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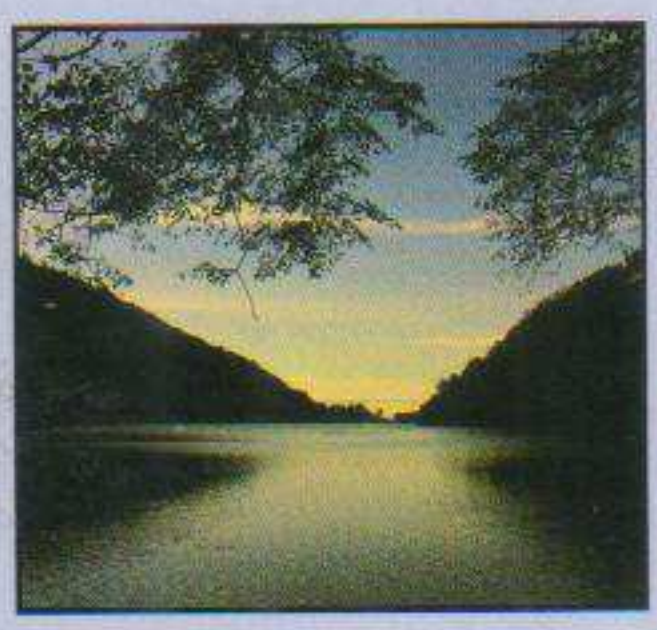

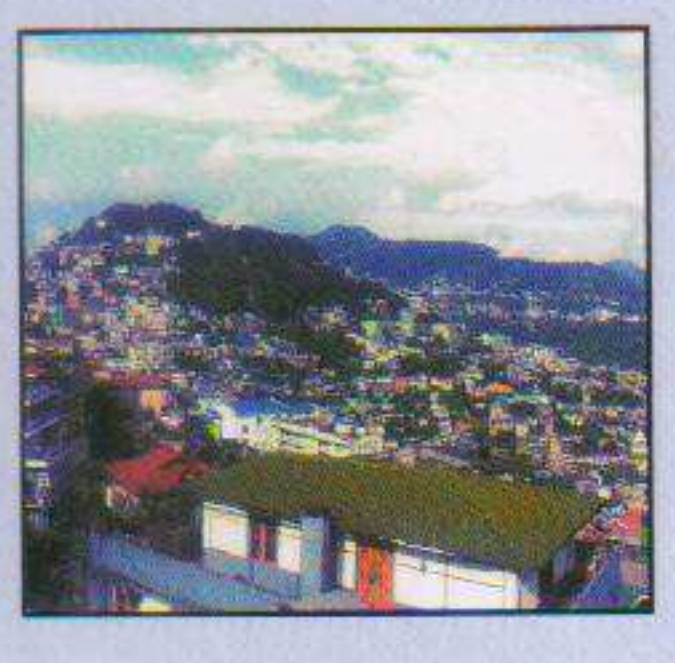

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Impact of climate change on heterotrophic bacterial communities in the water and sediment of kongsfjord in Norwegian Arctic

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Introduction

Cold environments represent a large part of the Earth's biosphere, and their microbiota are of increasing interest. Polar regions are of interest since they provide diverse terrestrial and marine habitats for psychrophilic and psychrotrophic microorganisms. The vast numbers of cold-adapted microorganisms which successfully inhabit these regions, are referred to as psychrophilic and psychrotolerant organisms, play vital roles in global elemental cycles and in the mineralization of organic matter (Jiao *et al*. 2010, Piontek, Borchard *et al*. 2012). Unlike deep oceans, polar marine environments are subject to large seasonal variations in sea-ice cover, greatly affecting the biology of organisms (Vadstein, 2011). Currently, the knowledge of polar microorganisms based on ecological and genomic perspectives is in the early phase of an exponential growth (Russo, R. *et al*. 2010, Iversen, K.R. & Seuthe, L., 2011).

The Arctic fjord Kongsfjorden located off the west coast of Spitsbergen have attracted the attention of scientists as a model site for studies on the impact of climate change in the Arctic (Piontek *et al*., 2011; Iverson and Seuthe, 2012) and they are regarded as key European sites for Arctic biodiversity monitoring[6]. Earlier studies in these fjords have focused both on the physical characteristics of the environment and the biotic components of the ecosystem (Hop, H., Pearson, T., *et al*. 2002, Shivaji S, Kiran M D & Chintalapati S 2007). Despite the fact that Arctic has a number of fjords comparatively little is known about the bacterial diversity of the fjords (Jankowska, K., Wlodarska-Kowalczuk, *et al*. 2005, Teske, A., Durbin, A. 2011).

Considering the extent of warming in the arctic region and the resultant changes in the dynamic marine environments there is a need to monitor the bacterial diversity in the fjord environments, especially in terms of cultivable bacteria. The present study reports the diversity of cultivable heterotrophic bacteria from the water and sediment samples of Kongsfjord, their growth responses to important environmental variables and ability to produce industrially important hydrolytic enzymes.

