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ORIGINAL ARTICLE

Bacterial diversity of the outflows of a Polichnitos (Lesvos, Greece) hot spring, laboratory studies of a *Cyanobacterium* sp. strain and potential medical applications

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Abstract The bacterial diversity of the outflows of Polichnitos (Lesvos, Greece) hot spring has been investigated. Cyanobacteria showing high sequence homologies with *Phormidium* sp. and *Cyanobacterium aponinum* were found. Members of the Alphaproteobacteria closely related to *Rhodobium* sp. *Albidovulum* sp., *Rhodobacter* sp., *Microvigra* sp., *Nitratireductor* sp. and *Phaeobacter* sp. Gammaproteobacteria, Betaproteobacteria and Firmicutes were represented by members of *Idiomarina* sp., *Marinobacter* sp., *Shinella* sp., *Bacillus* sp. and *Clostridium* sp. with sequence homologies ranging from 92% to 100%. Members of the Bacteroidetes and Planctomycetes were represented by sequences of novel phylogenetic linkages exhibiting 87–90% sequence homology with type strains. When the hot spring consortium was cultivated in bioreactor repeated batch culture under photo-autotrophic growth conditions at temperature < 30 °C, *Cyanobacterium* sp. dominated over *Phormidium* sp. *Cyanobacterium* sp. seems to have biotechnological potential since its extracellular broth exhibited a strong insecticidal activity against larvae of *Aedes aegypti* (a vector of important human diseases) and significant anticancer activity against the PC3 human prostate cancer cell line, while its toxicity against human endothelial cells was relatively low.

Keywords Polichnitos hot springs · 28 bacterial clones · *Cyanobacterium* sp. · Photobioreactor cultures · Insecticidal/anticancer activity

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Introduction

The investigation of microbial diversity in extreme habitats is of great significance for biological sciences since it enriches knowledge on the structure and function of ecosystems, and, together with deciphering of genomes representing deep branching lineages, is a valuable tool for evolutionary studies and providing blueprints for the functional diversity that structure the environment (Rinke et al. 2013). In addition, microorganisms isolated from extreme habitats are of great interest as these are putative sources of new metabolites and enzymes possessing exceptional properties with potential applications both in medicine and in chemical industry. Indeed, the unusual metabolism of extremophiles is presumably supported by proteins possessing exceptional properties, and may lead to uncommon metabolite synthesis.

Although investigations on the microflora of hot springs have been at the forefront of ecological research for many years (Castenholz 1969; Anagnostidis and Pantazidou 1988; Ward et al. 1998; Pentecost 2003), it is only in recent decades that microorganisms isolated from such extreme environments have been considered as candidates for biotechnological applications (Abed et al. 2009; Hu et al. 2015). Specifically, thermophilic microorganisms, being among the most studied extremophiles, have gained biotechnological interest mainly as sources of thermostable enzymes demonstrating a high catalytic efficiency over wide temperature and pH ranges (Bora et al. 2013). For instance, thermophilic microorganisms isolated from hot springs in North Western Spain showed remarkable extracellular lipase activity (Deive et al. 2013), while some thermophiles isolated from Al-Khoba and Al-Arida hot springs in Saudi Arabia have been characterized as lipase, cellulase, and amylase producers (Khalil 2013). Other thermostable enzymes, such as a polyphosphate kinase and restriction enzymes were produced by cyanobacterial strains of *Thermosynechococcus elongatus* (Sato et al. 2007) and *Phormidium* sp. (Piechula et al. 2001), respectively. Enzymes with potential applications in the manufacture of biofuel have been also produced by a *Geobacillus thermodenitrificans* strain isolated from the Mushroom Springs of Yellowstone National Park in the United States (i.e., a lipase that converted efficiently waste cooking oil and coconut oil into biodiesel) (Christopher et al. 2015) and by *Caldicellulosiruptor bescii* and *Caldanaerobius polysaccharolyticus* strains during their growth on lignocellulosic materials such as xylan and cellulose (Han et al. 2012; Su et al. 2013). Moreover, Koskinen et al. (2008) described a thermophilic consortium derived from a geothermal spring in Iceland, in which *Thermoanaerobacterium aotearoense* predominated, which was able to produce biohydrogen along with butyrate and acetate when cultivated on carbohydrates. Microorganisms isolated from hot springs have also been recently identified as sources of bioactive compounds (e.g., strain-specific antibacterial, antifungal and cytotoxic molecules) of potential interest in the pharmaceutical industry (Berry et al. 2008).

In Greece, a large number of hot springs are recorded (i.e., more than 750 thermal springs and more than 50 spas in operation) (Fytikas et al. 2005), as the deep tectonic structures and the young-to-recent volcanism in the region have created a large number of shallow geothermal fields of both low and high enthalpy (Hatziyannis 2007). Especially numerous hot springs are present in Lesvos Island located in the volcanic region of the North Aegean extending from Axios valley to the inactive Tertiary volcanoes of Samothraki, Lemnos, Agios Efstratios and Lesvos Islands (Fig. S1a, b). Polichnitos hot springs in Lesvos (39.073951, 26.200204) are considered to be among the hottest in Europe and worldwide, having a geochemical temperature in the range 115–125 °C (Fytikas et al. 1989); so far, no investigations concerning their microflora have been performed.

The aim of this paper was to investigate the bacterial diversity of the outflows of the Polichnitos (Lesvos) hot spring and to study the biological activity of a cyanobacterial strain identified as the dominant bacterial strain after repeated batch cultivation of the microbial consortium under autotrophic growth conditions. The strain, having 99% similarity with *Cyanobacterium aponinum* type strain, was studied under various conditions in laboratory bioreactor cultures, and the biological activity of its extracellular broth and cellular extract was investigated. It was found that the extracellular broth of this strain exhibited a strong insecticidal activity against larvae of *Aedes aegypti* (the vector of dengue fever, chikungunya, and yellow fever viruses) and a significant anti-cancer activity against the PC3 human prostate cancer cell line, while its toxicity against human endothelial cells (HUVECs) was relatively low. However, the above preparations did not show any anti-bacterial activity against either Gram negative or Gram positive bacteria.

Materials and methods

Sampling

Samples were taken from one of the outflows of Polichnitos hot spring and stored in 50 mL sterile falcon tubes at 4 °C before DNA extraction. The water temperature measured in situ was 80 °C at the time of sampling (Fig. S2).

