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REVIEW ARTICLE

Detection of Biofilm on Water Supply Technical Materials with the Application of an Impedance Sensor

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Abstract

In favourable environmental conditions microorganisms can adhere to surfaces and reproduce, forming biofilm. Such a structure causes biodeterioration, i.e. biological degradation of technical materials. The issue is of high importance in the case of distribution of treated water to end-point consumers. An important factor determining the formation of biofilm is the type and character of the surface which can stimulate or inhibit its growth. The article presents innovative results of research involving measurement of growth of biofilm on technical materials used for the construction of water supply networks by means of an impedance sensor. The research was conducted at a laboratory scale continuously for 6 months, reflecting actual conditions occurring in water distribution systems. After half a year culture of environmental microorganisms in the bioreactor, an almost 100% increase in the value of relative impedance was recorded by means of a sensor placed inside the bioreactor. A comparison of the surface coverage of technical materials and the sensor with bacteria (fluorescence in situ hybridization) showed that the sensor could be used for technical materials made of polybutylene, polypropylene, and polyvinyl chloride. Observations (scanning electron microscopy) of the surface of the plastics used to build the water supply network pipes (new materials, with biofilm present, and after detaching the biofilm) showed significant changes in the structures of the materials due to biofilm formation. The largest changes in the structure were observed on the polyethylene and polypropylene surfaces. The proposed sensor could be applied in the measurements of biofilm adhesion to selected technical materials.

Article Highlights

- Impedance measurement of biofilm composed of mixture of drinking water bacterial strains
- ITO selected as the most promising material for biosensor construction
- Impedance correlated with bacterial counts
- Biofilm detachments seen as the relative impedance changes
- Four technical materials (PB, PE, PP, PVC) tested in terms of biofilm coverage
- Biosensor suitable for the detection of biofilm on PB, PP, PVC

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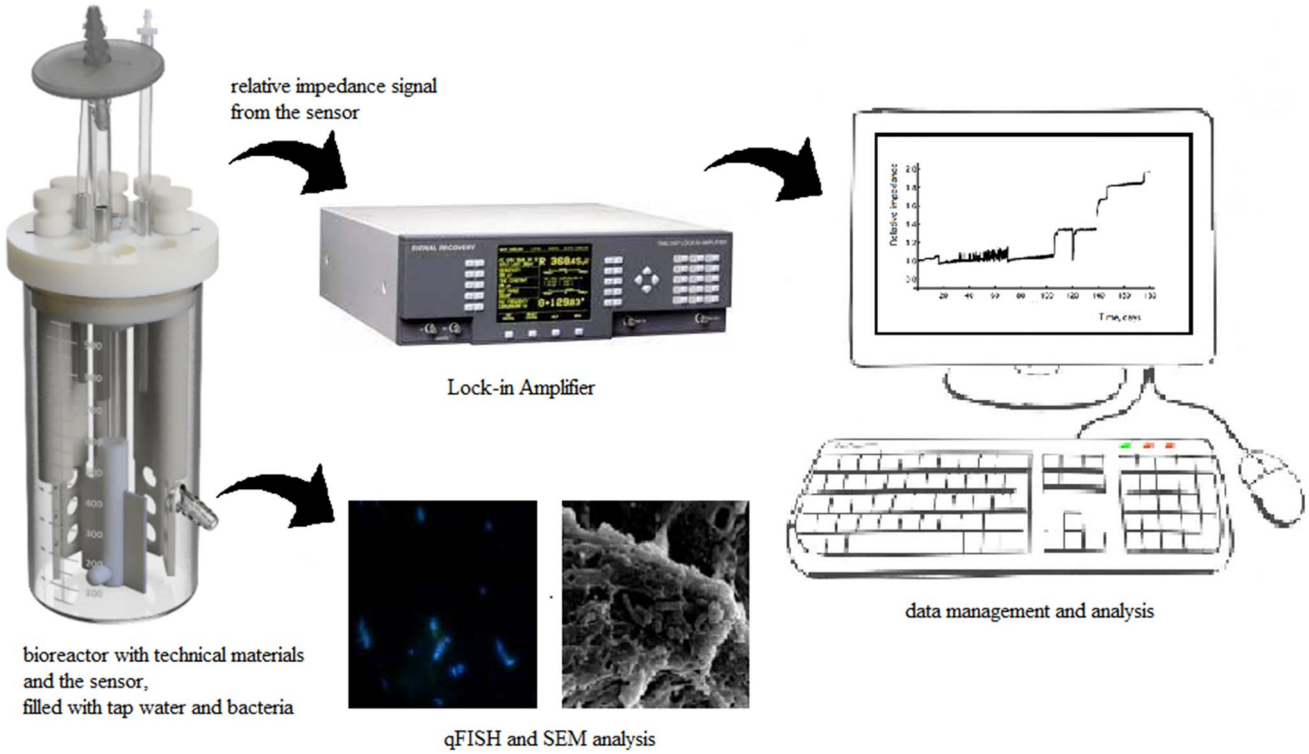
Graphical abstract



Keywords Bacterial adhesion · CDC bioreactor · Drinking water distribution system · Pipe construction materials

Introduction

Lack of biological stability of water introduced to a network results in its secondary contamination during the transport to consumers (Waller et al. 2018; Chan et al. 2019; Petersen and Hubbart 2020). Biologically unstable water provides perfect conditions for the formation of biofilm, i.e. the colonisation of the surfaces of pipelines by microorganisms, and accumulation of their metabolic products (Asghari et al. 2018; Simunič et al. 2020). Mature biofilm, also called biological membrane, is a complex structure comparable to an ecosystem (Di Pippo et al. 2018), generating strategies of survival in unfavourable environmental conditions (Ahamed et al. 2020; Gloag et al. 2020). Microorganisms developing in biofilms are suspended in extracellular polymers, and the exchange of matter and genes with the surroundings occurs through an expansive system of canals (Zhang et al. 2018; Reinhardt et al. 2020). Next to the possibility of detachment of fragments of biofilm and transport of microorganisms to further sections of the network, the survival or reproduction of opportunistic pathogens (e.g. *Legionella* sp.) (Wolf-Baca and Siedlecka 2020) in the structures of biological membrane, among others due to limited penetration of disinfectants, is also problematic (Fish and Boxall 2018).

The interaction between drinking water components and products of microbiological corrosion (of the internal surfaces of water pipelines) considerably reduces the quality of transported water (Fish et al. 2017; Douterelo et al. 2020). Therefore, fast and credible methods of biofilm monitoring are sought to immediately counteract its development and the related negative effects (Liu et al. 2016; Azeredo et al. 2017; Wolf-Baca and Piekarska 2020). It is worth to note that the standard methods of assessment of microbial quality of water are time-consuming and directed only to viable and cultivable microorganisms.

