Interactions between humic matter and bacteria when disinfecting water with UV light

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1   **ABSTRACT**

2   **Aims:** To investigate the impact of aquatic humic matter on the inactivation of *E. coli* and *B.*

3   *subtilis*, by 254 nm ultraviolet (UV) light, and to qualitatively investigate the interaction between

4   humic matter and the bacterial surfaces in water.

5   **Methods and Results:** A bench-scale study investigated the potential for humic matter to coat

6   the surface of bacteria (*E. coli* and *B. subtilis*) and consequently offer protection from low-

7   pressure UV light. A factorial experiment was completed in which two bacteria (*E. coli* and *B.*

8   *subtilis*) and two types of humic matter (Aldrich® humic acid (AHA) and Suwannee River

9   natural organic matter (SR NOM)) were exposed to UV light (5 and 14 mJ cm^{-2}). The UV light

10   was delivered using a collimated beam apparatus at four concentrations of humic matter (0, 10,

11   50, 120 mg L^{-1}) in reagent grade water. Both AHA and SR NOM were found to offer statistically

12   significant ($\alpha = 0.05$) protection of both *E. coli* and *B. subtilis* at concentrations of 50 and 120

13   mg L^{-1} for a UV dose of 14 mJ cm^{-2}. The level of protection from low-pressure UV light offered

14   by AHA or SR NOM was not sensitive to pH between 5 and 9. TEM images of *E. coli* and humic

15   matter suggest that the cells and humic matter share an affinity under non-aqueous conditions.

16   **Conclusions:** Both *E. coli* and *B. subtilis* are susceptible to coating by aquatic- and soil-derived

17   humic matter which can reduce the sensitivity of the cells to UV light.

18   **Significance and Impact of Study:** This study provides evidence for a mechanism whereby

19   microorganisms in the environment may acquire characteristics through interaction with humic

20   matter that render them more resistant to UV disinfection than would be predicted based on

21   laboratory inactivation studies using clean cells.

22

23   **Keywords:** *B. subtilis*, disinfection, *E. coli*, humic matter, particles, shielding, ultraviolet

2

1 **INTRODUCTION**

2 UV inactivation data are generally collected using microorganisms in high quality, humic-free

3 water. However this may not be representative of typical UV disinfection for drinking water

4 treatment, and in particular when treating water from surface sources where humic material is

5 common. This study explored the potential for humic matter in the natural environment to coat

6 the surfaces of bacteria, protecting them from UV disinfection.

7 Natural organic matter (NOM) from biota, vegetation, soil and other sources, is present in

8 all surface water. In one survey, 50% of the organic matter in surface water and 90% of

9 dissolved organic matter was humic substances (Thurman 1985). Humic substances can be

10 divided into hydrophobic (humic acids) and hydrophilic (fulvic acids) components (Thurman and

11 Malcolm 1981). The amount and composition of humic matter varies greatly depending on the

12 originating sources and changes due to biotic and abiotic processes within the water (Kitis *et al.*

13 2001; Thurman 1985). Most surface water contains less than 10 mg L^{-1} humic matter. Humic

14 acid molecules are multifunctional (*i.e.* have many different functional groups) and can display

15 affinity for various surfaces, such as aqueous metal cations (Hering and Morel 1988; Manunza *et*

16 *al.* 1995) and hydrophobic organic molecules (Fein *et al.* 1999). As a result, there is good reason

17 to suspect that humics might interact with microbial surfaces.

18 The extent to which humic matter interacts with viruses has been the topic of recent

19 study. Templeton *et al.* (2006) reported that soil-derived humic acid coated the surface of

20 viruses, protecting them from UV disinfection. This impact on UV disinfection was observed for

21 a humic layer coating the viruses that was thin enough to be able to pass through 0.45 μm pore-

22 size filters. The study reports statistically significant protection ($\alpha = 0.05$) at Aldrich[®] humic

23 acid concentrations of 50 and 150 mg L^{-1} for T4 phage and 150 mg L^{-1} for MS2 phage. Since

1   filtration (0.45 μm pore-size) had no effect on the inactivation trials, the authors hypothesized

2   that protection was due to humic acid coating the surfaces of the viruses. The current study will

3   explore whether a similar phenomenon is observed for bacteria.

4   Bacterial cell wall surfaces contain numerous functional groups (*e.g.* carboxyl,

5   phosphate, and phenolic) which results in bacterial surfaces displaying both hydrophilic and

6   hydrophobic regions (Harden and Harris 1953). Bacteria are commonly categorized by their

7   Gram designation, a test based on the cell staining properties (Prescott *et al.* 1999). By selecting

8   surrogates for this study from both categories, the effect of Gram designation on humic coating

9   and subsequent protection might be considered. Two surrogates were selected for this study:

10   *Escherichia coli* (*E. coli*) which is Gram negative, and *Bacillus subtilis* (*B. subtilis*) which is

11   Gram positive.

12   This study expanded on the earlier work of Templeton *et al.* (2006) in several ways. First,

13   it investigated whether soil-derived humics would coat bacteria as observed with viruses.

14   Second, in addition to using the soil-derived humics used by Templeton *et al.*, this study

15   considered humic matter extracted from a river. Aquatic humic matter is more representative of

16   the humics that might pass through a full-scale UV reactor used for drinking water treatment.

