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MARINE MICROALGAE *MICROCHLOROPSIS GADITANA* AND *PSEUDOCHLORIS WILHELMII* CULTIVATED IN OIL REFINERY WASTEWATER – A PERSPECTIVE ON REMEDIATION AND BIODIESEL PRODUCTION

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ABSTRACT

Industries are seen as centers of pollution, thus finding sustainable solutions for recycling their waste products such as chemicals, heat and CO_2 is of a high priority. Along these lines, marine microalgae *Microchloropsis gaditana* and *Pseudochloris wilhelmii* were selected and cultivated in 50% diluted oil refinery wastewater at 18°C and 80 µmol photon m^{-2} s^{-1} at the salinity of 19 psu with a CO_2 supply to study biomass quality for biofuel production. Between the two species, faster growth was observed for *P. wilhelmii* during the exponential phase, but after 10 days of growth its total lipid content (35.5%) was lower in comparison to *M. gaditana* (40.6%). Fatty acid methyl esters of a higher quality suitable for biodiesel production were produced in lag phase (within 48 h) for both species. Maximum lipid concentrations between the species were comparable, 115.3 mg l^{-1} for *M. gaditana* and 114.0 mg l^{-1} for *P. wilhelmii*. Both species have successfully removed ammonia/ammonium (~0.9 mM) from the wastewater within 8 days. *M. gaditana* had a considerably lower requirement for phosphorus. This indicates that *M. gaditana* could be more suitable for bioremediation of phosphorous-poor industrial wastewater due to lower production costs of its biomass with the high lipid content of good quality for biodiesel production.

KEYWORDS:
Marine microalgae, wastewater, nutrient uptake, biomass, lipids, biodiesel

INTRODUCTION

The increase in world population is tightly linked to higher consumption of non-renewable energy sources [1]. The availability of fertile land for agricultural production is crucial in satisfying the needs of a growing population. To lower the global ecological footprint of a modern-day human, it is pivotal to look for alternative and sustainable resources. From the pioneering ideas and practices of biofuel production by using agriculture crops (1st generation) [2] to biomass from residues without the need of extra arable land (2nd generation) [3], today more attention is given to studying biofuel production from microalgae (3rd generation biofuels) [4] that does not require fertile land areas.

Microalgal biomass is a suitable resource for different types of biofuels, among which biodiesel stands out. Biodiesel is produced from lipids that are major components of cell membranes and energy storage vesicles (liposomes) in microalgae cells. It is known that microalgae can accumulate considerable amounts of lipids, even more than half of their dry cell mass [5]. However, lipid quantity and quality depend on the environmental conditions that microalgae are exposed to. Microalgae are diverse group of microorganisms that vary in their preferences for the light intensity, temperature and salinity [6]. They also differ in features of nutrient uptake in oligotrophic and eutrophic environments [7], ammonia tolerance [8], sensitivity to heavy metals [9], physiological adaptability [10, 11, 12], type of metabolism (autotrophs, mixotrophs, heterotrophs) [13], usage of organic compounds [14] and capability to degrade crude oil [15]. Some species of microalgae can successfully utilize nutrients from wastewater for their growth, remediating polluted waters [16, and references therein]. Among them, marine and brackish species could be used to treat salty wastewater. They could also be grown in wastewater as a nutrient source in combination with seawater to adjust the salinity and, if necessary, dilute concentrations of pollutants in the final growth medium. Accordingly, wastewater that usually represents an environmental burden could also be a valuable source of nutrients. Moreover, excessive CO_2, another environmental burden, could be used as a carbon source to feed microalgae.

Microorganisms are extensively used in remediation of different kinds of wastewater that vary in their contents. It is especially challenging to cultivate microorganisms in industrial wastewater, such as oil refinery wastewater rich in phenols, toluene, xylene, benzene, thiols, sulfides, cyanides, heavy metals, ammonia etc. [17]. Thus, the resistance to toxic substances and adaptability to a wide range of environmental factors, such as salinity, temperature and light intensity, are critical to preselect algal species most suitable for production of high lipid content in their biomass, when fed by industrial wastewater in open raceway ponds. Bearing that in mind, mathematical modelling that incorporates nutrients' uptake rates, algal growth rates and content of lipids in the biomass in relation to environmental variables (salinity, temperature and light intensity) [18] is a valuable tool to calculate and determine key parameters that are important for bringing algal biodiesel to an industrial scale of production.

