

# An environmental $^1\text{H}$ NMR metabolomic study of the exposure of the marine mussel *Mytilus edulis* to atrazine, lindane, hypoxia and starvation

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**Abstract** Marine mussels are useful, robust model organisms that have been widely used as biomonitors. In the natural environment they can be subjected simultaneously to a mixture of environmental stresses (hypoxia, starvation) and to pollutants. In this study *Mytilus edulis* was used to investigate the effects of two pesticides, atrazine and lindane, which have different modes of action but produce similar changes in behaviour (depression of ventilation and feeding) in the mussels, and can cause starvation and mild hypoxia. Acetonitrile/ $^2\text{H}_2\text{O}$  (60/40% v/v) extracts of foot muscle from animals subjected to hypoxia, or starvation, or low or high doses of pesticide were analysed using  $^1\text{H}$  NMR spectroscopy to produce metabolic fingerprints associated with these treatments. Discriminant analysis based on metabolites that showed significant differences between treated and control animals gave a clear separation between all treatment groups. The fingerprints of atrazine treated animals were clearly separated from those of animals that were starved or subjected to hypoxia. The high and low doses of atrazine were also well separated. Lindane treatment was separated from control animals in a dose dependent way, and was associated with an increase in the concentration of alanine, and a decrease in all of the other identified metabolites (including

osmolytes). This is consistent with a general depression of activity. This limited study demonstrates the potential of the metabolomic approach to provide a separation of the effects of poisoning from those of environmental stress.

**Keywords** Environmental metabolomics · Pesticide toxicology · Marine mussel · NMR · Atrazine · Lindane

## 1 Introduction

Many aquatic invertebrates living in marginal environments such as the coastal fringe or estuaries are often subjected to stress because of changing environmental conditions due to natural factors such as tidal events. This is particularly marked for sessile organisms such as bivalve molluscs that depend on maintaining a flow of water over the gills for both feeding and external respiration. These organisms avoid the harmful effects of exposure to low salinities as found in some regions of estuaries at low-tide by closing their two shells (valves) together. There are indications that valve opening is also reduced when there is a low concentration of food suspended in the water column (Kramer et al. 1989; Newell et al. 2001). While the valves are closed the molluscs are unable to feed and are subjected to hypoxia. These organisms can also be subjected to hypoxia because of for instance increases in water temperature that reduce concentrations of dissolved oxygen (De Zwaan and Eertman 1996). In the natural environment mussels are not usually subjected to a single stressor at a time, and this contrasts with most laboratory-based investigations (Hamer et al. 2008). Any factors that limit feeding will impact on scope for growth and reproductive capacity, and hence the time available for normal activity will have a

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marked effect on the population size, and time necessary for development. This will ultimately limit the distribution of a species depending on their tolerance (Widdows et al. 2002).

Some anthropogenic pollutants are known to have a marked effect on the behaviour, physiology and biochemistry of bivalve molluscs (Donkin et al. 1997), and because of this, their ability to tolerate a range of environmental conditions, and sessile life style, they have been widely used in biological early warning systems (e.g. the Mussel-monitor) (Bayne 1989) and in biomonitoring (Hellou and Law 2003; Burton et al. 2005). In the latter, the accumulation of pollutants of concern in caged organisms over a fixed deployment period is measured to provide an estimate of bioavailability of substances of concern. Where organisms are sessile it is possible to use wild specimens to detect exposure to pollutants. This involves the identification of suitable biomarkers (physiological or biochemical changes in response to exposure to toxicants) that can be easily measured. The Mussel Watch programme involved the measurement of a fundamental parameter, scope for growth, as a means of assessing the water quality at test sites (Baldwin and Kramer 1994). Scope for growth is a measure of the overall energy status of an animal based upon the difference between energy intake (measured by food intake) and energy expenditure (measured from respiration rate). That is the amount of energy that is available for growth and reproduction after the basic needs of metabolism have been met. Recently a range of chromatographic and spectroscopic methods has been used in metabolomic studies to identify changes in the composition of the small molecular weight metabolites in either biofluids or tissues extracts (Lin et al. 2006). This has provided a new approach to assessing the health of organisms. When linked with pattern recognition protocols and data mining tools these methods can detect changes in the profile of metabolites in response to environmental stressors, diseases or exposure to toxicants. This approach, originally developed for human biomedical applications (Nicholson et al. 1995), has now been applied in a range organisms including micro-organisms, fungi, plants, invertebrates (e.g. annelids, molluscs), and fish (Fiehn 2002; Bundy et al. 2004; Kell 2004; Larsen et al. 2005; Viant et al. 2006). However, as highlighted by Hines et al. (2007a) relatively little work has been carried out on aquatic species. The studies have been undertaken to gain understanding of the biochemical basis of a wide range of changes in behaviour or physiology.

