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A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*

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SUMMARY

Interest in exploiting algae as a biofuel source and the role of inorganic nutrient deficiency in inducing triacylglyceride (TAG) accumulation in cells necessitates a strategy to efficiently formulate species-specific culture media that can easily be manipulated. Using the reference organism *Chlamydomonas reinhardtii*, we tested the hypothesis that modeling trace element supplements after the cellular ionome would result in optimized cell growth. We determined the trace metal content of several commonly used *Chlamydomonas* strains in various culture conditions and developed a revised trace element solution to parallel these measurements. Comparison of cells growing in the revised supplement versus a traditional trace element solution revealed faster growth rates and higher maximum cell densities with the revised recipe. RNA-seq analysis of cultures growing in the traditional versus revised medium suggest that the variation in transcriptomes was smaller than that found between different wild-type strains grown in traditional Hutner's supplement. Visual observation did not reveal defects in cell motility or mating efficiency in the new supplement. Ni^{2+}-inducible expression from the *CYC6* promoter remained a useful tool, albeit with an increased requirement for Ni^{2+} because of the introduction of an EDTA buffer system in the revised medium. Other advantages include more facile preparation of trace element stock solutions, a reduction in total chemical use, a more consistent batch-to-batch formulation and long-term stability (tested up to 5 years). Under the new growth regime, we analyzed cells growing under different macro- and micronutrient deficiencies. TAG accumulation in N deficiency is comparable in the new medium. Fe and Zn deficiency also induced TAG accumulation, as suggested by Nile Red staining. This approach can be used to efficiently optimize culture conditions for other algal species to improve growth and to assay cell physiology.

Keywords: algae, ionome, inductively coupled plasma mass spectrometry, zinc, iron.

INTRODUCTION

Physiologically diverse algae are being developed as potential biofuel feedstocks because of their ability to accumulate triacylglycerides (TAGs) in the form of 'lipid bodies' under stress conditions (Wijffels and Barbosa, 2010). The large-scale culturing of these diverse strains requires a strategy to efficiently develop growth media that are species specific and amenable to manipulation. These media should maximize cell density per volume and/or TAG accumulation while minimizing the cost and resources required to provide cells with essential mineral nutrients.

Chlamydomonas reinhardtii serves as a reference organism for studies of algal physiology, including photosynthesis (Levine, 1960; Grossman, 2000; Dent *et al.*, 2001; Eberhard *et al.*, 2008), sexual reproduction (Quarmby, 1994; Lee *et al.*, 2008; Nishimura, 2010), ciliary biology (Silflow and Lefebvre, 2001; Cole, 2003; Snell *et al.*, 2004), micronutrient deficiency (Merchant *et al.*, 2006), ecological metal accumulation (Lustigman *et al.*, 1995) and algal biofuel production (Moellering and Benning, 2010; Radakovits *et al.*, 2010). *Chlamydomonas* has a sequenced genome (Merchant *et al.*, 2007), and a rigorous genetic system has been developed, making it amenable to metabolic manipulation through techniques such as gene knock-down or overexpression (Grossman *et al.*, 2007, 2010; Merchant *et al.*, 2007; Wijffels and Barbosa, 2010).

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The composition of growth media in which *Chlamydomonas* is cultured affects the overall viability and physiology of cells, especially in the case of micronutrient concentrations, where optimal health is attained only in a narrow concentration range for each nutrient (Sunda *et al.*, 2005; Merchant *et al.*, 2006). In standard practice, the trace element solution usually used to culture *Chlamydomonas* was derived from a formulation proposed by Hutner and colleagues based on the growth of pseudomonads (Hutner *et al.*, 1950). Hutner's trace element solution is problematic, as Fe precipitation occurs during a 2-week equilibration period. The precipitate must be filtered out, leading to batch-to-batch variation, not only in Fe concentration but in other metal ion concentrations as a result of their adsorption to the Fe precipitates. Such changes in Fe and other metal ion bioavailability can have dramatic effects on photosynthetic activity and respiration (Moseley *et al.*, 2002; Naumann *et al.*, 2007; Terauchi *et al.*, 2010). A more ideal supplement would be modeled after the specific needs of *Chlamydomonas*, accounting for metal ion concentrations, chelation strategies, bioavailability and metal speciation. Previous optimization of ammonium, phosphate and sulfur concentrations in the growth medium resulted in improved growth and increased hydrogen production in *Chlamydomonas* (Jo *et al.*, 2006). Variations in ammonium concentration leading to nitrogen depletion have also been used to induce cell differentation in preparation for mating and genetic studies, and for the accumulation of lipid bodies, a stress response that could potentially be exploited for developing algae as a biofuel source (Wang *et al.*, 2009; Moellering and Benning, 2010; Radakovits *et al.*, 2010). To date, however, a systematic optimization of the trace element supplement used to culture *Chlamydomonas* has not been attempted.

Here, we test the hypothesis that a trace element supplement modeled after the ionome of *Chlamydomonas* will improve its growth. Our revised trace element supplement avoids the pitfalls of traditional recipes. This strategy can be applied to the culturing of other algae to quickly assess the nutritional requirements of each species and optimize growth conditions.

RESULTS

Analysis of Hutner's trace element solution

Multiple trace element recipes are presently in use for culturing *Chlamydomonas* (Table 1) (Hutner *et al.*, 1950; Sager and Granick, 1953; Kuhl, 1962; Bischoff and Bold, 1963). Concentrations of Fe and Cu in some of these recipes are now known to induce symptoms of deficiency, even though overall growth is not affected (Merchant *et al.*, 2006). A commonly used protocol for making a standard trace element supplement for *Chlamydomonas* was derived from a recipe presented by Hutner and colleagues for the general culturing of photosynthetic prokaryotes (Hutner *et al.*, 1950; Harris, 2009). The preparation of Hutner's stock solution requires the sequential pooling and heating of trace metals followed by an equilibration and oxidation process that can take up to 2 weeks. The resulting solution is filtered to remove precipitates, which results in batch-to-batch variation in the final composition, an important consideration given that even mild differences in Fe impact photosynthesis, and therefore impact data interpretation in both laboratory-to-laboratory comparisons and year-to-year comparisons in the same laboratory (Moseley *et al.*, 2002). Measurement of metal concentration in a batch of Hutner's supplement immediately upon dissolving the trace elements and 2 weeks later, after filtration of the sample, revealed slight reductions in Zn and Cu, and a 50% decrease of Fe (Table 2). These measured final concentrations also varied from those measured in a batch of the mix made 3 years earlier in 2007.

