SH5 HPL method talk

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Primary Application

- Analyze ~6500 samples/year collected around the world by multiple PIs
- sometimes we know the accuracy requirements of the PI, sometimes we don't

SeaHARRE-5 samples

- Date received: 2 February 2009
- Stored at -80˚C
- Sorted in -25˚C walk-in
- Dates extracted: 9,10 March 2009
- Complications:
  - After starting the SH samples, our Rs dropped from 1.3 to 1.1 (zea/lut)
  - Blunders-We had errors in reporting

    -due to changes in procedures from the ‘everyday’

Extraction

Procedures

- Extraction solvent - 2.5ml 100% acetone (w/ISTD) and 150 ul water
- Pulse sonic disruption with 1/8” Sonic probe for 8 sec (kept in ice/water)
  - Branson Digital 450-400W max
  - 25% amplitude
- Kept at -25˚C ~3 hrs
- filtered through 13 mm 0.45 um Teflon syringe filter

Considerations

- Final solvent concentration ~90% (2.5 ml 100% acetone + 100 ul water + 150 ul water contribution from water retained in sample filter)
- Extraction volume determination
  - ISTD (Vitamin E acetate)
  - This calculation checked against an assumed extraction volume
  - Accuracy of solvent delivery device checked daily, actual volume used in calculation

HPLC Hardware-Agilent 1100

- Quaternary pump
- Chilled autosampler (-4˚C) with 900 ul loop
- Diode-array detector with visible and UV lamps, 13 ul flow cell (10 mm)
  - 1 cm pathlength
  - Data collected at:
    - 450 +/- 10 nm (carotenoids and some chlorophylls)
    - 665 +/- 10 nm (chlorophyll a and associated products)
    - 222 +/- 5 nm (internal standard)
  - Spectral data collected 350-750 nm

HPLC method-Van Heukelem and Thomas 2001

- Column: Zorbax Eclipse XDB C8 kept at 60˚C
  - 150 x 4.6 mm, 3.5 um particle size
- Solvent A: 70% Methanol, 30% 0.028 M tetrabutyl ammonium acetate (pH 6.5)
- Solvent B: 100% Methanol
- Linear gradient 5%-95% solvent B over 22 minutes
- 1.1 ml/minute
- Re-equilibrate with 15 ml of initial conditions solvents before the next injection

Injection procedures

- **Same injection procedure for standards and samples**
- ~ 500 ul of sample or standard is placed in each sample vial
- All vials reside in the TCAS (4° C) for at least 1 hr before injection, the first injection of sequence is disregarded.
- Buffer is 10% Methanol, 90% 0.028M tetrabutyl ammonium acetate
- 525 ul total injection volume, 150 ul of sample loaded **(sample:buffer ratio = 150:375)**
- Injector program (using a 900 ul loop) steps:
  - Draw 150 ul buffer
  - Draw 75 ul sample
  - Rinse needle
  - Draw 75 ul buffer
  - Draw 75 ul sample
  - Rinse needle
  - Draw 150 ul buffer
  - Inject
- **HPLC vials limit evaporation** to no more than 0.7% maximum over 24 hrs
- Pre-slit septa are used with the more viscous buffer to prevent formation of vacuum within vial during withdraw of buffer

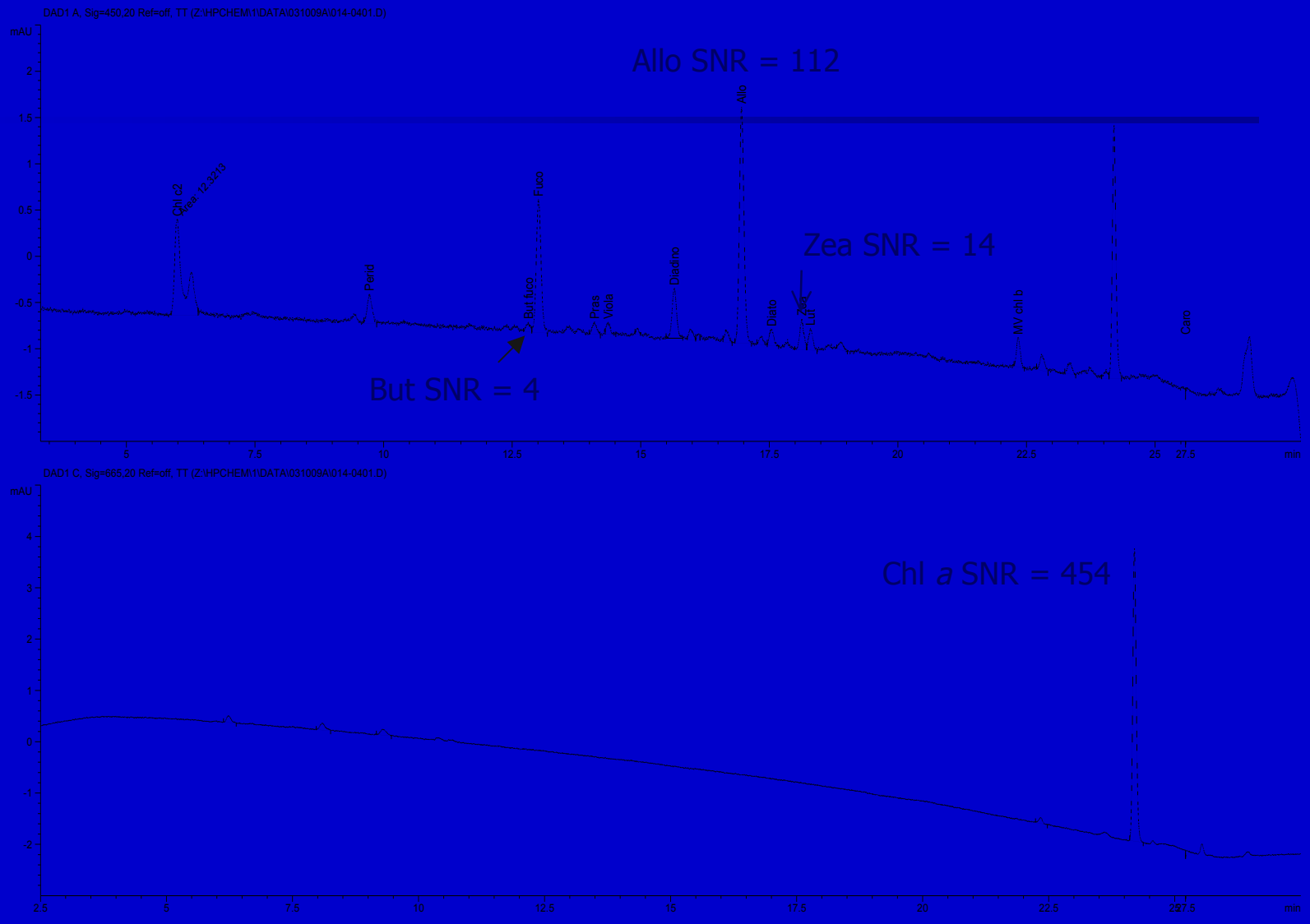
Quantitation Procedures

- **Chlorophyll *c*3-**add monovinyl and divinyl peaks together, report the sum as Chl c3
- **Peridinin isomer-**quantitate with the Peridinin main peak
- **Diadino** in the presence of dinoxanthin and diadinochrome-when there are a lot of Peridinin-containing dinoflagellates, we see diadinochrome and dinoxanthin on either side of the Diadino peak and we remove their contribution to the peak area of Diadino before quantifying
- **Chl *b*/DV Chl *b*-**quantify by peak height if both are present
- **Pheophorbides-**usually see 2-5 pheophorbide-like peaks, report the sum of these as pheophorbide

