# Discrimination of Marine Polychaete Species of Different Harvest Times Using FTIR Metabolomics

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Marine polychaetes have gained prominence in the study of their potential bioactivities and structurally intriguing compounds. However, studies on the chemical profile of polychaete species are still lacking. This study aimed to conduct the chemical profile of marine polychaete species subjected to different harvest times using Fourier transform infrared (FTIR) metabolomics and proton nuclear magnetic resonance ($^1$H NMR) analysis. In this study, two marine polychaetes, namely *Diopatra claparedii* and *Marphysa moribidii*, were collected and analyzed. Multivariate data analysis of the FTIR data via orthogonal partial least squares discriminant analysis (OPLS-DA) revealed four distinct clusters of the polychaetes were formed based on their different harvest times. Furthermore, organic acids, fatty acids, and aromatic compounds were found to be important in discriminating the polychaete species based on $^1$H NMR characteristic signals. FTIR metabolomics results suggest that the first and second quarters of the year are the best harvesting times due to their chemical profile consistency. FTIR metabolomics is a useful preliminary analytical tool to discriminate polychaete samples from different harvest times and predict new batches of samples. These results provide basic information on the metabolite variations in polychaetes from different species and harvest times, which might be useful for future applications (e.g., drug discovery and climate change study).

**Keywords**: Marine polychaetes; chemical profile; FTIR metabolomics; $^1$H NMR; model prediction

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Polychaete from phylum Annelida is a marine organism with enormous morphological diversity in the marine environment [1]. About 12,654 valid species have been discovered in 2023 that have a lot of potential as a new scientific development [2]. Different species of polychaetes have different behaviour, most living under rock or sediment as errantia species, while some gather materials to make permanent tubes (sedantaria) [3]. They also constitute a significant part of the ecological process through endo and epibenthos in marine environments.

The potential of marine polychaetes as a source of natural products has not been fully discovered. Other marine invertebrates, including sponges and molluscs, are gaining more attention compared to polychaetes [4]. Previously, antimicrobial and anti-oxidant activities have been found in marine polychaetes [5, 6]. Halogenated aromatics are the primary isolated substances in marine polychaetes [4]. A protein complex is also related to the marine organism system implicated in diseases such as inflammation [7]. This protein is also involved in the immune response activated by their environment's biotic and abiotic factors. Different environmental approaches, such as UV radiation, salinity, pH acclimatization, and symbiotic interaction, influenced complex proteins [8].

In addition, rising temperatures during different seasons in the 20th century have also impacted the environment [9]. Meanwhile, the chemical compositions of marine organisms, including proteins and fatty acids, can be influenced by light and temperature [10]. This implies different harvest times of polychaetes can be a crucial factor in their chemical constituents. Besides, documented seasonal variations of chemical constituents in marine polychaetes are poorly

discovered. Methods such as metabolomics can help comprehend polychaetes' interactions in different environments.

Many analytical tools can provide a detailed characterization of phenotypes and chemical structures to improve the range of metabolomic studies [11]. Metabolomics can also unravel the potential of natural products, such as metabolite fingerprints, metabolic changes, and toxicity, to be amplified as therapeutic agents [12]. Spectroscopy techniques are common tools used for metabolomics. For example, liquid chromatography (LC) has the flexibility to combine with other detection techniques, including nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) [11].

Previously, ATR-FTIR metabolomics was successfully applied to discriminate the chemical profile of two Malaysian marine polychaetes, namely *Diopatra claparedii* and *Marphysa moribidii* [6]. FTIR technique is a fast and cost-effective method [13]. In this study, the chemical profile of *D. claparedii* and *M. moribidii* subjected to different harvest times was investigated using ATR-FTIR metabolomics and $^{1}$H NMR analysis. The outcomes and methodological approaches used in this study may provide valuable information for future research and applications, such as drug discovery and climate change studies.

## EXPERIMENTAL

### Chemicals

The chemicals used for extraction and FTIR analysis were analytical-grade solvents, including methanol and acetone. Deuterated methanol ($CD_3OD$, 99.8 %) and 3-(trimethylsilyl)propionic-2,2,3,3 acid (TSP) sodium salt were used for $^{1}$H NMR analysis. All chemicals were purchased from Sigma Aldrich (Darmstadt, Germany).

### Sample Collection and Preparation

Two different species of polychaete samples, *D. claparedii* and *M. moribidii*, were collected from the Morib mangrove area at Selangor, Peninsular Malaysia (2°45'39"N 101°26'09"E). Both species were collected at four different times: batch 1 (April 2021), batch 2 (October 2021), batch 3 (May 2022), and batch 4 (February 2023). The habitat of the species *D. claparedii* is in the lower tidal flat zone area of the local mangrove during low tide, while *M. moribidii* is found in the upper tidal flat zone. After collection, the polychaete samples were rinsed with tap water to remove soil and then subjected to freeze drying to remove excess water. The freeze-dried samples were ground into powder and stored in a freezer (-80°C) prior to analysis. In this study, six biological replicates were used from each polychaete sample for the analysis.