In the microbiological analysis of water, the key issue is the time of detection, particularly with regard to the possibility of epidemiological threat for recipients (Van Nevel et al. 2017; Fu et al. 2021). A method that would reduce the time of obtaining results pointing to the presence of microorganisms showing a tendency for adhesion is measurement of the impedance value of the system. The value determines metabolic and adhesion changes in bacteria (Butler et al. 2019). Impedance microbiology is applied in different fields, from detection and monitoring of microorganisms, detection

of antibiotics, analysis of food preservatives, food hygiene, clinical and pharmaceutical microbiology, to environmental samples. The method determines the quantitative change in the content of microorganisms through measurement of a change in electric conductivity of the medium during growth of microorganisms (Varshney and Li 2008; Ghosh Dastider et al. 2015; Bancalari et al. 2016; Furst and Francis 2019; Nyhan et al. 2020). It is estimated that the minimum concentration of bacterial cells in a range from 10^3 to 10^7 CFU/mL can cause a recorded change in the signal (Felice and Valentinuzzi 1999). The impedance value is a response (a measure of the opposition, so-called resistance) of the system (an alternating current circuit) to attaching microorganisms. Measurement of the signal can depend on the frequency of the system and temperature the change of which by 1 °C corresponds to a disturbance of the conductance signal at a level of 1.8% (Lasik et al. 2013).

Literature data report the application of impedance spectroscopy in monitoring of adhesion of microorganisms. Such research, however, usually involves the application of pure strains, and the created system consists of a single species. The most frequently used strains are those clinically relevant, commonly found in hospitals, i.e. *Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Listeria monocytogenes,* and *Clostridium perfringens* ([CSL STYLE ERROR: reference with no printed form.]; Yang et al. 2004; Varshney et al. 2007; Paredes et al. 2012; Reich et al. 2017; Ward et al. 2018; Guła et al. 2020; Nyhan et al. 2020). It is also worth to mention that both in natural and anthropogenic environments, including the hospitals and drinking water distribution systems, practically no monospecific biofilms occur. Moreover, in many research, such single-species cultures are conducted in an intensified manner (elevated temperature, optimal for the selected strain), and the cultivation time is often in the range of 48–72 h (Van Duuren et al. 2017; Liu et al. 2018; Párraga-Niño et al. 2018; Guła et al. 2020; Nyhan et al. 2020). According to the authors' best knowledge, there are no long-term studies conducted on environmental strains isolated from drinking water, reflecting the actual conditions in water distribution networks and the time of biofilm formation. Therefore, the presented results are crucial for better understanding of the adhesion of bacteria on pipe construction materials. Control of adhesion of mixed cultures of microorganisms can find application not only in water distribution networks, but also in medicine or industry. Understanding the process of adhesion of microorganisms to abiotic surfaces would contribute to the development of a strategy of inhibition and prevention of the occurrence of the phenomenon.

The objective of the study was an attempt of continuous monitoring of the first stage of biofilm development (adhesion) through half-year long measurement of changes in impedance. The following questions were addressed: (i) Can impedance spectroscopy be used in control of biofilm development in water distribution networks? (ii) Can the impedance signal measured by the sensor be correlated with the adhesion of microorganisms? (iii) Can the sensor be used as a tool to measure biofilm growth for all technical materials? (iv) Are technical materials destroyed after removing biofilms?

Materials and Methods

Bacterial Strains

A mixture of model strains (MMS) was used as a starter in the bioreactor. It was a group of representative bacteria isolated from the water distribution network of Wroclaw, prepared in accordance with the procedure described in (Wolf et al. 2018). Pure colonies of the strains, grown on R2A medium, were sterilely transferred to nutrient broth (Merck) and incubated at 22 °C. Each strain was reproduced separately. For further research, 0.5 mL of a given strain suspension was each time sampled from each test tube, and mixed together. Based on optical density measured on a spectrophotometer T80 + (PG Instruments) at a wavelength of 550 nm, determined as 1.07, the concentration of bacterial cells in the mixture was estimated ($12.84*10^8$ cells/mL) and confirmed with the application of the McFarland scale (bioMerieux).

The Measurement System

The measurement system was composed of the bioreactor for biofilm culture, containing fragments of technical materials building the water distribution networks and the impedance sensor.

Bioreactor

Biofilm culture employed a CDC bioreactor (BioSurface Technologies Corporation) in the form of a 1-L cylindrical glass flask with a side opening serving as outflow. The working volume of the bioreactor was approximately 0.4 L. Its top featured a cover made of polycarbonate with openings holding polycarbonate fixtures supporting fragments of technical materials and the 3D-printed fixture for the sensor. An attempt was made to provide conditions inside the bioreactor corresponding to the actual conditions in water distribution networks. The bioreactor was filled with synthetic water prepared based on parameters and ingredients of treated drinking water in Wrocław and prepared MMS (as described in 2.1.).

Briefly, the input to the reactor was a distilled sterile water and the combination of minerals with the appropriate density ($NaHCO_3$, $MgSO_4{\cdot}7H_2O$, $CaSO_4{\cdot}2H_2O$, K_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NaCl, $FeSO_4$, $NaNO_3$, $CaCO_3$) (Wolf et al. 2018). The temperature of water in the reactor and the dosed synthetic water was around 10 °C (the value is the most similar to the conditions in the network). The pH and the temperature of synthetic water supplied to the bioreactor and inside the bioreactor was constantly measured and was in the range of 7.0–7.7 and 9–12 °C, respectively, what is in accordance with the average values of drinking water in the network provided by the local Municipal Water and Sewerage Company. Final concentration of bacteria in was $12.84*10^6$ cells/mL. The system was maintained in conditions of constant temperature (18 °C). For the purpose of provision of oxygen conditions in the bioreactor, a filter with a pore diameter of 0.45 µm was mounted on its top. The solution prevented the introduction of microorganisms from the air to the bioreactor.

The culture was conducted for half a year. For the first 45 days, the experiment was carried on in static conditions with continuous stirring at 300 rpm (Elpin), and then water flow was introduced with a velocity of 0.4 m/s. The selected water velocity (0.04 m/s) represents the average velocity near the household connections to the network (data from the local Municipal Water and Sewerage Company). Based on literature data and the researchers' own experience, the appropriate time to create a thin layer of biofilm is about 30–45 days (cell structure with a layer of external polysaccharides, and not just clusters of cells randomly joined together). Due to the fact that the tests were carried out on plastic materials, which are characterized by a lower roughness coefficient than other materials from which water pipes are made (e.g. cast iron), the time established in the current study was 45 days. A peristaltic pump (Cole-Parmer) supplied synthetic sterile water to the bioreactor through the clean water pipe. Excess culture was removed from the bioreactor through the outflow pipe. A flow regulator was also mounted in the system for the purpose of elimination of turbulent conditions.

