Radioisotope Techniques for measuring coccolithophore calcification

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Outline

- Overview of various methods to estimate coccolithophore calcification, with and without isotopes
- Sample collection
- Difference technique
- Micro-diffusion technique
- Blank vs time zero's
- Dark calcification
- Equations for calculating calcification and assumptions
- Foraminifera and pteropod calcification

Overview of Methods – Coccolithophore calcification

a) Isotope methods

1) ^{14}C (Paasche 1962, 1963)

"difference technique"- indirect- measures loss of activity after dissolution of ^{14}C-CaCO3.

"Microdiffusion technique"(Paasche & Brubak, 1994)-direct measures incorporation of ^{14}C into tissue and CaCO3

2) ^{45}Ca (Van der Wal 1995)-measures direct incorporation of ^{45}Ca (from CaCl) into $^{45}CaCO_3$.

b) Mass-based Culture estimates of acid-labile $CaCO_3$ mass at two time points

c) Microscope-based (e.g. Paasche, 1962; Taylor et al., 2007)-direct visualization but must know mass of coccoliths

Overview of coccolithophore calcification methods (…continued)

d) Chemical methods TCO_2, alkalinity and calculated pCO_2 (Bates et al, 1996) With calcification and uptake of HCO_3^- there should be a decrease in TA and TCO_2 in a ratio of 1:1 to 2:1. Value of 2:1, suggests carbonate alone is precipitated.
e) Sediment trap-based (e.g. Honjo, Dymond, etc.)
f) Satellite-based (e.g. Balch et al., 2007)-statistical approach based on field measurements

Strengths of isotope techniques

i) fairly simple, old techniques
ii) relatively high signal to noise (provided sufficient isotope addition)
iii) short incubations possible (reduced bottle effects)
iv)parallel photosynthesis and calcification possible
v) Isotope techniques focused on coccolithophores due to their abundance relative to other pelagic calcifiers (forams and pteropods)

Weaknesses

a) *Isotope techniques Blanks are tricky…*Not the same for different kinds of poisons. Blank issues greater for ^{45}Ca- is "sticky"

b) *Difference technique*- small difference between two large numbers (lower signal to noise).

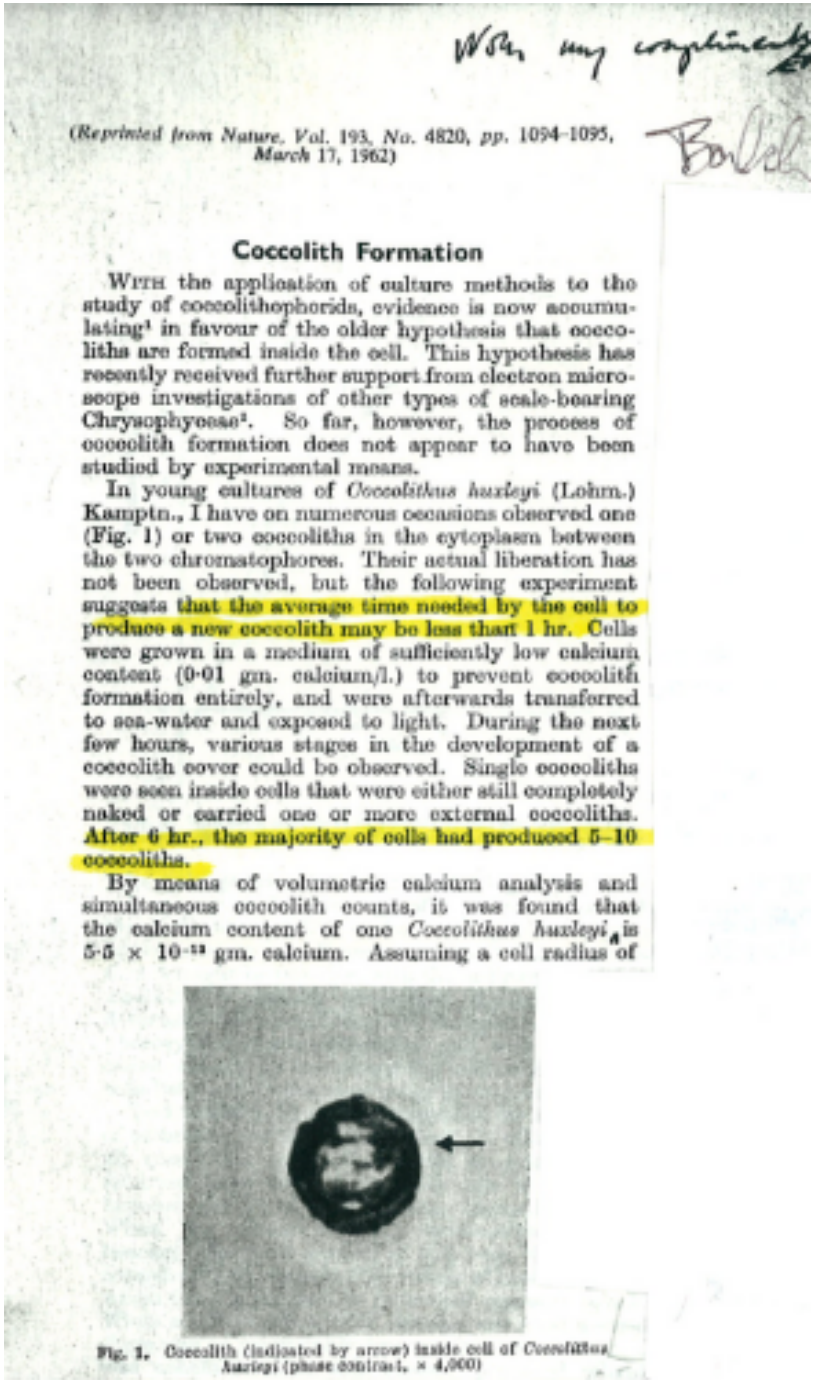
c) *Micro-diffusion technique*- laborious

Water sampling and handling for coccolithophore calcification measurements

- Clean Niskins or GoFlo bottles
- Must sub-sample quickly as large coccolithophores settle quickly in Niskin
- 300mL polycarbonate tissue culture bottles as primary incubation bottles from which triplicates are taken plus one blank
- Standard protocols for primary production apply to calcification (See JGOFS protocols)
  - Bottles acid soaked, ETOH rinsed, rinsed 5X with RO water; 3-5X rinse of bottle with sample
  - Prevention of light shock-preferably pre-dawn casts, manipulations under low light

Differencing Technique

Paasche, E. 1962.
Coccolith formation.
Nature. 193:1094-1095.



(Reprinted from Nature, Vol. 193, No. 4820, pp. 1094–1095, March 17, 1962)

Coccolith Formation

WITH the application of culture methods to the study of coccolithophorids, evidence is now accumulating[1] in favour of the older hypothesis that coccoliths are formed inside the cell. This hypothesis has recently received further support from electron microscope investigations of other types of scale-bearing Chrysophyceae[2]. So far, however, the process of coccolith formation does not appear to have been studied by experimental means.

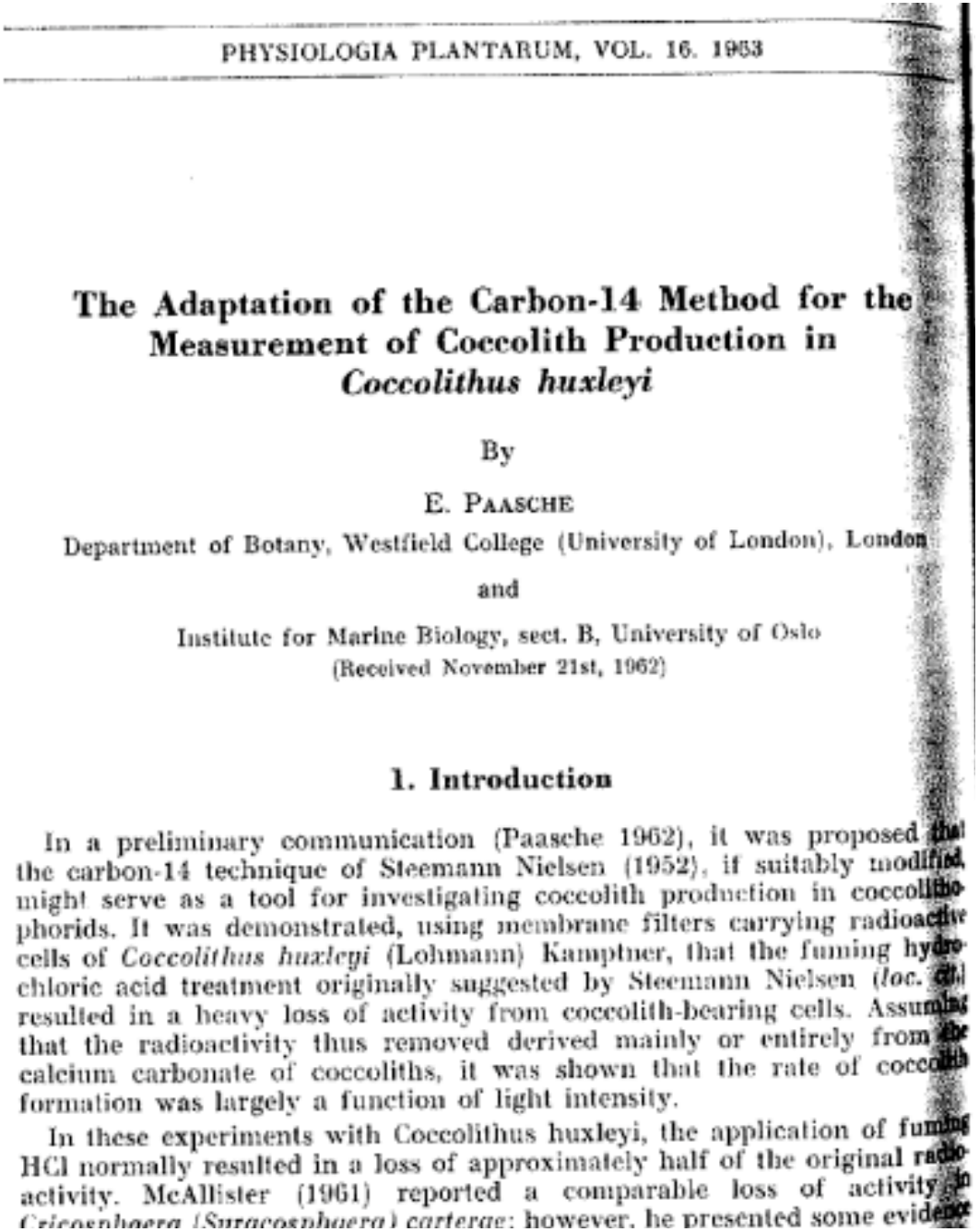
In young cultures of *Coccolithus huxleyi* (Lohm.) Kamptn., I have on numerous occasions observed one (Fig. 1) or two coccoliths in the cytoplasm between the two chromatophores. Their actual liberation has not been observed, but the following experiment suggests that the average time needed by the cell to produce a new coccolith may be less than 1 hr. Cells were grown in a medium of sufficiently low calcium content (0·01 gm. calcium/l.) to prevent coccolith formation entirely, and were afterwards transferred to sea-water and exposed to light. During the next few hours, various stages in the development of a coccolith cover could be observed. Single coccoliths were seen inside cells that were either still completely naked or carried one or more external coccoliths. After 6 hr., the majority of cells had produced 5–10 coccoliths.

By means of volumetric calcium analysis and simultaneous coccolith counts, it was found that the calcium content of one *Coccolithus huxleyi* is $5{\cdot}5 \times 10^{-13}$ gm. calcium. Assuming a cell radius of

Fig. 1. Coccolith (indicated by arrow) inside cell of *Coccolithus huxleyi* (phase contrast, × 4,000)

Differencing Technique

Paasche, E. 1963. The adaptation of the Carbon-14 method for the measurement of coccolith production in, *Coccolithus huxleyi*. *Physiologia Plantarum*. 16:186-200.



PHYSIOLOGIA PLANTARUM, VOL. 16. 1963

The Adaptation of the Carbon-14 Method for the Measurement of Coccolith Production in *Coccolithus huxleyi*

By

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and

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(Received November 21st, 1962)

1. Introduction

In a preliminary communication (Paasche 1962), it was proposed that the carbon-14 technique of Steemann Nielsen (1952), if suitably modified, might serve as a tool for investigating coccolith production in coccolithophorids. It was demonstrated, using membrane filters carrying radioactive cells of *Coccolithus huxleyi* (Lohmann) Kamptner, that the fuming hydrochloric acid treatment originally suggested by Steemann Nielsen (*loc. cit.*) resulted in a heavy loss of activity from coccolith-bearing cells. Assuming that the radioactivity thus removed derived mainly or entirely from the calcium carbonate of coccoliths, it was shown that the rate of coccolith formation was largely a function of light intensity.

