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# HRMAS NMR as a tool to study metabolic responses in heart clam *Ruditapes decussatus* exposed to Roundup®

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ABSTRACT

The essence of this study was to investigate the metabolic responses of heart tissues of carpet-shell clam *Ruditapes decussatus* after exposure to two doses (0.2 and 1 g/L) of Roundup® during 24 and 72 h. The main metabolic changes after Roundup® exposure were related to disturbance in energy metabolism and metabolic biomarkers such as alanine, succinate, acetate and propionate, suggesting the occcurence of anaerobiosis and the impairment of oxydative metabolism. Results showed also that peak intensities of amino acids used as biomarker of anaerobiosis in molluscs are time and dose dependent. In the opposite, phosphoarginine and ATP level are dependent to Roundup® concentration rather than to the time of exposure. We suggest that changes in energy demands require adjustements in the forward arginine kinase reaction rate. Therefore, the results demonstrate the high applicability of HRMAS NMR to elucidate the mechanism of toxicity of Roundup®. In addition, $^{31}$P HRMAS NMR appeared to be an effective and simple method to follow bioaccumulation of Roundup® formulation.



## 1. Introduction

The increasing use of herbicides in agriculture and forestry has become a growing hazard to our environment, especially to aquatic ecosystems [1,2].The glyphosate-based herbicide, Roundup®, is among the most used pesticides worldwide. This compound is a non selective herbicide widely used in agriculture and nonagricultural activities for non-selective weed control and it is already one of the most used xenobiotics in modern agriculture [3]. Due to its extensive use in the environment and its high water solubility (15.700 mg/L), the exposure of nontarget aquatic organisms to this herbicide is a concern for ecotoxicologists [4]. The commercial formulation of Roundup® consists of the glyphosate [N-(phosphonomethyl)glycine] as the active ingredient which is commonly used as isopropylamine salt and polyethoxylene amine added as a surfactant. Studies have shown that Roundup® is more acutely toxic to freshwater invertebrates and fishes than its active ingredient [5–7] and this may be due to the surfactant [8] or due to the possible synergy between glyphosate and Roundup® formulation product [3].

The use of bivalves as sensitive bioindicators of environmental changes has been established for a long time, due to their high filtration rate, their ability to bioconcentrate toxicants, their widespread distribution and abundance. It has been demonstrated for example that clam, an economically important bivalve in many countries, such as in France, Tunisia and Portugal, could be submitted to various pollutants of anthropic origin [9] and could accumulate a large number of organic and inorganic compounds reflecting the bioavailable fraction of contaminants in the environment [9–11]. So, if contaminated, this bivalve may represent a potential risk to human health [12].

Under stressful conditions aquatic animals can activate physiological responses that could counteract the imposed stress. Evaluation of the physiological status of the organism by monitoring its heart rate was considered as a rapid approach for the assessment of marine pollution and the determination of the ecological health of a marine ecosystem [13]. Our preliminary study showed alteration of beating rate of cells isolated from the heart of *Ruditapes decussatus* after exposure to Roundup® [14]. The increased heart rate of Roundup® exposed bullfrog tadpoles was associated with hyperactivity, which may represent an unfavorable avoidance response suggesting that Roundup® exposure shifts a considerable amount of energy from the morphogenetic processes to counteract the negative effects of this herbicide [15]. In addition it was reported that the use of phosphorus $^{31}$P magnetic resonance spectroscopy provides a unique non-invasive tool to investigate myocardial high-energy-phosphate metabolism [16]. It was also reported that clam showed a tolerance to pesticide, but less is known of their metabolic responses to contamination.

In this context, the objective of the present study was to detect changes in the metabolic profile of heart tissue of clam

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*R. decussatus* as a response to exposure to Roundup®, using HRMAS NMR spectroscopy method.

Indeed NMR tool has been proven useful and powerful in environmental toxicology [17]. NMR has several important advantages including its ability to overcome the constraints of traditional metabolic investigations during analysis of a wide range of compounds in intact tissues, cells, and biofluids. In particular, High-Resolution Magic Angle Spinning (HRMAS) spectroscopy, which is a rapidly non-destructive study of intact tissue, does not require any preliminary extraction but only a minimal sample preparation [18]. This technique is suited for monitoring the accumulation and disappearance of various classes of metabolites or of their intermediates following stress [19]. However, this is the first study on the application of HRMAS NMR spectroscopy to elucidate the toxicological effects of Roundup® in marine invertebrates.

## 2. Materials and methods

### 2.1. Chemicals

The formulated glyphosate used was Roundup® 3plus, a trademarked product from Monsanto (170 g/L glyphosate as isopropylamine salt) and was from a commercial source. Glyphosate 96% and Deuterium oxide ($D_2O$) 100% were purchased respectively from Aldrich and Euriso-top.

