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Optimization of sample preparation – filtration and DNA extraction – for the analysis of seawater samples

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Report summary

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<i>Ágríp á íslensku:</i>	<p>Lítið er vitað um örverur eða fjölbreytileika örverusamfélaga á Íslandsmiðum en þær gegna mikilvægu hlutverki í vistkerfi hafsins. Nauðsynlegt er að rannsaka örverufræði hafsins í kring um Ísland með nýjum og öflugum aðferðum sem byggja á sameindalíffræði. Við slíka vinnu skiptir gæði sýna og sýnaundirbúningur mjög miklu máli.</p> <p>Í þessari rannsókn var gerð forkönnun á sjósýnum, sýnatöku og sýna-meðhöndlun áður en sýni verða tekin í miklu magni. Fyrst voru sýni tekin úr smábátahöfninni í Reykjavík til forathugunar og svo var haldið lengra með sýnum úr rúmsjó. Skoðaðar voru heimtur með tilliti til DNA magns og hversu vel tókst til að magna upp erfðaeftni örveranna með PCR. Niðurstöðurnar sýndu að besta aðferðin var aðkeypt DNA einangrunarsett sem einangraði mest af DNA og var magnanalegt með PCR. Ódýrari og fljótvirkari aðferð með sjálfvirku einangrunartæki og heimatilbúnum hvarfefnum reyndist einnig mjög vel þar sem sambærilegar niðurstöður fengust úr PCR mögnun þó svo að lægri DNA heimtur fengust. Út frá þessum niðurstöðum er unnt að setja upp verkferla sem byggja á sjálfvirkri DNA einangrun sýna en notkun aðkeyptrar einangrunarsetta á erfiðari sýni. Fyrirhugað er að nota þessar niðurstöður við sjósýni úr vorralli Hafrannsóknarstofnunarinnar.</p>		
<i>Lykilorð á íslensku:</i>	<i>Íslandsmið, sjór, örverur, fjölbreytileiki, sýnaundirbúningur</i>		
<i>Summary in English:</i>	<p>The knowledge on microbial diversity and community structure in Icelandic seawater is scarce at present despite their important role in ocean ecology. The agenda is to increase our knowledge in this field by applying recent and powerful analytical tools. In order to do that it is essential to have access to high quality samples and sample preparation procedures.</p> <p>In the present study sea sample preparation was studied with aim of comparing different methods and optimizes the workflow. Samples from a harbour in Reykjavík and open sea samples were used for this purpose. The results showed that an extraction method based on an Epicentre kit gave the best results regarding DNA recovery from the samples and suitability in a PCR amplification. However, a method based on semi-automatic protocol and in house reagents proved to be more cost effective and showed comparable performance with PCR suitability of the samples although a lower DNA recovery was obtained. From these results it is now possible to establish an efficient work flow for microbial diversity analysis of sea samples using an automated method as a first choice with the option of more costly method for more challenging samples.</p>		
<i>English keywords:</i>	<i>Icelandic seawater, microbes, diversity, sample preparation</i>		

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1 Introduction

The exposure of marine microbial structures with the help of modern bioanalytic tools is becoming more and more important. At least the fast development of high efficient and comfortable technologies like real-time PCR, microarrays, and sequencing robots during the last decades were important steps for the realization of such ambitious analyses. At least cultivation methods are more time consuming and above all in many cases there is no knowledge about cultivation methods for some species. There are a vast number of microscopic life forms inhabiting the world's oceans. According to nominal cell counts more than 10^5 cells occupy one ml of surface sea water (Porter and Feig 1980, Hobbie *et al.* 1977) which means that projected $3.6 * 10^{29}$ microbial cells inhabit the oceans with a total cellular carbon content of $3 * 10^{17}$ g (Whitman *et al.* 1998). Bacteria, archaea, protists, and unicellular fungi are the biggest part of the oceanic biomass accounting 98% of primary production (Whitman *et al.* 1998, Atlas and Bartha 1993) and mediate all biogeochemical cycles in the oceans (Atlas and Bartha 1993).

In particular there is a substantial interest for the exploration of the microbial diversity around the Iceland Sea which so far has not been examined and thus represents a black box regarding to the prevailed marine ecosystem. The changing of the microbial flora with changing local external factors represents therefore an interesting part of marine exploration.

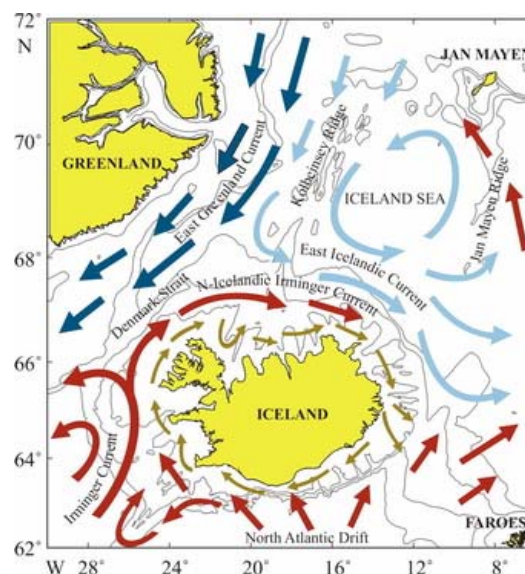


Fig.1.1 Ocean currents surrounding the Icelandic coast

One important factor that affects the marine life of Iceland is the differing water conditions around the island caused by the surrounding current system. Iceland is

located 63-67°N and 18-23°W. Nevertheless, the climate along the west and south coast is quite moderate because a branch of the Gulf Stream, the so called Irmiger Current, flows along these coast parts (see also Fig.1.1) leaving warm sea temperatures that rise to over 10 °C in the summer and bringing saline Atlantic water. On the other hand there is the East Greenland Current that flows west of Iceland bringing cold water with low salinity. A branch of that current, the East Icelandic Current, approaches the northeast- and east coasts (Fig.1.1) inducing water temperatures which only slightly rise to over 8 °C in the north and remaining 8 °C in the east (Jónsson and Valdimarsson 2007).

