

## Surveying a Diverse Pool of Microalgae as a Bioresource for Future Biotechnological Applications

Gisela Jakob<sup>1,2</sup>, Juliane Wolf<sup>1,2</sup>, Tony VL Bui<sup>1</sup>, Clemens Posten<sup>3</sup>, Olaf Kruse<sup>4</sup>, Evan Stephens<sup>1,2</sup>, Ian L Ross<sup>1,2</sup> and Ben Hankamer<sup>1,2\*</sup>

<sup>1</sup>Institute for Molecular Bioscience, The University of Queensland, Queensland, Australia

<sup>2</sup>Solar Biofuels Research Centre, The University of Queensland, Queensland, Australia

<sup>3</sup>Institute of Life Science Engineering, Bioprocess Engineering, University of Karlsruhe, Karlsruhe, BW D-76131, Germany

<sup>4</sup>Department of Biology, Algae Biotechnology & Bioenergy, University of Bielefeld, Bielefeld, NRW D-33501, Germany

### Abstract

Resource limitation is an escalating concern given human expansion and development. Algae are increasingly recognised as a promising bioresource and the range of cultivated species and their products is expanding. Compared to terrestrial crops, microalgae are very biodiverse and offer considerable versatility for a range of biotechnological applications including the production of animal feeds, fuels, high value products and waste-water treatment. Despite their versatility and capacity for high biomass productivity on non-arable land, attempts to harness microalgae for commercial benefit have been limited. This is in large part due to capital costs and energy inputs remaining high, the necessity of identifying 'suitable' land with proximal resource and infrastructure availability and the need for process and strain optimisation. Microalgae represent a relatively unexplored bioresource both for native and engineered strains. Success in this area requires (1) appropriate methods to source and isolate microalgae strains, (2) efficient maintenance of motherstocks, (3) rapid strain characterisation and correct matching of strains to applications, (4) ensuring productive and stable cultivation at scale, and (5) ongoing strain development (breeding, adaptation and engineering). This article illustrates a survey and isolation of over 150 local microalgae strains as a bioresource for ongoing strain development and biotechnological applications.

**Keywords:** Microalgae; Bioresource; Motherstocks

### Introduction

The global population is projected to increase from its current level of ~7 billion up to ~9 billion by 2050 [1]. This, together with unprecedented levels of lifestyle change in developing countries and policies designed to alleviate poverty (though global effect on addressing hunger appears to have recently stalled [2]), is by 2050 forecast to result in the requirement of ~70% more food [3] and ~50% more fuel [4], as well as ~50% more fresh water [5] and an increasing amount of chemical feedstocks. To supply these resources while simultaneously reducing global CO<sub>2</sub> emissions requires a transition away from fossil fuels, and towards renewable systems. The scale of this challenge should not be underestimated, given the urgent need for a very significant CO<sub>2</sub> emission reduction in this decade if we are to stay within the so called 'safe limit' (2°C) defined by the Intergovernmental Panel on Climate Change [6]. This is an ambitious target given recent claims that 80% of remaining fossil fuels must be left in the ground to prevent progressing past this threshold [7].

Fuel, food and water resources are all inextricably connected within our production-consumption cycles. For example, high levels of fertiliser use and water desalination are already required to support our existing population and will likely have to increase to provide food and water security. This in turn requires increased fuel consumption. More efficient means for utilising biological systems as sustainable bioresources to produce food, fuel chemical feedstocks and high value products are becoming increasingly important as consuming ancient fossil fuels becomes more controversial, and the necessity of CO<sub>2</sub> emission reductions becomes more widely represented in global policy.

Microalgae production systems are positioned at the nexus of these challenges as many species have high efficiencies relative to conventional crops in terms of using solar energy to drive the conversion of CO<sub>2</sub> to biomass (stored chemical energy). This biomass can subsequently be used to produce a broad range of downstream

products. It has been widely stated that microalgae have the advantage that they can be produced on a proportion of non-arable land (non-arable land is ~25% of global surface area vs. ~3% arable land area [8,9]) and in many cases can use saline and waste water streams. This theoretically opens up the opportunity to extend global photosynthetic capacity beyond arable lands and assist with a transition from the current *food vs fuel* position [10,11] to a more sustainable *food and fuel* future. However the simplicity of the concept has not progressed to commercial reality despite a significant international research effort. This is primarily due to the many interconnected challenges of optimising biology and engineering parameters for high efficiency production and integrating these into commercially viable systems. Newly emerging strategies for high efficiency microalgae production [12,13] may contribute significantly to a food and fuel future but they are not the panacea that some have promoted. Opposing opinions that microalgal production systems lack the appropriate production strains suitable to overcome the challenges of economic and environmental sustainability for competitively priced biofuel production may be valid at the present time, but such arguments are insubstantial given the early stage of technology maturity, the rapid ongoing development in the field currently, and the large microalgae biodiversity (~350,000 species)

**\*Corresponding author:** Ben Hankamer, Institute for Molecular Bioscience, The University of Queensland, Queensland, Australia, E-mail: [b.hankamer@imb.uq.edu.au](mailto:b.hankamer@imb.uq.edu.au)

**Received** July 16, 2013; **Accepted** September 16, 2013; **Published** September 23, 2013

**Citation:** Jakob G, Wolf J, Bui TVL, Posten C, Kruse O, et al. (2013) Surveying a Diverse Pool of Microalgae as a Bioresource for Future Biotechnological Applications. J Pet Environ Biotechnol 4: 153. doi:10.4172/2157-7463.1000153

**Copyright:** © 2013 Jakob G, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

and advanced genetic engineering techniques that can be tapped for strain optimisation [14-16]. Exploiting such a large biological resource is clearly an advantage but also presents a considerable undertaking, and high-throughput processes for strain isolation and maintenance are certainly required to increase the efficiency of traditionally laborious methods. This article describes the establishment of native Australian microalgae collections in terms of bioresource potential, and summarises the purification and cryopreservation protocols developed to efficiently isolate over 150 native strains from a range of water sources for ongoing strain development in a broad range of applications.

