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# IMOS Flow cytometry sampling comparison

## Pooled versus discreet depths

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# Acknowledgments

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# Executive summary

The Integrated Marine Observation System (IMOS) National Reference Station (NRS) network has been conducting seawater sampling from multiple locations around Australia since 2009. Depending on the NRS location, these samples have been collected on a monthly to quarterly basis. Flow cytometry has been employed to determine picophytoplankton abundances in these samples, with the photosynthetic cyanobacteria *Prochlorococcus* and *Synechococcus* being of interest due to their major contribution to oceanic primary productivity.

Prior to July 2017, the IMOS NRS flow cytometry sample consisted of one pooled or WC sample per NRS per sampling effort. As each NRS has a different depth profile, this sample consists of both different depths and a different overall number of depths depending on the NRS. This pooled sample has been referred to as an integrated depth sample in other fora, however this term is incorrect as the sample consists of a sub-sample from several discreet depth samples being mixed and then an aliquot being taken from this mixed sample, i.e. not truly integrating the whole of the water column over the sampled depth range. Since July 2017, the flow cytometry samples have consisted of sub-samples being taken directly from discreet depth water samples rather than combining several depths into one overall sample.

In order to determine the differences, if any, between pooled and discreet depth sampling at IMOS NRSs due to this changeover in sampling approach, parallel samples were collected from the Maria Island (MAI) NRS off Tasmania's east coast and measured using a CytoSub flow cytometer. The three main taxonomic groupings considered in these samples, being *Prochlorococcus, Synechococcus* and "Picoeukaryotes", showed a large range of measured cell abundances over the 15-month study period that followed seasonal cycles and variation with depth.

Overall, for both *Synechococcus* and Picoeukaryotes, estimated abundance differences were higher when absolute abundance was higher, whereas the opposite was apparent for *Prochlorococcus* i.e. estimated abundance differences were higher when absolute abundance was lower. Average abundance differences between pooled and discreet depth sampling for MAI were 1%, 4% and 4% for *Prochlorococcus*, *Synechococcus* and Picoeukaryotes, respectively. In addition, higher abundance differences were estimated by both sampling methods at different times. These results indicate very little difference between sampling methods and should alleviate any concerns with using the flow cytometry dataset where it spans the point where a changeover from pooled to discreet sampling occurred (June to July 2017).

# 1 Introduction

Starting in 2009, the Integrated Marine Observation System (IMOS) National Reference Station (NRS) network has been conducting regularly repeated (monthly to quarterly depending on the NRS) seawater sampling from multiple locations around Australia. These include station locations near Rottnest Island (WA), Ningaloo (WA), Albany (WA), Kangaroo Island (SA), Maria Island (TAS), Port Hacking Bay (NSW), North Stradbroke Island (QLD), Yongala (QLD), and Darwin Harbour (NT). Due to logistical complications, sampling at the Ningaloo and Albany NRSs ceased after approximately 3 years, however sampling continues at the remaining seven NRSs samples.

For every sampling effort at each NRS a suite of biogeochemical (BGC) samples are taken for subsequent analysis, including samples for flow cytometry analysis. Flow cytometers are particularly useful for measuring the picophytoplankton component of aquatic samples – photosynthetic cells that are less than 2 µm in size and extremely difficult to measure using traditional light microscopy techniques due to their small size– and also for measuring the microplankton component (2 – 100 µm) that can also be difficult to measure by other means due to their small yet highly-variable size and morphology. For IMOS, *Prochlorococcus* and *Synechococcus* are photosynthetic cyanobacterial genera of interest within the picophytoplankton as they contribute most of the primary productivity to the world's open oceans (Worden et al., 2004).

In order to measure these small cells, flow cytometers are designed to separate and align the particles in a given sample via flow hydrodynamics, whereby the sample is injected at a precise speed into the centre of a body of "sheath" fluid that is also moving at a precise speed. As the flow-concentrated sample passes through a flow cell – a critical point at which one or more fixed-wavelength lasers are being focussed – particles within the sample alter the laser beam such that the new beam properties can be measured by a series of detectors. The detected light wavelengths then give a signature of the properties of each particle. If cells containing light-harvesting pigments – such as phytoplankton – are excited by light, some of the excess light energy is emitted via autofluorescence. Discrimination of phytoplankton taxonomic groupings can therefore be assisted by understanding their intracellular pigment characteristics and the specific fluorescence emission properties of different pigments.

