Pilot study of metabolomic changes in marine sediments following spiking with a sea lice chemotherapeutant Emamectin Benzoate

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St. Andrews, NB, Canada

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PILOT STUDY OF METABOLOMIC CHANGES IN MARINE SEDIMENTS FOLLOWING SPIKING WITH A SEA LICE CHEMOTHERAPEUTANT EMAMECTIN BENZOATE

by

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Abstract

Shaikh, A.A., Davies, J., Jonah, L., Tahlan, K., Asnicar, D., Dufour, S.C., and Hamoutene, D. 2026. Pilot study of metabolomic changes in marine sediments following spiking with a sea lice chemotherapeutant Emamectin Benzoate. Can. Tech. Rep. Fish. Aquat. Sci. 3760: viii + 17 p. https://doi.org/10.60825/cqe5-p694

Sediments beneath salmon aquaculture cages may receive a variety of compounds including chemotherapeutants used to treat diseases or parasitic infestations. Emamectin benzoate (EMB), a drug used in finfish aquaculture for controlling sea lice in salmon, can accumulate in benthic habitats alongside its breakdown products which include the potentially more toxic metabolite desmethyl emamectin benzoate (DES). To contribute to our currently limited understanding of the potential metabolites of EMB other than DES in marine sediments, we undertook a pilot study of metabolomic changes in sediments after they were spiked with EMB over a 14-day incubation period. In both abiotic (autoclaved) and biotic (non-autoclaved) sediments considered separately, we found no significant change with time in the overall composition of detected metabolites although concentrations of some compounds (besides EMB and DES) varied significantly over time. The metabolite profiles were however significantly different when comparing abiotic and biotic spiked sediments. The presence of specific features in each treatment group may suggest distinct metabolic responses to EMB under abiotic versus biotic conditions, indicating that bacterial communities might be contributors. The observed features could potentially represent EMB degradation products other than DES however more work should be conducted to identify changes in microbial and other organismal communities.

Résumé

Shaikh, A.A., Davies, J., Jonah, L., Tahlan, K., Asnicar, D., Dufour, S.C., and Hamoutene, D. 2026. Pilot study of metabolomic changes in marine sediments following spiking with a sea lice chemotherapeutant Emamectin Benzoate. Can. Tech. Rep. Fish. Aquat. Sci. 3760: viii + 17 p. https://doi.org/10.60825/cqe5-p694

Les sédiments marins situés sous les cages de salmoniculture peuvent recevoir une variété de composés, y compris des agents chimio thérapeutiques utilisés pour traiter les maladies ou les infestations parasitaires. Le benzoate d'émamectine (EMB), un médicament utilisé en pisciculture pour lutter contre le pou du poisson chez le saumon, peut s'accumuler dans les habitats benthiques en même temps que ses produits de dégradation, notamment le métabolite potentiellement plus toxique, le benzoate de déméthyle d'émamectine (DES). Pour contribuer à la connaissance, de nos jours limitée, des métabolites potentiels de l'EMB autres que le DES dans les sédiments marins, nous avons entrepris une étude pilote sur les changements métabolomiques de sédiments enrichis avec de l'EMB sur une période d'incubation de 14 jours. Dans les sédiments abiotiques (autoclavés) et biotiques (non autoclavés) considérés séparément, nous n'avons trouvé aucun changement significatif avec le temps dans la composition globale des métabolites détectés, bien que les concentrations de certains composés (en plus de l'EMB et du DES) aient varié de manière significative au fil du temps. Les profils métaboliques étaient toutefois significativement différents lorsque l'on comparait les sédiments enrichis abiotiques et biotiques. La présence de caractéristiques spécifiques dans chaque groupe de traitement suggère des réponses métaboliques distinctes à l'EMB indiquant que les communautés bactériennes contribuent à la métabolisation. Les caractéristiques métaboliques observées pourraient représenter des produits de dégradation des EMB autres que le DES, mais des travaux supplémentaires devraient être menés pour mieux identifier ces composés ainsi que les changements dans les communautés microbiennes et autres organismes.

Introduction

The presence and impact of aquaculture chemotherapeutants entering the marine benthic environment is of increasing concern as more studies document spatial and temporal variation in their fate and deposition (e.g., Hamoutene et al., 2018; Kingsbury et al., 2023). The antiparasitic drug Emamectin benzoate (EMB, 4” deoxy- 4”-epi-methylaminoavermectin) is extensively used in Canadian salmon aquaculture to control sea lice infestations (Bright and Dionne, 2005; Hamoutene et al., 2022). EMB is an in-feed therapeutant marketed under the trade name SLICE®, containing 0.2% w/w of the active ingredient (Bright and Dionne, 2005; Roy et al., 2000) comprised at >90% of isomer EMB1a and <10% of isomer EMB1b (Ikonomou and Surridge, 2013). EMB is introduced to the marine environment through uneaten medicated feed, and via fish faeces and urine (Grefsrud et al., 2018). Due to its hydrophobic nature (log octanol/water partition coefficient Kow of 5.0 at pH 7), EMB sorbs easily to particulate matter and partitions quickly into sediment (Bright and Dionne, 2005; Jacova and Kennedy, 2022; Reddy, 2013; Strachan and Kennedy, 2021). The estimated half-life of EMB in sediment can range from 100 to >400 days (e.g., Mayor et al., 2008; Benskin et al., 2016; Strachan and Kennedy 2021; Hamoutene et al., 2023b). Due to its toxicity, mode of action (Burridge et al., 2010) and persistence in the sediment, EMB has the potential to affect the benthic community and other non-target organisms in the marine environment.

The breakdown products of EMB in the benthic marine environment should also be considered. Roberts and Hutson (1999) identified twelve molecular by-products of EMB degradation in soils, from photolysis, chemical degradation, and metabolization by plants and animals. EMB and its metabolites have been detected in the faeces of salmon post treatment (Kim-Kang et al., 2001, 2004). Desmethyl emamectin benzoate (DES, 4″-deoxy-4″-epi-aminoavermectin) is a common product of EMB degradation (Chukwudebe et al., 1996; Kim-Kang et al., 2004) that has the potential to be 10 times more toxic than the parent compound to Eohaustorius estuarius, a West Coast amphipod crustacean (Kuo et al., 2010). In a survey of sediment samples collected around Atlantic salmon aquaculture farms in Eastern and Western Canada, DES and EMB were measured at concentrations above the limit of quantification (LOQ) in at least 21% (DES) and 16% (EMB) of samples, a maximum concentrations of 19.7 ng/g for DES and 37.1 ng/g for EMB; the ecotoxicological relevance of these concentrations for benthic fauna remain unknown (Kingsbury et al., 2023).

