Metabolomics (2009) 5:95–107
DOI 10.1007/s11306-008-0140-4

ORIGINAL ARTICLE

NMR-based metabolomics using earthworms as potential indicators for soil health

Simone J. Rochfort · Vilnis Ezernieks · Alan L. Yen

Received: 31 May 2008 / Accepted: 30 October 2008 / Published online: 21 November 2008

Abstract Soil health is key for sustainable productivity and adaptation to climate change. Agricultural practice can significantly impact on soil health. The aim of this study was to examine the effect of two land management regimes on organisms (earthworms) that may be used as indicators for soil health via NMR metabolomics. Earthworms are important in the soil decomposition process and viewed as a sentinel species in soil. The presence/absence of earthworm species and their relative abundances provide a gross indication of the health of the soil and it is expected that land use would affect earthworm metabolism as the populations rose or declined in response to changing soil health parameters. In order to test this hypothesis metabolomics approaches were employed to determine if biological indicators of soil change can be detected. Two species of earthworms, an unidentified native species and the European species *Aporrectodea caliginosa*, were collected from properties in Victoria, Australia where the land was treated with either biological (organic) or conventional (chemical) treatment regimes. Both lipid and aqueous NMR metabolomics for earthworms was employed, demonstrating that class classifications can be achieved with both datasets and provide orthogonal, complementary, chemical information. The study indicates that land-use has a measurable effect on the biochemistry of worm populations. Potential biomarkers of land use and worm stress were found, including elevated levels of glucose, maltose, alanine and triacylglycerides. This study demonstrates the utility of NMR metabolomics approaches in detecting biomarkers related to land treatment regimes and potentially soil health attributes.

Keywords *Aporrectodea caliginosa* · Stress · Glyphosate · Soil health · NMR · Metabolomics

1 Introduction

Soil health is fundamental for sustainable productivity and to assist adaptation to future climate change. Climate change is expected to put increased stress on current production systems and, in the South Eastern Australian context, will include both increased drought and frost stress. A healthy soil enhances plant survival under normal conditions and will be increasingly important under the additional stressors felt through climate change. An indication of good soil health is a diverse and active soil biota that undertakes essential ecosystem functions such as maintaining soil structure, decomposition, nutrient recycling and providing mechanisms to inhibit or reduce the effects of pests and diseases. Healthy soils with high organic content retain more moisture, thus utilizing rainfall more efficiently and reducing leakage to the groundwater. A biological approach to land management incorporating minimal tillage and organic fertilizers is though to enhance soil health via increasing organic content and soil biota diversity including beneficial organisms such as earthworms.

Electronic supplementary material The online version of this article (doi:10.1007/s11306-008-0140-4) contains supplementary material, which is available to authorized users.

S. J. Rochfort (✉) · V. Ezernieks
Biosciences Research Division, Department of Primary Industries, 621 Sneydes Road, Werribee, VIC 3030, Australia
e-mail: simone.rochfort@dpi.vic.gov.au

A. L. Yen
Biosciences Research Division, Department of Primary Industries, 621 Burwood Highway, Knoxfield VIC 3180, Australia

The soil biota is very diverse and complex, ranging from the smallest microbes and fungi to the larger macro invertebrates and it is impractical to attempt to study all groups. One way to circumvent technical difficulties in studying soil biota is to use an ecologically important group as indicators. Earthworms are important in the soil decomposition process and may be an indicator of total biological activity. Due to their important role in soil biology earthworms have been the focus of several studies investigating the effect of potential agricultural toxins (Arnaud et al. 2000; Callahan et al. 1994; Roberts and Dorough 1984; Saint-Denis et al. 2001; Springer and Gray 1992).

The presence/absence of earthworm species and their relative abundances provide a gross indication of the health of the soil and it is expected that land use would affect earthworm metabolism as the populations rose or declined in response to changing soil health parameters. Longitudinal surveys of soil biota ('soil foodweb' analysis), plant productivity and plant nutritional value have been used to measure soil productivity or soil health. As such, indicators of environmental stress include declining numbers of soil organisms over the years or a fall in nutrient value of plant crops. Extreme or acute stress can result in the lack of earthworms (and other organisms normally present) but stress can result in a gradual decline. These current techniques can take several years of monitoring before the signs of soil health decline are obvious. The identification of subtle, pre-symptomatic biomarkers of environmental stress is an important goal for the monitoring of soil systems. Earthworms may be used as a sentinel species in such systems. The invertebrate biomarkers could then be employed to indicate general environmental stress in soils before otherwise detected (Weeks et al. 2004).

Nuclear magnetic resonance (NMR) metabolomics is a technique that can be used to study an organism's response to different environments and can detect pre-symptomatic signs of stress and disease. Though much work has focused on mammalian systems for environmental toxicology, in the last few years the technique has been increasingly applied to other organisms. One of the key group of organisms that have been employed in such studies are earthworms (Jones et al. 2008; Bundy et al. 2004; Bundy et al. 2003; Bundy et al. 2002; Bundy et al. 2001; Bundy et al. 2008; Gibb et al. 1997). This study aimed to compare the metabolic responses of both native Australian species (not examined by NMR methods before) and exotic species to determine if a different land management practice (which included different weed and nutrient treatments) could have a measurable effect on the worms. The treatments studied included two properties, properties 1 and 2, which have had sites managed in both a conventional (chemical based fertilizers and herbicides) and a biological (organic fertilizers, limited chemical use) manner for the past two years.

This is also one of very few studies to demonstrate the use of lipophilic NMR metabolomics. Recently Brown et al. (2008) carried out a study on the extraction of earthworms using various solvents. Not unexpectedly there was considerable overlap between the more polar solvents D_2O, MeOH, ACN and DMSO with the D_2O extract having higher levels of the polar metabolites including amino acids and sugars. The chloroform and benzene spectra were significantly different with the absence of these metabolites but with a predominance of the lipophilic metabolites, particularly fatty acids and triacylglycerides. The study by Brown et al. demonstrated that different solvent extracts yield different classes of metabolites. In a laboratory based study of copper toxicity Bundy et al. (2008) also demonstrated that the lipid spectra differed between treatments. In this study we confirm that liphophilic extracts can be used in field based metabolomics experiments to investigate different treatment effects on earthworms and that this information can provide additional biological information.

2 Materials and methods

2.1 General site description

Property 1. Oats. Located at McCartin Road, off Junor Road, Woodstock West, Victoria (Zone 55, 0233242–5922513). Upper slope in colluvium derived from metasediments. Residual low hills or peneplain. Soil type: Red Sodosol.

Property 2. Wheat. Located on the corner of Elmsford and Neivandts Roads, Woodstock West, Victoria Zone 55, 0235043–5923366). Alluvial plain. Soil type: Mottled Brown Sodosol.

The properties were evaluated in 2007 for a number of chemical and physical properties (MacEwan 2007). In brief, both properties have acidity (low pH) of surface soil and alkalinity (high pH) in the subsoil. Electrical conductivity (EC) was within tolerable limits at both sites in the upper half metre of soil, but increases with depth at both properties where it would impact on water availability. None of the sites or horizons are classified as saline. The sites were dry over the worm collection period. The treatment regime and soil nutrient properties are summarized in Table 1.