### 2.2. Clam exposure

Adult clams, *R. decussatus*, of 3–4 cm shell in length, were collected from a local fish farm. Animals were allowed to acclimate in aerated seawater for three days. After acclimatization, clams were transferred to three tanks: one control and two exposed groups with Roundup® (0.2 and 1 g/L) during one and three days. After treatments, clams were immediately dissected and the hearts were removed for HRMAS NMR analyses.

### 2.3. Metabolite extraction

Quickly after treatment the heart tissues were homogenized in methanol/water (v/v). The homogenate was centrifuged (4 °C, 10 min, 13.000 g) and the supernatant was removed and lyophilized.

### 2.4. NMR spectroscopy

All the acquisitions were recorded on a BRUKER DRX 500 spectrometer equipped with an indirect HRMAS $^1H/^{31}P$ probe. During the HRMAS study, one heart was loaded in a 4 mm $ZrO_2$ cylindrical rotor with 70 μL of sea water/$D_2O$ 3/1, at a spinning rate of 5.000 Hz, around an axis which is oriented at the so-called magic angle of 54.7° with regard to the magnetic field $B_0$. The spectra were performed with a 30° pulse, with a 2 s delay and a presaturation of the $H_2O$ signal. The temperature was controlled at 25 °C. The result was an NMR spectrum with resolution approaching that of a liquid sample which made it possible to analyze the metabolites of low molecular weight in solution inside the organisms.

For 1D $^1H$ NMR, the lyophylised powders were dissolved in 0.7 mL deuterium oxide. $^1H$ NMR spectra were acquired at 25 °C on a Bruker AVANCE 500 equipped with an inverse 5 mm TCI $^1H/^{13}C/^{15}N$ cryoprobe. $^1H$ NMR spectra were phased, baseline corrected and calibrated (TSP at 0.0 ppm). Some identifications of metabolites were confirmed by the COSY DQF (Double-quantum filtered $^1H$–$^1H$ correlated spectroscopy), HMQC $^1H$–$^{13}C$ (Heteronuclear multiple quantum coherence $^1H$–$^{13}C$) and HMBC $^1H$–$^{13}C$ (Heteronuclear multiple bond coherence $^1H$–$^{13}C$) sequences.

### 2.5. Statistical analysis

The effect of both Roundup® concentrations on the level of the phosphoarginine and on the energy metabolites was studied. Samples profiles were determined by principal component analysis (PCA) using stat Box 6.6 software (Stat Box logiciels, Grimmersoft, France).

## 3. Results and discussion

### 3.1. $^1H$ HRMAS NMR spectroscopy of clam hearts

Fig. 1 shows representative $^1H$ HRMAS NMR spectra obtained from control and exposed carpet shell clam to Roundup® 0.2 and 1 g/L for 24 and 72 h. No difference in major peaks was found between the control and the exposed groups in the part of specter above 4.5 ppm. However, clear differences can be observed in the spectral region between 4.5 and 0 ppm. After exposure to Roundup®, the metabolic profiles resulted in significant increase in alanine (peak 1) and in the appearance of isopropyl amine, succinate, acetate, propionate and glyphosate (peaks 2, 3, 4, 5 and 6 respectively). Those metabolites had been identified using 1D $^1H$ NMR obtained from clam heart tissue extracts (Table 1). Their peak intensities are dose and time dependent.

### 3.2. $^1H$ NMR spectroscopy of clam heart extracts

A representative $^1H$ NMR spectrum of heart tissue extracts from control clam is shown in Fig. 2. The NMR spectrum is dominated by organic osmolytes such as betaine (3.28 and 3.92 ppm), taurine (3.27 and 3.44 ppm) and glycine (3.57 ppm). However, the amount of this latest osmolyte is about 5 times lower than other two major metabolites. Several classes of metabolites were assigned, including amino acids (valine, leucine, isoleucine, glycine, arginine etc.) and organic osmolytes (e.g., betaine, homarine, hypotaurine, taurine) (Table 1).

These results are in agreement with those reported for clam *Ruditapes philippinarum* [20,21] showing the dominance of organic osmolytes such as betaine and taurine. It was indicated that these molecules play a key physiological role in the osmotic regulation of marine organisms via various metabolic pathways and were therefore detected at higher levels than other metabolites in clams [22]. These osmolytes can be actively accumulated or released when the salinity increases or decreases [17–22].

As shown in Fig. 3, exposure to the highest dose of Roundup® for 72 h did not show significant changes in the major compounds with respect to control. However, the most significant metabolic changes in heart tissues were the increase of alanine, and the accumulation of succinate and acetate. Those metabolites are the most common end products in marine species during environmental anoxia [23]. Moreover, our results showed that alanine is accumulated even under short time exposure (24 h) to Roundup® when acetate and propionate were observed only after prolonged time exposure (72 h). These observations are in agreement with many studies indicating the accumulation of succinate and alanine during the initial phase of anaerobiosis and of acetic acid and especially propionic acid after prolonged anoxia [24]. Thus, we can suggest that the metabolic responses of clam *R. decussatus* exposed to Roundup® are similar to those observed in animal tissues under anoxic stress.

