Research Paper

Electropermeabilization of nematode eggs for parasite deactivation

M. H. Dryzer, C. Niven, S. D. Wolter, C. B. Arena, E. Ngaboyamahina, C. B. Parker and B. R. Stoner

ABSTRACT

The eggs of parasitic helminth worms are incredibly resilient – possessing the ability to survive changing environmental factors and exposure to chemical treatments – which has restricted the efficacy of wastewater sanitation. This research reports on the effectiveness of electroporation to permeabilize ova of *Caenorhabditis elegans* (*C. elegans*), a helminth surrogate, for parasite deactivation. This technique utilizes electric pulses to increase cell membrane permeability in its conventional application, but herein is used to open pores in nonparasitic nematode eggshells – the first report of such an application to the best knowledge of the authors. A parametric evaluation of electric field strength and total electroporation duration of eggs and worms in phosphate-buffered saline was performed using a 1 Hz pulse train of 0.01% duty cycle. The extent of pore formation was determined using a fluorescent label, propidium iodide, targeting *C. elegans* embryonic DNA. The results of this research demonstrate that electroporation increases eggshell permeability. This treatment, coupled with existing methods of electrochemical disinfection, could improve upon current attempts at the deactivation of helminth eggs. We discuss electroporation treatment conditions and likely modification of the lipid-rich permeability barrier within the eggshell strata.

Key words | *Caenorhabditis elegans*, eggshell permeability, electroporation, parasitic helminth eggs, propidium iodide staining, wastewater sanitation

M. H. Dryzer (corresponding author)
C. Niven
S. D. Wolter
Department of Physics,
Elon University,
Elon, NC 27244,
USA
E-mail: *mdryzer@elon.edu*

C. B. Arena
Department of Biomedical Engineering and Mechanics,
Virginia Tech,
Blacksburg, VA 24061,
USA

E. Ngaboyamahina
C. B. Parker
B. R. Stoner
Department of Electrical and Computer Engineering,
Duke University,
Durham, NC 27708,
USA
and
Center for WaSH-AID,
Duke University,
Durham, NC 27708,
USA

INTRODUCTION

Posited as a public health risk by the World Health Assembly in 2001, helminths are a virulent family of parasites prominent in the developing world with various species together thought to have had infected over half of the world's population (Horton 2003). Helminth eggs are incredibly resilient, possessing the ability to survive changing environmental factors and exposure to various chemical treatments (Wharton 1983; Lysek *et al.* 1985) and while conventional sanitization methods (i.e., chlorination or oxidation) are able to inactivate the eggs (Alouni & Jemli 2001; Bandala *et al.* 2012), they are largely inefficient in doing so. Other studies have expanded the capabilities of conventional methods by enhancing and expediting their effects with photochemistry (Alouni & Jemli 2001; Bandala *et al.* 2012). The work presented here follows a similar

doi: 10.2166/washdev.2019.100

trend by combining traditional electrochemical treatments with electroporation with the prospect of finding a cost-effective and sustainable means of sanitization.

The use of electroporation for wastewater treatment and destruction of helminths offers a practical approach to parasite remediation. Electroporation is a highly versatile technique that utilizes pulsed electric fields to open pores in lipid bilayer membranes of eukaryotic cells (Ivorra & Rubinsky 2010; Rems & Miklavčič 2016) and Gram-negative and Gram-positive bacteria (Daly *et al.* 2011; Rauch & Leigh 2015). As such, this electrophysical technique has made headway in the medical, biomedical, and food sanitization industries as a method of pathogen elimination and cellular and tissue manipulation (Ivorra & Rubinsky 2010; Rems & Miklavčič 2016). The interest in exploring electroporation for helminth remediation stems from its potential impact on the lipid-rich permeability barrier within the eggshell of parasitic ova. While there are functional similarities to cell membranes, there presently exists no information on parasite eggshell electropermeabilization.

