Artificial dry surface biofilm (DSB) models for testing the efficacy of cleaning and disinfection

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Significance and impact

The widespread presence of biofilms on dry surfaces in healthcare settings has been recently documented. These dry surface biofilms (DSB) present an unprecedented challenge to cleaning and disinfection processes. Here we describe a practical efficacy protocol based on an in vitro *Staphylococcus aureus* DSB model. The protocol measures reduction in viability, transferability and biofilm regrowth post treatment to provide altogether a practical assessment of product efficacy against dry surface biofilms.

Abstract

Dry surface biofilms (DSB) harbouring pathogens are widespread in healthcare settings, difficult to detect and resistant to cleaning and disinfection interventions. Here, we describe a practical test protocol to palliate the lack of standard efficacy test methods for DSB.

Staphylococcus aureus DSB were produced over a 12-day period, grown with or without the presence of organic matter, and their composition and viability were evaluated. Disinfectant treatment was conducted with a modified ASTM2967-15 test and reduction in viability, transferability, and biofilm regrowth post treatment were measured. Dry surface biofilms produced over a 12-day period had a similar carbohydrates, proteins and DNA content, regardless the presence or absence of organic matter. The combination of sodium hypochlorite (1,000 ppm) and a microfiber cloth was only effective against DSB in the absence of organic load. With the increasing concerns of the uncontrolled presence of DSB in healthcare settings, the development of effective interventions is paramount. We propose that our DSB model in the presence of organic load is appropriate for the testing of biocidal products, while the use of three parameters, $\log_{10}$ reduction, transferability and regrowth, provides an accurate and practical measurement of product efficacy.

keywords: dry surface biofilm, cleaning, disinfection, efficacy test, test protocol, sodium hypochlorite

Introduction

Biofilms are microbial communities embedded in self-secreted extracellular polymeric substances (EPS). Biofilms are significantly more tolerant to antimicrobials when compared to their planktonic equivalents (Akinbobola et al., 2017). The vast majority of studies on biofilm resistance to disinfection concerns hydrated biofilms formed and consistently grown in liquid environments or in the presence of high level of moisture (Francolini and Donell, 2010; Bridier et al., 2011; Otter et al., 2015). Not much attention has been paid to 'dry' biofilms colonising surfaces with the presence of desiccated microorganisms, limited moisture and nutrient resource (Vickery et al., 2012; Almatroudi et al., 2015) despite their widespread presence on healthcare surfaces (Vickery et al., 2012; Hu et al., 2015; Ledwoch et al., 2018).

There is no standardized efficacy test against biofilm published by the European Norm. In the US, disinfectant efficacy tests against biofilms concern the treatment of hydrated biofilms (US Environmental Protection Agency, 2013). Among common test protocols the Calgary device enables the measurement of the minimal biofilm eradication concentration (MBEC) which corresponds to the lowest concentration of a biocide or biocidal formulation that kill a mono species bacterial (hydrated) biofilm (Ceri et al., 1999; Ali et al., 2006; Azaredo et al., 2017); The CDC reactor model and drip flow reactor model have also been used successfully to measure the efficacy of antimicrobials against hydrated biofilms. (Schwartz et al., 2010, Almatroudi et al., 2015). Other non-standardised biofilm efficacy methods have also been described (Pierce et al., 2008; Millhouse et al., 2014; Sherry et al., 2016). There are, however, no standard protocols to measure the efficacy of biocidal formulations against dry surface biofilms (DSB).

This paper proposes such a test and establishes test parameters to ensure the appropriate control of DSB in practice following cleaning/disinfection interventions.

Results and discussion

Dry surface biofilms are widespread on various surfaces in healthcare facilities (Vickery et al., 2012; Almatroudi et al., 2015), although their impact on healthcare associated infections has not been yet established. *Bacillus* spp. and *Staphylococcus aureus* were recently identified as the species most commonly associated with DSB formed on hospital surfaces (Ledwoch et al., 2018). Furthermore, Vickery and colleagues (2012*)* showed that DSB can persist on surfaces despite effective cleaning. In their study, samples isolated from ICU unit harboured pathogens including multidrug resistant microorganisms following terminal cleaning with neutral detergent followed by chlorine 500 ppm disinfection. Equipment and furnishing retrieved from hospital were also positive for the presence of VRE and MRSA (Vickery et al., 2012).

