INSTITUTE OF OCEAN SCIENCES PROTOCOLS FOR PHYTOPLANKTON PIGMENT ANALYSIS BY HPLC

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Monitoring changes in phytoplankton biomass and community composition is important for the evaluation of ecosystem function and status, as well as for the study of biogeochemical cycles. At the Institute of Ocean Sciences (IOS), phytoplankton pigments (chlorophylls and carotenoids) are being measured by High Performance Liquid Chromatography (HPLC) in order to monitor changes in the phytoplankton community in the northeast subarctic Pacific and coastal waters of British Columbia. This technique, although less precise than microscopic enumeration, is faster and more appropriate for the identification of pico- and nano-phytoplankton that dominate in open ocean samples. At present, twenty four phytoplankton pigments (four of which are degradation products of chlorophyll *a*) are separated and quantified following the method of Zapata et al. (2000). This report describes in detail the evolution of the method used to quantify phytoplankton pigment concentrations at IOS, detailing the steps for sample collection, filtration, and analysis, as well as calibration procedures and data processing. Several experiments were carried out to determine optimal filtration volumes for a range of phytoplankton concentrations and to identify the extraction method that best optimizes yields while minimizing variability between replicates. Results show that it is not necessary to mechanically disrupt the filters (by dicing, grinding and /or sonication) to improve extraction. Two intercomparisons with other labs have been carried out that validate both the extraction techniques and calibration parameters in use and show that our method reliably produces results that are at least quantitative, and at best state-of-the-art as defined by Hooker et al. (2005).

RÉSUMÉ

Le monitorage des changements qui se produisent dans la biomasse et la composition des communautés de phytoplancton est important pour évaluer le statut et le rôle d'un écosystème, ainsi que pour l'étude des cycles biogéochimiques. À l'Institut des sciences de la mer (ISM), les pigments de phytoplancton (chlorophylles et caroténoïdes) sont mesurés par chromatographie liquide à haute performance (CLHP) dans le but de suivre l'évolution des changements dans les communautés de phytoplancton du nord-est du Pacifique subarctique ainsi que dans les eaux côtières de la Colombie-Britannique. Cette technique, quoique moins précise que l'énumération à l'aide d'un microscope, est plus rapide et mieux adaptée pour l'identification du pico- et nano-phytoplancton en dominance dans les échantillons de haute mer. Jusqu'à présent, vingt-quatre pigments de phytoplancton (desquels quatre sont des produits de dégradation de la chlorophylle *a*) sont mesurés selon la méthode de séparation de Zapata *et al.* (2000). Le présent rapport décrit en détail l'évolution de la méthode utilisée à l'ISM pour quantifier les concentrations de pigments de phytoplancton, énonçant les étapes de collection d'échantillons, de filtration, et d'analyse, ainsi que les processus de calibration et de traitement des données. Plusieurs expériences ont été effectuées pour déterminer le volume optimal de filtration sur une gamme de concentrations de phytoplancton et pour identifier la méthode d'extraction qui optimise le mieux les résultats tout en minimisant la variabilité entre les échantillons pris en double. Les résultats montrent qu'il n'est pas nécessaire de perturber mécaniquement les filtres (soit par découpage, mouture et/ou sonication) pour améliorer l'extraction. Deux comparaisons corrélatives avec d'autres centres de recherche ont validé et les techniques d'extraction et les paramètres de calibration utilisés; elles démontrent aussi que notre méthode donne des résultats fiables qui sont, tout au moins quantitatifs, sinon le « nec-plus-ultra » tel que défini par Hooker *et al.* (2005).

1.0 INTRODUCTION

Quantifying variations in phytoplankton biomass and community composition is important for the evaluation of ecosystem function and status, as well as for the study of biogeochemical cycles. Traditionally, the composition and abundance of phytoplankton populations has been determined by microscopy; however this method has several limitations. Light microscopy requires taxonomic expertise, is very time consuming, can be subjective, and is associated with counting errors resulting in large variability in sample analysis between laboratories (Duarte et al. 1990). Cell counts can be converted to biomass using biovolume measurements and appropriate conversion factors, but such a conversion further reduces the precision of the analysis due to the high variability in biovolume of most species. Many of the smaller (pico- and nano-plankton) cells are very difficult to study. Their small size and absence of taxonomically useful morphological features often preclude identification by light microscopy, even to the class level, and many are too fragile to be adequately preserved for electron microscopy. Thus, since microscopic enumerations are complicated and time consuming, and the number of algal cells that can be counted is limited, the resultant coefficient of variation of cells counted using microscopy is high. Minor variations in the composition of phytoplankton communities are consequently not revealed using microscopic techniques.

An alternative approach is the characterization of phytoplankton communities based on the analysis of photosynthetic pigments (chlorophylls and carotenoids) which can identify taxonomic groups when it is difficult or impractical to identify and count individual cells. This technique is faster and more appropriate for the identification of pico- and nano-phytoplankton than microscopy being especially useful for samples from open ocean areas. Pigment analysis by High Performance Liquid Chromatography (HPLC) allows easy and accurate separation, identification and quantification of phytoplankton pigments. The larger number of samples that can be processed by HPLC allows a more thorough examination of the structure and dynamics of phytoplankton populations than has been possible by enumeration of phytoplankton under the microscope.

Analyses of phytoplankton pigments by HPLC are more reproducible than microscopic analyses (Schlüter et al. 2000) and are increasingly being used to monitor phytoplankton communities. However, reconstruction of the phytoplankton community based on pigment concentrations is not straightforward. While some pigments are unambiguous markers for particular phytoplankton classes (e.g peridinin), many markers are present in several classes. In order to relate the measured pigment composition to the distribution of phytoplankton groups, statistical techniques like multiple linear regression (e.g. Letelier et al. 1993) or matrix factorization, such as the well-known CHEMTAX software (Mackey et al. 1996) can be used. CHEMTAX results can correlate strongly with those from microscopy, and in some instances have revealed the presence of groups not detected with traditional enumeration methods (Wright et al. 1996).

Although not providing the taxonomic precision of microscopy, the analysis of pigments by HPLC and statistical tools such as CHEMTAX is rapid and objective, generating highly reproducible results (Wright et al. 1996). Thus, with appropriate precautions and some knowledge of potential populations within a sample region, this method is faster and more appropriate than the microscopic method for quantitative analysis of phytoplankton abundance and community composition.

At the Institute of Ocean Sciences (IOS), phytoplankton pigments are being measured by HPLC to monitor changes in the phytoplankton community in the northeast subarctic Pacific and coastal waters of British Columbia. These regions differ significantly in the abundance and type of phytoplankton present. In coastal regions, the phytoplankton community is dominated by diatoms especially during spring (Harris et al. 2009), whereas in the iron limited northeast subarctic Pacific community composition is more diverse and dominated by small phytoplankton cells (Booth et al. 1993). Because of this diversity, we have adapted existing literature methods to deal with the high variability in phytoplankton abundance and composition and the desire to minimize processing time. This report describes in detail the methods used to quantify phytoplankton pigment concentrations at IOS and presents results of several experiments carried out to optimize them. Results from recent intercomparisons with other labs, which validate both the extraction techniques and calibration parameters in use for all pigments are also presented.

2.0 PRINCIPLE OF ANALYSIS

A number of methods for the determination of phytoplankton pigments are available which have various levels of sophistication and accuracy. These range from widely used simple fluorometric methods for measuring chlorophylls and their derivatives (e.g. Strickland and Parsons, 1972) to chromatographic methods that separate an array of chlorophylls and carotenoids (see review by Roy, 1987). Several HPLC methods for the analysis of phytoplankton pigments have been published; however, no one method is ideal for separating all pigments. Most of these methods use reverse-phase liquid chromatography with C_8-C_{30} stationary phase columns and gradient elution from partially aqueous mobile phases to non-aqueous organic mixtures (Wright and Jeffrey, 2006). As such, pigments are primarily resolved on the basis of their polarity. General purpose methods that separate the maximum number of significant chlorophylls and carotenoids are those of Wright et al. (1991), Van Heukelem and Thomas (2001), and Zapata et al. (2000).

HPLC separations of algal extracts can yield dozens of pigments in highly variable amounts depending on the abundance and diversity of the phytoplankton community at the location and time of sampling. A recent book by Roy et al. (2011) summarizing the current knowledge on phytoplankton pigments describes the molecular structure, biological occurrence, and absorbance spectra for 47 carotenoids and 21 chlorophylls. In addition, well over 100 additional algal pigments are listed that have been identified but not fully characterized. Thus, prior to approaching this analysis consideration must be given as to which pigments should be identified and quantified in order to address the specific research questions posed. When the goal is determining phytoplankton community composition, the choice of which pigments to measure should be based on their ability to distinguish between the phytoplankton classes typically encountered in the area of study. For example, peridinin and dinoxanthin always co-occur in Type 1 dinoflagellates and neither pigment is found in any other group. Thus measuring dinoxanthin does not provide any additional information that could not be gleaned from peridinin alone. The availability of a commercial standard is also an important consideration when choosing which pigments to measure as these are required for quantification, and also aid significantly in identification by providing a reference absorbance spectrum in the solvent being used.

Three criteria are required for the positive identification of a given pigment in a chromatogram (Roy et al. 2011). To be certain of a match an unknown pigment must have: 1) the same absorbance spectrum, 2) the same retention time (RT), and 3) the same molecular mass as a known standard. In practice, it is not always possible to meet all 3 criteria when working with field samples leading to potential uncertainties in pigment identification. Most labs that measure phytoplankton pigments lack a mass spectrometer interfaced with the HPLC for determining the molecular mass of the analyte so at best only the first 2 criteria are attainable. However, most HPLC systems have a photodiode array (PDA) detector that produces an absorbance spectrum for each pigment as it elutes off the column. These spectra can be compared to a spectral library built from runs of commercial pigment standards during calibration, or compared to published absorbance spectra for pigments for which no standard is available. In the latter case however, identification can be more difficult as published absorbance spectra are dependent on the solvent used, and as a result absorbance maxima may be shifted and the shape of the spectrum affected in the HPLC eluent compared to published values. Even with a full pigment library specific to a given HPLC method, spectral matching might be impossible for the less abundant pigments as the PDA may not be able to produce an absorbance spectrum above the noise level. In the absence of a suitable absorbance spectrum, fluorescence detectors can aid in identification by distinguishing between chlorophylls (and their degradation products) which fluoresce, and carotenoids which do not. Often however, comparing the RT of the unknown peaks in a chromatograph to those of known standards is the only available means of pigment identification.

2.1 PIGMENTS MEASURED

At present, 24 phytoplankton pigments are measured at IOS, 4 of which are degradation products of chlorophyll *a* (Table 1). These pigments were chosen because they are usually present in both coastal and oceanic waters of the North Pacific and standards for almost all are commercially available through either DHI Lab products in Denmark or Sigma Aldrich in Canada. In addition, all but the chlorophyll *a* degradation products and *chl c1* are available together in a relatively inexpensive mixed pigment standard which serves as a daily RT check. This is particularly important since, as mentioned above, the RT is often the only available means of pigment identification. Standards are not available for *chl c1* or *Me-chlide a*, but concentrations can be estimated using the response factors for *chl c2* and *chlide a*, respectively, which are similar pigments with almost identical absorbance spectra.

Table 1: Pigments currently quantified at IOS with average retention time (RT) in min. Note the two closely eluting pairs *viola*/*hex-fuco*, and *zea*/*lut*.

Pigment Name	Abbreviation	average RT (min)	Standard source
chlorophyll *c3*	*chl c3*	8.9	DHI
chlorophyllide *a*	*chlide a**	11.8	DHI
Mg-2,4,-divinyl pheoporphyrin a_5 monomethyl ester	*MgDVP*	12.5	DHI
chlorophyll *c2*	*chl c2*	13.0	DHI
chlorophyll *c1*	*chl c1*	14**	--
methyl chlorophyllide *a*	*Me-chlide a**	15**	--
peridinin	*peri*	16.1	DHI
pheophorbide *a*	*phide a**	19**	DHI
19’-butanoyloxyfucoxanthin	*but-fuco*	20.0	DHI
fucoxanthin	*fuco*	21.1	DHI/Sigma
neoxanthin	*neo*	22.2	DHI
prasinoxanthin	*pras*	23.0	DHI
violaxanthin	*viola*	24.0	DHI
19’-hexanoyloxyfucoxanthin	*hex-fuco*	24.3	DHI
diadinoxanthin	*diadino*	26.7	DHI
alloxanthin	*allo*	28.8	DHI
diatoxanthin	*diato*	29.5	DHI
zeaxanthin	*zea*	30.0	DHI
lutein	*lut*	30.2	DHI
trans-β-Apo-8-carotenal	*TAC****	32**	Sigma
chlorophyll *b*	*chl b*	34.4	Sigma
divinyl chlorophyll *a*	*DV chl a*	35.9	DHI
chlorophyll *a*	*chl a*	36.3	DHI/Sigma
pheophytin *a*	*phytin a**	38.7	DHI
β-carotene	*β-car*	39.5	Sigma

*chl *a* degradation products; **not present in mixed pigment standard thus RT are approximate, *** internal standard

The distribution of each pigment among the 15 major classes of marine phytoplankton is shown in Table 2. For simplicity the common names of each group are used. Although the Haptophyta is actually a Division comprised of 2 classes: the Pavlovophyceae and the Prymnesiophyceae (or coccolithophorids), the sub-types of these 2 classes are designated as HAPTO1-8 based on their pigment signatures (Zapata et al. 2004), so we use this nomenclature for this group. Note that while most pigments such as *zea* have a widespread distribution occurring in 9 out of the 15 classes listed, others such as *peri* are unique to only one type of one class. The 3 most commonly occurring and abundant pigments (other than *chl a*) present in almost all of our samples are *fuco, chl c2*, and *diadino*. These pigments are characteristic of the "brown algae" (chl c containing) which include the diatoms and haptophytes. In contrast, less abundant and less frequently occurring pigments such as *neo, pras* and *lut* are characteristic of "green algae" (chl b containing) of the chlorarachniophyte, chlorophyte, and prasinophyte classes.

Table 2: Distribution of pigments among the 15 common classes of marine phytoplankton (from Roy et al. 2011). Numbers in brackets indicate that the pigment is confined to only those sub-types of the class.

Pigment	Occurrence
chl a	all classes except cyanobacteria (4)
DV chl a	cyanobacteria (4)
chl c1	diatoms (1, 3), silicoflagellates, chrysophytes, pelagophytes, raphidophytes, haptophytes (1-5), dinoflagellates (3)
chl c2	diatoms, silicoflagellates, chrysophytes, pelagophytes, raphidophytes, haptophytes, cryptophytes, dinoflagellates
chl c3	diatoms (2, 3), silicoflagellates, pelagophytes, haptophytes (4-8), dinoflagellates (2)
chl b	cyanobacteria (3), euglenophytes, chlorarachniophytes, chlorophytes, prasinophytes
MgDVP	cyanobacteria, diatoms, eustigmatophytes, haptophytes, cryptophytes, dinoflagellates (1,2), prasinophytes, chlorophytes, prasinophytes
fuco	diatoms, silicoflagellates, chrysophytes, pelagophytes, raphidophytes, haptophytes, dinoflagellates (2,3)
but-fuco	silicoflagellates, pelagophytes, haptophytes (6-8), dinoflagellates (2)
hex-fuco	haptophytes (6-8), dinoflagellates (2)
peri	dinoflagellates (1)
neo	euglenophytes, chlorarachniophytes, chlorophytes, prasinophytes
pras	prasinophytes (3)
viola	silicoflagellates, chrysophytes, eustigmatophytes, raphidophytes, chlorarachniophytes, prasinophytes
zea	cyanobacteria, silicoflagellates, chrysophytes, eustigmatophytes, raphidophytes, dinoflagellates (3), chlorarachniophytes, chlorophytes, prasinophytes
lut	chlorarachniophytes, chlorophytes, prasinophytes
allo	cryptophytes
diadino	diatoms, silicoflagellates, pelagophytes, haptophytes, dinoflagellates (1-3), euglenophytes
diato	diatoms, silicoflagellates, pelagophytes, haptophytes, dinoflagellates (1-3), euglenophytes

The 4 chlorophyll *a* degradation products listed in Table 1 are not used as markers for distinguishing between algal classes but instead provide information about the health of the community, as well as indicating possible damage caused during sample filtration and extraction. *Phide a* and *phytin a* are degradation products of *chl a* that have lost the central magnesium ion. This process occurs under slightly acidic conditions such as those found in the guts of zooplankton grazers. As a result these pigments are markers for grazing activity and are found in fecal pellets and algal detritus (Jeffrey et al. 1997). *Chlide a* formed by the dephytylation (loss of carbon tail) of *chl a*, is also found in damaged or senescent phytoplankton but is also commonly a filtration artefact particularly for species with high chlorophyllase activity such as diatoms (Jeffrey and Hallegraeff, 1987). *Me-chlide a* does not exist in cells naturally but is formed when *chlide a* combines with methanol used during the extraction process (see Section 4.1). Both of these compounds tend to scale with *chl a* concentration and are typically only seen in quantifiable amounts in high biomass samples, particularly when too much sample has been filtered and there are extraction efficiency problems (see Section 3.2). Therefore, high levels of *chlide a* and *Me-chlide a* in a sample are an indication that the sampling, filtration or extraction protocols may need adjusting.

2.2 HPLC METHOD AND INSTRUMENT SET-UP

Pigment separation at IOS is performed by HPLC according to the method of Zapata et al. (2000). This method was selected because it enables the separation of monovinyl and divinyl chlorophyll *a*, but more importantly in our local waters, because it facilitates the separation of the acidic chlorophylls *c2, c1* and *MgDVP*. This method is also practical in the absence of a column heater as it is optimized for a column temperature of 25 °C and can therefore be successfully utilized at room temperature. In contrast the more recent, popular method of Van Heukelem and Thomas (2001) which has the advantage of faster separation with very sharp peaks and only a 30 min sample run time requires a column temperature of 60 °C and cannot resolve *chl c2* from *MgDVP*, and *chl c1* from *chlide a*.

Sample extracts are analyzed using a Waters Alliance System HPLC consisting of a 2695 Separations Module equipped with both a 2996 Photodiode Array Detector (PDA) and a 2475 Multi-wavelength Fluorescence Detector (FD) connected in series, interfaced with a Windows PC running data acquisition and analysis software. The 2695 Separations Module integrates solvent and sample management into a single unit. The solvent delivery system consists of 2 independent piston pumps that drive eluent flow via a quaternary gradient proportioning valve (GPV) capable of mixing and delivering up to four solvents. Solvents are pumped from 1 L amber glass bottles held on top of the unit through an inline vacuum degasser that removes dissolved gases, improving eluent blending and detector performance. An inline filter downstream of the GPV acts as a last defence against any particles the solvents may contain. The system is plumbed with a seal wash reservoir that operates automatically rinsing and prolonging the life of the seals with a 20% methanol solution, as well as a 50% methanol needle wash system that rinses the needle between each sample draw to eliminate sample carryover.

The sample management system is comprised of 5 24-place carousels providing auto-sampling capabilities for up to 120 samples with a built-in programmable temperature controller allowing maintenance of sample temperatures between 4-40 ºC. Injections can be programmed with an auto-addition function allowing for dilution of the sample with buffer or water prior to injection to improve peak resolution and sensitivity. This dilution must be made immediately prior to injection to avoid losses of *chl a* and other late-eluting hydrophobic pigments (Wright, 2005). The sampling loop has a 2000 µl capacity with a maximum syringe draw for each auto-addition of 250 µl. Separations are performed using the method of Zapata et al. (2000) which employs gradient elution with a binary solvent system comprising 50%:25%:25% methanol:acetonitrile:aqueous pyridine (pH 5.0; Eluent A) and 60%:20%:20% acetonitrile:methanol:acetone (Eluent B) pumped at a constant flow rate of 1 ml/min through a Waters Symmetry C_8 column (150 mm x 4.6 mm, 3.5 µm particle size; Part #WAT200630). The 45 min elution gradient has the following program in terms of the percentage of Eluent A: 0 min at 100%, 22 min at 60%, 28 min at 5%, 38 min at 5%, and 40 min at 100% (Zapata et al. 2000). The maximum system pressure during the gradient is ~2500-3100 psi (column dependent) at the start of each injection or during equilibration in 100% Eluent A. Typical column lifetime is in the range of 800-2000 injections, after which chromatographic peak shapes tend to degrade, leading to tailing and poor resolution between adjacent peaks. A sacrificial guard column (Waters Part #WAT054250), located immediately upstream of the C_8 analytical column helps to prolong the life of the analytical column and is replaced every ~400 injections.

2.3 LABORATORY EQUIPMENT AND PRACTICES

All solvents used at IOS for separations, extractions and standard solutions are HPLC-grade. Aqueous solutions are made up using ultrapure Milli-Q water with a resistivity of 18.2 MΩcm. In the past, eluents were filtered through 0.45 µm PTFE membrane filters under vacuum into a glass collection flask before use. However, it was discovered that evaporation sometimes occurred during the vacuum filtration and the different boiling points of the solvents resulted in changes in eluent composition that negatively affected the chromatography. As a result, filtration of eluents was abandoned with no apparent detriment to the analysis. All graduated cylinders and funnels used for mixing eluents are rinsed with HPLC-grade methanol prior to use to remove any dust or other particles from their surfaces. Eluents are mixed in 4 L batches directly into amber glass solvent bottles that previously contained HPLC-grade solvent before being dispensed into 1 L amber glass bottles. Any solution being injected into the HPLC is first filtered through a 0.2 µm PTFE membrane filter to prolong the life of the column; this includes sample extracts, the vials of water used to buffer the samples, and the TAC internal standard reference solvent.

Equipment that is re-used for sample collection and extraction (sampling bottles, glass test tubes, plastic syringes) is soaked in a lab soap solution (Extran) for several hours, then rinsed 3 times in warm tap water and 3 times in Milli-Q water before being air dried. Care is taken to avoid contacting any of this equipment with acids which lead to degradation of chlorophylls. Standard solutions are prepared in Class A volumetric flasks and all standard additions are made with gas-tight syringes and volumes are confirmed gravimetrically (see Section 6.1). A calibrated bottle top dispenser specific for solvent delivery is used to add extraction solvent to tubes.

3.0 SAMPLING AND STORAGE

3.1 SAMPLE COLLECTION

Seawater samples for HPLC pigment analysis at IOS are collected in duplicate from the surface layer into 625 ml, 1 L, or 2 L LDPE bottles (actual volume of about 625 ml, 1050 ml and 1975 ml, respectively) depending on the expected biomass. Sample bottles are rinsed with sample water and filled all the way to the top of the neck directly from Niskin bottles, with the larger volume bottles (1 L and 2 L) filled using silicone tubing to minimize bubbles (which lead to reduced volumes) and cell disruption. Samples are filtered immediately following collection; however if a delay of more than 1 h is unavoidable, they are stored in the dark at 4 °C prior to filtration.

Many HPLC pigment protocols involve filtering “to colour” meaning that filtration is stopped when sufficient phytoplankton material is collected on the filter to produce colour on the white background. However, filtration “to colour” is subjective and will differ between individual samplers. In addition, filtering less than whole bottle volumes can lead to errors in the determination of sample volumes, inaccuracies resulting from cell settling, and under-sampling if some of the colour is due to detrital sediment particles rather than phytoplankton cells in coastal regions. Because sampling at IOS is carried out by several persons, it is preferable to standardize the sampling volume to avoid sampling variability. However, this is not straightforward since enough water should be filtered to detect most pigments but not so much as to prevent quantitative pigment yields during extraction.

3.2 FILTRATION VOLUME OPTIMIZATION

In the early years of HPLC sample collection at IOS, 625 ml bottles were used exclusively in coastal waters and 1250 ml or 2 L bottles were used offshore. However, these pre-determined volumes were not always found to be appropriate since, depending on the season or oceanic conditions, too little or too much water was sometimes being filtered. To determine optimal filtration volumes for a range of phytoplankton concentrations an experiment was carried out in May 2012 on a cruise off the west coast of Vancouver Island where a large range of biomass is typically encountered. The first part of the experiment aimed to compare pigment yields between samples filtered “to colour” and those filtered to a fixed volume: 4 replicates were collected for each of 5 eutrophic samples (*chl a* concentrations of 10-16.5 µg/L), with 2 filtered “to colour”, and 2 to a fixed volume of 1 L using 47 mm GF/F filters. For these samples, volumes filtered “to colour” ranged from 380 ml to 670 ml, or approximately 1/3 to 2/3 of the fixed volume, and were not always the same for both replicates. All samples were extracted using the same method (Method A; see Section 4.1) in 5 ml of solvent. Comparison of the pigment yields from replicates filtered to colour vs. 1 L are shown in Figure 1 in order of increasing *chl a* concentration as measured by fluorometry.

