Nucleic acids: indicators for dynamic processes of clogging in soil filter systems

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Abstract A high-performance liquid chromatography (HPLC)-method was established to quantify the development and degradation of DNA, rRNA and tRNA in the Langenalb soil filter fed with combined sewer overflow. The DNA served as a parameter for the microbial biomass and the RNA/DNA ratio for the growth rate. For nucleic acids, quantification samples were taken at different depths and sections of the clogged and the unclogged soil filter. Highest DNA concentrations were found at a depth of 0–2 cm in the clogged soil filter. During a starvation period of three months the DNA- and RNA-concentrations decreased twofold at depths between 0–8 cm. The higher nucleic acid concentrations at depths of 10–40 cm in the unclogged soil filter were attributed to a shift of the starved microorganisms. Similar RNA/DNA ratios in the clogged and the unclogged soil filter indicate similar growth rates and metabolic activities. It could be proven that the clogging of the soil filter was due to the microbial biomass and the DNA concentration, respectively.
Keywords Biomass; clogging; DNA; HPLC; RNA; soil filter

Introduction

The estimation of microbial biomass and their activity by nucleic acid determination is a significant challenge in the understanding of microbial processes in environs. But the use of nucleic acids for the quantification of biomass and activity, especially RNA/DNA determinations in natural environs is very limited. There is a variety of parameters which are influencing the quantitative recovery of both DNA and RNA from environmental samples. As described by Hurt *et al.* (2001) there are several criteria for an ideal procedure for the recovering of nucleic acids from environmental samples. Our optimized method allows the simultaneous extraction and quantification of DNA, rRNA and tRNA. The calculation of tRNA/DNA and rRNA/DNA ratios gives a deeper understanding of the metabolic activity of the microbial community in such habitats.

Under high wastewater loads and low temperatures such soil filters fed with combined sewer overflow (a mixture of wastewater and stormwater runoff) are clogging. The clogging is caused by chemical, physical or microbial processes (MacLeod *et al.*, 1988; Baveye *et al.*, 1998). In this article we focus on the microbial clogging and its reversibility which is called unclogging. In soil filter systems the clogging is disadvantageous because the decrease in hydraulic conductivity results in lower purification efficiency or in ponding. Up to now no field investigations are available that focus on the nucleic acids in the microbial clogging process. So far, the complex soil–wastewater matrix makes it difficult to quantify the microbial biomass in such environs.

The aim of this study was to quantify the depth-related DNA, rRNA and tRNA distribution (bacterial and protozoan) in a soil filter fed with combined sewer overflow and to calculate the RNA/DNA ratios. Therefore a nucleic acid extraction protocol from Dell'Anno *et al.* (1998) was adopted and optimized for the quantification of the depth-related nucleic acid distribution. The DNA served as a parameter for the microbial biomass, and the RNA as a parameter for the microbial activity. The RNA/DNA ratio served as a parameter for the

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microbial growth rate. The rRNA/DNA and tRNA/DNA ratios served as parameters for the metabolic activity of the microbial biomass.

This investigation was conducted in the clogged soil filter (February 2001, filter temperature 4 °C) up to this date intermittently fed with combined sewer overflow and in the unclogged soil filter (May 2001, filter temperature 16 °C). The soil filter was clogged and ponding occurred. Between the two samplings the soil filter was not fed with combined sewer overflow. After a three month starvation period (no feeding with combined sewer overflow) the soil filter was unclogged and fed again.

Methods

Sampling and nucleic acid quantification

Sand samples (9 g kg^{-1} organic matter per dry sand; pH 6.5; sand 100%) were collected from the University test soil filter (Langenalb near Karlsruhe, Germany). The samples were taken from three representative sites (inlet, middle, outlet) of the filter at different depths with a drilling stick (1.0-cm inside diameter; depth of penetration <70 cm). The soil filter has a total depth of 70 cm. The sand collected was placed immediately into cryo-vials and was frozen in liquid nitrogen after sampling. All samples were stored at -80 °C to minimize nuclease activity until nucleic acid extraction.

For the extraction of DNA, rRNA and tRNA an optimized protocol was used, based on a previously described HPLC-method (Coppella *et al.*, 1987; Dell'Anno *et al.*, 1998). At first, this optimized method was successfully applied to laboratory-scale soil filters for the extraction of DNA, rRNA and tRNA (Schwarz *et al.*, 2002; Schwarz, 2004). To overcome the degradation of RNA by ribonuclease it was necessary to modify the method published by Dell'Anno *et al.* (1998) and to add some precautionary steps to protect the RNA. To avoid nuclease contamination, all materials were washed carefully with 0.1% diethylpyrocarbonate (DEPC)-treated Milli-Q-water and were sterilized by heat as described by Moran *et al.* (1993). All solutions, including the HPLC eluents, were treated with DEPC and the glassware and utensils were sterilized by heat as previously described (Hurt *et al.*, 2001). The chemicals were purchased in molecular biology grade quality.