Aquatic organisms can be exposed to a wide range of pollutants including heavy metals, household products (e.g. detergents), pesticides and high volume, non-polar industrial chemicals (e.g. polyaromatic hydrocarbons, brominated flame retardants and fluorinated compounds). The levels of these compounds in water bodies can vary in time depending

on factors such as sporadic discharges, seasonal use, water flow and weather events. Usually organisms are exposed to a complex mixture of toxicants rather than to a single substance and so it is difficult to identify the impact of individual compounds (Altenburger et al. 1996). Accumulation of a body burden of non-polar organic compounds can lead to non-specific narcosis (Hermsen et al. 1994), even in the absence of toxicants with a specific mode of action. In a regulatory context and for risk assessments, the interest is in the toxicity and impact of single compounds, and metabolomics offers a way of identifying the mode of action that is characteristic of individual pollutants. Similar secondary lesions can be produced by a range of compounds, and in some cases the responses to a toxicant could be similar to those observed in healthy animals subjected to environmental stress. For instance if a pollutant has the effect of reducing ventilatory activity and hence feeding activity, then the animals would be subjected to a combination of starvation and hypoxia.

The aim of this study was to investigate the potential of metabolomic methods for identifying the impact of individual toxicants in an aquatic organism, and for separating those symptoms from the effects of hypoxia and starvation in individuals not subjected to a toxic insult. On the basis of published work, we selected two model toxicants, atrazine (6-chloro-*N*<sup>2</sup>-ethyl-*N*<sup>4</sup>-isopropyl-,3,5-triazine-2,4-diamine) and lindane [1,2,3,4,5,6-hexachlorocyclohexane (mixed isomers)] that have contrasting physicochemical properties and modes of action (Tomlin 1997); and the marine mussel *Mytilus edulis* was selected as the model test organism. Atrazine is a systemic herbicide that inhibits photosynthetic electron transport, and lindane is an insecticide that is an antagonist of the GABA receptor-chloride channel complex (Tomlin 1997). Both are persistent in the environment and can be found in natural waters. Regulatory concentrations of these two substances in surface waters vary from country to country but are generally in the low  $\mu\text{g l}^{-1}$  range (Akkanen et al. 2001; Basheer et al. 2002). In a study in which *M. edulis* was subjected to chronic (56 days) exposure to high sub-lethal (0.5 LC<sub>50</sub> at 60 days) concentrations of lindane (0.93 mg l<sup>-1</sup>), and atrazine (3.585 mg l<sup>-1</sup>) both compounds produced a decrease in feeding activity and absorption efficiency, and a marked decrease in scope for growth. However, there were differences between the profiles of responses produced by the two compounds. The oxygen consumption of lindane exposed individuals fell to 10% of control levels while for those exposed to atrazine it was 29% greater than that of controls (El-Shenawy et al. 2006). The impacts of exposure to lindane at two doses, one high, sub-lethal, and the other below the no effect level, were compared with control animals. In a second study, the effects of similar doses of atrazine were compared with control animals, and

with animals subjected to either starvation or hypoxia. It was not possible to compare the effects of lindane and atrazine directly since the two studies were conducted at different times of the year due to limited capacity of the holding facility.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Lindane (97%  $\gamma$ -isomer) and atrazine (pure analytical standard), and amino acids were purchased from Sigma Aldrich (Poole, UK). All solvents (chromatographic grade), salts (Analar grade), and acids (Analar or better grade) were obtained from Fisher Scientific UK Ltd (Loughborough, UK). Deuterium oxide [99.6% (v/v)] was purchased from Goss Scientific Instruments Ltd (Nantwich, UK). All of the water used in the laboratory was Purite deionised water. Bond Elut<sup>®</sup> C<sub>18</sub> solid-phase extraction cartridges were obtained from Varian Ltd (Oxford, UK). Artificial sea water salt mix (Red Sea) and artificial diet (Kent Marine Phytoplex) for the mussels was obtained from Maidenhead Aquatics (Havant, UK).

### 2.2 Mussel culture

*Mytilus edulis* were obtained from Solent Fish (Portsmouth, UK) being originally sourced from Scottish waters. The specimens were rope grown and gave a consistent age and size throughout the population. The culture was maintained at ambient temperature in an external flow-through holding tank system using unfiltered sea water (from Langstone Harbour, Portsmouth, UK) that contained a natural food source. The physiology of mussels changes during their reproductive cycle. During gametogenesis and spawning the animals deplete the energy stores built up during the inter-spawning period when gamete production is inhibited. The animals used in this study were all in the inter-spawning (non-reproductive) stage during which it is difficult to determine the gender of the animals. In this study the sex of the test animals was not known. Two morphologically similar species of *Mytilus* (*M. edulis* and *M. galloprovincialis*) and a viable hybrid now occur in populations with overlapping geographical distributions in the UK (Hellou and Law 2003). The identity of the species of the test animals was determined using polymerase chain reaction (PCR) techniques. The identification is based on variation in an adhesive protein gene sequence between these species. A method based on a sequenced set of oligonucleotide primers, was used to identify the test animals as *M. edulis* (Inoue et al. 1995; Wood et al. 2003).