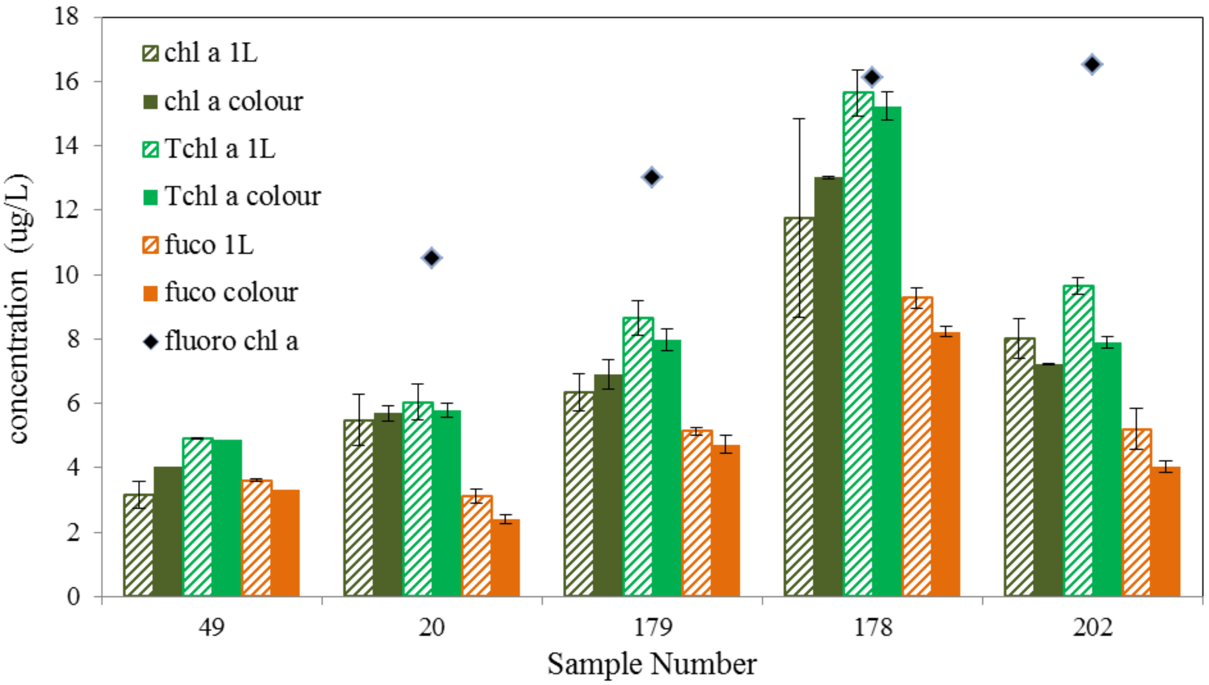

Figure 1: Comparison of yields of *chl a*, total *chl a*, and *fuco* between 1 L replicates and those filtered "to colour". Error bars represent standard deviations of duplicates. Diamonds are *chl a* concentrations measured fluorometrically (no value for sample #49).

In the 1 L fixed volume replicates, the mean concentration of *chl a* in 4 out of 5 samples was lower than the mean from replicates filtered "to colour", but total *chl a* (*Tchl a* = *chl a* + *chlide a* + *Me-chlide a*) was higher in all 5 samples due to significantly elevated (at least double and as much as 8 times higher) levels of *chlide a* and *Me-chlide a* (Fig. 1). This implied that the 1 L samples were likely not suffering from extraction problems, but rather that significant degradation of *chl a* was occurring during the filtration process itself. This observation is not surprising given that high biomass samples such as these are often dominated by diatoms (as indicated by the high *fuco* concentration; Fig. 1), a group known for its significant chlorophyllase activity (Jeffrey and Hallegraeff, 1987). Consistent with the results of total *chl a*, most of the other pigment concentrations (data not shown) were also higher and, on occasion, only quantifiable in the 1 L replicates. Only *β-car* had consistently greater yields in the "to colour" samples. It is also interesting to note that the "to colour" samples had less variability between replicates despite the fact that the filtration volumes often differed between the two replicates. The fact that the 1 L replicates contained more quantifiable pigments and produced higher yields than their "to colour" counterparts indicates that the presence of colour alone does not guarantee a good sample and more volume may be required for satisfactory detection of pigments present. On the other hand the increased *chl a* degradation observed in the 1 L samples shows that filtering too much water can lead to filtration and extraction artefacts.

The second part of the experiment aimed to determine what standard volumes should be used at different levels of *chl a* biomass to obtain sufficient material for a good signal without creating extraction problems. For the duration of the cruise, samples from 5 m were collected in duplicate in 2 L bottles and filtered onto 47 mm GF/F filters. Filtration was stopped at the first sign of colour on the filter with the remaining seawater in the bottle subtracted from the total bottle volume. Filtration volumes “to colour” ranged from a low of 370 ml to the full 2 L sample during this survey with extracted fluorometric *chl a* values ranging from 0.4-19.5 µg/L. The correlation between fluorometric *chl a* and HPLC total *chl a* was good with $r^2 = 0.90$ and no major outliers, indicating good extraction efficiency. To determine cut-off points for filtration volumes based on biomass, the “to colour” volumes for this cruise were plotted against their corresponding extracted fluorometric *chl a* values. As expected the relationship between *chl a* biomass and volume filtered roughly followed an exponential decay curve (Fig. 2a). It was immediately clear that at biomass levels >2 µg/L *chl a*, 1 L was the maximum volume required to attain colour on the filters, but it was also interesting that beyond ~8 µg/L *chl a* the volume filtered remained relatively constant with an asymptote around 400 ml, indicating this to be a minimum volume required to see colour on this filter size regardless of biomass. Because biomass levels need to be known prior to collecting samples for HPLC, the same filtration volumes were plotted against *in situ* chlorophyll fluorescence values from the Seapoint fluorometer (Serial #2228) mounted on the CTD package (Fig. 2b). Fluorescence values were obtained from the upcast when the Niskin bottles were fired. Although the two plots are similar, the relationship between volume filtered and *in situ* fluorescence showed considerably more scatter, and the maximum *chl a* value observed was lower (~14 µg/L vs. 20 µg/L; Fig. 2).

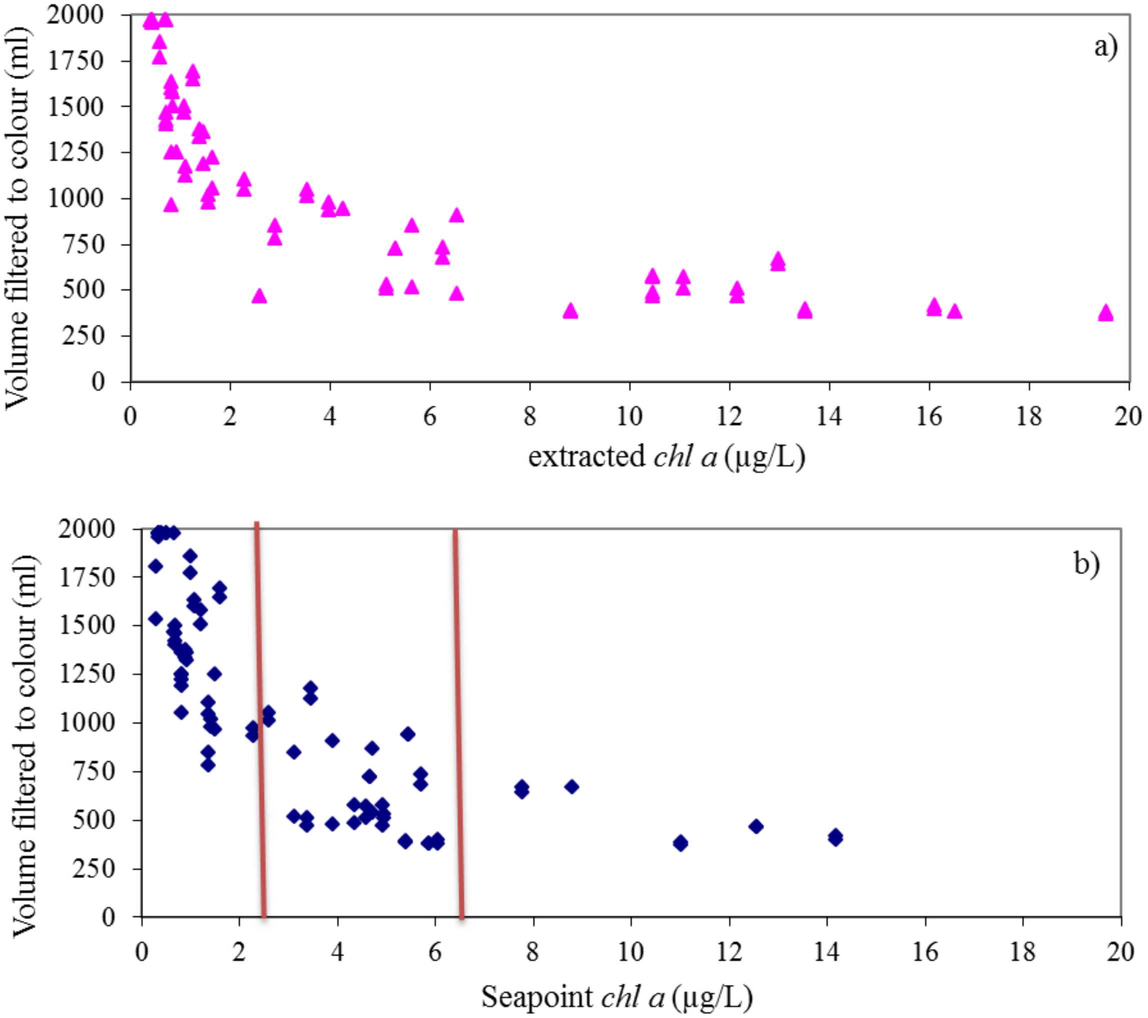

Figure 2: Volumes filtered to colour during cruise 2012-25 plotted against a) extracted fluorometric *chl a* and b) in situ *chl a* fluorescence from the Seapoint sensor. Vertical red lines in b) indicate empirically derived cut-off points for the three bottle sizes based on this dataset.

The relationship between Seapoint fluorescence and volume filtered was examined to determine the cut-off points for the three sample bottle sizes used at IOS. At fluorescence readings <2 µg/L *chl a*, filtration volumes ranged from the full 2 L to a minimum of 780 ml, although the majority of samples required more than 1250 ml to be filtered for colour to be seen (Fig. 2b). At fluorescence values above 2 µg/L *chl a*, only a few samples required more than 1 L to be filtered, indicating a natural cut-off point between the 2 L and 1 L sample bottles. Although the range in volumes required below 2 µg/L *chl a* was quite large potentially leading to more material collected than necessary for some samples, there were several samples with fluorescence readings close to 2 µg/L that still required almost the full 2 L volume for colour to be seen. The second cut-off point was not as obvious as the first; only 3 samples containing >5 µg/L *chl a* required filtration of more than 700 ml and many required less than 500 ml, so this level was subsequently chosen as the second cut-off point between the 1 L and 625 ml sample bottles (Fig. 2b). Table 3 summarizes the filtration volume cut-offs determined from this dataset for sampling in high biomass coastal waters. This scheme was subsequently used on coastal cruises with good results but its success depends on the stability of the relationship between the *in situ chl a* fluorescence and extracted *chl a*. This relationship is usually more variable at high chlorophyll concentrations (>5 µg/L), as shown in Fig. 3. Therefore, *in situ* and extracted *chl a* concentrations determined fluorometrically are compared before HPLC samples are analyzed in the lab to ensure the desired volume was filtered. If that is not the case, the extraction procedure is modified to optimize pigment yields for samples where the filtration volume was too high (see Section 4.2.4).

Table 3: Summary of the Seapoint fluorescence ranges that determine the sampling bottle size

Seapoint fluorometer (µg/L)	Bottle Size (ml)
<2	1975
2-5	1050
>5	625

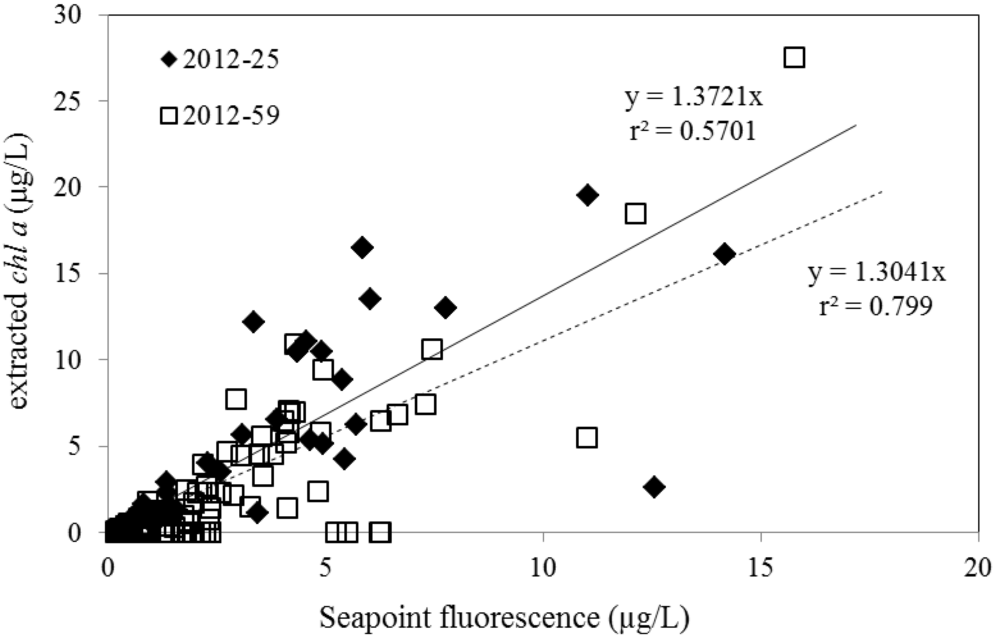

Figure 3: Linear regression between *in situ* Seapoint fluorescence readings and extracted *chl a* concentration shows the stability of the relationship for 2 cruises in 2012. Solid regression line is for cruise 2012-25 and dashed regression line is for cruise 2012-59.

3.3 FILTRATION

At present, water samples are vacuum filtered (pressure <100 mm Hg) under low light onto 47 mm GF/F filters (nominal pore size 0.7 µm; AMD Manufacturing Inc.). Filters are folded in half with the sample inside, blotted 3 times between sheets of acid-free blotting paper to remove excess water, and rolled into 5 ml cryovials. The filtration set-up consists of a 6-port manifold holding 47 mm Nalgene polysulfone filter funnels (Cat# DS0310-4000) connected to a vacuum pump. The filter funnel chambers are fitted with screw-on covers with 3 port openings. Two of these remain closed with silicone plugs during filtration and the third is connected by tubing to the sampling bottle. The 1 L and 2 L sample bottles are fitted with screw caps with an outlet port to which a piece of clamped tubing is connected. Bottles are inverted and held aloft in the rack above the filter cups, the free end of the tubing is connected to the open port in the lid of the filtration cups, a clamp on the tubing is released, and a vent on the bottom of the bottles (now the top) is opened to release the vacuum and start flow. The additional plug vents on the lids of the filter cups can be opened to bleed out air, preventing airlock. Once the system is bled in this way, the plug is resealed and the level in the filter cup remains constant as the flow self-regulates. The 625 ml bottles do not have bottom vents, so filter funnel covers are removed for these samples and the water is poured directly from the bottle into the open filter funnel.

Initially, HPLC samples were filtered onto 25 mm GF/F filters using a 6-port manifold holding open Nalgene filtration funnel cups connected to a vacuum pump, the same setup as used for regular chlorophyll *a* filtration. Seawater volumes of 625 ml (coastal regions) or 1250 ml (oceanic regions) were manually poured into the filter funnels. This filtration set-up was later modified to accommodate the larger sampling volumes (~2 L) needed for good detection in low biomass regions. The Nalgene filter cups were replaced with closed 25 mm Swinex filter holders connected on both ends to tubing leading to the vacuum on the outlet side, and to a spigot in the sampling bottle on the inlet side. This modification was made to protect the sample on the filter from light exposure during the longer time required to filter the larger volume. The 2 L sampling bottles were held in a rack above the filter holders and once the spigot was open, the flow was self-regulating requiring little intervention. A drawback of this setup was that it was trickier for the sampler to use as an airlock would sometimes form in the sealed filter holder, hindering filtration. With filtration taking more than 2 h for some samples (Table 4), this set-up was abandoned after being used on only 3 Line P cruises in 2008.

Table 4: Summary of the 2008 Line P cruise samples filtered using the closed 25 mm Swinex filtration set-up. For each cruise the number of samples (and percentage of total samples per cruise) that took between 1-2 h and greater than 2 h to filter is presented.

Cruise	**1-2 h filtration**	**>2 h filtration**	**Total samples**
2008-01	8 (24%)	5 (15%)	34
2008-26	10 (33%)	5 (17%)	30
2008-27	13 (43%)	12 (40%)	30

In 2009, the current filtration system using 47 mm filters was developed and has been exclusively used since. This filter size was recommended for open ocean samples in the JGOFS core measurement protocols (UNESCO, 1994). The much larger surface area of the 47 mm filters reduced filtration time significantly to between 8-15 min on average for 2 L oceanic samples, while some lower volume samples filter in as little as 5 min. The fast filtration allows for rapid collection of sufficient material with minimal sample degradation, offsetting the increased volume of solvent required to extract the larger filters. A summary of the filtration methods used at IOS over the years is presented in Table 5.

Table 5: Summary of the filtration methods used to collect HPLC samples at IOS.

Method	Filter (mm)	Volume (ml)	Setup	Dates Used
F1	47	625, 1050, 1975	closed Nalgene funnels	2009-present
F2	25	1975	closed Swinex holders	2008 Line P only
F3	25	625, 1250	open filter cups	1999-2007

3.4 COMPARISON OF FILTRATION METHODS

In order to determine that phytoplankton monitoring samples collected by the different filtration methods were comparable, an experiment designed to evaluate all three methods side by side was carried out at sea in August 2012. Seawater from 5 m depth was collected at each of 3 stations along Line P (P4, P12, P26) from replicate Niskin bottles and pooled into a 25 L carboy. Carboys were inverted to mix the sample well, and sampling bottles were filled such that the first replicate for all treatments was dispensed first, followed by the second and then the third in order to minimize any patchiness or effects of cell settling in the carboy amongst all treatments. The volumes that were routinely used with each filtration setup on past Line P cruises were used in this experiment: 1975 ml for methods F1 and F2, and both 1250 ml and 1975 ml for Method F3 (Table 5). Although Method F3 with a 1975 ml volume was not routinely used in the past, it was employed once during cruise 2007-15 as a comparison to the 1250 ml volume and was thus tested in this experiment (heretofore referred to as method F4 for simplicity). All samples were filtered under low light with vacuum pressure <100 mm Hg.

Chlorophyll *a* concentrations as determined by fluorometry as well as the duration of filtration for each method are presented in Table 6. Despite the large range in phytoplankton biomass between the stations studied, filtration with Method F1 took roughly the same length of time at each station. In contrast, when using the 25 mm filters, filtration lasted from a low of 30 min for a 1250 ml sample with method F3 to 2.5 h at P4 with Method F2 (Table 6). Due to high biomass at P4, Method F4 had to be abandoned when filtration ceased due to clogged filters after well over 3 h, despite significant volume still remaining in the funnel. All samples were analyzed back in the lab at IOS following a 24 h soak extraction in 95% methanol (Method A; see Section

4.1). Samples collected on 47 mm filters and 25 mm filters were extracted in 5 ml and 3 ml of solvent, respectively.

Table 6: Average filtration duration (h:mm) of triplicate samples for each filtration set-up tested during Line P cruise 2012-13.

		Filtration Method			
Station	Chl *a* (µg/L)	F1	F2	F3	F4
P4	5.3	0:14	2:29	1:35	aborted
P12	0.24	0:13	1:35	0:29	1:17
P26	0.49	0:10	1:49	0:33	1:17

Only *chl a* yields for each method are presented in Figure 4 as yields for the other pigments followed similar patterns at each station. Apart from the samples at P4 where extraction difficulties arising from high biomass affected all treatments (Fig. 4a), pigment yields from the 4 filtration methods were similar. At P4, despite incomplete extraction for all treatments as evidenced by green filters post-extraction, Method F1 produced the highest yield of *chl a* (Fig. 4a) and the lowest yields of *chlide a* and *Me-chlide a* likely because the short filtration time minimized pigment degradation, and the larger surface area to volume ratio for this filter size improved extraction efficiency. At P12 where biomass was lowest, Method F1 on the 47 mm filters produced the lowest *chl a* (Fig. 4b) and *chl b* yields (~10% lower than F3), but yields for the other pigments were very similar between the four treatments (data not shown). This result is thus likely attributable to the differences in yields observed between 25 mm and 47 mm filters for these late-eluting pigments (see Section 4.2.3) as opposed to variability arising from the filtration protocols themselves. At P26 where *chl a* yields were the most similar between treatments (<5%CV), the small differences in yields between filtration methods appeared to be strongly related to filtration duration, as the treatments with the highest yields (Methods F1 and F3) had the shortest filtration times (Fig. 4c). This pattern of higher yield with shorter filtration duration was even more pronounced in the yields of the accessory pigments (data not shown). Methods F2 and F3 may thus have suffered from minor pigment losses during filtration which took longer than 1 h for both treatments. Filtration times of less than 10 min are recommended for minimizing pigment degradation (Wasmund et al. 2006).

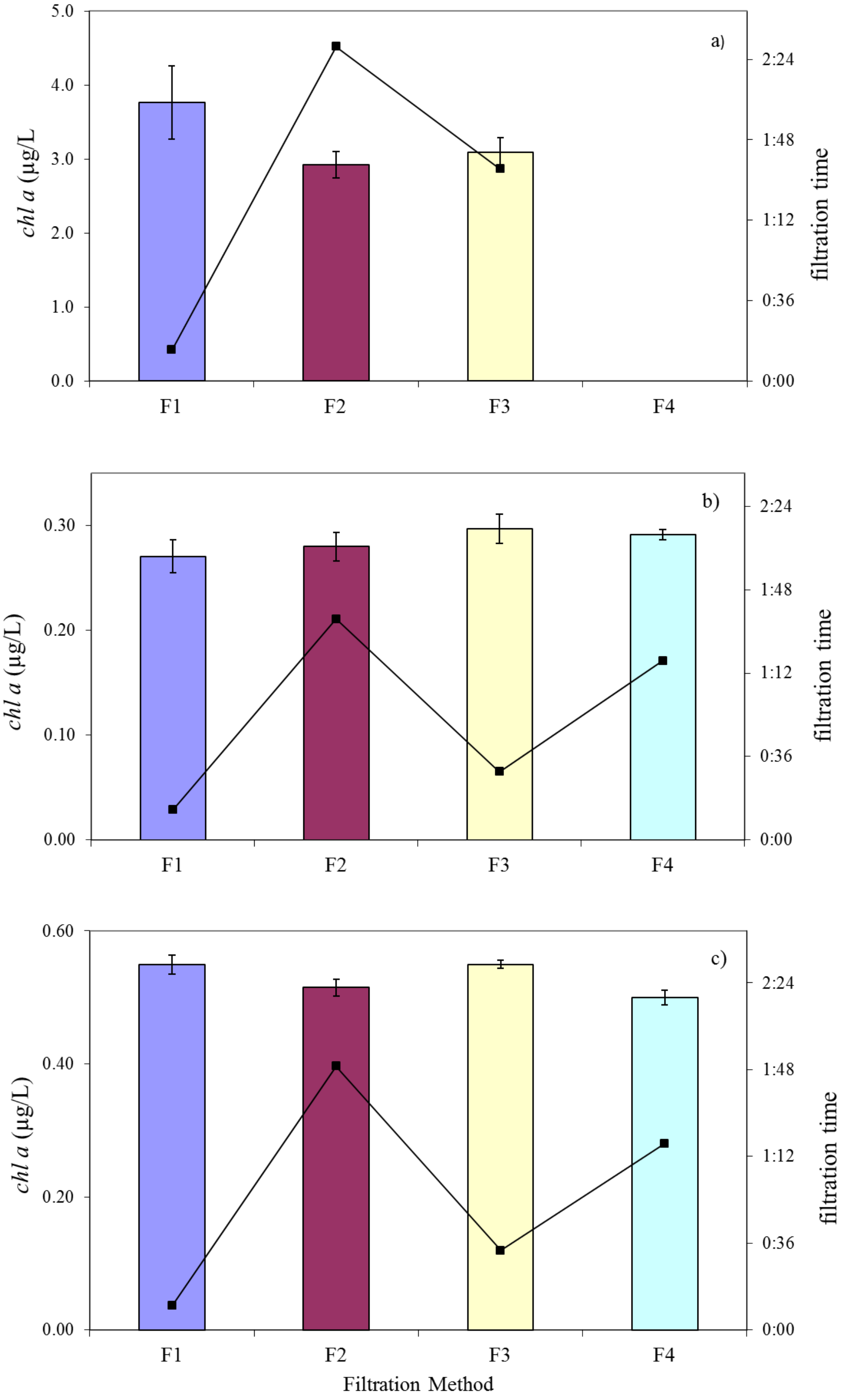

Figure 4: Mean *chl a* yields (histograms) and filtration time (black squares) from replicate samples collected with four filtration methods at stations: a) P4, b) P12, and c) P26. Error bars represent %CV of triplicate samples. Note the same filtration time scale for all three plots.

In summary, all 4 methods are quite comparable at the typical concentrations seen along Line P (<1 µg/L *chl a*) indicating that changes in filtration methodology over the years do not significantly influence the variability observed between samples for this time series. Filtration Method F1 which is currently in use seems to give the best results over the largest range of concentrations, offering fast filtration with high yields. However, as mentioned above, at high biomass, filtration volumes should be reduced to prevent extraction difficulties.

3.5 SAMPLE STORAGE

Following filtration, sample filters are folded in half, blotted, rolled into 5 ml cryovials and immediately placed in a -80 °C freezer or if unavailable, into liquid nitrogen. Once back in the lab, all samples are transferred to a -80 °C freezer pending analysis. Samples are typically analyzed within a few months and most samples have been analysed within 9 months of collection. However, some samples have been stored for several years either at -80 °C, in liquid nitrogen, or a combination of the two. No specific experiments have been conducted to investigate the degradation of pigments with time and a comparison of data from 3 cruises where the duplicate sets were analyzed approximately 1.5-2 years apart produces mixed results.

