili C, Morabito R. 2003. Butyltins concentration levels and imposex occurrence in snails from the Sicilian coasts (Italy). *Chemosphere* 50, 311-319

Cho SM, Jeong WG. 2005. Spawning impact on lysosomal stability of the Pacific Oyster, *Crassostrea gigas*. *Aquaculture* 244, 383– 387

Cornelissen G, Breedveld GD, Næs K, Oen AMP, Ruus A. 2006. Bioaccumulation of native polycyclic aromatic hydrocarbons from sediment by a polychaete and a gastropod: freely

dissolved concentrations and activated carbon amendment. *Environmental Toxicology and Chemistry* 25(9), 2349-2355

Costello MJ, Bouchet P, Boxshall G, Emblow C, Vanden Berghe E. 2004. European Register of Marine Species. Available online at http://www.marbef.org/data/erms.php (15/01/2010).

Croudace IW, Cundy AB. 1995. Heavy metal and hydrocarbon pollution in recent sediments from Southampton water, Southern England: a geochemical and isotopic study. *Environmental Science & Tecnology* 29(5), 1288-1296

Da Ros L, Meneghetti F, Nasci C. 2002. Field application of lysosomal destabilisation indices in the mussel *Mytilus galloprovincialis*: biomonitoring and transplantation in the Lagoon of Venice (north-east Italy. *Marine Environmental Research* 54(3-5), 817-22

Dailianis S, Domouhtsidou GP, Raftopoulou E, Kaloyianni M, Dimitriadis VK. 2003. Evaluation of Neutral Red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of Mytilus galloprovincialis (L.), in pollution monitoring. *Marine Environmental Research* 56, 443–470

Daren Z. 2001. QSPR studies of PCBs by the combination of genetic algorithms and PLS analysis. *Computer & Chemistry* 25, 197–204

Davies IM, Minchin A. 2002. Quality assurance of imposex and intersex measurements in marine snails. *Journal of Environmental Monitoring* 4, 788–79

Davies IM. 2000. Kinetics of the development of Imposex in transplanted adult dogwhelks, *Nucella lapillus*. *Environmental Pollution* 107, 445-449

De Lange RP, van Minnen J. 1998. Localization of the neuropeptide APGWamide in gastropod mollusks by *in situ* hybridization and immunocytochemistry. *General and Comparative Endocrynology* 109, 166-174

De Mora SJ, Pelletier E. 1997. Environmetal tributyltin research: part, present, future. *Environmental Science and Technology* 18, 1169-1177.

Deml E. 2000. I bifenili policlorurati. In: Greim H, Delm E. – Toxicology. Zanichelli, Bologna, pp. 324-330

Depledge MH, Billinghurst Z. 1999. Ecological significance of endocrine disruption in marine invertebrates. *Marine Pollution Bulletin* 39(1-12), 32-38

Depledge MH, Fossi MC. 1994. The role of biomarkers in environmental risk assessment (2). Invertebrates. *Ecotoxicology* 3, 161–172

Dhawan A, Bajpayee M, Parmar D. 2009. Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biology and Toxicology* 25(1), 5-32

Di Giulio RT, Benson WH, Sanders BM, Van Veld PA. 1995. Biochemical mechanisms: metabolism, adaptation, and toxicity. In: Rand GM (Ed.). Fundamentals of aquatic toxicology. Effects, environmental fate and risk assessment (II edition).Taylor & Francis, Washington DC, USA. pp. 523-561

Di Giulio RT, Washburn PC, Wenning RJ, Winston GW, Jewell CS. 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* 8, 1103-1123

Dissanayake A, Galloway TS, Jones MB. 2008. Physiological responses of juvenile and adult shore crabs *Carcinus maenas* (Crustacea: Decapoda) to pyrene exposure. *Marine Environmental Research* 66, 445–450

Dixon DR, Pruski AM, Dixon LRJ, Jha AN. 2002. Marine invertebrate eco-genotoxicology: a methodological overview. *Mutagenesis* 7(6), 495–507

Dolcetti L, Venier P. 2002. Susceptibility to genetic damage and cell types in Mediterranean mussels. *Marine Environmental Research* 54, 487–491

Downs CA, Dillon Jr RT, Fauth JE, Woodley CM. 2001. A molecular biomarker system for assessing the health of gastropods (*Ilyanassa obsoleta*) exposed to natural and anthropogenic stressors. *Journal of Experimental Marine Biology and Ecology* 259, 189–214

Fairbairn DW, Olive PL, O'Neill KL. 1995. The comet assay: a comprehensive review. *Mutation Research* 339, 37-59

Fang JK, Wu RS, Yip CK, Shin PK. 2009. Power analysis for biomarkers in mussels for use in coastal pollution monitoring. *Marine Pollution Bulletin* 58(8), 1152-1158

Fenech M. 2000. The *in vitro* micronucleus technique. *Mutation Research* 455, 81–95

Fent K, Looser PW. 1995. Bioaccumulation and bioavailability of tributyltin chloride: influence of pH and humic acids. *Water research* 29(7), 1631-1637

Fent K. 1996. Ecotoxicology of organotin compounds. *Critical Reviews in Toxicology* 26(1), 1-117

Fent K. 2003. Ecotoxicological problems associated with contaminated sites. Toxicology Letters 140-141, 353- 365

Féral C, LeGrall S. 1983. The influence of a pollutant factor (tributyltin)on the neuroendocrine mechanism responsible for the occurrence of a penis in the females of *Ocenebra erinacea*. In: Lever J, Boer HH (Ed.). Molluscan neuro-endocrinology. Amsterdam: North Holland Publ. Co. pp. 173-175

Fernàndez P, Grimalt JO. 2003. On the global distribution of persistent organic pollutants. *Chimia* 57(9), 514-521

Fernley PW, Moore MN, Lowe DM, Donkin P, Evans S. 2000. Impact of the Sea Empress oil spill on lysosomal stability in mussel blood cells. *Marine Environmental Research* 50, 451-455

Fillmann G, Watson GM, Howsam M, Francioni E, Depledge MH, Readman JM. **2004.** Urinary PAH Metabolites as Biomarkers of Exposure in Aquatic Environments. *Environmental Science and Technoogy*, *38*(9), 2649–2656

Fishbein L. 1973. Polychlorinated biphenyls. In: Chromatography of Environmental Hazards, Vol. II, Ed. Elsevier Scientific Publishing Company, New York, USA, pp. 529-577

Francioni E, Wagener AdLR, Scofield AL, Depledge MH, Cavalier B. 2007. Evaluation of the mussel *Perna perna* as a biomonitor of polycyclic aromatic hydrocarbon (PAH) exposure and effects. *Marine Pollution Bulletin* 54, 329-338

Frenzilli G, Nigro M, Scarcelli V, Gorbi S, Regoli F. 2001. DNA integrity and total oxyradical scavenging capacity in the Mediterranean mussel, *Mytilus galloprovincialis*: a field study in a highly eutrophicated coastal lagoon. *Aquatic Toxicology* 53, 19–32

Fretter V, Graham A. 1994. British prosobranch molluscs: their functional anatomy and ecology; revised and updated edition. London: The Ray Society

Gabbianelli R, Moretti M, Carpenè E, Falcioni G. 2006. Effects of different organotins on DNA of mollusk (*Scapharca inaequivalvis*) erythrocytes assessed by comet assay. *Science of the Total Environment* 367, 163-169

Galloway T, Sanger RC, Smith KL, Fillmann G, Readman JW, Ford TE, Depledge M. 2002. Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays. *Environmental Science & Tecnology* 36, 2219-2226

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Depledge MH, Jones MB. 2006. The ECOMAN project: a novel approach to defining sustainable ecosystem function. *Marine Pollution Bulletin* 53, 186–194

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Jones MB, Depledge MH. 2004. A multibiomarker approach to environmental assessment. *Environmental Science & Tecnology* 38, 1723-1731