Technical Materials

The research concerned plastic technical materials used for the construction of water distribution networks, namely: polybutylene (PB), polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC). These materials were selected to research due to the increasingly common application and interests in plastic pipelines (Rożej et al. 2015; Liu et al. 2017; Li et al. 2019). Plastics are considered resistant to both electrochemical and biological corrosion, and are easy to assemble and exploit, unlike pipes made of metals. The selected materials (PB, PE, PP, PVC) were delivered from three different Polish plastics producers. Each material included from 60 to 80% of the composition of the dry mix of pure plastic and the rest were additives such as chalk, stabilizers and modifiers, and microducts: dyes and waxes (the composition and proportion of additives for each material are the trade secret of the manufacturers—data not available).

Construction of the Sensor and Impedance Measurements

Impedance measurement was performed by means of an impedance sensor produced in the process of photolithography with conducting material indium tin oxide (ITO) in accordance with the diagram presented in Fig. S1. The material for sensor electrodes was selected based on literature and preliminary research among other materials (ITO, aluminum, gold, silver, palladium, platinum). Initial material selection studies included determining the material toxicity using the Microtox test (Microtox®), as well as the degree of adhesion of microorganisms present in the water supply network using acridine orange (0.01%, Sigma–Aldrich) and the general probe for bacteria EUBmix by means of fluorescence in situ hybridization (FISH)—data not shown. The sensor was composed of two electrodes with a branch configuration. Each electrode was equipped with 4 "branches" with a width of 300 µm, arranged at a distance of 300 µm from one another. The sensor was connected to the measuring device 7280 DSP Lock-in Amplifier (Signal Recovery, AMETEK Scientific Instruments), transmitting the signal through the GPIB connector (National Instruments) to a computer with software. Impedance measurements were conducted at a constant frequency of 100 Hz, selected based on literature reports (Paredes et al. 2012, 2014; Tubia et al. 2018). The measurements were performed at 10 s intervals. Due to the high amount of data, measurement results for 300 s intervals were presented.

Determination of the Relation Between the Received Impedance Signal and Bacterial Adhesion

Before the experiment, it was evaluated whether the read and recorded impedance signal is caused by bacteria inhabiting the system, and is not related to other factors disturbing the measurement. Such a confirmation involved measurement of the value of adenosine-5′-triphosphate (ATP) of microorganisms with simultaneous monitoring of changes in impedance in an intensive 48 h culture. ATP measurement was performed by means of Molecules Probes ATP Kit (ThermoFisher), in accordance with manufacturers' instructions.

The bioreactor for biofilm culture with synthetic water was inoculated with MMS (in the same manner as described in 2.2.1.). Six fragments of ITO (the selected construction

material of the sensors electrodes) were placed in the bioreactor and removed after 2 h, 4 h, 6 h, 8 h, 24 h, and 48 h of the duration of the experiment. The impedance value recorded by the sensor was simultaneously read. Bacteria which colonised the surface of the ITO fragments (2 cm^2) were washed out to the sterile physiological solution (0.85%, Sigma–Aldrich), and then subject to the determination of the ATP level, pointing to the presence of microorganisms. The experiment was conducted on a 96-well plate with introduced relevant samples (90 µL) and reagent containing luciferase (10 µL). The time of exposure of the sample and luciferase was 10 s. Measurements were performed in 2 s intervals. The experiment was conducted in two variants: with the application of trichloroacetic acid (TCA)—final concentration in a sample 4% (Sigma–Aldrich), and without the TCA. The applied acid destroys the cellular membrane of bacteria, permitting ATP measurement, which in the case of a mixture of microorganisms provides more credible results. Luminescence was measured in two repetitions on a Fluorescence Spectrophotometer F-4500 (HITACHI). Due to the fact that the solution was sterile water, measurement of free ATP in water was omitted. A standard curve was prepared in a range from 10^1 to 10^{15} pmol ATP from which the quantity of ATP in each analysed sample was read. The results of luminescence measured for the samples in arbitrary units were transferred to pmol of ATP with the use of the obtained curve formula. Next, the ATP were expressed by unit of mass and the quantity of bacterial cells on ITO fragments were estimated with the assumption that 10^{-15} g ATP represent one bacterial cell (Pistelok et al. 2016). The calculated bacterial cells on each ITO fragment were compared with received impedance signal measured by the sensor after each time (2 h, 4 h, 6 h, 8 h, 24 h, and 48 h).

The Measurement System Assembly and Conducting the Experiment

The sensor and fragments of technical materials used for the construction of water distribution networks with dimensions of 1 cm × 1 cm (such relatively small fragments of technical materials were applied due to the considerable radius of surface curvature of pipes) were carefully rinsed with deionised water, degreased with 70% ethyl alcohol (Sigma–Aldrich), and then sterilised under a UV lamp (254 nm) for 15 min (Kim et al. 2011). Then, the technical materials were placed into the bioreactor. The system with technical materials was sterilised in an autoclave for 20 min at a temperature of 121 °C. Next, the sensor was placed in the bioreactor, and connected to the measurement equipment (Lock-in-Amplifier Model 7280), continuously recording changes in impedance.

The sensor was mounted in the bioreactor on a fixture printed in a 3D printer (3D Systems, model 3510) with the application of Visijet M3 Crystal material. It is a biologically stable material—resistant to corrosion and applicable as a component of human implants due to its non-toxic properties (Zhu et al. 2015; Bhattacharjee et al. 2016). The design of the fixture was prepared in AutoCAD software (Autodesk Inventor 2016). The printed element was tightly fitted to the bioreactor. Its dimensions are presented in Fig. S2. The fixture was prepared in a way to separate the electronic elements from the liquid (routing the cables was designed inside the element—Fig. S2). The sensor was additionally glued to the fixture with conducting glue, to eliminate signal disturbances during water movements (caused by continuous stirring) and the fixture with the sensor was mounted by means of a screw in the bioreactor. The diagram of the entire measurement system is presented in Fig. S3.

After assembling the system and adjusting the conditions (constant temperature and pH) in the bioreactor, biofilm cultivation started with impedance measurement, lasting half a year. During this time, weekly microbiological monitoring of bacterial cells suspension (CFU/mL) inside the bioreactor and in its outflow was performed. This allowed to correlate the adhesion or removal of bacterial cells with impedance changes. The concentration of psychro- and mesophilic bacteria was in the suspensions were determined by means of the cultivation method. Briefly, the bacterial suspensions from the bioreactor and its outflow were diluted with sterile physiological solution (0.85%, Sigma–Aldrich) and appropriate dilutions (1 mL) were transferred on Petri dishes and poured with R2A medium (BTL) for deep inoculation. Dishes were incubated at conditions appropriate for psychro- and mesophilic strains (22 °C for 72 h and 37 °C for 48 h, respectively) in aerobic conditions. After the incubation time, the CFU/mL were counted.

After the period of the experiment, the surfaces of the technical materials and the sensor were analysed by means of the FISH (FISH). The structures of the technical materials (PB, PE, PP, PVC) with attached biofilms were also observed using a scanning electron microscope (SEM).

