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APPLICATION OF FT-IR FOR CHARACTERIZATION OF BIOMASS ISOLATED FROM SURFACE AND DRINKING WATER WITH HIGH CONCENTRATION OF NATURAL ORGANIC MATTER

Kristina Tihomirova[1], Marita Gavare[2], Linda Mežule[1], Māra Grube[2], Tālis Juhna[1]

[1, 3, 5]*Department of Water Engineering and Technology, Riga Technical University, Azenes street 16/20-263, Riga, LV 1048, Latvia.*
[2, 4]*Institute of Microbiology and Biotechnology, University of Latvia, Kronvalda blvd. 4, Riga, LV 1586, Latvia.*
E-mail: [1]kristina.tihomirova@rtu.lv,

Abstract. Fourier Transform Infrared (FT-IR) spectroscopy for identification of bacteria in raw and drinking water samples in which the biomass before the analyses was concentrated from about 0.4 m3 of water with crossflow ultrafiltration (CFUF) method was used. The species of the bacteria were identified based on the analyses of the main biochemical cell components: lipids – 2928 – 2856 cm^{-1}; proteins – Amide I and II – 1549 and 1655 cm^{-1}; nucleic acids – 1242 cm^{-1}, and carbohydrates – 1080 cm^{-1}. The applicability of the FT-IR methods was tested before and after the enrichment using low nutrient broth (R2A) of CFUF samples. The optimal final concentration for the bacteria identification was found to be in the range from 2×10^8 and 4×10^9 cells/ml. Results showed that if water samples after the concentration with CFUF were not enriched on R2A the identification of bacteria was not possible because the peaks (in region 1800 – 716 cm^{-1}) from humic substances (HS) overlapped the biochemical cell components peaks. However, after cultivation on R2A for 27 hours selected groups of *E. coli*, *P. fluorescens* and *B. subtilis* in the water samples could be identified applying the Cluster analysis. The study showed that FT-IR can be used for the characterization of biomass in oligotrophic water samples after concentration by ultrafiltration, however, prior the analyses enrichment was required. To decrease the time for analyses to less than one day due to enrichment of the samples methods for removing the HS from the concentrated biomass should be developed in the future.

Keywords: FT-IR, drinking water, biomass, *E. coli*, *P. fluorescens* and *B. subtilis*.

1. Introduction

To rapidly respond to deliberate contamination of drinking water a fast and efficient method for identification of microbial agents are needed. Fourier Transform infrared (FT-IR) spectroscopy has been proven as a rapid chemical method for characterization, identification and differentiation of microbes in food industry, biochemistry and biology (Ami *et al*. 2003, Filip and Hermann, 2001, Filip *et al*. 2004, Filip *et. al.* 2008, Helm *et al*. 1991, Lefier *et al.* 2007, Mariey *et al*. 2001). IR spectral analysis of microorganisms followed by chemometrics has advantages of speed and consistency (Lefier *et al*. 2007) in comparison with traditional cultivation methods. Up-to-date application of FT-IR in drinking water sector is limited to analyses of biofilm, due to high concentration of bacteria therein (Delille *et al*. 2007; Humbert *et al.* 2007), whereas in drinking water samples biomass concentration is not sufficiently high. Thus cross flow ultrafiltration (CFUF) has previously been applied to concentrate drinking water samples (Simmons *et al.* 2001) followed by the identification of pathogens (Veenendaal and Briuwer-Hanzens 2007). The natural organic matter (NOM), mostly humic substances (HS), in drinking water produce strong absorption peaks in IR spectrum which may overlap the peaks generated by the biomass, thus rendering the analyses of the latter one problematic. The cultivation step before the application of FT-IR for identification in drinking water should preferably be avoided, in order to shorten the time of analyses and allow the identification and quantification of viable but not culturables (VNBC) bacteria.

The aim of this study was to evaluate (i) whether the biomass concentration with CFUF followed by FT-IR spectral analysis can be used for identification of bacteria in surface and drinking water containing a high concentration of natural organic matter and (ii) whether the enrichment of the samples before processing with FT-IR is needed.

