Lee et al. Appl Biol Chem (2020) 63:11
https://doi.org/10.1186/s13765-020-00495-0

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Metabolite profiles of live or dead carp (*Cyprinus carpio*) exposed to endosulfan sulfate using a targeted GC–MS analysis

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Abstract

Endosulfan sulfate is a major oxidized metabolite of endosulfan, which is a broad-spectrum chlorinated cyclodiene insecticide. In this study, GC–MS-based metabolic profiles of dead or live carp (*Cyprinus carpio*) exposed to endosulfan sulfate were investigated to elucidate the molecular toxicological effects of endosulfan sulfate on carp. Three different extraction methods were compared, and a 50% methanol solution was chosen as an efficient extraction method. Carp was exposed to endosulfan sulfate at a concentration of 8 ppb for 2 days. After exposure, the whole body of the fish was homogenized with liquid N_2, extracted with the 50% methanol solution and dried before TMS derivatization for GC–MS analyses of the dead and live carp. A SIM (selected ion monitoring)-library of 373 metabolites was applied after GC–MS analysis to detect 146 metabolites in carp. Based on the one-way ANOVA results ($P < 0.001$) and fold changes of metabolites in dead carp versus control (fold change > 1.5 or < 0.667), 30 metabolites were identified as biomarkers that were significantly different in the metabolic profiles among the control, dead and live carp. A metabolic pathway analysis using MetaboAnalyst 4.0 revealed that those biomarkers were important for the living or death response to endosulfan sulfate. The pathways indicated by the metabolic pathway analysis included starch and sucrose metabolism, galactose metabolism, glycerolipid metabolism, the citrate cycle and linoleic acid metabolism. These results suggest that these pathways underwent significant perturbations over the exposure period.

Keywords: Metabolic profile, Metabolomics, Endosulfan sulfate, Carp, GC–MS

Introduction

The use of pesticides has improved agricultural productivity by controlling weeds, pests and pathogens that are harmful to agricultural products. However, long-term and intensive pesticide use has caused environmental pollution and ecological side effects [1].

Endosulfan, the broad-spectrum organochlorine insecticide, has been widely used on various crops for many years [2], and it produces endosulfan sulfate as a major oxidative metabolite. Due to the semivolatility and persistence of endosulfan and endosulfan sulfate, they are still currently detected in large areas of various environments [3, 4]. Endosulfan sulfate has a similar toxicity and similar or higher persistence than endosulfan [5–7].

Metabolomics explores the dynamic and broad response of organisms to reveal the effects and toxic mechanisms of certain chemical compounds, and enables understanding of the impacts of these chemicals on organisms and the environment [8]. Conventional toxicological assessment variables, such as behavioral changes and mortality, are inefficient in distinguishing among toxic substances that produce similar phenotypes [9]. Changes in metabolic products indicate toxicity, genetic modification, and disturbance due to environmental factors and disease conditions [10]. Metabolomics studies

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are typically performed using mass spectrometry or nuclear magnetic resonance (NMR) for the analysis of metabolites [11]. NMR is a relatively simple and convenient method but has low sensitivity and resolution [12, 13]. Mass spectrometry, on the other hand, is able to detect many metabolites with high sensitivity and selectivity [12].

Fish are often used as test organisms for measuring water pollution among various ecotoxicity models [14]. Carp (*Cyprinus carpio*) has a number of advantages over other small fish models, such as easy rearing over a long period [15]. In the present study, carp was exposed to endosulfan sulfate to understand its toxicological effects at the molecular level. Therefore, the metabolic profiles of dead or live carp were investigated using targeted GC–MS analysis, and the results were compared via metabolic pathway analysis.

Materials and methods

Chemicals and reagents

Endosulfan sulfate was obtained from ChemService Inc. (West Chester, PA). Ribitol and the MSTFA reagent (1% TMCS) were purchased from Wako (Tokyo, Japan) and Thermo Fisher Scientific Co. (Pittsburgh, PA), respectively. Methoxyamine hydrochloride, pyridine, dimethyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol of HPLC grade was from Merck (Darmstadt, Germany), and all of the reagents were the highest grade products.

Experimental animals

Carp approximately 3–5 cm in total length were acclimated for 2 weeks in a glass water tank with dechlorinated tap water at 26 ± 1 °C. The carp were fed a commercial fish food once a day during the acclimation period. They were not fed for 1 day prior to the chemical exposure. A constant 16:8-h light:dark cycle photoperiod was used during experiment.