The construct of helminth eggshells is quite complex, generally consisting of multilayer strata whereby each layer contributes to its overall resiliency (Wharton 1983; Lysek *et al.* 1985; Perry & Moens 2001; Jimenez-Cisneros & Maya-Rendon 2007). Recent work signifies the complexity of the eggshell, as reported for the nematode *Caenorhabditis elegans* (Stein & Golden 2015) – widely recognized as a model organism for parasitic nematodes (Holden-Dye & Walker 2014). Consistent with prior reports on the structure of nematode eggshells, the vitelline layer, a lipoprotein layer that acts as the first line of defense for the egg, and the chitinous layer, that provides the egg with structural support and mechanical strength, are believed to initially form which provide the physical basis for the establishment of additional layers (Stein & Golden 2015; Olson *et al.* 2018). In combination with a proteoglycan chondroitin layer, these three outer layers form the trilaminar outer eggshell (Stein & Golden 2015). Located below the trilaminar strata is the lipid-rich permeability barrier, situated between the extra-embryonic matrix and the peri-embryonic layer. This permeability barrier is crucial to the well-being of the embryo by resisting molecular intrusion while maintaining proper osmotic conditions and enabling function of signaling molecules during embryo development (Stein & Golden 2015). Approaches to increasing the permeability of the lipid-rich layer may be useful for chemical treatment and destruction of harmful parasites.

This paper reports on the application of electroporation for permeabilization of the ova of *C. elegans*, a nonparasitic helminth surrogate. It will be shown that pulsed electric fields may be used to increase the permeability of the nematode eggshell using fluorescence bioimaging. We overview electroporation parameters used for apparent pore formation in nematode eggshells performed in simple buffer solution.

MATERIALS AND METHODS

Caenorhabditis elegans were chosen for study as a suitable proxy for helminths given safety concerns regarding parasite handling. This nematode species has been exploited in prior work studying the effects of anthelmintic drugs since, while being nonparasitic, it is genetically close to the helminth family and possesses an eggshell morphology that is structurally similar to most helminths (Gilleard 2004; Kaminsky *et al.* 2008; Ferreira *et al.* 2015; Stein & Golden 2015; Olson *et al.* 2018). By definition, helminths are parasitic worms that include taxa of flatworms, tapeworms, flukes, and include nematode species. *C. elegans* were purchased from Carolina Biological Supply Co. and grown monoxenically in the laboratory using *Escherichia coli* strain OP50 as a food source on nematode growth medium (NGM) petri plates (following the general procedure reported in Stiernagle (2005)). Large *C. elegans* populations were produced through a process of 'chunking' samples from the NGM agar culture plate onto separate OP50-seeded plates. The seeded plates were stored at room temperature in the absence of light for 2 to 3 days. This resulted in a sizable population of eggs and worms as determined using optical microscopy.

Electroporation (EP) was performed *ex situ* in plastic cuvettes inserted into a BTX T820 Electro Square Porator. The cuvettes are fitted with two opposing stainless-steel electrodes positioned 0.4 cm apart and served as the reservoir for the *C. elegans* test solutions, as illustrated in Figure 1. *C. elegans* worms and eggs were harvested from a seeded plate and suspended in 3 mL of 1× phosphate-buffered saline (PBS). One milliliter of this nematode/PBS mixture