For extraction of DNA and RNA the samples were freeze-dried (freezing temperature below -20 °C; Christ Alpha 1–4, Germany). After weighing all procedures were carried out at room temperature to avoid precipitation of the extraction buffer. According to Dell'Anno *et al.* (1998), 0.4 g of the sand was added to a Tris-HCl extraction buffer (0.2% SDS, 10 mmol l^{-1} EDTA, 40 mmol l^{-1} Tris, 87 mmol l^{-1} NaCl, 74 mmol l^{-1} HCl [pH 8.0 with NaOH]). After soaking the sample for 5 min in the extraction buffer and sonicating the sample six times (10 s with 1-min intervals to avoid overheating; 130 W Vibracell Ultrasonicator, 2 output watts) with a 2 mm microtip, the sample was centrifuged and filtrated. The supernatant (100 µl) was injected into the HPLC (Hewlett Packard 1050 Ti), equipped with a Nucleogen® 4000–7 DEAE anion-exchange column (Macherey-Nagel), as previously described (Dell'Anno *et al.*, 1998). The nucleic acids which adsorbed to the anion-exchange column were eluted by applying urea buffer with an increasing KCl gradient. The separated nucleic acids were quantified at 260 nm (Hewlett Packard 1050 Multiple wavelength detector). Using DNA, rRNA and tRNA from *Escherichia coli* (all Sigma Chemicals) as standards, the three main peaks were identified as tRNA, rRNA and DNA (Figure 1). The peaks were additionally verified by adding DNase and RNase (data not shown). The integrated peak areas from standard solutions were used to conduct calibration curves for the calculation of DNA, rRNA and tRNA concentration in the sand samples. The RNA/DNA ratio was calculated by dividing the RNA concentration (rRNA + tRNA) by the DNA concentration. The recovery rates from the soil matrix determined with external nucleic acid standards from *Escherichia coli* resulted in recovery rates of 82%, 76% and 91% for DNA,

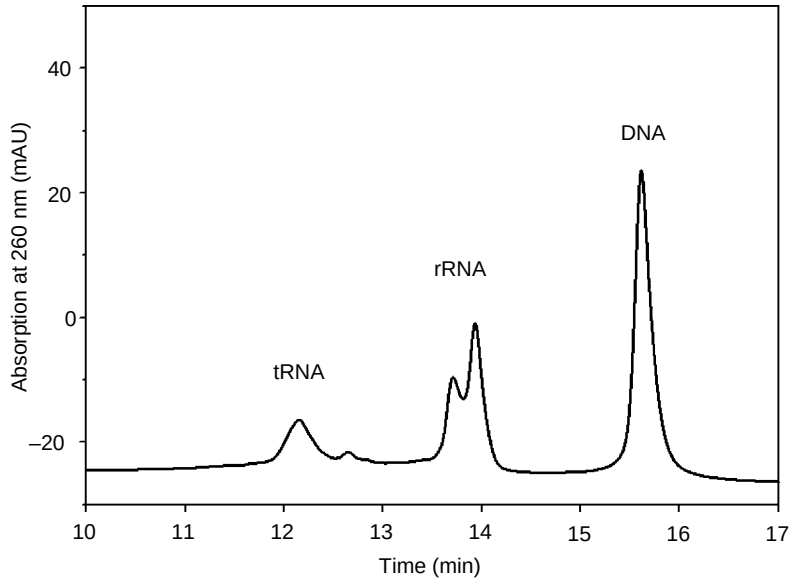

Figure 1 A typical HPLC chromatogram from a sand sample showing the tRNA-, rRNA- and DNA-peak

rRNA and tRNA, respectively. The absence of clay particles had a positive effect on nucleic acid recovery (Blum *et al.*, 1997; Frostegård *et al.*, 1999). Controls by scanning electron microscopy showed that more than 95% of the microorganisms were detached from the sand (Schwarz, 2004). The recovery rates of the HPLC-method in the investigation from Dell'Anno *et al.* (1998) were higher for DNA (95%) and RNA (90%). This is probably due to the different sediment or sand samples respectively.

Results and discussion

Nucleic acid distributions and RNA/DNA ratios

In contrast to Dell'Anno *et al.* (1998) it was possible to quantify the rRNA and tRNA separately. In the clogged soil filter an exponential decrease of all three nucleic acids with depth was found (Table 1 and Figure 2). The highest concentrations of DNA, rRNA and tRNA were found in the two uppermost layers whereas the lowest concentrations were found at 60 cm. For DNA, the concentration was about 25-fold higher in the first two centimetres compared to the depth at 60 cm (Figure 2). The lower RNA concentrations in the first layer are probably due to harsh environmental conditions because snow covered the clogged filter. In contrast to marine sediments (Dell'Anno *et al.*, 1999), much higher DNA concentrations and a steeper gradient with a greater depth-penetration of the DNA was found in the clogged filter. The average RNA/DNA ratio in the clogged and the unclogged soil filter was higher than the average RNA/DNA ratio of 0.2 ± 0.2 calculated from marine deep sea sediments (Dell'Anno *et al.*, 1998). This means that the microbial community in the clogged and the unclogged soil filter is growing faster than the microbial communities in deep-sea sediments. Between tRNA/DNA and rRNA/DNA no significant differences were found and there are no data in the literature to compare with.

