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„Approaches to generate axenic *Limnospira fusiformis* cultures and a comparison of methods to verify axenicity"

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Deutschsprachige Zusammenfassung für Nicht-Biologen

Cyanobakterien (auch Blaualgen genannt) sind photosynthesebetreibende Organismen ohne echten Zellkern. Ihre ökologische und evolutionäre Bedeutung ist immens. Die Sauerstoffproduktion durch Blaualgen ermöglichte vor über 2 Milliarden Jahren die Entstehung unserer heutigen Atmosphäre und somit auch die Eroberung von Landökosystemen. Cyanobakterien sind die Vorfahren der Chloroplasten, die in allen Pflanzen zu finden sind und in denen Photosynthese stattfindet. In der vorliegenden Untersuchung lag der Fokus auf Reinkulturen des fadenförmigen Cyanobakteriums *Limnospira fusiformis*, welches unter dem Handelsnamen „Spirulina“ allgemein bekannt ist, sei es als Superfood-Zutat in Smoothies oder schlicht als Lebensmittelfarbe für Gummibärchen. *Limnospira* gehört zu den kommerziell bedeutendsten Mikroalgen weltweit. Dies ist auch der Grund, weshalb *Limnospira fusiformis* im Fokus zahlreicher Forschungsprojekte liegt.

In der Phykologie unterscheidet man zwischen monoalgalen und axenischen Kulturen. Eine monoalgale Kultur beinhaltet einen einzigen Algenstamm. Kontaminationen durch andere Lebewesen, wie heterotrophe Bakterien oder Pilze, werden toleriert. Eine axenische Kultur hingegen hat größere Reinheitsansprüche. Eine axenische Kultur beschreibt eine Kultur, in der nur ein einziger Stamm und kein weiterer lebender Organismus nachweisbar ist. Die hierfür notwendige Aufreinigung der Kultur kann sehr zeitaufwändig und teuer werden. In manchen Forschungsbereichen, speziell in der Molekularbiologie und der Physiologie, ist es aber oftmals unerlässlich, mit axenischen Kulturen zu arbeiten. Bei der Identifizierung von Toxinproduzenten oder wertvoller bio-aktiver Substanzen ist es zumeist alternativlos. Ein fiktives Beispiel soll das Problem aufzeigen: Wir wollen herausfinden, ob unsere Mikroalge ein gefährliches Toxin erzeugt. Befinden sich in unserer Kultur neben dem Zielorganismus noch weitere 15 verschiedene Bakterien und Pilzarten, wird der eigentliche Toxin-Produzent nicht einwandfrei zu identifizieren sein. Bei axenischer Kultivierung der Mikroalge lässt sich hingegen eine eindeutige Aussage treffen. Ein weiterer Bereich ist die Erforschung von Mikroalgen-Bakterien Interaktionen. Ziehen wir nochmals unser fiktives Beispiel heran. Wir vermuten, dass unsere Mikroalge nur in Anwesenheit eines bestimmten Symbionten das Toxin produziert. In der ursprünglichen Kultur werden wir kaum Erkenntnisse gewinnen können, da hier zu viele verschiedene Spezies miteinander interagieren und bei unseren Untersuchungen letztlich interferieren. Haben wir

hingegen eine axenische Kultur unserer Mikroalge, können wir einzelne definierte Bakterienstämme zu der Kultur hinzufügen und die Interaktionen zwischen Alge und Bakterium ohne Interferenzen mit anderen Arten untersuchen.

Über die Jahre wurden zahlreiche Methoden entwickelt, um axenische Kulturen herzustellen. Generell kann man zwei Strategien unterscheiden: die Isolation durch physische Trennung und das Abtöten jeglicher Kontaminanten. Zu Ersterem zählt etwa das Ausstreichen auf Agarplatten und Isolieren von klonalen Kolonien, aber auch der Einsatz von Verdünnungsreihen oder Dichtegradient-Zentrifugation. Zu Zweiterem zählt der Einsatz von Antibiotika, das Bestrahlen der Kultur mit UV-Licht oder sogar der Einsatz von Hypochlorit-Lösungen. Zumeist reicht eine einzelne Methode nicht aus, um eine axenische Kultur zu generieren und eine Reihe an unterschiedlichen Behandlungen muss durchgeführt werden, um letztlich zum Erfolg zu gelangen.

Ein Fokus der vorliegenden Arbeit lag darin, axenische Kulturen des filamentösen Cyanobakteriums *Limnospira fusiformis* zu generieren. Der 2008 in Kenia isolierte Stamm Nakuru wurde hierfür herangezogen. Eine 2011 entwickelte und bewährte Strategie zur Aufreinigung von *Limnospira* wurde angewandt. Diese Strategie umfasst Waschschritte am Filter, Kultivierung in Nährmedium mit erhöhtem pH-Wert, eine Antibiotika Behandlung mit 4 verschiedenen Beta-Lactam-Antibiotika und eine abschließende Verdünnungsreihe. Diese etablierte Behandlungsreihe wird im weiteren Verlauf dieses Textes als „Standard treatment“ oder abgekürzt als StT bezeichnet. Der initiale Waschschritt dient dazu, die Kontaminanten auszudünnen. Die pH Behandlung ist hingegen sehr viel spezifischer. *Limnospira fusiformis* ist ein extremophiler Organismus und stammt aus Seen mit hohem Salzgehalt und hohem pH-Wert. Entsprechend überlebt *Limnospira fusiformis* temporär auch sehr hohe pH-Werte, die für viele andere aquatische Organismen bereits tödlich sind. Diesen Umstand macht man sich zunutze, indem man bei der Aufreinigung ein modifiziertes Nährmedium mit erhöhtem pH-Wert verwendet und die Alge für drei Tage darin kultiviert. Die Antibiotikabehandlung dient zum Abtöten diverser kontaminierender Bakterien. Man versucht hierbei mit Wirkstoffen zu arbeiten, die den Kontaminanten mehr schaden als den Cyanobakterien. Die Verdünnungsreihe dient letztlich dazu, die wenigen, nach der Behandlung verbleibenden Kontaminanten durch einen rein stochastischen Prozess zu entfernen. Zusätzlich zu StT wurden zwei modifizierte Varianten dieser Strategie getestet. In einer dieser Varianten wurde eine

Ultraschallbehandlung hinzugefügt. Zweck dieser Behandlung war es, auf den Filamenten sitzende Bakterien durch Ultraschallbeschuss abzulösen. Außerdem neigt der verwendete *Limnospira fusiformis* Stamm bei ungünstigen Umweltbedingungen zur Aggregation, also zur Bildung dichter Ansammlungen von Filamenten. Es entstehen quasi Knäuel aus Algen-Fäden. Aus der Biofilmforschung ist bekannt, dass ein Bakterium in solch einer Matrix bis zu tausendfach weniger sensitiv auf Antibiotika reagiert als ein Bakterium, das sich frei in einer Flüssigkeit befindet. Die Ultraschallbehandlung sollte die Knäuel auflösen und damit die Wirkung der Antibiotika erhöhen. Diese modifizierte Variante wird als StT+U (Standard Treatment + Ultrasonication) bezeichnet. Zusätzlich wurde eine weitere Adaption von StT getestet. Hierbei wurde der aus Ampicillin, Penicillin, Meropenem und Cefoxitin bestehende Antibiotikacocktail um Chloramphenicol erweitert. Im Gegensatz zu den vier erstgenannten Antibiotika ist Chloramphenicol kein Beta-Lactam-Antibiotikum. Chloramphenicol gilt als äußerst wirksamer Translationshemmer, wodurch die Proteinsynthese der Bakterien inhibiert wird. Diese Testreihe wurde als StT+C (Standard Treatment + Chloramphenicol) bezeichnet. StT+C unterscheidet sich allerdings nicht nur durch das Hinzufügen von Chloramphenicol von StT. Eine zweite Modifikation bei StT+C bestand aus der Zugabe einer kleinen Menge Glucose. Mehrere Studien deuten darauf hin, dass es sich bei den persistenten Zellen nach einer Antibiotika-Behandlung häufig um dormante Zellen handelt. Eine dormante (schlafende) Bakterienzelle befindet sich nicht im Wachstum, sondern in einer Art Stand-By-Modus. Ohne die Aufnahme von Nährstoffen und ohne Zellteilung haben sie oftmals bessere Chancen, eine Antibiotikabehandlung zu überleben. Durch die Zugabe von Glucose (ein sehr leicht aufnehmbarer Einfachzucker) können dormante Stadien reaktiviert werden. Gegenüber StT sollten die Modifikationen StT+U und StT+C eher zum Erfolg führen, da die modifizierten Strategien auch Problembereiche wie Aggregation oder dormante Zellen berücksichtigen.

Ein zweiter Fokus der Untersuchung lag auf dem Vergleich unterschiedlicher Methoden zur Verifizierung eines axenischen Zustands. Grob kann man hier vier unterschiedliche Herangehensweisen unterscheiden. (1) Eine visuelle Begutachtung der Kultur mittels Mikroskopie ist eine altbewährte Strategie. Die Techniken reichen hier von einfacher Lichtmikroskopie über Epifluoreszenzmikroskopie bis hin zum Elektronenmikroskop. Färbetechniken werden häufig genutzt, um Bakterien unter dem

Epifluoreszenzmikroskop besser sichtbar zu machen. Einige Methoden erlauben zusätzlich auch eine exakte Quantifizierung der Bakterien für den Fall, dass keine axenische Kultur vorliegt. (2) Eine weitere und sehr weit verbreitete Strategie ist der Einsatz von Testmedien. Agarplatten oder flüssige Medien werden hierbei mit kleinen Mengen der Kultur angeimpft. Bei den Plattentests wird im Anschluss beobachtet, ob sichtbare Bakterienkolonien heranwachsen. Bei den Tests mit Flüssigmedien wird beobachtet, ob es durch das Wachstum heterotropher Bakterien zu einer Trübung der Flüssigkeit kommt. (3) Ein moderner Ansatz ist die Anwendung von Durchflusszytometrie. Sämtliche Partikel in der Kultur (und somit auch Zellen potenzieller Kontaminanten) werden hierbei durch einen Lichtstrahl geschickt. Je nach Form und Größe des Partikels wird das Licht unterschiedlich abgelenkt und von Detektoren erfasst. Um eine lebende Zelle eines potenziellen Kontaminanten detektieren zu können, kommt zusätzlich noch DNA-Färbung zum Einsatz. Somit kann der Durchflusszytometer deutlich besser zwischen unbelebten Partikeln und Bakterien unterscheiden. (4) Zuletzt sind molekularbiologische Ansätze wie qPCR oder DNA-Sequenzierung zu nennen. Diese hochmodernen Methoden besitzen eine extrem hohe Empfindlichkeit. Bis auf Plattentests oder einfache lichtmikroskopische Überprüfungen sind die Methoden allesamt sehr aufwendig - sei es hinsichtlich Gerätschaften und auch Arbeitszeit.

