Picky with peakpicking: assessing chromatographic peak quality with simple metrics in metabolomics

William Kumler

2023-05-29

## Abstract

### Background

Chromatographic peakpicking continues to represent a significant bottleneck in automated LC-MS workflows. Uncontrolled false discovery rates and the lack of manually-calibrated quality metrics require researchers to visually evaluate individual peaks, requiring large amounts of time and breaking replicability. This problem is exacerbated in noisy environmental datasets and for novel separation methods such as hydrophilic interaction columns in metabolomics, creating a demand for a simple, intuitive, and robust metric of peak quality.

### Results

Here, we manually labeled four HILIC oceanographic particulate metabolite datasets to assess the performance of individual peak quality metrics and constructed a predictive model calibrated to the likelihood that visual inspection by an MS expert would include a given mass feature in the downstream analysis. We found that a simple logistic regression model built on two metrics calculated from the raw MS data reduced the fraction of false positives in the analysis from 80-90% down to 1-5% and showed minimal overfitting when applied to novel datasets. We then explored the implications of this quality thresholding on the conclusions obtained by the downstream analysis, concluding that the poor performance of peakpicking algorithms significantly reduces the power of both univariate and multivariate statistical analyses in detecting environmental differences. We finally show that depth is a major driver of variability in the metabolome and identify several interesting metabolite groups for future investigation.

### Conclusions

TBD

## Background

Liquid chromatography-mass spectrometry (LC-MS) is a powerful tool for exploring the molecular composition of biological samples. Its rapid sample processing (typically <1 hr run time), low limits of detection (pM-nM range), and ability to characterize novel molecules via fragmentation fingerprints make it a common workhorse for metabolomic research. In the past two decades, data-driven methods have established workflows for untargeted metabolomics but the imperfect performance of the core peakpicking algorithms continue to require manual oversight and curation. This problem has been exacerbated by the increased use of non-traditional chromatography such as hydrophilic interaction which tends to produce noisier peaks.

Noisy data and imperfect detection algorithms introduce a tradeoff between false positives (where background instrument or chemical noise is misclassified as biological signal) and false negatives (where real signals are undetected). Existing algorithms favor false positives because downstream analyses can always remove erroneous mass features, but false negatives cannot be later recovered. However, this approach creates two additional downstream problems. First, the abundance of false positives requires more time from the researcher as they manually evaluate a potentially enormous number of peaks, a task that scales combinatorially with the number of samples and compounds measured. Second, false negatives cannot be fully accounted for in the way false positives can because false negatives are introduced in every step of metabolomic analyses from sample collection through injection. Instead of minimizing false negatives, we believe that emphasis should be placed on allowing the experimenter to set a threshold for the false discovery rate and accept that this will inherently add to the number of peaks already lost in the data collection process.

Existing peak-detection softwares do not provide a clear way to do this. Typical outputs consistent across the different implementations consist of the *m/z* ratio, retention time, and area for each molecular feature, with some additional useful information occasionally provided such as the peak’s signal-to-noise ratio or degree of skew. None of these parameters answer the critical question about the likelihood that a given feature corresponds to a molecule present in the original sample. This parameter is crucial for downstream analysis because it represents the base rate for error propagation and acceptable thresholds should vary widely by the particular project’s goals. In an exploratory analysis, any molecular feature more than 50% likely to be real is perhaps worth considering, while in a confirmatory study this threshold may need to be above 99%. While significant effort has been invested in improving the peakpicking algorithms, very little has been done to quantify the accuracy and precision of their outputs across the wide variety of datasets to which they are applied.

A single parameter of peak quality also facilitates downstream analyses in multiple ways. This metric would improve statistical power by reducing the number of effective hypotheses tested and allow researchers to focus effort on features least likely to be noise. Additionally, this parameter could be optimized to improve peakpicking and chromatographic settings independently of the software used or inter-lab variability when scripted to provide consistent, reproducible results independent of the particular expert reviewing its performance. Constructing such a single comprehensive metric calibrated to likelihood is also more effective than multiple independent thresholds because it has meaningful units, does not require estimating the relative power of individual metrics, and allows a good peak to compensate for weak performance in one area with strong performance in other metrics.

[Where to talk about the peakpicking vs integration? Here we just looked at peakpicking]

An area particularly ripe for metabolomic analysis is that of the open ocean. Low compound and high salt concentrations make this area difficult to study [@BMIS] but its vast size and direct effect on the Earth’s carbon cycling make it critical that we understand its dynamics of energy and nutrients on a molecular scale. Metabolites are the currency of chemical exchange both intra- and inter-cellularly, serving as building blocks of larger molecules, regulators of osmotic balance and storage of nutrients, as well as important chemical signals on their own. These small molecules serve both as signposts for the complex biological landscape in this highly dynamic region and give a sense of not only who is present but also what ecological roles they’re serving and the niches they fill.

In this paper, we use open ocean marine metabolite LC-MS samples to develop and test a variety of chromatographic peak metrics. Here, we construct and validate multiple predictive models of peak quality based on metrics both common in the literature and custom implementations we’ve found useful in our own analysis. This allows us to connect the physical, chemical, and biological measurements taken regularly around the globe to a molecular-scale perspective of particulate organic matter in the ocean by linking the chemical currencies that fuel the planet to the environments in which they’re found.

## Results

### Dataset characterization

An average of 3,300 molecular features (MFs) were reported by XCMS across the 4 datasets, with the fewest (1495) in the Falkor data and the most in the Pttime samples (7781). In the Falkor and MESOSCOPE datasets that were fully labeled by an MS expert, approximately 70% (69% and 73%, respectively) of the features were given a “Bad” designation, corresponding to noise peaks that the expert would not have included in a downstream analysis. In both, 5% of the MFs were unable to be assigned confidently to either “Good” or “Bad” classes and 10% were identified as appearing only in the standards, leaving only ~15% of the features classified as “Good” (16% and 12%, respectively).

### Logistic regression performance

According to all three logistic regressions (see Methods), the majority of MFs were estimated to have a less than 1% chance of being good. The full model (containing all possible peak metrics) and the XCMS model (built on only those metrics calculated from the XCMS output) both displayed a strongly bimodal distribution, with a large number of peaks also exceeding a 99% chance of being good, while the two-parameter model (consisting of the novel SNR metric and the peak shape correlation metric) had a flatter distribution with fewer high-confidence MF assignments and more intermediate values (Fig. 3).

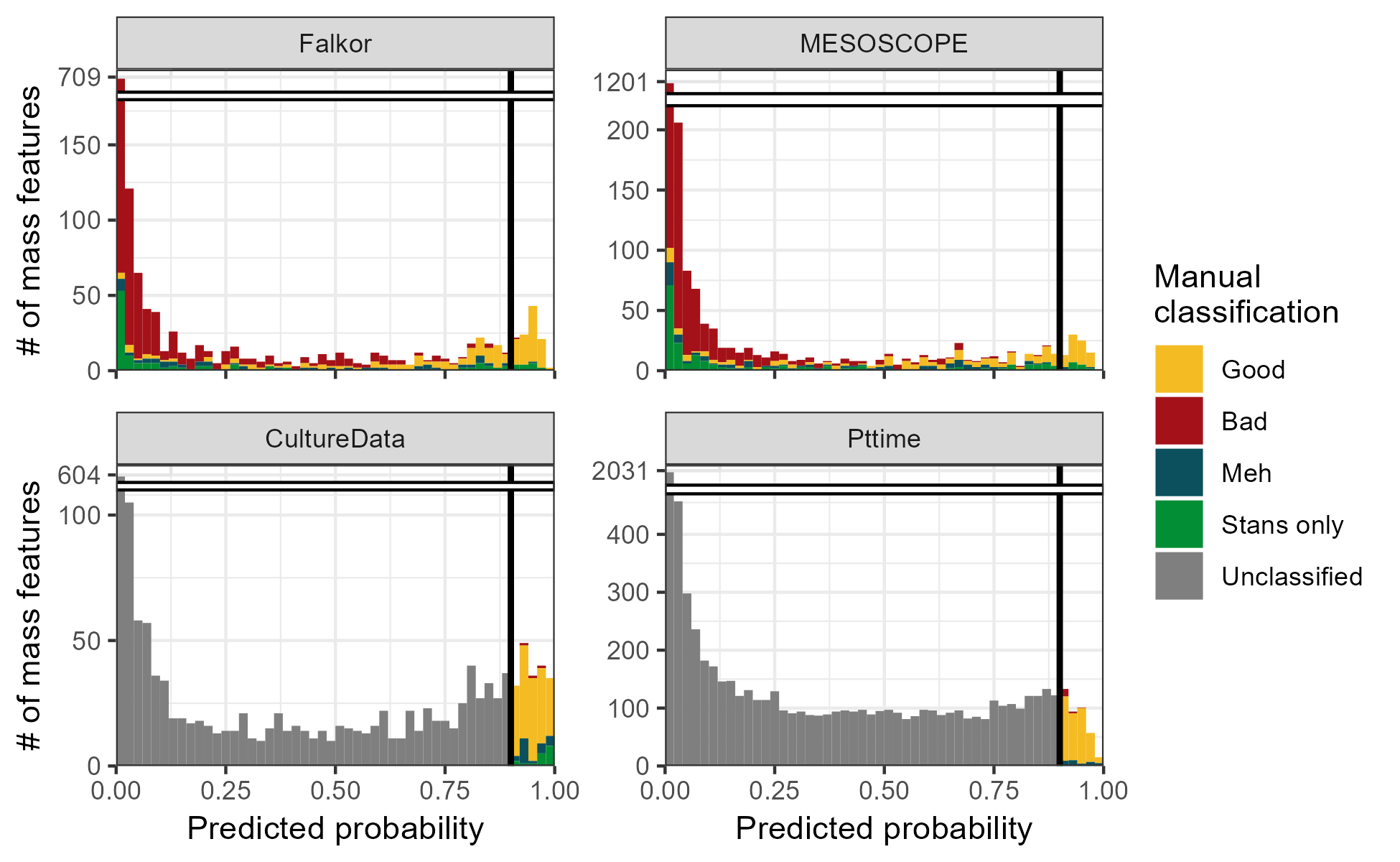


Figure 3: Histograms showing the estimated likelihood of a given mass feature being categorized as “Good” according to the two-parameter logistic model trained on the combined fully-labeled Falkor and MESOSCOPE datasets. Colors indicate the category in which each feature was manually assigned by an expert, with “Stans only” referring to a good peak that was only visible in the standards run alongside the samples. Datasets CultureData and Pttime were manually labeled only for those features with an estimated likelihood above 90% (black vertical line) and were otherwise unclassified.