2. **Materials and methods**

Glassware

All glass bottles, flasks and filtration systems used in the experiments were thoroughly cleaned with 10% solution of potassium dichromate in concentrated sulfuric acid, rinsed with hot tap water and sterile ultra pure water (Elga Pure Lab Ultra, Veolia Water Ltd.; UK), dried, covered with aluminum septum and sterilized for 20 minutes at +121 ºC.

Sampling

The water samples were taken from two drinking water plants (WTP) in Riga (Latvia). The Daugava WTP is supplied by surface water whereas the Baltezers WTP is supplied by groundwater. Daugava WTP abstracts water from the river Daugava and the water is treated with pre-ozonation, chemical coagulation, rapid sand filter filtration, main ozonation and biologically active carbon (BAC) filtration. The raw water from the lake Mazais Baltezers is pumped into infiltration ponds. Water is naturally purified by percolating through porous layers of ground, which also recharge groundwater resources, and about 40% of the water returns to the wells. The system provides water to the pump station of Baltezers WTP. Finally chlorine disinfectant is added for both water types.

Four types of samples were analyzed for characterization of biomass: 1) Daugava river water (RW), 2) drinking water Daugava WTP (DW) after chlorination, 3) Mazais Baltezers lake water (MB), 4) drinking water Baltezers WTP (BW) after chlorination. Five types of samples were analyzed for characterization of natural organic matter (NOM): 1) RW, raw water from pumping station, 2) MC, in mixing chamber after pre-ozonation, 3) RF, after sedimentation and rapid sand filtration, 4) OZ, in main ozonation chamber, 5) BAC, after biologically active carbon filter. Water samples (500 and 100 ml, respectively) were collected in glass bottles. The bottles were stored in a refrigerator at temperature 2 ºC to 5 ºC before the analyses.

Method of biomass concentration

The ultrafiltration technique using equipment with Hemoflow filter developed by Veenendaal and Brouwer-Hanzens (2007) was used – water was filled in a tank (10 l), and further pumped through the Hemoflow filter. The overpressure over the filter was increased until the fraction of water filtrate was slowly pressed through the straw walls (cross-flow). The concentrate was returned back into the tank, thus the concentration of micro-organisms in the tank was increased. The concentration process was stopped after 12 h when the tank was empty. The concentrate from the hose and filter was pumped into a sterile bottle. Four types of CFUF samples were analyzed: RW with concentration degree (CD) 1003, DW – CD = 720, MB – CD = 336 and BW – CD = 720. CFUF samples were additionally concentrated by centrifugation (Sigma 2-16KC, Germany) for 10 minutes at RCF = 4000 g, collected in sterile tubes and four types of concentrated CFUF samples were analyzed.

Cultivation of bacteria and water samples

Cultures of *Escherichia coli* ATCC 25922, *Pseudomonas fluorescens* LMKK 559 and *Bacillus subtilis* ATCC 6051 grown in liquid R2A media (30 °C, overnight) were inoculated into 100 ml of sterile liquid R2A medium (final concentration ~ 10^6 cells/ml) and incubated with constant shaking (150 rpm) at 30 °C. Samples were collected after 1, 6, 7, 23, 24, 27, 30, 44 and 48 hours and washed twice with sterile distilled water (Mini Spin Pluss, Sigma - Aldrich, USA) at RCF=11000 g for 10 min, resuspended in a small amount of sterile water and analyzed by FT-IR. To determine the cell growth intensity, samples were stained by DAPI (4', 6-diamidino-2- phenylindole) for 5 minutes. The cell number was determined by epifluorescence microscopy by counting 20 random fields of view (Ex: 545 ± 30 nm; Em. 610 ± 75 nm, dichromatic mirror 565 nm, Leica DMLB, Germany).

Samples from WTP grown in liquid R2A media at 30 °C were inoculated into 100 ml of sterile liquid R2A medium (1:1) and incubated with constant shaking (150 rpm) at 30 °C. After 27 and 48 hours samples were collected and washed thrice (10 min at RCF=11000 g) with sterile distilled water and analyzed by FT-IR.

