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Lipid Yield and Composition of *Azolla filiculoides* and the Implications for Biodiesel Production

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Abstract The aquatic fern *Azolla* is one of the fastest-growing nitrogen-fixing plants on Earth and therefore considered as a potential source of biomass for bioenergy production. The lipid fraction from *Azolla filiculoides* was analyzed to investigate whether it suited biodiesel production. Since the productivity of *Azolla* is further increased at higher CO_2 concentrations, *A. filiculoides* biomass was produced at 800 ppm CO_2 mimicking a cultivation system utilizing CO_2 waste from industry. The harvested biomass contained 7.92±0.14 % dry weight (dw) crude lipids. Drying conditions did not significantly affect lipid composition or yields, indicating that drying conditions may be energetically optimized without the risk of product loss. Total lipid extracts contained 4.2±0.38 % free fatty acids. Of the crude lipid fraction, 41±13 % consisted of fatty acids that were converted into fatty acid methyl esters upon saponification in methanol. Unique mid-chain (di)hydroxy compounds constituted 7.2±2.8 % of the crude lipids. Based on the fatty acid profile, it was estimated that *Azolla* biodiesel meets requirements set by the EN14214 standard on fuel density, cetane number, and iodine value. The cold filter plugging point (CFPP), however, is expected to be too high due to relatively high concentrations of lignoceric acid and the presence of the mid-chain (di)hydroxy compounds. To produce high-quality biodiesel from *Azolla* lipids, therefore, a fractionation step will be required removing these compounds. As an advantage, the long-chain alcohols and (di)hydroxy fatty acids obtained after fractionation may provide a valuable secondary product stream with applications to chemical industry and nutrition.

Keywords *Azolla* · Fern · Lipids · Feedstock · Biodiesel · Biochemicals

Introduction

Since fossil resources are finite and their large-scale use as fuels has a negative impact on global climate, alternative resources for energy and materials are needed. It is expected that, with the depletion of fossil resources, plants will need to provide an important proportion of our requirements for fuels and chemicals [1].

However, not all plant-based alternatives are equally sustainable. Current biodiesel feedstocks, i.e., soybean, rapeseed, palm, and sunflower oil, suffer from low land use efficiency, high input requirement [2], and competition for arable land with food production. Hence, to produce fuels and chemicals in a more sustainable way, novel crops are sought that are low-input, land-efficient, and highly productive. The floating water fern *Azolla* is one such potential novel crop. It can be cultivated in closed systems on non-arable land or in natural occurring freshwater basins. It is known for its high growth rates: In a 154-day outdoor growth experiment, Becerra et al. [3] estimated the annual productivity of *Azolla filiculoides* to be 39 t dry weight per hectare. Moreover, *Azolla* reaches high growth rates without inorganic nitrogen in its growth medium

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[4], due to its symbiotic relationship with nitrogen-fixing cyanobacteria (*Nostoc azollae*). Active CO_2 supply may increase the productivity of *Azolla* even further by 53 % at 760 ppm CO_2 compared to that at 340 ppm [5].

Until now, *Azolla* has been applied as biofertilizer in rice paddy fields, investigated as a biosorbent of heavy metals [6], and evaluated as raw animal feed [7–9]) or protein feed [10]. For biofuel or bioenergy production, so far, anaerobic fermentation to produce biogas [11, 12] and pyrolysis of whole *Azolla* biomass have been suggested [28].

In the present study, we focus on the lipid fraction of *Azolla* as a source of biodiesel. The lipid fraction contains ester-bound fatty acids (FAs) which may be converted into biodiesel using standard alkaline trans-methylation. Biodiesel quality can be predicted from the FA profile of the feedstock [13]. Therefore, various researchers have developed, and used, numerical models to estimate biodiesel quality indicators including density, cetane number, iodine value, and cold filter plugging point (CFPP), using FA profiles [14–19]. To assess the quality of biodiesel produced from A*zolla* using these numerical models, quantitative information on its lipid composition is required.

Existing reports on *Azolla* lipids either were incomplete [20] or did not focus on compound quantification [21]. Therefore, we aimed to analyze all major lipids of *A. filiculoides*. To mimic the situation in a production system utilizing CO_2 waste, biomass was obtained from productive cultures grown at two times the atmospheric CO_2 concentration, i.e., 800 ppm CO_2, comparable to Speelman et al. [5].

Quantification of compounds derived from dried biomass may be affected by the drying condition [22]. Since *Azolla* is an aquatic crop with a high water content, drying will be a likely step in any biomass processing system and needs to be performed with the least energy costs, without affecting extractability and composition of lipids. Therefore, this study first investigates the lipid yields and composition of *A. filiculoides* in relation to drying conditions. Second, we evaluate the implications for biodiesel production, using numerical models to estimate biodiesel quality.