Galloway TS. 2006. Biomarkers in environment and human health risk assessment. *Marine Pollution Bulletin* 53, 606-613

Garaventa F, Centanni E, Fiorini S, Noventa S, Terlizzi A, Faimali M, Pavoni B. 2008. New implications in the use of Imposex as a suitable tool for tributyltin contamination: experimental induction in *Hexaplex trunculus* (Gastropoda, Muricidae) with different stressor. *Cell Biology and Toxicology* 24, 563-571

Garaventa F, Centanni E, Pellizzato F, Faimali M, Terlizzi A, Pavoni B. 2007. Imposex and accumulation of organotin compounds in populations of *Hexaplex trunculus* (Gastropoda, Muricidae) from the Lagoon of Venice (Italy) and Istrian coast (Croatia). *Marine Pollution Bulletin* 54, 726–732

Garaventa F, Faimali M, Terlizzi A. 2006. Imposex in pre-pollution times. Is TBT to blame? *Marine Pollution Bulletin* 52, 696-718

Ghoshal S, Weber WJ, Rummel AM, Trosko JE, Upham BL. 1999. Epigenetic toxicity of a mixture of Polycyclic Aromatic Hydrocarbons on gap junctional intercellular communication before and after biodegradation. *Environmental Science & Tecnology* 33(7), 1044-1050

Gibbs PE, Bryan GW, Pascoe PL, Burt GR. 1987. The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *Journal of the Marine Biological Association of the United Kingdom* 67, 507-523

Gillesby BE, Zacharewski TR. 1998. Exoestrogens: mechanisms of action and strategies for identification and assessment. *Environmental Toxicology and Chemistry* 17(1), 3-14

Giovannelli L, Cozzi A, Guarnieri I, Dolara P, Moroni F. 2002. Comet assay as a novel approach for studying DNA damage in focal cerebral ischemia: differential effects of NMDA receptor antagonists and poly(ADP-ribose) polymerase inhibitors. *Journal of Cerebral Blood Flow & Metabolism* 22, 697-704

Gobbo Braz M, Fávero Salvadori DM. 2007. Influence of endogenous and synthetic female sex hormones on human blood cells *in vitro* studied with comet assay. *Toxicology in vitro* 21, 972-976

Gorbi S, Virno Lamberti C, Notti A, Benedetti M, Fattorini D, Moltedo G, Regoli F. 2008. An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic Sea. *Marine Environmental Research* 65, 34-49

Gorbushin AM, Iakovleva NV. 2006. Haemogram of *Littorina littorea*. *Journal of the Marine Biological Association of the United Kingdom* 86, 1175-1181

Gorbushin AM, Iakovleva NV. 2007. Functional characterization of *Littorina littorea* (Gastropoda: Prosobranchia) blood cells. *Journal of the Marine Biological Association of the United Kingdom* 87, 741-746

Griffin SP, Bhagooli R. 2004. Measuring antioxidant potential in corals using the FRAP assay. *Journal of Experimental Marine Biology and Ecology* 302, 201-211

Grundy MM, Moore MN, Howell SM, Ratcliffe NA.1996. Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology* 34, 273-290

Grundy MM, Ratcliffe NA, Moore MN. 1996. Immune inhibition in marine mussels by Polycyclic Aromatic Hydrocarbons. *Marine Environmental Research* 42(1-4), 187-190

Hagger JA, Jones MB, Lowe D, Leonard DRP, Owen R,. Galloway TS. 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: a case study. *Marine Pollution Bulletin* 56: 1111-1118

Hagger JA, Depledge MH, Galloway TS. 2005. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. *Marine Pollution Bulletin* 51, 811-816

Hagger JA, Depledge MH, Oehlmann J, Jobling S, Galloway TS. 2006. Is there a causal association between genotoxicity and the Imposex effect? *Environmental Health Perspectives* 114(1), 20-26

Hagger JA, Fisher AS, Hill SJ, Depledge MH, Jha AN. 2002. Genotoxic, cytotoxic and ontogenetic effects of tri-n-butyltin on the marine worm, *Platynereis dumerilii* (Polychaeta: Nereidae). *Aquatic Toxicology* 57, 243–255

Hagger JA, Galloway TS, Langston WJ, Jones MB. 2009. Application of biomarkers to assess the condition of European marine sites. *Environmental Pollution* 157(7), 2003-2010

Ham KD, Adams SM, Peterson MJ. 1997. Application of multiple bioindicators to differentiate spatial and temporal variability from the effects of contaminant exposure on fish. *Ecotoxicology and Environmental Safety* 37, 53–61

Handy RD, Galloway TS, Depledge MH. 2003. A proposal for the use of Biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotoxicology* 12, 331-343

Harvey RG. 1998. 1 Envoronmental Chemistry of PAHs. In: Neilson AH (Ed.). The Handbook of Environmental Chemistry, Vol 3, Part I, PAHs and related compounds. Springer-Verlag Berlin Heidelberg, pp. 1-54

Hauton C, Hawkins LE, Hutchinson S. 2001. Response of haemocyte lysosomes to bacterial inoculation in the oysters *Ostrea edulis* L. and *Crassostrea gigas* (Thunberg) and the scallop *Pecten maximus* (L). Fish & Shellfish Immunology 11(2), 143-53

Hoch M. 2001. Organotin compounds in the environment – an overview. *Applied Geochemistry* 16, 719-743

Huffman Ingwood A, Conners DE, Hoguet J. 1998. Effects of natural and anthropogenic stressors on lysosom destabilization in oysters *Crassostrea virginica*. *Marine Ecology Progress Series* 166, 163-171

Hutchinson TH, Shillabeer N, Winter MJ, Pickford DB. 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. *Aquatic Toxicology* 76, 69-92

Hwang H, Wade TL, Sericano JL. 2002. Relationship between lysosomal membrane destabilization and chemical body burden in eastern oysters (*Crassostrea virginica* from Galveston bay, Texas, USA. *Environmental Chemistry and Toxicology* 21(6), 1268-1271

ICES. 2004a. Biological effects of contaminants: Measurement of lysosomal membrane stability. By Moore MN, Lowe D, Köhler A. *ICES Techniques in Marine Environmental Sciences*, No. 36. 31 pp.

ICES. 2004b. Biological effects of contaminants: use of Intersex in the periwinkle (*Littorina littorea*) as a biomarker of tributyltin pollution. By J. Oehlmann. *ICES Techniques in Marine Environmental Sciences*, No. 37. 22 pp.

Inadera H. 2006. The immune system as a target for environmental chemicals: xenoestrogens and other compounds. *Toxicology Letters* 164, 191-206

Janer G, Sternberg RM, LeBlanc GA, Porte C. 2005. Testosterone conjugating activities in invertebrates: are they targets for endocrine disruptors? *Aquatic Toxicology* 71(3), 273-282

Kagley AN, Snider RG, Krishnakumar PK, Casillas E. 2003. Assessment of seasonal variability of cytochemical responses to contaminant exposure in the blue mussel *Mytilus edulis* (complex). *Archives of Environmental Contamination and Toxicology* 44(1), 43-52

Katalinic V, Modun D, Music I, Boban M. 2005. Gender differences in antioxidant capacity of rat tissues determined by 2,2' azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) and ferric reducing antioxidant power (FRAP) assays. *Comparative Biochemistry and Physiology*, Part C 140, 47-52

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM. 1995. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* 375, 581-585

Kelce WR, Wilson EM. 2001. Antiandrogenic effects of environmental endocrine disruptors. In: Metzler M (Ed.). The Handbook of Environmental Chemistry, Vol 3, Part L, Endocrine Disruptors, Part I. Springer-Verlag Berlin Heidelberg. pp. 39-61

Kester MHA, Bulduk S, Tibboel D, Meinl W, Glatt H, Falany CN, Coughtrie MWH, Bergman A, Safe SH, Kuiper GGJM, Schuur AG, Brouwer A, Visser TJ. 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology* 141(5), 1897-1900

