Development of a Raman Spectroscopic Sensor and Library for Classification of HAB Species and Toxins

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Harmful Algal Blooms (HAB)

Non-toxic but can cause anoxic conditions

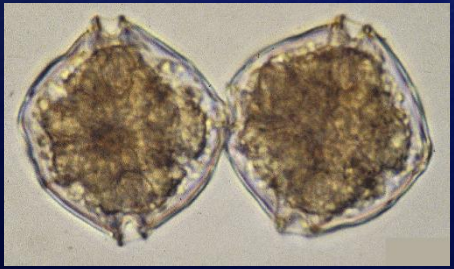
Noctiluca scintillans

Non-toxic to humans but harmful to fish

Cochlodinium polykrikoides

What is the efficacy of Raman spectroscopy for cell and toxin identification ?

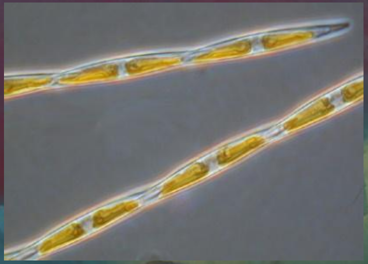
Paralytic Shellfish Poisoning (PSP)
Alexandrium catenella



Produce very potent toxins

Neurotoxic Shellfish Poisoning (NSP)
Karenia brevis

Domoic Acid
Pseudo-nitzschia spp.



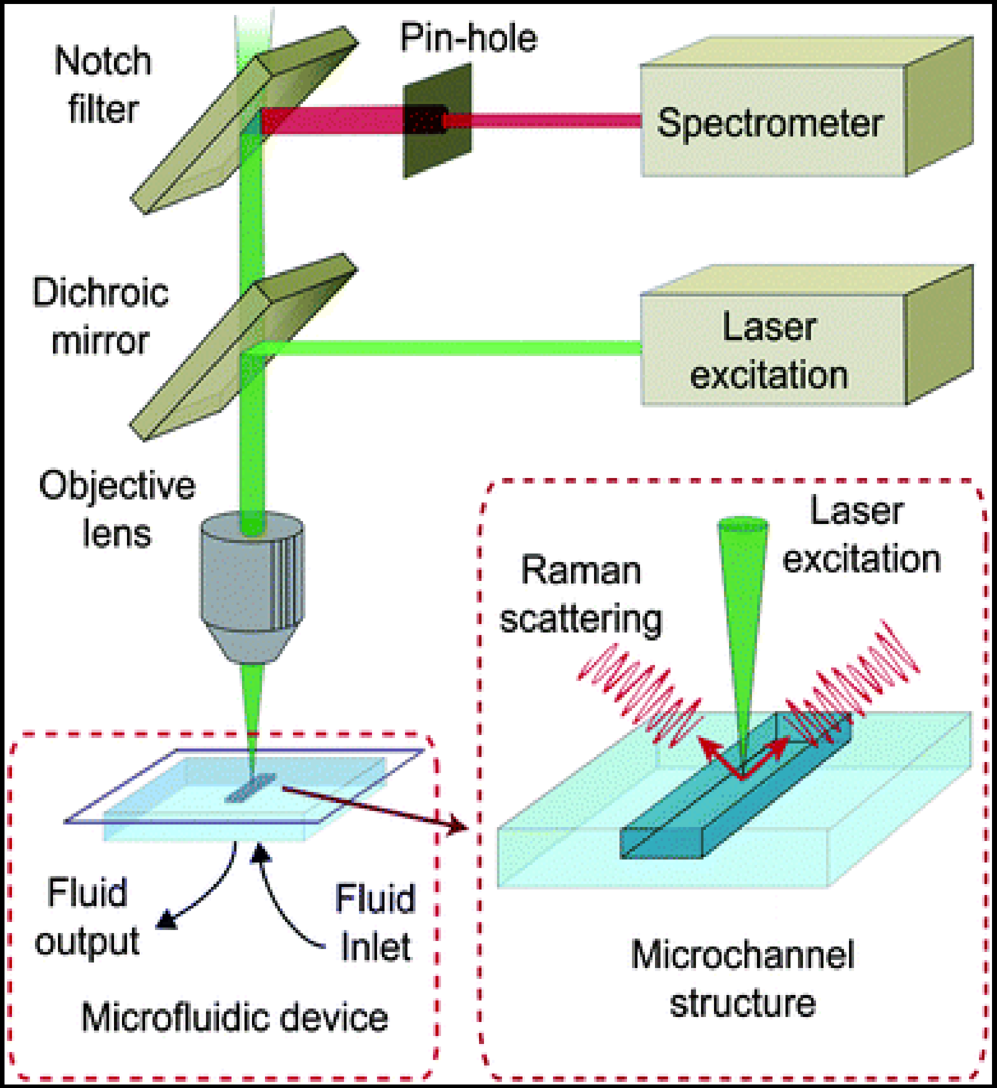
Images via World Register of Marine Species

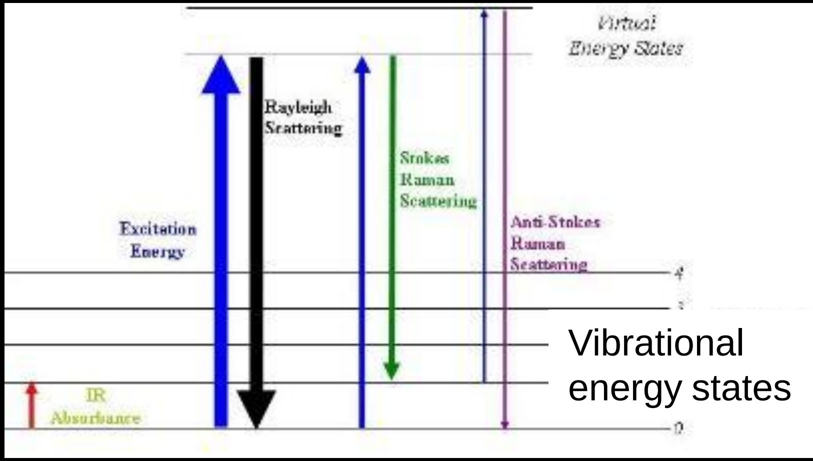
Objectives

1. To combine Raman spectroscopy with image analysis to discriminate between HAB species using Deep Learning

2. To miniaturize and package the sensor to allow hand held, in situ, and distributed arrays of real-time instruments

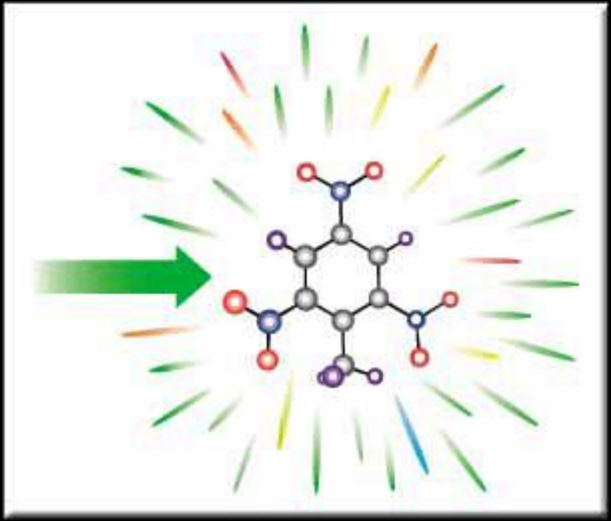
Raman Spectroscopy



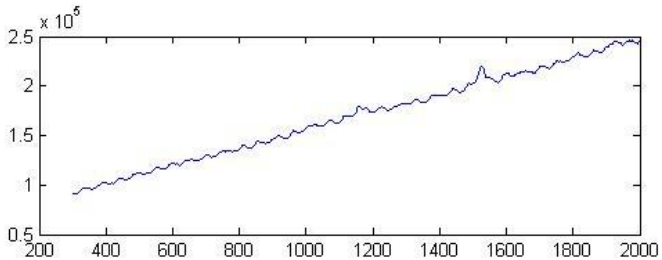


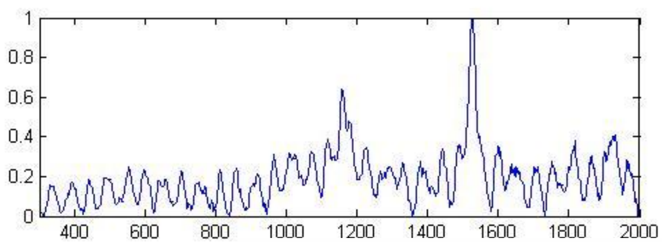
Raman Shift υ (cm^{-1})

$$\upsilon = \frac{1}{\lambda_{incident}} - \frac{1}{\lambda_{scattered}}$$



Processing a Raman Spectra

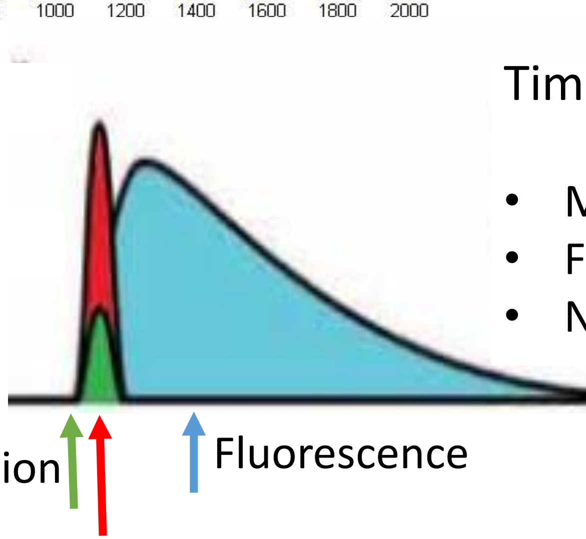




Standard Continuous Excitation Raman

Remove:

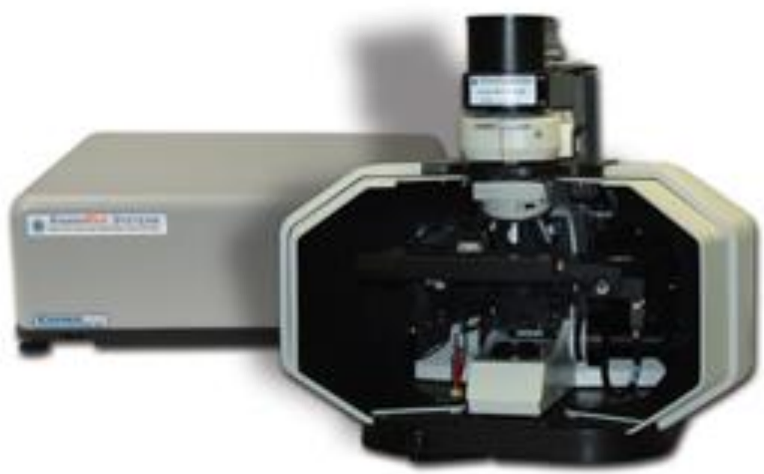
- cosmic ray events
- background fluorescence
- S/N depends on many variables



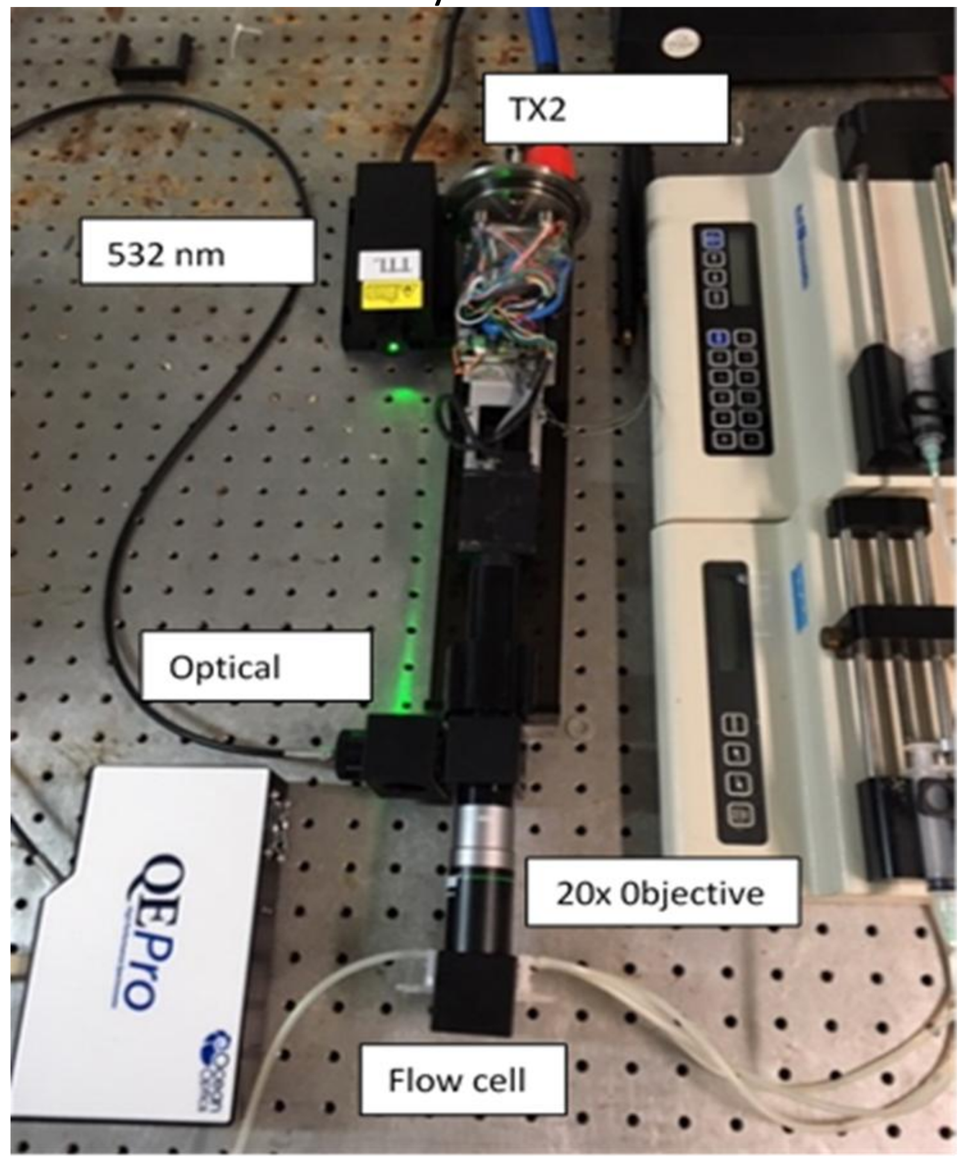
Time-Domain Excitation and Emission Raman

- Measure fluorescence separately from Raman
- Flattens baseline
- No need to use longer wavelength
  low energy laser (e.g., 785, 1025)