Comparison of the metabolite extraction efficiency of different extraction solvents

The acclimated carp without any treatment were frozen in liquid N_2 and then ground into a fine powder using a mortar and pestle. Each 100 mg of the homogeneous sample powder was vortexed for 10 min at 4 °C with 1 mL of 50% methanol, 80% methanol, or dichloromethane/methanol/water (2/5/2) solutions. The mixtures were then centrifuged at 13,000 rpm for 10 min, and 100 μL of the supernatant was dried using a speed vacuum concentrator (Hanil Modulspin 40). The dried residue was reacted with 50 μL of the methoxyamination reagent (20 mg/mL of methoxyamine hydrochloride in pyridine) at 37 °C for 90 min with shaking, and then treated with 50 μL of the MSTFA reagent (MSTFA + 1% TMCS) at 37 °C for 30 min.

Chemical exposure

Endosulfan sulfate was dissolved in DMSO and diluted to concentrations of 8 mg/L for the stock solution. Either 1 mL of the stock solution or 1 mL of DMSO as a control was added to 1 L of water in 1 L beakers. After 15 carp per test group were distributed into each beaker, the live carp in each group were sampled after 2 days, and the dead carp from the exposed group were sampled immediately after death for up to 2 days.

Sample preparation

The whole body of the live or dead carp sample was frozen in liquid N_2 and ground into a fine powder using a mortar and pestle as previously described [1]. 100 mg of the ground sample was vortexed with 1 mL of 50% methanol solution containing 0.2 μg/mL of ribitol as an internal standard for 10 min at 4 °C. After extraction of the samples, the derivatization process was conducted as described in the 'comparison of the metabolite extraction efficiency of different extraction solvents' section above.

Profiling and identification of metabolites by GC–MS

The metabolites were scanned using a GC–MS (Shimadzu QP2010) and a SIM-library of 373 metabolites (311 metabolites from the Smart Metabolites Database, Shimadzu, and 62 metabolites from an in-house library made by our group) was applied to identify metabolites from the live and dead carp. Split (10:1) mode was used on an injection of a 1.0 μL aliquot into a DB5 column (30 m × 0.25 mm i.d., 1 μm film thickness, Agilent). The GC–MS parameters were set to optimal conditions using an injector temperature of 280 °C, and ion source and interface temperatures of 230 °C and 280 °C, respectively. The initial oven temperature was 100 °C for 4 min, and the temperature was then increased to 320 °C at a rate of 4 °C/min and held there for 11 min. The flow rate of the helium carrier gas was 1 mL/min, and the electron ionization energy was 70 eV.

GCMS Solution software (version 4.3, Shimadzu) was used for data processing. The peak reconfirmation was

Table 1 Total peak area of 146 metabolites in each extraction solvent

Extraction solvent	50% MeOH	80% MeOH	DCM/MeOH/H_2O (2/5/2)
Total peak area	33,077,414	25,182,402	19,891,805

Table 2 Metabolites detected in whole-body carp samples by targeted metabolite analyses using GC–MS