Molecular identification and phylogenetic analysis

To isolate genomic DNA, water samples were successively filtered through sterile filters of decreasing porosity (3 μm and 0.2 μm glass fiber and membrane filters, respectively, Whatman, Florham Park, NJ). Total DNA was extracted as previously described (Katsaveli et al. 2012) and quantified with a Qubit fluorometer (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Near full-length 16S rRNA gene amplification for the construction of libraries was carried out using universal primers 27F and 1492R (Lane 1991). PCR amplifications were performed in 20 μL reactions containing ~20 ng DNA, 4 μL 5× reaction buffer (Promega), 1.6 μL $MgCl_2$ (25 mmol L^{-1}), 0.1 μL deoxynucleotide triphosphate mixture (25 mmol L^{-1} each), 0.5 μL of each primer (25 mmol L^{-1}), 0.1 μL *Taq* polymerase (Promega 1 U μL^{-1}) and 12.2 μL water. Amplification was performed in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA), with a denaturation step of 10 min at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 52 °C, and finally 90 s extension at 72 °C. The PCR was completed by a final extension at 72 °C for 10 min.

The size of the PCR products was determined by agarose gel electrophoresis using 1 kb size markers. The bacterial PCR products were precipitated with polyethylene glycol (PEG)

(Hartley and Bowen 2003), with the purified DNA being cloned into pGEM-T, according to the manufacturer's protocol (Promega, Madison, WI), and transformed into competent *Escherichia coli* DH5α cells. White colonies on ampicillin/X-gal plates were screened for inserts of the correct length by PCR with the pGEM-T compatible primers T7 and SP6. Inserts were fully sequenced with the same primers and with internal 16S rRNA gene-specific primers (Tsiamis et al. 2008). Sequencing was performed using an ABI3110 analyzer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The 16S rRNA gene sequences reported in the present study have been deposited in GenBank with accession numbers KX062015–KX062042.

Sequencing analysis was performed using the Mothur software package (Schloss et al. 2009). All clones were first screened for potential chimeric structures using USEARCH v7.0. The closest relative was assigned using the Ribosomal Database Project (Cole et al. 2005). Alignment of sequences was carried out using the program MUSCLE (Edgar 2004). A phylogenetic tree, based on the distance matrix method, was constructed using the software package MEGA v6.0. Evolutionary distances were calculated using the F84 model, and topology was inferred using the "neighbor-joining" method. Sequences of at least 1200 bp in length were used for tree constructions.

Bioreactor cultures

The microbial consortium was cultivated in a BioFlo/CelliGen 115 bioreactor (New Brunswick, Hamburg, Germany) of total volume 3 L and active volume 2.2 L on a modified artificial seawater (ASW) medium consisted of (g L^{-1}) NaCl (Sigma, Steinheim, Germany), 27; $MgSO_4 \cdot 7H_2O$ (Sigma), 6.6; $CaCl_2$ (Carlo Erba, Rodano, Italy), 1.5; KH_2PO_4 (Sigma), 0.02; KNO_3 (Merck, Darmstadt, Germany), 1; $FeCl_3 \cdot 6H_2O$ (BDH, Poole, UK), 0.014; Na_2EDTA (Sigma), 0.019 and 1 mL L^{-1} of a microelement solution containing (in mg L^{-1}) $ZnSO_4 \cdot 7H_2O$ (Merck), 40; H_3BO_3 (Fluka, Steinheim, Germany), 600; $CoCl_2 \cdot 6H_2O$ (Sigma), 1.5; $CuSO_4 \cdot 5H_2O$ (BDH) 40; $MnCl_2$ (Sigma), 400 and $(NH_4)_6Mo7O_{24} \cdot 4H_2O$ (Sigma), 370. The culture vessel was initially washed with 70% ethanol and filled with 2 L sterilized (at 121 °C for 30 min) medium, before being inoculated with the hot spring microbial consortium (200 mL). The culture was grown aerobically by providing air at 1.5 vvm, which was passing through a bacteriological filter with 0.2 μm pore size (Whatman, Kent, UK). Agitation was kept constant at 200 rpm, and pH was adjusted to 8.3 ± 0.2. Illumination of 480 μE m^{-2} s^{-1} was provided by 8 W fluorescent lambs, which were placed at a distance of 30 cm around the culture vessel.

Repeated batch bioreactor cultures, lasting 2 weeks each, were performed under full illumination regime (24 h light: 0 h dark) and different temperatures ranging from 19 °C to 55 °C for several months. At the end of each batch, 2 l of the culture broth was removed from the vessel and an equal volume of fresh medium was added.

Growth estimation of *Cyanobacterium* sp., biomass harvesting and chemical analyses

The growth of *Cyanobacterium* sp. was studied under different photoperiods, i.e., 24:0; 16:8 and 12:12 at 21 ± 1 °C, 26 ± 1 °C and 29 ± 1 °C. Growth was estimated by enumerating the number of cells per milliliter using a Neubauer improved Precicolor haematocytometer (HBG, Giessen, Germany). The parameters of growth were estimated using a modified Verhulst equation (Eq. 1):

$$\frac{dN'}{dt} = r_{max} \cdot \left[1 - \left(N' \Big/ K\right)\right] \cdot N' \quad (1)$$

Where N' is the normalized cell density (N/N_0), r_{max} (h^{-1}) is the maximum specific growth rate, and K is the carrying capacity of the system. For biomass determination, an adequate volume of the culture (i.e., 150 mL) was withdrawn from the bioreactor and the cells were harvested by centrifugation at 22,500 *g* for 15 min at 4 °C (Heraeus, Biofuge Stratos, Osterode, Germany), washed twice with NaCl 0.9%, dried at 80 °C to constant weight, and then determined gravimetrically.

Total sugars in *Cyanobacterium* sp. cells were determined according to Liang et al. (2010). Total intracellular protein, total lipids and fatty acid composition were determined as previously described in Bellou and Aggelis (2012).

Culture broth and cell extract preparation and analysis

About 1 L *Cyanobacterium* sp. culture was withdrawn from the bioreactor and the cells were harvested by centrifugation as above. The cyanobacterial cells were ruptured by one sonic burst of 90 W, lasting 2 min, and five sonic bursts of 90 W, lasting 1 min, at 0–4 °C using a Sonics Vibra cell CV188 sonicator (Newtown, CT), and the cell debris was removed by centrifugation at 40,000 *g* for 50 min at 4 °C. The supernatant was filtered through a Whatman 0.2 μm sterilized membrane to remove remaining cell debris (Papanikolaou et al. 2004), as well as to avoid bacterial contamination. The cell extract was kept in sterilized Eppendorf tubes. Similarly, the culture broth was filtered through Whatman 0.2 μm and kept under aseptic conditions in Eppendorf tubes. Soluble proteins in the culture broth and the cell-free extract were quantified according to Lowry et al. (1951).

