

**FA signatures of the diatom *Skeletonema* cf. *marinoi*: just how natural is culturing anyway?**

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## **Abstract**

Spatial subsidies including particulate organic matter (POM) play an important role in near-shore food web ecology. Fatty acid (FA) analysis has proven an effective tool for constructing these food webs, although the FA profiles of many marine autotrophs and the validity of culturing is still unknown. We cultured the diatom *Skeletonema cf. marinoi* using dialysis tubes and compared the FA profile to published batch culture FA profiles and to POM FA profiles. PERMANOVA analysis showed that FA profiles of dialysis replicates were not significantly different from each other ( $p=0.14$ ) but were different from batch culture ( $p=0.001$ ) and POM data ( $p=0.0001$ ). FA suites from cultured phytoplankton may delineate specific phytoplankton taxa, but further studies are necessary to determine the most accurate culturing method.

## **Introduction**

Many ecologists have recently considered the importance of energy transfer from one ecosystem to another in the form of food and nutrients (Polis et al. 1997, Nakano & Murakami 2001, Fagan et al. 2007). In marine environments, this energy exists in detritus from macroalgae and microalgae. Using stable carbon isotope analysis, kelp detritus has been shown to subsidize subtidal environments (Duggins et al. 1987). The extent of this subsidy is still unknown, but it may be more important than phytoplankton to deep water habitats. Biomarkers such as stable isotopes and fatty acids (FA) can be used to assess the contributions of different primary producers to food webs (Nichols et al. 1985, Kharlamenko et al. 2001, Hanson et al. 2010). In particular, these biomarkers may allow the determination of the relative contribution of macroalgae, microalgae and particulate organic matter (POM) to benthic consumers. Baseline values for stable isotope and FA

composition of many kelps already exist (Galloway et al. 2012) but pure phytoplankton values are difficult to obtain and thus it is hard to delineate their biomarkers.

Due to the difficulty of collecting individual plankton species or even just separating phytoplankton from zooplankton from the wild for FA and isotope analysis, profiles are typically created for a conglomerate of plankton (Hama 1999, Najdek et al. 2002, Maazouzi et al. 2008). In order to identify species specific FA and stable isotope profiles of phytoplankton, monocultures are necessary. Culturing, however, has long been criticized by some as uninformative due to the introduction of culture artifacts (Brutemark et al. 2009).

Diatoms are abundant phytoplankton that bloom rapidly in culture (Lv et al. 2010). While cultured FA profiles for the species *Skeletonema costatum* (Ackman et al. 1964, Volkman et al. 1989, Dunstan et al. 1994, Piveteau et al. 1999, Renad et al. 1999, Pennarun et al. 2003, Tremblay et al. 2007) are already known, the effect culturing has on FA and stable isotope composition is still unclear. In the study we wished to determine the validity of culturing as a method for FA and stable isotope profiling of phytoplankton.

Semi-permeable membranes such as dialysis tubing allow for the inflow and outflow of macronutrients and have been used for the culture of *Skeletonema costatum* (Yoder 1979). Here we employ this method to culture *S. cf. marinoi* to obtain its FA composition. The signature is then compared to previous studies utilizing batch culture and to FA signatures of POM obtained from (Lowe-in prep).

## **Methods**

We obtained *Skeletonema cf. marinoi* stock from the University of Washington Friday Harbor Labs, cultures of Dr. C. O'Kelley. Strain F162 was chosen for its rapid

growth. Three 25mL aliquots of stock culture were added to three flasks containing 100mL of sterile sea water and allowed to grow in a 15°C culture chamber with 16:8 light/dark regime. After two weeks, eight dialysis tubes were inoculated with ~25mL of culture. The tubes were then filled with ~2L of sterile sea water filling ~1.2m of tubing. The dialysis tubes were bathed in flowing sea water at 10°C under a 18:6 light:dark regime using four 100watt halogen bulbs. The dialysis tubes were regularly wiped to remove algal growth on the outsides. 5 mL aliquots were removed and preserved in IKI for cell counts daily beginning the ninth day after inoculation. Cell counts were later performed using an Improved Neubauer Hemocytometer and four replicate samples. After 15 days, the contents of the tubes were filtered through Whatman GF/F filters and stored at -28°C. The samples were later freeze dried in a lyophilizer for 48 hours and ground into a powder. We performed the FA extraction following Brett et al. (2009).