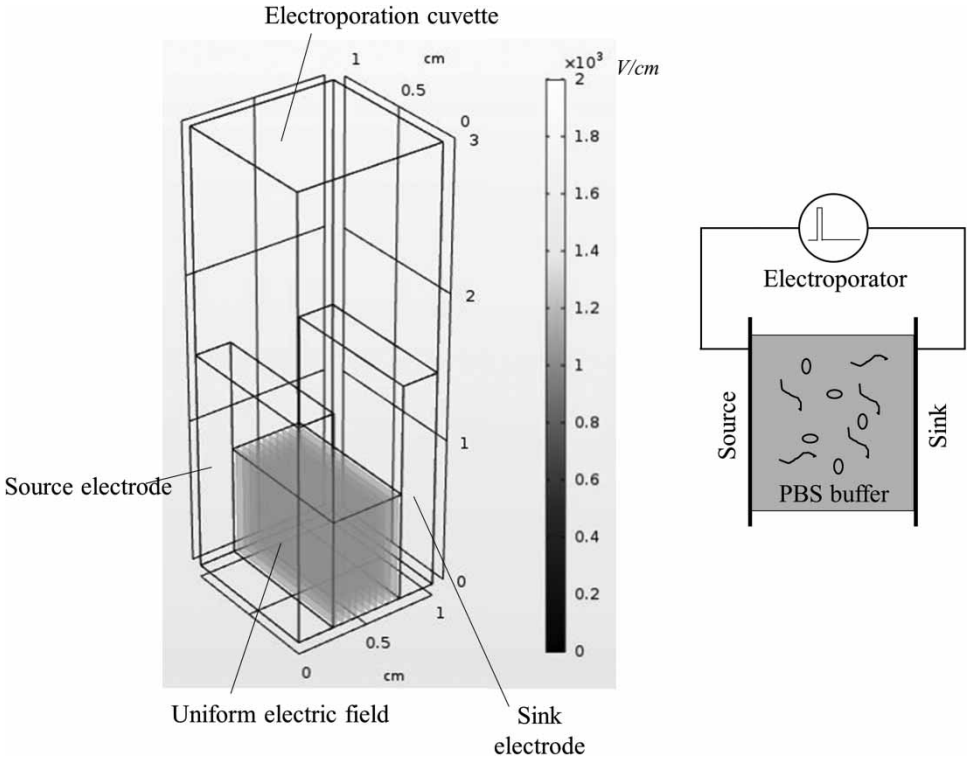

Figure 1 | Illustration of the test cell cuvette used for *C. elegans* electroporation. The image includes the electric field uniformity at an applied potential of 500 V using COMSOL Multiphysics® modeling software.

was pipetted into the cuvette test cell. A concentration of 200–300 nematode ova per milliliter was estimated using optical microscopy. Furthermore, the simple buffer solution is comparable in pH (7.4) and electrical conductivity (~160 mS/cm) to human wastewater (Rose *et al.* 2015). The cell-porator electroporation system produces pulse trains whereby the pulse repetition frequency, pulse amplitude, pulse duration, and total EP duration are tunable parameters. In this study, a 1 Hz pulse train of approximately 0.01% duty cycle (100 μsec pulse duration) was employed, a standard waveform used in cellular EP research, to mitigate bubble formation and heating (Davalos *et al.* 2010; Ivorra & Rubinsky 2010). Pulsed electric fields of 1,500 V/cm, 1,750 V/cm, and 2,000 V/cm were applied to the cuvette test cell (with COMSOL simulated currents of 18 A, 21 A, and 24 A, respectively); a 900 V pulse amplitude (2,250 V/cm electric field) was determined to be the upper limit because of excessive electrical arcing. The total EP duration was evaluated in the range from 1 min (60 pulses) to 8 min (480 pulses) for each of the pulsed electric fields. A compilation of the experimental electroporation parameters is shown in Table 1.

Table 1 | Electroporation parameters used for electropermeabilization of the nematode ova

Electroporation parameters	Experimental conditions	Values
Pulse repetition frequency	Fixed	1 Hz
Pulsed electric fields	Variable	1,500, 1,750, 2,000 V/cm
Pulse duration	Fixed	100 μs
Total electroporation duration	Variable	0–8 min

RESULTS AND DISCUSSION

The extent of pore formation was determined using a fluorescent label, propidium iodide (PI), targeting *C. elegans* embryonic DNA. Immediately following EP treatment (~1 min), 25 μL of the fluorophore was pipetted into the

1 mL nematode/PBS test solution. The solution was then pipetted onto a microscope slide into concave wells for *ex situ* characterization via fluorescence microscopy. The fluorophore served as an indicator of pore formation wherein it fluoresces red when it binds to DNA; the relative fluorescence intensity served as a measure of the amount of fluorophore entry into the eggs and cells. The intensity of the red fluorescence was captured using the same imaging settings for all experiments in bright field (at 60% illumination) and red filtered field (at 10% illumination) using a fluorescence microscope (EVOS® FL Cell Imagining System, Thermo Fisher Scientific). The image intensity as a function of pulse amplitude and total EP duration was quantified using image processing and analysis Image-J software. Fluorescence intensity associated with PI staining of DNA is regularly used as an indicator of non-viability of mammalian cells for cellular EP (Jones & Senft 1985; Sasaki *et al.* 1987). An important outcome of our work for eggshell strata EP was application of fluorescence microscopy as a means of monitoring eggshell permeabilization and gauging the effectiveness of EP parametric conditions for compromising the nematode eggshell.

