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Title: Evaluation of the ability of *Acinetobacter baumannii* to form biofilms on six different biomedical relevant surfaces

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Running Head: *A. baumannii* biofilms on six different surfaces

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SIGNIFICANCE AND IMPACT OF THE STUDY

In the hospital environment, *Acinetobacter baumannii* is one of the most persistent and difficult to control opportunistic pathogens. The persistence of *A. baumannii* is due, in part, to its ability

to colonize surfaces and form biofilms. This study demonstrates that *A. baumannii* can form biofilms on a variety of different surfaces and develops substantial biofilms on polycarbonate- a thermoplastic material that is often used in the construction of medical devices. The findings highlight the need to further study the *in vitro* compatibility of medical materials that could be colonized by *A. baumannii* and allow it to persist in hospital settings.

ABSTRACT

The human opportunistic pathogen, *Acinetobacter baumannii*, has the propensity to form biofilms and frequently causes medical device-related infections in hospitals. However, the physio-chemical properties of medical surfaces, in addition to bacterial surface properties, will affect colonization and biofilm development. The objective of this study was to compare the ability of *A. baumannii* to form biofilms on six different materials common to the hospital environment: glass, porcelain, stainless steel, rubber, polycarbonate plastic and polypropylene plastic. Biofilms were developed on material coupons in a CDC biofilm reactor. Biofilms were visualized and quantified using fluorescent staining and imaged using confocal laser scanning microscopy (CLSM) and by direct viable cell counts. Image analysis of CLSM stacks indicated that the mean biomass values for biofilms grown on glass, rubber, porcelain, polypropylene, stainless steel and polycarbonate were 0.04, 0.26, 0.62, 1.00, 2.08 and 2.70 $\mu m^3/\mu m^2$ respectively. Polycarbonate developed statistically more biofilm mass than glass, rubber, porcelain and polypropylene. Viable cell counts data were in agreement with the CLSM-derived data. In conclusion, polycarbonate was the most accommodating surface for *A. baumannii* ATCC17978 to form biofilms while glass was least favorable. Alternatives to polycarbonate for use in medical and dental devices may need to be considered.

Key words: Biofilms, *Acinetobacter baumannii*, medical device, infection control, environment, environmental surfaces

INTRODUCTION

A. baumannii can disseminate and persist in hospital environments, causing nosocomial outbreaks and serious disease in the critically ill (Towner 2009; Chen *et al*. 2015; Weber *et al*. 2015). Many of the infections caused by *A. baumannii* (ranging from urinary tract infections to ventilator-associated pneumonia) are associated with indwelling devices (Manchanda *et al*. 2010;

Patel *et al*. 2014) due to the formation of biofilm on these surfaces. Biofilms of *A. baumannii* are found on the surfaces of many types of medical devices including urinary catheters, central lines, surgical drains, ventilation equipment, dental water lines, and cleaning equipment as well as on a variety of other surfaces in the hospital environment (Donlan and Costerton, 2002; Cohen *et al*. 2014; Patel *et al*. 2014).

Biofilms are a dynamic, heterogeneous community of microorganisms within a complex matrix of extrapolymeric substance that have integrated metabolic activities and produce sessile phenotypes markedly different from their planktonic counterparts (Sutherland 2001; Stoodley *et al.* 2002); (Hall-Stoodley and Stoodley 2005). A critical step for biofilm formation is for the pathogen to adhere to a surface. Cell-surface associated structures on the surface of *A. baumannii* can enhance attachment via pili, encoded by the *csu*A/BABCDE chaperone-usher pilus assembly operon (Tomaras *et al*. 2003), and there is evidence to suggest that the $bla_{\text{PER-1}}$ gene also enhances substrate adhesion (Lee *et al*. 2008). In terms of surface chemistry, the physio-chemical properties of inanimate surfaces also play a key role in cell adhesion and biofilm development. Electrostatic forces, Lifshitz-van der Waals forces, and hydrophobic/hydrophilic forces positively or negatively influence microbial adhesion to a surface (Bos *et al*. 1999). Increased surface roughness can increase the hydrophobicity of the surface by effecting the surface contact angle (Patankar 2004). For example, *Staphylococcus epidermidis* has greater adhesion to hydrophobic surfaces compared to hydrophilic surfaces (Cerca *et al*. 2005).