Here, we present our findings on two marine nanoeukaryotes, *Microchloropsis gaditana* and *Pseudochloris wilhelmii*, originally isolated from the Adriatic Sea, revealing them as suitable source of lipids for biodiesel production even when grown under suboptimal conditions in 50% diluted oil refinery wastewater.

MATERIALS AND METHODS

Culture conditions. Marine algae *Microchloropsis gaditana* (SAG 2.99) and *Pseudochloris wilhelmii* (SAG 55.87) were obtained from the Culture Collection of Algae at Göttingen University (SAG). Both strains were cultured at pH 8.3 in aged seawater filtered through 0.2 µm, enriched with Guillard's f/2 medium without silicate, set at 35 psu for *M. gaditana*, and with Blue-Green medium (BG-11) set at 19 psu for *P. wilhelmii*. Cultures were cultivated under ambient temperature of 25±1°C, and exposed to cool white light of 70 µmol photons m^{-2} s^{-1} with light/dark cycle of 14 h/10 h.

Tests in microwells: salinity and toxicity. Salinity. To test the growth under different salinity conditions, experiments in microwell plates were performed. Cultures were grown in the appropriate media at following salinities: 0, 7, 14, 17.5, 21, 31.5 and 35 psu. Measurements for each species and blanks (media) were determined in triplicates for each salinity level. Wells containing 250 µl of media were inoculated with 20 µl of microalgae culture. Optical density at λ=690 nm (OD_{690}) was recorded daily as a proxy for growth using Multiskan Ascent Plate Reader. The triplicates were checked for outliers by using the Modified z-score method [19] and averaged after omitting the eventual outlier values. Daily OD_{690} values were calculated by subtracting the average OD_{690} of the blanks from the average OD_{690} values of inoculated wells for each salinity and species.

Toxicity. The balance between ammonia (NH_3) and ammonium (NH_4^+) in the seawater is controlled by pH, temperature and salinity [20]. Four concentrations of NH_4^+ (1.2, 1.6, 2.0 and 2.4 mM) were prepared in triplicates diluting the filtered (0.2 µm) oil refinery wastewater with artificial seawater (ASW) (~50:50 vol:vol) in microwell plates. Additions of NH_4^+ served to cover the common annual range of NH_4^+/NH_3 concentration in the oil refinery wastewater that was used in this study. Salinity of 19 psu obtained by dilution was appropriate for *P. wilhelmii*, whilst NaCl was added to reach salinity of 35 psu for *M. gaditana*. All nutrients' concentrations, namely DIN (dissolved inorganic nitrogen i.e. nitrate (NO_3^-), nitrite (NO_2^-) and NH_4^+) and DIP (dissolved inorganic phosphorus i.e. orthophosphate) were adjusted by addition of stock solutions (100 mM for DIP and 800 mM for NH_4^+) to achieve not only the precise concentrations of NH_4^+, but also the same DIN to DIP ratios of 24 and 98, simulating the DIN/DIP of f/2-Si and BG-11 media that are commonly used in cultivation of *M. gaditana* and *P. wilhelmii*, respectively. Since NH_4^+ contribution to DIN in wastewater was higher than 99.9% (Table 1), DIN values were almost the same as NH_4^+, whilst DIP concentrations were adjusted to 49.3, 65.7, 82.1 and 98.5 µmol l^{-1} in the case of *M. gaditana*, and to 12.3, 16.4, 20.4 and 24.5 µmol l^{-1} in the case of *P. wilhelmii*, from the lowest to the highest concentration of NH_4^+ and DIN, respectively. Considering the pH of 8.3 and ambient temperature of 25±1°C, NH_3 concentrations (toxic form of interest) in the prepared solutions were ~10% of the targeted NH_4^+ concentrations. Deionized water (270 µl) was used as a blank. The final calculations of daily OD_{690} measurements were identical to those described for the salinity test, including the Modified z-score method [19] for detecting outlier values.

