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FATTY ACID CHARACTERIZATION OF CYANOBACTERIAL STRAINS ISOLATED FROM VELA LAKE AND MONDEGO RIVER RICE FIELDS (CENTRAL-WESTERN, PORTUGAL)

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ABSTRACT

It is know that cyanobacterial taxonomic groups are characterized by particular lipid patterns that can be used as their biological markers. The present study examined the fatty acid composition of nostocacean heterocystous cyanobacterial strains isolated from Central-western Portuguese freshwater shallow water bodies, namely Vela Lake and rice fields from Mondego River Basin. Morphological characterization showed that strains from Vela Lake belonged to *Aphanizomenon gracile* (strains UADFA16 and UADFA18), *Aphanizomenon flos-aquae* (strain UADFA15) and *Anabaena cf. solitaria* (strain UADFA14) species, whereas rice field strains belonged to *Anabaena cylindrica* (strain UTAD_A212) and *Nostoc muscorum* (strain UTAD_N213). Biochemical characterization inferred from lipid analysis showed that predominant fatty acid methyl esters (FAMEs) in the lipids of the strains were palmitic, oleic and α-linolenic, with trace amounts of myristic and C_{20} polyunsaturated FAMEs. To our knowledge, there is almost no information about lipid composition in freshwater cyanobacterial species living in different habitats in Portugal. Therefore, this limnological study is a contribution to our investigation on freshwater diazotrophic cyanobacteria.

INTRODUCTION

Cyanobacteria are impressive ecosystem engineers with an evolutionary history stretching over at least 2.15 billion years. Formerly knew as blue-green algae, these oxygenic photoautotrophic prokaryotes are widely distributed in natural environments and constitute a major component of microbial populations in terrestrial and aquatic habitats worldwide;

Whereas a significantly number of beneficial cyanobacteria fix atmospheric N_2 thus contributing to the fertility of rice field soils, others behave as nuisance microorganisms of aquatic ecosystems due to their involvement in noxious and toxic bloom events;

The information about cyanobacteria characterization present in rice fields and other agro-ecosystems from the Iberian Peninsula is still scarce and differs significantly from that obtained from Asiatic countries, especially with regards to different climatic conditions;

On the other hand, the diversity, ecology, genetic and biochemical heterogeneity among planktonic *Anabaena*-like and *Aphanizomenon*-like morphospecies requires a more deep characterization since there is not a clear phylogenetic nor morphological separation;

The taxonomy of cyanobacteria has been traditionally based on morphological, physiological and ecological characteristics, but recently also on biochemical approaches (Li *et al.*, 2008; Zapomělová *et al.*, 2010);

Moreover, many discrepancies between morphological traditional/classic classification and phylogenetic analysis are still unresolved (Komárek, 2010);

Therefore, chemotaxonomic markers such as lipids and their fatty acids methyl esters (FAMEs) have been looked as complementary tools, since they provide interesting information about the taxonomic position assignment and some correlations with morphological properties of cyanobacteria.

AIM OF THE STUDY

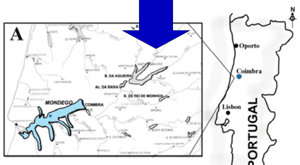
⇨ References about lipid composition of freshwater cyanobacteria inhabiting different natural ecosystems in Portugal are non-existent and polyphasic studies still scarce;

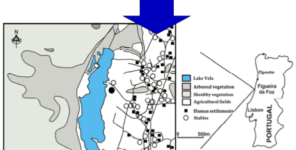
⇨ In the present work, morphological information from filamentous cyanobacterial strains isolated from two Portuguese freshwater ecosystems, namely rice fields and an eutrophic shallow lake, together with biochemical attributes obtained from fatty acid methyl esters (FAMEs) analysis, were used to characterize the strains;

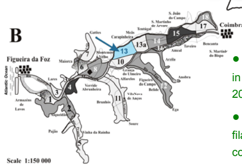
⇨ This work is a contribution for the investigation on diazotrophic cyanobacterial species in the framework of a comprehensive collaboration program concerning the taxonomy and phylogenetic relationships (e.g., de Figueiredo *et al.*, 2010), as well as the toxicological effects of agrochemicals and other xenobiotics (*e.g.*, Galhano *et al.*, 2010) on Portuguese freshwater cyanobacteria.

MATERIALS AND METHODS (Sampling Sites)

- The strains *Anabaena cylindrica* UTAD_A212 and *Nostoc muscorum* UTAD_N213 were collected from rice field soils, located near Montemor-o-Velho village at the Mondego's river basin (40º 10' N, 8º 41' W, Central-western POR), whereas *Aphanizomenon flos-aquae* UADFA15, *Anabaena* cf. *solitaria* UADFA14, *Aphanizomenon gracile* UADFA16 and *Aphanizomenon gracile* UADFA18 were isolated from the eutrophic shallow Vela Lake near Quiaios village, Figueira-da-Foz (44º 58' N, 5º 18' W, Central-western POR):







- All collected strains were cultivated in liquid N-free BG11 medium and kept in exponential growth conditions as previously described (Galhano *et al.*, 2009);

- The morphologic observations were made with fresh cyanobacteria filaments using a photomicroscope (Olympus mod. IX71), identified and compared based on standard/classical literature.

