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Cryptosporidium parvum decay during air drying and stockpiling of mesophilic anaerobically digested sewage sludge in a simulation experiment and oocyst counts in sludge collected from operational treatment lagoons in Victoria, Australia

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Short title: Decay of *C. parvum* in air-drying treatment of sewage sludge

ABSTRACT

The inactivation of *Cryptosporidium* species oocysts during sewage sludge treatment is important to protect human health when the residual biosolids are applied to agricultural land. Quantifying the decay of *Cryptosporidium* species during sludge treatment for microbiological assurance purposes is difficult if low numbers are present in wastewater. The rate of decay of *Cryptosporidium parvum* oocysts during solar/air drying treatment and in sludge stockpiles in temperate environment conditions was simulated in laboratory inoculation experiments using sludge sampled from a mesophilic anaerobic digester. Oocyst numbers were also determined in settled lagoon sludge samples collected from three operational rural wastewater treatment plant (WWTP). *C. parvum* oocysts were enumerated by immunomagnetic separation followed by staining with vital dyes and examination by confocal laser scanning microscopy. An air-drying/storage period equivalent to 11 weeks was required for a 1 $\log_{10}$ reduction of viable oocysts inoculated into digested sludge. Oocyst viability in air-dried and stored digested sludge decreased with time, but was independent of sludge desiccation and dry solids (DS) content. No oocysts were detected in sludge samples collected from the anaerobic digester, and the average concentration of oocysts found in settled lagoon sludge from the rural WWTP was 4.6 x 10^2 oocysts/g DS.

Keywords: air-dried sludge, *Cryptosporidium parvum*, biosolids, land application, microbial safety, confocal laser scanning microscopy.

INTRODUCTION

Infectious protozoa belonging to the genus *Cryptosporidium* are a significant cause of human diarrhoeal disease (Slifko et al. 2000) and are responsible for up to 20% of cases of gastroenteritis globally, depending on geographical location (WHO 2009). Most human infections are caused by *C. parvum* (which also infects cattle) or the human type, *C. hominis*. Oocysts excreted by infected humans and animals may persist in the environment and are highly resistant to chemical disinfection (WHO 2009). Human infection may be acquired directly by person-to-person contact, from ingesting food including raw vegetables, or water, contaminated faeces, or through zoonotic transmission, either directly or indirectly (Thompson et al. 2005, 2008; WHO 2009). The infective dose is low to very low and in the range 1 to 30 oocysts (DuPont et al. 1995). Infections in adults and children with normal host immunity can lead to self-limiting diarrhoea typically for about one week, although symptoms may be severe in children. Other symptoms may include nausea and vomiting, loss of weight and abdominal cramps. However, cryptosporidiosis in immunocompromised patients is potentially a much more serious condition, causing persistent diarrhoea (WHO 2009; Kortbeek 2009).

Infected humans may shed as many as $10^{5\text{-}7}$ oocysts (*C. parvum* or *C. hominis*)/g faeces during the acute phase of the illness (Chappell et al. 1999). Other animals and birds typically shed *Cryptosporidium* species that do not generally infect humans

(WHO 2009). Nevertheless, *Cryptosporidium* species that infect humans are important water-borne environmental parasites and international regulatory agencies require their monitoring and surveillance in potable water supplies (USEPA 2001, 2002; Health Canada, 2004; MHNZ, 2015; UKDWI, 2015). Numbers of oocysts of *Cryptosporidium* species reported in raw wastewater in different countries are highly variable and in the range from 0 to ~10^5/L (Robertson et al. 2000; Tonani et al. 2013; Kitajima et al. 2014; Marin et al. 2014; Halam-Nahovandi et al. 2015; Taran-Benshoshan et al. 2015; King et al. 2016; Ramo et al. 2017).

Wastewater treatment processes transfer and concentrate the oocysts of *Cryptosporidium* species into the sewage sludge (Stadterman et al. 1995). One of the main objectives of sewage sludge treatment is to reduce the viability of these and other enteric pathogens potentially present in sludge to protect human health when treated sewage sludge biosolids are used as an agricultural soil improver. The microbiological classification of biosolids for land application is determined by the extent of removal of specified indicator or other pathogenic organisms, but does not include *Cryptosporidium* (WHO 1981; ADAS 2001; USEPA 2003; EPA Victoria 2004; NRMMC 2004; Smith and Reimers 2006).

Mesophilic anaerobic digestion is widely adopted as a sewage sludge stabilisation process by the Water Industry internationally, and is moderately effective at inactivating *Cryptosporidium* oocysts, achieving approximately a ~2-3 $\log_{10}$ reduction rate (Stadterman et al. 1995; Horan and Lowe 2002; Kato et al. 2003). A risk analysis study based on infection and excretion rates in the general human population, with calculated pathogen transfer rates to wastewater and sludge,

predicted that the total number of *Cryptosporidium* oocysts in mesophilic anaerobically-digested sludge was in the range 1 to 100 total (viable plus non-viable) oocysts per g dry solids (DS) (Grant et al. 2012).

In metropolitan Melbourne, Australia, anaerobically digested sewage sludge is consolidated by air/solar drying in open pans, followed by storage for a specified minimum period in stockpiles (Rouch et al. 2011). Dewatering sewage sludge by solar/air drying can be a cost effective option and is a widely adopted process where the climate is suitable and space is available permitting this type of extensive sludge treatment (Hall and Smith 1997; Idris et al. 2002; Seginer and Bux 2006; Malack et al. 2007; Tamimi et al. 2007). In rural areas in Victoria, Australia, urban wastewater is typically treated in lagoon systems, consisting of two or three stabilization ponds in series, and the final effluent is often recycled for irrigation purposes (Rouch et al. 2013). The solid fraction settles and accumulates in the primary pond and the sludge is periodically removed, although usually this only occurs relatively infrequently. To our knowledge there are no published data available on the content of *C. parvum* in rural lagoon pond sludge.

Reliable, consistent and comprehensive inactivation of relevant pathogenic organisms during sludge treatment is critical to the advocacy of agricultural recycling programmes. Therefore, quantitative decay kinetics are required to determine minimum process conditions and retention times for the effective removal of important pathogen types. To our knowledge, no information is available in the literature on the rates of decay of viable *Cryptosporidium* oocysts during air drying treatment of sewage sludge. This is partly due to the practical difficultly of measuring

the decay rates of oocysts under operational conditions, since numbers in sludge entering drying beds may be too low to be detected.