Materials and methods

Sampling was carried out from 4 stations in the Kongsfjord (Fig. 1), Norwegian Arctic on 4th July 2009. Kongsfjorden (79°58′N, 12°E) is a small fjord with a wide opening to the open ocean via Kongsfjordrenna. A sill in the middle of the fjord divides it in an outer part, strongly influenced by West Spitsbergen Current, and an inner part that is under the impact of 4 different glaciers namely, Kronebreen, Kongsvegen, Conwaybreen and Blomstrandbreen (Svendsen, H., Beszczynska-Möller, *et al.* 2002).

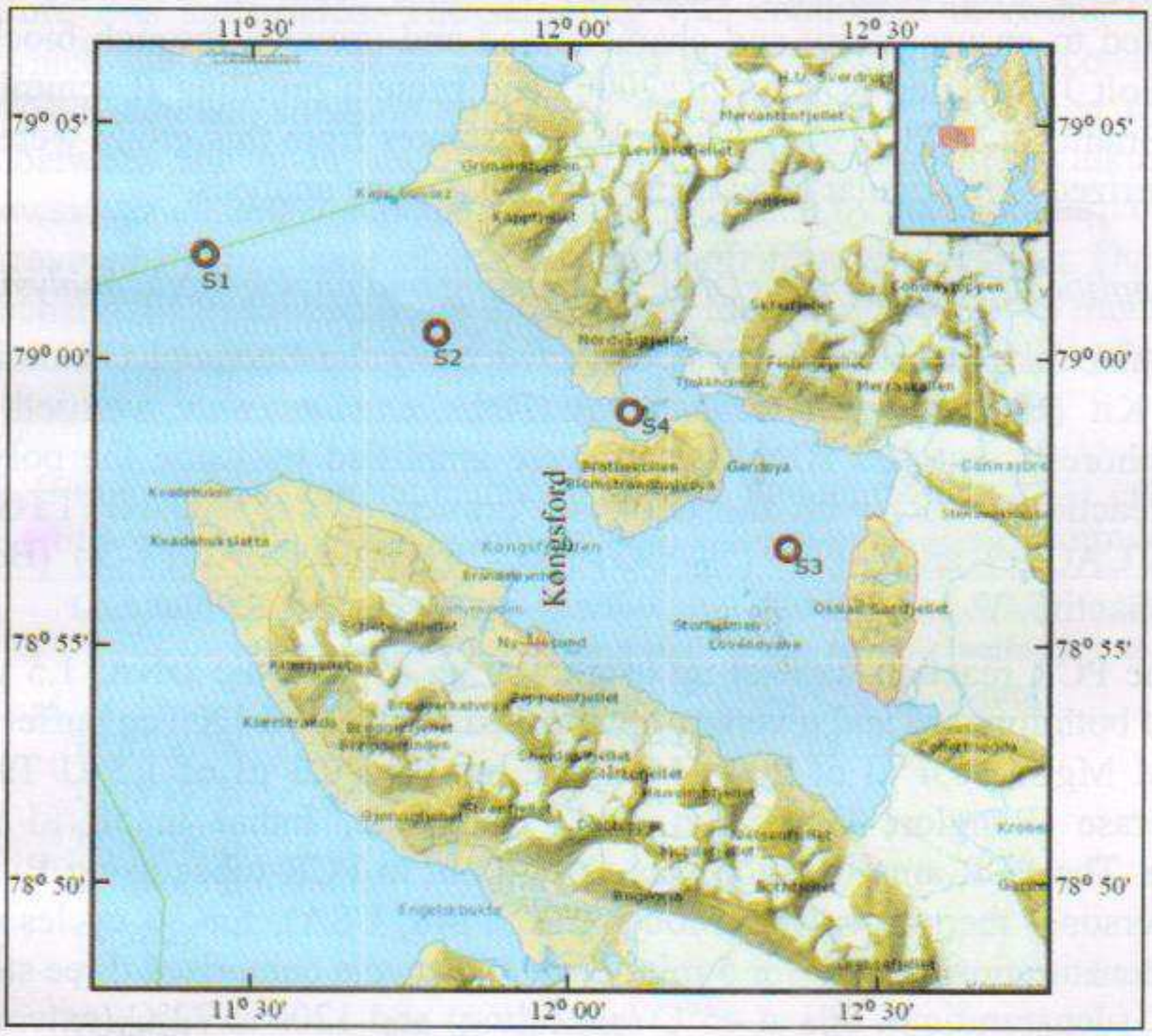

Fig. 1. Location of sampling sites in Kongsfjorden fjord, Ny Alesund, Arctic

The water and sediment samples were collected on board Research Vessel 'Teisten' and position of the sampling site was noted using the global positioning system (GPS, Raymarine E 120) system installed on-board Teisten. Water and sediment samples were collected with the help of Niskin sampler and Van Veen Grab (Model 12.320, KC, Denmark) respectively. Depth of the sampling site,

water temperature and salinity were measured using the STD/CTD instrument model SD204 (SAIV A/S Environmental Sensors and Systems, Norway). Soon after hauling up the sample on board, water and sediment temperature was checked by centigrade digital thermometer and salinity by refractometer (Atago, Japan). Water and sediment samples were collected aseptically in sterile Duran Schott glass bottles and polythene bags respectively. Samples were transported to the Kings Bay marine lab at the International Arctic Research Station within 4 hours of collection and processed for retrievable heterotrophic bacteria. Particle size analysis of the sediment samples was done by laser diffraction method using particle size analyzer (SympaTEC, Germany).