Above all the analysis of water samples taken directly after the eruption of Eyjafjallajökull volcano at the 18th of April 2010 along the south coast of Iceland where the molten glacial ice mixed with the volcanic ash flew into the surrounding sea, was of particular interest in order to gain new information about microorganisms of extreme habitats.

The 16 S rRNA gene is present in all living bacteria and archaea and its evolutionary rate is suitable to distinguish between genera and most species which makes it an ideal instrument for these ecological studies (Green and Noller 1997). 16 S rRNA plays an important role in the biosynthesis of living cells where it is a key factor in translation of mRNA to proteins (Green and Noller 1997). Highly conserved three dimensional structures such as hairpins and helices are important for binding of other molecules. These conserved areas can be used as priming sites for so called universal primers that are able to hybridize to most bacteria present in a sample which makes it possible to amplify the detected organisms for a successful analysis.

The initial point of this working part is a new sequencing device, the so called Roche 454 GS-FLX system using Titanium Chemistry (CD genomics) that promises unprecedented sensitivity and reliability for a range of high throughput sequencing applications. It is said to obtain up to 500 Mb per run with between one and sixteen samples with an average read length of 300 to 500 bp.

Aim of the present work

The aim of the present work is to find a reliable procedure of sample preparation that will allow a successful detection and analysis of microbial diversity, especially for bacteria and archaea that inhabit the Iceland Sea. The basis to identify microorganisms with the help of bioinformatics tools by 16°S clone-analysis and sequencing is a good, reliable and fast DNA extraction method that will guarantee a high yield. Therefore three different DNA isolation methods were tested and compared with each other to find the best opportunity.

2 Materials and Methods

2.1 Sampling spots

2.1.1 East Reykjavík harbor side

20 l of seawater had been taken from East Reykjavík harbor side which were provided as trying samples for the methods that are going to be used on the water samples taken from spots swayed by the volcano eruption of Eyjafjallajökull (subitem^o2)



Fig.2.1 Sampling spot (harbor side, East Reykjavík) for the harbor water samples used for pre-experiments

2.1.2 South coast of Iceland, district south of Eyjafjallajökull

Due to the eruption of the Eyjafjallajökull at the 18th of April 2010 water samples (each with a volume of 500 ml) had been taken at the south coast of Iceland on 13 different spots (Fig.2.2) at diverse depth (see Appendix Tab.A.1) where the molten glacial ice combined with the volcanic ash was flown into the sea and dispensed by prevail currents.

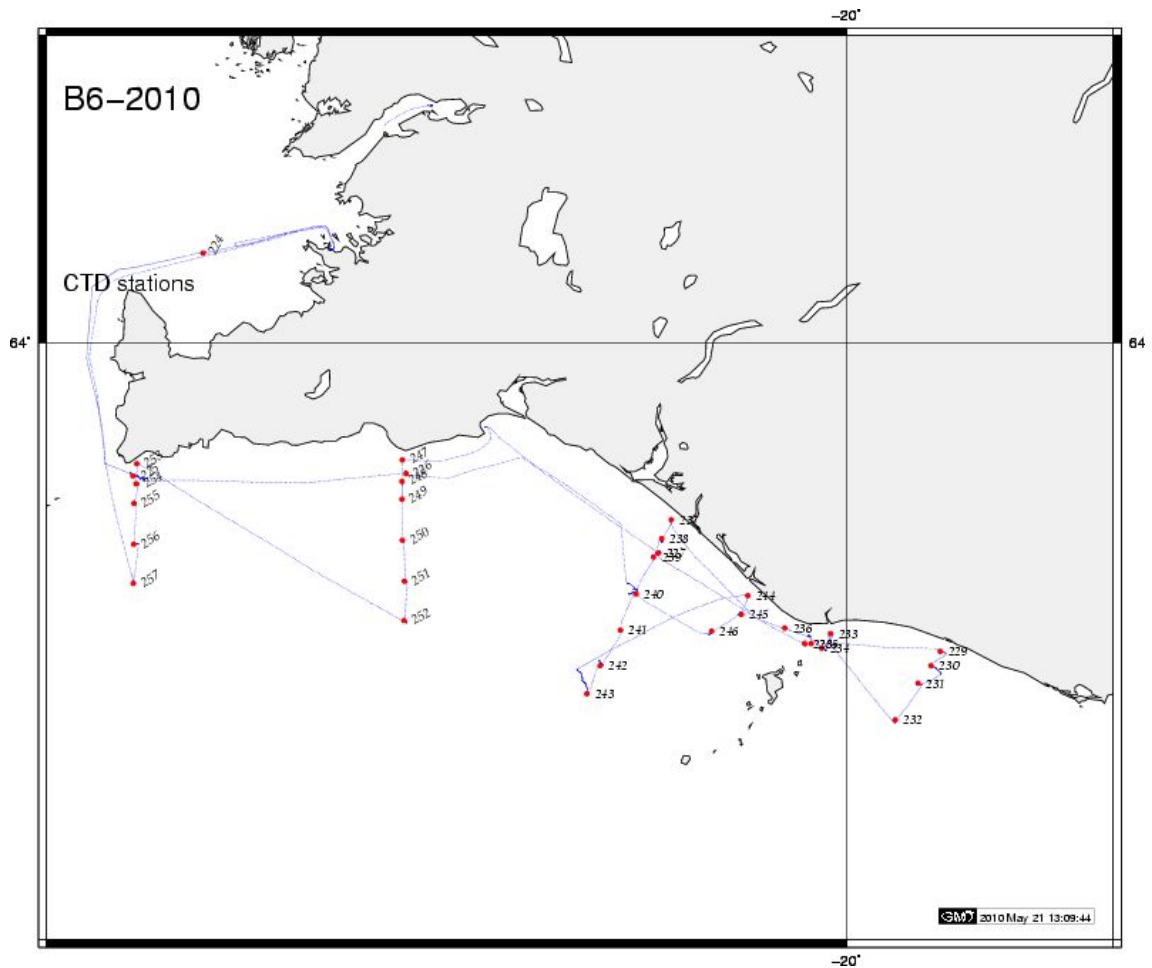


Fig.2.2 Sampling spots of the sea water samples taken from the South coast of Iceland after the volcano eruption of Eyjafjallajökull at 18th of April.