Based on these observations, we sought to develop a new medium supplement by determining the metal ion content

Table 1 Comparison of trace metal supplements

	Hutner	Sager-Granick	Kuhl	Bold	Kropat
Fe^{2+}	17.9	0	25	17.9	0
Fe^{3+}	0	370	0	0	20
Zn^{2+}	76.5	3.5	1.0	30.7	2.5
Cu^{2+}	6.3	0.25	0.01	6.3	2
Co^{2+}	6.8	0.84	0	1.7	0
Mn^{2+}	25.6	2.0	1.0	7.3	6
Mo^{6+}	6.2	0.82	0.07	4.9	0.2
BO_3^{3-}	184	16	1.0	184	0
Se^{4+}	0	0	0	0	0.1
EDTA	134	0	25	171.1	57.8

Comparison of various trace metal supplements used to culture *Chlamydomonas*, adapted from Harris (2009). Values shown are the final concentration (in μM).

Table 2 Metal concentrations in Hutner's trace element solution

		Measured		
		April 2010		October 2007
	Calculated	Fresh	>2 weeks	>2 weeks
Fe	17.9	22	12	9.6
Zn	76.5	76	68	78
Cu	6.3	6.6	5.8	6.4
Co	6.8	7.2	6.4	6.7
Mn	25.6	27	24	24
Mo	6.2	6.5	5.7	6.8
B	184	ND	ND	ND

Metal concentrations in Hutner's trace element stock solution (mM) as described in the protocol, and as measured before and after the 2-week equilibration period from a batch made in April 2010 and one made previously in October 2007.

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of healthy wild-type *Chlamydomonas* cells growing in the traditional recipe, and iteratively modifying the supplement to reduce medium concentrations without affecting intracellular stores.

Metal quotas within wild-type cells

We determined the metal quota of three commonly used wild-type *Chlamydomonas* strains, CC-3269 (2137), CC-125 (137c) and CC-1690 (21gr), as well as CC-425 (an *arg2 cw15* strain derived from CC-125), grown in Tris–acetate–phosphate (TAP) medium (Gorman and Levine, 1965), promoting photoheterotrophic growth, and grown in TP (without acetate) medium supplemented with Hutner's trace element solution, promoting phototrophic growth. Cells in the exponential growth phase were collected by centrifugation, washed with EDTA to remove extracellular bound metals and analyzed for metal content using inductively coupled plasma mass spectrometry (ICP-MS) (Figure 1). Over a broad range, metal profiles were similar among all four strains, although a statistically significant difference was calculated for the Fe content in strains CC-425 and 2137, as well as for the Mn and Cu content for 21gr relative to the other strains. Fe is the most abundant metal ion in cells, followed by Mn, Zn and Cu ions, respectively. Generally, the accumulation of Cu and Fe ions was higher in cells grown photoheterotrophically relative to cells grown phototrophically. In all instances Co and B ions were below detection limits in the cell samples, but not in the medium. Comparison of relative metal ion concentrations in the media with relative intracellular metal ion profiles suggests that a dramatic excess of Co, B and Zn ions may be present in the media, as well as an excess of Mn ions.

Development of a revised trace-element supplement

Based on the intracellular metal quotas measured in TAP medium, a new trace element supplement was formulated to provide cells with Cu, Fe, Mn and Zn ions to support a cell density of 2×10^7 cells ml^{-1}, representing the typical cell density of a healthy culture at stationary phase, in threefold excess (Figure S1; Table 1). The excess quantity was meant to address late phase culture conditions by ensuring that cells did not experience trace nutrient deficiency in the stationary phase. The excess also accommodates changes in nutritional demand under different physiological conditions, such as those that occur during the transition between respiratory growth and photosynthetic growth (Terauchi *et al.*, 2010). Extreme nutrient excess is not beneficial, as optimum concentrations for some nutrients like Se and Fe cover only a narrow range in humans (from about five to sixfold) (O'Dell and Sunde, 1997; Merchant *et al.*, 2006). Excess nutrient availability may also cause unwanted metal interactions (Allen *et al.*, 2007; Baxter, 2010). As molybdate concentration did not significantly affect final cell density in TAP-grown cultures (Figure S1), it was provided in twofold excess, corresponding to concentrations used by Fernández and co-workers who grew CC-1690 cells with nitrate as a sole nitrogen source (Tejada-Jiménez *et al.*, 2007). Boric acid and cobalt ions were excluded completely as they did not impact final cell density (Figure S1), and no boron or cobalt could be measured inside the cells (Figure 1). We also do not know of any biochemical requirement for cobalt or boron for *Chlamydomonas* (see Discussion). Selenate was added to the supplement to account for the known presence of selenoproteins in *Chlamydomonas* (Novoselov *et al.*, 2002; Grossman *et al.*, 2007; Merchant *et al.*, 2007).

