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RESEARCH

Quantification of phototrophically grown *Galdieria sulphuraria* and other microalgae using diphenylamine

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Abstract

Cell growth is an important parameter that requires accurate measurement during the cultivation of different microalgae and in biomass production for downstream processing. Also, the assessment of microalgal growth by optical density during process optimisation for wastewater treatment is sometimes confounded by suspended and/or particulate material and changes in medium turbidity. To address these challenges the current work sought to determine whether the one-step diphenylamine (DPA) method could be used reliably to quantify growth of the red alga, *Galdieria sulphuraria* and other microalgae. Results confirmed that the simplified DPA colorimetric method was sufficiently reproducible for the quantification of *G. sulphuraria* growth in phototrophic culture and that a linear relationship existed between an increase in cell culture dry biomass and the increase in cell density (OD_{750}) and DNA concentration (A_{595}). Similar results were obtained for laboratory cultures of locally sourced and isolated *Chlorella* sp., *Scenedesmus* sp. and the diatom, *Phaeodactylum* sp. For each isolate, the increase in algal dry biomass was linearly correlated with an increase in growth quantified either by monitoring OD_{750} or DNA concentration using DPA at A_{595}. These findings confirm DPA as a simple and relatively inexpensive colorimetric method for the rapid determination of cell growth in microalgal cultures.

Keywords Algal wastewater treatment · Aquaculture wastewater · Cell growth · Diphenylamine colorimetric assay · Microalgae · Quantification

Introduction

The unicellular red alga *Galdieria sulphuraria* is a cyanidiophyte, a class of microalgae that include species that are described as extremophilic (Merola et al. 1981; Gross 1998; Gross and Oesterhelt 1999; Oesterhelt et al. 2007). *Galdieria sulphuraria*, like other Cyanidiophyceae, survives harsh environments and can withstand low pH (~ 2) and elevated temperature, > 50 °C (Curien et al. 2021). Genomic studies have revealed that this species has a high level of metabolic flexibility (Barbier et al. 2005; Schönknecht et al. 2013; Rossoni et al. 2019) and can grow heterotrophically using several different carbon substrates (Sloth et al. 2017; Pleissner et al. 2021, 2023), mixotrophically by combining heterotrophy and photoautotrophy (i.e., light-catalysed assimilation of CO_2), and photoautotrophically using light as the sole energy source (Gross and Schnarrenberger 1995). Since this microalga shows a propensity for cultivation in organics-containing (waste)water outdoors in open ponds it is considered an emerging candidate for biotechnology applications (see Curien et al. 2021 and references therein). The latter suggests that *G. sulphuraria* is potentially also an ideal biocatalyst for use in wastewater treatment (Delanka-Pedige et al. 2019) and biomass production and that it may be particularly well-suited for use in dryland aquaculture and in water-scarce geographies where water recycle to re-use forms an integral part of fish production.

Microalgae together with commensal bacteria have the potential to facilitate nutrient removal in nature-based

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wastewater treatment systems and the resultant flocs, termed microalgal-bacterial flocs (MaB-flocs), settle readily yielding water for re-use (Jimoh and Cowan 2017; Laubscher and Cowan 2020). To derive a biomass for downstream use in feed production and to explore a circular economic approach for commercial-scale dryland aquaculture, *G. sulphuraria* is being grown phototrophically in aquaculture wastewater to determine its suitability for use as a biocatalyst in high-rate algal oxidation ponds (HRAOP) for wastewater treatment and recycle to feed production.

Optical density (OD) has been the method of choice for the determination of growth and estimation of biomass in cultures of various microalgae (Thiviyanathan et al. 2023). For the most part, OD at wavelengths of 600, 680 or 750 nm have been used and at least one study showed that there was consistency (Young et al. 2022). Even so, variability in the selection of the wavelength for OD measurement of algal cultures has been evident which sometimes, is related to culture condition e.g., OD at 678 nm where different light regimes were used (Abu-Ghosh et al. 2015). Thus, the OD-cell count relationship appears to be strong and well defined regardless of growth conditions and wavelength but, within the absorption range of chlorophyll, i.e., 550 and 665 nm, measurement does give higher regression coefficients under conditions that promote high cell chlorophyll content (Nielsen and Hansen 2019). This is perhaps why some authors have argued that there exists an error in the quantification of biomass concentration using OD, relative to actual dry biomass, in cells with a pigment content that varies with culture condition. Consequently, several studies suggest methodological strategies to mitigate this error by selecting a wavelength that minimizes absorbance where chlorophyll is the dominant pigment e.g., 750 nm (Griffiths et al. 2011; Chioccioli et al. 2014).