A variety of material types are used in medical equipment and in the hospital setting. Polycarbonate, a durable, low-cost plastic that can undergo autoclave sterilization is found in a variety of medical devices including urinary catheters, gastrointestinal tubes, and cardiopulmonary bypass circuits, blood oxygenators and flood filters used in the bypass circuit (Duty *et al*. 2013). Mesh prosthetics are often composed of polypropylene (Byrd *et al*. 2011) and porcelain is commonly used in many implants and dental crowns (Schroder *et al*. 2011; Ren and Zhang 2014). Stainless steel makes up the majority of surgical equipment and rubber has a number of uses, particularly rubber seals, such as that used in disposable plastic syringes (Hamilton 1987). Cells of *A. baumannii* can persist on most of these inanimate surfaces (Wendt *et al*. 1997) but studies comparing *A. baumannii* biofilms across various surface types is lacking.

A. baumannii biofilms have been demonstrated on a limited number of substrata such as glass (Vidal *et al*. 1996) and plastic surfaces (Tomaras *et al*. 2003). Thus, the aim of this study was to compare the ability of *A. baumannii* to form biofilm on six different material types: glass, porcelain, stainless steel, rubber, polycarbonate plastic and polypropylene plastic. Understanding the propensity for biofilm formation on various surfaces provides critical information to different parties for selecting low biofilm materials, which is essential for minimizing the risk of biofilm-associated infections.

RESULTS AND DISCUSSION

Biofilm formation by *A. baumannii* ATCC17978 varies across substrata

The material substratum is an essential factor that contributes to the ability of a pathogen to adhere to and form biofilm on a surface (Brandao *et al*. 2015; Fernandez-Delgado *et al*. 2015);. Aside from cellular properties and pathogen adhesion mechanisms, variations in surface roughness, hydrophobicity and chemical structure can impede or promote a pathogens ability to attach and populate on that surface. To evaluate if variations between these surface types influenced the development of biofilms, the biofilms of *A. baumannii* ATCC 17978 were developed on disc coupons of glass, rubber, porcelain, polypropylene, stainless steel and polycarbonate in a CDC reactor for 4 days and the mean biomass values for biofilms grown on each surface type was determined using fluorescent staining and imaging by confocal laser scanning microscope (Figure 1). We did not anticipate that the rubber surface would absorb the stain, which made it difficult to distinguish the biomass from the background. Therefore, the biomass and live/dead ratio data obtained for rubber using microscopy is presented for reference only and the viable cell count data (which does not rely on microscopy) should be relied upon to estimate the biofilm biomass on rubber. We report that *A. baumannii* ATCC17978 can readily form biofilms on polycarbonate. Polycarbonate, a hydrophobic type of plastic, developed statistically more biofilm mass than glass, rubber, porcelain and polypropylene. We confirmed these biomass results by estimating the mean CFU cm^{-2} for the biofilms grown on each of the surfaces using a serial dilution method that is independent of CLSM. The mean viable cells on each surface type is presented in Figure 2 and corroborate the mean biomass values determined using the confocal microscope. The biofilms growing on polycarbonate had a statistically significantly higher CFU cm^{-2} compared to all other surface types. Our finding of high biofilm

formation on polycarbonate is consistent with the finding of Brandao *et al*. who demonstrated that polycarbonate composite orthodontic brackets sustained the highest level of bacterial adhesion in the buccal cavity compared to metal and ceramic brackets (Brandao *et al*. 2015).

In contrast to polycarbonate, *A. baumannii* cells did not adhere to glass. On glass, which is a hydrophilic surface, *A. baumannii* weakly formed small, flat aggregates of biofilm. We found no statistically significant difference in biofilm mass on glass compared to porcelain and polypropylene, although higher biofilm mass was formed on these surfaces, which could also be visually seen (Figure 3). This is consistent with several other studies showing that biofilm formation by *A. baumannii* was less favorable on glass compared to plastic such as polystyrene, polypropylene and Teflon plastics (Tomaras *et al*. 2003; McQueary and Actis 2011) as well as polycarbonate (Pour *et al*. 2011). Surface roughness (Ra) measurements for glass, stainless steel and polycarbonate (only) were available from the supplier (BioSurface Technologies Corp., Bozeman, MT), which were 0.425, 20.20 and 50.95 µin, respectively. Recall that the mean biomass for these three surfaces was 0.043, 2.08 and 2.70 respectively. The increasing surface roughness and mean biomass, from glass to polycarbonate, suggests a positive trend between increased biofilm formation and rougher surfaces, although not statistically significant (Pearson correlation p value = 0.27).

