Using network component analysis to study axenisation strategies for phototrophic eukaryotic microalgae

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Abstract

Background: The axenisation of phototrophic eukaryotic microalgae has been studied for over a century, with antibiotics commonly employed to achieve axenic cultures. However, this approach often yields inconsistent outcomes and may contribute to the emergence of antibiotic-resistant microbes. A comprehensive review of microalgal species and the methods used to achieve axeny could provide insights into potentially effective workflows and identify gaps for future exploration.

Methods: Scholarly databases were systematically searched, supplemented by citation network analysis and AI-assisted tools, to collect studies on achieving axenic phototrophic eukaryotic microalgae cultures. Data about microalgal species, axenisation workflows, outcomes, and related factors (e.g., sampling locations, axenisation confirmation methods) were summarised. Network component analysis was used to identify clusters of commonly reported methods for diatoms, dinoflagellates, and green algae. A scoring framework was developed to assess the quality and reliability of evidence presented in the studies.

Results: Emerging patterns suggest that workflows involving filtration, washing, and micropicking are frequently reported for diatoms; micropicking, subculturing, and flow cytometry for dinoflagellates; and anoxy, photosensitisation, and streak plating for green algae. Evidence from the literature indicates that a combination of microscopy (e.g., epifluorescence), cell counting (e.g., agar plating), and sequencing (16S and/or 18S) could enhance confidence in confirming axeny.

Conclusion: While antibiotics dominate current practices, alternative pathways for achieving axenic cultures are identifiable through network component analysis. Higher confidence in these methods depends on improved experimental designs and high-quality reporting.

Keywords: Axenisation, microalgae, eukaryote, phototrophs, single culture

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1 Introduction

Background: Axenisation "describes the state of a culture in which only a single species, variety, or strain of organism is present and entirely free of all other contaminating organisms" (Thain & Hickman, 2004). Limiting the influence of confounders is crucial to test hypotheses in an experimental setup, and in the case of microalgal research, factors such as clonality, strain variance, and axenicity requires significant consideration when reporting or interpreting outcomes. Popular culture collections and banks host a wide collection of microalgae, but many; if not most, of the offered species are xenic. They contain bacteria, amoebae, or fungi that grow in consort in the natural habitat from where they were sampled and stored. While characterising these organisms provides important clues about the community where these microalgae thrive, hypotheses focusing on the metabolic capabilities of these organisms requires axeny.

Additionally, there is a resurgence of interest in community interactions between microalgae and other organisms (Pushpakumara et al., 2023), either as interactions in non-associative biological mixtures in an experimental system Grossart (1999) and Solomon et al. (2023), or indeed in symbiotic periphyton (Francoeur et al., 2003), and subsequently their emergent metabolic properties in relation to the wider ecology. Consequently, teasing out the individual contribution from a group effect, or attributing metabolic properties to an organism remains challenging (Fouilland et al., 2018).

The matter is further complicated by the polyphyletic grouping of organisms with varying trophic capabilities under the umbrella term "algae". Although a popular definition was provided by Cavalier-Smith (2007) (reiterated in Cavalier-Smith (2016)) as "oxygenic photosynthesisers other than embryophyte land plants", databases such as AlgaeBase include organisms from all domains of life that do not necessarily satisfy this definition. Terminologies notwithstanding though, even within the set of well defined and widely accepted algal species, most reports fail to specify / confirm if the cultures had non-algal organisms such as fungi or bacteria growing in consort with the algae (xenic cultures). Thus, as more biotechnological uses of microalgae are identified, the demand for purer cultures is expected to rise.

Rationale: Axenisation for algae; particularly microalgae, has been reported for more than a century, and a careful and comprehensive analyses of the available literature would shed some light on important trends that can help establish monocultures effectively. Microalgae are widely used for research owing to their robust physiology and the ability to generate a wide range of commercially valuable bioproducts (Ullmann & Grimm, 2021). While some aspects of the microalgal physiology; such as the ability of some species to overproduce lipids under nitrogen deficiency (Russell et al., 2022) has been a topic of great scientific interest (Sajjadi et al., 2018; Udayan et al., 2023), other areas associated with their physiology and stress acclimatisation, such as the study of the secretome, remain under-investigated (Dixit et al., 2021). Such endeavours are highly sensitive to inconsistencies in the media composition, and the presence of other organisms, that may influence the metabolic processes.

Objectives: The primary focus of this work was to examine which method, or combination of methods can reliably achieve axenic eukaryotic microalgal cultures. The secondary focus was to identify if specific methods were better suited for certain types of microalgae. The goal of this work is to identify a reliable axenisation process that can be incorporated in routine laboratory growth experiments before the mother culture is inoculated into growth experiments or transfer of inoculum cultures in upscaling activities under industrial settings.

2 Materials and methods

2.1 Search protocol

The search protocol used a hybrid method of structured and systematic querying using bibliometric databases, followed by citation network analyses, and artificial intelligence (AI) assisted services to discover and collect publications. The search term `(algae AND (axen* OR purif*))` was queried in PubMed, Google Scholar, and Scopus bibliometric databases and filtered based on title and abstract using an awk script. The publications were then subjected to citation network analysis using the online tool Local Citation Network (https://localcitationnetwork.github.io/) and ResearchRabbit (www.researchrabbit.ai) to discover other publications based on input publications. The final list of recovered publications is available online at: https://www.researchrabbitapp.com/collection/public/0ZGR9O5YLQ. Publications in all languages were included and relevant information was extracted from the text using online translation tools. The working definition of microalgae for the purposes of this review was organisms that are single-cellular, phototrophic, eukaryotic, free-living, and capable of asexual reproduction. The exhaustive list of parameters, exclusion criteria and rationale is available in Supplementary Table S1.

2.2 Data items and synthesis

The following information was extracted from the literature and is provided in a master table as a .csv file (Supplementary Table S2):

1. Year of publication
2. Country of the research institution(s)
3. Microalgal species: The species, including the culture code / accession number if provided by the author.
4. Purification method(s): The method described by the publication to isolate microalgae or decrease the contaminant load. The order in which the methods are written represent the order in which they were applied in the described experimental procedure.
5. Screening method(s): Methods used to verify if the method used to achieve axeny was successful. Alternatively, this also refers to methods to quantify the contaminant load and check the degree of success of the treatment.
6. Media used: The media used to grow the microalgae. In cases where the plates are used to test axeny, the media composition of the agar plates was noted here as well.
7. Screening method(s): Methods used to verify successful axeny. Alternatively, this also refers to methods to quantify the contaminant load.
8. Quality score: Refer to the subsection 2.3 below.

Data extraction procedures detailed further in Supplementary Text S3.
The various axenisation methods noted in the publications were classified either as Physical, Chemical, and Biological, where Physical methods involved the use of techniques such as colony picking to isolate a

group of axenic cells from a Petri-dish for further culturing, or density gradient centrifugation to separate microalgae based on relative cellular densities. Chemical methods referred to the use of antibiotics or special compounds that inhibit the growth of unwanted microbes to achieve axenic microalgal cultures. Biological methods included those where unique abilities of the microalgae is used to isolate them from bacteria and fungi, for example, the use of phototaxis to isolate microalgae (Imai & Yamaguchi, 1994), or removing carbon source and depleting the nitrogen content of the medium, to give the microalgae a competitive advantage. Another example includes the addition of specific microbes that overgrow or inhibit the existing contaminating microbes, and then subsequently removing the introduced microbe using antibiotics or through physical methods.

2.3 Evidence quality

Using the approach described by Jadad et al. (1996), five questions were framed based upon which, the experimental quality of each publication describing the axenisation procedure was scored by AI, MM and QT. The final score was the rounded average of the three scores. The questions are noted below:

1. Does the publication report the source of the organism(s)?
   Yes: 1, No: 0
   Explanation: The contaminating organism(s) may change based on the location from where the microalgae was sampled. In the case of purchase from culture collections, in-house processing protocols, long-term storage (such as cryopreservation), and handling may alter the composition or presence of other organisms cultured along with the microalgae.

2. Are technical details of the axenisation method explained to facilitate reproducibility?
   Yes: 1, Incompletely: 0, No: -1
   Explanation: The question pertains to whether sufficient details of the chemicals or components used in the axenisation process is provided to easily procure or purchase them, or accurately construct, assemble or fabricate them.

3. Was growth medium clearly described?
   Yes: 1, Referenced: 0, No (either not mentioned, or only mentioned without proper reference): -1
   Explanation: The growth medium works in synergy with the purification to support growth of the microalgae which may be stressed after any purification step. Corresponding changes made to the growth media would help ensure successful regeneration of the isolated cultures. Besides, numerous variants of media have been described without labelling or classifying them as an entirely different composition mix.

4. Was axeny verified after treatment?
   With multiple methods: 1, With only one method: 0, No: -1
   Explanation: Veracity of the experimental purification method success validates treatment efficacy.

5. Were contaminating organisms identified?
   Yes: 1, No: 0
   Explanation: A method's efficacy may change depending in the nature of the species contaminating

the microalgal culture. Characterisation or the identification of the contaminating microbes would help establish relevance to the purification method employed.

2.4 Data visualisation and availability

Data was processed using R (4.4.0) (R Core Team, 2024) and preliminary analyses was performed using the tidyverse package (Wickham et al., 2019).
Phylogenetic trees from the species names were constructed using the R packages metacoder (Foster et al., 2017), ggtree (Yu et al., 2017), taxize (Chamberlain & Szöcs, 2013), and rentrez (Winter, 2017). Further network component analysis was performed using the iGraph (Csárdi et al., 2024) and tidygraph (Pendersen, 2024) packages. Subsequent visualisation of the network and the phylogenic tree was performed using the TikZ (Tanau, 2021) and pgfplots (Feuersänger, 2023) packages in LaTeX (Lamport, 1994).

Network component analysis: For the network analysis, two main outcomes were noted, firstly, the success rates for each method (number of times axenisation was successfully achieved / number of times the method was used), and secondly, the grouping / clustering of methods in a network for each microalgal division. To calculate success rates, only those methods that were reported in more than six species were included to remove over-representation of a single method or species within the division level analysis. Next, the clusters within each network were identified using "optimised modularity algorithms" (see documentation of iGraph) and the largest clique (methods commonly used together across the publications) was then obtained. The codes used to process the information is presented in Supplementary_codes.pdf. The awk code used to filter additional irrelevant titles is provided in Supplementary Materials, Supplementary Text, Section 2. All data and resources necessary to reproduce the analyses is available in the OSF repository: https://osf.io/8cmk7/.

