Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713926090

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To cite this Article Woolverton, Christopher J. , Gustely, Erin , Li, Lanfang and Lavrentovich, Oleg D.(2005) 'Liquid crystal effects on bacterial viability', Liquid Crystals, 32: 4, 417 — 423
To link to this Article: DOI: 10.1080/02678290500074822
URL: http://dx.doi.org/10.1080/02678290500074822

Liquid Crystals, Vol. 32, No. 4, April 2005, 417–423

Liquid crystal effects on bacterial viability

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(*Received 5 October 2004; accepted 21 November 2004*)

The primary objective of this research was to test the hypothesis that lyotropic chromonic liquid crystals (neutral grey, red 14, blue 27, cromolyn) are not toxic to bacteria as compared with surfactant-based lyotropic (CPCl and CsPFO) or thermotropic (5CB and E7) liquid crystals. Biocompatibility of most liquid crystals is currently unknown and is required for the development of systems interfacing liquid crystals and biological systems. Potential liquid crystal toxicity was evaluated by two methods. The first examined bacterial survival measured by bacterial growth over 24 hours, after exposure to various liquid crystals. The second toxicity method evaluated liquid crystal effects on bacterial membrane permeability using two fluorescent dyes. Three different types of bacteria were evaluated to assess bacterial structure differences with respect to liquid crystal toxicity. The results of this study indicate that lyotropic chromonic liquid crystals are not toxic to bacteria, whereas thermotropic and surfactant-based lyotropic liquid crystals are toxic to one or more forms of bacteria. We conclude that lyotropic chromonic liquid crystals may be the preferred material in designing liquid crystal-based systems that interact with biological systems, especially in the use of liquid crystal-based biosensors.

1. Introduction

The importance of the liquid crystalline state in the development and sustainability of life on earth was recognized long ago in the pioneering works by Bernal and others [1–5]. Liquid crystals (LCs) are found naturally in biologicial systems. For example, biopolymers such as chitin, collagen and DNA form ordered associations that are structurally similar to LCs; the biomolecular lipid membrane that encloses all cells resembles the structure of a smectic LC. Another facet of the LC–biology interface is that the structural organization at the supramolecular level that is so well studied for LCs might offer a path for consideration of morphogenesis in biological systems. Finally, there has been a development in using LCs as the working medium for biomedical applications such as drug delivery with controlled release [6–8], gene delivery [9], protein capture [10], lipid labelling [11] and detection of pathogens [12–18].

The recent reports of microbial biosensors based on liquid crystals [12–18] require data that define parameters around which the LCs, as the detecting medium, can be used without altering or destroying the organisms to be detected. In one embodiment [12, 17, 18], the biosensor technology exploits the director distortions and the corresponding change in light transmission when the inclusion alters LC alignment. The biological part of the approach is that each microbe has characteristic molecular groups (antigens) at the surface, to which the corresponding antibody can bind, thus 'recognizing' and 'detecting' it [19]. The physical problem is to amplify this highly selective binding. Amplification can be achieved if the binding triggers some other process accessible to optical detection: for example, a reorientation of the LC director. It is well known that the antigen–antibody binding can cause aggregation of the microbes into immune complexes (each antibody molecule has two binding sites) [19]. If such a complex can grow in the LC bulk and become larger than some critical size d_c defined as the ratio $d_c = K/W$ of the characteristic LC elastic constant K to the anchoring coefficient at the complex–LC interface, W, then it should cause director distortions and thus light transmittance through the properly chosen optical set-up. In other words, detection is based on the director distortions around immune complexes that grow inside the LC above the critical size d_c [17].

Clearly, this mechanism of detection, as well as others such as that based on surface anchoring transitions [10]

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Liquid Crystals
ISSN 0267-8292 print/ISSN 1366-5855 online © 2005 Taylor & Francis Group Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/02678290500074822

requires a certain compatibility of the species to be detected and the LC medium. Compatibility with the microbial antigens is essential for aggregation and thus for detection to occur. Loss of structural integrity resulting from microbial damage or lysis may prevent antigen recognition and significantly decrease the effectiveness of a biosensor. This study was therefore designed to evaluate the effect of eight liquid crystalline materials on the viability of three very different prokaryotes.

