1 **Synopsis:**

2 The mechanism of bacterial cell death by the action of halogenated compounds is still
3 not clear. The aim of this research work is to use metabolomics to differentiate
4 between apparently dissimilar mechanisms of bacterial cell death by the action of
5 halogenated compounds. Two halogenated compounds (sodium hypochlorite and N-
6 halamine polymers) were selected for this study and their effect was examined against
7 *Escherichia coli*. Changes in the intracellular metabolite pools of bacterial cells
8 treated with different formulations of these compounds were analysed using FT-IR
9 spectroscopy and LC-MS. Principal component analysis (PCA) was used to generate
10 metabolic profiles.

11 The study proved that the halogen containing compounds had an effect on the
12 bacterial intracellular metabolite profile. In addition, an effect was noticed for the
13 non-halogenated heterocyclic polymers. The mode of action of N-halamine polymer
14 is assumed to be a combination of contact, release and changes in the nature of the
15 medium around the cells. We think that the non-halogenated polymers may disturb
16 the ionic equilibrium between intra- and extra-cellular positive charges which result
17 in a deleterious effect on the bacteria cells.

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1 Metabolomic profiling can differentiate between 2 bactericidal effects of free and polymer bound 3 halogen

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18 **ABSTRACT:** The effects of two halogenated compounds (sodium hypochlorite and
19 N-halamine polymers) on the *Escherichia coli* metabolome were investigated.
20 Changes in the intracellular metabolite pools of bacterial cells treated with different
21 formulations of these compounds were analysed using FT-IR (Fourier Transform
22 Infra Red) spectroscopy and LC-MS (Liquid Chromatography Mass Spectroscopy).
23 Principal component analysis (PCA) was used to generate metabolic profiles of the

intracellular metabolites in order to investigate the effect of sub-lethal concentrations on the metabolome of treated cells. The effect of treatment with sodium hypochlorite was quantitatively dependent on the exposure time. The resulting metabolic profiles supported our previous hypothesis that the mode of action of some halogenated compounds, such as N-halamine polymers, can be initiated by release of halogen ions into the aqueous environment, in addition to direct contact between the solid polymer material and the bacterial cells. Moreover, the metabolic profiles were able to differentiate between the effect of free and polymer-bound halogen. Our metabolomic approach was employed for hypothesis generation in order to distinguish apparently different bactericidal effects of free and polymer-bound halogen.

Keywords: N-halamine; hypochlorite; metabolite; cells; LCMS; PCA.

INTRODUCTION

Chlorinated compounds have been used for disinfection purposes since the early 1900s due to their effective bactericidal action.¹ They can act either as free small species such as HOCl or NaOCl (which are considered to be the most effective disinfectants for water and clinical purposes),²⁻⁵ attached to amino containing compounds (N-halamines) such as halogenated isoxazoles and halogenated imidazoles,⁶⁻¹¹ or attached to high molecular weight compounds (N-halamine polymers).¹²⁻¹⁶

The precise bactericidal mode of action of these compounds has not been investigated systematically. It has been suggested that HOCl can denature bacterial DNA by pyrimidine base halogenation¹⁷ and reduce ATP levels, resulting in immediate cell

1 death.¹⁸ It has also been reported that treating bacterial cultures with NaOCl may
2 result in amino acid neutralization¹⁹ and protein denaturation due to halogen
3 transfer.¹⁹⁻²¹ Modifying the structure of the amino-containing compounds to N-Cl will
4 also result in the oxidation of SH groups of essential enzymes and alteration of the
5 intracellular pH, ultimately leading to global disruption of cell metabolism.¹⁹

6 It has been proposed that the action of halogenated heterocyclic compounds (N-
7 halamines) depends on halogen ion exchange between these compounds and the
8 bacterial cell membrane by contact, resulting in cell-wall oxidation.⁶⁻¹¹ The same
9 explanation has been suggested for the mode of action of N-halamine biocidal
10 polymers, but it is still not clear whether the halogen ion is delivered to the bacterial
11 cells by contact,^{12,13} halogen ion release²² or a combination of both.¹⁶ We have
12 hypothesized in previous publications that the mode of action of the N-halamines is
13 based on a combination of contact, release and changes in the cell environment.^{15,16,19}

14 The objective of this work was to investigate the effect of sub-lethal concentrations of
15 halogenated compounds on the bacterial metabolome, so that metabolomic profiling
16 could be applied to differentiating between the two halide delivery mechanisms. An
17 important application of this approach could be the investigation of the precise
18 bactericidal mode of action of halogenated compounds and the reason for resistance
19 of some species. Cultures of *E. coli* were treated with halogenated compounds
20 (NaOCl and a novel N-halamine polymer, Scheme 1). Intracellular metabolites were
21 extracted and subjected to Fourier Transform Infra Red (FT-IR) and Liquid
22 Chromatography-Mass Spectrometry (LC-MS), and the resulting data analysed using
23 principal component analysis (PCA) to provide metabolic profiles characteristic of the
24 different treatments.