Examples of other, widely used methods for measuring microalgal growth include the use of counting chambers such as haemocytometers, the Sedgwick-Rafter chamber (Woelkerling et al. 1976) and the Neubauer-improved counting chamber with or without densitometer (Krediet et al. 2015; Ma'mun et al. 2022). Also used for measurement of microalgal growth are Coulter counters, flow cytometers, computational imagers (Krediet et al. 2015; Salmi et al. 2021), protein homogenates (Krediet et al. 2015) and optical detection of glucose (Thiviyanathan et al. 2023). An alternative and perhaps more accurate approach is to measure cell growth by quantification of total nucleic acid as has been achieved for other microorganisms (Thompson and Dvorak 1989). Indeed, DNA, considered the most accurate indicator of cell number, has been quantified spectrophotometrically using diphenylamine (DPA) since the mid 1950 s (Burton 1956; Kissane and Robbins 1958; Gendimenico et al. 1988; Thompson and Dvorak 1989; Zachleder 1989) and more recently, the method elaborated into a sensitive, simple, reliable, and rapid application for determination of bacterial cell growth (Zhao et al. 2013). While, DNA concentration within microalgal cells can change as a response to growth stage, nutrient status and availability, abiotic and biotic stress, and cell lysis, quantification of key biomolecules such as DNA remains key in determining the quality of microalgal cultures (Elisabeth et al. 2021).

The DPA method is a colorimetric assay for quantifying DNA based on the reaction of this dye with deoxyribose sugar under acidic conditions, producing a blue coloured complex that can be measured spectrophotometrically at 595 nm. Although data from Burton (1956) indicated linearity in the range 10–120 µg mL^{-1} with a limit of quantification somewhere between 3 and 10 µg (Burton 1956; Gendimenico et al. 1988), it was considered outdated due to its apparent lower sensitivity and the assay being too time-consuming. This contrasts with data from a detailed comparative assessment of three rapid colorimetric assays for DNA presented by Patterson and Mura (2013). These authors demonstrated that colorimetric assays for measuring DNA, including DPA, are rapid, can be used to differentiate between nucleic acid types (i.e., DNA from RNA) with no need to isolate the components, and be used to detect the presence of reducing sugars. Furthermore, a comparative study of DNA measurement methods revealed that the DPA method gave the highest accuracy (Li et al. 2014) and, when used to measure DNA in the complex matrices found in many types of soil, this method was shown to be specific and not compromised by extraneous organic matter (Lajmi et al. 2020). It was these more recently reported beneficial aspects of the DPA assay coupled with its apparent versatility that prompted the experiments described here. Thus, in this study, we set out to determine the suitability of the DPA colorimetric method for quantifying the growth of *G. sulphuraria* and other laboratory-cultured microalgae. A secondary objective was to illustrate quantification of algal growth in a complex matrix such as an aquaculture wastewater-based medium.

Materials and methods

Algal strains and culture conditions

The isolate, *Galdieria sulphuraria* strain SAG 21.92, used in this study was obtained from the Institute for Food and Environmental Research (ILU), Nuthetal, Germany. Cells were recovered from the agar plates and inoculated into a modified Allen medium prepared as described elsewhere (Allen and Stanier 1968; Barone et al. 2020). Cultures were incubated on an orbital shaker in the laboratory at 25 °C in continuous light supplied by two 15 W cool daylight bulbs (Eveready Battery Company, Inc., USA) each providing a

total amount of visible light equivalent to 825 lm for periods up to 30 days. Sub-culturing was by transfer of an appropriate volume of cells to fresh Allen medium to make a 2% inoculum and was carried out every 30 days.

For the purposes of this study, aquaculture wastewater was sourced from a pilot-scale system located at the Department of Ichthyology and Fisheries Science, Rhodes University. This demonstration-scale aquaponics system was designed and configured for the coupled cultivation of catfish (CF-WW) and spinach (CF-S-WW) as shown in the schematic of the process flow (Fig. 1). Grab samples of wastewater (5-L aliquots) were from the sampling points indicated (see Fig. 1) and the resulting composites, filtered through 16 layers of miracloth (Merck, Millipore, South Africa) and the pH, electrical conductivity (EC), chemical oxygen demand (COD), metal ion, ammonium nitrogen (NH_4^+-N); nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) concentrations determined (Table 1) and the water stored in darkness at 4 °C until needed.

Prior to use for the acclimatization and enrichment of this microalga, the aquaculture (catfish, CF-) and aquaponics (catfish-spinach, CF-S-) wastewaters were autoclaved (121 °C for 15 min), cooled to room temperature and 50 mL *G. sulphuraria* SAG 21.92 seed culture in modified Allen medium inoculated into Fernbach flasks containing 490 mL of either full strength or half-strength filter-sterilized aquaculture wastewater (CF-WW) or full-strength aquaponics wastewater (CF-S-WW). Following several enrichment passages i.e., subculture in the various wastewaters using a 2% inoculum, cultivation proceeded under the conditions described above and aliquots of cell culture were removed at the specified intervals for biomass determination and spectrophotometric determination of culture density and cell growth.