Kim IY, Hyun CK. 2006. Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms. *Ecotoxicology and Environmental Safety* 64, 288–297

Knerr S, Schrenk D. 2006. Carcinogenicity of "non-dioxinlike" polychlorinated biphenyls. *Critical Reviews in Toxicology* 36, 663-694

Koukouzika N, Dimitriadis VK. 2008. Aspects of the usefulness of five marine pollution biomarkers, with emphasis on MN and lipid content. *Marine Pollution Bulletin* 56, 941-949

Laffon B, Rábade T, Pásaro E, Méndez J. 2006. Monitoring of the impact of Prestige oil spill on *Mytilus galloprovincialis* from Galician coast. *Environment International* 32, 342-348

Lam PKS, Gray JS. 2003. The use of biomarkers in environmental monitoring programmes. *Marine Pollution Bulletin* 46, 182-186

Langston WJ, Chesman BS, Burt GR, Hawkins SJ, Readman J, Worsfold P. 2003. Plymouth sound and Estuaries, (candidates) Special Area Conservation, Special Protection Area. In: Site characterisation of the south west European marine sites. *Marine Biological Association of the U.K*, occasional publication n°9, 1-202

Larade K, Storey KB. 2004. Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate. *Journal of Experimental Biology* 207, 1353-1360

Large AT, Shaw JP, Peters LD, McIntosh AD, Webster L, Mally A, Chipman JK. 2002. Different levels of mussel (*Mytilus edulis*) DNA strand breaks following chronic field and acute laboratory exposure to polycyclic aromatic hydrocarbons. *Marine Environmental Research* 54, 493-497

Lee RF, Steinert S. 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research* 544(1), 43-64

Levasseur A, Shi L, Wells NC, Purdie DA, Kelly-Gerreyn BA. 2007. A three-dimensional hydrodynamic model of estuarine circulation with an application to Southampton Water, UK. *Estuarine, Coastal and Shelf Science* 73, 753-767

Livingstone DR. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine pollution bulletin* 42(8), 656-666

Lowe DM, Moore MN, Evans BM. 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Marine Ecology Progress Series* 91, 135-140

Lowe DM, Moore MN, Readman JW. 2006. Pathological reactions and recovery of hepatopancreatic digestive cells from the marine snail *Littorina littorea* following exposure to a polycyclic aromatic hydrocarbon. *Marine Environmental Research* 61, 457-470.

Lowe DM, Pipe RK.1994. Contaminant induced lysosomal membrane damage in marine mussels digestive cells: an in vitro study. *Aquatic Toxicology* 30, 357-363

Lowe DW, Fossato VU. 2000. The influence of environmental contaminants on lysosomal activity in the digestive cells of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon. *Aquatic Toxicology* 48, 75-85

Magni P, De Falco G, Falugi C, Franzoni M, Monteverde M, Perrone E, Sgro M, Bolognesi C. 2006. Genotoxicity biomarkers and acetylcholinesterase activity in natural populations of *Mytilus galloprovincialis* along a pollution gradient in the Gulf of Oristano (Sardinia, western Mediterranean). *Environmental Pollution* 142, 65-72

Maguire RJ. 2000. Review of the persistence, bioaccumulation and toxicity of tributyltin in aquatic environments in relation to Canada's toxic substances management policy. *Water Quality Research Journal of Canada* 35(4), 633-679

Mamaca E, Bechmann RK, Torgrimsen S, Aas E, Bjørnstad A, Baussant T, Le Floch S. 2005. The Neutral Red lysosomal retention assay and Comet assay on haemolymph cells

from mussels (*Mytilus edulis*) and fish (*Symphodus melops*) exposed to styrene. *Aquatic Toxicology* 75, 191-201

Maran C, Centanni E, Pellizzato F, Pavoni B. 2006. Organochlorine compounds (polychlorinated biphenyls and pesticides) and polycyclic aromatic hydrocarbons in populations of *Hexaplex trunculus* affected by Imposex in the Lagoon of Venice, Italy. *Environmental Toxicology and Chemistry* 25, 486-495

Martins LK, Nascimento IA, Fillmann G, King R, Evangelista AJ, Readman JW, Depledge MH. 2005. Lysosomal responses as a diagnostic tool for the detection of chronic petroleum pollution at Todos os Santos Bay, Brazil. *Environmental Research* 99(3), 387-396

Matthiessen P, Gibbs PE. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in molluscs. *Environmental Toxicology and Chemistry* 17(1), 37-43

McFarland VA, Inouye LS, Lutz CH, Jarvis AS, Clarke JU, McCant DD. 1999. Biomarkers of oxidative stress and genotoxicity in livers of field-collected brown bullhead, *Ameiurus nebulosus*. *Archives of Environmental Contamination and Toxicology* 37(2), 236-241

McKinlay R, Plant JA, Bell JNB, Voulvoulis N. 2008. Endocrine disrupting pesticides: Implications for risk assessment. *Environment International* 34, 168-183

Metzler M, Pfeiffer E. 2001. Chemistry of natural and antropogenic endocrine active compounds. In: Metzler M (Ed.). The Handbook of Environmental Chemistry, Vol 3, Part L, Endocrine Disruptors, Part I. Springer-Verlag Berlin Heidelberg. pp. 63-80

Minelli A, Ruffo S, La Posta S. (Eds). 1993. 2006. Checklist delle specie della fauna italiana. Fascicoli 1-110. Edizioni Calderini, Bologna.

Mitchelmore CL, Birmelin C, Chipman JK, Livingstone DR. 1998. Evidence for cytochrome P-450 catalysis and free radical involvement in the production of DNA strand breaks by benzo[a]pyrene and nitroaromatics in mussel *(Mytilus edulis* L.) digestive gland cells. *Aquatic Toxicology* 41, 193-212

Moore MN, Allen JI, McVeigh A. 2006B. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Marine Environmental Research* 61, 278-304

Moore MN, Allen JI, Somerfield PJ. 2006. Authophagy: role in surviving environmental stress. *Marine Environmental Research* 62, S420-S425

Moore MN, Depledge MH, Readman JW, Leonard DRP. 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *Mutation Research* 552, 247–268

Moore MN. 2004. Diet restriction induced autophagy: a lysosomal protective system against oxidative- and pollutant-stress and cell injury. *Marine Environmental Research* 58, 603-607

Morales-Caselles C, Martín-Díaz ML, Riba I, Sarasquete C, Del Valls TA. 2008. The role of biomarkers to assess oil-contaminated sediment quality using toxicity tests with clams and crabs. *Environmental Toxicology and Chemistry* 27(6), 1309-1316

Morcillo Y, Porte C. 1997. Interaction of tributyl- and triphenyltin with the microsomal monooxygenase system of molluscs and fish from the Western Mediterranean. *Aquatic Toxicology* 38, 35-46

Mueller SO, Korach KS. 2001. Mechanisms of estrogen receptor–mediated agonistic and antagonistic effects. In: Metzler M (Ed.). The Handbook of Environmental Chemistry, Vol 3, Part L, Endocrine Disruptors, Part I. Springer-Verlag Berlin Heidelberg. pp. 63-80

Mukerjee D. 1998. Health risk of endocrine-disrupting ortho-substituted PCBs emitted from incinerators. *Environmental Engineering Science* 15(2), 157-169

Nigro M, Falleni A, Del Barga I, Scarcelli V, Lucchesi P, Regoli F, Frenzilli G. 2006. Cellular biomarkers for monitoring estuarine environments: transplanted versus native mussels. *Aquatic Toxicology* 77(4), 339-347

Oberdörster E, McClellan-Green P. 2000. The neuropeptide APGWamide induces Imposex in the mud snail, *Ilyanassa obsoleta*. *Peptides* 21, 1323-1330

Oehlmann J, Bauer B, Minchin D, Schulte-Oehlmann U, Fioroni P, Markert B. 1998. Imposex in *Nucella lapillus* and Intersex in *Littorina littorea*: interspecific comparison of two TBT-induced effects and their geographical uniformity. *Hydrobiologia* 378, 199-213

