

## **Culturable bacterial flora associated with the dinoflagellate green *Noctiluca miliaris* during active and declining bloom phases in the Northern Arabian Sea**

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### **Abstract**

A massive algal bloom of the dinoflagellate *Noctiluca miliaris* (green) was located in the Northern Arabian Sea by IRS-P4-2 (OCM-II) for microbiological studies, during two consecutive cruises of Feb-Mar 2009. Culturable bacterial load during bloom were ~2-3 fold higher in comparison to non-bloom waters and ranged from  $3.20 \times 10^5$  -  $6.84 \times 10^5$  CFU ml<sup>-1</sup>. An analysis of the dominant heterotrophs associated with *Noctiluca* bloom resulted in phylogenetic and a detailed metabolic characterization of 70 bacterial isolates from an overlapping active and declining bloom phase location near north-central Arabian Sea. The active phase flora was dominated by gram-positive forms (70.59%), a majority of which belonged to *Bacillus* (35.29%) of *Firmicutes*. As the bloom declined, Gram-negative forms (61.11%) emerged dominant and these belonged to a diverse  $\gamma$ -proteobacterial population consisting of *Shewanella* (16.67%) and equal fractions of a *Cobetia-Pseudomonas-Psychrobacter-Halomonas* population (36.11%). An Unifrac based PCA of partial 16SrDNA sequences showed significant differences among the active and declining phase flora and also with reported endocytic flora of *Noctiluca* (red). A Non-parametric multidimensional (NMDS) scaling of antibiogram helped differentiation among closely related strains. The organic matter synthesized by *Noctiluca miliaris* appears to be quickly utilized and remineralized as seen from the high efficiency of isolates to metabolize various complex and simple C/N substrates such as carbohydrates, proteins/amino-acids, lipids, sulphide production from organic matter and solubilize phosphates. The ability of a large fraction of these strains (50%- 41.67%) to further aerobically denitrify indicates their potential for Nitrogen removal from these high-organic micro-niches of the *Noctiluca* bloom in the Arabian Sea, also known for high denitrification activity. The results indicate that culturable euphotic bacterial associates of *Noctiluca* are likely to play a critical role in the biogeochemical ramifications of these unique seasonally emerging tropical open-water blooms of the Northern Arabian Sea.

**Key Words:** *Noctiluca miliaris* (green) bloom, Northern Arabian Sea, culturable Bacteria, 16SrDNA diversity, metabolism

## Introduction

*Noctiluca miliaris* Suriray 1836 (synonym *N. scintillans* Macartney 1810) are large sized dinoflagellates (dia. ~ 400-1200 µm), and are an important bloom forming species [24]. They have two distinct physiological forms: (i) The heterotrophic red *Noctiluca* and (ii) the mixotrophic green *Noctiluca* containing endosymbiotic photosynthetic prasinophytes, the *Pedinomonas noctilucae* (dia. ~5µm) [13]. Being a high-biomass harmful algae [16], the ability of both these physiotypes to form mucilagenous blooms can cause fish-kills by depleting oxygen or accumulation of ammonia [56]. Distributed globally, blooms of *Noctiluca* also show an environmental preference as orange-red tides of *Noctiluca*(red) are more restricted to cooler (10-25 °C), temperate and sub-tropical coasts, whereas, the green-tides of *Noctiluca* are favored in the warmer waters of South-east Asia (25-30°C) [24].

The Arabian Sea is however, an important habitat of both these forms of *Noctiluca*. While sporadic blooms of red and green forms are reported from coastal India, Pakistan as well as Oman waters [24], a large-scale bloom of *Noctiluca* (green) has been recently observed to emerge across the open-waters of Northern Arabian Sea (NAS), during the Northeast winter-monsoon (NEM) period of Jan-Mar [20, 47]. The bloom is favored by the cool-dry Northeast Monsoon (NEM) of Dec-Mar, which absorbs heat from surface waters of NAS to drive convective-mixing and replenish 'new' nutrients from the base of thermocline to the surface waters [11]. Although, such winter-cooling mechanisms, which lead to supply of nutrients in euphotic zone have been known to traditionally favor productivity of diatoms [44, 69], this systemic shift to a high biomass *Noctiluca* (green) bloom covering the entire northern basin of the Arabian Sea remains unexplained [20]. The ramifications of these events can be serious as the Arabian Sea also harbors an extensive meso-pelagic oxygen-minimum zone, with bacteria playing an important role in nitrogen removal through contrasting mechanisms of denitrification or 'annamox' [31, 67].

The emergence and seasonal expansion of such eutrophic organic levels created by *Noctiluca* (green), therefore, calls for an examination of the dominant heterotrophic bacterial associates, which play a critical biogeochemical role in the water-column [4]. Earlier studies of the Arabian Sea underlines the importance of bacteria in the euphotic zone [12] and a strong microbial-loop operating following the winter monsoon period which can stimulate/sustain the zooplankton biomass [44]. Phylogenetic composition of these bacteria during two consecutive NEM of Jan and Dec 1995 shows that the euphotic flora of NAS consisted mostly of the SAR-11 clade of oligotrophic unculturable  $\alpha$ -*proteobacteria*, complete absence of any  $\gamma$ -*proteobacteria* and *Cytophaga-Flavobacteria-Bacteroidetes* as well as unusual detection of Magnetotactic bacterial clones in pelagic waters [59].

The bacterial flora developing with algal-blooms are known to be highly specific [60]. The resulting community can largely determine the fate of bloom organic matter: from a more general perspective of extracellular hydrolysis of bloom based dissolved organic matter and solubilization of particulate/detrital organic matter [3], to a more specific role in being toxic to bloom forming algae [50] as well as promoting bloom formation itself through photo-sensitive siderophores [2] or even promoting cyst formations [51].

Earlier, bacteriological examination of red *Noctiluca* reveals a high predominance of endocytic  $\gamma$ -*proteobacterial* forms [54, 62] some of which are also toxic to  $\text{Na}^+$  channels [35]. Bacteria algicidal to green *Noctiluca* have been recently known from an aquaculture pond in Gulf of Thailand [36]. Our preliminary assessment of this bloom in NAS during the spring – inter monsoon cruise of March 2007, shows a ~2-3 fold higher culturable bacterial load in bloom waters, as well as presence of bacteria that grew over a range of low and high pH, nutrients and salinity indicating growth of osmotically flexible forms in the bloom eco-niche[6].

The present study is an attempt to dissect the phylogenetic composition of the dominant culturable bacteria associated with green *Noctiluca miliaris* during its unique open-ocean bloom in the NAS. The succession/shift in the bacterial flora at a location during two consecutive cruises of Feb-Mar 2009 in NAS is reported and the paper is discussed in light of their detailed metabolic potentials and relation to water-column characteristics.

## Methods

### Algal Bloom description

Massive blooms of the dinoflagellate green *Noctiluca miliaris* were observed in the open-waters of the northeastern Arabian Sea (NAS) during two consecutive ship-cruises from Feb-Mar 2009: ORV *Sagar Kanya* 256 (9<sup>th</sup> - 23<sup>rd</sup> Feb) and FORV *Sagar Sampada* 263 (1<sup>st</sup> – 14<sup>th</sup> Mar) (Fig. 1).

On 17<sup>th</sup> Feb-09, bloom was sighted at 20.986 N and 66.186 E nearing north-central Arabian Sea. It was of a thick soup like consistency, highly mucilaginous with a musky-odor. The bloom gave a deep-green coloration to the open-ocean waters as actively buoyant *Noctiluca* (green) cells remained well distributed in the water column. On 2<sup>nd</sup> Mar-09 during the cruise SS263, the bloom at this same location was declining as cells of green *Noctiluca* formed massive clumps of aggregates at the surface (Fig. 1a). Intact *Noctiluca* (green) counts decreased from 9600 cells  $\text{L}^{-1}$  on 17<sup>th</sup> Feb-09 to 80 cells  $\text{L}^{-1}$  on 2<sup>nd</sup> Mar 09, within a span of 13 days, confirming the demise of the bloom. This overlapping location (St-O1) ideally represented two distinct growth phases of the bloom, hereafter referred to as the 'active' and the 'declining' phase throughout the study.

### Water-column characteristics

*In situ* wind-speed and direction were recorded from the automatic weather station (AWS) installed onboard during the cruises of Feb-Mar, 2009. At bloom and non-bloom stations (Fig. 1), the depth of euphotic zone was established using a Secchi-disk and water-column samples were collected using 1.7-5L Niskin sampling bottles mounted onto Sea Bird Electronics 911-plus® Conductivity-Temperature-Density (CTD) profiler rosette. Microscopic cell counts of *Noctiluca miliaris* and HPLC estimations of Chlorophyll *a* (Chl *a*) were done as exactly detailed by Parab et al.[57]. Dissolved oxygen was estimated by a modified Winkler titration using Metrohom Dosimat Titrator onboard [21]. pH was recorded using an automated pH meter (Lab India). Dissolved inorganic nutrients (nitrate, nitrite, phosphate and silicate) were analyzed using standard procedures using a Skalar Autoanalyzer [21].

Rates of Photosynthetic fixation of organic carbon were estimated onboard using the  $\text{H}^{14}\text{CO}_3$  ( $5\mu\text{Ci}$ ) tracer method as detailed by Parab et al. [58]. Primary-productivity in terms of  $\text{mgC m}^{-3} \text{day}^{-1}$  was determined using a Wallac Scintillation counter (Perkin Elmer, model no. 1409) after quench corrections [41].

Chromophoric-dissolved organic matter (C-DOM) was estimated using a Perkin Elmer  $\lambda 40$  UV/Vis spectrophotometer using a quartz cuvette of 10 cm path-length as exactly described earlier by Basu et al. [5]. Surface and average C-DOM absorption coefficients at 300 nm was calculated as a proxy of the dissolved organic load during the bloom [55].

### **Collection of bloom for bacteriological study**

Multiple sub-samples of surface waters containing the *Noctiluca miliaris* bloom were collected using Niskin water sampler at St-O1. Samples were also collected from other observable bloom waters as well during both the phases (Fig. 1b). *Noctiluca* (green) cells were screened on a 100  $\mu\text{m}$  nytex mesh from 1-2 L of water-samples and the thick green biomass was collected in sterile 50 ml conical centrifuge tubes for further plating (Tarsons, India). Non-bloom surface water samples below  $19^\circ\text{N}$  at stations SS1 and SK-10 (Fig. 1b) were also collected for bacteriological analysis.

### **Isolation of bacteria**

Triplicate sub-samples of bloom-biomass were serially diluted in filtered-sterilized sea-water prepared from the bloom itself and plated on: (a) Zobell's marine agar 2216E (ZB) (b) 1/10 strength ZB (ZB1/10) and (c) Sea-water agar (SWA) [Peptone  $2.5 \text{ gL}^{-1}$ ; Yeast Extract  $1.5 \text{ gL}^{-1}$ ; Filtered Sea-water 50% (v/v)]. Plates were incubated at RT  $25^\circ\text{C}$  for 72-120 hours, but incubations were also prolonged upto two weeks to retrieve any slow growing types. 70 well isolated colonies (34 isolates from active and 36 isolates from declining bloom phase) were selected from the  $10^{-5}$  dilution to represent the dominant growing bacteria at St-O1 (Table S1). Isolate morphotypes were recorded, further purified by streaking and maintained on their respective medium of isolation. All active phase isolates were coded with the prefix 'SK256' and declining phase isolates as 'SS263'.

### **Phenotypic characterization**

All isolates were subjected to a battery of 103 phenotypic/metabolic tests. Key phenotypic characters of cell morphology, gram's reaction, endospore staining, catalase, oxidase, anaerobic growth in thioglycollate broth, Nitrate reduction and motility were examined as described previously [5, 61]. Tests for citrate utilization, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, urease and  $\text{H}_2\text{S}$  production were determined using KB-002 biochemical-kit of Himedia Lab Pvt Ltd., India. Acid from the carbohydrates D-Glucose, D-Adonitol, D-Lactose, D-Arabinose, L-Arabinose, D-sorbitol, D-maltose, D-fructose, D-Galactose, D-Raffinose, D-Trehalose, D-Melibiose, Sucrose, D-mannose, inulin, sodium gluconate, D-Salicin, D-Dulcitol, D-glycerol, D-inositol, D-sorbitol, D-Mannitol, D-Arabitol, D-Erythritol,  $\alpha$ -methyl-d-glucoside, D-Rhamnose, D-Cellobiose, D-xylitol and D-Sorbose were determined using KB-009 kit of Himedia labs Pvt Ltd. , India following manufacturer's instructions.

