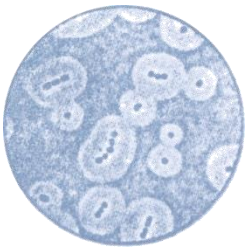
Eötvös Loránd University Faculty of Science

PhD School of Environmental Sciences

Environmental Biology Programme



Monitoring and elimination of microbes from the water purification system of a Hungarian power plant

- PhD thesis -

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INTRODUCTION

High purity, so-called ultrapure water is manufactured and used by several industries (pharmaceuticals, analytical chemistry, in laboratory equipment, engineering applications, power plant systems). Though the ultrapure water and its production and consumption environment are heavily deficient in nutritional sources, due to the strong survival capabilities and metabolic flexibility of the microorganisms they manage to subsist and breed in such oligotrophic environments and even start processes that are able to damage the systems severely. In most cases the survival and the reproduction of such organisms are secured by forming biofilm. The biofilm makes the organisms comprising it resistant to external physical and chemical effects thus their full eradication becomes almost impossible. Among other negative impacts they may cause fouling in the systems of a plant (biofouling) or the corrosion of various materials (MIC - microbiologically influenced corrosion). There are numerous chemical and physical methods known today that are used with a varying degree of success in order to avoid microbial contamination or to remove an existing one. The application and combination of these methods are adjusted according to the particularities of certain industries and the diverse utilization areas of ultrapure water.

The supply water preparation unit of the power plant studied in this PhD research was tasked with the production of ultrapure water for the various water systems of the plant. This unit frequently suffered from operational issues. The associates working in the water purification plant identified severe corrosion in some technological units. The chemical and physical parameters of the water measured on location have not explained this corrosion. Knowing the wide proliferation of microbes in similar systems, it was presumed that the corrosion in the pipe systems and the degradation of the water quality were caused by the microbial contamination of the system and the subsequent biofilm formation.

In order to clarify this issue, the Department of Microbiology at ELTE started an investigation in 2005 regarding the identification of the microbiological condition of the primary and secondary water circles, the pipe systems of the water supply system feeding them, other operational units and the chemicals used in these processes. The microbial communities in water and biofilm samples collected from multiple locations at the power plant were identified by using cultivation and cultivation independent, DNA-based methods. Further investigations focused on the water supply system of the power plant based on the results of the analyses carried out between 2005 and 2008. During this process the water purification system was

mapped in detail and we have established the microbiologically most contaminated (critical) point(s). Then we have designed a microbe eradication method that can be safely implemented in the plant in question.

OBJECTIVES

1. Sampling and analysis of critical points

The critically contaminated areas of the water purification system of the power plant was mapped by plate count, cell counts and cultivation independent DNA-based tests. We have taken into account the impact of the processes of the plant, e.g. large volumes of water suddenly released during the utilization of water filtration gravel system, tanks used for the temporary storage of water after the various water purification steps, chemicals used to regenerate the ion-exchange resin columns, etc.

2. Biocide treatment

Testing diverse biocides including the establishment of their concentration and exposure time that safely fit the operating conditions of the studied power plant.

The detailed preparation of the operating conditions of the chemical treatment.

3. Special cultivation analyses

The microbial communities of the ultrapure water produced in the studied plant were identified by newly designed cultivation methods on special media.

4. Polyphasic taxonomic tests, description of new taxa

Polyphasic taxonomic research and description of newly identified bacteria isolated from the ultrapure water produced in the water purification system.

TEST METHODS

1. Sampling

In totality we have taken samples from 12 locations in the water purification system for later cultivation and cultivation independent analyses. We have sampled the water of the Danube (raw water) once, the raw water tank, the lime softener, and the water of the gravel filtration unit daily for a week. Simultaneously we have followed the full exhaustion cycle of the ion-

exchange resin columns. Regarding the primary desalination block this cycle meant 16-20 hours with sampling every 4 hours, in the case of the mixed-bed ion-exchange resin it was 4-5 weeks with sampling once a week. In addition we have sampled the rinsing water of a recently exhausted mixed-bed ion-exchange resin column for 5 weeks, once a week. (There are 4 such columns in operation.) The goal of the serial sampling was to identify the condition of the water purification system and to establish the microbiologically critical areas. A further goal of the research was the establishment of the exact locations of future microbe eradication treatment and the proper design and preparation of the particulars of the treatment (chemical, exposure time, concentration).

