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# Proton Nuclear Magnetic Resonance ($^1$H NMR) profiling of isolated organs in the snail *Helix aspersa maxima*

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ABSTRACT

*Helix aspersa maxima* is a terrestrial snail living at the air-ground interface, which makes it an ideal bioindicator candidate to evaluate environmental pollution. In this study, Proton Nuclear Magnetic Resonance ($^1$H NMR) based metabolomics approach was applied on diverse tissue extracts isolated from *Helix aspersa* individuals. This omics technique allows to characterize in one single spectroscopic measurement the global metabolic changes caused by pollution on the organism and to assess the potential of this species as a pollution indicator. In a first set of experiments conducted on naïve individuals, the main endogenous metabolites were identified. Those included amino and organic acids, Krebs cycle intermediates and a series of osmolytes. Next, a second group of animals was exposed to methomyl, a pesticide used in agriculture, in order to reveal what metabolic pathways were potentially affected by the pollutant. Our results indicate important fluctuations in energetic metabolism and several biomarkers of oxidative stress. In conclusion, this work demonstrates the potential of combining invertebrates models to metabonomics as a powerful tool to predict adverse effects of chemicals.

## 1. Introduction

Ecotoxicology is accustomed to the exploitation of invertebrate models as bioindicators species in terms of their sedentary lifestyle, their bioaccumulation capacity, their distribution and abundance in the environment, their many offspring and their breeding in the laboratory (Beeby and Richmond, 2002; Itziou and Dimitriadis, 2011; Laskowski and Hopkin, 1996; Regoli et al., 2005). In order to cover the different ecosystems, several species are usually explored such as earthworms for soil pollution (Antunes et al., 2008; Lanno et al., 2004; Paoletti, 1999; Velki et al., 2014); mussels for the monitoring of either marine (Musselwatch projects) (Cantillo, 1998; O'Connor 1998; Melwani et al. 2014) or continental waters (Beckvar et al., 2000; Elder and Collins 1991; Kraak et al., 1991) and honeybee to appreciate the effect of pesticides on plants (Badiou et al., 2008; Badiou-Benéteau et al., 2013; Porrini et al., 2002). Terrestrial snails, on the other hand, turn out to be encouraging bioindicators at the air-ground interface.

Numerous biomarkers of toxicity have already been described in these organisms such as enzymatic activities (Glutathion-S-transferase, catalase, acetylcholinesterase) (Larba and Soltani, 2014; Parveen et al., 2001; Salama et al., 2005; Regoli et al., 2006; Radwan and Mohamed, 2013), genotoxic effect (micronuclei, comet assay) (da Silva et al., 2013; De Souza et al., 2015; Ianistcki et al., 2009) and oxidative stress evaluation (lysosomal membrane stability) (Itziou et al., 2011; Itziou and Dimitriadis, 2012; Regoli, 1992). Interestingly, Metabonomics, one emergent omics family member, supplies a global view of the metabolic changes occurring very early in tissues exposed to toxicants. The metabolic signature obtained by this approach is characteristic of a particular toxic effect and may lead to the discovery of new biochemical biomarkers. Indeed, Proton Nuclear Magnetic Resonance ($^1$H NMR) is already commonly applied in xenobiotic risk assessment in vertebrates, mostly in rodents but also in humans. Such studies are based on a rapid and non-destructive analytical method applied to biofluids like urine and blood (Bollard et al., 2005; Conotte and Colet, 2014; Holmes et al., 2000; Potts et al., 2001) or on tissue extracts (Beckonert et al., 2007; Nicholson et al., 2002). Considering the very promising results obtained in vertebrates, metabonomics was more recently transposed to invertebrate species such as *Eisenia fetida* (Ji et al., 2013; Lankadurai et al., 2011, 2015; McKelvie et al., 2009), *Lumbricus rubellus* (Jones et al., 2007) or *Arion subfuscus* (Gibb et al., 1997).

*Helix aspersa maxima* (Taylor, 1883), also called "Gros gris" is a 40–45 mm garden snail from North Africa weighing 20–30 g. This

*Abbreviations:* AchE, acetylcholinesterase; ADME, absorption, distribution, metabolism and excretion; CV-ANOVA, cross-validation analysis of variance test; $D_2O$, deuterium oxide; DMSO, dimethyl sulfoxide; $^1$H NMR, proton Nuclear Magnetic Resonance; LMW, low molecular weight; PLS-DA, partial least square discriminant analysis; ROS, reactive oxygen specie; SAM, sulfur-adenosylmethionine; TSP, 3-trimethylsilyl propionate

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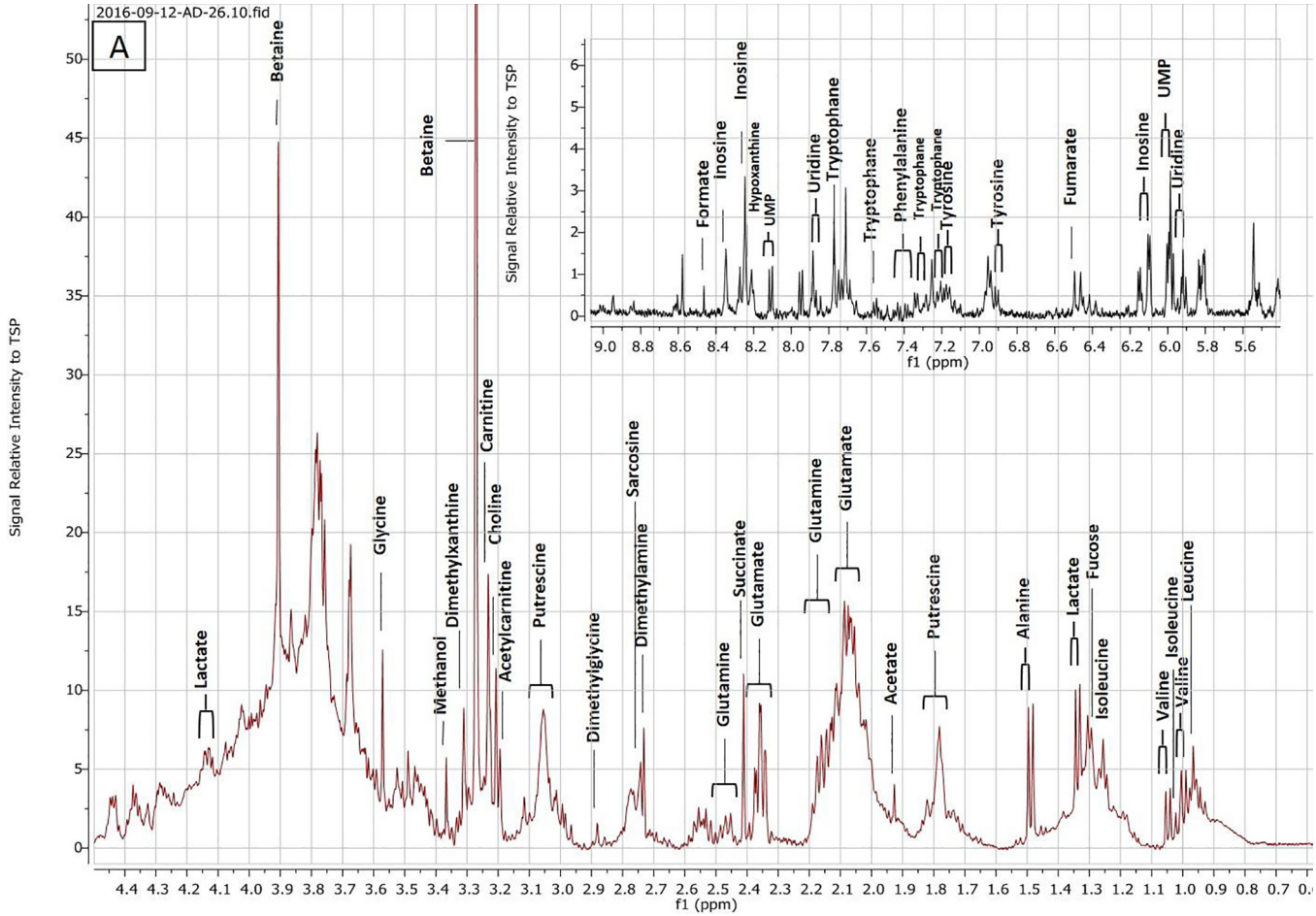