Materials and Methods

Biomass Production and Pre-treatment

A. filiculoides was obtained from the International Rice Research Institute (IRRI) under accession number 1052. Details on the original site of collection are provided in the IRRI germplasm collection catalogue [23]. Plants were cultivated in a growth chamber at Wageningen University. Growth in continuous culture was performed, in five separate 30-L containers, at a CO_2 concentration of 800 ppm, a day/night temperature of 23/20 °C, a 16-h day length, and a photosynthetic flux density of 325 μmol m^{-2} s^{-1}. Fresh nutrients were supplied every 3 days with the following concentrations: 0.7 mM KNO_3, 0.1 mM $Ca(NO_3)_2$, 0.13 mM KH_2PO_4, and 0.1 mM $MgSO_4 \cdot 7H_2O$ of macronutrients and 4.7 μM Fe-EDTA, 2.2 μM $MnSO_4 \cdot H_2O$, 0.1 μM $Na_2Mo_4 \cdot 2H_2O$, 8.1 μM H_3B_3, 0.06 μM $CuSO_4 \cdot 5H_2O$, and 3.1 μM $ZnSO_4 \cdot 5H_2O$ of trace elements.

The entire medium was refreshed every 2 weeks. Every 3 or 4 days, one third of the surface area of each container was harvested. From all five containers, a batch of fresh *Azolla* biomass was harvested for the analysis. Each batch was divided into three aliquots which were dried for 3 days at 30 °C, dried for 1 day at 65 °C, or freeze-dried, respectively. After drying, the biomass was ground using a Retsch ZM200 grinder equipped with a 1-mm sieve.

To determine the dry weight of each aliquot after drying and grinding, 1 g of biomass was pre-weighed in alumina cups, which were placed into an oven set at 105 °C. They were left to dry for 24 h after which they were transferred into an exsiccator. After cooling for 1 h inside the exsiccator, the alumina cups were taken out and immediately weighed using a Bosch SAE 200 scale with a sensitivity of 0.1 mg.

Total Lipid Extraction and Saponification

The freeze-dried and ground *Azolla* biomass was Soxhlet-extracted with a 7.5:1 dichloromethane (DCM) to methanol (MeOH) solution for 24 h. The collected extracts were dried using a rotary evaporator and weighed to determine the amount of "crude lipids" obtained. Subsequently, these total lipid extracts (TLEs) were re-dissolved in 9:1 DCM/MeOH and ran over a Na_2SO_4 column to remove any traces of water. Afterward, the TLEs were dried under continuous nitrogen flow.

For TLE analysis, an aliquot was methylated using diazomethane at room temperature, which was subsequently purified over a silica (60-Å pore size) column. Next, the extract was silylated by bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine at 60 °C for 20 min. Five to ten micrograms of squalane was added as an internal standard.

A selection of lipid extracts was saponified in 2 N KOH in MeOH (96 %) at 70 °C for 2 h. After this, the solution was acidified to pH <5.5 using 2 N HCl. One milliliter of ultrapure water (from Milli-Q water purifier) was added, and the solution was three times extracted by 1 ml of DCM. The DCM fractions were combined and dried under continuous nitrogen flow. From these saponified extracts (SEs), traces of water were removed using a Na_2SO_4 column, after which the same derivation steps were conducted as for the TLEs.

Lastly, TLEs and SEs were run on a Hewlett Packard Gas Chromatograph–Flame Ionization Detector (GC-FID) and Thermo-Finnigan TraceGC ultra-Trace DSQ Gas Chromatograph–Mass Spectrometer (GC-MS). Both machines were

equipped with a Varian CP-Sil5CB column (30 m, 0.32 mm i.d., and film thickness of 0.10 μm). The GC was operated at constant pressure of 100 kPa, whereas the GC-MS was operated at constant flow of 1.6 ml min^{-1}. Temperature profiles were identical for each analysis, i.e., 70 °C during on-column injection, increasing to 130 °C at a rate of 20 °C min^{-1}, then increasing to 320 °C at a rate of 4 °C min^{-1}, and finally, an isothermal hold for 20 min. The MS operated within a scanning range of *m/z* 50–800.