### 2.3 Procedures for pesticide exposure

For experimental exposures (30 days), under a static renewal regime (Ernst 1977), specimens were transferred to glass beakers (2 l) containing artificial sea water (salinity 35‰) and maintained at 15°C (16 h light:8 h dark) in a temperature controlled room. Two mussels were used in each tank, and were fed daily with 200  $\mu$ l of phytoplanktonic food. The artificial sea water containing each treatment was renewed daily. Two concentration regimes (a high sub-lethal dose and a no effect dose) were used for each pesticide. For lindane these were 1 mg l<sup>-1</sup> and 5  $\mu$ g l<sup>-1</sup>, and for atrazine 3 mg l<sup>-1</sup> and 5  $\mu$ g l<sup>-1</sup>. Stock solutions of lindane (1 g l<sup>-1</sup> and 5 mg l<sup>-1</sup>) and atrazine (3 g l<sup>-1</sup> and 5 mg l<sup>-1</sup>) were prepared in acetone and stored in the dark at -20°C. Fresh incubation solutions in sea water were prepared from the stock solutions daily for replenishment of the beakers. These working concentrations of the pesticides were produced by the addition of 2 ml of the appropriate stock solution in acetone to 2 l of sea water in order to maintain a constant concentration (1 ml l<sup>-1</sup>) of acetone. The acetone was used to aid solvation and ensure uniform distribution of the test chemicals in the tank. Controls were exposed to the same concentration of acetone alone. Artificial sea water used for the replacement solutions was cooled and held at 15°C for at least 48 h before use. The spent artificial sea water was filtered through activated charcoal before disposal. In the experiment to investigate the effects of lindane, eight mussels were allocated to each treatment (control, high dose and low dose). For atrazine seven mussels were allocated to each of three treatments (control, high dose and low dose), and five to the other two treatments (hypoxia and starvation). The numbers of animals used was determined by the space available in the constant temperature facility, and there was no mortality during the experiment.

High, sub-lethal doses of lindane (1 mg l<sup>-1</sup>) and atrazine (3 mg l<sup>-1</sup>) were equitoxic (0.5 LC<sub>50</sub>) and were selected on the basis of earlier work (El-Shenawy et al. 2003; 2006) that demonstrated a clear impact on the metabolism, physiology and behaviour of *M. edulis*. Lower doses (lindane 5  $\mu$ g l<sup>-1</sup>, and atrazine 5  $\mu$ g l<sup>-1</sup>) were in the region of reported no effect concentrations in chronic exposure to lindane (daphnid and fish) and much lower than those for atrazine (chironomid and fish) (Girling et al. 2000). These levels were designed to investigate effects on metabolism in the absence of gross externally detectable toxicological modifications of physiology and behaviour. Use of these model pesticides has been heavily restricted in many countries in recent years, and as a consequence both of these concentrations are higher than those found currently in surface waters including estuaries in many regions of the world (Graymore et al. 2001). Hypoxic conditions

were imposed by exposing mussels to air for 10 h prior to tissue extraction, and for the starvation treatment animals were left unfed for 10 days prior to sacrifice. Water quality (pH, oxygen and ammonia concentrations) was monitored throughout the exposure period, and remained constant. Nominal concentrations of the pesticides were checked regularly by analysing aliquots (1 l) of the exposure media. The water was extracted using C<sub>18</sub> solid-phase extraction cartridges, with *n*-hexane (1 ml) as eluant. Extracts were analysed using standard gas chromatography-mass spectrometry methods with single ion monitoring (atrazine  $m/z = 173.10$ , 200.10, 215.20, and lindane  $m/z = 111.10$ , 181.00, 219.00). The levels of both pesticides fell over the 24 h periods between replenishment both in control (with no mussels) and test beakers. For lindane the concentration fell to between 63–71% of the nominal levels in the absence of animals and to 47–64% where mussels were present. The equivalent figures for atrazine were 68–75, and 61–69% respectively. These losses could be attributed to adsorption to the glass surfaces and particulate matter, evaporation and photo-degradation as well as uptake by the mussels. It has been shown previously (El-Shenawy et al. 2006) that under similar exposure conditions the mussels bioaccumulated significant amounts (concentration on a wet weight basis in foot tissues of 13  $\mu\text{g g}^{-1}$  of atrazine, and 60  $\mu\text{g g}^{-1}$  of lindane after 28 days exposure).

#### 2.4 Tissue extraction

Tissue (foot muscle) samples (approximately 1 g wet weight) were taken from live mussels. Shells were opened by cutting the anterior and posterior adductor muscles, and the foot removed using scissors and forceps. Tissues were wrapped in aluminium foil and immediately plunged into liquid nitrogen to stop metabolism. Each frozen sample was then homogenised to a fine powder using a pre-cooled (in liquid nitrogen) ceramic pestle and mortar. Additional liquid nitrogen was used as required in order to ensure the sample remained brittle during the grinding process. The homogenised samples were transferred to pre-cooled Eppendorf tubes (2 ml) and lyophilised for 24 h to bring them to dryness. In order to be able to infer changes in metabolite concentrations between individuals on the basis of the NMR data, a standard weight (100 mg) of each dry sample was then transferred to a clean Eppendorf tube for solvent extraction. Acetonitrile (600  $\mu\text{l}$ ) was added, and thoroughly mixed. This was followed by the addition of <sup>2</sup>H<sub>2</sub>O (400  $\mu\text{l}$ ), mixing, and then centrifugation in a micro-Eppendorf desk centrifuge (13,000 rpm for 10 min) to remove cell debris and precipitated protein. The supernatant was transferred, without disturbing the pellet, to a pre-washed (<sup>2</sup>H<sub>2</sub>O) Eppendorf tube (2.0 ml) and frozen in liquid nitrogen and lyophilised (24 h) to remove all of the solvent. The dried

samples were stored under anhydrous conditions until NMR analysis. This extraction procedure was selected following trials using a range of ratios of <sup>2</sup>H<sub>2</sub>O to acetonitrile (100:0, 70:30, 60:40, 50:50, 40:60 and 30:70% v/v). The selected regime gave an efficient extraction of small metabolites without significant contamination with lipids and proteins (Lin et al. 2007).

