Algal pigments in benthic organisms and fish:
development of biomarkers to trace food-web relationships

Katherine T. Alben, Maxime Bridoux, Monika Sobiechowska

Wadsworth Center, New York State Department of Health
Dept. Environmental Health Sciences, SUNY-Albany

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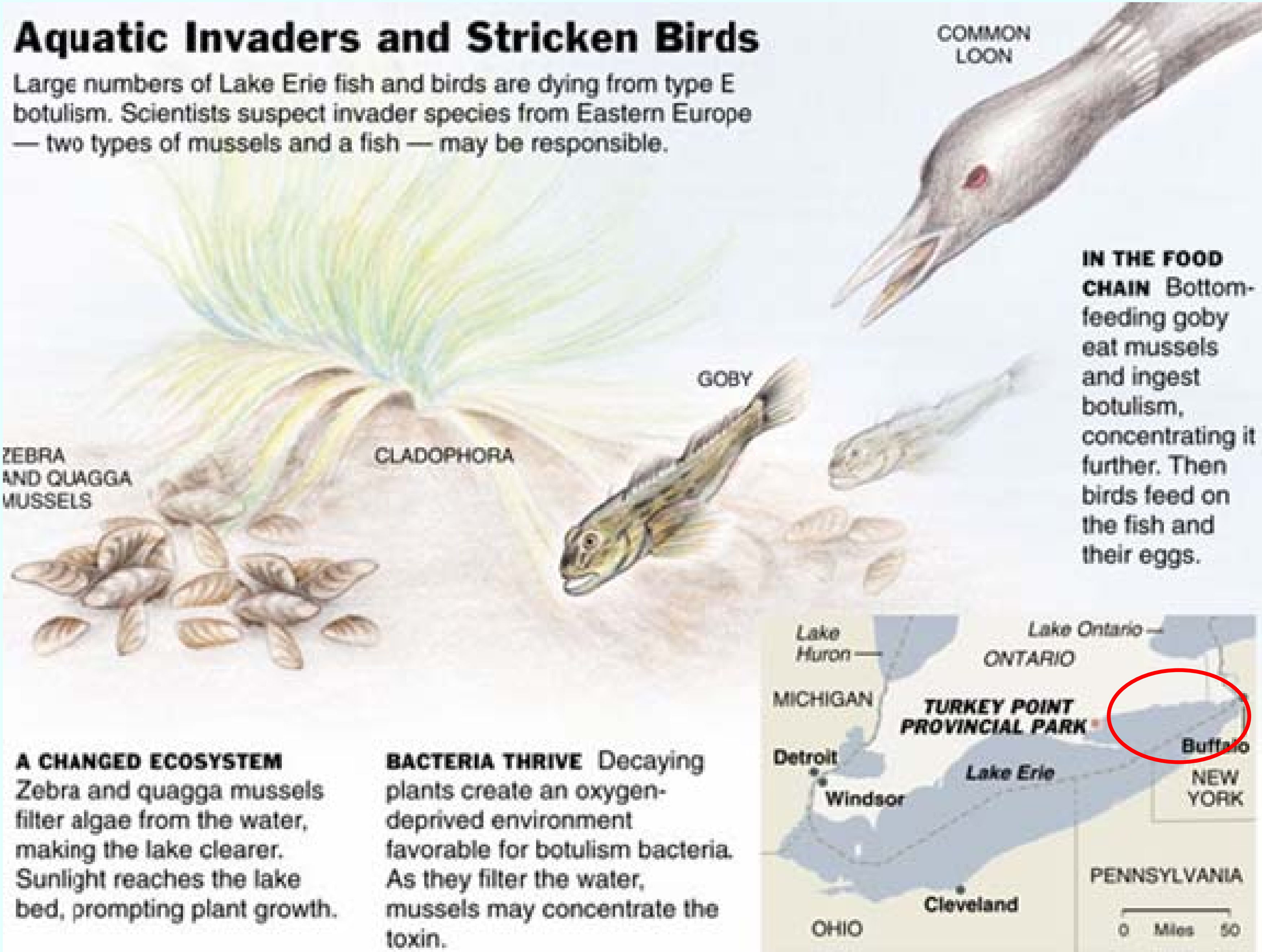
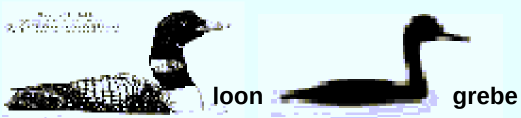
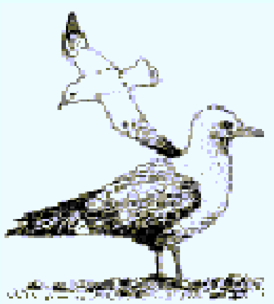
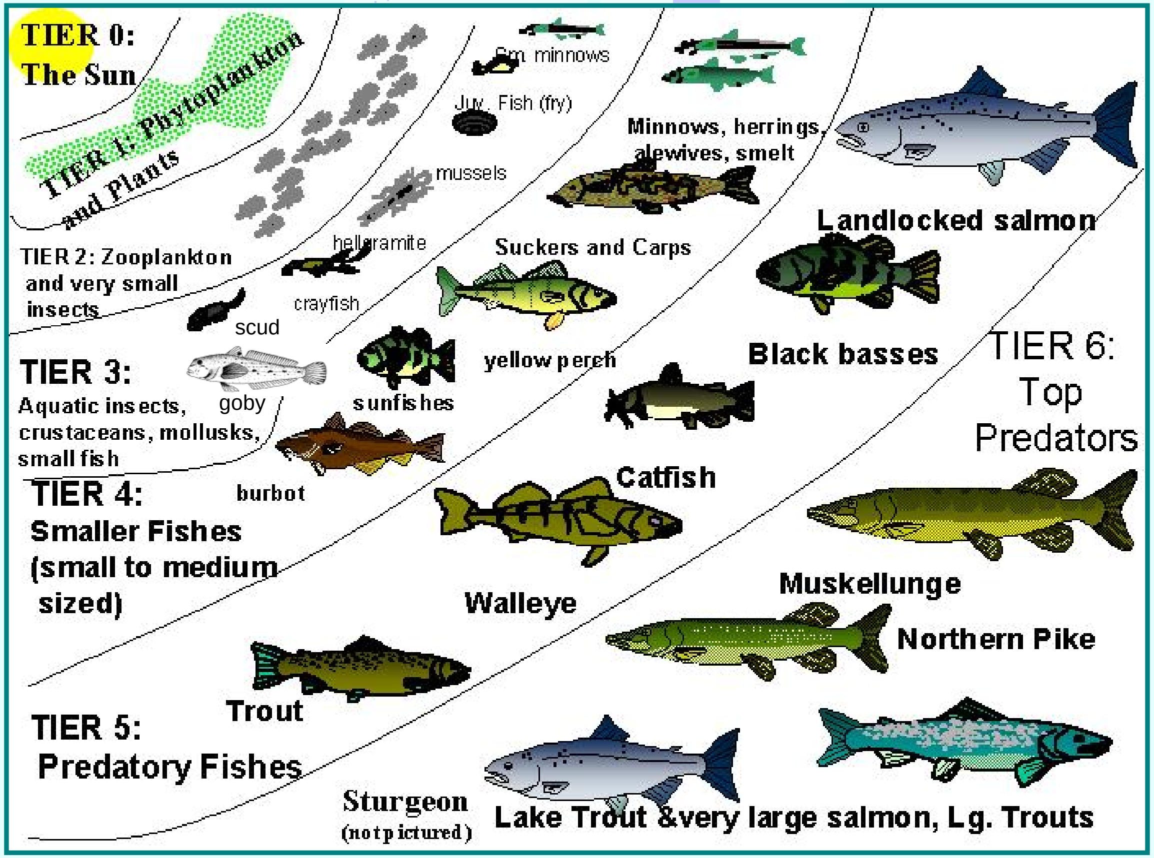


From R. Ruffin, with permission

Type E botulism:
what are the food-web pathways?







Hypothesis:
algal pigments
yellow
orange
red
can be used to trace food-web connections

http://www.combat-fishing.com/lakepondbalance.htm#coldwaterlglake
http://www.dnr.state.mn.us/exotics/aquaticanimals/roundgoby/index.html
http://www.vancouverisland.com/021wildl&cons/wildlife/birds/cw/cw_herringgull.html
http://www.admiraltyaudubon.org/

lneifert@olympus.net

Sea Grant New York

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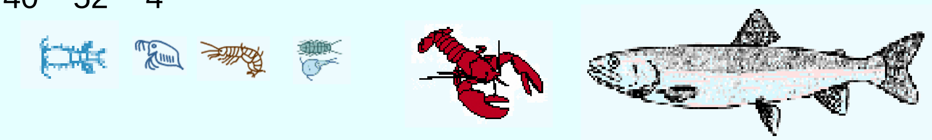
Algal carotenoids found in the food web

diatoms &	fucoxanthin	$C_{42}H_{60}O_6$	☼	☼		
chrysophytes	diatoxanthin	$C_{40}H_{54}O_2$		☼	☼	
cryptophytes	alloxanthin	$C_{40}H_{52}O_2$		☼	☼	
chlorophytes	lutein	$C_{40}H_{56}O_2$	(☼)	☼	☼	☼
cyanobacteria	zeaxanthin	$C_{40}H_{56}O_2$	☼	☼	☼	☼
	cantha-	$C_{40}H_{52}O_2$			☼	☼
	β-crypto-	$C_{40}H_{56}O$			☼	☼
	echinenone	$C_{40}H_{54}O$	☼		☼	☼
euglenophytes	neoxanthin	$C_{40}H_{56}O_4$			☼	
dinoflagellates	peridinin	$C_{39}H_{52}O_7$	NF	NF	NF	NF
crustacean metabolism	astaxanthin	$C_{40}H_{52}O_4$	☼	☼	☼	☼



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Method Development

Standards – high resolution separations

Quantitation – NIST, reference materials, MDLs

Sample prep – microanalytical procedures, SPE, enzymatic hydrolysis

Applications –

Phytoplankton

Gastropods

Dreissenids

Fish – round gobies, drum, perch, bass

Standards - chlorophyll & carotenoids

1 - 5 mg amounts

chlorophyll *a*	SA	ubiquitous	astaxanthin	SA	crustacean metabolism
chlorophyll *b*	SA	chlorophytes	β-carotene	SA	ubiquitous
pheophorbide *a*	WA	chl *a* degradation	lycopene	SA	cryptophytes
pheophytin *a*	WA	ubiquitous	lutein	CD	chlorophytes
			β-cryptoxanthin	CD	cyanobacteria
			zeaxanthin	CD	cyanobacteria
			6'R-α-carotene	CN	cryptophytes
			antheraxanthin	CN	chlorophytes, chrysophytes
			canthaxanthin	CN	cyanobacteria
			echinenone	CN	cyanobacteria
			fucoxanthin	CN	diatoms, chrysophytes
			neoxanthin	CN	chlorophytes, euglenophytes
			violaxanthin	CN	chlorophytes

2.5 mL @ 1.0 μg/mL

chlorophyll *c2*	DHI	cryptophytes	alloxanthin	DHI	cryptophytes chrysophytes
			diadinoxanthin	DHI	diatoms, dinoflagellates
			diatoxanthin	DHI	chrysophytes, diatoms