Despite these studies, there is a paucity of data on EMB and its metabolites in marine sediment, especially concerning the potential degradation of EMB in sediments in light of its estimated long half-life (Benskin et al., 2016; Mayor et al., 2008; Strachan and Kennedy, 2021; Hamoutene et al., 2023b). A recent study by Hamoutene et al. (2023a) explored the transformation of EMB into DES in marine sediments experimentally enriched with EMB (with no added DES) under biotic (i.e. including bacterial communities) or abiotic (autoclaved sediment) conditions over a 16 h incubation period, and documented DES:EMB transformation ratios ranging from 0.16 to 0.39% of EMB across samples, with transformation rates being greater under biotic conditions. In a follow-up study examining EMB transformation in sediments during a longer incubation period (318 days), DES:EMB transformation ratios as elevated as 4.4% were observed (Hamoutene et al., 2023b).

Metabolomics is the study of all metabolites present in an organism, cell, or environment (Bundy et al., 2009). Environmental metabolomics characterize changes in compound profiles and how organisms respond to their environment or stressors (Bundy et al., 2009; Viant, 2007).

Metabolomics have been used to reveal changes in specific marine organisms in the presence of stressors (as reviewed in Bayona et al., 2022), or to look for specialized metabolites that might show potential as novel antimicrobial, anticancer, antiviral, or anti-inflammatory compounds, sometimes within complex environmental matrices (e.g., Aminov, 2022; Gerwick and Moore, 2012; Molinski et al., 2009, Shaikh et al., 2023). Knowledge gaps remain on the transformation of EMB into DES and other metabolites in the marine environment, as well as around sedimentary metabolomic changes that might result from exposure to these chemotherapeutants. In this study, we describe a pilot trial that explores changes over time in marine sediment metabolomes under biotic and abiotic conditions, following exposure to EMB.

Materials and Methods

Standards and reagents

Emamectin benzoate ((4″R)-4″-Deoxy-4″-(methylamino) avermectin B1 benzoate (CAS# 155569-91-8)) was purchased from Thermo Scientific, Catalog number 463050010, lot #: A0412341 at 92 %, 1.2% water content, and stored between 2-8°C. Methanol was purchased from Fisher Scientific. A 2500 ng/µL stock solution of EMB was prepared in methanol and further diluted to 25 ng/µL.

Spiking of Sediment and Set-up of incubations

Mud was collected at low tide, below the mean tide mark, near Chamcook Harbour, New Brunswick, Canada (45.11208N, 67.05124W) using a shovel and stored in 3-gallon plastic storage buckets with sealing lids. Substrate physical characteristics are representative of some of the Atlantic salmon aquaculture sites in Southwest New Brunswick. Sediment was refrigerated at 4°C, sieved (2 mm) upon arrival at the St. Andrews Biological Station, thoroughly mixed and kept at 10°C for 1 hour prior to subsampling (replicating natural sediment and water temperatures from the collection site).

Sediment was subsampled (20.00 ± 0.08 g wet weight) into individual 50 mL Falcon tubes and designated as either biotic or abiotic, with three tubes for each incubation timepoint: 18 hours, 24 hours, and 14 days. All tubes in the abiotic condition were autoclaved (Tuttnauer, model 3850 E-B/L, Heidolph) at 121°C for 15 minutes, using standard sterilizing settings that significantly reduce microbial Colony Forming Units, DNA and RNA (Otte et al., 2018). The tubes of abiotic sediment were cooled in a water bath to 10°C and all tubes were kept at 10°C in a dark room for all further processing.

Seawater (20 mL) was added to the tubes to recreate field sediment conditions and allow a more even distribution of solution after sediment spiking; small water aliquots are typically added to substrates with cohesive properties to increase fluidity and mixing (Ditsworth et al., 1990; Murdoch et al., 1997). Unfiltered seawater from the Huntsman Marine Science seawater supply, sourced directly from Brandy Cove (45.08488N, 67.0812W; New Brunswick), and acclimated to 10°C was added to all biotic samples, while autoclaved seawater (following the above autoclaving conditions) was added to all abiotic samples. All tubes were then mixed by vortexing (Talboys Advanced Vortex Mixer).

Sediment samples were spiked with 48 µL of 25 ng/µL emamectin benzoate for a final concentration of 60 ng/g (60 ppb) EMB. All tubes were vortexed thoroughly and placed in a dark room, with the tube caps removed, at 10 °C to allow the spiking solution time to settle and penetrate the matrix. After incubation, tubes from both conditions were frozen at -80°C at 18

hours, 24 hours or 14 days (336 hours) post spiking. Exposure to light was kept to a minimum during the spiking process, incubation, sampling, and freezer storage to avoid photodegradation of the compounds (Chukwudebe et al., 1997; Roberts & Hutson, 1999). It is important to note that airborne bacteria present in the laboratory environment could have been introduced into both biotic and abiotic samples as they were not manipulated or incubated in a laminar flow hood prior to freezing; contamination was not assessed in this study.

Sample processing for metabolomics

Extraction of EMB and DES from sediments was performed at the Health Canada Pesticide laboratory as previously described (Hamoutene et al. 2023a). Briefly, a QuEChERS sample preparation approach was taken using a C18 clean-up. After the addition of 20 mL acetonitrile, samples were homogenized and phase separation was achieved by adding 8 g of NaCl and centrifugation at 4500 rpm for 5 minutes. Aliquots of acetonitrile supernatant were transferred to tubes containing C18 (Sigma-Aldrich) for solid substrate cleanup then evaporated to a final volume of 1 mL using an Xcel-Vap. As only two replicates were available for the 24 hours abiotic condition, one of the samples was used twice in the subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and served as a pseudo-triplicate, to maintain the integrity of the analysis. The samples were sent to Memorial University of Newfoundland for liquid chromatography method selection using either a Hypersil Gold C18 column (50 x 2.1 mm, 1.9 µm, Agilent Technologies) or a Scherzo SM-C18 column (2 × 250 mm, 3 µm, 130 Å; Imtakt) and were then subjected to LC-MS/MS at the metabolomics service facility at the University of Toronto. All shipping was carried out on dry ice to preserve sample integrity as much as possible.

LC-MS/MS and Metabolomics analysis

Separation and analysis were performed using an ultra-high performance liquid chromatography (UHPLC) system-coupled Q to a Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, United States). Chromatographic separation employed a Hypersil Gold C18 column (50 x 2.1 mm, 1.9 µm) maintained at 40°C using 10 µL of each sample at a flow rate of 0.4 mL/min with a mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The following program was used: 0-1 min, 5% B; 1-7 min, gradient to 98% B; 7-10 min, 98% B; 10-10.5 min, gradient to 5% B; 10.5-15 min, 5% B.