We report here the results of evaluation of six lyotropic liquid crystalline materials and two thermotropic materials on the viability (toxicity) of *Staphylococcus aureus* (a Gram-positive coccus), *Escherichia coli* (a Gram-negative rod) and *Bacillus atrophaeus* (a Gram-positive spore-forming rod). These model bacteria were chosen as examples of bacteria with thick cell walls (Gram-positive), thin cell walls (Gram-negative) and very thick cell walls complexed with calcium and dipicolinic acid (Gram-positive spores). (The bacterial cell wall is essentially carbohydrate 'scaffolding' around the cell that prevents osmotic lysis in hypo-osmotic environments.) We focus particularly on the lyotropic chromonic liquid crystals (LCLCs) as they are non-surfactant, aqueous LCs that represent a more physiologic environment to the microbes as compared with their surfactant-based lyotropic and their thermotropic LC counterparts.

LCLCs embrace a wide variety of dyes, drugs, and nucleic acids [20]. In contrast to surfactant-based lyotropic LCs, LCLC molecules are usually plank-like rather than rod-like, aromatic rather than aliphatic, and rigid rather than flexible. Face-to-face stacking of these flat molecules produces elongated aggregates that align parallel to each other once in water and thus form the simplest type of the liquid crystalline order, the so-called nematic phase [20]. LCLCs can be used to form new molecular architectures, such as nanometer-thick films with long range orientational order [21]. Additionally, these lyotropic LCs with chromonic mesophases may also be used to enhance the performance of standard twisted nematic display devices [22].

2. Experimental

2.1. *Materials*

Nutrient agar and trypticase soy agar supplemented with 5% sheep blood (SBA) were purchased from Becton Dickson Co. (Sparks, MD). Nutrient sporulation agar was prepared according to Dang [23] with all chemicals purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS, pH 7.4) and isopropyl alcohol were purchased from Sigma Chemical Co. Spore releasing buffer was made by adding 0.05% Tween 20 (Mallinckrodt Baker, Inc., Paris, KY) to PBS.

The liquid crystals Red 14, Blue 27 and Neutral Grey were obtained from Optiva (San Francisco, CA). Cromolyn (disodium cromoglycate) was purchased from Spectrum Chemical Manufacturing Corp., Gardena, CA. The LCs materials, 4-*n*-pentyl-4′-cyanobiphenyl (5CB) and the eutectic mixture of biphenyls and terphenyls (E7) were purchased from Merck (Whitehouse Station, NJ). Cetylpyridinium Chloride (CPCl) was purchased from Aldrich, and Cesium pentadecafluorooctanoate (CsPFO) was prepared at the Liquid Crystal Institute, Kent, OH, from chemicals purchased from Aldrich. The structure, phase and composition of these LCs used in this study are listed in table 1. All liquid crystals were used at room temperature (20°C).

2.2. *Bacteria and spores*

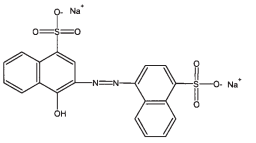
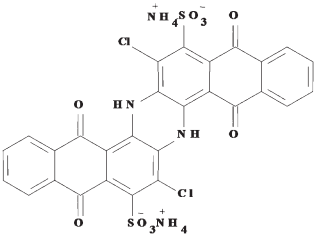
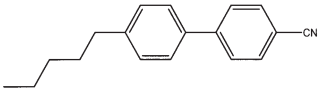
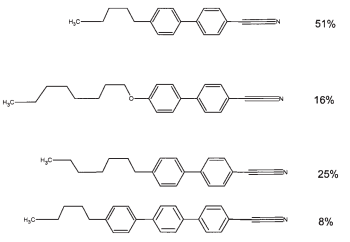
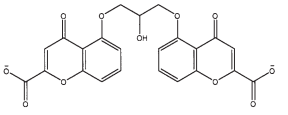
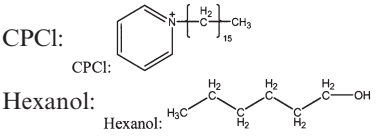
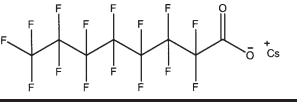
Staphylococcus aureus (# 49976), *Bacillus atrophaeus* (# 9372) and *Escherichia coli* K99 (# 31616) were purchased from the American Type Culture Collection, Manassas, VA. Each organism was sub-cultured daily on nutrient agar. Bacteria were cultured on nutrient agar at 36°C for 18 h. Bacteria were washed from the agar surface with PBS into sterile tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged at $2740 \times g$ for 10 min (IEC Centra MP4R, Needham Hts., MA). Bacteria were suspended as $10^8\,ml^{-1}$ in PBS and used within 1 h. The purity of each culture was confirmed by microscopy and colonial morphology; optical images of the bacteria and spores are shown in figure 1.

