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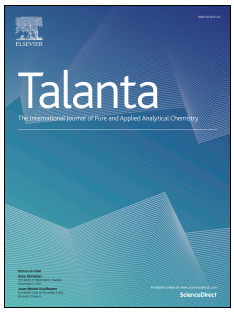

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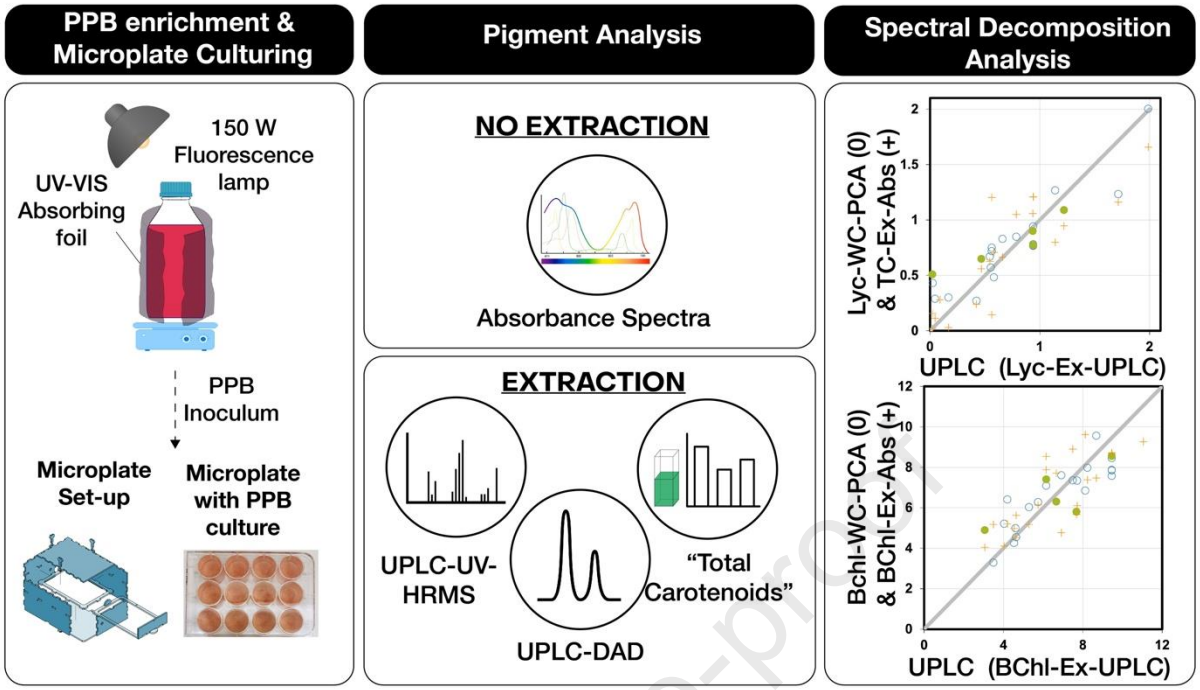
Graphical abstract



Method development for PPB culture screening, pigment analysis with UPLC-UV-HRMS vs. spectrophotometric methods, and spectral decomposition-based analysis

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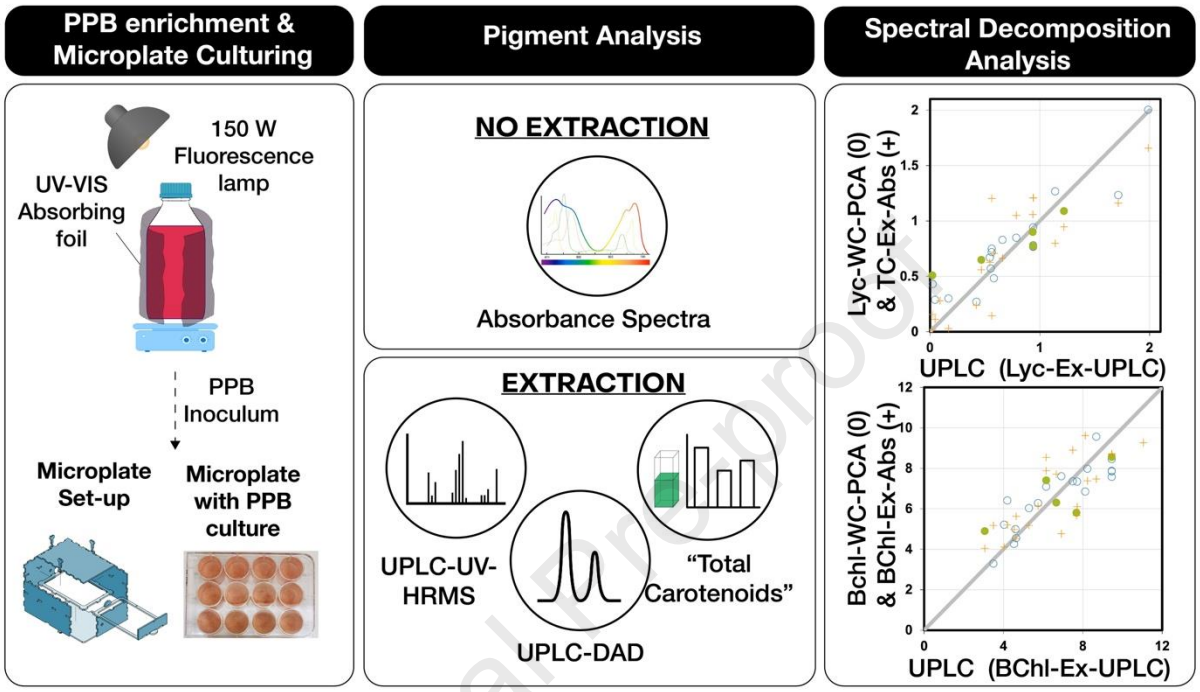
Abstract

PPB carotenoids are usually measured through spectrophotometric analysis, measuring total carotenoids (TCs) which has low accuracy and cannot identify individual carotenoids or isomers. Here, we developed an ultra-performance liquid chromatography method with ultraviolet and high-resolution mass spectrometry detection (UPLC-UV-HRMS) to quantify neurosporene, lycopene, and bacteriochlorophyll a contents in PPB cultures. The method exhibited satisfactory recoveries for individual pigments (between 82.1% and 99.5%) and was applied to a range of mixed PPB cultures. The use of a C_{30} column also enabled the detection of three different isomers of lycopene. In addition, a method for anaerobic photoheterotrophic PPB cultivation to acquire live-cell spectrophotometric information was developed and tested by modifying a standard microbial culture microplate system. A rapid, and relatively low effort principal component analysis (PCA) based decomposition of the whole-cell spectra for pigment analysis in the microplates was also developed. Analysing whole-cell spectra via PCA allowed more accurate prediction of individual pigments compared to absorption methods, and

can be done non-destructively, during live-cell growth, but requires calibration for new media and microbial matrices.