Here, we report the development of artificial mono-species DSB grown in the presence of organic load or not, for biocidal product testing. To date there are no such tests reported in the literature and limited existing protocols refer to testing against hydrated biofilms. The number of bacteria recovered from CL-DSB dry-biofilm or OL-DSB was the same after the formation of DSB (Fig. 1). There was no statistically significant difference (Two-way ANOVA, $p=0.08821$) in viable count of bacteria ($\log_{10}$ CFU/ml = 7.60 ± 0.60) recovered from each disc between 20 environmental DSB replicates. The average bacterial concentration in CL- and OL-DSB was 7.38 ± 0.58 and 7.89 ± 0.60 $\log_{10}$ CFU ml^{-1}, respectively. *S. aureus* DSB composition consisted mainly of proteins (96 ± 1%) with some carbohydrates (4 ± 1%), with overall little DNA (Fig. 2). No lipids were detected by the colorimetric sulfo-phospho-vanillin method suggesting lipid level was below the detection limit of 35 µg ml^{-1} (Anschau et al., 2017). The amount of proteins and carbohydrates remains constant throughout the dry surface biofilm cycles with 95-97% of proteins and 3-5% carbohydrates in both CL and OL- DSB. Surprisingly the addition of BSA during the OL-DSB production did not impact on the composition of the biofilm (Fig. 2). Indeed, there was no statistical difference ($p=0.5317$) between the

carbohydrate and proteins ratio of CL- and OL-DSB. More DNA (ANOVA; $p<0.05$) was extracted from DSB grown in the presence of organic load (21 ± 7 ng/disc) compared to without BSA (17 ± 9 ng/disc). Hydrated *S. aureus* biofilms are also mostly composed of proteins, although carbohydrate concentration is higher. Abdallah et al. (2014) reported *S. aureus* 24h and 48h hydrated biofilms formed on stainless steel coupons were composed of 70% and 78% proteins and 30% and 22% of carbohydrates, respectively.

The composition, appearance and viability of our dry surface biofilms were homogeneous (Fig. 3) with 8.0 ± 0.6 $\log_{10}$ CFU cm^{-2} after the 12 days process. Abdallah et al. (2014) reported similar findings with hydrated *S. aureus* biofilms following 24h and 48 h incubation, 8.4 ± 0.2 and 8.2 ± 0.2 $\log_{10}$ CFU cm^{-2}, respectively. After the first dry phase, the majority of bacteria were viable (90% and 98% of all bacteria in CL- and OL-DSB, respectively) (Fig. 3). However, in the course of growth and after sequential dry phases, the number of dead bacteria increased (Fig. 3). At the end of dry surface biofilm formation cycle, 42% and 75% of bacteria were viable in CL- and OL-DSB, respectively. SEM of 12-day DSB showed homogenous cluster of bacteria embedded in a matrix and separated by channels. OL-DSB appeared to contain more matrix (Fig. 4). Although uniformity is a positive attribute for reproducibility, these biofilms differ somewhat to DSB isolated from healthcare surfaces. Indeed, environmental DSB form clusters of unevenly scattered bacteria through the colonised surface, thus making disinfectant testing using in situ dry surface biofilms inappropriate (Ledwoch et al., 2018).

Considering potential product usage in practice (Sattar and Maillard, 2013), it seemed appropriate to combine the use of the wiperator (ASTM26987-15, 2015) with our DSB. The ASTM2697-15 (2015) was preferred to the EN1665-15 (2015) protocol, as it was recently shown to be a more stringent protocol (Wesgate et al., 2018). To provide a sensible and useful measurement of product efficacy against DSB, we decided to measure several criteria indicative of product efficacy: i) reduction in viability as a result of bactericidal activity or/and removal of bacteria from the surface, ii) transferability of bacteria post-wiping and iii) DSB regrowth indicating the frequency of product application needed to render the surface safe.

