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RESEARCH ARTICLE



# MRM-driven metabolomic workflow for early detection of antibiotic-triggered sub-lethal toxicity using Q-TRAP and zebrafish model

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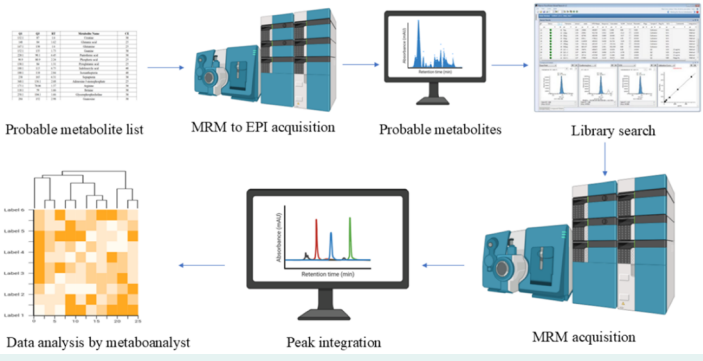
**ABSTRACT**

1. This study aimed to develop a targeted metabolomics workflow using low-resolution tandem mass spectrometry (MS/MS) to identify metabolic alterations in zebrafish (*Danio rerio*) embryos exposed to environmentally relevant concentrations of the antibiotic's amoxicillin and clarithromycin.
2. Zebrafish embryos were exposed to the lowest concentrations (1 µg/L) and the highest concentration (1 mg/L) of amoxicillin and clarithromycin. A library-assisted multiple reaction monitoring–enhanced product ion (MRM–EPI) approach was applied using a QTRAP LC–MS/MS system, enabling the detection and structural confirmation of 108 endogenous metabolites. A targeted MRM–EPI method was subsequently optimised for sensitivity, reproducibility, and specificity. Multivariate statistical analyses, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were performed to assess metabolic differences between exposed and control groups.
3. Exposure to both antibiotics resulted in significant alterations in amino acid, purine, lipid, and energy metabolism, indicating that even sub-lethal concentrations can disrupt vital physiological processes in zebrafish embryos. These findings highlight the sensitivity of metabolomics for detecting early biochemical perturbations and support the use of zebrafish embryos as a practical and ethically suitable model for environmental toxicity assessment. The developed MRM-driven workflow provides a reproducible platform for predictive toxicology and ecological risk evaluation.

**GRAPHICAL ABSTRACT**

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LC-MS/MS; MRM–EPI workflow; environmental metabolomics; biomarkers exposure; MS/MS library

## Introduction

Detection of pharmaceutical contaminants in aquatic systems has drawn growing concern, especially regarding antibiotics that persist in the environment. Since the 1990s, advancements in analytical methods have revealed the presence of such compounds in wastewater, surface water, and groundwater, often at nanogram to microgram-per-liter levels (Akhter et al. 2024). The increasing release of pharmaceuticals into the aquatic environment has raised global concerns about chemical safety, necessitating comprehensive toxicological assessments (Bambino and Chu 2017). These pharmaceuticals are categorised by their intended biological activity, such as antibiotics, analgesics, antihistamines, and anti-inflammatories. Among these, antibiotics are particularly problematic due to their biological activity and potential to cause short and long-term effects in non-target organisms even at sub-lethal concentrations. These sub-lethal

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exposures rarely result in visible developmental deformities in early life stages of aquatic organisms such as zebrafish. However, they may trigger subtle metabolic disruptions that escape conventional toxicity assessments. This threatens non-target organisms due to their possible ecotoxicological consequences (De Oliveira et al. 2020). Therefore, their is a critical need for analytical strategies that can sensitively detect such hidden biochemical changes and link them to environmental exposures.

Metabolomics, the comprehensive study of endogenous small molecules (typically <1000 Da), is an emerging field that provides deep insights into physiological and toxicological responses (Olesti et al. 2021). It allows for the detection of metabolic changes in response to chemical stressors, offering a powerful complement to traditional toxicological endpoints (Qin et al. 2020). Environmental metabolomics, in particular, has proven useful in linking chemical exposure to phenotypic changes in aquatic species, notably the zebrafish (*Danio rerio*), a widely used vertebrate model due to its transparent embryos, rapid development, and genetic similarity to humans (Aguilar et al. 2022). Environmental metabolomics, a high-throughput molecular approach, offers a promising strategy to investigate the sub-lethal effects of pharmaceuticals on aquatic organisms, particularly where conventional toxicity endpoints fail to reveal functional disturbances. Its application in evaluating antibiotic toxicity in zebrafish has gained attention, leveraging the model's physiological similarity to mammals in metabolism and endocrine function (Bajad and Shulaev 2007; Viant 2008; Peterson and Macrae 2012; De Sotto et al. 2016; Da Silvaet al. 2021). Zebrafish at different life stages – embryos, larvae, and adults – serve as robust models for acute and chronic toxicity assessments. In recent years, combining zebrafish embryo assays (ZFEs) with metabolomics has emerged as a powerful platform to elucidate the mechanisms of toxicity at the metabolic level (Vogs et al. 2019; Menger et al. 2020). Despite its promise, metabolomics faces analytical challenges, including the need for accurate metabolite identification and quantification across diverse metabolic pathways. Non-targeted approaches using high-resolution MS can detect thousands of features but often lack definitive identification. Conversely, targeted metabolomics, particularly using triple quadrupole in multiple reaction monitoring (MRM) mode, offers improved quantitation and sensitivity but is limited by the availability of chemical standards and validated transitions (Ortiz-Villanueva et al. 2018). However, some antibiotics, like tetracyclines, exhibit minimal metabolic transformation, making biomarkers essential for legally distinguishing drug-treated animals (Liesenfeld et al. 2020), and obtaining all necessary chemical standards for the metabolites of interest is challenging, which generally limits the coverage of detected metabolites in targeted metabolomics.

To overcome these challenges, especially in targeted studies of known metabolites, we implemented a QTRAP-based method combining MRM (for targeted detection) and enhanced product ion (EPI) scans (for structural confirmation) using an information-dependent acquisition (IDA) strategy (Guo et al. 2022), MRM transitions serve as the survey scan to trigger EPI for structural confirmation. This hybrid approach allows real-time structural validation *via* spectral library matching using NIST library using SCIEX LibraryView software, significantly increasing confidence in metabolite identification. Metabolite lists were curated from chemical databases such as PubChem. Following compound confirmation and retention time alignment, a refined semi-quantitative MRM–EPI method was developed to track the metabolic alteration of selected metabolites. To demonstrate the applicability of metabolomics in environmental toxicology, we employed our advanced analytical method to investigate the metabolic responses of zebrafish embryos exposed to lowest concentrations (1 µg/L) and highest concentration of (1 mg/L) of the antibiotic's amoxicillin and clarithromycin. This study highlights the power of metabolomics in capturing subtle biochemical changes in early developmental stages. Utilising an integrated MRM–EPI workflow, we achieved accurate metabolite identification, positioning this strategy as a valuable tool for elucidating toxicity mechanisms and identifying potential metabolomic biomarkers of environmental contaminants in aquatic models like zebrafish embryos in Figure 1.