The first set of samples collected during a 2005 summer cruise in the Strait of Georgia (2005-14) was analyzed 8 months after collection, with replicates analyzed 2 years later. The mean difference between replicates across all samples showed a slight decrease in concentration for all pigments after the additional 2 years storage. Approximately 80% of the samples had higher *chl a* values for the earlier analyzed replicates with an average decrease of 0.067 µg/L (-4.7%) for the longer stored samples. A similar trend was evident for replicates collected in the North Pacific along Line P (2007-01), many of which were also analyzed 2 years apart following an initial 5 months of storage. In this case, most major pigments were also lower in concentration after the additional 2 year storage time for the majority of samples (90% of samples for *chl a*). For the 4 major pigments found along Line P (*chl a*, *fuco, hex-fuco,* and *but-fuco*) the replicates analyzed 2 years later were on average 10%, 7%, 7%, and 13% lower respectively, than their counterparts. Replicate sets from the same cruise that were analyzed at the same time had a mean difference between duplicates close to zero (ranging from ±0.002 µg/L for all pigments), as expected when there is no directional bias between duplicates. In contrast, for the replicate sets analyzed 2 years apart, mean differences between duplicates ranged from +0.008 µg/L for *zea* to -0.060 µg/L for *chl a*.

However, the results for both cruises are potentially confounded by changes in methodology which occurred in the interim between analyses of sets of replicates. These include differences in extraction protocols and soak times, injection volumes, and ways of calculating the final extraction volume (see Section 4). In particular, the inability to calculate an accurate final extraction volume (V_x) for the earlier analyzed replicates, as many of these samples were run prior to the introduction of the internal standard normalization, is perhaps the largest obstacle to accurate quantification of changes in pigment concentration with storage duration. Using either a fixed constant V_x of 3.22 ml (Bidigare et al. 2003), or the mean V_x from samples normalized to a TAC run gives slightly different pigment concentrations which are large enough to shift the direction of the small changes observed over time between the replicates.

The individual chromatograms from both sets of replicates were examined for the presence of *chl a* degradation products that could have formed over time with storage. Chlorophyll *a* allomers and epimers were only observed in the longer stored samples. The degradation product peaks in both cases were small (~3-5% of the *chl a* peak, on the order of the decline observed in *chl a* concentrations) and could have been indicative of pigment degradation during sample storage. However, *chl a* allomers and epimers are also known to form in the presence of alcohols (particularly methanol) and are commonly extraction artefacts (Jeffrey et al. 1997), thus their presence in the later analyzed samples may have resulted from longer methanol exposure stemming from a change in the extraction protocol. It should be noted though that these *chl a* transformation products were not observed in experiments comparing several extraction methods with variable soak durations so there is no evidence to suggest that the duration of exposure to methanol played a role in the observed differences (see Section 4.2.1). Alternatively, the *chl a* degradation products may have been present equally in both sets of replicates but only visible in the longer stored replicates due to the increased sensitivity from the larger injection volume used.

There is only one dataset (2010-16) where a significant time gap occurred between analysis of replicates, but where both sets were processed using the same extraction and analysis protocols. The first set of replicates from this coastal Strait of Georgia cruise was analyzed 2 months after collection while the second set was analyzed after an additional 16 months storage. In this case the added storage time did not appear to have any effect on the pigment concentrations. About half of the samples (7 out of 15) had higher *chl a* concentrations in the longer stored replicate. In addition, *chl a* allomers were present in both sets of replicates at levels of 1-25% of the *chl a* peak area and were unrelated to storage time. In fact, in 3 out of 4 samples examined in detail, allomer peak areas were higher in the earlier analyzed replicates compared to the later ones. This indicates that chlorophyll *a* allomers and epimers are likely not useful for assessing pigment degradation resulting from sample storage and are more likely present prior to sampling, or formed during sample collection (e.g. long filtration times), or extraction. The data from this cruise also suggest that there is little difference in quality between samples stored for 2 months and samples stored for 18 months at -80 °C. Other investigators have found that filter samples stored in liquid nitrogen or in low temperature freezers at -80 °C are stable for at least a year (Roy et al. 2011). Storage for longer than 2-3 years may, however, be a problem as seen above. Nonetheless, to put the magnitude of the observed differences in perspective, the mean *chl a* variability between replicates for the 2005-14, 2007-01, and 2010-16 datasets (6.7%, 6.9%, 5.1% CV, respectively) was comparable to that from other cruises where both sets of replicates were analyzed at the same time. This suggests that whatever possible pigment degradation occurred in the longer stored replicate it was relatively minor, and differences in pigment yields between replicates analyzed many months apart were within the range of natural sampling variability and precision of the current extraction and analytical methodology (see Section 7.4.3).

In June 2013, 21 replicates were collected at both P4 and P26 with the aim of conducting a long-term sample storage experiment to assess the time course of pigment degradation with storage time. All samples were stored at -80 ºC both at sea and once back in the lab. Triplicate samples from each station were analyzed immediately following the cruise (approximately 2 weeks after collection). A second set of triplicates was run 4 months after collection and the third set 3.5 months after that. The remaining replicates will be run at different intervals spanning the next several years with the aim of quantifying the changes in pigment concentrations with long term storage.

4.0 SAMPLE EXTRACTION

4.1 EXTRACTION PROTOCOLS

Sample extraction procedures have evolved over the years in conjunction with changing filtration techniques, and based on the results of several extraction efficiency experiments. In the current protocol, whole, folded GF/F filters are extracted by placing them in glass test tubes (Pyrex Vista rimless culture tubes 16 mm x 100 mm; VWR Part#89090-310) pre-filled with extraction solvent consisting of 95% methanol with 17 µM TAC used as an internal standard. The TAC primary standard is a 7 µg/ml solution of which 1 ml is added to each 100 ml of 95% methanol to make a batch of extraction solvent. Batches of extraction solvent are prepared on roughly a weekly basis to fill enough extraction tubes for a week's run. At the same time, individual HPLC vials are filled with the same extraction solvent for each daily sample set and stored at -20 °C until analysis. These are used as internal standard references for normalizing TAC peak areas in the samples for accurate extraction volume determinations. Extraction procedures are performed under green light in the fume hood and the filters are added directly to pre-dispensed cold solvent (-20 °C) to limit pigment exposure to both heat and light. Test tubes are quickly capped (VWR Safe-T-Flex caps; Part# 60828-704), vortexed to aid in opening the filter, and left to extract by soaking for 24 h at -20 °C (Method A; Table 7). The volume of methanol extraction solvent is typically 5 ml for 47 mm filters (3 ml for 25mm filters) but is sometimes increased or decreased based on expected biomass. Larger volumes (e.g. 10 ml) seem to improve extractability of high biomass samples as seen from the colour of the filters following extraction (see Section 4.2.4), whereas a reduced volume of 4 ml (requiring the filter to be cut in half for submersion) is sometimes used to improve detection when biomass is expected to be particularly low. After the 24 h soak period, the sample extracts are vortexed again to ensure homogeneity and are filtered through 0.2 µm 13 mm PTFE luer-lock syringe filters (Canadian Life Sciences Part #SF6500-06) directly into amber glass HPLC vials for immediate analysis.

Table 7: Methods employed for sample extraction at IOS. Method A produced consistently better results and was adopted as the standard in late 2007.

Extraction Method	Filter (whole/diced)	Filter Treatment	Soak Time
A	whole	soak only	24 h
B	diced	soak only	24 h
C	diced	sonication 60 sec.	2-4 h
D	diced	manual grinding	2-4 h

In the past, samples were extracted by mechanical disruption of the filter followed by a short soak period (2-4 h) following Zapata et al. (2000). This protocol (Method D; Table 7) involved placing the diced filter into a glass test tube with 3 ml of pre-chilled 95% methanol and manually grinding it using a metal spatula. The grinding method was later modified to sonication (Method C; Table 7), where the test tube with the diced filter in solvent was placed in a darkened beaker containing ice slurry and the sample was sonicated for 60 seconds by inserting an ultrasonic probe (Omni-Ruptor 4000 Ultrasonic Homogenizer) directly into the tube. Tubes were then placed in the freezer at -20 °C to extract for 2-4 h, before being filtered as above and analyzed. Neither Methods C nor D employed an internal standard for accurate determination of final extraction volume. These 2 mechanical methods were initially chosen as many sources recommend sonication, grinding, or other forms of cell disruption to aid in pigment extraction (Bidigare et al. 2003; Hooker et al. 2005). However, because this process is time-consuming (limiting the number of samples that can be analyzed per day) and the extra manipulation of the filter has the potential to lead to pigment degradation or losses, extraction methods that do not mechanical disrupt the filter were investigated. The results of the various extraction experiments outlined below led to the adoption of Method A (Table 7).

4.2 EXTRACTION EFFICIENCY EXPERIMENTS

4.2.1 Comparison of Extraction Methods on 25 mm filters

In order to identify the extraction method that best optimizes yields while minimizing variability between replicates, and to potentially avoid the laborious extraction process of sonication, the first extraction efficiency experiment was conducted in October 2007. Seawater was collected at the surface from the IOS dock in Patricia Bay (48 39.11ºN, 123 26.59 ºW) and pooled into a carboy before being dispensed into replicates. Due to the unexpectedly high chlorophyll biomass (~80 µg/L), small volumes of 100-150 ml were filtered using 25 mm GF/F filters. All but 2 replicates were immediately frozen in liquid nitrogen and stored for approximately 1 week prior to analysis. The 2 fresh samples were extracted and analyzed the day of collection. All replicates were extracted in 3 ml of solvent and analysis was performed on 100 µl injections, but 11 different treatments were tested in duplicate (Table 8).

Table 8: Treatments used for the extraction efficiency experiment in Oct. 2007

Treatment	Storage	Soak Time (h)	Filter	Method	Solvent
A	No	1.7	whole	vortex only	95% methanol
B	Yes	2	diced	sonication	95% methanol
C	Yes	4	diced	sonication	95% methanol
D	Yes	4	whole	vortex only	95% methanol
E	Yes	4	diced	vortex only	95% methanol
F	Yes	4	whole	vortex only	90% acetone
G	Yes	4	diced	sonication	90% acetone
H	Yes	6	diced	sonication	95% methanol
I	Yes	6	whole	vortex only	95% methanol
J	Yes	24	whole	vortex only	95% methanol
K	Yes	24	whole	vortex only	90% acetone

The treatments varied by their method of extraction, duration of soak time, and type of solvent used. Treatments B and C most closely resembled the standard extraction procedure in place at the time of the experiment (Method C; Table 7), whereas treatment J is the current extraction protocol (Method A; Table 7). Most treatments employed 95% methanol as the extraction solvent following the method of Zapata et al. (2000). Methanol produces sharper, taller and better resolved peaks for the early-eluting compounds (Zapata and Garrido, 1991), it is inexpensive, and it has better extraction capabilities compared to acetone for some algal groups (Jeffrey et al. 1997). However, *chl a*, *b* and *c* are known to be less stable in methanol over time with the potential of allomerization (Jeffrey et al. 1997; Hooker et al. 2005). This can be a factor during overnight soak periods and during longer sample runs where extracts can sit in the autosampler for up to 24 h (see Section 7.4.2). The remaining treatments employed 90% acetone as the extraction solvent as this is used by several pigment analysis labs (Hooker et al. 2005), and was recommended in the JGOFS Core Measurement protocols (UNESCO, 1994). Pigment yields and the variability between duplicates for each treatment are presented in Tables 9 and 10.

Table 9: Pigment yields by treatment (in µg/L) for the 15 pigments measured. Variability in yield across treatments is expressed as %CV (stdev/mean) and excludes treatment A. The highest yield for each pigment is highlighted in bold. Acetone treatments are highlighted in blue.

Pigment	A	B	C	D	E	F	G	H	I	J	K	%CV
chl c3	6.51	7.32	7.47	7.18	7.55	6.44	7.01	7.27	7.15	**7.73**	7.44	**4.9%**
chl c2	12.6	14.6	14.4	14.2	14.9	13.9	15.1	14.9	14.6	**15.5**	**15.5**	**3.6%**
peri	3.94	4.37	4.82	4.65	4.75	4.40	4.45	**4.89**	4.70	4.54	4.59	**3.9%**
but-fuco	14.3	16.3	16.3	16.0	16.8	15.8	16.3	16.9	16.7	**17.2**	17.0	**2.8%**
fuco	32.8	37.5	37.3	36.8	38.6	36.2	37.8	39.0	38.4	**39.5**	39.3	**2.9%**
pras	0.29	0.40	0.36	0.39	0.42	0.39	0.33	0.42	0.39	**0.43**	0.36	**8.1%**
viola	0.20	0.23	**0.27**	**0.27**	0.25	0.25	0.26	0.26	0.25	0.26	0.25	**4.2%**
hex-fuco	0.43	0.48	0.46	0.47	0.47	0.43	0.45	0.43	0.45	**0.51**	0.48	**5.1%**
diadino	5.06	5.57	5.48	5.46	5.74	5.56	5.82	5.85	5.87	**6.11**	6.05	**4.0%**
allo	0.42	0.54	0.56	0.56	0.54	0.55	0.54	**0.57**	0.51	0.55	**0.57**	**3.4%**
diato	**0.69**	0.63	0.60	0.60	0.62	0.60	0.66	0.66	0.64	0.68	0.64	**4.5%**
lut	0.58	0.58	0.59	0.65	0.70	0.67	0.66	0.74	0.66	0.70	**0.75**	**8.0%**
chl b	0.26	0.46	0.47	0.45	0.50	0.51	0.48	0.52	0.44	**0.53**	0.52	**6.8%**
chl a	47.4	81.9	80.6	79.1	84.1	81.7	86.8	86.5	82.3	**90.4**	89.8	**4.6%**
β-car	0.69	1.57	1.49	1.42	1.65	1.50	**2.24**	1.79	1.46	1.55	2.18	**17.6%**

Table 10: Variability between duplicates for each treatment expressed as %CV (stdev/mean). Values greater than 5% are highlighted in bold. Acetone treatments are highlighted in blue.

Pigment	A	B	C	D	E	F	G	H	I	J	K
chl c3	4.9%	4.5%	**7.5%**	2.3%	0.3%	2.0%	1.3%	1.9%	3.5%	0.5%	2.2%
chl c2	4.4%	4.6%	**9.7%**	1.9%	1.2%	2.9%	2.4%	0.8%	3.2%	1.8%	1.0%
peri	0.9%	4.8%	**9.4%**	**5.3%**	4.4%	2.6%	**7.8%**	**6.7%**	4.2%	3.1%	1.6%
but-fuco	3.7%	4.1%	**7.5%**	2.6%	1.9%	2.8%	1.2%	0.9%	4.3%	1.3%	0.2%
fuco	4.1%	**5.0%**	**7.0%**	1.5%	0.7%	2.3%	4.7%	2.3%	3.3%	0.2%	1.4%
pras	1.7%	1.8%	**18.3%**	**12.1%**	**6.8%**	4.8%	**8.9%**	4.4%	2.8%	**9.0%**	0.2%
viola	3.3%	4.9%	**9.6%**	**6.7%**	**5.5%**	**6.2%**	0.7%	2.1%	**9.4%**	4.4%	3.4%
hex-fuco	**9.4%**	2.6%	**6.9%**	2.8%	4.9%	**5.0%**	4.1%	3.4%	**18.5%**	0.8%	0.9%
diadino	**7.0%**	**10.1%**	4.6%	2.4%	1.4%	2.1%	1.6%	1.8%	**12.0%**	3.6%	**5.8%**
allo	**8.5%**	**5.6%**	**11.4%**	4.4%	3.5%	2.6%	4.4%	0.6%	**5.3%**	1.3%	0.4%
diato	**7.1%**	**7.4%**	**29.0%**	0.8%	**7.7%**	0.1%	0.8%	**6.4%**	**12.4%**	**5.7%**	4.8%
lut	4.9%	4.5%	**7.5%**	2.3%	0.3%	2.0%	1.3%	1.9%	3.5%	0.5%	2.2%
chl b	4.4%	4.6%	**9.7%**	1.9%	1.2%	2.9%	2.4%	0.8%	3.2%	1.8%	1.0%
chl a	0.9%	4.8%	**9.4%**	**5.3%**	4.4%	2.6%	**7.8%**	**6.7%**	4.2%	3.1%	1.6%
β-car	3.7%	4.1%	**7.5%**	2.6%	1.9%	2.8%	1.2%	0.9%	4.3%	1.3%	0.2%
Average	5.0%	5.0%	**11.0%**	3.9%	3.5%	3.0%	3.4%	2.8%	**7.2%**	2.9%	2.0%

In general, pigment yields increased, and variability between duplicates decreased with longer soak times regardless of the method used (Table 9, 10). Treatment A yielded the lowest concentrations of most pigments (Table 9, Fig. 5) likely because it consisted of a very short soak time, no mechanical disruption of the filter, and a lack of low-temperature freezing, which is known to rupture cell walls and aid in pigment extraction (Bidigare et al. 2003). This treatment was employed more out of curiosity and not as a viable method alternative since field samples will always require some frozen storage at sea and in the lab while awaiting analysis. Due to the significantly lower yields from this treatment compared to the others, it was excluded from the coefficient of variation (%CV) calculation in Table 9. With the exception of *β-car*, the yields of the other pigments were relatively insensitive to the methodological differences between treatments (Table 9). Very little difference in yield was observed for most pigments between treatments employing a 2 h vs. 24 h extraction, and methanol vs. acetone as the solvent. The variability in yield across all treatments was no more than 8%, and typically less than 5% for all pigments except for *β-car* (Table 9).

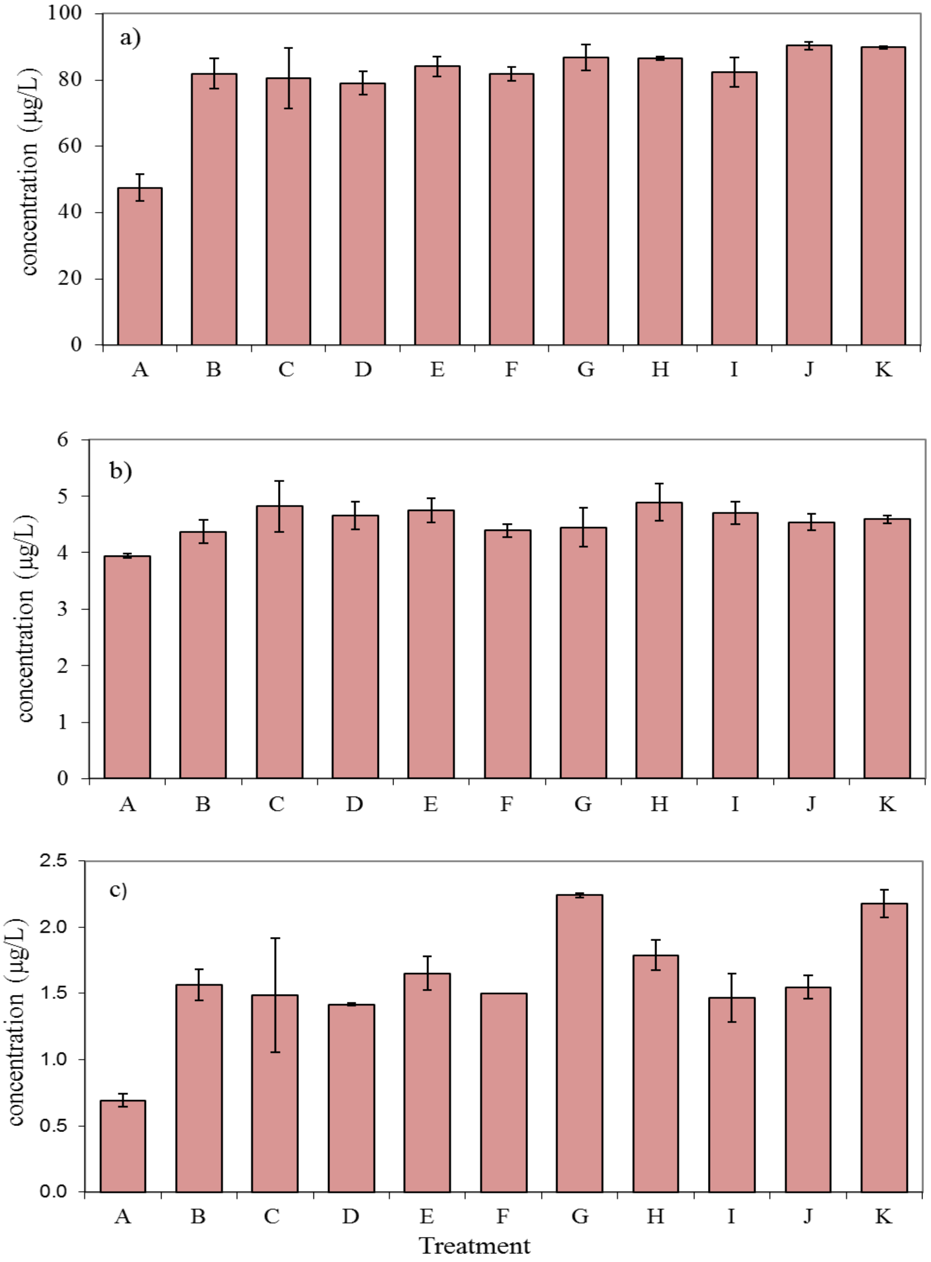

Figure 5: Pigment yields by treatment for a) *chl a*, b) *peri*, and c) *β-car*. Error bars represent %CV of duplicate samples.

Concentrations of *chl a* increased in conjunction with soak duration, while at the same time variability between replicates decreased (Fig. 5a). Compared to the other pigments, *peri* seemed to benefit most from sonication as the highest yields were in treatments C and H (Fig. 5b); however these treatments were also associated with the highest variability between duplicates. Yields for *β-car* (Fig. 5c) were considerably higher in acetone compared to methanol treatments for the 24 h soak period (treatment J vs. K), but only marginally higher for the 4 h soak period (treatment D vs. F). They also increased significantly with sonication compared to simple soaking when in 90% acetone for the same 4 h period (treatment F vs. G). This implied that *β-car* may be one of the more difficult pigments to extract fully. In contrast, *but-fuco* and *fuco* may be among the easiest pigments to extract as they were the least sensitive to differences in treatment with the smallest variations in yield (Table 9). Although acetone seemed to be a better extraction solvent for some pigments (e.g. *β-car* and *lut*), due to its associated peak shape distortions of the early-eluting pigments it was not considered as a replacement for 95% methanol (Fig. 6). Furthermore, the chromatograms from the 24 h extractions in methanol were examined in detail and there were no chlorophyll degradation peaks present, contrary to the notion that methanol promotes allomerization/epimerization (Jeffrey et al. 1997).

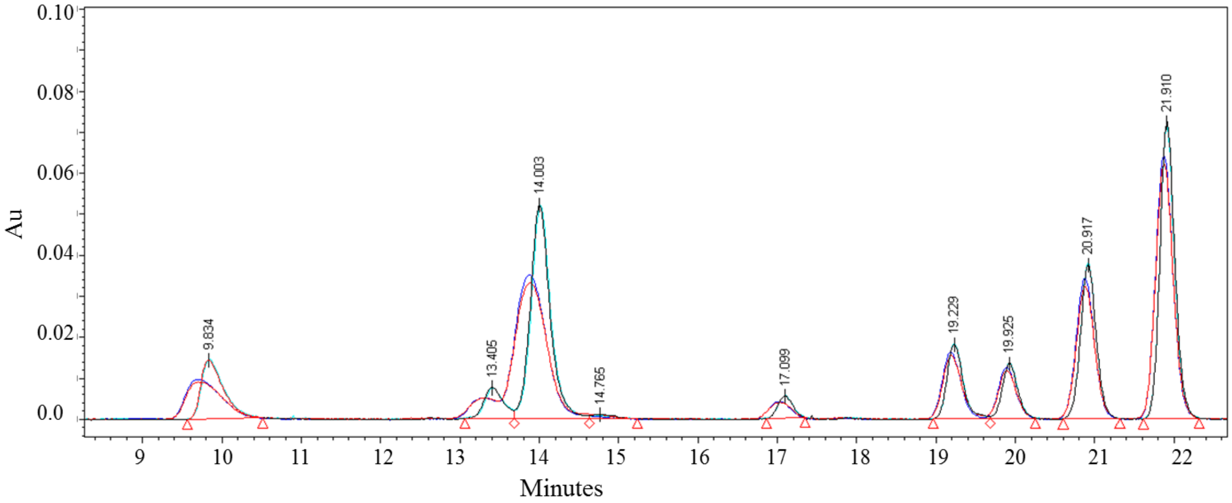

Figure 6: Overlay of partial chromatograms showing the early-eluting peaks. Samples extracted in 95% methanol (black and turquoise lines) produce sharper, more-symmetrical peaks than those extracted in 90% acetone (red and blue lines).

It is clear that no single extraction method will prove optimal for every pigment under all conditions. Nonetheless, treatment J produced the highest yields in 9 out of the 15 pigments quantified (Table 9), along with the third lowest variability between replicates (Table 10), and favourable chromatographic performance (Fig. 6). This was an encouraging finding as it indicated that pigment yields could be maintained, and even potentially improved over the extraction method in use at the time (treatments B and C; Table 9) with a simpler method that did not require mechanical disruption of filters. Moreover, by eliminating additional sample handling that could lead to pigment losses, this method produced tighter replicates. The 24 h soak extraction was also less labour-intensive, allowing an increase in the number of samples that could be processed in a given day (from 12 to 25). Thus, following the results of the above experiment, treatment J was selected as the standard extraction protocol in late 2007 and is subsequently referred to as Method A (Table 7).

4.2.2 Comparison of extraction methods on replicate field samples

To compare the pigment yields from the two extraction protocols used most extensively at IOS (Methods A and C; Table 7) on more typical concentrations than those used in the experiment above, replicate samples from 2 cruises (2004-21 in the Strait of Georgia, *chl a* range 0.54-6.7 µg/L, and 2007-15 along Line P, *chl a* range 0.16-1.0 µg/L) were extracted by each of these two methods. Both set of replicates were analyzed under the same HPLC conditions and within a week of each other and differed only by their extraction method. On average, Method A produced higher yields for all pigments, consistent with the findings from the above experiment (Fig. 7). For cruise 2004-21, 79% of samples (22 out of 28) had higher yields of *chl a* in the method A extraction with an average increase in *chl a* concentration of 6% and an average increase in *fuco* concentrations of 10%. For cruise 2007-15 all samples had higher yields of *chl a* in the method A extraction with an average increase of 18% and an average increase in *fuco* concentrations of 9.5%.