TABLE 1
Dissolved inorganic nutrients and DOP* concentrations with DIN/SRP* ratio in the filtered undiluted oil refinery wastewater.

	SRP	DOP	NO_2^-	NO_3^-	NH_4^+/NH_3	DIN	DIN/SRP
(µmol l^{-1})	1.37	3.90	0.13	0.29	1819.75	1820.17	1328.6

*DOP-dissolved organic phosphorus; *SRP-soluble reactive phosphorus

Experimental set-up in photobioreactors. Photobioreactors (PBRs), i.e. double-layered glass containers with capacity of 2.6 l were employed to grow microalgae at controlled temperature, pH, light intensity, light/dark cycle and CO_2/air mixing. PBRs are equipped with: LED 3000 K (warm white) lights, air/CO_2 supply for bubbling, water pump associated with the cooler/heater and sensors for pH, temperature, conductivity and light intensity. The system is operated by Supervisory Control and Data Acquisition (SCADA), a software adapted by Comprehensive Water Technology ltd., Faculty of chemical engineering, University of Zagreb, Croatia.

Depending on the density of the cultures (measured as OD at λ=720 nm), appropriate volumes of the cultures were centrifuged for 10 min at 4900 rpm and the pellets were resuspended in the associated PBRs. The initial cell density corresponded to ~30 mg of dry weight (dw) biomass l^{-1} in each PBR. A mixture, a total of 2.6 l, 1:1 vol:vol, of filtered (0.2 µm) oil refinery wastewater and filtered (0.2 µm) ASW was prepared by adjusting salinity to 19 psu at pH 8.3. The resulting salinity was a consequence of dilution with the ASW, and the pH was adjusted by SCADA system via CO_2 supply and by manual addition of NaOH (1 M) to the value used for culturing selected microalgae species. Controlled conditions accounted for a light/dark cycle of 12h/12h, temperature of 18°C, light intensity of 80 µmol photon m^{-2} s^{-1}, and bubbling by sterile air supply of 0.99 l min^{-1} and CO_2 supply of 0.01 l min^{-1}. The temperature of 18°C selected for the experimental set-up in PBRs is considered to be suboptimal for both species [21, 22], whilst light intensity of 80 µmol photon m^{-2} s^{-1} was within the range of 50-100 µmol photon m^{-2} s^{-1}, commonly applied in their cultivation [23, 24].

Nutrients were adjusted so that the initial DIN (910 µmol l^{-1}) to DIP (165 µmol l^{-1}) ratio equals ~5.5, aiming to achieve N-limited growth of microorganisms while approaching the stationary phase after expected depletion of DIN. Concentration of DIP was adjusted by addition of 4.3 ml of phosphate buffer (100 mM PO_4^{3-}). Lower temperature (18°C) provoked even lower contribution of NH_3 (6.4%) within NH_4^+/NH_3 at time=0 in comparison to the toxicity test at 25°C in microwell plates.

Biomass. Triplicates of 5 ml of cultures were daily sampled and filtered on a pre-weighed 0.4 µm pore size Whatman® polycarbonate filters. During filtration, the biomass was rinsed with distilled water to remove the salt. The filters were dried at 60°C for a minimum of 2 h to reach a constant weight. The biomass concentration was calculated by subtracting the blank filter mass from the mass of the filter with dry sample, and dividing the resulting mass by the sampled volume. The triplicates were checked for outliers by using the Modified z-score method [19] and averaged for the final dw biomass concentration.

Nutrients and elemental analysis. Aliquots (50-100 ml) were sampled daily for dissolved inorganic nutrients, DOP and cellular C, H, N and P determination. NO_3^-, NO_2^-, NH_4^+/NH_3, SRP and DOP were analyzed from the supernatant after the immediate centrifugation (10 min, 5000 rpm) of the collected samples. The procedures described in [25, 26, 27] were followed by using the appropriate dilutions of the samples to fit the range of spectrophotometric determination (Shimadzu UV 1800, path length 1 cm) for each method. Orthophosphate measurements in fact represent SRP because acid-labile organic P (a fraction of DOP) may contribute to the formation of blue complex [28]. This distinguishes DIP from SRP. Centrifuged pellets from 10-20 ml aliquots were used for particulate P analysis (final dilution 10x) as described in [26]. Centrifuged pellets from 40-80 ml aliquots were rinsed with deionized water after the first centrifugation, resuspended in deionized water (5 ml) after the second centrifugation (10 min, 5000 rpm), transferred into the polyethylene bottles and kept at -80°C until lyophilization that was carried out for 48 h (-51°C, pressure <0.030 mbar). Biomass was weighed immediately upon completion of lyophilization. Cellular P mass contribution was calculated according to dw biomass concentration, whilst C, H, N contributions in biomass were analyzed by using PerkinElmer 2400 Series II CHNS/O Elemental Analyzer.

