An evaluation of the application of CHEMTAX to Antarctic coastal pigment data

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A B S T R A C T

Presented is an evaluation of the application of CHEMTAX (CHEMical TAXonomy) to Antarctic coastal pigments collected along the western Antarctic Peninsula (wAP). Overall analytical error is <20% for all pigments involved in the analysis. CHEMTAX was stable within a range of input pigment ratios; data were analyzed in three bins based on light depths, with each year’s data run independently. Results were validated by comparison to those from CHEMTAX methods that included randomized error, feedback loops and additional diagnostic pigments. Blooms during mid-summer (chlorophyll a concentrations >5 μg L−1) were dominated primarily by either diatoms or cryptomonads. Mixed flagellates can also be abundant and Pheocystis spp. and prasinophytes are frequently present in low concentrations. Comparison with microscopy shows CHEMTAX to give superior results in identifying Pheocystis spp. with favorable results for other groups. This analysis shows CHEMTAX to be a reliable and stable tool for providing estimations of the main phytoplankton taxa in wAP waters based on long-term data collected during a 12-year time series.

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1. Introduction

Common methods for estimation of phytoplankton abundance and composition include microscopy (e.g., Comi et al., 2007), flow cytometry (e.g., Smith et al., 2007; Sosik and Olson, 2007), genetic analysis (e.g., Medlin et al., 2006; Countway and Caron, 2006) and several versions of methods that can be categorized as chemotaxonomic, i.e., groupings based on the presence of chemical markers. The latter category includes the use of single or multiple pigment markers and multiple linear regression analysis to determine contribution by various groups to the total chlorophyll a (chl a) pool (Gieskes et al., 1988; Peeken, 1997), the application of inverse methods to develop a least-squares solution to a matrix algorithm (Tarantola, 1987; Letelier et al., 1993), and an iterative method of matrix factorization for determination of algal class abundance (Mackey et al., 1996).

Composition and distribution of phytoplankton communities can be documented using high performance liquid chromatography (HPLC) analysis of photosynthetic pigments (Jeffrey, 1980). Chemotaxonomic methods, based on the presence of characteristic pigments, mostly carotenoids, in algal phyla, are an extension of this approach (Gieskes and Kraay, 1986). The CHEMTAX (CHEMical TAXonomy) method has been widely used to determine phytoplankton composition, including in the Southern Ocean (e.g., Wright and van den Enden, 2000). Along with benefits such as having the ability to be reprocessed if needed, CHEMTAX has provided a means of estimating phytoplankton composition in datasets that might not have otherwise been analyzed for more traditional means of phytoplankton enumeration. CHEMTAX results have correlated strongly with those from microscopy, and in some instances have revealed the presence of groups not detected with traditional enumeration methods (e.g., cryptophytes, Wright et al., 1996; Havskum et al., 2004). Primary concerns regarding use of chemotaxonomic methods center around non-unique pigment markers (Schlüter and Mehlberg, 2003; Zapata et al., 2004) and potential fluctuations of the pigment ratios both at a species and at a cellular level under various physiological stressors (Jeffrey, 1981; Goericke and Montoya, 1998; DiTullio et al., 2007). However, with appropriate precautions, awareness of impacts and implications of physiological stressors, and some knowledge of potential populations within a sample region (Irigoien et al., 2004), CHEMTAX is considered a viable method for determination of phytoplankton composition (Mackey et al., 1998).
Although many studies have examined variability of Antarctic phytoplankton structure and dynamics, our understanding of the large-scale geographic and long-term temporal variability remains weak. Previous studies examining phytoplankton composition and distribution have primarily focused on short term, limited scale sampling. The Palmer Long Term Ecological Research (Pal LTER) project has as its focus the marine ecosystem of the western Antarctic Peninsula (wAP). Pal LTER has been collecting data in this region since 1991, providing a temporally and spatially extensive dataset for better understanding of the ecology of the area. HPLC analysis was chosen at the project's onset as a combined approach to taxonomy and optical studies related to phytoplankton under the premise that pigment analysis provides high consistency between users and laboratories with low variability and high reproducibility (Hooker et al., 2005), requires less time and labor than microscopy, and is a lower cost alternative to genetic studies and flow cytometry. However, the long-term nature of the Pal LTER data brings challenges not usually present in analysis of single cruises. Variability in methods and instruments as well as the large size of the dataset make the development of a consistent method for determination of phytoplankton composition particularly imperative.

The specific goal of this study was to establish and apply to the Pal LTER HPLC dataset a robust means of determining phytoplankton composition. In this paper, we report the results of phytoplankton community composition in the wAP from an analysis of 12 years of pigment data using CHEMTAX. Methods used for CHEMTAX processing CHEMTAX are reviewed, evaluated and validated. Results for three years are compared to microscopic classification and possible impacts of variation in light regimes on CHEMTAX output are presented. Examples of the resolution in phytoplankton group distribution as estimated by CHEMTAX are included.

2. Methods

2.1. Study area and sampling regime

Data was collected as part of the Pal LTER project along the wAP, from 63.6°S to 68.2°S and from 64 W to 73 W, bordering the eastern boundary of the Bellingshausen Sea to the South (Fig. 1). The region is characterized by a glacially sculpted coastline containing a series of islands, bays and passages (Anderson, 1999), and is often divided into three sub-regions, coastal, shelf and slope, based on bathymetry and associated biological and physical dynamics (Martinson et al., 2008).

Pal LTER large-scale sampling stations are laid out on a grid system (Waters and Smith, 1992), with transects (“grid lines”) 100 km apart running roughly south-east (onshore) to north-west (offshore) across the Antarctic continental shelf, approximately perpendicular to the coast. “Grid stations” are spaced 20 km apart along these lines (Fig. 1b). Sampling used in this analysis was limited to five cardinal lines, 200–600, which cover the region from Southern end of Anvers Island (600 line) to Marguerite Bay (200 line), and included all cardinal grid stations as well as other off-grid stations (e.g., near Palmer Station on Anvers Island, stations north and inland of Renaud Island and stations in the vicinity of southern Adelaide Island in the mouth of Marguerite Bay) occupied during yearly sampling efforts.

Water collection was done as part of the Pal LTER annual austral summer cruises from 1995 to 2007. Stations were occupied in most years between the first week in January and the first week of February (Table 1). Sampling was carried out from the R/V Polar Duke in 1995–1997 and from the ASRV Laurence M. Gould from 1998 to 2007. Not all stations were sampled on all cruises due to ice, weather or scheduling issues. HPLC data from January 2002 is not available.

2.2. Sampling and processing methods

At all stations sampled, water was collected in 10 L Go-Flo (1995–1997) or 12 L Niskin (1998–2007) bottles, at the surface and at depths corresponding to 50 (±6), 25 (±3), 10 (±2), 5 (±1) and 1 (±1) percent of surface photosynthetically available radiation (PAR, 400–700 nm). Light depths were determined immediately prior to the water collection using a Biospherical
Instruments QSP 200L4S sensor from 1995 to 2000 and 2003 and the PAR channel from a Biospherical Instruments Profiling Reflectance Radiometer during the remaining study years (600 Series 2001–2002, 800 Series 2004–2007). To ensure adequate light availability for instrumentation, water sampling was limited to daytime hours when the ambient PAR irradiance (4π) was greater than 100 μE m−2 s−1. This typically bound water sampling hours to between 0730 and 0100 GMT (next day). For purposes of this study, the euphotic zone depth (Zₑ) is defined as the 1% irradiance depth.

2.3. HPLC methods

From each depth sampled, 0.5–2 L of seawater was filtered, at <12 psi vacuum, under dim light conditions, onto 25 mm Whatman glass fiber filter (pore size nominally 0.7 μm; hereafter referred to as “GF/F”). Filters were stored in cryovials in liquid nitrogen until analysis. Samples were extracted and injected as soon as possible after collection (minimum of 24 h in liquid nitrogen); for years other than 2000, time between filtration and analysis was typically less than one month and no more than three months. Samples from January 2000 were transported (in liquid nitrogen) to Scripps Institution of Oceanography and analyzed within thirteen months of collection.

