

# **A cryogel-based bioreactor for water treatment applications**

Dmitriy A. Berillo\*<sup>1</sup>, Jonathan L. Caplin<sup>2</sup>, Andrew B. Cundy<sup>3</sup> and Irina N. Savina<sup>1</sup>

<sup>1</sup> School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, UK.

<sup>2</sup> School of Environment and Technology, University of Brighton, Brighton, UK.

<sup>3</sup> School of Ocean and Earth Science, University of Southampton, Southampton, UK.

\*Corresponding author e-mail: dmitriychemist@gmail.com

## *Abstract*

*The aim of this study was to develop and test a non-diffusion limited, high cell density bioreactor for biodegradation of various phenol derivatives. The bioreactor was obtained using a straightforward one-step preparation method using cryostructuration and direct cross-linking of bacteria into a 3D structured (sponge-like) macroporous cryogel composite material consisting of 11.6% (by mass) cells and 1.2-1.7% polymer, with approximately 87% water (in the material pores). The macroporous cryogel composite material, composed of live bacteria, has pore sizes in the range of 20-150  $\mu\text{m}$  (confirmed by SEM and Laser Scanning Confocal Microscopy). The enzymatic activity of bacteria within the cryogel structure and the effect of freezing on the viability of the cross-linked cells was estimated by MTT assay. Cryogels based on *Pseudomonas mendocina*, *Rhodococcus koreensis* and *Acinetobacter radioresistens* were exploited for the effective bioremediation of phenol and m-cresol, and to a lesser extent 2-chlorophenol and 4-chlorophenol, utilising these phenolic contaminants in water as their only source of carbon. For evaluation of treatment scalability the bioreactors were prepared in plastic "Kaldnes" carriers to improve their mechanical properties and allow application in batch or fluidised bed water treatment modes.*

*Keywords: bacteria immobilisation, bioremediation, phenol, cresol, chlorophenols.*

26

## 27 **Introduction**

28 Bioremediation of contaminated water and soil is an effective approach for the removal of  
29 xenobiotics and other contaminants from the environment. It utilises the ability of  
30 microorganisms to degrade, or reduce the toxicity or mobility of, specific target compounds,  
31 and is considered one of the more efficient, cost effective and eco-friendly remediation  
32 methods. An extensive body of research has focused on utilising this method for a wide range  
33 of contaminants, including pesticides, heavy metals and aromatic hydrocarbons. For water  
34 treatment applications, the use of bacteria immobilised on a substrate has numerous  
35 advantages over the use of bacterial suspensions, such as higher biomass density, high  
36 metabolic activity, and higher resistance to toxic chemicals, allowing continuous operation  
37 processes(Villegas et al., 2016; Chen et al., 2007; Börner et al., 2014; Zaushitsyna et al.,  
38 2014; Gonzalez et al., 2001). Several bacterial immobilisation methods have been developed  
39 and various synthetic and natural carriers are known(Anku et al., 2017; Dzionek et al., 2016;  
40 Chiellini et al., 1999). The degree of bacterial immobilisation depends on the structure, pore  
41 size and surface area of the support, as well as the nature of the material (i.e. its hydrophobi-  
42 city, charge, etc.) and environmental conditions, such as pH, and temperature( Hailei et al.,  
43 2016; Cortez et al., 2017). Adsorption (to the substrate) or biofilm formation is often a long  
44 process however and represents the main cost, and one of the major limitations, of  
45 bioremediation using immobilised bacteria. Immobilisation methods via encapsulation of  
46 cells inside a polymer matrix (e.g. polyvinylalcohol (PVA), carrageenan and agar gel) can  
47 produce relatively high cell densities (Cortez et al., 2017; El-Naas et al., 2009; Sinha et al.,  
48 2011), however such materials tend to have diffusion limitations due to the use of high  
49 concentrations of polymer (6-10%) which coats the cells and restricts their interaction with  
50 contaminated water. Polyethylenimine(PEI) derivatives are a popular immobilising agent

which have restricted application for bacterial immobilisation due to high toxicity issues (Virgen-Ortíz et al., 2017; Milović et al., 2005). In this study, we suggest a novel approach to the design of immobilised bacteria-based bioreactors for water treatment applications. To overcome existing limitations, a minimum concentration of polymers (1.2%) was used for cross-linking an 11.6% wt. suspension of bacterial cells in a 3D macroporous bacterial “sponge”, produced via a facile one-step cryostructuration process. The morphology of the developed macroporous materials, based on *Acinetobacter radioresistens*(*Acn*), *Pseudomonas mendocina*(*Pse*) and *Rhodococcus koreensis*(*Rho*) with six commonly used immobilising polymers (and their combinations – PVA, PEI, chitosan, Gellan Gum and glutaraldehyde), was characterised and the materials tested for their bioremediation efficiency against phenol derivative compounds (phenol, *m*-(*p*-)cresols and CPs).

## **2. Materials and Methods.**

### **2.1 Synthesis of aldehyde containing polymers**

A combination of PVA-al and PEI-al was utilised as a cross-linking agent for bacteria (*Pse*, *Acn* and *Rho*). Polyvinyl alcohol was modified with aldehyde groups (PVA-al). Briefly, 0.6 mL of GA (50% v/v) was added to a 2% acidified PVA solution in distilled water. Note that the PVA modification is inefficient at neutral conditions. The reaction was terminated by pH adjustment to pH 6-7.5. Unreacted GA was removed by dialysis utilising a dialysis bag (cut off 12 kDa) against water. Polyethyleneimine (PEI) was modified in similar manner by adding 8.8 mL of GA (20 v/v %) to 2% polymer solution in water. The pH was adjusted to 7 prior to GA addition to avoid aldol condensation of aldehyde under alkaline conditions, and therefore to achieve a more consistent chemical composition of the polymer. The reaction mixture was incubated under stirring for 2h at room temperature, the pH adjusted to seven, and then the mixture was dialysed against water (a dialysis bag cut off 1 kDa).The obtained polymer

solution was filtered through a 0.45µm membrane and stored at 4 °C until subsequent use. The concentration of the polymer in the solution was estimated by using a freeze drying method and measuring the final dry weight of the polymer.