The full model performed very well when tested internally on the same dataset both during 80/20 cross validation and when [predicting] using the full dataset, with FDR values in the 5-10% range and 80-90% GPF values implying that a large majority of the good peaks passed the threshold. The XCMS metrics performed slightly worse, with FDR values in the 10-15% range and GPF values closer to 75%. The two-parameter model performed worst when tested internally, with an FDR of about 20% and %GPF also around 75% (Figure 4). However, when the models were trained on a different dataset than the one they were used to predict classifications for, nearly all models had similar performance with %FDR around 10-25 and %GPF around 60-80. The model trained on MESOSCOPE and tested on Falkor had consistently higher values, indicating that it was favoring more peaks recovered at the cost of a higher FDR, while the reverse was true for the model trained on Falkor and tested on MESOSCOPE. (Figure 4)

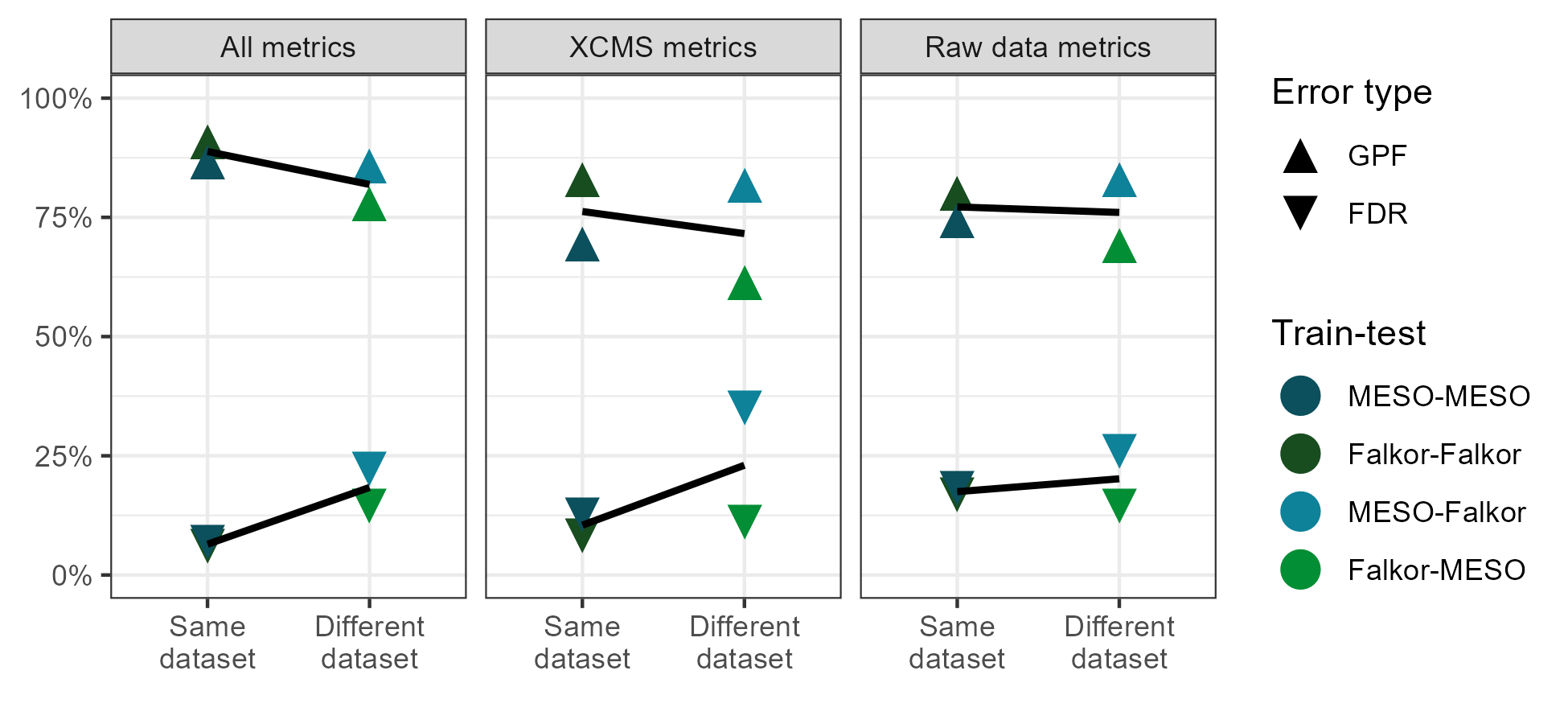


Figure 4: False discovery rate (%FDR) and fraction of good peaks found (GPF) plotted across different subsets of model parameters. Lower %FDR indicates a smaller fraction of false positives among those peaks the model categorized as “Good” using a threshold of 0.5, and higher %GPF indicates a larger fraction of the total good peaks were found using the same threshold. Points are colored by the model used for training and testing, with internal validation (using the same dataset for training as prediction) in the darker colors on the left and external validation (using a different dataset for training than prediction) in the lighter colors on the right of each panel. Lines of best fit have been estimated and plotted in black on top of the data points.

### Model stability under different training sets and subset of labels

We found that the predictions made from a Falkor-trained dataset consistently differed from a MESOSCOPE-trained dataset for the full and XCMS-only models. In the raw probability space, the two-parameter models had the highest value of 0.996, while the full models and the XCMS-trained models had values of 0.856 and 0.896, respectively. When compared in ranked space, we found an intensification of the effect described previously, with an even higher for the two-parameter model of 0.998 but lower values for the full and XCMS-trained model of 0.818 and 0.849, respectively (Figure 5).

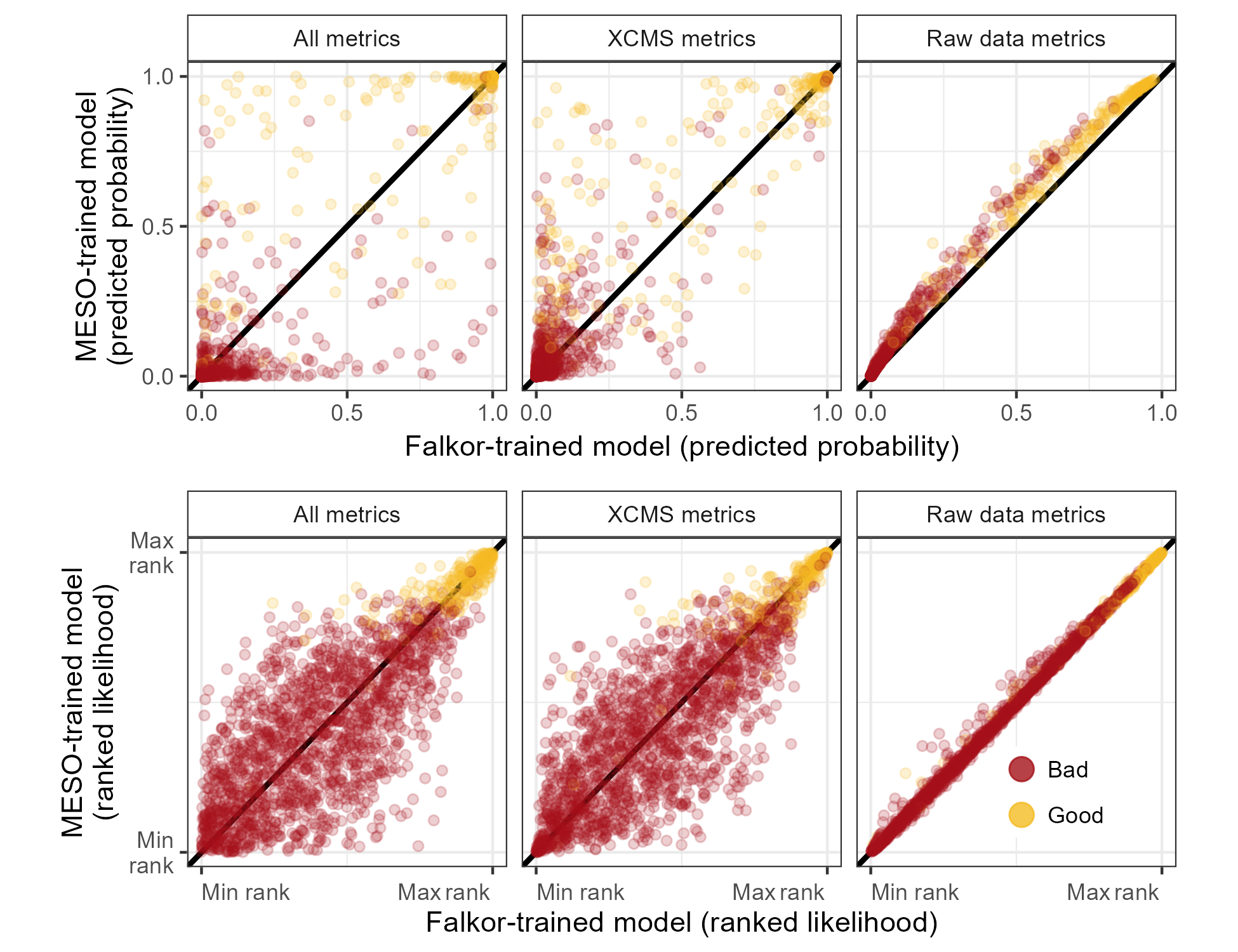


Figure 5: Predicted likelihood of a feature being “Good” according to a model trained on the MESOSCOPE dataset vs a model trained on the Falkor dataset. The top row of plots show the exact likelihood predicted by the logistic model across three different subsets of parameters, while the bottom row shows the estimates ranked from least likely to most likely. Points are colored by their manually-assigned quality according to an expert.