Visualisation and Quantification of Biofilms by Means of FISH

An analysis of bacteria attached to the technical materials and the sensor was conducted with the application of FISH. This microscopic method detects sequences of nucleic acids by means of a fluorescence-labelled probe which directly hybridises with the complementary target sequence in a cell. Before each analysis of biofilm samples, the degree of autofluorescence and bond non-specificity was determined. The verification of autofluorescence involved performing hybridisation with no application of probes. The bond non-specificity of the applied probes was observed with the application of probe NON338. The determination

employed probes: EUB338 (majority of bacteria), EUB338 II (*Planctomycetales),* EUB338 III (*Verrucomicrobiales*), BET42 (*Betaproteobacteria),* GAM42 (*Gammaproteobacteria*), and HGC69 (*Actinobacteria).* The probes EUB338, EUB338 II and EUB338 III were labelled with Fluo dye and mixed together (EUB338mix). The BET42 and GAM42 probes were labelled with Cy3 dye and the HGC69 probes were labelled with UV-excited fluorochrome. All labelled probes were supplied by Future Synthesis (Poland).

The FISH was performed in accordance with the procedure described previously (Wolf et al. 2018). Analyses of cells marked by labelled probes usually boils down to summing up surface areas (pixels) occupied by the cells. The images (20 fields of view in each prepared sample) were taken by Eclipse 90i epifluorescence microscope (Nikon) equipped with DS-Ri2 camera (Nikon), with the use of immersion objective lens 100 × and filters for UV extinction, Fluo and Cy3 dyes. The obtained images were analysed by means of software IMAGEJ (Nielsen et al. 2009), determining the bond of labelled probes EUB338mix, BET42 and GAM42, and HGC69 to specific bacteria. The means, medians, standard deviations, maximum and minimum values of pixels, as well as % surface coverages were calculated.

Visualisation of Technical Materials by Means of SEM

The topography and structures of biofilms formed on the surfaces of the technical materials taken out the bioreactor at the end of the half a year experiment were analyzed with a SEM.

Additionally, to demonstrate the negative effects of biofilm adhesion on PB, PE, PP, and PVC, the clean surfaces of commercially new technical materials delivered from manufacturers were compared with the same technical materials immersed in MMS suspension for 3 years. Precisely, the technical materials were incubated in the MMS suspension (prepared as described in 2.1.) diluted to $12.84*10^6$ cells/mL for 3 years, under the conditions of continuous stirring at 300 rpm (Elpin) and the temperature of 18 °C, identical as for the bioreactor. After this period, the technical materials were subjected to ultrasounds for 15 min at 35 kHz (POLNED) to detach biofilms. The degradation of surfaces of technical materials after 3-year biofilm adhesion was assessed by means of a SEM.

In the first stage for SEM analysis, the technical materials were fixed with 3% glutaraldehyde (Sigma–Aldrich) in 0.1 M phosphate buffer of pH 7.2 (Sigma–Aldrich) at 4 °C for 24 h. After this time, the samples were washed three times in 0.1 M phosphate buffer of pH 7.2, each time for 10 min. In the next stage, the samples were dehydrated in an increasing water alcohol series of 50%, 60%, 70%, 80%, 90%, 96% (MERCK), the holding time at each concentration was 15 min. The samples were coated with gold using a sputtering machine (Edwards S150 Sputter Coater) and then the images were viewed with a SEM (JSM-6380 LV; JEOL, Japan), as described in (Dohnalkova et al. 2011; Vidal et al. 2020).

Impedance Data Management and Statistical Analyses

Impedance values and phase angle were read by the signal detector and saved in the Microsoft Excel (Microsoft Office 2018) format. Due to the high number of measurement points, the impedance diagrams were prepared in Mathematica 10 software (Wolfram). The obtained results were presented as diagrams of relative impedance in comparison to the initial value at the beginning of the process (synthetic water with inoculated microorganisms).

All calculations and basic statistical analyses: means, medians, standard deviations, maximum and minimum values were performed in Microsoft Excel (Microsoft Office 2018).

Results

Determination of the Relation Between the Received Impedance Signal and Bacterial Adhesion

The results of calculated bacterial cells on each ITO fragment (taken out of the bacterial suspension at a given time) are presented in Table 1. Quantity of bacterial cells was determined based on ATP standard curve and the assumption that 10–15 g ATP represent one bacterial cell (Pistelok et al. 2016), as described in Materials and

Table 1 Calculated quantity of bacterial cells on ITO fragments depending on the time of keeping the material in the bacterial suspension

Time, h	2	4	6	8	24	48
Quantity of bacterial cells without TCA	18 270	458 531	605 284	1 559 182	4 567 628	11 244 911
Quantity of bacterial cells with TCA	1 045 545	1 926 065	2 659 830	2 806 585	11 538 415	38 320 925

Methods. Table 1 presents total cell counts (all bacteria washed from the same surfaces of ITO samples).

During the progressing time, increasing adhesion of bacterial cells to the surface of the ITO material was observed. The values changed from 18 270 to 11 244 911 bacterial cells for the variant without TCA, and from 1 045 545 to 38 320 925 bacterial cells with TCA. Cells in the second variant were subject to lysis with the application of the acid. It caused extraction of cellular ATP from the microorganisms, consequently causing an increase in luminescence.

The quantity of bacterial cells was correlated with impedance measurement the relative value of which over the first 24 h of the experiment varied in a range from 0.992 to 1.014 (Fig. 1). It could have been caused by the stabilisation of the process and adhesion conditions. Initially, single cells adhere to the sensor. They are easily detached, which may cause considerable variations in the impedance value. Then, in the following hours, the proper process of forming of biofilm commenced, as manifested by a successive increase in the value of relative impedance. The obtained results point to the proper functioning of the sensor, and a change in signal is caused by attaching bacterial cells, as confirmed by the obtained changes in ATP values.

The biological membrane at the beginning of the colonization process differs significantly from that of the final phase. In the beginning, the high enzymatic activity of cells (EPS production) may occur and is related to the adhesion of individual bacteria to the substrate and the formation of cell aggregates. In the initial phase, when there is no EPSs produced yet, the cells do not have strong (permanent) connections to the substrate, forming the so-called reversible adhesion, which causes large impedance jumps. Each burst of single cells causes changes in impedance values perceived as changes presented in the graph.

Impedance Measurement in the Bioreactor

The phase angle at the beginning of the measurements showed a volumetric character of impedance—the system was composed of two separated electrodes on which bacteria only commenced to adhere. After a certain time, the system gained an almost resistive character as a result of accumulation of a thick layer of biofilm between the electrodes. Further measurements can involve only one parameter (relative impedance or phase angle), because changes on both diagrams occur in exactly the same time (Figs. 2 and 3).