The observed changes in the metabolic profiles may support our previous hypothesis that the effect of N-halamine polymers on bacterial cells is due to a combination of factors (contact, release and changing the nature of the culture medium)^{15,16} and may help to differentiate between the mechanisms of action of free and polymer-bound halogen.

An imide N-halamine polymer was selected for this study to ensure a rapid effect of the polymer on the cells; as it is the N-halamine with lowest stability.^{14,23} The imide N-halamine dissociation constant is 1.6×10^{-12} to 8.5×10^{-4} , while for amide it is 1.6×10^{-8} and for amino $<10^{-12}$.^{14,23}

EXPERIMENTAL

Strain and culture media

The strain used was *Escherichia coli* K12 substrain MG1655. Cultures were grown in Nutrient broth and Nutrient agar (Oxoid, UK). Primary cultures were maintained on nutrient agar slopes and subcultures on nutrient agar plates stored at 4°C.

Reagents

m-Phenylenediamine, cyanuric acid, sodium hypochlorite, perchloric acid, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), disodium salt of ethylenediaminetetracetic acid (EDTA), sodium hydrogen carbonate and guanosine-3',5'-cyclic monophosphate (cGMP) were supplied by Sigma Aldrich Chemicals, UK. Ethanol, methanol, acetonitrile, ammonium acetate, N,N-dimethylformamide (DMF),

sodium hydrogen carbonate, monobasic sodium phosphate, dibasic sodium phosphate and potassium carbonate were supplied by Fisher Chemicals, UK. All chemicals were used as obtained from suppliers without extra purification.

Metabolite detection and analysis

FT-IR metabolite detection was performed using a Perkin Elmer System 2000 FT-IR (PerkinElmer LAS Ltd, UK). LC-MS was performed using an Acquity TQD UltraPerformance LC (Waters Ltd, UK) for separation and a Q-ToF Premier Micromass mass spectrometer (Waters Ltd, UK) for mass detection. LC-MS chromatography details were as follows: an Acquity UPLC BEH C18 3.0 x 100mm column (Waters Ltd., UK.) was used, Solvent A = water + 0.1% w/v formic acid, Solvent B = acetonitrile and the flow = 0.2ml/min. The gradients were: 0min = 100%A - 0%B; 5min = 100%A - 0%B; 25min = 10%A - 90%B; 35min = 10%A - 90%B; 36min = 100%A - 0%B; 45min = 100%A - 0%B.

Principal component analysis (PCA) was performed to identify FT-IR and LC-MS markers. LC-MS markers were created using Markerlynx software while PCA was performed using SIMCA-P+ software (MKS Instruments UK Ltd - Umetrics UK).

Preparation of cross-linked Polyepicyanurohydrin (1)

Polyepichlorohydrin (2 g) was dissolved in dimethylformamide (DMF) (30 ml). *m*-phenylenediamine (0.2 g, 10% w/w) and sodium hydrogen carbonate (0.3 g) were added and the reaction mixture heated at 120°C for 24h. Cyanuric acid (3.2 g) and sodium hydrogen carbonate (1.9 g) were added and the heating was continued for

1 24hr. The product was added to water and washed with water (80°C, 100 ml) to
2 remove any cyanuric acid contaminating the polymer (Scheme 1). The polymer was
3 dried and ground into small particles of 1-2 mm diameter. Analysis: FT-IR (KBr):
4 ν_{max} (cm^{-1}), 1739, 1702, 1646 (C=O, heterocyclic ring), 1589 (C=N), 1260 (C-N and
5 C-O), 2842, 2992 (CH aliphatic), 3210 (NH), 3434 (OH). Solid state ^{13}C NMR, 30-40
6 (aliphatic moiety), 155, 162 (C=O, heterocyclic ring).

