Cellular localization of anthropogenic contaminants and related biological effects in the blue mussel *Mytilus edulis*

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of

**Doctor of Philosophy
in Cell Biology**

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Date of Defense: July 10. 2007

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Publications

Einsporn, S., Broeg, K., Koehler, A. 2005. The Elbe flood 2002 - toxic effects of transported contaminants in flatfish and mussels of the Wadden Sea. Marine Pollution Bulletin 50: 423-429.

Einsporn, S., Koehler, A. 2006. Electron microscopic localization of lipophilic chemicals by an antibody-based detection system in cells of the blue mussel *Mytilus edulis*. Environmental Toxicology and Chemistry, submitted.

Einsporn, S., Koehler, A. Accumulation, localization and detoxification of the polycyclic aromatic hydrocarbon phenanthrene in cell fractions and organelles in digestive gland of the blue mussel *Mytilus edulis,* in preparation.

Einsporn, S., Bressling, J., Koehler, A. Ultrastructural localization of lead (Pb) using an antibody-based detection system and modulation of biotransformation enzyme activities in gills and digestive gland of the blue mussel *Mytilus edulis*, in preparation.

Einsporn, S., Koehler, A. Cytopatholgies and subcellular targets of the chemicals phenanthrene, aroclor 1254 and lead (Pb) in gills and digestive gland of the blue mussel *Mytilus edulis,* in preparation.

Poster presentations

Title: **Multi xenobiotic resistance related transport in two mussel species at different temperature and contamination situations** Sonja Einsporn & Angela Koehler

"Challenges in Environmental Risk Assessment and Modelling", 12th Annual Meeting of the Society of Environmental Toxicology and Chemistry, SETAC Europe meeting 2002 Vienna, Austria.

Title: **Species differences in biomarker response in wrasse and blue mussel at differently polluted Norwegian fjord sites** Sonja Einsporn & Angela Koehler

"Understanding the complexity of environmental issues -A way to sustainability", 13th Annual Meeting of the Society of Environmental Toxicology and Chemistry, SETAC Europe 2003 Hamburg, Germany.

Title: **Lysosomal storage disorders and membrane destabilisation during toxic exposure** Angela Köhler, Sonja Einsporn, Bjarne Lauritzen, Ute Marx

"Ultrastructure in the era of proteomics", 45th Symposium, Society for Histochemistry, 17 – 20 September 2003 , Les Diablerets, Switzerland.

Title: **Lysosomal storage disorders and membrane destabilisation during toxic exposure: An ultrastructural, immuno- and enzyme histochemical study**
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"New blood in ecotoxicology", SETAC Europe-German Language Branch e.V., 8. Jahrestagung, 21- 23 September 2003, Heidelberg, Germany.

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12th International Congress on Histochemistry and Cytochemistry, 24-29 Juli 2004, San Diego, California, USA.

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Title: **Health effects in fish and mussel after the Elbe flood in August 2002**

"Understanding the complexity of environmental issues -A way to sustainability", 13th Annual Meeting of the Society of Environmental Toxicology and Chemistry, SETAC Europe 2003 Hamburg, Germany.

Title: **Uptake and cellular trafficking of anthropogenic toxic chemicals in the blue mussel Mytilus edulis**

"The Raison d´Etre of Environmental Toxicology and Chemistry", 15th Annual Meeting of the Society of Environmental Toxicology and Chemistry, SETAC Europe 2005 Lille, France.

Abstract

Understanding the uptake, intracellular transport processes and fate of chemicals as well as their biotransformation and elimination is essential to provide information about potential health risks of environmental pollutants for exposed organisms. Moreover, this knowledge helps to identify biomarkers suitable for the use in monitoring studies of health effects of chemicals in marine organisms.

To address this issue, an immunocytochemical approach was developed for the cellular and subcellular detection of chemicals. Based on the newly implemented antibody-based detection techniques the intracellular trafficking of lipophilic organic pollutants and the heavy metal lead (Pb) was studied. Induction of relevant biotransformation and pathological alterations at the subcellular level were investigated in parallel.

Various approaches for the detection of the lipophilic contaminants phenanthrene, a polycyclic aromatic hydrocarbon (PAH) and oil component, and aroclor 1254, a polychlorinated biphenyl (PCB) mixture used in various industrial processes e.g. softening agents, were applied at the ultrastructural level using monoclonal antibodies against PAHs and PCBs. For this purpose, we tested different immunogold-labelled secondary antibodies and a suite of post- and preembedding procedures. The Gold-Substituted Silver-intensified Peroxidase (GSSP) detection system in combination with Catalysed Signal Amplification (CSA) was successfully used for the detection of the lipophilic compounds. Accumulation of PAHs and PCBs in association to subcellular compartments could be demonstrated under the electron microscope. Furthermore, the newly developed antibody-based detection method was successfully used to identify interactions of the heavy metal Pb with subcellular compartments. Accumulation of PCBs, PAHs and the metal Pb were shown in the endo-lysosomal system of the cell. Also, mitochondria and chitinous cell elements were identified as subcellular targets for the PCB mixture. The heavy metal Pb was also localized in mitochondria and the cell nucleus. Thus, we could evidence that the contaminants accumulate in cell organelles which are known to play an important role in metabolic regulation as well as sequestration and detoxification processes. Moreover, we also localized the chemicals in association to subcellular structures and organelles such as mitochondria and nuclei where accumulation of those pollutants is particularly hazardous.

Biotransformation enzyme activities are used as biomarkers in monitoring studies of the marine environment. They provide information on early contaminant-induced effects. In this regard, xenobiotic metabolizing enzymes of phase I and II have shown a high potential to be used as biomarkers of exposure to chemicals in many field and laboratory studies. Nevertheless, interpretation of results is often difficult due to their sensitivity to confounding biotic and abiotic factors

as well as partially opposing effects of mixtures of pollutants. Our laboratory studies with phenanthrene as a lipophilic organic contaminant and with Pb as a heavy metal showed distinct responses of phase I (NADPH cytochrome c reductase, NADPH cyt c red) and phase II (glutathione S-transferase, GST) enzymes as well as catalase (CAT), a general marker enzyme for oxidative stress: Increased activities of phase I and II enzymes (NADPH cyt c red, GST) were measured after exposure to PAHs. Antioxidative enzyme activity of CAT also increased after PAH exposure, but not after Pb exposure. GST activities were elevated after Pb exposure in gills whereas no changes were observed in digestive gland.

Histopathology as an integrative tool to monitor health effects of chemicals has been widely used in monitoring programmes using mussels and fish. In the present thesis, pathological changes were investigated at the subcellular level. In parallel, the distribution of the contaminants was analysed with the developed antibody-based detection technique to elucidate direct toxicant-induced effects. Our results revealed specific accumulation sites in the cells for the environmental pollutants which were related to specific pathological alterations of these cell organelles.

The antibody-based detection technique for contaminants is also a very promising tool for applications in medical and pharmaceutical research. It may be used to identify subcellular interaction sites and effects of various chemicals and drugs.

List of selected abbreviations

ABC	avidin biotin complex
Ah receptor	aryl hydrocarbon receptor
AMG	autometallography
BSA	bovine serum albumin
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
CSA	catalysed signal amplification
CYP	cytochrome P450 monooxygenase
DAB	3,3-diaminobenzidine tetrahydrocloride
DG	digestive gland
DDT	dichlor diphenyl trichlorethan
DNA	desoxyribonucleic acid
dw	dry weight
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immuno-sorbant assay
EPA	environmental protection agency
EPMA	electron probe micro analysis
FSW	filtered sea water
GA	glutaraldhyde
GSH	glutathione
GST	glutathione S-transferase
GPX	glutathione peroxidase
GSSP	gold-substituted silver-intensified peroxidase
HB	homogenization buffer
H&L	heavy and light chains
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
ICES	international council for the exploration of the seas
IgG	immunoglobulin G
LM	light microscopy
log Kow	logarithmic octanol-water partition coefficient
3-MC	3-methylcholanthrene
MFO	mixed-function oxygenase
MXR	multixenobiotic resistance
NADPH cyt c red	nicotinamide adenine dinucleotide phosphate cytochrome c reductase
NaOH	sodium hydroxide
PA	paraformaldehyde
PAHs	polycyclic aromatic hydrocarbons
Pb	lead
PBS	phosphate buffered saline
PCBs	polychlorinated biphenyls
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SIMS	secondary ion mass spectrometry
SOD	superoxide dismutase
TEM	transmission electron microscopy
UNEP	united nations environmental programme

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CHAPTER 1

1 General Introduction

Increasing rates of substances of industrial, agricultural and municipal origin have been charged into marine environments due to human activities. Estuaries, in particular, are contaminated by complex mixtures of contaminants such as agricultural fertilizers and pesticides, large quantities of heavy metals, and organic synthetic compounds from industry and shipping and/or urban effluents from human settlements (Quintaneiro et al. 2006). Contaminants accumulate in marine organisms and further in the food web where they cause a variety of adverse effects to animal and finally human health. Therefore, understanding the routes of uptake and distribution of different chemicals in tissues and cells is a prerequisite for the identification of toxicological mechanisms. Hazardous environmental compounds that accumulate in biota require the development of new techniques to detect smallest amounts of these molecules in cells. These techniques help to gain an important insight into subcellular interactions between toxins and target cell structures with the overall aim to elucidate different pathways of toxicity of various environmental contaminants.

1.1 Environmental pollutants

Lipophilic organic compounds such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are widespread in the aquatic environment. Sources of PAHs can be categorized into two groups: pyrogenic and petrogenic. Pyrogenic (pyrolytic) PAHs are generated through incomplete combustion of organic matter (e.g. coal, petroleum, wood). Pyrogenic sources include industrial operations and power plants using fossil fuels, smelting, waste incinerators, exhaust from vehicles powered by gasoline or diesel fuel, and forest fires. Most pyrogenic PAHs are emitted to the atmosphere as soot or gas and are finally brought to marine environments through direct atmospheric fallout and surface run-off. Crude oil and petroleum products contain PAHs and form another major primary source of PAHs (petrogenic source). Petroleum products include kerosene, gasoline, diesel fuel, lubricating oil, and asphalt. They are emitted directly to marine environments through oil spills and routine tanker operations (e.g. discharge of ballast water) (Boonyatumanond et al. 2006). Due to their hydrophobic character and low biodegradability, accumulation in organisms often exceeds concentration levels in the marine environ-

ment. Some of the PAHs are known mutagens and/or carcinogens that require metabolic activation to exert their genotoxicity. The amount of the ultimate carcinogen produced is the result of competing activation and detoxification pathways (Pisoni et al. 2004). Phenanthrene is a low molecular weight, 3-ring polyaromatic hydrocarbon (PAH), and is classified as a priority pollutant by the United States Environmental Protection Agency (US EPA). Phenanthrene occurs as a major component of the total content of PAH compounds in the environment and is an important petroleum-source PAH, usually found in higher amounts in refined oil products than in crude oil.

In contrast to PAHs, PCBs have been banned in the United States (1977) and in Germany (1989). Despite this ban on their manufacture, PCBs are still found in significant quantities in the marine environments of these countries. Although usage of PCBs was banned in Germany, this ban is not strictly followed because 50 mg/kg are allowed to be mixed with other chemicals. Due to their environmental persistence and biologically nearly non-degradable nature, PCBs are still found in biological samples of wildlife and ecosystems. PCBs are mixtures of different congeners of chlorobiphenyl. The degree of chlorination determines persistence and environmental fate mechanisms. In general, the persistence of PCBs increases with an increase in the degree of chlorination. Mono-, di- and trichlorinated biphenyls biodegrade relatively rapidly, tetrachlorinated biphenyls biodegrade slowly, and higher chlorinated biphenyls are resistant to biodegradation (http://www.epa.gov/). Mixtures of PCBs, such as the commercially available aroclor 1254 are comprised of 60 or more congeners combined to yield a product containing a certain percentage of chlorinated compounds (54% in the case of aroclor 1254). PCB mixtures, based on their degree of chlorination, were used for a variety of industrial purposes in heat conductors, transformers and electrical capacitors, softening agents in paints, sealants and plastics (e.g. cable sheathing). PCBs are among the persistent organic pollutants (POPs) and fall under the Stockholm Convention on Persistent Organic Pollutants of May 2001, where twelve of the most persistent environmental pollutants were listed to be banned. Various chemical substitutes for PCBs exist e.g. phtalates, alkylated aromatics and diphenyls, but toxicological and ecotoxicological properties of many of those substances are also not fully characterized or are even too hazardous to be used instead.

Research from several laboratories has found that many of the toxic effects linked to PCB exposure including immunotoxicity, endocrine and reproductive toxicity, and carcinogenicity are determined by the high affinity for the aryl hydrocarbon (Ah) receptor. In general, PCBs with coplanar structural configuration have a high affinity for the Ah receptor, while noncoplanar PCB congeners, particularly those that are ortho-substituted, have a weak or no Ah receptor affinity.

Non-dioxin-like, ortho-substituted PCBs increased intracellular calcium concentrations and inhibited calcium sequestration by mitochondria and microsomes (Tilson et al. 1998).

Metals and their compounds, both inorganic and organic, are released to the environment as a result of a variety of human activities. The main anthropogenic sources of heavy metals are various industrial point sources, including present and former mining activities, foundries and smelters, and diffuse sources such as piping, constituents of products, combustion by-products and traffic. Relatively volatile heavy metals and those that become attached to air-borne particles can be widely dispersed on very large scales even globally. Heavy metals conveyed in aqueous and sedimentary transport (e.g., river run-off) enter the normal coastal biogeochemical cycle and are largely retained within near shore and shelf regions. Heavy metals are stable and persistent environmental contaminants, which cannot be degraded or destroyed. They accumulate, partly absorbed to organic and anorganic components, in soils and sediments. Marine invertebrates accumulate high levels of various heavy metals in their tissues (Rainbow 1990). Arsenic, cadmium, chromium, copper, nickel, mercury and lead are the most common heavy metal pollutants (http://www.unep.org/). Lead (Pb) is a heavy metal with a high toxicity at very low exposure levels and has acute and chronic effects on health. Pb is not degradable in nature and will thus, once released to the environment, stay in circulation.

Pb and its compounds as wells as PAHs are on the list of priority substances under the EU Water Quality Framework directive (2000/60/EC) (http://ec.europa/environment/water/water-framework/priority_substances.htm) The Directive aims the progressive reduction of discharges, emissions and losses of hazardous substances within 20 years after the adoption of these proposals by the European Parliament and the Council. The ultimate goal is to achieve concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances (Green et al. 2003). In addition to the 33 listed priority hazardous substances of the Directive, many more contaminants exist of which improved and consistent data are missing to evaluate the impact on marine waters and biota. Thus, there is a great need for new monitoring strategies and tools for future assessments of hazardous substances (for a detailed report see: ICES Marine Habitat Committee (ICES 2004)).

1.2   Mussels in environmental monitoring

Mussels are widely used as sentinel species for monitoring in coastal environments. Chemical analyses of pollutant concentrations in bioindicator organisms have been used for decades to assess water quality in "Mussel Watch"-like programmes (Goldberg et al. 1978, Farrington et al.

1983, Goldberg et al. 1983, Mee et al. 1995, Goldberg and Bertine 2000). Mussels have a number of characteristics, which make them useful bioindicators of chemical pollution: They have a wide geographical distribution, are easy to collect and are abundant in estuarine waters which are submitted to high contamination levels. They are filter-feeding organisms, which are able of withstanding baseline levels of pollution; consequently, they may be exposed to large amounts of chemical pollutants even if those compounds are present in fairly diluted concentrations. Mussels are also capable of bioconcentrating xenobiotics (foreign compounds) up to many thousand times of the biotope background. They are sessile species, a characteristic which is particularly desirable for bioindicators since they are likely to reflect changes in pollution status of the sampling area (Manduzio et al. 2004). Mussels are known to accumulate high levels of metals and organic contaminants including PAHs and PCBs in their tissues with observable cellular and physiological responses (McDowell et al. 1999, Livingstone et al. 2000). Biological markers or biomarkers indicate that the organism has been exposed to pollutants (exposure biomarker) and / or the magnitude of the organism's response to the pollutant (effect biomarker or biomarker of stress) (Cajaraville et al 2000). Alterations in cellular metabolism are used as biomarkers for the detection of pollutant induced cellular effects and serve as early warning signals of exposure to contaminants in environmental monitoring.

1.3   Biology of the blue mussel *Mytilus edulis*

The mussel *Mytilus edulis* is a widely distributed species in the northern hemisphere. It occurs in all European waters south to the Mediterranean and North Africa, and in arctic waters extending south to California and Japan on Pacific coasts and to North Carolina on the Atlantic coast (Sunila 1987). *Mytilus edulis* is a sessile suspension feeder that acquires nutrients using mucociliary mechanisms on the gills and labial palps to ingest suspended particles. Ingested material is subjected to extracellular digestion throughout the gut and supplemented by intracellular digestion of selected particles within the tubules of the digestive gland (Gosling 1992).

In the present study, we focus on tissues of the mussel *Mytilus edulis* which are considered as important target organs for bioaccumulation and biotransformation of pollutants: the gills and the digestive gland.

The pair of gills (ctenidia) consists of ciliated filaments through which branchial blood vessels pass. The opposite face is called ventral or frontal.) The functions of the gills are various: i.e. respiratory gas exchange, blood hematosis, capture of blood particles, uptake of nutrients and dis-

solved organic particles. The ciliary and mucous cells of the gill epithelium mainly perform all these functions (Gosling 1992, Auffret et al. 2003).

The digestive system of *Mytilus* is adapted to process particulate food. The mouth is located between the outer and inner labial palps, and proceeds into the oesophagus that opens into the stomach. The stomach extends into the gastric intestine corresponding to the style sac/midgut complex. The digestive gland (digestive diverticula) surrounds the stomach and part of the intestine. It is a loose branched tissue which consists of a series of primary and secondary ducts opening into the stomach and ending in tubules. The main (primary) ducts are partly ciliated, while the secondary ducts from which the tubules are formed are not. After several steps of food sorting, small particles enter the digestive gland via a brush-border of the ducts and are transported to the tubules. In the lumen of the tubules digestive enzymes are involved in extracellular digestion. However, intracellular digestion is the main digestive process that takes place in the digestive cells. Therefore, digestive cells contain a well-developed endo-lysosomal system (Dimitriadis et al. 2003). Residues of digestive particles are stored in residual bodies and lysosomes until release from the digestive cells into the lumen of the tubules (Gosling 1992, Auffret et al. 2003).

1.4 Uptake and distribution of contaminants

In polluted environments organisms exhibit several strategies at the cellular level to protect themselves from the toxic effects of organic and metallic compounds. The major ones are the antioxidant defence systems, sequestration capacity, binding to specific proteins, and detoxification processes including metabolization and exclusion by multixenobiotic resistance transporters. Steps in contaminant processing within tissues and cells are uptake, sequestration and excretion. Contrary to organic substances, much more detailed references are available for cellular and subcellular uptake of heavy metals. Dissolved forms of metals are mainly taken up via the gills whereas particulate-bound metals enter through the gut. In the gills metals are incorporated in hemocytes and brown cells as well as associated with blood plasma ligands (e.g., high molecular weight proteins). Metallothioneins are involved in the cellular metabolic regulation of essential metals and the binding and detoxification of toxic heavy metals. They are low molecular weight cysteine-rich proteins which sequester specific metallic ions (Cd, Cu, Hg and zinc) via their high proportion of –SH groups (Simkiss 1998, Marigomez et al. 2002).

Subsequently, these metals are transported via hemocytes, pore cells, and blood plasma to the digestive cells of the gut or to nephrocytes of the kidney. Pathways for elimination of metals are: 1) excretion from the kidney as urine, 2) excretion of digestive cell lysosomes as faeces, and 3)

excretion after diapedesis of hemocytes and brown cells across the epithelia of digestive gland, gut, and kidney as both urine and faeces. Other processes for metal detoxification are sequestration and immobilization of metals in clusters of brown cells in the interstitial connective tissue in foot, stomach, intestine, and digestive gland tissue and incorporation into the shell by mantle epithelial cells (for a schematic overview of metal processing see figure 1).
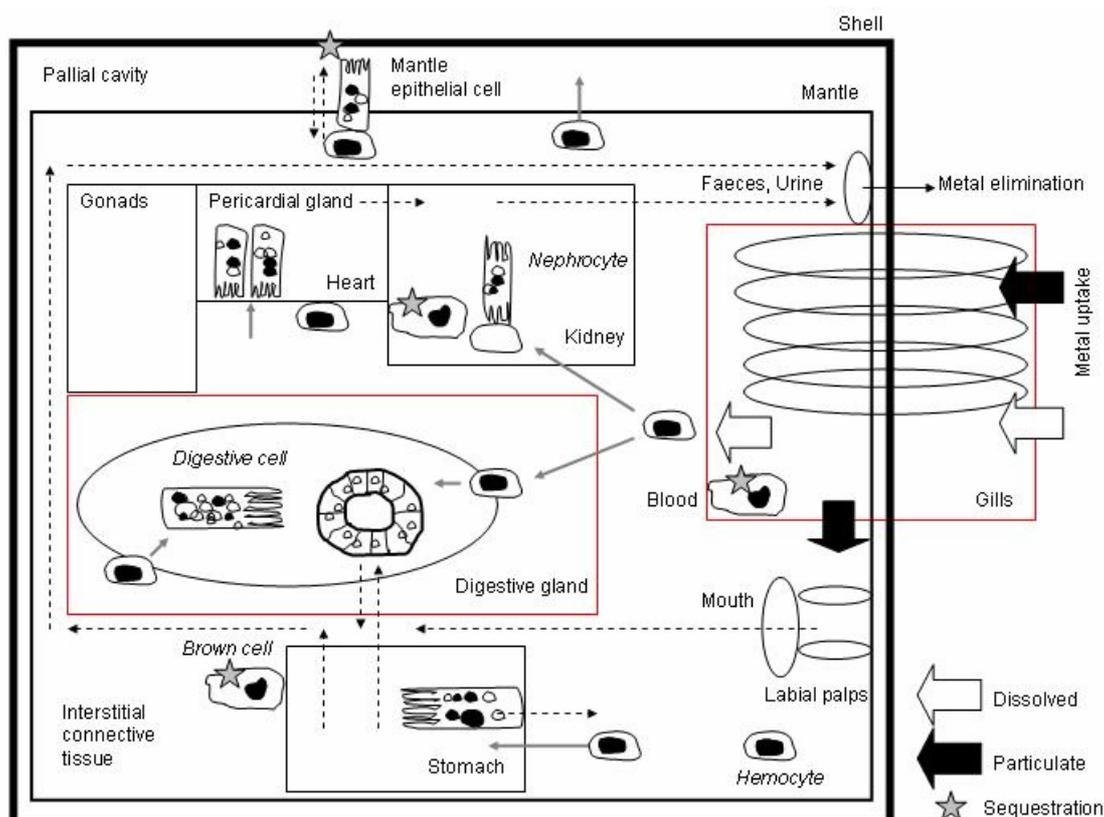
Fig. 1: **Schematic overview of the model routes for metal processing throughout the tissues of mussels (*Mytilus galloprovincialis*), with indication of tissues and cells involved in metal uptake, sequestration, and excretion (modified from Marigomez et al. 2002).**

The uptake and accumulation of organic micropollutants and metals by marine organisms is based on their physical chemical characteristics (Moore et al. 2006) (figure 2). Lipophilic compounds such as PAHs and PCBs are easily taken up into tissues and accumulate in concentrations above those in the surrounding environment. Important factors for tissue distribution of chemicals are lipid levels of tissues and route of uptake. In general, bioaccumulation is higher in tissues which are lipid-rich and contain high amounts of lysosomes such as the digestive gland. Uptake occurs mainly from the water column and/or via particulate material. Uptake rates of organic contaminants are initially linear until equilibrium between tissue concentrations and surrounding environment is attained. Depuration of chemicals is dependent on pre-exposure times. Long-time

or chronic exposure conditions may result in slower or incomplete elimination of contaminants than short term-exposure. This phenomenon is interpreted as compartmentalization of contaminants into different membrane-limited vesicles (Livingstone and Pipe 1992). Lysosomes are membrane-limited vesicles which are highly conserved cell organelles from yeast to humans. In eukaryotic animal cells lysosomes are the major degrading organelles. These compartments contain a range of hydrolases that are able to degrade essentially any cellular constituents into proteins, lipids, nucleic acids and carbohydrates. In addition, regulated turnover of cell organelles is confined to the lysosome. Cytoplasmic components are degraded within the lysosome by autophagic processes (microautophagy, chaperone-mediated autophagy, and macroautophagy) (Klionsky and Emr 2000). Lysosomes sequester and accumulate diverse chemicals and pharmaceuticals which range from metal ions, transuranics, asbestos, PAHs, heterocyclics, and antipsycotic drugs to nanoparticles (Moore et al. 2006).
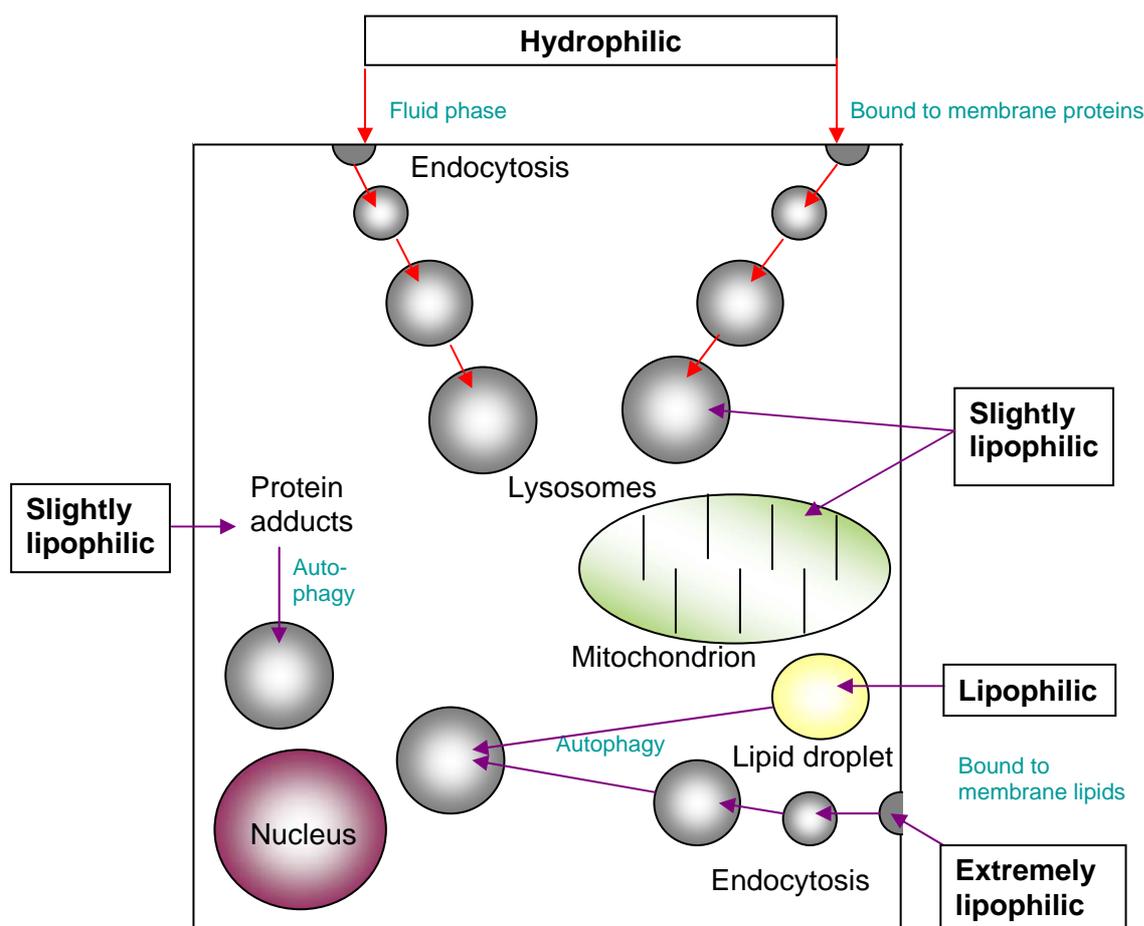
Fig. 2: Hypothetical scheme of the various routes of uptake (diffusion and endocytotic) of contaminants into the cell that result in their accumulation in the lysosomal compartment, dependent on their physical chemical characteristics (modified from Moore et al. 2006).

1.5 Biotransformation processes

In general, the cell possesses various biotransformation enzymes for the metabolism of contaminants. Biotransformation can be divided into functionalization (Phase I) and conjugative processes (Phase II) (figure 3). During phase I metabolism the foreign compound is chemically modified (e.g. oxidation, reduction, hydrolysis etc.) to obtain a reactive group for the biotransformation in phase II. In phase I, one enzyme is of particular interest in the biotransformation of organic contaminants: Cytochrome P450 monooxygenase. Cytochrome P450 monooxygenase (CYP) or mixed-function oxygenase (MFO) has been identified and characterized in mussels. A previous study indicated at least five CYP forms in digestive gland of *Mytilus* sp (Pisoni et al. 2004). Although different isoforms of CYP exist in bivalves, enzyme activities are often very low and rarely detectable compared to vertebrates such as fish (Parant 1998).
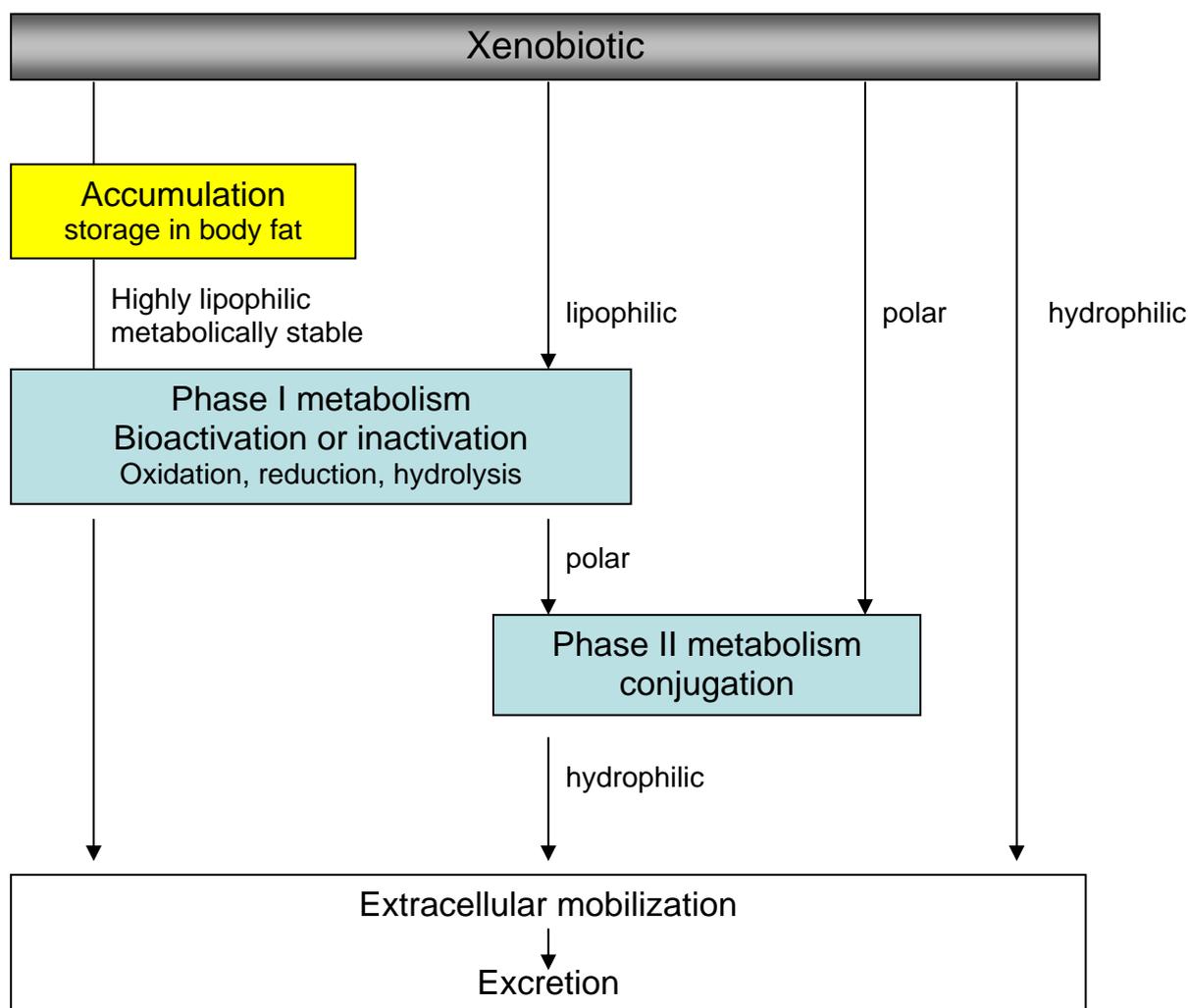
Fig. 3: **Diagrammatic representation of xenobiotic metabolism and processing in the body, based on their lipophilicity and polarity (modified from http://aquaticpath.umd.edu/appliedtox/metabolism.pdf).**

Phase II enzymes catalyze the conjunction between the xenobiotic compounds containing electrophilic sites and endogenous molecules. Glutathione S-transferases (GSTs), sulfo-transferases, and other phase II enzymes allow the excretion of metabolites from phase I of biotransformation via specific transmembrane transporters. The activity of GSTs has been shown in organisms as a function of xenobiotic concentration in the medium. GST induction has been confirmed in the field on either organisms collected from polluted areas or caged organisms.

The potential of oxygen free radicals (e.g. O_2^-, HOO ; OH) and other reactive oxygen species (ROS) to damage tissue and cellular components in biological systems is called oxidative stress. Chemical toxic pollutants are important sources of ROS, especially metabolites of xenobiotics that are capable of redox cycling (eg. the quinones menadione, the herbicide paraquat, the transition metal cadmium). The antioxidative system of organisms can be subdivided into enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) and non-enzymatic antioxidants such as glutathione, vitamin E, ascorbate, and ß-carotene. Catalase is not corresponding to a specific group of contaminants. Catalase removes hydrogen peroxide from cells during basal aerobic metabolism or after pollution-enhanced oxyradical generation that can be within the limit of tolerance of the organism. Catalase is nearly ubiquituous among organisms that can grow in the presence of oxygen (Khessiba et al. 2001).

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Online tools

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http://www.epa.gov/

United Nations Environment Programme
http://www.unep.org/

Priority substances and other pollutants
Last updated: 19/01/2007
http://ec.europa.eu/environment/water/water-framework/priority_substances.htm

Applied Toxicology Nurs 735 Andrew S. Kane, Ph.D. UM Program in Toxicology Director, UM Aquatic Pathobiology Center, UMCP
http://aquaticpath.umd.edu/appliedtox/metabolism.pdf

CHAPTER 2

2 Scope of the thesis

Chemical analysis of contaminants in marine organisms provides information about concentration of hazardous chemicals in tissues. However, little information is revealed through classical chemical analysis regarding the toxic effects of these substances to the health of organisms. Furthermore, chemical analysis needs relatively high amounts of samples, comprises costly and time consuming methods and detects only a small range of chemicals for which the analytical procedure is implemented. The use of biomarkers for the detection of contaminant-induced detrimental health effects in marine organisms is an important supplement and alternative for marine monitoring programmes and has attracted public attention during the last ten years.

Commonly-used biomarkers such as lysosomal stability, induction/inhibition of specific enzyme activities and "oxidative stress" parameters have been well-established in environmental research and are routinely applied in environmental monitoring studies.

In this thesis new visualization techniques were developed for the detection of chemicals, even at low concentrations, inside of cells and tissues. These detection techniques help to understand the interaction of the toxins with molecules and structures within cells and tissues, explain observed effects of contaminants at the cellular level, and close the gap between chemical monitoring and biomonitoring in environmental research.

2.1 Objectives of the study

The aim of the present study is to investigate the fate of anthropogenic chemicals (phenanthrene, aroclor 1254, Pb) in cells of the mussel *Mytilus edulis* so as to gain insights into the mechanisms of toxic effects of different environmental toxins. This knowledge is essential for the development of diagnostic tests for determination of specific contaminant-induced effects in tissues and cells.

For this purpose an antibody-based visualization technique for lipophilic contaminants was developed at the ultrastructural level as described in chapter 3. This study demonstrates the accumulation of the organic contaminants, phenanthrene (PAH) and aroclor 1254 (PCB), at the subcellular level and their interactions with different cell organelles. A detailed study of accumulation and fate of phenanthrene at the subcellular level was performed in chapter 4. Additionally, we

focused on biotransformation enzymes involved in PAH metabolism (chapter 4). Interactions of the heavy metal Pb with subcellular compartements were identified with the developed antibody-based detection method as well as effects on cellular defence mechanism with respect to recovery (chapter 5). Chapter 6 describes cytopathological alterations at the ultrastructural level in gills and digestive gland after exposure to the different contaminants (PAHs, PCBs and Pb) in parallel to their subcellular localization. The discussion includes recommendations for the use of the developed antibody-based detection technique and investigated biomarkers for future research and application for environmental studies (chapter 7).

The following questions in particular were addressed:

- Where are the subcellular target sites for the accumulation of diverse anthropogenic contaminants (phenanthrene, aroclor 1254, and the heavy metal Pb) in gills and digestive gland of the mussel *Mytilus edulis*?

- Are subcellular localization sites pollutant-specific?

- How do detoxifying enzymes (NADPH cyt c red, GST) and the antioxidative enzymes CAT respond to different classes of pollutants and are the responses quantifiable for use as biomarkers of exposure?

- What kind of cytopathological effects after contaminant exposure can be found?

- Is there a relationship between ultrastructural histopathological alterations and subcellular contaminant accumulation sites?

The approaches taken to address these questions were the following:

1. Development and implementation of an antibody-based detection method for the ultrastructural localization of lipophilic contaminants (phenanthrene, aroclor 1254) –Chapter 3;

2. Laboratory studies were carried out and mussels were exposed to the contaminants. – Chapters 3, 4, 5, 6;

3. Transfer and application of the antibody-based detection method for the ultrastructural localization of the heavy metal Pb –Chapter 5;

4. Subcellular detection of the contaminants in cells (TEM, chemical analysis of cell fractions) – Chapters 3, 4, 5, 6;

5. Effects of pollutants (phenanthrene, Pb) on biotransformation enzyme activities were studied – Chapters 4, 5;

6. Recovery responses of detoxifying enzymes were studied –Chapters 5;

7. Cytopathological effects of pollutants were studied –Chapter 6

CHAPTER 3

3 Electron microscopic localization of lipophilic chemicals by an antibody-based detection system in cells of the blue mussel *Mytilus edulis*

3.1 Abstract

Lipophilic organic chemicals such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are widespread pollutants in the environment. Understanding their cellular uptake and subcellular localisation is a prerequisite for the investigation of their mode of toxic action, either in pharmaceutical or environmental research. Identification of lipophilic chemicals in biota requires the development of new techniques to detect these compounds at the cellular and subcellular level. To address this issue, we have developed a combinational protocol using antibodies directed against chemicals together with various immunohistochemical techniques to capture the lipophilic compounds at their subcellular sites of accumulation. As a model organism, we exposed the blue mussel *Mytilus edulis* to the PAH phenanthrene and the PCB mixture aroclor 1254 for up to 10 days. The aim of these exposure experiments was to analyse the routes of uptake, the cellular targets and sites of accumulation in the digestive system at the subcellular level by transmission electron microscopy. Monoclonal mouse antibodies directed against PAHs and PCBs were applied with various immunogold-labelled secondary antibodies and immunoperoxidase in pre- and postembedding procedures. Of all combinations tested, only the preembedding technique using the GSSP (Gold-Substituted Silver-intensified Peroxidase) detection system in combination with Catalyzed Signal Amplification (CSA) successfully scavenged the lipophilic chemicals at specific subcellular sites for visualization. The endo-lysosomal system of digestive cells and mitochondria of epithelial cells appeared to be the preferred accumulation sites for the lipophilic compounds studied. The antibody-based approach to detect lipophilic chemicals provides an important insight into the pathways of uptake and accumulation as well as mechanisms of toxicity.