Für die vorliegende Arbeit wurde aus allen vier Überprüfungsmöglichkeiten je eine Methode herangezogen. Vor Anwendung der Tests auf Kontamination wurden *Limnospira*-Filamente durch Filtration entfernt (3µm Porengröße). Für die visuelle Überprüfung mittels Epifluoreszenzmikroskopie wurde das 3µm-Filtrat nochmals über 0,2 µm Membranfilter abfiltriert (Bakterien werden am Filter zurückgehalten), die Filter mit DAPI (einem DNA-Färbemittel) behandelt und anschließend mikroskopiert. Der Vorteil dieser Methode ist neben der Überprüfung des axenischen Zustands auch die zusätzliche Quantifizierung etwaiger Kontaminanten. Als Plattentest wurde der weit verbreitete LB-Agar Plattentest herangezogen. Die Platten wurden wiederholt über einen Monat lang auf Koloniebildung von Bakterien gescreent. Ein Durchflusszytometer kam ebenfalls zum Einsatz. Hierfür wurden die 3µm-Filtrate mit SYBR Green 1 eingefärbt (Fluoreszenzfarbstoff zur DNA-Färbung) und automatisiert Partikel gezählt. Schließlich wurde auch DNA extrahiert und danach „16S rRNA-Gen Amplikon-Sequenzierung“ angewandt.

Je nach angewandter Überprüfungsmethode wurden durch die Behandlungen axenische Bedingungen festgestellt oder auch nicht, was die Problematik des Axenie-Nachweises deutlich macht. Unter Einbeziehung aller Testmethoden konnte letztlich keine einzige axenische Kultur nachgewiesen werden, jedoch wurden die Abundanz der Kontaminanten und die Anzahl unterschiedlicher kontaminierender Taxa durch die Behandlungsschritte signifikant gesenkt. Es soll an dieser Stelle auch erwähnt werden, dass der Zeitabstand zwischen Behandlung und Tests auf Axenie extreme Auswirkungen auf das Ergebnis haben kann, da das Wachstum einer exponentiellen Funktion folgt. Dies kann dazu führen, dass nach wenigen Tagen noch keine Kontamination angezeigt wird, da die Kontaminantenzahl unter dem gerätespezifischen Detektionslimit liegt, während nach zwei Wochen deutliche Kontamination nachgewiesen werden kann. Die behandelten Kulturen wurden zum Teil ein Monat gezogen bevor auf Axenie getestet wurde. Für Kontrollen auf axenische Kulturen stellt dies eine sehr lange Periode dar. Weitere Erklärungen, weshalb keine hundertprozentig axenische Kultur hergestellt werden konnte: (1) Im Gegensatz zu dem *Limnospira maxima* Stamm, für den die etablierte Methode 2011 entwickelt wurde, besitzt der für diese Studie verwendete *Limnospira fusiformis* Stamm eine stark spiralige Form und neigt unter bestimmten Umständen zur Aggregation, was wiederum die Antibiotikawirkung abschwächt. (2) Die Stabilität von Beta-Lactam-Antibiotika ist stark temperatur- und pH-abhängig. Eventuell hatten die Antibiotika-Lösungen bereits einen Teil ihrer Effizienz verloren, da diese nicht für jedes Experiment neu zubereitet wurden. (3) Die 2011 entwickelte Methode empfahl eine Verdünnungsreihe bis zu einer Verdünnung von 1:10.000. In der vorliegenden Studie wurde 1:500 als stärkste Verdünnung benutzt.

Bezüglich des Minimierens von Kontaminanten durch StT, StT+U und StT+C konnte eine annähernd gleiche Effizienz festgestellt werden. Überraschenderweise waren es letztlich die StT behandelten Kulturen, die die geringste Anzahl an Kontaminanten aufwiesen. Eine potenzielle Erklärung dafür wäre das mögliche Vorhandensein höherer Nährstoffkonzentrationen in StT+U und StT+C behandelten Kulturen. Durch den Ultraschallbeschuss bei StT+U wurden zusätzlich *Limnospira fusiformis* Zellen aufgebrochen und der ausgetretene Zellinhalt wirkte wachstumsfördernd auf heterotrophe Bakterien. Bei StT+C wurde Glucose hinzugefügt, die ebenfalls einen idealen Nährboden für zahlreiche Taxa darstellt.

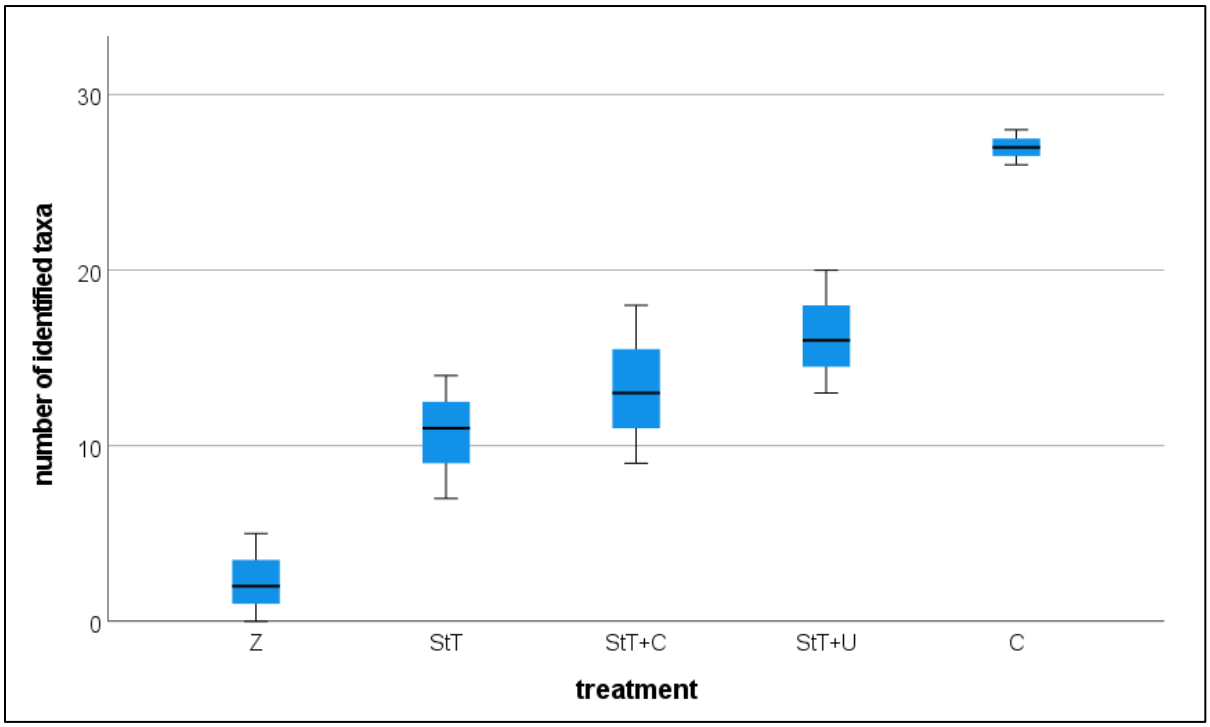

Abbildung 1: In den unbehandelten Kulturen (C) wurden mittels 16S rRNA-Gen Amplikon-Sequenzierung zwischen 25 und 30 Taxa detektiert. Bei den 1:500 verdünnten, behandelten Kulturen (StT, StT+C und StT+U) lag die Zahl im Bereich zwischen 10 und 20. Selbst in dem sterilen Nährmedium (Z) konnten DNA-Sequenzen einiger weniger Taxa detektiert werden. Die Anzahl an reads war allerdings so extrem gering (zwischen 2 und 14), dass es trotzdem als praktisch steril angesehen werden kann.

Der zweite Teil der Arbeit beschäftigte sich mit Methoden zum Nachweis axenischer Kulturen. Hier konnte eine interessante Beobachtung gemacht werden: LB-Agar Plattentests erwiesen sich als nicht zuverlässig. Während alle anderen Methoden Kontaminanten detektierten, zeigten die Plattentests nicht den geringsten Hinweis auf Kontamination durch heterotrophe Bakterien. Nicht nur die behandelten Kulturen, selbst die unbehandelten Kulturen wären als axenisch einzustufen, würde man den Plattentests Glauben schenken. Das Wissen, dass zahlreiche Mikroorganismen nicht einfach kultivierbar sind und auf Platten kein Wachstum zeigen, ist nicht neu. Die Anwendung verschiedener Platten-Medien macht die Methode effizienter, da die Wahrscheinlichkeit erhöht wird, dass potenzielle Kontaminanten geeignete Bedingungen für das Wachstum vorfinden. Trotzdem kann es sein, dass einige Taxa durch solche Tests nicht detektierbar sind. Interessanterweise ist der LB-Agar Plattentest nach wie vor sehr weit verbreitet. Algenkultursammlungen und renommierte Arbeitsgruppen nutzen ihn, um auf axenische Bedingungen zu testen.

Sowohl Epifluoreszenzmikroskopie als auch Durchflusszytometrie erwiesen sich als effiziente Methoden zur Verifizierung axenischer Kulturen und der Quantifizierung der

Kontaminanten. Die Zellzählung am Durchflusszytometer geht allerdings deutlich schneller und objektiver als das Auszählen unter dem Mikroskop. Nachteil dieser Methoden ist, dass sie nichts über die Vitalität der Bakterien sagen: es werden Partikel gezählt. Die 16S rRNA-Gen Amplikon-Sequenzierung zeigte sich ebenfalls als geeignetes Werkzeug zur Verifizierung nicht-axenischer Bedingungen und bietet gleichzeitig die Möglichkeit, Kontaminanten zu identifizieren. Auch hier gilt, dass DNA in der Kultur amplifiziert und schließlich sequenziert wird, die auch von Bruchstücken oder toten Organismen stammen könnte.

Letztlich stellt sich die Frage, ob die Arbeit mit einer axenischen Kultur für die jeweilige Studie überhaupt notwendig oder wünschenswert ist. In vielen Fällen stellen Bakterien wichtige Lebenspartner der Alge dar, ohne die die Alge deutlich schlechter wachsen würde. Oft sind saubere, vitale Kulturen mit nur einer geringen Anzahl an Kontaminanten vorzuziehen. Ist Axenie essentiell für die Forschungsfrage, so muss im Anschluss auch ein vertrauenswürdiger Test auf Axenie angewandt werden. Die aus der vorliegenden Studie resultierende Empfehlung ist die Kombination aus Durchflusszytometrie und 16S rRNA-Gen Amplikon-Sequenzierung. Die Reinheit der Kultur wird damit einer doppelten Kontrolle unterzogen. Sollte sich die Kultur als nicht axenisch herausstellen, erhält man zusätzlich die Abundanz und die Identität der Kontaminanten, wodurch man zusätzliche spezifisch wirkende Antibiotika anwenden könnte. Als weiterer Schritt wäre noch eine Methode zur Untersuchung der Vitalität der Kontaminanten zu empfehlen.

