Title: Cross-study analysis of factors affecting algae cultivation in recycled medium for biofuel production

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Abstract

Current high costs of commercial-scale algal biofuel production prevent its widespread use as a renewable fuel source. One cost-saving approach is the reuse of algae cultivation water after biomass harvesting, which reduces water pumping and treatment costs. However, dissolved compounds, cell debris, and microorganisms remaining in the water could affect subsequent algae generations. Previous studies demonstrate a variety of effects of recycled medium on algae growth, yet their results have not been collectively analyzed. Here we integrate data across 86 studies to determine the relative importance of different factors influencing algae growth in recycled medium. We found that algae taxa can have the greatest influence, while the harvesting method is less influential on growth outcomes. This meta-analysis identifies favorable taxa and thus provides a tool for algae cultivation decision-making when medium reuse is an important driver. Results can also aid in estimating relative algae yield and growth rates for technoeconomic assessments that incorporate water recycling.

Keywords: Algal biofuel; Microalgae cultivation; Growth medium recycling; Crop rotation; Algae harvesting; Meta-analysis

1. Introduction

While benefits of using microalgae as a feedstock for biofuels and co-products are numerous (e.g., carbon capture, ability to use non-arable land and non-freshwater resources, higher areal productivity than other oil-based crops [1]), algal biofuels are currently not cost-competitive with conventional fuels [2] and have an impractical energy return on investment [3]. Increasing the profitability of algal fuels requires improvements to algae cultivation, among other production processes.

Using wastewater or recycled growth medium for algae cultivation is necessary for financially and environmentally sustainable algae production, especially for low-value products such as biofuels [4, 5]. Using a variety of wastewater sources for cultivation has been extensively researched to reduce freshwater and nutrient demand [6]. Some studies demonstrate energetic feasibility of such systems [7, 8], although obstacles remain such as biological contaminants in wastewater [9] and co-location of cultivation facilities with wastewater sources [1]. An alternative approach is to reuse cultivation water to reduce costs and energy associated with pumping input water, adding nutrients, and treating discharged cultivation water [4].

After harvesting, however, remaining water can contain dissolved compounds, cell debris, and microorganisms that may affect algae growth. The composition and concentration of extracellular organic matter in recycled medium will depend on the previously grown algae strain, its growth phase when harvested, the harvesting process, microbial co-habitants, and growth conditions [4, 10-13]. Algal organic matter is released by excretion and cell lysis and is composed of all major macromolecules, but is mostly polysaccharides by weight [14]. These compounds can be photosynthetic waste products, signaling molecules, allelochemicals, colony formation and motility aids, or metal chelators, among others [15-18]. Released organic compounds could be directly inhibitory, such as fatty acids [19, 20] released from lysed cells, or

may alter physical and chemical aspects of the water (e.g., viscosity [21]) that could influence nutrient availability and uptake, motility, gas exchange, cell aggregation, and shading [22, 23].

Algae growth experiments in spent, or conditioned, medium date back to the 1940s [24], while similar experiments for biotechnological applications have been reported mostly within the last five years. Collectively these studies provide a wide range of conclusions about the effects of recycled medium and extracellular compounds on algae growth, likely because of the vast array of algal exudates and their relative concentrations across studies. Recently, Farooq et al. [4] reviewed the topic of water use in algae cultivation, and described about a dozen studies testing algae growth in recycled medium. The review identified harvesting methods and organic matter as influential factors affecting growth in recycled water, but a comprehensive, quantitative analysis of many more studies and factors is warranted.

Here, we present a meta-analysis to evaluate how recycled growth medium affects algae growth and what factors affect suitability of the recycled medium. The objectives of this cross-study analysis are to 1) determine which cultivation and harvesting factors explain variation in algae growth response in recycled medium, and 2) provide a recommendation of cultivation and harvesting strategies for successful reuse of cultivation water.

We hypothesize that factors strongly influencing dissolved organic matter concentration and composition explain variation in algae growth success in recycled medium. These factors include a) harvesting method, which affects the removal of organic matter [25, 26] and potential contaminants [27] from the water, as well as the degree of algae cell disruption during harvest; b) taxa of microalgae previously grown in the medium (hereafter termed “source algae”); and c) growth stage when harvested.. We predict that relative algae growth success is negatively correlated with the number of medium reuses, because dissolved waste products or toxins may accumulate and affect growth.

Commodity industry needs recommendations for cultivation strategies that make long-term, low-cost reuse of growth medium a reality. Although a demonstration facility has successfully performed long-term water recycling [27], understanding how different factors influence algae growth in recycled medium can provide recommendations to make medium recycling more widespread. This analysis can also provide relative algae growth data for life cycle and technoeconomic assessments so that they reflect any changes in algae yield caused by recycled medium.

2. Materials and Methods

2.1 Resource search and inclusion criteria

Relevant data sources are defined as those that: a) include an experimental treatment in which microalgae are grown in water that was previously used to grow microalgae, b) provide quantitative growth results (e.g., biomass concentration, specific growth rate, biomass productivity) from both the experimental treatment and a control in fresh medium, and c) use microalgae taxa that are not associated with harmful algae blooms and are not dinoflagellates or *Prochlorococcus* (which are unlikely to be used for biofuel production). Sources that sterilized the recycled medium (by autoclaving, ozonation, or filtering with ≤ 0.2 μm pore size) were common (39% of total experiments analyzed) and were included, though experiments that used outdated methods such as boiling were excluded. Sources that used activated carbon filtration to treat the recycled medium were included as a comparison to other harvesting methods.

Comprehensive searches were done using Google Scholar and Web of Science databases with the terms: [conditioned OR spent OR recycled OR reuse] AND medium AND [algae OR microalgae OR phytoplankton]. Database searches accounted for 51 relevant sources used in the meta-analysis. Manual forward and backward citation searches were conducted on all relevant

sources, which revealed 31 additional relevant sources. Three sources not found through these methods were either recommended by colleagues (e.g., student theses) or acquired at conferences, and an additional source was found as a citation in Wu, et al. [13]. Searches were performed up until November 12, 2016. A total of 86 sources were included in the meta-analysis from both ecological and biotechnological sources (Table S1 and Figure 1) [15, 17, 22-26, 28-106]. An additional 67 sources deemed potentially relevant were excluded because they did not meet the inclusion criteria or the full text in English was unavailable (Table S2).

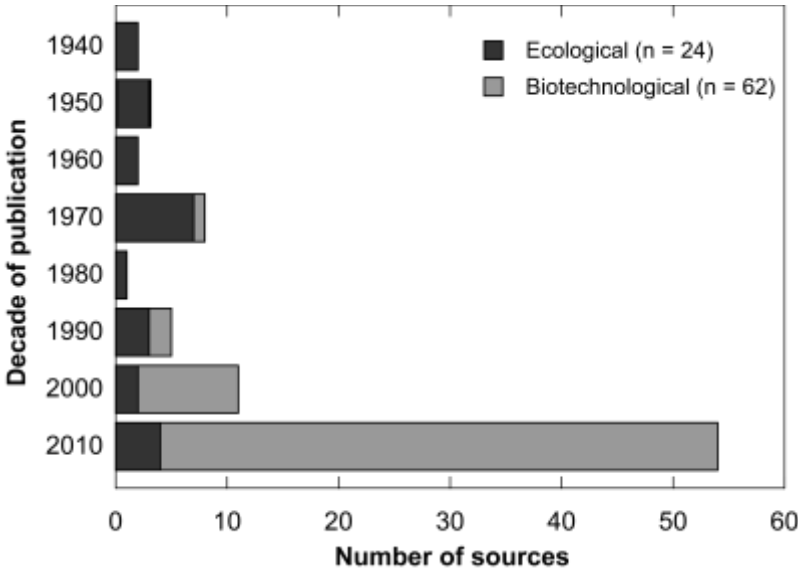


Figure 1: Sources used for meta-analysis grouped by publication decade and research purpose (ecological or biotechnological).

2.2 Response variables and data extraction

Three response variables were chosen to represent algae growth: 1) biomass concentration (cells/mL, g/L dry weight, or optical density), 2) specific growth rate (day^{-1}), and 3) biomass productivity (g/L/day). Biomass measurements for all variables were based on cell counts (via microscopy, flow cytometry, or Coulter counters), dry weight, or optical density.

