Eötvös Loránd University Faculty of Sciences
Biology PhD School
Experimental Plant Science PhD program

Veronika Bohus

Microbiological investigation of an industrial ultra pure supply water plant using cultivation-based and cultivation-independent methods

– PhD Thesis –

Supervisor:

Dr. Erika Tóth

Assistant professor, PhD

(ELTE Biological Institute, Department of Microbiology)

Head of the PhD School:

Dr. Anna Erdei

Professor, DSc

(ELTE Biological Institute, Department of Immunology)

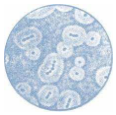
Program leader:

Dr. Zoltán Szigeti

Professor, DSc

(ELTE Biological Institute, Department of Plant Physiology and Molecular Plant Biology)

ELTE Biological Institute, Department of Microbiology, 2011



INTRODUCTION

Many industries suffer from the microbial contamination of ultra pure water (UPW), e.g. in semiconductor, pharmaceutical, food and beverage production, or industrial heating systems, power plants. UPW is mostly used as raw material, feed or cooling water, etc. Since high purity water is important component of many manufactured type environments, the investigation of their bacterial diversity has attained global importance.

Ultra oligotrophic aquatic ecosystems (both natural and industrial) are extreme environments. As part of their adaptation, microbes tend to form biofilms on different surfaces. In the biofilm, nutrients are more available and give the chance for microbes to persist in a nutrient-deprived environment. Biofilm formation and the metabolic activity of microorganisms may bring forth biofouling and microbially influenced corrosion (MIC) of metals and other surfaces, even leading to critical (metal) failures. Although UPWs' generally contain extremely low quantities of organic (TOC <3 mg/L) and inorganic compounds (conductivity <1 mS/cm), it was discovered that a wide range of bacteria- mainly oligotrophic bacteria- have the ability to grow in such low nutrient containing environments.

The supply water system of a Hungarian power plant has produced marks of biofouling and biocorrosion, causing reduced lifetime, effectiveness and yield, and increased operational costs as a result. These phenomena developed on contrary the fact that the produced supply water has a CODpa < 0,1 mg/L and conductivity < 0,1 mS/cm. The objective of the present study was to characterize the bacterial community structure of this ultra pure water system (supply water itself, biofilms formed in the pipelines and on ion exchange resins, water purifvation systems, water storage tank) by using cultivation and cultivation independent methods, in order to help control operations.

OBJECTIVES

Ultra pure waters (UPW), characterized by extremely low salt and nutrient concentrations, can suffer from microbial contamination which causes biofouling and biocorrosion, possibly leading to reduced lifetime and increased operational costs. The aim of the present study was to reveal the microbiological contamination of different parts of a Hungarian industrial ultra pure supply water plant and to reveal its microbiological community structure regarding the role of microbes in corrosion processes.

1. At the beginnig of or work, little was known about the microbial community structure in the system. Thus, in the first step, we had to choose a medium suitable for cultivation of oligotrophic bacteria based on the literature. In the next step, our aim was to reveal the bacterial community structure of the refined saltless water derived from the exhausted and the freshly regenerated mixed bed ion exchange resin in the refined saltless water production plant.
2. The raw river water is purified in many steps befor using it as boiler, feed or cooling water. The last step of the purification process is a purification with a mixed bed ion exchange unit. The construction material of the system is carbon steel coverd with a butyl rubber inner coat in the stainless steel in the refined saltless water production plant and in the secondary cycle but stainless steel in the primary cycle. To get a whole picture of the bacterial community structure of the system it is important to know the microbial contamination of the used resin and the forming biofilm on different surfaces (resin, pipe).
3. The system can be devided into two main units: primary cycle/steam generator/ boiling cycle and the secondary cycle/turbines. Our further aim was to get a glamp on the microbiological community structure inside these systems as follows:
   3.1. to reveal the bacterial community structure of the volume compensatory tank (TK) which is to store the refined saltless water before it goes to the primary system;
   3.2. to reveal the bacterial community structure of the water purification units of the primary and secondary cycles including the examination of the inlet and outlet waters of these units.