Determination of Michaelis-Menten i.e. Monod kinetics constants. Half saturation concentrations (K_m) of NH_4^+ and SRP and their maximal uptake rates per biomass (V_{max}) were estimated for each species by extrapolation of the logarithmic regression trend lines fitting the relations between substrates' concentrations and their uptake rates per average biomass within each period of 24 h between the start of the exponential growth and the end of the experiment.

Lipids and FAME profile. Aliquots (30-50 ml) of cultures were filtered onto pre-combusted and pre-weighed Whatman® GF/F filters to determine the total lipid content and fatty acid methyl ester (FAME) profile. Filters were dried for 12 h at 60°C and weighed again. For the lipid extraction, filters were homogenized and lipids were extracted as described in [29]. Grinded filters were soaked in 30-50 ml of dichloromethane:methanol=2:1 (vol:vol) mixture, and sonicated for 1 h in ultrasonication bath Aquasonic 750D VWR Scientific Product. The extraction was repeated three times. The extracts in dichloromethane were combined, then evaporated using a rotary evaporator and weighed at the end. As follows, total extracts were saponified by adding 2 ml of 1.2 M NaOH (methanol:water=1:1 vol:vol), acidified by adding 1 ml of 6 M HCl, methylated by adding 2 ml of 14% BF3 (in methanol) and extracted

in dichloromethane [30]. FAME profiles were measured using Agilent 6890N gas chromatography system equipped with a 5973 Network Mass Selective Detector, capillary column (25 m×0.3 mm×0.25 µm, cross-linked 5% phenylmethyl siloxane) and ultra-high purity helium as the carrier gas. The gas-liquid chromatography settings were programmed for column temperature to rise from 70°C by 4°C min^{-1} up to a ramp of 4 min at 205°C and continue rising up to 270°C by 4°C min^{-1} at constant column pressure of 2.17 kPa. Chromatographic spectra were analyzed by Chemstation software while the FAME profiles were determined by mass spectral data. Family plot of equivalent chain length data for GC standards (F.A.M.E. Mix C4-C24 Sigma-Aldrich, F.A.M.E. Mix C18-C20 Sigma-Aldrich) for the GC column was used. Lipid content was expressed as a portion in dw biomass. Lipid yields were calculated as a difference in lipid content in time.

Biodiesel properties. From analyzed FAME profiles, the unsaturation/saturation ratio between total unsaturated and saturated FAME (UNS/SAT), and the average degree of unsaturation were calculated as described in [31], by summing up all the products of mass fractions of each unsaturated fatty acid with their number of double bonds. Biodiesel characteristics, described by kinematic viscosity (KV), specific gravity (SG), cloud point (CP), cetane number (CN), iodine value (IV) and higher heating value (HHV) were calculated as proposed in [32] and compared to EN 14214 European Standards [33].

RESULTS

Salinity and toxicity tests. *M. gaditana* grew significantly slower under freshwater conditions (0 psu), indicating clearly its marine nature i.e. dependence on higher salinity (Fig 1 b). In contrast, *P. wilhelmii* grew notably even at 0 psu in the period of 6 days (Fig 1 a). Both species showed considerable growth in a wide range of salinities. However, *P. wilhelmii* reached highest OD_{690} value, and thus highest biomass at 7 psu, which was similar to the highest OD_{690} value for *M. gaditana* detected at 17.5 psu (Fig 1).