A second culture treatment used the batch culture method. Two ~1.2m wide plastic tubes were sealed using clips and placed in a wire cage. The tubes were then filled with 25.5L of sterile sea water. 3.5 mL/L of Guillard's (F/2) Marine Enrichment excluding vitamins was added after filtering through a 0.2µm syringe filter. The sealed tubes were then inoculated with 1500mL of *S. cf. marinoi* culture. Aliquots (5mL) were removed as above for cell counts beginning the fourth day after inoculation. We performed the experiment in and outside of the Fernald building at the University of Washington's Friday Harbor Labs, Friday Harbor, WA.

We identified 22 FAs in the *S. cf. marinoi* from the dialysis culture. Of these, 17 were also reported by the majority of the papers reporting values for *Skeletonema spp.* The additional FAs we found are: 15:0, 16:1n-5, iso-17:0, 20:3n-6 and 22:5n-3. 16:4n-1

was not found in our sample but existed in other studies. It is possible that other studies have recorded iso-18:0 as 16:4n-1 and vice versa, therefore we considered them to be the same FA and included the values in our statistical analyses. Initially we identified 25 FAs using an 85 minute program which others may not use. We removed only FAs that did not constitute more than 1% of the total FA composition. FA extraction was performed at the University of Washington, Seattle, WA.

We used PERMANOVA (Anderson 2001) to evaluate multivariate differences in FA among samples in three comparisons: 1. Pure *S. cf. marinoi* values from four of the dialysis replicates versus the four contaminated replicates. 2. All dialysis FA against published batch data. 3. Both dialysis FA and published batch FA versus POM FA data. In PERMANOVA, statistical significance is determined by repeated  $n=(999 \text{ or } 9,999)$  permutations. Similarly calculations used Euclidean Distances for all FA data.

## **Results**

### *Population Growth.*

The cell count data from both dialysis and early batch-culture treatments are shown in Fig. 1 with means plotted in Fig. 2. Four of the dialysis tubes were found to be contaminated. Tube 2 was contaminated with the diatoms species *Thalassiosira sp.* and *Nitzschia sp.* Tubes 4, 5 and 6 were contaminated with an unknown flagellated alga. Contaminate cells accounted for < 1% of all cells.

### *Fatty Acid Analyses of Dialysis Cultures.*

The results of PERMANOVA analyses on the FA composition of various culture and POM datasets are shown in Table 1. Both the pure and contaminated cultures were

compared. For the first comparison, FA composition did not vary between contaminated and uncontaminated tubes ( $p = .14$ ).

Fig. 3. shows this high overlap between the FA of the pure and contaminated cultures. A stress value of 0.01 reinforces the robustness of the plot. Transforming the data with Log (X+1) transformation did not affect the visual output. Replicates 1, 2, 3 and 4 overlap in the center of the plot while replicates 6 and 8 lie at outer edges, indicating somewhat different FA compositions.

Additional PERMANOVA analyses (Table 1) compared the dialysis culture FA with published batch culture FA for various strains of *Skeletonema*. General information about the culture methods, growing mediums, sources, *Skeletonema* strains and harvest times are included in Table 2. For this comparison (illustrated in Fig. 4), FA of the datasets were significantly different ( $P=0.001$ ).

A stress value of 0.01 reinforces the robustness of the plot. Transforming the data with Log (X+1) transformation or 4<sup>th</sup> root transformation did not affect the visual output. FA profiles from our dialysis culture replicates are tightly clustered compared to the scattered published culture values, showing a high degree of inconsistency among FA in batch cultures. Figure 5 shows the importance of specific FAs to this analysis. FAs 16:4n-1 / iso-18:0 play a role in differentiating samples 9, 13 and 14. FAs 22:6n-3 and 16:2n-4 contribute to differentiating samples 10, and 17 and 18 respectively.

For comparisons of *Skeletonema* cultures with FA of POM samples (Lowe in-prep) we included three additional FAs (18:3n-6, 20:4n-6 and 20:4n-3) that were not present in the POM data were added for comparison with values entered as 0.00. A PERMANOVA (dataset 3, Table 1) comparing FA in the dialysis culture, published batch

culture and POM showed these three groups to be significantly different ( $P=0.0001$ ). Figure 6 illustrated these differences. A stress value of 0.09 reinforces the robustness of the plot. Transforming the data with Log (X+1) transformation did not affect the visual output. Both POM samples and batch culture samples had variable FA signatures compared with the very consistent dialysis culture data.