Table 1 Physicochemical parameters of the catfish (CF-WW) and catfish-spinach wastewater (CF-S-WW) used for cultivation of *Galdieria sulphuraria* strain SAG 21.92

Parameter (unit)	CF-WW	CF-S-WW
pH	7.2	7.7
Electrical conductivity (mS m^{-1})	251	304
Sodium adsorption ratio (SAR, $mmol_c$ L^{-1})	7.5	8.8
Dissolved sodium (mg L^{-1})	345	444
Dissolved calcium (mg L^{-1})	55.4	67.2
Dissolve magnesium (mg L^{-1})	63.1	77.0
Dissolved potassium (mg L^{-1})	3.8	< 1.0
Chemical oxygen demand (COD, mg L^{-1})	15.0	48.0
Ammonium-N (mg L^{-1})	13.9	14.6
Nitrate–N (mg L^{-1})	302.1	68.7
Nitrite-N (mg L^{-1})	67.3	2.1

Seed cultures of the chlorophytes *Chlorella* sp. and *Scenedesmus* sp. and the diatom, *Phaeodactylum* sp., previously isolated from HRAOP of the demonstration-scale IAPS located at the Belmont Valley Wastewater Treatment Works (WWTW), Makhanda, South Africa (33° 19′ 07″ S; 26° 33′ 25″ E) as described elsewhere (Johnson 2010; Jimoh 2021; Keshinro et al. 2024), were inoculated into flasks containing Bold Basal medium (prepared according

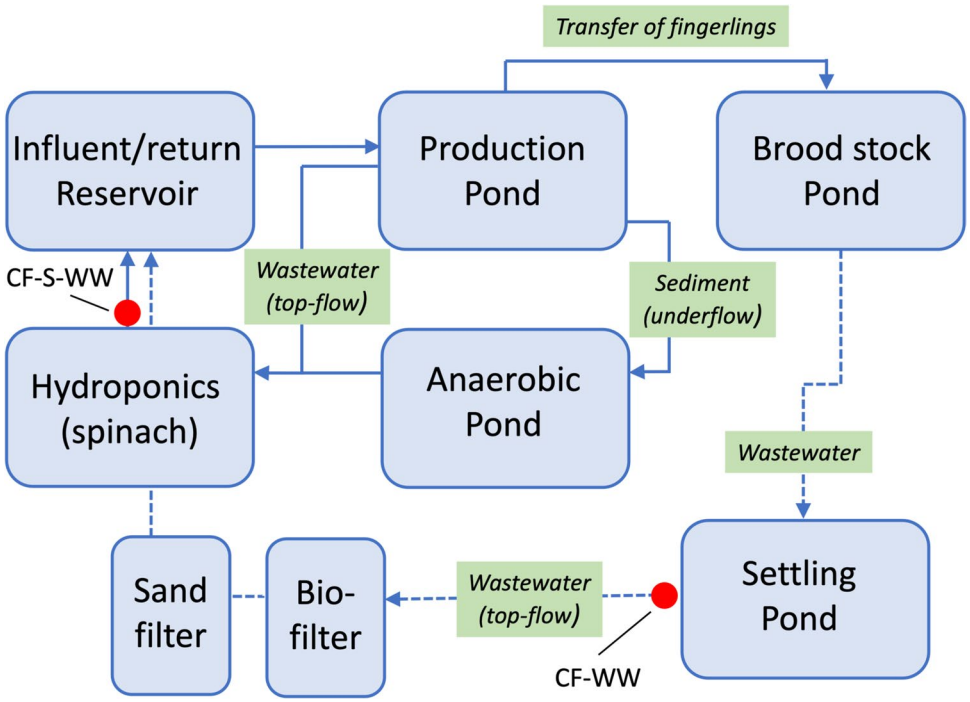

Fig. 1 Schematic of the configuration and process flow of the experimental demonstration-scale recirculating aquaculture-aquaponics system for catfish and spinach production. Stippled lines distinguish the aquaculture train from the aquaponics train of this laboratory-scale demonstration system. Closed red circles indicate the wastewater sampling points. CF-S-WW = catfish-spinach (i.e., aquaponics) wastewater; CF-WW = catfish (i.e., aquaculture) wastewater

to the UTEX Algal Culture Media recipe, 2019) and incubated using an orbital shaker in the laboratory as described above. At intervals, samples of algal culture were harvested and used to investigate the relationship between OD_{750}, biomass, and growth measured at 595 nm using DPA.

Analytical methods

Analysis of aquaculture and aquaponics wastewater

Physicochemical parameters determined for aquaculture and aquaponics wastewater included EC, measured using an EC Testr 11 Dual range 68 × 546,501 m (Eutech Instruments, Singapore) and pH, determined using a Hanna HI8 424 pH meter (Hanna Instruments, USA). For determination of chemical oxygen demand (COD) and dissolved Na, Ca, Mg, and K, water samples were collected and shipped on the same day to Bemlab, Gant's Centre, Strand, South Africa, for analysis by a SANAS-accredited testing laboratory in accordance with ISO 17025:2005, for chemical analyses of soil, leaves, fruit, and water.

Ammonium-nitrogen (NH_4^+-N) was determined using Nessler's reagent (Masudi et al. 2024) and the amount of free ammonia (NH_3) quantified spectrophotometrically at 430 nm followed by interpolation from a standard curve for NH_4Cl (Jeong et al. 2013).