Single Cell Analysis on commercial micro-Raman Spectrometer

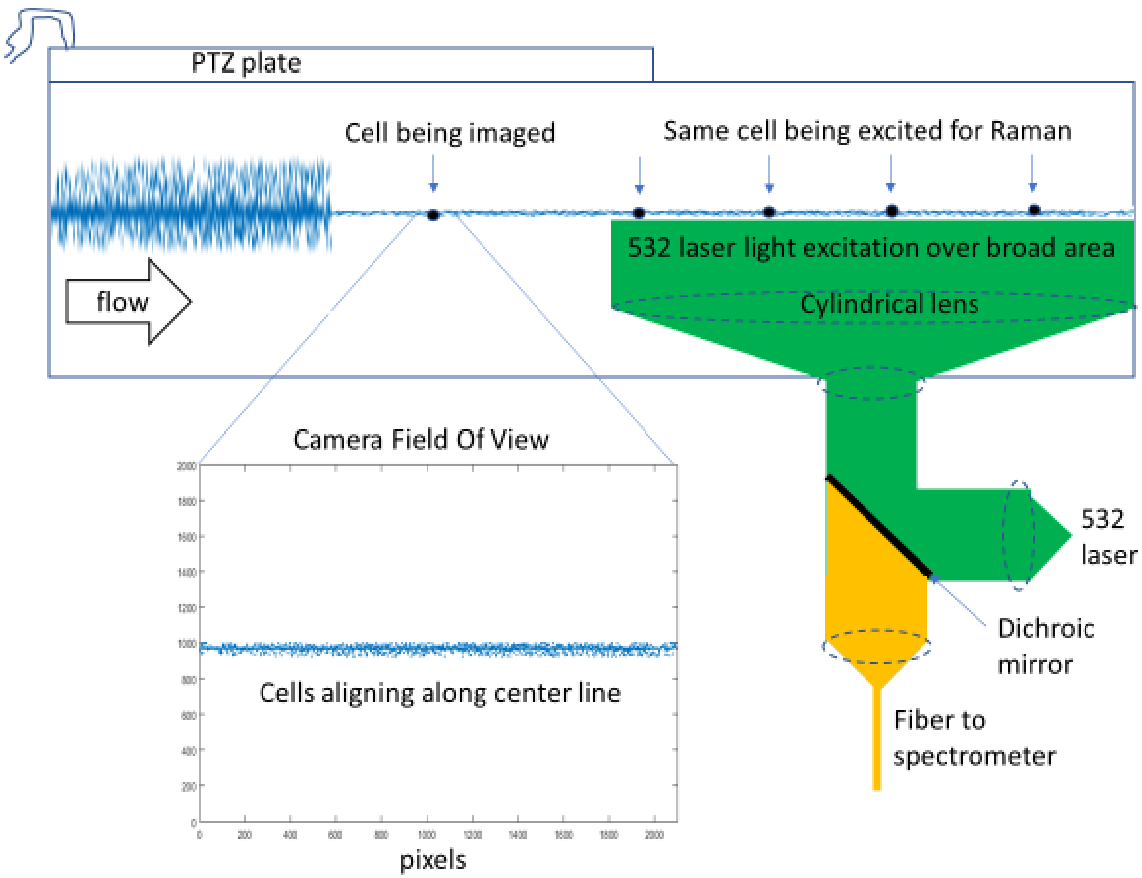


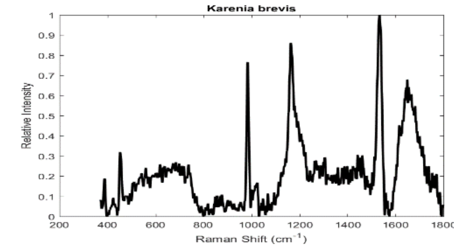
Phase I SBIR:
Prototype time-domain Raman Flow Cell Cytometer



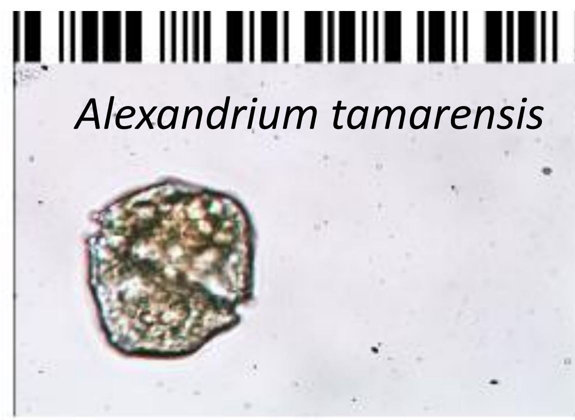
Phase II SBIR Goal:
Population level
Analysis @ 532 nm
in a hand held instrument
or distributed network of sensors

Raman flow cytometer is very different from standard flow cytometer

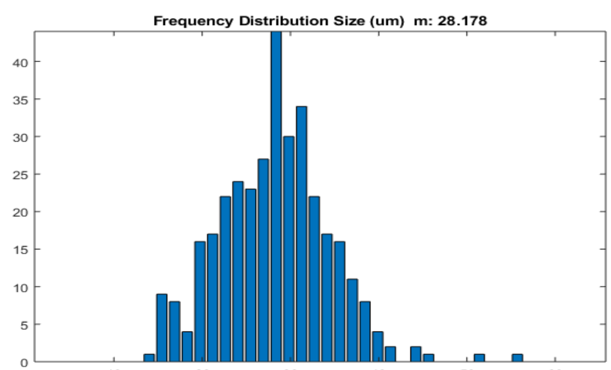




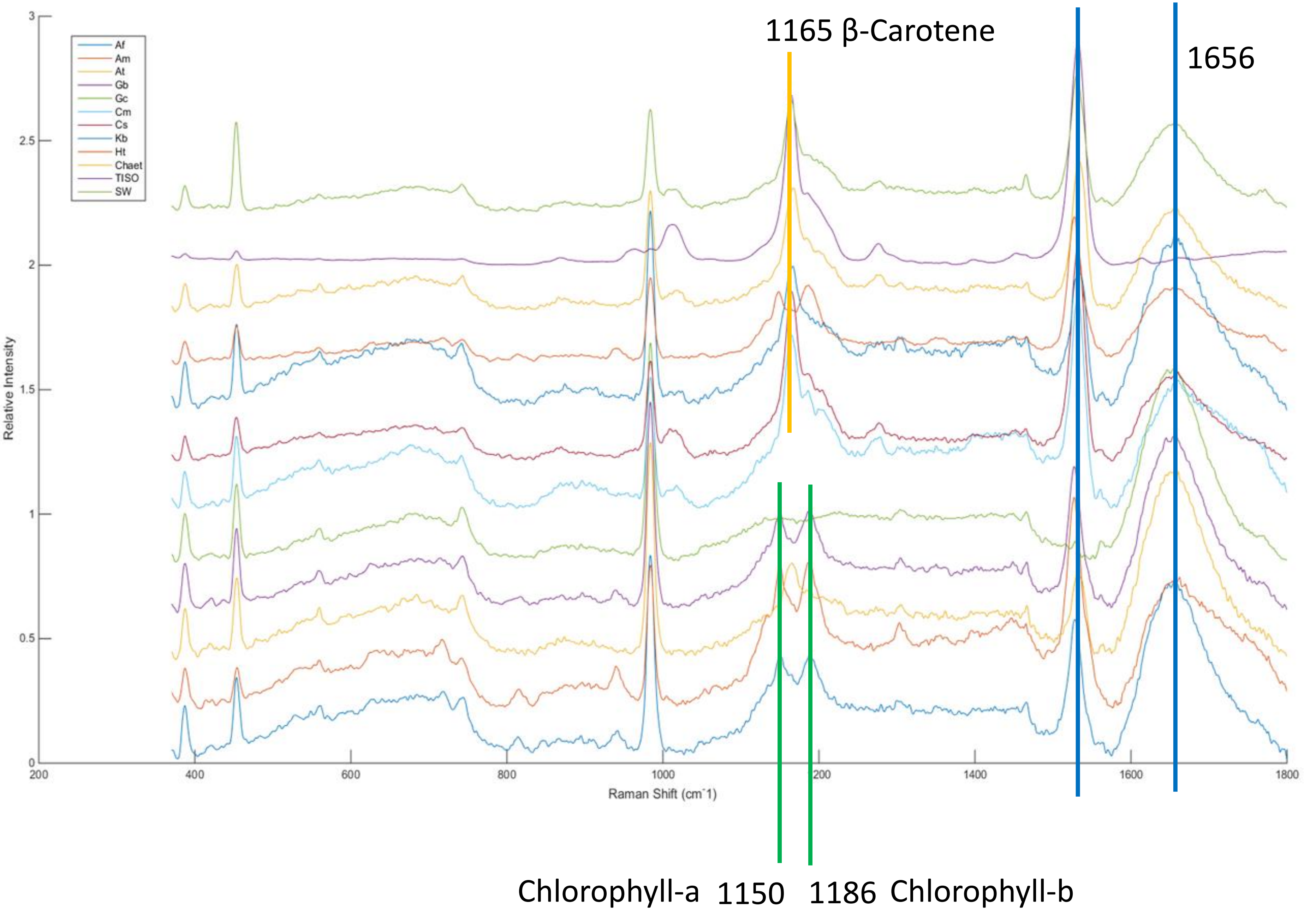
Raman spectrum bar code



Size frequency distribution

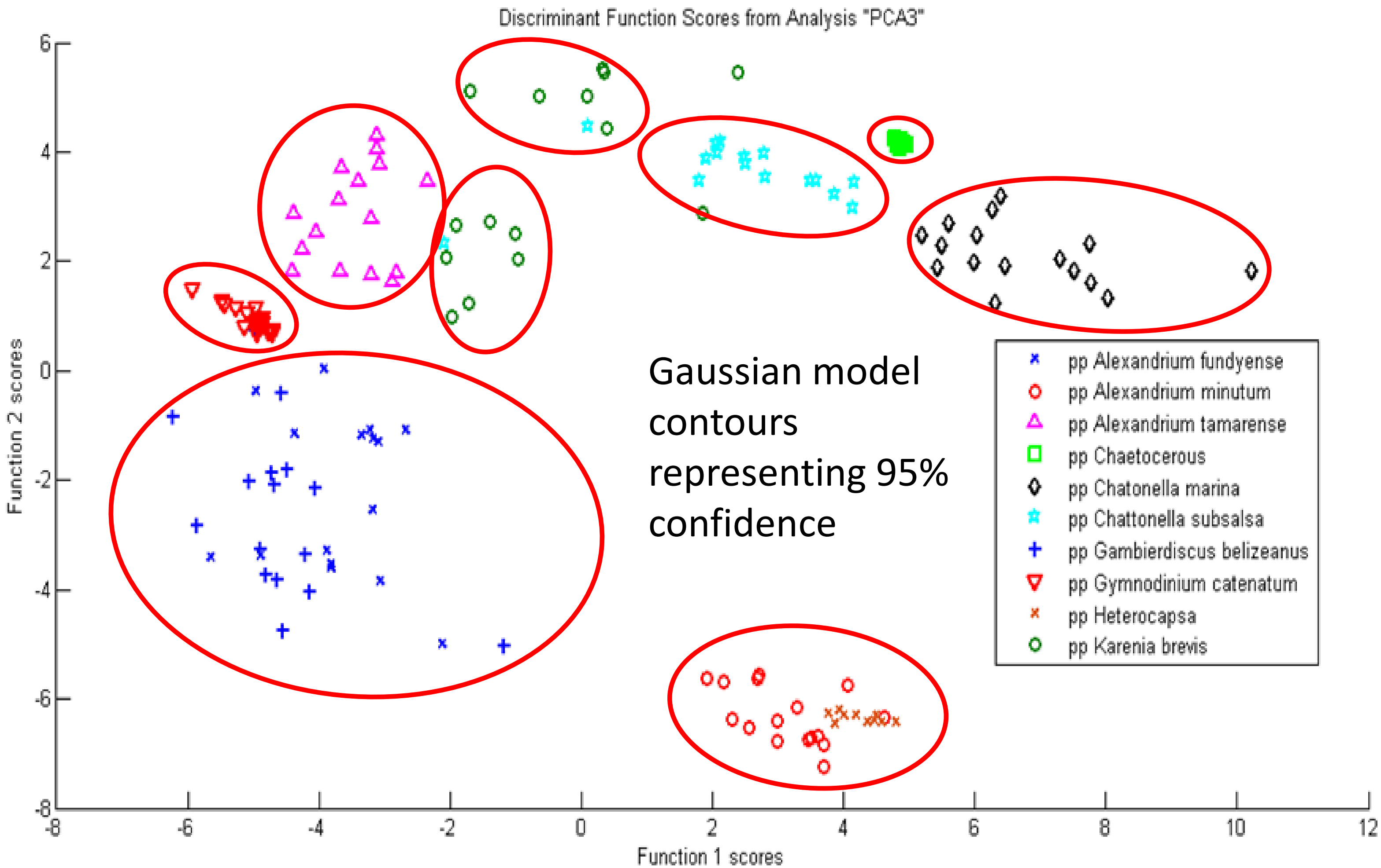


Characteristics of 12 Species at 532 nm



Raman Shift	Gambierdiscus belizuanus	Alexandrium catenella ATMP7	Alexandrium catenella 1911	Alexandrium minutum	Heterocapsa sp.	Chaetoceros sp.	Alexandrium tamarense	Chattonella subsalsa	Chattonella marina	Isochrysis galbana	Karenia brevis	Pseudo-nitzschia subcurvata 92	Pseudo-nitzschia subcurvata 13	Gymnodimium catenatum	Compound	Molecular assignments
388	0.23	0.26	0.26	0.20		0.04	0.25	0.13	0.19	0.05	0.24			0.22		v2 bending reduction in symmetry of the
454	0.38	0.39	0.39	0.21		0.08	0.39	0.21	0.35	0.06	0.41			0.36		
744	0.29	0.47	0.47	0.33			0.32	0.19	0.37		0.22			0.26	chlorophyll a-b	
940				0.21	0.09											v(C-C), alpha-helix
987	0.95	0.94	0.94	1.00		0.12	0.98	0.46	0.62		0.92			1.00	chlorophyll a-b	
1000	0.03	0.04	0.05	0.12	0.02	0.08	0.04	0.07	0.03	0.12	0.13	0.13	0.15	0.07	chlorophyll a-b	v3
1017											0.86				Gyroxanthin	
1150	0.45	0.47	0.48	0.66	0.41										β-Carotene	v2 C14-C15 δC15-H peridinin?
1165						0.70	0.45	0.78	0.81	0.77	0.67	0.54	0.59			
1186	0.45	0.47	0.48	0.68	0.45				0.56						chlorophyll a-b	20δ(CH), vN21-C4
1190						0.32	0.22	0.35	0.52	0.31	0.44	0.32	0.27		β-Carotene	δ(C10-H), δ(C11-H)
1200						0.28	0.26	0.33	0.51	0.26	0.38	0.31	0.25			
1278						0.12						0.17	0.11		fucoxanthin	
1289															chlorophyll a-b	CH3 bend,(10+5)δ(CH), vN23-C14
1303				0.41			0.26		0.30		0.35			0.24		Carbonate antisymmetric stretching
1348					0.07										chlorophyll a-b	CH3 bend,(20+5+10)δ(CH), vN21-C4
1467	0.25	0.33	0.33	0.54	0.05	0.06	0.24	0.15	0.36	0.04	0.24	0.06	0.04	0.25	triglyceride	C-C saturated
1495		0.12	0.12	0.12		0.12			0.12						chlorophyll a-b	vC12-C13, vC1-C20, vC5-C6(81+82+121)CH3 b
1520									0.12		0.12				β-Carotene	v(C13=C14), v(C11=C12)
1529	0.66	0.64	0.65	0.98	1.00	1.00	0.40	0.97	0.97	0.99	0.64	0.97	1.00	0.10	chlorophyll a-b	
1656	0.86			0.82				0.44	0.73						triglyceride	cis C=C stretching vibration unsaturated

Can Raman distinguish between species?