Oocyst measurements in environmental samples are generally reported as total numbers, and do not distinguish between viable and non-viable oocysts. Since non-viable oocysts are not infectious to humans, an assay for viable oocysts is necessary to accurately assess the risk to health. However, the standard methods available to count viable oocysts are unsuitable for routine testing of sewage sludge. The published methods for enumerating viable *Cryptosporidium* species oocysts were reviewed by Qintero-Betancourt et al. (2002) and include: neonatal mouse infectivity (Enriquez and Sterling,1991); *in vitro* excystation (Robertson et al. 1993); *in vitro* cell culture (Slifko et al. 1997) and exclusion of fluorogenic dyes, e.g., propidium iodide (PI) (Campbell et al. 1992); Syto-9 or Syto-59 (Neumann et al. 2000). The neonatal mouse assay detects infectious *C. parvum* oocysts and has become established as the reference method for quantifying infectivity (Belosevic et al. 1997; Bukhari et al. 2000). However, this assay is time consuming and expensive to perform and is therefore unsuitable for routine application to wastewater sludge samples. Moreover, some genotypes of *C. parvum* may be specific to humans and unable to infect mice (Peng et al. 1997). The excystation method is simple to perform, but requires expertise in interpretation (Belosevic et al. 1997; Bukhari et al. 2000). *In vitro* cell culture provides similar results to the mouse assay, but is also expensive, time consuming and requires significant expertise, limiting its application to routine testing (Rochelle et al, 2002). By contrast, vital dyes offer a simple, cost effective alternative method for differentiating oocyst viability. This is achieved by the selective exclusion of vital dyes by viable, or penetration and inner staining of non-viable,

oocysts depending on the integrity of oocyst membranes (Qintero-Betancourt et al. 2002).

The aim of the research described here was to quantify the numbers of viable *Cryptosporidium* oocysts in air-dried, anaerobically digested sewage sludge, in controlled experiments to simulate field conditions, and in sludge collected from operational wastewater treatment lagoons. We selected a vital dye assay to enumerate *Cryptosporidium* species above the other possibilities as the analytical problems paralleled those experienced in detecting viable eggs of *Ascaris suum* in biosolids samples, where a similar methodology was successfully applied (Karkashan et al. 2015). The specific objectives were to: (i) isolate inoculated oocysts from sewage sludge using magnetic bead separation and detection with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (FITC-C-mAb) , (ii) evaluate the viability of *C. parvum* oocysts isolated from sludge using the vital dye Syto-59, (iii) examine the decay of *C. parvum* oocyst viability during simulated air-drying and stockpiling of mesophilic anaerobically digested sludge from a major metropolitan wastewater treatment plant (WWTP) in Melbourne, Victoria, Australia, (iv) estimate the retention time required for oocyst inactivation in air dried/stockpiled sludge, and (v) enumerate viable oocysts in sludge from rural wastewater treatment lagoons.

MATERIALS AND METHODS

Sludge collection and preparation

Fresh samples of liquid mesophilic anaerobically digested sludge were collected (in May 2011) from a WWTP in metropolitan Melbourne, Australia (WWTP A) for air-drying and stockpile simulation under laboratory conditions. Sludge samples were also collected (during 2012) from the primary ponds of three rural wastewater lagoon stabilization treatment plants (WWTP B, C, D) for direct enumeration of viable oocysts. In the case of WWTP B and C, desludging of the primary pond occurred at a frequency of 10 to 20 years; however, no sludge had been removed from WWTP D, which began operation 40 years ago. The sample collection coincided with the operational removal and dewatering (by portable centrifuge) of sludge at WWTP B and ~60 kg of mechanically dewatered sludge was collected for investigation. Sludge samples from the other WWTPs (C and D) were collected using a positive displacement pump (with a flow rate of 10 L/min) attached to a 10 m length of 40 mm diameter polyethylene pipe. The end of the sampling pipe was submerged at the bottom of the pond and the output was pumped to a 300 L open container. The sludge was mixed with a spade to ensure homogeneity and 22 individual aliquots of sludge, each consisting of ~15 L, were pumped to separate 20 L plastic jerry cans with sealable lids. Sludge samples were transported to the laboratory at ambient temperature on the day of collection and upon arrival were settled for 4 days at 20 ^{o}C and decanted to increase the DS content simulating field conditions. This procedure raised the sludge DS to 3 to 4% for WWTP A and C and to 9% for WWTP D. The sludge sample collected from WWTP B was mechanically dewatered and contained 15% DS, therefore no further consolidation measures were required prior to microbiological analysis in this case.

Laboratory simulation of air drying and stockpiling of mesophilic anaerobically digested sludge

Source of oocysts and microscopy

A stock inoculum of *C. parvum* oocysts (3.2 x 10^6/mL) was supplied by Professor Una Ryan (Murdoch University, Western Australia). Oocysts were harvested from laboratory-infected mice and transported to RMIT University in a medium consisting of sterile phosphate buffered saline (PBS), penicillin (10000 IU) and streptomycin (10000 μg/mL); the oocyst inoculum was stored at 4 °C for a maximum period of 4 months. The total oocyst concentration in the stock inoculum was confirmed (in triplicate) by phase contrast microscopy at x40 magnification on each occasion oocysts were removed for the experimental work.

Experimental conditions

The average duration of air-drying treatment of liquid sludge at WWTP A in Melbourne, Australia, is ~150 days, with a range of 52 to 410 days, depending on seasonal conditions (Rouch et al. 2012). Two air drying simulation experiments were completed using sludge collected from WWTP A on two separate occasions: Simulation 1 (S1) was conducted during May to September 2011 and had a total duration of 140 days, and Simulation *2* (S2) was conducted from July to October 2011 for a period of 112 days. Sludge was incubated at 20 °C to represent the average daily maximum mid-summer (January) temperature in Melbourne, Australia. This regime was selected to provide a relatively rapid desiccation rate, to simulate the shortest and therefore potentially most conservative operational air-drying

treatment retention time during the summer period for pathogen and indicator organism decay in air dried sewage sludge.

The air-drying simulation experiments were conducted in a pair of modified class II biological safety cabinets, fitted with infrared heaters. The heaters were operated for 7.6 h per day to provide a controlled radiant heat flux of 383 watts/m^2/h, equivalent to the mean infrared exposure rate received during the summer period in Melbourne. The average linear air flow rate in the cabinets was maintained at 0.42 m/s. The heating and air circulation treatment was performed on a weekly cycle; the heaters were switched off and the cabinets were closed to minimise air flow during each alternate week to control and reflect the rate of sludge desiccation observed under field drying conditions.

In both simulation experiments, three 5 L plastic containers were placed in each of the two cabinets and filled with 4.5 L of settled digested sludge (DS content after settling was 3.0% - 3.1%) to a depth of approximately 20 cm. After a drying treatment period of 91-92 days the DS content had increased to 12.5 – 15.0% and the sludge was formed into piles at one end of the containers to a height of about 10 cm to represent the stockpile condition.

