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Source of Vitamin B_{12} in plants of the Lemnaceae family and its production by duckweed-associated bacteria

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ABSTRACT

While most plants are not known to contain significant levels of the essential corrinoid cobalamin (vitamin B_{12}), considerable levels of vitamin B_{12} were found in the duckweed clone *Wolffia globosa* Mankai. Although endophytes were speculated to be the source of this vitamin B_{12}, it has not been tested yet. In this study, we carried out comparative analysis of vitamin B_{12} content in 15 duckweed accessions across 11 species. Significant but variable levels of vitamin B_{12} were found in all unsterile duckweed cultures tested. In contrast, disinfected duckweed cultures with lower levels of bacteria, as monitored by PCR-based methods, contained little vitamin B_{12}. Importantly, two strains of duckweed-associated bacteria were shown to produce vitamin B_{12}. This work provides evidence for bacteria as the source of vitamin B_{12} in duckweeds. Bioinformatics-aided identification of vitamin B_{12} producers among duckweed-associated bacteria could facilitate their systematic incorporation into duckweed-based foods to support a planetary health diet.

1. Introduction

Corrinoids are a group of compounds consisting of a corrin ring with a highly complex structure that contains a central cobalt ion (Roth et al., 1996). The main physiologically important corrinoid for human health is vitamin B_{12} (cobalamin), which is essential for various biological processes including DNA synthesis, red blood cell formation, neurological function, and amino acid metabolism. Due to its role in these key functions, a deficiency of this vitamin causes significant health risks (Table 1). The planetary health diet, as proposed by the EAT-Lancet Commission, is a groundbreaking concept that intertwines human health with the health of the planet (Tulloch et al., 2023). It represents a sustainable and nutritious dietary framework aimed at addressing both the rising global population and the environmental challenges associated with climate change and food production. It is hypothesized that plant-based diets generally require fewer natural resources. A systematic review of observational and intervention studies was conducted by Neufingerl and Eilander (2021) from 2000 to 2020 concerning the nutrient intake in adults who are on plant-based diets as compared to that of meat consumers. It revealed that the mean vitamin B_{12} intake was highest in meat consumers (5.6 μg/day) compared to vegetarians (2.1 μg/day) and vegans (1.5 μg/day).

Vegans often consume macroalgae and fermented vegetables to improve their vitamin B_{12} status, even though these foodstuffs are not potent sources of vitamin B_{12}. Yet, considerable quantities of vitamin B_{12} were found in the macroalga *Porphyra* (Miyamoto et al., 2009) and microalga *Chlorella* (Kumudha et al., 2015). Eukaryotic algae cannot synthesize vitamin B_{12} and must instead obtain it from certain vitamin B_{12}-producing prokaryotes. For example, Croft et al. (2005) showed that vitamin B_{12} in algae originates from symbiotic or contaminating

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Table 1
Risk factors involved in vitamin B_{12} deficiency.

Risk Factor	Specificity/Causality	Vitamin B_{12} Supply Status
Planetary health diet	Shift towards more plant-based food	Plants contain no or only traces of this vitamin
Vegan diet	No intake of animal foods	Algae, fermented vegetables, and mushrooms are ineffective sources of cobalamin, contain mostly Pseudo-B_{12}
Aging population	Deterioration of vitamin B_{12} absorption with increasing age	Deficiency in older people causes e.g., neurodegenerative diseases and pernicious anemia
Diseases of the gastrointestinal tract	Crohn's and celiac diseases, gastric or small intestinal resection restrict vitamin B_{12} absorption, increased risk of gastritis with increasing age	Diseases and age-related changes of the gastrointestinal tract increase the risk of vitamin B_{12} deficiency

bacteria. It should be noted that the estimated digestion rate of vitamin B_{12} from these dried macroalgae under *in vitro* conditions at pH 2 (as a model for stomach digestion) was about 50 %.

The main problem of non-animal vitamin B_{12} sources is that they can contain a relatively large proportion of pseudo vitamin B_{12} (Pseudo-B_{12}) that is inactive in humans. In food analysis, the method commonly used for measuring vitamin B_{12} is the microbiological assay. However, since the microbiological assay can measure both vitamin B_{12} and Pseudo-B_{12}, there is a risk of overestimating the vitamin B_{12} content. A gold standard to diagnose B_{12}-status is the determination of vitamin B_{12} in the blood samples of plant consumers using competitive immunoassays. To accurately characterize the vitamin B_{12} status in people consuming plant-based diets, the determination of laboratory markers such as holo-transcobalamin, total homocysteine, and methylmalonic acid in the serum is also required (Harrington, 2017). Another approach to measure the "true" vitamin B_{12} content is the more laborious determination of true vitamin B_{12} using LC/electrospray ionization-tandem mass spectrometry (Watanabe and Bito, 2018).

Several reviews listed non-animal foods that contain corrinoids (Watanabe et al., 2014; Koseki et al., 2023; Marques de Brito et al., 2023). In the last decade, the Lemnaceae plant family has come into focus as a valuable nutrient source (Appenroth et al., 2017; Appenroth et al., 2018). These plants are the smallest and fastest growing flowering plants that predominantly propagate by vegetative means with a doubling time of approximately one day when grown under optimal conditions (Sree et al., 2015; Ziegler et al., 2015). This feature makes these plants interesting for biotechnological and food applications (Acosta et al., 2021). Moreover, duckweed has been used for many generations as part of human nutrition in countries such as Thailand, Laos, and Cambodia (Bhanthumnavin and McGarry, 1971). In ancient China and medieval Europe, the plant was used even for medicinal purposes (Edelman et al., 2022; Fan et al., 2023). More recently, several clinical investigations showed consumption of duckweeds can improve several health indicators (Diotallevi et al., 2021; Kaplan et al., 2019; Yaskolka Meir et al., 2019). The duckweed clone *Wolffia globosa* Mankai was reported to contain bioactive vitamin B_{12} (Sela et al., 2020). More recently, Hoffmann et al. (2023) reported a high capacity of the Mankai duckweed to affect the epigenome of humans, after studying the blood methylome and transcriptome. Until now, the presence of vitamin B_{12} in duckweed has been reported only in clone *W. globosa* Mankai (Kaplan et al., 2019). Whether B_{12} is synthesized by the plant itself or by any associated bacteria remains unclear. Watanabe and Bito (2018) have stated that "Vitamin B_{12} has been found to be synthesized only by certain bacteria and archeon, but not by plants". The presence of vitamin B_{12} in certain plants (e.g., *Hippophae rhamnoides, Elymus repens, Inula helenium*) has also been assumed to result from microbial associations.