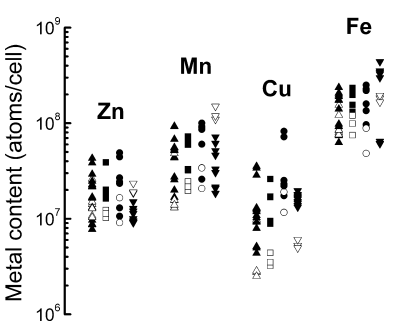

Figure 1. Intracellular micronutrient content.
Chlamydomonas strains CC-3269 (upright triangles), CC-125 (squares), CC-1690 (circles) and CC-425 (inverted triangles) were grown in TAP (filled symbols) and TP (open symbols) media amended with Hutner's mineral nutrient supplement and collected by centrifugation. The cell pellet was washed with 1 mM EDTA to remove extracellular bound metal and dissolved in 30% nitric acid by incubation in 65°C. The metal content was measured by ICP-MS. The *y*-axis plots the range of metal content on a per-cell basis under the various conditions or for different strains.

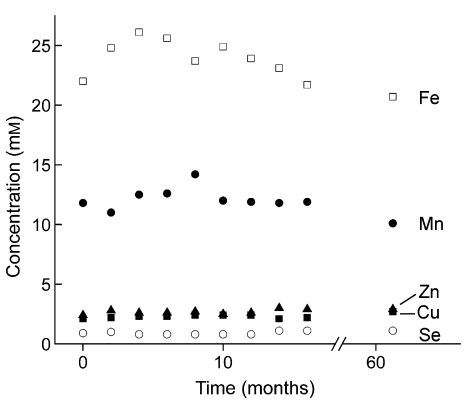

Figure 2. Stock solutions are stable over 5 years.
The concentrations of Cu (filled squares), Fe (open squares), Mn (filled circles), Se (open circles) and Zn (filled triangles) in stock solutions from a primary iteration of the revised trace element composition were measured by ICP-MS over a period of 61 months. Each solution was stored at 25°C in the dark. Before each sampling, the solution was filtered through a 0.22-μm filter into a new container. Each metal solution was diluted to an appropriate concentration prior to injection into the ICP-MS.

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The components of the revised trace elements were formulated as individual stock solutions. EDTA was included with Cu, Fe, Zn and Mn ion stocks to prevent precipitation of the salts. Additionally, 25 μM EDTA was added to the final medium to serve as a trace metal ion buffer (Sunda *et al.*, 2005). The EDTA supplement resulted in more reproducible growth curves when we tested the impact of variation in trace mineral concentrations. Solutions were stored in the dark at 25°C. Cu^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+} and Se^{6+} solutions from an early iteration of the recipe were found to be stable over a 5-year period under these conditions (Figure 2).

Physiological comparison of cells

To compare growth rates and maximum cell densities of cultures grown in medium supplemented with either the traditional trace element solution or the revised solution, CC-3269 was inoculated into bioreactors containing TAP medium with the two trace element supplements. Cells were initially followed during growth to stationary phase without the input of additional medium. During this time course, cells were collected at various optical densities (ODs) and analyzed for cell density, size and metal content. Under these conditions, cells supplemented with the revised trace elements showed an increase in growth rate and in maximum cell density (Figure 3a,b). Measurement of intracellular metal content at each time point during the initial growth curve showed no significant differences between the two supplements (Figure 3c–f). During the early period of growth the cell diameter averaged 9.1 μm, with a range of 4.4–11.8 μm, for cells grown with the traditional supplement, and the cell diameter averaged 8.5 μm, with a range of

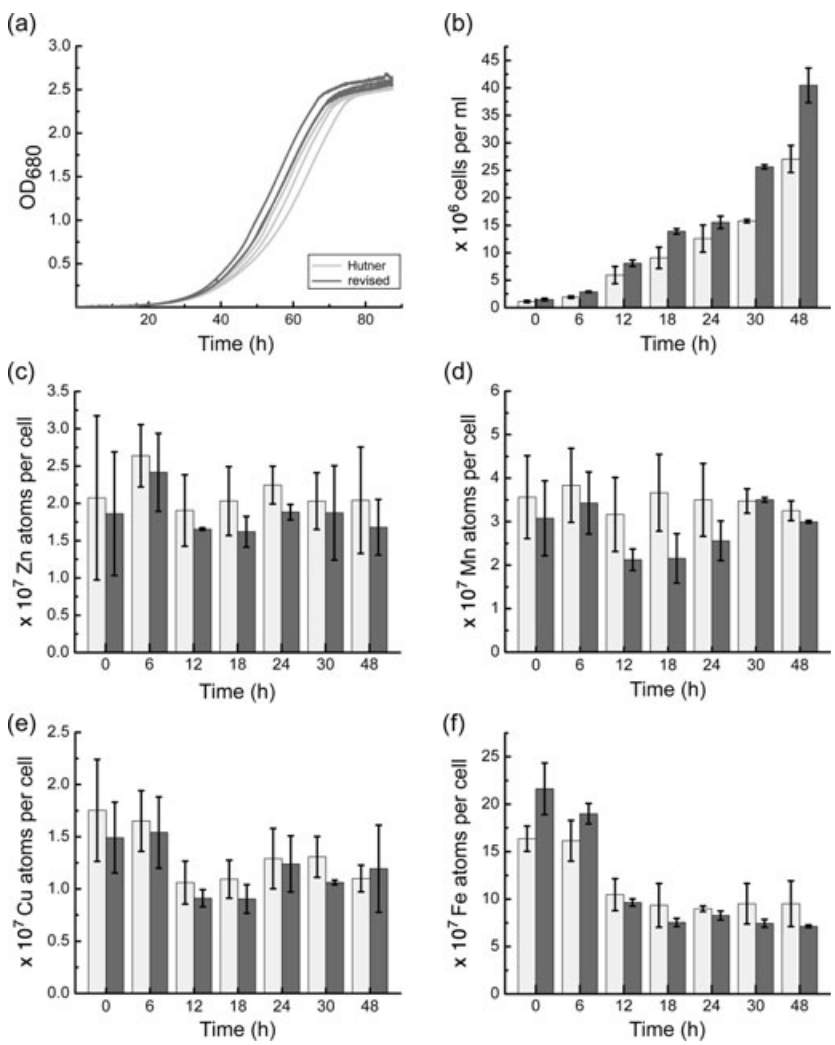

Figure 3. Supplementation with a revised trace element composition improves cell growth.
Strain CC-3269 was grown in TAP medium supplemented with Hutner's trace elements (light gray) or the revised composition (dark gray). Cell growth was measured by monitoring optical density at 680 nm (a) and by counting cells using a hemocytometer (b). Intracellular Zn (c), Mn (d), Cu (e) and Fe (f) were measured by ICP-MS, as described in Figure 1. The average of biological triplicates is shown and is representative of experimental duplicates. Cell counts were performed by two independent researchers for each duplicate without prior knowledge of the source of the supplement.