SA Sigma-Aldrich (Sigma, BioChemika) CD ChromaDex WA Wako (Japan)
CN CaroteNature (Switzerland) DHI (Denmark)

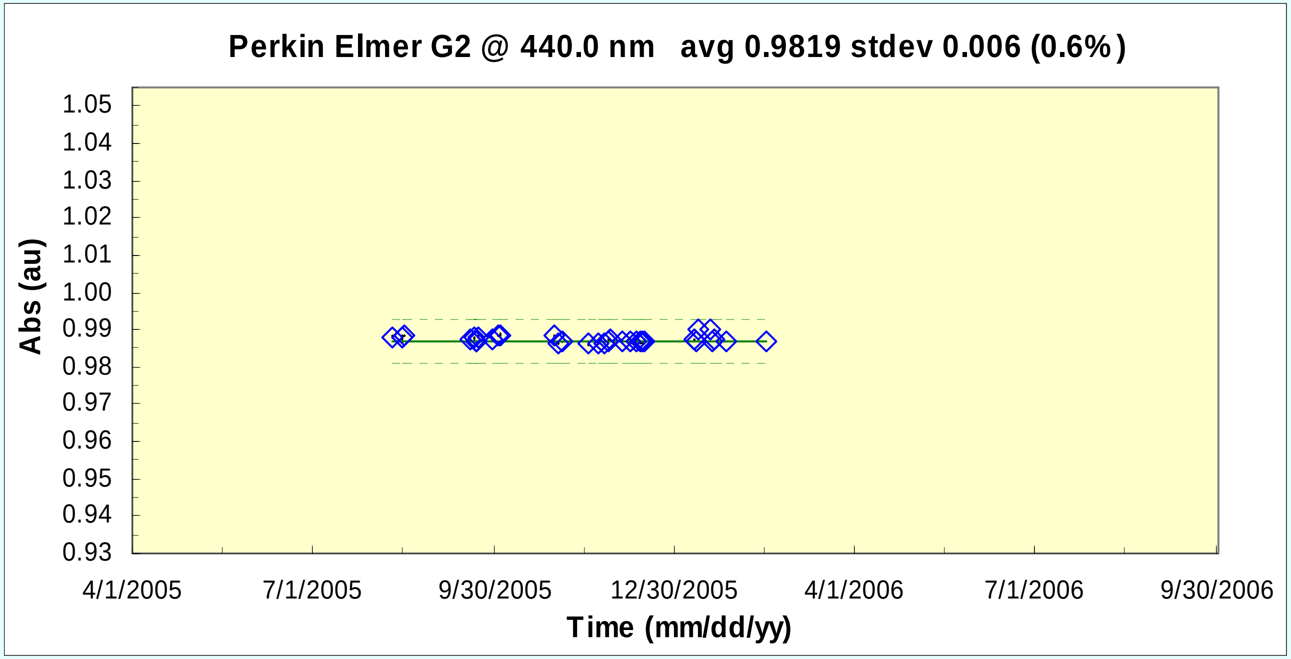
Spectrophotometric calibration of pigment stock solutions

Conc (μg/mL) = Abs/(εb)

ε (L/g-cm) extinction coefficient at λ_{max} for designated solvent

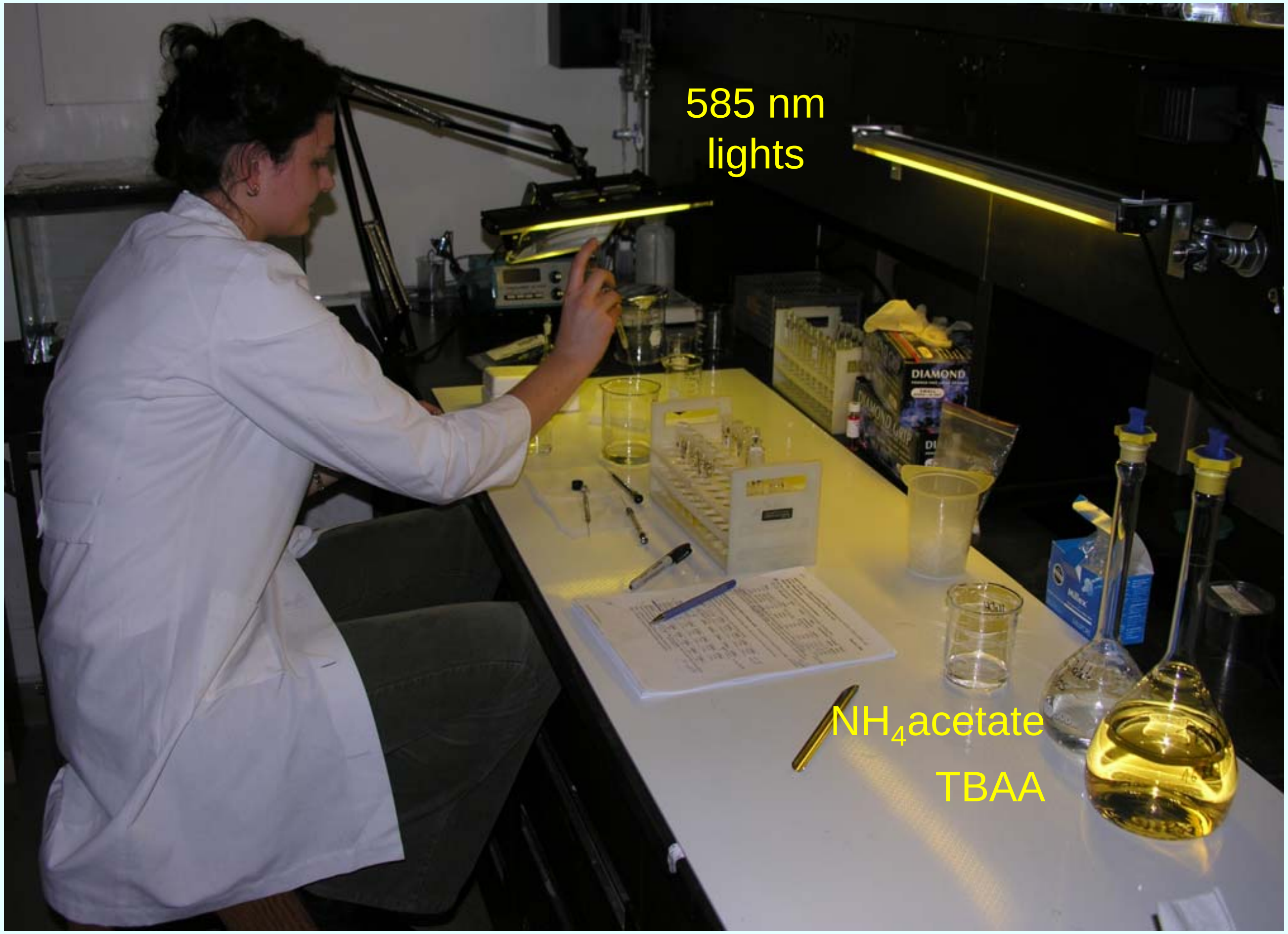
Spectrophotometer accuracy: NIST-traceable standard reference materials

absorbance measurements, average error < tolerance limit 0.006 au-cm			
Grey-glass filters	@440 nm	@546 nm	@635 nm
G1 0.3 au-cm	0.0003	0.0000	0.0037
G3 0.5 au-cm	- 0.0033	-0.0030	- 0.0029
G2 0.9 au-cm	0.0005	0.0009	0.0006



Preparation - mixed standards, 21 pigments

final additions:	*large scale*	300 µL HPLC buffer	50 µL IS	per 4 mL
	small scale	45 µL HPLC buffer	7.5 µL IS	per 600 µL



Analyses - Mixed Standards and Tissue Extracts

Waters HPLC-PDA

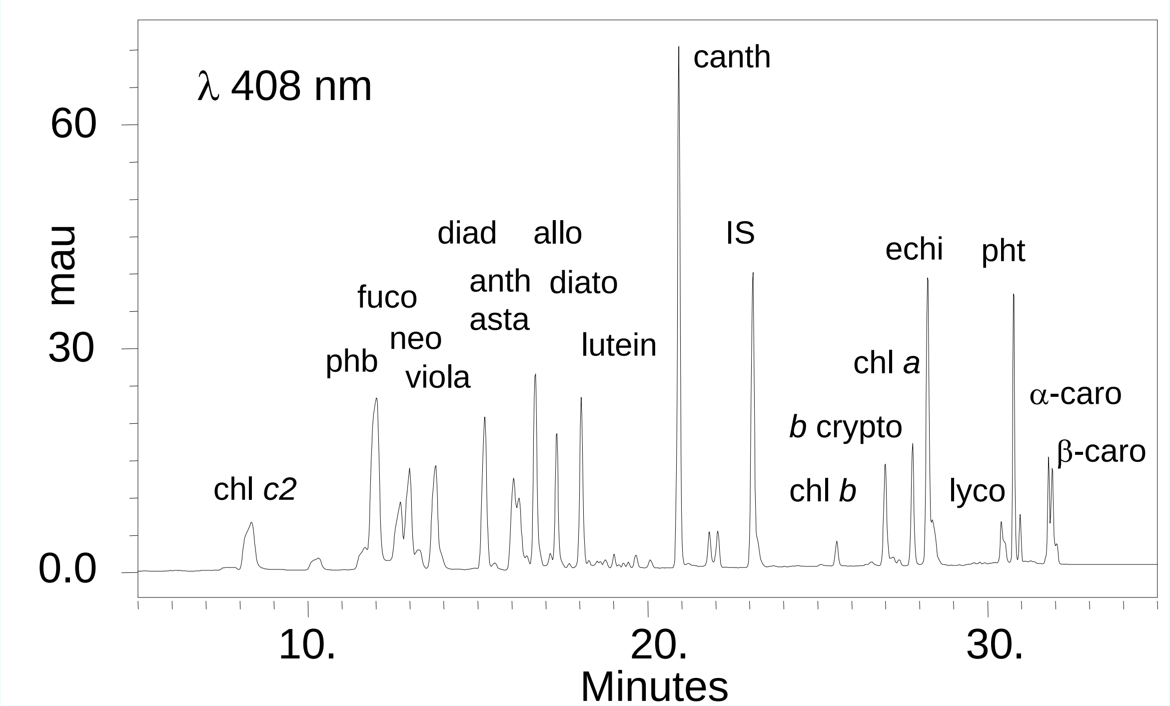
625	pump controller (ternary)
717	autosampler
996	photodiode array detector 200 - 800 nm
474	fluorescence detector
	Empower data system

Agilent-Thermoquest LC-MS

1100	pump system (binary)
1100	autosampler
1100	column heater
1100	single wavelength detector
LCQ	ion-trap mass spectrometer with ESI (electrospray) and APCI (atmospheric pressure-chemical ionization) sources
	Xcaliber data system