Schließlich ist der Begriff „axenisch“ zu hinterfragen, denn es gibt unterschiedlichste Definitionen dafür. Eine etwas realitätsferne Definition beschreibt eine axenische Kultur als eine Kultur, in der sich nur ein einziger lebender Stamm eines Lebewesens und kein weiterer lebender Organismus oder Virus befinden darf. Eine leichte Abwandlung dieser Definition, die häufiger zu finden ist, lässt Viren außer Acht. Eine pragmatische Definition beschreibt eine axenische Mikroalgen-Kultur als eine Kultur, in der sich nur ein einziger Algen Stamm befindet und keinerlei Kontaminanten detektierbar sind. Der Autor der vorliegenden Studie richtet sich nach dieser Definition.

Ob Axenie nachgewiesen wird, ist in hohem Maße auch von der Verifizierungsmethode und ihrer Sensibilität abhängig. Alle Methoden haben ihre Grenzen. Vom Fehlen eines Nachweises bakteriellen Wachstums kann keinesfalls geschlossen werden, dass

keine Bakterien vorhanden sind. Selbst hochsensible molekularbiologische Methoden stoßen an Limits. Zum Abschluss soll Pinevich et al. (2018) zitiert werden: „An absolutely pure culture belongs to the sphere of abstract theory: in practice, ‘pure culture’ refers to a certain probability level” (p. 1517).

Pinevich, A. V., Andronov, E. E., Pershina, E. V., Pinevich, A. A., & Dmitrieva, H. Y. (2018). Testing culture purity in prokaryotes: criteria and challenges. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *111*(9), 1509–1521. https://doi.org/10.1007/s10482-018-1054-4

Abstract

Axenicity is defined as the state of a culture with only a single strain present, free of any other organism. Obtaining axenic cultures can be tough and many strategies have been developed over the years. We searched for methods to generate axenic cultures of *Limnospira fusiformis*, which is commercially exploited and which shows a constantly increasing market value. We applied filtration-washing, increased pH, β-lactam antibiotics and dilution series, which are established treatments. Furthermore, we tested modified versions of this approach by adding an ultrasonication step and by expending the antibiotics cocktail. In addition, we assessed techniques to verify axenicity and highlighted their advantages and disadvantages. Flow cytometry, 16S rRNA gene amplicon sequencing, tests on LB-agar-plates and epifluorescence microscopy were compared. Even if we could not achieve an axenic culture, our treatments were able to significantly reduce the abundance and taxa richness of contaminants. We found the classic LB-agar-plate test as inappropriate to verify axenicity in *L. fusiformis*. Plate tests did not indicate any contamination, contrarily to the other methods applied. We found a linear relationship between total cell counts of contaminants achieved by flow cytometry and epifluorescence microscopy, with flow cytometry counts being consistently higher. 16S rRNA gene amplicon sequencing demonstrated its superiority by not only being an efficient tool for axenicity-verification, but also for identification of persistent contaminants.

Keywords: Antibiotics · *Arthrospira* · Axenic cultures· Flow cytometry · Purification · *Spirulina* · Ultrasonication treatment

Introduction

Unialgal cultures contain a single algal species and usually also heterotrophic bacteria. In general, algae collections maintain unialgal cultures (Brand et al., 2013). For various research questions, the use of unialgal cultures is sufficient or even advantageous over axenic ones. Some microalgal strains are not even viable as axenic cultures, probably to the lack of obligate symbionts (Barsanti & Gualtieri, 2005). Nevertheless, for some research fields, especially in molecular biology, biochemistry and physiology, axenicity is urgently needed (Doppler et al., 2021). An axenic culture can be defined as a culture containing no other living organism except the strain of interest (Brand et al., 2013). A more pragmatic approach defines an axenic (or pure) algal culture as a culture without any detectable contaminants (Guillard, 2005). Extremely strict definitions include not only living beings but even viruses (Vu et al., 2018). Guillard (2005) stated that “the goal of purification methods is to obtain a viable culture of a single species, free of all other species (“contaminants”) whether eukaryotes, prokaryotes, or viruses” (p. 117). The term “axenic” was first introduced by Baker et al. (1942). They declared that “*Axenic* is derived from two Greek words: *A*- meaning *without* or *free from*, and *Xenos*- denoting a *stranger* or *foreign life*. An axenic organism, as here defined, is a species free from any life apart from that produced by its own protoplasm” (Baker et al., 1942, p. 116).

In the past, various protocols for algae purification have been published, which can be assigned into two general approaches: (1) separating the strain of interest and its contaminants physically and (2) killing all living organisms except the species of interest (Guillard, 2005). In most cases, applying just one purification method is insufficient to achieve axenicity, because different contaminants have to be tackled with different approaches. Therefore, treatments need to be combined to generate an axenic culture. The most common method is based on cell dispersion on agar-plates by either streaking or spraying via atomizer (Vu et al., 2018). This isolation technique can be improved by repeated transfers of young colonies to obtain axenic cultures (Stanier et al., 1971). Other established methods belonging to the approach of physical separation include micro-manipulation (Bowyer & Skerman, 1968), filtration (Lee et al., 2013), selective gas vesicle collapse (Hicks et al., 2021), cell washing by micro-pipetting (Lee et al., 2013), serial dilution (Guillard, 2005), separation by density gradient centrifugation (Whitelam et al., 1983) and ultrasonication (Guillard, 2005).

Vortexing can be used as a supporting technique to shake off contaminants attached to the surface of algal cells (Hicks et al., 2021). Vaara et al. (1979) took advantage of the gliding motility of certain strains for generating axenic cyanobacteria cultures. One of the most advanced methods is fluorescence-activated cell sorting (FACS; Doppler et al., 2021; Sensen et al., 1993). Unfortunately, the application of FACS is only possible for single-celled organisms with simple shapes. For filamentous cyanobacteria like *Limnospira*, *Phormidium, Anabaena* or *Nostoc* other purification methods need to be used.

Techniques belonging to the killing-approach include lysozyme treatments (Kim et al., 2002), antibiotic treatments (Choi et al., 2008; Rippka, 1988; Sena et al., 2011; Vázquez-Martínez et al., 2004), short-term UV irradiation (Lee et al., 2013) and thermal treatments (Wieringa, 1968). Vaz et al. (2014) published an inexpensive and fast method for generating axenic *Nostoc* cultures without the use of antibiotics. Instead, the bleach sodium hypochlorite is applied as a disinfectant to eliminate all contaminants. As a result, only the extremely resistant and tough akinetes (resting spores) are able to survive (Vaz et al., 2014). Such methods of surface disinfection are commonly used for seeds sterilisation of higher plants.

Limnospira fusiformis (Nowicka-Krawczyk et al., 2019) is a filamentous cyanobacterium and placed in the order Oscillatoriales. Its diverse morphology includes densely coiled filaments, wavy formed trichomes and even completely straight forms (Zhi & Zhao, 2005). The taxonomy is quite complex; the species was formerly treated under the name *Spirulina platensis*, then transferred to *Arthrospira fusiformis*, and only recently the new genus *Limnospira* was established (Nowicka-Krawczyk et al., 2019). *Limnospira* is used as dietary supplement and food source since the 16th century (Ciferri, 1983). *Limnospira* not only provides carotenoids, minerals, vitamins, essential fatty acids, but also high concentrations of readily bioavailable proteins (Belay, 2007). Beside the profitable sale as health food for humans, *Limnospira* is utilized as food additive especially in aquaculture (Vonshak, 2002). It is cultivated worldwide at large scale and commercially sold under the trade name “Spirulina” (Sili et al., 2012). It needs to be pointed out here that the genus *Spirulina* and the genus *Limnospira* are not closely related to each other and – beside the coiled filament morphology – do not share other characteristics (Komárek et al., 2014). One of the causes for this curiosity can be traced back to the famous phycologist Lothar Geitler,

who refused to accept *Arthrospira* as a distinct genus (Geitler, 1925). While *Chlorella* is still the most important commercial microalgae regarding annual trading volume in US dollar, the produced biomass of *Limnospira* per year is expected to be more than double the amount of *Chlorella* (Sili et al., 2012). *L. fusiformis* is thriving in East African soda lakes, which offer extreme environmental conditions such as, high pH, high salinity and high turbidity (Schagerl, 2016). Nevertheless, they are extremely productive ecosystems with *L. fusiformis* as its main primary producer (Melack & Kilham, 1974; Oduor & Schagerl, 2007).

In general, purity of cultures maintaining extremophile algal species is easier achieved compared to cultures of non-extremophiles (Barclay & Apt, 2013), because potential contaminants often do not survive the extreme conditions. The strategy to obtain axenic cultures of *Limnospira maxima* ("*Arthrospira maxima*") developed by Sena et al. (2011) took advantage of this fact by including a quite challenging pH-treatment. Their recommended series of treatments involved a washing step, a pH 12 treatment, the use of four different antibiotics and a dilution series. The approaches of the current study follow this strategy for the most part.

L. fusiformis sometimes shows aggregations during unsuitable conditions, which might be related to excretion of polysaccharides, which is a well-known problem for generating axenic cultures of filamentous cyanobacteria (Vázquez-Martínez et al., 2004). These carbohydrate-rich substances are an optimal substrate for heterotrophic bacteria (Piontek et al., 2011). In addition, cyanobacteria often feature complex and very sticky cell envelopes (Vázquez-Martínez et al., 2004).

The region directly surrounding algal cells is called phycosphere – it can be compared to the rhizosphere of higher plants and is characterized by an intense exchange of metabolites (Seymour et al., 2017). In this busy microenvironment, several biological interactions between algae and other organisms take place such as mutualism, antagonism, parasitism, commensalism and competition. The tight association between host and embedded organisms is also true for *L. fusiformis*, which complicates treatments against contaminants. Besides physical treatments, also chemical ones are aggravated, especially if extended polysaccharide excretion occurs. Bacteria embedded in this so-called extracellular polymeric substance (EPS) matrix are less sensitive to antimicrobial agents such as antibiotics (Ceri et al., 1999). We

therefore introduced an additional ultrasonication step between the pH and the antibiotic treatment to remove contaminants. Ultrasonication to get rid of contaminants from cell walls has already been applied successfully in diatoms (Shishlyannikov et al., 2011). An ultrasonication treatment before using antibiotics was also recommended by Vázquez-Martínez et al. (2004) for generating axenic strains of the filamentous cyanobacterium *Phormidium*. Hicks et al. (2021) suggested the combination of chemical and physical treatments to obtain axenic *L. fusiformis* cultures. Additionally to their newly established method of selective gas vesicle collapse, vortexing was used in their study to remove contaminants attached to the filaments (Hicks et al., 2021).