A majority of the time, the estimates from the two models disagreed by more than 2x the standard error of the estimate. Some parameters disagreed not only in magnitude but also in sign, with the Falkor-trained full model increasing peak goodness likelihood with larger ppm variation and a wider peak width, while the MESOSCOPE-trained full model had negative estimates for each of these parameters. Notably, the peak shape and novel SNR parameters used in the two-parameter model were among the most robust to training model variation, potentially explaining the consistency described above (Supp. figure 1).

When testing model stability under a smaller sample size, we found reasonably good convergence in a dataset containing half the mass features with most values falling within two standard errors of the estimate for the XCMS and two-parameter model, while the full model required closer to 80% of the mass features to produce estimates consistent with the original model (Supp. figure 2).

### Regularized regression and random forests perform about the same

None of the penalized regression models significantly improved cross-validated performance between the MESOSCOPE and Falkor datasets when measured by both initial performance and the performance drop when applied across datasets. All three regularized regression models had similar behavior, with ridge regression (α = 0) had the lowest rates for both GPF and FDR, with lasso (α = 1) obtaining higher ones and representing a less-stringent false negative acceptance while, as expected, the elastic net (α = 0.5) fell in between the two (Supp. figure 3). The random forest model, interestingly, had perfect predictive capacity when tested internally on the training data (FDR=0%, GPF=100%, for both MESOSCOPE and Falkor) but showed a significant drop in improvement when applied across datasets (Supp. figure 3). In each case, the performance drop when applied to a novel dataset was more extreme than the simple two-parameter model described above.

### Performance of a stricter threshold on novel datasets

We settled on a 90% likelihood threshold for application to novel datasets because it struck a nice balance between the number of peaks we estimated to be necessary for robust testing while still remaining reasonable to manually label. For the CultureData dataset, we obtained 1,790 total mass features, 192 of which had predicted likelihoods above 0.9. Of these, 151 were identified manually as “Good”, 21 were given “Ambiguous” designations, and only 3 were flagged as “Bad”, with the remaining 17 appearing only in the standards. For the Pttime dataset, 7,781 were obtained with 400 flagged by the model as “Good”. 348 were truly good peaks, 35 were ambiguous, and 17 were “Bad”. No standards were run during this analysis, so there were no features in that category.

With the stricter threshold, we obtained FDR rates consistently below 5% even on the novel datasets, with values of 1.0%, 0.0% (truly zero false positives), 2.0%, and 4.6% for the Falkor, MESOSCOPE, CultureData, and Pttime respectively [Figure 6]. Of course, this low error rate required that we miss out on additional potentially valuable features, with only a fraction of the total good peaks making it past this threshold. In both the Falkor and MESOSCOPE datasets, the fraction of good peaks found was below 40% with actual values of 39.4% and 26.5%, respectively. Since we did not label the complete dataset for CultureData and Pttime, we cannot accurately calculate the %GPF but expect it to be in a similar range (Figure 6).

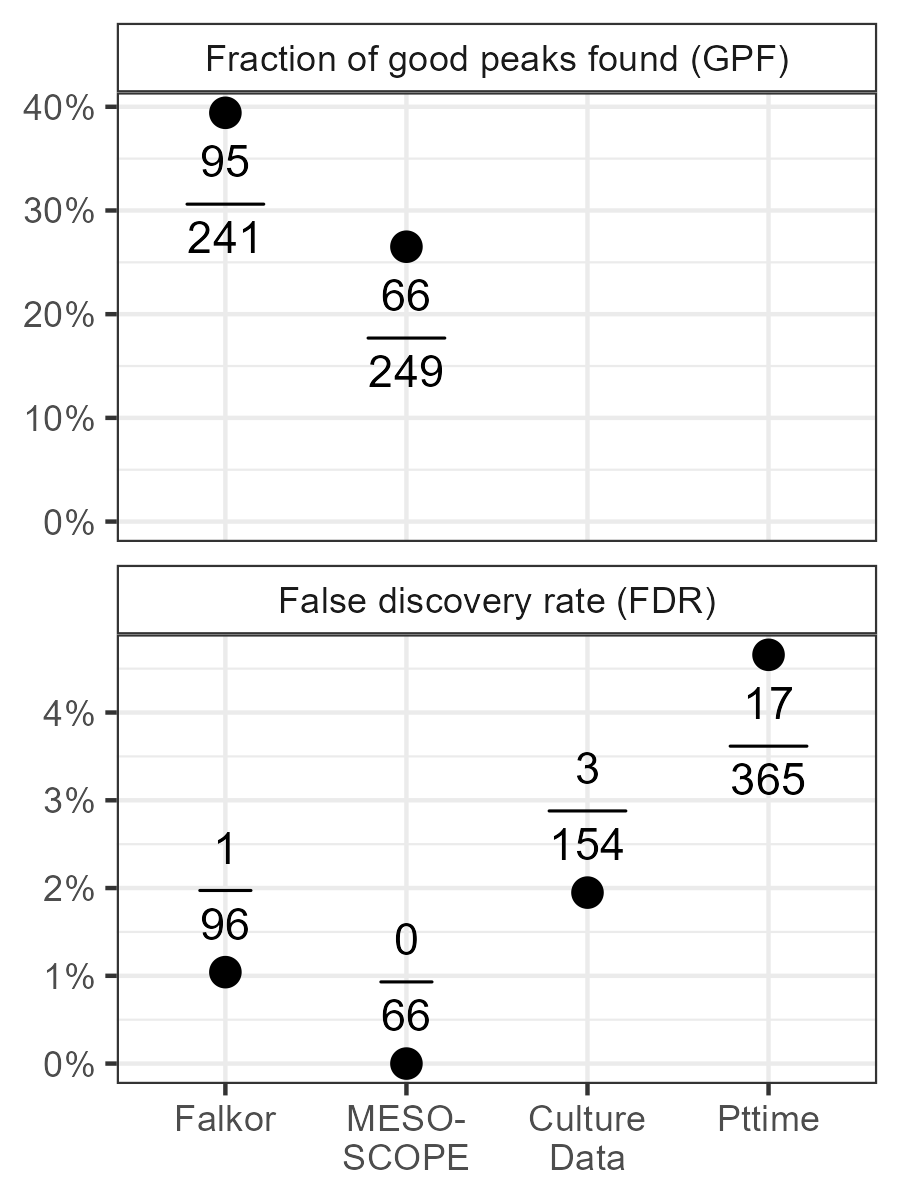


Figure 6: False discovery rate and proportion of total good peaks identified as good by the two-parameter model trained on the combined MESOSCOPE/Falkor dataset and applied to each dataset individually with a threshold of 0.9. FDR is calculated by dividing the number of false positives by the total positives produced by the model and GPF is calculated by dividing the number of true positives by the total number of good peaks as identified manually. Points correspond to the calculated percentage and absolute numbers are provided above/below the point.

### Implications for biological conclusions

#### Univariate techniques

A majority of the features (1,323 of 1,832 total) in the MESOSCOPE dataset had no significant trend with depth, with FDR-controlled Kruskal-Wallis p-values exceeding 0.05 (Figure 7). The largest category that did have a trend with depth was the 15m = DCM > 175m category, containing 118 molecular features, with largest peak areas distributed evenly between the 15 meter and deep chlorophyll maximum (DCM) samples, while the 175 meter samples had significantly smaller areas. The similar but statistically distinct categories of 15m > DCM > 175m and DCM > 15m > 175m had 68 and 35 features, respectively, and together indicate that many molecules are highly abundant throughout the surface ocean, down to the DCM layer and decrease in concentration thereafter. A surprising number of features were also found to have DCM minima (DCM < 15m = 175m, 26 features) or linear increases with depth (15m < DCM < 175m, 12 features) given the few environmental parameters that have these trends (Figure 7).

A different story emerged, however, when the bad peaks were removed from this analysis. Good features were most commonly found to have their highest concentrations at the DCM or the surface, rather than being fixed with respect to depth. Of the 182 good peaks, less than a fifth had no trend with depth (44/249) and a majority had unequivocally lowest values in the 175 meter samples (those with 15m/DCM > 175m, 145 features). The two-parameter model, when applied with a 50% likelihood threshold, also recovered this general feature distribution and classified many of the features with no significant depth signal as likely to be bad (Figure 7B, currently in Slack?).