## **Discussion**

Even with mild amounts of contamination, common in culturing, FA composition is still dominated by the desired species, *S. cf. marinoi*.

Many factors appear to affect the FA profiles of cultured species. Even cultures of the same species can yield different signals. Light quality and quantity, nutrient levels, temperature and strain may account for this. Ackman (1964) showed that FA composition changes through time. Batch culture Sample 9 (Fig. 6) also has a very distinct FA signal. This study (Piveteau et al. 1999) performed FA analysis earlier in the culture process than the other studies, during mid-log phase. Some strains were isolated to grow faster in culture. Because of this, the growth phase at the time of FA analysis must be known.

POM samples 23-28 had very different FA signatures from both the dialysis and the batch culture samples. The phytoplankters dominating these POM samples were dinoflagellates, not diatoms suggesting that dinoflagellates have distinctly different FA signatures. With such a distinct signal, in the future it may be possible to determine the presence of specific phytoplankter taxa by the FA signal, although additional culture work of pure phytoplankters is needed before such conclusions can be drawn. Galloway et al. (2012) showed similar promising results with taxonomic differences in FA of macrophytes. A suite of FAs are signaled more strongly in the dialysis cultures compared

to all the batch cultures (as illustrated in Fig. 5). Certain FAs may even be used to delineate specific strains.

The variation in POM and batch culture FA signatures suggests that dialysis culture may be an excellent method for getting clear FA signatures for particular phytoplankton. Once attained, this data will be essential in identifying the composition of POM. With the FA profiles of both macroalgae and microalgae well established, their individual importance as subsidies can be better described.

## **Conclusion**

Several forms of culturing phytoplankton exist. Dialysis culturing to obtain FA signatures is promising, but requires more testing. Future studies are necessary to determine the effect different variables have on these profiles. FA profiles may be used to distinguish taxa and species within POM and may even hold enough precision to differentiate strains.

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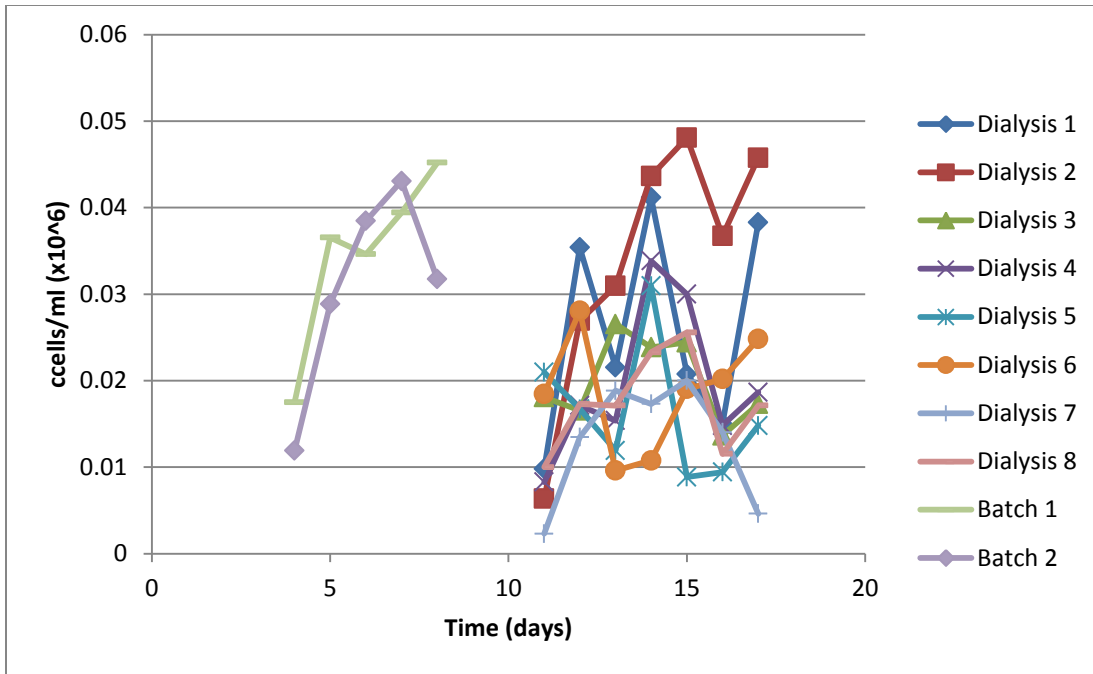


