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Measurement of bacterial adhesion to metal surfaces with different chemical composition – evaluation of different methods

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ABSTRACT

In the current study attention has been focused on the evaluation of different methods of measurement of bacterial adhesion to the metal surfaces with different chemical composition. The direct methods, which included determination of the number of bacterial cells using fluorescence microscopy and Colony Forming Units (CFU) on agar medium, and indirect methods using Alamar Blue (AB) and MTT assays, were evaluated. The chemical compositions of the metal surfaces included: copper, iron, chromium and nickel. Interaction effects of assay and metal compound have been specifically demonstrated in this study. It was found that metal ions reacted with components of the indirect colorimetric tests used in this study (AB and MTT assay). Consequently, those tests gave positive false results. In contrast to the indirect methods, direct counting methods such as microscopy techniques and CFU counting, were successfully applied for evaluation of bacterial adhesion to the metal surfaces. However, considering the limits for the surfaces of the examined samples for microscopy, the determination of the CFU was found to be the best method for testing the adhesion to metal surfaces. The method, combined with the appropriate detachment procedures allowed for a precise determination of the number of bacteria on the entire surface of the evaluated metal samples.

INTRODUCTION

Bacterial adhesion to metal surfaces lead to biofilm formation, biofouling and biocorrosion problems, which result in significant costs of cleaning and maintenance in engineered systems in industries (Beech and Sunner, 2004; Coetser and Cloete, 2005; Sheng et al., 2008). Considering the number of industries negatively affected by bacterial adhesion and also the increasing restrictions on the use of biocides, prevention of bacterial adhesion to metal surfaces seems to be a good strategy (Garrett et al., 2008; Klemm et al., 2010; Kumar and Anand, 1998). Current research concerning modification of the surface properties, and characteristics of new complex anti-adhesive alloys are now considered as a tool for preventing the adhesion of bacteria to metal surfaces. To develop these kind of materials, it is essential to precisely evaluate the bacterial adhesion to the metal surfaces.

Although evaluation of bacterial adhesion is important itself, a relative small number of techniques can be used in the measurement of adhesion. These techniques are not well validated and there is lack of studies where those methods are compared to each other (Vesterlund et al., 2005). According to Ofek and Doyle (1994) no experimental model has been developed that can be used to adequately evaluate bacterial

adhesion and biofilm formation, taking into account all interactions occurring between the bacteria and the surface.

Bacterial adhesion can be analyzed by different methods. The conventional methods such as microscopy techniques or Colony Forming Units (CFU) counting and are classified as direct methods. These methods are simple and do not require sophisticated instrumentation, so they can easily be performed in most laboratories (Vesterlund et al., 2005). However, they are less sensitive and do not give information concerning cell viability (Frioni et al., 2010). Spectrophotometry is one of the most useful indirect methods for quantitative analysis which can be applied for evaluation of bacterial adhesion (Frioni et al., 2010). There are several dyes that can be applied, such as crystal violet, safranin, congo red (Martin and An, 2000). Additionally, tetrazolium salts and resazurin sodium salt can be used to determine the cell viability of adherent cells (An and Friedman, 1997; Vesterlund et al., 2005). Spectrophotometric methods are rapid, sensitive, and high throughput, but cannot be used when adhesion of one bacterial strain is studied in an environment where other bacteria are present. In order to distinguish bacteria in a mixed population, radiolabels (Ahearn et al., 2000; Jin et al., 1998), fluorochromes (Bosch et al., 2003; Drudy et al., 2001), or bacteria-specific anti-bodies (Sanchez et al., 1993) can be used. Radiolabels are regarded as undesirable due to safety and cost concerns. Fluorochromes are used to replace radio-labels, but they may alter the surface properties of bacteria or affect the viability of bacteria (Fuller et al., 2000).

Since detection of bacterial adhesion to the metal surfaces is important (e.g., in research of new complex anti-adhesive alloys), much more studies should be done where different methods are evaluated. Because of complicated electro- and physicochemical characteristics of metal surfaces the evaluation of the degree of bacteria adhesion can be difficult. Therefore, in the current study attention has been focused on the evaluation of different methods of measurement of bacterial adhesion to the metal surfaces with different chemical composition. The direct methods which included bacterial cells counting using fluorescence microscopy and Colony Forming Units counting on agar medium, and indirect methods using Alamar Blue (AB) and MTT assays, which are based on the determination of the metabolic activity of adhered cells, were evaluated. The chemical compositions of the metal surfaces included: copper, iron, chromium and nickel. Metals composed of these elements are among the most widespread in industry, due to the fact that they are readily available, relatively cheap, but also protect surfaces against corrosion and biocorrosion (Beech and Sunner, 2004).