Determination of total and organic carbon

Total organic carbon (TOC) and dissolved organic carbon (DOC) measurements were performed with a TOC-5000A Analyzer and auto sampler ASI-5000 (Shimadzu Corporation, Kyoto, Japan) based on high temperature and acidification of sample and by the difference of the total carbon and inorganic carbon measurement, according to the European Standard EN 1484:1997. For determination of DOC samples were filtered thought the 0.45 μm pore size membrane filters (Millipore Corporation, USA), which were carefully rinsed, first with sterile ultra pure water and then with the water sample. Each sample was tested in duplicate and the mean values were calculated (CV≤2 %). The blank and control solutions were analyzed with each series of sample in order to verify the accuracy of the results obtained by the method. The detection limit was 560 μg/l.

FT-IR analysis

FTIR spectra of pure cultures, CFUF and enriched CFUF samples formed by drop-drying of liquid samples (10-20 μl) and of NOM liquid samples (25-175 μl) were recorded on a microplate reader HTS-XT (Bruker, Ger-

many). Spectra were collected over a wavelength range 4000-600 cm-1, with a resolution of 4 cm-1. Each spectrum was produced by coaddition of 32 scans, CO2 and H2O bands excluded. Evaluation of spectral data – baseline correction by rubber band method, vector normalization, calculation of first derivates integration and cluster analysis (Ward's algorithm, vector normalization and first derivative, spectral regions 3045 – 2809 and 1700 – 726 cm^{-1} was processed by OPUS 6.5 software (Bruker, Germany).

Quantitative analysis

The cell concentration which is necessary for rapid and high-quality spectra was determined using Lambert–Bouger–Beer law (absorbance of Amide I band in range of 0.35 – 1.25 absorption units (a.u.)). Quantitative analysis was carried out using the determined molar extinction coefficients, and applying a system of Vierordt equations to evaluate the concentrations of the principal cell components (Grube *et al.;* 2002). As the characteristic absorption bands were used: 1080 cm^{-1} for carbohydrates; 1550 cm^{-1} for proteins; and 2930 cm^{-1} for lipids. Several repetitions of samples (n_{min}=3) were analyzed to check the reproducibility of IR spectra.

3. Results

FT-IR analysis of pure-culture strains of bacteria. Prior to the analyses of water samples FT-IR spectra of pure-culture strains of *E. coli*, *P. fluorescens* and *B. subtilis* grown in low nutrient broth were examined. This was done later to check whether certain bacterial species can be identified in water samples and to test whether growth phase affects IR spectrum. The absorption spectra of each bacterial strain did not show significant qualitative difference regardless the growth phase they were spiked (data not shown). The maximum cell concentration was reached after 27 h and the measurements of this time were used as specific cultivation time in the experiments. The highest cell density that was reached after 27 h was 6.0×10^8, 1.1×10^9, 7.7×10^8 cells/ml for *E. coli*, *P. fluorescens* and *B. subtilis* respectively.

To determine the cell concentration that ensures a qualitative FT-IR absorption spectrum the biomass of each culture was concentrated and then by decimal dilutions of the pure culture were prepared to determine the maximum and the minimum absorption range. It was concluded that for obtaining FT-IR spectral data interpretation the concentration of cells should be in range of 2×10^8 to 4×10^9 cells/ml.

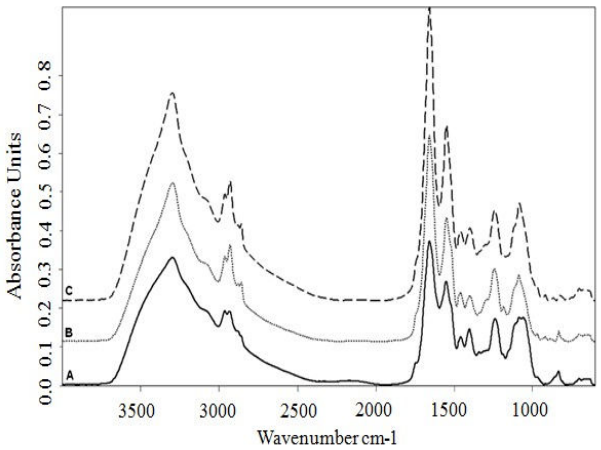

Fig 1. FT-IR spectra of E. coli (A), P. fluorescens (B) and B. subtilis (C) harvested after 27h growth in R2A broth

