SELDI-TOF-MS ANALYSIS OF CIRCADIAN EXPRESSION OF PROTEINS FROM *KARENIA BREVIS*, A HARMFUL ALGAL BLOOM CAUSING DINOFLAGELLATE

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Introduction

Issues related to harmful algal blooms (HABs) are of significant concern to coastal marine researchers, as well as ecologists and public health officials worldwide. HAB causing microorganisms produce toxins that can have debilitating effects on fish and mammalian tissues. The photosynthetic marine dinoflagellate *Karenia brevis* is primarily responsible for the "red-tide" blooms observed throughout Florida and the Gulf coast. Blooms of *K. brevis* occur through vegetative cell division regulated by a circadian rhythm entrained to the diel cycle. Further knowledge of the nature of circadian entrained diel cycling in *K. brevis* may prove useful to researchers and public health officials seeking to understand the mechanisms underlying HAB occurrences.

A number of methods have been used to characterize the chronobiological behavior of marine microorganisms. Diel phasing in *K. brevis* has been determined by flow cytometry of DNA-stained cultures (Van Dolah and Leighfield, 1999). 2D-PAGE has proven successful for the determination of biorhythmicity in the marine dinoflagellate *Lingulodinium polyedrum* (Akimoto, *et al.*, 2004). Here, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) was combined with cosinor-rhythmometry to analyze diel cycling of proteins expressed in *K. brevis* cultures.

SELDI-TOF-MS (Ciphergen Biosystems, Fremont, CA) is a chip-based variant of MALDI-TOF-MS ideally suited for biomarker analysis of marine and freshwater microorganisms (Yuan and Carmichael, 2004; Gregson *et al.*, submitted). Cosinor-rhythmometry is a calculation-intensive mathematical procedure to evaluate the complex characteristics of "rhythms" from sample datasets (Nelson, *et al.*, 1979). A robust and user-friendly adaptation has been developed that uses commonly available spreadsheet software to simplify the process of analyzing basic biological and physiological circadian data (Bourdon, *et al.*, 1995). The advanced biomarker functionality of SELDI-TOF-MS was combined with the analytical capabilities of a cosinor-rhythmometry spreadsheet to further characterize diel cycling in *K. brevis.* In lysates from diurnally maintained cultures, a substantial number of protein peaks were detected that exhibited circadian patterns of expression.

Methods

Sample preparation

Cultures of *K. brevis* were maintained in diurnal (12 hours light, 12 hours dark), constant dark or constant light conditions for approximately 3 weeks. Samples were harvested every 2 hours for 24 hours and concentrated by gentle centrifugation. Cell pellets were resuspended in a small volume of buffer and lysed by vigorous vortexing. Sample lysates were desalted and adjusted to equal concentrations of total protein. Replicate aliquots of desalted lysate supernatants were applied to prepared Q10 anion-exchange ProteinChip® (Ciphergen Biosystems) spots and washed with a low stringency buffer according to the manufacturer's recommendations. Fifty-percent saturated sinnapinic acid (SPA) was deposited twice on each spot, with drying between applications. TOF-MS laser desorption/ionization was performed using standard parameters on a Ciphergen PBS IIC+ with ProteinChip® software v.3.2.1 (Ciphergen Biosystems).

Data analysis

Spectra from all sample timepoints were copied to a single ProteinChip software experiment. Biomarker Wizard Software (embedded in ProteinChip Software) was run to identify common peaks that differed from those of the control cultures.

Peak data from the Biomarker Wizard analysis was exported as comma-saved value files and subsequently imported into an Excel 2003 (Microsoft, Redmond, WA) spreadsheet. The data were sorted by individual peaks. Peak intensity data for each timepoint were copied into spreadsheets embedded and validated with the necessary formulae to perform cosinor rhythmometry (Bourdon, *et al.*, 1995; Figures 3, 4). Resulting plots were generated and datasets with critical F-values greater than the value corresponding to a 95% confidence level were considered to exhibit significant patterns of circadian rhythmicity.