Test organisms/cell line and treatment

The antibacterial activity of both extracellular culture broth and cell extract of *Cyanobacterium* sp. was assayed using the well diffusion method as described in Sayegh et al.

(2016). The Gram negative strains *Escherichia coli* ATCC BAA1001, *Klebsiella pneumoniae* ATCC 700603, *Moraxella catarrhalis* ATCC 25238, *Neisseria gonorrhoeae* ATCC 49226, *Salmonella* sp. and the Gram positive strains *Enterococcus faecalis* ATCC 29212, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43330, *S. aureus* subsp. *aureus* ATCC 25923, and *S. epidermidis* ATCC 12228 were used as test organisms.

The larval susceptibility test was conducted according to the method recommended by World Health Organization (2005). Treatments were carried out by exposing early fourth instar larvae of *A. aegypti* to various concentrations of *Cyanobacterium* sp. extracellular broth and cellular extract for 24 h, in groups of glass beakers containing 100 mL tap water. Five replicates of 20 larvae each per concentration were run for control trials. The larvae were fed with the usual larval food during these experiments. Larval mortalities were recorded at 24 h post-treatment. Dead larvae were identified when they failed to move after being probed by a needle on the cervical region.

The extracellular culture broth and the cellular extract of *Cyanobacterium* sp. were tested against human prostate cancer epithelial cell lines PC3 and HUVECs. The PC3 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin under 5% CO_2 and 100% humidity at 37 °C. The HUVECs were grown in complete M199 medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin under 5% CO_2 and 100% humidity at 37 °C.

Cells were seeded in a 96-well plate (2000 cells per well) in the absence or presence of either the extracellular culture broth or the cellular extract used in various concentrations, and incubated for 3 days. At the end of the incubation period, cell viability was determined using the crystal violet assay. Briefly, adherent cells were fixed with formaldehyde and stained with 0.5% crystal violet in 25% methanol for 20 min. After gentle rinsing with water, the retained dye was extracted with 30% acetic acid and the absorbance was measured at 590 nm in a spectrophotometric microplate reader.

Additionally, cell viability as a function of redox potential was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay (Mosmann 1983). Briefly, cells were seeded at 2000 cells per well in 96-well tissue culture plates in the absence or presence of either the extra-cellular culture broth or cellular extract at various concentrations for 3 days. MTT stock (5 mg mL^{-1} in PBS) at a volume equal to 10% of the medium was added, and plates were incubated at 37 °C for 4 h. Subsequently, the medium was removed, the cells were washed with PBS pH 7.4 and acidified isopropanol (0.1 mol L^{-1} HCl, 10% Triton-X in isopropanol) was added to a volume equal to the original medium volume to all wells and agitated thoroughly to solubilize the dark blue formazan crystals formed. The absorbance of the solution was read immediately on a microplate reader at a wavelength of 570 nm.

Microscopy

Cell morphology was observed using an optical microscope (Zeiss, Gottingen, Germany) at 1000× magnification, and pictures were taken using a digital camera (Exwave HAD, Sony, Tokyo, Japan) adjusted to the microscope and connected to a computer.

Statistical analysis

All cultures and biochemical analyses were carried out at least in duplicate. The experimental data were treated statistically using OriginPro 9.0.0 SR2©, 1991–2013. The hypothesis of a dose–response effect of cyanobacterial preparations on cancer cells and larvae was tested using linear regression analysis.

Results

Water chemical composition

Data on the chemical composition of the Polichnitos hot spring water (Z. Aggelides, personal communication), indicate a high content of Na^+ (146.28 mmol L^{-1}) and Ca^{2+} (13.77 mmol L^{-1}) and the presence of K^+ (6.55 mmol L^{-1}) and Mg^{2+} (4.77 mmol L^{-1}) in significant concentrations. Cl^- predominated among anions (182.26 mmol L^{-1}) while SO_4^{2-} was also found at 3.90 mmol L^{-1}. Nitrogen compounds were detected, mainly in the form of NH_4^+ and NO_3^- (0.46 and 0.36 mmol L^{-1}, respectively). The water conductivity was found to be 16.4 mS cm^{-1} (at 20 °C) and pH 7.5 (at 20 °C).

Bacterial community of the outflows of Polichnitos hot springs

The 16S rRNA libraries from Polichnitos hot springs covered 16 bacterial operational taxonomic units (OTUs), with the Alphaproteobacteria (25.0%), Gammaproteobacteria (17.9%), Cyanobacteria (21.4%) and Firmicutes (17.9%) being the most dominant. Betaproteobacteria, Bacteroidetes, and Planctomycetes were also detected but to a lesser degree (Fig. 1, Table 1).

Fig. 1 Phylogenetic relationships based on 16S rRNA gene sequences obtained from a microbial consortium developed at Polychnitos hot spring (PHS), Greece. Evolutionary distances were calculated using the method of Jukes and Cantor; the topology was inferred using the neighbor-joining (NJ) method. The numbers at each node represent bootstrap proportions based on 1000 replications for both the NJ (*upper branch*) and the maximum parsimony and the maximum-likelihood (*lower branch*) analysis.

