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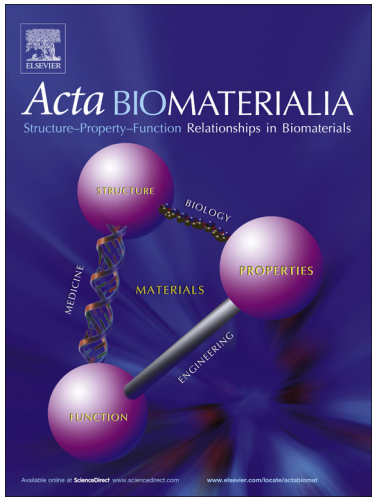

Review article

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PII: S1742-7061(18)30071-0
DOI: https://doi.org/10.1016/j.actbio.2018.02.001
Reference: ACTBIO 5306

To appear in: *Acta Biomaterialia*

Received Date: 27 October 2017
Revised Date: 12 January 2018
Accepted Date: 1 February 2018

Please cite this article as: Sjollema, J., Zaat, S.A.J., Fontaine, V., Ramstedt, M., Luginbuehl, R., Thevissen, K., Li, J., van der Mei, H.C., Busscher, H.J., *In vitro* methods for the evaluation of antimicrobial surface designs, *Acta Biomaterialia* (2018), doi: https://doi.org/10.1016/j.actbio.2018.02.001

REVIEW

In vitro methods for the evaluation of antimicrobial surface designs

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ABSTRACT

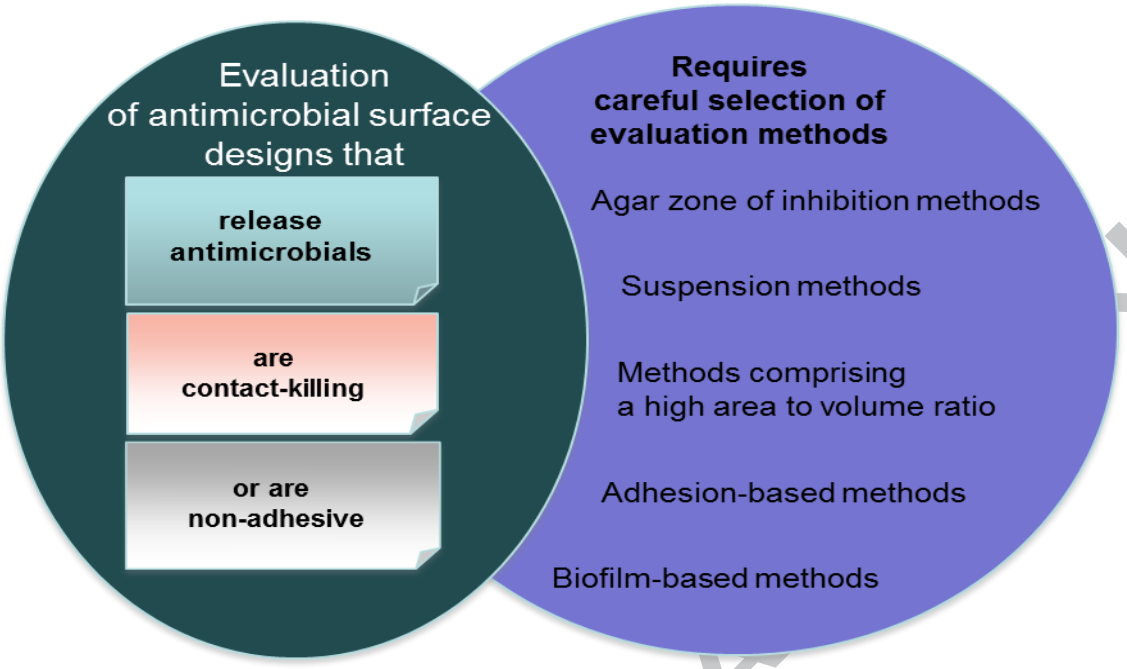
Bacterial adhesion and subsequent biofilm formation on biomedical implants and devices are a major cause of their failure. As systemic antibiotic treatment is often ineffective, there is an urgent need for antimicrobial biomaterials and coatings. The term "antimicrobial" can encompass different mechanisms of action (here termed "antimicrobial surface designs"), such as antimicrobial-releasing, contact-killing or non-adhesivity. Biomaterials equipped with antimicrobial surface designs based on different mechanisms of action require different *in vitro* evaluation methods. Available industrial standard evaluation tests do not address the specific mechanisms of different antimicrobial surface designs and have therefore been modified over the past years, adding to the myriad of methods available in the literature to evaluate antimicrobial surface designs. The aim of this review is to categorize fourteen presently available methods including industrial standard tests for the *in vitro* evaluation of antimicrobial surface designs according to their suitability with respect to their antimicrobial mechanism of action. There is no single method or industrial test that allows to distinguish antimicrobial designs according to all three mechanisms identified here. However, critical consideration of each method clearly relates the different methods to a specific mechanism of antimicrobial action. It is anticipated that use of the provided table with the fourteen methods will avoid the use of wrong methods for evaluating new antimicrobial designs and therewith facilitate translation of novel antimicrobial biomaterials and coatings to clinical use. The need for more and better updated industrial standard tests is emphasized.

Statement of Significance

European COST-action TD1305, IPROMEDAI aims to provide better understanding of mechanisms of antimicrobial surface designs of biomaterial implants and devices. Current industrial evaluation standard tests do not sufficiently account for different, advanced antimicrobial surface designs, yet are urgently needed to obtain convincing *in vitro* data for approval of animal experiments and clinical trials. This review aims to provide an innovative and clear guide to choose appropriate evaluation methods for three distinctly different mechanisms of antimicrobial design: (1) antimicrobial-releasing, (2) contact-killing and (3) non-adhesivity. Use of antimicrobial evaluation methods and definition of industrial standard tests, tailored toward the antimicrobial mechanism of the design, as identified here, fulfill a missing link in the translation of novel antimicrobial surface designs to clinical use.