No.	Metabolite	No.	Metabolite	No.	Metabolite	No.	Metabolite
	Alcohols		*Carbohydrates*	78	Xylose	115	Citric acid
1	1,2-Propanediol[a]	38	Arabinose		*Esters*	116	Courmaric acid[a]
2	2-Aminoethanol	39	Arabitol	79	*O*-Phosphoethanolamine	117	Fumaric acid
3	Octadecanol	40	Ascorbic acid		*Fatty acids*	118	Glycolic acid
	Amides	41	Dihydroxyacetone	80	2-Methyl-3-hydroxyvaleric acid	119	Glyoxylic acid
4	Creatinine	42	Dihydroxyacetone phosphate	81	5-Aminovaleric acid	120	Hypotaurine
5	Pantothenic acid	43	Fructose	82	Arachidonic acid	121	Isocitric acid
6	Urea	44	Fructose 1-phosphate	83	Caproic acid	122	Lactic acid
	Amines	45	Fructose 6-phosphate	84	Decanoic acid	123	Maleic acid
7	Cadaverine	46	Galactose	85	Docosahexaenoic acid[a]	124	Malic acid
8	Putrescine	47	Galacturonic acid	86	Eicosapentaenoic acid	125	Methylsuccinic acid
	Amino acids	48	Glucaric acid	87	Elaidic acid	126	Oxalic acid
9	2-Aminoisobutyric acid	49	Gluconic acid	88	Heptanoic acid[a]	127	Phosphoenolpyruvic acid
10	3-Aminoisobutyric acid	50	Glucose	89	Lauric acid	128	Pyruvic acid
11	3-Aminopropanoic acid	51	Glucose 6-phosphate	90	Linoleic acid	129	Succinic acid
12	3-Sulfinoalanine	52	Glucuronic acid	91	Myristic acid	130	Taurine
13	4-Aminobutyric acid	53	Glucuronic acid lactone	92	Nonanoic acid	131	Trichloroacetic acid
14	4-Hydroxyproline[a]	54	Glyceraldehyde	93	Octanoic acid		*Phenols*
15	Acetylglycine	55	Glyceric acid	94	Oleic acid	132	Coniferyl alcohol
16	Alanine	56	Glycerol	95	Palmitic acid	133	Coniferyl aldehyde
17	Aspartic acid	57	Glycerol 3-phosphate	96	Palmitoleic acid	134	Phenol
18	Citrulline	58	Inositol	97	Pentanoic acid[a]		*Phosphoric acids*
19	Cysteine	59	Isomaltose	98	Stearic acid	135	Phosphoric acid
20	Glutamic acid	60	Lactitol	99	Undecanoic acid[a]		*Purines*
21	Glutamine	61	Maltose		*Glycerides*	136	Adenine
22	Glycine	62	Maltotriose[a]	100	Monostearin	137	Hypoxanthine
23	Homocysteine	63	Mannitol		*Organic acids*	138	Inosine
24	Leucine	64	Mannose	101	2-Hydroxybutyric acid	139	Xanthine
25	Lysine	65	N-Acetylmannosamine	102	2-Hydroxyglutaric acid		*Pyridines*
26	Methionine	66	Rhamnose	103	2-Hydroxyisobutyric acid	140	3-Hydroxypyridine[a]
27	Methylcysteine[a]	67	Ribose	104	2-Ketoglutaric acid	141	Niacinamide
28	*N*-Acetylaspartic acid	68	Ribose 5-phosphate	105	2-Phosphoglyceric acid		*Pyrimidines*
29	*N*-Acetyl-Ornithine	69	Ribulose	106	3-Hydroxybutyric acid	142	Cytosine
30	*N*-Acetylserine	70	Ribulose 5-phosphate	107	3-Hydroxyisobutyric acid	143	Thymine
31	Ornithine	71	Sedoheptulose 7-phosphate	108	3-Hydroxyisovaleric acid	144	Uracil
32	Phenylalanine	72	Sorbitol	109	3-Phosphoglyceric acid		*Steroids*
33	Proline	73	Sorbose	110	5-Oxoproline	145	Cholesterol
34	Sarcosine	74	Sucrose	111	Allo-isoleucine		*Tocopherols*
35	Serine	75	Threonic acid	112	Azelaic acid	146	Alpha-tocopherol[a]
36	Threonine	76	Trehalose	113	Benzoic acid		
37	Valine	77	Xylitol	114	Boric acid		

[a] Metabolites from the in-house library

carried out by manually examining the selected ions and the peak retention times of the corresponding metabolites. The relative area of the individual metabolites was calculated by comparing the peak areas of the metabolites and the internal standard, ribitol.

Metabolic pathway analysis

The processed data were analyzed using a one-way ANOVA (analysis of variance) and the fold changes of metabolites in dead carp versus control was calculated to identify the metabolites that were significantly affected ($P < 0.001$, fold change > 1.5 or < 0.667) by exposure to