We additionally tested a modified version of the standard treatment by adding a fifth antibiotic and glucose to the proven antibiotic cocktail. In addition to ampicillin, penicillin, cefoxitin and meropenem the antibiotic chloramphenicol and glucose were added. Bacteria surviving antibiotic treatments are usually cells in a stationary phase (Balaban et al., 2004). This non-growing (dormant) state cannot be removed by antibiotic treatments which rely on active growth (Levin-Reisman et al., 2017). Adding glucose as a readily bioavailable carbohydrate boosts dormant bacteria towards the active state, which increases the efficiency of the antibiotic treatment. The addition of glucose to stimulate bacterial growth and antibiotic susceptibility while treating *Limnospira* cultures was already applied successfully by Choi et al. (2008). Chloramphenicol as a potent inhibitor of protein synthesis (Weisberger, 1967) was chosen to extend the spectrum of antibiotics, otherwise maintaining only β-lactam antibiotics. The original cocktail of β-lactam antibiotics was chosen by Sena et al. (2011) based on their predominant efficacy against gram-positive bacteria, whereas cyanobacteria (belonging to the gram-negative bacteria) are less effected (Davis, 2018). β-lactam antibiotics inhibit the synthesis of the peptidoglycan layer of bacterial cell walls by acting on enzymes called penicillin-binding proteins (PBPs; Davis, 2018). Therefore β-lactam antibiotics are only efficient against bacteria in active growth (Davis, 2018). β-lactam antibiotics belong to the class of bactericidal agents: by binding irreversibly to the PBPs, the bacterial cell wall is weakened or even ruptured and the cell is highly susceptible to lysis (Lima et al., 2020). To bind to the PBPs, the active agent has to penetrate the bacterial cell wall by diffusion (Davis, 2018).

In addition to the development of approaches to obtain axenic cultures of *L. fusiformis*, this study assessed methods to verify axenicity. Axenicity tests can be grouped into four categories. (1) The oldest but still most popular technique are contamination tests on solid media (agar-plates) and in liquid media (Vu et al., 2018). Enriched media like LB, R2A and potato dextrose agar are still widely used (Atlas, 2010). Basal algal growth media, enriched with organic substances, can be used as well (Guillard, 2005). In liquid media, grey colouring is considered as evidence for contamination growth, while plate tests mainly focus on the screening of colony forming units (Vu et al., 2018). (2) Microscopic examination is another widely used test method. This direct visual approach includes different methods of light microscopy (Vu et al., 2018) including epifluorescence techniques with staining agents such as 4′,6-diamidino-2-phenylindole (DAPI) staining (Zafiriou & Farrington, 1980). Even scaning electron microscopy (SEM) is used to check for axenicity as shown by Vu et al. (2018) for *Ettlia* sp. cultures. (3) A highly advanced technique to verify axenicity and quantify potential contaminants is flow cytometry (FCM; Hyka et al., 2013). (4) Sequence-based methods like qPCR or high-throughput sequencing are currently the most sensitive methods for axenicity confirmation (Heck et al., 2016).

We tested four approaches for their applicability and informative value: (1) LB-agar-plate tests, (2) epifluorescence microscopy using DAPI staining, (3) FCM using SYBR Green 1 staining and (4) 16S rRNA gene amplicon sequencing. Advantages and disadvantages of the respective method are identified and discussed. In addition, we included a survey, where we contacted algae culture collections around the world and asked for their method to check axenicity of cultures.

Methods

Survey

We asked 58 algae culture collections around the world if and how they verify axenicity of cultures (i.e., which specific methods they use to test for axenic conditions). Interviewees were contacted via email in November 2020.

Strain and long-term cultivation conditions

We used the unialgal, clonal *Limnospira fusiformis* strain ASW 01 100 (Algensammlung Wien, algae culture collection of the University of Vienna) with maintenance culture conditions provided 25°C and a day:night cycle of 12:12 (warm-white fluorescence tubes, 25 µmol photons $*m^{-2}*s^{-1}$) in Zarrouk cultivation medium (Zarrouk, 1966).

Treatments

For the approaches, a dense preculture was raised in Zarrouk medium. Cultivation was conducted in bottles on an orbital shaker (85rpm) in a greenhouse for two weeks at 26°C and a light:dark cycle = 16h:8h at an intensity of 50 $\mu mol*m^{-2}*s^{-1}$. Triplicate controls were taken from the preculture before any treatments were conducted. If not stated otherwise, cultivation followed preculture conditions.

The experiment started with a washing step on filters. Algal biomass was put on membrane filters (Isopore hydrophilic polycarbonate, 2µm pore size) and rinsed with sterile bicarbonate-free Zarrouk medium. The filaments were then transferred into bicarbonate-free Zarrouk medium and split into 9 flasks (Fig. 1). Three slightly different approaches were accomplished, each of them was performed in triplicates (Fig. 2).

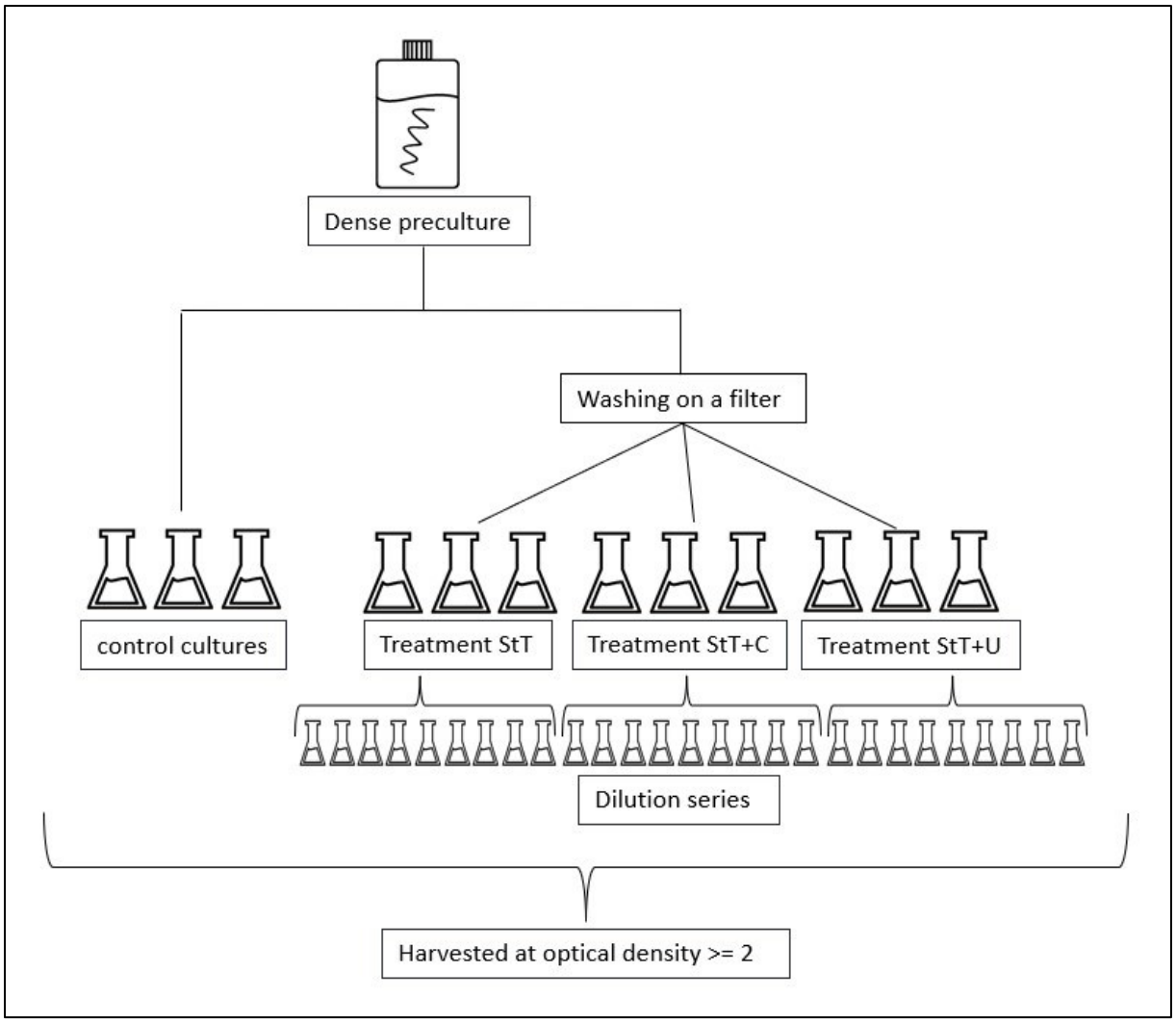

Figure 1: Schematic drawing of the experimental setup

The standard treatment (=StT) followed the recommendations of Sena et al. (2011)(Fig. 2). First, the rinsed cultures were cultivated at pH 12 for 72h. The pH was raised by slowly adding 1M NaOH. After 72h, the modified medium was replaced with Zarrouk medium with three centrifugation cycles (each with 2880 rcf for 10min at 20°C). After each centrifugation cycle including medium replacement, cultures were briefly vortexed until pellets were dipersed. Subsequently, the cultures were treated with antibiotics for 48h in the dark. The cocktail contained as follows: ampicillin (61.6 µg*ml^{-1}), penicillin (85.8 µg*ml$^{-1)}$, cefoxitin (76.9 µg*ml^{-1}), meropenem (38.9 µg*ml^{-1}). The antibiotic-enriched medium was then again replaced by Zarrouk medium as explained previously, followed by serial dilution (undiluted, 1:50, 1:500).

Treatment StT+U was performed exactly as treatment StT, but with adding ultrasonication between pH- and antibiotic treatment (Fig. 2). Each sample was treated

with an ultrasonicator (Sonifier 250, Branson) for 12 cycles of 10 s each at the lowest intensity. Between each cycle samples were cooled down for 30 s (bottom half of the Greiner tube immersed in cold water, ~10°C). After ultrasonication, an additional medium replacement was performed as explained previously. Treatment StT+C was performed exactly as treatment StT, but with adding chloramphenicol (6.8 µg*ml^{-1}) and glucose (100 µg*ml^{-1})(Fig. 2).