## 2.2 Characterisation of polymers

FT-IR analysis of freeze dried polymers (PVA-al and PVA and PEI-al) was performed using a Bruker Alpha P instrument with attenuated total reflectance. Spectra were acquired in the 4000–400 cm<sup>-1</sup> range with a resolution of 4 cm<sup>-1</sup> for 24 scans to confirm the presence of aldehyde groups.

<sup>1</sup>H-NMR spectra of solutions of PEI-al and PVA-al (10 mg/mL) and non-modified PEI and PVA dissolved in D<sub>2</sub>O were produced using a Bruker DSX 400 MHz spectrometer.

The molecular weight distribution of the modified polymers (PVA-al and PEI-al) was estimated via photon correlation spectroscopy, using a particle sizer (Zeta sizer 3000 HAS, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Analysis was performed at 25°C using a measurement angle of 90°, with 11 runs for each measurement (n=3). Zeta potential measurements of the polymers were also carried out (in triplicate) to estimate surface charge, using a Zeta sizer 3000 HAS (Malvern Instruments Ltd., Malvern, Worcestershire, UK, 1mL aqueous dip cell, cuvette DTS1070 (n=3)).

## 2.3 3D macroporous bioreactor preparation.

A significantly improved streamlined preparation method for 3D macroporous bioreactors (here termed cryobacteria reactors, or CBRs prepared within a special plastic carrier) is presented compared to one recently published by our group (Al-Jwaid et al., 2018). Briefly, 120 mL of media after cell cultivation OD<sub>600</sub> 1.0-1.4 was centrifuged at 10 000 rpm for 10 minutes at 4 °C. According to literature data for *Pseudomonas sp.*, one unit OD<sub>600</sub> corresponds to 2.08x10<sup>8</sup> CFU and has a cell density of 2.085 mg/mL (Kim et al., 2012). The wet weight of the pelleted cells was calculated accordingly. For calculation of the bacterial cell density of

101 *Rho* and *Acn* the following ratio was applied: an OD<sub>600</sub> of one resulted in a cell wet weight of  
 102 1.7 g/L and 0.39 g/L of dry weight, respectively (Glazyrina et al., 2010). The pelleted cells  
 103 (wet mass of 0.29g) were mixed gently with 2.5 mL of cross-linker solution in a phosphate  
 104 buffer(PB) (pH 7.2) (or water or PB and 2% glucose) at various ratios (PEI-al-PVA-al 0.6:0.6%  
 105 or 1.7% PVA-al), avoiding generation of bubbles in the cell suspension (all percentages shown  
 106 in material compositions are based on % mass in the final composite material). This solution  
 107 was transferred into a glass tube (diameter 9 mm) and frozen rapidly in a cryobath at -12 °C,  
 108 and kept at that condition for 3 days. CBRs based on *Pse* (11.6 %)-PVA-al-PEI-al 0.6:0.6%  
 109 (Cryo-*Pse*), *Rho* (11.6 %)-PVA-al-PEI-al 0.6:0.6% (Cryo-*Rho*), *Acn* (11.6 %)-PVA-al-PEI-al  
 110 0.6:0.6% (Cryo-*Acn*) obtained were thawed at room temperature and used for bioremediation  
 111 process testing directly.

112 Cryogels without bacteria were prepared according to the aforementioned procedure and were  
 113 based on the combination of PVA-al-PEI-al 0.6:0.6 % or PVA-al-PEI-al 1:1 and PVA-al 1.7%.  
 114 These cryogels were used as a control to estimate nonspecific adsorption of phenol derivatives  
 115 by the polymer matrix, and for physico-chemical characterisation.

116 Cryogels prepared from a combination of polymers PVA, PEI, chitosan(CHI), Gellan  
 117 Gum(Gel) and glutaraldehyde(GA), as well as only GA(0.5%) and were prepared analogously  
 118 to the above mentioned procedure. Prior to the addition of chitosan to the cells suspension, a  
 119 stock solution was dissolved in 1% acetic acid. The stock solution of Gellan Gum (1.5%) was  
 120 prepared in distilled water. CryoPho-PVA-PEI-GA 11.6:1:1:0.25%, CryoPho-PVA-PEI-GA  
 121 11.6:0.6:0.6:0.25%, CryoPho-CHI-GA 11.6:1:0.25%, CryoPho-Gel:PVA-al 11.6:0.3:1.0%,  
 122 CryoPho-Gel:PVA-al 11.6:0.52:0.32%, CryoPho-Gel 11.6:0.5% CryoPse-Gel 11.6:0.5% were  
 123 prepared at -12 °C and their bioremediation activity tested.

124 Cryobacteria reactors based on *Acn*, *Pse* and *Rho* were also prepared in polypropylene  
 125 ‘Kaldnes’ carriers (d=11mm and h=7mm, with the volume of cell suspension 0.55 mL) using

the same method as detailed above. Incorporation of the CBRs into plastic carriers improves their mechanical stability and decreases the possibility of mechanical damage under intensive mixing or application of high flow rate, or during the recharging of solutions between bioremediation cycles (Önnby et al., 2010, Rusten, et al., 2006). CBRs based on: *Pse* (pellet of 100 mL, OD<sub>600</sub> 1.06 or 221mg), PVA-al-PEI-al 0.6:0.6% (CBR-*Pse*); *Rho* (pellet of 100 mL, OD<sub>600</sub> 1.4 or 238mg), PVA-al-PEI-al 0.6:0.6% (CBR-*Rho*); and *Acn* (adapted on the TSB plate to 2CP) (pellet of 100 mL, OD<sub>600</sub> 1.04 or 170mg) PVA-al-PEI-al 0.6:0.6% (CBR-*Acn*) were prepared in PB with final concentration of bacteria of 11.6%. The bacterial suspension was mixed with cross-linker and placed into a glass tube 11.5 mm in diameter containing plastic carriers. The glass tube was frozen at -12 °C for 3 days. Then CBR samples in the carrier (*Pse* PVA-al-PEI-al 11.6:0.6:0.6% (CBR-*Pse*) (44mg of cells/carrier), *Rho*-PVA-al-PEI-al 11.6:0.6:0.6% (CBR-*Rho*) (47.6mg of cells/carrier), and *Acn*-PVA-al-PEI-al 11.6:0.6:0.6% (CBR-*Acn*) (34mg of cells/carrier)) were thawed at room temperature and used for bioremediation process testing directly.