**Fig. 1A.** Identification of resonances from $^{1}$H NMR spectra acquired at 500 MHz (128 scans) on the aqueous phase from extracts of digestive gland in *Helix aspersa maxima*.

pulmonate gasteropod is usually exploited in snail farming for food and pharmaceutical industries (Bonnemain, 2005). Nevertheless, their size and ease of reproduction show interest as possible bioindicator models. Bigger than the *Helix aspersa aspersa* species, its use facilitates the sampling of different organs such as pulmonary membrane, kidney, digestive gland and heart during dissection. The pulmonary membrane has the important role of oxygen uptake, followed by its distribution through the circulatory system (Krogh, 1941). It forms a pocket considered as a lung describing snails as a pulmonate organisms. The heart being a chambered heart composed of a ventricle and auricle corresponds to a primitive organization of vertebrates (Sodikdjon, 2011). The digestive gland, formerly called hepatopancreas, shares the same role that liver in vertebrates, as well as the kidney. These organs are particularly important in toxicological studies since respiratory system represents a major absorption pathway of environmental toxic compounds; digestive gland and kidney play key roles in biotransformation processes and elimination routes (Ilowite and Laxer, 2011) and heart is a mandatory target organ in risk assessment.

In the present work, we first investigated the possibility to obtain metabolic profiles of those four selected organs separately using $^{1}$H NMR spectroscopy in naïve individuals and to characterize their possible specific metabolomes. Next, we verified that these profiles were sensitive to xenobiotic-induced toxic effects expecting to unveal predictive biomarkers. This second goal was evaluated by exposing snails to the prototypical pollutant Methomyl, a carbamate pesticide. The impact of Methomyl on the metabolome of different organs of interest demonstrated the value of combining NMR-based metabonomics to invertebrate models in risk assessment.

## 2. Materials and methods

### 2.1. Animal housing

Terrestrial gastropods of the species *Helix aspersa maxima* were purchased from the helicicole farm of Avesnois (France). They were housed in plastic boxes at 20 ± 2 °C and exposed to natural photoperiod of the day through windows with permanent access to water and food (Helinove product, France). Only adults from same age and similar body weight (about 20 g) were investigated. In this study, a total of 16 individuals were used, 8 per group.

### 2.2. Chemicals

The following compounds were used for the preparation and the NMR analysis of the samples:

Phosphate buffer prepared with $Na_2HPO_4{\cdot}2H_2O$ 0.2 M and $NaH_2PO_4{\cdot}H_2O$ 0.04 M in water-deuterium oxide ($D_20$) solution (80:20, v:v); Methanol; Chloroform; TSP (Deuterated sodium 3-trimethylsilyl propionate); DMSO (Dimethyl sulfoxide 99,5%); Methomyl (1-methylthioacetaldehyde o-methylcarbamoyloxime, PESTANAL® analytical grade, Sigma-Aldrich BVBA, Overijse, Belgium).

### 2.3. Acquisition and identification of metabolic profiles by $^{1}$H NMR in the four organs of Helix aspersa maxima

Metabonomics with spectroscopic methods such as $^{1}$H NMR has proved to be part of the most powerful predictive techniques for

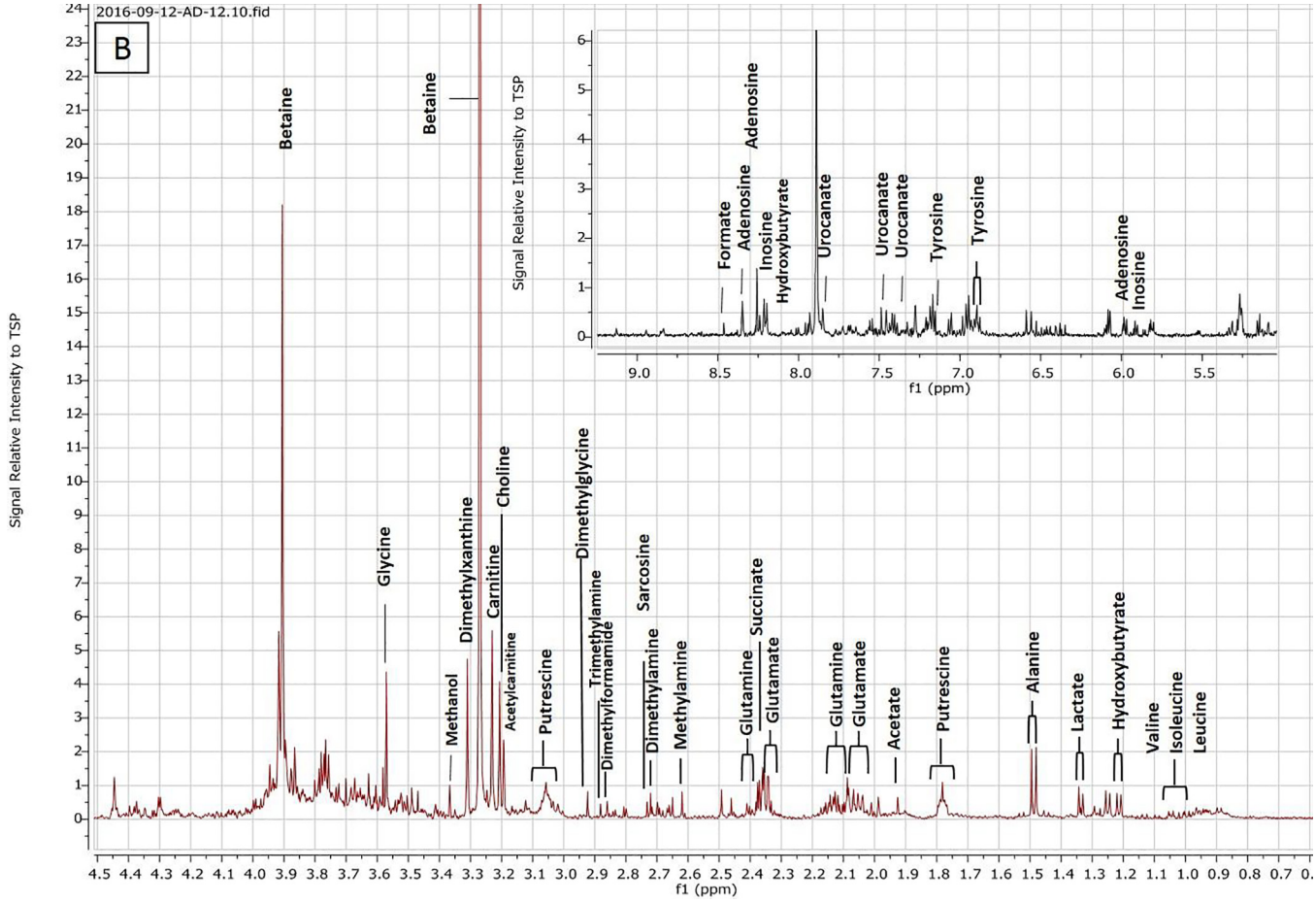


**Fig. 1B.** Identification of resonances from $^{1}$H NMR spectra acquired at 500 MHz (128 scans) on the aqueous phase from extracts of kidney in *Helix aspersa maxima*.

investigating the response of organisms to xenobiotic (Shockcor and Holmes, 2002). The procedures of sample preparation and acquisition by NMR spectroscopy of tissue extracts used in this study, were based on a validated protocol described by Beckonert et al. (2007).