2.2 Worm sampling

Mature worms were collected from four sites in two properties (named property 1 and property 2 in this paper,

Table 1 Site treatment regime and soil properties for 2007

Class/property/treatment	Class 1/property 1/biological	Class 2/property 1/conventional	Class 3/property 2/biological	Class 4/property 2/conventional
2007 Field inputs (2006 inputs where the same except where noted) (CCCo = Camperdown Compost Co.) Sowing July 2007	Echidna oats, *Composting* pure chook manure (caged birds- no chemicals) 1 l eMCA per 5 cm of manure 1 l eMCHc per 5 cm of manure 3 l Compost extract (CCCo.) per 5c/m of manure (production method as per attachment) *Seed Dressing* (CCCo.) 6 l per tonne of seed @ 80 kg of seed per ha *Fertilizer* Nutrismart/MAP 70/30 @ 67 kg per ha *Foliar Spray* 1 l Worm juice per ha 1 l VRM Cal booster per ha 2 l Sea minerals per ha 1 kg Rainbow seaweed per ha Echidna oats, treated with biological seed treatment, sown at 65 kg/ha with 60 kg/ha Nutrismart + MAP and 2 m^3 composted chicken manure	Echidna oats, treated with Vincit C, sown at 65 kg/ha with 60 kg/ha Nutrismart + MAP (roundup was applied with Roundup, 1 l/ha, in 2006 but not 2007)	Chara Wheat, *Composting* pure chook manure (caged birds- no chemicals) 1 l eMCA per 5c/m of manure 1 l eMCHc per 5 cm of manure 3 l Compost extract (CCCo) per 5 cm of manure (production method as per attachment) Seed Dressing (Camperdown Compost Co.) 6 l per tone of seed @ 80 kg of seed per ha *Fertilizer* Nutrismart/MAP 67 kg per ha *Foliar Spray* 1 l Worm juice per ha 1 l VRM Cal booster per ha 2 l Sea minerals per ha 1 kg Rainbow seaweed per ha Chara Wheat sown at 91 kg/ha with 80 kg/ha Nutrismart MAP. The site also received 2 m^3 of composted chicken manure and a late biological foliar spray Weeds were controlled pre-sowing with 1 l/ha Roundup, 90 ml/ha Goal & 166 ml/ha K Fulvate., Composted chook manure 2 cm/ha, Nutrismart/MAP 70/30 @ 80 kg/ha	Chara Wheat sown at 91 kg/ha with 85 kg/ha MAP-Triple Super. The site also received 2 m^3 of chicken manure. Weeds were controlled pre-sowing with 1 l/ha Roundup plus 90 ml/ha Goal
2007 Rainfall (mm)	395.5	395.5	229	229
Soil analysis (units)				
Total soil moisture (mm)	113.0	127.1	96.5	102.5
Nitrate 0–10 (mg/kg	28	25	42	23[a]
Ammonium 0–10 (mg/kg)	23	22	29	na
Phosphorus Colwell (mg/kg)	38	35	40	23[a]
Potassium (mg/kg)	482	451	431	364[a]
Organic carbon (%)	1.68	1.8	1.74	1.2[a]

[a] 2007 Data unavailable, 2006 reported

see Table 1 for details) in central Victoria which used biological and conventional treatment regimes at separate sites within the properties. The properties were part of the Mid-Loddon Sub-Catchment Management Group and located in Woodstock West.

Property 1 was sampled on 8 Oct 2007 and property 2 sampled on 9 October 2007. In all cases, the sampling sites were covered with a hessian bag and the bag kept wet. The bags were set up 2–3 weeks before collection. Worms were collected by digging up soil from beneath the hessian bag. They were collected by forceps and placed into a container. The worms were frozen on site by dropping the container straight into a liquid nitrogen dry shipper. Samples collected Oct 8 were kept in the dry shipper container overnight, transferred and kept on dry ice until Oct 9. The samples were kept in the laboratory at −80°C until analysis. The samples were snap frozen rather than taken back to the laboratory for depuration to avoid any acclimatisation to laboratory conditions, particularly important in this case since it was expected that the differences between metabolite profiles due to land use would be minor.

A total of 42 mature earthworms were collected: property 1 (14 from the biological treatment and 8 from the conventional treatment) and property 2 (15 from the biological treatment and 5 from the conventional treatment). The unequal number of earthworms collected from the different treatments reflected relative abundances of earthworms at the sites. There were significantly less earthworms in the property 2 conventional treatment sites, despite searching.

Two species of earthworms were collected. A European species, *Aprorrectodea caliginosa*, was the dominant species (39 individuals) across all the sites. Three specimens of an unidentified native species were collected (samples 2, 15 and 16) on the property 1.

2.3 Tissue extraction

Individual whole worms were ground in liquid nitrogen and approximately 100–110 mg extracted in methanol/chloroform/water. The ground worms were weighed into plastic centrifuge tubes and ice cold methanol/water (1.6:0.6), 2.2 ml added. The solutions were vortexed thoroughly; chloroform (0.8 ml) added and mixed in an ice bath for 10 min. After this time chloroform (0.8 ml) and water (0.8 ml) were added and the solutions vortexed before centrifugation at 5000 rpm, 4°C, for 10 min. The aqueous and organic layers were separated into glass vials and dried under reduced pressure (Savant SpeedVac System®, full vacuum, low heat). The samples were stored with desiccant at −20°C until analysis. The aqueous extracts were dried and reconstituted in approximately 600 µl D_2O with 0.1% TSP to equivalent concentrations. i.e. Samples were individually made to a concentration equivalent to 100 mg worm to 600 ul D_2O, except where there was insufficient initial mass to do so, in which case the sample was dissolved in 500 ul solvent (samples 12, 14 and 18). 500 µl was transferred to 5 mm NMR tubes for analysis. A number of duplicates were prepared to assess the robustness of approach.

The organic extract was dried down and reconstituted in approximately 600 µl $CDCl_3$ with volume adjusted for each sample to maintain a concentration of 100 mg worm to 600 µl $CDCl_3$, except as noted above. 500 µl was transferred to 5 mm NMR tubes for analysis. A number of duplicates were prepared to assess robustness of approach.

2.4 NMR spectroscopy

Proton spectra were obtained on a Bruker 800 MHz instrument equipped with a cryoprobe. Standard Bruker zg30 pulse sequence was used over −3 to 13 spectral range with 256 scans collected after 2 dummy scans, total acquisition time was 2.55 s. A line broadening of 0.3 Hz was applied to all spectra prior to Fourier transformation. Spectra were manually phased and baseline corrected in Topspin. Aqueous samples were zeroed to TSP and the organic samples were referenced to residual solvent ($CHCl_3$) at δ 7.26.

2.5 Data analysis

Aqueous Extracts: Spectra were imported into MatLab R2007a (Version 7.4.0.287) using ProMetab_v1_1 script (courtesy M. Viant, (Viant 2003)). Data were imported over 0.2–10.5 ppm range (26798 data points). The data points covering the residual water peak were removed 4.7–4.9 ppm). This modified data was used for all subsequent analysis without binning. Initial inspection of the NMR data showed all the spectra demonstrated excellent overlap with minimal peak shifting reducing the need for binning the data. Without binning the loading plots more closely resembled NMR spectra and so were easier to interpret. Data were statistically analysed in MatLab using PLS-Toolbox (Eigenvector Research, Inc., Version: 4.1.1). Two PCA analyses were carried out; one using normalized (the total intensity of each spectrum was normalized to one) and mean centred data and the other using mean centred data. Both analyses gave similar separation; however, the separation between classes one and two was improved when mean centred data was used with the low weight samples (12, 14 and 18) excluded. When duplicates are removed from the model both the loadings and PCA plots remain very similar indicating that the duplicates are not causing undue bias in the model. The duplicates also cluster together demonstrating that the sample preparation and data

acquisition is robust. This data set was used to examine the differences between the biological sites and conventional sites for the two properties. A PLSDA model was also constructed. The first used four classes (corresponding to the two sites and two management regimes) with X-block data normalized (the total intensity of each spectrum was normalized to one) and mean centred as for PCA, Y-block was autoscaled. The PLSDA model was built with 34 spectra, cross validation was employed (Venetian blind, maximum levels 16, 6 splits) and tested against 7 spectra withheld from the initial model. The PLSDA model had a cross validation class prediction accuracy of between 94 and 87%. An independent data set (7 samples) was correctly assigned for all but one sample.

2.5.1 Organic extracts

Spectra were imported into MatLab R2007a (Version 7.4.0.287) using ProMetab_v1_1 script. Data were imported over 0.0–10.5 ppm range. The data points covering the residual solvent peak were removed (6.98–7.47 ppm). The modified data (20298 data points) were used for all subsequent analysis. Data were statistically analysed in MatLab using PLS-Toolbox (Eigenvector Research, Inc., Version: 4.1.1). PCA analysis was carried out using mean centred data. Four spectra had to be excluded based on results of the Hotelling T2 test and were clear outliers. These were 2, 13, 14 and 18. Three of these were for technical reasons—the spectral quality of sample 13 was very poor and 14 and 18 were two of the very low mass samples. Sample 2, from a native worm, was a clear outlier, possibly due to species. Since it was not possible to obtain more native worms from this site the single spectrum was removed to ensure robustness of the PCA model. Outliers in PCA can significantly distort the model unless there are additional 'like' samples to be added.