Assay chamber construction

Inoculated oocysts were confined within an assay chamber to: (i) manage and control the amount of inoculum required and the volume of inoculated sludge; (ii) improve control of the inoculation procedure and oocyst density in the sludge; (iii) increase the uniformity of sampling and treatment exposure; and (iv) provide for the

general containment of infective material in the laboratory. The inoculation chamber had an internal volume of 0.5 mL and was constructed from a microcentrifuge tube fitted with a 0.45 µm spin filter (Merck Millipore, Kilsyth, Australia) and with the tip removed (Fig. 1). The open end of the spin filter was also capped with a 0.45 µm filter. Each batch of filters was leak tested before use. Assay chambers were designed to prevent the movement of bacteria and parasites, but sludge components <0.45 µm could exchange and move freely between the internal and external environments. The assay chamber was validated by Rouch et al. (2012) and provided similar decay coefficients of indigenous *Escherichia coli* and *Enterococcus* species, added bacteriophage P22, and DS values compared to external sludge samples in open containers during air-drying simulation in the radiant heater cabinets.

Inoculation and sampling procedure

The concentrated inoculum of *C. parvum* (3.2×10^6 oocysts/mL) was mixed by vortexing for ~20 sec with sludge to give a final oocyst concentration of 3.2×10^5/mL. Aliquots (0.5 mL) of the inoculated sludge were transferred to the assay chambers using a 1 mL pipette with a sterile Low Retention Aerosol Barrier (LRAB) Tip (Interpath Services Pty Ltd). The tips were autoclaved and the fine point of the tip was removed aseptically using a hot blade, to provide an internal diameter of ~3 mm to avoid blockage by solid sludge particles. Sufficient assay chambers for the duration of each experimental monitoring period were suspended, using monofilament resistant plastic yarn, and immersed in the liquid sludge at a depth of approximately 5 cm from the base of the sludge containers. When the sludge was

formed into piles, assay chambers were detached and mixed into and completely covered by the solid sludge matrix.

Three assay chambers were collected for oocyst recovery immediately after seeding (time zero control). Triplicate sets of assay chambers were subsequently collected at intervals of 2 to 4 weeks during the simulation period. The sampling interval was extended on an iterative basis as the experiment progressed following the theoretical decay profile of the organism (see equation 1 below). The contents of each assay chamber were examined for DS content and viable count of *C. parvum* oocysts.

Collection and extraction of sludge from assay chambers

Assay chambers were removed from the sludge containers and washed to remove adherent material from the external surface. For extracting wet content (sludge incubation period <100 days), as much sludge as possible (at least 95%) was removed using a pipette with a LRAB tip with the end removed, and the remainder was collected by rinsing several times with PBS (Sigma Aldrich, Castle Hill, New South Wales, Australia). A different procedure was necessary to extract dry sludge from assay chambers (sludge incubation period >100 days). In this case, a small sterile spatula was used to break up and remove the solid material. Dry sludge was carefully transferred to a 1.5 mL centrifuge tube avoiding damage to the filter to prevent leakage; residual material in the chamber was collected by washing several times with PBS to maximise the extraction of visible sludge solids. Dry sludge samples were prepared for microbiological analysis by vortexing to disperse aggregated particles. The volumes of wet sludge and dispersed dry sludge were

made to 1 mL with PBS, and the samples were stored at 4 °C for a maximum period of 2 h prior to microbiological examination.

Immunomagnetic separation of *C. parvum* oocysts

Cryptosporidium parvum oocysts were obtained from sludge by immunomagnetic separation (IMS) using the Dynabeads anti-*Cryptosporidium* kit (Invitrogen Life Technologies, Mulgrave, Australia), Dynal Magnetic Particle Concentrator™ (MPC-1) and Dynal MX-1, which specifically isolates oocysts of *C. parvum*. This involved adding sludge samples to anti-*Cryptosporidium*-coated beads, magnetic separation of coated beads from sludge and, finally, the dissociation of oocysts from the Dynabeads. The IMS technique is designed for separation of *C. parvum* oocysts from water samples, therefore, the standard bead separation and dissociation protocols were adapted to improve recovery from sludge. Previously (data not shown), we evaluated several detergents, with and without glass beads, to disintegrate sludge particles and assist the recovery efficiency of *C. parvum* oocysts from sludge. A combination of sodium docecyl sulphate (SDS) and Sigmacote-coated glass beads consistently increased the recovery of oocysts from the original inoculum suspension by a factor of two compared to the recommended protocol (Kong 2011), although the differences were not statistically significant. The physical properties of liquid sludge samples with low DS contents were relatively homogeneous for the first two sampling times of S1 and, in this case, the standard method was used for oocyst separation. Subsequently, however, the adapted procedure was followed for drying sludge by adding 8.0 mL sterile PBS, 1 mL of 1% SDS solution and 1g Sigmacote-coated glass beads (Sigma-Aldrich, Australia) to the sludge/oocyst suspension obtained following the IMS separation procedure (1.0 mL

volume) to provide a final volume of 10 mL. The acid dissociation phase was also modified by replacing the recommended 1X SL™ buffer with PBS.

Enumeration of viable and non-viable oocysts

Oocysts obtained by IMS were stained with FITC-C-mAb (Life Research Pty Ltd, Melbourne, Australia) and Syto-59 (Invitrogen Life Technologies, Mulgrave, Australia) and examined by confocal laser scanning microscopy (CLSM). A combination of Syto-59 with FITC-C-mAb vital dyes was used to distinguish between viable and non-viable oocysts. Syto-59 penetrates the cell wall of non-viable oocysts and stains the inner contents red, but is excluded from viable oocysts which take up FITC to stain the outer membrane green (Belosevic et al. 1997; Neumann et al. 2000).

An aliquot of 5 µL of FITC-C-mAb (undiluted) was added to 55 µL of purified oocysts in a 1.5 mL centrifuge tube. The suspension was vortexed for 5 s to ensure uniform mixing and reaction with the vital stain, and the centrifuge tubes containing the stained oocysts were wrapped in aluminium foil and incubated at 37 °C for 30 min. The tubes were removed from the incubator and 5 µL of Syto-59 (1:10 dilution in sterile deionised water) was added by pipette and the vortex mixing and incubation steps were repeated. Stained, incubated samples were vortexed for a further 5 s and 8 µL of the oocyst suspension was transferred onto a haemocytometer and observed with the 60x objective lens of a confocal microscope (Nikon Eclipse Ti-E A1 Laser Scanning Confocal System, Nikon Instruments Inc, Japan). Red fluorescence (Syto 59) was observed with a 561 nm laser and 570-620 nm emission filter, and green fluorescence (FITC) was observed with a 468 nm laser and 500-550 nm emission

filter. Spectral interferences between the vital dye, Syto-59, and FITC, used for specific detection of *C. parvum* oocysts, were minimal, since the fluorescence spectra of Syto-59 and FITC do not overlap (Neumann et al. 2000).