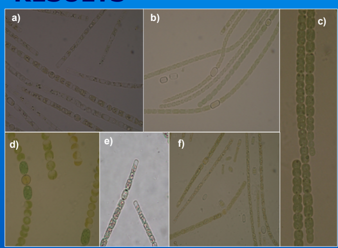
MATERIALS AND METHODS (Total FAME Analysis)

- Extraction of total lipids was according to Sato & Murata (1988), with some modifications. The $CHCl_3$ phase was vortex-mixed with a double volume of KCl solution (0.1 M). After centrifuging for 5 min at 3000 rpm, the lower phase was collected and dried under a stream of N_2. The preparation of FAMEs was achieved using 5% H_2SO_4 in CH_3OH as the derivatising reagent, at 70 ºC for 2 h. The different lipid classes were transmethylated after addition of heptadecanoic acid (C:17) as internal standard. The *n*-hexane layer was dried over anhydrous Na_2SO_4 and evaporated to dryness;

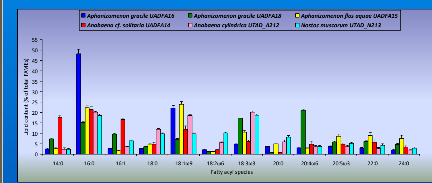
- FAMEs were analysed by gas chromatography (Trace GC 2000, Thermo-Finnigan; Thermo Electron Corporation, Austin TX, USA) with *He* as the carrier gas (flow rate of 40 cm/s at 140 ºC). A 4 µL sample was injected in split injection mode. We used an Agilent J&W Scientific (Agilent Technologies, Inc., USA) capillary column (DB-225MS; 30 m × 0.25 mm ID × 0.25 µm) and the temperature program of the GC oven was 2 min at 140 ºC, with a casting ramp of 4 ºC/min until 220 ºC, being this temperature maintained for 5 min. The temperature of the injector and FID were kept at 250 ºC and 270 ºC, respectively;

- Total FAMEs were identified by co-chromatography of known authentic standards. The relative concentrations of different fatty acids were calculated by the percentage area method. Peak area integration was done by using an appropriate data processor software. Results were expressed as average of at least three independent experiments ± standard error. Hierarchical cluster analysis of the fatty acids data set were numerically analyzed using the version 7.0 of Statistica (Statsoft Inc., Tulsa OK, USA).

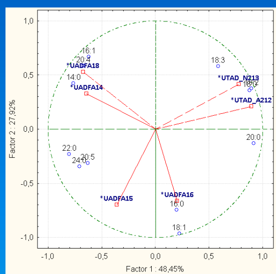
RESULTS



a) *Aphanizomenon flos-aquae* UADFA15; **b)** *Anabaena cylindrica* UTAD_A212; **c)** *Nostoc* sp. UTAD_N213; **d)** *Anabaena* cf. *solitaria* UADFA14; **e)** *Aphanizomenon gracile* UADFA16; **f)** *Aphanizomenon gracile* UADFA18.



Fatty acid composition of the *Nostocaceae* freshwater cyanobacterial strains. The values were expressed in % of total fatty acids and are means ± SE of at least three independent experiments.



FAME properties	Cyanobacterial strains					
	Aph. gracile A16	*Aph. gracile* A18	*Aph. flos.aquae* A15	*An. cf. solitaria* A14	*An. cylindrica* A212	*N. muscorum* N213
Saturated	61.74 ± 1.51	37.74 ± 0.35	51.29 ± 1.35	53.55 ± 0.84	44.95 ± 0.60	46.04 ± 0.41
Monounsaturated	24.78 ± 1.11	16.79 ± 0.13	25.58 ± 1.58	28.65 ± 1.25	22.08 ± 0.37	16.18 ± 0.43
Polyunsaturated	13.48 ± 0.58	45.47 ± 0.48	23.13 ± 0.43	17.79 ± 1.23	32.97 ± 0.57	37.79 ± 0.50
Sat./Unsat.	1.62 ± 0.10	0.61 ± 0.01	1.06 ± 0.06	1.15 ± 0.04	0.82 ± 0.02	0.85 ± 0.01
MUFA/PUFA	1.84 ± 0.07	0.37 ± 0.01	1.11 ± 0.08	1.63 ± 0.16	0.67 ± 0.02	0.43 ± 0.02
Unsat. Index	73.21 ± 3.39	184.80 ± 1.13	113.50 ± 1.45	94.23 ± 3.25	126.21 ± 2.03	133.41 ± 1.96

Analysis of fatty acid properties and important ratios of the cyanobacterial strains (MUFA/PUFA, ratio of monounsaturated/polyunsaturated fatty acids; Unsat. Index, Unsaturation Index, see Rosa & Catalá, 1998 for UI determination).