The number of live bacteria in the CBRs was assessed using a MTT assay(Hao et al., 2002), following standard methods.

#### 2.4 3D macroporous bioreactor characterisation

The elastic moduli of the cryogel bacteria reactors (CBRs) were studied using a TA-XT2 instrument (Stable Micro Systems, Godalming, Surrey, UK). The compression test was performed at room temperature. The cryogels were prepared with a diameter of 8 mm and height of 9 mm. Samples were compressed up to a deformation level of 50 % at a speed of 0.05 mm/s. The elastic modulus was calculated at a deformation of 5% using the equation

$$E = (F/S)/(\Delta h/h)$$

where E is the elastic modulus (Pa), F is the force applied (N), S is the cross-sectional area of the sample ( $\text{m}^2$ ),  $\Delta h$  is the height (m) at compression, and h is the original height (m) (Kirsebom et al. 2013).

Three samples were used for each type of composite cryogel.

Scanning electronic microscopy (SEM) images were obtained using a Zeiss Sigma field emission gun SEM (Zeiss NTS). Slices of CBRs with thickness of 1-2 mm were washed with PBS, and then samples were fixed in 5% v/v GA in PBS overnight following by washing with PBS and water. Then, slices were frozen at  $-20\text{ }^{\circ}\text{C}$  overnight and freeze dried in a Christ ALPHA 2-4 freeze-dryer for 24 hours. Finally, freeze-dried samples were coated with a layer of platinum using a Quorum (Q150TES) coater.

Confocal laser scan microscopy (CLSM) images were obtained using a Leica TCS SP5 using objective lens x10, x20, x40 and x63. CBRs were fixed in 5 v/v % GA in PBS buffer overnight and then cut into slices with a thickness of 1 mm. Samples were washed with distilled water to remove non-crosslinked bacteria and stained with Rhodamine B solution overnight. Unbound Rhodamine B was washed out with water. Another batch of CBRs was stained with FITC solution (0.02 mg/mL in sodium phosphate buffer, pH 9.0) overnight. Non-reacted FITC was washed out with water. The 488 and 530 nm excitation and emission wavelengths were applied. Images were produced by optical sectioning in the xy-planes along the z-axis with 30-70 optical sections with 1  $\mu\text{m}$  intervals.

Evaluation of the viability of crosslinked bacteria within the cryogel was performed using standard Live/Dead assay. Cryogels were cut into thin slices with a thickness of 1 mm and washed out with NaCl 0.9% solution to remove uncross-linked cells. Samples were stained using Live/Dead Bac Light kit containing SYTO 9 stain at wavelength 480/500 nm and propidium iodide at wavelength 490/635 nm for 15 minutes at room temperature under dark conditions (according to the protocol of the LIVE/DEAD <sup>®</sup> Bacterial viability kit of staining).

## 2.5 Bioremediation by suspensions of bacteria and CBRs.

The degradation of phenol, *m*-cresol and 2-chlorophenol (2-CP) or 4-chlorophenol (4-CP) by a suspension of non-crosslinked bacteria and by various CBRs was studied. In the case of bioremediation by a bacterial suspension(planktonic state) the same amount of bacteria (measured based on OD<sub>600</sub>) as applied in the cryogel preparation was used . Forty mL of phenol derivative were added to the pellet of bacteria in a sterile 50 mL plastic falcon tube. The tube was regularly opened for sampling (207 µL at each time point) under sterile conditions. Note that the tube contained approximately 10 mL of air. The tubes were shaken at 150 rpm at 30°C in the dark(dynamic mode). The number of bacteria in the solution during the bioremediation process was monitored by measurement of OD<sub>600</sub> at each sampling point.

CBRs were placed in 40 mL of phenol derivative (phenol, *m*-cresol, 2-CP and 4-CP) in minimum salt media (MSM) buffer at pH 7.0 in a static mode(without additional shaking) at 30°C. Control sterile solutions (phenol, *m*-cresol, 2-CP and 4-CP in MSM) were prepared under the same conditions and tested to estimate stability against degradation over time in a static mode. Nonspecific adsorption of 4-CP on the PEI-al-PVA-al and PVA-al control cryogels (without bacteria, see section 2.3) was evaluated in MSM at 30°C in dynamic mode. Due to observable biofilm formation in the glass bottle during the bioremediation process, each cycle was performed in a new set of sterile bottles to eliminate the effect of biofilm formation on the following bioremediation cycles. The efficiency of the bioreactors was calculated as previously discussed (Börner et al. 2014), based on the change of contaminant concentration at a given time point (48 and 220h) divided by the experimental duration (i.e. the number of hours of bioreactor or bacteria exposure to the target contaminant) (mg/L/h) according to the equation:  $\text{Efficiency} = (C_0 - C_t) / t$  where  $C_0$  is initial concentration,  $C_t$  concentration at a time point.

The error was calculated according to the standard equation:



Error = (StDev(A1:Ai))/( $\sqrt{\text{COUNT}(A1:Ai)}$ ), where StDev is standard deviation,  
COUNT(A1:Ai) number of replicates.