## Founding A Microalgae Strain Library

International microalgae collections such as the Culture Collection of Algae and Protozoa (CCAP), Culture Collection of Algae at Göttingen University (SAG) and the University of Texas (UTEX) algae collection already offer a valuable resource for the provision of microalgae reference, research and breeding stocks. However international strain collections have their limitations and would benefit from augmentation with complementary local native strain collections which can offer a number of advantages. First, indigenous species are less likely to trigger local quarantine regulations (e.g. some imported strains are considered invasive 'weed' species or contain compounds undesirable for introduction into natural ecosystems). Second, indigenous species are generally more adapted to local climate conditions (e.g. light and temperature) and local biology (e.g. competitors and predators). Third, if correctly maintained and preserved (e.g. cryopreserved) wild type collections of indigenous species can be prevented from adapting to laboratory conditions (i.e. low selection pressures) which over time can result in a loss of culture robustness and suitability for large scale outdoor mass cultivation. Fourth, many strain collections are encumbered with intellectual property restrictions which specific local strain collections can avoid, although governments, national parks and private land owners can exert certain rights over commercially interesting strains isolated in such owned areas. The establishment of a phenotypically broad collection of local strains provides both a motherstock suited for further strain development and optimisation, and an improved understanding of competitor species that can invade aspiring monocultures of local or imported species. The aim of this article is to assist others with the establishment of similar local collections.

## Methods

Capturing a broad range of phenotypic diversity from natural water sources requires collection from a broad range of environmental conditions. In this study, saline and fresh water sources, as well as photoautotrophic and mixotrophic environments were sampled. Sampling from extreme environmental conditions is possible and can reveal extremophile species which continue to yield significant potential. 'Moderate' extremophiles like *Dunaliella* or some *Tetraselmis* strains (growing in hyper saline ponds) or *Arthrospira* (growing in alkaline ponds) are relatively easy to cultivate using these methods, but 'extreme' extremophiles generally require more advanced facilities (e.g. 60°C cultivation systems) that are not discussed here.

Isolation of strains from water samples is indelibly influenced by the isolation process design, and furthermore both passive analytical screens (e.g. productivity and compositional monitoring) and active biological response screens (e.g. selection pressure applied through cultivation) can be used to guide the strain selection processes and the subsequent development of databases of strain characteristics. A

flow diagram of the strategies used for microalgae isolation is shown in Figure 1.

In the strategy presented here the collection of crude water samples was followed by microscopic analysis (Figure 1 Native water samples) and subsequent incubation of the sampled species both in 'sterile source water' (to maintain species diversity) and in 'nutrient enriched water' samples supplemented with artificial medium for selection of the most adaptable species (Figure 1 Pretreatment). Following incubation several isolation techniques were employed including micromanipulation (Figure 1 Microman.), fluorescence activated cell sorting (Figure 1 FACS) and dilution (Figure 1 Dilution). Once isolated the method of choice for long-term storage was cryopreservation (Figure 1 Cryo) while serial cultivation on agar plates and in liquid media (Figure 1 Serial) was used for storage of sensitive strains. These isolates were identified via 18S [17,18] and 16S ribosomal sequencing [19] in conjunction with morphological classification (Figure 1 Identification) [20,21]. They were subjected to further screening to improve cultivation conditions and identify species for specific traits of interest (Figure 1 Screening) and to evaluate commercial cultivation capacity (Figure 1 Scale-up) to assist with strain selection and development for specific biotechnological applications. Each method step is described below.

## Water samples

500 mL samples were collected from a broad range of local water sources in the east and south of Australia (Table 1). At the location site, samples were taken between the water surface and 10 cm depth. Samples from biofilms on plant and rock surfaces were also obtained. Microscopic analysis (Nikon Ti-U fitted with a Nikon Digital Sight DS-U2, 5mp colour head; 200x and 400x magnification) was performed prior to further treatment to record microorganism diversity and provide an initial basis for morphological classification (e.g. Figure 1 Native water samples).

## Pre treatment

**Sterile source water cultivation:** The 'sterile source water' strategy was used to maintain maximum biodiversity. Although original water samples were non-sterile, the source water was sterilised (0.2 µm Supor® Membrane Syringe Filter, Acrodise® 32 mm, Pall Life Sciences) to produce a natural water supply for subculture. Sterile technique was practiced throughout the purification process to preserve initial biodiversity and prevent further contamination. The microalgae were cultivated (100 rpm, C10 Platform Shaker, New Brunswick Scientific; illumination at 10 to 100 µE m<sup>-2</sup>s<sup>-1</sup> cool white fluorescent light, relative to cell density) to increase the microalgae concentration.

**Nutrient enriched water based cultivation:** In this scenario water samples were enriched with nutrients to favour the selection of strains capable of fast nutrient uptake and fast growth. For nutrient enrichment, TP medium (TAP media [22] without acetate) was added to base water at a 1:3 enrichment ratio with subsequent cultivation for 4-7 days. Following initial enrichment and isolation, strains were transitioned to a range of fully artificial media including TP, TP +250 mM NaCl, TP +500 mM NaCl, TP + vitamins (3.9 µM thiamine, 7.5 nM cyanocobalamin, and 0.16 µM biotin, and these same vitamin concentrations were maintained as constant for all vitamins included media in this work, denoted as +V), TAP+V, 3NBBM+V [23], BG11+V for cyanobacteria [23], and DM+V for diatoms [23]. TAPY (TAP + 0.35% yeast extract) was used to encourage growth of contaminating microorganisms to confirm establishment of axenic cultures. Reagents were supplied by *Sigma-Aldrich*, *Chem-Supply* and *Amresco*.