## Materials and methods

### Chemicals

LC-MS grade methanol, acetonitrile, and water were obtained from J.T. Baker (Mumbai, India). Formic acid (99%) was supplied by Thermo Scientific (Mumbai, India). Analytical-grade acetic acid and syringe filters (PTFE, 0.22 µm pore size) were sourced from Phenomenex (Bengaluru, India) and Tarsons (Kolkata, India), respectively. Whatman filter papers (0.22 µm) were provided by Millipore (Mumbai, India).

### Sample preparation

Zebrafish (*Danio rerio*) embryos were collected at 5 d post-fertilisation (dpf). Six batches of thirty zebrafish embryos each were collected at snap-frozen, and stored at −80 °C until analysis. As per European Directive 2010/63/EU, ethical approval is not required for experiments involving zebrafish embryos up to 5 dpf.

Homogenisation was performed using a Precellys 24 Dual homogeniser at 6500 rpm for two 10-second cycles with a 15-s rest period. For homogenisation, 150 µL of water was added to each sample, followed by 150 µL of methanol, and it was incubated on ice for protein precipitation. Samples were centrifuged at 14,000 rpm for 5 min at 4 °C. An aliquot of 250 µL of the supernatant was collected, centrifuged again, dried in a refrigerated CentriVap concentrator, and reconstituted in 100 µL methanol/water (90:10, *v/v*) for Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method development. A pooled quality control (QC) sample was prepared by combining 10 µL aliquots from each biological replicate while evaluating the method. In this study, a sample volume of 50 µL was analysed, by the 'dilute and shoot' protocol (Cao et al. 2015; Dong et al. 2015) *via* direct injection. All samples, including the pooled QC, were stored at −80 °C until analysis.

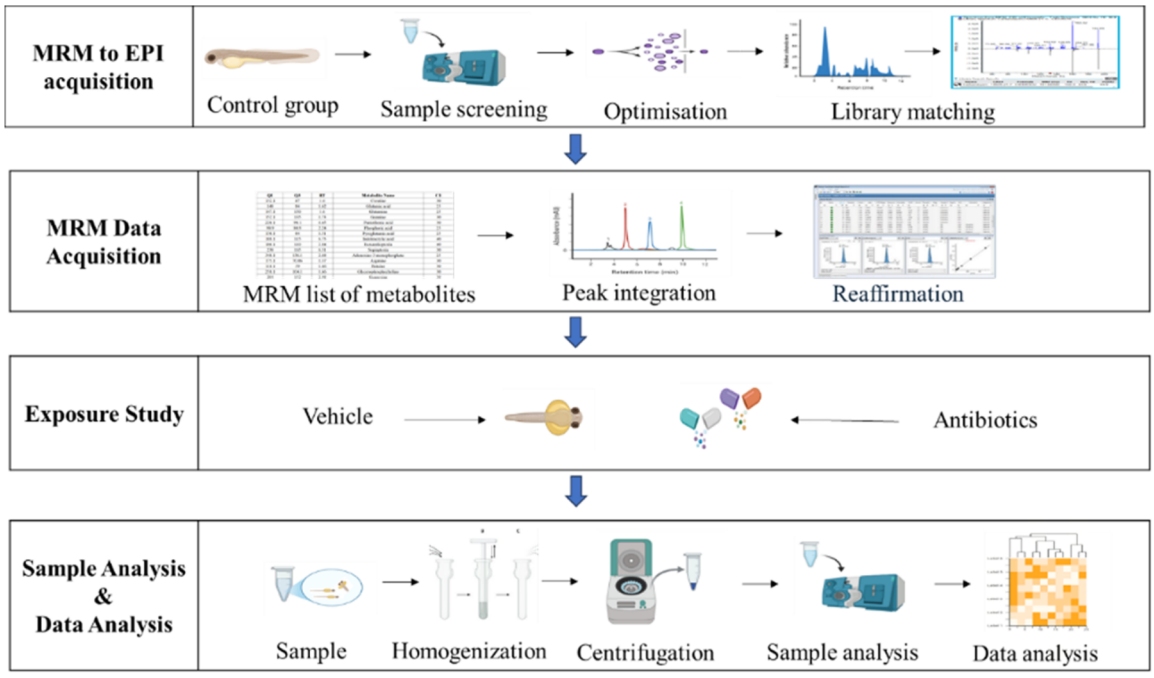


Figure 1. MS based workflow for detecting sub-lethal toxicity at the metabolic level induced by environmental antibiotics.

### LC-MS method conditions

The 4500 Q TRAP® mass spectrometer (Sciex, Darmstadt, Germany), equipped with an electrospray ionisation (ESI) source, was integrated with Shimadzu LC-20AD unit featuring binary solvent control, a CTO-20AC column oven, an SIL-20AC autosampler, a DGU20A3 degasser, and a CBM-20A system controller. Data acquisition and processing were managed using Analyst software (version 1.4.2) and Sciex OS software. Separation was achieved on a reversed-phase C18 column, specifically a Phenomenex Luna-C18 (100 mm × 4.6 mm, 3 µm particle size), maintained at 40 °C. The UHPLC mobile phase comprised solvent A (0.001% formic acid in acetonitrile) and solvent B (0.001% formic acid in water). The gradient began with 95% solvent B, which decreased linearly to 10% by 3 min, reaching 10% at 15 min and holding steady with a flow rate of 0.6 mL/min for an additional 5 min. Initial conditions were re-established within 0.5 min and held for 2 min to ensure system re-equilibration. The total analysis time was 22 min, with an injection volume of 50 µL.

### MRM–EPI method development

The MRM–EPI method was developed to incorporate IDA in both positive and negative ESI modes, aiming to identify the maximum number of metabolites. For precise mass accuracy, the 4500 Q TRAP was calibrated semi-annually using polypropylene glycol (PPG). Major mass parameters, including declustering potential (DP) and collision energy (CE), were optimised.

A list of metabolites like amino acids, nucleotides, and lipid metabolism was curated from PubChem and other databases. These formed the basis of a targeted screening method developed on a Sciex 4500 QTRAP® mass spectrometer equipped with an ESI source. An initial MRM method was established to act as the survey scan, designed to monitor up to 190 metabolite transitions in both positive and negative ionisation modes. Each transition was assigned optimised CE values (20, 35, or 50 eV) and dwell times. To enable structural confirmation, EPI scans were triggered using IDA when ion intensity exceeded 500 counts per second. The EPI was acquired using a CE spread (CES ± 15 eV) to capture a broader range of fragment ions. Instrument conditions included curtain gas at 30 psi, ion source voltage at 5.4 kV, source temperature at 550 °C, gas 1 at 55 psi, gas 2 at 50 psi, CAD gas set to high, and CE at 35 eV. A TurbolonSpray source, which operated in EPI scan mode with a DP of 30 V was used in the MS/MS circumstances. The Q1 resolution unit was utilised, with a trap (Q3) fill time of 3 ms and a LIT scan rate of 10,000 amu/s. An MRM scan was performed using a DP of 60 and a CE of 35, covering a mass range of 100–1000 Da. Enhanced resolution (ER) was applied to the three most intense peaks. In negative ionisation mode, the MRM scan utilised parameters of −60 DP, −35 CE, −10 entrance potential (EP), and 15 CES. IDA was employed to identify metabolites based on their fragmentation patterns, referencing the Library View software and database. EPI scans were conducted with ±15 CE to maximise metabolite detection. Transitions were monitored with 3 ms dwell time and 5 ms pause time. A dynamic exclusion window of 60 s was applied to prevent repeated acquisition of the same precursor ion.