### 3. Results and discussion

#### 3.1 Cryobacteria reactor (CBR) preparation and characterisation

In our recent work we used combinations of polymers GA(0.385%); GA & PVA (0.385:0.77%); PVA-al & PEI-al(0.385:0.46%); PVA-al & PEI-al(0.77:0.19%), keeping the concentration of bacteria at 23% (by mass) for preparation of cryogels(Al-Jwaied et al., 2018). Here, to select the best performing bioremediation system, cryobacteria reactors based on three bacterial strains and six immobilising polymers (and their combinations) were screened. PVA and PEI modified with aldehyde groups were used as cross-linkers. The presence of aldehyde group in the modified polymers was confirmed by FTIR and  $^1\text{H}$ -NMR spectroscopy (Fig. S2(a-c)). As expected the zeta potentials of the PVA and PVA-al were close to zero, illustrating the electroneutrality of the cross-linking agent, whereas PEI-al showed a zeta potential of  $32 \pm 4$  mV indicating that the cross-linker is positively charged (Fig. S3) and therefore can additionally interact with cell membranes via electrostatic interactions. Additionally, cryogels with chitosan and Gellan Gum were prepared to analyse the bacteria immobilisation as result of electrostatic interaction between the cell membrane and these polymers.

In previous work, bacteria capable of the bioremediation of phenol were immobilised on cryogel surfaces in three steps; overall the cryogel preparation and the following biofilm formation was completed in 10 days and required the use of additional equipment such as UV lamps (400 W)(Satchanska et al. 2015). The subsequent bioreactor contained a large amount of bulk polymer leading to issues of disposal after its use, and this technique therefore is less practical for industrial application.

Here, suspensions of *P. mendocina* (*Pse*), *R. koreensis* (*Rho*) and *A. radioresistens* (*Acn*) (11.6 w/w%) were cross-linked by a combination of PEI-al-PVA-al (0.6-0.6 w/w%) prepared in PB

in a one-step cryostructuration process. When the suspension of cells and cross-linker freezes under semi-frozen conditions ( $-12^{\circ}\text{C}$ ) ice crystals form, which leads to phase separation of the cells and the cross-linker suspension and ice (Fig. 1). The growing ice crystals expel all solutes and bacteria into a non-frozen liquid microphase, which remains non-frozen even at  $-12^{\circ}\text{C}$  (Kirsebom et al., 2013). The bacteria and cross-linker therefore concentrate in the thin non-frozen liquid microphase, and the bacteria become densely packed and cross-linked by functional polymers interacting with their cell membranes. Thawing of samples results in the melting of the ice crystals, producing water-filled voids - pores. This results in a 3D structured macroporous material, formed in a one-step preparation method, with the pore walls composed mostly of bacterial cells tightly attached to each other, surrounding a well-developed system of interconnected large pores with size  $20\text{-}150\text{ }\mu\text{m}$  (Fig. 2). The obtained cryogels were self-supporting, mechanically stable (Fig. S4), and withstand processing in a static and a dynamic mode (i.e. shaking at 150 rpm) for at least 4 weeks (Fig. S5(A-D)).

Rapid aggregation of cells was observed upon addition of glutaraldehyde. The Cryo-*Acn*-PVA-al has no obvious cells in the cryogel wall due to use of a relatively high concentration of cross-linking agent (1.7%, Fig. 2i(A-C)). It was observed that the change of composition of the buffer did not change the morphology of the material significantly (Fig. 2ii). Nevertheless, the use of PB or PB & glucose has advantages compared to cryogels prepared in pure water, as these decrease the osmotic stress during cryogel preparation and keep the pH constant.

To visualise the efficiency of mixing of the bacteria with the polymer, as well as to assess the distribution and amount of live cells, CBRs were stained with Live/Dead stains. The hydrogel based on Gellan was characterised by the absence of large pores in the structure of the composite hydrogels, and a homogeneous distribution of cells within the material (Fig. 3(E-H)). A strong fluorescence background due to electrostatic binding of the positively charged

249 dyes with the negatively charged polymer can be observed under CLSM. Nevertheless, at  
 250 magnification x100 the images clearly distinguish between cells and background (Fig. 3(H)).  
 251 The material has a predominantly microporous structure however and therefore can be used  
 252 only in batch application in a dynamic mode (Fig. 3(E-H)). We focused on preparation and  
 253 assessment of the bioremediation activity of macroporous materials, which potentially could  
 254 be used additionally in a flow through mode.

255 Some of the key beneficial properties of cryogels relate to their elastic strength, flexibility and  
 256 shape recovery. In our recent study we comprehensively investigated the rheological properties  
 257 of cryogels based on bacteria (Al-Jwaid et al., 2018), however the elastic modulus was not  
 258 measured. The elastic modulus of the cryogels *Acn*-PVA-al (1.0%) and *Acn*-PVA-al-PEI-al  
 259 (1.0:0.25(%)) was  $3.52 \pm 0.7$  and  $4.64 \pm 0.1$  kPa, respectively. These values were similar to  
 260 those previously reported for cryogels made of *E.coli* and activated polyethylenimine, which  
 261 had an elastic modulus of  $3.1 \pm 0.39$  kPa,(Zaushitsyna et al., 2014) or porous material *C.*  
 262 *saccharolyticus* cross-linked by glutaraldehyde which showed a modulus in the range 8 - 29  
 263 kPa (Kirsebom et al., 2009). Our data were also comparable with the elastic modulus of  
 264 cryogels based on polymers such as enzymatically cross-linked casein (1.3 - 7 kPa) and gelatin  
 265 (0.95 - 1.9 kPa)(Kirsebom et al., 2013) or combinations of polymeric cross-linking agent  
 266 aldehyde dextran and gelatin (0.6-2.8 kPa), respectively(Berillo and Volkova 2014). Cryo-  
 267 Rho-PVA-al-PEI-al-Gel (11.6:0.5:0.6:0.3(%)), Cryo-Rho-PVA-al-Gel (11.6:1.0:0.3(%))  
 268 prepared in water, and Cryo-Rho-PVA-al-Gel (11.6:1.0:0.3(%)) formed in phosphate buffer  
 269 had elastic moduli of  $16.1 \pm 1.7$ ,  $87.7 \pm 16.3$ ,  $28.7 \pm 17.0$  kPa, respectively. As expected, an  
 270 increase of CHI concentration from 0.5 to 1.0% at constant polymer cross-linking mass ratio  
 271 (2:1) led to a significant increase in the toughness of the material (Fig. S4). Cryogels based on  
 272 *Rho*(11.6%) in combination with polymers CHI-GA 0.5:0.25% and CHI-GA 1.0:0.5%, PEI-  
 273 GA1.0:0.25%, and 1.0:0.5%, PEI-PVA-GA 1.0:1.0:0.25% showed an elastic modulus of 34.7

