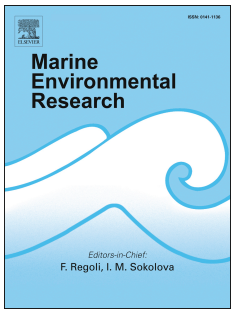

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Oxidative stress, lysosomal damage and dysfunctional autophagy in molluscan hepatopancreas (digestive gland) induced by chemical contaminants

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Two line capsule of paper:

Oxidative stress was induced by a range of contaminants in two marine molluscs resulting in dysfunctional lysosomal autophagy in digestive gland cells.

Keywords: *lysosomal membrane stability; dysfunctional autophagy; lipofuscinosis; lipidosis; oxidative stress; molluscs; hepatopancreas/digestive gland; chemical pollutants.*

Abstract

Autophagy is a highly conserved evolutionary survival or defence process that enables cells and organisms to survive periods of environmental stress by breaking down cellular organelles and macromolecules in autolysosomes to provide a supply of nutrients for cell maintenance. However, autophagy is also a part of normal cellular physiology that facilitates the turnover of cellular constituents under normal conditions: it can be readily augmented by mild environmental stress; but becomes dysfunctional with severe oxidative stress leading to cellular pathology. The molluscan hepatopancreas or digestive gland provides a versatile and environmentally relevant model to investigate lysosomal autophagy and stress-induced dysfunctional autophagy. This latter process has been implicated in many animal and human disease conditions, including degenerative and neurodegenerative diseases, as well as obesity related conditions. Many environmental pollutants have also been found to induce dysfunctional autophagy in molluscan hepatopancreatic digestive cells, and in this study, the marine blue mussel *Mytilus galloprovincialis* was exposed for 7 days to: 0.1 µM, 1 µM and 10 µM concentrations of fluoranthene and phenanthrene (PAHs); chlorpyrifos and malathion (organophosphorus compounds); atrazine (triazine herbicide); copper (transition metal) and dodecylbenzene sulphonic acid (LAS, surfactant). The marine snail or periwinkle, *Littorina littorea*, was also exposed to phenanthrene, chlorpyrifos and copper. Indices of oxidative stress, cell injury and dysfunctional autophagy were measured (i.e., lysosomal membrane stability, protein carbonyls, lipofuscin, and lysosomal accumulation of lipid or lipidosis). Evidence of oxidative stress, based on the elevation of lipofuscin and protein carbonyls, was found for all compounds tested; with chlorpyrifos being the most toxic to both species. Dysfunctional autophagy was induced by all of the compounds tested in both species, except for atrazine in mussels. This failure of normal autophagy was consistently associated with oxidative stress. Autophagic dysfunction is an important emerging feature in the aetiology of many disease conditions in animals and humans; and an explanatory conceptual mechanistic model has been developed for dysregulation of autophagy in response to oxidative stress.

Introduction

Autophagy is a highly conserved evolutionary survival or defence process that enables cells and organisms to survive periods of environmental stress by breaking down cellular organelles and macromolecules in autolysosomes to provide a supply of nutrients for cell maintenance (Cuervo, 2004, 2008; Klionsky et al., 2016). However, autophagy is also a part of normal cellular physiology that facilitates the turnover of cellular constituents under normal conditions: it can be readily augmented by mild environmental stress; but becomes dysfunctional with severe stress, such as oxidative stress, leading to cellular injury, with consequent tissue atrophy and organ pathology and reduction in physiological scope for growth (Boya, 2012; Cuervo, 2004, 2008; Lowe, 1988; Moore, 1982, 1988; Moore et al., 2006a, 2007a, b, 2015; Numan et al., 2015; Sforzini et al., 2018; Shaw et al., 2011). The molluscan hepatopancreas or digestive gland is a liver analogue that provides a versatile and environmentally relevant model to investigate lysosomal autophagy and stress induced dysfunctional autophagy (Lowe et al., 2006; Moore et al., 2006a, b, 2007b; Sforzini et al., 2018; Shaw et al., 2011). This latter process has been implicated in many animal and human disease conditions, including fibromyalgia, degenerative and neurodegenerative diseases, as well as obesity related conditions (Chen et al., 2012; Colacurcio et al 2018; Jiang & Mizushima, 2014; Namkoong et al., 2018; Nixon, 2013; Numan et al., 2015; Oezel et al., 2016).

Molluscan digestive cells have a highly developed lysosomal-vacuolar system that is essential for the digestion of endocytosed food particles by the process of intracellular digestion: the major component of digestion in mussels and periwinkles (Moore et al., 2006a; Moore, 1988). In addition to this heterophagic lysosomal digestion, digestive cells also have a highly developed capacity for lysosomally mediated autophagic digestion of cellular constituents including organelles and macromolecules (Koukouzika et al., 2009; Lowe, 1988; Moore et al., 2006a, 2007a, b; Sforzini et al., 2018). Effective performance of the autophagic processes is essential for the normal recycling of damaged and redundant proteins and organelles, as well as mobilisation of stored reserves for gametogenesis (Bayne et al., 1978; Lowe et al., 1982). Autophagy has a protective role in physiological responses to low-level environmental stress: however, more severe stress results in functional perturbation of autophagy leading to the pathological reactions described as dysfunctional autophagy (Boya, 2012; Cuervo, 2004; Dimitriadis

et al., 2004; Domouhtsidou et al. 2001; Moore, 2008; Moore et al., 2006b, 2008; Numan et al., 2015; Sforzini et al., 2018; Shaw et al., 2011).

Many environmental pollutant chemicals can induce oxidative stress that, in turn, can contribute to autophagic responses (Moore et al., 2006a, 2007b). If the oxidative stress becomes severe then the initial protective autophagic response can lead to a pathological dysfunctional autophagic reaction (Moore, 2008, 2010). Industrial and domestic incomplete combustion processes and oil spills, as well as vehicle emissions and road/tyre dust contribute to the influx of polycyclic aromatic hydrocarbons in air pollution and into the aquatic environment (Cajaraville et al., 1995; Numan et al., 2015; Shaw et al., 2011). Pest control and agricultural use are major contributors to the release of herbicides and pesticides into the aquatic environment. Environmental contaminants tested in the current study of oxidative stress, lysosomal damage and dysfunctional lysosomal autophagy include polycyclic aromatic hydrocarbons (phenanthrene & fluoranthene), pesticides (malathion & chlorpyrifos) a herbicide (atrazine), a surfactant (sodium dodecylbenzene sulphonate - LAS) and the transition metal copper (Moore et al., 2018; Readman et al., 2002). Copper, a known inducer of oxidative stress, has been, and is still, a global priority pollutant of fresh and marine waters (Hamed et al. 2006; Langston et al., 1999). These chemicals have been chosen on the basis of their well-documented toxicity and their continued presence in the environment in various parts of the world (Cassee et al., 1998; LeBlanc & Olmstead, 2004).

The aim of this investigation was to test for the induction of dysfunctional autophagy in molluscan hepatopancreatic digestive cells, associated with oxidative stress, by deploying biomarkers of cellular health in the marine blue mussel *Mytilus galloprovincialis* treated with fluoranthene and phenanthrene (PAHs); chlorpyrifos and malathion (OP - organophosphorus compounds); atrazine (triazine herbicide); copper (transition metal) and LAS (surfactant), at concentrations of 0.1 µM, 1 µM and 10 µM, for 7 days. In addition, marine snails or periwinkles (*Littorina littorea*) were also treated with chlorpyrifos, phenanthrene and copper at the same concentrations and for the same time period as the mussels. Animal health was assessed by measuring indices of oxidative damage and autophagy (protein carbonyls and lysosomal lipofuscin - lipofuscinosis), cellular health (lysosomal membrane damage) and fatty change (equivalent to steatosis in mammalian liver); with a pathological dysfunctional autophagic

accumulation of triglyceride (lipidosis) in abnormally enlarged autolysosomes (Domouhtsidou et al. 2001; Lowe, 1988; Moore, 1988; Moore et al., 2006a, 2007b). Data for lysosomal and oxidative stress biomarkers was synthesised using multivariate analysis, with the first principal component being used as an integrated index of "health status" (Allen & Moore, 2004; Moore et al., 2006a; Sforzini et al., 2018).

Materials & Methods

Chemicals

Most chemicals were obtained from Sigma-Aldrich, unless stated otherwise. Anthracene and phenanthrene were > 99% pure; LAS (dodecylbenzene sulphonate) was Pharmaceutical Secondary Standard - Certified Reference Material; pesticides and herbicides were analytical standard grade; DMSO (> 99.9%); and neutral red powder (N4638) was graded as suitable for cell culture. Other reagents used were of ANALAR grade. Cytochemical substrates for lysosomal hydrolases, naphthol-AS-BI-N-acetyl-β-gluosaminide and naphthol-AS-BI-β-glucuronide, were obtained from Sigma-Aldrich. The diazonium coupler fast red-violet LB was obtained from Difco Laboratories. The anti-DNPH (dinitrophenyl) primary antibody (A6435), Nanogold secondary antibody (A-24926) and the silver enhancement kit (L24919) were purchased from Molecular Probes, UK.