MATERIALS

In this study 3 metal plates with different chemical composition prepared according to the ISO 1456:2009 and ISO 4525:2003 international standards were used. Their chemical compositions are shown in Table 1. The diameter and thickness of the metal plate used in this experiment were 1 cm and 5 mm respectively (Figure. 1).

Prior the experiment metal plates were washed in hot water with detergent in an ultrasonic cleaner for 15 min, and then were soaked for 15 min in 70% ethanol. After rinsing in sterile distilled water the plates were dried in UV radiation.

Table 1. The chemical composition of the metal plates used for examinations.

No.	Basis metal	Plating type	Grade	Minimum plating thickness (µm)	Symbol
1.	Steel	copper plating	1	0.3 of copper	Fe/Cu
2.	Steel	nickel - chrome plating	3	10 of nickel, 0.3 of chrome	Fe/Nib10Cr
3.	Steel	copper-nickel plating	2	5 and 10 of nickel, 10 of copper	Fe/Ni5Cu10Nib10

Figure 1. Metal plates: Fe/Cu, Fe/Nib10Cr, Fe/Ni5Cu10Nib10, respectively.

METHODS

Bacterial adhesion

All analysis were performed using *Staphylococcus aureus* ATCC (American Type Culture Collection) 6538. Prior the experiment *S. aureus* was incubated for 24 h at 37oC in the Brain Heart Infusion (BHI) broth medium (BioMaxima, Poland). In the next step, bacterial cells from 24 h cultures were separated by centrifugation for 20 min at 3.300 x g, washed and suspended in phosphate buffered saline (PBS, Sigma-Aldrich, Germany) to obtain bacteria concentration equals 2°McFerland (DEN-1, BIOSAN, Latvia). Subsequently, metal plates were transferred to sterile 12 - wells culture plate (Becton Dickinson and Co., USA) and 1 mL of bacterial suspension was added to each well. The plates were incubated for 3 h at 25°C (Galaxy R PLUS CO2 Incubators, RS Biotech, UK). After incubation metal plates were used in the assays described below (the adhesion procedure was the same regardless the test performed).

Direct methods

Fluorescence microscopy

After incubation metal plates were washed three times in PBS and dried fixed with methanol and stained with 0.01% acridine orange (AO) solution for 2 min. The number of bacteria cells attached to the metal plates were investigated by fluorescence microscope under magnification of 1000x (field of view 0.08 mm). The results are presented as the mean number of bacteria adhered to the metal plates.

Plate counting method

After incubation metal plates were transferred to 12 - wells culture plate. Then, to each wells 1 mL of 0.2% saponin solution was added, and the plate was shaken for 30 min in room temperature (Amersham, UK). In a further step, serial dilutions of bacterial suspensions obtained after shaking was perform. The number of adhered cells was determined by performing quantitative plating on Petri dishes containing BHI agar medium, which next was incubated for 24 h at 37°C (Galaxy R PLUS CO2 Incubators, RS Biotech, UK). After incubation, the grown colonies were counted and the number of Colony Forming Units (CFU) per 1 mL, taking into account the volume of buffer used for making bacterial suspension and the volume of suspension added to the Petri dishes, were determined. To confirm complete detachment of all the bacterial cells from the metal plates, after shaken metal plates with 0.2% saponin solution, there were rinsed in PBS and then flooded with BHI medium agar at temp. 45^{o}C and then incubated at 37^{o}C for 24 h an incubator.

Indirect methods

Alamar Blue assay

Alamar Blue (AB) assay was performed in two different modifications: AB1 - which determined the number of bacteria adhered to metals plates; AB2 - which determined the number of non-adhered bacteria that remain in suspension as planktonic form.