Results

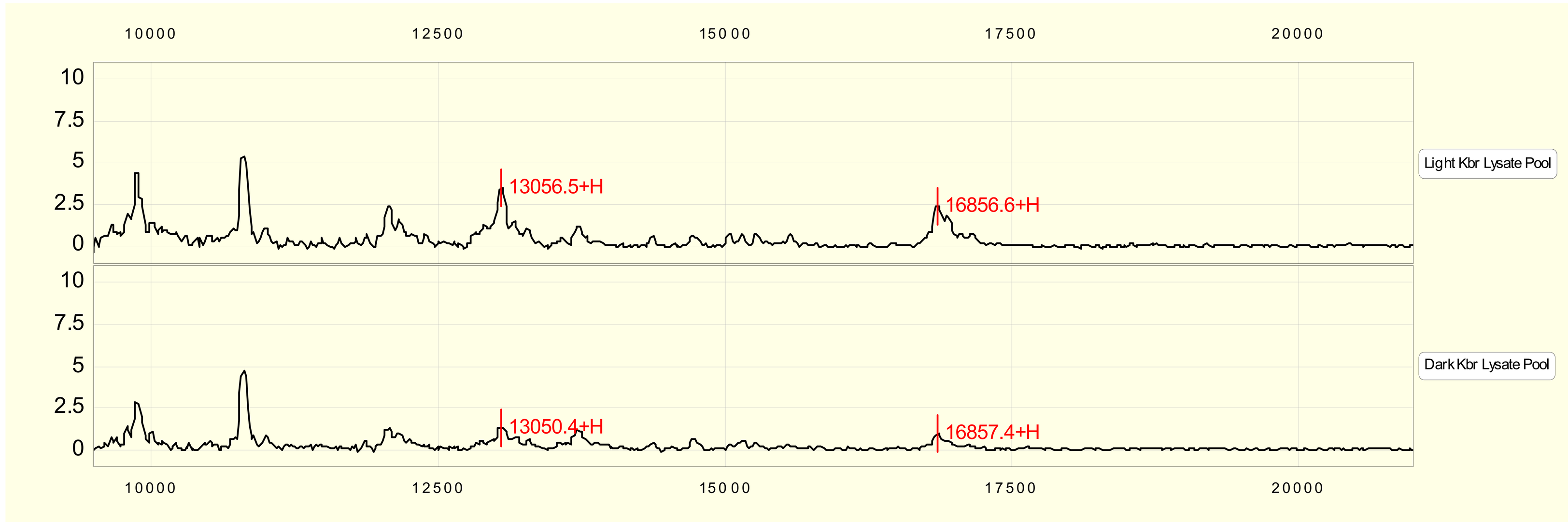


Figure 1. SELDI-TOF-MS spectra of a preliminary experiment comparing *K. brevis* cultures maintained in conditions of constant light or constant dark. For the peaks indicated in red (M/Z = 13059, 16863), the culture maintained in constant light exhibits substantially greater peak intensity (2.356 vs. 1.853 and 1.816 vs. 1.244, respectively)