Animal Husbandry and Experimental Treatments

Mussels *Mytilus galloprovincialis* (4.8 – 5.2 cm) were collected from Trebarwith Strand, North Cornwall, U.K. (avoiding the spawning period); and periwinkles (littorinids) *Littorina littorea* (2 - 2.5 cm) were collected from Port Quin, North Cornwall, U.K. The animals were placed in 34 psu, 15 ± 1ºC, 1 µm filtered sea water (aerated) to acclimate for one week. Sea water was replaced daily. Mussels were fed 30 mg/animal/day of dried kelp continuously via a peristaltic pump throughout the exposures and periwinkles were fed green sea lettuce *Ulva* (collected from Port Quin) *ab libitum*. The animals were divided into five treatments: control (C), solvent or vehicle control (VC; 0.02% DMSO), and 0.1 µM, 1 µM and 10 µM initial chemical concentrations, which were renewed on a daily basis. Mussels were treated in 10L polypropylene tanks (10 animals/tank and natural daylight regime) with atrazine, chlorpyrifos, copper, fluoranthene, dodecylbenzene sulphonic acid (a linear alkylbenzene sulphonate, LAS), malathion and phenanthrene. Periwinkles were exposed as with the mussels to chlorpyriphos, copper and

phenanthrene. Neither Cu nor malathion required the use of a solvent (DMSO). Animals were dosed with the contaminant each day for 7 days and sampled on day 0 and day 7. Digestive glands were carefully removed, plunged into liquid nitrogen and stored at -40ºC.

Chemical Analyses – Water and Digestive Gland

Fluoranthene, phenanthrene, chlorpyrifos, malathion and atrazine analyses

Half an hour after the contaminant was added to the exposure vessels, 250 ml water samples were removed, taking care to avoid the putative surface microlayer, and placed in acid washed, hexane rinsed, stoppered glass vessels. 50 ml of dichloromethane (DCM) was added to stabilize the samples before being kept at 4ºC until analysis (DCM was omitted for Cu samples). The samples were analysed for organic contaminants essentially as described by Kelly et al. (2000). The clean-up was derived from Readman et al. (2002), and was rigorously tested for performance/recoveries using spiked standards.

Water samples (including the DCM) were spiked with an internal deuterated standard, mixed and then transferred directly to a glass separating flask fitted with a PTFE tap. The phases were shaken vigorously for 2 minutes and allowed to settle for a further 2 min before the solvent phase was run off into round bottomed flasks. This extraction procedure was repeated twice with additional DCM. The combined DCM fraction was dried using a small amount of anhydrous sodium sulphate (Na_2SO_4). To remove the Na_2SO_4, the extracts were transferred to a second series of round bottom flasks with rinsings from the original flasks. The extracts were concentrated down to ~ 2 ml using a BUCHI rotary evaporator before being transferred to the final analysis vials and blown down to 1 ml. Analysis was performed using gas chromatography mass spectrometry. 1 µl of sample was injected into a 30 m x 0.250 DB-5MS column with helium used as the carrier gas. The injector was set on splitless, with a temperature of 250ºC. The oven had an initial temperature of 40^{o}C which was held for 2 mins before being ramped at 6ºC per min to reach a final temperature of 300ºC.

Digestive glands and remainder tissues were stored at -80ºC prior to analysis (Law et al., 1999). The samples were allowed to defrost at room temperature. A sub-portion was freeze dried to obtain moisture content. The mussel tissues were weighed into round

bottom flasks, spiked with a deuterated internal standard and digested by adding 20 g of potassium hydroxide pellets and 100 ml of methanol (saponification). The samples were refluxed for 4 hours and filtered through glass fibre filters. The filtrate was placed into separating flasks with 50 ml *n*-pentane and shaken for 2 min and allowed to settle and separate for a further 2 min after which the phases were separated by running off the lower layer and collecting the upper *n*-pentane layer. This extraction procedure was carried out a second time and the *n*-pentane layers combined and dried over Na_2SO_4. The samples were reduced to ~5ml by rotary evaporation. Due to the release of lipids and co-extractives a clean up using alumina and silica was required.

To ensure the quality of the analytical results, reagent blanks and recoveries were run with each batch. Good quantitative data was achieved using relevant deuterated internal standards. Instrument calibration and instrument response factors were run and calculated for each batch of analyses.

LAS analysis in water

LAS was determined by fluorimetry, using a Perkin Elmer LS-50B fluorometer and FLWinlab program. New standards were prepared on a daily basis to prevent standard degradation. The standard range was 0 – 2.5 mg/L and quenching occurred around 3 mg/L. No sample preparations were necessary and both standards and samples were analysed using a quartz cuvette under the following conditions: excitation wavelength – 231 nm; emission wavelength – 301 nm; excitation slit width -10 nm; emission slit width – 10 nm; emission filter – 350 nm cut off.

Chlorpyrifos, malathion and LAS were not measured in tissue because they are comparatively polar, are difficult to extract and isolate, and tend not to bioaccumulate.

Cu determination

Copper was determined using a combination of flame and graphite furnace atomic absorption spectrophotometry, depending on concentration. Analysis of tissues was performed on freeze-dried samples following microwave digestion in high purity (Aristar) nitric acid. (Langston et al. 1999).

Cytochemical Determinations for Lysosomal Stability, Lipofuscin and Lysosomal Neutral Lipids

The following assays were all performed on 10 µm frozen sections of mussel and periwinkle digestive gland. Lipofuscin was determined *via* the Schmorl assay as described by Moore (1988). Lysosomal membrane stability was measured by determining the latency period for N-acetyl-β-hexosaminidase activity (mussels), and β-glucuronidase (periwinkles) (Moore, 1988; Moore et al., 2008; Sforzini et al., 2018). Lysosomal accumulation of neutral lipids (triglycerides) was determined using the Oil Red O method (Moore et al., 2008). Lipofuscin and lysosomal neutral lipid were assessed using series of micrographs based on increasing relative absorbance of their respective cytochemical reaction product (Moore 1988; Moore et al., 2006a, 2007b, 2008).

Immunocytochemical Determination for Protein Carbonyls

Protein carbonyls were determined immunocytochemically in 10 µm frozen sections of digestive gland method using a modified 2,4-dinitrophenylhydrazine detection method (Alamdari et al., 2005; Frank et al., 2005; Smith et al., 1998; Shaw et al., 2011). Following sectioning, the sections were mounted on slides and air dried at room temperature for 1 hour. Subsequently, the sections were fixed in Baker's calcium formal fixative solution (10% formaldehyde, 2.5% sodium chloride, 1% calcium chloride) for 5 minutes at 4^{o}C. The slides were rinsed in distilled water before being incubated in 2,4-dinitrophenylhydrazine (10 mM DNPH in 6M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5) solution for 45 min at room temperature in a humidity box in the dark (Alamdari et al., 2005; Frank et al., 2005; Smith et al., 1998). Following this, the slides were rinsed in distilled water followed by PBS for 10 minutes, and a blocking solution was applied (non-fat dried milk 1:10 in PBS) for 10 minutes. The slides were placed in anti-DNPH (1:100 in PBS) overnight at 4^{o}C. The slides were then rinsed in PBS before being incubated in nano-gold secondary antibody overnight at 4^{o}C. Following this, the slides were washed in sodium citrate buffer (3 M sodium chloride, 300 mM sodium citrate, pH 7) before silver enhancement. Protein carbonyls were assessed by construction of a series of micrographs based on increasing protein carbonyl immunocytochemical reaction product density (Moore 1988; Moore et al., 2008; Shaw et al., 2011).

Univariate Statistical Analysis

The non-parametric Mann-Whitney *U*-test was used to compare the data from treated animals with those of the controls (Sforzini et al., 2018).

Multivariate Analysis

Biomarker data for mussels and periwinkles exposed to the seven test chemicals were analysed using non-parametric multivariate analysis software, PRIMER v 6 (PRIMER-E - University of Auckland, New Zealand; Clarke, 1999; Clarke & Warwick, 2001; Moore et al., 2006a). All data were log transformed [$\log_n(1 + x)$] and standardised to the same scale. Correlations between biomarkers were tested using a scatter plot matrix (PRIMER v 6, Draftsman Plot). Principal component analysis (PCA) and hierarchical cluster analysis, derived from Euclidean distance similarity matrices were used to visualise dissimilarities between sample groups. The results were further tested for significance using analysis of similarity (PRIMER v6 - ANOSIM), which is an approximate analogue of the univariate ANOVA and reflects on differences between treatment groups in contrast to differences among replicates within samples (the *R* statistic). Under the null hypothesis H_0 ("no difference between samples"), $R = 0$ and this was tested by a non-parametric permutations approach; there should be little or no effect on the average R value if the labels identifying which replicates belong to which samples are randomly rearranged.

Finally, in order to map integrated biomarker data onto "health status space", measured primarily as the first principal component - PC1 for the biomarker data, the individual biomarkers were correlated with PC1 from the integrated evaluation (Allen and Moore, 2004; Moore et al., 2006a; Sforzini et al., 2015, 2017, 2018).

Results

***Chemistry* (see Table 1)**

No nominal water values matched the dose concentrations. This probably resulted from several factors. Water samples were not taken for half an hour after sampling, to allow dispersal, however during this period the contaminants may have sorbed onto the exposure tanks surfaces, suspended particulate matter or the animals themselves. Alternatively, they may have been ingested, in particular by the filter feeding mussels.

Tissue concentrations of the xenobiotics measured indicated that the two PAHs were accumulated in mussel digestive gland (Table 1). Phenanthrene accumulated in the periwinkle digestive gland; but to a lesser extent than in the mussels (Table 1). Copper accumulated in both species, and the concentrations in the periwinkle digestive gland were greater than in the mussels (Table 1).

Biomarkers **(see Table 2: summary of results; Figures 1-5)**

Lipofuscin

Lipofuscin in mussels was increased significantly ($p \leq 0.05$) at all exposure concentrations for both phenanthrene and fluoranthene (Fig. 1). Lipofuscin was also elevated at 0.1 µM and 10 µM for malathion (Fig. 1). Liposfuscin was only significantly higher within the 10 µM exposure mussels for LAS, copper and chlorpyrifos, while there were no significant changes following atrazine exposure (Fig. 1).