We performed biofilm imaging using the CLSM for each material type and select images are shown in Figure 4. Differences in the formation of biofilm can be visually seen. Biofilms grown on polypropylene and porcelain displayed a flat architecture. Polycarbonate best supported biofilm growth followed by stainless steel, as evidenced by the formation of mushroom structures on these two surfaces (Figure 4). Stainless steel had statistically significantly more biofilm mass compared to porcelain and glass. We used a brushed stainless steel, which has a striated surface structure. While the high surface energy of stainless results in a more hydrophilic surface (Fernandez-Delgado *et al*. 2015), the roughness of the surface increases surface hydrophobicity (Patankar 2004), which may contribute to the increased adhesiveness of cells. The surface groves also increase the surface area and enhance microbial colonization. This may also account for the high live/dead ratio seen for stainless steel (Figure 3) as cells adhere within the grooves, forming a strong base onto which live cells attach and subsist (Figure 4). A qualitative comparison of microscan images with studies by Nan *et al.* who compared the

biofilms of *Staphylococcus aureus* on stainless steel with copper treated stainless steel (Nan *et al*. 2015) and by Fernandez-Delgado *et al*. who evaluated the biofilms of *P. mirabilis* on stainless steel (Fernandez-Delgado *et al*. 2015) reveals similarity in biofilm development with regard to this metal.

Study limitations

We evaluated the biofilm forming ability of a single, clonal species of *A. baumannii*, which makes it difficult to generalize our results to other microorganisms. Additional studies using diverse species are needed. Different strains/isolates may have different abilities to form biofilms on the materials we tested and this will be the subject of future studies to determine if our conclusions can be generalized to other *A. baumannii* strains/isolates. In addition, biofilms are known to exist as mixed species in nature and mixtures of colonizing species will influence bacterial attachment and the formation of biofilms (McEldowney and Fletcher 1987). Therefore, the level of biofilm we observed may be over or underestimated from what might occur in the natural environment. In addition to these considerations, this study focused on growing biofilms under dynamic (versus static) conditions. Dynamic conditions result in less biofilm formation when compared to static conditions (Tomaras *et al*. 2003). Therefore, our measures of biofilm mass do not represent biofilm that would form in the open environment lacking shearing stress. Of note, the hydrophobicity parameters of each substratum were not determined prior to use in this study, so we cannot definitively correlate differences in biofilm development on the basis of surface hydrophobicity.

Summary

We have demonstrated that there are differences in biofilm formation by *A. baumannii* ATCC17978 across different substrata. Specifically, we found that the formation of biofilm by *A. baumannii* ATCC17978 readily developed on polycarbonate followed by stainless steel. Glass was least favorable for biofilm formation. The differences in biofilm formation across different material types may be due to variations in surface roughness and porosity, ionic charge, and hydrophobicity and the extent to which the material surface influences attachment and biofilm formation warrant further investigation. Understanding these differences at the molecular level will deepen our understanding of how microorganisms are able to colonize and persist on medical devices, which is important for the development of new materials that will

inhibit microbial attachment and reduce biofilm related infections. In this regard, research on polycarbonate alternatives or on how polycarbonate used in the manufacture of invasive devices could be treated/modified to inhibit microbial attachment and biofilm formation is warranted.

MATERIALS AND METHODS

Bacterial strain and culture conditions: *Acinetobacter baumannii* ATCC 17978 (American Type Culture Collection, Manassas, VA) was used for all biofilm tests. A single colony on Mueller Hinton II (MHII) agar plate was sub-cultured into MHII broth (Becton, Dickinson and Co., Sparks, MD) and incubated for 15-18h at 37ºC, which was then used to create the inoculum for the biofilm development.