Nitrate–N (NO_3^--N) and nitrite-N (NO_2^--N) were quantified using a method adapted from Miranda et al. (2001). In brief, NO_3^--N was quantified spectrophotometrically by adding to 10 mL of wastewater 2.5 mL of sulfanilic acid solution (1.7 g sulfanilic acid dissolved in 500 mL of 15% acetic acid) which was incubated for 5 min before the addition of 2.5 mL of *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) solution (0.7 g of NEDD dissolved in 500 mL of 15% acetic acid) followed by dilution to 100 mL with distilled water and measurement of the absorbance at 540 nm. Nitrate–N determination was by addition of 3.5 mL of wastewater sample to 96.5 mL of distilled water. Then, 10 mL of this diluted sample was added to 2.5 mL sulfanilic acid solution which was incubated for 5 min prior to addition of 2.5 mL of NEDD solution. This was made up to 100 mL with distilled water and the absorbance measured at 540 nm. Nitrate and nitrite quantities were determined by interpolation from the nitrate and nitrite standard curves.

Determination of culture density, biomass and algal growth

Culture density (OD_{750}) was determined spectrophotometrically at 750 nm using a UV-1280 UV–VIS Spectrophotometer (Shimadzu). All values were determined in the linear range and within the standard error of OD_{750} measurements.

For biomass determination and quantification of growth, cell pellets were collected from 1 mL aliquots of cell culture by centrifugation at 10,000 × *g* for 10 min and then washed twice with phosphate-buffered saline (PBS, 0.1 M, pH 7). Algal biomass was determined by measuring the dry mass of oven-dried (~ 50 °C until constant mass was achieved) aliquots of cell culture, after centrifugation and removal of the supernatant, using an analytical balance (Sartorius TE3102S) with readability: 0.01 g.

Quantification of algal growth was achieved using the diphenylamine (DPA) colorimetric assay which was performed essentially as described by Burton (1956) and Zhao et al (2013). The DPA solution was prepared by dissolving 1.5 g DPA (Sigma-Aldrich, USA) in 100 mL of glacial acetic acid followed by addition of 1.5 mL of concentrated H_2SO_4 and the colorless solution stored in darkness. Immediately prior to use, 1.6% (by vol.) aqueous acetaldehyde was added. For quantification by DPA, pellets were resuspended in 2 mL of this reagent by vortexing and incubated at 60 °C for 60 min. The supernatant from individual reactions was collected after centrifugation and the absorbance at 595 nm was measured spectrophotometrically using a UV-1280 UV–VIS Spectrophotometer (Shimadzu). All manipulations were carried out in an ordered sequence and where possible duplicate samples were analyzed. Growth data are representative of three or more determinations, each from an independent 30-day culture cycle.

Determination of cell viability

Cell viability of algal cultures was routinely determined at the conclusion of each growth cycle, and prior to either subculture or destructive sampling and analysis, using the non-toxic and ready-to-use alamarBlue Cell Viability Reagent. AlamarBlue is a resazurin-based assay and indicator of cell health which is determined by quantifying the viable reduction of resazurin to resorufin ($\lambda_{max} = 570$ nm) as described by Lavogina et al. (2022).

An aliquot (200 µL) of the algal sample was placed into wells of microtiter plates, 20 µL alamarBlue Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific) added to each well, mixed using an autopipette and the reference absorbance measured at 570 nm using a BioTek Epoch 2 microplate reader (Agilent Technologies, USA) and then incubated at 37 °C for periods of 4-h, after which the absorbance was again measured at 570 nm. The difference in absorbance was calculated and corrected for controls comprising the appropriate dilution of the respective aquaculture or aquaponics wastewater.

Statistical analysis

Data were computed using the statistical function in Sigma Plot version 11 (Systat Software Inc., USA) or Excel (version 16, Microsoft Corporation, USA). Where necessary,

the linear regression operator within Sigma Plot was used to establish R^2 values from lines of best fit.

Results and discussion

To innovate an algae-based bioprocess for converting and recirculating side streams (i.e., settled sludge and wastewater) in dryland aquaculture, the integrated algal pond system (IAPS) was selected as a technology of choice. As elaborated by Laubscher and Cowan (2020), an appropriately scaled IAPS has the potential to generate > 75 m^3 day^{-1} water, > 9 kg day^{-1} biomass for processing, and > 0.5 m^3 day^{-1} high nitrogen-containing (> 1 g L^{-1}) liquid organic fertilizer. In the IAPS process, the alga is inoculated into and cultivated in paddlewheel-mixed HRAOP. The growth medium used in these shallow channels is the decant from an advanced facultative pond (AFP) containing, in its base, a submerged anaerobic digester designed to operate as an up-flow anaerobic sludge blanket (UASB) reactor. These reactors are based on a three-phase separator principle, which enables the separation of gas, water, and sludge in turbulent conditions. Effluent from fish grow-out ponds enters the UASB at the base of the facultative pond with flow directed upwards at a rate not exceeding 1–1.5 m day^{-1} and the overflow from the AFP directed under gravity into the HRAOPs (Laubscher and Cowan 2020). Continuous operation is facilitated by balanced inflow into the IAPS and outflow to settlers where separation of the algae from the treated water occurs by gravity sedimentation (Dube and Cowan 2023). A pilot-scale version of this concept circular approach for dryland aquaculture was used as a source of wastewater for the cultivation, acclimatization, and enrichment of *G. sulphuraria* SAG 21.92. And to facilitate quantification of algal growth in a complex matrix such as an aquaculture wastewater-based medium, the suitability of the DPA colorimetric method was examined.