Isolation and characterization heterotrophic bacteria

The water and sediment samples were serially diluted and 0.2 ml of various dilutions was spread plated on Zobell marine agar (ZMA). Plates were incubated at 10°C for 1-2 weeks. Plates with 25-250 colonies from ZMA were selected for enumeration and expressed as cfu/ml or g of water and sediment samples respectively. Morphologically different colonies from ZMA were isolated, restreaked to ensure purity and characterized and grouped through biochemical tests (Holt J G, Krieg N R, *et al.* 2000) and protein profiling (Laemmli, U.K., 1970, Bradford, M.M., 1976). The selected isolates from this group were further characterized at molecular level using 16s rRNA gene analysis.

PCR amplification of the 16S rDNA, sequencing and phylogenetic analysis

Total bacterial genomic DNA was extracted using the Bacterial Genomic DNA (prep) Kit (Chromous Biotech, India). DNA extracts were verified by gel electrophoresis and 16S rDNA genes were amplified by using the polymerase chain reaction (PCR) with the universal primers 27f (5'-AGAGTTTGATCC-TGGCTCAG-3') and 1492r (5'- GGTTACCTTGTTACGACTT-3') (Bosshard, P.P. & Santini, Y. *et al.* 2000).

The PCR reaction was set up using 100 ng of genomic DNA, 1.5 µl of 10 pmol of both forward and reverse primers, 5 µl of standard 1X Taq buffer, 3 µl of 1.5 mM $MgCl_2$, 0.4 µl of 200 µM dNTP mix and 0.3 µl of 1.5 U Taq DNA polymerase (Banglore Genei Pvt. Ltd., Bangalore, India) in 50 µl reaction volume. The PCR amplification was carried out in PCR tubes using Biorad MJ Mini personal thermal cycler (Model PTC 1148, USA), for 30 cycles after an initial denaturation at 94°C for 5 min. Each PCR cycle comprised three steps: 30s at 95°C (denaturation), 30s at 45°C (annealing) and 120s at 72°C (extension). A final extension of 10 min was given at 72°C. PCR products were checked by gel electrophoresis and purified using the PCR Clean-Up Kit (Chromous Biotech, India) and sequenced using an ABi 3730 XL Genetic Analyser (Applied Biosystems, USA).

The almost complete 16S rRNA gene sequences (1418-1542 bases) obtained was subjected to BLAST sequence similarity search (http://blast.ncbi.nlm.nih.gov/BLAST) to identify the nearest taxa. 16S rRNA gene sequences were aligned

using the CLUSTAL W and phylogenetic trees were constructed using two tree making algorithms, maximum likelihood (ML) and Neighbour-joining (NJ) methods using MEGA version 5 (Tamura, K., Peterson, D., *et al.* 2011). Two consensus trees were finally constructed to place isolates from water and sediment samples.

Nucleotide sequence and accession numbers

All the 16S rRNA gene sequences of the strains selected from each group were deposited in GenBank with accession numbers JX262392 to JX262406.

Result and discussion

Exact geographical co-ordinates of the sampling station, depth, surface and bottom water temperature, sediment temperature and salinity of water are given in Table 1. While surface water temperature ranged from 3.61 (station III) to 6.13 (station I), bottom water temperature was around 1°C. Stations III and IV were much closer to the front moraines of two major glaciers and the lower temperature was anticipated. The sampling was conducted in Arctic summer, during which the oceanography of the fjord undergoes rapid and considerable change. The summer situation suggested an unobstructed exchange of water masses between the open sea, the shelf and the fjord itself (Walkusz, W., Kwasniewski, *et al.* 2009). Kongsfjord was influenced by Atlantic-derived water masses covered by thin layer of freshwater runoff from the glaciers. Due to the great discharge of freshwater from the glaciers, the stations closer to them shows strong stratification in temperature and salinity with the advancement of summer season. However, this gradient diminishes towards the fjord opening.

Table 1. Geographical location of sampling sites and physiochemical properties of water and sediment samples from Kongsfjord during summer 2009.