2.2 Sample preparation (filtration)

Materials

- Water samples
- Sterile Filters (pore size: 0,45 μm)
- Petri dishes
- Seawater
- Motor driven pump connected with the filtering device
- Plastic hopper

Procedure

1. East Reykjavík harbor side

The filtration was implemented under sterile conditions with a filtering device actuated by a motor driven pump. Different volumes were tested, including: 50, 100, 250 and 500 ml. After filtration the filter was put into a Petri dish where it was spiked with four ml of sterile seawater. The filter coating was removed from the filter by pipetting up and down or by carefully scraping with the pipet tip and mixed with the seawater. The volume of 4 ml was subdivided into two 2 ml portions from which one was directly centrifuged at 8000 x g for 10 min at 4 °C. After the supernatant had been discarded the fluid of the second vessel was added to the pellet and a further centrifugation under the same conditions followed. The supernatant was discarded and the pellet stored at – 20 °C for later extraction.

2. South coast of Iceland, district south of Eyjafjallajökull

From each sample 10 ml of liquid were taken out of the bottle, transferred to a separate vessel and stored for later experiments. The entire remaining volume was filtered under sterile conditions with a filtering device actuated by a motor driven pump. After filtration the filter was put into a Petri dish where it was spiked with 4 ml of sterile seawater. The filter coating was removed from the filter by pipetting up and down or by carefully scraping with the pipet tip and mixed with the seawater. The total volume of 4ml was subdivided into two equal portions from which one was used for fixation. The other sample was centrifuged at 8000 x g for 10 min at 4 °C, the supernatant discarded and the pellet frozen at –80 °C for later extraction.

2.3 Extraction

Three different extraction methods have been employed and checked against each other to find out the best method for the analysis of the seawater samples.

2.3.1 Extraction with Kingfisher instrument

Materials

- Sample material (water sample pellets)
- Magnesil Kf genomic DNA extraction lysis buffer (Promega, item MD 1521)
- Nanoparticles (UK beads)
- Wash solution 1 (50 mM Tris-HCl, 750 mM NaCl, pH 7.0)
- Wash solution 2 (25% ethanol, 25% isopropanol, 100 mM NaCl, pH 7.0)
- Sterile ddH₂O
- Kingfisher nucleic acid extraction instrument (Thermo Fisher Scientific Inc)
- 100 µl trays for Kingfisher extraction instrument (Thermo Fisher Scientific Inc)

Procedure

After thawing 200 µl of lysis buffer were added to each pellet, vortexed vigorously and incubated at RT for 45 min. During incubation time a 96 well tray was prepared for the DNA extraction with Kingfisher instrument as shown in Tab.2.1. The UK beads (1°mg/sample) had been washed twice with sterile ddH₂O before they were placed into the tray. After lysis the sample was centrifuged briefly to collect potential drops from the rim of the tube at the bottom and then dispersed in wells A, B and C of the corresponding column. For extraction the tray was placed into the Kingfisher instrument and the appropriate program was selected. Extraction time took approximately 40 min.

Tab. 2.1 Allocation example for a 12 samples tray. One sample is processed through one column by the Kingfisher instrument. The respective sample volume (~ 200 µL) was dispensed into 3 wells (e.g. A1, B1, C1 for sample 1). Wells of row D and E were filled with 100 µL wash solution 1, wells of row F and G with 100 µL wash solution 2. For the elution step (row H) 50 µl ddH₂O were put into each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample
B	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample
C	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample	25 µL beads + 1/3 sample
D	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1
E	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1	Wash solution 1
F	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2
G	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2	Wash solution 2
H	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O	Sterile ddH ₂ O

2.3.2 Extraction with MasterPure™ DNA Purification Kit

(Method of gentle salt-precipitation)

Materials

- Sample material (water sample pellets)
- MasterPure™ DNA Purification Kit (Epicentre Biotechnologies) including:
 - Tissue and Cell Lysis Solution
 - MPC Protein Precipitation Reagent
 - TE Buffer
 - Rnase A (5 µg/µl)
 - Proteinase K (50 µg/µl)
- Isopropanol (absolute)
- Ethanol (75%)

Procedure

For each sample 1 µl of Proteinase K was first diluted in 300 µl Tissue and Cell Lysis Solution. 300 µl of this mixture were added to the thawed pellet and mixed thoroughly. While incubating at 65 °C for 15 min each sample was mixed every 5 min. After having been cooled down to 37 °C 1 µl of Rnase A was added to each sample and mixed thoroughly followed by a further incubation at 37 °C for 30 min. After lysis the samples had been placed on ice for 3 – 5 min. For precipitation 175 µl of MCP Protein Precipitation Reagent were added to 300 µl of lysed sample and mixed vigorously for 10 s. The cell debris was spun down by centrifugation at 10000 x g for 10 min and the supernatant transferred to a clean new microcentrifuge tube. 500 µl of isopropanol were given to each supernatant and every tube was inverted 30 - 40 times. To form a DNA pellet the samples were centrifugated at 4 °C for 10 min. Without dislodging the pellet the isopropanol was carefully pipetted off followed by two rinsing steps with ethanol (75%). After all residuals of ethanol had been removed the DNA was resuspended in 50 µl of TE Buffer.