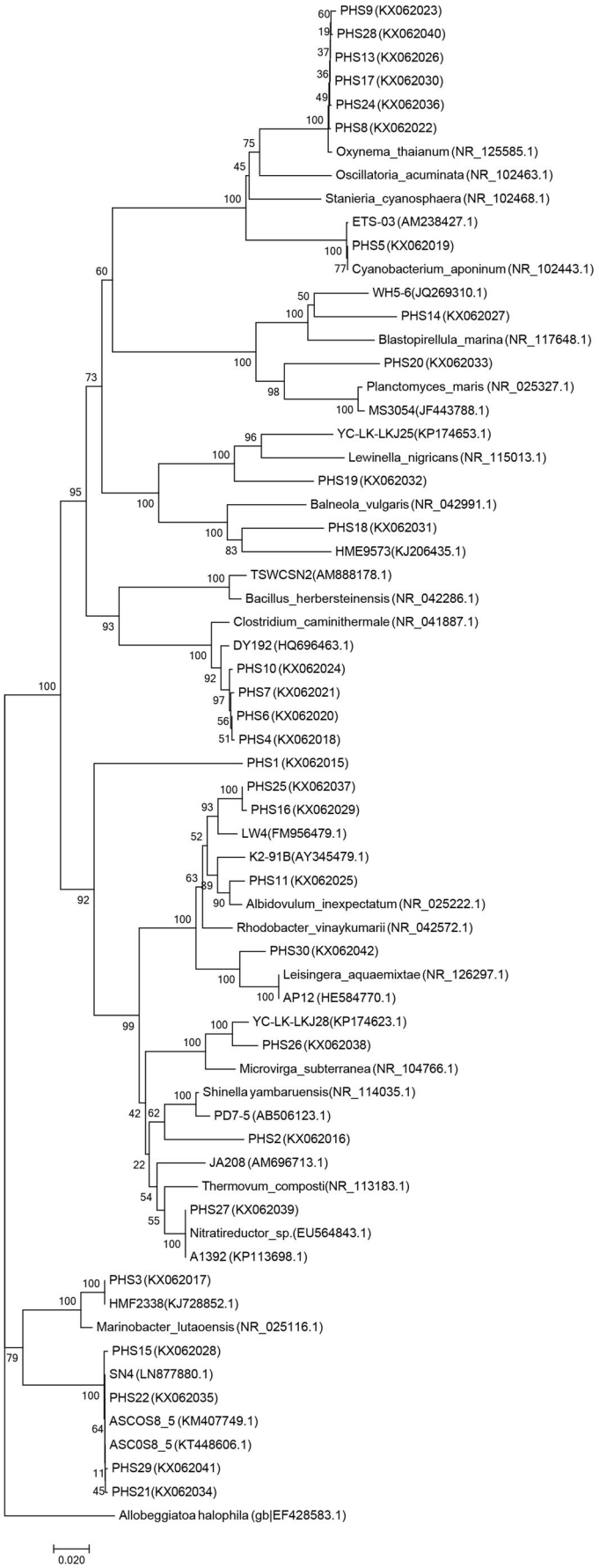
PHS9 (KX062023)
PHS28 (KX062040)
PHS13 (KX062026)
PHS17 (KX062030)
PHS24 (KX062036)
PHS8 (KX062022)
Oxynema_thaianum (NR_125585.1)
Oscillatoria_acuminata (NR_102463.1)
Stanieria_cyanosphaera (NR_102468.1)
ETS-03 (AM238427.1)
PHS5 (KX062019)
Cyanobacterium_aponinum (NR_102443.1)
WH5-6(JQ269310.1)
PHS14 (KX062027)
Blastopirellula_marina (NR_117648.1)
PHS20 (KX062033)
Planctomyces_maris (NR_025327.1)
MS3054(JF443788.1)
YC-LK-LKJ25(KP174653.1)
Lewinella_nigricans (NR_115013.1)
PHS19 (KX062032)
Balneola_vulgaris (NR_042991.1)
PHS18 (KX062031)
HME9573(KJ206435.1)
TSWCSN2(AM888178.1)
Bacillus_herbersteinensis (NR_042286.1)
Clostridium_caminithermale (NR_041887.1)
DY192 (HQ696463.1)
PHS10 (KX062024)
PHS7 (KX062021)
PHS6 (KX062020)
PHS4 (KX062018)
PHS1 (KX062015)
PHS25 (KX062037)
PHS16 (KX062029)
LW4(FM956479.1)
K2-91B(AY345479.1)
PHS11 (KX062025)
Albidovulum_inexpectatum (NR_025222.1)
Rhodobacter_vinaykumarii (NR_042572.1)
PHS30 (KX062042)
Leisingera_aquaemixtae (NR_126297.1)
AP12 (HE584770.1)
YC-LK-LKJ28(KP174623.1)
PHS26 (KX062038)
Microvirga_subterranea (NR_104766.1)
Shinella yambaruensis(NR_114035.1)
PD7-5 (AB506123.1)
PHS2 (KX062016)
JA208 (AM696713.1)
Thermovum_composti(NR_113183.1)
PHS27 (KX062039)
Nitratireductor_sp.(EU564843.1)
A1392 (KP113698.1)
PHS3 (KX062017)
HMF2338(KJ728852.1)
Marinobacter_lutaoensis (NR_025116.1)
PHS15 (KX062028)
SN4 (LN877880.1)
PHS22 (KX062035)
ASCOS8_5 (KM407749.1)
ASC0S8_5 (KT448606.1)
PHS29 (KX062041)
PHS21(KX062034)
Allobeggiatoa halophila (gb|EF428583.1)
0.020

Table 1 Closest phylogenetic relatives of isolates obtained from Polichnitos hot springs (PHS), Greece

Phylogenetic linkage	Number of clones (PHS)	Similarity range (%)	Closest 16S rDNA accession number	
			Non type strains	Type strains
Alphaproteobacteria				
Rhodobium orientis	1	88	AM696713.1	NR_113183.1
Albidovulum inexpectatum	1	95–97	AY345479.1	NR_025222.1
Rhodobacter sp.	2	96–97	FM956479.1	NR_042572.1
Microvirga subterranea	1	95–96	KP174623.1	NR_104766.1
Nitratireductor sp.	1	99–100	KP113698.1	EU564843.1
Phaeobacter daeponensis	1	96	HE584770.1	NR_126297.1
Betaproteobacteria				
Shinella zoogloeoides	1	92	AB506123.1	NR_114035.1
Gammaproteobacteria				
Idiomarina sp.	4	99–100	KT448606.1	KM407749.1
Marinobacter sp.	1	97–100	LN877880.1 KJ728852.1	NR_025116.1
Bacteroidetes/chlorobi group				
Gracilimonas sp.	1	88	KJ206435.1	NR_042991.1
Lewinella nigricans	1	87–88	KP174653.1	NR_115013.1
Planctomycetes				
Planctomyces maris	1	88	JF443788.1	NR_025327.1
Blastopirellula marina	1	88–90	JQ269310.1	NR_117648.1
Cyanobacteria				
Cyanobacterium aponinum	1	99	AM238427.1	NR_102443.1
Phormidium sp.	5	91–99	HQ730084.1	NR_102458.1 NR_102463.1 NR_125585.1
Firmicutes				
Clostridium sp.	5	97–100	HQ696463.1	NR_041887.1

The most dominant genera exhibited sequence homologies 91–99%, 99–100% and 97–100 with *Phormidium* sp. (Cyanobacteria), *Idiomarina* sp. (Gammaproteobacteria) and *Clostridium* sp. (Firmicutes), respectively. A strain showed 99% homologies with *C. aponinun* type strain was also present. Members of the Alphaproteobacteria were closely related to *Rhodobium* sp. *Albidovulum* sp., *Rhodobacter* sp., *Microvigra* sp., *Nitratireductor* sp., and *Phaeobacter* sp. with the *Rhodobium* sp. sequences representing a novel phylogenetic linkage of Rhodobiaceae. Gammaproteobacteria, Betaproteobacteria, and Firmicutes were represented by members of *Idiomarina* sp., *Marinobacter* sp., *Shinella* sp., and *Clostridium* sp. with sequence homologies ranging from 92% to 100%. Moreover, members of the Bacteroidetes and Planctomycetes were represented by sequences of novel phylogenetic linkages exhibiting 87–90% sequence homology (Fig. 1; Table 1). Finally, two bacterial strains were present in the hot spring consortium, having 88% and 90% sequence similarities with unclassified bacteria WH5–6 (JQ269310) and YC-LK-LKJ25 (KP174653), respectively.