Lipofuscin in littorinid snails was significantly ($p \leq 0.05$) elevated in all exposure concentrations for phenanthrene (Fig. 5). Lipofuscin was significantly higher in the 1 µM and 10 µM animals exposed to chlorpyrifos, while the 10 µM concentration of copper induced a significant increase (Fig. 5).

Lysosomal membrane stability (based on latency of N-acetyl-β-hexosaminidase for mussels & β-glucuronidase for littorinids)

Mussel lysosomal stability was significantly decreased ($p \leq 0.05$) in all concentrations for chlorpyrifos (Fig. 2), and in the 1 µM and 10 µM concentrations for atrazine (Fig. 2), fluoranthene, phenanthrene (Fig. 2) and malathion (Fig. 2). Lysosomal stability was only seen to significantly decrease in 10 µM copper and LAS treatments (Fig. 2).

Periwinkle lysosomal stability significantly decreased at all concentrations for chlorpyrifos, at 1 µM and 10 µM for phenanthrene, and only at the 10 µM copper concentration (Fig. 5).

Fatty change & autolysosomal lipids (triglycerides)

Mussel neutral lipids (triglycerides), within swollen autolysosomes (i.e., increase in lysosomal/cytoplasmic volume ratio – L/C vol), were increased significantly ($p \leq 0.05$) in all chlorpyrifos exposure treatments (Fig. 3). Lipids were also significantly elevated in the

10 µM LAS, 1 µM fluoranthene and 0.1 µM copper treatments (Fig. 3). However, neutral lipids were significantly decreased in the 1 µM and 10 µM malathion exposures (Fig. 3); and there were no differences in lipids in mussels exposed to atrazine (Fig. 3).

Periwinkle neutral lipids, again within swollen autolysosomes, were significantly elevated ($p \leq 0.05$) in all exposure treatments for chlorpyrifos and copper; and in the 1 µM and 10 µM phenanthrene treatments Fig. 5).

Protein carbonyls

Mussel protein carbonyls were significantly ($p \leq 0.05$) elevated in the 1 µM and 10 µM atrazine, copper, fluoranthene and LAS exposure treatments and in the 10 µM chlorpyrifos, malathion and phenanthrene treatments (Fig. 4).

Periwinkle protein carbonyls were significantly ($p \leq 0.05$) increased in the 1 µM and 10 µM chlorpyrifos and phenanthrene treatments; and at all three concentrations of copper treatment (Fig. 5).

Multivariate analysis

Principal component (PCA) and hierarchical cluster analysis of all the biomarker reactions showed that the various treatments had a detrimental effect on the digestive cells of both mussels and periwinkles (Fig. 6, 7 & 8). Analysis of similarity showed that these clusters were significantly different (ANOSIM, $p \leq 0.001$); and clear separation of the effects of different treatments was apparent in the PCA and Cluster plots (Fig. 6, 7 & 8), and particularly for the periwinkles (Fig. 8). Regression analysis (Scatter Plot matrices, not shown) of the mussel biomarker data for the four most toxic treatments (i.e., chlorpyrifos, copper, fluoranthene & phenanthrene) indicated that LMS, was inversely correlated with lipofuscin, lysosomal lipid and protein carbonyls; and that lipofuscin was correlated with lysosomal lipid, except for the phenanthrene treatment, and protein carbonyls (Table 3). However, lysosomal lipid and protein carbonyls were not correlated (Table 3).

When all chemical treatments were analysed together for both species of mollusc, all four biomarkers were strongly correlated ($p \leq 0.001$) with the first Principal Components (Table 4).

Discussion

Indices of oxidative stress, cell injury and dysfunctional autophagy were assessed in both species of mollusc (i.e., lysosomal membrane stability, lipofuscin, protein carbonyls, and fatty change involving lysosomal accumulation of lipid [triglyceride] or lipidosis). Evidence of oxidative stress, based on the elevation of lipofuscin and protein carbonyls, was found for all compounds tested; with chlorpyrifos being the most toxic to both species (Sohal & Brunk, 1989, Sforzini et al., 2018; Shaw et al., 2011). Dysfunctional autophagy was induced by all of the compounds tested in both species, except for atrazine in mussels. Furthermore, dysfunctional autophagy was characterised by reduced lysosomal membrane integrity, increased lipofuscin or lipofuscinosis (indicative of oxidative stress) and increased lysosomal lipid or lipidosis (Moore et al., 2006a, 2007b; Terman & Brunk, 2002). This failure of normal autophagy was generally associated with oxidative stress (Moore et al., 2006b, 2007a, b, 2008).

All of the chemicals tested in this study induced some degree of oxidative stress (Table 2). ROS can damage lipoprotein membranes, and cellular proteins producing protein carbonyls and other oxidative products (Shaw et al., 2011; Sforzini et al., 2018). The damaged proteins and membranes are normally removed by autophagy; however, if the level of intracellular damage becomes excessive due to severe oxidative stress, then the autophagic processes are overwhelmed and become dysfunctional (Fig. 9). Lipofuscin build-up as a result of lipid peroxidation of autophagocytosed lipoprotein membranes (e.g., mitochondria and endoplasmic reticulum) is an effective biomarker for moderate to severe oxidative stress. However, lipofuscin, long thought of as an inert waste product of “cellular housekeeping”, is an effective generator of ROS due to Fenton reactions mediated by lipofuscin-bound iron, thereby, inhibiting lysosomal degradation (Grune et al., 2004; Terman & Brunk, 2004). The inference is that abnormal accumulation of lipofuscin in autolysosomes (i.e., lipofuscinosis) will result in excessive ROS production and oxidative stress (Fig. 9). ROS generation *in vivo* hepatopancreatic digestive cells has been demonstrated by Winston et al. (1991), indicating that there is a highly reactive oxidative intra-lysosomal environment in these cells (Terman & Brunk, 2002).

Fatty change is often a cellular pathological feature of a number of diseases including those resulting from exposure to a variety of organic xenobiotics (Fig. 9; Klaunig et al.,

2018). However, somewhat paradoxically starvation can also induce fatty change in mammals (Kneeman et al., 2012; Ohama et al., 1994), although there is no evidence for this pathological reaction in molluscs (Moore et al., unpublished data). This excess cytoplasmic lipid (triglyceride) can be autophagocytosed (lipophagy) and accumulates in autolysosomes: a condition known as lipidosis and effective autophagic degradation of intracellular material is inhibited by fatty change (Cingolani & Czaja, 2016; Koukouzika et al., 2009; Moore, 1988; Moore et al., 2007b; Ward et al., 2016; Yan et al., 2017).

The overall consequence is that oxidative stress results in reduced food uptake by inhibition of endocytosis, and augmented autophagy of damaged organelles, lipoprotein membranes and proteins (Fig.9; Flinn & Backer, 2010; Han & Wang, 2018; Sforzini et al., 2018). As the severity of oxidative stress increases, normal lysosomal and autophagic function is dysregulated, with reduced lysosomal membrane stability, intralysosomal build-up of triglyceride, phospholipid and lipofuscin; and further generation of ROS from the lipofuscin (Moore et al., 2006a, 2007b; Terman & Brunk, 2002).

This pathological syndrome of oxidative stress-linked dysfunctional autophagy is a major contributor to programmed cell death (PCD; Fig. 9), probably mediated by both apoptosis (PCD Type 1) and autophagic cell death (PCD Type 2) (Cuervo, 2008; Das et al., 2012; Lowe, 1988; Shimizu et al., 2014). The consequences for the animal will be digestive gland tissue atrophy as described by Lowe (1988), loss of fecundity and overall functional deterioration leading to death (Bayne et al., 1978; Lowe et al., 1982).

PAHs are known to accumulate in molluscan digestive cell lysosomes and cause damage to the membrane (Moore et al., 2004, 2006a, 2007a, b; Sforzini et., 2018). Moore (1988) reported enlarged lysosomes, lipidosis, lipofuscinosis and a large reduction in lysosomal stability in periwinkles exposed to PAHs and other contaminants; and Lowe et al. (2006) also saw a reduction in lysosomal membrane stability in periwinkles exposed to 2 µM fluoranthene for 5 days. Lysosomal membrane disruption may also result from the ability of PAHs, due to their lipophilicity, to penetrate and incorporate phenanthrene and fluoranthene into phospholipid membranes resulting in increased membrane lipid unsaturation and hydrophobicity (Nelson et al., 1990).

Furthermore, PAHs can be biotransformed in mussels by cytochromes P450 (a large family of proteins that biotransform exogenous and endogenous molecules) and hydroperoxides (Garcia Martinez, Livingstone, 1995; Lemaire et al., 1993). These hydroperoxides can catalyse PAH metabolism, forming cation radicals which can also bind to DNA and other macromolecules (Livingstone et al. 1990).

Chlorpyrifos and malathion are both known to induce oxidative stress in molluscs, and this reaction was associated with lysosomal injury and autophagic dysfunction, particularly with chlorpyrifos treatment in mussels and periwinkles (Canesi et al., 2011; Khalil, 2015; Livingstone, 2003). Oxidative damage as a result of malathion exposure has been reported in the freshwater snail *Stagnicola* sp., including elevation of MDA (Martinez-Tabche et al., 2002). Periwinkles were not treated with malathion.

Atrazine can induce oxidative stress in Pacific oysters (*Crassostrea gigas*), and some evidence for oxidative stress was observed in treated mussels (Table 2; Lee et al., 2017). However, this was limited to increased protein carbonyls and lysosomal destabilisation; and there was no evidence for dysfunction of autophagy (Table 1).