Identification of the compounds was carried out by their mass spectra using a NIST library or by interpretation of the spectra, by their retention times, and/or by comparison with literature data. Quantification was conducted by integration of peak areas of each individual peak/compound relative to that of the internal standard. In the case of co-eluting peaks, peak separation was achieved by comparing relative intensities of specific fragment ions and using their ratio to quantify each of the compounds identified. Dotriacontane was added as a tracer before Soxhlet extraction to analyze losses during the extraction and subsequent workup procedure and allow to correct compound concentrations obtained from GC and GC-MS analysis. In total, 11 TLEs were analyzed, 4 of which were extracted from biomass batches dried at 30 °C, 4 extracted from batches dried at 65 °C, and 3 extracted from freeze-dried batches. Nine SEs were analyzed, three for each drying condition.

Analysis of Variance

To determine whether drying conditions affect lipid yields and composition, a one-way analysis of variance (ANOVA) was performed using SPSS 20 statistics software. As we were interested in the specific effect of predefined drying conditions, i.e., freeze-dried, 3 days at 30 °C, and 1 day at 65 °C, we used a straightforward fixed effects model:

$$\gamma_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where γ_{ij} = lipid or compound concentration (%), μ = common value, α_i = treatment effect, and ε_{ij} = residual error due to variation between biomass batches and variations introduced during analysis. For determining the effect of drying conditions on crude lipid yield, all five replicates were used (j=5), whereas for determining the effect of drying conditions on individual compounds, a random subset of at least three replicates were used (j=3). For individual compounds, computing was done on the basis of normalized concentrations to eliminate the effect of the variance in total compound detection. In cases that the F test provided a value with $p<0.05$, Tukey's test was used to determine which drying condition showed significant deviation.

Estimation of Biodiesel Properties

Fatty acid methyl ester (FAME) composition was used to estimate biodiesel properties, i.e., density, cetane number, iodine value, and cold filter plugging point (CFPP) of *Azolla* biodiesel. To estimate these properties, numerical models are available in the literature. The density (ρ) was calculated by Eq. (1) by assuming a perfect mixture of FAMEs. The densities of pure FAMEs were estimated using Eq. (2) [16]:

$$\rho_B = \sum X_i \rho_i \tag{1}$$

$$\rho_i = 851.471 + \frac{250.718db + 280.899 - 92.180(m-1)}{1.214 + n} \tag{2}$$

where X_i = relative content of the FAME (%), db = number of double bonds in the FAME, m = number of carbon atoms in the alcohol used for esterification, and n = number of carbon atoms in the original fatty acid. Similarly, the cetane number (CN) is calculated in Eq. (3) by assuming a perfect mixture of FAMEs, and cetane numbers of pure FAMEs are estimated using the formula in Eq. (4) [15]:

$$CN_B = \sum X_i CN_i \tag{3}$$

$$CN_i = -21.157 + \left(7.965 - 1.785db + 0.235db^2\right)n - 0.099n^2 \tag{4}$$

where CN_B = cetane number of the blend and CN_i = cetane number of pure FAME. Iodine value and cold filter plugging point were estimated using formulas derived by Ramos et al. [14]. Equation (5) provides the iodine value as a function of the degree of unsaturation (DU), whereas DU is given by Eq. (6) [14]:

$$Iodine\ value = 87.396 \cdot DU + 1.6691 \tag{5}$$

$$DU = (monosaturated\ Cn:1,\ wt.\%) + 2 \cdot (polysaturated\ Cn{:}{>}1,\ wt.\%) \tag{6}$$

The CFPP was estimated with Eq. (7) using the long chain saturation factor $LCSF(A)$, as defined in Eq. (8) [14]:

$$CFPP = 8.9243 \cdot LCSF(A) - 19.325 \tag{7}$$

$$LCSF(A) = MP_{C18} \cdot C18(wt.\%) + MP_{C20} \cdot C20(wt.\%) + MP_{C22} \cdot C22(wt.\%) + MP_{C24} \cdot C24(wt.\%) \tag{8}$$

where MP_i = melting point of the saturated FAME (in degrees Celsius).

For comparison with other biodiesel feedstocks, cetane number, iodine value, and CFPP of rapeseed, soybean, and palm were obtained from Ramos et al. [14], whereas reported FAME distributions were used to calculate biodiesel density. For the green algae *Scenedesmus incrassatulus*, all biodiesel parameters were calculated using the FAME distribution

reported by Arias-Peñaranda et al. [17]. European standards for biodiesel properties were drawn from EN 14214 [24].