Fig. 1. Population growth of *S. cf. marinoi* in both dialysis culture and batch culture. Eight dialysis and two batch replicates are included. Dialysis cultures were stopped at day 17, during suspected late log phase. Batch cultures continued beyond day 8.

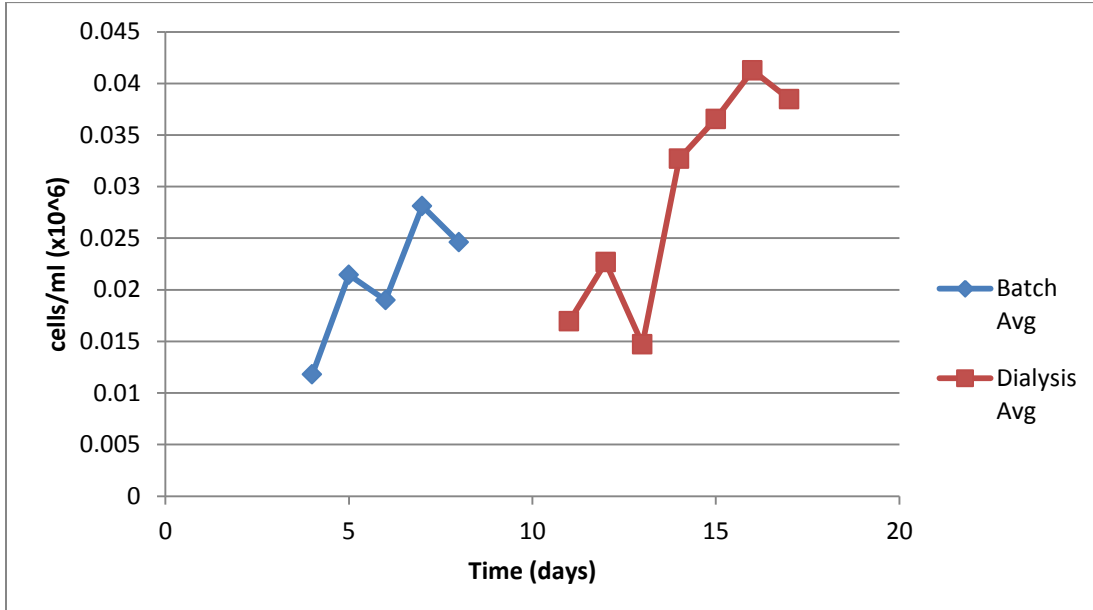


Fig. 2. Average population growth of *S. cf. marinoi* in both dialysis culture and batch culture. Eight dialysis and two batch replicates are included. Dialysis cultures were stopped at day 17, during suspected late log phase. Batch cultures continued beyond day 8.

Table 1. Results of PERMANOVA testing for differences in FA percent composition of *Skeletonema* spp. and POM. Dataset 1 includes the 8 dialysis culture replicates of *S. cf. marinoi*. Dataset 2 uses the 8 dialysis replicates and published batch culture values. Dataset 3 again includes the 8 dialysis replicates, the published batch culture values and POM values from (Lowe-in prep).

Dataset							
Source		df	SS	MS	Pseudo-F	P(perm)	Unique perms
1.	22FA Dialysis Only	1	6.2402	6.2402	2.1978	0.1416	35
	Residual	6	17.036	2.8393			
	Total	7	23.276				
2.	17 FA Dial + Lit	1	1779.7	1779.7	12.102	0.001	984
	Residual	16	2352.9	147.05			
	Total	17	4132.6				
3.	17 FA Dial + Lit + POM	2	4207.9	2103.9	12.41	0.0001	9932
	Residual	25	4238.3	169.53			
	Total	27	8446.1				