Sampling stations	Location	Depth of the station (m)	Surface Water temp. (°C)	Bottom Water temp. (°C)	Water salinity (ppt)	Sediment temp (°C)
Station I	79°02'36" N 11°19'14" E	320	6.13	0.8	33.69	1.0
Station II	79°01'22" N 11°43'06" E	185	6.51	2.3	33.56	1.5
Station III	78°57'29" N 12°19'27" E	45	4.14	0.8	33.13	-0.6
Station IV	79°00'01" N 12°02'50" E	110	3.61	1.2	33.11	-0.8

Due to the large scale land run-off of the melt water the inner basins of the Kongsfjord were found to be reddish brown, indicating massive influx of terrogenous sediments. The outer basins and the sampling station I at fjord opening were characterized by clear blue waters. Similar observations were reported previously at (Kongsfjorden Zajaczkowski, 2008, Piwosz, Walkusz *et al.* 2009).

Grain size analysis of the sediment samples revealed silty-clay nature of the sediments from all stations, except that from station III, which was of silty sand (Fig. 2). While silt fraction ranged from 67% at station III to 82% at station I, clay fraction ranged from 12% (station II) to 23% (station IV). Fairly large proportion of sand was noticed in the sediments of station III. As this station is closer to the front moraine of glacier, the sand transport mediated through the glacier might have contributed to this fairly significant sand fraction in the sediments at this station. The sediments from all the four stations were visually distinguishable in their color which varied from light brown to dark chocolate. The color variation may be due to variation in the organic content of the sediment and oxygenation at the bottom (Jorgensen *et al.* 2005).

Retrievable bacterial count

Retrievable bacterial load of four water and sediment samples were ranged from 1.22×10^3 to 1.68×10^3 cfu/ ml and 1.35×10^5 to 8.45×10^6 cfu/g respectively. The sediment samples from station III (inner to the fjord) showed highest retrievable count of bacteria than other stations. As expected more heterotrophic bacteria were retrieved from the sediment samples, which were nearly 2 log higher than the load encountered in the water samples. In general sediments act as a repository of bacteria and offer much more stable and protective environment for the survival of them. To the contrary, the conditions in the water column are highly dynamic, especially in the summer when there is a large scale influx of melt water, which can seriously alter the salinity, at least at the surface water, and pose stress and injury to the microbial community. Such injured cells, though viable, might not develop on the media, which in general have some inhibitory substances. Though the land run-off during the summer result in large scale influx of terrogenous organic carbon, which are more or less recalcitrant and offer little help by way of providing nutrition (Kirchman *et al.* 2009). The retrievable count encountered in the sediment samples were higher than those reported (Srinivas *et al.* 2009). Relatively higher density of cultivable bacteria were reported from inner shelf sediments (Zheng *et al.* 2009), which is similar to our observations.

Benthic bacterial communities in the ocean environment play a significant role in ecological and global biogeochemical cycle, because they can rapidly degrade and utilize particulate organic matter (Teske *et al.* 2011, Bowman *et al.* 2003, Michaud *et al.* 2004) and regulating the transformation of biogenic elements such as C, N, P, Fe, O, and S (Kirchman *et al.* 2009, Vandieken *et al.* 2006, Selje *et al.* 2004). Microbial community structure analysis is important for an understanding of ecosystem processes and in defining the roles that bacteria play in overall oceanic processes and bacterioplankton components is related to oceanic water masses and controlled by their environmental and biogeochemical properties (Cottrell *et al.* 2000). It is important to know which phylogenetic groups of bacteria dominate marine bacterioplankton communities because abundant groups may be proportionally more influential in carbon cycling and

other biogeochemical processes (Zengler *et al.* 2009). In culture-dependent methods, the bacteria obtained allow for detailed studies and provide more information about the physiological and metabolic characteristics of bacteria, and microbial response to environment change etc (Zeng *et al.* 2011, Denton *et al.* 1998).