Graphical abstract



Keywords

Purple phototrophic bacteria; Pigments; Carotenoid; Lycopene; Bacteriochlorophyll

Highlights

- A UPLC-UV-HRMS method to detect and quantify PPB pigments was developed
- A C_{30} column enabled detection and quantification of two lycopene isomers
- A microplate method for photoheterotrophic PPB cultivation was developed
- Pigment contents can be determined by PCA on the whole-cell spectra

Acronyms and Symbols

APCI	Atmospheric pressure chemical ionization
ASV	Amplicon sequencing variants
BChl	Bacteriochlorophyll
BHT	Butylated hydroxytoluene
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
$E_{\overline{X}}$	Estimated error at 95% confidence level
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography-mass spectrometry
HRMS	High-resolution mass spectrometry
IR	Infrared
LC	Liquid chromatography
LED	Light-emitting diode
LHC	Light harvesting complexes
MeOH	Methanol
MP	Mobile phase
MTBE	Methyl-tert-butyl ether
OD	Optical density
PCA	Principal component analysis
PC	Principal component
pCOD	Particulate COD

PCG	Photosynthetic gene cluster
PPB	Purple phototrophic bacteria
R^2	Coefficient of determination
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RSD	Relative standard deviation
SCOD	Soluble chemical oxygen demand
SCP	Single-cell protein
S_{Xi}	Standard deviation
TC	Total carotenoids
TCOD	Total chemical oxygen demand
UPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
VIS	Visible
$\overline{X}$	Average value

1 Introduction

The production and extraction of natural carotenoids from microbial cultures is an area of research receiving increasing attention. To date, around 600 naturally occurring carotenoids with various functional properties have been identified [1, 2]. Natural carotenoids are significantly superior to synthetic compounds in terms of beneficial properties [3] but also incur significantly higher production costs compared with synthetic products. This is mainly due to harvesting and extraction, which account for up to 90% of the production costs [4, 5]. For

example, the β-carotene extraction costs from *Dunaliella salina are* 10.38 ± 0.99 USD kg^{-1} when using organic solvents and 7.7 ± 0.83 USD kg^{-1} with supercritical carbon dioxide extraction [6]. As an alternative, carotenoids contained in pure or mixed microbial cultures can be used as whole-cell products (*e.g.* as carotenoid-rich single-cell protein (SCP)) [7]. In this context, purple phototrophic bacteria (PPB) are a group of anoxygenic phototrophic microorganisms that generate bacteriochlorophyll (BChl) and carotenoids during photoheterotrophic growth under anaerobic-illuminated conditions. Without dedicated content manipulation, carotenoid contents of PPB biomass were reported in the range of 1.0 to >12 $mg \cdot VS^{-1}$ [8], but can reach >1.7 $g_{carotenoids} \cdot L^{-1}$ in concentrated biofilms [9].

The most commonly used method to quantify the carotenoid content in PPB biomass has been solvent (acetone/methanol) extraction combined with ultraviolet-visible (UV–VIS) absorption detection, measuring the TC content. However, the accuracy of this quantification method for PPB with an unknown carotenoid composition is questionable because the extinction coefficients are unknown, especially for carotenoid mixtures. However, PPB samples normally consist of an unknown, complex matrix of more than one carotenoid, which differs depending on the species, growth stage, and growth conditions [10].

Separation through liquid chromatography on pigment extract is required to determine the carotenoid profile. The use of analytical standards in High Performance Liquid Chromatography (HPLC) studies on PBB carotenoids is rare and the identification is usually based on the retention time and UV spectra [11-13]. The main reason for this is that carotenoid standards are expensive and notoriously unstable due to UV light, temperature, and oxygen enhanced degradation [14, 15]. A further limitation of PPB studies using HPLC is the widespread use of C_{18} columns, which are not able to resolve geometrical isomers and inefficiently resolve positional isomers [11-13]. Yet, in nature carotenoids exist in different isomer forms (the main difference with synthetic products), which results in different bioavailability and effects when ingested with the diet [16, 17]. A non-endcapped reverse phase (RP)-HPLC column with triacontyl (C_{30}) can be used to maximise chromatographic

resolution and selectivity of carotenoids and their isomers [18, 19]. High-performance and ultra-performance liquid chromatography coupled with mass spectrometry (HPLC-MS, UPLC-MS) and using suitable analytical standards are the most effective tools for the detection and quantification of PPB carotenoids [20, 21]. However, methods based on these techniques are expensive, somewhat slow, and cannot be used *in* or *ex-situ* during growth experiments (*e.g.*, to determine maximum carotenoid extent), since an extraction and analysis process is required.

This study aims to address pigment identification and quantification in PPB by using ultra-performance liquid chromatography with UV and high-resolution mass spectrometry detection (UPLC-UV-HRMS), to analyse and quantify neurosporene, lycopene, and BChl a. We also developed a modified high-throughput microplate culture protocol to enable anaerobic photoheterotrophic PPB cultivation with live-cell absorption spectrum collection (in a plate reader). Whole (live) cell, as well as extract spectra are decomposed by principal component analysis, and the principal components analysed for the ability to predict UPLC extract analysis of lycopene and BChl. This provides a non-destructive, rapid pigment analysis method for PPB cultures, also assessing the relevance of spectrophotometric methods for pigment quantification.

2 Material and methods

2.1 Inoculum, media composition, and enrichment procedure

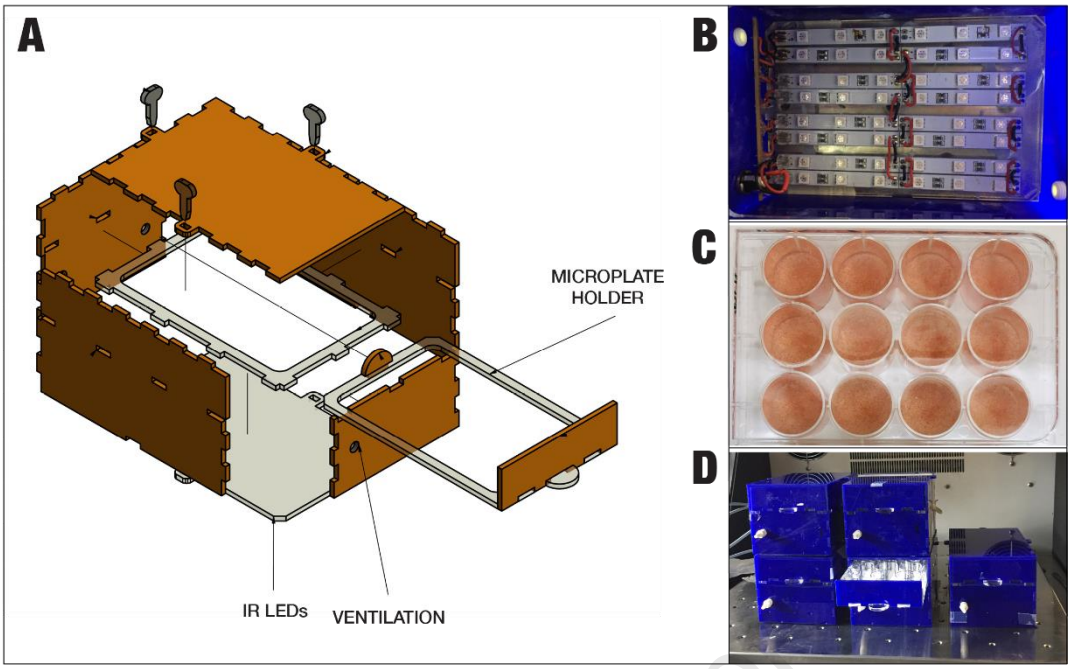
Six enrichments cultures were started using inoculum sources from sediments harvested from the Brisbane River (Tuckean Broadwater, -28.987226, 153.407759) and Ballina Mangrove Habitat (28.84159418, 153.5703618) in New South Wales (Australia), as well as samples of poultry and domestic wastewaters (Brisbane, Australia). NaCl was added to the enrichments in different concentrations to increase the salinity to 30 and 70 $mS \cdot cm^{-1}$, aiming to select for halotolerant and halophilic PPB genera [22] and thus increase the overall microbial diversity,

which would allow to test and validate the proposed pigment quantification method (see Section 2.4) under different scenarios. The resulting enrichments were designated as E1-E6, corresponding to sediments from Tuckean Broadwater and sea salt addition of 30 $mS \cdot cm^{-1}$ (E1), Ballina Mangrove Habitat, and salt addition of 30 $mS \cdot cm^{-1}$ (E2) and 70 $mS \cdot cm^{-1}$ (E3), Brisbane River (E4), poultry processing wastewater (E5) and domestic wastewater (E6).