Considering this background, we hypothesize that duckweeds could form commensal relationships with bacteria that are capable of synthesizing vitamin B_{12} and thus fortify these aquatic plants with this essential vitamin. To test this hypothesis, we address the following questions in this report: 1) Is the presence of vitamin B_{12} restricted to the duckweed clone *W. globosa* Mankai, or to other species as well within the genus *Wolffia*; or is it a general property of plants in the duckweed family? 2) Is vitamin B_{12} synthesized by duckweed itself or by endophytic or surface-associated bacteria? 3) Can regular consumption of duckweed, as part of a plant-based diet, improve vitamin B_{12} supply of humans?

2. Material and methods

2.1. Plant material and cultivation

The majority of duckweed clones used in this study were obtained from the stock collection at the Friedrich Schiller University of Jena – Plant Physiology and were cultivated for many years under laboratory conditions in N medium as previously described (Appenroth, 2015). The remaining strains were sourced from the Rutgers Duckweed Stock Cooperative (RDSC) at Rutgers University, NJ, USA. Species identity of the clones was originally defined by E. Landolt, ETH Zurich, Switzerland, on the basis of morphological markers, confirmed by one of the authors (KSS), and in most cases also by plastidic barcoding and fingerprinting by amplified fragment length polymorphism (Bog et al., 2013; Bog et al., 2019). Plants were pre-cultivated in sterile N medium for 4 weeks at 25 °C and 100 μmol m^{-2} s^{-1} continuous white light to ensure reproducible results after adaptation of the plants to these conditions. Nutrient medium was changed every week. Thereafter, cultured plants were transferred to plastic trays (area 60 ×40 cm) filled with autoclaved N medium (12.5 L in each) and then covered with glass plates. During this period, interaction with airborne microorganisms in the cultivation room was possible. The main cultivation phase lasted for 14 days, during which the size of the inoculum ensured that the fronds did not overcrowd, thus avoiding a possible stress response. Biomass was harvested with a nylon net, rinsed carefully with deionized, sterile water and lyophilized for analysis of vitamin B_{12} content. These samples were labelled as "unsterile".

To remove surface-associated microorganisms, plants were surface sterilized using a commercial bleaching agent (Danklorix, containing 2.8 g NaOCl/ 100 mL; Colgate-Palmoliv, Hamburg, Germany), diluted with sterile distilled water to 2 – 10 % (V/V). After treatment for 5 – 30 min, plants were transferred to 6-well-plates filled with N medium containing 25 mM glucose. Possible contaminations were visible after 10 days under these cultivation conditions. These cultures were labelled with "DK" for Danklorix. To stop the growth of possible endophytic contaminations, surface-sterilized plants were transferred onto petri dishes containing agar medium (0.9 %) prepared with N medium plus the antibiotic cefotaxime, 1 mg/10 mL (GoldBio; Catalog # C-104) (Lam and Acosta, 2019). Plants were inoculated at the center of the 9 cm-plate and cultivated for 10 days. Fronds from the outer part of the grown population were used for further investigations (labelled as "CEF"). Surface-sterilized or cefotaxime-treated plants were transferred into glass flasks containing 2.5 L sterile N-medium, closed with cotton-wool stoppers, and cultivated for 10 days under conditions as described above. This plant material was also freeze-dried and used for determination of vitamin B_{12} and in some cases also used for bacterial DNA fingerprinting assays as described below.

2.2. Analysis of corrinoids

2.2.1. Extraction and bioassay of vitamin B_{12}

Each lyophilized duckweed sample (0.2 g) was homogenized with 5.0 mL of distilled water using a mortar and pestle. Then, the homogenate was treated three times for 10 s each at 4°C using a mini-bead beater homogenizer (Biospec Products Inc., Bartlesville, OK, USA). When extracting vitamin B_{12} from freeze-dried bacterial cells, each

sample, ranging from 1.4 to 3.3 mg, was suspended in 4 mL of distilled water. The suspension was then disrupted by sonication at 10 kHz for 5 seconds at 0°C, repeated ten times. Total vitamin B_{12} extraction from each homogenate followed the KCN-boiling method (described by Bito et al., 2016). For each sample, 4.0 mL of cell homogenate was mixed with 1.0 mL of 0.57 M acetic acid buffer (pH 4.5) and 40 μL of 0.05 % (w/v) KCN. Boiling for 30 min converted the various vitamin B_{12} forms to cyanocobalamin. After cooling to room temperature (25°C), 60 μL of 10 % (w/v) metaphosphoric acid solution was added. The mixtures were diluted to 10 mL with distilled water. For total vitamin B_{12} assays (Extract A), 5.0 mL aliquots of the diluted solution were adjusted to pH 6.0 with 1 M NaOH and further diluted to 10 mL with distilled water. For alkali resistance factor assays (Extract B), 5.0 mL aliquots were adjusted to pH 11.0–12.0 using 6 M NaOH, autoclaved at 121°C for 30 min to degrade vitamin B_{12} forms, and diluted to 10 mL with distilled water. The extraction procedures were conducted in a draft chamber (Dalton Co., Tokyo, Japan). Vitamin B_{12} content was determined with a microbiological method using *Lactobacillus delbrueckii* ATCC 7830, which uses vitamin B_{12} as an essential nutrient. Cyanocobalamin was used to generate a calibration graph for converting the relative bacteria growth observed to cobalamin concentrations (Fig. S1). Alkali resistance factors (nucleotides and deoxyribonucleotides) exhibit vitamin B_{12} activity in this bacterium. Therefore, an accurate vitamin B_{12} content calculation involved subtracting the alkali resistant factor values from the total vitamin B_{12} content. The vitamin B_{12} content was independently determined three times for each sample and is presented as mean ± SE. The results were either investigated by Student's t-test (comparison of two samples) or by one-way ANOVA (three samples).