Keywords: Biofilm, biomaterial-associated infection, antimicrobial-releasing, contact-killing, non-adhesive

Graphical abstract



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1. Introduction

Bacterial adhesion and subsequent biofilm formation on biomedical implants and devices are the main cause of their failure [1]. The incidence rates of biomaterial-associated infection (BAI) depend on the application considered, i.e. for urinary catheters the risk of acquiring an infection rises with 3–7% per day [2,3], central venous catheters (CVC) infections occur 2 to 4 times per 1000 CVC days [4,5], vascular prosthesis infection rates are between 0.5 and 5% [6], aortic endografts (stents) 0.2 to 0.7% [7], total hip and knee arthroplasties have infection rates from 1% in primary replacements to 5% in revision surgery [8], while being higher for plates and screws in trauma patients [9] and infections rates for abdominal wall meshes range between 1% and 2% [10]. BAI is difficult to treat with antibiotics since bacteria are on the one hand protected by their biofilm mode of growth and on the other hand not effectively targeted by a compromised host immune system at the site of the implanted biomaterial or device [11–13]. Dental implants, though placed in an unsterile environment, have a relatively low infection rate of around 1% [14], suggested to be due to adaptation of the immune system to the presence of bacteria and biomaterials. BAI often requires surgical replacement of the implant or device, typically accompanied by great discomfort to the patient, loss of quality of life, and high treatment costs [15]. Not seldom, the difficulties associated with the proper diagnosis of BAI lead to severe morbidity and mortality [16]. For instance, cases with low-grade BAI can easily be misdiagnosed as a touch of the flu, with fatal consequences for patients [17].

BAI develops frequently through transfer of commensal bacteria from the skin to an implant or device surface during surgery (“early per-operative contamination”) or hospitalization prior to complete wound closure (“late per-operative contamination”) [18]. Apart from the per-operative route, bacteria can reach the biomaterial surface through hematogenous spreading from infections elsewhere in the body [19]. Importantly, bacteria may survive in surrounding tissue even after revision surgery, posing novel requirements to treatment strategies [20]. Because therapeutic measures to control BAI

often fail and have severe consequences to the patient, emphasis has shifted to prevention of BAI by designing novel antimicrobial biomaterials or coatings for implants and devices [21].

The term “antimicrobial” is rather loosely used in the current literature encompassing different mechanisms of action that, in one way or another, may contribute to the prevention of BAI. Three distinctly different mechanisms of antimicrobial action can be distinguished based on (1) surfaces that release antimicrobials, (2) surfaces that kill adhering bacteria directly upon adhesion without release of antimicrobials (contact-killing) (3) surfaces that are non-adhesive towards bacteria (non-adhesivity). (Note that we carefully avoid the more general term “antifouling”, because it is highly non-specific. “Fouling” refers to any unwanted deposition of material onto a surface, ranging from microorganisms on biomaterials implants and devices to barnacles and mussels in a marine environment, while “anti” encompasses all possible mechanisms that prevent or reduce fouling.) Local release of antimicrobials, like gentamicin and amoxicillin, from biomaterial beads or coatings has been applied in cardiovascular stents, surgical meshes, urinary catheters, orthopedic implants, or trauma devices [22–24]. In addition, antiseptics like chlorhexidine and silver sulfadiazine have been used in drug-releasing central venous catheters [25,26]. Antimicrobial-releasing coatings may be depleted when antimicrobial-release is most needed, while as a second drawback sustained low-level tail-release will contribute to the development of antibiotic-resistance [27]. Therefore contact-killing [28–33] and non-adhesive [34,35] surfaces are considered advantageous for long-term antimicrobial activity. Long-term efficacy of contact–killing surfaces, however, has been questioned because of a potential coverage of the surface by adsorbed proteins from blood, serum or other body fluids or development of a layer of dead bacteria [36]. Yet, efficacy of contact-killing quaternary ammonium-coated surfaces has been demonstrated over a time period of several days in rats [37] and up to a month in sheep [38]. Herewith contact-killing and non-adhesive surfaces bear promise in applications where hematogenous spreading of bacteria forms a

long-term threat as for joint replacements [44], pace-makers or intravenous catheters which are under continuous risk of colonization by cutaneous or subcutaneous bacteria [45,46]. Surfaces that are non-adhesive to bacteria however, are often also non-adhesive to tissue cells, making them less suitable for biomaterial implants and devices requiring tissue integration. Addition of RGD-peptides to a non-adhesive polymer-brush, however, enhanced tissue cell attachment without affecting non-adhesiveness towards *Staphylococcus aureus* [39,40]. Thus recent advances go beyond the level of a single mechanism of antimicrobial action and comprise dual- or multi-functional antimicrobial coatings combining advantages of both releasing, non-adhesiveness and/or contact-killing designs with features to improve in tissue integration [39]. Also nanotechnology-based antimicrobial strategies are rapidly emerging [41-43], but these too can be classified as working according to either of the three mechanisms of action distinguished in this paper or combinations thereof.

Many experimental antimicrobial surface designs for biomaterials or coatings reported in the literature never get translated to clinical use, mainly because industry requires simple, robust and cheap manufacturing processes for antimicrobial surface designs while regulatory agencies require costly, often statistically impossible, large clinical trials before allowing market introduction [44]. In addition, it becomes harder and harder to obtain approval for animal studies and in most countries approval for animal experiments is subject to convincing *in vitro* evidence of efficacy. This puts emphasis on the design of proper *in vitro* evaluation methods for antimicrobial surface designs, tailored towards specific mechanisms of action [45]. Available industrial standard evaluation tests (see Table 1) are mostly intended to assess the antimicrobial efficacy of non-medical products, such as chemical disinfectants and antiseptics for food and domestic appliances (European Standard, EN 13697), antibacterial plastics (International Organization for Standardization, ISO 22196) or for textiles with improved hygiene, odor control and protection from microbial attack and products for disinfection [46–48]. Industrial standard

evaluation tests often do not consider differences in antimicrobial mechanism of a design. Accordingly, many industrial standard tests have been modified over the past years, adding to the myriad of methods available in the literature to evaluate antimicrobial biomaterials or coatings. The aim of this review is to categorize fourteen presently available methods including industrial standard tests for the *in vitro* evaluation of antimicrobial surface designs for biomaterials implants and devices according to their specific antimicrobial mechanism of action. Suggestions are made for a comprehensive and versatile set of evaluation methods for specific antimicrobial surface designs, enabling cross-laboratory comparison.