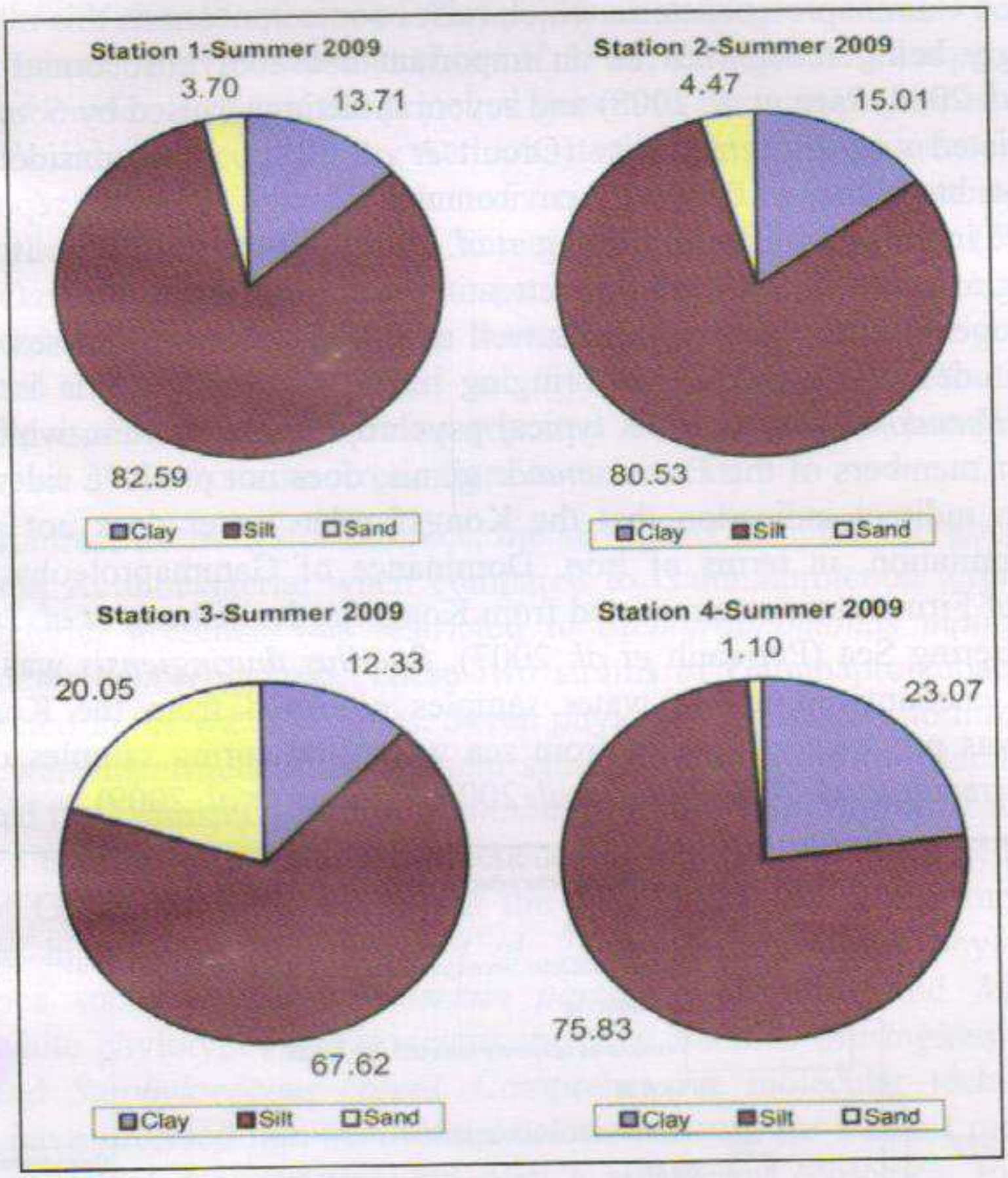

Fig. 2. Percentage particle size analysis of sediment samples from Arctic Ocean during summer 2009

Major groups of bacteria

A total of 298 bacterial strains were characterised from water and sediment samples and based on the biochemical test and protein profiing, the isolates could be categorised into 33 groups. The nearest phylogenetic neighbour of the selected 33 isolated strains from each group were identified following BLAST analysis of the 16S rRNA gene sequence. Sequence similarity of the strains compared to the nearest phylogenetic neighbour ranged from 97 to 100% (Table 2). Based on 16S rRNA gene sequence similarity, the isolates were categorised into 8 phylotypes belonging to two phyla (Fig. 3) groups from water samples. Gammaproteobacteria and Bacilli were the two bacterial phyla identified from the surface water samples of Kongsfjord in the present study (Fig. 3). Gammaproteobacteria was

the major group with more than 80% of occurrence. The Gammaproteobacteria group also showed good diversity with various heterotrophic bacteria such as *Enterobacter ludwigi, Enterobacter cancerrogenous, Halomonas boliviensis, Pseudomonas sabulinigri, Pseudomonas fragi, Pseudomonas koreensis* and *Stenotrophomonas maltophila. Stenotrophomonas* strains formed major share of the isolated Gammaproteobacteria which raises some concern as this organism is increasingly being recognized as an important cause of nosocomial infection (Kwa *et al.* 2008, Paez *et al.* 2008) and severe infections caused by *S. maltophila* are associated with high mortality (Orcutt *et al.* 2011). It is considered as an uncommon bacterium in the arctic environment, though there are reports of its occurrence in the marine realm (Zheng *et al.* 2009, Zeng *et al.* 2011). It would be interesting to study whether the fast retreating ice cover of the Arctic Ocean and resultant opening up of sea routes as well as mixing of water masses from the lower latitudes playing a role in bringing more mesophilic fauna into higher latitudes. *Pseudomonas fragi* is a typical psychrophilic bacterium, which unlike most other members of the *Pseudomonas* genus, does not produce siderophores. This is an indirect indication that the Kongsfjorden water does not pose any nutrient limitation, in terms of iron. Dominance of Gammaproteobaceria and presence of Firmicutes were reported from Kongsfjorden (Zheng *et al.* 2009) and northern Bering Sea (Perreault *et al.* 2007). *Bacillus thuringiensis* was the sole Firmicute encountered in the water samples collected from the Kongsfjord. *Bacillus* was previously reported from sea water and spring samples of Arctic Ocean (Perreault *et al.* 2008, Zeng *et al.* 2004, Srinivas *et al.* 2009).