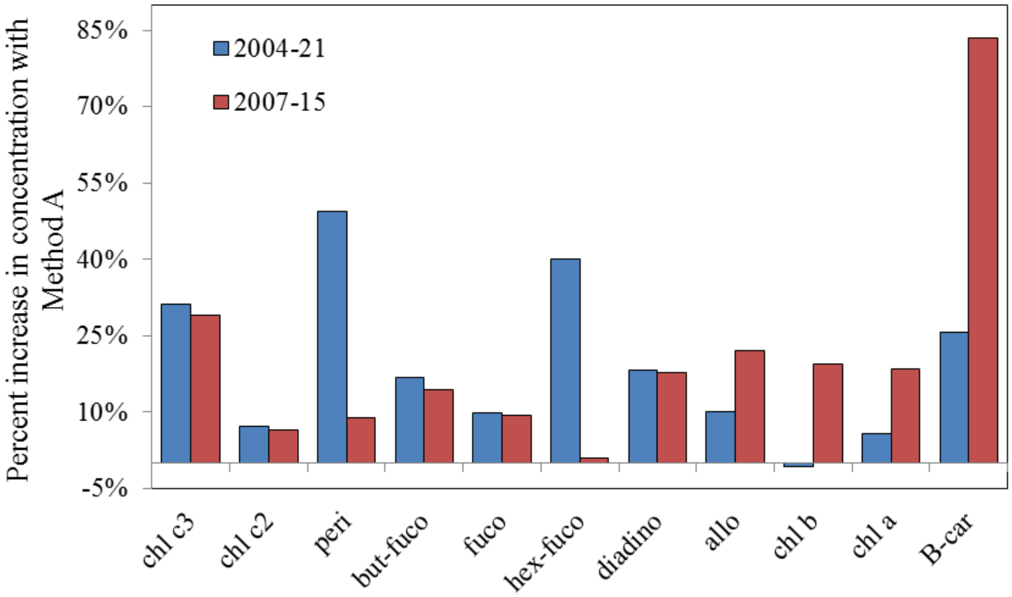

Figure 7: Average percent increase across all samples for replicates extracted with Method A compared to Method C for a coastal (2004-21) and offshore (2007-15) cruise.

A follow-up comparison of the two extraction methods was conducted in August 2012 with samples collected at Stations P16 and P26 during Line P cruise 2012-13. During this cruise, 6 replicates (1250 ml volume, 25 mm filters) were collected from the euphotic zone at each station with triplicates extracted with each of Method A and C back in the lab. All samples were analyzed on the same day under the same conditions. For the majority of pigments, Method A produced marginally higher yields than Method C but the differences were well within the methodological precision of replicate filters (Fig. 8). In contrast, *chl a* yields were significantly higher (~20%) with Method A compared to Method C similar to the results from Line P cruise 2007-15 above. These results agree with the findings of the initial extraction experiment where a 24 h soak extraction (treatment J; Table 9) yielded higher *chl a* concentrations than a shorter soak with sonication (treatment C; Table 9). This indicates there may have been a slight underestimation in *chl a* levels in field samples extracted prior to 2008 using Method C.

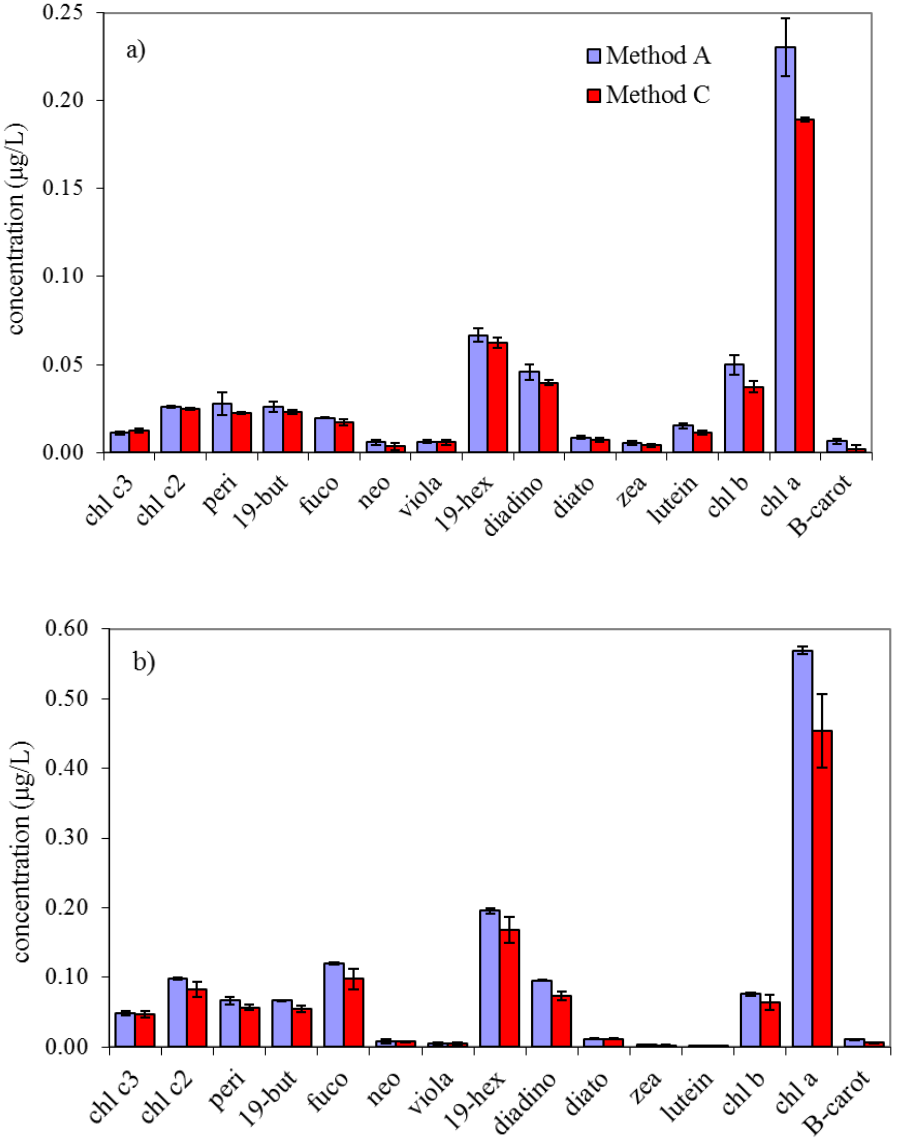

Figure 8: Mean pigment yields obtained from replicate samples extracted with Method A (blue histograms) and Method C (red histograms) for (a) station P16 and (b) station P26. Error bars represent the standard deviation of triplicate samples.

4.2.3 Comparison of yields from 25 mm vs 47 mm GF/F filters

A number of experiments were carried out to ensure pigment yields were comparable between the 25 mm vs. 47 mm GF/F filters when the switch was made to the larger size to accommodate increased filtration volumes. In the first experiment, water was collected into a carboy from the dock in Pat Bay in early September 2008 (*chl a* ~ 3.5 µg/L). Duplicate samples were collected onto both 25 mm and 47 mm filters, using the two filtration volumes typically used for these filter sizes in offshore waters: 1250 ml and 1975 ml, respectively. Both sample volumes filtered onto 47 mm filters took on average 9 min to filter, whereas with the 25 mm filters it took 20 min and 50 min to filter the 1250 ml and 1975 ml volumes, respectively. For analysis, all samples were extracted by soaking filters for 24 h in 3 ml 95% methanol in 4 ml amber glass vials. The 25 mm filters were soaked intact (Method A; Table 7), while the 47 mm were diced up to allow complete submersion of the filter in the small volume of solvent used (Method B; Table 7).

The lowest yields for most pigments were generally from the 1975 ml samples on 25 mm filters likely because this was too much volume for this filter size at this biomass level. This was not a concern as this high a volume was rarely used for sample collection with 25 mm filters. An encouraging finding was the *chl a* yield from the 47 mm filters was very close to the value from the 25 mm filters when using the typical volume for each filter size for offshore samples (1975 ml and 1250 ml, respectively; Fig. 9a). Except for *β-car*, yields for most of the other pigments were comparable, but typically slightly higher from the 47 mm filter compared to the 25 mm, especially for the 1975 ml standard volume (Fig. 9). Chlorophyll *c3* showed high variability when only 1250 ml was filtered likely because of detection issues and had almost a doubling of yield in the 1975 ml, 47 mm replicates. *β*-carotene showed the opposite trend with yields from the 47 mm filters nowhere near what they were from the 25 mm regardless of volume filtered (Fig. 9b). Only one Line P cruise was collected on 47 mm filters and extracted with Method B (Table 7) before dicing of the 47 mm filters was abandoned and these results suggest that pigment yields for that cruise would be comparable to earlier cruises where the 25 mm filters were used, except perhaps for *β-car*.

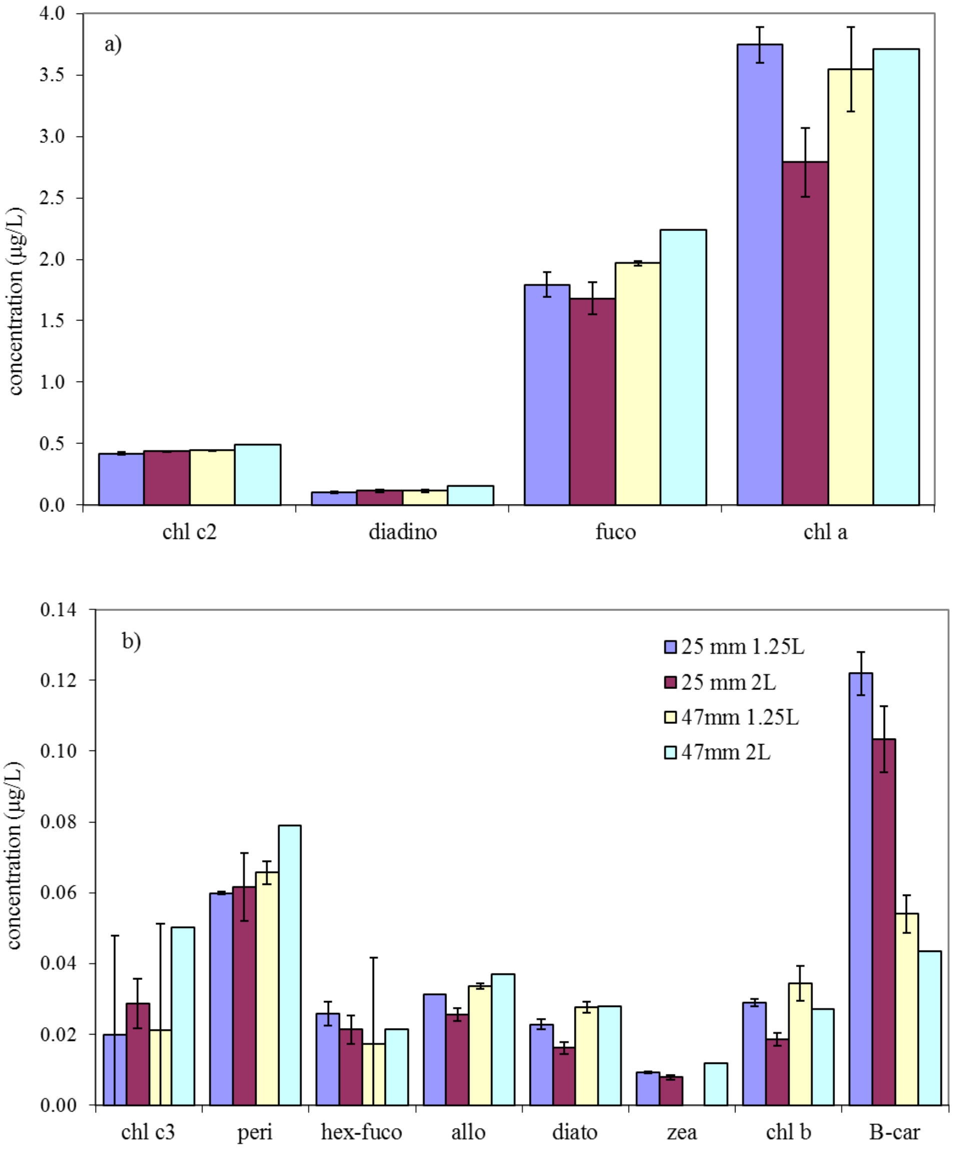

Figure 9: Pigment yields by treatment for a) the 4 most abundant pigments, and b) the minor pigments. Error bars represent standard deviations of duplicate samples. Only a single replicate was available for the 47 mm 2 L filtration.

The experiment was repeated with samples collected in November 2008 in the Strait of Georgia (*chl a* ~ 0.5 µg/L). These samples were filtered in triplicate onto both sizes of filter using only 1250 ml volumes. All filters were extracted with a 24 h soak in 3 ml 95% methanol with the 25 mm filters soaked whole (Method A) and the 47 mm filters diced (Method B). At this lower biomass (*chl a* <1 µg/L), yields for most pigments were also comparable between the 2 filter sizes (Fig. 10). As in the previous experiment this was not the case for *β-car* which was not even detectable on the 47 mm filter (Fig. 10a). However, in contrast to the previous experiment, concentrations of *chl a* and *chl b*, like *β-car*, were also lower on the 47 mm filters compared to the 25 mm ones. Interestingly, these 3 pigments have the longest retention times indicating that this extraction difficulty may be related to the polarity of these compounds, the higher volume of water retained by the 47 mm filters, and the corresponding reduction in extraction solvent strength.

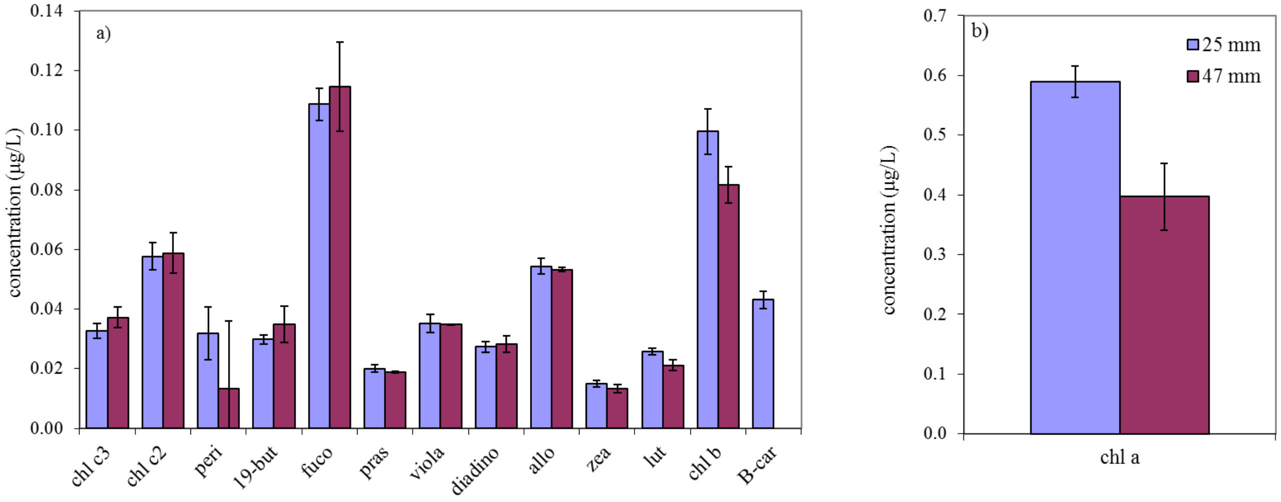

Figure 10: Comparison of pigment yields from low biomass, 1250 ml samples collected on 25mm and 47mm filters for a) accessory pigments, and b) *chl a*. Error bars denote standard deviations of triplicate filters.

Although these two experiments showed that significant differences in yields between the 2 filter sizes were only found in a couple of pigments, further experiments were carried out because of the few shortcomings of these earlier experiments. In the first experiment, the phytoplankton biomass was higher than that typical of Line P where the larger filtration volumes are used. In the second experiment, the filtration volume used for the 47 mm filter was lower than optimal for this biomass level (see Section 3.2). In both experiments, the vessels used for extractions, which had been effective for the 25 mm filters, were too small to adequately fit the 47 mm filters making it necessary to dice the filters to ensure submersion in 3 ml of solvent (Method B; Table 7). Thus, the comparison of yields from the two filter sizes may have been influenced by differences in yield between extraction methods A and B. In subsequent experiments, larger glass extraction tubes (Pyrex Vista rimless culture tubes 16 mm x 100 mm; VWR Part#89090-310) with plastic caps (VWR Safe-T-Flex; Part# 60828-704) were used, that could hold the filters upright fully submerged in 5 ml of solvent and with sufficient vessel width to allow them to vortex easily and open fully.

Four coastal and one oceanic field samples ranging in biomass from 0.2-2 µg/L *chl a* were used to compare pigment yields between the 25 mm and 47 mm filters in 2 separate experiments carried out in November and December 2009. Since the larger filters were adopted to accommodate a 2 L filtration volume, this volume was used for all 47 mm treatments and different extraction methods were employed in an attempt to increase the *chl a* yield from these filters relative to the 25 mm. The control for both experiments consisted of duplicate 25 mm filters with 625 ml filtered for the coastal samples and 2 L for the oceanic one, extracted by standard Method A. The 625 ml coastal control samples took approximately the same time to filter as the 2 L volume onto 47 mm filters. Three different treatments were then used on duplicate 2 L 47 mm test samples with slight variations in extraction treatments between the November and December runs. Treatment A for both experiments was extracted by Method A as for the 25 mm control except with 5 ml of solvent to compensate for the larger filter size. In the November experiment, the filters were diced for the other two treatments with treatment B consisting of a 24 h soak (Method B; Table 7) and treatment C employing cell disruption with either an ultrasonic bath (with 6 h soak) or sonicator probe with 2 h soak (Method C; Table 7). In the December experiment all filters were extracted whole; the control and treatment A were the same as above, while treatment B consisted of a 5 min ultrasonic bath immersion prior to the 24 h soak, and treatment C used the sonicator probe with only a 4 h soak period.

For the first time, although still slightly lower, *β-car* yields from the 47 mm filters were closer to those from the control 25 mm filters for all 5 samples from both experiments (Figs. 11a, 12a). Yields from treatment A were comparable but generally slightly higher for most pigments compared to the 25 mm control (Figs. 11, 12). The November experiment clearly showed that dicing the filters leads to both pigment losses and increased variability as treatments B and C had significantly lower yields of most pigments than treatment A in all 3 samples. Chlorophyll *a* in particular was significantly lower in the diced treatments with a concomitant increase in *chlide a* concentrations, and the largest variability between replicates indicating destruction of this pigment during filter handling (Fig. 11). This result could explain the *chl a* differences observed between the two filter sizes, extracted using Methods A and B above (Fig. 10.)

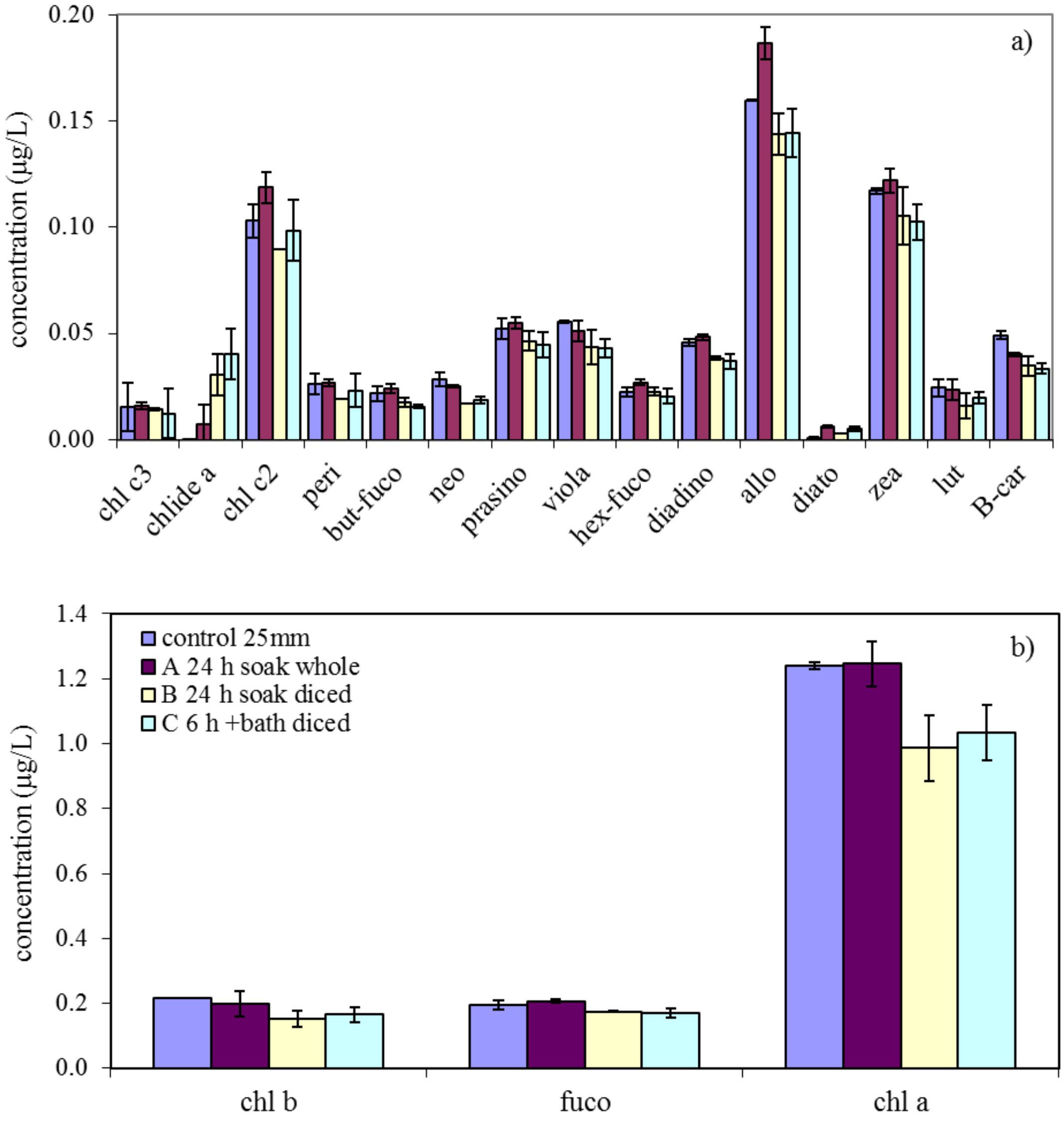

Figure 11: Results from 1 of 3 samples in the Nov 2009 experiment showing the pigment yields by treatment for a sample from the Strait of Georgia for a) the minor pigments and b) the 3 most abundant pigments. Error bars represent standard deviations of duplicate samples.

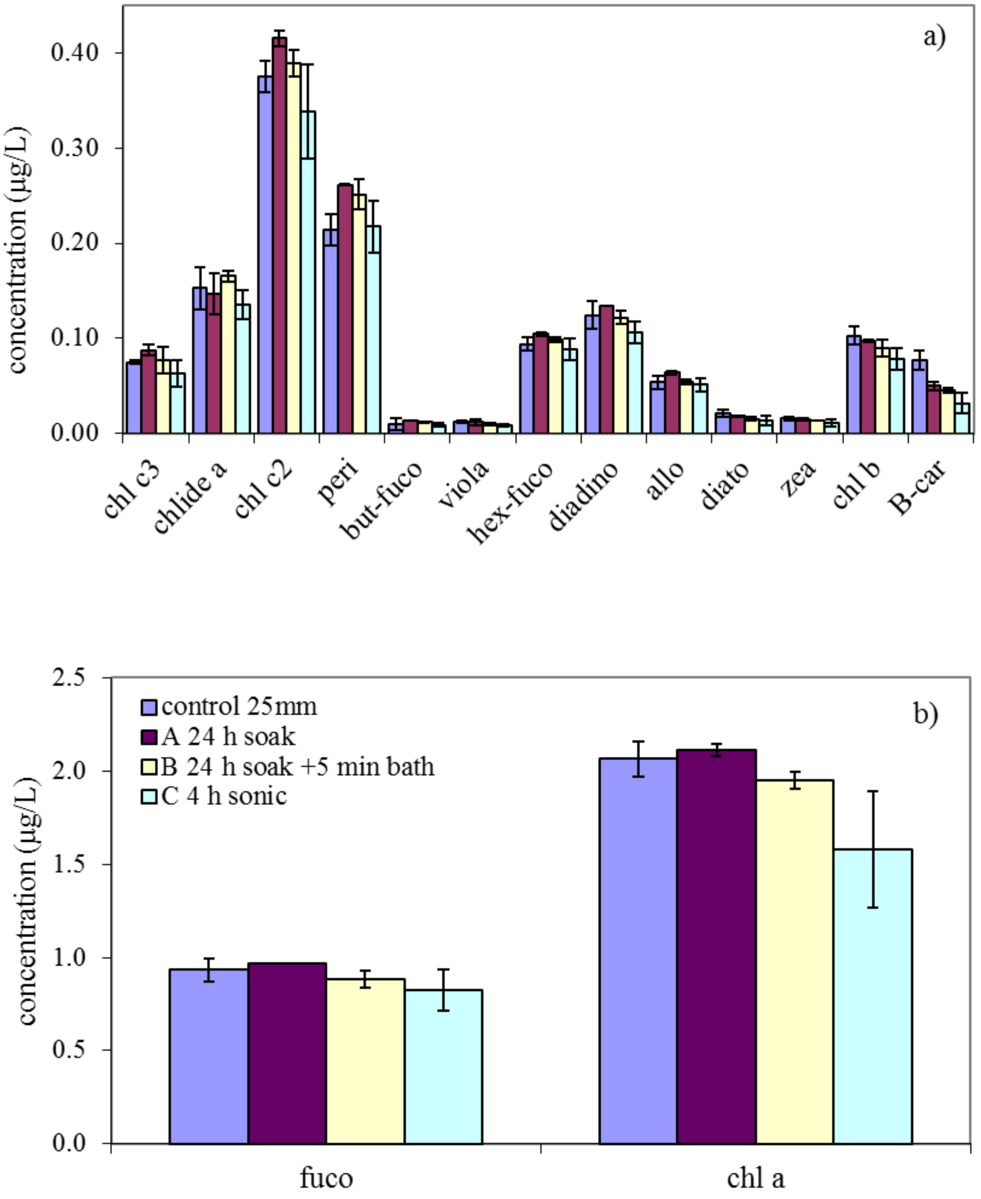

Figure 12: Results from 1 of 2 samples in the Dec 2009 experiment showing the pigment yields by treatment for a sample from Saanich Inlet for a) the minor pigments and b) *fuco* and *chl a*. Error bars denote standard deviations of duplicate samples.