AB1

After the adhesion process, the metal plates were washed with PBS to removed non-adherent bacteria calls and transferred to new sterile 12 - wells culture plate. Then, 1 mL of PBS and 100 L of AB assay (AlamarBlue® Cell Viability Reagent, Invitrogen Life Technologies, Belgium) was added. The metal plates were incubated at 37°C for 1 h. After incubation 200 µL of obtained bacterial suspension was transferred to 96 - well plate (Becton Dickinson and Co, USA). The absorbance was measured using microplate reader (Tecan Infinite NanoQuant M200 Absorbance Microplate Reader, Switzerland) at wavelength of 570 nm and reference wavelength of 600 nm. The positive control (PC) was a suspension of bacteria used for the adhesion. The results of AB1 are given as a correction factor: RO, which was calculates by formula: RO = AOLW / AOHW

Where: AOLW = absorbance of oxidized form at lower wavelength; AOHW = absorbance of oxidized form at higher wavelength.

AB2

After the adhesion process, 200 µL of bacterial suspension from each well were transferred to 96 - well plate and 20 µL of AB was added. The plates were incubated for 1 h at 37°C. The absorbance was measured in the same way as described for AB1.

The MTT assay

The MTT assay was done as previously described by Walencka et al. (2006) with slight modifications. Similarly, to the AB assay, the MTT assay was also performed in two modifications (designated as MTT1 and MTT2).

MTT1

The metal plates were transferred to sterile 12 - wells culture plate. In the next step, 1 mL of PBS and 100 L of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (3 mg/mL in PBS, Sigma-Aldrich, Germany) were added to each well. The plate was incubated at 37°C for 1 h. Then, 1 mL of isopropanol (Sigma-Aldrich, Germany) was added to each well, and the plates were vigorously shaken for 15 min. The amount of MTT formazan formed during the incubation was measured at a wavelength of 540 nm and reference wavelength of 630 nm in 96 - well plates with 200 µL of each sample using microplate reader (Tecan Infinite NanoQuant M200 Absorbance Microplate Reader, Switzerland).

MTT2

After adhesion, 100 µL of bacterial suspension from each well was transferred to 96-well plate, and supplemented with 10 µL of MTT solution. The plates were incubated for 1 h at 37°C. In the next step, 100 µL of isopropanol was added to each well, and the plates were vigorously shaken for 15 min. The amount of MTT formazan formed during the incubation was measured in the same way as described for MTT1 assay.

Statistical analysis

Data are presented as the means ± standard errors of the means (SEM) calculated from at least 5 measurements. The statistical significance of the differences between experimental and control samples were analyzed by Student's t test. The relationship between result obtained with all method was analyzed using Pearson's correlation coefficient. A two-tailed analysis was conducted and *P* value of less than 0.05 was considered statistically significant. The statistical analyses were conducted with Statistica 9.0 (StatSoft, Poland).