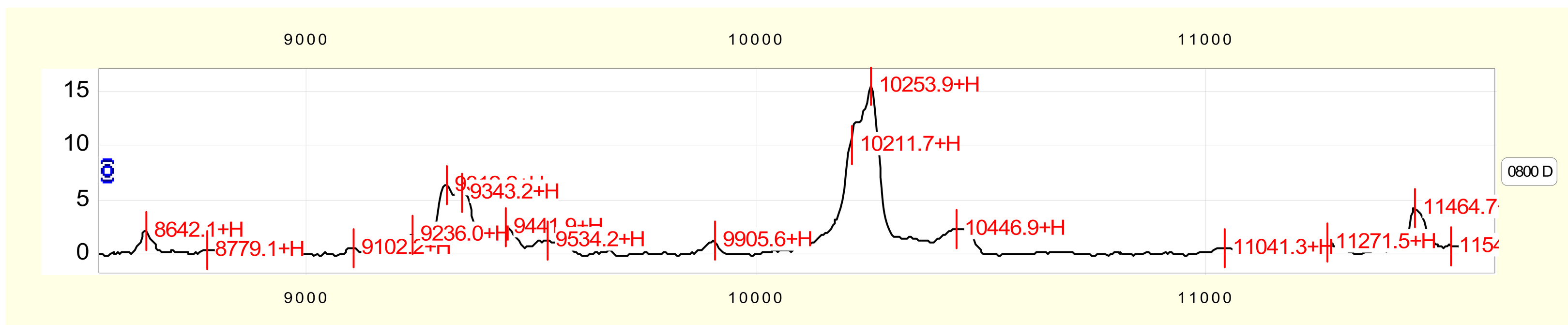


Figure 2. Representative SELDI-TOF-MS spectrum of a sample from one timepoint (t=8 hours). For clarity, the M/Z region from ca. 8500-12000 has been expanded, with significant peaks common to all timepoints indicated in red. In all, 42 protein peaks common to 12 bi-hourly timepoints were identified that were significantly different from the control culture maintained in conditions of constant light.