Mass spectrometry data was collected in polarity switching mode using an electrospray ionization source with a heater capillary temperature of 320°C along with the following parameters: ± 3500.0 V; S-lens RF, 55; sheath gas flow rate, 20; and auxiliary gas flow rate, 5. MS1 and MS2 scans were acquired for the 70–1000 m/z range at resolutions of 70,000 and 17,500, respectively. The maximum injection time and automatic gain control target values were set at 50 ms and 1 × 105. Higher-energy collision-induced dissociation was performed with a normalized collision energy of 35 eV. The obtained raw LC-MS/MS data were processed through Thermo Scientific Compound Discoverer 3.2 for molecular formula and compound predictions with ChemSpider (Pence, 2010), mzCloud (Thermo Scientific, 2023), and KEGG (Kanehisa et al., 2016) databases. For further analysis, the raw files were converted to mzXML format in ProteoWizard (Adusumilli and Mallick, 2017), with 32-bit binary encoding precision, zlib compression, peak peaking, and polarity was changed to either positive or negative mode. The converted files were uploaded to the global natural products social (GNPS) molecular networking webserver (Wang et al., 2016), and metabolite annotations were done using the previously reported parameters (Shaikh et al., 2021). All raw positive-ion mode data were processed in MetaboAnalyst (Pang et al., 2021) for comprehensive metabolomics comparisons, relative feature quantification, and statistical analyses, using preset parameters in the platform as described (PMID: 21637195 and PMID:

35715522). The corresponding spiked time-zero (T0) samples from both biotic and abiotic treatments were used as blanks for subtraction during analysis to remove background features from subsequent time points.

Results and Discussion

Abiotic and biotic samples (at 18, 24, and 336 hours) were analyzed using LC-MS/MS. The initial analysis detected emamectin benzoate B1a (EMB1a) and suggested the presence of either emamectin B1b (EMB1b) or desmethyl emamectin B1a (DES-B1a) in all spiked abiotic and biotic samples. EMB1a was identified using the GNPS platform (Wang et al. 2016). In contrast, EMB1b and DES-B1a were not identified by GNPS, likely because their concentrations were below the threshold required for precursor ion selection for MS/MS fragmentation, upon which GNPS relies. It is also important to note that EMB1b and DES-B1a share the same precursor ion mass (m/z 872); therefore, in the absence of informative MS/MS spectra, it was not possible to differentiate between these two compounds, and the presence of one could confound interpretation of the other. Consequently, in the current analysis, the presence of EMB1b/DES-B1a was inferred based solely on MS1 detection of the [M+H]+ precursor ion (m/z 872.51, retention time 7.04 min) rather than MS/MS spectra. For EMB1a, the [M+H]+ precursor ion (m/z 886.53, retention time 7.2 min) was additionally used to confirm its presence alongside GNPS-based identification.

To perform a comparative metabolomics analysis, raw data were processed and analyzed using the MetaboAnalyst suite (Pang et al. 2021). Principal component analysis (PCA) on mean centered data divided by standard deviation grouped samples from abiotic and biotic conditions (across all time points) into distinct clusters, indicating significant differences in spectral features between the treatment groups (Figure 1A). However, PCA did not reveal differential clustering of samples when comparing post-spike timepoints (18h, 24h, and 336h) within each treatment group (Figure 1B and Figure 1C). This suggests that variations in metabolomic profiles are not strongly influenced within each treatment group over the timeframe analyzed. Further investigation using correlation heat maps again revealed differences in spectral features, particularly between abiotic and biotic conditions (Figure 2A). Although overall metabolomic profiles did not vary significantly over time within each treatment group, specific spectral features did show differences (Figure 2B). Chemical degradation rates and presence of metabolites in sediments depend on several factors including temperature, light, pH, organic matter content, bacterial communities, and anaerobic/aerobic conditions (Farghaly et al., 2013; Benskin et al., 2016). The presence of specific features in each treatment group suggests distinct metabolic responses to EMB exposure under abiotic versus biotic conditions, indicating that bacterial communities and metabolism might be influenced by EMB.

Comparative analysis of EMB and DES abundance showed no substantial variations over time under both conditions (Figures 3A and 3B). Further analysis did not reveal significant differences in detected metabolites (beyond a 5-fold change threshold) when comparing samples from abiotic and biotic conditions at each time point (Supplementary Figures 1A-C) or when aggregating them into two separate treatment groups (Supplementary Figure 1D). Statistical analysis using an unpaired two-sample t-test with equal variance supported these findings (p-value > 0.05; Supplementary Figures 2A-D). Additionally, volcano plots, which combine fold change and t-test results, also indicated no significant differences in EMB and DES abundance between abiotic and biotic conditions at each time point (Supplementary Figures 3A-C) or across the overall treatment groups (Supplementary Figure 3D). In contrast, other spectral features exhibited significant variations in abundance during the analysis (Supplementary Figures 1-3). However, these

features warrant further investigation because their levels were below the threshold required for selecting ions for MS2 spectra acquisition and library matching, which also prevented predictive analysis.