In the first 15 days, the impedance reading pointed to the stabilisation of the system. After day 15 until approximately day 40, a plateau phase occurred—changes in impedance were inconsiderable. After 45 days of the experiment, when flow of synthetic water was initiated, a gradual, even increase in relative impedance was observed from a value of 1.1 to approximately 1.25 (before day 45, only single impedance fluctuations were recorded). After the period of static culture, bacteria commenced producing extracellular polymers, facilitating adhesion of subsequent cells floating in the bioreactor. Then, a rapid decrease in relative impedance was observed to a value of approximately 1.1 around day 70. The change in the value may be caused by partial detachment of bacterial cells accumulated on the surface of the sensor. The quantitative research on bacteria involving cultivation methods (deep inoculation on R2A plates) showed accordance with the observed changes (Figs. S4 and S5). The quantity of bacteria in the bioreactor increased during the time, and on the following days an increase in the quantity of bacteria in the outflow occurred. Changes in the relative impedance value pointed to simultaneous quantitative changes in microorganisms suspended in the bioreactor and its outflow. Each recorded decrease in relative impedance was equivalent to an increase in the quantity of microorganisms in the bioreactor and its further increase in the outflow (bacteria detached

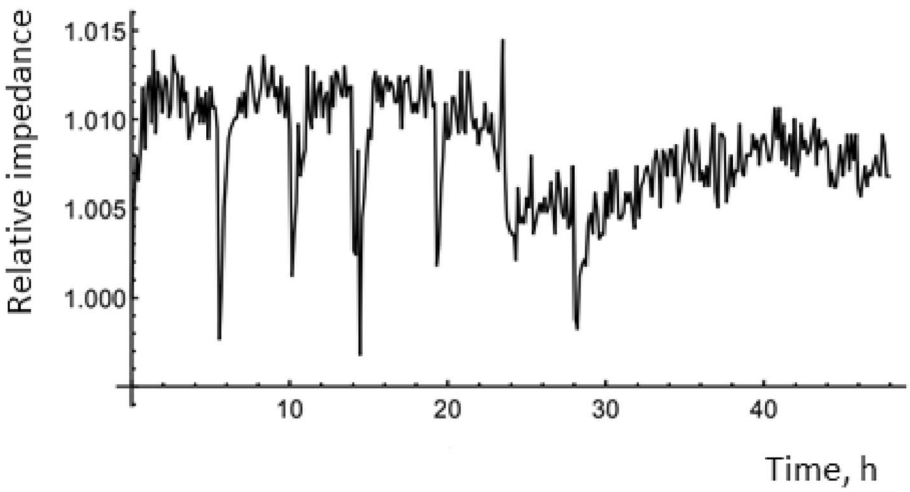


Fig. 1 Measurement of relative impedance in 48 h culture

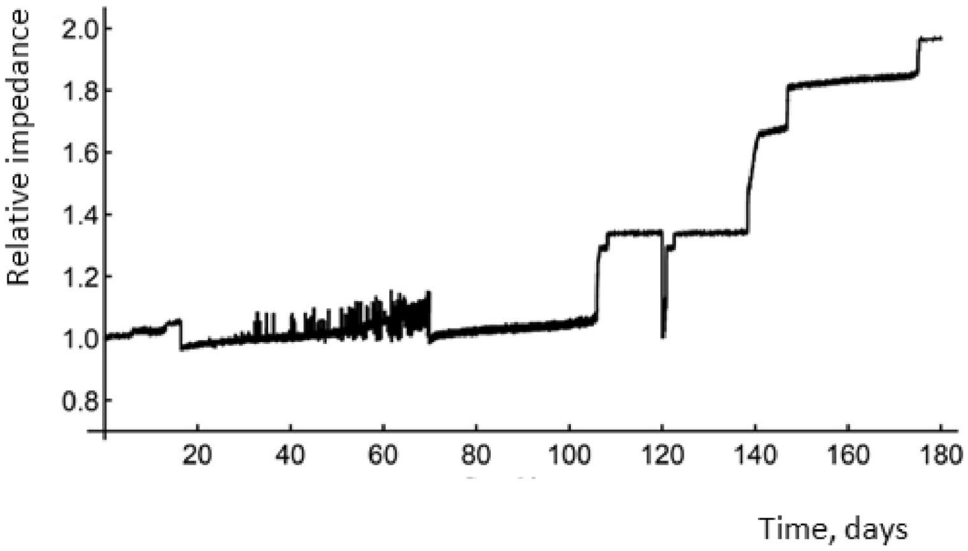


Fig. 2 Relative impedance value measured in the bioreactor during 180 day culture

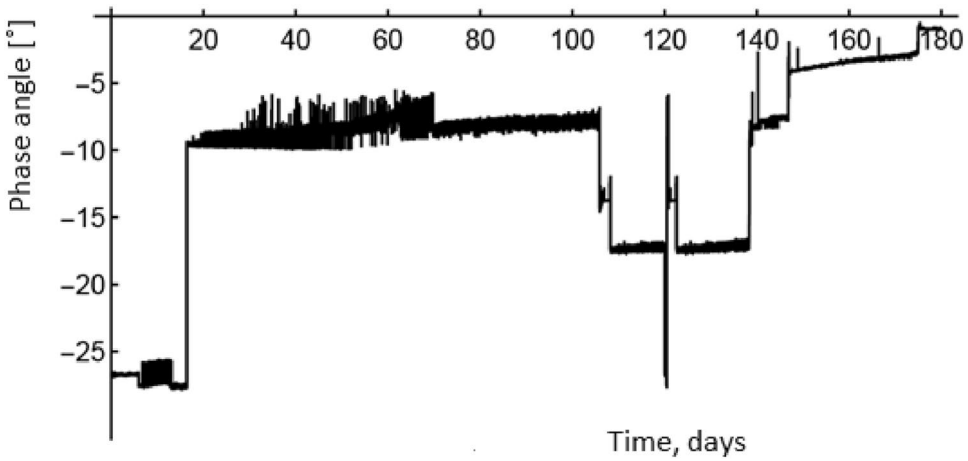


Fig. 3 Phase angle measured in the bioreactor during 180 day culture

from the sensor and were suspended in the bioreactor and washed from the bioreactor to the outflow in the following days).

Such a phenomenon imitates the natural process of formation and detachment of biofilm in water distribution networks. Relatively low flow rate does not cause immediate washing out bacteria from the bioreactor. A decrease in the quantity of bacteria suspended in the bioreactor occurs after several days, when they are washed out and occur in outflow. The observed phenomenon could have a negative effect particularly in full-scale drinking water distribution systems, where detached biofilm fragments could reach end-point consumers.

Visualisation and Quantification of Biofilms by Means of FISH

The surface coverage of the technical materials and the sensor by probe EUB338mix (corresponding to the majority of bacteria) were as follows in decreasing order: PVC > PB > SEN > PP > PE (Fig. 4). The sensor was characterised by an approximately 56% surface cover by all bacteria, i.e. an amount approximate to that for PVC, PB, and PP. Therefore, for these technical materials, the impedance sensor with an ITO layer could find application at a larger scale. In the case of a pipeline made of PE, the threshold conditions of the experiment would have to be determined again, and material for sensor construction showing more fitness would need to be selected.