#### 2.5 <sup>1</sup>H NMR spectroscopy

The dried extracts were reconstituted in <sup>2</sup>H<sub>2</sub>O (700  $\mu\text{l}$ ) and Sorensen's phosphate buffer (1 M, KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> prepared using <sup>2</sup>H<sub>2</sub>O, pH 7.2) (35.5  $\mu\text{l}$ ) and vortexed (30 s). The solution was centrifuged (13,000 rpm) briefly to remove any remaining particulate matter and the supernatant transferred to a 5-mm diameter NMR tube. Sodium 3-(trimethylsilyl)-2,2,3,3-d<sub>4</sub>-propionate (TMSP) (3  $\mu\text{l}$ , 0.25 mM) was added as an internal reference standard to which the chemical shift axis can be calibrated (0.00 ppm) and the instrument was locked to the <sup>2</sup>H<sub>2</sub>O frequency.

All analyses were performed on a Unity Inova 600 NMR Spectrometer (Varian Ltd, Oxford, UK) equipped with a cryoprobe (held at 25 K) and operating at a spectral frequency of 599.80 MHz at 300 K. One-dimensional <sup>1</sup>H NMR spectra were obtained using a pre-solvent saturation pulse sequence with a saturation delay of 1.5 s, followed by a 90° pulse (7  $\mu\text{s}$ ) and then an acquisition time of 7.5 s. All spectra were recorded with a spectral width of 8,000 Hz, post 16 steady state pulses succeeded by 256 transients that were collected into 60,000 data points over a period of 15 min. In order to improve the base line flatness and thereby integration of peaks a delay of 30  $\mu\text{s}$  was set between the end of the 90° hard pulse and the start of the acquisition period. The resultant data-sets were zero-filled to 131,072 points and then a Fourier transformation was applied with a weighted exponential line broadening factor of 0.5 Hz. The spectra were manually phased and base line corrected before being calibrated (TMSP at 0.00 ppm) using ACD/Specmanager (ACD/Labs, version 9.0). Peaks were assigned by reference to known chemical shifts (Wishart 2007) using the ACD/Specmanager software. Some shifts were confirmed by spiking samples with standard amino acids (5  $\mu\text{g}$  of each) and comparing the original and spiked spectra.

#### 2.6 Spectral pre-processing and statistical analysis

Each NMR spectrum was segmented into 0.01 ppm bins between 0.80 and 9.00 ppm using the ACD/Specmanager software, and exported to a spreadsheet (Microsoft Excel). Any pH-sensitive resonances were compressed into single bins to remove this variability using the 'looseness of fit'

operation within the ACD/Specmanager software. Bins at 4.70–4.90 ppm (water), 1.91 ppm (residual acetonitrile) and 3.36 ppm (contaminant) were removed from all spectra before analysis. The broad signal at 0.85–0.88 ppm (residual lipid) was manually reduced during baseline correction procedures. The resultant non-square matrix was analysed using routines in the Minitab (version 14) and GenStat (version 10.1) software packages. For lindane the matrix comprised 24 samples by 671 bins, and for the atrazine, hypoxia, and starvation experiments it comprised 31 samples by 658 bins. The small difference in the number of bins between the two experiments is due to the fitting software that combines (on the basis of the integration slope) adjacent bins that overlap a single peak. The samples by bins matrix was transposed to give the samples in rows and variables in columns. The principal components (PCs) analysis routine in GenStat was used to reduce the original standardised data to a number of PCs. A linear discriminant analysis (LDA) was used to identify those PCs that were important in separating the treatment groups (see supplementary information). The loadings of the original variables on those PCs were used to identify those bins that contained information that was correlated (loading score > 0.05) with those components. Bins that did not load on to those components were removed, and the chemical shifts corresponding to the remaining bins were investigated to assign them, where possible, to specific metabolites. Standard normal probability plots (Minitab) were used to check for normality of the distributions of each metabolite, and where necessary Box–Cox transformations were used to identify appropriate normalising transformations. This was followed by analysis (one-way ANOVA) of the transformed variables to identify those exhibiting a statistically significant effect of the treatments. A LDA (GenStat) was used to optimise the separation of the treatment groups on the basis of the sets of metabolites (transformed variables). Plots of the first two discriminant functions were used to map the distances between the various group means, and the correlations between the original variables and the discriminant scores were used to identify those metabolites that were important in the separation of the treatment groups, and to indicate the directions of the changes with treatment.