To evaluate the effects of LCs on chemically-resistant bacterial spores, *B. atrophaeus* cultures were grown on SBA at 36°C for 48 h, transferred to nutrient sporulation agar, incubated at 36°C for 48 h and then at 20°C for 2 weeks, to induce spore formation. Bacteria containing spores were washed from the agar surface with PBS into sterile tubes and centrifuged at 2740 g for 10 min. The bacterial pellet was suspended in spore releasing buffer, mixed thoroughly for 5 min and washed copiously with sterile, 18 MOhm deionized water (DW). The resulting spore suspension was adjusted to $10^9\,ml^{-1}$ and stored at 4°C until used. *B. atrophaeus* spores were also germinated into their vegetative form when cultured on nutrient agar overnight at 36°C. These Gram-positive rods were washed from the agar surface and prepared as described above, just before reacting with LCs.

2.3. *Viabilityltoxicity bioassay*

E. coli, S. aureus, B. atrophaeus or *B. atrophaeus* spores were added to sterile, 1 ml tubes and centrifuged

Table 1. Structure and composition of liquid crystal materials evaluated for toxicity to bacteria.

Liquid Crystal	Structure/chemical formula	Composition/wt%
Neutral Grey	Neutral Grey is a combination of several different dyes: Blue 27, Red 14, and Violet 20 in weight proportion 10:4:2.	12.09% Neutral Grey, 87.91% H_2O; nematic at r.t. [30]
Red 14		93.0% Red 14, 7.0% H_2O; hexagonal/isotropic biphasic at r.t. [30]
Blue 27		92.93% Blue 27, 7.07% H_2O; nematic at r.t. [30]
5CB		100% 5CB; nematic at r.t.
E7		100% E7; nematic at r.t., information from manufacturer.
Cromolyn		13% Cromolyn, 87% H_2O; nematic at r.t. [20]
CPCl/Hexanol/Brine		3.5% CPCl, 2.8% Hexanol (Hexanol/CPCl=0.8), 93.7% Brine (1% NaCl aqueous solution); L_α (lamellar) at r.t. [31]
CsPFO		33.5% CsPFO, 66.5% H_2O. Nematic at 17°C ± 5 [32, 33]

(13 000 rpm, 15 min). The bacterial or spore pellets were suspended in 0.1 ml of liquid crystal (or control) and the system reacted at room temperature for 15 min. The bacteria or spores were then centrifuged and the liquid crystal removed. The pellets were washed with sterile DW and resuspended to their original volume with DW. Bacteria and spores were diluted serially into sterile DW and aliquots transferred to nutrient agar for culture (18 h at 36°C). Bacterial colonies were counted at each plated dilution to determine the number of organisms remaining after exposure to the LCs. Toxicity was determined by significant decrease in bacterial growth (an indicator of viability). Colony count results were transformed as log colony count for statistical analyses. Additionally, log colony counts obtained for each LC treatment were subtracted from the log colony counts of the negative (DW) control. That difference was divided by the log DW counts and the quotient multiplied by 100 to represent % dead bacteria due to LC exposure. Each LC was tested on each organism at least in

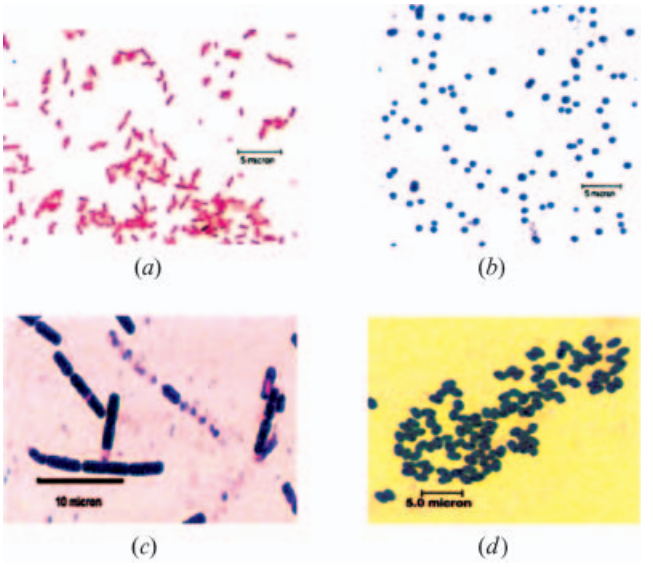


Figure 1. Optical images (bright field) of the three bacteria and the bacterial spores used in this study: (*a*) *E. coli* (Gram stain); (*b*) *S. aureus* (Gram stain); (*c*) *B. globigii* (Gram stain); (*d*) *B. globigii* Spores (Spore stain).

triplicate. DW was used as a sham treatment to control for microbe manipulations; 10% hypochlorous acid (in H_2O) was used as a positive control, killing 100% of the bacteria or spores.