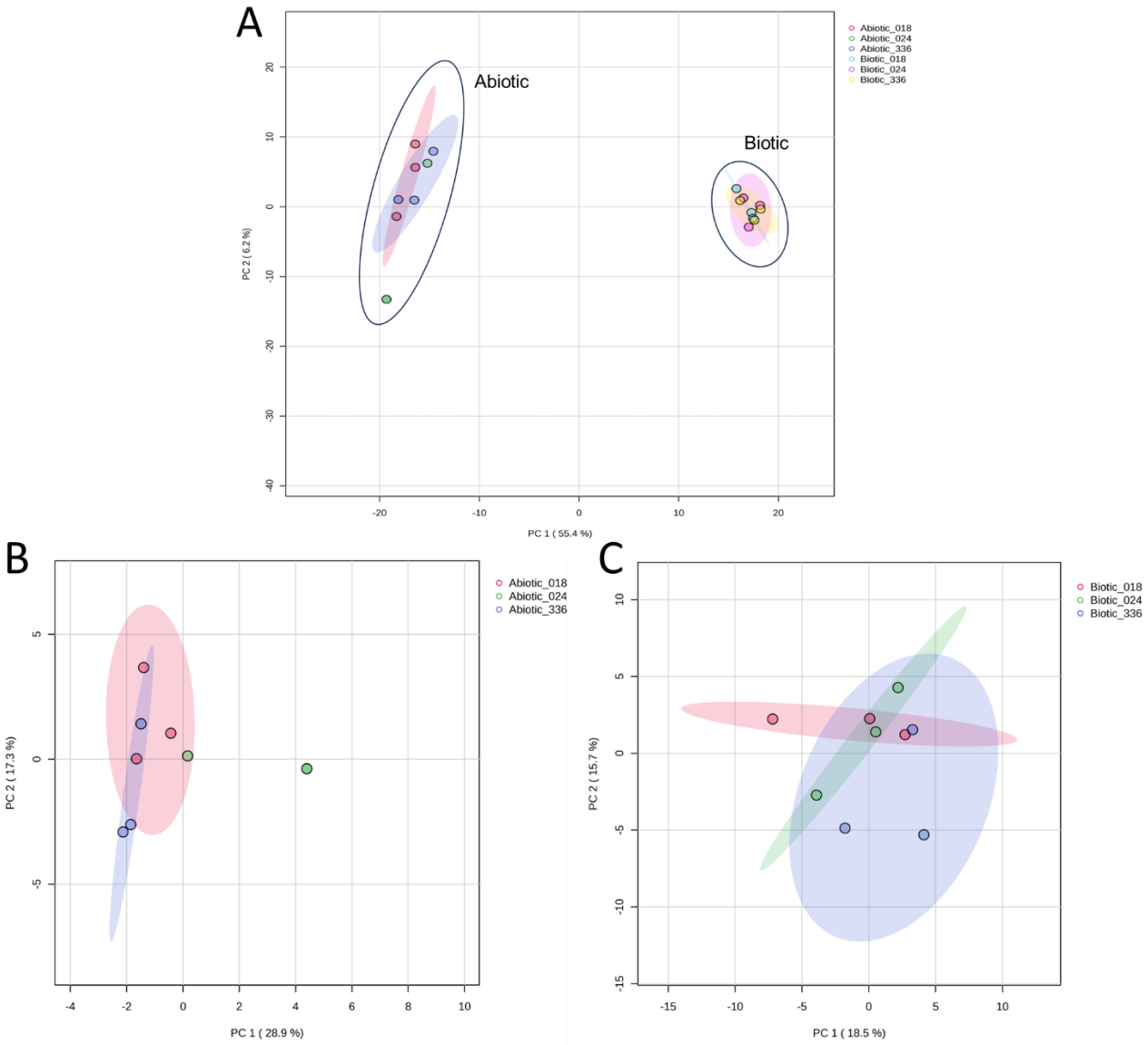


Figure 1. Principal Component Analysis (PCA) of EMB-spiked ocean sediments under abiotic and biotic conditions. The plots show clustering of overall metabolomic responses based on multivariate differences between the three time points (18, 24, and 336 hps) in the designated treatment groups (A) abiotic and biotic, (B) abiotic only, and (C) biotic only. All samples were analyzed in triplicate except for the 24 hours abiotic condition.

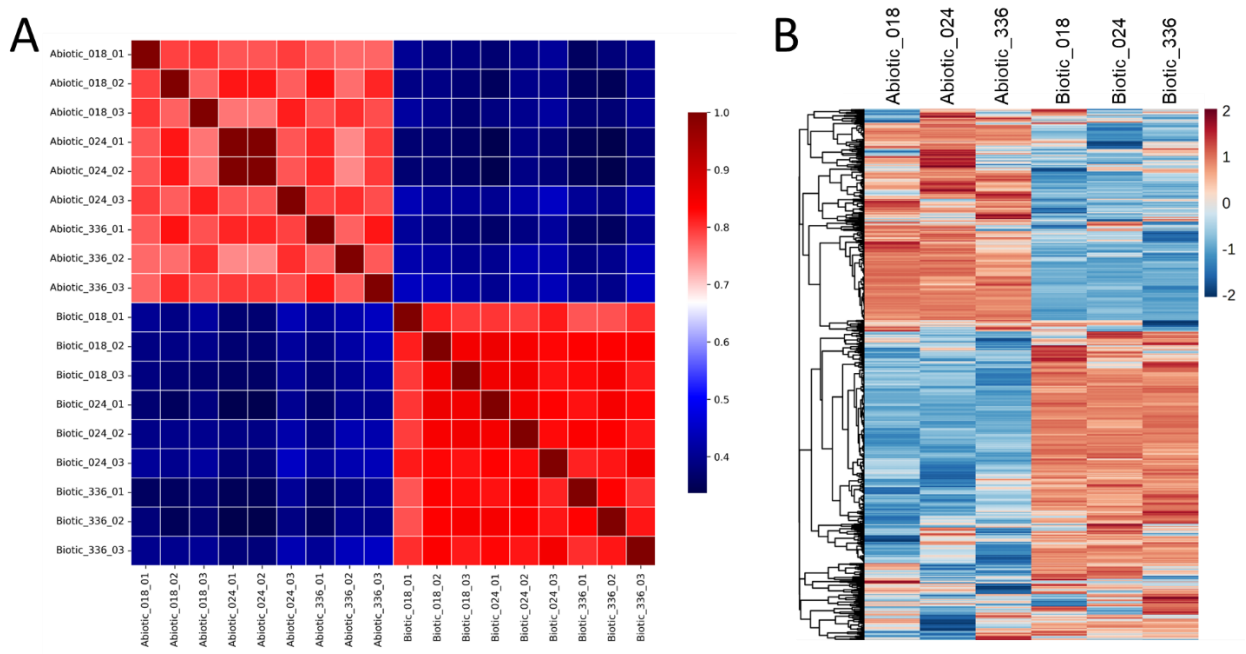


Figure 2. Heatmaps using Pearson correlations to illustrate differences in the abundance of spectral features in abiotic and biotic samples. (A) Overall metabolome correlations where each square represents a comparison between the respective samples. Three replicates for all time points from both treatment groups (except for the 24 hours abiotic condition which had only two) were used to create the heatmap. (B) Abundance of individual spectral features, where each line represents a spectral feature, and the tree shows the relationship between them. The average abundance of each feature from triplicate samples was used in the analysis. (A and B) The bars on the right of each heatmap represent the Pearson correlation coefficient legend. The samples are labelled with the treatment condition and time point of sample collection (18, 24, and 336 hps).

