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ORIGINAL RESEARCH

Detection of weed algae in open pond cultures of *Cyanobacterium aponinum* using PAM

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Abstract The potential use of non-arable land in the al-Wusta region of the Sultanate of Oman for the production of algae biomass was examined. Brackish cleaned production water from oil production supplemented with commercial fertilizer was used as growth medium. The indigenous isolate *Cyanobacterium aponinum* WP7(1) was grown in open ponds using batch or semi-continuous cultivation. Biomass production rates of 15–24 g/m^2/day were achieved. The change of salinity due to evaporation, which was thought to be a major challenge, did not exceed 35 ppt. All cultures showed contaminations with weed algae. Contaminations with green algae or diatoms were detectable using fluorescence pattern excited by four different wavelengths using a pulse-amplitude-modulation chlorophyll fluorometer (PAM). It is possible to estimate the health level and the mayor groups of which a culture is composed using the PAM method. Therefore, the fluorescence of the photosynthetically inactive sample is compared with the fluorescence after all copies of photosystem II were closed by exposing the sample to a high-intensity light beam. A detection limit of one weed algae cell in a hundred cells was achieved.

Keywords PAM · *Cyanobacterium aponinum* · Evaporation · Open pond · Arid environment · Produced water · Weed algae

Introduction

Over the course of the past decades microalgae and cyanobacteria were considered to be a useful tool for numerous applications starting with water treatment (Cabanelas et al. 2013), biomass production for the use as nutritional supplements (Gantar and Svirčev 2008) or the production of biofuels (Slade and Bauen 2013). It was shown that especially for commodity products such as biofuels a stand-alone algae production facility could not be economically feasible. But the integration into existing procedures may result in an overall increase in revenue (Lam and Lee 2014).

Up to 800,000 m^3 of production water (PW) is built up per day during the production of crude oil in the al-Wusta region of the Sultanate of Oman (Breuer and Al-Asmi 2010). Approximately 100,000 m^3 of it is treated

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in a reed bed water treatment plant. The cleaned water [cleaned PW (cPW)] is free of hydrocarbon but of brackish nature. The location of the treatment plant on non-arable land in a region with a high annual temperature regime, maximum light exposure, low cloud coverage, and an already existing infrastructure (Grobbelaar 2009) led to the consideration of the establishment of an algae production facility despite the distance to potential customers.

The *Cyanobacterium aponinum* (CA) strain WP7(1) used was sampled at the reed bed and isolated. It is said that the use of indigenous organisms may circumvent problems arising with abiotic location factors and they may be able to out-compete weed algae (Mutanda et al. 2011). A high lipid content of CA was reported (Karatay and Dönmez 2011) and, therefore, a possible use as biofuel source and marketability was assumed.

The development of a stable process providing biomass and a reliable product outcome is a crucial step for a viable algae production facility. The process control should allow the exploitation of physiological properties of the target species. The maximum photosynthetic rate is a marker for the overall efficiency of the photon capture and possible damage to the photosystem reaction centers (Grobbelaar 2009).

Two major concerns regarding the production stability were addressed. The first was the evaporation of brackish growth media leading to increasing salt content and possible salt stress. The dilution with fresh water is not favored as water is an economic factor to make a production feasible (Guieysse et al. 2013). Laboratory tests have shown CA's ability to survive up to 45 °C (Moro et al. 2007; Winckelmann et al. 2015) and increasing salt content (Winckelmann et al. 2015). Experiments to limit the salinity in the open pond cultures below 35 ppt were performed. For this reason the differences of a partial exchange of the culture with fresh cPW on a regular basis (semi-continuous cultivation) was compared to cultivations where only the evaporated water was replaced (batch cultures).

Furthermore, the possible introduction of weed algae might lead to unwanted competition for nutrients and sunlight resulting in an unfavorable shift in overall biomass composition. In a process aiming at a high oil content weed algae with a lower lipid content will have an advantage because they require less photosynthetically derived reductant per unit biomass (Fulbright et al. 2014).

The main aim of this study was to determine the detectability of weed algae in CA cultures using a multichannel pulse-amplitude-modulation chlorophyll fluorometer (Kolbowski and Schreiber 1995).