Packed cell volume and long-term carbon uptake were also accepted as biomass measurements, used in three data sources. A Data Extraction Protocol was developed to extract data and information objectively and consistently from each source, and is available in the Supplementary Information (SI). Response variable data were recorded directly from text or tables when available, but were mostly available in figures only. The web-based application Webplot Digitizer [107] was used to extract data from figures, including standard deviations when error bars were present and distinguishable.

The first response variable, 'Biomass concentration,' is defined here based on operation mode of the culture. For batch cultures (and semicontinuous cultures if a full growth curve is available), 'biomass concentration' is defined as the maximum biomass concentration reached in the growth curve, or the final biomass concentration if maximum is unavailable. Because some studies likely stopped experiments before algae reached their maximum biomass, the difference between biomass concentration in recycled and fresh medium could be influenced by experiment duration. Relatively short experiments are still included and may even be more realistic, however, since industrial-scale cultures are typically harvested on relatively short time scales to prevent biological contamination and maximize harvest yield [108]. For continuous cultures, 'biomass concentration' is the available biomass concentration data averaged over the experimental period. For semicontinuous cultures harvested on short time-scales (e.g., once a day), 'biomass concentration' is the peak biomass concentrations averaged over the experimental period.

The 'specific growth rate' response variable applies to batch cultures, as well as semicontinuous cultures with a full growth curve available. When figures of growth curves were available, digitized data were used to calculate the specific growth rate. Complete details on growth rate calculation are available in the Data Extraction Protocol in the SI.

140 ‘Biomass productivity’ applies to continuous cultures and semicontinuous cultures
141 harvested on short time-scales. When biomass productivity was obtained from ‘biomass
142 concentration versus time’ figures, it was calculated from digitized biomass concentration data
143 and averaged across the experimental period.
144 Many study descriptors were recorded where available, including growth conditions such
145 as initial nitrogen (N) and phosphate (P) concentrations, initial pH, initial biomass concentration,
146 light level, hours of light per day, temperature, culture volume, aeration, number of reuses of the
147 medium, and percent dilution of the recycled medium with fresh medium. Categorical variables
148 were also recorded such as the growing algae strain, the source algae strain, growth stage when
149 harvested from the medium, harvesting method, growth stage of the inoculum, sterilization
150 method of recycled medium if applicable, whether the culture was axenic or not, and the growth
151 measurement method.
152 The number of medium reuses in semicontinuous and batch culture experiments was
153 recorded as reported in the text if there were no dilutions with fresh medium or water. However,
154 when dilution occurred, the effective number of reuses was calculated as a weighted average
155 because portions of recycled water were reused less than other portions. See the Data Extraction
156 Protocol in SI for complete details on the *times_reused* variable.
157
158 2.3 Effect size calculation
159 The effect size in a meta-analysis measures the impact of a treatment [109]. Here, the
160 effect size represents the impact of recycled medium on microalgae growth. Effect sizes were
161 calculated for each experimental treatment (recycled medium) versus its control (fresh medium).
162 We chose to use log-transformed response ratios [110] as the effect size:
163 $$\text{Effect size} = \ln(G_R/G_F)$$

where G_R and G_F are the mean values of a growth response variable (biomass concentration, specific growth rate, or biomass productivity) in recycled medium and fresh medium, respectively. The null value of an effect size is therefore 0, when mean growth is equal in both media. Because negative values and zero values cannot be log-transformed, five growth rate effect sizes and one productivity effect size could not be calculated. These are discussed qualitatively in Section 3.5.3.

The three growth response variables were each analyzed separately in univariate meta-analyses. We hereafter refer to their effect sizes as ES_C for biomass concentration, ES_μ for specific growth rate, and ES_P for biomass productivity. Most data sources had multiple experimental treatments, from which more than one response variable could be extracted. A total of 518 experimental treatments were included from the 86 sources. Of these 518 treatments, ES_C was available from 414, ES_μ was available from 310, and ES_P was available from 104, for a total of 828 effect sizes (see Table S3 for complete distribution of available response variables among treatments).

When calculating mean effect size, effect sizes are typically weighted by their sample size and variance so that more precise results are weighted more heavily [109]. However, 69% of the 828 effect sizes did not have standard deviations available from the recycled medium treatment, fresh medium control, or both. Number of replicates was also unavailable from 18% of the 518 treatments. Lack of reporting such information is a common problem in ecological meta-analyses and makes traditional meta-analysis methods infeasible [111]. Because of this high degree of missing information and the generally high replicability of laboratory experiments, it was not considered appropriate to weight experiments based on standard deviations and number of replicates. Instead, an alternative weighting scheme combined with a resampling technique was used to calculate 95% confidence intervals of mean effect size [112].

188 The weight of a treatment was decided to be n^{-1}, where n is the number of treatments
189 sharing the same original source of recycled medium (see Data Extraction Protocol in the SI for
190 further details). This weighting scheme discounts non-independent treatments and gives a full
191 weight of 1 to treatments with an exclusive source of recycled medium. Experimental treatments
192 compared to the same control are also considered non-independent in traditional meta-analysis
193 methods [109]. However, most experiments were conducted in controlled laboratory settings
194 where replicability of controls is theoretically high, so non-independence due to shared controls
195 was not accounted for in these analyses.
196 Confidence intervals of weighted mean effect size were calculated by bootstrapping with
197 5000 repetitions [112] using the 'boot' package in R with the 'adjusted bootstrap percentile'
198 interval type [113-115]. Mean effect sizes whose confidence intervals do not contain the null
199 value 0 are considered significant. Unweighted analyses (equal weight for each treatment) were
200 performed to determine if weighting affected conclusions of the analysis.
201 All information and data used in analyses, including effect sizes, weights, and other
202 variables, are included in the Recycled Medium Database provided in the SI. Definitions of
203 database variables are available in the Data Extraction Protocol in the SI. The database and
204 extraction protocol are also available publicly at figshare.com [116].
205
206 2.4 Data Assessment
207 2.4.1 Growth response variable correlations
208 Before analysis, correlations between effect sizes of different response variables were
209 computed to check for dependence, because dependent growth response variables cannot be
210 analyzed separately in univariate analyses [117]. If two response variables were correlated ($R^2 >$
211 0.60), only the response variable with the greater number of treatments was analyzed.

212

213 2.4.2 Nutrient addition analyses

214 Not all studies measured or replenished nutrients in the recycled medium. The
215 appropriateness of using all treatments, even if researchers did not re-supply nutrients, was tested
216 before conducting analyses. Initial N and P concentration for each recycled medium treatment
217 were categorized into one of three nutrient-addition subgroups: "higher," "lower," or "same"
218 compared to its fresh medium control (Table S4). See the Data Extraction Protocol in the SI for
219 further details on how these were categorized.

220 Confidence intervals of mean effect size for each nutrient-addition subgroup were
221 calculated. Significant differences between subgroups were tested by calculating the between-
222 group heterogeneity, or Q_B statistic. This statistic was computed with the Q_B equation provided
223 by Adams, et al. [112] (note that Koricheva, et al. [117] refer to this same heterogeneity statistic
224 as Q_M.). Significance of the Q_B statistic was tested by performing 5000 randomizations and
225 calculating the percent of times that Q_B from randomized data was higher than the actual Q_B
226 [112]. The actual Q_B was included as a potential randomization outcome in this percentage
227 calculation [117]. If the outcome was less than 5% ($p < 0.05$), Q_B was considered significant and
228 this was interpreted as a significant difference in effect sizes between subgroups. If Q_B was not
229 significant, this meant nutrient-addition groups were not associated with algae growth response
230 in recycled medium, and all nutrient-addition subgroups were retained in subsequent analyses.

231

232 2.5 Effect of continuous variables on algae growth response

233 To test the association between continuous variables and mean effect size, we conducted
234 weighted least squares regression in R [113]. Because of multiple different units of reported light
235 levels, these were compared separately based on their unit of either μmol photons m^{-2} s^{-1} or lux

(lux cannot be converted to µmol photons m^{-2} s^{-1} without specific details about the light source; W m^{-2} also cannot be converted but were not analyzed because of too few treatments). The following continuous variables were log-transformed before regression to improve their normality: treatment's initial N:P ratio (for treatments that reported nutrient concentrations in recycled medium), light level (both µmol photons m^{-2} s^{-1} and lux), culture volume, and number of growth medium reuses.