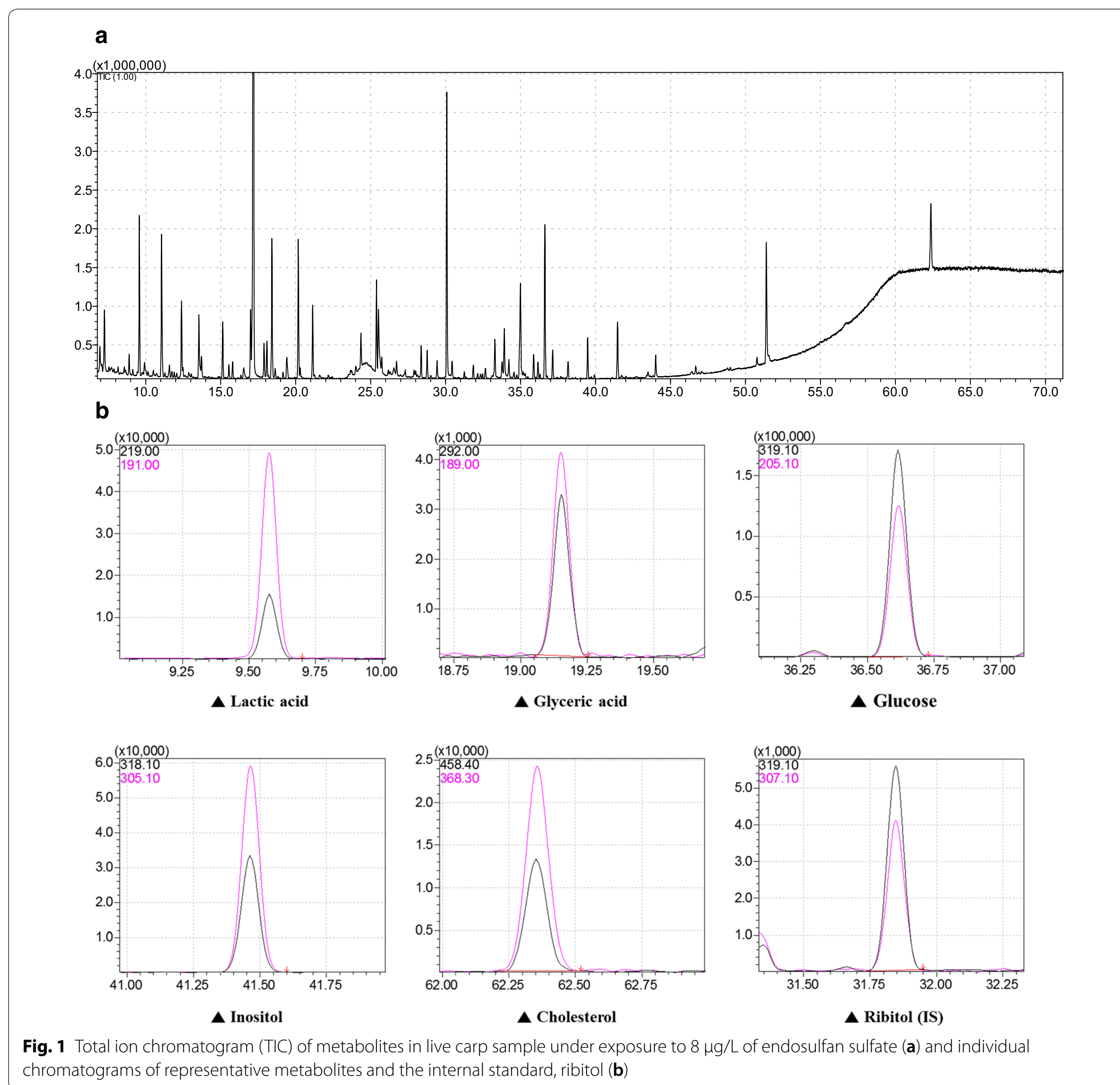

Fig. 1 Total ion chromatogram (TIC) of metabolites in live carp sample under exposure to 8 μg/L of endosulfan sulfate (**a**) and individual chromatograms of representative metabolites and the internal standard, ribitol (**b**)

endosulfan sulfate. A heat map was generated with MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) using the relative areas of the metabolites that had been selected as biomarkers in the ANOVA analyses. Metabolic pathway analysis plots were produced with MetaboAnalyst 4.0 and the metabolic pathways were identified based on the *Cyprinus carpio* KEGG library.

Results and discussion

Chemical exposure level

The exposure concentration of endosulfan sulfate was set at 8 μg/L to ensure the death of carp and understand the toxicological phenomenon. This concentration was twice the concentration of the 48-h LC_{50} value (2.6–5.0 μg/L) for four fish species [5].