2.2.2. *Escherichia coli 215 bioautography analysis*

Bioautography of corrinoid compounds using vitamin B_{12}-dependent *E. coli* 215 was conducted following the procedures outlined by Tanioka et al. (2008). A 2-μL aliquot each of previously described extract A samples, standard vitamin B_{12}, and standard Pseudo-B_{12} was applied to a silica gel 60 thin-layer chromatography (TLC) sheet. The TLC sheet was developed in the dark at 25°C using 2-propanol/NH_4OH (28 %)/water (7:1:2 v/v) as the solvent. Subsequently, the TLC sheet was dried. Agar containing basal medium with pre-cultured *E. coli* 215 was overlaid on the dried TLC sheet, which was incubated at 30°C for 20 h. After incubation, the sheet was removed from the agar plate, and the gel plate was sprayed with a 2,3,5-triphenyltetrazolium methanol solution. Corrinoid compounds were visualized by the presence of a reddish-brown color which indicated bacterial growth (Fig. S2).

2.2.3. *Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis*

Selected lyophilized duckweed samples including unsterile *W. microscopica* 2007 (Wm2007), unsterile *S. polyrhiza* 9509 (Sp9509), and unsterile, surface-sterilized, and cefotaxime-treated *L. minor* 9606 (Lm9606) at 2.0 g each were homogenized with 30 mL of distilled water. Homogenization was done using a mortar and pestle and further treated three times for 10 s at 4°C using a mini-bead beater homogenizer (Biospec Products Inc.). The resulting cell homogenate was centrifuged at 10,000 x *g* for 10 min, and the supernatant was used for further analysis. Total vitamin B_{12} was extracted from each homogenate using the KCN-boiling method. Specifically, 20 mL of cell homogenate was mixed with 5.0 mL of 0.57 M acetic acid buffer (pH 4.5) and 0.2 mL of 0.05 % (w/v) KCN, followed by boiling for 30 min. After cooling to 25°C, vitamin B_{12} compounds were partially purified using a Sep-Pak C_{18} 20cc (5 g) cartridge (Water Corp., Milford, MA, USA) that were washed with 20 mL of 75 % (v/v) ethanol and equilibrated with 20 mL of distilled water. The C_{18} cartridge was washed with 30 mL of distilled water, and vitamin B_{12} related compounds were eluted with 5 mL of 75 % (v/v) ethanol. Eluates were evaporated to dryness under reduced pressure, dissolved in 2.0 mL of distilled water, and loaded onto an immunoaffinity column [EASE-EXTRACT vitamin B_{12} Immunoaffinity Column (P80), R-Biopharm AG, Darmstadt, Germany] for further purification, following the manufacturer's protocol. Purified vitamin B_{12} compounds were dissolved in 100 μL of distilled water and used as samples for LC-MS/MS, which was performed under the same analytical conditions using the same LC system as described previously (Bito et al. 2016).

2.3. *PCR-based method for detecting the presence of bacteria associated with duckweeds using ribosomal intergenic spacer analysis (RISA)*

Nucleic acid was isolated from sterile and unsterile duckweed using a previously developed bead-beating protocol (Acosta et al., 2023). To detect duckweed-associated bacteria, PCR was performed on nucleic acids using primers (16S-e1390f: 5'-TGYACACACCGCCCGTCA-3' & 23S-e130r: 5'-GGGTTBCCCCATTCRG-3') targeting the ribosomal intergenic spacer region as previously described (Acosta et al., 2023). Sample quality was validated and duckweed DNA was compared using primers (5'-TTAGCATTTGTTTGGCAAG-3' & 5'-ACTCGCACACACTCCCTTTCC-3') targeting the plastid *psbK-psbI* intergenic region (Borisjuk et al., 2015). The *psbK-psbI* PCR reactions were prepared in a total volume of 25 μL consisting of: 1X PCR buffer with Mg^{2+} (1.5 mM $MgCl_2$, 10 mM KCl, 8 mM $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 9.0, 0.05 % NP-40; Thomas Scientific, Swedesboro, NJ, USA), 4 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer, 2 units of ChoiceTaq DNA polymerase (Thomas Scientific, Swedesboro, NJ, USA; catalog # CB4050–2), and 2 μL nucleic acid template (~ 100 ng/μL). The *psbK-psbI* PCR was carried out using the following 3-stage thermocycler program: (1) denaturation stage of 95°C for 5 min; (2) 35 cycles consisting of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and (3) a final extension stage of 72°C for 5 min. RISA and *psbK-psbI* PCR products were visualized on a 1.0 % (w/v) agarose gel stained with ethidium bromide.

2.4. *Bioinformatics analysis of genomes from duckweeds and duckweed-associated bacteria for potential of vitamin B_{12} requirement and B_{12} biosynthesis, respectively*

To gain insight into duckweed vitamin B_{12} requirements, the following primary proteomes were retrieved from lemna.org (accessed February 11, 2024) (Ernst et al., 2023): *Wolffia australiana* 8730 (REF-CSHL-1.0_CSHL2022v1), *Lemna minor* 7210 (REF-CSHL-1.0_CSHL2022v1), *Lemna gibba* 7742a (REF-CSHL-1.0_CSHL2022v1), and *Spirodela polyrhiza* 9509 (REF-OXFORD-3.0_CSHL2022v1). Proteomes were analyzed for cobalamin-independent (MetE) and cobalamin-dependent (MetH) methionine synthase (Table S1; Fig. S4). MetE (Genbank accession XP_001702934.1) and MetH (Genbank accession XP_001696420.1) from *Chlamydomonas reinhardtii* were used as BLASTP queries against duckweed proteomes with an e-value cutoff of 0.0001. For further comparison, *Arabidopsis thaliana* MetE orthologs were retrieved from Genbank (AtMS1 = NP_197294.1, AtMS2 = NP_850507.1, AtMS3 = NP_001078611.1). MetE orthologs were then scanned for protein domains using InterProScan v5.55–88.0 (Jones et al., 2014). MetE phylogeny was constructed using IQ-TREE v1.6.12 (Nguyen et al., 2015) with a JTT+R2 substitution model (Kalyaanamoorthy et al., 2017) and bootstrap approximation (Hoang et al., 2018) using the following parameters: -bb 1000, -bbni. Proteins were classified as MetH if they contained an activation domain, cobalamin/B_{12}-binding domain, homocysteine binding domain, and N5-methylTHF-binding domain (Table S1). The vitamin B_{12} biosynthesis potential of duckweed-associated bacteria (Table S2) was assessed by reviewing previous work of Shelton et al. (2019).