2.6 Effect of categorical variables on algae growth response

To test the hypotheses that harvest method, taxa of source algae, and culture age are responsible for variation in algae growth responses in recycled medium, we conducted subgroup analyses. Categorical variables (e.g., harvest method) were divided into subgroups (e.g., centrifugation, flocculation, filtration). For taxa of source algae, genus-level was the lowest taxonomic rank available from enough treatments to make subgroups. The 95% confidence interval of mean effect size was calculated for each subgroup using the bootstrapping technique described in Section 2.3. If a subgroup's confidence interval did not contain the null value 0, its impact on algae growth in recycled medium was considered significant. Subgroup data were plotted with the forest plot package in R [113, 118].

To determine whether a given categorical variable was responsible for variation in growth responses across experiments, we tested whether subgroups of the variable had significantly different mean effect sizes. The between-group heterogeneity, or Q_B statistic, was computed and tested for significance (detailed in Section 2.4.2). To ensure adequate sample sizes for Q_B analyses, a chosen minimum of 10 treatments ($n \geq 10$) were required for a subgroup to be included in an analysis. For comparison, analyses were also performed including all subgroups regardless of the number of treatments they contained.

2.7 Limitations of the meta-analysis

As with all meta-analyses, this study is limited by availability of published results, and does not include proprietary data. Combining available data from many studies means that meta-analysis results are observational, representing associations and not necessarily causal relationships. Since this is an analysis of experiments performed by many different researchers in various settings, all variables are not controlled across experiments. In many cases there could be confounding factors, as will be discussed in Sections 3.4.1 through 3.4.3, where results may be influenced to a greater degree by variables other than the one being analyzed.

With these caveats, this study defined algae growth response in recycled medium as significantly inhibited if the mean effect size confidence interval was less than 0. However, successful and unsuccessful growth in recycled medium could be defined in different ways. For example, a biomass concentration in recycled medium that is 97% of that in fresh medium leads to a negative effect size, but perhaps a company is willing to accept this lower biomass if the water-recycling energy savings greatly offset the decreased yield. Additionally, combining effect size data to create 95% confidence intervals does not completely capture the spread of individual growth responses within a subgroup. Alternative methods could be used to account for individual growth responses, such as determining the percent of treatments in each subgroup that performed worse in recycled medium than in fresh medium.

3. Results and Discussion

3.1 Overall effect size results

Figure 2 shows the overall distribution of effect sizes for each response variable. Algae biomass concentration and specific growth rate in recycled medium were significantly

unfavorable compared to growth in fresh medium, as indicated by their mean effect size confidence intervals of [-0.23, -0.07] and [-0.17, -0.03], respectively. Although the distribution peaks of ES_C and ES_μ center around the null value of 0, there is considerable variation with some effect sizes spreading past -2 and 2, which equate to an 86% decrease and a 739% increase, respectively, in algae growth in recycled medium compared to fresh medium. The wide range of effect sizes within each response variable might be attributed to differences in cultivation and harvesting variables, including factors such as algae strain, which will be discussed in Sections 3.3 and 3.4.

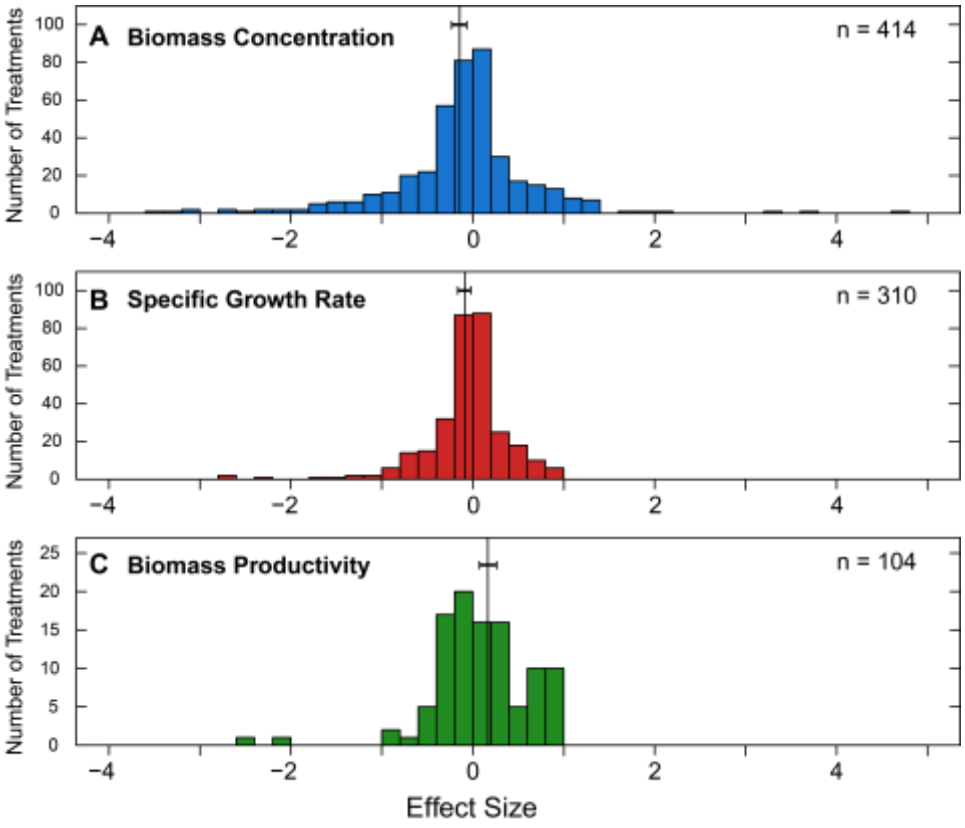


Figure 2: Distribution of A) ES_C, B) ES_μ, and C) ES_P. Weighted mean effect size is indicated by a vertical solid line and its 95% confidence interval is a horizontal line.

298 ES_P has a confidence interval of [0.07, 0.26], meaning this growth parameter was
299 significantly improved in recycled medium. The conflicting result for this latter response
300 variable compared to the former ones is likely because about half of the 104 treatments used to
301 calculate mean ES_P treated the recycled medium with activated carbon, which may have
302 improved growth responses (see Section 3.4.1). Less than 15% of treatments contributing to
303 mean ES_C and ES_μ used activated carbon.
304
305 3.2 Data Assessment
306 3.2.1 Growth response variable correlations
307 Correlations among response variables were checked to determine if response variables
308 were dependent, in which case the variables could not be analyzed separately in univariate
309 analyses. There were 232 treatments that had both ES_μ and ES_C available (Table S3). ES_μ and
310 ES_C were only weakly correlated ($R^2 = 0.19$; Figure S1), so separate analyses of these response
311 variables were deemed acceptable. However, a strong correlation was observed between ES_C and
312 ES_P ($R^2 = 0.96$) from the 82 treatments that had both response variables available. Productivity
313 data from these 82 treatments were therefore withdrawn from the ES_P data pool, leaving only 22
314 treatments remaining with productivity data. However, this number of treatments was too low to
315 include in subsequent categorical analyses, where the treatments are separated into even smaller
316 subgroups. ES_P was therefore not studied further.
317
318 3.2.2 Nutrient addition analyses
319 Another issue with productivity data that contributed to the decision to exclude them
320 from subsequent analyses was the difference between mean effect size in treatments that did and
321 did not resupply N to the recycled medium (Table S5, Figure S2). Mean ES_P was significantly

higher in treatments that contained higher N in the treatment that the control, compared to treatments with lower N in the treatment than the control (Table S5). ES_P results therefore may not have been primarily caused by the recycled water but could have been explained by nutrients. Removing treatments with lower N than their control would decrease the ES_P data pool even further, so ES_P was definitively not used in categorical or regression analyses.