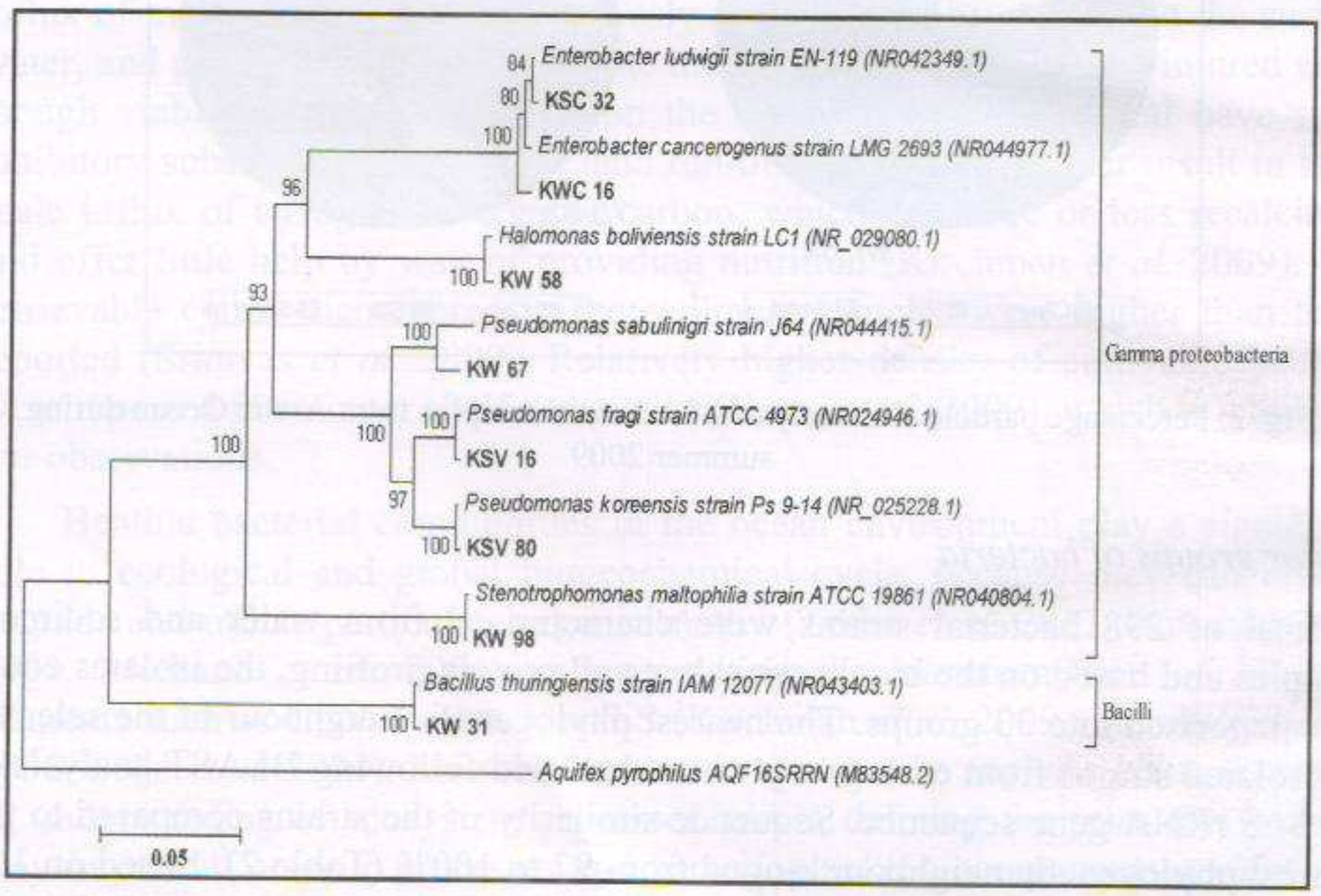

Fig. 3. Phylogenetic trees based on 16S rRNA gene sequences showing the relationship of bacterial strains obtained from water samples collected from Kongsfjord, Arctic, with their nearest phylogenetic relatives

Predominance of Gammaproteobacteria group has been reported from the Arctic fjords of Spitzbergen and from spring samples of Arctic was reported previously (Fogg 1986, Groudieva *et al.* 2004). Reported five different ARDRA profiled *Pseudomonas* from sea ice and sea water from fjords of Spitzbergen, Arctic Ocean. These reports are in agreement with present findings and we have isolated three different species of *Pseudomonas* namely *P. sabulinigri, P. fragi* and *P. koreensis*. Previous studies (Li *et al.* 1999, Yu *et al.* 2006) support the idea that *Pseudomonas* is one of the principal genera of bacteria present in the Arctic Ocean. We did not encounter any Actinobacteria, though the sequences affiliated with the Actinobacteria were detected both in surface and bottom water in Kongsfjorden (Zheng *et. al.* 2009). Three species of *Pseudomonas* (*P. fragi, P. koreensis and P. sabulinigri*) and two species of *Bacillus* (*B. thuringiensis and B. flexus*) encountered in this study were not reported previously from the Kongsfjord. Interestingly we could not find any *Cytophaga – Flavobacterium* group, the isolation of which was reported in some of the previous studies (Reddy *et al.* 2009) from the Arctic fjord environments.