THE INVESTIGATED SYSTEM

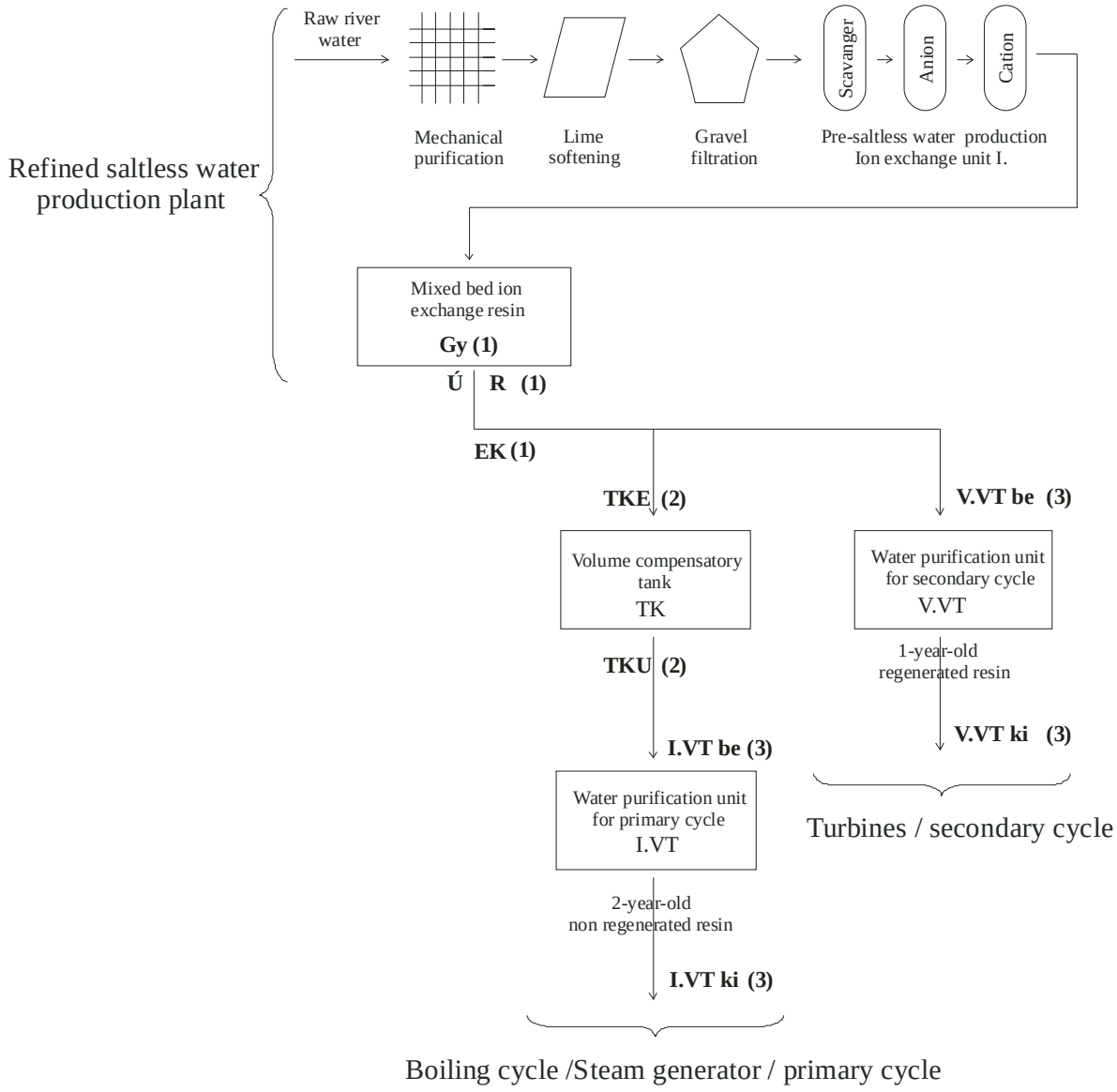


Figure 1. The schematic representation of the investigated supply water production system and the sampling points during 2005-2007.

(1) First sampling (04. 05. 2005.): **R-** refined saltless water from the exhausted mixed bed ion exchange resin, **Ú**- refined saltless water from the freshly regenerated mixed bed ion exchange resin, **EK**- rich biofilm formed on the surface of the rubber coated low flow rate elbow part of the product water collection pipe, **Gy**- the mixed bed ion exchange resin sample in the refined saltless water production plant.