Methods

Pre-cultivation

CA WP7(1) was isolated and characterized similar to CA PB1 (Winckelmann et al. 2015). The culture was maintained on algae culture broth (Sigma-Aldrich Chemie GmbH, Germany) agar slants (1 % w/v agar). The initial inoculum was cultured in Wuxal-media consisting of sterilized tap-water containing 0.05 % (v/v) Wuxal universal fertilizer, 1 % (w/v) NaCl and 0.002 % (w/v) $MgSO_4$ (Winckelmann et al. 2014, Wuxal® Universaldünger liquid plant fertilizer (8 % N, 8 % P_2O_5, 6 % K_2O, 0.01 % B, 0.004 % Cu, 0.02 % Fe, 0.012 % Mn, 0.004 % Zn), Wilhelm Haug GmbH & Co. KG, Germany) until a biomass of above 0.5 g dry weight/L was reached. The cultures were transferred to 1.5 L bottle reactors which were placed outside in full sunlight until midday and covered in shade afterwards. The cultures were aerated with a rate of 30 L/h. For the first 4 days of outdoor cultivation unsterilized bottled water with 0.05 % (v/v) Wuxal was used as growth media. The cultures were divided and diluted with unfiltered (2013) or filtered (2014) cPW (440–550 mg/L SO_4, NH_3 0.3–0.6 mg/L, NO_3 0.2–1.0 mg/L, $PO_4 < 0.2$ mg/L, salinity 7–12 ppt; analysis kindly provided by Bauer Nimr LLc) containing 0.5 % (v/v) Wuxal each time the biomass exceeded 0.5 g dry weight/L. Inoculates were grown in cPW for 14 (CA 13 batch), 5 (CA 14 batch) or 25 days (CA 14 semi-continuous).

Pond construction

Algae ponds were constructed on site of the Bauer Nimr water treatment plant (Abed et al. 2014). Open ponds (4 m long, 80 cm wide and 38 cm deep), were constructed on a levelled area. Building bricks (39 cm long, 19 cm wide and 19 cm deep) were used for the outer walls and 2 mm thick high-density polyethylene (HDPE) sheet was used as liner. A centrifugal pump (2 inch diameter; Pedrollo, HFm 5AM) was used for mixing. The

mass transfer coefficient (k_La) was determined using the dynamic method (Doran 1995). Culture velocity was measured using a current meter (C2 with a 30 mm blade (gradient 0.1) and counter Z30 OTT Hydromet GmbH, Germany).

Growth experiment

cPW was left for sedimentation in 1 m^3 container for 2 days and used as growth medium. Only the top 70 % was used. 0.05 % (v/v) Wuxal was added directly into the pond. The ponds were filled to a depth of 20 cm and inoculated with 18 L inoculum (biomass content of at least 1 g dry weight/L). The cultures were mixed 24 h per day with breaks due to human or systematic error. Evaporated water was replaced in the morning with cPW. Growth was measured by means of optical density at 660 nm (Lovibond MaxiDirect photometer, Tintometer GmbH, Germany). Culture composition was determined microscopically (using a Zeiss Axiostar Plus light microscope, Carl Zeiss GmbH, Germany and a Thoma Neu chamber with a depth of 0.1 mm Paul Marienfeld GmbH & Co. KG, Germany). For the determination of bio dry weight (dw) part of a culture was washed and filtered. The filter was dried at 105 °C until the weight was constant. Increase of salinity was measured by means of conductivity (Water quality meter U-52, Horiba Ltd., Japan). The relative fluorescence and the maximum photosynthetic efficiency (Y) were measured using a phyto-PAM with phyto-EDF (Heinz Walz GmbH, Germany) as described previously (Winckelmann et al. 2015). Temperature and light intensity were measured by an adjacent weather station.

The cultures were either grown as batch culture (CA 13 batch and CA 14 batch) or half of the culture was exchanged on a regular basis (CA 14 semi-continuous). Therefore, the culture was removed until the level was 10 cm. Fresh cPW containing 0.05 % v/v fertilizer was added until a depth of 20 cm was reached.

Biomass for further analysis was separated from the excess liquid via flocculation. Therefore, 600 ml flocculant (0.2 % w/v, PK 55 H Separ Chemie, Germany; at least matured for 1 day) was added to 180 L culture. After mixing the culture was left to stand for 4 h. The supernatant was discharged and the biomass frozen immediately.