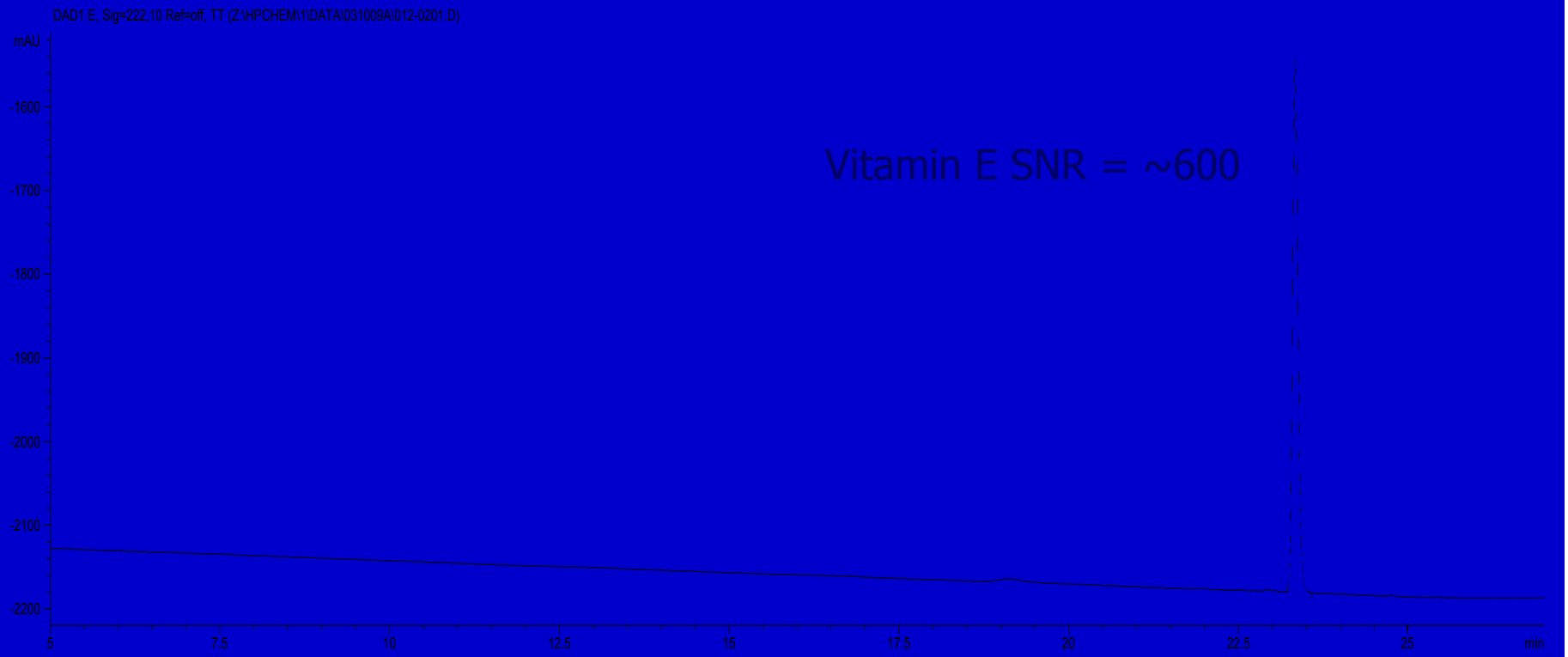
Calibration procedures

- single point calibration
  - Samples within linear range, previous dilution series showed y-intercept is near zero
  - Calibration accuracy is regularly checked, dilution series periodically reassessed
- Use absorption coefficients in common with DHI

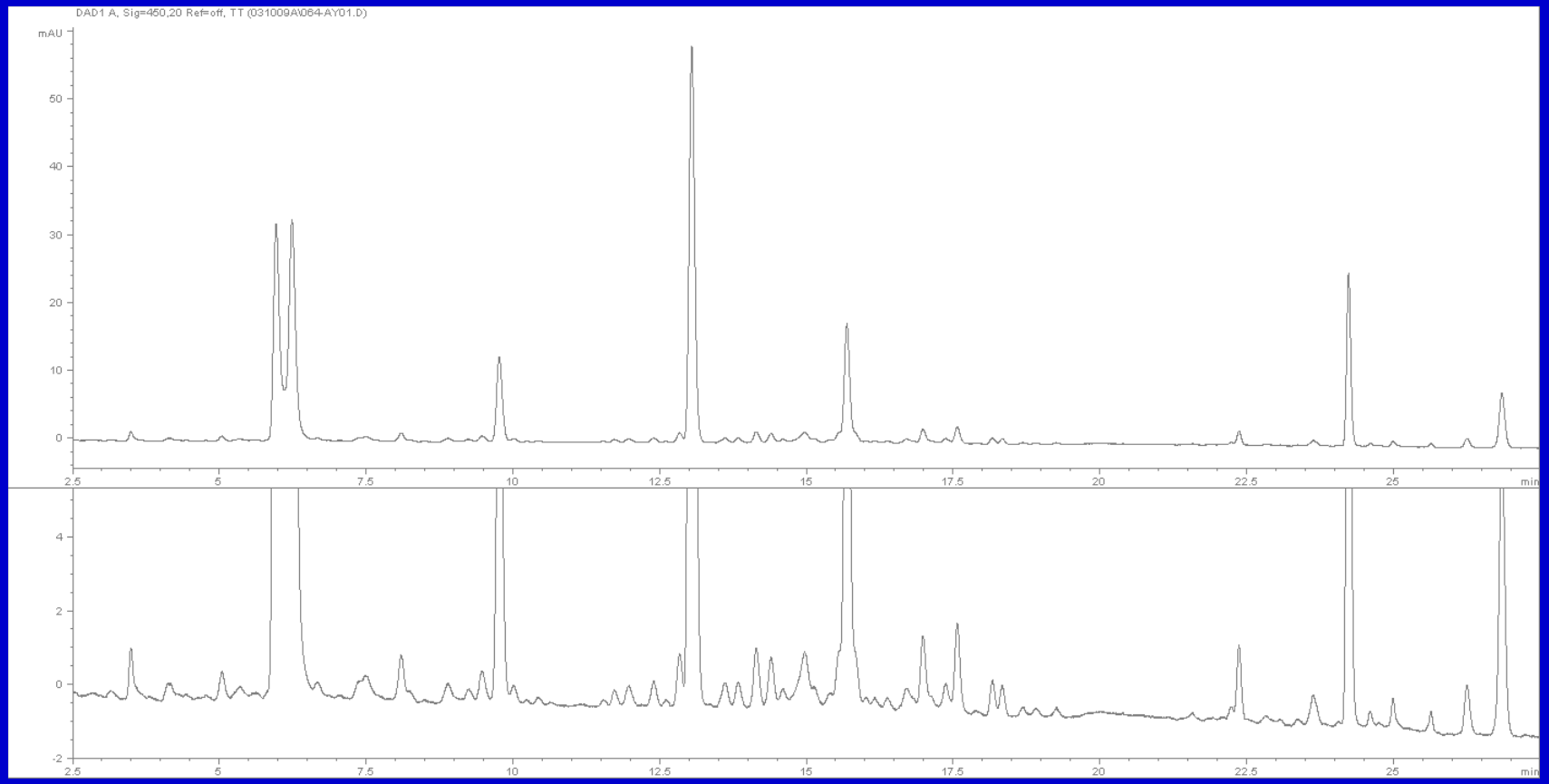
Site A – weak chromatogram



Internal Standard Chromatogram-Vitamin E



Challenging SH5 chromatogram-AK



Example QC Measurements

Measurement	Average	Tolerances
Daily		
Accuracy-Chl *a*		WL =2.4%,CL=4.7%
Injector precision	0.5%	WL=1.0%, CL=1.5%
Resolution		Minimum Rs=1.0
Carryover		< 0.1%
Repipette calibration	Accuracy = 1.0% Precision = 0.3%	WL=1.9%,CL=2.8% WL=0.4%,CL=0.7%
Weekly		
Calibration accuracy-other than Chl *a*	1.3%	WL=3.9%, CL=5.9%