Utilization of carbohydrates and amino-acids were determined by suitably modifying a micro-titre well plate method incorporating the respiratory dye tetrazolium chloride (TTC) as indicator. 150 $\mu\text{l}$  of a mineral salts

basal media (containing in grams per liter:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  - 20g;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  - 13g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  - 1g; KCl - 4g;  $\text{NaHCO}_3$  - 0.2 g;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  - 0.005g;  $\text{KH}_2\text{PO}_4$  - 0.5 g;  $\text{NH}_4\text{Cl}$  - 2g; pH7) was dispensed into the micro-titre wells and filter-sterilized (sterile 0.2 micron hydrophilic HDPE syringe filters) stocks of carbohydrates (D-glucose, D-fructose, D-ribose, D-mannose, D-xylose, D-maltose and D-sucrose), carboxylic acids (succinate, citrate), and complex organics (starch, casein, tween-80 and carboxy methyl cellulose) were added at 0.5% v/v final concentrations to the basal medium. For studying utilization of amino-acids as sole source of carbon and Nitrogen, the  $\text{NH}_4\text{Cl}$  was excluded from basal media and 2mM amino-acids (L-alanine, L-methionine, L-leucine, L-isoleucine, L-threonine, L-lysine, L-tryptophan, L-serine, L-phenylalanine, L-histidine) were added to each well. Micro-titre plates were inoculated with 2% culture suspension in 1% sterile saline with an OD of 0.8-0.9 at 600 nm. All micro-titre plates were incubated for 36 hours and 20 $\mu\text{l}$  of 1% TTC dye (Hi-media, India) were added to each well and incubation continued overnight. Positive wells showed pinkish-red color against negative control wells without any added substrates. Positive controls of the same tests using 5ml volume of broth were run randomly to verify the results using standard protocol [61].

Denitrifying potential among the isolates were screened on a Giltay-Nitrite (GN) medium [48]. Isolates were inoculated in GN broth and left shaking at 220 rpm and 25°C to provide adequate aeration. The change in color of GN broth from blue to green with gas collected in Durham's tubes indicated denitrifying potential among the isolates. Growth was also checked using 0.2% Acetamide as sole carbon and nitrogen source on Verstraete and Alexander broth (VA) [48].

The ability of isolates to produce extracellular enzymes amylase, protease, lipase, cellulase and phosphatase were determined by spotting cultures on prepared plates of starch agar, casein agar, tributyrin agar, CMC (carboxymethyl cellulose) agar and Pikovskaya's media [61], respectively. All plates were incubated at 25°C. Zones of amylase and protease were observed by flooding starch plates with iodine solution and casein plates with 3% tricarboxylic acid. Cellulose degradation were checked after a week's incubation by flooding plates with 1% Congo-red for 15 mins and then destaining the agar with 1.5M NaCl solution. Lipase and phosphatase activity could be determined by visible zones of clearance against turbid background of media. Liquefaction of gelatin stabs at 4°C indicated gelatinase activity [61].

Resistance of isolates to 18 different antibiotics were checked by spreading a growing suspension of each culture on Mueller-Hinton agar plates (Himedia-India) with 3.5 % w/v NaCl over which antibiotic discs (Ampicillin - 10mcg; Ciprofloxacin - 5 mcg; Chloramphenicol - 30 mcg; Clindamycin - 2 mcg; Erythromycin - 15mcg; Furazolidone - 50 mcg; Gentamycin - 10 mcg; Kanamycin - 30 mcg; Methicillin - 30 mcg; Norfloxacin - 10 mcg; Novobiocin - 5 mcg; Neomycin - 30 mcg; Nalidixic acid - 30 mcg; Oxytetracycline - 30 mcg; Polymyxin B - 30 mcg; Streptomycin - 300 mcg; Tetracycline - 30 mcg and Penicillin-G - 50U) manufactured by Hi media Labs, India were placed. Appearance of zones of inhibition were recorded after 36-48 hours of incubation at 25°C and referred using the Kirby-Bauer susceptibility chart to determine resistance.

## **Molecular characterization**

A single colony of each purified cultures were inoculated in Zobell's marine broth 2216E and genomic DNA was extracted from a 24 hour growing culture (OD ~1) using the Gen-Elute bacterial genomic DNA spin extraction kit NA2110 (Sigma-Aldrich), following manufacturer's instructions. Genomic DNA was amplified using the universal bacterial primers 27f (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' ACGGCTACCTTGTTACGACTT 3') [22]. 50µl PCR reaction mixture was prepared with 25 µl of 2X red-dye PCR master-mix (Genei, India), 20 µl PCR-grade water (Genei, India), 10pM each of primer (8f and 1522r) and 10-30 ng of DNA template. The thermocycler (Takara-Japan) was programmed to denaturation at 95 °C for 3 minutes, 35 cycles at 95 °C for 1 min, primer annealing at 48 °C for 1 min and strand extension at 72 °C for a brief period of 90s. This was followed by a final elongation step at 72 °C for 5 mins. The amplified DNA was purified using the PCR-purification kit (Genei, India). 16SrDNA amplicons corresponding to ~ 1.5 kb were then bi-directionally sequenced using the Big-dye terminator sequencer method (Applied Biosystems) in Bangalore Genei, India. The reactions were analyzed through automated sequencer (ABI-3730 DNA analyser) and electropherograms were imported in Chromas-pro, ver 1.5.

## **16SrDNA Sequence Analysis**

Partial sequences (300 - 1000 bp) were obtained for all 70 isolates. Among these sequencing of 18 isolates were repeated to obtain near-full-length (≥1300 bp) sequences to ensure procedural reliability and quality-control. All sequence electropherograms were manually curated to remove few extra nucleotides flanking the sequences, checked for their query coverage (~100%) using BLASTn and then uploaded in the Ribosomal Database Project [9] for alignment using 'infernai' secondary structure alignment of RDP-10.

## **Bacterial identification**

To reach the best possible generic/species level taxonomic assignments and select the closest phylotype/s to our sequences, we followed a common frame-work based upon similarity/dissimilarity in 16SrDNA homology and phenotypic charactersitics. Based on this approach, isolates were referred reliably to 'species' level in cases of >97 % 16SrDNA sequence homology to a single nearest described Type strain in both RDP-II and EZtaxon-e [38] database with supporting phenotypic characteristics. In cases where 16SrDNA homology is >97% but key phenotypic characters were dissimilar, isolates were referred to genus level. At >97% homology, isolates were further referred to 'groups' of genus when generic level distinction based on 16SrDNA and phenotypic characters were unreliable. 16SrDNA sequences showing <97% match with nearest Type strains were referred to as unclassified family/genus level for further characterization.

## **Phylogenetic and community analysis**

A neighbor-joining phylogenetic tree was constructed after multiple-alignment of only partial sequences using Clustal-X and MEGA-5. The UniFrac distance metric [43] web-based test was run to compare whether 16SrDNA sequences representing active and declining bloom phases differed significantly. Further, partial sequences of the cultured red *Noctiluca* endocytic bacterial community reported from Helgoland roads, North Sea

[62] were also incorporated in the phylogenetic tree to examine phylotype differences with our present study and represented as a UNIFRAC computed PCA-biplot. The diversity of bacterial 'genus' occurring during the bloom phases were compared from the Shannon-wiener diversity index ( $H'$ ), calculated using Primer-6. Strain level proximity/dissimilarity of isolates showing similar biochemical characteristics were deciphered based on a Non-metric Multidimensional scaling of zone –sizes using NMDS module of XLStat<sup>2012</sup> (Addinsoft).

### **Accession number**

Sequences submitted to Gen-Bank were assigned accession numbers JX429828-JX429861 (Active bloom) and JX429792 – JX429827 (Declining bloom).

## **Results**

### **Water-column characteristics**

The water column characteristics conditioning the active and declining bloom at St-O1 are given in Table 1. The active bloom was characterized by  $\sim 0.4 \text{ m sec}^{-1}$  higher wind-speed in comparison to the declining phase of Mar-09. At this overlapping station, sea-surface temperature during active bloom were  $\sim 2.5^{\circ}\text{C}$  cooler in comparison to the declining phase. Surface dissolved oxygen and pH decreased from  $3.06 \text{ mL}^{-1}$  and 8.3 in the active bloom to  $2.71 \text{ mL}^{-1}$  and 8.11 as the during declining bloom, respectively. Dissolved nutrients in water-column showed unusually higher Nitrite concentration of  $0.77 \pm 0.13 \text{ }\mu\text{M}$  during active phase which decreased to  $0.12 \pm 0.05 \text{ }\mu\text{M}$  during bloom decline. Average Nitrate concentrations in water column however, substantially increased from  $0.79 \pm 0.27 \text{ }\mu\text{M}$  during active bloom to  $6.86 \pm 7.78 \text{ }\mu\text{M}$  in the declining bloom. Dissolved inorganic phosphate at surface during active bloom was as high as  $5.23 \text{ }\mu\text{M}$  and this decreased to  $0.64 \text{ }\mu\text{M}$  during bloom decline. However, water column average remained similar and varied from  $1.82 - 1.29 \text{ }\mu\text{M}$ . Average euphotic column silicate was also  $\sim 3$ times higher during the active bloom ( $14.8 \pm 3.58 \text{ }\mu\text{M}$ ) in comparison to the declining bloom ( $5.56 \pm 5.09 \text{ }\mu\text{M}$ ).

Significantly higher organic matter production rates by the active bloom of Feb-09 was indicated from both primary-productivity estimates and C-DOM absorption coefficient. The active bloom was characterized by a  $\sim 3$  times higher surface primary-productivity rate of  $1426.93 \text{ mgC m}^{-3} \text{ day}^{-1}$  in comparison to the declining bloom. Average C-DOM Ac300 recorded in the euphotic zone was also  $\sim 3$  times higher and decreased from  $2.48 \pm 1.45 \text{ m}^{-1}$  during active phase to  $0.81 \pm 0.32 \text{ m}^{-1}$  as the bloom declined.

### **Bacteria load**

Cultivable bacterial counts during active and declining phases of *Noctiluca* (green) bloom did not differ significantly (Fig. 2a). Culturable bacterial load at the overlapping station St-O1 was highest among all other stations during both the active and declining bloom phases. Plate counts at this location during active bloom varied from  $6.84 \times 10^5 \text{ CFU ml}^{-1}$  on ZB,  $5.21 \times 10^5 \text{ CFU ml}^{-1}$  on ZB1/10 and  $5.2 \times 10^5 \text{ CFU ml}^{-1}$  on SWA medium. During declining bloom a culturable load of  $6.73 \times 10^5 \text{ CFU ml}^{-1}$  obtained on ZB was similar to that of active phase. Plate counts however, slightly increased to  $5.51 \times 10^5 \text{ CFU ml}^{-1}$  on ZB1/10 and  $5.42 \times 10^5 \text{ CFU ml}^{-1}$  on SWA during the declining phase. Counts at other active bloom waters of Feb-09 (Fig. 1) varied from  $4.73 \times 10^4 - 5.1 \times 10^5 \text{ CFU}$

ml<sup>-1</sup> on ZB, 3.23x10<sup>3</sup> – 5.3x10<sup>5</sup> CFU ml<sup>-1</sup> on ZB1/10 and 8.7 x10<sup>4</sup> - 3.95x10<sup>5</sup> - CFU ml<sup>-1</sup> on SWA. As the bloom declined by Mar-09 plate counts varied from 3.73x10<sup>4</sup> – 6.7x10<sup>5</sup> CFU ml<sup>-1</sup> on ZB, 2.96x10<sup>4</sup> – 5x10<sup>5</sup> CFU ml<sup>-1</sup> on ZB1/10 and 3.97x10<sup>4</sup> – 4.9x10<sup>5</sup> CFU ml<sup>-1</sup> (Fig. 2a). In comparison, a significantly ~2-3-fold lower culturable count was obtained at the non-bloom locations which ranged 2.1x10<sup>3</sup> – 1.1x10<sup>3</sup> CFU ml<sup>-1</sup> on ZB, 5.1x10<sup>3</sup> – 5.8x10<sup>3</sup> CFU ml<sup>-1</sup> ZB1/10 and 5.65x10<sup>3</sup> – 4.99x10<sup>2</sup> CFU ml<sup>-1</sup> on SWA (Fig. 2a).

### **Description of flora: active and declining phase**

Of the total 70 bacterial isolates retrieved, several were pigmented, which increased from 25% in the active bloom to 38.7% in the declining bloom. Pigmentation varied from yellow (SK256-34; SK256-15; SK256-28; SK256-35; SK256-37; SK256-N8; SS263-17), pink (SK256-5), orange (SK256-17; SS263-26; SS263-4; SS263-2; SS263-9; SS263-14; SS263-28; SS263-29; SS263-30; SS263-33), brick-red (SS263-19); reddish-pink (SS263-23) to cream (SK256-8; SK256-12; SK256-16; SK256-29; SS263-5; SS263-10; SS263-12; SS263-24; SS263-31) and the rest were white. While the dominant isolates during the 'active phase were Gram-positive (70.59%), Gram-negative forms (61.11%) increased in the declining phase. Majority of these isolates were motile during both the bloom phases (Table 5).

