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ANALYSIS OF APPLICATION OF BACTERIAL DIVERSITY METHODS IN WASTEWATER MICROBILOGY RESEARCH

Theses of PhD dissertation

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INTRODUCTION

In these days among the countless environmental protection challenges the protection of natural waters and potable water reservoirs is an extremely important issue. Developing of the capacity and efficacy of wastewater treatment is inevitable to avoid the threat of agricultural, industrial and communal pollution. Microbes have an overall role in global carbon, nitrogen and phosphor cycles, as well as in the degradation of several pollutants. Therefore the importance of microbial research of wastewater treatment process is obvious. These results reveal the "black box" of biological treatments and allow the wastewater technologists to operate the reactors not only based on empirical information, but influencing specifically the microbial communities.

Wastewater treatment plants besides their technical and environmental role are a perfect environment for microbial ecology studies as very few similar complex microbial habitats exist where most of the conditions are well-defined, controlled and regularly checked. In the last decades cultivation-independent methods have developed in extremely fast way, however quick and easy microbial monitoring methods suitable for wastewater treatment analyses are demanded.

In this study the application of a molecular fingerprinting method as a monitoring tool was analyzed through the examination of the microbial community of two different wastewater treatment plants. The two analyzed systems differed both in the influent wastewater and in the way of treatment.

One of them was the Biofor nitrogen-removal system of a communal wastewater treatment plant (Dél-pest Wastewater Treatment Plant = DpWWTP) consisting of fixed bed reactors where microbial processes take place in biofilms formed on the carriers provided. In the Biofor system nitrogen-removal happens in two steps. In the first one, oxidation of ammonia to nitrate (nitrification) is enhanced by intensive aeration of the basins. In the next step denitrification is enhanced by anoxic conditions and addition of methanol. Most of the year the process works perfectly, however sometimes effluent ammonium concentrations exceeds the environmental limits. Therefore the microbes catalyzing the first step (oxidation of ammonia to nitrite) where studied in this work. This process is achieved by the ammonia-oxidizing bacteria (AOB) corresponding to the β-*Proteobacteria* subdivision

The other studied system was an industrial wastewater treatment plant treating the effluent of a coking plant (ISD Coking Ltd. = ISD-C). Coke is an essential material in modern metallurgy, however a high strength wastewater is produced as a byproduct of coking process. This wastewater is characterized by high concentration of phenols, cyanate, thiocyanate and ammonia. This wastewater following several pretreatment steps is treated in aerated activated sludge reactors where many of the pollutants (phenols) are removed effectively, while the pH value decreases. Ammonium concentration increases through the process and the removal of thiocyanate is unbalanced.

In activated sludge systems microbial cells are located in flocs produced by the process called bioflocculation. While communal wastewater treatment plants are extensively studied microbiologically, little is known about the microbes inhabiting the sludge treating the special toxic effluent of coke plants. Therefore in the case of this plant we examined the whole bacterial community.

OBJECTIVES

In the case of DpWWTP our goal was to develop a monitoring method suitable for the follow up of the AOB community and to identify the microbial changes behind the nitrogen-removal inefficacies. While in the case of the ISD-C WWTP our aim was to reveal the microbial composition of the activated sludge and follow up its changes to recognize the microbial process behind the thiocyanate removal inefficacies.

In the first case our goals can be summarized as follows:

1. Create a database suitable for the identification of the dominant AOB groups based on their molecular fingerprint;
2. Testing the efficacy of AOB identification based on our database;
3. The use of the monitoring system for the follow up of the seasonal microbial changes.

In the case of the coking wastewater treatment plant:

1. Reveal the composition of the microbial community of the activated sludge;
2. Follow up the changes of the composition and activity of the microbial community to identify the reasons of the unbalanced efficacy of thiocyanate removal.

APPLIED METHODS

For the monitoring of wastewater treatment's microbial communities a quick and inexpensive microbial ecology method is needed which allows the comparison of the proportion of the main bacterial groups and the long term follow up of the community by providing comparable results.

Among the cultivation-independent methods terminal restriction fragment length polymorphism (TRFLP) analysis resulted to be the most adequate for our goals, because this method is:

- by optimal conditions properly fast allowing sample analysis in 2 days;
- simple and therefore inexpensive;
- due to applied capillary electrophoresis and internal standards effects more comparable fingerprints than gel based methods like DGGE;
- as the fingerprints are based on direct sequence data (restriction enzymes recognition sites), it allows to identify the phylotypes behind the TRFLP peaks;
- as a semi-quantitative method makes it possible to compare the proportion of the different groups of the community.