In contrast to the water samples, the sediments samples had good share of Bacilli and Actinobacteria, when compared to Gammaproteobacteria. Gammaproteobacterial presence was restricted to *Stenotrophomonas maltophila* and *Enterobacter cancerogenous*. These two strains of Gammaproteobacteria were also detected in the water samples. Seven phylotypes belonging to three phyla of bacteria were identified from sediment samples (Fig. 4). While Bacilli (42.8%) dominated the sediment samples, *Actinobacteria* and Gammaproteobacteria were equal (28.6%) (Fig. 4). Culture independent methods also reported predominance of Gammaproteobacteria from the cold saline sediment samples of the Canadian high Arctic (Perreault *et al.* 2008) Actinobacteria phyla included phylotypes such as *Brachybacterium paraconglomeratum* and *Micrococcus luteus*, while phylotypes of Firmicutes included *Bacillus thuringiensis, Bacillus flexus* and *Staphylococcus cohnii*. Comprehensive molecular techniques and surveys have revealed that the Actinobacteria account for a small proportion in marine microbial communities, but play a significant ecological role and are ubiquitous component of marine microbial communities. Occurrence of Actinobacteria, Bacilli and Gammaproteobacteria phyla were reported from the fjord sediments of Arctic. However, phylotypes showed variations which could be expected in a highly dynamic environment such as marine environment. The phylotypes of Gammaproteobacteria encountered in the present study included *Stenotrophomonas maltophila* and *Enterobacter ludwigii*. Considerable share of *Stenotrophomonas maltophila* calls for further studies on its virulence and antibiotic resistance properties as it is fast emerging as a bacteria of public health significance. We are in the process of microbial source tracking studies to look at the molecular markers in this organism. Since most of the species identified from water and sediment samples of present study are not reported earlier from Kongsfjorden, present findings give additional knowledge in culturable bacterial diversity of Kongsfjord.