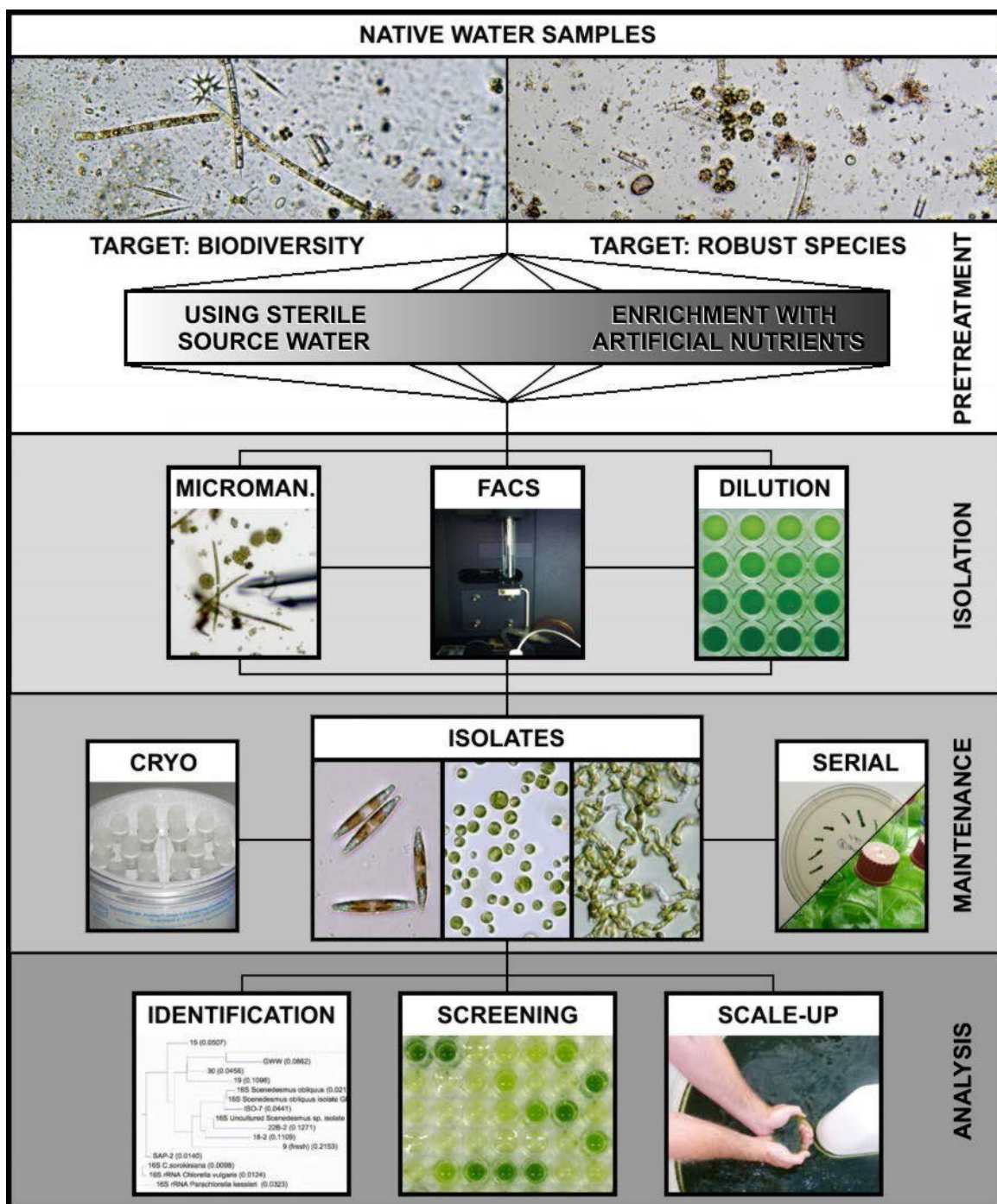


Figure 1: Flow chart of the isolation, maintenance and analysis connected to the establishment of a mid size microalgae strain collection for biotechnological applications.

## Isolation

**Fluorescence activated cell sorting (FACS):** FACS offers a rapid isolation technique to purify microalgae from the original sample or from contaminants. FACS has become increasingly popular in freshwater and marine ecology studies [24-26], and for these isolation procedures [27,28] due to the efficacy and high throughput aspects of this process. Success in this approach relies on several factors including the algal cell density and composition of the sample. Dominating

species are more likely to be successfully obtained, and therefore the algal diversity of purified cells can be compromised. The size and shape of individual algae cells also has an influence on the success rate of sorting, and the survival rate differs from species to species because of sensitivity to physical stress. Fragile diatoms for example had lower survival rates than chlorophytes. For FACS analysis 5 mL samples of the *sterile source water* and *nutrient enriched water* based cultivations were pre-filtered (40 µm, Nylon Cell strainer, BD Falcon) into a FACS