### **Phylogenetic composition**

Based on 16SrDNA sequence relatedness, the taxonomic assignments of isolates were determined (Table 2 and 3) with supporting phenotypic characteristics (Fig. 3). The neighbor-joining boot-strap consensus tree grouped all 70 isolates, belonging to 21 classified genera and 5 unclassified genera under 4 different phylum of *Firmicutes*, *Actinobacteria*, *γ-proteobacteria* and *α-proteobacteria*. Members of *Firmicutes* were represented by the genera *Bacillus* spp. (SK256-8; SK256-12; SK256-13; SK256-14; SK256-S9; SK256-16; SK256-17; SK256-21; SK256-5; SK256-22; SK256-32; SK256-S7; SK256-20; SS263-20; SS263-5; SS263-34), *Oceanobacillus* sp. (SK256-21), *Brevibacillus* sp. (SS263-26), *Virgibacillus* spp. (SK256-18; SK256-19; SS263-37); *Staphylococcus* spp. (SK256-30; SS263-12), *Exiguobacterium* sp. (SK256-25/2) and unclassified members of Planococcaceae and order Bacillales (SK256-38; SS263-7; SS263-10). Representative genus of phylum *Actinobacteria* belonged to *Micrococcus* spp. (SK256-35; SK256-37), *Microbacterium* spp. (SK256-N8; SS263-4), *Brachybacterium* sp. (SK256-N4; SS263-16), *Dietzia* spp. (SS263-17; SS263-19), *Kocuria* sp. (SS263-23), *Leucobacter* sp. (SS263-27), Unclassified Micrococcaceae (SK256-15) and Unclassified Intrasporangiaceae (SK256-28). The Phylum *γ-proteobacteria* were represented by members of the *Pseudomonas* spp. (SK256-25/1; SK256-N3; SK256 N5; SK256 N7; SK256 N6; SK256 N2; SK256-N1; SK256-33; SS263-3; SS263-6; SS263-8), *Halomonas* spp. (SK256-29; SS263-24; SS263-38; SS263-30), *Psychrobacter* spp. (SS263-1; SS263-21; SS263-36), *Shewanella* spp. (SS263-2; SS263-9; SS263-14; SS263-N4; SS263-28A; SS263-33), *Cobetia* spp. (SS263-N5; SS263-29; SS263-13; SS263-11), *Vibrio* sp. (SS263-31), *Pseudoalteromonas* sp. (SS263-N4) and *Providencia* sp. (SK256 16/1). Members of Phylum *α-proteobacteria* was represented by the sole genus *Ochrobactrum* sp. (SS263-38A).

A compilation of the relative abundance of these bacterial genera showed that bacteria belonging to *Bacillus* spp. were by far the most dominant during the active phase and comprised 35.29% of the flora with several other genera constituting the minor fractions (Table 4). During the declining phase, *Shewanella* spp.



emerged as the dominant forms, constituting 16.67% of the flora, followed by *Cobetia* spp. constituting 11.11% and an equal dominance of species belonging to *Pseudomonas*-*Psychrobacter*-*Halomonas* consortium comprising 24.9 % of the flora (Table 4). Species belonging to the genus *Micrococcus*, *Exiguobacterium*, *Providencia* and the unclassified bacteria of families *Intrasporangiaceae* and *Micrococcaceae* were exclusively present during the active phase of bloom; whereas, those belonging to genera *Ochrobactrum*, *Shewanella*, *Cobetia*, *Psychrobacter*, *Pseudoalteromonas*, *Vibrio*, *Dietzia*, *Leucobacter*, *Kocuria*, *Brevibacillus* and unclassified *Planococcaceae* were retrieved exclusively during the declining bloom at St-O1. Common species present during both phases of the bloom were *Bacillus flexus*, *Bacillus cereus*, *Virgibacillus halodentrificans*, *Brachybacterium paraconglomeratum* and *Halomonas meridiana*. The common phylotypes of the dominant genera *Pseudomonas* belonged to the highly coherent 'Fluorescent *Pseudomonas*' cluster of bacteria, and hence their unclear distinction at the species level restrained further conclusions (Table S1).

### Community diversity analysis

The overall Shannon-Wiener diversity index ( $H'$ ) of genera increased from 2.66 during declining phase to 2.07 during the active phase (Fig. 4a). At the phylum level, this diversity shift was more prominent. Generic diversity of *Proteobacteria* increased from 0.64 during the active phase of bloom to 1.74 during the declining bloom with an increase in richness from 0.87 to 2.04. Although population size of Firmicutes during the active phase was numerically dominant ( $N = 18$ ), diversity in genera was as low as 1.16. In comparison, a low-population size of Firmicutes ( $N=5$ ) during declining phase was more diverse with an index of 1.33. Diversity of Actinobacterial genus remained unchanged during both phases as 1.56 (Fig. 4a).

The Unifrac 'P-test' showed that bacterial phylotypes based on 16SrDNA sequences associated with *Noctiluca* (green) bloom phases did differ significantly from each other as  $p = 0.03$ . This was further reflected in the web-based Unifrac-PCA plot which showed spatial separation of bacterial communities at St-O1 during active and declining phases of the bloom as well as with that of the reported endocytic bacterial flora of the red *Noctiluca* (Fig. 4b).

### Antibiogram

The strain level differentiation of the flora was aided from their dissimilarity in their antibiograms as seen in a NMDS plot (Fig. 5). Although, high resistance (>25% of isolates) to several antibiotics was exhibited by the micro-flora during both the phases of the bloom, decaying phase microflora showed a comparatively lower resistance to the antibiotics tested. During the active phase highest resistance of micro-flora was to the antibiotics Nalidixic acid (94.12%), Penicillin (85.29%), Ampicillin (79.41%) and Oxytetracycline (73.53%). In the declining flora of the bloom, resistance to both Nalidixic acid and Ampicillin decreased to 47.22%. Penicillin-G resistance also decreased to 61.1%, whereas resistance to Oxytetracycline remained similar. However, the  $\gamma$ -*proteobacteria* dominated declining flora showed higher resistance than active phase community to four different antibiotic classes: Clindamycin (beta-lactam), Kanamycin (amino-glycoside), Furazolidone (nitro-furan) and Novobiocin (amino-coumarin).

## Metabolic preference

The possible functional role of these diverse bacteria occurring during the two -bloom phases was indicated from their metabolic characteristics to utilize a host of carbohydrates, proteins and amino-acids, lipids, urea, solubilize phosphates, produce H<sub>2</sub>S from organic-matter and reduce nitrate/denitrify (Table 5 and Fig. 6). The ability to degrade complex organic substrates was over-whelming as several of these bacteria expressed multiple hydrolytic enzymes (Fig. 6).

### Carbohydrate degradation

Amylase producers were frequent and decreased from 50% during the active to 38.89% during the declining phase of the bloom (Table 5). Most frequent amylase producers belonged to the phylum *Actinobacteria*. All Actinobacterial members except *Dietzia schimae* (SS263-19) produced amylase during both-phases of the bloom. It was interesting to note that all members belonging to *Bacillus cereus* (SK256-17; SK256-22; SK256-20 and SS263-34) failed to produce amylase (Fig. 3). Several of  $\gamma$ -*proteobacterial* amylase producers in the declining bloom phase were more strongly amylolytic and belonged to *Cobetia marina* (SS263-N5), *Halomonas axialensis* (SS263-24) and *Vibrio campbellii* (SS263-31) which also exhibited the highest zone (4.1cm) on starch plates.

Frequency of cellulose degraders were low and varied from 20.59% during active phase to 13.89% during the declining phase. Dominant cellulose producers in the active phase were Firmicutes members belonging to *Bacillus* spp. In the declining phase cellulose was hydrolyzed by *Pseudomonas* sp. (SS263-6), *Shewanella* sp. (SS263-9) and *Ochrobactrum* sp. (SS263-38A) of the  $\alpha$ -Proteobacteria (Fig. 3)

### Protein degradation

Proteolytic bacteria were frequently detected during both bloom phases (Table 5). Isolates expressing caseinase were equally dominant (55.88% - 58.34%) during both phases of bloom. Species belonging to *Bacillus* including all isolates of the *Bacillus cereus* group, *Virgibacillus*, *Oceanobacillus* and *Brevibacillus* were strongly proteolytic in the active phase (Fig. 6). As the bloom declined, members of the genus *Pseudomonas*, *Cobetia*, *Psychrobacter* and *Halomonas* were important protease producers along with *Bacillus* spp. (Fig. 6).

In comparison, gelatin liquefiers decreased from 61.76% in active to 38.89% during the declining phase. Ability to degrade gelatin remained dominant among members of Firmicutes during both the active (36%) and declining (20%) phases of the bloom (Fig. 6)

### Lipids degradation

Frequency of Tributyrin degraders were low (26.47%-30.56%) in comparison to Tween-80 degraders (52.94%-61.11%) during the bloom phases (Table 5).  $\gamma$  -*proteobacterial* forms were strong lipase producers and majority of them belonged to the Fluorescent *Pseudomonas* spp. group (Fig. 6).

### Solubilization of inorganic phosphates

A large fraction of the flora (52.94%-63.89%) could solubilize Tricalcium-phosphate during both the bloom phases and dominant phosphate solubilizers belonged to  $\gamma$ -*proteobacterial* forms (Fig. 6). Members of *Cobetia*

*marina* along with the Fluorescent *Pseudomonas* group were detected as strong phosphatase producers. However, as many as 8 out of 12 Actinobacterial isolates also produced phosphatase during both bloom phases and were also important (Fig. 6).

#### *Nitrogen metabolism*

Percentage of isolates able to break-down urea to produce ammonium in organic medium increased from 35.29% during active phase to 52.78% during the bloom declining phase. Dominant urea degrading bacteria of the active phase were *Bacillus* and *Pseudomonas*. In the declining phase, urea hydrolysis was exhibited by several other genera of *Pseudomonas*, *Shewanella*, *Halomonas* and *Psychrobacter* of  $\gamma$ -proteobacteria, *Dietzia* of the Actinobacteria and also *Bacillus* (Fig. 6).

Ability to reduce nitrate (73.53%-83.33%) was wide spread (Table 5) and more significantly a large fraction (50% - 41.67%) of both the active and declining phase strains were denitrifying under aerobic conditions as well (Table 6). Utilization of the amino-acids D-Alanine, L-Histidine and L-serine as sole source of carbon and nitrogen were more frequent (Table 5 and Fig. 6)

#### *Desulfurase activity*

H<sub>2</sub>S production from organic matter increased from 11.76% during active to 27.78% during declining phase as all members of *Shewanella* produced H<sub>2</sub>S (Fig. 6).

### **Discussion**

The bloom of *Noctiluca miliaris* persists for a period of ~3 months, during the winter-monsoon period of Jan-Mar and is a recent finding in the Arabian Sea [20, 47]. During consecutive cruises of Feb-Mar 09, we observed case-I area under thick *Noctiluca* (green) bloom at several locations across the entire Northeastern Arabian Sea, covering ~1 lac sq Km of open-waters between 19-23°N in the NAS (Fig. 1). At St-O1, sea surface temperature remained low (~2°C) during active bloom. Higher wind-speed recorded during this phase (Table 1) can be attributed due to the Northeast monsoon and winter convective mixing which makes nutrients available in the euphotic zone for triggering of the bloom [11]. The decline of bloom observed on 2nd-Mar at this location is accompanied with elevated sea-surface temperature (27.62°C) slowing down the mixing process, thereby constraining nutrients availability at surface [11]. Both the active bloom and declining bloom are also associated with low dissolved oxygen as has been observed earlier [20].

#### **Culturable bacterial load**

The plate counts revealed ~ 2-3 fold increase in culturable bacterial load during active and declining phases of *Noctiluca* (green) bloom against 'non-bloom' waters, on both nutrient-rich (ZB and SWA) as well as nutrient-poor (ZB 1/10 strength) media (Fig. 2). In comparison, the red *Noctiluca* bloom of April 1993 in the coastal waters off Mangalore gave reasonably lower plate counts, varying between  $6.5 \times 10^3$  CFU ml<sup>-1</sup> -  $3.3 \times 10^2$  CFU ml<sup>-1</sup> [54]. Examination of red *Noctiluca* from Helgolands, North Sea further shows that a high population of bacteria can actually exist within red *Noctiluca* cells, referred to as the 'turbid' *Noctiluca* [62] which also feeds on

bacterial cells [39]. The higher plate counts also supports our preliminary assessment of bloom waters during March 2007 in NAS [6] and therefore, can only be explained as a '*natural enrichment*' of certain bacterial community during the course of the bloom. Further, as our flora is directly retrieved from the bloom-biomass, screened from water-samples using a 100µm nytex-mesh, these isolates should be more intimate associates (phycospheric and/or intracellular) of the green *Noctiluca* cells itself. Hence, these bacteria must be important, not only to initially process the fresh exudates of the bloom and contribute to supplying this DOM to the free-living bacterial community in the surrounding water-column [4], but also as they are valuable and relevant forms which must be further examined for their role in the process of bloom formation and bloom-termination as well.