Toxicity test further showed evident difference between the two species. Both species showed an acceptable tolerance to 50% of pollutants present in the wastewater in the whole range of NH_4^+ concentrations. *M. gaditana* growth was slightly inhibited at the highest NH_4^+ concentration (2.4 mM) compared to lower concentrations, but still having similar growth to *P. wilhelmii* at 2.4 mM NH_4^+. At lower NH_4^+ concentrations (Fig. 2), *M. gaditana* showed more than two-fold higher growth compared to *P. wilhelmii.*

Experiment in photobioreactors. Growth curves. *P. wilhelmii* achieved higher biomass concentration (0.321 g l^{-1}) than *M. gaditana* (0.284 g l^{-1}) at the end of the experiment (day 11) (Fig. 3), whereas the relative multiplication of the biomass between the start and the end of the experiment was similar, 7.19 for *P. wilhelmii* and 7.22 for *M. gaditana.* Maximal observed specific growth rates were notably different between the species, 0.567 day^{-1} for *P. wilhelmii* and 0.405 day^{-1} for *M. gaditana* (Table 2). Exponential growth of *M. gaditana* was delayed for 2 to 3 days, with μ_{max} in the period of day 7 to day 8, in comparison to the exponential growth of *P. wilhelmii*, reaching μ_{max} in the period of day 4 to day 5 (Fig. 3).

Nutrients and C/H/N/P stoichiometric ratios in the biomass. *P. wilhelmii* showed notably higher affinity for SRP uptake than *M. gaditana*, whilst the affinities for NH_4^+ uptake were similar between the species (Table 2). Almost complete depletion of the NH_4^+ (day 9) as the major portion of DIN was delayed for 1 day in the case of *M. gaditana* in comparison to *P. wilhelmii* (day 8) (Fig. 4).

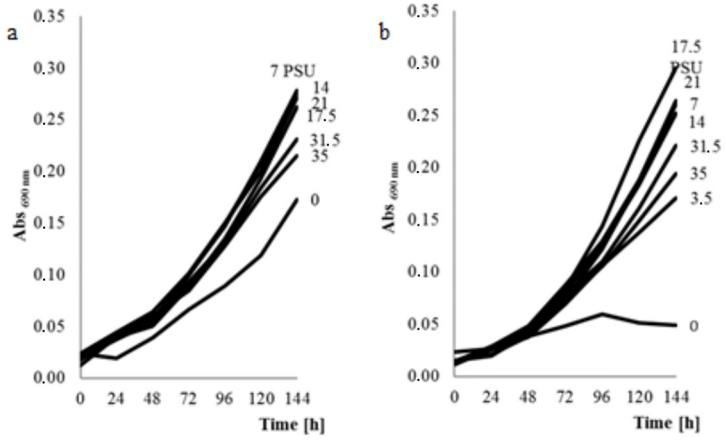

FIGURE 1
Growth curves based on OD_{690} of: (a) *P. wilhelmii* and (b) *M. gaditana* at different salinities.