Classification of weed algae was performed as described earlier (Winckelmann et al. 2015) using primer pair ITS4 and ITS5 (Connell 2000) and EukA and EukB without GC-clamp (Medlin et al. 1988).

Results

The sequences gained for the 16 s RNA gene sequence and the internal transcribed spacer region of *C. aponinum* WP7(1) were identical to the sequences gained for *C. aponinum* PB1 (Winckelmann et al. 2015).

The ponds showed a k_La value of 0.04/s (SD 0.01) and a current velocity of between 0.2 and 2.3 m/s.

Both cultures grown in batch culture showed a similar linear growth (Fig. 1) with a biomass increase as measured in OD of 0.075 and 0.08 g/L/day (15–16 g/m^2/day, respectively). The maximum biomass concentration was 0.6 g dw/L (SD 0.03) on day 11. The daily maximum temperature during the cultivation of CA 13 batch was between 26 and 31 °C with daily maximum light intensities between 820 and 940 W/m^2. While the maximum temperature during the cultivation of CA 14 batch was between 32 and 38 °C with daily

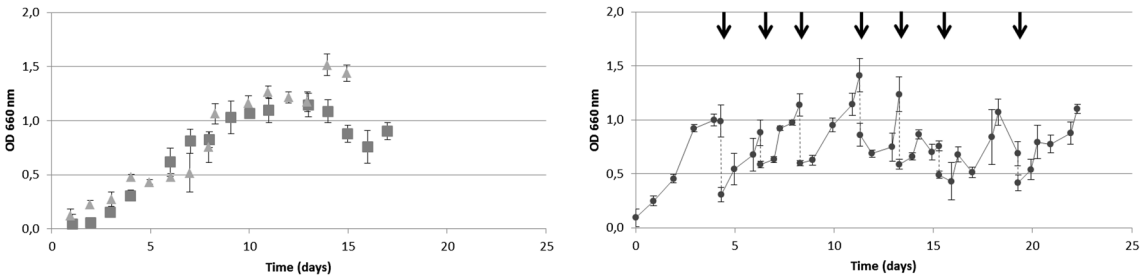

Fig. 1 Growth of *C. aponinum* WP7(1) in pre-cleaned PW with fertilizer added. *Left* batch cultures [*squares* CA WP7(1) 13; *triangle* CA WP7(1) 14]; *right* semi-continuous culture (point of dilution is indicated with a *black arrow* and a *dotted line*). *Error bars* represent three times the standard deviation

maximum light intensities between 820 and 960 W/m^2 (Fig. 2). The salinity rose due to evaporation and replacement of 45 L of water per day in 2013 from 8 to 26 ppt. During the batch cultivation in 2014 55 L of water had to be replaced daily and the conductivity rose from 9 to 24 ppt (Fig. 3).

The culture grown with a semi-continuous procedure was diluted every 2–3 days (Fig. 1). It showed the highest maximum biomass concentration of 0.7 g dw/L (SD 0.04) on day 11. The increase of biomass as measured in optical density was 0.12 g/L/day (SD 0.03; or 24 g/m^2/day) over the whole 23 days. After removal of data influenced by power outage and pump failure the growth was 0.14 g/L/day (SD 0.01; or 28 g/m^2/day) per day. The maximum daily temperature during the cultivation was between 34 and 41 °C, while the maximum light intensities were between 810 and 870 W/m^2 (Fig. 2). The conductivity was between 10 and 16 ppt and only rose up to 18 ppt at the end of the cultivation (Fig. 3). In average 220 L of water were moved daily; this includes the replacement of evaporated water and the exchange of part of the culture.

Y of the CA 13 batch rose to 0.4 on day 2, dropped to 0.3 on day 3 and stabilized afterwards between 0.39 on day 12 and 0.51 on day 14. For CA 14 Batch the Y rose to 0.45 on day 3 and decreased to 0.3 on day 4. After day 4 the Y increased again to 0.45 on day 5 and stabilized from day 6 onwards between 0.65 and 0.71. The Y for 14 semi-continuous increased to 0.35 on day 3 and decreased slightly to 0.3 on day 4. On day 5 it dropped to below 0.1 but increased again to 0.3 on day 7. It increased slowly to 0.4 on day 9 and remained the same up to day 16, after which it decreased again until it reached its low point at 0.15 on day 19. A steep increase to 0.6 followed by stabilized values for the rest of the cultivation that occurred on day 20 (Fig. 4).