For these enrichments, six reactors (Schott bottles with a working volume of 500 mL) were filled with 400mL of the different environmental sources and inoculated with 500 mg $COD \cdot L^{-1}$ of acetic acid, and the different NaCl concentrations for 10 days, until a visible colour shift developed. Subsequently, the enrichments were maintained and fed every 5 days with fresh, modified Ormerod medium [23], containing 500 mg $COD \cdot L^{-1}$ of acetic acid and 405 $mg \cdot L^{-1}$ of NH_4Cl. The headspace of the bottles was flushed with N_2 to remove oxygen traces. Afterwards, the pH was adjusted to 6.8 (2M NaOH) and the bottles were closed and covered with UV-VIS light absorbing foil (ND 1.2 299, Lee Filter, Transformation Tubes) to exclude wavelengths lower than 790 nm. The bottles were incubated at ambient temperature (23-25 ^oC) mixed with magnetic stirrers at 150 rpm, and they were illuminated from the side with a 150 W fluorescence lamp (Nelson Clamp Flood Light), resulting in an incident intensity of ~90 $W \cdot m^{-2}$.

2.2 Microplate set-up and tests for in vivo and extracted spectral data acquisition

A series of custom-made stackable, non-transparent acrylic boxes (139 x 92 x 80 mm) were used to fit 12-well, transparent polystyrene microplates in an integrated, removable plate holder. This system allowed to minimise external light effects, avoid light attenuation issues and enable easy access for sampling (Figure 1).



159

160 **Figure 1**. (A) Schematic of the acrylic box, (B) LED stripes attached on the bottom of the
161 boxes, (C) an example of PPB cultures grown in the set-up, and (D) picture of the set up
162 showing a microplate.

163 Microplates were clear flat-bottom polystyrene plates with 12 wells, each with a volume of 6.9
164 mL (Costar®, Bio-Strategy Pty Limited, Tullamarine, Australia). The plate material did not
165 attenuate the light based on measurements (data not shown). Each box encased 4 rigid strips
166 containing 12 infrared (IR; 850 nm/940 nm) light-emitting diodes (LEDs, LEDLightsWorld LTD)
167 placed at the bottom, at a distance of 50 mm from the microplate holder, to ensure optimal
168 light distribution (Figure 1B) (Note, NIR LED could be exchanged with other light sources to
169 study the effects). A dimmer enabled light intensity changes between 0 and 40 $W \cdot m^{-2}$ for each
170 set-up. The light intensity was measured with a StellarNet BLUE-Wave Spectrometer
171 (StellarNet Inc, Florida, USA). Specifically, the emitted irradiances were measured at 12
172 coordinates, corresponding to each of the microplate's wells. Irradiation levels were recorded
173 via SpectraWiz spectrometer software (OS v5.33, 2014, StellarNet Inc, Florida, USA). The
174 obtained grid point values were interpolated cubically over the irradiated area to calculate the
175 incident irradiance.

To start the test, a glass bead (3 mm) was placed inside each microplate well to ensure proper mixing within the shaker before the wells were filled to maximum capacity with liquid (and inoculum) to minimise the presence of oxygen. The microplate was carefully sealed with a transparent, non-O_2 permeable, adhesive film (MicroAmp™ Optical Adhesive Film, In Vitro Technologies, Australia), avoiding the formation of bubbles between the liquid and the film to ensure correct optical readings. The boxes containing the microplates were placed in a temperature-controlled Shaker (ThermoFisher-MAXQ4000-Incubator-110, Thermo Fisher Scientific, Massachusetts, and the USA), and experiments were conducted at ~28 °C and 150 rpm.

Microplate tests were performed to acquire in-vivo UV spectra data and to extract samples for carotenoid extraction followed by spectrophotometric and UPLC-UV-HRMS analyses. Five microplates were inoculated with a 10 vol% of PPB inoculum, fed with Ormerod media containing 500 mg COD·L^{-1} of acetic acid. The microplates were illuminated with different IR light intensities (dimmable LEDs) (*i.e.,* at 1.7, 5.7, 14.2, and 33.1 W·m^{-2} (later referred to as 5 batch tests in microplates). A non-illuminated dark control microplate was executed in equivalent non-illuminated setups. Full wavelength scans (1-1000 nm) were performed every 5 h approximately, in a plate reader (CLARIOstar Plus, BMG Labtech) of the whole-cell (WC), in-vivo and previous to any pigment extraction. To follow biomass growth, the optical density at 660 nm was used as a PPB biomass proxy (OD_{660}) (Figure S2). Wells were sacrificially sampled from random locations at t_{10}, t_{20}, t_{30} h after incubation, and at t_{final} (~55 h). The sample at t_0 was taken from the bulk initial mixture. Samples were extracted from the wells with a syringe with a needle, to avoid removing the film covering the microplate. Approximately 2 mL of sample was immediately filtered with a 0.45 mm membrane filter (Millipore, Millex®-HP) for SCOD analysis. The remaining unfiltered sample was stored at -20 ℃ and later used to measure TCOD, TC (via spectrophotometry), and BChl a, neurosporene, and lycopene contents, through UPLC-MS. TCOD and SCOD were determined by COD cell test (Merck, 1.14541.0001). TS/VS were determined according to

Standard Methods [24].

2.3 Pigment analysis

2.3.1 Analytical standards preparation

BChl a and lycopene were acquired from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia). Neurosporene was purchased from Novachem (Heidelberg West, VIC, Australia). To prepare stock solutions, lycopene and neurosporene analytical standards were dissolved in hexane/2% CH_2Cl_2 and BChl was dissolved in methanol. To ensure preservation, 1% butylated hydroxytoluene (BHT) was added to the standards, and the stock solutions were flushed with argon and stored in amber vials at -20 ℃. The exact concentration of each stock solution was determined by UV photometry, using the absorption coefficients ($mM^{-1} \cdot cm^{-1}$) 185, 159.4, and 54.8 for lycopene, neurosporene, and BChl a respectively, and by measuring the absorbance of each compound at 470 nm, 438 nm and 771 nm using a UV-VIS spectrophotometer (Cary 50 conc, Varian) [25-27].