Prior to the electropermeabilization studies, the buffered test solution was evaluated for impact on nematode ova permeability in the absence of EP treatment. *C. elegans* worms and eggs were placed in separate methanol and PBS solutions excluding EP to gauge susceptibility to fluorophore uptake in the two chemical environments. Methanol immersion was adopted from Ferreira *et al.* (2015) which is known to affect the lipid layer in *C. elegans* cell membranes enabling PI fluorophore labeling, while immersion in the PBS solution demonstrated whether the eggshells would be permeable to the PI in our test solution. Both the PI/methanol and PI/PBS solutions were prepared with an identical ratio to our experimental electroporation test solution (25 μL PI/1 mL methanol or PBS buffer solution). As expected, the eggshells fluoresced red after exposure to the methanol solution indicating that both the eggshell and cell membrane lipid layers were compromised in the organic solvent and showed no observable red emission upon exposure to the PI/PBS solution. It is important to acknowledge that the eggshell was permeable to the PI label in the methanol solvent, likely due to compromising the lipid-rich permeability barrier.

The outcome of our electroporation studies indicates the feasibility of electropermeabilizing nematode eggshells. No obvious change in the geometric size or shape of the ova was observed for all EP parametric conditions evaluated, suggesting modification to existing eggshell strata that does not compromise structural integrity. Fluorescence microscopy showed increasing dye labeling and red emission intensity with EP pulse amplitude, as shown in Figure 2. The fluorescence images in Figure 2(d)–2(f) show increasing red emission intensity for *C. elegans* electroporated for 3 min of total EP duration using 1,500 V/cm, 1,750 V/cm, and 2,000 V/cm pulsed electric fields, respectively. Fluorophore uptake was observed for all pulse amplitudes showing greater reaction-diffusion kinetics with electric field magnitude and total EP duration, as shown in Figure 3. The standard deviation error bars shown in this figure reveal greater variability for the shorter EP treatment times, possibly attributed to different developmental stages of the eggshells and embryos and aggregation of the eggs and worms in the test solution given that the electric field within the cuvette test cell is uniform (shown in Figure 1 via COMSOL Multiphysics® modeling). Data variability decreased with total EP duration as prolonged exposure to the electric fields negated these effects and rendered all ova equally permeable to dye uptake. It is noted that the standard deviation variability was similar for all the pulsed electric fields evaluated and only shown for the 2,000 V/cm results for clarity. Another important observation was that active and healthy *C. elegans* worms prior to EP were destroyed upon exposure to the intense electric fields. Dye uptake was observed in permanently immobilized worms and showed similar fluorophore uptake kinetics to ova fluorescence, while stunned worms displayed no fluorescence and regained mobility shortly after the treatment. Fluorescent eggs were differentiated from fluorescent worms based off morphological and geometrical differences.

Fluorophore nucleic acid labels, such as the PI label, may be used to assess the stability of lipid layer modifications and, ultimately, cell viability (Bill *et al.* 2003; Chan *et al.* 2012; Ferreira *et al.* 2015). Electroporated modifications to membrane lipids are defined by two processes: those that exhibit reversible EP (RE) and those that exhibit irreversible EP (IRE) attributes. In the former process,