Additionally, a large number of features manually identified as bad nonetheless had significant differences with depth. This was surprising because we had assumed that bad peaks corresponded to instrument noise, which we did not expect to have any biological trend. Further investigation of a few randomly selected bad features with a biological difference revealed the reason behind this: most of those investigated were actually tails of other peaks. Integrating just the tail of a peak retains the biological signal of the full peak while still looking visually like instrument noise, thereby introducing pseudoreplication in the feature space.

The model did fail to recover some interesting biological variation, however. Two features of particular interest were those good peaks with a DCM minimum (DCM < 15m = 175m), both of which were missed by the two-parameter model. These features possess an unexpected biological signal that does not track with depth or other common oceanographic parameters, thereby potentially representing an interesting biomarker or pathway that’s downregulated in excess of the biomass effect. [Maybe better for discussion?]

[MAA paragraph?] Several mass features that were found dominantly at the surface were putatively identified as mycosporine-like amino acids (MAAs), which have been shown to have UV photoprotective properties.[@ref] Although we were unable to calculate their exact concentrations due to a lack of authentic standards, peak areas were approximately 100 to 1000 times higher at 15 meters than their values averaged between the DCM and 175 meter samples. The abundance of MAAs in this particular category indicates that there may be other MAA molecules with this depth pattern that have not yet been characterized also among these unknowns.

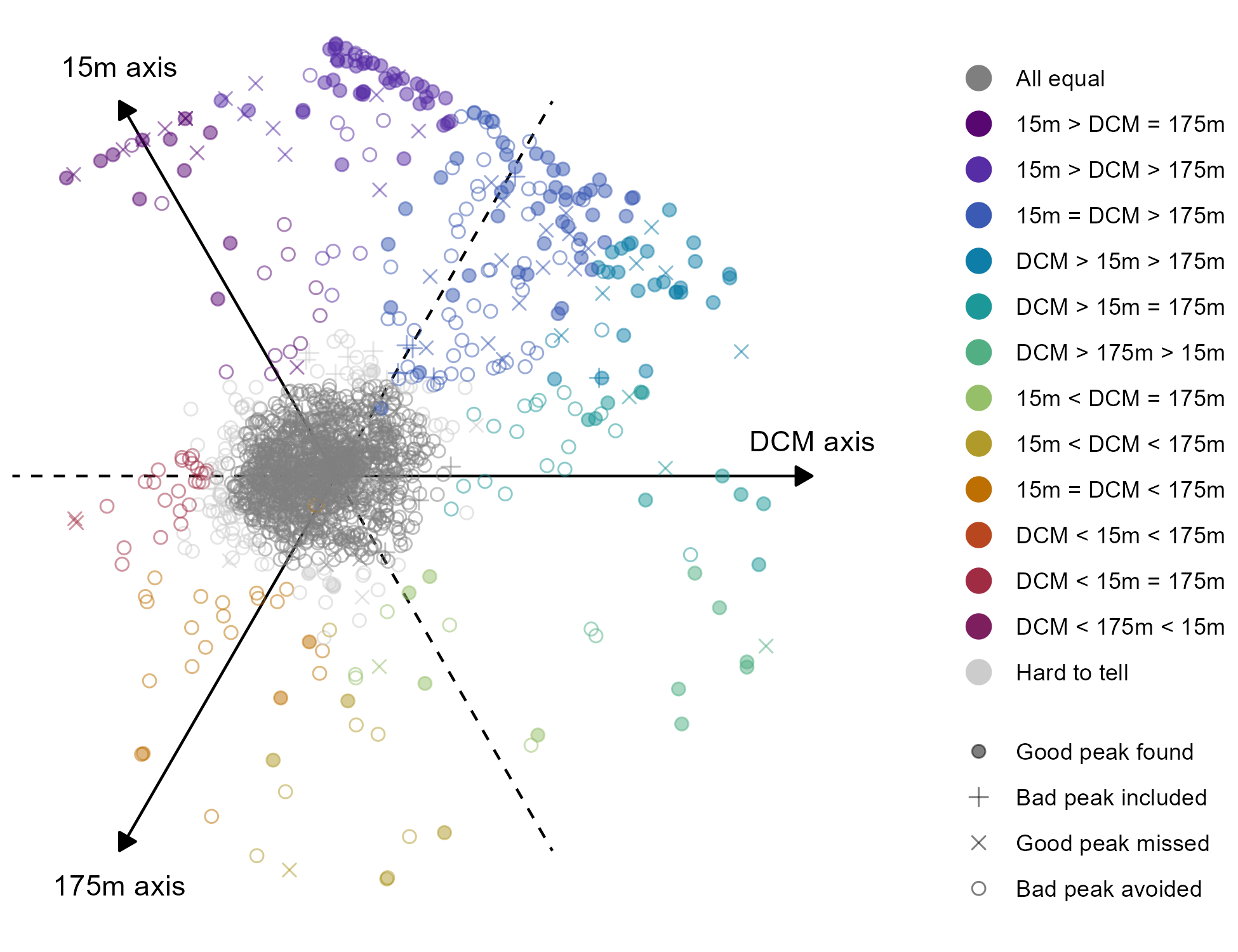


Figure 7: Plot of metabolite response to depth shown across three axes using the rank-normalized median value at each depth as the coordinate for that axis. Each molecular feature corresponds to a point in the plot, and their position on the plot describes the shape of their depth profile. Compounds aligning with the 15m axis correspond to compounds with most of their abundance found in the surface ocean; points far to the right side correspond to compounds that are found only at the deep chlorophyll maximum; points found at the bottom of the plot are those compounds that increased more or less linearly with depth.

#### Multivariate techniques

Multivariate statistics also benefitted from the reduced FDR when applying the two-parameter model. For the PERMANOVAs, we found that the proportion of variance explained (R) and the pseudo-F statistic increased monotonically with the likelihood threshold used to subset the data (Table 1).

In each test, the permutational p-value obtained was less than 0.001, indicating that the differences between samples due to depth were unlikely to be due to chance in all cases. However, the pseudo-F was much larger with higher thresholds, scaling from around 8.5 when thresholding at a 1% likelihood to 42 when thresholded at a 90% likelihood.

|  | n | R2 | F | Pr(>F) |
| --- | --- | --- | --- | --- |
| mat\_good | 249 | 0.44 | 38.0 | 0.001 |
| mat\_90 | 75 | 0.46 | 42.0 | 0.001 |
| mat\_50 | 287 | 0.34 | 25.3 | 0.001 |
| mat\_10 | 516 | 0.25 | 16.0 | 0.001 |
| mat\_01 | 1129 | 0.15 | 8.5 | 0.001 |
| mat\_all | 2086 | 0.10 | 5.5 | 0.001 |

*Table 1: Number of mass features (n), variance explained (R2), pseudo-F statistic (F), and permutational p-value (Pr(>F)) estimates obtained from performing a permutational MANOVA on subsets of the full mass feature selection according to variable likelihood thresholds. [Needs cleaning, will fix]*

We also tested the inclusion of all the features identified with XCMS (corresponding to a 0% threshold) and the results when only the manually-identified “Good” features were included. The default XCMS output continued the trend observed above, as expected, with the least variance explained and the lowest R value. Subsetting for the “Good” peaks only, however, did not actually return the highest F-ratio or R, instead falling between the 50% and 90% thresholds for these two metrics. In large part this is due to the much smaller number of features: 249 features were manually labeled as Good, while only 75 exceeded the 90% likelihood threshold.

The relative power of identifying only the very best peaks was also illustrated visually with non-metric multidimensional scaling (NMDS) plots (Figure XX). In these common exploratory plots, the features with likelihoods above 50% strongly separated by depth while lower thresholds disguised the true signal and had higher stress values. Performing an NMDS on the manually-identified Good peaks resulted in output nearly indistinguishable from those of the 90% and 50% thresholds, while the 0.1 and 0.01 thresholds looked almost identical to the same analysis performed on all the XCMS features.



Figure XX: NMDS plots of metabolite similarity according to sample depth across multiple likelihood thresholds. Triplicate samples are represented by the vertices of the triangles and colored by the depth from which they were sampled (DCM = deep chlorophyll maximum, ~110m). “Only good” refers to those features manually labeled as “Good”. NMDS stress values are reported to 3 significant figures in the upper right of the plot.

## Discussion

### Simple logistic regression models show good performance and minimal overfitting

* Recap performance metrics
  + Discuss the relative performance and compare to assumptions in lit for
    - Large peak area
    - Has an isotope
    - p-values
  + Discuss use of summary statistics and the potential need for a blocked model by sample type
* Emphasize the best performance of the two-parameter model
  + Full model and XCMS model definitely overfit, same for regularized/random forest
  + XCMS alone does not provide enough information to accurately distinguish good from noise
    - Likely true for all peakpickers
    - Plea: report mz/rt bounding box for individual peaks so additional metrics can be calculated
  + These params very simple (and fast!) to estimate
    - Easy to do better with fancier models (e.g. loess smoothing for SNR)
  + Why do all the models seem to approach ~25% FDR and ~75% GPF on the novel datasets?
* Limited to HILIC data at the moment but does seem to perform well across labs

### Unable to create strict control of the FDR but a single threshold still makes life easier

* Initial goal: set an FDR, choose a likelihood threshold based on it
  + Not doable here but maybe in future work?
  + Pivoted to recommending step-down manual labelling
* Plea: report your FDR! Especially if you’re manually labelling!
  + Necessary for error propagation anyway
* Future work: alter chromatography/peakpicking params to maximize # of peaks above 0.9 threshold

### Biological results do depend on the threshold used

* Large amounts of noise reduce the power of both univariate and multivariate stats
  + Trade-off with loss of interesting signals at higher thresholds (cmpds on clockplot left)
  + Possible to get wildly different results with different thresholds (% variance explained)
  + Manual evaluation for interesting signals still strongly recommended
* Depth is a major driver of metabolome composition in the gyre
* Majority of metabolites track with biomass metrics
* Some interesting metabolites diverge from dominant biomass trend
  + Some new MAAs in clockplot top left? (Still need to check this w MSMS)
  + Degradation products / bacterial specialties at bottom?