2.3.3 Lysis Extraction

Materials

- Sample material (water sample pellets)
- Lysis buffer
(50 mM Tris, 1 mM EDTA buffer (pH 7.6), 0.5% (v/v) Tween 20)
- Proteinase K (20 mg/ml)

Procedure

200 μ l of lysis buffer were added to each pellet, thoroughly mixed and spiked with 2 μ l of Proteinase K. During incubation in the water bath for 2 h at 55 °C all reaction vessels had been mixed thoroughly every 30 min. Subsequently a final incubation for 5 – 7 min at 95 °C was implemented in the thermoblock.

2.4 Concentration determination with the NanoDrop

Concentration and purity of the extracted nucleic acid have been determined with the NanoDrop ND-1000 Spectrophotometer. 1 μ l of each sample was pipetted onto the sensor, a fiber optic cable, and the photometric measurement was automatically conducted by the device. The relative elution reagent was used for blank measurement. The concentration of nucleic acid is measured in ng/ μ l by the device. Moreover the purity of the sample is determined by calculating the quotient of the sample absorption at 260 nm and 280 nm, as well as 260 nm and 230 nm.

2.5 Gel electrophoresis

Materials

- Prepared Agarose gel solution (2%) (stored at 60 °C)
- Ethidium bromide
- TAE (Tris-acetate-EDTA) buffer (1 x working solution: 40 mM Tris acetate, 1 mM EDTA)
- 100 bp DNA ladder (100 bp – 2000 bp separated as follows: 100, 200, 300, 400, **500**, 600, 700, 800, 900, **1000**, 1500, 2000)
- 1 kbp DNA ladder (0.5 kbp – 10.0 kbp separated as follows: 500, 1000, 1500, 2000, 2500, 3000, 4000, 5000, 6000, 8000, 10000 bp)
- loading dye
- mould and comb
- Gel electrophoresis chamber
- Voltage supply source
- Transilluminator

Procedure

100 ml of the ready to use agarose gel solution had been transferred with 3 µl of ethidium bromide and gently spilled into the mould. Possible disturbing bubbles were carefully shifted to the side with a pipet tip. A comb was placed into the mould which had been removed after 15 min of hardening time. During hardening time 5 µl of each DNA sample had been mixed with 2 µl loading dye. The solidified gel was placed into the electrophoresis chamber and TAE buffer was added to cover the gel. The wells were filled with the prepared DNA samples as well as 8 µl of each 100 bp and 1 kbp DNA ladder. Gel run was carried out at 100 V, 400 mA for 40 min. The result was visualized with the transilluminator under ultraviolet light and documented by camera.

2.6 PCR (Teg-System)

Materials

- Mastermix (for 35 μ l)
 - ddH₂O 25,00 μ l
 - Buffer (contains 1,5 mM MgCl) 4,00 μ l
 - dNTPs 1,60 μ l
 - BSA (20 mg/ml) 2,00 μ l
 - Primers
 - forward F9 (10 μ M) 0,80 μ l
 - reverse R 805 (10 μ M) 0,80 μ l
 - Length of amplicon: 800 bp
 - Teg polymerase (3 U/ μ l) 0,80 μ l
(Matis-Prokaria, Reykjavík, Iceland)
- PCR plastic consumables
(Tubes with flat lids or tray with sealable film)
- Real-time PCR instrument (Mx3005 Stratagene)
- Template DNA 5,00 μ l

Procedure

According to the number of reactions the needed volumes for the mastermix had been calculated and the components were mixed together under the PCR hood. Each well was filled with 35 μ l of the mastermix. 5 μ l of DNA template were added to the accordant reaction vessel (outside of the PCR hood) and well mixed. To monitor any kind of cross contamination 5 μ l of ddH₂O were taken instead of sample material as no template control (NTC). After sealing the tray/stripe was carefully centrifuged to bring all reagents to the bottom of the tube. Assuring that there were no bubbles within the mixture the plate/stripe was positioned inside the thermocycler instrument.

The used thermal profile was as follows:

Initial temperature	95 °C (3 min)
Cycling (x 35)	
Denaturation	95 °C (50 s)
Annealing	52 °C (50 s)
Extension	72 °C (60 s)
Finale elongation	72 °C (10 min)

2.7 Gel Band Purification

Material

- Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) including:
 - Capture buffer type 3
(Contains pH indicator. Yellow or pale orange color: Capture buffer sample mix is at optimal pH for efficient DNA binding to the silica membrane. Dark pink or red color: pH is too high to achieve efficient DNA adsorption to the silica membrane.)
 - Wash buffer type 1
 - Elution buffer type 6 (sterile nuclease free water)
 - Illustra™ GFX™ MicroSpin™ columns
 - Collection tubes
- Cutted Gel Band

Procedure

After the wished gel band was cut out the agarose gel with a clean scalpel and put into a new, clean 1.5 ml microcentrifuge tube ca. 400 µl of capture buffer type 3 were added till the gel slice was completely covered. The components were mixed by inversion and then incubated at 60 °C for 15-30 min and 350 rpm until the agarose was completely dissolved. For each purification, one GFX MicroSpin column was placed into one Collection tube. The Capture buffer type 3-sample mix was centrifuged briefly to collect all liquid at the bottom of the tube, transferred onto the column and incubated for 1 min at RT followed by one centrifugation step at 16,000 x g for 30 s. The flow through was discarded and 500 µl of Wash buffer type 1 solution pipeted onto the column. The column containing tube was spun once again for 30 s at 16,000 x g and after it the collection tube was discarded and the column transferred to a new clean one. 50 µl of elution buffer type 6 were added to the center of the membrane, incubated for 1 min at RT and then centrifuged at 16,000 x g for 1 min to recover the purified DNA. The purified samples were stored at -20 °C for further experiments.