### **Nature of bloom-organic matter**

The biochemical nature of the bloom organic matter plays an important role in selection of its bacterial associates [3,4]. The present study dealing with blooms of the green variant of *Noctiluca* are hosting *Pedinomonas noctilucae* as symbionts, which are Chl *a* and Chl *b* bearing prasinophytes [57] and this makes green *Noctiluca* mixotrophic. The organic matter produced by this symbiont will be similar to the labile photosynthetic products rich in carbohydrates, amino-acids and fatty-acids. This is clearly indicated from the high photosynthetic carbon fixation rates based on <sup>14</sup>C tracer estimates and subsequently high dissolved organics production seen from the C-DOM estimates (Table 1). Signatures of mycosporine like amino-acids are also evident from the UV absorption peak of C-DOM at 337 nm, similar to red *Noctiluca* [7].

A detailed biochemical analysis of the mucilage and C/N content of the green *Noctiluca* has neither been performed by us nor at present available elsewhere. However, few studies show that the lipids of red *Noctiluca* consists of sterols, free-fatty acids, triglycerides and ~26% phospho-lipids [10]. Further, *Noctiluca miliaris* by itself (non-symbiotic and red variant) is reported to have a CN content varying from 123-627 ngC cell<sup>-1</sup> and 36-232 ngN cell<sup>-1</sup> with a C:N ratio of 2.3- 4.4. These wide variations are attributed to their available phagotrophic feeding habitat [63].

### **Taxonomic diversity of bacteria**

Several factors are known to determine the bacterial-flora, which are specific to algal-blooms and can also change at different stages of the bloom [60]. Since, a large fraction of bacteria in the active phase are motile (76.47%), they can be chemotactic to the *Noctiluca* (green) DOM during its initial growth. The enzymatic repertoire of 'bacterial-forms' to grow on and utilize organic exudates [26], along with 'antagonistic' interactions among them [42] possibly shapes up the succession in bacterial community at later stages of the bloom.

The growth of bacteria on all the plating media conspicuously shows a stark contrast in colony morphologies appearing during the two bloom phases (Fig. 2a inset). While bacterial colonies appearing during active phase are mostly non-pigmented types, the declining phase consists of dominant orangish-mucoid colonies on all the plating medium used for isolation. Such a shift in bacterial flora has been distinctly emphasized in the results, referred to as the 'active' and 'declining' phase. In this regard, it is further important to note that an apparent peak-phase of bloom occurs when actively buoyant *Noctiluca* (green) cells rise to surface waters and

form dense patches, after which the bloom can decline very quickly [24]. This is exactly what we observed at the overlapping station St-01, when the massive active bloom spreading over a large area on 17<sup>th</sup> Feb-09 suddenly crashed to aggregated lysed *Noctiluca* cells (declining' phase) on 2<sup>nd</sup> Mar-09. This makes the active phase bacterial community, just prior to bloom decline a strong suspect in the possible termination through 'algicidal' bacteria [51]. This may also be intricately timed with an aging *Noctiluca* (green) population as north-east monsoon cooling weakens, creating unfavorable conditions for the growth of the bloom [11].

However, the most important features of the bacterial community representing these two distinct phases of the bloom are: (a) dominance of *Firmicutes* in the active phase, exclusively belonging to order *Bacillales* just before the bloom declines, (b) the emergence of a dominant and more diverse  $\gamma$ -*proteobacterial* forms in the declining bloom, (c) a diverse and consistent Actinobacterial population in both the bloom phases, although represented by mostly different species, and (d) culturable *alpha-proteobacteria* comprising a minor fraction and detected only during the declining phase.

The UNIFRAC-PCA plot based on partial 16SrDNA sequences clearly shows that flora of *Noctiluca* (green) differed significantly from the active to the declining phase as well as the one reported from the endocytic bacteria of red-*Noctiluca* from Helgoland roads, North-Sea [62]. The community also differed from the coastal red-*Noctiluca* bloom (April-May, 1993) off Mangalore, reporting a dominant *Moraxella* spp. population comprising 33%-54% of the flora and a dramatic decline in *Bacillus* spp. from 37.29% during the 'peak' stage to 3.92% on the subsequent day [54]. Although *Moraxella* spp. phylotypes remained undetected, those belonging to *Psychrobacter* spp. (8.33%) of the family *Moraxallaceae* are the only phylogenetically close population recorded in this study during the declining phase of *Noctiluca* (green).

The low frequency of cultured *alpha-proteobacterium* is surprising. Overwhelming evidence from culturable and molecular analysis of bacterial community of several bloom forming dinoflagellate species(s) *Gymnodinium catenatum*, *Alexandrium tamarense*, *Karenia brevis*, *Scropsiella trachoides*, the diatom *Pseudonitzschia* spp. as well as the green-algae *Enteromorpha prolifera* bloom off Qingdao in Yellow Sea show *alpha-proteobacterium* to be the dominant community with a high frequency of retrievable aerobic-anoxygenic phototrophic (AAP) bacteria, commonly referred to as the *Roseobacter* clade, also implicated in the DMSP cycle [15, 19, 22-23, 30, 65]. A recent red *Noctiluca* bloom event in South China Sea also showed a similar high predominance of AAP's [8]. However, the absence of such pigmented AAP bacterial morphotypes on both nutrient rich and nutrient poor media, amended with filtered sea-water from the bloom itself, does show absence of culturable types, atleast as the dominant culturable forms. The only retrieved isolate belonging to  $\alpha$ -*proteobacteria* is referred to *Ochrobactrum* sp., SS263-38A. Closest relatives of this strain in RDP.10 database shows ability to mineralize phenolic compounds and their derivatives (Strain As-12, AY662685) and also produce siderophores (Strain Sp-18) isolated from a coastal phytoplankton bloom [45].

Although a minor component of the *Firmicutes* flora, the isolate SK256-25B referred to as *Exiguobacterium aurantiacum* also belongs to an important group of bacterial species capable of degrading such complex hydrocarbons, phenolics and poly-aromatic hydrocarbons [32]. These bacteria are important as they may

break down complex hydrocarbons in the blooms and pave way for the other members in a consortium to grow on their products of metabolism.

Of the total 70 isolates, 21.42% belongs to *Bacillus*. Among these, 12 isolates belong to the active phase itself, whereas only 3 isolates are retrieved from the declining phase. This is further confirmed by the dominance of *Bacillus* on all plated medium in active phase. Occurrence of *Bacillus* is nearly ubiquitous in the marine environment, and they play an important role in biogeochemical cycling [14]. However, their strong association as a dominant culturable community with an open-ocean algal bloom is a very new finding. The high efficiency of the fast growing *Bacillus* community to degrade complex carbohydrates and their highest abilities to reduce nitrate perhaps made them important constituents of the "active" phase bloom, rich in mucilage and fresh photosynthates. Further, the tropical environment of the Arabian Sea may also be responsible for natural selection of such forms. Recent studies in the mid-west coastal estuaries of India also show *Bacillus* as dominant with high abilities to efficiently degrade carbohydrates [37]. Their role as an important fraction of the phosphate mineralizing bacteria in the coral-reefs of the Gulf- of Mannar, in southern peninsular India, has been documented quite recently [33]. As the neighbor joining boot-strap consensus tree brings out their phylogenetic heterogeneity (Fig. 3), the source of nearest isolates to the retrieved *Bacillus* forms are also seen to be equally diverse: from extreme deep-sea sediment habitat to highly efficient bio-remediating strains. Further, as many as five isolates (SK256-S7; SK256-5; SK256-38 from active bloom and SS263-10 from declining bloom) are potential candidates for new species designations, all retrieved from 1/10 strength Zobell's medium. A surprising result among this group however, is the inability of all *Bacillus cereus* isolates to express amylase. The closest phylogenetic relatives to our *Bacillus cereus* in RDP-II database indicated toxin "cereulide" producing phylotypes [RIVM BC00068 (AJ577283); OH599 (AJ577286); BC00067 (AJ577282); A4-20-12 (AB591768)] and B204 (AJ577292)]. A review of *Bacillus* literature led us to confirm their inability to produce acid from salicin, a key phenotypic identifying feature of the toxic 'cereulide' producing emetic *Bacillus cereus* biovars, although tentative [1, 27]. The role of these possible emetic *Bacillus* biovars therefore, needs to be further screened for possible toxic effects on cultured *Noctiluca* (green). We opine that if bacteria are to play a decisive role in terminating such a high-biomass open-water bloom, covering such large area in the Arabian Sea, the relative abundance of those species should be also be substantially higher, if not the dominant flora. Retrieved flora from green *Noctiluca* of NAS is however, conspicuous in the absence of the major reported algicidal genera from *Noctiluca* (green) belonging to *Marinobacter* spp. [36], atleast as the dominant culturable flora. We however detected the occurrence of *Pseudoalteromonas* spp. and *Vibrio* spp. with a very low frequency in the declining phase.

Members of *Pseudomonas* spp. form the next major group of bacteria in the active phase (24%) and with a low relative abundance (8.3%) in the declining 'phase' of the bloom. The close phylogenetic and phenotypic relatedness among various species refrains us from referring most of these isolates to species level. However, a comparison of the recent examination of detailed *Pseudomonas* 16SrDNA phylogeny shows that our isolates belongs to 2 distinct lineages [49]: (a) *P aeruginosa* lineage, represented by the *P stutzeri* group with two species identifiable as *P stutzeri*; SK256-33 and *P xanthomarina*; SK256-25-1; and (b) *P fluorescens* lineage, represented by the *P fluorescens* group with 9 phylotypes. Based on their closest relatives, the most probable placement of

these phylotypes should be under any of the three subgroups: the *P fluorescens* subgroup, *P gessardii* subgroup and the *P chlororaphis* subgroup. The closest relatives of SS263-N1 of active and SS263-3 of declining bloom shows that *Pseudomonas* sp. PIL-H1, a siderophore producing strain having 99.59% similarity with *P chlororaphis* subsp. *aurantiaca* and capable of producing the broad-spectrum antibiotic 2,4-diacetylphloroglucinol (PHL), a major determinant in the biological control of a range of plant pathogens by many fluorescent *Pseudomonas* spp. [35]. Further, closest members belonging to our Fluorescent *Pseudomonas* group are capable of producing bio-active compounds [28], degrade a host of organic hydrocarbons as indicated from their nearest phylotypes and are well-known to produce siderophores [28].

The dominant forms during the declining phase of the bloom were the *Shewanella* spp. (16.7%), phylogenetically close with *S algae* and *S haliotis* group, forming mucoid orange to beige colonies on Zobell's and NASW medium. All isolates of *Shewanella* produced H<sub>2</sub>S and failed to produce amylase. Members of the genus *Shewanella* are known to be highly versatile in respiring over a suite of organic and inorganic compounds including several radionuclides and metals and therefore play an important role in carbon cycling [17]. *Shewanella* sp. are mostly retrieved from chemically stratified communities with changing redox environments [25]. As Nawata et al. [53] reports a highly acidic pH of *Noctiluca* flotation vacuole, a gradient in redox-potentials created during *Noctiluca* cell lysis can be highly suitable for members of *Shewanella* sp. to out-compete other microbes [17]. A drop in the water-column pH from 8.3 - 8.4 during the active bloom phase to 8.1-7.9 during the declining bloom phase (Table 1) further supports such a succession in favor of *Shewanella*.

Although, *γ-proteobacteria* remained the dominant class during the declining phase, the most commonly reported dominant members from algal blooms belonging to *Alteromonas* sp., *Alcanivorax* spp. and *Marinomonas* spp. [22] were missing among our isolates. Instead, members of the genera *Cobetia*, *Halomonas*, *Psychrobacter* and *Pseudomonas* were identified in almost equal fractions during the “declining” phase.

Both *Cobetia* and *Halomonas* belong to the family *Halomonadaceae*. The high ability of the *Cobetia marina* isolates to produce phosphatase corroborates with published strains which also produces alkaline-phosphatase with a high-specific activity and are also regarded as 'specialized bacteria' appearing during algal-thallus degradation, due to their versatility in assimilating several by-products of polysaccharides [29]. Retrieval of several *Halomonas* isolates were interesting and their phylogenetic closeness to those strains occurring in deep-sea hydrothermal vents (group-2B) and other extreme deep-sea habitat provokes further interest to study these osmotically flexible physiotypes [34].

### **Antibiogram**

Apart from a taxonomic use of the antibiogram to study strain level proximity or dissimilarity among closely related isolates (Fig. 5), the results also show that these bacteria tend to display strong resistance to several antibiotics (Table 5). The change in the overall bacterial community from a dominant gram positive to gram negative forms should have essentially different antibiograms and this is also reflected in the overall resistance to different antibiotics (Table 5). Multiple resistance to antibiotics can affect bacterial population dynamics [46] and are also known to spread through horizontal /lateral gene transfers [18]. Thus, the seasonal emergence of this bloom can

give rise to strongly drug resistant flora in the Arabian Sea. As the bloom spreads across the entire Northern Arabian Sea basin - from coastal Oman to the shelf off Gujrat, such antibiotics-resistant microbes may also spread from fishing activities and be of human-concern.