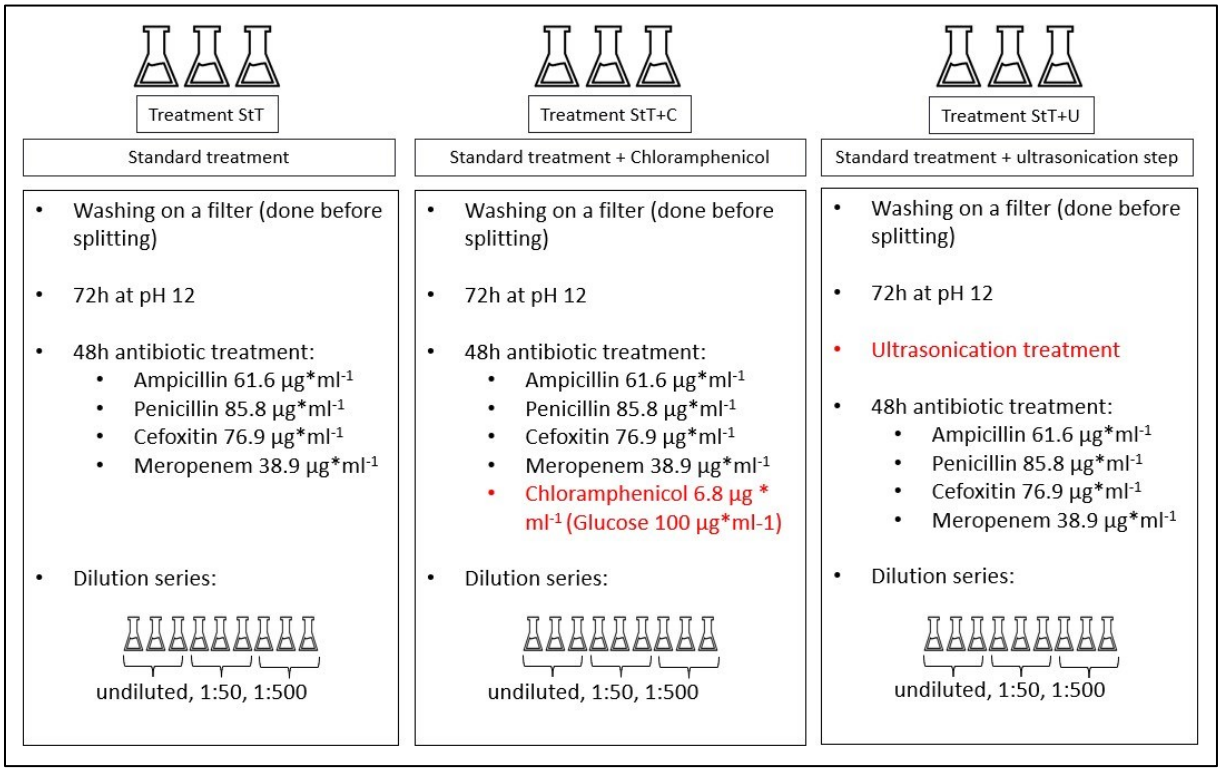

Figure 2: A comparison of the experimental settings of StT, StT+C and StT+U. Differences to StT are marked in red.

Harvest, sample fixation and storage

Controls and treated cultures were harvested at optical density >= 2 (measured at 750nm; U-2000 photometer, Hitachi; quartz cuvette: 10mm light path) and *in-vivo*-chlorophyll a fluorescence reached at least 2.5 (measured at EX 410nm and EM 670nm; RF-5301 PC Spectrofluorophotometer, Shimadzu; quartz cuvette: 10mm light path). This procedure ensured a comparison of treatments with similar initial density. As a result, harvesting of different approaches was performed at different times with controls harvested first, followed by treatments. Harvested cultures were microscopically checked for monoalgal purity and the remaining part was filtrated (3µm pore size) in order to remove filaments. A subsample of the filtrate was immediately used for the plate test. Another part was fixed with formaldehyde (final concentration 2%) and stored at 4°C for microscopy. Another subsample of the filtrate was fixed with glutardialdehyde (final concentration 0.5%) for 10 min at room temperature, shock frosted in liquid nitrogen for 30 min and stored for FCM at -80°C. The remaining part of the filtrate was kept for prospective DNA isolation and 16S rRNA gene amplicon sequencing: 2ml of the respective sample were centrifuged for 30 min at 21000 rcf at 4°C, the supernatant carefully removed, the microcentrifuge tube again filled with 2ml sample, followed by a second and third step (same conditions as before). The visible cell pellet obtained from a total of 6ml filtrate was frozen in liquid nitrogen for 30 min and stored at -80°C until DNA extraction and sequencing.

LB-agar-plate tests

Screening for heterotrophic contaminants was tested by inoculation of 100µl filtrate on LB-agar-plates (NaCl 1%, tryptone 1%, yeast extract 0.5%, agar 1.5%). Plate tests were done in triplicates. Inoculated agar-plates were kept in the dark at room temperature for one month and were observed in regular intervals.

FCM analysis

Prokaryotic contamination was quantified using a Luminex Amnis CellStream Flow Cytometer equipped with a 488nm blue light laser. Samples were thawed and diluted 1+99 with sterile Milli-Q water (except for blanks and pure Zarrouk medium where dilution was not appropriate). Subsequently, they were stained with SYBR Green I nucleic acid stain (Invitrogen) at a volume ratio of 1:10000. Samples were incubated

in the dark in a heat block at 37°C for 13 min. Detector channel settings used were: side scatter at 100%, forward scatter at 100%, trigger channel laser (488nm) at 100%. Speed was adjusted to “fast” (14.64 µL*min^{-1}). Every particle count was recorded. Cell counts were conducted in technical triplicates and from each measurement four records were taken. A gated area was counted using a plot of the two parameters 488-611/31-C5 versus 488-528/46-C3. Samples featuring an event rate higher than 800 events*sec^{-1} were diluted 1+199 with sterile MQ, stained, incubated and measured again. Calculations were performed with Amnis CellStream Acquisition and Analysis software.

Cell count by epifluorescence microscopy

DAPI stained cells were counted on a filter using an epifluorescence microscope. Formaldehyde-fixed filtrate was processed within 24 h after harvest. First, 1ml of the sample was diluted with 10ml sterile Milli-Q water to ensure a homogenous distribution during subsequent gentle vacuum filtration on a Whatman Cyclopore track etched membrane filter (0.2µm pore size). A cellulose nitrate filter (0.8µm pore size) served as support filter; a hand pump was used to keep vacuum at a maximum of -20kpa. The membrane filters were then air dried for 15 min and stored at -20°C until further investigation. For bacterial counts, filters were thawed, mounted on a slide and the ready-to-use VECTASHIELD mounting medium with DAPI H-1200 was applied for staining (approximately 25µl per slide). After putting a cover slip onto the filter, random squares were counted with a minimum of 100 cells per filter by means of a Zeiss AXIO Imager M1 Epifluorescence microscope (Zeiss EC Plan-Neofluar 100x/1,3 oil objective; at least 10 squares per filter). Cell number was then extrapolated to the total filter area.

16S rRNA gene amplicon sequencing

DNA extraction, 16S rRNA gene amplicon sequencing and bioinformatical analysis of the sequencing data were conducted by the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna (JMF). For DNA extraction, the DNeasy PowerSoil Pro Kit from QIAGEN was used. The JMF Team used Illumina MiSeq-based highly multiplexed 16S rRNA gene amplicon sequencing as described in the methodical publication by Pjevac et al. (2021). Target gene (first-step) PCR was used to amplify the V4 regions of the 16S rRNA genes. The following 16S rRNA gene-

targeted oligonucleotide primers were used: FWD: 515F Parada; REV: 806R Apprill; FWD: GTGYCAGCMGCCGCGGTAA; REV: GGACTACNVGGGTWTCTAAT (Apprill et al., 2015; Parada et al., 2016). Barcoding (second-step) PCR including the unique dual barcoding approach (UDB-H12) was applied: two barcoding primers each consisting of a distinct 12 nt barcode and one of the two using 16 nt head sequences (5'-BC12_1-H1- 3' and 5'-BC12_2-H2-3') were used for amplification. Following this, sequencing library preparation, sequencing on an Illumina MiSeq, sequence processing and sequence analysis were performed. All of these standardized workflows were performed according to protocols included in Pjevac et al. (2021). They are implemented as standard operating procedures (SOPs) for amplicon sequence generation and analysis at the JMF (Pjevac et al., 2021). The total abundance of each taxon in a sample was calculated by multiplying the total cell count measured by FCM and the fraction of detected reads of each amplicon sequence variant (ASV).

Statistics

Statistical analyses were performed with IBM SPSS Statistics (version 28.0.0.0) to compare the efficiencies of StT, StT+C and StT+U. Only data from 1:500 diluted, treated cultures (considered as our "end products") and controls were taken into account for statistics. An ANOVA and a Bonferroni post hoc test were performed for FCM data (cells*ml^{-1}) to investigate if differences in total cell counts are significant (total n=12; control n=3, StT n=3, StT+C n=3, StT+U n=3). An ANOVA and a Bonferroni post hoc test were performed for taxa richness data (numbers of detected ASVs) to investigate if differences in contaminants taxa richness are significant (total n=12; control n=3, StT n=3, StT+C n=3, StT+U n=3).

Results

From 58 algae culture collections 21 responded to our inquiry. Six collections do not maintain axenic strains, two collections did not answer to the specific question but explained methods for the removal of contaminants instead. The remaining 13 answers are shown in table 1.

Table 1: Results of the survey. The table shows how many algae culture collections are using which approach to test for axenicity. Only applicable answers from culture collection who maintain axenic cultures are included.

Used test methods	Single test medium	Multiple test media	Single test medium + microscopy	Multiple test media + microscopy	FCM + Multiple test media + microscopy
Number of collections	5	2	2	3	1

According to the LB-agar-plate tests, all *L. fusiformis* cultures including the controls were axenic even after one month of incubation. FCM data, microscopical cell counts and sequencing however indicated contamination in all cultures including the controls, irrespective of treatment and any dilutions.

A high and significant linear relationship (R^2=0.916, n=28) between different counting techniques was found (Fig. 3). To avoid redundancy, further analyses were based on FCM results.