3.2   Introduction

Mode of uptake and subcellular localisation of chemicals determine their toxicity as well as their pharmaceutical effects. Lipophilic drugs such as organochlorines and polycyclic aromatic hydrocarbons that accumulate in biota require the development of new techniques to detect smallest amounts of these molecules in target cells. Polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) are ubiquitous lipophilic contaminants in marine and terrestrial ecosystems with a high persistence and ability to accumulate in organisms and in the food web. Most of these pollutants are waste products from industrial processes and transportation activities that enter the marine environment through the atmosphere or rivers. These contaminants are of major concern because they are known to cause a variety of adverse effects to animal and human health. Whereas cytotoxic and mutagenic effects have been linked to tissue concentrations in the micro-molar range (Baumard et al. 1999, Corsolini et al. 2005), certain chemicals have already been shown to exert their endocrine effects modulating the immune response, fertility and development in the nanomolar range (Kerr Lobel and Davis 2002, Binelli and Provini 2003, Canesi et al. 2003). The properties of lipophilic chemicals result in preferential storage and bioaccumulation in fatty tissues and lipids of detoxification organs, with increasing biomagnification in the food chain (Birdsall et al. 2001).

We selected the blue mussel *Mytilus edulis* as a sessile suspension feeder that rapidly takes up and accumulates diverse contaminants. Therefore, it serves as a suitable model organism for the study of routes of chemical uptake, storage and elimination and their adverse cellular effects.

Many contaminants are bound to particulate material and enter the digestive tract with food material. Particulate-associated pollutants are accumulated and further redistributed in the digestive gland, which is a major site for the detoxification of contaminants. Intracellular digestion of food particles, taken up by endocytosis, takes place in a well-developed lysosomal vascular system of digestive cells (Pipe and Moore 1986, Dimitriadis et al. 2003).

Immunocytochemistry and immunohistochemistry are techniques to detect antigens in cells and tissues using specific antibodies and are widely used in human pathology (for reviews see Heyderman 1980, Neville et al. 1982, Howell et al. 1998). A relatively new field of research is the detection of toxic substances and pharmaceuticals with immunohistochemical approaches. Antibodies against chemicals have already been successfully applied in immunoassays to detect pollutants in environmental samples such as soil, sediments and water (Zajicek et al. 1996, Strandberg et al. 1998, Zajicek et al. 2000, Fillmann et al. 2002, Kerr Lobel and Davis 2002, Chuang et al. 2003). In biota, antibodies against PCBs have been successfully applied in damselfish embryos and in clams from contaminated sites at the light microscopic level (Strandberg et al. 1998, Kerr

Lobel and Davis 2002). Embryos of damselfish *Abudefduf sordidus* show positive immunostaining with anti-PCB antibodies in the lateral line organs, outside of the oil globule and around the blastodisc in a yolk ring (Kerr Lobel and Davis 2002).

In contrast to the above mentioned studies, ultrastructural immunolocalization of lipophilic and hydrophobic chemicals is problematic because solvents used during tissue dehydration in most embedding techniques tend to extract the lipophilic chemicals out of the tissue sections. Alternative techniques using cryo ultramicrotomy are available only in few laboratories and need relatively sophisticated methods. Another problem may arise after immunolabelling because in conventional labelling procedures the reaction product itself is too small to allow visualization of minimal quantities of chemicals.

In our study, mussels were exposed to phenanthrene (PAH) and aroclor 1254 (PCB) in the laboratory and two different monoclonal antibodies, one against PAHs and the other against PCBs congeners, were used to identify cellular sites of toxin accumulation. Water solubility and vapor pressure are the key physical/chemical characteristics of PAHs which influence their distribution amongst the soluble and particle components of the atmosphere, hydrosphere and biosphere. Phenanthrene is a three-ring hydrocarbon with a low water solubility (1.1 mg/L) and vapor pressure (0.02 Pa). Thus, phenanthrene is relatively insoluble, has low volatility, and has a logarithmic octanol-water partition coefficient (log Kow) of 4.57 which is moderately lipophilic compared to other PAHs. The PCB mixture aroclor 1254 is almost water insoluble (0.021 mg/L), low volatile (0.01 Pa) and highly lipophilic (log Kow 6.2) (WHO 1992, Douben 2003). Both substances phenanthrene and aroclor 1254 are characterized by their lipophilic and hydrophobic properties which make them preferentially accumulate in lipid rich tissues of organisms.

During the past century, diverse techniques have been developed for immuno electron microscopy. The oxidative polymerization of 3,3-diaminobenzidine (DAB) is widely used in histochemistry, cytochemistry, immunology, virology and neuroanatomy for the demonstration of enzymes, proteins and other biologic materials labelled with peroxidase or intrinsic peroxidase activity (Gallyas et al. 1982). Horseradish peroxidase (HRP) is often used in preembedding immunocytochemistry as it is characterized by a low molecular size and displays, therefore, sufficient tissue penetration properties. If required, staining intensity may be additionally intensified with diverse post-intensification methods by the use of metal-ions such as silver, gold and osmium (Gallyas et al. 1982, Liposits et al. 1982, Newman et al. 1983, Merchenthaler et al. 1989). Immunogold followed by silver enhancement is routinely used for electron microscopic studies. Immunogold reagents such as nanogold and ultra small colloidal gold probes which are covalently bound to

Fab fragments of IgGs are small enough to penetrate into tissues and cells and give a high signal for immunolocalization.

Most of the postembedding methods result in high resolution immunolocalizations, but antigenicity and labelling intensity are often reduced by dehydration and embedding steps (Sawada and Esaki 1994), and preservation of cell morphology is often not satisfying. Therefore, preembedding techniques with low size labelling molecules (HRP, nanogold, Fab fragments of IgGs) provide a good alternative to postembedding procedures.

Gallays and co-workers (1982) developed an immunocytochemical method which provides a high intensification of a diaminobenzidine (DAB) reaction product by silver/gold-toning. In our study, the gold-substituted silver-peroxidase (GSSP) intensification method by Gallays et al. (1982) was combined with catalyzed signal amplification (CSA) which enhances the sensitivity of immunohistochemical methods by deposition of biotinylated tyramide. CSA has been developed for ELISA, in situ hybridisation and Western blotting and adapted to light and electron microscopy (Landry and Hokfelt 1998, Koehler et al. 2000, Murakami et al. 2001). The CSA system relies on high amplification of the biotin molecules and the linkage of streptavidin-peroxidase conjugates. As a final step of the CSA method, incubation with DAB and H_2O_2 gives a brown precipitate at the antigen site. Continuing the incubation with the GSSP technique, a silver intensification is performed and the existing insoluble DAB polymers catalyze the deposition of metal salts onto itself (Gallyas et al. 1982, Van den Pol and Gorcs 1986).

The above-mentioned embedding and labelling methods were applied and compared in our study for the detection of lipophilic organic toxins at the electron microscopic level. The use of antibodies allows the direct localization of chemicals in tissues and cells and may elucidate the interaction and accumulation of toxins within organelles and other cell compartments. The objectives of the present study were to establish a combinational approach using various methods for the ultrastructural detection of chemicals in tissues and cells with the aim to localize lipophilic organic substances in the digestive system of the bivalve mollusc *Mytilus edulis*. To our knowledge, this study describes for the first time the detection of lipophilic contaminants in tissues and cells at the electron microscopic level.

3.3   Methods

Conditions for maintenance and exposure of mussels

Mytilus edulis individuals were sampled at the Wadden Sea Station Sylt and transferred to the Alfred Wegener Institute Foundation for Polar and Marine Research (AWI) laboratory in Bremerhaven. Specimens ranging from 4.0-6.0 cm shell length were selected and maintained in aerated filtered sea water (FSW) at 12-14°C for up to 14 days for depuration until the start of the exposure experiments.

Mussels were exposed via the seawater (one mussel per liter) to 150 µg/l phenanthrene (dissolved in acetone) or 20 µg/l aroclor 1254 (dissolved in ethanol) for up to 10 days. Relatively high concentrations of contaminants which correspond to point-source inputs into the marine environment from industrial outfalls and in the vicinity of contaminated sediments were chosen (WHO 1992, Douben 2003). Controls were maintained in FSW alone and with the solvent (acetone or ethanol). Seawater was changed every 24 hours and contaminants in the respective concentration or vehicle concentration for the controls were added. Mussels were fed every 2 days 2 hours before re-dosing of contaminants. After 10 days mussels were dissected and samples of digestive gland tissue were taken for the immunohistochemical studies. Tissues pieces were immediately prepared for ultrastructural immunocytochemistry as described in the following paragraphs.

Antibodies

Monoclonal mouse anti-phenanthrene and mouse anti-PCB antibodies were purchased from Research Diagnostics Inc. (Flanders, NJ, USA). The Catalyzed Signal Amplification (CSA) system was obtained from DAKOCytomation (Glostrup, Denmark). Nanogold®-Streptavidin (1.4 nm) was purchased from Nanoprobes (Yaphank, NY, USA).

Goat anti-mouse IgG (H&L), UltraSmall (0.8 nm), F(ab')2 fragment of goat anti-mouse IgG (H&L) UltraSmall (0.8 nm), goat anti-mouse IgG (H&L) (10 nm) were purchased from Aurion (Wageningen, NL).

Immunocytochemical procedures

For ultrastructural detection of contaminants we applied post- and preembeddings with various immunolabelling procedures including a) immunogold-silver enhancement after postembedding, b) preembedding combined with immunogold-silver enhancement and c) preembedding with Catalyzed Signal Amplification (CSA) and Gold-Substituted Silver-intensified Peroxidase (GSSP).

The named detection systems are described in detail in the following section. Figure 4 gives a general overview of the different steps for tissue treatment in pre- and postembedding approaches.

a) Immunogold-silver enhancement after postembedding

In the postembedding procedure (for general overview see figure 4) the tissue was sectioned with a tissue chopper (Linton Instrumentation, Norfolk, United Kingdom) in pieces of 0.5 x 0.5 mm and fixed in 2% paraformaldehyde (PA) and 0.1% glutaraldehyde (GA) in phosphate buffered saline (PBS) (pH 7.4) for 2 hours at 4°C and in 2% PA in PBS at 4°C overnight. After washing in PBS for 60 min at 4°C, tissue sections were dehydrated in 30, 50, 70, 80, 96 and two times in 100% methanol for 20 min followed by an embedding procedure with LR Gold. Thereafter, tissue pieces were incubated for 2 hours in LR-Gold and 100% acetone (1:1) and in LR-Gold with 0.1% benzil overnight. Polymerisation was done in Beam-Capsules under UV light (360 nm) at 4°C for 3-5 days. After curing the tissue blocks, semi-thin sections were cut with an ultracut microtome, dried in 40% acetone on slides, stained with 0.5% toluidine blue and examined by light microcopy to get an overview of tissue morphology. For electron microscopy ultra-thin sections (60-90 nm) were cut with an ultramicrotom (Reichert-Jung, Wetzlar, Germany) and collected on formvar coated nickel grids which had been pre-cleaned in chloroform.

Ultra-thin sections on nickel grids were rinsed on a drop of distilled water (25 μl) for 5 min in a 24 well microtiterplate which was placed on a magnetic stirrer. To neutralize eventual formaldehyde excess the grids were incubated in 0.05 M glycine for 15 min. All incubation steps were done at room temperature (RT), followed by a washing step (3 times for 5 min) with PBS (pH 7.4). Endogenous biotin and peroxidase activities were blocked consecutively with the biotin blocking system (DAKOCytomation, Glostrup, Denmark) and a solution of 3% hydrogen peroxide in water for 20 min. After washing, the sections were incubated in a blocking solution (1% BSA, 0.2% fish gelatine, 0.05% saponin) for 20 min followed by incubation with the primary antibodies (anti-phenanthrene 1:50 = 10 μg/ml, anti-PCB 1:50 = 25 μg/ml) in the blocking solution at 4°C overnight in a humidity chamber. Controls were incubated in 1% BSA, 0.2% fish gelatine and 0.05% without the first antibody.

The second detection step was performed with different antibody systems: 1) F(ab')2 fragment of goat anti-mouse IgG ultrasmall (1:100) for 2 hours at RT, 2) a goat anti-mouse IgG 10 nm (1:100) for 2 hours at RT, 3) a goat anti-mouse IgG ultrasmall (1:100) for 2 hours and 4) a biotinylated link-antibody (anti-mouse, DAKOCytomation, Glostrup, Denmark) for 2 hours before

adding nanogold-Streptavidin 1.4 nm (1:60) at RT for 60 min. These secondary detection steps were chosen to test different sized gold particles and amplification systems in the postembedding approach. After the secondary incubation step 2% GA in PBS (pH 7.4) was added to the sections for gold complex stabilization. After washing in distilled water, silver enhancement was performed according to Koehler et al. (2000) under UV free light at 21-25°C for 7 min in a freshly prepared silver solution (2 ml 1 M citric acid buffer pH 3.7, 3.3 ml 50% gum arabic in distilled water, 1.5 ml distilled water containing 0.019 g silver lactate, 1.5 ml distilled water containing 0.09 g hydroquinone). After silver enhancement, sections were washed twice for 5 min in distilled water and for 3 min in 2.5% sodium thiosulfate in distilled water, and again in distilled water for three times for 5 min. Sections were contrasted shortly in uranyl acetate (2.5 min) and lead citrate (30 sec). Observations were made under a Zeiss EM109 transmission electron microscope and digital analysis system (Soft Imaging System's iTEM, Muenster, Germany).

b) Preembedding combined with immunogold-silver enhancement

A preembedding approach was performed with immunogold (10 nm, 1.4 nm and ultrasmall) conjugated to IgGs (for general overview see figure 4). Freshly prepared mussel digestive glands were cut with a tissue chopper (Linton Instrumentation, Norfolk, United Kingdom) in pieces of 0.5 x 0.5 mm and fixed in 2% paraformaldehyde (PA) and 0.1% glutaraldehyde (GA) for 2 hours at 4°C and in 2% PA overnight. All washing steps between incubations were done with phosphate buffered saline (PBS) (pH 7.4). After washing, unspecific antibody binding sites were blocked for 2 hours with 1% bovine serum albumin, 0.2% fish gelatine and 0.05% saponin in PBS. Tissue sections were incubated with the monoclonal antibodies against the antigens (anti-phenanthrene 1:100 = 20 µg/ml, anti-PCB 1:100 = 50 µg/ml) at 4°C overnight. Controls were run without addition of the first antibody in the blocking solution. The second detection step was performed with three different secondary antibody systems conjugated to immunogold: 1) a goat anti-mouse IgG 10 nm (1:100), 2) a goat anti-mouse IgG ultrasmall (1:100) and 3) a biotinylated link antibody (anti-mouse, DAKOCytomation, Glostrup, Denmark) followed by a nanogold 1.4 nm streptavidin antibody (1:60). These secondary detection steps were chosen to test differently sized gold particles and amplification systems in the preembedding procedure. After incubation for 2 hours, 2% GA in PBS (pH 7.4) was added to the sections for gold complex stabilization. After washing in distilled water, silver enhancement was performed as described in the postembedding section. After silver enhancement, sections were dehydrated in 30, 50, 70, 80, 96 and 100% ethanol for 20 min and embedded in LR Gold. Sections were contrasted shortly in uranyl acetate (2.5 min) and

lead citrate (30 sec). Observations were made under a Zeiss EM109 transmission electron microscope and digital analysis system (Soft Imaging System's iTEM, Muenster, Germany).

c) Preembedding with Catalyzed Signal Amplification (CSA) and Gold-Substituted Silver-intensified Peroxidase (GSSP)

A preembedding technique was applied with a signal amplification system based on Catalysed Signal Amplification (CSA) according to Koehler et al. (2000) and Gold-Substituted Silver-intensified Peroxidase (GSSP) according to Van den Pol and Gorcs (1986). Figure 5 shows a scheme of the constructed immuno-sandwich with the CSA and GSSP techniques. Freshly prepared mussel digestive glands were sectioned with a tissue chopper (Linton Instrumentation, Norfolk, United Kingdom) in pieces of 0.5 x 0.5 mm and fixed in 2% paraformaldehyde (PA) and 0.1% glutaraldehyde (GA) for 2 hours at 4°C and in 2% PA overnight. All washing steps between incubations were done with phosphate buffered saline (PBS) (pH 7.4). After washing, unspecific antibody binding sites were blocked for 2 hours with 1% bovine serum albumin, 0.2% fish gelatine and 0.05% saponin in PBS. Tissue sections were incubated with the monoclonal antibodies against the antigens (anti-phenanthrene 1:100 = 20 µg/ml, anti-PCB 1:100 = 50 µg/ml) at 4°C overnight.

The second detection step was performed with the CSA system with diaminobenzidine tetrahydrochlorid (DAB) from DAKOCytomation (Glostrup, Denmark) for monoclonal mouse antibodies. CSA-DAB staining was performed according to the manufacturer's protocol for mouse primary antibodies with a few modifications. All incubation steps were done at RT, followed by a washing step (3 times for 5 min) with PBS (pH 7.4). First, endogenous peroxidase activity was blocked with a solution of 3% hydrogen peroxide in water for 20 min. Endogenous biotin and avidin were quenched with biotin-avidin blocking reagent (DAKOCytomation, Glostrup, Denmark). The secondary biotinylated link-antibody (anti-mouse-antibody) was added for 2 hours to the sections before adding the streptavidin-biotin for 1 hour and the amplification reagent (biotinyl tyramide and hydrogen peroxide in PBS) for 30 min.

Controls were run without addition of the first antibody or DAB. Streptavidin-peroxidase incubation for 2 hours was followed by fixation in 1% GA in PBS for 30 min and the DAB chromogen incubation for 5-8 min. After washing in PBS to stop the DAB reaction, the tissue sections were washed in 2% sodium acetate. Afterwards, the sections were washed for 3 h in 10% sodium thioglycolate. All following washing steps were done with 2% sodium acetate. After washing sections were incubated with the freshly prepared silver solution consisting of solution A

(5% sodium carbonate), B (0.05% ammonium, 0.25% tungsten silicide, 0.05% silver nitrate) and C (37% formalin) (A:B:C = 1:1:0.004) up to 8 min. The reaction was stopped with 1% acetic acid and samples were washed. The gold labelling was done with 2% gold chloride for 8 min. After washing and fixation with 3% sodium thiosulphate followed by a washing step, the sections were transferred to 0.1 M sodium cacodylate buffer (pH 7.4) and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. After a post-fixation with 0.5% osmium tetroxide and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 15 min sections were washed several times in distilled water and dehydrated in 30, 50, 70, 80, 96 and 100% ethanol for 20 min followed by an Epon-embedding. Semi-thin sections were cut with an ultramicrotome (Reichert-Jung, Wetzlar, Germany), dried in 40% acetone on slides, stained with 0.5% toluidine blue and examined by light microcopy to get an overview of tissue morphology.

For transmission electron microscopy ultra-thin sections were cut with an ultracut microtome and collected on formvar copper grids. Contrasting with uranyl acetate and lead citrate was omitted due to osmification staining and to avoid overstaining of the final reaction product. Observations were made under a Zeiss EM109 transmission electron microscope and images were taken with a digital analysis system (Soft Imaging System's iTEM).
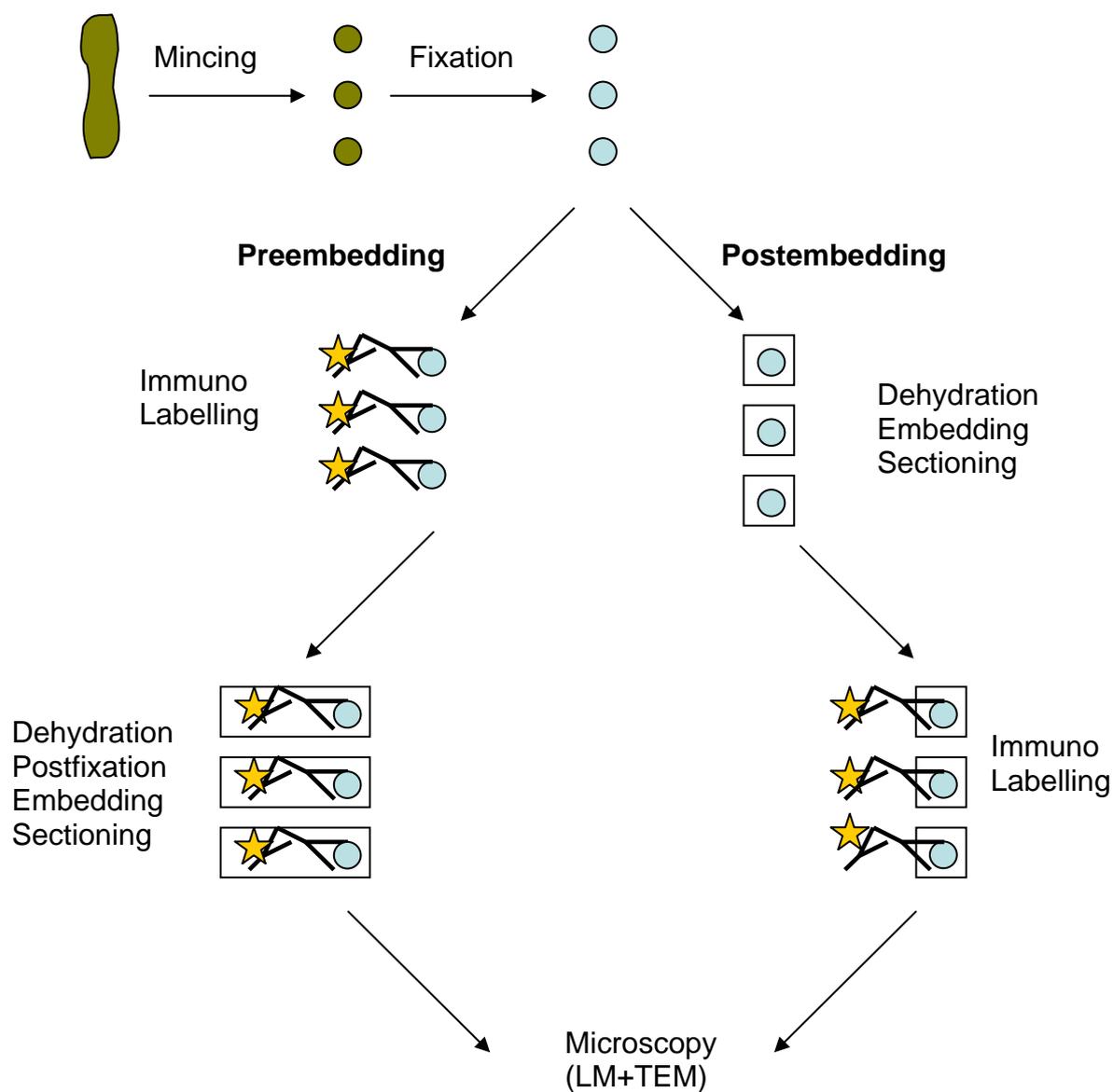
Fig. 4: **Schematic illustration for pre- and postembedding and subsequent detection procedures for electron microscopy.**
Left: For preembedding, after mincing and fixation, tissues were incubated with the anti-toxin antibodies and detection was performed with the different immunolabelling systems followed by dehydration, postfixation and embedding in Epon. Embedded tissues were sectioned and observed under the TEM. Right: For postembedding, after mincing and fixing, tissues were dehydrated, embedded in LR gold and cut into ultra-thin sections. The ultra-thin sections were incubated with the anti-toxin antibodies and processed with the different detection systems and observed under the TEM.

streptavidin-
peroxidase
conjugate

streptavidin-biotin-
peroxidase complex

biotinylated
bridge antibody

biotinylated
thyramine

primary antibody
(eg. mouse-anti-PAH)

antigen (eg. PAH)

DAB + H_2O_2

silver nitrate

gold chloride

electron dense
endproduct
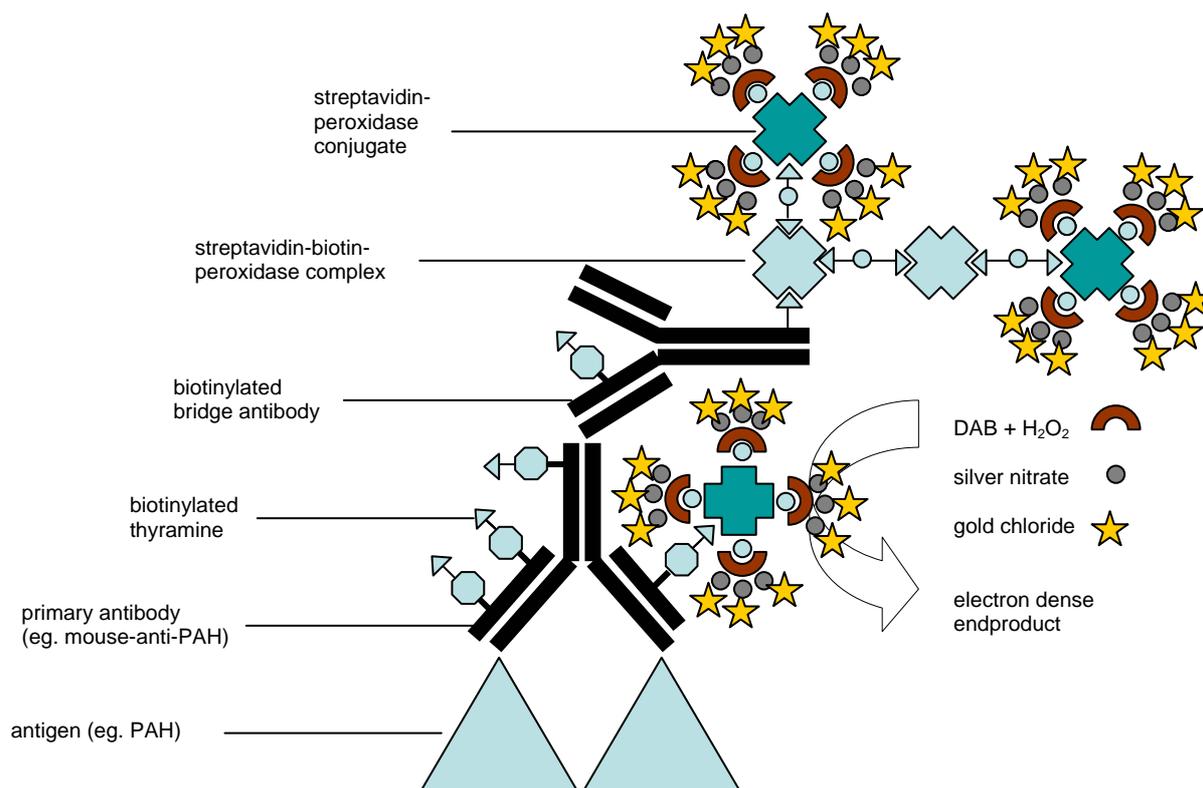
Fig. 5: **Schematic illustration of the constructed "immuno-sandwich" with Catalyzed Signal Amplification (CSA) and Gold-Substituted Silver-intensified Peroxidase (GSSP) (modified from manufacture`s protocol DAKOCytomation, Glostrup, Denmark).**
After silver-gold enhancement sections were fixed in 3% sodium thiosulphate and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer followed by post-fixation with 0.5% osmium tetroxide and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer. Sections were washed several times in distilled water and dehydrated in graded series of ethanol followed by Epon-embedding (for a detailed description see text).

3.4   Results

Various immunocytochemical detection systems using different pre- and postembedding techniques and secondary antibodies were applied to localize lipophilic anthropogenic chemicals in digestive cells. Table 1 gives an overview of the different methods that were applied in our study and the positive or negative results of the immunodetection.

Tab. 1: **Comparison of various pre- and postembedding approaches at the ultrastructural level of LR Gold and Epon embedded material**

Embedding resin	1. Antibody dilution	2. Antibody dilution	Embedding procedure	Results Post-/ preembedding
LR Gold	1:100 anti-PAH 1:100 anti-PCB	1:100 Anti-mouse IgG (H&L) 10 nm	Postembedding/ Preembedding	-/-
LR Gold	1:100 anti-PAH 1:100 anti-PCB	1:100 Anti-mouse IgG (H&L) US	Postembedding/ Preembedding	-/-
LR Gold	1:100 anti-PAH 1:100 anti-PCB	1:100 F(ab)2 Anti-mouse US	Postembedding	-/ not applied *
LR Gold	1:100 anti-PAH 1:100 anti-PCB	Biotinylated link antibody & 1:60 Nanogold Streptavidin 1.4 nm	Postembedding/ Preembedding	-/-
Epon	1:100 anti-PAH 1:100 anti-PCB	CSA/GSSP	Preembedding	+

Silver amplification was performed for all detection systems. +/- = positive/negative immunolabelling, H&L = heavy and light chains, US = ultra small * not applied due to negative results in a postembedding approach

Nanogold-Silver Enhancement Method (post- and preembedding)

The use of immunogold reagents (nanogold, colloidal gold) for the visualisation of lipophilic chemicals was not effective in any of the preembedding or postembedding approaches. At the ultrastructural level a positive immunolabelling was only observed rarely in some sections after the preembedding procedure with the biotinylated anti-mouse antibody and streptavidin labelled with nanogold, but labelling intensity seemed too weak and randomly distributed to be taken into further analysis (not shown, table 1).

Gold-Substituted Silver-intensified Peroxidase (GSSP) (preembedding)

Using the GSSP method we were able to capture and fix contaminants inside of the cells. The results showed different distribution patterns for the lipophilic compounds phenanthrene and aroclor 1254.

Figure 6 shows successful ultrastructural immunolabelling with the GSSP preembedding method which resulted in a granular deposition of silver and gold particles with high electron density. Preservation of tissue morphology was excellent and post-fixation with osmium gave enough contrast to visualize subcellular structures.

The PCB mixture aroclor 1254 was observed inside several mitochondria of duct cells of digestive gland. Figure 6 A-C demonstrates cells of the epithelial layer of the digestive duct. The PCB mixture aroclor 1254 was detected in the mitochondria of those cells. The immuno positive labelled mitochondria were mainly located in the apical part of the duct cells. A few GSSP reaction products were also visible on the apical surface of the cells in association with cilia and microvilli. Controls incubated without the anti-PCB antibody or DAB did not show any GSSP labelling (not shown).

Immunolabelling for phenanthrene was found in the endo-lysosomal system of the digestive cells. Phenanthrene was detected in lysosomal structures and various smaller vesicles of digestive cells (figure 6 D-F). Immunolabelling was prominent inside of heterolysosomes (figure 6 D), in membranes of vesicles and in a few cases at the granular content of vesicles (figure 6 E). Close to those vesicles, larger digestive vacuoles were labeled at their membranes (figure 6 E). Granular silver-gold precipitates were located centrally in vesicular inclusions of dense lysosomes of digestive cells after phenanthrene exposure (figure 6 F). Controls incubated without the anti-phenanthrene antibody or DAB did not show GSSP labelling (not shown). Immunolabelling of non-exposed organisms for aroclor 1254 and phenanthrene did not result in the appearance of any GSSP reaction product (not shown).

Lysosomes, endosomes and smaller vesicles of digestive cells which are all part of the cellular uptake, transport and storage system for toxins are the sites where the lipophilic chemical phenanthrene was detected. In contrary, the PCB mixture aroclor 1254 was observed inside several mitochondria of digestive duct cells.

Fig. 6: Electron microscopic views of contaminant detection in cells of *Mytilus edulis*.
Figure 6 shows electron microscopic pictures after aroclor 1254 (A, B and C) and phenanthrene exposure (D, E and F) and immunodetection of the lipophilic toxins in the digestive system of *Mytilus edulis* using the CSA/GSSP preembedding method. Electron dense particles represent the granular immunoprecipitates after labelling with GSSP.
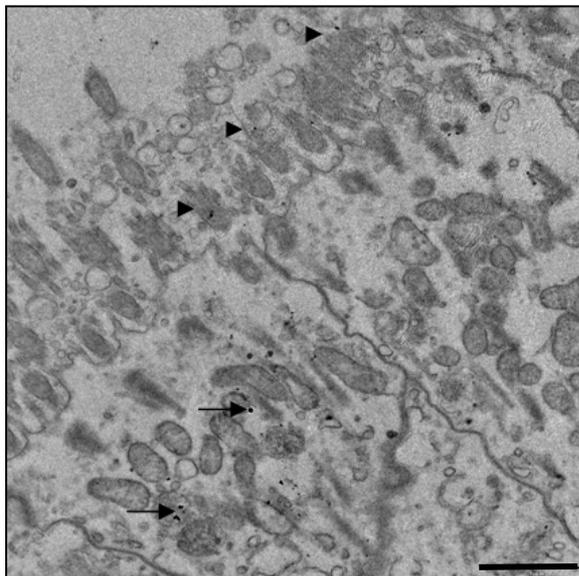
A) Epithelial cells of a digestive duct with positive immunolabelling of aroclor 1254 in mitochondria (arrows), as well as GSSP labelling on the cell surface (arrowheads). Bar 1μm
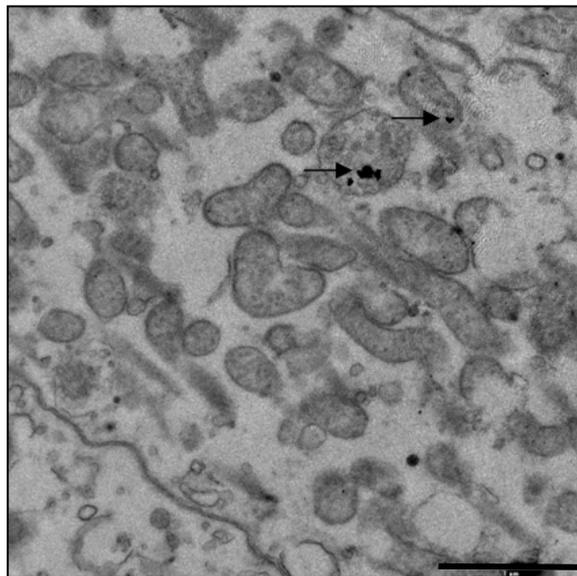
B) Higher magnification of a selected area of showing GSSP labelling inside of mitochondria. Bar 1μm
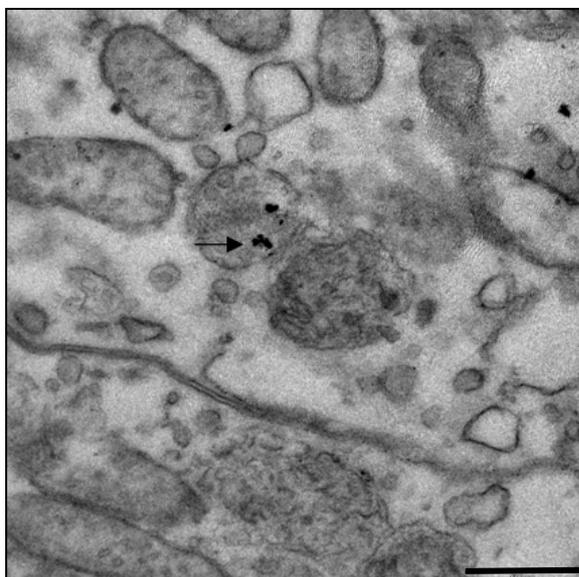
C) Higher magnification of a selected area of showing GSSP labelling inside of a mitochondrion. Bar 500 nm
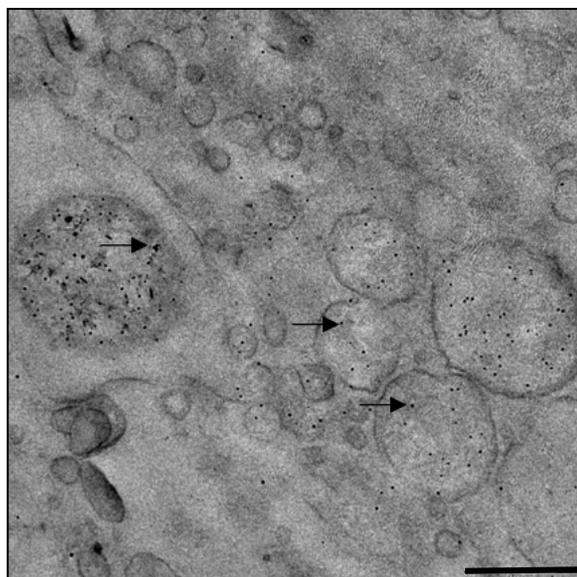
D) Immunolabeling inside of lysosomes (arrows). Bar 500 nm
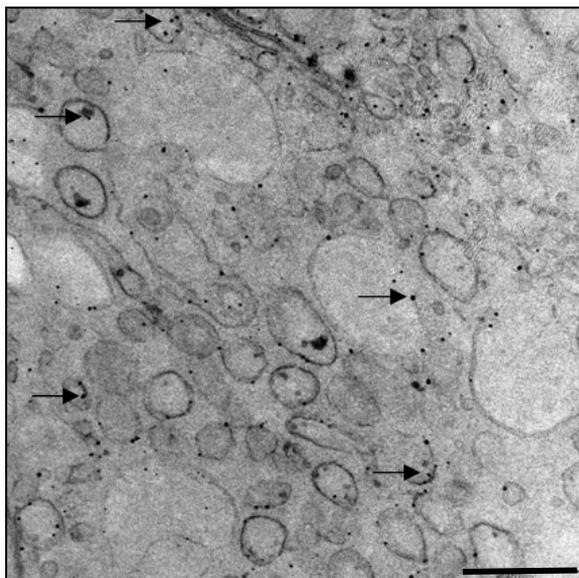
E) GSSP immunolabelling with the anti-phenanthrene antibody was observed in membranes and at granular inclusions of vesicles as well as in membranes of larger vacuoles in a digestive cell (arrows). Bar 500 nm
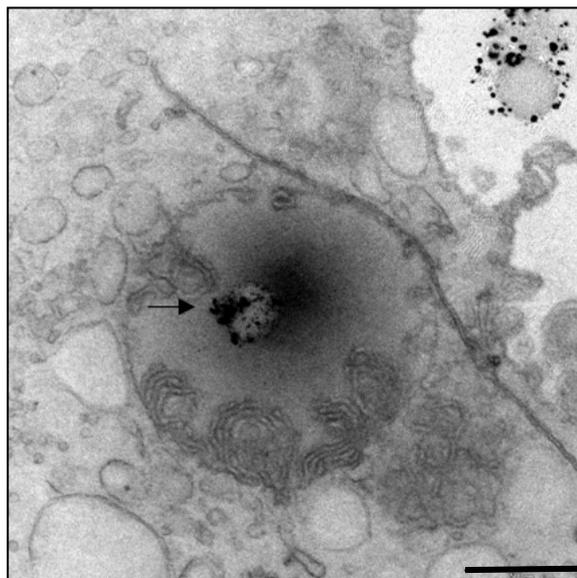
F) Lysosome with centrally located granular silver-gold particles associated to enclosed vesicles (arrow). Note the incorporation of membranous material. Bar 500 nm

3.5 Discussion

In the present study, a first approach taken was to use antibodies against chemicals with immunogold-labelled IgGs for the detection of toxins under the transmission electron microscope. None of the immunogold-labelled antibodies used in combination with silver enhancement was successful in detecting the toxins in the cells neither in the pre- nor in the postembedding approach (table 1). For postembedding, the reason is likely due to the various dehydration and embedding steps which tend to extract lipids and other lipophilic cell compounds which might as well include the accumulated lipohilic substances. To circumvent extraction of chemicals to be visualized, preembedding procedures with various nanogold conjugates were tested, but none of the applied immunogold-conjugates not even ultrasmall gold particles gave a positive immunostaining. The appearance of some weak reaction product in the preembedding approach with a biotinylated bridge antibody and a streptavidin-labelled nanogold antibody which was not taken into analysis gave an indication to attempt a more sensitive and higher amplification technique in combination with a preembedding system.

Therefore, a second approach based on an insoluble DAB reaction product catalyzed by peroxidases was applied. The GSSP intensification method, which we selected for the preembedding approach, is often used in neuro-histochemical studies for the detection of very low levels of antigens. Additionally, it appeared promising to overcome negative effects of the dehydration and

embedding procedures by the DAB coupling forming an insoluble polymer attached to the chemical to be demonstrated (Goercs et al. 1986, Van den Pol and Gorcs 1986). With the choice of the DAB technique to form a strong insoluble antigen-antibody complex with high amplification, we hoped to overcome the above mentioned obstacles of successful visualization of the lipophilic chemicals. The GSSP technique was applied in a preembedding approach to enhance the fixation, ultrastructural preservation and intensification of lipophilic contaminants and resulted in positive immunoreactions.

To obtain a signal multiplication, immuno-sandwich methods, such as Avidin-Biotin-Complex (ABC) or CSA can be applied. High signal amplification of 20-200 times higher sensitivity can be achieved with the CSA system as compared to other conventional immuno enzymatical detection systems (Luttmann et al. 2004). Furthermore, the GSSP technique assures an excellent cell preservation and morphology by post-fixation with osmium tetroxide and the use of Epon embedding. Additionally, osmium gives enough contrast for transmission electron microscopy, and therefore staining with lead citrate and uranyl acetate is not absolutely required, anymore. In our study, contrasting with lead citrate and uranyl acetate was omitted in order to be able to identify the positive immunoreaction.