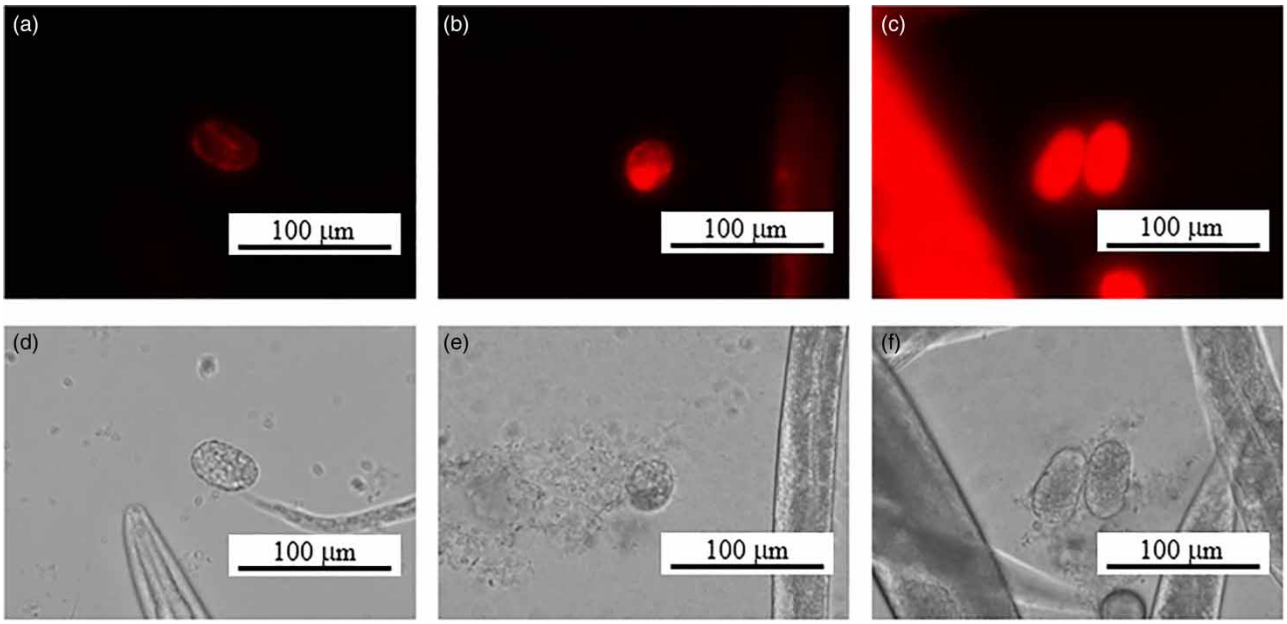

Figure 2 | Optical and fluorescent images of *C. elegans* eggs post-EP. The images display increasing red fluorescence for nematode eggs electroporated for 3 minutes at three different field amplitudes: 1,500 V/cm (a) and (d), 1,750 V/cm (b) and (e), and 2,000 V/cm (c) and (f).

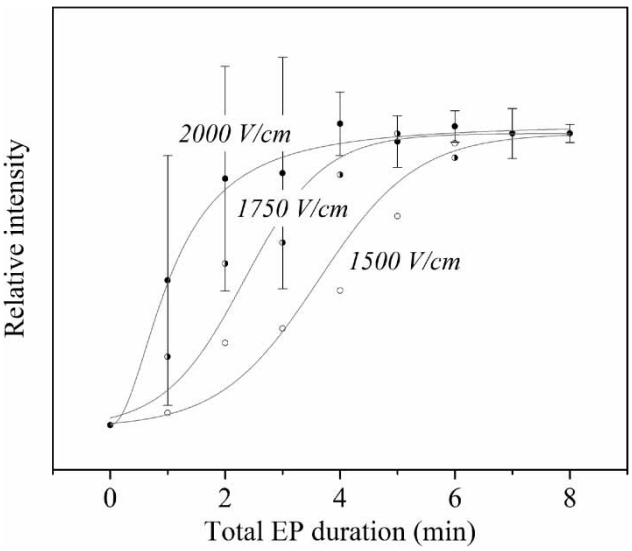

Figure 3 | Fluorescence uptake as a function of total EP duration. The rate of dye uptake was observed to increase as a factor of the electric field strength and the treatment duration. Standard deviation error bars show greater variability for shorter treatment times.

pores are essentially healed following EP treatment, only allowing a temporary increase in cell permeability. In the latter process, pores become permanent and indicate IRE-induced cell death. Ongoing work is evaluating EP parameters under which RE and/or IRE occurs by adding the PI label to separate test solutions following 1 min (as described previously) and 30 min of EP treatment. While 1 min PI labeling may not discriminate between RE and IRE, 30 min labeling is generally believed sufficient for IRE determination. Preliminary results show similar fluorophore uptake kinetics for both post-EP labeling times suggesting that for our parametric conditions under study, irreversible EP is operative. This is suggestive of embryo death from EP in simple buffer solution. The authors are currently conducting work to harvest post-EP treated eggs, in the absence of fluorophore chemistry, and providing conditions suitable for larvae hatching. The absence of viable worms will be used to fully evaluate conditions for *C. elegans* destruction.