Preparation of material coupons: All material coupons were round discs of one cm in diameter and approximately 3 mm thick. The following non-porous material coupons were used to grow *A. baumannii* biofilms: medical grade stainless steel (RD128-304), AHW BUNA-N Rubber (RD128-BUNA), porcelain (RD128-PL), polycarbonate plastic (RD128-PC), polypropylene plastic (RD128-PP) and borosilicate glass (RD128-GL) (all material coupons from BioSurface Technologies, MO). Before use, all material coupons were washed with soap and water, followed by a 70% ethanol bath, and then autoclaved for sterilization.

Biofilm development: A CDC biofilm reactor (Biosurface Technologies, Bozeman, MT) was used for the biofilm growth. The CDC biofilm reactor and its coupon holders were autoclaved before use. Material coupons (3 of each material type) were mounted on the coupon holders and the reactor was supplemented with 10% LB medium by a peristaltic pump with a continuous flow rate of 100 mL per h. Overnight cultures of *A. baumannii* ATCC 17978 (grown under shaking conditions at 37°C) were diluted by 1:100 for an initial concentration of approximately $4x10^8$ CFU and inoculated into the glass vessel of the CDC reactor aseptically for a final concentration of approximately $1x10^6$ CFU/mL. The liquid growth medium was circulated through the vessel and a magnetic stir bar rotated by a magnetic stir plate generated a shear force. The CDC biofilm reactor was placed on bench and biofilms were grown at room temperature to mimic a natural environment. After four days of growth, the coupons were aseptically removed

for biofilm imaging and viable bacteria plate counting. Three duplicate CDC biofilm chamber experiments were performed.

Bacterial count determination: Biofilms on the coupons were recovered by homogenizing the coupon in 3 mL of 1× phosphate buffered saline (PBS, 10 mM, pH7.2) solution for 1 min using Omni-Tip™ disposable probes (OMNI International, Kennesaw, GA). Samples were serially diluted, 50 µl of each dilution were plated onto an MHII agar plate and incubated overnight at 37^{o}C for colony enumeration and the mean colony forming units (CFU) per cm^{2} was calculated.

Microscope Analysis: Coupons were used for fluorescent staining and imaging by confocal laser scanning microscope (CLSM). Coupon with adhered biofilm was stained with LIVE/DEAD BacLight Bacterial Viability kit (L7012, Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Fluorescent images were acquired with an inverted CLSM (Olympus 1X71, Center Valley, PA) equipped with a Fluorescence Illumination System (X-Cite 120, EXFO) and filters for SYTO-9 (excitation = 488 nm/emission = 520 nm) and propidium iodide (excitation = 535 nm/emission = 617 nm). Images were obtained using an oil immersion 60× objective lens and for each location, images were scanned at 1µm intervals. After acquiring images, a 3-D image was re-constructed by using IMARIS 7.3.1 software. Five different surface areas of each material coupon were randomly chosen for imaging in order to better represent biofilms. Biofilm biomass was calculated based on microscopic images using Comstat 2 (Heydorn *et al*. 2000; Vorregaard 2008). The surface of the rubber absorbed the live/dead stain making it difficult to differentiate the biomass from the background. Therefore, data on the biomass and live/dead ratio obtained for rubber using microscopy was presented for reference only and the viable cell count data (which does not rely on microscopy) is reliable to determine biofilm biomass developed on the rubber.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism 6 for Windows (Version 6.01, Graph Pad Software, Inc., La Jolla, CA). Statistical significance was assessed using one-way ANOVA with multiple comparisons using t-test and a significance level of 0.05.

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Potential conflicts of interest

All authors report no conflicts of interest.