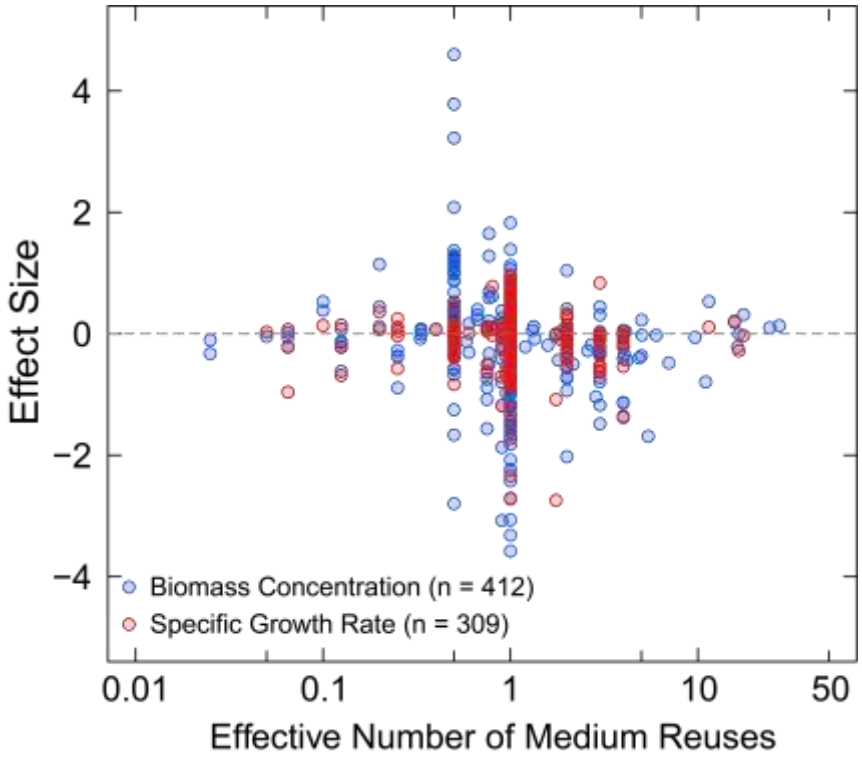
The three P-addition groups (lower, higher, or same as control) also had significant between-group heterogeneity in mean ES_C (Table S5). However, the "lower" group had a significantly higher mean ES_C than the "higher" group. In this situation, removing treatments was not justified since it was not relevant to the concern that treatments with relatively lower nutrient concentrations might have overall lower effect sizes. All ES_C and ES_μ were therefore included in subsequent analyses because there were no conflicting differences between nutrient-addition groups (Table S5).

3.3 Effect of continuous variables on algae growth response

Regression analyses revealed no associations between continuous variables (temperature, light, pH, aeration rate, initial N:P ratio, culture volume) and ES_C or ES_μ, with R^2 values all below 0.25 when calculated both by weighted and unweighted least squares regression (Table S6 and Figure S3). Lack of correlation means there are likely no interaction effects between these environmental variables and the recycled medium.

Interestingly, effect sizes were also not correlated with the number of medium reuses (Figure 3), with an R^2 of 0.003 for ES_C and <0.001 for ES_μ (Table S6). Growth inhibition (or lack of inhibition) may be more strongly influenced by other factors (such as those presented in Section 3.4), and not some universal inhibitor that accumulates with each reuse. Even so, several studies observed declining growth performance with increasing medium reuses and/or organic

346 matter accumulation [23, 37, 103], though these results are outweighed by other experiments in
347 the regression. A limitation of this analysis, however, is that the experimental duration of each
348 medium reuse is different across studies. In Section 3.4.3 the effect of culture age at harvest is
349 therefore inspected. Additional experiments are also warranted to determine a feasible number of
350 medium reuses, since there were not many treatments with more than 1 reuse (only 42 with ES_μ
351 and 72 with ES_C). The five treatments with zero or negative specific growth rate response ratios
352 (and thus incalculable ES_μ) all had an effective reuse of 1, so these "pond crashes" were not
353 caused by many water reuses.
354



355
356
357 **Figure 3:** ES_C and ES_μ versus number of previous uses of the growth medium.
358
359
360

3.4 Effect of categorical variables on algae growth response

Subgroup analyses were conducted to determine whether harvest method, taxa of source algae, and culture age were responsible for variation in algae growth success in recycled medium across studies, and whether certain subgroups are associated with favorable growth responses.

3.4.1 Harvesting methods

Harvesting methods (centrifugation, filtration, flocculation, centrifugation & filtration, flocculation & filtration) did not exhibit significantly different mean ES_C and ES_μ, as determined by their overlapping confidence intervals and non-significant Q_B statistic (Figure 4 and Table 1). Harvest method was therefore not associated with algae growth response in recycled medium. Experiments that treated the recycled medium by activated carbon filtration and dialysis were not included in Q_B significance tests, though are still shown in Figure 4 for comparison.

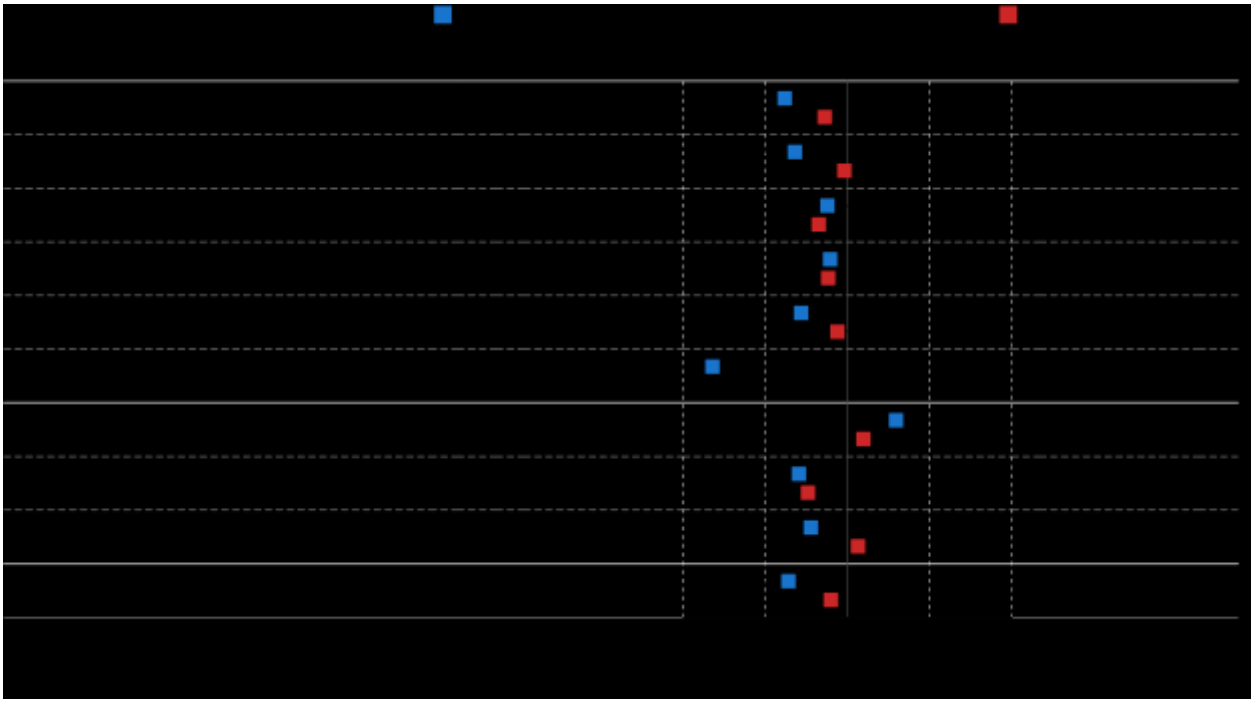


Figure 4: ES_C and ES_μ weighted means (boxes) and confidence intervals (95% CI) when grouped by algae harvesting method. Groups containing less than 3 experiments (n < 3) show

weighted mean effect size only. Experiments that treated the recycled medium by activated carbon and dialysis are separated from those that did not perform such treatments.

Table 1: Between-group heterogeneity (Q_B) significance levels for categorical variables. Asterisk indicates Q_B is significant based on 5000 randomization tests ($p < 0.05$).