### Sample Extraction

Approximately 100 mg of freeze-dried sample in powder form was extracted with 1 mL of methanol at a ratio of 1:10 (w/v) and sonicated for 30 min at room temperature. The supernatant was then filtered using a 0.45 µm PTFE membrane filter. The extraction procedure was repeated three times, resulting in six individual extractions for each polychaete species to obtain six biological replicates. The samples were concentrated using a miVac centrifugal concentrator. The dried extracts were subsequently stored in the freezer (4 ºC) prior to analysis.

### ATR-FTIR Analysis

Fourier transform infrared (FTIR) analysis was conducted in IRTracer-100 Shimadzu coupled with a MIRacle attenuated total reflection (ATR) accessory. The ATR crystal was cleaned, and the infrared background spectrum was first collected before the analysis. About 10 µL of the sample (30 mg/mL, acetone) was applied onto the crystal, and the measurement was started. The FTIR spectrum was recorded between the wavelengths of 4000–600 $cm^{-1}$ with 40 interferograms at a resolution of 4 $cm^{-1}$. Six biological replicates and three technical replicates were performed for all analyzed samples. All spectra data were converted to ASCII files before the multivariate data analysis.

### $^{1}$H NMR Analysis

Sample preparation was done by adding 700 µL of deuterated methanol ($CD_3OD$) containing 0.1% of 3-(trimethylsilyl)propionic-2,2,3,3 acid (TSP) sodium salt to 20 mg of sample. The sample was vortexed and centrifuged at 13000 rpm for 10 min. The supernatant was then transferred to an NMR tube (17.78 cm × 5 mm × 4.2 mm) and submitted for $^{1}$H NMR analysis. $^{1}$H NMR analysis of the different batches of polychaete extracts was conducted on a 400 MHz Bruker NMR machine. The parameters used in this $^{1}$H NMR analysis included a pulse width (PW) of 21.0 µs (90°), relaxation delay (RD) of 2.0 s, and number of scans of 256. $^{1}$H NMR spectral pre-processing was done using MNOVA software version 6.0, which included baseline correction and phasing. Spectral alignment was done according to the TSP signal at 0.00 ppm. Metabolite identification was carried out using Chenomx Profiler software version 7.62, which was further confirmed by comparing the $^{1}$H NMR signals of the identified compounds with an available database at https://hmdb.ca/ and published literature.

### Multivariate Data Analysis

The multivariate data analysis (MVDA) of FTIR spectral data was carried out using SIMCA-P 14.1 (Umetrics AB, Umeå, Sweden), employing a MVDA model such as orthogonal partial least squares

discriminant analysis (OPLS-DA). The OPLS-DA model was used to determine the chemical profile and prediction of the batches of samples, respectively. For model prediction purposes, 36 samples (batch 1) from *D. claparedii* and *M. moribidii* were used as the training dataset, while 24 samples (batch 3 and 4) from *D. claparedii* and *M. moribidii* were used as the testing dataset.