Filters were extracted in 90% HPLC grade acetone and either (a) manually crushed with a clean Teflon pestle, stored at −80 °C for 24 h, and pre-filtered through another GF/F before injection (1995–2001) or (b) ultrasonicated while held in a −20 °C benchtop cooler for 10 s, stored at −80 °C for 24 h and pre-filtered through a 1 μm syringe tip glass fiber filter (2003–2005) or a 0.45 μm Whatman nylon Puradisk filter (2006–2007) before injection. Testing confirmed that the changes in pre-injection filter type affected only pre-column and column longevity, not pigment separation or retention times (data not shown).

For cruises between 1995 and 1999, pigments were separated using a Hitachi system (D-6500 in 1995; D-7000 from 1996 to 1999) with L-4250 fixed wavelength (440 nm) and L-4500 diode array (DAD, scanning 350–650 nm) detectors. HPLC grade mobile phase eluents followed the method of Wright et al. (1991):

Solvent A: 80:20 methanol:0.5 M aqueous ammonium acetate (pH 7.2);
Solvent B: 90:10 acetonitrile:water;
Solvent C: 100% ethyl acetate.

The analytical gradient was modified to reduce spreading of the early eluting, highly polar chlorophyll c₃ (chl_c₃) (Table 2). As explained later, the chl_c₃ data was not used in the CHEMTAX analysis due to this peak spreading and the associated difficulties of calibration. Solvents were degassed by sparging with 99% pure Helium gas, and separations were performed on a Waters Resolve C18, 300 nm × 3.9 μm, 5 μm column. Hitachi’s ConcertChrome software package was used for system control and data collection, and peaks were quantified at 440 nm on the DAD.

January 2000 cruise data was analyzed on a Waters 600 controlled system with a Thermo Separation Products (TSP) AS3000 sampler, a TSP Spectra 1000 variable wavelength detector (VVD) for peak quantification and a Waters 470 Scanning fluorescence detector for peak identification when applicable (excitation at 440 nm, emission at 665 nm). Data was collected using the Waters Millennium 32 software package, and peaks were quantified at 440 nm on the VWD.

For cruises between 2001 and 2007, samples were separated using an Agilent Technologies (Hewlett-Packard) 1100 Series HPLC system, equipped with G1314A variable wavelength (fixed at 440 nm), G1315A diode array (scanning 330–800 nm) and G1321A fluorescence (440 nm excitation, 650 nm emission) detectors. Solvents were degassed using a vacuum degasser and column temperature was maintained at 25 °C with a G1316A column thermostat. Agilent Technologies ChemStation for LC 3D software was used for system control and data collection and peaks were quantified at 440 nm on the VWD.

From 2000 to 2007 (both Waters and Agilent Technologies systems), HPLC grade mobile phase eluents followed the method of Zapata et al. (2000):

Solvent A: 50:25:25 methanol:acetonitrile:0.25 M aqueous pyridine;

The analytical gradient was modified slightly from Zapata et al. (2000) to allow adequate separation of the mid-chromatogram range xanthophylls (Table 2). Separations with this method were performed on Waters Symmetry C8, 150 mm × 4.6 μm, 3.5 μm columns, and samples were injected as either (a) a 2:1 sample: water mixture in 2000 or (b) a 5:4 sample:water mixture from 2001 to 2007.

Table 3 lists the pigments quantified in this study, their average retention times, and extinction coefficients used for determining concentrations of standards. In 1995 and 1996, chl_a, chlorophyll b (chl_b), alpha- and beta-carotene were quantified with measured spectral absorbance and published extinction coefficients; all other standards were isolated and quantified by Moss Landing Marine Laboratory previous to the cruises and were injected as external standards to determine signal response and retention times. From 1997 to 2007 commercially produced plant pigment standards were used for system calibration (Sigma Chemical for chl_a and chl_b and alpha- and beta-carotene until 2000; DHI (formerly VKI), Denmark (http://www.c14.dhi.dk/PhytoplanktonPigmentStandards.htm) for all others). Four to six point response curves were built using standard peak areas measured at 440 nm, and daily injections of chl_a, as well as random single-point injections of all other pigments, were made to confirm system stability and monitor column degradation throughout the duration of sample processing. Sample peaks were identified based on retention times and confirmed spectrally with the diode array and fluorescence detectors, and pigment concentration was calculated by regression in Microsoft Excel.

### Table 2

<table>
<thead>
<tr>
<th>HPLC system</th>
<th>Time (min)</th>
<th>% Solvent A</th>
<th>% Solvent B</th>
<th>% Solvent C</th>
</tr>
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<tr>
<td>Hitachi</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0</td>
<td>100</td>
<td>0</td>
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<td></td>
<td>3</td>
<td>100</td>
<td>0</td>
<td>0</td>
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<td></td>
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<td>30</td>
<td>70</td>
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<td>80</td>
</tr>
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<td></td>
<td>26</td>
<td>0</td>
<td>100</td>
<td>0</td>
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<td></td>
<td>29</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Waters²/Agilent²</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>60</td>
<td>40</td>
<td>n/a</td>
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<td>100</td>
<td>n/a</td>
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<td>38</td>
<td>100</td>
<td>0</td>
<td>n/a</td>
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<td>40</td>
<td>100</td>
<td>0</td>
<td>n/a</td>
</tr>
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<td></td>
<td>42</td>
<td>100</td>
<td>0</td>
<td>n/a</td>
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<tr>
<td></td>
<td>45</td>
<td>100</td>
<td>0</td>
<td>n/a</td>
</tr>
</tbody>
</table>

* A modification of the Zapata et al. (2000) method was used for both the Waters and Agilent systems; minor adjustments were made based on system configuration to minimize total run time while allowing adequate post-run equilibration.
than on values was used because the data failed to meet the assumptions of normality of the residuals for linear regression. Similar to parametric tests of correlation, Spearman Rank results in a correlation coefficient (ρ or rho) of one (or −1) if the data are a perfect function of one another.

2.5. CHEMTAX

Phytoplankton composition was determined using CHEMTAX (Mackey et al., 1996), a program that uses factor analysis and a steepest descent algorithm to determine the best fit to the data with a given input matrix of pigment ratios. Using an iterative process for a given input matrix, the software optimizes the pigment ratios for each group and applies the final ratio to the total chl_a in each sample to determine the proportion of chl_a concentration attributed to each phytoplankton group in the community. For this study, the Microsoft Excel based CHEMTAX version 1.95, a beta version received directly from the CHEMTAX authors, was used. In addition, CHEMTAX provides the “final” optimized ratio and results once that final ratio is applied to the pigment data for calculation of the phytoplankton groups. The results are output in terms of absolute amounts (µg L⁻¹) of chl_a attributed to each phytoplankton group, and as a relative amount (percentage) of the total chl_a in a sample.