Probe BET42 and GAM42 permitted detection of bacteria from *Betaproteobacteria* and *Gammaproteobacteria* classes. The highest value of cover by this group concerned PB (38.9%) and the smallest—PE (0.8%). Intermediate cover values occurred for the sensor (14.2%) as well as PVC (6.1%) and PP (3.7%). Probe HGC69 permitted detection of bacteria from *Actinobacteria* phylum. The highest value of cover was observed on the surface of the sensor (31.2%). Relatively high coverage by *Actinobacteria* was observed

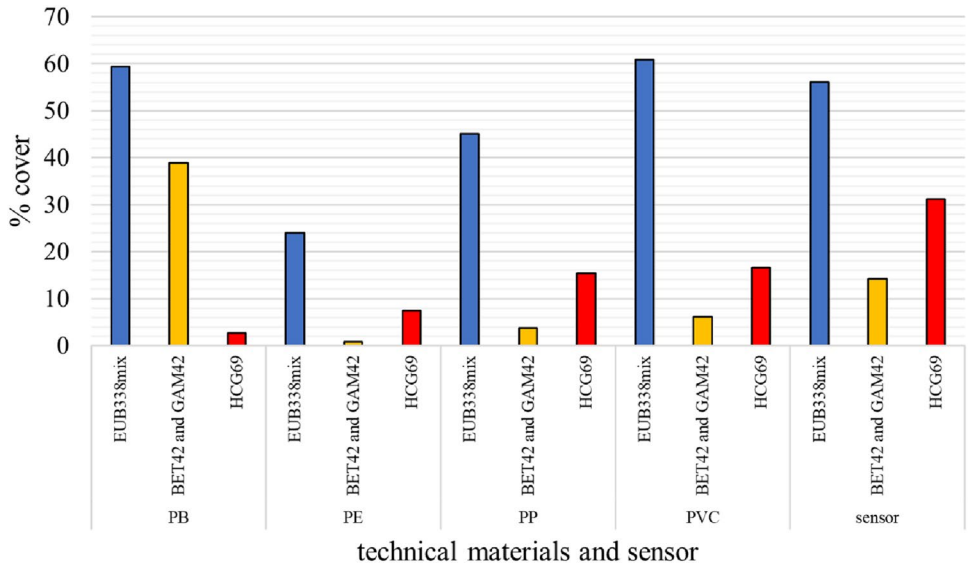


Fig. 4 Percent coverage of technical materials and sensor by fluorescent probes labelled with different fluorochromes

on PVC (16.6%) and PP (15.4%), while the smallest—on PB (2.6%).

Detailed results of the percentage of coverage are presented in Table S1 and Fig. 4.

Visualisation of Technical Materials by Means of SEM

The analysed technical materials (PB, PE, PP, PVC) are characterized by a different production method and a different chemical composition, therefore, they could be inhabited and destroyed by microorganisms to a different extent. Differences in the structure and composition of the new, clean materials have a direct impact on the formation of biofilm. These structure differences are presented in Fig. 5. The surfaces of technical materials covered with biofilm after a half a year experiment carried out in the bioreactor are presented in Fig. 6, and the surfaces of PB, PE, PP and PVC after the removal of 3-years old biofilm in Fig. 7.

The PB surface, like the PVC surface, had numerous scratches, probably as the result of the production process

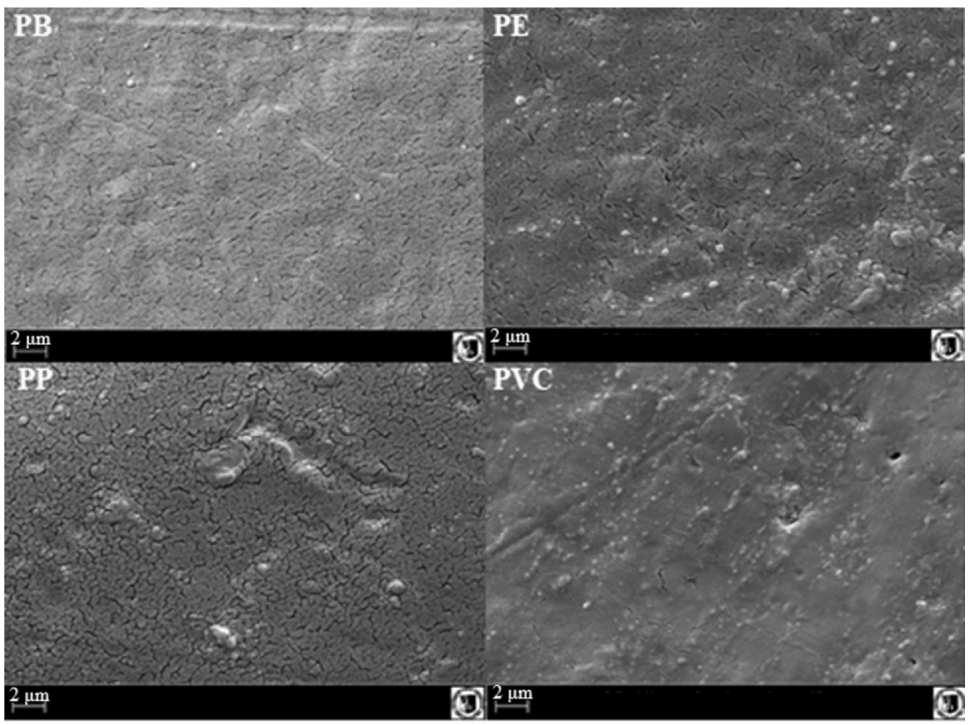


Fig. 5 Clean technical materials surfaces observed under the SEM

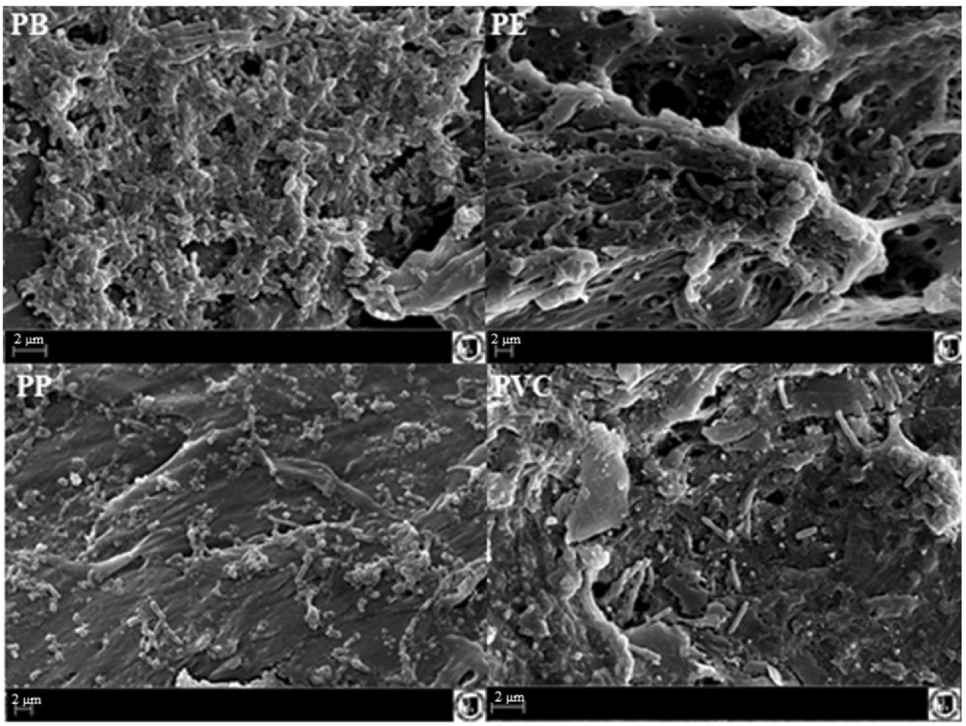


Fig. 6 Technical materials surfaces covered by biofilm after a half a year culture in the bioreactor observed under the SEM