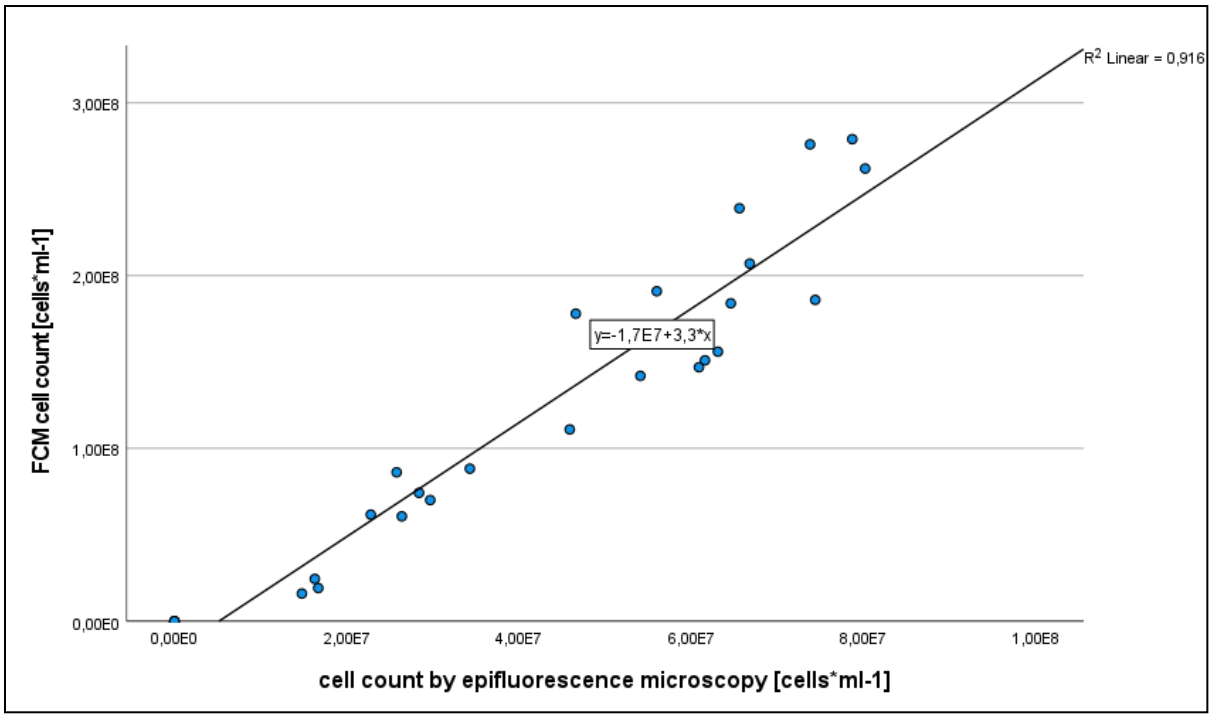

Figure 3: Linear regression of cell count data determined by epifluorescence microscopy and flow cytometry

All treated cultures contained lower numbers of contaminants than the control (Fig. 4). In all treatment types, 1:500 diluted cultures were the least contaminated ones. It should be mentioned here that controls and treated cultures including dilution series were harvested at a comparable optical density, which means different harvest times. A statistical comparison including controls and 1:500 dilutions revealed that StT and StT+C contained significantly less contaminants than controls (ANOVA: $p = 0.013$; Bonferroni: StT to control, $p = 0.019$; StT+C to control $p = 0.050$). StT+U featured no significant differences regarding total cell counts, neither to controls nor StT or StT+C (Bonferroni: StT+U to control $p = 0.056$; StT+U to StT $p = 1.000$; StT+U to StT+C $p = 1.000$).

Sequence data revealed the same pattern. We compared the total count of identified ASVs in each group (1:500 dil. StT, 1:500 dil. StT+U, 1:500 dil. StT+C, control, pure Zarrouk medium; Fig. 5) and found a significantly lower taxa richness in the 1:500 dilutions of StT, StT+U and StT+C than in the control (ANOVA with $p = 0.002$; Bonferroni: control to StT $p = 0.002$; control to StT+C $p = 0.007$; control to StT+U $p = 0.029$). None of the treatments turned out as significantly more advantageous compared to the others (Bonferroni: StT to StT+C $p = 1.000$; StT to StT+U $p = 1.000$; StT+C to StT+U $p = 1.000$).

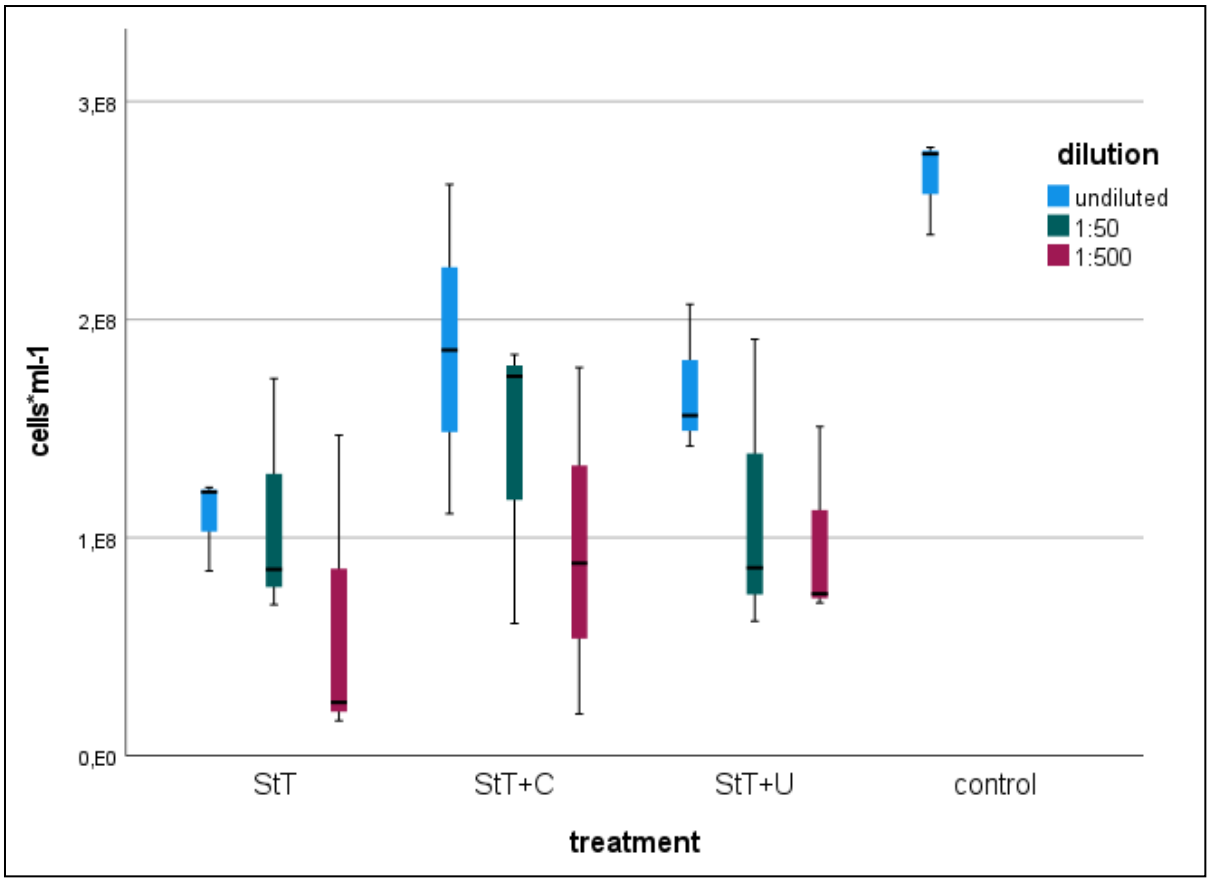

Figure 4: Box-plot presenting FCM cell counts of treated and untreated (control) cultures. Treated groups include cell counts of undiluted, 1:50 diluted and 1:500 diluted cultures. StT = Standard Treatment; StT+C = Standard Treatment + Chloramphenicol; StT+U = Standard Treatment + Ultrasonication. n for each group = 3.

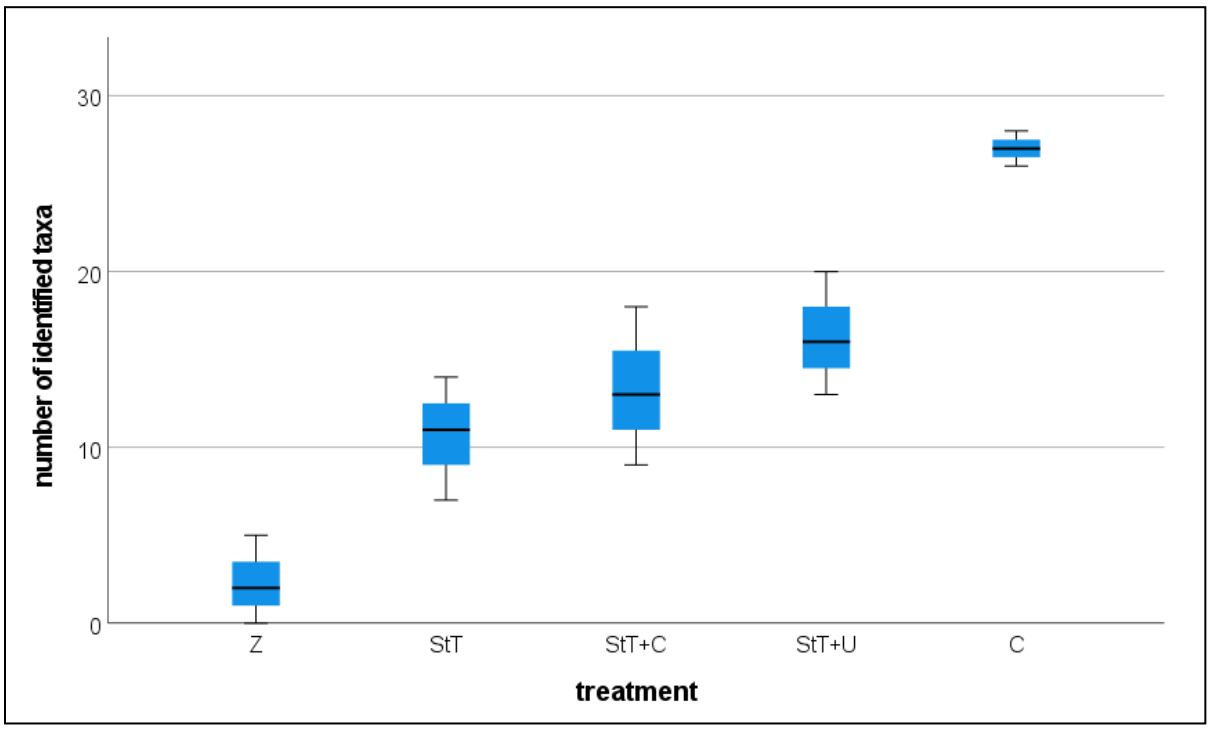

Figure 5: Box-plot presenting taxa richness in pure Zarrouk medium (Z), untreated cultures (C) and 1:500 dilutions of treated cultures: Standard Treatment (StT), Standard Treatment + Chloramphenicol (StT+C), Standard Treatment + Ultrasonication (StT+U). n for each group = 3.