Copper accumulates in mussel lysosomes and probably exerts its toxicity by ROS attack on lysosomal membranes with subsequent reduction in membrane stability (Ringwood et al., 1998; Shepard & Bradley, 2000). However, in this study lysosomal stability did not prove to be more sensitive than induction of either lipofuscin, or protein carbonyls in either mussel or snails (Table 2). Elevated mussel lipofuscin and protein carbonyls, and reduced lysosomal membrane stability, were seen only in the 10 µM exposure. No elevated lipofuscin was seen in any periwinkle copper treatment (Table 2; Fig. 5). However increased protein carbonyl formation was detected in both the 0.1 µM and 1 µM exposures; and lysosome stability was seen to decrease only in the 10 µM treatment.

LAS, in common with other surfactants, has a strong affinity for sediments and it is thus postulated that filter feeders may have increased vulnerability to its toxic effects (Da Ros et al., 1995; Sanderson et al., 2006). However, LAS was only found to exert a free radical mediated toxic effect at the highest exposure concentration of 10 µM in this study (Fig. 1-4), suggesting that it is far less toxic than all the other compounds assessed in

the study, excepting atrazine. LAS probably exerts its toxic effect through the disruption of biological membranes via intercalation into the membrane bilayer. This can result in the solubilisation and substitution of the component proteins and lipids consequently disrupting membrane functions such as endocytosis and phagocytosis (Jackson et al. 1977). These observations suggest that the reduction of lysosomal membrane stability seen within this study may also result from LAS intercalation as well as ROS activity, as shown by the elevation of protein carbonyls (Table 2; Fig. 4). However, the absence of any increase (or even reduction) of lipofuscin indicated that oxidative stress was probably limited to the 10 µM LAS treatment (Table 2; Fig. 1).

In summary, the patterns of toxic cell pathological reactions were very similar between mussel and periwinkle as previously demonstrated (Moore, 1988; Moore et al., 1987a, b). However, the snails were generally more susceptible to the toxic effects of chlorpyrifos and copper, which agrees with previous work demonstrating the robustness of mussels (*M. edulis*), compared to the common limpet (*Patella vulgata*) and crab (*Carcinus maenas*), on exposure to copper (Brown et al. 2004); and also the mussel *Amblema plicata*, compared to the oyster *Crassostrea virginica,* following exposure to chlorpyrifos (Borthwick et al. 1981; Doran et al. 2001). The main difference between the two test species in this investigation was that evidence for fatty change and lipidosis was more pronounced in the snails (see Table 2; Fig. 5).

Principal component analysis (PCA) is an effective method for integrating biomarker data for lysosomal pathology and oxidative stress into a “health status space”, reducing the multi-dimensionality of the problem to a simple two dimensional representation (Allen and Moore, 2004; Chatfield and Collins, 1980). PCA is commonly used as a cluster analysis tool and effectively captures the variability in a dataset in terms of principal components. Previously PCA has facilitated modelling the integrated responses of multiple biomarkers in the context of “health status space” (Allen and Moore, 2004; Moore et al., 2006a, Sforzini et al., 2015, 2017, 2018). These models have shown that there is a strong direct relationship between LMS, as an indicator of cellular health, and the first principal components for the combined biomarker responses (Fig. 6, 7 & 8; Table 4; Allen & Moore, 2004; Moore et al., 2006a; Sforzini et al., 2018). The other biomarkers (lipofuscin, lysosomal lipid & protein carbonyls) are inversely correlated with the first principal components (Table 4).

Previous PCA and cluster analysis, which does not integrate the various biomarkers in a functionally meaningful way, was previously employed as the first stage in developing numerical and network models for environmental impact on the health of sentinel animals such as mussels and earthworms (Allen & McVeigh, 2004; Allen & Moore, 2004; Moore et al., 2015; Sforzini et al., 2016, 2017, 2018). Network models particularly, encapsulate the cellular physiological processes that enable the construction of a logical functional framework through the interconnections between the biomarker data; and, such models have repeatedly demonstrated the importance and utility of the set of biomarkers used in this investigation (Figs. 6, 7 & 8). The main output from the network models is a measure of system complexity (connectance %) and this is very strongly correlated with the first principal components and lysosomal membrane stability (LMS) as measures of functional integrity. Consequently, in this investigation, the first principal component is considered to be an effective functionally integrated representative index of “health status” (Moore et al., 2006a, 2007b; Sforzini et al., 2015, 2017, 2018).

A conceptual mechanistic model for stress reactions in molluscan hepatopancreatic digestive cells related to lysosomal functions autophagy has been developed to explain the findings from this investigation and the links between reactive oxygen species (ROS) generation, oxidative stress and autophagy (Fig. 9). The model also draws on previous studies (Moore et al., 2006a, 2007b; Shaw et al., 2011), including the effects of benz[a]pyrene on the mTOR (mechanistic target for rapamycin) cell signalling system (Sforzini et al., 2018). mTOR complex 1 (mTORC1) is one of the key controlling foci in the cellular regulatory network, where it regulates many aspects of cell growth. mTORC1 specifically regulates lysosomal membrane stability (permeability), lipogenesis and endocytosis; while inhibition by nutrient stress or oxidative stress triggers augmented physiological lysosomal autophagy (Flinn & Backer, 2010; Han & Wang, 2018; Laplante & Sabatini, 2012; Moore et al., 2015; Tan & Miyamoto, 2016). The model describes the interactive relationships between ROS, oxidative stress, mTORC1 inhibition, endocytosis, augmented physiological autophagy, lysosomal membrane stability, lipogenesis, fatty change, lysosomal lipidosis, lipofuscin generation, lysosomal lipofuscinosis, dysfunctional autophagy and programmed cell death (PCD Types 1 & 2) and digestive gland tubule tissue atrophy (Lowe, 1988). The mechanistic links between

these various pathological reactions have been described previously using quantitative network models for mussels and periwinkles (Moore et al., 2015; Sforzini et al., 2018).

Conclusions

Despite the diverse array of chemicals (i.e., PAHs, organophosphate insecticides, herbicide, transition metal and surfactant) used within this investigation, all have demonstrated a common initial mechanism of toxicity in the two test species. Oxidative stress, assessed via products of damage, appears to be a useful non-specific measurement of cellular perturbation. Lysosomal and autophagic dysregulation involving fatty change and lipid accumulation within the lysosomes of digestive cells also appears to be a useful integrated indicator of general chemical-induced stress as confirmed by previous work (Moore 1988; Domouhtsidou et al. 2001; Sforzini et al., 2018).

The toxic effects of the majority of compounds used within this study tended to be more prominent at the higher treatment concentrations (i.e., $\geq$1 µM), and so, perhaps less environmentally relevant. However, animals were only exposed for 7 days; and much, if not most, of aquatic pollutant toxicity is chronic in nature. Consequently, the detrimental impact of exposure to these contaminants, at even the lowest levels used within this investigation, is likely to increase in severity over time.

The loss of normal and augmented autophagic function in the hepatopancreatic digestive cells, as a result of autophagic dysregulation, will have profound effects on the physiology of the mussels and periwinkles. The hepatopancreas or digestive gland is the major organ for digestion, assimilation of nutrients and storage of energy reserves (i,e. glycogen and lipids); and detoxication of toxic metals and organic xenobiotics (Moore et al., 2006a, 2007b). As such, its normal and physiologically augmented autophagic functions are essential for the wellbeing of the whole animal; and if these become dysfunctional then the health of the animal declines as demonstrated by the related decline in physiological scope for growth (Moore et al., 2006a).

Failure of normal and physiologically augmented autophagic function as a pathological reaction to chemically induced stress appears to be a widespread phenomenon in many eukaryotic species, including molluscs, annelids and mammals, perhaps indicating that this type of reaction is generic. Evidence for the generic nature of this pathological

reaction, is supported by the observations from the present investigation and builds on previous studies (Cuervo, 2004, 2008). Dysfunctional autophagy is characteristic of a number of animal and human diseases, including neurodegenerative diseases, fibromyalgia, ocular pathologies, cardiovascular disease and obesity related conditions (Cajaraville et al., 1995; Chen et al., 2012; Colacurcio et al 2018; Cuervo, 2004; Domouhtsidou et al. 2001; Jiang & Mizushima, 2014; Moore et al., 2006a, 2007b; Namkoong et al., 2018; Nixon, 2013; Numan et al., 2015; Oezel et al., 2016; Sforzini et al., 2018; Yan et al., 2017). Some of these disease conditions have been linked to uptake of harmful nanoscale materials derived from road traffic (Bai et., 2016; Chen et al., 2016; Numan et al., 2015).

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Figure Legends

Fig. 1. Lipofuscin in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; lipofuscin (absorbance).

Fig. 2. Lysosomal membrane stability (LMS - based on N-acetyl-β-hexosaminidase latency) in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; LMS is shown in minutes.

Fig. 3. Neutral lipids (triglycerides) in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; neutral lipids (absorbance).

Fig. 4. Protein carbonyls in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; protein carbonyls (density).

Fig. 5. Lipofuscin, lysosomal membrane stability (based on β-glucuronidase latency), lysosomal lipids and protein carbonyls in digestive gland of periwinkles (2 – 2.5 cm) exposed to 0.1 µM, 1 µM and 10 µM of chlorpyrifos, copper and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL.

Fig. 6. Combined principal component and cluster analysis (i.e., elliptoid boundaries based on Euclidean distance) for all of the test compounds, based on the data for the four biomarkers in mussels. Vectors for the individual biomarkers are shown; and the large arrow indicates increasing cellular pathology. Exposure times are noted in key as 0 or 7 (days). PC1 and PC2 captured 44.5% and 24.3% of the variation respectively.