Stabilization of the immunoreaction product by an insoluble oxidative polymerization product (phenazine molecule) (Van den Pol and Gorcs 1986) minimizes the risk of release of antigens, in our case the contaminant, during the following embedding process which involves several steps of dehydration with organic solvents.

In general, persistant organic pollutants have been shown by analytical chemistry to concentrate in marine organisms including invertebrates ((Nelson et al. 1995, Baumard et al. 1999, Narbonne et al. 1999, Baussant et al. 2001, Birdsall et al. 2001). Mussels rapidly accumulate lipophilic chemicals whether water soluble, dispersed, or in particles adsorbed in their tissues. Multiphasic uptake of organic pollutants has been observed and interpreted in terms of contaminants entering a multicompartment system (Livingstone and Pipe 1992). Different compartments for potential toxin accumulation are various lipid pools such as membrane lipids, depot lipids, or circulating lipids.

By the application of an antibody-based detection system, phenanthrene was found in various transport vesicles, endososmes and lysosomes in cells of the digestive tubules. These observations coincide with previous light microscopic studies of Moore et al. (2006) which showed lysosomal accumulation of PAHs in the digestive gland using 3-methylcholanthrene (3-MC) as a fluorescent marker. Moore and Willows (1998) suggested a simplified model for the uptake and fate of pollutants into cells, in which lipophilic organic contaminants enter cells by diffusion across the plasma membrane and / or bound to particulates (e.g. particulate organic carbon and

lipoprotein in body fluids). Contaminants, bound to particulates, enter the cells via endocytotic or phagocytotic mechanisms and are transported to endosomal and lysosomal compartments. The author hypothesizes that inside the lysosomes the particulate material is degraded and, in case of lysosomal damage, the released xenobiotics will be able to interact with the membrane, lipids and proteins, and may cause toxicity.

In the present study, PCBs were found on the cell surface in association to microvilli and cilia and in the epithelial cells of the digestive tract. In the clam *Mya arenaria* PCBs were detected at the light microscopic level on cilia of the gill epithelium, in the renal ductular epithelium and in intestinal contents as well as in hemocytes (neoplastic and normal) and ovaries (Strandberg et al. 1998). A new and important finding of the present study is the detection of aroclor 1254 at the subcellular level inside of mitochondria. The observed localization of aroclor 1254 corresponds with several reported effects of aroclor 1254 on signal transduction systems such as calcium homeostasis, phosphoinositol hydrolysis, and protein kinase C in mitochondria (Tilson et al. 1998) as well as with reported effects on permeability changes of the inner membrane or respiratory inhibition (Ebner and Braselton 1987).

At the tissue level, lipophilic toxins are absorbed and accumulated in the epithelia of intestinal organs. In summary, the present study demonstrates the accumulation of organic contaminants inside of cell organelles at the subcellular level. Positive immunolabelling of phenanthrene was found in the endo-lysosomal system of digestive gland cells. Aroclor 1254, on the other hand, was localized inside of mitochondria of epithelial cells.

For future research, we propose the application of the immunological CSA/GSSP technique as described in the present study for the detection of chemicals from tissue levels to subcellular components. This method is a promising approach to localize various environmental contaminants in biota and to study their toxic effects. Moreover, antibody-based detection of contaminants in combination with signal amplification systems such as CSA and GSSP provide the high resolution and high sensitivity necessary for the detection of very low amounts of chemicals in small tissue samples and single cells. The use of preembedding procedures in electron microscopy is a successful approach that allows for the optimal conservation of cell structures and subsequent detection of antigens.

3.6   References

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CHAPTER 4

4 Accumulation, localization and detoxification of the polycyclic aromatic hydrocarbon phenanthrene in cell fractions and organelles in the digestive gland of the mussel *Mytilus edulis*

4.1   Abstract

Subcellular distribution in cell fractions and ultrastructural localization of phenanthrene in the digestive gland of the mussel *Mytilus edulis* was investigated in a laboratory exposure study. Concomitantly, various detoxifying enzyme activities which have been shown to respond to organic contamination were determined. Mussels were exposed to 150 μg/L phenanthrene and samples of digestive gland tissue were taken after ten days of exposure for biochemical and immunocytochemical analyses. Concentrations of the polycyclic aromatic hydrocarbon phenanthrene in subcellular fractions of digestive glands were measured chemically by gas chromatography/mass spectrometry (GCMS). Accumulation of the contaminant was observed in all subcellular fractions. In the heavy mitochondrial fraction 3561 μg/kg dry weight phenanthrene were determined. The light mitochondrial fraction contained 695 μg/kg dry weight of phenanthrene. In the cytosol fraction 295 μg/kg of the hydrocarbon were detected whereas the phenanthrene content of the microsomal fraction (10 μg/kg dry weight) was close to the detection limit. Subcellular localization of the hydrocarbons at the light microscopic and ultrastructural level was investigated by an immunohistochemical approach. Phenanthrene was detected in the endo-lysosomal system of the cell and found in association with membranes of various smaller transport vesicles in the digestive tissue. Additionally, lysosomal membrane stability as an integrative biomarker of cellular dysfunction was significantly reduced after phenanthrene exposure. Activities of detoxifying enzymes NADPH cytochrome c reductase (NADPH cyt c red) as a first response of phase I enzyme detoxification system, glutathione S-transferase (GST) as an indicator of conjugation activities of phase II and catalase (CAT) as an indicator of defence against oxidative stress were determined in homogenates of mussel digestive gland. NADPH cyt c red and CAT activities were significantly increased after 10 days of phenanthrene exposure while GST showed only slightly increased activity compared to controls.

4.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous hydrophobic contaminants from a variety of industrial activities with a high persistence and ability to accumulate in marine organisms. PAH concentrations in coastal areas and harbors with high anthropogenic activities may reach up to 505 µg/L in water and 232 mg/kg in sediments (Grundy et al. 1996). In tissues of aquatic organisms reported values range from undetectable quantities (approximately 0.01 µg/kg dry weight) to values in excess of 5000 µg/kg dry weight for individual PAHs.

The major source for uptake of PAHs and other hydrophobic contaminants are sediments and the water column. Phenanthrene is a low molecular weight, three-ring aromatic hydrocarbon which occurs as a major component of the total content of PAH compounds in the marine environment and is declared as a priority pollutant by the Environmental Protection Agency (EPA). Phenanthrene is a toxic pollutant classified as a non-carcinogen to humans on the basis of no human and inadequate data from animal studies by EPA. Nevertheless, studies regarding the metabolic activation of polycyclic aromatic hydrocarbons suggest a carcinogenic potential for phenanthrene (Wood et al. 1979). Phenanthrene often occurs together with other PAHs, and a typical complex mixture of aromatics may be more toxic or hazardous in general than a single compound alone (Irwin et al. 1998). PAHs can elicit toxicity through several mechanisms including adduct formation, the generation of reactive oxygen radicals and hormonal disturbances (Olmstead and LeBlanc 2005). In general, the toxic, mutagenic, and carcinogenic action of PAHs is caused by formation of reactive PAH metabolites and intermediates by the oxidative metabolism in the mixed-function oxygenase (MFO system) (Kalf et al. 1997).

Bivalve molluscs are sessile suspension feeders which take up large volumes of water for respiration, ion exchange, and feeding. They can rapidly accumulate PAHs and have a relatively low rate of PAH metabolism (1-2 orders of magnitude) compared to fish (Stegeman and Lech 1991). Therefore, most of the PAH measurements have been made in bivalve molluscs which are useful bioindicators of persistent organic pollutants (McElroy et al. 1985). When mussels are transferred to clean seawater after contaminant exposure, they are able to eliminate accumulated hydrocarbons, but depuration times vary between species and depend on previous exposure time (Rantamaki et al. 1997).

Mussels process large volumes of water (filtration rate 52-196 $l\,g^{-1}\,d^{-1}$) and therefore uptake from the dissolved phase may significantly contribute to contaminant accumulation (Green et al. 2003). Uptake of contaminants occurs via passive diffusion through membranes of exposed tissues and via nutrition. Food particles are transported by the gut system for metabolization to the digestive

gland. The digestive cells contain a lysosomal vacuolar system dealing with the uptake and the intra-cellular digestion of endocytosed substances (Dimitriadis et al. 2003).

In the present study, we applied an antibody-based detection system for localization of phenanthrene in cryostat sections at the light microscopic level. In a previous study, antibodies against environmental pollutants were successfully used to detect organic contaminants at the ultrastructural level in mussels (Einsporn and Koehler 2006, submitted). This newly developed immunohistochemical approach was also applied for localization of phenanthrene in association to cell organelles and other subcellular components in mussel digestive gland. Additionally, phenanthrene was measured by chemical analysis in subcellular fractions of digestive gland.

Phenanthrene treatment of molluscan digestive cells results in oxidative stress, probably by direct oxidative attack on intra-lysosomal PAH by oxyradicals normally generated in the lysosomal compartment (Moore et al. 2006). Therefore, lysosomal membrane stability was investigated as a marker of cellular stress in digestive gland after phenantrene exposure.

Different enzymes are involved in the biotransformation and detoxification process of organic compounds. Biotransformation processes include functional reactions (phase I) and conjugative reactions (phase II). In phase I metabolism oxidative reactions are catalyzed by the cytochrome P450-dependent MFO system. The MFO system is inducible by exposure to organic xenobiotics and has been used as a specific biomarker of exposure to organic contaminants in marine invertebrates (Petushok et al. 2002). It consists of at least two protein components: Cytochrome P-450 and NADPH-ferrihemoprotein reductase (NADPH cytochrome P-450 or neotetrazolium reductase) (Mohammed and Agard 2004). NADPH cytochrome c reductase (NADPH cyt c red) is a microsomal flavoprotein which transfers electrons in two separate one electron reductions from NADPH to cytochrome P-450 (Buhler and Williams 1989). NADPH cyt c red activity is associated with the endoplasmic reticulum. Increased activity of NADPH cyt c red has been reported in molluscs after exposure to hydrocarbons such as phenanthrene and benzo(a)pyrene. In general, activity rates of the whole MFO system are relatively low in marine invertebrates compared to fish (Bayne et al. 1985, Suteau and Narbonne 1988, Okay et al. 2000) resulting in high accumulation rates of contaminants. Phase I oxidation renders more reactive products for further transformation during phase II, in which the PAH metabolites are conjugated to various endogenous cell components such as sugar derivatives, peptides or sulphates, thereby increasing water solubility and facilitating excretion of compounds. Phase I products such as diols and phenols can be further oxidized to diol- and phenol-epoxides, which are highly reactive and bind to DNA while other products which produce free radicals and/or reactive oxygen species (ROS) may induce DNA strand breaks (Bach et al. 2005), lipid peroxidation of cellular membranes, protein degrada-

tion, and enzyme inactivation (Regoli 2000). The most important intracellular system preventing the formation of diol epoxide-DNA adducts is the glutathione S-transferase (GST)-catalyzed conjugation of the intermediates with glutathione (GSH) (Sundberg et al. 1998). GSTs from phase II act as catalysts of a wide variety of conjugation reactions of glutathione with xenobiotic compounds containing electrophilic centers (Regoli and Principato 1995).

Catalase (CAT) is part of the cellular defense system against toxicity originating from active oxygen forms (Regoli and Principato 1995). The enzyme decomposes hydrogen peroxide which is a toxic product of both normal aerobic metabolism and pathogenic reactive oxygen species. Increased CAT activities have been observed in *M. edulis* exposed to organic xenobiotics such as benzo(a)pyrene and menadione (Livingstone et al. 1990).

The use of biotransformation enzyme activities as biomarkers in environmental monitoring studies using mussels is complex due to seasonal changes, high individual variability, and adverse effects of contaminants mixtures. Laboratory studies are useful to describe effects of selected environmental chemicals on enzyme activities and investigate the ranges of enzyme activities of marine mussels under different exposure conditions.

In the present study, detoxifying enzyme activities were investigated in digestive gland homogenates after laboratory exposure to the hydrocarbon phenanthrene. NADPH cyt c red, GST and CAT were used as measures for phenanthrene exposure because of their important role in xenobiotic transformation and detoxification of organic contaminants.

The objectives of this work were (1) to clarify compartmentalization of phenanthrene in different subcellular fractions of mussel digestive gland, 2) to identify subcellular targets of phenanthrene interaction at the ultrastructural level and (3) to quantify the response to phenanthrene exposure using detoxifying enzymes such as NADPH cytochrome c reductase, catalase and GST in digestive gland homogenates as well as the lysosomal membrane stability as a general marker of cellular stress.

4.3   Methods

Experimental animals

Mytilus edulis (shell length 40-60 mm) were collected at the Wadden Sea Station Sylt, a relatively unpolluted area with low background concentrations of organic pollutants in *M. edulis* (Einsporn et al. 2005). Mussels were transported within a few hours to the laboratory in Bremerhaven where they were acclimated in aerated filtered sea water at 12-14°C for up to four weeks until the start

of the exposure experiment. Mussels were fed every 2 days with a solution of zoo- and phyto-plankton for marine invertebrates (Dohse Aquaristik, Gelsdorf, Germany).

Exposure experiment

After the acclimation period, mussels were transferred to glass aquaria (one mussel per liter) and incubated in aerated sea water containing 150 μg/l phenanthrene dissolved in acetone for up to 10 days. Sea water temperature was 15±1°C during the whole experimental period. Sea water was renewed every 24 hours with re-dosing of contaminants or clean sea water as controls. After 10 days of exposure, mussels (seven individuals per treatment) were dissected and samples of diges-tive gland tissue were taken for chemical, biochemical and immunohistochemical analysis.

Immunolocalization of phenanthrene at the light microscopic level in cryostat sections

For light microscopy, 10 μm cryostat sections of digestive gland were cut with a microtome (Mi-crom, Walldorf, Germany) at -20 °C, air dried and dipped in freshly prepared 4% paraformalde-hyde (PA) for 2 min. Sections were washed in PBS, placed in 1% Triton in PBS for 5 min and washed again in PBS. Slides with tissue sections were placed in a humidified chamber and incu-bated with the first monoclonal antibodies (anti-phenanthrene 1:50 = 10μg/ml RDI, Flanders USA) in 3% BSA in PBS for 120 min at RT. Controls were incubated without the first antibodies in 3% BSA in PBS. After washing the sections in PBS, the Catalyzed Signal Amplification (CSA) system from DAKOCytomation (Glostrup, Denmark) for monoclonal mouse antibodies was applied. CSA-DAB staining was performed according to the manufacture`s protocol (DAKOCy-tomation, Glostrup, Denmark) for mouse primary antibodies with a few modifications. All incu-bation steps were done at RT, followed by a washing step (3 times for 5 min) with PBS (pH 7.4). First, endogenous peroxidase activity was blocked with a solution of 3% hydrogen peroxide in water for 20 min. Endogenous biotin and avidin were quenched with biotin-avidin blocking re-agent (DAKOCytomation, Glostrup, Denmark). The secondary biotinylated link-antibody (anti-mouse-antibody) was added for 2 hours to the sections before adding the streptavidin-biotin for one hour and the amplification (biotinyl tyramide and hydrogen peroxide in PBS) reagent for 30 min. Incubation with streptavidin conjugated to horseradish peroxidase in PBS was performed for 2 hours before the DAB chromogen solution was added for 5 min for the final staining reac-tion. Counterstaining was performed with Gill`s hematoxylin. Sections were mounted in glycerin gelatin and images were taken with a Zeiss Axiovert microscope and the digital imaging software Axiovision (Zeiss, Germany).

Immunocytochemistry at the electron microscopic level

For ultrastructural localisation of phenanthrene a preembedding technique with signal amplification system based on Catalysed Signal Amplification (CSA) according to Koehler et al. (2000) and Gold-Substituted Silver intensified Peroxidase (GSSP) according to Van den Pol and Gorcs (1986) was combined as described in detail elsewhere (Einsporn et al. 2006, submitted). Freshly dissected and chopped mussel digestive glands were fixed in 2% paraformaldehyde (PA) and 0.1% glutaraldehyde (GA) for 2 hours at 4°C and in 2% PA over night. Unspecific antibody binding sites were blocked for 2 hours with 1% bovine serum albumin, 0.2% fish gelatine and 0.05% saponin in PBS. Tissue sections were incubated with a monoclonal antibody against the antigen (anti-phenanthrene 1:100 = 20 µg/ml) at 4°C over night. The second detection step was performed with the CSA system with diaminobenzidintetrahydrochlorid (DAB) from DAKOCytomation (Glostrup, Denmark) for monoclonal mouse antibodies. Controls were run without addition of the first antibody or DAB. The streptavidin-peroxidase incubation for 2 hours was followed by fixation in 1% GA in PBS for 30 min and the DAB chromogen incubation for 5-8 min. After washing in PBS to stop the DAB reaction, the tissue sections were washed in 2% sodium acetate. Afterwards, the sections were washed for 3 h in 10% sodium thioglycolate. After washing, sections were incubated with the freshly prepared silver solution consisting of solution A (5% sodium carbonate), B (0.05% ammonium, 0.25% tungsten silicide, 0.05% silver nitrate) and C (37% formalin) (A:B:C = 1:1:0.004) up to 8 min. The reaction was stopped with 1% acetic acid. The gold labelling was done with 2% gold chloride for 8 min. After fixation with 3% sodium thiosulphate, the tissue pieces were transferred to 0.1 M sodium cacodylate buffer (pH 7.4) and fixed in 2% GA in 0.1 M sodium cacodylate buffer. After a post-fixation with 0.5% osmium tetroxide and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 15 min, tissue pieces were washed several times in distilled water and dehydrated in 30, 50, 70, 80, 96 and 100% ethanol for 20 min followed by an Epon-embedding. For electron microscopy, ultra-thin sections were cut with an ultracut microtome (Reichert-Jung, Wetzlar, Germany) and collected on formvar copper grids. Contrasting of sections was omitted to avoid over-staining of the immuno-reactions. Observations were made under a Zeiss EM109 transmisssion electron microscope and by digital analysis system (Soft Imaging System`s iTEM, Muenster, Germany).

Subcellular fractionation

Digestive gland samples were pooled (~0.5 g / 5 ml) and homogenized in ice-cold homogenization buffer (containing 250 mM sucrose, 20 mM hepes, 1 mM EDTA, 1 mM of protease inhibitor Pefabloc) at pH 7.0. Tissues were homogenized using a rotor homogenizer (Ultra-Turrax,

Janke & Kaukel KG, Staufen i. Breisgau, Germany) with two strokes at level 2. Subcellular fractions were obtained by differential centrifugation of the homogenate according to the protocol of Cajaraville et al. (1992) with slight modifications. The homogenate consisting of pooled digestive glands from seven individuals was centrifuged at 700 x g for 10 min to remove gross cell debris and unbroken tissue fragments. A sample of 1 ml of the total tissue supernatant was collected and stored for determination of enzyme activities on ice. The rest of the obtained supernatant was centrifuged for 10 min at 1900 x g and the pellet was re-suspended in 2 ml homogenization buffer (HB) and stored as the heavy mitochondrial fraction (b) on ice. This crude fraction is composed of nuclei, mitochondria, cell membranes and cell debris. The supernatant was centrifuged for 45 min at 39000 x g and the obtained pellet re-suspended in HB and stored as the light mitochondrial fraction (d) on ice which contained lysosomes, mitochondria and peroxisomes. The supernatant was centrifuged for 60 min at 170000 x g. The obtained pellet was re-suspended in 2 ml HB to yield the microsomal fraction (g) and stored with the supernatant (cytosol fraction f) on ice until further analyses or at -80°C for long-term storage times (maximal four weeks). The microsomal fraction contains small pieces of the plasma membrane, fragments of Golgi apparatus, ribosomes and microsomes (disrupted endoplasmic reticulum forming closed vesicles). The cytoplasm (cytosol fraction) contains a large number of enzymes and other proteins.

Chemical analysis of phenanthrene

Phenanthrene accumulation in subcellular fractions and total homogenates was determined by gas chromatography/mass spectrometry by the environmental analysis laboratory IBEN (Bremerhaven, Germany). Sample extraction for Phenanthrene analysis was performed using cyclohexane for 2 hours at 50 °C in an ultrasonic bath. For analysis of PAHs, a Shimadzu GC-MS (Duisburg, Germany) with an auto sampler was used. Quantification was done with external standards from Ehrenstorfer (Augsburg, Germany). Quality control was done with a certified calibration standard (BAM) and gas chromatograms were recorded in the SIM modus.

Activity of marker enzymes

In order to determine the relative amount of the cell organelles in the obtained fractions, activities of marker enzymes for mitochondria, lysosomes and plasma membranes were measured. Enzyme activities were measured with a microtiter plate reader (Tecan, Crailsheim, Germany) as described in the section regarding the biochemical analyses.

Total protein contents were determined according to the Bradford method and with bovine serum albumin (BSA) as a standard using a commercial kit from Bio-Rad Laboratories (Hercules, USA).

Biochemical analyses of enzyme activities

Enzyme activities (succinate dehydrogenase, ß-galactosidase, alkaline phosphatase, NADPH cytochrome c reductase, glutathione S-transferase, catalase) were determined spectrophotometrically in a microtiter plate reader at constant temperature ($20 \pm 1°C$). The actual extinction coefficients of the substrates were adjusted according to the path length of the solution in the well.

Succinate dehydrogenase

Succinate dehydrogenase (EC 1.3.5.1) activities were analysed according to Graham (1993) at 490 nm using p-iodonitrotetrazolium violet (INT). The wells contained 30 µl assay mixture (0.01 M sodium succinate in 0.05 M phosphate buffer pH 7.5) and 5 µl sample or blank. The mixture was incubated for 15 min and again for 10 min after addition of 10 µl INT (2.5 mg/ml in 0.05 M phosphate buffer, pH 7.5). The reaction was stopped by adding 100 µl ethyl acetate: ethanol: trichloracetic acid in the ratio 5:5:1 (v/v/w).

ß-Galactosidase

ß-Galactosidase (EC 3.2.1.23) activities were measured according to Graham (1993) at 405 nm using o-nitrophenyl ß-D-galactopyranoside. The wells contained 50 µl assay mixture (0.2 M citrate-phosphate buffer pH 4.3, 2% Triton X-100, 12 mM o-nitrophenyl ß-D-galactopyranoside in the ratio 1:1:2 by volume) and 5 µl sample. The mixture was incubated for 30 min and the reaction was stopped by adding 100 µl 0.25 M glycine-NaOH (pH 10). For blanks the assay mixture was incubated alone, and then 0.25 M glycine-NaOH (pH 10) was added, followed by sample. The absorbance was measured at 405 nm against the blank.

Alkaline phosphatase

Alkaline phosphatase (EC 3.1.3.1) activities were determined according to Graham (1993) at 410 nm ($\epsilon = 18.2$ mM^{-1} cm^{-1}) by the formation of para-nitrophenol from para-nitrophenol phosphate (PNPP) as substrate. The wells contained 40 µl assay buffer (16 mM p-nitrophenyl phosphate,

250 mM sodium borate-NaOH pH 9.8, 1.0 mM $MgCl_2$ in the ratio 1:1:10000 by volume) and 5 μl sample or blank. The mixture was incubated for 20 min and the reaction was stopped by adding 120 μl of 0.25 M NaOH. The absorbance was read at 405 nm against a blank in which the 0.25 mM NaOH is added before the assay mixture.

NADPH cytochrome c reductase

NADPH cytochrome c reductase (EC 1.6.2.4) activity was measured according to Graham (1993) at 550 nm. The wells contained a mixture of 100 μl 50 mM phosphate buffer pH 7.7 containing 0.1 mM EDTA, 5 μl cytochrome c (25mg/ml in 50 mM phosphate buffer) and 5 μl sample. The absorbance was recorded at 550 nm until the baseline was steady and 10 μl NADPH (2 mg/ml in 50 mM phosphate buffer) were added. After mixing, the absorbance readings were continued until a linear increase in values was measured.

Glutathione S-transferase

Glutathione S-transferase (GST) (EC 2.5.1.18) activities were determined according to Habig et al. 1974 with a commercial GST assay kit (Cayman Chemical Company, Michigan, USA) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The GST-catalyzed formation of CDNB-GSH produces a dinitrophenyl thioether which can be detected by spectrophotometrically at 340 nm. The wells contained 150 μl assay buffer (100 mM potassium phosphate, 0.1% Triton X-100, pH 6.5), 20 μl glutathione (GSH) and 20 μl sample or blank (HB). The reaction was initiated by adding 10 μl of CDNB and the absorbance was read every minute for 10 min at 340 nm.

Catalase

Catalase (CAT) (EC1.11.1.6) activities were measured according to Johansson and Borg (1988) with a commercial catalase assay kit (Cayman Chemical Company, Michigan, USA) using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. The assay utilizes the peroxidase function of CAT to measure the enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal H_2O_2 concentration. Purpald forms a bicyclic heterocycle with the produced formaldehyde, which changes from colourless to a purple colour upon oxidation. Final concentrations of formaldehyde standards ranged from 0-75 μM. Wells contained 100 μl assay buffer (diluted), 30 μl methanol and 20 μl sample or formaldehyde standards, respectively. The sample wells contained 100 μl assay buffer, 30 μl methanol, and 20 μl

sample. The reaction was initiated by adding 20 µl H_2O_2 and incubated 20 min at room temperature (RT). The reaction was terminated by adding 20 µl Purpald. After further 10 min of incubation on a shaker at RT, 10 µl potassium periodate were added to each well. After a further incubation of 5 min on a shaker at RT the absorbance was read at 540 nm.

Lysosomal membrane stability

For the lysosomal membrane stability test (N-acetyl-ß-hexosamidase histochemistry) pieces of digestive gland were taken from five mussels from each treatment (phenanthrene and control) and frozen immediately in liquid nitrogen. Tissue samples were stuck on aluminium cryostat chucks, wrapped into parafilm and aluminium foil and stored at -80°C until sectioning.

The lysosomal membrane stability test was performed according to the method of Moore (1976) and Koehler et al. (2002). Briefly, serial cryostat sections were incubated in acid buffer to destabilise the membrane at varying time intervals (2-50 min). After labialisation, the sections were incubated in medium containing the substrate for the lysosomal marker enzyme. To visualize the enzyme-substrate complex, a post-coupling reaction with a diazonium salt was performed and analysed by microscopic examination. A long labialisation time means high lysosomal membrane integrity and vice versa.

Statistics

Differences between exposure and control group for detoxifying enzyme activities and lysosomal membrane stability were tested with the Mann-Whitney Rank Sum Test. The criterion of significance was $p < 0.05$. Statistical analyses were performed using Sigma Stat 3.0 (SPSS).

4.4 Results

Detection of phenanthrene at the light microscopic level

At the light microscopic level the application of a monoclonal antibody against phenanthrene in combination with the CSA system resulted in a positive immunolabelling of the chemical. A strong brown reaction product was observed in lysosomes of the digestive gland treated with the CSA system after phenanthrene exposure (figure 7 A) while the controls (figure 7 B) incubated without the first antibody did not show any reaction product in lysosomes of the tubules.

Fig. 7: Detection of phenanthrene in mussel digestive gland at the light microscopic level.
A) Digestive gland section showing tubules after incubation with the anti-phenanthrene antibody. Note the strong brown DAB reaction product. Magnification x400
Inset: Zoom of A showing positive labelled lysosomes of digestive gland tubules.
B) Digestive gland section showing tubules incubated without the first antibody (control). Magnification x400
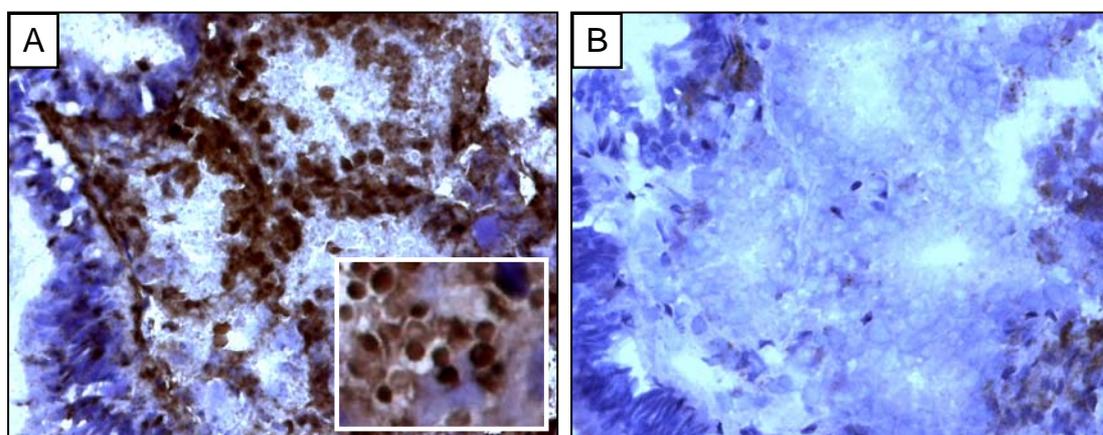
Subcellular localization of phenanthrene in the digestive gland

At the ultrastructural level positive immmolabelling with the anti-phenanthrene antibody was found in various vesicles, vacuoles and parts of the endo-lysosomal system of the digestive cells. Figure 8 A and B shows examples of subcellular locations where the reaction product enhanced with silver-gold in digestive cells was detected. Different types of the endo-lysosomal system showed the GSSP reaction product (see also Einsporn et al. 2006, submitted). Immunolabelling was present in the membranes of lysosomes and at their granular incluions. Secondary lysosomes with heterogeneous contents such as lipid inclusions and membrane residues had a more centrally located immunolabelling (figure 8 A) whereas electron-dense secondary lysosomes were labelled at their membranes (figure 8 B). Controls incubated without the anti-phenanthrene antibody or DAB did not show any GSSP labelling (not shown). Immunolabelling of non-exposed organisms for phenanthrene did not result in the appearance of the GSSP reaction product.

Fig. 8: Electron microscopic views of the subcellular distribution of phenanthrene in cells of mussel digestive gland after 10 days laboratory exposure.
A) Secondary lysosomes with positive immunolabelling (arrows). Note the fusion of smaller vesicles with the lysosomes. Bar 1 μm. **B)** Immunolabelling of membranes of secondary lysosomes (arrows). Bar 1 μm
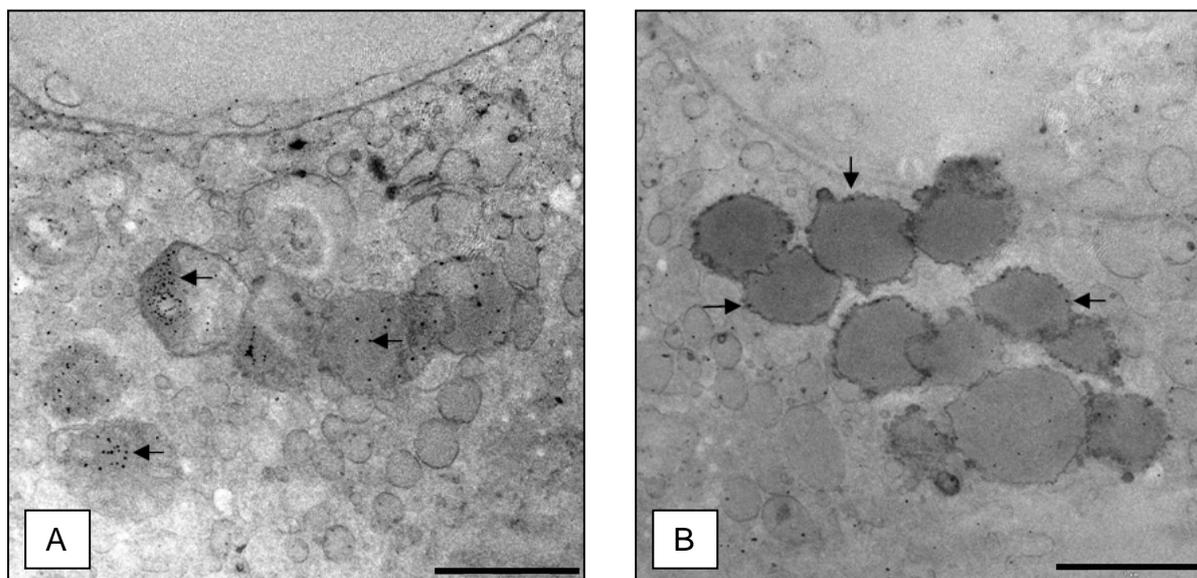
Activity of marker enzymes in subcellular fractions

Identification of marker enzyme activities showed that the light mitochondrial fraction (d) contained high amounts of lysosomes as indicated by high ß-galactosidase activity (table 2). This fraction also had the highest activity of the membrane bound alkaline phosphatase (table 2). The heavy mitochondrial fraction (b) was enriched in mitochondria as indicated by enhanced succinate dehydrogenase activity (table 2). Relatively high activities of succinate dehydrogenase were also found in the microsomal fraction (g) (table 2) probably due to contamination with mitochondria during centrifugation procedures (Mee et al. 1995). Only low levels of all organelle marker enzyme activities were found in the soluble fraction (f) which indicates a relatively clear separation of the subcellular fractions by the differential centrifugation method used in the present study.

Tab. 2: **Activities of organelle marker enzymes in main fractions obtained by differential centrifugation of mussel digestive gland homogenates**

Enzyme	Fraction	Mean activity (nM / min x mg protein) ± Standard error (SE) n=6	
Alkaline Phosphatase	heavy mitochondrial	9.97	±4.57
	light mitochondrial	32.74	±9.94
	cytosol	4.51	±1.42
	microsomal	3.03	±1.75
ß-Galactosidase	heavy mitochondrial	203.30	±117.58
	light mitochondrial	4225.48	±1176.08
	cytosol	74.77	±9.47
	microsomal	70.23	±46.46
Succinate dehydrogenase	heavy mitochondrial	15.07	±5.74
	light mitochondrial	5.86	±3.38
	cytosol	0.52	±0.27
	microsomal	13.89	±5.26

Subcellular distribution of phenanthrene

PAH distribution was determined for four fractions: the heavy mitochondrial fraction (b) (contains nuclei, membranes, mitochondria and cell debris), the light mitochondrial fraction (d) (contains mitochondria, lysosomes and peroxisomes), the microsomal fraction (g) (contains small pieces of the plasma membrane, fragments of Golgi apparatus, ribosomes and disrupted endoplasmic reticulum forming closed vesicles) and the cytosol fraction (f) (contains proteins). Table 3 shows phenanthrene accumulation in subcellular tissue fractions (b-g) of digestive gland. After 10 days of phenanthrene exposure high amounts of 3561 µg/kg dry weight (dw) phenanthrene were found in the heavy mitochondrial fraction (b). The light mitochondrial fraction (d, lysosomal fraction) contained 695 µg/kg dw and the cytosol fraction (f) 259 µg/kg dw of phenanthrene. In the microsomal fraction (g) only low levels (10 µg/kg dw) of the hydrocarbon were detected.

Tab. 3: Distribution of phenanthrene in different subcellular fractions of mussel digestive gland exposed to 150 µg/L phenanthrene for 10 days

Fraction	Cellular components	Phenanthrene exposure µg/kg dw	Control µg/kg dw
b	Nuclei, membranes, mitochondria, cell debris	3561	323
d	Mitochondria, lysosomes, peroxisomes	695	13
f	Proteins	259	<10
g	Microsomes, Golgi vesicles, ribosomes, ER	10	12

Effects of phenanthrene exposure on detoxifying enzyme activities and lysosomal membrane stability in mussel digestive gland

In pilot experiments, detoxifying enzyme activities for NADPH cyt c red and CAT showed less variability in total homogenates than in subcellular fractions. Therefore, detoxifying enzyme activities for NADPH cyt c red and CAT were determined in total homogenates of digestive gland tissue, although enzyme activities were lower in total homogenates than in subcellular fractions. GST was determined in the cytosol fraction where highest activities were found with low deviations. Activities of detoxifying enzymes after phenanthrene exposure and effects on lysosomal membrane stability are shown in figure 9 A-D. NADPH cyt c red activity was significantly increased (mean=5.59, S.E.=1.3, n=6) after phenanthrene exposure compared to the control (mean=1.28, S.E.=0.58, n=6) (figure 9 A). Also, GST activities were increased (mean=13.82, S.E.= 2.0, n=4) in phenanthrene exposed mussels compared to the control group (mean=10.4, S.E.= 0.33, n=4), but this increase was not significant (figure 9 B). CAT activities were significantly higher (mean=20.49, S.D.= 1.07, n=4) in phenanthrene treated groups compared to the controls (mean=13.92, S.D.= 0.77, n=4) (figure 9 C). Significant changes in membrane stability of lysosomes were found in digestive gland tissue of mussels after 10 days laboratory exposure to phenanthrene compared to the controls. Lysosomal membrane destabilization time was significantly reduced from 30 min (control) to 7 min after phenanthrene exposure (figure 9 D).
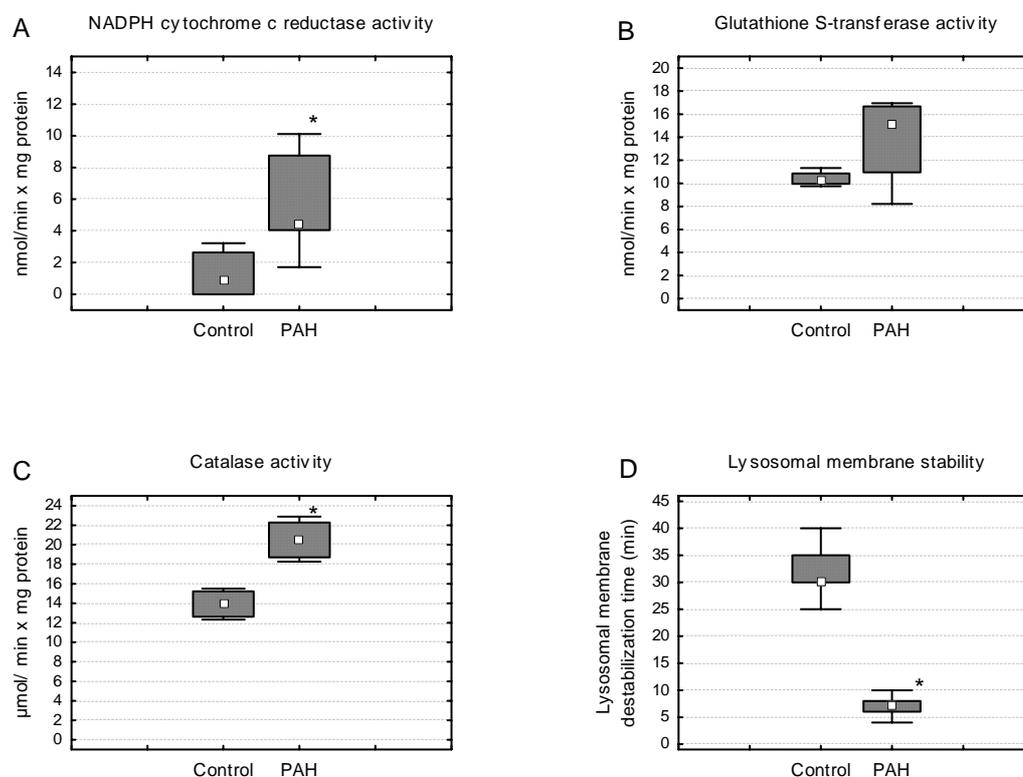
Fig. 9: **Detoxifying enzyme activities and lysosomal membrane stability in mussel digestive gland in unexposed control mussels and after 10 days of phenanthrene exposure.**
A) NADPH cyt c red activity (nM/min x mg protein), B) GST activity (nM/min x mg protein), C) CAT activity (μM/min x mg protein) D) lysosomal membrane stability (destabilization time in min). Box and Whisker Plots present the medians, with 25.-75. percentiles and minima and maxima. *significant differences ($p < 0.05$)

4.5 Discussion

The digestive gland of bivalve molluscs is part of the digestive system and is composed of several cell types. These cells are rich in lysosomes which are involved in intracellular digestion and sequestration of organic xenobiotics (Viarengo et al. 1987, Livingstone et al. 1989, Winston et al. 1991, Moore and Willows 1998). Once chemical substances such as polycyclic aromatic hydrocarbons enter the cell by diffusion across the plasma membrane or endocytotic / phagocytotic mechanisms (if the lipophilic compounds are bound to particulates) (Moore and Willows 1998), biotransformation enzymes are activated to metabolize the chemicals and form better excretable water-soluble metabolites. During the first step of biotransformation of PAHs by the MFO system reactive intermediates such as quinones, dihydrodiols, hydroxyl and phenols are generated

giving rise to reactive oxygen species (ROS) which may cause toxic effects to various subcellular components (Livingstone et al. 1989). Antioxidant enzymes protect against the destructive action of oxyradicals which are continuously produced in biological systems and are elevated under exposure to redox cycling contaminants (Eertman et al. 1995).