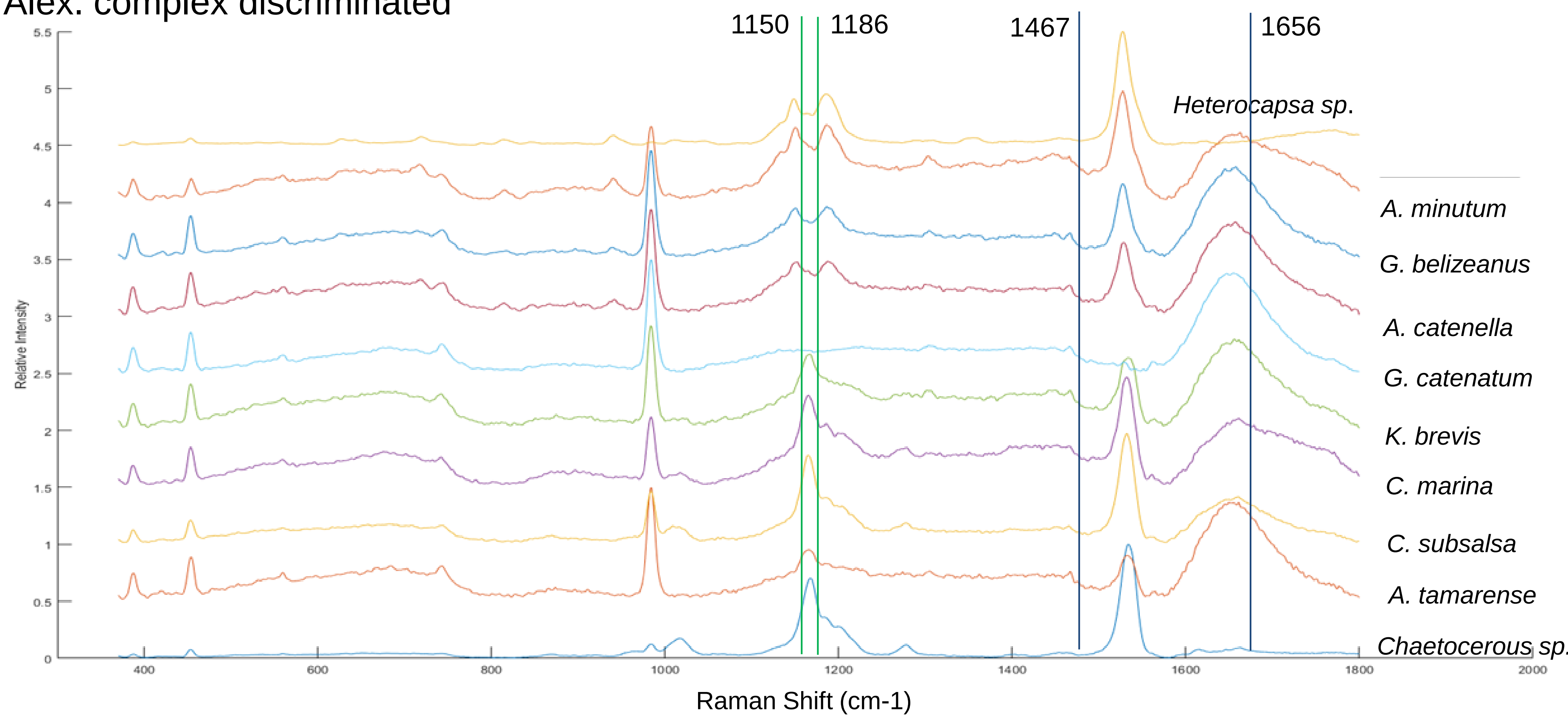
Gaussian model contours representing 95% confidence

LOO-Cross Validation of Linear Discriminant Function

- 8 toxic, 3 non-toxic species

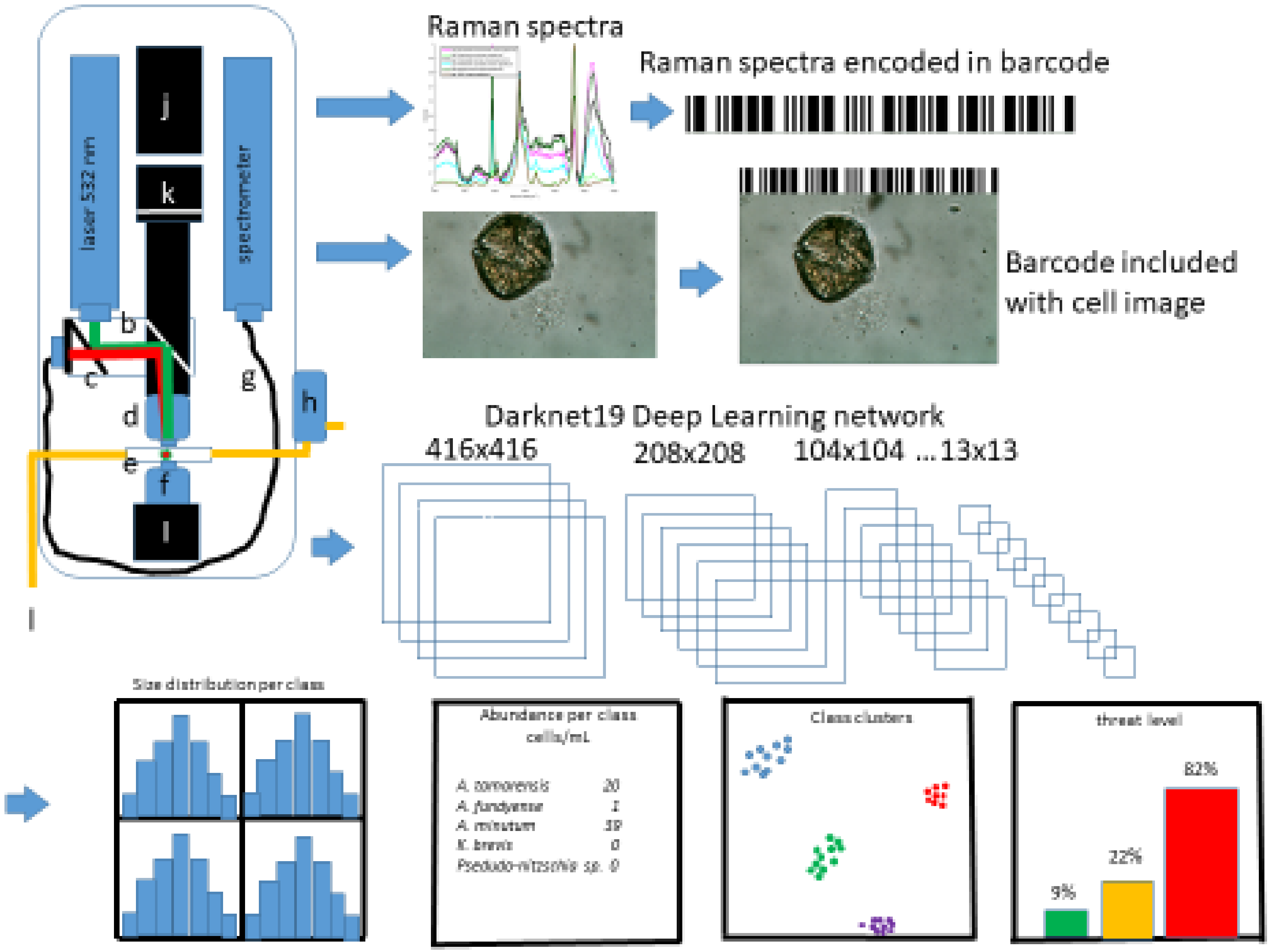
Result: 100% discrimination between toxic and non-toxic
Alex. complex discriminated

Species												% Accuracy
Alexandrium catenella	16	0	0	0	0	0	0	0	0	0	0	100
Alexandrium minutum	0	16	0	0	0	0	0	0	0	0	0	100
Alexandrium tamarense	0	0	15	0	0	0	0	0	0	1	0	94
Chaetocerous sp.	0	0	0	16	0	0	0	0	0	0	0	100
Chatonella marina	0	0	0	0	16	0	0	0	0	0	0	100
Chattonella subsalsa	0	0	0	0	1	13	0	0	0	2	0	81
Gambierdiscus belizeanus	0	0	0	0	0	0	14	2	0	0	0	88
Gymnodinium catenatum	0	0	0	0	0	0	1	15	0	0	0	94
Heterocapsa sp.	0	0	0	0	0	0	0	0	16	0	0	100
Karenia brevis	0	0	0	0	1	1	0	0	0	14	0	88
Isochrysis galbana TISO	0	0	0	0	0	0	0	0	0	0	16	100



Feature Sets for Training and Classifying Cells using Deep Learning

Feature Type	Feature	Source	Number Features
Morphology	Geometry	3-D image	128
	Shape (FFT)		>1000
	Texture		256
	Invariant Moments		128
	Color pattern		156
	Fractal index		>128
	Cell volume		1
	Cell length		1
	Cell area		1
Chemical Content	Pigmentation (β-Carotene, Chlorophylla,b,c, Fucoxanthin, Xanthrophylls, Phycobilliproteins etc.)	Raman & Florescence	~128
	Triglycerides	Raman	21
	Toxins (saxitoxin, ciguatera, Domoic Acid, etc.)	Raman	>100



laser 532 nm
j
k
spectrometer
b
c
d
e
f
g
h
l
Raman spectra
Raman spectra encoded in barcode
Barcode included
with cell image
Darknet19 Deep Learning network
416x416
208x208
104x104 ...13x13
Size distribution per class
Abundance per class
cells/mL
A. tamarensis 20
A. fundyense 1
A. minutum 19
K. brevis 0
Pseudo-nitzschia sp. 0
Class clusters
threat level
82%
22%
9%