Prior to July 2017, the IMOS NRS flow cytometry samples were taken from one pooled sample (in triplicate) per NRS per sampling effort. The pooled sample has been referred to as an integrated depth sample, although this term is incorrect as the sample consists of a sub-sample from several discreet depth samples being mixed and then an aliquot being taken from this mixed sample, i.e. not truly integrating the whole of the water column over the sampled depth range. As each NRS has a different depth profile (Table 1), this sample consisted of both different depths and a different overall number of depths depending on the NRS. Since July 2017 the flow cytometry samples have consisted of sub-samples (in triplicate) being taken directly from discreet depth water samples (Table 1). Depending on the NRS, this means between three and six discreet depth samples are measured.

In parallel with the discreet depth sampling protocol, the collection of pooled samples continued between August 2017 and December 2018 for the Maria Island (MAI) NRS only. The comparison provided in this report serves to use these MAI samples to determine the degree of similarity between the pooled and discreet depth approaches and gives some context as to the range of measurements covered over the comparison study time period.

**Table 1 Details of IMOS NRS pooled and discreet depth samples used for flow cytometer analysis**

| NATIONAL REFERENCE STATION (NRS) | SAMPLING DEPTHS (M)* |
|---|---|
| Darwin | 0, 10, ~20 (tidal bottom) |
| Kangaroo Island | 0, 10, 25, 50, 75, 100 |
| Maria Island | 0, 10, 20, **30(P), 40(P)**, 50, **75(D), 85(D)** |
| Nth Stradbroke Island | 0, 10, 20, 30, 40, 50 |
| Port Hacking Bay | 0, 10, 25, 50, 75, 100 |
| Rottnest Island | 0, 10, 20, 30, 40, 46 |
| Yongala | 0, 10, 20, 26 |

*MAI depths followed by (P) denote sampling depths that were included in pooled (integrated) samples only. Depths followed by (D) denote sampling depths that were included in discreet samples only.

# 2 Sample details

All samples used in this comparison study were collected from the MAI NRS (42° 35.80 S, 148° 14.00 E) off Tasmania's east coast.

Seawater samples for pooled and discreet samples consisted of different depths, with some depths being taken from different Niskin casts due to equipment limitations associated with the cast line. Each of the two casts were made within one hour of each other and from the same GPS location. The cast from which pooled depth samples were taken included depths of 0, 10, 20, 30, 40 and 50m, as this cast also serviced several other subsample types that required these sample depths. Discreet depth samples were taken from a combination of casts including 0, 10, 20 and 50m from the same cast as pooled depth samples as well as 75 and 85m from a second cast. The difference in sampling depths is due to the alignment of samples with microbial community profiling being undertaken as part of the Australian Marine Microbial Biodiversity Initiative (AMMBI; Brown et al. 2018). AMMBI was introduced to NRS sampling for the MAI, PHB and NSI NRSs from mid-2012, followed by the remaining NRSs in mid-2015. The need to cover as much of the water column as possible (within the limitations of the sampling equipment) determined AMMBI sampling depths, hence MAI is the only NRS that changed some of the depths when sampling moved from pooled to discreet depths.

After collection, samples were dealt with according to recognised and published protocols (Marie et al, 2000, 2005; Ribeiro et al, 2016) and the National Reference Stations Biogeochemical Operations Manual (Davies et al., 2019). In brief, 1 mL aliquots were taken from each sample in triplicate and transferred into 2 mL cryovials, to which 10 µL of 25% glutaraldehyde solution was added. Samples were then gently mixed and stored at room temperature for 15 minutes before being placed in either liquid nitrogen or in a -80 $^{o}$C ultrafreezer. Samples requiring shipping were done so in a fully saturated liquid nitrogen dry shipper and then quickly placed in a -80 $^{o}$C ultrafreezer until analysis. Before being analysed, samples were thawed in a 25 $^{o}$C water bath for no more than 15 minutes and then kept in low or no light until measurement.

All samples were measured using a CytoSub flow cytometer situated at CSIRO Marine Laboratories in Hobart. The CytoSub has two excitation lasers (488 nm and 552 nm) and six detection channels covering dual forward- and side-scatter as well as red, orange and yellow fluorescence. Measurements were taken in closed-sheath mode with a measurement volume of 100 µL. Measurements were analysed using CytoClus 4.0 analysis software, with taxonomic discriminations based on the marine cyanobacterial genera *Prochlorococcus* and *Synechococcus* along with the "Picoeukaryotes" – all non-cyanobacterial auto-fluorescing cells.