2.3.2 Pigment extraction

Pigment extraction was carried out following Ruivo et al. (2012) [12], using a mixture of acetone/methanol (7:2 v/v), and with the following modifications: i) the first supernatant obtained by a first liquid-liquid extraction was discarded, as it resulted in no pigment recovery. However, the addition of this step decreased variability between replicates in the following extractions, indicating that this first extraction may serve as a wash-up step to remove other polar components in the mixture that may interfere with the detection of carotenoids; (Figures S1A and S1B); ii) wet biomass extract was used, as higher pigment recovery was obtained in comparison to freeze-dried biomass extracts. Both wet and freeze-dried biomass samples were kept at -20 ^{0}C until extraction. There was also less variability between replicates when using wet biomass compared to freeze-dried biomass (data not shown); iii) samples were centrifuged at a centrifugal force of 3270 x g using an Allegra X-12 centrifuge (Beckman Coulter, Australia) and ultasonicated with a 10 L bath sonicator (100 W ultrasonic power, FXP14, Unisonics, Australia) at a frequency of 40 kHz. Ultrasonication and centrifugation

times were fixed to 10 and 20 minutes, respectively, as longer times did not result in a significant increase in extracted pigments (data not shown); iv) the number of full extraction cycles from one sample was fixed to three, as it was found to be sufficient to recover most of the contained pigments (Figure S1C).

2.3.3 Pigment analysis by UPLC-UV-HRMS of BChl a (BChl-Ex-UPLC) and lycopene (Lyc-Ex-UPLC)

A UPLC-UV-HRMS method for separating, detecting and quantifying pigments was developed, including BChl a of the extract by UPLC (BChl-Ex-UPLC) and lycopene of extract by UPLC (Lyc-Ex-UPLC). The UPLC separation was carried out on an UltiMate 3000 Rapid Separation (RS) UPLC system (Thermo Fisher Scientific, Bremen, Germany), equipped with an RS pump, a temperature control column compartment, an autosampler, and a variable wavelength detector. A Thermo Fisher Scientific AcclaimTM C30 (2.1 mm x 250 mm; 3.0 µm) column was used for the liquid chromatography (LC) separation. The LC parameters consisted of 0.1% formic acid and 10 mmol ammonium formate (NH_4COO^-) in methanol (MeOH) as mobile phase A (MP A), and 100 % methyl-tert-butyl ether (MTBE) as mobile phase B (MP B), running at 0.2 $mL \cdot min^{-1}$, column oven temperature at 20.0 °C, and an injection volume of 15.0 µL. The starting mobile phase condition was 95%:5% (MP A:MP B) at 0.0 min and hold for 2.0 min before gradually increase to 90%:10% (MP A:MP B) at 4.0 min, followed by 60%:40% at 6.0 min, 50%:50% at 13.0 min, 40%:60% at 18.0 min and eventually to 1%:99% (MP A:MP B) at 22.0 min and hold for 1.0 min until 23.0 min. Then, the solvent composition was adjusted back to the starting condition by increasing the MP A composition from 1% at 23.0 min to 50% at 24 min and 95.0% at 26.0 min. Finally, MP A: MP B was maintained at 95%:5% for the last 4 min before completing the chromatography at 30.0 min. A Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with an Atmospheric Pressure Chemical Ionization (APCI) probe. The MS data were collected in the full scan positive mode at 70,000 FWHM mass resolution, scanning from 150 to 2000 m/z. The automatic gain control target was set at $3.0 \cdot 10^6$ and the maximum injection time at 200 ms. The source settings were as

follow: spray voltage at 3,500 (+), the capillary temperature at 256 °C, sheath gas at 48 arbitrary units (arb), auxiliary (aux) gas flow rate at 11 arb, aux gas heater temperature at 413 ℃, S-lens radio frequency level at 50, and sweep gas flow rate at 2 arb. A 5 $\mu g \cdot mL^{-1}$ mass accuracy window was set for the mass extraction and detection setting. The UPLC-UV-HRMS system was controlled by XcaliburTM 4.1, and the MS data were processed using TraceFinderTM 4.1 software. Peaks were identified by comparing the retention times, MS, and UV–VIS spectral data with those of the corresponding standards. For lycopene, the 537.4455 m/z was used as the quantification peak and 538.4489 m/z (the ^{13}C isotope peak) as the confirmation peak. UV was quantified at 470 nm. The developed method was validated in terms of recovery. For the validation, and to test recovery, samples of pigment-free PPB biomass, which pigments had been previously extracted, were spiked with low, medium, and large concentrations of a mixed solution of known concentrations of analytical standards. The spiked sample with low concentration contained 0.3 $\mu g \cdot mL^{-1}$ lycopene and 3.6 $\mu g \cdot mL^{-1}$ BChl, the medium concentrations sample had 1 $\mu g \cdot mL^{-1}$ lycopene and 10 $\mu g \cdot mL^{-1}$ BChl, and the high concentration samples had 3 $\mu g \cdot mL^{-1}$ lycopene and 25 $\mu g \cdot mL^{-1}$ BChl. Lower concentrations of neurosporene (1 $ng \cdot mL^{-1}$, 2 $ng \cdot mL^{-1}$, and 20 $ng \cdot mL^{-1}$) were spiked based on the amount detected in PPB samples. The spiked samples were then extracted as described before. After performing UPLC-UV-HRMS analysis, the recovery of each carotenoid was calculated. The UPLC-DAD analysis was carried out on an Agilent 1290 Infinity UPLC system equipped with a 1290 binary pump, an auto-sampler, a column oven, and a diode array detector (DAD). The chromatography conditions and column used for the UPLC-DAD analysis were identical to the UPLC-UV-HRMS analysis mentioned earlier. The DAD data were collected from 190-640 nm range.

2.3.4 Pigment analysis by spectrophotometry of TC (TC-Ex-Abs) and BChl (BChl-Ex-Abs)

TC and BChl extracts were determined by absorbance (TC-Ex-Abs and BChl-Ex-Abs) using previously published methods [28] and a Quartz cuvette for measurements. The TC content

was calculated using the absorbance of the extracted pigments at 475 nm and using the spirilloxanthin extinction coefficient (94 $mM^{-1}\cdot cm^{-1}$). The molecular weight of spirilloxanthin (596.94 $g\cdot mol^{-1}$) was used to obtain the concentration of TC in $mg\cdot g^{-1}$. BChl content was calculated by measuring absorbance at 771 nm, assuming a molar extinction coefficient of 65.3 $mM^{-1}\cdot cm^{-1}$ [29]. The cuvette path length of 1 cm was used for all calculations.

2.4 Microbial analysis

The microbial composition of samples collected at the end of each batch test was analysed via genomic sequencing by the Australian Centre for Ecogenomics (ACE). DNA extraction as per Qiagen DNeasy Powersoil Kit and amplification were conducted by ACE. The universal primer pair Univ_ SSU_ 926F-1392wR was used, targeting regions of the 16S and 18S rRNA genes [30]. Sequencing was conducted using the Illumina® platform. Reads identified as a single read, with a relative abundance of less than 0.05% or sequence identity less than 60% were discarded. Raw Illumina amplicon sequencing reads across samples were processed using the DADA2 package (version 1.16) in R (version 4.0.2) to infer amplicon sequencing variants (ASV) and their relative abundance. The taxonomic affiliation of the ASVs was assigned in DADA2 with the SILVA rRNA gene database (version 138).

2.5 Data processing and statistical analysis

The measured results are presented as averages, and their variability is expressed as standard deviation, given as $\overline{X}(S_{Xi})$ where $\overline{X}$, is the average value for the data X_i, and S_{Xi} is the corresponding standard deviation. The calculated parameters are presented as average values, with uncertainty expressed as uncertainty in the mean value based on a two-tailed t-test at the 95% confidence level and with an appropriate number of degrees of freedom. Thus, values are given below as $\overline{X} \pm E_{\overline{X}}$, where $E_{\overline{X}}$ is the estimated error at a 95% confidence level.