Instrument website

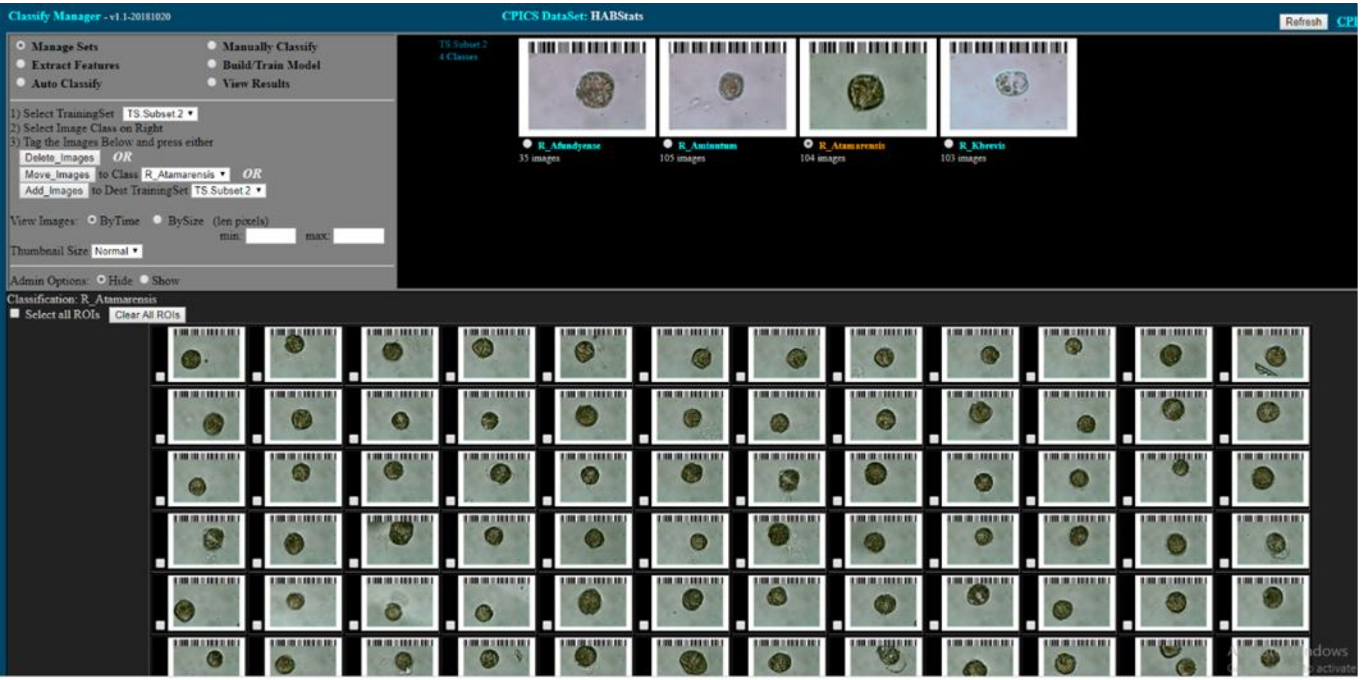
*sort images with Raman bar codes

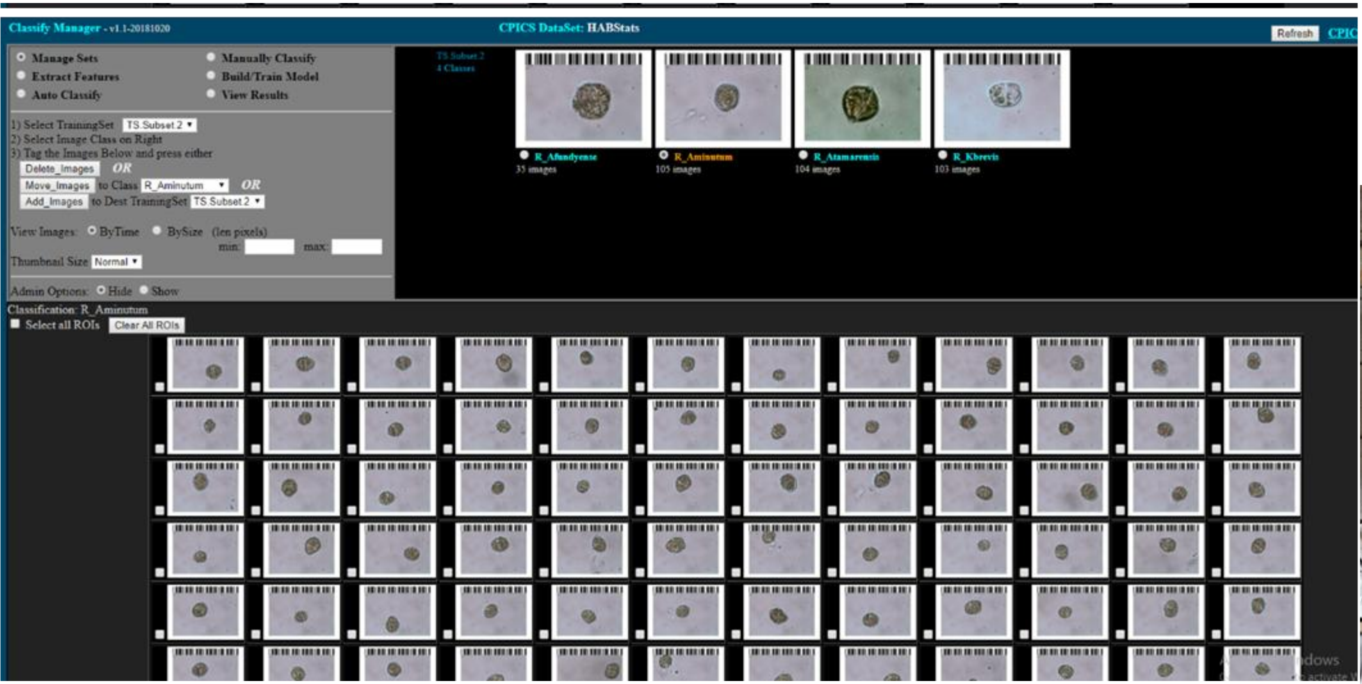
*build training sets

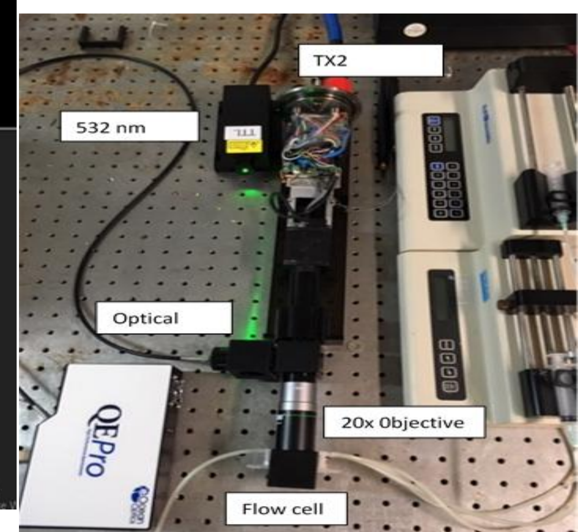
*build Deep Learning models

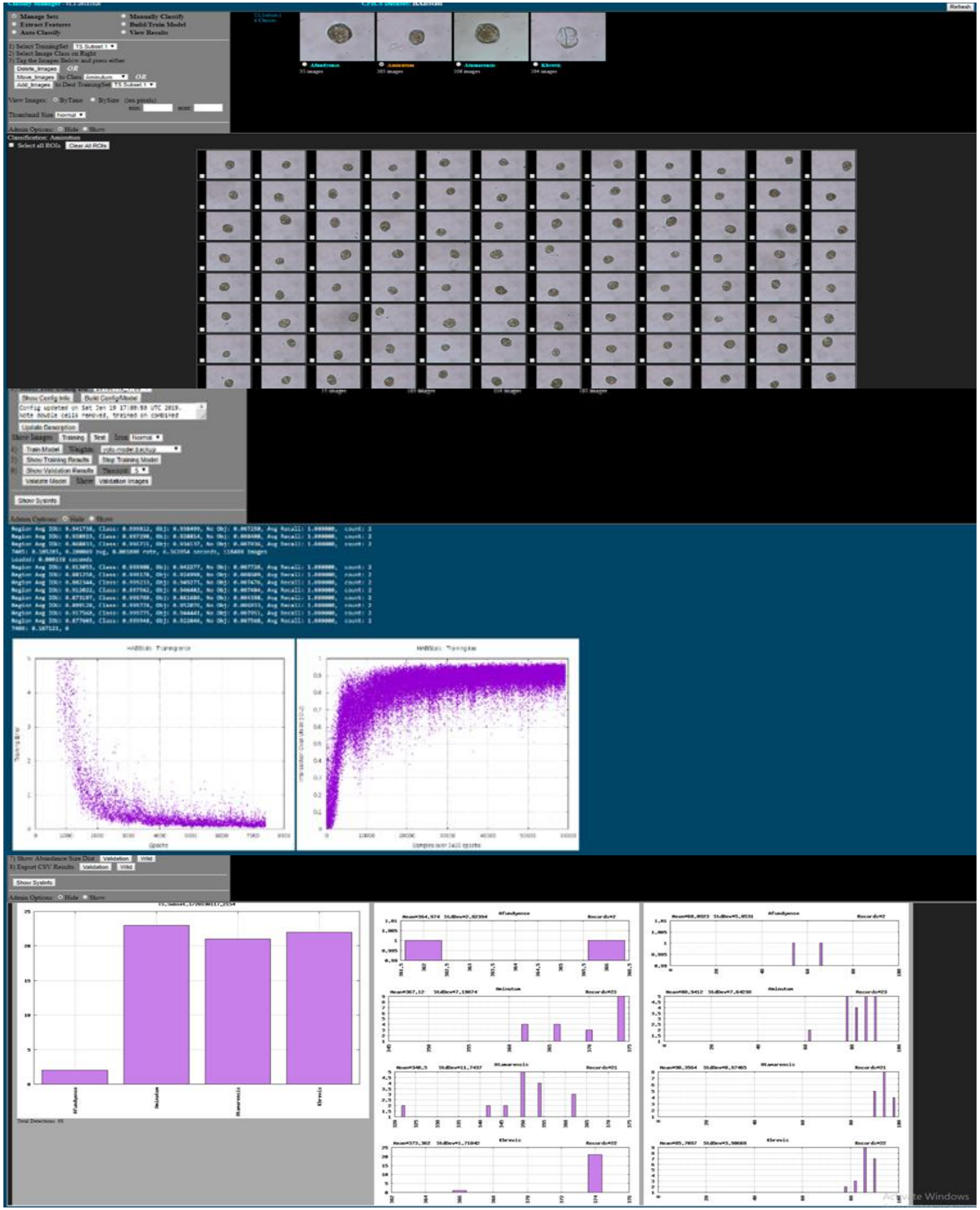
*run cross-validations

*classify unknown cells









Instrument website

*build Deep Learning models

*run cross-validations

*classify unknown cells by matching against spectral library in real-time at 10 to 50 cells /sec

Marine Raman and Image Library

1	Alexandrium tamarense		saxitoxin
2	Alexandrium tamarense		saxitoxin
3	Alexandrium tamarense	1771	saxitoxin
4	Alexandrium tamarense	1493	saxitoxin
5	Alexandrium minutum	1598	saxitoxin
6	Gambierdiscus belizeanus	ATSW01-2	ciguatera
7	Gambierdiscus belizeanus	113	ciguatera
8	Alexandrium fundyense	401	saxitoxin
9	Alexandrium fundyense	401-K	saxitoxin
10	Pyrocystis noctiluca	ATMP7-E8	
11	Karenia brevis	1911-K	brevitoxins
12	Karenia brevis	Florida, 08/02/18	brevetoxins
13	Karenia mikimotoi	2229-K	brevitoxins
	Karenia mikimotoi	718	brevitoxins
14	Gymnodimium catenatum	429	gonyautoxin
15	Chattonella subsalsa	430	brevetoxin-like
16	Chattonella subsalsa	1937	brevetoxin-like
17	Chattonella marina	217	brevetoxin-like
18	Chattonella marina	217-K	brevetoxin-like
19	Pseudonitschia subcurvata	2050-K	domoic Acid
20	Aureococcus amophageffreus	2050	non-toxic but produces fish kills through anoxia

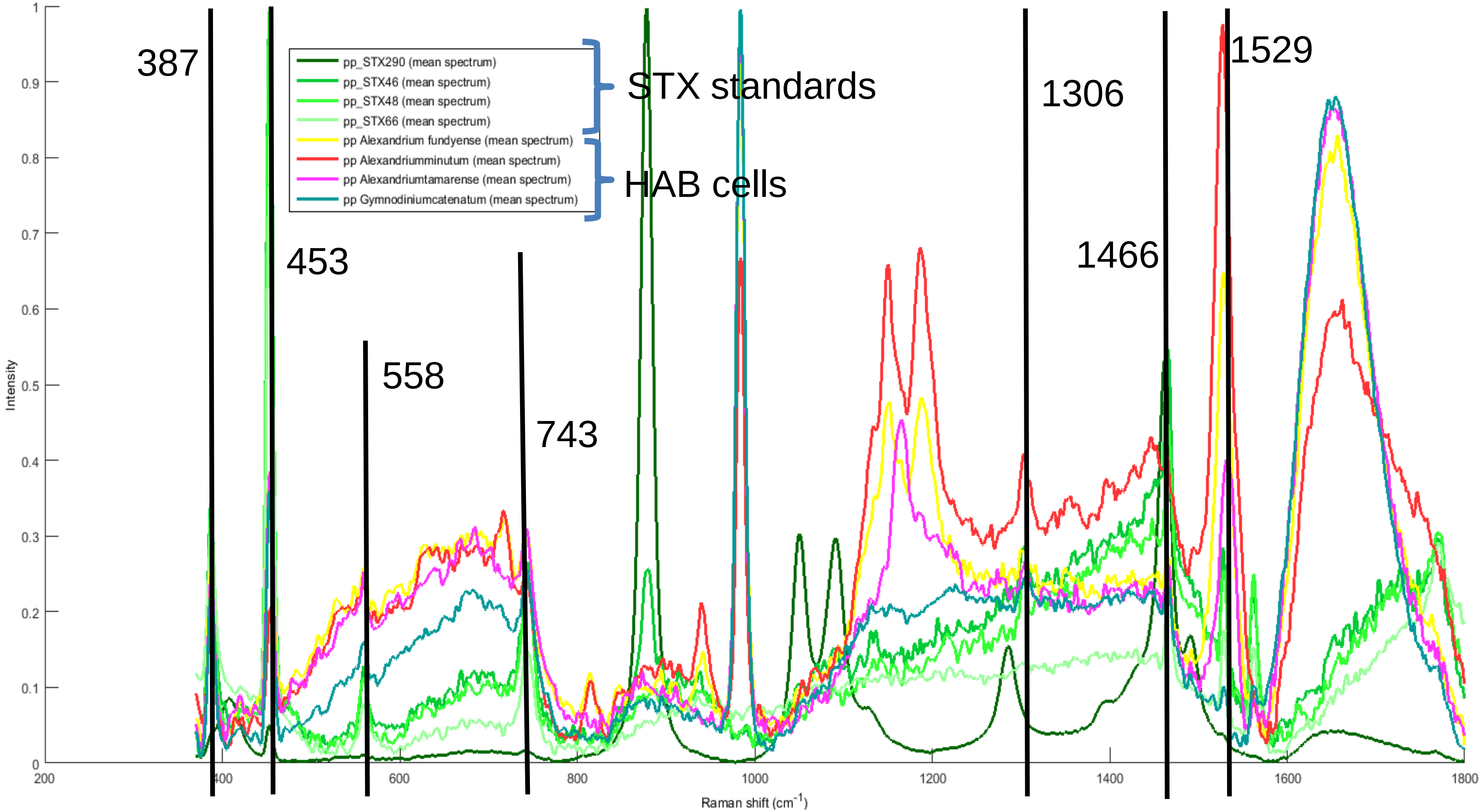
	Non-Toxic	
1	Isochrysis galbana	Tiso
2	Chaetoceros	chaet
3	Tetraselmis suecica	tet
4	Rhodomonas salina	1319-rhodo
5	Heterocapsa sp.	Het

Freshwater Raman and Image Library

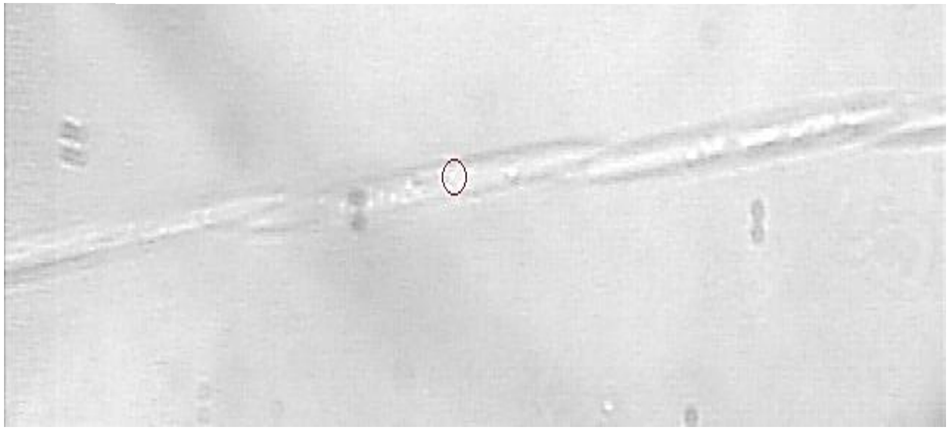
1	Anabaena cylindrica	B 1611 UTEX	various cyanotoxins- microcystins
2	Anabaena variabilis	B 377 UTEX	nodularin
3	Anabaena sp.	1448 UTEX	anatoxins
4	A. spiroides	B 1552 UTEX	saxitoxins
5	Microcystis aeruginosa		cylindrospermopsins
6	M. aeruginosa	LB 2061 UTEX	lyngbytoxin a
7	M. aeruginosa	LE3 S. 3462	aplysiatoxins
8	M. flos-aquae	LB 2673 UTEX	
9	Nostoc		
10	Nostoc sp.	LB 2210 UTEX	
11	Nostoc sp.	B 2211 UTEX	
12	Oscillatoria lutea	B 1814 UTEX Texas	
13	Volvox	Carolina biological	not toxic
14	Cylindrospermopsis raciborskii		cylindrospermopsin, anatoxin-a
15	C. raciborskii	LB 2897 UTEX	
16	C. raciborskii	Thai W. Carmichael Toxic?	
17	Nodularia harveyana	B 2093 UTEX Washington	
18	Calothrix parietina	LB 1952 UTEX swimming pool	
19	Microcoleus	LB 2220 UTEX	
20	Aphanizomenon flos-aquae	AFA S.Wilhelm	
21	A. flos-aquae	LB 2557 UTEX Toxic Hebgen Lake, MT	
22	A. flos-aquae	LB 2558 UTEX NON-TOXIC Hebgen Lake, MT	
23	A. flos-aquae	B 1444 UTEX Not Listed Mississippi	
24	Microcystis flos-aquae	LB 2673 UTEX Wisconsin	
25	Cyanophora paradoxa	LB 555 UTEX not listed Not listed	
26	Cylindrospermum sp.	LB 942 UTEX not listed California	
27	A. catenula	B 375 UTEX Netherlands	
28	Synechocystis sp.	2470 UTEX not listed California	
29	Synechococcus sp.	LB 2537 UTEX not listed Vancouver Island	
30	Planktothrix	Tim Davis Lab	

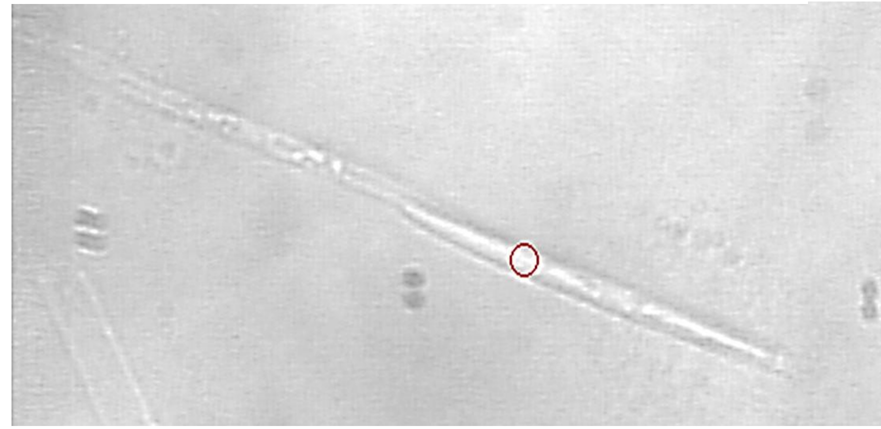
Can toxins be detected in single HAB cells?