2.4. *Viability/toxicity fluorescence assay*

The Live/Dead® Backlight™ Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR) was purchased and used according to the manufacturer's instructions to monitor the viability of bacteria as a function of cellular membrane integrity. Briefly, *E. coli* (8.3 log bacteria) were treated for 1 h with sterile DW (negative control) or 70% (v/v) isopropyl alcohol (positive control). Bacteria were washed with sterile DW and mixed proportionally to determine the % live bacteria once stained with fluorescent markers of viable and dead cells [24]. Live bacteria were treated with LC samples for 15 min, washed free of the LC (as described above) and also stained according to kit instructions. LC-treated cells were determined as percent viable when % viable bacteria was extrapolated from the curve of fluorescence intensity versus proportionally live cells. Fluorescence was measured with a 7620 Microplate Fluorometer (Cambridge Technologies, Inc., Cambridge, MA) using 485 nm (excitation) with 530 and 630 nm (emission), respectively for green and red fluorescence.

2.5. *Statistical analyses*

The log colony count data were evaluated by the One-way Analysis of Variance to determine variation between data set means [25]. The analysis determines the probability (P) that the data set means are equal within a confidence interval of 95% ($P \leqslant 0.05$). The Tukey-Kramer Multiple comparisons test [25] was used *post hoc* to determine significant differences between treatment groups and the DW control.

3. Results and discussion

3.1. *Bioassay*

The development of liquid crystal-based biosensors that exploit the physical and chemical properties of liquid crystalline materials to report the detection of harmful bacteria, requires that the LCs not interfere with the mechanism(s) by which bacteria are detected. The use of lyotropic liquid crystals to report and amplify receptor-mediated microbe detection presents an interesting application of LC anisotropy [17]. However, LC induced lysis, surface alteration and/or removal of microbial ligands (to which biosensor receptors bind) would obviate the use of LCs in the biosensor. The direct exposure of bacteria to potentially toxic agents and their subsequent lack of growth (in a nutrient medium) represent a biological assay of toxicity. Bacteria that are injured beyond survival will not replicate and will not form colonies in the agar medium. Viable bacteria will replicate in the agar medium and form countable colonies. The differences between the LC-treated and the DW-treated bacteria (log transformed counts) represent the absolute decrease in bacteria resulting from LC effects.

Of the *c*. 8 log *E. coli*, *S. aureus* and *B. atrophaeus* spores treated with water, 8.08 ± 0.172 log bacteria, 7.76 ± 0.168 log bacteria and 7.54 ± 0.220 log bacteria were recovered, respectively. Ten percent hypochlorous acid destroyed all bacteria and spores, resulting in no growth in nutrient agar after treatment (data not shown). The results of LC toxicity to bacteria using the bioassay are reported in figure 2. Of note is the fact that treatment with CPCl and CsPFO significantly inhibited the growth of *E. coli* ($P<0.001$) and *S. aureus* ($P<0.001$). Germination and growth of *B. atrophaeus* spores were not inhibited by CPCl (7.75 ± 0.272 log spores) as compared with the control (7.54 ± 0.220 log spores). Figure 3 reports that germination and growth of *B. atrophaeus* spores were inhibited by 5CB ($P<0.01$) and E7 ($P<0.001$). In other experiments, *B. atrophaeus* spores were germinated in nutrient agar prior to exposure to 5CB and E7. Interestingly, the vegetative form of *B. atrophaeus* treated with 5CB or E7 grew equally well as the DW-treated control bacteria (figure 3) suggesting that 5CB and E7 are selectively toxic to the spore form of *B. atrophaeus*.