C. parvum oocysts were recorded as viable when there was no internal staining and the outer membrane was stained green with FITC; by contrast, oocysts with red internal staining with Syto 59 were recorded as non-viable (Fig. 3). Oocyst numbers per g DS of sludge were calculated at each sampling point from the total oocyst counts per mL in assay chambers, adjusting for the differences in DS contents initially and at each specific sampling time (based on the DS of the external sludge material, obtained by drying at 105 ^{o}C for 24 h in a forced-air oven). The numbers of viable oocysts were estimated from the proportion of viable to non-viable oocysts counted at each time, assuming that the recovery efficiency of viable and non-viable oocysts were equivalent. The limit of detection of *C. parvum* oocysts in sludge obtained by the IMS-vital staining technique was approximately 10^2/g DS.

Viability determination of *C. parvum* oocysts using the IMS method was compared with the standard excystation method, using fresh stocks of oocysts. Excystation is a process where sporozoites are released from viable oocysts under controlled conditions. The excystation test was completed as follows: 1% sodium deoxycholate (DOC) solution in Hank's Minimum Essential Medium (HMEM) (Invitrogen, Mulgrave, Australia) and 2.2% sodium hydrogen carbonate solution in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Mulgrave, Australia) was prepared 30 minutes before use; 10 µl of each of 1% DOC and 2.2% sodium hydrogen carbonate solution was added to 100 µl of a 1:10 dilution of *C. parvum.* The sample was incubated in a water

bath at 37 °C for 1 h and 10 µl was pipetted onto a slide and observed under a 100x objective lens of a phase contrast microscope. Images were captured using an attached Nikon DS Camera Control Unit DS-L2.

The proportion of viable oocysts assessed by the IMS method was 95.8 ± 3.6% (mean of three replicate tests), and duplicate tests by the excystation method gave slightly smaller viability rates equivalent to 91% and 92%.

Data management and calculations

The decay relationship for viable *C. parvum* in air-dried sludge was described by the exponential equation:

$$y = a * e^{-k*t}$$

where: y is the number of viable *C. parvum* oocysts per g DS at treatment time, t, in days; a is the initial number of viable *C. parvum* oocysts per g DS; k is the decay coefficient.

Decay coefficients for viable *C. parvum* oocysts were calculated using natural log (Ln) transformed data as there is a mathematically linear relation between Ln(y) and t. This allowed robust linear regression analysis techniques to be applied to the statistical examination of the experimental data.

Numbers of *Cryptosporidium parvum* in rural lagoon sludge

The detection method was modified to increase sensitivity to enumerate small numbers of *C. parvum* expected in sludge sampled from treatment lagoons at rural operational WWTPs (B, C, D). The alternative approach involved the removal and settling of large particles of sludge, followed by extraction of oocysts from the supernatant using a sucrose gradient. Larger particles were disrupted by a procedure modified from Iacovski et al. (2004). Wet sludge (1 g DS) was added to 15 mL of freshly prepared extraction buffer (PBS, 1% bovine serum albumin, 0.1% SDS, 0.05% sodium azide); the mixture was vortexed at high speed for 2 min, settled for 20 min at room temperature and the supernatant (15 mL) was decanted and retained. A further 15 mL of extraction buffer was added to the sludge deposit and the process was repeated twice more. Collected supernatant was centrifuged for 30 min at 1000 g if the initial DS content of the original sample was <6% DS (WWTP C, WWTP D) or at 1500 g for 15 min if the initial DS was >6% D (WWTP B). The pellet (3 – 5 mL) was retained and suspended in 50 mL of PBS, Tween-80 was added to a concentration of 0.1% and the suspension was vortexed for 10 s before centrifuging at 1500 g for 15 min. The pellet was resuspended in PBS to a volume of 15 mL forming a concentrated sludge extract for oocyst extraction. Sucrose solution with a specific gravity of 1.18 at 4 °C (10 mL) was transferred to a 50 mL clear plastic centrifuge tube and underlayed with sludge extract. The mixture was centrifuged at 1500 g for 15 min and oocysts were harvested by gently pipetting approximately 2 mL of solution from the slightly brownish layer just above the interface. The solution was transferred to a centrifuge tube and PBS was added to a total volume of 50 mL. After vortexing for 10 s, the suspension was centrifuged and the supernatant was discarded. The deposit (optimal volume 0.5 mL) was washed with 50 mL PBS, centrifuged for 15 min at 1500 x g, and the deposit was made up to a volume of 10

mL with PBS. Purification of *C. parvum* oocysts was conducted by IMS as described above. If the volume of sludge obtained was >2 mL, two separate suspensions were prepared for IMS separation. The total number of oocysts/g DS was calculated.

The recovery efficiency of the modified extraction method used for lagoon sludge was estimated by inoculating 1 g DS of sludge from WWTP B (15% DS) with 2.15 x 10^5 oocysts. The recovery efficiency was expressed as the percentage of oocysts obtained by the modified method compared with the original inoculum. The limit of detection was estimated as the minimum countable number of oocysts and was equivalent to approximately 43 oocysts/g DS.

RESULTS

Isolation of *C. parvum* oocysts (total, viable and non-viable) from sludge by IMS

Preliminary experiments in which oocysts were seeded into PBS indicated the recovery efficiency of oocysts of *C. parvum* from PBS under controlled conditions on average was 65.0% ± 4.0%. In contrast, the recovery rate, defined as the total numbers of oocysts (viable plus non-viable) present in sludge at each time point during the simulation experiments relative to the initial controlled inoculum size, varied from 10% to 49%. This recovery rate accounted for loss of oocysts during treatment as well as recovery efficiency. However, smaller recovery rates were generally observed for drier sludge samples compared to samples with higher water contents (Fig. 2). Thus, the overall mean recovery rate from wet samples (3% to 13% DS) was 32.5% ± 13.3%, and the average value from drier sludge (DS content

26% to 77%) was equivalent to 17.3% ± 9.7%. Differences in oocyst recovery rates observed between the simulation experiments may be due to the variation in properties of the collected digested sludge samples.

Appearance of viable and non-viable oocysts by CLSM

Selective staining with vital dyes and examination by CLSM readily distinguished viable oocysts (outer membrane stained green with FITC and no internal staining) and non-viable oocysts (stained red with Syto 59) in digested sewage sludge (Fig. 3). Intact particles of sludge were also visible containing magnetic beads and oocysts (Fig. 3). Incomplete sludge dispersion could therefore represent potentially an important physical limitation to the staining, separation and detection of oocysts embedded within intact sludge particles (Fig. 3). Extracts from dried sludge with DS contents of approximately 70% frequently contained objects resembling unstained oocysts that appeared crushed and deformed. All damaged, but recognisable oocysts and red-stained oocysts were classified as non-viable; however, significant numbers of oocysts were so extensively damaged that they resembled sludge particulate material and could not be clearly differentiated or counted. Intact oocysts (round with internal organelles visible) with outer membranes and stained green were counted as viable.