2.5.1 Analysis of whole-cell spectra (Lyc-WC-PCA, BChl-WC-PCA)

The spectrum ranges of 450-550 nm (1 nm spacing), obtained across the 25 observations, corresponding to the 5 light conditions (0, 1.7, 5.7, 14.2, and 33.1 $W\cdot m^{-2}$) at 5 time-points each, were decomposed across the 25 observations by PCA on the 100 evenly spaced

spectral amplitudes. The top 20 principal components (of 100) were regressed (see below). Both the whole-cell and extract spectra were analysed, but only the whole-cell results are presented here. Spectrum PCs were assessed for their ability to predict UPLC results on all samples analysed for extracted BChl (BChl-Ex-UPLC) and extracted lycopene (Lyc-Ex-UPLC). Similarly, the capability of spectrophotometric-absorbance measures of extracted TC (TC-Ex-Abs) and BChl (BChl-Ex-Abs) to predict UPLC-UV-HRMS results was assessed. TC-Ex-Abs and BChl-Ex-Abs were centred on the lycopene and BChl means (*i.e.*, TC and BChl means and slopes were normalised) to best estimate their ability to predict BChl and Lyc contents, and allow a consistent comparison to PCA based predictions (see below). This was done on all 25 whole-cell samples, and on the 20 extracted samples.

PCAs (with centred covariance) were performed using Matlab 2020b (function pca()). Correlation analysis (function Correl()) in Excel 16 was used to determine which combination of PCs could be used as a predictor for measured values, and the general linear model in Excel 16 (function list()) was used to build models for UPLC (BChl-Ex-UPLC) and Lycopene (Lyc-Ex-UPLC) concentrations from PCs without considering interaction or parabolic effects.

3 Results and discussion

3.1 Neurosporene, lycopene and BChl detection and quantification through UPLC-UV-HRMS: method development and recovery efficiency

The mean lycopene recovery from 6 spiked samples at the three different spike levels (0.30, 1.02, and 3.00 ug·g^{-1}) were 97.5%, 85.0%, and 83.8% analysed under full scan HRMS and 98.7%, 82.2 %, and 87.0% at UV 470 nm (Table 1). Both HRMS and UV detection gave very similar average recoveries across the three different spiked levels at 88.8% and 89.3%. The average relative standard deviations (RSD) calculated from the mean recoveries for the HRMS, and UV detections were 8.6% and 9.6%, suggesting that both methods have good precision and reproducibility (average RSDs <10%). The mean neurosporene recoveries were 88.5%, 96.3%, and 113.7% at the three different spiked levels (0.002, 0.005, and 0.020 ug·g^{-1}), respectively for HRMS detection (UV detection was not sensitive enough at the spiked

levels). BChl showed a lower mean recovery (73.6% and 82.1% for HRMS and UV, respectively), which may be linked to its rapid degradation or an insufficient extraction.

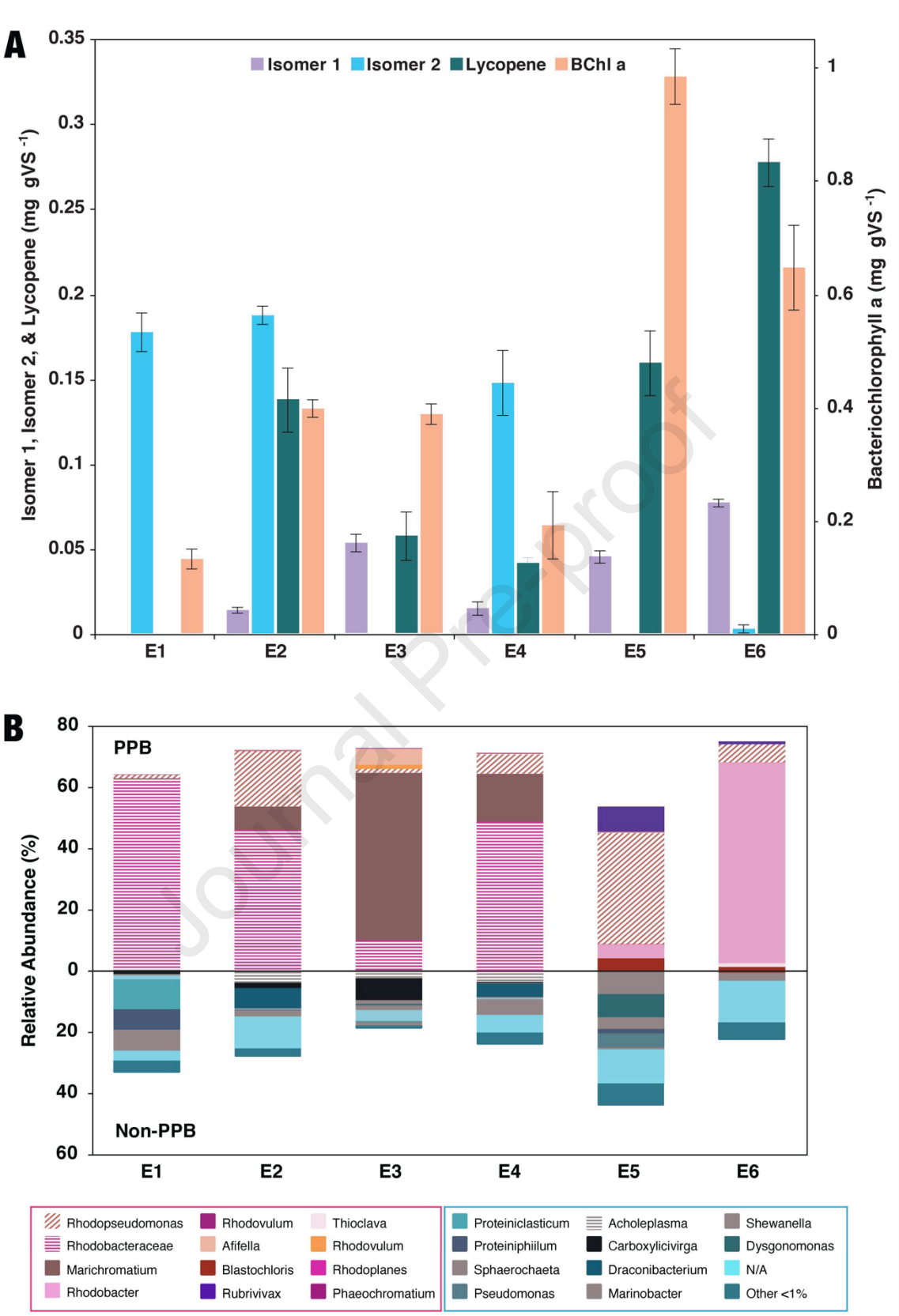
Table 1. Recovery of neurosporene, lycopene, and BChl as measured by UPLC-UV_HRMS (n = 6 at each spiked level).