Two different groups of photosynthetic active organisms were found in the culture CA 13 batch from the beginning. The first one consisted of *C. aponinum*-like cells (Fig. 5 left) and was thought to be the desired organism, while the second group consisted of green spherical cells (Fig. 5 right), which were identified as belonging to the Chlorophyceae (For 18S rRNA-gene sequences and internal transcribed spacer see GenBank KM873328 and KM873329). On the third day, diatoms (Fig. 5 middle) were found and reoccurred over the duration of the experiment in changing numbers. The overall share of the weed algae started with 4 % and increased to up to 10 % on day 15 (Fig. 6, top row, left). The pattern of relative fluorescence excited by different wavelengths changed over the course of the experiment. The fluorescence excited by 645 nm long light was the highest and was used as point of comparison. In the beginning all other signals were 0.4 times as high. The signals excited by 470 nm decreased to day 9 and increased till the end and reached a value of 0.75. The fluorescence assigned to 520 nm decreased slowly to a value of below 0.2 at day 6 and fluctuated around 0.2 with the exception of day 16, on which it was above 0.3. The values excited by 665 nm increased on day 2–0.65, decreased on day 3–0.45 and stayed stable till day 8, after which it increased till it was above 0.95 on day 14 (Fig. 6, top row, right).

During the batch cultivation in 2014 single diatoms were found in the pond 1 day after inoculation. In the following days no diatoms were found until day 7 after which they were found during each examination until the end of the experiment. Green algae were found on the third day and increased until day 14 when the weed algae had taken over the culture with a combined share of 62 % (Fig. 6, middle row, left). The relative fluorescence was dominated by light excited by 645 nm for most of the cultivation except for the days 6–9 and

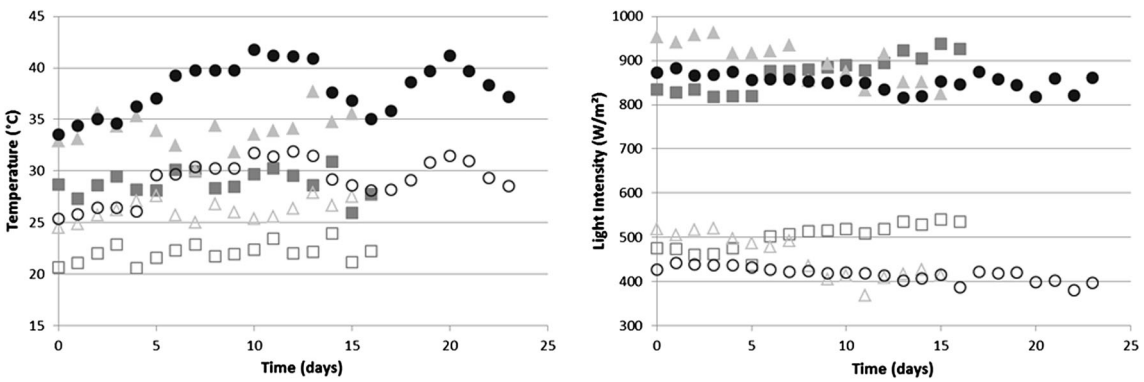

Fig. 2 Development of temperature and light intensity over time [*squares* CA WP7(1) 13 batch; *triangle* CA WP7(1) 14 batch; *circle* CA WP7(1) 14 semi continuous]; *left*: *solid symbols* maximum temperature; *open symbols* mean temperature; *right solid symbols* maximum light intensity; *open symbols* mean light intensity (only daylight hours used for calculation)