3 Experiments and Results

3.1 Comparison of three different extraction methods using the example of harbor water

With the ambition to find a good, fast and efficient extraction method that yields to a high amount of isolated nucleic acid and thus is useful for the DNA extraction of the volcano water samples from the South coast of Iceland, three different extraction methods were tested and compared with each other. For this test the partially automated “Kingfisher extraction” method based on the use of paramagnetic particles was chosen on the one hand. Further the precipitation based “MasterPure™ DNA Purification Kit” (EPICENTRE) and a Lysis extraction that only includes lysis buffer and Proteinase K as chemical reagents were consulted.

The harbor water was used as sample material for this pre test to avoid loss of the precious water samples from the South coast. To get an impression of the sensitivity of each extraction method four different volumes (50 ml, 100 ml, 250 ml and 500 ml) were filtrated and treated as described in Materials and Methods 2.2. In case of the first implemented extraction with the Kingfisher instrument a fit volume of 750 ml was also tested. But as it took a long lasting filtration time this volume was excluded for the other extraction methods. The pellets were used for the extraction which was carried out as described in Materials and Methods 2.3.

As can be seen in the digital image of the pre PCR agarose gel run (Fig.3.1) the “Kingfisher extraction method” leads to clearly visible high molecular DNA containing bands at the filtration volumes 500 and 750 ml. In addition one hardly recognizable band was observed in case of 250 ml filtration volume. In the case of the sample volumes 50 and 100 ml no band can be seen. This result is reflected by the concentration values established with the spectrophotometric measurement with the NanoDrop (Tab.3.1). Reliable values were achieved in case of the 500 and 750 ml samples. The other values are beneath the detection limit. With the “Epicentre Kit” clearly visible bands of high molecular DNA were obtained throughout all filtration volumes. Beyond an increase of band intensity with increasing filtration volume can be noticed. This increase of intensity with rising volume is supported by the NanoDrop values (Tab.3.1). In case of the “Lysis Method” no bands were detected on the agarose gel despite relatively high concentration values shown in Tab.3.1. But in case of the Lysis extraction the values for the ratio of sample absorbance at 260 and 280 nm were lower than 2.0, the value for pure DNA. The values for the ratio of sample absorbance

at 260 and 230 nm were beneath the range of 1.8 – 2.0 and therefore beneath the purity value for nucleic acid.

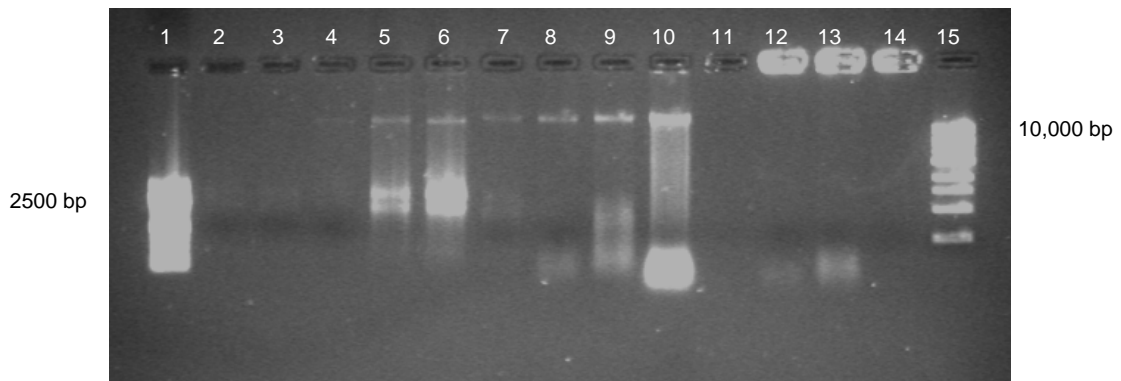


Fig. 3.1 Digital image of a 2% agarose gel run (pre PCR) showing the comparison of the three different extraction methods “Kingfisher”, “MasterPure™ DNA Purification Kit” (EPICENTRE) and “Lysis Method” using the example of harbor water in different volumes. Line 1: 100 bp ladder. Line 2 – 6: “Kingfisher instrument”. 50, 100, 250, 500, and 750 ml (from left to right). Line 7 – 10: “Epicentre Kit”. 50, 100, 250 and 500°ml (from left to right). Line 11 – 14: “Lysis Extraction”. 50, 100, 250 and 500°ml (from left to right). Line 15: 1 kbp ladder.

Tab.3.1 Determination of concentration and purity of the extracted nucleic acid from the harbor water samples with the NanoDrop ND-1000 Spectrophotometer. (Values beneath detection limit are marked as cursive)

	ng/µl	260/280	260/230
Kingfisher instrument			
50 ml	0.8	-1.52	1.39
100 ml	1.2	1.54	-1.39
250 ml	0.8	-4.68	0.01
500 ml	23.0	2.37	1.10
750 ml	125.2	2.23	0.72
Epicentre Kit			
50 ml	9.2	1.68	0.67
100 ml	12.2	2.19	0.95
250 ml	28.2	2.15	1.61
500 ml	117.7	2.21	1.87
Lysis			
50 ml	15.4	0.97	0.17
100 ml	42.2	1.20	0.29
250 ml	56.1	1.22	0.33
500 ml	148.7	1.31	0.46

After the extraction all samples were used for amplification with universal bacterial primers (Materials and Methods 2.6). The results of the PCR run are shown in Fig.3.2 Thus the “Kingfisher” extraction led to good visible bands at the wished height of 800 bp for all sample volumes. Just the band of the 50 ml water sample is of less intensity. The band intensity of the samples 100, 250, 500 and 750 ml is comparable. The amplification of the samples extracted with the “Epicentre Kit” led to good visible bands with the same intensity in all cases. Not as intensive but still good detectable are the bands for 100, 250 and 500 ml of the “Lysis Extraction”. In case of the 50 ml sample no band can be seen.