### 2.3.1. Sample preparation

Snails were killed by freezing them at −20 °C and dissected on ice to sample the pulmonary membrane, the digestive gland, the kidney and the heart. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis by $^{1}$H NMR. Hearts were prepared with two different ways, either by pooling three samples or individually. Each organ was crushed in a mortar with liquid nitrogen into powder and were extracted following a methanol-water-chloroform extraction method using precooled solutions (volumes used per gram of organ): homogenization of the organ powder with 4 mL of methanol and 850 µL of water with vortex, 2 mL of chloroform was added before extrahomogenization with 2 mL of chloroform and 2 mL of water. The homogenates were placed on ice during 10 min before centrifugation (15 min. at 1000 × *g*, 4 °C). The aqueous phase on the top was recovered and methanol was removed *in vacuo* during 6 h for the digestive gland and kidney, 2 h for pulmonary membrane and 1 h for heart. Each extract, except for individual heart, was reconstituted into 700 µL phosphate buffer 0.1 M before final centrifugation (10 000 × *g*, 10 min), 50 µL of TSP 14 mM was added to 650 µL supernatant for the digestive gland and TSP 7 mM for kidney and pulmonary membrane. TSP is an external reference added to each sample for the calibration of the NMR spectra. The TSP resonance was arbitrarily fixed at 0.00 ppm and prepared in 100% deuterium oxide. Finally, 650 µL were transferred into individual tubes (5 mm diameter) for NMR analysis. Individual heart extract was reconstituted with $D_20$ (100%) and TSP 7 mM in 80 µL final volume before introducing 70 µL into 2 mm diameter capillary tube for NMR analysis.

### 2.3.2. $^{1}$H NMR spectroscopy and data analysis

One-dimensional NMR spectra of extracts were acquired on a Bruker 500 Avance spectrometer (11,8T corresponding to a proton Larmor frequency of 500 MHz) at 297 K using a NOESYPRESAT-1d pulse sequence. 128 free induction decays (FID) (except for individuals hearts for which 256 scans were used), with 65.536 data points per FID were collected for each tissue extract using a spectral width of 10.330,578 Hz, an acquisition time of 3.17 s and a pulse recycle delay of 3 s. All spectra were referenced to the chemical shift of TSP (0.0 ppm) and were phase and baseline-corrected using MestReNova 11.0 software (Mestrelab Research, Santiago de Compostela, Spain), before being Fourier transformed and submitted to a line broadening of 0.3 Hz. Spectral regions distorted by imperfect water peak saturation were removed. Remaining areas (Area under the curve, AUC) were normalized against the total sum of spectral integrals, reducing the spectral region from 0.08 to 10.00 ppm to 248 integrated regions (buckets) with 0.04 ppm width each, before multivariate analysis in SIMCA-P + 12.0 software (Umetrics, Umea, Sweden). After mean-centering of the data, an unsupervised analysis by principal component analysis (PCA) was used to identified possible outliers and evaluate the degree of homogeneity. However, since this analytical method does not allow statistical partitioning between groups, a Partial Least Square Discriminant Analysis (PLS-DA) was performed to analyze clustering models by one-way cross–validation analysis of variance (CV-ANOVA) and possibly discriminate different metabolic patterns among tissues. The quality of the model was described by $R^2$ parameter which represents the explained variation in the data set and the reliability was described by $Q^2$

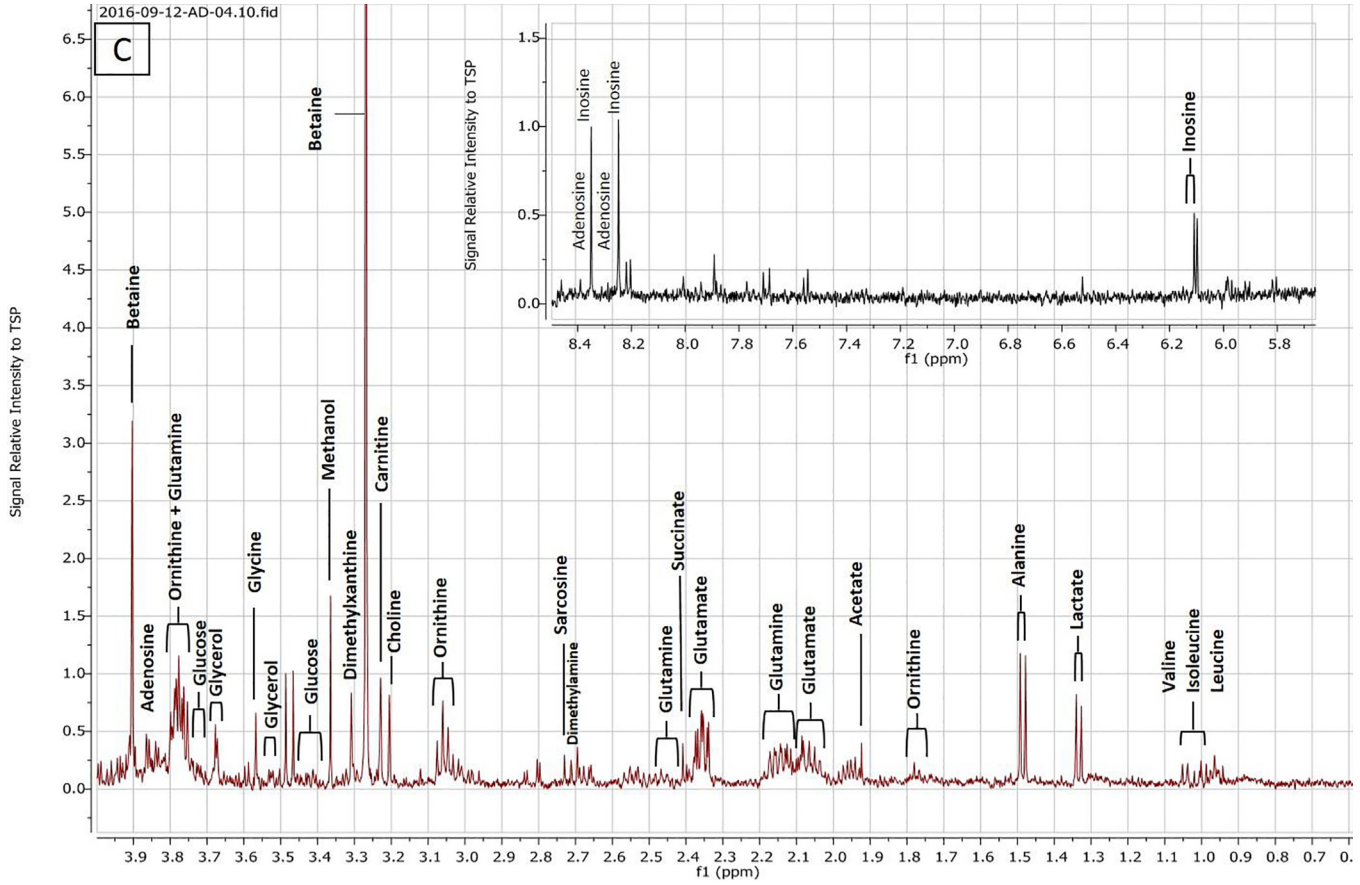


**Fig. 1C.** Identification of resonances from $^{1}$H NMR spectra acquired at 500 MHz (128 scans) on the aqueous phase from extracts of pulmonary membrane in *Helix aspersa maxima*.

parameter which uses cross-validation to estimate the goodness of prediction of the model. The most efficient representation of the groups clustering given by the PLS-DA is the score plot and the corresponding loading plot was used to observe metabolites responsible of this cluster. Metabolites were identified with a database of $^{1}$H NMR spectra based on chemical shift and multiplicity of peaks.