274  $\pm 15.5$ ;  $1955 \pm 268$ ;  $12.4 \pm 8$ ;  $10.8 \pm 0.9$ ;  $7.2 \pm 1.5$  kPa, respectively. As expected the highest  
275 elasticity modulus was found for samples based on CHI, which is related to the rigid structure  
276 of this polysaccharide. The incorporation of Gel to the structure significantly improves the  
277 mechanical properties of the material due to formation of a physical gel. In figure S4 an  
278 illustrated comparison of the elastic properties of composite cryogels based on *Rhodococcus*  
279 *sp* is given.

280 The analysis of live and dead bacteria using live/dead kit indicates that most of the bacteria  
281 after cross-linking were viable (stained green, Fig. 3(e-h)) even after undergoing freeze-  
282 thawing conditions during the cryogelation process. Potentially, cryogenic conditions  
283 (particularly ice-crystal formation) as well as the cross-linker and the osmotic stress itself could  
284 damage the bacterial cells and reduce viable cell numbers. To distinguish the potential harmful  
285 effects of the polymer cross-linker from those caused by cryogenic conditions the experiments  
286 were performed at  $4^{\circ}\text{C}$  /  $-12^{\circ}\text{C}$ , respectively. Incubation of *Rho* suspension in the solution of  
287 PBS containing 0.25 or 0.5 % of GA (negative control) at  $4^{\circ}\text{C}$  for 24 h led to a significant  
288 decrease in the number of live bacteria, to 27 % and 22 % of initial numbers respectively, due  
289 to the toxicity of GA. The use of PEI-al (0.365 w/v %) in PBS resulted in a 25 % of live *Acn*  
290 bacteria. PEI-al was more toxic compared to unmodified PEI (where 50 % of bacteria  
291 survived). PVA-al 1.2 % in PBS buffer at  $4^{\circ}\text{C}$  revealed a high survival of *Acn* (92.4 %)   
292 compared to a bacterial suspension stored in phosphate buffer without polymer (100 %)   
293 (positive control) (Table S1).

294 The issue of the relatively high toxicity of PEI-al was overcome by the use of a combination  
295 of nontoxic PVA-al with PEI-al, leading to 72.5% active bacteria. The use of only PVA-al led  
296 to formation of a material with low water permeability, while using a combination of PVA-al-  
297 PEI-al (1:1) resulted in formation of a material with desirable porosity characteristics and  
298 mechanical properties. A small amount of glucose, which acted as a cryoprotector and

potentially as a source of carbon, was added to the bacterial suspension to decrease the effect of cell bacteria damage due to ice crystal formation and osmotic shock (Moslemy et al., 2002). PVA-al 1.2% dissolved in PBS buffer in the presence of 2 % glucose showed some growth of the *Acn* bacteria at 4°C (109 %) after 24h (Table S1). The frozen suspension of *Acn* in PVA-al 1.2 % and PBS buffer with 2 % glucose added showed 88.6 % survival from the initial number of cells. Thus, a decrease of 11.4% in live bacteria was related to bacterial damage by growing ice crystals. The effect of 1.0% glucose in PBS buffer on bacterial viability was also investigated (Table S1). The incubation of *Pse* suspension in PBS buffer containing 1.0% glucose over 24h at 4°C led to a 13.8% decrease in cell populations compared to the PBS buffer. The presence of glucose did not significantly improve the viability of *Pse* during the cryogelation process either (Table S1). It can be concluded that addition of glucose in combination with PVA-al-PEI-al (1:1) did not significantly improve the survival of the bacteria or even had some negative effect on *Pse*, but conversely it had a positive effect on *Acn* during incubation for 24h at 4°C. It can also be concluded that the 23% *Pse* bacteria population decrease observed after incubation at 4°C for 24h was due to cumulative toxicity from the PVA-al-PEI-al polymers, while the 53% loss of bacteria after cryogelation was presumably due to damage of the bacteria by growing ice crystals during the cryo- structuration process (Table S1).

### 3.2 Bioremediation of phenol and cresols

Recently we illustrated the bioremediation efficiency of cryogels based on *Pse*, *Rho* and a *Pse:Rho* mixture for 50ppm phenol in carbonate buffer in a dynamic mode (Al-Jwaid et al., 2018). In this study phenol and m-cresol bioremediation by Cryo-*Rho*, Cryo-*Pse*, and Cryo-*Acn* cryogels was examined in dynamic and static mode in MSM buffer (50 ppm (40 mL) and compared with bacterial suspensions (Fig. S5(D) and S6). Suspensions of bacteria of *Pse* or *Acn* degraded 50 ppm (40 mL) of phenol in 7 days (166h), whereas *Rho* completed the