In these experiments with Coccolithus huxleyi, the application of fuming HCl normally resulted in a loss of approximately half of the original radioactivity. McAllister (1961) reported a comparable loss of activity in *Cricosphaera (Syracosphaera) carterae*; however, he presented some evidence

Basics of the differencing technique

- Samples filtered just like standard productivity samples (GF/F filters fine)
- Everything is done in duplicate
  - One filter is put straight into fluor
  - One filter is put in a desiccater containing a petri dish with concentrated HCl for 2-4 minutes, then into fluor
- Calcification estimate is based on the difference between the unfumed (total ^{14}C fixation) and fumed (organic ^{14}C fixation) [and it is the difference between two big numbers…never good!]

Statistics of the differencing technique

- OK for cultures but challenging in the field
- Error propagation for a difference of X and Y with std dev of S_X and S_Y, respectively:
- $S_z = SqRt[S_X^2 + S_Y^2]$
- Thus, you do counts to 1% precision (Std dev) for each, the std dev of the difference is 1.4%. Calcification typically only contributes 1-5% of the total carbon fixation anyway.
- Thus, the difference is barely above the signal to noise of the counting!

Microdiffusion Technique

Paasche, E., and S. Brubak. 1994. Enhanced calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) under phosphorus limitation. *Phycologia*. 33:324-330.

Phycologia (1994) Volume 33 (5), 324–330

Enhanced calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) under phosphorus limitation

E. PAASCHE AND S. BRUBAK

Section for Marine Botany, Department of Biology, University of Oslo, P.O. Box 1069, Blindern, 0316 Oslo, Norway

E. PAASCHE AND S. BRUBAK. 1994. Enhanced calcification in the coccolithophorid *Emiliania huxleyi* (Haptophyceae) under phosphorus limitation. *Phycologia* 33: 324–330.

The effect of phosphorus limitation on calcification in the coccolithophorid *Emiliania huxleyi* (Lohmann) Hay et Mohler was investigated by chemical Ca and C analyses, as well as in short-term ^{14}C uptake experiments. The latter made use of a newly developed microdiffusion method to separate ^{14}C in coccolith carbonate from photosynthetically assimilated ^{14}C. In comparison with exponentially growing cells in a nutrient-replete medium, cells grown in P-limited chemostats at half the maximum growth rate produced *c.* 60% more $CaCO_3$ relative to organic carbon. In the short-term incubations, cells from P-limited chemostats showed a relative increase in the capacity for calcification under reduced irradiance and in the dark. Nutrient effects on calcification are of potential interest in considerations of the impact of *E. huxleyi* blooms on the sea-air CO_2 interchange, and they deserve further study.

Microdiffusion Technique- Basics

GFA wetted with NaOH

Membrane filter with ^{14}C-labeled coccolithophores adhered to side

1% phosphoric acid

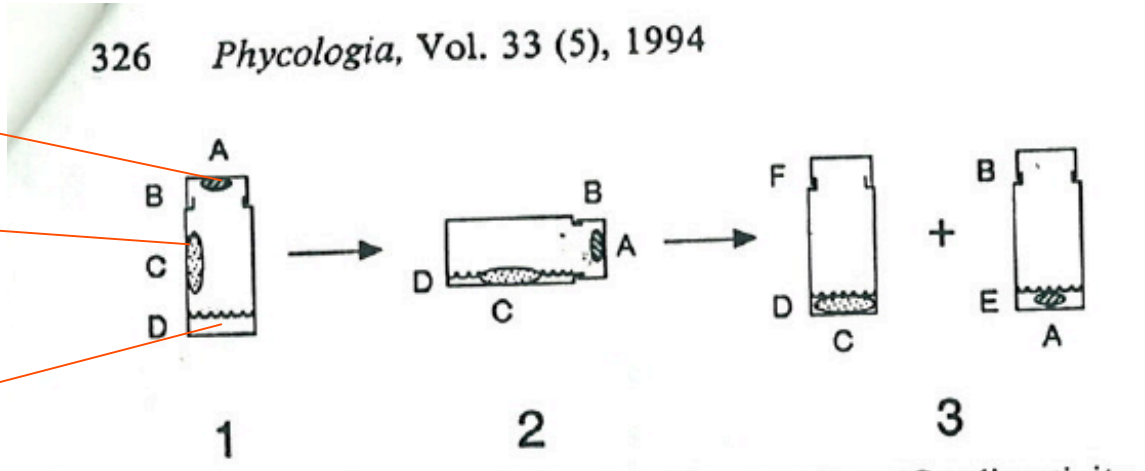


Fig. 2. Essential features of procedure for separating ^{14}C radioactivity in coccolith carbonate from that in photosynthetic products by CO_2 microdiffusion. 1: A scintillation vial is supplied with small glass-fibre filter (A) wetted with sodium hydroxide solution and adhering to inside of screw cap (B); membrane filter (C) with coccolithophorid cells and sodium carbonate carrier adhering to side wall; and dilute phosphoric acid (D) placed on bottom. 2: The membrane filter (C) is bathed in acid (D) while vial is agitated in horizontal position. 3: The screw cap (B) is transferred to a second vial containing water (E) into which the glass-fibre filter is shaken down. The first vial is provided with new screw cap (F). Scintillation cocktail is added to both vials and radioactivities from A (acid-labile carbon) and C (residual non-labile carbon) are counted separately.

Not a sea-going technique!

Tips for success with the microdiffusion technique

- Fastidiously clean manipulation is critical!
- Interstitial water in sample filters with ^{14}C activity must be rinsed away
- Blanks should be run for every sample
- Controls for micro-diffusion efficiency should be done for each experiment
- Checks of isotope specific activity to make sure what fraction is acid-labile (should be 99.99%)
- Controls for reagent contamination (e.g. Phenethylamine CO_2 trap)

Overview of the microdiffusion technique
(Paasche and Brubaak, 1994)
a) Water sample incubated with 20-40uCi ^{14}C-HCO_3 per sample
b) Sample filtered onto 0.4um poresize polycarbonate filter
c) Filter rinsed 5X with “cold” filtered-seawater
d) Filter placed in base of scintillation vial, capped with rubber septum with GF/A filter suspended in bucket, wetted with 0.2mL PEA (CO_2 absorbent).

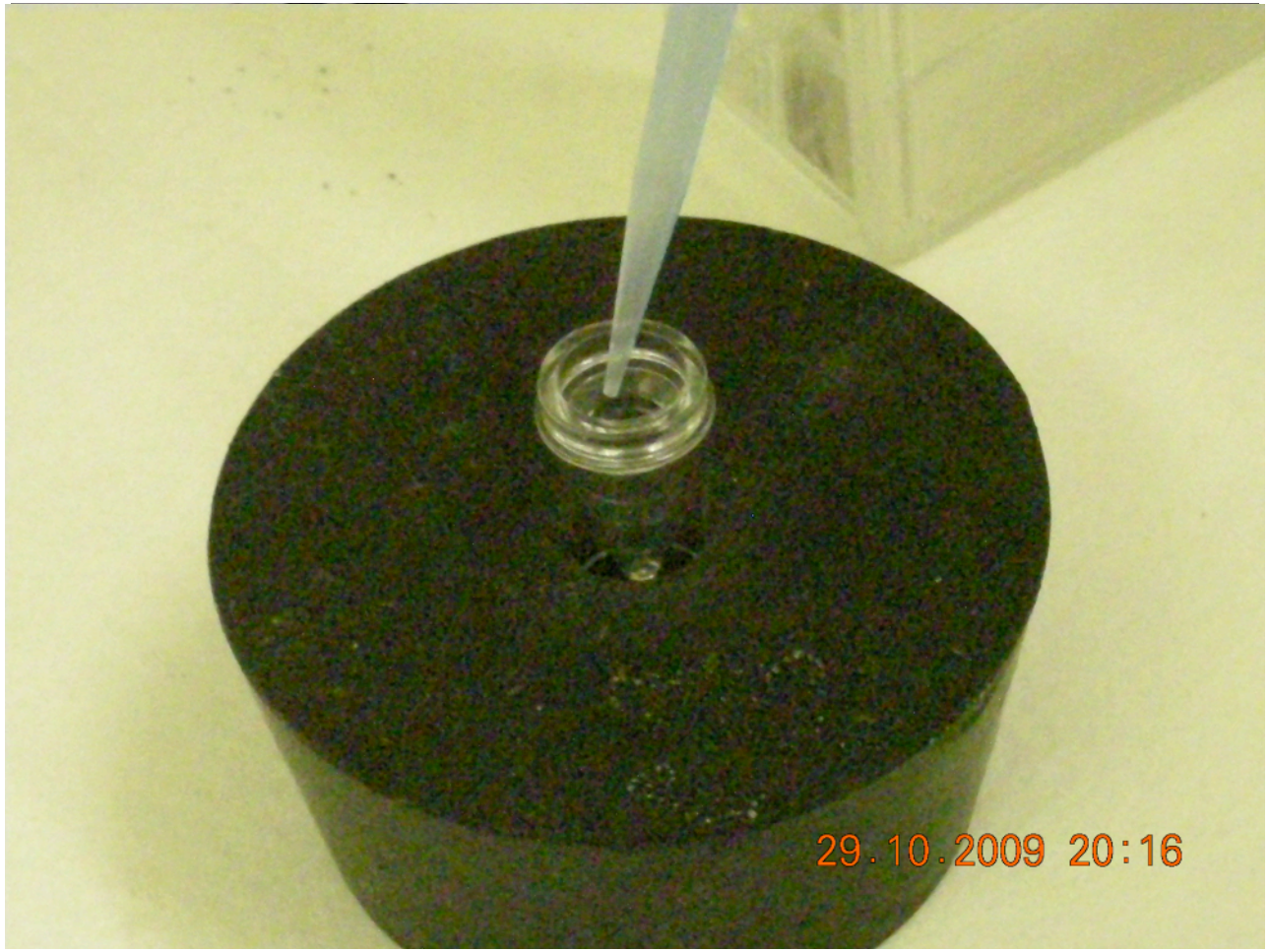
Microdiffusion overview (continued)

e) 1.0mL 1% HPO_3 injected through septum, past bucket, onto filter in base of vial, drives ^{14}C-$CaCO_3$ into ^{14}C-CO_2 in headspace.
f) Vials on shaker table for 24h
g) Vials opened. Bottom filter gets fluor, top PEA bucket with filter placed into separate vial, with 1 mL of water plus scintillation cocktail.
h) Vials sit 24h prior to counting in high sensitivity scintillation counter—lowers blanks
i) Calculations involve standard ^{14}C equations, quenching (channels-ratio method) subtracting a "blank", using 5% isotope discrimination factor, concentration of DIC (corrected for salinity)

During sampling, water screened through 200um mesh to remove large grazers

Work done under low intensity red light (inoculating, filtering)

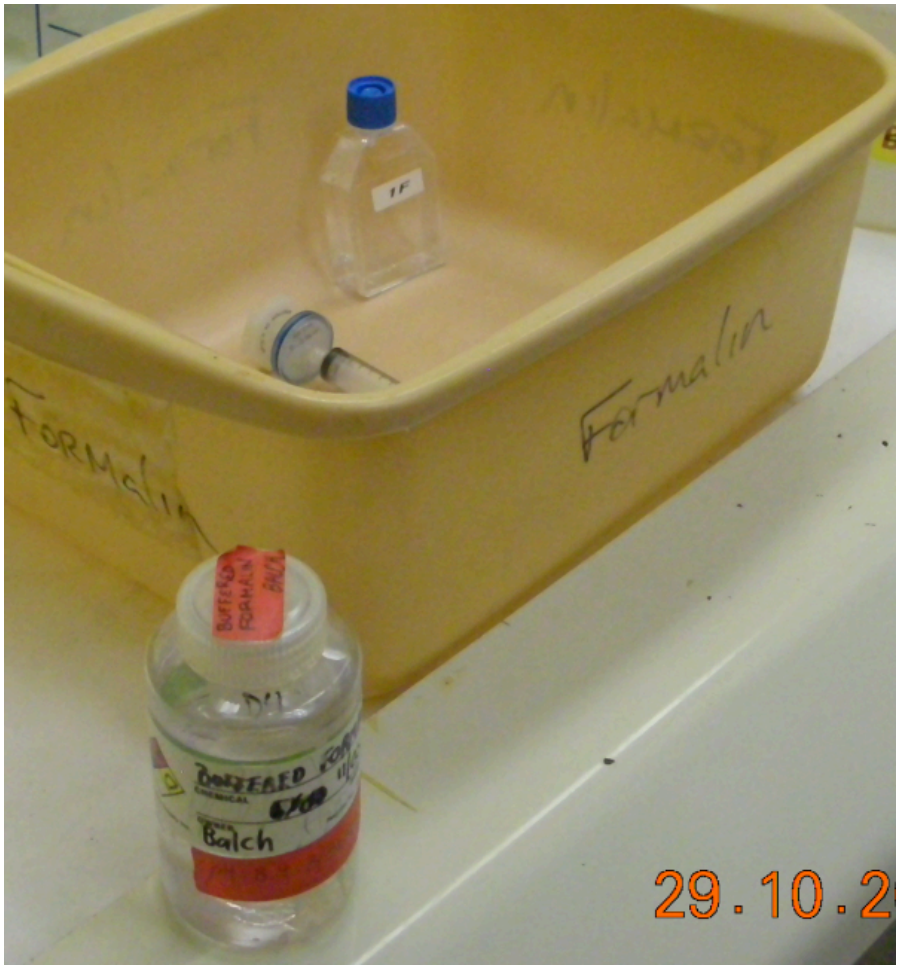
^{14}C stock for experiment is placed into a micro-vial for subsequent addition to bottles…