The occurence of anaerobiosis metabolism as consequence of the high level of alanine and succinate was also reported in clam

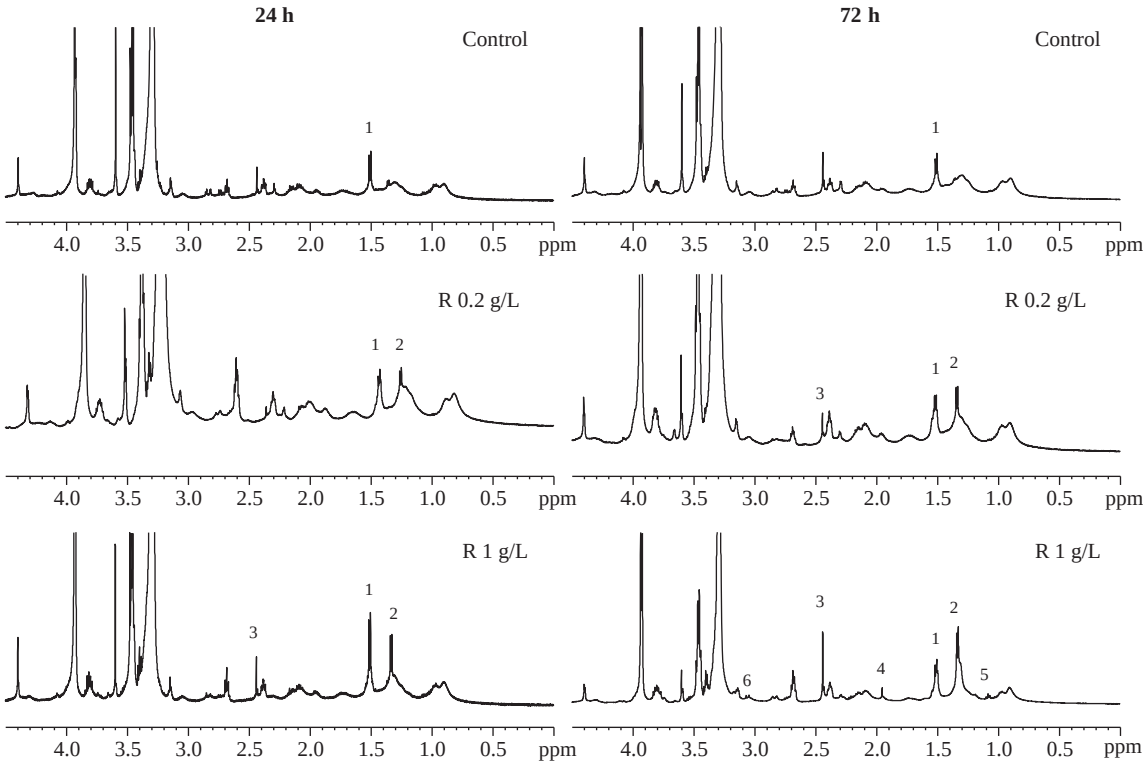


**Fig. 1.** 500 MHz $^{1}$H HRMAS NMR spectra of carpet shell clam (*Ruditapes decussatus*) heart after 24 and 72 h Roundup® (R) exposure. Alanine (1), Isopropyl amine (2), Succinate (3), Acetate (4), Propionate (5) and Glyphoate (6) evolve according to time and concentration of exposure. 4 experiments were carried out ($N$=3 in each group).

**Table 1**
List of compounds identified in $^{1}$H NMR spectrum of heart tissue extracts, and their respective chemical shifts (s=singlet, d=doublet, t=triplet, q=quadruplet, m=multiplet).

| Peak(s) | $^{1}$H $\delta$(ppm) | Metabolites |
|---|---|---|
| **1** | 1.49 d<br>3.80 q | Alanine |
| **2** | 1.31 d<br>3.51 m | Isopropyl amine |
| **3** | 2.42 s | Succinate |
| **4** | 1.93 s | Acetate |
| **5** | 1.07 t | Propionate |
| **6** | 3.04 d<br>3.74 s | Glyphosate |
| **7** | 3.28 s<br>3.92 s | Betaine |
| **8** | 3.27 t<br>3.44 t | Taurine |
| **9** | 3.57 s | Glycine |
| **10** | 1.33 d | Lactate |
| **11** | 2.14 m<br>2.36 m | Glutamic acid |
| **12** | 2.66 t<br>3.37 t | Hypotaurine |
| **13** | 4.37 s<br>7.98 t<br>8.04 d<br>8.55 t<br>8.72 d | Homarine |
| **14** | 1.68 m<br>1.90 m | Arginine |

gills exposed to 0.2 μM of benzo(a)pyrene for 24 h [20]. The accumulation of succinate is a clear biomarker of facultative anaerobiosis in molluscs [25]. Higher levels of succinate in clam exposed to Roundup® can be due as indicated by Spann [26] to an impairement of the oxidative metabolism in Asian clam exposed to sediment spiked with zinc and cadmium. Inhibition of the enzyme succinate dehydrogeanse in the citric acid cycle of Anodonta cygnea in response to cadmium exposure was also hypothesized by Hemelraad [27]. Peixoto [3] indicate that succinate dehydrogenase and succinate cytochrome *c* reductase are significantly inhibited by Roundup® in rat liver mitochondria, suggesting that this herbicide affects the redox electron transfer chain at the level of complex II and III. Similar effects were also suggested for Pentachlorophenol (PCP) which uncouples mitochondrial oxidative phosphosphorylation and causes complete inhibition of the electron transport chain in red abalones at sublethal concentrations [28].