Decay of viable *C. parvum* oocysts during simulated air drying treatment

The initial DS content of liquid digested sludge was approximately 3% at the beginning of the simulation experiments and the DS value increased slowly for approximately 70 days when a pronounced rise in the drying rate was observed (Fig. 4). Thus, the sludge DS content increased to ~10% after drying for a period of 70

days and increased markedly to a maximum value of 70-80% over the following 40 day period. The drying period was extended to a total duration of 140 days in experiment S1, but the sludge DS value did not increase above this maximum range. The simulated drying profile of the sludge was consistent with the operational air drying treatment process observed by Rouch et al. (2012) at WWTP A.

In both simulation experiments, the estimated numbers of viable oocysts per g DS decreased relatively slowly, generally following a log-linear decay pattern, irrespective of the extent of sludge desiccation (Fig. 4). The decay coefficient, *k*, and 95% confidence limits were consistent in both simulation experiments and were -0.030 (lower confidence limit range: -0.036 to -0.035; upper confidence limit range -0.026 to -0.025). Both decay relationships were highly statistically significant ($P < 0.005$), with r^2 values for S1 and S2 of 0.88 and 0.85, respectively (Fig. 4). Numbers of viable oocysts declined overall by approximately 1 - 2 $\log_{10}$ during the course of the simulated drying and storage experiments. Calculations based on the decay coefficients showed that 1.0 $\log_{10}$ removal of *C. parvum* viability in digested sewage sludge required an air-drying period equivalent to 11 weeks at ambient summer temperatures in the Melbourne region of Victoria. Over the same period, the numbers of detectable non-viable oocysts decreased at a similar rate to the decline in viable oocysts. This was attributed to the destruction of non-viable oocysts during sludge desiccation, which was consistent with the observed appearance of crushed oocysts.

Numbers of *C. parvum* oocysts in rural lagoon sludge

The recovery rate of inoculated *C. parvum* oocysts from lagoon sludge from WWTP B was 34.4 ± 5.5%. The estimated numbers of viable plus non-viable oocysts in sludges from the rural lagoon treatment systems were similar, ranging from 6.9 x 10^3 to 8.7 x 10^4 oocysts per L, equivalent to 1.7 x 10^2 to 9.5 x 10^2 oocysts/g DS (Table 1).

DISCUSSION

Recovery of oocysts from sludge by IMS

A simple, reliable and accurate method is required to measure numbers of viable oocysts of *Cryptosporidium* in sewage sludge. The mean recovery efficiency of spiked *C. parvum* oocysts (~10^5/mL) measured here in PBS by IMS (65%) was within the typical range reported (62 to 100%) for different water sources (Bukhari et al. 1998; Rochelle et al. 1999; McCuin et al. 2001; Feng et al. 2003). However, sewage sludge presents a more challenging matrix for detecting *C. parvum* oocysts, due to the presence of solid organic matter and other microorganisms and, consequently, poorer recoveries were obtained. For example, Rochelle et al. (1999) used a Dynal IMS bead assay to detect oocysts in seeded bovine faeces and found recovery efficiencies were considerably smaller (4.5%) than from water samples (62 - 100%), depending on the turbidity and oocyst seeding density. Here, the mean recovery efficiency for lagoon sludge from WWTP B (15% DS) spiked with oocysts of *C. parvum* was 34%. The recovery rate of total oocysts (viable plus non-viable relative to the initial inoculum density) of *C. parvum* inoculated into liquid digested sludge from WWTP A (DS 3% to 13%) measured at different time points during simulated solar/air drying was up to 49%, but could be as low as approximately 10% with an overall mean value of 33%. These values were within similar ranges of

oocyst recovery efficiencies reported by Molloy et al. (2006) for liquid (4-5% DS) and dewatered (19-20% DS) primary sludge, equivalent to 22 ± 3.5% and 43 ± 11.4%, respectively.

Microscopic observations suggested that the decay of oocysts undergoes several stages: viable, non-viable with intact surface, non-viable with damaged surface, and finally, disintegration. Indeed, we observed extensive amounts of physical damage to the parasite, such as partially crushed and disintegrating oocysts, and damaged outer membranes, particularly in the later stages of air drying treatment. The total number of oocysts observed at each sampling time included viable oocysts (stained) and non-viable oocysts. However, the presence of damaged (unstained) oocysts or oocysts that failed to bind with the IMS reagents, because they were embedded within intact sludge particles, would reduce the apparent recovery. Therefore, we took the approach of using the recovery of all observable oocysts to quantify the inactivation rate rather than separately determining the recovery of viable and non-viable oocysts. Thus, calculated decay rates for viable oocysts represented conservative estimates.

This is the first time that Syto-59 staining following FITC-C-mAb detection has been applied to the enumeration of viable *C. parvum* oocysts to quantify inactivation rates during air drying treatment of sewage sludge. The enumeration of viable *C. parvum* oocysts in sludge by IMS, followed by staining with Syto-59 and examination by CLSM was adopted for several reasons. Firstly, viable oocysts (stained green with FITC) could be readily differentiated from non-viable oocysts (stained red with Syto-59) by CLSM. Moreover, the ability to switch between different images of the sample

by CLSM, to select single dye, double dye or unstained views, also assisted in differentiating and classifying oocysts as viable or non-viable, based on their physical appearance and staining reaction.

Our results suggested that vital dye staining can determine the viability of *C. parvum* oocysts in sewage sludge treated, for example, by air drying and storage, where the inactivation mechanism is linked to physical and membrane damage. However, it is less likely to be applicable where oocyst decay is linked to DNA integrity without physically damaging the outer membrane structure. For example, UV light or γ-irradiation in drinking water treatment processes give false-positive reactions to vital dye staining because the outer membrane remains intact and does not allow entry and staining of the oocyst interior (Bukhari et al. 1999; Kato et al. 2001). Further studies are required to compare and confirm the measurement of *Cryptosporidium* oocyst viability in sewage sludge by dye permeability with the standard viability assay based on the neonatal mouse method. Nevertheless, from a water industry and microbiological risk assessment perspective, the use of vital dye staining may offer a suitable, practicable and cost-effective approach to monitor oocyst inactivation by common sludge treatments where biological and/or physical environmental factors promote decay of pathogens, such as lagoon and air-drying treatment processes and storage.

Decay of *C. parvum* in simulated pan-drying and stockpiling treatment

The rate of oocyst inactivation measured in the air drying simulation for digested sludge at 20 ^{0}C was highly statistically significant, with a decay coefficient of ~ -0.030. This value is comparable to previously reported decay data for the bacterial

indicators: *E. coli* and *Enterococcus* species, which exhibited average decay coefficients of 0.031 and 0.0305, respectively, in operational sludge drying pans at WWTP A (Rouch et al, 2011). This suggested that standard enteric bacterial indicator organisms could also be used to track the decay of *C. parvum* by air drying and storage treatment of sewage sludge. The loss of viability during pan drying was primarily related to storage time, and was generally independent of sludge desiccation and the increase in sludge DS content (Fig. 3). Furthermore, regression analysis, utilizing single decay rate models for *C. parvum* oocysts, provided statistically robust r^2 values, 0.88 and 0.85, with $P = 0.005$. Therefore, a single log-linear function gave a representative description of the overall decay of *C. parvum* for the experimental time periods applied in the air drying simulation experiments.