Using such an approach we first identified that DSB produced in the presence of OL, were more difficult to control than those produced in the absence of organic load, despite that NaOCl (1,000 ppm) in combination with the microfiber cloth decreased *S. aureus* number in DSB by >4 $\log_{10}$ (Table 1). More bacteria within DSB could be removed/killed when the biofilm was grown in the absence of organic load. Almost 6 $\log_{10}$ reduction was achieved which could be compared to results showed by Almatroudi *et al.* (2016) where more than 7 $\log_{10}$ of bacteria in DSB were removed/killed by treatment with 1,000 ppm NaOCl. Although, NaOCl treatment significantly lowered (Two-way ANOVA; $p<0.05$) the transfer of bacteria from dry surface biofilms compared to the absence of treatment, it was significantly less effective when DSB were formed in the presence of organic load (Table 1). Likewise, time for regrowth post NaOCl exposure was much shorter in the presence of organic load (Table 1). Hence, measuring additional parameters to the traditional viability one, provide additional stringency. Transferability post-treatment is particularly important to consider notably in relation to hand hygiene compliance. Indeed, DSB have been shown to be widespread in healthcare settings (Hu et al., 2015; Ledwoch et al., 2018), acting as a potential transmission reservoir. As median hand hygiene rate from 96 empirical studies is only 40% (Erasmus et al., 2010), the risk of transmitting pathogens from DSBs is high. It is thus conceivable that, despite the reduction in viability following, here, exposure to NaOCl (1,000 ppm), bacteria embedded in a dry surface biofilm can still be easily transferred. Chowdhury *et al*. (2018) also reported on the persistent nature of DSB; in their study, treatment with neutral detergent had a little effect on bacterial transferability from DSB. The regrowth parameter, although linked somewhat to a reduction of viability, provides information on how long the surface would be biofilm-free post treatment. We are proposing that the dry surface biofilm model formed and grown in the presence of organic load, as well as the parameters investigated, are suitable to measure the efficacy of cleaning and/or disinfectant treatments.

Materials and methods

Bacterial growth and maintenance

Staphylococcus aureus NCTC107888 was propagated in tryptone soya broth (TSB; Oxoid Limited, Hampshire, UK) at 37°C overnight and washed in tryptone sodium chloride following centrifugation at 1,400 xg. Tryptone sodium chloride was prepared by mixing 1 g of tryptone (Oxoid Limited, Hampshire, UK) and 8.5 g of sodium chloride (Sigma- Aldrich®, Dorset, UK) in 1 l of distilled water followed by autoclaving. The bacterial suspension was adjusted to 1 x 10^6 cfu ml^{-1}.

Dry biofilm models

The bacterial growth approach in our DSB model is based on alternating hydrated (growth) phases with desiccation phases as described by Almatroudi et al. (2015). In our model we utilised a sedimentation protocol to form and grow DSB, as described below. Bacteria were initially cultured in normal hydrated conditions to allow initial adherence and biofilm formation. This was followed by cycles of dry and hydrated phases for a total duration of 12 days (Fig. 1).

Stainless steel discs AISI 430 (0.7 ± 0.07 mm thickness; 10 ± 0.5 mm diameter, Goodfellow Cambridge Limited, Huntington, UK) were used as a support. Sterile discs were placed in Corning™ Costar™ flat bottom cell culture plates (Fisher Scientific, Loughborough, UK), and each well was inoculated with 1 ml of TSB containing 5% anhydrous D-glucose (Fisher Scientific, Loughborough, UK) with 10^6 CFU ml^{-1} washed S. *aureus* suspension. Bacteria were first allowed to attach and form a biofilm on the disc surface over 2 days period at 25°C under gentle agitation using an Orbit P4 plate rocker (Labnet International, Edison, USA); i.e. the hydrated phase. The solution was then drained from the wells and plates were incubated at 37°C for 48 h. Following this dry phase, 1 ml of TSB was added into each well containing