Laboratory bioreactor cultures of *Cyanobacterium* sp.

Initially, the microbial consortium was cultivated in repeated batch photobioreactor cultures. At incubation temperature 40–55 °C, strain(s) of *Phormidium* sp. dominated in the reactor, forming filaments (Fig. 2a). However, at temperatures below to 30 °C, a single-celled cyanobacterial strain dominated over the autotrophic microflora (Fig. 2b), while after five cycles the population stabilized, including only the above cyanobacterium and some bacterial cells as well.

The dominated cyanobacterium was identified using morphological, biochemical (i.e., fatty acid composition) and molecular data. Analysis of ten transconjugant plasmids revealed that the *Cyanobacterium* sp. shares a 99% identity with *C. aponinum* type strain. Stock cultures were established in 250 mL conical flasks containing 50 mL ASW medium. The flasks were incubated at $T = 28 \pm 1$ °C under constant illumination of 180 μE m^{-2} s^{-1} in a rotary shaker working at 100 rpm, and were renewed twice a month. Single cyanobacterial colonies were obtained on a modified ASW agar medium.

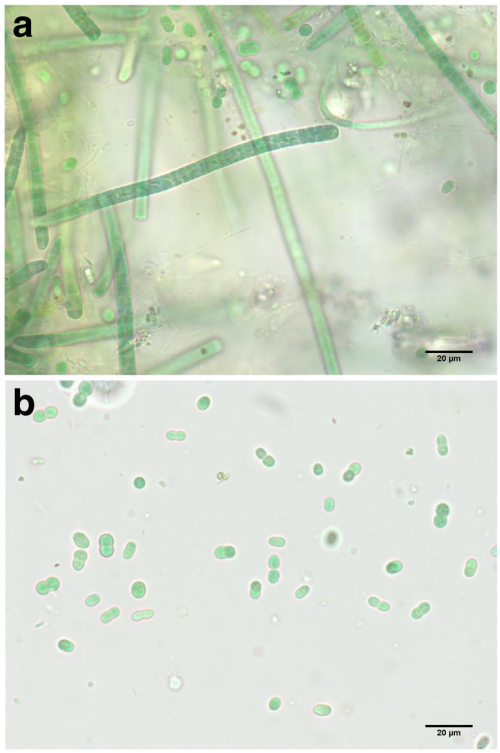

Fig. 2a,b Photomicrograph of batch photobioreactor cultures. **a** *Phormidium* filaments. Cells of *Cyanobacterium* sp. are also present. **b** *Cyanobacterium* sp. cells

Unicyanobacterial, but not aseptic, cultures were established in the photobioreactor, and the growth of the *Cyanobacterium* sp. was studied on ASW in three different photoperiods: 24:0; 16:8 and 12:12 at 21 ± 1 °C. The photoperiod 24:0 (light:dark) resulted the maximum cell growth, and therefore seemed most appropriate for this strain. The strain growing under full illumination in different temperature regimes (i.e., 19–29 °C; Fig. S3) produced 21.88–29.75 × 10^6 cells mL^{-1} (Fig. 3a–c). The growth parameters estimated for each run using Eq. 1 indicated that the maximum growth rate was achieved at 25 °C under full illumination, while the maximum carrying capacity was observed at 29 °C (Table 2). Under these conditions, the *Cyanobacterium* sp. synthesized remarkable amounts of intracellular polysaccharides and proteins, up to 25.52% and 65.93%, w/w, respectively (Table 3). Extracellular proteins were also produced at concentrations ranging from 0.16 g L^{-1} to 0.39 g L^{-1}. Low quantities of lipids (i.e., 1.5–2.5%, w/w), probably structural, were synthesized. Myristic acid (C14:0) was the predominant fatty acid, followed by palmitic (C16:0) and palmitoleic (C16:1) acids. The rather unusual myristoleic acid (C14:1) was also present in non-negligible concentrations (Table 4).