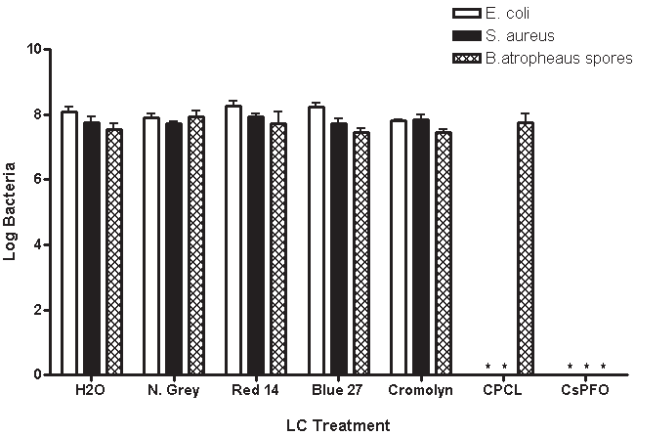


Figure 2. Cytotoxic effects of lyotropic liquid crystals on three bacterial species. *E. coli* (white bars), *S. aureus* (black bars) and spores of *B. atrophaeus* (hatched bars) were exposed to LLCs for 15 min at r.t., washed free of LLCs, serially diluted and cultured on nutrient agar at 36°C for 18 h. Asterisk (*) indicates statistical difference from the water control ($P<0.001$).

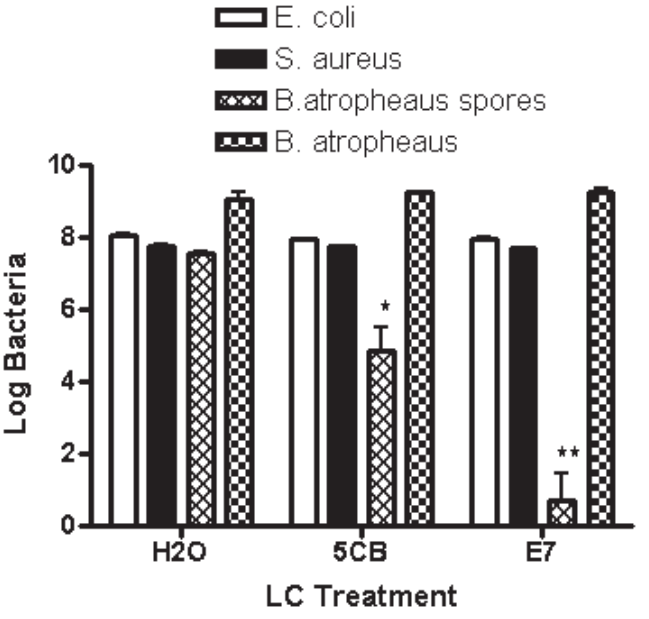


Figure 3. Cytotoxic effects of thermotropic liquid crystals on three metabolically active bacterial species and the dormant spore stage of one species. *E. coli* (white bars), *S. aureus* (black bars), spores of *B. atrophaeus* (hatched bars), and *B. atrophaeus* (checkered bars) were exposed to LCs for 15 min at r.t., washed free of LCs, serially diluted and cultured on nutrient agar at 36°C for 18 h. Asterisks indicates statistical difference from the water control (*$P<0.01$, **$P<0.001$).

3.2. *Fluorescence assay*

The Live/Dead® Backlight™ Bacterial Viability Kit quantitatively distinguishes live cells from dead by using two nucleic acid stains. These fluorescent dyes (SYTO 9 and propidium iodide) differ in their ability to penetrate bacterial cells. SYTO 9 stains both live and dead cells. Propidium iodide (PI) only stains cells with damaged membranes. In cells containing both SYTO 9 and PI, the green SYTO 9 fluorescence is masked by the red PI fluorescence. Thus, viable bacteria fluoresce green while damaged and dead bacteria fluoresce red. Extrapolation of live/dead data from the green/red fluorescence regression line is then used to determine viability of LC treated bacteria. The results of treating *E. coli* with various LCs prior to testing with the viability kit are presented in table 2, and are expressed as percent (%) dead. The Live/Dead® Backlight™ Bacterial Viability assay also documented that CPCl and CsPFO were toxic to *E. coli* while the chromonic LCs were not toxic.

