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Isolation and characterization of cyanobacteria strains based on the compositional approach of fatty acids: case of drinking water reservoirs in the region of Tetouan (Northern Morocco)

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Abstract

The aim of this study is isolation and characterization of cyanobacteria strains from drinking water reservoirs in the region of Tetouan. The isolation was carried out using two synthetic culture media BG13 and BG130 and the strains of cyanobacteria were harvested during the stationary phase. The characterization isolated species was carried out based on fatty acids contents using GC-MS analysis. We have isolated eleven strains of cyanobacteria from three main aquatic reservoirs in the region of Tetouan (Nakhla, M.H.Belmehdi and SMIR dams). The morphological aspect, the specific composition in fatty acids and the total lipids contents allowed the identification of five species of cyanobacteria (*Microsystis aeruginosa, Pseudanabeana galeata, Oscillatoria tenuis, Anabeana sp,* and *Nostoc sp.*). GC-MS analysis showed variation in n-saturated, unsaturated and long chain branched fatty acids. More than 60% of detected fatty acids belong to polyunsaturated fatty acids. The study also found that palmitic acid (C16: 0) was found in all strains, followed by stearic acid (C18: 0), and linoleic acid (C18: 2). In some other strains, the long chain fatty acids (C20: 1 and C24: 0) were found with lower concentrations.

Key words: Cyanobacteria; fatty acids; GC-MS; dams.

1. Introduction

In Morocco, several water reservoirs are experiencing degradation of their water quality due to various sources of pollution including the over enrichment with nutrients such as N and P which indicate an advanced stage of eutrophication. This phenomenon breaks the balance of an aquatic ecosystem with an unwanted development of algae and an intense consumption of oxygen in the low layer of the reservoirs (El Ghachtoul, 2005). Another consequence is the proliferation of harmful cyanobacteria blooms which are a diverse group of photosynthetic organisms found in different aquatic habitats. Cyanobacteria thrive in eutrophic water and pose a problem for water treatment through dense cell accumulation ; some species pose a health risk as a result of the production of intracellular toxins that are fatal when ingested in large quantities by humans or other animals (Matthews, 2015). In freshwater, they form an important component of primary producers in the food chain, and thus used in aquaculture and mainly in mariculture practices. Their diversity and potential applications were developed by Tajuddin and Subramanian. (2005). They store reserve foodstuffs which are the source of pigments, lipids, vitamins and proteins (Rastogi and Sinha, 2009). In eutrophic conditions, they produce secondary metabolites which usually are toxic to aquatic fauna (Bendarz and al., 2002; Carmichael and al., 2001).

Cyanobacteria contain significant quantities of lipids and some of them are also rich in essential fatty acids such as linoleic and gamma linolenic acids (Sharathchandra and al, 2011). Environmental growth conditions determine the fatty acid profile of lipid in microalgae and cyanobacteria (Cuellar-Bermudez, 2016). In addition to their nutritional value, cyanobacteria fatty acids are generally used to clarify taxonomic problems (Li et al., 2001). According to Kenyon et al. (1972), four types of fatty acids exist in cyanobacteria and are linked to morphological characteristics. The composition of fatty acids in marine microalgae was studied more widely (Volkman et al. 1989; Renaud et al., 1999; Caudales et al., 2000; Tran et al., 2009) then the fresh water ones (Rezanka et al., 1982; Caudales and Wells, 1992; Kruger et al., 1995). This composition is a useful analytical tool for bacterial taxonomy determination (Rezanka et al., 1982; Welch, 1991). Some studies have proposed that four types of fatty acids in cyanobacteria demonstrated high correlation with their morphological properties (Kenyon, 1972; Kenyon et al., 1972; Murata et al., 1992). Caudales and Wells, (1992) studied the cellular fatty acids of benthic cyanobacteria belonging to *Nostoc* and *Anabeana* and found significant differences between these two genera.

Krüger et al. (1995) assessed the taxonomic importance of fatty acid composition at the genus and subgenus levels by analyzing the fatty acid composition of different isolates of *Microcystis* and other members of the *Chroococcales* order. However, there is little information on fatty acid composition and chemotaxonomy of freshwater cyanobacteria species. Fatty acid profile is used as an effective taxonomic tool for many species (Kruger et al., 1995; Li and Watanabe, 2004).