2. Critical point analyses

Heterotrophic plate count

The heterotrophic plate count of the water samples collected from various locations at the water purification system using R2A medium was determined.

Cell count estimation using epifluorescence microscopy

Cells filtered (0.2 μm isopore-diameter polycarbonate membrane) and fixed from the water samples were dyed by DAPI (4', 6-diamidino-2-phenylindole) (1mg*ml^{-1}) then we have established the cell counts of the individual samples using a Nikon80i epifluorescence microscope and the ImageProPlus software package.

Biofilm and resin samples analysed by scanning electron microscopy

In order to establish the microbial contamination of the individual ion-exchange resin columns we have used a scanning electron microscope to examine all resin types used in the studied water purification unit as well as the biofilm samples taken from the pipes leading to and from the mixed-bed ion-exchange columns and the post mixed-bed connector pipes and filters.

Later we have analysed the surfaces of resin treated by biocides with this method.

Molecular research (DGGE-Denaturing Gradient Gel Electrophoresis)

The bacterial communities of the water samples flowing off the ion-exchange resin columns used in the plant were compared using DGGE method. There were 25 samples in total taken at various points in time from the 4 types of ion-exchange resin (scavenger, anion-exchange, cation-exchange, mixed-bed ion-exchange).

Later, microbial communities of the water originated from the resins treated by chemicals in the laboratory model system as well as the resin and water samples taken from mixed-bed ion-exchange columns treated as per operational conditions by Kathon WT biocide were also compared by the DGGE method.

3. Biocide treatment

Establishing the susceptibility of bacterial strains isolated during earlier analyses and from the special media to biocides

The susceptibility of bacterial strains to biocides were tested against ProClin 150 (Supelco), Kathon WT (Rohm and Haas) and Bronopol (Sigma). A dense suspension of the 24-hour cultures of the strains were prepared, their optical density were set to standard values, then various concentrations (0.1-250 $mg*l^{-1}$) of biocide solutions were added to the bacterial suspensions distributed on 96-well microtiter plates. The change in cell count (proliferation of bacteria, the change in optical density) was measured every 24 hours for 5 days at 620 nm wavelength using a Tecan Sunrise (Tecan Austria GmbH) plate reader.

Biocide treatment of resins in the laboratory model system

Based on our results from the bacterial strains, we have evaluated the Kathon WT biocide as most capable of the treatment of ion-exchange resins. We have created a laboratory model system that allowed us to analyze the impact of biocides on resins (all resin types used in the plant) and on the biofilms formed on the surface of the resins.

After biocide treatment (different exposure time and concentration) the supernatant originated from the resins, similarly to the suspension of bacterial strains, were studied on 96-well microtiter plates. Variations in the cell numbers were cheked in alignment the description above. In this case the biocide concentrations of 25 and 100 $mg*l^{-1}$ were tested in detail, monitoring if the killing effect on microbes could be increase by further rise of the concentration.

Biocide treatment of resins under operational conditions

Based on our earlier test results and discussion with staff at the plant we have finally carried out the treatment of all 4 mixed-bed ion-exchange resin columns of the water purification system. The biocide treatment we used was a Kathon WT solution at 25 $mg*l^{-1}$ concentration. Strict regulations approved by plant staff were followed in all instances during the treatment process.

It should be noted that after the chemical treatment, the plant ordered toxicological analyses before the release of water off the resin columns into the sewer system or live water.

Analysis of the ion-exchange capacity of resins

In order to monitor the impact of chemical treatment on resins, we have examined the ion-exchange capacity of the resin samples before and after treament both in the laboratory and at the plant.

4. Special cultivation, identification of bacterial strains

We have created 5 different specially combined media for the cultivation of bacteria orginating from water samples from the refined saltless water tank of the water purification system. We have attempted to replicate the extremely oligotrophic environment of the refined saltless water tank for the composition of the newly formulated media (synthetic and complex). As complex media we have used an "extract" of the bacterial strains isolated from samples originated from the same plant during earlier researches. The enriched samples were distributed onto the proper medium and incubated at 28 ^{o}C for one week. Bacterial strains were isolated randomly from the media, cleaned thoroughly as per standard procedures used in microbiology, then maintained on R2A medium for future research.