Variable	Biomass Concentration	Specific Growth Rate
Source algae genus	0.0002 *	0.0002 *
Source algae broad taxonomic group	0.0002 *	0.055
Harvest method	0.188	0.555
Growth phase at harvest	0.034 *	0.971
Crop rotation	0.772	0.370

Flocculation was predicted to be more favorable than other methods because of its potential to remove organic matter [25, 26]. Flocculation can occur after adding chemicals, changing pH, applying electric current, or culturing with certain microbes that induce cell aggregation [119, 120], and allows algae to settle or, if air is supplied, float for removal by skimming [25]. Fon Sing, et al. [26] attributed the long-term success of their recycled medium cultures partly to electro-flocculation, because it did not break cells during harvesting and removed about 25% of dissolved organic carbon (DOC) from the water on average. Farooq, et al. [25] also observed that $FeCl_3$ flocculant removed proteins and carbohydrates from the medium.

However, flocculation alone did not show significantly higher mean effect sizes than other harvesting methods (Figure 4). Certain flocculants such as alum can be unfavorable for water reuse [25, 57], so mean effect sizes for flocculation likely vary across different flocculation methods. For example, flocculation by pH decrease with $NaNO_3$ was a feasible harvest method for water reuse. Additionally, it did not lose efficiency with increased cell or released

polysaccharide concentration, and the $NaNO_3$ in recycled medium could be used by algae as a nutrient source [61].

In treatments where activated carbon filtration was combined with flocculation, however, algae growth was stimulated by recycled medium (Figure 4). Activated carbon removes dissolved organic matter [71], though it is unclear why growth was higher in recycled medium than in fresh medium. Growth may have been promoted by residual iron flocculant in the medium [25]. Additionally, the 51 treatments using flocculation with activated carbon were all performed with *Arthrospira* by the same research group [71-73], so results may be more variable if additional studies used activated carbon with other algae strains. Indeed, in experiments where activated carbon was combined with filtration, growth was overall unfavorable in recycled medium (Figure 4). Rijstenbil [81] found that activated carbon did not remove inhibitory compounds from the filtrate of marine diatom cultures. Zhang, et al. [23] showed that activated carbon removed organic matter and could improve specific growth rate of *Nannochloropsis* in recycled medium, but biomass concentration was still below that in fresh medium.

While harvesting at high speeds could remove particulate matter and bacteria from cultures along with the algae [121], centrifugation is energy-intensive and costly, and therefore unlikely to be implemented on an industrial scale for biofuel production [122]. Based on studies used in this meta-analysis, most of which were small-scale and conducted in a lab, centrifugation is also not associated with favorable growth in recycled medium (Figure 4). Rodolfi, et al. [84] harvested *Nannochloropsis* sp. by centrifugation and observed that recycled medium still contained particulate matter that slowed algae growth. Moreover, Farooq, et al. [25] measured no decrease in extracellular carbohydrates or proteins after centrifugation of a *Chlorella vulgaris* culture, although recycled medium with added nutrients still stimulated growth.

There are no overall clear winners among the harvesting techniques investigated here, in terms of creating an optimal reusable growth medium. Nevertheless, certain methods of flocculation may be most practical and economical for harvesting on a large scale [123]. Sapphire Energy, Inc. has successfully used recycled medium in demonstration-scale semi-continuous open ponds for two years by harvesting with dissolved air flotation preceded by the addition of a polymer flocculant. The polymer was designed specifically for the target algae strain, and the algae adapted over time to the recycled medium [27]. This long-term study also stressed that harvesting techniques must remove unwanted organisms from the water to prevent their proliferation in subsequent cultures [27]. In future developments of harvesting techniques for large-scale algae cultivation, the effect of the harvest method on water reusability must be evaluated.

3.4.2 Taxa of Source Algae

Genus of source algae was significantly associated with ES_C and ES_μ,explaining effect size variation across studies (Q_B $p = 0.0002$, Table 1). Recycled medium from *Desmodesmus*, *Dictyosphaerium*, *Hormidium*, *Hormotila*, *Tetraselmis*, and *Arthrospira* supported favorable growth based on multiple experiments (Figure 5). Most or all data for *Desmodesmus* [39, 40], *Dictyosphaerium* [29], *Hormidium* [29], *Hormotila* [68, 69], and *Arthrospira* [70-73] were each published by a single research group, however, so additional experiments are advisable to corroborate results.

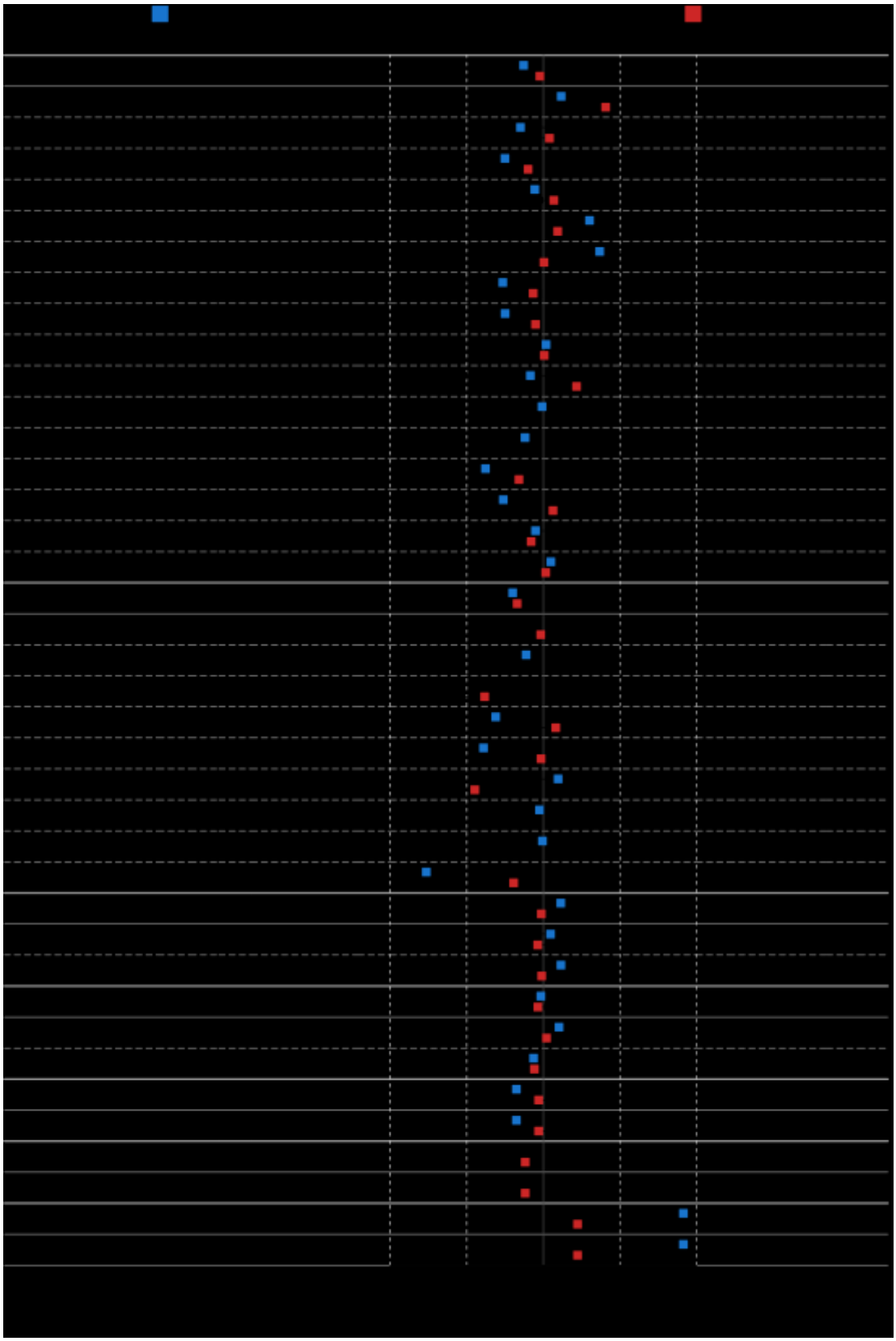


Figure 5: ES_C and ES_μ weighted means (boxes) and confidence intervals (95% CI) when grouped by taxa of source algae. Indented rows are algae genera, and the broader groups they belong to appear in bold. Groups containing less than 3 experiments (n < 3) show weighted mean effect size only.