Analysis of the nitrifying basins of Dél-pest WWTP

The development of the method suitable for monitoring of the AOB communities of the DpWWT nitrifying basins was started by the creation of a database consisting of selected sequences of ammonia-monooxigenase enzyme coding (*amoA*) gene which is a group-specific gene of AOB. The database contained the most important properties of the sequences (environmental origin) and the size of the TRFs (terminal restriction fragments) produced by the use 6 different enzymes.

The next step was the evaluation of the identification of AOB based on our database. For this the fingerprint of the first samples (2002) was created. In parallel, from the same isolated DNA samples *amoA* clone libraries were created and grouped by RFLP. The sequences of the selected clones were compared with the results of the identification based on the database.

Next year (2003) the developed system was used for monitoring of the AOB community with the aim of detecting the microbial changes behind the fluctuations of ammonium removal efficacy.

Analysis of the activated sludge reactors of ISD-K

The microbial study of the biological treatment unit of ISD-C WWTP was conducted by the analysis of the 16S rRNA gene which being a universal gene allows the exploration of all of the members of *Bacteria* domain including by the time uncultured or even unknown phylotypes.

First the six parallel activated sludge tanks were compared by TRFLP fingerprints made by the use of four different restriction enzymes. As the basins resulted to be adequately similar on the following sampling dates only the fingerprint of one of them was created by the use of the two enzymes providing the most diverse patterns.

The monitoring of the community (July-November 2004) was done not only by TRFLP fingerprints based on 16S rRNA gene amplified from isolated DNA but by fingerprints created from isolated RNA. The latest method allowed comparing the activity profile of the community as more active bacteria usually have more ribosomes. Activity analysis is supposed to show smaller changes of the community.

For the identification of the members of this almost unknown community the use of databases was hopeless. Therefore four clone libraries were created from the most distinct samples and the individual TRFLP pattern of the clones was compared with community fingerprints to identify their peaks. Selected clones were sequenced and sequences were compared with public databases. By this method not only the specific "hunting" for missing community TRFs was possible but the recognition of pseudo-TRFs too.

RESULTS AND DISCUSSION

Results of the analyses of nitrifying basins

1. Formation of TRF database of *amoA* sequences

A database based on more than 1000 *amoA* sequences was successfully created. The phylogenetic analysis of these sequences supported the phylogenetic relationship of AOB suggested by previous studies. Furthermore it allowed the recognition of several phylospecies consisting of only uncultured bacteria.

2. Efficacy of phylotype identification based on our database

The identification of dominant community members based on solely TRFLP pattern comparison with our database resulted to be problematic as in the case of the first 2002 samples 4-5 different phylotypes, belonging to *Nitrosomonas europaea* and *Nsm. oligotropha* lineage were identified by community TRFLP, but the analysis of clone libraries confirmed the presence of only two of them. Among the possible reasons of these differences are the possible biases of cloning and the weakness of RFLP based grouping of the clones

A further difficulty was the presence of pseudo-TRFs. In the case of samples dominated by only one phylotype (2003 samples) the recognition of the pseudo-TRFs was possible but in the case of more complex communities (2002) the situation was more complicated. To avoid the problems caused by pseudo-TRFs the screening of clones by their individual TRFLP pattern was suggested. This way the pseudo-TRFs of the community fingerprints can be recognized. Furthermore more accurate selection of clones is needed because as the analyses of 2002 samples showed, even by the use of five restriction enzymes TRFLP pattern groups can cover polyphyletic groups.

Even though creation of clone libraries from every sample is not needed as the slow growing AOB communities change there composition (except for drastic impacts) very slowly in the course of months in case of smaller changes in their environment.

Based on our results the following procedure can be suggested for the monitoring of AOBs in wastewater treatment systems:

1. At the beginning of the monitoring *amoA* TRFLP fingerprints should be created by the use of many (4-6) restriction enzymes;
2. TRFLP patterns should be compared with our database and the dominant phylotypes should be determined;
3. Clone libraries should be created from the most diverse samples, or in the case of appearance of new peaks in the future samples;
4. Individual TRFLP pattern of clones should be compared with community fingerprints to identify the peaks and if it is needed selected clones should be sequenced;
5. The examined system should be checked by TRFLP created by the most proper enzymes every one or two months.

3. Results of the monitoring of nitrifying basins

Monitoring of the nitrifying basins samples presented two groups differing in their chemical parameters (nitrogen-removal) as well as microbiological properties (AOB community composition). These two groups were the samples from 2002, and the samples from 2003.

In 2002, apart form nitrification, the basins showed only little nitrogen-removal (4 %), while the AOB community was more complex showing two groups based on cloning results, and five groups based on TRFLP. The two groups identified by both methods consisted of one-one third of the community and both had only uncultured closest relatives. So their properties can be deduced only from the habitats of their closest relatives.