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3.7–11.1 μm, for cells grown with the revised trace-element solution. The differences were not statistically significant. The pH did not differ significantly in the two conditions, ranging on average from 7.3 to 8.4 (as acetate was consumed by the cells) for medium supplemented with the revised trace elements, and from 7.4 to 8.4 for medium supplemented with Hutner's trace elements, over the course of the experiment. In a second approach, cultures were maintained in turbidostat mode with 5% tolerance, producing a regular oscillating turbidity pattern introduced by cell growth (an increase in OD) and by medium input (a decrease in OD). Rates of growth were measured over these oscillating patterns for 16-h intervals. From this, a doubling time of 8.8 h was calculated for Hutner's solution versus 7.9 h for the revised supplement. All experiments were performed in experimental duplicate, and biological triplicates and cell counts were performed concurrently by two individual researchers without prior knowledge of the trace element supplement identity of the samples. Further analysis revealed that mating efficiency was similar for cells supplemented with either trace element solution. Moreover, the changes in medium metal composition did not result in differences in cell motility, phototaxis or presence of cilia.

Pairwise comparisons of RNA abundance levels from CC-3269 (2137) cells growing in traditional medium (H_{2137}), 2137 cells grown in an early iteration of the revised medium (mK_{2137}) or D66 cells growing in traditional medium (H_{D66}) (González-Ballester *et al.*, 2010) were performed to evaluate gene expression in the various media (Figure 4). D66 is similar to 2137 except that it lacks a cell wall and contains a mutation in the *NIT2* gene encoding a positive regulator of the nitrate assimilation pathway (Schnell and Lefebvre, 1993). Variation between identical strains growing in the different media (H_{2137} versus mK_{2137}) is less pronounced than variation between different strains grown in the same traditional medium (H_{2137} versus H_{D66}). Of the twelve genes encoding selenoproteins in the *Chlamydomonas* genome, transcript abundance of three genes, encoding MsrA, a functional methionine-*S*-sulfoxide reductase, SelW1 and SelH, the functions of which are unknown (Kim *et al.*, 2006; Grossman *et al.*, 2007), increased in the revised medium relative to the traditional medium. The increased abundance of these transcripts probably results from the introduction of selenate ions in the revised supplement.

Because a threefold excess of nutrients was supplied in the revised recipe, we characterized cells growing in a one to threefold dilution of the supplement, representing sufficient but not excessive nutrient conditions. No difference in cell density was observed relative to the normal supplement, and at late exponential phase, genetic markers for Fe deficiency (*FRE1*), Mn deficiency (*NRAMP1*) and Zn deficiency (*ZRT3*) were also not upregulated relative to cells grown with the normal supplement, showing that cells did not reach deficient conditions at the end of the growth phase (Figure S2).

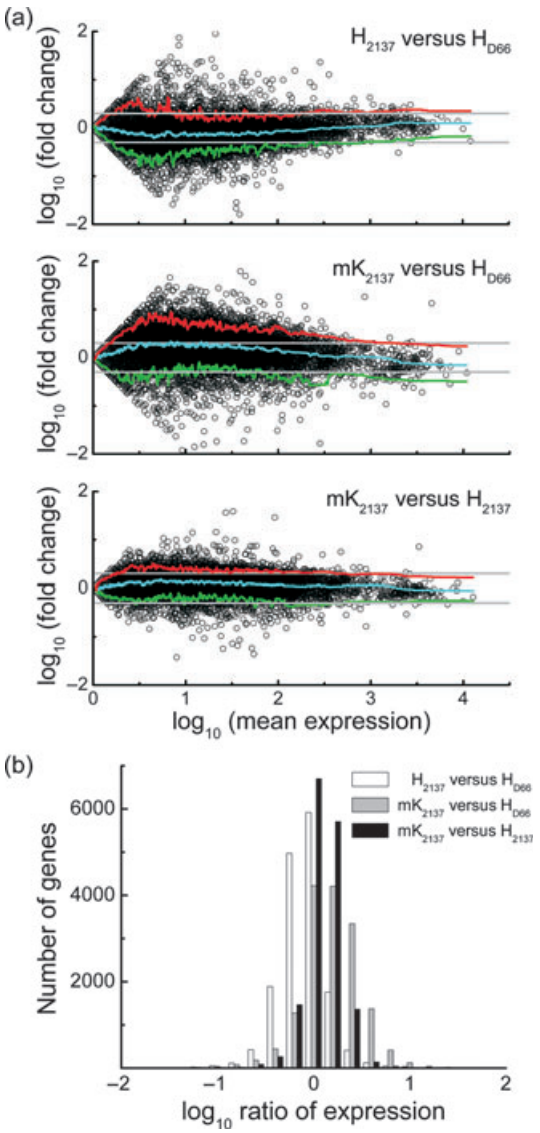

Figure 4. Pairwise comparison of transcriptomes from cells supplemented with different trace element recipes.
(a) Logarithmic mean-difference scatter plots of normalized expression fold change (*y*-axes) versus mean expression (*x*-axes). The plots were generated from pairwise transcriptome comparisons of wild-type strain 2137 supplemented with Hutner's trace element recipe (H_{2137}) (Castruita *et al.*, 2011), wild-type strain D66 supplemented with Hutner's trace element recipe (H_{D66}) (González-Ballester *et al.*, 2010) and wild-type strain 2137 supplemented with an early iteration of the revised trace element mix (mK_{2137}). Cyan lines in scatter plots represent the average fold change. Red and green lines represent the 95th and 5th percentile of fold changes, respectively. Gray lines represent a twofold difference in fold change.
(b) Histogram of expression fold changes for each pairwise comparison grouped into clusters of $\log_{10}$ 0.2.