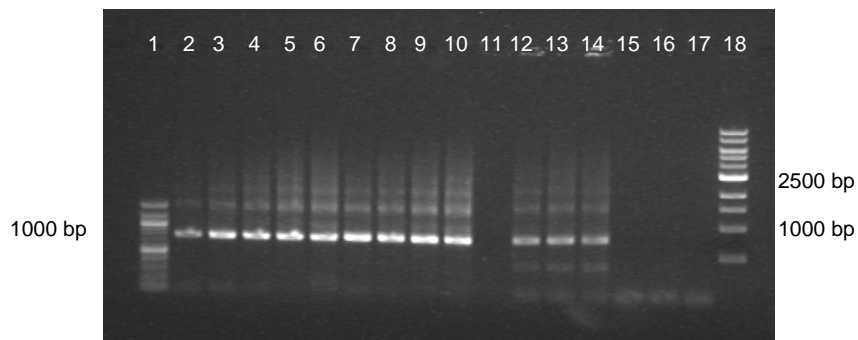


Fig. 3.2 Digital image of a 2% agarose gel run (post PCR) showing the comparison of the three different extraction methods “Kingfisher”, “MasterPure™ DNA Purification Kit” (EPICENTRE) and “Lysis Method” using the example of harbor water in different volumes. Line 1: 100 bp ladder. Line 2 – 6: “Kingfisher instrument”. 50, 100, 250, 500, and 750 ml (from left to right). Line 7 – 10: “Epicentre Kit”. 50, 100, 250 and 500°ml (from left to right). Line 11 – 14: “Lysis Extraction”. 50, 100, 250 and 500°ml (from left to right). Line 15, 16 and 17: empty. Line 18: 1 kbp ladder.

3.2 Extraction test with the “MasterPure™ DNA Purification Kit” (EPICENTRE) using the example of one water sampling spot of the South coast of Iceland

After the comparison of the three different extraction methods on the basis of the harbor water sample used in different volumes, a test was made with the “Epicentre Kit” using the water samples from one sampling spot of the Icelandic South coast (# 252, see also Tab.A.1). With the “Epicentre Kit” even the smallest filtration volume of the harbor water was detected in the pre PCR gel (Fig.3.1) and all samples led to the same band intensity in the post PCR gel as well. Thus this experiment was carried out, to get an impression, if it is possible to detect nucleic acid extracted from the volcano water samples with the “Epicentre Kit”. This sampling spot (# 252) included sampling depth of 0, 10, 20, 40 and 100 m. The extraction was accomplished as described in

Materials and Methods 2.3.2. After the extraction the samples were amplified by Tegu-real-time PCR with universal bacterial primers (Materials and Methods 2.6). Pre as well as post PCR results are shown in Fig.3.3. Thus high molecular DNA was extracted in all cases (Line 2 –6). The band for the 10 m sample shows the highest intensity. 0, 20 and 40 m are of nearly the same intensity and 100 m is of lowest intensity and hardly visible. These optical results are supported by the concentration values measured with the NanoDrop spectrophotometric device (Tab.3.2). The PCR result (Line 7 – 11) shows clearly visible bands throughout all samples. The thickest band (marked in red, Fig.3.3) is the wished amplification at a height of 800 bp which was cut of the gel in each case and used for gel band purification. The purified DNA was stored for at –20 °C for later planned experiments. No bands are detected in case of the negative template controls (Line°13°15).

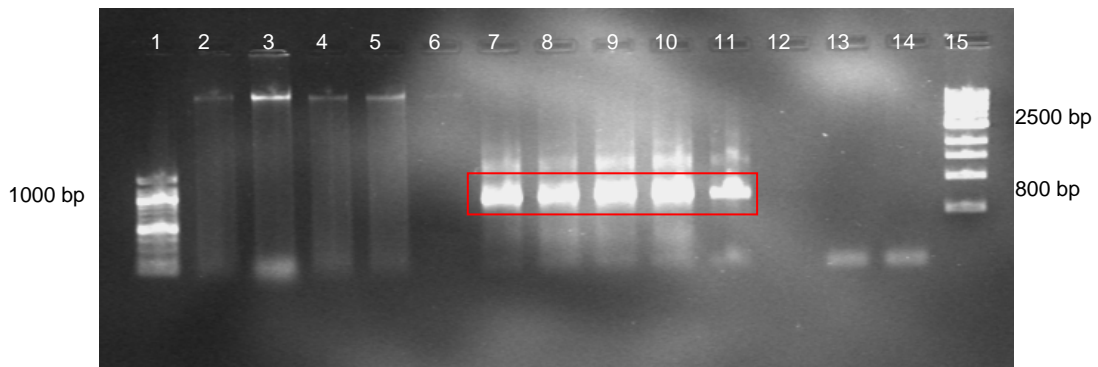


Fig.3.3 Digital image of a 2% agarose gel run (**pre and post PCR**) showing the results of the extraction with the “MasterPure™ DNA Purification Kit” (EPICENTRE) using the example of one water sampling spot (# 252) from the Southcoast/Iceland. Line 1: 100 bp ladder. Line 2 – 6: **pre PCR** results. Sampling depth: 0 m, 10 m, 20 m, 40 m, 100°m (from left to right). Line 7 – 11: **post PCR** results. Sampling depth: 0 m, 10 m, 20°m, 40 m, 100°m (from left to right). Line 12: empty. Line 13 – 14: NTC. Line 15: 1°kbp ladder.