2.5. *Detection of Vitamin B_{12} production by duckweed-associated bacteria*

Glycerol stocks of the bacterial strains, *Aeromonas sp.* RU39B (DAB 39B) and *Pseudomonas alcaligenes* RU36E (DAB 36E), stored at −80°C

were streaked out on agar media containing Miller's lysogeny broth (LB) and incubated at 28°C for 24–48 hours. Single colonies were then picked to inoculate 5 mL of liquid LB cultures. These cultures were gently shaken at 28°C for 48 hours. To generate sufficient bacterial populations for experiments, 5 mL bacterial cultures were used to inoculate 100 mL LB liquid cultures and were then incubated at 28°C with shaking (220 rpm) on an orbital shaker (IKA Works Inc., Wilmington NC, Model KA260) for another 3 days. Then, 50 mL aliquots of the bacterial cultures were centrifuged for 10 minutes at 4°C and 7000 rpm (6000 X rcf) to pellet the bacteria. Pellets of each bacterial strain were rinsed twice with sterile 0.5X Schenk and Hildebrandt (SH) medium and re-suspended in 500 mL of 0.5X SH medium at an optical density (OD_{600}) of 0.2. Bacterial cultures (500 mL) were then inoculated with or without *Spirodela polyrhiza* 9509, at 70–80 % coverage of the liquid surface, and incubated in a growth chamber at 25°C with 50 μmol m^{-2} s^{-1} of photosynthetically active radiation using fluorescent lamps under long day conditions. After 7 days, bacterial pellets were collected from the media by centrifugation, flash frozen in liquid nitrogen, and stored at −80°C until processing.

3. Results

3.1. Survey of vitamin B_{12} content across multiple genera within the Lemnaceae family

To assess whether vitamin B_{12} is found among different duckweed species, fresh biomass for 15 duckweed accessions was freeze-dried and used for the determination of apparent vitamin B_{12} by the *Lactobacillus delbrueckii* ATCC7830 bioassay. The results are summarized in Table 2. Vitamin B_{12} was detected in all samples, but the results of two runs were quantitatively different for some of the samples. This observation illustrates the technical variability of this bioassay that could arise in one or more steps during sample extraction or during the bacterial growth assay.

In a subsequent experiment, unsterile samples of five clones from three different species were compared with samples that were surface sterilized with bleach (Danklorix) or additionally treated with cefotaxime (CEF). It should be noted that the unsterile plant samples were cultivated in open trays, and the surface-sterilized and cefotaxime-treated samples were cultivated in closed 2.5 L glass bottles under sterile conditions. The results shown in Fig. 1A revealed detectable vitamin B_{12} in each duckweed sample tested; however, the vitamin B_{12} content was reproducibly decreased in most cases by surface sterilization and CEF treatment. Compared to the others, the clone *W. australiana* 7540 (Wa7540) was particularly difficult to decrease the apparent content of bacteria based on the PCR assay (see below), and only showed a decrease of B_{12} content by 50 % when treated with bleach and CEF (Fig. 1). For three of the samples (Wm2007, Wa7540 and Sp9509), bioautography analysis was carried out using *E. coli* 215 to compare these samples with authentic vitamin B_{12} and Pseudo-B_{12}. The corrinoid compounds found in these duckweed samples were analyzed with the *E. coli* 215 bioautogram after being separated by silica gel 60 TLC (Fig. S2). The majority of the corrinoids in Wm2007, Wa7540, and Sp9509 extracts were found as a single spot with R_f values identical to those of standard vitamin B_{12}. These results demonstrated the presence of bioactive vitamin B_{12} in all three species of duckweeds examined and thus indicate the presence of vitamin B_{12} is not unique to *the W. globosa* clone Mankai.

3.2. High Resolution Characterization of vitamin B_{12} and Pseudo-B_{12} using LC-MS/MS

To ascertain whether the corrinoid compounds present in duckweed samples are B_{12} or Pseudo-B_{12}, corrinoid compounds were purified from the extracts of three different duckweed species (Wm2007, Sp9509, and Lm9606), and their identification was conducted using LC–MS/MS. Standard B_{12} (*m/z* 678.29) was observed as a peak with a retention time of 9.7 min (Fig. S3A). The mass spectrum (MS) of standard B_{12} showed a prominent doubly charged ion with an *m/z* 678.2892 $[M + 2H]^{2+}$ (Fig. S3A). The MS/MS spectrum of standard B_{12} revealed that its dominant ion at *m/z* 359.0989 was consistent with the nucleotide moiety containing 5,6-dimethylbenzimidazole as a base (Fig. S3A). Similarly, standard Pseudo-B_{12} (*m/z* 672.77) appeared as a peak with a retention time of 9.4 min (Fig. S3B). A prominent doubly charged ion with an *m/z* 672.7767 $[M + 2H]^{2+}$ was observed (Fig. S3B). The MS/MS spectrum of standard Pseudo-B_{12} indicated that the dominant ion at *m/z* 348.0792 was attributable to the nucleotide moiety containing adenine as a base (Fig. S3B). These MS and MS/MS data were identical to those of standard B_{12} and Pseudo-B_{12} described previously (Bito et al., 2016).

Purified corrinoid compounds from unsterile Lm9606 samples were eluted as a single peak with *m/z* 678.29 while no peak with *m/z* 672.78 was detected (Fig. 2A). The mass spectrum of the ion peak with *m/z* 678.29 indicated the formation of the doubly charged ion at *m/z* 678.2929 (Fig. 2A). The MS/MS spectrum of the compound showed the singly charged ion at *m/z* 359.0998, identical to that of standard B_{12} (Fig. S3A). Similar results were obtained for purified corrinoid compounds from Wm2007 unsterile sample and surface sterilized and cefotaxime-treated samples also contained B_{12} but not Pseudo-B_{12} (data not shown). These findings showed that B_{12} is the predominant corrinoid in Lm9606 and Wm2007.