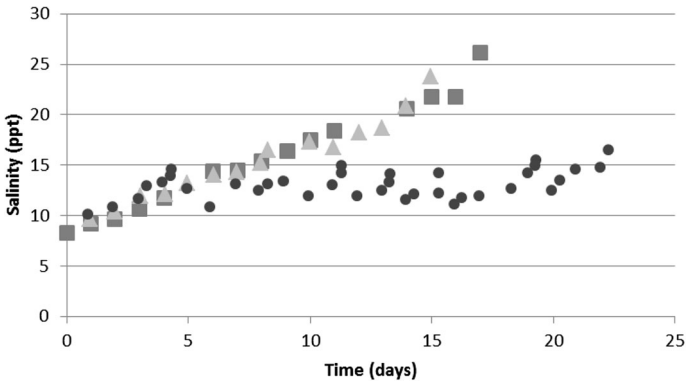

Fig. 3 Development of salinity over time [*squares* CA WP7(1) 13 batch; *triangle* CA WP7(1) 14 batch; *circle* CA WP7(1) 14 semi-continuous]; the standard deviations were below 0.09 mS/cm for all measurements and are not shown

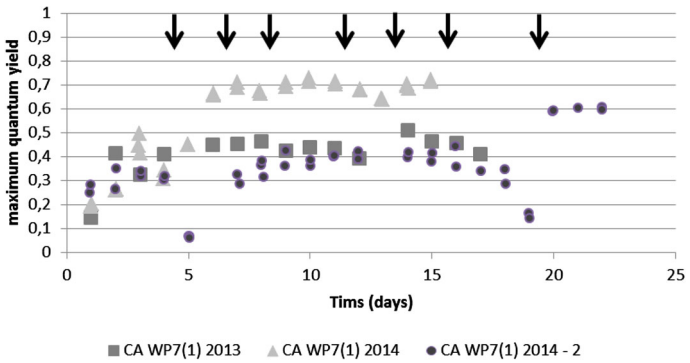

Fig. 4 Development of maximum quantum yield over time; samples were dark adapted for 5 min. Light of a wavelength of 645 nm was used for excitation; *squares* CA WP7(1) 13 batch; *triangle* CA WP7(1) 14 batch; *circle* CA WP7(1) 14 semi-continuous (point of dilution is indicated with a *black arrow* and a *dotted line*); each measurement was performed twice; both data point are shown

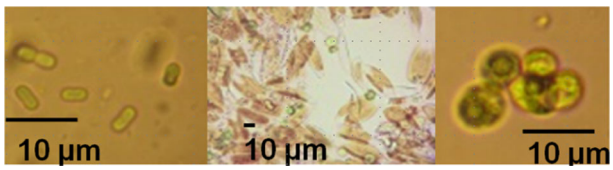

Fig. 5 Photosynthetic active organisms found in all growth experiments (*left C. aponinum*, *middle* diatoms, *right* green algae)

day 14 when the fluorescence excited by 470 nm was stronger. The light excited from 470 nm started at 0.2 and decreased below 0.15 on day 2. On days 4 and 5 the value was between 0.4 and 0.5 and increased to 1 on day 6. Afterwards it was stable between 0.95 and 1. The 520 nm dependent signal started at 0.2 decreased slightly and increased over time to a value of 0.35 on day 6. It alternated between 0.33 and 0.42 for the remaining time. The signal excited by 665 nm was around 0.2 till day 2 and increased to 0.3 for days 3 and 4, after which a steep increase to 0.6 occurred on day 6. It increased further until it reached values of just below 0.8 on day 10 and stabilized (Fig. 6, middle row, right).

The culture composition of the semi-continuous culture showed no weed algae hours after the inoculation. A small amount of green algae and diatoms with a cell number of below 10^5 cells/m was found during each examination until day 6 where the cell count increased. The cell amount stayed below 2×10^5 cells/mL till day 13 after which a steady increase to 6×10^5 cells/mL on the last day occurred. The green algae were the most abundant weed algae till day 13. Afterwards, the amount of diatoms was equal or above the amount of green algae found (Fig. 6 lower row, left). The relative fluorescence excited by 645 nm was used as point of