Identify Pigments - HPLC retention times

high-resolution separations: 20 pigments

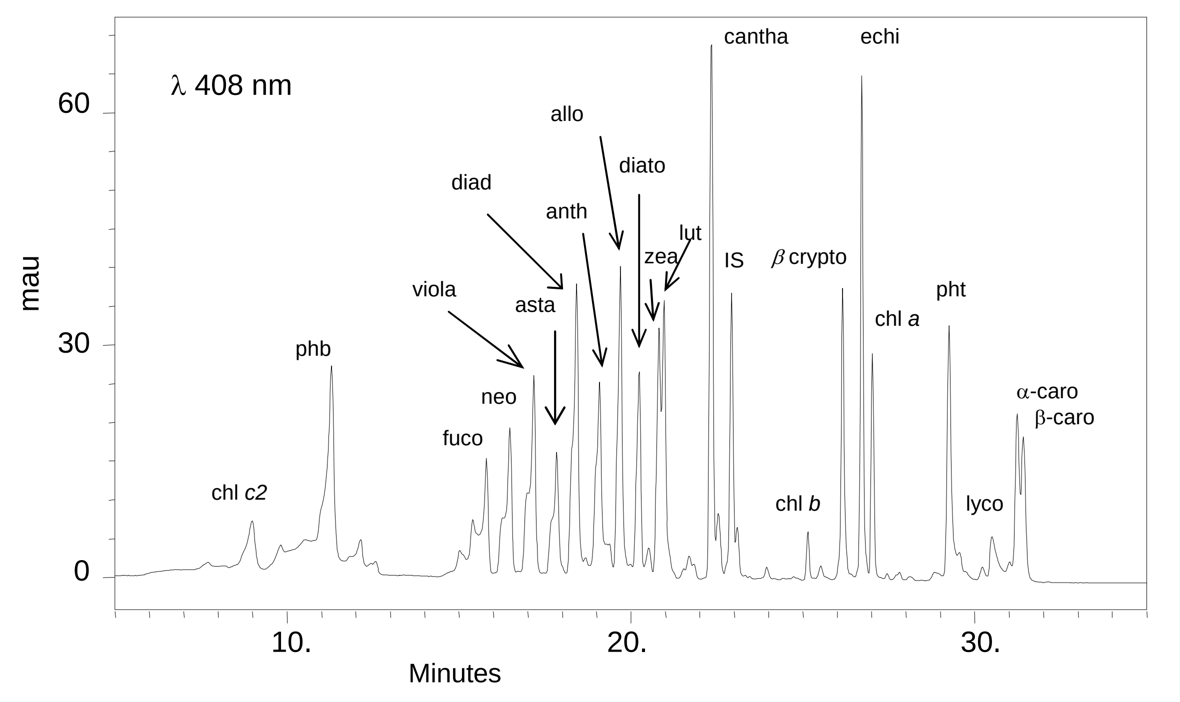


Agilent 3 μm C18 25 cm
60 °C

HPLC solvent program
modified Wright & Jeffrey
(1991, 1997)

A 80:20 MeOH:
0.5 M NH_4acetate:H_2O
B 90:10 MeCN:H_2O
C ethyl acetate

Identify Pigments - HPLC retention times
high-resolution separations: 21 pigments

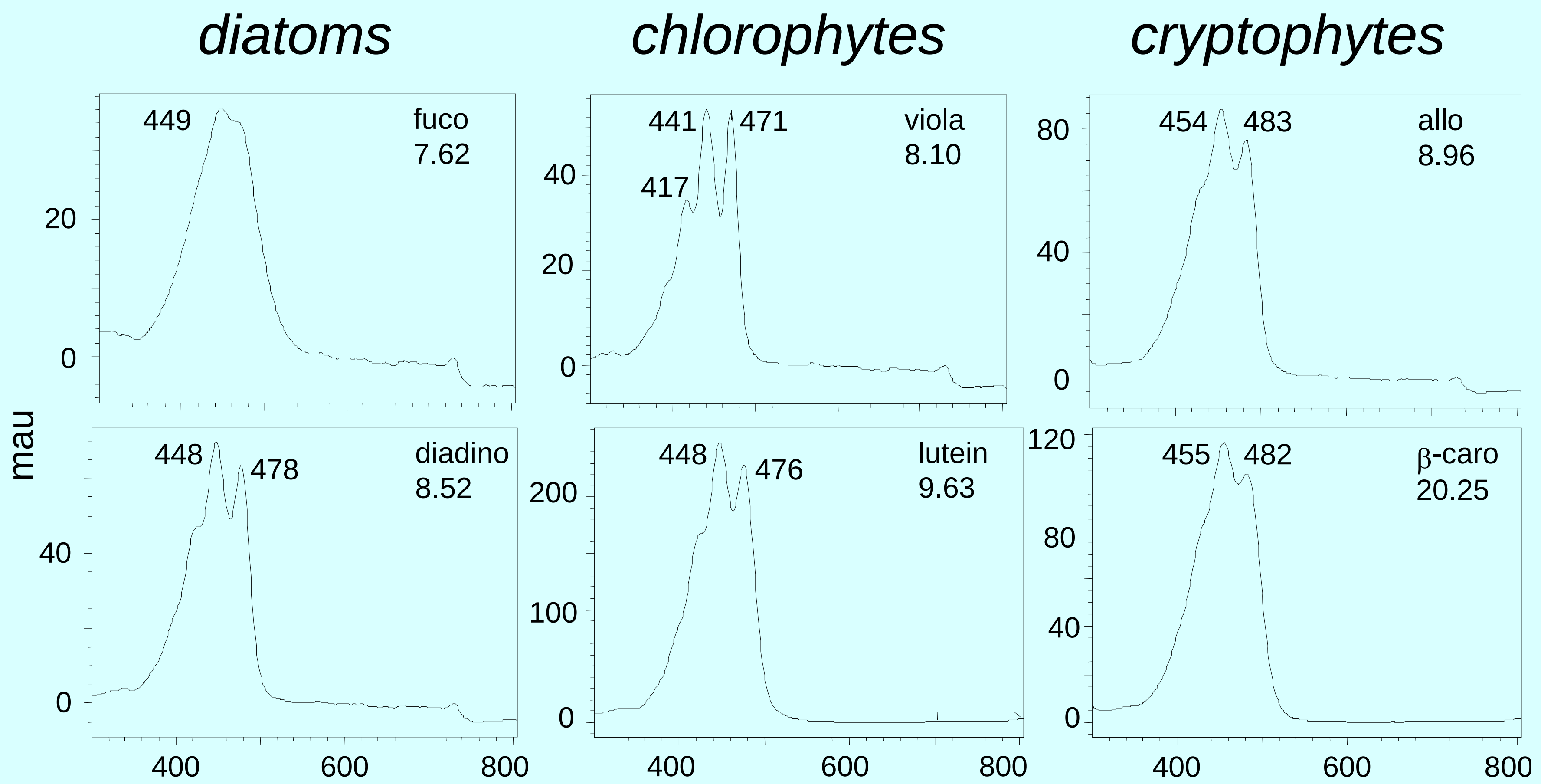


Agilent 3 μm C8 25 cm
60 °C

HPLC solvent program
modified Van Heukelem
Thomas 2001

A 70:30 MeOH:
28 mM TBAA:H_2O
B MeOH

Identify Pigments - PDA spectra 300 - 800 nm



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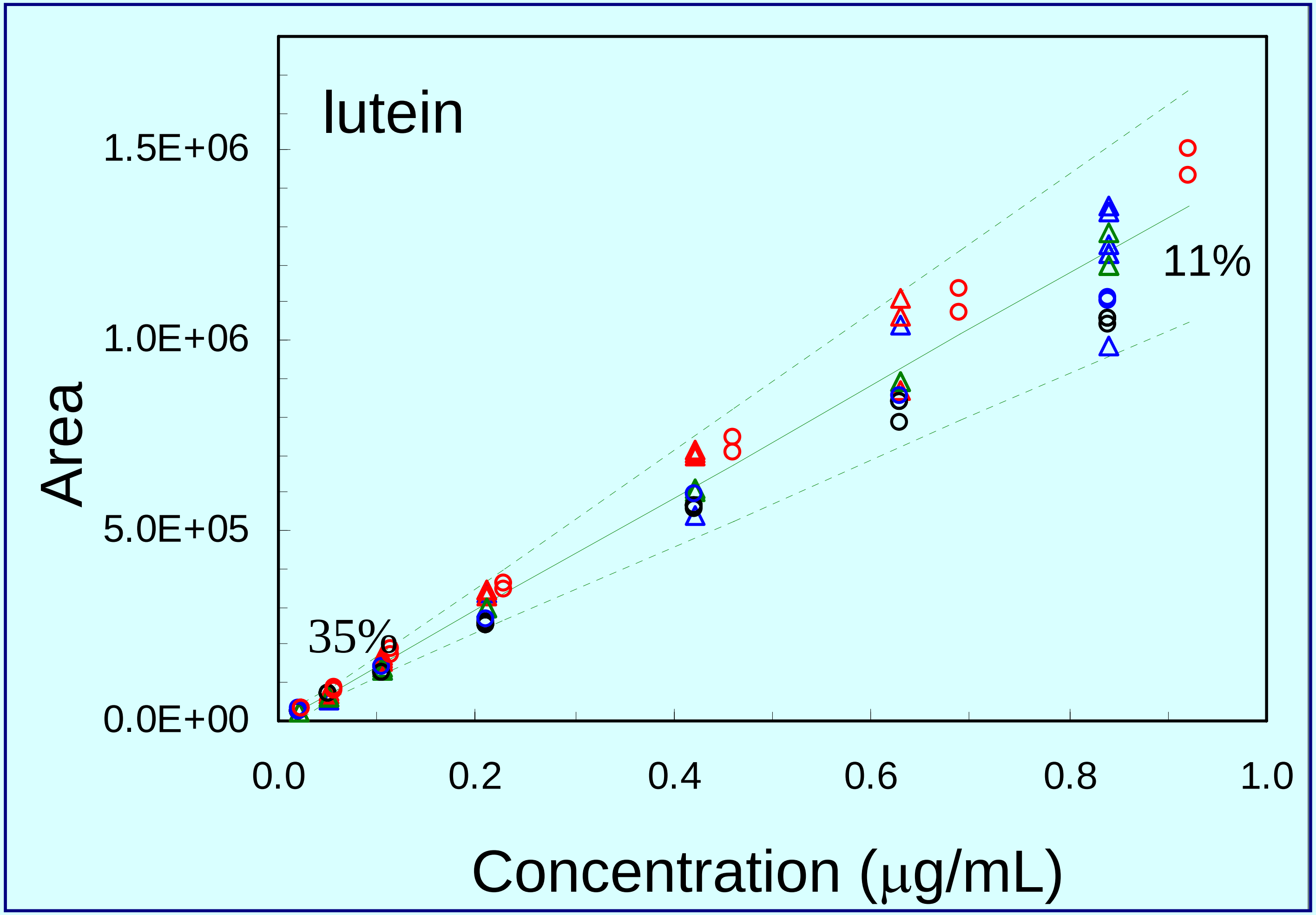
Quantify pigments