The aqueous and lipophilic duplicates clustered closely together in both PCA and PLSDA, indicating the extraction and NMR technique is reproducible and the statistical analysis is differentiating samples based on real biochemical differences.

3 Results

NMR spectra of the aqueous extracts of the worms were complex, however, a number of metabolites can be identified from the 1D spectra (Bundy et al. 2003; Jones et al. 2008). The spectra are dominated by the presence of sugars (glucose and maltose), organic acids and amino acids and 2-hexyl-5-ethyl-3-furansulfonate (HEFS) previously identified by Bundy et al. as a major metabolite in *E. veneta* (Bundy et al. 2002). Despite the complexity of the spectra the individual samples overlay well without binning (Fig. 1). Visual inspection of the spectra reveals obvious differences between the classes. Figure 1 shows the mean spectra for each class with expansions demonstrating differences in the anomeric methine region (Fig. 1b) and in the aliphatic region (1c).

The lipophilic extracts were somewhat simpler with major metabolites including fatty acids, triacylglycerides and sterols as well as a unidentified metabolites (Fig. 2). As with the aqueous spectra there are obvious concentration differences for several metabolites between the classes.

Unsupervised analysis of the NMR spectra of the aqueous tissue extracts via principal components analysis (PCA) revealed that the worms from Property 1 living under the two different management conditions were clearly distinguishable from each other (Fig. 3). Class 4 is also well separate but class 3 is not. These results are mirrored in the PCA analysis of the lipophilic extracts. Again classes 1 (property 1 biological) and 2 (property 1 conventional) and 4 (property 2 conventional) are quite distinct, with class 3 (Property 2 biological) being the most diverse and overlapping with each of the other classes (Fig. 4). The Figs. 3a and 4a demonstrate that the duplicate samples cluster near each other supporting the robustness of the tissue extraction and data acquisition procedure. The presence of the few duplicates employed in this study did not significantly affect the PC1 loadings of either the aqueous or lipophilic models (Figs. 5 and 6).

The models portrayed in Figs. 3 and 4 demonstrate that the separation between classes is largely in PC1.

The analysis was further broken down to a comparison by treatment and by location. Figure 7 compares the aqueous spectra from property 1 (classes 1 and 2) by PCA analysis. Analysis of the loadings plots in PC1 clearly identifies a series of metabolites contributing to the class separation. The expansion plots reveal the advantage of using unbinned data or very small bins—the resolution of the loadings is comparable to a NMR spectrum and the coupling pattern was used to confirm metabolite identity. It also demonstrates the resolution advantage of PCA analysis compared to direct spectral analysis; in this analysis lactate is clearly separate from HEFS due to their opposite influence in the loadings. Worms from the biologically treated site of property 1 (class 1) had elevated levels of phenylalanine, tyrosine, HEFS and leucine. By comparison, the worms from the conventional site (class 2) demonstrated elevated levels of inosine, maltose, glucose, alanine and the organic acids lactate and formate. Similar analyses for property 2, classes 3 and 4, resulted in essentially the same loadings and metabolite trends for the conventional and biological treatments although there was some overlap between the classes (Figure S1, supplemental data). Comparison of the biological treatments from the two

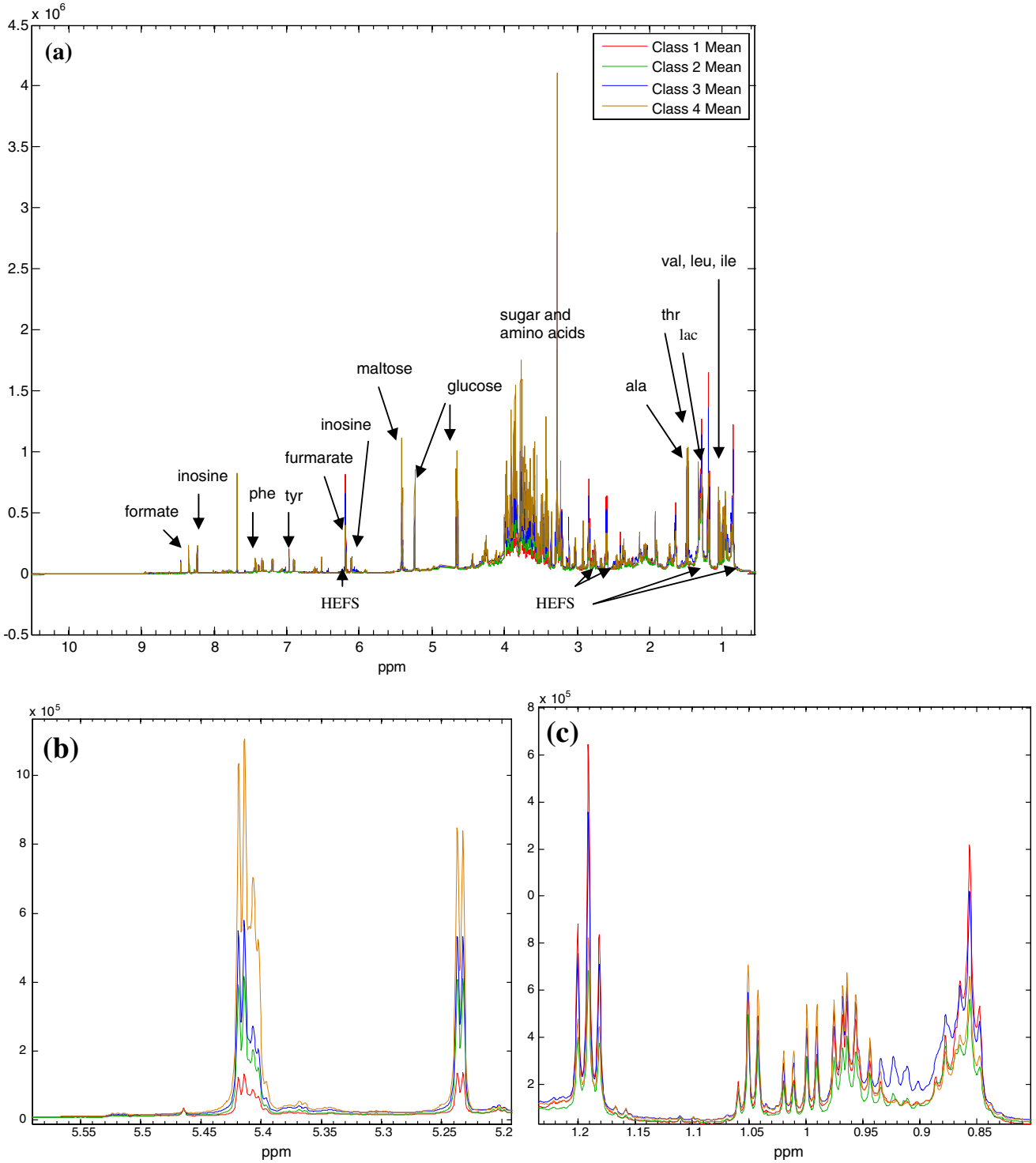

Fig. 1 **a** Mean aqueous proton spectra presented by class **b** Expansion of the anomeric sugar region **c** Expansion of aliphatic region (phe = phenylalanine, tyr = tyrosine, HEFS = 2-hexyl-5-ethyl-3-furansulfonate, ala = alanine, thr = threonine, lac = lactate, val = valine, ile = isoleucine, leu = leucine)

properties, classes 1 and 3 (Figure S2, supplemental data), also resulted in similar loadings plots but with inosine, maltose, glucose and lactate at higher levels and HEFS at lower levels in class 3 compared to class 1. Compared to the earlier analysis the influence of the amino acids was significantly less important in differentiation of the biological treatments. Comparison of the conventional treatments from the two properties, classes 2 and 4 (Figure