(2) Second sampling (18. 06. 2006.): **TKE**- inlet refined saltless water of the volume compensatory (TK) tank, **TKU**- outlet refined saltless water of the volume compensatory (TK) tank.

(3) Third sampling (08. 03. 2007.): **I. VT**- its main function to purify the refined saltless water coming from the primary cycle. It contains non regenerated mixed bed ion excjange resin. The investigated water derived from a 2-year-old, non regenerated resin: **I. VT be- inlet water**, **I. VT ki- outlet water**; **V. VT** - its main function to purify the refined saltless water coming from the secondary cycle. It consists of a mechanical filtration and a mixed bed ion exchange resin. The investigated water derived from a 1-year-old, regenerated resin: **V. VT be- inlet water**, **V. VT ki- outlet water**.

MATERIALS AND METHODS

Microscopy - scanning electron microscopy (SEM) and epifluorescent microscopy

Scanning electron microscopy (SEM) is an effective method that is generally used for either revealing the morphological diversity of microbial communities or investigating biofilm sructures. Our biofilm samples was derived from the rich biofilm formed on the surface of the rubber coated low flow rate elbow part of the product water collection pipe. Direct cell count estimation could be carried out on water samples during the second (TKE, TKU) and the third (inlet and outlet waters of I. VT and V. VT) sampling which was performed by using epifluorescent microscopy after DAPI staining.

Cultivation

Three different media, such as tryptic soy agar (TSA), M-27, and R2A were used to enumerate aerobic heterotrophic bacteria from water samples (Ú, R, TKE, TKU, I. VT outlet, V. VT outlet). In addition to direct plating, enrichment cultures were prepared. All plates and enrichment cultures were incubated at 28 °C for up to 5-7 days. Enrichment cultures were serially dilutedand plated onto the same medium used for enrichment and the plates were incubated again at 28 °C for up to 5-7 days. Separate bacterial colonies growing on the plates were randomly isolated, then purified and maintained on R2A agar slants.

The strains were grouped with ARDRA by using AluI (AG$^{\downarrow}$CT) and Hin6I (G$^{\downarrow}$CGC) restriction enzymes. The group representatives and the ungrouped strains were subjected to 16S rDNA sequence analysis. The search for phylogenetically related type strains was carried out by using the NCBI BLAST program and the EzTaxon Server (version 2.1).

Novel taxa from I. VT sample could be detected. Designation of the isolated strains are I/28 and I/32.

A novel actinobacterium *Aquipuribacter hungaricus* gen. nov., sp. nov. with strain IV-75^{T} (DSM 21674^{T}; NCAIM B 02333^{T}) could be described. The search for phylogenetically related type strains was carried out by the BLAST and the EzTaxon Server (version 2.1). The 16S rRNA gene sequence of strain IV-75T was manually aligned with the BioEdit program against sequences available from EMBL (http://www.ncbi.nlm.nih.gov/). The alignment was used to calculate the distance matrix

corrected by the Kimura 2-parameter method and to construct a phylogenetic tree using the neighbour-joining method of the CLUSTAL X software package. Bootstrap analysis was based on 1000 resamplings.

T-RFLP and molecular cloning

As it is well known that small amount of bacteia could be detected by using cultivation methods, samples (water samples: Ú, R, TKE, TKU; I. VT and V. VT inlet and outlet); biofilm sample (EK); resin sample (GY) were subjected to molecular cloning and T-RFLP analysis as well.

Water samples (14 L) were membrane filtered and total DNA was extracted from the filters using Ultra Clean Water DNA. From the biofilm and ion exchange resin samples, DNA extraction was made by the Ultra Clean Soil DNA Kit. The 16S rRNA gene fragments were amplified with universal primers 27F-TET and 534R. PCR reaction and T-RFLP analysis were carried out according to Sipos et al. (2007) using AluI (AG$^{\downarrow}$CT), Hin6I (G$^{\downarrow}$CGC) restriction enzymes. T-RFLP profiles were analysed by hierarchical clustering. The similarity of patterns was displayed as dendrograms on the basis of binary data of the presence or absence of T-RFs (SM coefficient). ShannoneWeaver diversity indices were calculated from T-RFs representing >1.0% of the total peak area of the given sample.