### **Relationship of metabolic-potentials to water-quality**

The metabolic potentials of the flora (Fig.5) throws light on two important physico-chemical characteristics of the bloom water-column. As seen in Table 1, high dissolved inorganic phosphate is recorded from surface waters of active bloom in comparison to the water-column. This indicates the impact of the large fraction of Phosphate solubilizing bacteria to release inorganic phosphates as they utilize/grow on the active bloom originated organics (Table 5). Such trends in phosphate regeneration has also been noticed during red *Noctiluca* blooms [52]. These bacteria predominantly belonged to the genera *Pseudomonas*, *Kocuria*, *Halomonas*, *Micrococcus*, *Microbacterium*, *Brachy bacterium*, *Bacillus* and unclassified members of the family Intrasporangiaceae and Micrococcaceae (Fig. 6).

Several species of the active phase are able to aerobically denitrify nitrate (Table 6), a trait which is coming to light only in recent times [66]. A strong indication of such denitrification is also seen from the increase of water-column nitrite which significantly remained higher than Nitrate in the active surface bloom waters of Feb-09 at the overlapping station (Table 1). Relative abundance of such denitrifying bacterial isolates are also seen to increase during the active bloom in comparison to the declining bloom (Table 4). Strains of three such denitrifying species among our isolates, belonging to *Providencia rettgeri*, *Pseudomonas stutzeri* and *Bacillus subtilis* are also reported with the unique ability to heterotrophically nitrify and simultaneously aerobically denitrify and this finds important applications in waste-water treatments for nitrogen-removal from sludge [64, 70-71]. The role of our retrieved aerobic denitrifiers therefore, needs to be further studied for their nitrifying ability as they may not only provide important clues to Nitrogen-flux via bloom but also may find important industrial applications for nitrogen-removal from contaminated sites.

### **Summary**

In summary, the culturable bacterial associates of green *Noctiluca* bloom of NAS shows a ~2-3 fold increase in plate counts against non-bloom waters and is significant for an open-ocean ecosystem. These bacteria are shown to form a specific consortium during two distinct bloom phases (active and declining). During the active bloom phase, the microflora was less diverse but more antibiotics resistant members of a dominant *Firmicutes* population belonging mostly to the genus *Bacillus*. During declining bloom, the microflora became more diverse and less antibiotics resistant. The declining phase is dominated by members of *γ-proteobacteria* belonging to *Shewanella* spp. and a mixed consortium of *Cobetia*-*Psychrobacter*-*Halomonas*-*Pseudomonas*. Similarly, the Actinobacterial forms are also seen to be more diverse during both the bloom phases and represented by at least 2 novel uncharacterized genera. Common species detected during both phases of the bloom belongs to the genus *Bacillus flexus*, *Bacillus cereus*, *Virgibacillus halodentrificans*, *Brachy bacterium paraconglomeratum* and the Fluorescent *Pseudomonas* group of bacteria. Extracellular enzyme potentials of these bacteria indicates their abilities to degrade complex organics like carbohydrates, proteins and lipids. While



cellulose and gelatin degraders predominated among active phase members of *Firmicutes*, lipid degraders predominated among declining phase members of  $\gamma$ -*proteobacteria*. H<sub>2</sub>S production from declining bloom organic matter is detected among dominant members of *Shewanella* and *Halomonas*. Further, the ability of these bacteria to solubilize phosphates and aerobically denitrify is supported from water-column characteristics of the active bloom. The role of these bacteria however, needs to be further evaluated for their ability to: (a) remove nitrogen through simultaneous aerobic nitrification and denitrification, and (b) to produce toxic ionophoretic compounds in light of the suspected toxic *Bacillus cereus* which may implicate crucial roles of bacteria during blooms of *Noctiluca miliaris* green.

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## Legends for Figures

**Fig. 1** Map of sampling stations showing bloom-phases in the Northeastern Arabian Sea during Feb-Mar 2009: (a) active and declining phase images of *Noctiluca miliaris* (green) bloom at overlapping station (St-O1) with (inset) light-microscopic examination(40X) showing green *Noctiluca miliaris* cells in active phase and clumps of lysed *Noctiluca(green)* cells in declining phase (b) Cruise-tracks of ORV Sagar Kanya-256 (Feb-09) show sampling stations located outside 1000m depth contour in open-waters. Data from sampling stations considered in present study are listed in the map and marked with colored legends.

**Fig. 2** Statistical box (25-75 percentile) and whisker (min-max) plots shows response of open-ocean bacteria to *Noctiluca* (green) bloom of NAS in Feb-Mar 2009: (a) Plate counts of active and declining bloom against 'non-bloom' waters; (inset) plate images of flora at St-O1 on ZB and SWA (b) Shift in percent gram-character of flora.

**Fig. 3** Neighbor joining boot-strap (50% cut-off) consensus tree of partial 16SrDNA sequences depicts phylogenetic relationship among bacterial associates of *Noctiluca miliaris* (green) during active (*SK256*) and declining (*SS263*) bloom. Major generic clusters are indicated from tree. Key phenotypic characteristics of strains are merged as a heat-map of positive/negative tests.

**Fig. 4** Community analysis of bacterial-flora retrieved from *Noctiluca(green)* bloom phases at St-O1 **(a)** Diversity of bacterial genera **(b)** Diversity of bacterial genera based on phylum representations **(c)** Unifrac drawn PCA plot showing bacterial communities based on 16SrDNA partial sequences. Bacterial community of *Noctiluca* (red) cells of Helgolands, North Sea (Seibold A et al., 2001) was included in the phylogenetic tree for comparison with active and declining phases of NAS *Noctiluca* (green) bloom in the present study.

**Fig. 5** Multi-dimensional scaling showing strain-level proximity/ dissimilarity based on antibiotics resistance among bacterial isolates. Identity of isolates is shown in Table1 and 2.

**Fig. 6** Metabolic potentials of active and declining phase bacterial flora: (a) Utilization of carbon/nitrogen as sole sources (b) Hydrolysis of extracellular compounds and H<sub>2</sub>S production on organic-matter breakdown (c) Utilization of carbohydrates with acid production under aerobic conditions.

**Table 1** Water-column characteristics at the overlapping 'active' and 'declining' *Noctiluca miliaris* bloom station in Northeastern Arabian Sea

Hydrological characteristics	Active phase	Declining phase
	Feb-09 Surface (Mean±SD)*	Mar-09 Surface (Mean±SD)*
Wind-speed (m sec <sup>-1</sup> )	4.1	3.7
Temperature (°C)	25.12 (24.91±0.13)	27.62(25.57±0.69)
Salinity (psu)	36.48 (36.51±0.01)	36.58(36.55±0.03)
pH	8.3 (8.35±0.2)	8.11 (8.17±0.16)
Dissolved Oxygen (ml L <sup>-1</sup> )	3.06(2.95±0.07)	2.71(2.94±0.28)
Dissolved Inorganic Nutrients:		
Nitrate (µM)	0.54 (0.79±0.27)	0.02 (6.86±7.78)
Nitrite (µM)	0.65 (0.77±0.13)	0.08 (0.12±0.05)
Phosphate (µM)	5.23 (1.82±1.64)	0.64 (1.29±0.86)
Silicate (µM)	19.79 (14.8±3.58)	1.77 (5.56±5.09)
Primary-Productivity (mgC m <sup>-3</sup> day <sup>-1</sup> )	1426.93 (798.32±1112.96)	469.53 (160.24±179.75)
C-DOM (Ac 300 m <sup>-1</sup> )	1.14 (2.48±1.45)	0.61 (0.81±0.32)
Chlorophyll a (mg m <sup>-3</sup> )	27.7 (7.46±9.36)	0.99 (0.65±0.28)
<i>Noctiluca</i> counts (cells L <sup>-1</sup> )	9600 (2459±3588)	80 (45±41)

\*(Mean±SD) is Euphotic zone average.

**Table 2** Identification of bacteria isolated from 'active' *Noctiluca* (green) bloom phase of Feb-2009 in NAS (cruise SK256).

Isolate code*	Species identity	Accession	Nearest strain/s in RDP-II and/or Eztaxon-e database	Pair-wise similarity (%)
SK256-25A	<i>Pseudomonas xanthomarina</i>	JX429828	<i>Pseudomonas xanthomarina</i> ; KMM 1447 <sup>T</sup> [AB176954]	99.6 (2/512)
SK256-N3	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429829	<i>Pseudomonas chlororaphis</i> subsp. <i>chlororaphis</i> ; 4.4.1[FR682804]	100.0 (0/226)
SK256-N5	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429830	<i>Pseudomonas merdiana</i> ; KJPB15 [FM213379]	99.0 (4/384)
SK256-16A	<i>Providencia rettgeri</i>	JX429831	<i>Providencia rettgeri</i> ; IITRP2 [GU193984]	100.0 (0/867)
SK256-29	<i>Halomonas meridiana</i>	JX429832	<i>Halomonas meridiana</i> ; DSM 5425 <sup>T</sup> [AJ306891]	99.8 (1/826)
SK256-N7	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429833	<i>Pseudomonas</i> spp. [AJ492830; AY509898; AF374472]	100.0 (0/326)
<b>*SK256-N6</b>	<i>Pseudomonas gessardii</i>	JX429834	<i>Pseudomonas gessardii</i> ; CIP105469 <sup>T</sup> [AF074384]	99.6 (5/1405)
SK256-N2	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429835	<i>Pseudomonas</i> spp.; [AJ492830; AY509898; AF268029]	100.0 (0/366)
SK256-N1	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429836	<i>Pseudomonas</i> sp., PILH1 ; AY456708	99.7 (0/394)
SK256-33	<i>Pseudomonas stutzeri</i>	JX429837	<i>Pseudomonas stutzeri</i> ATCC 17588 <sup>T</sup> [CP002881]	99.0 (4/409)
SK256-N4	<i>Brachybacterium paraconglomeratum</i>	JX429838	<i>Brachybacterium paraconglomeratum</i> ; CT24 [EU660352]	99.3 (3/417)
SK256-N8	<i>Microbacterium oxydans</i>	JX429839	<i>Microbacterium oxydans</i> ; NJ6 [DQ403811]	100.0 (0/476)
SK256-37	<i>Micrococcus luteus</i>	JX429840	<i>Micrococcus luteus</i> ; CV39 [AJ717368]	100.0 (0/696)
<b>*SK256-35</b>	<i>Micrococcus luteus</i>	JX429841	<i>Micrococcus</i> sp.; HB241 [GU213502]	99.9 (1/1323)
SK256-28	Unclassified <i>Intrasporangiaceae</i> <sup>b</sup>	JX429842	<i>Phycoccus bigeumensis</i> ; MSL03 <sup>T</sup> [EF466128]	97.3 (17/640)
<b>*SK256-15</b>	Unclassified <i>Micrococcaceae</i> <sup>b</sup>	JX429843	<i>Micrococcaceae</i> K2-66; [AY345408]	93.7 (89/1418)
SK256-25/2	<i>Exiguobacterium aurantiacum</i>	JX429844	<i>Exiguobacterium aurantiacum</i> DSM 6208 <sup>T</sup> ; [DQ019166]	99.4 (5/844)
SK256-20	<i>Bacillus cereus</i>	JX429845	<i>Bacillus cereus</i> ; ATCC 14579 <sup>T</sup> [AE016877]	100.0 (0/1014)
SK256-S7	<i>Bacillus</i> sp. <sup>b</sup>	JX429846	<i>Bacillaceae bacterium</i> ; KVD-unk-34 [DQ490422]	97.2 (11/386)
SK256-32	<i>Bacillus subtilis</i> subsp. <i>subtilis</i>	JX429847	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> ; GH44 [AB301013]	99.3 (3/418)
SK256-22	<i>Bacillus cereus</i>	JX429848	<i>Bacillus cereus</i> ; CICC10185 [AY842872]	99.8 (1/419)
<b>*SK256-5</b>	<i>Bacillus</i> sp. <sup>b</sup>	JX429849	<i>Bacillus</i> sp.; 53-11 [FJ607050]	99.9 (2/1413)
SK256-38	Unclassified <i>Planococcaceae</i> <sup>b</sup>	JX429850	<i>Bacillus</i> sp.; M71_N104b [FM992794]	99.8 (2/920)
SK256-30	<i>Staphylococcus arlettae</i>	JX429851	<i>Staphylococcus arlettae</i> ; P2S4 [EU221385]	100 (0/765)
SK256-19	<i>Virgibacillus halodenitrificans</i>	JX429852	<i>Virgibacillus halodenitrificans</i> ; DSM 10037 <sup>T</sup> [AY543169]	99.5 (3/701)
SK256-18	<i>Virgibacillus halodenitrificans</i>	JX429853	<i>Virgibacillus halodenitrificans</i> ; DSM 10037 <sup>T</sup> [AY543169]	99.8 (1/535)
SK256-21	<i>Oceanobacillus</i> sp.	JX429854	<i>Oceanobacillus</i> sp.; VE-2-10-82 [EU604320]	100.0 (0/817)
SK256-17	<i>Bacillus cereus</i>	JX429855	<i>Bacillus cereus</i> ; RIVM BC00068 [AJ577283]	100.0 (0/833)
SK256-16	<i>Bacillus flexus</i>	JX429856	<i>Bacillus flexus</i> ; MDLD1 [FJ861081]	99.3 (3/429)
SK256-S9	<i>Bacillus</i> sp.	JX429857	<i>Bacillus firmus</i> ; XJSL1-9 [GQ903388]	99.8 (1/474)
SK256-14	<i>Bacillus</i> sp.	JX429858	<i>Bacillus firmus</i> ; 87-7 [FJ607055]	99.6 (1/284)
SK256-13	<i>Bacillus mycoides</i>	JX429859	<i>Bacillus mycoides</i> ; BGSC1 <sup>DN3</sup> [EU285664]	100.0 (0/793)
SK256-12	<i>Bacillus</i> sp.	JX429860	<i>Bacillus</i> sp.; BA-54 [AY557616]	100.0 (0/821)
<b>*SK256-8</b>	<i>Bacillus</i> sp.	JX429861	<i>Bacillus</i> sp.; ARCTIC-P30 [AY573046]	99.5 (7/1490)

\*a' Fluorescent *Pseudomonads* group (14.7%)

\*b' Unclassified strains with nearest Type strain match <97% and significantly different phenotypic characteristics from nearest Type strain  
Near complete (> 1200bp) sequences obtained for 'bold-faced' isolate codes



**Table 3** Identification of bacterial isolates retrieved from 'declining' *Noctiluca* (green) bloom phase of Mar-2009 in NAS (cruise SS263).