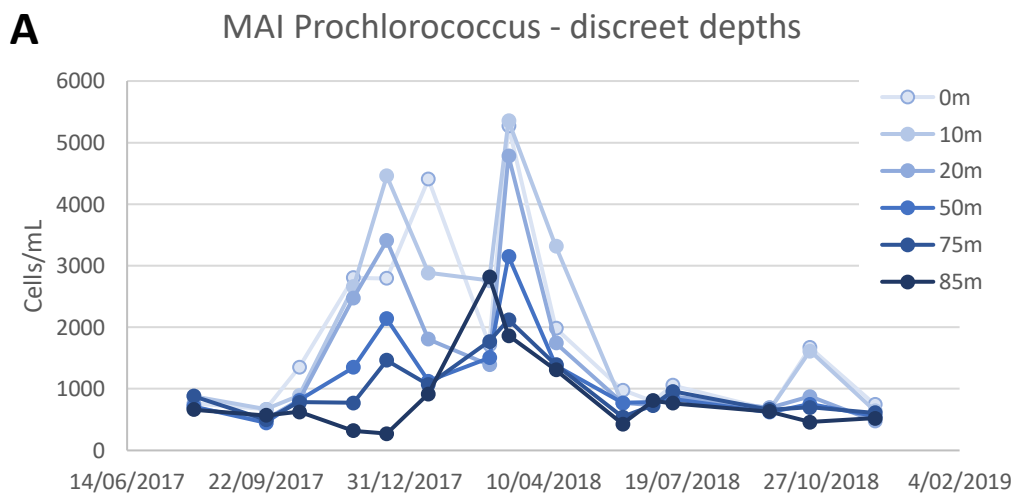
# 3 Results

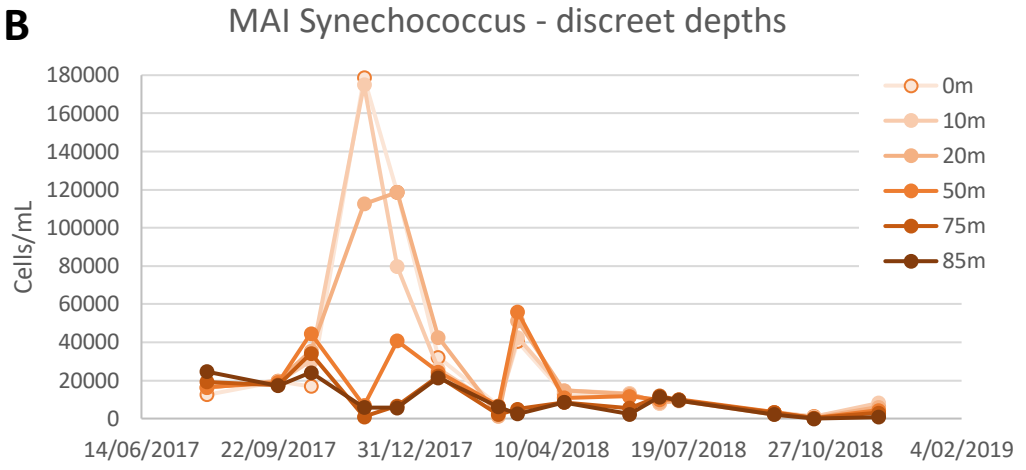
A large range of cell abundances were measured from the MAI NRS over the 15-month study period, as observed from discreet depth samples (Fig. 1). *Prochlorococcus*, commonly occurring in tropical marine environments when salinity is high, had typically low abundances between May and October, with two "pulses" of increased abundance in November 2017 to January 2018 and again in March. During this November to January pulse, an approximately five-fold increase in abundance occurred mainly for the shallower depths (0 – 20m), with no or only a minor change occurring for the lower depths (50 – 85m). In February, between the two pulses, only the lower depths showed an increase in abundances, while during the March pulse all depths had an approximate six-fold increase in *Prochlorococcus* abundance.