### 2.3.3. Statistical analysis between tissue extracts

In 8 snails, variations between four types of organ were assessed by CV-ANOVA and metabolites responsible of differences were extracted with the Variable Important Parameter (VIP) of the PLS-DA model. This VIP list identify the metabolites mainly contributing to the cluster with cut-off value of VIP ≥ 1. Since AUC measurements are semi-quantitative data, a non-parametric test had to be applied. In addition, since the comparison concerned more than two groups, a Kruskal-Wallis (K-W) statistical test was chosen. For each extracted discriminant metabolite (VIP ≥ 1), a one-way analysis of variance was applied by a K-W test on normalized AUC values to determine significant differences between organs (p-value $<$ 0.05 as significant level). If K-W test was significant, a multiple comparison procedure was performed with a Dunn Test between each group taken by pairs (p-value $<$ 0.05 as significant level).

## 2.4. Sensibility to toxicological effects of metabolites detected by $^{1}$H NMR approach

### 2.4.1. Methomyl exposure

The dose of Methomyl compound was chosen based on $LD_{50}$ (=32 mg/Kg, oral route, US-EPA 81-1) in rats and from a literature report (Patil et al., 2008) carried out on rats weighing 200 g and exposed for 3 days at 4 mg/Kg Methomyl by intraperitoneal administration. In this study, a decrease in the CYP450 activity and increases in plasma transferases were noticed (Patil et al., 2008). This selected dose allowed to observe minimal mortality while ensuring measurable metabolic effects in survivors.

*Helix aspersa maxima* snails (n = 8) housed in plastic boxes at 20 ± 2 °C and exposed to natural photoperiod of the day, without any food or water, were exposed to methomyl at 800 µg/snail (equivalent of 40 mg/Kg) in 20 µL of solution applied on the roof of the mantel cavity by a hole drilled in the shell. The rate of survival of the snails consecutively to the drilling of the hole was checked for 24 h before the compound was applied. At the same time, control individuals (n = 8) were exposed using the same procedure (by a hole in the shell) but to DMSO, the solvent used for the preparation of the methomyl solution. Only adults from same age and similar body weight (20 ± 2 g) were investigated. All individuals were euthanized 48 h after exposure by freezing at −20 °C. Sample preparation of tissue extracts and $^{1}$H NMR spectroscopy were performed as already explained.

### 2.4.2. Statistical analysis between snails exposed to Methomyl and control batch

PLS-DA plots were built to compare the metabolic response observed in the Methomyl-exposed snails and controls after NMR analysis of their tissue extracts (digestive gland, kidney and pulmonary membrane separately). A CV-ANOVA approach was applied on metabolic profiles to test the significance of intergroup differences (p-value $<$ 0.05 significance level). Discriminant metabolites were extracted on the basis of variable importance (VIP $>$ 1) of the PLS-DA model and their normalized AUC values were used to evaluate possible changes in their levels after Methomyl exposure with a Wilcoxon test (p-value

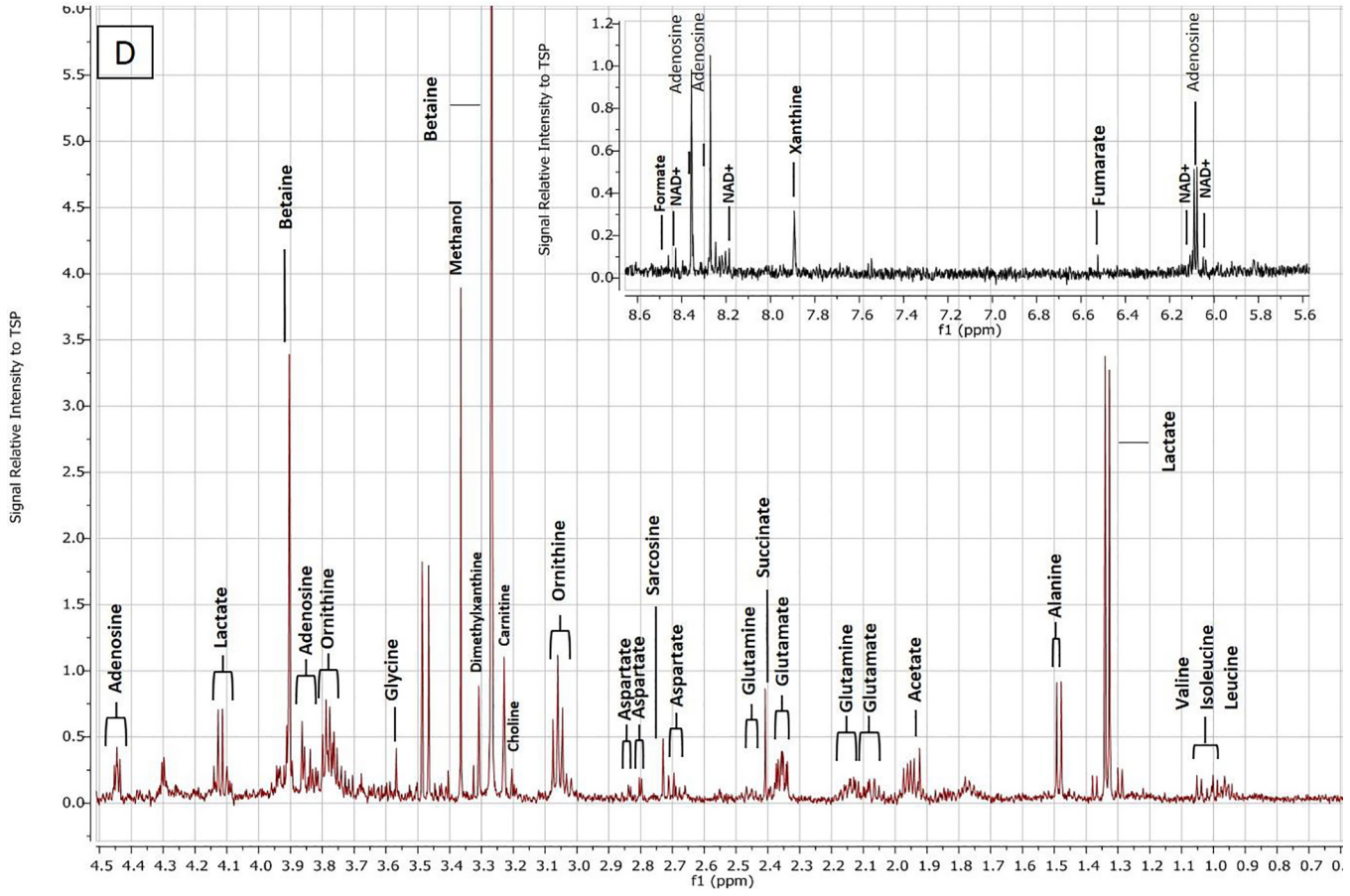


**Fig. 1D.** Identification of resonances from $^{1}$H NMR spectra acquired at 500 MHz (128 scans) on the aqueous phase from extracts of pooled heart in *Helix aspersa maxima*.