2.5 Contact with culture collections

The culture collections of CCAP, Bigelow, CSIRO and NIES were contacted to enquire about the number of unique microalgal species they house, and how many of them are available only in xenic forms, how many are available as axenic as well as xenic. In addition, a subjective question was asked on the challenges they face when trying to make some strains axenic. Their answers are quoted and added to the arguments made in the discussion section.

3 Results

3.1 Summarisation of data

The systematic search yielded 63 publications from which data was extracted and analysed (See Supplementary Figure 1 for the literature recovery schema). Only four publications lacked any DOIs or similar permanent identifiers. Among the selected publications, 34 reported only one microalgae while the other 29 reported multiple microalgal species. Across the search workflow, 52 relevant articles using the traditional search method were recovered, with an additional 7 using citation network analysis, and another 4 using AI assisted methods. Thus lateral search methods could contribute to 17.7 % of the total recovered relevant publications. A summary of all the information collected from 63 publications is provided in Table 1. A consolidated master table with the greater details of each study is provided in Supplementary Table S2.

Across the publications, there were 24 unique axenisation methods that were broadly classified as Biological (comprised of Bacterial co-culturing (CoCu), Selective predation (SePd), and Phototaxis (PhoX) reported in eight publications), Chemical (comprised of Antibiotics (AntB), Lysozyme (LysZ), Hypochlorite (Chlo), Surfactants (Dtrg), Phenol (Phen), Anoxy (Anox), Rose Bengal / Photosensitisation (PhoS), and Salt solution (Salt), reported in 52 publications) and Physical (comprised of Centrifugation (Ctfg), Streak plating (StPl), Washing (Wash), Micropicking (Mkpk), Filtration (Fltr), Density gradient (DenG), Ultrasonication (UltS), Subculturing (SubC), Microfluidics (Mifl), French Press (FrPr), Serial Dilution (SrDl), and Adsorptive resin (Resn), reported in 61 publications. A brief description of these methods are provided in Supplementary Text 1, Sections 1.1 to 1.3.

In the majority of cases method types were applied in concert with others, to achieve varying levels of effectiveness (see “Success” column in Table 1). The six most commonly used methods grouped according to the report quality and axenicity outcome is shown in Supplementary Figure S2 and the comprehensive counts of methods used for each species grouped according to according to axenicity outcome is provided in Supplementary Table S3.

Table 1: Summary table of the major data-points extracted from the recovered publications. Resolution of the information is at a publication level.

Division[a]	**Method type** [b]	**Genera**	**Media**[c]	**Country**[d]	**Success**[e]
Diatoms (Pubs = 11)	Biological = 1, Chemical = 1, Physical = 8	Phaeodactylum sp., Ulnaria sp., Achnanthes sp., Amphora sp., Navicula sp., Cocconeis sp., Cymatosira sp., Cymbella sp., Diploneis sp., Dimeregramma sp., Encyonema sp., Fallacia sp., Fragilaria sp., Gyrosigma sp., Hantzschia sp., Pinnularia sp., Nitzschia sp., Petrodictyon sp., Staurophora sp., Coscinodiscus sp., Chaetoceros sp., Hemidiscus sp., Licmophora sp., Skeletonema sp., Tabularia sp., Caloneis sp., Stephanocyclus sp., Gomphonema sp., Staurosira sp., Synedra sp., Achnanthidium sp., Cylindrotheca sp., Actinocyclus sp., Detonula sp., Ditylum sp., Hemiaulus sp., Lithodesmium sp., Odontella sp., Trieres sp., Thalassiosira sp.	Bacillariophycean medium (n=1), Cramer-Myers medium (n=1), Diatom medium (n=2), DM medium (n=1), f+Si medium (n=1), F/2+Si medium (n=2), MP1 (n=1), SWM-3 (n=1), SWES (n=1).	China (n=1), Germany (n=3), Japan (n=2), Mexico (n=1), Russia (n=2), USA (n=1).	Yes=8, No=2, Multi/Part=1

Dinoflagellates (Pubs = 15)	Biological = 2, Chemical = 3, Physical = 8	Symbiodinium sp., Alexandrium sp., Cochlodinium sp., Karenia sp., Gymnodinium sp., Heterocapsa sp., Prorocentrum sp., Scrippsiella sp., Peridinium sp., Pyrodinium sp., Amphidinium sp., Akashiwo sp., Coolia sp., Fragilidium sp., Karlodinium sp.	Artificial seawater (n=1), Cramer-Myers medium (n=1), DM medium (n=1), f/2 (n=6), K medium (n=1), L1 medium (n=2), MES-volvox (n=1), MP1 (n=1), SWM-3 (n=1), Not mentioned (n=1)	China (n=1), France (n=1), Japan (n=2), New Zealand (n=1), Australia (n=1), Philippines (n=1), South Korea (n=2), Taiwan (n=1), USA (n=6).	Yes = 7, No = 5, Multi/Part = 3

Green algae (Pubs = 33)	Biological = 2, Chemical = 6, Physical = 10	Amphikrikos sp., Auxenochlorella sp., Chlamydomonas sp., Chlorella sp., Chloroidium sp., Chloromonas sp., Choricystis sp., Coelastrella sp., Coenochloris sp., Cosmarium sp., Desmodesmus sp., Dunaliella sp., Ettlia sp., Haematococcus sp., Mesotaenium sp., Micromonas sp., Monomastix sp., Nannochloris sp., Platymonas sp., Pseudopediastrum sp., Pyramimonas sp., Scenedesmus sp., Scherffelia sp., Spermatozopsis sp., Stichococcus sp., Tetradesmus sp., Tetraedron sp., Tetraselmis sp.	Artificial seawater (n=2), BBM (n=3), BG11 (n=5), Cramer-Myers medium (n=1), Enriched artificial seawater (n=1), f/2 (n=6), HGC (n=1), K medium (n=1), Knop medium (n=1), L1 medium (n=1), Mann & Myers (n=1), MP-1 (n=1), Not mentioned (n=2), SWEWS f/2 (n=2), TAP medium (n=2), Waris solution (n=2)	Austria (n=2), Canada (n=2), China (n=7), Ecuador (n=1), France (n=2), Germany (n=2), Hong Kong (n=1), India (n=1), Indonesia (n=1), Ireland (n=1), Israel (n=1), Korea (n=3), Malaysia (n=1), Taiwan (n=1), UK (n=1), USA (n=5)	Yes=22, No=7, Multi/Part = 3

Other eukaryotes (Pubs = 22)	Biological = 5, Chemical = 24, Physical = 24	Chattonella sp., Chromulina sp., Chrysotila sp., Cryptomonas sp., Cyanophora sp., Emiliania sp., Euglena sp., Eutreptiella sp., Fibrocapsa sp., Florenciella sp., Hemiselmis sp., Heterosigma sp., Isochrysis sp., Nannochloropsis sp., Rhodomonas sp., Porphyridium sp.	Artificial seawater (n=1), Cramer-Myers medium (n=3), Enriched artificial sewater (n=1), f medium (n=1), F/2 medium (n=6), f/2-Si (n=1), Hall's medium (n=1), Heterotrophic growth complex, BBM (n=1), K medium (n=3), L1 medium (n=3), NMB3 (n=1), Not Mentioned (n=4), SWEWS f/2 (n=1), SWM-3 (n=2), TYG medium (n=1), Waris solution (n=1),	Canada (n=1), China (n=3), France (n=1), Germany (n=1), Ireland (n=1), Japan (n=1), Korea (n=4), Mexico (n=1), Norway (n=1), Poland (n=1), UK (n=1), USA (n=6)	Yes = 15, No = 5, Multi/Part = 2

a. "Division" is the trivial/non-taxonomic grouping of microalgae. 'Pubs' indicate the number of publications where organisms belonging to this division were investigated.

b. The 'Method type' column categorizes the purification methods described in the publications into Biological, Physical, or Chemical, based on their mode of action. The values indicate the count of unique methods employed in each category, with most publications using multiple methods in concert to achieve axenic cultures.

c. “Media” notes the growth medium used to culture the microalgae.

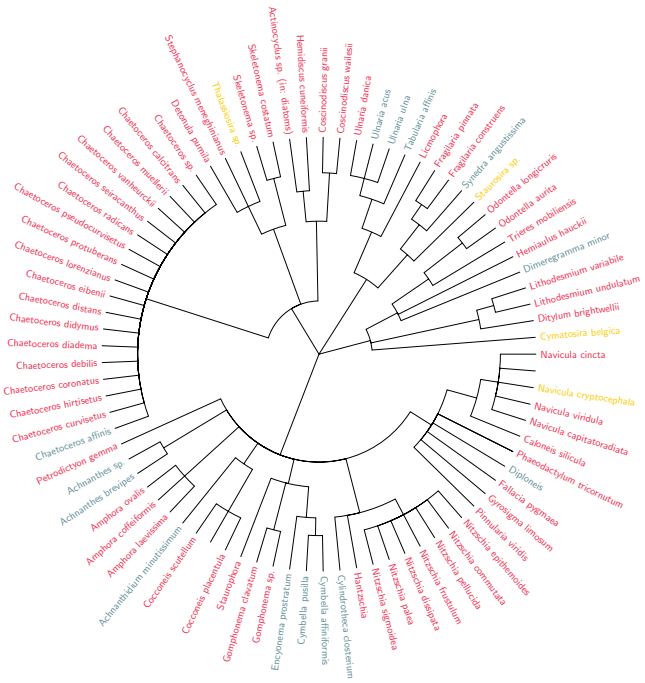
d. “Country” refers to the geographical location where the samples were collected or the research was conducted. Note that some publications had international partners and thus, the number of countries counted do not correspond to the number of publications.

e. “Success” refers to whether axenic cultures could be achieved using the described protocol. Yes, indicates successful achievement of axeny using the applied methods, No indicates continued presence of contaminants in the final culture, Part/Multi indicates that across the multiple species investigated, some remained contaminated.