7 The reaction was performed without cross-linking (linear polymer) to facilitate both
8 ^1H NMR and ^{13}C NMR analysis. The following data were obtained for the linear
9 polymer: FT-IR (KBr): ν_{max} (cm^{-1}), 1736, 1702, 1646 (C=O, heterocyclic ring), 1587
10 (C=N), 1260 (C-N and C-O), 2839, 2962 (CH aliphatic), 3130 (NH), 3428 (OH). ^1H
11 NMR (DMSO, 500 MHz): δ 2.5 ($\text{CH}_2\text{-N}$, singlet), 3.6 ($\text{CH}_2\text{-CH}$, broad band, aliphatic
12 part), 10.1 (NH, broad singlet). ^{13}C NMR (DMSO, 125 MHz): ppm 45 (CH_2), 69
13 (CH), 79 ($\text{CH}_2\text{-N}$), 155, 162 (C=O).

14 Cross-linked polyepicyanurohydrin (**1**) was chlorinated using sodium hypochlorite
15 (10% w/w). The polymer (1.0 g) was soaked overnight in sodium hypochlorite (10%
16 w/w, 15 ml) and distilled water (5 ml). The product was filtered, washed with 100 ml
17 distilled water and dried. The amount of halogen loaded onto to the polymer was 115
18 ± 20 ppm determined by iodometric titration.²³

19

20 **Effect of hypochlorite concentration on bacterial growth, viability and** 21 **intracellular metabolite profile**

22 Seven conical flasks (250 ml) each containing nutrient broth (50 ml) were inoculated
23 with 0.1 ml of bacterial suspension (*E. coli*, previously grown for 17h at 37°C) and
24 incubated at 37°C for 17 h. One of these flasks was used as a bacterial control while

1 the others were treated with a range of sodium hypochlorite concentrations (0.002%,
2 0.004%, 0.008%, 0.012%, 0.016% and 0.02% w/w). The bacterial viability was
3 followed by viable counts at timed intervals using the 'Miles and Misra' method.^{16,24}

4 Similar procedures were followed to determine the effect of different hypochlorite
5 concentrations on bacterial growth. Hypochlorite treatment was performed before
6 incubating the flasks at 37°C and growth was followed spectrophotometrically by
7 determining the optical density of the culture at 540 nm (OD) at timed intervals.
8 Growth was followed until OD = 0.4, while the cells were still in logarithmic growth
9 phase. The experiment was also performed both with and without pH control (using
10 phosphate buffer, 39.0 ml 0.2M monobasic sodium phosphate and 61.0 ml 0.2M
11 dibasic sodium phosphate diluted to 200ml, to control pH at 7).¹⁶

12 The same method was used to determine the effect of hypochlorite on intracellular
13 metabolites by using FT-IR as a metabolite detection method.^{25,26} Hypochlorite
14 treatments were performed before incubation, and growth was stopped by transferring
15 the bacterial suspensions from each flask to 50 ml plastic vials held on ice.
16 Metabolites were extracted as follows: bacteria were harvested by centrifugation (10
17 min at 2851g) at 4°C, and the pellet washed twice with fresh ice-cooled nutrient
18 broth. The collected cells were re-suspended in 1 ml distilled water and lysed by
19 adding 2 volumes of hot ethanol (50°C).²⁷ Excess ethanol was removed by boiling for
20 30 min. Debris was removed by centrifugation in an Eppendorf centrifuge (9300g) for
21 10 min at 4°C. Samples were freeze-dried and the resulting powders were investigated
22 by FT-IR.^{25,26}

23

24

**1 Comparing the effects of N-halamine biocidal polymer (2) and sodium
2 hypochlorite treatments on bacterial viability.**

3 Five universal bottles containing nutrient broth (10 ml each) were inoculated with 0.1
4 ml of *E. coli* suspension, previously grown for 17 h at 37°C, and incubated at 37°C
5 until OD 0.6 (while the cells were actively growing in logarithmic phase). The
6 bacterial suspensions were treated with either chlorinated polymer (0.8 g), non-
7 halogenated polymer (0.8 g) (as the polymer control), halogenated polymer (0.8 g)
8 contained within a semi-permeable membrane, or sodium hypochlorite (0.008 %
9 w/w). A bacterial control was also included. The vessels were stirred and growth was
10 estimated by viable counts at timed intervals. The experiment was repeated using 0.9
11 g of polymer and changing the sodium hypochlorite concentration to 0.01 % (w/w) to
12 determine the concentration of polymer that would produce an equivalent effect to
13 sodium hypochlorite concentrations between 0.008% - 0.01% w/w.