Typical growth response curves for seed cultures of this microalga cultivated in 1-L Fernbach flasks containing either modified Allen medium, full strength, or half-strength filter-sterilized aquaculture wastewater (i.e., catfish wastewater, CF-WW) or full-strength aquaponics wastewater (i.e., catfish-spinach wastewater, CF-S-WW) are shown in Fig. 2.

Growth in modified Allen medium was, as expected, superior and continuous to a maximum at 32 days after culture initiation. Although the response of *G. sulphuraria* was similar in 100% CF-WW, cell growth was slightly reduced. Growth in half-strength CF-WW was slow presumably due to nutrient, more specifically nitrogen limitation (Pancha et al. 2014; Zarrinmehr et al. 2020). Growth of *G. sulphuraria* in CF-S-WW mirrored growth in 100% CF-WW up to 10 days after culture initiation. Thereafter, algal growth in undiluted CF-S-WW remained suppressed with a protracted lag period, possibly related to the higher COD concentration of CF-S-WW most likely due to the well-known exudation of spinach-derived organic acids coupled with nutrient (i.e., NO_3^--N) limitation (Yang et al. 2005; Joshi et al. 2021). This was confirmed for the CF-S-WW cultured *Galdieria* prior to completion of growth using the alamarBlue Reagent. While cell viability was apparently unaffected and sustained for *G. sulphuraria* cultivated in CF-WW, the viability of *G. sulphuraria* cells in cultures produced in CF-S-WW was markedly reduced (Table 2).

Measurement of cell growth by quantification of DNA using the diphenylamine (DPA) colorimetric method is an apparently reliable and sufficiently sensitive method, with a limit of detection in the range 1 to 3 µg (Burton 1956; Gendimenico et al. 1988). And quantification of cell growth of model microorganisms including the bacteria *Escherichia coli* and *Streptomyces clavuligerus* and the fungi

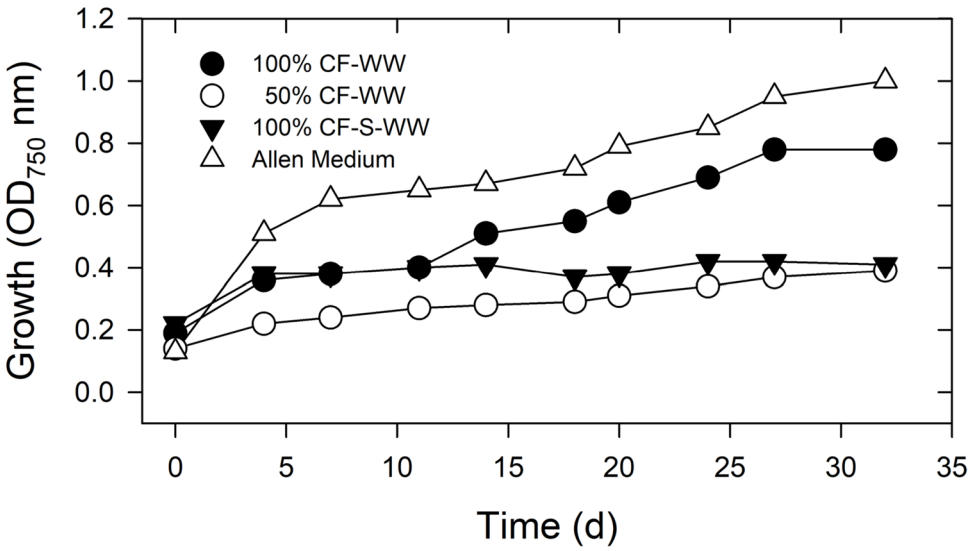

Fig. 2 Growth of *Galdieria sulphuraria* strain SAG 21.92. Cells from stock culture were inoculated into modified Allen medium, 100% and 50% catfish wastewater (aquaculture) and into 100% catfish-spinach wastewater (aquaponics) and growth monitored for approximately 4 weeks by measurement of OD at 750 nm

Table 2 Cell viability of *Galdieria sulphuraria* cultivated in aquaculture and aquaponics wastewater. Algal cultures were established in full strength or half-strength filter-sterilized aquaculture wastewater (i.e., catfish wastewater, CF-WW) or full-strength aquaponics wastewater (i.e., catfish-spinach wastewater, CF-S-WW) as described for Fig. 2 and cell viability determined using alamarBlue Reagent

Time (h)	CF 100% A_{570} nm (% change)	CF 50%	CF-S
t = 0	0.878 ± 0.005 (0)	0.730 ± 0.045 (0)	0.769 ± 0.068 (0)
t = 4	1.533 ± 0.654 (74.6)	1.228 ± 0.032 (68.2)	1.035 ± 0.155 (34.6)

Saccharomyces cerevisiae and *Trichoderma reesei*, using DPA, has been successfully demonstrated (Zhao et al. 2013). To determine whether this colorimetric method could be applied to and adapted for the measurement of microalgal growth we sought to verify its reliability and sensitivity under our conditions using cultures of *G. sulphuraria*.