2. Definition of antimicrobial activity and efficacy

In order to assess antimicrobial activity of biomaterials and coatings, a proper definition of the term "antimicrobial" is needed. "Antimicrobial" activity according to ISO 20743 is "*the activity of an antibacterial finish used to prevent or mitigate the growth of bacteria, to reduce the number of bacteria or to kill bacteria*". The Japanese Industry Standard (JIS) defines "antimicrobial" in JIS Z 2801 as "*the condition inhibiting the growth of bacteria on the surface of products*". Importantly, whereas ISO 20743 mentions both growth inhibition and bacterial killing in their definition of antimicrobial activity, JIS Z 2801 only mentions growth inhibition. However, no distinction is made between killing and growth inhibition in their definition of antimicrobial activity. Both organizations judge the efficacy of antimicrobial products based on the difference in the logarithmic value of viable cell counts between antimicrobial test products and inert controls after incubation in the presence of a bacterial inoculum

$$A = \log (C_t/C_0) - \log(T_t/T_0) \qquad \text{(Eq. 1a)}$$

in which C_0 and T_0 are the challenge numbers of bacteria before incubation on the control and antimicrobial test sample respectively, while C_t and T_t are the number of bacteria obtained typically after 16 - 24 h incubation from the control and test sample respectively. Ideally, C_0 and T_0 should be equal numbers, although this is difficult to achieve when evaluating non-adhesive surfaces. In the ideal conditions, Eq. 1a reduces to its more well-known form

$$A = log\ (C_t/T_t) = log(C_t) - log\ (T_t) \quad \text{(Eq. 1b)}$$

Most available standard methods represented in Table 1 judge efficacy based on the value of antimicrobial activity according to Eq. 1b. It should be noticed however, that due to the use of a logarithmic scale, values for antimicrobial activity greatly depend on the bacterial challenge numbers applied: i.e. a log reduction of 2 involves a much larger reduction in bacterial numbers, when from 10^8 to 10^6 than when from 10^4 to 10^2. Eq. 1b can also be applied in its linear, analogous form [28,49–52] in which case it reads

$$A\ (\%) = 100 - \left(\frac{T_t}{C_t}\right) * 100 \quad \text{(Eq. 2)}$$

Antimicrobial activity values A can be evaluated purely on the basis of statistically significant differences, but from a microbiological perspective, including the criteria in JIS Z 2801, differences are only meaningful when more than two log-units or linearly expressed, more than 99% [53].

3. The choice of bacterial species and challenge number

The microbial species causative to BAI differ greatly among the different sites of functional restoration or support across the human body. Table 2 gives an overview of species causative to BAI of different

implants and devices. Many organisms causative to BAI are commensals of the skin, the intestines or the oral cavity, especially after short-term use of an implant or device. Opportunistic pathogens may become involved, especially after long-term use, such as *Prevotella intermedia* and *Porphyromonas gingivalis* in BAI associated with dental implants [59]. Infection after short-term use of urinary catheters is often due to *Staphylococcus epidermidis, Escherichia coli* or *Enterococcus faecalis*, while after long-term catheterization *Pseudomonas aeruginosa, Proteus mirabilis* and *Klebsiella pneumoniae* come into play [62,63]. In relation with studies into BAI and standard industrial tests, it is advisable to use well-known laboratory strains in combination with fresh clinical isolates. The use of laboratory strains offers the advantage of better allowing comparison of results obtained in different institutions and their specific properties are generally well studied. Compared with clinical isolates however, repeated culturing of laboratory strains may yield loss of their virulence, ability to produce EPS and form biofilms, including quorum sensing [64–66]. Many antimicrobial surface designs in the literature are not geared in their pre-clinical development stage towards a specific implant or device, in which case it is advisable to choose strains and species generally occurring in BAI. From Table 2, it is clear that such a collection of strains should minimally involve Gram-positive species such as *S. aureus* and *S. epidermidis*. Since the cell wall architecture of Gram-negative and Gram-positive bacterial strains differs considerably [67] with a possible impact on the efficacy of antimicrobial surface designs, at least one Gram-negative strain should be included too. Accordingly, in JIS Z 2801, it is suggested to use both a Gram-negative *E. coli* and a Gram-positive *S. aureus*. Depending on the application aimed for, pathogenic yeasts like *Candida* spp. should be included in the evaluation of antimicrobial surface designs, as they have antimicrobial susceptibilities that are very different from bacteria [68].

Antimicrobial biomaterials and coatings can be challenged with different numbers of microorganisms to evaluate their efficacy. Therapeutic antimicrobial surface designs are mostly applied when a patient shows clear signs of infection and a mature biofilm is present. Therapeutically aimed

antimicrobial surface designs such as antibiotic-loaded beads and spacers for the treatment of osteomyelitis [8,69,70] therewith face a much higher microbial challenge than designs aimed to prophylactically counteract the consequences of generally low levels of per-operative microbial contamination. In absence of well-documented data on the numbers of organisms clinically found on biomaterial implants and devices [71], industrial standard tests usually instruct to employ a defined inoculum that is expressed as the number of colony forming units (CFUs) per unit suspension volume, in which a material with a specified surface area is placed. Inoculum concentrations instructed in JIS Z 2801 for instance (2.5×10^6 CFU/mL), reflect the concentration of bacteria found in urine of patients with a catheter-associated-infection (10^5 even up to 10^8 CFU/mL [62]), but these bear no relation with the number of bacteria found on the catheter surface itself. In a measure of clinically relevant numbers of bacteria per unit area, the Infectious Diseases Society of America [72] states in its guideline on the diagnosis and management of intravenous catheter associated infections, that more than 100 CFUs on a 5 cm catheter tip reflect catheter colonization. This would be roughly equivalent to 30 bacteria per cm^2. However, much higher localized numbers, i.e. more than 10^6 bacteria per cm^2 (roughly equivalent to 1% of full, bacterial mono-layer coverage), can be inferred from electron micrographs and fluorescent *in situ* hybridization images of biofilms on biomaterial implants and devices retrieved from patients with BAI [71,73–75], but these represent bacterial numbers found in clinical infections, rather than much lower initial per-operative bacterial contamination numbers. Moreover, bacteria involved in per-operative contamination do not yet exhibit a mature biofilm architecture. Here too unfortunately, reliable numbers are absent. Typically, only less than 1 CFU per cm^2 per hour is detected on a surface under a downward airflow in a ventilated operating theatre [76]. In other studies, 270 bacteria per cm^2 of bacteria were found to contaminate a wound during surgery [77].