Extrapolating the decay profiles measured here for oocysts of *Cryptosporidium* species to pan-drying of sewage sludge under ambient environmental conditions in the State of Victoria indicated that 1 $\log_{10}$ removal may be achieved after 11 weeks drying treatment. At WWTP A, sludge is treated by mesophilic anaerobic digestion followed by air drying. Log_{10} removal values for *Cryptosporidium* oocysts reported for sludge anaerobic digestion processes are in the range of 2.15 to 3.20 (Stadterman et al. 1995; Horan & Lowe 2002; Kato et al. 2003). Therefore, a combination of anaerobic digestion followed by air drying for a minimum period of 11 weeks is forecasted to provide an overall reduction in *Cryptosporidium* oocysts equivalent to 3 to 4 $\log_{10}$ under ambient summer temperate conditions representative of Melbourne, Australia.

Oocyst numbers in lagoon sludge samples from rural WWTPs

Sucrose gradient extraction reduced the limit of oocyst detection in sludge to 43/g DS compared to approximately 130/g DS for the standard IMS method. Physical settlement is a major mechanism for removal of *Cryptosporidium* oocysts from wastewater, as the oocysts transfer to the settled sludge solids in sedimentation tanks or lagoons and we measured up to approximately 3 $\log_{10}$ oocysts/g DS in lagoon sludge sampled from rural WWTPs (Table 1). The results therefore suggested further treatment of sludge from lagoon treatment pond systems at rural WWTP in Victoria would be necessary to eliminate viable *Cryptosporidium* oocysts.

The relatively large numbers of *C. parvum* oocysts found in sludge lagoons tested in rural Victoria during the study period could be due to unrecognised or asymptomatic infections occurring in the community at that time. For example, a large increase in notifications of cryptosporidiosis occurred in 2013 and coincided with large increases in oocyst numbers detected in sewage sludge in Southern Australia (King et al. 2016). Alternatively, as water birds may transport and deposit viable *C. parvum* oocysts in waterways (USEPA 2001), they may also have been an important source of oocyst inputs to the lagoons as water birds were commonly present in large numbers at all the lagoon sites investigated. Nevertheless, any potential occurrence of *C. parvum*, irrespective of the source, requires appropriate management of the wastewater treatment system (e.g. by excluding wild animals and birds) and/or sludge to ensure microbiological safety if application to land is the selected end-use as an agricultural fertiliser.

CONCLUSIONS

Immunomagnetic separation, staining with vital dyes and examination by CLSM provided an effective approach to the isolation and enumeration of viable *C. parvum* oocysts in liquid anaerobically digested sewage sludge and after air-drying treatment. The limit of detection of *C. parvum* oocysts was ~130/g DS and was reduced to ~40/g DS by sucrose gradient separation. Inoculated oocysts of *C. parvum* decayed relatively slowly, with decay coefficients of approximately 0.030, in laboratory experiments simulating the solar/air drying and stockpiling of digested sewage sludge under temperate conditions. The main factor influencing the overall extent of oocyst inactivation was the duration of the drying/storage period. Air-drying treatment of digested sludge for 11 weeks provided a 1.0 $\log_{10}$ reduction in the viability of *Cryptosporidium* oocysts under temperate summer conditions experienced in the region of Melbourne, Australia. Numbers of oocysts are typically small or undetectable in sludge in regions with high standards of public health management and well developed waste treatment systems. However, open lagoon wastewater treatment systems may also receive inputs of *Cryptosporidium* oocysts from other environmental sources and sludge management practices must ensure adequate microbiological safety. Nevertheless, the results presented here provide assurance that a combination of mesophilic anaerobic digestion and air drying for 11 weeks together ensure an overall inactivation rate of viable *Cryptosporidium* oocysts equivalent to 3.0 $\log_{10}$. Based on the predicted numbers of total oocysts in raw sludge (<100/g DS) estimated from regional epidemiological infection statistics in Australia (Grant et al. 2012), this degree of oocyst inactivation is sufficient to effectively control the risk of human infection by *Cryptosporidium* species from agricultural utilisation of the treated biosolids.

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Table 1

Background numbers of viable *Cryptosporidium parvum* oocysts from environmental sources detected in lagoon sludge from rural WWTPs in regional Victoria

Sludge source	***Cryptosporidium parvum*^**	
	(oocysts / g DS ± sd)	**(oocysts / L ± sd)**
WWTP B	$2.60 \times 10^2 \pm 2.60 \times 10^2$	$3.90 \times 10^4 \pm 3.90 \times 10^4$
WWTP C	$1.70 \times 10^2 \pm 1.99 \times 10^2$	$6.93 \times 10^3 \pm 7.94 \times 10^3$
WWTP D	$9.53 \times 10^2 \pm 3.97 \times 10^2$	$8.68 \times 10^4 \pm 3.61 \times 10^4$