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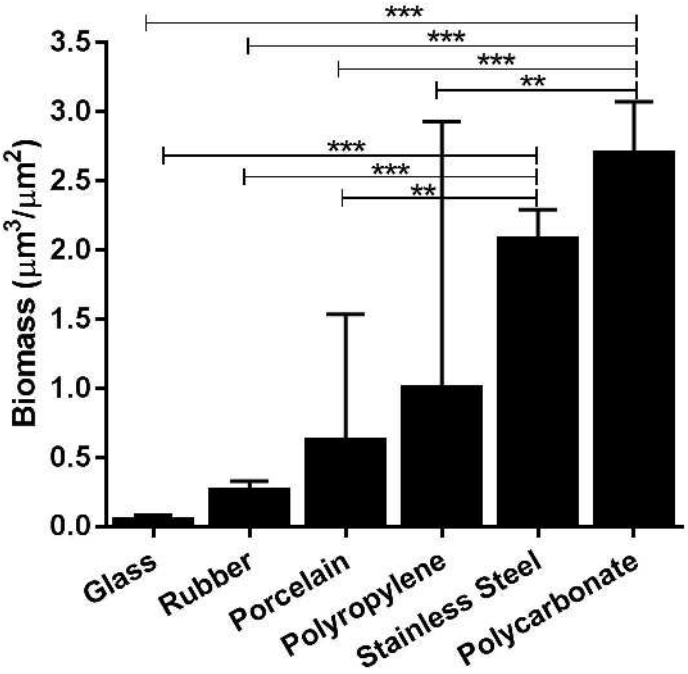

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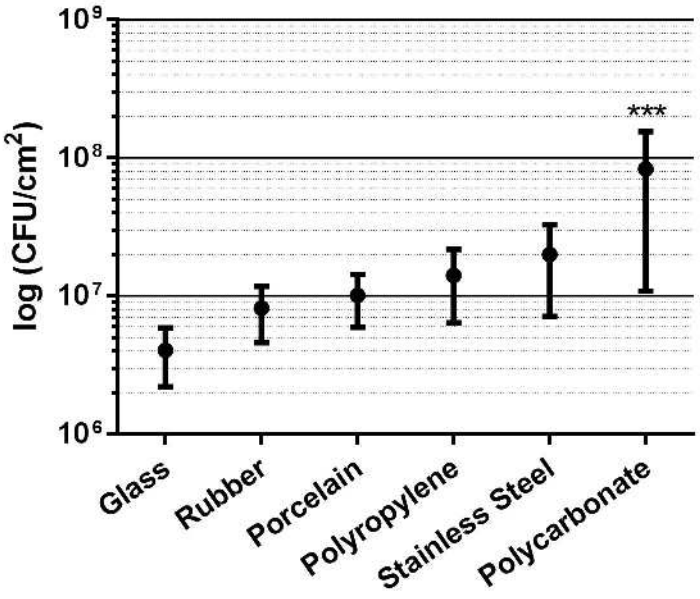

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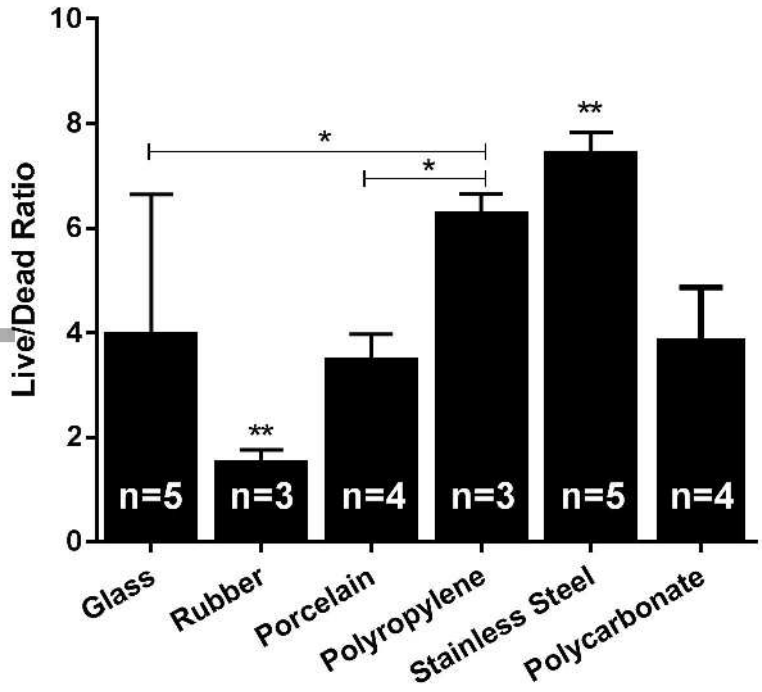