Contaminants were identified by 16S rRNA gene amplicon sequencing. Most identified contaminants belong to gram-negative taxa. Two taxa belonging to the phylum of Actinobacteria constitute an exception, but their total read count is extremely low. ASV_hz1_v13 is dominant in controls and all treated cultures (including all dilutions, Fig. 6). This ASV belongs to the family of Cyclobacteriaceae. Absolute abundances of identified taxa were calculated by multiplying total cell counts by FCM and fractions of reads of each ASV per sample (Fig. 7). The heatmap emphasizes the dominance of ASV_hz1_v13, followed by a taxon belonging to the family Rhizobiaceae and a member of the genus *Lentimonas.*

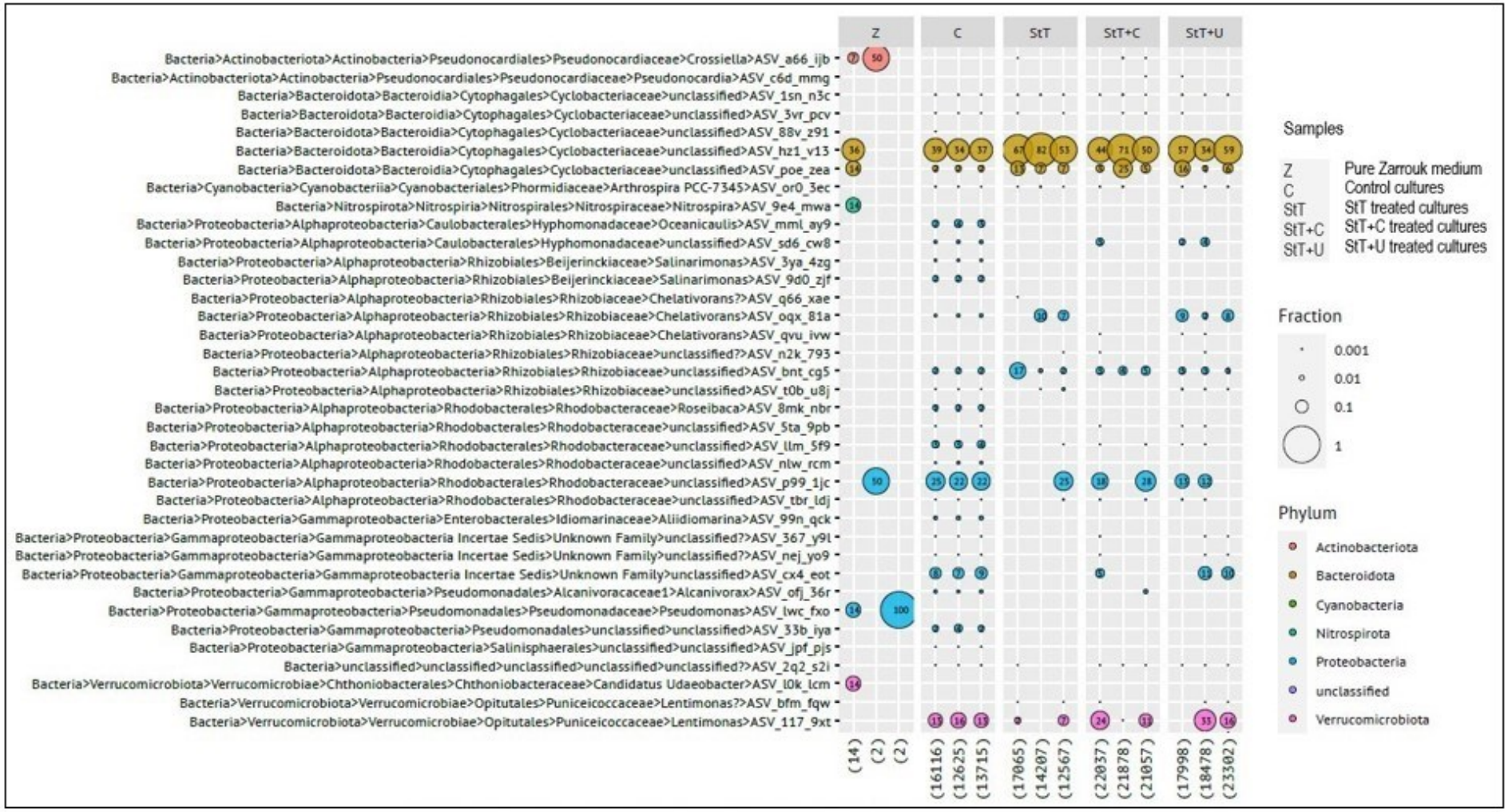

Figure 6: Numbers in bubbles are percentages when more abundant than 1%. Numbers in parentheses are total read counts (depth) per library (i.e., per sample). The diameter of a bubble corresponds with the fraction of reads per library. Fractions shown for higher taxonomic ranks are exclusive of the fractions for separately shown lower taxonomic ranks. The colour within a bubble indicates the phylum an ASV belongs to. Solid bubbles are single ASVs. Faded bubbles are the sum of multiple ASVs at different taxonomic ranks.

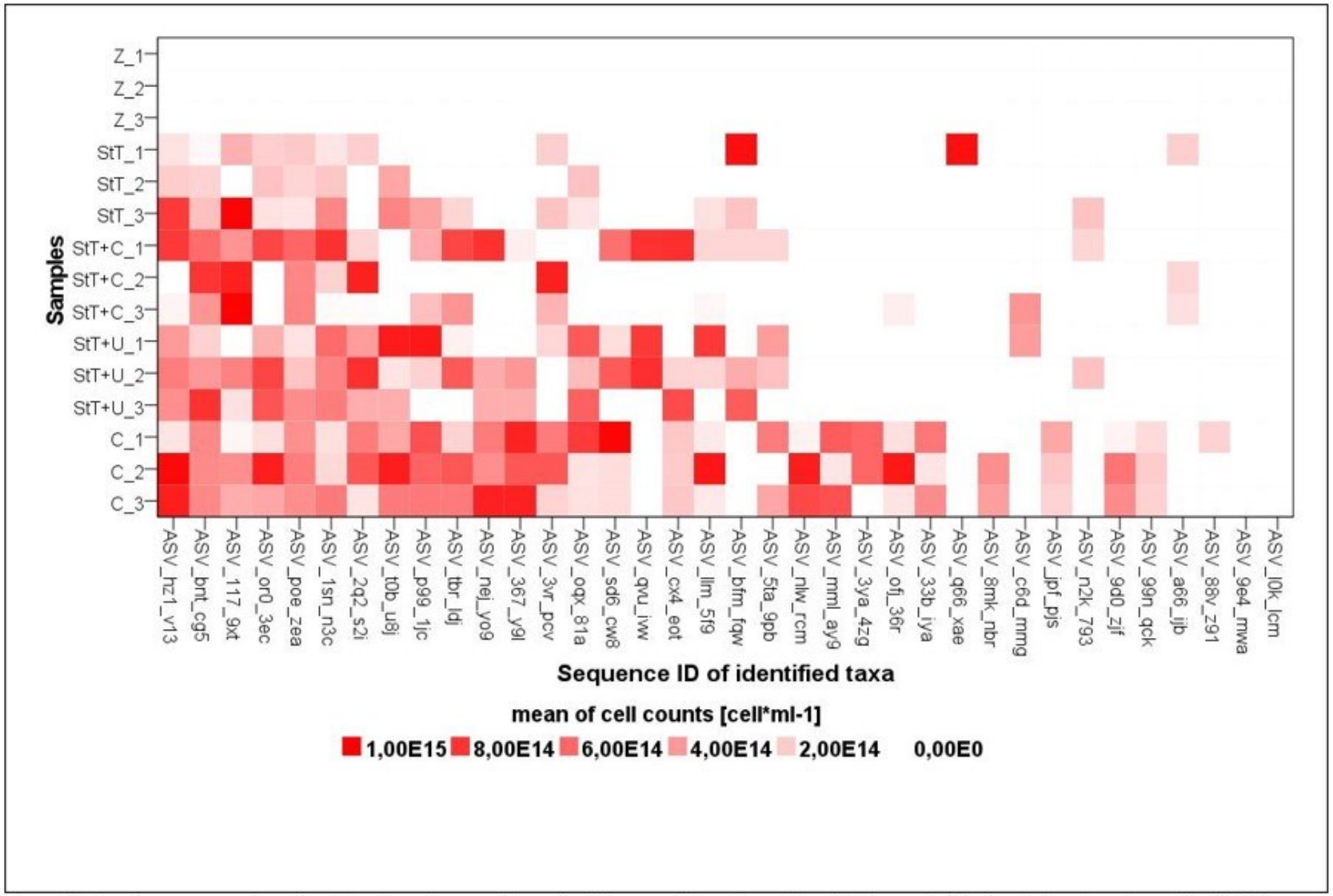

Figure 7: Heatmap presenting absolute abundances of taxa in Z (= pure Zarrouk medium), C (= control/untreated cultures) and in 1:500 dilutions of treated cultures: Standard Treatment (StT), Standard Treatment + Chloramphenicol (StT+C), Standard Treatment + Ultrasonication (StT+U). ASVs are organized from overall most abundant (left) to overall least abundant (right).

Discussion

5 out of 13 culture collections which maintain axenic cultures are testing axenicity on a single test medium. According to our study the use of just the single test medium LB-agar resulted in a wrong conclusion of the purity-state of our culture. Only a single algae culture collection stated that FCM is used for monitoring axenicity. Another culture collection announced that they do not maintain axenic cultures but that they use FCM regularly to check the abundance of contaminants. High-throughput sequencing or qPCR are generally still not used as purity-tests (or at least it was not mentioned).

Our tests for axenicity revealed somehow contradictory results. LB-agar-plate tests indicated axenic conditions. FCM data, microscopical examinations or 16S rRNA gene amplicon sequencing still showed contamination after treatments, although with a significantly lower taxa richness compared to the control. Furthermore, StT and StT+C treated cultures contained significantly fewer contaminating cells. The method comparison revealed that at least for *L. fusiformis* cultures, the classic LB-agar-plate test is an inappropriate method to verify axenicity. This result is not surprising as most microbes are hard to cultivate. This phenomenon was already described 40 years back as ‘great plate anomaly’ (Staley, 1985). However, axenicity verification using only LB-agar-plate tests is still common in many algae culture collections and is even present in recent publications (Doppler et al. 2021).