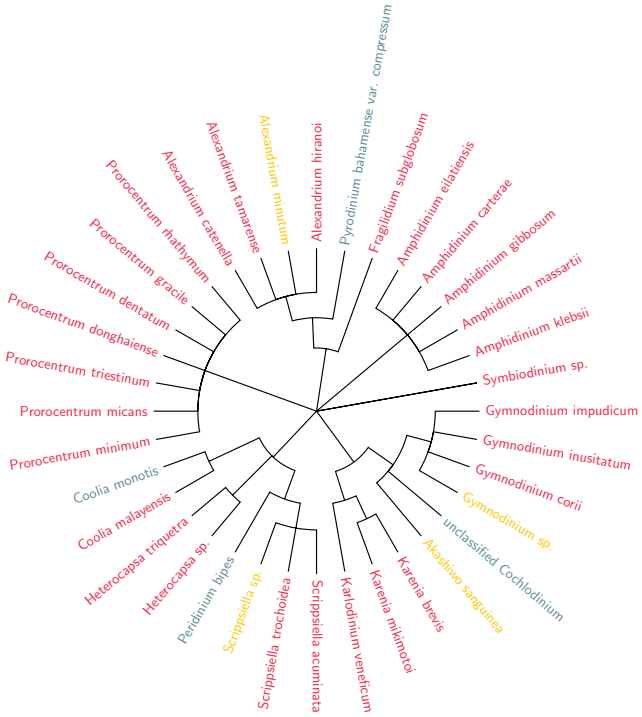
3.2 Reported species and sampling locations

Across the literature 174 unique species were identified. Species were split into their non-taxonomic divisions such as diatoms, dinoflagellates, etc., and sub-grouped as freshwater, marine and brackish water species. In all instances where microalgae were purified from soil, it was found that they could grow in freshwater bodies as well and thus were coloured as freshwater. Across the literature, organisms from the divisions of cryptomonads (n= 4), diatoms (n= 81), dinoflagellates (n= 36), euglenoids (n= 5), golden algae (n= 1), eukaryotes (n= 10), green algae (n = 31), haptophytes (n= 4), red algae (n= 1), and green plants (n=1) were noted. While diatoms comprised the largest number of reported species, they were generally subjected to only 11 of the total 24 methods. Conversely, all 24 methods were applied to the green algae species. Many of the reported organisms such as *Nannochloris* sp., and *Chlorella* sp., have significant commercial value and thus the investigation of newer methods to rapidly and effectively achieve axenic cultures is incentivized.

A



B



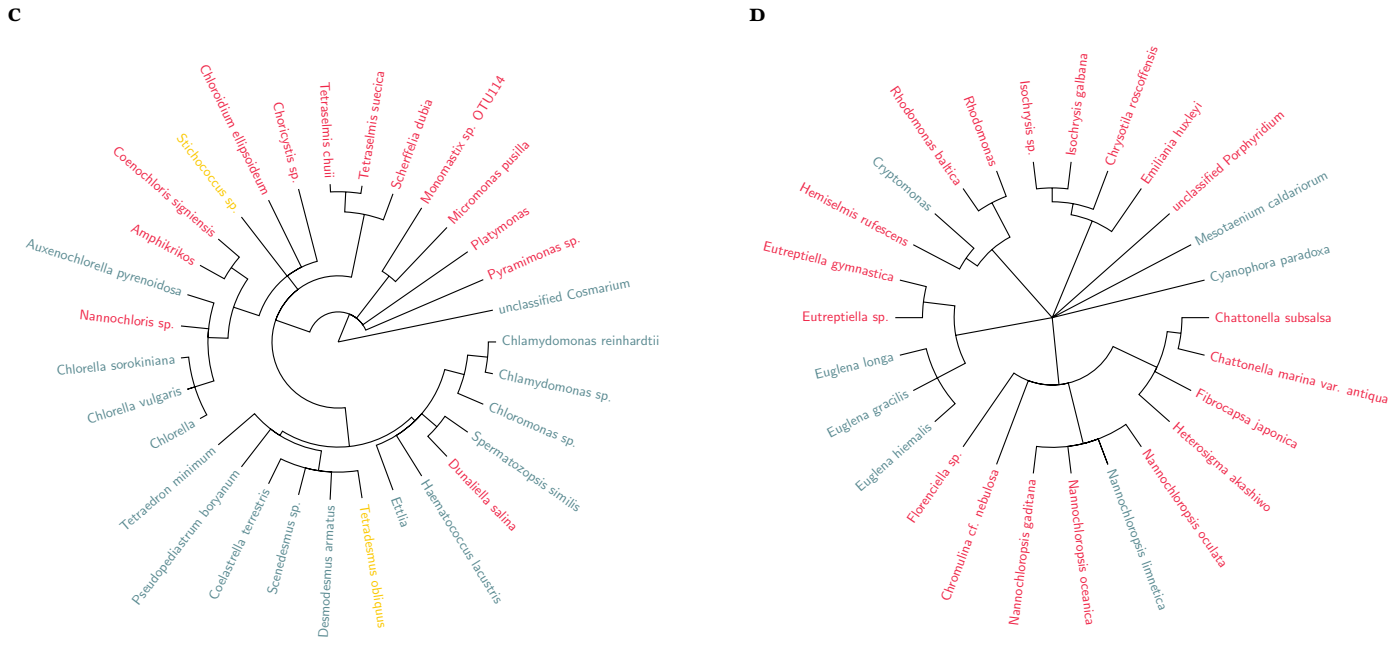


Figure 2: Phylogenetic tree of the species noted in the collected publications. Species names are coloured red for marine, sunset-yellow for brackish, and steelblue for freshwater species. (a) Taxonomic tree of 81 diatom species identified across 11 publications. (b) Taxonomic tree of 36 dinoflagellate species identified across 15 publications. (c) Taxonomic tree of 31 green algae species identified across 33 publications. (d) Taxonomic tree of 26 microalgal algae species identified across 22 publications.

Among the 63 publications, 19 publications collected samples from natural water sources to identify and isolate microalgae. With the exception of sampling from Ecuador (Aray-Andrade et al., 2018), sample collection from natural sources were mostly limited to the northern hemisphere in the available reports. Among the publications, 27 used samples from culture banks (public or private), while 17 publications provided no information on where the microalgal samples were obtained. Although many microalgal species such as *Nannochloropsis* sp. or *Nitzschia* sp. are cosmopolitan / global in distribution, success in achieving axenic cultures was variable. While this of course is largely attributed to the difference in the treatment, the effect of the contaminating microbes cannot be discounted (See further discussion in Section 4.4.4). Moreover, this highlights other regions in the world where potential sampling can be performed to identify and isolate unique microalgal species. For instance, Sharma and Rai (2011) note the systematic failures in global sample collection and species screening; potentially leading to a large number of species remaining undiscovered, and mutualistic relations between known species and their environment being poorly understood. The sites where microalgal sampling was reported is shown in Figure 3.

3.3 Antibiotics – the *de facto* starting point

Antibiotics is by far the most preferred initial approach when attempting to generate axenic cultures are they are convenient to apply and the outcomes are generally rapid. Across the 63 publications systematically collected, 42 reported the use of antibiotics that spanned across 12 classes as shown in Table 2. In almost all cases, these antibiotics were applied as a cocktail and the combinations are shown in Figure 4. As the number of components in a cocktail increases, the range and potency to kill contaminants also rises. However, it comes at the risk of adversely affecting the viability of the microalgal culture itself.

Aminoglycosides, and Penicillins were the most popular classes of antibiotics applied in axenisation procedures

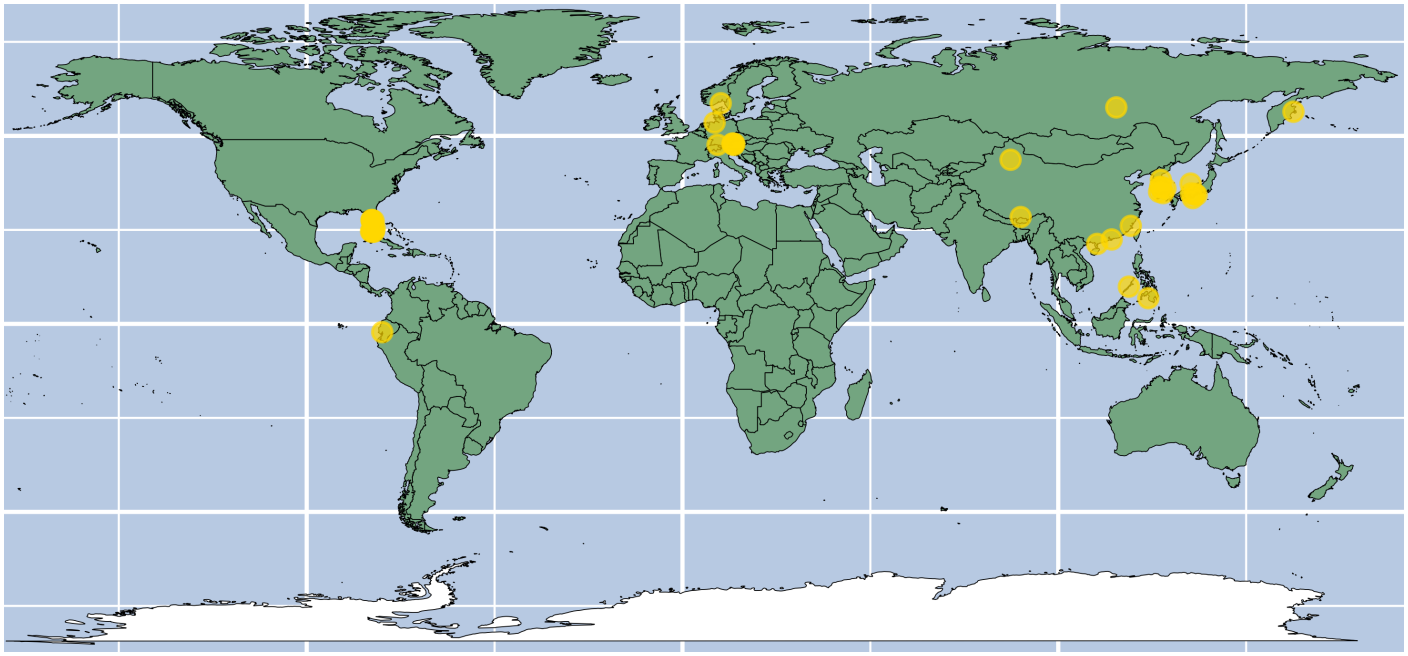


Figure 3: Geographic locations of microalgal samples collected from locations noted in 19 publications.

across all microalgal classes. Application of the antibiotics appeared to be cognisant of the microalgal class as well, with diatoms being exposed to the fewest classes of antibiotics; predominantly Aminoglycosides and Penicillins. Conversely, Dinoflagellates and Green algae appear to have been experimented upon with almost all the classes of antibiotics. The three most commonly employed antibiotics included two aminoglycosides; namely, Streptomycin (n=21; median conc. = 100 mg L^{-1}), Kanamycin (n=17; median conc. = 200 mg L^{-1}) and one penicillin; namely, Ampicillin (n=16; median conc. = 100 mg L^{-1}. Neomycin + Kanamycin (n=7), Streptomycin + Penicillin-G (n=7) and Streptomycin and Neomycin (n=6) were the most common cocktail pairs employed in the publications. The complete list of antibiotics used and the corresponding antibiotic classes are provided in Supplementary Table S4.