Results and Discussion

Lipid Yield, General Composition, and Effects of Drying Conditions

Cultures of *A. filiculoides* grown at 800 ppm CO_2 had an average productivity of 13.24 g m^{-2} day^{-1} over a period of 40 days. The harvested biomass was either dried for 3 days at 30 °C, dried for 1 day at 65 °C, or freeze-dried, before lipid extraction. Table 1 provides the crude lipid yields from biomass dried at each of these conditions. Small differences were observed between drying conditions, but differences between groups were not found to be significant by ANOVA as shown in Table 2. The average crude lipid yield of all extractions equals 7.92±0.14 % of the dry weight (dw).

Online Resource 1 provides the average compound concentrations of the TLEs and SEs. Based on the SEs, 4.6±1.4 % dw of lipids could be accounted for using quantification on the GC/GC-MS, whereas only 0.84±0.40 % dw of lipids are detected in TLEs. Differences between the TLEs and the SEs are summarized in Fig. 1 for all major (groups of) compounds in *Azolla*. Saponification in methanol converted the ester-bound fatty acids into fatty acid methyl esters (FAMEs). Similarly, phytol was derived from chlorophyll upon saponification. The amount of sterols did not change significantly. Saponification further resulted in the detection of higher amounts of mid-chain (di)hydroxy compounds, due to the hydrolysis of esters of C26-C36 (di)hydroxy fatty acids ((di)OH FAs).

Variations in the concentrations of individual compounds can mainly be explained by the variation in total compound detection, as indicated by the much lower variations in normalized concentrations. The ANOVA test only showed a significant ($p<0.05$) difference between drying conditions for 9,10-dihydroxynonacosane (C29 20,21(ω9,ω10)diol) in TLEs and phytol in SEs, as indicated with an asterisk in Online Resource 1. All results of the ANOVA test on TLEs and SEs are provided in Online Resource 2. For SEs, most phytol was detected in batches dried at 65 °C and least in freeze-dried batches. This may indicate that extraction of chlorophyll and derivatives is less efficient from freeze-dried material. Overall, the effect of drying conditions on the lipid yield and composition appears to be of minor importance within the scope of this study. Therefore, it was decided not to distinguish between drying conditions for the further analysis.

Table 1 Crude lipid yield of *Azolla* batches exposed to different drying conditions and results of analysis of variance

Drying condition	*N*	Crude lipid yield (% dw)
30 °C, 3 days	5	7.75±0.49
65 °C, 1 day	5	7.94±0.22
freeze drying, 3 days	5	8.16±0.30
Average	5	7.92±0.14

N denotes the number of replicates. Crude lipid yield is given as average ± standard deviation

Table 2 Results of analysis of variance (ANOVA) in crude lipid yield

	Sum of squares	Degrees of freedom	Mean square	*F*	*p* value
Between groups	$4.53 \cdot 10^{-5}$	2	$2.27 \cdot 10^{-5}$	1.936	.187
Within groups	$1.40 \cdot 10^{-4}$	12	$1.17 \cdot 10^{-5}$		
Total	$1.86 \cdot 10^{-4}$	14			

F provides the result of the Fisher test. The *p* value gives the likelihood of the found *F* value for the given degrees of freedom

Fatty Acid Distribution

The FAs were estimated to constitute 3.2±1.0 % dw of *Azolla* biomass and 41±13 % of the crude lipid fraction. The average FA profiles of TLEs and SEs of *Azolla* are given in Fig. 2. Palmitic acid (C16:0 FA), linoleic acid (C18:2 FA), and linolenic acid (C18:3 FA) are by far the dominating FAs. Furthermore, the concentration of lignoceric acid (C24:0 FA) in *Azolla* is relatively high compared to that in other plant species, such as soy, palm, and rapeseed [14].

In TLEs, free fatty acids (FFAs) amounted to 4.2±0.38 % of the crude lipid fraction. Many of the FAs identified in SEs could not be detected in TLEs. These include pentadecylic acid (C15:0 FA), palmitoleic acid (C16:1 FA), hexadecatrienoic acid (C16:3), arachidic acid (C20:0 FA), eicosatrienoic acid (C20:3 FA), nervonic acid (C24:1 FA), and cerotic acid (C26:0 FA).