14 The semi-permeable membrane was dialysis tubing, nominal molecular weight cut-off
15 12-14 kDa. The tube (10-20 cm length) was first boiled with a mixture of 1 mmole of
16 the disodium salt of ethylenediaminetetracetic acid and 2% w/w sodium hydrogen
17 carbonate (20 ml) for 10 min, then removed and cleaned by boiling with distilled
18 water (20 ml) for 10 min. The cleaning process was repeated twice, followed by
19 sterilization by autoclaving in a glass bottle.

**20 Determination of the changes in metabolite profile induced by treatment with the
21 halogenated polymer**

22 Bacterial suspensions were prepared as described above; three 100 ml bottles, each
23 containing 30 ml nutrient broth were treated with the following: chlorinated polymer
24 (3 g), non-halogenated polymer (3 g) (polymer control), chlorinated polymer (3 g)

1 within a semi-permeable membrane (as above), sodium hypochlorite (0.008% w/w).
2 One bottle was used as a bacterial control. All vessels were stirred and samples (5 ml)
3 were taken at different time intervals (15, 30 and 60 min). All treatments were
4 performed at ambient temperature.

5 Metabolites were extracted as follows: Samples (5 ml) were taken using pre-cooled
6 syringes (-50°C) containing the quenching fluid (15 ml 60% v/v aqueous methanol
7 containing 70 mM HEPES). The samples were centrifuged for 5 min at 9300g and
8 4°C, and the supernatant removed and stored at -20°C. The remaining cell-pellet was
9 resuspended by vortexing in a methanol-water mixture (500 µl) containing 0.5 mole
10 cGMP. Perchloric acid (35%; 2 ml) was added, and the pellets frozen at -80 °C.
11 Samples were thawed and centrifuged at 9300g and 4°C for 30 min to remove
12 proteins and cell debris. The clear supernatant was transferred to a fresh vial and
13 neutralized by adding 1 ml of 5 M potassium carbonate solution. The suspensions
14 were centrifuged at 9300g and 4°C for 5 min to remove the precipitated perchlorates
15 and the resulting supernatant was freeze-dried.²⁸

16 A fraction of the freeze-dried samples was weighed and used for FT-IR analysis. The
17 remaining freeze-dried material was extracted with acetonitrile/ammonium acetate
18 buffer solution (9:1) (v/v). The samples were vortexed and then centrifuged at 9300g
19 at 4°C. The supernatant was collected for LC-MS analysis.

20

21 **RESULTS AND DISCUSSION**

22

23 **Effect of the exposure of bacterial cultures to sodium hypochlorite**

1 The effect of sodium hypochlorite on the viability of *E. coli* cultures is greatly
2 dependent on concentration and exposure time (Figure 1). No viable cells were
3 detected after a 1hr exposure to 0.02% hypochlorite; however, cultures exposed to a
4 concentration of 0.016% or lower continued to be viable, albeit at decreased levels.
5 These viable hypochlorite-treated cells were subjected to metabolomic analysis.

6 7 **Assessment of the effects of different forms of halogen ions delivery on bacteria** 8 **using metabolic profiling (FT-IR)**

9 We investigated the use of FT-IR analysis as an approach that could be used to
10 determine the effect of sub-lethal hypochlorite doses on the intracellular metabolite
11 profile. No significant qualitative changes due to increasing concentrations of sodium
12 hypochlorite were observed in the FT-IR spectrum in the wavelength region 500-4000
13 cm^{-1} (Figure 2). However, small quantitative differences between treated and
14 untreated cell lysates were observed at 3280 cm^{-1} (OH and NH), 2200-3400 cm^{-1} (OH
15 carboxylic acid), 2952 cm^{-1} (CH aliphatic), 1654 cm^{-1} (C=O, amide) 1520 cm^{-1} (C=C
16 alkene and C=N imino), and 1112 cm^{-1} (C-O, ether, and C-N, amines). A Principal
17 Components Analysis (PCA) was carried out for the FT-IR spectra of lysates of cells
18 exposed to a range of concentrations of sodium hypochlorite. Taking the first
19 principal component (which accounted for the majority of the variance between the
20 data sets) indicated that the effect of sodium hypochlorite, at different concentrations,
21 was progressive and qualitatively consistent (Figure 3). Increasing the hypochlorite
22 concentration increased the difference between the positions of the clusters in a
23 directly proportional relationship.