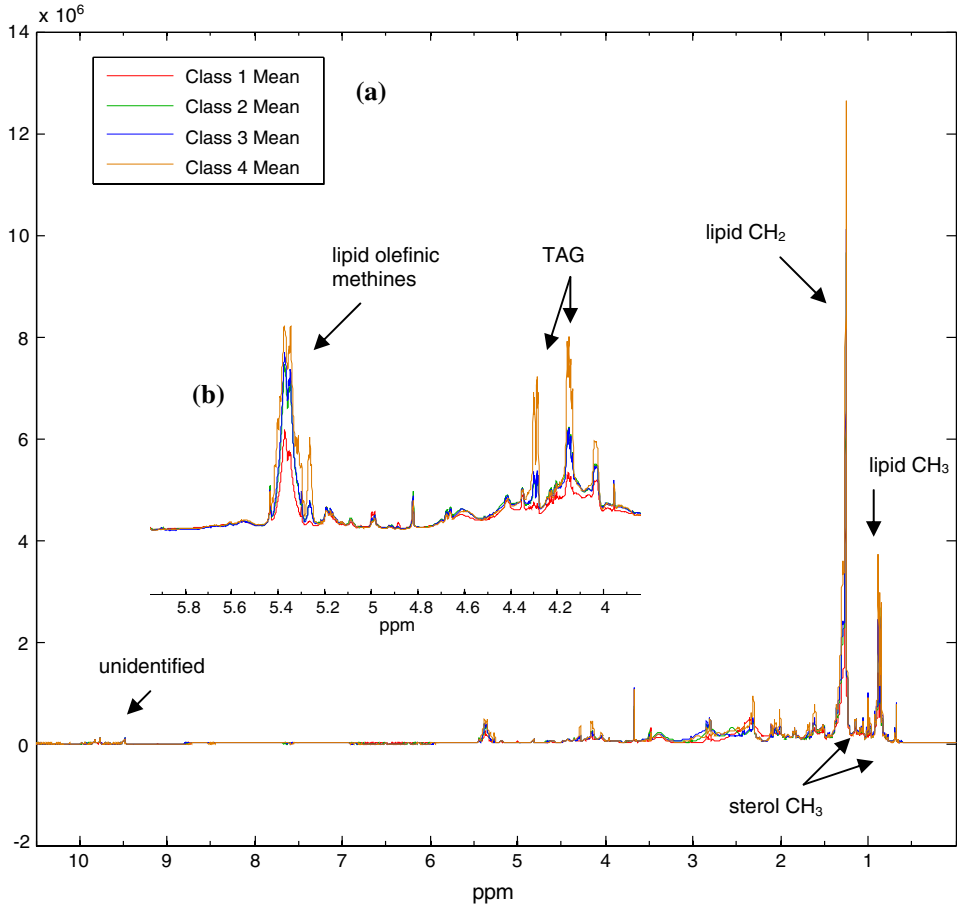

Fig. 2 **a** Mean lipid proton spectra presented by class **b** Inset showing expansion of the olefinic region (TAG = triacylglycerides)

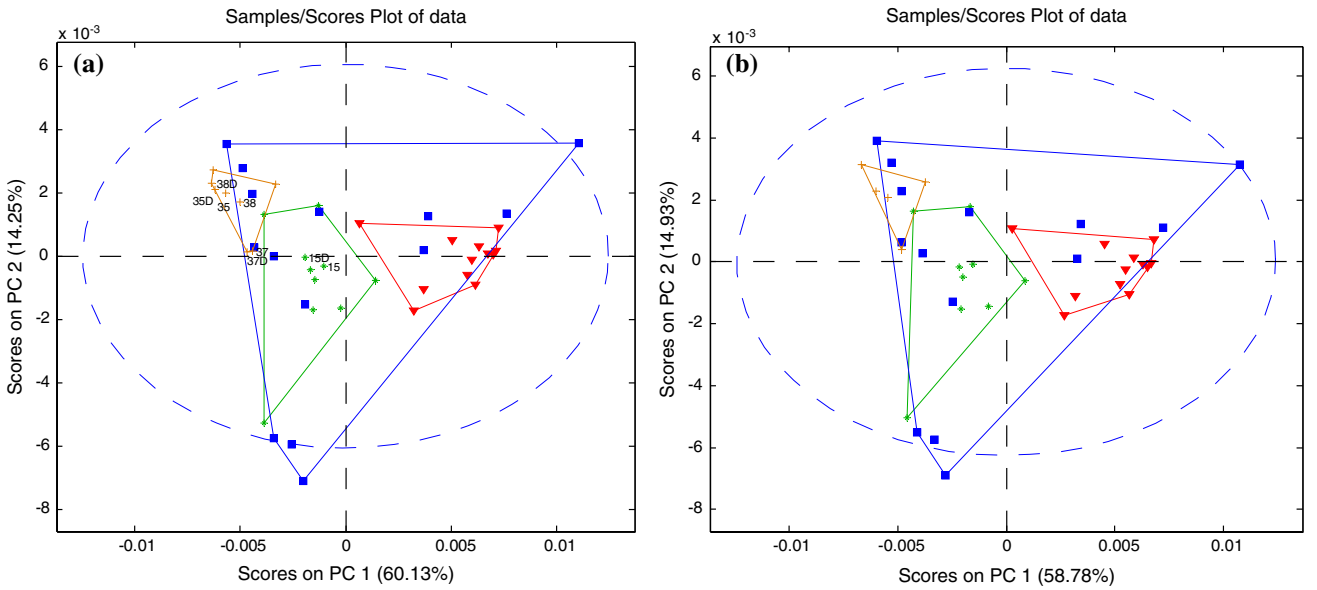

Fig. 3 Analysis of duplicates **a** PCA entire data set **b** PCA analysis with duplicates removed. Class 1 ▼: 1–14 Property 1 biological treatment. Class 2 *: 15–24 Property 1 conventional treatment. Class 3 ■: 25–33, 39–43 Property 2 biological treatment. Class 4 +: 34–38 Property 2 conventional treatment

S3, supplemental data), also resulted in similar loadings plots but with the major discriminating factors due to the sugars (with an apparent elevation in class 4 over class 2 and HEFS mcuh less significant but influencing class 2 to a greater extent. Two unidentified metabolites with singlet peaks at 7.68 ppm and 3.27 ppm had a strong positive

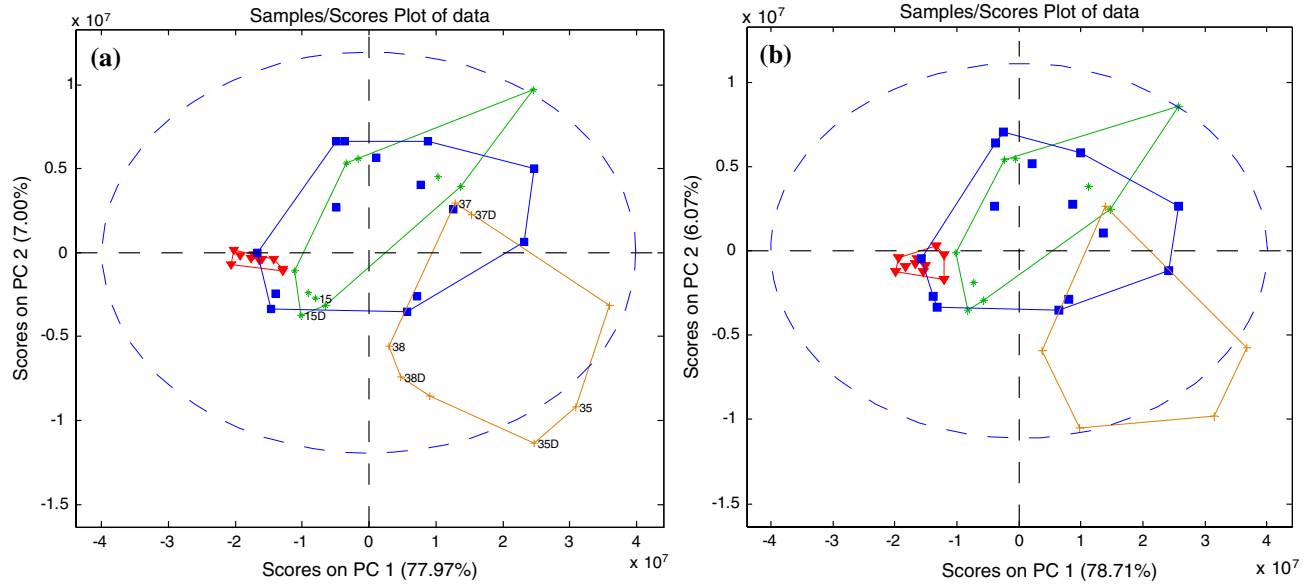

Fig. 4 Analysis of duplicates for lipophilic data **a** PCA entire data set **b** PCA analysis with duplicates removed. Class 1 ▼: 1–14 Property 1 biological treatment. Class 2 *: 15–24 Property 1 conventional treatment. Class 3 ■: 25–33, 39–43 Property 2 biological treatment. Class 4 +: 34–38 Property 2 conventional treatment