The taxonomic identification of the isolated bacteria strains

DNA was isolated from the strains using a G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Inc.) then we amplified the 16S rRNA gene subunit of the bacterial DNA with 27F and 1492R primers with a GeneAmp PCR System 2700 Thermal Cycler (Applied Biosystems). The amplified 16S rRNA gene subunits were grouped by ARDRA (Amplified Ribosomal DNA Restriction Analysis) method. The group representative and the single strains underwent 16S rRNA gene sequence analysis and we carried out their taxonomic identification using the NCBI BLAST (Basic Local Alignment Search Tool) Program and the EzTaxon Identification service.

5. Polyphasic taxonomy, description of novel bacterial taxon

We have managed to cultivate some bacterial taxa new to science during the special cultivation examination from the refined saltless water tank of the studied water purification system. During the polyphasic taxonomic research, we have identified the morphological, biochemical and physiological characteristics of the bacterial strains (PI_21^{T}, PI_31, PI_25) isolated by us and the closest relatives as per the 16S rRNA gene sequence. Furthermore, we

have established the chemotaxonomic markers of the strains. Finally, we have performed API diagnostic tests and DNA-based tests with our strains.

RESULTS AND CONCLUSIONS

1. As per the results of the critical point analysis of the studied water purification system we have ascertained that several locations of the plant are heavily contaminated by microbes. The results of the heterotrophic plate count and the cell count by microscope also identified the gravel filtration unit and the mixed-bed ion-exchange resin columns as the most contaminated spots of the system. Volumes of slack water occur in these locations, due to the design and operational aspects of the system and this slack water allows intense microbial growth.

2. The SEM analyses of the surface of the untreated resin samples showed that some of those (scavenger, mixed-bed ion-exchange resins) are heavily contaminated, occasionally biofilm is growing over them. (**Image 1.**).

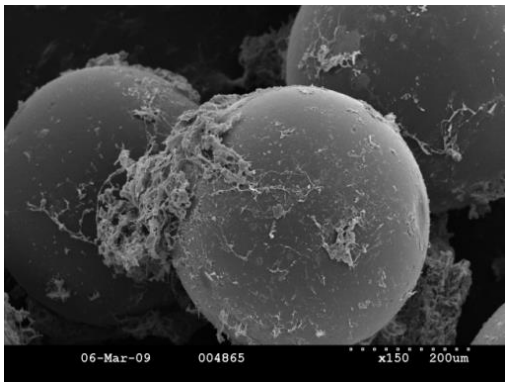

Image 1. The SEM photo of the mixed-bed ion-exchange resin before treatment

3. The results of the cultivation independent tests using the DGGE method established that resin columns of the primary desalination block have diverse microbial communities. The mixed-bed resin columns that are newly regenerated or about to be used have similar microbial communities while the mixed-bed ion-exchange that is close to exhaustion has a modified bacterial community: during the utilization of the resin a succession is noticeable in the composition of the bacterial community. The mixed-bed column that has been in prolonged use along with the water sample of rinsing water running off the mixed-bed show a difference in the microbial community compared to the rest of the samples.

Taking into account all of these preliminary results we have targeted the chemical treatment of the most contaminated mixed-bed ion-exchange resin columns.

4. Testing the susceptibility of the bacterial strains originating from the refined saltless water tank of the water purification system ProClin and Kathon WT seemed effective in all instances. The Kathon WT was successful in decreasing the bacterial growth even in very small concentration (5 mg*l^{-1}). It should be noted that due to the construction of their cell wall the tested *Mycobacterium* strains showed resistance against the highest concentrations (100 mg*l^{-1}) of biocide. Despite the treatment they have exhibited cell count growth, though at a reduced pace. The complete elimination of microorganisms from already contaminated systems is impossible, however, the applied biocides have reduced their numbers significantly.

5. The laboratory model system proved capable of testing the Kathon WT biocide at the concentration (25 mg*l^{-1}) chosen by us.

6. The results of the ion-exchange capacity were adequate after examinations in both small volume and the laboratory model system. The chemical has not caused significant ion-exchange capacity variance for any of the resin types even in the largest applied concentration (100 mg*l^{-1}).

The successful reduction of the bacterial growth and the tests checking the impact of the applied chemical on the ion-exchange capacity allowed utilization of the selected Kathon WT biocide under operational conditions.