Effect of the different mechanisms of chlorine ions delivery

Previous work from us and other researchers^{12,13,15,16,22,23} suggested that different cytotoxic mechanisms can occur, depending on whether bacterial cells come into physical contact with halogenated polymer (contact mechanism) or are simply exposed to released halogen ions from the polymer. We observed different rates of bactericidal effect depending on whether the polymer was enclosed in a semi-permeable membrane (cell death due to released halogen ions alone) or was free to make contact with the cells (Figure 4). It can be seen that the effect was dependent on the concentration of polymer. At a concentration of 80 mg.ml⁻¹ little difference was observed between the biocidal activity of enclosed or exposed polymer, which was very low at the first 1 h of contact (Figure 4a). However, at a concentration of 90 mg.ml⁻¹, a significantly greater bactericidal rate was observed with the exposed polymer (Figure 4b). This observation is consistent with the existence of a separate contact mechanism, which requires a threshold concentration of polymer to become apparent. Cultures exhibited the expected progressive susceptibility to sodium hypochlorite at different concentrations with a very close effect to that of the halogenated polymer in the first hour of contact (Figure 4a). These results determine the most effective sub-lethal concentrations of the halogenated polymer and sodium hypochlorite on the intracellular metabolite profiles.

To investigate this phenomenon further taking sodium hypochlorite as example, cultures were subjected to a range of concentrations of sodium hypochlorite with and without pH control, and the growth was followed with time (Figures 5a and 5b). The lowest effective concentration on *E. coli* growth, with and without pH control was found to be 0.08 mg ml⁻¹ (Figures 5a and 5b).

FT-IR analysis was carried out on these cultures and the resultant spectra subjected to PCA. This analysis enabled the resolution of the various treatments in PCA space, and samples exposed to halogenated polymer were clearly distinguishable as a separate class (Figure 6, class 3). Interestingly, PCA was able to discriminate between cells exposed to non-halogenated polymer and the control (Figure 6, classes 1 and 2). Since cell viability was unaffected by exposure to non-halogenated polymer (Figure 4), we speculate that the resolution of these samples must have been due to a secondary effect (*i.e.* other than cytotoxicity) consistent with the influence of the second principal component (PC2) on the separation of these samples.

LC-MS analysis

PCA of the results obtained from the LC-MS analysis resulted in overlapping plots of the spectra from cultures treated with polymer enclosed in the semi-permeable membrane (Figure 7, class 4) and cultures treated with sodium hypochlorite (Figure 7, class 5). This is presumably more reflective of the *in vivo* situation than the clustering obtained with FT-IR, since no qualitative difference between the effects of enclosed polymer and free hypochlorite would be expected. Cultures treated with enclosed polymer were assumed to be affected by hypochlorous acid, formed by the chlorine ions released to water, which can affect the cells by a similar mechanism as sodium hypochlorite (Equation 1), possibly reflecting the greater potential of LC-MS for providing distinguishing data, derived from resolving the samples into a series of fractions. Treatments with both halogenated and non-halogenated polymer resulted in overlapping clusters (Figure 7, clusters 2 and 3), providing further evidence for an effect on the metabolome, derived from the polymer itself. Differentiation using FT-IR spectra is based on a comparison of the intensity of functional groups within a

1 population of metabolites, whereas LC-MS-based differentiation derives from
2 differences in molecular masses and chromatography. This may help explain why LC-
3 MS appears to provide more effective discrimination of the different treatments when
4 PCA is applied. Using this assumption, LC-MS data can be used to recognize peaks
5 that may correspond to potential biomarker molecules.

7 **Putative potential biomarkers**

8 The LC-MS analysis performed on the samples provides a preliminary set of potential
9 biomarkers that could be used to elucidate the mode of action of the halogenated
10 polymers. Some of the peaks show significant differences between the control and the
11 treated cells, suggesting that those differences were due mainly to the effect of the
12 polymers on the metabolism of the cells, and not to the direct chlorination of
13 metabolites. Among them, the peaks corresponding to molecular masses of 101.1, 172
14 and 242 Da are of particular interest as those showed the most significant changes in
15 peak intensity.