Collection sites	Salinity	Collected samples	Isolation technique	Non-axenic isolates	Axenic isolates	Acetate utilising isolates	Isolates lost	Isolates cryopreserved	Identified isolates
Australia, QLD, Brisbane, rain water tank	fresh	2	TAP Enrichment + FACS	12	12	12	0	12	<i>Chlorella</i> sp., <i>Scenedesmus</i> sp., <i>Desmodesmus</i> sp., <i>Desmodesmus intermedius</i>
Australia, QLD, Brisbane, Nursery 1	fresh	3	TAP Enrichment + FACS	12	12	10	2	2	<i>Chlorella</i> sp., <i>Chlorella sorokiniana</i> , <i>Micractinium pusillum</i>
Australia, QLD, Brisbane, Nursery 2	brackish/fresh	3	TAP Enrichment + FACS	18	18	14	4	14	<i>Chlorella</i> sp., <i>Chlorella sorokiniana</i> , <i>Micractinium pusillum</i> , <i>Scenedesmus</i> sp.
Australia, QLD, Brisbane, Nursery 3	fresh	1	TAP Enrichment + FACS	6	6	6	1	2	<i>Chlorella</i> sp., <i>Micractinium</i> sp.
Australia, NSW, rainforest waterfall	fresh	1	TAP Enrichment + FACS, Micro-manipulation	15	15	15	0	6	<i>Chlorella</i> sp., <i>Chlorococcum</i> sp., <i>Chlamydomonas</i> sp., <i>Desmodesmus</i> sp.
Australia, QLD, SE Townsville, port, seaside pond	salt	1	Micro-manipulation, FACS	2	2	1	2	0	No confirmed identifications
Australia, QLD, Townsville, river outlet	salt	4	Micro-manipulation, FACS	27	20	18	3	8	<i>Chlorella</i> sp., <i>Chlorella sorokiniana</i> ,
Australia, QLD, NE Townsville, pond	salt	1	Micro-manipulation, FACS	13	4	4	9	0	<i>Chlorella</i> sp.
Australia, QLD, Townsville, lake	brackish	2	FACS in Liquid	9	3	5	3	0	<i>Chlorella</i> sp., <i>Micractinium</i> sp., <i>Navicula pelliculosa</i> sp.
Australia, QLD, Gold coast, fish tank	fresh	2	Dilution, FACS in Liquid	18	11	4	3	7	<i>Stichococcus</i> sp., <i>Merismopedia</i> sp., <i>Elakatothrix</i> sp., <i>Ankistrodesmus</i> sp., <i>Chlorella</i> sp.
Australia, QLD, UQ, pond	fresh	3	Dilution, Micro-manipulation	9	5	9	0	3	<i>Chlorella</i> sp., <i>Scenedesmus</i> sp.
Australia, QLD, D'Aguilar, river	fresh	1	Micro-manipulation	7	7	0	1	3	<i>Ankistrodesmus</i> sp., <i>Chlorella</i> sp., <i>Scenedesmus abundans</i>
Australia, QLD, Central coast lake (1)	fresh	1	Micro-manipulation	3	1	0	0	0	<i>Chlamydomonas</i> sp., <i>Chlorella</i> sp.
Australia, QLD, Central coast lake (2)	fresh	1	Micro-manipulation	4	1	0	1	0	<i>Euglena</i> sp., <i>Chlamydomonas</i> sp., <i>Chlorella</i> sp.
Australia, SA, Waikerie, Murray River	fresh	1	Micro-manipulation	5	3	0	1	0	No confirmed identifications
Australia, NSW, Yanga, storm water	fresh	1	Micro-manipulation	4	1	0	1	0	<i>Anabaena</i> sp., <i>Staurastrum</i> sp., <i>Coleastrum</i> sp., <i>Nannochloris</i> sp.
Australia, QLD, Goondiwindi, creek	fresh	1	Micro-manipulation	3	0	0	0	0	<i>Aulacoseira</i> sp., <i>Closterium</i> sp.,

**Table 1:** Statistical analysis of algae isolation success from crude water samples. Collection sites, water characteristics, the number of water samples and strains isolated to increasing levels of purity are provided, together with their ability to utilise acetate, storage characteristics and species identification.

tube and analysed in a BD FACS Aria unit (BD Biosciences). The samples were then probed with a laser to detect individual 'events' corresponding to specific particles (e.g. algae cells or bacteria). The resultant dot plots present individual algae cells as population clusters (Figure 2a) which can be analysed in terms of parameters such as forward and side scatter (which represent cell size and granularity). In

addition chlorophyll fluorescence was monitored (488 nm excitation wavelength, 695 ± 40 nm transmitting filter) to distinguish between bacteria and dead/stressed algal cells (low fluorescence) and healthy algae cells (high fluorescence). This is achieved through the application of gating thresholds (Figure 2a delineated regions) which define different subpopulations based on size and fluorescence (e.g. P1-P6).

In order to maximise species diversity it is important to select cells and discrete regions to avoid oversampling dominant species, a process that is simplified by FACS.

Single or multiple events (e.g. individual or multiple cells) with different cell size and chlorophyll content were sorted into 96 well plates at a gating setting of one or more events per well (Figure 2b), containing 150  $\mu\text{L}$  of solid agar media, 150  $\mu\text{L}$  of liquid media, or 150  $\mu\text{L}$  solid agar media topped with 50  $\mu\text{L}$  liquid media (media as defined above). After sorting, microalgal growth was monitored via inverted microscope (Figure 2c), and success rates were ranked for each strain on the basis of colony formation and contamination status. Using a setting of three sorting events per well into liquid media yielded the highest success rate of single species recovery (>63%). Using a lower events/well setting or sorting on solid agar media resulted in a lower success rate (<10%) and less diverse algae populations constant with [29] who also reported a relationship between sorting success, culture media and culture vessel size of the micro well plate.