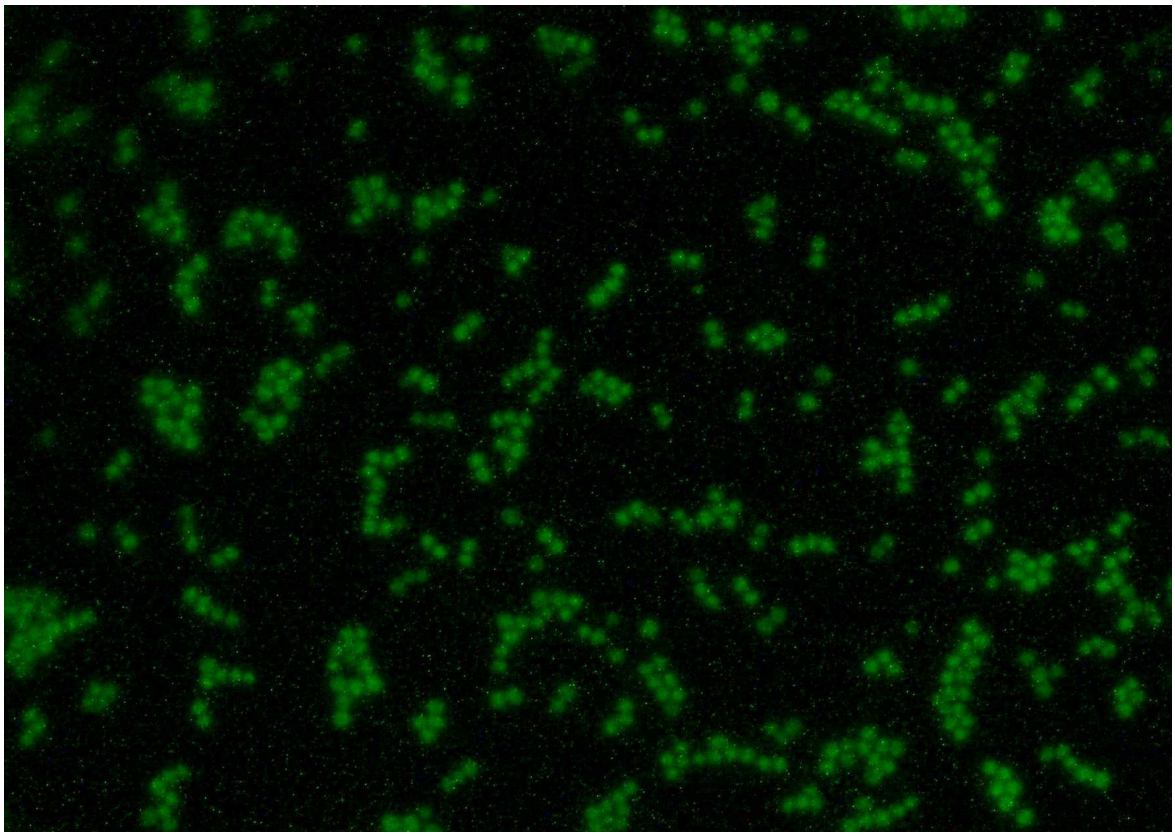
RESULTS

Direct methods

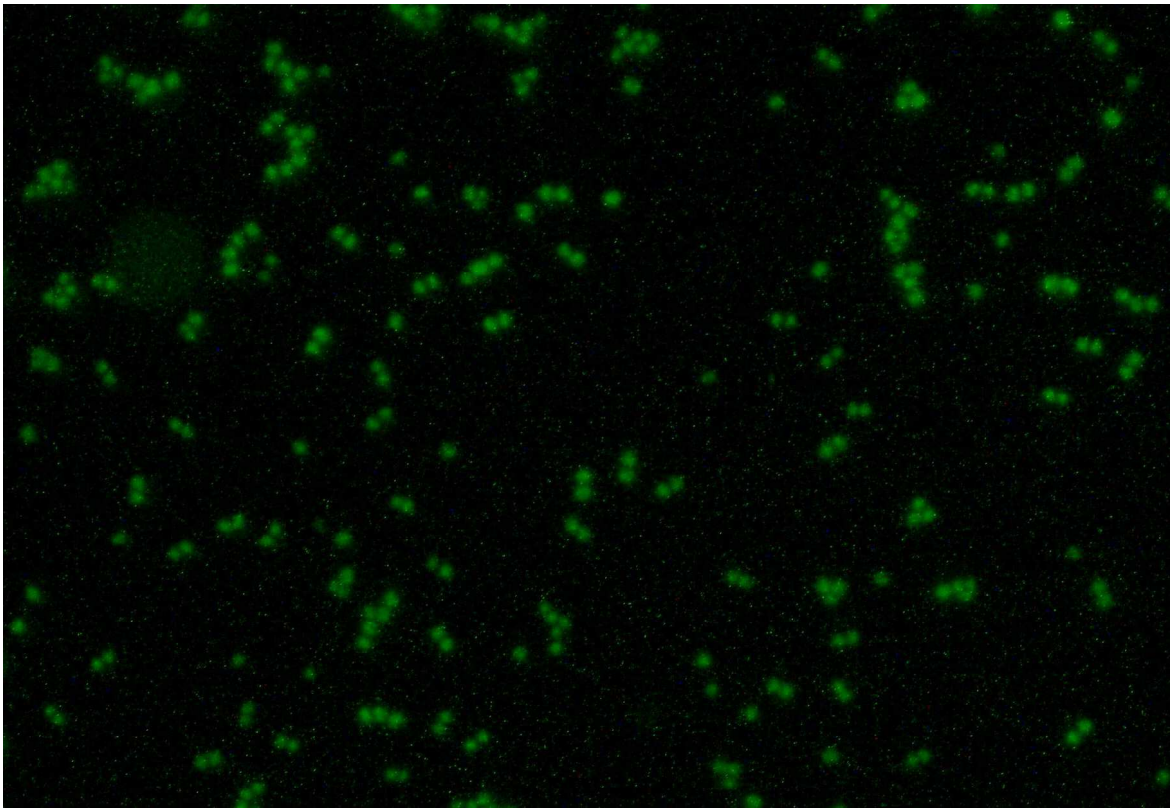
Fluorescence microscopy was used to determine the number of adhere bacteria cells to the surface of the metal plates. Example of the image which was taken using the fluorescence microscope was shown in Figure 2. The acridine orange (AO) staining procedure used in this study allowed for complete staining of all bacterial cells. It was observed, that in the case of samples with irregular surface, AO used for staining, accumulated in the grooves and crevices of metal plates, which greatly impeded the counting (Figure 2C).