Initially, actively growing phototrophic cultures of this microalga were chosen and diluted (with culture medium) to within the linear range and, cell density, DNA concentration, and biomass were then quantified from the same sample. Quantifying these parameters in the same sample was required to cement the relationship between culture biomass as the independent variable and the dependent variables as indicators of growth obtained by measuring culture density at OD_{750} or DNA concentration using DPA at A_{595}. In short, volume was transformed to biomass which was in turn, related to culture density and DNA concentration. Measurement of culture density was by spectrophotometry at OD_{750}. Biomass of the algal culture was established by determining the dry mass of the algal pellet after centrifugation and removal of any residual culture medium. The DNA concentration, as a function of the amount of purine-released 2-deoxyribose, in the precipitated biomass was assayed using DPA at A_{595} nm and a summary of the results from this series of studies is shown in Fig. 3.

It was determined, using Pearson's correlation analysis that there was a strong linear relationship between OD_{750} ($r^2 = 0.986$, $p < 0.01$), A_{595} using the DPA method ($r = 0.989$, $p < 0.01$) and dry biomass ($r^2 = 0.994$, $p < 0.01$) as a function of culture volume following cultivation of *G. sulphuraria* in CF-WW (Fig. 3a). These findings indicate that the simplified DPA colorimetric method is potentially a sufficiently reproducible and rapid colorimetric method for the quantification of *G. sulphuraria* growth in phototrophic culture. Results in Fig. 3b confirmed the linear relationship between an increase in cell culture dry biomass and the increase in either cell density (OD_{750}) or an increase in DNA concentration (A_{595}). Furthermore, the DPA colorimetric method appeared to be reliable for the quantification of microalgal growth in early (dilute) and late-stage mature cultures, viz. for *G. sulphuraria* at 4- and 20-days after culture initiation (Fig. 3c).

To further establish the appropriateness of the DPA method for quantifying growth of *G. sulphuraria* in wastewater matrices, the change in culture density at OD

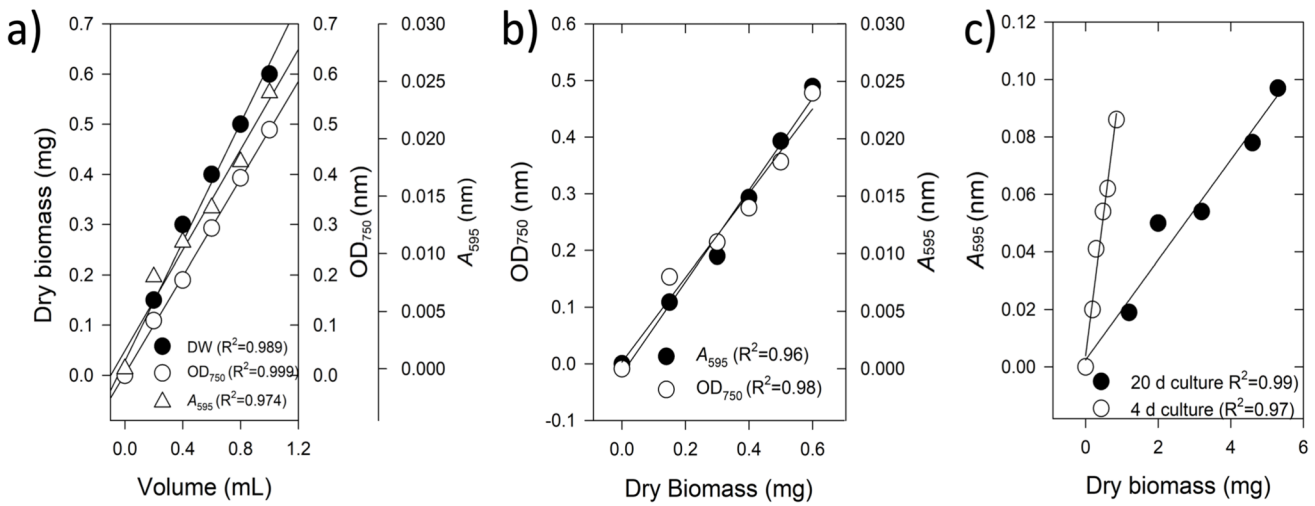

Fig. 3 Relationship between culture volume and dry biomass and between culture volume and growth quantified at OD_{750} and by determination of DNA concentration (**a**), dry biomass and growth quantified at OD_{750} and by determining DNA concentration (**b**) and dry biomass and growth in cultures of different age (**c**), of *Galdieria sulphuraria* cells cultivated in aquaculture wastewater. Dry biomass, OD_{750} and the concentration of DNA were quantified in increasing volumes of cultures of *G. sulphuraria* during the linear phase of growth. Dry biomass was determined gravimetrically. OD was measured at 750 nm while the concentration of DNA was determined colourimetrically at A_{595} following the addition of DPA. Regression analysis is representative of several independent experimental determinations