In the present study, changes in detoxifying enzyme activities of NADPH cyt c red, GST and CAT were observed in mussel digestive gland after exposure to phenanthrene. A significant increase of phase I NADPH cyt c red activity was observed which is probably associated with a higher production of phase I metabolites of phenanthrene such as monohydroxy derivates and trans-phenanthrene-dihydrodiols (Irwin et al. 1998). A previous study has shown that elevated levels of NADPH cyt c red in the digestive cells of *Mytilus edulis* and *Littorina littorea* directly correlated with total PAH concentration (Mohammed and Agard 2004). Okay et al. (2000) also measured an increase of microsomal NADPH cyt c red activity after short time exposure to benzo(a)pyrene in digestive gland of *M. edulis*. Phase I metabolism leads to the production of substrate metabolites for phase II enzymes such as GST (Akcha et al. 2000). GST activity is described as a potential biomarker of phase II metabolism of lipophilic organic contaminants (Amiard-Triquet et al. 1998). In the present study, GST showed increased activity after phenanthrene exposure. Contradictory results have been reported on GST enzyme activity in bivalve molluscs: Increased GST activities have been reported after short-term laboratory exposure to organic pollutants and activities were positive correlated with increasing tissue concentrations of chemical compounds (Cheung et al. 2004). Other authors, however, reported decreased levels of GST activities after acute PAH exposure (Michel et al. 1993). Studies on the effects of organic pollutants on GST activity are often conflicting, showing induction, no change, or inhibition of enzyme activity (Petushok et al. 2002).

Oxyradicals produced during phase I PAH metabolism are scavenged by antioxidative enzymatic activities of the cell. CAT is one of those protective enzymes against oxidative stress which catalyses hydroperoxide to molecular water. In the present study, CAT activities showed an increase after 10 days phenanthrene exposure. This observation coincides with previous studies of CAT activity in mussels exposed to PAHs from field and laboratory investigations in which increased activities or positive correlations between CAT activities and PAH concentrations were reported (Porte et al. 1991, Akcha et al. 2000).

Comparisons of enzyme activities measured in different laboratory studies are complicated because of differences between contaminant concentrations, exposure times, sample preparation methods and the use of different units to describe data. In field studies, reported effects are even more difficult to interpret since mixtures of PAHs and other chemicals possibly interfere with

enzyme inductions. Moreover, various parameters such as temperature, salinity and food availability additionally affect enzyme activities. A databank of reported activities of various detoxifying enzymes of marine invertebrate species after exposure to single pollutants might be helpful to validate detoxifying enzyme activities as suitable and reliable biomarkers of exposure to contaminants for environmental monitoring studies. Furthermore, there is an urgent need for standardized protocols for biomarker activities to compare measurements and for quality assurance.

Differences in biotransformation abilities also reflect differences in the subcellular distribution of lipophilic compounds (Bach et al. 2005). In the subcellular fractionation approach of the present study, the lipophilic hydrocarbon phenanthrene accumulates mainly in the heavy mitochondrial fraction which is a crude fraction, similar to the homogenate, because it consists of cell membranes, nuclei, mitochondria and other cell fragments. This fraction also contains amounts of lysosomes indicated by ß-galactosidase activity. Detection of phenanthrene in the light mitochondrial fraction (lysosomes) also displays interactions with the endo-lysosomal system of the cell whereas phenanthrene in the cytosol fraction points to an association of the chemical with enzymes or other proteins that are enriched in this fraction. When PAHs are not biotransformed, they are highly lipophilic and tend to accumulate within lipid-rich cell membranes whereas transformed metabolites are found in non-membrane cell compartments. Some PAH metabolites and intermediates are known to covalently bind to cellular macromolecules such as DNA, RNA, and proteins (Kalf et al. 1997).

In the present study, lysosomal membrane stability decreased significantly after ten days of phenanthrene exposure. Reduced lysosomal membrane stability in digestive gland cells is a well reported effect after phenanthrene exposure (Nott et al. 1985, Pipe and Moore 1986, Moore and Viarengo 1987). Therefore, the relatively high cytosolic contaminant content, measured in the subcellular fractions, might also be an effect of the observed lysosomal destabilization and leakage of the accumulated phenanthrene into the cytoplasm.

For heavy metals a much deeper knowledge of their fate and biological sequestration in marine invertebrates exists compared to organic contaminants. There are several models describing cellular and subcellular pathways for metal detoxification (see Marigomez et al. 2002). Chelomin et al. (2005), for example, studied the subcellular distribution of cadmium in mussels and observed a shift of the heavy metal concentration from the membrane fraction (containing intracellular membrane-formed compartments) to the cytoplasm, putatively due to structure destabilization of biomembranes and release of the content of lysosomes including metals into the cytoplasm. The same situation might occur with lipophilic compounds.

In the immunohistochemical part of this study the endo-lysosomal system of digestive cells with its numerous lysosomal structures and transport vesicles is the dominant site where the lipophilic chemical phenanthrene was detected. Lysosomes of the digestive tubules were positively labelled for phenanthrene in cryostat sections at the light microscopic level. Moreover, ultrastructural immunolabelling of phenanthrene was found in membranes and inside of these organelles. Distribution of PAHs within the lysosomal systems of digestive cells has also been demonstrated at the light microscopic level in other studies using a fluorescent marker hydrocarbon or micro-autoradiography (Magnusson et al. 2000, Moore et al. 2006). In the present study, cellular trafficking of phenanthrene seems to occur via various transport vesicles of the dynamic endo-lysosomal system and probably via fusion events of lysosomes. Possible mechanisms of lysosomal injury by organic chemicals are the production of reactive intermediates after first bio-transformation with phase I enzymes and the formation of reactive oxygen species (ROS) inside of lysosomes leading to disruption and lipid peroxidation of membranes. Moreover, phenanthrene accumulation inside of the lysosomes and their membranes provokes destabilization of the lipid bilayer and finally results in the release of the toxic compounds into the cytosol where cellular macromolecules and other cell organelles are targets for toxic actions of the chemical compounds and their metabolites. In contrast to the relatively high phenanthrene concentration measured chemically in the cytosol fraction, the chemical was not detected in the cytoplasm by immunolabelling. Therefore, affinity of the anti-PAH antibody towards metabolites of phenanthrene or the chemical bound to cellular macromolecules should be investigated in future studies. However, disruption of the lysosomes during homogenization and centrifugation processes might also explain this observation.

The results of the present study lend support for the application of immunocytochemistry to detect contaminants from tissue level to subcellular components and to clarify toxic effects inside the organism. Immunolocalisation of uptake and cellular trafficking of chemicals seems to be a very promising tool in toxicological research.

4.6   References

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CHAPTER 5

5 Ultrastructural localization of lead (Pb) using an antibody-based detection system and modulation of biotransformation enzyme activities in gills and the digestive gland of the blue mussel *Mytilus edulis*

5.1 Abstract

Lead (Pb) exposure experiments were conducted to study its intracellular fate in gills and the digestive gland of the mussel *Mytilus edulis*. For this purpose an antibody-based detection method for ultrastructural localization and a subcellular fraction approach for chemical analysis of Pb were used. Mussels were exposed to 2.5 mg/l lead nitrate for ten days. Afterwards they were maintained in clean sea water up to ten days for depuration. Additionally, effects of Pb on enzyme activities such as the conjugative enzyme glutathione S-transferase (GST) and the antioxidative enzyme catalase (CAT) were determined after exposure and depuration time in gills and digestive gland. In the ultrastructural part of the study, Pb was mainly detected in lysosomes of gill epithelial cells and digestive cells. Furthermore, Pb was detected in cell nuclei and granular hemocytes. Higher metal concentrations were measured by chemical analysis in subcellular fractions of gills compared to digestive gland. Increased activities of GST were found in gills after exposure and remained elevated during the depuration period whereas GST activity remained unaffected in the digestive gland. CAT activities showed no changes after Pb exposure neither in the gills nor in the digestive gland. We conclude that gill cells are major sites of uptake and accumulation for dissolved Pb and are involved in sequestration and detoxification of this metal in *Mytilus edulis*.

5.2 Introduction

Marine organisms are continuously exposed to heavy metals in their environment. Bivalve molluscs such as the blue mussel *Mytilus edulis* accumulate high levels of heavy metals and, therefore, are used as biological indicators of trace-metal pollution (Odzak et al. 1994).

Pb is a widely distributed heavy metal in marine areas with a high toxic potential to wildlife and, therefore, listed as a priority substance under the EU Water Framework Directive (2000/60/EC).

Pb is known to cause a variety of adverse health effects including immunosupression, neurotoxicity as well as reproductive and developmental defects. Subcellular sites for Pb toxicity are various structures including membranes, nuclei, mitochondria, and polyribosomes (Nash et al. 1981). Moreover, Pb activates cellular functions due to its calcium mimicking effect and inhibits a number of enzyme systems through its binding to sulphydryl (SH) groups of amino acids (De la Fuente et al. 2002).

Although the metabolism of metals (Cd, Cu, and Zn) in *Mytilus edulis* has been extensively studied, less is known about the cellular pathways and mechanisms of Pb toxicity in the marine mussel. In the present study, subcellular targets were identified and cellular detoxifying mechanisms measured in order to provide information on Pb distribution patterns and detoxification in cells of *Mytilus edulis*.

The gills and the digestive gland have been identified as the major organs for metal accumulation in mussels (Marshall and Talbot 1979, Marigomez et al. 2002, Dimitriadis et al. 2003). The gills exhibit various functions in aquatic organisms. They are the sites for respiratory gas exchange, osmoregulation, nutrient uptake and transport of food material (Gosling 1992, Auffret et al. 2003, Gomez-Mendikute et al. 2005). Dissolved and particulate metals may enter the gills by two mechanisms: 1) transport across membrane channels, or 2) endocytosis followed by incorporation into lysosomes or vesicles for subsequent excretion and transport via the blood. Additionally, transport of metals is described to occur with brown or pore cells (phagocytes) that can infiltrate various tissues (Marigomez et al. 2002).

The digestive gland of molluscs is primarily adapted to process particulate food (Auffret et al. 2003). Furthermore, it is involved in metabolic regulation, the mechanisms of immune defence and homeostatic regulation of the internal medium (calcium, hemolymphatic pH, cell volume, etc.), as well as in the processes of detoxification and elimination of xenobiotics (Marigomez et al 2002). The epithelia of the digestive diverticula are composed of two main cell types: The digestive cells and the basophilic cells. Digestive cells consist of a well-developed endo-lysosomal system for intracellular food digestion. In contrast, basophilic cells are mainly involved in extracellular digestion and have secretory function (Dimitriadis et al. 2003, Marigomez et. al. 2002)

The digestive gland tissue is known to be a major site of metal accumulation (Marigomez et al. 2002). Metals arrive either from the internal medium (via blood plasma or hemocytes) or from the external medium (via food particles) and can be taken up by endocytosis or phagocytosis into digestive cells. Digestive gland cells synthesize metallothioneins for metal binding and endocytose metal–protein complexes through the apical or the basal plasma membranes. Endocytotic vesicles subsequently fuse with primary lysosomes to form heterophagolysosomes in which the bio-

logical material is hydrolyzed (Marigomez et al. 2002). Heavy metals can be classified by their Lewis acidity as Class A (hard), Class B (soft) and borderline which indicates the form of bonding in their complexes (Duffus 2001). Most Class B metal cations such as Ag, Cd, Cu, Hg and Zn are partially available for the cell and partially bound to the undigested material that remains in the endo-lysosomal system. These metal cations may be finally eliminated from the cell via exocytosis of residual bodies (Marigomez et al. 2002). At the subcellular level, lysosomes of the digestive cells and gill epithelial cells are described as the main cell organelles for metal accumulation (e.g. Cd, Mn, Fe, V, Zn, Cu, Hg and Ag) (Marigomez et al. 2002, Dimitriadis et al. 2003). Borderline metals such as Pb can be found associated with both oxygen and sulphur donors in cells.

Metal binding to membrane functional proteins such as receptors, G proteins, signalizing pathways and ion channels has been described as an early response to exogenous agents. Precipitation of metals within the cell as insoluble salts and compartmentalization into lysosomes prevents further toxic effects inside of the cell (Giannaccini et al. 2004).

Exposure to metals has been shown to induce oxidative stress in marine molluscs through the formation of reactive oxygen species and lipid peroxidation (De Almeida 2004). To protect against oxidative stress the cell activates various antioxidative defence mechanisms. Glutathione S-transferases (GST) catalyze a wide variety of conjugation reactions of glutathione with xenobiotic compounds containing electrophilic centres. The enzyme catalase (CAT) is also part of the cellular defense system which acts against toxicity originating from active oxygen forms (Regoli 1995).

Localization of contaminants in tissues and cells is essential for understanding their mechanisms of toxicity. Detection of chemicals at the subcellular level allows the tracing of their cellular uptake, distribution and elimination pathways. In the present study, we analyzed the localization of Pb at the ultrastructural level with an antibody-based detection system for contaminants in major biotransformation tissues such as gills and digestive gland. The same detection technique was developed in a previous study (Einsporn and Koehler 2006, submitted) for the visualization of organic contaminants. The technique is very sensitive with a high amplification potential and allows the localization of the chemicals under study by electron microscopy. Moreover, distribution of Pb in subcellular fractions was analyzed and compared to the results of the electron microscopic investigations. Effects of Pb on activities of the conjugative enzyme GST and the oxidative stress enzyme CAT were investigated after metal exposure. Furthermore, we examined the cellular recovery potential after a depuration period following Pb exposure.

5.3   Methods

Exposure experiments

Mytilus edulis individuals were transfered from the Wadden Sea Station Sylt to the laboratory. Specimens ranging from 4.0-6.0 cm shell length were selected and maintained in aerated filtered sea water (FSW) at 12-14°C for up to 14 days until the start of the exposure experiments.

Mussels were exposed to Pb nitrate for up to 10 days in seawater containing 2.5 mg Pb nitrate and for further 10 days in clean sea water for recovery. Controls were maintained in FSW only. Sea water was changed every 24 hours with addition of contaminant concentration or sea water for the controls.

Sample processing for electron microscopic detection of Pb was done after 10 days of exposure. For subcellular fractionation, biochemical analyses of enzyme activities and chemical analysis of Pb content, samples of gills and digestive gland were taken after 10 days Pb exposure and after 10 days recovery time. Control samples were taken in parallel at the same time periods for the above described investigations.

Antibody-based detection of Pb at the ultrastructural level

For ultrastructural localisation of the heavy metal Pb we applied the preembedding technique and a signal amplification system based on Catalysed Signal Amplification (CSA) according to Koehler et al. (2000) and Gold-Substituted Silver intensified Peroxidase (GSSP) according to Van den Pol and Gorcs 1986. Tissues were sectioned with a tissue chopper (Linton Instrumentation, Norfolk, United Kingdom) in pieces of 0.5 x 0.5 mm and fixed in 2% paraformaldehyde (PA) and 0.1% glutaraldehyde (GA) for 2 hours at 4°C and in 2% PA over night. All washing steps between incubations were done with phosphate buffered saline (PBS) (pH 7.4). After washing, unspecific antibody binding sites were blocked with 1% bovine serum albumin, 0.2% fish gelatine and 0.05% saponin in PBS for 2 hours. Tissue sections were incubated with the monoclonal antibodies against the antigens (anti-Pb 1:1000 = Lot specific) at 4°C over night. The secondary detection step was performed with the CSA (Catalyzed Signal Amplification) system from DAKO-Cytomation (Glostrup, Denmark) for monoclonal mouse antibodies. The streptavidin-peroxidase incubation for 2 hours was followed by fixation in 1% GA in PBS for 30 min and diaminobenzidintetrahydrochlorid (DAB) incubation for 8 min. After washing in PBS to stop the DAB reaction the tissue sections were washed in 2% sodium acetate. Subsequently the sections were washed for 3 h in 10% sodium thioglycolate. All following washing steps were done with 2% sodium acetate. After washing sections were incubated with the freshly prepared silver solution

consisting of solution A (5% sodium carbonate), B (0.05% ammonium, 0.25% tungsten silicide, 0.05% silver nitrate) and C (37% formalin) (A:B:C = 1:1:0.004) for up to 8 min. The reaction was stopped with 1% acetic acid and samples were washed. The gold labelling was done with 2% gold chloride for 8 min. After washing and fixation with 3% sodium thiosulphate followed by a washing step, the sections were transferred to 0.1 M sodium cacodylate buffer (pH 7.4) and fixed in 2% GA in 0.1M sodium cacodylate buffer. After a post-fixation with 0.5% osmium tetroxide and 1.5% potassium ferricyanide in 0.1M sodium cacodylate buffer for 15 min, sections were washed several times in distilled water and dehydrated in 30, 50, 70, 80, 96 and 100% ethanol for 20 min followed by an Epon-embedding. Semi thin sections were cut with an ultracut microtome (Reichert-Jung, Wetzlar, Germany) dried in 40% acetone on slides, stained with 0.5% toluidine blue and examined by light microcopy to get an overview of tissue morphology. For electron microscopy ultrathin sections were cut with an ultracut microtome (Reichert-Jung, Wetzlar, Germany) and collected on formvar copper grids. Observations were made under a Zeiss EM109 electron microscope and images were taken with a digital analysis system (Soft Imaging System's *iTEM*).

Subcellular fractionation and analysis of protein

Digestive gland samples were pooled (~0.5 g / 5 ml) and homogenized in ice-cold homogenization buffer (containing 250 mM sucrose, 20 mM hepes, 1 mM EDTA, 1 mM of protease inhibitor Pefabloc) at pH 7.0. Tissues were homogenized using a rotor homogenizer (Ultra-Turrax, Janke & Kaukel KG, Staufen i. Breisgau, Germany) with two strokes at level 2. Subcellular fractions were obtained by differential centrifugation of the homogenate according to the protocol of Cajaraville et al. (1992) with some modifications. The homogenate consisting of pooled digestive glands from seven individuals was centrifuged at 700 x g for 10 min to remove gross cell debris and unbroken tissue fragments. A sample of 1 ml of the total tissue supernatant was collected and stored for determination of enzyme activities on ice. The rest of the obtained supernatant was centrifuged for 10 min at 1900 x g and the pellet was re-suspended in 2 ml homogenization buffer (HB) and stored as the heavy mitochondrial fraction (b) on ice. This crude fraction is composed mainly of nuclei, mitochondria, cell membranes and cell debris. The supernatant was centrifuged for 45 min at 39000 x g and the obtained pellet re-suspended in 2 ml HB and stored as the light mitochondrial fraction (d) on ice which contains lysosomes, mitochondria and peroxisomes. The supernatant was centrifuged for 60 min at 170000 x g. The obtained pellet was re-suspended in 2 ml HB to yield the microsomal fraction (g) and stored with the supernatant (cytosol fraction f) on ice until further analyses or at -80°C for longer storage times (maximal four weeks). The microsomal fraction contains small pieces of the plasma membrane, fragments of

Golgi apparatus, ribosomes and microsomes (disrupted endoplasmic reticulum forming closed vesicles). The cytoplasm (cytosol fraction) contains a large number of enzymes and other proteins.

Total protein contents were determined according to the Bradford method and with bovine serum albumin (BSA) as a standard using a commercial kit from Bio-Rad (Hercules, USA).

Chemical analysis

Pb accumulation in subcellular fractions of gills and digestive gland was measured by chemical analysis with gas chromatography/mass spectrometry by a laboratory for environmental analysis IBEN (Bremerhaven, Germany). Samples for lead analysis were dried, weighted and solubilised in nitrohydrochloric acid. Analysis was performed with AAS Varian (Darmstadt, Germany) using a graphite tube.

Biochemical analyses of enzyme activities

Enzyme activities of glutathione S-transferase and catalase were determined spectrophotometrically in a microtiter plate reader (Tecan, Crailsheim, Germany) at constant temperature ($20 \pm 1°C$). The actual extinction coefficients of the substrates were adjusted according to the path length of the solution in the well.

Glutathione S-transferase

Glutathione S-transferase (GST) (EC 2.5.1.18) activities were determined according to Habig et al. (1974) with a commercial GST assay kit (Cayman Chemical Company, Michigan, USA) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GST activity with CDNB was assayed at 340 nm ($\varepsilon = 9,6$ mM^{-1}cm^{-1}). The wells contained 150 µl assay buffer (100 mM potassium phosphate, 0.1% Triton X-100, pH 6.5), 20 µl glutathione (GSH) and 20 µl sample or blank (HB). The reaction was initiated by adding 10 µl of CDNB and the absorbance was read every minute for 10 min.

Catalase

Catalase (CAT) (EC1.11.1.6) activities were measured according to Johansson and Borg (1988) with a commercial catalase assay kit (Cayman Chemical Company, Michigan, USA) using 4-

amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. The assay utilizes the peroxidase function of CAT to measure the enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal H_2O_2 concentration. Purpald forms a bicyclic heterocycle with the produced formaldehyde, which changes from colourless to a purple colour upon oxidation. Final concentrations of formaldehyde standards ranged from 0-75 μM. Wells contained 100 μl assay buffer (diluted), 30 μl methanol and 20 μl sample or formaldehyde standards, respectively. The sample wells contained 100 μl assay buffer, 30 μl methanol, and 20 μl sample. The reaction was initiated by adding 20 μl H_2O_2 and incubated 20 min at RT. The reaction was terminated by adding 20 μl Purpald. After further 10 min of incubation on a shaker at RT, 10 μl potassium periodate were added to each well. After a further incubation of 5 min on a shaker at RT the absorbance was read at 540 nm.

Statistics

Differences between the groups were tested with the Mann-Whitney Rank Sum Test and as a Post hoc test for all pairwise multiple comparisons procedure the Dunn`s test. The criterion of significance was $p < 0.05$. Statistical analyses were performed using Sigma Stat 3.0 (SPSS).

5.4   Results

Ultrastructural detection of Pb

Immunolabelling with the Pb-antibody was found mainly at the granular content of large-sized lysosomal structures of the apical part of the gill epithelial cells and digestive cells. Figures 10-15 show positive immunolabelling after Pb exposure in gills and digestive gland tissue of *M. edulis*. Reaction product also appeared inside of the cell nucleus and in association to the nuclear membrane (figure 10). Cells of the digestive gland also showed Pb deposition in vesicles containing membrane residues (figure 11 and 12). Positive reaction product was observed close to vesicles at the microvilli surface of the gill epithelium suggesting a direct incorporation inside of lysosomal structures after endocytotic uptake of Pb across the cell membrane (figure 13 and 14). Furthermore, intense labelling for Pb was also found in granules of hemocytes of exposed mussels (figure 15).
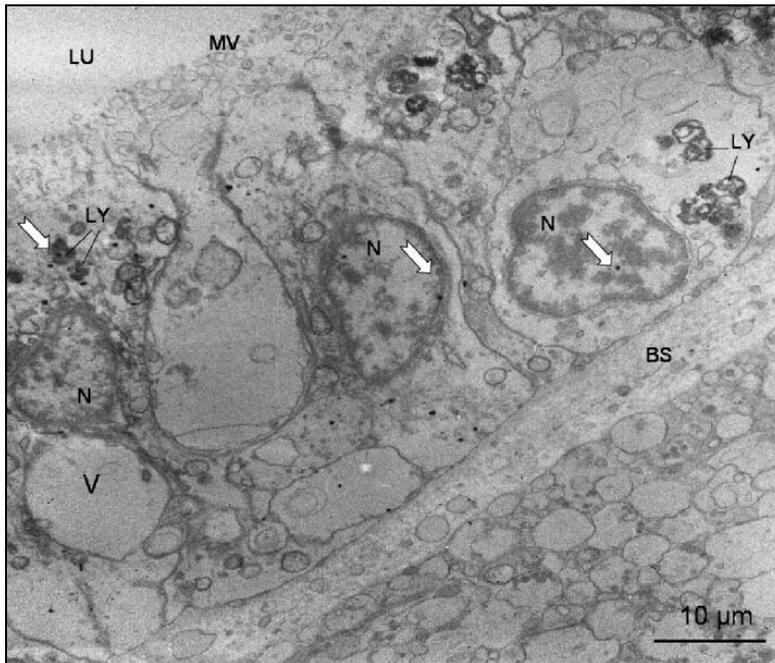
Fig. 10: Digestive epithelial cells of a mussel exposed to Pb.

Intense accumulation of Pb is noted in lysosomal structures (arrow). Moreover Pb appeared in cell nuclei and at the nuclear membrane (arrows). BS (basal lamina), LU (lumen), LY (lysosome), MV (microvilli), N (nucleus)
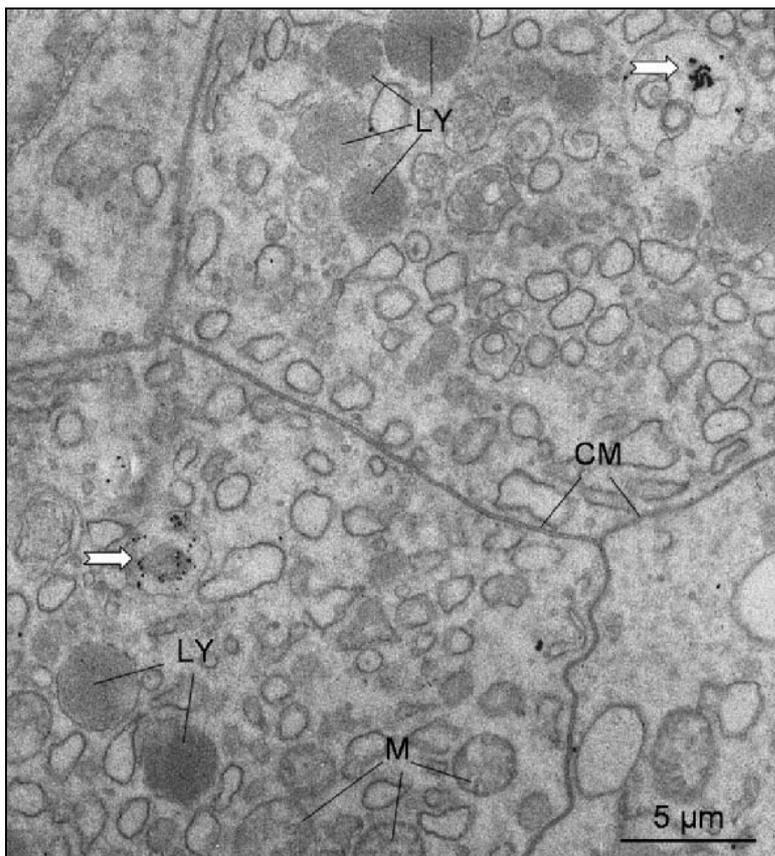
Fig. 11: Digestive gland cells of a mussel exposed to Pb.

Metal deposition is shown in multi-membrane vesicles (arrows). CM (cell membrane) LY (lysosome), M (mitochondria)
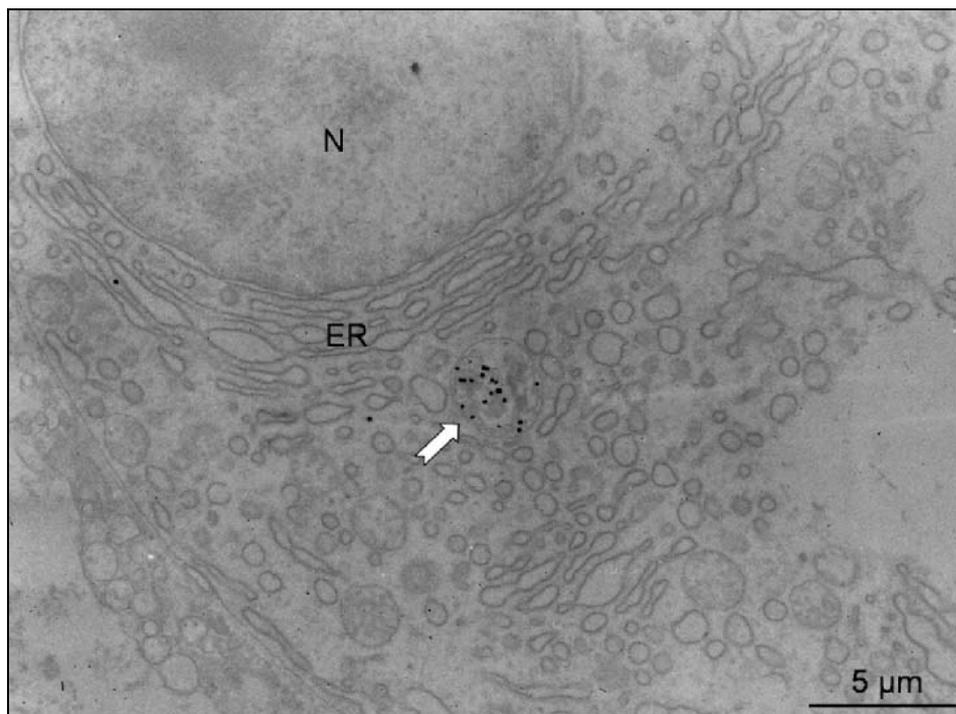
Fig. 12: Digestive gland cell of a mussel exposed to Pb.
Close to the nucleus a vesicle shows Pb deposition (arrow). ER (endoplasmic reticulum), N (nucleus)
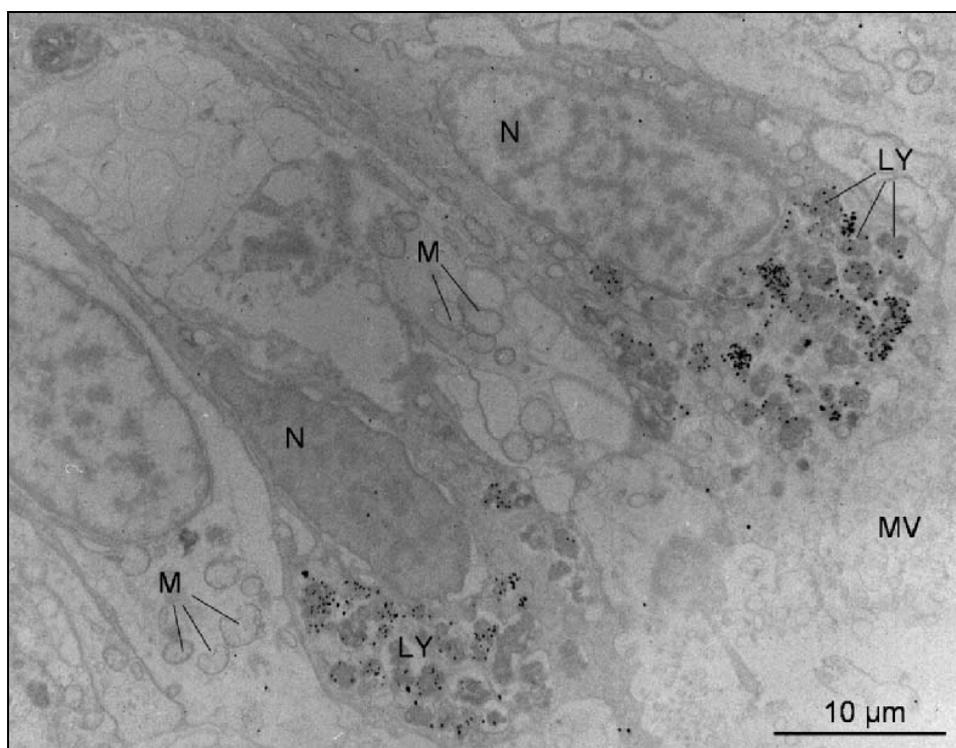
Fig. 13: Gill epithelium of a mussel exposed to Pb.
Epithelial cells of the postlateral zone show Pb accumulation inside of lysosomes which are located at the apical part of the cells. LY (lysosome), M (mitochondrion), MV (microvilli), N (nucleus)
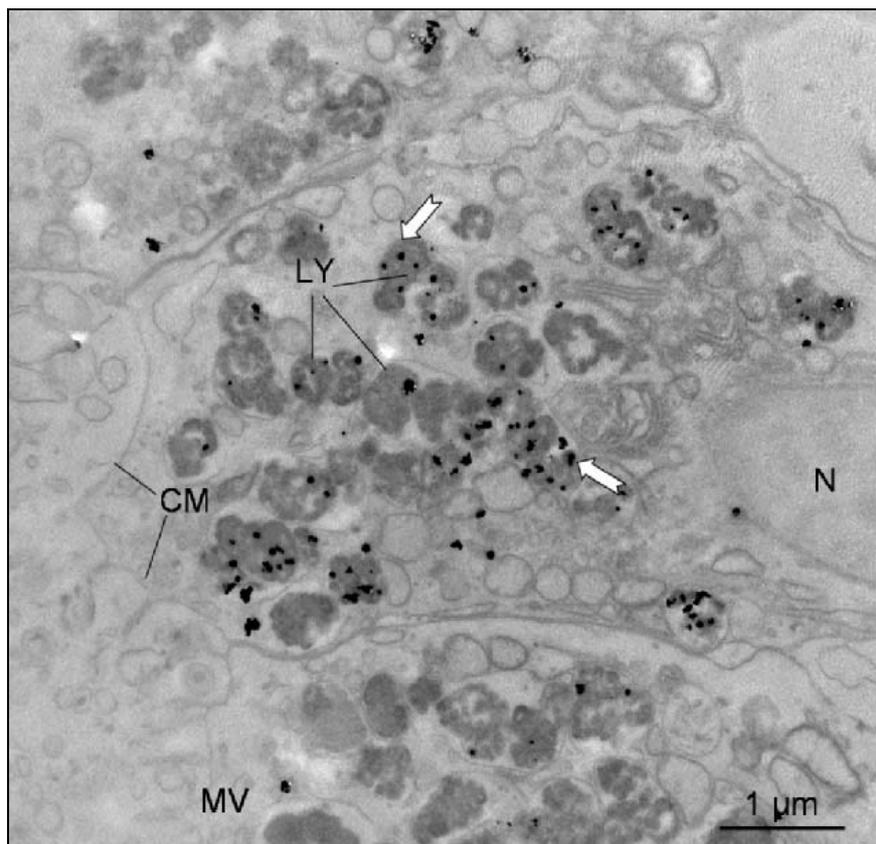
Fig. 14: Higher magnification of the gill epithelium of a Pb-exposed mussel.
Pb accumulation appears at electron-dense granular inclusions inside of lysosomes (arrows). CM (cell membrane), LY (lysosome), MV (microvilli), N (nucleus)
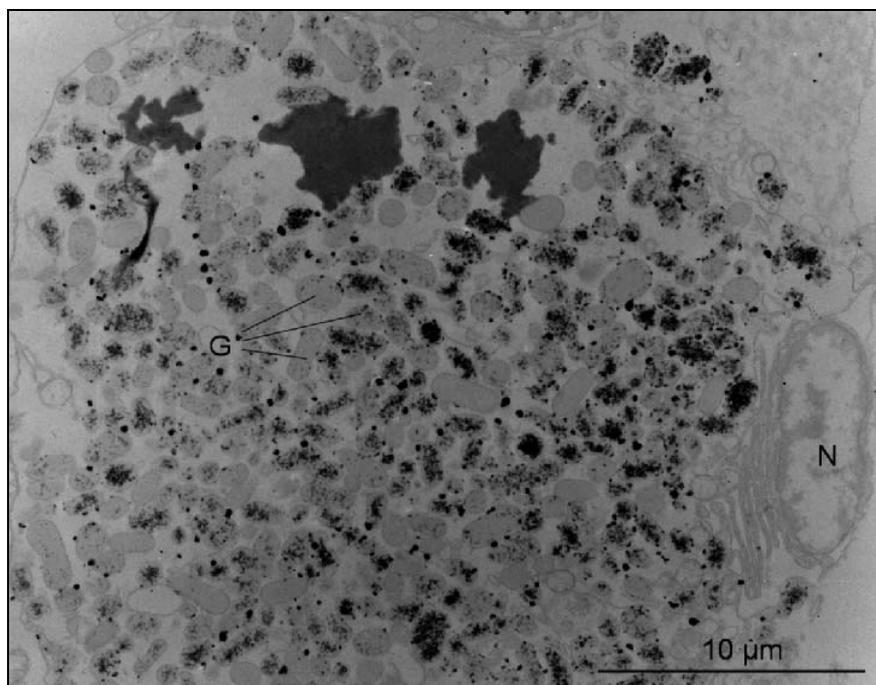
Fig. 15: Granular hemocyte of a Pb-exposed mussel.
Granules of the hemocyte show intense Pb deposition. G (granule), N (nucleus)

Detoxifying enzyme activities

GST activity was measured in gills and digestive gland tissue after 10 days exposure to Pb and again after ten days of recovery in clean sea water. Control mussels obtained clean sea water over the whole period. Figure 16 shows the results of the mean GST activity measured in both tissues after exposure and recovery. No significant differences in enzyme activities between Pb-exposed mussels and individuals sampled after depuration time were found for both digestive gland and gills ($P < 0.05$, N=4). Control mussels showed lower enzyme activities, but these were only significantly different in gills. Significant differences were observed for GST activity between gill and digestive gland tissue. Gill tissue showed higher levels of conjugative enzyme activities compared to digestive gland tissue.
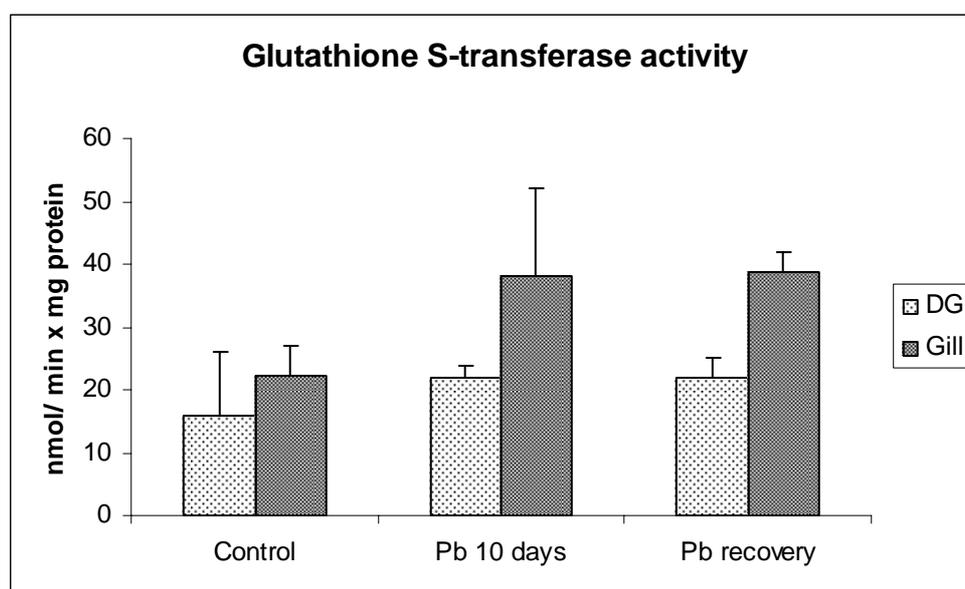
Fig. 16: GST activity in gills and digestive gland under control conditions, after 10 days Pb-exposure and after a recovery time of 10 days.