Figure 2. *Staphylococcus aureus* adhered to the metal plates: A) Fe/Cu, B) Fe/Nib10Cr, C) Fe/Ni5Cu10Nib10.

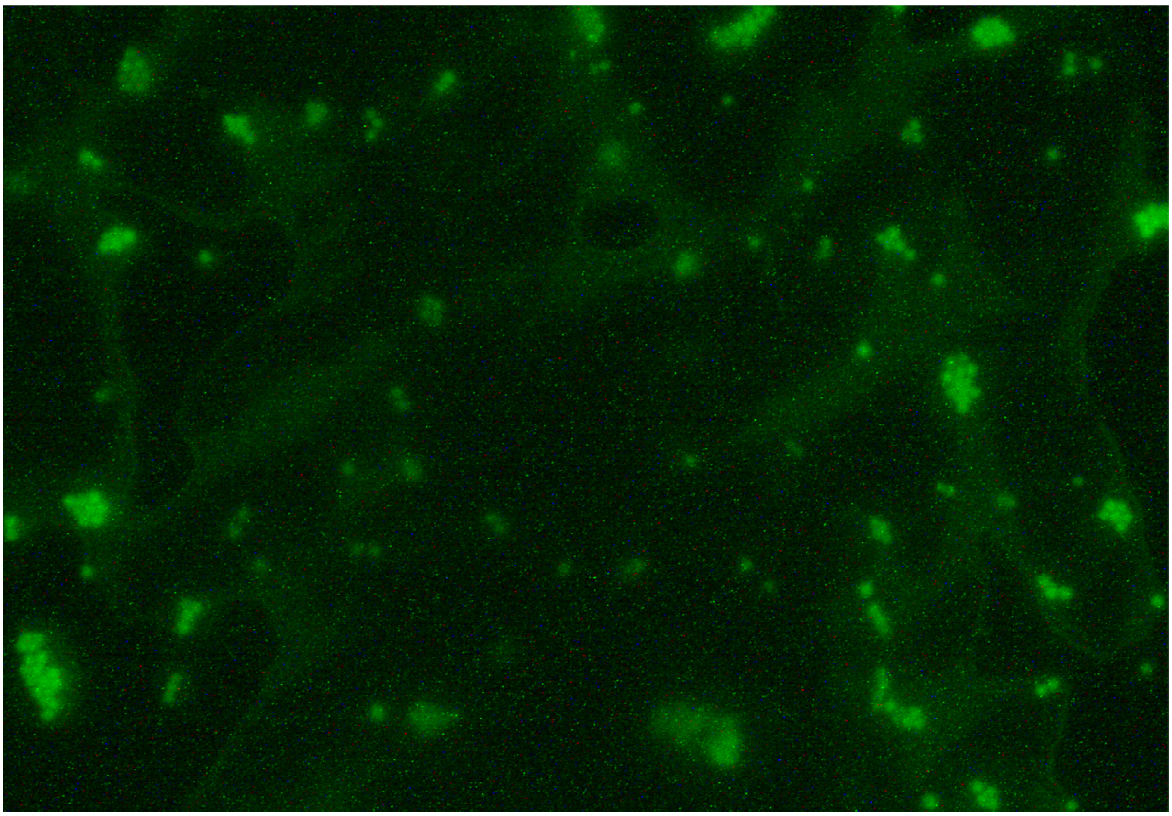
A)



B)



C)



Microscopy does not give information regarding the number of bacteria on the edge of the metal plates. Therefore, the total number of adhere bacteria cells, which was established only indirectly, only covers the surface area of the plates (Table 2). The number of adhered cells was also determined byperforming quantitative plating of CFU on Petri dishes, taking into account the volume of buffer used for making bacterial suspension and the volume of suspension added to the Petri dishes (Table 2). Results obtained through plate counting method were consistent with the results obtained with the fluorescence microscope.