One of these groups called HM3 (based on its TRFLP pattern) was member of the *Nitrosomonas europaea* lineage and was closely related to uncultured *Nitrosococcus oceani* phylotypes. Most of their closest relatives were detected in wastewater treatments, especially in biofilm reactors showing the tolerance of higher amounts of ammonium- and organic materials, higher oxygen affinity, and the preference of biofilm life form.

The other group was HM 5.7 phylotype which belongs to the *Nsm. oligotropha* lineage and its closest cultured relative is *Nsm. ureae*. This group is also connected to wastewater environments, however its preference of biofilms is not guaranteed as half of its members originate from biofilms, and half from activated sludge.

In the 2003 samples a nitrogen-removal rate of 25-30 % was measured in the nitrifying basins, while the AOB community consisted only of members of the HM3 phylotype. The evidence of nitrogen-removal in the aerated tanks supposes the presence of anoxic zones in the biofilms where denitrification happened. This is possible only in the case of more structured biofilms. The solely dominance of the biofilm preferring HM3 also supports these presumption.

Denitrification in the aerated tanks makes the overall nitrogen-removal of Biofor system more effective and inexpensive as fewer methanols should be added to the denitrifying basins. The development of more structured biofilm was possible due to an operational change in the plant: the washing periods of the biofilm was improved by pressure drop control.

Results of the analyses of the coke plant activated sludge reactors

1. Revealing the composition of the bacterial community of the activated sludge reactors

Because of our experience in the analysis of AOB, in the case of the coking WWTP the identification of community TRFLP peaks was done by the comparison of the TRFLP patterns of monomolecular clones to the ones of the community. This way, pseudo-TRFs were easily recognized and most of the peaks (90 % of the community) were identified.

The community comprised of members of *Proteobacteria* and *Bacteroidetes* divisions. In the case of optimal function the members of β-*Proteobacteria* subdivision were dominant, especially one phylotype, the slow growing, K-strategist, phenol-degrading and floc-forming *Comamonas badia*. Besides members of the thiocyanate-degrading *Thiobacillus* genus were also present in these samples. The latest phylotypes decomposes thiocyanate by the production of ammonia and hydrogen-sulfide. The production of ammonia explains the increase of its concentration through the biological treatment, while *Thiobacilli* also oxidize hydrogen-sulfide to sulfate with accompanying pH fall explaining the pH fall experienced.

By the malfunction of the activated sludge process the members of γ-*Proteobacteria* subdivision, especially *Pseudomonas putida* phylotypes became dominant. This fast growing, typical r-strategist group is also able to degrade phenol, but it can't produce flocs.

Besides the most dominant phylotypes several aromatic compound degrader bacteria were identified like many members of α-*Proteobacteria* subdivision (*Bosea thioxidans*, *Sphingomonas spp.*) or the γ-*Proteobacteria* belonging *Rhodanobacter lindanoclasticus* phylotype which previously have been detected in different thiocyanate degrading communities.

Nitrate reducer, denitrifier phylotypes were also detected like *Bosea thioxidans*, *Comamonas denitrificans*, *Diaphorobacter nitroreducens*, *Thiobacillus denitrificans*, and *Thialkalivibrio thiocyanodenitrificans*. This explains the nitrate removal capacity of the plant.

2. Results of the monitoring of the activated sludge reactors

Our results proved the sensitivity of RNA based fingerprinting as the samples resulted to be more diverse in time based on the TRFLP fingerprints of the ribosomes. The DNA fingerprints showed remarkable differences only in the cases of drastic environmental changes. All these prove the assumption that microbial communities in case of smaller impacts change only their functional activity profile and not there number. Therefore if it is possible, RNA based monitoring is suggested.

During our sampling period the influent of the ISD-C plant was properly balanced. Only two October sampling dates showed higher influent nitrate concentration. The efficacy of biodegradation of most of the pollutants was the same as earlier, but in the case of thiocyanate an even more drastic drop in removal happened before our last sampling. The fall-back of thiocyanate removal was accompanied by an operational conversion of the plant (change from one-stepped to two-stepped operation) and by a steep decrease of temperature (6°C decrease in 1 week). Furthermore the usual pH fall through biological treatment stopped and the dry matter content of the tanks decreased.

The microbial examinations showed that the thiocyanate removal drop was accompanied by a remarkable community composition change. The abundance of the previously dominant, phenol-degrading, floc-forming *C. badia* phylotype decreased, while the phenol-degrading *P. putida* increased in number and became totally dominant in activity profile. Besides, the phylotypes belonging to *Thiobacillus* genus disappeared from the tanks.