degradation in 9 days (220h)(Fig. S5(D)). Increasing the phenol concentration to 100 ppm resulted in 39.5 % phenol degradation by a suspension of *Acn* over 220h (bioremediation efficiency 0.123 mg/L/h). *Rho* formed a biofilm on the glass surface in the solution of phenol (100 ppm, 200 mL), and did not degrade phenol over 12 days of incubation (data not shown). CBR-*Pse*, CBR-*Acn* and CBR-*Rho* were more efficient for phenol degradation compared to a suspension of equivalent bacteria(Table 1). Bacterial suspensions of *Pse* or *Acn* degraded 50 ppm (40 mL) phenol in 166h, whereas *Rho* completed the degradation in 220h (Fig. S5(C)). Cryogel *Pse*-Gel (0.5%) showed slow bioremediation of m-cresol (300ppm), with 21% degraded over 17 days in a static mode. The presence of polysaccharide Gel in the structure of the cryogel decreased the bioremediation efficiency of the material, which might be related to its consumption as a source of carbon. A cryogel composed of a combination of *Rho*, 0.13% of PVA-al and 0.5% Gel was not as effective for phenol bioremediation as cryo-*Rho* PVA-al-Gel (1.0:0.3%) (Fig. 4A). Other polymer compositions were used for cryogels preparation based on PEI-PVA-GA(1:1:0.25%); PEI-PVA-GA(0.6:0.5:0.25%), CHI-GA(1:0.25%) (Fig. 4B), which revealed complete bioremediation of m-cresol (50 ppm) in 116 and 225h, respectively.

**Table 1.** Bioremediation efficiency (mg/L/h) of cryogels(1mL) and suspensions of bacteria *Pse*, *Rho* and *Acn* for Phenol(Ph), m-cresol and 4CP for the 1<sup>st</sup> cycle otherwise specified, volume of solution 40mL otherwise specified.

Bioremediation efficiency, mg/L/h			
CBRs prepared within plastic carriers	CBR- <i>Rho</i>	CBR- <i>Pse</i>	CBR- <i>Acn</i>
Cresol 50 ppm at 48h	0.0964 ± 0.029	0.234 ± 0.019	0.307 ± 0.064
Cresol 50 ppm at 220h	0.166 ± 0.051	0.535 ± 0.125	0.60 ± 0.10
Ph 100 ppm at 48h, 2 <sup>nd</sup> cycle	2.18 ± 0.15	2.250 ± 0.166	0.761 ± 0.098

Ph 100 ppm at 48h, 3 <sup>rd</sup> cycle	$1.84 \pm 0.089$	-	$1.860 \pm 0.240$
Ph 50 ppm at 48h (V 200mL)	$0.091 \pm 0.021$	$0.60 \pm 0.10$	$0.363 \pm 0.0424$
Ph 50 ppm at 120h (V 200mL)	$0.468 \pm 0.007$	$0.50 \pm 0.01$	$0.517 \pm 0.016$
Free suspensions of bacteria	<i>Rho</i> suspension	<i>Pse</i> suspension	<i>Acn</i> suspension
Cresol 50ppm at 120h (V 200mL)	0.0074	-	$0.068 \pm 0.073$
Phenol 50 ppm at 48h	$0.028 \pm 0.003$	$0.159 \pm 0.080$	$0.42 \pm 0.080$
Phenol 50 ppm at 120h	$0.213 \pm 0.045$	$0.317 \pm 0.080$	$0.352 \pm 0.050$
Phenol 100ppm at 48h, 2 <sup>nd</sup> cycle	$0.048 \pm 0.039$	-	$0.276 \pm 0.044$
Phenol 100ppm at 120h 2 <sup>nd</sup> cycle	$0.039 \pm 0.011^d$	-	$0.184 \pm 0.026$
CBRs without plastic carriers	<i>Cryo-Rho</i>	<i>Cryo-Pse</i>	<i>Cryo-Acn</i>
Cresol 50ppm at 48h (V 200mL)	$0.086 \pm 0.022$	$0.386 \pm 0.086$	$0.0833 \pm 0.099$
Phenol 50ppm at 48h	$0.371 \pm 0.022$	$0.151 \pm 0.020$	-
Phenol 50ppm at 120h	$0.874 \pm 7 \times 10^{-4}$	$0.237 \pm 0.050$	-
4CP 50ppm at 48h	-	$0.333 \pm 0.038$	-

344 The suspension of bacteria *Acn* produced only 10 % degradation of *m*-cresol (50ppm, 200mL,  
 345 a static mode) within 12 days. There was no significant difference in *m*-cresol biodegradation  
 346 by *Pse* and *Rho*. The suspension of *Rho* degraded only 11 % of *m*-cresol (50ppm, 200mL) after  
 347 12 days (data not shown). The first bioremediation cycle of *m*-cresol (50 ppm, 200mL) by  
 348 CBR-*Acn* ( $9 \times 10^6$  CFU) was completed within 5 days. The addition of fresh phenol solution  
 349 (100 ppm, 200 mL) to the CBR-*Acn* instead of *m*-cresol resulted in only 50% consumption of  
 350 phenol within 8 days, indicating that the same batch of CBR-*Acn* might be utilised for  
 351 bioremediation of mixed or complex mixtures, but with similar (by structure) contaminants,  
 352 i.e. it can be acclimatised to a new contaminant, however it took a longer time compared to the  
 353 model system(Fig. 4C).

354 The bioremediation of the *m*-cresol was slower over the first 90h of testing for the three  
 355 bacteria cryogels of cryo-Rho-PEI-PVA-GA, cryo-Rho-PEI-PVA-GA and cryo-Rho-CHI-GA  
 356 due to bacterial acclimatisation prior to *m*-cresol consumption (Fig. 4B). Bioremediation with  
 357 CBR-*Rho* and CBR-*Pse* consumed 71% of *m*-cresol (40 mL, 50 ppm) after 10 days (Fig. 4C).  
 358 The bioremediation using CBR-Rho at higher *m*-cresol concentrations (40 mL, 100ppm)  
 359 indicated only a 30% decrease in *m*-cresol concentration(data is not shown). CBR-*Acn* revealed  
 360 better ability to degrade *m*-cresol compared to CBR-*Rho* and CBR-*Pse*. The 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup>  
 361 bioremediation cycles of *m*-cresol (50ppm) by CBR-*Acn* were completed within 265, 90 and  
 362 46h, respectively. Regardless of which bacterial strains were used (*Pse*, *Rho* and *Acn*), the CBR  
 363 had an adaptation period to phenol and *m*-cresol of 48h and 90h, respectively. CBR-*Acn*  
 364 showed the best degradation efficiency for *m*-cresol among the studied bacterial strains.