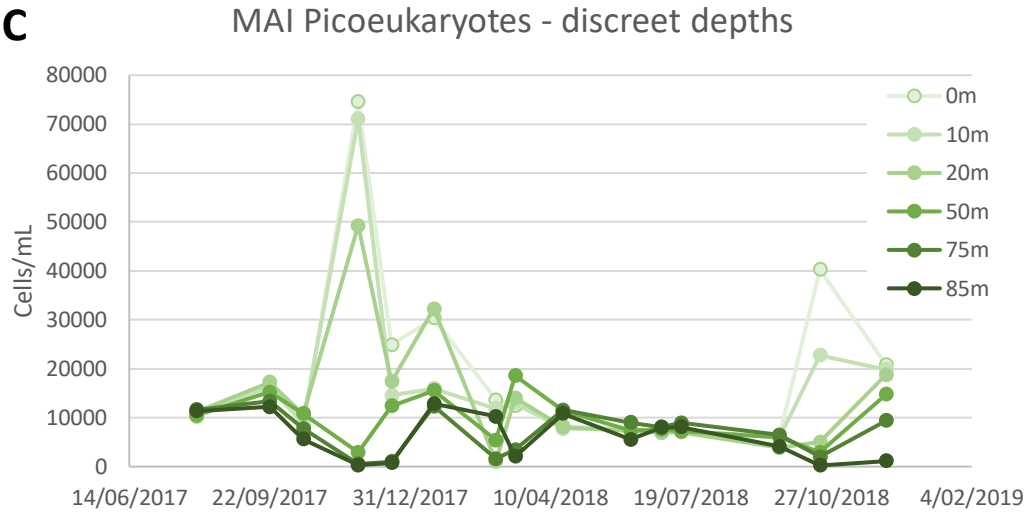
*Synechococcus* similarly had two pulses of increased abundance at the same times as *Prochlorococcus*, although with quite different characteristics. Over November and December 2017, shallower depths (0 – 20m) had a 12- to 20-fold increase in *Synechococcus* abundance, while the March pulse only had an approximate six-fold increase in the 0 – 50m depths, with the 50m depth sample having the highest abundance.

For the picoeukaryotes, a five- to eight-fold increase in abundance was seen in surface depths (0 – 20m) during November 2017, slightly elevated abundances in these depths through to January, then a smaller increase seen in only the upper 10m in October 2018.

In relative terms, *Synechococcus* and picoeukaryotes had similar abundances of approximately 10,000 cells.mL$^{-1}$ during the colder months with *Prochlorococcus* having abundances of below 1000 cells.mL$^{-1}$ during these same months. During warmer months, especially during the November to January pulse, abundances increased up to 180,000 cells.mL$^{-1}$, 70,000 cells.mL$^{-1}$ and 5000 cells.mL$^{-1}$ for *Synechococcus*, picoeukaryotes and *Prochlorococcus*, respectively.





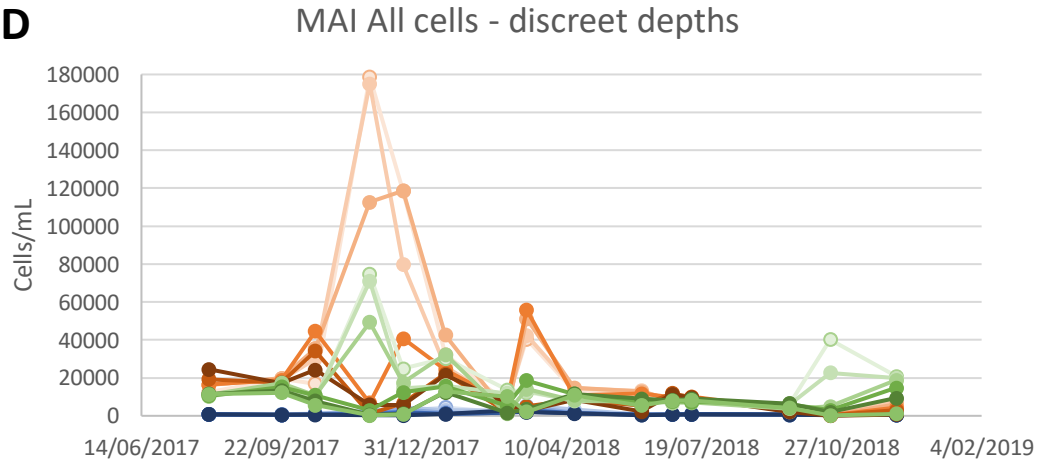




**Figure 1 Cell abundances measured for discreet depth samples over the 15-month study period. A = *Prochlorococcus*; B = *Synechococcus*; C = Picoeukaryotes; D = all taxonomic groups**

To make the closest comparison of discreet versus pooled depth sampling methods, the upper 50m of discreet depth samples (0, 10, 20 and 50m) were averaged and compared with the pooled depth sample (a mixed aliquot from each of 0, 10, 20, 30, 40 and 50m depth samples). Figure 2 shows the differences in estimated absolute abundance for each of these methods. While some differences were seen for measurements between sampling methods, the overall trend is closely followed. Differences in abundance were most obvious for the November 2017 sample when *Synechococcus* and picoeukaryotes were at their highest abundances.