The two samples analyzed in December whose replicates were all extracted whole had more comparable concentrations for the 4 treatments but yields were still highest in the standard method A extractions (Fig. 12). Across both experiments sonication (treatments B and C) only increased pigment yields in 1 out of the 5 samples tested and generally did not offer an improvement over the standard 24 h soak extraction. The one sample in which it did produce higher yields had an unusual pigment profile with a relatively high *chl b* concentration and a population likely dominated by green algae atypical of the communities usually sampled (data not shown). It must be noted that while these experiments indicate that results comparable to those seen on the 25 mm filters with a 625 ml volume (Method F3; Table 5) are obtainable with the 47 mm filters and a 2 L filtration volume (Method F1; Table 5), the experiments only spanned a *chl a* range of 0.2-2.5 µg/L; it was not yet clear how Method F3 compared to F1 at higher biomass. Thus, while filtering a 2 L volume on 47 mm filters produced good results in low biomass waters (e.g. *chl a* biomass of ~2.5 µg/L), the extraction method needed to be adapted to improve the extractability of samples where more than the recommended volumes had been filtered based on estimated biomass (see Table 3, Section 3.2).

4.2.4 Optimization of extraction method at high biomass

Although filtration volumes are now determined by *in situ chl a* fluorescence, occasionally too much sample is inadvertently filtered either because strong vertical gradients in phytoplankton biomass create an offset between the sensor reading and water collected in the bottle above, because of variations in the relationship between *in situ* fluorescence and extracted *chl a* (Fig. 3), or simply as a result of sampler error. To determine an extraction method that improves the extractability of these dense samples, further tests with the 47 mm filters were carried out on high biomass samples. Three 25 L high biomass samples were collected in early spring from the underway sampling loop on the *CCGS John P. Tully* in Saanich Inlet during cruise 2012-14. From each sample, two different volumes (2 L, 1 L or 625 ml) filtered on 47 mm filters were compared to controls collected with Method F3 (25 mm filter, 625 ml volume), the previous standard protocol for coastal water sampling. The goal of the experiment was to test modified extraction procedures that could improve yields from the 47 mm filters when too much material had been collected.

Since prior experiments have shown that it is not necessary to mechanically disrupt the filters (dicing, grinding and/or sonication) during extraction, the aim was to leave the filters whole and handle them as little as possible, but to see how the soak method could be modified to improve yields. Two modifications to the method were tried. In the first, the filters were placed in the solvent tubes for extraction and the entire rack was sonicated in an ice bath for 5 min prior to the soak period (4.5 h or 24 h). In the second modification termed the "double soak", filters were sonicated as above and left to extract for 5 h, after which time the extract was decanted off into a separate tube and a second 5 ml aliquot of extraction solvent was added to each filter. The tubes were then stored at -20 ºC for a further 19 h to complete the 24 h extraction cycle and the two aliquots were merged prior to analysis. The controls and one set of each of the two test volumes were extracted with the standard Method A with no further manipulation of the sample.

The first striking observation consistent across all 3 samples was that the pigment concentrations from the standard Method A extraction of the 2 L samples were comparable to those from the 25 mm control. Chlorophyll *a* biomass in the three samples was 10, 12 and 16 µg/L as determined by fluorometry suggesting that at least up to this biomass range there is little difference in yields when using the standard filtration volumes on the two filter sizes with extraction Method A. This was an encouraging result as it greatly extended the range of comparability between the two filter sizes beyond those tested in the previous section and validated comparisons of datasets collected with these different methods. There were however differences in the yields of some pigments when comparing the reduced filtration volumes and the modified extraction methods to the standard ones. Most of the yield differences in the past between the 25 mm and 47 mm filters have affected just 3 pigments: *β-car, chl b* and *chl a* and the same was true for this experiment.

In the 12 µg/L *chl a* test sample, only the modification adding the 5 min sonication was used for both volumes (1 L and 2 L) and for both soak periods (4.5 h and 24 h). In this case it was clear that the biggest increase in yields over Method A for *chl a*, *chl b,* and *β-car* occurred with filtering less material regardless of the extraction method or soak time used (Fig. 13). *Chl a* yields were 21-30% lower and variability between replicates higher in the 2 L samples when compared to their 1 L counterparts for each extraction treatment tested. It seems a large fraction of this *chl a* loss can be attributed to degradation to *chlide a* and *Me-chlide a* (produced from the former in the presence of methanol during extraction) as the concentrations of total *chl a* (defined as *chl a* + *chlide a* + *Me-chlide a*) were much more comparable between treatments (Fig. 13a). Concentrations of *chlide a* were approximately double in the control and the standard 2 L samples (26% and 30% of the total *chl a*, respectively) compared to the 1 L samples extracted for the same duration (15% of the total *chl a*). Concentrations of these degradation products were reduced by adding sonication, and with shorter soak times (Fig. 13b). It makes sense that a shorter soak time would limit the breakdown of *chl a* but it is unclear why adding sonication prior to the 24 h soak period also helps preserve the original pigment.

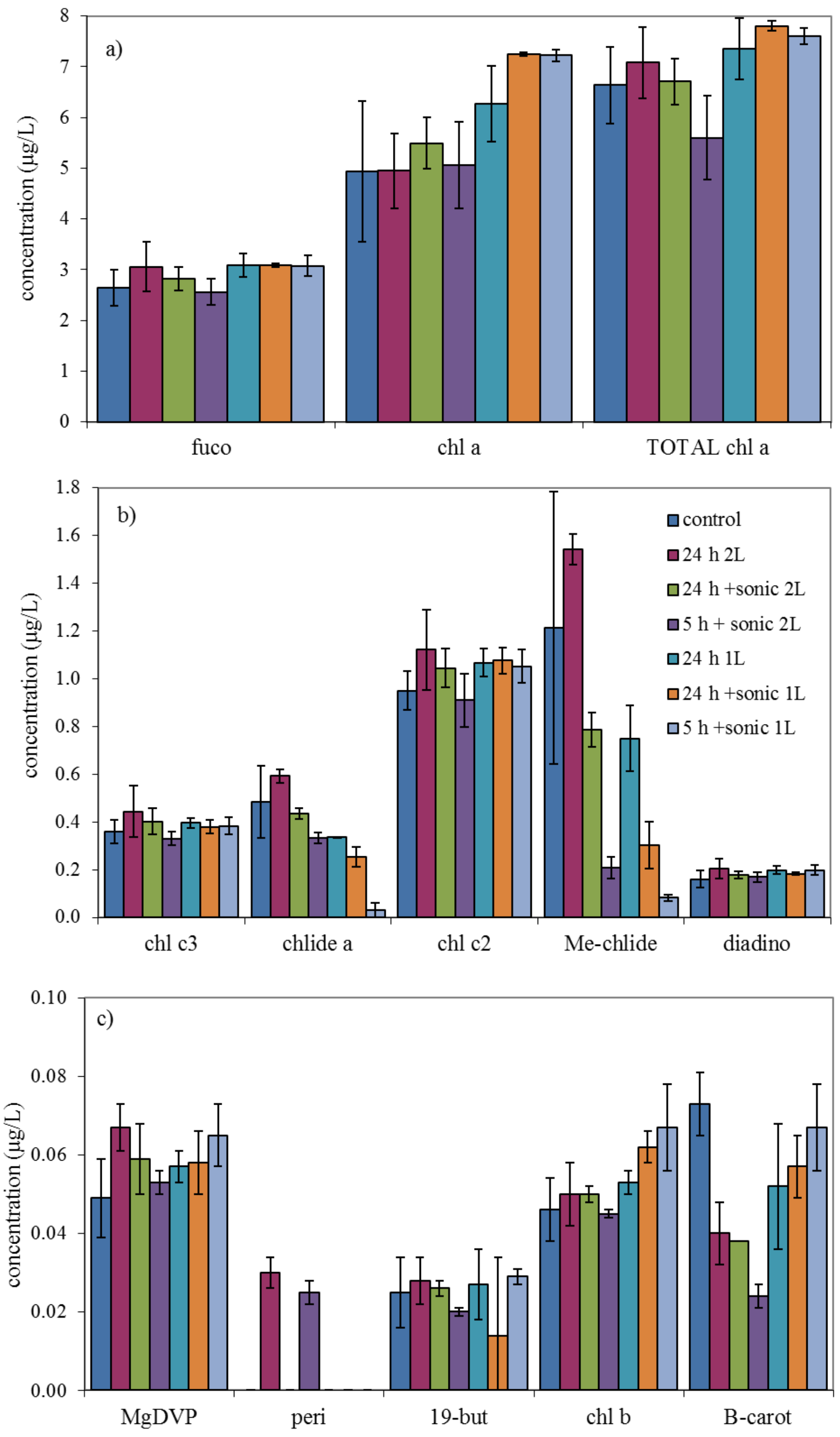

Figure 13: Comparison of pigment yields for 2 L and 1 L filtration volumes and 2 soak durations on 47 mm filters for the 12 µg/L *chl a* sample. Controls on 25 mm filters are the mean of triplicate samples. All 47 mm filter treatments were analyzed in duplicate. Error bars represent standard deviations of the mean.

Similar patterns were observed in the yields of *chl b* and even more dramatically in *β-car* (Fig. 13c). Concentrations of *β-car* on the 47 mm filters with a 1 L filtration volume greatly exceeded those where 2 L was filtered, but none reached the levels seen in the 25 mm control. It is puzzling why it is so difficult to get comparable yields from the 25 mm and 47 mm filters for this pigment. As *β-car* has the greatest affinity for the column, and is the last to elute off of it during analysis, it is possible that the solvent strength relative to the amount of pigment present and the amount of water on the filter plays a role. The remaining pigments measured were largely unaffected both by filtration volume and tweaks to the extraction method. The addition of sonication did not increase yields for *fuco* or any of the other pigments and a reduced filtration volume negatively affected *peri* which was present in concentrations close to the limit of quantification (LOQ; see Section 6.2) and thus not measurable with a 1 L filtration volume.

For the 16 µg/L *chl a* sample, volumes of 625 ml and only 1 L were used for the 47 mm filters due to higher biomass and the double soak treatment was added. In this case, the difference between the two filtration volumes was too small to impact the pigment yields on the 47 mm filters, but yields of *chl a* and *b* were higher in almost all treatments compared to the control indicating that at this biomass level it was the 25 mm filters that were over-saturated with material (Fig. 14). Once again, the reduced *chl a* yield in the controls was due to higher levels of *chlide a* and *Me-chlide a* since total *chl a* was more comparable between filter sizes. The double soak treatment used for the 1 L samples did however produce significantly higher *chl a* yields which were not simply the result of less degradation since total *chl a* was also significantly higher than in any of the other treatments. The double soak also produced much higher yields of *β-car* that for the first time even exceeded those on the 25 mm filters. This implies that more solvent is required at high biomass to fully extract some pigments which may be saturated at these concentrations. The downside of the addition of the extra 5 ml extraction solvent was that many minor pigments were diluted out to the point of being below LOQ and thus not reportable (Fig. 14b).

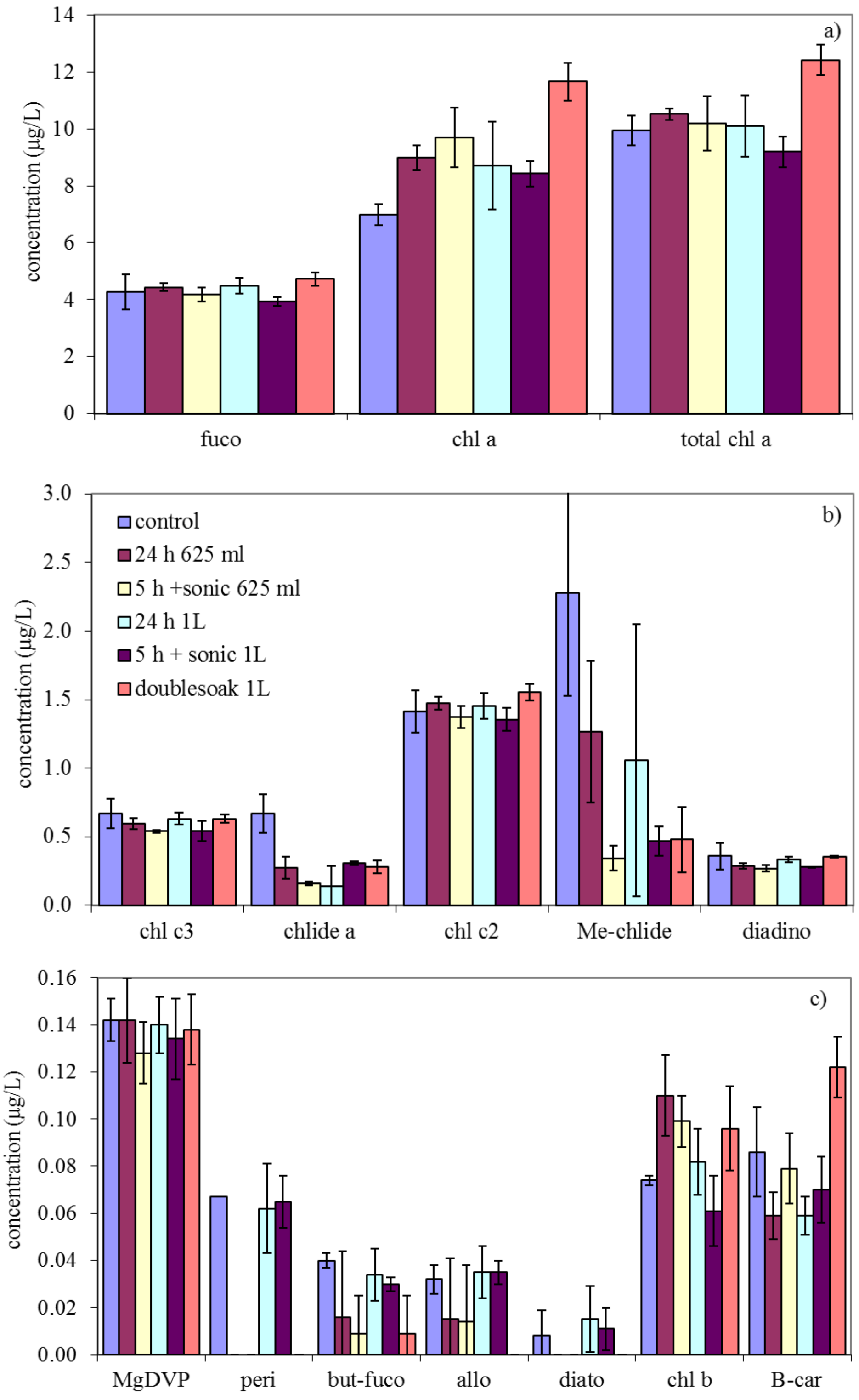

Figure 14: Comparison of pigment yields for 625 ml and 1 L filtration volumes and 2 soak durations on 47 mm filters, plus double soak extraction. Controls on 25 mm filters are the mean of duplicate samples. All 47 mm treatments analyzed in triplicate. Error bars represent standard deviations of the mean.

For the last 10 µg/L *chl a* sample only 24 h extractions were compared as the previous results had shown slightly reduced pigment yields with the shorter soak periods (Fig. 13). Two litre and 625 ml volumes were compared and the double soak was tested again on the 2 L samples but this time compared to a 24 h extraction with sonication in 10 ml of solvent to determine whether it was the extra solvent volume or the 2 step process that was increasing yields. The results showed that once again pigment yields from the 625 ml samples were much higher than those where 2 L was filtered regardless of the extraction method used, and significantly higher than those from the controls which differed only by their filter size (filtered volume and extraction method were exactly the same). Yields from the double soak were comparable to those from the 10 ml extraction but the latter had slightly less variability indicating it could simply be the increased solvent volume that is aiding extraction (data not shown).

Two clear points emerged from these experiments: 1) filtering less volume at high biomass is favoured for producing optimal yields, especially for *chl a*, and 2) if too much volume is filtered the standard extraction method can be adapted by adding the 5 min sonication bath and doubling the extraction volume, with or without the double soak modification to improve yields.

5.0 SAMPLE ANALYSIS AND DATA PROCESSING

5.1 ANALYSIS PROCEDURES

Immediately following extraction, pigment samples are analyzed on a Waters Alliance HPLC equipped with a 2996 Photodiode Array Detector (PDA) and a 2475 Multi-wavelength Fluorescence Detector (FD) connected in series. Separations are performed according to Zapata et al. (2000) using a Waters Symmetry C_8 column (150 mm x 4.6 mm, 3.5 µm particle size) running a 45 min elution gradient of 50%:25%:25% methanol:acetonitrile:aqueous pyridine (pH 5.0) and 20%:60%:20% methanol:acetonitrile:acetone at a constant flow rate of 1 ml/min. The column is not thermostatted and operates at room temperature. Samples are typically extracted in sets of 25 with each sample set taking ~24 h to analyze, enabling continuous, efficient analysis throughout the week minimizing solvent usage for startup and shutdown procedures.

Sample vials are held in the autosampler carousels at 4 °C prior to analysis. This is the lowest temperature attainable on most instruments and is the standard for this type of analysis (Zapata et al. 2000; Wright 2005). Separations are performed on a 200 µl injection of sample which is layered with water (stored in the autosampler in a separate vial) at a ratio of 5:1 (200 µl sample:40 µl water) in 4 alternating draws into the sampling loop by the auto-addition function on the HPLC. The sample-water "sandwich" is then injected onto the column. The water increases peak height and improves resolution and separation of the early-eluting, polar pigments (Zapata and Garrido, 1991), and is added just prior to injection to minimize loss of non-polar pigments (Zapata et al. 2000). A mixed pigment standard (DHI Lab Products) containing most of the peaks of interest is run daily to determine RT for individual peaks for identification purposes, and to monitor the stability of the PDA detector and validity of response factors in between full calibrations (see Section 7).

An internal standard (TAC) reference vial is run at the end of each sample set to normalize the TAC peak in each sample. This provides a more accurate measure of the final extraction volume (V_x) accounting for volume changes caused by seawater retention on the filters, and/or evaporation of solvent from the sample tubes during both storage and extraction. The internal standard also corrects for any injection anomalies. For example, if the auto-addition function is not programmed properly, and only half of the intended sample is injected, normalizing the TAC peak in the sample to the TAC peak in the reference will give an apparent extraction volume that is double what it should be, but the final pigment concentrations calculated using this V_x will be in line with those of the replicate. The extraction solvent is dispensed into HPLC vials at the same time as it is dispensed into extraction tubes for each week's run and both are stored at -20 °C until analysis. Since the HPLC vials have screw caps (as opposed to looser snap caps for the test tubes), evaporation from these is assumed to be negligible over the course of storage (<1 week). Because they only contain a single peak, these TAC reference runs are also useful for quantifying signal noise and drift, and for identifying potential sample carryover from a previous run. Prior to the use of the TAC reference, a constant V_x value of 3.22 ml was assumed for all samples as 0.22 ml was determined to be the average volume of seawater retained on a 25 mm GF/F filter (Bidigare et al. 2003). Only water retention in the filters was accounted for with this fixed value since evaporation was likely negligible on soak times of <4 h (Methods C and D; Table 7).

Once analysis is complete, pigment peaks are identified and quantified using Waters Empower 2 Pro software. The 3D UV-VIS absorbance spectrum from the PDA is extracted at 440 nm for 2D chromatographic processing. The RT for each pigment, which can show slight day-to-day fluctuations, is updated daily in the processing method based on that of the mixed pigment standard run on that day. These fluctuations result from minor variations in lab temperature, eluent composition, and/or system pressure. Identification is achieved by comparing sample peak RT to those of the mixed pigment standard. Peak identifications are corroborated by comparing their absorbance spectra to those in a library created by running individual pure pigment standards during calibration; although this is only possible for peaks that are well above the limit of quantification (LOQ; see Section 6.2). In addition the fluorescence detector aids in identification by distinguishing chlorophylls (and their degradation products) which fluoresce, from the other pigments which do not. Integration is manually optimized for each chromatogram and the peak table is then exported into Microsoft Excel for quantification and data post-processing.

5.2 PIGMENT QUANTIFICATION

Chromatograms processed in Empower software are exported into Microsoft Excel as peak tables which contain the list of identified pigments for each sample along with their corresponding RT, peak heights and peak areas. The peak area for each pigment is used to calculate the amount that was injected onto the column and from that the *in situ* concentration in the original water sample. Peak areas for each pigment are first screened to ensure they are above the LOQ peak area for each pigment. Because peak size and shape changes during the gradient due to the chromatographic properties of each pigment, the peak area cutoff value is unique for each pigment, and is determined during calibration by multiple replicate injections of low concentrations of standard (see Section 6.2.2). Pigments with peak areas above the LOQ are quantified and distinguished from those pigments which are below the LOQ or not detected at all. Pigment amounts in nanograms are determined by dividing the peak area of each pigment by its corresponding response factor (Rf, determined during calibration from the slope of the calibration curve). The concentration is then calculated according to:

$\mathbf{C = (A/V_{inj.}) \times (V_x/V_f)}$ where

C = concentration of pigment in µg/L
A = amount of pigment in ng loaded onto column
V_{inj} = injection volume of sample in ml

V_f = volume filtered in ml
V_i = initial extraction volume added to filters in ml
$V_x = V_i$ x (TAC peak area in reference ÷ TAC peak area in sample)

Final pigment concentrations for each sample are reported as the average of duplicates and sample precision is assessed as the %CV equivalent to the standard deviation divided by the mean.

6.0 CALIBRATION

6.1 CALIBRATION PROCEDURES

A full pigment calibration of any HPLC system is not a trivial undertaking. Independent multi-point calibration curves need to be generated for each pigment being measured and this is both an expensive and time-consuming operation. Fortunately, full calibration typically only needs to be performed every couple of years as the response factors (Rf) remain relatively constant over time despite hardware changes such as new columns and detector lamps. In between full calibrations, daily and/or weekly checks are used to monitor system performance and detector stability over time to ensure data quality (see Section 7). There are several factors that need to be considered when performing these instrument calibrations that can affect the quality of the results. Examples include whether or not to subtract the intercept for each linear regression or force the line through zero, determining how to optimize calibration mixes to avoid overlap of closely eluting pairs, adjusting the water auto-addition amount dependent on the solvent strength in the standard mix, and accounting for the purity of the standards when calculating concentrations, just to name a few.

6.1.1 Pigment standards

Standards for *chl a, chl b, β-car* and more recently *fuco* are available inexpensively from Sigma-Aldrich in powdered form (typically 1 mg ampules). The contents of the ampule are dissolved in 250 ml HPLC-grade solvent (90% acetone for the chlorophylls and 100% ethanol for the carotenoids) in an amber-glass volumetric flask to make a primary stock (~ 4 mg/L). Secondary stocks can be diluted from this primary stock producing a seemingly unlimited supply of standard in a large range of concentrations. In contrast, standards for all of the other pigments are only available from DHI Lab products in Denmark. These are produced by preparatory HPLC from algal cultures and come pre-dissolved in a small volume of solvent (2.5 ml), have a fixed upper concentration rarely exceeding 1.5 mg/L, and are expensive with individual vials costing \$200-\$600. Furthermore, they need to be shipped from overseas on dry ice which adds to their expense. The chlorophylls are dissolved in 90% acetone solution while all carotenoids are in 100% ethanol and all come in crimp sealed vials under an argon atmosphere. DHI standards are accompanied by a document of certification listing their measured concentration and chromatographic purity. DHI certifies stability of their standards for 3 years stored unopened in the dark at -20 ºC.

6.1.2 Determination of pigment standard concentrations

Whether obtained commercially or produced in the lab, prior to calibration the concentration of each standard solution should be measured using a spectrophotometer with its corresponding specific extinction coefficient (α) for the solvent in which it is dissolved (Table 11). For lab standards this step is necessary as the precise amount of the powdered standard in each ampule varies from batch to batch. Although the DHI standards come with published concentrations, it is still good practice to confirm these values prior to use especially if these vials have been stored for some time (Hooker et al. 2005). In addition, once the standard vials are opened they are subject to evaporation and potential degradation rendering them useless for calibrations unless their concentrations and purity can be re-measured. Measuring the concentration of the DHI standards can prove difficult due to the limited volume available. Spectrophotometer cells with a 1 cm path length which are used for the large volume lab-made standards are not appropriate since they require 2-4 ml of sample for a measurement (often more than the entire volume of a DHI standard). Instead, microvolume (50-100 µl) spectrophotometer cells are used but it is critical that they are correctly matched to the characteristic beam height of the spectrophotometer for accurate concentration calculations.

Table 11: The specific absorption coefficients (α) and the wavelength of maximum absorbance (λmax) for each pigment in the solvent listed (from DHI certificate of analysis).