182 stainless steel disc and a new ‘hydrated phase’ began for 48 h. Hydrated and dry phases
183 alternate every 48 h for a period of 12 days, ending with biofilm in a dry phase (Fig. 1).
184 Two models of *S. aureus* dry surface biofilms were developed: i) a clean (CL) DSB grown in
185 TSB only, and ii) an organic load (OL) DSB grown in 3 gl^{-1} bovine serum albumin (BSA;
186 Sigma® Life Science, Dorset, UK). BSA was added to each wet phase during the 12 days
187 period.
188
189 *Biofilm composition*
190 Carbohydrate: Carbohydrate content in DSB models was measured by dinitrosalicylic (DNS;
191 Fisher Scientific Ltd, Loughborough, UK) colorimetric assay (Miller, 1959). Briefly, disc
192 containing biofilm was placed in McCartney bottle with 1 g of glass beads and 3 ml of sterile
193 water. The disc was vortexed with Fisherbrand® vortex shaker (Fisher Scientific,
194 Loughborough, UK) for 10 min prior to the analysis. Two gl^{-1} of phenol (Fisher Scientific Ltd,
195 Loughborough, UK) was added to DNS reagent to intensify the colour density and increase
196 the sensitivity of the method. Three ml of DNS reagent was added directly to vortexed culture
197 or directly to an overnight planktonic suspension of *S. aureus* and covered with lid to prevent
198 liquid evaporation. The mixture was heated for 15 min at 90°C in Fisherbrand water bath
199 (Fisher Scientific, Loughborough, UK) until the colour developed. Colour was stabilised by
200 adding 1 ml of 40% potassium sodium tartrate solution (Fisher Scientific Ltd, Loughborough,
201 UK). Absorbance at 575 nm was read after the mixture cooled down to the room temperature
202 and compared against the standard curve to evaluate carbohydrates content.
203
204 *Protein analysis:* Discs with *S. aureus* DSB were placed in sterile 30 ml capacity flat bottom
205 glass bottles containing 1g of glass beads and 2 ml of sterile water and vortexed for 10 min.
206 The suspensions were then centrifuged in Biofuge Primo R centrifuge (Heraeus, Thermo
207 Fisher Scientific, Newport, UK) at 1,400 g and 20°C for 10 min. The supernatant was then
208 discarded, and the remaining bacterial pellets were weighted. Proteins were extracted using

the Total Protein Extraction Kit (Chemicon®, Millipore Limited, Watford, UK). Briefly, 2.5 ml TM buffer was added to 1 g of the pellet and put on ice for 5 min. Bacterial cells were homogenised three times by vortexing for 20 sec and incubation on dry ice for 15 sec. The mixture was then centrifuged at 11,000 g at 4°C for 20 min using Avanti™ J-20 XP centrifuge (Beckman Coulter, High Wycombe, UK). Collected supernatant was quantified for proteins content. Quantification was carried out with Folin–Ciocalteu reagent (Lowry et al., 1951) by using Pierce™ Modified Lowry Protein Assay (Thermo Scientific™, Loughborough, UK). One ml of Modified Lowry Reagent was added to each tube containing 0.2 ml of supernatant. The mixture was well homogenised with vortex shaker (Fisherbrand®, Fisher Scientific Ltd, Loughborough, UK) and incubated at room temperature for 10 min. One hundred µl of 1X Folin-Ciocalteu Reagent was then added and the sample vortexed for 5 seconds. Sample was covered and incubated for 30 min at room temperature. The absorbance at 750nm was measured and the amount of the proteins in the sample was evaluated by using standard curve prepared according to manufacturer instructions using diluted albumin (BSA) standards.

Lipid analysis: Lipids were extracted with the chloroform-free Lipid Extraction Kit (Abcam®, Cambridge, UK). Discs with *S. aureus* DSB were vortexed for 10 min in Mccartney bottles containing 1 gr of glass beads and 2 ml of sterile water. The suspensions were then centrifuged at 1,000 g at 20°C for 5 min. Supernatant was discarded, and the pellets were washed and resuspended in 25 µl of phosphate buffer saline (PBS; 8 g of sodium chloride (Sigma- Aldrich®, Dorset, UK), 0.2 g of potassium chloride (Fisher BioReagents®, Fisher Scientific Ltd, Loughborough, UK), 1.44 g of sodium phosphate dibasic heptahydrate (Thermo Fisher Scientific, Newport, UK) and 0.24 g of potassium phosphate monobasic (Thermo Fisher Scientific, Newport, UK) in up to 1 l water). pH was adjusted to 7.4. Five hundred µl of Abcam extraction buffer containing 60% hexane and 40% isopropanol (Abcam®, Cambridge, UK) was added to the samples which were vortexed for 2 min. The mixture was agitated on Orbit P4 plate rocker (Labnet International, Edison, USA) at room temperature for 20 min. The tubes