QC measurements

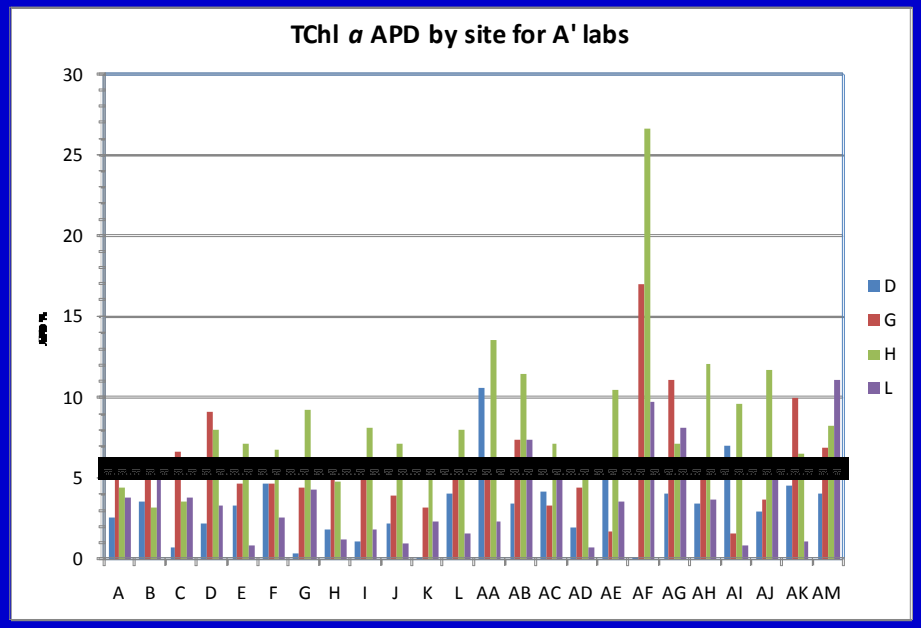
- Monitored primarily through 95% and 99% confidence limits (warning limits and control limits)
  - ***If QC measurements indicate the method is 'out of control' *we do not run until we can fix the underlying problem* ***

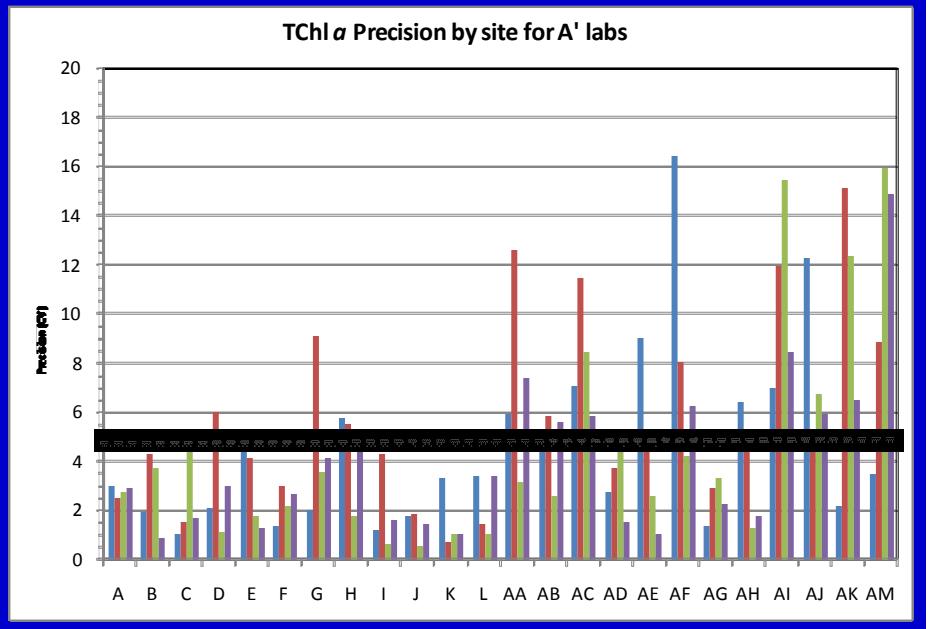
Advantages/disadvantages of our method

- Advantages:
  - consistent results across multiple SeaHARRE activities for most primary pigments (average accuracy is 19.7% and some pigments are, on average, regularly better than this PPIG average)
  - Uncertainties with variables in governing equation are known
- Disadvantages:
  - consistently poor accuracy with:
    - Chl *c*3 - could be due to differences among laboratories regarding reporting practices
    - Pras - problematic because of possible co-elution
    - Chlide *a* - formation during extraction
  - A high bias for Caro in Australian SH-5 samples for which cause is unknown at this time.

Investigations

- Had poorer CV% in Australian samples, started looking at data in different ways to ascertain possible reasons why


TChl a APD by site for A' labs
30
25
20
15
10
5
0
A B C D E F G H I J K L AA AB AC AD AE AF AG AH AI AJ AK AM
D
G
H
L