Fig. 7. Individual principal component analysis and cluster analysis (i.e., elliptoid boundaries based on Euclidean distance) for each of the test compounds, based on the data for the four biomarkers in mussels. Vectors for the individual biomarkers are shown; and the large arrow indicates increasing cellular pathology. Exposure times are noted in key as 0 or 7 (days).

Fig. 8. Combined principal component analysis and cluster analysis (i.e., elliptoid boundaries based on Euclidean distance) for all of the test compounds, based on the data for the four biomarkers in periwinkles. Vectors for the individual biomarkers are shown; and the large arrow indicates increasing cellular pathology. Exposure times are noted in key as 0 or 7 (days). PC1 and PC2 captured 54.8% and 22.4% of the variation respectively.

Fig. 9. Conceptual mechanistic model for the role of oxidative stress in dysregulation of autophagy and heterophagy in hepatopancreatic digestive cells. Processes outlined in narrow red are considered to be potentially adverse; and those outlined in thick red constitute cell injury and programmed cell death (PCD). mTORC1 is the mechanistic target for rapamycin complex 1 – a key component of the cellular signalling system. Dotted arrows indicates generic process, although not yet confirmed in molluscs.

Table 1. Table of water (Day 0) and tissue chemistry for mussel and periwinkle following 7 days exposure treatments to contaminant chemical concentrations of 0.1 µM, 1 µM and 10 µM.

Treatment		*Mussel*		*Periwinkle*	
		Water (µM/L)	**Tissue (µg/g dry weight)**	**Water (µM/L)**	**Tissue (µg/g dry weight)**
Atrazine	Control	0	-	-	-
	0.1µM	0.029468	-	-	-
	1 µM	0.45563	-	-	-
	10 µM	3.557744	-	-	-
Chlorpyrifos	Control	0	-	0	-
	0.1 µM	0.018	-	0.002	-
	1 µM	0.068	-	0.056	-
	10 µM	0.685	-	0.621	-
Copper	Control	0.004	0.77	0.005	1.55
	0.1 µM	0.043	0.89	0.068	1.8
	1 µM	0.41	2.85	0.57	4.67
	10 µM	8.2	2.8	9.83	4.2
Fluoranthene	Control	0	0.3	-	-
	0.1 µM	0.022	39.2	-	-
	1 µM	0.810	833.6	-	-
	10 µM	5.073	4273.9	-	-
LAS	Control	0.09	-	-	-
	0.1 µM	0.3261	-	-	-
	1 µM	0.8098	-	-	-
	10 µM	4.2055	-	-	-
Malathion	Control	0	-	-	-
	0.1 µM	0.085	-	-	-
	1 µM	0.911	-	-	-
	10 µM	3.193	-	-	-
Phenanthrene	Control	-	8.69	0.001	0.02
	0.1 µM	-	85.72	0.022	4.26
	1 µM	-	870.53	0.184	86.52
	10 µM	-	2061.13	1.297	605.68

Water chemistry results are presented as µM/L. Tissue chemistry results are presented as µg/g dry weight. Chlorpyrifos, LAS and malathion cannot be quantified in tissue; *L. littorea* was not exposed to atrazine, fluoranthene, LAS or malathion.

Table 2. Summary of biomarker responses and the inferred cellular pathological reactions in mussel and periwinkle digestive cells. Mussels were exposed to atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene, and periwinkles were exposed to chlorpyrifos, copper and phenanthrene at concentrations of 0.1 µM, 1 µM and 10 µM for 7 days.

Treatment	*Lysosomal lipofuscin*	*Lysosomal stability*	*Lysosomal lipids*	*Protein carbonyls*	*Oxidative stress*	*Dysfunctional autophagy*
Atrazine						
0.1 µM						
1 µM		⬇	⬇	⬆	✓	
10 µM		⬇	⬇	⬆	✓	
Chlorpyrifos						
0.1 µM		⬇⇩	⬆⇧			✓✓
1 µM	⇧	⬇⇩	⬆⇧	⇧	✓	✓✓
10 µM	⬆⇧	⬇⇩	⬆⇧	⬆⇧	✓✓	✓✓
Copper						
0.1 µM			⬆⇧	⇧		
1 µM			⇧	⬆⇧		
10 µM	⬆	⬇⇩	⇧	⬆⇧	✓✓	✓✓
Fluoranthene						
0.1 µM	⬆				**?**	
1 µM	⬆	⬇	⬆	⬆	✓	✓
10 µM	⬆	⬇		⬆	✓	✓
LAS						
0.1 µM				⬆	**?**	
1 µM				⬆	**?**	
10 µM	⬇	⬇	⬆	⬆	✓	✓
Malathion						
0.1 µM	⬆				**?**	
1 µM		⬇	⬇			
10 µM	⬆	⬇	⬇	⬆	✓	✓
Phenanthrene						
0.1 µM	⬆⇧				**?** ?	
1 µM	⬆⇧	⬇⇩	⇧	⇧	✓✓	✓✓
10 µM	⬆⇧	⬇⇩	⇧	⬆⇧	✓✓	✓✓

⬆ & ⬇ represent significant ($p \leq 0.05$) increases and decreases in mussels; and ⇧ & ⇩ represent significant ($p \leq 0.05$) increases and decreases in periwinkles, from the day 7 control (or carrier

control, where applicable); ✓ - represents the presence of oxidative stress and dysfunctional autophagy in mussels; ✓ - represents the presence of oxidative stress and dysfunctional autophagy in periwinkles; **?** - represents possible mild oxidative stress in mussels; and ? - represents possible mild oxidative stress in periwinkles.

Table 3. Correlations between biomarkers from regression matrices for fluoranthene, phenanthrene, chlorpyrifos and copper treatments in mussels.

Biomarker	***LMS***	***Lipofuscin***	***Lipid***
Fluoranthene			
Lipofuscin	-0.689***		
Lipid	-0.406**	0.497***	
Protein carbonyls	-0.567***	0.382**	0.159
Phenanthrene			
Lipofuscin	-0.632***		
Lipid	-0.254*	0.081	
Protein carbonyls	-0.510***	0.323**	0.237
Chlorpyrifos			
Lipofuscin	-0.286*		
Lipid	-0.761***	0.420***	
Protein carbonyls	-0.421***	0.289**	0.368
Copper			
Lipofuscin	-0.689***		
Lipid	-0.406**	0.497***	
Protein carbonyls	-0.567***	0.382**	0.159

Values are Pearson's Coefficient of Correlation R. *p < 0.05; **p < 0.01; ***p < 0.001; 58df.

Table 4. Correlation coefficients for the first Principal Component derived from all treatments as a "measure of health status" *versus* the individual biomarkers for mussels and periwinkles.

First Principal Component	***Lysosomal Membrane Stability***	***Lipofuscin***	***Lipid***	***Protein Carbonyls***
PC 1 - Mussels	0.71***	-0.60***	-0.59***	-0.75***
PC 1 - Littorinids	0.79***	-0.64***	-0.79***	-0.75***

Values are Pearson's Coefficient of Correlation R. Mussels - n = 400; Periwinkles – n = 170; *** $p \leq 0.001$

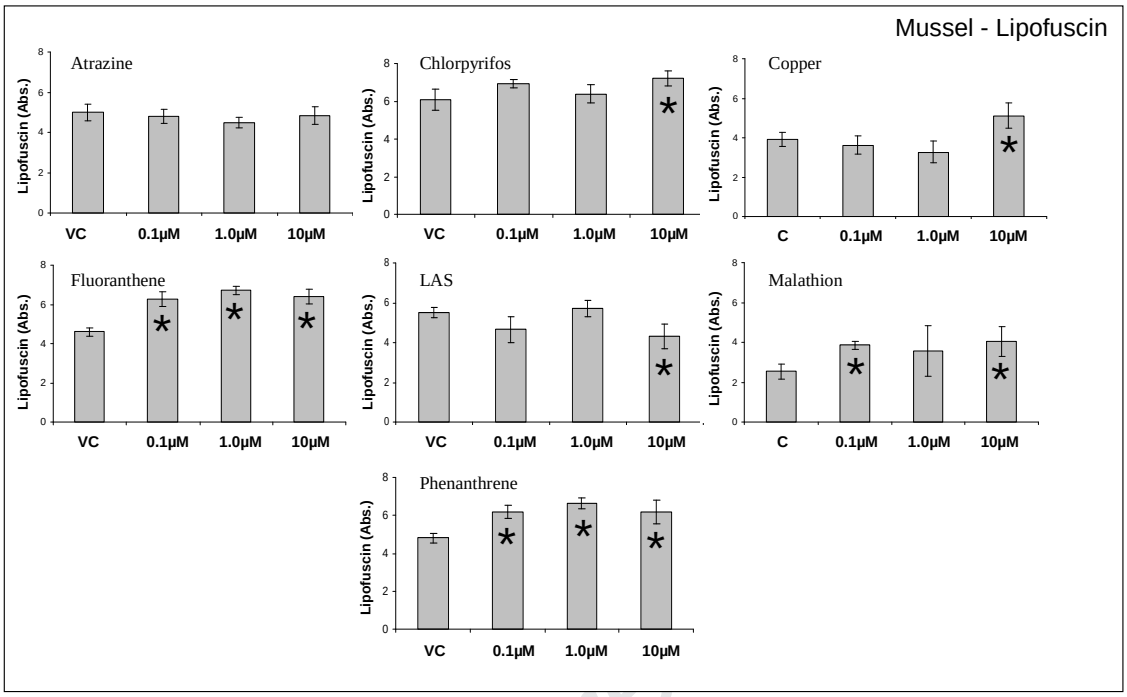

Fig. 1. Lipofuscin in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; lipofuscin (absorbance).