Chlamydomonas is a reference organism for fundamental studies of chloroplast and cilia biology, and a metal-responsive promoter has been developed to drive

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ectopic gene expression (Quinn *et al.*, 2003; Surzycki *et al.*, 2007; Ferrante *et al.*, 2008). The promoter was developed for cells growing in traditional media (Quinn *et al.*, 2003; Ferrante *et al.*, 2008), and we therefore tested how it would work in the revised supplement. A standard concentration of 25 μM Ni ions was required for induction in medium supplemented by the traditional Hutner recipe, whereas a concentration of 50 μM Ni ions was required to elicit the response in medium supplemented with the new recipe (Figure 5). This increase corresponds to the 25 μM EDTA added to the medium to serve as a divalent metal ion buffer. A modification of the revised recipe with 25 μM less EDTA resulted in *CYC6* induction at the standard concentration of 25 μM Ni ions. Because of the known toxicity of Ni ions, using the variation of the new supplement with decreased EDTA is recommended for experiments using this promoter.

Characterization of deficient cell phenotypes

There have been many studies of nutrient stress in *Chlamydomonas*, including S-stress (Wykoff *et al.*, 1998; Laurinavichene *et al.*, 2004; Fouchard *et al.*, 2005), P-stress (Wykoff *et al.*, 1999; Yehudai-Resheff *et al.*, 2007; Moseley *et al.*, 2009), N-stress (Peltier and Schmidt, 1991; Beck and Acker, 1992; Abe *et al.*, 2004) and our studies on micronutrient deficiencies (Merchant and Bogorad, 1986; Moseley *et al.*, 2002; Allen *et al.*, 2007; Haas *et al.*, 2009). For macronutrient stresses such as N-deficiency, the accumulation of TAGs has been observed, and is presently of great interest because of its utility as an energy source (Wang *et al.*, 2009; Moellering and Benning, 2010; Radakovits *et al.*, 2010).

To characterize the physiology of nutrient-deficient cells under the new supplement regime, medium components were individually excluded and cells were grown in deficient conditions into stationary phase (Figure 6). We observed that the new medium allows for the study of individual nutrient deficiencies in a similar manner to our previous studies using the traditional trace element mix, and we see lipid body accumulation under N deficiency as predicted. Nile Red staining of cells to visualize lipid body accumulation (Cooksey *et al.*, 1987) also revealed an increase in lipid bodies in Fe- and Zn-deficient conditions. As these two conditions coincide with conditions that also inhibit growth, these results suggest that lipid body accumulation may be a general stress response. Cell size or morphology also appeared to be significantly different in several deficiencies, including Zn, Mn and N. As previously reported, Fe deficiency resulted in a chlorotic phenotype (La Fontaine *et al.*, 2002).

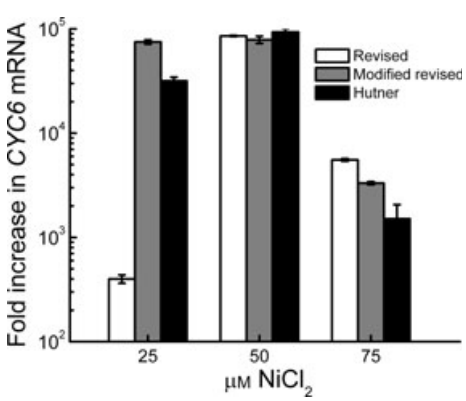

Figure 5. Optimization of the quantity of Ni^{2+}required to induce *CYC6* expression in the revised trace elements.
RNA was isolated from cells grown in TAP medium supplemented with the revised mineral nutrient mix, a modification of the revised recipe with EDTA reduced so that the molar levels of free species were comparable with the levels in Hutner's (2.5 μM), or Hutner's trace elements 5 h after the addition of the indicated quantity of $NiCl_2$. Transcript abundance was measured by RT-PCR and normalized to *EIF5A* expression. The average C_T value was calculated from technical triplicates. Expression was calculated by the $2^{-\Delta\Delta C_T}$ method. The average of experimental triplicates is shown.

DISCUSSION

Traditionally, the growth of *Chlamydomonas* relied on a trace element recipe developed for prokaryotes. We present a revised recipe based on wild-type *Chlamydomonas* ionomic data that reduces the time and metal salts required to make the medium, and results in faster growth and higher biomass production. Most importantly, the composition is reproducible and stable for 5 years. This general strategy, outlined in Figure 7, for developing species-specific media for algal culturing can potentially be applied to novel strains and mutants, including large-scale cultures used for biofuel development.

The *Chlamydomonas* ionome provides insight into its physiology. Marine algae *Bangia* and *Porphyra* and the freshwater green alga *Chlorella* reportedly require boron (Henkel, 1952; McIlrath and Skok, 1958), and boron deficiency in the cyanobacterium *Nostoc* was shown to lead to chlorosis (Eyster, 1952). The lack of a boron requirement in *C. reinhardtii* may result from the fact that it was isolated from soil, a typically boron-poor environment (Li *et al.*, 2008). *Chlamydomonas* also lacks rhamnogalacturonan II in the cell wall, a component that requires boron for dimer formation in plants (Kobayashi *et al.*, 1996). Although *Chlamydomonas* secretes biological compounds that interfere with the Al-2 furanosyl borate diester-mediated quorum sensing system of *Vibrio* spp., the chemical structures of these 'mimics' have yet to be identified (Chen *et al.*, 2002; Teplitski *et al.*, 2004).