Selection of the metabolite extraction solvent

Three extraction solvents, 50% methanol, 80% methanol and dichloromethane/methanol/water (2/5/2), have been frequently used in metabolomics studies of fish [1, 16, 17]. Therefore, we selected those three extraction solvents to compare their extraction efficiency of metabolites in carp. The 50% methanol was chosen as the extraction solvent in this study because it showed better peak shape and

Table 3 Significantly altered metabolites in carp exposed to endosulfan sulfate

Metabolite	P-value	Fold change	
		Live	Dead
Isomaltose	5.73E−07	0.15	0.02
Phosphoenolpyruvic acid	8.10E−07	0.82	0.14
Courmaric acid	1.04E−06	0.33	0.00
Galactose	1.29E−06	0.76	0.12
3-Phosphoglyceric acid	1.90E−06	0.76	0.11
Glucose	2.04E−06	0.36	0.03
Linoleic acid	3.02E−06	0.36	0.25
Trehalose	3.03E−06	0.28	0.03
Maltose	1.04E−05	0.28	0.03
Lactic acid	1.75E−05	0.63	0.36
Lysine	2.17E−05	0.52	11.87
3-Aminopropanoic acid	2.78E−05	0.88	4.39
3-Aminoisobutyric acid	2.83E−05	1.04	3.63
Trichloroacetic acid	5.03E−05	0.59	0.39
Mannose	8.84E−05	0.68	0.41
2-Phosphoglyceric acid	1.09E−04	0.69	0.10
Glycerol 3-phosphate	1.40E−04	0.80	0.35
Myristic acid	2.35E−04	0.60	0.56
Inosine	2.58E−04	1.10	0.40
Azelaic acid	2.79E−04	0.97	0.46
Maleic acid	2.80E−04	0.46	0.30
Palmitoleic acid	3.47E−04	0.41	0.36
4-Hydroxyproline	3.48E−04	0.47	0.32
Lactitol	3.59E−04	0.29	0.04
Citric acid	4.81E−04	1.07	0.59
Mannitol	5.42E−04	1.11	0.46
Ornithine	6.57E−04	0.73	1.53
Arachidonic acid	6.64E−04	0.86	2.22
Taurine	6.83E−04	0.95	0.36
Glyceric acid	7.16E−04	0.66	0.42

extraction efficiency than the other two solvents when peak shape and area were compared (Table 1). A previous study comparing the extraction method of metabolites in fish also showed that the yield of metabolites was higher with 50% methanol compared with the other various solvents [18].

Metabolite profiling using GC–MS

After the GC–MS analyses, metabolites were identified using an SIM-library of 373 metabolites included 16 alcohols, 7 amides, 7 amines, 63 amino acids, 74 carbohydrates, 2 esters, 44 fatty acids, 1 glyceride, 4 indoles, 8 nucleosides, 107 organic acids, 9 phenols, 1 phosphoric acid, 13 purines, 4 pyridines, 3 pyrimidines, 6 steroids, 1 terpene and 3 tocopherols. In the live and dead carp, 146 metabolites were detected as confirmed metabolites (Table 2, Fig. 1). The relative area of the individual metabolites compared to ribitol was subjected to a one-way ANOVA and fold change calculation for the determination of biomarkers.

Metabolic pathway and function analysis

ANOVA analyses ($P < 0.001$) of the 146 metabolites was performed and fold change of them in dead carp compared to control (fold change > 1.5 or < 0.667) was calculated to obtain a total of 30 metabolites as biomarkers that contributed significantly to distinguish the metabolic profiles among the test groups (Table 3). The heat map with dendrograms of the 30 metabolites was generated using MetaboAnalyst 4.0 software, allowing for an intuitive identification of changes in the altered metabolite levels between the control and live and dead carp after exposure (Fig. 2). The metabolite levels are shown