Comparison of FT-IR spectra of bacterial biomass of *E. coli*, *P. fluorescens* and *B. subtilis* harvested after 27 h growth in R2A liquid broth is shown in Fig 1. Interpretation of FT-IR spectra of culture samples was based on previously published data (Filip and Hermann, 2001, Filip *et al.;* 2004, Filip et. al.; 2008). Results of quantitative analysis of the cultures showed, that *E. coli* (as dry weight) contains 12.42 % of carbohydrates, 57.98 % of proteins and 5.62 % of lipids; *P..fluorescens* - 8.68 % of carbohydrate, 54.08 % of proteins and 9.15 % of lipids and *B. subtilis* – 10.93 % of carbohydrates, 59.27 % of proteins and 3.33 % of lipids.

FT-IR analysis biomass in water. The biomass in water samples from raw water and drinking water of Riga after concentration with ultrafiltration method was analyzed. Aliquots were either analyzed directly or enriched on low nutrient broth R2A for 27 hours (specific cultivation time) to increase the intensity of the peaks typical of biomass. The results from FT-IR spectra of isolated bacterial biomass of samples from WTP are shown in Fig 2. In all spectra the most intensive absorption bands were Amide II and Amide I, thus these bands were used for identifying bacterial biomass in the concentrates.

In IR spectra of CFUF concentrated non-enriched samples (Fig 2(A)) not all typical peaks of the main cell components are seen: the absorption intensity of lipid peak ranged from 0 to 0.008 a.u. in all samples; that of proteins – from 0.009 to 0.044 a.u. and that of carbohydrates – from 0 to 0.089 a.u. Amide II and Amide I peaks were not observed. Thus it can be assumed that these samples do not contain biomass or its concentration is too low for the detection by FT-IR. On the other hand, other peaks with the main absorption bands at 2920, 2850, 1800, 1600, 1500, 1400, 1250, 1180, 1040, 876, 831 and 716 cm^{-1} indicate the presence of various dissolved organic substances in water which may hide the peaks representative for biomass.

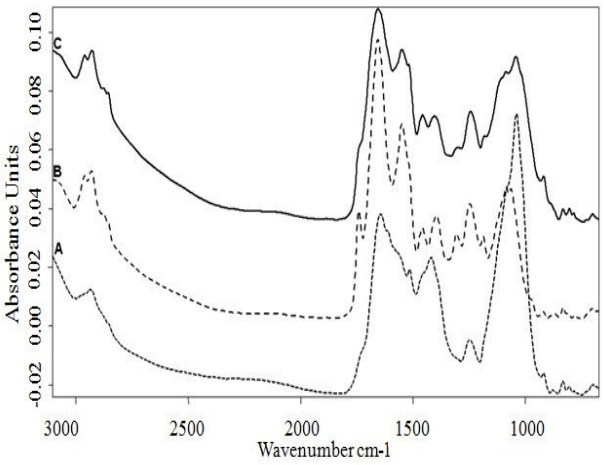

Fig 2. Normalized FT-IR spectra of BW samples. Legends: CFUF sample (A), 27 h enriched grab sample (B) and 27 h enriched CFUF sample (C)