The aim of this study is to isolate and identify freshwater cyanobacterial species from three mains reservoirs in the region of Tetouan - northern Morocco namely Nakhla, M.H. Belmehdi, and SMIR, based on the morphological aspect and the specific composition of cyanobacteria cell membranes fatty acids, in order to identify their taxonomy and describe their degree of toxicity.

2. Materials and methods

2.1. Study Site

- Nakhla reservoir is located 35km south of Tetouan with a capacity of 4.32 M m^3, surface of 110 km^2, and 30m depth. It was entered into operation in 1959. It also supports the supply of drinking water to the city of Tetouan.
- Smir reservoir is located about 30 km north of Tetouan, with a capacity of 40.7 M m^3, surface of 4.7 km^2, and about 30 m of depth. It was inaugurated in 1991 intending to supply drinking water to the city of Tetouan and its coastal touristic zone.
- M.H Belmehdi reservoir is located about 25 km from Tetouan, with a capacity of 28.8 M m^3, surface of 4.8 km^2, and about 50 m deep. It was inaugurated in 2005 to support the supply of drinking water to the city of Tetouan.

2.2. Conditions of cultivation

Samples of cyanobacteria collected from the aforementioned three water reservoirs of Tetouan were subjected to consecutive laboratory culture in an aseptic and photo-autotrophic manner on BG13 (Ferris and Hirsch, 1991) and BG130 (BG13 without NaNO3) (Figure 1). These media are exclusively used for heterocysts cyanobacteria isolation which thanks to their ability to fix atmospheric nitrogen can more easily dominate other algae. However, unlike the BG13 medium, the BG130 medium is deficient of nitrogenous nutrients (NaNO3). Liquid cultures are bubbled with sterile air and maintained at room temperature (22 - 27°C) and under constant illumination provided by cold white fluorescent tubes illuminating radiations of about 20 µmol m^{-2} s^{-1} on the surface of the growth glass container. The biomass was harvested during the stationary phase by centrifugation (4000 g, 20 min, then lyophilized and stored at -20°C until further use (Řezanka et al., 2003).

Figure 1: Culture and Extraction of fatty acids from cyanobacteria samples.

2.3. Extraction of fatty acids

The extraction of fatty acids was carried out according to the protocol described by Zhu et al. (2002). It is a modification of the wet extraction method of Bligh and Dyer (1959). The cells are recovered after centrifugation at 8500 g for 5 min and washing with distilled water (Figure 1). After drying, the samples are crushed in a mortar

and extracted with a mixture of chloroform- methanol (v / v), Chloroform–methanol 1:1 was shown to be the best solvent mixture for extraction of total lipids from microalgae (Ryckebosch, 2012) . After that, samples underwent a mechanical soft agitation for 5h and then sonification for 30min to disrupt cellular membranes. Finally, the samples were centrifuged at 3000g for 10min. The solid phase is separated carefully using a filter paper. The filtrate is evaporated in a rotary evaporator under vacuum at 60°C. The extraction of residues is repeated three times until all the lipids are extracted (Widjaja, 2009). The fatty acids are released after reaction with 2N KOH (saponification). Then, the free fatty acids are converted to methyl esters. The methyl ester derivatives are in the form of complex mixtures which are volatile and can be injected in GC-MS. The various individual mass spectra obtained are used to determine the structures of the main saturated and unsaturated fatty acids. The positions of the unsaturations are identifiable in GC-MS thanks to the N-acylpyrrolidide derivatives directly obtained from the methyl esters.

2.4. Gas chromatography coupled with mass spectrophotometry

In gas chromatography coupled with mass spectrophotometer, the methyl esterified samples (40 µL + FAME sample 960 µl HPLC grade n-hexane) were diluted in vials with micropipettes. The sample vials then were placed in autoclave vial tray. One µL was injected into the gas chromatograph (GC-MS Hewlett Packard (Palo Alto, CA, USA) HP-1100 LC / MSD series system) equipped with solvent binary pump, auto-sampler, and MSD Coupled to an analytical workstation. The MSD consists of a standard API source configured as ES and a single quadrupole Eluents were detected on the flame ionization detector (FID). The temperature of the GC column was set to 1000°C for 3min and then increased to 2800°C. The temperature injector was maintained at 22.50°C. The flow rate of carrier gas (nitrogen) was 1.29 mL/min. The amplified signals were transferred and saved in a computer with GC-solutions software.

3. Results and discussion

3.1. Morphological study

The cultivation of the cyanobacterial samples on the solid medium BG13 and BG130 and the successive cultures made it possible to purify genus of cyanobacteria representing the same characteristics over the studied reservoirs:

1. *Microcystis*: unicellular cyanobacteria whose colonies are spherical, grouped in an sheath and which are floating with the aid of gaseous vacuoles,
2. *Pseudanabaena*: filamentous cyanobacterium whose trichomes are solitary, mobile and without sheath. The cells are distant from one another and joined by a gelatinous bridge. There are no akintes or heterocysts,
3. *Oscillatoria* is a filamentous cyanobacterium whose trichome is free, solitary and devoid of sheath. The movement and the helical displacement of the apex are characteristic of this kind,
4. *Anabaena*: a cyanobacterium whose trichomes are simple, regular and of equal thickness. It has intermediate heterocysts and the absence of sheath is noted,
5. *Nostoc*: a cyanobacterium whose thallis is gelatinous and contains, in a common jelly, dishevelled trichomes with intercalary heterocysts.

3.2. Total lipid content

The total lipid content in the eleven species of cyanobacteria is presented in figure 2, table 1 and 2. The total lipid content is considerably high for the majority of the cyanbacterial species. This is due to maintain favorable growing conditions to ensure rapid growth of cyanobacteria. The specific fatty acid composition of all species is presented in table 2.

Table 1: Total lipid content in cyanobacteria strains in mg/mg of dry biomass.

Site	Cyanobacteria Strains	Total lipid content mg/l of dry weight
Smir	S1: *Microsystis aruginosa*	39.22
	S2: *Pseudanabeana galeata*	44.87
	S3: *Oscillatoria tenuis*	51.54
M.H Belmehdi	S1: *Microsystis aruginosa*	34.89
	S2: *Pseudanabeana galeata*	35.55
	S3: *Oscillatoria tenuis*	13.90
Nakhla	S1: *Microsystis aruginosa*	45.39
	S2: *Pseudanabeana galeata*	69.79
	S3: *Oscillatoria tenuis*	13.90
	S10: *Anabeana sp.*	37.89
	S11: *Nostoc sp.*	33.47

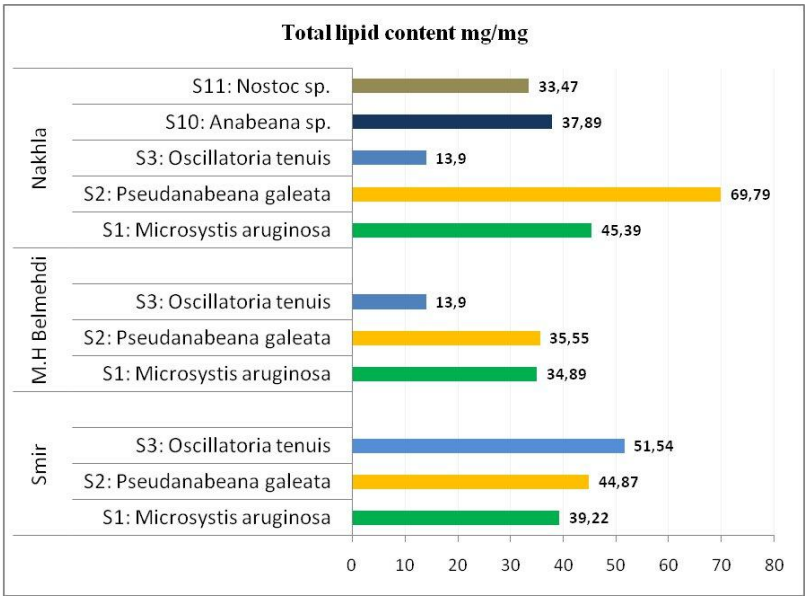

Figure 2: Total lipid content in cyanobacteria strains in mg/mg of dry biomass

Table 2: Profiles of fatty acids contents in different species of cyanobacteria isolated from the three water reservoirs

	Barrage Smir			Barrage M.H.Belmehdi			Barrage Nakhla				
Strains	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Saturated longue chain Fatty Acid											
Lauric acid (C: 12)	-	-	-	-	-	-	-	-	-	-	-
Tridecanoic acid (C13:0)	+	-	-	+	-	-	+	-	-	-	-
Myristic acid (C14:0)	-	-	-	-	-	-	-	-	-	+	-
Palmitic acid (C16:0)	+	+	+	+	+	+	+	+	+	+	+
Palmitoleic acid (C16 :1)	-	-	-	-	-	-	-	-	-	+	+
Heptadecanoic acid (C17:0)	-	-	-	-	-	-	-	-	-	-	-
Stearic acid (C18:0)	+	+	+	+	+	+	+	+	+	+	-
Oleic acid (C18 :1)	-	-	-	-	-	-	-	-	-	+	+
Linoleic acid (C18:2)	-	+	-	-	+	-	-	-	-	-	+
α linoleic acid (C18:3)	+	+	-	+	+	-	+	+	-	-	+
γ-linolenicacid (C18:3)	+	-	-	+	-	-	+	-	-	-	+
Cis-11- Eicosenoic acid (C20:1)	+	-	-	+	-	-	+	-	-	-	-
Cis- 13-Eicosenoic acid (C20 :1)	-	-	-	-	-	-	-	-	-	-	-
Lignoceric acid (C24:0)	-	-	-	-	-	-	-	-	-	-	-
insaturated longue chain Fatty Acid											
Tetracosanoic acid (C24:0)	-	-	-	-	-	-	-	-	-	-	-

Saturated Fatty Acid											
9-hexadecenoic (9-16:1)	-	-	-	-	-	-	-	-	-	-	-
9-octadecenoic (9-18:1)	-	-	-	-	-	-	-	-	-	-	+
9, 12-octadecadienoic (9,12-18:2)	-	-	-	-	-	-	-	-	-	-	+
all-*cis*-9 12 15-octadecatrienoic	-	-	-	-	-	-	-	-	-	-	-
(+) Present; (-) Absent *Ma : Microsystis aeruginosa (S1,S4,S7); Ot : Oscillatoria tenuis (S3,S6,S9); Nsp : nostoc sp (S11)* *Pg : Pseudanabeana galeata (S2,S5,S8); Asp :Anabeana sp.(S10)*											

Fatty Acids are easy to use in GC-MS since they are very polar and very volatile. In addition, they meet a number of GC-MS exploitable criteria such as; the conversion reaction must be complete and selective; it must be able to be done on a micro-preparative scale; the derivatives must be separable in GPC with excellent resolution; each chromatographic peak can lead to specific SM fragmentations; and the quantitative analysis of each of the peaks can be made. Some (saturated) fatty acids are fully identified by the GC-MS analysis of the corresponding EMAGs alone, whereas the unsaturated ones are totally identifiable only from the mass spectra of the pyrrolidide derivatives (NAPs). These acid derivatives possess more structural affinities with the column. This type of derivatives chosen to supplement fatty acid identifications is widely used in the laboratory (Barnathan, 1993), but other authors such as Yu et al. (1988) developed 4,4-dimethyloxazoline derivatives (DMOX) instead to localize the branches of fatty acids. Thus, they have the advantage of being stable under electron impact and of producing a series of homologous fragments from the functional start of the chain up to the molecular ion.

In order to identify certain fatty acids or to ascertain their structure, we used the calculation of different LCEs. The equivalent chain length is described as early as 1956, James and Martin have shown a relationship between the log retention time (Tr) and the n carbon number of normal chain saturated fatty acids, log Tr = a*n + b, equivalent chain length is, by definition, "the number of carbon atoms in a saturated normal fictive fatty acid that would have the same retention time (Tr) as the fatty acid under consideration" (Barnathan, 1993).

From a GC-MS analysis of a mixture of EMAG, we obtain a result in a chromatogram (total ion current) on which different peaks stand out, each corresponding to a detected molecule. The chromatogram of the GC-MS analysis carried out on the cyanobacteria isolated species are presented in figure 3 (*M. aeruginosa*), figure 4 (*Pseudanabeana galeata*), figure 5 (*Oscillatoria tenuis*), figure 6 (*Anabeana sp*) and figure 7 (*Nostoc sp*). Each peak corresponds to a mass spectrum and from these spectra the corresponding GAs can be determined. The desired peaks are the peak of Mac Lafferty m/z74 (base peak) and the peak m/z87 which are characteristic of the methyl esters. The carbon chain length is deduced by the molecular ion M+ (possesses the highest m/z ratio) and by the interpretation keys of the mass spectra of the EMAGs. Thus, the results of fatty acid composition identified by GC-MS are presented in Table 2. The strains of cyanobacteria isolated in this study were identified on the basis of their morphological aspect and then on the basis of their composition in fatty acids.

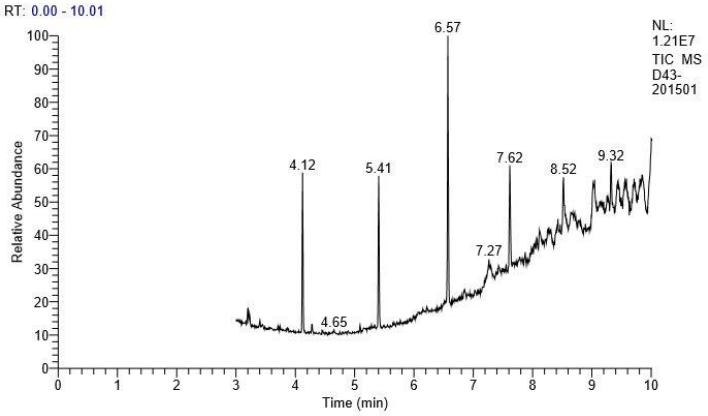

Figure 3: Chromatogram of the GC-MS analysis carried out on the *M. aeruginosa* culture extract. The strain was isolated from the DAMS (Nakhla, Smir and Moulay el Hassan Belmehdi) and cultivated at the laboratory.

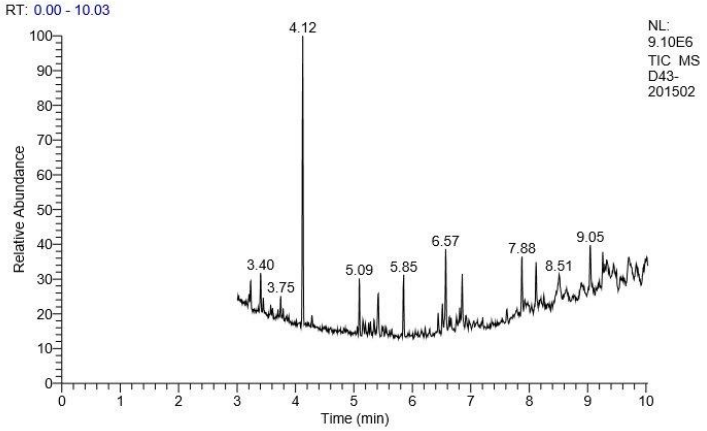

Figure 4: Chromatogram of the GC-MS analysis carried out on the *Pseudanabeana galeata* culture extract. The strain was isolated from the DAMS (Nakhla, Smir and Moulay el Hassan Belmehdi) and cultivated at the laboratory.

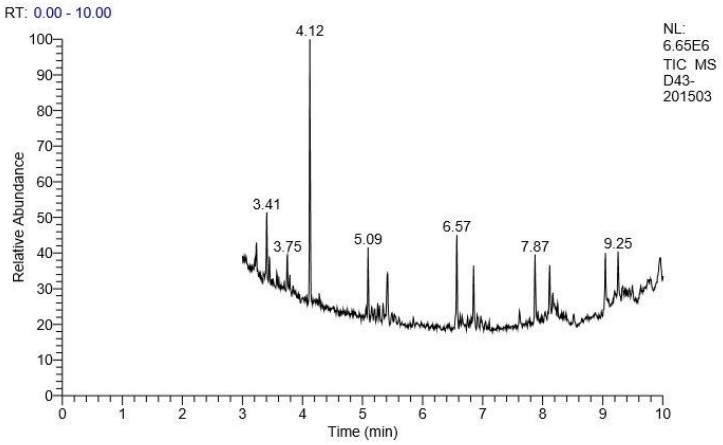

Figure 5: Chromatogram of the GC-MS analysis carried out on the *Oscillatoria tenuis* culture extract. The strain was isolated from the DAMS (Nakhla, Smir and Moulay el Hassan Belmehdi) and cultivated at the laboratory.

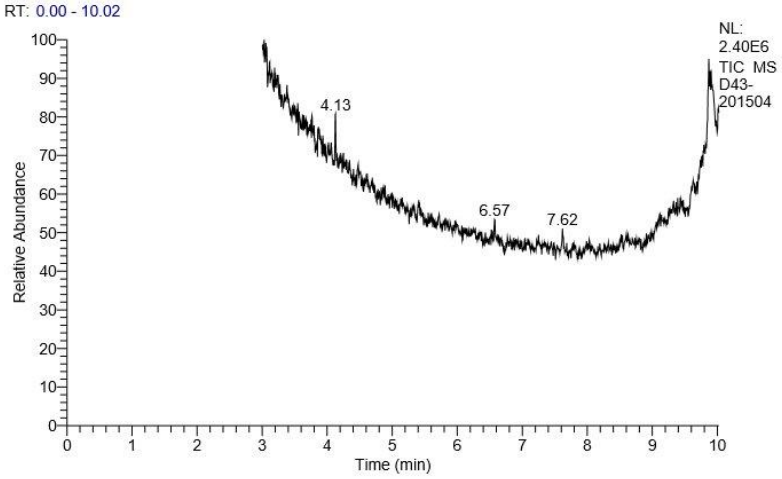

Figure 6: Chromatogram of the GC-MS analysis carried out on the *Anabeana sp* culture extract. The strain was isolated from the DAMS (Nakhla, Smir and Moulay el Hassan Belmehdi) and cultivated at the laboratory.

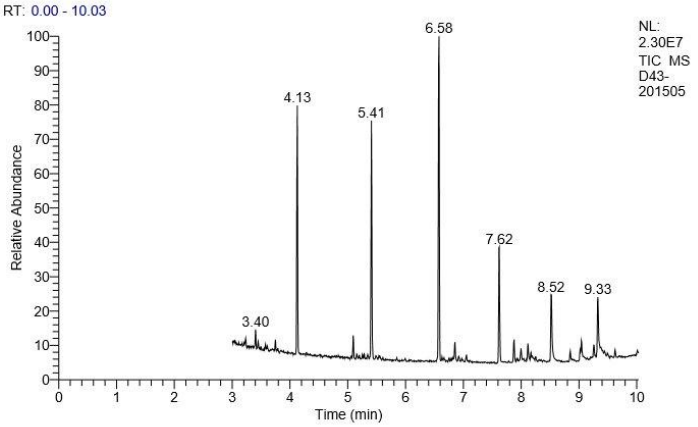

Figure 7: Chromatogram of the GC-MS analysis carried out on the Nostoc sp culture extract. The strain was isolated from the DAMS (Nakhla, Smir and Moulay el Hassan Belmehdi) and cultivated at the laboratory.

The strain S1, isolated from Smir reservoir, belonging to *Chroococcaceae* family and the genus *Microsystis;* it contains the following fatty acids: tridecanouic (C13: 0), palmitic acid (C16: 0), stearic acid (C18: 0) A-linoleic acid (C18: 3), γ-linoleic acid (C18: 3) and acid cis11-ecosenoic acid (C20: 1), this composition refers to *Microsystis aeruginosa*. The same composition was seen for the strains S4 and S7 isolated respectively from Nakhla and M.H Belmehdi reservoirs.

The strain S2, isolated from Smir dam, belongs to the family of *Oscillatoriaceae* and genus *Pseudanabaena;* it contains the following fatty acids: palmitic acid (C16: 0), stearic acid (C18: 0), α linoleic acid (C18: 3), cis11-ecosenoic acid (C20: 1) and cis-13-eicosenoic acid (C20: 1), this composition refers to the species *Pseudanabeana galeata*. The same observation was made for the composition of strains S5 and S8 isolated respectively from the Nakhla and M.H Belmehdi dams.

The strain S3, isolated from Smir dam, belongs to the family of *Oscillatoriacea* and genus *Oscillatoria* and it contains the following fatty acids: (C13: 0), (C16: 0), stearic acid (C18: 0), this composition refers to the species *Oscillatoria tenuis*. The same observation was made for the composition of strains S6 and S9 isolated respectively from Nakhla and M.H Belmehdi dams.

Strains S10 and S11 are cyanobacteria collected exclusively from the Nakhla dam. Two genus were isolated; *Anabeana* and *Nostoc* belonging to the family *Nostocaceae*. *Anabeana* fatty acid composition includes lauric acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid Linoleic (C18: 2) and linoleic α (C18: 2). While, *Nostoc* genus fatty acids includes only palmitic acid (C16: 0), palmitoleic acid (C16: 1) and oleic acid (C18: 1), α-linoleic acid C18: 3).

The palmitic acid (C16: 0) was found in all isolate, polyunsaturated compounds such as α-linolenic acid (C18: 3) (ALA) and γ-linolenic acid (C18: 3) were found only in *Microcystis aeruginosa* (up to 5-14%), *Pseudanabeana,* and *Nostoc*. Similarly, cis-11-eicosenoic acid (C20: 1) was found only in *Microcystis aeruginosa*, while, 9-Octadecanoic acid (9-18: 1) and 9,12-Octadecadienoic acid (9,12-18: 2) were found only in the *Nostoc* species.

In this study, it was also observed that the long chain saturated fatty acid, for instance, lignoceric acid (C24: 0) was not found in any isolate with low concentration. Total lipids have been recognized as essential elements in food and feed and are used as food additives in aquaculture. Microalgae are traditionally used for bivalves in mollusc hatcheries. They are the primary producers in mariculture, to feed consumers such as rotifers, copepods, daphnia, artemia, which are fed to fine crustacean larvae and juvenile fish (Fraser et al., 1989; Langdon et al., 1981; Pernet et al., 2003; Viso et al., 1993).

The fatty acids of cyanobacteria are either saturated or unsaturated. They can also tolerate environmental stresses such as heat, cold, desiccation, and salinity (Agarwal et al., 2000; 2002; Benjamin et al., 2008; Sinha et al., 1996; Tandeau et al., 1993).

Conclusion

This study shows the lipid and fatty acid profile of 11 freshwater cyanobacterial isolates from the 03 dams in the town of Tetouan and indicates that the total lipid content and composition of fatty acids differed in quality and quantity. Some of these freshwater cyanobacteria are a source of essential fatty acids in the food industry, of medical and commercial interest; they are oleic, cis-linoleic, linolenic, γ-linolenic and arachidonic acids among others. In addition, fatty acids are an important food for aquatic animals; their survival and growth rates are related to the availability of fatty acid content.

The analysis of fatty acids is a valuable tool in research studies to characterize cyanobacterial species, this biochemical approach allowed to identify 11 isolates within the 03 dams studied and is well suited to the morphological, physiological and Ecological problems.

The difficulty in the process of the current taxonomic system based on morphology usually results from several reasons, including the various morphological criteria among the various researchers, the occasional lack of certain diacritical characters (akintes), and the change in characteristics Morphological conditions by culture conditions. Investigating the lipids in 11 isolates of cyanobacteria found that the unsaturated fatty acid content correlates well with the morphology and classification based on the physiology of unicellular and filamentous Cyanobacteria.

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