Table 2
Vitamin B_{12} content of 15 clones belonging to 13 species and 3 genera of Lemnaceae. The results (μg vit. B_{12} / 100 g dry weight) were obtained by bioassay using *Lactobacillus delbrueckii* ATCC7830. In both runs, plants from the Jena stock cultures were used as starting material, inoculated onto autoclaved nutrient medium, and grown in open trays inside a climate chamber for 10 days. Vitamin B_{12} contents were compared by Student's t-test between measurement 1 and measurement 2 for each duckweed sample. * and ** indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

Species	Clone ID	Origin	Vitamin B_{12} (measurement 1)	Vitamin B_{12} (measurement 2)
Wolffia angusta	8878	Malaysia	0.79 ± 0.03	0.15 ± 0.09**
Wolffia arrhiza	8618	Kenya	0.66 ± 0.01	0.21 ± 0.03*
Wolffia arrhiza	8853	Brazil	0.71 ± 0.02	0.52 ± 0.07*
Wolffia arrhiza	9528	Germany	0.80 ± 0.01	0.25 ± 0.02**
Wolffia australiana	7540	New Zealand	7.12 ± 0.50	7.07 ± 2.08
Wolffia borealis	9123	USA (CA)	0.51 ± 0.01	0.19 ± 0.02*
Wolffia brasiliensis	7925	Argentina	0.51 ± 0.01	0.38 ± 0.44
Wolffia cylindracea	9056	Zimbabwe	0.92 ± 0.00	0.01 ± 0.02**
Wolffia elongata	9188	Colombia	0.42 ± 0.02	0.36 ± 0.06
Wolffia globosa	5514	Thailand	0.68 ± 0.02	0.36 ± 0.00*
Wolffia globosa	5516	Thailand	0.60 ± 0.02	0.33 ± 0.01**
Wolffia globosa	9498	India	0.76 ± 0.08	0.33 ± 0.02*
Wolffia microscopica	2007	Bangaldesh	1.81 ± 0.02	1.26 ± 0.49
Spirodela polyrhiza	9509	Germany	15.50 ± 0.50	25.5 ± 0.39*
Lemna minor	9606	Switzerland	1.17 ± 0.02	0.72 ± 0.09

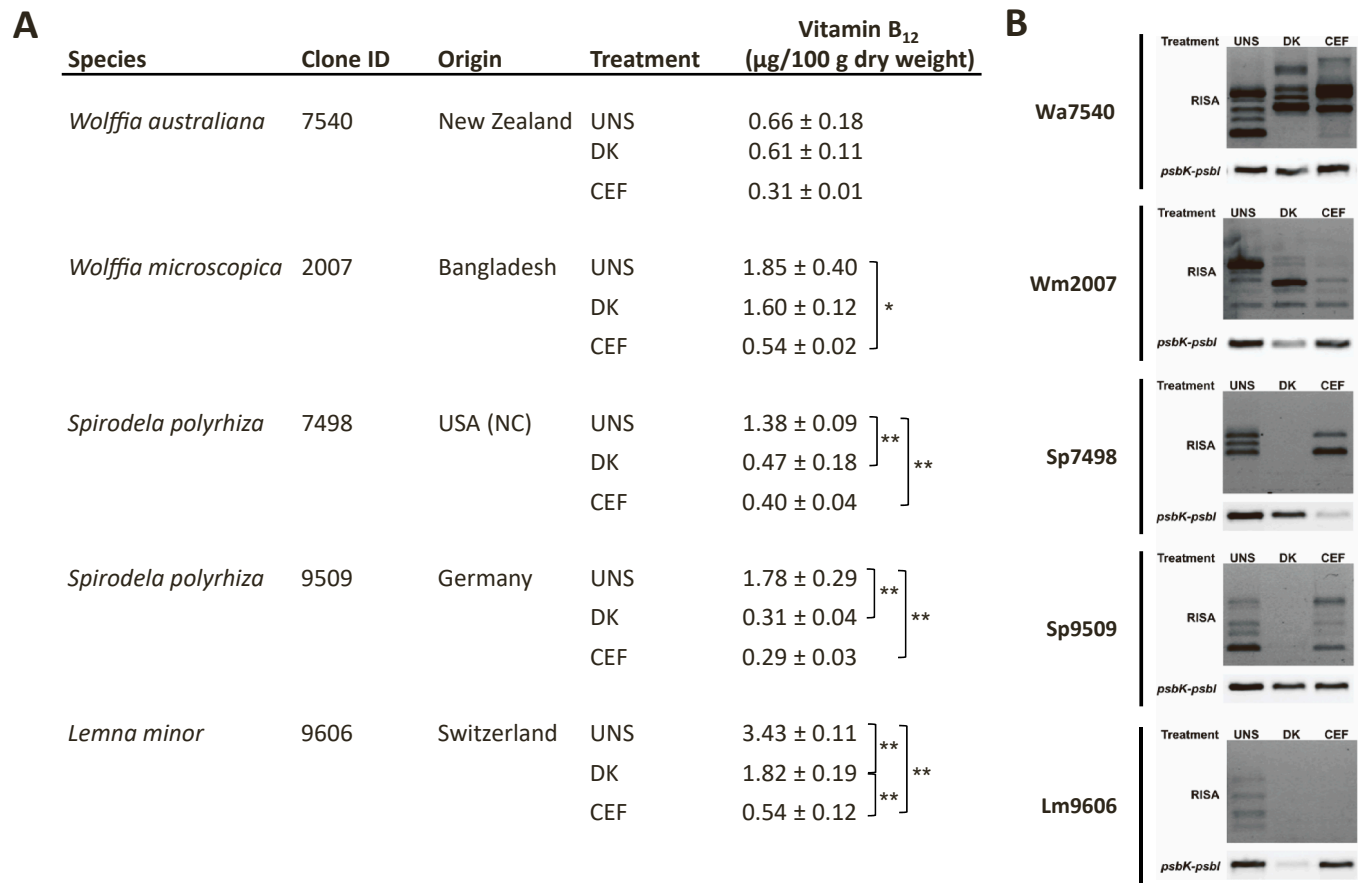

Fig. 1. Correlation between antimicrobial treatment and lower vitamin B_{12} content in five clones of duckweed. A) Vitamin B_{12} content of duckweed clones before and after surface sterilization or antibiotic treatment. UNS = no treatment, DK = surface sterilization, CEF = cefotaxime treatment. Vitamin B_{12} content in controls (UNS), after surface sterilization, and after additional antibiotic treatment were compared by one-way ANOVA. * and ** indicate significant differences between treatments (*, $p < 0.05$; **, $p < 0.01$). B) Detection of duckweed-associated bacteria and duckweed DNA by RISA and *psbk-psbI* PCR respectively. Wa7540 = *W. australiana* 7540, Wm2007 = *W. microsopica* 2007, Sp7498 = *S. polyrhiza* 7498, Sp9509 = *S. polyrhiza* 9509, Lm9606 = *L. minor* 9606.