7. The results of the SEM analyses assessing the efficacy of the chemical microbe eradication program in the plant have established that the surface of resins that were heavily contaminated and occasionally overgrown by biofilm were cleaned up completely after treatment and the multiple refined saltless water washes. Microbe cells were often undetectable, not even in traces (**Image 2.**) The ion-exchange capacity of the treated mixed-bed resin columns was reinstated to almost original condition thanks to the enlarged free resin surface. After the treatment it was possible to put in operation a resin column and source good quality ultrapure water off it (in 2-3x volume than before) even though the column had not been in use for more than one and a half year because the quantity and quality of the water produced by the column were not adequate. Despite the successful antimicrobial treatment the resin column was re-contaminated with time. After 3 months several cells could be detected again though the formation of compact biofilm was not noticeable. For this reason we have suggested the infrequent (every 3-6 months) chemical antimicrobial treatment of the resin columns and their frequent refined saltless water washes.

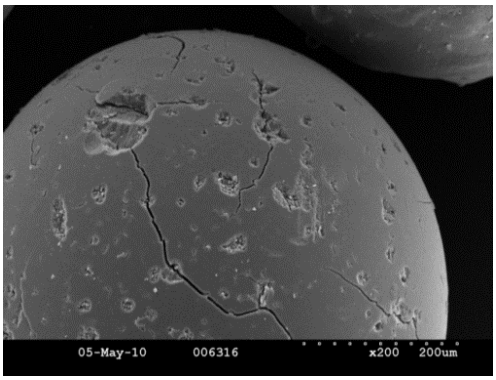

Image 2. The mixed bed ion-exchange resin column after chemical treatment, multiple refined saltless water washes and agitation by air

The surface of the resins is fractured, damaged but microbes are not detectable on them anymore.

8. The special cultivation tests of the water sample originated from the refined saltless water tank have made a new section of cultivable diversity visible compared to the earlier cultivation tests using samples from the same plant. We were able to detect several bacterial strains that, based on data in literature, normally occur in aqueous environments and are key organisms for biofilm formation there. A number of these strains characterized by diverse metabolic ways may be capable of stripping the rubber coating of pipe systems, corroding metals and assisting corrosive processes. By using special media we managed to cultivate different bacteria from previously described communities, thus our experience strengthens the view that the composition of the given medium influences the type of organisms one is capable of identifying by cultivating from a given environment.

9. Several isolated organisms represent new taxa to science. We have carried out the polyphasic taxonomic examination and the publication of the new taxon in scientific paper. The new species was named *Phreatobacter oligotrophus* (**Figure 1.**).