In summary, in the evaluation of antimicrobial surface designs, a challenge concentration should be applied that is in line with the intended application. Considering that most designs are

intended for prophylactic use, their evaluation against bacterial challenge numbers derived from biomaterial implants and devices retrieved from patients with clinical signs of infection, will put any prophylactically intended design at a disadvantage. Therefore we here suggest that antimicrobial surface designs intended to negate the potential development of infection arising from per-operative bacterial contamination, should be evaluated at challenge numbers of 1000 CFU per cm^2 or less. Experimental conditions, especially in the evaluation of non-adhesive designs, often dictate working at concentrations of around 10^8 bacteria per mL, which is far higher than observed for instance, in urine of patients with a catheter associated infection [62]. These considerations should be thoroughly taken into account in any evaluation method of antimicrobial surface designs.

4. Sterilization and application of conditioning films prior to evaluation

In most evaluation methods, antimicrobial surfaces are challenged by bacterial suspensions without further pre-treatment of the sample surfaces. Sterilization of biomaterials may leave residuals on their surface, affecting the surface chemistry designed. In the case ethylene oxide sterilization, such residuals can be irritating, mutagenic and at high levels leading to organ damage and carcinogenicity [78]. Moreover, sterilization residuals may interfere with an antimicrobial surface design, regardless of its mechanisms [79,80].The relevance of evaluating sterilization effects is most evident in translational studies as sterilization is a prerequisite for implants or devices that are in direct contact with the human body. In particular safety aspects around ethylene oxide use are addressed in the ISO 10993 standard that guides biocompatibility evaluation of medical devices.

Also, in nearly all clinical settings, biomaterial implants or devices are exposed to urine, saliva, tear fluid, sweat, blood, etcetera posing another challenge to antimicrobial surface designs affecting their surface chemistry. Adsorption of macromolecular components such as proteins from these body fluids proceeds much faster than adhesion of bacteria to form a so-called “conditioning film” [81]. Depending

again on the application aimed for, the potential presence of a conditioning film on an antimicrobial coating should be taken into account, as for instance many pathogens have specific receptors for salivary and blood-borne protein films [21,82–85]. Moreover, conditioning films may potentially impair the efficacy of antimicrobial-release and contact-killing designs [86,87].

Conditioning films can also form during evaluation of antimicrobial surface designs, especially when the bacterial challenge originates from suspensions in culture medium. Tryptic Soy Broth, for instance, contains enzymatically digested soy bean proteins, whereas Nutrient Broth contains peptones of often undefined animal sources and beef extract. Culture media can also be supplemented with serum, from which many different proteins can sequentially adsorb to a sample surface [88] prior to or during evaluation. Industrial tests provide conflicting instructions on the use of undiluted (e.g. AATCC 100) or diluted (e.g. ISO 20743 and JIS Z 2801) culture media to suspend bacteria, which can subsequently lead to different evaluation results.

Summarizing, it is suggested here that for evaluation of antimicrobial surface designs sterilization or application of an appropriate conditioning film geared to the application aimed for should always be taken into account.

5. How dead are killed bacteria?

There is a plethora of evaluation methods that have been developed and adapted to evaluate different antimicrobial surface designs, that at the end of the assay require enumeration of dead and live bacterial numbers. Whereas most methods are based on culturing and enumeration of the number of CFUs (see Figs. 1-3), many others include live/dead staining, fluorescence *in situ* hybridization (FISH), MTT (based on the reduction of the MTT dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to the purple formazan by NAD(P)H) or use of bioluminescent strains to demonstrate bacterial death. It is beyond the scope of this review to extensively discuss the merits of the different methods to

demonstrate bacterial death. However, different types of antimicrobials can yield highly different types of damage to a bacterium with variable outcomes of a bacterial death quantification. Live/dead staining (e.g. LIVE/DEAD® Bacterial Viability Kit (BacLight™)) composed of two nucleic acid-binding stains: SYTO 9 (green-fluorescent) and propidium iodide (red-fluorescent) [89] in principle demonstrates the presence or absence of cell wall damage, but it has been shown that cell wall damaged, red-fluorescent bacteria usually presumed dead, may sometimes turn out to be culturable as well [90]. Bioluminescence and MTT heavily rely on metabolic activity, but absence of metabolic activity does not necessarily mean bacterial death. Moreover, with respect to culturing as the generally accepted "gold standard", many bacterial strains are not culturable, while specific antimicrobials may bring bacteria in a reversible "dead" state, called "viable-but-not-culturable" or in an irreversible "dead" state, such as by lysis [91]. This makes demonstration of bacterial death one of the main challenges that microbiologists are facing across all fields of applications on a daily basis [92]. This challenge is extended greatly in the context of BAI. Bacteria in biofilms on biomaterials implants and devices are often subject to programmed cell death or the generation of a hibernation state such as in "persister" or "dormant" cells [93]. Due to their extremely low metabolic activity, persister or dormant cells are less susceptible to antimicrobials and easy to miss in death evaluation methods. With the ongoing discussion on when a bacterium can be declared death in absence of cell lysis as the most evident sign of death, it is advisable to evaluate bacterial death by culture based methods, extended with minimally one other method.

6. Evaluation methods for antimicrobial surface designs

In the forthcoming sections we will briefly describe the most common methods to evaluate antimicrobial surface designs, as schematically depicted in Figs. 1-3, in which we also indicate their suitability with respect to designs based on mechanisms of antimicrobial release, contact-killing or non-adhesivity.

6.1. Agar zone of inhibition methods

In agar zone of inhibition methods [27,47,94–98], samples are placed with their antimicrobial side down on an agar plate inoculated with microorganisms (see Fig. 1a.1). Possible antimicrobials released from a sample subsequently diffuse into the agar yielding a concentration gradient away from the sample. As long as the concentration in the agar is above the minimal inhibitory concentration (MIC), a zone in which bacterial growth is inhibited can be observed, the width of which is taken as a measure of antimicrobial activity. It is recommended that zones of inhibition be measured in different directions or locations, depending on the sample geometry. Typically, a 24 to 48 h incubation time is applied after which the width of the inhibition zone is measured, as a measure of both the amount of antimicrobial released and the susceptibility of the bacterial strain involved towards the antimicrobial. The method does not provide an antimicrobial activity value as defined in section 2, while it has been suggested that only a zone of inhibition with a minimal width of 10-15 mm indicates potential clinical significance [99].