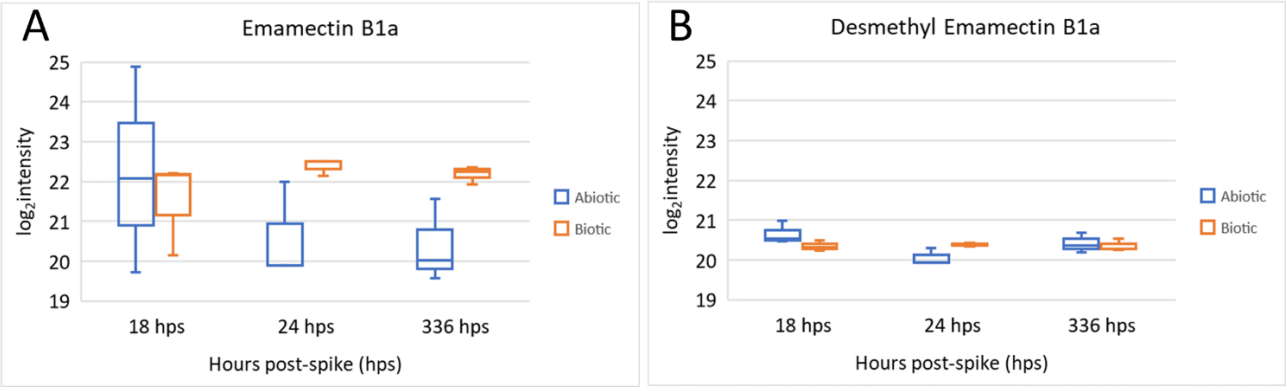
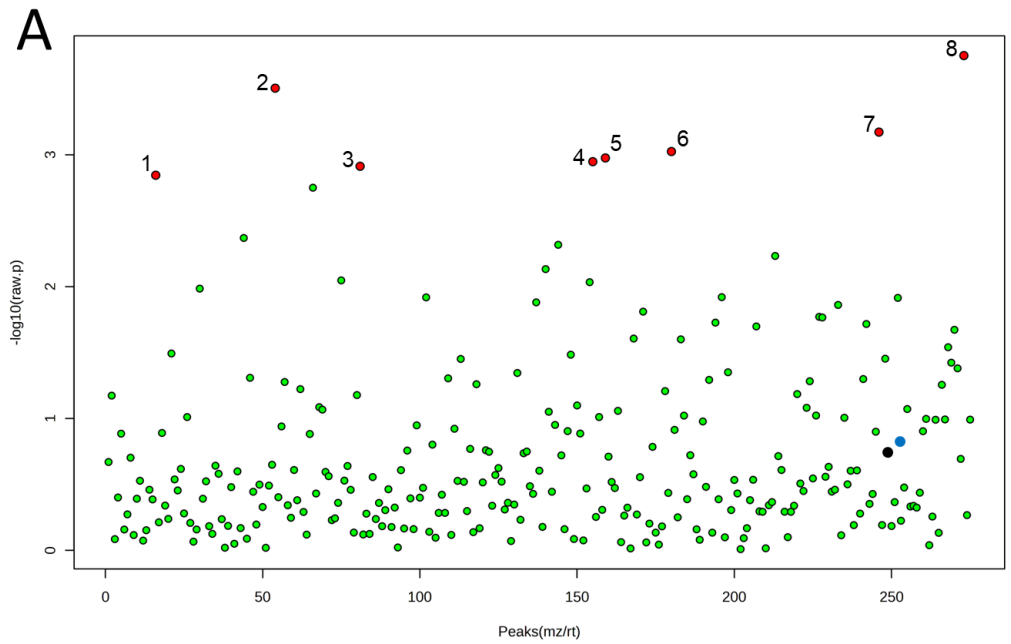
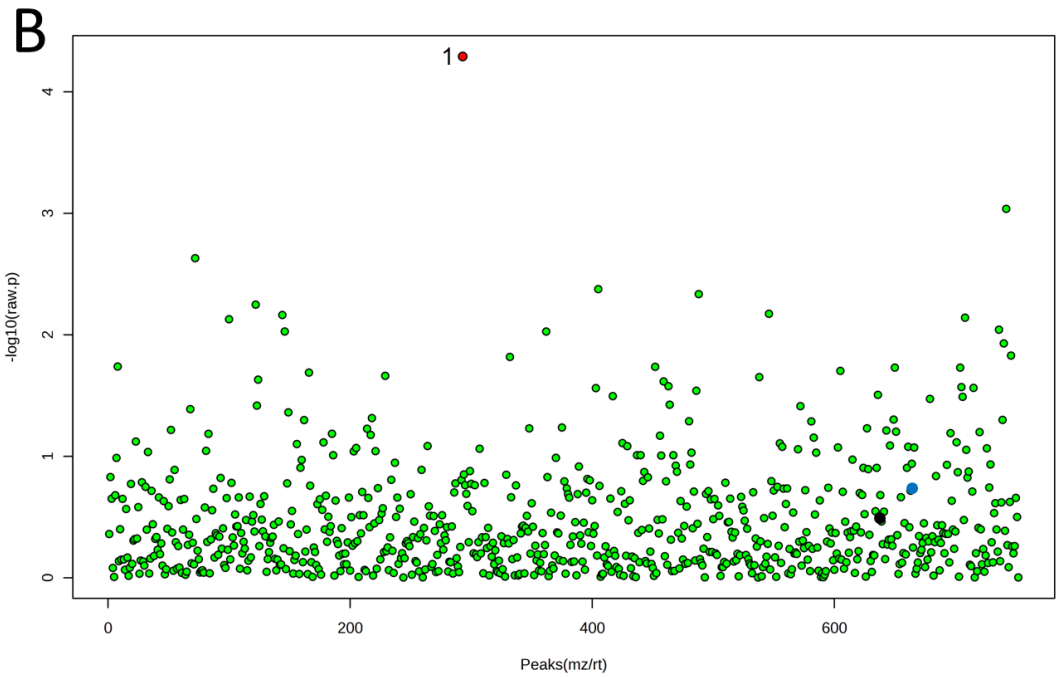


Figure 3. Comparison of abundance of (A) Emamectin B1a and (B) Emamectin B1b/Desmethyl Emamectin B1a in abiotic and biotic samples at each sampling time point. All samples were analyzed in triplicate except for the 24 hours abiotic condition.

Considering only abiotic samples, some spectral features exhibited significant differences in abundance using ANOVA across post-spike time points, after blank subtraction (T0) (Figure 4A). In contrast, the same analysis of biotic samples revealed only one spectral feature with significant changes in abundance over time (Figure 4B). Additionally, the top 25 varying spectral features across time points from both abiotic and biotic treatments, respectively, including those showing significant differences by ANOVA, were further analyzed using Pearson correlation (Figures 5A and 5B). Among these, five features continually increased in abundance over time under each condition considered separately (Figures 5A and 5B). It is important to note that not all these features demonstrated significant differences in the ANOVA analysis. These five features, along with those showing significant differences in each condition, were plotted with their respective abundance at three post-spike time points after blank subtraction (T0) (Figures 6A-L and 7A-E). The predicted molecular formulas for the top 25 features are listed in Supplementary Tables 1 (abiotic) and 2 (biotic), although further structural characterization was not possible in the current study.



Feature	m/z	Retention time (min)	Designation	Predicted Molecular Formula
1	142.933	0.5845	Ab1	$C_3HOClNaS$
2	269.054	13.902	Ab2	$C_{13}H_9ON_3Na_2$
3	297.083	4.3542	Ab3	$C_{10}H_{23}N_2KNaS_2$
4	452.251	5.7322	Ab4	No Prediction
5	462.293	5.2743	Ab5	No Prediction
6	538.242	7.7957	Ab6	$C_{31}H_{10}ON_{10}$
7	790.586	7.5337	Ab7	$C_{15}H_2O_9N_2Br_2ClKNa_2PS_4$
8	1261.8	9.318	Ab8	$C_{74}H_6O_6N_2BrK_2NaS_2$



Feature	m/z	Retention time (min)	Designation	Predicted Molecular Formula
1	389.3403	9.159	Bio1	No Prediction

Figure 4. ANOVA analysis of the abundance of individual spectral features in (A) abiotic and (B) biotic samples. All three time points were compared after subtracting the blank, where the red and green circles depict spectral features showing significant (p-value < 0.05) and non-significant differences in abundance, respectively. The blue and black dots represent Emamectin B1a and Desmethyl Emamectin B1a, respectively. The table at the bottom provides information on significantly different features depicted by red circles, which are marked with a number.