365 Following this, the bioremediation process was scaled up in a static mode, and the volume of  
 366 the solution was increased to 200 mL, revealing a significant decrease of concentration within  
 367 100-120h for the studied bacterial strains(Fig. 5). The biodegradation of phenol by CBR-*Pse*,  
 368 CBR-*Rho* and CBR-*Acn* was studied with addition of a fresh portion of phenol after each



369 bioremediation cycle. CBR-*Pse* and CBR-*Acn* consumed 60ppm of phenol within 200h  
 370 whereas CBR-*Rho* completed the bioremediation in approximately 100h for the first cycle. It is  
 371 important to note that CBR-*Pse*, CBR-*Rho* and CBR-*Acn* revealed similar phenol degradation  
 372 activity for following 2-10 cycles, which were completed within approximately 60-65h (Fig  
 373 S6(A-C)). To the best of our knowledge there is still no information about the efficiency of  
 374 *Rho* to degrade phenol, cresols and chlorophenols. *Pseudomonas spp.* has been previously used  
 375 for bioremediation of benzene and toluene into phenol and cresol (Tao et al., 2004 and 2005).  
 376 Calculation of degradation efficiency showed that CBR-*Pse* caused a more rapid  
 377 bioremediation of phenol compared to CBR-*Rho* or CBR-*Acn* (Fig. 5 and Table 1). CBR-*Rho*  
 378 needed an acclimatisation period of approximately 48h before the bacteria became active for  
 379 bioremediation. CBR-*Pse*, CBR-*Acn* and CBR-*Rho* were more efficient for phenol degradation  
 380 compared to a suspension of equivalent bacteria (Table 1) and phenol bioremediation  
 381 efficiencies were 0.6, 0.363 and 0.0915 mg/L/h (48h), respectively (Fig. 5). It can be concluded  
 382 that the selected strains *Pse*, *Rho* and *Acn* preferably degrade phenol while the biodegradation  
 383 of m-cresol took place at least 3 times more slowly (Fig. 4C).  
 384 Overall, the CBRs show resistance to high concentrations of phenol derivatives and  
 385 significantly faster bioremediation (Fig. 5 & Fig. S5(A-C)) compared to equivalent suspensions  
 386 of bacteria in a static mode (Fig. S5(D)). Moreover, the recovery of the bioreactors is  
 387 straightforward and does not require centrifugation to separate suspensions of bacteria from  
 388 the aqueous solution, making the whole bioremediation process considerably simpler to apply.  
 389 The same CBR-*Pse*, CBR-*Rho*, CBR-*Acn* bioreactors were used over 10 bioremediation cycles  
 390 without showing any decline in activity over more than one month of exploitation,  
 391 demonstrating the ability for reuse of the material over repeat cycles (Fig. S5(A-C)).  
 392 As noted above, suspensions of bacteria cannot be used instantly for bioremediation of high  
 393 phenol concentrations (>60 ppm). An increase in phenol concentration resulted in prolongation

of the bioremediation process required for its complete degradation (Fig. 5(A-C)). There was no delay of bioremediation at high concentrations of contaminant for the 2<sup>nd</sup> (and following) bioremediation cycles. MTT assay after 17 days of the bioremediation process showed, for CBR-*Pse*, a significant increase in bacterial numbers compared to initial numbers (Table 2), indicating their existence in an exponential growth phase. In contrast, CBR-*Acn* and CBR-*Rho* showed a decrease in bacterial numbers following treatment (Table 2) indicating that these bacteria had entered a senescence phase. Based on figure 5 one can assume that 300 ppm is not the limit for the bioremediation process, however there are few examples where such high concentrations would be encountered in a real case scenario therefore higher phenol concentrations were not tested further. Usually the concentration of phenols in waste water is quite low and adaptation of the CBR is unnecessary, but in cases where faster bioremediation of high concentrations of phenols (200-300 ppm) is required, CBRs may be pre-adapted to phenol at concentrations of 25-50 ppm.

**Table 2.** Number of viable cells in initial cryogel and after 10, 28 and 40(\*) days of the bioremediation process (n=3) estimated using MTT assay. CBR-*Pse* control (without contaminant) the number of cells was  $18.9 \pm 2.5 \times 10^6$  CFU after 10 days in MSM at 4 °C.

Contaminant concentration and volume	Before remediation.	2CP, 40 mL, 50 ppm	m-cresol, 40 mL, 50ppm	m-cresol, 40 mL 100 ppm	Phenol, 200 mL, 300 ppm
Time point, days	0	10	10	10	28
Number of viable cells x 10 <sup>6</sup>					
CBR- <i>Acn</i>	39.7 ± 1.42	10.4 ± 3.12	-	-	17.6 ± 0.96
CBR- <i>Pse</i>	12.2 ± 3.64	-	34 ± 2.54	-	38.1 ± 8.3
CBR- <i>Rho</i>	2088 ± 99	653 ± 64	716 ± 122	453 ± 105	576 ± 52

Cryo-RhoGel 1.0% *	-	70.46±18.6	-	-	-
Cryo-RhoGel 0.5%*	-	76.0±5.5	-	-	-
Cryo-PseGel 0.5% *	-	82.1±10.8	-	-	-
Cryo-PseGel 1.0%*	-	60.8 ±3.4	-	-	-
<i>Pse</i> suspension *	-	46.8±8.8	-	-	-