Several variations of the above exists, most notably including the regular transfer of samples to a freshly inoculated agar plate in order to study the kinetics of antimicrobial release [100]. Other variations include placing entire catheter sections in holes punched into the agar [26,101] or the use of 3D agar molds to evaluate antimicrobial surface designs on real- or miniaturized implants or devices [102].

The zone of inhibition method is the most commonly used method for evaluating antimicrobial-releasing designs. It can also be used for contact-killing designs by studying bacterial growth directly underneath a sample. Several industrial standard evaluation tests (see Table 1) relate with the zone of inhibition assay, in which inoculated agar is poured over an antimicrobial surface, implant or device before solidification, as e.g. in ASTM 2180 (see Fig. 1a.2). Although culturing on agar counts as the gold standard in antimicrobial evaluation, the method has as a drawback that not all bacterial strains

and species are culturable [103], while furthermore zones of inhibition may depend on the rate of diffusion of antimicrobials through the agar [104]

6.2. Suspension methods

In suspension methods such as ASTM E2149 [96,97,105–107], a known challenge number of microorganisms in a suspension volume is exposed to an antimicrobial-releasing sample for defined time periods after which the numbers of CFU in the suspension are assessed and related with those found for control samples (see Fig. 1b.1). Samples are placed in capped glass tubes containing a defined volume of a microbially inoculated suspension medium. After overnight growth under agitation at 37°C, aliquots are drawn from the suspension medium for agar plating and CFU counting [103,104] to facilitate calculation of the antimicrobial activity according to section 2. Drawback is the use of microorganisms in their planktonic state, in which they are much more susceptible to antimicrobials than organisms in their biofilm-mode of growth. Although optical density measurements are also done to asses antimicrobial activity [42, 110], optical density reflects both live and dead bacteria without distinction. The sample area to fluid volume is critical in these methods and mostly small. This implies that the build-up of a high antimicrobial concentration may be slow or never occurring, particularly not when antimicrobial release is from coatings with low housing capacities.

Immersion of porous, antimicrobially loaded materials such as textile fabrics [47] into a suspension, as in JIS L1902, SN 195924, AATCC 100, and ISO 20743 gives rise to a higher area to volume ratio (see Fig. 1b.2). Absorption of a bacterial suspension in growth medium into a porous material is allowed for a given period of time after which the suspension is removed, assuming bacteria remain entrapped in the medium absorbed in the pores. Next, porous samples are incubated, typically up to 24 h at 37°C, while entrapped bacteria become exposed to the antimicrobials released. After incubation, bacteria are removed from the porous material by vortexing or sonication followed by serial

dilution, agar plating and CFU enumeration and final calculation of antimicrobial activity. To prevent that possible antimicrobial release during vortexing or sonication influences bacterial viability, use of a neutralizing broth has been recommended in EN 1040 [105,111].

Suspension methods are also advocated like in ASTM E2149, to evaluate bacterial contact-killing designs. In such an application of suspension methods, a contact-killing surface is placed in a suspension volume under shaking. Bacterial killing upon contact or adhesion to the sample surface is enumerated from reductions in the number of viable bacteria in suspension. Extensive experimental comparison of methods geared towards evaluation of contact-killing designs has shown this method to be unsuitable for evaluation of contact-killing surfaces, as mass-transport towards the surface is poorly controlled and usually small [112].

6.3. Methods comprising a high area to volume ratio

Suspension methods are generally carried out at small (sample) area to (fluid) volume ratios, which, pending on the clinical application aimed for, is not always a realistic clinical scenario. This can have a severe impact in the evaluation of antimicrobial-releasing designs. Therefore a variety of methods has been developed that allows to work under conditions of high area to volume ratios enabling the rapid build-up of a high concentration of antimicrobials in case of antimicrobial-releasing designs.

In JIS Z 2801 or ISO 22196, high area to volume ratios are established by sandwiching a microbial suspension in 0.2% medium between a sample surface and a cover slip, confining the suspension by capillary forces to around 250 μm thickness (see Fig. 2.1). After incubation for 24 h, bacteria are retrieved by sonication or extensive washing of both the sample material and the cover slip, and subsequently cultured on agar plates followed by enumeration to yield antimicrobial activities according to section 2 [112-116]. For the evaluation of antimicrobial-releasing bone cements, it has been suggested to grow bacteria in small gaps, cut in the cement. Importantly, bacteria surviving

antimicrobial-release from bone cements in suspension methods with a small area to volume ratio, were killed in a gap model [117].

The Petrifilm® all-in-one-plating system (3M, St. Paul, MN, USA) [39,112,118-120] originally developed for fast screening of bacterial contamination of food products, is a commercial, ready to use system, consisting of a thin agar coat on a transparent foil that must be folded over a sample surface in presence of a small bacterially contaminated liquid volume of 20 - 50 µL (see Fig. 2.2). During an incubation time of up to 48 h at 37°C, bacterial colonies are formed. Apart from nutrients, the foil is also loaded with a stain (tetrazolium chloride) to visualize bacterial colonies that can subsequently be enumerated and used to calculate antimicrobial activity according to section 2 without any further processing. The combination of agar and stain in an all-in-one-plating method avoids washing or sonication and leaves relatively little waste. The possibility to enumerate bacteria implies applicability of antimicrobial activity values as defined in section 2, but cannot be directly applied when using high challenge concentrations or when the antimicrobial activity is low. In these cases the foil turns completed stained over the entire sample surface. This drawback can be circumvented for high challenge numbers by diluting the bacterial suspension from which challenge aliquots for inoculation are taken. By determining the number of CFUs of a diluted suspension on samples known to be non-antimicrobial, challenge numbers in more concentrated challenge suspensions can then be easily calculated.