## Conclusions

TBD

## Methods

### Sample collection

Environmental samples were collected from the North Pacific Subtropical Gyre near Station ALOHA during two research cruises that targeted strong mesoscale eddy features during June/July 2017 and March/April 2018, traversing an area between 28 °N, 156 °W and 23 °N, 161 °W. An eddy dipole off the coast of Hawaii was detected using sea-level anomaly (SLA) satellite data and targeted for both a transect across the cyclonic and anticyclonic poles of the eddy dipole. The cyclonic pole of the eddy had a maximum negative SLA anomaly of -15 cm in 2017 and -20 cm in 2018, while the anticyclonic center reached +24 cm in 2017 and +21 cm in 2018. The 2017 cruise samples were taken along a transect across the eddy dipole while the 208 cruise targeted only the center of each eddy.

Environmental samples were obtained using the onboard CTD rosette to collect water from 15 meters, the deep chlorophyll maximum (DCM), and 175 meters during the 2017 MESOSCOPE cruise and from 25 meters and the DCM during the 2018 Falkor cruise. The DCM was determined visually from fluorometer data during the CTD downcast and Niskin bottles were tripped during the return trip to the surface. Seawater from each depth was sampled in triplicate by firing one Niskin bottle for each sample. Samples were brought to the surface and decanted into prewashed (3x with DI, 3x with sampled seawater) polycarbonate bottles for filtration. Samples were filtered by peristaltic pump onto 142mm 0.2 µm Durapore filters held by polycarbonate filter holders on a Masterflex tubing line. Pressures were kept as low as possible while still producing a reasonable rate of flow through the filter, approximately 250-500 mL per minute. Samples were then removed from the filter holder using solvent-washed tweezers and placed into pre-combusted aluminum foil packets that were then flash-frozen in liquid nitrogen before being stored at -80 °C until extraction. A methodological blank was also collected by running filtrate through a new filter and then treated identically to the samples.

Culture samples used as the validation sets for this paper were obtained from existing [blah] and have been previously described by [@Bryn] and [@Pttime paper?].

### Sample processing

Extraction of the environmental samples followed a modified Bligh & Dyer approach as detailed in [@BMIS]. Briefly, filters were added to PTFE centrifuge tubes with a 1:1 mix of 100 µm and 400 µm silica beads, approximately 2mL -20 °C Optima-grade DCM, and approximately 3mL -20 °C 1:1 methanol/water solution (both also Optima-grade). Extraction standards were added during this step. The samples were then bead-beaten three times, followed by triplicate washes with fresh methanol/water mixture. Samples were then dried down under clean nitrogen gas and warmed using a Fisher-Scientific Reacti-Therm module. Dried aqueous fractions were re-dissolved in 380 µL of Optima-grade water and amended with 20 µL isotope-labeled injection standards. Additional internal standards were added at this point to measure the variability introduced by chromatography and ionization, and the reconstituted fraction was syringe-filtered to remove any potential clogging material. This aqueous fraction was then aliquoted into an HPLC vial for injection on the HILIC column and diluted 1:1 with Optima-grade water. A pooled sample was created by combining 20 µL of each sample into the same HPLC vial, and a 1:1 dilution with water half-strength sample was aliquot from that to assess matrix effects and obscuring variation [@BMIS]. Also run alongside the environmental samples were two mixes of authentic standards in water and in an aliquot of the pooled sample at a variety of concentrations for quality control, annotation, and absolute concentration calculations. HPLC vials containing the samples were frozen at -80 °C until thawing shortly before injection.

The CultureData samples were re-run from the frozen aliquots for this paper. The Pttime sample processing is documented [on MW? in their paper?].

### LC conditions

For the MESOSCOPE, Falkor, and CultureData samples a SeQuant ZIC-pHILIC column (5 um particle size, 2.1 mm x 150 mm, from Millipore) was used with 10 mM ammonium carbonate in 85:15 acetonitrile to water (Solvent A) and 10 mM ammonium carbonate in 85:15 water to acetonitrile (Solvent B) at a flow rate of 0.15 mL/min. The column was held at 100% A for 2 minutes, ramped to 64% B over 18 minutes, ramped to 100% B over 1 minute, held at 100% B for 5 minutes, and equilibrated at 100% A for 25 minutes (50 minutes total). The column was maintained at 30 °C. The injection volume was 2 µL for samples and standard mixes. When starting a batch, the column was equilibrated at the starting conditions for at least 30 minutes. To improve the performance of the HILIC column, we maintained the same injection volume, kept the instrument running water blanks between samples as necessary, and injected standards in a representative matrix (the pooled sample) in addition to standards in water. After each batch, the column was flushed with 10 mM ammonium carbonate in 85:15 water to acetonitrile for 20 to 30 minutes. LC conditions for the Pttime samples are documented [on MW? in their paper?].

### MS conditions

Environmental metabolomic data was collected on a Thermo Q Exactive HF hybrid Orbitrap (QE) mass spectrometer. The capillary and auxiliary gas heater temperatures were maintained at 320°C and 100°C, respectively. The S-lens RF level was kept at 65, the H-ESI voltage was set to 3.3 kV and sheath gas, auxiliary gas, and sweep gas flow rates were set at 16, 3, and 1, respectively. Polarity switching was used with a scan range of 60 to 900 m/z and a resolution of 60,000. Calibration was performed every 3-4 days at a target mass of 200 m/z. DDA data was collected from the pooled samples for high-confidence annotation of knowns and unknowns. All files were then converted to an open-source mzML format and centroided via Proteowizard’s msConvert tool. For the Pttime samples, files were pulled directly from Metabolomics Workbench via Project ID [blah] and used in their existing mzXML format.

### Peakpicking, alignment, and grouping with XCMS

The R package XCMS was used to perform peakpicking, retention time correction, and peak correspondence. Files were loaded and run separately for each dataset (Falkor, MESOSCOPE, CultureData, and Pttime) using the new “OnDiskMSnExp” infrastructure. Default parameters for the CentWave peakpicking algorithm were used except for: ppm, which was set to 5; peakwidth, which was widened to 20-80 seconds; prefilter, for which the intensity threshold was raised to ; and integrate, which was set to 2 instead of 1. snthresh was set to zero because there are known issues with background estimation in this algorithm [@myers2017], and both verboseColumns and the extendLengthMSW parameter were set to TRUE. For retention time correction, the Obiwarp method was used except for the CultureData dataset, which was visually inspected and determined not to require correction. For the Obiwarp algorithm, the binsize was reduced to 0.1 but all other parameters were left at their defaults or equivalents.

Peak grouping was performed on the two environmental datasets and the Pttime data with a bandwidth of 12, a minFraction of 0.1, binSize of 0.001, and minSamples of 2 but otherwise default arguments. CultureData’s minFraction was raised to 0.4 but was otherwise identical. Sample groups were constructed to consist of the biological replicates for all datasets. After peak grouping, peak filling was performed using the fillChromPeaks function with the ppm parameter set to 2.5. Finally, mass features with a retention time less than 30 seconds or larger than 20 minutes were removed to avoid interference from the initial and final solvent washes. [Check this with Laura]

### Manual inspection and classification

After the full XCMS workflow was completed, the molecular features were visually inspected by a single qualified MS expert. For the Falkor and MESOSCOPE datasets, every molecular feature was inspected, while only those features with a predicted probability of 0.9 or higher according to the two-parameter model produced below were for the CultureData and Pttime datasets. Inspection consisted of plotting the raw intensity values against the corrected retention-time values for all data points within the *m/z* x RT bounding box determined by the most extreme values for the given feature. For this step, we decided to plot the entire feature across all files simultaneously rather than viewing each sample individually to both accelerate labelling and to more accurately represent what MS experts typically do when assessing the quality of a given peak. [Fig 1.] We also decided to ignore missing values and linearly interpolate between known data points rather than filling with zeroes. These EICs were then shown to an MS expert for classification into one of 4 categories: Good, Bad, Ambiguous, or Stans only if the peak appeared to only show up in the standards. A few randomly-chosen features from the manually-assigned Good and Bad classifications are shown in [Fig. 1].