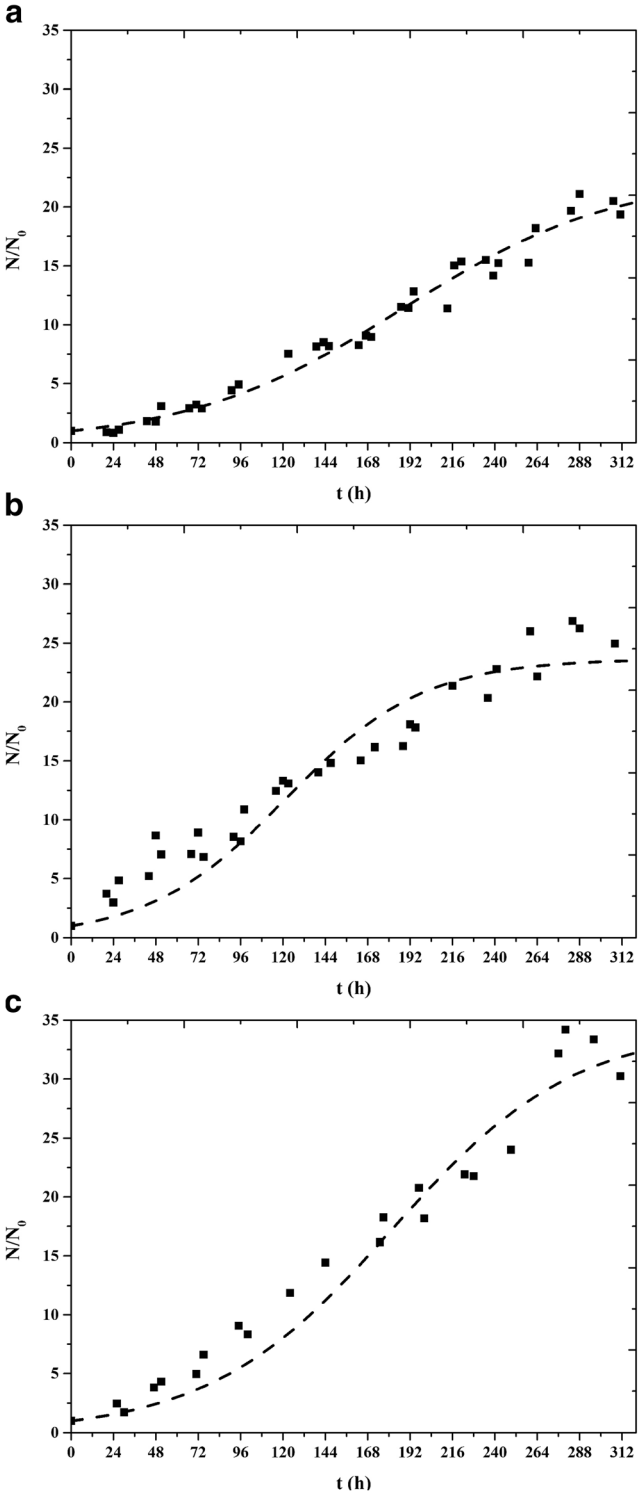

Fig. 3a–c Growth kinetics of *Cyanobacterium* sp. cultivated under full illumination (24:0 light:dark) at different temperatures. **a** 21 °C, **b** 26 °C, **c** 29 °C

Antimicrobial activity of culture broth and cell extract

The *Cyanobacterium* sp. culture broth and the cellular extract were tested against several important human pathogens as described in Materials and methods. However, none of the above mentioned strains showed sensitivity against the *Cyanobacterium* sp. extracts, even when used at very high concentrations (i.e., containing 0.4 mg mL^{-1} and 21.7 mg

Table 2 Growth parameters of *Cyanobacterium* sp. cultivated under full illumination (24:0 light:dark) estimated by the Verhulst model fitted on the experimental data. R_{max} (h^{-1}) represents the maximum specific growth rate of the cyanobacterial cells and K the carrying capacity of system

Temperature (°C)	r_{max} (h^{-1})	K	R^2
21 ± 1	0.016 ± 0.001	22.74 ± 0.94	0.9767
26 ± 1	0.026 ± 0.001	23.63 ± 0.99	0.8953
29 ± 1	0.019 ± 0.001	34.47 ± 1.95	0.9587

mL^{-1} protein for culture broth and the cellular extract, respectively).

Insecticidal activity of culture broth and cell extract against *A. aegypti* larvae

The extracellular broth of the *Cyanobacterium* sp. cultures showed a strong insecticidal activity against *A. aegypti* larvae that was linearly correlated ($R^2 = 0.98$) to dose (Fig. 4a). The LC50 value, estimated by the diagram of mortality versus protein concentration, was 8.77 μg mL^{-1}. The cellular extract of *Cyanobacterium* sp. also demonstrated an insecticidal activity against *A. aegypti* larvae, although this was much weaker than that of the extracellular broth (Fig. 4b). The LC50 value was estimated to be 560.9 μg mL^{-1}.

Anticancer activity of culture broth and cell extract against PC3 cell line

The activity of the culture broth against the PC3 cancer cell line was revealed using both crystal violet and MTT assays. According to the crystal violet assay, the extracellular broth showed a strong toxicity against PC3 cells, which was linearly correlated to dose (Fig. 5a). Similar results were obtained using the MTT assay (Fig. 5b). Moreover, the toxicity of the culture broth against HUVECs, using the MTT assay, was found to be much weaker than that exhibited against the PC3 cell line (Fig. S4). On the contrary, the cellular extract showed no effect against both PC3 cell line and HUVECs.

Table 3 Biomass (x, g l^{-1}) and intracellular sugar (S/x %, w/w) and protein (P/x %, w/w) content of *Cyanobacterium* sp. cultivated under full illumination (24:0 light:dark) at different temperatures

Temperature (°C)	t (h)	x (g L^{-1})	S/x%	P/x%
21 ± 1	144	0.28 ± 0.02	25.52 ± 0.17	30.38 ± 3.29
	311	0.57 ± 0.01	16.85 ± 0.58	63.68 ± 3.08
26 ± 1	144	0.38 ± 0,02	12.18 ± 2,01	60.49 ± 3.86
	308	0.70 ± 0,03	14.63 ± 0,93	65.93 ± 0.15
29 ± 1	144	0.33 ± 0.01	18.49 ± 0.54	42.03 ± 2.74
	311	0.80 ± 0.01	14.42 ± 1.11	43.53 ± 3.71

Discussion

The outflows of Polichnitos hot springs constitute a highly selective habitat, due to their very high temperature and to their chemical composition (containing high amounts of Na^+ and Cl^- and being limiting in P), determining the structure of the microbial community. Previous geochemical studies reported that thermal fluids in three areas (including Polichnitos) of Lesbos Island originate by slow percolation of meteoric waters (Fytikas et al. 1989), while other studies have shown that thermal fluids may result from mixing between meteoric and sea water (Michelot et al. 1993). Independently of the origin of these waters, their chemical composition seems to be stable over time, since the chemical composition reported for Polichnitos hot spring waters both by Fytikas et al. (1989) and Michelot et al. (1993) is very similar to that determined over 20 years later, which indicates a stable physicochemical environment that predisposes a long-term co-existence of different species.

Phormidium sp. Kutzing ex Gomont 1892 (Oscillatoriales, Cyanobacteria), which participates in Polichnitos hot springs with six strains, is a polyphyletic genus, members of which have been isolated from freshwater, marine and terrestrial habitats. Several species of the genus have been also isolated from outflows of hot springs in various parts of the world, such as Greece (Anagnostidis and Pantazidou 1988; Kanellopoulos et al. 2016), the United States (Walter 1976), Thailand (Sompong et al. 2008), New Zealand (Jones et al. 2005), etc. Stromatolites formed in the shallow waters of hot springs are often composed of *Phormidium* sp. with the participation of other cyanobacteria as well (Jones et al. 2005). Some species (e.g., *Ph. bijugatum*, *Ph. molle*, *Ph. papyraceum*, *Ph. uncinatum*, *Ph. autumnale*) are considered to be producers of bioactive compounds having toxic effects on animals, but also on human cancer cell lines, presenting therefore a potential as sources of therapeutic agents (Teneva et al. 2005).

The second autotrophic strain of the population, identified as a *Cyanobacterium* sp. strain with high sequence identity with *C. aponinum* (Chroococcales), which is a newly recognized species first isolated from cyanobacteria mats in Euganean thermal springs (Padua, Italy) (Moro et al. 2007, 2010). The exopolysaccharides of *C. aponinum* stimulate synthesis of the immunosuppressive cytokine IL-10, which can potentially lead to the treatment of psoriasis, in dendritic cells (Gudmundsdottir et al. 2015). On the other hand, it seems that strains of *C. aponinum* were able to accumulate significant lipid quantities, and they are thus of interest to the biofuel industry (Karatay and Dönmez 2011).