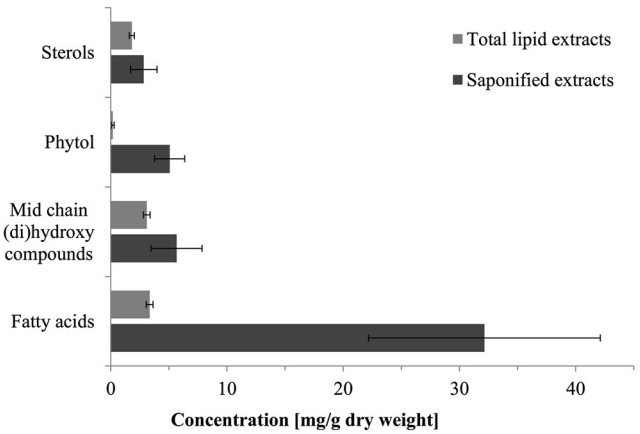

Fig. 1 Concentrations of major (groups of) compounds. *Error bars* indicate standard deviation (*N*=5, *N*=4 for TLEs and SEs, respectively)

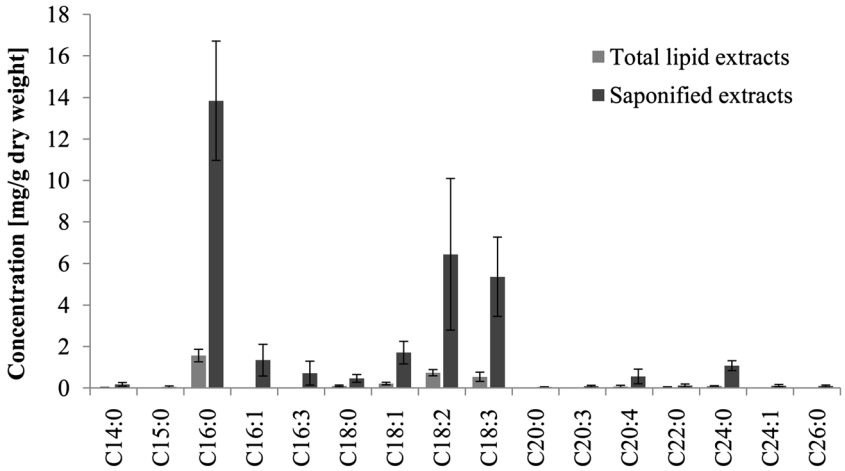

Fig. 2 Fatty acid concentrations in TLEs and SEs. *Error bars* indicate the standard deviation, *N*=5 for TLEs and *N*=4 for SEs

Mid-chain (Di)hydroxy Compounds

The mid-chain (di)hydroxy compounds in *Azolla* are characterized by their long chain, i.e., 26 to 52 carbon atoms, and the presence of at least one mid-chain hydroxy group located at either the ω20 or ω9 position on the carbon chain. This allows the methyl and trimethylsilyl (TMS) derivatives to be identified using typical fragment ions at *m/z* 369 and *m/z* 215, respectively [21]. As illustrated in Fig. 3, they can be further subdivided in (di)OH alkanes and (di)OH alcohols, and (di)OH FAs and (di)OH wax esters. For each of these classes, examples of structural formulas are given in Fig. 3a–h. The (di)OH alkanes consist of alkanols and diols with solely mid-chain hydroxy groups and thus have an alkane backbone. In contrast, the (di)OH alcohols are diols and triols, both have a hydroxy group at the 1 position as mid-chain hydroxy group(s) and are therefore termed (di)OH alcohols. Similarly, the (di)OH fatty acids possess a carboxylic acid group at the 1 position and mid-chain hydroxy group(s). All (di)OH wax esters identified are palmitate esters of (di)OH alcohols. Four of the mid-chain (di)hydroxy compounds identified were not previously reported [21]. In Online Resource 3, the mass spectra of the methyl ester and TMS ethers of these compounds are displayed. Some compounds identified by Speelman et al. [21] were not detected in the TLEs or in SEs, including 7-hexacosanol (C26 7(ω20) alkanol), 9-octacosanol (C28 (ω20) alkanol), and 17-hydroxyhexadotriacontane palmitate ester (C52 (ω20) OH wax ester). Small quantities of odd-numbered OH FAs were detected but provided too little signal to be quantified.

In Fig. 3i, the concentrations are summarized for all types of mid-chain (di)hydroxy compounds. In TLEs, 0.31±0.028 % dw of mid-chain (di)hydroxy compounds were detected, consisting of even amounts of (di)OH alkanes and (di)OH alcohols and a slightly lower amount of (di)OH wax esters. The most abundant compounds in TLEs are the 20,21-dihydroxynonacosane palmitate ester (C45 (ω9,ω10) diOH wax-esters) and the C29 20,21(ω9, ω10) diol.