**Micromanipulation:** Micromanipulation is a laborious but powerful technique which allows the manual targeting of specific cells within a complex mixture. This is useful for sensitive strains and to increase biological diversity. Individual target cells were identified by microscopy (Olympus BX 41, 100x magnification) and extracted with a micromanipulator MM33 (Maerzhauser Wetzlar). Replaceable glass capillaries (Drummond Scientific, length 3.5", outer diameter 1.14 mm, inner diameter 0.53 mm) were used to select and transfer the cell into either *sterile source water* or *nutrient enriched water* as defined above. Individual cells were directly selected from these water samples. An alternative strategy involved spreading the microalgae sample (50  $\mu\text{L}$ ) onto agar and selecting cells after they had settled [29].

**Dilution technique:** Dilution either in liquid or solid media can be used as an alternative technique to resolve and purify individual algae strains. Achieving effective dilution on solid media [30] involves streaking of a small volume of the original sample onto agar plates (TP or original sterile water source media) with an inoculation loop in a three- or four phase streaking pattern. Plates were then incubated (conditions as above) until colonies appeared (some originating from a single isolated cell) which could then be manipulated individually. Re-streaking was repeated until pure cell colonies were observed. In parallel, liquid serial dilution was performed using 96 well plates.

Enriched as well as untreated water samples were serially diluted (4:1) through 48 wells filled with 500  $\mu\text{L}$  of the appropriate medium. Samples were incubated under low light conditions ( $\sim 50 \mu\text{E m}^{-2} \text{ s}^{-1}$  cool white fluorescent light) and examined daily (Nikon Ti-U inverted microscope).

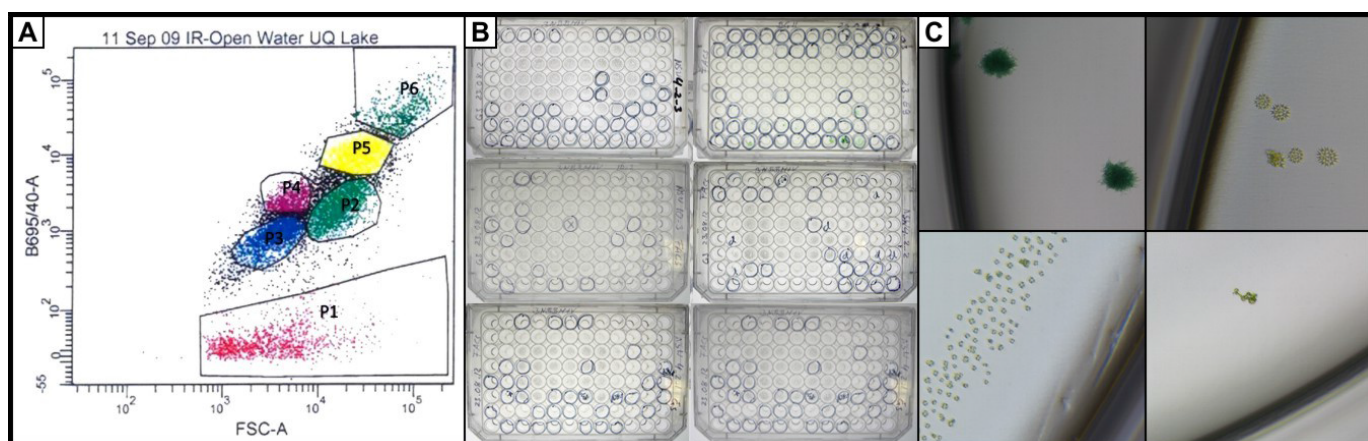
## Maintenance

**Enrichment and maintenance of established isolates:** Established isolates were enriched further with artificial media and incubated in larger volumes (10 mL) to increase cell number and concentration. For some microalgal isolates a stepwise increase of the concentration of artificial medium was found to be beneficial and was applied, with growth monitored microscopically and by optical density ( $\text{OD}_{750}$ ) measurements. For long term storage triplicate samples of each isolate were cryopreserved using  $3\text{--}5 \times 10^6$  cells per cryo-vial using a refined two-step freezing protocol developed for microalgae [31]. The final volume (1 mL containing 6.5 % DMSO and 0.2 M sucrose (Sigma-Aldrich, Chem-Supply)) was stored at  $-80^\circ\text{C}$  for at least 4 hours before being transferred to  $-196^\circ\text{C}$  for long-term storage in liquid  $\text{N}_2$  vapour phase. Strains that could not be efficiently cryopreserved were maintained through serial cultivation using both liquid and solid media.