Table 2. Number of bacterial cells adhered to the metal plates.

No.	Fluorescence microscope	Plate counting method
1.	$83*10^4 \pm 21^{acd}$	$120*10^5 \pm 12^{ac}$
2.	$27*10^4 \pm 33^{d}$	$97*10^5 \pm 9$
3.	$30*10^4 \pm 27^{d}$	$85*10^5 \pm 13$

Data are presented as mean number of adhered cells ± standard error of the mean (SEM) calculated from three repetitions of the experiment; a - statistically significant difference between the number of adhered cells to the metal plate no. 1 and 2; b - statistically significant difference between the number of adhered cells to the metal plate no. 2 and 3; c - statistically significant difference between the number of adhered cells to the metal plate no. 1 and 3; d - statistically significant difference between the number of adhered cells determined using fluorescence microscope and plate counting method. *P* value less than 0.05 was considered statistically significant.

Indirect methods

AB1 determine the number of viable cells adhered to the metal plates. Results of the present study showed a strong interaction of all metal plates with the dye. The absorbance of blank (metal plates incubated in PBS instead of bacterial suspension) was close or higher than absorbance's of positive control (Table 3). The strongest reactivity was observed in the case of results obtained for metal plate no. 1 (Fe/Cu). As a modification of the AB assay AB2 was also performed. The AB2 determinated the number of bacteria remaining in the suspension, which were not adhere to the metal plates. However, in case of the AB2 strong interaction of AB assay with metal plates was also observed.

Table 3. Correction factor (R_O) for Alamar Blue assay.

No.	*AB 1*		*AB 2*	
	Blank	**Sample**	**Blank**	**Sample**
1.	0.932	1.212	0.804	0.730
2.	0.557	0.794	0.425	0.570
3.	0.734	0.881	0.644	0.739
PC			0.811	

Data are presented as mean correction factor (R_O). As blank, PBS incubated with metal plates instead of bacterial suspension was used. The positive control (PC) constituted a suspension of bacteria used for the adhesion.

In case of MTT1 also strong interaction of metal plates with the dye was observed. The absorbance obtained in MTT1 are shown in Table 4. The strongest interaction was also observed in the case of results obtained for metal plate no. 1 (Fe/Cu). In MTT2, the reactivity of metal plates with dye was not observed. The results obtained with this test allowed to determine the number of bacteria adhered to the surface of the metal plates by comparison to a standard formazan curve derived by adding MTT to known concentrations of bacteria (Table 5).

Table 4. The values of absorbance obtained in the MTT assay.

No.	*MTT 1*		*MTT 2*	
	Blank	**Sample**	**Blank**	**Sample**
1.	1.429	1.346	0.059	1.718
2.	0.859	1.310	0.052	0.994
3.	1.012	1.328	0.056	1.361
PC	0.457			

Data are presented as mean absorbance. As blank, PBS incubated with metal plates instead of bacterial suspension was used. The positive control (PC) constituted a suspension of bacteria used for the adhesion.

Table 5. Number of bacteria adhered to the metal plates.

No.	*MTT2* assay
1.	$96*10^7 \pm 33^{ac}$
2.	$59*10^7 \pm 23$
3.	$63*10^7 \pm 34$

Data are presented as mean number of adhered cells ± standard error of the mean (SEM) calculated from three repetitions of the experiment; a - statistically significant difference between the number of adhered cells to the metal plates no. 1 and 2; b - statistically significant difference between the number of adhered cells to the metal plates no. 2 and 3; c - statistically significant difference between the number of adhered cells to the metal plates no. 1 and 3. *P* value less than 0.05 was considered statistically significant.

The relationship between result obtained with all method was analyzed using Pearson's correlation coefficient (Table 6.). There was a high correlation between microscope and the plate counting method ($r = 0.8311$, $P < 0.05$) and the correlation was statistically significant. Statistical analysis revealed a negative correlation between plate counting method and MTT ($r = -0.6118$, $P < 0.05$), and between microscope and MTT ($r = -0.5989$, $P < 0.05$, $R2=0.3576$).