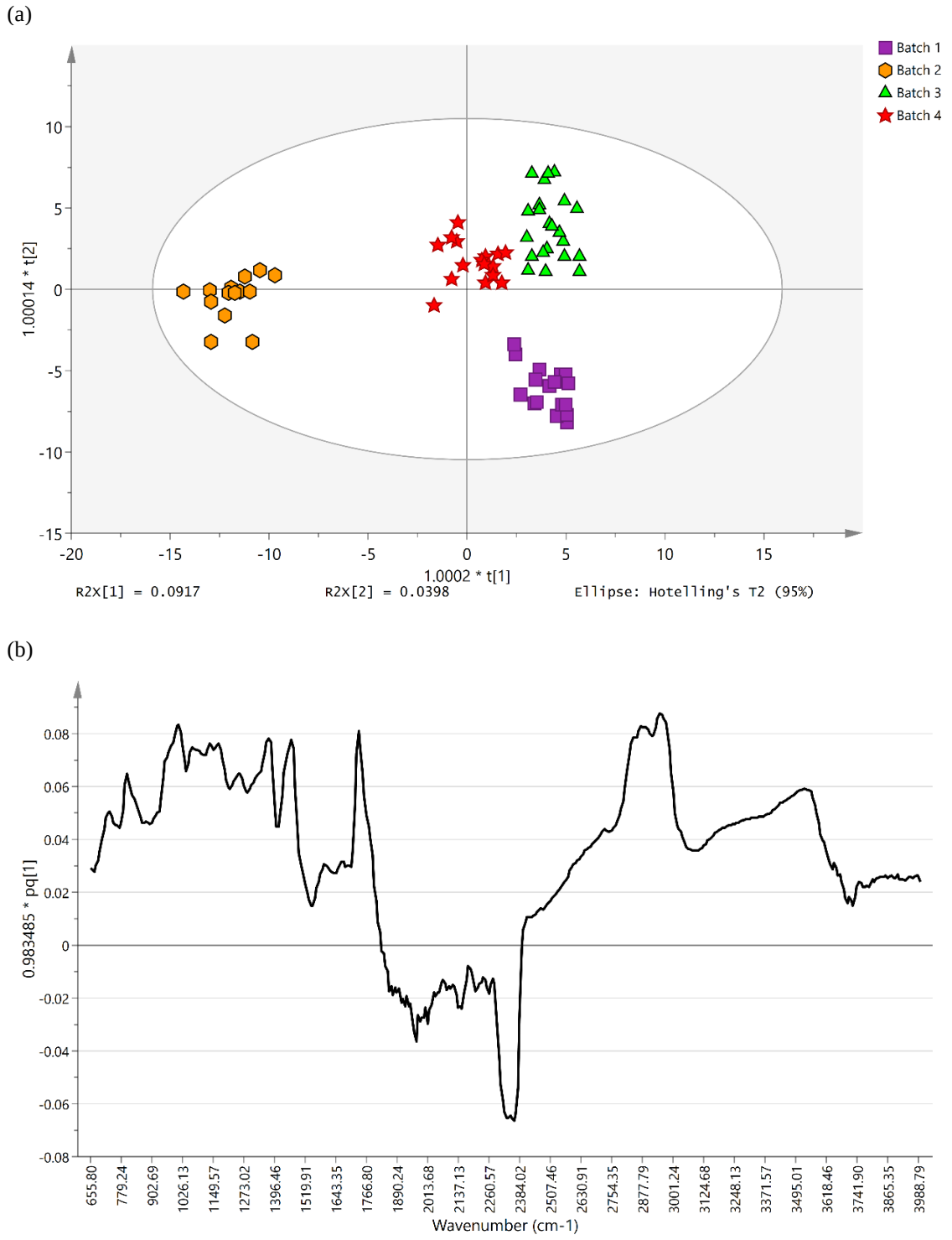



**Figure 1.** OPLS-DA scores (a) and loadings (b) plots of the FTIR data of *D. claparedii*.

## RESULTS AND DISCUSSION

### Chemical Profile of *D. claparedii* at Different Harvest Times

In this study, the chemical profile of four batches (batch 1 in April 2021, batch 2 in October 2021, batch 3 in May 2022, and batch 4 in February 2023) of *D. claparedii* samples was evaluated using the OPLS-DA model (Figure 1). The OPLS-DA showed cumulative $R^2X$, $R^2Y$ and $Q^2$ values of 0.999, 0.925, and 0.815, respectively, indicating good model performance [14]. Further model validation was conducted using permutation tests and CV-ANOVA. The permutation tests (200 permutations) resulted in $R^2$ values ranging from 0.336 to 0.339 and $Q^2$ values ranging from -0.743 to -0.854, while the CV ANOVA test showed statistical significance (P-value < 0.001) (Supplementary Figure S1 and Table S1). This indicates the validity and reliability of the OPLS-DA model.

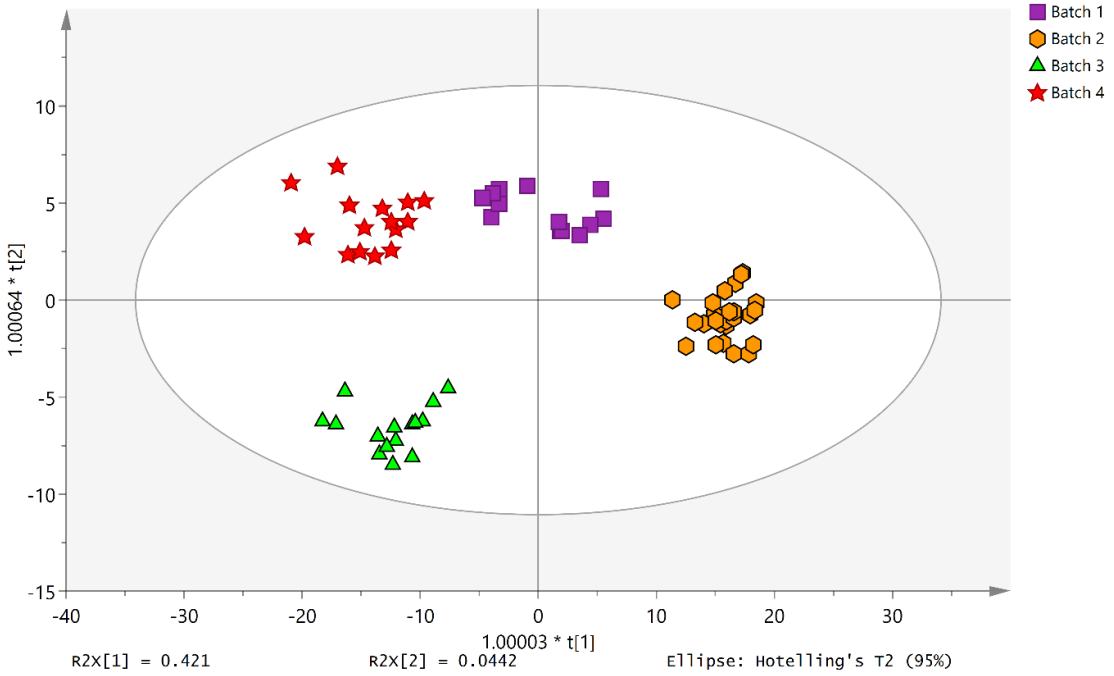
The OPLS-DA scores plot displays four distinct clusters representing different batches of *D. claparedii* samples, indicating metabolite variations in different harvest times (Figure 1a). Samples of batch 2 were situated far from other batches on the left side of the OPLS-DA scores plot, as indicated by PC1. Meanwhile, samples from batch 1, 3, and 4 were relatively close to each other on the right side of the OPLS-DA scores plot. The separation between batch 1 from batch 3 and batch 4 was also shown by PC2, indicating a slight metabolite variation between them. According to the OPLS-DA loadings line plot (Figure 1b), most of the FTIR signals (at the upper positive side) were prominent in batch 1, 3, and 4, harvested in February, April, and May, respectively. In contrast, batch 2, harvested in October, showed the least prominent FTIR signals (at the lower negative side). The metabolite variations of different batches were also observed on their FTIR spectra (Figure S2).