multilevel mixed standards - reproducibility, 3 mos



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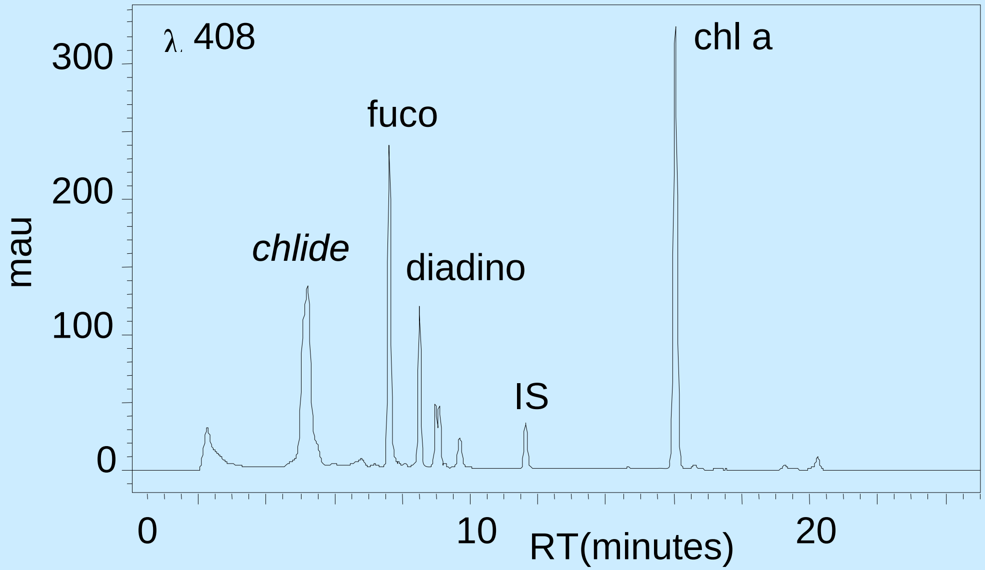
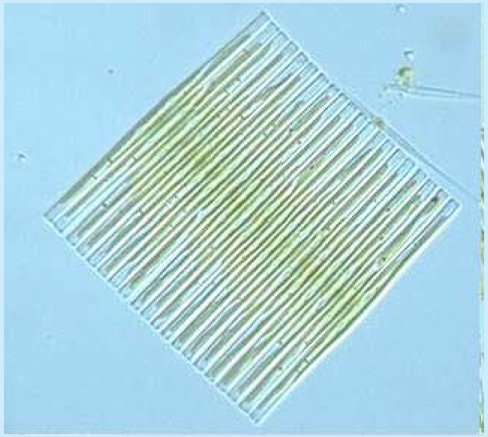
Reference culture: diatom

UTCC 542 *Fragilaria crotonensis*

	μg/10⁶ cells		μg/10⁶ cells
chlorophyll a	1.60	fucoxanthin	1.05
chlorophyllide	1.66	diadinoxanthin	0.33
pheophytin a	0.02	β-carotene	0.07

0.31 - 5.3
Roy et al
1996
St Lawr R





http://protist.i.hosei.ac.jp/PDB/Images/Heterokontophyta/Araphidineae/Fragilaria/Fragilaria.jpg

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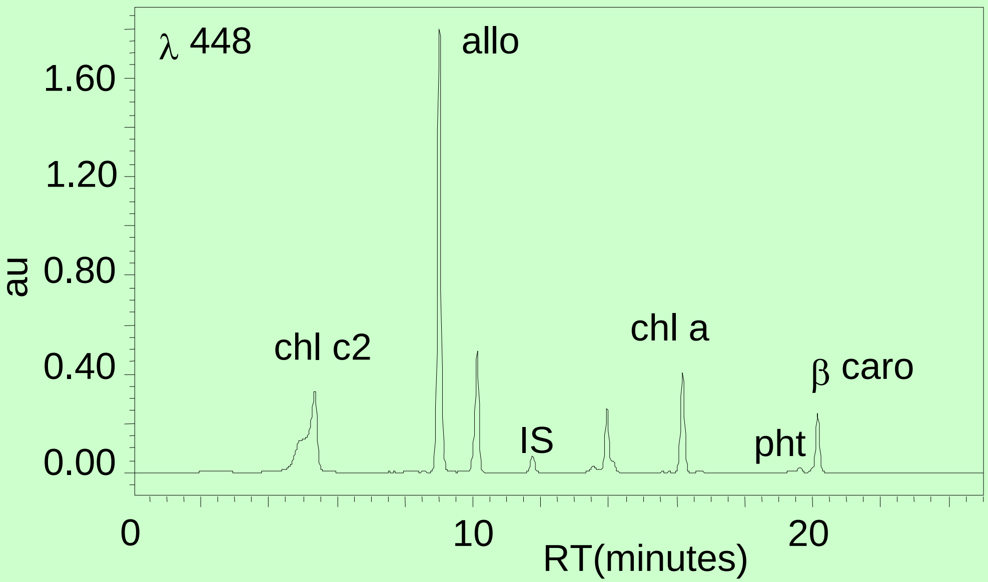
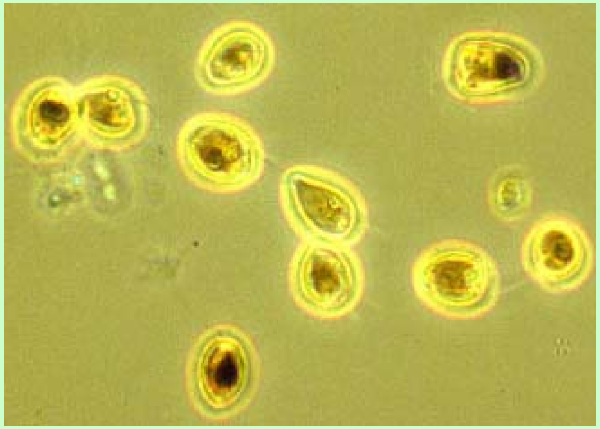
Reference culture: cryptophyte

UTCC 344 *Rhodomonas minuta*

	$\mu g/10^6$ cells		$\mu g/10^6$ cells
chlorophyll a	0.349	alloxanthin	0.101
chlorophyll c2	0.042	β-carotene	0.019
pheophytin a	0.004		

0.19 -0.28
Roy et al
1996
St Lawr R





http://huey.colorado.edu/LTER/images/pictures/lakes/plankton/rhodomonas/Rhodomonas_891204_T2D_8.0.html

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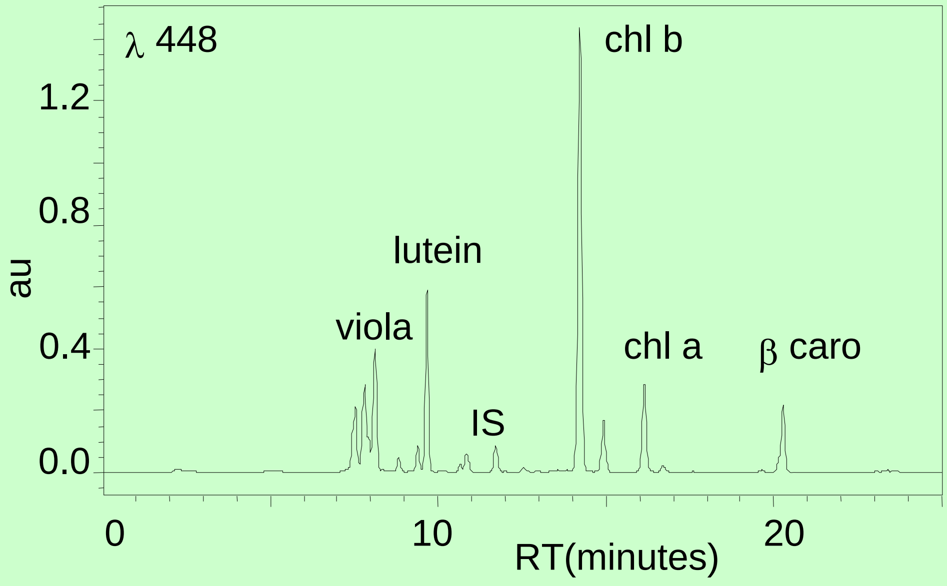
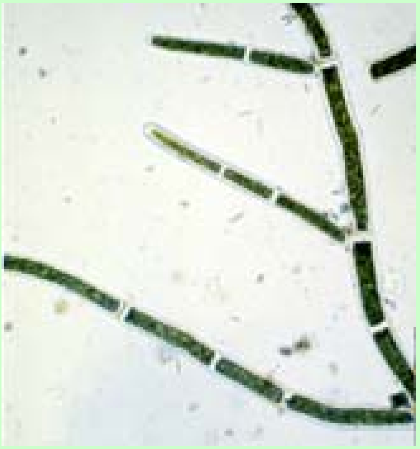
Reference culture: filamentous chlorophyte

UTCC 13 *Cladophora glomerata (L.) Kutzing*

	μg/g ww		μg/g ww
chlorophyll a	1079	lutein	100
chlorophyll b	721	violaxanthin	133
pheophytin a	12	β-carotene	91





http://vis-pc.plantbio.ohiou.edu/algaeimage/pages/Cladophora.html

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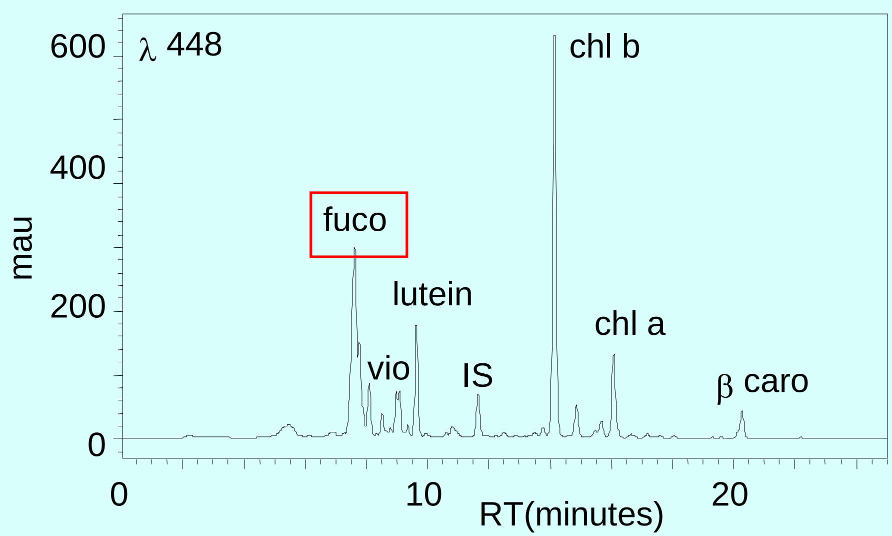
Lake Ontario: *Cladophora glomerata*