were centrifuged for 5 min at 10,000 g and the supernatant was collected and weighted. The tube with supernatant was dried overnight in Thermo Heraeus Herasafe™ safety cabinet (Thermo Fisher Scientific, Newport, UK) at 37°C. The analysis of lipids was carried out following the colorimetric sulfo-phospho-vanillin (SPV; Fisher Scientific Ltd, Loughborough, UK) method (Cheng et al., 2011). One ml of chloroform: methanol solvent was added per 15 mg of sample followed by 100 µl of sulfuric acid (Fisher Scientific Ltd, Loughborough, UK). Samples were heated at 90°C for 10 min on a stirring hotplate (Fisher Scientific Ltd, Loughborough, UK) and then placed on ice to cool them down to room temperature. One hundred µl vanillin- phosphoric acid reagent was added to the sample to develop the colour. Absorbance at 540nm was measured after 5 min to determine the lipid content.

DNA analysis: Discs with DSB were placed in 30 ml capacity flat bottom glass bottle with 1 g glass beads and 0.5 ml TSB and vortexed for 10 minutes to remove the biofilm from disc surface. 0.5 ml of 4 mol l^{-1} guanidine isothiocyanate (UltraPure™, ThermoFisher Scientific, Newport, UK) was added to the sample and further vortexed for 1 min. One ml of mixture was transferred to a 2 m l tube with cap and Fisherbrand® O-ring (Fisher Scientific, Loughborough, UK) with 1 g of 0.1 mm diameter zirconia/silica beads (Thistle Scientific, Glasgow, UK) and homogenised in bead bug (Benchmark Scientific, Cole-Parmer®, St Neots, UK) at 2,800 rpm. DNA amplification was carried out with Maxwell® 16 Instrument (Promega, Southampton, UK). The amount of extracted DNA was quantified with Quibit® 3.0 fluorometer (ThermoFisher Scientific, Newport, UK).

Scanning Electron Microscopy (SEM) imaging

S. aureus DSB samples were prepared by overnight incubation of discs in 2.5% glutaraldehyde solution (Fisher Scientific, Loughborough, UK) followed by immersion in successive concentrations of 10%, 25%, 50%, 70%, 90% and 100% ethanol (Honeywell, Fisher Scientific Ltd, Loughborough, UK) for 10 min each. Prior to SEM scanning, samples

were coated with 20 nm AuPd coating with sputter coater (SC500, Biorad, UK). Secondary electron images were acquired with a beam energy of 5kV using an in-lens detector on a Sigma HD Field Emission Gun Scanning Electron Microscope (Carl Zeiss Ltd., Cambridge, UK) at 10,000x magnification and 5-7 mm working distance. SEM images were false-coloured to help visualisation and contrast using GNU Image manipulation program (GIMP 2.8) software. Images were not otherwise altered.

Live/dead staining

Staining of dry surface biofilm was carried out with LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Thermo Fisher Scientific, Newport, UK) with Syto 9 and propidium iodide in 1:1 ratio. Prior to staining, each disc was mildly washed with 1 ml sterile water for 5 sec to remove any planktonic or loosely adhered cells. Stained discs were imaged with Zeiss LSM880 Airscan Confocal Microscope (Carl Zeiss Ltd., Cambridge, UK).

LIVE/DEAD cells ratios were evaluated using BioFilmAnalyzer v. 1.0 software with the procedure developed by Bogachev et al. (2018). Prior to analysis, non-homogenous colour distribution of obtained images in the studied colour channels was resolved by preliminary image colour normalization using GNU Image manipulation program (GIMP 2.8).

ASTM E2967-15 test

The effectiveness of sodium hypochlorite (NaOCl 1,000 ppm; Fisher Scientific Ltd, Loughborough, UK) combined with Rubbermaid® HYGEN™ disposable microfiber cloth (Rubbermaid Products, Surrey, UK) allowing 2.5 ml of disinfectant per 1 g of wipe was evaluated against CL- and OL-DSB controls consisted of untreated samples. Disinfection tests were performed according to a modified ASTM E2967 test (2015). The surface of the disc was wiped with the Wiperator (Filtaflex Ltd, Ontario, Canada) from both sides using separate wipes. Dry surface biofilms were wiped for 10 sec under 500g pressure, left at room temperature for 2 min, and then the wiped discs neutralised by placing each disc into 1 ml