The small volume of the all-in-one-plating method also enforces direct contact of bacteria with the sample, making the method not only suitable for antimicrobial-releasing but also for contact-killing designs. It has been strongly recommended however, that when an all-in-one-plating method is used for evaluating contact-killing surfaces, it should first be ascertained, for instance using a zone of inhibition method, that there are no antimicrobial components leaching out of the sample. Due to the small volume involved in an all-in-one-plating method, leachables may easily interfere with contact-killing

mechanisms. Also JIS Z 2801 has been suggested for evaluation of contact-killing designs [112,113]. In a slight modification of JIS Z 2801, a bacterially inoculated filter is placed on a contact-killing surface on which a 20 μL droplet is positioned containing 1% Tryptic Soy Broth after which the procedure is similar to the JIS Z 2801 test [121,122] (see Fig. 2.3). This so called "printing" of bacteria onto a sample to establish direct contact between a contact-killing surface and bacteria has also been prescribed in the ISO 20743 where a standard force of 4 N is applied to press the filter on a sample surface. The modified JIS method however, has yielded bacterial growth on surfaces, indicated in all-in-one-plating and JIS Z 2801 methods as being contact-killing [112], possibly demonstrating more favorable conditions for bacterial growth in the sheltered environment of the filter, as applied in the modified JIS test.

To evaluate contact-killing designs towards bacterial aerosols, spraying has been proposed [28,123,124] (see Fig. 2.4). This method was particularly applied to evaluate contact-killing surfaces that prevent growth of contamination by air-borne pathogens. After spraying a diluted bacterial suspension on test samples, samples are subsequently air-dried for 2 min at room temperature after which agar slabs of growth medium with the size of the sprayed surface are placed on top of the samples and covered with Parafilm® to prevent their drying-out during overnight incubation. Bacterial colonies grown on the sample surfaces in the agar are counted by visual inspection without any further processing of the samples.

In an extensive comparison of methods to evaluate bacterial contact-killing methods against Gram-positive and Gram-negative bacterial strains [112], it has been concluded that Petrifilm® all-in-one-plating and JIS Z 2801 methods are most suitable to this end, provided that they are complemented with a zone of inhibition assay to exclude that leachables out of a sample add an additional killing mechanism.

6.4. Adhesion-based methods

Adhesion of bacteria to a substratum surface is one of the first steps in biofilm formation. Methods to study initial bacterial adhesion usually involve adhesion of bacteria from a static or flowing fluid suspension. In static systems, mass transport of microorganisms to substratum surfaces mostly occurs through sedimentation [125], while in flow perfusion systems convective-diffusion contributes to mass transport. Since adhesion implies intimate contact between bacteria and substratum surfaces, adhesion-based methods are excellently suited for evaluating contact-killing designs, especially because contact-killing designs by cationic surfaces will yield electrostatic attraction of bacteria to the surface with an impact on adhesion numbers [126].

Static adhesion assays are relatively easy to carry out, e.g. test samples are exposed to a droplet of a bacterial suspension from which bacteria settle [50,127] (see Figs. 3a.1 and 3a.2) or are placed in well plates under mild shaking conditions [128] while keeping the number of bacteria allowed to sediment on a substratum surface below monolayer coverage in case of contact-killing designs. After a specific time period in which bacterial adhesion takes place, e.g. 1-4 h, the number of adhering viable bacteria is assessed by first carefully washing off all non-adhering bacteria, and then collecting adhering bacteria by sonication and subsequent CFU counting. By using live/dead staining of adhering bacteria, the ratio of killed bacteria can be determined by fluorescence microscopy. Alternatively, after careful washing off non-adherent bacteria, the sample is covered with a nutrient agar slab (see Fig. 3a.2) and subsequently incubated. No steps to dislodge the bacteria are needed in this case, and individual viable bacteria adhering to the surface grow out as colonies, which are easily counted [92,129].

Drawback of static assays is that enumeration requires washing off non-adherent bacteria, implying that removal of the fluid phase above the substratum should be done extremely careful in order to prevent inadvertent removal of adhering bacteria by passing liquid-air interfaces or flowing

fluid in general. Especially passing liquid-air interfaces are notorious for exerting high detachment forces on adhering bacteria causing their removal [130,131].

Static adhesion-based methods are predominantly applied in testing antimicrobial-releasing or contact-killing surfaces, but are less well suited for anti-adhesive designs due to the lack of control of mass transport conditions and fluid flow forces operative during sedimentation and fluid removal. Flow perfusion methods not only allow to control mass transport, but also offer the possibility to accurately calculate the fluid flow forces on the adhering bacteria and fine-tune them to those occurring for instance in urinary catheters, vascular grafts, around artificial heart valves or extraluminal surfaces of cardiovascular catheters or to flow of therapeutic fluids as in intravenous catheters [132]. Different models of flow perfusion systems exist of which the parallel flow chamber is the most common one, particularly since fluid flow forces and mass transport are relatively easy to calculate (see Figs. 3a.3 and 3a.4) [125,133,134]. When combined with *in situ* observation of adhering bacteria, "washing" or "slight rinsing artefacts" can be fully avoided making the method extremely suitable to evaluate non-adhesive antimicrobial designs to which bacteria usually adhere very weakly making them amenable to inadvertent removal.

Flow perfusion systems are also highly suitable to evaluate contact-killing designs, in which case the flow of the bacterial suspension after the adhesion phase, is subsequently switched to a flow of nutrient media, that only allows surviving bacteria to grow out and form a biofilm from which bacteria are removed and analyzed by CFU counting and live/dead staining [135]. Alternatively, after the adhesion phase and after flushing out non-adherent bacteria, the chamber can be injected with live/dead stain after which fluorescence microscopy can directly be applied to obtain the antimicrobial efficacy of the sample (without exposing the adhering bacteria to a passing liquid-air interface) [136] (see Figs. 3a.3 and 3a.4). Flow perfusion systems are less suitable to evaluate antimicrobial-releasing designs as the antimicrobials released are rapidly washed out of the system. In fact, growth of bacteria adhering to

antibiotic-releasing bone cements has been observed in flow perfusion systems, that was absent in static systems comprising a high area to volume ratio [137].

6.5 Biofilm-based methods

Biofilm-based methods are in essence similar to adhesion-based methods but are carried out over much longer periods of time and must involve the presence of nutrients. Biofilm-based methods can rely on a plethora of different systems, that include all common static and flow perfusion systems, modified Robbins devices, drip flow reactors including the constant depth film reactor [139,140] rotary biofilm reactors and microfluidic devices [103,141,142].