Oehlmann J, Di Benedetto P, Tillmann M, Duft M, Oetken M, Schulte-Oehlmann U. 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. *Ecotoxicology* 16, 29-43

Oehlmann J, Schulte-Oehlmann U, Tillmann M, Markert B. 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. *Ecotoxicology* 9, 383-397

Oehlmann J, Schulte-Oehlmann U. 2003. Endocrine disruption in invertebrates. *Pure and Applied Chemistry* 75(11-12), 2207-2218

Orlando EF, Guillette LJ. 2002. Developmental and reproductive abnormalities associated with endocrine disruptors in wildlife. In: Metzler M (Ed.). The Handbook of Environmental Chemistry, Vol 3, Part M, Endocrine Disruptors, Part II. Springer-Verlag Berlin Heidelberg. pp. 249-270

Ostling O, Johanson KJ. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communication* 123, 291-298

Palmqvist A, Selck H, Rasmussen LJ, Forbes VE. 2003. Biotransformation and genotoxicity of fluoranthene in the deposit-feeding polychaete *Capitella sp*. *Environmental Toxicology and Chemistry* 22(12), 2977-2985

Pampanin DM, Andersen OK, Viarengo A. 2006. Background for the BEEP Stavanger workshops: biological effects on marine organisms in two common, large, laboratory experiments and in a field study comparison of the value (sensitivity, specificity, etc.) of core and new biomarkers. *Aquatic Toxicology* 78S, S1-S4

Pavoni B, Centanni E, Valcanover S, Fasolato M, Ceccato S, Tagliapietra D. 2007. Imposex levels and concentrations of organotin compounds (TBT and its metabolites) in *Nassarius nitidus* from the Lagoon of Venice. *Marine Pollution Bulletin* 55, 505-511

Peharda M, Morton B. 2006. Experimental prey species preferences of *Hexaplex trunculus* (Gastropoda: Muricidae) and predator–prey interactions with the Black mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae). *Marine Biology* 148(5), 1011–1019

Pellizzato F, Centanni E, Marin MG, Moschino V, Pavoni B. 2004. Concentrations of organotin compounds and Imposex in the gastropod *Hexaplex trunculus* from the Lagoon of Venice. *Science of the Total Environment* 332, 89-100

Pereira CD, Abessa DM, Bainy AC, Zaroni LP, Gasparro MR, Bícego MC, Taniguchi S, Furley TH, De Sousa EC. 2007. Integrated assessment of multilevel biomarker responses and chemical analysis in mussels from São Sebastião, São Paulo, Brazil. *Environmental Toxicology and Chemistry* 26(3), 462-469

Perugini M, Cavaliere M, Giammarino A, Mazzone P, Olivieri V, Amorena M. 2004. Levels of polychlorinated biphenyls and organochlorine pesticides in some edible marine organisms from the Central Adriatic Sea. *Chemosphere* 57, 391-400

Petrović S, Semencić L, Ozretić B, Ozretić M.2004. Seasonal variations of physiological and cellular biomarkers and their use in the biomonitoring of north Adriatic coastal waters (Croatia). *Marine Pollution Bulletin* 49, 713-720

Poletta GL, Larriera A, Kleinsorge E, Mudr MD. 2009. Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and the Micronucleus test. *Mutation Research* 672, 95-102

Porte C, Janer G, Lorusso LC, Ortiz-Zarragoitia M, Cajaraville MP, Fossi MC, Canesi L. 2006. Endocrine disruptors in marine organisms: approaches and perspectives. *Comparative Biochemistry and Physiology*, 143 Part C, 303-315

Prime M, Peharda M, Jelic K, Mladineo I, Richardson CA. 2006. The occurrence of imposex in *Hexaplex trunculus* from Croatian Adriatic. *Marine Pollution Bulletin* 52(7) 810-812

Pytharopoulou S, Kouvela EC, Sazakli E, Leotsinidis M, Kalpaxis DL. 2006. Evaluation of the global protein synthesis in *Mytilus galloprovincialis* in marine pollution monitoring: seasonal variability and correlations with other biomarkers. *Aquatic Toxicology* 80, 33-41

Raccanelli S, Pavoni B, Maroli L, Sfriso A. 1994. One step cleanup and separation of chlorinated, aliphatic, and polycyclic aromatic hydrocarbons in environmental samples, prior to gas chromatographic quantification. *Toxicological & Environmental Chemistry* 45(1-2), 121-137

Ramòn M, Amor MJ. 2001. Increasing Imposex in populations of *Bolinus brandaris* (Gastropoda: Muricidae) in the north-western Mediterranean. *Marine Environmental Research* 52, 463-475

Regoli F, Frenzilli G, Bocchetti R, Annarumma F, Scarcelli V, Fattorini D, Nigro M. 2004. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic Toxicology* 68, 167-178

Regoli F, Gorbi S, Fattorini D, Tedesco S, Notti A, Machella N, Bocchetti R, Benedetti M, Piva F. 2006. Use of the land snail *Helix aspersa* as sentinel organism for monitoring ecotoxicologic effects of urban pollution: an integrated approach. *Environmental Health Perspectives* 114(1), 63-69

Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S. 2003. Integrating enzymatic responses to organic chemical exposure with Total Oxyradical Absorbing Capacity and DNA damage in the European eel *Anguilla Anguilla*. *Environmental Toxicology and Chemistry* 22(9), 2120-2129

Regoli F, Winston GW. 1999. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicology and Applied Pharmacology* 156, 96-105

Reid DJ, MacFarlane GR. 2003. Potential biomarkers of crude oil exposure in the gastropod mollusc, *Austrocochlea porcata*: laboratory and manipulative field studies. *Environmental Pollution* 126(2), 147-155

Ringwood AH, Conners DE, Hoguet J. 1998. Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassotrea virginica*. *Marine Ecology Progress Series* 166, 163-171

Rittshof D, Mc Clellan-Green P. 2005. Molluscs as a multidisciplinary models in environment toxicology. *Marine Pollution Bulletin* 50, 369-373

Rolán E, Luque AA. 1994. *Nassarius reticulatus* (Linnaeus,1758) and *Nassarius nitidus* (Jeffreys, 1867) (Gastropoda, Nassariidae), two valid species from the European seas. *Iberus* 12 (2), 59–76

Roméo M, Gharbi-Bouraoui S, Gnassia-Barelli M, Dellali M, Aïssa P. 2006. Responses of *Hexaplex* (*Murex*) *trunculus* to selected pollutants. *Science of the Total Environment* 359, 135-144

Ronis MJJ, Mason AZ. 1996. The metabolism of testosterone by the periwinkle (*Littorina littorea*) *In Vitro* and *In Vivo*: effects of tributyltin. *Marine Environmental Research* 42(1-4), 161-166

Ross JA, Nesnow S. 1999. Polycyclic aromatic hydrocarbons: correlations between DNA adducts and ras oncogene mutations. *Mutation Research* 424, 155-166

Rüdel H. 2003. Case study: bioavailability of tin and tin compounds. *Ecotoxicology and Environmental Safety* 56, 180-189

Ruus A, Shaanning M, Øxnevad S, Hylland K. 2005. Experimental results on bioaccumulation of metals and organic contaminants from marine sediments. *Aquatic Toxicology* 72, 273-292

Safe SH. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Critical Reviews in Toxicology* 24(2), 87-149

Safe SH. 2001. Hydroxilated polychlorinated biphenyls (PCBs) and organochlorine pesticides as potential endocrine disruptors. In: Metzler M (Ed.) - The Handbook of Environmental Chemistry, Vol. 3, Part L, Endocrine Disruptors, Part I. Springer-Verlag Berlin Heidelberg, pp. 155-167

Santos MM, Vieira N, Reis-Henriques MA, Santos AM, Gomez-Ariza JL, Giraldez I, ten Hallers-Tjabbes CC. 2004. Imposex and butyltin contamination off the Oporto coast (NW Portugal): a possible effect of the discharge of dredged material. *Environment International* 30, 793-798

Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J, Watermann B, Scherf S. 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the Laboratory. Part II: triphenyltin as a xeno-androgen. *Ecotoxicology* 9, 399-412.

Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J, Watermann B, Scherf S. 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: triphenyltin as a xeno-androgen. *Ecotoxicology* 9, 399-412

Scientific Committee on Food (SCF). 2002. *Opinion of the Scientific Committee on Food on the Risks to Human Health of Polycyclic Aromatic Hydrocarbons in Food, SCF/CS/CNTM/PAH/29 Final.* <http://europa.eu.int/comm/food/fs/sc/scf/index_en.html>

Seo JY, Choi JW , Shim WJ, Kim GB. 2008. Field application of a method for measuring DNA damage in polychaete blood cells exposed to Masan Bay sediment extracts. *Marine Pollution Bulletin* 56, 348-379

Sfriso A, Marcomini A, Pavoni B. 1994. Annual nutrient exchanges between the central Lagoon of Venice and the northern Adriatic Sea. *The Science of Total Environment* 156, 77-92

Shaw JP, Large AT, Chipman JK, Livingstone DR, Peters LD. 2000. Seasonal variation in mussel *Mytilus edulis* digestive gland cytochrome P4501A- and 2E-immunoidentified protein levels and DNA strand breaks (Comet assay). *Marine Environmental Research* 50, 405-409

Shaw JP, Large AT, Livingstone DR, Doyotte A, Renger J, Chipman JK, Peters LD. 2002. Elevation of cytochrome P450-immunopositive protein and DNA damage in mussels (*Mytilus edulis*) transplanted to a contaminated site. *Marine Environmental Research* 54, 505-509

Shugart LR. 2000. DNA damage as a biomarker of exposure. Ecotoxicology 9, 329-340

Siboni N, Fine M, Bresler V, Loya Y. 2004. Coastal coal pollution increases Cd concentrations in the predatory gastropod *Hexaplex trunculus* and is detrimental to its health. *Marine Pollution Bulletin* 49, 111-118

Singh NP, McCoy M, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175, 184-191

Siu SYM, Lam PKS, Martin M, Caldwell CW, Richardson BJ. The use of selected genotoxicity assays in green-lipped mussels (*Perna viridis*): a validation study in Hong Kong coastal waters. *Marine Pollution Bulletin* 57, 479-492

Sjögren M, Ehrenberg L, Rannug U. 1996. Relevance of different biological assays in assessing initiating and promoting properties of polycyclic aromatic hydrocarbons with respect to carcinogenic potency. *Mutation Research* 358, 97-112

Smith BS. 1971. Sexuality in the American mud snail, *Nassarius obsoletus*: abnormalities in the reproductive system. *Proceedings* in *the Malacological Society of London* 39, 377-378

Soclo HH, Garrigues P, Ewald M. 2000. Origin of polycyclic aromatic hydrocarbons (PAHs) in coastal marine sediments: case studies in Cotonou (Benin) and Aquitanie (France) areas. *Marine Pollution Bulletin* 40(5), 387-396

Solé M, Morcillo Y, Porte C. 1998. Imposex in the commercial snail *Bolinus brandaris* in the north-western Mediterranean. *Environmental Pollution* 99, 241-246

Solidoro C, Pastres R, Cossarini G, Ciavatta S. 2004. Seasonal and spatial variability of water quality parameters in the Lagoon of Venice. *Journal of Marine System* 51, 7-18.

Sonnenschein C, Soto AM. 1998. An updated review of environmental estrogen and androgen mimics and antagonists. *Journal of Steroid Biochemistry & Molecular Biology* 65(1-6), 143-150

Sotil G, Tarazona J, Francia RAJC, Shiga B. 2008. Comparative evaluation of the DNA damage response in two Peruvian marine bivalves exposed to changes in temperature. Helgoland Marine Research 62(S1), S101-S105

Sousa A, Genio L, Mendo S, Barrosoi C. 2005. Comparison of the acute toxicity of tributyltin and copper to veliger larvae of *Nassarius reticulatus* (L.). *Applied organometallic chemistry* 19, 324-328

Spooner N, Gibbs PE, Bryan GW, Goad LJ. 1991. The effect of tributyltin upon steroid titres in the female dogwhelk, *Nucella lapillus*, and the development of Imposex. *Marine Environmental Research* 32, 37-49

Stewart C, De Mora SJ. 1990. A review of the degradation of tri(n-butyl)tin in marine environment. *Environmental Technology* 11, 565-570

Strickland JDH, Parson TR. 1972. A pratical handbook of seawater analyses. *Fisheries Research Board of Canada*, Bull. No. 167, Ottawa. 310 pp

Stroben E, Oehlmann J, Bettin C. 1991. TBT-induced Imposex and the role of steroids in marine snails. In *Proceedings*, Tenth World Meeting of the Organotin Environmental Programme Association, Berlin, September 26-27, pp 68-73

Stroben E, Oehlmann J, Fioroni P. 1992A. The morphological expression of Imposex in *Hinia reticulata* (Gastropoda: Buccinidae): a potential indicator of tributyltin pollution. *Marine Biology* 113, 625-636

Stroben E, Oehlmann J, Fioroni P. 1992B. *Hinia reticulata* and *Nucella lapillus*. Comparison of two gastropod tributyltin bioindicators. *Marine Biology* 114, 289-296

Taban IC, Bechmann RK, Torgrimsen S, Baussant T, Sanni S. 2004. Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. *Marine Environmental Research* 58, 701-705

Terlizzi A, Delos AL, Garaventa F, Faimali M, Geraci S. 2004. Limited effectiveness of marine protected areas: imposex in *Hexaplex trunculus* (Gastropoda, Muricidae) populations from Italian marine reserves. *Marine Pollution Bulletin* 48 (1-2), 188-192

Terlizzi A, Geraci S, Gibbs PE. 1999. Tributyltin (TBT)-induced imposex in the Neogastropod *Hexaplex trunculus* in Italian coastal waters: morphological aspects and ecological implications. *Italian Journal of Zoology* 66, 141-146

Terlizzi A, Geraci S, Minganti V. 1998. Tributyltin (TBT) pollution in the coastal waters of Italy as indicated by imposex in *Hexaplex trunculus*. *Marine Pollution Bulletin* 36(9), 749-752.

Terlizzi A. 2000. Imposex (Pseudoermafroditismo) in Molluschi Gasteropodi mediterranei: aspetti morfologici e considerazioni ecologiche. *Bollettino Malacologico* 36, 155-158

Thomas RE, Lindeberg M, Harris PM, Rice SD. 2007. Induction of DNA strand breaks in the mussel (*Mytilus trossulus*) and clam (*Protothaca staminea*) following chronic field exposure to polycyclic aromatic hydrocarbons from the Exxon Valdez spill. *Marine Pollution Bulletin* 54, 726-732

Tillmann M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J. 2001. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III: Cyproterone acetate and and vinclozoline as antiandrogens. *Ecotoxicology* 10, 373-388

Tucker JD, Preston RJ. 1996. Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges and cancer risk assessment. *Mutation Research* 365, 147-159

UNEP/RAMOGE: Manual on the biomarkers recommended for the MED POL Biomonitoring Programme. UNEP, Athens, 1999

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety* 64, 178-189.