At least 7 peaks for Saxitoxin (STX) standards align with cells known to be toxic

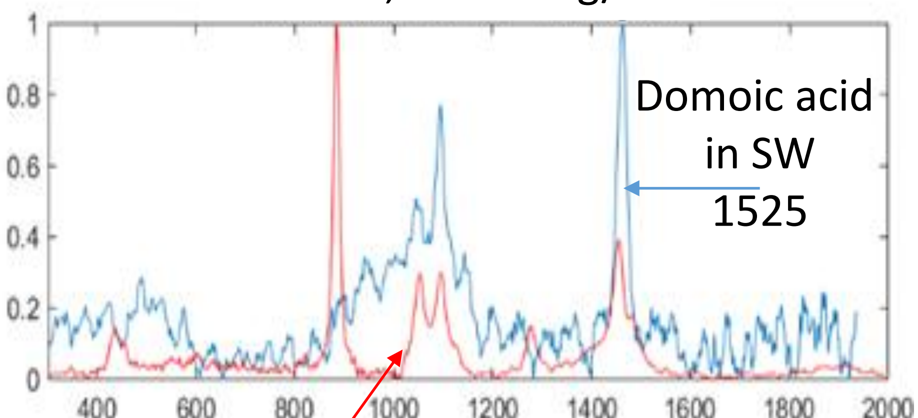


Single cell analysis of *Pseudo-nitzschia* from Woods Hole harbor February 2017



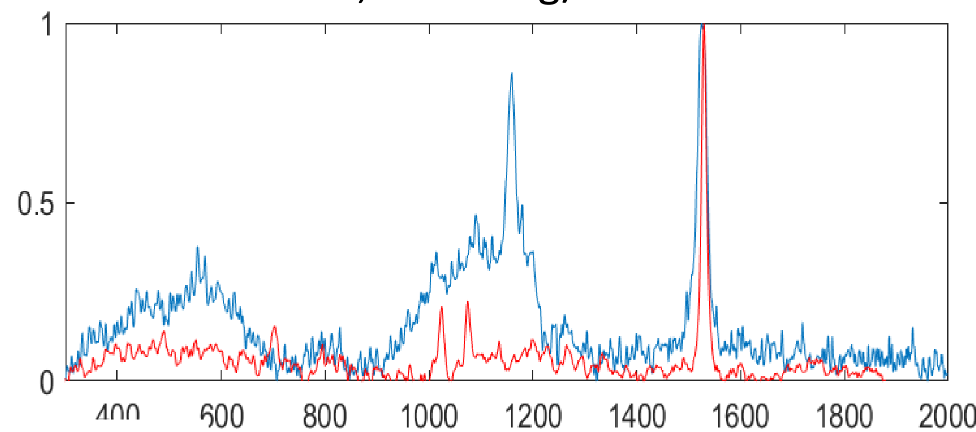


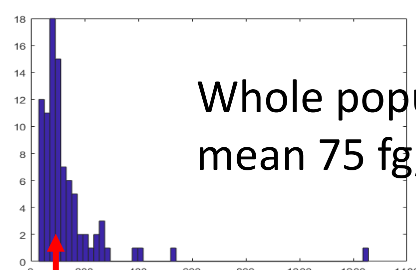
Cell # 28,473 9.5 fg/cell



PN cell

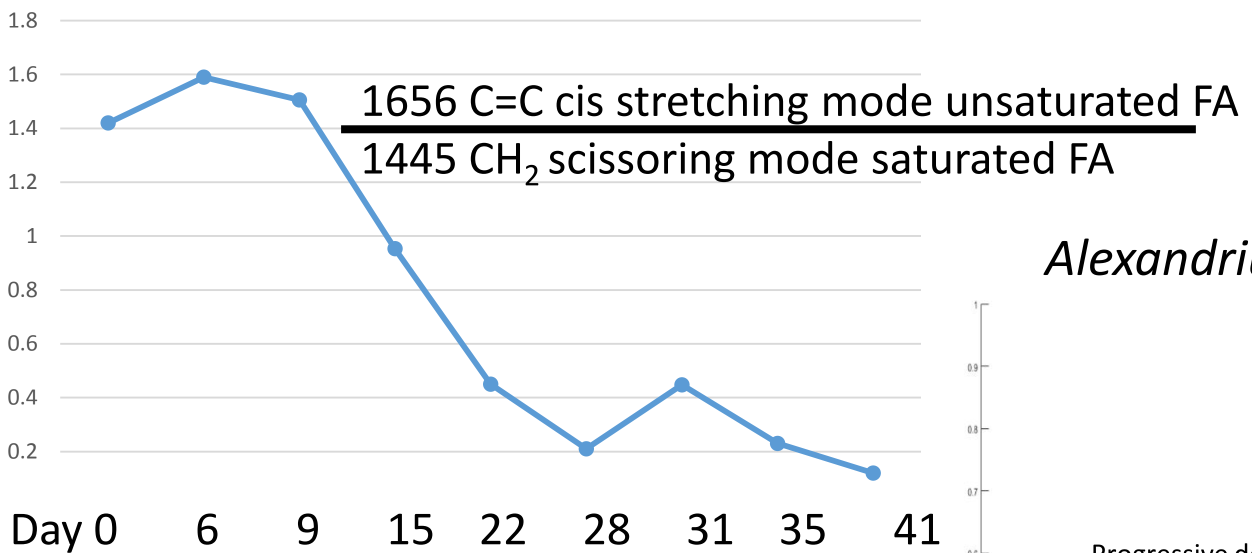
Cell # 4,274 84 fg/cell

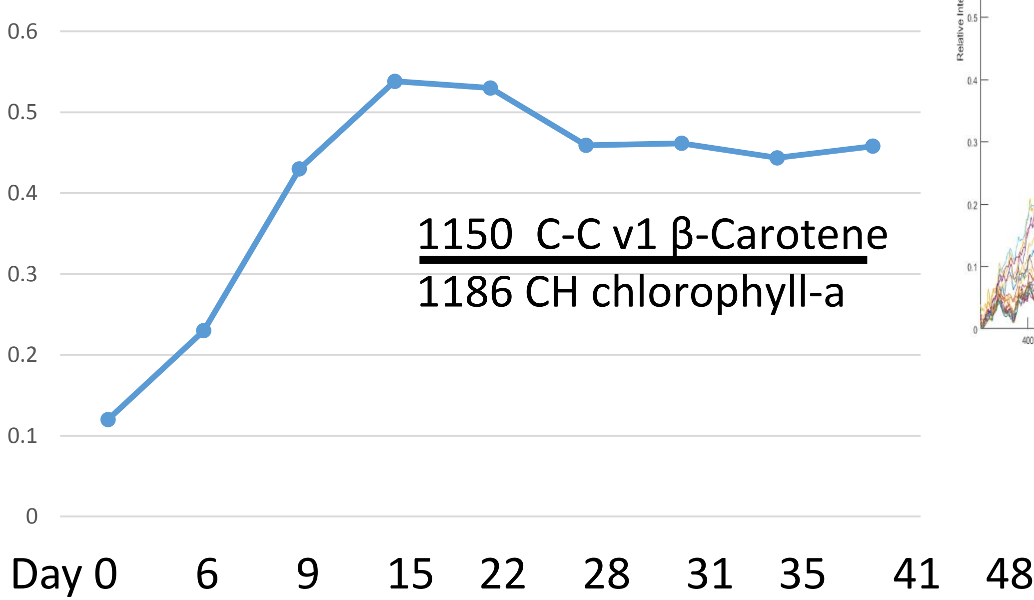




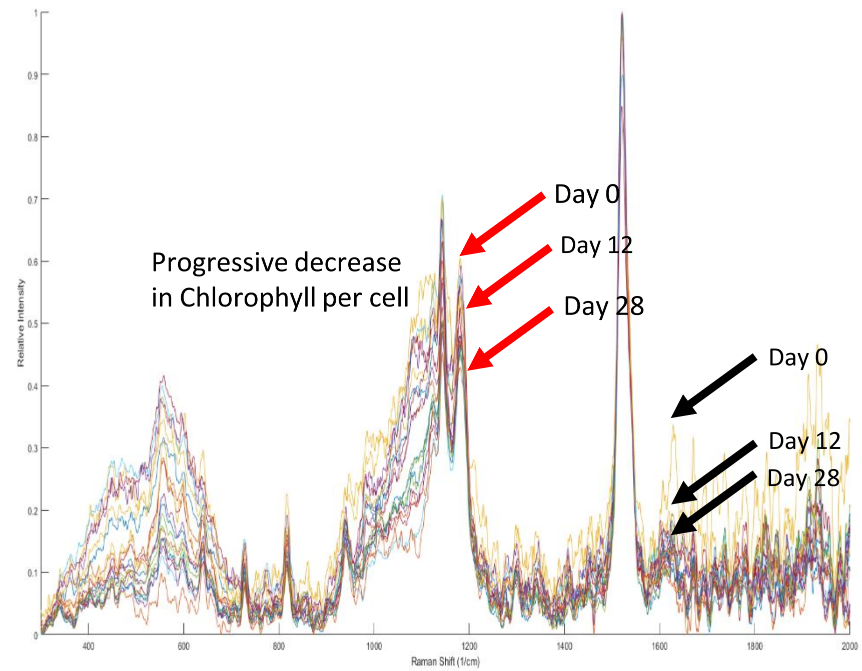
Whole population distribution
mean 75 fg/cell   166 cells

Can We Track Cell Dynamics in a Bloom?





Alexandrium catenella ATMP7-E8



Progressive decrease
in degree of unsaturation

Conclusions and Implications for Further Research

- Raman spectroscopy can be used directly in natural seawater to discriminate between HAB species and non-toxic cells, and some toxins.

- Real-time, automated distributed array of sensors to detect HABS on large scales when HAB species are present before they form blooms.

- Future work: continue peak database, miniaturize flow cytometer

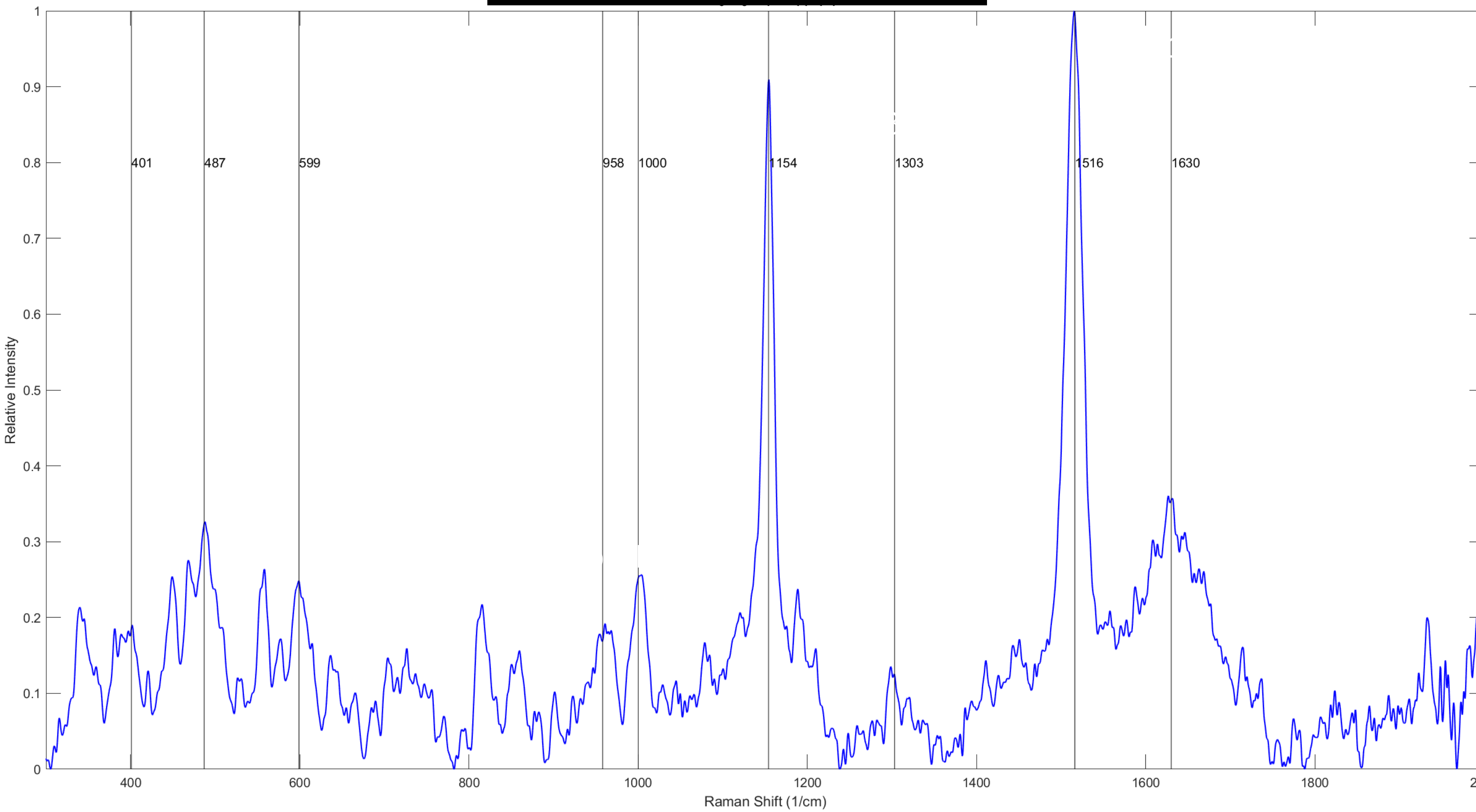
- Need beta testers to use instrument under various conditions- this summer

Acknowledgements

SBIR program

STTR program

Microcystis aeruginosa 346

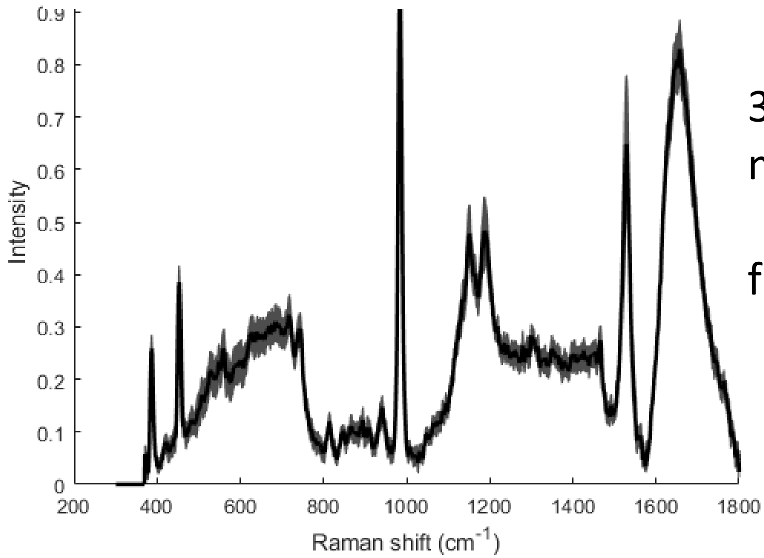


How much variability cell to cell?