	µg/g ww		µg/g ww
chlorophyll a	107.3	lutein	7.0
chlorophyll b	70.6	violaxanthin	5.7
pheophorbide a	15.7	β-carotene	4.1
pheophytin a	4.8	fucoxanthin	37.5
chlorophyllide	*1.2*	diadinoxanthin	2.0

diatoms ←



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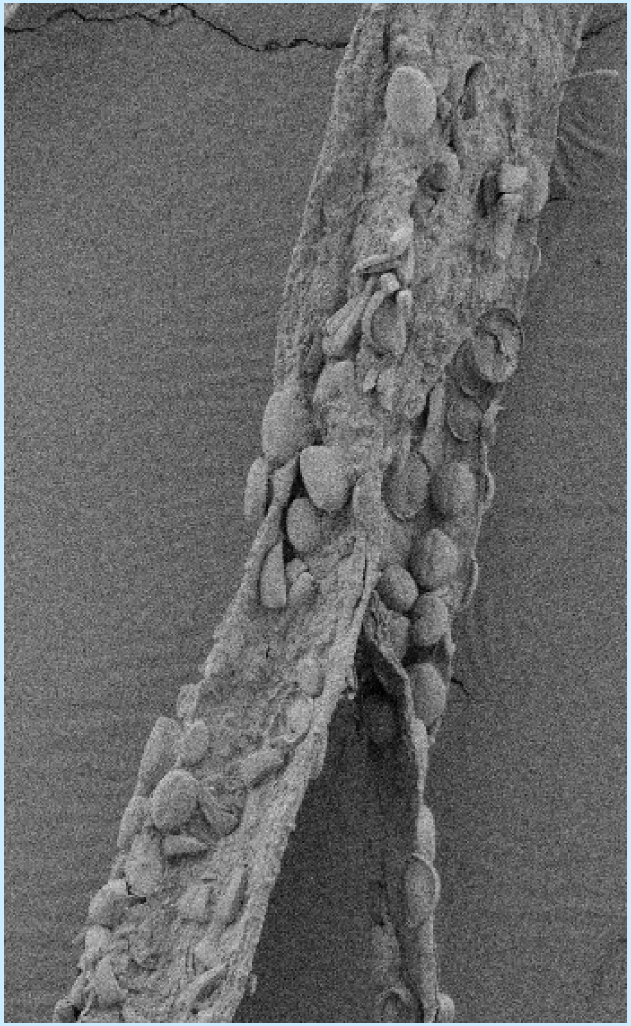
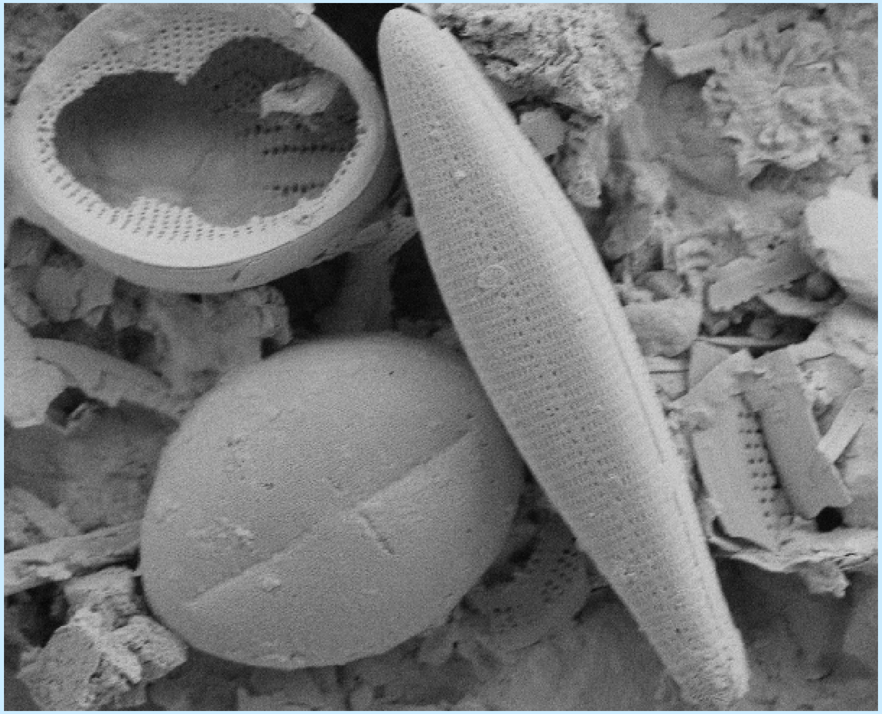
Epiphytic diatoms



100 μm



3 μm

Cocconeis pediculus on fiber of *Cladophora glomerata*

Stevenson Stoermer 1982

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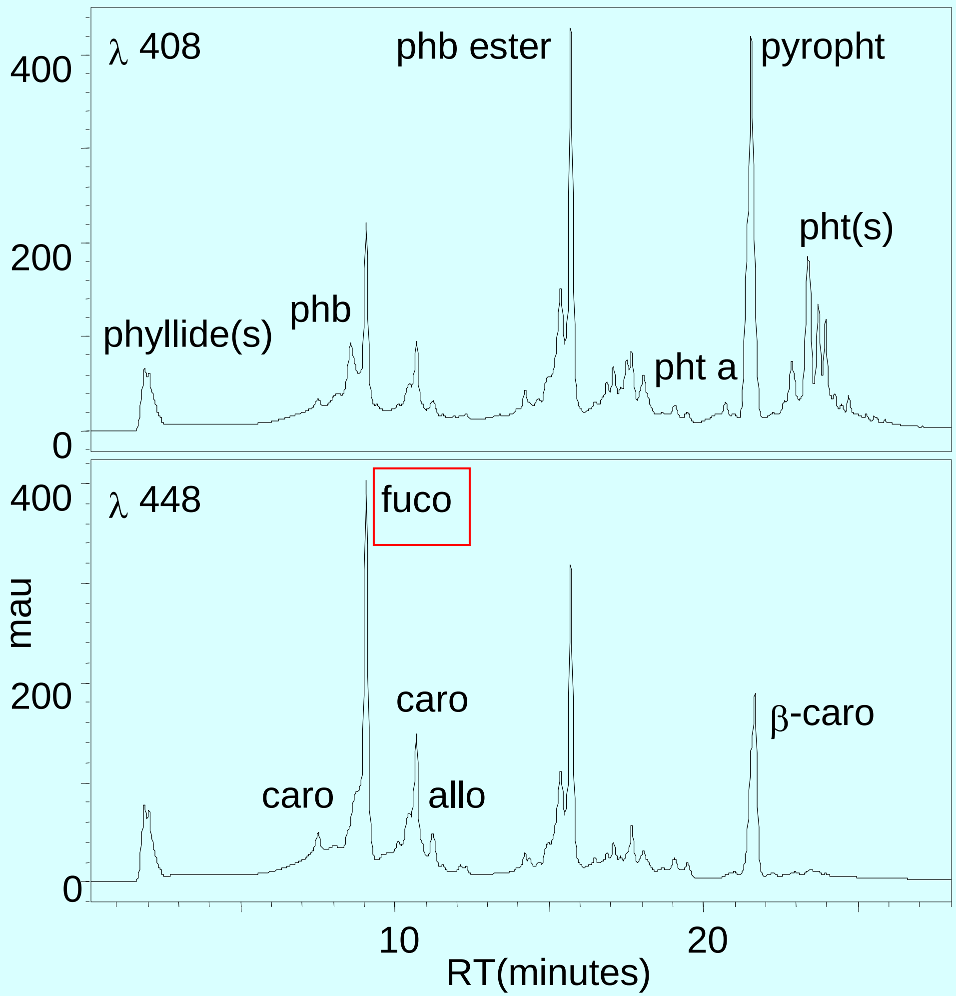
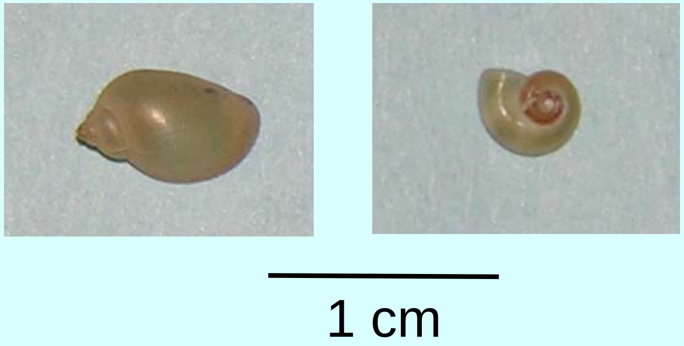
Lake Ontario gastropods





	μg/g ww
pheophytin a	1.5
pyropheophytin	26.2
fucoxanthin	29.0
alloxanthin	4.4
β-carotene	11.2

diatoms ←

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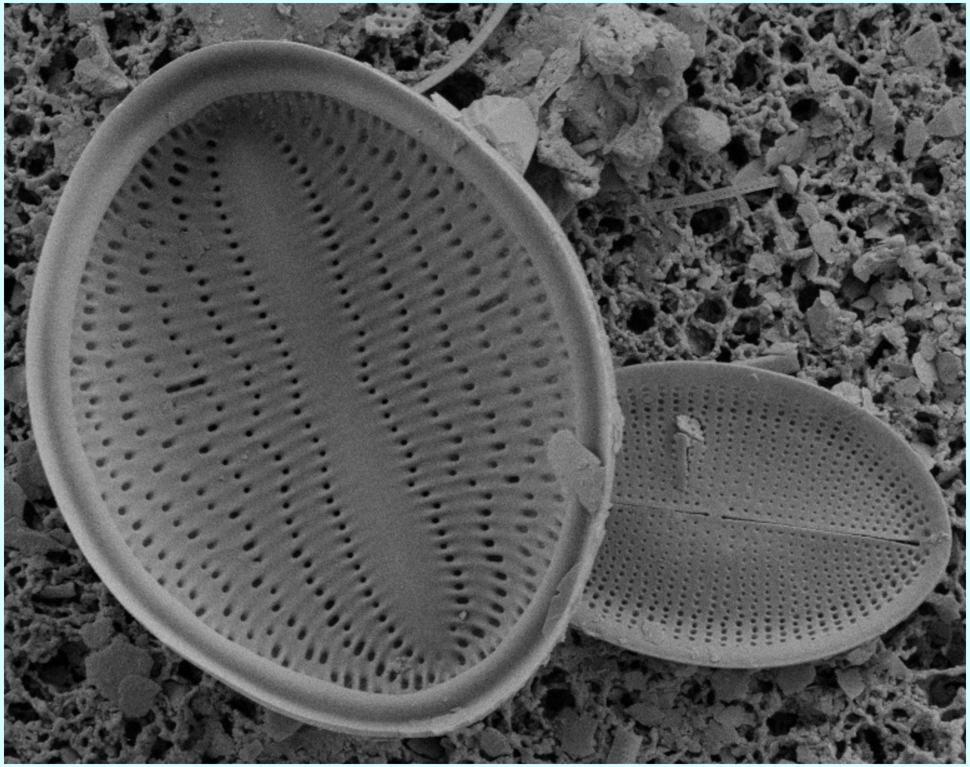
Epiphytic diatoms → ingested diatoms

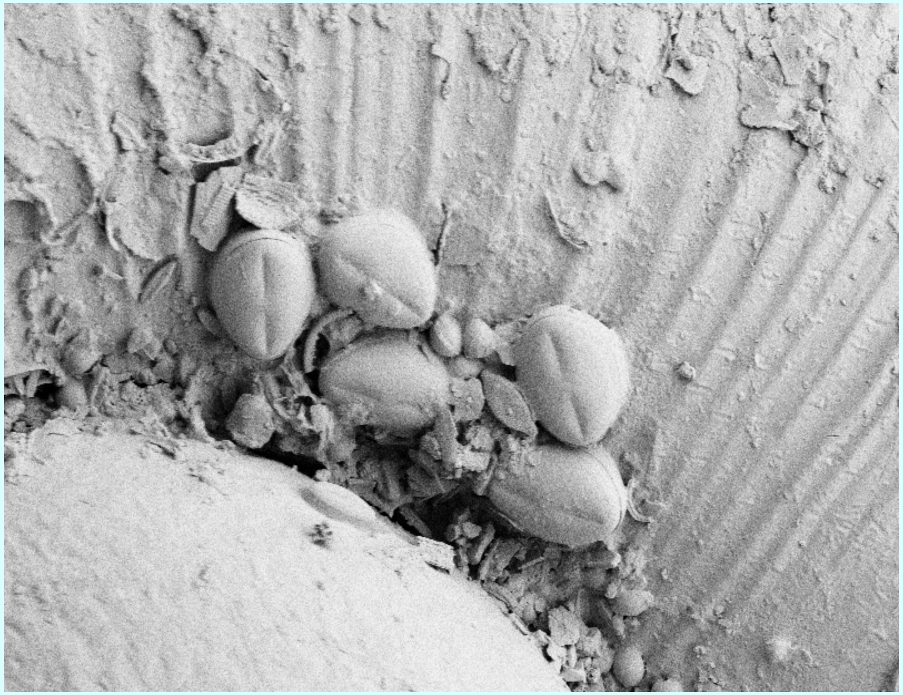


3 μm

Cocconeis pediculus on snail shell

→

← *Cocconeis pediculus* in snail tissue ∴ *ingested*



10 μm

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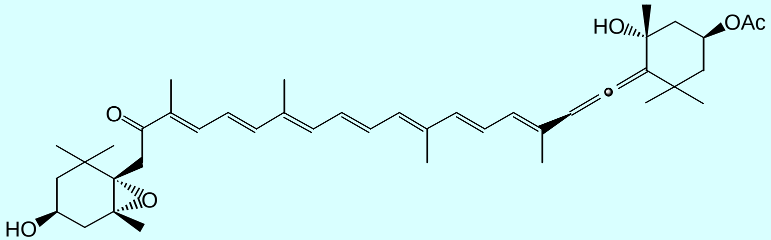
Case study #1: conclusions