Van den Broeck H, De Wolf H, Backeljau T, Blust R. 2007. Effects of environmental stress on the condition of *Littorina littorea* along the Scheldt estuary (The Netherlands). *Science of the Total Environment* 376, 346-358

Vasseur P, Cossu-Leguille C. 2006. Linking molecular interactions to consequent effects of persistent organic pollutants (POPs) upon populations. *Chemosphere* 62, 1033-1042

Venier P, Maron S, Canova S. 1997. Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo[a]pyrene. *Mutation Research* 390, 33-44

Verrengia Guerrero NR, Taylor MG, Wider EA, Simkiss K. 2003. Influence of particle characteristics and organic matter content on the bioavailability and bioaccumulation of pyrene by clams. *Environmental Pollution* 121, 115–122

Viarengo A, Burlando B, Giordana A, Bolognesi C, Gabrielides GP. 2000. Networking and expert-system analysis: next frontier in biomonitoring. *Marine Environmental Research* 49, 483-486

Viarengo A, Lowe D, Bolognesi C, Fabbri E, Koehler A. 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology*, Part C 146, 281-300

Viarengo A, Ponzano E, Dondero F, Fabbri R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusc. *Marine Environmental Research* 44, 69-84

Wake E. 2005. Oil refineries: a review of their ecological impacts on the aquatic environment. *Estuarine, Coastal and Shelf Science* 62, 131-140

Walker K, Vallero DA, Lewis RG. 1999. Factors influencing the distribution of Lindane and other hexachlorocyclohexnes in the environment. Environmental Sciences and Technology 33(24), 4373-4378

Watanabe N, Sakai S, Takatsuki H. 1992. Examination for degradation paths of butyltin compounds in natural water. *Water Science and Technology* 25(11),117-124

Wayner DDM, Burton GW, Ingold KU, Locke SJ. 1985. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. *FEBS Letters* 187, 33-37

Wenzl T, Simon R, Kleiner J, Anklam E. 2006. Analytical methods for polycyclic aromatic hydrocarbons (PAHs) in food and the environment needed for new food legislation in the European Union. *Trends in Analytical Chemistry* 25(7), 716-725

White ME, Waldock MJ, Thain JE, Smith DJ, Milton SM. 1991. Reduction in TBT concentrations in UK estuaries following legislation in 1986 and 1987. *Marine Environmental Research* 32, 89-111.

White PA. 2002. The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures. *Mutation Research* 515, 85-98

White RD, Shea D, Stegeman JJ. 1997. Metabolism of the aryl hydrocarbon receptor agonist 3,3',4,4'-tetrachlorobiphenyl by the marine fish scup (*Stenotomus chrysops*) *in vivo* and *in vitro*. *Drug Metabolism and Disposition* 25(5), 564-572

Wilson SP. 2001. Imposex levels in selected gastropods from Venice and the Veneto region. Report to the Gladys Krieble Delmas Foundation of New York, pp 15

Winston GW, Moore MN, Kirchin MA, Soverchia C.1996. Production of reactive oxygen species by hemocytes from the marine mussel, *Mytilus edulis*: lysosomal localization and effect of xenobiotics. *Comparative Biochemistry and Physiology. Part C: Pharmacology, Toxicology and Endocrinology* 113(2), 221-229

Winston GW, Regoli F, Dugas AJ, Fong JH, Blanchard KA. 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Radical Biology & Medicine* 24(3), 480-493

Xue W, Warshawsky D. 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. *Toxicology and Applied Pharmacology* 206, 73-93

Zander AG. 1980. Polycyclic aromatic and heteroaromatic hydrocarbons. In: Hutzinger O (Ed.). The Handbook of Environmental Chemistry, Vol 3, Part A, Anthropogenic compounds. Springer-Verlag Berlin Heidelberg, pp. 109-131

Zanon F, Rado N, Centanni E, Zharova N, Pavoni B. 2009. Time trend of butyl- and phenyl-tin contamination in organisms of the Lagoon of Venice (1999-2003). *Environmental Monitoring and Assessment* 152, 35-45

Zhang Z, Li X, Vandepeer M, Zhao W. 2006. Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in pacific oysters, *Crassostrea gigas* (Thunberg). *Aquaculture* 256, 502-509

Zhou B, Liu W, Siu WHL, O'Toole D, Lam PKS, Wu RSS. 2006. Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. *Aquatic Toxicology* 77, 136-142

Zitko V. 2003a. Chlorinated Pesticides: Aldrin, DDT, Endrin, Dieldrin, Mirex. In: Fiedler H (Ed.). The Handbook of Environmental Chemistry, Vol. 3, Part O, Persistent Organic Pollutants. Springer-Verlag Berlin Heidelberg, pp. 48-90

Zitko V. 2003b. Hexachlorobenzene. In: Fiedler H (Ed.). The Handbook of Environmental Chemistry, Vol. 3, Part O, Persistent Organic Pollutants. Springer-Verlag Berlin Heidelberg, pp. 91-121

# ANNEX I

Annex I reports the whole dataset of Experiment 1 and Experiment 2 chemical analyses and of Experiment 3 chemical and biological analyses.

**Table 1.I Experiment 1 chemical results: PAHs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | MOTHECOMBE ng/g dw | BRIXHAM ng/g dw | MOUNT BATTEN ng/g dw | TORPOINT ng/g dw | CALSHOT ng/g dw | CRACKNORE HARD ng/g dw |
|---|---|---|---|---|---|---|
| **Naphthalene** | 0,05(*) | 0,05(*) | 0,05(*) | 0,05(*) | 0,05(*) | 0,5(*) |
| **Acenaphthylene** | 0,03(*) | 0,03(*) | 0,03(*) | 0,03(*) | 3,64±0,05 | 0,03(*) |
| **Acenaphthene** | 0,2(*) | 0,2(*) | 0,2(*) | 0,2(*) | 0,2(*) | 25,6 ± 0,2 |
| **Fluorene** | 0,08(*) | 0,08(*) | 0,08(*) | 0,08(*) | 0,08(*) | 13,7 ± 0,8 |
| **Phenanthrene** | 12,6 ± 0,9 | 11±4 | 10,2 ± 1,7 | 12±4 | 18,9±0,6 | 94 ± 6 |
| **Anthracene** | 0,2(*) | 0,2(*) | 0,33 ± 0,08 | 0,2(*) | 4,9±0,5 | 15 ± 3 |
| **Fluoranthene** | 2,0 ± 0,5 | 2,6±0,9 | 3(*) | 3,8±0,5 | 39±3 | 156 ± 7 |
| **Pyrene** | 3(*) | 3(*) | 3(*) | 3(*) | 25±1 | 135 ± 8 |
| **Benz[a]anthracene** | 7,4 ± 0,3 | 2(*) | 3,28 ± 0,05 | 1,86±0,05 | 4,60±0,10 | 64 ± 2 |
| **Chrysene** | 3,3 ± 0,1 | 2(*) | 8,0 ± 0,1 | 5,0±0,2 | 28±3 | 56 ± 5 |
| **Benz[b]fluoranthene** | 6 ± 6 | 0,01(*) | 0,01(*) | 32±20 | 14±2 | 96 ± 5 |
| **Benz[k]fluoranthene** | 6 ± 5 | 2(*) | 2(*) | 10,9±0,4 | 14,4±0,5 | 75 ± 2 |
| **Benz[a]pyrene** | 0,8(*) | 12,2±0,7 | 0,8(*) | 0,8(*) | 0,8(*) | 17 ± 4 |
| **Indeno[1,2,3-cd]pyrene** | 5 ± 5 | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 30 ± 4 |
| **Dibenzo[a,h]anthracene** | 0,04(*) | 0,04(*) | 0,04(*) | 0,04(*) | 0,04(*) | 0,04(*) |
| **Benz[ghi]perylene** | 0,02(*) | 0,02(*) | 0,02(*) | 0,02(*) | 0,02(*) | 34,1 ± 0,3 |