In SEs, the mid-chain (di)hydroxy compounds amounted to 0.57±0.22 % dw of the biomass and 7.2±2.8 % of the crude lipid fraction, respectively. Upon saponification, the (di)OH wax esters were efficiently hydrolyzed, which is apparent from their absence from SEs and the increase in the concentration of their breakdown products: C16:0 FA and (di)OH alcohols, i.e., C30-C34 1,ω20 diols, C30-C32 1,ω20,ω21 triols, and in particular, 1,20,21-nonacosanetriol (C29 1,20, 21 (1,ω9,ω10) triol). Surprisingly, (di)OH FAs turned out to be the dominant compounds in SEs, whereas in the TLEs, only C29 20,21 (ω9,ω10) diOH FA was faintly detected. The increase in (di)OH FAs after saponification suggests that they are part of lipid esters that are too large to be GC amendable and thereby undetected in TLEs. The estimated mass of all (di)OH FAs was approximately 0.22±0.092 % dw. Overall, the compounds with a hydroxy group at position ω20 occur in more diverse chain lengths compared to the compounds with hydroxy groups at positions ω9 and ω10, whereas both types occur in equal amounts.

Implications for Biodiesel Production

The ester-bound FAs are by far the dominant lipids in *A. filiculoides*, i.e., 3.1±1.0 % dw. Other *Azolla* species might have a higher lipid content, such as *A. caroliniana*, for which a crude lipid fraction of 12.7–16.4 % dw was reported [20]. However, this difference may as well be caused by adjustment of the biomass composition to the growth rate and/or a shift in carbon allocation under high CO_2 concentrations, as observed in higher plants [25]. Due to its continuous productivity, considerable amount of oil can be produced from an *Azolla* production system on an annual basis. In a controlled production system with active CO_2 supply, we achieved an oil production equivalent to 1500 kg ha^{-1} $year^{-1}$. In an outdoor system, Becerra et al. [3] obtained a biomass productivity of 39,

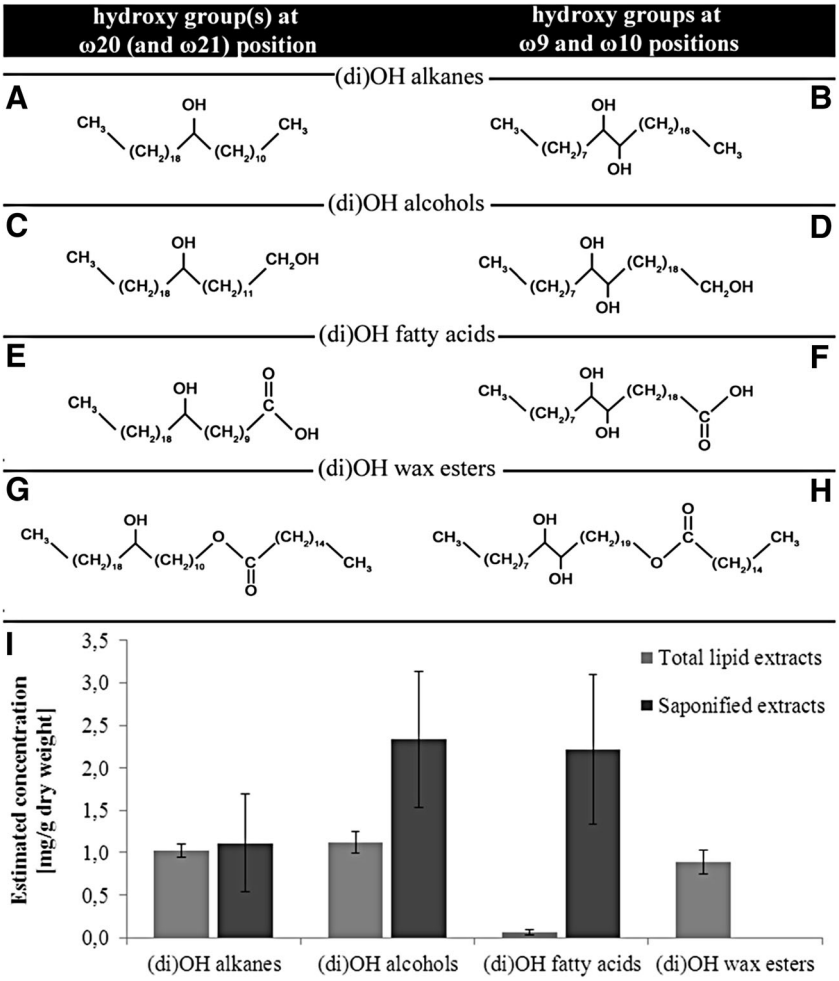

Fig. 3 **a**–**h** Examples of structural formulas of each compound type and class: **a** 12-hentriacontanol (C31 12(ω20) alkanol), **b** 9,10-dihydroxynonacosane (C29 20,21(ω9,ω10) diol), **c** 1,13-dotriacontanediol (C32 1,13 (1,ω20) diol), **d** 1,20,21-nonacosanetriol (C29 1,20,21 (1,ω9,ω10) triol), **e** 11-hydroxytriacontanoic acid (C30 11(ω20) OH FA), **f** 20,21-dihydroxynonacosanoic acid (C29 20,21(ω9,ω10) diOH FA), **g** 11-hydroxytriacontane palmitate ester (C46 (ω20)OH wax ester), **h** 20,21-dihydroxynonacosane palmitate ester (C45 (ω9,ω10) diOH wax ester). **i** Concentrations of major classes of mid-chain (di)hydroxy compounds in TLEs and SEs. *Error bars* indicate the standard deviation, N=5 for TLEs and N=4 for SEs