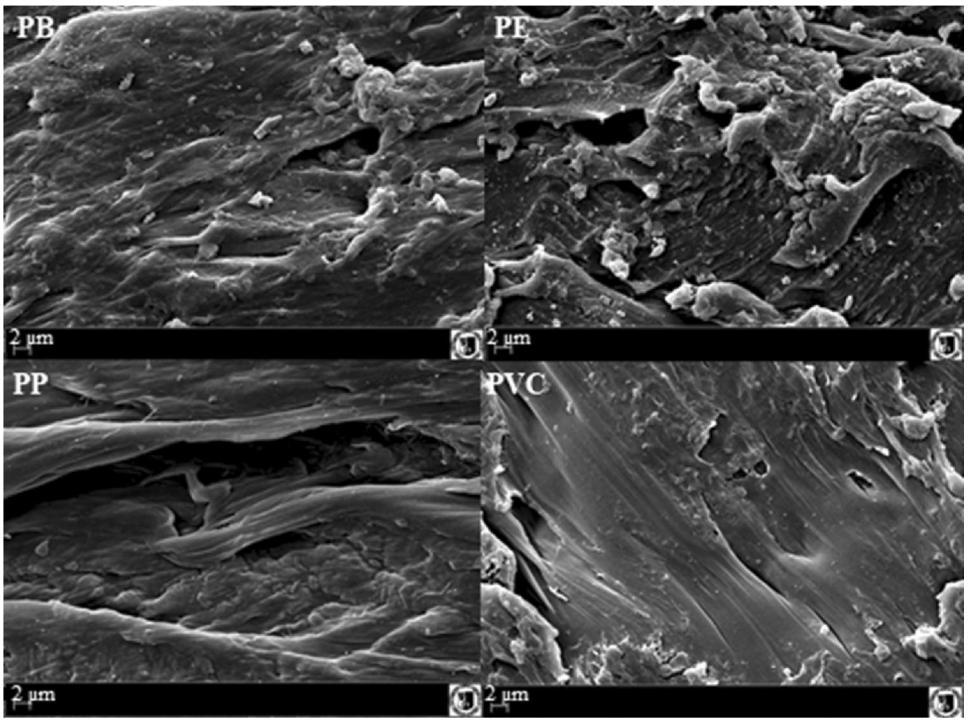


Fig. 7 Technical materials surfaces after 3-year biofilm removal observed under the SEM

(Fig. 5). PB had finer cracks without visible protrusions and indentations. After removing the microorganisms, significant scratches of the material were observed, conditioning the increase in roughness (Fig. 7). It was not possible to remove all bacteria from the PB surface, which may mean that the microorganisms settled on this surface will be more difficult to eliminate under real conditions and may constitute the basis for the development and adhesion of other microorganisms.

After the production process, the surface of the pipeline made of PE (Fig. 5) shows significant changes in structure (cracks on the entire observed surface). There were also minor

"swellings" which may have an influence on the increase in roughness. The surface is not perfect and changes in structure may enhance the colonization with microorganisms. After removing the bacteria from the polymer structure (Fig. 7), a significant change was observed in the form of numerous protrusions and broken fragments of the outer layer. There were also visible folds in the structure and significant unevenness, which created more favourable conditions for the colonization of new microorganisms.

On the surface of the produced PP (Fig. 5), a regular structure arranged into polygons with larger visible depressions was observed along with lumpy irregularities arranged irregularly with a size of approx. 0.5 µm. There were also numerous fine spaces (2 µm) on the surface. After the removal of microorganisms (Fig. 7), PP showed a "fibrous" structure, where long sheets of plastic were visible, arranged in a folded whole with numerous channels. Such a structure increases the surface roughness, and the spatial arrangement of large, irregular fibers creates ideal conditions for bacterial adhesion.

Microscopic observations indicated the regular construction of the new PVC (Fig. 5). There were numerous scratches on the surface along with small cracks that formed into larger groups. Significant protrusions as well as cavities in the entire structure also appeared on the surface. After sonication of microorganisms from PVC, it turned out that they caused a significant change in the structure of the material (Fig. 7). Microorganisms present on the surface of PVC in conditions of starvation can use the substrates released into the water from the pipelines and thus damage its top layer. Therefore, the presence of biofilm could cause the deletion of such large entire external sheets of the material and numerous porosities, significantly increasing the surface roughness.

Observation of the surfaces under the SEM after a 3-year culture showed significant changes in each material (Fig. 7). They were different due to the differences in new materials structures. Despite the long-lasting effect of ultrasounds, still single microorganisms could be observed on the PE or PB surfaces. These materials were only exposed to the action of microorganisms in laboratory conditions—in actual drinking water distribution systems, the pipes are exposed to physical–chemical damage, as well. It is worth to note that the observed surface damages are caused because bacteria could use the materials as a nutrition source. Moreover, the contact of technical materials with extracellular polymeric substances, enzymes, and other metabolites of microorganisms, could also contribute to its biocorrosion.

Discussion

The presence of biofilm in drinking water distribution systems could considerably decrease the microbial quality of treated water. Therefore, it is necessary to conduct so-called in situ analyses to control biofilm growth directly in the places of its formation. Such monitoring is essential to fully understand the factors and mechanisms of biofilm development (Douterelo et al. 2016; Liu et al. 2016; Xu et al. 2020). This paper discusses the possibility to apply the impedance sensor in the monitoring of biofilm development in water supply networks (assuming that environmental biofilms are multi-species). Although there are some literature reports concerning measurements of biofilm growth by sensors, the majority of them was focused on single-species biofilms and conducted at elevated temperatures during relatively short periods of time. Nevertheless, Bimakr et al. (Bimakr et al. 2018) have successfully applied electrochemical impedance spectroscopy for biofilm detection in dam fresh water at 22 ± 2 °C while Yang and Reyes-De-Corcuera (Yang and Reyes-De-Corcuera 2020) reported that the method could work with relatively low bacterial suspensions, reaching 10^4 CFU/mL.