290 Dey-Engley (DE) neutralising broth (Neogen® Corporation, Ayr, UK) for 2 min. Inoculated
291 broth was then incubated overnight at ambient temperature. Transfer of viable bacteria from
292 used wipes to clean a sterile disc was not performed.
293 *Log_{10} reduction in bacteria embedded in DSB*: Reduction in bacterial viability (Log_{10} reduction
294 in CFU ml^{-1}) gave the number of bacteria that were removed or and killed following wiping.
295 Following wiping, samples were placed in a solution containing 1 g of glass beads (Fisher
296 Scientific, Loughborough, UK), 2 ml DE neutralising broth and 100 µg ml^{-1} proteinase K (Fisher
297 Bioreagents™, Fisher Scientific, Loughborough, UK) for 1 h at 37°C. After incubation, samples
298 were vortexed for 2 min, serially diluted and 3 x 10 µl drops of each dilution plated onto tryptone
299 soya agar (TSA; Oxoid, Thermo Fisher Scientific, Newport, UK). Log_{10} reduction was
300 calculated as the difference between the number of bacteria recovered from untreated
301 (control) and treated samples.
302
303 *Transferability test following disinfection*: Transfer test was conducted to investigate the
304 transferability of surviving bacteria from the dry surface biofilm following wiping. The test was
305 designed to imitate the touch of a finger onto treated surface. Following wiping and 2 min
306 contact time, discs were pressed 36 separate times with 100 g pressure on the surface of DE
307 agar. Following the transfer test, DE agar was incubated overnight at 37°C. Positive
308 growth/adpression was recorded and transferability calculated as the number of positive
309 contact/number of adpressions.
310
311 *Dry surface biofilm regrowth test following treatment*: Regrowth measures the time needed for
312 the DSB to recover following treatment. Wiped samples were placed in 30 ml capacity flat
313 bottom glass bottle with 2 ml of DE neutralising broth (Acumedia®, Neogen® LabM,

Lancashire, UK). The number of days for the DE broth colour to change from purple to yellow indicative of bacterial growth was recorded.

Statistical analysis

Statistical significance of data sets was evaluated with GraphPad PRISM® (version 7.04) using two-way ANOVA. All measurements, if not stated otherwise, were performed in triplicates. The sample standard deviation was evaluated with Bassel's correction.

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Conflict of interest

K Ledwoch is employed by GAMA Healthcare on a part time basis. P Norville is an employee of GAMA Healthcare and a Honorary Lecturer at Cardiff University

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Table 1. Effectiveness of NaOCl (1,000 ppm) on clean (CL) and organic load (OL)-DSB. Impact of disinfectant on reduction in bacteria, transferability and regrowth.

	NaOCl 1,000 ppm	No treatment
Log_{10} reduction in bacteria (cfu cm^{-2}) ± SD		
CL-DSB	5.83 ± 1.25	-
OL- DSB	4.26 ± 1.26	-
Transferability (%) ± SD		
CL- DSB	1 ± 2	95 ± 8
OL- DSB	68 ± 37	100 ± 0
Regrowth (days)		
CL- DSB	5.4 ± 3.3	1 ± 0
OL- DSB	2.8 ± 0.8	1 ± 0

Figure 1 Scheme of dry surface biofilm formation and growth

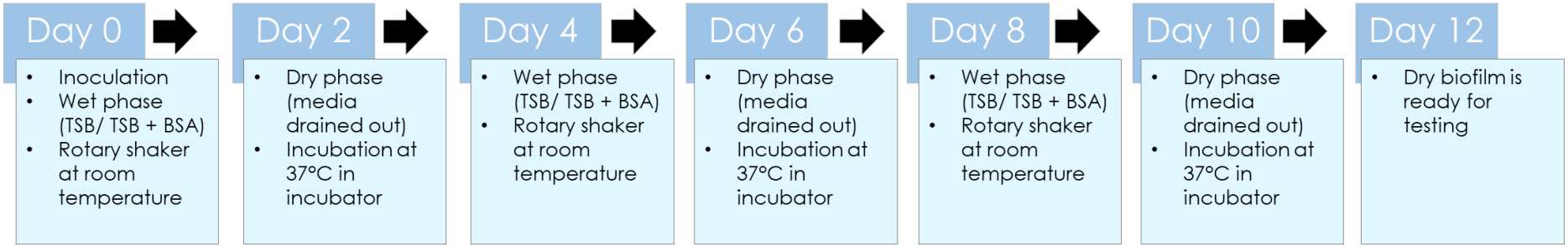

Figure 2. Composition of clean (CL; left) and organic load (OL; right) dry biofilms. DNA: ■, Carbohydrates: ■, Proteins: ■. Time "0" indicates planktonic bacteria. No lipids were recoved with the protocol used in this study.