This study also showed that lactate is not accumulated after treatments with Roundup® contrary to results obtained in Zebra clams exposed to mercury [29]. Our results are consistent with previous studies showing that this compound does not accumulate during anoxia in oysters [30] and mussel [24]. This also agrees with studies showing low activity of lactate dehydrogenase (LDH), enzyme involved in carbohydrate metabolism, and no lactate accumulation during environmental anaerobiosis in intertidal molluscs [31,32]. It was reported that LDH activity may be a sensitive criterion for pesticide exposure [33]. The LDH activity was sligtly affected in wistar rats treated with Roundup® [34] but it was not altered in mosquitofish exposed to commercial glyphosate formulation [35].

### 3.3. $^{31}$P HRMAS NMR spectroscopy of clam heart extracts

The heart performs mechanical movement using chemical energy stored in phosphoanhydride bond of ATP. As environmental anaerobiosis commences, phosphoarginine hydrolysis represents the main source of energy [36,37]. This process is catalyzed by arginine kinase, which exchanges a phosphate from phosphoarginine to ADP [38]. Therefore, phosphoarginine serves as a critical buffer when mitochondrial mechanisms are unable to meet ATP demand due to elevated metabolism [39] or when their

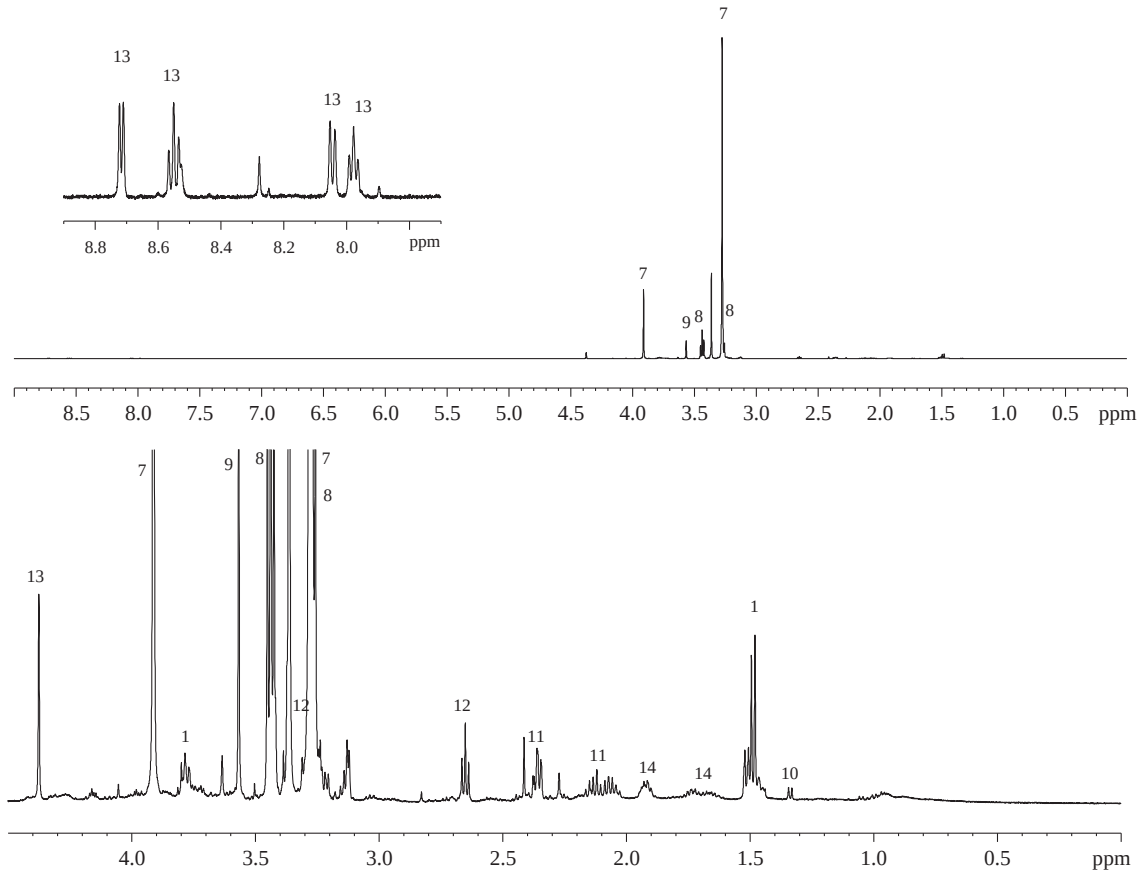


**Fig. 2.** A representative one dimensional 500 MHz $^{1}$H NMR spectrum of heart tissue extracts from a control clams ($N=15$). Keys: Betaine (7), Taurine (8), Glycine (9). The assignment of minor compounds is also reported: Alanine (1), Lactate (10), Glutamic acid (11), Hypotaurine (12), Homarine (13), Arginine (14).

function is compromised by toxic stressors [38]. For this reason we have studied the effect of Roundup® in phosphoarginine and ATP.