Three strains of the phylum Alphaproteobacteria having similarities with *Albidovulum inexpectatum*, *Rhodobacter* sp. and *Phaeobacter daeponensis* represent the Rhodobacteraceae family. *Albidovulum inexpectatum* and *Rhodobacter* sp. can be isolated from various aqueous habitats including hot springs

Table 4 Fatty acid composition of *Cyanobacterium* sp. cultivated under full illumination (24:0 light:dark) at different temperatures. Analyses were performed in duplicate (SD < 5%)

Temperature (°C)	t (h)	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	Others
21 ± 1	144	41.83	3.87	10.68	33.26	2.03	1.01	3.01	4.30
	311	38.03	4.19	7.04	43.86	0.57	3.50	1.61	1.19
26 ± 1	308	42.03	7.87	12.09	33.05	0.86	2.28	0.74	1.08
29 ± 1	144	38.67	5.09	10.88	38.06	0.68	2.35	2.26	2.02
	311	40.21	3.99	11.44	38.89	0.64	1.95	0.69	2.19

and hypersaline environments (Albuquerque et al. 2002; Nuianzina-Boldareva et al. 2014). *Phaeobacter* sp., including *P. daeponensis*, can be found in abundance in marine environments but they have never been found before in hot habitats. Therefore, the presence of *Phaeobacter* sp. genome in the Polichnitos hot spring may indicate mixing of the freshwater of geothermal origin with seawater—a process that occurs in this particular hot spring according to Michelot et al. (1993). The three remaining bacteria presented similarities individually with *Rhodobium orientis* (Rhodobiaceae) (Hiraishi et al. 1995) and *Nitratireductor* sp. (Phyllobacteriaceae), which have been isolated from various environmental samples including hot spring water (Petursdottir et al. 2009) and *Microvigra subterranea* (Methylobacteriaceae) (Kanso and Patel 2003).

A strain having 92% similarity with the type strain of *Shinella zoogloeoides* (An et al. 2006) (formerly *Zoogloea ramigera*) (Rhizobiaceae) was the only representative of the Betaproteobacteria section in the outflows of Polichnitos hot spring. So far there are no references to the presence of *S. zoogloeoides* in hot springs.

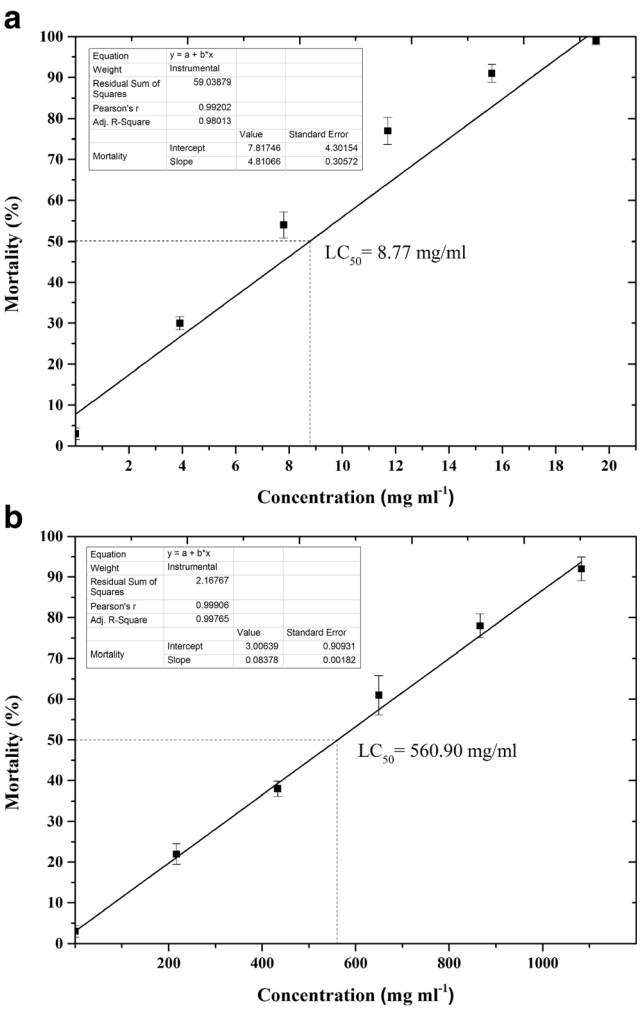

Fig. 4 Insecticidal activity against *Aedes aegypti* larvae of **a** extracellular broth and **b** cellular extract of *Cyanobacterium* sp. cultures

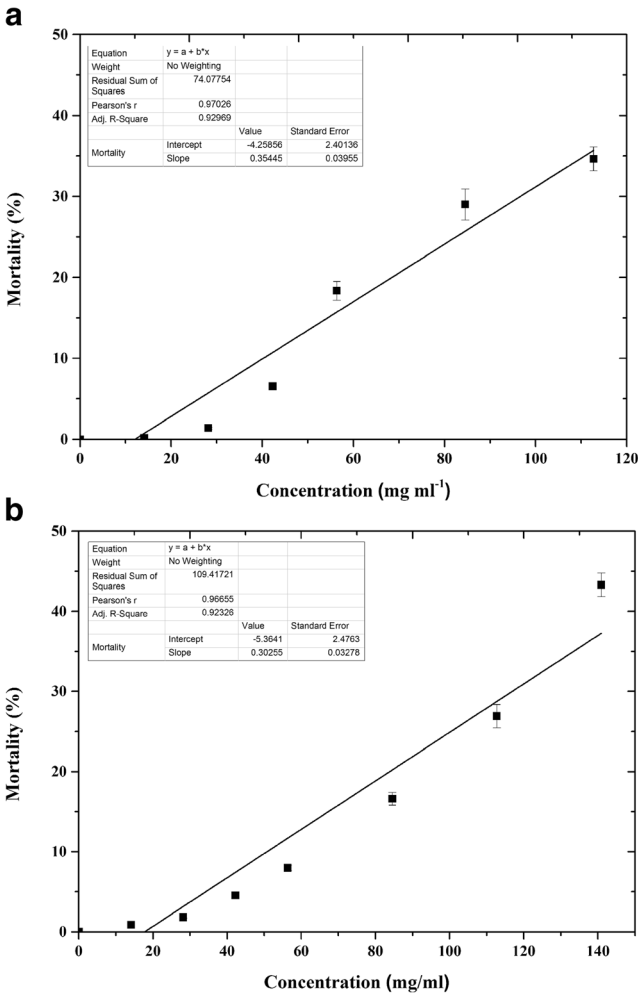

Fig. 5a,b Anticancer activity of culture broth against the cell line PC3. Cell viability was determined using two different assays: **a** crystal violet, **b** MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- dimethyltetrazolium bromide)

Gammaproteobacteria were represented in Polichnitos hot springs by two strains, similar by 99% with *Idiomarina* sp., and one strain, similar by 100% with *Marinobacter* sp. Species of *Idiomarina* sp., i.e. *I. abyssalis* and *I. zobellii*, were isolated initially from seawater samples collected from the north-western Pacific Ocean at a depth of 4000–5000 m (Ivanova et al. 2000). The presence of *Idiomarina* sp. genome in the Polichnitos hot spring may reflect mixing of freshwater of geothermal origin with seawater. *Marinobacter lutaoensis* has been isolated from a hot spring on the coast of Lutao, Taiwan (Shieh et al. 2003).