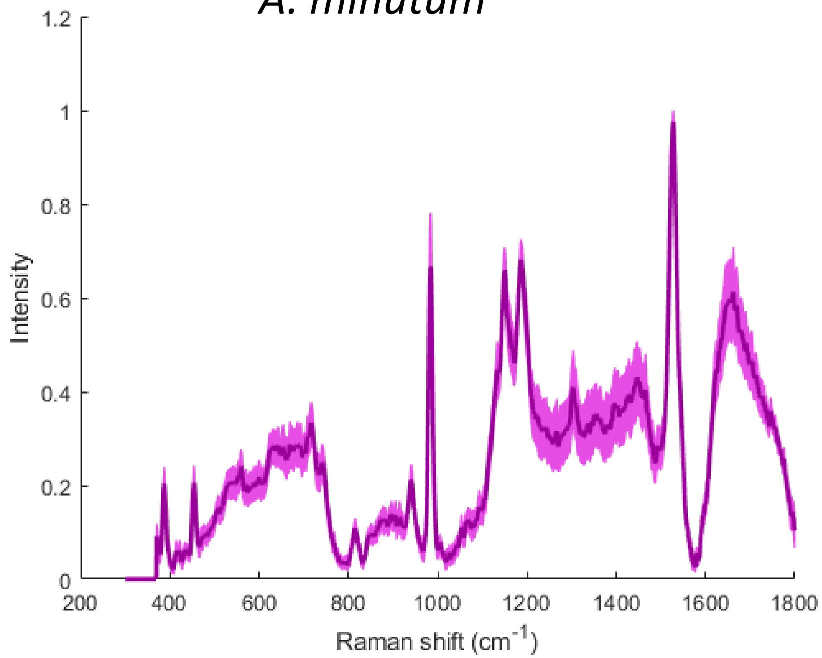
3 species
mean and SE of 16 replicate single cells

fingerprint analysis- peak location and area

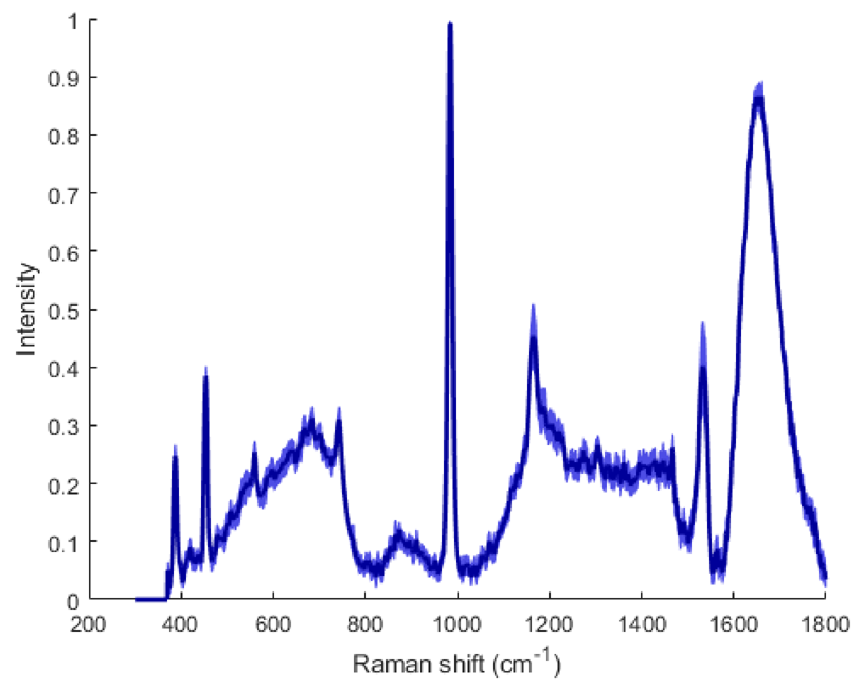
Alexandrium catenella
(mean & SE)



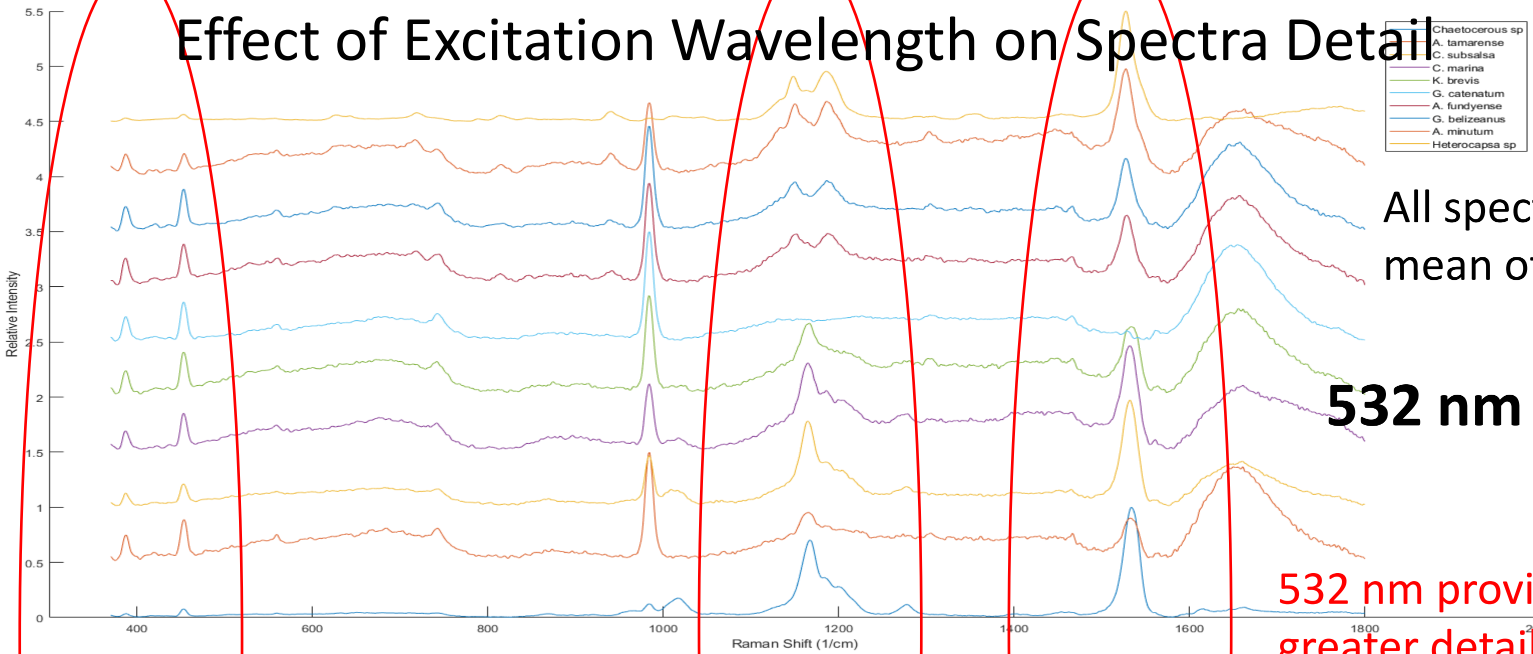
A. minutum



A. tamarense 1771 -non-toxic



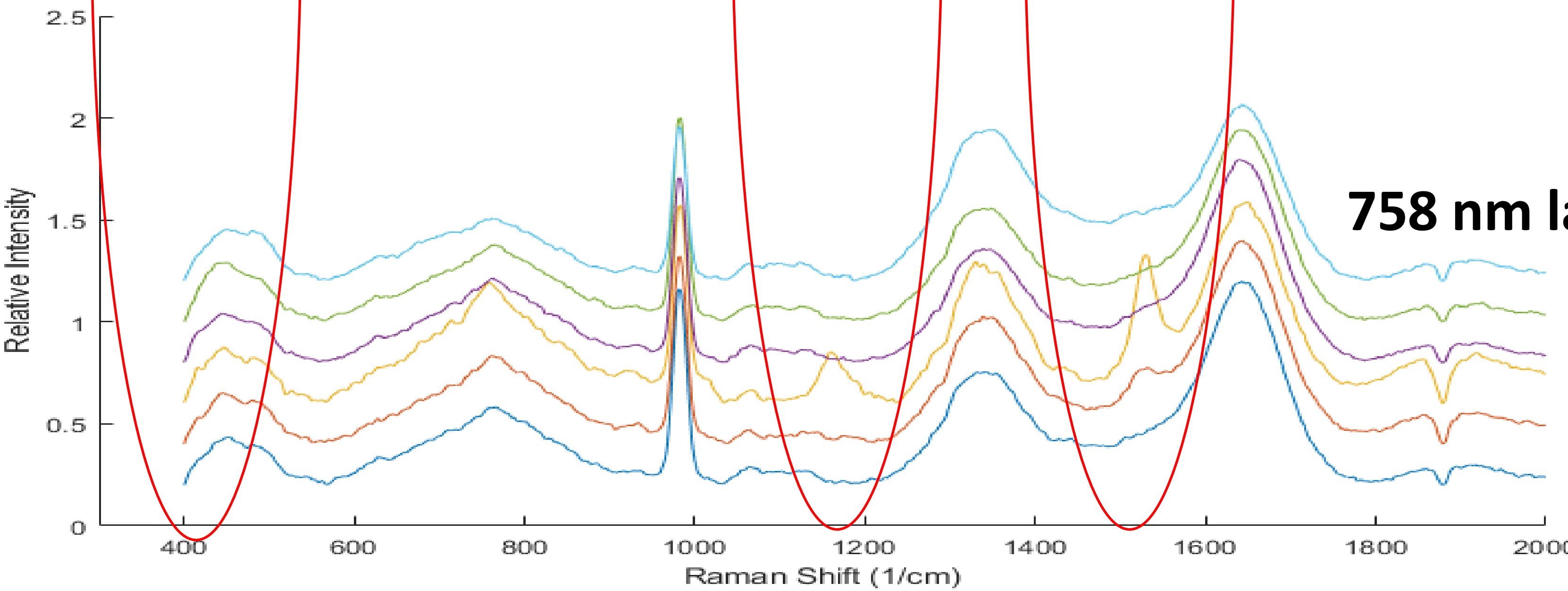
Effect of Excitation Wavelength on Spectra Detail



All spectra are mean of 16 cells

532 nm laser

532 nm provides greater detail, more information



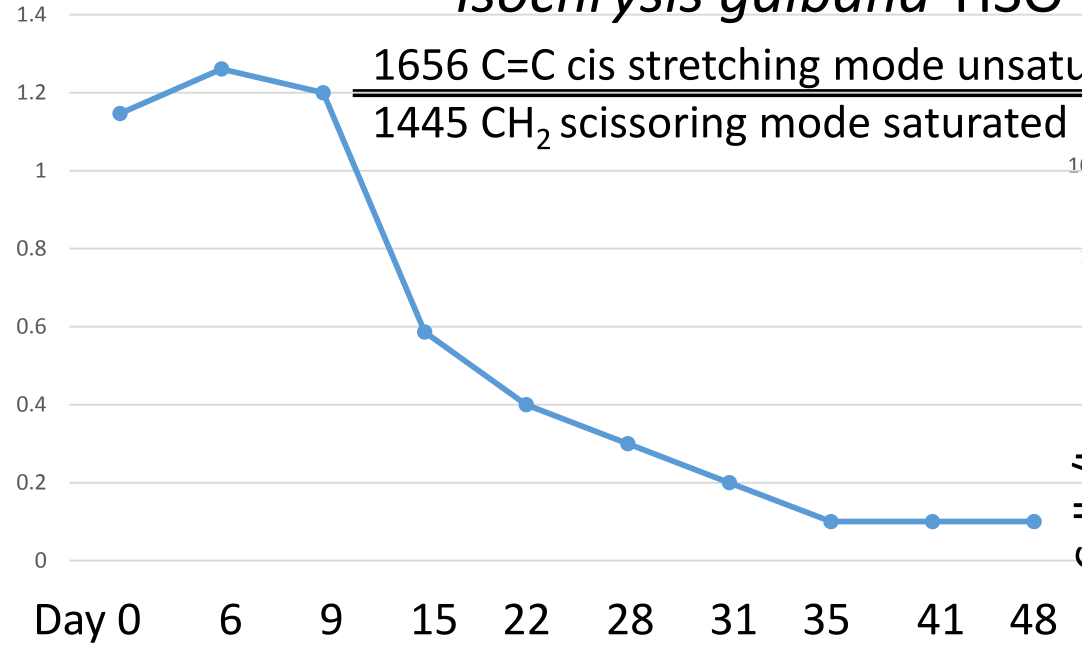
758 nm laser

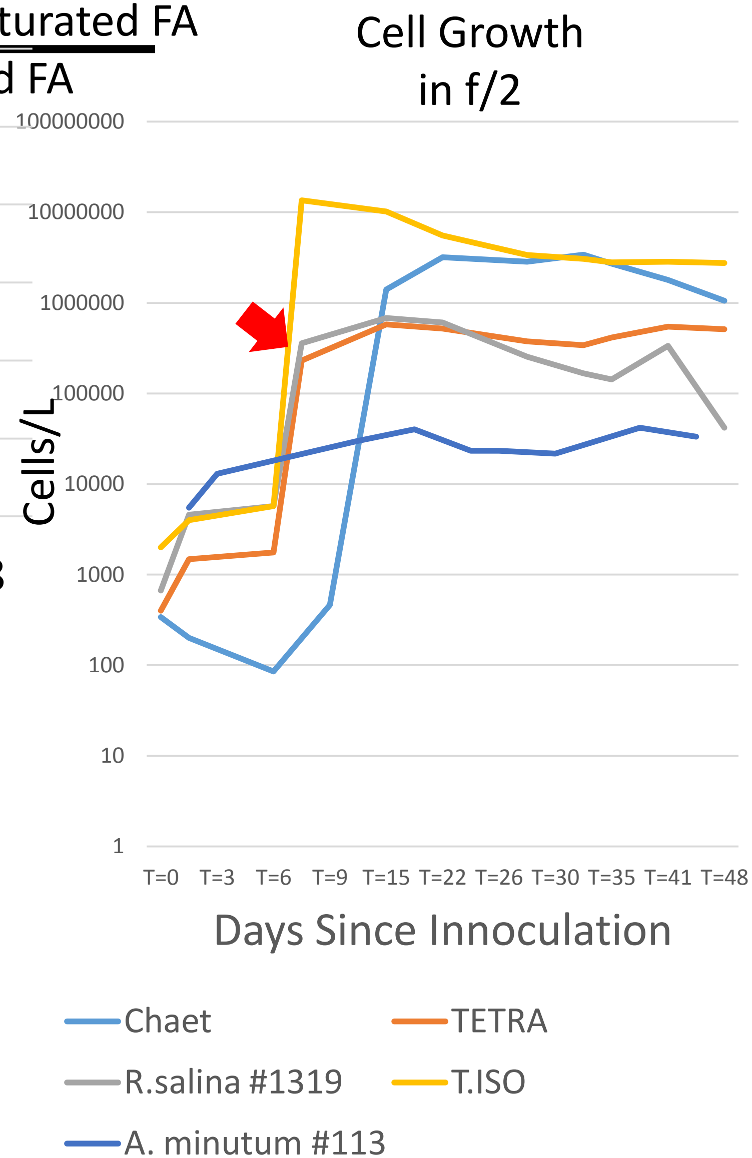
Can we use indices of cell physiology to assess bloom dynamics?