In contrast to those of *L. minor* and *W. microscopica*, purified corrinoid compounds from *Sp*9509 unsterile samples were eluted as respective peaks with *m/z* 678.29 and *m/z* 672.78 (Fig. 2B, top panel). The mass spectrum of the ion peak with *m/z* 678.29 indicated the formation of a doubly charged ion at *m/z* 678.2876 (Fig. 2B). The MS/MS spectrum of this compound showed the singly charged ion at *m/z* 359.0998, identical to that of standard B_{12} (Fig. S3A). The mass spectrum of the ion peak with *m/z* 672.78 indicated the formation of a doubly charged ion at *m/z* 672.7747 (Fig. 2B). The MS/MS spectrum of this peak showed the singly charged ion at *m/z* 348.0656, identical to that of standard Pseudo-B_{12} (Fig. S3B). The relative content (%) of B_{12} and Pseudo-B_{12} in the extracts calculated from each peak area against the total peak area by measuring the absorbance at 361 nm using HPLC indicated that Sp9509 contained both B_{12} (approximately 74 %) and Pseudo-B_{12} (approximately 26 %). These results are quantified and presented in Table 3.

3.3. Effects of tissue sterilization on vitamin B_{12} content in duckweed tissues

We suspected that the residual vitamin B_{12} detected in sterilized duckweed tissues originated from duckweed-associated bacteria partially resistant to bleach treatment and culture on cefotaxime. Therefore, we tested the unsterile duckweed strains before and after their sterilization treatments for the presence of bacteria using a previously described DNA-fingerprinting assay targeting the ribosomal intergenic sequence (RISA) between the 16 S and 23 S rRNA genes in bacteria (Acosta et al., 2023; Huang et al., 2020). While RISA cannot inform on the vital status of the bacteria that may be present, it could provide a reasonable proxy for the approximate bacteria load in the tissues of interest. Fig. 1B shows the RISA fingerprint from corresponding freeze-dried tissue samples of the five species studied in Fig. 1A. It is apparent that bleach treatment and further exposure to the antibiotic cefotaxime significantly decreased the bacteria levels, but varying quantities of bacteria still remain in these duckweed samples. Our data are consistent with the speculation (Sela et al., 2020) that endophytic microbes, most likely vitamin B_{12}-producing bacteria, in duckweed are the source for the observed presence of this corrinoid in these plants.

3.4. Curation of duckweed genomes indicates no requirement for vitamin B_{12} as essential cofactor for methionine biosynthesis

One of the key functions of vitamin B_{12} in organisms ranging from bacteria to animals is its cofactor role for conversion of homocysteine to methionine via the vitamin B_{12}-dependent methionine synthase (MetH) enzyme (Shelton et al., 2019). The model photosynthetic microalga *C. reinhardtii* is known to contain genes encoding both vitamin B_{12}-dependent (MetH) and B_{12}-independent (MetE) methionine synthases. Using the predicted methionine synthase protein sequences for *C. reinhardtii*, we searched duckweed genomes available for a total of four species from the *Lemna*, *Spirodela*, and *Wolffia* genera via BLASTP for methionine synthase homologs and used InterProScan to scan the resulting genes for vitamin B_{12}-binding domains (Fig. S4, Table S1). BLASTP revealed at least 3 or more MetE homologs in each of the three