750 nm and DNA concentration using DPA at A_{595} were measured for cells cultured in wastewater sourced from the demonstration aquaculture process stream for catfish (CF) production and from the aquaponics process stream for catfish and spinach (CF-S). Results show that values for OD_{750} are routinely higher and as expected, that growth in full strength CF-WW (Fig. 4 a) was almost double that of the response in 50% CF-WW (Fig. 4 b). In the aquaponics wastewater however, i.e., CF-S-WW derived after passage of the CF effluent via a spinach hydroponics cultivation train, algal growth measured as absorbance at OD_{750} was distinctly separate from the colorimetric measurement of growth using DPA (Fig. 4c). This separation was most likely due to background absorption which limits resolution of spectrophotometric assays by interfering with or masking relevant absorption features. In this example, the contributing factors are presumably root exudates from the growing seedlings and in particular, the well-studies efflux of oxalates from spinach root tips (Yang et al. 2005; Joshi et al. 2021). This result supports and extends other reports on the use of the DPA colorimetric method for DNA quantitation in samples from complex matrices (D'Abzac et al. 2010; Bourven et al. 2015; Lajmi et al. 2020).

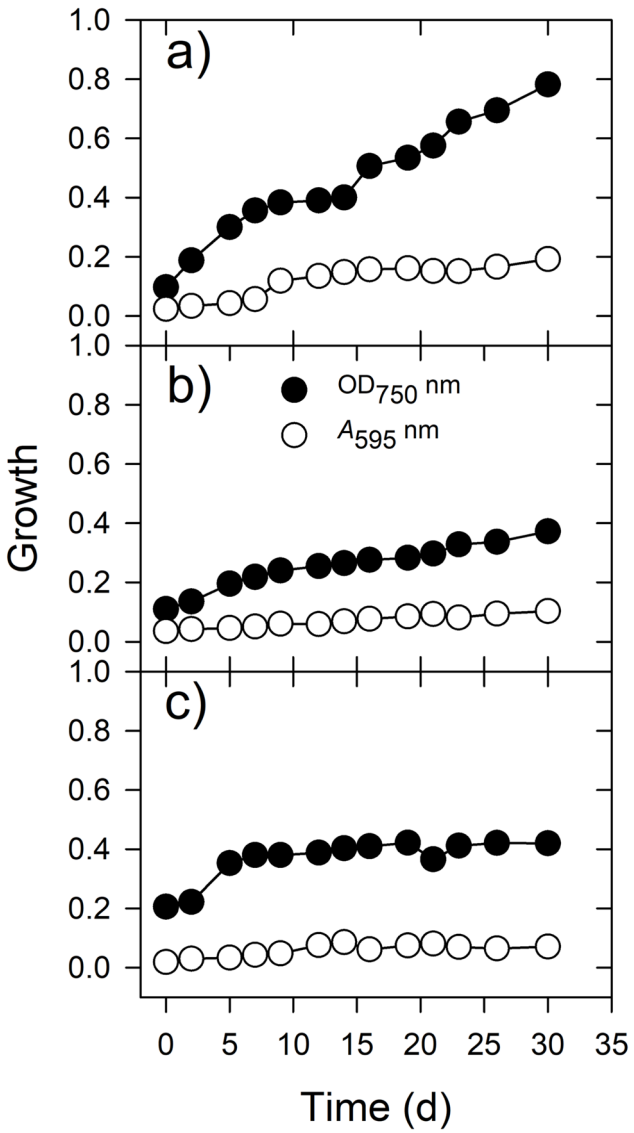

Fig. 4 Contribution of the culture medium to estimations of microalgal growth measured as OD_{750} or DNA concentration at A_{595} for *Galdieria sulphuraria* in 100% CF-WW (**a**), 50% CF-WW (**b**), and 100% CF-S-WW (**c**). OD was measured at 750 nm while the concentration of DNA was determined colourimetrically at A_{595} following the addition of DPA. Results are a typical representation of the growth response of *G. sulphuraria* in these wastewaters

To further investigate the applicability of the DPA colorimetric assay for estimating growth in microalgae, actively growing cultures of strains of locally sourced and isolated *Chlorella* sp., *Scenedesmus* sp. and the diatom, *Phaeodactylum* sp., from HRAOP and cultivated in Bold Basal medium, were used to test the relationship between cell culture dry biomass, culture density (OD_{750}) and cell culture DNA concentration (A_{595}). For each isolate, and as shown in Fig. 5a, b and c, an increase in algal dry biomass was linearly corelated with the increase in growth quantified either by monitoring OD_{750} ($r^2 = 0.97$, 0.96 and 0.90 respectively) or DNA concentration using DPA at A_{595} ($r^2 = 0.96$, 0.96 and 0.96). Taken together, these findings appear to confirm the suitability of DPA as a simple and relatively inexpensive method for the rapid determination of cell growth in microalgal cultures.