Fig. 4 shows representative $^{31}$P HRMAS NMR spectra obtained from control and exposed clam to Roundup® 0.2 g/L and 1 g/L for 24 h and 72 h. Spectral assignements of γ-P(ATP) ($\delta=-5.2$ ppm) and β-P (ADP) ($\delta=-10.2$ ppm), α-P(ATP+ADP) and β-P(ATP) ($\delta=-18.8$ ppm) can readily be assigned on the basis of their chemical shift as characterized by previously published data [40]. The resonance peaks of inorganic phosphate, phosphonates, phosphomonoesters, phosphodiesters and phosphoarginine (PA) are also easy to identify in heart clam (Fig. 4).

### 3.3.1. *PCA profiles of responses to exposure to different Roundup® concentrations*

In order to evaluate the effect of Roundup® on energy metabolism, intensities of $^{31}$P NMR peaks have been integrated. The ratio of the peak area of ATP to the total area of all phosphorus peaks and the ratio of the phosphoraginine peak area to the total area of all phosphorus peaks were determined and analyzed with PCA (Fig. 5). Because of our $^{31}$P HRMAS NMR conditions (time experiment, T1, integration) and the fast degradation of ATP, ADP and AMP after heart extraction, the calculated ratios are approaching values of energy metabolites quantities which evolution can be followed according to Roundup® exposure conditions.

The effect of exposure to different concentrations of Roundup® (0, 0.2 and 1 g/L) on phosphoarginine and energy metabolites after 24 and 72 h was profiled by Principal Component Analysis (PCA). Fig. 5A shows the correlation circle where all treatments are very well represented. Fig. 5B represents the two principal components (PC) where PC1 and PC2 accounted for 96% of the total variation. The first axis (PC1) indicates the effect of Roundup® concentrations on phosphoarginine and energy metabolites while the second (PC2) distinguishes between the two metabolic parameters studied.

Based on PCA analysis, four profiles could be identified: I (*a* and *b*), II, III and IV. Profile I consisted in samples with similar high energy values due to exposure to 0.2 g/L of Roundup®. Profile II consisted in samples with similar low energy values due to exposure to 1 g/L of Roundup®. Profile III was made up of high phosphoarginine in samples exposed to 0.2 g/L of Roundup® while Profile IV contained samples exposed to 1 g/L of Roundup®. This analysis showed that the time of Roundup® exposure is not important since the profiles obtained included samples incubated for 24 and 72 h. Phosphoarginine and energy metabolites were more influenced by the concentration of Roundup® with high concentration inducing minimal responses and low concentrations inducing high responses.

Values of phosphoarginine and energy metabolites obtained for samples exposed to 0.2 g/L of Roundup® were higher than control. This may be due to the arginine kinase (AK) rates increase during exposure to Roundup®, indicating an increased metabolic demand for ATP production through phosphoarginine hydrolysis as suggested for red abalone exposed to hypoxia [41]. Elevation in the forward arginine kinase rate constant over the basal value was observed in abalone exposed to copper [38]. Thus, it is possible that carpet shell clam exposed to 0.2 g/L accumulated bigger reserves of ATP which could be utilized under toxic stress to meet the increased energy demands for detoxification.