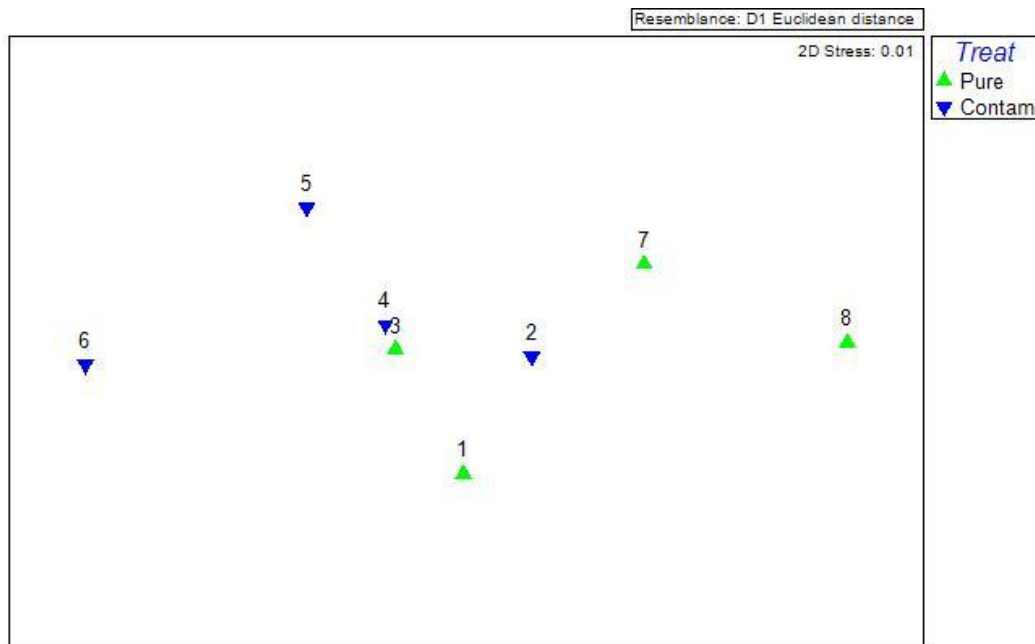


Fig. 3. Nonmetric Multidimensional Scaling (NMDS) on raw Euclidean distance plot of *S. cf. marinoi* FA composition data. 22 FAs are included comparing the pure dialysis cultures to the contaminated dialysis cultures.

Table 2. Dialysis, published batch and in-prep POM FA data. Quality of sample (Dialysis), culture medium (Batch) and sample number (POM) listed as Details. Sample name (POM) listed as Strain#. Percentage of phytoplankters in sample (POM) as Harvest Time.

Dialysis data in context					
ID	Method	Details	Source	Strain#	Harvest Time
1	Dialysis	Pure	This study	F162	LateLog
2	Dialysis	Contam	This study	F162	LateLog
3	Dialysis	Pure	This study	F162	LateLog
4	Dialysis	Contam	This study	F162	LateLog
5	Dialysis	Contam	This study	F162	LateLog
6	Dialysis	Contam	This study	F162	LateLog
7	Dialysis	Pure	This study	F162	LateLog
8	Dialysis	Pure	This study	F162	LateLog
9	Batch	NA	Piveteau-1999	NA	MidLog
10	Batch	Con+Sil	Tremblay-2007	NA	LateLog
11	Batch	f/2	Renaud-1999	GOC36	LateLog
12	Batch	f/2	Renaud-1999	GOC27 (sp)	LateLog
13	Batch	f/2	Dunstan-1994	CS-181	LateLog
14	Batch	f/2	Dunstan-1994	CS-252 (sp)	LateLog
15	Batch	f/2	Volkman-1989	CS-181	MidLog
16	Batch	NA	Pennarun-2003	NA	NA
17	Batch	NA	Ackman-1964	NA	LateLog
18	Batch	NA	Ackman-1964	NA	LateLog
19	POM	2	Lowe-in prep	25APR11_SKP1	Diat>40
20	POM	3	Lowe-in prep	25APR11_PTC1	Diat>40
21	POM	8	Lowe-in prep	2MAY11_SKP3	Diat>40
22	POM	13	Lowe-in prep	OS_30JUL11	Diat>40
23	POM	21	Lowe-in prep	23AUG11_PIL1	Dino>50
24	POM	23	Lowe-in prep	23AUG11_PTC1	Dino>20
25	POM	24	Lowe-in prep	26AUG11_PIL2	Dino0.06
26	POM	25	Lowe-in prep	26AUG11_SKP2	Dino0.04
27	POM	26	Lowe-in prep	26AUG11_PTC2	Dino0.12
28	POM	29	Lowe-in prep	2SEP11_PTC3	Dino>20