In theory, electroporation offsets the electrochemical gradient that exists in cell membranes pertaining to cellular EP. The applied field leads to an increase in transmembrane potential, and above a critical threshold, naturally occurring gaps in the lipid bilayer (hydrophobic pores) transition to nanoscale pores lined by phospholipid headgroups (hydrophilic pores). It is speculated that similar modifications occur within the lipid-rich nematode eggshell permeability barrier due to similarities in their physical and chemical construct. Evidence suggests the relevance of biosynthetic/

modification fatty acid and carbohydrate enzymes operative in the formation of this lipid barrier and the likelihood of glycolipids comprising the permeability layer (Stein & Golden 2015; Olson *et al.* 2018). While there are numerous types of lipids (van Meer & de Kroon 2011), the three major kinds, and applicable to our work, are phospholipids, glycolipids, and cholesterol chemistry that exist in bacterial, archaeal, and eukaryotic cells. Therefore, it is not surprising that eggshell EP kinetics show similarity to that of cellular EP.

CONCLUSION

This research marks the first known application of electroporation for increasing nematode eggshell permeability. The kinetics of pore formation can be controlled by altering the pulse parameters for cellular EP but also, as reported herein, for eggshell strata EP. Varying the strength of the pulsed electric field and the total EP duration was shown to affect the extent of pore formation within the eggshell strata and embryo cell membranes using fluorophore labeling. *C. elegans* species served as a suitable surrogate for helminth worms, and therefore, this research offers insight into the effects of electroporation on the broader class of helminth parasitic ova. Future work will involve the application of EP to the deactivation of helminth eggs in wastewater. Following the examples established by Bandala *et al.* (2012) and Alouni & Jemli (2001), electroporation will be performed in the presence of commercial chemical disinfectants and those synthesized via electrochemical modification of human waste in order to evaluate the feasibility of destroying parasites.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge funding for this work provided by the Bill & Melinda Gates Foundation (OPP ID: OPP1148486, Duke University: Improved understanding and use of generated oxidizing species in liquid waste disinfection) and by the Lumen Prize at Elon University.

REFERENCES

Alouni, Z. & Jemli, M. 2001 Destruction of helminth eggs by photosensitized porphyrin. *Journal of Environmental Monitoring* **3** (5), 548–551.

Bandala, E. R., González, L., Sanchez-Salas, J. L. & Castillo, J. H. 2012 Inactivation of *Ascaris* eggs in water using sequential solar driven photo-Fenton and free chlorine. *Journal of Water and Health* **10** (1), 20–30.

Bill, M. S., Olsen, A., Sampayo, J. N. & Lithgow, G. J. 2003 An automated high-throughput assay for survival of the nematode *Caenorhabditis elegans*. *Free Radical Biology & Medicine* **35** (6), 558–585.

Chan, L. L., Wilkinson, A. R., Paradis, B. D. & Lai, N. 2012 Rapid image-based cytometry for comparison of fluorescent viability staining methods. *Journal of Fluorescence* **22**, 1301–1311.

Daly, K. E., Huang, K. C., Wingreen, N. S. & Mukhopadhyay, R. 2011 Mechanics of membrane bulging during cell-wall disruption in Gram-negative bacteria. *Physical Review E* **83** (4), 041922.

Davalos, R. V., Garcia, P. A. & Edd, J. F. 2010 Thermal aspects of irreversible electroporation. In: *Series in Biomedical Engineering: Irreversible Electroporation* (B. Rubinksy, ed.). Springer-Verlag, Berlin, Germany, pp. 123–154.