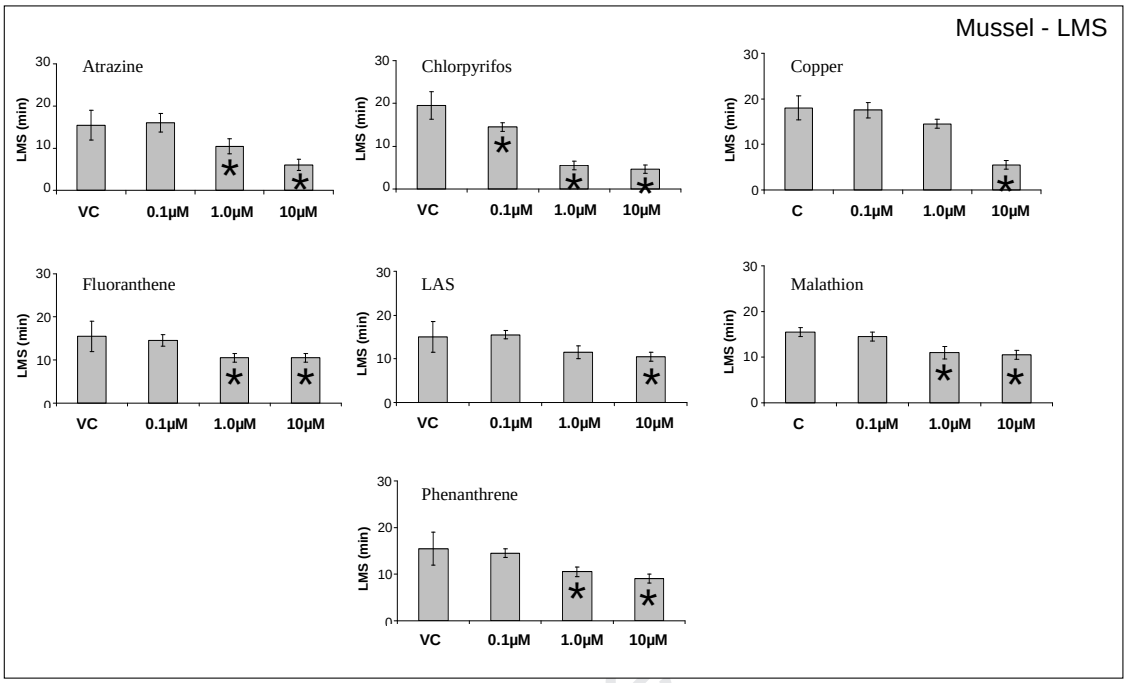

Fig. 2. Lysosomal membrane stability (LMS - based on N-acetyl-β-hexosaminidase latency) in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; LMS is shown in minutes.

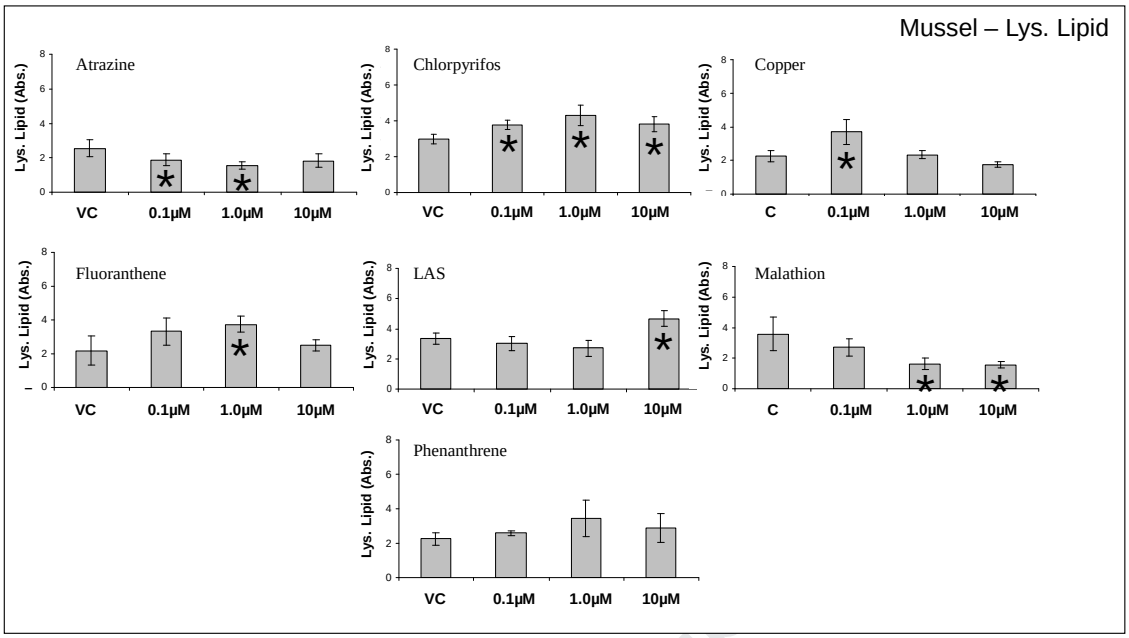

Fig. 3. Neutral lipids in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; neutral lipids (absorbance).

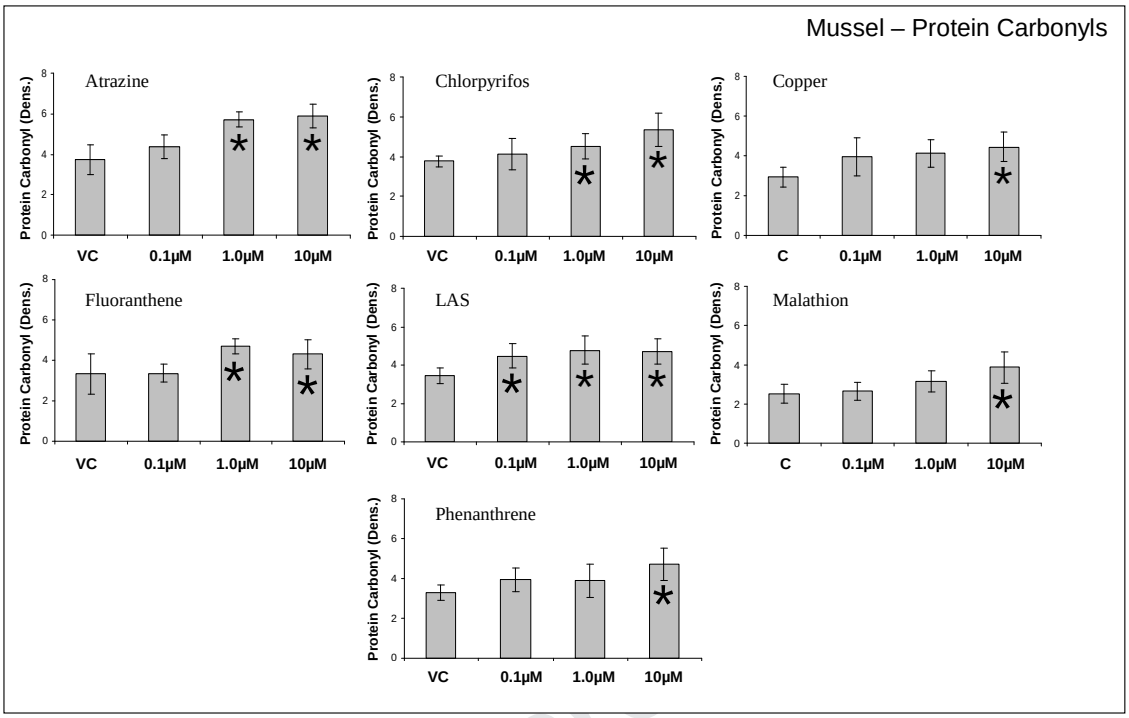

Fig. 4. Protein carbonyls in digestive gland of mussels (4.8 – 5.2 cm) exposed to 0.1 µM, 1 µM and 10 µM of atrazine, chlorpyrifos, copper, fluoranthene, LAS, malathion and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL; protein carbonyls (density).