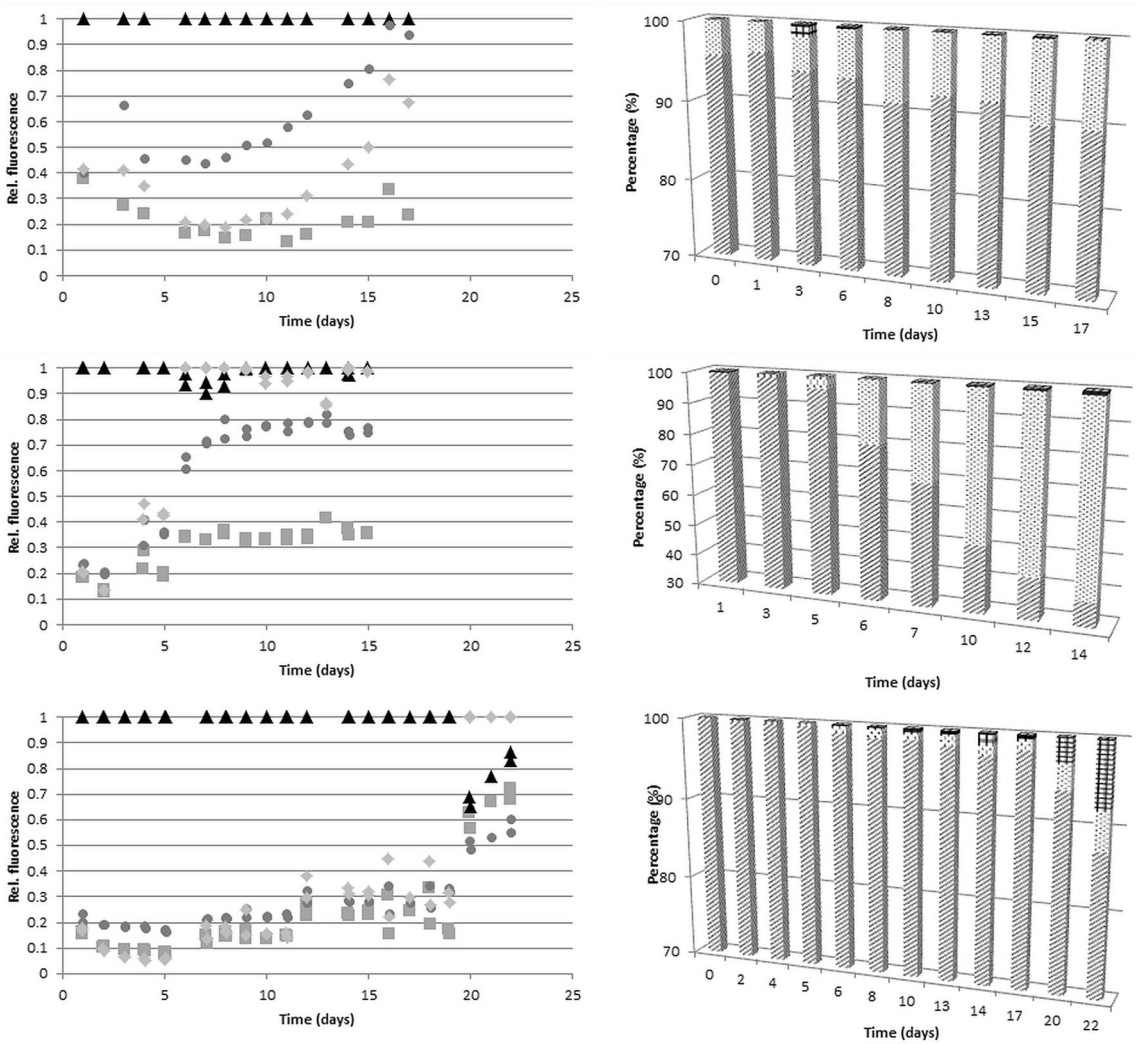

Fig. 6 Development of relative fluorescence versus the share of different photosynthetic active organism groups over time. Graphs on the left show the relative fluorescence after 5 min dark adaption [excitation wavelength were 470 nm (*diamond*); 520 nm (*squares*), 645 nm (*triangle*) and 665 nm (*circles*). On the right is the microscopically determined share of *C. aponinum* (*stripes*, bottom), green algae (*dots*, middle) and diatoms (*squares*, top)]. *First row* WP7(1) 13 batch; *middle row* CA WP7(1) 14 batch; *last row* CA WP7(1) 14 semi-continuous

comparison until day 20. During the first 5 days all fluorescence excited by other wavelength was below 0.2. All signals increased but stayed below 0.25 during day 7 and remained stable. After day 12 all values increased and fluctuated between 0.15 and 0.45. On day 20 and later the fluorescence excited by light of a wavelength of 470 nm was the highest. Compared to the excitation at 470 nm the other fluorescence values started at 0.5 (665 nm), 0.6 (520 nm) and 0.65 (645 nm) and increased by up to 0.2 points during the following days (Fig. 6, lower row, right).