The lack of a cobalt requirement is consistent with our current understanding of the absence of vitamin B_{12} biosynthesis in eukaryotic cells (Croft *et al.*, 2006). Cobalt has been shown to inhibit chlorophyll synthesis and induce iron deficiency in photosynthetic organisms (Csatorday *et al.*, 1984). Cobalt toxicity in *Chlamydomonas* was observed at a level of 10 ppm cobalt nitrate, and was significant at 20 ppm (Lustigman *et al.*, 1995). The elimination of non-essential trace elements decreases the

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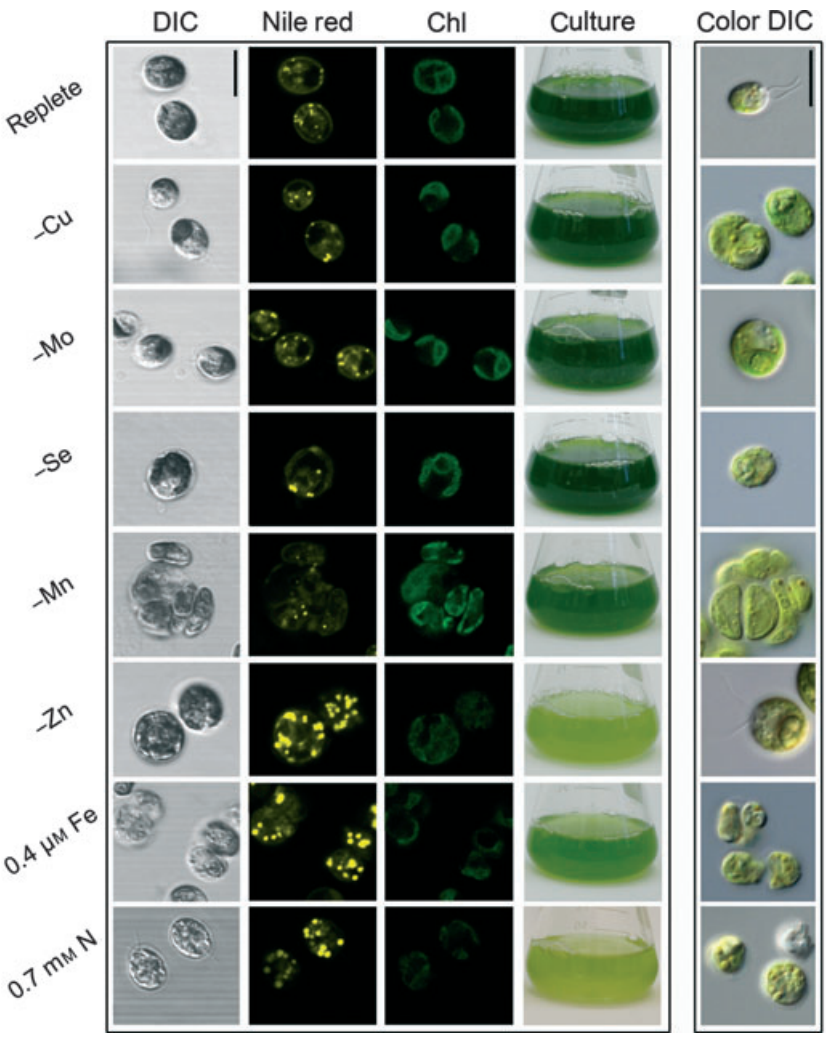

Figure 6. Comparison of lipid body formation, cell morphology and culture appearance of different growth conditions.
Cells were grown under replete and deficient nutrient regimes as indicated in the figure. Confocal micrographs of Nile Red stained cells in Photomultiplier Tube Trans and Photomultiplier Tube 1 (Nile Red fluorescence) channels are shown in columns 1 and 2, respectively. Chlorophyll autofluorescence is shown in column 3. The overall appearance of the cultures is shown in column 4. Additionally, cells were imaged on a Zeiss AxioImager Z1 microscope equipped with a color camera (AxioCam HRc) in DIC mode (column 5). Representative cells are shown. Scale bars: 10 μm.

likelihood of contamination of algal cultures by microbes that may require these nutrients to grow.

The Se^{6+} ion was included in our revised supplement to account for the known selenoproteins present in *Chlamydomonas* (Novoselov *et al.*, 2002). Although it was hypothesized that selenium atoms may cause toxicity by substitution for sulfur atoms in sulfoproteins, exposing *Chlamydomonas* to 0.1 μM selenate was not toxic with 8 or 80 μM sulfate (Fournier *et al.*, 2010), and exposing *Chlamydomonas* to Se^{6+} in a synthetic freshwater medium did not inhibit growth at a concentration of 0.1 μM (Geoffroy *et al.*, 2007). These data suggest that no adverse effects should arise from the inclusion of a low quantity of selenate in our supplement. Comparison of gene expression levels of cultures supplemented with Hutner's trace element recipe versus the revised recipe revealed the increased accumulation of transcripts encoding three of the 12 previously identified selenoproteins in the *Chlamydomonas* genome associated with the revised recipe. In each case, the Sec-encoding codon, UGA, is upstream of an intron. In the absence of a Sec-charged tRNA, the corresponding genes would be subject to non-sense-mediated decay (Moriarty *et al.*, 1998). In previous work on the *PCY1* locus, we confirmed that non-sense mediated decay operated in *Chlamydomonas* (Li *et al.*, 1996). Frame-shift mutations leading to premature stop codons at the 5′ end (upstream of an intron) in *pcy1-ac208* and *pcy1-2* resulted in reduced mRNA abundance. The addition of Se^{6+} may be beneficial under conditions of stress, when some members of the *Chlamydomonas* selenoproteome are thought to be required (Grossman *et al.*, 2007).