### Chemical Profile of *M. moribidii* at Different Harvest Times

Next, the evaluation of the chemical profile of four batches (batch 1 in April 2021, batch 2 in October 2021, batch 3 in May 2022, and batch 4 in February 2023) of *M. moribidii* was conducted, and the results are shown in Figure 2. The OPLS-DA used in this study showed cumulative $R^2X$, $R^2Y$ and $Q^2$ values of 0.992, 0.943, and 0.925, respectively, indicating an excellent model. Validation via permutation tests and CV-ANOVA further confirmed the validity of the OPLS-DA model. The permutation tests (200 permutations) showed that the $R^2$ values of the model ranged from 0.14 to 0.124, while the $Q^2$ values ranged from -0.393 to -0.423. The CV ANOVA test further confirmed the validity of the model, demonstrating statistical significance (P-value < 0.001) (see Supplementary Figure S3 and Table S2).

Based on the observation of the OPLS-DA scores plot, samples from batch 2 were also far from the other three batches by PC1, indicating differences in their chemical compositions (Figure 2a). It's worth noting that batch 3 exhibits slight metabolite variations from batches 1 and 4, as observed in the PC2 scores plot. According the OPLS-DA loadings line plot, the wavenumbers at 2878-2900 $cm^{-1}$ (CH stretching) and 1396-1400 $cm^{-1}$ (CH bending) corresponding to fatty acids [15] were more prominent in batch 2, while the wave-numbers at 3495-3618 $cm^{-1}$ (OH stretching) and 1026 (CO stretching) probably attributed to aromatic compounds [16] were more intense in other batches (Figure 2b). The FTIR spectra of different batches of *M. moribidii* are shown in Supplementary Figure S4. In addition, the chemical profile of *M. moribidii*, which was subjected to different harvest times, showed a similar trend to the chemical profile of *D. claparedii*. These findings suggest that harvest time in the first and second quarters of the year results in a more consistent chemical profile compared to the fourth quarter. Polychaetes are one of the dominant benthic groups that live in muddy estuarine environments and are extremely influenced by wet and dry seasons. Polychaetes highly adapt to large fluctuations in salinity during these different seasons. During the wet season, late-flood salinity is low and increases in the dry season [17]. These biotic and abiotic influences by seasonal changes significantly affect their allelochemicals [18].

(a)




(b)