Tab.3.2 Determination of concentration and purity of the extracted nucleic acid with the “Epicentre Kit” from the volcano water samples/Southcoast Iceland with the NanoDrop ND-1000 Spectrophotometer. (Values beneath detection limit are marked as cursive)

# 252	ng/µl	260/280	260/230
0 m	36.8	1.96	1.41
10 m	92.8	2.23	1.87
20 m	41.0	2.21	1.27
40 m	36.1	2.09	1.57
100 m	6.3	2.27	0.51

3.3 Optimization of the “Kingfisher” extraction including a Proteinase K treatment step using the example of one water sampling spot of the South coast of Iceland

Due to the fact that the “Kingfisher” extraction method is half automated and thus faster and furthermore cheaper than the manually extraction with the “Epicentre Kit” but on the other hand of less yield, an optimization experiment was implemented inserting an additional Proteinase K treatment step. Each filtration sample pellet was spiked with 2.5°µl of Proteinase K (20 µg/ml) and incubated at 55 °C for 1 h under mixing. After the incubation 200 µl of lysis buffer were added to each sample and the extraction was continued as described in Material and Methods 2.3.1. A PCR with universal primers was implemented after the extraction. Both pre and post PCR results are shown in Fig.3.4. No bands throughout all sampling depth were detected in case of the pure applied DNA (Line 2 – 6). The appropriate concentration values calculated by NanoDrop measurement are relatively low, too (Tab.3.3). The values for the ratio of sample absorbance at 260 and 280 nm (Tab.3.3) were lower than 2.0, the value for pure DNA with the exception of the surface value at 0 m. All values for the ratio of sample absorbance at 260 and 230 nm (Tab.3.3) were beneath the range of 1.8 – 2.0 and therefore beneath the purity value for nucleic acid. The amplification was effective and clearly visible, thick bands of the same intensity were detected on the gel at the expected size of 800 bp (Line 7 – 11, Fig.3.4).

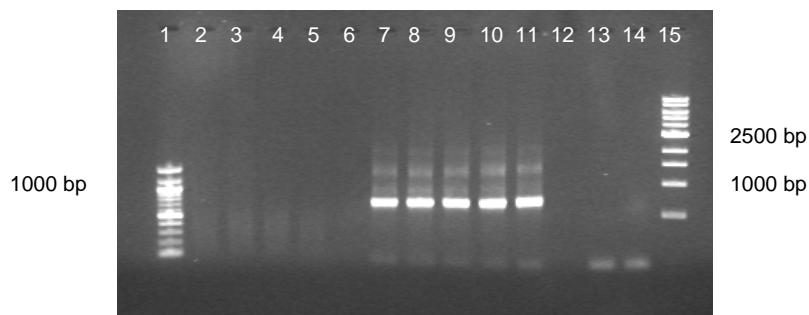


Fig. 3.4 Digital image of a 2% agarose gel run (**pre and post PCR**) showing the results of the extraction with the “Kingfisher instrument” tested with an additional Proteinase K treatment step, using the example of one water sampling spot (# 252) from the South coast/Iceland. Line 1: 100 bp ladder. Line 2 – 6: **pre PCR** results. Sampling depth: 0 m, 10 m, 20 m, 40 m, 100°m (from left to right). Line 7 – 11: **post PCR** results. Sampling depth: 0 m, 10 m, 20°m, 40 m, 100°m (from left to right). Line 12: empty. Line 13 – 14: NTC. Line 15: 1°kbp ladder.

Tab.3.3 Determination of concentration and purity of the extracted nucleic acid with the “Kingfisher instrument + Proteinase K” from the volcano water samples/South coast Iceland with the NanoDrop ND-1000 Spectrophotometer. (Values beneath detection limit are marked as cursive)

# 252	ng/μl	260/280	260/230
0 m	10.1	3.73	1.22
10 m	12.7	1.63	1.38
20 m	11.8	1.52	1.19
40 m	7.4	1.40	1.45
<i>100 m</i>	2.2	2.96	<i>0.04</i>

4 Discussion

4.1 Comparison of three different extraction methods using the example of harbor water

With the aim to find a good, fast, efficient and reliable DNA-extraction method that can be used for the analysis of the water samples taken after the eruption of the Eyafjallajökull volcano at the south coast of Iceland, three different extraction methods have been tested. The comparison between the partially automated “Kingfisher extraction” based on the use of magnetic beads, the “MasterPure™ DNA Purification Kit (EPICENTRE)” and the simple “Lysis method” showed that the best results are obtained with the “Epicentre-Kit”. In this case thick bands were obtained throughout all sample volumes (50, 100, 250 and 500 ml) in the pre as well as in the post PCR agarose gel. The pre PCR gel shows that high molecular DNA was extracted in case of all volumes. Moreover with this kit reliable predications regarding to sensitivity seem to be possible because a good visible increasing of the band intensity with increasing sample volume was detected in the pre PCR gel. This statement is supported by the results obtained with the NanoDrop spectrophotometric measurement. The increase of concentration with increasing sample volume reflects the increase of band intensity. Nevertheless, with the “Kingfisher extraction” method high molecular DNA bands are good visible in the case of sample volumes 250 and 500 ml in the pre PCR gel. This is also reflected by the obtained NanoDrop concentration values. In the case of the sample volumes 50 and 100 ml these values were not significant because they seem to be under the detection limit. But in the post PCR gel bands were obtained as well for these volumes. Only the band intensity of the Kingfisher extraction is lower than with the Epicentre kit. With the Epicentre Kit all bands were of the same strong intensity in the post PCR gel. In case of the Kingfisher extraction the intensity for sample volume 50 ml is lower than with the other higher concentrations. Consequently the use of the “Epicentre kit” leads to a higher rate of yield than the “Kingfisher extraction method”. In case of the Lysis method no visible bands were detected in the pre PCR gel. On contrary the concentration results obtained by NanoDrop measurement were relatively high. This can be explained by the obtained values for the ratio of 260/280 and 260/230 which were very low and thus depart from the desired values off 2 and 1.8-2.0. Therefore contamination caused by proteins and other cell impurities that inhibit the gel run are thinkable. But even in this case bands were detected at the expected size of

800 bp in case of the samples 100, 250 and 500 ml. But the band intensity was lower as with the other two methods.