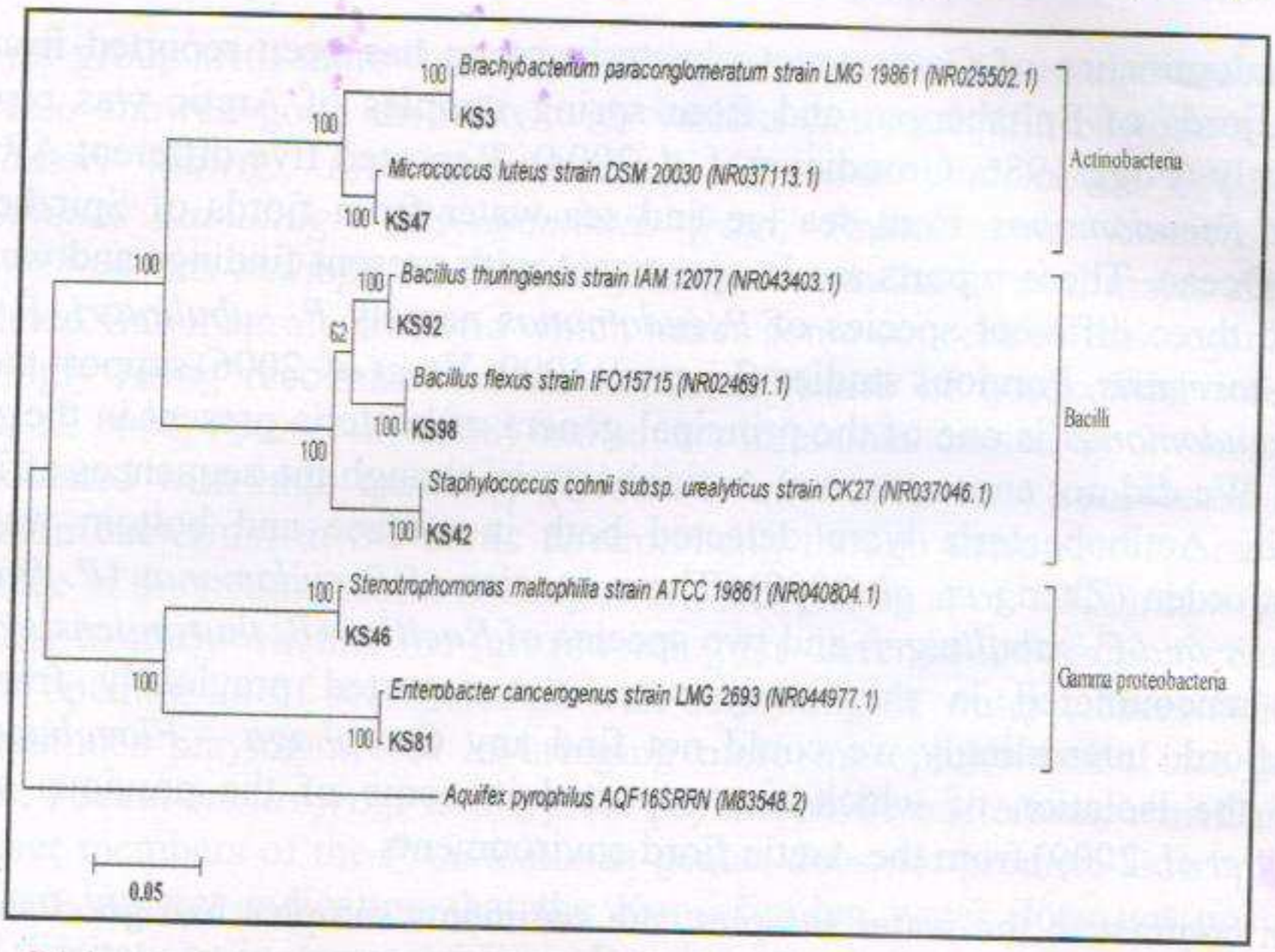

Fig. 4. Phylogenetic trees based on 16S rRNA gene sequences showing the relationship of bacterial strains obtained from sediment samples collected from Kongsfjord, Arctic, with their nearest phylogenetic relatives.

Two *Bacillus* sps. and one *Staphylococcus* sps were identified as Firmicutes from sediment samples (Table 2). Actinobacteria represented by *Brachybacterium* and *Micrococcus* sps. and Gammaproteobacteria by *Stenotrophomonas* and *Enterococcus* (Figure 4). *Bacillus* was also identified from spring samples of Arctic (Perreault *et al.* 2008, Groudieva *et al.* 2004) and melt water stream sediment of Arctic glacier. Though *Staphylococcus* from the spring samples of high Arctic (Groudieva *et al.* 2004), *Stenotrophomonas maltophilia* was not reported from the Arctic Ocean environment.

Table 2. Identification of the bacterial strains isolated from water and sediment samples of Kongsfjord during summer 2009 based on BLAST analysis of the 16S rRNA gene sequences.

S N.	Strain number	Gene accession number	Nearest phylogenetic relative	16S rRNA gene sequence similarity (%)
1	KWC 3, **KWC 16***, KSC 17	JX262394	*Enterobacter cancerogenus* LMG 2693 (NR044977)	98.59
2	KSC 32, KSV 1, KSV 33, KW 17, KW 42, **KW 93**	JX262395	*Enterobacter ludwigii* EN-119 (NR042349)	99.64
3	**KSV 16**	JX262396	*Pseudomonas fragi* strain ATCC 4973 (NR024946.1)	99.71
4	**KSV 80**	JX262397	*Pseudomonas koreensis* strain Ps 9-14 (NR025228)	99.85

5	KW 27, KW 43, KW 44, KW 45, **KW 98**	JX262398	*Stenotrophomonas maltophilia* strain ATCC 19861 (NR040804)	99.78
6	**KW 31**	JX262401	*Bacillus thuringiensis* strain IAM 12077 (NR043403)	99.43
7	**KW 58**	JX262399	*Halomonas boliviensis* strain LC1 (NR029080)	99.43
8	**KW 67**	JX262400	*Pseudomonas sabulinigri* strain J64 (NR044415)	97.7
9	KS 1, KS 7, KS 15, KS 27, **KS 46**	JX262392	*Stenotrophomonas maltophilia* strain ATCC 19861 (NR040804)	99.71
10	**KS 3**	JX262402	*Brachybacterium paraconglomeratum* strain LMG 19861 (NR025502)	99.78
11	**KS 42**	JX262403	*Staphylococcus cohnii* subsp. urealyticus CK27 (NR037046)	100.00
12	**KS 47**	JX262404	*Micrococcus luteus* strain DSM 20030 (NR037113)	99.63
13	KS 51, **KS 92**, KS 96, KS 101	JX262405	*Bacillus thuringiensis* strain IAM 12077 (NR043403)	99.50
14	**KS 81**	JX262393	*Enterobacter cancerogenus* strain LMG 2693 (NR044977)	99.32
15	**KS 98**	JX262406	*Bacillus flexus strain* IFO15715 (NR024691)	100.00

*Bold strain numbers are selected for sequencing

Conclusion

Retreivable heterotrophic bacteria from the water and sediment samples of Kongsfjord were dominated by psychrotolerant strains, indicative of more warm water intrusion into the Kongsfjord. Some species of bacteria identified from Kongsfjord are not reported from earlier; especially three species of *Pseudomonas* and two species of *Bacillus*. Relatively high level of isolation of *Stenotrophomnas maltophila* calls for further studies on this organism, especially in terms of source tracking as well as related to virulence and antibiotic resistance.

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