Of course, the application of these antibiotics also depended on whether a culture needed to be decontaminated, or if the axenic culture required to be maintained. For example, Kan and Pan (2010) and (Han et al., 2016) profess the use of highly concentrated cocktails of antibiotics to achieve axenic cultures, while Youn and Hur (2007) and (Wang et al., 2016) employ antibiotics at moderate to low concentrations to achieve axenic cultures over multiple rounds to treatment and washes, and maintain the axenic status by incorporating the antibiotics in the growth medium.

3.4 Alternate axenisation pathways with network component analyses

Axenisation workflows for three of the most commonly reported divisions, diatoms, dinoflagellates and green algae are provided in Figure 5a, 5b and 5c respectively. Workflow for microalgal families with low representation grouped under “others” is available in Supplementary Figure S3.

In Figure 5b, the network of the various methods used to achieve axenic diatom cultures is shown. Analyses included identifying clusters and cliques between the methods employed. Clusters represent the collection of nodes (here, methods) with similar network characteristics such as the edge density (number of connections to other nodes), and relative position from the central node - Incubation (i.e: they are directly connected to incubation or first connect to other nodes / methods before reaching incubation), within the network space (See iGraph documentation by Csárdi et al. (2024) and more general works by Clauset et al. (2004)

Table 2: Antibiotic classes used to achieve axenic cultures of the commonly reported microalgal groups.

Class	Diatoms	Dinoflagellates	Green algae	Others
Aminoglycosides	135 (10 to 600)	160 (8 to 2000)	600 (8 to 10000)	600 (8 to 2000)
Carbapenems	98 (98 to 98)	10 (10 to 10)	5 (5 to 5)	-
Fluoroquinolones	5 (5 to 5)	36.5 (23 to 50)	13 (10 to 16)	-
Penicillins	250 (50 to 600)	100 (50 to 1000)	600 (2.5 to 2000)	100 (10 to 2000)
Tetracyclines	10 (10 to 10)	12 (1 to 12)	50 (5 to 500)	50 (12 to 500)
Cephalosporins	-	60 (20 to 100)	400 (10 to 500)	30 (30 to 400)
Macrolides	-	6 (6 to 6)	54 (8 to 100)	6 (6 to 6)
Polypeptides	-	14 (12 to 16)	3.3 (3.3 to 5000)	16 (3.3 to 30)
Rifamycins	-	10 (10 to 10)	-	30 (25 to 30)
Nitrofurans	-	-	10 (10 to 10)	250 (250 to 250)
Antifungal Agents	-	5 (5 to 15)	20 (0.01 to 400)	15 (1 to 15)
Others	27 (27 to 27)	125 (50 to 200)	35 (5 to 60)	20 (2.5 to 68)

Values are expressed in mg L^{-1} as median (minimum to maximum) values.

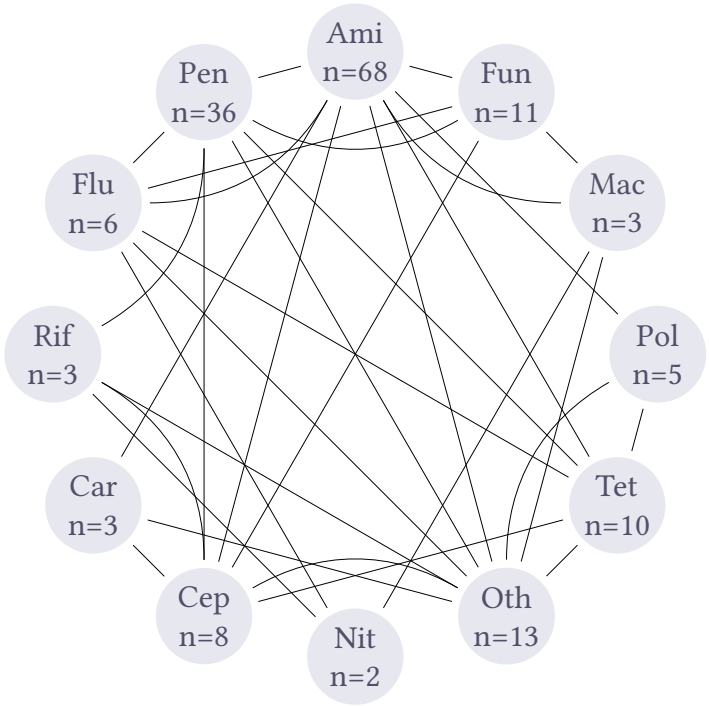


Figure 4: Antibiotic class combinations reported in the literature. Ami = Aminoglycosides, Fun = Antifungal agents, Mac = Macrolides, Pol = Polypeptides, Tet = Tetracyclines, Oth = Others, Nit = Nitrofurans, Cep = Cephalosporins, Car = Carbapenems, Rif = Rifamycins, Flu = Fluoroquinolones, Pen = Penicillins, n= number of publications reporting the use of the antibiotic class.

for deeper analysis). In context to the work presented here, clusters could help identify a set or collection of methods with similar usage frequencies across the collected publications, and other methods that are commonly reported to be used along with them. Cliques, on the other hand comprise of adjacent nodes that are all directly interconnected to form subgraphs within the network space (Eppstein et al., 2010). In the context of the work presented here, the largest cliques were sought within each cluster to identify workflows using methods of similar usage and reportage.

Details of the analyses and codes for Figure 5, are provided in Supplementary Codes (Code Chunk 6). General

network parameters for methods used on diatoms were also calculated, such as the overall network density was 0.21; (i.e.: ratio of actual number of connections to the theoretical maximum number of connections). Clustering analyses found that the network could be sub-graphed into three clusters an overall modularity score of 0.37 and a median connectivity of 4 connections (incoming or outgoing) per node. Details of the overall network, and the clusters and cliques for diatoms, dinoflagellates and green algae are summarised in Table 4.

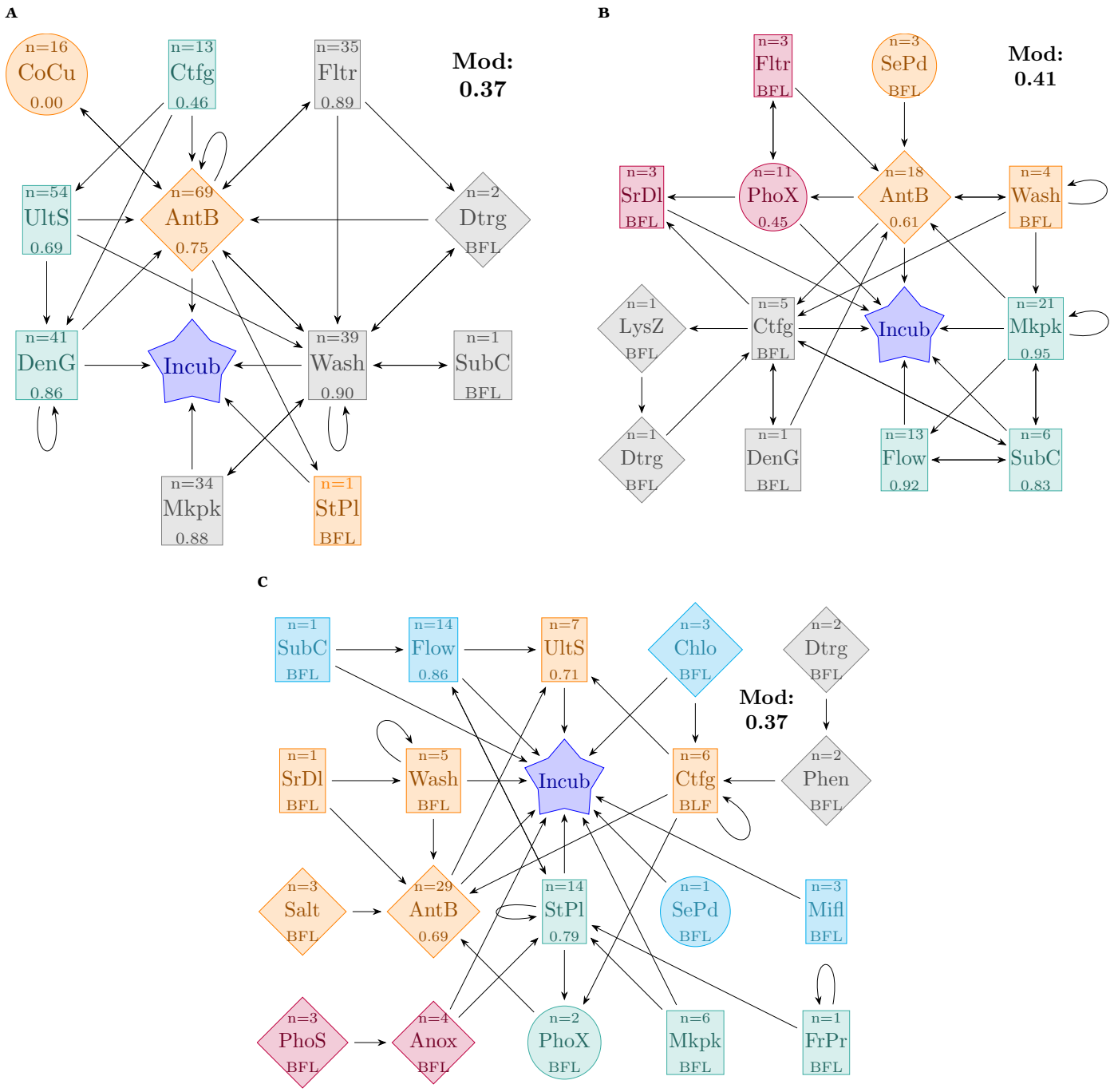


Figure 5: Clustering of the axenisation workflows. In each panel, methods belonging to the same cluster are assigned the same colour. Panel A shows the workflows incorporating the 11 methods used for 81 diatoms species in 11 publications. Panel B shows workflows incorporating 13 methods for 36 dinoflagellate species in 15 publications. Panel C shows the workflows incorporating 13 methods for 26 green algae species in 22 publications. Numbers in the network components represent success rates when number of reports is n>6; for n≤6 reports, success rates are below the filter limit (BFL) and not calculated. Node shapes denote method types: squares for "Physical," circles for "Biological," and diamonds for "Chemical." Nodes of the same colour indicate they belong to the same cluster. The analysis is presented at the species level. Mod value represents the modularity value (range between 0= no clustering to 1 = perfect clustering) of the nodes in the network space.