**Table 1.II Experiment 1 chemical results: OTCs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | MOTHECOMBE ng Sn/g dw | BRIXHAM ng Sn/g dw | MOUNT BATTEN ng Sn/g dw | TORPOINT ng Sn/g dw | CALSHOT ng Sn/g dw | CRACKNORE HARD ng Sn/g dw |
|---|---|---|---|---|---|---|
| **TBT** | 2(*) | 5 ± 5 | 2(*) | 14 ± 4 | 12 ± 2 | 93 ± 12 |
| **DBT** | 0,8(*) | 18 ± 6 | 8±2 | 22 ± 5 | 15 ± 3 | 86 ± 9 |
| **MBT** | 0,4(*) | 18 ± 1 | 1,7±0,1 | 19 ± 1 | 12 ± 2 | 81 ± 7 |
| **TPhT** | 0,06(*) | 3 ± 4 | 0,08(*) | 3 ± 3 | 0,08(*) | 5 ± 1 |
| **DPhT** | 0,6(*) | 0,6(*) | 0,003(*) | 0,4(*) | 0,003(*) | 0,4(*) |
| **MPhT** | 0,08(*) | 0,08(*) | 0,08(*) | 0,2(*) | 0,5(*) | 0,2(*) |

**Table 1.III Experiment 1 chemical results: OCPs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | MOTHECOMBE ng/g dw | BRIXHAM ng/g dw | MOUNT BATTEN ng/g dw | TORPOINT ng/g dw | CALSHOT ng/g dw | CRACKNORE HARD ng/g dw |
|---|---|---|---|---|---|---|
| **$\alpha$-HCH** | 0,28±0,01 | 0,4±0,3 | 0,002(*) | 0,3 ± 0,3 | 0,002(*) | 0,002(*) |
| **HCB** | 0,9±0,2 | 1,0±0,2 | 1,9 ± 0,5 | 1,0 ± 0,9 | 1,5±0,2 | 0,4 ± 0,2 |
| **$\gamma$-HCH** | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) |
| **o,p-DDE** | 0,01(*) | 0,01(*) | 0,5 ± 0,6 | 0,01(*) | 0,01(*) | 0,01(*) |
| **p,p'-DDE** | 0,01(*) | 1,5±0,2 | 0,7 ± 0,6 | 1,7 ± 0,1 | 0,01(*) | 0,5 ± 0,7 |
| **o,p-DDD** | 0,01(*) | 0,01(*) | 1,2 ± 0,1 | 3,3 ± 0,6 | 0,01(*) | 3 ± 3 |
| **p,p'-DDD** | 0,02(*) | 1,26±0,08 | 0,02(*) | 0,4 ± 0,1 | 0,02(*) | 2 ± 2 |
| **o,p-DDT** | 0,01(*) | 1,132±0,008 | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) |
| **p,p'-DDT** | 0,002(*) | 2,0±0,2 | 0,002(*) | 0,53 ± 0,06 | 0,002(*) | 0,002(*) |

**Table 1.IV Experiment 1 chemical results: PCBs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | **MOTHECOMBE** ng/g dw | **BRIXHAM** ng/g dw | **MOUNT BATTEN** ng/g dw | **TORPOINT** ng/g dw | **CALSHOT** ng/g dw | **CRACKNORE HARD** ng/g dw |
|---|---|---|---|---|---|---|
| **cb 18** | 0,005(*) | 0,005(*) | 0,005(*) | 0,005(*) | 0,005(*) | 0,005(*) |
| **cb 28** | 0,03(*) | 0,03(*) | 0,03(*) | 0,03(*) | 0,03(*) | 0,03(*) |
| **cb 52** | 0,06(*) | 0,06(*) | 1,5 ± 0,2 | 1,6 ± 0,3 | 0,8 ± 0,7 | 0,06(*) |
| **cb 155** | 0,003(*) | 0,003(*) | 0,003(*) | 0,003(*) | 0,003(*) | 0,003(*) |
| **cb 101** | 0,07(*) | 0,74 ± 0,10 | 1,4 ± 0,2 | 2,72 ± 0,32 | 0,07(*) | 6 ± 1 |
| **cb 77** | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) |
| **cb 123** | 1,89 ± 0,02 | 0,8 ± 0,2 | 1,5 ± 0,4 | 2,2 ± 0,2 | 0,2 ± 0,3 | 7 ± 1 |
| **cb 118** | 1,4 ± 0,1 | 0,7 ± 0,3 | 1,1 ± 0,1 | 2,7 ± 0,4 | 0,2 ± 0,4 | 4,2 ± 0,6 |
| **cb 153** | 1,5 ± 0,2 | 1,2 ± 0,2 | 2,7 ± 0,2 | 4,93 ± 0,05 | 1,3 ± 0,3 | 16,0 ± 0,8 |
| **cb 105** | 0,002(*) | 0,002(*) | 0,002(*) | 0,002(*) | 0,4 ± 0,3 | 0,0(*) |
| **cb 138** | 0,80 ± 0,09 | 0,8 ± 0,2 | 2,1 ± 0,3 | 2,8 ± 0,2 | 0,7 ± 0,2 | 8,0 ± 0,5 |
| **cb 126** | 0,001(*) | 0,001(*) | 0,001(*) | 0,001(*) | 0,001(*) | 0,001(*) |
| **cb 185** | 0,001(*) | 0,001(*) | 0,001(*) | 0,001(*) | 0,001(*) | 0,001(*) |
| **cb 156** | 0,01(*) | 0,01(*) | 0,01(*) | 0,24 ± 0,04 | 0,01(*) | 0,4 ± 0,4 |
| **cb 157** | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) | 0,01(*) |
| **cb 180** | 0,02(*) | 0,02(*) | 0,68 ± 0,26 | 0,67 ± 0,02 | 1,2 ± 0,5 | 3,7 ± 0,4 |
| **cb 198** | 0,003(*) | 0,003(*) | 0,003(*) | 0,003(*) | 0,3 ± 0,1 | 0,003(*) |
| **cb 169** | 0,01(*) | 0,01(*) | 0,1 ± 0,1 | 0,27 ± 0,07 | 0,01(*) | 2,1 ± 0,2 |
| **cb 170** | 0,004(*) | 0,004(*) | 0,004(*) | 0,004(*) | 0,004(*) | 0,004(*) |
| **cb 194** | 0,01(*) | 0,01(*) | 0,01(*) | 0,18 ± 0,07 | 0,01(*) | 0,51 ± 0,03 |
| **cb 209** | 0,02(*) | 0,02(*) | 0,02(*) | 0,02(*) | 0,02(*) | 0,02(*) |

**Table 2.I Experiment 2 chemical results: PAHs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | ADRIATIC<br>ng Sn/g dw | S.M. MARE<br>ng Sn/g dw | CERTOSA<br>ng Sn/g dw |
|---|---|---|---|
| **Naphthalene** | 0,23 ± 0,02 | 3,0 ± 0,7 | 4,2 ± 0,5 |
| **Acenaphthylene** | 0,02(*) | 0,6 ± 0,2 | 0,52 ± 0,06 |
| **Acenaphthene** | 0,2(*) | 1,9 ± 0,6 | 3,0 ± 0,5 |
| **Fluorene** | 0,6 ± 0,2 | 2,4 ± 0,2 | 2,86 ± 0,03 |
| **Phenanthrene** | 5,47 ± 0,02 | 8 ± 2 | 11 ± 2 |
| **Anthracene** | 0,177 ± 0,001 | 1,40 ± 0,06 | 1,8 ± 0,3 |
| **Fluoranthene** | 1,29 ± 0,07 | 2,2 ± 0,5 | 2,1 ± 0,2 |
| **Pyrene** | 2,3 ± 0,3 | 4,25 ± 0,05 | 4,6 ± 0,8 |
| **Benz(a)anthracene** | 0,04 ± 0,02 | 0,15 ± 0,02 | 0,23 ± 0,07 |
| **Crysene** | 0,29 ± 0,08 | 0,7 ± 0,2 | 0,63 ± 0,05 |
| **Benzo(b)fluorantene** | 0,32 ± 0,03 | 8 ± 2 | 9,33 ± 0,02 |
| **Benzo(k)fluoranthene** | 1(*) | 1,1141 ± 0,0005 | 1,10 ± 0,02 |
| **Benzo(a)pyrene** | 0,8(*) | 0,7842 ± 0,0003 | 0,77 ± 0,01 |
| **Indeno(1,2,3-cd)pirene** | 0,01(*) | 0,01(*) | 0,01(*) |
| **Dibenz[a,h]anthracene** | 0,03(*) | 0,03(*) | 0,03(*) |
| **Benzo[ghi]perylene** | 0,02(*) | 0,02(*) | 0,02(*) |