Ferreira, S. R., Mendez, T. A. O., Bueno, L. L., Araújo, J. V., Bartholomeu, D. C. & Fujiwara, R. T. 2015 A new methodology for evaluation of nematode viability. *BioMed Research International*. **2015**, 879263. Available at: https://www.hindawi.com/journals/bmri/2015/879263/abs/.

Gilleard, J. S. 2004 The use of *Caenorhabditis elegans* in parasitic nematode research. *Parasitology* **128** (S1), S49–S70.

Holden-Dye, L. & Walker, R. J. 2014 Anthelmintic drugs and nematicides: studies in *Caenorhabditis elegans*. *WormBook* **16**, 1–29.

Horton, J. 2003 Human gastrointestinal helminth infections: are they now neglected diseases? *Trends in Parasitology* **19** (11), 527–531.

Ivorra, A. & Rubinsky, B. 2010 Historical review of irreversible electroporation in medicine. In: *Series in Biomedical Engineering: Irreversible Electroporation* (B. Rubinksy, ed.). Springer-Verlag, Berlin, Germany, pp. 1–22.

Jimenez-Cisneros, B. E. & Maya-Rendon, C. 2007 Helminths and sanitation. In: *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* (A. Méndez-Vilas, ed.). Formatex, Badajoz, Spain, pp. 60–71.

Jones, K. N. & Senft, J. A. 1985 An improved method to determine cell viability by simultaneous staining with fluorescein diacetate–propidium iodide. *The Journal of Histochemistry and Cytochemistry* **33** (1), 77–79.

Kaminsky, R., Ducray, P., Jung, M., Clover, R., Rufener, L., Bouvier, J., Weber, S. S., Wenger, A., Wieland-Berghausen, S., Goebel, T., Gauvry, N., Pautrat, F., Skripsky, T., Froelich, O., Komoin-Oka, C., Westlund, B., Sluder, A. & Mäser, P. 2008 A new class of anthelmintics effective against drug-resistant nematodes. *Nature* **452**, 176–180.

Lysek, H., Malinsky, J. & Janisch, R. 1985 Ultrastructure of eggs of *Ascaris lumbricoides* Linnaeus, 1758 I. Egg-shells. *Folia Parasitological* **32** (4), 381–384.

Olson, S. K., Greenan, G., Desai, A., Muller-Reichart, T. & Oegema, K. 2018 Hierarchical assembly of the eggshell and permeability barrier in *C. elegans*. *Journal of Cell Biology* **198** (4), 731–748.

Perry, R. N. & Moens, M. 2001 Survival of parasitic nematodes outside the host. In: *Molecular and Physiological Basis of Nematode Survival* (D. A. Wharton, ed.). CABI, Wallingford, Oxfordshire, UK, pp. 1–27.

Rauch, C. & Leigh, J. 2015 Theoretical evaluation of wall teichois acids in the cavitation-mediated pores formation in Gram-positive bacteria subjected to an electric field. *Biochimica et Biophysica Acta* **1850** (4), 595–601.

Rems, L. & Miklavčič, D. 2016 Tutorial: electroporation of cells in complex materials and tissue. *Journal of Applied Physics* **119** (20), 201101. doi:10.1063/1.4949264.

Rose, C., Parker, A., Jefferson, B. & Cartmell, E. 2015 The characterization of feces and urine: a review of the literature to inform advanced treatment technology. *Critical Reviews in Environmental Science and Technology* **45** (17), 1827–1879.

Sasaki, D. T., Dumas, S. E. & Engleman, E. G. 1987 Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. *Cytometry* **8** (4), 413–420.

Stein, K. K. & Golden, A. 2015 The *C. elegans* eggshell. *WormBook* **2018**, 1–36. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5603424/.

Stiernagle, R. 2005 Maintenance of *C. elegans*. http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html (accessed 1 October 2017).

van Meer, G. & de Kroon, A. I. P. M. 2011 Lipid map of the mammalian cell. *Journal of Cell Science* **124**, 5–8.

Wharton, D. A. 1983 The production and functional morphology of helminth egg-shells. *Parasitology* **86** (4), 85–97.

First received 6 July 2018; accepted in revised form 18 October 2018. Available online 2 January 2019