### 3 Results and discussion

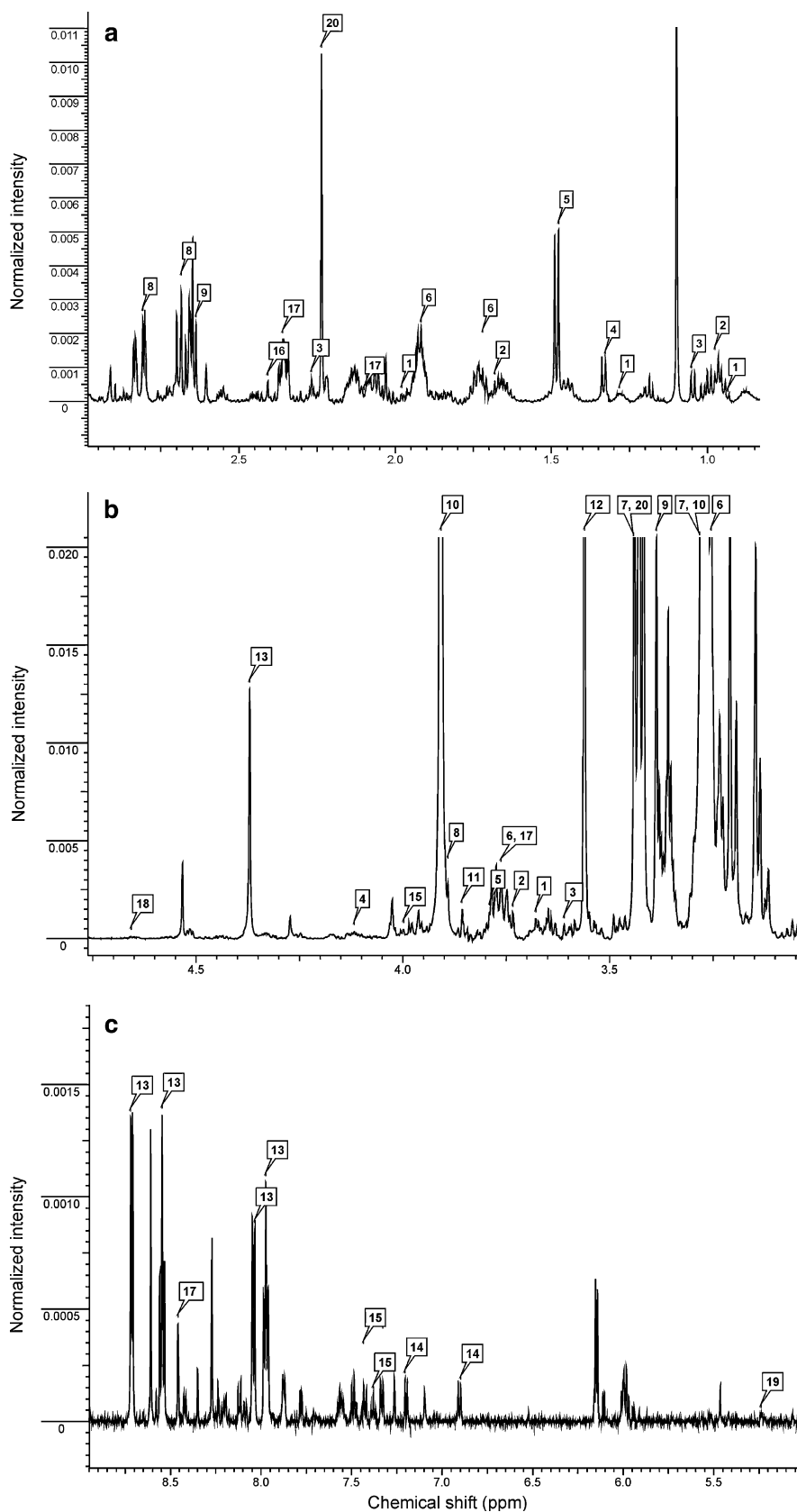
NMR spectroscopy provides a means of obtaining a profile of metabolites present in body fluids or tissues of organisms, and these can be compared between healthy individuals and those subjected to a toxic insult (Bundy et al. 2001). The changes produced in the pattern of metabolites can provide clues as to the secondary lesions caused by various toxicants. These have the potential to

identify useful biomarkers of exposure to different classes of toxicants, including industrial chemicals and pesticides. One significant difficulty in this work is separating the effects of specific stressors from natural biological variation and seasonal changes in metabolism (Viant et al. 2003). This background variability can be increased significantly where there are marked differences in metabolite profiles between sexes and closely related species of test organisms (Hines et al. 2007b). Despite these difficulties NMR-based metabolomics using multivariate analytical techniques has been used with some success in identifying changes in metabolism associated with biological, chemical and physical stressors in different species (Viant et al. 2003). In this study we show changes in the metabolite profile that are associated with poisoning with the herbicide atrazine, and the pesticide lindane, and with environmental stress (hypoxia and starvation). The latter were studied because the toxicants cause a decrease in rates of feeding and ventilation, and these may mimic the effects of the environmental stressors. The pesticides have been shown to have different modes of action but in this study the two were investigated at different times of year when there were differences between the profiles of control animals. Thus it is not possible to compare the two directly, but only with the appropriate controls.