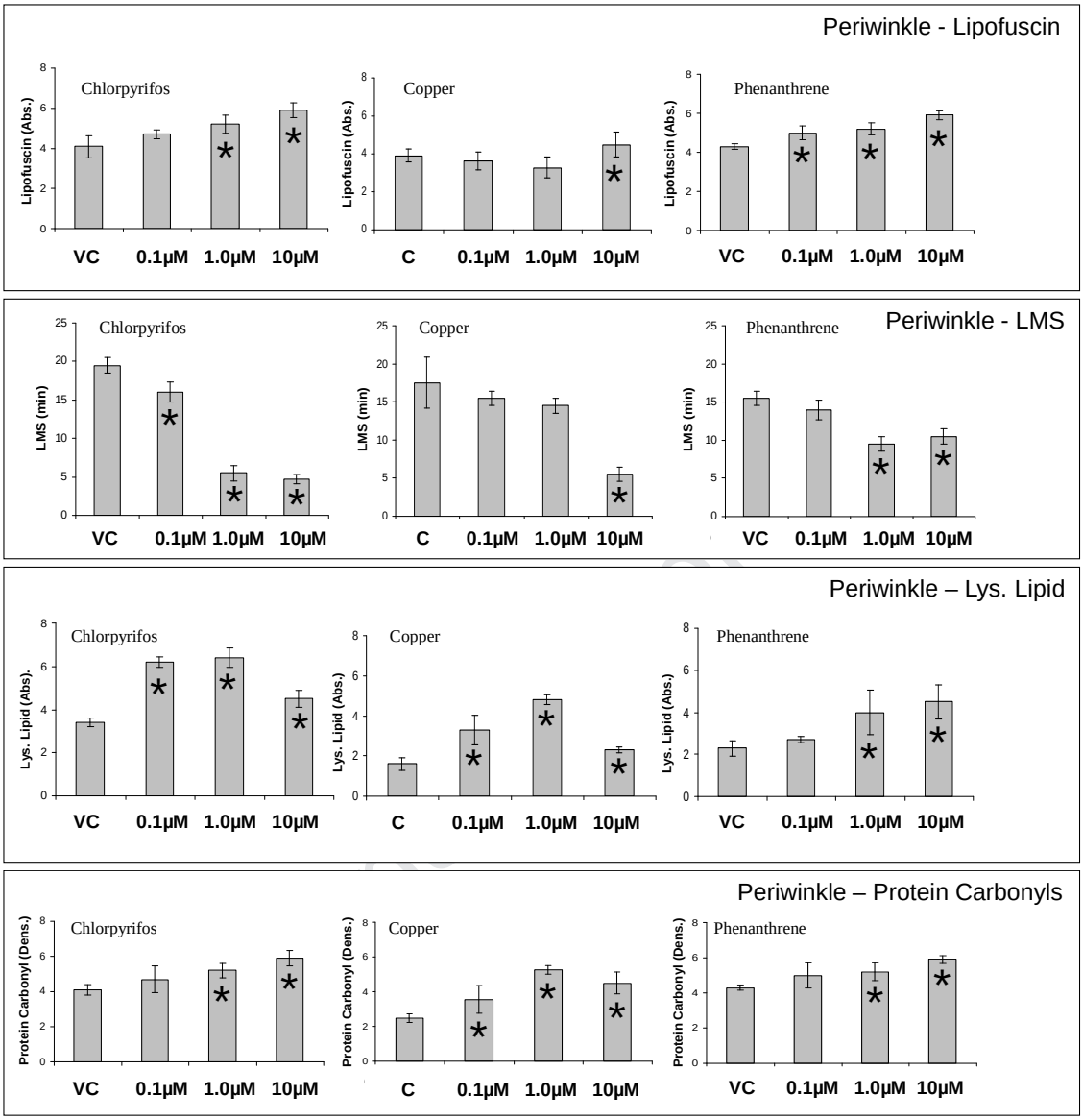

Fig. 5. Lipofuscin, lysosomal membrane stability (based on β-glucuronidase latency), lysosomal lipids and protein carbonyls in digestive gland of periwinkles (2 – 2.5 cm) exposed to 0.1 µM, 1 µM and 10 µM of chlorpyrifos, copper and phenanthrene for 7 days. The carrier or vehicle control (VC) solvent was DMSO. Data is presented as the mean ± 95 % CL.

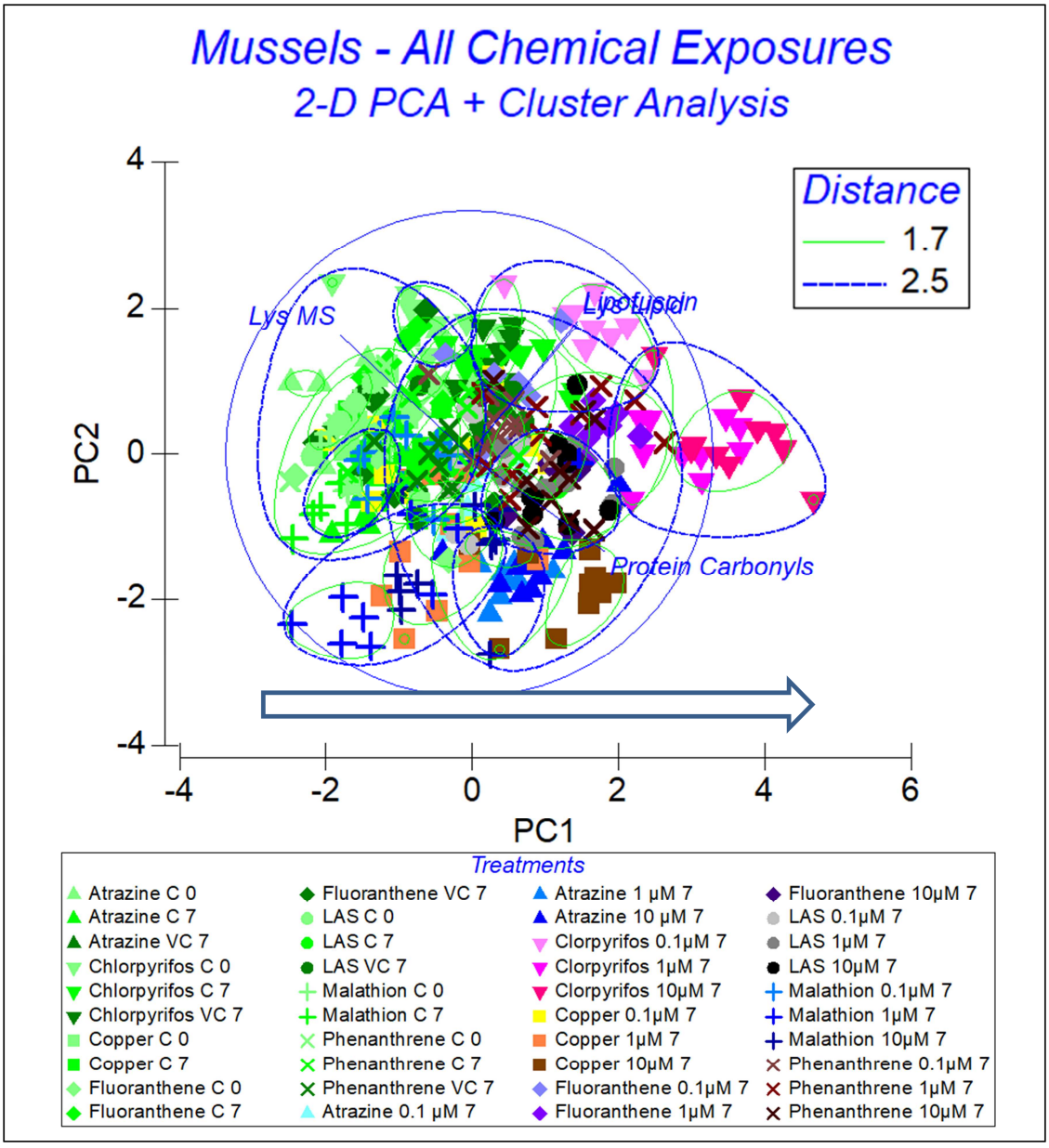

Fig. 6. Combined principal component and cluster analysis (i.e., elliptoid boundaries based on Euclidean distance) for all of the test compounds, based on the data for the four biomarkers in mussels. Vectors for the individual biomarkers are shown; and the large arrow indicates increasing cellular pathology. PC1 and PC2 captured 44.5% and 24.3% of the variation respectively.

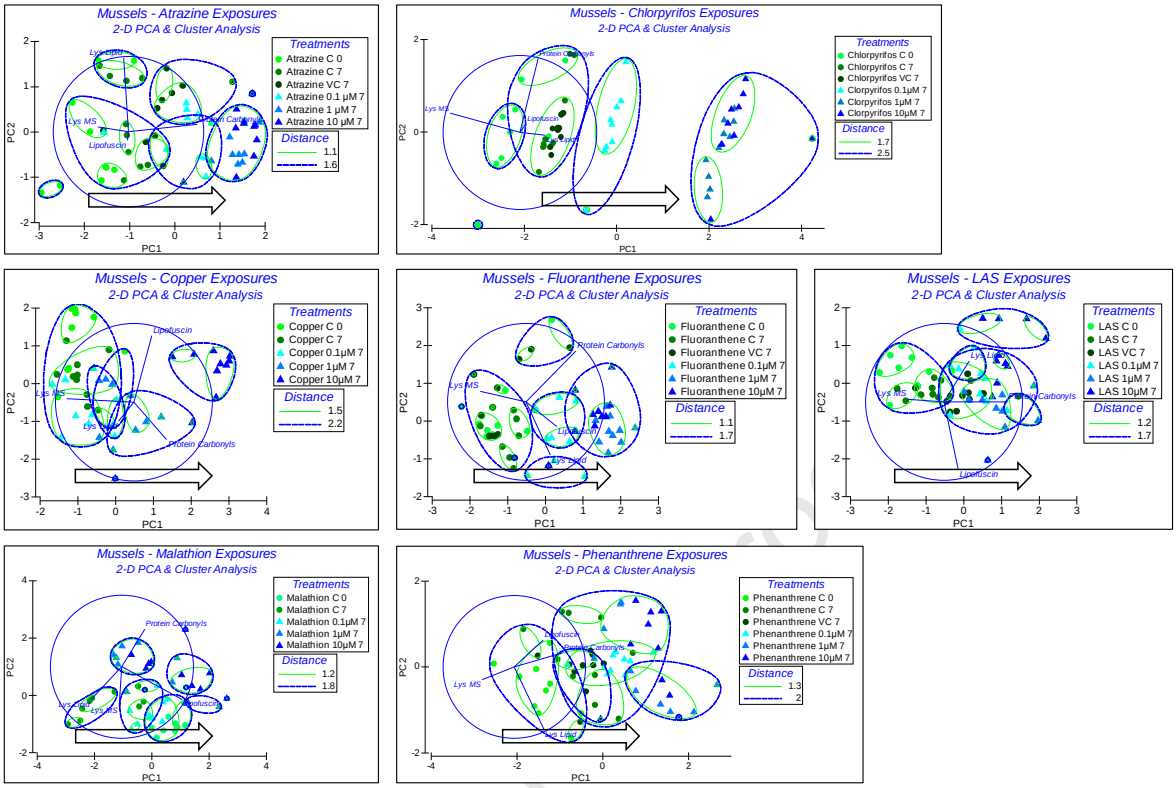

Fig. 7. Individual principal component and cluster analysis (i.e., elliptoid boundaries based on Euclidean distance) for each of the test compounds, based on the data for the four biomarkers in mussels. Vectors for the individual biomarkers are shown; and the large arrow indicates increasing cellular pathology.