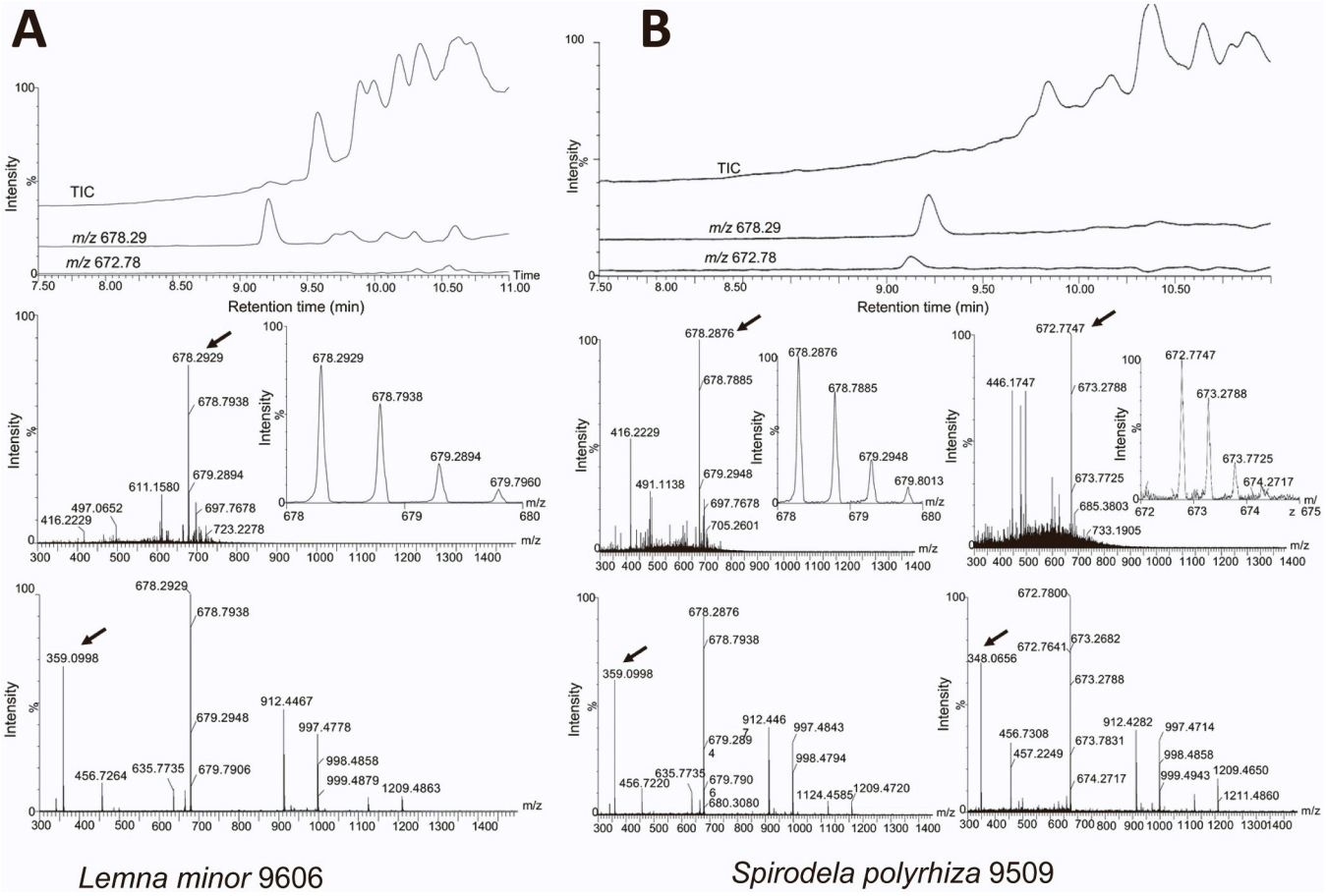

Fig. 2. Detection and identification of vitamin B_{12} and Pseudo-B_{12} present in two duckweed species using LC-MS/MS methods. A) LC−MS/MS chromatograms of corrinoid compounds purified from unsterile *Lemna minor* (9606) samples. Panel 1 (top): total ion chromatogram (TIC) and selected ion monitoring chromatograms of *m/z* 678.29 (vitamin B_{12}) and *m/z* 672.77 (Pseudo-B_{12}). Panel 2 (middle): mass spectrum of the peak from the sample at *m/z* 678.29. Magnified spectrum from *m/z* 678 to *m/z* 680 is presented as an inset in Panel 2 (middle). Panel 3 (bottom): MS/MS spectrum of the peak of the sample at *m/z* 678.29. The arrows are showing signal intensities at *m/z* characteristic of vitamin B_{12} in MS and MS/MS. B) LC−MS/MS chromatograms of corrinoid compounds purified from unsterile *Spirodela polyrhiza* (9509) samples. Panel 1 (top): total ion chromatogram (TIC) and selected ion monitoring chromatograms of *m/z* 678.29 (vitamin B_{12}) and *m/z* 672.77 (Pseudo-B_{12}). Panel 2 (middle left): mass spectrum of the peak from the sample at *m/z* 678.29. Magnified spectrum from *m/z* 678 to *m/z* 680 is presented as an inset in Panel 2. Panel 3 (bottom left): MS/MS spectrum of the peak of the sample at *m/z* 678.29. Panel 4 (middle right): mass spectrum of the peak from the sample at *m/z* 672.77. Magnified spectrum from *m/z* 672 to *m/z* 675 is presented as an inset in Panel 4. Panel 5 (bottom right): MS/MS spectrum of the peak from the sample at *m/z* 672.77. The arrows indicate ions showing signal intensities at *m/z* characteristic of vitamin B_{12} or Pseudo-B_{12} in MS and MS/MS analyses. The quantitative results of measurements are shown in Table 3.

duckweed genera we examined. No significant MetH homologs were found in the duckweed genomes examined (Table S1). This result indicates that there is likely no obligate interactions between duckweed and vitamin B_{12}-producing bacteria since no vitamin B_{12}-dependent enzyme is present to provide essential functions in these plants.

3.5. Production of vitamin B_{12} by duckweed-associated bacteria

To test if there are duckweed-associated bacteria (DAB) which can produce vitamin B_{12}, we reexamined a previous study that surveyed the distribution of vitamin B_{12} biosynthesis capabilities among 11,000 bacterial species and isolates (Shelton et al., 2019). In this study, bacteria were classified into 7 vitamin B_{12} biosynthesis phenotypes: "very likely cobamide producer", "likely cobamide producer", "possible cobamide producer", "tetrapyrrole precursor salvager", "cobinamide salvager", "likely non-producer", and "very likely non-producer". Importantly, this work characterized the vitamin B_{12} biosynthetic potential for 26 DAB isolates with publicly available genomes and annotations at the time (Table S2). Out of the 26 DAB isolates for which the predicted proteomes were examined, *Aeromonas sp.* RU39B (DAB 39B) and *Pseudomonas alcaligenes* RU36E (DAB 36E) were classified as "very likely corrinoid producers" (Table S2). To confirm this prediction, vitamin B_{12} measurements were taken of DAB 36E and DAB 39B bacteria cultivated in the presence and absence of Sp9509 plants on 0.5X SH culture medium (Table 4). Vitamin B_{12} was found in DAB 36E and DAB 39B cells under both conditions. Moreover, the vitamin B_{12} content of DAB 36E cells seems to be significantly altered by the presence of Sp9509 plants in co-culture. These data show that DABs from certain Pseudomonadota genera, such as *Aeromonas* and *Pseudomonas,* are indeed vitamin B_{12} producers.

4. Discussion

In this study we intended to determine whether significant levels of vitamin B_{12} found in the duckweed clone *W. globosa* Mankai are a unique characteristic of this particular duckweed species or a general feature of plants in the Lemnaceae family. Therefore, we surveyed eight other *Wolffia* species as well as two additional duckweed species from the genera of *Spirodela* and *Lemna*, which are two of the most common duckweeds worldwide. Our results clearly showed that the association of

Table 3
Investigation of vitamin B_{12} and Pseudo-B_{12} content. Vitamin B_{12} contents (μg B_{12} / 100 g dry weight) were obtained by bioassay using *Lactobacillus delbrueckii* ATCC7830. Vitamin B_{12} content in control group (unsterile *Lemna minor* 9606), after surface sterilization, and after additional antibiotic treatment was compared by one-way ANOVA. ** indicates significant differences between treatments ($p < 0.01$). Vitamin B_{12} and Pseudo-B_{12} present in plant samples were identified using LC-MS/MS methods.