#### 3.1 Metabolic profiles of healthy control animals

In this study the species of the mussel used was confirmed by PCR as *M. edulis* (Inoue et al. 1995; Wood et al. 2003). However, because the animals were in inter-spawning condition, the gender was not identified, and profiles were from a mixture of male and female animals. This will have contributed to the background noise in the separation of control and treated individuals (Hines et al. 2007a). The foot tissue was selected because it is easily identifiable, and separable from the hepatopancreas (to avoid contamination with ingested material), and gives sufficient material for extraction and metabolite analysis. This tissue provides a representative picture of the state of the metabolic pool. In other NMR studies in marine molluscs, mantle tissue, digestive gland and haemolymph have also been used (Hines et al. 2007a; Viant et al. 2001). A constant weight (100 mg) of dry tissue was extracted for use in all experiments to enable comparisons on a dry weight concentration basis. The extraction procedure developed here was designed to extract as many polar metabolites as possible whilst minimising contamination with lipids and proteins that obscure the spectra of the other low molecular weight compounds of interest. The use of acetonitrile in combination with water for the extraction of mussel foot tissue provided clean spectra (Fig. 1) in which numerous individual metabolites could be assigned (Table 1). Some of

**Fig. 1** Representative one-dimensional  $^1\text{H}$  NMR spectrum of the foot muscle from a healthy marine mussel (*Mytilus edulis*). The figure has been enlarged into three sections for better clarity with **a**, **b** representing the aliphatic region and **c** the aromatic region with identified metabolite peaks numbered. Key to metabolites: 1 isoleucine, 2 leucine, 3 valine, 4 lactate, 5 alanine, 6 arginine, 7 taurine, 8 aspartate, 9 hypotaurine, 10 betaine, 11 serine, 12 glycine, 13 homarine, 14 tyrosine, 15 phenylalanine, 16 succinate, 17 glutamate, 18  $\beta$ -glucose, 19  $\alpha$ -glucose, 20 acetoacetate



**Table 1** Metabolites present in *Mytilus edulis* foot muscle with corresponding peak values (ppm) and peak shapes

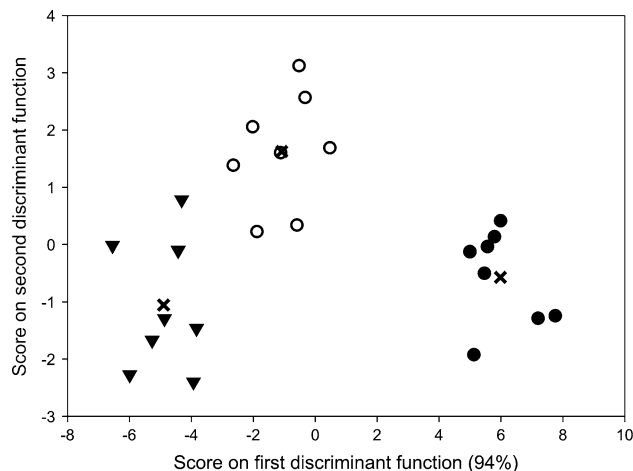
Metabolites	Chemical shift and peak shape	Peak identification in Fig. 1
<i>Amino acids</i>		
Alanine	1.48 (d), 3.78 (m)	5
Arginine/arginine phosphate	1.70 (m), 1.92 (m), 3.25 (t), 3.76 (t)	6
Aspartate	2.68 (dd), 2.81 (dd), 3.89 (dd)	8
Glutamate	2.10 (m), 2.36 (m), 3.76 (t)	17
Isoleucine	0.94 (t), 1.02 (d), 1.28 (m), 1.98 (m), 3.68 (d)	1
Leucine	0.96 (d), 0.98 (d), 1.68 (m), 3.73 (t)	2
Phenylalanine	4.00 (m), 7.33 (d), 7.38 (t), 7.43 (m)	15
Serine	3.86 (m), 3.98 (m)	11
Tyrosine	6.90 (d), 7.21 (d)	14
Valine	1.00 (d), 1.05 (d), 2.27 (m), 3.61 (d)	3
<i>Organic acids</i>		
Lactate	1.33 (d), 4.12 (q)	4
<i>Energy related</i>		
$\alpha$ -Glucose	5.24 (d)	19
$\beta$ -Glucose	4.66 (d)	18
<i>Osmolytes</i>		
Betaine	3.27 (s), 3.91 (s)	10
Glycine	3.56 (s)	12
Hypotaurine	2.66 (t), 3.38 (t)	9
Taurine	3.27 (s), 3.43 (t)	7
<i>Kreb cycle intermediate</i>		
Succinate	2.41 (s)	16
<i>Micellaneous</i>		
Acetoacetate	2.24 (s), 3.43 (m)	20
Homarine	4.37 (s), 7.97 (dd), 8.04 (d), 8.55 (dd), 8.72 (d)	13

*s* singlet, *d* doublet, *t* triplet, *dd* double doublet, *m* multiplet

these were confirmed by spiking with chemical standards. The spectra included amino acids, carbohydrates, glycolytic products, Krebs cycle intermediates, nucleotides, organic acids and osmolytes. The latter were the most dominant compounds in the spectrum. The range of metabolites is comparable with that found in marine molluscs by other workers (Hines et al. 2007a, b; Viant et al. 2001).