### Compound confirmation using library matching

To filter and screen the raw data, it was loaded into the SCIEX OS program and matched against the NIST MS/MS library through LibraryView. Smart confirmation library search algorithms were used to first match the component names and then search using the spectra if no match is found. Results were sorted using purity, precursor mass tolerance 0.4 Da and intensity threshold (0.05). The comprehensive score weights of mass deviation (<300 ppm), database matching degree (>70),

were set to 50% each, respectively. The SCIEX OS program examined the potential elemental composition and chemical structure in conjunction with the literature research. Confirmed metabolites were then aligned based on retention time (Rt) using QC samples. This confirmed list served as the basis for constructing the final semi-quantitative method.

### Semi-quantitative MRM–EPI method for differential metabolite expression

An MRM–EPI workflow was developed targeting confirmed metabolites with optimised CE and Rt values. Semi-quantitative data were collected in the form of relative peak intensities from MRM transitions and normalised against pooled QC samples. These normalised intensity values were used to assess fold changes, allowing the detection of upregulated and downregulated metabolites in treatment groups.

### Application of the method in zebrafish embryo exposure

To demonstrate method applicability in accordance with the OECD 236 standards (No, 2013), zebrafish embryos were exposed to amoxicillin and clarithromycin at the lowest concentrations (1 µg/L) reflecting those detected in our previous environmental monitoring studies and consistent with reports from Indian river waters (Yamuna River; hospital effluents in Ujjain) (Akhter et al. 2023) and clarithromycin in aquatic environment (Vumazonke et al. 2020). The highest level, 1 mg/L, was a sub-lethal concentration used to evaluate potential toxicity. Embryos were exposed from 4 h post-fertilisation (hpf) to 120 hpf in 6-well plates (30 embryos/well), maintained at room temperature with a 12 h light/12 h dark cycle. DMSO concentration was fixed at 0.05% for all groups. Post-exposure, embryos were extracted and analysed using the established MRM–EPI method. Raw data were filtered and normalised using SCIEX OS and converted into a csv format for further analysis.

### Statistical analysis

Metabolite intensity data were processed in MetaboAnalyst version 6.0. Unsupervised (principal component analysis [PCA]) and supervised (partial least squares discriminant analysis [PLS-DA], sparse PLS-DA [sPLS-DA]) multivariate analyses, along with hierarchical clustering (heatmaps), were used to visualise metabolic differences. Missing values were imputed using K-nearest neighbours. Significance of individual metabolites was assessed using ANOVA with false discovery rate (FDR) correction ($p < 0.05$). In addition, permutational multivariate analysis of variance (PERMANOVA) was performed with 999 permutations to statistically evaluate the explanatory power of treatment effects on overall metabolic variance. PERMANOVA $R^2$ values and associated p-values were used to quantify the proportion of variance explained by antibiotic exposure.

## Results

### Analytical method development and screening workflow

To enable robust metabolic profiling from complex biological matrices, a two-tiered analytical strategy was implemented using a QTRAP LC-MS/MS system. First, a targeted MRM approach was established based on a curated list of 190 metabolites. Curated list compiled from PubChem and existing metabolomics literature, and aligned with an NIST MS/MS library using retention time and fragment pattern matching. Survey scans in both positive and negative ionisation modes were configured with optimised DP, CE, and dwell times to detect a wide range of metabolites. EPI scans were triggered via IDA based on signal intensity thresholds. The EPI spectra were matched to the NIST MS/MS spectral library using SCIEX OS software and LibraryView, ensuring structural confirmation of detected compounds. Based on spectral purity, precursor tolerance, and retention time consistency across QC samples, 108 metabolites were confidently identified. Confirmed metabolites were then transitioned into a final MRM panel shown in Table 1.

This MRM–EPI hybrid approach demonstrated high sensitivity, selectivity, and reproducibility across pooled QC and biological replicates. Sensitivity was assessed based on the ability to detect metabolites consistently at low signal intensities across replicates. Selectivity was ensured by optimising precursor–product ion transitions with compound-specific CE and retention time coupled with EPI spectra for structural confirmation, which minimised interference from isobaric compounds and ensure that detected peaks corresponded specifically to the target metabolites. Reproducibility was evaluated by monitoring retention time stability and relative peak intensity consistency across biological replicates and pooled QC samples. QC clustering in PCA and low variability in relative standard deviation (RSD < 5%) for metabolites confirmed high reproducibility of the workflow. Retention times were aligned with QC samples to ensure chromatographic reproducibility. These identified metabolites were then subjected to analysis using optimised MRM transitions. The total ion chromatogram of the control group used in the identification of metabolites is shown in Figure 2. While this study was focused on qualitative and semi-quantitative profiling, the consistent ion responses and chromatographic reproducibility indicate that the method is well-suited for future quantitative validation, including linearity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), and matrix effect assessments.

### MRM metabolomic profiling of zebrafish embryos exposed to antibiotics

Metabolomic profiling of zebrafish embryos after exposure to amoxicillin and clarithromycin was analysed using a Q-TRAP. Data pre-processing in MetaboAnalyst involved log transformation and Pareto scaling to normalise variance while keeping biological differences. Strict QC measures confirmed the high reproducibility of the analytical workflow. Pooled QC samples, injected at regular intervals, showed stable retention times (variation <0.1 min) and peak intensity CVs below 15%, indicating minimal instrument drift. PCA score plots including both QC and biological samples (Supplementary Figure S1) demonstrated tight clustering of QC injections, confirming instrument stability and analytical reproducibility. Unsupervised multivariate analysis using PCA showed clear clustering of the experimental groups, control, high-concentration (HC) exposed, and low-concentration (LC) exposed, for both antibiotics. In the amoxicillin dataset, PC1 and PC2 together explained about 75.6% of the variance. In the clarithromycin dataset, the first two components

**Table 1.** MRM transitions, Rt, and collisional energy values of metabolites.

| Q1 | Q3 | RT | Metabolite | CE |
|---|---|---|---|---|
| 202.10 | 84.10 | 1.12 | Glu-Ser | 25 |
| 154.10 | 84.10 | 1.17 | 2-Hexenoylcholine | 25 |
| 162.11 | 85.03 | 1.20 | Carnitine | 40 |
| 181.00 | 89.00 | 1.22 | Galactose | 25 |
| 157.10 | 85.10 | 1.22 | 4-Hydroxynonenal | 25 |
| 204.10 | 84.10 | 1.22 | *N*-acetyl-b-glucosaminylamine | 25 |
| 204.10 | 147.10 | 1.22 | N8-acetylspermidine | 25 |
| 224.10 | 84.10 | 1.22 | Gln-Thr | 25 |
| 130.10 | 84.00 | 1.31 | Pyroglutamic acid | 25 |
| 227.10 | 110.10 | 1.32 | Carnosine | 30 |
| 156.10 | 110.10 | 1.32 | Histidine | 25 |
| 176.10 | 70.10 | 1.32 | Citrulline | 25 |
| 175.10 | 130.10 | 1.32 | *N*-Acetyl asparagine | 30 |
| 175.10 | 70.06 | 1.37 | Arginine | 30 |
| 170.00 | 124.00 | 1.37 | Monomethylphophate | 30 |
| 62.00 | 44.00 | 1.42 | Ethanolamine | 20 |
| 208.10 | 105.00 | 1.42 | Phenylpropionylglycine | 40 |
| 104.10 | 69.00 | 1.45 | 4-Amino-butyric acid | 30 |
| 260.10 | 98.00 | 1.45 | D-Glucose-6-phosphate | 30 |
| 90.10 | 44.00 | 1.56 | Alanine | 25 |
| 106.10 | 60.10 | 1.56 | Serine | 20 |
| 147.00 | 102.00 | 1.56 | Oxoglutaric acid | 25 |
| 198.10 | 152.10 | 1.56 | L-Dopa | 25 |
| 198.10 | 136.10 | 1.56 | Phenylalanine betaine | 30 |
| 133.00 | 74.00 | 1.57 | Asparagine | 30 |
| 132.10 | 87.00 | 1.60 | Creatine | 30 |
| 133.00 | 115.00 | 1.60 | Oxalacetic acid | 20 |
| 241.10 | 152.10 | 1.60 | Cystine | 19 |
| 184.10 | 86.10 | 1.61 | Phosphocholine | 25 |
| 120.10 | 74.10 | 1.61 | Threonine | 25 |
| 148.10 | 84.10 | 1.61 | L-Glutamic acid | 25 |
| 120.10 | 102.10 | 1.61 | L-Threonine | 20 |
| 180.10 | 72.10 | 1.61 | D-Glucosamine | 25 |
| 148.00 | 84.00 | 1.62 | Glutamic acid | 25 |
| 139.10 | 121.00 | 1.65 | Urocanic acid | 25 |
| 118.10 | 59.00 | 1.66 | Betaine | 30 |
| 258.10 | 104.10 | 1.66 | Glycerophosphocholine | 30 |
| 229.30 | 171.30 | 1.66 | Myristic acid | 30 |
| 162.10 | 98.10 | 1.68 | Aminoadipic acid | 35 |
| 122.00 | 76.10 | 1.71 | Cysteine | 25 |
| 154.10 | 137.10 | 1.71 | Dopamine | 25 |
| 154.10 | 127.10 | 1.71 | 3,4-Dihydroxybenzylamine | 25 |
| 152.10 | 135.00 | 1.73 | Guanine | 30 |
| 61.00 | 44.00 | 1.76 | Urea | 15 |
| 308.10 | 170.00 | 1.80 | *N*-acetylneuraminic acid | 20 |
| 134.00 | 88.00 | 1.81 | Aspartate | 25 |
| 118.10 | 72.10 | 1.81 | Valine | 25 |
| 251.20 | 102.10 | 1.81 | Leu-Arg | 30 |
| 324.00 | 112.00 | 1.84 | CMP | 35 |
| 268.10 | 180.10 | 1.90 | Prolylyphenylalanine | 30 |
| 268.10 | 136.00 | 1.95 | Adenosine | 25 |
| 150.10 | 104.10 | 1.95 | Methionine | 25 |
| 138.10 | 91.10 | 1.95 | 2-Hydroxyphenethylamine | 20 |
| 166.10 | 148.10 | 1.95 | (*S*)-Homostachydrine | 25 |
| 175.10 | 129.10 | 1.95 | 5-Aminovaleric acid betaine | 25 |
| 150.20 | 104.00 | 1.99 | Methionine | 15 |
| 133.00 | 115.00 | 2.03 | Malic acid | 25 |
| 98.90 | 80.90 | 2.24 | Phosphoric acid | 25 |
| 113.03 | 94.90 | 2.28 | 2-Furancarboxylic acid | 35 |
| 182.10 | 136.10 | 2.64 | Tyrosine | 25 |
| 123.00 | 80.00 | 2.64 | Niacinamide | 20 |
| 348.10 | 126.00 | 2.64 | Inosine-monophosphate | 25 |
| 167.10 | 124.10 | 2.64 | Dopaquinone | 25 |
| 348.10 | 136.10 | 2.68 | Adenosine-5-monophosphate | 25 |
| 136.00 | 119.00 | 2.68 | Adenine | 30 |
| 113.00 | 70.00 | 2.71 | Uracil | 20 |
| 133.10 | 70.00 | 2.73 | Ornithine | 20 |
| 180.10 | 110.00 | 2.84 | Isoxanthopterin | 40 |
| 284.00 | 152.00 | 2.98 | Guanosine | 30 |
| 137.03 | 110.05 | 3.06 | Hypoxanthine | 35 |
| 269.00 | 137.00 | 3.08 | Inosine | 25 |
| 383.20 | 193.20 | 3.17 | Gadusol | 30 |
| 268.10 | 152.10 | 3.27 | Deoxyguanosine | 25 |
| 93.10 | 75.10 | 4.00 | Glycerol | 20 |
| 203.16 | 86.04 | 4.40 | L-Alanyl-L-norleucine | 35 |

(*Continued*)