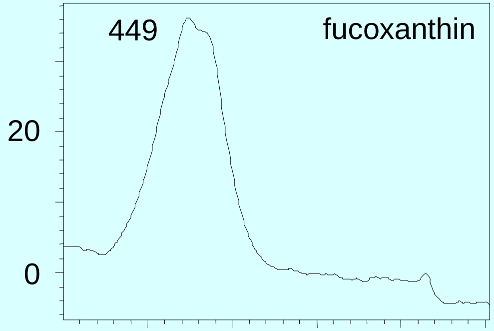
Carotenoid biomarkers for diet

epiphytic gastropods ~ grazers ~ epiphytic diatoms

first trophic level ← primary productivity (diatoms)



$C_{42}H_{60}O_6$ *fucoxanthin*

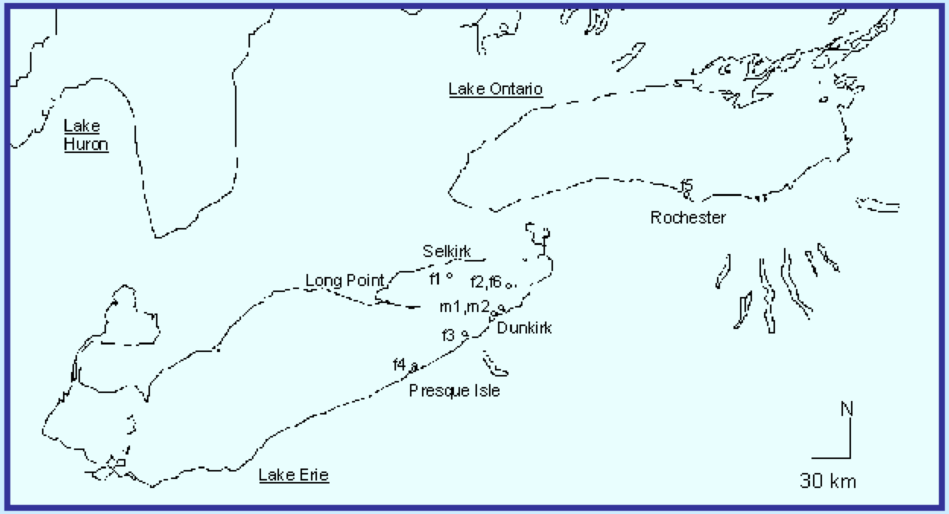


__on-going studies__

Analysis of other benthic organisms (oligochaetes, tubificids, diptera etc) - senescent phytoplankton

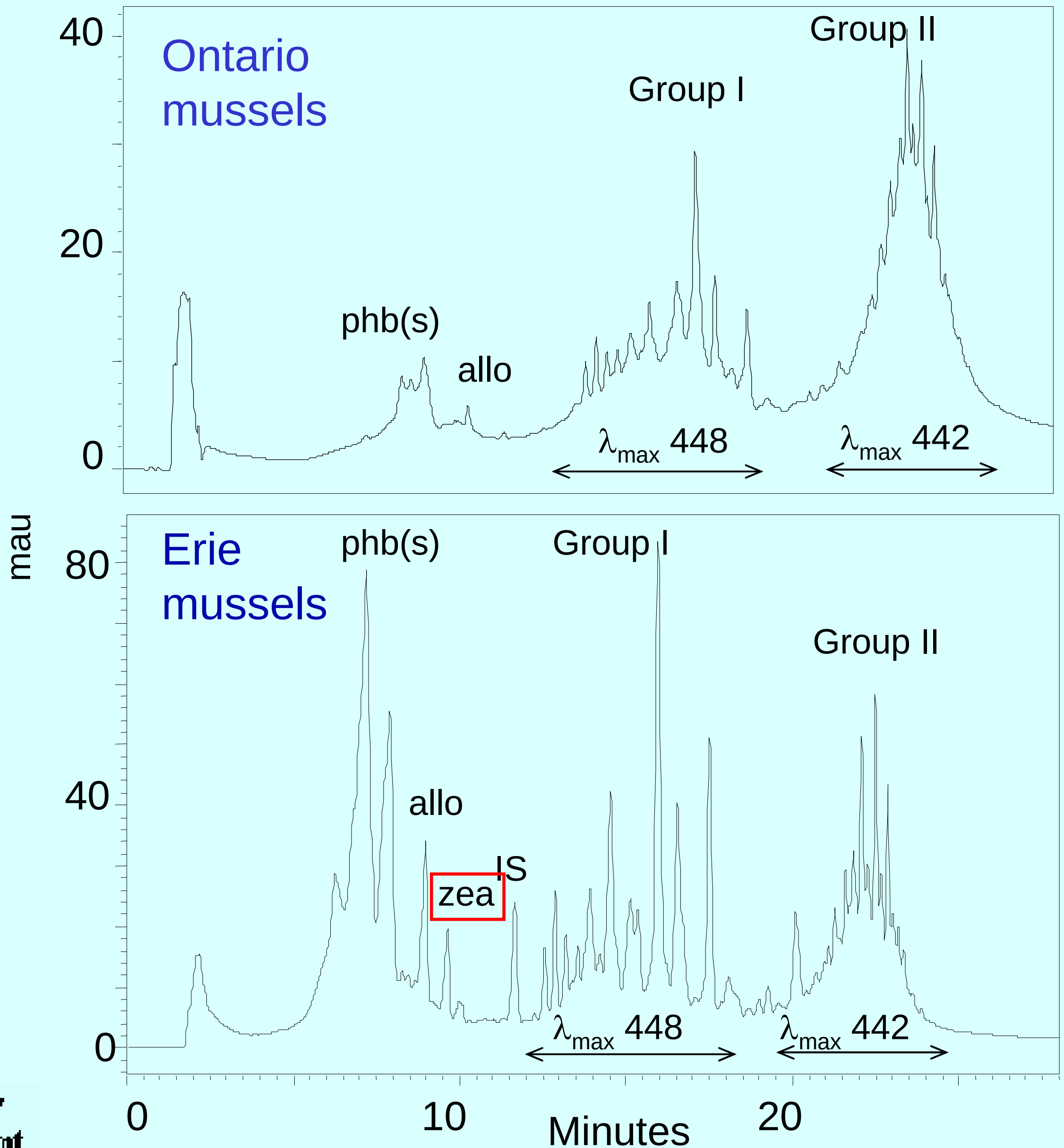
Analysis of fish (and birds) that eat snails

Case study #2: quagga mussels & phytoplankton



dreissenid invasion of Great Lakes: benthic filter feeders (~1 L/day/mussel) reduced chlorophyll a, phytoplankton biomass (MacIssac et al 1992; Horgan & Mills 1997; many others)

HPLC-photodiode array analysis



cyanobacteria ←

Barbiero Tuchman 2000

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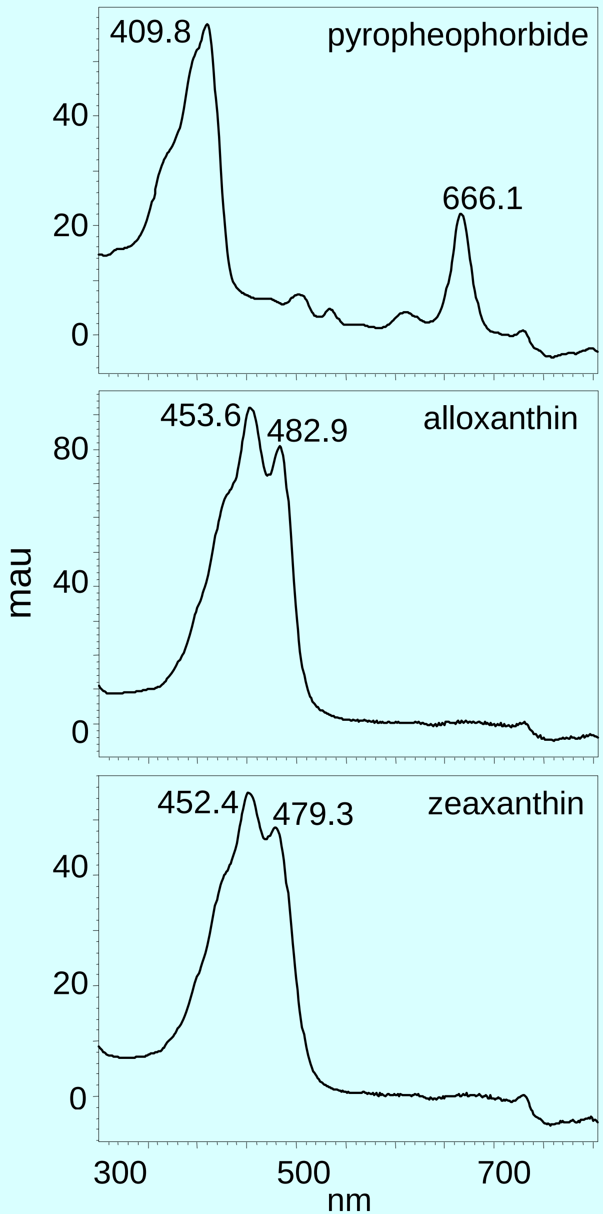
Algal biomarkers in mussel tissue