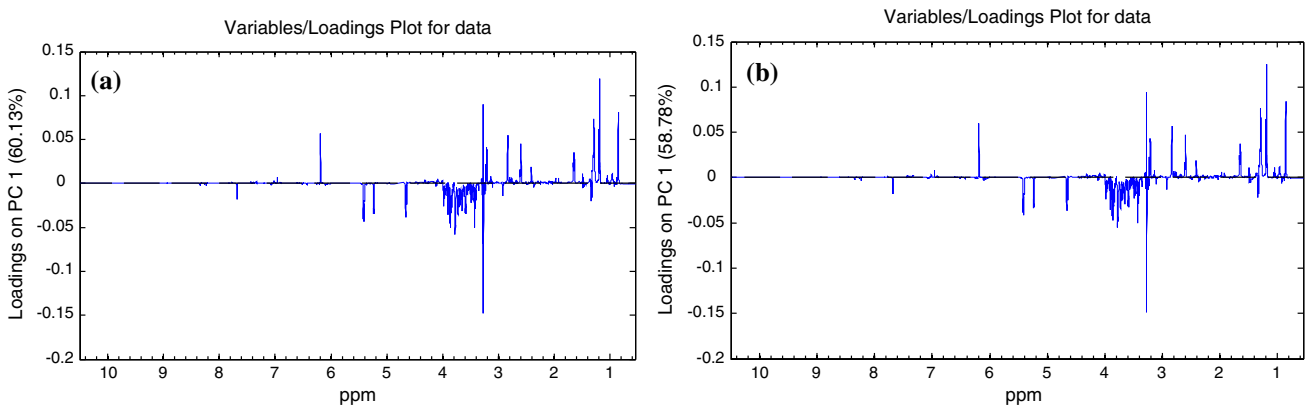

Fig. 5 **a** Loadings plot for PC1 for aqueous model with duplicates and, **b** with duplicate spectra removed

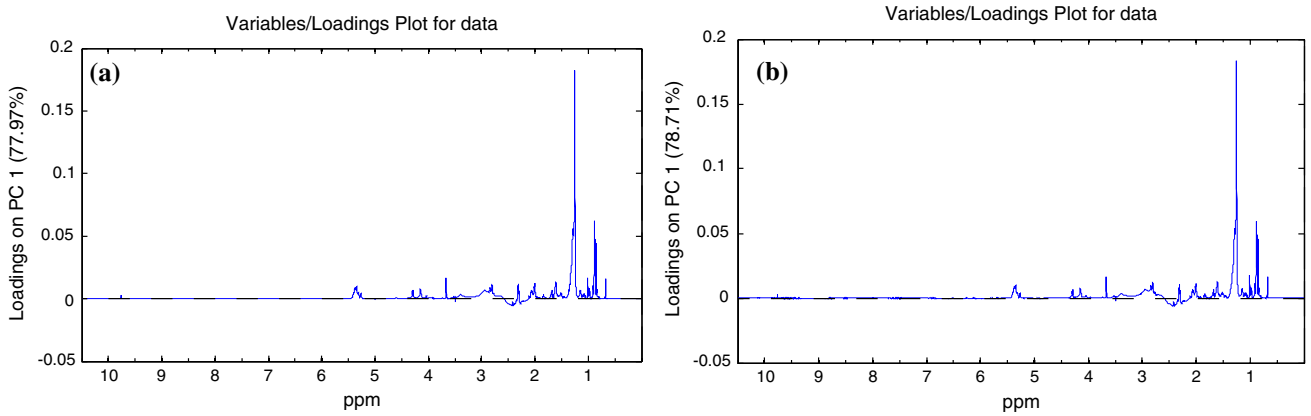

Fig. 6 **a** Loadings plot for PC1 for lipophilic model with duplicates and, **b** with duplicate spectra removed

influence on PC1. These metabolites are tentatively identified as xanthine and betaine (second peak obscured in sugar region) respectively. Lactate and alanine are not discriminating metabolites in this analysis.

A similar analysis was carried out for the lipophilic extracts (Figures S4–S7, supplemental data). Essentially unsaturated fats and triacylglyceride levels were responsible for the discrimination on PC1, with levels increasing across the classes; $1 < 2 < 3 < 4$. Triacylglyerides were most abundant in worms collected from property two (classes 3 and 4).

To test if the data could be use to predict class classification of unknown worm extracts a PLSDA model was constructed. The PLSDA model allowed greater separation of the four classes (Fig. 8). One model was constructed using 36 spectra (X-block normalized with mean centering and Y-block autoscaled) and cross validated using a venetian blind technique (Specificity for four classes (CV): 0.93 0.78 0.74 0.75; Class. Err (CV): 0.12 0.34 0.25 0.26). The model was tested against the remaining 7 spectra. This model accurately predicted the class in all cases but one. The model inaccurately classified spectra 36 as class 3 (70% probability) as well as, correctly, class 4. This is not surprising given the diversity of class 3 (as seen in Fig. 3). Nonetheless, this does suggest that with a larger dataset worm extracts could be accurately classified into treatment classes.

4 Discussion

These statistical analyses suggest that the environment of the worms has had a biochemical effect on the worms' metabolism. Since the land management regime is the largest environmental differentiator between the two sites at property 1 it appears that land treatment has had an effect on the worms. The biological sites have been managed differently from the conventional sites (see Table 1) for two years prior to this analysis and this has had a measurable effect on the biochemistry of the worms, as demonstrated by the class separation. The worms from the conventional treatment at property 2 are also separable from the two populations from the property 1 sites, probably representing a between site difference. Interestingly, those worms collected at the property 2 biological site varied more than any other group and could not be separated by simple PCA analysis. This may be due to the pre-treatment of this property with herbicides. It is possible that the observation is indicative of chemical toxicity and a combination of the