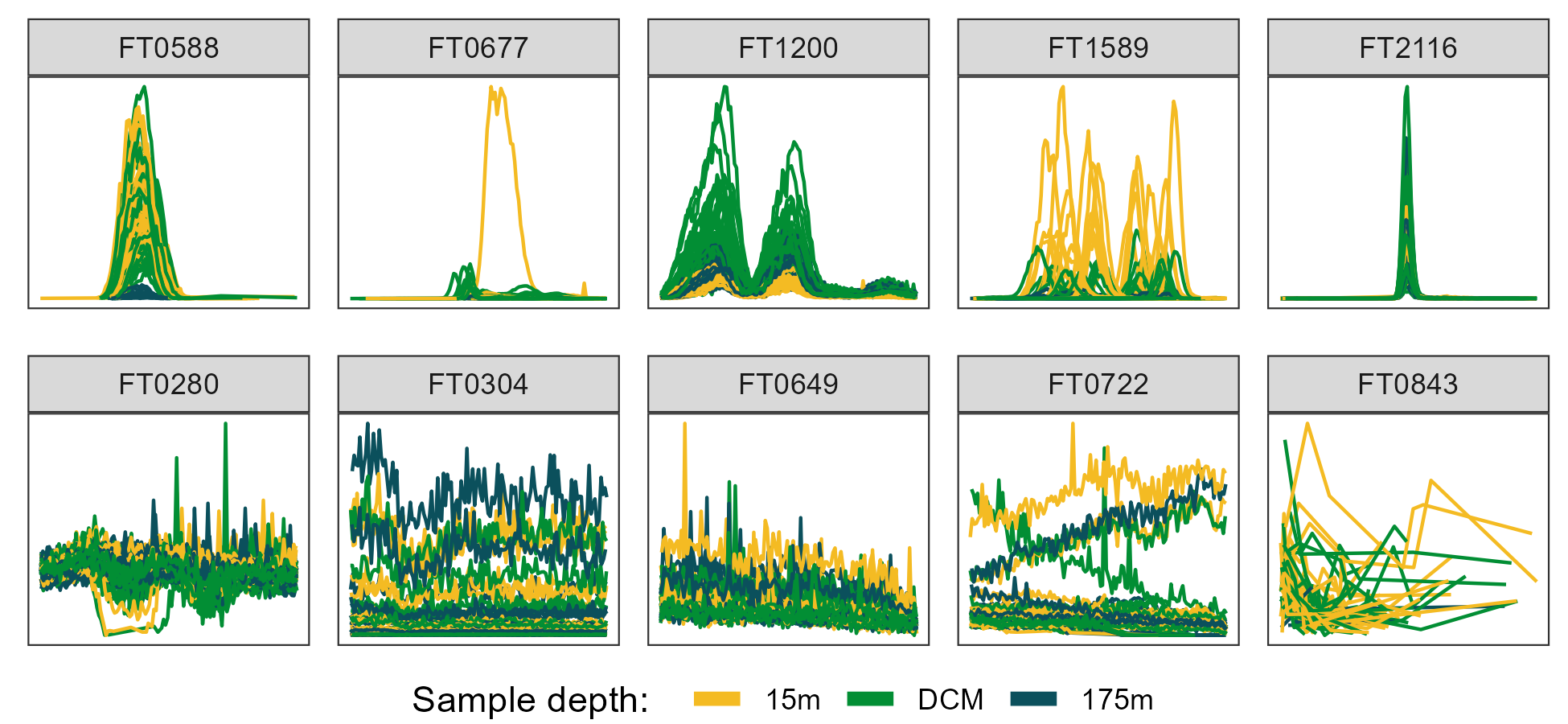


Figure 1: Randomly selected ion chromatograms from both “Good” (top row) and “Bad” (bottom row) manual classifications, colored by the depth from which the biological sample was taken. DCM = deep chlorophyll maximum, approximately 110 meters.

### Peak feature extraction and metric calculation

Our process of feature engineering was divided into three categories of features: those that could be calculated using a single peak trace, those that required access to the entire file, and those that could only be calculated across multiple files. [Rewrite without the “three categories” business ugh] For metrics belonging to the first two categories, summary statistics were used (mean/median, standard deviation) to create a single consensus value for the mass feature as a whole. Missing values were ignored during these summary statistic calculations by setting na.rm=TRUE. Distributions were visually inspected and highly abnormal metrics were transformed using log-scaling if necessary. Using a pairs plot, highly correlated (above a Pearson’s r ~ .9) had one of the correlated metrics removed.

The first category consisted largely of information produced directly by XCMS: the very typical *m/z* ratios, retention times, peak widths, and integrated areas as well as the less common and poorly-documented sn, f, scale, and lmin parameters. Although we requested additional parameters from XCMS with the verboseColumns = TRUE argument, all of the values returned were NAs and could not be used in the model building so were dropped. The total number of peaks found was calculated as the number of peaks divided by the total number of files, and the total number of files in which a peak was initially detected was calculated by subtracting the number of NAs in the peak baseline estimate (which is NA if the peak was filled in) from the total number of files and then dividing by the number of files to normalize across datasets. This calculation were performed for the Falkor, MESOSCOPE, and CultureData datasets to estimate the proportion of sample and standard files in which a given peak was found, but could not be supplied for the Pttime dataset because no standard files were available. The sn metric contained a large number of zero values to begin with that then became negative infinities when log-scaled, so we replaced those values with zeroes and functionally equated an sn of 0 with an sn of 1.

We also calculated several metrics from the raw mz/rt/int values. Using the R package RaMS we extracted the data points falling within each individual peak’s mz and retention time bounding box (values between the XCMS-reported min and max) separately for each file. The data points were then linearly scaled to fall within the 0-1 range by subtracting the minimum RT and dividing by the maximum RT, then each scaled RT was fit to a beta distribution with α values of 2.5, 3, 4, and 5, and a fixed β value of 5. This approach allowed us to approximate a bell curve with increasing degrees of right-skewness and the beta distribution was chosen because it is constrained between 0 and 1 and simple and speedy to generate in R. For each α value, Pearson’s correlation coefficient was calculated between the beta distribution and the raw data, with the highest value returned as a metric for how peak-shaped the data were [Fig. 2]. The beta distribution with the highest *r* was also then used to estimate the noise level within the peak by also scaling both the beta distribution densities and the raw data intensity values as described above, then subtracting the scaled beta distribution from the scaled intensity values, producing the residuals of the fit [Fig. 2]. The signal-to-noise ratio (SNR) was calculated by dividing the maximum original peak height by the standard deviation of the residuals multiplied by the maximum height of the original peak. This method of SNR calculation allowed us to rapidly estimate the noise within the peak itself rather than relying on background estimation using data points outside the peak, which may not exist or may be influenced by additional molecular signals. [@myers?] If there were fewer than 5 data points, a missing value was returned and dropped in subsequent summary calculations. Accessing the raw data values also allowed us to calculate the proportion of “missed” scans in a peak for which a retention time exists at other masses in the same sample but for which no data was produced at the selected *m/z* ratio.

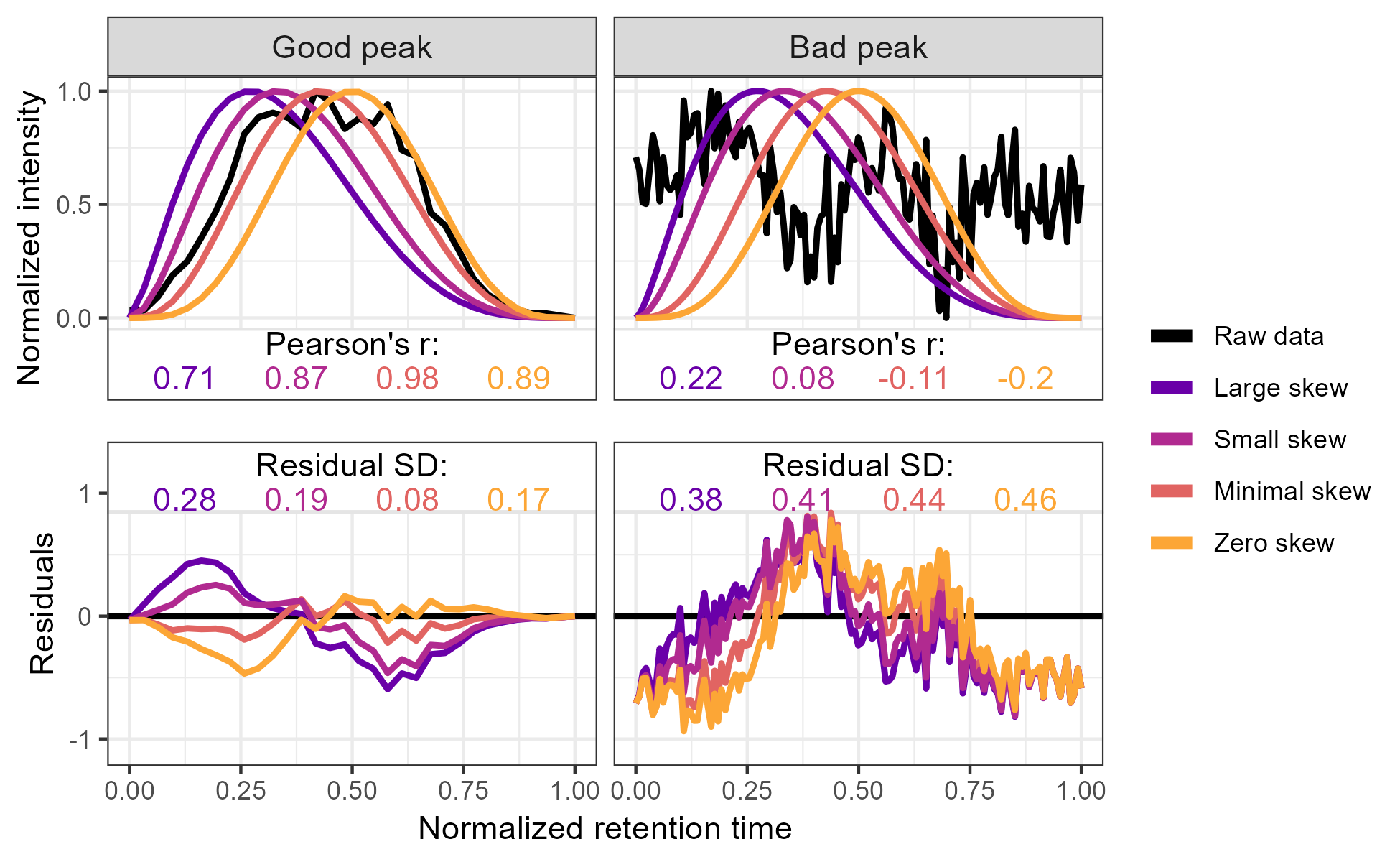


Figure 2: Method used to calculate the metrics for the two-parameter model from the raw data via comparison to an idealized pseudo-Gaussian peak for both manually identified “Good” and “Bad” peaks. Normalization was performed by linearly scaling the raw values into the 0-1 range by subtracting the minimum value and dividing by the maximum. Peak shape similarity was measured with Pearson’s correlation coefficient and the noise level is estimated as the standard deviation of the residuals after the raw data is subtracted from the idealized peak.