Table 3: Workflow Network Analysis of Different Microalgae Divisions

Division[a]	**Cluster**[b]	**Nodes**[c]	**Density**[d]	**Degree**[e]	**Cliques**[f]
Diatoms	Overall	Antibiotics, Co Culture, Streak plating, Filtration, Surfactants, Washing, Subculturing, Micropicking, Ultrasonication, Density gradient, Centrifugation	0.21	4	1. Ultrasonication, Antibiotics, Centrifugation, Density gradient.
					2. Antibiotics, Filtration, Washing, Surfactants.
	Cluster 1	Streak plating, Antibiotics, and Co Culture	0.44	2	1. Streak plating, Antibiotics.
					2. Antibiotics, Co Culture.
	Cluster 2	Filtration, Surfactants, Washing, Subculturing, Micropicking	0.36	2	1. Filtration, Surfactants, Washing
	Cluster 3	Ultrasonication, Density gradient, Centrifugation	0.44	2	Ultrasonication, Density gradient, Centrifugation
Dinoflagellates	Overall	Selective predation, Antibiotics, Washing, Micropicking, Subculturing, Flow Cytometry, Centrifugation, Lysozyme, Surfactants, Density gradient, Filtration, Phototaxis, Serial dilution	0.17	4.5	Subculturing, Incubation, Flow Cytometry, Micropicking
	Cluster 1	Selective predation, Antibiotics, Washing	0.44	3	1. Selective predation, Antibiotics.
					2. Antibiotics, Washing.
	Cluster 2	Micropicking, Subculturing, Flow Cytometry	0.67	4	Micropicking, Subculturing, Flow cytometry
	Cluster 3	Centrifugation, Lysozyme, Surfactants, Density gradient	0.31	2	Centrifugation, Lysozyme, Surfactants

Continued on next page

Division[a]	Cluster[b]	Nodes[c]	Density[d]	Degree[e]	Cliques[f]
	Cluster 4	Filtration, Phototaxis, Serial dilution	0.33	2	1. Serial dilution, Phototaxis.
					2. Phototaxis, Filtration.
Green algae	Overall	Anoxy, Photosensitisation, Antibiotics, Salt solution, Ultrasonication, Washing, Centrifugation, Serial dilution, Streak plating, Phototaxis, Micropicking, French Press.	0.11	2.5	Centrifugation, Antibiotics, Phototaxis, Streak plating
	Cluster 1	Anoxy, Photosensitisation	0.5	2	Anoxy, Photosensitisation
	Cluster 2	Antibiotics, Salt solution, Ultrasonication, Washing, Centrifugation, Serial dilution	0.33	4	1. Serial dilution, Antibiotics, Washing.
					2. Antibiotics, Centrifugation, Ultrasonication.
					3. Antibiotics, Centrifugation, Washing.
	Cluster 3	Streak plating, Phototaxis, Micropicking, French Press	0.37	2.5	1. Micropicking, Streak plating.
					2. Phototaxis, Streak plating.
					3. Streak plating, French press.
	Cluster 4	Flow Cytometry, Predation, Subculturing, Hypochlorite, Microfluidics	0.04	0	Flow cytometry, Subculturing
	Cluster 5	Surfactants, Phenol	0.25	1	Surfactants, Phenol

a. Division is the non-phylogenic grouping of the microalgae with shared characteristics.

b. Cluster represents the number of method groups identified in the workflow network.

c. Nodes enlist the methods grouped in each cluster.

d. Density is the edge density of the cluster and is calculated as the ratio of the number of actual connections versus the theoretical maximum number of connections possible between the nodes, including loops/self-connections.

e. Degree is the median number of connections (incoming and outgoing) from each node in the cluster.

f. Cliques represent the largest cliques of methods, which is a subgroup of the cluster of methods that could influence each other's effectiveness.

- The details are collected and summarised from code chunks 6, 13, and 19 in supplementary codes.

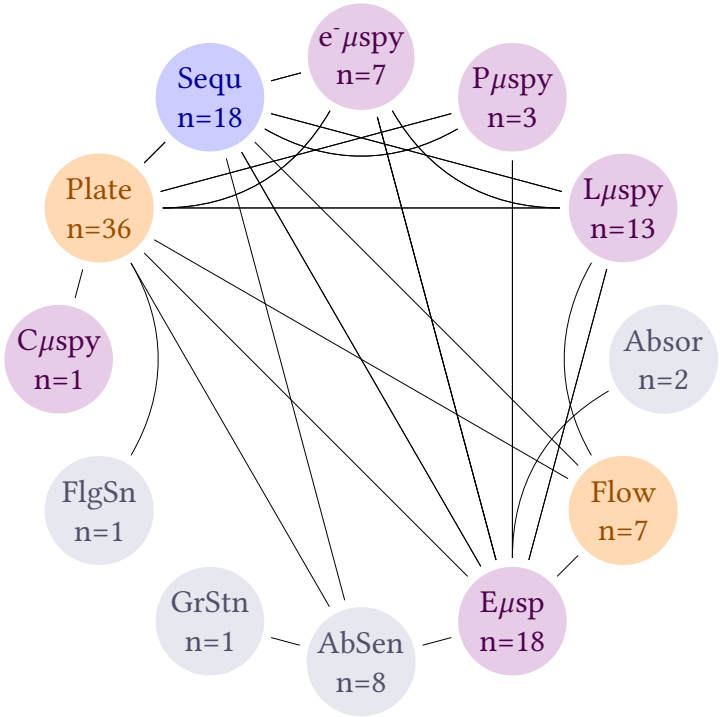


Figure 6: Methods reported in literature used either individually, or in combination to assess axeny in microalgal cultures. Resolution of information is at publication level. e-μspy = electron microscopy, Pμspy = Phase contrast microscopy, Lμspy = light microscopy, Absor = Absorbance, Flow = Flow cytometry, Eμspy = Epifluorescence microscopy, AbSen = Antibiotic sensitivity, GrStn = Gram staining, FlgSn = Flagella staining, Cμspy = Confocal microscopy, Plate = Plate counting, Sequ = 16S/18S sequencing. Methods marked in purple are microscopy-based methods. Methods marked in orange are cell counting methods. Methods marked in blue are sequencing methods. Methods marked in gray are physiology specific methods.

Beyond generating the workflow network, further analyses to identify clusters and cliques for other eukaryotic microalgae (Supplementary Figure S3) was not performed as they did not belong to any distinct or discernable division / grouping.

3.5 Status confirmation and publication quality

Information on cross verification methods of the final results, and the characterisation of contaminating microbes in the culture were crucial to providing the context and information necessary to reproduce outcomes. An objective method of assessment using a scoring system was introduced to gauge reporting quality of the available evidence. The scoring across the publication timeline is shown in Supplementary Figure S4. The highest number of relevant publications were available between 2011 to 2020 (n=29) with seven publications scoring >0. Across the 63 relevant publications, 17 publication (26.0 %) had a score <0. This was largely contributed by poor description of methods and lack of information on axenisation validation. For instance, across the 63 publications, 13 distinct methods were reported to check the validity of their claim of achieving axeny. Only six publications did not report a follow-up axeny verification, 23 reported the use of only one method to check for axeny, 12 used a combination of two methods to test axeny, while the remaining 22 employed three or more methods to assess axeny. Method combinations to verify axeny is shown in Figure 6. The most common verification method was plate counting using different media compositions. The literature reported five different kinds of microscopy, namely, confocal microscopy, electron microscopy, epifluorescent microscopy, phase contrast microscopy and light microscopy that were used to test for axeny, of which

epifluorescent microscopy was most commonly used for verification. Collectively, microscopy in all its forms were reported in 42 publications. Methods leveraging specific aspects of the microalgal physiology such as light absorbance spectrum, flagella staining, or antibiotic sensitivity were among the seldom used method, collectively reported in 12 publications. Cell counting methods such as Flowcytometry and plate counting was reported in 43 publications and lastly, sequencing (both 16S/18S) methods were used in 18 publications.

4 Discussion

Many high quality and detailed reviews such as those by Fernandez-Valenzuela et al. (2021), McCracken (1969), Molina-Cárdenas et al. (2016), and Vu et al. (2018), have previously described axenisation procedures for microalgae. While these publications provide critical insights in applying the investigated methods, their approach was narrative and a top-down perspective of the various strategies adopted as a combination of axenisation methods has not been presented thus far. The approach adopted in this study was to collect methodologies described across the various publications and visualise them as "networks" of the methods connected to each other depending on the sequence in which they were applied, that ultimately led to the final step of "incubation" where the culture is presumed to be axenic. This allows for methods to clusters within the network based on key characteristics such as edge density and relative node position. Edge density refers to the number of connections a node shares with others, influencing how tightly integrated a method is within the network. Another important factor is the relative position of a method relative to Incubation – final step and the central node. This position determines whether a methodology is single step (i.e: only method directly connected to Incubation) or sequential (many methods used in sequence) before generating an axenic culture.

The methods described in the publications were grouped according to their mode of action into Physical, Chemical and Biological; with each presenting their characteristic advantages and shortcomings that need to be considered when designing an axenisation workflow. Physical methods require the most initial investment in the form of equipment as well as manual skill to successfully decontaminate the culture. By themselves physical methods such as density gradient, washing, or filtration do not generate axenic cultures, can contribute to the wider process to achieving them. Micropicking techniques require specialised skills to carefully separate microalgal cells from the contaminating organisms, without damaging cells. Flowcytometry on the other hand can automate the precise sorting process, but are generally ineffective against physical aggregates of microalgal cells and bacteria.