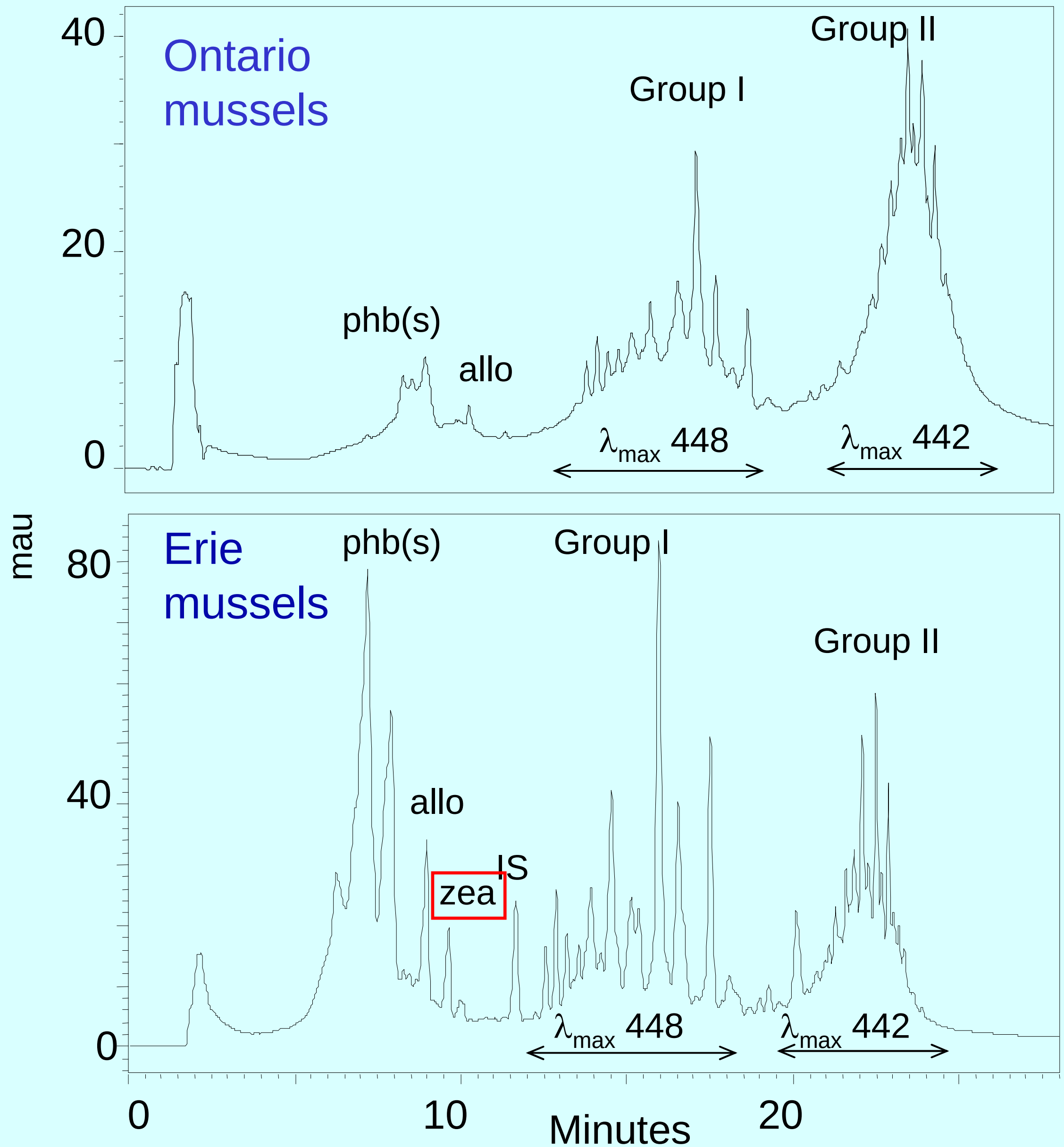
degradation of chlorophylls
- digestion by mussels

cryptophytes

cyanobacteria - *Lake Erie*

Barbiero Tuchman 2000

Group I & II pigments: hydrophobic (late eluting)



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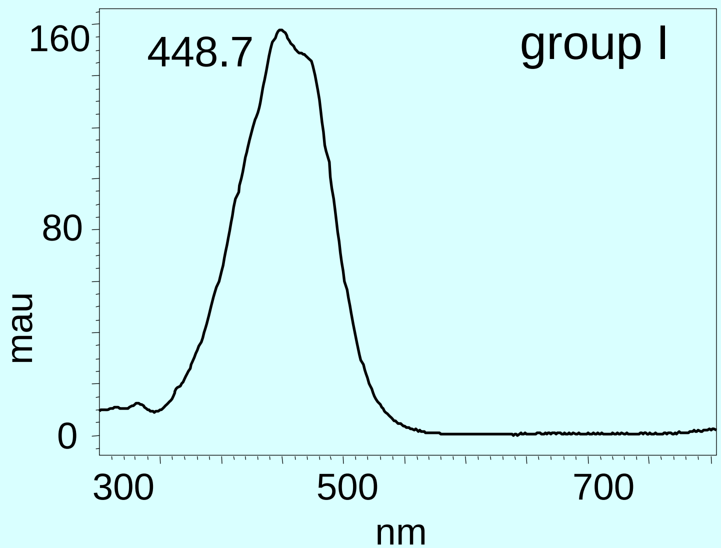
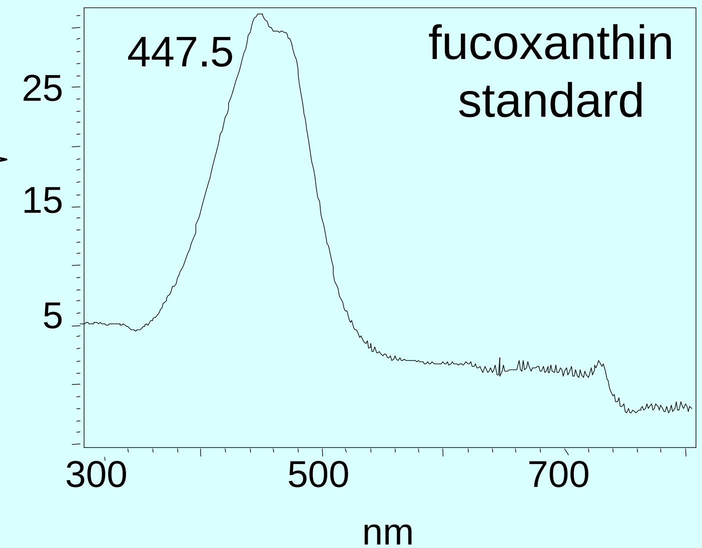
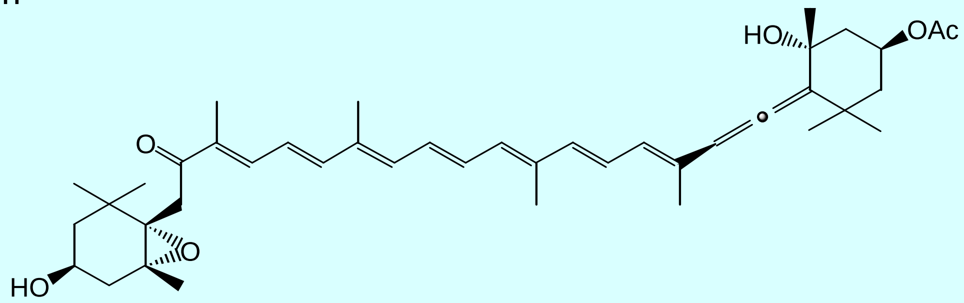
Group I cluster: identification



Library:
fucoxanthin
chromophore



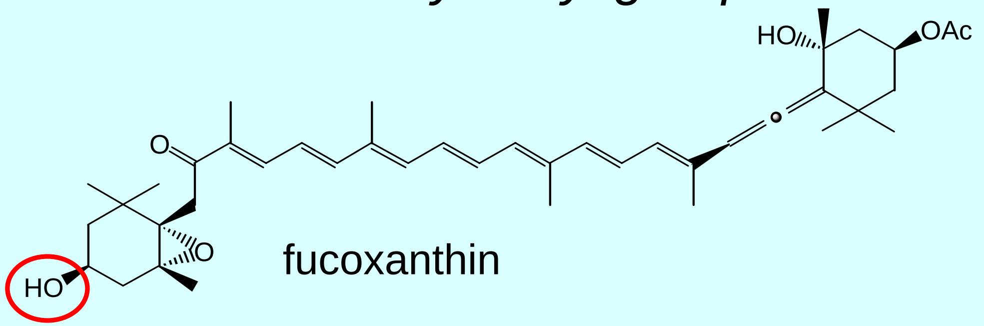


pigment fingerprint of *diatoms*

Group I: carotenoid fatty-acid monoesters

Xanthophyll pigments: 1 or more *hydroxyl groups*

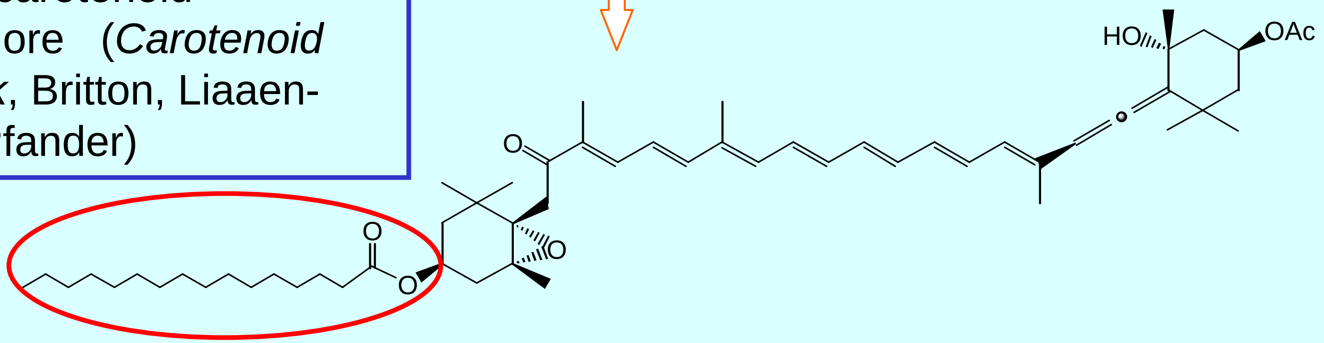
Hydroxyl available to be esterified by fatty acids



fucoxanthin

esterification

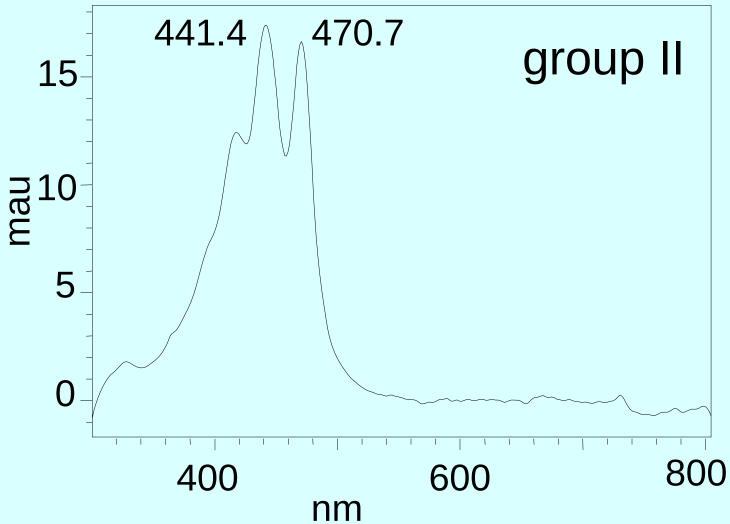
esters typically have spectrum of parent carotenoid chromophore   (*Carotenoid Handbook*, Britton, Liaaen-Jensen, Pfander)