Table 6. Peterson's correlation coefficient between results obtained in fluorescence microscope, plate counting method and MTT2.

Fluorescence microscope vs. plate counting method			Fluorescence microscope vs. MTT			Plate counting method vs. MTT		
R^2	P value*	correlation coefficient [r]	R^2	P value*	correlation coefficient [r]	R^2	P value*	correlation coefficient [r]
0.84	0.0001	0.9178	0.36	0.0186	−0.5979	0.48	0.0154	−0.6118

***P value according to the Student's t test. *P* value less than 0.05 was considered statistically significant.**

DISCUSSION

Bacterial adhesion onto metal surfaces is of importance in a wide spectrum of problems in various industrial applications. One of the solutions of those problems is searching anti-adhesive materials that can delay or completely avoid the adhesion of microorganisms. However, proper assessment of the bacterial adhesion to the metal surfaces can be difficult because of the physicochemical properties of metal (Sheng et al., 2008). Therefore, in the current study attention has been focused on the evaluation of different methods of measurement of bacterial adhesion to the metal surfaces with different chemical composition. The following methods were analyzed: direct method which included bacterial cells counting using fluorescence microscopy and CFU counting on agar medium, and indirect methods using Alamar Blue (AB) and MTT assays.

Microscopy techniques including light, fluorescence, scanning, electron microscope, and atomic force microscope are suitable for the investigation of bacterial adhesion (An and Friedman, 1997). Light microscope is applied to observe and enumerate bacteria in translucent surfaces and usually it is preceded by staining the bacteria with dyes, such as Gram stain and crystal violet (Frioni et al., 2010). However, this method provides no information on the actual number of living bacteria and therefore it is less useful to evaluate the anti-adhesive efficacy of antimicrobial substances (Pantanella et al., 2010).

In the current study the fluorescence microscopy was used to evaluate bacterial adhesion. The method is suitable for assessment of bacterial adhesion on smooth and opaque surfaces, e.g. metal, plastic, or ceramic surfaces (An and Friedman, 1997). Acridine orange, used in this study, is a sensitive dye for staining living bacteria cells. The method is inexpensive and easy to perform. However, roughness of the tested surfaces is here particularly important. It was observed that bacteria cells or the dye may accumulate in the grooves and crevices in case of samples with irregular surfaces. This may impede determination of the number of adhered bacteria. Additionally, roughness can be cause of uneven adhesion on the surfaces of the metal plates (An and Friedman, 1997), which explains high variation of the results. Although, this method is laborious and time consuming, still is one of the most used techniques to evaluated bacterial adhesion (Vesterlund et al., 2005).

Colony Forming Units counting is another direct method, which is simple and do not requires additional reagents or equipment. However, the method is time-consuming and can be less useful, when many plates have to be enumerated (Pantanella et al., 2013). The greatest difficulty in this method is the selection of an appropriate dilution, which should take into account the type of bacteria and the size of their colonies. This is particularly important in the case of mixed cultures of bacteria, because the adjacent or overlapping colonies of

different bacteria can stimulate or inhibit the growth of other microorganisms (An and Friedman, 1997).

Another weak point of the method is the detachment procedure. A soft and non-invasive detachment procedure does not ensure a complete detachment of all the bacterial cells. Moreover, the detached bacteria appear as aggregates of different size and not as independent cells, therefore the relative CFU counting show a very high standard deviation value as an index of low reliability (Frioni et al., 2010). In our study, it was confirmed, that used procedure allows complete detachment of all the bacterial cells from the metal plates. In addition, compare to fluorescence microscope, the method let to specify the number of cells that adhered to the entire surfaces of the metal plate. Results obtained using CFU counting method were reliable, reproducible and statistically significantly correlated with the results from the fluorescence microscope correlation coefficient equals 0.945.