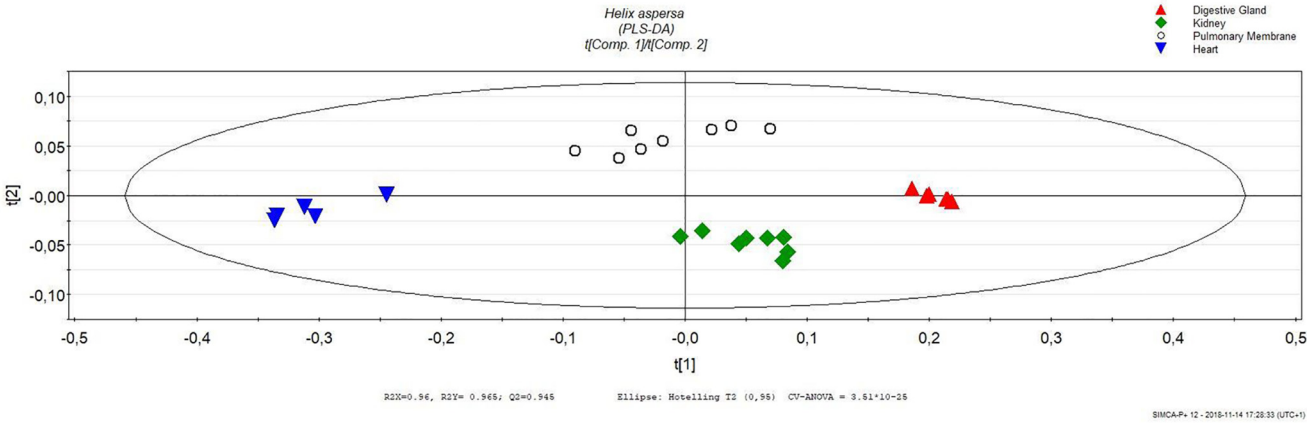


**Fig. 2.** Scores scatter plot obtained after PLS-DA on $^{1}$H NMR spectra of tissues extracts of digestive gland (red triangle), kidney (green diamond), pulmonary membrane (black circle) and individual heart (blue inverted diamond) of *Helix aspersa (N = 8)*.

adjusted with Holm-Bonferroni significance level) (Olejnik et al., 1997). Wilcoxon test is a non-parametric statistical analysis used in the comparison between two groups (Methomyl-exposed and DMSO-exposed as control groups).

## 3. Results

### 3.1. Acquisition and identification of metabolic profiles obtained by $^{1}$H NMR spectroscopy of isolated tissues of Helix aspera maxima

$^{1}$H NMR Spectroscopic method allowed to obtain exploitable spectrum in each of the selected tissues. Many endogenous metabolites were identified, including a variety of amino acids such as alanine, valine, leucine, isoleucine, glutamine, glutamate, tyrosine and tryptophan; sugars (i.e., glucose); Krebs cycle intermediates such as succinate and

**Table 1**
Major metabolites assigned and semi-quantified by normalized AUC allowing characterization of each type of tissue extracts obtained by $^{1}$H NMR profiling in *Helix aspersa* (N = 8).

| METABOLITES | AUC [a] Digestive Gland | AUC [a] Kidney | AUC [a] Pulmonary Membrane | AUC [a] Heart | *K-W p* value [b] $\alpha < 0.05$ |
|---|---|---|---|---|---|
| Acetate | 0.0455 $^{\#\$}$ | 0.0053 | 0.0027 $^{*}$ | 0.0098 $^{\#}$ | $2^{*}10^{-4}$ |
| Acetylcarnitine | 0.0712 $^{*\#}$ | 0.0361 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $2^{*}10^{-5}$ |
| Adenosine | 0.0000 $^{*\#}$ | 0.0000 $^{*\#}$ | 0.0040 $^{*\$}$ | 0.1953 $^{\#\$}$ | $2.1^{*}10^{-5}$ |
| Alanine | 0.2212 $^{\#}$ | 0.0507 | 0.0279 | 0.0603 | $4^{*}10^{-4}$ |
| Betaine | 1.4495 $^{\#}$ | 1.3459 $^{\#}$ | 0.1935 $^{*\$}$ | 3.7486 $^{\#}$ | $1.1^{*}10^{-4}$ |
| Carnitine | 0.2454 $^{\#}$ | 0.1503 $^{\#}$ | 0.0239 $^{*\$}$ | 0.1354 $^{\#}$ | $1.2^{*}10^{-4}$ |
| Choline | 0.1423 $^{*\#}$ | 0.0572 $^{\#}$ | 0.0097 $^{\$}$ | 0.0069 | $5.8^{*}10^{-5}$ |
| Dimethylamine | 0.0045 $^{*\#}$ | 0.0096 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $1.5^{*}10^{-5}$ |
| Dimethylglycine | 0.0031 | 0.0078 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $7.6^{*}10^{-5}$ |
| Dimehylxanthine | 0.1016 $^{*\#}$ | 0.0924 $^{*\#}$ | 0.0134 $^{\$}$ | 0.0000 $^{\$}$ | $5.7^{*}10^{-5}$ |
| Formate | 0.0058 $^{*\#}$ | 0.0024 $^{*}$ | 0.0008 $^{*}$ | 0.0000 $^{\#\$}$ | $7^{*}10^{-5}$ |
| Fucose | 0.1738 $^{*\#\$}$ | 0.0000 | 0.0000 | 0.0000 | $2.4^{*}10^{-5}$ |
| Fumarate | 0.0030 $^{*}$ | 0.0019 $^{*}$ | 0.0010 | 0.0000 $^{\$}$ | 0.012 |
| Glutamate | 0.5183 $^{\#}$ | 0.0874 | 0.0359 | 0.1491 | $5^{*}10^{-4}$ |
| Glycine | 0.0628 $^{*\#}$ | 0.0348 $^{\#}$ | 0.0055 $^{\$}$ | 0.0081 | $4^{*}10^{-4}$ |
| Hydroxybutyrate | 0.0000 $^{\$}$ | 0.0374 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $1.18^{*}10^{-5}$ |
| Hypoxanthine | 0.0369 $^{*\#}$ | 0.0835 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $3^{*}10^{-5}$ |
| Inosine | 0.0493 $^{*\#}$ | 0.0235 $^{*\#}$ | 0.0085 $^{*\$}$ | 0.0000 $^{\#\$}$ | $2^{*}10^{-5}$ |
| Isoleucine | 0.0398 $^{*\#}$ | 0.0070 $^{*}$ | 0.0024 | 0.0000 $^{\$}$ | $6.5^{*}10^{-5}$ |
| Lactate | 0.1674 $^{\#\$}$ | 0.0346 | 0.0152 $^{*}$ | 0.0875 $^{\#}$ | $8.1^{*}10^{-5}$ |
| M1 (1.24–1.26. d) | 0.0000 $^{\$}$ | 0.0469 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $1.1^{*}10^{-5}$ |
| Methylamine | 0.0000 $^{\$}$ | 0.0077 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $1.1^{*}10^{-5}$ |
| Ornithine | 0.0000 $^{*\#}$ | 0.0000 $^{*\#}$ | 0.0280 $^{\$}$ | 0.4821 $^{\$}$ | $1.6^{*}10^{-5}$ |
| Putrescine | 0.5521 $^{*\#}$ | 0.0851 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $1.1^{*}10^{-5}$ |
| Sarcosine | 0.0438 $^{\#}$ | 0.0058 | 0.0017 $^{*}$ | 0.0550 $^{\#}$ | $3.3^{*}10^{-5}$ |
| Succinate | 0.1109 $^{\#}$ | 0.0185 $^{\#}$ | 0.0028 $^{\#\$}$ | 0.0178 $^{*}$ | $1.7^{*}10^{-4}$ |
| Trimethylamine | 0.0144 $^{*\#}$ | 0.0045 $^{*\#}$ | 0.0000 $^{\$}$ | 0.0000 $^{\$}$ | $1.5^{*}10^{-5}$ |
| Valine | 0.0598 $^{*\#}$ | 0.0141 $^{*}$ | 0.0050 | 0.0000 $^{\$}$ | $6.4^{*}10^{-5}$ |
| Xanthine | 0.0125 $^{*\#}$ | 0.0018 $^{*}$ | 0.0009 | 0.0000 $^{\$}$ | $1.2^{*}10^{-4}$ |