TChl a Precision by site for A' labs
20
18
16
14
12
10
8
6
4
2
0
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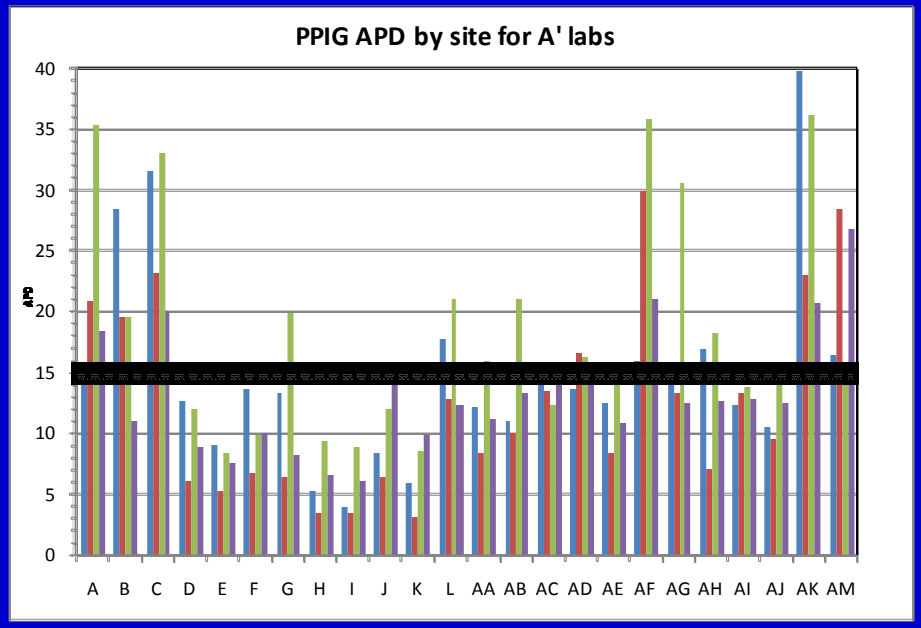
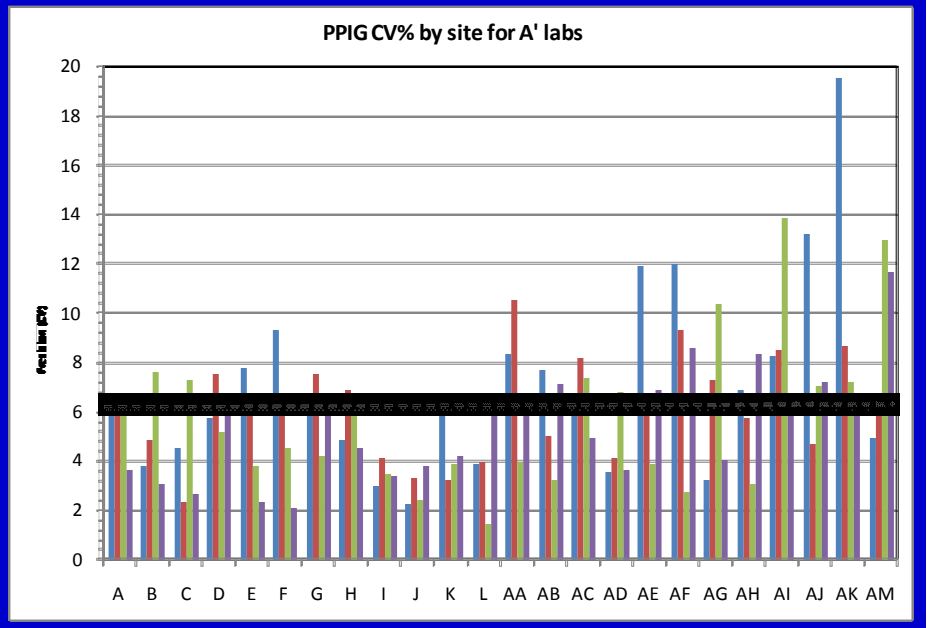
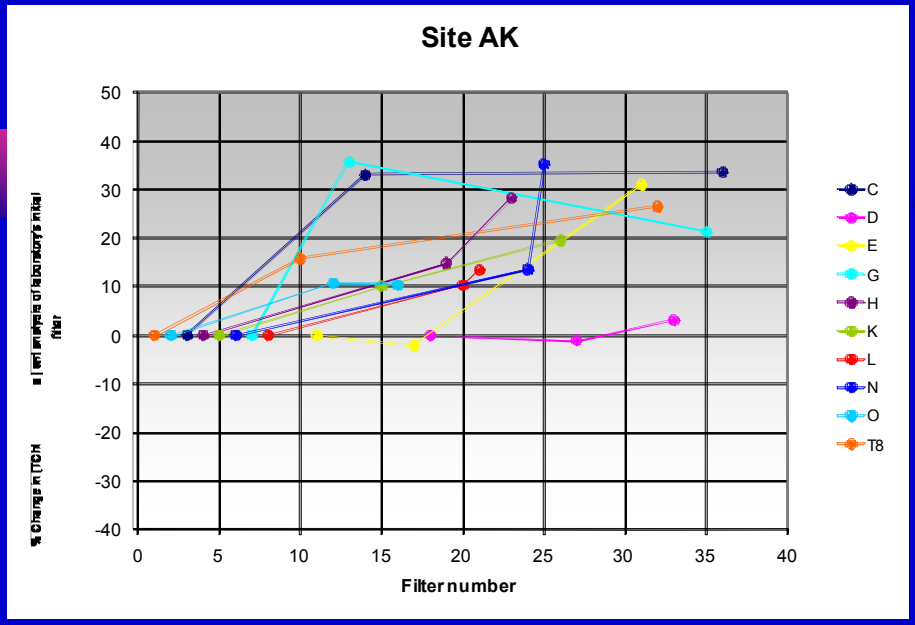
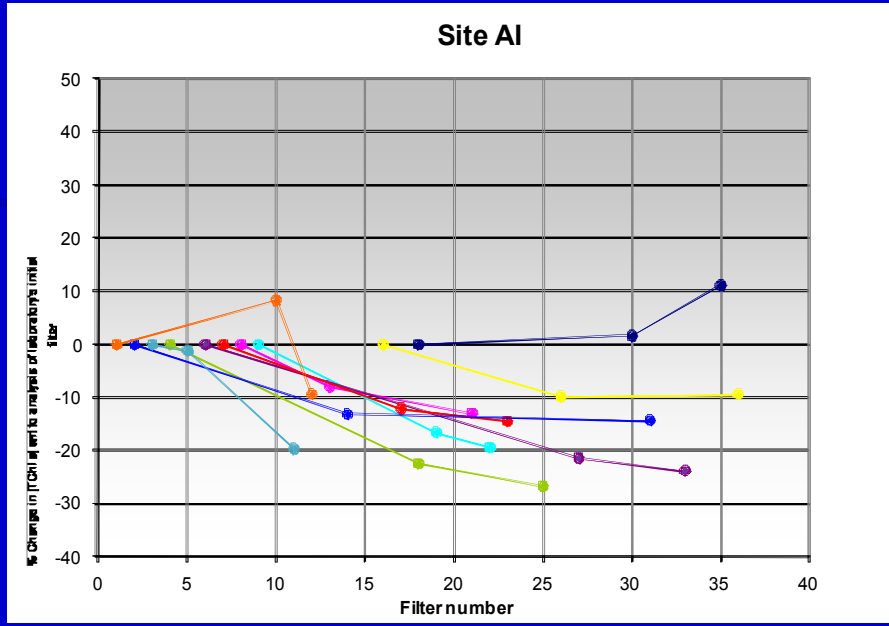
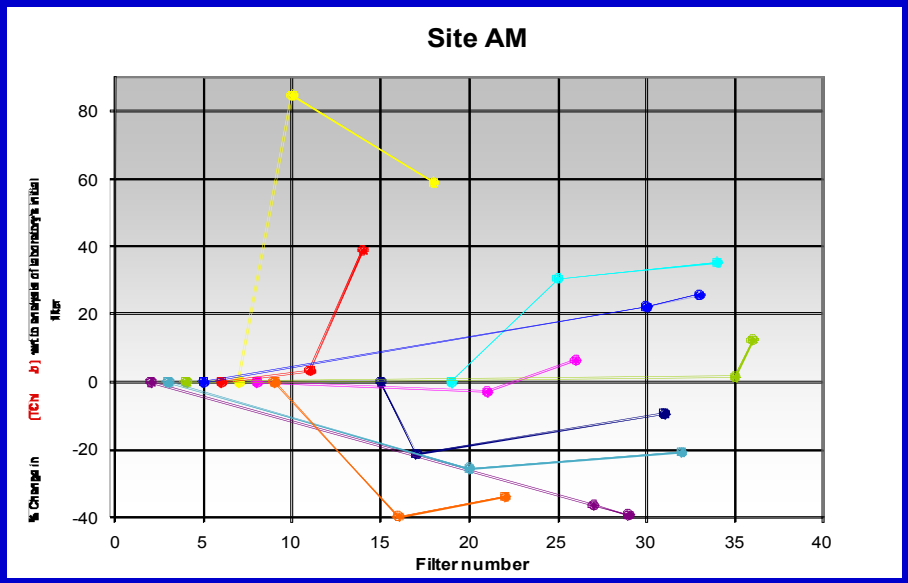

PPIG APD by site for A' labs
40
35
30
25
20
15
10
5
0
A B C D E F G H I J K L AA AB AC AD AE AF AG AH AI AJ AK AM


PPIG CV% by site for A' labs
20
18
16
14
12
10
8
6
4
2
0
A B C D E F G H I J K L AA AB AC AD AE AF AG AH AI AJ AK AM