We predict that no symptoms of sulfur deficiency should arise from the revised medium, although the final sulfate

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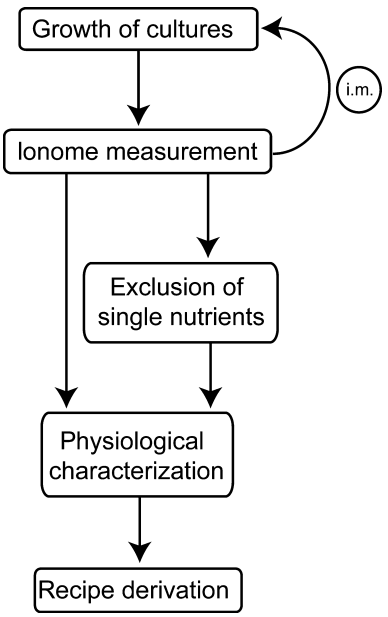

Figure 7. A strategy for deriving species-specific culture media; i.m., iterative modification of nutrient concentrations to accommodate the cell ionome.

concentration did decrease from 496 to 402.5 μM, in part because of the decrease in $ZnSO_4$. The inclusion of excess EDTA as a trace metal ion buffer resulted in more reproducible growth curves, suggesting that the growth of *Chlamydomonas* cells is sensitive to small fluctuations in mineral nutrient availability. This excess EDTA is not essential, and under situations where accurate accounting of metal nutrients is not critical, or in experiments relying on the Ni^{2+}-inducible gene promoter, may be unnecessary.

We note that the determination of metal quotas does not distinguish between absolute nutritional requirements and apparent requirements affected by the kinetics of nutrient uptake and bioavailability. Although lower absolute metal ion concentrations may be sufficient to allow the proper function of all required metalloenzymes in the cell, the rates of uptake by transporters may not be fast enough to provide optimal health, and the intracellular sequestration of metal ions may also affect nutrient requirements. The potential for interaction between elements also increases the complexity of nutritional studies (Merchant, 2010). A multivariable study of the effect of NO_3^-, PO_4^{3-}, K^+, Na^+ and Cl^- concentrations on *Chlorella vulgaris* and *Peridinium cinctum* growth determined that multiple nutrient profiles could result in similar growth rates (Evens and Niedz, 2010). In *Chlamydomonas*, our previous studies have documented mineral nutrient interactions. For instance, manganese deficiency results in secondary phosphorous and iron deficiency, and deletion of the C-terminal cysteine-rich region of the Copper response regulator Crr1 in *Chlamydomonas* disrupts Zn homeostasis (Allen *et al.*, 2007; Sommer *et al.*, 2010).

Studies of nutrient deficiency contributed to the discovery of acclimation mechanisms in *Chlamydomonas* (Merchant *et al.*, 2006). Here, we characterized cells growing under various deficient conditions and determined that there are no dramatic differences in acclimation responses as a result of the supplement revision. It is well established that stress caused by N and S deprivation or drastic pH changes leads to a change in lipid profile and an accumulation of total lipid content in microalgae, a physiological response that may be useful in the development of biofuels (Cooksey *et al.*, 1987; Guckert and Cooksey, 1990; Sheehan *et al.*, 1998; Sato *et al.*, 2000; Wang *et al.*, 2009). A hypothesis to explain this observation proposed that accumulation was a passive result of cell division inhibition caused by stress combined with the steady production of new lipids (Sheehan *et al.*, 1998). Consistent with this hypothesis, we observed that iron-deficient cells accumulated TAG in parallel with inhibition of cell division (E.I. Urzica, D. Casero, L. Adler, S.J. Karpowicz, S.I. Hsieh, J.A. Loo, S.G. Clarke, M. Pellegrini and S.S. Merchant, unpublished data), and a recent study noted that salt stress also resulted in growth inhibition and TAG accumulation in *Chlamydomonas* strain CC-124 (Siaut *et al.*, 2011). Here, we broadened our study to investigate the intracellular lipid accumulation response under conditions of deficiency in other trace metals. Increased lipid accumulation in N-, Fe- and Zn-deficient situations all corresponded to growth inhibition (Figure 6).

The revised medium and the work presented here establish baseline conditions for future studies focused on the interaction of multiple deficiencies and/or toxicities that require the ability to measure intracellular metal quotas and compare them between experiments and between laboratories.

EXPERIMENTAL PROCEDURES

Culturing and strains

Chlamydomonas reinhardtii wild-type strains CC-3269, CC-425, CC-125 and CC-1690 were cultured under 50–100 μmol m^{-2} sec^{-1} illumination in TAP and TP media with the specified trace element supplements. These strains may be obtained from the Chlamydomonas Resource Center at the University of Minnesota. For metal-free studies, all glassware was freshly washed in 6 N hydrochloric acid and medium was made in Milli-Q (Millipore, http://www.millipore.com) water (Quinn and Merchant, 1998).

Trace element solutions

Hutner's trace element solution was made according to the protocol described in Harris (2009). A detailed protocol for the revised trace element supplement is available in Appendix S1. Briefly, stock solutions of 25 mM EDTA-Na_2, 28.5 μM $(NH_4)_6Mo_7O_{24}$, 0.1 mM Na_2SeO_3, 2.5 mM $ZnSO_4$ in 2.75 mM EDTA, 6 mM $MnCl_2$ in 6 mM EDTA, 20 mM $FeCl_3$ in 22 mM EDTA and 2 mM $CuCl_2$ in 2 mM EDTA were made individually in Milli-Q water, and were then stored away from direct sunlight at room temperature. Each stock solution was diluted 1:1000 in the final growth medium.