**Table 1.** Continued.

| Q1 | Q3 | RT | Metabolite | CE |
|---|---|---|---|---|
| 233.18 | 74.02 | 4.42 | Thr-Leu | 35 |
| 120.11 | 77.05 | 4.65 | *S*-(2-Aminoethyl)isothiourea | 35 |
| 149.00 | 103.00 | 4.68 | Cinnamic acid | 25 |
| 166.10 | 120.10 | 4.83 | Phenylalanine | 25 |
| 231.18 | 83.12 | 5.97 | DL-Leu-DL-Val | 35 |
| 231.27 | 86.05 | 6.02 | Val-Ile | 35 |
| 258.10 | 120.10 | 6.05 | Leu-Phe | 25 |
| 238.00 | 165.00 | 6.31 | Sepiapterin | 30 |
| 220.10 | 90.10 | 6.45 | Pantothenic acid | 30 |
| 398.10 | 250.10 | 6.49 | *S*-Adenosylhomocysteine | 30 |
| 231.19 | 71.90 | 6.54 | Val-Leu | 35 |
| 363.10 | 203.10 | 6.54 | Trisaccharide1 | 30 |
| 229.23 | 116.10 | 6.56 | Ile-Pro | 35 |
| 204.10 | 114.10 | 6.69 | Ala-Asp | 25 |
| 205.10 | 118.10 | 6.74 | Tryptophan | 25 |
| 188.10 | 115.00 | 6.75 | Indoleacrylic acid | 40 |
| 245.27 | 86.07 | 7.22 | Leu-Leu | 35 |
| 245.27 | 84.04 | 7.22 | Ile-Leu | 35 |
| 279.17 | 120.09 | 7.35 | Phe-Leu | 35 |
| 229.18 | 69.96 | 7.35 | Pro-Leu | 35 |
| 114.10 | 86.10 | 7.57 | Creatinine | 24 |
| 544.41 | 184.10 | 14.76 | 1-(5Z,8Z,11Z,14Z-Eicosatetraenoyl)-sn-glycero-3-phosphocholine | 35 |
| 550.43 | 184.10 | 14.86 | 1-*O*-Hexadecyl-2-*O*-(2E-butenoyl)-sn-glyceryl-3-phosphocholine | 35 |
| 520.47 | 184.10 | 15.03 | 1-(1Z,12Z-Nonadecadienyl)-sn-glycero-3-phosphocholine | 35 |
| 496.46 | 181.10 | 16.06 | 1-Palmitoyl-sn-glycero-3-phosphocholine | 35 |
| 496.46 | 184.10 | 16.06 | 1-*O*-Hexadecyl-2-*O*-methyl-sn-glyceryl-3-phosphorylcholine | 35 |
| 343.10 | 163.00 | 16.30 | Maltose | 25 |
| 482.40 | 341.40 | 17.67 | 1-Stearoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine | 35 |
| 364.00 | 89.00 | 18.55 | Dehydroascorbic acid dimer | 25 |
| 146.00 | 117.00 | 21.82 | Indole-3-carboxaldehyde | 20 |
| 144.10 | 115.10 | 21.82 | Indoline | 25 |
| 162.10 | 117.10 | 21.82 | 5,6-Dihydroxyindole | 25 |
| 103.00 | 57.00 | 21.84 | 2-Ketobutyric acid | 20 |

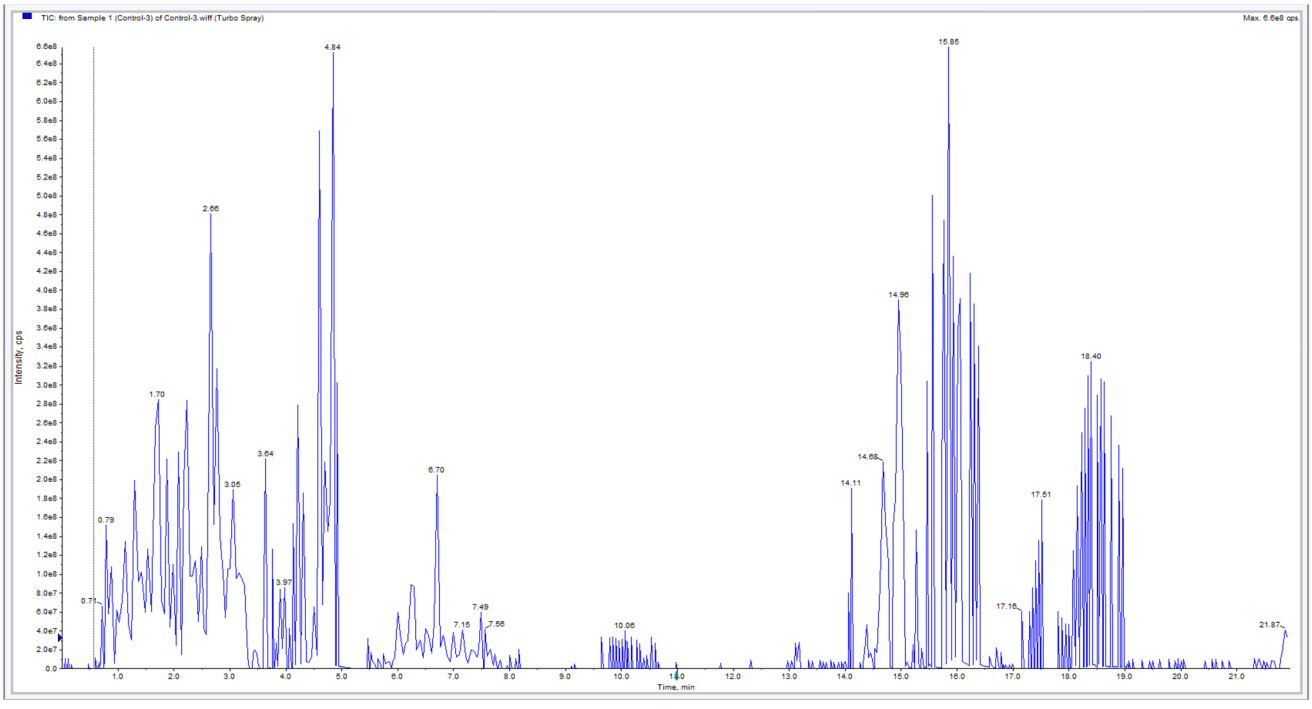


**Figure 2.** Total ion chromatogram of control group embryos.

captured more than 70% of the variance. The score plots displayed a close grouping of replicates within each treatment. The loadings plots indicated that distinct sets of metabolites drove most of the variance. PCA loading plots were generated (Supplementary Figure S2). These revealed that amino acid and purine-related metabolites were the primary drivers of variance, consistent with our pathway-level interpretations. Supervised classification with partial least squares discriminant analysis (PLS-DA) and sPLS-DA refined these observations. Both models achieved perfect or nearly perfect

separation between the control and exposed groups, with over 90% classification accuracy in cross-validation. Supervised classification with PLS-DA achieved clear separation between the control and exposed groups. Model validation using a 1000-iteration permutation test confirmed that the observed separations were statistically significant ($p \leq 0.01$) as shown in Figure 3, indicates the robustness of the model and ruling out overfitting. Despite the limited number of biological replicates ($n = 3$ per group), this statistical significance demonstrates that the supervised PLS-DA model accurately reflects the actual group differences in the metabolic profiles of control and exposed groups. sPLS-DA improved separation by selecting only the most discriminative metabolites (Supplementary Tables S1, S2 and Supplementary Figure S3). These modifications led to less group overlap and better interpretability. Although PCA, as an unsupervised method, already revealed clear clustering of the groups, we further applied supervised approaches (PLS-DA and sPLS-DA) to strengthen statistical interpretation and biological insight. PLS-DA enabled the calculation of Variable Importance in Projection (VIP) scores, highlighting the metabolites most responsible for group separation, while sPLS-DA reduced model complexity by selecting only the most discriminative features. This complementary use of unsupervised and supervised methods ensured that the observed separation was both statistically validated and biologically interpreted. To further examine the metabolites contributing to group separation. PERMANOVA results showed that the treatment factor had a strong explanatory power for both drugs ($R^2 = 0.96$ and 0.92) indicating a strong impact. This finding confirms that the metabolic variance was mainly due to antibiotic exposure instead of random differences. The hierarchical clustering heatmaps offered a complementary view, showing that samples grouped by treatment instead of random patterns. These results visually confirmed the reliability of the analysis. These converging statistical outcomes, PCA, PLS-DA in Figure 3, sPLS-DA, PERMANOVA, and clustering in Figure 4, validate that the analytical pipeline is both sensitive and specific. They also provide confidence that the patterns observed are real biological effects of antibiotic exposure instead of artefacts of sample processing or analysis.