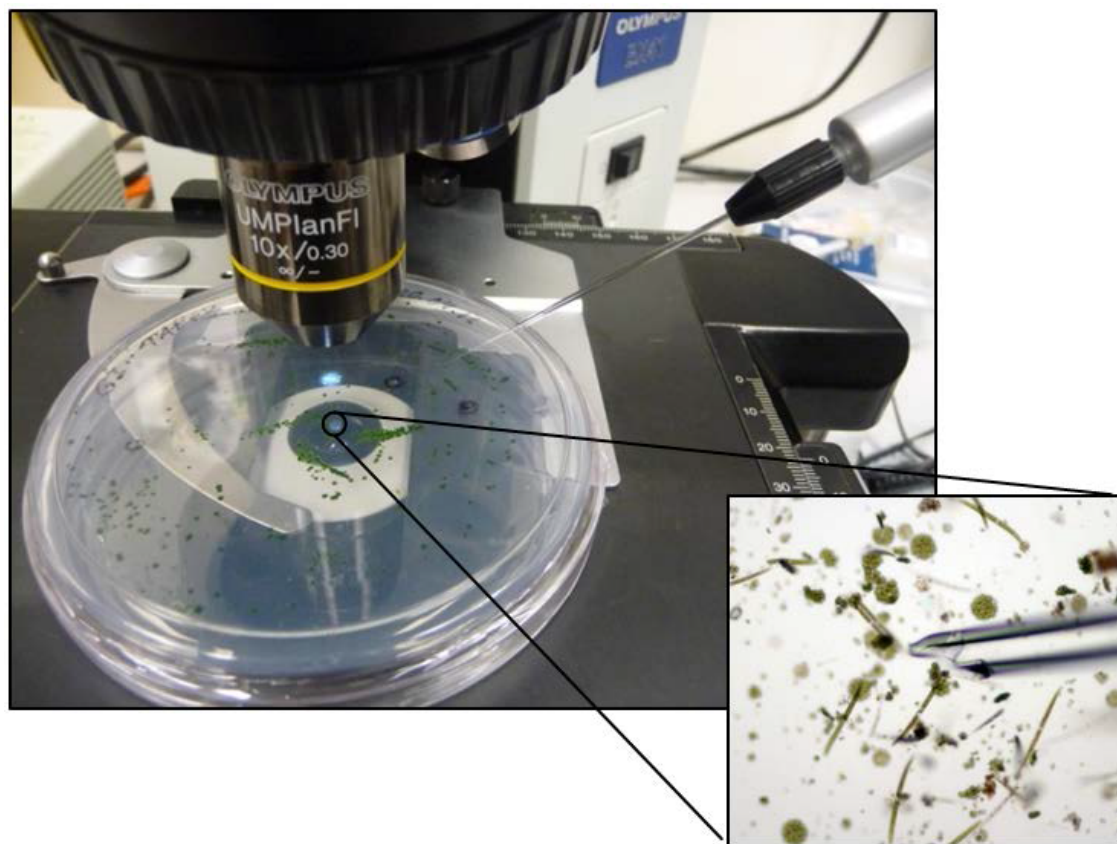
## Analysis

**Screening:** Screening for desirable properties is an ongoing process that can be repeated once a microalgal collection has been established. The isolates can be re-screened for a variety of applications, and where breeding is not possible, rapidly advancing methods for engineering microalgae can enable further advancement. The screening characteristics used here are therefore illustrative only. The principles, however are universal – very specific screens are usually time consuming so early rapid screening for indicative traits can be utilised first, followed by specific screening on a smaller subset of parameters. The isolates obtained in this work were initially screened on the basis of biomass productivity, and have already been subjected to a rigorous set of secondary screens and this work will be reported in the near future.

**Identification:** Only a subset of  $\sim 20\%$  of strains, which performed well in early screens, were selected for full identification (though this is clearly flexible). Identification consisted of morphological investigation (Olympus BX42 and Nikon Ti-U, 200x and 400x magnification) [20,21]



**Figure 2:** FACS isolation process. (A) FACS dot plot based upon laser excitation (y-axis) and forward scatter (x-axis) of cell mixtures which facilitates population analysis and selection of defined regions (P1-P6) for sorting; (B) 96 well plates containing purified algae; (C) microalgal isolate growth in individual wells monitored at 100x magnification.



**Figure 3:** Micromanipulation setup. Cells are imaged on agar plates. The insert shows individual cells at the tip of the needle used for aspiration (needle ID 0.53 mm).

and molecular classification by rDNA analysis. For the latter, DNA was isolated according to [32] though a 10 min sonication step was required to break open the cell walls of numerous wild type strains. Both 18S and 16S ribosomal DNA analysis was performed. The amplification of 18S rDNA and its sequencing was outsourced to the Australian Genome Research Facility (AGRF). The analysis of 16S rDNA was performed in house using two 'universal' primers [19] that specifically target cyanobacteria and eukaryotic photosynthetic plastids. PCR amplicons were sequenced at AGRF. Sequences were aligned using nucleotide BLAST (NCBI, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the 'nucleotide collection (nr/nt)' database.

## Results and Discussion

To establish this subset of our local microalgae strain library, water samples were sourced from 17 locations which included rainforest water sources, creeks, ponds and rivers, brackish/saline seaside ponds and river outlets, as well as artificial systems such as rainwater tanks, plant nurseries and fish tanks (Table 1). Clearly the biodiversity recovered from any biodiscovery program is dependent upon the biodiversity of the original water samples. The number of species recovered from a given water sample may be related to the trophic status of the source (i.e. oligo-, meso- or eutrophic) [33]. It has been previously reported [34] that oligotrophic conditions can have a higher level of species biodiversity for algae and while eutrophic water sources may contain more algae, species biodiversity is often lower as fewer species tend to dominate. Our observations supported this, and isolation processes yielded more isolates from eutrophic water sources.

In this study the 17 locations sampled, initially yielded a total of 167 non-axenic isolates. Of these ~95% were Chlorophytes (e.g. *Chlorella*, *Chlorococcum*, *Scenedesmus* and *Chlamydomonas*), ~4% cyanobacteria (e.g. *Anabaena* and *Merismopedia*) and diatoms (e.g. *Navicula*), and ~1% were unidentified cell types. 104 strains were recovered using the *sterile source water* method and 63 were obtained using the *nutrient enrichment water* method. Although source and treatment specific, the fact that the *sterile source water* approach generally yielded a greater biodiversity suggests that it may be the better standard method for the establishment of bio-diverse local microalgae culture collections. For the isolation of fast growing strains higher levels of nutrient enrichment were beneficial.