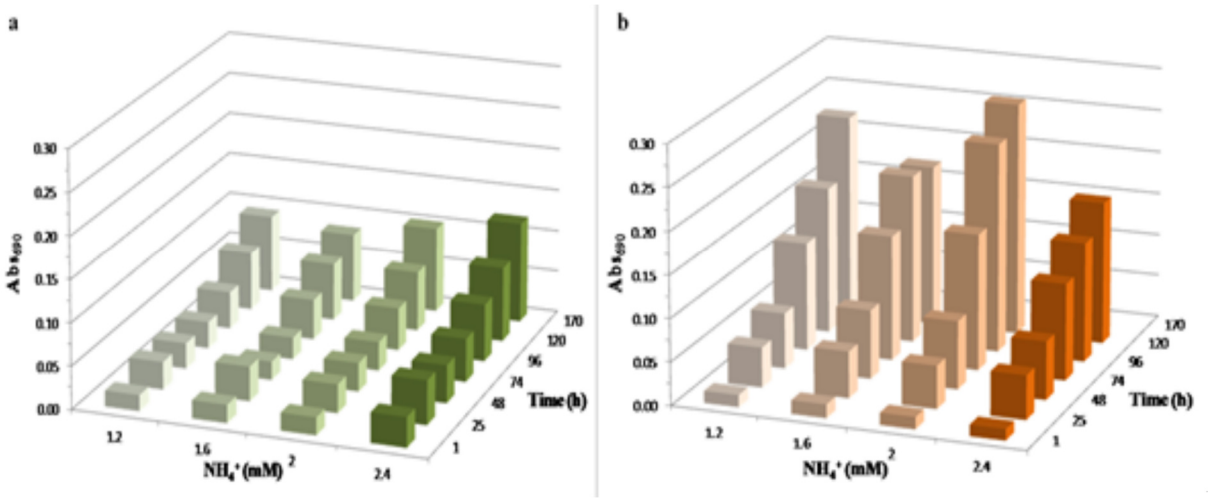

FIGURE 2

Toxicity tests for: (a) *P. wilhelmii* and (b) *M. gaditana* in a range of NH_4^+ concentrations from 1.2 mM to 2.4 mM with 50%:50% contributions of oil refinery wastewater and artificial seawater.

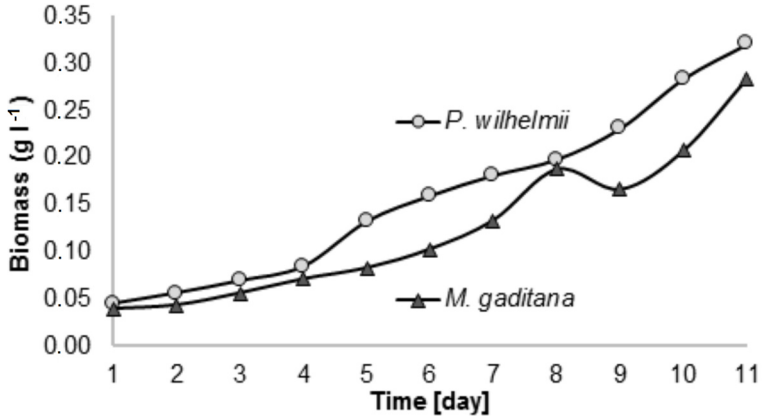

FIGURE 3

Growth curves of *M. gaditana* and *P. wilhelmii* in 50% diluted oil refinery wastewater.

TABLE 2

Nutrient uptake kinetics and maximum specific growth rates of *M. gaditana* and *P. wilhelmii.*

	V_{max} (µmol SRP g^{-1} d^{-1})	K_m (µmol SRP l^{-1})	Affinity for SRP (V_{max}/K_m) (l g^{-1} d^{-1})	V_{max} (µmol NH_4^+ g^{-1} d^{-1})	K_m (µmol NH_4^+ l^{-1})	Affinity for NH_4^+ (V_{max}/K_m) (l g^{-1} d^{-1})	μ_{max} (observed) (d^{-1})
M. gaditana	360	229.89	1.57	2400	205.21	11.70	0.405
P. wilhelmii	500	159.21	3.14	2000	210.80	9.49	0.567

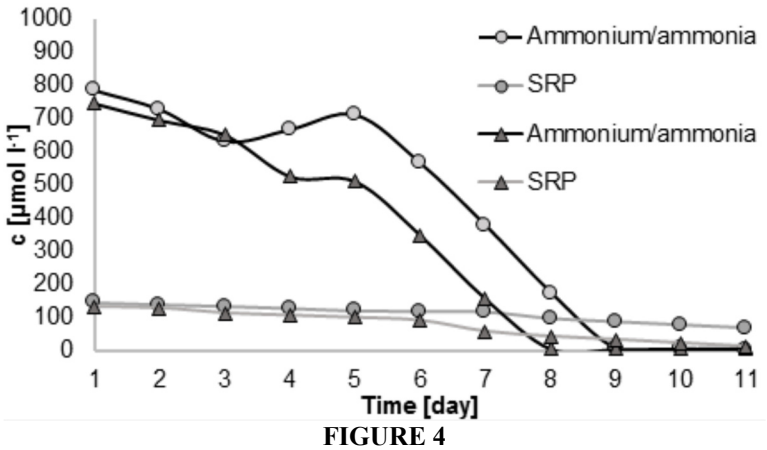

FIGURE 4

Nutrient dynamics in photobioreactors with *M. gaditana* (o) and *P. wilhelmii* (Δ).