Analyte	Spiking Level, µg·g^{-1}	Detected ($\overline{X}(S_{Xi})$), µg·g^{-1}	Recovery ($\overline{X}(S_{Xi})$), %	Avg Recovery ($\overline{X}(S_{Xi})$), %	RSD, %
Lycopene					
HRMS	0.30	0.29 (0.04)	97.5 (13.3)		
	1.02	0.87 (0.12)	85.0 (11.6)	**88.8 (7.6)**	**8.6**
	3.00	2.52 (0.49)	83.8 (16.3)		
UV	0.30	0.30 (0.04)	98.7 (12.8)		
	1.02	0.84 (0.13)	82.2 (12.3)	**89.3 (8.5)**	**9.6**
	3.00	2.61 (0.48)	87.0 (16.0)		
Neurosporene					
HRMS	0.002	1.77 (0.24)	88.5 (11.8)		
	0.005	4.82 (0.23)	96.3 (4.5)	**99.5 (12.5)**	**12.6**
	0.020	22.7 (0.32)	113.7 (1.6)		
BChl a					
HRMS	5.15	4.52 (0.55)	87.7 (10.6)		
	20.13	14.03 (2.21)	69.7 (11)	**73.6 (12.6)**	**17.1**
	50.14	31.78 (3.83)	63.4 (7.6)		
UV	5.15	5.52 (1.66)	107 (32.2)		
	20.13	14.52 (2.95)	72.1 (14.7)	**82.1 (21.7)**	**26.4**
	50.14	33.69 (4.48)	67.2 (8.9)		

3.2 UPLC-UV-HRMS detection and quantification of pigments in PPB enrichments

Several PPB cultures enriched from domestic wastewater, poultry processing wastewater, Brisbane River, and sediments (E1-E6; described in 2.1) were analysed for their pigment contents. Representative isotropic profiles of lycopene (M+H) ($C_{40}H_{56}$), and a comparison of the chromatograms from the lycopene standard (retention time of 20.7 min), from the lycopene present in a PPB sample monitoring the lycopene molecular ion (537.4455 m/z, black peak), and the isotope ion (538.4489 m/z, red peak), are presented in Figure S3. All the enrichments except for E1 contained lycopene, eluting at 20.8 min as in the analytical standard (all-trans lycopene). However, the method also enabled the detection of two other lycopene-like compounds, eluting at 16.38 and 17.6 min and designated as isomer 1 and isomer 2 (Figure

2A). Although different lycopene isomers have been widely studied in dietary sources such as tomato, and human serum and tissues, this is the first time that the detection and quantification of different lycopene isomers in PPB are described in the literature. The isomers content varied between samples, most likely because of the different PPB communities resulting from the different inoculum sources and growth conditions (Figure 2B). While *Rhodopseudomonas sp.* and *Rhodobacter sp.* (dominant in piggery wastewater (E5) and domestic wastewater (E6)) showed a similar isomer distribution (mainly the all-trans lycopene), samples with dominant *Rhodobacteraceae* also showed the presence of an isomer 2 peak, in higher concentrations than all-trans lycopene. Although neurosporene was detected in negligible amounts in PPB enrichments, the method may be relevant in the future to enable the analysis of this compound under different growth conditions. While other illumination sources including UV-VIS might impact individual carotenoids, NIR generally produces both, BChl and carotenoids as relevant genes for their synthesis are grouped in photosynthetic gene cluster (PGC) [31-34]. In fact, carotenoids are essential for the formation of the light harvesting complexes (LHC) [11]



368

369 **Figure 2.** (A) Distribution of the three major lycopene isomers detected at RT: 16.38 (isomer

370 1), 17.6 (isomer 2), and 20.8 min (lycopene) among 6 PPB mixed cultures enriched from

371 sediments from Tuckean Broadwater and sea salt addition of 30 mS (E1), Ballina Mangrove
372 Habitat and salt addition of 30 mS (E2) and 70 mS (E3), Brisbane River (E4), poultry
373 processing wastewater (E5) and domestic wastewater (E6). (B) Relative abundance of
374 different microbial communities corresponding to each of the enriched PPB cultures.

375 The UV spectra for lycopene, isomer 1, and isomer 2 were obtained by running the samples
376 (E1-E6) through UPLC coupled with a DAD detector. The UPLC-DAD chromatogram at 470
377 nm, the UPLC-UV chromatogram, and the UPLC-HRMS chromatogram of each PPB
378 enrichment sample were compared to identify the peaks with m/z corresponding to lycopene
379 and thus be able to access the individual UV spectra, as shown in Figure 3 for sample E3.
380 Three different UV spectra profiles were observed for each compound (lycopene, isomer 1,
381 and isomer 2) when matching the UV chromatogram from the two different instruments (MS
382 and DAD) in all the PPB enrichment samples (see Table 2 and Figure S4). Isomer 2 and
383 lycopene showed a very similar UV spectrum; however, their distinct elution times (16.3 and
384 20.8 min) make isomer 2 a lycopene isomer candidate (see Figure S5 for an example with
385 isomer 2). Isomer 1 had a more distinct UV spectrum, with maximum absorbance peaks
386 shifted to shorter wavelengths when compared to isomer 2 and lycopene. Although this
387 method enabled the detection of different lycopene isomers, there are no available analytical
388 standards for accurate identification of the isomers and further isolation and structural
389 elucidation (*e.g.* by using NMR) should be performed to fully elucidate these compounds.

390

391 **Table 2.** Identification data for lycopene isomers.

Lycopene Isomer	RT (min)	λ max (nm)			
Isomer 1	16.3	280	430	454	487
Isomer 2	17.6	294	446	472	504
Lycopene	20.8	294	446	470	502