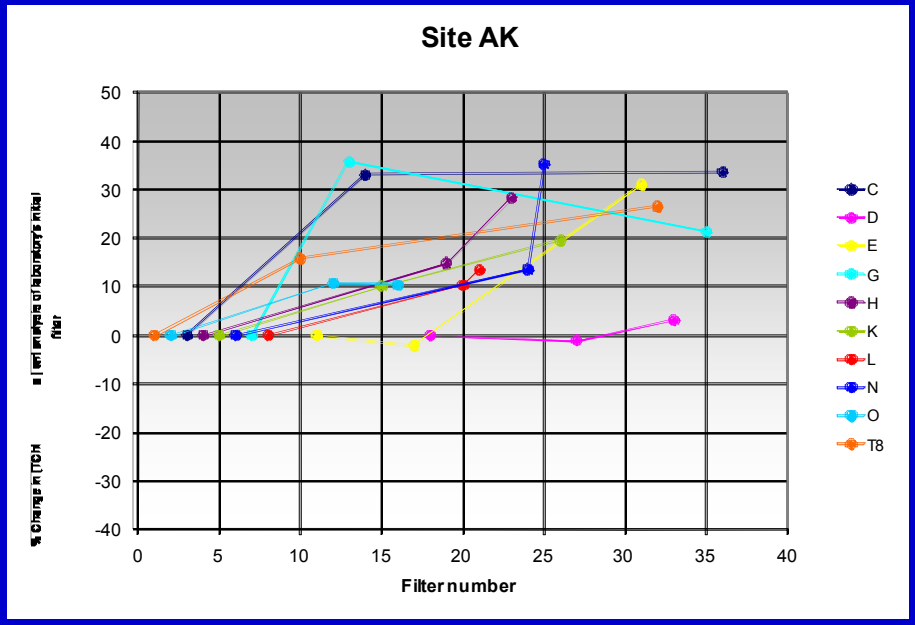
Plotted data according to filter

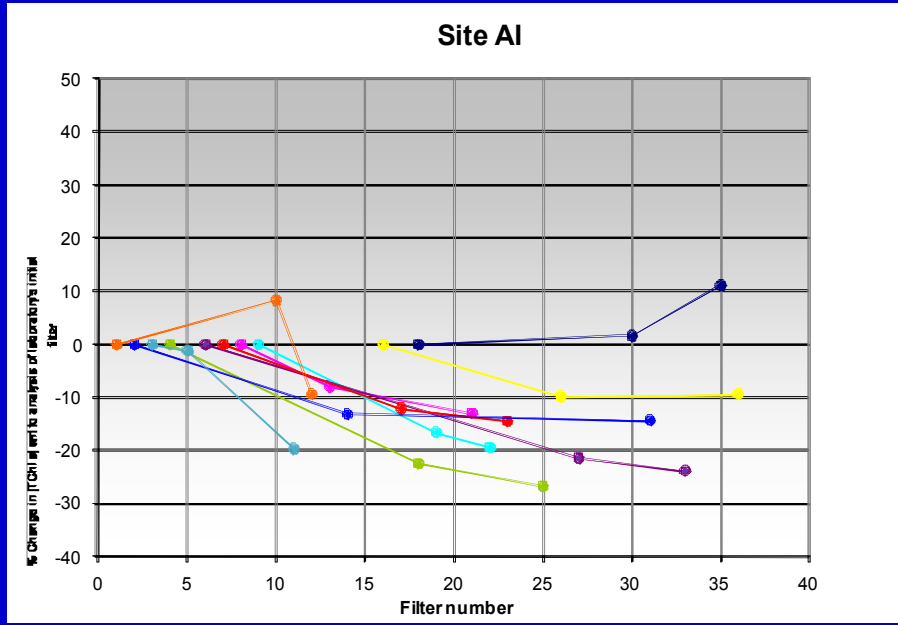


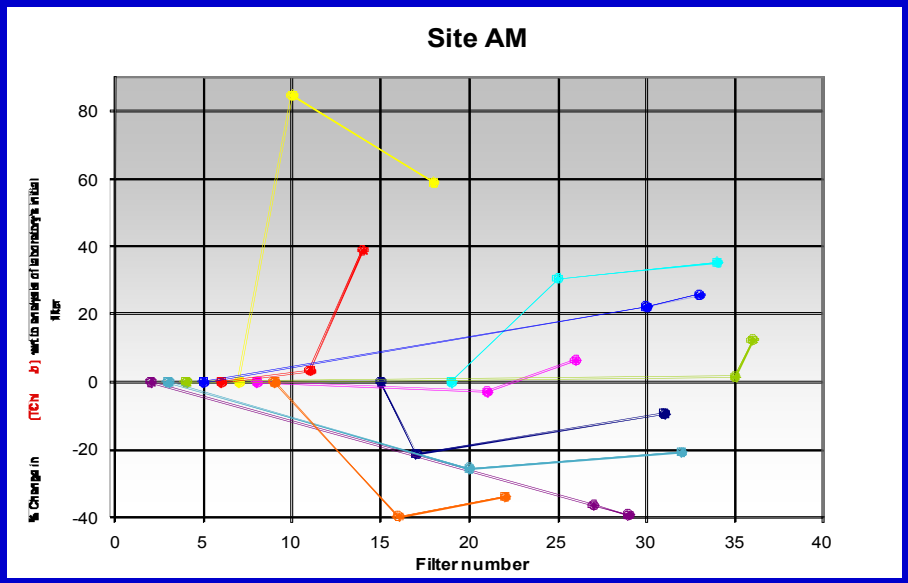


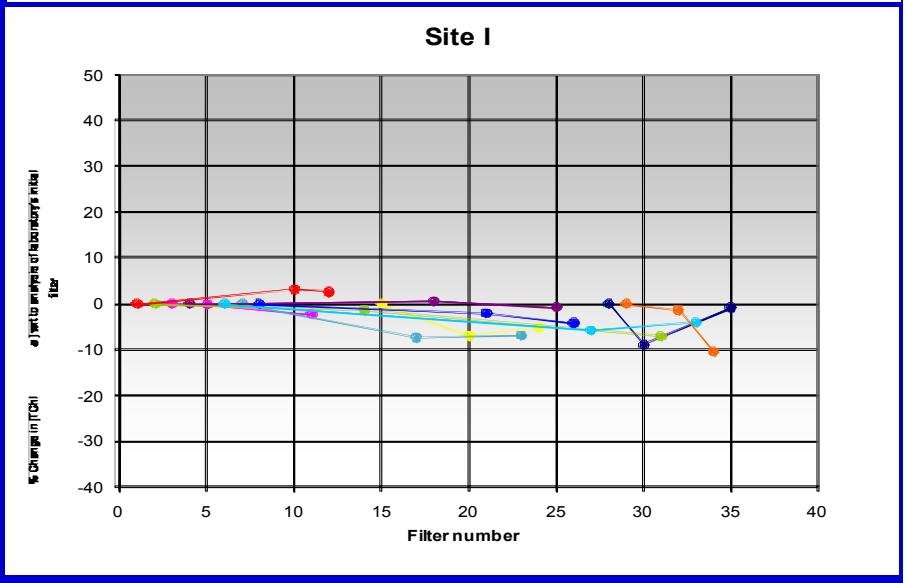


Plotted data according to filter