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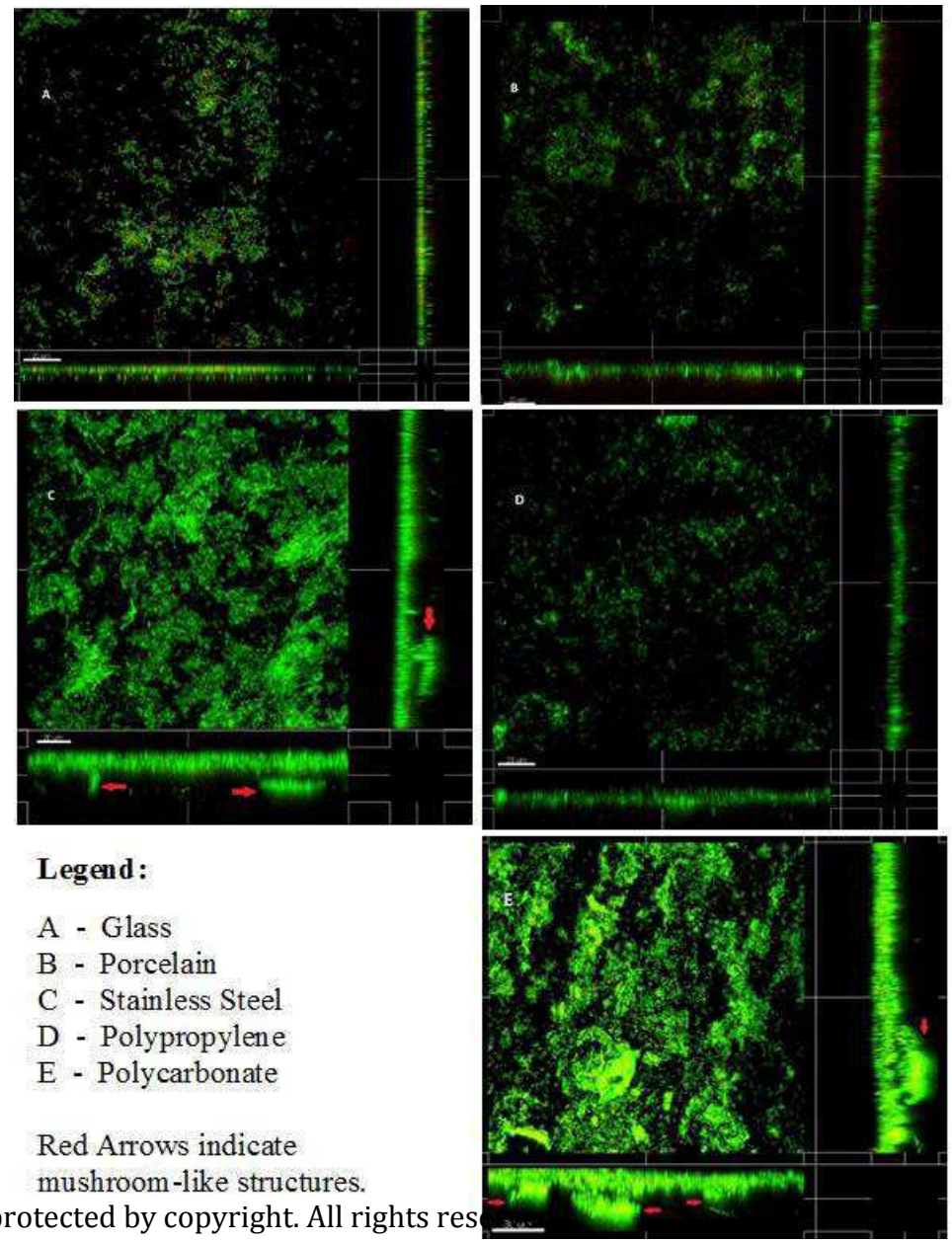

Legend:

A - Glass
B - Porcelain
C - Stainless Steel
D - Polypropylene
E - Polycarbonate

Red Arrows indicate mushroom-like structures.