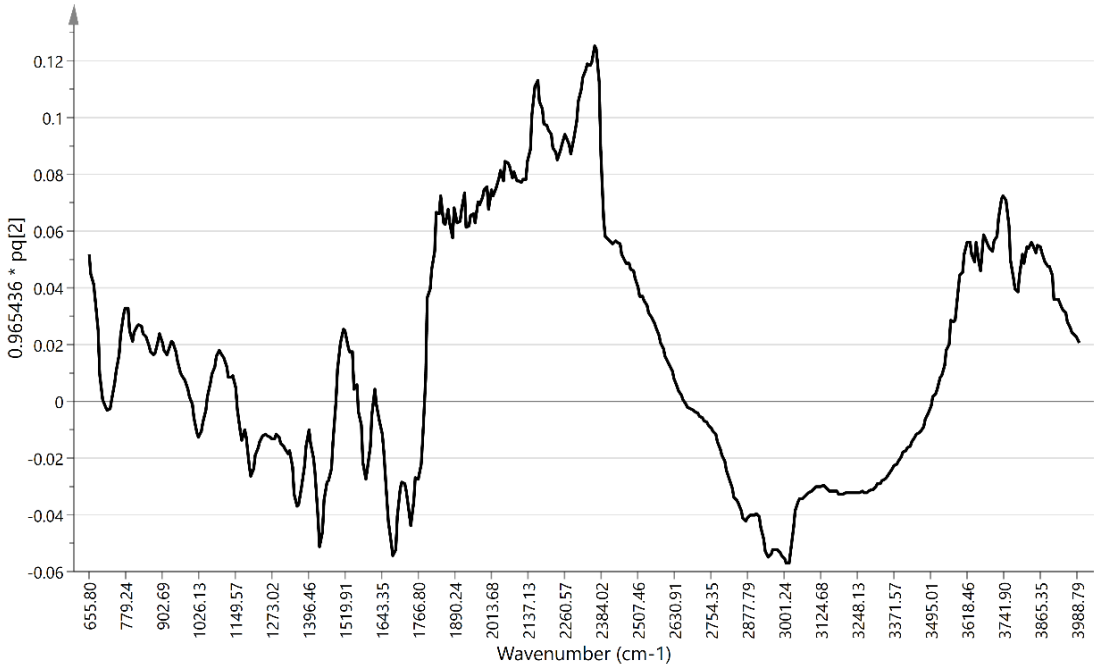



**Figure 2.** OPLS-DA scores (a) and loadings (b) plots of the FTIR data of *M. moribidii*.

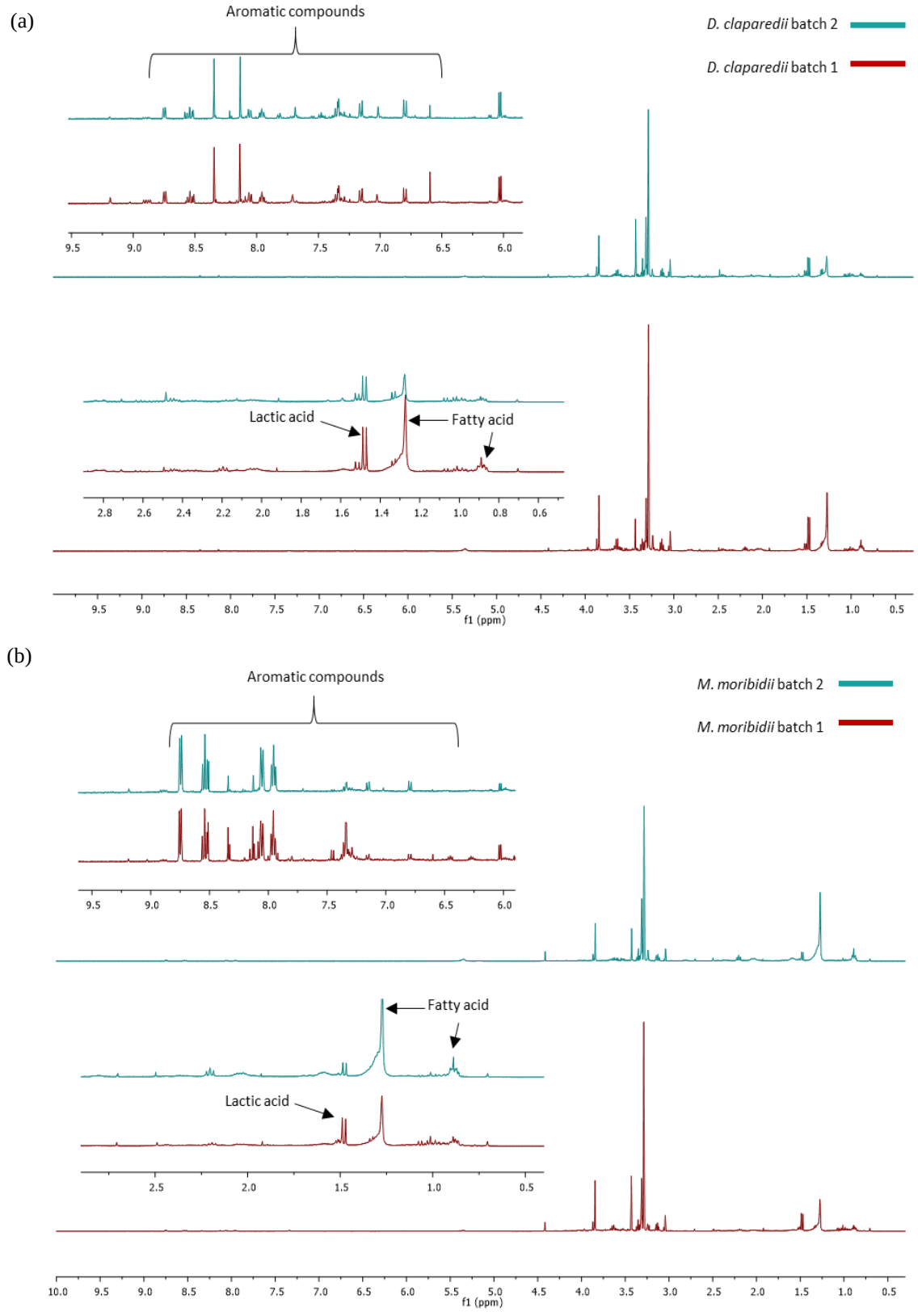



**Figure 3.** $^{1}$H NMR spectra of different batches of *D. claparedii* (a) and *M. moribidii* (b). Batch 1: April 2021; Batch 2: October 2021.