Clone library was constructed from the R water sample. This sample was chosen, because it has been supposed to contain the most complex microbiota, harboring all possible contaminants of the system. 16S rRNA genes were amplified. After purification, the amplicons were inserted into a pGEM-T Easy Vector and transformed into E. coli JM 109 competent cells following the manufacturer's instructions. From the randomly selected clones, recombinant plasmids were extracted, and the inserts were amplified by the M13F and M13R primers. Grouping was based on ARDRA profiles (by using AluI and Hin6I restriction enzymes). Sequence analysis and identification were performed as above. T-RFLP analysis of the clones and strains was carried out to match the peaks of the community fingerprints.

RESULTS AND DISCUSSION

Summary of the main results

1. The most suitable medium for the cultivation of the bacteria persisting in the system is proven to be R2A medium which contains the organic and inorganic compounds in nearly equal quantities.
2. In spite of the fact that the samples were taken from different points of the system at different times, more isolates can be isolated from the low nutrient containig media (M27 and R2A) in case of samples derived from those parts of the system that are less contaminated with microbes (Ú, TKE, I. VT outlet) and visa verse. More isolates can be isolated from the high nutrient containig medium (TSA) in case of samples derived from those parts of the system that are strongly contaminated with microbes (R, EK, TKU, V. VToutlet).
3. The heterotrophic germ counts (CFU) meet the criteria of potable water / (ultra) pure water category except sample TKU where 40 times higher CFU value could be detected. In general, the CFU counts are smaller with 2 or 3 orders of magnitude in comparison with the results from the direct cell count method. Direct cell counts of inlet waters are smaller with 1 order of magnitude as compared to the ones of outlet waters except in the case of samples TKU-TKE, where the order of magnitude remained the same but was followed with a 3 times increasement in total cell counts.
4. The main conclusion of our work related to the examined ultra pure water supplying Hungarian power plant is that complex microbial communities can be revealed in any parts of the whole system:
   - **4.1.** The resin, rubber covered carbon steel surfaces and the refined saltless water samples are microbially contaminated even in the refined saltless water production plant.
   - **4.2.** Despite of special parameters (high temperature, low nutrient, strong flow rate, etc.) existing in the primary and secondary cycles, complex microbiological communities can be revealed.
5. Bacteria found in the product water of the supply water plant derive from the used raw water, but many uncultured bacteria seem to be indigenous planktonic species.
6. Much of the bacteria detected in the system are aerob or anaerob chemolitotrophic

autotrophs, mainly H_2-autotrophs. These organisms not only fix CO_2 producing organic matters thus increasing the amount of inner organic content but also can adhere to surfaces forming biofilms and can contribute to the corrosion processes. Some photolitotrophic autotroph microorganisms (cyanobacteria, *Rhodopseudomonas* species) also could be observed. They can be not only passive survivors but also could proliferate or could contribute to the inner loading of the system.

7. Most of the microorganism are chemoheterotrops. They use the compounds of died microbes, the organic compounds produced by autotrophs, the structural elements of the system as nutrients. Most of them are aerob or facultatively anaerob. Strictly anaerobes can be hardly detected. Their main characteristic is that they are degraders of hardly degradable compounds, such as xenobiotics, rubbers, polycyclic aromatic compounds and different kinds of resins.

8. Methylotrophs, the one-carbon-compound using microorganisms, could also be detected. They convert the hardly degradable organic compounds into accessible forms.

9. The nitrogen source is not limiting in the system: most of the revealed microorganisms are able to fix nitrogen.

10. The detected microorganism can take part in inducing / accelerating / contributing to the corrosin processes by using the structural aromatic compounds, producing corrosive metabolic by-products or forming biofilms. The hydrogen-consumers that accelerate the corrosion processes and the methylotrophs that use the aromatic components of inner coates and rubber surfaces act a main role in these processes.

11. Novel taxa are also could be revealed and a novel actinobacterium is described: *Aquipuribacter hungaricus* gen. nov., sp. nov. with strain IV-75^T (DSM 21674^T; NCAIM B 02333^T).

12. The revealed microbiological community structure and contamination of the system can be a basis of further investigations that can be carried out in the future.