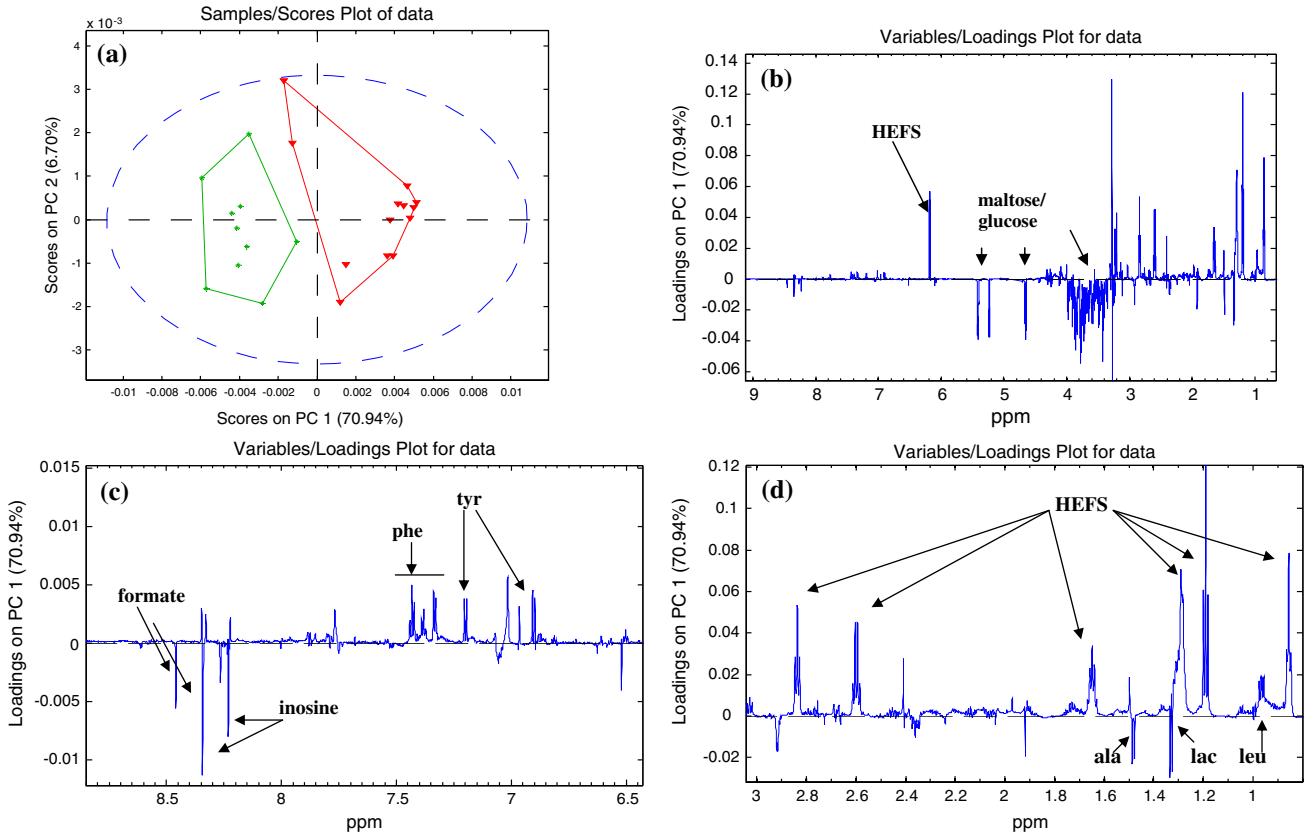

Fig. 7 **a** PCA analysis of aqueous for class 1 and 2 (Property 1) datasets. Class 1 ▼: 1–14 Property 1 biological treatment. Class 2 *: 15–24 Property 1 conventional treatment. **b** Loadings plot for PC1 **c** expansion of downfield region **d** expansion of upfield region

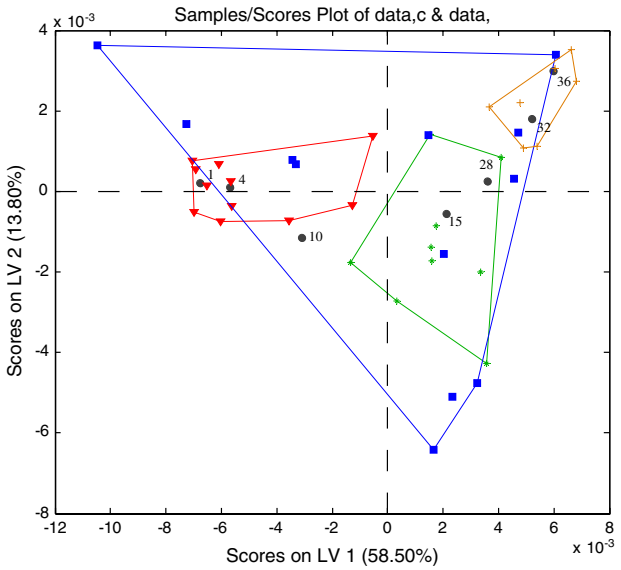

Fig. 8 PLSDA prediction for unknowns (weight outliers excluded) Test data 1,4,10 class 1; 15, class 2; 28, 32 class 3; 37 class 4

worm's individual exposure and response to the initial dose of these chemicals, i.e. a dose recovery response which is greater for the biological site compared to the conventional site. Additional longitudinal studies would be required to test this hypothesis. There are mixed reports in the literature about the toxicity of glyphosate formulas to earth worms. Dalby et al. (1995) reported there were no lethal effects after one dose of glyphosate to four earthworm species *A. trapezoides*, *A. rosea*, *A. caliginosa* or *A. longa* (Dalby et al. 1995). However other research suggests that repeated low doses has a significant effect on earthworm condition (as measured by weight gain) and some toxicity (Springer and Gray 1992). Most studies are laboratory based rather than field trials and do not cover the time periods that are relevant in this study (i.e. five months from initial chemical application). It is also important to note that glyphosate itself may not be the toxic agent but rather the surfactant or wetting agent may cause or enhance toxicity, as has been demonstrated for several aquatic species (Brausch and Smith 2007; Howe et al. 2004). The co-administered chemical, oxyfluorfen, also has reported toxicity to worms (Srivastava and Palta 2002).

Inspection of the loadings for the aqueous spectra revealed that changes in sugars, insoine, HEFS and some amino acid levels were important discriminators in the PCA model, along with the organic acids lactate and formate. Two trends were observed: for lactate, formate, insoine, alanine, glucose and maltose levels class 1 < class 2, class 3 < class 4 i.e. property 1 biological worms had the lowest concentrations. Conversely HEFS, phenylalanine, tyrosine and leucine levels were highest in class 1 and the opposite trend was observed: class 1 > class 2, class 3 > class 4.

Toxicology studies in rodents have noted changes in amino acids and organic acids in rodents where a pollutant exposure stress event lead to a general stress related event and to reduced feeding (Connor et al. 2004). Increased glucose levels have been linked to stress of various kinds in many different organisms. In humans, anxiety can cause elevated glucose (Armario et al. 1996). In fish, a similar blood glucose response is observed due to acute stress caused by transport, handling, netting and confinement (Kubļay and Uluköy 2002), lice infestation (Mustafa et al. 2000) and elevated temperatures (Viant et al. 2003). Glucose in crustaceans can be elevated in response to heavy metals (Lorenzon et al. 2000) and in sea hares due to atmospheric exposure (Carefoot 1994). Similarly, when subjected to freezing stress worm extracts display elevated glucose levels, the levels of which varied according to species and freeze-tolerance (Bundy et al. 2003). More recently, gluconeogensis has been noted as a response to sub lethal doses of pyrene where enhanced levels of amino acids are seen as an indication of response to starvation (Jones et al. 2008). The other major sugar present is maltose and, like glucose, is elevated in the worms from the conventionally treated sites. Elevated levels of maltose have been noted in previous studies on earthworms, where elevated concentrations of toxic metals were correlated with increased levels of maltose (Bundy et al. 2004). The metabolite 2-hexyl-5-ethyl-3-furansulfonate (HEFS) has been described as a negative biomarker of toxicity (Bundy et al. 2002) and the opposite concentration trends of this metabolite compared to the glucose tend to support that conclusion.

Analysis of the lipid fractions show an increase in lipids, particularly triacylglycerides in line with the trend observed for sugars with the concentration trend: class 1 < class 2, class 3 < class 4 observed. Recently, Bundy et al. (2008) demonstrated an increased lipid level in response to copper toxicity. Lipid levels have been observed as indicator of stress in a number of systems. Lipid levels have been shown to be an indicator of environmental stress in mussels (Hellou and Law 2003). Hellou and Law investigated the stress response in 60 groups of mussels of two species. Lipid content and condition indices were higher within *Mytilus edulis* and *Mytilus trossulus* that displayed shorter survival times than those from other sites. Moore et al. have carried out studies in mussels, focusing on the lysosome and have been able to demonstrate an increase in neutral lipids as a response to various stressors (Moore et al. 2006, 2007). Lysosomes are highly conserved in almost all cells of eukaryotic organisms including worms. Lysosomes break down intracellular organelles and play a crucial role in disease processes, toxicant removal as well as normal physiological maintenance (including those involved in nutrient restriction). Gastaldi et al. (2007) investigated the stress responses of

the earthworm *Eisenia andrei* including the potential biomarkers lysosomal membrane stability of coelomocytes, lysosomal accumulation of lipofuscin in chloragogenous tissue and of neutral lipids in coelomatic cells (Gastaldi et al. 2007). The authors investigated response to varying concentrations of both inorganic (copper and cadmium) and organic (benzo[α]pyrene) toxins. The pattern of neutral lipid accumulation varied depending on toxin but there was a consistent increase initially followed by a return to control levels. All the tests were on filter paper so over the duration of the experiments (7 days) starvation would start to play a role, though the authors believed this effect was not significant. It would be interesting to compare the response to these toxins in a more complex environment such as soil. The authors suggest that neutral lipid content could be a good indicator of mild environmental stress. The results presented here would seem to support this conclusion, though clearly the worms from the chemically treated sites are not seeing the return to a control state (in our case Property 1 biologically treated sites) as was observed by Moore et al. using the filter paper assays. The complementary observations of increased glucose levels and increased neutral lipids in the worms from conventionally treated land compared to those from the biologically treated land suggests that the worms collected from the sites using conventional land treatments are experiencing greater environmental stress.