In 51 of the 66 *Arthrospira* experiments contributing to mean ES_C, cultures were harvested by flocculation followed by activated carbon treatment, which likely contributed to the favorable growth response. Excluding treatments with any activated carbon treatment decreases *Arthrospira*'s weighted mean ES_C to -0.09 (n = 7) and ES_μ to -0.24 (n = 8). Other favorable taxa did not have their recycled medium treated with activated carbon, and in most cases the favorable growth responses can be attributed to released organic compounds. Grabski, et al. [39] identified *Desmosdesmus subspicatus* growth promoters as hydrophilic molecules less than 0.5 kDa that are excreted during exponential growth. Adding support to *Desmodesmus*'s successful growth, the alga that Sapphire Energy grew in recycled medium for two years was a strain of *Desmosdemus* [27]. Monahan and Trainor [69] note that *Hormotila blennista* produces a "gelatinous sheath," and conclude that this strain's filtrate is stimulatory due to extracellular, heat-labile, low-molecular weight organic compounds. Burkiewicz and Synak [15] also note that *Dictyosphaerium pulchellum* is covered in mucilage, or exopolysaccharides. Extracellular carbohydrates isolated from *D. pulchellum* filtrate stimulated growth of *Scenedesmus armatus*, which the authors concluded was mixotrophic. Similarly, Fon Sing, et al. [26] attributed stimulated growth of *Tetraselmis* in its own recycled medium to mixotrophy, concluding that the algae must have benefitted from the recycled medium's higher DOC concentration. Several other studies have reported favorable growth of *Tetraselmis* under mixotrophic conditions (e.g., [124, 125]).

Recycled medium from *Chlorella*, *Ditylum*, and *Dunaliella* cultures led to especially poor mean growth responses when considering both ES_C and ES_μ (Figure 5). Individual growth responses to *Dunaliella* recycled medium varied from neutral to completely inhibited, though some severe inhibitory responses were caused by unfavorable flocculation methods [33, 75] rather than by exudates from the algae themselves. Rijstenbil [81] demonstrated that *Ditylum*

cellular extracts caused more severe inhibition than *Ditylum* filtrate, so the toxic compounds in recycled medium had to be from cellular secretion or cell lysis. The mean negative effect sizes of *Chlorella*'s recycled medium are largely driven by the early work of Pratt et. al [24, 76], who found that *Chlorella vulgaris* secretes autoinhibitors which are heat-labile organic bases that accumulate as the culture ages [126]. However, another study did not observe *Chlorella vulgaris*'s autoinhibition [64], and other strains of *Chlorella* may not secrete identical metabolites.

Recycled medium from *Skeletonema* cultures led to a particularly unfavorable mean ES_{μ}, although biomass concentrations exhibited less inhibition. *Skeletonema*'s mean ES_{μ} was entirely from the strain *S. costatum*. Several studies have observed evidence of an allelochemical produced by this strain that inhibits both its own growth and that of other algae [30, 52, 81]. Kustenko [55] also observed stimulatory properties of the recycled medium, however, which suggests the effects of *Skeletonema* exudates might depend on its growth phase.

Broader taxonomic levels shown in Figure 5 (e.g., green algae) were responsible for significant variation in mean ES_C but not ES_{μ} (Table 1), conveying that significant differences in mean ES_{μ} across genera tend to cancel out when grouping algae at broader levels. Among these broader taxonomic groups, recycled medium from diatom cultures significantly reduced overall mean specific growth rate and biomass concentration. Diatoms typically release higher concentrations of organic carbon per biomass because they secrete polysaccharides for motility [18] and have relatively large diffusive boundary layers [127], which may play a role in their less suitable recycled medium compared to other broad taxonomic groups.

Not all studies grew the source algae strain in its own recycled medium. A total of 113 experimental treatments grew an algae strain in recycled medium that differed from the source algae strain. Studies that alternated algae strains were mostly performed to test ecological

hypotheses, and therefore did not specifically use biofuel-promising algae. To our knowledge, no published biofuel-focused studies have experimented with algae crop rotation combined with medium recycling, though some have suggested crop rotation to deal with climate perturbations and parasites [128, 129].

Alternating algae strains in recycled medium may maximize nutrient uptake and prevent build-up of the same algal exudates, since phylogenetically distant strains produce DOC with distinct compositions [10]. However, mean ES_C and ES_μ were not better when crop rotation was performed (Figure 6), and there were no significant differences between mean effect sizes of treatments that did or did not alternate algae strains (Table 1).

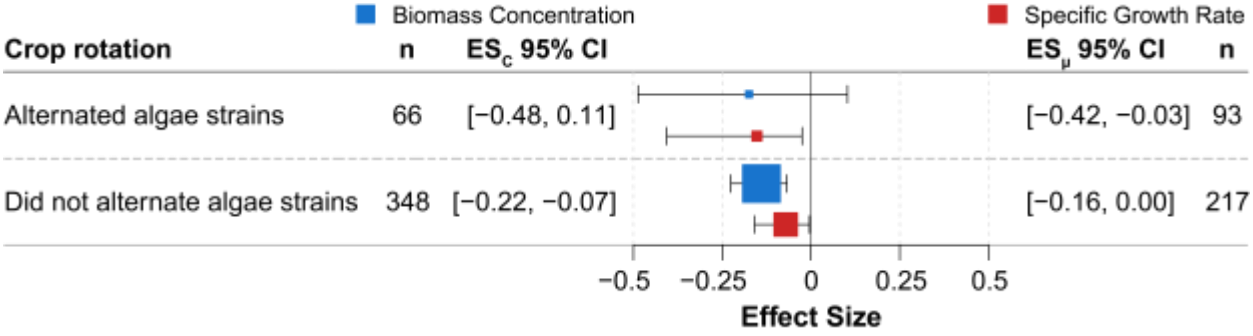


Figure 6: ES_C and ES_μ weighted means (boxes) and confidence intervals (95% CI) when grouped by whether the algae strain growing in recycled medium differed from the source strain. Box sizes reflect the number of treatments n in a subgroup.

Interestingly, recycled medium produced from certain algae genera induced favorable growth responses for some genera but not others, such as recycled medium produced from *Chlorella*, *Scenedesmus*, and *Phaeodactylum* cultures (Figure 7 for ES_C and Figure S7 for ES_μ). Such variability could be caused by many factors, including differences in experimental design across studies and differences among strains within a genus. We propose that a genus's suite of extracellular DOC (which is influenced by growth conditions, growth stage, and microbes [11-13]), combined with algae strains' unique responses to the same DOC, highly influenced the

variability observed in Figures 7 and S7. For example, Bednarz and Cierniak [29] found that extracellular compounds in *D. pulchellum* filtrate induced favorable growth responses for several green algae strains and *Anabaena*, yet inhibited its own growth.