2.5.1. Phytoplankton groups

For this study, phytoplankton groups included in the CHEMTAX analysis were based on those used by Rodriguez et al. (2002) (hereafter referred to as RVZ02), who worked along the wAP during 1995 and 1996. Microscopy datasets (Garibotti et al., 2003a) confirmed these groupings, and were allocated as described in the microscopy section, with the “dinoflagellate” and “unidentified phytoflagellate” groups combined to form the derived “mixed flagellates” group. Other studies completed in the region confirm the consistent dominance of diatoms, cryptophytes, Phaeocystis, flagellates and prasinophytes (Varela et al., 2002). For this study, only pigmented autotrophs are considered in the phytoplankton groups. The chosen groups likely do not include all taxa present in the ecosystem nor are they necessarily

### Table 3
HPLC pigment information. Pigments quantified and abbreviations as used in this text, average retention time (RT) for each method, and for those pigments mixed from powdered standards, extinction coefficients (ε) applied and application wavelengths used.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Abbreviation</th>
<th>Retention time</th>
<th>Extinction coefficient (µg⁻¹ cm⁻¹)</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wright et al., 1991</td>
<td>Zapata et al., 2000</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll c3</td>
<td>chl_c3</td>
<td>6.2</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll c2⁺</td>
<td>chl_c2⁺</td>
<td>7.1</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Chlorophyllide a</td>
<td>chlide-a</td>
<td>n/a</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>Peridinin</td>
<td>per</td>
<td>9.1</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>19’ Butanoyloxyfucoxanthin⁻</td>
<td>19’-but</td>
<td>9.3</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>Fucoxanthin⁻</td>
<td>Fuc</td>
<td>10.1</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>19’ Hexanoyloxyfucoxanthin⁻</td>
<td>19’-hex</td>
<td>10.4</td>
<td>21.2</td>
<td></td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>neox</td>
<td>10.8</td>
<td>19.1</td>
<td></td>
</tr>
<tr>
<td>Prasinoxanthin</td>
<td>pras</td>
<td>11.3</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>Diadinoxanthin</td>
<td>dd</td>
<td>12.9</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>Alloxantin⁻</td>
<td>allox</td>
<td>13.8</td>
<td>24.2</td>
<td></td>
</tr>
<tr>
<td>Diatoxanthin</td>
<td>dr</td>
<td>14.4</td>
<td>24.6</td>
<td></td>
</tr>
<tr>
<td>Luteinb</td>
<td>lut</td>
<td>14.8</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>Zeaxanthinb</td>
<td>zeax</td>
<td>15.1</td>
<td>24.9</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll b⁺</td>
<td>chl_b⁺</td>
<td>18.2</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>viol</td>
<td>n/a</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>Crocoxanthin</td>
<td>croc</td>
<td>n/a</td>
<td>27.4</td>
<td></td>
</tr>
<tr>
<td>Echinonene</td>
<td>ech</td>
<td>n/a</td>
<td>28.1</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll e⁺</td>
<td>chl_e⁺</td>
<td>19.3</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>α,β-Carotene</td>
<td>alpha</td>
<td>21.8</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>βc-Carotene</td>
<td>beta</td>
<td>21.9</td>
<td>31.4</td>
<td></td>
</tr>
</tbody>
</table>

Denotes pigments used in CHEMTAX input ratios for composition determination.

Note elution order change between lutein and zeaxanthin with the uncertainty (L g⁻¹ C0⁻¹ cm⁻¹) of chl_a.

### Table 4
HPLC detection ranges and uncertainties. Detection ranges used (injection volumes limited to these masses) and relative uncertainties in the calibration regressions, calculated as average intercept divided by average slope for the pigments used in the determination of diatom and cryptophytes abundances.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Hitachi range (µg)</th>
<th>Agilent range (µg)</th>
<th>Agilent uncertainty (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chl_c2</td>
<td>0.005–0.071</td>
<td>0.002–0.069</td>
<td>0.14</td>
</tr>
<tr>
<td>fuc</td>
<td>0.007–0.132</td>
<td>0.004–0.085</td>
<td>0.27</td>
</tr>
<tr>
<td>19’ but</td>
<td>0.001–0.072</td>
<td>0.002–0.049</td>
<td>0.72</td>
</tr>
<tr>
<td>19’ hex</td>
<td>0.006–0.126</td>
<td>0.002–0.068</td>
<td>0.84</td>
</tr>
<tr>
<td>allox</td>
<td>0.001–0.077</td>
<td>0.002–0.053</td>
<td>0.20</td>
</tr>
<tr>
<td>chl_b</td>
<td>0.003–0.285</td>
<td>0.009–0.518</td>
<td>0.34</td>
</tr>
<tr>
<td>chl_e</td>
<td>0.011–1.268</td>
<td>0.008–1.131</td>
<td>0.44</td>
</tr>
</tbody>
</table>

2.3.1. HPLC limits of detection

Table 4 lists for each detection system, the mass ranges of the pigments used in the CHEMTAX analysis and the uncertainty of the minimum detector response, for each pigment, calculated from the regressions as intercept (b) divided by slope (m). Sample injection volumes were constrained to these limits to ensure linear response of the detectors.

2.4. Microscopy

In 1996, 1997 and 1999 water was sampled for quantitative microscopic analysis from the 50% light depth and preserved with 2% Lugol’s iodine solution (Parsons et al., 1984). Samples were analyzed following methods described in Garibotti et al. (2003a). For purposes of this study, microscopic data was binned for the main phyta: diatoms, cryptomonads, Phaeocystis spp. (hereafter, “Phaeocystis”), prasinophytes, dinoflagellates and unidentified small phytoflagellates.

The final CHEMTAX output from this study was compared to the microscopy data using Spearman Rank correlation. This non-parametric version of linear regression based on ranks rather than on values was used because the data failed to meet the assumption of normality of the residuals for linear regression. Similar to parametric tests of correlation, Spearman Rank results in a correlation coefficient (ρ or rho) of one (or −1) if the data are a perfect function of one another.

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broken down into more conventional taxonomic groups, but rather are intended to be "representations of typical pigment patterns" found in the study region (Wright and van den Enden, 2000).

2.5.2. Initial pigment ratios

To accurately allocate chl \(_a\) to phytoplankton groups, CHEMTAX requires an input matrix containing the expected classes and initial ratios of pigments in those classes. Along the wAP during the time frame of the study, interannual variability in the abundance and relative proportions of groups present can be expected, but diversity of the primary contributing phytoplankton groups in the region are consistent (Garibotti et al., 2005a). Thus, the same initial ratio was used in all years of this analysis, but each year was run separately to allow potential variation in optimization to be expressed. Additionally, a test of the impacts of irradiance on the matrix for this study.

When using CHEMTAX with phytoplankton cultures, it is ideal to determine initial input ratios measured from regionally specific taxon grown under known experimental conditions. This was not an option in this study, as all samples were mixed field samples. However, CHEMTAX has been used extensively in the Southern Ocean, including East Antarctica (South West of Australia; Wulff and W. A. Kozlowski et al. / Deep-Sea Research I 58 (2011) 350–364 Kozlowski et al., 2005a; DiTullio et al., 2007), the eastern Atlantic region (South of South Africa; Wulff and Wängberg, 2004), the Southern Pacific sector (South East of New Zealand; DiTullio et al., 2003) and in the Gerlache and Bransfield Straits along the Antarctic Peninsula (RVZ02). Given the geographic proximity of the RVZ02 dataset to the current study, and the similarity in included groups to those found in the Pal LTER microscopy datasets (Garibotti et al., 2005a), the initial matrix from RVZ02 was used as the starting point from which to build the matrix for this study.

The RVZ02 initial ratios included pigments that were either not quantified through the entire time series (chlorophyll c1, violaxanthin, Non-Polar chlorophyll c2), or had low peak quality in the early years of the study (chl_c3). The initial pigment ratio matrix used in this study is listed in Table 5a.

Initial ratios used in the Southern Ocean by others have also included pigments such as peridinin (per) and chl_c3. To evaluate the impact of these two pigments on the resulting final ratios and composition output, CHEMTAX was run for 1996, 1997, 1999 and 2007 with both chl_c3 (added to the mixed flagellate and Phaeocystis ratios) and per (added to the mixed flagellate ratios) included in the initial ratios. The final ratios from these CHEMTAX runs were analyzed using ANOVA, with each year considered a replicate. Among the major diagnostic carotenoids, we found per to be uncommon and when present, was frequently in extremely low amounts (average 0.019 ± 0.039 µg L\(^{-1}\), \(n=4508\)), and as explained later, not included in the initial ratios.