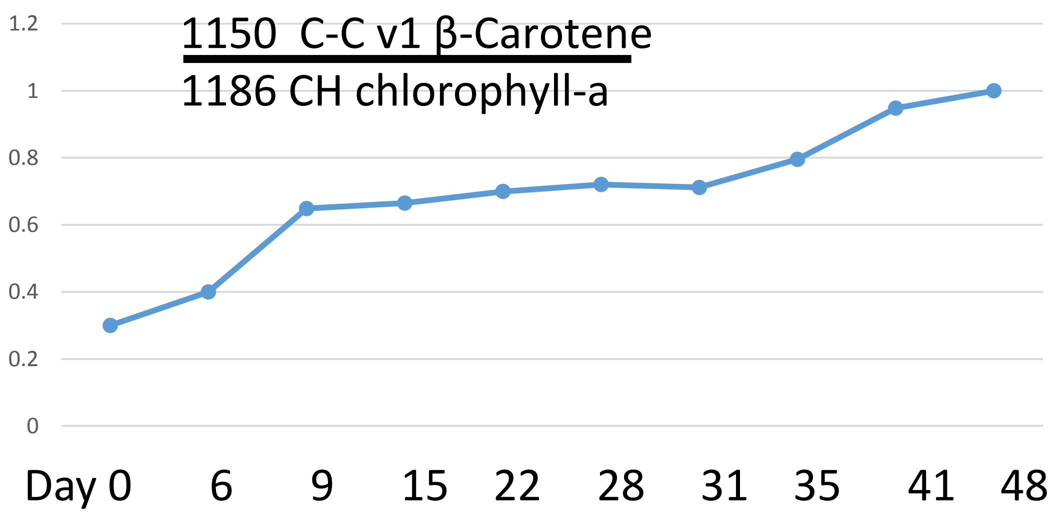
Pigment ratios:
β-carotene / chlorophyll a,c

Triacylglycerols:
unsatuarated / saturated Iodine Value: Samek, et al. 2010 in biofuels