### 3.2 Metabolic effects of exposure to lindane

An LDA was conducted on the set of variables (transformed where necessary on the basis of Box–Cox plots) that showed a statistically significant effect of treatment. The score plot (Fig. 2) resulting from LDA showed a clear separation of the controls, low dose ( $5 \mu\text{g l}^{-1}$ ) of lindane, and high dose ( $1 \text{ mg l}^{-1}$ ) of lindane. The first discriminant function (94% of variation) separates the controls from the lindane treated metabolic profiles. The models produced gave 100% correct classification on the basis of leave one out. The low dose is nearer to the controls than the higher dose and this is consistent with the expected dose–response relationship. On the basis of the correlations of the original



**Fig. 2** The scores on the two discriminant functions based on the metabolites that were significantly different between the treatments (*closed circles* indicate control, *open circles* indicate low dose of lindane, *closed inverted triangles* indicate high dose of lindane). The positions of the treatment means are indicated by *crosses*

variables with the LDA axes, exposure to lindane is associated with an increase in the concentration of alanine and decreases in all of the other metabolites (the amino acid

aspartate; the osmolytes betaine, homarine, taurine; the Krebs cycle intermediate succinate; and three unknowns at 1.10, 6.10 and 8.27 ppm). The second dimension accounts for only a small proportion (6%) of the variation, and will not be interpreted here.

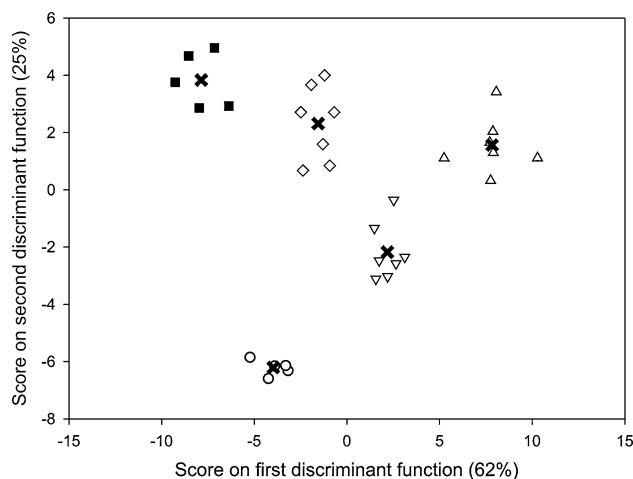
Animals treated by El-Shenawy et al. (2006) with the higher concentration ( $0.935 \text{ mg l}^{-1}$ ) of lindane were observed after 28 days of exposure to exhibit a fivefold increase in ammonia production compared with controls. This was associated with a sixfold decrease in energy consumption, and a tenfold decrease in energy absorption. This was consistent with a fivefold decrease in feeding rate, a 60-fold decrease in the ratio of valve open to valve closed time, and a reduced gape. In that study there was a 12-fold increase in the oxygen to nitrogen ratio indicating that the treated animals were utilising more protein relative to carbohydrates and lipids than the controls (El-Shenawy 1999). This combination of symptoms indicates that the treated animals are starving. These animals were also showing reduced ventilation rates and so the effects of starvation may have been combined with those of reduced oxygen supply.

An increase in levels of alanine have been observed in a range of invertebrate species [*Deroceras* species (Storey et al. 2007), *Crassostrea gigas* (Michaelidis et al. 2005), crayfish (Abe 2002), *Patella caerulea* (Santini et al. 2001), *M. galloprovincialis* (Bacchiocchi and Principato 2000; Isani et al. 1995), *Macoma balthica* (Ahmad and Chaplin 1984) and *Limulus polyphemus* (Carlsson and Gäde 1986)] when subjected to hypoxic conditions for different periods of time. In some of these studies the increase in alanine caused by anoxia was associated with an increase in succinate. Accumulation of succinate is a clear indication of facultative anaerobiosis in molluscs (De Zwaan et al. 1976). This was not observed in the lindane poisoned mussels in the current study where the increase in alanine was accompanied by a decrease in the other analytes (including succinate) that contributed to the discrimination. These differences may be attributable to wide range of exposure regimes used in the various studies. Alanine is an important osmolyte in many invertebrates (Abe et al. 2005), and is found in high concentrations, as well as being produced in anaerobic metabolism (Carlsson and Gäde 1986). The mussels in the current study were exposed to lindane over a 28-day period, and at the end of this at the higher dose were shown (El-Shenawy 1999) to have negative scope for growth. This is consistent with the general decrease in metabolites from a range of classes observed in this metabolomic study. It is difficult to associate this with the known specific mode of action of lindane in insects, and the observed symptoms may be associated with non-specific toxicity caused by accumulation of this non-polar compound in the body lipids of the mussel, and the prolonged decrease in feeding efficiency and ventilation rate.

### 3.3 Metabolic effects of hypoxia, starvation and exposure to atrazine

The first discriminant function (62% of the variation) separates starvation and hypoxia from the controls, and low and high doses of atrazine from each other and from the controls and the starved animals (Fig. 3). The second function (25% of the variation) separates hypoxia from the controls, and starvation and high and low doses of atrazine. The last three fall in a similar position on this axis. The controls fall between the low and high doses of atrazine. The models produced gave 100% correct classification on the basis of leave one out. The original variables with the highest correlations with the scores on the second discriminant function indicate that hypoxia (relative to controls) is associated with increased levels of succinate and valine, and decreased levels of isoleucine, leucine and a number of unidentified peaks (at 4.02, 6.10 and 8.24, 8.27 and 8.61 ppm). For atrazine treatment and starvation that fall above the controls on this axis, the opposite applies. Correlations of the original variables with the scores on the first discriminant function indicate that the high dose of atrazine (relative to the low dose, hypoxia and starvation) is associated with increased levels of isoleucine, leucine and a number of unidentified peaks (at 4.02, 4.58, 8.27 and 8.61 ppm). Starvation and hypoxia relative to the controls are associated in this dimension with increased aspartate and some unknowns (at 6.10 and 8.24 ppm).