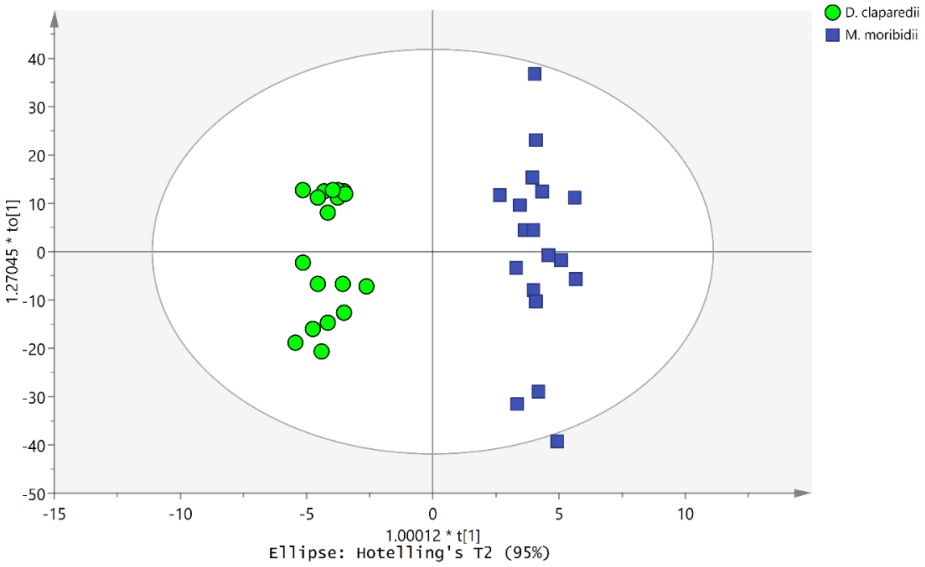
## $^{1}$H NMR Analysis of Polychaete Species

Although FTIR metabolomics successfully discriminated the significant batches (1, 3, and 4) from batch 2, the information is limited to the chemical fingerprint related to its functional groups. Thus, to better understand the influence of different harvest times, $^{1}$H NMR spectroscopy was used to

compare metabolite variations in representative samples from the significant batch 1 (April 2021) and batch 2 (October 2021). The $^{1}$H NMR spectra showed characteristic signals of several classes of metabolites that can be found in the polychaetes, including amino acids, organic acids, fatty acids, sterols, and aromatic compounds. The results clearly showed that fatty acids (δH 1.28-1.32 and 0.87 ppm) [19] and lactic acid (δH 1.32 ppm) [20] were more prominent in *D. claparedii* sample of batch 1 compared to batch 2 (Figure 3a). High precipitation during the wet season in batch 2 (October) may decrease salinity concentration in the environment. Polychaetes have a significant response to changes in environmental salinity that may affect their metabolic processes [21]. In response to low salinity, polychaete mortality rate and stress increase [22]. Some marine organisms at lower salinity levels have lower fatty acid concentrations than those in higher salinity environments [23].

The signals of aromatic compounds (δH 6.00-9.00 ppm) [5] were found clearly in *D. claparedii*; however, a similar pattern of aromatic compounds was observed in batch 1 and batch 2 (Figure 3a). Interestingly, *M. moribidii* samples from batch 1 exhibited more aromatic compound signals in the range of 7.00-7.50 ppm compared to batch 2 (Figure 3b). Aromatic compounds could be potential chemical markers of *M. moribidii*. Observation on the $^{1}$H NMR spectra of *M. moribidii* showed that the sample from batch 1 had higher levels of fatty acids, while batch 2 had higher lactic acid (Figure 3b). Various classes of metabolites detected in $^{1}$H NMR spectra of polychaetes, such as aromatic compounds, fatty acids, and organic acids, might have the potential for applications in drug discovery and as indicators of climate change.

(a)




(b)