In the unclogged filter no exponential decrease of DNA, rRNA and tRNA with depth was observed (Table 1 and Figure 2). The highest concentrations of DNA and rRNA were found in the top layer of the filter whereas the highest tRNA concentration was at the depth of 10 cm. The lowest concentrations for DNA, rRNA and tRNA were found at 60 cm and are similar to those observed in the clogged filter. This indicates that the high

Table 1 Depth-related distribution of nucleic acids and RNA/DNA ratios in the clogged and unclogged soil filter

Depth (cm)	Clogged soil filter							UnClogged soil filter						
	Mean concn ± SD (μg g^{-1})[a]				RNA/DNA ratios			Mean concn ± SD (μg g^{-1})[a]				RNA/DNA ratios		
	tRNA	rRNA	RNA	DNA	tRNA/DNA	rRNA/DNA	RNA/DNA	tRNA	rRNA	RNA	DNA	tRNA/DNA	rRNA/DNA	RNA/DNA
0	28.2 ± 9.2	33.4 ± 16.9	61.7 ± 26.0	167.6 ± 17.6	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	7.0 ± 6.6	25.3 ± 20.3	32.2 ± 26.9	65.3 ± 18.6	0.1 ± 0.3	0.4 ± 0.3	0.5 ± 0.3
2	34.3 ± 20.8	59.7 ± 31.8	94.0 ± 52.6	120.8 ± 53.8	0.3 ± 0.1	0.5 ± 0.2	0.8 ± 0.3	9.7 ± 5.8	14.3 ± 4.1	24.0 ± 9.9	60.8 ± 34.3	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.2
4	17.0 ± 4.7	26.6 ± 3.9	43.6 ± 8.6	63.1 ± 13.2	0.3 ± 0.1	0.4 ± 0.2	0.7 ± 0.3	6.3 ± 8.0	10.9 ± 9.6	17.3 ± 17.6	28.4 ± 16.9	0.2 ± 0.2	0.4 ± 0.2	0.6 ± 0.3
6	7.3 ± 2.6	20.2 ± 5.2	27.5 ± 7.7	44.4 ± 7.2	0.2 ± 0.1	0.5 ± 0.2	0.6 ± 0.3	7.8 ± 5.5	14.2 ± 9.9	22.1 ± 15.3	34.9 ± 15.5	0.2 ± 0.2	0.4 ± 0.2	0.6 ± 0.3
8	3.7 ± 2.2	13.5 ± 5.4	17.2 ± 7.6	30.6 ± 7.8	0.1 ± 0.1	0.4 ± 0.2	0.6 ± 0.2	6.7 ± 5.3	7.7 ± 4.8	14.4 ± 10.2	29.8 ± 16.6	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.2
10	2.3 ± 1.3	10.7 ± 4.6	13.0 ± 5.9	25.2 ± 8.9	0.1 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	18.7 ± 13.2	16.1 ± 7.9	34.8 ± 21.1	45.6 ± 17.4	0.4 ± 0.2	0.4 ± 0.2	0.8 ± 0.3
20	1.7 ± 0.3	5.4 ± 1.3	7.1 ± 1.7	15.1 ± 3.2	0.1 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	5.8 ± 2.2	9.3 ± 2.2	15.1 ± 4.4	33.0 ± 12.6	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.2
40	0.9 ± 0.2	2.7 ± 0.5	3.6 ± 0.6	8.2 ± 0.9	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	1.1 ± 0.1	3.5 ± 0.5	4.6 ± 0.6	12.8 ± 3.8	0.1 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
60	0.8 ± 0.2	2.1 ± 0.8	2.9 ± 0.9	6.5 ± 2.0	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.7 ± 0.1	1.5 ± 0.5	2.2 ± 0.6	6.7 ± 1.4	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
Avg	10.7 ± 4.6	19.4 ± 7.8	30.0 ± 12.4	53.5 ± 12.7	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	7.1 ± 5.2	11.4 ± 6.6	18.5 ± 11.8	35.2 ± 15.2	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.2