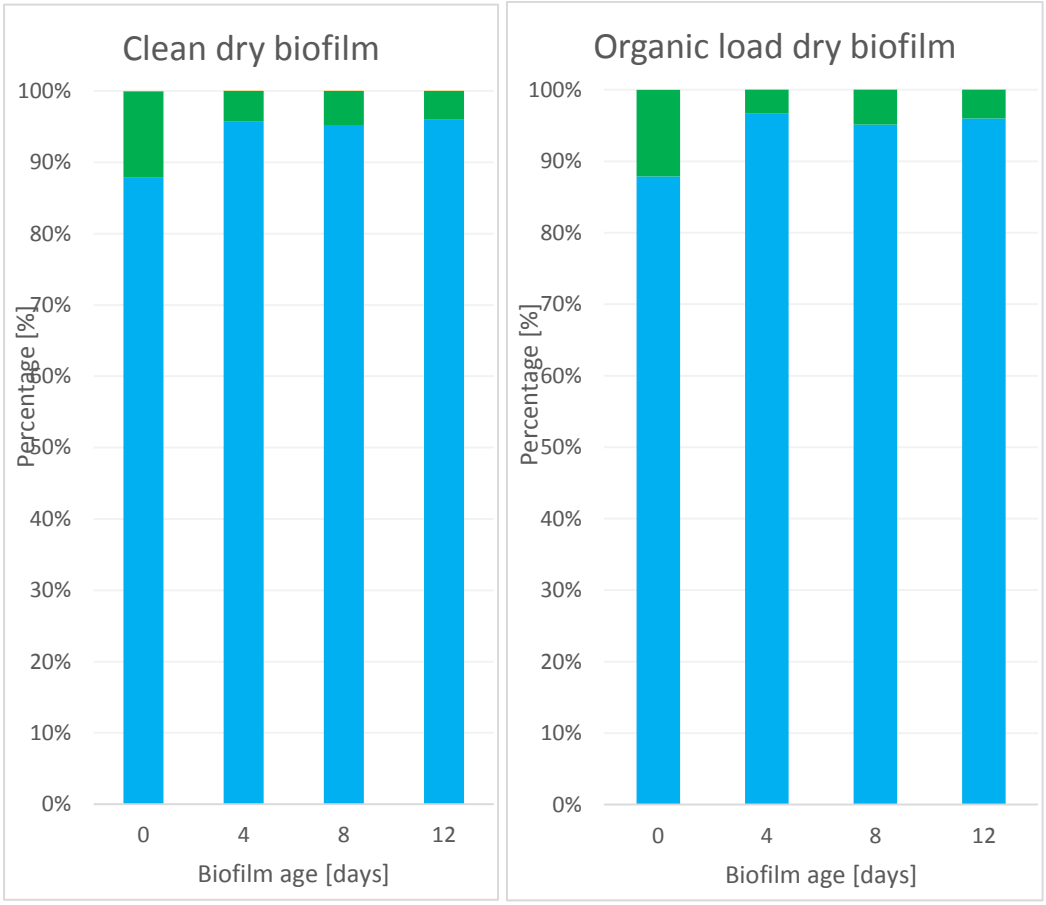

Figure 3. Live (green)/dead(red) fluorescence images of Syto 9/propidium iodide stained clean dry biofilm (CL, top) and Syto 9/propidium iodide stained organic load dry biofilm (OL, bottom) after 4, 8 and 12 days of cultivation. x63 magnification, Zeiss LSM880 Airscan Confocal Microscope. Representative images from 3 fields of 2 samples. CL-DSB after 4 (A), 8 (B) and 12 (C) days; OL-DSB after 4 (D), 8 (E) and 12 (F) days.