We additionally estimated the existence of a C isotope using a similar method to extract the raw mz/rt/int values within the peak bounding box, then searched the same RT values at an *m/z* delta of +1.003355. In places where more than 5 data points existed at both the original mass and the C mass, we again used Pearson’s correlation coefficient to estimate the similarity between the two mass traces and used a trapezoidal Riemann sum to estimate the area of the original and isotope peaks. The overall feature isotope shape similarity was calculated by taking the median of the correlation coefficients. We also calculated the correlation coefficient of the ratio of the across multiple files, expecting that a true isotope would have a fixed ratio. Both the isotope shape similarity and the isotope area correlation were used as metrics in the downstream analysis. Peaks for which no isotope signal was detected or had too few scans to calculate the above metrics were imputed with NA values that were again dropped in the calculation of summary statistics.

Finally, we calculated several metrics using a design-of-experiments (DoE) approach for the Falkor and MESOSCOPE datasets which were expected to have a large difference in metabolite composition between those samples collected at the surface (15-25 meters) and those collected deeper in the water column. We used ANOVAs to compare the integrated peak area with depth and extracted the p-values of each test, which became an additional potential peak quality metric. We also calculated the difference between the mean sample area and the mean blank area and the difference between the mean sample area and the mean area of the standards which also became metrics. When there were too many missing values to perform the ANOVA or sample averages, we replaced the calculated statistic with a value a single order of magnitude outside the most extreme value.

### Regressions and model development

We used three different multiple logistic regression models to predict the likelihood of each MF being categorized as “Good”. The first model included all metrics calculated as described above in Methods, the second contained only those parameters immediately available from the XCMS output without revisiting the raw data (the four core peak metrics *m/z*, RT, peak width, area and their standard deviations plus the mysterious lmin, f, and scale values as well as the fraction of peaks, samples, and standards found), and the final model was a simple two-parameter model using only the peak shape and novel SNR metrics.

In each case, we categorized each molecular feature as a true positive (TP) if it was predicted to be Good and was manually classified as Good, a true negative if both predicted and classifed as Bad, a false positive if predicted to be Good but manually classified as Bad, and a false negative if predicted to be Bad but was in fact manually classified as Good. This allowed us to additionally define two useful measures of success, the traditionally-defined false discovery rate (FDR, defined as 1-precision or the number of false positives divided by the total number of predicted positives) and the percentage of good peaks found (GPF, also known as the recall or sensitivity and defined as the number of true positives divided by the total number of positive predictions).

To further explore questions of model stability and the potential for overfitting, we compared the predictions from a Falkor-trained model to a MESOSCOPE-trained model. This comparison was done in both the raw probability space as well as a rank-ordered space to test whether the most extreme likelihood (i.e. very best and very worst) peaks were consistently found to be most extreme independently of the actual likelihood predicted. For the raw probability space we compared the predictions using Pearson’s correlation coefficient, while Spearman’s rank-ordered coefficient was used for the ranked space. We also looked at the estimates produced by these two models and compared them with the combined model trained on both datasets combined to assess the model stability directly.

We also tested the robustness of the model under a smaller training set, emulating a situation in which only a fraction of the data was available or only a portion of the mass features had been labeled. This allowed us to test the required sample size for the different models, with a larger sample size presumably required for the models with more parameters. Because no parameter was present in all 3 models, we looked at the top 2 most significant parameters from each model: average *m/z* and peak shape for the full model, average peakwidth and the standard deviation in retention time for the XCMS model, and peak shape and SNR for the two-parameter model.

We also tested whether the performance could be improved with elastic net regression or random forest models. These models handle correlated variables better than ordinary least squares regression, so we also included several additional implementations of the peak shape and novel SNR parameters when summarizing across multiple files, using a max and a median of the top-three best values rather than just the overall median as well as a log-transformed version of the median peak shape calculated as where is Pearson’s correlation coefficient, as described above (Figure 2). Cross-validation was used to select the optimal tuning parameter with glmnet package’s cv.glmnet for an elastic net penalty (α) of 0, 0.5, and 1. Random forests were implemented using the randomForest package with default settings and a factor-type response vector to ensure classification was used rather than regression.

### Application of the model to novel datasets

After exploring the different models described above and determining that the two-parameter model would likely perform most consistently on novel datasets, we applied this trained model on two additional datasets that differed significantly from the training data. The CultureData dataset was produced in the Ingalls lab like MESOSCOPE and Falkor, but represent data from a variety of phytoplankton and bacterial cultures in fresh and salt water rather than environmental samples.

The Pttime dataset was discovered on Metabolomics Workbench [@MW id] and consists of *Phaeodactylum tricornutum* cultures collected at a variety of timepoints from both pelleted cells and the released exudate. This dataset was chosen because of the similar LC-MS setup used as a benchmark comparison for the performance other labs with similar setups may expect to achieve using the trained model directly. Each of these datasets were only fractionally labeled, with those peaks above the 0.9 likelihood threshold according to the two-parameter model reviewed manually and categorized.

This stricter threshold was chosen because we felt less comfortable interpreting results based on mass features that were only 50% likely to be real, but did not feel the need to be so strict with this exploratory analysis that we wanted to limit it to 99+% likelihood peaks.

### Using variable thresholds to determine effects on biological conclusions

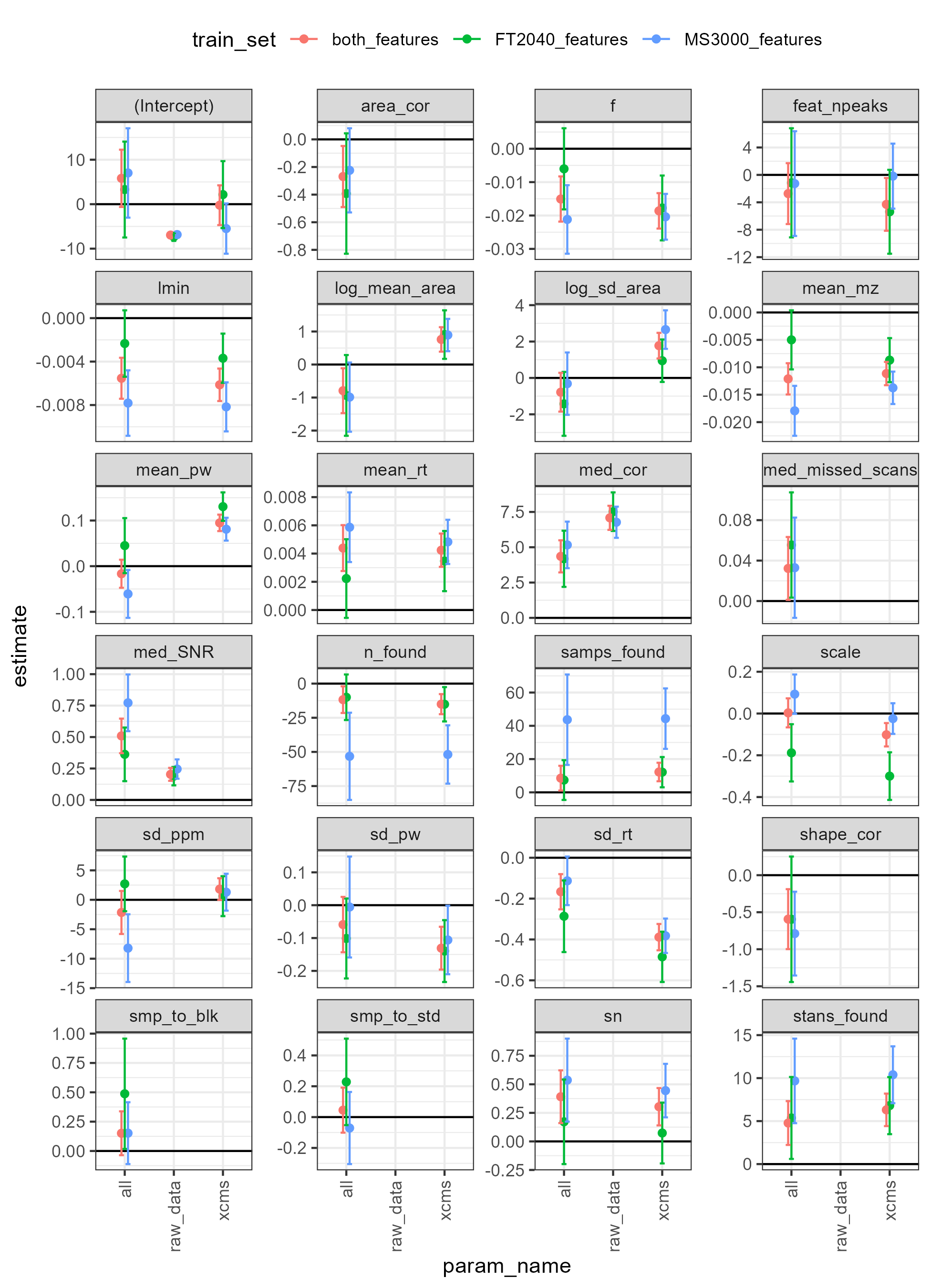
We explored the implications of applying this model to the MESOSCOPE dataset at a variety of thresholds. In univariate space, we used nonparametric Kruskal-Wallis analyses of variance to measure the difference between the surface (15m), DCM (~110m), and 175m samples because the metabolite peak areas could not be assumed to be normally distributed. These univariate tests were then controlled for multiply hypothesis testing using R’s p.adjust function with method fdr.[@ref] We also performed post-hoc Dunn tests provided by the rstatix package to categorize the response to depth for those mass features for which the KW test was significant, with responses falling into one of the 14 classes possible when permuting the sign and significance of the Dunn test.[@ref] p-values obtained from the Dunn tests were not FDR controlled because it was used as a categorization tool rather than a null hypothesis test. In multivariate space, we used a permutational MANOVA (PERMANOVA) [@ref] provided by the vegan package’s adonis2 function to test for multivariate differences in structure of the metabolome with depth.[@ref] We ran multiple PERMANOVAs with a different subset of mass features included each time, corresponding to using the output from XCMS directly, likelihood thresholds of 0.01, 0.1, 0.5, 0.9, and finally only those peaks manually annotated as good.