4.2 Extraction test with the “MasterPure™ DNA Purification Kit” (EPICENTRE) using the example of one water sampling spot of the South coast of Iceland

The first experiment showed that the “Epicentre kit” leads to the best results regarding to high yield and sensitivity. Even the lowest concentration was detected in the pre as well as post PCR gel. Thus this method was adducted for one extraction experiment with one water sampling point of the Icelandic South coast. Sampling spot #252 (see Tab.A.1 and Fig.2.2) was chosen for this analysis. This sampling point is characterized by a farther distance from the coastline where lower concentrations may be expected than at sampling spots close to the coast. 0, 10, 20, 40 and 100 m were the chosen sampling depth. The extraction was successful in every case because good visible bands of high molecular DNA were detected throughout all samples in the pre PCR gel. In case of the 10 m water sample the band intensity was the highest and lowest for the surface and the deepest (100 m) sample. This result is supported by the NanoDrop values. Additionally this result seems to be realistic because the most material is expected beneath the surface around the 10 m level. With increasing depth a decrease of concentration may be expected and this is also reflected and supported by the obtained results regarding to the low intensity band at 100 m sampling depth. Thus the “Epicentre kit” seems to be a very reliable extraction method for these valuable sample material because even from low concentrated samples DNA can be extracted and verified. Furthermore the PCR implemented with the universal bacterial primers was successful in all cases and therefore seems to be an adequate tool for the analysis.

4.3 Optimization of the “Kingfisher” extraction including a Proteinase°K treatment step using the example of one water sampling spot of the South coast of Iceland

So far the “Epicentre kit” was posed to be the method of choice regarding to the analysis of the volcano water samples from the south coast of Iceland. But still this method is disadvantageous regarding to cost-effectiveness. Furthermore the handling is much more elaborate and time consuming than the half automated “Kingfisher extraction method”. For this reason an optimization experiment was implemented to

see if there is an opportunity to improve the “Kingfisher extraction method” regarding to the rate of yield and sensitivity. In this optimization experiment a proteinase K treatment step that is also used in case of the “Epicentre extraction” was attached to the procedural method. For a reliable comparison to the results obtained by “Epicentre extraction” the same sampling spot was chosen for the implementation. In contrast to the “Epicentre kit” results there were no bands visible in the pre PCR gel throughout all samples. Moreover the concentration values obtained by NanoDrop measurement were nearly three to four times lower than the ones of the “Epicentre method”. The concentration value for 100 m sampling depth seems to be under the detection limit. The values for 260/280 and 260/230 measurement are lower than the desired values of 2 and 1.8 – 2 which is an indicator for protein and other cell contaminations. But nevertheless after PCR thick bands with the same intensity throughout all samples were detected in the gel which means that the extraction was successful. Only the concentration of pure DNA may be insufficient for pre PCR gel detection. Or inhibitory effects caused by contaminants prevent the gel run. But still there is enough DNA material for amplification. And even at 100 m sampling depth a very good visible band could be seen in the gel. In conclusion with this additional proteinase K step an optimization was achieved regarding to sensitivity because even at sampling points of low concentration good visible results were obtained.

As consequence this faster and cheaper method can also be used for the analysis of the volcano seawater samples. Only in exceptional cases of maybe very low concentrations that might not be detected by “Kingfisher extraction” it seems to be useful to repeat the extraction manually with the “Epicentre kit”.

5 Future Prospects

With the pre tests exposed afore a reliable procedural method was elaborated that can now be used for further experiments. A 16 S clone analysis is intended, to gain new valuable information about the different communities, the allocation as well as the diversity of marine bacteria and archaea inhabit the Icelandic sea which has not been done before. With this favorable method it should be possible to implement the 16°S°rRNA gene cloning, sequencing and identification of the gained sequences by bioinformatics tools (alignment). Also with the background to test the new sequencing device Roche 454 GS-FLX system using Titanium Chemistry (CD genomics) mentioned in the introduction.

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7 Appendix

Tab.A.1 Sampling locations and corresponding sampling depth of the seawater samples taken from the South coast of Iceland belonging to Fig.2.2

Sample name	Sampling depth (m)
# 229	0
# 229	10
# 230	0
# 230	10
# 230	20
# 230	30
# 230	50
# 231	0
# 231	10
# 231	20
# 231	30
# 231	50
# 232	0
# 232	10
# 232	20
# 232	50
# 232	150
# 238	0
# 238	10
# 238	20
# 238	30
# 238	50
# 240	0
# 240	10
# 240	20
# 240	30
# 240	50
# 242	0
# 242	10
# 242	20
# 242	30
# 242	50
# 245	0
# 245	10
# 245	20
# 245	30

# 245	47
# 247	0
# 247	10
# 247	20
# 247	30
# 247	50
# 249	0
# 249	10
# 249	20
# 249	30
# 249	50
# 252	0
# 252	10
# 252	20
# 252	30
# 252	50
# 254	0
# 254	10
# 254	20
# 254	30
# 254	50
# 254	130
# 256	0
# 256	10
# 256	20
# 256	30