Pigment	Standard source	Solvent	α (L/g/cm)	λmax (nm)
chl c3	DHI	90% acetone	346	453
chlide a	DHI	90% acetone	127	664
MgDVP	DHI	90% acetone	318	439
chl c2	DHI	90% acetone	374	444
peri	DHI	100% ethanol	132.5/135*	472/475*
phide a	DHI	90% acetone	74.2	667
but-fuco	DHI	100% ethanol	160	446
fuco	DHI/Sigma	100% ethanol	160	449
neo	DHI	100% ethanol	224.3/233*	439/437*
pras	DHI	100% ethanol	160	454
viola	DHI	100% ethanol	255	443
hex-fuco	DHI	100% ethanol	160	447
diadino	DHI	100% ethanol	262	446
allo	DHI	100% ethanol	262	453
diato	DHI	100% ethanol	262	449
zea	DHI	100% ethanol	254/245*	450/453*
lut	DHI	100% ethanol	255	445
chl b	Sigma	90% acetone	51.4	647
DV chl a	DHI	90% acetone	87.7/88.3*	664/663*
chl a	DHI/Sigma	90% acetone	87.7	664
phytin a	DHI	90% acetone	51.2	667
β-car	Sigma	100% acetone	250	454

* values from Roy et al. 2011 where they differ from those used by DHI.

At IOS, all pigment standards are measured on a Thermo Scientific Evolution 201 UV-VIS spectrophotometer with an 8.5 mm beam height. DHI standards are measured with a quartz 50 µl masked low volume microcell with a 1 cm path length and a matched 8.5 mm height window (Thermo Scientific Cat# 268-809900), whereas lab-made standards are measured with regular 1 cm glass cuvettes typically in triplicate. Concentrations of *chl b* determined using both the large cuvette and microcell confirm good agreement between these two cells. Standards are brought to room temperature in the dark prior to being measured spectrophotometrically. All measurements are conducted in a dim room under green light to minimize pigment degradation. A matched solvent blank is run prior to each set of acetone or ethanol standards. Absorbances are scanned in the visible light range from 350-800 nm with a 0.1 nm data interval. For the lab-made pigment solutions the exact batch of solvent used to make up the standard is used for the blank. For the DHI standards this is not possible, but the blank solvent is matched as closely as possible to that of the standard. The same cuvette is used for both blank and standard measurements to minimize any differences resulting from the specific optical properties of the cells. The large glass cuvettes are rinsed with solvent in between standards and then rinsed with standard before being filled for measurement to avoid any carryover or dilution. For the DHI standards there is not enough volume to allow for rinsing so instead the microcell is rinsed out well with blank solvent and then air-dried using a directed flow of nitrogen gas to ensure all rinsing solvent that could dilute the standard is removed before the pigment standard is dispensed. The spectrophotometer automatically subtracts the absorbance of the blank from the absorbance spectrum of each pigment at every wavelength. However, in order to zero the absorbance spectrum, the absorbance at 750 nm (a wavelength at which no pigments absorb light) is subtracted from the absorbance at λmax. Pigment concentration is thus calculated as:

$$\mathbf{C = ((Abs_{\lambda max} - Abs_{750nm.}) / \alpha) * 1000}$$

where
C = concentration of pigment in standard in mg/L
$\mathrm{Abs}_{\lambda max}$ = absorbance at wavelength of max absorbance
$\mathrm{Abs}_{750\ nm}$ = absorbance at 750 nm
α = specific extinction coefficient in L/g/cm

Table 11 lists the specific extinction coefficients and wavelengths of maximum absorbance used for calculating concentrations from absorbance spectrums for each pigment. The absorption coefficients shown are those provided by DHI that are used for calculating pigment concentrations, but more recently published values are also indicated by asterisks where they differ from those of DHI (Table 11; Roy et al. 2011). Once calculated the standard concentrations need to be purity corrected. DHI lists the chromatographic purity of each standard as a percentage on its certificate of analysis but it can, and should, be independently verified particularly for standards that have previously been opened. This is easily done by analyzing a 100 or 200 µl aliquot of pure standard on the HPLC (using a low-volume insert in a standard

HPLC vial). The area of the standard peak is expressed as a percentage of the total peak area of all compounds present in the chromatogram. The calculated concentration from above is then multiplied by this purity percentage to get a purity corrected concentration which is used for all subsequent calibration calculations.

6.1.3 Preparation of calibration mixes

A 5-point calibration curve needs to be generated for all pigments being measured to determine the response factor (Rf) for each. Calibration curves need to bracket the typical pigment amounts encountered in field samples, and the linearity of the response as well as a sufficiently small y-intercept (to allow regression lines to be forced through zero) need to be established. Once a multi-point calibration curve that meets these criteria is produced, only single point injections can be used for verification of Rf. Injecting dozens of pigment standards individually at 5 levels in order to generate a calibration curve for each wastes both time and solvents. To avoid this, calibration is done by grouping 4 or 5 pigments all mixed in varying amounts into each of 5 calibration vials. In this way linear regression curves can be generated for several pigments at once with only 5 injections. Although the injector can be used to deliver varying volumes of a single standard mix thus producing a multi-point curve, this procedure only establishes the precision of the injector with all points being dependent on the accuracy of the single standard mix. By creating a separate mix for each level, one has 5 independent measurements and is able to verify the accuracy of the procedures that went into creating each mix.

Table 12 shows the template used for creating calibration mixes adapted from Wright (2005). Pigment combinations are chosen based on their retention time to avoid combining any overlapping or adjacent peaks with low resolution. The appropriate calibration range in nanograms is first determined for each pigment by examining the minimum and maximum amounts encountered in typical field samples under the same injection conditions. Once these ranges are known, the target volume of each standard for the mix can be determined using its corresponding concentration. To keep the volume equal between all 5 vials of the mix, each one contains pigments at different levels and all are made up to 1 ml final volume with 95% methanol. Because solvent standards evaporate quickly, cannot be pipetted accurately, and tiny volumes are dispensed, all standard additions are done with gas-tight syringes and are verified by weight.

Table 12: Example template for pigment calibration mixes used to determine the standard addition volumes and final amounts of pigment injected for each vial, based on Wright (2005).

Target	vol std in mix (ul) nominal				
Pigment	CAL1	CAL2	CAL3	CAL4	CAL5
but-fuco	25	50	200	100	75
neo	200	150	100	50	25
diato	25	50	75	100	200
viola	500	300	150	75	25
chl c2	25	75	150	300	400
95% MeOH	225	375	325	375	275
Total:	1000	1000	1000	1000	1000

Calculation of pigment injected:

	Weight of pigment in vials						vol std in mix (ul)				
Pigment	CAL1	CAL2	CAL3	CAL4	CAL5	density	CAL1	CAL2	CAL3	CAL4	CAL5
but-fuco	0.01869	0.03799	0.15484	0.07651	0.05678	0.78504	23.81	48.39	197.24	97.46	72.33
neo	0.15418	0.11480	0.07770	0.03717	0.01798	0.78392	196.68	146.44	99.12	47.42	22.94
diato	0.01838	0.03830	0.05748	0.07660	0.15695	0.77768	23.63	49.25	73.91	98.50	201.82
viola	0.38972	0.23651	0.11514	0.05901	0.01863	0.77944	500.00	303.44	147.72	75.71	23.90
chl c2	0.01960	0.06114	0.12119	0.24828	0.32917	0.80308	24.41	76.13	150.91	309.16	409.88
95% MeOH	0.17802	0.30092	0.25963	0.29959	0.21705	0.79952	222.66	376.38	324.73	374.71	271.48
Total							991.18	1000.03	993.63	1002.95	1002.34

	vol std in injection (ul)						ng pigment in injection				
Pigment	CAL1	CAL2	CAL3	CAL4	CAL5	adj. conc	CAL1	CAL2	CAL3	CAL4	CAL5
but-fuco	2.40	4.84	19.85	9.72	7.22	0.957	2.299	4.631	18.996	9.299	6.905
neo	19.84	14.64	9.98	4.73	2.29	1.402	27.822	20.533	13.987	6.629	3.208
diato	2.38	4.92	7.44	9.82	20.13	0.879	2.095	4.327	6.536	8.629	17.692
viola	50.44	30.34	14.87	7.55	2.38	0.874	44.091	26.521	12.994	6.598	2.084
chl c2	2.46	7.61	15.19	30.82	40.89	0.847	2.086	6.449	12.865	26.111	34.638

For each calibration solution, 5 amber HPLC vials are capped and labelled with the mix number. Then 500 µl of each standard is injected into a separate tared HPLC vial and weighed. This mass is doubled to get a density for each solution in g/ml which is used to convert the weight of each standard addition to a volume. If more than 500 µl of a given standard is needed to make up all 5 mixes, this latter vial is topped up and then used to dispense the appropriate amounts into each calibration mix vial. Each vial is tared prior to every pigment injection and the weight recorded after each addition. Additions are performed through the septum to minimize evaporation but the cap is kept loose during injection to avoid pressurizing the vial. Any solvent that ends up on the top of the septum is blotted off before the weight is recorded. Syringes are rinsed with an appropriate solvent between standards and dried by pumping air through them to avoid diluting the next standard. Once all standards have been added to each vial and the volume made up to 1 ml with 95% methanol, the vials are tightly capped, vortexed to mix thoroughly and analyzed immediately on the HPLC using the same injection conditions as for samples (200 µl aliquot with water auto-addition). Water amounts sometimes need to be increased for mixes with a high proportion of acetone standards to improve peak height and resolution of early-eluting peaks, nonetheless matrix effects will always remain due to differences between field sample extracts and standards (salt and water content, lipids, solvent composition, etc.).

The following steps are used to calculate the amount of each pigment injected for each calibration mix in the template in Table 12:

1. The weight of the pigment (g) ÷ the density (g/ml) x 1000 = volume of standard added to each vial (µl)
2. The volume of standard added (µl) ÷ total volume of the vial (µl) x injection volume (µl) = the volume of each standard injected onto the column (µl).
3. The volume of standard injected (µl) x purity corrected concentration (ng/µl) = amount of pigment injected (ng)

6.1.4 Pigment response factors

The integrated peak area of each pigment from all 5 calibration mixes is plotted against the amount of pigment injected in nanograms. A linear regression of the points is forced through zero (provided the y-intercept is sufficiently small) and the slope of the regression becomes the response factor for each pigment. The r^2 values for all calibration curves are always >0.99. Table 13 lists the current Rf values for all pigments as well as the upper end of the calibration range and limits of detection (LOD) and limits of quantification (LOQ). No LOD/LOQ values have been obtained for *phide a* and *phytin a* as the method for obtaining these values was determined after the last calibration for these pigments was carried out. These two pigments are of lesser importance for us as they are not algal group markers and thus not used for phytoplankton community composition determinations. In the near future these two *chl a* degradation products will be recalibrated and LOD/LOQ calculated.

Table 13: Current response factors (Rf) for all calibrated pigments, except for *phide a* and *phytin a*, presented with the maximum amount of pigment (ng) used for calibration and the limit of detection (LOD) and limit of quantification (LOQ).

Pigment	Rf	max (ng)	LOD (ng)	LOQ (ng)	Cal date
chl c3	7314	26	0.50	1.66	Feb 2013
chlide a	4332	45	0.43	1.45	Feb 2013
MgDVP	13771	34	0.16	0.53	Feb 2013
chl c2	12781	179	0.21	0.70	May 2011
peri	5668	77	0.45	1.50	Feb 2013
but-fuco	8085	22	0.16	0.52	Feb 2013
fuco	8432	1488	0.20	0.66	May 2011
neo	10082	10	0.25	0.82	Feb 2013
pras	8047	14	0.19	0.63	Feb 2013
viola	12034	29	0.22	0.75	Feb 2013
hex-fuco	9067	38	0.23	0.76	Feb 2013
diadino	12105	68	0.20	0.66	Feb 2013
allo	10823	23	0.21	0.69	Feb 2013
diato	9867	15	0.16	0.52	Feb 2013
zea	8385	18	0.22	0.73	Feb 2013
lut	11017	23	0.16	0.53	Feb 2013
chl b	3311	146	0.37	1.23	May 2011
DVchl a	4580	13	0.36	1.20	Feb 2013
chl a	3552	1848	1.05	3.51	May 2011
β-car	9865	14	0.24	0.81	Feb 2013

For most pigments the most recent calibration conducted in Feb 2013 produced the best results likely due to our ability to confirm and/or re-measure the published DHI standard concentrations with the matched beam height microcell for the spectrophotometer. In a few cases where the change in Rf was negligible (*chl c2, fuco, chl a*), an older calibration was used if the corresponding pigment range was deemed more appropriate. The maximum calibrated pigment amounts in Table 13 cover the observed ranges for >95% of all field samples analyzed to date. Occasionally, some samples will have pigment amounts that exceed these limits particularly if injection and/or filtration volumes change, or if a mono-specific bloom is encountered. For example, extremely high *viola* levels (up to 75 ng) have been observed in *Heterosigma* blooms in the Strait of Georgia and *peri* amounts upwards of 2000 ng (well outside the typical range) have been measured in dinoflagellate blooms off the west coast of Vancouver Island. The maximum observed pigment amounts in field samples are noted when each dataset is processed so that calibration ranges can be extended in the future. Range extension is often achieved by using the HPLC injector to deliver fixed volumes of pure standard which can then be added to the existing calibration curves. For example, the datapoint at 1848 ng for *chl a* (Table 13) was obtained by injecting 200 µl of a 10 mg/L solution and the curve was still found to be linear up to this amount. As mentioned above, the upper limits achievable with the DHI standards are fixed at a much lower level due to the concentration limitations of the standards. If pigment amounts in samples routinely exceed the calibration ranges possible with the standards, filtration or injection volumes of samples should be decreased. The majority of samples where pigment amounts fell outside the calibration range came from high biomass regions such as the west coast of Vancouver Island. Since the use of fluorescence readings to determine filtration volumes was adopted to avoid extraction problems (see Section 3.2), these occurrences are now rare.

6.2 LOD/LOQ DETERMINATION

To provide assurance in the reported pigment values, LOD and LOQ should be determined for every HPLC system and only pigments that exceed these levels can be confidently reported. Many different approaches for determining these values exist in analytical chemistry and they can generally be grouped into two classes: instrumental and non-instrumental (Roy et al. 2011). Instrumental approaches do not take into account the quality of the calibration or method related procedures and simply look at the signal to noise ratio (SNR) inherent to the instrumentation. This was the method recommended in the SeaHARRE-2 report (SeaWIFS HPLC Analysis Round-Robin Experiment), a multi-lab intercomparison exercise aimed at establishing quality metrics for pigment data being used for satellite calibration (Hooker et al. 2005), and was the first method tried at IOS.

6.2.1 LOD based on SNR

In this instrumental approach, Empower software is used to calculate the short term detector noise over a 1 min region of the chromatogram where no peaks are present. Empower calculates the root mean square noise (RMS) which is equivalent to one standard deviation of the baseline; this was doubled to get the 2σ noise criterion of Hooker et al. (2005). Noise was calculated and averaged for almost 100 chromatograms obtained over a one year period and was found to be relatively constant at 90 ± 10 Au (Absorbance units). By definition, the LOD is equivalent to an SNR of 3 (270 Au in this case) and the LOQ has an SNR of 10 (900 Au). Thus, any pigments with peak heights less than 270 Au are considered below the LOD and not significantly different from zero, whereas only pigments with peak heights >900 Au are considered to be quantifiable with acceptable confidence. All chromatograms to be processed are then screened by peak height, with integrated peaks shorter than 270 Au being assigned a default value equal to approximately half the LOD value of each pigment in ng to differentiate these pigments from ones in other samples which were not detected at all.

To determine the LOD/LOQ for individual pigments in terms of ng, the peak heights from the 5-point calibration injections were converted to SNR values by dividing each by the noise amplitude (90 Au). To force the plots through the noise origin, 1 was subtracted from each SNR value and these were plotted against the injected pigment amounts. The LOD for each pigment was then computed by dividing 3 by the slope of the linear regression of the plot forced through zero (Fig. 15).

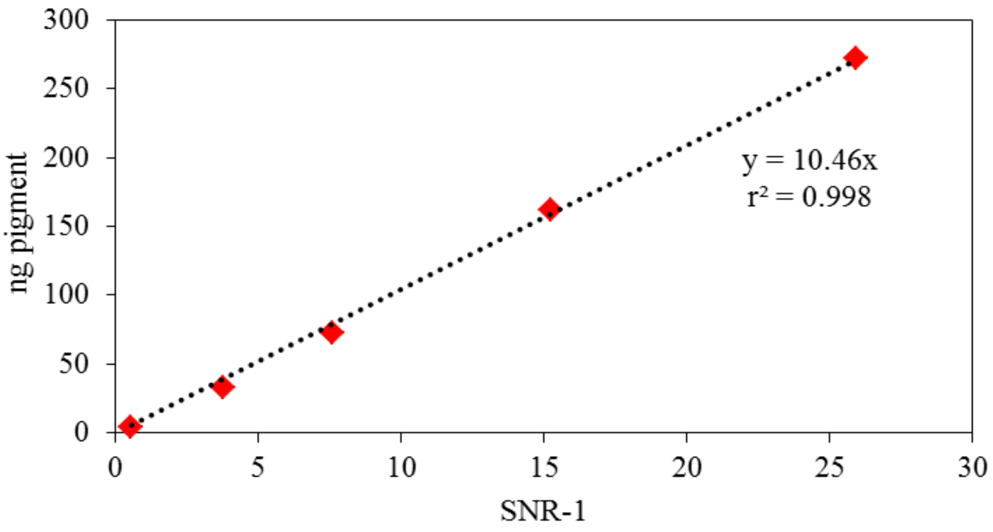

Figure 15: Example plot of the graphical determination of LOD for *diadino* based on SNR from Dec 2008 calibration data. LOD =3/slope or 0.29 ng.

LOD calculated in this manner ranged from a low of 0.2 ng for lutein to a high of 1.23 ng for peridinin. The difference in magnitude of the LOD between these two pigments was not surprising as peridinin is one of the early eluting polar compounds that are most affected by solvent strength and water content and typically exhibit short, broad peaks resulting in a low SNR based on peak height. In contrast, lutein is late eluting and relatively unaffected by the chromatographic conditions always producing narrow, sharp peaks and a correspondingly high SNR. This difference also highlights a problem with using peak height as the basis for setting detection limits. For example a standard mix containing a high percentage of acetone (which leads to broader peaks) was run with the regular water addition (20 µl) and then rerun with 3 times the water added (60 µl). Peaks were distinctly narrower and taller in the latter injection leading to much lower LOD values based on SNR for those pigments despite the fact that the peak areas, and by extension the response factors remained constant between the two runs. Since it is very difficult to match the sample and standard matrices this creates an uncertainty in applying SNR based LODs determined from calibration standards to field samples in a different matrix.

More troubling however was the magnitude of the LODs themselves which were almost an order of magnitude larger than those reported by Horn Point lab in the SeaHARRE-2 report (Hooker et al. 2005). At first a calculation error was suspected and to rule this out the short term detector noise was manually recalculated as described in the SeaHARRE-2 report and confirmed to agree with the automated noise calculation made by Empower. Thus it appears our method has an inherently larger noise signal than that at Horn Point labs (considered a “state-of-the-art” lab; Hooker et al. 2010) which may result from a number of instrument factors including lamp energy, detector performance, lack of proper solvent mixing, column temperature fluctuations, etc. In particular, the HPLC methods used may be a big factor in this difference as Horn Point lab uses the Van Heukelem and Thomas (2001) method which employs a high efficiency column and elevated column temperature resulting in very sharp peaks (and thus a low SNR). The lack of a column heater on the system at IOS prevents adoption of this method and results in a higher inherent SNR. As pointed out in the SeaHARRE-2 report, HPLC hardware and software can be the limiting factors for some methods despite the best efforts of the analysts, thereby limiting their ability to produce state-of-the art results (Hooker et al. 2005). It should be noted however, that only a handful of labs worldwide are likely considered state-of-the-art and we had to compare our values to those of Horn Point as the other labs participating in the SeaHARRE-2 intercomparison did not provide LOD/LOQ values. Our intercomparison results below in Sections 7 and 8 demonstrate that we can achieve quantitative or state-of-the-art results in most of the other performance metrics.

6.2.2 LOD/LOQ based on standard deviation of response

Non-instrumental approaches for calculating LOD/LOQ look at the calibration and methodological data themselves. One such method is based on the standard deviation of the residuals to the linear fit of the calibration points. The principle here is that with very small residuals, the calibration curve is capable of producing accurate results at very low pigment amounts even when forced through zero. Thus, the tighter the fit and the smaller the residuals, the smaller the LOD. The LOD and LOQ are calculated by dividing the standard deviation of the residuals by the slope of the regression multiplied by 3 and 10, respectively (Suzanne Roy, Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski, Québec G5L 3A1, pers. comm.). This approach applied to the same calibration data above produced values very similar to the SNR approach, validating the previous calculations. However, both of these methods require that at least the lowest injected amounts fall close to the LOD. For many of the pigments, the calibrated ranges were quite large with the lowest injected amounts having SNR values > 10 leading to potential inaccuracies in the LOD value.

To avoid having to re-create the full calibration curves with adjusted pigment amounts a third, simpler approach for calculating LOD was later employed. This method takes the standard deviation of the peak areas for 7 replicate injections of the same low pigment standard and multiplies it by 3 or 10 to get the LOD and LOQ, respectively (Bidigare et al. 2005). It has several advantages over the previous two methods. First, as opposed to relying on multi-point calibrations only a single low standard needs to be injected for each pigment allowing for a dozen or more pigments to be combined in one LOD mix with very little standard used and fewer injections required. Second, by taking the standard deviation of the peak area of multiple injections of the same sample, one is able to incorporate the precision or repeatability of the manual integration into the LOD value. Third, the precise amount of standard used does not need to be known for the calculations, although it is useful to prepare the standard gravimetrically as the calculated and measured amounts can be compared to evaluate the Rf accuracy near the LOD. The injected amounts should not exceed 5 times the LOD and this is achievable with approximately 1 ng of each pigment.

The limits of detection and quantification currently in use that are presented in Table 13 were calculated using this method. The LOD/LOQ values presented in ng are determined by dividing the LOD/LOQ peak areas by the Rf for each pigment. These values are fixed and limited by the instrumental conditions, however the effective LOD/LOQ in terms of *in situ* pigment concentrations are fluid and can be adjusted by changing the filtration, extraction and injection volumes. Thus, although the LOD/LOQ values in Table 13 apply to all datasets collected, the effective LOD/LOQ values are different for each sample; values can be lowered by increasing the filtration and extraction volumes and/or decreasing extraction volume. The LOD and LOQ peak areas are used to screen sample peaks during processing. Only pigments with peak areas that exceed the LOQ cut-off are quantified, all other peaks are assigned a zero value whether they were detected or not. This is in contrast to the original SNR based formulations which assigned a small non-zero value to peaks below the LOD to distinguish them from peaks that were not detected at all. However, deciding what this arbitrary non-zero value should be was a problem as depending on the filtration, extraction and injection volumes of individual samples the effective LOD would change. These variable values thus created problems when using CHEMTAX to determine community composition as analytically speaking these pigments were not “detectable” and thus should not have values even though they were detected. We no longer distinguish between pigments that fall below the LOQ and those not detected at all. In both cases they are assigned a zero value.

7.0 QUALITY ASSURANCE/QUALITY CONTROL PROTOCOLS

7.1. ALLIANCE HPLC MAINTENANCE AND MONITORING

Since full pigment calibrations are only performed every couple of years, it is crucial to maintain and monitor both HPLC system performance and detector response on a regular basis to ensure method validity and applicability of response factors over time. A detailed log is kept of all HPLC maintenance and parts replacement or repairs, and a full Performance Maintenance service is done by a Waters technician every 1-2 years. This service replaces the majority of the parts that suffer wear with regular usage such as the injector needle, syringe and associated seals, the inline filter, solvent, needle and seal wash filters and seals, and pump plungers, seals and check valve cartridges. Usage logs are kept for system parts such as columns and detector lamps. A running tally is maintained of the number of injections performed on each new column with guard columns replaced every 400 injections to prolong the life of the primary separations column. The separations column is replaced when the resolution between adjacent peaks begins to deteriorate (especially between the *viola/hex-fuco* pair) and/or when peaks begin to tail or become asymmetrical. Depending on the concentration of the sample extracts analyzed on the column, this occurs at anywhere between 800-2000 injections. The PDA deuterium lamp has a lifetime of ~2000 h and the lamp energy is checked periodically to ensure sufficient output. A mixed pigment standard is run before and after each hardware change to ensure consistency in the response. Lamp changes have shown no effect on the detector response with pigment peak areas before and after the change differing by 1.7% across all pigments on average which is much less than the 5% average variability across all pigments in the mixed standard runs over a 3 year period (see Section 7.4). With column changes there is a marked improvement in peak shape with taller, narrower peaks and improved resolution on the new columns but peak areas remain constant. This highlights the robustness of using peak area for quantification and for LOD/LOQ determination as opposed to peak height which shows more variability with changing chromatographic conditions.

At the start of each week’s analysis, the HPLC runs through a roughly hour long start-up procedure consisting of a series of self-tests. These self-tests alert the user to any system troubles such as pressure leaks, or injector or vial homing problems prior to them becoming an issue during sample analysis. During the start-up protocol the column is equilibrated in 100% methanol and then conditioned by running the elution gradient. The system pressure in methanol is recorded. A continuous log of these pressures provides a good baseline against which any pressure increases that may indicate a clog or block in the inline filter or columns, or decreases, which may indicate a leak can be investigated. This is a particularly useful check as it is independent of any eluent composition changes which lead to minor pressure variability. Apart from the fact that pressure variations can indicate hardware problems, they also cause RT to shift so it is ideal to keep operating pressure as constant as possible. The biggest shift in system pressure typically occurs with each new column that has an inherent operating pressure signature.