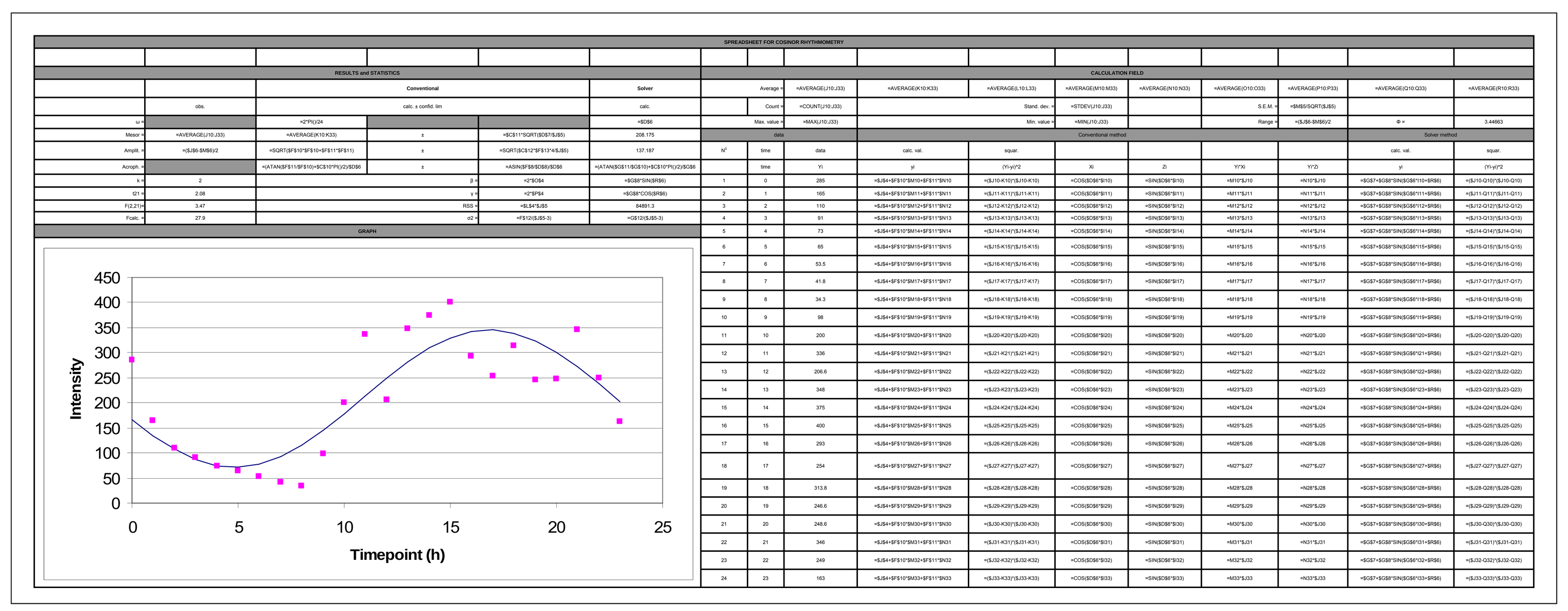
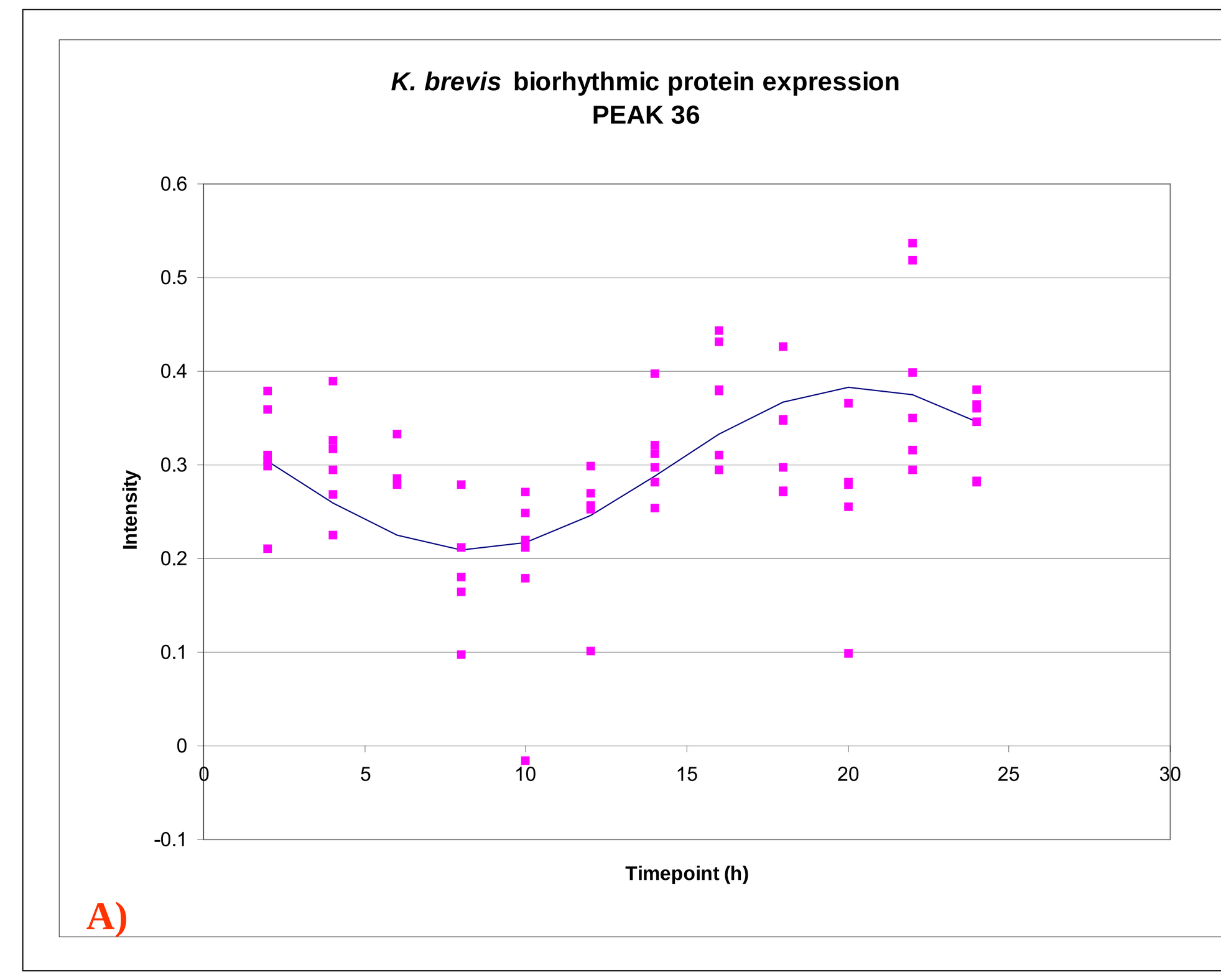