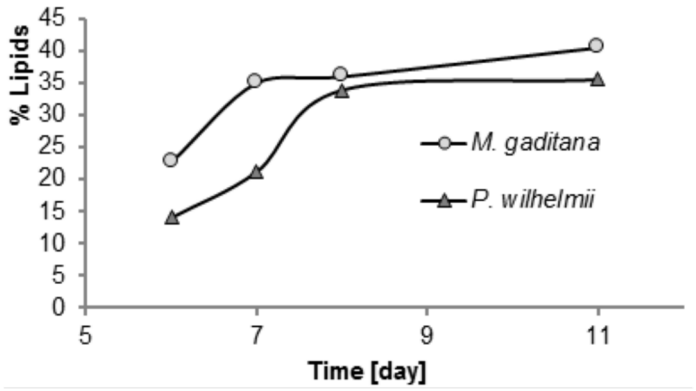

FIGURE 5
Change in total lipid content of *M. gaditana* and *P. wilhelmii* from day 6 to day 11, covering the exponential growth phase and the period after the depletion of DIN for both species.

TABLE 3
Biodiesel properties of microalgae in comparison to the proposed values for biodiesel.

	P. wilhelmii		*M. gaditana*		Biodiesel
	Lag phase	**N-depletion**	**Lag phase**	**N-depletion**	EN 14214 [33]
KV	4.48	4.16	5.07	4.13	3.5-5.0
SG	0.87	0.86	0.87	0.86	0.86-0.90
CP	4.59	-2.13	17.14	-2.87	-
CN	55.19	51.83	61.45	51.46	≥ 51
IV	98.46	*135.88*	28.59	*140.05*	≤120
HHV	40.56	41.45	38.91	41.55	41.28*
UNS/SAT	1.04	2.71	0.23	1.69	-

*Soybean [34]

M. gaditana showed significantly lower P needs for the build-up of biomass which resulted in much higher residual concentration of SRP at day 9 (86.3 µmol l^{-1}) in comparison to *P. wilhelmii* (SRP at day 8 equalled 43.1 µmol l^{-1}). During the first 8 days of the growth (day 1 to day 9) *M. gaditana* consumed 900.9 µmol DIN l^{-1} and 78.9 µmol SRP l^{-1}, resulting in the average DIN/SRP uptake of 11.4. *P. wilhelmii* consumed 900 µmol DIN l^{-1} and 122 µmol SRP l^{-1} during first 7 days (day 1 to day 8), resulting in the average DIN/SRP uptake of 7.37.

The average stoichiometric elemental composition of the biomass throughout the experiment was $C_{45.62}H_{88.05}N_{6.26}P$ for *P. wilhelmii* and $C_{47.28}H_{96.92}N_{7.76}P$ for *M. gaditana.* The N/P stoichiometric ratios in the biomass of *M. gaditana* were slightly higher (average ± SD=7.76±3.94) than those in *P. wilhelmii* (6.26±2.32) (data not shown).

Lipids. Lipid content did not differ significantly (unpaired two tailed t-test; n=4, p=0.146) between two species (Fig. 5), neither did the final biomass yield. Final lipid content reached 40.6% in *M. gaditana* and 35.5% in *P. wilhelmii* (Fig. 5). The final lipid concentration for *M. gaditana* at day 11 was 115.3 mg l^{-1} and for *P. wilhelmii* 114 mg l^{-1}. *M. gaditana* achieved maximum lipid yield between day 6 and day 7 with 23.5 mg lipids day^{-1} l^{-1} whereas *P. wilhelmii* had the maximum lipid yield of 28.8 mg lipids day^{-1} l^{-1} between day 7 and day 8.

Biodiesel properties. For both species, slightly better biodiesel properties were observed within the first 48 h i.e. during the lag phase in comparison to growth after depletion of DIN. The only parameter that indicated an unfavorable change in the biodiesel properties was iodine value that exceeded the recommended maximum limit value of 120 by the end of the experiment for both species.