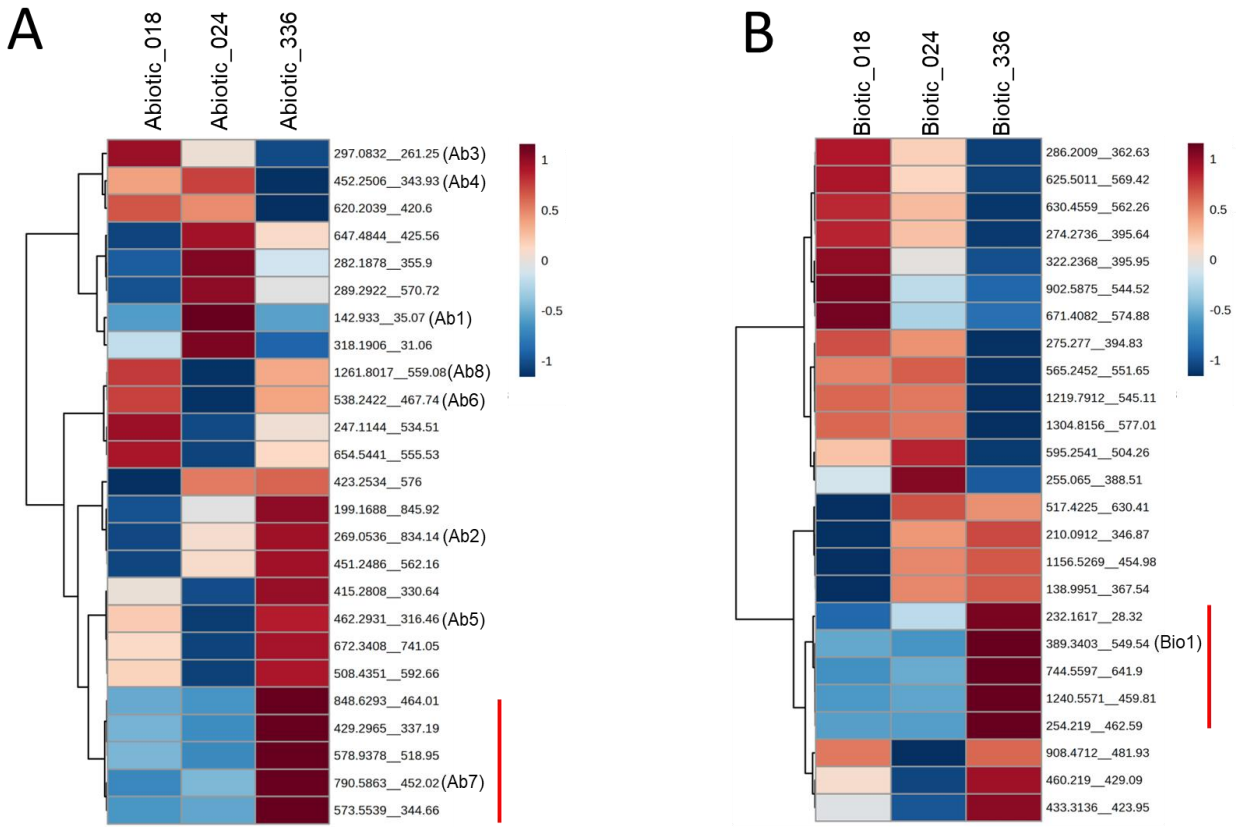
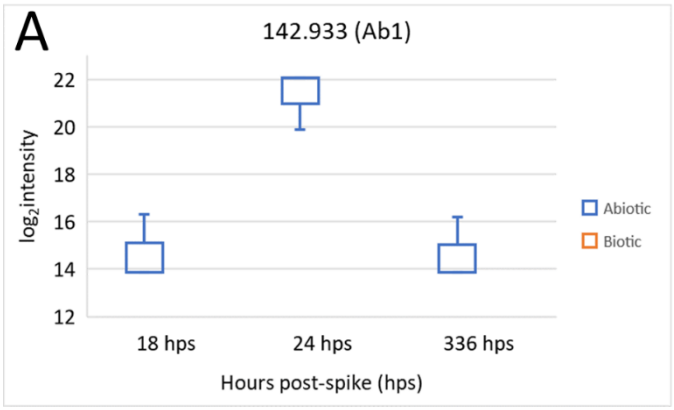
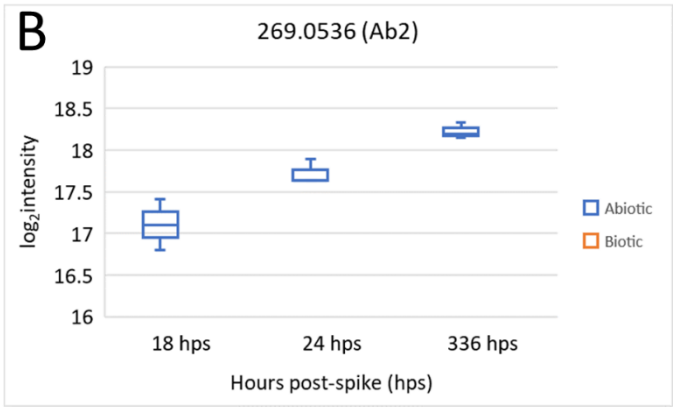
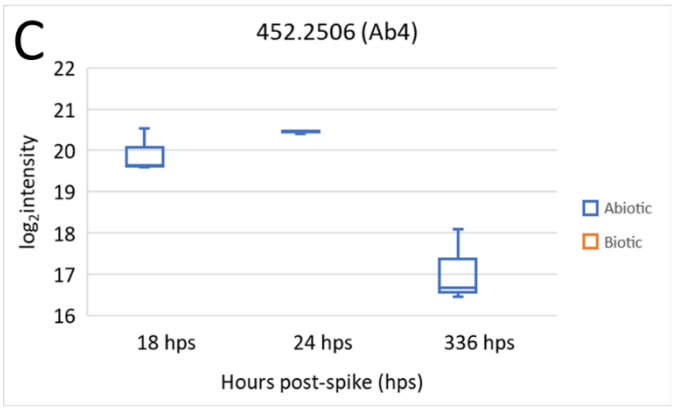
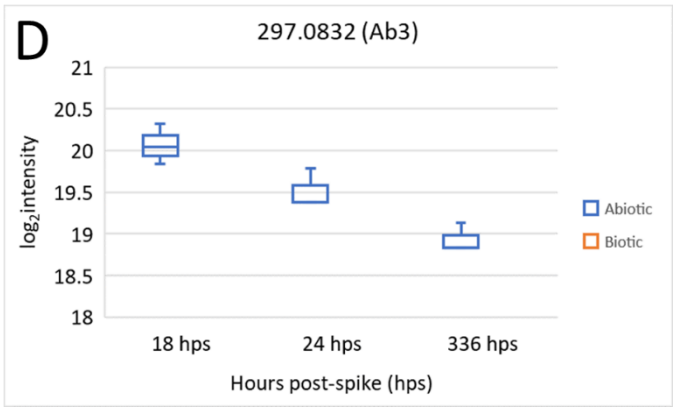