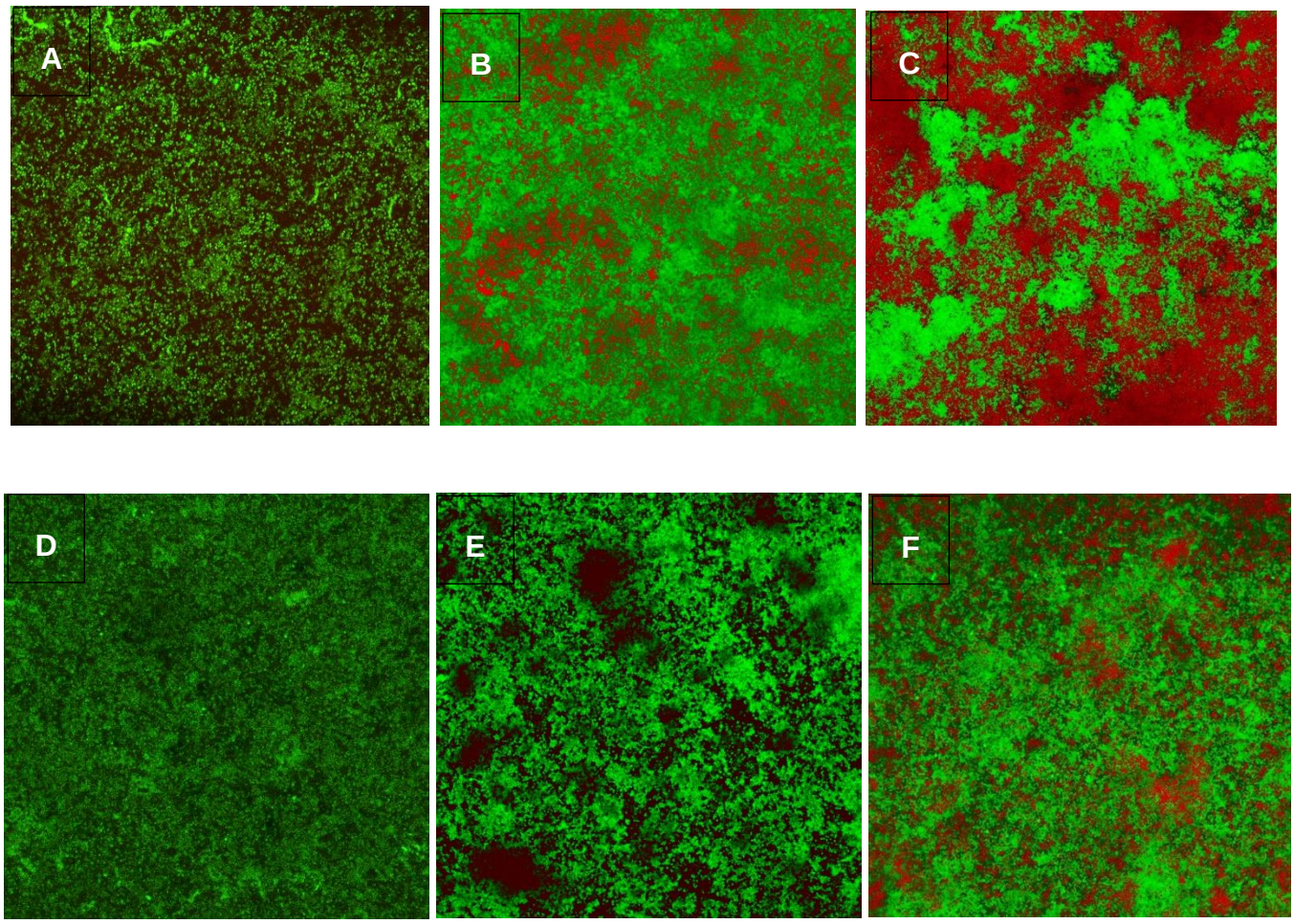

Figure 4 Scanning electron microscope images of clean (CL) dry biofilm and organic load (OL) dry biofilm, x2,000 and x5,000 magnifications. Images presented are representative for the whole disc surface. CL-DSB at x2,000 (A) and x5,000 (B) magnification, OL-DSB at x2,000 (C) and x5,000 (D) magnification.