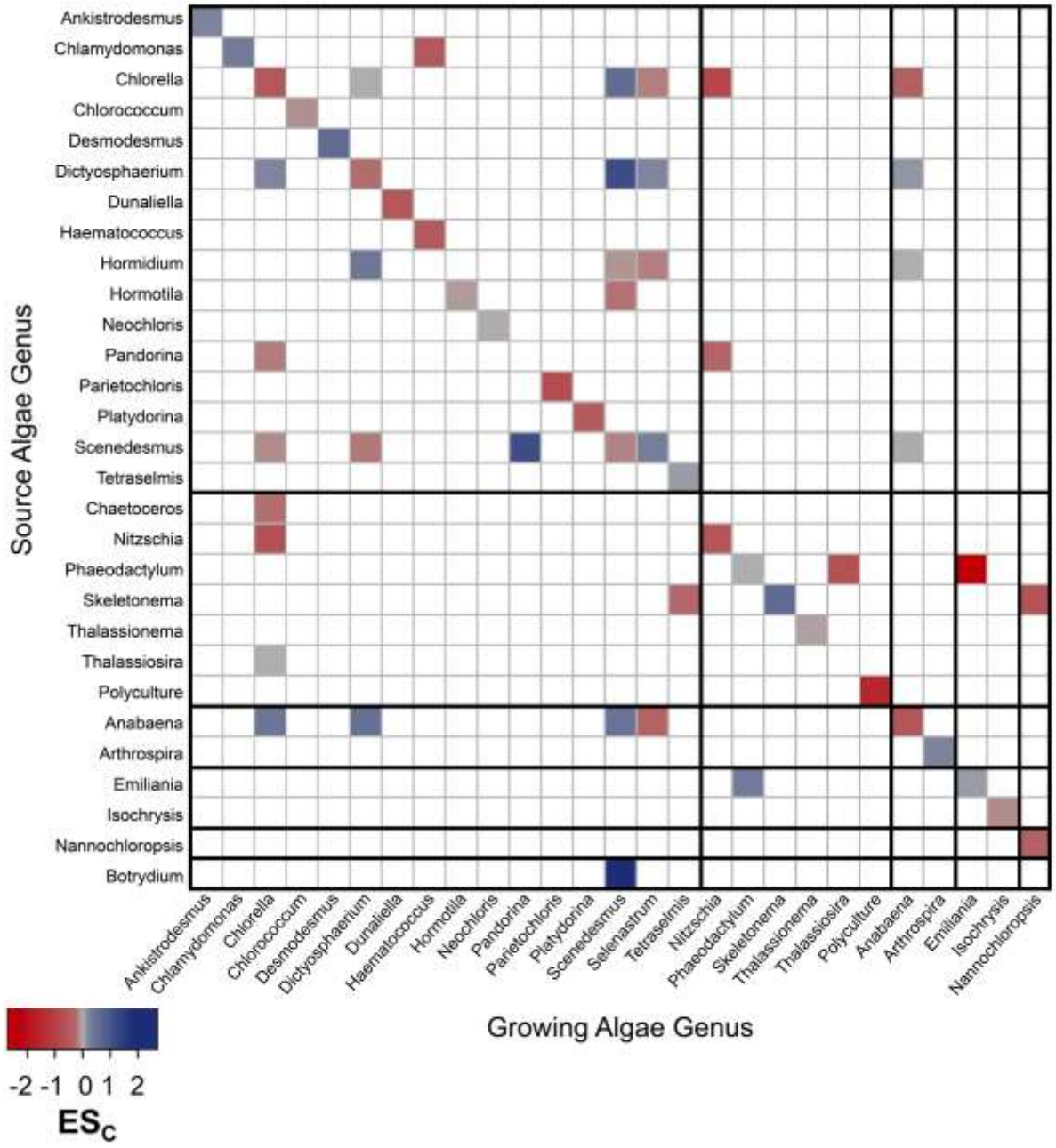


Figure 7: Heatmap of weighted mean ES_C, grouped by genus of source algae and genus of algae grown in the recycled medium. A cell can represent 1 or more experimental treatments. White cells indicate no data. Black lines separate genera into the same broader taxonomic groups as in Figure 5.

521 Exudates have ecological significance in determining species dominance in natural
522 ecosystems, and allelochemicals [130] may explain some unfavorable results in Figures 7 and
523 S7. Mixotrophy, as discussed previously, explains some of the favorable growth responses.
524 Complete characterization of algal exudates and their mechanisms is an area of active research
525 (e.g., [14, 16, 131-133]), and in the future may further elucidate the findings here.
526
527 3.4.3 Culture Age at Harvest
528 Algae culture age when harvested explained variation in ES_C but not ES_μ, based on Q_B
529 significance (Table 1). Growth phase subgroups showed no differences in mean ES_μ, and only
530 late stationary phase was associated with significant growth rate inhibition in recycled medium
531 (Figure 9). Recycled medium produced from early exponential and exponential phase cultures
532 supported significantly greater biomass concentration effect sizes than medium harvested from
533 late exponential and stationary phase cultures. This could be because less stressed cultures in
534 exponential phase likely have lower concentrations of released DOC [134], which could be
535 inhibitory. However, there was no overall trend of declining growth responses with culture age,
536 despite previous reports of differential DOC release with growth phase [12, 13].

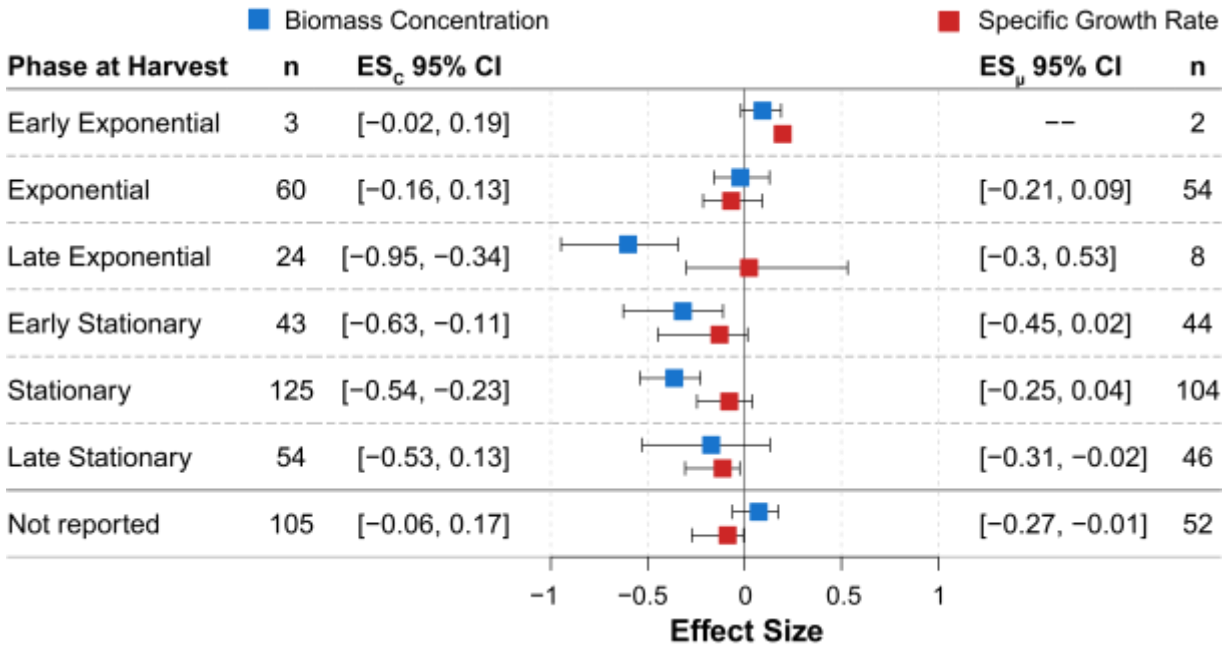


Figure 8: ES_C and ES_μ confidence intervals, grouped by algae growth phase when the source culture was harvested to create recycled medium. Groups containing less than 3 experiments (n < 3) show weighted mean effect size only.

Another explanation for the observed results could be that peak DOC release occurs mostly in late exponential phase, and heterotrophic bacteria consume potentially growth-inhibiting DOC as the cultures age. If this were true, we would expect recycled medium from xenic cultures be more favorable than that from axenic cultures. However, there were no significant differences between ES_C or ES_μ in axenic versus xenic cultures, or in sterilized versus non-sterilized recycled medium (Figures S10 and S11, respectively). The data suggests that harvesting algae in exponential phase may produce the most suitable medium for reuse, whereas later phases may be more likely to cause inhibition.