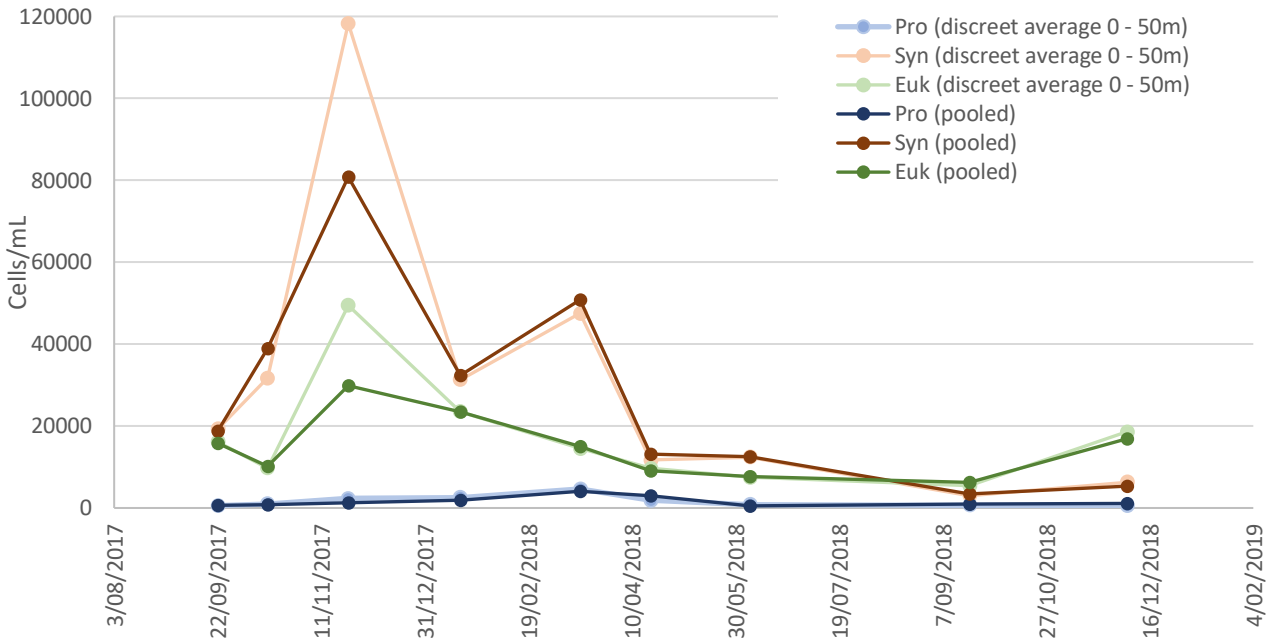



**Figure 2 Comparison of absolute cell abundances between discreet and pooled depth samples.**

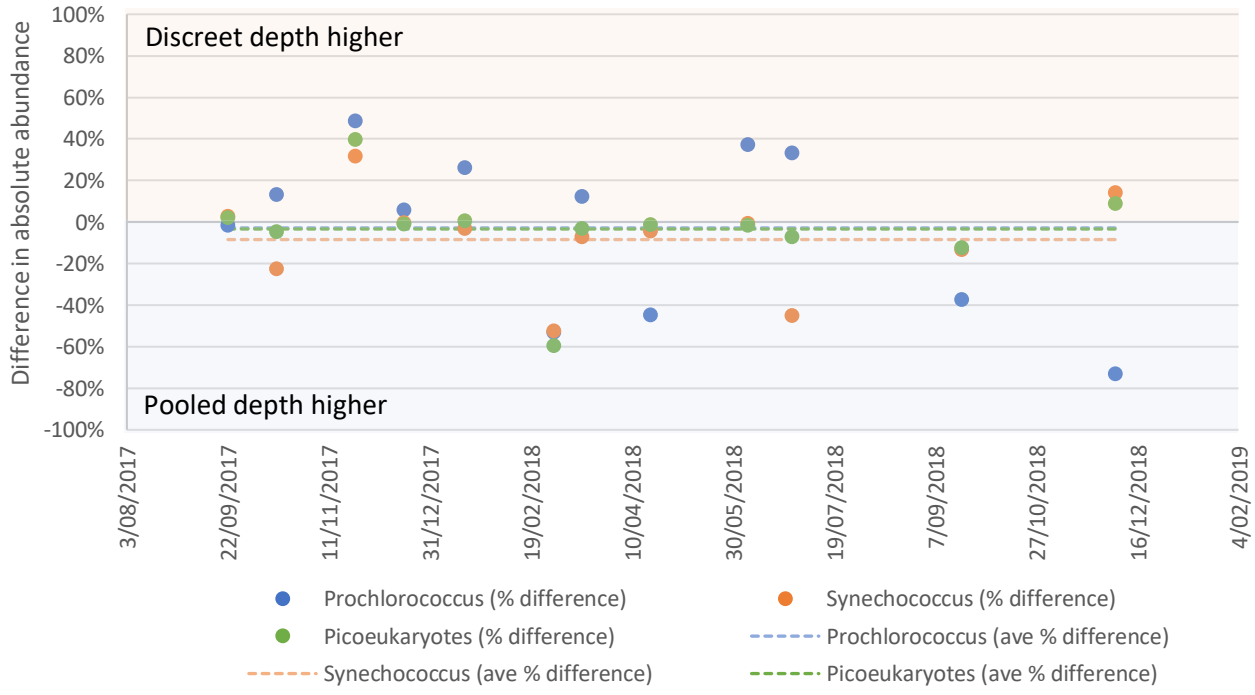


**Figure 3 Comparison of estimated cell abundances between averaged discreet and pooled depth samples as expressed in percentage difference terms.**

When estimated abundances for each of the sampling methods is expressed as a percentage, greater resolution in differences can be seen (Fig. 3). *Prochlorococcus* had the lowest average percentage difference of 2.3% (higher estimation by pooled sample) despite having the greater range in percentage difference (35%). This large range in differences for *Prochlorococcus* is not surprising given the overall low abundances measured over most of the study period which are typical for cool temperate locations such as the MAI NRS. Owing to the small size and low pigment content of *Prochlorococcus* in high light intensity surface waters, it's signal can be particularly difficult to discriminate from the background instrument noise. When abundances are low, for instance below 2000 cells.mL$^{-1}$ (as determined in report "IMOS/FC-1_2019"), this high noise:signal can magnify differences in estimated abundance. As such, abundances under 2000 cells.mL$^{-1}$ were removed for this comparative analysis.

For *Synechococcus*, abundance differences up to 52% were measured, while the average difference was 8.4% (higher estimation by discreet depth). For the picoeukaryotes, abundance differences of up to 60% were measured, while the average difference was only 3.4% (higher estimation by discreet depth).

Overall, for both *Synechococcus* and picoeukaryotes, estimated abundance differences were higher when absolute abundance was higher e.g. November 2017 sample, whereas the opposite was apparent for *Prochlorococcus* i.e. estimated abundance differences were higher when absolute abundance was lower. Average abundance differences between pooled and discreet depth sampling for MAI of 1%, 4% and 4% for *Prochlorococcus*, *Synechococcus* and picoeukaryotes, respectively, indicate very little