2.5.3. Additional testing and uncertainty calculation

Because CHEMTAX is known to be sensitive to the seed values in the initial ratio matrix, two additional ratio optimization methods were run. In all methods, data was binned by light as described above. First, following the recommendation of Latasa (2007), six sets of ten CHEMTAX runs each were made on the 2007 data, with the first runs using Table 5a initial ratio up to a ±75% random error added to the initial ratio values. Each subsequent run applied the output ratio of the previous run as its initial ratio, and the output calculated from the tenth runs were considered "final". In seven cases, (primarily chlorophyll c2 (chl_c2) and fucoxanthin (fuc) for the mixed flagellates and Phaeocystis) the process was repeated an additional five times.

<table>
<thead>
<tr>
<th>Class/pigment</th>
<th>chl_c2</th>
<th>fuc</th>
<th>19_rbut</th>
<th>19_hex</th>
<th>allox</th>
<th>chl_b</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Initial pigment ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prasinophytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td>Cryptophytes</td>
<td>0.174</td>
<td></td>
<td>0.228</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed flagellates</td>
<td>0.126</td>
<td>0.290</td>
<td>0.122</td>
<td>0.315</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatoms</td>
<td>0.183</td>
<td>0.745</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeocystis spp.</td>
<td>0.144</td>
<td>0.011</td>
<td>0.080</td>
<td>1.111</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Final pigment ratios applied*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100–50%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prasinophytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.969±0.071</td>
</tr>
<tr>
<td>Cryptophytes</td>
<td>0.152±0.062</td>
<td></td>
<td>0.443±0.125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed flagellates</td>
<td>0.183±0.087</td>
<td>0.440±0.188</td>
<td>0.299±0.143</td>
<td>0.209±0.156</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatoms</td>
<td>0.193±0.049</td>
<td>0.714±0.160</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeocystis spp.</td>
<td>0.155±0.058</td>
<td>0.011±0.002</td>
<td>0.062±0.023</td>
<td>1.110±0.212</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25–10%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prasinophytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.941±0.089</td>
</tr>
<tr>
<td>Cryptophytes</td>
<td>0.166±0.075</td>
<td></td>
<td>0.428±0.117</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed flagellates</td>
<td>0.205±0.127</td>
<td>0.439±0.211</td>
<td>0.261±0.130</td>
<td>0.239±0.203</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatoms</td>
<td>0.191±0.063</td>
<td>0.719±0.206</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeocystis spp.</td>
<td>0.156±0.044</td>
<td>0.011±0.002</td>
<td>0.066±0.026</td>
<td>1.117±0.331</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–1%</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Prasinophytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.008±0.240</td>
</tr>
<tr>
<td>Cryptophytes</td>
<td>0.198±0.105</td>
<td></td>
<td>0.416±0.224</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mixed flagellates</td>
<td>0.245±0.324</td>
<td>0.620±0.710</td>
<td>0.467±0.545</td>
<td>0.289±0.223</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatoms</td>
<td>0.175±0.067</td>
<td>0.705±0.194</td>
<td>0.007±0.014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeocystis spp.</td>
<td>0.165±0.034</td>
<td>0.011±0.001</td>
<td>0.165±0.263</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Presented here by light bin, normalized to chl_a, as an average of all 12 years of the study ± one standard deviation of all years.
until convergence occurred (see Latasa, 2007) and the output from the fifteenth run was considered “final”. Second, based on the method by Wright et al. (2009) 60 repeat runs of the software were completed. Each run had as its initial matrix the pigment ratios listed in Table 5a, and again, to each non-zero number a random amount up to ±75% of the ratio value was added. During optimization, CHEMTAX determines the pigment content explained by the solution for each run (residual or root mean square error (RMSE)). Of the 60-run batch, the run with the lowest RMSE was considered “final”. Uncertainties of the final CHEMTAX pigment ratios were calculated as the average RMSEs for each light bin for the 12-year dataset.

Results of the various methods (i.e., the method with no error added and no feedback runs which will be referred to hereafter as the “wAP” method, the “Latasa” method with error added and feedback runs, the “Wright” method with error added and no feedback runs, and the “chl_c3+per” method where chl_c3 and per were added to the “wAP” method ratios) and the microscopy results were analyzed and compared several ways. First, since randomization could have altered the initial CHEMTAX ratios to be taxonomically incorrect, the final ratios were examined to be sure the results were ecologically realistic, and if not, those results were removed from the comparison. Second, because the final ratios may not necessarily show the ecological impacts on the system until they are applied to the actual data, the resulting composition outputs (final ratios applied to the pigment data, 1996, 1997, 1999 and 2007) were analyzed using the Mann–Whitney U test to check for differences in means. This non-parametric version of the ANOVA was used because no one transformation could be applied to satisfactorily normalize all the datasets.

Comparison of the means of all the samples from each year focuses on the overall composition tendencies, but could potentially miss localized but distinct differences in the datasets. To focus instead on this perspective, a third method was used on the 2007 data. Differences were calculated between the “Latasa”, “Wright”, “chl_c3+per” and the “wAP” method (i.e., for each sample, resulting relative (%) composition output from each alternate method was subtracted from the “wAP” method), and the average, minimum and maximum differences were calculated. An accuracy criterion threshold (see Hooker et al., 2005) was set at fifteen percent, and all data differing by more than that amount was flagged and noted. Methods were considered to be not equal if the accuracy criterion was exceeded in more than ten percent of the stations in a given group.

2.5.4. Configuration parameters

CHEMTAX was run using the initial matrix listed in Table 5a, and data was binned by light and year to optimize the final matrix ratios applied to the data. Configuration parameters required by CHEMTAX were set as follows: (a) ratio limits = 500 (allowed ratio (r) to vary from r/6 to 6r), (b) weighting = bounded relative, weighting = 3 and weight bound = 30 (used to specify error distribution in the ratio matrix), (c) iteration limit = 500, (d) epsilon limit = 0.0001, (e) initial step size = 10, (f) step ratio = 1.3 (used to set size and amount by which it can change with each iteration), (g) cutoff step = 1000 (limits size of iteration step), and (h) elements varied = 7 (all pigments). See Mackey et al. (1997) for a complete explanation of each setting. All settings were individually modified by one order of magnitude in either direction but no significant difference was seen in the output (data not shown), and all final settings were left as described above.

2.6. Data processing and analysis

After initial processing in HPLC software, data handling was primarily done in Microsoft Excel 2007. Full pigment datasets for all cruises are available online at: http://oceaninformatics.ucsd.edu/datazoo/data/palmeter/datasets. Additional plotting and statistics were carried out using Ocean Data View (Alfred-Wegner Institute for Polar and Marine Research, http://odv.aw.de; Schlitzer, 2010), Systat Version 12 (Systat Software, Inc., www.systat.com) and Matlab 2009b (Mathworks, Inc., www.mathworks.com). For all statistical testing significance was set at the α = 0.05 level.

3. Results

3.1. HPLC uncertainties

HPLC uncertainties are addressed from within the confines of the relevant quality assurance (QA) practices of the sample processing. Across the thirteen-year study, protocol dictated that all water samples be gently mixed and measured to within 5 mL of 1.0 L for filtration. Filters themselves were examined to assure proper placement on the funnel bases and filters were stored folded in half and rolled into 2.5 mL cryovials to be sure material was not lost before sample processing. Though extraction in methanol is a commonly used method (Wright et al., 1997; Zapata et al., 2000), extraction in 90% acetone has been found to be a more stable extraction solvent, with lower degradation rates for all chlorophylls (Hooker et al., 2005). Extraction efficiency was maximized by freezing the samples for 24 h and adding sonication. Precision of injection volume was confirmed by linearity of results from standards injections, and peak areas for field samples were limited to the linear calibration range of the detectors. Despite the long time frame of data collection, internal consistency for the dataset is high due to a limited number of individual technicians processing both samples (n = 5) and data (n = 2).