[a] $n = 3$. From the inlet, middle and outlet section of the filter

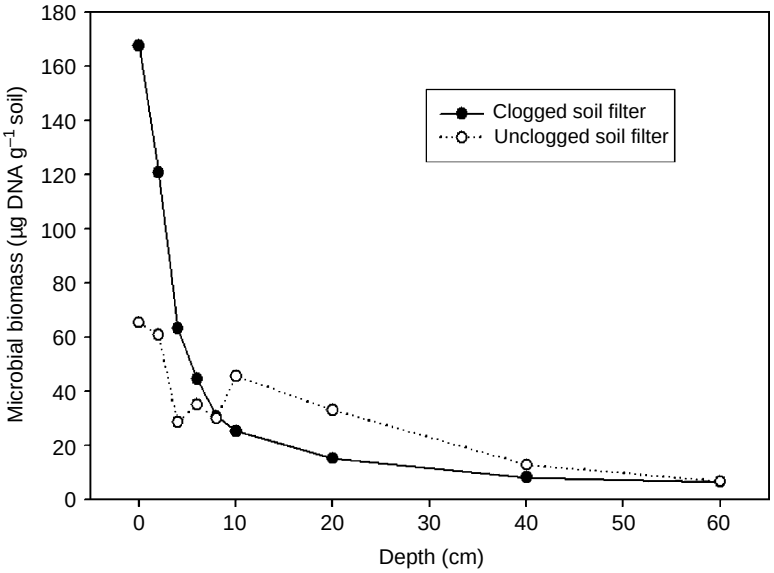

Figure 2 DNA concentration as a parameter for the microbial biomass in the clogged soil filter and three months later in the unclogged soil filter

nucleic acid concentrations and their changes occurred above 60 cm in the soil filter. In comparison to the clogged filter, all three nucleic acid concentrations decreased about twofold in the first 8 cm. Interestingly, in the depth between 10 and 40 cm, higher DNA and RNA concentrations occurred (Figure 2). We assume that the starved and dead microorganisms in the upper sections were transported into deeper filter layers by rain. The microorganisms in the deeper filter layers metabolized the organic matter and synthesized DNA and RNA or incorporated the nucleic acids directly (Novitsky, 1986; MacLeod *et al.*, 1988).

The results from the clogged and the unclogged filter show that the nucleic acids vary highly by concentrations but comparatively not in their RNA/DNA ratios. This means, that the microorganisms at different depths in the clogged and unclogged filter have had a similar growth rate. In contrast to RNA/DNA ratios derived from marine deep sea sediments (Dell'Anno *et al.* 1998), these ratios show higher values. This indicates higher microbial growth rates in the investigated soil filter than in marine sediments. The comparison of RNA/DNA ratios with other studies is difficult because different nucleic acid quantification methods were applied and mostly pure cultures were investigated (Kemp *et al.*, 1993; Hurt *et al.*, 2001; Milner *et al.*, 2001). Similar nucleic acid concentrations in activated sludge, 53–131 $\mu g\,ml^{-1}$ for DNA and 27–51 $\mu g\,ml^{-1}$ for RNA, were reported by Yu and Mohn (1999). Muttray and Mohn (1998) found RNA/DNA ratios of 0.5–3.2 for resin acid-degrading bacteria. In contrast to Dell'Anno *et al.* (1998) no detrital nucleic acids were found. Under wastewater-conditions degradation processes can be assumed. This is indicated by high nucleic acid concentrations and RNA/DNA ratios. We also found good correlations between DNA and the colony forming units in laboratory soil filters operated under similar conditions (Schwarz, 2004) which confirm these findings.

The results of this study demonstrate that our optimized method is an appropriate tool for the simultaneous extraction of DNA, rRNA and tRNA from matrices penetrated by wastewater. The comparison of the nucleic acid distributions of the clogged and the unclogged filter implicates the hypothesis that the nucleic acids were transported into greater depths by rain. In contrast to the high variations in the nucleic acid

concentrations, the RNA/DNA ratios in all samples were similar and therefore a good parameter for the microbial growth rate. Our further research will focus on the RNA/DNA ratios influenced by different environmental conditions like aerobic or anaerobic conditions. This will also include a special focus on the rRNA/DNA and the tRNA/DNA ratio. Another topic will be the vertical transport of the nucleic acids in a native or a detrital form.

Conclusions

A HPLC-method was successfully applied for the simultaneous quantification of DNA, rRNA and tRNA in a soil filter. The nucleic acids were separated from other substances in the wastewater matrix by means of anion exchange chromatography. The DNA concentration is a good parameter for estimating biomass in soil filter systems.

The highest nucleic acid concentrations were found in the uppermost layer of the microbiologically clogged soil filter showing an exponential decrease with depth. In the unclogged soil filter higher nucleic acid concentrations were found at depths of 10–40 cm. This implicates that the clogging process in soil filter systems is considerably influenced by microbiological growth and hence reversible. The RNA/DNA ratios in the clogged and unclogged filter showed only little variations indicating a similar growth rate.

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