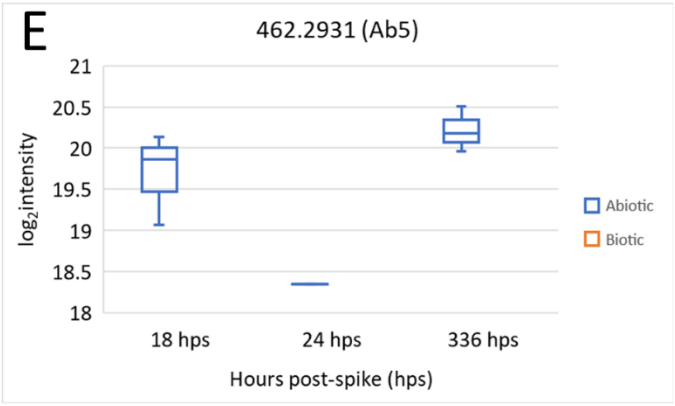
Figure 5. Heatmap of top 25 spectral features displaying differences in abundance in the three-time points examined (18, 24 and 336 hps) under (A) abiotic and (B) biotic conditions. The samples are labelled with the treatment condition and time point of sample collection (18, 24 and 336 hps). The m/z, retention time (separated by underscore) of each spectral feature, and the legend bar indicating the Pearson coefficient are shown on the right. The average abundance of each feature from triplicate samples was used in the analysis. Spectral features whose abundance is increased after 336 hps as compared to 18 and 24 hps are indicated by the red line, whereas those that showed significant differences in abundance through ANOVA analysis were given specific designations (shown in parenthesis).

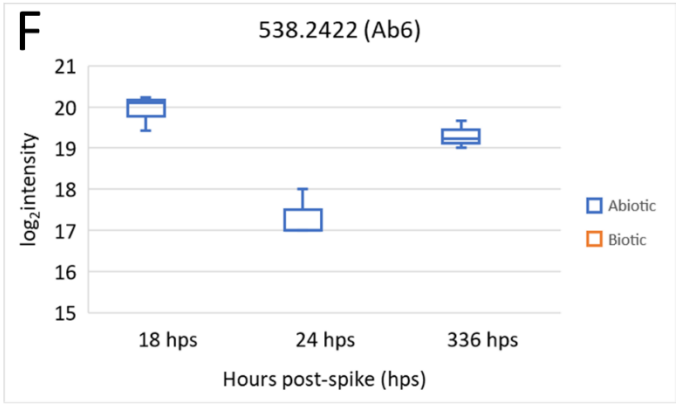

A
142.933 (Ab1)
log2intensity
22
20
18
16
14
12
18 hps
24 hps
336 hps
Hours post-spike (hps)
Abiotic
Biotic


B
269.0536 (Ab2)
log2intensity
19
18.5
18
17.5
17
16.5
16
18 hps
24 hps
336 hps
Hours post-spike (hps)
Abiotic
Biotic


C
452.2506 (Ab4)
log2intensity
22
21
20
19
18
17
16
18 hps
24 hps
336 hps
Hours post-spike (hps)
Abiotic
Biotic


D
297.0832 (Ab3)
log2intensity
21
20.5
20
19.5
19
18.5
18
18 hps
24 hps
336 hps
Hours post-spike (hps)
Abiotic
Biotic


E
462.2931 (Ab5)
log2intensity
21
20.5
20
19.5
19
18.5
18
18 hps
24 hps
336 hps
Hours post-spike (hps)
Abiotic
Biotic


F
538.2422 (Ab6)
log2intensity
21
20
19
18
17
16
15
18 hps
24 hps
336 hps
Hours post-spike (hps)
Abiotic
Biotic