392

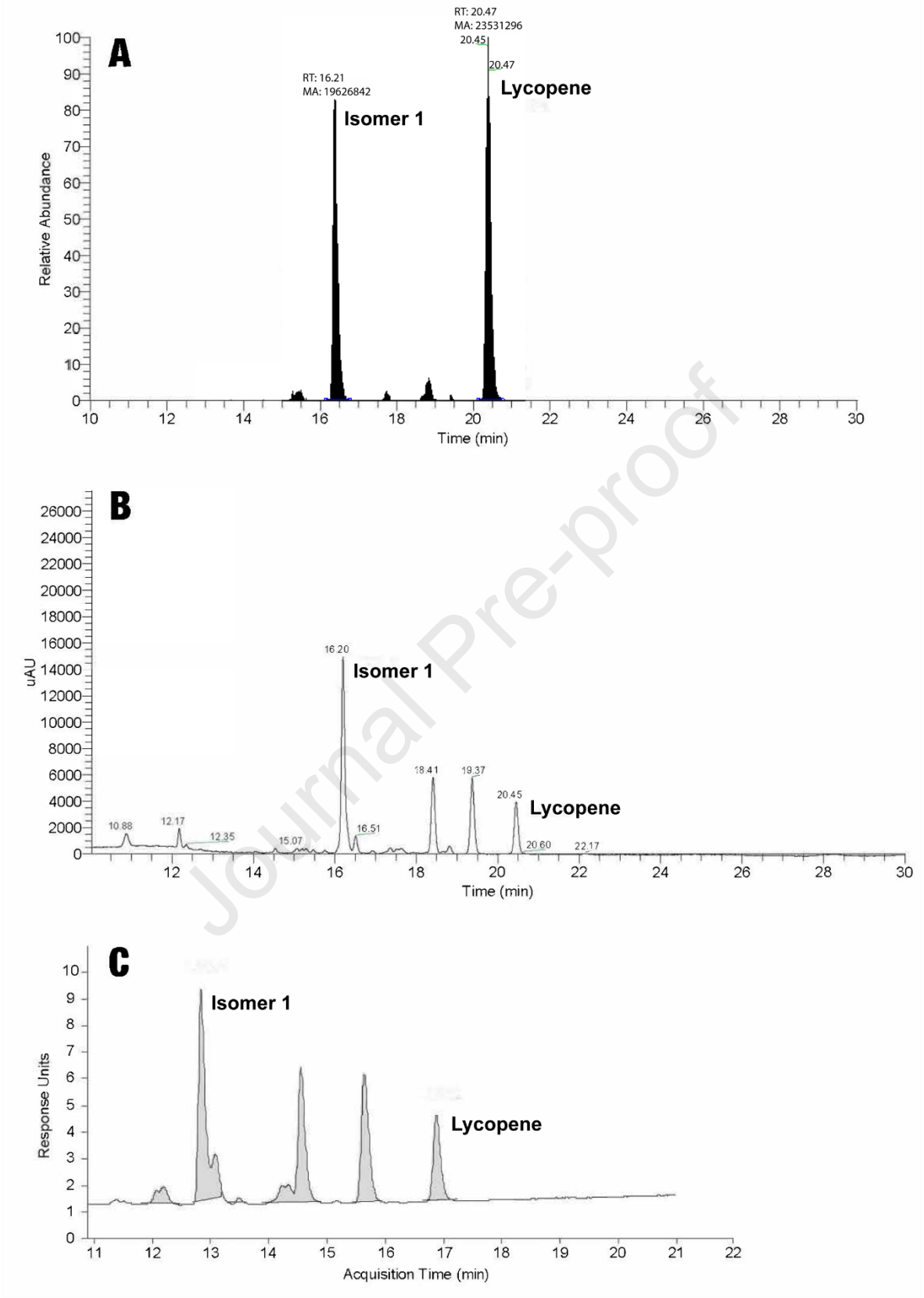
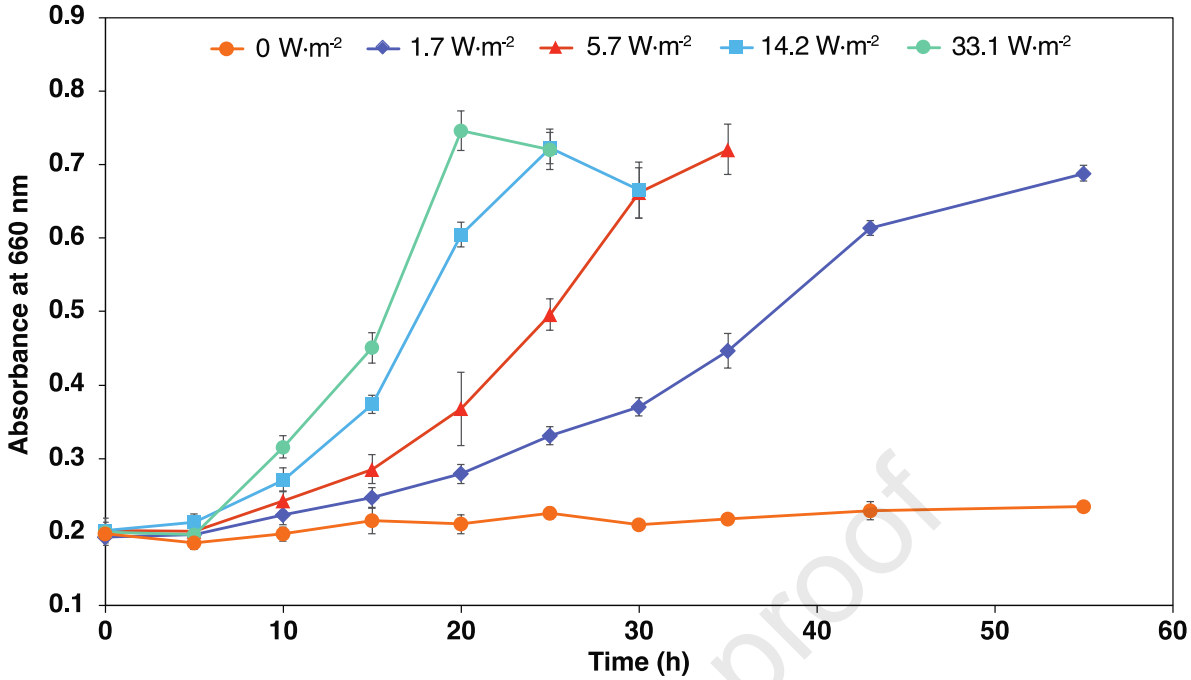


Figure 3. Composition analyses of lycopene in E3. (A) UPLC-MS chromatogram at 537 m/z, (B) UPLC-UV chromatogram at 470 nm, and (C) UPLC-DAD chromatogram at 470 nm.

The combined lycopene contents did not exceed 0.3 mg lycopene·g VS^{-1} (combined isomers), which is on the lower end of literature values where up to 2 mg g^{-1} (dry weight) was reported for the purple phototrophic bacterium *Rhodospirillum rubrum* after genetic manipulation [35], and up to 18 mg g^{-1} (dry weight) were reported in other genetically engineered microorganisms [36]. At this stage, we cannot state standard lycopene contents of mixed PPB cultures under non-engineered conditions. The data show that the UPLC-UV-HRMS method can be used to analyse the lycopene content in environmental samples, with greater accuracy compared with spectrophotometric methods. Due to the use of a polymeric C_{30} column, the developed method also enables lycopene isomers separation, which cannot be resolved by the commonly used C_{18} columns [37]. Different isomers have varying functions and potencies [38, 39], which makes the isomer identification relevant to assess the potential absorption and health benefits of the lycopene produced in PPB cultures, *e.g.* when used as SCP in feed. For example, the antioxidative properties of cis-lycopene have been reported inferior to all-trans-lycopene [38]. On the other side, cis-isomers have repeatedly been shown to be substantially more bioavailable than all-trans lycopene [16].

3.3 Plate reader tests and spectral decomposition-based analysis

The microplate set-up enabled the recording of PPB photoheterotrophic growth in 12 well microplates. Growth curves were recorded by measuring OD_{660} in a plate reader (with an acceptable CI95%). OD_{660} measurements for the four light intensities over time are shown in Figure 4. This is an effective predictor for particulate COD (R^2 = 0.9908), shown in Figure S2, which can be used as biomass proxy, especially in clean substrate tests (without native particulate COD due to serial dilutions). Additionally, each well's absorbance spectrum was scanned to monitor characteristic peaks of carotenoids (400-500 nm) and BChl a (800-900 nm).



420

421 **Figure 4.** PPB growth curves measured as OD_{660} in microplates using different light intensities
422 (0, 1.7, 5.7, 14.2, and 33.1 $W \cdot m^{-2}$).

423 Time series whole-cell spectra collected during experiments in the microplates were
424 decomposed via covariance PCA as noted in the methods (2.5.1). The vast majority of
425 covariance was held in PC1 (99.97%), which was related to change in overall spectrum
426 amplitude. This effectively removes the background of general cellular material. Of the
427 remaining covariance, 99.999% was contained in PCs 2-10.