In our study two indirect methods were evaluated: AB and MTT assay. AB is a fluorescent reduction oxidation indicator that responds quantitatively to the viable cells (Sakum et al., 2011). Its main component is resazurin, which is irreversibly reduced to pink resorufin by viable cells. Resazurin is easily soluble in water, which eliminates the additional step of dissolution, as in the case of MTT reduction based method. Due to the fact that AB it is extremely stable, non-toxic to the cells and sensitive, this test has been considered superior to classical tests for cell viability (Fields and Lancaster, 1993). It was proved that resazurin-based assay can be also used for assessment of bacterial adhesion and biofilms quantification (Peeters et al., 2008b; Sakum et al., 2011). Fluorescent assay based on resazurin was also used for detection of activity of disinfectants against bacterial biofilm (Mariscal et al., 2009).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay measures cell viability in terms of reductive activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells (Walencka et al., 2008). MTT assay is standard methods used to assess cytotoxicity of compounds, determining the number of bacteria and different kind of cells and also to evaluate the degree of adhesion (Hamid et al., 2004, Walencka et al., 2008).

There is a number of factors that can affect the sensitivity and accuracy of the metabolic assays, e.g. pH, cell density, incubation temperature, chemical interactions between the media components, test compound and assay chemistry, dosage and exposure time to the test compound, test compound and assay reagent stability for time-course monitoring. (Rampersad, 2010). As reported by several authors, there are a number of substances which may enhance or decrease the reduction of AB and MTT assay, including in particular substances having reducing ability and can affect the metabolic activity of the cells (Maioli et al., 2009; Mariscal et al., 2009; O'Brien et al., 2000; Sims and Plattner, 2009). Based on literature review, research concerning the interaction of the cell viability assays with metal alloys has not been performed yet. Although, the results of the present study showed that the metal ions interact with components of the dye (AB and MTT assay). According to Wataha et al. (1993) presence of metals can cause no non-specific interactions with assay chemistry which result in false positive signals.

In addition, Wataha et al. (1993) point out that the metal ions may penetrate into the medium and could be retained by the cells during the incubation period, which can affect the results. This can explain why in case of blank (PBS incubated with metal plates instead of bacterial suspension) in AB2, the strong interaction of metal ions with assay components was observed. In the case of MTT2 the interaction of metal ions with assay components was not observed. This may be due to a higher sensitivity of AB compared to MTT assay (Hamid et al., 2004; Patel et al., 2013). The MTT2 modification allowed to determine the number of bacteria adhered to the metal plates. However, there was no correlation between MTT, fluorescence microscopy method and CFU counting. It can be assumed that the presence of ions in the medium could affect the high side results obtained in case of AB and MTT.

The chemical compositions of the tested metal plates included: copper, iron, chromium and nickel. The strongest reactivity with both assays was observed in the case of metal plates contained Fe/Cu. The weaker reactivity with AB and MTT assay components was observed in the case of the sample no. 2 (Fe/Nib10Cr), which, compared to the two other metal plates, did not included copper in its composition. This is consistent with previous reports from Rudzok et al., (2011) who demonstrated adverse effects in present of Cu for MTT.

Although several tests are available for evaluation of bacterial adhesion, their applicability can be limited by the physicochemical properties of the metal surfaces. Regarding the experimental methods, there is no standard method available for the study of microbial adhesion on the metal surfaces. Results of our study showed, that metal ions reacted with components of the indirect colorimetric tests used in this study (AB and MTT assay). Consequently, those tests give positive false results. Since the application of AB and MTT assays used for evaluation of adhesion and biofilm formation on the metal surfaces is increasingly growing, a critical examination of their advantages and limitations is justified. Negative and positive controls must be empirically determined to ensure that there are no non-specific interactions with assay chemistry which would result in artifacts or false positive signals (Rampersad, 2010).

Except to AB and MTT assay, also other Direct coating method, including microscopy techniques and CFU counting, were successfully applied for evaluation of bacterial adhesion to the metal surfaces. Both methods showed high correlations. However, considering the limits for the surfaces of the examined samples for microscopy, the determination of the CFU was found to be the best method for testing the adhesion to metal surfaces. The method, combined with the appropriate detachment procedures allowed for a precise determination of the number of bacteria on the entire surface of the evaluated metal samples.

The physicochemical properties of metal surfaces should be taken into account while method for evaluation of bacterial adhesion is selected. It was showed, that application of indirect colorimetric tests such as AB and MTT assay can be limited because of the interaction effects of the assay and metal compound. Although, direct counting methods such as microscopy techniques and CFU counting method are more laborious and time consuming, both were successfully applied for evaluation of bacterial adhesion to the metal surfaces.

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