Optical density remains a rapid, convenient and popular method for the estimation of microbial cell growth. Although optical density of a cell culture is accepted as a measure of the relative number of photons of light from a source that pass through a 1 cm path i.e., the reciprocal of transmittance (Koch 1968) and, which for bacteria is measured at 600 nm (OD_{600}) and for most microalgae 750 nm (OD_{750}), its use and accuracy have recently been questioned (Mira et al. 2022). These authors argue that cells mL^{-1} rather than OD is a more accurate metric for the direct comparison of results across experiments, instruments, and species. Likewise, results from the present study show that quantification of DNA in microalgal cell cultures using the one-step DPA reagent assay is a more accurate measure than OD_{750}, reliable, easy to perform, and appropriate as a measure of growth in the mass cultivation of microalgae. Furthermore, interference by membrane-bound pigments including chlorophylls and carotenoids, and phycocyanin and phycoerythrin which absorb significantly at 595 nm, contrary to the caution expressed by Zhao et al. (2013), did not interfere with quantitation. Removal of residual cell debris by centrifugal filtration (0.22 µm) rendered a colourless supernatant suitable for spectrophotometric quantification at A_{595} and which routinely showed a linear relationship ($r^2 > 0.9$) between this metric and either cell culture volume or dry biomass.

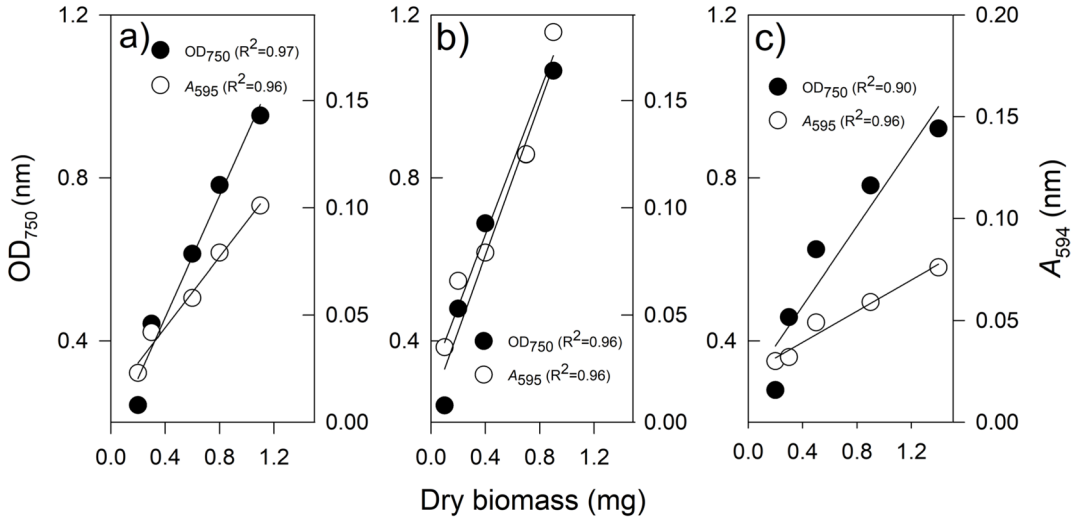

Fig. 5 Relationship between microalgal dry biomass and growth in cultures of *Chlorella* sp. **a**), *Scenedesmus* sp. (**b**), and *Phaeodactylum* sp. (**c**) cultivated in Bold basal medium. Dry biomass, OD_{750} and the concentration of DNA were quantified for increasing mass amounts of each microalga during the linear phase of growth. Quantification was as described in Fig. 3. Regression analyses represent one of several independent experimental determinations for each microalgal species

Conclusion

Methods for the determination of culture density in both indoor laboratory- and outdoor commercial-scale microalgal systems have been long debated and fraught with challenges. Of concern has been the wavelength for measurement of OD, interference by other organics extruded into the medium by actively growing cells that change both the turbidity and spectrophotometric properties of the culture solution, the contribution of nutrients and salts in the interstitial volume to dry biomass estimates, and the skills level required to operate most mechanised cell counting techniques. By comparison, besides access to a spectrophotometer no specialized laboratory equipment is needed to carry out the DPA assay. However, where culturing is outdoors and without imposition of selective pressures (i.e., osmotic, alkalinity, nutrient, light stress, etc.), consideration must be given to the occurrence of contaminating species i.e., bacteria, fungi, protozoans, etc. Where near axenic laboratory algal cultures are used, as in the present study, results appear to confirm DPA as an appropriate alternative for the rapid colorimetric quantification of microalgal growth for cultures of the red alga, *G. sulphuraria*, the chlorophytes *Chlorella* sp. and *Scenedesmus* sp., and the diatom *Phaeodactylum* sp.

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Data availability Datasets generated and/or analysed during the study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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