Species	Clone ID	Condition	Vitamin B_{12} (μg/100 g dry weight)	Identified corrinoid compounds
Wolffia microscopica	2007	unsterile	1.97 ± 0.03	All corrinoid B_{12}
Spirodela polyrhiza	9509	unsterile	1.33 ± 0.13	vitamin B_{12} and Pseudo-B_{12} in ratio 3:1
Lemna minor	9606	unsterile	2.74 ± 0.03	All corrinoid B_{12}
Lemna minor	9606	surface sterilized	0.93 ± 0.08	All corrinoid B_{12}
Lemna minor	9606	cefotaxime treated after surface sterilization	0.55 ± 0.07	All corrinoid B_{12}

Table 4
Vitamin B_{12} production by duckweed-associated bacteria. *Aeromonas sp.* RU39B (DAB 39B) and *Pseudomonas alcaligenes* RU36E (DAB 36E) were inoculated with (+Sp9509) or without *Spirodela polyrhiza* 9509 in plant growth media under axenic conditions. Bacterial cells were collected from the media, lyophilized, and used in bioassay with *Lactobacillus delbrueckii* ATCC7830 for vitamin B_{12} quantification. The influence of *S. polyrhiza* 9509 (+ Sp9509) plants on the vitamin B_{12} content, compared to the control without *S. polyrhiza* was tested using Student's t-test. * indicates a statistically significant difference by the presence of the plants ($p < 0.05$).

Bacterial Cell Sample	Apparent B_{12} (μg /100 g)	Alkali-resistant Factor (μg /100 g)	Vitamin B_{12} (μg /100 g)
DAB 39B	205.8 ± 28.5	15.1 ± 2.9	190.7 ± 27.8
DAB 39B + Sp9509	381.4 ± 92.6	36.1 ± 10.7	345.3 ± 94.5
DAB 36E	234.9 ± 34.5	16.7 ± 3.0	218.2 ± 35.2
DAB 36E + Sp9509	152.5 ± 12.9	60.6 ± 21.0	91.9 ± 23.0*

vitamin B_{12} with duckweeds is prevalent across this plant family, but the quantities could be variable. This conclusion is consistent with findings reported in a preprint on quantification of cobalamin content in nine different duckweed species in addition to the Mankai clone (Klamann et al., 2023), in which variable levels of up to 20-fold could be found between the samples analyzed using two different assays. In our work, using both biological activity-based assays and the more definitive LC-MS/MS method, we showed that authentic vitamin B_{12} is indeed associated with plants of different duckweed genera and species apart from that already reported for *W. globosa* Mankai (Sela et al., 2020). In the case of *S. polyrhiza*, we also found a significant amount of Pseudo-B_{12} (approximately 25 % out of the total vitamin B_{12} content).

To address the potential microbial origin of the detected vitamin B_{12} in the duckweed species examined, we carried out surface sterilization and antibiotic treatment to eliminate microbes from the plant tissues. Using a sensitive, bacterial specific DNA fingerprinting assay, we demonstrated the presence of bacteria in untreated duckweed samples, while much less bacterial DNA amplification was observed after bleach and antibiotic treatments in most cases. The level of vitamin B_{12} present in duckweed also decreased concomitantly. Presence of duckweed associated bacteria (DABs) is well known from the bacterial collection of the Lam lab (Rutgers University, USA), and we examined whether vitamin B_{12} producers can indeed be present. Out of the 26 DABs with fully annotated genomes that were examined, Shelton et al. (2019) identified two "very likely vitamin B_{12} producers" while five were designated as "likely vitamin B_{12} producers". For this study, we directly validated this prediction by demonstrating the ability of the two "very likely vitamin B_{12} producer" bacterial strains to produce B_{12} *in vitro*. We also note that the five other "likely B_{12} producers" are bacteria in the *Azospirillum* and *Rhizobium* genera that are very commonly found associated with duckweeds (Acosta et al., 2020; Huang et al., 2020). These observations indicate that vitamin B_{12}-producing bacteria may be common among DABs, which then result in the common presence of vitamin B_{12} in duckweed plants.

Our studies here show that harsh treatments (like bleach and antibiotics) are required to minimize bacteria that can produce vitamin B_{12} in the duckweed tissues. This is consistent with the previous suggestion that the association could be endophytic (Sela et al., 2020). Together with our preliminary bioinformatics screening of sequenced DABs for vitamin B_{12} producers, this work may enable the identification of duckweed endophytes capable to fortify duckweed strains of choice with high vitamin B_{12} to improve the plant's nutritional quality. The recommended daily allowance (RDA) for adults in the United States is 2.4 μg of vitamin B_{12} (National Institutes of Health NIH Office of Dietary Supplements, 2024). Our studies show that the vitamin B_{12} content in duckweeds could vary between 0.5 and 10 μg/100 g of dry matter. Targeted inoculation of duckweed with certain bacterial strains, either alone or as a microbial consortium, could therefore provide vitamin B_{12} at stable levels of up to 8 μg/100 g dry plant matter (if the bacteria of interest could successfully colonize duckweed plants). Consuming 150 g of fresh duckweed per person (corresponding to 10–15 g of dry material) is possible, for instance in a *Wolffia* spicy salad containing *Wolffia*, tomatoes, onions, celery as well as herbs and spices. Alternative dishes can be a *Wolffia* smoothie with a touch of orange or a *Wolffia* spicy omelette (Fig. S5). An intake of 10–15 g *Wolffia* dry matter via this single meal corresponds to a vitamin B_{12} intake of 0.8 μg, i.e. about a third of the RDA for vitamin B_{12}. We can conclude that supplying a quarter to a third of the daily recommended vitamin B_{12} intake per one meal with duckweed-based food is readily feasible. In a further perspective, duckweed could be an important part of the planetary health diet to significantly reduce the worldwide vitamin B_{12} deficiency. By further optimizing the symbiosis between vitamin B_{12}-producing bacteria and suitable duckweed species, at least half of the RDA for vitamin B_{12} may be achieved in the future.

CRediT authorship contribution statement

Shawn Sorrels: Writing – review & editing, Investigation, Data curation. **Fumio Watanabe:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Gerhard Jahreis:** Writing – review & editing, Conceptualization. **Kyohei Koseki:** Writing – review & editing, Formal analysis, Data curation. **Naho Okamoto:** Writing – review & editing, Data curation. **Sowjanya Sree:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation. **Kenneth Acosta:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Eric Lam:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Klaus Appenroth:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eric Lam reports financial support was provided by US Department of Energy. Eric Lam reports a relationship with Gordon and Betty Moore Foundation that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106603.

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