It could be argued that earlier growth stages are associated with more favorable mean ES_C because these cultures carry over relatively higher nutrient concentrations in the recycled medium. However, we compared the distribution of the three nutrient addition groups ("lower", "higher", and "same" as the control) for N and P among the growth phase subgroups and did not

554 observe such a phenomenon (e.g., the late stationary phase subgroup had a relatively low
555 proportion of treatments with "lower" nutrient concentrations in the treatment than the control)
556 (Figure S9). Additionally, we previously ruled out the possibility that treatments with higher
557 initial N or P concentrations than their control might have significantly higher mean ES_C and ES_μ
558 than treatments with lower, or the same, N and P concentrations as their control (Table S5,
559 Figure S2).
560
561 3.5 Robustness of analyses
562 3.5.1 Weighting treatments
563 Unweighted analyses were conducted to verify there were no major differences from
564 conclusions drawn from weighted analyses (Section 2.3). Nutrient addition analyses (Table S5)
565 and continuous variable regressions (Table S6) show no major differences in results
566 interpretation between unweighted and weighted analyses. For categorical analyses, between-
567 group heterogeneity (Q_B) significance levels led to the same conclusions whether using
568 unweighted or weighted analyses (Table S7 and Table 1), except the broad taxonomic group of
569 source algae had a significant Q_B for ES_μ when treatments were not weighted. Figures S4, S5,
570 S6, and S8 are the unweighted compliments to Figures 4, 5, 6, and 8, respectively. Although
571 there are minor differences between the subgroup confidence intervals, main conclusions drawn
572 from the weighted and unweighted approaches are the same.
573
574 3.5.2 Minimum subgroup size for Q_B analyses
575 For categorical analyses we computed Q_B only with subgroups that contained at least 10
576 experimental treatments, to ensure adequate sample sizes (Table 1). For comparison we
577 computed Q_B values and their significance when using all subgroups within a categorical

variable, regardless of n. There were no differences in Q_B significances between "$n \geq 10$" and "all n" analyses when using the weighted method, and only minor differences were evident when using the unweighted method (data not shown). The chosen subgroup minimum of n=10 for between-group heterogeneity analyses was thus upheld.

3.5.3 Zero or negative response ratios

ES_μ could not be calculated for 5 treatments because their response ratio was zero or negative (could not be log-transformed), indicating the growth rate was extremely low in recycled medium. Within these 5 treatments, the harvest methods varied and so did the source algae genera (*Chlamydomonas*, *Dunaliella*, *Ochromonas*, and *Nitzschia*). Figure 5 shows that *Dunaliella*'s recycled medium induced significant inhibition of specific growth rate based on available effect sizes, so the unusable data lends more support to this result. Available ES_μ from *Chlamydomonas* did not show significant growth inhibition in recycled medium overall, which might change if the unusable data were incorporated. Lastly, 3 of these 5 treatments used recycled medium harvested from exponential phase algae cultures, which means the conclusion that optimal recycled medium comes from exponential phase cultures may be conditional, though the degree to which these 3 treatments could alter the ES_μ confidence interval is likely minor, especially since their weights only sum to 0.75.

3.6 Recommended areas for future research

Life cycle and technoeconomic analyses should be performed to compare different scenarios of recycled medium use and determine how many times medium can be recycled for optimal biofuel production with a given input of resources and energy. Currently available growth data from both biotechnological and ecological publications, analyzed here, is a starting

point for designing future experiments and executing economic analyses for algae cultivation in recycled medium.

Once optimal growth conditions, algae strains, and harvesting methods are chosen, long-term and large-scale experiments with recycled medium must be carried out. Less than 5% of the 518 experimental treatments used in this meta-analysis had culture volumes of at least 1000 liters. Data will likely vary more widely once algae are grown at larger scales and in outdoor ponds, where water will accumulate dissolved and particulate matter not just from the target algae strain but also from algae weeds, bacteria, algae predators, and environmental debris. Given that seawater is one of the more sustainable water sources for cultivation [135], and that only 23% of the total 518 treatments in this meta-analysis grew marine algae, more marine algae stains should also be tested in recycled medium. Here, marine algae were associated with lower mean ES_C and ES_μ than freshwater algae, though the results are not significant(Figure S13).

For studies that observed inhibitory growth effects in recycled medium, further experiments are needed to determine the mechanisms of inhibition if the negatively affected strains are otherwise promising for biofuel production. Most studies did not measure DOC or other compounds leftover in recycled medium, which should be done in the future to correlate concentrations of these compounds with growth response. Additionally, released compounds can decrease efficiency of certain harvesting methods, thereby decreasing the harvested biomass and/or increasing harvesting costs if DOC accumulates in recycled medium [97, 136].

Of the studies that did measure organic compounds in recycled medium (including [15, 17, 22, 23, 25, 26, 37, 38, 52, 61, 69, 86, 89, 97, 103]), most measured polysaccharides, the most abundant algal exudate, and observed an accumulation [15, 22, 25, 37]. Depraetere, et al. [37] confirmed that although polysaccharides in recycled medium inhibited *Arthrospira* growth to some degree, there were other factors intensifying the inhibition. Hadj-Romdhane, et al. [22]

626 grew C*hlorella vulgaris* in continuous culture with medium recycling and found that while high
627 molecular weight compounds (likely polysaccharides) first increased then decreased in
628 concentration, small compounds (likely proteins) continued accumulating. The authors
629 hypothesized that bacteria were consuming polysaccharides and worried that bacteria may harm
630 the algae culture [22]. However, bacteria could prevent DOC accumulation in the medium,
631 which could improve growth conditions and should be studied in further experiments (though
632 currently available data shows no difference between xenic and axenic cultures in recycled
633 medium, Figure S10).

634 More research is also needed to determine if mixotrophic strains can reliably achieve
635 higher biomass yields in recycled medium than photoautotrophic strains can. The ability to
636 photosynthesize and use organic carbon simultaneously can stimulate algae growth rates and
637 biomass productivities [26, 137]. As discussed in Section 3.4.2, authors of several studies
638 concluded that strains stimulated by recycled medium were likely mixotrophic [15, 26] .
639 Furthermore, pairs of algae that grew well in each others' recycled medium (as seen in Figure 7
640 and S7) can be researched further for biofuel-promising properties and robustness in outdoor
641 cultivation.

642 This meta-analysis analyzed algae biomass concentration and specific growth rate in
643 recycled medium because growth measurements were most widely available in published
644 studies. Assessment of algae for fuel products relies on many measurements, however, and these
645 may not necessarily follow the same trends as biomass concentration and specific growth rate.
646 Specific compounds of interest should be measured in future recycled medium experiments.
647 Studies that did measure algae composition collectively showed that effects of recycled medium
648 on lipid accumulation are situation-specific, but protein content may decrease while carbohydrate
649 content increases if algae are stressed (see [5, 25, 37, 38, 60, 66, 70, 83, 89, 103, 104]).

4. Conclusions and recommendations for algae cultivation in recycled medium

Specific growth rate and biomass concentration are significantly reduced in recycled medium based on available experiments, but there are many cases where algae grew favorably in recycled medium compared to a fresh medium control. While effects of recycled medium on algae growth are often situation-specific and do not associate with environmental variables or harvesting methods, there are some genera and growth phases that appear to be more suitable for medium recycling. On the genus level, *Arthrospira*, *Desmosdemus*, *Hormotila*, and *Tetraselmis* cultures are especially promising for their production of suitable recycled medium. On a broader taxonomic level, diatoms were more likely to create growth-inhibiting recycled medium. Harvesting cultures while they are still in exponential phase is recommended for producing suitable recycled medium, because recycled medium from this phase was the only case in which neither biomass concentration nor specific growth rate were significantly inhibited. Harvesting at exponential phase could be achieved by either short-lasting, fast-growing batches or by continuous cultures. Although harvesting method did not appear to influence responses, other more detailed studies suggest that harvesting by certain flocculation methods may be optimal based on costs, practicality, and evidence of favorable growth responses (e.g., [26, 27, 56, 61, 95]). Treating the medium with activated carbon after flocculation was associated with even more favorable growth responses in recycled medium, yet activated carbon treatment is likely infeasible for production of low-value biofuels. Future research can apply the optimal factors leading to growth medium reusability, as determined by this study, to determine if these are also well-suited to large-scale outdoor cultivation and production of biofuel precursors. This research can then inform life cycle and economic analyses to determine the optimal degree of medium recycling.

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Author Contributions

SEL and ZIJ conceived of the study; SEL collected and analyzed the data and drafted the paper; SEL and ZIJ edited the paper; SEL and ZIJ approved the final version of this paper. SEL (sarah.e.loftus@gmail.com) takes responsibility for integrity of the work as a whole.

Conflict of Interest

The authors declare no conflict of interest.

Appendix: Supplementary Information (SI)

SI_A. Supplementary Tables and Figures

SI_B. Data Extraction Protocol

SI_C. Recycled Medium Database

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