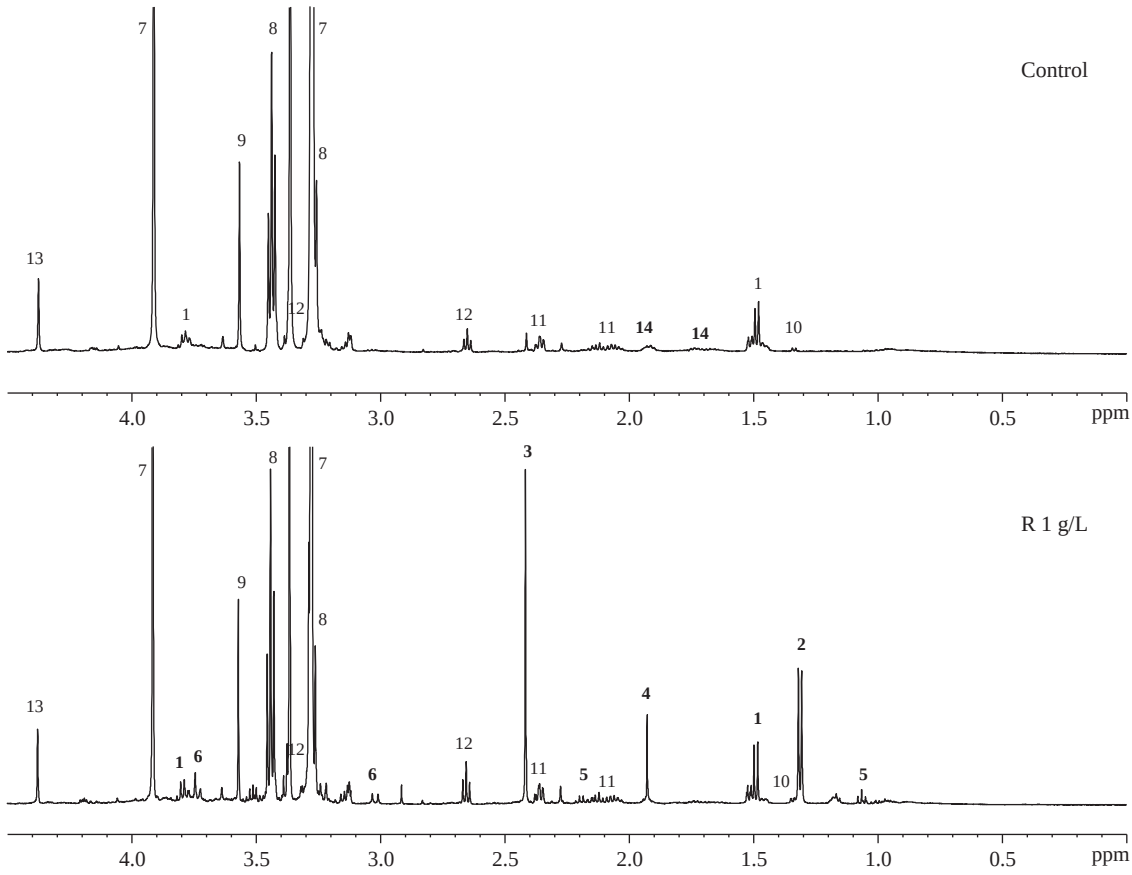


**Fig. 3.** A 500 MHz $^{1}$H NMR spectrum of heart tissue extracts from control and Roundup® 1 g/L groups after 72 h exposure ($N$=15 in each group). Keys: alanine (1), isopropyl amine (2), succinate (3), acetate (4), propionate (5) and glyphosate (6), Betaine (7), Taurine (8), Glycine (9), Lactate (10), Glutamic acid (11), Hypotaurine (12), Homarine (13), Arginine (14).