⬅ PCA diagram based on the fatty acyl species of the six studied strains under laboratory controled conditions. The first and the second canonical axes explain 27.92% and 48.45% of the total variance, respectively.

DISCUSSION AND CONCLUSIONS

- Strains were identified as *Aphanizomenon gracile*, *Aph. flos-aquae*, *Anabaena* cf. *solitaria*, *Anab. cylindrica* and *Nostoc muscorum*;
- Predominant cellular FAMEs in lipids of the six strains were palmitic (16:0), oleic (18:1ω9) and α-linolenic (18:3ω3). Additionally, myristic (14:0), palmitoleic (16:1), stearic (18:0) and linoleic (18:2ω6) acids were present. With few exceptions, the strains presented trace amounts of C_{20} PUFAs. Therefore, according to the Kenyon-Murata classification system, FAME composition of the strains matched to Group II;
- Surprisingly, the two *Aph. gracile strains* (UADFA16, UADFA18) had different FAME composition as further confirmed by PCA. Probably, the UADFA18 population could be intermixed with unicellular cyanobacteria and this could explain the relevant high content (21.1%) of arachidonic acid (20:4ω6). The significantly high 16:0 content (48.2% of total FAMEs) of UADFA16 needs further biochemical and molecular analysis. This strain could be a potential source of commercially interesting fatty acids;
- The high concentration (17.7 %) of 14:0 detected in *An.* cf. *solitaria* UADFA14 could be indicative of hepatoxicity (Gugger *et al.*, 2002);
- Comparison of quantitative and qualitative FAME composition of the strains isolated from different habitats with different local environmental conditions could explain the close relationship between e.g. *An. cylindrica* and *N. muscorum*, both collected from rice fields, as confirmed by PCA analysis. However, the same approach could not be applied to *Aph. gracile* strains isolated from Vela Lake;
- The geographical origin of *Aph. flos-aquae* UADFA15 must be re-checked again since it appeared isolated in the PCA analysis. Other biochemical indicators are currently under investigation with this strain (Hugo Santos *et al.*, submitted for publication);
- In conclusion, fatty acid analysis is a valuable tool in polyphsic research studies, as shown by the differentiation of the genera *Anabaena*, *Nostoc* and *Aphanizomenon*, which agree well with morphological analysis;
- In order to identify and authenticate different freshwater cyanobacterial strains from diverse Iberian Peninsula habitats by using chemical profiling *e.g.* FAME analysis, it is necessary to obtain many strains as possible representative of each genus.

REFERENCES

- de Figueiredo, D.R., Alves, A., Pereira, M.J. & Correia, A., 2010. Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). *J. Plankton Res.*, **32:** 239-252.
- Galhano, V., Peixoto, F. & Gomes-Laranjo, J., 2010. Bentazon triggers the promotion of oxidative damage in the Portuguese ricefield cyanobacterium *Anabaena cylindrica:* response of the antioxidant system. *Environ. Toxicol.*, online first, DOI: 10.1002/tox.20597.
- Gugger, M., Lyra, C., Suominen, I., Tsitko, I., Humbert, J.-F., Salkinoja-Salonen, M.S., Sivonen, K., 2002. Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena*, *Aphanizomenon*, *Microcystis*, *Nostoc* and *Planktothrix* (cyanobacteria). *Int. J. Syst. Evol. Microbiol.*, **52:** 1007-1015.
- Komárek, J., 2010. Modern taxonomic revision of planktic nostocacean cyanobacteria: a short review of genera. *Hydrobiologia*, **639:** 231-243.
- Li, R., Wilhelm, S.W., Carmichael, W.W. & Watanabe, M.M., 2008. Polyphasic characterization of water bloom forming *Raphidiopsis* species (cyanobacteria) from central China. *Harmful Algae*, **7:** 146-153.
- Rosa, D. & A. Catalá, 1998. Fatty acid profiles and non enzymatic lipid peroxidation of microsomes and mitochondria from bovine liver, kidney, lung and heart. *Arch. Physiol. Biochem.*, **106:** 33-37.
- Sato, N. & Murata, N., 1988. Membrane lipids. *Methods Enzymol.*, **167:** 251-259.
- Zapomělová, E., Řehaková, K., Jezberová, J. & Komárková, J., 2010. Polyphasic characterization of eight planktonic *Anabaena* strains (Cyanobacteria) with reference to the variability of 61 *Anabaena* populations observed in the field. *Hydrobiologia*, **639:** 99-113.

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