All analyses were run in R, version 4.2.2, and code is available on the Github at <https://github.com/wkumler/MS_metrics>.[@ref]

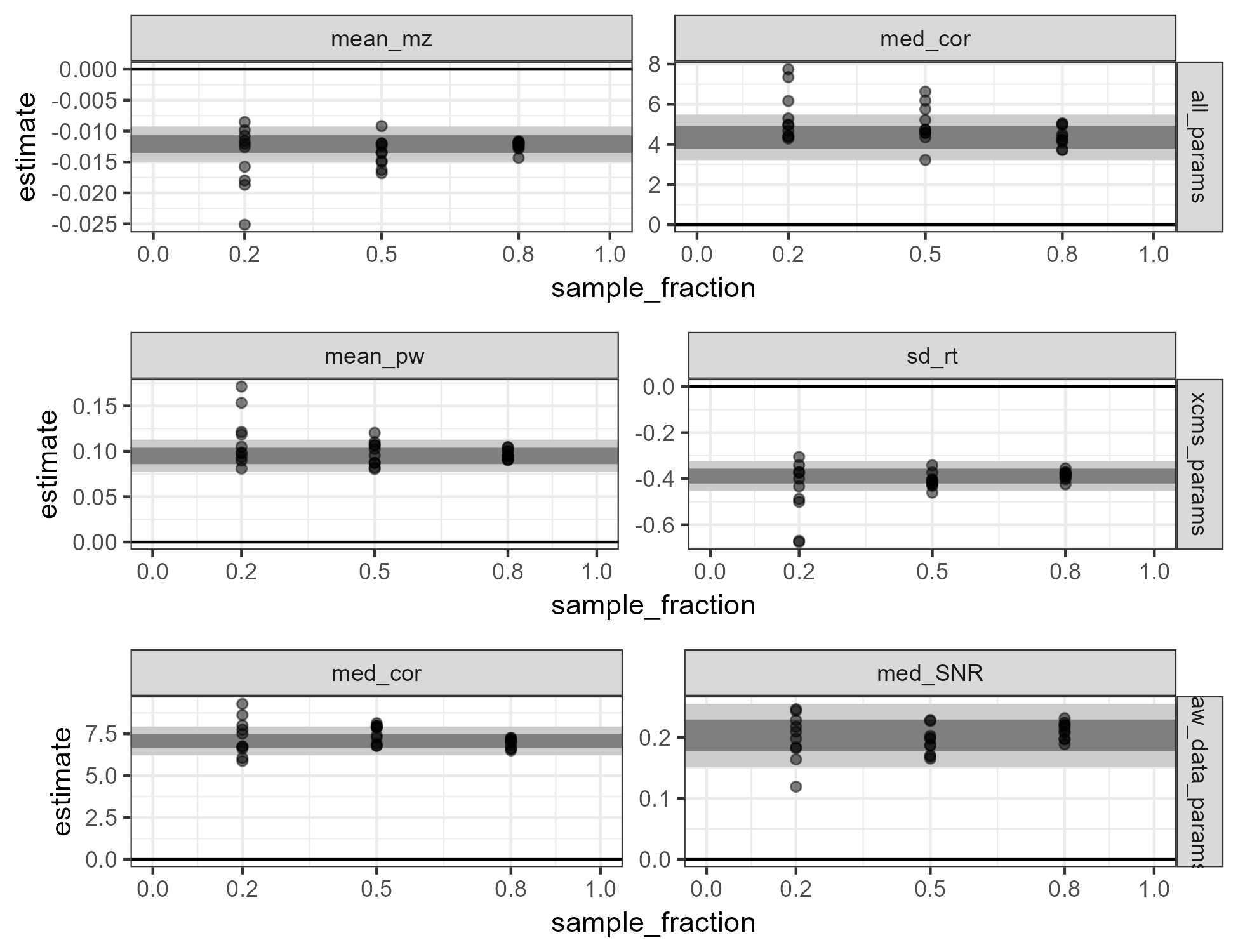
## Acknowledgements

## References

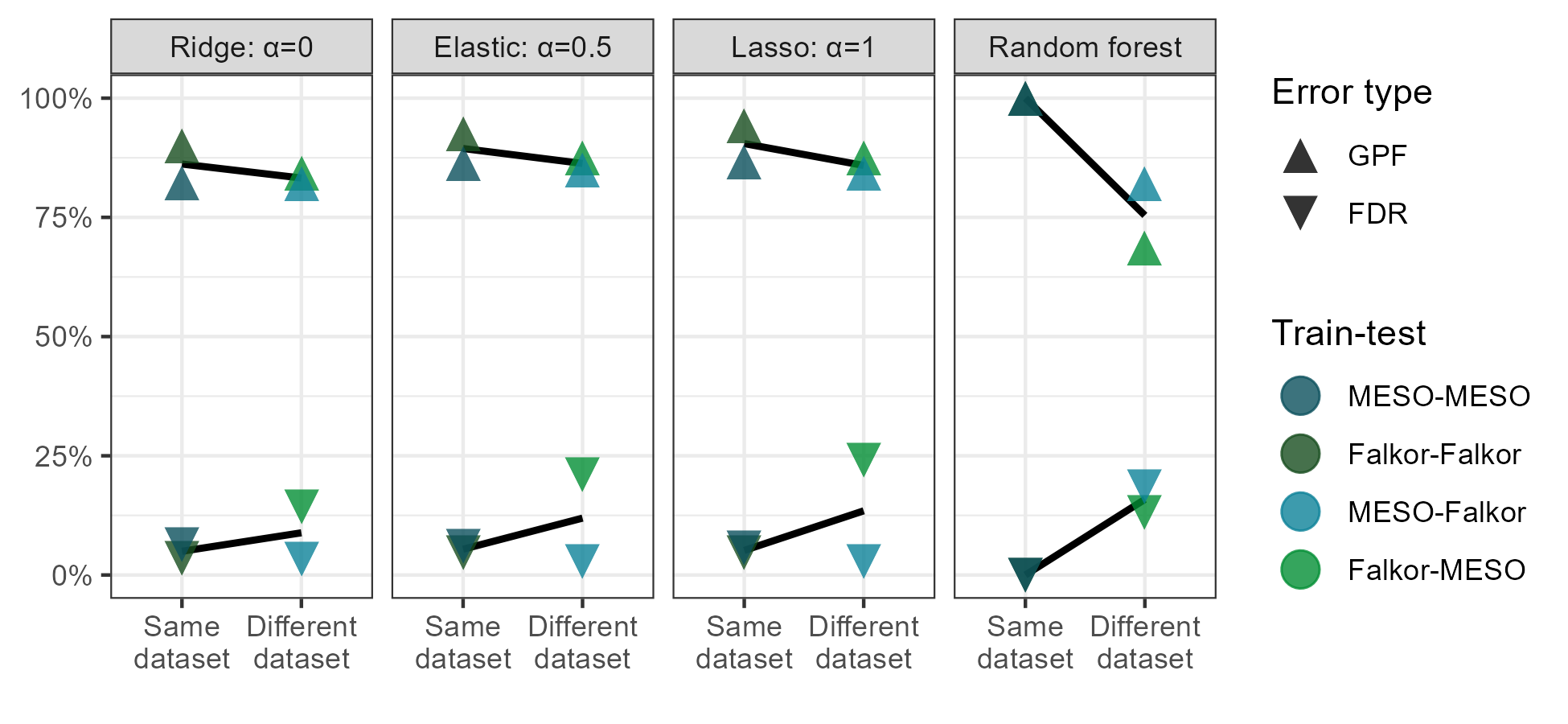
## Supplement



Supplemental figure 1: Model parameter estimates for each of the metrics in the full model, additionally broken down by their inclusion in the two-parameter (raw\_data) and XCMS-exclusively models. Colors correspond to the dataset used to train the logistic regression model, with “both” indicating a combined model using all manually-labeled features across both datasets. <Explanations of the actual facet names forthcoming.>



Supplemental figure 2: Robustness of the two most significant metrics across the full (all\_params), XCMS-only (xcms\_params), and two-parameter (raw\_data\_params) models. The x-axis corresponds to the fraction of the data used to train the model and the y-coordinate shows the estimated value for the specified term in the subset across 10-fold replicated subsampling. The grey bar in the background corresponds to the estimate of the full model +/- 1SE (thinner dark grey bar) and 2SE (thicker light grey bar).



Supplemental figure 3: Performance of regularized regression and random forest models on internally (same train-test) and externally (different train-test) validated datasets.