Semi-quantification was evaluate to TSP reference. a: Area Under the Curve (AUC) normalized by total spectrum area, b: Kruskal-Wallis One Way Analysis of Variance on Ranks and if significant Multiple Comparison Procedures (Dunn Test, $\alpha < 0.05$ as significant level) ($^{\$}$: significant compared to kidney, $^{\#}$: significant compared to pulmonary membrane, $^{*}$: significant compared to individual heart). Underlined null values for heart indicate that the metabolite is only detectable after pooling.

fumarate, organic acids such as lactate and acetate and osmolytes (betaine). The $^{1}$H NMR spectra showed characteristic profiles for each examined organ. Figs. 1A–1D shows the assignments of $^{1}$H NMR spectra of the aqueous phase of extracts of digestive gland (Fig. 1A), kidney (Fig. 1B), pulmonary membrane (Fig. 1C) and pooled heart (Fig. 1D) of *Helix aspersa maxima*, respectively.

$^{1}$H NMR spectrum is defined by chemical shift in abscissa (ppm) and signal strength in ordinate. Each spectrum is divided into two regions: the aliphatic region from 0.6 to 4.5 ppm (in red) and a non-aliphatic region from 5.5 to 9 ppm (in black color). $H_2O$ signal from 4.5 to 5.5 was artificially suppressed. Metabolite identification was based on the chemical shift and multiplicity of the corresponding peaks by comparison to home and -web databases of the $^{1}$H NMR spectra.

As shown in Fig. 2, representing the scores plot obtained from the PLS-DA analysis of the NMR data (N = 8), clear organ-specific clustering based on different metabolic compositions are observed. These results suggest that specific metabolic pattern can be associated to each individual organ.

The PLS-DA results showed good qualities from the retrieved $R^2$ and $Q^2$ values ($R^2x = 0.960$; $R^2y = 0.965$; $Q^2 = 0.945$) and the significance of the clustering observed is confirmed by the p-value of $3.51^{*}10^{-25}$ ($p < 0.05$) obtained with a CV-ANOVA statistical test. For each symbol of plots, each point represents a sample. The ellipse represents Hotelling's with 95% confidence.

Among the metabolites responsible for the variations between tissue extracts highlighted by VIP lists, many were found in the four organs but not at the same levels as: acetate (1.93 ppm, singlet), alanine (1.48 ppm, doublet), betaine (3.27 ppm, singlet; 3.91 ppm, singlet), carnitine (3.23 ppm, singlet), choline (3.21 ppm, singlet), glutamate (2.35 ppm, multiplet), glycine (3.57 ppm, singlet), lactate (1.33 ppm, doublet), sarcosine (2.73 ppm, singlet) and succinate (2.41 ppm, singlet). Others were only detected in digestive gland and kidney extracts as putrescine (3.05 ppm, multiplet; 1.8 ppm, multiplet) trimethylamine (2.9 ppm, singlet), acetylcarnitine (3.19 ppm, singlet), dimethylamine (2.72 ppm, singlet), dimethylglycine (2.93 ppm, singlet), and hypoxanthine (8.17 ppm, singlet; 8.2 ppm, singlet); while hydroxybutyrate (1.2 ppm, doublet), methylamine (2.6 ppm, singlet) and unidentified peaks at 1.24 ppm (doublet) were only found in kidney extracts. Adenosine (6.08 ppm, doublet) and ornithine (3.05, triplet) were just observed in pulmonary membrane and heart tissues.

Based on statistical differences, some major metabolites were selected as tissue representative and listed in Table 1 which contains normalized AUC values for each metabolite in each tissue and p-value of Kruskal-Wallis test allowing to distinct a statistical group separation for the corresponding metabolite (p-value < 0.05 significant level). Statistical Dunn-Test determined differences between groups taken by pairs for each metabolite.

### 3.2. Metabolic changes with methomyl exposure

All control snails survived the study, however 44% of mortality was observed after 48 h of exposure to methomyl. After sacrifice, the $^{1}$H NMR spectra of each tissue collected from survivals were analyzed and compared to controls. Plots in Fig. 3 show clusters between treated snails with 800 µg/snail of Methomyl (blue diamonds) and control snails identically exposed to DMSO (black squares) for 48H. Digestive gland tissues extracts on the top, kidney tissues extracts in the middle, pulmonary membrane tissues extracts in the bottom. For each symbol of plots, each point represents a sample. The ellipse represents Hotelling's with 95% confidence.

The discriminant analysis clearly separated the treated group from controls when kidney and pulmonary membrane extracts were