Discussion

The discovery of contaminating weed algae in cultures of cyanobacteria was possible by analysis of the photosynthetic yield excited with 645 nm: but only as certain preconditions were met. To be distinguishable weed algae had to have a higher maximum yield. The addition of weed algae with a lower maximum yield showed a quenching effect after a ratio of 1:1 was reached (based on optical density, data not shown). The detection limit discovered was one of five cells for CA batch 14, in which the weed algae were mainly green

algae. The maximum Y of CA WP7(1) in laboratory studies was 0.45 (Winckelmann et al. 2015). In CA batch 13 where green algae were found in a ratio of 1:10 or even less abundant Y stayed below 0.45 except on day 14. Therefore, with the Y alone a classification as healthy culture or contaminated culture was not possible. CA semi-continuous 14 had a Y below 0.45 over the majority of cultivation time. It alternated around 0.3 for the first 5 days where contamination by green-algae was <1 in 250 cells. It stayed below 0.45 when the ratio of weed algae to CA was above 1–20. On day 20 the overall abundance of weed algae increased above 3 % with a high ratio of diatoms and Y increased above 0.5. The sudden drop of Y on day 5 and day 19 can be attributed to the culture dilution the day before and light-induced photoinhibition (Schreiber and Klughammer 2013).

It was concluded that Y, which is influenced by several different factors like light (Schreiber and Klughammer 2013), culture density (Schreiber and Klughammer 2013), salt stress (Lu and Vonshak 2002) and nutrient availability (Lippemeier et al. 2001) is not sensitive enough to be utilized for weed detection.

Cyanobacteria, green algae and diatoms contain different light-capturing pigments which are excited by light of different wavelengths. According to Kolbowski and Schreiber (1995) the excitation of fluorescence using different wavelengths allows the differentiation between cyanobacteria, green algae and diatoms. By using the minimal fluorescence of a dark-adapted sample (F0) the interdependence between photosynthetic processes and fluorescence is minimized; the fluorescence is influenced by biomass alone (Geel et al. 1997). The typical cyanobacterial fluorescence pattern of a culture showing no detectable contamination, growing in cPW and on site during the spring resembles the pattern shown by CA 14 semi-continuous from day 2 to day 5 (Fig. 5, last row, left; Data of growth in bottle reactors not shown). From comparing the developing fluorescence patterns in the different cultures over a specific time period, it can be concluded that CA 13 batch was contaminated from day 0, CA 14 batch showed signs of contamination from day 3 and CA 14 semi-continuous from day 7. The fluorescence pattern of CA 14 batch and CA 14 semi-continuous on the first day was also different from the pattern of a clean culture. It is assumed that the change in pattern was caused by the culture density in combination with light of high intensity, which led to photoinhibition severe enough to not be repairable during the short time of dark adaption but not lethal to the culture as whole.

Using the fluorescence pattern for weed algae detection is favorable compared to using the Y. But the applicability as an online sensor depends on the development of an automatic dark adaption step. The use of other fluorescence parameter (fm, fm′ and f′) for the calculation of fluorescence pattern indicating biomass is less reliable and F0 was shown to be linear with biomass (Serôdio et al. 2006). If a dark adaption step can be established in a routine measurement the change of pigment composition within a species over time has to be considered. The method has to be able to distinguish between change of fluorescence pattern due to physiological change or photoinhibition (Kromkamp and Forster 2003) or weed algae (Kolbowski and Schreiber 1995). It was shown that the fluorescence excited by different wavelengths changes due to photoinhibition caused by light of different wavelengths (Schreiber and Klughammer 2013). The light utilization of algae communities changes from season to season with the changing wavelengths of the sunlight (Sutherland et al. 2013). It is most likely that with the change of season and sunlight exposure the fluorescence pattern of an algae culture will also change. Whether the pigment composition of the target algae changes over an annual cycle or whether the same reference pattern can be used over the course of a whole year has to be established for each site and algae separately.