Catalase activity as a marker for oxidative stress in gill and digestive gland tissue was measured after 10 days exposure to Pb and again after ten days recovery in clean sea water. Figure 17 shows the results of the mean CAT activity measured in both tissues after exposure and recovery. Catalase activities showed similar levels for gills and digestive gland for all exposure conditions. No significant differences between exposed individuals, recovered mussels and controls were found ($P < 0.05$, N=4).
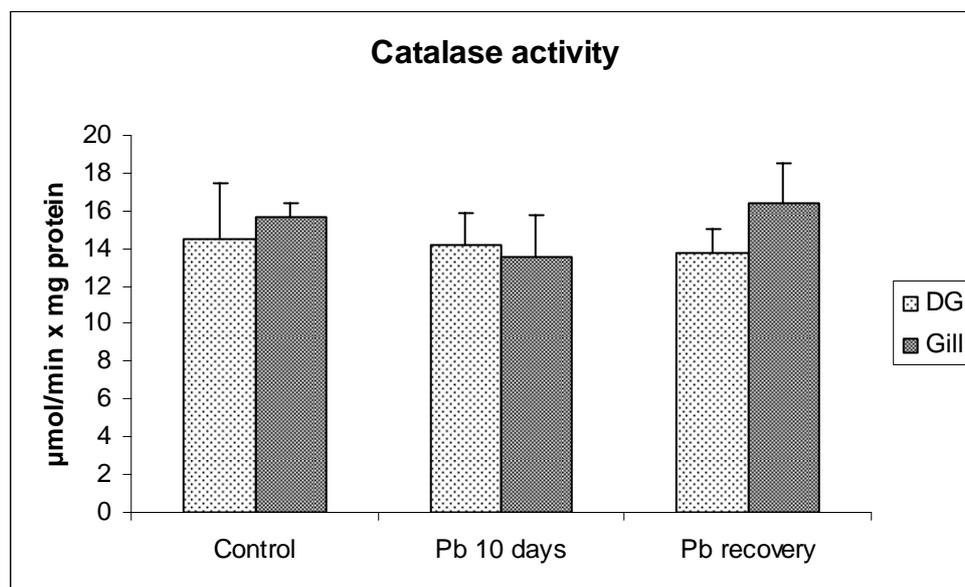
Fig. 17: CAT activity in gills and digestive gland under control conditions, after 10 days Pb-exposure and after a recovery time of 10 days.

Chemical analysis of Pb accumulation in subcellular fractions of gills and digestive gland after Pb exposure and recovery

Accumulation of Pb was higher in subcellular fractions of gills than of digestive gland after 10 days of Pb exposure (table 4). Highest amounts of Pb in the cytosol fraction were found for both digestive gland and gill tissue (f) with 117.04 mg/kg dw and 165.58 mg/kg dw respectively, followed by the heavy mitochondrial fraction (b, 67.39 mg/kg dw and 99.37 mg/kg dw respectively) and the light mitochondrial fraction (d, 34.88 mg/kg dw and 67.11 mg/kg dw respectively) after 10 days Pb contamination. The microsomal fractions (g) contained lowest levels for both tissues (9.93 mg/kg dw and 22.40 mg/kg dw respectively). After ten days of recovery in clean sea water from Pb exposure, Pb content decreased for all subcellular fractions of gills and digestive gland. Elimination of Pb was more efficient in subcellular fractions of gills than of digestive gland. Controls had lower Pb levels in all subcellular fractions of gills and digestive gland compared to the tissue fractions of mussels from both the Pb exposure and recovery study. Distribution of Pb between the fractions was similar for gills and digestive gland under all three conditions of treatment, with the gill fractions tending to contain higher Pb levels after exposure and depuration compared to the respective fractions of the digestive gland.

Tab. 4: **Pb accumulation in subcellular fractions of gills and digestive gland of controls, after 10 days Pb exposure and 10 days depuration**

Organ	Fraction	Control (mg/kg dw)	Pb exposure (mg/kg dw)	Depuration (mg/kg dw)
gills	b	0.193	99.370	73.766
gills	d	0.103	67.109	34.383
gills	f	0.266	165.580	66.552
gills	g	0.045	22.400	7.209
Digestive gland	b	0.808	67.385	57.305
Digestive gland	d	0.127	34.875	13.754
Digestive gland	f	0.619	117.043	55.428
Digestive gland	g	0.025	9.932	3.204

5.5 Discussion

Analysing the target sites of metals in tissues and cells can clarify their toxic effects and the underlying mechanisms of toxicity. Furthermore, it helps to interpret and understand different responses of detoxifying enzymes and antioxidative processes at specific tissue sites. Antibody-based detection techniques have been previously applied for the detection of organic contaminants at the light and electron microscopic level (Strandberg et al. 1998, Kerr Lobel and Davis 2002, Einsporn and Koehler 2006, submitted, Einsporn and Koehler 2006, in preparation). In the present study, antibodies against Pb were successfully used for the visualization of the metal at the subcellular level. Pb was observed in lysosomal structures in the direct vicinity of the brush-border site of the epithelial cells of gills and digestive gland in *Mytilus edulis*. Furthermore, we observed reaction product in association to microvilli, in numerous small vesicles distributed from the epithelial cells across the basal lamina to the proximal portion of the cells. Thus, an incorporation of the metal from the uptake site at the microvilli border into lysosomes or further transport to the basal lamina is likely to occur in cells of *Mytilus edulis*. Metal ions enter the cytosol either across membrane channels or by endocytosis (Mason and Simkiss 1983). They can be incorporated into lysosomes or transported in vesicles across the epithelial cells to be exocytosed basally into the blood and incorporated into circulating hemocytes. Granular hemocytes showed a strong positive immunolabelling in the present study, suggesting transport of soluble Pb be-

tween different tissues via the blood. Pb ions are known to compete with calcium ions and Pb may also enter the cell membrane via specific calcium binding proteins. Passive transport of Pb ions occurs with a concentration gradient maintained by the formation of complexes between Pb ions and other macromolecules within the cell (Nash et al. 1981).Those metal transport processes appear to be cell- and metal-specific (Mason and Simkiss 1983).

In pulse labelling experiments, Pb was also detected in endocytotic vesicles of epithelial cells in the gills of mussels, *Mytilus edulis* (Coombs and George 1978). Both ciliated and flattened cells of the gill epithelium are rich in cytoplasmic granules, which can be carbonate-, carbonate/phosphate- or phosphate-containing granules (Marigomez et al. 2002). Those granules are specifically involved in metal sequestration processes. Electron Probe Micro Analysis (EPMA) for Pb detection showed that Pb was localized in lysosomes combined mainly with Ca, S, and P and other minor trace elements. Nonessential metals appear to be stored transiently in gill epithelial cells. For example, in the marine gastropods (*Busycon canaliculatum, Littorina littorea, L. saxatilis*), Cu and Cd were localized in lysosomes of gill epithelial cells (Herwig et al. 1989). In the study of Marshall and Talbot (1979) Pb was also found in extracellular deposits in the basal lamina that forms the capillary walls of the gill lamellae in *Mytilus edulis*. All the above-mentioned studies showed a major role of the lysosomal granules of gill epithelial cells in uptake and sequestration of Pb. In the present study, water dissolved Pb seems to be affiliated to gill cells since it was detected in high amounts in lysosomes of gill epithelial cells. Chemical analysis of Pb accumulation also showed higher levels in gills than in digestive gland tissue fractions.

Subcellular distribution of Pb indicated highest Pb content in the cytosol fraction of both gill and digestive gland where the metal could be associated to enzymes or other proteins that are enriched in this fraction. In contrast, we detected Pb-labelling in relatively low amounts in the cytosol in the ultrastructural part of our study. Thus, accumulation of Pb in the cytosol fraction might also be an effect of disruption of lysosomes during homogenization and centrifugation processes which has been described by other authors (Livingstone et al. 1989). Accumulation of Pb in nuclei which was shown in the ultrastructural part of the present study might contribute to the relatively high amounts of Pb in the heavy mitochondrial fraction also containing the nuclei. Mitochondria which are also enriched in this fraction were identified as targets for Pb accumulation in a previous study (Einsporn and Koehler 2006, in preparation). Pb concentrations in the light mitochondrial fractions of gills and digestive gland confirm the ultrastructural results which show that lysosomes are involved in Pb sequestration.

During the experiments decrease of Pb concentrations was more pronounced in gills than in digestive tissue, suggesting a highly efficient elimination and detoxification potential of this organ.

Thus, in the present study the gills were the primary sites for uptake and elimination of dissolved Pb. This could be clearly shown in the ultrastructural study where Pb accumulated in epithelial cells and their lysosomal vacuolar system as well as by chemical analysis of Pb concentrations in gills.

In our study, Pb was observed in nuclei of the gill and digestive epithelial cells. Pb has a high affinity to bind to DNA and intranuclear accumulation, suggesting a carcinogenic potential of this metal. Pb is known to form complexes with phosphate groups of nucleotides and nucleic acid which leads to non-enzymatic hydrolysis of nucleoside triphosphates. Increments of chromosomal aberrations and malformation rates after Pb exposure have been reported in mammals such as hamsters and mice (Vallee and Ulmer 1972). However, nuclear accumulation of Pb in mussels has not been described until now to our knowledge. Though, in *Crassostrea gigas* the metal germanium has been detected in the nuclei of gill epithelial cells by electron probe micro analysis (EPMA) (Ballan-Dufrancais et al. 1997).

Biochemical analysis of GST activity, involved in metal detoxification processes, demonstrated higher activities in gills than in digestive gland homogenates. This corresponds to the higher accumulation of Pb in gill than in digestive tissue observed in the present study. Moreover, significant higher values of GST activities were found in the gill tissue of exposed mussels and these were still increased after a recovery period compared to the controls. In contrast, Pb treatment had no effects on GST activities in the digestive gland. Correspondingly, no effects in digestive gland tissue of *Perna perna* after Pb exposure were observed in the studies of Dafre et al. (2004) and De Almeida et al. (2004) whereas in a laboratory study with the metals copper and mercury an increase of GST activity was reported for both gills and digestive gland tissues of *Mytilus edulis* (Canesi et al. 1999). In a field and laboratory study with the metals As, Se, Mn, Fe, Pb, Cu, Zn, Cr and Ni no trend for GST activity was found either in the gills nor in the digestive gland of *Mytilus galloprovincialis* (Regoli and Principato 1995). Thus, GST activities in mussels seem to be affected differently by various metals. Moreover, enzyme activities are tissue dependent.

The oxidative stress marker enzyme CAT showed similar activities for both gills and digestive gland tissue as well as for exposed, recovered and control mussels in the present study. Similarly, in the study of Dafre et al. (2004) no effects of Pb treatment on enzyme activities of CAT were found in digestive gland tissue of *Perna perna*. In contrast, De Almeida et al. (2004) found increased activities of CAT in digestive gland of *Perna perna* after exposure to the metals Pb, Ag, Cd and Co. In gills of the mussel *Bathymodiolus azoricus*, exposed to the metal cadmium, oxidative stress and biotransformation enzyme activities indicated that the gills are first affected by metal

toxicity. Results revealed a decrease of CAT and superoxide dismutase activities (Company et al. 2006). Regoli and Principato (1995) reported an increase of CAT activity in gills and a slight decrease in digestive gland after exposure to a mixture of diverse metals (As, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn) in *Mytilus galloprovincialis*. Comparisons of various studies show that the response of CAT activity to metals is dependent on exposure times, metal concentrations, tissues, and mussel species. CAT activities are not affected in our study, probably due to effective metal detoxification and sequestration processes such as the observed incorporation of the Pb metal in lysosomes and rapid elimination processes, particularly in gills, protecting the cells against the generation of ROS.

Mussels are known to tolerate relatively high amounts of heavy metals and toxic effects are not apparently obvious until high accumulation levels are reached. It should be mentioned here, that the Pb concentration was not lethal despite the extremely high Pb tissue levels. Although, *Mytilus edulis* has developed an efficient detoxification and elimination system for Pb and can cope with high concentrations of Pb, the severe hazardous potential (carcinogenic?) of this metal in mussels was clearly shown by the immunocytochemical localization of Pb inside the cell nucleus in our study.

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CHAPTER 6

6 Cytopathologies and subcellular targets of the chemicals phenanthrene, aroclor 1254 and lead (Pb) in gills and the digestive gland of the mussel *Mytilus edulis*

6.1 Abstract

A cocktail of various classes of hazardous chemicals contaminate oceans and end up in marine organisms by bioaccumulation and biomagnification in the foodnet. In dependence of their chemical properties these pollutants impair animal health by various mechanisms and interactions of toxicity. Cell and tissue pathology of both gill and digestive tissue has been the object of many research studies for the elucidation of contaminant-induced biological effects. In the present study, cellular pathological alterations were linked to subcellular sites of chemical accumulation in gills and digestive gland tissues. For this purpose, mussels were exposed to the organic contaminants aroclor 1254 (PCB), phenanthrene (PAH) or the metal lead (Pb) in environmentally relevant concentrations. The localization of chemicals at the subcellular level was analysed with an antibody-based detection system by the use of commercially available antibodies specifically directed against the chemicals. Pathological changes were analysed in parallel in identical samples by transmission electron microscopy. After exposure to the different contaminants, cell organelles such as mitochondria, the endo-lysosomal system as well as the endoplasmic reticulum showed clear evidence of chemical-induced alterations. Large number of crystalline inclusions was found in mitochondria and autophagic lysosomes as well as multilamellated whorls after phenanthrene and aroclor 1254 exposure. Immunocytochemical detection of the chemicals showed that they accumulate inside of various cell organelles such as lysosomes, mitochondria, and nuclei. Additionally, chemicals were localized in association to membranes and microvilli of gill and digestive gland cells. Furthermore, the chitinous rod and mucous secretions of gill epithelial cells were positively labelled for contaminants indicating their role in protection. Localization of contaminants by immunodetection in combination with pathological diagnosis gives insights into the cellular targets of chemical attack.

6.2   Introduction

Marine invertebrates and bivalve molluscs in particular, accumulate high amounts of various environmental chemicals such as organic contaminants and heavy metals in specific cellular compartments for further metabolization and elimination. Light and electron microscopic diagnosis have been linked with histo- and cytochemical studies for the elucidation of contaminant-induced pathological effects in tissues and cells (Moore and Clarke 1982, Pipe and Moore 1986, Nott and Moore 1987, Sunila 1987, Livingstone et al. 2000), but direct evidence of interaction of chemicals with specific cell structures is still missing. These studies have been performed on mussels from different contaminated field sites or laboratory exposure experiments. Histopathology as a tool to monitor health effects of pollution in various species has been most intensively studied in the international Mussel Watch Program (Yevich and Barszcz 1983). Results from research and monitoring applications reveal that histopathology has an important role in marine pollution monitoring and shows possible carcinogenic effects of pollutants as well as reduced reproduction success (Weddeburn et al. 2000, Stentiford et al. 2003, Au 2004, Koehler 2004, Cajaraville et al. 2006). Histopathology integrates the effects of various classes of chemicals in field studies. In order to analyse specific responses to a single type of chemical exposure laboratory research studies are needed.

In general, histopathological changes can be observed at different levels a) at the tissue, b) cell and c) subcellular level. Histopathology of both gill and digestive gland tissue of Mytilidae has been the object of many field and laboratory studies and clearly reflected the hazardous potential of environmental contaminants in both organs (Nott et al. 1985, Pipe and Moore 1986, Sunila 1986, Sunila 1988, Ballan-Dufrancais et al. 1990, Domouhtsidou and Dimitriadis 2000, Livingstone et al. 2000, Dimitriadis et al. 2003). Indeed, direct linkages between subcellular localization sites of toxins and subsequent specific pathological changes have not been established in detail until now. In the present work, we applied both the ultrastructural immunolocalization of chemicals and the pathological diagnosis simultaneously to study subcellular contaminant-induced effects in gills and digestive gland tissue. This detailed analysis of responses allows the diagnosis of early stages of pollutant-induced cell injury in various tissues at the subcellular organization and permits the prediction of impact on the organ, the organism and finally, the population level (Koehler 1990). In our study, mussels were exposed to the organic contaminants aroclor 1254, phenanthrene or the metal lead and organs involved in uptake and detoxification have been examined for cytopathological lesions in relation to sites of chemical accumulation. Therefore, an immunocytochemical approach, developed in our laboratory to localize different environmental chemicals in tissues and cells to study subcellular localization sites and mechanisms of detoxifica-

tion, was applied in parallel to histopathological diagnosis of subcellular changes in the gills and digestive gland of the mussel *Mytilus edulis.*

The immunodetection system can be applied for the localization of various classes of contaminants by using specific antibodies for the respective toxins. In parallel, histopathological studies at the ultrastructural level can be conducted in the same sample to elucidate toxic effects of the detected contaminants in tissues and cells.

6.3 Materials and Methods

Conditions of maintenance and experimental procedures

Mussels, *Mytilus edulis*, were sampled at a relatively non-polluted site at the isle of Sylt (55°01'34 N, 08°26'45 E) and transferred to the laboratory. Specimens ranging from 4.0-6.0 cm shell length were selected and maintained in aerated filtered sea water (FSW) at 14 ±1 °C for 14 days to provide acclimatization to laboratory conditions until the start of the exposure experiments.

Afterwards, mussels were exposed via the seawater (one mussel per liter) to 150 μg/l phenanthrene (dissolved in acetone), 20 μg/l aroclor 1254 (dissolved in ethanol) or 2.5 mg lead nitrate for up to 10 days. Control series without the contaminants were carried out in seawater and with the solvent (acetone or ethanol). Seawater was changed every 24 hours and contaminants in the respective concentration or vehicle concentration for the controls were added. Mussels were fed every two days with a solution of zoo- and phytoplankton for marine invertebrates (Dohse Aquaristik, Gelsdorf, Germany) for 2 hours. After 10 days mussels were dissected and samples of gill and digestive tissue were taken for immunocytochemisty and cytopathological studies. For electron microscopy, gill and digestive tissue collected from three mussels from each treatment and tissue pieces for ultrastructural investigations were immediately prepared as described in the following chapter.

Antibodies

Monoclonal mouse anti-phenanthrene and mouse anti-PCB antibodies were purchased from Research Diagnostics Inc. (Flanders, NJ, USA) and mouse anti-lead from Biodesign International (Maine, USA). The Catalyzed Signal Amplification (CSA) system was obtained from DAKOCytomation (Glostrup, Denmark).

Ultrastructural immunocytochemistry with Gold-Substituted Silver-intensified peroxidase (GSSP) and cytopathology

For ultrastructural localisation of the organic contaminants and the metal lead we applied a preembedding technique and a signal amplification system based on Gold-Substituted Silver-intensified Peroxidase (GSSP) according to (Van den Pol and Gorcs 1986) and described in detail in a previous study (Einsporn and Koehler 2006, submitted). Tissues were sectioned with a tissue chopper (Linton Instrumentation, Norfolk, United Kingdom) in pieces of 0.5 x 0.5 mm and fixed in 2% paraformaldehyde (PA) and 0.1% glutaraldehyde (GA) for 2 hours at 4°C and in 2% PA over night. All washing steps between incubations were done with phosphate buffered saline (PBS) (pH 7.4). After washing, unspecific antibody binding sites were blocked with 1% bovine serum albumin, 0.2% fish gelatine and 0.05% saponin in PBS for 2 hours. Tissue sections were incubated with the monoclonal antibodies against the antigens (anti-phenanthrene 1:50 = 10 µg/ml, anti-PCB 1:50 = 25 µg/ml, anti-lead 1:1000 = Lot specific) at 4°C over night. The secondary detection step was performed with the CSA (Catalyzed Signal Amplification) system from DAKOCytomation (Glostrup, Denmark) for monoclonal mouse antibodies. The streptavin-peroxidase incubation for 2 hours was followed by fixation in 1% GA in PBS for 30 min and diaminobenzidintetrahydrochlorid (DAB) incubation for 8 min. After washing in PBS to stop the DAB reaction, the tissue sections were washed in 2% sodium acetate. The sections were washed for 3 h in 10% sodium thioglycolate. All following washing steps were performed with 2% sodium acetate. After rinsing, sections were incubated with the freshly prepared silver solution consisting of solution A (5% sodium carbonate), B (0.05% ammonium, 0.25% tungsten silicide, 0.05% silver nitrate) and C (37% formalin) (A:B:C = 1:1:0.004) up to 8 min. The reaction was stopped with 1% acetic acid and samples were washed. The gold labelling was done with 2% gold chloride for 8 min. After washing and fixation with 3% sodium thiosulphate followed by a washing step, the sections were transferred to 0.1 M sodium cacodylate buffer (pH 7.4) and fixed in 2% GA in 0.1 M sodium cacodylate buffer. After a post-fixation with 0.5% osmium tetroxide and 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 15 min sections were washed several times in distilled water and dehydrated in 30, 50, 70, 80, 96 and 100% ethanol for 20 min followed by Epon-embedding. Semi-thin sections were cut with an ultracut microtome, dried in 40% acetone on slides, stained with 0.5% toluidine blue and examined by light microcopy to get an overview of tissue morphology. For electron microscopy ultrathin sections were cut with an ultracut microtome (Reichert-Jung, Wetzlar, Germany) and collected on formvar copper grids. Visualization of chemicals and ultrastructural examinations were performed with a Zeiss EM109 electron microscope. From each mussel, five views were photographed using digital image analysis system (Soft Imaging System's iTEM, Muenster, Germany) and histopathological alterations

as well as contaminant distribution were analysed in a semi-quantitative manner, using a four-grade evaluation scheme.

6.4 Results

General gill morphology

The pair of gills (ctenidia) of *Mytilus edulis* comprises each two demibranchs and each demibranch in turn consist of two lamellae. Lamellae comprise ciliated filaments through which branchial blood vessels pass. Each filament has a frontal and an abfrontal zone (figure 18). The frontal zone consists of frontal and laterofrontal cells with well-developed cilia whereas the abfrontal cells have fewer and shorter abfrontal cilia. A branchial vein passes inside of the filament and a chitinous rod together with frontal and abfrontal muscles stabilizes the whole structure. Laterally the branchial vein is surrounded by small, nonciliated endothelia cells.
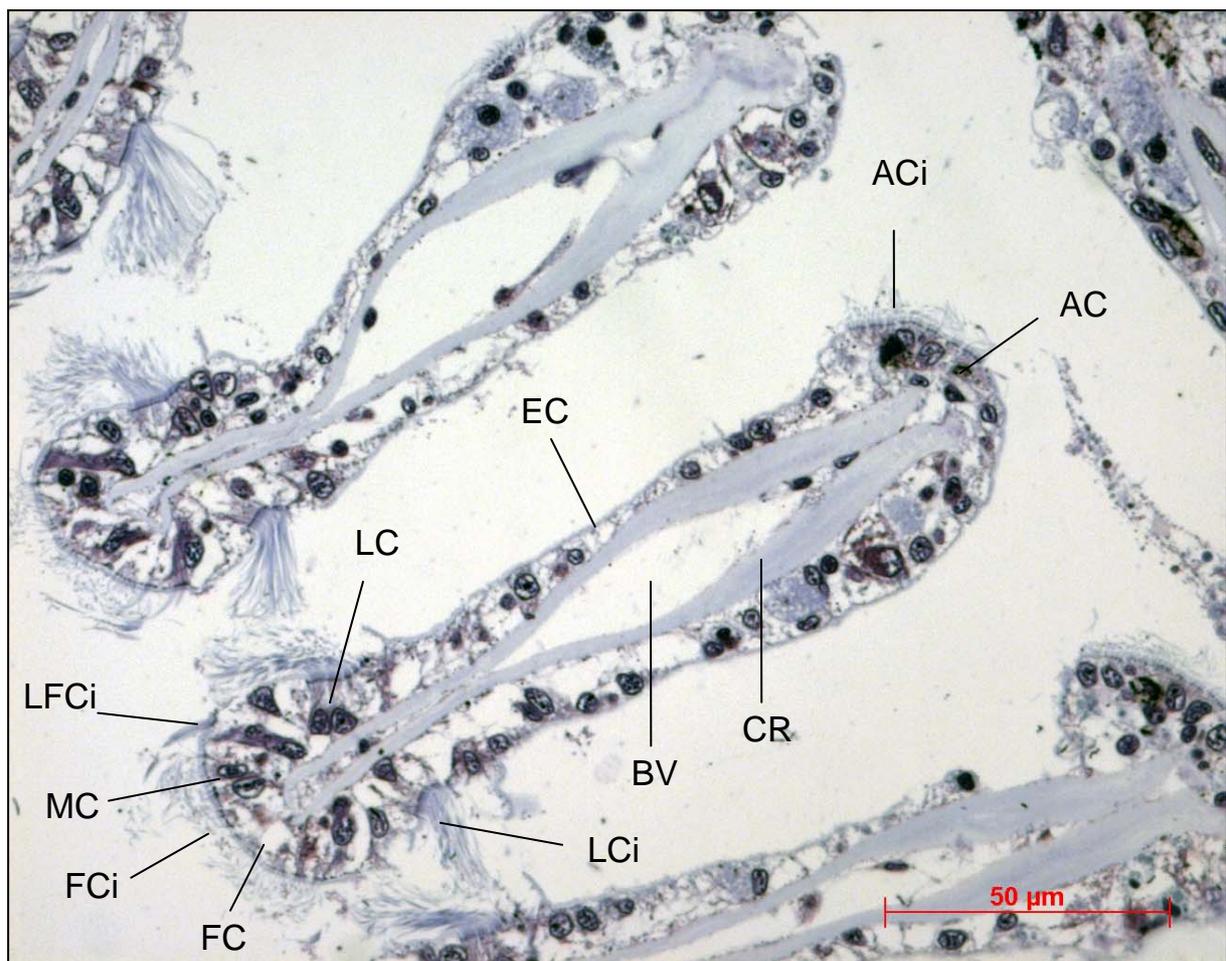
Fig. 18:   **General overview of filament structure of gills of the mussel *Mytilus edulis.***

Semi-thin section of Epon-embedded gill tissue was stained with toluidine blue. AC (abfrontal cells), ACi (abfrontal cilia), BV (branchial vein), CR (chitinous rod), EC (endothelia cells), FC (frontal cells), FCi (frontal cilia), LFCi (laterofrontal cilia), MC (mucous cells). Magnification x400

General digestive gland morphology

Food particles are transported from the stomach via the primary and secondary ducts to the digestive tubules (figure 19). The digestive tubule epithelium consists mainly of two cell types: Digestive cells and basophilic cells. Basophilic cells have a well-developed granular endoplasmic reticulum and a cup-shaped Golgi zone above the nucleus that produces membrane bound vesicles. Digestive cells are the sites where the intracellular digestion takes place and contain a well-developed lysosomal vacuolar system (Domouhtsidou and Dimitriadis 2000).
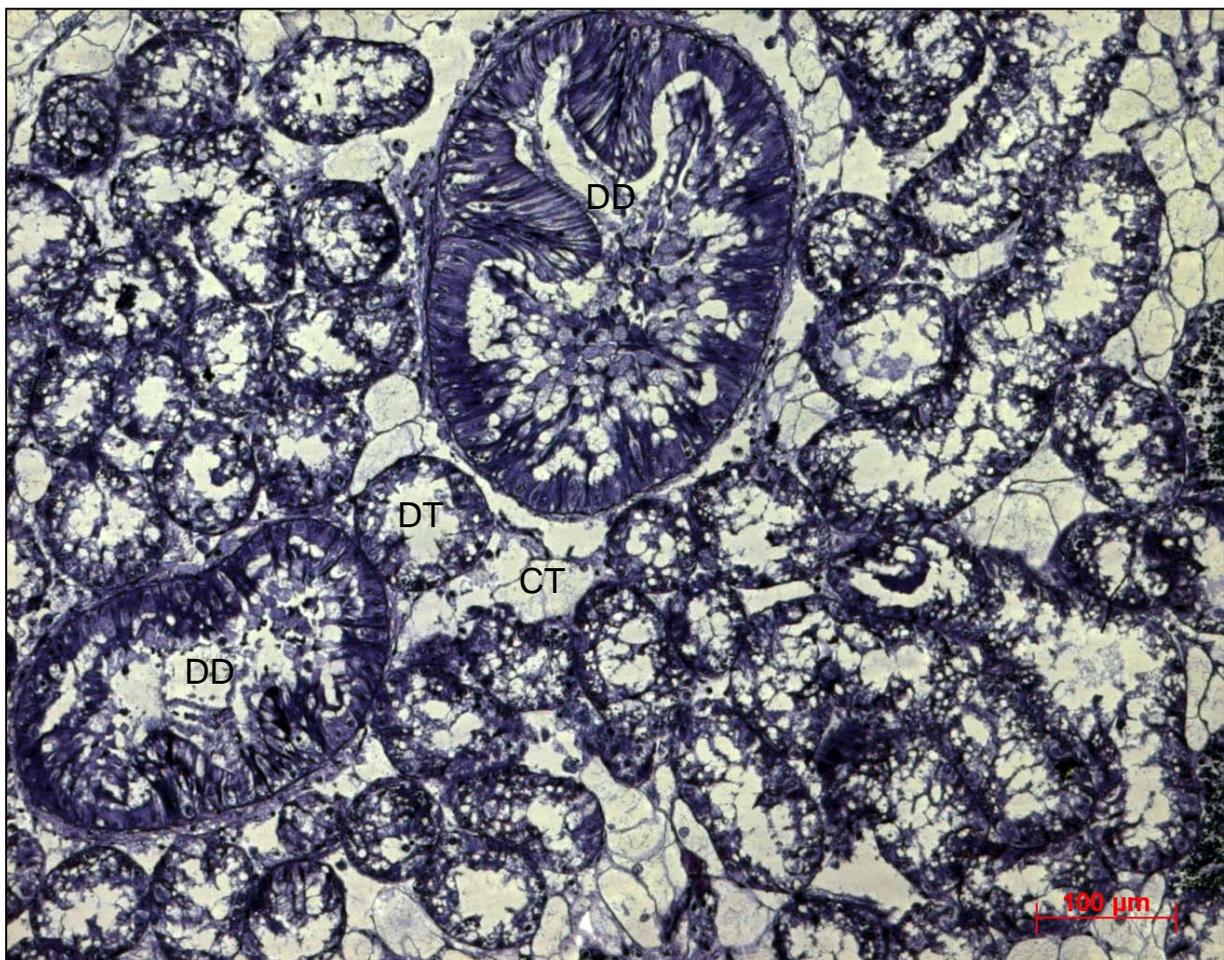
Fig. 19:   **Cross section of the digestive gland of *Mytilus edulis* showing digestive ducts and digestive tubules.**

Semi-thin section of Epon-embedded digestive gland tissue was stained with toluidine blue. DD (digestive duct), DT (digestive tubule), CT (connective tissue). Magnification x400

Subcellular localization of organic chemicals and the metal lead in gills and digestive tissue

Accumulation of contaminants was detected in association to various subcellular components of gills and digestive tissue (table 5). In intestinal tissue, aroclor 1254 was observed in epithelial cells of the digestive duct. There, the PCB mixture was detected in the mitochondria in the apical part (figure 23). The gills exhibited GSSP reaction product for PCBs in association to mucous secretions on the surface (figure 24). Furthermore, the chitinous rod of the gill filaments which consists of collagen and associated muscle fibers showed positive immunolabelling for aroclor 1254 (figure 25).

Immunolabelling for the lipophilic chemical phenanthrene was found in the endo-lysosomal system of the digestive cells. There, phenanthrene labelling was located centrally in vesicles inside of electron dense lysosomes of the digestive cells (figure 30).

Immunolabelling with the lead-antibody was observed mainly in lysosomes with granular contents of the apical part of the gill epithelial cells and digestive cells or in association to multi-lamellated aggregates (figure 39 and 40). Lead labelling also appeared inside the cell nucleus and at the nuclear membrane (figure 39 and 40). In summary, the results showed different accumulation sites for the lipophilic compounds phenanthrene and aroclor 1254 as well as for the heavy metal lead.

The endo-lysosomal system of the digestive cells including endocytotic vesicles which are all part of the cellular compartment involved in uptake, transport, and storage of foreign compounds are the sites where the lipophilic chemical phenanthrene was detected. Reaction product of the PCB mixture aroclor 1254 was observed inside of mitochondria of digestive duct cells and associated to mucous secretions and the chitinous rod of gill filaments. Lysosomes and smaller transport vesicles were labelled only in a few cases. Lead accumulation was mainly detected in granules inside of lysosomes of both gill and digestive epithelial cells. The endoplasmic reticulum and Golgi fields did not show any immunolabelling after exposure to the organic contaminants or the metal Pb.

Tab. 5: Subcellular detection of the organic contaminants aroclor 1254 and phenanthrene and the heavy metal lead in gills and digestive tissue of *Mytilus edulis* after laboratory exposure

Conta-minant	Organ	Microvilli	Chitinous rod	Mucous secretions	Mitochondria	Lysosomes	Nucleus	ER/ Golgi
PCB	Gills	-	+++	++	-	+	-	-/-
	Digestive system	+		-	++	+	-	-/-
PAH	Gills	+	-	+	-	+	-	-/-
	Digestive system	-		-	-	+++	-	-/-
Pb	Gills	+	-	+	+	+++	++	-/-
	Digestive system	+		-	-	+++	++	-/-

By using a four-grade examination scheme (-, +, ++, +++) the appearance and amount of immuno-labelled contaminants is evaluated.

Pathological ultrastructural changes after laboratory exposure to organic chemicals and metals in gills and digestive tissue

Whereas unexposed control mussels showed normally developed cell organelles such as ly-sosomes, mitochondria, and Golgi fields (figure 20-22) without any appearance of pathological alterations, several ultrastructural disturbances were observed after exposure to the different toxicants in gills and digestive tissue (table 6).

An increase of electron-dense vesicles and mucous granules was found in the gills after PCB exposure (figure 24 and 25). Mitochondria of digestive cells had abnormal crystalline inclusions and the number of cristae was reduced (figure 26). In general, subcellular mitochondrial alterations such as reduction of cristae, dissolution of the membrane and abnormal shapes were similar in digestive tissue and gills whereas crystalline inclusions were only observed in the digestive epithelium.

Additionally, the endo-lysosomal system of the digestive cells revealed severe changes. Digestive cells contained high amounts of residual bodies which aggregated. Autophagosomes containing cellular debris such as degenerated mitochondria or exogenous material accumulated in the digestive cells. Lipofuscin-like material was excreted by epithelial cells of digestive ducts (figure 27). The Golgi complexes were augmented and enlarged; increased numbers of primary and secondary lysosomes as well as large-sized multivesicular bodies were observed nearby (figure 28 and 29).

After exposure to phenanthrene, similar observations were made as for the PCB-exposed group, but subcellular alterations of the lysosomal system were even more striking. High amounts of autophagosomes, secondary lysosomes and residual bodies and formation of membrane whorls indicated an increased digestive activity directed against toxically injured cell components (figure 30-35). Large numbers of lysosomes and mitochondria contained crystalline inclusions (figure 31, 36 and 37) and mitochondrial cristae were reduced (figure 38).

After Pb exposure, epithelial cells of gills and digestive gland showed high amounts of secondary lysosomes containing electron-dense granular material (figure 39 and 40). Large numbers of residual bodies and autophagosomes accumulated in the digestive cells. In contrast to PCB- and PAH-exposed mussels, formation of crystalline structures in mitochondria and lysosomes did not appear after Pb treatment.

Deformation of the nuclear envelope was observed for the toxicants phenanthrene and Pb in both gill and digestive gland. After aroclor 1254 exposure digestive cells showed augmented active nucleoli.

Tab. 6: **Ultrastructural changes after laboratory exposure to aroclor 1254 (PCBs), phenanthrene (PAH) and the metal lead (Pb) in gills and digestive gland of _Mytilus edulis_**

		Lysosomes			Mitochondria		Golgi apparatus	ER	Nucleus	
Contaminant	Tissue	Proliferation of autophagosom es	Aggregations of residual bodies	Crystalline inclusions in lysosomes	Reduction of mitochondrial cristae	Crystalline inclusions in mitochondria	Augmentation of Golgi complexes	Formation of ER whorls	Deformation of nuclear envelope	Augmentation of nucleolus
PCB	Gills	-	-	-	++	-	-	+++	-	-
	Digestive epithelium	++	+++	-	++	+++	++	+	-	++
PAH	Gills	-	-	-	++	-	-	-	+	-
	Digestive epithelium	+++	+++	+++	++	+++	++	+++	+	-
Pb	Gills	-	-	-	+	-	-	-	++	-
	Digestive epithelium	++	+++	-	++	-	-	++	++	-

By using a four-grade examination scheme (-, type of reaction not observed; +, little developed; ++, moderately developed; +++, strongly developed) the status of a variety of subcellular parameters is evaluated.
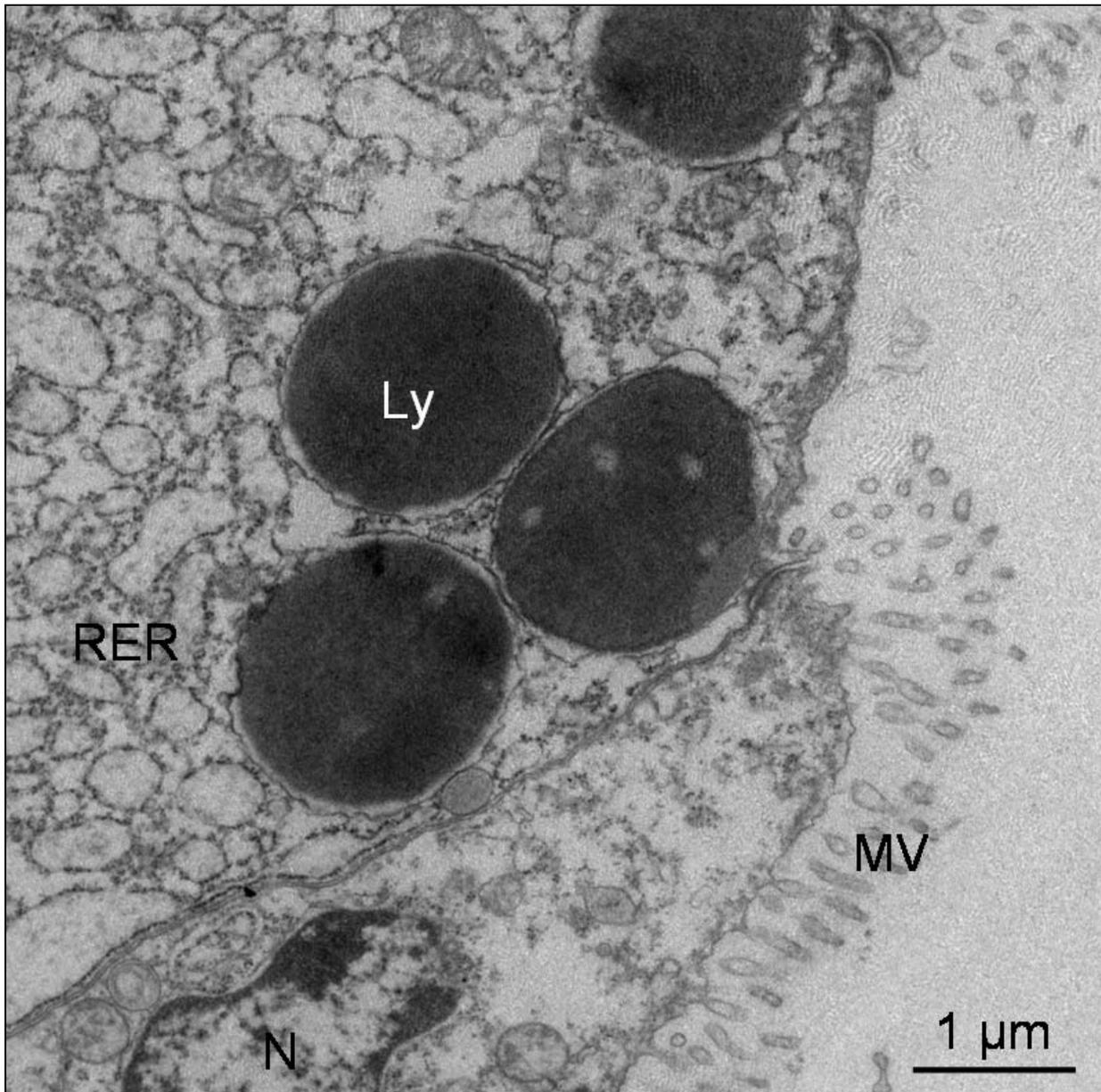
Fig. 20: **Lysosomes in the gill epithelium of an unexposed control mussel.**
LY, (lysosome), MV (microvilli), N (nucleus), RER (rough endoplasmic reticulum)
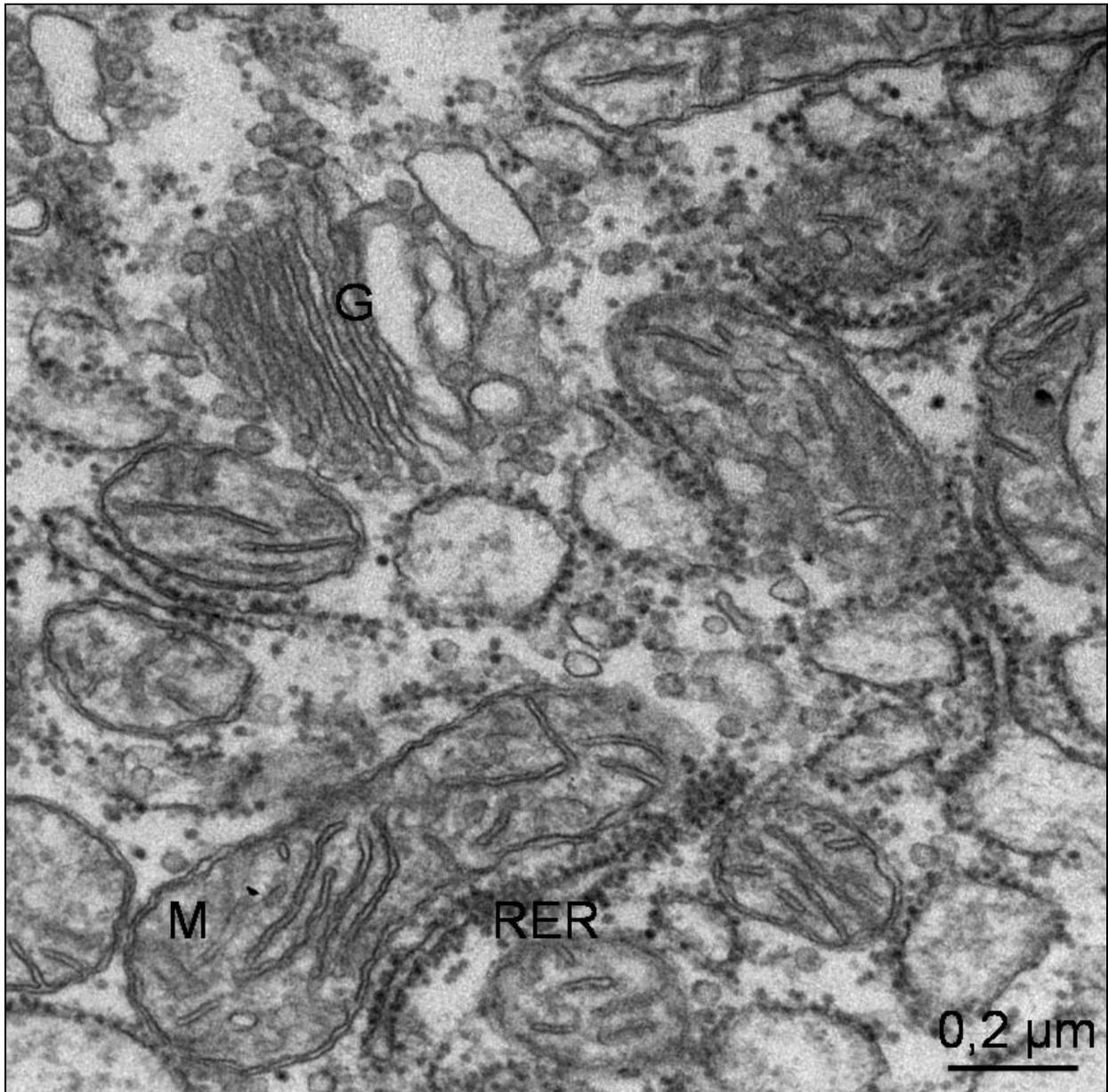
Fig. 21: **Mitochondria of the cristae type and a well-developed Golgi apparatus in the gill epithelium of an unexposed control mussel.**

G (Golgi apparatus), M (mitochondrion), RER (rough endoplasmic reticulum)
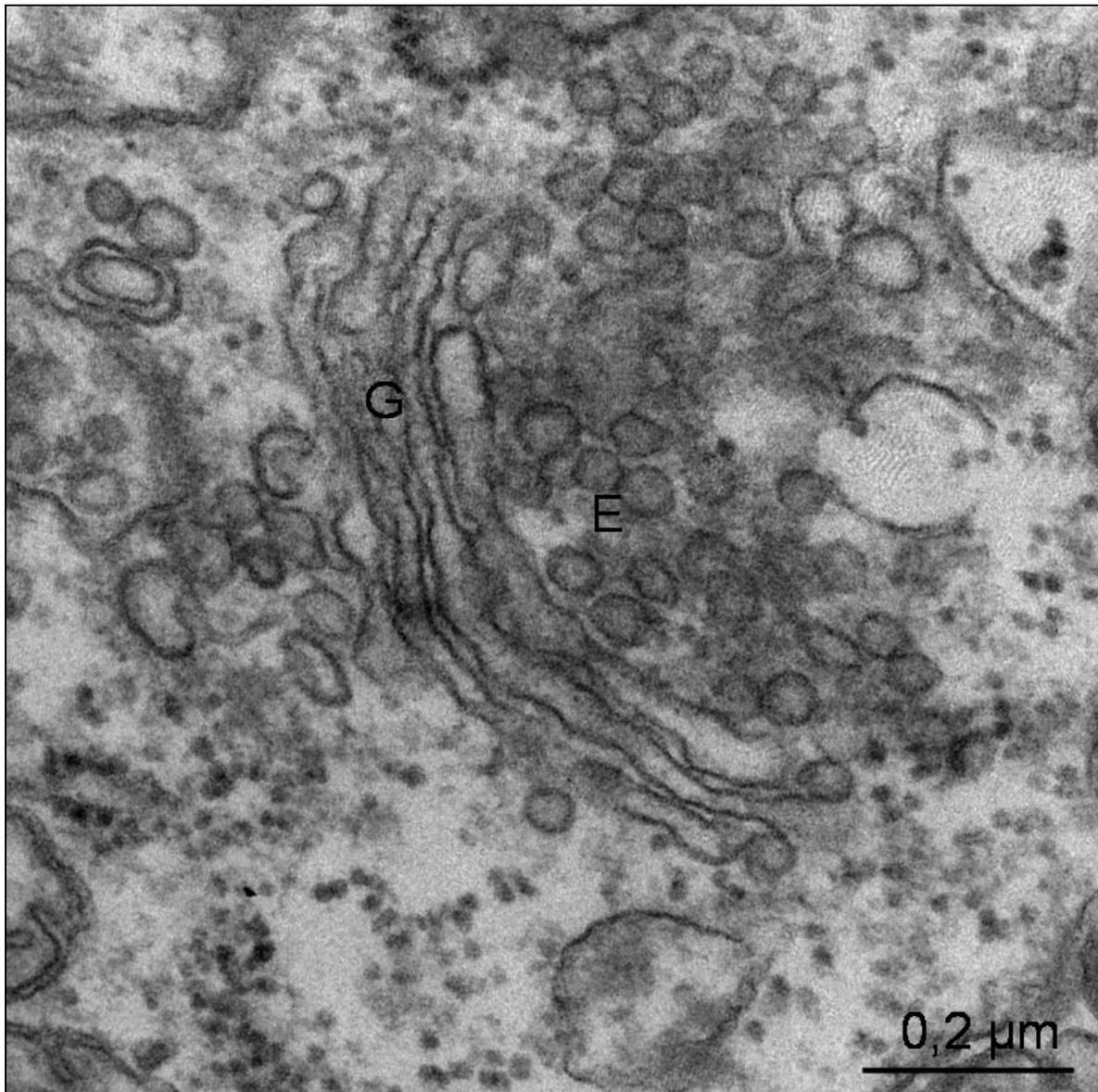
Fig. 22: Golgi apparatus producing early endosomes in the gill epithelium of an unexposed control mussel.
E (early endosomes), G (Golgi apparatus),
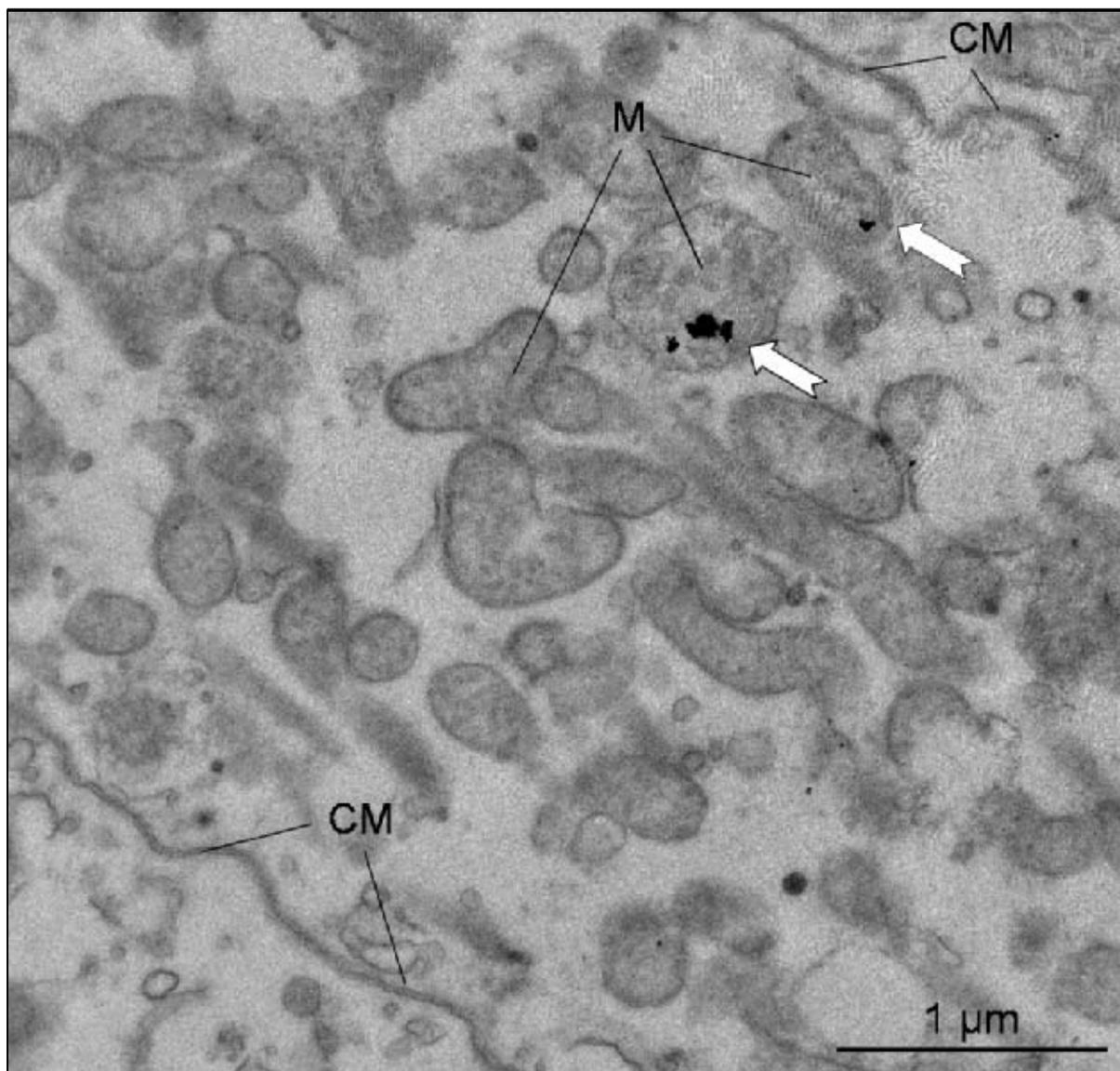
Fig. 23: Digestive duct epithelial cell of a mussel exposed to aroclor 1254 after treatment with the anti-PCB antibody.

Mitochondria show a high variability in size and shape, reduction of cristae and partly dissolution of the outer membrane. The PCB mixture is located inside of some mitochondria (arrows). CM (cell membrane), M (mitochondrion),
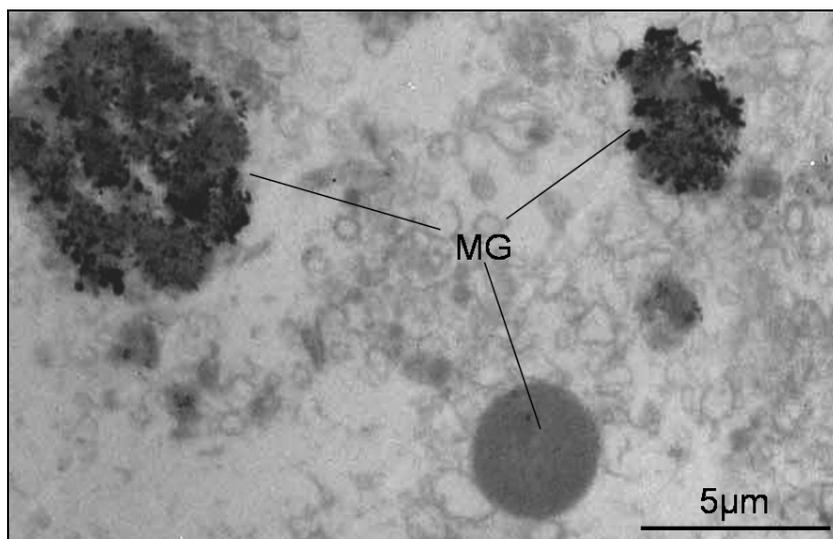
Fig. 24: Gill filament of a mussel exposed to aroclor 1254 after incubation with the anti-PCB antibody.
Some mucous granules show strong positive immunolabelling for aroclor 1254. MG (mucous granule)
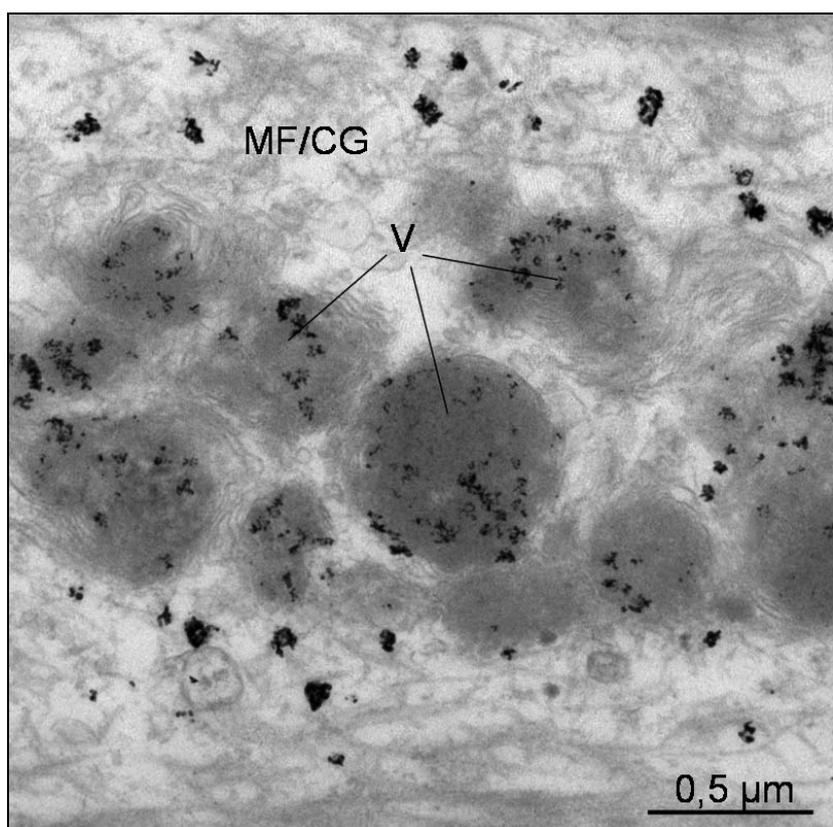
Fig. 25: Gill filament of a mussel exposed to aroclor 1254 after incubation with the anti-PCB antibody.
Dense vesicles show positive immunolabelling for PCBs. Immunolabelling also appears in association to collagen and muscle fibres of the chitinous rod. MF/CG (muscle fibres/collagen), V (vesicle)
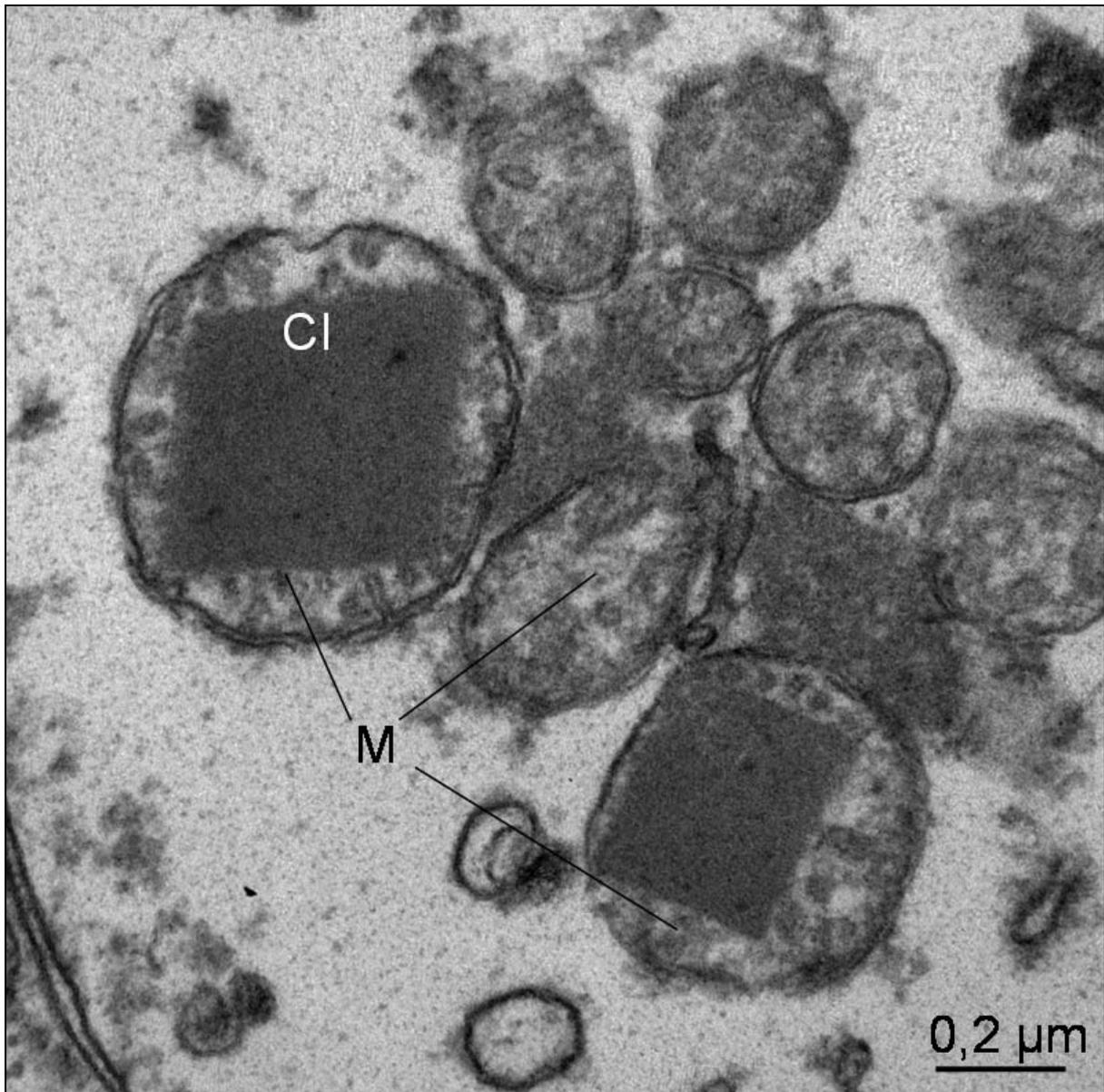
Fig. 26: **Formation of crystals inside of mitochondria of a digestive duct epithelial cell of a mussel exposed to aroclor 1254.**
Note the reduction of cristae inside of the mitochondria. CI (crystalline inclusion), M (mitochondrion),
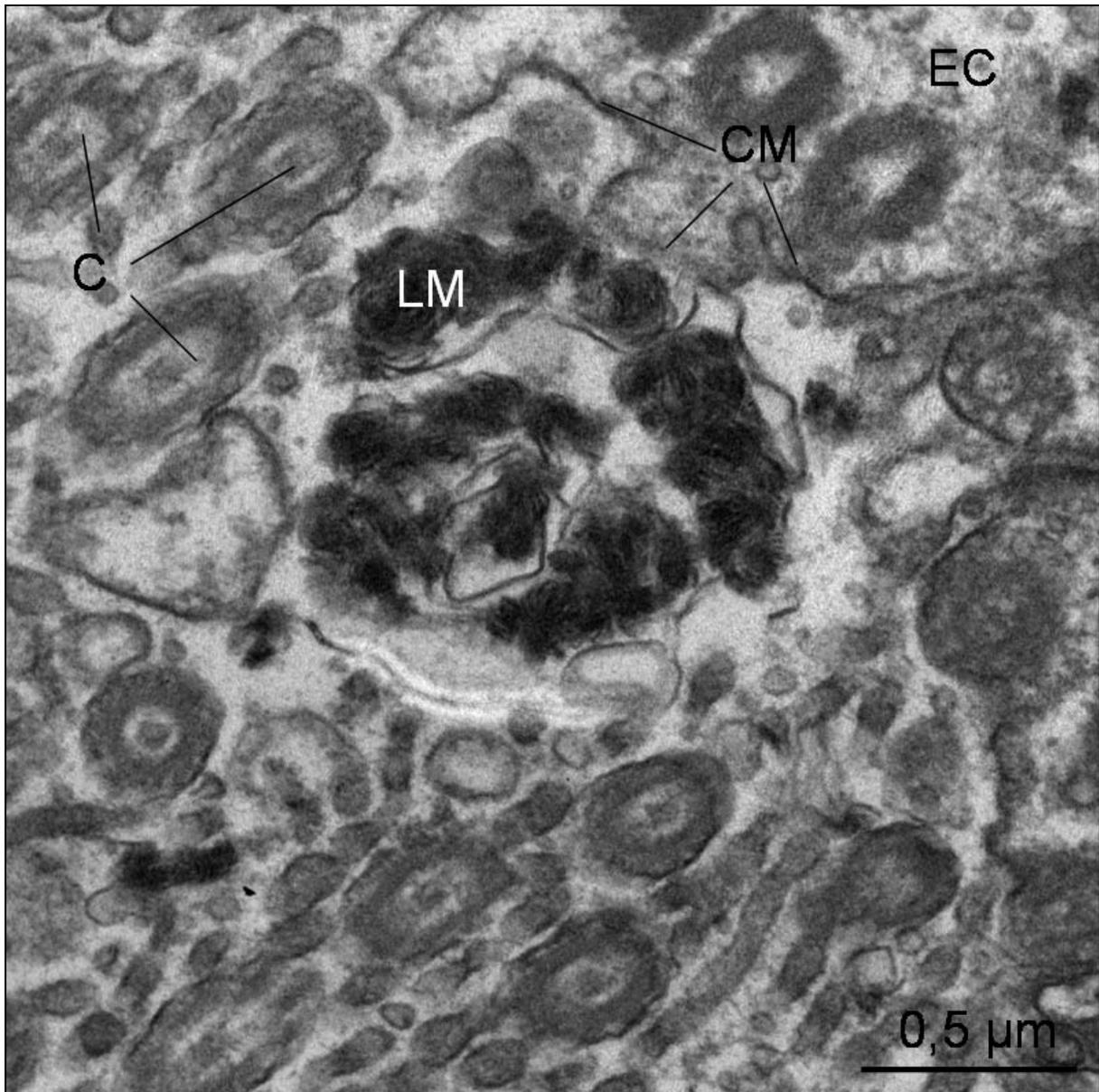
Fig. 27: Excretion of lipofuscin-related material from a digestive duct epithelial cell of a mussel exposed to aroclor 1254.

C (cilia), CM (cell membrane), EC (epithelial cell), LM (lipofuscin-like material)
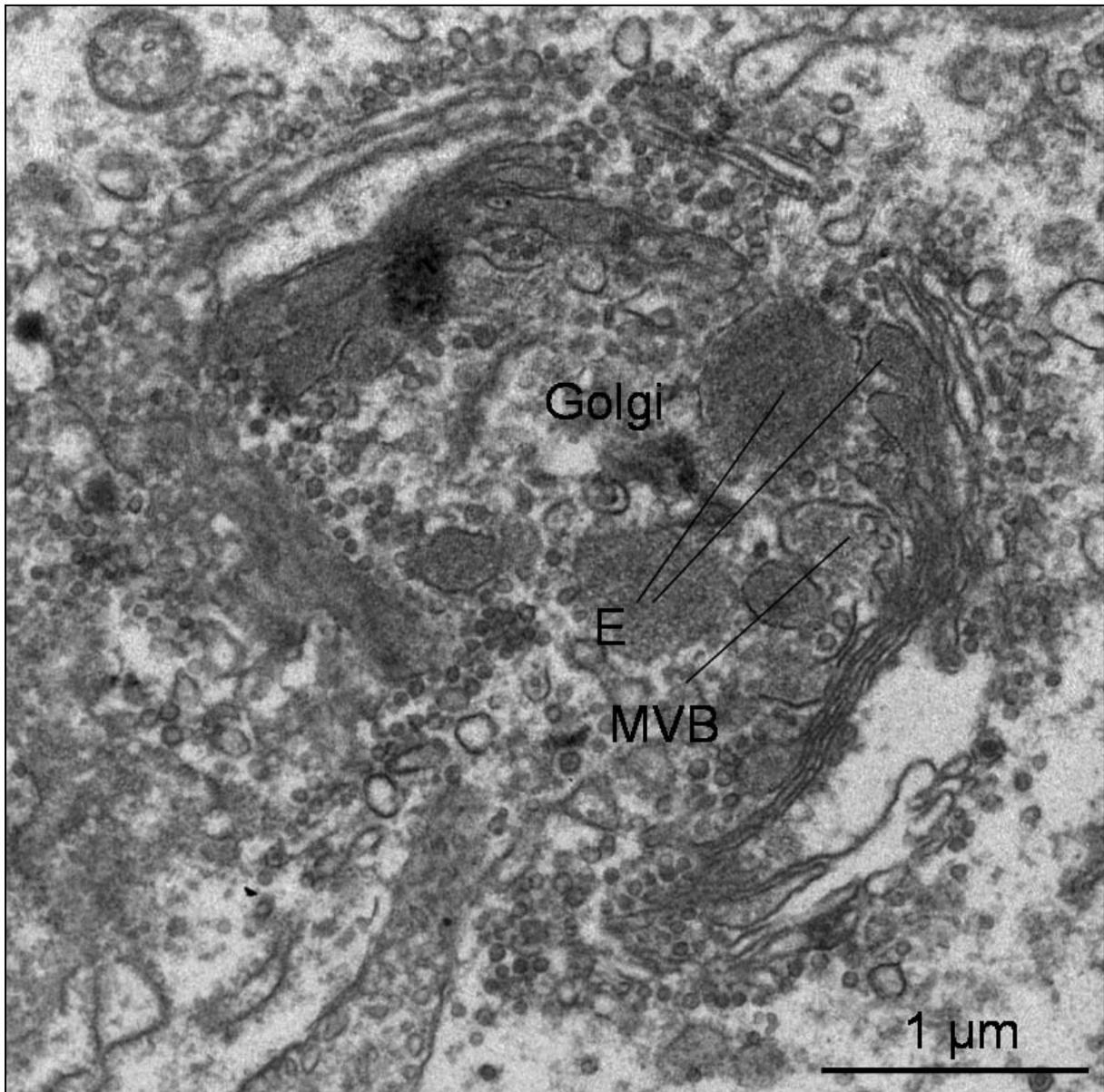
Fig. 28:   Enlarged Golgi complex of a digestive duct epithelial cell of a mussel exposed to aroclor 1254.
Note the formation of endosomes and multivesicular bodies. E (endosome), MVB (multivesicular body)
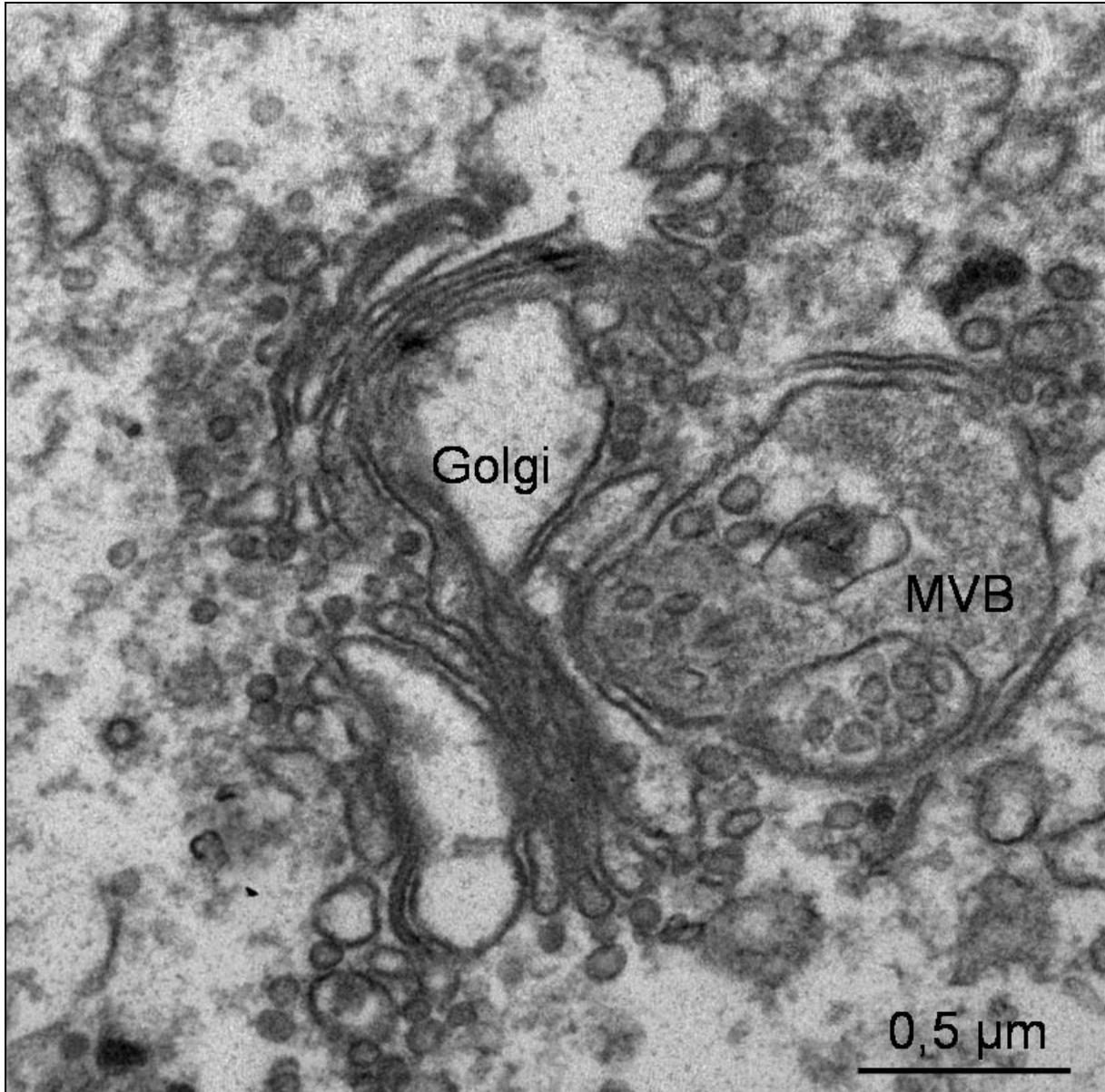
Fig. 29: Golgi apparatus with a large-sized multivesicular body of a digestive duct epithelial cell of a mussel exposed to aroclor 1254.
MVB (multivesicular body)
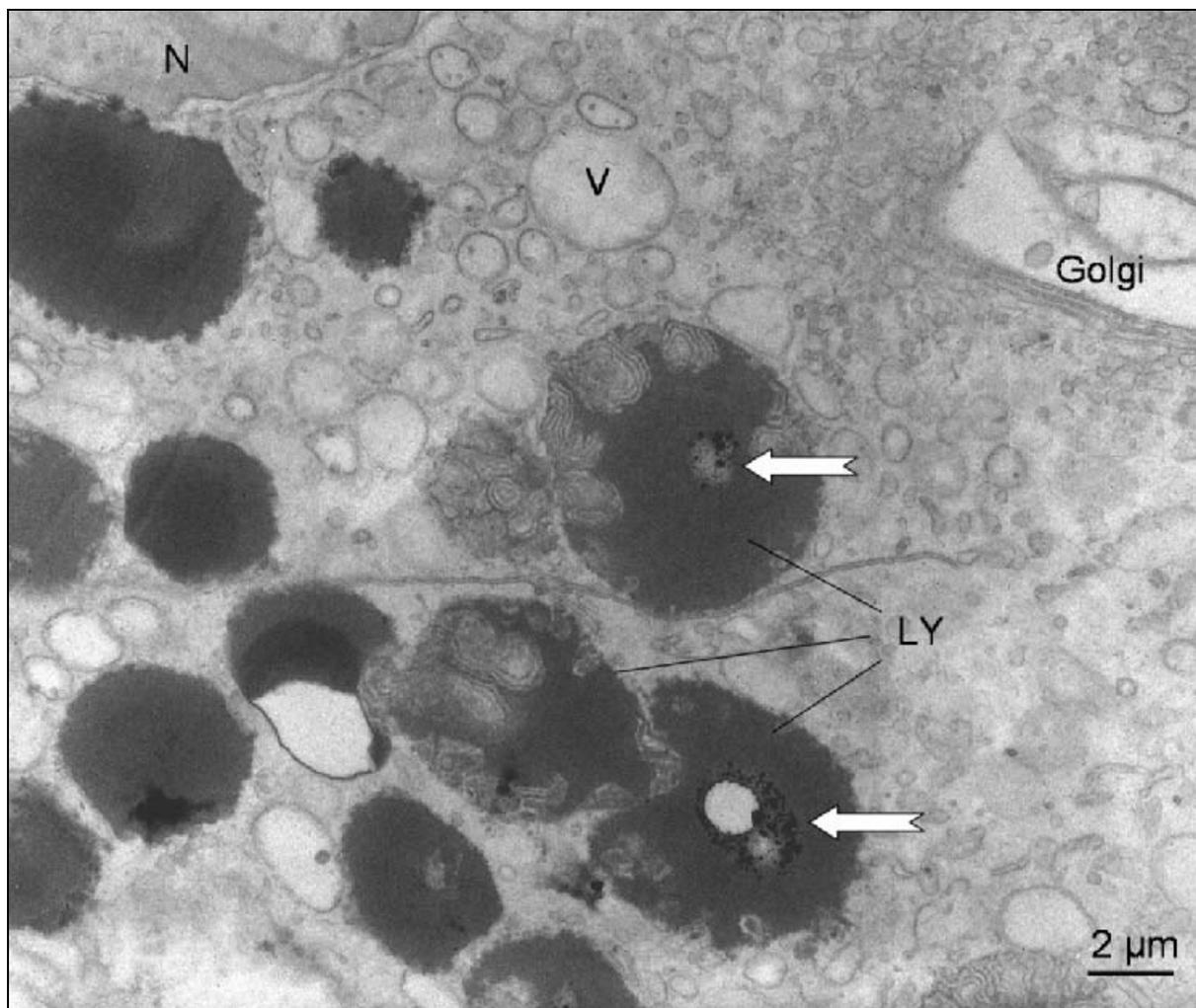
Fig. 30: Digestive gland of a mussel exposed to phenanthrene after incubation with the anti-PAH antibody.