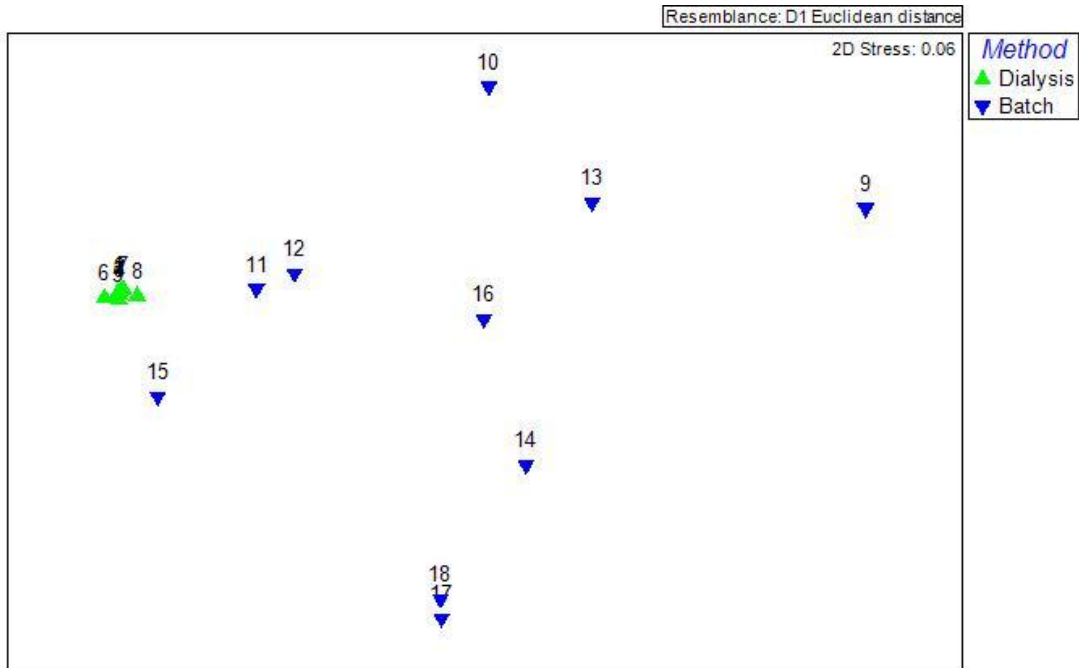


Fig. 4. Nonmetric Multidimensional Scaling (NMDS) on Euclidean distance plot of *S. cf. marinoi* raw FA composition data. 17 FAs are included comparing all dialysis cultures to published batch cultures.