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Monitoring growth

Cells were inoculated to a density of 1×10^4 cells ml^{-1} into TAP medium in a Photobioreactor FMT150 device (Photon Systems Instruments, http://www.psi.cz) with a cultivation chamber volume of 450 ml. The culture was kept at a stable temperature of 24°C and a light intensity of 190 μE m^{-2} sec^{-1} (split equally between red and blue wavelengths). Cells were mixed by aeration. Cell size, growth rate and metal content were determined during the course of growth. Cell size was calculated by using the arithmetic average of the length and width of individual cells measured on a Cellometer (Nexcelom, http://www.nexcelom.com) as a proxy for cell diameter. Growth rate was measured both by counting under a microscope with a hemocytometer and by automated counting with a Cellometer after correcting for cell clusters. At each time point, counts were performed by two independent researchers who did not have prior knowledge of the identity of the trace element mix in the culture. The growth rate was also calculated for cultures grown in turbidostat mode set for an OD of 0.7 (approximately 3×10^6 cells ml^{-1}), with 5% tolerance, at 680 nm. Peaks in OD values created by this tolerance were used to compute doubling time using the formulae $k = \log_2 (O_t/O_0)/dt$ and $T = 1/k$, where k is the number of doublings per hour, O_t and O_0 are the OD values at the end and the beginning of the time interval, dt is the duration of the interval in hours and T is the doubling time in hour (Adl *et al.*, 2005). Data for doubling time computation were collected for 16 h from the time when an OD of 0.7 was reached.

Measurement of intracellular metal content

Cells were collected by centrifugation at 1700 ***g*** for 5 min. Pellets were washed once in 1 mM EDTA, to remove cell surface-associated metals, and once in Milli-Q water. The washed cell paste was overlain with nitric acid corresponding to a final concentration of 24% in 1 ml, and digested at 65°C. To obtain a corresponding blank, the volume of the cell paste was replaced by deionized water and treated as described above. Total metal and phosphorous content was measured by ICP-MS.

Mating efficiency

Fresh mt^+ and mt^- cells from TAP medium supplemented with Hutner's trace elements were plated onto nitrogen-deficient agar plates supplemented with either Hutner's trace elements or the revised formulation for 3 days. Cells were resuspended into sterile water and allowed to shake for 1 h prior to mixing. Upon mixing, cells were allowed to mate for 1 h or overnight. Cell density before and after mixing as well as clumping of cells was used to estimate mating efficiency.

Motility

Motility in cells was verified by the microscopic observation of exponentially growing cells from triplicate cultures being ability to move away from a light source and by the presence of cilia.

RNA analysis

Total *Chlamydomonas* RNA was prepared as described by Quinn and Merchant (1998).

Pairwise transcriptomic comparisons

Probability plots were generated as described in González-Ballester *et al.* (2010). In brief, RNA-Seq data was mapped to the genome, filtered to keep unambiguous alignments and assigned to annotated genes (Au5 gene models, 2007 *Chlamydomonas* genome assembly). For each experiment i, we compute a whole-transcriptome vector of normalized expression E_{ij}, with units of alignment hits per million reads. This vector is used to compute, for each pairwise comparison (i, i') and each gene j, expression fold-changes ($E_{ij}/E_{i'j}$) and overall expression measures ($\sqrt{E_{ij}/E_{i'j}}$).

Quantitative real-time PCR (RT-PCR)

Genomic DNA was removed from the total RNA preparation by treatment with Turbo DNase (Ambion, http://www.ambion.com) according to the manufacturer's instructions with the following modifications: 3 U of enzyme was used per 10 μg nucleic acid, and incubation at 37°C was held for 90 min. Complementary DNA, primed with oligo(dT), was generated with reverse transcriptase (Invitrogen, http://www.invitrogen.com) according to manufacturer's instructions. Amplification was carried out with reagents from the iQ SYBR Green Supermix qPCR kit (Bio-Rad Laboratories, http://www.bio-rad.com). Each reaction contained the vendor's master mix, 0.3 μM of each primer and cDNA corresponding to 20 ng input RNA in the reverse transcriptase reaction. The reaction conditions for the Opticon 2 from MJ Research were: 95°C for 5 min, followed by cycles of 95°C for 10 s, 65°C for 30 s and 72°C for 30 s, up to 40 cycles. The fluorescence was measured at each cycle at 72 and 83°C. The $2^{-\Delta\Delta C_T}$ method was used to analyze the database on the fluorescence at 83°C (Livak and Schmittgen, 2001). Melting curves were performed after the PCR reaction to assess the presence of a unique final product.

Ni^{2+}-induced CYC6 expression

CC-125 was grown in TAP medium supplemented with the revised trace elements, a modification of the revised recipe containing approximately 22.5 μM less EDTA, or with Hutner's trace elements. When the culture reached the late exponential phase, $NiCl_2$ was added. RNA was extracted 5 h later. Expression of *CYC6* was determined by RT-PCR. Transcript abundance was normalized to *EIF5A*, and all data are shown relative to time 0, immediately prior to the addition of Ni^{2+}.

Fluorescence and light microscopy

Cells were grown to stationary phase in replete medium, media lacking Cu, Zn, Mn, Mo and Se ions, or media containing low levels of Fe (0.4 μM) or N (0.7 mM). Cells were stained with Nile Red (10 μg ml^{-1} final concentration; Sigma-Aldrich, http://www.sigma-aldrich.com) for 15 min at room temperature, before being collected by centrifugation at 500 ***g*** for 3 min. Cells were mixed with low melting agarose (Bethesda Research Laboratories, now Life Technologies, http://www.lifetechnologies.com) in phosphate buffer and viewed by confocal microscopy on a Leica TCS SPE with an ACS APO 63× oil objective lens (numerical aperture 1.30). The Nile Red signal was captured using a laser excitation line at 488 nm; and emission was collected between 554 and 599 nm (gain 700; offset 0). Chlorophyll autofluorescence was excited at 635 nm and captured between 620 and 700 nm. Differential interference contrast (DIC) images were acquired in the PM Trans channel (gain 371; offset 0). Images were colored using Leica confocal software (Leica, http://www.leica.com).

A Zeiss AxioImager Z1 microscope equipped with a color camera (AxioCam HRc; Zeiss, http://www.zeiss.com) and a 63× oil immersion objective lens was used in DIC mode to capture morphological changes induced by various micronutrient deficiency regimes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of variations in Mo, Co, B and EDTA concentration on maximum cell density.

Figure S2. A reduction of trace elements by one-third does not result in a deficiency response.

Appendix S1. Revised trace elements recipe.

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