All biofilm-based methods have to start with an adhesion step (see Fig. 3b), preferentially carried out from a low nutrient suspension as the presence of high nutrient concentrations in suspension eliminates the need for planktonic bacteria to adhere to a surface, where they "know" most nutrients accumulate [31,143] a condition that they have in common with e.g. the JIS Z 2801 method. In the subsequent growth step, nutrient availability can be readily controlled in flow displacement systems which is important to avoid artefacts that might arise for instance from nutrient depletion of the environment in which biofilm growth of adhering bacteria is pursued. Biofilm-based methods seldom yield full eradication of biofilm by any antimicrobial design, and the best that can be achieved by any design is reduced amount of biofilm or delayed growth. In case of antimicrobial-releasing designs, growth inhibition or killing heavily depends on accumulation possibilities of antimicrobials released in the biofilm. This requires an appropriate biofilm structure that prevents wash-out of the antimicrobials, in which respect it is important to notice that biofilms grown in absence of any mechanical environmental stimulus such as under static conditions [31,144] (see Fig. 3b2), are usually fluffy and highly aqueous, which is opposed to biofilms grown in presence of applied compression or under fluid flow [145,146]. Since contact-killing surfaces seldom kill all adhering bacteria, biofilm-based methods

will always show biofilm growth on antimicrobial contact-killing designs [135]. Similarly, even on the most non-adhesive poly(ethylene)glycol polymer brush coating, bacteria have been demonstrated to adhere and form a biofilm very slowly, as compared to other surfaces [147]. This puts special emphasis on the inclusion of proper control surfaces in biofilm-based methods and the duration of time allowed for growth. Calculation of antimicrobial activity values according to Eq. 2 is well possible in most cases, but will decrease over time.

7. Concluding comments

This reviews clearly indicates (see Figs. 1-3), that there is no single method or industrial test that allows to distinguish antimicrobial designs according to the three mechanisms identified here. However, these figures clearly indicate that for each of the three antimicrobial mechanisms distinguished in this review, suitable methods are available. It is anticipated that use of this review will avoid the use of wrong methods for evaluating new antimicrobial designs and therewith facilitate downward clinical translation. Yet, further method standardization is needed. In particular, simple industry standards should be established that allow adhesion under flow conditions in which shear rates are quantifiable and in a range that complies with shear rates occurring in different clinical applications. In order to create the necessary high through-put, micro-fluidic systems [103, 141] may become useful, although their versatility with respect to the materials that can evaluated is limited. Moreover, considering the development of multi-functional coatings [39] that are not only equipped with an antimicrobial functionality but also with tissue integrating moieties, academic developments of co-culture systems [148] should be standardized into industrial tests, preferably encompassing host immune cells as well [149]. In an era in which animal experiments become increasingly difficult to

obtain permission for [21,53], these standardizations are extremely important and may reduce the need for animal experiments.

Acknowledgements

This project is funded by the research program of the BioMedical Materials institute (BMM, Project P4.01 NANTICO, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation). Parts of the paper reflect the outcomes of the Patras and Bratislava meetings organized in May 2015 and February 2016, funded by the European Commission (Cost Action TD1305, IPROMEDAI). HJB is also director of a consulting company SASA BV. The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organization or their respective employer(s).

Fig. 1. Schematics of agar zone of inhibition (a) and suspension (b) methods, as discussed in this review to evaluate the activity of antimicrobial surface designs, together with an indication of whether the method is identified as being suitable for antimicrobial surface designs based on release of antimicrobials, contact-killing or non-adhesivity.

Fig. 2. Schematics of methods comprising a high area to volume ratio, as discussed in this review to evaluate the activity of antimicrobial surface designs, together with an indication of whether the method is identified as being suitable for antimicrobial surface designs based on release of antimicrobials, contact-killing or non-adhesivity.

Fig. 3. Schematics of adhesion (a) and biofilm (b) methods, as discussed in this review to evaluate the activity of antimicrobial surface designs, together with an indication of whether the method is identified as being suitable for antimicrobial surface designs based on release of antimicrobials, contact-killing or non-adhesivity.

Table 1. Industrial standard evaluation tests of antimicrobial surface designs and their possible relation with the different methods distinguished in Fig. 1, together with their intended application. (AATCC: American Association of Textile Chemists and Colorists, ASTM: American Society for Testing and Materials, EN: European Standard, ISO: International Standard Organisation, JIS: Japanese Industrial Standard, SN: Schweizerische Normen Vereinigung).

Standard	Application area
AATCC 30 Antifungal Activity, Assessment on Textile Materials	Textiles/Fabrics
AATCC 90 Antibacterial Activity, Assessment of Textile Materials	Textiles/Fabrics
AATCC 100 Assessment of Antibacterial Finishes on Textile Materials	Textiles/Fabrics
AATCC 147 Antibacterial Activity Assessment of Textile Materials	Textiles/Fabrics
ASTM E2149 Standard Test Method for Determining the Antimicrobial Activity of Antimicrobial Agents Under Dynamic Contact Conditions	Textiles/Fabrics
ASTM E2180-07 Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials	Non-porous materials
ASTM E2722 Standard Test Method for Using Seeded-Agar for the Screening Assessment of Antimicrobial Activity in Fabric and Air Filter Media	Textiles/Fabrics
EN 1104 Paper and Board Intended to come into Contact With Foodstuffs - Determination of the Transfer of Antimicrobial Constituents	Paper and board
ISO 16869 Assessment of the Effectiveness of Fungistatic Compounds in Plastics Formulations	Plastics
ISO 20645 Textile fabrics - Determination of Antibacterial Activity	Textiles/Fabrics
ISO 20743 Textiles-Determination of Antibacterial Activity of Antibacterial Finished Products	Textiles/Fabrics
ISO 22196 Plastics – Measurement of Antibacterial Activity on Plastics Surfaces	Non-porous materials
JIS L 1902 Testing for Antibacterial Activity and Efficacy on Textile Products	Textiles/Fabrics
JIS Z 2801 Antimicrobial Products-Test for Antimicrobial Activity and Efficacy	Non-porous materials
SN 195920 Textile Fabrics - Determination of the Antibacterial Activity	Textiles/Fabrics
SN 195924 Textile Fabrics; Determination of the Antibacterial Activity	Textiles/Fabrics

Table 2. Microbial species involved in BAI among different sites of functional restoration or support across the human body.