^ limit of detection ~43 oocysts/g DS

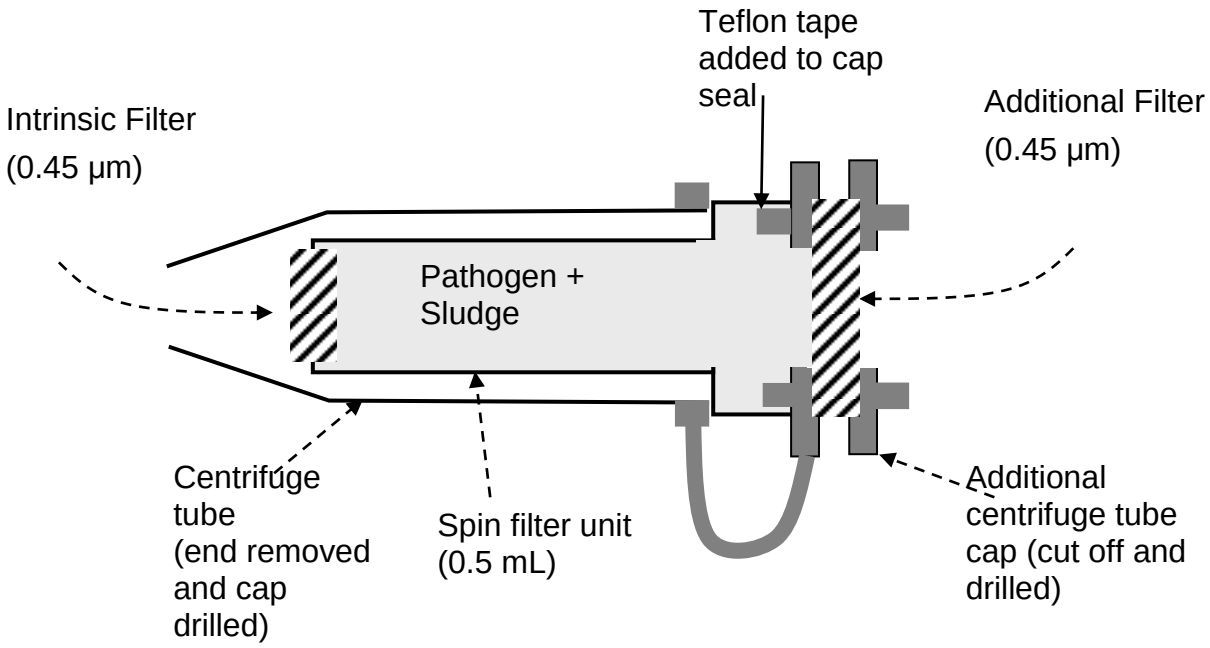

Figure 1

Assay chamber for investigating *Cryptosporidium* inactivation

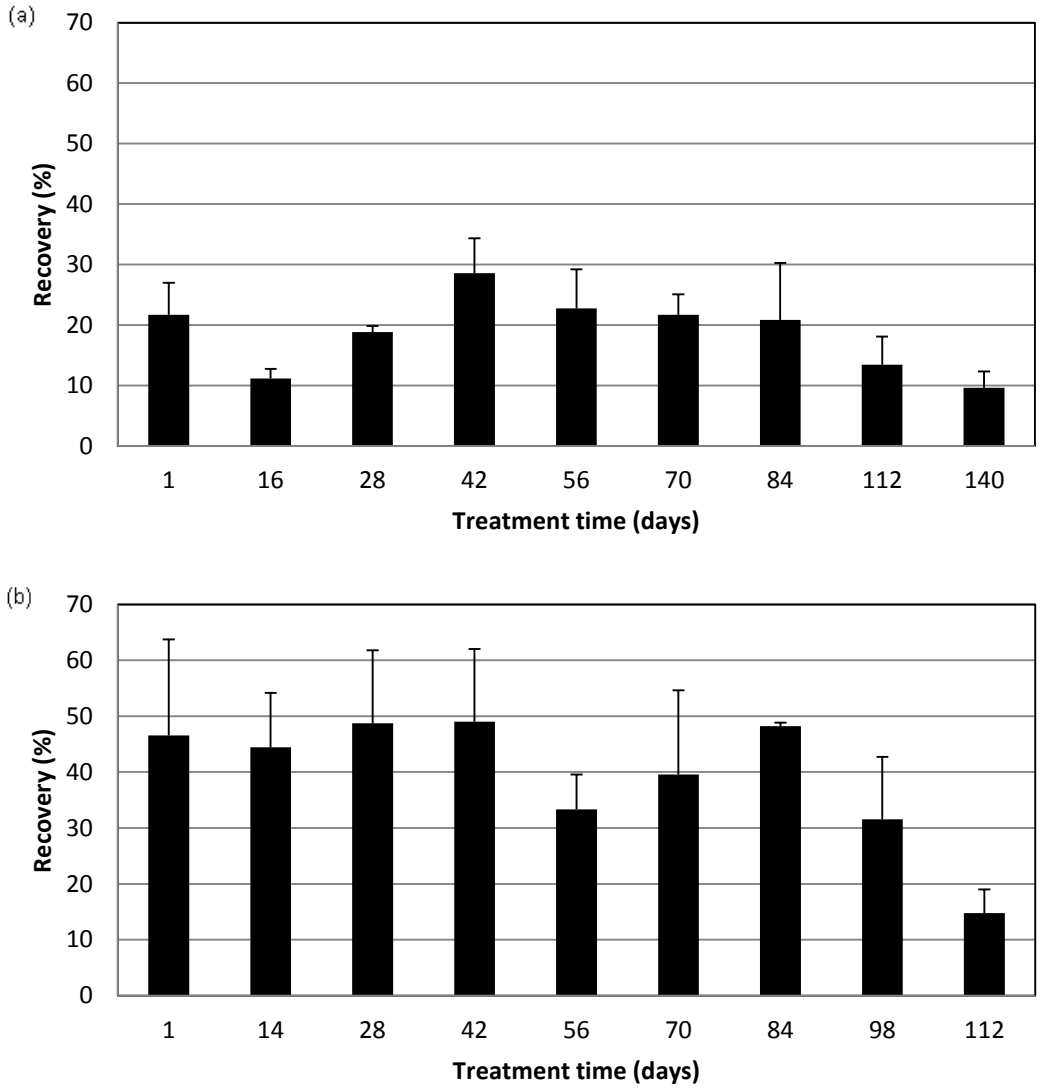

Figure 2

Recovery rate of total oocysts (% relative to the initial inoculum size) during simulated solar/air drying treatment of liquid mesophilic anaerobically digested sewage sludge: (a) Simulation Experiment 1 (S1) and (b) Simulation Experiment 2 (S2). Error bars indicate the sd