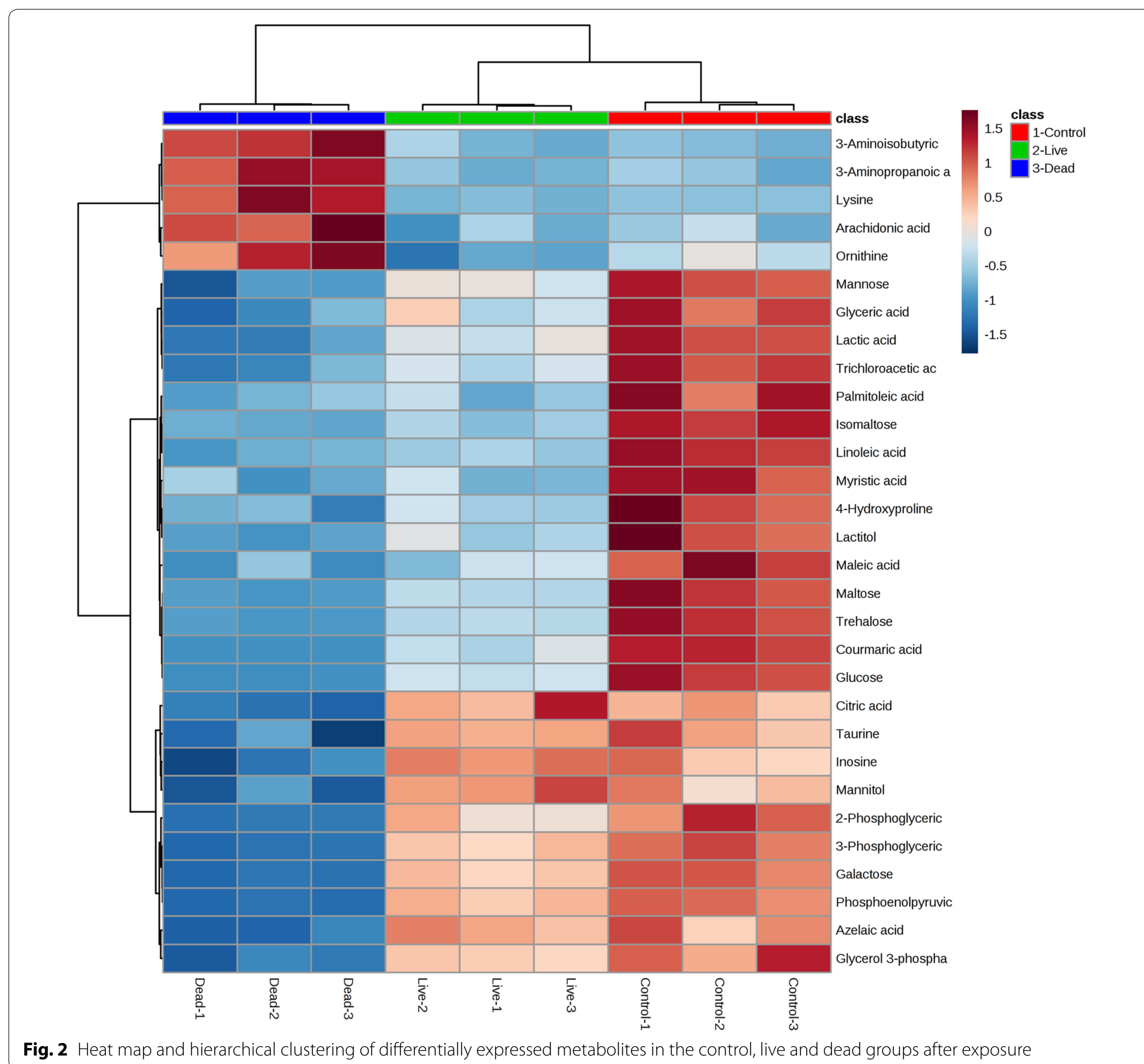

Fig. 2 Heat map and hierarchical clustering of differentially expressed metabolites in the control, live and dead groups after exposure

ranging in color from red (highest) to blue (lowest). In the hierarchical clustering analyses of the differential levels of metabolites associated with those groups, which were performed by the sample (columns) and variables (rows), a clear separation between test groups was confirmed.

Metabolic profiling is able to show changes in each metabolite level and reveal a comprehensive interpretation of the metabolic processes due to toxic xenobiotics [19]. The pathway analysis module of MetaboAnalyst 4.0 contains new algorithms and concepts with the high-quality Kyoto Encyclopedia of Gene and Genomes (KEGG) and well-established methods [16]. In this study, metabolic pathway analyses were performed with 30 biomarkers using the pathway analysis module