Isolate code	Species identity	Accession	Nearest strain/s in RDP-II and/or Eztaxon-e database	Pair-wise similarity (%)	
SS263-38A	<i>Ochrobactrum</i> sp.	JX429792	<i>Ochrobactrum</i> spp. [AY776289; CP000758; AY457038]	99.3	(2/305)
SS263-2	<i>Shewanella</i> sp.	JX429793	<i>Shewanella</i> sp.; KJW23 [HM016085]	99.7	(2/645)
SS263-24	<i>Halomonas axialensis</i>	JX429794	<i>Halomonas axialensis</i> ; Althf1 <sup>T</sup> [AF212206]	99.5	(2/409)
SS263-28A	<i>Shewanella</i> sp.	JX429795	<i>Shewanella</i> sp.; W3 [GQ280385]	99.5	(2/692)
<b>*SS263-N5</b>	<i>Cobetia marina</i>	JX429796	<i>Cobetia marina</i> ; DSM 4741 <sup>T</sup> [AJ306890]	99.8	(2/1413)
<b>*SS263-N4</b>	<i>Shewanella haliotis</i>	JX429797	<i>Shewanella haliotis</i> ; DW01 <sup>T</sup> [EF178282]	100.0	(0/1414)
<b>*SS263-38</b>	<i>Halomoans meridiana</i>	JX429798	<i>Halomonas meridiana</i> ; DSM 5425 <sup>T</sup> [AJ306891]	99.8	(2/1374)
SS263-31	<i>Vibrio campbellii</i>	JX429799	<i>Vibrio campbellii</i> ; ATCC 25920 <sup>T</sup> [X74692]	99.7	(1/430)
SS263-36	<i>Psychrobacter</i> sp.	JX429800	<i>Psychrobacter</i> sp.; B-5151 [DQ399761]	98.8	(7/613)
SS263-33	<i>Shewanella</i> sp.	JX429801	<i>Shewanella upenei</i> ; 20-23R <sup>T</sup> [GQ260190]	99.8	(1/630)
<b>*SS263-30</b>	<i>Halomonas venusta</i>	JX429802	<i>Halomonas venusta</i> ; DSM 4743 <sup>T</sup> [AJ306894]	99.8	(2/1430)
<b>*SS263-29</b>	<i>Cobetia marina</i>	JX429803	<i>Cobetia marina</i> ; DSM 4741 <sup>T</sup> [AJ306890]	99.7	(3/1305)
<b>*SS263-28</b>	<i>Pseudoaltermonas</i> sp.	JX429804	<i>Pseudoalteromonas elyakovii</i> KMM162 <sup>T</sup> [AF082562]	99.3	(13/1437)
SS263-21	<i>Psychrobacter</i> sp.	JX429805	<i>Psychrobacter</i> sp.; B-5151 [DQ399761]	99.0	(6/629)
SS263-14	<i>Shewanella</i> sp.	JX429806	<i>Shewanella algae</i> ; ATCC 51192 <sup>T</sup> [AF005249]	97.6	(7/296)
SS263-13	<i>Cobetia marina</i>	JX429807	<i>Cobetia marina</i> DSM 4741 <sup>T</sup> [AJ306890]	99.6	(2/633)
SS263-11	<i>Cobetia marina</i>	JX429808	<i>Cobetia marina</i> DSM 4741 <sup>T</sup> [AJ306890]	99.3	(4/648)
<b>*SS263-9</b>	<i>Shewanella</i> sp.	JX429809	<i>Shewanella</i> spp. [EF028091; EU252498]	100.0	(0/1452)
SS263-8	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429810	<i>Pseudomonas</i> spp. [AY486386; AF094729]	100.0	(0/619)
SS263-6	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429811	<i>Pseudomonas</i> spp. [AY492830; AY509898; AF268029; AF37447]	99.4	(2/389)
SS263-3	<i>Pseudomonas</i> sp. <sup>a</sup>	JX429812	<i>Pseudomonas</i> sp. PILH1 [AY456708]	100.0	(0/392)
SS263-1	<i>Psychrobacter</i> sp.	JX429813	<i>Psychrobacter marincola</i> ; KMM277 <sup>T</sup> [AJ309941]	98.5	(6/402)
SS263-27	<i>Leucobacter komagatae</i>	JX429814	<i>Leucobacter komagatae</i> IFO 15245 <sup>T</sup> [AJ746337]	100.0	(0/631)
<b>*SS263-23</b>	<i>Kocuria</i> sp.	JX429815	<i>Kocuria</i> sp.; C20 [AB330815]	100.00	(0/1396)
SS263-16	<i>Brachybacterium paraconglomeratum</i>	JX429816	<i>Brachybacterium paraconglomeratum</i> ; CT24 [EU660352]	99.7	(2/798)
SS263-19	<i>Dietzia schimae</i>	JX429817	<i>Dietzia schimae</i> ; YIM 65001 <sup>T</sup> [EU375845]	100.0	(0/433)
SS263-17	<i>Dietzia timorensis</i>	JX429818	<i>Dietzia timorensis</i> ; ID05-A0528 <sup>T</sup> [AB377289]	100.0	(0/513)
SS263-4	<i>Microbacterium</i> sp.	JX429819	<i>Microbacterium</i> spp. [AJ853910; X77444]	99.6	(1/316)
SS263-26	<i>Brevibacillus borstellensis</i>	JX429820	<i>Brevibacillus borstelensis</i> ; NRRL-NRS-818 <sup>T</sup> [D78456]	99.8	(1/775)
SS263-12	<i>Staphylococcus cohnii</i>	JX429821	<i>Staphylococcus cohnii</i> subsp. cohnii; ATCC29974 <sup>T</sup> [D83361]	99.7	(1/440)
SS263-37	<i>Virgibacillus halodenitrificans</i>	JX429822	<i>Virgibacillus halodenitrificans</i> ; DSM 10037 <sup>T</sup> [AY543169]	99.8	(1/663)
SS263-10	Unclassified Bacillales <sup>b</sup>	JX429823	<i>Bacillus</i> sp.; Pd3T [GU391528]	96.2	(8/215)
<b>*SS263-34</b>	<i>Bacillus cereus</i>	JX429824	<i>Bacillus cereus</i> ; B204 [AJ577293]	100.0	(0/1503)
<b>*SS263-5</b>	<i>Bacillus flexus</i>	JX429825	<i>Bacillus flexus</i> IFO 15715(T)	99.86	(2/1461)
SS263-20	<i>Bacillus</i> sp.	JX429826	<i>Bacillus firmus</i> IMAUB1032; BT5-2 [FJ641034]	99.6	(3/726)
SS263-7	Unclassified Bacillales <sup>b</sup>	JX429827	<i>Chryseomicrobium imtechense</i> ; MW 10(T) [GQ927308]	92.57	(20/269)

<sup>a</sup> Fluorescent Pseudomonads group ( 8.33%)

<sup>b</sup> Unclassified strains with nearest Type strain match <97% and significantly different phenotypic characteristics

Near complete (> 1200bp) sequences obtained for 'bold-faced' isolate codes

**Table 4** Relative frequency of bacterial genera occurring during 'active' and 'declining' bloom phases of *Noctiluca* (green).

Phylum	Bacterial Genera	Relative abundance (%)	
		Active phase (N=34)	Declining phase (N=36)
<b>α-proteobacteria</b>	<i>Ochrobactrum</i>	-	2.78
<b>γ-proteobacteria</b>	<i>Shewanella</i>	-	16.67
	<i>Halomonas</i>	2.94	8.33
	<i>Providencia</i>	2.94	-
	<i>Cobetia</i>	-	11.11
	<i>Psychrobacter</i>	-	8.33
	<i>Pseudoalteromonas</i>	-	2.78
	<i>Vibrio</i>	-	2.78
	<i>Pseudomonas</i>	23.53	8.33
<b>Actinobacteria</b>	<i>Leucobacter</i>	-	2.78
	<i>Kocuria</i>	-	2.78
	<i>Brachybacterium</i>	2.94	2.78
	<i>Dietzia</i>	-	5.56
	<i>Microbacterium</i>	2.94	2.78
	<i>Micrococcus</i>	5.88	-
	Unclassified <i>Micrococcaceae</i>	2.94	-
	Unclassified <i>Intrasporangiaceae</i>	2.94	-
<b>Firmicutes</b>	<i>Brevibacillus</i>	-	2.78
	<i>Staphylococcus</i>	2.94	2.78
	<i>Virgibacillus</i>	5.88	2.78
	<i>Exiguobacterium</i>	2.94	-
	<i>Oceanobacillus</i>	2.94	-
	<i>Bacillus</i>	35.29	11.11
	Unclassified <i>Planococcaceae</i> and <i>Bacillales</i>	2.94	2.78

- not detected / absent

**Table 5** Phenotype and metabolic comparison of the 'active' and 'declining' phase bacterial flora

Phenotypic/Metabolic characteristics	Percentage of flora	
	Active (N=34)	Declining (N=36)
Gram-Character (+ve)	70.59	38.88
Motility	76.47	58.33
Endospores	44.12	19.44
Anaerobic growth in thioglycollate	50.00	47.22
Catalase	100.00	97.22
Oxidase	41.18	55.56
$\beta$ -galactosidase (ONPG)	20.59	13.89
Lysine decarboxylase	8.82	8.33
Ornithine decarboxylase	26.47	38.89
Phenylalanine deaminase	5.88	8.33
H <sub>2</sub> S (Desulfurase)	11.76	27.78
Nitrate reduction	73.53	83.33
Denitrification (Aerobic)	50.00	41.67
<b>Extracellular Hydrolysis of:</b>		
Urea	35.29	52.78
Esculin	47.06	30.56
Starch	50.00	38.89
Carboxy-methyl Cellulose	20.59	13.89
Casein	55.88	58.34
Gelatin	61.76	38.89
Tributylin	26.47	30.56
Tween-80	52.94	61.11
Tricalcium Phosphate	52.94	63.89
<b>Carbon source utilization:</b>		
Citrate	41.18	61.11
Succinate	82.35	86.11
Acetamide	44.12	63.90
D-Glucose	100.00	80.56
Sucrose	44.12	75.00
Maltose	73.53	83.33
D-Xylose	38.24	47.22
D-Fructose	76.47	83.33
L-Arabinose	23.53	27.78
D-Mannose	32.35	50.00
Trehalose	29.41	61.11
<b>Amino-acids as sole C&amp;N source:</b>		
Alanine	47.06	41.67
Methionine	17.65	19.44
Lysine	41.18	27.78
Threonine	32.35	33.33
Leucine	38.24	66.67
Iso-leucine	35.29	22.22
Valine	26.47	44.44
Phenylalanine	20.59	52.78
Histidine	41.18	41.67
Serine	55.88	63.89
Tryptophan	14.71	13.89
<b>Acid from Carbohydrates (Aerobic)</b>		
Glucose	64.71	66.67
Adonitol	17.65	8.33
Lactose	11.76	19.44
L-Arabinose	35.29	27.78
Sorbitol	2.94	13.89
Xylose	20.59	33.33
Maltose	44.12	30.56
Fructose	41.18	44.44
Galactose	29.41	16.67
Raffinose	8.82	ND
Trehalose	44.12	22.22
Melibiose	14.71	8.33
Sucrose	41.18	16.67
Mannose	47.06	25.00
Inulin	8.82	ND
Na-gluconate	11.76	13.89
Glycerol	29.41	22.22
Salicin	11.76	5.56
Glucosamine	23.53	5.56

**Table 5** Continued

Phenotypic/Metabolic characteristics	Percentage of flora	
	Active (N=34)	Declining (N=36)
Dulcitol	8.82	ND
Inositol	14.71	5.56
Sorbitol	5.88	8.33
Mannitol	41.18	13.89
alpha-methyl D Glucoside	2.94	ND
Ribose	52.94	30.56
Rhamnose	11.76	2.78
Cellobiose	20.59	8.33
Melezitose	8.82	5.56
alpha-methyl D Mannoside	5.88	ND
Xylitol	2.94	19.44
Sorbose	2.94	2.78
Erythritol	14.71	ND
Arbutin	17.65	2.78
<b>Antibiotics Resistance:</b>		
Ampicillin	79.41	47.22
Ciprofloxacin	11.76	2.78
Chloramphenicol	26.47	16.67
Clindamycin	58.82	77.78
Erythromycin	50.00	22.22
Furazolidone	50.00	58.33
Gentamycin	11.76	11.11
Kanamycin	41.18	52.78
Methicillin	58.82	33.33
Norfloxacin	17.65	16.67
Novobiocin	38.24	58.33
Neomycin	14.71	16.67
Nalidixic acid	94.12	47.22
Oxytetracycline	73.53	75.00
Polymyxin B	52.94	38.89
Streptomycin	2.94	5.56
Tetracycline	55.88	47.22
Penicillin	85.29	61.11

Nd –not detected

**Table 6** List of retrievable denitrifying (aerobic) bacterial strains associated with *Noctiluca miliaris* during its bloom of Feb-Mar 2009 in NAS.