difference between sampling methods and should alleviate any concerns with using a dataset that spans the point where a changeover from pooled to discreet sampling occurred (June/July 2017). This assumes the findings for MAI are applicable for other NRSs – indeed they should be, as MAI is the only station where a change in depths occurred in the transition from pooled to discreet depths.

# 4 Conclusions

The main aim of this comparison study was to determine the differences, if any, between pooled and discreet depth sampling at IMOS NRSs due to a changeover in sampling approach starting in July 2017. MAI is the main NRS potentially affected by this change, with the essential differences being the 30m and 40m sample depths which are not sampled and included in the determination of discreet depth average abundance.

Over a 15-month study period, from August 2017 to December 2018, it has been determined that average abundance differences between pooled and discreet depth sampling for MAI were 2.3%, 8.4% and 3.4% for *Prochlorococcus*, *Synechococcus* and picoeukaryotes, respectively, with pooled depth sampling having the higher average abundance for each taxonomic category. Overall, this indicates that there are differences in estimated abundances between sampling methods, however these differences are minor.

For both *Synechococcus* and picoeukaryotes, differences in estimated abundance were higher when absolute abundance was higher, whereas the opposite was apparent for *Prochlorococcus* i.e. estimated abundance differences were higher when absolute abundance was lower. While each of the three taxonomic groupings show variation in absolute abundance over seasonal cycles and with depth, these variations were much more marked in *Synechococcus* and picoeukaryotes. It is highly likely that when overall abundances are high, lower abundances in the 30m and 40m components of the sample relative to the 0, 10 and 20m components has resulted in the discreet depth average estimated abundance being slightly higher compared with the pooled depth abundance.

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