**Table 2.II Experiment 2 chemical results: OTCs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | ADRIATIC<br>ng Sn/g dw | S.M. MARE<br>ng Sn/g dw | CERTOSA<br>ng Sn/g dw |
|---|---|---|---|
| **TBT** | 1(*) | 11±1 | 13±3 |
| **DBT** | 7±2 | 4±3 | 24±2 |
| **MBT** | 4,3±0,6 | 2±3 | 5,0±0,6 |
| **MPhT** | 0,8(*) | 0,2(*) | 0,2(*) |
| **DPhT** | 2(*) | 0,9(*) | 0,9(*) |
| **TPhT** | 0,6(*) | 0,6(*) | 0,6(*) |

**Table 2.III Experiment 2 chemical results: OCPs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | ADRIATIC<br>ng Sn/g dw | S.M. MARE<br>ng Sn/g dw | CERTOSA<br>ng Sn/g dw |
|---|---|---|---|
| **α-HCH** | 0,002(*) | 0,002(*) | 0,002(*) |
| **HCB** | 0,03(*) | 0,03(*) | 0,03(*) |
| **γ-HCH** | 0,01(*) | 0,01(*) | 0,01(*) |
| **o,p-DDE** | 4,4±0,8 | 0,01(*) | 0,01(*) |
| **p,p'-DDE** | 8,9±0,1 | 10 ± 1 | 17,5 ± 0,3 |
| **o,p-DDD** | 4,4±0,8 | 7,0 ± 0,8 | 13,0 ± 0,2 |
| **p,p'-DDD** | 0,02(*) | 2,0 ± 0,2 | 4,8 ± 0,6 |
| **o,p-DDT** | 0,007(*) | 0,007(*) | 0,007(*) |
| **p,p'-DDT** | 0,002(*) | 1,98 ± 0,10 | 4,9 ± 0,6 |

**Table 2.IV Experiment 2 chemical results: PCBs tissue concentrations in organisms' pools (20 specimens each). The simbol (*) marks the concentration values below the limit of detection (LOD): the values reported correspond to half of the respective LOD.**

| | **ADRIATIC** ng Sn/g dw | **S.M. MARE** ng Sn/g dw | **CERTOSA** ng Sn/g dw |
|---|---|---|---|
| **cb 18** | 0,005(*) | 0,005(*) | 0,005(*) |
| **cb 54** | 0,01(*) | 0,01(*) | 0,01(*) |
| **cb 28** | 0,03(*) | 0,03(*) | 0,03(*) |
| **cb 52** | 1,0 ± 0,1 | 2,9 ± 0,7 | 5,2 ± 0,4 |
| **cb 155** | 0,003(*) | 0,003(*) | 0,003(*) |
| **cb 101** | 3,8 ± 0,5 | 11 ± 3 | 21,8 ± 0,3 |
| **cb 77** | 0,01(*) | 0,01(*) | 0,01(*) |
| **cb 123** | 5,3 ± 0,6 | 11 ± 3 | 16,8 ± 0,4 |
| **cb 118** | 3,7 ± 0,4 | 11 ± 3 | 17,4 ± 0,4 |
| **cb 153** | 22 ± 3 | 43 ± 12 | 64 ± 3 |
| **cb 105** | 4,8 ± 0,5 | 0,002(*) | 0,002(*) |
| **cb 138** | 14 ± 2 | 24 ± 7 | 43 ± 3 |
| **cb 126** | 0,001(*) | 0,001(*) | 1,0 ± 0,9 |
| **cb 185** | 0,001(*) | 0,001(*) | 0,001(*) |
| **cb 156** | 1,67 ± 0,08 | 2,0 ± 0,5 | 3,1 ± 0,6 |
| **cb 157** | 0,009(*) | 0,009(*) | 1,7 ± 0,1 |
| **cb 180** | 7 ± 2 | 11 ± 4 | 14 ± 1 |
| **cb 198** | 0,003(*) | 0,003(*) | 2,0 ± 0,6 |
| **cb 169** | 6 ± 1 | 4 ± 1 | 5,3 ± 0,4 |
| **cb 170** | 0,004(*) | 0,004(*) | 0,4 ± 0,4 |
| **cb 194** | 0,009(*) | 0,009(*) | 3,2 ± 0,7 |
| **cb 209** | 0,02(*) | 0,02(*) | 0,02(*) |

# EXPERIMENT 3

**Table 3.I Decontamination test**

| | **TBT** ng Sn/g dw | **DBT** ng Sn/g dw | **MBT** ng Sn/g dw | **ΣBTs** ng Sn/g dw | **cb 153** ng/g dw | **cb138** ng/g dw | **cb180** ng/g dw | **ΣPCBs** ng/g dw | **Mean Female Penis Length** mm | **VDSI** | **RPLI** % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRE-EXPOSURE** | 65 ± 4 | 20,8 ± 0,8 | 4 ± 1 | 90 ± 4 | 4,21 ± 0,7 | 1,9 ± 0,4 | 0,54 ± 0,08 | 6,6 ± 0,8 | 0,35 ± 0,51 | 0,9 ± 1,3 | 2,4 ± 3,5 |
| **POST-EXPOSURE** | 7 ± 4 | 3 ± 2 | 4 ± 3 | 13 ± 5 | 8 ± 3 | 4 ± 1 | 0,9 ± 0,2 | 13 ± 3 | 0,28 ± 0,25 | 0,6 ± 0,5 | 1,5 ± 1,5 |

**Table 3.II *Ricepe A* poisoning test**

| | **TBT** ng Sn/g dw | **DBT** ng Sn/g dw | **MBT** ng Sn/g dw | **ΣBTs** ng Sn/g dw | **cb 153** ng/g dw | **cb138** ng/g dw | **cb180** ng/g dw | **ΣPCBs** ng/g dw | **Mean Female Penis Length** mm | **VDSI** | **RPLI** % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRE-EXPOSURE** | 65 ± 4 | 20,8 ± 0,8 | 4 ± 1 | 90 ± 4 | 4,21 ± 0,7 | 1,9 ± 0,4 | 0,54 ± 0,08 | 6,6 ± 0,8 | 0,3 ± 0,5 | 1 ± 1 | 2,4 ± 3,5 |
| **POST-EXPOSURE** | 81 ± 2 | 79 ± 8 | 19 ± 7 | 179 ± 11 | 7,59 ± 1,0 | 3,4 ± 0,6 | 1,0 ± 0,2 | 12 ± 1 | 0,3 ± 0,2 | 0,9 ± 1,0 | 2,08 ± 1,5 |

**Table 3.III *Recipe B* poisoning test**

| | **TBT** ng Sn/g dw | **DBT** ng Sn/g dw | **MBT** ng Sn/g dw | **ΣBTs** ng Sn/g dw | **cb 153** ng/g dw | **cb138** ng/g dw | **cb180** ng/g dw | **ΣPCBs** ng/g dw | **Mean Female Penis Length** mm | **VDSI** | **RPLI** % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRE-EXPOSURE** | 7 ± 4 | 3 ± 2 | 4 ± 3 | 12,5 ± 5 | 8 ± 3 | 4 ± 1 | 0,9 ± 0,2 | 13 ± 3 | 0,3 ± 0,2 | 0,6 ± 0,5 | 1,6 ± 1,5 |
| **POST-EXPOSURE** | 74 ± 3 | 36,9 ± 0,4 | 15,1 ± 6 | 126 ± 7 | 8,0 ± 0,1 | 4,0 ± 0,2 | 1,023 ± 0,008 | 13,1 ± 0,2 | 1 ± 2 | 2 ± 2 | 3,8 ± 8,6 |