12.1. The total eradication of microbes from the system seems to be impossible but the germ count can be radically decreased by using and combining different purification methods, like i) mechanical purification of the pipe system; ii) eradication of deposites from the surfaces by using chemical agents; iii) it is advisable to change the structural components of the system which can be ideal for microbial adhesion or proliferation (eg. rubber coatings).

12.2. It would be advisable to produce low cell count containig waters by using and combining different water purification methods.

12.3. Further investigations can be carried out on the usage of microbial proliferation inhibitory agents like biocides: setting a modell system and performing *in situ* applicability studies.

PUBLICATIONS RELATED TO THE THESIS

Articles in IF journals

Bohus, V., Tóth, E. M., Székely, A. J., Makk, J., Baranyi, K., Patek, G., Schunk, J., Márialigeti, K. Microbiological investigation of an industrial ultra pure supply water plant using cultivation-based and cultivation-independent methods. Water Research, 44, 2010, pp. 6124-6132.

E. M. Tóth, Zs. Kéki, **V. Bohus**, A. K. Borsodi, K. Márialigeti and Peter Schumann. *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. April 22, 2011 ijs.0.032672-0. Published ahead of print April 22, 2011.

OTHER PUBLICATIONS

Articles in scientific journals

Bohus, V., Székely, A., Makk, J., Márialigeti, K., Tóth, E. M. Ultra tiszta vizek bakteriológiai vizsgálata tenyésztésen alapuló és tenyésztéstől független módszerekkel. Hidrológiai Közlöny 87(6), 2007, pp. 58-61.

Abstracts

Bohus, V., Tauber, T., Tóth, E. M. Microbial communities of the sediment of two Hungarian shallow lakes (Hévíz and Balaton) studied by chemotaxonomical methods. Acta Microbiologica et Immunologica Hungarica, Akadémiai Kiadó, Budapest, 52, 2005, pp. 16.

Bohus, V., Tóth, E. M., Székely, A., Makk, J., Márialigeti, K. Bacteriological study of ultra pure water by cultivation based and molecular methods. International Symposium on Environmental Biotechnology ISEB ESEB JSEB Book of Abstracts, 2006, pp. 125.

Veronika Bohus, Anna Székely, Judit Makk, Károly Márialigeti, Erika M. Tóth. Bacteriological diversity of an ultra pure water evaluated by cultivation and cultivation independent methods. Acta Microbiologica et Immunologica Hungarica, Akadémiai kiadó, Budapest, 53(3), 2006, pp. 253.

Tóth, E., **Bohus, V.**, Székely, A., Márialigeti, K. Study of microbial communities of high purity water of a power plant based on cultivation and cultivation independent molecular methods. Biospektrum, 2007, pp. 66.

Bohus, V., Szoboszlay, M., Márialigeti, K., Tóth, E.M. Study of the bacterial community of industrial cooling waters by molecular methods. Acta Microbiologica et Immunologica Hungarica, Akadémiai Kiadó, Budapest, 54, 2007, pp. 16.

Tóth, E. M., **Bohus, V.,** Makk, J., Székely, A., Márialigeti, K. Microbial community of the high purity water system of a power plant. Acta Microbiologica et Immunologica Hungarica, Akadémiai Kiadó, Budapest, 54, 2007, pp. 136.

Main conference lectures

Veronika Bohus, Erika M. Tóth, Anna Székely, Judit Makk, Károly Márialigeti. Bacteriological study of ultra pure water by cultivation based and molecular methods. ISEB, ESEB, JSEB Konferencia, Lipcse, 2006. július 9-14.

Erika M. Tóth, **Veronika Bohus**, Judit Makk, Anna J. Székely, Károly Márialigeti (2007) Microbial community of the high purity water system of a power plant. 15th International Congress of the Hungarian Society for Microbiology, Budapest, 2007. június 18-20.

Bohus, V., Szoboszlay M., Márialigeti K., M. Tóth, E. Bacteriological characterisation of three ultra pure cooling water purification tanks by means of cultivation and cultivation independent methods. MMT 2008. évi Nagygyűlése és a XI. Fermentációs Kollokvium, Keszthely, 2008. Október 15-17.

Tóth, E., Szoboszlay, M., **Bohus, V.**, Makk, J., Márialigeti, k. Studies of biofilms formed in ultra pure water pipelines of a power plant. FEMS Conference, Göteborg, Svédország, 2009. június 28 - július 2.