411

412 Analysis of treated water via HPLC confirmed the absence of phenol or release of its  
413 derivatives, such as p-hydroxybenzoic acid, hydroquinone, catechol and protocatechuate (Tao  
414 et al., 2005; Martínková et al., 2009). A few peaks with retention times in the range of 1 & 2  
415 minutes were observed which most probably relate to the peak of the solvent, or to the final  
416 degradation products of phenol, possibly tri-carbonic acids. The biodegradation of *p*-cresol  
417 performed using suspensions of *Pse* and *Rho* revealed a trace amount of *p*-cresol, and some  
418 trace amounts of its derivatives were detected after 2 weeks of bioremediation by suspensions  
419 of bacteria *Pse*, *Rho* and the corresponding cryobacteria reactors. The bioremediation results  
420 for *p*-cresol and m-cresol were comparable. Indirect evidence of the release of tri-carbonic  
421 acids was given by a pH shift from 7.1 to 6.5-6.4 which is in agreement with previously  
422 described pathways of degradation of phenols and cresols (Kolomytseva et al., 2007).

### 423 3.3 Biodegradation of chlorophenols

424 Chlorophenols (CPs) are relatively stable products of the biodegradation of triclosan and  
425 triclocarban, which are widely used as antimicrobial agents in toothpaste, soaps and detergents  
426 and are present in most waste waters (Dhillon et al., 2015). Therefore, the efficiency of the  
427 developed Cryo-*Rho*, Cryo-*Pse*, Cryo-*Acn* reactors for degrading various CPs was examined.  
428 The viability of bacteria via monitoring of turbidity as well as their biodegradation efficiency  
429 for 4CP and 2CP in carbonate buffer and minimum salt media (MSM) at pH 7.2 was estimated  
430 (Fig. S7 & S9). Data indicated that bioremediation efficiency for suspensions of *Acn*, *Rho* and

*Pse* were negligible (40mL 50ppm 4CP) in carbonate buffer at 48 and 120 h, respectively. Bioremediation efficiencies for suspension of the *Rho* and *Pse* in MSM were  $0.044 \pm 0.019$  mg/L/h and  $0.032 \pm 0.025$  mg/L/h (40mL 50ppm 4CP) at 48 and 220h, respectively (Fig. S8). From the cryobacteria reactors tested, only cryo-*Pse* PVA-al or PVA-al-PEI-al showed consistent ability to degrade 4CP (Fig. 6 and Table 1), although cryo-*Rho* showed some degradative

The 4CP degradation efficiency was much lower compared to bioremediation efficiencies for phenol or m-cresol using the same bacterial strains. Biodegradation of 2CP (60 ppm) resulted in only a 9% decrease after 6.6 days (Table S2). Control cryogels of PEI-al-PVA-al and PVA-al (without bacteria) did not reveal adsorption of phenols or CPs or self-decomposition of the contaminant within 1000h of incubation in the 4-CP (50ppm) solution (40mL).

One can observe that Gel based cryogels cross-linked by magnesium ions were less efficient in the bioremediation process compared to sodium cross-linked physical gel, which may be related to some diffusion restriction, as Gel forms a hydrogel such faster. Estimation of the final concentration of 4CP was performed using HPLC, illustrating that suspensions of bacteria (*Pse* and *Rho*) and cryo-*Pse* and cryo-*Rho* can slowly degrade 4CP (Table S2, Fig. 6). It was observed that *Rho* immobilized within the cryogel structure is capable of degrading chlorophenol without additional sources of carbon. These data indicate that cryo-*Pse* and cryo-*Rho* bioreactors, while less effective for 4CP and 2CP, warrant further study in their application for treatment of waste water from hospitals, which has high concentrations of recalcitrant chlorophenols and their metabolites which require removal.

## Conclusion

3D structured bioreactors based on live bacteria, suitable for the bioremediation of a range of phenol derivatives (phenol, cresols, chlorophenols), were prepared by cryogelation in a one-step process, using water as a solvent. The macroporous material consisted of 11.6% live

bacteria, 1.2% polymers and 87% voids/ pores. For the first time toxicity levels of novel aldehyde containing polymers – cross-linking agents (PEI-al and PVA-al) were estimated and their suitable combination proposed. The cryobacteria reactors maintain their degradation activity over at least 10 cycles. The efficiencies of cryobacteria reactors of *Pse*, *Rho* and *Acn* for degradation of four concentrations of phenol derivatives revealed complete, rapid, bioremediation in dynamic as well as static mode. Moreover, the developed material exhibited significantly better performance for phenol degradation than recently reported by our group, where the bioremediation of 50ppm of phenol(40 mL) in a dynamic mode was studied (Al-Jwaid et al., 2018). Bioremediation cycles (2<sup>nd</sup>-10<sup>th</sup> cycle) were completed within 2.6 days, compared to about 10 days for equivalent cell suspensions. The purification of 200 mL of 300 ppm of phenol by *Pse*, *Rho* and *Acn* within cryobacteria reactors took approximately 7 days. The use of plastic carriers showed slightly slower bioremediation of cresols compared to conventional cryobacteria reactors. Overall, six polymers and their combinations were utilised to produce materials for bioremediation purposes using three bacterial strains, the activities of which were tested using four model phenol derivatives. The developed technology preserves the native structure and activity of the bacteria. Cryobacteria reactors have several advantages. Cryogels based on live bacteria can be easily reprocessed after inactivation due to low content of a polymeric cross-linker (<10%) in the structure and therefore the technology can be considered as “green”. There is no diffusion restriction as materials are macroporous with well-developed connected micro channels that improve interaction of the cells with contaminated water. The macroporous structure of interconnected pores also creates the possibility of exploiting the bioreactor as a flow through column or filter system with no requirement to separate the bacteria after the bioremediation process. The technique is faster and simpler than a previously published three-step cryogel surface immobilisation techniques for phenol

degrading bacteria, which also contain a large bulk content of polymer resulting in more complex processing after use.

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