Lysosomes of digestive cells contain multiple inclusions of membranous material and vesicles. Note the incorporation of membrane vesicles and fusion of lysosomes. Phenanthrene accumulated in association to centrally located vesicles inside the lysosomes (arrows). LY (lysosome), N (nucleus), V (vacuole)
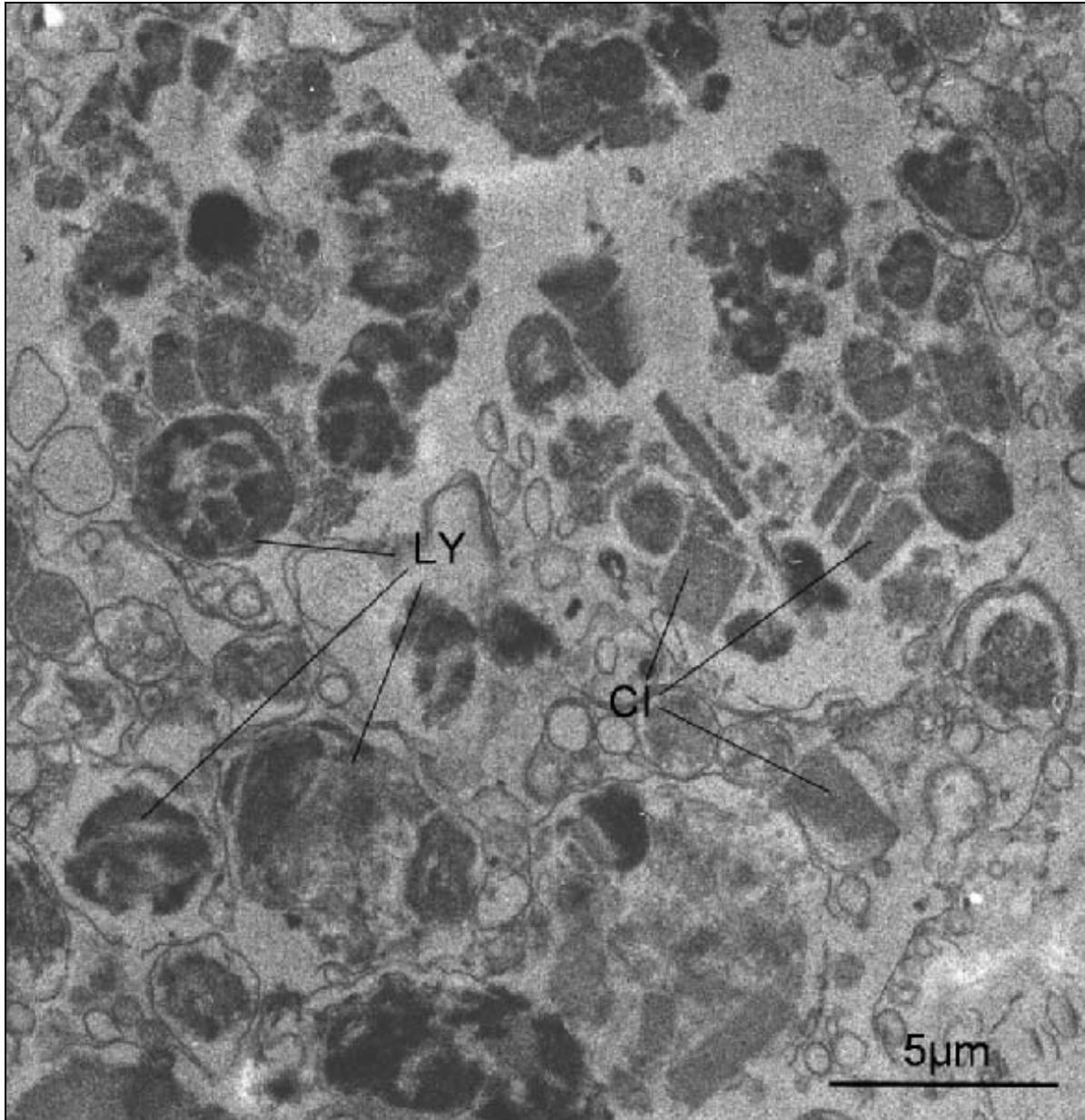
Fig. 31: **Aggregations of autophagic lysosomes and numerous crystalline inclusions of various shape in a digestive cell of a mussel exposed to phenanthrene**.
CI (crystalline inclusion), LY (lysosome),
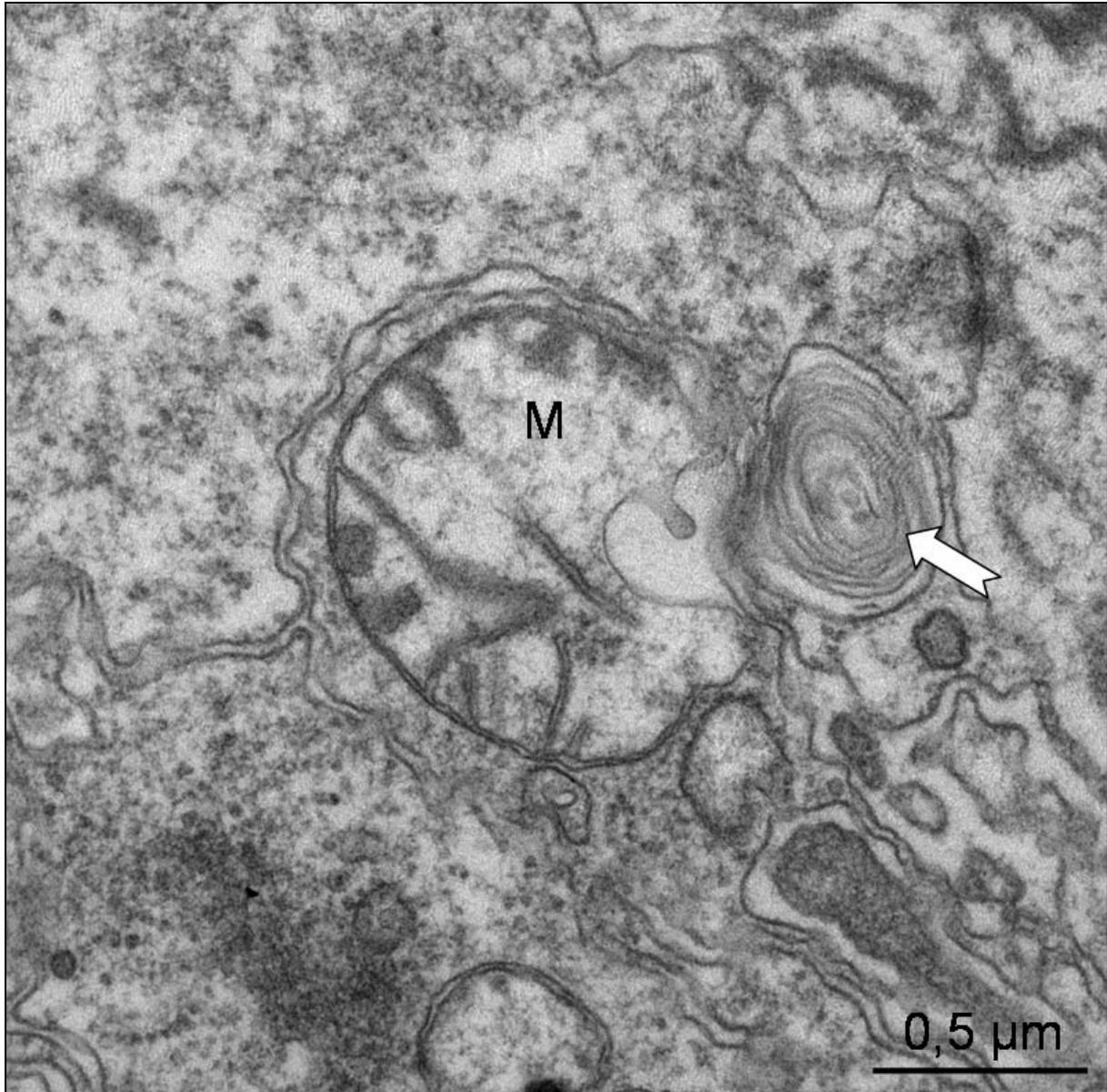
Fig. 32: **Degenerated mitochondrion in association to a vesicle containing membrane whorls in a digestive cell of a mussel exposed to phenanthrene.**
M (mitochondrion), arrow = membrane whorls
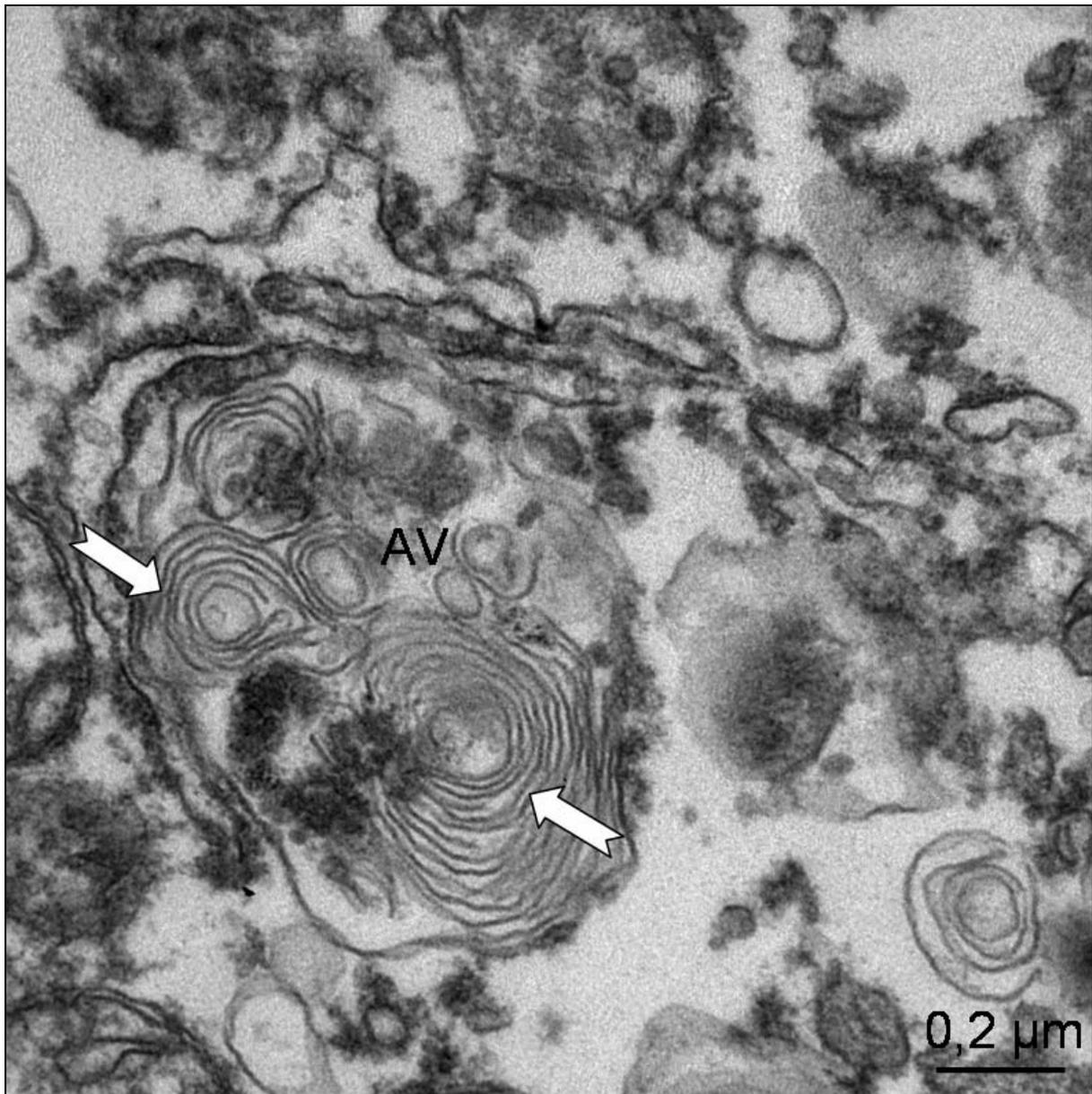
Fig. 33: Autophagic vacuole containing membrane whorls in a digestive cell of a mussel exposed to phenanthrene.
AV (autophagic vacuole), arrows = membrane whorls
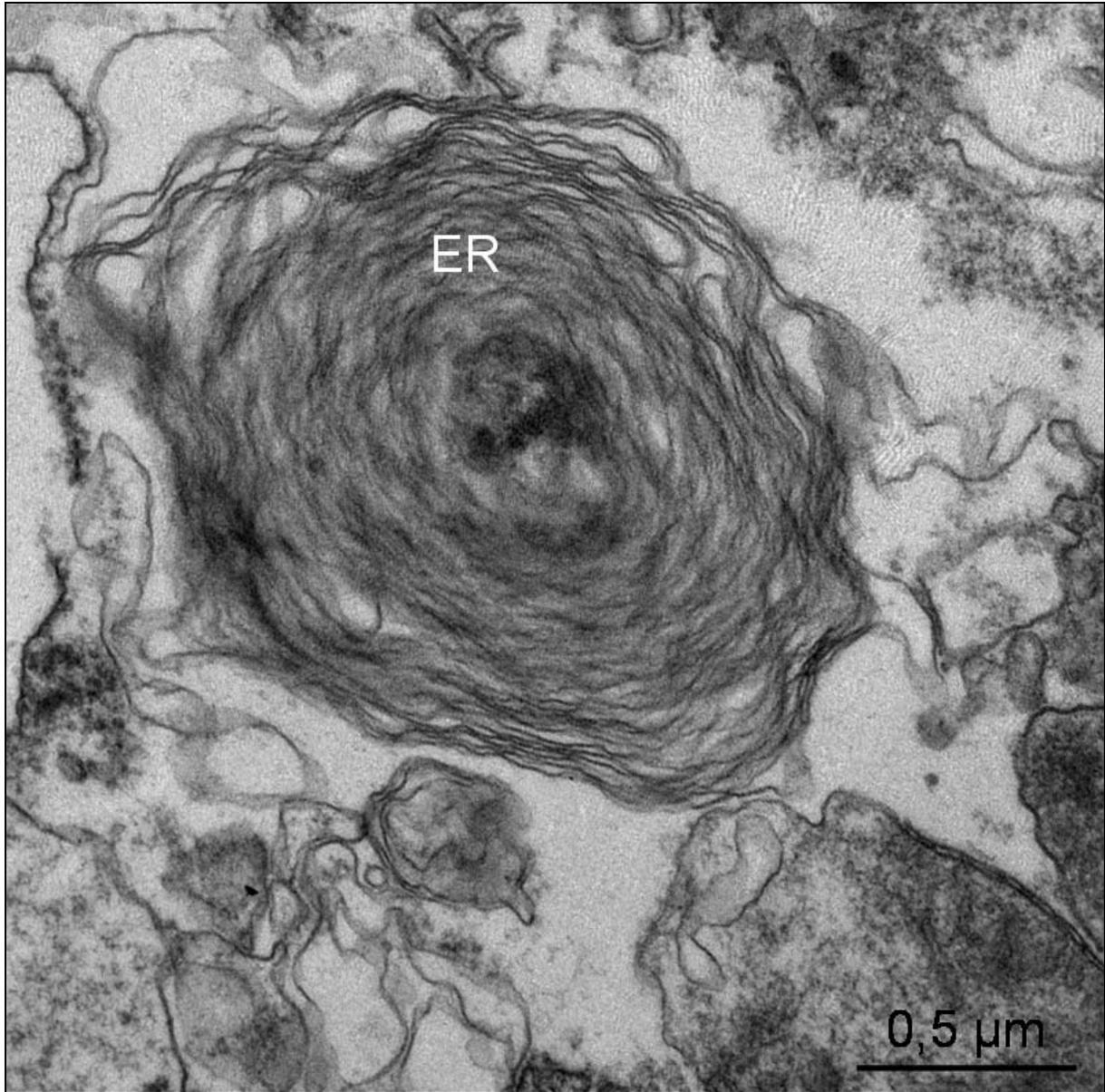
Fig. 34: Multi-lamellated aggregate of degenerated ER in a digestive cell of a mussel exposed to phenanthrene.
ER (endoplasmic reticulum)
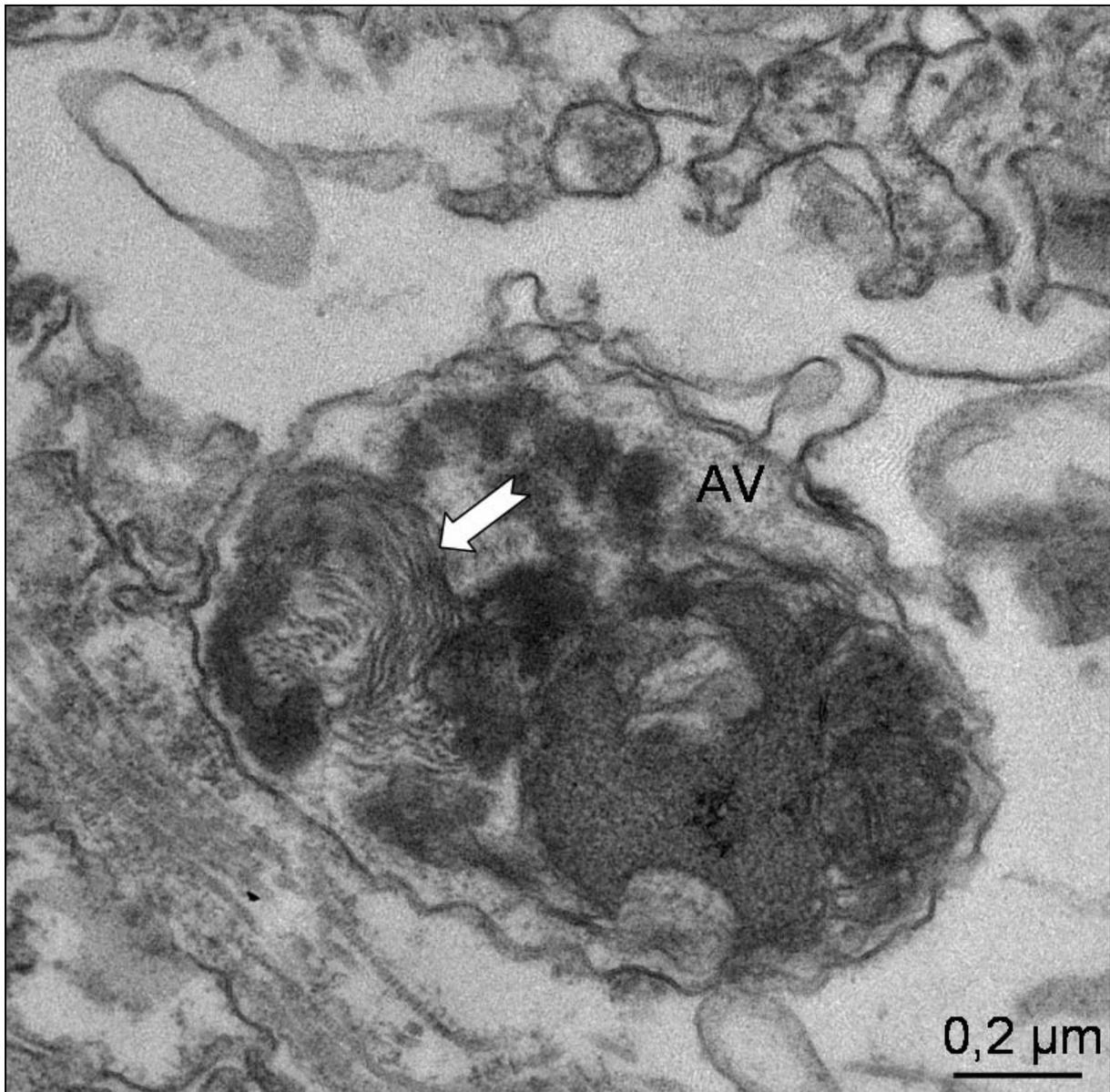
Fig. 35:   Autophagic vacuole containing inclusions of cellular debris in a digestive cell of a mussel exposed to phenanthrene.
AV (autophagic vacuole), arrow = membrane whorls
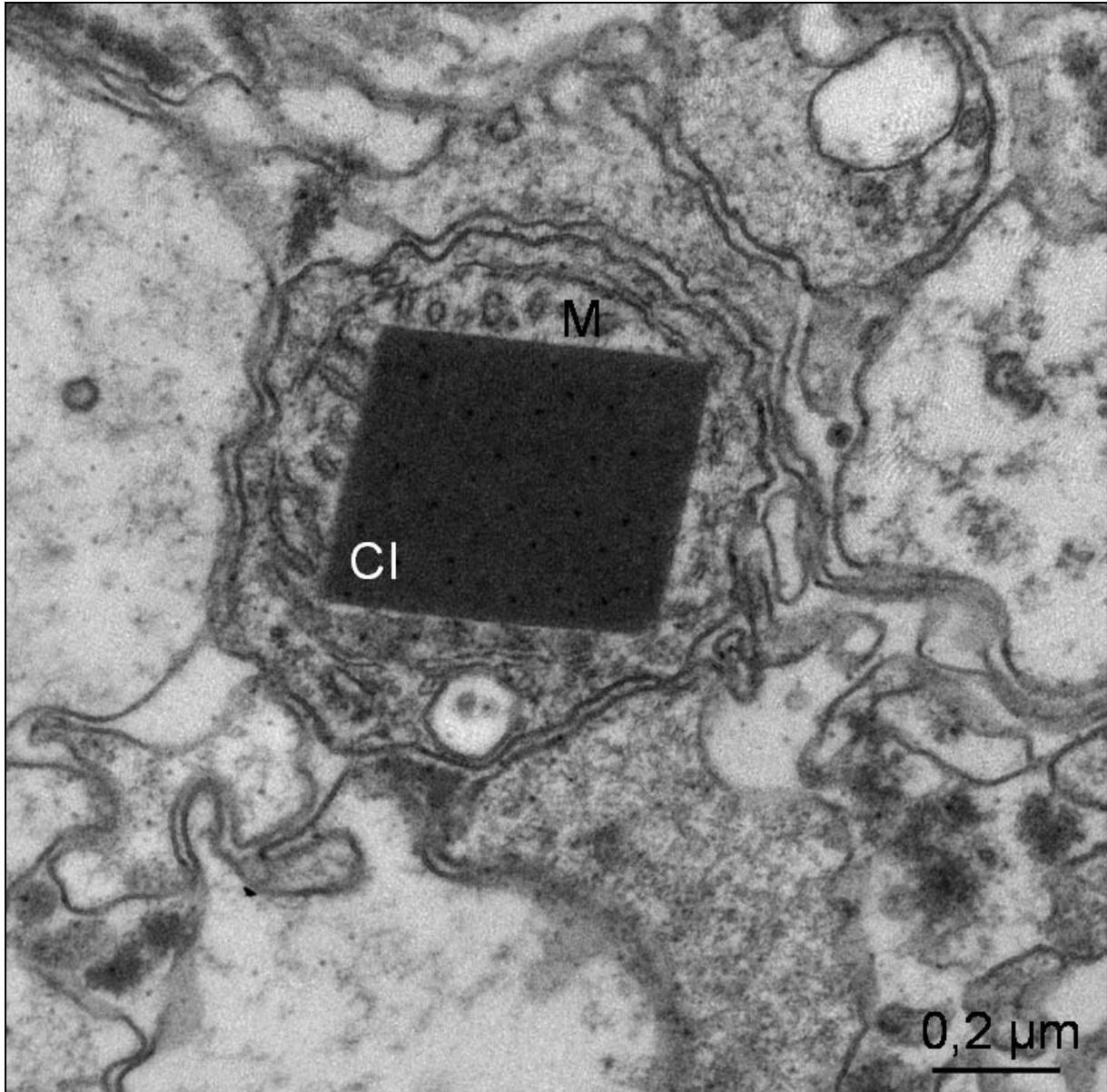
Fig. 36: Crystalline inclusion inside a mitochondrion of a digestive cell of a mussel exposed to phenanthrene.
CI (crystalline inclusion), M (mitochondrion),
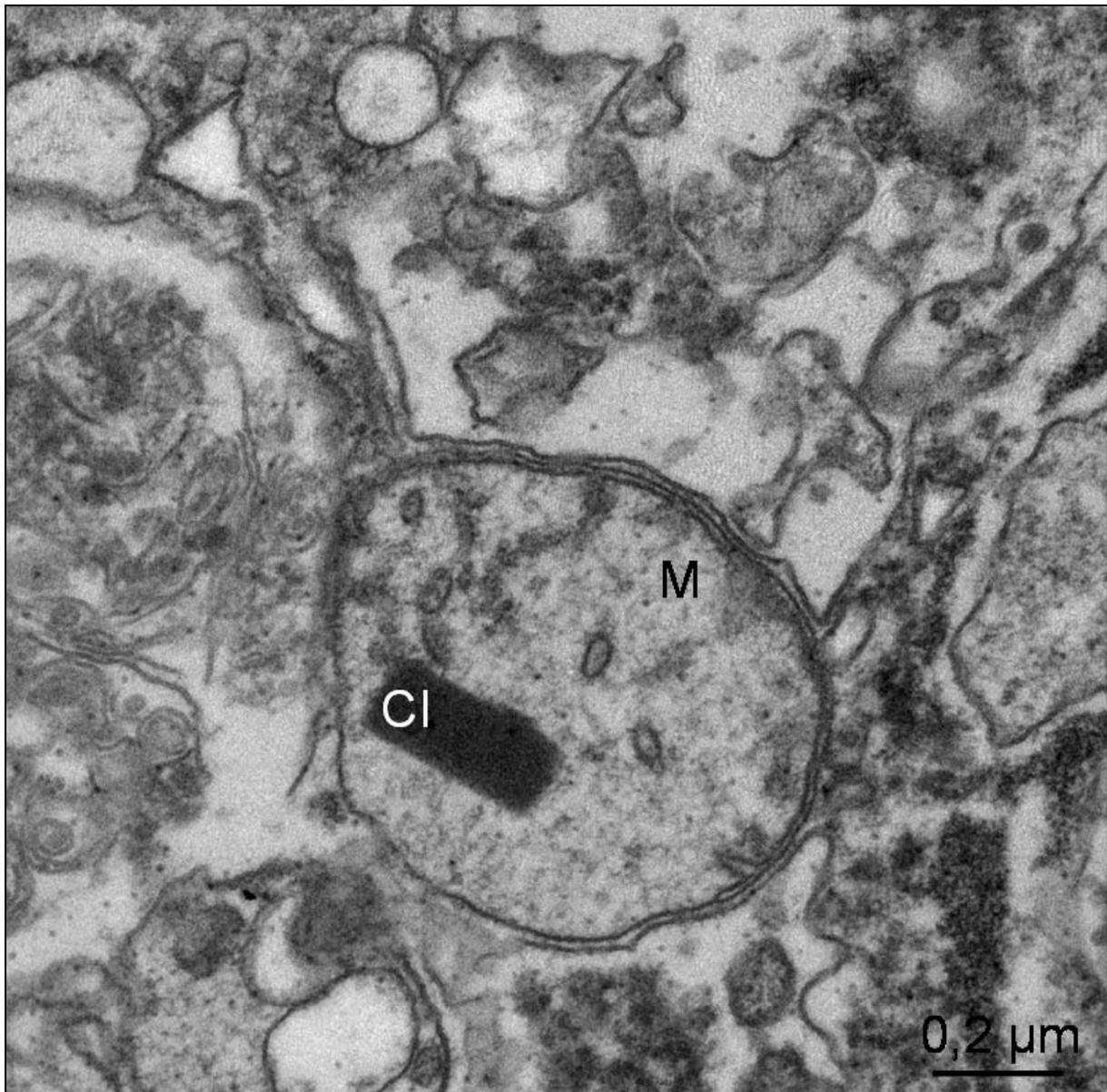
Fig. 37: Crystalline inclusion inside a mitochondrion of a digestive cell of a mussel exposed to phenanthrene.
Note the nearly complete loss of cristae inside the mitochondrion. CI (crystalline inclusion), M (mitochondrion)
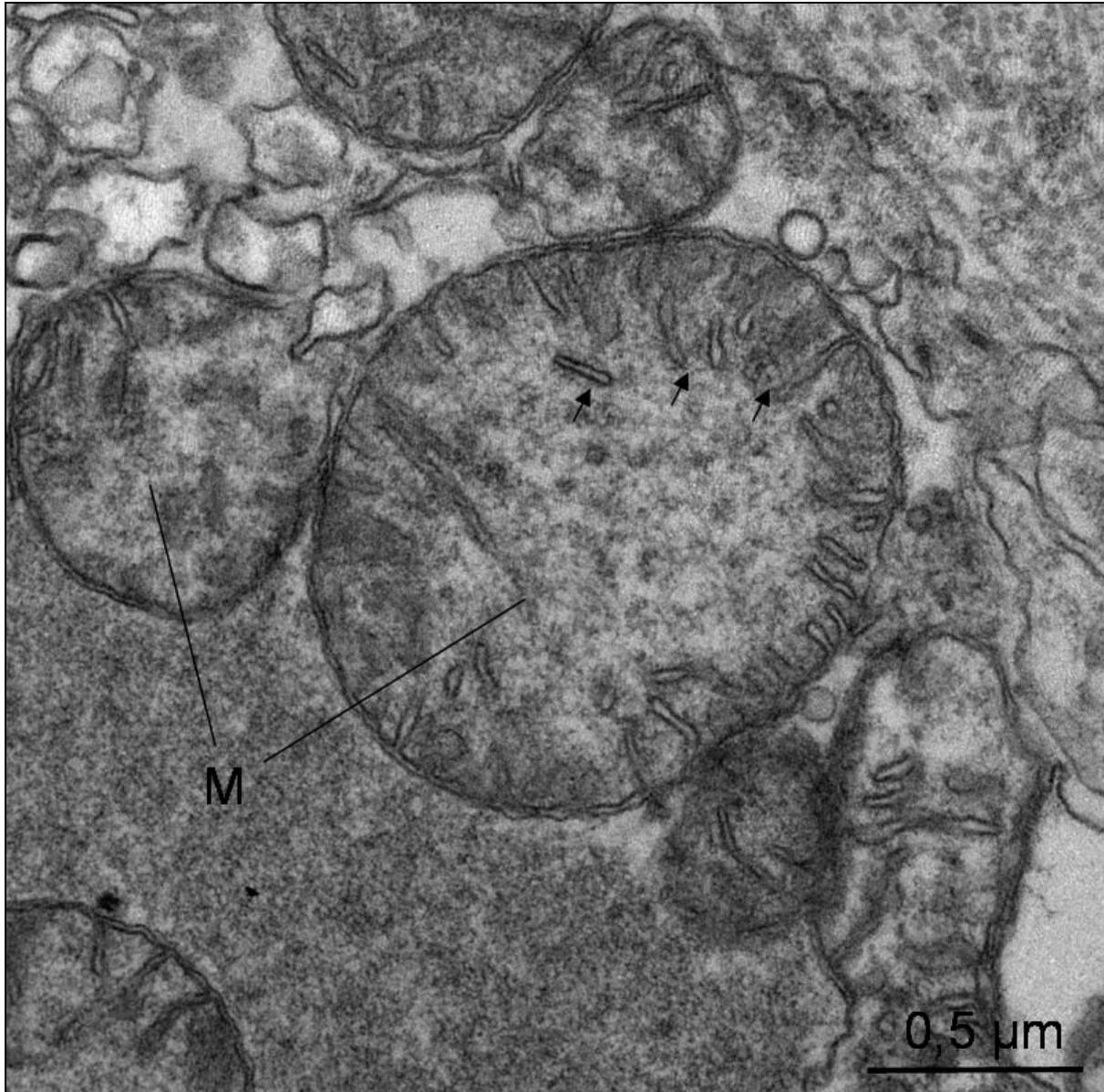
Fig. 38:   Mitochondria of a digestive cell of a mussel exposed to phenanthrene.
Note the reduction of cristae inside of the mitochondria. (M, mitochondrion)
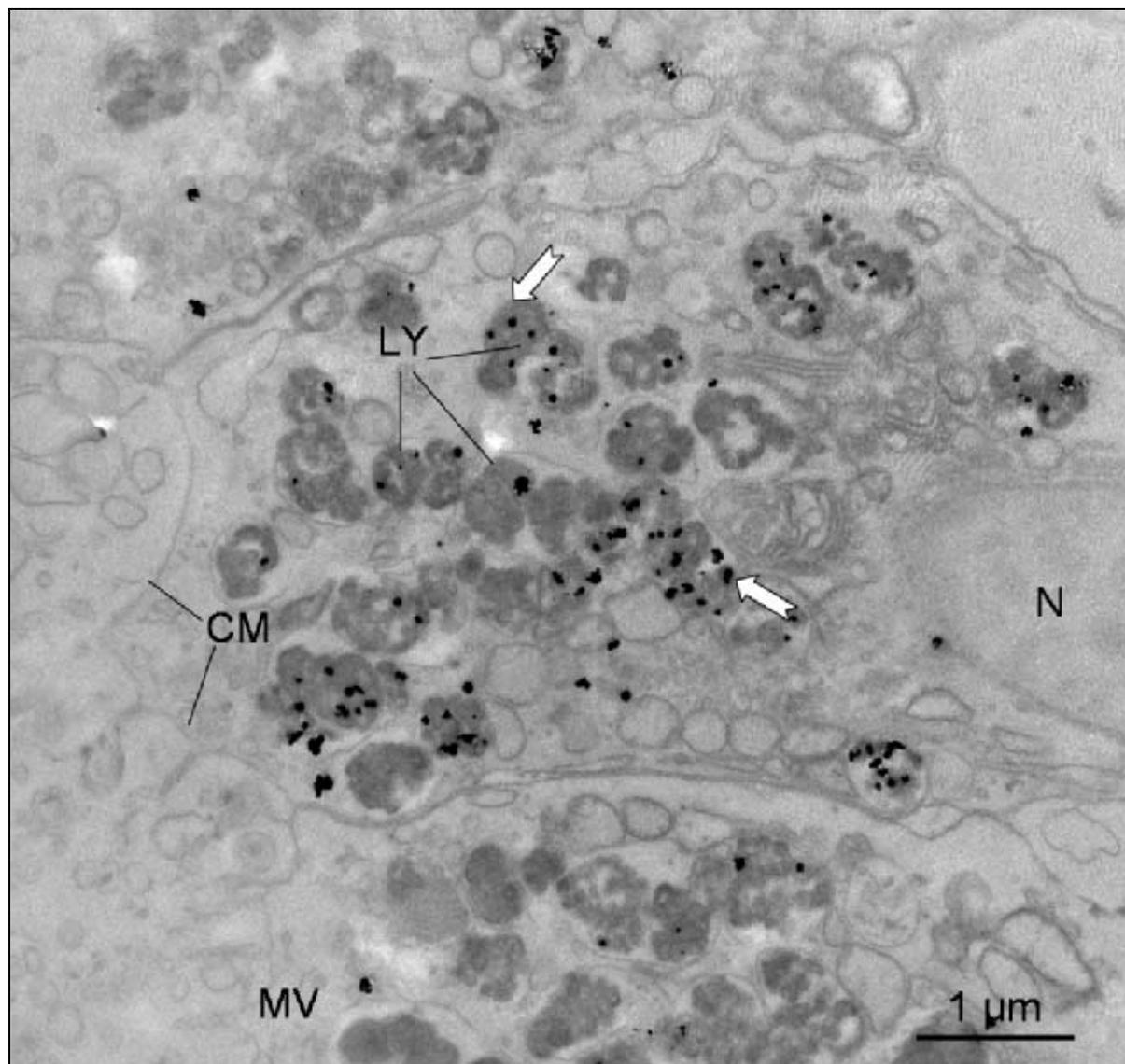
Fig. 39: Gill epithelium of a Pb-exposed mussel after incubation with the anti-lead antibody.
Pb accumulation appears at granular inclusions inside of lysosomes (arrows). Note the reduction of microvilli appearance and vesicular extrusions at the apical surface of the cells. CM (cell membrane), LY (lysosome), MV (microvilli), N (nucleus)
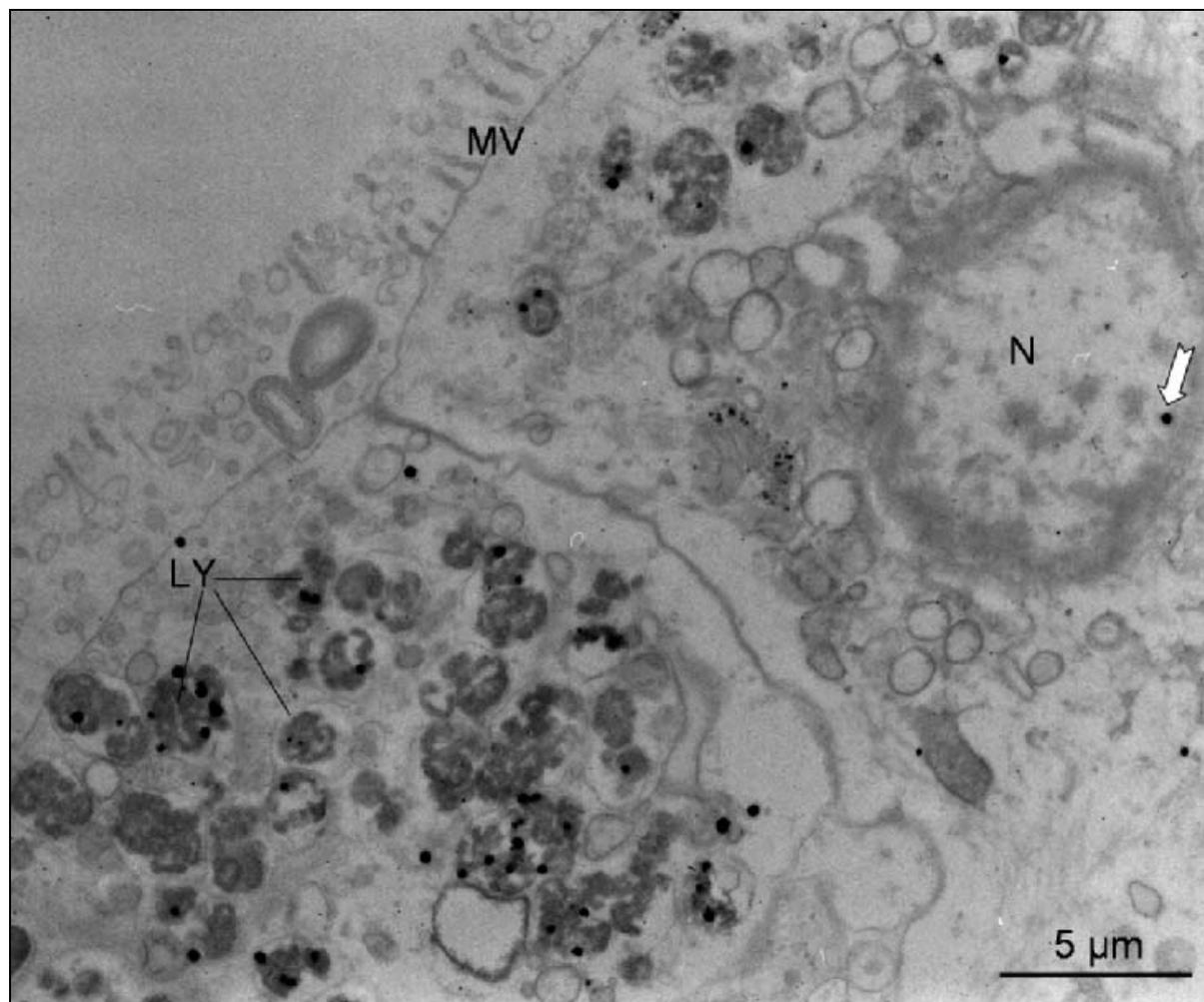
Fig. 40: Epithelium of the digestive duct of a mussel exposed to Pb after treatment with the anti-Pb antibody.
The cells contain numerous granular lysosomes with Pb accumulation. Note the appearance of Pb inside of the nucleus (arrow). LY (lysosome), MV (microvilli), N (nucleus)

6.5   Discussion

The immunodetection technique applied in the present study showed the accumulation of organic contaminants (aroclor 1254 and phenanthrene) and the metal Pb in epithelial cells of gills and digestive gland of the mussel *Mytilus edulis* in coincidence with several pathological alterations of cell organelles. Mucous granules play an important role as a first barrier in the detoxification process by absorption of organic contaminants as shown in our study for aroclor 1254 and thereby prevent the penetration of chemicals inside of the cells. Whereas the metal Pb was not

detected in mucous granules in the present study, Dimitriadis et al. (2003) localized Pb in mucous granules of the gill epithelium and Mason and Simkiss (1983) also detected the metal zinc in association to extracellular mucous on the branchial lamellae.

Changes of mitochondrial morphology such as reduction of cristae, dissolution of the outer membrane, and crystalline inclusions were a common pathological feature after exposure to the organic contaminants phenanthrene and aroclor 1254. Morphological alterations of mitochondria might be caused by the direct interaction of the lipophilic toxins with mitochondrial membranes. Structural changes of membranes lead to the interruption of membrane-associated mitochondrial functions such as fatty acid oxidation and oxidative phosphorylation as well as biosynthesis of pyrimidines, amino acids, phospholipids, nucleotides and heme. Mitochondrial disorders such as enlargement and abnormal shape, reduction and variations of cristae, and abnormal inclusions have also been observed in vertebrates after drug treatment (Pavelka and Roth 2005). Formation of crystalline deposits may be induced by the xenobiotic substance or precipitation of the compound itself. The fact that the crystalline structures were not positively labelled for aroclor 1254 or phenanthrene in our study contradicts the assumption that they are formed by the chemicals themselves. Crystalline mitochondrial inclusion bodies have been found in relation to mitochondrial myopathies, but their chemical composition is quiet unknown. O`Gorman et al. (1997) could show that mitochondrial creatine-kinase cristallises with another component in mitochondria of creatine depleted rat muscles. Formation of crystalline inclusions by mitochondrial enzymes as an adaptive response to metabolic disorders was also discussed by other authors (Kashiwagi et al. 1999).

Various contaminants such as hydrocarbons and organochlorines stimulate the formation of autophagic vacuoles and processes indicating an increased turnover of cellular components (Nott and Moore 1987, Braunbeck 1998). In the present study, accumulation of membrane whorls and crystalline structures was observed inside of the lysosomes. Enhanced autophagy and overloading of lysosomes by non-digestible material such as crystalline inclusions is indicative of lysosomal disorders induced by lysosomothrophic chemicals (Schneider et al. 1997). Accumulation of phospholipids as whorled arrays or lamellar myelin-like structures in lysosomes is a lipid storage disorder which has been reported to be induced by cationic amphiphilic drugs (Sawada et al. 2004).

Ultrastructural changes such as deformations of mitochondria in gills have been found in the present study after exposure to all chemicals, the organic contaminants and the metal lead. Particularly, mitochondrial alterations such as the reduction of cristae inside of the mitochondria are a common feature for both gills and digestive gland cells observed in the present study and might be induced by direct interactions of the contaminants with the inner mitochondrial membrane.

Ballan-Dufrancais et al. (1990) observed mitochondrial changes such as swollen membranes and the lack of cristae amongst other cellular alterations in mussels exposed to titanium and iron and detected the metals by EPMA in several organs (gills, internal pallial mantle epithelium, digestive gland, kidney, male genital cells, epithelium of the food, byssal glands). The described impairments of normal mitochondrial homeostasis by intoxification of chemical substances may disrupt normal physiology and function of the entire organ with severe consequences for the whole organism.

Lysosomal elements of the digestive gland showed specific alterations such as aggregation of residual bodies, proliferation of autophagosomes and an increase in the numbers of multi-lamellated aggregates. Autophagic activity of lysosomes is responsible for the degradation of various macromolecules and organelles and is activated in response to stress, promoting cell survival (Terman and Brunk 2005). On the other hand, enhanced autophagy along with lysosomal swelling destabilizes the lysosomal system which results in leakage of the lysosomal content into the cell causing severe cell injury or cell death (Moore et al. 2004). An excess of chemical substances which is stored inside of the lysosomes, e.g. in crystalline form, also leads to proliferation of lysosomes and may result in various lysosomal storage diseases with impairment of lysosomal functions (Schneider 1992). In the study of Domouhtsidou and Dimitriadis (2000) subcellular alterations after heavy metal exposure (Hg, Ag, Pb, and Cu) such as fusion events of residual bodies and enlarged dense granules in the digestive gland cells were similar to our observations. In the study of Lowe and Clarke (1989), alterations of the digestive epithelial cells after exposure to a mixture of hydrocarbons and copper were analysed and quantified, and showed, among others, alterations of the lysosomal vacuolar system such as enlarged lysosomes and residual bodies.

After PCB treatment of the mussels in the present study, augmentation of Golgi fields and enhanced formation of endosomes was observed which might be an adaptive response indicating detoxification processes (Koehler 1990). Expansion of the Golgi apparatus and formation of trans-Golgi networks indicate enhanced endocytotic activity (Pavelka and Roth 2005) which might be directed towards the toxicants in the present study. Impressive swelling and vesiculation of the Golgi apparatus have also been reported in the marine prosobranch *Littorina littorina* after exposure to the hydrocarbon naphthalene (Cajaraville et al. 1990) and in fish hepatocytes after exposure to the insecticide diazinon (Braunbeck 1998).

For the detection of heavy metals in cellular and subcellular compartments of molluscs different microscopic techniques have been successfully used (table 7). Application of these metal detection methods in field studies is often limited due to their non-specificity to a single metal. Therefore, they have to be combined with other analytical techniques to increase their sensitivity.