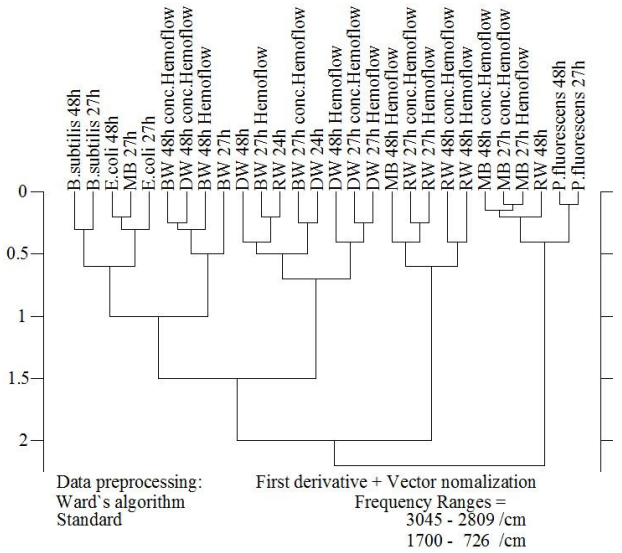

Fig 3. Discrimination and grouping of E. coli, P. fluorescens and B. subtilis biomass, enriched CFUF and enriched grab samples by FT-IR spectra cluster analysis

After the enrichment all typical peaks of the main biochemical cell components: lipids – 2928 – 2856 cm^{-1} (CH2, CH3 symetric/asymetric stretching vibrations); proteins - Amide I and II – 1549 and 1655 cm^{-1} ; nucleic acids – 1242 cm^{-1} (P=O), and carbohydrates - 1080 cm^{-1} (C-O-C, C-O, C-O-H, P=O of PO^{2-}, valent stretching vibrations of COC groups and ring vibration modes in the composition of cyclic structures) appeared (Fig 2 (B and C)). Moreover, the absorption band at 1735 cm^{-1} is specific for nitrogen fixing bacteria and indicates the accumulation of reserve biopolymers. During the enrichment the amount of biomass increased and the absorption of Amide I band increased from 0.4 to 0.8 a.u. Results of quantitative analysis of enriched RW grab samples showed, that the dry weight (dw) of biomass in the samples contained 17.70 % of carbohydrates, 57.06 % of proteins and 2.64 % of lipids whereas the enriched MB grab samples - 15.95 % of carbohydrate, 60.26 % of proteins and 3.95 % of lipids.

FT-IR spectra cluster analysis of these samples was used to discriminate between enriched CFUF samples from grab water samples, grouping the selected samples and cultures of *E. coli*, *P. fluorescens* and *B. subtilis* (Fig 3). All samples in this study were grown on the same medium therefore the biochemical composition difference that may arise due to the growth on different media could be excluded.

All of the samples tested formed a cluster which can be subdivided into three subclusters of pure culture, surface water and drinking water after chlorination. These clustering indicates that the samples differ significantly since the degree of difference is higher than 1.0. FT-IR spectra analysis showed high similarity between the enriched BW and DW CFUF samples (H<0.5) and essential difference between enriched RW, MB, DW, BW grab samples and enriched RW, MB, DW, BW CFUF samples (Fig 3). The spectra of grab MB sample after 27 h growth was more similar to that of E. coli biomass after 27 and 48 h cultivation (H <0.5) and less similar to B. subtillis after 27 h and 48 h growth (H <1.0). Figure 3 shows that the FT-IR spectra of MB CFUF samples after 27 h and RW grab sample after 48 h enrichment are similar to the P. fluorescens biomass spectrum (H<0.5). Cluster analysis revealed similarities between BW CFUF enriched for 27 h, RW grab sample after 24 h enrichment and DW grab sample after 48 h enrichment (H<0.5).

Influence of humic substances (HS) on the FT-IR spectra of water samples. The catchments of River Daugava and Lake Mazais Baltezers are largely covered in swamps and forests, therefore the concentration of HS in the water is high. In monitoring period in 2007 the concentration of DOC in the river Daugava was 15.34±3.84 mg/l and in Lake Mazais Baltezers - 13.45±1.16 mg/l. After the water treatment process in Daugava WTP the DOC concentration decreased by 65% and the average concentration in drinking water was 5.33±0.45 mg/l. After the infiltration process in Baltezers WTP the DOC concentration decreased by 64% and the average concentration in the drinking water was 4.79±0.19 mg/l.