As part of the NASA SeaHARRE-5 project, our chromatographic results were compared to eleven other international laboratories. Preliminary results from this inter-laboratory comparison show that our average uncertainty for the pigments used in CHEMTAX was 18.2% ± 11.1%. See Hooker, et al. (2005) for calculation of pigment uncertainties.

3.2. Pigment distributions

Table 6 lists concentrations of pigments, as measured by HPLC, used to determine diatom and cryptophytes abundance by CHEMTAX. The average chl_a concentration for the wAP region in January was 1.229 ± 2.373 μg L⁻¹, characteristic of summer phytoplankton in coastal Antarctica (Prézelin et al., 2000; Garibotti et al., 2003a, 2003b; RV202) and an order of magnitude higher than blue waters of the Antarctic circumpolar current (Hewes et al., 2008). Minimum average concentration in the region was measured in 1998 and the maximum in 1996, with 0.328 and 4.288 μg L⁻¹, respectively. The large yearly standard deviation is indicative of the two, and up to three, orders of magnitude difference observed each season, with an overall range of three orders of magnitude, a minimum chl_a concentration 0.022 μg L⁻¹ in 1997 and a maximum of 19.315 μg L⁻¹ in 1996. Similar variability was observed in the main accessory pigments (chl_c2, fuc and alloxanthin (allox)). Chl_a and fuc were always in measurable concentrations (average regional concentration of fuc is 0.222 μg L⁻¹ and over a range of 0.004 to 10.169 μg L⁻¹) while chl_c2 and allo could be below detection limits (as defined in Table 4) to a maximum of 0.789 and 3.983 μg L⁻¹, respectively. Maximum concentrations of these major pigments were seen in the year 1996.

Accessory pigments concentrations in these phytoplankton populations were similar to those of chl_a, as indicated by the
Table 6
Pigment data. Minimum, maximum, mean and standard deviation of pigments used to quantify contribution to diatoms and cryptophytes. Units are µg L⁻¹.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1995</td>
<td>1.528 ± 1.487</td>
<td>0.907 ± 0.892</td>
<td>0.091 ± 0.109</td>
<td>0.040 ± 0.081</td>
<td>0.358 ± 0.272</td>
<td>0.078 ± 0.092</td>
<td>0.498 ± 0.310</td>
<td>0.206 ± 0.212</td>
</tr>
<tr>
<td>1996</td>
<td>3.772 ± 5.587</td>
<td>2.088 ± 3.062</td>
<td>0.178 ± 0.057</td>
<td>0.033 ± 0.039</td>
<td>0.327 ± 0.135</td>
<td>0.109 ± 0.107</td>
<td>0.486 ± 0.157</td>
<td>0.167 ± 0.105</td>
</tr>
<tr>
<td>1997</td>
<td>0.594 ± 0.600</td>
<td>0.333 ± 0.288</td>
<td>0.123 ± 0.051</td>
<td>0.037 ± 0.040</td>
<td>0.182 ± 0.095</td>
<td>0.178 ± 0.089</td>
<td>0.412 ± 0.163</td>
<td>0.232 ± 0.146</td>
</tr>
<tr>
<td>1998</td>
<td>0.265 ± 0.225</td>
<td>0.233 ± 0.109</td>
<td>0.257 ± 0.167</td>
<td>0.016 ± 0.028</td>
<td>0.428 ± 0.349</td>
<td>0.335 ± 0.175</td>
<td>0.889 ± 0.453</td>
<td>0.269 ± 0.185</td>
</tr>
<tr>
<td>1999</td>
<td>0.780 ± 1.143</td>
<td>0.457 ± 0.381</td>
<td>0.085 ± 0.041</td>
<td>0.024 ± 0.017</td>
<td>0.244 ± 0.138</td>
<td>0.229 ± 0.125</td>
<td>0.545 ± 0.239</td>
<td>0.212 ± 0.171</td>
</tr>
<tr>
<td>2000</td>
<td>1.006 ± 1.196</td>
<td>0.761 ± 0.679</td>
<td>0.330 ± 0.176</td>
<td>0.028 ± 0.036</td>
<td>0.536 ± 0.394</td>
<td>0.085 ± 0.085</td>
<td>0.719 ± 0.474</td>
<td>0.368 ± 0.272</td>
</tr>
<tr>
<td>2001</td>
<td>0.873 ± 0.943</td>
<td>0.900 ± 0.843</td>
<td>0.233 ± 0.129</td>
<td>0.101 ± 0.098</td>
<td>0.685 ± 0.423</td>
<td>0.148 ± 0.140</td>
<td>0.913 ± 0.480</td>
<td>0.297 ± 0.188</td>
</tr>
<tr>
<td>2002</td>
<td>0.363 ± 0.360</td>
<td>0.355 ± 0.287</td>
<td>0.189 ± 0.052</td>
<td>0.047 ± 0.068</td>
<td>0.318 ± 0.168</td>
<td>0.285 ± 0.155</td>
<td>0.678 ± 0.302</td>
<td>0.453 ± 0.206</td>
</tr>
<tr>
<td>2003</td>
<td>0.470 ± 0.318</td>
<td>0.364 ± 0.205</td>
<td>0.140 ± 0.032</td>
<td>0.072 ± 0.065</td>
<td>0.209 ± 0.115</td>
<td>0.251 ± 0.124</td>
<td>0.531 ± 0.207</td>
<td>0.305 ± 0.168</td>
</tr>
<tr>
<td>2004</td>
<td>0.720 ± 0.716</td>
<td>0.577 ± 0.614</td>
<td>0.176 ± 0.051</td>
<td>0.038 ± 0.051</td>
<td>0.272 ± 0.202</td>
<td>0.129 ± 0.086</td>
<td>0.459 ± 0.263</td>
<td>0.371 ± 0.197</td>
</tr>
<tr>
<td>2005</td>
<td>2.601 ± 3.012</td>
<td>2.671 ± 2.856</td>
<td>0.185 ± 0.055</td>
<td>0.051 ± 0.137</td>
<td>0.691 ± 0.234</td>
<td>0.152 ± 0.233</td>
<td>0.881 ± 0.266</td>
<td>0.195 ± 0.086</td>
</tr>
<tr>
<td>2006</td>
<td>0.935 ± 1.671</td>
<td>0.874 ± 1.498</td>
<td>0.149 ± 0.043</td>
<td>0.029 ± 0.090</td>
<td>0.428 ± 0.212</td>
<td>0.291 ± 0.251</td>
<td>0.775 ± 0.227</td>
<td>0.267 ± 0.140</td>
</tr>
<tr>
<td>All</td>
<td>1.229 ± 2.373</td>
<td>0.914 ± 1.618</td>
<td>0.165 ± 0.105</td>
<td>0.043 ± 0.075</td>
<td>0.374 ± 0.282</td>
<td>0.184 ± 0.166</td>
<td>0.619 ± 0.336</td>
<td>0.267 ± 0.193</td>
</tr>
</tbody>
</table>

* Accessory pigments include both PSC and PPC carotenoids.

* Photosynthetic carotenoids (PSC) include: fucoxanthin, 19’ hexanoyloxyfucoxanthin, 19’ butanoyloxyfucoxanthin.