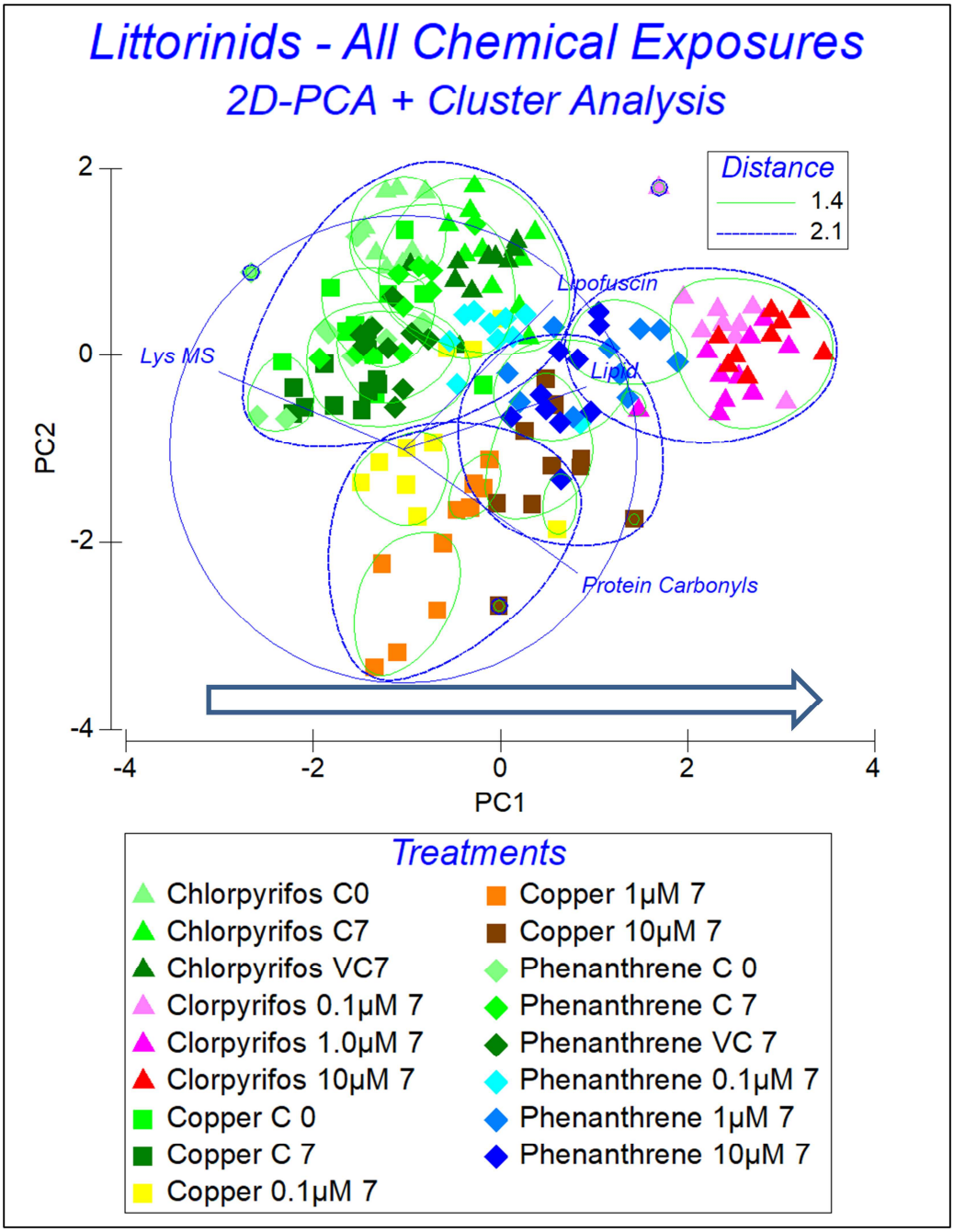

Fig. 8. Combined principal component and cluster analysis (i.e., elliptoid boundaries based on Euclidean distance) for all of the test compounds, based on the data for the four biomarkers in periwinkles. Vectors for the individual biomarkers are shown; and the large arrow indicates increasing cellular pathology. PC1 and PC2 captured 54.8% and 22.4% of the variation respectively.

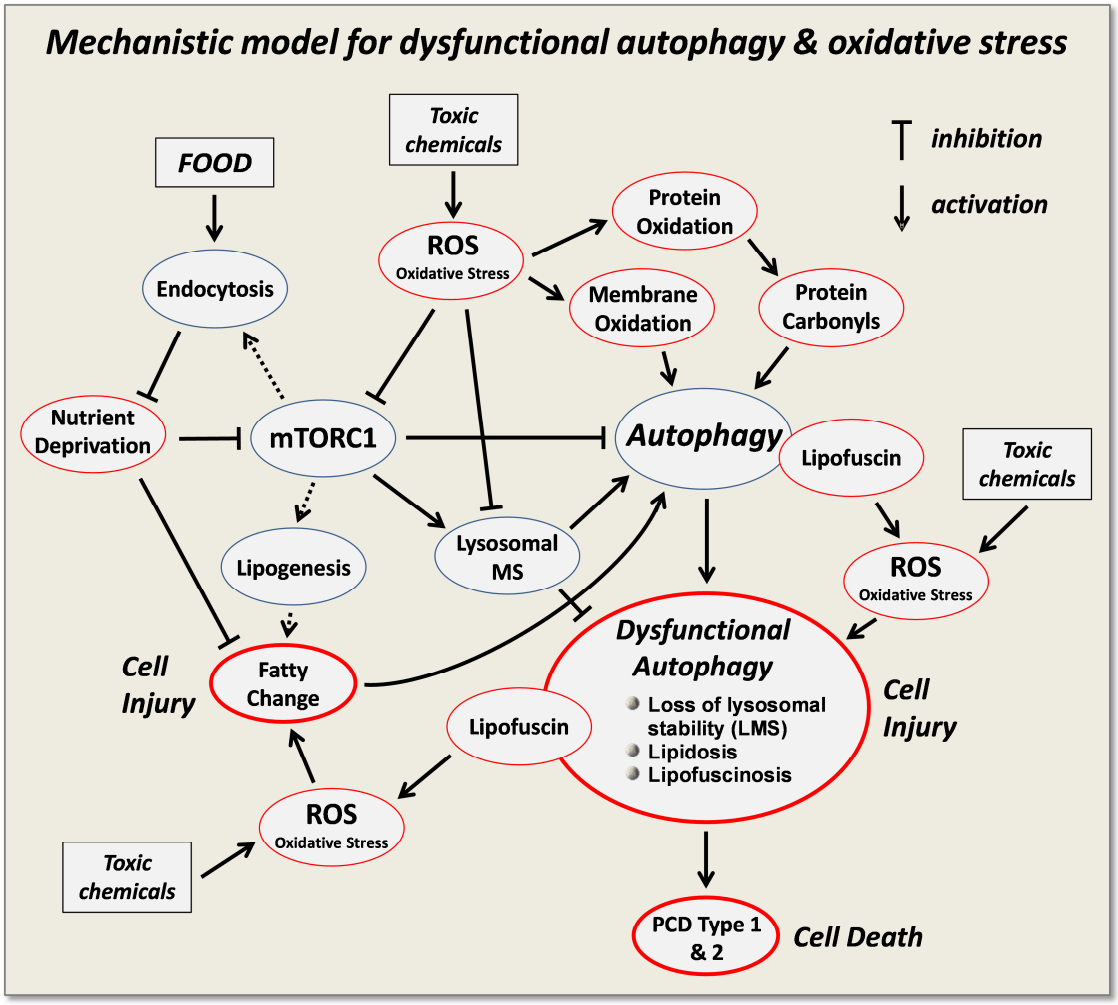

Fig. 9. Conceptual mechanistic model for the role of oxidative stress in dysregulation of autophagy and heterophagy (endocytosis of food) in hepatopancreatic digestive cells. Processes outlined in narrow red are considered to be potentially adverse; and those outlined in heavy red constitute cell injury and programmed cell death (PCD). mTORC1 is the mechanistic target for rapamycin complex 1 – a key component of the cellular signalling system. ROS – reactive oxygen species; dotted arrows indicate a generic process, although not yet confirmed in molluscs.

Highlights:

Failure of normal and physiologically augmented autophagic function as a pathological reaction to chemically induced stress appears to be a widespread phenomenon in many eukaryotic species, including molluscs, annelids and mammals, perhaps indicating that this type of reaction is generic. Evidence for the generic nature of this pathological reaction is supported by the observations from the present investigation and builds on previous studies. Dysfunctional autophagy is characteristic of a number of animal and human diseases, including neurodegenerative diseases, fibromyalgia, ocular pathologies, cardiovascular disease and obesity related conditions. Some of these disease conditions have been linked to uptake of harmful nanoscale materials derived from road traffic.

There are no conflicts of interest.