3.3. *Liquid crystal chemistry and toxicity*

Considering the substantial use of liquid crystal devices, it is noteworthy that relatively few studies examining liquid crystal toxicity are in the public domain. Weber and Richter evaluated the acute toxic effects of the liquid crystalline 3-beta-chlorosteroids (i.e. cholesteryl beta-chloride and sitosteryl beta-chloride) in mice. After feeding LCs to mice over 3 months, they found no indication of irreversible cell damage in the stomach, intestine, liver, kidneys or spleen. However, they did not test for cytotoxicity or chronic toxicity [26]. Luk *et al.* [27] examined several thermotropic materials, e.g. 1-(4-ethylcyclohexyl)-4-[2-(4-pentylcyclohexyl)ethyl]benzene and 1-ethyl-4-(4-pentylcyclohexyl)benzene to which ether or ester groups were added, for acute (4 h exposure) cytotoxicity to eukaryotic cells using a fluorescent indicator of membrane integrity. They also tested 5CB, E7 and several LC materials having fluorinated functional groups. They found that cytotoxicity of the eight LCs studied correlated with the chemical functional group(s) of the LCs. All of the olefin-based thermotropic materials were acutely toxic to the cultured cells. Of note, 5CB and E7 were also cytotoxic while the fluorophenyl compounds were not.

Table 2. Summary of LC toxicity using The Live/Dead® Backlight™ Bacterial Viability Kit.

Liquid Crystal	% Dead
CPCl	91.3
CsPFO	85.4
Blue 27	0
Red 14	0
Cromolyn13% wt %	0

Direct correlation with this current study is not possible due to the markedly different assay conditions. However, it is worth noting that both prokaryotes and eukaryotes should be evaluated for toxicity, as both cell types are to be used in LC biosensor systems. Furthermore, systematic evaluations of structure–function relations should be completed on each LC family, for each biological system to be used.

We also evaluated two surfactant-based lyotropic LCs. CPCl has been used for many years as a broad spectrum biocide in hospitals, personal hygiene products and the food industry. CPCl is a quaternary ammonium compound and cationic surfactant, containing a long, hydrophobic aliphatic chain. The bactericidal activity of CPCl results from cell disruption and protein denaturation at the membrane. CPCl is also toxic to many animal species (by oral and inhalation routes) and can cause severe irritation to cells at mucus membranes [28]. The CPCl concentration of 0.8% used in this study is analogous to concentrations (0.1–1.0%) used to control bacterial growth on food or humans [28]. Thus, CPCl toxicity to bacterial cells was expected. The antibacterial activity of CsPFO, however, was previously unknown. The extensive fluorine substitutions of the aliphatic chain, along with its ionic cesium, suggest that CsPFO should be highly reactive toward biological membranes and their proteins, undermining cell integrity. The data herein suggest that CsPFO is quite toxic to bacteria. In comparing the fluorescence and the biological assays evaluating CPCl and CsPFO, the data suggest that a 15 min exposure to CPCl and CsPFO is sufficient to kill 85–90% of *E. coli* (by fluorescence staining) and that this exposure initiates a complete loss of *E. coli* viability (measured by bioassay).

The sporicidal effects of 5CB and E7 were also previously unknown. The toxicity of 5CB (and of E7) is suggested by the material safety data sheet [29]. But no public data regarding acute toxicity, mutagenicity, chronic exposure or ecological implications are available. The mechanisms that lead to the difference in the observed action of thermotropic materials 5CB and E7 on bacterial spores and on metabolically viable, vegetative bacteria remain undetermined. One possibility would be a different membrane and wall structure of spores and vegetative bacteria; 5CB and E7 may uniquely bind or enter bacterial spores and thus inhibit spore germination and growth of the vegetative cell.

4. Conclusions

In summary, we have evaluated the effects of eight LCs on the viability of three prokaryotic species. Bacteria were exposed to LCs, washed free of the LC and examined for membrane integrity by fluorescence and for survival in a bioassay. We find that the lyotropic chromonic LCs (neutral grey, red 14, blue 27 and disodium cromoglycate) are not toxic to *S. aureus*, *E. coli* or the spore form of *B. atrophaeus*. In contrast, CPCl and CsPFO were toxic to vegetative cells but not to the spores. Furthermore, 5CB and E7 were toxic to the spores but not to vegetative bacteria. The bactericidal activities of CsPFO and the sporicidal activities of 5CB and E7 are unknown and are the subject of future research.

Acknowledgements

We acknowledge Shannon Helfinstine for technical assistance. This research was partially supported by the National Science Foundation and the Intelligence Technology Innovation Center through the joint 'Approaches to Combat Terrorism' Program Solicitation NSF 03-569 (DMR-0346348) and by an Ohio Board of Reagents Research Challenge Grant. We gratefully acknowledge the generous donation by Mrs Glenn H. (Jessie) Brown in support of this research. Summer undergraduate research of E. Gustely was supported by the US Depatment of Education fund for Center for Liquid Crystal Science and Education at the Liquid Crystal Institute.

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