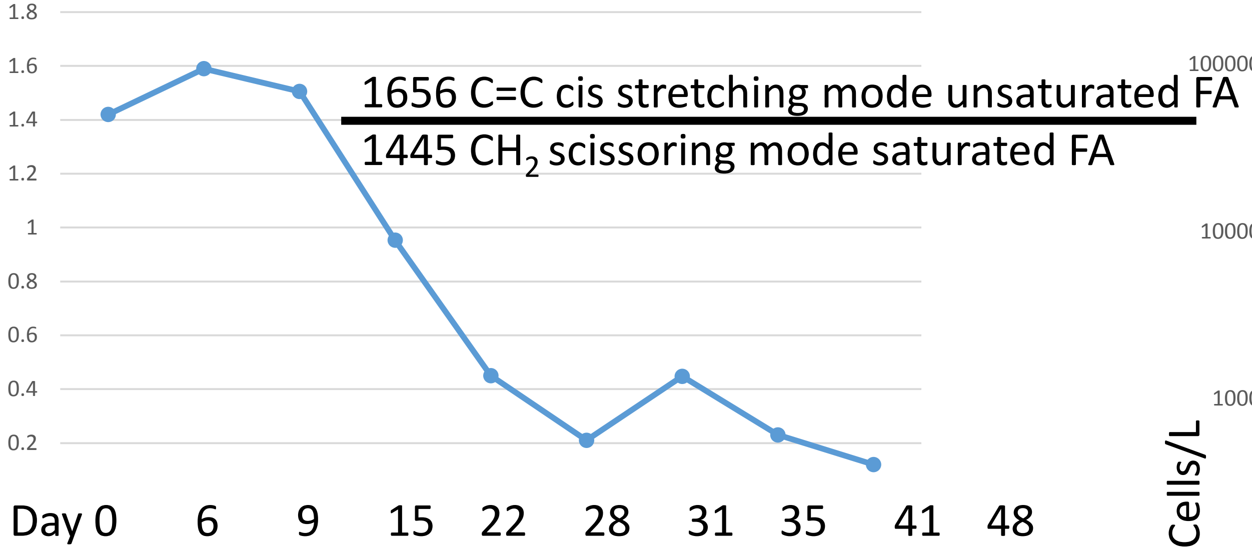
Isochrysis galbana TISO

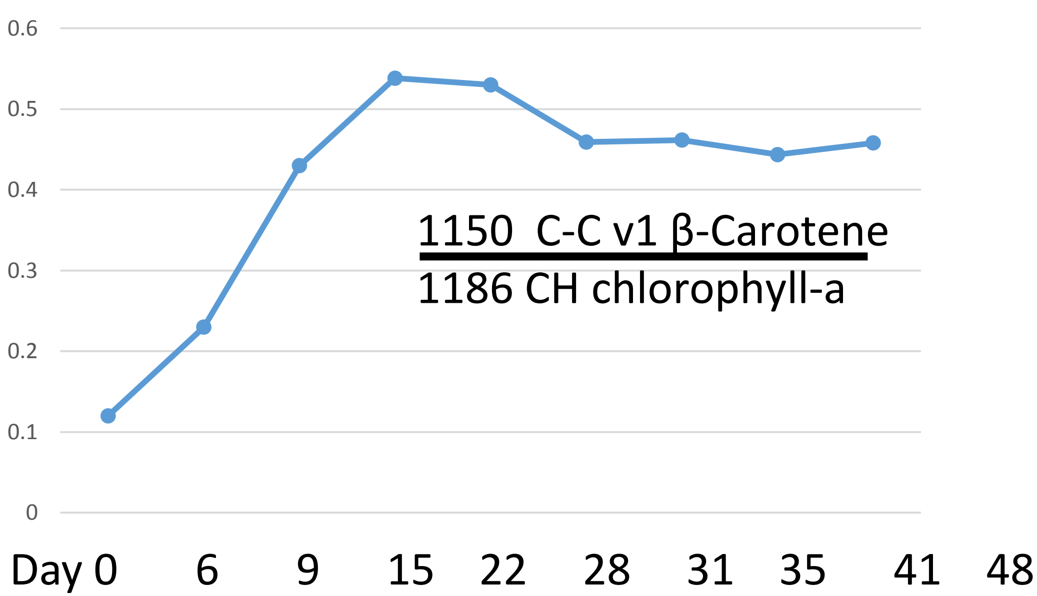


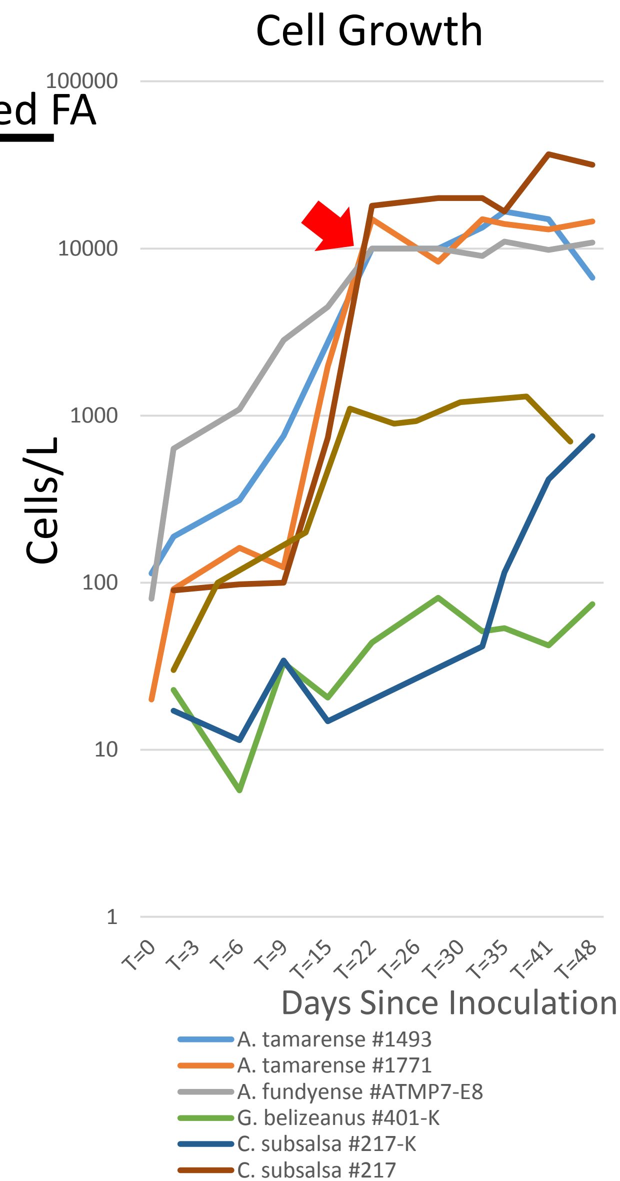




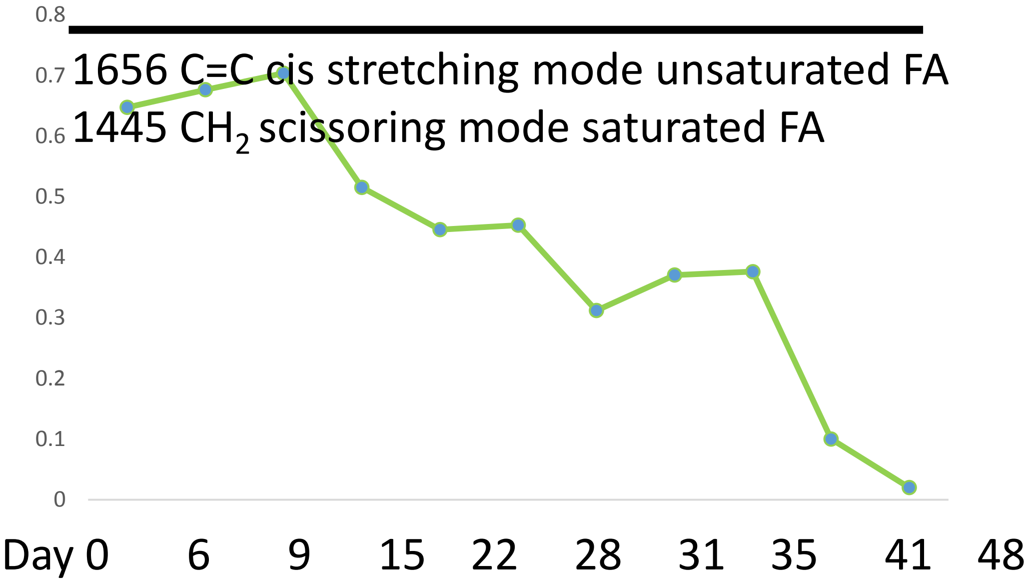
Alexandrium catenella ATMP7-E8

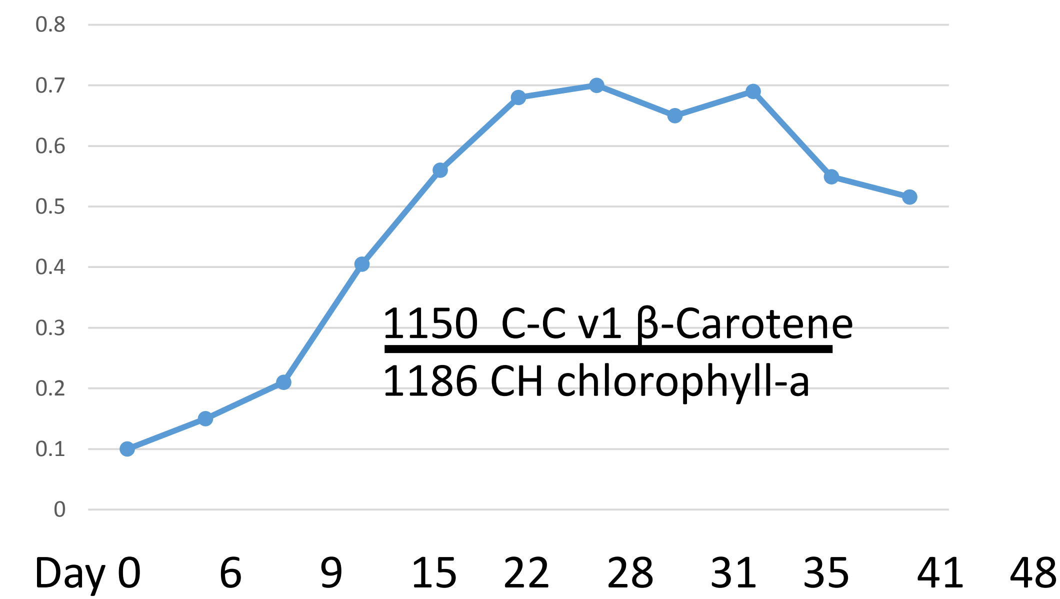


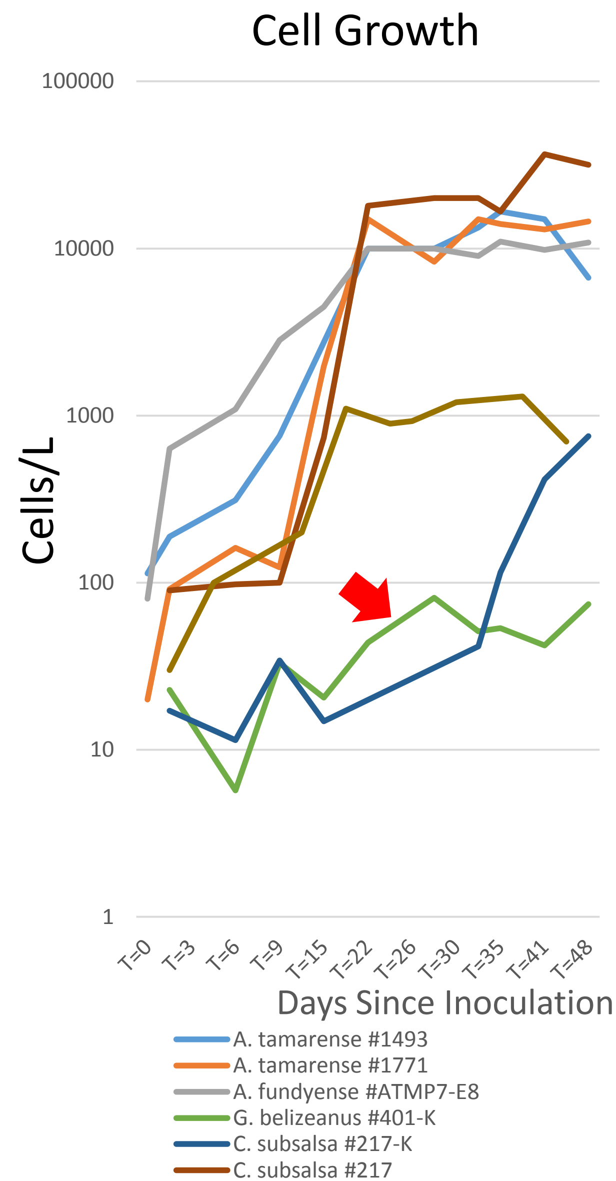




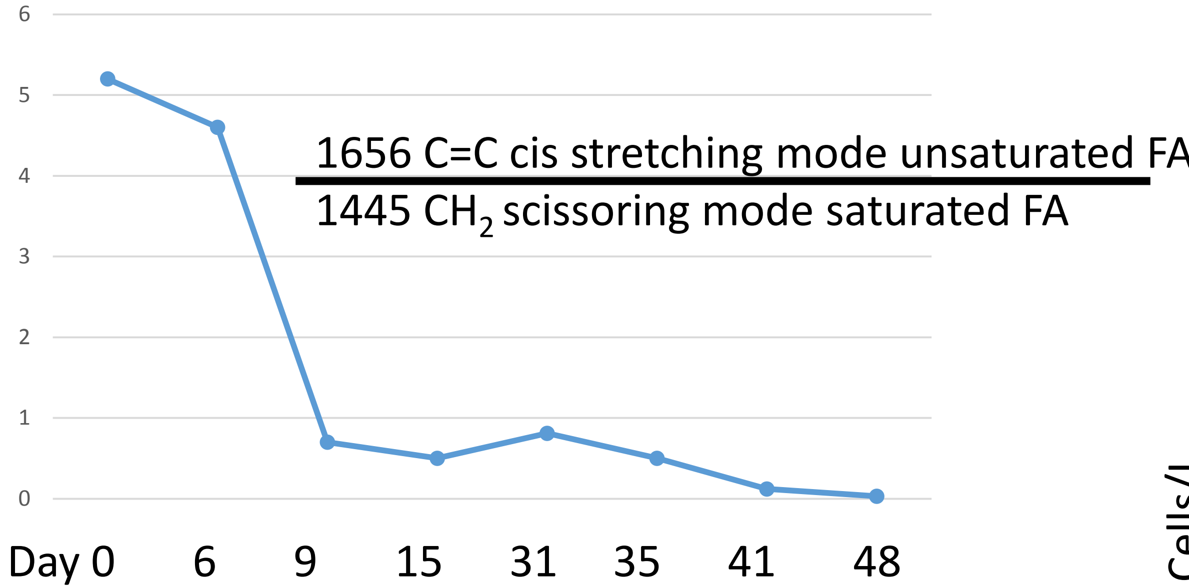
Gambierdiscus belizeanus 401-k

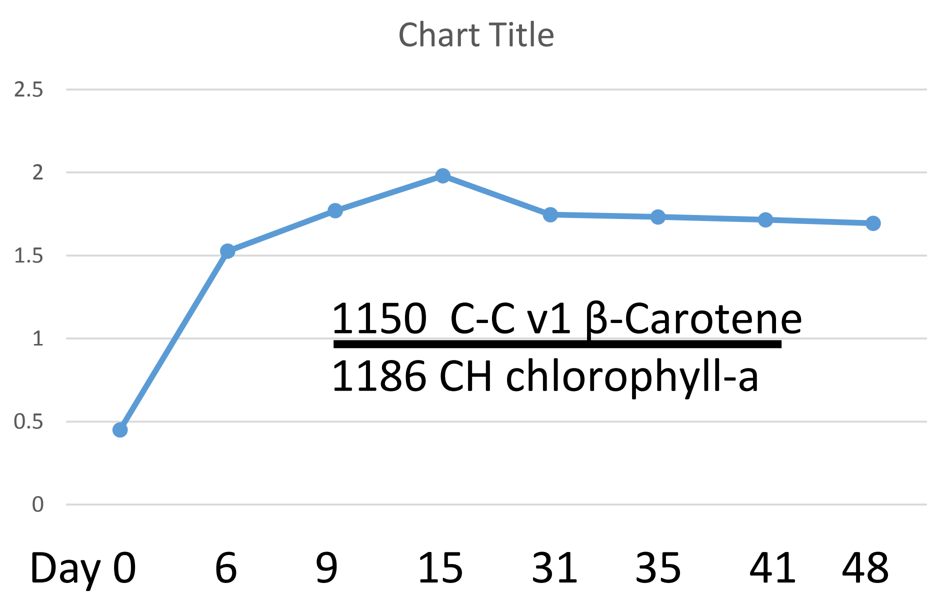


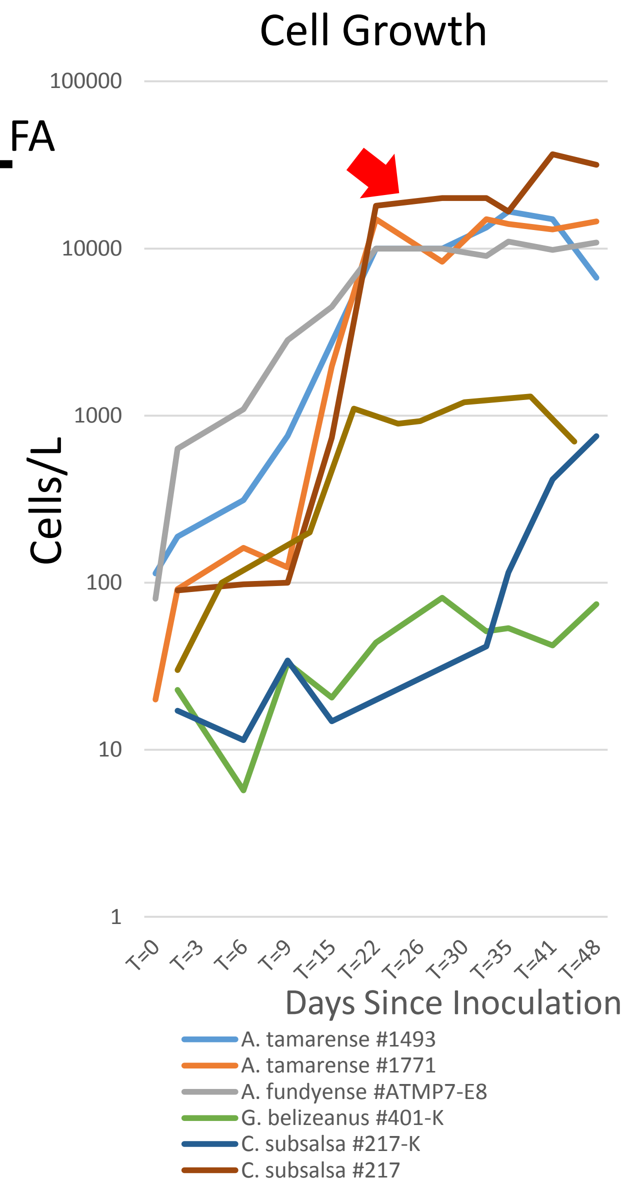




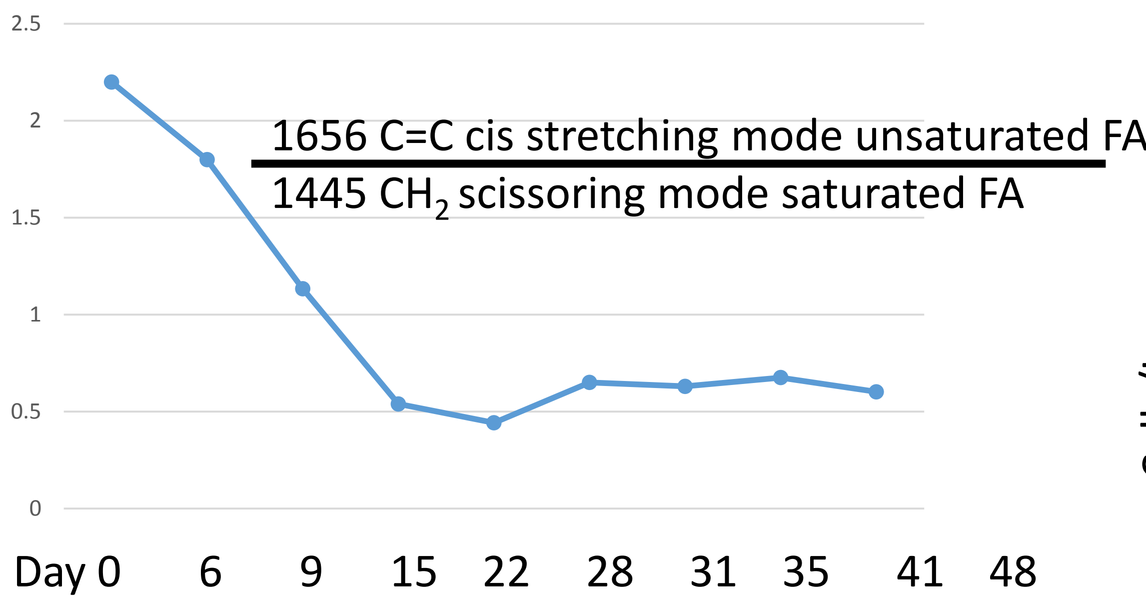
Chattonella subsalsa 217

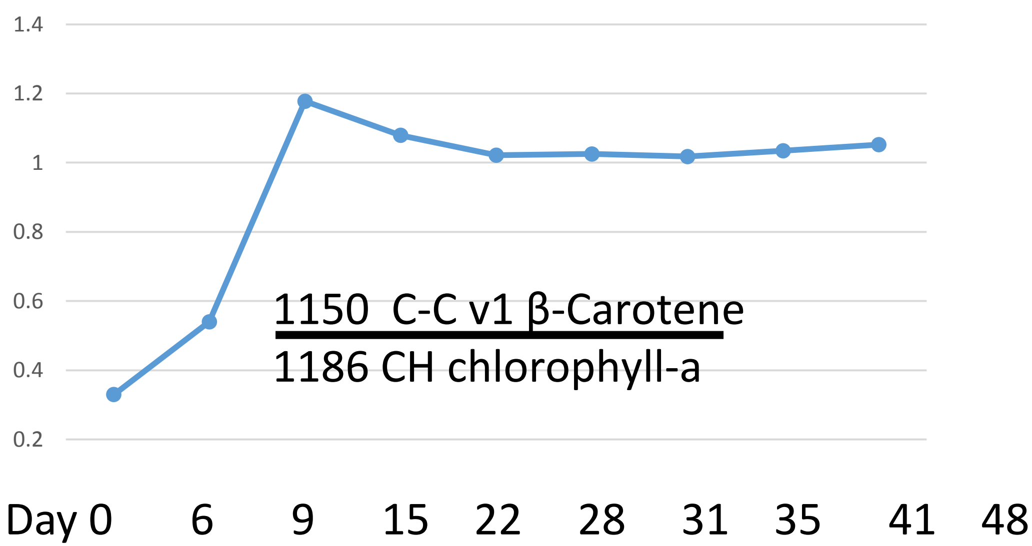


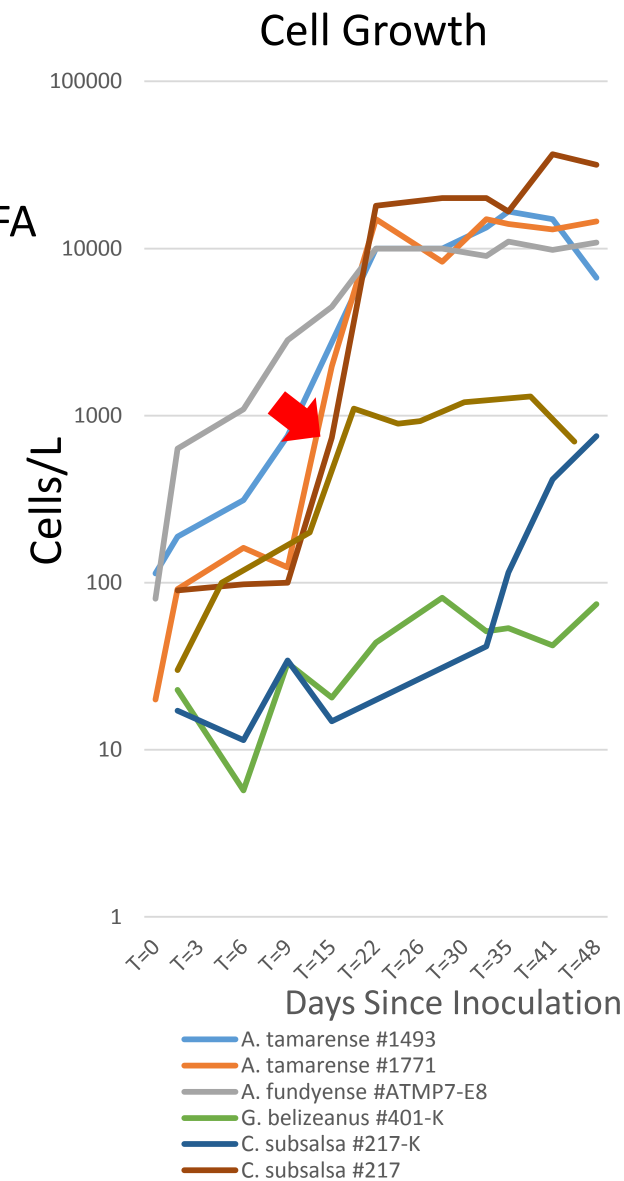




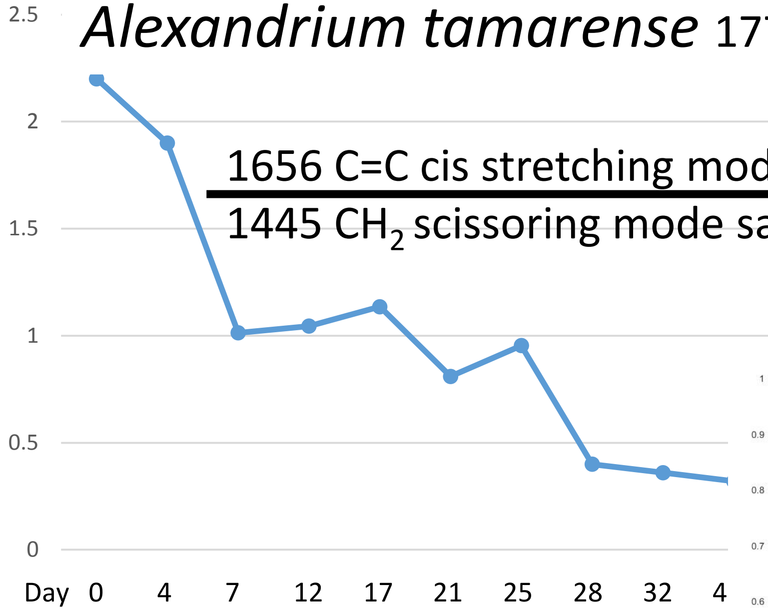
Alexandrium tamarense 1771







Alexandrium tamarense 1771 during 28 days of nutrient depletion



At Day 10 of normal growth
Cells rinsed 3x
Replaced in N-free media