16 The peak corresponding to a molecular mass of 101.1 was detectable after 30 min
17 exposure to all of the experimental treatments but not in the untreated control (Figure
18 8). Although it has been reported that the antimicrobial effect of HOCl is due to the
19 chlorination of different cellular components (such as amino acids, peptides, lipids,
20 etc.)^{19,30} it is unlikely that this metabolite is a chlorinated derivative of an existing
21 metabolite: this peak was also detected in cultures treated with the non-halogenated
22 polymer, so it presumably indicates significantly elevated levels of a metabolite with
23 a molecular mass of 101.1 Da that might have been released when the cells were
24 exposed to a sub-lethal treatment. It has to be noted that most of the studies in the

1 literature were performed with lethal concentrations of chlorinating agents ¹⁹ or in
2 experimental systems reproducing the killing of bacteria by phagocytosis ³¹ while the
3 purpose of our system was the study of sub-lethal concentrations of the antimicrobial
4 agent.

5 The exposed halogenated polymer specifically caused increased levels of ionic
6 species such as the compound represented by the ion peak with molecular mass 172
7 (consistent with the glycolytic triose phosphates glyceraldehyde-3-phosphate and
8 dihydroxyacetone phosphate) (Figure 9). The other treatments such as exposure to the
9 halogenated polymer contained by the semi-permeable membrane reduced the
10 concentration of this metabolite to non-detectable levels.^{32,33}

11 A hypothesis which may explain this finding is that the effect of sub-lethal
12 concentrations of HOCl inhibits the activity of glyceraldehyde 3-phosphate
13 dehydrogenase (GAPD). It has been long known that inhibiting this enzyme causes an
14 accumulation of its substrate, glyceraldehyde 3-phosphate ³² The mechanism of
15 inhibition of GAPD by oxidative stress has been identified in yeasts and other
16 eukaryotic cells.^{34,35} In those cases, the inactivation of this enzyme serves the function
17 of “metabolic switch”, through a mechanism that redirects the metabolic fluxes to
18 counteract oxidative stress, causing an increase in the concentration of those
19 glycolytic metabolites, glyceraldehyde-3-phosphate and dihydroxy acetone
20 phosphate.³⁴ Our results are in agreement with those reports.

21 All chlorinating treatments resulted in a reduction in the metabolite corresponding to
22 a molecular mass of 242 (consistent with thymidine) (Figure 10). Halogenation of
23 thymidine would, presumably, inhibit DNA synthesis, resulting in bactericidal
24 activity.

1 The interaction of HOCl and DNA causes a complex series of reactions.^{36,37}
2 Pyrimidine nucleotides are chlorinated by a fast reaction, and the chlorinated
3 derivatives of thymidine obtained are very reactive towards different intracellular
4 substrates, such as glutathione, disulfides, aliphatic amines and amino acids and
5 NADH. The oxidation of the latter may lead to the redirection of metabolic fluxes
6 (and eventually cell death) that is reflected in the changes observed in the pool of
7 intracellular metabolites.

8 We have previously reported the effect of the non-halogenated polymer on bacterial
9 growth.¹⁶ We speculated that the effect could be due to attachment of bacterial cells to
10 the polymer surface.¹⁶ However, the studies reported here are consistent with an effect
11 of the non-halogenated polymer on the metabolome. The non-halogenated polymer
12 contains amide bonds which, in theory, would be able to complex with metal ions
13 exchanged through the cell wall. It also has the potential to consume hydrogen ions
14 easily which could disturb the equilibrium between intra- and extra-cellular protons,
15 potentially having a deleterious effect on numerous metabolic processes. We
16 succeeded in preparing some poly quaternary ammonium salts from similar structures
17 by acidification, demonstrating the bactericidal activity of hydrogen ions.¹⁴

18 The previous results indicated that the halogenated polymer enclosed in a semi-
19 permeable membrane has an effect on the bacterial metabolites consistent with our
20 assertion that the halogenated polymer is able to affect the cells by halogen ions
21 release and not by contact only as assumed by other authors.^{12,13,23} At the same time,
22 the distinguishable difference between the clusters, in both FT-IR- and LCMS-PCA of
23 metabolites treated with the halogenated polymer directly and the enclosed
24 halogenated polymer indicated that there is an additional effect from the polymer *per*
25 *se*. This also contrasts with the assertion that the mode of action of this type of

polymer derives solely from halogen ions release into the aqueous environment.²²

These results support our previous hypothesis that the mode of action of N-halamine polymers is a combined effect of contact, release and changes in the cell environment.^{15,16}

These results encourage further efforts to explain the actual mechanism of the bacterial cells' death by the action of halogen containing compounds as well as the free halogen. In addition, they suggest that an investigation on the effect of the non-halogenated heterocyclic polymers on the ionic equilibrium through the cell could explain the mechanism of action of these compounds.