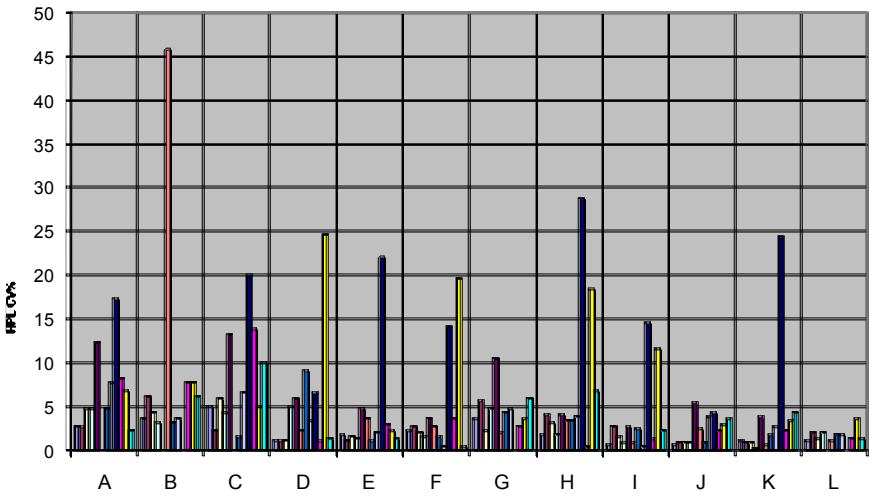


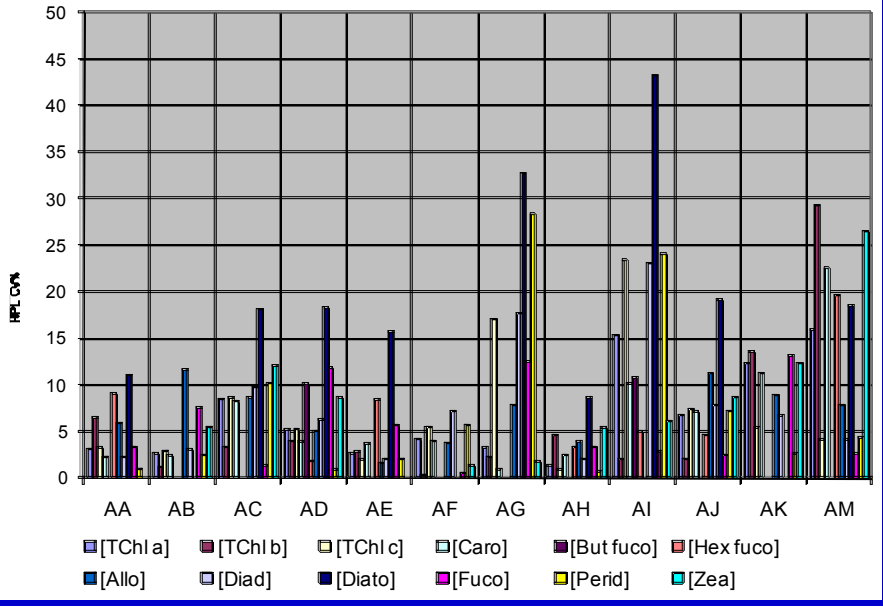


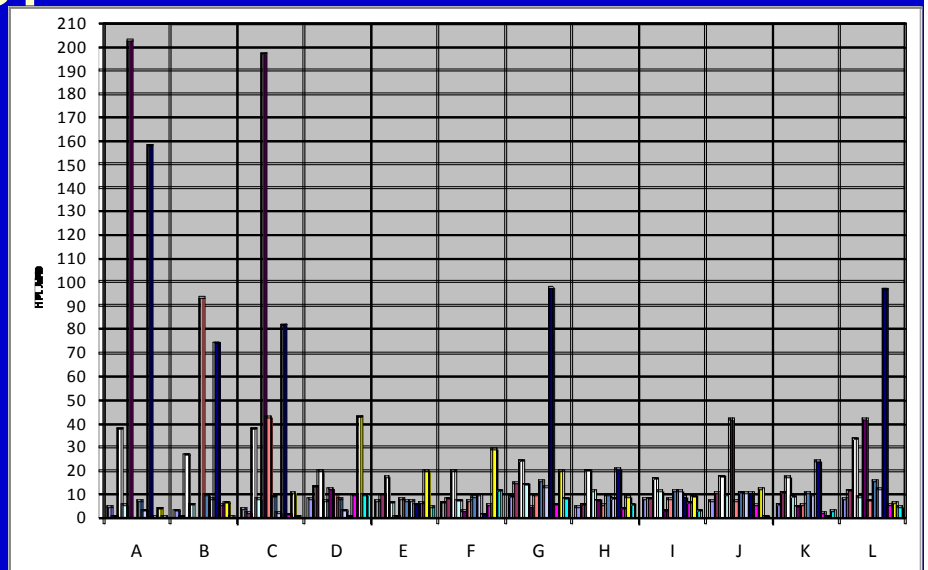


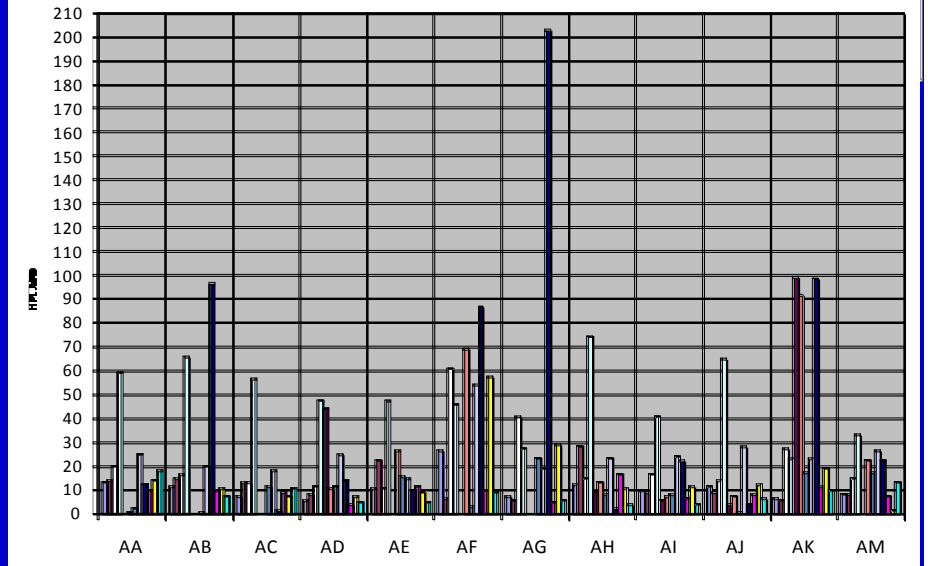


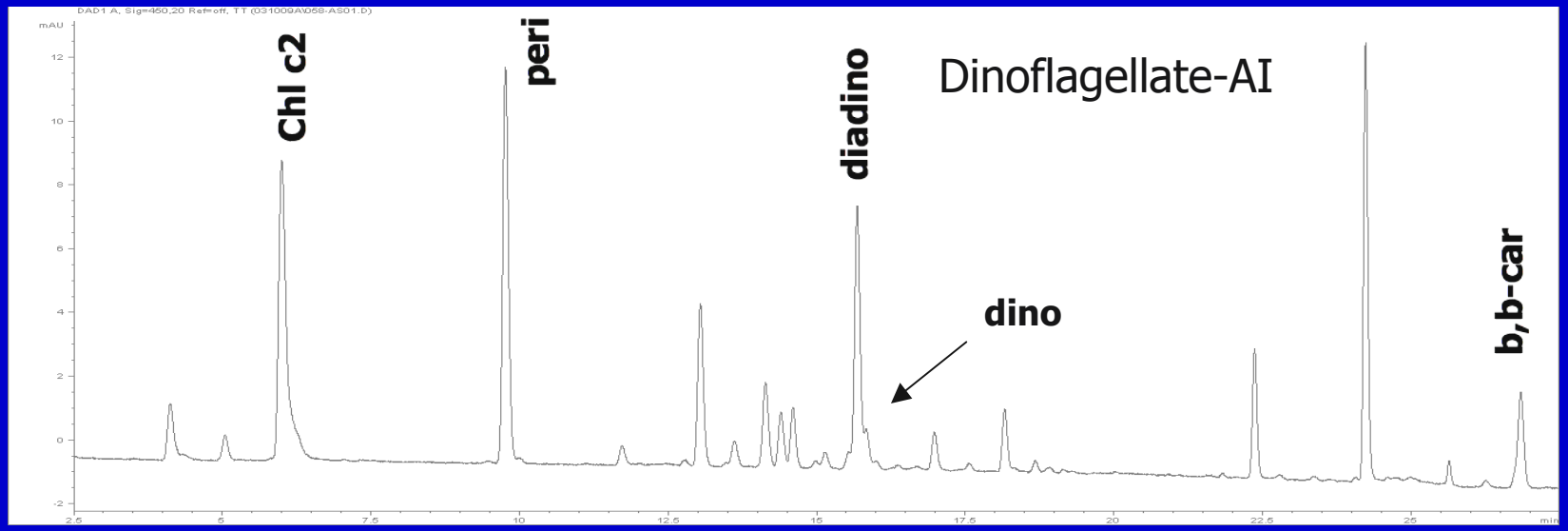
At these ‘bad’ sites, was behavior the same for all pigments?