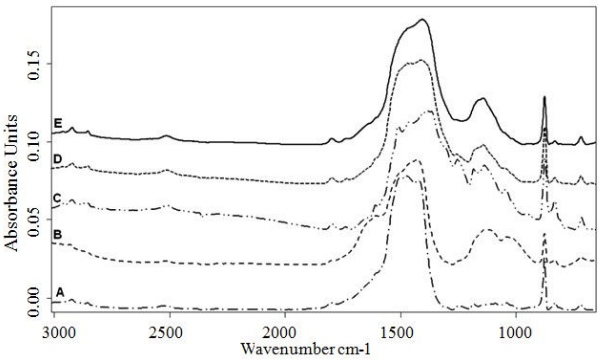

Fig 4. Normalized FT-IR spectra of samples from Daugava WTP. Legends: RW, raw water from pumping station Daugava (A); MC, in mixing chamber after pre-ozonation (B); RF, after sedimentation and rapid sand filtration (C); OZ, in main ozonation chamber (D); BAC, after biologically active carbon filter (E)

To assess the effects of different concentrations of NOM on the IR spectra the grab samples from the WTP Daugava were used. As an example, the IR spectra of water samples from Daugava WTP are shown in Fig 4. The FT-IR absorption band assignment based on published data (Chen *et al.* 2002, Dai *et al.* 2006, Hay and Myneni 2007, Kim and Yu 2005, Rocha *et al.* 2000, Simmons *et al.* 2001, Veenendaal and Broewer-Hanzens, 2007). FT-IR spectra did not show significant composition differences between samples thus for qualitative FT-IR spectral analysis it was necessary to concentrate the grab samples by centrifugation. Only by integration of spectra it was observed that some spectral bands of raw water were slightly shifted after conventional treatment and the area of each peak increased or decreased through the water treatment process. Peaks at 1140 – 1043, 876 and 716 cm^{-1} were practically constant during the treatment (carbohydrate/alcohol functions, the occurrence of polysaccharides).

The DOC concentration in CFUF of RW and MB samples was 275.8±19.8 mg/l and 115.4±5.6 mg/l, respectively. The concentration of DOC decreased to 91.2±4.8 mg/l (67%) in DW and to 11.3±1.5 mg/l (90%) in BW. IR spectra of samples RW, DW, MB and BW showed strong absorption at 1140 – 1043, 876 and 716 cm^{-1} (Fig 2) indicating the relatively high concentration of carbohydrates.

4. Discussions

FT-IR spectroscopy is widely used for discrimination of Gram+ and Gram bacteria and rapid screening of strains as well as a quick screening of environmental samples (Filip and Hermann 2001, Filip *et al.* 2004, Filip *et. al.* 2008, Hay *et al.* 2007). However the concentration of pathogenic microorganisms in drinking water is usually too low for detection and quantification with FT-IR. One of possible techniques for monitoring of biofilms, where biomass concentration is relatively high, is the attenuated total reflection – FT-IR (Delille *et al.* 2007, Humbert *et al.* 2007), but implementation of this technique in distribution system is difficult due to the high background noise and the low signal generated by the bacterial biomass (Humbert *et al.* 2007). Hence a sufficient biomass concentration is needed for the application of FT-IR spectroscopy for the drinking water analysis. A method for concentration of microorganisms in different types of water samples with CFUF has been described earlier (Veenendaal and Broewer-Hanzens 2007) which has showed that the recovery rate is sufficiently high and reproducible for most microorganisms in all water types. This study evaluated the possibilities of FT-IR spectroscopy for the characterization of biomass isolated from drinking water and concentration by cross flow ultrafiltration (CFUF).

For the identification and recognition of microorganisms in CFUF water samples it was necessary to compare and evaluate the FT-IR spectra of sample and several bacterial strains, such as three cultures tested in this study, *Pseudomonas fluorescens* LMKK 559 widely spread in surface and drinking water (Humbert *et al.* 2007); *Escherichia coli* ATCC 25922 as indicator of the presence of many pathogens (Filip *et. al.* 2008) and *Bacillus subtilis* ATCC 6051 as etiological agent for a variety of clinical infections (Filip *et al.* 2004).