Bloom phase	Genera	* Strains detected Positive for Aerobic Denitrification	Percentage of flora
'Active'	<i>Pseudomonas</i>	SK256-33; SK256-25A; SK256-N1; SK256-N2; SK256-N3; SK256-N5; SK256-N6; SK256-N7	<b>50.00%</b>
	<i>Bacillus</i>	SK256-8; SK256-12; SK256-17; SK256-20; SK256-32; SK256-14	
	<i>Virgibacillus</i>	SK256-18; SK256-19;	
	Unclassified <i>Intrsporangiaceae</i>	SK256-28	
'Declining'	<i>Pseudomonas</i>	SS263-3; SS263-6; SS263-8	<b>41.67%</b>
	<i>Shewanella</i>	SS263-2; SS263-14; SS263-33; SS263-28A	
	<i>Psychrobacter</i>	SS263-1; SS263-36; SS263-21	
	<i>Ochrobactrum</i>	SS263-38A	
	<i>Brevibacillus</i>	SS263-26	
	<i>Virgibacillus</i>	SS263-37	
	<i>Bacillus</i>	SS263-5; SS263-34	

\* Identity and closest relatives of strains as provided in Table 1 and 2.

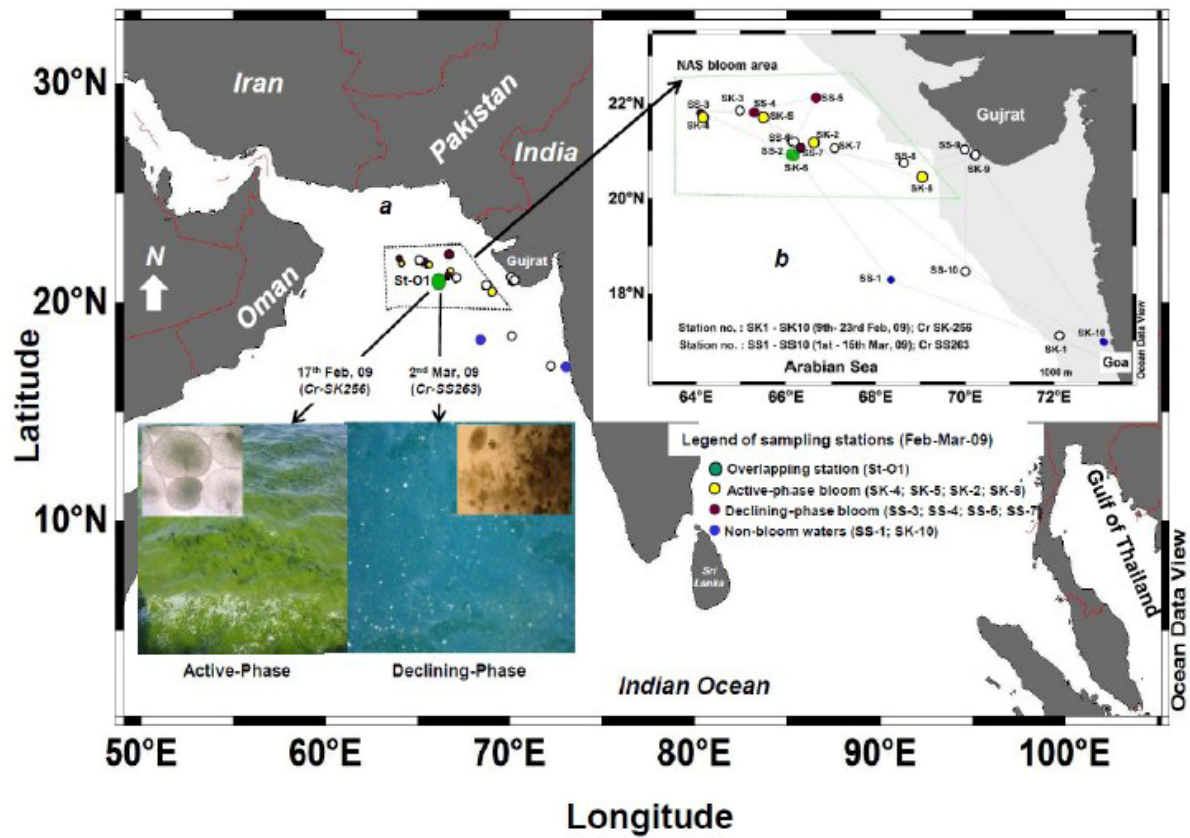


Fig. 1

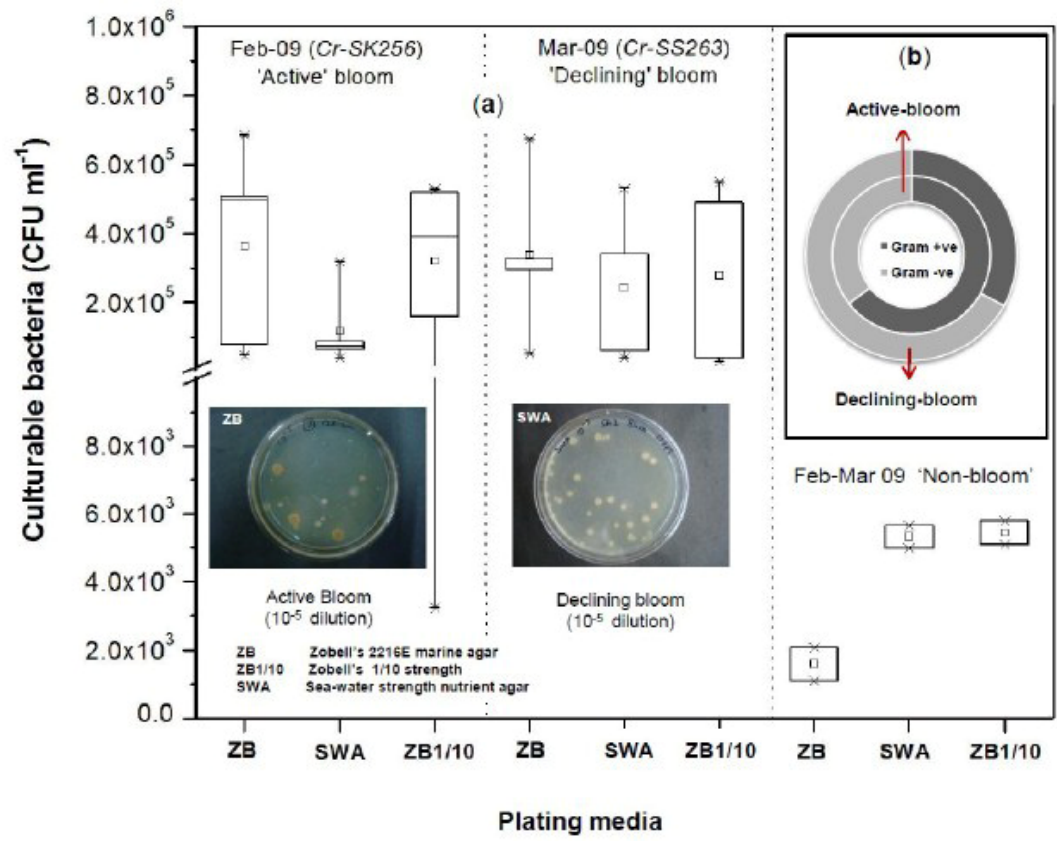


Fig. 2

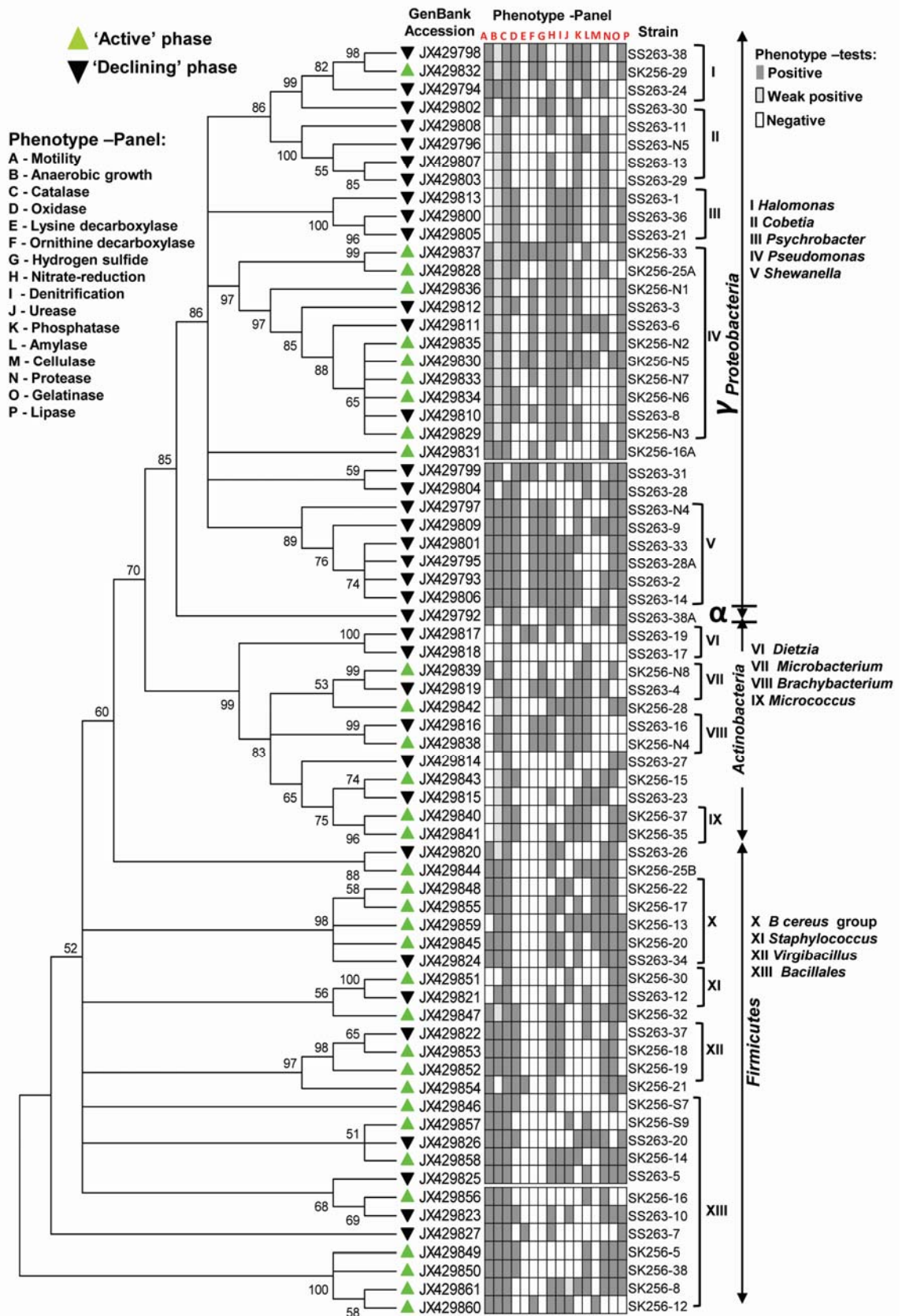
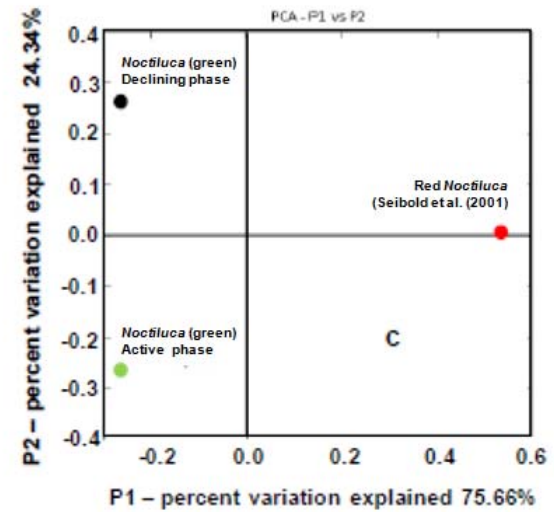
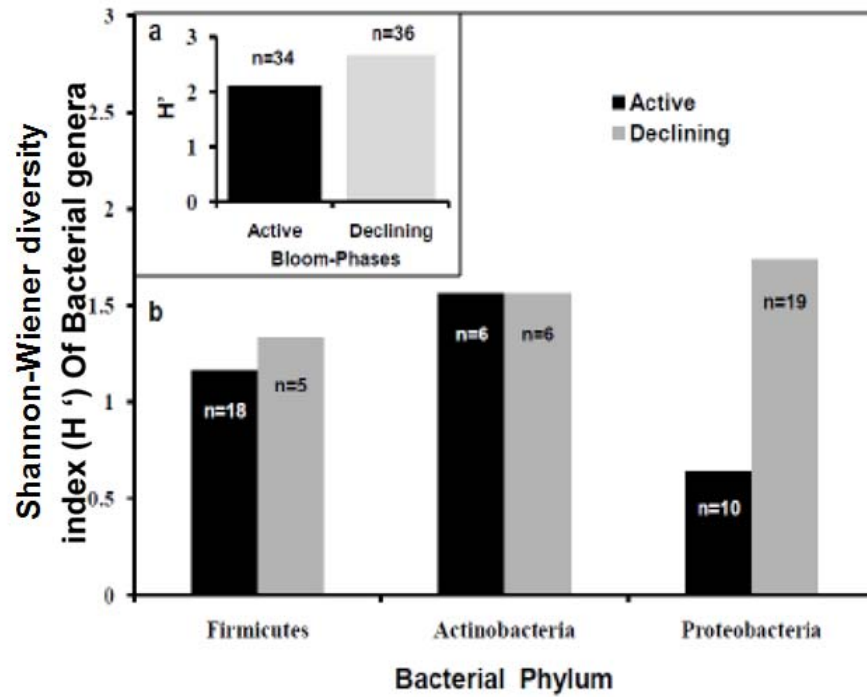