Implant or device	**Species causative to BAI**	**References**
Joint prostheses	*Staphylococcus aureus* *Staphylococcus epidermidis* *Streptococci*	[54]
Vascular grafts	*Staphylococcus epidermidis* *Staphylococcus aureus*	[6]
Central venous catheters	*Coagulase-Negative Staphylococci* *Enterococci* *Klebsiella pneumonia*	[55]
Pace makers	*Coagulase-Negative Staphylococci* *Staphylococcus aureus*	[56]
Contact lenses	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Serratia marcescens*	[57]
Biliary stents	*Enterococcus faecium* *Escherichia coli* *Enterobacter cloacae* *Klebsiella pneumonia*	[58]
Dental implants	*Streptococcus mutans* *Streptococcus sanguinis* *Streptococcus mitis* *Actinomyces viscosus* *Prevotella intermedia* *Porphyromonas gingivalis*	[59,60]
Abdominal wall meshes	*Staphylococcus aureus* *Streptococcus pyogenes* *Coagulase-Negative Staphylococci* *Escherichia coli*	[10]
Voice prostheses	*Candida albicans* *Streptococci* *Staphylococcus epidermidis*	[61]
Urinary catheters	*Escherichia coli* *Enterococci* *Klebsiella pneumoniae* *Coagulase-Negative Staphylococci* *Pseudomonas aeruginosa* *Candida albicans* *Proteus mirabilis*	[2,62,63]

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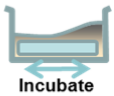
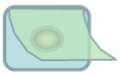
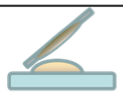
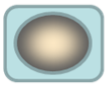
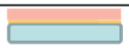
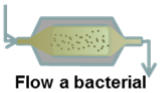
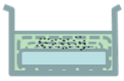
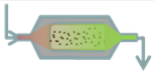
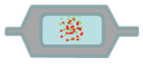
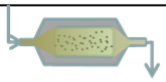
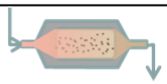
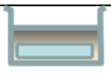
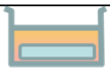
Statement of Significance

European COST-action TD1305, IPROMEDAI aims to provide better understanding of mechanisms of antimicrobial surface designs of biomaterial implants and devices. Current industrial evaluation standard tests do not sufficiently account for different, advanced antimicrobial surface designs, yet are urgently needed to obtain convincing *in vitro* data for approval of animal experiments and clinical trials. This review aims to provide an innovative and clear guide to choose appropriate evaluation methods for three distinctly different mechanisms of antimicrobial design: (1) antimicrobial-releasing, (2) contact-killing and (3) non-adhesivity. Use of antimicrobial evaluation methods and definition of industrial standard tests, tailored toward the antimicrobial mechanism of the design, as identified here, fulfill a missing link in the translation of novel antimicrobial surface designs to clinical use.

	Main steps	Suitable for designs based on			References
		Antimicrobial release	Contact killing	Non-adhesivity	
(a) Agar zone of inhibition methods					
1	 Samples on bacteria inoculated nutrient agar  Measure zones of inhibition after incubation	√			AATCC 147, DIN EN 1104, ISO 20645, SN 195920 [27, 47, 94-98]
2	 Bacteria inoculated nutrient agar on top of the sample  Measure zone of inhibition after incubation  Determine number of CFUs in suspension after dissolving agar	√			AATCC 30, AATCC 90, ISO 16869, ASTM E2722-4 EN 1104 ASTM 2180
(b) Suspension methods					
1	 Submerge sample in bacterial suspension in medium  Incubate under shaking  Determine number of CFUs in suspension	√			ASTM E2149 [42, 96,97,103-107, 110,112]
2	 Submerge porous sample in bacterial suspension  Remove liquid, incubate, detach bacteria by vortexing or sonication  Determine number of CFUs in suspension	√			JIS L 1902, SN 195924, AATC 100, ISO 20473 [47]

	Main steps			Suitable for designs based on			References
				Antimicrobial release	Contact killing	Non-adhesivity	
Methods comprising a high area to volume ratio							
1	 Inoculate sample with bacterial suspension with diluted medium, cover with polyethylene film and incubate	 Detach bacteria from sample and cover film by sonication or extensive washing	 Determine number of CFUs in suspension/ washing fluid	√	√		JIS Z 2801 ISO 22196 [112–116]
2	 Inoculate sample with bacterial suspension, close top film and incubate 48 h		 Determine number of CFUs on Petrifilm	√	√		[39,112,118-120]
3	 Put droplet of suspension in diluted medium on sample, add bacterial inoculated filter on sample and incubate	 Detach bacteria from sample and filter by sonication	 Determine number of CFUs in suspension	√	√		[112,121,122]
4	 Spray diluted bacterial suspension onto the samples	 Cover sample with solid nutrient agar slab and incubate	 Determine number of CFU		√		[28,112,123,124]

	Main steps	Suitable for designs based on: Antimicrobial release	Suitable for designs based on: Contact killing	Suitable for designs based on: Non-adhesivity	References
(a) Adhesion methods					
1	 Inoculate test sample with bacterial suspension (from droplet or in microtiterplate) during 1-4 h  Carefully rinse samples to remove non-adhering bacteria. Detach adhering bacteria through sonication  Determine number of CFU in PBS aliquots	√	√		[50,127,128]
2	Inoculate test sample with bacterial suspension (from droplet or in microtiterplate) during 1-4 h  Or: Carefully rinse samples to remove non-adhering bacteria Cover sample with solid nutrient agar slab and incubate  Determine number of CFU				[92,129]
3	 Flow a bacterial suspension through the chamber Count deposited bacteria  Take sample out flow chamber. Detach adhering bacteria through sonication  Determine number of CFU in PBS aliquots		√	√	[125,133-135]
4	Flow a bacterial suspension through the chamber Count deposited bacteria  Or: Flow with LIVE/DEAD staining  Determine ratio live/dead bacteria (dead bacteria: red-, live bacteria: green fluorescent)				[136]
(b) Biofilm methods					
1	 Flow with bacterial low nutrient suspension during 1-4 h. Flush with buffer to remove non-adhering bacteria  Flow with nutrient medium to grow a biofilm in 24-48 h  Take sample out flow chamber. Detach biofilm bacteria through sonication. Determine number of CFU		√	√	[103,135,136,141, 142,147]
2	 Submerge sample in bacterial low nutrient suspension during 1-4 h. Carefully rinse sample to remove non-adhering bacteria  Incubate in medium during 24-48 h to grow a biofilm  Take sample out medium. Detach biofilm bacteria through sonication. Determine number of CFU	√	√	√	[31,144]