In this study, the material of choice for the sensor construction was ITO and the frequency of 100 kHz was established. Bayoudh et al. (Bayoudh et al. 2008) also conducted research on impedance measurement during adhesion of bacterial cells on an ITO plates (for a variable frequency range), but the study employed only one strain, i.e. *Pseudomonas stutzeri*, and was conducted for only 140 min. In terms of frequency, Ben-Yoav et al. (Ben-Yoav et al. 2011) tested its range from 100 to 400 kHZ and evidenced that better reading effects were obtained for lower frequencies. The same conclusions were made by Parades et al. (Paredes et al. 2012) and Yang et al. (Yang et al. 2004). However, in the case of lower frequencies, a higher level of disturbance of the system can be detected. Therefore, it is necessary to reach a compromise between measurement effects and disturbances. Hence, the frequency of 100 kHz was chosen based on own preliminary research (data not shown) and seems to be the most appropriate choice for the relative impedance measurement in conditions simulating drinking water distribution systems.

Other types of sensors for bacterial adhesion monitoring were also described in the literature. Beyond ITO, stainless steel was proposed as a practical material for biosensor construction (Bimakr et al. 2018). Pires et al. (Pires et al. 2013) presented results of measurements of biofilm developed by *Pseudomonas aeruginosa* with the application of a measurement electrode (with attached cells), and a second reference one (not exposed to the effect of microorganisms). This approach permitted revealing the difference in the received signal, caused by adhesion of *P. aeruginosa* cells. Similar research conducted by Estrada-Leypon et al. (Estrada-Leypon et al. 2015), concerning facilitated biofilm growth developed by *Staphylococcus aureus*, subjected to the effect of strong shear stress, also confirmed the justification of the application of the measurement and reference

electrodes. In their 24 h culture, Estrada-Leypon et al. (Estrada-Leypon et al. 2015) evidenced the possibility of assessment of growth of the aforementioned strain with the application of impedance measurements, and the change of signal was caused by adhesion of the bacterial cells, as confirmed by SEM and fluorescence microscope observations. The current study also shows differences in the received signal when the sensor surface was populated with bacteria, even though the system did not include a reference electrode. The change in the relative impedance revealed to be correlated with bacterial adhesion on ITO material, as demonstrated by ATP measurement. Moreover, simultaneously with relative impedance measurements, the concentrations of bacteria in the bioreactor as well as in its outflow were measured, what confirms reliability of the recorded relative impedance results.

In their research, Parades et al. (Paredes et al. 2012) presented impedance measurement during growth of biofilm developed by *S. epidermidis* in a CDC bioreactor. The entire culture was conducted in conditions of increased temperature of 37 °C for 24 h. The signal was measured by 18 sensors placed in the bioreactor. During the first 7 h of the experiment, no changes in the system were observed. The greatest increase was recorded at the second stage and at the end of the process (17–22 h). The results obtained by the aforementioned authors, as well as observations presented in the current study, point to the justified application of impedance spectroscopy in the analysis of bacterial adhesion and biofilm growth. However, it is worth to mention that the experiment of Parades et al. concerned a single-species biofilm cultivated at elevated temperature and was performed for only 24 h, while in the current study the change in relative impedance was tested in multi-species biofilm cultured at 18 °C for half a year, as an attempt to reflect conditions prevailing in water distribution systems. Despite these differences, both papers (Parades et al. (Paredes et al. 2012) and the present paper) demonstrated a more considerable change in impedance at the second stage of the experiment, after the stabilisation of the system. According to this, Ben-Yoav et al. (Ben-Yoav et al. 2011) measured the impedance value in culture of biofilm developed by *E. coli* and evidenced that the obtained results depended on the phase of growth of bacteria.

Dheillyl et al. (Dheilly et al. 2008) conducted research concerning monitoring of cell aggregation and forming of biofilm developed by pure Gram-negative (*Pseudomonas aeruginosa PAO1*) and Gram-positive (*Bacillus subtilis*) strains on the surface of metal. The obtained electrochemical spectre of impedance during adhesion of cells and initial phase of growth for both strains points to an increase of total resistance with culture time, and its rapid decrease during detachment of cells from the surface. The study results confirm the possibility of application of the sensor in research on the development of mixed-species biofilm. The conclusions made by Dheillyl et al. (Dheilly et al. 2008) seem to be in accordance with the results presented in the current paper, where a change in relative impedance value was evidenced in a mixed-species system.

A literature review demonstrated that the majority of papers concerned monospecific biofilms and relatively pathogenic strains, particularly related with the colonisation of medical materials (Guła et al. 2020; Romero et al. 2021) or food infection (Yang et al. 2004). In addition, such experiments were usually limited in time to short-term studies (few hours or days) and were conducted in conditions suitable for mesophilic, potentially pathogenic strains (elevated temperature, dosing of the nutrients) (Bayoudh et al. 2008; Ben-Yoav et al. 2011; Paredes et al. 2012; Pires et al. 2013; Estrada-Leypon et al. 2015; Gutiérrez et al. 2016).This paper discusses an attempt of measurement of biofilm in water supply networks. The bacterial strains used in the current study originated from the actual drinking water distribution system in Wrocław, Poland (Wolf et al. 2018). Moreover, in contrast to the previous papers, the attempt was made to create hydraulic conditions as close as possible to the real conditions prevailing in the water supply networks, and the experiment was conducted for much longer time, i.e. half a year. The presented results point to the adhesion of the tap water bacteria not only to the sensor, but also to technical materials (PB, PE, PP, PVC) commonly applied in the construction of drinking water distribution networks. It was also evidenced that adhesion of biofilm to the technical materials could destroy their surfaces. Moreover, the observed abrupt decreases in relative impedance could indicate on the detachment of biofilm fragments, what seems to be confirmed by the subsequent increases in bacterial concentration in the suspension inside the bioreactor.

Conclusions

This study presents results of the long-term measurement of relative impedance values during biofilm formation on technical materials. A mixture of environmental strains, isolated from an actual water supply system, was used for the analysis. It is very important to understand the mechanisms of biofilm formation on different technical materials. The investigation of the process of adhesion permits monitoring of biofilm development. Moreover, accidental biofilm detachment observed in the current study points to the potential risk for tap water consumers and highlights the need of a development of a reliable method for biofilm detection, which would provide immediate results.

The presented study confirms that the tested ITO impedance sensor can be potentially used in control of biofilm development in water distribution networks, as the relative

impedance signal measured by the sensor was correlated with the adhesion of microorganisms. The impedance sensor seems to be an appropriate solution for this purpose in the case of PB, PP, and PVC, as these technical materials revealed to be less colonised by bacteria than the tested sensor.

It was also shown that technical materials commonly used in the construction of water supply networks deteriorate after long-term adhesion of microorganisms due to the release of their metabolites and extracellular polymeric substances. Observation of the surface of the tested materials revealed significant deletions of plastic fragments and changes in the continuity of the structure. The damaged structure facilitates (promotes) the adhesion of subsequent cells, which has a negative effect on the quality of transmitted water.

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Declarations

Conflict of Interest The authors declare that they have no conflicts of interest.

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