Chemical methods on the other hand are the most popular though the use of antibiotics. But as noted before, this has led to the wider problem of generating antibiotic resistant bacteria / fungi, while also adversely affecting the growth of the microalgal cells. In some cases, microalgae are known to metabolise the antibiotics and thus, antibiotic application may inadvertently become ineffective. Besides, chemical methods such as the use of detergents of oxidising agents such as chlorate, can significantly hamper the viability of the culture and incessant use can become mutagenic.

Lastly, biological methods have not been widely reported across the literature owing to the highly specific nature of their mode of action. Phototaxis is not a global microalgal ability, and is mostly restricted to certain dinoflagellates and green algae. Biological methods offer a more sustainable method of contaminant removal by leveraging physiological properties of the microalgae and selectively promoting their growth at the cost of the contaminants.

4.1 Clusters and cliques from network component analysis

The network component analysis revealed variations in methodological approaches and interconnections across microalgal divisions, with key differences in network density, clustering, and reliance on specific methods. Workflows described to achieve axenic diatoms cultures comprising of 11 nodes (methods) exhibited a moderate network density of 0.21 (ratio of the number of connections to the theoretical maximum number of connections possible) and a median node degree of 4 (i,e: number of connections; incoming and outgoing, for each node), and an evenly distributed number of methods in each clusters. Workflows for dinoflagellates comprising of 13 nodes had a lower network density of 0.17, despite a higher median degree of 4.5, suggesting a broader range of methods applied, but with weaker interconnections. For example, methods as phototaxis, or selective predation were used in limited combinations with other methods. The sparseness in the workflow network is demonstrated further in the methods described for green algae. With 19 nodes, the network density was only 0.11, and had a median degree of 2.5. This indicates that a large number of specialised methods were used, largely in isolation. For example, selective predation and microfluidics were used in isolation without using them in concert with other methods.

4.1.1 Cluster characteristics

Diatoms: Two method-specific clusters of similar comprising of cliques with similar network densities of 0.44 emerged, namely Cluster 1 comprising of streak plating, antibiotics (success rate= 0.75), and co-culture (success rate= 0.0), and Cluster 3 comprising of ultrasonication (success rate= 0.69), density gradient (success rate= 0.86), and centrifugation (success rate= 0.46). Cluster 1 uses a mix of physical, chemical, and biological methods to achieve axenic cultures, while Cluster 3 comprises exclusively of physical methods and consequently, offers a workflow that circumvents the use of antibiotics.

Dinoflagellates: Among the clusters identified for Dinoflagellates, Cluster 2 comprised of a clique with the highest network density of 0.67, and exclusively involved physical methods of micropicking (success rate= 0.95), subculturing (success rate= 0.83), and flow cytometry (success rate= 0.92). Micropicking and flowcytometry are high precision methods and showed high success rates for diatoms. Other clusters of interest were Cluster 3 and Cluster 4 that used methods leveraging the specificity of enzyme action (lysozyme) and motility (phototaxis), but had low network densities and insufficient number of publications to calculate success rates.

Green algae: Identifying effective workflows for green algae is challenging as no clear trend with reliable metrics were found from the network component analysis. For instance, Cluster 1 comprising of only anoxy and photosensitisation had the highest network density of 0.5, but none of the methods were sufficiently represented in the literature to calculate a success rate. In comparison, Cluster 2 comprising of a wider collection of methods such as antibiotics (success rate= 0.69), salt solution, ultrasonication (success rate= 0.71), washing, centrifugation, and serial dilution, and an overall network density of 0.33, had two methods with sufficient representation to calculate success rates, but involved the use of antibiotics.

Key comparisons and trends: Across the workflows described to achieve axenic microalgal cultures, clusters and cliques could be identified that avoided the use of antibiotics and instead rely on other strategies.

For example, highly dense clusters for methods like micropicking, subculturing, and flow cytometry in dinoflagellates reflects the option to adopt precision techniques. Similarly, with diatoms, filtration, washing and micropicking or ultrasonication and density gradient have been reported to attempt axenisation. In contrast, green algae does not show any preferred approach towards achieving axenisation, although there is preliminary evidence showing the use of anoxy and photosensitisation to be effective. Across all divisions, antibiotics emerged as a common bridging method, appearing in high-degree nodes and multiple cliques. Mechanical methods like ultrasonication and centrifugation were consistently clustered in diatoms and green algae, but were less central for dinoflagellates, reflecting divisions-specific adaptations to their cell structures and ecological traits. Dinoflagellates had the highest reliance on precise isolation methods, likely due to their complex morphology and their own susceptibility to antibiotics and harsh chemical methods. Diatoms showed a balance between mechanical and chemical techniques, leveraging synergies within and across clusters. Green algae utilised a blend of unique (e.g., photosensitisation) and shared methods (e.g., antibiotics), indicating a diverse yet less interconnected network of approaches.

4.2 Verification methods

Across the recovered literature, six publications did not properly verify the axenicity of their final culture, while eight publications only used one method to declare axeny. This is important as each verification method has inherent limitations that make it insufficient when used as the sole test. For example, plate counting is a well-established technique for detecting viable bacteria, as it requires microbial growth on a solid substrate. However, its limit of detection varies depending on the bacterial species and the growth conditions, typically ranging from >8 to 100 living cells / mL (Cappuccino & Sherman, 2008). Some bacteria may fail to grow on standard media but can persist in microalgal cultures, leading to false confirmation of axenicity. On the other hand. molecular approaches such as 16S rRNA gene sequencing are highly sensitive, with detection limits as low as ~10 cells/mL (Brandt & Albertsen, 2018). However, while sequencing can confirm the presence of bacterial DNA, it cannot differentiate between live bacteria and lysed cells. This means that sequencing alone may overestimate contamination levels by detecting residual DNA from non-viable cells. Microscopy methods, such as epifluorescence or differential interference contrast microscopy, provides direct visualization of potential contaminants but suffers inherent subjectivity in detecting the type and level of contaminants. The accuracy of this method depends on the experience of the observer and prior knowledge of expected contaminants in a given culture. Moreover, microscopy may fail to distinguish bacterial cells if they are present at very low densities or embedded within algal sheaths. Flow cytometry, while highly effective at detecting single cells and distinguishing between populations based on size and fluorescence properties, has limitations in identifying bacteria that are closely associated with microalgae. Some microalgal-bacterial aggregates (e.g., diatoms with tightly bound bacteria within the silica frustule) may not be detected as separate entities. Additionally, while live/dead staining can be incorporated into flow cytometry protocols to assess bacterial viability, this approach may still fail to detect bacteria in intimate symbiotic relationships with microalgae.

Given these limitations, a combination of complimentary testing methods is necessary to deduce axenicity with high confidence.. Plate counting provides confirmation of viable bacteria, sequencing ensures highly sensitive detection, and microscopy serves as a cross-validation tool for detecting bacteria that might be overlooked due to primer biases in PCR or aggregation with microalgae. Flow cytometry could serve as a complementary tool, particularly if live/dead staining is included, but its efficacy depends on the strength of microalgal-bacterial

associations. Based on the method combinations shown in Figure 6, a robust approach could involve using plate counting (or flow cytometry if live/dead staining is implemented) alongside sequencing and microscopy to maximize detection sensitivity while minimizing false negatives.

4.3 Perspective from microalgal culture collection

Culture collections are critical towards curating and maintaining microalgal cultures and was identified as a prime-source to gain insight from the expertise and knowledge drawn from regular practical work. A query was made to these culture collections to enquire about their practices to achieve axeny, and the tips and important considerations when maintaining axeny in working culture. The number of phototrophic eukaryotic microalgae along with the number of axenic species held in four major culture banks is provided in Supplementary Table S5. Upon enquiry, personnel from CSIRO stated that axeny was achieved through the use of antibiotics, while NIES exclusively used washing and micro-picking, and do not use antibiotics. The respondent from Bigelow did not mention any specific method, but acknowledged that the process can be labour intensive. All the culture collection respondents emphasised on the use of good sterile lab techniques to ensure axeny with the respondent from Bigelow noting that

> "Maintaining axenic cultures is not difficult if proper working conditions are maintained. We do all work in a flow hood and practice sterile technique."

The emphasis of the importance of considering the role of commensal/phycospheric microbes was also universally echoed, with the respondent from CCAP stating,

> "... however we don't try to make other axenic (unless necessary for a particular project) as many strains don't like to grow well as axenic cultures. "

, and from CSIRO,

> "We find many do not grow if axenic, possibly due to the need for vitamins etc produced by bacteria. Streptomycins and G-penicillin etc can be used - some species will not tolerate them but most will."

The respondent from NIES stated,

> "One of the challenges to maintain axenic strains is that not a few axenic strains grow poorly. This is likely due to the removal of benefit from coexisting bacteria, for example."[sic]

It is unclear how many of the microalgae in the culture collection are obligate symbionts, and if there has been a systematic attempt to achieve and maintain axenic cultures. The responses from the culture collection institutes suggest that there is currently insufficient interest in the researchers requesting the cultures to generate and maintain axeny; likely ascribed to common understanding that beneficial and stable symbiosis between microalgae and pychospheric bacteria is needed to achieve high biomass productivity. The lack of interest can be due to the heavy initial investment and time/labour required for axenisation, lack of knowledge of the media composition to facilitate cultivation of microalgae without the commensal bacteria, the lack of need for high purity cultures for the translational, or the more application-based research that researchers are generally interested.

4.4 Potential research gaps

Across the collected literature, many points and inconsistencies in the reportage were noted, that were of significant importance when trying to study and assess axenisation procedures.