* Photoprotective carotenoids (PPC) include: diadinoxanthin, alloxanthin, diatoxanthin, zeaxanthin, alpha carotene and beta carotene.

ratio of accessory to chl_a of 1.229 ± 2.373 (Table 7). From the major accessory pigments, the fuc:chl_a ratio was always the highest, chl_c2:chl_a and 19’ hexanoyloxyfucoxanthin (19’_hex): chl_a were intermediate and chl_b:chl_a was the lowest. Photosynthetic carotenoids presented an average ratio to chl_a of 0.619 ± 0.336 for the region, with a factor of two variability (minimum of 0.412 ± 0.163 in 1997 and a maximum of 0.913 ± 0.480 in 2001) (Table 7). Photoprotective carotenoids presented on average half the cellular concentration of photosynthetic carotenoids, with a regional average ratio to chl_a of 0.272 ± 0.193 and a variability of a factor of 2 (minimum of 0.167 ± 0.105 in 1996 and a maximum of 0.453 ± 0.206 in 2003). The pigment ratios and inter-year variability from this dataset are within the range of previously published studies, for both a global average, and for Southern Ocean specific samples (Trees et al., 2000; Bidigare et al., 1996).

3.3. Evaluation of initial pigment ratios in CHEMTAX

The initial ratios provided to CHEMTAX for this study included six accessory pigments combined in thirteen ratios. We tested that the exclusion of chl_c3 and per, two pigments considered characteristic indicators of common phytoplankton taxa, did not affect our group determinations. Both the final ratios and the resulting compositional output from the years with microscopy data (1996, 1997, and 1999) and 2007 data were examined. After the addition of these two pigments to the initial ratios, four of the 39 final pigment ratios (twelve pigments in three light bins) were found to be significantly different (ANOVA, p < 0.05). The significantly different ratios were 19’_hex but in the 100–50% light bin of mixed flagellates and 19’_hex in all light bins for Phaeocystis. An evaluation of the differences in composition output for the 2007 data between the two methods revealed that the mixed flagellate, Phaeocystis and diatom groups were affected by the addition of chl_c3 and per (Table 8). On average the difference in relative group abundance was close to 10% for Phaeocystis and mixed flagellates, with CHEMTAX assigning more biomass to the mixed flagellates and less to Phaeocystis when chl_c3 and per were in the initial ratios. On average, diatoms show a 5% change in relative group abundance, and also had more than 10% of the stations exhibiting a change of 15% or greater, but there was no consistent directional shift observed (Table 8). In summary, by adding chl_c3 and per to the initial ratios we obtain a higher percentage of Phaeocystis, a lower percentage of mixed flagellates and a
slightly lower percentage of diatoms. These results would increase the differences observed between CHEMTAX and microscopy (see also Section 3.6).

### 3.4. Light bin comparisons and final ratios applied

As a check of potential irradiance impacts on cellular pigment content that would affect pigment ratios (Wright et al., 1996), the final ratios for each year were analyzed for differences in three light depth bins (100–50%, 25–10% and 5–1%). The final ratio applied to the data by CHEMTAX for each light bin is listed in Table 5b. There are no statistically significant differences in the final ratios between the three light bins in any output matrix values (ANOVA results for all pigments in all groups: $p \geq 0.2888$), indicating a similarity of actual pigment ratios in the samples regardless of collection depth. However, in order to have a protocol that allows for possible future changes in pigment ratios with changing light regimes, the data was run as three separate light depth bins (100–50%, 25–10% and 5–1%) light bins, respectively.

### 3.5. CHEMTAX method comparisons and uncertainties

The comparison of the different CHEMTAX optimization methods (“wAP”, “Latasa” and “Wright”) to test whether CHEMTAX properly optimized the final pigment ratios was done in several steps. After any output containing non-realistic final pigment ratios were removed from the “Wright” runs, the final ratios for the three methods were compared graphically to examine if they all converged upon the same ratio. A selection of these graphs is shown in Fig. 2. Convergence of the three methods on the same, or very close to the same number was confirmed for 27 out of 39 pigments in all groups. The exceptions were the pigment ratios for chl_c2, fucoxanthin and 19′_hex for the mixed flagellates and fucoxanthin of Phaeocystis. Lack of convergence in all of these cases except fucoxanthin for the mixed flagellates, was due to the “Latasa” method converging upon non-ecologically realistic results. For example, in all three light bins for the mixed flagellates in the “Latasa” method, the chl_c2 final contribution was nearly zero ($0.004 \pm 0.004$), which is unrealistic (initial ratio value 0.126). Similarly, in all three light bins for Phaeocystis in the “Latasa” method resulted in near zero contribution of fucoxanthin (0.0003 ± 0.0004), essentially making it a non-contributor to the group.

Slight differences were seen in the final ratios between the methods for allo and 19′ butanoyloxyfucosanin (19′_but) (Fig. 2) but a Mann–Whitney U test revealed no statistical difference between the final ratios in any of the groups ($p \geq 0.1770$), and thus were considered the same. In addition, the differences in the applied compositional output from these three methods were compared and less than 1% of the samples had larger than a 15% differences in group estimation (Table 8).

Uncertainties resulting from the calculation of the CHEMTAX solutions using the listed initial ratio and the “wAP” method were $0.080 \pm 0.054$, $0.076 \pm 0.054$ and $0.080 \pm 0.051$ for the 100–50%, 25–10% and 5–1% light bins, respectively.

### 3.6. Microscopy vs CHEMTAX

Direct comparison of the estimated biomass of phytoplankton groups using microscopy and CHEMTAX showed consistently significant correlation between methods for diatoms and cryptophytes. The strongest correlations were seen in the 1996 and 1999 data for the cryptophytes ($p=0.84$ and 0.83, respectively). Correlation between methods was lower and less consistent between years for prasinophytes and mixed flagellates. Phaeocystis was rarely recorded in the microscopy dataset compared to CHEMTAX; in 1999 Phaeocystis was documented at only one station using microscopic methods, where CHEMTAX indicated relative concentrations of 10% or higher at 45 of 57 stations. Plots of percent carbon estimated from microscopy data vs. relative composition from CHEMTAX are shown in Fig. 3.

### 3.7. Phytoplankton composition

An example of the distribution of phytoplankton taxa in the wAP between 64 and 68°S is shown as integrated concentration through the $Z_{eu}$ for the year 1999 (Fig. 4). In this year, CHEMTAX showed that crypytomonads were most dominant, with as much as 151 µg m$^{-2}$ chl_a associated to this group, with a localized distribution in the northern coastal region. Diatoms and mixed flagellates had similar maximum concentrations with the former showing a more coastal distribution, while diatoms were more

### Table 8

<table>
<thead>
<tr>
<th>Method difference</th>
<th>Diatoms</th>
<th>Cryptophytes</th>
<th>Mixed flagellates</th>
<th>Phaeocystis spp.</th>
<th>Prasinophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>wAP-chl_c3 and peridinin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>5.2</td>
<td>-2.0</td>
<td>9.4</td>
<td>-12.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Minimum</td>
<td>-79.2</td>
<td>-60.8</td>
<td>-41.4</td>
<td>-83.7</td>
<td>-56.9</td>
</tr>
<tr>
<td>Maximum</td>
<td>-78.9</td>
<td>53.4</td>
<td>73.1</td>
<td>62.1</td>
<td>75.1</td>
</tr>
<tr>
<td>% ≥ 15% Low</td>
<td>13.3</td>
<td>7.1</td>
<td>2.0</td>
<td>35.7</td>
<td>0.6</td>
</tr>
<tr>
<td>% ≥ 15% High</td>
<td>18.4</td>
<td>4.5</td>
<td>24.1</td>
<td>4.0</td>
<td>1.1</td>
</tr>
<tr>
<td>wAP-Latasa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>-1.3</td>
<td>0.9</td>
<td>0.7</td>
<td>-0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Minimum</td>
<td>-16.6</td>
<td>-0.7</td>
<td>-7.2</td>
<td>-14.7</td>
<td>-2.9</td>
</tr>
<tr>
<td>Maximum</td>
<td>3.6</td>
<td>19.5</td>
<td>14.7</td>
<td>8.2</td>
<td>1.8</td>
</tr>
<tr>
<td>% ≥ 15% Low</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>% ≥ 15% High</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>wAP-Wright</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>-1.6</td>
<td>0.6</td>
<td>1.2</td>
<td>-0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Minimum</td>
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<td>-0.7</td>
<td>-5.2</td>
<td>-3.4</td>
</tr>
<tr>
<td>Maximum</td>
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<td>14.1</td>
<td>29.9</td>
<td>6.0</td>
<td>27.3</td>
</tr>
<tr>
<td>% ≥ 15% Low</td>
<td>0.3</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>% ≥ 15% High</td>
<td>0.3</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>
abundant offshore and towards the south. *Phaeocystis* and pras-
ionphytes were less abundant, representing up to only 12.8 and
2.1 \( \mu g \cdot m^{-2} \) chl\(_a\), respectively.