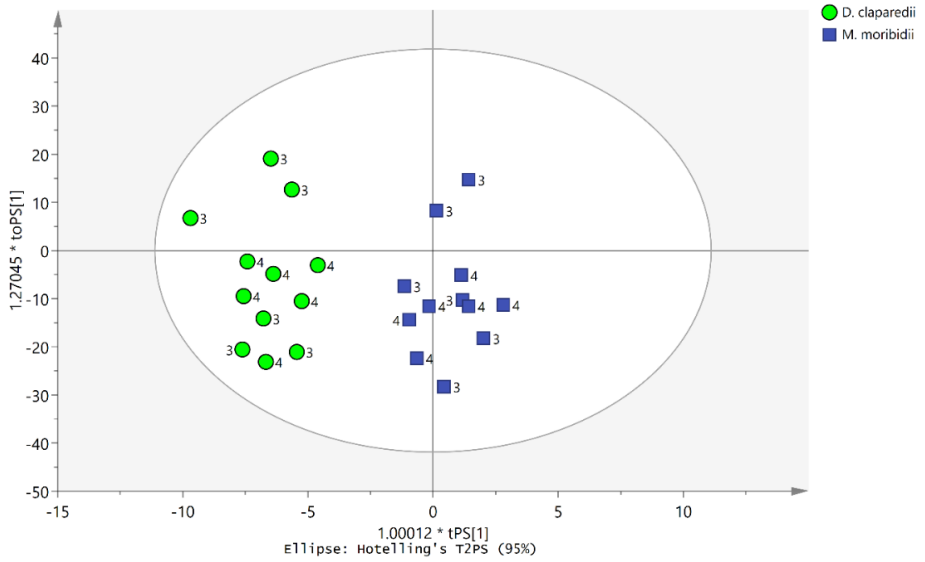



**Figure 4.** OPLS-DA scores plot of FTIR training dataset (a) and testing dataset (b) of polychaete species.

**OPLS-DA Model for the Prediction of New Batches of Polychaete Species**

Multivariate data analysis, commonly used for classification, is also useful and capable of prediction purposes [21]. In this study, the OPLS-DA model was developed to predict the chemical profile of new batches of two polychaetes (*D. claparedii* and *M. moribidii*) samples harvested in the first and second quarters of the year. First, samples from batch 1 (April 2021) were used as training datasets. The model's performance on the training dataset, including cumulative $R^2X$, $R^2Y$ and $Q^2$ values, showed excellent results of 0.997, 0.970, and 0.934, respectively, indicating a validated model. The OPLS-DA scores plot of the training dataset successfully separated the polychaetes according to their species. However, one of the samples from *M. moribidii* species was situated outside of the confident ellipse (95%) (Figure 4a).

Further evaluation showed no indication of strong outliers according to Hotelling's T2 plot (Figure 5a), although the DModX plot analysis suggested the presence of one potential moderate outlier in the samples (Figure 5b). Permutation test results also confirmed the validation of the OPLS-DA model on the training dataset (Figure 5c and Figure 5d). Subsequently, the OPLS-DA model was tested to predict the two polychaetes' chemical profiles of batch 3 (May) and batch 4 (February). The testing dataset of *D. claparedii* formed the same cluster as the training dataset, indicating good capability in model prediction. The testing dataset of *M. moribidii* also clustered in a similar position as the training dataset; however, some samples were shifted to the left side of the quadrant of the OPLS-DA scores plot (Figure 4b). Overall, the developed OPLS-DA model is considered good and reliable for predicting new batches of polychaetes samples.

To evaluate the important functional groups that contribute to the separation of the two polychaete species, as shown in Figure 4, an OPLS-DA loading line plot was used (Figure 6). Based on the observation, some characteristic signals of fatty acids (CH stretching and CH bending) and aromatic compounds (OH stretching and C=C bending) were higher in *D. claparedii* than in *M. moribidii*. Meanwhile, *M. moribidii* was characterized by higher signals of functional groups C=O and C-O compared to *D. claparedii*. These results align with the $^1$HNMR spectra of both polychaete species (Figure 3), indicating that *D. claparedii* has a higher concentration of fatty acids and a lower concentration of aromatic compounds than *M. moribidii*. However, the FTIR profile in this study is slightly different from those of the previous study [6], indicating that seasons greatly affect metabolite variations of the polychaetes.