In this study cell numbers were determined by epifluorescence microscopy and showed that $10^8 – 10^9$ cells per milliliter of *E. coli*, *P. fluorescens* or *B. subtilis* cells are necessary for the FT-IR analyses in this study, which is relatively high. Some earlier studies have defined that for FT-IR analysis the cells of 500 CFU per ml are required (Hamzal *et al.* 2008). FT-IR spectra difference between the cell mass harvested after different cultivation time showed that maximum cell density was reached after 24 h of *E. coli* and *B. subtilis* cultivated in Minimum Nutrient Broth (MNB) as well as starved cell mass of *E. coli* developed identical spectra (Filip *et al.* 2004, Filip *et. al.* 2008). In this study the highest *E. coli*, *P. fluorescens* and *B. subtilis* cell density was reached after 27 h.

FT-IR spectra analysis showed that enriched CFUF MB samples were similar to the *P. fluorescens* biomass, but grab B samples after enrichment were more similar to *E. coli* biomass thus *P. fluorescens* was the dominant culture in CFUF samples and after enrichment prevailed on IR spectra. By this the high spectral distance between enriched of grab and CFUF MB samples, the high similarity between *E. coli* and enriched grab MB sample and the low heterogeneity between *P. fluorescens* and enriched CFUF samples can be explained.

FT-IR spectra of grab, CFUF samples and CFUF samples after concentration by centrifugation showed that there are no typical biomass peaks. All typical peaks of main biochemical cell components in FT-IR spectra appeared only after enrichment of samples. Simultaneously with Amide I peak absorption was reached the optimal conditions (based on Lambert–Bouger–Beer law), DOC level in CFUF samples was up to 9 - 18 times higher for RW, MB and DW; and only to 2.4 times higher than in a sample from BW after concentration with cross-flow ultrafiltration. These organic compound concentrations are

sufficiently high to create disturbing background for FTIR spectra recording. The absorption peaks at 1140 – 1043, 876 and 716 cm^{-1} are very evident (carbohydrate including polysaccharides) in CFUF samples. RW grab sample after 24 h similarity to BW CFUF sample enriched for 27 h (H<0.5) can be explained by the fact that these samples are similar regarding the DOC concentrations (about 15.3 and 11.3 mg/l respectively). Results of quantitative analysis showed that ratio of carbohydrates/proteins/lipids can be calculated for enriched RW and MB grab samples, where biomass initial concentration was relatively high, comparing with DW and BW grab samples, and NOM concentration was relatively low, comparing with CFUF samples.

In further studies other bacteria stains from CFUF filtrate should be identified and enrichment conditions chosen for more precise evaluation of the drinking water microflora. Application of other FT-IR spectroscopy methods may benefit to create optimal chart for biomass identification and quantification in different water types. The combination of CFUF and FT-IR method can be used for a quick characterization of NOM composition in each treatment step on WTP and could be useful for the improvement of the treatment process to enhance the NOM removal and to reduce the generation of contaminants in drinking water supply system in regions with high humic substance concentrations.

5. Conclusions

The following conclusions were produced in this study: (i) it was possible to identify biomass in raw and drinking water with FT-IR spectroscopy after the sample concentration with crossflow ultrafiltration methods followed by enrichment on low nutrient broth; (ii) *P. fluorescens* was the dominant species in CFUF samples both before and after the enrichment on low nutrient broth. Due to the high concentration of *P. fluorescens* identification of other bacteria such as *E. coli* and *B. subtilis* was difficult; (iii) to compare enriched sample and *P. fluorescens*, *E. coli* and *B. subtilis* species under study by cluster analysis, enrichment on similar low nutrient broth (R2A) at similar time (27 hours) was necessary; (iiii) quantitative analysis showed that the ratio of carbohydrates/proteins/lipids can be calculated for enriched and grab samples if the initial concentration of biomass is relatively high and NOM concentration is relatively low; (iiii) high concentration of DOC in surface and drinking water in Riga (Latvia) create disturbing background for FT-IR spectra recording in the CFUF samples.

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