DISCUSSION AND CONCLUSION

In the present study, the experiment in PBRs was performed without a period of adaptation of the microalgae on the specific composition of the wastewater, e.g. pollutants such as heavy metals, phenol, thiols and cyanides. As a result, growth rates of the tested species might be different from those if the adaptation phase would have been introduced. Two species are phylogenetically and physiologically different, thus to proliferate successfully, they require different optimum conditions. It is known that *P. wilhelmii* is able to survive relatively low pH values and that its optimal pH range is between 5 and 7 [35, 36], whereas the proposed optimal pH for *M. gaditana* is 8 [37]. This implies that applied pH value

of 8.3 was in favor of *M. gaditana*. Toxicity test showed that both species grow in wastewater containing up to 2.4 mM of NH_4^+/NH_3. Nevertheless, *M. gaditana* appeared to be two times more successful in growth at lower concentrations of NH_4^+/NH_3 (1.2-2 mM) than *P. wilhelmii*. Both species tolerate NH_4^+/NH_3 up to 16 mM (examined upper limit) in the appropriate media at pH 8.3 and temperature of 25°C (unpublished data). The total toxicity of wastewater decreased according to ammonia depletion. It is possible that microalgae have converted other pollutants to their less toxic forms but this was outside the scope of this study. Tested species expressed significantly different growth at salinity of 0 psu in microwells, indicating a better adaptation of *P. wilhelmii* to low salinity, being in line with recommended cultivation of this species in the brackish medium. Therefore, we assume that brackish conditions might have had a notable impact on a delayed exponential growth phase of *M. gaditana*. On the other hand, salinity stress is known to be beneficial for lipid production in microalgae [38]. This might have impacted higher content of lipids in the biomass of *M. gaditana*.

Significantly lower DIN/DIP than 16, commonly considered to represent an average stoichiometric N/P ratio of the phytoplankton needs [39, 40], was adjusted at the start of the experiment in PBRs to provoke N limitation by the end of the experiment even in the case of potentially lower N/P uptake ratio by selected species during fast metabolism and growth [11]. Namely, N-limitation favors neutral lipids production [41, 42, 43, 44], which was one of the goals of the experiment. Our results indicated that both species were exposed to conditions leading to N-limited growth towards the end of the experiment. The extent to which they adjusted their metabolism towards the lipid accumulation was probably dependent on the nitrogen quota in their cells [45].

It has been shown that *M. gaditana* is able to accumulate up to 45% of total lipids when grown at optimal temperature and continuous illumination [46]. Our results confirmed that *M. gaditana* is a rich oleaginous species even in suboptimal conditions concerning the temperature, moderate illumination and exposure to industrial wastewater. Also, our results demonstrated that *P. wilhelmii*, which was not considered the most prominent microalgae species for biofuel production up to now [47], did not differ considerably from *M. gaditana* in the lipid quality, content and yield.

Tested species produced FAME contents of similar biodiesel quality, which fit the recommended values by EN14214 [33]. Decrease in biodiesel quality according to parameter IV, indicated by higher unsaturation of FAME profiles, was observed during N-depleted growth of both species. Better quality of lipids was observed for the biomass obtained in lag phase of the experiment. Prior to the experiment, algae inoculum was cultivated in the optimal conditions and suitable growth media. Hence, cells in lag phase could have had better fitness because they were exposed to the wastewater in a shorter period than those at the end of the experiment. This implies that more research should be done to understand which factors have a direct impact on FAME quality.

The two tested species exhibited several notable differences while growing on 50% diluted and filtrated oil refinery wastewater. *P. wilhelmii* grew slightly faster than *M. gaditana* in the exponential phase, while both species showed similar multiplication of the biomass between the initial uptake of nutrients and the end of the experiment after 10 days. The most obvious difference between the two species was the uptake of SRP where *P. wilhelmii* showed notably higher demand for phosphorus to build up the biomass. Production of biomass by *M. gaditana* appears to be more cost-effective in comparison to *P. wilhelmii* because *M. gaditana* requires lower orthophosphate quantity when grown on this type of wastewater that are adjusted to provoke N-limited growth. Besides bioremediation, another benefit of growing microalgae is the usage of excessive heat and CO_2 produced by the industries, which could further reduce the costs of biofuel production.

Further experiments on the impact of light intensity, duration of light and dark periods, concentration of nutrients, salinity, CO_2 supply and temperature are needed in order to provide more detailed and economically responsible decision on the choice between these two species for production of biodiesel.

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