## Identification of differential metabolites

Differential metabolite expression was assessed using semi-quantitative relative intensity values obtained from the

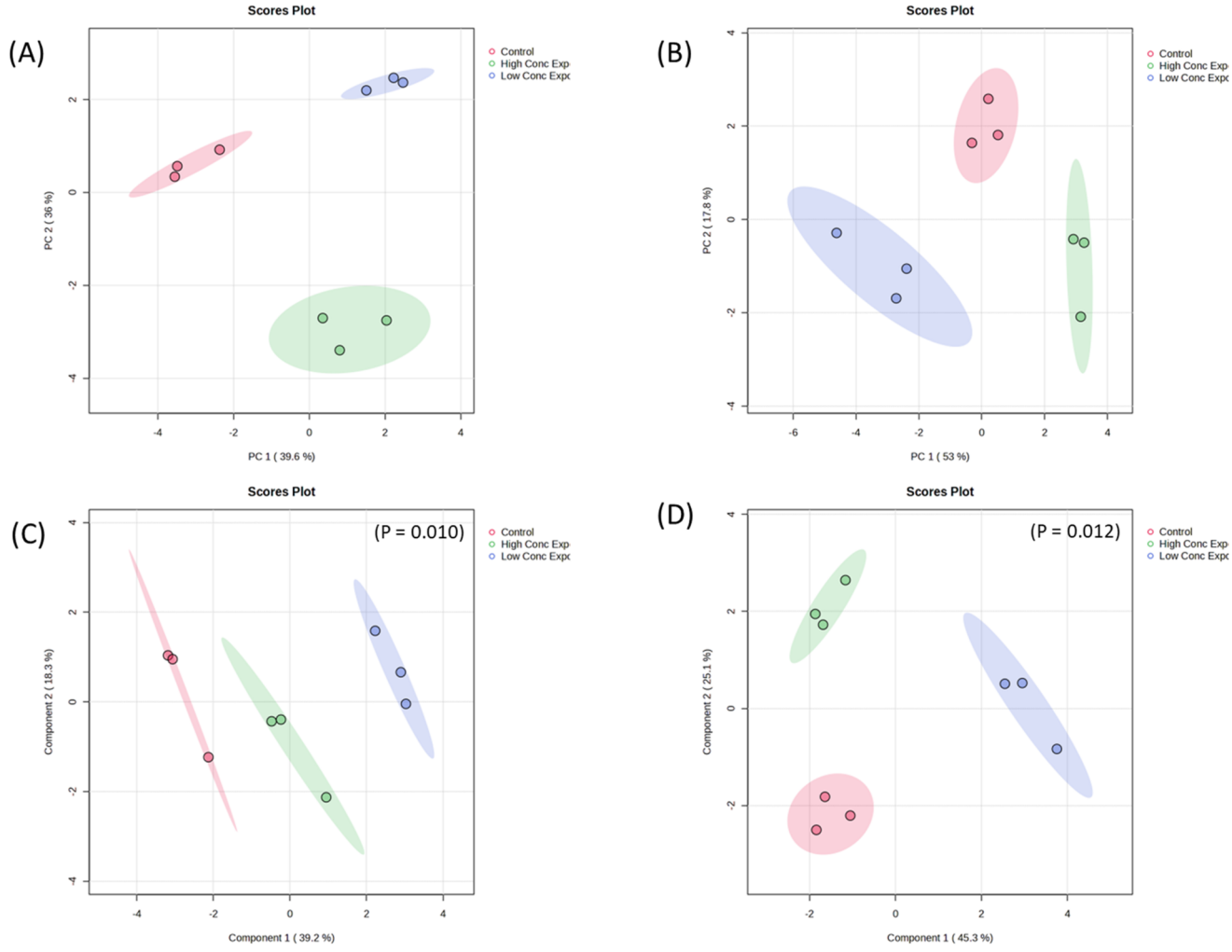


Figure 3. PCA plot of control group vs exposed group amoxicillin (A), and clarithromycin (B) and PLS-DA plot of control group vs exposed group amoxicillin (C), and clarithromycin (D).

MRM–EPI workflow. These values represent normalised peak areas adjusted to pooled QC samples and the total ion current, ensuring that comparisons reflect relative metabolite abundances rather than absolute concentrations. The normalised intensities were used to calculate fold-changes between control, LC, and HC groups, and were subjected to statistical testing. A mix of univariate (ANOVA with FDR correction) and multivariate (VIP scores from PLS-DA) analyses was used to find metabolites significantly changed by antibiotic exposure. Selection criteria included ANOVA $p < 0.05$, FDR-adjusted significance, and VIP $\geq 1.0$. In the amoxicillin-treated groups, the analysis showed several key metabolites with strong statistical significance. Based on normalised relative intensity values, glutamic acid was significantly decreased in both LC and HC exposure groups compared to controls (FDR < 0.001, VIP = 1.6), indicating disruption of excitatory amino acid metabolism under antibiotic exposure. Similarly, significant decreases in 2-ketobutyric acid, adenosine, arginine, and cysteine, indicating alterations in amino acid metabolism, while glycerophosphocholine and phospholipids exhibited non-monotonic changes. U-shaped and non-monotonic patterns in urocanic acid and gadusol suggest dose-dependent adaptive responses. In the case of clarithromycin exposure, major metabolite changes affected amino acid and nucleotide metabolism, with decreases in alanine, methionine, pantothenic acid, and creatinine, and increases in adenosine, along with non-monotonic changes in deoxyguanosine, 1,5-diaminonaphthalene, and dehydroascorbic acid dimer. Lipid metabolism was also perturbed, as indicated by decreased glycerophosphocholine. These results indicate that while both antibiotics disrupt amino acid and lipid metabolism, amoxicillin predominantly reduces metabolite levels with compensatory U-shaped responses, whereas clarithromycin shows mixed patterns, including increases and non-monotonic trends.

## *Biological and toxicological interpretation*

Metabolomic data from zebrafish exposed to amoxicillin and clarithromycin showed important changes in key biochemical networks linked to amino acid and energy metabolism. For amoxicillin, affected pathways included amino acid metabolism, with significant changes in the glutamate, and methionine cycles. There were also changes in nucleotide biosynthesis, as seen in altered adenosine levels, which may indicate cellular stress affecting purine turnover. Additionally, we observed patterns consistent with oxidative stress response, suggesting a greater need for reactive oxygen species (ROS) defence mechanisms. In embryos exposed to clarithromycin, notable metabolic effects appeared in the biosynthesis of aromatic amino acids. Reduced precursor metabolites suggest these pathways are inhibited. The metabolism of sulphur-containing amino acids, methionine and cysteine, was disrupted, and the depletion of methionine could potentially impact methyl group transfer and antioxidant capacity. Furthermore, shifts in energy metabolism, particularly in tricarboxylic acid (TCA) cycle intermediates, point to mitochondrial dysfunction or changes in bioenergetics. The observed decrease in glutamic acid levels in both LC and HC exposure groups suggests impairment of excitatory amino acid metabolism, which may reflect altered neurotransmitter balance and disrupted energy metabolism in developing zebrafish embryos under antibiotic stress. The loss of methionine is especially troubling because it may hinder methylation processes and glutathione production, weakening cellular antioxidant defences. Elevated adenosine during clarithromycin treatment might indicate responses to cellular stress, low oxygen, or immune modulation. From an environmental toxicology view, these metabolic disruptions could harm zebrafish growth, reproductive health, and resilience to environmental stress, especially with long-term or high doses of antibiotics. This raises concerns about ecological impacts in aquatic environments. Overall, these findings highlight how amoxicillin and clarithromycin affect biological and toxicological processes at the metabolome level.

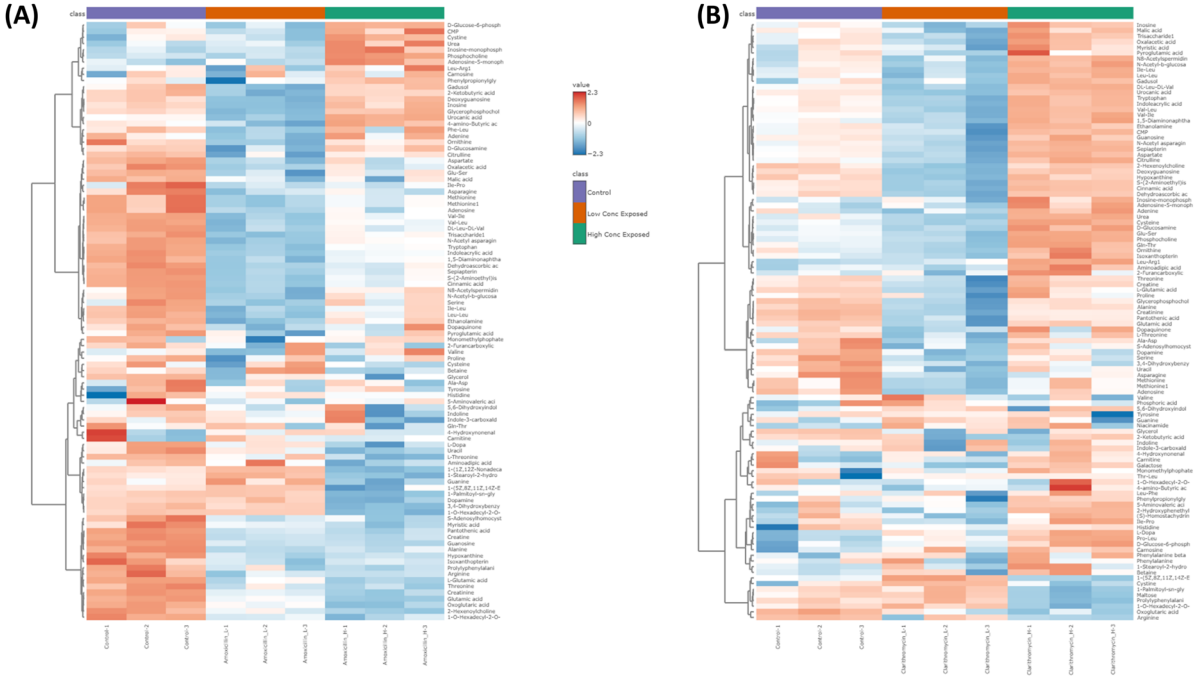


Figure 4. A comparative view of metabolite abundance of control group across the exposed groups amoxicillin (A), and clarithromycin (B).

## Discussion

Understanding the biochemical effects of environmental pollutants at sub-lethal concentrations is essential for early detection of toxicity and ecological risk assessment. The development and application of the MRM–enhanced product ion (EPI) workflow significantly advances metabolomics method development by integrating the high sensitivity and specificity of MRM with the structural confirmation power of EPI. MRM provides selective monitoring of precursor-to-product ion transitions, enabling sensitive and reproducible quantitation of targeted metabolites. However, MRM alone may face challenges such as isobaric interference or ambiguous peak identification. The addition of EPI triggers MS/MS spectra acquisition for targeted ions within the same run, allowing structural verification through spectral library matching. This dual approach not only enhances confidence in metabolite identification covering pathways associated with amino acid, energy, and purine metabolism highlighting its utility for sub-lethal toxicity screening and reduces false positives without compromising throughput or sensitivity. While previous studies have utilised high-resolution mass spectrometry for non-targeted metabolomics in similar models (Ortiz-Villanueva et al. 2018; Da Silva et al. 2021), our study demonstrates that the MRM–EPI workflow with spectral matching, can reliably detect and structurally confirm metabolites relevant to toxicity pathways. The analytical pipeline developed with MRM followed by EPI scanning enabled robust, high-throughput metabolite screening and confirmation from complex environmental and biological matrices. This approach capitalised on the sensitivity of MRM and the structural confirmation power of EPI spectra, in line with existing strategies for reliable metabolomics analysis using Low resolution MS/MS (Want et al. 2013). This significantly lowers operational costs while maintaining analytical confidence, making it more accessible for routine screening and environmental labs. The application of EPI spectra for structural confirmation represents an essential layer of confidence, particularly for closely eluting compounds, a concern highlighted in metabolomics validation frameworks (Matraszek-Zuchowska et al. 2016). Targeted metabolomics provides distinct advantages over the non-targeted approach. Typically, targeted metabolomics uses a triple quadrupole mass spectrometer (QqQ, also known as MS/MS) in MRM mode. This setup is often more affordable and robust than time-of-flight (TOF) mass spectrometry

Data analysis becomes easier when MRM transitions are optimised for target metabolites using genuine standards (Ferrario et al. 2016). Additionally, MRM-based targeted metabolomics is inherently more sensitive, selective, and quantitative, offering a dynamic range of 5–6 orders of magnitude greater than that achieved with non-targeted TOF or QTOF instruments in complex biological samples (Dillen et al. 2012; Morin et al. 2013). This methodological rigour ensured the reliable detection of exposure-responsive metabolites in both control and treated samples, setting the foundation for meaningful statistical and biological interpretations. Studies have shown developmental shifts in zebrafish embryos' metabolome, and integrating metabolomic data with transcriptomic information has revealed novel gene functions in metabolic pathways (Huang et al. 2013; Alfaro and Young 2018). The metabolic alterations observed following amoxicillin and clarithromycin exposure provide insights into antibiotic-specific effects on cellular physiology. Amoxicillin-induced decreases in 2-ketobutyric acid, adenosine, arginine, and cysteine suggest impaired protein synthesis, reduced antioxidant capacity, and altered immune function, while changes in glycerophosphocholine and phospholipids indicate modulation of membrane integrity and signalling pathways (Jijie et al. 2021). Non-monotonic and U-shaped responses in metabolites such as urocanic acid and gadusol reflect dose-dependent adaptive mechanisms under antibiotic stress (Rice et al. 2023). In contrast, clarithromycin affected both amino acid and nucleotide metabolism, with decreases in alanine, methionine, pantothenic acid, and creatinine, and increases in adenosine, highlighting effects on energy metabolism, oxidative stress regulation, and DNA/nucleotide turnover (Pereiro et al. 2022). The decrease in glycerophosphocholine further suggests impacts on membrane stability and cellular signalling (Kenny et al. 2025). Overall, these patterns indicate that while both antibiotics perturb core metabolic pathways, the nature of the perturbation differs: amoxicillin primarily induces reductions with compensatory responses, whereas clarithromycin shows mixed patterns, reflecting distinct adaptive and stress-response mechanisms. These metabolic signatures may serve as potential biomarkers for monitoring antibiotic exposure and predicting clinical or environmental outcomes (Jijie et al. 2021; Vivekaa et al. 2025). The key metabolite alterations observed in this study can be linked to biological processes with potential clinical implications. The decrease in glutamic acid suggests disruption of excitatory neurotransmitter balance, a pathway closely associated with neurotoxicity and impaired synaptic signalling in vertebrates (Zhang et al. 2024). Methionine depletion indicates perturbation of one-carbon metabolism and methylation capacity, raising the possibility of epigenetic dysregulation during embryonic development (Xu and Sinclair 2015). In contrast, elevated adenosine reflects an imbalance in purine metabolism and cellular energy turnover, conditions often associated with inflammation and hypoxia-like metabolic stress. Taken together, these changes suggest that sub-lethal antibiotic exposure interferes with fundamental amino acid, purine, and carbohydrate metabolism pathways, echoing mechanisms reported in clinical and toxicological studies and underscoring the translational relevance of our findings. Currently, the method provides semi-quantitative insights, but due to its reproducibility, signal consistency, and optimised transitions, it holds strong potential for quantitative application. With further validation – including assessments of linearity, accuracy, precision, and matrix effects – this method could be extended to absolute quantification of key biomarkers. Our findings are consistent with prior environmental metabolomics studies, which report similar patterns of redox imbalance, purine dysregulation, and mitochondrial stress in response to antibiotics and other contaminants (Long et al. 2020; Zhang et al. 2023). The ability of this MRM–EPI workflow to distinguish exposed groups, even in the absence of morphological changes,

underscores its sensitivity and early detection capability, aligning with modern goals in regulatory toxicology and chemical safety evaluation (Olesti et al. 2021). Evidence from the literature shows that MRM–EPI workflows, especially on platforms like QTRAP, enable simultaneous quantitative and qualitative analyses by dynamically acquiring EPI spectra after MRM detection thresholds are met. This ability allows for the identification of positional isomers and related metabolites that traditional MRM may miss. As a result, it improves coverage and reliability in complex biological samples like plasma or tissue extracts. Moreover, MRM–EPI workflows maintain strong quantitative performance similar to standard MRM while offering additional qualitative information that is important for metabolite annotation and confirmation. However, there are some limitations to consider. This study used a limited number of exposure concentrations. Future studies should include longitudinal time-course data, multiple dose groups, and integration with transcriptomic or microbiome data. This will help provide a better understanding of exposure responses and host–environment interactions.

## Limitations

This study has certain limitations that should be acknowledged. First, the number of biological replicates was relatively low ($n$ = 3 per group). Although each replicate consisted of 30 pooled embryos thereby integrating responses from 90 embryos per condition and reducing individual variability the nominal sample size remains limited and may restrict statistical power. The present MRM–EPI workflow was applied in a semi-quantitative manner; full quantitative validation, including calibration curves, LOD/LOQ determination, and matrix effect evaluation, was beyond the scope of this work. Finally, the study focused on zebrafish embryos only; additional work in later life stages and across species would strengthen the ecological relevance. Despite these limitations, this study establishes a methodological framework that can be extended in future investigations. The combination of pooled sampling, stringent QC, and multi-layered statistical validation provides confidence in the robustness of the observed metabolic alterations. The findings should therefore be viewed as a proof-of-concept demonstration of the MRM–EPI approach, with future studies incorporating larger sample sizes, expanded dose ranges, and quantitative validation to further build on these results.

## Conclusion

This study presents a workflow that combines multiple MRM with EPI scanning to detect metabolic changes in zebrafish embryos exposed to relevant levels of antibiotics. By curating and confirming 108 biologically relevant metabolites through library-matched EPI spectra, we created a strong MRM–EPI method that can identify sub-lethal biochemical responses to amoxicillin and clarithromycin exposure. Developing and applying the MRM–EPI workflow marks a significant improvement in targeted metabolomics and analytical chemistry, especially for complex biological samples. This hybrid approach combines the high specificity and sensitivity of MRM with the structural confirmation provided by EPI, allowing for both quantitative and qualitative analysis in one run. Although the current implementation mainly focuses on qualitative and semi-quantitative analysis, the method's high reproducibility, optimised transitions, and consistent retention times make it well-suited for future quantitative applications after full analytical validation. This positions the method as a cost-effective and scalable tool for environmental toxicology, particularly in areas where high-resolution equipment may not be available. Our metabolomics-driven approach enhances our detection capabilities and offers greater insight into how organisms interact with their chemical environment.

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## Credit authorship contribution statement

Patharaj Gokul: Investigation, Writing – original draft, Methodology, Data curation. Rajesh Pamanji: Zebrafish toxicity studies, Writing – review & editing. Ragothaman Prathiviraj: Methodology, Writing – review & editing. Hari Krishna Kumar S: Methodology, Writing – review & editing. Murugesan Sobanaa: Methodology, Writing – review & editing. Aseem Setia: Writing – review & editing. Medapati Nikitha Lakshmi Suseela: Writing – review & editing. Joseph Selvin: Supervision, Writing – review & editing. Madaswamy S. Muthu: conceptualisation, project administration, supervision, writing – review & editing.

## Disclosure statement

There are no recognised conflicts of interest, either financially or in terms of personal connections, among the authors that would have affected the results presented in this research.

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## Data availability statement

The data will be made available on request.

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