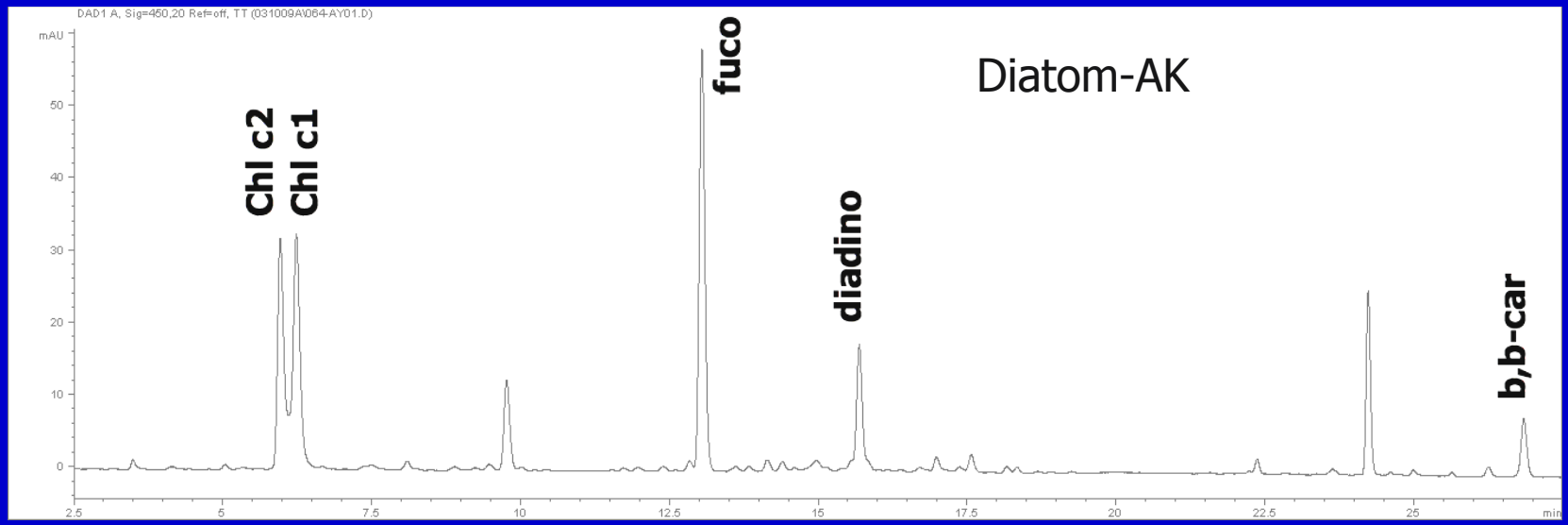









DAD1 A, Sig=450,20 Ref=off, TT (031009A\068-AS01.D)
mAU
Chl c2
peri
diadino
Dinoflagellate-AI
dino
b,b-car
min


DAD1 A, Sig=450,20 Ref=off, TT (031009A\064-AY01.D)
mAU
Chl c2
Chl c1
fuco
Diatom-AK
diadino
b,b-car
min

What can cause poor filter homogeneity?

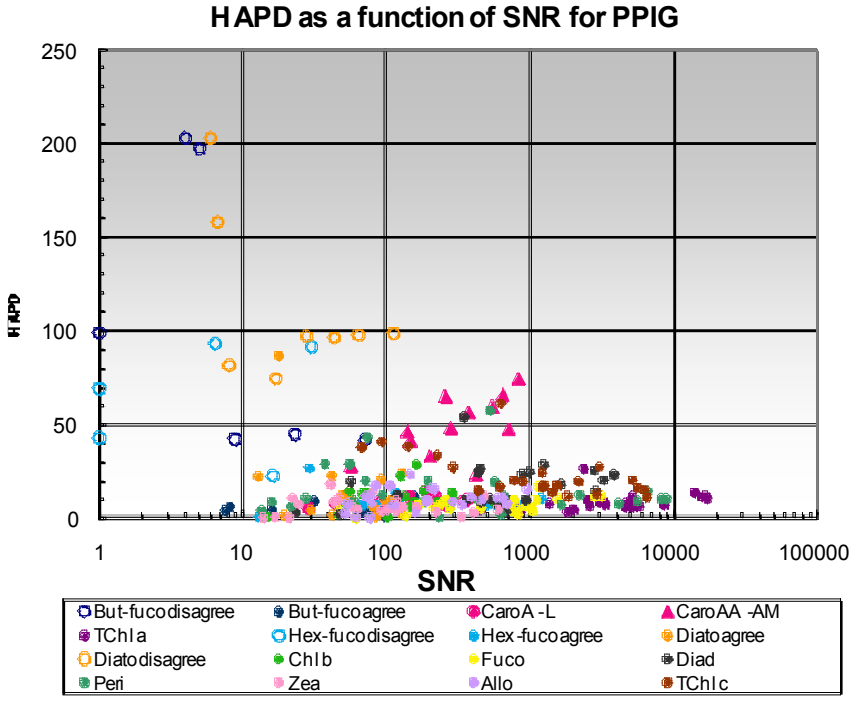
- Blooms
- Settling or growth in the jug
- Photoinhibition
- Globular algal communities

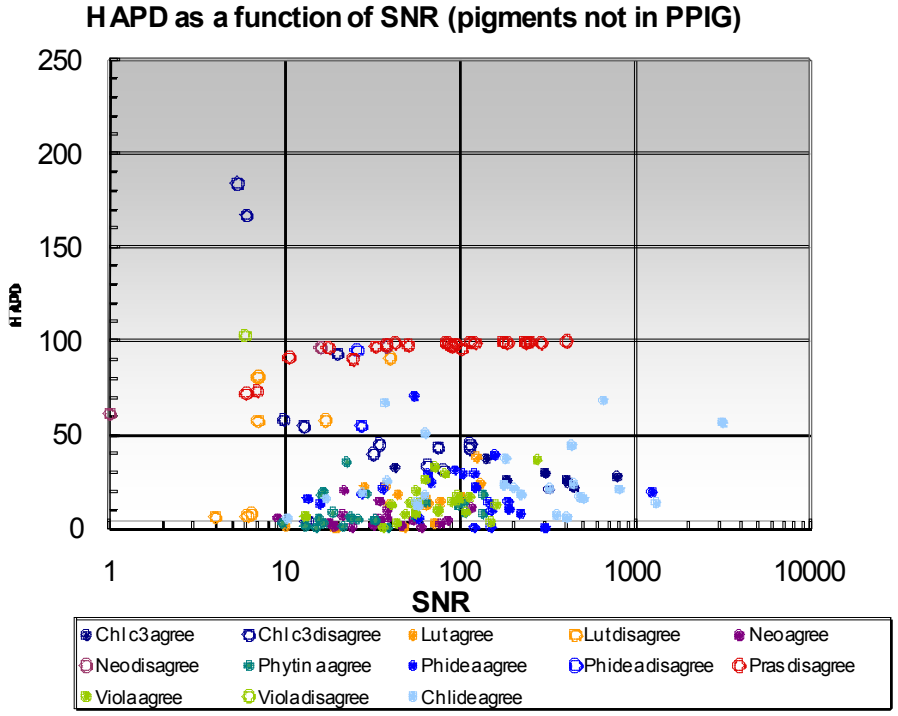
- Is this only a problem in samples with higher overall biomass?
- Does this mean that at sites with certain concentration levels or algal classes, it may be more difficult to get agreement at levels that have come to be expected
- Possible fix-Speed up filtration time?
  - Positive filtration
  - Decrease filtration volume (lose some small peaks, but gain precision of pigs that you still get?)

APD as a function of SNR

- When investigating how to improve agreement, fixes are different for:
  - Pigs at high SNR-chromatography, data interpretation
  - Pigs at low SNR-detection, small peak acceptance/rejection criteria

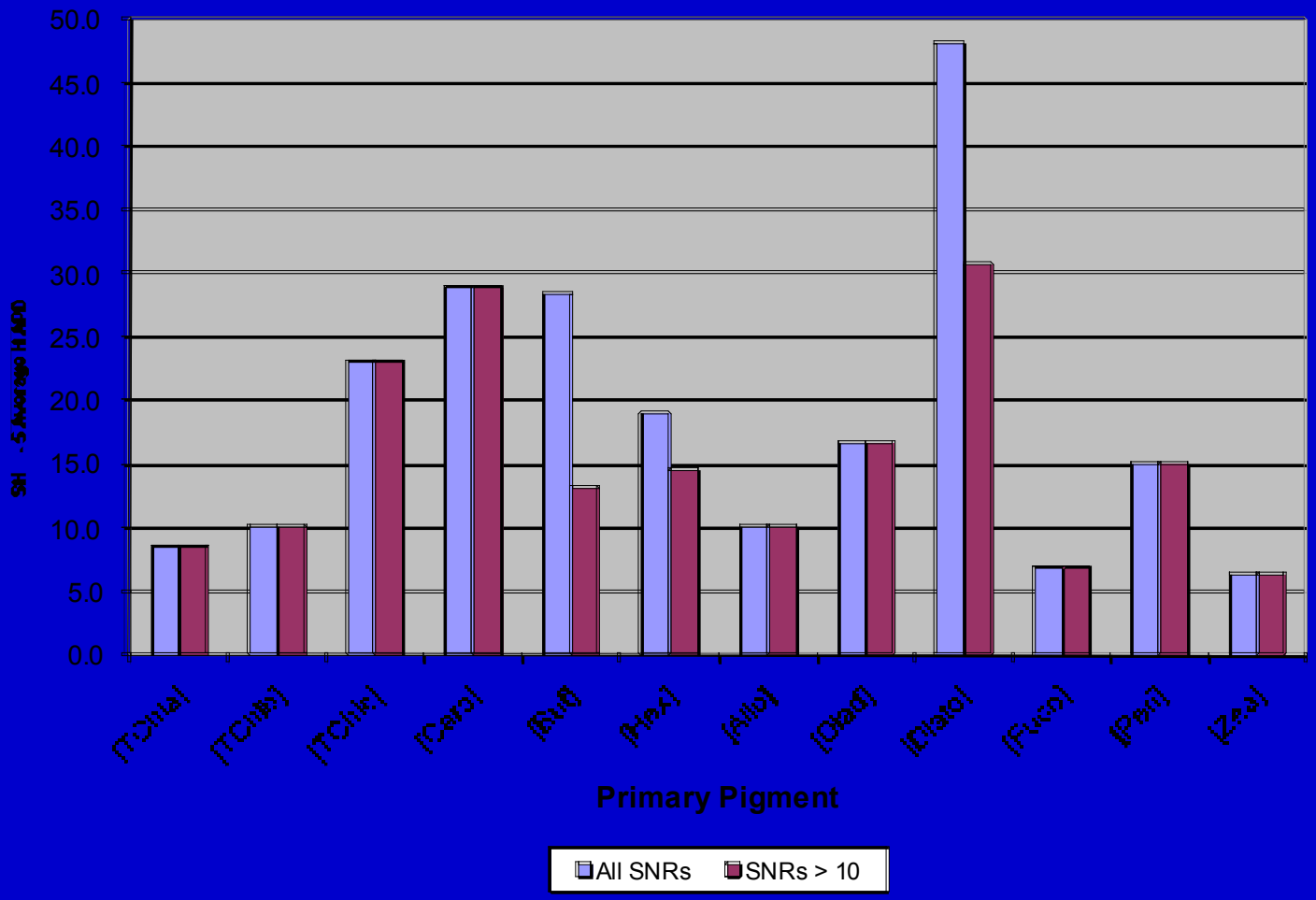
HPL APD as a function of SNR



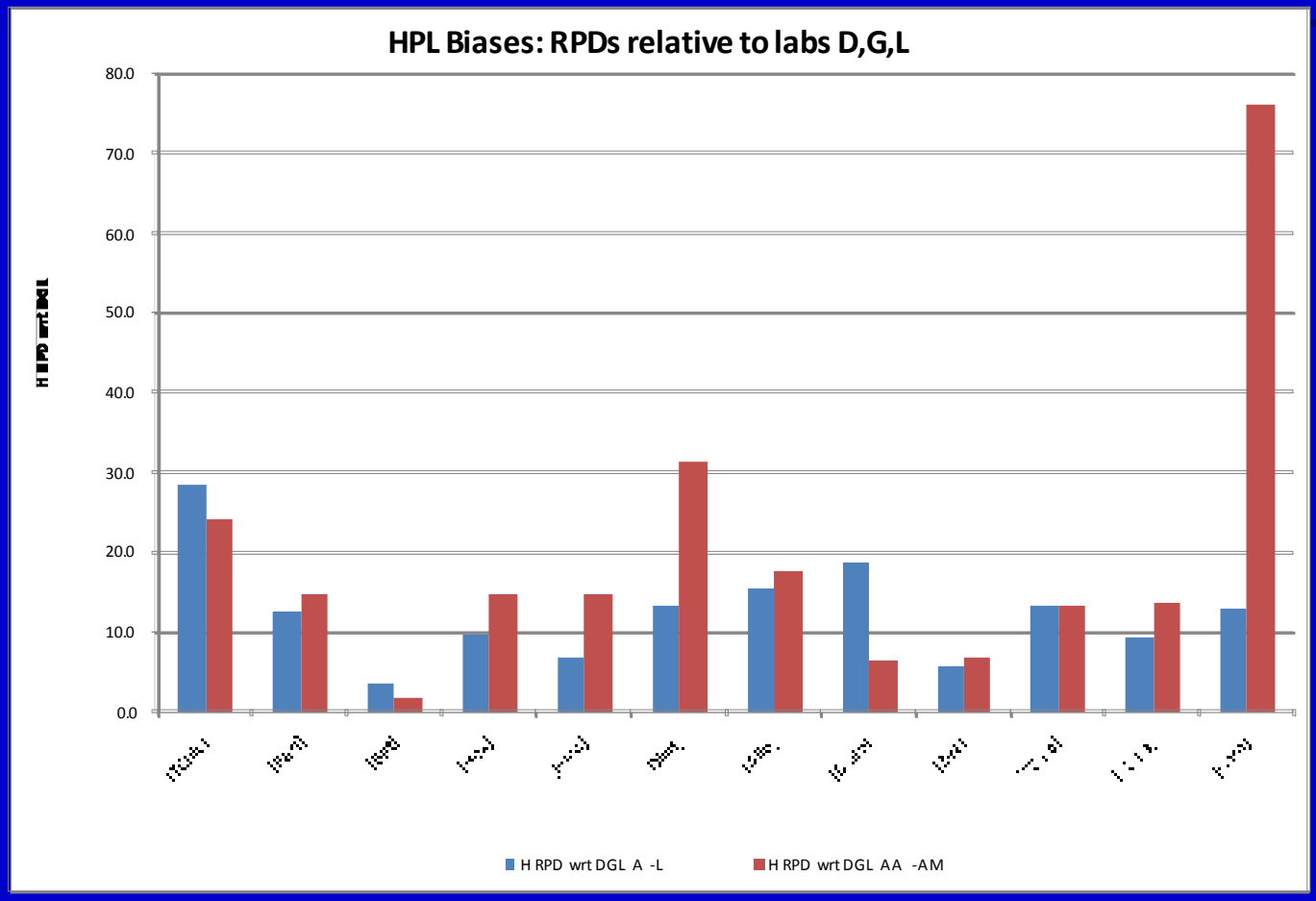


Agree - all A' labs agreed with regard to presence/absence
Disagree - at least one A' lab reported differently than other labs

HPL APD by SNR for each pig



RPD bias- HPL compared to A' labs



Ideas for improving results

- Define a threshold below which identity based on absorption spectra cannot be confirmed
- Adopt a "digit-of-precision" that is realistic to uncertainties expected
- Collaborate on how to quantify difficult pigments (Phide *a*, Chl *c*3, Diato, etc.)
- For database management-lab has to prove it has similar capabilites as current contributors to add their data

Many, many thanks to Laurie!!