000 kg ha^{-1} year^{-1}, corresponding to an oil production of approximately 1200 kg ha^{-1} year^{-1}, assuming a similar lipid content. Common biodiesel feedstocks such as soybean, rapeseed, and palm have annual oil productivity of 406, 1307, and 5462 kg ha^{-1} year^{-1}, respectively [26]. As an example of other novel biodiesel sources, the annual oil productivity of the microalgae *S. incrassatulus* can be estimated between 2300 and 3900 kg ha^{-1} year^{-1}, mainly depending on annual productivity [27]. Hence, with oil as the sole product, *Azolla* cultivation is likely outcompeted by palm oil and microalgae. Utilizing whole *Azolla* biomass can yield higher amounts of bioenergy. Muradov et al. [28] obtained 33 % of biooil after pyrolysis of whole *Azolla* biomass that can be directly used as diesel fuel supplement. In their approach, all biomass components, including protein, polyphenols, and carbohydrates, are converted into pyrolysis products. The advantage of using only the lipid extract for biodiesel production is that other biomass components can be extracted separately and commercialized as higher-value products. This holds in particular for the protein fraction which is a major (20–25 %) component of *Azolla* biomass and potentially valuable as feed [8–10]. If we compare *Azolla* to current protein crop soy, the amount of biodiesel that can be produced from the *Azolla* lipid fraction is nearly three times higher.

Production of biodiesel from *Azolla* lipids will require a conversion process that tolerates a high FFA content. As with many other biodiesel feedstocks, the FFA content of *Azolla*, i.e., 4.2±0.38 %, is higher than the limit, i.e., 2.5 %, for base-catalyzed conversion [29]. The FFA content can be reduced by using acid-catalyzed methylation of the FFAs prior to alkaline hydrolysis [30]. Alternatively, high FFA tolerating methylation methods can be employed, such as the use of sulphonated synthetic carbon catalysts [31].

Biodiesel quality indicators—density, cetane number, iodine value, and cold filter plugging point (CFPP)—estimated using FA profile and available numerical models are provided in Fig. 4, along with (estimated) values for a selection of other biodiesel sources and the limits set by the EN 14214 standard. Values were calculated from the average fatty acid profile in SEs (n=4). The biodiesel has an estimated density of 880±2.9 kg m^{-3}, a cetane number of 63±4.0, and an iodine value of 80±15. The values are all well within limits set by the EN 14214 standard. Although no European-wide limits are provided for the CFPP, it can be seen that the estimated CFPP for