4.1 Native earthworms

There were only three native worms specimens collected across the properties, all at property 1. One (sample 2) was collected from the biological treatment and two from the conventionally treated site (samples 15 and 16). They were the same species but the species identification is yet to be confirmed. Although in the aqueous analysis the extracts of these specimens cluster with the non-native worms on PCs 1 and 2, the native collected from the biologically managed site is very different from all others in PC 3 of the PCA model (Fig. 9). In the lipophilic PCA, worm 2 is also an outlier; but even more significantly different than in the aqueous case. Investigation of PC3 loadings and the spectra in class 1 revealed that the native worm, 2, has significantly higher levels of alanine (×7), sugars (×5), lactate (×2), acetate (×2) and triacylglyceride compared to other worms from this site (though the levels of these metabolites were not as high as for those worms collected in the traditionally managed sites). There are a number of possible explanations for this. It may be that the native worm species naturally have a greater phenotypic diversity that encompass this response. It is also possible that this is a real species phenotypic difference between the natives and European species. It may also be that this represents the

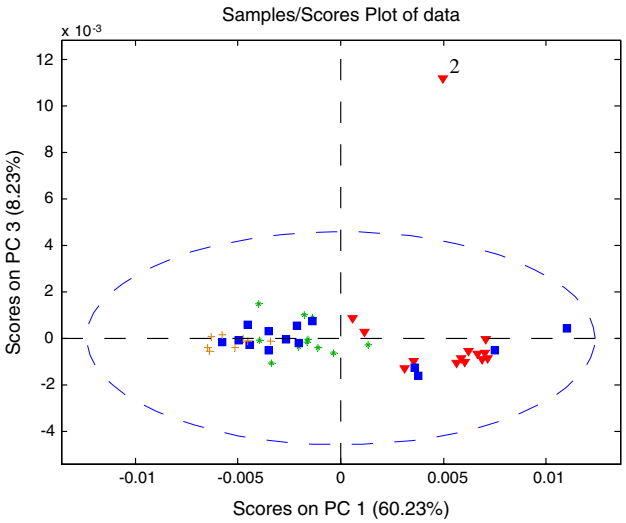

Fig. 9 The native worm (2) clearly differentiates in the third PC. Class 1 ▼: 1–14 Property 1 biological treatment. Class 2 *: 15–24 Property 1 conventional treatment. Class 3 ■: 25–33, 39–43 Property 2 biological treatment. Class 4 +: 34–38 Property 2 conventional treatment

additional stress displayed by native Australian worms to any form of intensive land use. Interestingly the natives collected at the conventionally managed site cannot be separated from non-natives by PCA analysis in either the lipid or aqueous analysis. It is tempting to speculate that the chemical treatment of the land is having a greater effect on the worm's metabolism than species identity but a greater number of native worms would need to be collected from both sites to confirm these hypotheses. The overall low number of native worms collected may be an indicator that the natives are not as well adapted as the European species to farmed land. Greater numbers of native worms than analyzed in this study would be required to gain further insight into the attributes of native species. One interesting observation is that the native worm also contains the HEFS metabolite (Fig. 10), suggesting that this metabolite is important for worms of significantly different origins. A focus of future work will be to survey matched remnant (native) and farmed land for native worms to assess stress and survival parameters.

5 Conclusion

Tissue extraction and NMR of the worm extracts revealed that the worm populations from property 1 living under the two different management conditions were statistically different and distinguishable from each other. That is, the land treatment appears to have had a significant effect on the worm's metabolism. Similarly the worms from conventional treatment at property 2 are distinctly different.

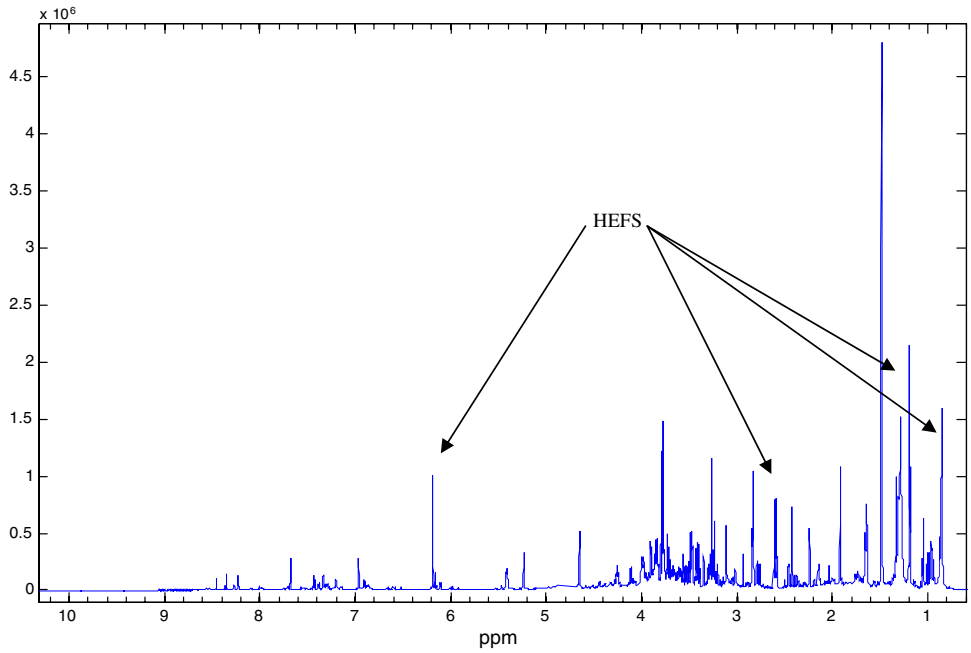

Fig. 10 Spectrum of the native worm (2)

The scattered data from the biological treatment at property 2 is intriguing and may be the result of initial herbicide treatment on the land, though further study is required to explore this possibility.

In general there is an increase in sugars, some amino acids (particularly alanine) and organic acids (including lactate and formate) in worms on conventionally treated land. Similarly analysis of the lipophilic extract suggested increased environmental stress with elevated levels of neutral lipids indicating increased lysosomal activity. Lysosomal activity and elevated sugar levels have previously been linked to environmental stress including toxic response. This study suggests that the worms in the conventionally managed sites may be experiencing greater stress and so may be an indicator of decreased soil health. Further work, including longitudinal studies, is required to validate these results; however this study suggests that earthworm biomarkers may be useful as an indicator for the overall health of the soil environment. The soil analysis in Table 1 reveals that there is little difference in traditional soil health parameters at this time (e.g. organic carbon levels are similar) and plant productivity data must be acquired over several years to assess treatment impact. Nonetheless it does appear that there is already a measurable effect on the health of the earthworms after just two years of adoption of the different treatment regimes.

The differences between the native and European worms are intriguing and may suggest that the natives are possibly less suited to managed land. This hypothesis would require further worm sampling across a range of soil types and across varied land management regimes, including land in its native state.

For the first time NMR, metabolomics has successfully been applied to Australian environmental questions and demonstrated that land management regimes can have a significant effect on the biochemistry of earthworms. This is also the first environmental comparison of lipid and aqueous NMR metabolomics for worms and demonstrates that similar class classifications can be gained with both datasets but providing orthogonal chemical information. With additional research such biochemical differences could be developed for biomarkers of soil health.

Acknowledgements This project was funded by the Mid-Loddon Sub-Catchment Management Group from a 2nd Generation State Government Grant allocated by the North Central Catchment Management Authority. It would not have been possible without the efforts of Judy Crocker, and we wish to thank Judy for her organization and her assistance. We also thank the owners of the two properties on which this work was conducted (Howard Hepburn and Lachlan Ralton and Peter and Steven Stone). The authors would also like to acknowledge the assistance of Dr David Keizer at Bio21, University of Melbourne for access to NMR instrumentation. The assistance of Monica Evani (La Trobe University) for loan of the dry shipper is also acknowledged.