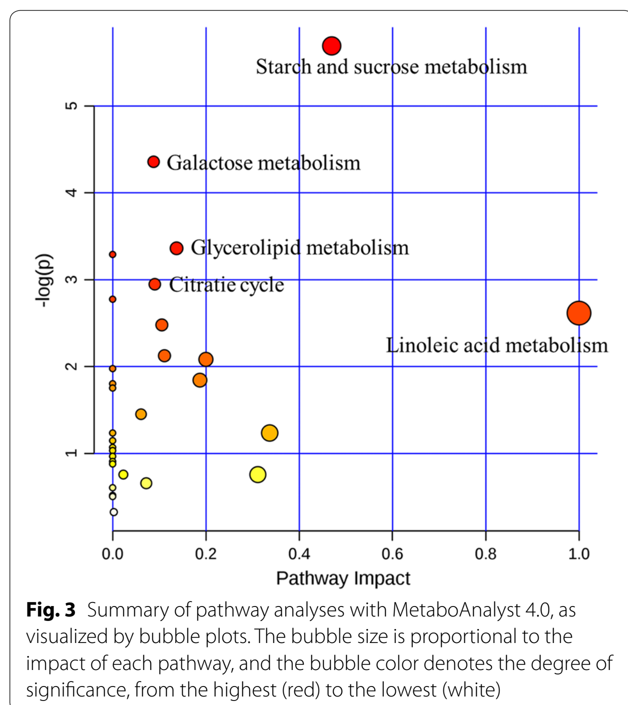

Fig. 3 Summary of pathway analyses with MetaboAnalyst 4.0, as visualized by bubble plots. The bubble size is proportional to the impact of each pathway, and the bubble color denotes the degree of significance, from the highest (red) to the lowest (white)

of MetaboAnalyst 4.0 (Fig. 3) [20]. As a result, five metabolic pathways (starch and sucrose metabolism, galactose metabolism, glycerolipid metabolism, the citrate cycle and linoleic acid metabolism) were identified as the major disturbance pathways. To compare the relative amounts of the metabolites that showed significant changes between the groups, the mean relative area of the individual metabolites that related to each metabolic pathway were graphed as histograms (Fig. 4). In the live and dead carp exposed to endosulfan sulfate, the metabolites associated with energy metabolism were significantly altered, and all of the metabolites associated with each metabolic pathway have a consistent trend that decreases from the control group to the dead group.

In starch and sucrose metabolism, the levels of glucose, maltose and trehalose were decreased in the live carp after endosulfan sulfate exposure. This significant downregulation in the live carp showed that their energy production increased to try to survive in the toxic environment [9]. However, those metabolites were almost exhausted in the dead group (Fig. 4a).

The levels of galactose and mannose in galactose metabolism decreased in the exposure groups (Fig. 4b). These sugars may be broken down into lactate and malate for producing additional energy [21]. Reduction of these sugars has also been demonstrated in the lungs of asthmatic mice, which may contribute to the development of airway inflammation and the severity of the disease [21].

In glycerolipid metabolism, glyceric acid and glycerol-3-phosphate were reduced in the carp after endosulfan sulfate exposure (Fig. 4c). Glyceric acid is a branch point between the glycerolipid and carbohydrate metabolic pathways, and this metabolite might be utilized to mobilize glycerolipids as an energy source through β-oxidation [22, 23].

With respect to the citrate cycle, citric acid and phosphoenolpyruvic acid were significantly decreased in the dead carp, while in the live carp there were no significant differences compared to the control group (Fig. 4d). Citric acid actually increased slightly in the live group, indicating that the citrate cycle was affected by endosulfan sulfate exposure [19]. In a previous study of zebrafish infection [24], citrate cycle intermediates showed the same tendency to decrease in the dead group and increase in the live group. These interesting results suggested that the status of the citrate cycle could be a biomarker for the survival or death of carp.

In linoleic acid metabolism, linoleic acid was significantly reduced in both the live and dead groups (Fig. 4e). Linoleic acid plays important physiological and biochemical functions in freshwater fish [25]. In a study of gilthead seabream, low levels of linoleic acid in plasma were related to the lean muscle phenotype [26]. Therefore, decreasing levels of linoleic acid indicated the dysfunction of energy metabolism.