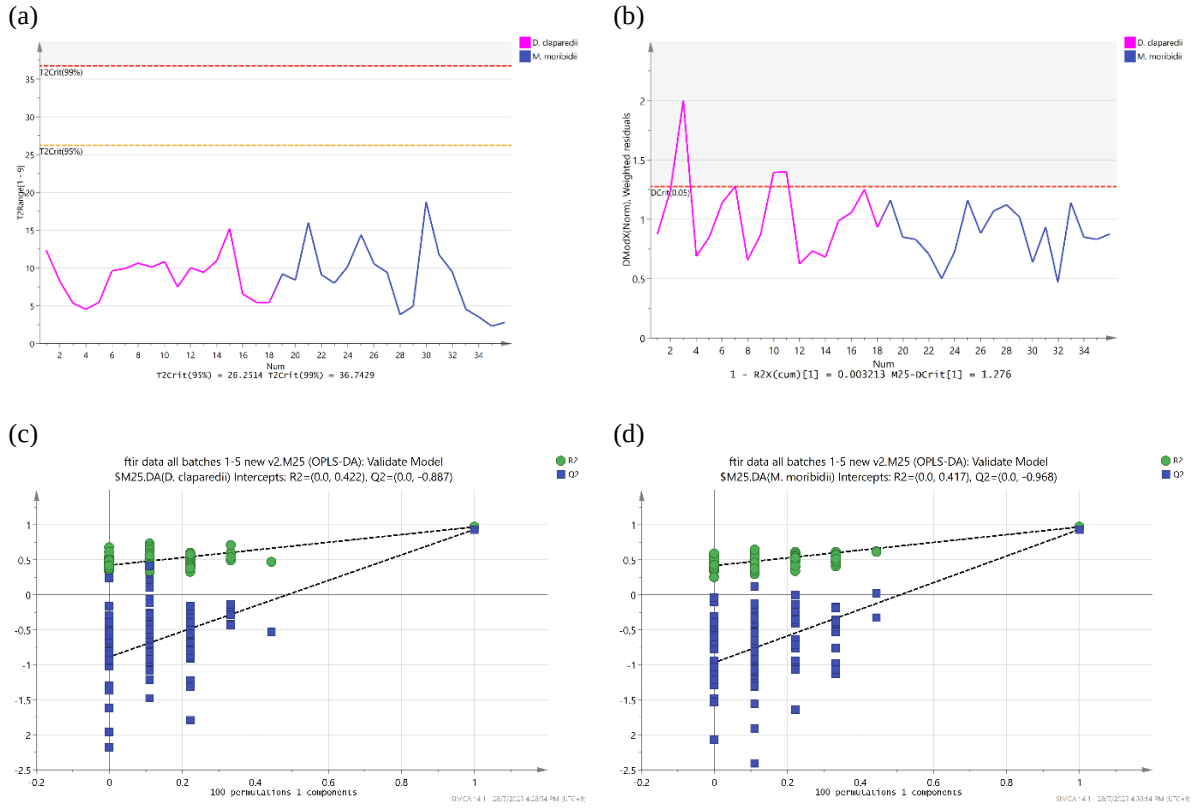



**Figure 5.** OPLS-DA model validation of FTIR training dataset of polychaete species: Hotelling's T2 plot (a), DModX plot (b), and permutation tests of *D. claparedii* (c) and *M. moribidii* (d) groups.

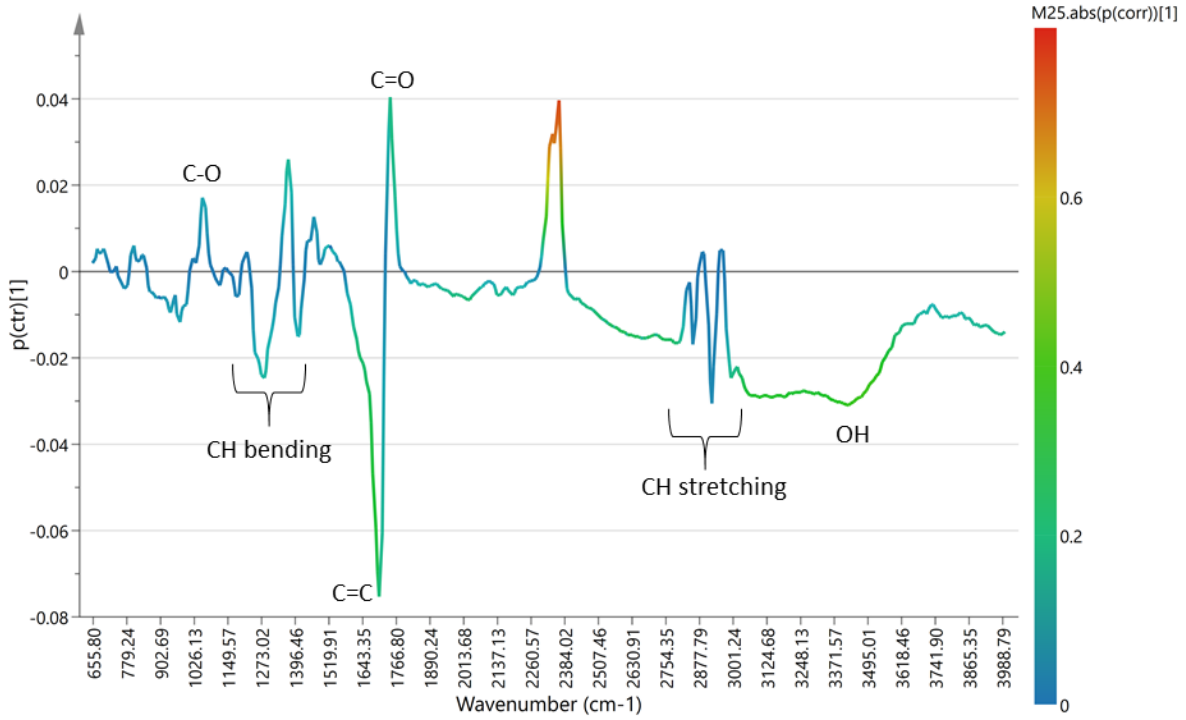



**Figure 6.** OPLS-DA loadings line plot of FTIR training dataset of polychaete species.

## CONCLUSION

FTIR metabolomics and $^{1}$H NMR analysis were successfully applied to discriminate between two species of marine polychaetes, *D. claparedii* and *M. moribidii*, at different harvest times. Multivariate data analysis via orthogonal partial least squares discriminant analysis (OPLS-DA) revealed four distinct clusters of polychaetes based on their different harvest times. According to the study, aromatic compounds and fatty acids were more prominent in batch 1 (April) than in batch 2 (October), contributing to the discrimination of polychaetes based on different harvest times. For consistency in the chemical profile, it is suggested that the first and second quarters are the best harvesting times for the polychaete species. Further study using other analytical tools such as NMR and LCMS is worth conducting to comprehensively understand the metabolite variations in different harvest times of the marine polychaetes.

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## SUPPLEMENTARY DATA

The supplementary materials of this manuscript are available and can be found at https://doi.org/10.5281/zenodo.10806787.

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