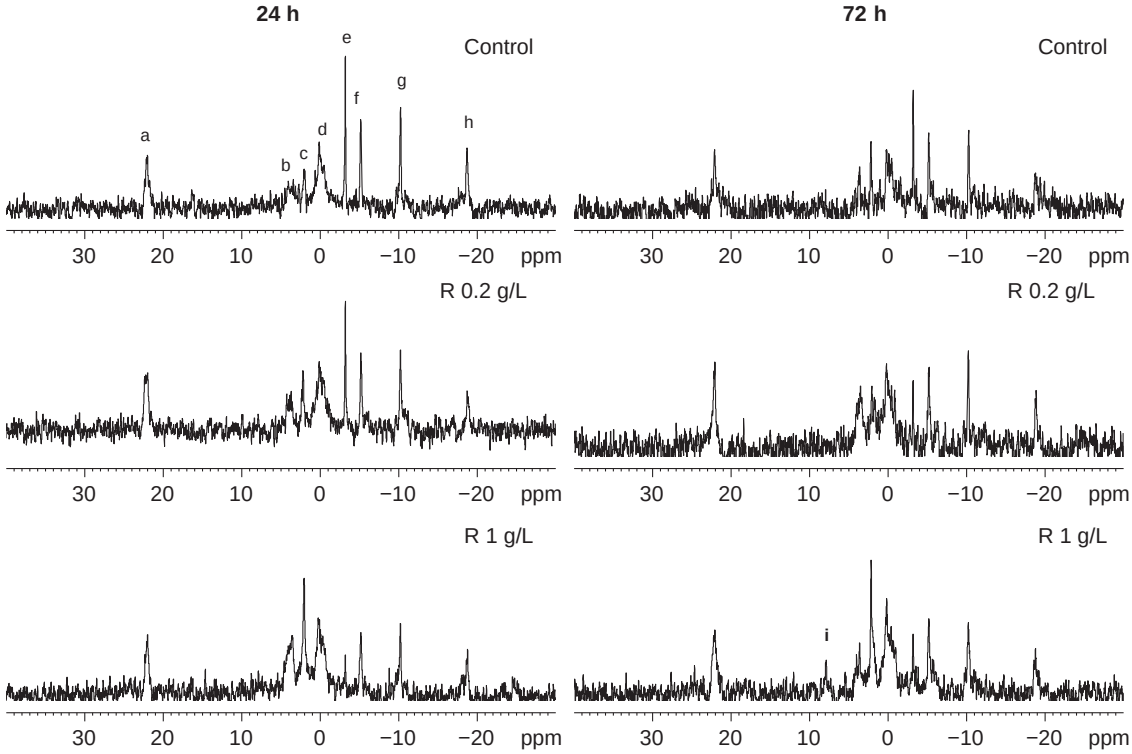


**Fig. 4.** $^{31}$P HRMAS NMR spectra of clam heart after 24 h and 72 h Roundup® (R) exposure. The peaks correspond to phosphonates (a), phosphomonoesters (b), inorganic phosphate (c), phosphodiesters (d), phosphagens (mostly phosphoarginine) (e), γ- and β-phosphates of ATP and ADP (f), α-phosphates of ATP and ADP (g), β-phosphate of ATP (h) and glyphosate (i). 4 experiments were carried out ($N$=3 in each group).

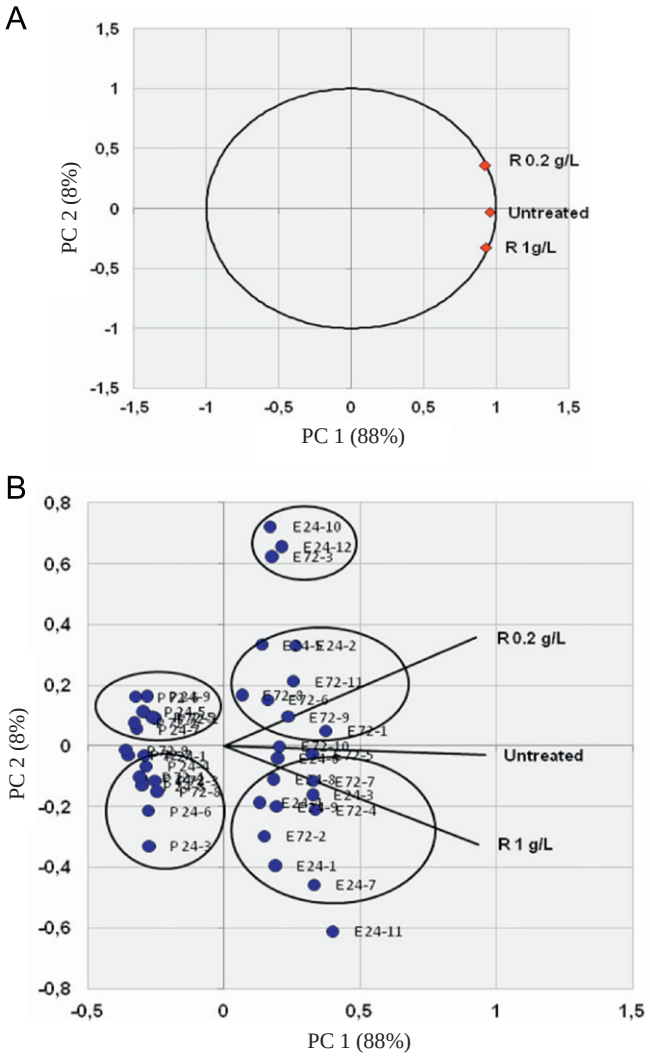


**Fig. 5.** (A) shows the correlation circle and (B) Principal Components Analysis (PCA) on the control and Roundup® (R) exposed clams. P 24-6 represents the concentration of phosphoargine measured for sample 6 after 24 h exposure to a given Roundup® concentration. E 24-1 represents the energy metabolites measured for sample 1 after 24 h exposure to a given Roundup® concentration.

A reverse feature was noted for samples exposed to 1 g/L of Roundup®. Moreover, our results showed that both arginine (Fig. 3) and ATP decreased in the heart of clams. This finding was consistent with previously published report indicating that the pentachlorophenol (PCP) at high concentration induce a decline in ATP prior to the complete utilization of PA suggesting that it may inhibit arginine kinase, which catalyzes phosphate transfer from PA to ADP to produce new ATP (PA+ADP→arginine+ATP) [42]. In the opposite, results obtained in the gill tissue of abalone exposed to copper show that ATP and arginine would be elevated with a depletion of phosphoarginine [43], this could be accounted for the various toxicological mechanisms between copper and Roundup® which seems at this high concentration to possess similar mechanisms to that involved in PCP exposure. The decrease of ATP could explain mortality observed after prolonged exposure of 7 day.