The RNA profiles showed that the abundance of temperature-sensitive *C. badia* already decreased in two earlier samples with lower temperature. As a consequence of the operational change the load of phenol slightly increased in the tanks. This, together with the slower phenol-degrading activity of *C. badia*, led to locally higher phenol concentration in the tanks. In this conditions the members of *Pseudomonas* genus, which are less sensitive to higher phenol concentrations and changes in their environment became the dominant phenol-degrading bacteria instead of *C. badia*. As a consequence phenol-degradation remained stable all the time, but *Pseudomonas* was not able to form flocs and maintain the optimal sludge structure. In the badly structured flocs many bacteria like the thiocyanate degrader *Thiobacilli* loss there optimal habitat, which led to the drop of thiocyanate removal efficacy.

To avoid similar drops of efficacy the improvement of temperature control of the tanks and the planning of operational changes during stable environmental conditions was suggested. The temperature control system of the activated sludge basins of ISD-C was carried out already next year (2005), and since that no similar fall back of thiocyanate removal was encountered proving that our results were remarkable not only from the microbial ecology point of view but by promoting a technical improvement which allowed a more stable operation.

SUMMARY OF THE MOST IMPORTANT RESULTS

On the whole our results justified the importance of the use of cultivation independent microbial methods in the study of wastewater treatment systems. Our most important achievements were the follows:

1. Development of a relatively fast and simple ammonia-oxidizing community monitoring process;
2. Contribution to the knowledge of uncultured ammonia-oxidizing phylotypes;
3. Identification of members of a previously unknown activated sludge degrading phenols, thiocyanate and nitrate;
4. Description of microbial ecology phenomena like the substitution of a K-strategist phylotype (*Comamonas badia*) occupying a given niche (phenol-degradation) by an r-strategist (*Pseudomonas putida*) due to drastic changes in environmental conditions;
5. Confirmation of the microbial, and efficacy benefits of an operational upgrade (pressure drop control of biofilm washing periods) and suggestion of a technical improvement (temperature control of activated sludge tanks), which led to more stable wastewater treatment.

PUBLICATIONS BASED ON THE RESULTS OF THE DISSERTATION

Research journal papers:

Székely A.J., Gorál R., Barkács K., Felföldi T., Márialigeti K. RNA and DNA based microbial community analysis of the effect of environmental changes on microbial community of coke plant wastewater activated sludge. *In preparation.*

Székely A.J., Sipos R., Berta B., Vajna B., Hajdú Cs., Márialigeti K. 2008. DGGE and T-RFLP analysis of bacterial succession during mushroom compost production and sequence aided T-RFLP profile of mature compost. Microb Ecol. 2008 Jul 25. *Epub ahead of print*.

Nikolausz M., Kappelmeyer U., **Székely A.**, Rusznyák A., Márialigeti K., Kästner M. 2008. Diurnal redox fluctuation and microbial activity in the rhizosphere of wetland plants. Eur J Soil Biol. 2008 May-June; 44(3):324-333.

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Tauber T., Berta B., **Székely A.J.**, Gyarmati I., Kékesi K., Márialigeti K., Tóth E.M. 2007. Characterisation of community structure of bacteria in parallel mesophilic and thermophilic pilot scale anaerobe sludge digesters. Acta Microbiol Immunol Hung. 2007 Mar; 54(1):47-55.

Nikolausz M., Sipos R., Révész S., **Székely A.**, Márialigeti K. 2005. Observation of bias associated with re-amplification of DNA isolated from denaturing gradient gels. FEMS Microbiol Lett. 2005 Mar 15; 244(2):385-90.

Selected conference participations:

Székely A.J. 2006. Molekuláris fingerprint a mikrobiális taxonómiában. A Magyar Mikrobiológiai Társaság 2006. évi Nagygyülése, Keszthely, Magyarország. *Felkért előadó.*

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Székely A.J., Gorál R., Barkács K., Táncsics A., Révész S., Márialigeti K. 2006. Microbial community analysis of activated sludge treating industrial wastewater. AXIOM Spring School, Lipcse, Németország. *Poszter* - ***Best Scientific Poster Award.***

Székely A.J., Felföldi T., Sipos R., Márialigeti K. 2004. Seasonal monitoring of the ammonia oxidizer bacteria of sewage treatment plant by TRFLP and real-time PCR. ISME-10, Cancun, Mexikó. *Poszter.*

Sipos R., **Székely A.J.**, Bujdosó L., Hajdú Cs., Márialigeti K. 2004. Tracking microbial communities during mushroom compost maturing with the prospect of selecting potential inoculants. ISME-10, Cancun, Mexikó. *Előadás.*

Székely A.J., Felföldi T., Sipos R., Nikolausz M., Márialigeti K. 2003.Comparison of ammonia oxidizing community structure in different sewage treatment strategies by *amoA* TRFLP analysis. 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred, Hungary. *Előadás* ***–Best presentation award.***