Figure 3. Excel spreadsheet formulae for cosinor-rhythmometry analysis of each peak identified by SELDI-TOF-MS. The spreadsheet was validated using a mock dataset with known results, contrived to represent hourly timepoints ("time") and Peak Intensity ("Y1"). The critical F-value for the 95th percentile of an F-distribution with (2,n-3) degrees of freedom is shown ($F_{(2,21)}$), along with the corresponding t-value (t_{21}). Values for F_{calc} greater than the critical value are considered significant. A resultant graph of this mock data demonstrates a significant circadian rhythm. Adapted from Bourdon *et al.* (1995).



PEAK #	M/Z	F(.05,2,64) > 3.15	F(.05,2,64) < 3.15	Circadian?
1	4038	22.91434814		YES
2	4640	35.8151765		YES
3	4845	18.45583146		YES
4	4933	31.40903023		YES
5	5392	27.87800325		YES
6	6394		2.501446416	NO
7	6464		0.297842607	NO
8	6710	32.15277886		YES
9	6780	32.95477639		YES
10	6896	19.47136912		YES
11	7046	75.18983831		YES
12	7134	10.69518604		YES
13	7356	5.754180876		YES
14	7496	11.46996181		YES
15	7702	13.34344665		YES
16	7966		2.231378766	NO
17	8081	63.99502244		YES
18	8301	45.47658153		YES
19	8654		1.735066091	NO
20	8775		1.998785864	NO
21	9103		1.082000362	NO
22	9231	8.549454009		YES
23	9306	4.694907002		YES
24	9344	4.758293651		YES
25	9439	5.451643384		YES
26	9567	6.675071157		YES
27	9903		2.075627927	NO
28	10212		0.189619926	NO
29	10255	38.43276894		YES
30	10448	19.64956905		YES
31	11015		2.343078199	NO
32	11266	18.29974956		YES
33	11464	63.14167857		YES
34	11547	14.01169527		YES
35	11676	23.54298122		YES
36	13115	19.12778564		YES
37	16228	13.74398947		YES
38	16229	17.16969226		YES
39	16521	15.57831093		YES
40	20989		2.348171213	NO
41	28030		1.03608337	NO
42	35153	20.07659197		YES

B)

Figure 4. A) Circadian expression of a representative protein peak from diurnally maintained *K. brevis* culture lysate, identified by SELDI-TOF-MS and analyzed using the cosinor-rhythmometry spreadsheet ($F_{(.05, 2,64)}=19.13$, $p < 0.05$). **B)** For each timepoint sampled from the diurnal cultures, 42 common protein peaks were identified to have significantly different levels of expression compared with the reference culture maintained in constant light. Of those, 31 peaks exhibited significant circadian biorhythmicity when tested using the cosinor-rhythmometry spreadsheet.

Discussion

Using the pattern recognition data analysis feature embedded in the SELDI ProteinChip software, mass spectra of culture lysates obtained from the different timepoints were compared for differences in expression of detectable protein peaks. Early experiments comparing cultures maintained in conditions of constant light or constant dark demonstrated consistent differences in the levels of expression of selected spectral peaks (Figure 1). Additional experiments were conducted to evaluate the nature of such expression differences. When comparing "diurnal" with "light" cultures, 42 peaks were detected that exhibited significantly different levels of expression (Figure 2). The peaks were tested by cosinor rhythmometry for evidence of non-linear rhythmic patterns (Figures 3, 4a). Thirty-one peaks demonstrated significant "circadian" oscillations over a 24-hour time period (Figure 4b). Although the peaks have not yet been identified, it is likely that some of the circadian proteins are involved in regulation of the *K. brevis* cell cycle and/or toxin synthesis. Additional related studies should provide greater knowledge of the role diel cycling plays in the metabolic life cycle of *K. brevis* and related algal blooms.

Literature cited

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