Fig. 3





- Active *Noctiluca* (green) bloom; Feb-09, NAS (Present study)
- Declining *Noctiluca* (green) bloom; Mar-09, NAS (Present study)
- *Noctiluca* (red) cells; Helgoland roads, North Sea (Seibold et al., 2001)

Fig. 4

Clusters represent antibiogram of isolates from:

- ▲ 'Active' bloom phase
- 'Declining' bloom phase
- Common to both bloom phases

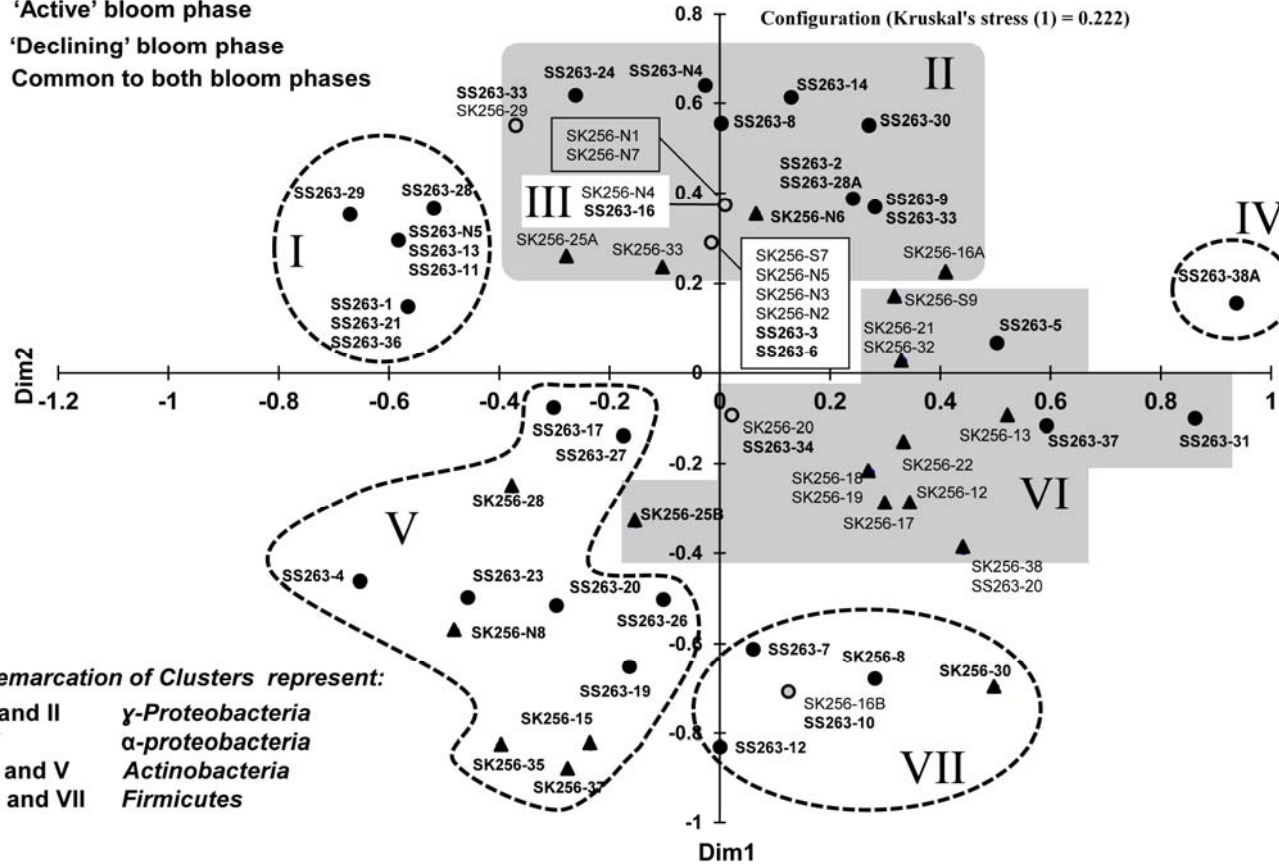


Fig. 5

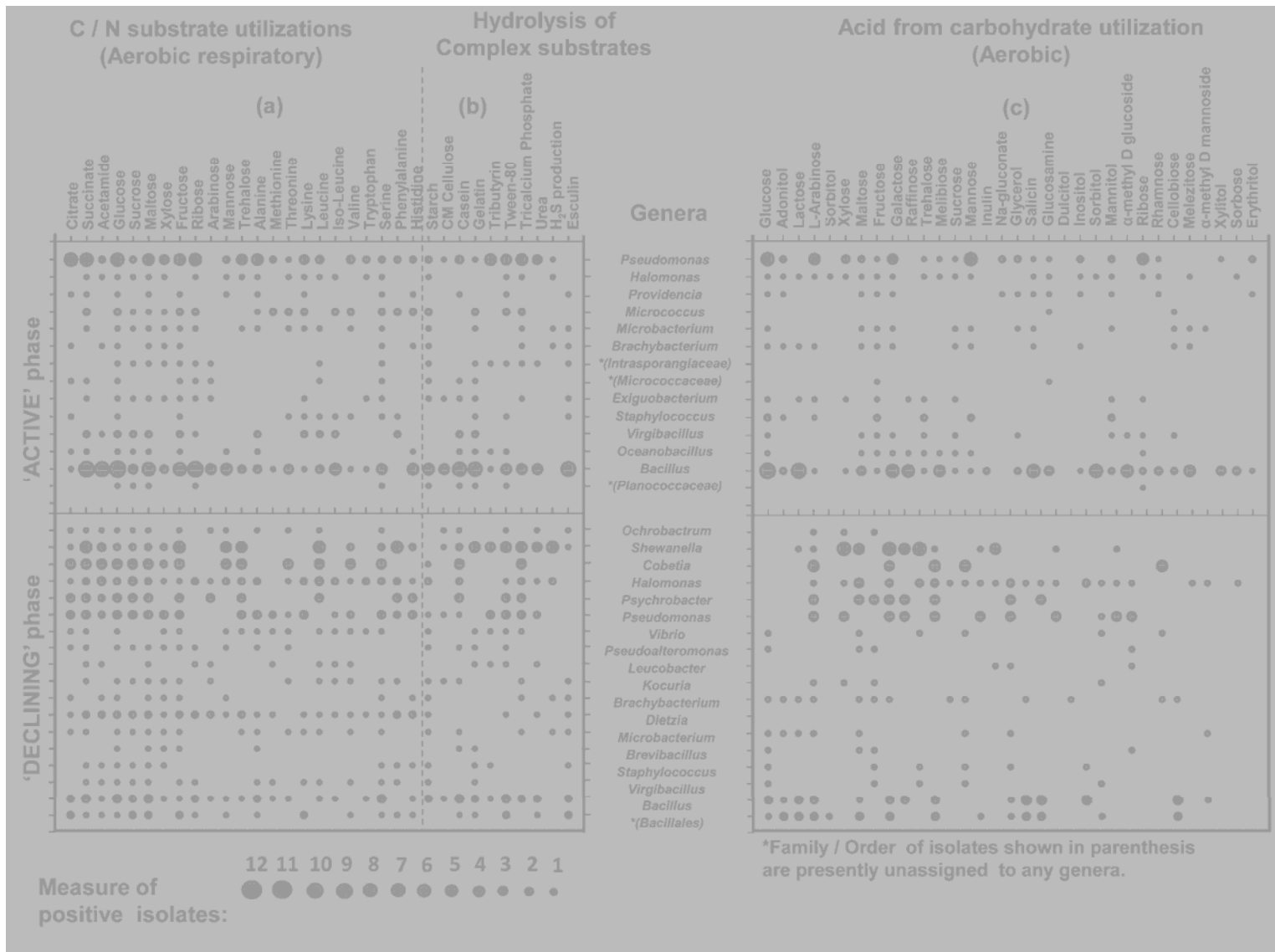


Fig. 6

1  
2

**Table S1** List of bacteria species from 'active' and 'declining' phases of the bloom at St-O1.

List of Bacterial species	Isolate codes	
	Active bloom (Cruise SK256) N-34	Decaying bloom (Cruise SS263) N=36
<i>Bacillus cereus</i>	SK256-17; SK256-22; SK256-20	SS263-34
<i>Bacillus mycoides</i>	SK256-13	Nd
<i>Bacillus flexus</i>	SK256-16	SS263-5
<i>Bacillus subtilis subsp. subtilis</i>	SK256-32	Nd
<i>Bacillus</i> sp.	SK256-8; SK256-12; SK256-14; SK256-S9; SK256-S7; SK256-5	SS263-30
<i>Oceanobacillus</i> sp.	SK256-21	Nd
Unclassified Planococcaceae	SK256-38	SS263-7
<i>Virgibacillus halodenitrificans</i>	SK256-18; SK256-19	SS263-37
<i>Brevibacillus borstellensis</i>	Nd	SS263-26
<i>Staphylococcus cohnii</i>	Nd	SS263-12
<i>Staphylococcus arlettae</i>	SK256-30	Nd
<i>Exiguobacterium aurantiacum</i>	SK256-25B	Nd
<i>Microbacterium oxydans</i>	SK256-N8	
<i>Microbacterium</i> sp.		SS263-4
<i>Micrococcus luteus</i>	SK256-37, SK256-35	
<i>Leucobacter komagate</i>	Nd	SS263-27
<i>Brachybacterium paraconglomeratum</i>	SK256-N4	SS263-16
<i>Kocuria</i> sp.	Nd	SS263-23
<i>Dietzia schimae</i>	Nd	SS263-19
<i>Dietzia timorensis</i>	Nd	SS263-17
Unclassified Micrococcaceae	SK256-15	Nd
Unclassified Intrasporangiaceae	SK256-28	Nd
<i>Shewanella haliotis</i>	Nd	SS263-N4
<i>Shewanella</i> spp.	Nd	SS263-2; SS263-28A; SS263-33; SS263-14; SS263-9
<i>Cobetia marina</i>	Nd	SS263-11; SS263-13; SS263-N5; SS263-29
<i>Providencia rettgeri</i>	SK256-16A	Nd
<i>Psychrobacter</i> spp.	Nd	SS263-36; SS263-21; SS263-1
<i>Halomonas meridiana</i>	SK256-29	SS263-38
<i>Halomonas axialensis</i>	Nd	SS263-24
<i>Halomonas venusta</i>	Nd	SS263-30
<i>Pseudoaltermonas</i> sp.	Nd	SS263-28
<i>Vibrio campbelli</i>	Nd	SS263-31
<i>Pseudomonas stutzeri</i>	SK256-33	Nd
<i>Pseudomonas xanthomarina</i>	SK256-25A	Nd
<i>Pseudomonas gessardii</i>	SK256-N6	Nd
Fluorescent <i>Pseudomonas</i> group	SK256-N1; SK256-N2; SK256-N3; SK256-N5; SK256-N7	SS263-3; SS263-6; SS263-8
<i>Ochrobactrum</i> sp.	Nd	SS263-38A

Nd - Not detected