120 of the initial non-axenic strain samples were recovered using FACS, 42 using micromanipulation, and 5 by dilution. This clearly shows the benefit of using FACS as a platform for developing local microalgae strain collections as it can sort and dispense over 500 events per hour, particularly if augmented with strains isolated using micromanipulation to increase biodiversity. Using a combination of FACS and micromanipulation yielded an average of approximately  $10 \pm 7$  strains per water sample. Using a FACS setting of 3 sorting events instead of 1 per well resulted in only slightly higher bacterial contamination levels, but increased the success rates of recovering algal cell isolates. Despite this it was noted that the survival rate of sorted algae cells rose when 3 events per well were used and so this is suggested as a sensible starting point for FACS purification. It was also noted that the use of 96 well plates instead of 384 well plates improved species recovery, with 150  $\mu$ L solid agar media topped with 50  $\mu$ L liquid media being the preferred media configuration.



Of the 167 non-axenic isolates, 121 were purified to the axenic level. This was confirmed by microscopic investigation of cultures supplemented with acetate and yeast extract as a carbon source to encourage heterotrophic growth and demonstrating the absence of contamination. Antibiotic treatment in some cases was able to assist with the production of axenic cell lines, but in many cases proved toxic to the algae themselves and so was of limited utility. Overall approximately 90% of the 121 axenic strains were purified from bacteria simply by using FACS or through repeated subcultivation on carbon-free agar media. The remaining 46 non-axenic isolates could not be successfully purified from contaminating bacteria. This may indicate the presence of either strong adhesion of the bacteria to the algae cells or the presence of endogenous bacteria. The observation that most of the non-axenic algae cultures visibly exhibited a white biofilm around the cells, suggests that the former was predominantly the case. Furthermore certain species having complex shapes (e.g. constricted symmetrical arrangements, spiral twisted, colonial or filamentous) such as the Chlorophyte *Staurastrum* proved more difficult to purify from bacterial contamination. Whether these strong interactions between the bacteria and algae are simply physical or represent a form of symbioses remains to be established, however it is commonly noted in our open pond trials and by others that in healthy and relatively stable raceway pond systems many bacteria and algae can coexist effectively. Indeed one benefit to their presence may be that the bacteria use the dissolved oxygen in the culture produced through the photosynthetic reactions of microalgae. The importance of this is that dissolved oxygen levels become increasingly inhibitory to algae photosynthetic processes. A further benefit of bacterial interactions might be the synthesis of essential vitamins required by certain algae (e.g. Vitamin B12 [35]), as well as some other beneficial compounds [36].

Of the 121 axenic cultures 57 were successfully cryopreserved using the method of Bui et al (47% success rate) [31]. Strains having a diameter of 3 to 50  $\mu\text{m}$  were effectively recovered although some of the very large strains proved difficult, acceptable this step of the process would clearly benefit from improvement. Critical parameters include the optimisation of light level as high light can result in oxidative damage, as well as in the optimisation of nutrient conditions for specific strains.

Of the 64 strains that could not be cryopreserved 24 were lost during serial subcultivation. One reason for this is that the standard media used may not be sufficiently specific to the needs of individual species. Ongoing research is therefore required to optimise media composition.

Initial species identification was based on morphological classification but was refined through ribosomal sequencing. Ribosomal sequence analysis can be based on 18S rDNA and 23S rDNA analysis (derived from the nuclei of eukaryotes), or on 16S rDNA analysis (derived from chloroplasts and mitochondria) present in both eukaryotic microalgae and prokaryotic cyanobacteria. In this study 18S rDNA analysis was used as the primary rDNA analysis method but was supplemented with 16S rDNA analysis, contributing to the expansion of this resource. The 18S rDNA sequencing approach has the advantage that corresponding databases (e.g. NCBI) are more advanced than those for 16S rDNA, making it possible to achieve a higher quality of strain identification. Furthermore the 18S rDNA approach can currently enable identification to the species level in many cases. In practice our analysis typically yielded sequence identities of >95% but less than 100%, suggesting that while closely related to some strains in the online database, many of these wild isolates have not been previously

catalogued. Exact matches occurred at low frequency and in some cases two or more hits with a similar identity greater than 95% were noted. Theoretically the combined use of 18S and 16S rDNA sequence analysis may facilitate improved identification and could also resolve the origin of specific plastids within a given species, contributing not only to species identification but the evolutionary relationships between specific nuclear and plastid genomes.

## Conclusion

In this paper we have demonstrated a streamlined process for microalgae recovery from a broad range of water sources and used this to conduct a mid-scale survey of species native to Australian waters. Typically the water sources collected yielded ~10 strains of microalgae per sample, of which approximately half could be effectively cryopreserved to minimise maintenance costs and genetic drift, with most of the remainder being amenable to traditional subculture. Through the use of rDNA sequence analysis and morphological examination the resultant isolates were identified, either to the genus or species level providing a solid basis to assist the international research community with the establishment of multiple local strain collections to maximise microalgae species recovery as a breeding stock for cell lines beneficial for a wide range of biotechnological applications including the production of food, fuel, chemical feedstocks, high value products and for applications for wastewater treatment and bioremediation. Although there are already large international algae collections, the benefit of local strains collections include the establishment of robust, well adapted and locally derived breeding stocks that are often without the IP encumbrance associated with commercial strain collections. These can be used for the development of improved cell lines for a wide range of biotechnological applications. At a time when the global population is expanding from ~7 to ~9 billion people by 2050 and food, fuel and water demands are predicted to increase by 70%, 50% and 50% respectively the importance of establishing such diverse stocks becomes apparent. The ongoing exploration of the diversity of microalgal biology is already yielding advances in high performance wild types with commercial potential and genetic characteristics that could enable improvements for engineered strains. Initial screens focused on biomass productivity as a primary criteria (being a critical economic driver for commercialisation) but ongoing strain development will require further screens for a range of other useful characteristics including oil composition and profile, predator resilience, flocculation and other traits that enhance harvestability (e.g. floatation or sedimentation), and capacity for wastewater systems and bioremediation.