7.2 SPECIFICTY

An important consideration when measuring multiple pigments in a complicated sample matrix is that of specificity, or how accurately one can isolate and identify the analyte of interest from other interferences. As mentioned in Section 2, to be certain of a positive pigment identification three rigorous criteria need to be met (same absorbance spectrum, same retention time and same mass as known standard), however in practice, the RT is often the only available means of pigment identification and this can lead to false positives, false negatives and/or mis-identifications for pigments with similar RT especially if they have similar absorbance spectra. In particular, there is a cluster of minor pigments that elute between *but-fuco* and *hex-fuco* (RT=20-25 min) many of which have overlapping RT, are often rare with no available standards, and either have similar spectra or are present in low amounts making spectral matching difficult and thus mis-identifications possible. This error is minimized with the use of the mixed pigment standard (DHI) which contains most of these pigments and is run daily allowing RT to be updated for each day's operating conditions and most importantly for each eluent batch. The early eluting pigments (*chl c3*, *chl c2*, *peri*) show the most variability in RT both day to day, and during a given day's analyses with RT variability generally decreasing with increasing retention time to a minimum at the RT of *chl b* (Table 1). However, for all pigments the variability in RT across all samples in a single sample set is about one quarter of that observed day to day (0.9%CV vs. 3.8%CV for *chl c3* and 0.1%CV vs. 0.4%CV for *chl b*, respectively). This significant reduction in the RT variability over a 24 h period compared to over multiple days illustrates the improvement in specificity obtained with the use of updated retention times from the daily mixed pigment standard.

Nonetheless even with accurate RT false positives can occur when RT is the only criteria used for identification. For example, an interfering pigment sometimes occurs at almost the precise RT of *pras* which can make identification difficult, fortunately the peak is often large enough to generate a good absorbance spectrum which clearly excludes it as being *pras*. Knowledge of which pigments tend to co-occur is also useful in minimizing errors associated with false positives or negatives. For example, in several samples during one cruise a large peak was observed at the same retention time as *but-fuco* but it was showing a spectral match to *fuco*. The peak was clearly not *fuco* but since these two pigments have similar absorbance spectra and Empower spectral matching can often be incorrect, it was not entirely clear if this pigment was indeed *but-fuco* or some other pigment entirely. Examination of the rest of the peaks in the chromatogram indicated the absence of *hex-fuco*. Since *but-fuco* and *hex-fuco* almost always co-occur in type 6-8 haptophytes (Table 2), the absence of the latter peak indicated the mystery peak was likely not *but-fuco* and was later determined to most likely be 4-keto-fucoxanthin, a rarer pigment with an absorbance spectrum very similar to *fuco* known to occur exclusively in type 5 haptophytes (Zapata et al. 2004).

Carryover from previous sample runs can also cause interferences that affect specificity or lead to overestimates of pigments in subsequent samples. This can occur through retention of pigments on the column at the end of a run or via contamination of the needle or sample loop. The needle wash cycle that runs during the injection process rinses the needle between each draw of sample or water making contamination of the needle very unlikely. The TAC reference standard that is run daily to normalize internal standard peak areas is ideal for assessing cross-contamination as it only contains a single peak so any additional peaks carried over from the previous sample would be clearly visible. In all the years of pigment analysis at IOS we have yet to witness sample carryover regardless of factors such as column age.

7.3 MONITORING DETECTOR STABILITY

Since full calibrations are only performed every couple of years, the stability of the detector response needs to be monitored on a regular basis to ensure applicability of the response factors (Rf) over time. Fortunately the DHI mixed pigment that is run daily to update RT values for peak identification serves a dual purpose as a calibration check for all pigments. Many vials of a single batch are purchased at once to allow Rf monitoring over periods of 1-3 years. Although several daily injections over the course of a week or more are obtained from one vial for RT checks, only the first injection from sealed vials is used to monitor response since pigment concentrations change rapidly due to evaporation from open vials. Concentrations calculated for each pigment are plotted against time on a process control chart which is a useful tool for temporal monitoring of various performance metrics. These charts quantify 95% (warning limits, WL) and 99% (control limits, CL) confidence intervals and track trends in performance identifying points that are out of control. Out of control points demand immediate attention and indicate a problem with the analysis that has the potential to affect all samples run on that day.

Figure 16 displays the process control chart for *fuco* concentrations from a single batch of the mixed pigment standard for analyses which occurred between November 2010 and December 2013. The random scatter of points about the mean verifies consistency in the response factors as well as good stability of the standards over this 3 year time period when stored sealed at -20 ºC. Most of the other pigments in the mix showed the same behaviour as *fuco* with an average absolute deviation from their mean concentration of 5%. Only *chl a* showed a slight decreasing trend in concentrations over time although average absolute deviation from the mean was still only 5.5% (data not shown). This decline was caused by the poorer stability of this pigment with long term storage as opposed to a change in the detector response since independent checks of the *chl a* response factor show it to be very constant (see below).

Two points over the 3 year period fell outside of the control limits for all pigments highlighting potential problems with some aspect of the analysis on these dates (Fig. 16). The point that fell below the control limit occurred on a day when pigment calibrations were being carried out so no sample analyses were affected. Column age was suspected as a possible contributor to lower response in this instance as 1200 injections had already been run on this column and peaks were starting to get very broad and asymmetrical. The column was pulled from service and replaced the next day and a fresh mixed pigment standard vial was run with concentrations falling back in line with the mean (Fig. 16). The calibration standards were also rerun but peak areas were the same as the previous day which was consistent with past observations that column changes actually have little effect on signal response. Thus it seems the analytical problem that led to the out of control point only affected the mixed pigment run and not those of the calibration standards, pointing to the possibility of an injection error. The point that fell above the control limits occurred on a day when samples were being analyzed and therefore potentially compromised. Precision of pigment concentrations from replicate samples analyzed on this and another day was compared to that of replicated samples not analyzed on the affected day and found to be comparable, although there was more variability in the V_x values between replicates. Thus, it is likely that the issue on this date was also injector related and may have affected some sample runs but did not affect final calculated concentrations owing to the correction available with the internal standard normalization.

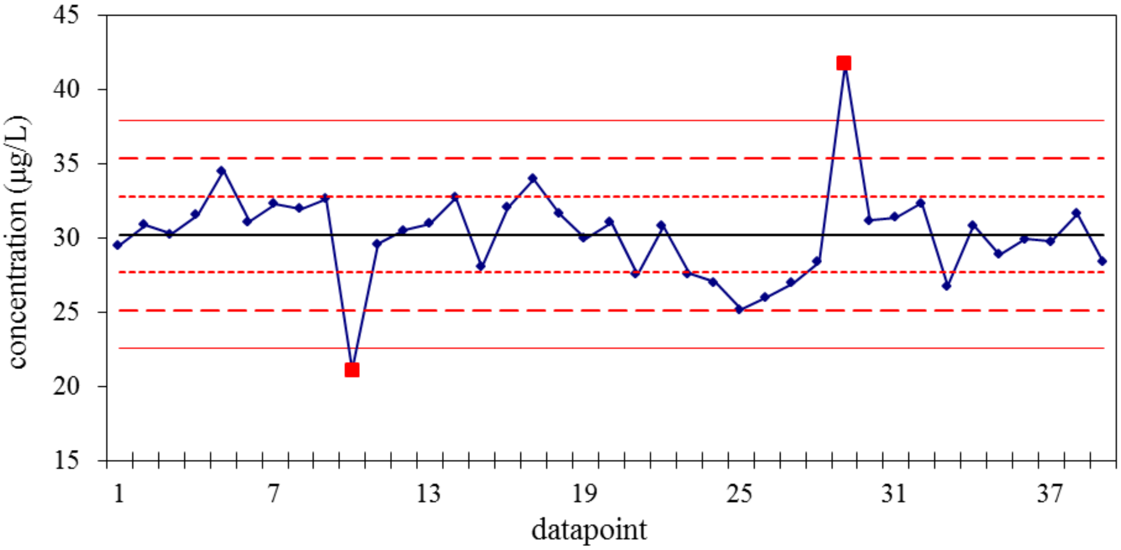

Figure 16: Process control chart for *fuco* from the DHI mixed pigment batch #108 over a 3 year period from Nov 2010-Dec 2013. Solid red lines indicate the control limits (99% CI), long dashed lines indicate the warning limits (95% CI); solid black line is the mean *fuco* concentration over that time period. The two points in red indicate concentrations falling outside of the control limits.

Since *chl a* is known to degrade in the mixed pigment standard over the time frame of a couple of years, it is necessary to confirm stability of the response factor for this pigment using standards whose concentrations and purity can be measured. Whenever batches of chlorophyll *a* standard are measured spectrophotometrically to determine precise concentrations, aliquots are dispensed into a dozen or more HPLC vials, sealed tightly and stored at -20 °C. These are then analyzed approximately weekly during sample analysis to confirm stability of the *chl a* response factors. Since no other pigments are present in these solutions it is simple to account for any chlorophyll degradation by calculating a new purity each time a vial is analyzed. The original concentration at the time the vials were dispensed is then purity corrected based on the present run to determine the injected amount of pure *chl a*. Injected amounts are typically ~200 ng or ~800 ng which fall in the middle of the *chl a* working range. Resultant peak areas are divided by the amount injected to get a single point Rf. This Rf value can be tracked on a process control chart along with those generated during multi-point calibrations. These regular single point calibrations confirm the stability of the *chl a* response factor as they have a relative percent difference (RPD) of only 2.2% on average and rarely exceed 4.7% (the 95% confidence interval for these measurements). This is in line with the 4.1% RPD for *chl a* calibration that represents the 95% CI for Horn Point lab, a state of the art facility (Roy et al. 2011). Any points that exceed the 95% CI warning limit are usually the result of a standard that has degraded below an acceptable purity for calibration (typically <90%), or has concentrated from evaporation as a result of being stored too long, as opposed to an actual change in the response. Freshly prepared and analyzed standards always fall back in line with the mean Rf.

7.4 EVALUATING METHOD PRECISION

7.4.1 Multiple injection precision

A number of different performance metrics can be used to assess an overall HPLC method and many are based on the precision of replicate injections. The retention time variability noted above in the context of specificity is between different samples analyzed on the same day over a 24 h period using the same eluent batches compared to variability in the RT of the mixed pigment standard over multiple days with different solvent batches. Retention time repeatability, a performance metric as defined in the SeaHARRE-2 intercomparison (Hooker et al. 2005) is the retention time precision of triplicate injections of the same sample, run sequentially under the same conditions. To satisfy the requirements of a quantitative HPLC method the retention time repeatability should not exceed 0.07% (Hooker et al. 2005). Our retention time repeatability is limited by our inability to maintain constant column temperature due to a lack of a column heater on our HPLC system producing at best a 0.1%CV which can be as high as 0.4%CV averaged across all pigments in a chromatogram. Nonetheless, this performance metric is probably the least important of all as it only affects specificity, and not accuracy or precision of pigment concentrations. As noted above, a number of steps are taken to ensure specificity and to minimize the impact of shifting retention times. Excellent precision in RT repeatability would be required for pigment identification in labs not running a mixed pigment standard to verify daily RT changes.

Triplicate injections of mixed standards or samples also allow calculation of injector precision which in our case inherently contains a measure of integration precision since all integrations are performed manually. Replicate TAC reference injections are performed intermittently to ensure that single injections are representative when used to normalize internal standard peaks in samples for extraction volume calculations. Precision of these replicate injections is always <1%CV as is the average precision for *chl a* meeting the requirements of a state-of-the-art method for this pigment (Hooker et al. 2005). Triplicate injections from the same sample vial give an average precision across all peaks of <3%CV meeting the requirements of a quantitative method. This precision is reduced slightly in complicated or concentrated extracts mainly as a result of resolution problems and integration difficulties from large peaks overlapping and merging together as opposed to injection precision per se. Thus finding the optimal concentration of extracts by adjusting filtration and extraction volumes is important for maintaining precision.

7.4.2 Short-term pigment stability

Samples are analyzed in sets of 25 over a 24 h period with all samples being loaded into the temperature-controlled autosampler compartment of the HPLC at the beginning of the run. This means that some samples are analyzed immediately after extraction whereas others spend almost 24 h at 4 ºC prior to analysis. Phytoplankton pigments are known to be highly labile, sensitive to temperature and light changes and thus can be expected to degrade in the autosampler especially in the presence of cellular enzymes in the sample extracts. Since all samples are run in duplicate but duplicates are always randomized and not analyzed in sequence or necessarily on the same day, this variability in the time spent in the autosampler has the potential to affect the overall precision of replicates.

To investigate the magnitude of this impact, the first sample extracted on 6 separate days was split into 2 HPLC vials, with one vial analyzed at the start of the sample set and the other analyzed at the end. Chlorophyll *a* concentrations for these samples ranged from 0.4-6.4 µg/L. Across all 5 samples, concentrations of all the chlorophylls (*a, b, c*) were lower on average after the 24 h period compared to those analysed at the start of the run whereas average changes in carotenoid concentrations were mixed (Table 14). Averaged across all pigments, across all samples, there was a mean decrease in pigment concentrations of 1.4% after the 24 h period. Interestingly, the reduced pigment concentrations in the longer stored aliquots were largely the result of reduced final extraction volumes in these aliquots compared to their earlier run counterparts as in some cases concentrations had decreased while peak areas had actually increased. In all 5 cases TAC peak areas were higher after 24 h likely indicating that some evaporation is occurring from the sealed HPLC vials over this time-frame leading to the larger TAC peaks in the samples and resultant reduced V_x. Although *chl a* concentrations decreased in all 6 samples, the reductions were relatively minor ranging from 0.2-7.6% (average -4.1%; Table 14). The largest decline in pigment concentrations was observed for *chl c3* with the peak area falling below LOQ for the lowest biomass sample after 24 h resulting in an average change across all 6 samples of –28.5%. *Chl b* also showed a significant average decline of -13.0%, which is consistent with the findings from SeaHARRE-2 where *chl b* and *c* were found to be the least stable pigments (Hooker et al. 2005). However, the authors did not distinguish between *chl c3* and *c2,* and the latter showed relatively good stability with an average decrease of -4.8% similar to that of *chl a*. Only *β-car* showed an increase in peak area and concentration for all six samples (only data above LOQ is shown in Table 14), and along with many of the carotenoids (*fuco, neo, pras, allo*, *diato*, *zea*) had overall positive changes in pigment concentration for the later run aliquot (Table 14).

Pigments are thought to be less stable in methanol compared to acetone and this trend was observed in short-term stability experiments during SeaHARRE-2 with average changes of -4.1% and -7.2% for the primary pigments (PPig) in acetone (PPig= *chl b, chl c, peri, fuco, diadino, allo, hex-fuco, zea, but-fuco, diato,* and *β-car*) and Tchl (*a, b, c*), respectively compared to -10.0% and -34.7% for the PPig and Tchl in methanol (Hooker et al. 2005). Our average changes were only -3.2% for the PPig which is less than the changes observed in acetone in that study. The average change in Tchl was –12.6%, about a third of that reported in SeaHARRE-2 for methanol extracts and largely influenced by the decreases in *chl c3*, including one -100% value. If this value near the LOQ is excluded then the average decrease for all the chlorophylls reduces to -8.1%. In conclusion, although some pigments show definite changes with time spent in the autosampler, for many pigments the differences were small and on the order of those for injector precision. Although our samples are all extracted in methanol, the short-term stability of pigments in this solvent observed here was markedly better than that reported in SeaHARRE-2 (Hooker et al. 2005).

Table 14: Percent differences in pigment concentrations after 24 h at 4 ºC in the autosampler for 6 samples in order of initial *chl a* concentration. Values in blue are for pigments not part of the PPig group according to Hooker et al. 2005.

Sample	1	2	3	4	5	6	**avg.**
chl c3	-100.0%	5.1%	-11.5%	-4.2%	-44.5%	-15.8%	**-28.5%**
MgDVP				-13.0%		-8.0%	**-10.5%**
chl c2	-12.6%	-0.1%	3.7%	-2.0%	-10.3%	-7.2%	**-4.8%**
peri		10.7%		-32.1%	-4.6%		**-8.7%**
but-fuco		-6.2%	-18.8%	-8.5%	-5.3%		**-9.7%**
fuco	11.2%	-3.2%	0.4%	-2.5%	-1.2%	-2.4%	**0.4%**
neo				5.3%	21.6%		**13.5%**
prasino			25.5%	-2.9%	-1.5%		**7.0%**
viola			0.0%	-10.7%	0.5%		**-3.4%**
hex-fuco	-13.5%	1.7%	-0.4%	-6.7%	-10.5%		**-5.9%**
diadino	10.5%	0.3%	0.6%	-1.8%	-8.8%	-5.9%	**-0.9%**
allo				4.8%	3.5%		**4.1%**
diato		2.1%		26.0%	1.7%		**9.9%**
zea	4.8%		-10.1%	-0.5%	12.9%		**1.8%**
chl b	-28.1%	-5.0%	-17.9%	13.2%	-27.1%		**-13.0%**
chl a	-7.6%	-3.7%	-0.2%	-2.6%	-5.9%	-4.8%	**-4.1%**
B-car				14.5%	15.2%	27.8%	**19.1%**
chl a µg/L	0.42	0.53	0.79	2.52	2.80	6.39	

7.4.3 Precision of replicate field samples

Perhaps the most useful precision measurement is the one that compares the pigment yields from replicate filters as it provides the overall effective precision of the entire method that takes into account all of the relevant steps involved in sample collection including sampling, filtration, handling, storage, and extraction, in addition to the various analytical precision factors discussed above. It is this effective precision that is most important to constrain as the variability involved with sample collection, handling and extraction can be much higher than that of the analysis, rendering analytical precision essentially meaningless. For example, phytoplankton are known to exhibit patchiness in the water column which can lead to low sampling precision even from the same Niskin bottle especially when biomass is high, collected volumes are low, and cells are large chain-forming diatoms or aggregates. Spills can occur during filtration which are difficult to estimate and adjust for in terms of lost volume, and filtration duration and filter handling can lead to degradation that can also affect precision. One of the biggest contributors to large variability between replicates is poor extraction efficiency, particularly when too much volume has been filtered (see Section 4.2.4).

The %CV of duplicate filters for *chl a* for all 58 cruises analyzed to date composed of just over 7000 samples ranges from 2.7-34.3% with an average of 7.8%. The dataset with the 34.3% CV was highly anomalous and this poor precision was almost exclusively the result of poor extraction efficiency resulting from too much volume being filtered despite high phytoplankton biomass. This situation is now avoided with the adoption of variable filtration volumes as described in Section 3.2 and improved extraction protocols for high biomass samples. Precision for other common pigments (*fuco, chl c2, hex-fuco, diadino, chl b*) in field samples is also <10% when these pigments are >LOQ in the majority of samples in a dataset. Across all 58 cruises, the two most common and abundant accessory pigments with the best precision are *fuco* and *hex-fuco* with 8.5%CV and 9.0%CV, respectively. Only 8 out of 58 cruises have *chl a* precision that is worse than 10% and with these excluded the mean reduces to 6.4% CV. It should be noted that this average precision for *chl a* encompasses datasets of field samples with highly variable biomass levels, collected over the span of 15 years by multiple samplers, filtered, handled and stored in varying ways, and with many datasets having replicates extracted by different protocols as our methods have evolved (see Section 4.2.2), and thus is lower than that considered quantitative by the SeaHARRE-2 guidelines (Hooker et al. 2005). However, such guidelines are set with the use of carefully collected and standardized intercomparison samples usually with optimum pigment detectability. Furthermore, many labs do not routinely collect replicate field samples for assessing precision, let alone collect all samples in duplicate preventing comparison of our values to other labs. When our method is evaluated with optimal intercomparison samples the precision for *chl a* is <1% and satisfies state-of-the-art requirements as per the SeaHARRE-2 guidelines (see Section 8.2). The biggest analytical obstacle to achieving good precision occurs for pigments present in low amounts near the LOQ where in one replicate they are quantifiable, and in the other they are not. Short term stability in the autosampler likely plays a role for reducing precision in some pigments, most notably *chl c3* as seen above.

8.0 PIGMENT ANALYSIS INTERCOMPARISONS

The precision of an HPLC method can be determined quite easily. Replicate injections of a single sample or standard can give a precision value that includes both injector and integration precision. Analysis of replicate sample filters gives a measure of precision that includes extraction techniques as well as the natural variability in the environment. Accuracy however, is much more difficult to assess as there is no way to know the "true" concentration of a given pigment. Even when best laboratory practices are followed, good calibration curves are established, extraction methods are tested, and quality assurance, quality control procedures are followed, uncertainties will exist. The best way to validate an existing method and get a measure of accuracy is through intercomparisons with other labs. With enough participant labs analyzing replicate samples an average consensus value can be reached that can be assumed to approximate the "true" value to which an individual lab's value can be compared.

8.1 UQAR INTERCOMPARISON

We conducted a two lab intercomparison to evaluate our methods and calibration parameters against those of the established phytoplankton pigment lab at Université du Quebec à Rimouski (UQAR) under the supervision of Dr. Suzanne Roy. Triplicate or quadruplicate filters were collected at several stations along Line P as part of the regular sampling program during August cruise 2012-13. Duplicate samples were analyzed at IOS in October 2012 and the remaining filters as well as split sample extracts were shipped to UQAR on dry ice for analysis there in November 2012. This intercomparison aimed to differentiate between calibration and extraction effects on pigment values by comparing a) filters extracted and analyzed independently by both labs (calibration and extraction), b) extracts prepared at IOS and analyzed by both labs (calibration only), and c) filters extracted by each lab's standard method and analyzed at UQAR (extraction only).

The extraction method used at UQAR differs significantly from that used at IOS but the HPLC method is very similar. The instrument is an Agilent 1200 series that uses the same Waters column and the same Zapata et al. (2000) separations method as ours. Calibrations are performed in a similar manner using DHI standards, concentrations of which are confirmed spectrophotometrically with multi-point calibration curves generated from a single mix using the injector to deliver varying volumes. An internal standard is also used to correct for extraction volume changes and injector variability.

8.1.1 Extraction method comparison

To compare the extraction protocols of both labs independent of pigment calibration differences, two sets of duplicate filters from Station P26 were extracted and analyzed at UQAR with each of the two standard methods. At the UQAR lab, the standard extraction protocol involves sonication. Diced filters are placed in 95% methanol and the sonicator probe is used to manually grind the filter against the side of the plastic centrifuge tube during sonication, which occurs in two short bursts of approximately 15 seconds each. The additional grinding action helps to break down the filter material into a thick slurry. Once all samples are sonicated they are immediately centrifuged, clarified by filtration and analyzed with no additional soak time. In contrast, previous IOS methods used a 60 second sonication with no mechanical action followed by a 2-4 h soak (Method C; Table 7).

Surprisingly, in both cases the standard UQAR sonication method produced marginally higher yields for most pigments than the standard IOS 24 h soak method contrary to all tests previously conducted at IOS. Differences were typically <10% though sonication produced a 21% higher yield in *chl b* in the 40 m sample and a 21% higher yield of *but-fuco* in the 25 m sample (Fig. 17). Why the results in this experiment were contrary to every sonication vs. soaking experiment carried out at IOS is unclear, but may be related to differences in the power and type of sonicator used, sonication duration, the extra grinding action used by UQAR, and/or soak time or lack thereof. Thus, it appears even seemingly subtle nuances in methodology can lead to varying results. For most pigments however, the differences in yield were small and within the range of natural replicate variability. In addition, only a single replicate was extracted with each method so we were unable to compare the precision of each method. Our past results have always shown increased variability with the additional sample handling associated with dicing and sonication. Given this, and our need to process many more samples in a given day with a simpler, less labour-intensive method, there was not a strong enough case to consider changing our extraction method based on this result.

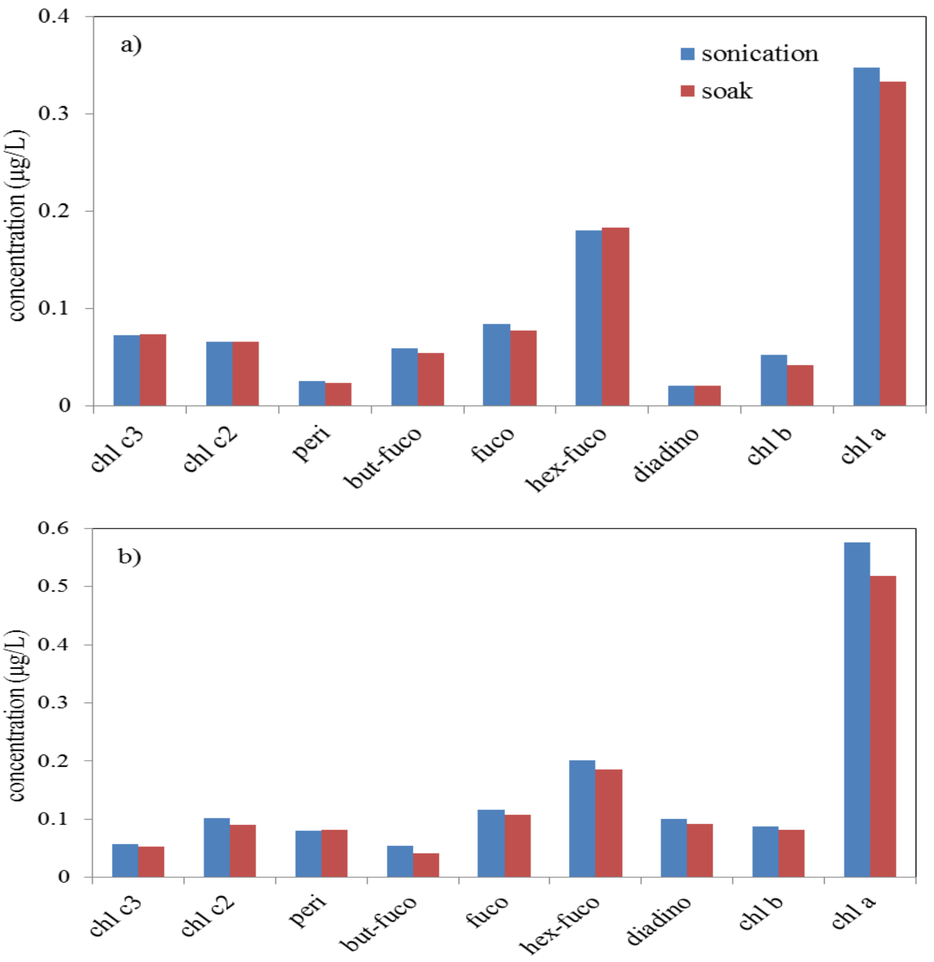

Figure 17: Comparison of pigment yields from replicates of 2 samples collected in August 2012 at Station Papa and analyzed at UQAR, a) from 40 m depth, and b) from 25 m depth. Blue bars represent the standard UQAR extraction protocol and red bars represent standard IOS Method A.

8.1.2 Calibration

To subtract the effects of extraction method differences on yields, 3 extracts that were prepared at IOS and split in 2 for analysis by each lab, and 2 replicate sample filters that were extracted with the IOS soak method in both labs were analyzed to determine differences in calibration factors only. Unfortunately, only 9 of the 24 pigments we measure were present in the samples analyzed, limiting the scope of the comparison. Six out of the 9 pigments were in very good agreement between the two labs with an absolute relative percent difference (RPD) in concentrations of <6% averaged across the 5 samples (Table 15). The other 3 pigments (*peri*, *but-fuco* and *diadino*) had poor agreement on average with concentration differences ranging from 15-29%, although the *peri* concentrations were similar in one sample and different in the other (Table 15). These differences in the calculated concentrations of these pigments indicate an error in the calibration parameters of one of the labs, which can become magnified with the concentration of the pigment. For example, at very low biomass there is ~10% difference in the *but-fuco* concentration which increases to 46% as the *chl a* concentration approaches 1 µg/L.

Table 15: RPD by sample number between calculated concentrations of each pigment on filter extracts prepared at IOS and then analyzed at both IOS and UQAR (single split extract), and separate replicate filters extracted with the IOS method and then analyzed in both labs (same extraction). A positive percentage indicates the IOS value was higher. The *chl a* concentration is given for each sample in µg/L.

	single split extract			same extraction method		
Pigment	568B	576A	833A	570	571	avg. ABS RPD
chl c3		5.4%	7.5%	1.2%	7.6%	**5.4%**
chl c2	-10.2%	6.3%	2.8%	-6.3%	3.8%	**5.9%**
peri				2.5%	-27.5%	**15.0%**
but-fuco	-10.1%	-19.8%	-46.0%	-27.9%	43.2%	**29.4%**
fuco	-2.0%	5.8%	1.1%	-3.0%	12.9%	**4.9%**
hex-fuco	1.3%	0.1%	-1.3%	-2.9%	2.3%	**1.6%**
diadino	-17.5%	-19.3%	-15.8%	-52.3%	-4.1%	**21.8%**
chl b	-11.2%	-2.6%	-3.5%	4.4%	-5.7%	**5.5%**
chl a	-2.2%	3.6%	-2.5%	-5.4%	5.6%	**3.9%**
chl a µg/L	0.25	0.33	0.95	0.32	0.55	

8.1.3 Calibration and extraction combined

The majority of the intercomparison samples were filters which were analyzed separately in each lab by their current respective methods to compare the combined extraction and calibration differences between the two labs. Comparing the average RPD for each pigment between the two types of comparisons (calibration only vs. calibration and extraction combined) gives us an idea of which pigments are suffering from extraction problems. In this case, the concentration differences for *chl c2*, *fuco* and *hex-fuco* increased slightly but the results were still in good agreement with <10% difference between the two labs (Table 16). The average absolute RPD for *peri*, *but-fuco*, and *diadino* remained largely unchanged from the calibration only comparison, indicating that there was little effect from differences in extraction methodology. However, the agreement for *chl b* and *chl a* which was good when only the calibration was considered, became considerably worse, especially for *chl b* with UQAR values consistently higher than those measured at IOS by ~50% on average (Table 16). This indicates low extraction efficiency for these pigments with our method compared to the UQAR one. However, this figure is not as alarming as it seems, since it appears to be a factor mostly at very low biomass (0.2-0.3 µg/L *chl a*), and the vast majority of field samples we routinely encounter have biomass levels much higher than this. At *chl a* >0.5 µg/L, *chl a* concentrations were within 5% of each other with higher concentrations reported for the IOS sample at 10 µg/L, and *chl b* differences also reduced dramatically. This extraction problem with *chl b* may be more a function of low recovery of this pigment from 47 mm filters for reasons yet unknown as seen in the various extraction efficiency experiments (see Figs. 10, 11) as opposed to indicating the need for mechanical filter disruption. Overall, the exercise was very useful and the results of this intercomparison encouraging as they confirmed good results for a number of important pigments (*chl c3, chl c2, fuco, hex-fuco*), identified pigments whose calibrations should be re-examined (*peri, but-fuco* and *diadino*) and pointed to extraction issues with *chl a* and *chl b* at low biomass.

Table 16: RPD between calculated concentrations of each pigment for replicate filters analyzed at IOS and UQAR by their respective methods in order of increasing *chl a* concentration (µg/L). A positive percentage indicates the IOS value was higher.

Chl a conc.	chl c3	chl c2	peri	but-fuco	fuco	hex-fuco	diadino	chl b	chl a
0.22		-8.8%		-26.1%	-12.8%	-9.2%	-26.5%	-51.6%	-18.5%
0.25	-15.0%	-21.5%		-42.4%	-13.6%	-12.0%	-12.9%	-68.9%	-30.1%
0.26	1.4%	-14.7%		-50.9%	-13.6%	-5.9%	-26.7%	-55.1%	-21.1%
0.27	-5.6%	-12.6%		-28.0%	-11.3%	-6.3%	-28.9%	-65.0%	-22.4%
0.31	0.4%	3.1%		-16.8%	6.6%	3.8%	-22.4%	-37.4%	-12.9%
0.32	2.6%	-6.3%	-5.5%	-37.5%	-10.6%	-1.2%	-52.3%	-19.4%	-9.9%
0.34	1.6%	-1.6%		-23.0%	-5.4%	0.2%		-75.9%	-19.4%
0.40	-0.5%	-5.3%		-30.5%	-4.4%	-3.4%	-24.7%	-59.0%	-13.6%
0.55	0.4%	-8.8%	-24.8%	21.4%	5.9%	-5.5%	-13.7%	-14.3%	-4.8%
10.49		4.0%	19.5%		12.3%		-13.0%		3.5%
avg. ABS	**3.4%**	**8.7%**	**16.6%**	**30.7%**	**9.6%**	**5.3%**	**29.8%**	**49.6%**	**15.6%**

8.2 DHI INTERCOMPARISON

Around the same time of the UQAR intercomparison, we were already committed to participating in an international pigment intercomparison coordinated by DHI, the commercial pigment supplier in Denmark. This presented a good opportunity to further evaluate the accuracy of our methods and calibration and possibly shed light on the few discrepancies seen between our results and those of UQAR. Fourteen participant labs from 11 countries were shipped triplicate 25 mm GF/F filters containing mixed algal cultures. HPLC separations and extraction methods varied greatly among the participants. Three labs used the same Zapata et al. (2000) HPLC method as us; 4 others used Wright et al. (1991), while the most popular method used by 5 labs was Van Heukelem and Thomas (2001). Differences in extraction protocols were even more varied. Interestingly, ours was the only lab to not employ any kind of mechanical filter disruption. Two labs used a cell homogenizer whereas the rest used some form of sonication with either a sonic probe (10 sec-2 min durations) or a sonic water bath (5-10 min durations). Soak times also varied greatly ranging from 0-30 h. Our samples were analyzed using the standard method A extraction protocol.

The compiled results showed that there were varying degrees of competency and experience between the participant labs with some reporting as few as 2 pigments. Of the 14 labs, 9 including ours provided values for the 17 major pigments present in the sample and were thus used as the reference labs (Table 17). The mean concentrations and 95% confidence intervals (CI) for each of the pigments reported by the reference labs were used as the “proxy of truth” to which each individual lab’s results were compared. All three of the common HPLC methods were represented in this reference group indicating good results are possible with any of these methods (Table 17). Of the 14 labs, we placed 4th overall in terms of accuracy with an average absolute percent deviation (APD) from the mean across all pigments of 7.35% (Table 17). Three of the pigments (*chl a*, *chl c2*, *chl c3*) agreed to within 1% of the mean reference value.

Table 17: Accuracy and precision results for all 14 participant labs. Accuracy is given as the absolute percent deviation (APD) from the reference value for all pigments and precision as the %CV of triplicates. Labs in grey were not part of the reference group. Our lab, XK is highlighted in red.

		accuracy (APD)		precision (%CV)	
Lab code	HPLC method	all	*chl a*	Ppig	all
MP	Zapata	30.22	2.18	5.05	5.71
WT	Wright	22.97	1.91	4.78	6.46
YL	Zapata	7.26	2.48	1.47	1.99
ZB	Wright	11.89			
FE	Wright	14.21	1.00	3.54	3.48
XK	**Zapata**	**7.35**	**0.43**	**1.95**	**2.87**
HI	Van Heukelem	6.52	0.88	1.68	1.77
NQ	Schmid	19.51	2.79	5.79	6.05
CS	Zapata	14.71	6.54	5.65	5.45
SJ	Wright	11.31	0.95	1.89	1.89
AS	Van Heukelem	7.45	1.59	1.67	2.52
LK	Van Heukelem	4.97	0.37	1.33	1.50
WJ	Van Heukelem	8.92	0.69	2.54	2.52
PN	Van Heukelem	9.71	3.26	2.97	3.01
avg. ref. labs		**9.04**	**1.91**	**2.53**	**2.80**
IOS rank		4th	2nd	6th	6th

Performance metrics established during SeaHARRE-2 (Hooker et al. 2005) state that for a lab`s results to be considered state-of-the-art in terms of precision, the %CV between replicate filters should be <2% for *chl a* and <3% for the other primary pigments (*chl b, chl c, peri, fuco, diadino, allo, hex-fuco, zea, but-fuco,* and *diato*). Eight out of the 14 labs including ours were able to achieve this performance level (Table 17). We placed 2nd overall for *chl a* precision with a 0.43%CV and in the middle of the pack at 6th overall with an average 1.95%CV for the primary pigments and a 2.87% CV across all pigments, mainly due to a high 14%CV in the *viola* value. The latter is always a tricky pigment to quantify since it is present in low amounts and elutes very close to *hex-fuco*, making resolution between the two pigments low.

Our intercomparison samples were analyzed in Dec 2012 while we were in the process of completing a full pigment calibration. As such submitted results were mostly based on the Rfs from the previous May 2011 calibration. Response factors for about half the pigments changed with the completion of the calibration in Feb 2013. For our interest, and in order to re-evaluate our performance in this exercise we present the pigment concentrations as they were submitted and as they changed along with the reference values in Table 18.

Table 18: Pigment concentrations in the DHI intercomparison sample in µg/L as submitted, and as they changed (bold) following the Feb 2013 calibration compared to the average derived from the 9 reference labs. Checkmarks indicate our values fall within the 95% CI of the reference (black as submitted, red only after recalibration, grey as submitted but not after recalibration).

Pigment	submitted	updated	reference	95% CI
chl c3	0.88	0.88	0.88	√
chl c2	1.96	1.96	1.96	√
peri	2.13	2.13	2.24	√
19-but	0.58	**0.54**	0.52	√
fuco	0.54	**0.53**	0.60	√
neo	0.37	0.37	0.33	
prasino	1.65	**1.62**	1.70	√
viola	0.12	**0.11**	0.15	
19-hex	1.29	**1.23**	1.21	√
diadino	0.74	**0.64**	0.71	√
allo	0.87	**0.85**	0.84	√
diato	0.26	**0.23**	0.24	√
zea	0.32	**0.25**	0.27	√
lutein	0.12	0.10	0.11	√
chl b	3.15	3.15	3.01	√
chl a	13.33	13.33	13.43	√

As shown above in Table 17, we placed high in terms of overall accuracy but there were 5 pigments whose values fell outside the 95% CI of the reference values (*but-fuco*, *neo*, *hex-fuco*, *zea*, and *viola*). Of these, the response factors for *but-fuco*, *hex-fuco* and *zea* all changed post-calibration bringing the re-calculated values within the 95% CI (Table 18). The revised concentrations for all pigments whose Rf changed had little effect on the reference means and standard deviations of these pigments, at most changing them by 0.01 µg/L. It was encouraging that in most instances this recalibration improved our results for the DHI intercomparison bringing more pigments within the 95% CI of the mean and improving our overall accuracy beyond that reported in Table 17. However, the *diadino* Rf changed significantly enough to fall outside the 95% CI of the reference lab mean (Table 18). Why the Rf changed so much for this pigment and why this updated value yielded poorer results is unclear since standard concentrations were measured spectrophotometrically prior to calibration, an unopened standard vial was used (though 4 years old), and injections from the standard mix fell in line with those of the pure standard. Given the discrepancy between the Feb 2013 *diadino* Rf and the UQAR and DHI intercomparison values, recalibrating *diadino* in the near future is a priority.

8.3 SUMMARY OF INTERCOMPARISON FINDINGS

Participation in both the UQAR and DHI intercomparisons proved very useful and informative and there were strengths and weaknesses in each study. The advantage of the DHI sample was that it contained all of the pigments quantified at IOS (plus a few unknowns) in sufficiently high amounts such that detection was not a problem allowing a comparison of yields for most of the pigments typically encountered in field samples. This was an excellent way to assess calibration parameters against other labs as well as compare across a myriad of different extraction protocols and HPLC methods. However, the concentrations of some pigments in the sample were well above levels seen in typical field samples and it would have been useful to have a second sample with lower concentrations more representative of typical field samples to assess performance near the LOD/LOQ where most of the challenges lie. It would also have been advantageous to have a sample extract as well as filters in order to differentiate between the effects of calibration and extraction on yields as was done with the UQAR exercise. Having a triplicate sample was useful for assessing precision though, something that was lacking in the UQAR exercise.

In the DHI intercomparison the participant labs were all assigned codes to ensure anonymity but transparency of the participants as in the SeaHARRE exercises (Hooker et al. 2005, 2010) would have been preferred in order to compare our results with labs such as Horn Point and DHI that have proven state-of-the-art performance. Having no information about the participants, their experience and whether their methods had been validated provided little confidence in some of the results. However, as noted in the final DHI report, this intercomparison coincided with the SeaHARRE-6 intercomparison with 4 of the participants from the latter exercise participating in this one. Horn Point lab has participated in every previous SeaHARRE intercomparison and DHI has been involved since SeaHARRE-2 thus we assume they were also SeaHARRE-6 participants. By comparing their published HPLC and extraction methods (Hooker et al. 2010) to those provided by the DHI participants we are fairly confident in deducing that lab LK is Horn Point and lab HI is DHI. This gives us greater confidence in our methods given that our results compare favourably to those of two validated, state-of-the-art labs with proven performance records (Table 17), one of which is the main provider of pigment calibration standards. It was also encouraging to see our results falling in line with the mean for most pigments despite being the only lab in the exercise to not use any form of sonication during extraction. This validates our previous experiments that have all shown good results with just soaking compared to sonication. In the UQAR intercomparison we did have lower yields of *chl a* and *chl b* but this was at much lower biomass and may be related to poor recovery of these pigments from the 47 mm filters for yet unknown reasons. In the DHI intercomparison our *chl a* and *chl b* values were well within the 95% CI of the reference and on the higher side of the mean indicating extraction efficiency was likely not an issue. Our *but-fuco* and *peri* results were accurate in the DHI study but very different from those of UQAR in that intercomparison, suggesting a possible problem with the UQAR calibration for these two pigments, however the concentrations were 10 times higher in the DHI sample than in the Line P samples analyzed at UQAR so this assessment is not straightforward. Overall the results of both of these exercises prove that we have made great strides in the past 7 years in establishing a proven protocol for phytoplankton pigment analysis by HPLC that gives at least quantitative results or better. Participating in an upcoming SeaHARRE exercise involving labs with validated methods and a larger range of pigment concentrations would be a useful next step in assessing our current performance and potentially improving on it in the future.

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REFERENCES

Bidigare, R.R., Van Heukelem, L. and Trees, C.C. 2003. HPLC phytoplankton pigments: sampling, laboratory methods and quality assurance procedures. *In* Ocean optics protocols for satellite ocean color sensor validation, revision 4, volume V: Biogeochemcial and bio-optical measurements and data analysis. Edited by: J.L. Mueller, G.S. Fargion and C.L. McClain. NASA Goddard Space Flight Center, Greenbelt. pp 5-14.

Bidigare, R.R., Van Heukelem, L. and Trees, C.C. 2005. Analysis of algal pigments by high-performance liquid chromatography. *In* Algal Culturing Techniques. Edited by R. Andersen. Elsevier Academic Press, Burlington, pp 327-345.

Booth, B.C., Lewin, J. and Postel, J.R. 1993. Temporal variation in the structure of autotrophic and heterotrophic communities in the subarctic Pacific. Prog. Oceanog. 32, 57-99.

Duarte, C.M., Marrasé, C., Vaqué, D. and Estrada, M. 1990. Counting error and the quantitative analysis of phytoplankton communities. J. Plankton Res. 12, 295-304.

Harris, S.L., Varela, D.E., Whitney, F.W. and Harrison, P.J. 2009. Nutrient and phytoplankton dynamics off the west coast of Vancouver Island during the 1997/98 ENSO event. Deep-Sea Res. II 56, 2487-2502.

Hooker, S.B., Van Heukelem, L, Thomas, C.S., Claustre, H., Ras, J., Barlow, R., Sessions, H., Schluter, L., Perl, J., Trees, C., Stuart, V., Head, E., Clementson, L., Fishwick, J., and Aiken, J. 2005. The second SeaWiFS HPLC analysis round-robin experiment (SeaHARRE-2). NASA Tech. Memo. 2005-212785, NASA Goddard Space Flight Center, Greenbelt, Maryland. 112 p

Hooker, S.B., Thomas, C.S., Van Heukelem, L, Schluter, L., Russ, M.E., Ras, J., Claustre, H., Clementson, L., Canuti, E., Berthon, J-F., Perl, J., Normandeau, C., Cullen, J., Kienast, M., and Pinckney, J.L. 2010. The fourth SeaWiFS HPLC analysis round-robin experiment (SeaHARRE-4). NASA Tech. Memo. 2010-215857, NASA Goddard Space Flight Center, Greenbelt, Maryland. 74 p

Jeffrey, S.W and Hallegraeff, G.M. 1987. Chlorophyllase distribution in 10 classes of phytoplankton- a problem for chlorophyll analysis. Mar. Ecol. Prog. Ser. 35, 293-304.

Jeffrey, S.W., Mantoura, R.F.C., and Wright, S. W. (Eds.). 1997. Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods, UNESCO monographs on oceanographic methodology, Vol. 10. UNESCO, Paris. 667 p.

JGOFS. 1994. Protocols for the Joint Global Ocean Flux Study Core Measurements. Intergovernmental Oceanographic Commission, Scientific Committee on Oceanic Research. Manual and Guides vol. 29. UNESCO Publishing, Paris. pp 111-117.

Letelier, R.M., Bidigare, R.R., Hebel, D.V., Ondrusek, M., Winn, C.D. and Karl, D.M. 1993. Temporal variability of phytoplankton community structure based on pigment analysis. Limnol. Oceanogr. 38, 1420-1437.

Mackey, M.D., Mackey, D.J., Higgins, H.W. and Wright, S.W. 1996. CHEMTAX-a program for estimating class abundance from chemical markers: application to HPLC measurements of phytoplankton. Mar. Ecol. Prog. Ser. 144, 265-283.

Roy, S. 1987. HPLC analysis of chloropigments. J. Chromatogr., 391, 17-34.

Roy, S., Llewellyn, C.A., Egeland, E.S., and Johnsen, G. (Eds.). 2011. Phytoplankton Pigments: Characterization, Chemotaxonomy, and Applications in Oceanography. Cambridge University Press, New York. 845 p.

Schlüter, L., Møhlenberg, F., Havskum, H. and Larsen, S. 2000. The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll a ratios. Mar. Ecol. Prog. Ser. 192, 49-63.

Strickland, J.D.H and Parsons, T.R. A Practical Handbook of Seawater Analysis. 1972. Fisheries Research Board of Canada, Bulletin 167, Ottawa. 310 p.

Wasmund, N., Topp, I. and Schories D. 2006. Optimising the storage and extraction of chlorophyll samples. Oceanologia. 48, 125-144.

Wright, S.W., Jeffrey, S.W., Mantoura, R.F.C., Llewellyn, C.A., Bjornland, T., Repeta, D., and Welschmeyer, N. 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. Mar. Ecol. Prog. Ser. 77, 183-196.

Wright, S.W., Thomas, D.P., Marchant, H.J., Higgins, H.W., Mackey, M.D. and Mackey, D.J. 1996. Analysis of phytoplankton of the Australian sector of the Southern Ocean: comparison of microscopy and size frequency data with interpretations of pigment HPLC data using the 'CHEMTAX' matrix factorisation program. Mar. Ecol. Prog. Ser. 144, 285-298.

Wright, S.W. 2005. Analysis of phytoplankton populations using pigment markers. course notes from: Pigment Analysis of Antarctic Microorganisms workshop. pp 1-49.

Wright, S.W. and Jeffrey, S.W. 2006. Pigment markers for phytoplankton production. *In* Marine Organic Matter: Biomarkers, Isotopes and DNA. Edited by J.K. Volkman. Springer Press, Berlin, pp 71-104.

Van Heukelem, L. and Thomas, C.S. 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. J. Chromatogr., 910, 31-49.

Zapata, M. and Garrido, J.L. 1991. Influence of injection conditions in reverse-phase high-performance liquid chromatography of chlorophylls and carotenoids. Chromatographia 31, 589-594.

Zapata, M., Rodriguez, F., Garrido, J.L. 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed-phase C8 column and pyridine-containing mobile phases. Mar. Ecol. Prog. Ser. 195, 29-45.

Zapata, M., Jeffrey, S.W., Rodriguez, F., Clementson, L., Garrido, J.L., and Wright, S.W. 2004. Pigment variability in 37 species (65 strains) of Haptophyta: implications for phylogeny and oceanography. Mar. Ecol. Prog. Ser. 270, 83-102.

APPENDIX 1: SUMMARY OF CURRENT IOS HPLC METHOD FOR PHYTOPLANKTON PIGMENT ANALYSIS

Seawater samples for HPLC pigment analysis are collected in duplicate from the surface layer into 625 ml, 1 L, or 2 L LDPE bottles. The fluorescence trace from the Seapoint sensor mounted on the CTD package is examined during the downcast, and fluorescence values at the desired sampling depths are noted. Bottle sizes are then chosen based on these *in situ* fluorescence readings according to Table 3, such that larger volumes are collected at low biomass and smaller volumes at high biomass. Sample bottles are rinsed with seawater and filled all the way to the top of the neck directly from Niskin bottles, with the larger volume bottles (1 L and 2L) filled using silicone tubing to minimize bubbles and cell disruption. Samples are generally filtered immediately following collection. HPLC samples are vacuum filtered (pressure <100 mm Hg) under low light onto 47 mm GF/F filters. The filters are carefully folded in half with the sample inside, blotted 3 times between sheets of acid-free blotting paper to remove excess water, and rolled into 5ml cryovials. Cryovials are immediately placed in a -80 ºC freezer or into liquid nitrogen, and are transferred to a -80 ºC freezer once back on land for storage pending analysis. Samples are typically analyzed within 1-3 months.

Just prior to analysis, filters are removed from the freezer and extracted for 24 h in 95% methanol containing TAC as an internal standard. Whole, folded filters are removed from cryovials with forceps and placed directly into glass test tubes pre-filled with 5 ml extraction solvent that had been stored at -20 ºC. Tubes are capped, quickly vortexed and then placed back in the freezer to extract for 24 h at -20 ºC. After the 24 h soak period, the sample extracts are vortexed again to ensure homogeneity and are filtered through 0.2 µm 13mm PTFE luer-lock syringe filters directly into amber glass HPLC vials for immediate analysis.

HPLC vials are placed into the 4 ºC autosampler compartment of a 2695 Waters Alliance HPLC equipped with a 2996 Photodiode Array Detector (PDA) and a 2475 Multi-wavelength Fluorescence Detector (FD) connected in series. Analysis is conducted using the HPLC method of Zapata et al. (2000) with a Waters Symmetry C_8 column (150 mm x 4.6 mm, 3.5 µm particle size) running a 45 min elution gradient of 50%:25%:25% methanol:acetonitrile:aqueous pyridine (pH 5.0) and 20%:60%:20% methanol:acetonitrile:acetone at a constant flow rate of 1 ml/min. Separations are performed on a 200 µl injection of sample which is layered with water (stored in the autosampler in a separate vial) at a ratio of 5:1 (200 µl sample:40 µl water) in 4 alternating draws into the sampling loop by the auto-addition function on the HPLC to improve peak shape of the early-eluting polar pigments. A mixed pigment standard containing most of the peaks of interest is run just prior to the samples to update the retention time of individual peaks for identification purposes, and to monitor the stability of the PDA detector and its associated response factors. An internal standard (TAC) reference vial is run at the end of each sample set to normalize the TAC peak in each sample for accurate final extraction volume calculations. Chlorophyll *a* standards are run approximately weekly to verify the Rf for this pigment. Chromatograms are processed using Waters Empower 2 software. Integration is manually optimized and peaks are identified based on their retention times and spectral matching to a custom library of pigment absorbance spectra. Results are exported into Microsoft Excel as peak tables which contain the list of identified pigments for each sample along with their corresponding RT, peak heights and peak areas. Peak areas are first screened to ensure they are above the LOQ peak area for each pigment. The amount of pigment in the sample is then calculated by dividing the peak area by the response factor for that pigment. Filtration, extraction and injection volumes are used to convert the amount of each pigment in ng to the *in situ* concentration in µg/L present in the original water sample. Final pigment concentrations for each sample are reported as the average of duplicates and sample precision is assessed as the %CV equivalent to the standard deviation divided by the mean.