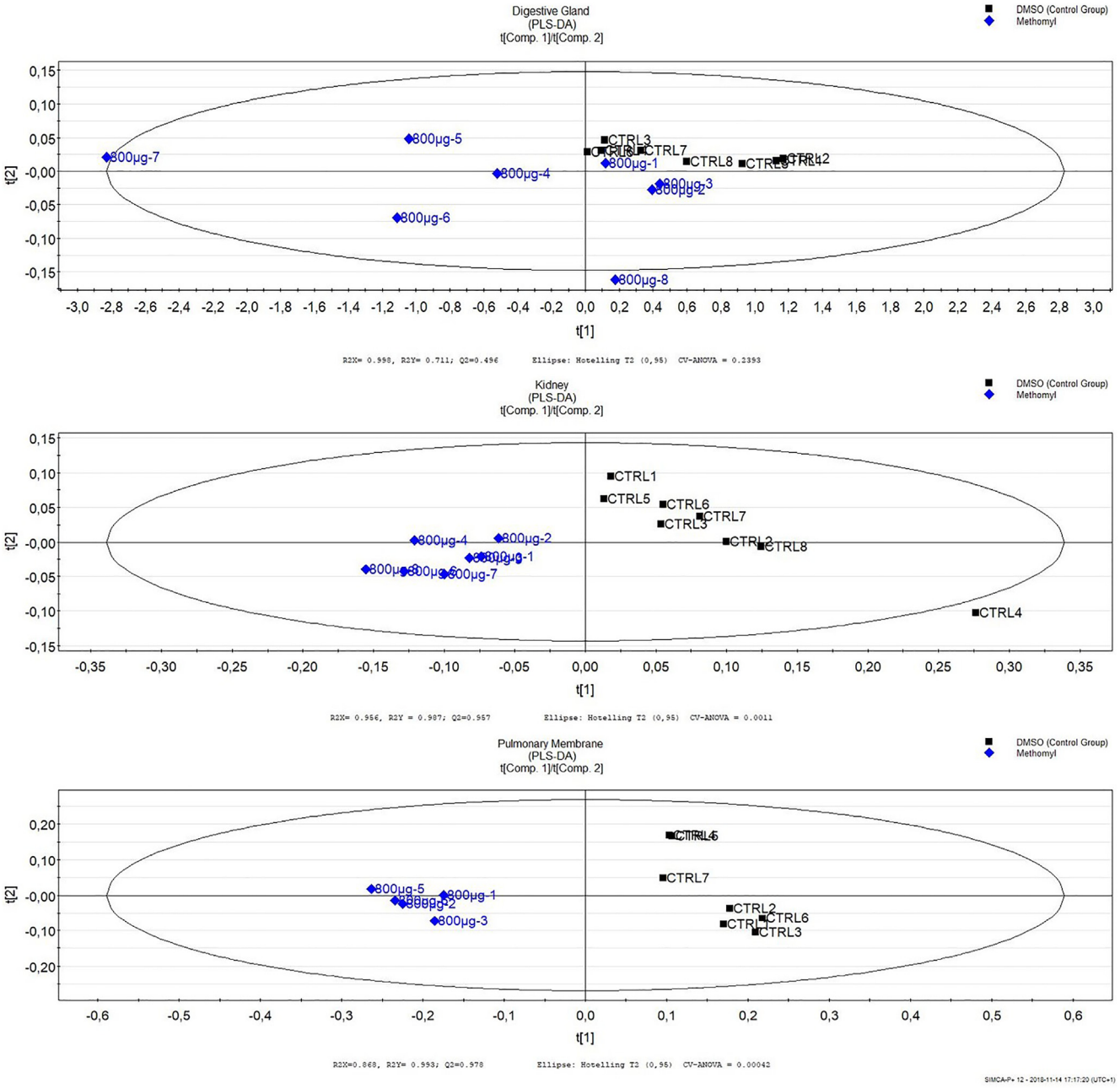


**Fig. 3.** Scores scatter plot obtained after PLS-DA on $^{1}$H NMR spectra of tissues extracts of *Helix aspersa* to identify differences in the metabolic profiles following Methomyl exposure. Two proposed principal components (x-axis and y-axis).

**Table 2**
Changes of discriminating metabolites from $^{1}$H NMR profiles in each tissue of *Helix aspersa* following 800 µg/snail of Methomyl exposure for 48H. Arrows (↑, ↓) describe significant increased or decreased levels of the metabolite as compared to control snails exposed to DMSO. (Wilcoxon test, Bonferroni correction significant p-value).

| Metabolite | Lactate | Alanine | Acetate | Succinate | Dimethyl-amine | Dimethyl-glycine | 3.16 ppm | Choline | Betaine |
|---|---|---|---|---|---|---|---|---|---|
| Digestive Gland | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ | ↓ | = | ↓ |
| Kidney | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ | ↓ | ↑ | ↑ |
| Pulmonary Membrane | ↑ | ↑ | = | ↑ | ↑ | = | ↓ | = | = |

compared, with respective *p*-values of 0.0011 and 0.00042 ($p < 0.05$) as provided by CV-ANOVA (Fig. 3).

A VIP list ranking the observations according to their contribution to the separation was examined to identify the metabolites mainly contributing to the cluster of exposed and control snails in the PLS-DA scores plot. Metabolites responsible of the dispersion were alanine (1.48 ppm, doublet), succinate (2.41 ppm, singlet), lactate (1.33 ppm, doublet), acetate (1.93 ppm, singlet), dimethylamine (2.72 ppm, singlet), betaine (3.27 ppm, singlet; 3.91 ppm, singlet), choline (3.21 ppm, singlet), dimethylglycine (2.92 ppm, singlet) and an

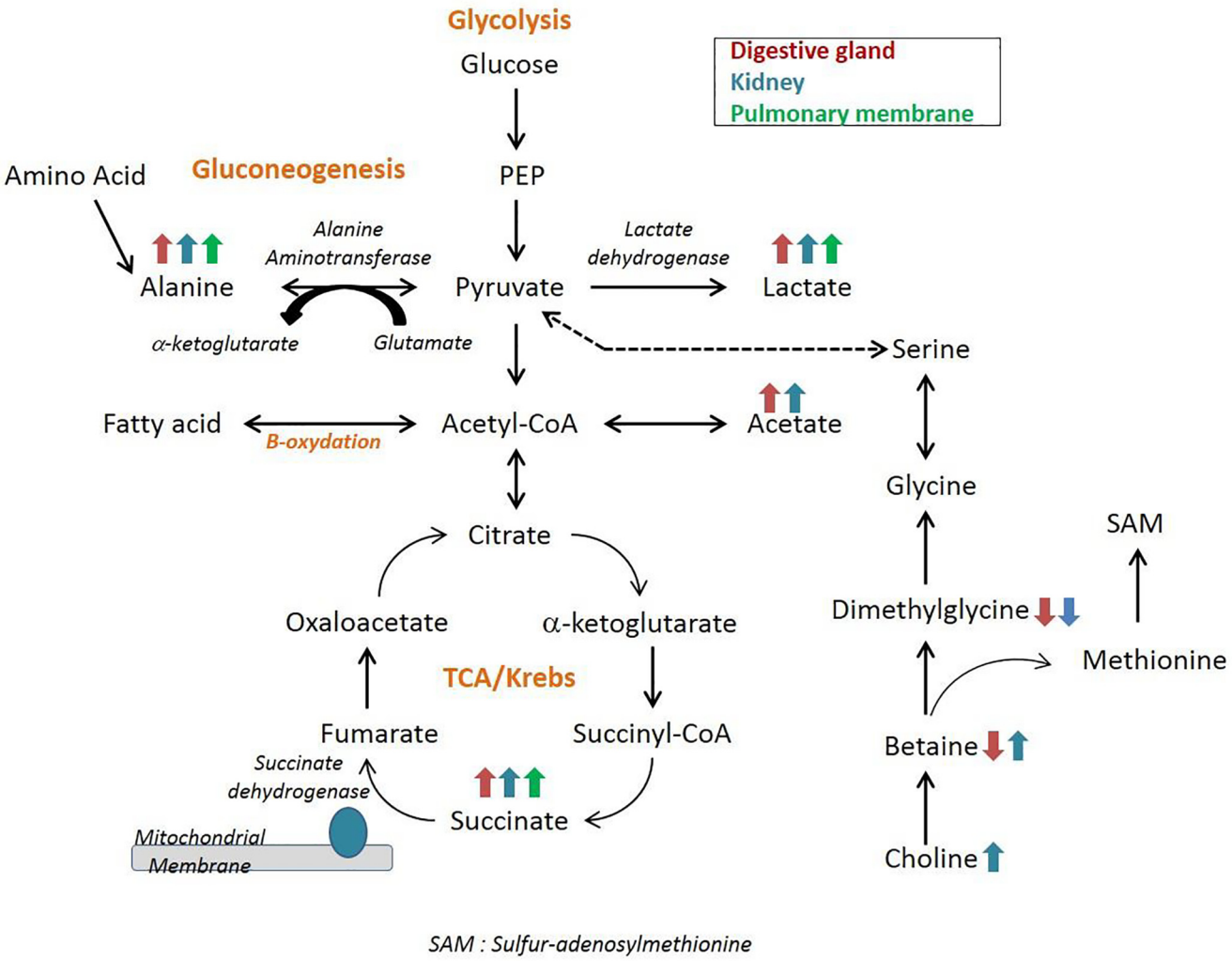


**Fig. 4.** Overview of Methomyl-induced metabolic changes in tissue extracts of *Helix aspersa maxima*. Arrows (↑,↓) describe significant increased or decreased levels of the metabolite as compared to control snails exposed to DMSO. Red, blue and green arrows represent changes in digestive gland, kidney and pulmonary membrane, respectively.

unassigned peak (3.16 ppm, singlet). These changes shown in Table 2 were statistically evaluated with a Wilcoxon test (p-value adjusted with Holm-Bonferroni), based on AUC (area under the curve) values normalized by total spectrum area values. No additional metabolites were observed in $^{1}$H NMR spectra of methomyl-exposed snails as compared to controls.

## 4. Discussion

### 4.1. Acquisition and identification of $^{1}$H NMR spectra of isolated tissues

In this work, separate $^{1}$H NMR profiles were obtained from selected tissues isolated from a terrestrial invertebrate model, *Helix aspersa maxima*. Killing by freezing at −20 °C preferred to liquid nitrogen, inappropriate to a shell-protected organism. Indeed, in preliminary tests, liquid nitrogen did not reach quickly enough internal organs involving acute stress often accompanied by widespread haemolymphatic loss. Moreover, dissection was compromised by exploding organ after exposure to liquid nitrogen. Killing at −20 °C took less than 1 h and each animal was killed the same way at the same time allowing intergroup comparison in a relative manner. Next, as described in material and methods Section 2.3.1, dissected tissues were frozen in liquid nitrogen to be stored at −80 °C.