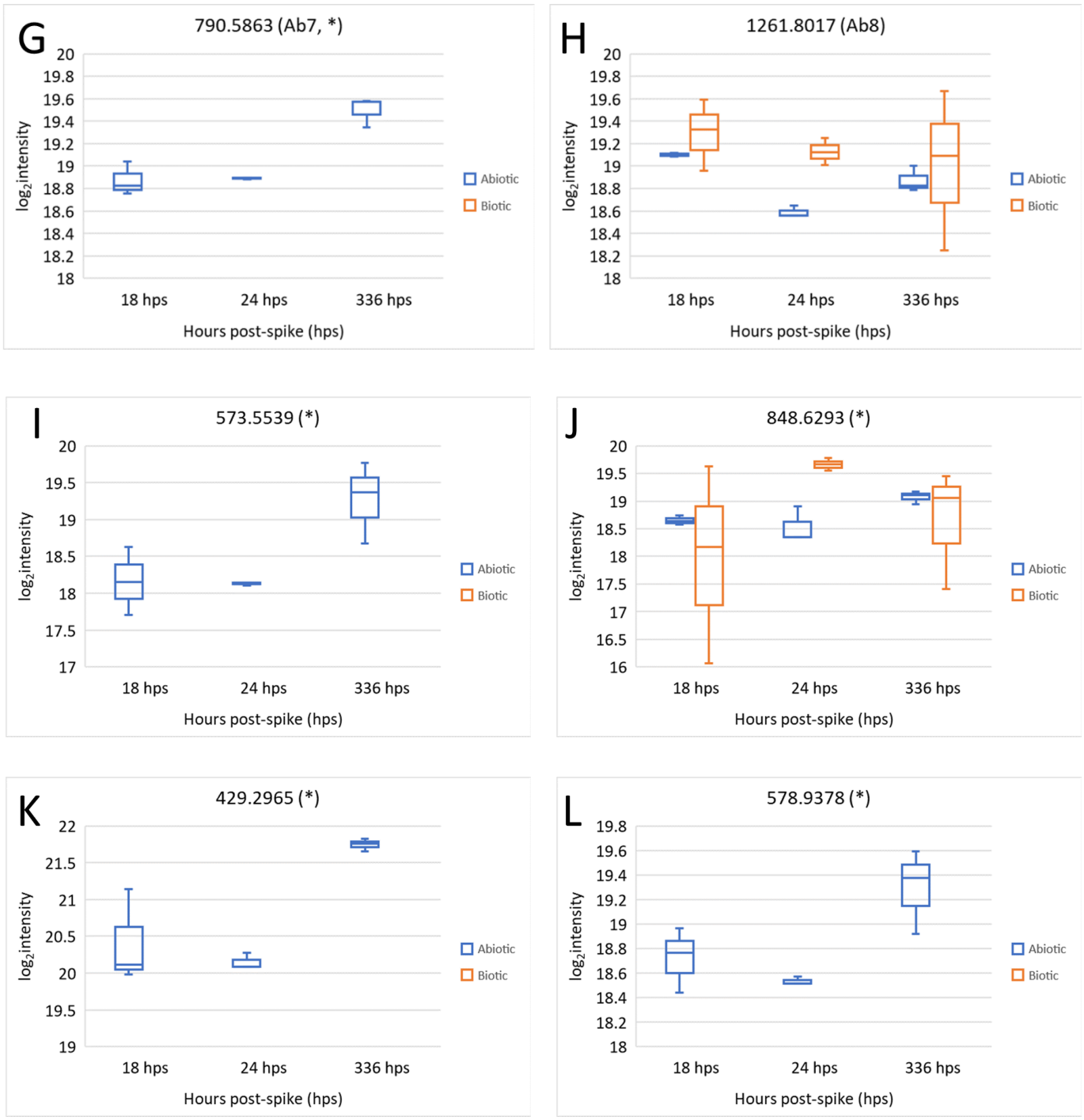


Figure 6. Changes in the abundance of twelve spectral features discussed in Figure 5A under abiotic and biotic conditions. (A-H) Spectral features whose abundance is increased after 336 hps as compared to 18 and 24 hps are indicated by (*). (G, I-L) spectral features that showed significant differences in abundance through ANOVA analysis and were given specific designations (shown in parenthesis). All the samples were analyzed in triplicate except for the 24 hours abiotic condition.

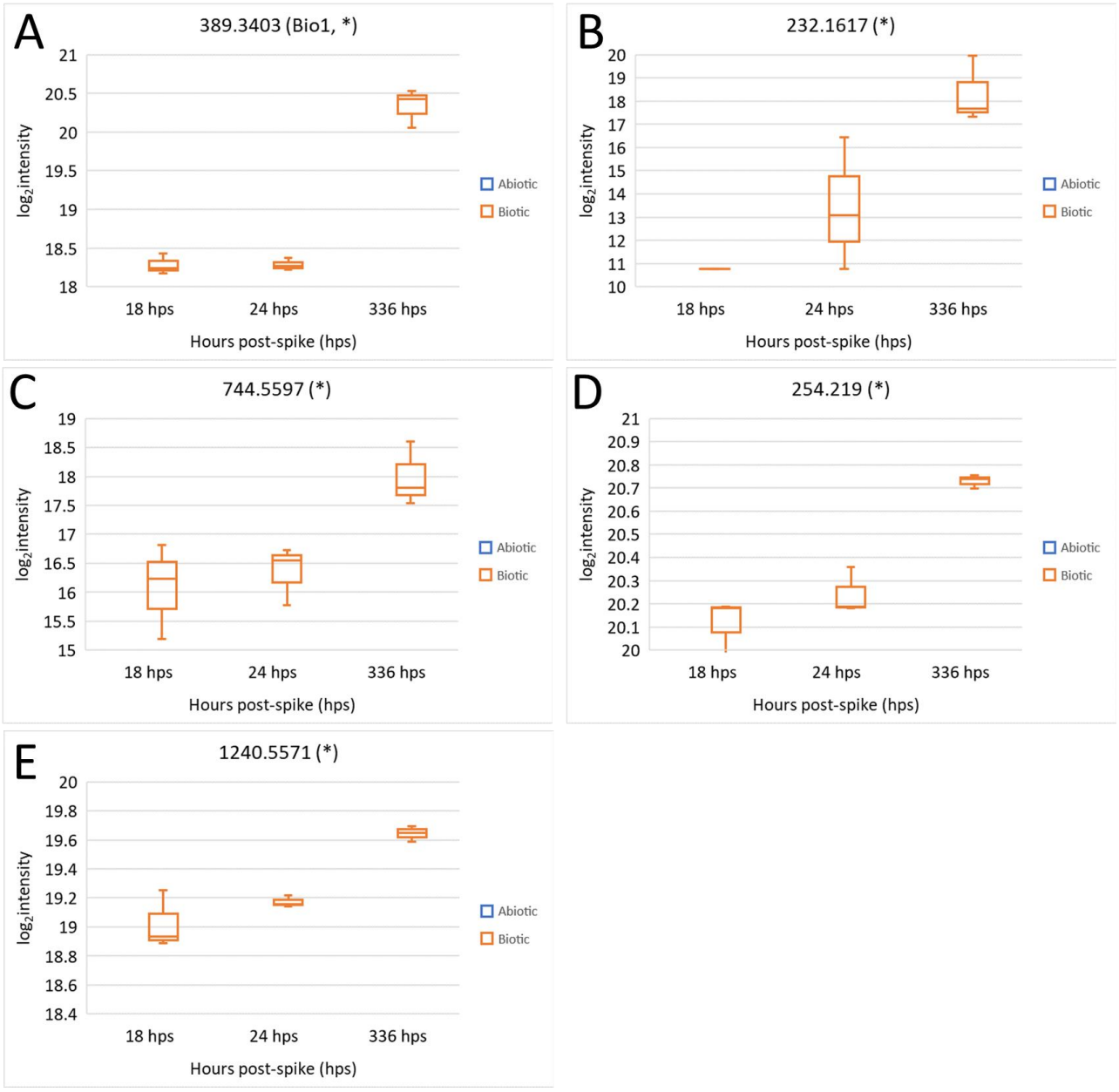


Figure 7. Changes in the abundance of five spectral features discussed in Figure 5B under abiotic and biotic conditions. (A-E) Spectral features whose abundance is increased after 336 hps as compared to 18 and 24 hps are indicated by (*). (A) Spectral features that showed significant abundance differences through ANOVA analysis were given specific designations (shown in parenthesis). All the samples were analyzed in triplicate except for the 24 hours abiotic condition.

In summary, sediments spiked with Emamectin benzoate under both abiotic and biotic conditions showed differential metabolomic responses with time when considered separately. While some compounds, such as EMB and DES, remained unchanged, other spectral features exhibited significant variations, highlighting the need for more in-depth analysis. The features observed in abiotic samples could potentially represent EMB degradation products other than DES. However,

for biotic samples it is currently unknown whether features result from the metabolism of EMB or from changes in microbial and other organismal communities. Further studies using matched spiked and un-spiked samples under both biotic and abiotic conditions, along with lower thresholds for MS2 spectra acquisition, are recommended to provide more in-depth information.

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