In the case of organic contaminants only a few methods have been developed for their tracing at the cellular and subcellular level (table 7). For example, the use of the fluorescent marker 3-methylanthracene demonstrated the accumulation of the hydrocarbon inside of lysosomes of the digestive gland (Moore et al. 2006). Similar results were obtained after treatment of *Mytilus edulis* with ^{3}H-benzo(a)pyrene in digestive gland and gill epithelial cells (Magnusson et al. 2000). The application of antibodies against PCBs in the clam *Mya arenaria* demonstrated the accumulation of PCBs in hemocytes (Harper et al. 1991, Strandberg et al. 1998). In the study of Harper et al. (1991) the presence of PCBs was directly correlated with leukemia in the soft shell clam *Mya arenaria*.

Various cellular and subcellular pathological alterations in Mytilid mussel have been observed after laboratory exposure to Pb, PCBs and PAHs (table 8). In the present study, cytopathological alterations after Pb, PCBs and PAHs exposure in cell organelles and compartments suggest a close cause-effect relationship to the subcellular localization of the contaminants. Phenanthrene accumulation strongly appears to be associated with the endo-lysosomal system. Phenomena, such as lysosomal membrane destabilization, lipofuscin accumulation, and multiple membrane vesicles, occuring after addition of membrane bound material to lysosomes, are reported effects during exposure of mussels to hydrocarbons (Pipe and Moore 1986) and lead to the assumption of a direct molecular interaction of the lipophilic phenanthrene with membrane lipids. Lysosomes of epithelial cells containing granular material have been shown to accumulate various metals and are important sequestration compartments for toxic metals (Dimitriadis et al. 2003).

In the present study, the distribution of different chemicals was shown at the subcellular level by the use of antibodies against these toxins. The detection of the contaminants in association to cellular and subcellular compartments such as lysosomes, mitochondria, nuclei and various transport vesicles of gill and digestive gland cells might explain most of the observed ultrastructural histopathological alterations.

In summary, immunocytochemical methods are promising tools for the detection of various classes of environmental chemicals and the identification of their cellular targets, provided that antibodies are available or can be manufactured for the contaminants of interests. This technique is highly specific to the selected chemical and can be theoretically applied for the tracing of all kinds of metals, organics, natural toxins, and pharmaceuticals in tissues and cells. Moreover, the simultaneous application of antibodies against different chemicals may be used to study combinatory effects (antagonistic or synergistic) of contaminant mixtures.

Our results show that the combined use of cytopathology and tracing of contaminants by immunodetection are successful tools for the subcellular localization of contaminants and description

of potential pathological effects at their target sites. Nevertheless, more studies in this field of research are needed to clarify the hazardous potential of different contaminants inside organisms and to understand their detailed mechanisms of toxicity.

Tab. 7: Localization of Pb, PCBs and PAHs in cells and subcellular compartments of bivalve molluscs by the use of various microanalytical techniques

Contaminant	Method	Species	Accumulation site	References
Pb	AMG	*Mytilus galloprovincialis*	Gill epithelial cells Dense bodies of hemocytes Dense bodies of digestive cells	Domouthtsidou and Dimitriadis 2000
Pb	AMG	*Mytilus galloprovincialis*	Dense bodies, residual bodies, heterolysosomes of digestive cells Dense bodies of gill epithelial/endothelial cells Dense bodies of hemocytes Mucous granules of gill filament	Dimitriadis et al. 2003
Pb	EPMA	*Mytilus edulis*	Extracellular granules Epithelial cells Hemocytes	Marshall and Talbot 1979
Pb	EPMA	*Dreissena polymorpha*	Hemocytes	Giamberini et al. 1996
Pb	EPMA	*Crassostrea gigas*	Digestive cells Gill epithelial cells	Amiard et al. 1995
Pb	SIMS/EPMA	*Hyridella depressa*	Granular concretions (gonad/mantle)	Adams et al. 1997
PCBs	Antibodies	*Mya arenaria*	Hemocytes	Strandberg et al. 1998
PCBs	Antibodies	*Mya arenaria*	Hemocytes	Harper et al. 1994
PAHs	Microautoradiography	*Mytilus edulis*	Epithelial cells of digestive gland Ciliated epithelial cells of gills	Magnusson et al. 2000
PAHs	UV-spectrofluorimetry	*Mytilus edulis*	Lysosomes of digestive cells Ovarian oocytes	Moore et al. 2006
Pb	Immunolocalization	*Mytilus edulis*	Microvilli, mucous secretions, mitochondria, lysosomes, nuclei of gill cells Microvilli, lysosomes, nuclei of digestive cells	Present study
PCB	Immunolocalization	*Mytilus edulis*	Chitinous rod, mucous secretions, lysosomes of gill cells Microvilli, mitochondria, lysosomes of digestive cells	Present study
PAH	Immunolocalization	*Mytilus edulis*	Microvilli, mucous secretions, lysosomes of gill cells Lysosomes of digestive cells	Present study

Tab. 8: **Cellular and subcellular pathological alterations in Mytilid mussel after laboratory exposure to Pb, PCBs and PAHs**

Contaminant	Pathological alterations	References
Pb	Fusion of residual bodies in digestive cells	Domouhtsidou and Dimitriadis 2000
Pb	Fusion and detachment from the basement membrane of gill cells	Dimitriadis et al. 2003
PCBs	Increase in lipid content in digestive tissue	Krishnakumar et al. 1997
PCBs, DDT, Dieldrin	Loosening of intercellular connections in the gill epithelia and shrinkage of the cells.	Sunila 1988
PAHs	Enlargement of digestive cell lysosomes Structural disturbance of digestive cell lysosomes	Moore and Clarke 1982
PAHs	Proliferation of SER in digestive cells Changes in lysosomal membrane configuration	Nott et al. 1985 Nott and Moore 1987
PAHs	Enlargement of lysosomes and residual bodies in digestive epithelium	Lowe and Clarke 1989
Pb	Proliferation of autophagosomes and aggregations of residual bodies in digestive epithelium Reduction of cristae in mitochondria of gills and digestive epithelium Formation of ER whorls in digestive epithelium Deformation of nuclear envelope in gills and digestive epithelium	Present study
PCBs	Proliferation of autophagosomes and aggregations of residual bodies in digestive epithelium Reduction of cristae in mitochondria of gills and digestive epithelium Crystalline inclusions in mitochondria of digestive epithelium Augmentation of Golgi complexes in digestive epithelium Formation of ER whorls in gills and digestive epithelium Augmentation of nucleoli in digestive epithelium	Present study
PAHs	Proliferation of autophagosomes and aggregations of residual bodies in digestive epithelium Crystalline inclusions in mitochondria of digestive epithelium Reduction of cristae in mitochondria of gills and digestive epithelium Augmentation of Golgi complexes in digestive epithelium Formation of ER whorls in digestive epithelium Deformation of nuclear envelope in gills and digestive epithelium	Present study

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CHAPTER 7

7   Synthesis

Increasing amounts of hazardous chemicals in the environment and related effects on organism's health require the rapid development of new analytical detection methods and biomarkers for impacts identification and quantification. Marine invertebrates have developed defence mechanisms to protect themselves against the toxic action of man-made environmental contaminants such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals. Important detoxification mechanisms in marine invertebrates include the sorting and sequestration of the xenobiotic compounds in subcellular compartments, metabolism of the chemicals by specific biotransformation enzymes (e.g. mixed function monooxygenase system (MFO) and glutathione conjugation), detoxification by elimination processes, and protection against redox-cycling and oxidative stress by antioxidative capacities. Investigations of those protective mechanisms and quantitative measurement of their responses are used in biological effect-monitoring as biomarkers to predict the risk for animal health. Moreover, biomarkers provide information about acute exposure situations and toxic health effects as well as prognosis of potential long-term impacts. Many potential biomarkers have been investigated and proposed for marine pollution monitoring using vertebrates and invertebrates. The "biomarker problem" is that many of them are susceptible to confounding factors such as biotic and abiotic variables and therefore are not solely pollutant-specific. Additionally, a "cocktail" of various pollutants is introduced into the marine environment that -when taken together- may show accumulating, interactive or compensatory effects. Thus, interpretation of field study results is often highly complex because of these "uncontrolled" conditions.

The main objective of the present study was to analyse the uptake, accumulation and fate of anthropogenic contaminants by an immuncytochemical approach and disclose pollutant-specific biological effects in the mussel *Mytilus edulis*. Therefore, an antibody-based detection system was developed for the cellular and subcellular detection of contaminants. The new immunocytochemical approach may serve as a tool for future application in environmental monitoring studies; it provides important information of toxin-specific interaction with cellular and subcellular components and allows the analysis of toxic mechanisms of various contaminants. Additionally,

it may be very useful for the differentiation between specific contaminant-induced biological effects and alterations induced by natural stress parameters.

The blue mussel *Mytilus edulis* was chosen as a model organism for our study, because it is a sessile suspension feeding invertebrate that rapidly takes up and accumulates various particulate or soluble contaminants from the water column to concentrations in excess of those in the surrounding environment. In contrast, biotransformation and elimination mechanisms are relatively slow in molluscs compared to fish. Thus, depuration time is relatively long in bivalves and the xenobiotic compounds are often detectable for a long time after exposure.

Field and laboratory studies have shown a great potential in using biotransformation enzymes of phase I and II, as early warning signals in bivalves for pollution events. On the other hand, the use of xenobiotic metabolizing enzymes of bivalves as biomarkers in marine pollution monitoring is restricted due to a lack of controlled laboratory studies to correlate responses of enzymes under defined conditions to complex field studies where changing environmental factors may influence the biomarker reaction. Furthermore, cytochrome P450 and the associated MFO system are present in bivalves, but their levels of specific activities are generally lower and less inducible in comparison to marine vertebrates, which make the interpretation of results often difficult, particularly in field studies where complex mixtures of contaminants and confounding environmental factors interact.

Glutathione S-transferase (GST) isoenzymes have been identified in *Mytilus edulis* and *M. galloprovincialis*. However, their activities vary or are sometimes opposite in field and laboratory studies. In the present study, enzyme activities of both phases I and II, NADPH cytochrome c reductase and GST, were investigated after contaminant exposure to evaluate their potential as prognostic biomarkers in mussels under defined pollution conditions. Additionally, catalase (CAT) activity as a marker enzyme for oxidative stress was analysed.

Pathological endpoints after toxicant exposure are histo- and cytopathological changes of tissues and cells integrating the effects of complex mixtures of contaminants. In the present study, pathological cell changes were analysed in relation to contaminant localization sites at the subcellular level, and the newly implemented immunodetection technique was applied to localize the contaminants in cell organelles and compartments in parallel to the analysis of cytopathological alterations in the same samples.

The newly developed immunodetection technique and the use of immunoanalytical methods in marine pollution monitoring in relation to responses of enzymes are discussed in the following chapters.

7.1 Antibody-based detection of contaminants

Production of antibodies against anthropogenic contaminants is a promising field in environmental research. Antibodies against organic pollutants were used for both extraction and detection of trace amounts of chemicals from environmental and biological samples such as water, semen, urine, plant extracts, food, soil and citrus fruits (Zajicek et al. 1996, Zajicek et al. 2000, Churchill et al. 2002, Fillmann et al. 2002, Chuang et al. 2003).

Antibodies can be produced by three different techniques, yielding polyclonal antibodies (animal's antiserum), monoclonal antibodies (hybridoma cell lines), and recombinant antibodies (genetic engineering) including the Fabs (entire L chain with first variable and constant region of H chain), Fvs (variable regions of both H and L chains) and single-chain scFv fragments (Fv is stabilized by a flexible amino acid linker) (Churchill et al. 2002).

Specific monoclonal anti-toxin antibodies were used for immunohistochemical detection of pollutants in the present study. Monoclonal antibodies are produced by fusing a B lymphocyte, usually derived from the spleen of an immunized animal (mouse), with a myeloma cell. Monoclonal antibodies contain a unique defined IgG molecule and, theoretically, an unlimited amount of an antibody with identical affinity for an antigen can be provided. For future monitoring studies plant-derived recombinant antibodies against environmental pollutants would offer several advantages: First, the use of animals for antibody production is not needed any longer; second, high amounts of antibodies can be obtained and third, antibodies are characterized by their high specificity and affinity for a single compound and its metabolites (Churchill et al. 2002).

The anti-PAH antibody is generated predominantly to phenanthrene and its affinity to other PAHs decreases with increasing structural differences (phenanthrene 100%, anthracene 100%, fluorene 65%, benzo(a)anthracene 64%, chrysene 84%, fluoranthene 73%, pyrene 29%, benzo(b)fluoranthene 22%, benzo(a)pyrene 12%, acenapthene 10%, acenapthylene 10%, indeno(,2,3-c,d)pyrene 9%, napthalene 0.5%). The fit of the hapten to the binding pocket of the antibody depends on the position of the aromatic rings as well as the size and functional groups of the aromatic core (Scharnweber et al. 2001). The anti-PCB antibody detects to 112% congeners of the PCB mixture aroclor 1260, while affinity for other commercial PCB mixtures declines, respectively (aroclor 1254 100%, aroclor 1248 67%, aroclor 1242 40%, aroclor 1232 40%, aroclor 1221 8%). Additionally, antibodies relatively broad cross-reactivity may allow the simultaneous detection of both the parent compound and its metabolites. Antibody-based methods for the detection of contaminants can be applied for analysis of toxin accumulation at the light microscopic level as shown in this (chapter 4) and other studies (Strandberg et al. 1998, Kerr Lobel and

Davis 2002) and for detailed subcellular analysis also at the ultrastructural level (chapter 3-6). A further advantage of the antibody-based detection technique is its high specificity to a single organic pollutant or metal. Thus, this technique can be principally applied under field conditions to detect the presence of contaminants in relation to their specific effects. Detection of PCBs in field samples of fish embryos has been demonstrated in the study of (Kerr Lobel and Davis 2002) and in clams from PCB contaminated sites at the tissue level by light microscopy (Strandberg et al. 1998). In these studies, pathologies could be correlated to contaminant localization sites. PCBs were predominantly localized in hemocytes of clams showing symptoms of the disease hemocytic neoplasia and PAH concentrations were highest in malformed embryos.

The immunohistochemical technique developed in the present study enables the detection of contaminants at the ultrastructural level by applying a combinational preembedding approach to provide enhanced fixation and high intensification of the antigens (chemicals) as well as a good ultrastructural preservation of cell morphology. Antigen fixation, intensification and preservation processes have been adopted from neuro-immunohistochemical studies for optimal conservation of low antigen levels (Gallyas et al. 1982, Liposits et al. 1982, Van den Pol and Gorcs 1986, Merchenthaler et al. 1989, Sawada and Esaki 1994). It is not known from our study if the detected immuno-reaction product is the parent compound (phenanthrene or aroclor 1254) or its metabolites. Although, the accumulation of phenanthrene was shown by parallel chemical analysis in whole tissue homogenates and subcellular fractions, phenanthrene metabolites may have been detected with the anti-PAH antibody, too.

Our studies concerning the heavy metal Pb showed that the antibody-based detection technique is also a promising tool for investigation and elucidation of heavy metal metabolism in bivalves. In future studies, labelling of metals with specific antibodies would allow the cellular tracing of single elements to analyse their distribution and fate in exposed organisms. The immunodetection method using an anti-Pb-antibody is characterized by its high specificity to this single type of metal. This is an advantage in comparison to other microscopic metal detection techniques such as autometallography (AMG) which demonstrates the deposition of various metals simultaneously, and has to be specified by the use of other microanalytical techniques such as X-ray microanalysis.

An immunocytochemical technique for detection of organic contaminants was developed for the application at the subcellular level for TEM. The immunocytochemical technique showed the distribution of organic contaminants such as aroclor 1254 and phenanthrene at the electron microscopic level in the mussel *Mytilus edulis*. Furthermore, an anti-Pb antibody was successfully used for the specific ultrastructural localization of the heavy metal Pb.

7.2   Identification of cellular and subcellular targets for contaminants

Organic contaminants show various cellular sites of accumulation and storage predominantly associated with lipid-rich cellular compartments due to their lipophilic character. Therefore, the potential hazard and toxicity of organic environmental pollutants is severe. By uptake into membrane lipids, structural modifications such as the degree of permeability or fluidity can affect integrity and physiological function of the cell and its organelles. Furthermore, inside cellular compartments chemicals may provoke oxidative stress by generation of AH-quinones which cause redox cycling (Moore et al. 2006).

Only a few studies exist where the localization of organic contaminants could be directly shown at the microscopic level. For example, accumulation of organic contaminants was demonstrated inside of lysosomes of the digestive gland by endogenous fluorescence of an artificial contaminant as well as in epithelial cells of gills and digestive glands by microautoradiography (Magnusson et al. 2000, Moore et al. 2006). To our knowledge, further cellular accumulation sites for lipophilic organic contaminants have not been previously detected microscopically.

In the present study, it was also shown that the lipophilic contaminants aroclor 1254 and phenanthrene accumulated in the endo-lysosomal system of the cell. Whereas phenanthrene was solely localized inside of lysosomes, the PCB mixture was detected additionally in association to collagen fibres and mitochondria. Inside the lysosomes, degradation processes may occur and contaminants interact with membranes, lipids and proteins, which lead to lipid peroxidation and production of reactive oxygen species. Disturbances of the lysosomal membrane caused by phenanthrene were shown in the present study (chapter 4). Impairment of lysosomal membrane stability after hydrocarbon exposure has also been reported by other authors (Nott et al. 1985, Pipe and Moore 1986, Moore and Viarengo 1987). Accumulation of PCBs inside of mitochondria and related mitochondrial alterations such as the reduction of cristae and appearance of crystalline inclusions inside of the mitochondria were observed in the cytopathological part of the present study (chapter 6). In vertebrate studies, physiological effects of the commercial PCB mixture aroclor 1254 are phosphokinase c translocation and inhibition of Ca^{2+}-uptake by subcellular organ-

elles such as mitochondria (Tilson et al. 1998, Sharma et al. 2000). In the context of our results, perturbation of intracellular signalling mechanisms may be an effect of direct interaction of the lipophilic PCB mixture with mitochondrial membranes. In particular, interactions of PCBs with mitochondrial membranes might cause interruption of mitochondrial homeostasis and lead to energy deficiencies by inhibition of oxidative phosphorylation (Sivalingan et al. 1973). Another site of interference of PCBs was the chitinous element in gill lamellae as shown in chapter 6 and might be responsible for morphological deformations of gill lamellae structures occurring after exposure to organic contaminants, which were not investigated in the present study, but have been detected in other studies. For example, during histopathological studies implemented in the mussel watch program (Yevich and Barszcz 1983), pathological alterations such as swelling of the gill interlamellar connective tissue related to chemical stress have been reported. In laboratory exposure studies of mussels (*Mytilus edulis*) cellular changes in gills were observed after exposure to the organic contaminants Clophen A 60 (PCB) and DDT (ppl-DDT, Aroclor). Frontal cells appeared granular, endothelia cells showed atrophy and adjacent cells lost their connections (Sunila 1988).

Sequestration of metals by lysosomes and binding to specific ligands are well known as parts of the metal metabolism and detoxification process which also prevent interactions of the toxin with other cell organelles (Domouhtsidou and Dimitriadis 2000, Marigomez et al. 2002, Dimitriadis et al. 2003). In our study, Pb accumulated in specific lysosomal structures in association to granular inclusions. Metals have a different affinity to specific metal binding ligands (see also chapter 5). Class "a" metals such as Cs, K, Na, Li, Ba, Sr, Ca, Mg, Be, La, Gd, Y, Lu, Sc and Al are localized in cells with granules composed of carbonate, oxalate, phosphate, and sulfate (oxygen donors), whereas class "b" metals such as Au, Cu, Hg, Se, Pd, Pt, Bi, Tl, Ag and Pb are associated with those cell types rich in sulfur and nitrogen ligands (sulfur donors). Borderline metals such as Cd, Sn, Ti, Mn, Fe, V, Cu, Co, Zn, Ni, Cr, In, Ga, Sb, As and Pb have an intermediate position between both classes and have been found in lysosomes (for review see Marigomez et al. 2002).

Transport of the dissolved metals Cd, Fe and Zn in *Mytilus galloprocincialis* occurs via circulating hemocytes, brown cells or blood plasma ligands to the digestive gland and kidney where excretion and elimination takes place as urine and faeces (Marigomez et al. 2002). In the present study, granules of hemocytes could be identified as specific transport vesicles for Pb inside of the blood cells (chapter 5), for transport between organs and elimination processes. In the freshwater mussel *Dreissena polymorpha* dissolved Pb is directly incorporated into freely moving granulocytes and associated to blood plasma ligands in the gills (Marigomez et al. 2002). Thus, similar transport mechanisms of dissolved Pb as for other borderline metals could be identified in *Mytilus edulis* in

our study. In summary, *Mytilus edulis* contains a subcellular system for accumulation and transport of the metal Pb, mainly based on sequestration and compartmentation of Pb in lysosomes and lysosomal-like granules. Moreover, binding to specific ligands inside of the multi-compartment system and excretion via exocytosis of residual bodies enables the organism to cope with metals. In contrast to other studies where authors describe elimination of metals such as Hg, Cu, Cd, Fe, and Zn by excretion of residual bodies of digestive cells (Ballan-Dufrancais et al. 1985, Marigomez et al. 2002), also known as tertiary lysosomes, we neither found Pb in residual bodies nor did we observe this process for Pb metabolism in *Mytilus edulis*. In the study of Dimitriadis et al. (2003) Pb was also detected to a very low extent in residual bodies of digestive cells compared to Hg and in the study of Domouhtsidou and Dimitriadis (2000) Pb was also not detectable in residual bodies by AMG. Therefore, both studies together with our observations argue for a minor role of sequestration and elimination of dissolved Pb via tertiary lysosomes of digestive cells. New findings of the present study are the detection of Pb inside of mitochondria and nuclei (chapter 5). Accumulation inside of these cell organelles indicates the hazardous potential and toxicity of this heavy metal.
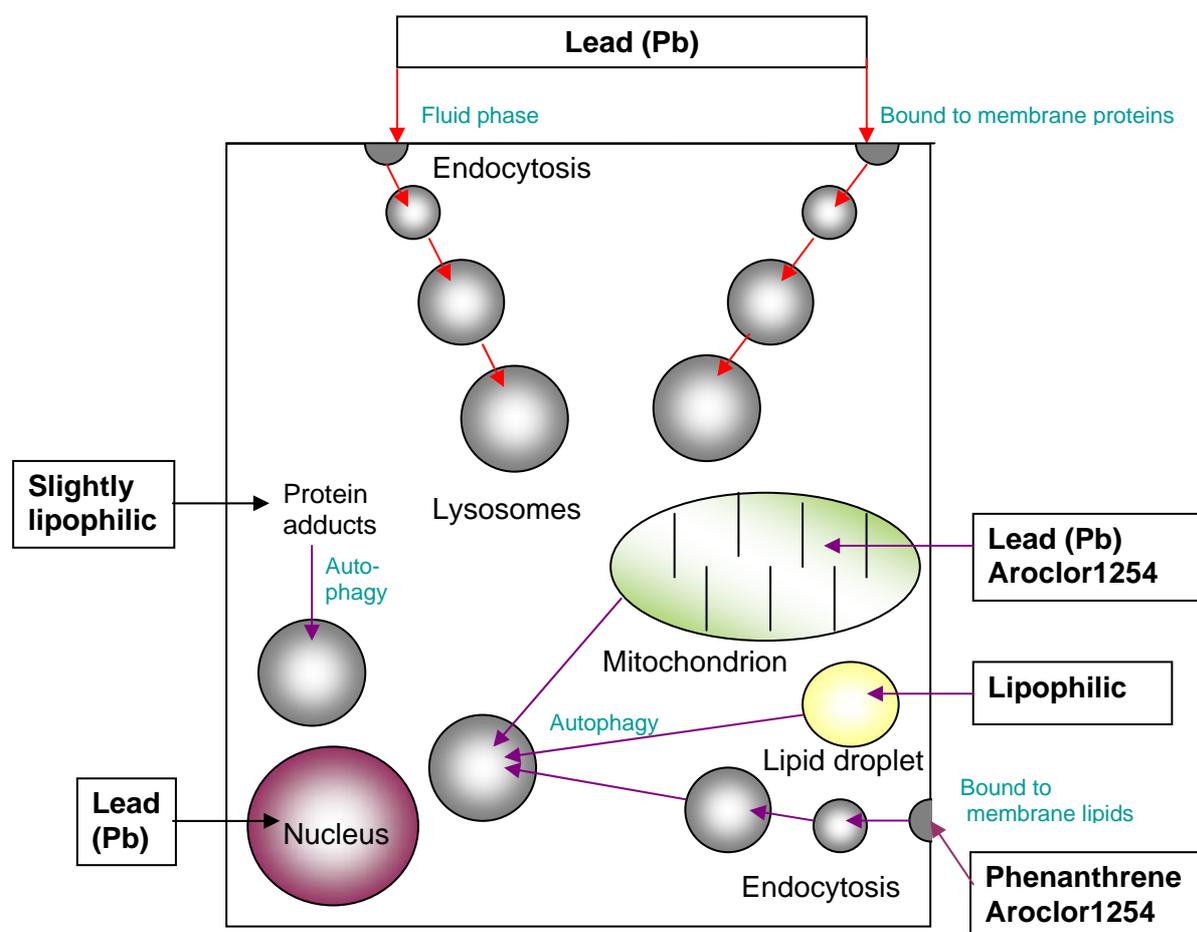

Fig. 41: Scheme illustrating routes for cellular trafficking of lipophilic organic contaminants (PCBs, PAHs) and the heavy metal Pb as shown in this thesis in reference to the hypothetical model of Moore et al. (2006).

Lysosomes showed accumulation of PCBs, PAHs and heavy metals. The endo-lysosomal system plays an important role in contaminant accumulation, sequestration and detoxification of the investigated anthropogenic toxins. Mitochondria were identified as further subcellular target sites for PCBs and subsequent pathological alterations of these cell organelles were observed. Additionally, PCBs were detected in association to the chitinous rod of the gill lamellae. Dissolved Pb was mainly detected in specific lysosomal structures of epithelial cells. Transport of Pb occurred via hemocytes in which the metal was localized in association to lysosomal granules. Other localization sites for Pb were identified as nuclei and mitochondria which demonstrates the severe hazardous potential of Pb.

7.3   Detoxification and biotransformation pathways in *Mytilus edulis*

Evaluation of enzyme activities as biomarkers of exposure to organic or metal contamination has been the objective of many research studies in the field of marine pollution monitoring (table 9, 10 and 11). Such biomarkers provide information on early toxic effects at the cellular level and help to predict potential long-term effects. In vertebrates, toxicity of organic pollutants is mediated through binding of the contaminant to the cytosolic aryl hydrocarbon receptor (Ah) receptor and subsequent changes in gene expression. In invertebrates, such as clams and mussels, Ah receptor homologues have been identified, but it is unknown if their functional mechanism in mediating toxicity is similar to those in vertebrates (Butler et al. 2001, Wiesner et al. 2001, Danis et al. 2004). It could have been shown that exposure to hydrocarbons tends to induce the MFO system (Parant 1998). In *Mytilus edulis* NADPH cytochrome c reductase activity, a component of the MFO system was sensitive to PAHs exposure (Livingstone et al. 1985, Livingstone 1987, Livingstone et al. 1989, Nasci et al. 1989, Michel et al. 1993, Okay et al. 2000, Mohammed and Agard 2004). In the present study, we could also report an increased activity of NADPH cytochrome c reductase activity after phenanthrene exposure. This shows the significance of phase I biotransformation of hydrocarbons and the potential to use this enzyme as a biomarker of exposure to hydrocarbons also in the marine mussel *Mytilus edulis*.

Glutathione S-transferases are a group of isoenzymes identified in bivalves and measured by utilization of CDNB (1-chloro-2,4-dinitrobenzene) as substrate. In *Mytilus edulis*, four GST isoenzymes have been characterized and the two major proteins are able to metabolize CDNB (Fitzpatrick et al. 1995). GST activity is tissue dependent with higher activities reported in gills than in digestive gland (Parant 1998). Higher activities in gills in comparison to digestive gland tissues were also found in the present study. Contrasting results concerning GST activity have been obtained in field and laboratory research studies, and often levels of activity changes are too low and not significant to show that GST activity is affected by a specific type of pollution. Aging of bi-

valves decreases GST activity in gills whereas activity in digestive gland remains unchanged (Parant 1998). In the present study, we demonstrated that GST activity is inducible in tissues of *Mytilus edulis* under controlled laboratory conditions after PAH and Pb exposure. Although it is not realistic presently to use GST as a biomarker of specific pollution in field studies due to variable results concerning GST activity, we showed that this enzyme could be used in future studies for biomonitoring of hydrocarbons and metals if more information is available about individual factors (e.g. age, size), confounding environmental factors, and substrate metabolization of isoforms with (CDNB) influencing GST activities.

Induction of antioxidative biomarkers as early warning indicators in field studies are susceptible to factors unrelated to the chemical exposure conditions such as reproduction, food availability, and water temperature (Cheung et al. 2004). In the present thesis induction of catalase activity shows a high potential to be used as a biomarker of oxidative stress after laboratory PAH exposure. In laboratory studies confounding factors are controlled and an antioxidative response can be directly related to a specific contaminant. Therefore, laboratory studies are helpful to interpret data from field investigations and should be carried out in parallel.

> Our study showed increased activities of phase I and II enzymes after exposure to PAHs (NADPH cyt c red, GST). Antioxidative enzyme activity of catalase was elevated after PAH exposure, but not after Pb exposure. GST enzyme activities in gills and the digestive gland displayed different levels of activity after Pb exposure. GST activities increased after Pb exposure in gills whereas no effects were observed in the digestive gland. Laboratory studies are important for the use of detoxifying enzymes as biomarkers of exposure in pollution monitoring. Enzyme activities should be examined in tissues where highest levels of activities are normally found. Laboratory investigations should be performed as internal controls in parallel to field studies where interactions of various contaminants as well as natural stress factors (e.g. water temperature, salinity, and food availability) might additionally affect enzyme activities.

Tab. 9: **Responses of NADPH cytochrome c reductase measured in the present thesis and by other authors**

Enzyme	Exposure	Contaminant	Species	Organ	Activity	Reference
NADPH cyt c red	Laboratory	PAH	*Mytilus edulis*	DG	Increase	Present thesis
NADPH cyt c red	Laboratory	Diesel oil	*Mytilus edulis*	DG	Increase	Livingstone et al. 1985
NADPH cyt c red	Laboratory	Diesel oil	*Mytilus edulis* *Littorina littorina*	DG	Increase	Livingstone 1987
NADPH cyt c red	Laboratory	Menadione	*Mytilus edulis*	DG	Increase	Livingstone et al. 1989
NADPH cyt c red	Field	Hydrocarbons	*Mytilus* sp.	DG	No change	Nasci et al. 1989
NADPH cyt c red	Laboratory	B(a)p (benzo(a)pyrene) TCB (tetrachlorobiphenyl) HCN (hexachlorobiphenyl)	*M. galloprovincialis*	DG	Increase Increase No change	Michel et al. 1993
NADPH cyt c red	Laboratory	Tributyltin (TBT) Triphenyltin (TPhT)	*M. galloprovincialis* *Tapes decussata* *Thais haemastoma*	DG	Inhibition Little effect	Morcillo and Cinta 1997
NADPH cyt c red	Laboratory	B(a)p	*Mytilus edulis*	DG	Increase (transient)	Okay et al. 2000

Tab 10: **Responses of glutathione S-transferase measured in the present thesis and by other authors**

Enzyme	Exposure	Contaminant	Species	Organ	Activity	Reference
GST	Laboratory	PAH	*Mytilus edulis*	DG	Increase	Present thesis
GST	Laboratory	Pb	*Mytilus edulis*	gills	Increase	Present thesis
GST	Laboratory	Pb	*Mytilus edulis*	DG	No change	Present thesis
GST	Field Mesocosm	PCBs/PAHs Diesel oil & copper	*Mytilus edulis*	DG DG	No change No change	Lee 1988, Suteau et al. 1988
GST	Field	PAHs	*Mytilus edulis*	DG	Little or no change	Sole et al. 1998
GST	Laboratory	B(a)p TCB HCN	*M. galloprovincialis*	DG	Decrease No change Increase	Michel et al. 1993
GST	Field	Hydrocarbons/PCBs/ organochlorines	*M. galloprovincialis*	DG	Slight decrease	Fitzpatrick et al. 1995
GST	Field/Laboratory	Metals (As, Se, Mn, Fe, Pb, Cu, Zn, Cr, Ni)	*M. galloprovincialis*	DG/gills	No trend	Regoli and Pricipato 1995
GST	Laboratory	Copper mercury	*M. galloprovincialis*	DG/gills	Increase	Canesi et al. 1999
GST	Laboratory	B(a)p	*M. galloprovincialis*	DG/gills	No change/ Decrease	Akcha et al. 2000
GST	Field	PCBs	*Perna viridis*	DG/gilsl	Correlation	Cheung et al. 2002
GST	Field	PAHs	*Mytilus edulis*	DG	Correlation	Gowland et al. 2002
GST	Laboratory	B(a)p/Aroclor	*Perna viridis*	DG	Correlation	Cheung et al. 2004
GST	Laboratory	Pb/Paraquat Pb Paraquat	*Perna perna*	DG	No effect No effect No effect	Dafre et al. 2004

Tab. 11: Responses of catalase measured in the present thesis and by other authors

Enzyme	Exposure	Contaminant	Species	Organ	Activity	Reference
CAT	Laboratory	Pb	*Mytilus edulis*	DG/gills	No change	Present thesis
CAT	Laboratory	PAH	*Mytilus edulis*	DG	Increase	Present thesis
CAT	Laboratory	Paraquat	*Geukensia demissa*	DG	Increase	Wenning et al. 1988
CAT	Field	PAHs	*M. galloprovincialis*	DG	Good correlation	Porte et al. 1991
CAT	Field	PAHs	*Mytilus edulis*	DG	Little or no change	Sole et al. 1998
CAT	Field/Laboratory	Metals (As, Se, Mn, Fe, Pb, Cu, Zn, Cr, Ni)	*M. galloprovincialis*	DG gills	Constant/decrease Increase	Regoli and Principato 1995
CAT	Laboratory	B(a)p	*M. galloprovincialis*	DG/gills DG/gills	Increase	Akcha et al. 2000
CAT	Field	PCBs/chlorinated pesticides	*Perna viridis*	DG/gills	No correlation	Cheung et al. 2002
CAT	Laboratory	Aroclor	*Perna viridis*	gills	Correlation	Cheung et al. 2004
CAT	Laboratory	Pb/Paraquat Pb Paraquat	*Perna perna*	DG	No effect No effect Decrease	Dafre et al. 2004

7.4 Contaminant-induced cytopathological alterations

Application of microscopic techniques for the localization of contaminants in association to cellular and subcellular components is a relatively new field in ecotoxicologial monitoring studies. Combination of toxin immunolocalization and pathological diagnosis by microscopic techniques constitutes an early warning measure with promising applications in future environmental monitoring studies.

Histopathological techniques have been widely used to describe and identify pollution induced effects in tissue and cells of marine invertebrates and vertebrates. In parallel to histopathology, chemical analysis of contaminants has been applied to provide information of chemical contents in whole organisms or specific tissues of the test animals. In general, chemical analysis, which often requires sophisticated, expensive and time-consuming techniques, provides no information about cellular distribution or interactions of chemicals with subcellular components.

Chapter 3-6 demonstrate that the use of antibodies directed against contaminants in microscopic diagnostics allows the study of pollutant-induced toxicity at the subcellular level and investigation of underlying mechanisms of toxicity leading to pathological alterations of cells and tissues. Lo-

calization sites of contaminants such as PCBs, PAHs and the metal Pb in cells of *Mytilus edulis* could be linked to observed pathological alterations. Specific localization sites were found for the different classes of contaminants. The endo-lysosomal system, in particular, plays an important role for sequestration and detoxification of all investigated contaminants, and early subcellular changes can be detected in these cell organelles. Many induced changes are not pollutant-specific in the early exposure phase and the cellular reaction to different toxicants consists of similar unspecific compensatory responses which are reversible. Manifestation of cellular alterations is associated with pathologies such as degenerative and irreversible processes, and is more specific to a type or a class of pollutants. Compensatory responses are of ecotoxicological significance and may serve as early warning biomarkers (Braunbeck 1998). Our study revealed several early responses at the subcellular level such as morphological alterations of mitochondria and ER, autophagic vacuoles or aggregation of residual bodies. Based on our results it is possible to identify target sites for the different contaminants and uncover subsequent cytopathological alterations. The combined application of cytopathology and microscopic detection of contaminants offers great potential for the elucidation of cause effect relationships in pharmaceutical and medical research as well as in pollution monitoring studies.

> Cytopathological alterations were identified at the subcellular level in relation to contaminant localization sites. Whereas specific routes and target sites for the chemicals could be identified and correlated to pathological effects, some of the observed subcellular changes should be considered as a general response to contaminant induced stress.

7.5 Perspectives

Information gathered in the present thesis shows a great potential and efficiency in the use of antibody-based methods for the discovery and identification of the presence of contaminants such as organic chemicals and metals in experimental exposure studies and marine environments. The implementation of this method in routine monitoring studies would provide a new tool for the identification of cause-effect relationships. Interactions of specific contaminants with various cell structures could be determined by imuno-detection and related to observed biological effects.

However, there are also limitations in the use of electron microscopical detection methods in routine monitoring programmes. Sample preparation for immuno-electron microscopy is a relatively time-consuming and sophisticated process at present. From sampling to visualization of results many fixation, incubation, dehydration, embedding, and sectioning steps are necessary, which take several days. For this reason, electron microscopic analysis of contaminants can only

be performed if manpower and microscopic equipment are available (which is generally not the case) and is restricted to a relatively low number of random samples. Nevertheless, this method offers great possibilities for the identification of contaminant induced-effects and underlying mechanisms of toxicity, when required on a case-by-case basis.

Cryo microscopic techniques are a further successful tool for immunological methods. A great advantage of cryo ultra microscopy is that multiple embedding and dehydration steps are circumvented, thereby protecting tissues, cells and antigens. Combination of the immunodetection technique, developed in the present thesis, with cryo ultra microscopy would safe time and money for sample processing and allows the preparation of a higher number of random samples.

In medical and pharmaceutical research application of the immunological CSA/GSSP technique is a promising approach to study uptake and accumulation sites of various drugs in tissues and cells, and their subcellular interactions and effects at specific localization sites. Combinatory effects of multiple drug treatments could be identified by using different antibodies and labelling system for the parallel detection of several drugs. Theoretically, this could be done in parallel tissue sections or with different secondary labelling techniques in the same section. Antagonistic or synergistic effects of multiple medical treatments could be investigated which would extremely increase the effectiveness and specifity of therapies.

Future research is required to validate and determine effectiveness of the immunodetection technique in field studies and for the use in other species. For field studies it seems to be a very promising tool due to its high sensitivity necessary for the detection of the presence of lowest amounts of chemicals. Application of the immunodetection system in other species and tissues as in the present thesis is under investigation in our laboratory at present.

7.6   References

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Vielen Dank an…

- **Frau Prof. Dr. Angela Köhler** für die Möglichkeit diese Arbeit am Alfred-Wegener-Institut anfertigen zu können und für die Betreuung der Arbeit.

- **Frau Prof. Dr. Klaudia Brix**, **Herrn Prof. Dr. Allan Cembella** und **Prof. Dr. Angela Köhler** für die Begutachtung der Dissertation.

- die gesamte Arbeitsgruppe Zellbiologie und Toxikologie insbesondere an **Sieglinde Bahns** (danke für alles!)**, Dr. Katja Broeg, Ute Marx** und **Jana Bressling**, die mir immer mit Rat und Tat zur Seite standen.

- **Sabine Schäfer** für ihre tatkräftige Unterstützung im Labor und beim Korrekturlesen der Arbeit sowie für ihre immer während Hilfsbereitschaft in allen Lebenssituationen.

- **Bjarne Lauritzen** für seine grossartige Einführung in die Welt der Antikörper und der Elektronenmikroskopie.

- **Herrn Prof J.J.L. van der Want** und seiner AG, Laboratory for Cell Biology and Electronmicroscopy, University of Groningen, für die Hilfe bei der Probenbearbeitung für die Immunzytochemie.

- **Frau Birgit Hussel** von der Wattenmeerstation Sylt für das Sammeln und Versenden unzählbarer Mengen an Miesmuscheln.

- **Dr. Ute Postel** für die vielen wertvollen Tipps und Anregungen sowie **Jeff Ardron** für seine „native speaker corrections".

- **Susanne Steck** für ihre Hilfe insbesondere in der Endphase der Doktorarbeit und eine langjährige Freundschaft.

- **meine Mutter** für ihre Liebe und Unterstützung. Danke!

- alle lieben Menschen, die mich auf diesem Weg begleitet haben.