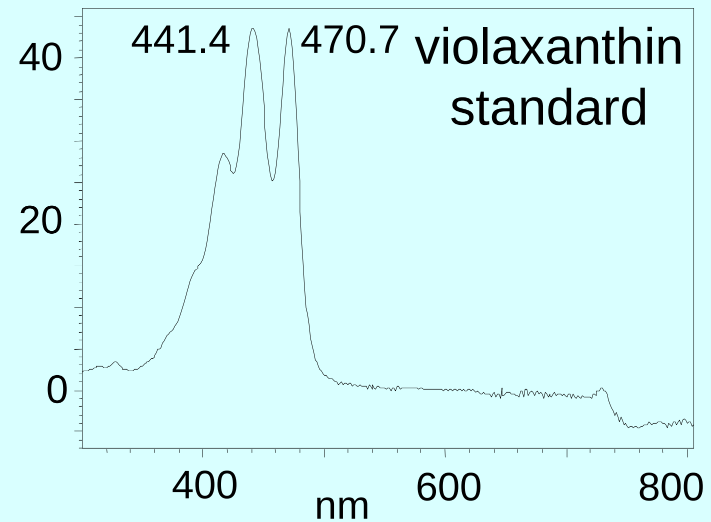
fatty acid monoester (eg C16 - palmitate)

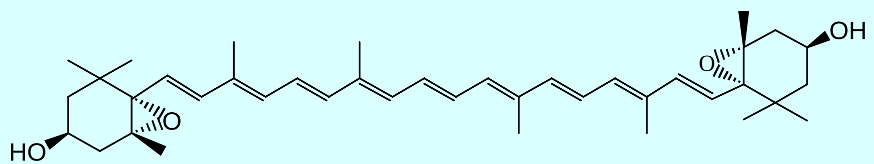
FAs	*saturated*	*monounsaturated*	*PUFAs*
	C14:0, <u>C16:0</u>, C18:0, C20:0	C18:1n9	C18:3ω3, C18:2ω6, C20:5ω3 C20:4ω6, C22:6ω3

Group II cluster: carotenoid fatty-acid *di*esters

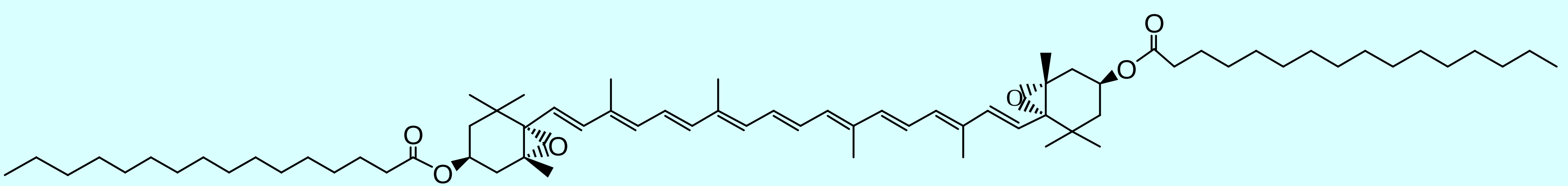


Library
violaxanthin
chromophore





pigment fingerprint of *chlorophytes*



violaxanthin diester

(C16:0)

(C16:0)

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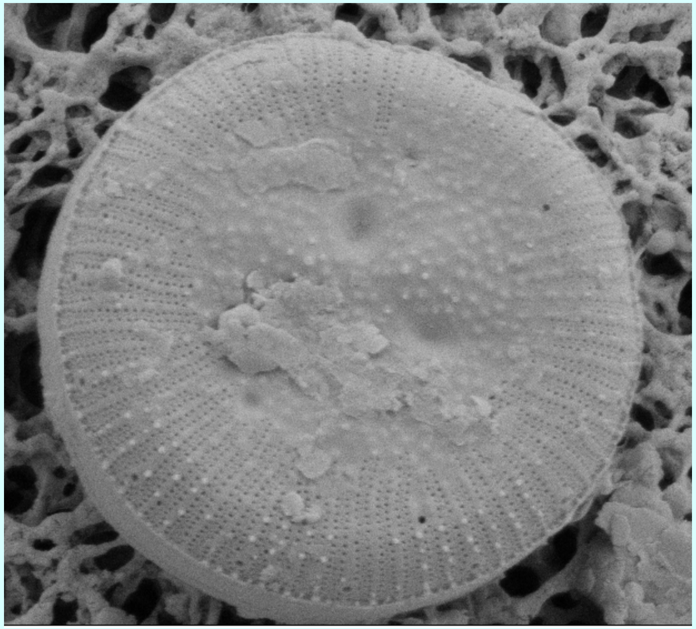
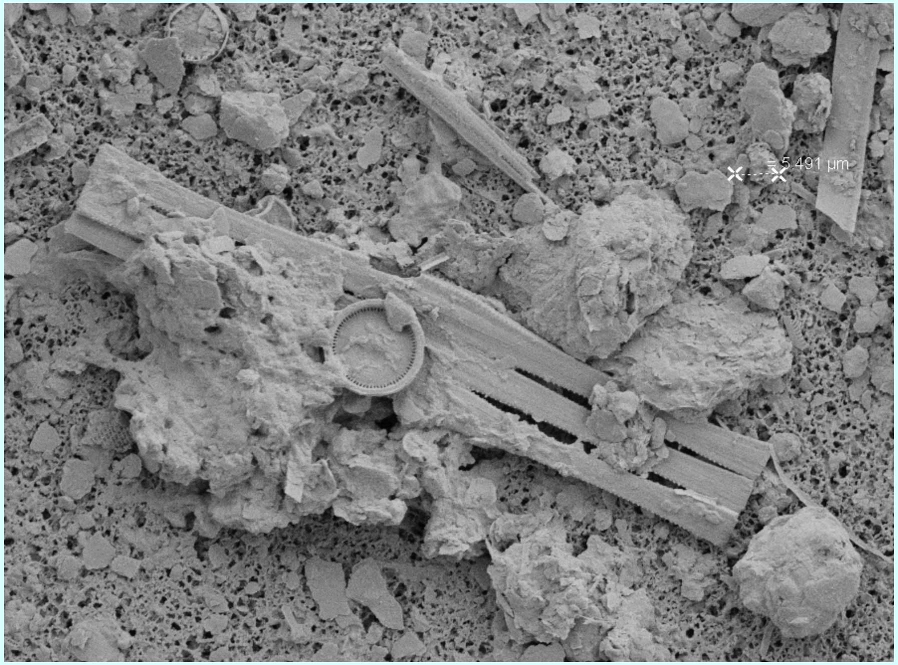
SEM: *ingested* diatoms, from mussel tissues



2 μm centric

pennate (*Fragilaria*) & centric



10 μm

Credit:Monika Sobiechowska 2005

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Quantitation

pigment	Lake Ontario (μg/g ww)	Lake Erie (μg/g ww)
pyropheophorbide (*phb*)	1.00 ± 0.56	8.57 ± 2.18
alloxanthin	0.04 ± 0.02	1.87 ± 0.65
zeaxanthin	NF	0.70 ± 0.26
monoesters *(fucoxanthin)*	7.45 ± 3.79	23.33 ± 4.97
diesters (*violaxanthin*)	4.60 ± 2.38	6.50 ± 2.95

Case study #2: conclusions

http://www.noaa.gov

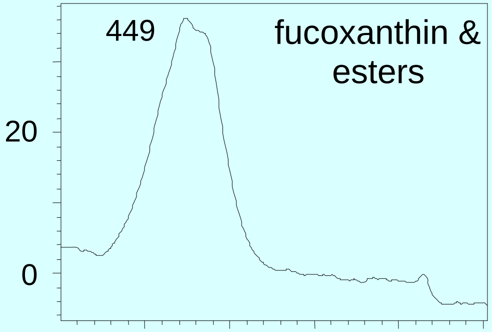
Carotenoid biomarkers for diet

mussels ~ filter-feeder ~ phytoplankton

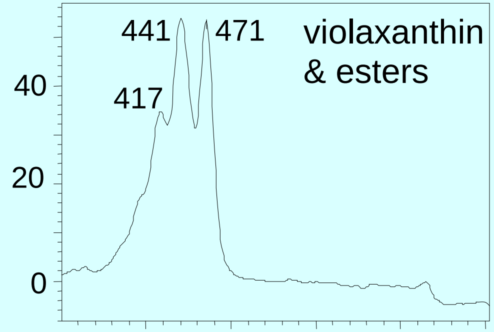
first trophic level ← primary productivity

from *chromophores in tissues*: can differentiate algal food sources

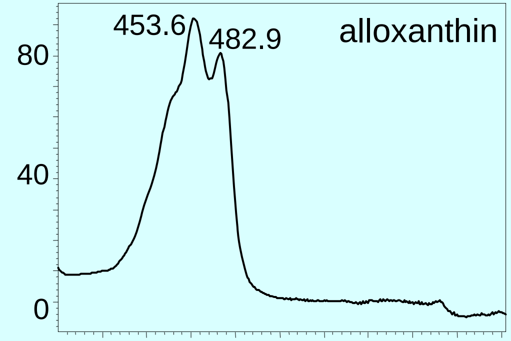
diatoms



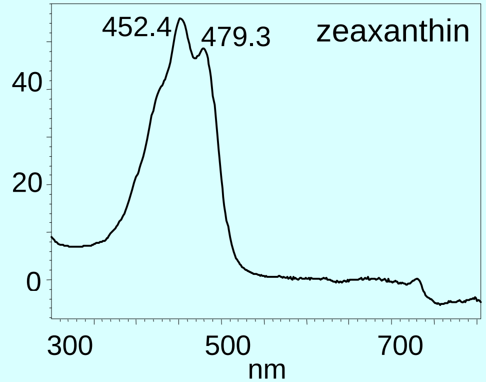
chlorophytes



cryptophytes



cyanobacteria



and differentiate mussels from Lake Ontario and Lake Erie

Sample preparation

Filamentous algae, sediments, tissue

wet weight → homogenize → freeze dry → dry weight

Phytoplankton

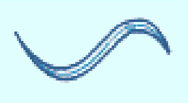
cell counts → filter GF/F

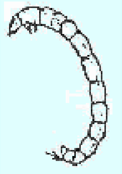
Extracts

sonicate *acetone*	→	concentrate *dry N_2*	→	filter *0.2 μm*		
	→	solid phase extraction *silica (hexane, acetone)*	→			reconcentrate *dry N_2*
	→	enzymatic hydrolysis *diethyl ether*	→			reconcentrate *dry N_2*

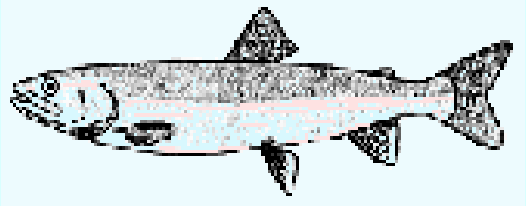
2005-6 studies: carotenoid biomarkers & diet

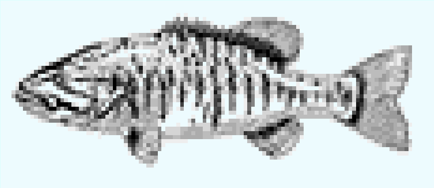
benthic organisms - oligochaetes, chironomids

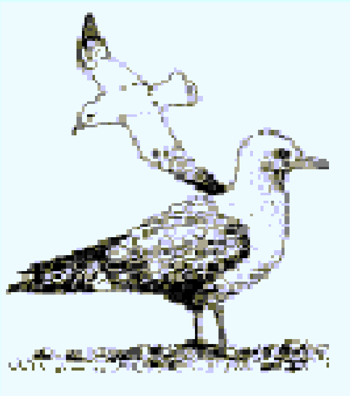




higher trophic levels - fish and birds







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