In an earlier investigation (El-Shenawy et al. 2006) of the impact of a high dose ( $3.585 \text{ mg l}^{-1}$ ) of atrazine on the scope for growth of *M. edulis* a range of physiological and biochemical symptoms were observed after a 28-day



**Fig. 3** The scores on the first two discriminant functions based on the metabolites that were significantly different between the treatments (open circles indicate hypoxia, open inverted triangles indicate control, closed squares indicate starvation, open diamonds indicate low dose atrazine, open triangles indicate high dose atrazine). The crosses indicate the treatment means



exposure. These included a 24-fold increase in ammonia production compared with controls, a halving of energy consumption, and a threefold decrease in energy absorption. This was consistent with a 60-fold decrease in the ratio of valve open to valve closed time, and a reduced gape. Overall a 20-fold decrease in the oxygen to nitrogen ratio was observed (El-Shenawy 1999). Again this syndrome is indicative of starvation. However, there was a marked contrast between the effects of this herbicide and lindane. The former had a far greater impact on scope for growth than the latter, and this was associated with an increase (fourfold) in total maintenance energy and a decrease in energy absorption. In contrast in lindane treated mussels the total maintenance energy was similar to that of controls but the energy absorption was much lower.

In the current study the effects of hypoxia and starvation operated in different directions relative to the control. Hypoxia was associated with an increase in succinate and valine, and a decrease in leucine and isoleucine. In these experiments the expected association with increased levels of alanine was not evident in the discrimination function. The effect of atrazine relative to the controls was in the direction of starvation. The animals poisoned with atrazine were subjected to a combination of starvation and reduced ventilatory activity, as well as to the biochemical lesions produced by atrazine (El-Shenawy et al. 2003, 2006). Insight into the specific toxic response to the herbicide can be gained from the first discriminant function that separates the high and low doses. The higher dose is associated with increases in leucine and isoleucine. This is consistent with the demonstrated (Ciummo 2006; El-Shenawy et al. 2006) stimulation of metabolic activity leading to an unnecessary high output of energy. Ciummo (2006) found this to be associated with increased citric acid cycle oxidation of succinate in fresh water mussels exposed to concentrations (1.5–150  $\mu\text{g l}^{-1}$ ) of atrazine over a period of 7 days. McCarthy and Fuiman (2008) found a large increase in both protein catabolism and anabolism in larvae of the fish *Sciaenops ocellatus* exposed to atrazine (40 and 80  $\mu\text{g l}^{-1}$ ) and this was consistent with the doubling of metabolic rate observed by del Carmen Alvarez & Fuiman (2005) in these larvae exposed to the same dose regime. Information on the chronic toxicity of atrazine in invertebrates is sparse. A recent GCxGC/TOF-MS metabolomic study (Ralston-Hooper et al. 2008) in an amphipod crustacean exposed to atrazine (from 0.03 to 3,000  $\mu\text{g l}^{-1}$ ) showed that L-aspartic acid was the only significant down regulated metabolite. The highest dose was similar to that used in the current study, and the observation is consistent with the association of the scores of the high atrazine group with decreased aspartate.

In this study we investigated the toxic effects of two contrasting pesticides; a herbicide and an insecticide that would be expected to have different modes of action. The

method produced a clear separation between the treatment groups, and controls, and identified some key metabolites that were associated with the effects of the toxicants. The utility of this approach to contribute to the identification of potential markers of exposure of organisms to pollutants has been demonstrated. However, interpretation of the metabolomic data is not straightforward, and is complicated by the interactions between natural environmental stressors and toxic insults due to anthropogenic pollutants. A clearer picture may be possible if the contribution of a range of biological factors including sex, age and seasonal effects, and previous history of exposure to pollutants and environmental stressors could be removed (Viant 2007). There is a need for further studies using this approach to provide statistically sound measurements of the normal metabolic operating range (Viant 2007) of this important model species taking into account the sources of variation described above.

#### 4 Concluding remarks

In the current study a clear separation between all of the treatment groups and controls has been achieved. Although the complex interactions between the factors investigated made it difficult to identify specific metabolic affects caused by the treatments, there is evidence that lindane causes a general depression of activity, whilst atrazine stimulates metabolic activity, and this is consistent with published biochemical and physiological data.

*Mytilus edulis* is a model organism that has been widely used for biomonitoring and as an indicator of pollution. This work using model compounds and a simple extraction procedure that yielded clean spectra in which metabolites from a range of classes could be identified indicates the potential utility of the metabolomic approach for assessing the potential impact of substances of emerging concern (e.g. pharmaceuticals) where mode of action in invertebrates is not known or uncertain.

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