For the four tissue extracts investigated in the present study, the metabolic profile was characteristic of the examined tissue. The low molecular weight (LMW) metabolites retrieved in these profiles are known to be also present in the intermediary metabolism of vertebrates. Since those metabolites have been linked to key metabolic pathways which are known to be affected in case of pollutants-induced toxicities (Lindon et al., 2004; Beger et al., 2010), it is expected that their relative variations could be further used as biomarkers to report on important cellular events such as oxidative stress, energetic imbalances, changes in protein catabolism or osmotic deregulation.

### 4.2. Sensibility of metabolites obtained by $^{1}$H NMR to Methomyl

Using the same metabonomic approach, Methomyl exposure was demonstrated to cause metabolic variations suggesting a stimulation of the metabolic activity to fulfil the energy need of the cells. Numerous studies have already explored the toxicity of Methomyl on several tissues like liver, kidney, heart and lung in rats (Radad et al., 2009). In the present study, kidney and lung seem to be the main target organs. Indeed, in rodents and humans, it is now accepted that kidney susceptibility to toxic insult is partly a consequence of a high blood volume. This was confirmed for Methomyl in mice evidencing renal dysfunction (El-Demerdash et al., 2013) as well as in workers exposed to this toxic substance (Khan et al., 2008). It has also been reported that in Methomyl-exposed mice, Methomyl extensively accumulated in lungs (El-

khawaga, 2005). In vertebrates, the non-essential amino acid **alanine** is one of the major substrates of gluconeogenesis, corresponding to glucose synthesis. The same glucose pathway has already been associated to changes in alanine, aspartate or glutamate in gastropods (Ladd Prosser, 1991). Alanine is metabolized in muscle cells from pyruvate by transaminase and reaches the liver through the bloodstream to reform glucose after reconversion to pyruvate by mitochondrial transaminase. It is known to increase during periods of stress (Felig, 1973; Mizock, 1995) and has already been described as a potential biomarker of stress related to phenanthrene exposure in invertebrates such as *Eisenia fetida* (Brown et al., 2009; Forcella et al., 2007; Lankadurai et al., 2011) or with other compounds such as DDT, endosulfan, naphthalene or pyrene (Brown et al., 2009; Jones et al., 2007; McKelvie et al., 2009, 2011; Warne et al., 2000). As the most abundant amino acid constituting proteins, the increase of alanine due to methomyl exposure could be linked to protein degradation leading to a release of free amino acids (Jones et al., 2007; Nath et al., 1997). Furthermore, changes in tissue levels of alanine in response to acetylcholinesterase (AChE) inhibition has also been described in Diptera insect (Forcella et al. 2007). Methomyl is a neurotoxic pesticide and, due to its AChE-inhibiting activity, the impact on alanine following methomyl exposure deserves further investigation. It has to be stressed out that, according to Forcella et al. (2007), alanine accumulation accompanied by AChE inhibition was not observed with carbamate exposures, although methomyl belongs to this class of pollutants.

Classical metabolic pathways of vertebrates such as glycolysis, Krebs cycle and respiratory chain, have also been described in mollusks (Bacca, 2007). **Succinate** is known as an intermediate of the Krebs cycle, a crucial pathway in energy metabolism described in vertebrates. The increase of this metabolite could reflect a slowing down of the Krebs cycle activity. Since its oxidation is catalyzed by succinate dehydrogenase, an enzymatic complex bound to the mitochondrial membrane, accumulation of succinate could be due to membrane damages caused by Reactive Oxygen Species (ROS). In this case, we can assume that the mitochondrial electron transport chain is impaired, jeopardizing the energy production from the aerobic respiration. This could ultimately lead to a switch to anaerobic respiration (Xu et al., 2015). According to Livingstone (1983), succinate is also the final product of glucose-succinate and asparate-succinate pathways, which take over the anaerobic metabolism described in invertebrates. These pathways related to the lactate pathway found in vertebrates has also been described in molluscs, such as *Mytilus edulis* (Livingstone, 1983; Zwaan, 1977), and could be supported by increasing acetate, lactate and alanine, which also are products of these anaerobic pathways.

Osmolytes are small charged molecules which act in maintaining the osmotic balance in cells (Conotte and Colet, 2014; Lankadurai et al., 2011). As a major osmolyte, the increase of **betaine** relative to controls reveals a defense mechanism against oxidative stress and represents a response to toxic exposure. **Choline** is one component of phospholipids and, as such, is important for structural integrity of cell membranes. Its increased level could be associated to membrane damages induced by ROS and free radicals generated by lipid peroxidation (Li et al., 2014; Xu et al., 2015). Choline, betaine and **dimethylamine** are also sources of methyl groups. The increase of these three metabolites in kidney could indicate a need for methylation process helping organisms to respond to stress, to detoxify xenobiotic as well as for cell regeneration (Li et al., 2013). **Dimethylglycine,** a product of choline as well as a source of methyl group during liver detoxification could be linked to a need of sulfur-adenosylmethionine (SAM), which is acting in liver regeneration.

A schematic representation summarizing all those metabolic changes induced by methomyl exposure is given in Fig. 4. According to all of these metabolic variations, a shift from aerobic to anaerobic respiration was suspected, most likely due to cellular hypoxia. The level of the metabolic indicators of oxidative stress (betaine, choline) were increased after exposure to methomyl. Moreover, some metabolic changes could be associated to membrane damages. Such alterations were most likely due to an increased production of ROS consecutively to the oxidative stress caused by the pesticide and/or its degradation products (Mansour and Mossa, 2010; Maran et al., 2010).

## 5. Conclusions

$^{1}$H NMR spectroscopy allowed to characterize metabolic profiles of *Helix aspersa maxima* in an organ-specific way. The main methomyl-induced changes were observed in kidney and pulmonary membrane compared to digestive gland after 48 h. In conclusion, this work demonstrated the potential of combining invertebrate models to metabonomics as a powerful tool to predict adverse effects of chemicals. Invertebrate models, already used in the environmental risk assessment, could be transposed from ecotoxicology to the evaluation of human health risks. Indeed, the results obtained in invertebrates indicate the involvement of cellular mechanisms similar to those found in mammals, evidencing their conservation through evolution. Furthermore, ADME information (Absorption, Distribution, Metabolism and Excretion) could be derived from the spectral analysis of specific tissues.

Following a legislative (DIRECTIVE, 2010) and current societal demand, it becomes necessary to reduce the experimental use of vertebrate models, in toxicological studies. Indeed, since 1959, the bioethical principle of the 3Rs law exists, aimed at Reducing the number of mammals, Refining the methodology used, or Replacing the current mammal models with alternative methods as described in the Article 2.8 of the Walloon government decree of 30 november 2017 (M.B. 21.12.2017) linked to the European Directive 2010/63/UE and to the Council of 22 september 2010 on the protection of animals in experimentation. The use of invertebrate models, as such alternative methods, could therefore be applied to meet the demand of the 3Rs law by replacing vertebrate organisms. Indeed, these models, except for cephalopods, are not subject to legislation for the protection of experimental animals according to the Article R214-88 of decree n°2013-118 of first February 2013 (AGRG1231951D) incorporating the European Directive 2010/63/UE. Therefore, combining invertebrates and metabonomics to predict potential adverse effects in humans without vertebrates testing could be a promising approach.

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## Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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