We compared automated FCM with contaminants stained with SYBR Green 1 and manual epifluorescence counts with bacteria stained with DAPI. In accordance to other studies (Duhamel & Jacquet, 2006; Felip et al., 2007), we found a very high correlation of the counts, although FCM consistently reported higher cell numbers. Vu et al. (2018) stated that DAPI and SYBR Green 1 unselectively penetrate and stain the DNA of both living and dead cells. With this in mind, both counting methods used in this study bear the risk of overestimation. The precision of FCM counts is assumed to be 10 times higher compared to counts by epifluorescence microscopy and sample processing time is reduced by even more than tenfold (Del Giorgio et al., 1996). Taking this into account, FCM is the recommended technique for quantification of contaminants, because the result is statistically much more reliable. Problems may occur when bacteria form filaments or aggregates. Nevertheless, bacterial quantification and

biomass estimation by FCM is even used for samples from activated sludge, but with physical or chemical disaggregation before counting (Brown et al., 2019). Even the valid point of criticism that SYBR Green 1 is staining both living and dead cells could be addressed by using live/dead staining (Vu et al., 2018). 16S rRNA gene amplicon sequencing demonstrated its superiority as an efficient tool for axenicity-verification and for identification of persistent contaminants. A strategy combining FCM and 16S rRNA gene amplicon sequencing is a solid way to double-verify axenicity. Simultaneously to the axenicity-check, quantification and identification of contaminants will be obtained for the case that axenicity was not achieved. In our study a member of the family Cyclobacteriaceae could be identified as most abundant contaminant. Based on this information future approaches might include modified treatments to tackle this dominant contaminant in our culture. The limitation of 16S rRNA amplicon gene sequencing for microbial community analyses is the inability to distinguish if DNA originated from a living or a dead cell (Li et al., 2017), what might result in false-positive findings in axenicity tests. Li et al. (2017) recommended the use of RNA-based sequencing instead of DNA-based methods for a reliable detection of living cells. The sensitivity of molecular biological approaches is extremely high and the slightest contamination in post-harvest processes may result into false-positive findings. We even detected ASVs in sterile medium, read counts were however low between 2 and 14 (Fig. 6).

Another issue which must not be neglected for axenicity verification is the duration between treatment and start point of tests. Sena et al. (2011) monitored cultures microscopically before and immediately after each treatment, which bears the risk of simply overlooking contaminants. Numbers might be below the detection limit of the chosen method of axenicity verification. The risk for this bias is especially high if dilution series are included and tests are conducted before reaching a high algal density. Duration of the tests is another issue. Choi et al. (2008) published an approach to achieve axenic *Limnospira* cultures including washing steps and two different antibiotic treatments, the first one with imipenem and cycloheximide and the second including neomycin and cycloheximide. After each purification step subsamples were stained with DAPI and observed microscopically. After the last treatment the established presumptive axenic cultures were grown in SOT medium with 0.1% glucose for 1 day at 30°C. Axenicity was tested by inoculation in different test media (1/10 NA, LB and

R2A), on both solid agar-plates and in liquid media. Test media were examined by naked eye and microscopically. Cultures were assessed to be axenic after only two more days without evidence of contamination in any of the test media (Choi et al., 2008). For visible colony formation of slowly growing bacteria, this period is certainly too short and might result in wrong conclusions. Contradictory to the duration (two days) specified in the method chapter, Choi et al. (2008) claimed in the discussion that "Purity verification of axenic *A. platensis* was confirmed for 1 week by test agar plate" (p. 92). Guillard (2005) recommended that "tubes and plates should be examined frequently beginning ca. 48 hours after inoculation and left for at least 21 days, or 1 month for cold-water organisms or methylaminotrophs" (p. 129). Sena et al. (2011) evaluated the efficiency of different antibiotic treatments by inoculation on AO agar-plates (glucose 1%, peptone 0.5%, yeast extract 0.3%) but did not specify test duration.

For StT, a slightly lower taxa richness and a lower total cell count was observed than for StT+C and StT+U. Addition of glucose in StT+C supported not only activation of dormant contaminants, but obviously boosted growth of heterotrophic bacteria as well. Likewise, ultrasonication applied in StT+U increased dissolved organic matter by detaching and dissolving EPS from the cell surface of *L. fusiformis* and by breaking some of its cells. Medium replacement applied directly after the treatment should have minimized this effect, but a fraction of damaged/weakened cells might have leaked with some delay. Hicks et al. (2021) demonstrated vortexing as an approach to detach bacteria from the surface of *L. fusiformis* before performing the initial washing step and before starting their newly established method of selective gas vesicle collapse. Short-term vortexing was also applied in our experiment (in StT, StT+U and StT+C). Longer and more intense vortexing between the pH and the antibiotic treatment should be considered as a slightly less aggressive alternative to ultrasonication. The application of vortexing even before performing the initial washing step on the filter as described by Hicks et al. (2021) might be an improvement to our purifying approach as well.

The fact that no axenic culture could be obtained with the strategy of Sena et al. (2011) could be a result of the affinity of the strain to form aggregates during unfavourable conditions. A bacterium embedded in such an aggregate or in a biofilm is significantly less sensitive to antibiotic treatments compared to the same species living as plankton (Ceri et al., 1999). Another reason might be use of β-lactam antibiotic stocks after long storage. Half-life periods of ampicillin and many other β-lactam antibiotics significantly

decrease at inadequate storage temperature and pH (Hwang et al., 1986). Even in StT, minor modifications to the strategy established by Sena et al. (2011) were already implemented. (1) Rinsing on the filter was accomplished by rinsing with sterile bicarbonate-free Zarrouk medium instead of sterile carbonate and bicarbonate buffer. (2) The modified medium was completely replaced after the pH treatment and not just adjusted to pH 9 by pouring carbonate and bicarbonate buffer. (3) Vortexing was applied and (4) medium replacement after the antibiotic treatment was slightly different. Moreover, our highest dilution was 1:500. More importantly, Sena et al. (2011) investigated a different species (*Limnospira maxima*). Therefore, also long-term cultivation conditions and applied medium differ. In the approach of Hicks et al. (2021) to establish axenic *L. fusiformis* cultures the pH treatment and the antibiotic treatment were not performed as two separate working steps, but merged as a one-step chemical treatment. Antibiotics were added to the medium and pH was adjusted to 12.15. Physically treated cultures were inoculated into this medium and incubated in the dark for 4 days at 150 rpm and 30°C (Hicks et al., 2021). By merging the two chemical treatments into one working step handling is noticeable reduced. It needs to be considered that additional handling always increases the risk of secondary contamination.

Before much time and money is invested in generating axenic cultures, the need of axenicity needs to be scrutinized (Vu et al., 2018). In most cases, monoalgal cultures will fit perfectly to the research question. In some cases, axenic cultures are not even viable, probably due to killing obligate symbionts (Barsanti & Gualtieri, 2005). Gao et al. (2020) reported for *Microcystis aeruginosa* a significantly slower growth at axenic conditions compared to cultures with associated heterotrophic bacteria. In order to prove that the promoting effect originates from extracellular substances of the heterotrophic strain B905–1, a cell-free filtrate of strain B905–1 was added to an axenic *Microcystis aeruginosa* culture resulting in increased growth (Gao et al., 2020). Another issue that needs to be considered is that each application of antibiotics in the establishment of axenic cultures for scientific or economic usage increases the risk of generating and spreading antibiotic-resistant microbes (Vu et al., 2018). However, there are certain fields of research where axenicity is essential “to avoid any immeasurable effects influencing the experiment’s outcome” (Doppler et al., 2021, p. 1). Working with axenic cultures will result in less noise and a better sequence

coverage in the field of full-length genome assembly, investigations of microalgae-bacteria interactions without interference by non-target bacteria and an easier identification of the producer of a toxin or a valuable bioactive component (Vu et al., 2018).

Conclusions

If axenicity is needed, reliable assessment tools are obligate. It is mandatory to specify the verification methods used to test for axenicity. We highly recommend the combination of FCM and 16S rRNA gene amplicon sequencing for axenicity tests. As these methods might be too time-consuming and too expensive for monitoring purposes of algae culture collections, they can be combined with other tests such as, multiple agar-plate tests and microscopy. Routine screening can be performed by the easy-to-go methods and at regular intervals, these tests are complemented by FCM and 16S rRNA gene amplicon sequencing.

The question arises, if it is possible to prove real axenicity. The absence of bacterial growth cannot ensure axenicity, because many bacteria do not respond to standard enrichments (Barsanti & Gualtieri, 2005). There exists no general method of choice, all methods have their limits. Even deep sequencing could fail to detect a contaminant (Pinevich et al., 2018). Barsanti & Gualtieri (2005) noticed that, “there is no way of demonstrating that a microalgal culture is completely axenic” (p. 212) and therefore axenicity describes a culture condition without demonstrable prokaryotic and eukaryotic contaminants. Pinevich et al. (2018) stated that “an absolutely pure culture belongs to the sphere of abstract theory: in practice, ‘pure culture’ refers to a certain probability level” (p. 1517). If no contaminants could be detected, you can only conclude that “according to your test methods, your culture is axenic” (Campbell, 2005, p. 2).

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Zusammenfassung

Eine axenische Kultur beschreibt eine Kultur, in der nur ein einziger Stamm und keine weiteren Organismen nachweisbar sind. Die notwendige Aufreinigung hierfür ist äußerst zeit- und arbeitsaufwändig. Über die Jahre wurden zu diesem Thema zahlreiche Strategien veröffentlicht. Das filamentöse Cyanobakterium *Limnospira* ist eine wirtschaftlich äußerst bedeutende Mikroalge und daher im Fokus aktueller Forschung. Wir untersuchten Methoden, um eine axenische Kultur eines *Limnospira fusiformis* Stammes aus dem Nakuru See in Kenia zu generieren. Eine bereits etablierte Strategie, bestehend aus einer Serie unterschiedlicher Behandlungen, wurde hierfür angewandt. Die Strategie umfasst Waschschritte (am Filter), die Kultivierung in Medium mit erhöhtem pH-Wert, β-Lactam-Antibiotikabehandlungen und eine abschließende Verdünnungsreihe. Zusätzlich wurden modifizierte Varianten dieser Strategie getestet. In einer der modifizierten Testreihen wurde eine Ultraschall-Behandlung hinzugefügt, in einer weiteren wurde das Antibiotika-Spektrum erweitert. Der zweite Fokus dieser Arbeit lag darin, vier unterschiedliche Methoden zur Verifizierung axenischer Bedingungen zu testen und im Anschluss zu vergleichen. Hierfür wurden Durchflusszytometrie, Epifluoreszenzmikroskopie, LB-Agar Plattentests und 16S rRNA-Gen Amplikon-Sequenzierung angewandt. Eine axenische Kultur konnte letztlich nicht generiert werden, jedoch konnten die Behandlungen die Abundanz der Kontaminanten und die Anzahl unterschiedlicher kontaminierender Taxa signifikant senken. Wir zeigten, dass der klassische LB-Agar Plattentest eine ungeeignete Methode der Verifizierung axenischer Bedingungen darstellt, da auch Plattentests deutlich kontaminierter Kulturen keinerlei koloniebildende Einheiten aufwiesen. Zellzählungen durch Durchflusszytometrie und Epifluoreszenzmikroskopie zeigten ein lineares Verhältnis, wobei die Durchflusszytometrie stets höhere Zählungen generierte. Die 16S rRNA-Gen Amplikon-Sequenzierung zeigte sich als effizientes Werkzeug zur Verifizierung axenischer Bedingungen und gleichzeitig als Möglichkeit, überlebende Kontaminanten zu identifizieren.