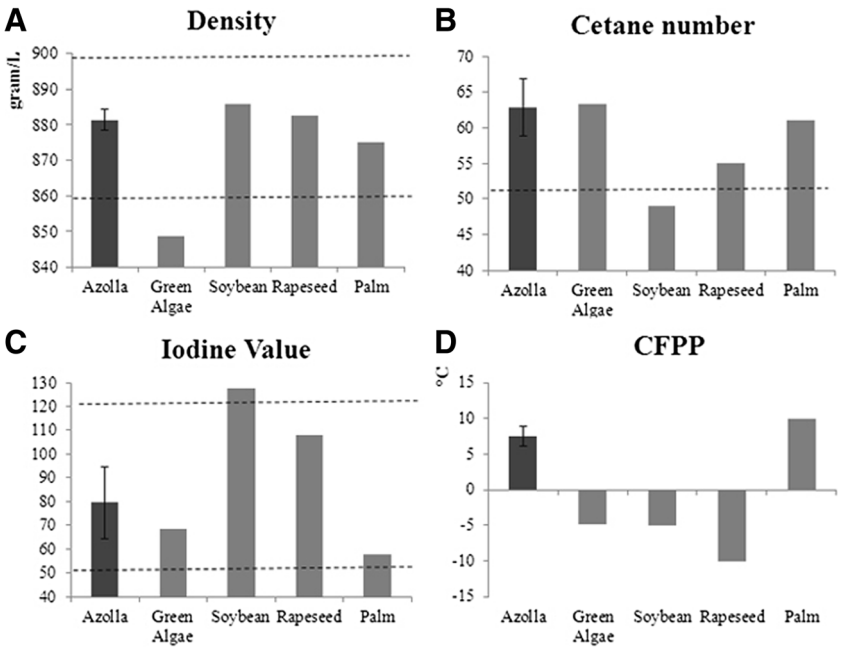

Fig. 4 Estimated density, cetane number, iodine value, and cold filter plugging point (CFPP) for biodiesel derived from *Azolla* compared to other feedstock sources. *Dotted lines* indicate limits set by the EN 14214 European standard. Parameters for rapeseed, soybean, and palm were taken from Ramos et al. [14] and parameters for green algae (*Scenedesmus incrassatulus*) were calculated from the fatty acid distribution obtained from Arias-Peñaranda et al. [17]

Azolla biodiesel, i.e. 7.5±1.4 °C, is too high for general operating conditions. This is mainly the result of the relatively high content of lignoceric (C24:0) in *Azolla* biomass, i.e., 3.4 ±0.32 % of the FAME fraction.

As the numerical models employed are limited to the FA profile, they do not take into account the effects of other lipid constituents. The mid-chain (di)hydroxy compounds amount to 7.2±2.8 % of the crude lipid fraction. Although exact temperature characteristics are unknown, the high chain length, lack of double bonds, and GC behavior indicate that these compounds have high melting points and can therefore be a serious source of filter plugging and wax settling issues. Hence, fractionation into a FAME fraction and a fraction containing the high melting point mid-chain (di)hydroxy compounds will be necessary to ensure biodiesel quality in terms of cold temperature characteristics. To enhance the CFPP, this fractionation step may be extended to remove the C24:0 FA. Removal of C24:0 by crystallization fractionation and solvent fractionation was demonstrated by Pérez et al. [32] in the case of peanut biodiesel. In the case of *Azolla*, full removal up to C24:0 FA would lead to a drastic decrease in CFPP to −12± 0.83 °C, while density, cetane number, and iodine value would change only slightly and remain within EU limits at 880±5.2 kg m^{-3}, 61.0±3.9, and 82±16, respectively. Hence, with added processing, high-quality biodiesel can be produced from the *Azolla* lipid fraction. Although an additional fractionation step infers additional costs, the second fraction rich in mid-chain (di)hydroxy compounds may also provide a second product stream, when the unique long-chain fatty alcohols, i.e., (di)OH alcohols, and (di)OH FAs find applications as biochemicals. Surfactants, often applied as detergents in washing and cleaning formulas, and various specialty chemicals are manufactured on the basis of fatty alcohols [33, 34]. However, the alcohols in *Azolla* have multiple hydroxy groups and a typical chain length of C26–C36, which is much longer than that of the long-chain alcohols produced from FAs or petroleum (C14–C24). In food industry, fatty alcohols with a more similar chain length, such as octacosanol, are currently sold as food additive, due to their cholesterol-lowering effect in animals and humans [35]. If the *Azolla* fatty alcohols exhibit a similar function, such an application could be of commercial interest.

OH FAs are, among others, used as surfactants, lubricants, and synthetic precursors in the polymer industry [36, 37]. They are difficult to synthesize via chemical routes due to inertness of the fatty acid chain [37]. Currently, the main commercially produced OH FA is ricinoleic acid (12-hydroxy-9-octadenenoic acid), which is extracted from castor oil and used for, among others, the synthesis of polyurethanes (PUs) and diacids [36].

Hence, various possible applications of these compounds exist. Whether, in addition to biodiesel production, further separation of the fatty alcohols and (di)OH FAs is worthwhile depends on the value of the biochemical versus its processing costs. The fact that they have longer chain length and more hydroxy groups compared to currently commercialized compounds may result in a high product value but also makes it difficult to assess the commercialization potential of these compounds beforehand and therefore requires further research.

Conclusions

The lipid fraction of *Azolla* was investigated in the context of biodiesel production. Biomass drying conditions do not affect lipid yield or composition, indicating that drying conditions may be freely chosen so that they are energetically optimal. After saponification in methanol 3.2±1.0 % dw of FAMEs are obtained. From the lipid composition, it is predicted that high-quality biodiesel can be produced from the *Azolla* lipid fraction but requires an additional fractionation step to decrease the CFPP. The unique long-chain (di)hydroxy fatty acids and fatty alcohols that are separated in this fractionation step may provide a valuable secondary product stream when purified into biochemicals, with possible applications to chemical industry and nutrition.

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