CONCLUSION

We have used metabolomic analysis for hypothesis generation concerning the bactericidal effect of chlorine ions, in various delivery systems. Although the bactericidal effect of halogens has been known for many years, it had not, hitherto, been characterized in this way. Halogen containing compounds had an effect on bacterial metabolites as well as the non-halogenated heterocyclic polymers. The effect of sodium hypochlorite on the bacterial metabolome was quantitatively dependent on the hypochlorite concentration. The mode of action of N-halamine polymer is now assumed to reflect a combination of contact, release and, possibly, a modification of the nature of the cell environment. We hypothesise that the non-halogenated polymers as well as the non-halogenated repeating units in the N-halamine polymers may affect the metabolome, perhaps by disturbing the ionic equilibrium between intra- and extra-cellular positive charges.

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2 **Scheme 1** Preparation of cross-linked polyepicyanurohydrin and its halogenation.

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2 **Figure 1** Effect of exposure to sodium hypochlorite on viability of 17h *E. coli*
 3 cultures.

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2 **Figure 2** FT-IR spectra (500-4000 cm⁻¹) of *E. coli* cell lysates previously treated
 3 (with 0.008% sodium hypochlorite, w/w) compared to non-treated cells metabolites.

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2 **Figure 3** Principal Components Analysis of the FT-IR spectra of cell lysates of *E. coli*
 3 cultures exposed to a range of concentrations of sodium hypochlorite.

1 **Figure 4** Effect of different treatments on *E. coli* viability using two different

2 concentrations of each treatment; halogenated polymer 80 mg.ml⁻¹ (a) and 90 mg.ml⁻¹
3 (b), direct (T1 and T2) and enclosed in semi-porous membrane (TS1 and TS2), and
4 sodium hypochlorite 0.008 % (a, TH1) and 0.01 % (b, TH2) compared to bacterial
5 control (BC) and non-halogenated polymer control (PC). In cases where the standard
6 errors are too small to display, the relevant error bars have been omitted.

1 **Figures 5a and 5b** Effect of different sodium hypochlorite concentrations (% w/w)
2 on time interval required to obtain OD = 0.4 for *E. coli*, (a) without, and (b) with pH
3 control. In cases where the standard errors are too small to display, the relevant error
4 bars have been omitted.

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2 **Figure 6** Principal components analysis of the FT-IR spectra of cell lysates of
 3 cultures exposed, for 30 minutes, to various bactericidal treatments. Class 1: untreated
 4 control culture; Class 2: culture treated with non-halogenated polymer; Class 3:
 5 culture treated with halogenated polymer; Class 4: culture treated with the
 6 halogenated polymer enclosed in a semi-permeable membrane; Class 5: culture
 7 treated with sodium hypochlorite. Points with the same symbol represent replicates.

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15 **Equation 1** HOCl formation due to chlorine ions released from the N-halamines.

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Figure 7 Principal components analysis of the LC-MS data of cell lysates of cultures exposed, for 30 minutes, to various bactericidal treatments. Class 1: untreated control culture; Class 2: culture treated with non-halogenated polymer; Class 3: culture treated with halogenated polymer; Class 4: culture treated with the halogenated polymer enclosed in a semi-permeable membrane; Class 5: culture treated with sodium hypochlorite. Points with the same symbol represent replicates.

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Figure 9 Comparison between the intensity of the ion peak of a compound with molecular mass 172 at different treatments. Where: BC is the bacterial control, PC non-halogenated polymer, T halogenated polymer, TS halogenated polymer enclosed in a semi-permeable membrane and TH is the sodium hypochlorite.

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2 **Figure 10** Comparison between the intensity of the ionic species with a molecular
3 mass of 242 at different treatments. Where: BC is the bacterial control, PC non-
4 halogenated polymer, T halogenated polymer, TS halogenated polymer enclosed in a
5 semi-permeable membrane and TH is the sodium hypochlorite.

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