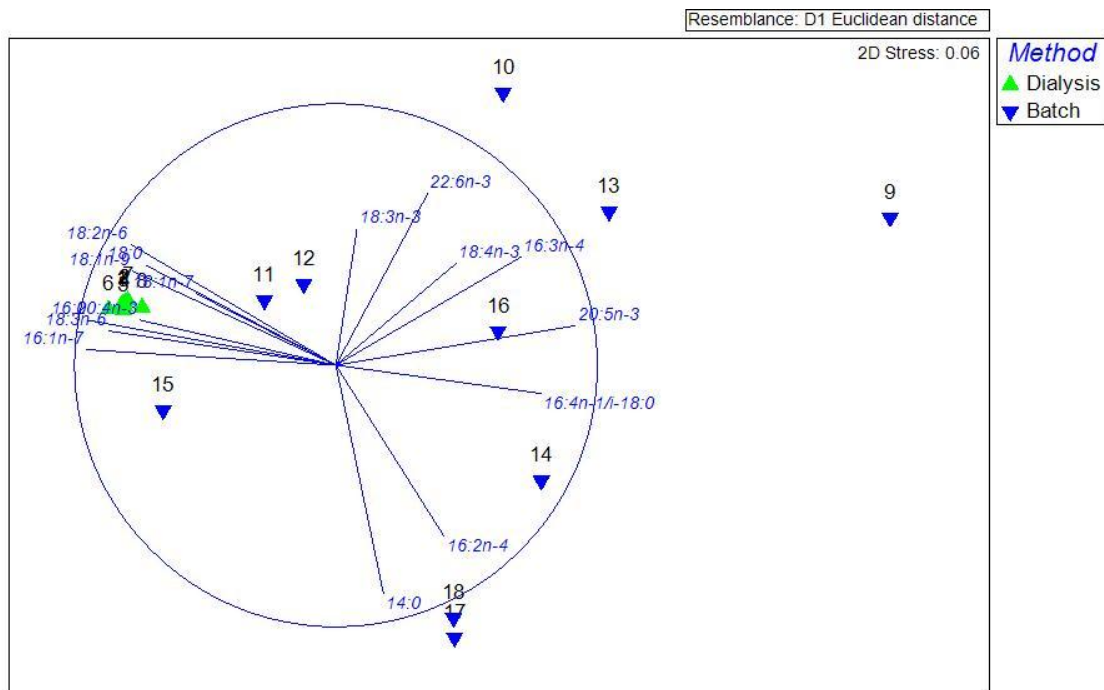


Fig. 5. Nonmetric Multidimensional Scaling (NMDS) on raw Euclidean distance plot of *S. cf. marinoi* FA composition data. 17 FAs are included comparing all dialysis cultures to published batch cultures. Vectors included to show strain of specific FAs.

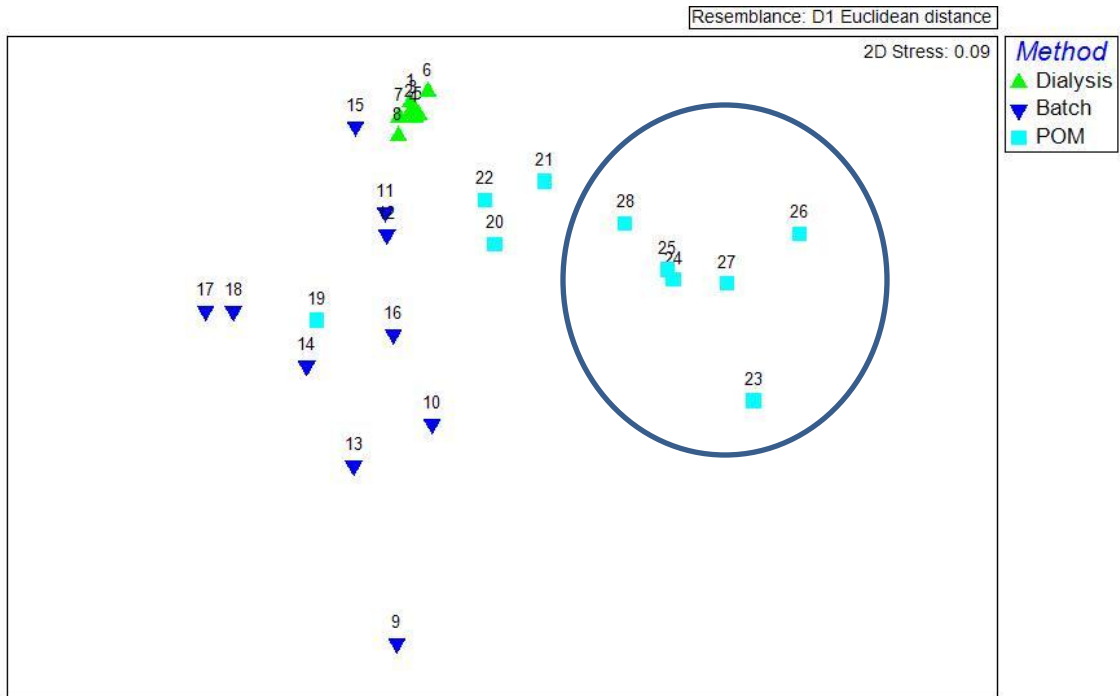


Fig. 6. Nonmetric Multidimensional Scaling (NMDS) on raw Euclidean distance plot of *S. cf. marinoi* FA composition data. 17 FAs are included comparing all dialysis cultures to published batch cultures and in-prep POM data. POM compositions with dominate phytoplankters as dinoflagellates circled.