## Acknowledgements

The authors would like to thank Eugene Zhang and Lou Brillault for their support in maintaining the microalgae collection. BH gratefully acknowledges the financial support of the Australian Research Council (LP0883380) as well as from a National and International Research Alliance Project (NIRAP) grant supported by Queensland State Government and project partners (KBR, Neste Oil, Siemens, Cement Australia, The University of Queensland, University of Bielefeld and Karlsruhe Institute of Technology).

## References

1. Population Reference Bureau (2009) World Population Data Sheet.
2. Food and Agriculture Organization of the United Nations (2012) Economic growth is necessary but not sufficient to accelerate reduction of hunger and malnutrition. The State of Food Insecurity in the World.
3. Food and Agriculture Organization of the United Nations (2009) How to Feed the World in 2050.
4. International Energy Agency (2010) World Energy Outlook 2010.

5. OCED (2012) OECD Environmental Outlook to 2050: The Consequences of Inaction.
6. Stocker TF (2013) The Closing Door of Climate Targets. *Science* 339: 280-282.
7. Climate Commission (2013) The Critical Decade 2013: Climate Change Science, Risks and Responses.
8. Food and Agriculture Organization of the United Nations (2010) FAO Statistical Yearbook 2010.
9. Central Intelligence Agency (2013) The World Factbook.
10. Pimentel D, Marklein A, Toth MA, Karpoff MN, Paul GS, et al. (2009) Food Versus Biofuels: Environmental and Economic Costs. *Hum Ecol* 37: 1-12.
11. Gomiero T, Paoletti MG, Pimentel D (2010) Biofuels: Efficiency, Ethics, and Limits to Human Appropriation of Ecosystem Services. *J Agric Environ Ethics* 23: 403-434.
12. Vasudevan V, Stratton RW, Pearlson MN, Jersey GR, Beyene AG (2012) Environmental Performance of Algal Biofuel Technology Options. *Environ Sci Technol* 46: 2451-2459.
13. Stephens E, Ross IL, Hankamer B (2013) Expanding the microalgal industry--continuing controversy or compelling case? *Curr Opin Chem Biol* 17: 444-452.
14. Norton TA, Melkonian M, Andersen RA (1996) Algal biodiversity. *Phycologia* 35: 308-326.
15. Larkum AW, Ross IL, Kruse O, Hankamer B (2012) Selection, breeding and engineering of microalgae for bioenergy and biofuel production. *Trends Biotechnol* 30: 198-205.
16. Georgianna DR, Mayfield SP (2012) Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature* 488: 329-335.
17. Huss VAR, Frank C, Hartmann EC, Hirmer M, Kloboucek A, et al. (1999) Biochemical Taxonomy and Molecular Phylogeny of the Genus *Chlorella* Ssensu Lato (Chlorophyta). *J Phycol* 35: 587-598.
18. de-Bashan LE, Trejo A, Huss VA, Hernandez JP, Bashan Y (2008) *Chlorella sorokiniana* UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater. *Bioresour Technol* 99: 4980-4989.
19. Stiller JW, McClanahan (2005) Phyto-specific 16S rDNA PCR primers for recovering algal and plant sequences from mixed samples. *Molecular Ecology Notes* 5: 1-3.
20. Entwisle TJ, Sonneman JA, Lewis SH (1997) *Freshwater Algae in Australia A Guide to Conspicuous Genera*. Sainty & Associates Australia.
21. Hallegraef GM, Bolch CJS, Hill DRA, Jameson I, Leroi JM, et al. (2010) Algae of Australia: Phytoplankton of Temperate Coastal Waters. ABRS, Canberra & CSIRO Publishing Melbourne, Canberra, Melbourne 432.
22. Gorman DS, Levine RP (1965) Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci USA* 54: 1665-1669.
23. CCAP (2012) Culture Collection of Algae and Protozoa.
24. Reckermann M (2000) Flow sorting in aquatic ecology. *Sci Mar* 64: 235-246.
25. Cellamare M, Rolland A, Jacquet S (2010) Flow cytometry sorting of freshwater phytoplankton. *J Appl Phycol* 22: 87-100.
26. Crosbie ND, Teubner K, Weisse T (2003) Flow-cytometric mapping provides novel insights into the seasonal and vertical distributions of freshwater autotrophic picoplankton. *Aquat Microb Ecol* 33: 53-66.
27. Andersen RA (2005) *Algal Culturing Techniques*. Academic Press London UK.
28. Pienkos, PT (2008) Update on Algal Biofuel Research at NREL. 2nd Algal Biomass Summit. Seattle, WA, USA.
29. Sieracki M, Poulton N, Crosbie, N (2005) Automated Isolation Techniques for Microalgae, in *Algal Culturing Techniques*, Anderson RA Elsevier Academic Press London UK 101-116.
30. Anderson RA, Kawachi M (2005) Traditional Microalgae Isolation Techniques. *Algal Culturing Techniques* Anderson RA Elsevier Academic Press London 83-100.
31. Bui TVL, Ross IL, Jakob G, Hankammer B (2013) Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae.
32. Melanie O (2009) Chloroplasts as bioreactors : high-yield production of active bacteriolytic protein antibiotics.
33. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, et al. (2008) Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *BioEnergy Research* 1: 20-43.
34. Rawson DS (1956) Algal Indicators of Trophic Lake Types. *Limnol Oceanogr* 1: 18-25.
35. Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature* 438: 90-93.
36. Gonzalez LE, Bashan Y (2000) Increased Growth of the Microalga *Chlorella vulgaris* when Coimmobilized and Cocultured in Alginate Beads with the Plant-Growth-Promoting Bacterium *Azospirillum brasilense*. *Appl Environ Microbiol* 66: 1527-1531.