## 4. Bioaccumulation of glyphosate and its formulation Roundup®

After exposure to Roundup® 1 g/L for 3 day, the $^{1}$H NMR spectra (Figs. 1 and 3) showed the appearance of the glyphosate and isopropylamine peaks. Identification of those compounds by $^{1}$H and $^{31}$P NMR in biological fluids after poisoning with Roundup® was also reported [33]. Glyphosate is characterized by two signals: a doublet at 3.04 ppm assigned to the $CH_2$-P group ($^{2}J_{C-P}$=11.6 Hz) and a singlet at 3.74 ppm corresponding to the $CH_2$–N group. Isopropylamine is also characterized by two signals: a doublet at 1.31 ppm assigned to the $CH_3$ groups and a multiplet at 3.51 ppm corresponding to the CH group. The $^{31}$P HRMAS NMR spectrum (Fig. 4) also showed the appearance of glyphosate peak ($\delta$=7.85 ppm). This peak is also present in the extract (Fig. 6) and its assignment was confirmed by addition of the commercial product in this extract.

It was reported that phosphorus chemical shifts of glyphosate and AMPA, its major degradation product, are pH dependent. In the range $0 < pH < 14$, glyphosate chemical shift varies between 8 and 16 ppm whereas AMPA chemical shift varies between 10 and 20 ppm. At pH 6.8, pH of our study, the glyphosate chemical shift is slightly over 8 ppm whereas that of AMPA is 10 ppm [34], confirming the presence of glyphosate but not AMPA in our samples. Thus, our results showed clearly that carpet shell clam

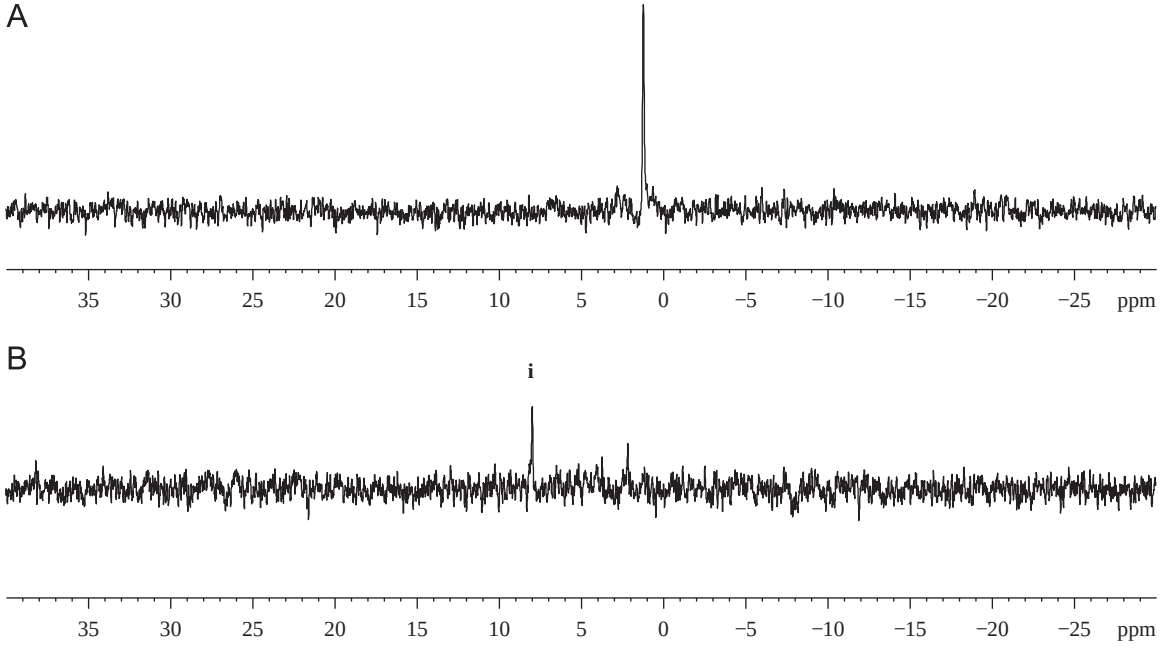


**Fig. 6.** $^{31}$P NMR spectrum of heart tissue extracts from control clam (A) and exposed clam to Roundup® 1 g/L for 72 h (B) ($N$=15 in each group). Appearing peak is glyphosate (i).

can bioaccumulate glyphosate and isopropylamine in their tissues. Glyphosate was also detected in the fillets and eggs of fish exposed to 2.0 mg/L of Roundup® [35].

## 5. Conclusion

In summary, this study focused on the metabolic responses and toxicological effects in the heart tissues of clam *R. decussatus* induced by two doses (0.2 and 1 g/L) after exposure to Roundup® after 24 and 72 h, demonstrate that HRMAS NMR is a useful technique to elucidate the toxicological mechanisms of Roundup® using metabolic biomarkers. In addition, this method allowed us to identify and characterize the bioaccumulation of Roundup® formulation specially, glyphosate and isopropylamine in the heart tissue of carpet shell clam. As found in previous metabolomic studies looking at different stressors in a variety of species, the main perturbations induced by Roundup® exposure were seen in amino acid and energy metabolism which appear to be a general stress response. We showed that high dose of Roundup® induced an increase of alanine, the appearance of succinate, acetate and propionate (metabolic biomarkers of anaerobiosis) and the decrease of energy metabolites. However, further experiments could be carried out in order to elucidate specific stress response involved in Roundup® toxicity.

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