References

Armario, A., Marti, O., Molina, T., de Pablo, J., & Valdes, M. (1996). Acute stress markers in humans: Response of plasma glucose, cortisol and prolactin to two examinations differing in the anxiety they provoke. *Psychoneuroendocrinology, 21*, 17–24. doi:10.1016/0306-4530(95)00048-8.

Arnaud, C., Saint-Denis, M., Narbonne, J. F., Soler, P., & Ribera, D. (2000). Influences of different standardized test methods on biochemical responses in the earthworm *Eisenia fetida andrei*. *Soil Biology and Biochemistry, 32*, 67–73. doi:10.1016/S0038-0717(99)00130-3.

Brausch, J. M., & Smith, P. N. (2007). Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, *Thamnocephalus platyurus*. *Archives of Environmental Contamination and Toxicology, 52*, 217–221. doi:10.1007/s00244-006-0151-y.

Brown, S. A. E., Simpson, A. J., & Simpson, M. J. (2008). Evaluation of sample preparation methods for nuclear magnetic resonance metabolic profiling studies with *Eisenia fetida*. *Environmental Toxicology and Chemistry, 27*, 828–836. doi:10.1897/07-412.1.

Bundy, J. G., Lenz, E. M., Bailey, N. J., Gavaghan, C. L., Svendsen, C., Spurgeon, D., et al. (2002). Metabonomic assessment of toxicity of 4-fluoroaniline, 3, 5-difluoroaniline and 2-fluoro-4-methylaniline to the earthworm Eisenia veneta (Rosa): Identification of new endogenous biomarkers. *Environmental Toxicology and Chemistry, 21*, 1966–1972. doi:10.1897/1551-5028(2002)021<1966:MAOTOF>2.0.CO;2.

Bundy, J. G., Osborn, D., Weeks, J. M., Lindon, J. C., & Nicholson, J. K. (2001). An NMR-based metabonomic approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress. *FEBS Letters, 500*, 31–35. doi:10.1016/S0014-5793(01)02582-0.

Bundy, J. G., Ramlov, H., & Holmstrup, M. (2003). Multivariate metabolic profiling using 1H nuclear magnetic resonance spectroscopy of freeze-tolerant and freeze-intolerant earthworms exposed to frost. *Cryo Letters, 24*, 347–358.

Bundy, J. G., Spurgeon, D. J., Svendsen, C., Hankard, P. K., Weeks, J. M., Osborn, D., et al. (2004). Environmental metabonomics: Applying combination biomarker analysis in earthworms at a metal contaminated site. *Ecotoxicology (London, England), 13*, 797–806. doi:10.1007/s10646-003-4477-1.

Bundy, J. G., Sidhu, J. K., Rana, F., Spurgeon, D. J., Svendsen, C., Wren, J. F., et al. (2008). 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. *BMC Biology, 6*, 25. doi:10.1186/1741-7007-6-25.

Callahan, C. A., Shirazi, M. A., & Neuhauser, E. F. (1994). Comparative toxicity of chemicals to earthworms. *Environmental Toxicology and Chemistry, 13*, 291–298. doi:10.1897/1552-8618(1994)13[291:CTOCTE]2.0.CO;2.

Carefoot, T. H. (1994). Effects of environmental stressors on blood glucose levels in sea hares, *Aplysia dactylomela*. *Marine Biology (Berlin), 118*, 579–583. doi:10.1007/BF00347504.

Connor, S. C., Wu, W., Sweatman, B. C., Manini, J., Haselden, J. N., Crowther, D. J., et al. (2004). Effects of feeding and body weight loss on the 1H NMR based urine metabolic profiles of make Wistar Han rates: Implications for biomarker discovery. *Biomarkers, 9*, 156–179. doi:10.1080/13547500410001720767.

Dalby, P. R., Baker, G. H., & Smith, S. E. (1995). Glyphosate, 2, 4-DB and dimethoate: Effects on earthworm survival and growth. *Soil Biology and Biochemistry, 27*, 1661–1662. doi:10.1016/0038-0717(95)00091-R.

Gastaldi, L., Ranzato, E., Capri, F., Hankard, P., Peres, G., Canesi, L., et al. (2007). Application of a biomarker battery for the evaluation of the sublethal effects of pollutants in the earthworm *Eisenia andrei*. *Comp Biochem Physiol C Toxicol Pharmacol, 146*, 398–405.

Gibb, J. O. T., Holmes, E., Nicholson, J. K., & Weeks, J. M. (1997). Proton NMR spectroscopic studies on tissue extracts of invertebrate species with pollution indicator potential. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology, 118B*, 587–598. doi:10.1016/S0305-0491(97)00063-1.

Hellou, J., & Law, R. J. (2003). Stress on stress response of wild mussels, *Mytilus edulis* and *Mytilus trossulus*, as an indicator of ecosystem health. *Environmental Pollution, 126*, 407–416. doi:10.1016/S0269-7491(03)00231-8.

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., & Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry, 23*, 1928–1938. doi:10.1897/03-71.

Jones, O. A. H., Spurgeon, D. J., Svendsen, C., & Griffin, J. L. (2008). A metabolomics based approach to assessing the toxicity of the polyaromatic hydrocarbon pyrene to the earthworm *Lumbricus rubellus*. *Chemosphere, 71*, 601–609.

Kubjay, A., & Uluköy, G. (2002). The effects of acute stress on rainbow trout (*Oncorhynchus mykiss*). *Turk J Zool, 26*, 249–254.

Lorenzon, S., Francese, M., & Ferrero, E. A. (2000). Heavy metal toxicity and differential effects on the hyperglycemic stress response in the shrimp *Palaemon elegans*. *Archives of Environmental Contamination and Toxicology, 39*, 167–176. doi:10.1007/s002440010093.

MacEwan, R. (2007). *Soil assessment for the mid loddon land management group, biological farming sites* (p. 13). Victoria, Australia: Department of Primary Industries.

Moore, M. N., Icarus Allen, J., & McVeigh, A. (2006). Environmental prognostics: An integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Marine Environmental Research, 61*, 278–304. doi:10.1016/j.marenvres.2005.10.005.

Moore, M. N., Viarengo, A., Donkin, P., & Hawkins, A. J. (2007). Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. *Aquatic Toxicology (Amsterdam, Netherlands), 84*, 80–91. doi:10.1016/j.aquatox.2007.06.007.

Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G. A., & Burka, J. F. (2000). Effects of sea lice (*Lepeophtheirus salmonis* Kroyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology, 10*, 47–59. doi:10.1006/fsim.1999.0229.

Roberts, B. L., & Dorough, H. W. (1984). Relative toxicities of chemicals to the earthworm *Eisenia foetida*. *Environmental Toxicology and Chemistry, 3*, 67–78. doi:10.1897/1552-8618(1984)3[67:RTOCTT]2.0.CO;2.

Saint-Denis, M., Narbonne, J. F., Arnaud, C., & Ribera, D. (2001). Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil. Effects of lead acetate. *Soil Biology and Biochemistry, 33*, 395–404. doi:10.1016/S0038-0717(00)00177-2.

Springer, J. A., & Gray, R. A. J. (1992). Effect of repeated low doses of biocides on the eathworm *Aporrectodea Caliginosa* in laboratory culture. *Soil Biology and Biochemistry, 24*, 1739–1744. doi:10.1016/0038-0717(92)90180-6.

Srivastava, C., & Palta, R. (2002). Effect of atrazine and oxyfluorfen against earthworm (*Eisenia fetida*). *Annals of Plant Protection Sciences, 10*, 145–147.

Viant, M. R. (2003). Improved methods for the acquisition and interpretation of NMR metabolomic data. *Biochemical and Biophysical Research Communications, 310*, 943–948. doi:10.1016/j.bbrc.2003.09.092.

Viant, M. R., Werner, I., Rosenblum, E. S., Gantner, A. S., Tjeerdema, R. S., & Johnson, M. L. (2003). Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead trout (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. *Fish Physiology and Biochemistry, 29*, 159–171. doi:10.1023/B:FISH.0000035938.92027.81.

Weeks, J. M., Spurgeon, D. J., Svendsen, C., Hankard, P. K., Kammenga, J. E., Dallinger, R., et al. (2004). Critical analysis of soil Invertebrate biomarkers: A feld case study in Avonmouth, UK. *Ecotoxicology (London, England), 13*, 817–822. doi:10.1007/s10646-003-4479-z.