4.4.1 Ambiguous glossary

Among the collected literature (including reviews), there is significant ambiguity in the use of terms that essentially allude to various aspects of an organism rather than its culture. Terms such as "culture", "species", "strain" and "clonal" have been used interchangeably across the literature although they mean very different things Belcher and Swale (1988). Even within the context of the collated research, the term species refers to the unique taxonomic identity of the organism being investigated. The term strain refers to a subpopulation of a species that have a property (or set of properties) of interest; for example, the strain IMET1 of *Nannochloropsis oceanica* is capable of higher lipid production although it is taxonomically identical to other populations of *N. oceanica* (Y. Ma et al., 2014). Clonal refers to the population that has been derived from a single microalgal cell through asexual reproduction; sharing identical genetic copies and expression. These three terms therefore refer to the investigated organism itself, but not the conditions in which it is grown. The term "culture" refers to the enclosed system in which the organism is grown. Belcher and Swale (1988) explains that, "A culture, as applied to algae, refers to the complex of culture medium, the organism(s) growing in the medium, and the vessel enclosing these." Unfortunately, referral to the culture itself remains inconsistent and synecdochical, with terms such as "unialgal", "single-strain", and "single-culture" used synonymously to imply axeny. These terms may refer to the fact that only one algal species is grown in the culture, but does not necessarily imply the absence of non-algal entities (e.g. bacteria). Thus, inconsistent usage of terms across the discipline contributes to the uncertainty and inaccuracy in the interpretation of the textual reports.

4.4.2 Lack of attention to the medium

The growth medium conditions are inherently different from the natural conditions from where the microalgae samples were collected and consequently, the growth dynamics of the microalgae and the contaminating microbes is expected to change. This is particularly true when employing nutrient-rich heterogenous medium such as Lysogeny Broth which provides a carbon source, and tends to favour bacterial growth over microalgae. Conversely, carbon-deficient medium such as the f medium relies on the ability of the microalgae to use photosynthesis to assimilate inorganic carbon and achieve dominance over heterotrophic bacteria. Corresponding increase in metabolic stress when using chemical methods such as hypochlorite or antibiotics during axenisation process, however, could significantly affect microalgal cell viability and decrease the chances of successful subculturing. This can be demonstrated using the example of *Euglena gracilis*, where, in all three reported cases, high antibiotic concentrations were used to achieve axenicity. However, the two studies that successfully established axenic cultures; namely by Gumińska et al. (2018) and Pappas and Hoffman (1952) employed Cramer-Myers's medium and Hall's medium respectively. Both these media recipes include a carbon source to allow microalgal cells to tap into their mixotrophic pathways. In contrast, Jones et al. (1973), who used artificial seawater, found that the microalgae became unviable before the bacteria were affected. Antibiotics caused chloroplast bleaching in Euglena, thereby impairing their ability to fix carbon. The supplementation of a carbon source, as provided by the media used in Gumińska et al. (2018) and Pappas and Hoffman (1952) may have mitigated the antibiotics' deleterious effects.

Even the manner in which a method is applied in tandem with the medium can affect outcomes. For example, if anoxy is achieved through flushing the medium with sterile CO_2 (as described by M. Ma et al. (2017)), it can begin to alter the pH of the culture medium. In such instances, consideration to the buffering capacity, for example through the use of TAP medium has a significantly higher buffering capacity owing to the presence of phophate buffer salts compared to BBM, and correspondingly, the effectiveness of the applied technique can vary significantly.

4.4.3 Lack of identification of co-growers

Across the 63 publications only three supplied sufficient information about the contaminating microbes. This is particularly relevant in cases where chemical methods such as phenol or antibiotics were used to achieve axeny as the effectiveness of the applied method is contingent on the relative susceptibility of the contaminating microbe to the treatment in comparison to the microalgae. In other words, if the contaminating / co-growing microbes are more resilient to the applied treatment in comparison to the microalgae, subsequent efforts to reproduce the outcome may fail even if the described procedure is diligently followed. This is particularly relevant in the case of diatoms as demonstrated by (Grossart, 1999) that even within the same culture different micro-communities of microalgae and bacteria can exist.

4.4.4 Lack of diversity in reportage

The diversity in the sources of the collected information is important to ensure robust representation of techniques used to achieve axeny, particularly when used across different laboratory settings. To wit, the 81 diatom species noted in Figure 2 was obtained from just 11 publications in which 11 methods were described to achieve axenic cultures. Likewise for dinoflagellates, the 36 species were noted in 15 publications and described 13 methods that were used to achieve axenic cultures, while for green algae, 31 species subjected to 19 different methods were described in 32 publications. Finally in the matter of microalgal divisions represented in the literature, cryptomonads, eugalenoids, red algae, etc. that were grouped as “other eukaryotes” in Figure 2d, were poorly represented in the collected literature and indicates a potential direction for further research work.

A more focused approach towards testing novel methods that is subsequently corroborated by other worker groups / laboratories would significantly increase confidence in the success rates noted for each method. Moreover, since each method (or its combination) has its intrinsic false positive rates, using the same workflow over multiple species, even within the same Division can result can affect subsequent efforts to reproduce the results.

More work is also required on systematic sampling, identification and purification of microalgae from natural sources. Although axenisation research has been carried out in many countries (Table 1), on-field sampling was described in only 19 publications (Figure 3). Type-sampling, archiving and perseveration of microalgae from natural water sources has become increasingly relevant as their local populations have become increasingly sparse and the chance of obtaining rarer species has become more precarious owing to the effects of climate change (Gimenez Papiol, 2017).

4.5 Limitations of this study

The work presented here attempted to systematically collect and curate relevant publications, and the extract information based upon which cogent axenisation workflows and strategies could be developed. However, there are aspects of this work that require careful interpretation and requires further research to furnish missing pieces of data.

Table 4: Limitations and caveats of the presented work

Limitations	Consequence	Mitigation
Narrow and stringent organism selection: Microalgae is an umbrella term that covers a vast number of organisms across multiple domains of life. The work presented here focused exclusively on phototrophic eukaryotic microalgae in order to provide a rational framework and context within which comparisons and analyses could be made to achieve axenic microalgae.	Many divisions such as cyanobacteria were excluded from the study even though they are an important member of the microalgae umbrella, and is of significant scientific and industrial relevance.	The workflow presented here, particularly the division-wise approach to study the workflows, is modular and can be extended to other organisms. Further work can build upon the existing code base (supplementary codes) and generate similar network analyses to identify robust workflows.
Network components do not account for success rates: The success rates of individual methods when applied individually or in specific pairs was not noted in all cases. The suggested clusters did not account for success rates.	Intrinsic success rates of each method involved in the axenisation workflow were not calculated and consequently, the success rates of known and potential hypothetical workflows cannot be calculated.	More high-quality experimental evidence of underreported methods is required to ascertain success rates of individual methods and their combinations to construct robust efficacy models of the workflow networks.

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Limitations	Consequence	Mitigation
Enquiry to culture banks was not systematic: The information obtained from culture banks was unstructured, subjective, and not systematic.	Comparisons between culture banks on important techniques, method workflows, and caveats when working with certain microalgae were missed. It remains unclear which microalgae have formed obligate symbiotic relations with bacteria and if it is possible to supplement the necessary growth factors to grow the microalgae in an artificial axenic culture.	National culture banks can be systematically identified and a questionnaire can be designed to identify workflows, methods, and important caveats when working with certain microalgae.

5 Conclusion

The available literature appears to focus heavily on workflows using the method set of Filtration, washing, and micropicking or Antibiotics, co culture and streak plating for diatoms, micropicking, subculturing, and flow cytometry for dinoflagellates, and lastly, anoxy, photosensitisation, and streak plating for green algae. More high quality reports with other methods and microalgal groups are required to estimate success rates and suggest alternate workflow strategies to achieve axenic eukaryotic microalgal cultures. A combination of at least three tests is recommended to verify claims of axeny that could include cell counting (using growth colonies on agar or flow cytometry), sequencing (16S and/or 18S), and microscopy (epifluorescence, light, confocal, etc.)

Supplementary materials

Supplementary Text 1:	Details on data collection and synthesis when assessing each publication.
Supplementary Text 2:	Details of the questions used to assess the evidence quality of each publication.
Supplementary awk code:	The script was used to further filter irrelevant titles recovered from database search. This is available in Supplementary Materials, Supplementary Text, Section 2.
Supplementary Table 1:	Table detailing the parameters, examples, and the rationale of each exclusion criteria. It is available in the document Supplementary materials, Section 3.
Supplementary Table 2:	Master table of all the data extracted from the publications upon using which network component analyses were performed. This is available as Supplementary_table2.csv.
Supplementary Table 3:	Species levels counts of positive or negative axenicity outcomes for each method described in literature.
Supplementary Table 4:	Number of species currently held in four major culture collections.
Supplementary Figure 1:	PRISMA-Sc flow schema of the search strategy and the recovered publications included herein.
Supplementary Figure 2:	Quality score distribution and outcome share of the six most commonly used methods used in experimental workflows. Data presented is at a species level of resolution.
Supplementary Figure 3:	Overview of species and axenization workflows for other eukaryotic microalgal groups noted from literature.
Supplementary Figure 4:	Quality score profile of publications across the timeline.
Supplementary codes:	This is available in file named Supplementary_codes.pdf, It contains the codes and methods used to process the data extracted from literature.

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Conflicts of interest

The authors have no conflicts of interest to declare.

References

Aray-Andrade, M. M., Uyaguari-Diaz, M. I., & Bermúdez, J. R. (2018). Short-term deleterious effects of standard isolation and cultivation methods on new tropical freshwater microalgae strains. *PeerJ*, *6*, e5143. https://doi.org/10.7717/peerj.5143

Belcher, H., & Swale, E. (1988). *Culturing algae: A guide for schools and colleges*. Institute of Terrestrial Ecology.

Brandt, J., & Albertsen, M. (2018). Investigation of Detection Limits and the Influence of DNA Extraction and Primer Choice on the Observed Microbial Communities in Drinking Water Samples Using 16S rRNA Gene Amplicon Sequencing. *Frontiers in Microbiology*, *9*, 2140. https://doi.org/10.3389/fmicb.2018.02140

Cappuccino, J. G., & Sherman, N. (2008). *Microbiology: A laboratory manual* (8th ed). Pearson/Benjamin Cummings.

Cavalier-Smith, T. (2007, November). Evolution and relationships of algae: Major branches of the tree of life. In J. Brodie & J. Lewis (Eds.), *Unravelling the algae* (0th ed., pp. 41–76). CRC Press. 10.1201/9780849379901-10.