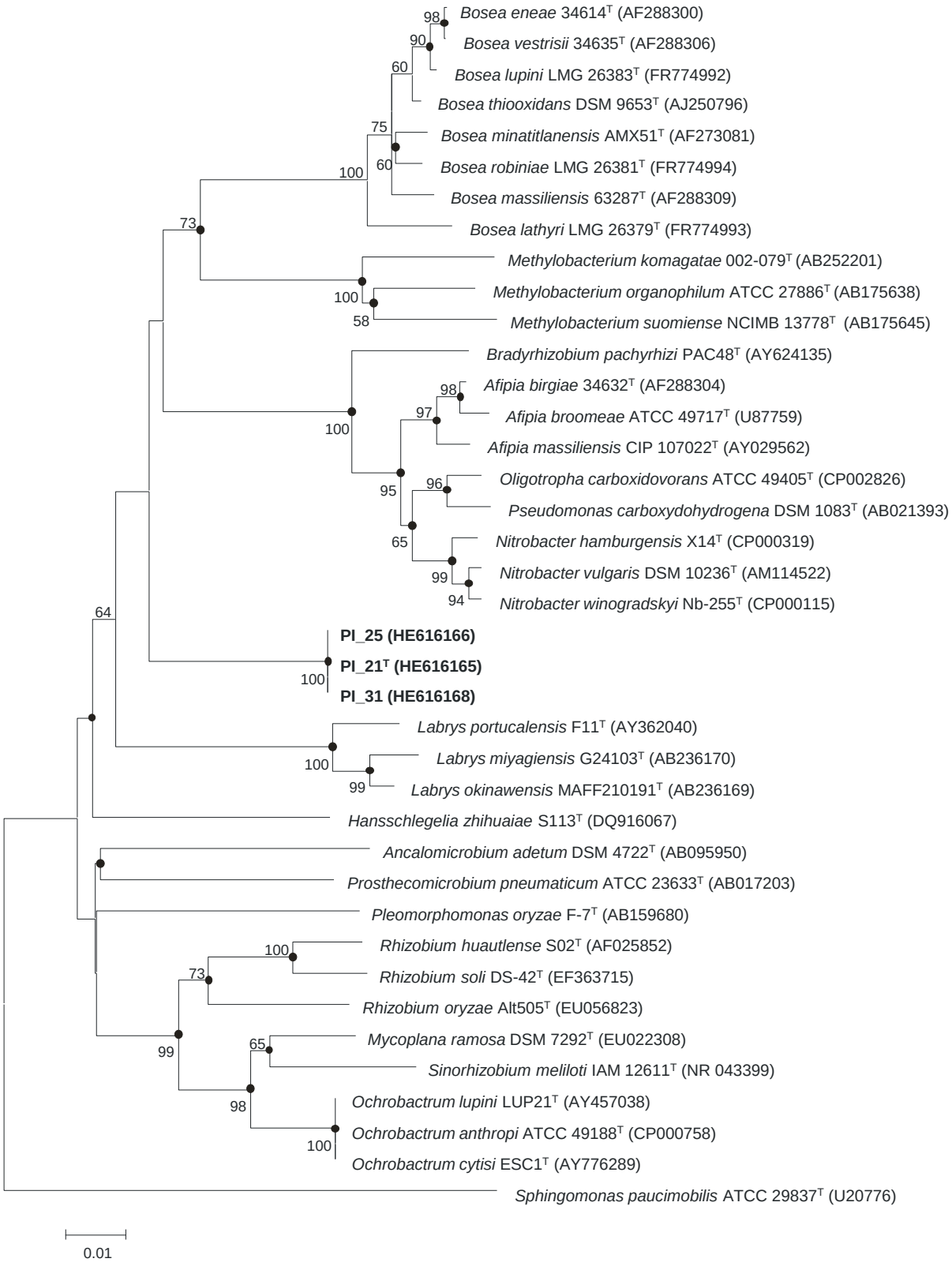

Figure 1. The neighbour-joining tree based on 16S rRNA describing the bacterial strains isolated in our laboratory (PI_21^T, PI_25, PI_31) and their nearest relatives

We have marked the bootstrap values based on 1000 repetitions on the tree (only those above 50%). The points signify the locations that resulted in the same branching with each tree editing method. The bar indicates one substitution per 100 nucleotides.

PUBLICATION LIST IN CONNECTION WITH THE THESIS

1. BOHUS, V., **KÉKI, ZS.**, MÁRIALIGETI, K., BARANYI, K., PATEK, G., SCHUNK, J., TÓTH, E. M.: Bacterial communities in an ultrapure water containing storage tank of a power plant. *Acta Microbiologica et Immunologica Hungarica* 58, 371-382 (2011).

2. **KÉKI, ZS.**, GRÉBNER, K., BOHUS, V., MÁRIALIGETI, K., TÓTH, E. M.: Application of special oligotrophic media for cultivation of bacterial communities originated from ultrapure water. *Acta Microbiologica et Immunologica Hungarica* 60, 345-357 (2013).

3. TÓTH, E. M., **KÉKI, ZS.**, BOHUS, V., BORSODI, A. K., MÁRIALIGETI, K., SCHUMANN, P.: *Aquipuribacter hungaricus* gen. nov., sp. nov., a novel actinobacterium isolated from the ultra-pure water system of a Hungarian power plant. *International Journal of Systematic and Evolutionary Microbiology* 62, 556-562 (2012).

4. TÓTH, E. M., VENGRING A., HOMONNAY, Z.G., **KÉKI, ZS.**, SPRÖER, C., BORSODI, A.K., MÁRIALIGETI, K., SCHUMANN, P.: *Phreatobacter oligotrophus* gen. nov., sp. nov., a novel *Alphaproteobacterium* isolated from the ultrapure water of a water purification system of a Hungarian power plant. *International Journal of Systematic and Evolutionary Microbiology* 64, 839-845 (2014).