It was possible to detect contaminations due to weed algae as low as 1 %.

Using other techniques even lower detection limits were achieved. It was shown that flow-cytometry was reliable down to an amount of 0.01 % weed algae, as long as the weed algae had slightly different shapes (Fulbright et al. 2014). It was shown that flow-cytometry is capable of monitoring the growth and for algae changing their morphology during their life cycle it also can detect the growth state (Havlik et al. 2013). It also can be used to detect other non-photosynthetic contaminations like grazers (Day et al. 2012). A recent publication using qPCR showed that weedy algae can be detected at a contamination level of 0.000001 % (Fulbright et al. 2014).

For procedural reasons and conservation of equipment involved the salinity of seawater should not be exceeded. The increase in salinity in the different cultures did not reach the threshold of 3 %. CA was shown to be able to grow uninhibited in Blue-Green Medium (BG11) with 34 g/L NaCl (Moro et al. 2007) and laboratory experiments have shown that its growth was not affected until the growth media contained more than 90 g/L NaCl (Winckelmann et al. 2015). This is an indicator that CA is part of the functional group of moderately halotolerant cyanobacteria as defined by Reed and Steward (1985). The use of halotolerant

cyanobacteria as a production strain in production systems without addition of carbon dioxide and in brackish water might lead to an economically viable productivity. Alkalization of growth medium due to carbon dioxide depletion was shown in laboratory studies (Shiraiwa et al. 1993) and was observed in natural ecosystems (Reed and Stewart 1985). Some cyanobacteria capable of growing in high alkaline environments have shown the need of sodium to maintain intracellular acidic conditions using Na^+/H^+-antiporter (Pogoryelov et al. 2003). This and other physiological traits might lead to a competitive advantage of cyanobacteria at high pHs (Paerl et al. 2001).

The elevated average temperature during the cultivation of the semi-continuous culture in 2014 might have promoted higher biomass production. It was shown that growth of some cyanobacterial species was promoted by higher temperatures (O'Neil et al. 2012). Using the basic local alignment search tool (BLAST) (Altschup et al. 1990) to compare the known sequences for the 16 s RNA gene (GenBank entry JN584264.1) with known *Synechococcus* sp. sequences a query coverage of 100 % with 88 % identical sequence was found. For the ITS region of CA PB1 (GenBank entry KF982001) a query coverage of 56 % with 100 % identical sequence was found. This might indicate a relationship between both species but further gene sequences of CA should be obtained to enable further phylogenetic analyses.

Assuming a year-round productivity as measured in either pond, the biomass yield in an open pond facility with 80 % pond surface, 300 working days per pond per year and a depth of 20 cm would be between 36 and 58 tons per hectare and year. This results in a caloric value of 72,000–116,000 MJ per year (Quintana et al. 2011). With a reported lipid content of up to 45 % and a high yield of methyl esters with 16 and 18 carbon molecules (Karatay and Dönmez 2011) the prospects of biofuels as a suitable product have to be investigated. Karatay and Dönmez (2011) performed all experiments in BG11; therefore, the biomass composition of CA grown in cPW might differ. It was shown that lipid content of Cyanobacteria increases with increased NaCl levels (Araujo et al. 2011; Bhadauriya et al. 2008) but is also influenced by the growth stage, temperature and light intensity (Liu et al. 2005). Assuming no change in biomass and the same esterification efficiency as reported (Karatay and Dönmez 2011) a lipid yield of 16,000–26,000 kg per year would result in 11,000–17,500 kg of methyl esters.

Assuming a water loss of 14–15 mm per day due to evaporation, the amount of water needed would be 33,000-36,000 m^3 per hectare per year. Adding the 1600 m^3 water used for the initial growth medium, and supposing that 20 % of the volume is harvested per day, the total water demand of a hectare would be 131,000–134,000 m^3 per year. The fertilizer demand would be 4900 m^3 per year.

For a final statement of economic feasibility: the energy consumption, nutrient recycling and costs, usability of all algae residues, and work demand have to be entered into the equation (Collet et al. 2013).

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