Fig. 5 shows an example of each of the main phytoplankton
groups’ vertical distribution in the southern region (200 line,
see Fig. 1) in 1996. Diatoms and *Phaeocystis* were the least
stratified. Diatom maximum concentration was in the coastal
stations, whereas *Phaeocystis* abundance was highest mid-shelf
and in slope regions. Cryptomonads and prasinophytes had the
highest stratification; cryptomonads were most abundant at the
surface in the mid-shelf and highest abundance of prasinophytes
was seen offshore at depth. Mixed flagellates presented inter-
mediate stratification compared to the other groups.

### 4. Discussion

#### 4.1. CHEMTAX for estimating wAP phytoplankton composition

The goal of this study was to develop and apply a means of
determining phytoplankton composition to the class level along
the wAP. The samples analyzed included those from twelve
trips encompassing a span of thirteen years, with an average
of 340 samples collected per year at six different depths, across an
80,000 km\(^2\) grid. The scope of the project required that the
method used be efficient enough to process the large volume of
samples at sea, be flexible in order to accommodate potential
physiological differences between sampling locations, yet be
robust enough to tolerate some uncertainty in input parameters
and provide realistic results with a low computational margin of
error. Used with proper consideration, CHEMTAX proved a viable
method for these estimations.

To assure high quality output from the CHEMTAX software,
there are two main requirements: that the input data is of high
quality and that the initial parameters (i.e., expected groups and
seed ratios) are realistic for the region of study. Both issues were
thoroughly addressed in this study.

First, to assure that the data being fed to CHEMTAX was
representative of the pigments present, several QA measures
were implemented. Sample collection and processing adhered to
strict protocol guidelines. Similarly, the HPLC analysis and data
handling methods assured limited and well defined uncertainties.
For example, observations of anomalous chl\(_c3\) data in the 1995
and 1996 datasets led to elimination of this pigment in the input
ratios. Comparison of our wAP dataset to others collected in the
region confirmed that both the range of concentrations as well as
the ratios of primary to accessory pigments was as expected for
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The second fundamental assumption of the CHEMTAX meth-
odology is that the input information, including groups expected
to be found in the samples and initial pigment ratios, is realistic
and representative of the physiological state of the organisms

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Fig. 2. Plots of selected comparisons of the three CHEMTAX optimization methods tested in this study. Note that the “final” ratios for the “wAP” (●), initial “wAP” ratio displayed as +) and “Wright” (●) methods are plotted with the tenth run of the “Latasa” method (six random start ratios displayed as: ● ○ × △) for comparison.
It has been recommended to build initial ratios from cultures or to collect data from local populations (Mackey et al., 1996), but others have concluded that literature-derived pigment ratios are a more reliable estimate of pigment data (Goericke and Montoya, 1998). Previous studies have employed initial ratios from literature based solely on the region under study (Wulff and Wängberg, 2004; Delizo et al., 2007). The initial ratios used in this study were adapted from previously published work, but the phytoplankton groups to be included in those ratios were confirmed via microscopic analysis.

In this study, CHEMTAX is robust in its tolerance to reduced pigment sets, and to introduced error (up to ±75%) in the initial pigment ratios, supporting the idea that precise a priori knowledge is not mandatory for the analysis to provide reliable and consistent estimations of phytoplankton composition.

A comparison of the Wright et al. (2009) and Latasa (2007) methods with a subset of the Pal LTER pigment data showed subtle but non-significant differences, and these modifications are not routinely applied. The authors of the CHEMTAX program make clear to the users that CHEMTAX should not be used as a “black box” (Mackey and Higgins, 2001), where initial pigment ratios are fed in without regard to geography or ecology, and results are taken verbatim as correct without evaluating the resulting final ratios. Methods such as those suggested by Wright et al. (2009) and Latasa (2007) are tools by which users of CHEMTAX can improve confidence in their results, but all results must still be examined carefully. For instance, in the wAP study, the lowest RMSE in the 100–50% light bin resulted in a chl_b value for prasinophytes 65% higher than the average of the next best six results, and so was taken to be incorrect. Similarly, although all six start ratios in all light depth bins for the “Latasa” method did converge on a single value for fuc in Phaeocystis, that value (0.0003 ± 0.0004) was too low and taken to be unrealistic and incorrect (Zapata et al., 2004).

Different questions require resolution on a range of scales and have differing tolerances for error, and it is critical that the above issues are not ignored when selecting a method and protocol for determining phytoplankton composition. In our case, deletion of some pigments because of analytical limitations and lack of inclusion of several modifications provided a robust method. However, if additional information is learned about the system or pigments involved one final advantage of the use of CHEMTAX over microscopy is the ability to re-analyze the data at any point to improve composition estimates.

4.1.1. Depth effects in CHEMTAX output

The CHEMTAX method routinely employed for the wAP study includes separation of samples in 3 light bins, including depths at 100–50%, 25–10% and 5–1% of surface PAR (Table 5b). This division of data is routinely applied in waters of the Southern Ocean (e.g., Wright et al., 2010). Our data, collected by light
intensity has an advantage over sampling in fixed depths, with expected increase in signal-to-noise ratio with respect to ability to detect phytoplankton pigment adaptation to irradiance.

Pigment ratios can be affected by both the depth of the mixed layer as well as by the stratification found within. The extent of vertical mixing within the MLD is related to the stability of the water column, and is heavily influenced by duration and intensity of wind events and storms (Holm-Hansen et al., 1989; Prézelin et al., 1991). A key factor is the relationship between the depth of the mixed layer and the depth of the $Z_{eu}$. Within the $Z_{eu}$ itself, it is possible for cells to be in high light when close to the surface, in low light at depth, or in variable light when mixing is high.

Fig. 4. Phytoplankton groups, 1999. Values are integrated through the $Z_{eu}$ and are in units of mg m$^{-2}$. X-axis is longitude (°W) and Y-axis is latitude (°S).
the MLD is deeper than the $Z_{eu}$, phytoplankton are exposed to irradiance levels which can lead to low-light stress. A MLD which is shallower than the $Z_{eu}$ keeps phytoplankton populations within well lighted waters and no low-light photoadaptation is expected with a high rate of mixing.

If photoadaptation is present, it can lead to variation in pigment ratios with depth and thus affect the CHEMTAX calculations leading to the suggestion that data be binned based on water column depth (Mackey et al., 1996). A particular concern involves low-light stress (Hammer et al., 2002). However, no such changes in pigment ratios
were detected in this dataset when different light depths were analyzed separately with CHEMTAX. This lack of stratification in phytoplankton composition within the Zn, observed in the 12-year wAP time series is consistent with previous analysis of pigment data subsets in the area (Ross et al., 2000; Garibotti et al., 2003a). In spite of not finding changes in pigment ratios with depth, we chose to analyze the data in the three light bins to allow for more flexibility in detection of phytoplankton response to changes in light regime within the wAP area.

The selection of the pigments included in the initial ratios may not be ideal to reflect changes associated with light adaptation. The region does exhibit some variations in carbon per unit chl\_a (Garibotti et al., 2005b), characteristic of photoadaptation. However, the "wAP" method initial ratios are not dependent on photoprotective pigments such as diadinoxanthin and diatoxanthin or violaxanthin, antheraxanthin and zeaxanthin, which are known to undergo rapid but short-term shifts under high-light stress (Lohr and Wilhelm, 1999; Moisan et al., 2006).

It is possible that the range of light intensity in this region of the Southern Ocean is not sufficient to produce low-light adaptation ("too light" scenario), or conversely, all the cells regardless of light depth are already low light adapted ("too dark" scenario). In general, Antarctic phytoplankton in coastal waters is considered to be low light adapted due to the high cellular pigment concentration (e.g., Mitchell and Holm-Hansen, 1991), though it has also been suggested that photodamage can occur due to high irradiance, particularly in areas of deep MLDs when cells are mixed from deep, low-light conditions to the surface (Alderkamp et al., 2010). The later study however, present fuc:chl\_a ratios not changing significantly with depth (Alderkamp et al., 2010, Fig. 3; ANOVA on fuc:chl\_a dependent on depth and fuc:chl\_a dependent on being above or below the MLD: \( p = 0.948 \) and 0.457, respectively), with similar fuc:chl\_a ratios as those measured in this study (see Table 7).

### 4.1.2. Potential effects of iron stress in CHEMTAX data

In addition to light conditions, water column micronutrient levels, specifically low iron levels for \textit{P. antarctica}, have been documented as an environmental stressor on phytoplankton physiology (DiTullio et al., 2007). Although limiting levels of iron have been recorded in the Southern Ocean (Martin et al., 1990), there is little evidence of iron stress on phytoplankton along the wAP below 64\(^\circ\)S in early to mid austral summer between 1995 and 2007, with some exceptions (Vernet et al., 2008). Studies done with \textit{P. antarctica} cultures have documented an increase in the concentration of fuc relative to both 19\_hex and chl\_a under iron replete conditions (van Leeuwe and Stefels, 2007; Feng et al., 2010). Of the samples in this study found to have > 50% \textit{Phaeocystis} (\( n = 92 \), or 2.3% of the samples), the fuc:19\_hex ratio was 0.300 ± 0.140 and the 19\_hex:chl\_a ratio was 0.700 ± 0.290. This fuc:19\_hex ratio is similar to those reported for cultures treated with additional iron and much higher than that reported for cultures grown under iron limiting conditions. However, the 19\_hex:chl\_a ratio falls somewhere between the numbers reported for ambient water conditions and high iron-conditions (DiTullio et al., 2007; van Leeuwe and Stefels, 2007). The observed disparity in these ratios could also be a result of (a) the pigment differences between natural assemblages and cultures, (b) an inconsistent contribution to the pigment pool of both fuc and 19\_hex by diatoms and by \textit{P. antarctica} (DiTullio et al., 2007) or (c) bias in the selection of \textit{Phaeocystis} samples in this study due to the CHEMTAX ratios applied. Future analysis of photosynthetic efficiency parameters measured during austral summer cruises will help to better understand the role of physiological stressors on phytoplankton in the wAP region.

### 4.2. Microscopy vs CHEMTAX

This study benefited from the microscopy data analysis for the sub-surface waters of the 1996, 1997 and 1999 datasets (Garibotti et al., 2005a), which provided confirmation of phytoplankton groupings to include in the CHEMTAX initial input ratios. This information was specific to the samples in the study, but also coincided well with previously published literature for main species found in the vicinity of the study region (Kang and Lee, 1995; Rodriguez et al., 2002). However, CHEMTAX showed the presence of five, not three, main taxonomic groups in the wAP and provided additional information with depth.

Qualitative observation during sample collection (Kozlowski, 2008, unpublished) and other work done in the region provided the impetus to include the \textit{Phaeocystis} group in the study. The presence of the marker-pigment 19\_hexanoyloxyfucoxanthin confirmed that this group frequently existed in wAP waters during the time frame of this work. Additionally, underestimation by microscopy has been documented (Perrin et al., 1987). Our samples indicate that \textit{Phaeocystis} is mostly in the flagellate stage in the summer in the wAP region below 64\(^\circ\)S, with the exception of Marguerite Bay. Due to its small size, it is also possible that \textit{Phaeocystis} cells were misidentified under the microscope and assigned to the mixed flagellate group. Although microscopy was invaluable to confirm the range of taxa to include in the CHEMTAX algorithms, it alone did not provide a complete picture of the phytoplankton composition.

The strongest relationship between the two methods was seen in the estimated cryptophyte and diatom abundances (Fig. 3). The strength of the correlation decreased when small cells or flagellated cells were present, as seen by the weaker coefficient of determination (\( p \)) in the mixed flagellate, \textit{Phaeocystis} and prasinophyte estimates. Mixed flagellates were underestimated by CHEMTAX in comparison to microscopy in all three years; this discrepancy supports the idea that \textit{Phaeocystis} cells might have been misclassified in the microscopic analysis. This possible source of error was confirmed by comparing the two methods with pooled enumeration of \textit{Phaeocystis} and mixed flagellates. Correlation between CHEMTAX and microscopy with this pooled data became significant for 1997 and 1999, without completely explaining all of the differences between the methods. Per was an infrequent pigment in the chromatograms and its concentration was consistently low and thus had relatively high uncertainty. As mentioned by Mackey et al. (1997), CHEMTAX is less tolerant to pigment uncertainties when that pigment is a major contributor to the suite of pigments associated with a taxonomic group, as would be the case for per in the mixed flagellate group (Wright et al., 2009). Thus we chose to not include this pigment in the initial ratios.

Independent estimation of dinoflagellates as a group is further complicated by the presence of non-peridinin containing species (Garibotti et al., 2003a; RV202). In our data when per was included in the initial ratios, even more biomass was attributed to the \textit{Phaeocystis} group, further confounding our ability to determine other flagellates. This suggests that the failure of the CHEMTAX and microscopy methods to agree on biomass assignment to \textit{Phaeocystis} originates in the limitations of microscopy. Subjectivity in microscopic identification of small flagellates has also been speculated to cause disparity between these methods in the past (Schlüter et al., 2000). It is also possible that the differences could be caused by improper allocation of carbon to these groups in the microscopic analysis as suggested for communities of high vs low biomass by Garibotti et al. (2003a). The large positive intercept values seen for diatoms in this study supports this idea; if carbon was under-allocated to diatoms in regions of low biomass, it would explain the overall correlation...
5. Conclusions

A robust means of determining phytoplankton composition was tested and applied to data collected between 1995 and 2007 along the wAP region. We chose photosynthetic pigments analyzed by HPLC for possible taxonomic determination as it is used also to determine optical properties in seawater. The strength of these findings resides in the large number of cruises and samples included in this dataset that allows for the observation of spatial and interannual variability. For group level resolution of questions being asked, CHEMTAX proved to be a robust means for estimation of phytoplankton contribution along the WAP. Results from the CHEMTAX analysis of HPLC data show five groups which commonly contribute to the austral summer phytoplankton in this area: diatoms, cryptophytes, mixed flagellates (dinoflagellates and unidentified photoflagellates), Phaeocystis spp. and prasinophytes.

The CHEMTAX procedure that proved most robust for the wAP data included six pigments combined in thirteen ratios, based on the previous method of Rodriguez et al. (2002) for the area with the exclusion of chlorophyll a and peridinin, and divided in three light depth bins. Final pigment ratios were sufficiently optimized without additional runs to minimize the RMSE and without the addition of error. All these options were carefully tested. Compared to microscopy, CHEMTAX provides better resolution of the small groups, mainly mixed flagellates, _Phaeocystis_ spp. and prasinophytes.

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