428 A full correlation table of Lycopene and BChl via UPLC-UV-HRMS (Lyc-Ex-UPLC and BChl-
429 Ex-UPLC), to the individual spectral PCs for whole-cell and extract is given in Table S1. Lyc-
430 Ex-UPLC was correlated with PC2, PC3, and PC12, while BChl-Ex-UPLC was correlated with
431 PC2, PC8, and PC13 (and to a lesser extent, PC1 and PC10). PC2 therefore likely contains
432 common features to both BChl and Lyc, but it is important to note that the other PCs are
433 different, and BChl and Lyc can be fitted separately using different features. To develop a
434 predictive model for Lyc-Ex-UPLC and BChl-Ex-UPLC from the spectral PCs, data were
435 randomly split into fitting (20 points) and validation (5 points). Increasing the number of
436 predictors (PCs) as inputs to the model improved the R^2 to the fit points (up to 0.99) but caused

validation to become excessively poor, likely because PCs were used to improve fits on individual points with excessive empirical inputs. To address this, the number of predictors was limited to achieve similar R^2 values between fitting and validation data sets, and in each case, this resulted in 3 predictors. The optimised models and individual parameter p-values are shown in Table 3. The low p-value for PC8 indicates it could be eliminated in the model for BChl-Ex-UPLC, but this resulted in the fit R^2 value dropping to 0.73 (from 0.77) without an improvement in validation R^2. Analysis of the extract PC spectroscopy resulted in similar model qualities (with an increased contribution from PC1 and decreased variance in PC1). Because it was not superior to the whole-cell PCA and is considerably more effort (requiring solvent extraction), it is not presented here (but included as Table S1).

Table 3. Whole-cell PCA models for Lycopene and BChl by UPLC (Lyc-Ex-UPLC and BChl-Ex-UPLC).

PCA-Lycopene Model				
Predictor	PC2	PC3	PC12	Intercept
Parameter	-6.06±0.67	-15.20±4.9	199±137	0.70
p-value	$1x10^{-14}$	$3x10^{-6}$	$8x10^{-3}$	
PCA-BChl Model				
Predictor	PC2	PC8	PC13	Intercept
Parameter	-25.2±5.0	-520.9±419	-1627.2±690	6.87
p-value	$8x10^{-10}$	$2x10^{-2}$	$1x10^{-4}$	

Uncertainty in parameters (±) are 95% confidence intervals

The correlation table for the three methods (Ex-UPLC, Ex-Abs, WC-PCA) and two target compounds are shown in Table 4. The Ex-Abs values required significant correction by regression to be effective predictors for the Ex-UPLC measures (particularly TC to Lyc) and even then, had significant issues. Correlation between extractive absorption (TC-Ex-Abs, BChl-Ex-Abs) and the UPLC methods are reasonable at r=0.84 and r=0.85 for target compounds. However, TC-Ex-Abs is also a good predictor for BChl-UPLC (*i.e.*, non-specific), which means that BChl is potentially a strong interference for the TCA method. In addition, the correlation between the two extraction-absorption (TC-Ex-Abs, BChl-Ex-Abs) was 0.91, which means that these methods interact strongly. That is, both are largely measuring the same

compound (total pigments) rather than separate pigments. In contrast, the correlation coefficient for WC-PCA methods was higher than for the Ex-Abs methods in both cases (to UPLC), and PCA was more specific to the target compound. UPLC (Ex-UPLC) results vs predictions from the whole-cell spectral PCA (WC-PCA) are shown in Figure S6A for TC, and S5B for BChl.

As noted in the methods, due to loss of some extracted individual replicates (due to limited sample volumes), averages of technical triplicates for spectra, UPLC measurements, etc. were used. To validate this for individual samples, the same procedure was also done on individual replicates excluding zero lycopene reads (total n=63). The results were consistent, with PC2 and PC3 dominating correlation, and with an overall R^2 of 0.79. As seen in supplementary Figure S7 (r=0.89), most of the errors are at very low lycopene levels (where the relative uncertainty in replicate analysis was highest). Due to the increased observations, p-values were far lower ($x10^{-3}$) than shown in Table 3.

Table 4. Comparison of different methods to assess Lycopene and BChl (Pearson correlation coefficient of parity model).

	Lyc-Ex-UPLC	BChl-Ex-UPLC
Lyc-Ex-UPLC	**1.00***	0.66
BChl-Ex-UPLC	0.66	**1.00**
TC-Ex-Abs	**0.84**	0.87
BChl-Ex-Abs	0.69	**0.82**
Lyc-WC-PCA	**0.88**	0.60
BChl-WC-PCA	0.68	**0.86**

*Target of analytical method is underlined and bold. Correlation between TC-Ex-Abs and BChl-Ex-Abs is 0.92. Colours indicate correlation.

The absorbance of extract, while effective for plant samples, where the carotenoid profile is known, is non-selective in microbial cultures. Limited improvement in the method is unlikely, while whole-cell PCA has been analysed with only 25 (averaged triplicate) samples from 5 microplates batch tests (described in section 2.2 at different wavelengths) and will improve

substantially as more samples can be analysed. Both are subject to matrix issues, which may include the flanking community, background coloured compounds (e.g., humics, cellular polymers, etc), but this can be corrected in PCA. PCA on whole-cell spectrum can be done on live cells, during the experiment, or for a pigment production platform, at an industrial scale, and does not require pigment extraction. When the matrix changes, a large number of whole-cell spectra can be fit using a relatively small number of UPLC measurements, and this can be continuously updated, with error analysis continuously applied. As far as we are aware, this is the first instance of full spectra decomposition by PCA and comparison against chemical analysis. Other methods, such as the Gauss-peak spectra method, have been applied to qualify and quantify individual pigments from total absorbance spectra from higher plants, brown algae, *Euglena*, *Trichodesmium*, and *Anabaena*. However, standards are necessary to calibrate the methods (a spectrum fitting library is available) [40], which limits the application for specific PPB pigments.

While UPLC remains the “best” technique, it is limited in terms of analytes. This is because other carotenoids produced by PPB such as spirilloxanthin or okenone do not have readily available commercial standards, which is a prerequisite for their reliable detection and quantification e.g. with UV-HRMS detectors [21]. Increased interest in the PPB platform will likely drive the availability of standards that can be used to calibrate the PCA method for various individual carotenoids. This would enable high throughput methods *e.g.* to screen the best conditions to accumulate specific carotenoids or control industrial production in mixed or pure cultures. We note the PCA calibration will be required to include matrix effects of different wastewaters and different communities and variability need to be further assessed.

4 Conclusions

Studies to optimise the biosynthesis of carotenoids in purple phototrophic bacteria require a reliable analytical method that enables the detection and quantification of individual carotenoids and their isomers. The UPLC-UV-HRMS method presented here was reliable for the quantitative determination of neurosporene, lycopene, and BChl a. These compounds

were separated on a reverse-phase C_{30} carotenoid column, enabling the detection of three different lycopene isomers. Compared to traditional spectrophotometric methods, this method has the advantages of detecting individual carotenoids and of providing accurate identification due to the use of analytical standards. Full identification of other carotenoids present in the sample requires suitable standards, but the m/z and UV Spectra data could be used for tentative identification of the other carotenoids. The classic extraction-absorption method was not particularly effective in separating different pigments and had matrix effects. However, analysing whole-cell spectra via PCA allowed more accurate prediction of individual pigments, and can be done non-destructively, during live-cell growth. It does require calibration for media and microbial matrices, but once calibrated, is a rapid, and relatively low effort method.

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638

Highlights

- A UPLC-UV-HRMS method to detect and quantify PPB pigments was developed
- A C_{30} column enabled detection and quantification of two lycopene isomers
- A microplate method for photoheterotrophic PPB cultivation was developed
- Pigment contents can be determined by PCA on the whole-cell spectra

Declaration of interests

☑The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: