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ORIGINAL PAPER

Anoxygenic phototrophic bacterial diversity within wastewater stabilization plant during 'red water' phenomenon

A. Belila · I. Fazaa · A. Hassen · A. Ghrabi

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Abstract The molecular diversity of the purple photosynthetic bacteria was assessed during temporal pigmentation changes in four interconnected wastewater stabilization ponds treating domestic wastewater by denaturant gel gradient electrophoresis method applying *puf*M gene. Results revealed high phylogenetic diversity of the purple phototrophic anoxygenic bacteria community characterized by the presence of the purple non-sulfur, purple sulfur, and purple aerobic photosynthetic anoxygenic bacteria. This phototrophic bacterial assemblage was dominated by the purple non-sulfur bacteria group (59.3 %) with six different genera followed by the purple sulfur community (27.8 %) with four genera and finally 12.9 % of the *puf*M gene sequences were assigned throughout the aerobic anoxygenic phototrophic bacterial group. The purple phototrophic bacterial community was characterized by the presence of salt-dependant bacterial species belonging to the genus *Marichromatium, Thiorhodococcus*, *Erythrobacter*, and *Roseobacter*. The wastewater treatment plant performances were unsatisfactory, and the biological and chemical parameters suggested that the purple photosynthetic bloom was correlated with the eutrophic state.

Keywords Functional diversity · Eutrophic state · Domestic wastewater · Denaturant gel gradient electrophoresis · *puf*M gene

A. Belila (✉) · I. Fazaa · A. Hassen · A. Ghrabi
Water Treatment and Reuse Laboratory, Water Researches and Technologies Center, Bordj-Cedria, BP. 273, 8020 Soliman, Tunisia
e-mail: belilaziz@yahoo.com; belilaabdelaziz@gmail.com

Introduction

Wastewater stabilization ponds (WSPs) are an extremely effective, natural form of wastewater treatment. They combine simplicity, robustness, low cost, and a very high degree of disinfection. WSPs are usually designed as one or more series of anaerobic, facultative, and maturation ponds. Their low operation and maintenance costs have made them a popular choice for wastewater treatment, particularly in developing countries since there is little need for specialized skills to operate the system. One of the major problems which can cause malfunction of the WSP is the massive growth of purple color producing bacteria (Belila et al. 2009; Sirianuntapiboon and Srikul 2006). When ponds were overloaded under anoxic and/or facultative conditions, the wastewater color appears purple in color due to the toxic effects of mainly sulfide on the algae and the consequent predominance of purple anoxygenic photosynthetic bacteria (Veenstra et al. 1995; Villanueva et al. 1994). As a result of this massive growth, chemical and physical wastewater effluent qualities such as color, smell, and suspended solid concentration can be seriously affected (Nair 1992).

The phototrophic purple bacteria are extremely heterogeneous on basis of morphological, physiological, and molecular data. Among the six phyla belonging to the photosynthetic prokaryotes, the purple phototrophic anoxygenic bacteria are the most metabolically diverse group. Currently, three groups of anoxygenic phototrophic bacteria are established: the purple sulfur bacteria (PSB), the purple non-sulfur bacteria (PNSB), and the aerobic anoxygenic phototrophic purple bacteria. Due to the wide distribution of this physiological bacteria group through several class of the *Proteobacteria* and due to their close phylogenetic relationship to non-phototrophic bacteria, the

use of general bacterial 16S rRNA gene primers often underestimates the diversity of phototrophic bacteria especially those present in rare numbers. The cultivation-dependent approaches also are critical and may lead to significant gaps in microbial diversity studies (Donachie et al. 2007). Different molecular methods based on the *puf*M gene encoding the M subunit of the photosynthetic reaction center have been used to explore the phototrophic bacterial diversity (Jiao et al. 2007) such as gradient gel electrophoresis (Lehours et al. 2010), terminal restriction fragment length polymorphism (Ranchou-Peyruse et al. 2006), and shotgun sequencing (Yutin et al. 2007). Although these DNA-based approaches have given insight into the diversity of anoxygenic phototrophic bacteria (APB), analysis of relevant functional communities is still a challenge.

So far, most studies on WSP systems have concentrated on the fate and removal of pathogenic microorganisms, such as fecal enterococci (Reinoso and Bécares 2008) and helminthes eggs (Tyagi et al. 2008). However, this study presents for the first time detailed taxonomic and phylogenetic information concerning the purple phototrophic microbial community composition during this bacterial photosynthetic bloom. For this purpose, a PCR-DGGE based approach targeting specific structural gene *puf*M and photopigments analysis were realized within four wastewater stabilization ponds situated in the North East of Tunisia exhibiting periodically red water phenomenon in April 2009. The ecophysiological factors enhancing this bacterial event were also discussed.

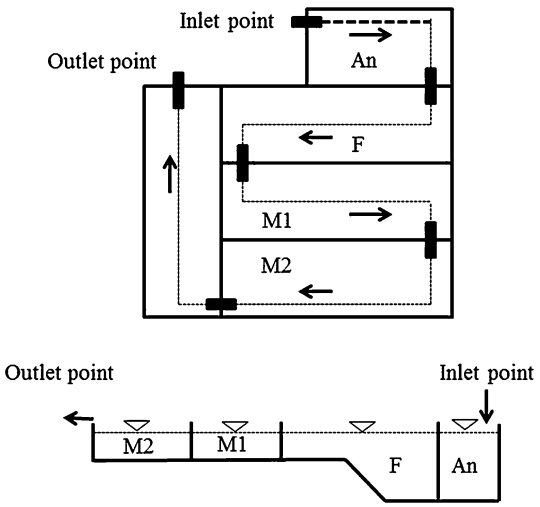

Fig. 1 Schematic representation of the wastewater stabilization plant. *AP* anaerobic pond, *FP* facultative pond, *MP* maturation pond

Table 1 Geometric characteristics of the wastewater stabilization ponds

	Anaerobic pond	Facultative pond	Maturation pond 1	Maturation pond 2
Surface (m^2)	30	100	122	129
Depth (m)	3.5	2.4	1.34	1.22
Volume (m^3)	96	180	164	147
Water depth (m)	3.3	2	1.15	1

Materials and methods

Wastewater stabilization plant description

This study was carried out in four WSPs located in Mutuelleville urban City (36°49′ N, 10°10′ E) in the North East of Tunisia. The wastewater stabilization system consists of four inter-connected ponds: the anaerobic pond followed by the facultative and two maturation ponds (Fig. 1). Size and depth of these ponds are summarized in Table 1. Effluents are essentially of domestic origin and result essentially from black waters (waters used for toilets) as well as grey waters (domestic sewage). Preliminary and primary treated wastewater fills up the first pond (anaerobic pond: An) and then enters the secondary facultative (F) pond through an outflow, and finally to the maturation (M) pond.

Sampling procedure

A total of 12 samples were collected from the wastewater stabilization ponds in April 2009. Four sediment samples were extracted from the anaerobic (S_{A1}–S_{A4}) and the facultative (S_{F1}–S_{F4}) ponds with Plexiglas core tubes (5 cm diameter, 25 cm length). The maturation pond sediment layer was very thin and could not be sampled. Four water samples were also collected (W_A, W_F, W_{M1} and W_{M2}) and were filtered using 0.2 μm pore size filters (Millipore). For molecular analysis, all samples were frozen at −50 °C.

Physical and chemical parameters

In situ measurements of the temperature and pH were performed using WTW Handheld Meters 340i model (WTW, Weilheim, Germany). Dissolved oxygen (DO) concentration was carried using a Multiline F/set P4 universal meter (WTW). Five-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspended solids (TSS), and sulfate and sulfide concentrations were carried out according to the analytical methods described in standard methods for the examination of water and wastewater (APHA 1995). The concentration of the chlorophyll a was estimated by the methanol extraction method (Pearson 1986).

Enrichment of the phototrophic purple bacteria and photopigments analysis

Aliquot volumes (1 ml) of wastewater collected from each wastewater stabilization pond were used to inoculate enrichment cultures for anoxygenic phototrophic bacteria. Two enrichment media were prepared according to the method of Pfennig and Trüper (1992) and Weaver et al. (1975) to enrich purple sulfur and non-sulfur bacteria, respectively. The enrichment cultures were incubated at 30 °C with light intensity of 500 lux from a tungsten lamp (16 h light and 8 h dark). In-vivo absorption spectra of living cells were determined using a Unicam Helios, a scanning spectrophotometer of a suspension of the cell pellets prepared in a sucrose solution (Pfennig and Trüper 1992).

Nucleic acid extraction and PCR amplification

Total genomic DNA from sediment and water samples was extracted using an Ultra Clean™ Soil and Ultra Clean™ water DNA extraction kits (Mo Bio Laboratories, Inc., USA) respectively using 0.5 g of sediments and 250 ml of filtered and centrifuged wastewater. The quality and quantity of the extracted DNA was checked by standard agarose gel electrophoresis.

PCR amplification of the *puf*M gene

The *puf*M gene was amplified using the primers puf557F and puf750R (Karr et al. 2003; Ranchou-Peyruse et al. 2006). PCR amplification mixtures contain 5 µl of 10× PCR buffer without $MgCl_2$, 3 µl of 25 mM $MgCl_2$, 1 µl of each deoxynucleoside triphosphate at 10 mM, 0.5 µl of each forward and reverse primer at 125 µg/ml, 1 µl of DNA template (ca. 100 ng), 1 U of Taq DNA polymerase and 37.75 µl of molecular water. Reactions were cycled in a Perkin-Elmer GeneAmp 2400 thermocycler at the following parameters: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The resulting amplification products were electrophoresed in 0.7 % (w/v) Tris-acetate-EDTA agarose gels and photographed. Although a GC clamp is typically attached to one of the PCR primers for subsequent use in DGGE, a high resolution of the DGGE bands was consistently obtained without a GC clamp.

Denaturant gel gradient electrophoresis analysis

Denaturant gel gradient electrophoresis (DGGE) experiment was performed using the D-Code system (Bio-Rad Laboratories, CA). Amplified fragments were electrophoresed on a 10 % polyacrylamide gel with 20–80 % denaturant (100 % denaturant was 7 M urea and 40 % (v/v) formamide) at 130 V for 8 h at 60 °C (Karr et al. 2003). DGGE gel was stained with Sybr GreenI (Sigma-Aldrich Corporation, St. Louis, MO, USA) and was visualized on a UV transilluminator. Individual bands were excised, re-suspended in 20 µl of Milli-Q water, and stored overnight at 4 °C. A volume of 3–5 µl of the supernatant was used for re-amplification using the original PCR conditions and the same primer pair without a GC clamp and then photographed using a GBox iChemi 2D gel image analyzer using Genesnap software 7.03 (Syngene, Synoptics, Ltd, Cambridge, UK).

Sequencing of DGGE bands

Prior to the sequencing reactions, primers were enzymatically removed from the reamplified DGGE bands using the ExoSAP-IT™ kit (Amersham Bioscience, Roosendaal, The Netherlands) following the descriptions of the manufacturer. Cycle sequencing reactions were performed with the ABI Prism Big Dye Terminator V3.0 kit (Applied Biosystems, CA, USA) using the forward or reverse primer (at a final concentration of 0.2 µM, and 10 ng of template DNA. The reaction volume was adjusted to a volume of 20 µl using molecular grade water (Sigma).

Phylogenetic analysis

The *puf*M sequences of the DGGE bands selected were first compared to the sequences stored in the publicly accessible database using the NCBI BLAST search tool. Subsequently, the sequences were imported into the ARB software package and aligned using the automatic aligner function. *Puf*M sequences obtained herein were inserted into the pre-established tree using the ARB parsimony tool. The base tree was calculated using neighbor-joining algorithm on the 632 position of long sequences within the *puf*M gene. Felsenstein correction was used with a filter ignoring the third base pair. The sequences derived from the DGGE were added after manual correction using ARB parcimony.

Comparative analysis of *puf*M gene sequences

The partial sequences were analyzed using BLAST in the NCBI database (http://ncbi.nlm.nih.gov/BLAST) and added together with the most important BLAST hits, to an alignment of homologous *puf*M gene sequences using the aligning tool of the ARB software package. The tree was generated by neighbor-joining, with the correction method of Felsenstein as implemented in ARB. Bootstrap analysis (1,000 replicates) was performed in PAUP version 4. Generated sequences were deposited in the GenBank database under accession numbers HQ222675–HQ222729.

Results and discussion

General characteristics of the wastewater and stabilization ponds performances

The performances of the wastewater stabilization plant investigated in April 2009 were unsatisfactory regarding to the BOD and the COD removal efficiency, the algal productivity and odor release (hydrogen sulfide) (Table 2). The chemical and biological parameters within the stabilization plant gave evidence of the eutrophic state as defined by the Urban Wastewater Treatment Directive (91/271/ECC) (Doering et al. 2006). The stabilization ponds performances decreased during spring and summer seasons and the percentage removal of the total suspended solid (TSS), biological and chemical oxygen demands (DBO_5 and DCO) was 50, 40 and 43 %, respectively. These results were in accordance with previous observed by Veenstra et al. (1995) and Alabaster (1991) who observed an unsatisfactory BOD and TSS removal rates, while Houghton and Mara (1992) noticed that photosynthetic purple bacterial blooming did not affect the effluent quality.

Both facultative and maturation ponds were characterized by a high productivity, and the chlorophyll *a* concentration reached 3,456 ± 265 µg/l in the first maturation pond and 2,634 ± 268 mg/L in the facultative pond. The dissolved oxygen concentration within the water column increased moderately from 0.2 mg/L in the anaerobic pond to 3.1 mg/L in the last maturation pond (BM_2). The proliferation of the purple red color throughout the water column, reaching surface of the four lagoons supports the prevalence of anoxic conditions within the whole system (Llorens et al. 1992). Wastewater pH varied between 6.9 ± 0.2 (anaerobic pond), 7.6 ± 0.2 (facultative pond) and 7.4 ± 0.2 (maturation pond 2) and the temperature ranged between 14.7 ± 2 °C (anaerobic pond) and 12.9 ± 2 °C (maturation pond 2). The biological oxygen demand varied between 473 ± 19.3 mg/L (anaerobic pond) and 280.6 ± 7.5 mg/L (maturation pond 2), while chemical oxygen demand varied between 908 ± 13 mg/L (anaerobic pond) and 505 ± 11 mg/L (maturation pond 2).

The increase of the sulfate removal rate from 23.4 % within the facultative pond to 81 % within the first maturation pond (M1) reveals distinctive development of sulfate-reducing conditions in this second stabilization pond. Sulfate concentration then stabilized at approximately 48 mg/L providing a final sulfate removal rate of 83.7 %. During spring and summer seasons, the sulphate reduction process—stimulated by temperature increase—becomes the dominant terminal process within sediments of the anaerobic and facultative ponds stimulating the production and the diffusion of the hydrogen sulfide which reaches the maturation ponds M_1 and M_2 where we recorded 9 ± 3.2 and 10 ± 0.7 mg/L, respectively.

Photopigments analysis

Spectrum patterns from sulfur and purple non-sulfur bacteria enrichment were characterized with maxima situated at 390 (393), 596 (593), 802 (805), and 856 nm, indicating the presence of Bchl *a*, while maxima situated at 486 (485) and 515 (556) indicate the presence of carotenoids of the normal spirilloxanthin series (Fig. 2) (Pfennig and Trüper 1974). These photopigments explain the origin of the red purple color. These results are comparable with those obtained by Veenstra et al. (1995) from the wastewater stabilization ponds affected by red water phenomenon with characteristic absorption peaks for bacteriochlorophyll *a* which match the absorption spectra of purple phototrophic bacteria as recorded by Trüper and Pfennig (1981).

Interpretation of the DGGE profile

The visual analysis of the DGGE profile reveals relative high diversity of the *puf*M gene as shown on Fig. 3, where the number of DGGE bands reaches 21 within the sediment of the facultative pond (SF_4). Also, DGGE bands position, intensity, and number differ between each sample (Fig. 3). In total, 53 DGGE bands were selected and sequenced for phylogenetic analysis (Fig. 3). The DGGE banding patterns obtained from the sediments and water samples were significantly different from each other. These findings are in accordance with previous results (Belila et al. 2009) and

Table 2 Stabilization ponds wastewater characteristics (April 2009)

	T (°C)	pH	DO (mg/L)	TSS (mg/L)	BOD_5 (mg/L)	COD (mg/L)	Chl a (µg/L)	SO_4^{2-} (mg/L)	S^{2-} (mg/L)	Salinity (mg/L)
BA	14.7 ± 2	6.9 ± 0.2	0.20 ± 0.2	533 ± 18	473.7 ± 19.3	908 ± 13	156 ± 28	320 ± 33	40.8 ± 0.7	2 ± 0.4
BF	13.5 ± 1	7.6 ± 0.2	2.50 ± 0.4	342 ± 38	360.0 ± 19.6	720 ± 17	2,634 ± 268	245 ± 43	25 ± 0.9	1.4 ± 0.6
BM1	12.2 ± 1	7.5 ± 0.1	3.10 ± 0.3	247 ± 18	283.3 ± 11.6	510 ± 26	3,456 ± 265	45 ± 15	9 ± 3.2	1.1 ± 0.5
BM2	12.9 ± 2	7.4 ± 0.1	3.10 ± 0.9	262 ± 18	280.6 ± 7.5	505 ± 11	3,288 ± 345	52 ± 17	10 ± 0.7	1.1 ± 0.3

T temperature, *DO* dissolved oxygen, *TSS* total suspended solid, BOD_5 5-day biochemical oxygen demand, *COD* chemical oxygen demand, *Chl a* chlorophyll a, SO_4^{2-} sulfates, S^{2-} sulfides, *BA* anaerobic pond, *BF* facultative pond, *BM1* and *BM2* maturation ponds

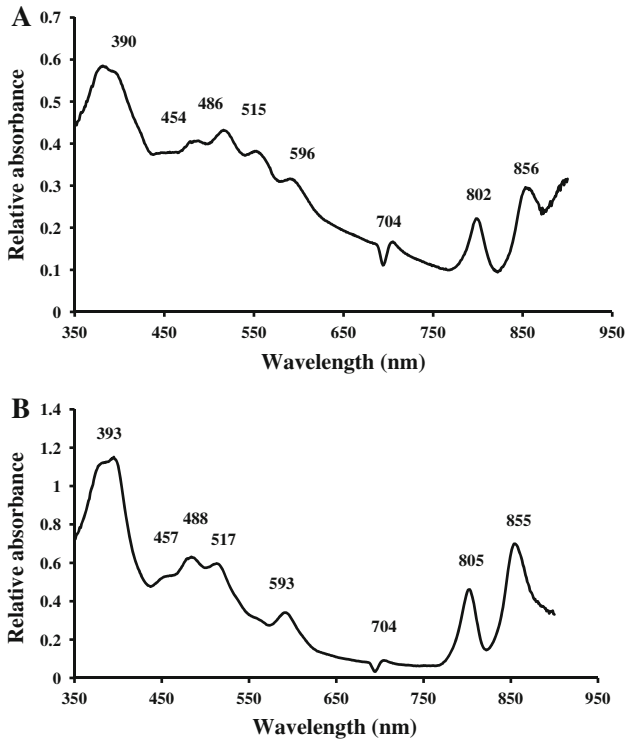

Fig. 2 Photopigments analysis. **a** Enrichment culture of purple sulfur bacteria, **b** enrichment culture of purple non-sulfur bacteria

the electrophoretic profile within water and sediment samples exhibits relatively different banding patterns (Fig. 3). Five DGGE bands were common in all sediment samples (C_4, D_5, E_5, F_3 and G_3) and four bands were common in all water samples (G_9, E_{10}, H_{10} and A_{12}).

Phylogenetic diversity of the purple phototrophic bacteria

The microbial community structure within the wastewater stabilization plant during the bacterial photosynthetic bloom appeared to be complex. The phylogenetic analysis of the selected DGGE bands revealed that 59.3 % of the total *puf*M sequences were assigned throughout the purple non-sulfur bacteria group (α and β *Proteobacteria*), while 27.8 % of the *puf*M DGGE bands sequences were related to purple sulfur bacteria (Chromatiaceae) (γ *Proteobacteria*). The rest of the *puf*M gene sequences (12.9 %) were assigned through the anoxygenic phototrophic bacteria group belonging to α- and β-*Proteobacteria* classes.

Purple non-sulfur bacterial community

The *puf*M analyzed sequences were affiliated with six purple non-sulfur bacterial genera: *Rhodospirillum, Rhodoferax, Rhodobaca, Rhodobacter, Rhodoplanes and Phaeospirillum* (Fig. 4). The taxonomic composition of the PNSB community was dominated by the *Rhodospirillum* genus with ten related *puf*M DGGE sequences followed by *Rhodoferax* with seven associated *puf*M DGGE sequences. The *puf*M gene sequences assigned to the *Rhodospirillaceae* belonged to three different phylogenetic groups: *α-1, α-2* and *α-3*. The DGGE bands A_4 and A_{11} related sequences were affiliated to phototrophic PNSB species of the α-3 group *Rhodobacter blasticus* and *Rhodobacter veldkampii*, respectively. The DGGE bands G_3, A_2, A_3, C_1 and D_6 sequences were identified to *Phaeospirillum molischianum* and *Phaeospirillum* sp species of the α-1 *Proteobacteria* group. Also, the *pufM* sequences of DGGE bands H_9, A_{10}, F_{10}, C_{11}, E_{10} C_6, D_{10}, C_{12}, B_4 and G_{10} were related to the *Rhodospirillum* genus (α-1 cluster group). DGGE band C_7 related sequence was affiliated to the α-2 group species *Rhodoplanes elegans*. Seven *puf*M DGGE bands sequences (B_2, D_8, D_9, C_3, C_{10}, B_{10} and D_7) were affiliated with relatives belonging to the *Rhodoferax* clade of the *Betaproteobacteria* class, while six *puf*M DGGE band sequences (D_{11}, H_{11}, B_{12}, E_{12}, B_8 and D_5) were related to the PNSB of the *Rhodobaca* clade.

The predominant purple non-sulfur bacteria belonging to the *Rhodospirillum* and *Rhodoferax* genera are known for their key role in carbon and nitrogen metabolism (Imhoff 2000; Tabita 1995) and were frequently found in sewage, activated sludge, and wastewater treatment plant (Li et al. 2009; Wan et al. 2010).Their frequent occurrence in these man-made ecosystems points towards their eco-physiological function (Fang et al. 2005; Jopia et al. 2009; LaPara et al. 2000).

Purple sulfur bacteria community

A total percentage of 27.8 % of the total *puf*M sequences were assigned through the Chromatiaceae family. The phototrophic purple sulfur bacteria (PSB) community composition was characterized by the presence of both marine (i.e. *Thiorhodococcus* and *Marichromatium*) and freshwater (i.e. *Thiocapsa roseopersicina* and *Chromatium weissei*) phototrophic purple bacterial branches. The sequences of the DGGE bands A_6 and E_6 were closely related to phototrophic purple sulfur bacteria *Thiocapsa imhoffi*, while DGGE band A_1 sequence was affiliated with *Thiocapsa roseopersicina*. The *puf*M-G_5 sequence belonged to *Thiorhodococcus* sp and DGGE band C_4 sequence was related to the γ-*Proteobacteria Chromatium weissei*. Four DGGE bands (D_4, E_4, F_5 and E_9) were identified to a Chromatiaceae bacterium. The *puf*M sequences of the DGGE bands: B_1, G_9, E_8, F_8, B_{11}, E_{11} and F_4 were closely related to the marine bacteria species of the *Marichromatium* genus (i.e. *Mch gracile, Mch indicum* and *Mch purpuratum*) (Fig. 4). No representative's of the

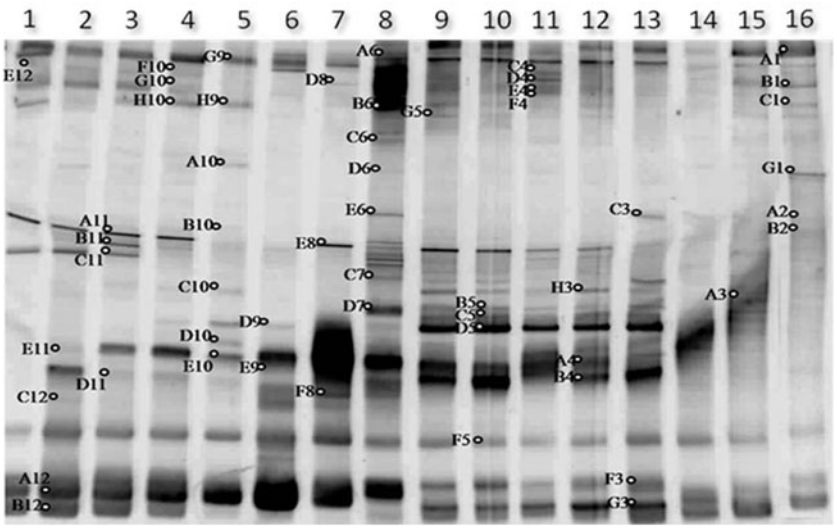

Fig. 3 DGGE fingerprint of the *puf*M gene. *Lanes* 1 and 2, water samples from maturation pond M2. *Lanes* 3 and 4 water samples from maturation pond M1. *Lane* 5 water sample from the facultative pond. *Lane* 6 water sample from the anaerobic pond. *Lanes* 7–11 sediment samples from the facultative pond (*F*). *Lanes* 12–16 sediment samples from the anaerobic pond (*An*)

Ectothiorhospiraceae family were identified among the purple phototrophic sulfur bacterial community.

Both hydrogen sulfide and oxygen gradients limit the distribution of the PSB within the WSP to the anaerobic treatment stage or more to the anaerobic microniches developed by these bacteria. Although the Chromatiaceae are basically anaerobic, some species are considerably tolerant to oxygen and flexible in their metabolism (Imhoff 2005) such as *Thiocapsa roseopersicina*, which is equipped to take advantage of changing conditions from oxic/dark to anoxic/light conditions by driving respiration and photosynthesis simultaneously (Imhoff 2001; Schaub and Van Gemerden 1994).

Based on their metabolic proprieties and their sulfide oxidizing capacities, the spatial distribution of the purple sulfur bacteria within the WSP should be limited to the lower water layer of the anaerobic and facultative ponds where the highest hydrogen sulfide concentrations were registered.

Aerobic anoxygenic phototrophic bacteria

Eight *puf*M sequences representing 12.9 % of the total analyzed sequences were assigned throughout the aerobic anoxygenic phototrophic purple bacterial group (AAnPB). Both DGGE bands C_5 and B_5 sequences were assigned through the *Roseateles* group of the β-*Proteobacteria* class (freshwater branch), while H_3 DGGE band sequence was affiliated with *Roseobacter* sp. (α-3 group) and F_3 DGGE band sequence was related to *Erythrobacter* sp (α-4 group). The *puf*M sequence of the DGGE band H_{10} was affiliated with *Bradyrhizobium denitrificans*, while that of DGGE band A_{12} was affiliated with uncultured aerobic anoxygenic phototrophic bacteria clone (Fig. 4). AAnPB are well adapted to a broad range of trophic conditions and are abundant in eutrophic and oligotrophic environments (Waidner and Kirchman 2007). Their higher growth rates and efficiency in organic carbon utilization over strict heterotrophs are likely to make them dynamic and significant contributors to the organic carbon production and cycling within the stabilization ponds (Gasol et al. 2008) and it has been shown that these bacteria play a key role in a diverse range of oxic ecosystems (Buchan et al. 2005; Oz et al. 2005). Among the aerobic anoxygenic phototrophic bacterial group, some representatives may be able to grow under anaerobic conditions (Ranchou-Peyruse et al. 2006).

Wastewater stabilization ponds are without doubt the most important method of wastewater treatment in developing countries where sufficient land is normally available and where the temperature is most favorable for their operation. The deepest ponds (anaerobic and facultative) generally stratify as a result of warm climatic conditions and with the absence of artificial aeration between March and September. During this period, the sulfate reduction process becomes the dominant terminal process within sediments of the anaerobic and facultative ponds stimulated by the increase of the temperature and thus stimulates sulfide production by sulfate reduction. The hydrogen sulfide spontaneously reduces oxygen expanding the anaerobic zone throughout the water column within the facultative pond and may diffuse throughout the maturation ponds. As a consequence, all ponds appear transiently purple in color under the toxic effects of mainly sulfide on the algae and the consequent predominance of purple photosynthetic bacteria.

The original objective of this work was to use a molecular approach based on the *pufM* gene analysis to study the diversity of the purple photosynthetic bacteria within four eutrophic wastewater stabilization ponds exhibiting red water phenomenon. This molecular approach was the first to investigate this microbial event. In fact, all previous studies attempts to identify bacteria

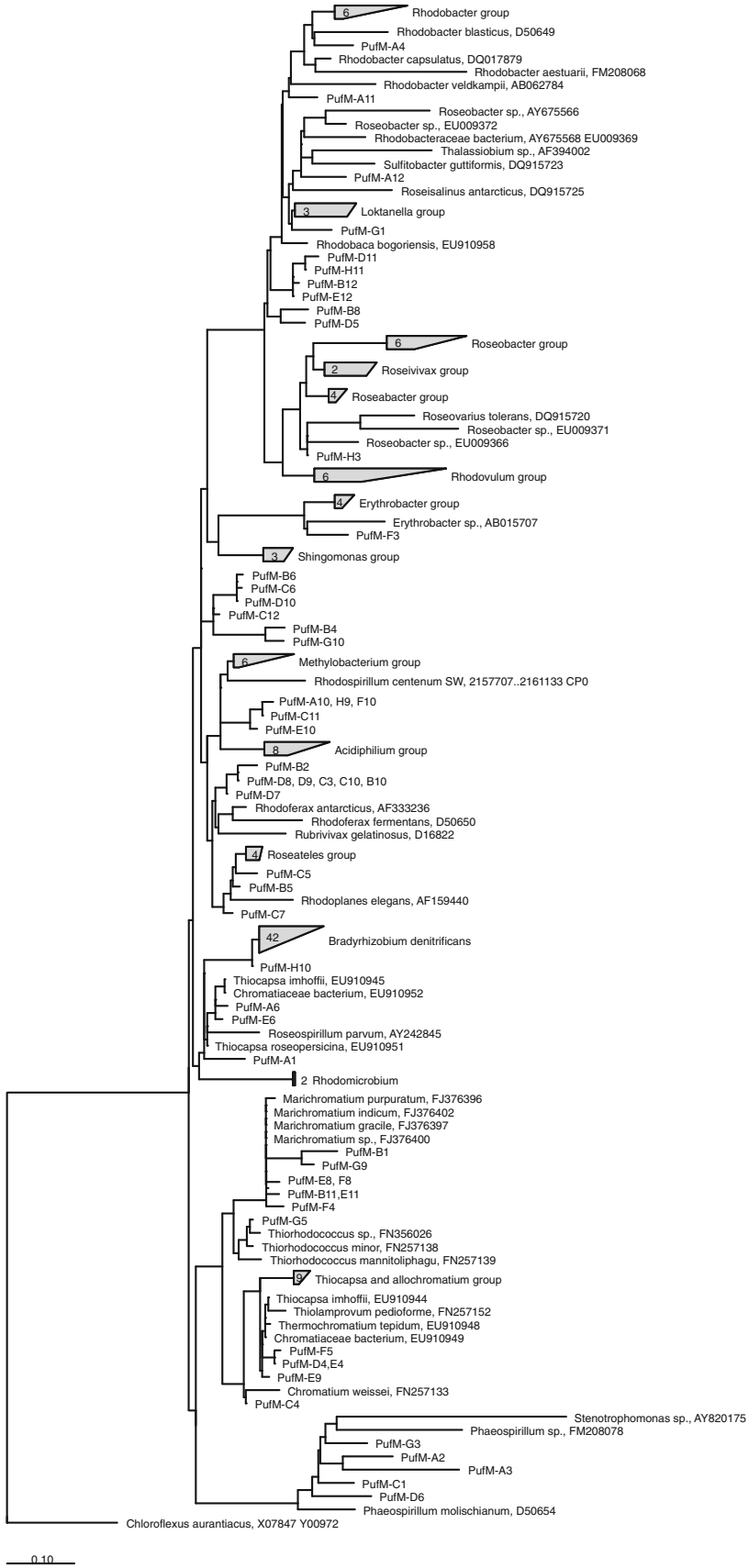

Fig. 4 Neighbor-joining tree based on 229 nucleotide positions of the *pufM* gene, obtained using *Chloroflexus aurantiacus* as the outgroup. *Scale bar* indicates the number of substitutions per site. The sequences derived from the DGGE gel were added after manual correction using ARB parsimony

responsible of this biological event were commonly based on culture-dependant approaches such as enrichment culture, isolation, biochemical, and physiological characterization methods (Siefert et al. 1978; Sirianuntapiboon and Srikul 2006). Due to the limitation of the culturable approaches and 16S rRNA genes to reveal the real phototrophic bacterial diversity (Donachie et al. 2007), targeting functional gene will allow to combine phylogenetic information and physiological traits indications. This was successfully demonstrated for *puf*M gene as for the *fmo*A gene for the green sulfur bacteria (Alexander and Imhoff 2006). The *puf*M gene proved as excellent molecular marker for revealing the purple phototrophic bacterial diversity and was successfully applied to reveal the diversity of the PNSB, the PSB (Tank et al. 2009), and the AAnPB diversity (Cho et al. 2007; Salka et al. 2008).

The metabolic plasticity of the PNSB constitutes an advantage over the phototrophic sulfur bacteria which exhibit strict metabolism especially regarding dissolved oxygen and sulfide concentrations. The PNSB are metabolically the most versatile among all the prokaryotes (Kim et al. 2004). They are ubiquitous in wastewater treatment systems (Hoogewerf et al. 2003; Okubo et al. 2006) where their great metabolic versatility constitutes a selective advantage in these habitats. Indeed, they can grow both photoautotrophically and photoheterotrophically under anaerobic-light or micro aerobic-light conditions (Imhoff 2006). Many PNSB grow anaerobically in the dark using fermentation processes and others aerobically in darkness using respiration. Consequently, these bacteria can thrive at all wastewater purification stages. In addition, the Rhodospirillaceae are better adapted to life under oxygen fluctuation (Pfennig and Trüper 1989).

Previous investigation of the red water phenomenon demonstrated that this temporal pigmentation change was attributed to the mono-specific massive growth of the purple sulfur bacteria such as *Thiopedia rosea* (Wenke and Vogt 1981) or *Chromatium* sp. (Caldwell and Tiedge 1975) or to the mono-specific blooming of the purple non-sulfur bacteria like *Rhodobacter* (Do et al. 2003) or *Rhodopseudomonas* (Okubo et al. 2006; Veenstra et al. 1995). Interestingly, the complex purple phototrophic anoxygenic bacterial assemblage reported herein was not described before in wastewater systems during this biological phenomenon, especially since the coexistence of these phototrophic bacterial groups is difficult in nature. The purple phototrophic bacterial diversity points out on the key role of the light quality on their ecology. The light spectrum quality stimulates the differential distribution of these photosynthetic bacteria through the differentiation of different ecological niches along the light spectrum resources (Stomp et al. 2007), which may promote their coexistence (Rueffler et al. 2006; Stomp et al. 2007). Montesinos et al. (1983) have demonstrated that light quality determines the species composition among phototrophic bacteria community by acting as a selective agent, which may explain the dominance of some phototrophic bacterial species within each stabilization pond.

In fact, in aquatic ecosystems, the underwater light spectrum depends on light absorption of the physical properties of the water, of the chemicals in solution, and of the suspended particles (gilvin, tripton, algae); consequently, each pond was characterized by specific light spectrum composition. Many studies have revealed a close correspondence between the phototrophic bacteria absorption spectrum and the prevailing underwater light spectrum (Bouman et al. 2006; Kühl et al. 2005; Sabehi et al. 2007). The photosynthetic purple bacterial assemblage reported herein was characterized by the presence of salt-tolerant purple sulfur (PSB) and purple aerobic phototrophic anoxygenic bacteria. Eight *puf*M phylotypes were associated with PSB of the *Marichromatium* genus (i.e. B_1, G_9 and B_{11}) and *Thiorhodococcus* genus (DGGE band G_5). In addition, the purple aerobic anoxygenic phototrophic bacteria *Erythrobacter* (DGGE band F_3) and *Roseobacter* (DGGE band H_3) genera were detected within the stabilization ponds. In Tunisia, the quality of treated wastewater varies spatially with the lowest salinity found in the northwest (average 1.3 mg/L) owing to the good quality of surface water resources and the low level of industrial activity in that region. By contrast, the wastewater treatment plant in the south exhibits alarmingly high concentrations of salt due to the salinity of the distribution waters and the presence of important industries that dispose of their wastes in certain stations (min. 2.7, max. 8.9, average 4.1 mg/L). In addition, the wastewater effluent salinity is conditioned by the proportion of industrial effluents, by the infiltration of brackish water, and by the quality of drinking water. In Tunisia, the wastewater salinity varies generally from 1 to 6 g/L from wastewater treatment plant to another (Al Atiri et al. 2002) which may explain the occurrence of such halophilic purple sulfur bacteria within the investigated stabilization ponds. The occurrence of such salt-tolerant photosynthetic bacteria under freshwater conditions points out that these bacteria have probably the potential to succeed at low salt concentrations or even in the absence of salt (case of *Thiorhodococcus*) (Kumar et al. 2007; Rabold et al. 2006); also purple sulfur bacteria have varying tolerances for salinity. In fact, some relative species persist in a wide salinity range (Pfennig and Trüper 1989).

Conclusion

Complex bacterial communities in wastewater ecosystems should be monitored in order to maintain their operation to

improve their efficiency and to guarantee their stability. Herein, the PCR-DGGE based approach revealed that the red water phenomenon is due to the presence of complex purple photosynthetic bacterial community dominated by non-sulfur bacteria species and also characterized by the presence of aerobic photosynthetic purple bacteria. This bacterial diversity reflects a high metabolic plasticity and points on eco-physiological role of these bacteria within the stabilization ponds during the red water phenomenon. Regarding their sulfur-oxidizing capabilities, the phylogenetic diversity of the purple phototrophic bacterial community may traduce probably an active biological sulfur cycle enhanced by the activity of the sulfate-reducing bacteria within the sediments of the anaerobic and the facultative stabilization ponds.

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