20-40 uCi per sample are added into 300mL polycarbonate bottles with seawater sample…

300mL polycarbonate bottles are gently mixed to disperse isotope stock then decanted into 3x75mL tissue culture bottles…

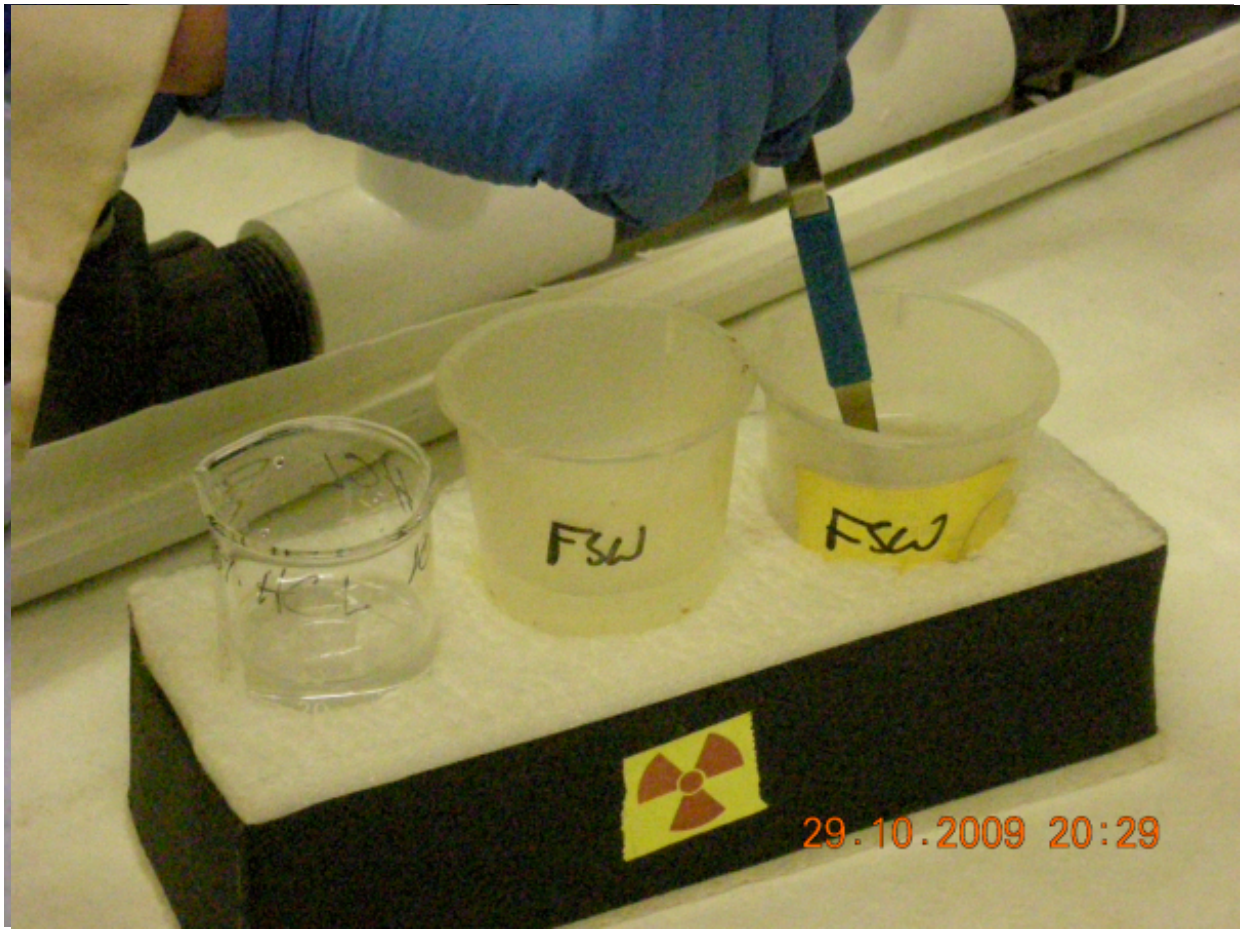
1.2mL concentrated, buffered formalin (0.2μm-filtered) is added to fourth “blank”, formalin bottle for 2% final conc. (NOT anywhere live samples!).



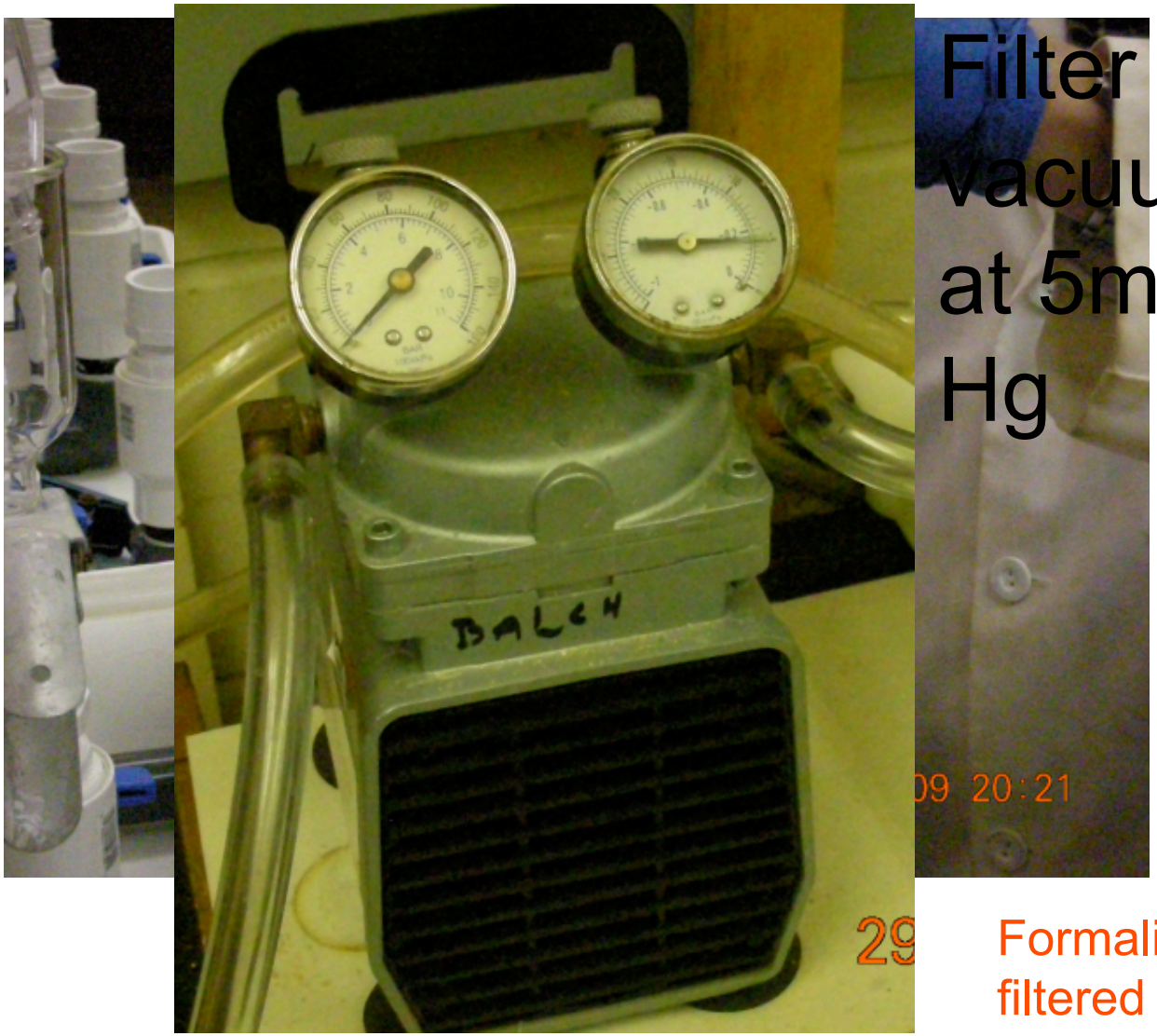
Formalin blanks are left in tub for incubation (NOT put in incubator with live samples!!)

Samples are placed in incubator (or deck simulated-*in situ* incubator or *in situ* drifter

A note on cleanliness…you must insure all surfaces are free of ^{14}C activity…including forcep rinses in 10% HCl followed by two rinses in FSW before picking up anything!



After incubation period (preferably 24h), sample is decanted into filter funnel with 0.4μm poresize polycarbonate filter.



Filter with vacuum at 5mm Hg

Formalin samples filtered separately

Rinsing…very important: first the bottle rinse with FSW…

Rinsing…then 3 cup rinses with FSW

There are many samples, done in triplicate… how do you keep track of the number of rinses?

Rinsing…lastly the rim rinse followed by “filter calisthenics” to remove interstitial water containing $^{14}C\text{-}HCO_3^-$

“Diffusion chamber”

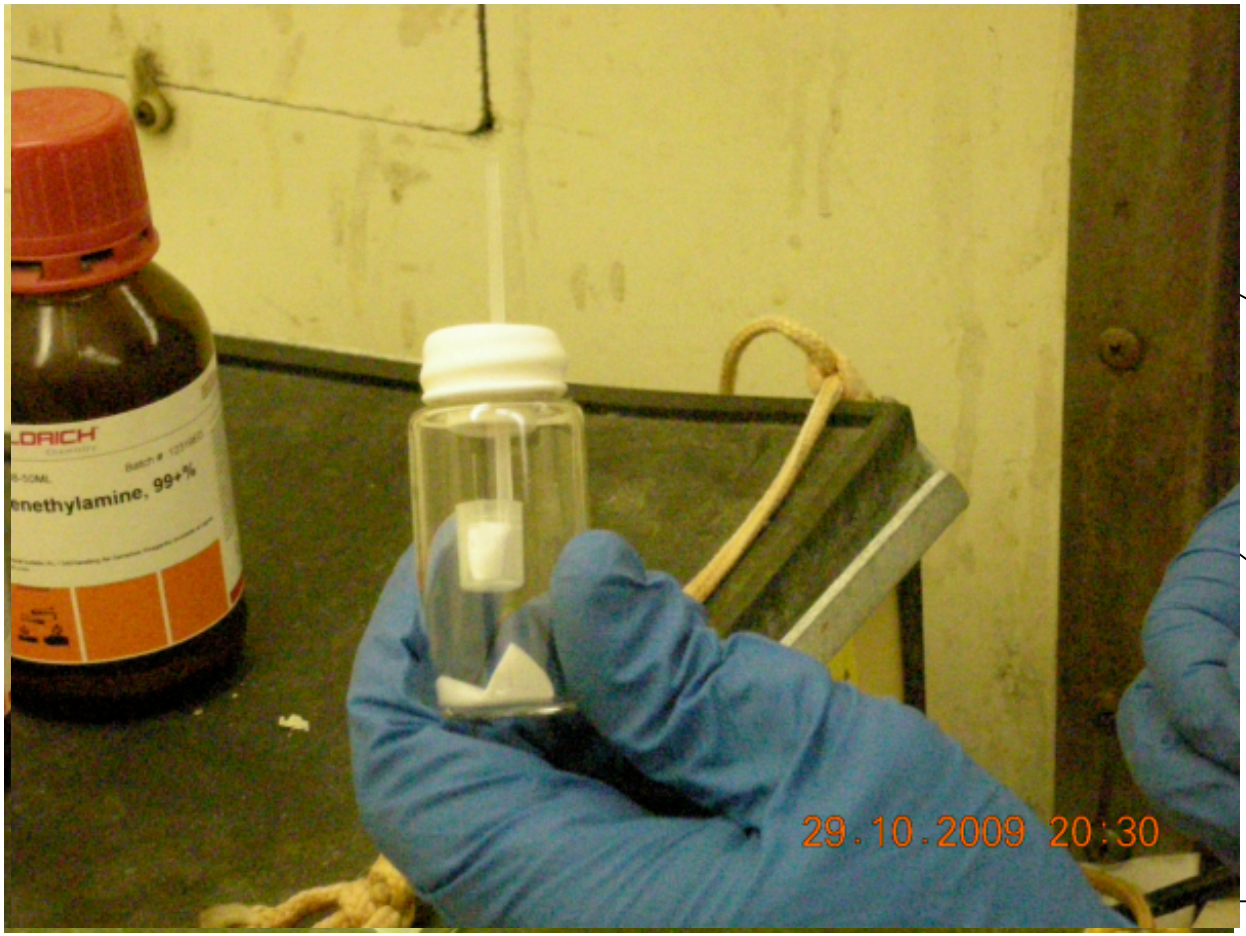
ber septum

ilter
rbant for

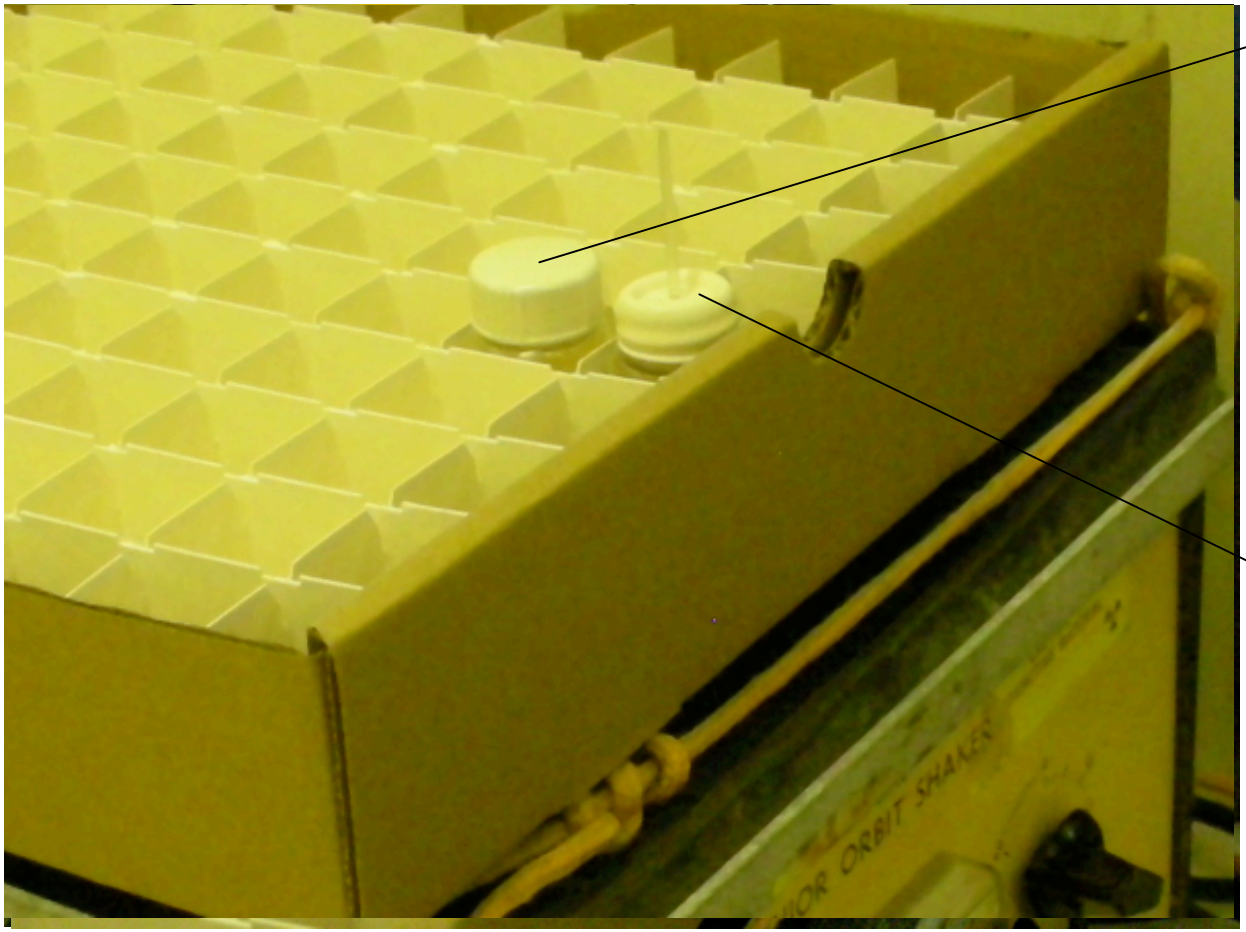
ng bucket

tilation

Add 0.2ml of the CO_2 scrubber (Phenethylamine) to the bucket with GFA filter-



Inject one mL of 1% phosphoric acid past bucket, into base of sealed vial

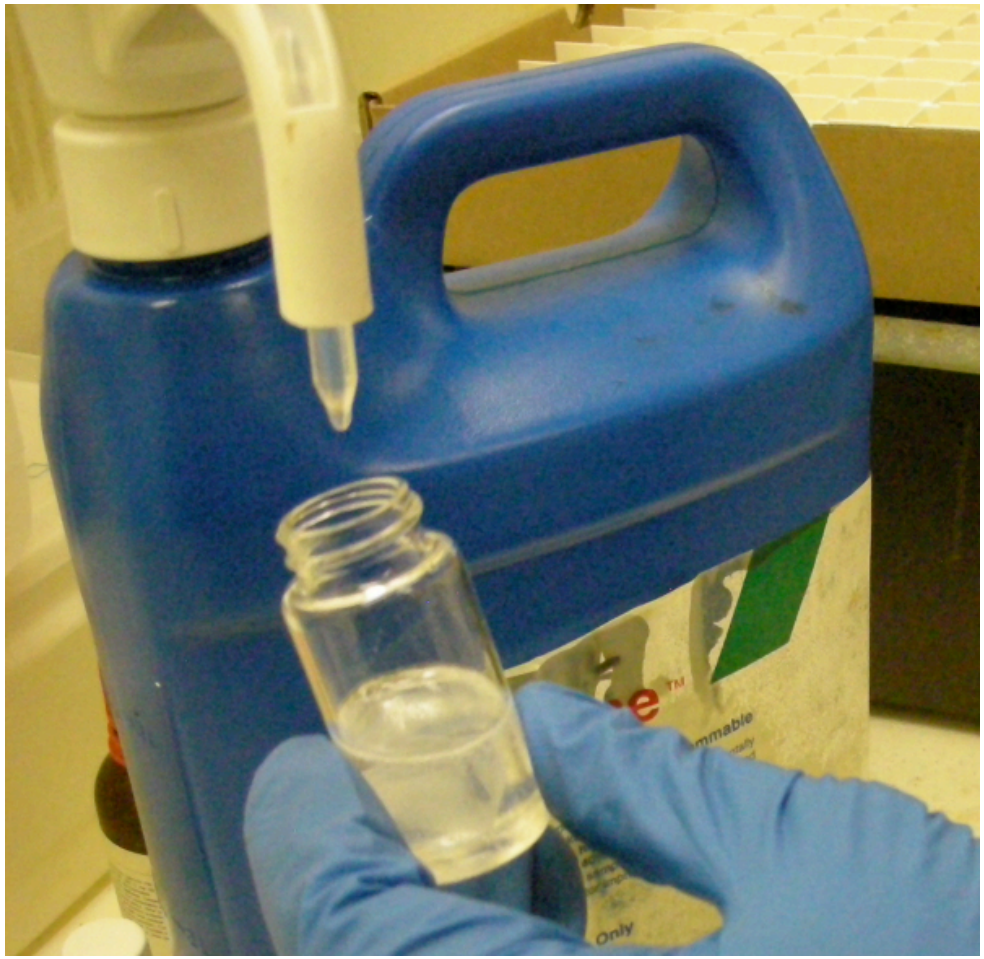


Will become the “C” vial into which the bucket is placed

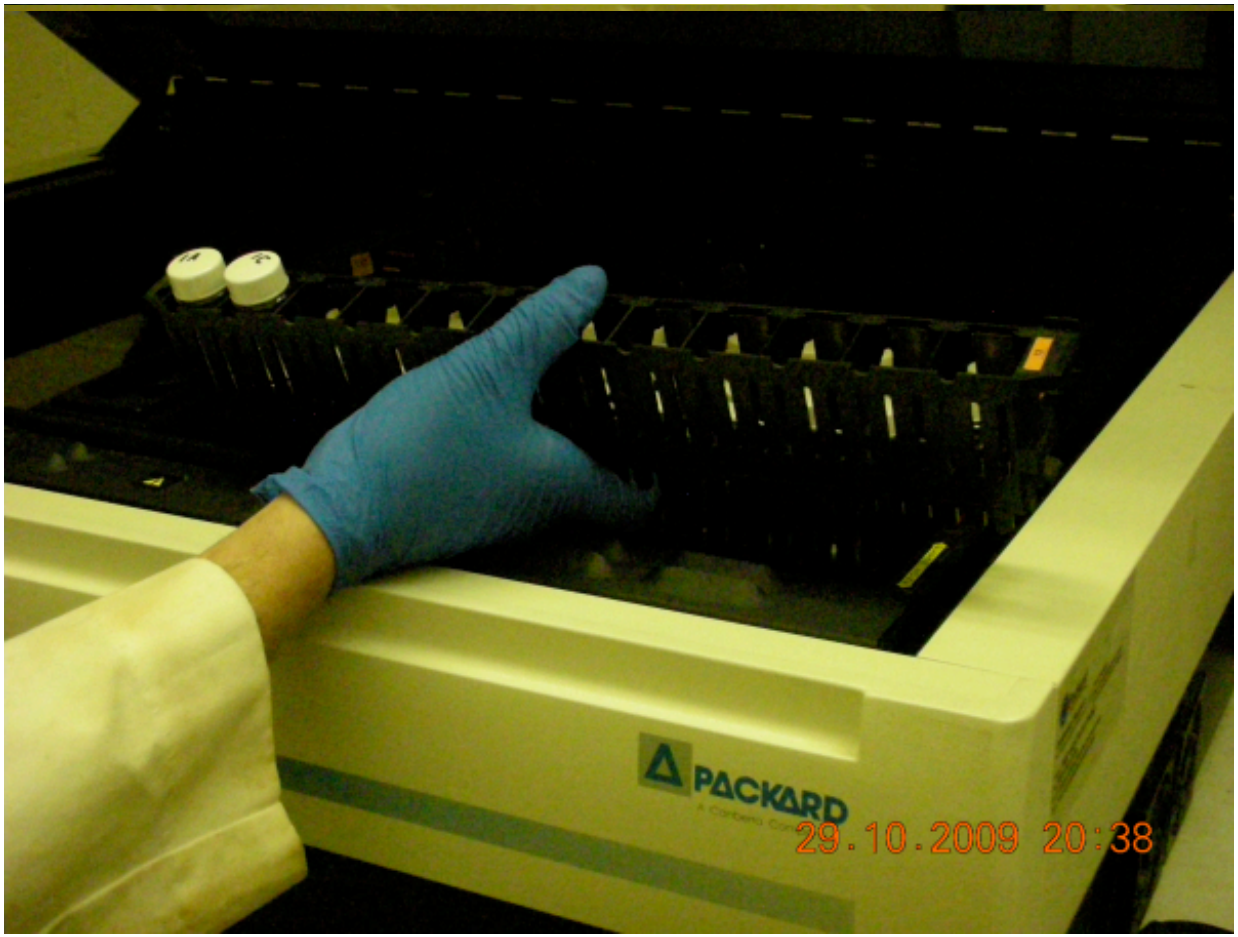
Diffusion Chamber; will become the “A” or “acidified” vial

Shake for 24h

Unseal “A” vial containing acidified filter plus 1mL HPO4. Remove septum/bucket, add 10mL Ecolume scintillation cocktail…



Snip bucket with acid-cleaned wire cutters into “C” vial. Add 1 mL water and 10mL Ecolume cocktail; Ready to count activity



^{14}C -Calcification techniques – The issue of blanks

a) Historical precedent for formalin blanks: Paasche 1962; Nature 193: 1094-1095;

He used the ^{14}C differencing technique…
"*Under the conditions used, the uptake of carbon-14 in cultures killed with formalin corresponded to less than 1 per cent of the coccolith uptake in living cells at maximum photosynthesis*"

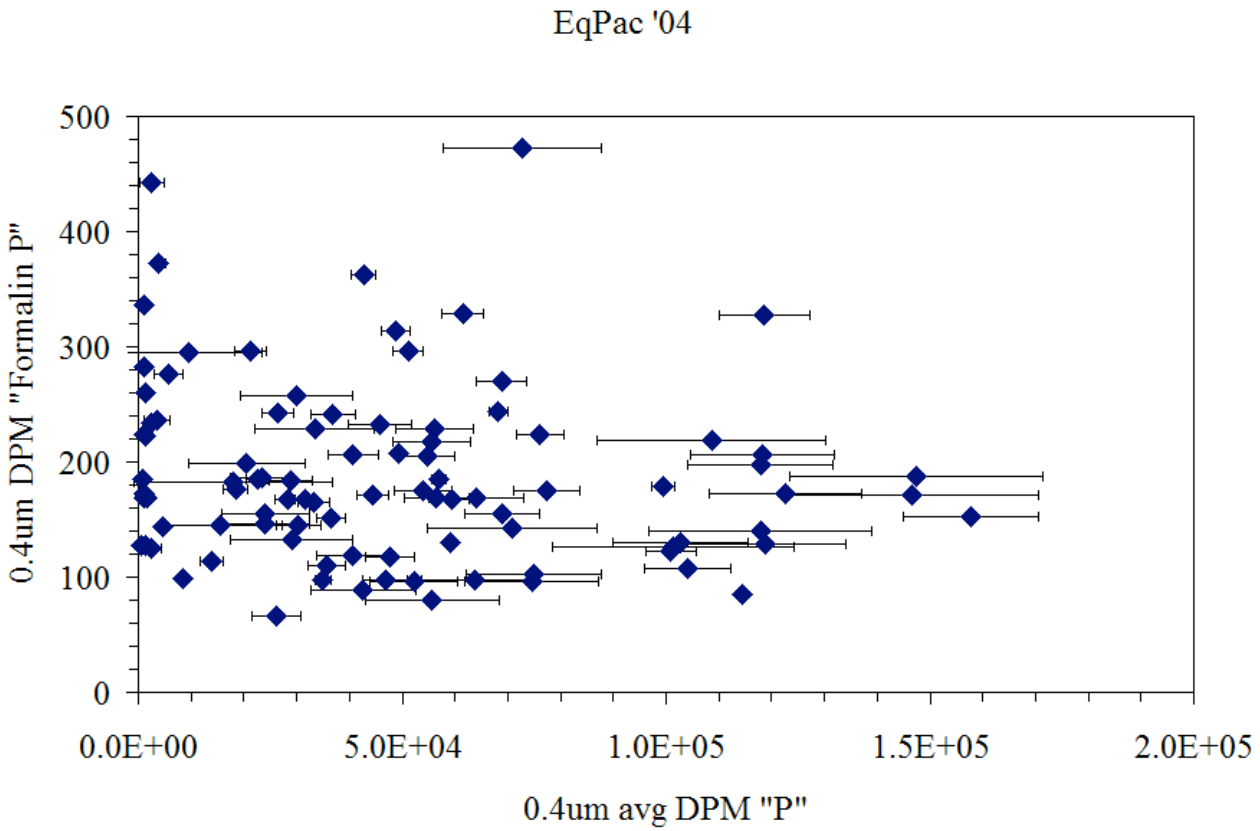
b)Paasche, 1963; Physiol. Plantarum 16: 186-200. ^{14}C differencing technique

"*A certain amount of non-biological isotope exchange will occur between the medium and the coccoliths. This was measured by incubating formalin-killed cultures under normal experimental conditions. The uptake of carbon-14, practically all of which was in the acid removable fraction, amounted to 0.5-4 per cent of the uptake in coccoliths in living cultures at light saturation. It was consistently higher in artificial medium than in natural sea-water.*"

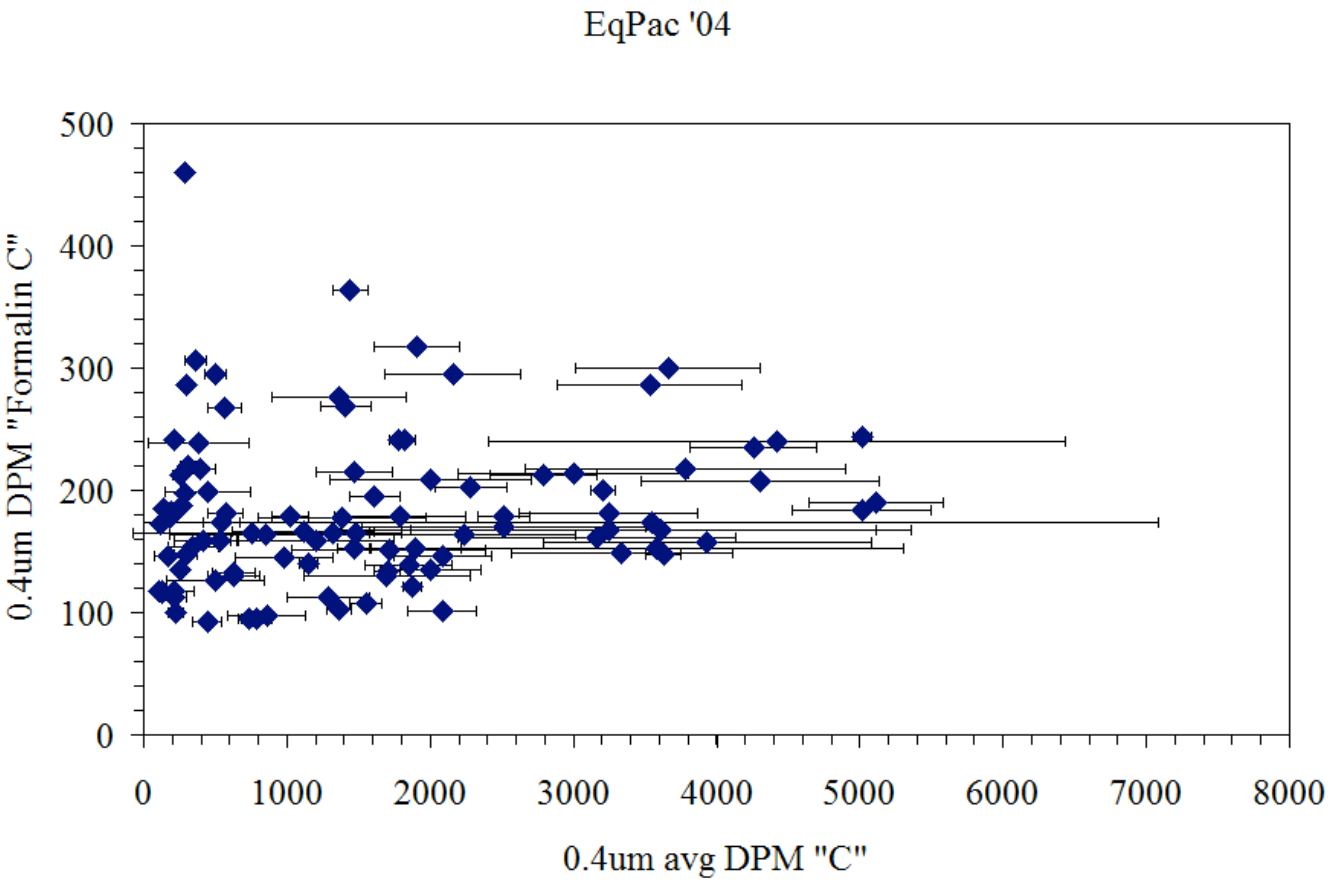
Our experience with blanks

a) Time-zero samples highly variable!
b) Dark incubations are not a good blank; they show significant calcification (known since the 60's)!
c) Mercuric chloride- works but not ideal to use at sea
d) Buffered formalin (2% final conc) shows best, most consistent results
e) In one summer, Gulf of Maine samples were characterized by high blanks for months…we never found the source.
f) We always run totals, filter efficiency tests, and checks of isotope activity in PEA to verify various potential sources of error.

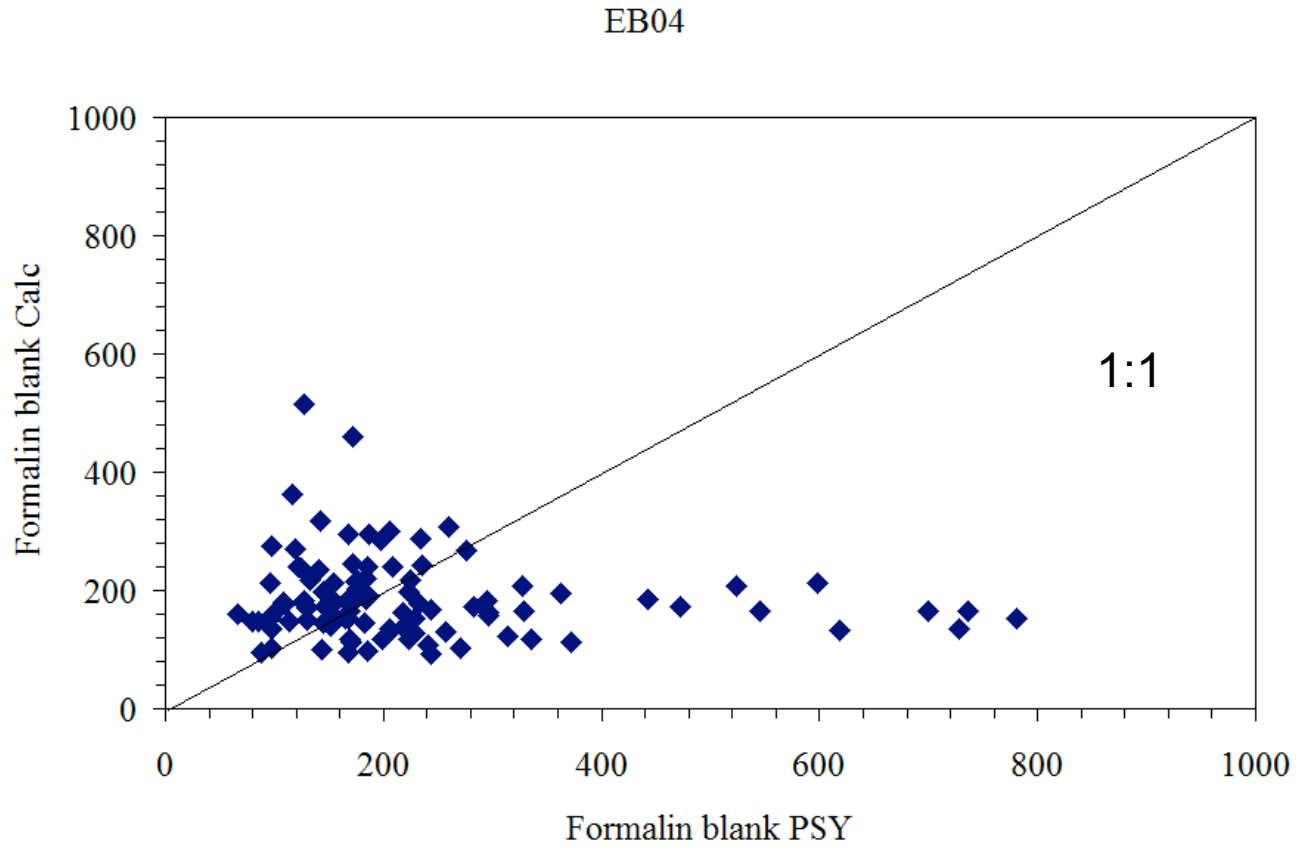
A case study on formalin blanks in the micro-diffusion technique
Formalin P DPM versus untreated P DPM.



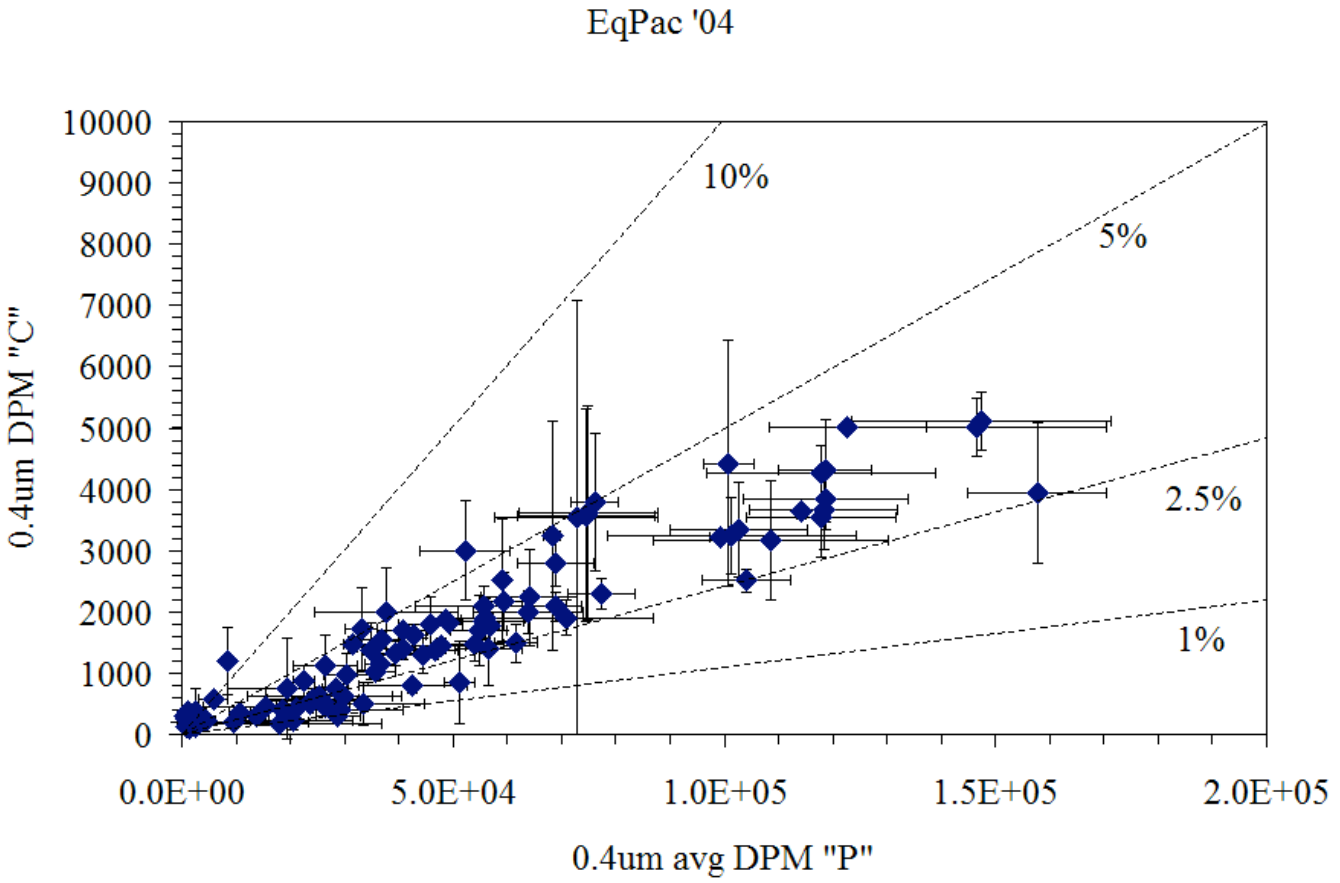
Micro-diffusion technique formalin DPM vs raw calcification DPM



Formalin P and Formalin C blanks for same water sample covary?

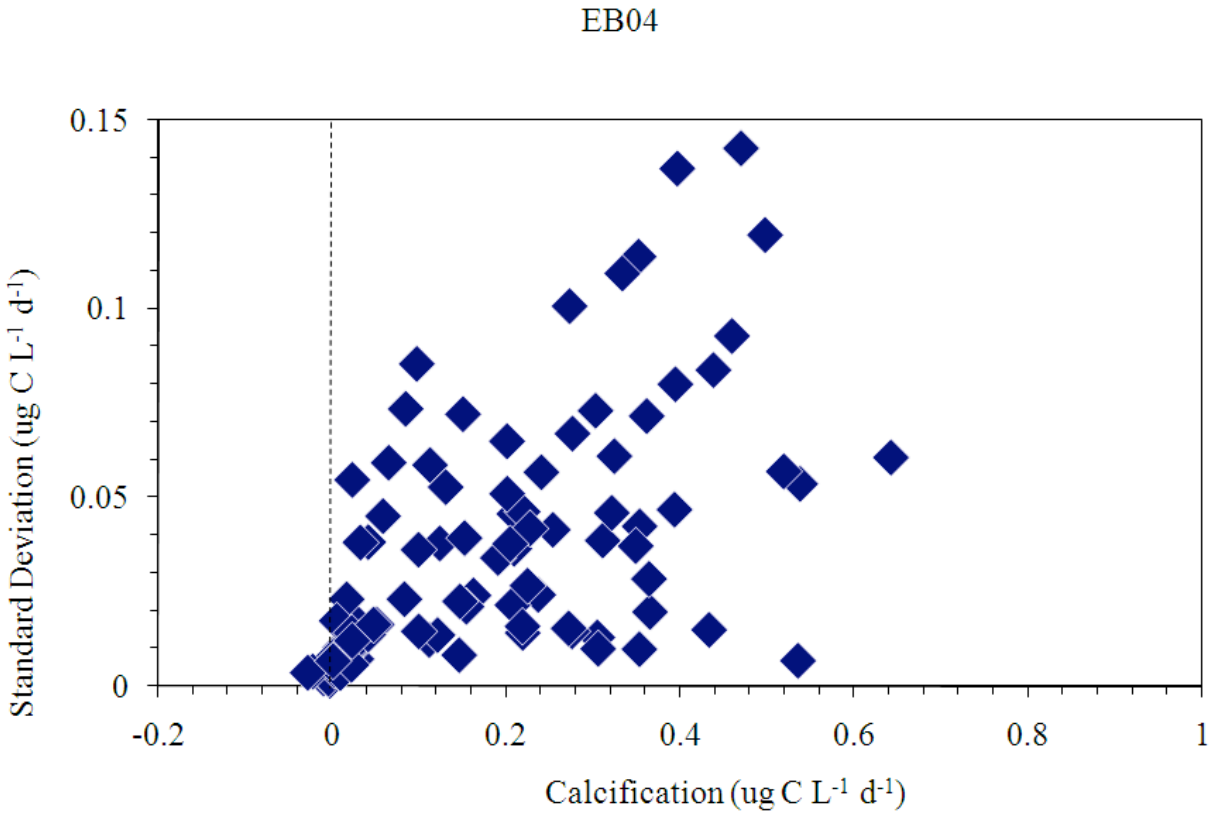


Equatorial Pacific 2004
Do P and C DPMs covary?



Equatorial Pacific-calcification

Average error of microdiffusion technique…
0.05 (range 0 to 0.15) ugC L^{-1} d^{-1}



Equations for estimating calcification and primary productivity

- Estimate W, the total dissolved inorganic carbon concentration in seawater (~25,000 ug C L-1 in most oceanic environments)
- In non-oceanic environs, is approximated as:

W =(0.96*((S*0.067)-0.05))*1000*12

Parsons, T.R., Y. Maita, and C.M. Lalli. 1984. *A manual of chemical and biological methods for seawater analysis*. Pergamon Press Inc., New York. 173 pp.

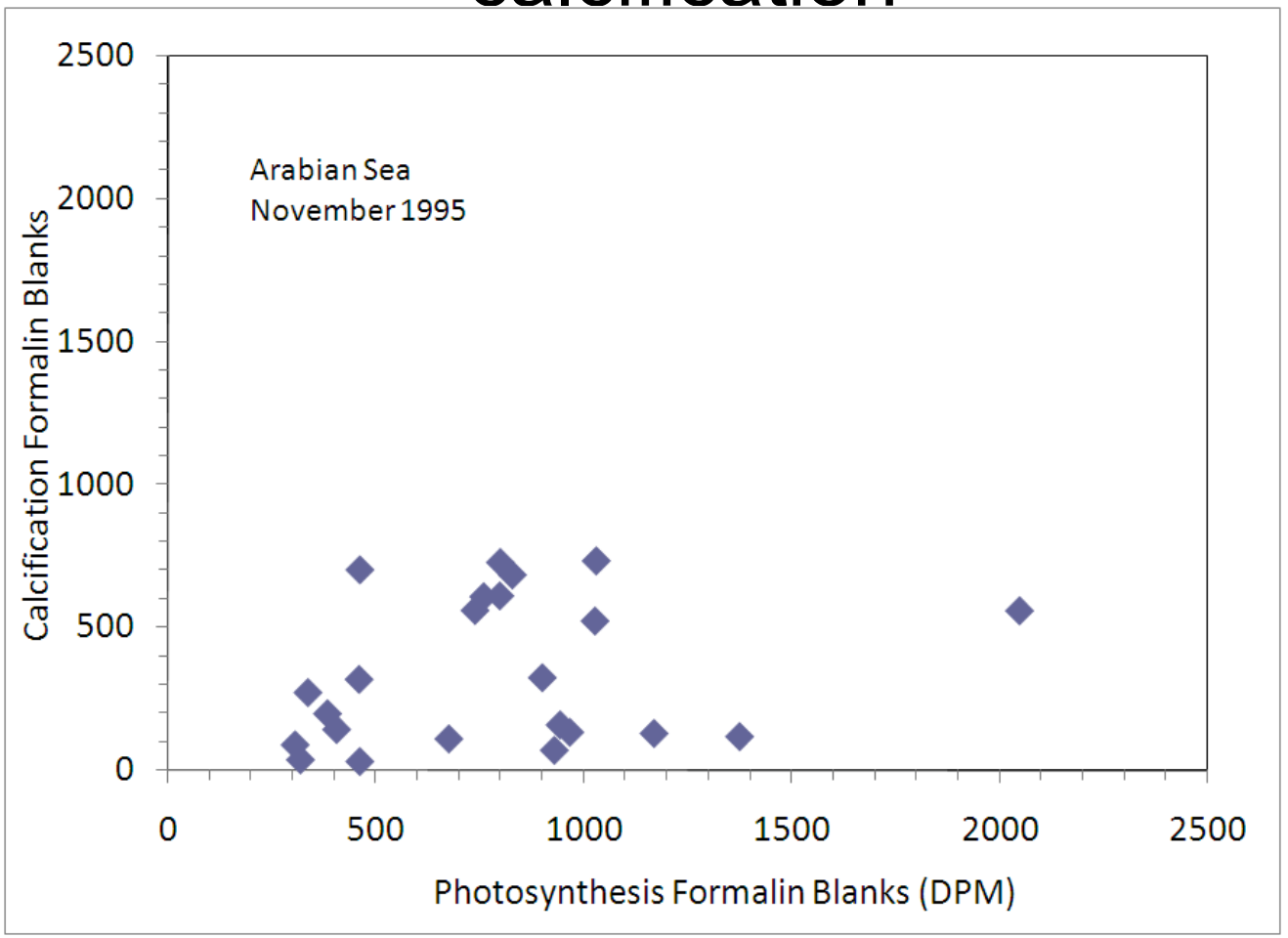
Equations for estimating calcification and primary productivity…

- C fixation (ug C L^{-1} d^{-1}) = [(DPM_{sample}-DPM_{blk})/V] * [W/DPM_{tot}]* [1.05/T]

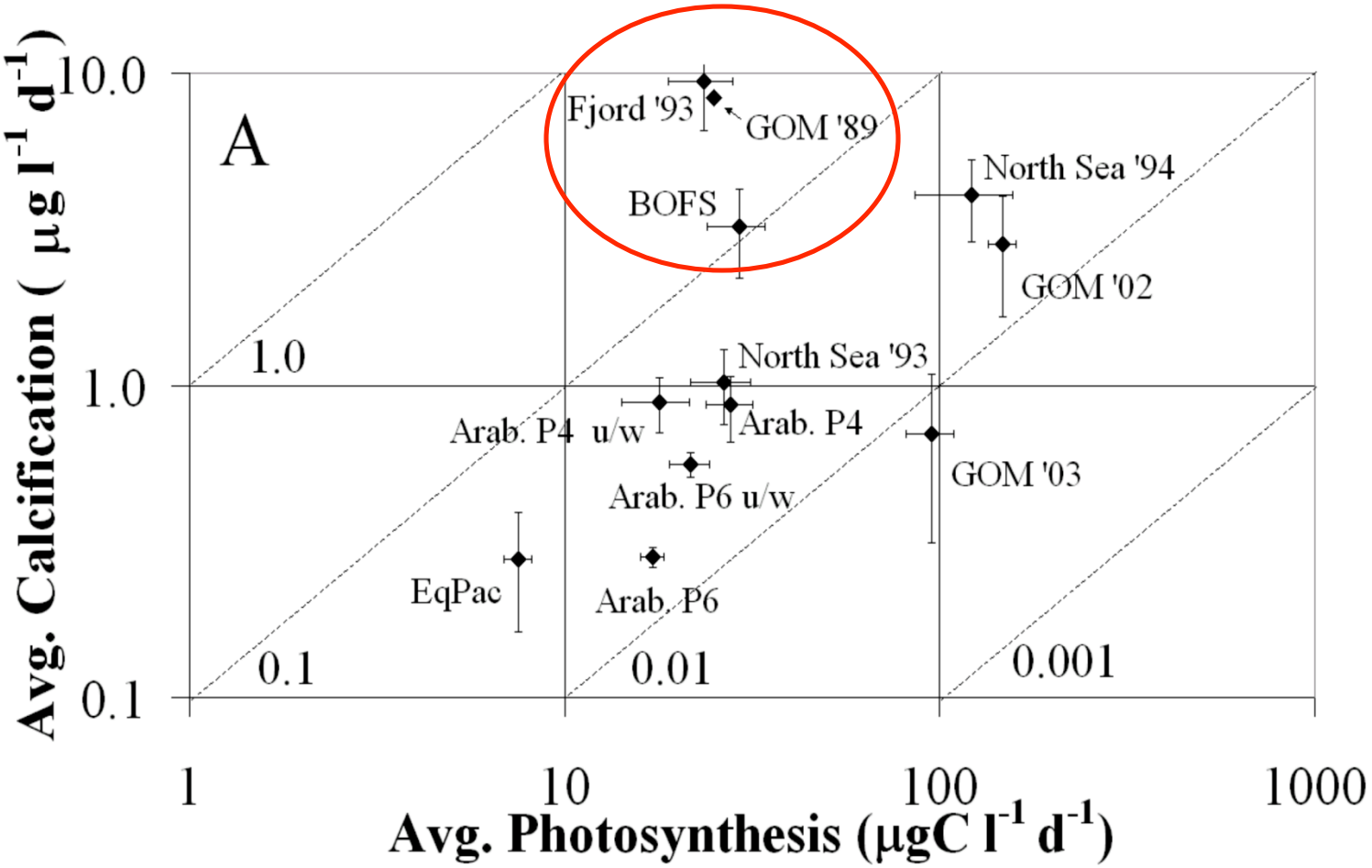
- Where DPM_{sample}= average of triplicate DPM counts in sample;
- DPM_{blk}=DPM in formalin blank;
- V = sample volume filtered (L);
- DPM_{tot}= total DPM per sample (measured in 100uL of seawater sample);
- 1.05 is the correction factor for lower uptake of ^{14}C compared to ^{12}C;
- T = time incubated (days)

Another example…Arabian Sea

Formalin blanks for photosynthesis and calcification

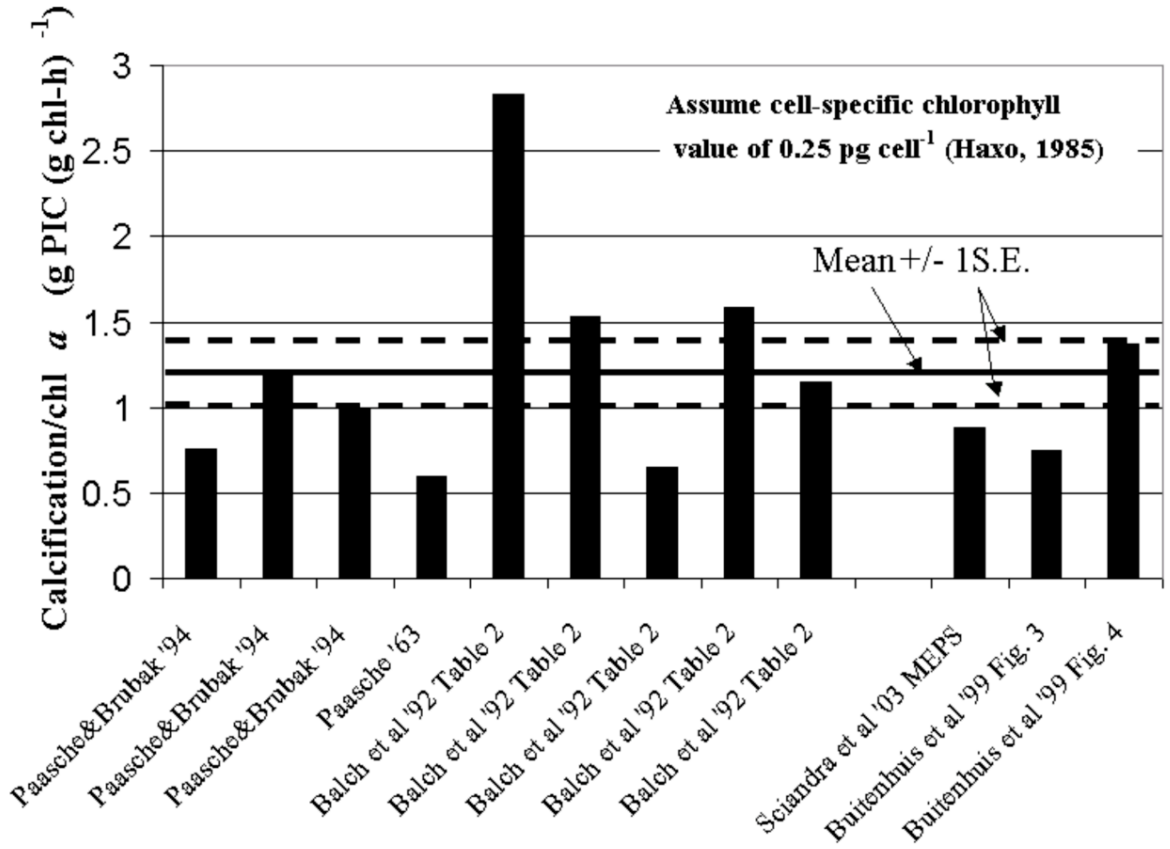


Globally, calcification and phytosynthesis roughly covary except in blooms



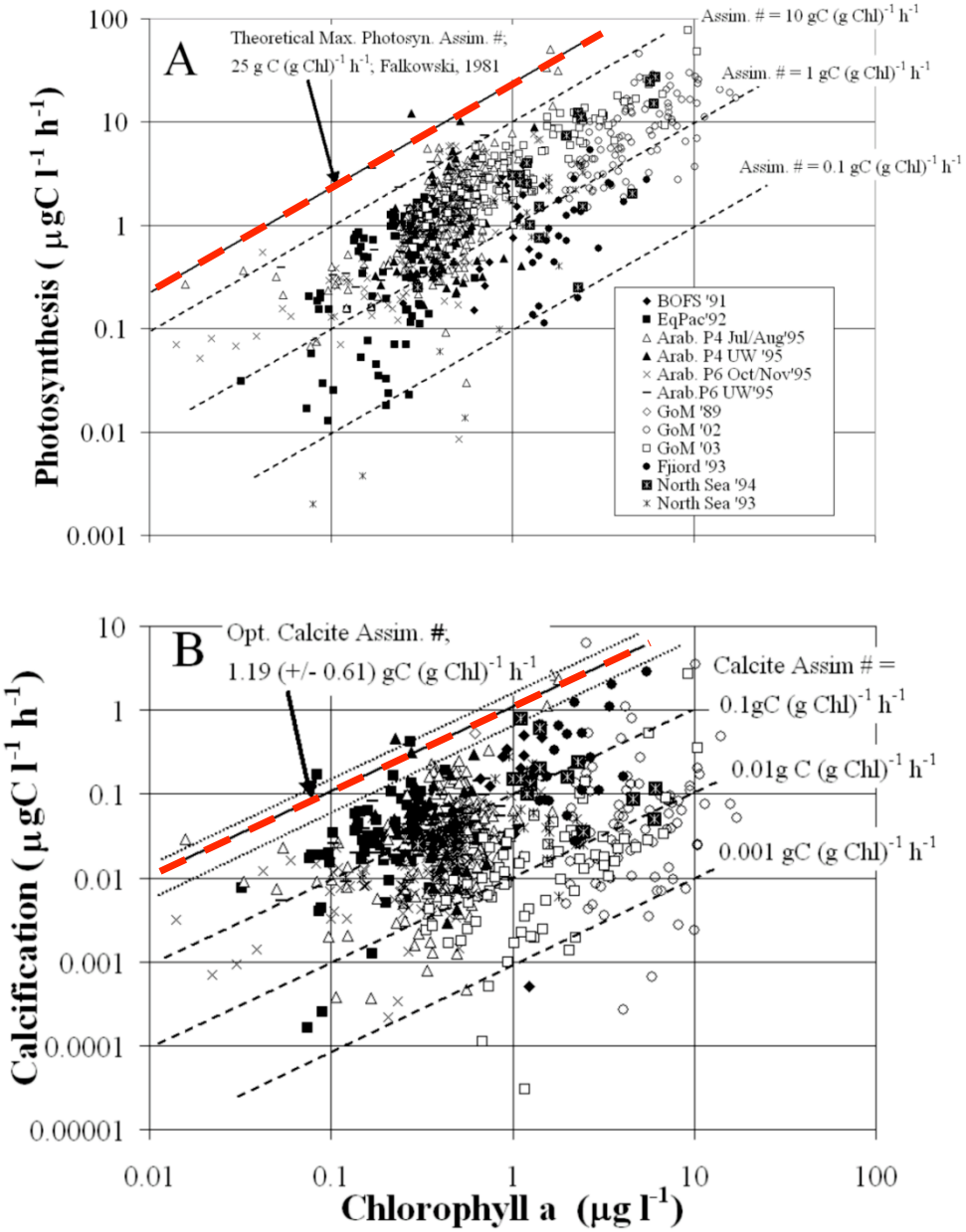
Balch et al., 2007; DSRII

Maximum calcification per unit chlorophyll is not constant in culture but predictable based on a number of reports…



Balch et al., 2007

Chlorophyll concentration provides some information about carbon fixation rates (as measured with ^{14}C)



Balch et al., 2007

Methods – Animal calcification (e.g. Foraminifera/Pteropod calcification

a) isotope methods (note units: ugCa/mg Ca shell-t)

1) ^{14}C (e.g. Erez and others)

2) ^{45}Ca (e.g. Fabry, 1989)

b) mass-based- must digest away tissue or do indirect estimate of acid-labile mass

c) chemical techniques (based on TCO_2, alkalinity)

c) sediment trap-based (Honjo; Prell; Deuser; Conte)

Strengths

Can remove the animal from the ^{14}C or ^{45}Ca solution and rinse away activity (allows pulse-chase experiments…very important difference between coccolithophore experiments). Allows estimates of isotope exchange.

Weaknesses

Volumes sampled for Forams and Pteropods must be larger than for coccolithophores (pre-pick to concentrate?)

Must deal with isotope exchange with tissue (high blanks)

Same blank issues that occur with coccolithophores

Realistic simulated in situ incubation conditions?

Potential Pitfalls for all techniques

1) Observing “Net” calcification…dissolution may, and likely is, occurring simultaneously.
2) rare big calcifying animals mixing in with abundant coccolithophores can increase variance.
3) Grazers in incubation vessels also consume calcifying plants, dissolving $CaCO_3$
4) Must be fastidious with direct isotope estimates of calcification!!
5) Bottle effects

Recommendations for standards/guidelines

1) As with isotope-based primary production measurements (bio-assays), with bottle incubations, there is no true “standard”, (except the standards in the scintillation counter!).

2) Blanks are critical to establish a true zero. Buffered formalin blanks best (but certainly room for more research).

3) Direct mass methods with coccolithophores will always suffer from poor signal to noise.

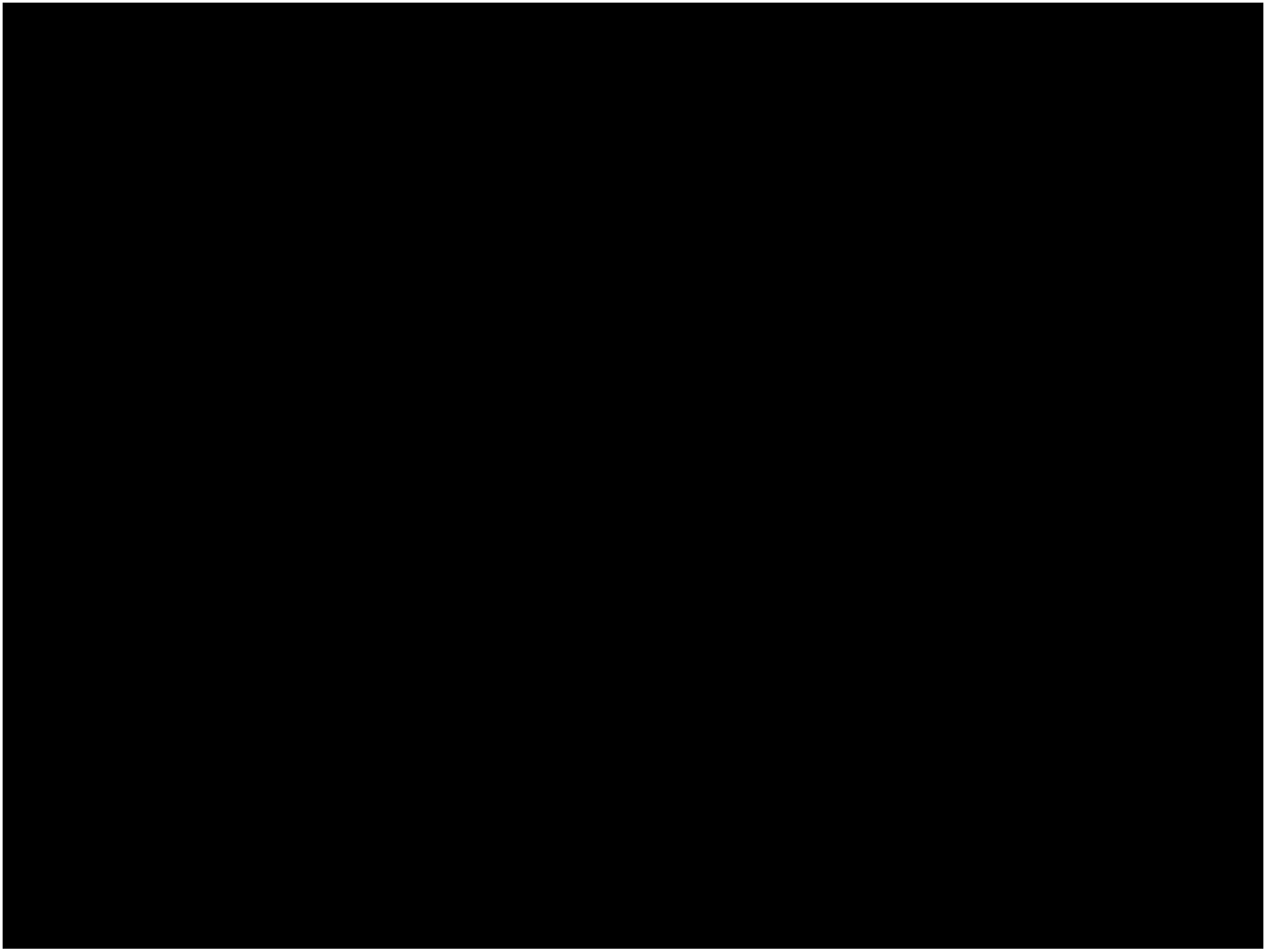
4) We are measuring “net calcification” (dissolution and calcification happening simultaneously…)!

In closing…

Accuracy– Somewhat problematic to define with bottle incubations. Is accuracy acceptable to answer the question? (e.g. what is the effect of OA on global coccolithophore calcification? …cellular calcification levels?). Comparison of different methods (some of which can be calibrated absolutely!) will help quantify the overall accuracy of all the methods.

Precision- +/- 0.05-0.1 mg PIC m^{-3} d^{-1} currently achievable. Is this sufficient to for future ocean acidification studies?

Thank you!



Suggestions for improvements

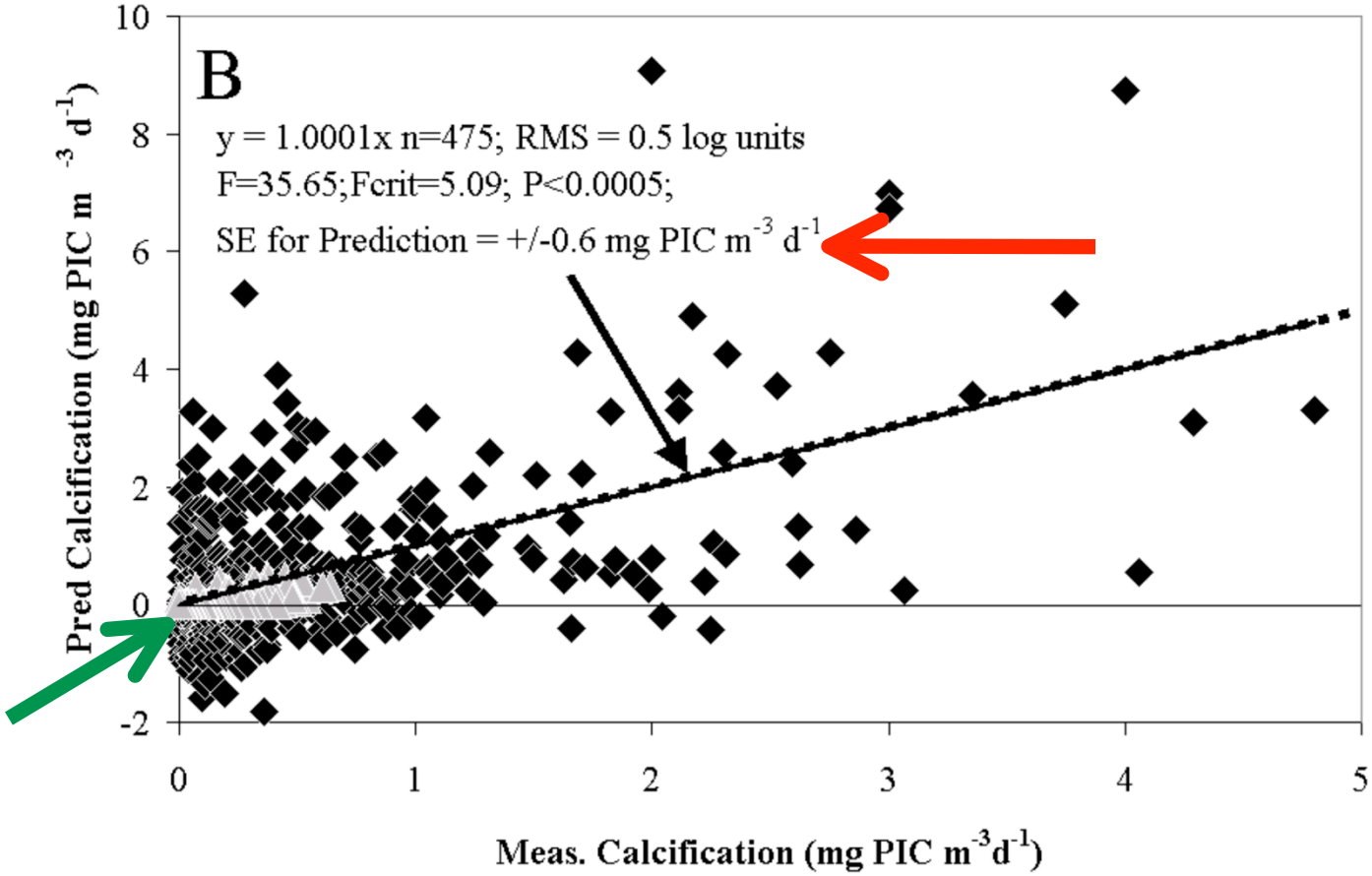
1) More studies of blanks and isotope exchange within calcification methods

2) More methods comparisons within and across different functional groups

3)Dark calcification- long known, but rarely measured in the field

4) While standardization of techniques should be a goal, actually finding suitable standards will be difficult for isotope techniques

Predicting ^{14}C production rates from temperature, chlorophyll, PIC, depth and daylength (Balch et al., 2007; DSRII)



Balch et al., 2007

A comparison of calcification estimates with different methods

Author	Technique	Global CaCO3 production (Pg PIC y^{-1})
This study	14C measurements and remote sensing algorithm	1.6 +/-0.3
Feely et al..(2004)	Seasonal cycle of euphotic zone alkalinity	0.8-1.4
Wollast(1994)	Chemical state of the carbonate system	1.1
Morse and Mackenzie (1990)	Geochemistry of sedimentary carbonates	1.0
Archer et al.(1994; 1996)	Gridded maps of calcite and diagenetic model of CaCO3 preservation	1.0
Moore et al.(2002)	global marine ecosystem mixed-layer model	1.1
Milliman (1993)	Historical accumulation rates and sediment trap data	0.6
Milliman et al.(1999)	Historical accumulation rates and sediment trap data	0.7

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With remote sensing you can reduce the standard error through large sample sizes (via space/time binning)…

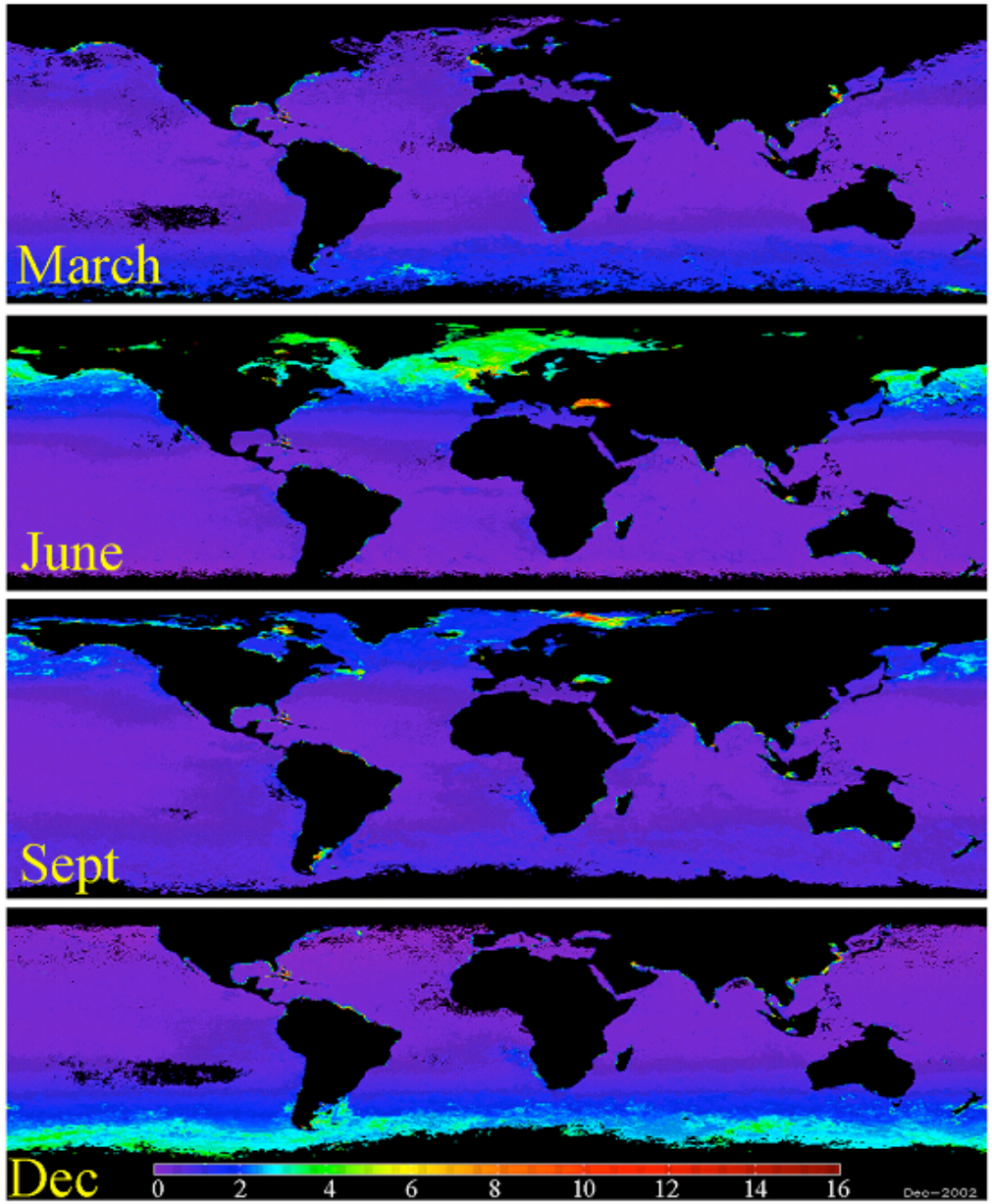
Table of standard errors for satellite-derived calcification ($\mu gC\ L^{-1}\ d^{-1}$)

Time Bins (d) \ Space Bins (km)	1	4.63	36	111.2
1	0.596	0.277	**0.099**	**0.057**
8	0.211	**0.098**	**0.035**	**0.020**
30	0.109	**0.051**	**0.018**	**0.010**
365	**0.031**	**0.014**	**0.005**	**0.003**

Bold numbers represent errors <0.1 ugC L^{-1} d^{-1}

Balch et al., 2007

Global predictions of surface calcification based on purely statistical approach. An integration of these over the euphotic zone, over all months, gives 1.6 Gtons PIC fixed/year



mg PIC m^{-3} d^{-1}

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