Two strains presenting 88% and 87–88% similarities with *Gracilimonas* sp. and *Lewinella nigricans*, respectively, may be considered as representatives of the Bacteroidetes/chlorobi group. The participation of these genera in hot spring microbial communities has not been reported so far. Planctomycetes group was represented by two strains having 88% and 88–90% sequence similarities with *Planctomyces maris* and *Blastopirellula marina*, respectively. All Planctomycetes were originally isolated from aquatic habitats, including sewage treatment plants carrying out anaerobic ammonium oxidation (ANAMMOX). Finally, one strain of the Firmicutes group having 97–100% similarity with *Clostridium* sp. was present. *Clostridium* sp. members are obligate anaerobes, and their presence in hot springs has been previously reported (Jin et al. 1988; Chan et al. 2015).

Research on microbial diversity in hot springs is of high interest to basic science and ecology, and may aid the discovery of new biological materials for exceptional biotechnological applications. The most famous thermophilic organism is *Thermus aquaticus* (Brock and Freeze 1969), with *Taq* polymerase having become a key reagent in PCR. Strains of *T. aquaticus* have been isolated from a variety of thermal springs in Yellowstone National Park and from a thermal spring in California (Brock and Freeze 1969). Since the beginning of systematic investigations by Brock in Yellowstone National Park in the early 1960s, a number of biotechnological applications have been arisen using thermophilic organisms. Among them, photosynthetic microorganisms are considered to be of high interest for environmental, biotechnological and medical applications (Abed et al. 2009; Bellou et al. 2014; Economou et al. 2015; Vijayakumar and Menakha 2015; Bellou et al. 2016).

In this report, we provide evidence that the extracellular broth of a *Cyanobacterium* sp. strain, member of the bacterial community of a Polichnitos hot spring, possesses a strong insecticidal activity against larvae of *A. aegypti*. Moreover, the extracellular broth showed a significant toxicity against the cancer cell line PC3 while its toxicity against HUVECs was low. However, no antibacterial activity of either the extracellular broth or the cellular extract of this strain has been observed against Gram negative and Gram positive human pathogens.

The finding that the extracellular culture broth of the newly isolated strain of *Cyanobacterium* sp. demonstrated insecticidal activity against *A. aegypti* is of high interest since this mosquito is the principal vector of several viral diseases and human parasites (protozoa, nematodes) in many parts of the world. The control of *A. aegypti* is currently performed by application of chemical insecticides, such as organochlorines, organophosphates, carbamates and pyrethroids, which are toxic for numerous organisms, while mosquito resistance has been reported in a number of cases where chemical insecticides are repeatedly used. Alternatively, natural bioactive substances, such as plant essential oils (Magalhães et al. 2010; Aguiar et al. 2015), fatty acids (Harada et al. 2000), and cyanobacterial extracts (Berry et al. 2008), have been tested against *A. aegypti* larvae. Specifically, extracts of cyanobacteria species belonging to *Microcystis*, *Oscillatoria*, *Nodularia*, *Nostoc* and *Anabaena*, have been considered as bioactive agents against *A. aegypti* (Kiviranta et al. 1993) but, with few exceptions (Nassar et al. 1999; Rao et al. 1999), their activity was rather associated with the presence of hepatotoxic and neurotoxic compounds (i.e., microcystins, anatoxin-a), which limit potential for large-scale applications. However *Cyanobacterium* species have not been reported to be toxin producers, while only the extracellular broth of *Cyanobacterium* sp. reported in this study demonstrated larvicidal activity, which enhances the potential for large scale applications. The spreading of *Cyanobacterium* sp. in natural habitat of *A. aegypti*, and its installation, which may be achievable judging from the ability of this strain to dominate over *Phormidium* sp. in bioreactor cultures, may provide an efficient and environmental friendly way to target mosquitos.

Besides larvicidal activity, the *Cyanobacterium* sp. culture broth demonstrated a significant anti-cancer activity against the PC3 cancer cell line. Many cyanobacterial species/strains belonging to genera *Calothrix*, *Lyngbya*, *Phormidium*, *Symploca*, *Oscillatoria*, *Nostoc* and so on have been reported as sources of bioactive compounds, such as cyclic and linear peptides, lipopeptides, fatty acid amides and glycosylated swinholide, with potential anticancer properties (Costa et al. 2012). According to Tan (2010), cyanobacterial compounds target essential proteins of the eukaryotic cytoskeleton, such as tubulin and actin microfilaments, provoking cell damage. On the other hand, *C. aponimum*, having 99% similarity with the *Cyanobacterium* sp. studied in this paper, was able to produce exopolysaccharides having in vitro immunomodulatory effects (Gudmundsdottir et al. 2015). Furthermore, the current paper is the first report demonstrating the ability of a *Cyanobacterium* sp. strain, similar to *C. aponimum*, to produce extracellular compounds having a cytotoxic activity against PC3 cancer cells but not against HUVECs. Under the specific growth conditions of the *Cyanobacterium* sp,. extracellular polysaccharides were not detected in the growth medium and therefore the biological activity of the extracellular broth should be attributed to the presence of peptides detected at concentrations 0.16 g/L to 0.39 g/L.

In conclusion, most of the bacterial strains detected in the outflows of a Polichnitos hot spring are related with general

species commonly isolated from hot springs in various parts of the world. However, some strains, i.e., those closely related to *Phaeobacter* sp. and *Idiomarina* sp., have never been found before in hot habitats. Instead, they have been found in abundance in marine environments, and therefore the presence of their genome in the Polichnitos hot spring may indicate mixing of freshwater of geothermal origin with seawater—a process that probably occurs in this particular hot spring. A *Cyanobacterium* sp. strain cultivated in a laboratory photo-bioreactor produced bioactive compounds, probably peptides, having a significant activity against *A. aegypti* larvae and against the cancer cell line PC3.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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