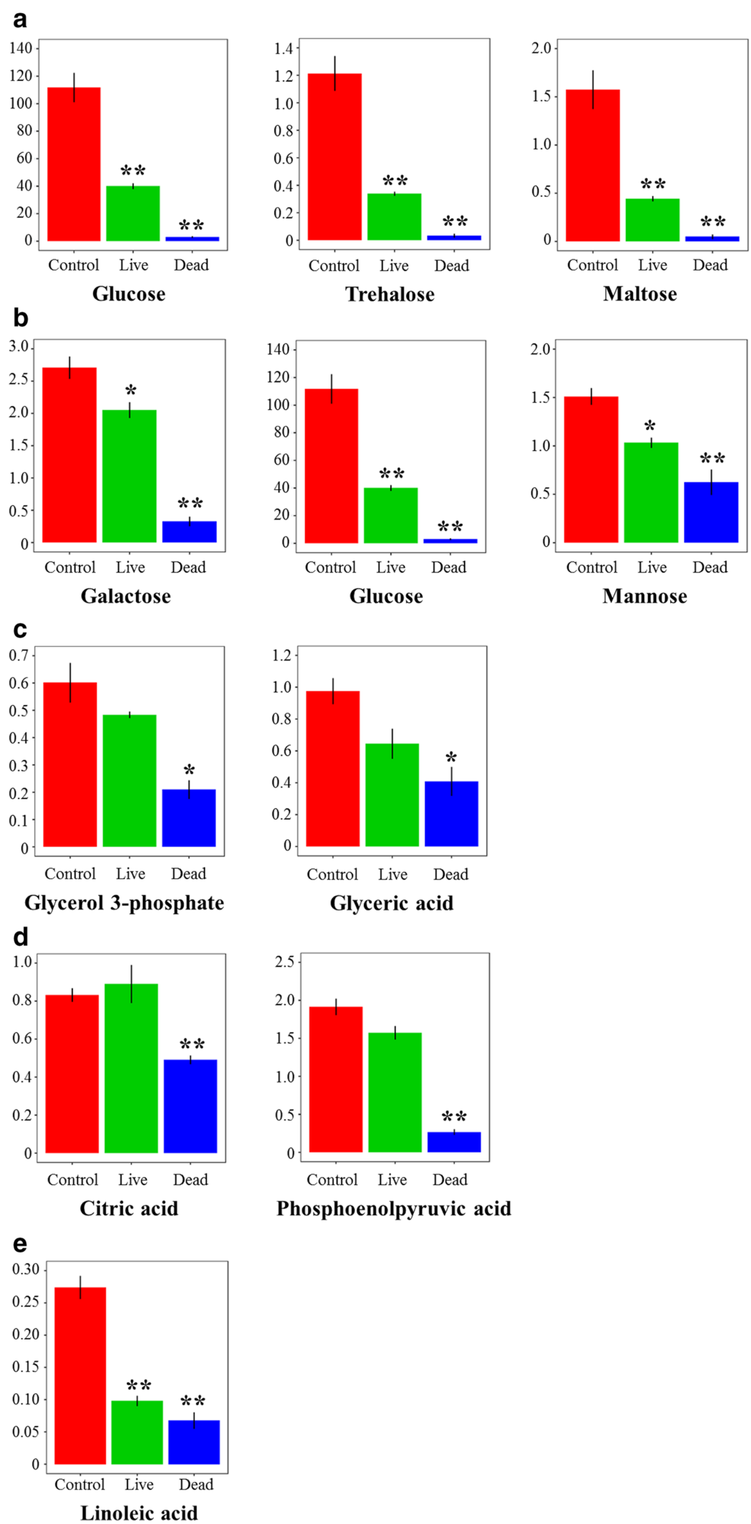

Fig. 4 Average relative areas of typical significant metabolites that are related to each metabolic pathway: starch and sucrose metabolism (**a**), galactose metabolism (**b**), glycerolipid metabolism (**c**), the citrate cycle (**d**), and linoleic acid metabolism (**e**). In the graphs, the red bars are the control groups, the green bars are the live groups and the blue bars are the dead groups. Significantly different changes of $P < 0.01$ and $P < 0.001$ in the relative areas are labeled by "*" and "**", respectively

Authors' contributions
HKL performed the experiment, data analysis and interpretation, and wrote the final manuscript. JL designed and set up the experimental conditions for the instrumental analysis and performed the data analysis. JL contributed to the design of the experimental conditions. HL assisted with the design and conduct of the experiment. JHK supervised the project and revised the final manuscript. All authors read and approved the final manuscript.

Funding
Funding information is not applicable/No funding was received.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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Received: 23 November 2019 Accepted: 6 February 2020
Published online: 13 February 2020

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