Cavalier-Smith, T. (2016). Higher classification and phylogeny of Euglenozoa. *European Journal of Protistology*, *56*, 250–276. https://doi.org/10.1016/j.ejop.2016.09.003

Chamberlain, S. A., & Szöcs, E. (2013). Taxize: Taxonomic search and retrieval in R. *F1000Research*, *2*, 191. https://doi.org/10.12688/f1000research.2-191.v2

Clauset, A., Newman, M. E. J., & Moore, C. (2004). Finding community structure in very large networks. *Physical Review E*, *70*(6), 066111. https://doi.org/10.1103/PhysRevE.70.066111

Csárdi, G., Nepusz, T., Müller, K., Horvát, S., Traag, V., Zanini, F., & Noom, D. (2024, October). Igraph for R: R interface of the igraph library for graph theory and network analysis. https://doi.org/10.5281/ZENODO.7682609

Dixit, R. B., Raut, B., Manjre, S., Gawde, M., Gocher, C., Shukla, M. R., Khopkar, A., Prasad, V., Griffin, T. P., & Dasgupta, S. (2021). Secretomics: A biochemical footprinting tool for developing microalgal cultivation strategies. *World Journal of Microbiology and Biotechnology*, *37*(11), 182. https://doi.org/10.1007/s11274-021-03148-6

Eppstein, D., Löffler, M., & Strash, D. (2010, June). Listing All Maximal Cliques in Sparse Graphs in Near-optimal Time. https://doi.org/10.48550/arXiv.1006.5440

Fernandez-Valenzuela, S., Chávez-Ruvalcaba, F., Beltran-Rocha, J. C., San Claudio, P. M., & Reyna-Martínez, R. (2021). Isolation and Culturing Axenic Microalgae: Mini–Review. *The Open Microbiology Journal*, *15*(1), 111–119. https://doi.org/10.2174/1874285802115010111

Feuersänger, C. (2023). Pgfplots – A TeX Package to Create Normal and Logarithmic Plots in Two and Three Dimensions.

Foster, Z. S. L., Sharpton, T. J., & Grünwald, N. J. (2017). Metacoder: An R package for visualization and manipulation of community taxonomic diversity data (T. Poisot, Ed.). *PLOS Computational Biology*, *13*(2), e1005404. https://doi.org/10.1371/journal.pcbi.1005404

Fouilland, E., Galès, A., Beaugelin, I., Lanouguère, E., Pringault, O., & Leboulanger, C. (2018). Influence of bacteria on the response of microalgae to contaminant mixtures. *Chemosphere*, *211*, 449–455. https://doi.org/10.1016/j.chemosphere.2018.07.161

Francoeur, S. N., Espeland, E. M., & Wetzel, R. G. (2003). Short-term effects of nitrogen and extracellular protease amendment on algal productivity in nitrogen-deprived periphyton. *Journal of Freshwater Ecology*, *18*(1), 105–113. https://doi.org/10.1080/02705060.2003.9663956

Gimenez Papiol, G. (2017). Climate conditions, and changes, affect microalgae communities… should we worry? *Integrated Environmental Assessment and Management*, *14*(2), 181–184. https://doi.org/10.1002/ieam.2009

Grossart, H. (1999). Interactions between marine bacteria and axenic diatoms (*Cylindrotheca fusiformis*, *Nitzschia laevis*, and *Thalassiosira weissflogii*) incubated under various conditions in the lab. *Aquatic Microbial Ecology*, *19*, 1–11. https://doi.org/10.3354/ame019001

Gumińska, N., Płecha, M., Walkiewicz, H., Hałakuc, P., Zakryś, B., & Milanowski, R. (2018). Culture purification and DNA extraction procedures suitable for next-generation sequencing of euglenids. *Journal of Applied Phycology*, *30*(6), 3541–3549. https://doi.org/10.1007/s10811-018-1496-0

Han, J., Wang, S., Zhang, L., Yang, G., Zhao, L., & Pan, K. (2016). A method of batch-purifying microalgae with multiple antibiotics at extremely high concentrations. *Chinese Journal of Oceanology and Limnology*, *34*(1), 79–85. https://doi.org/10.1007/s00343-015-4288-2

Imai, I., & Yamaguchi, M. (1994). A simple technique for establishing axenic cultures of phytoflagellates. *Bulletin of Japanese Society of Microbial Ecology*, *9*(1), 15–17. https://doi.org/10.1264/microbes1986.9.15

Jadad, A. R., Moore, R., Carroll, D., Jenkinson, C., Reynolds, D. M., Gavaghan, D. J., & McQuay, H. J. (1996). Assessing the quality of reports of randomized clinical trials: Is blinding necessary? *Controlled Clinical Trials*, *17*(1), 1–12. https://doi.org/10.1016/0197-2456(95)00134-4

Jones, A., Rhodes, M. E., & Evans, S. C. (1973). The use of antibiotics to obtain axenic cultures of algae. *British Phycological Journal*, *8*(2), 185–196. https://doi.org/10.1080/00071617300650211

Kan, Y., & Pan, J. (2010). A one-shot solution to bacterial and fungal contamination in the green alga *chlamydomonas reinhardtii* culture by using an antibiotic cocktail [1]. *Journal of Phycology*, *46*(6), 1356–1358. https://doi.org/10.1111/j.1529-8817.2010.00904.x

Lamport, L. (1994). *LATEX: A document preparation system: User's guide and reference manual* (2nd ed). Addison-Wesley Pub. Co.

Ma, M., Yuan, D., He, Y., Park, M., Gong, Y., & Hu, Q. (2017). Effective control of Poterioochromonas malhamensis in pilot-scale culture of Chlorella sorokiniana GT-1 by maintaining CO2-mediated low culture pH. *Algal Research*, *26*, 436–444. https://doi.org/10.1016/j.algal.2017.06.023

Ma, Y., Wang, Z., Yu, C., Yin, Y., & Zhou, G. (2014). Evaluation of the potential of 9 Nannochloropsis strains for biodiesel production. *Bioresource Technology*, *167*, 503–509. https://doi.org/10.1016/j.biortech.2014.06.047

McCracken, I. (1969). Purifying algal cultures - a review of chemical methods. *Proceedings of the Nova Scotian Institute of Science*, *38*(1), 145–168.

Molina-Cárdenas, C. A., Sánchez-Saavedra, M. D. P., & Licea-Navarro, A. F. (2016). Decreasing of bacterial content in Isochrysis galbana cultures by using some antibiotics. *Revista de biología marina y oceanografía*, *51*(1), 101–112. https://doi.org/10.4067/S0718-19572016000100010

Pappas, G., & Hoffman, H. (1952). The Use of Antibiotics for Obtaining Bacteria- Free Cultures of Euglena. *The Ohio Journal of Science*, *52*(2), 102–105.

Pendersen, T. L. (2024). Tidygraph: A Tidy API for Graph Manipulation.

Pushpakumara, B. L. D. U., Tandon, K., Willis, A., & Verbruggen, H. (2023). Unravelling microalgal-bacterial interactions in aquatic ecosystems through 16S rRNA gene-based co-occurrence networks. *Scientific Reports*, *13*(1), 2743. https://doi.org/10.1038/s41598-023-27816-9

R Core Team. (2024). R: A Language and Environment for Statistical Computing.

Russell, C., Rodriguez, C., & Yaseen, M. (2022). Microalgae for lipid production: Cultivation, extraction & detection. *Algal Research*, *66*, 102765. https://doi.org/10.1016/j.algal.2022.102765

Sajjadi, B., Chen, W.-Y., Raman, A. A. A., & Ibrahim, S. (2018). Microalgae lipid and biomass for biofuel production: A comprehensive review on lipid enhancement strategies and their effects on fatty acid composition. *Renewable and Sustainable Energy Reviews*, *97*, 200–232. https://doi.org/10.1016/j.rser.2018.07.050

Sharma, N. K., & Rai, A. K. (2011). Biodiversity and biogeography of microalgae: Progress and pitfalls. *Environmental Reviews*, *19*(NA), 1–15. https://doi.org/10.1139/a10-020

Solomon, W., Mutum, L., Janda, T., & Molnár, Z. (2023). Potential benefit of microalgae and their interaction with bacteria to sustainable crop production. *Plant Growth Regulation*, *101*(1), 53–65. https://doi.org/10.1007/s10725-023-01019-8

Tanau, T. (2021). The TikZ and PGF Packages. https://doi.org/https://tikz.dev/

Thain, M., & Hickman, M. (2004). *The Penguin dictionary of biology* (11th ed). Penguin Books.

Udayan, A., Pandey, A. K., Sirohi, R., Sreekumar, N., Sang, B.-I., Sim, S. J., Kim, S. H., & Pandey, A. (2023). Production of microalgae with high lipid content and their potential as sources of nutraceuticals. *Phytochemistry Reviews*, *22*(4), 833–860. https://doi.org/10.1007/s11101-021-09784-y

Ullmann, J., & Grimm, D. (2021). Algae and their potential for a future bioeconomy, landless food production, and the socio-economic impact of an algae industry. *Organic Agriculture*, *11*(2), 261–267. https://doi.org/10.1007/s13165-020-00337-9

Vu, C. H. T., Lee, H.-G., Chang, Y. K., & Oh, H.-M. (2018). Axenic cultures for microalgal biotechnology: Establishment, assessment, maintenance, and applications. *Biotechnology Advances*, *36*(2), 380–396. https://doi.org/10.1016/j.biotechadv.2017.12.018

Wang, L., Yang, F., Chen, H., Fan, Z., Zhou, Y., Lu, J., & Zheng, Y. (2016). Antimicrobial cocktails to control bacterial and fungal contamination in *Chlamydomonas reinhardtii* cultures. *BioTechniques*, *60*(3), 145–149. https://doi.org/10.2144/000114392

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., … Yutani, H. (2019). Welcome to the Tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Winter, D. J. (2017). Rentrez: An R package for the NCBI eUtils API The R Journal. *The R Journal*, *9*(2), 520–526.

Youn, J.-Y., & Hur, S.-B. (2007). Antibiotics and their optimum concentration for axenic culture of marine microalgae. *ALGAE*, *22*(3), 229–234. https://doi.org/10.4490/ALGAE.2007.22.3.229

Yu, G., Smith, D. K., Zhu, H., Guan, Y., & Lam, T. T.-Y. (2017). GGTREE : An R package for visualization and annotation of phylogenetic trees with their covariates and other associated data (G. McInerny, Ed.). *Methods in Ecology and Evolution*, *8*(1), 28–36. https://doi.org/10.1111/2041-210X.12628