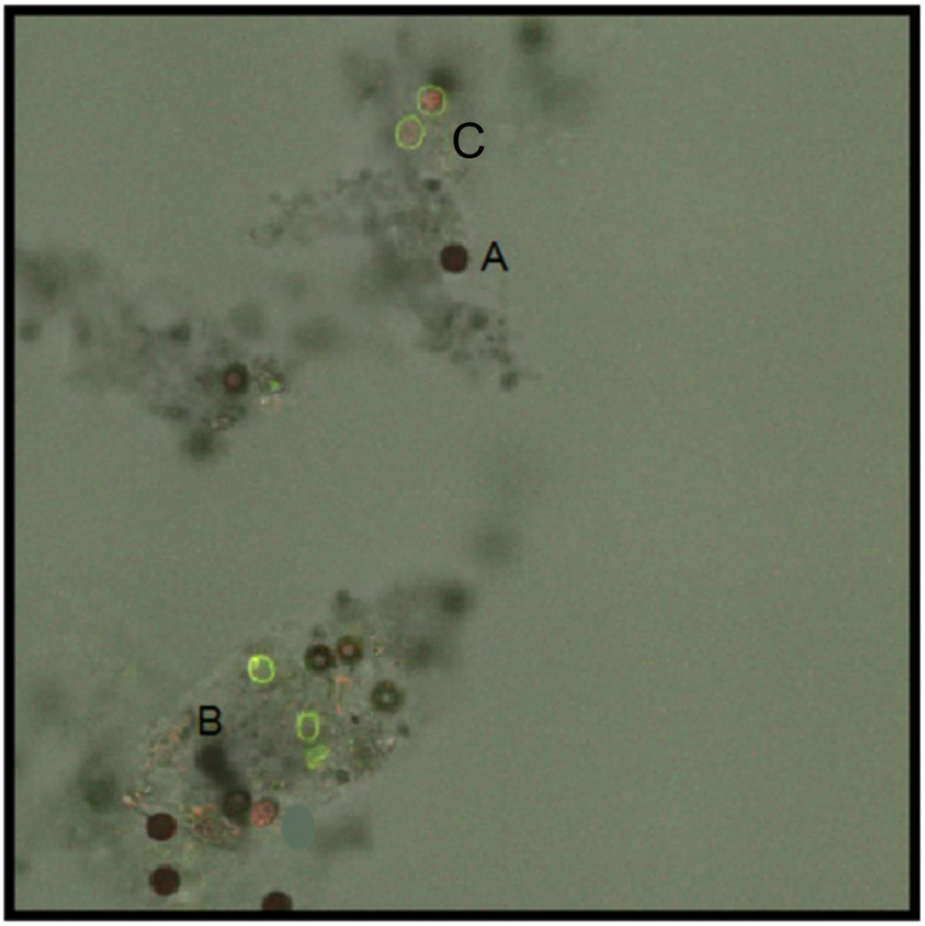

Figure 3

Appearance of *Cryptosporidium parvum* oocysts embedded in sludge particles by confocal laser scanning microscopy following immunomagnetic separation and vital staining. The sludge was sampled on day 56, Simulation 1. A, is a magnetic bead used to recover *C. parvum* oocysts from sludge. B, is a sludge particle with viable *C. parvum* oocysts embedded within the particle (surface stained green without internal stained red). C two non-viable *C. parvum* oocysts (surface stained green and internally stained red)

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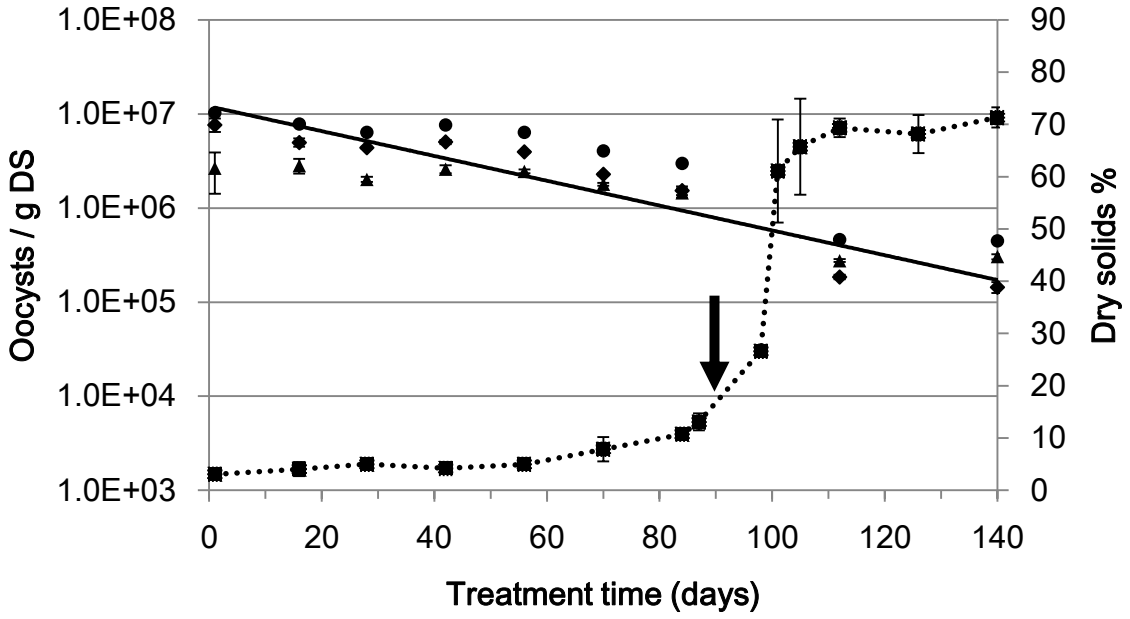
(a)



884

(b)

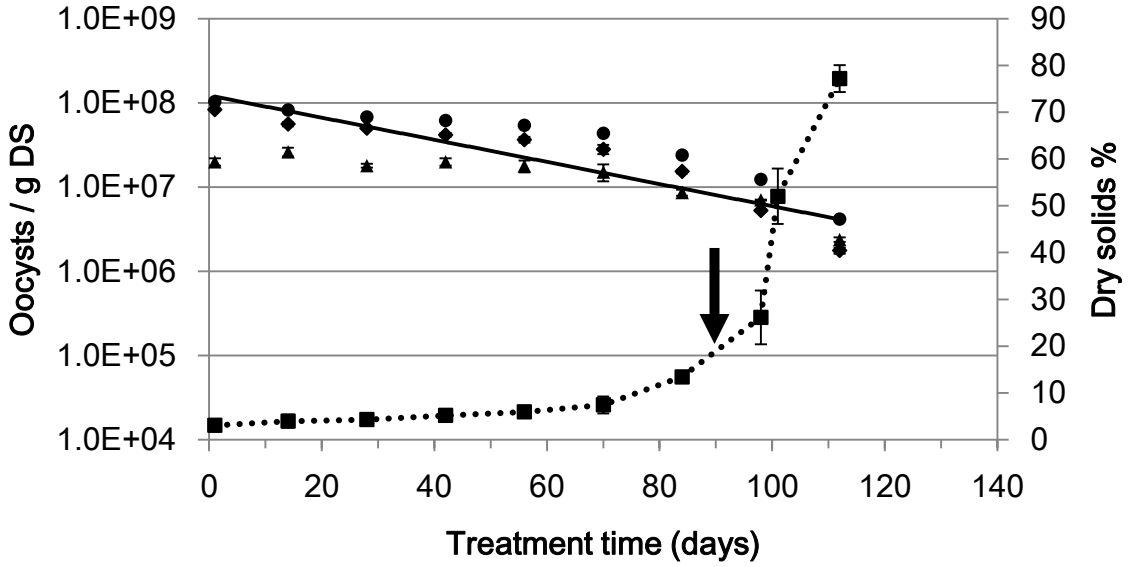


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Figure 4

Decrease in *Cryptosporidium parvum* oocyst viable counts (♦), non-viable counts (▲), and total counts (●), in mesophilic anaerobically digested sewage sludge during simulation air drying treatment and in relation to dry solids (%DS) content (■) in (a) Simulation Experiment 1 (S1) and (b) Simulation Experiment 2 (S2). Each data point represents the mean of three replicate values; error bars show one sd. Vertical arrows indicate the switch from liquid to solid (stockpile) sludge management phase. Regression relationships for decay of viable *C. parvum* are shown: number of viable oocysts per g DS, $y = 1.20 \times 10^{8*} e^{-0.03*t}$ for S1, and $1.22 \times 10^{8*} e^{-0.03*t}$ for S2, where t is the treatment time in days; statistical values: $r^2 = 0.88$, $P = 0.005$ (S1) and $r^2 = 0.85$, $P = 0.005$ (S2)