17   This study also assessed the impact of pH on observed protection offered by humic

18   matter. The pH of a sample can alter the surface chemistry of both humic matter and bacterial

19   surfaces (Harden and Harris 1953, Fein *et al.* 1999). This might impact the affinity between

20   bacteria and humic matter.

21   The objectives of this study were to (i) identify any surface-coating and consequent

22   protection of *E. coli* and *B. subtilis* from low-pressure UV light with the addition of Aldrich[®]

23   humic acid (AHA) or Suwannee River natural organic matter (SR NOM); (ii) determine the

1 effect of pH (5, 7, and 9) on AHA and SR NOM protection of *E. coli* from UV light; and (iii)

2 qualitatively investigate the interaction between humic matter and the bacterial surfaces in water

3 using microscopy.

4

5 **MATERIALS AND METHODS**

6 **Experimental conditions**

7 Synthetic water matrices were considered in this study so that humic matter type and

8 concentration, and pH, could be controlled to answer fundamental questions concerning the

9 interactions of humic matter and bacteria in water. The sample pH was controlled to 5, 7, or 9

10 with buffer solutions made with ratios of boric acid (H_3BO_3), citric acid ($H_3C_6H_5O_7 \cdot H_2O$) and

11 trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) (EMD Chemicals Inc., Gibbstown, NJ) (Shugar and

12 Ballinger 1996). For sample pH of 5, 7, and 9, the final concentrations of boric acid, citric acid

13 and tertiary sodium phosphate were 13, 3, and 7 mmol L^{-1} (pH 5), 10, 2.5 and 5 mmol L^{-1} (pH 7),

14 and 7, 2, and 3 mmol L^{-1} (pH 9). The buffer concentrations were determined based on the

15 minimum chemical concentrations able to buffer the sample pH to within 0.1 units after the

16 addition of 150 mg L^{-1} of AHA. The sample pH was measured using a VWR pH meter Model

17 8015 (VWR, Mississauga, ON).

18   Sample mixing was performed in 200 mL Nalgene® bottles. After the addition of humic

19 matter and bacteria to the buffer solution, the 100 mL samples were mixed by vortexing (Vortex

20 Genie 2, Fisher Scientific, Pittsburgh, PA) at a speed setting of 8 for 15 seconds. UV inactivation

21 trials with low-pressure UV light (5 and 14 mJ cm^{-2}) were completed with *E. coli* and *B. subtilis*.

22 Each disinfection trial was replicated 5 times (n = 5). All experiments were conducted at 20°C.

23

Humic matter

Two types of humic matter were considered for these experiments. Aldrich® humic acid, sodium salt (AHA) (Sigma-Aldrich Inc., St. Louis, MO), and aquatic natural organic matter (NOM) isolated from the Suwannee River by reverse osmosis (International Humic Substances Society (cat no. 1R101N, Golden, CO). AHA is not the ideal humic surrogate since it is derived directly from soil and not from aquatic sources (Malcolm and MacCarthy 1986; Latorre 2003) but is convenient for bench-scale studies and the coating effect has been shown with AHA and viruses (Templeton *et al.* 2006). SR NOM was therefore also used to represent natural aquatic humic matter. Stock solutions of 10 g L^{-1} were prepared by dissolving precisely weighed amounts of dried AHA and SR NOM in Milli-Q® water. These solutions were stored in the dark at 4^oC and prepared fresh every 28 days.

Experiments were conducted with humic concentrations of 0, 10, 50, and 120 mgL^{-1} (AHA or SR NOM). The AHA material was 65% carbon by mass so the carbon equivalent concentrations for AHA were 0, 6.5, 32.5, and 78 mg L^{-1} C, and the SR NOM was 51% carbon by mass so the carbon equivalent concentrations for SR NOM were 0, 5.1, 25.5, and 61.2 mg L^{-1} C. While typical source waters for drinking water treatment often have organic carbon concentrations less than 10 mg L^{-1}, the concentrations used in this study were selected with the intention of magnifying any humic coating effect that may exist to allow the fundamental principle to be observed with confidence. Also, such concentrations are representative of other treatment scenarios such as treatment of greywater (TOC: 35 to 160 mg L^{-1}) and wastewater (DOC: 20 to 40 mg L^{-1}; TOC: 50 to 1,000 mg L^{-1}) (Kavanaugh 1978; Cairns *et al.* 1993; Ramon *et al.* 2004; Winward *et al.* 2007).

1 **UV Collimated Beam Exposures**

2 The response of a microorganism to UV light is typically determined with the aid of a collimated

3 beam UV apparatus. The ultraviolet energy emitted by a UV lamp is directed onto a horizontal

4 exposure surface. The UV source is separated from the exposure surface by a cylindrical tube

5 that acts to collimate the UV light so that the UV light reaching the exposure surface is

6 orthogonal to the surface. Samples are placed on the collimator, mixed well and exposed to UV

7 light for predetermined lengths of time.

8   Two low-pressure mercury lamps (λ = 253.7 nm) (model 9328-L24, Photoscience/

9 Advanced UV Inc., Torrance, CA), were the source of UV radiation. The lamps were powered

10 using a standard 120 V outlet through a ballast system and housed in a collimating apparatus

11 constructed with 4-inch schedule 80 PVC tubing. The apparatus was constructed by Suntec

12 Environmental (Concord, ON). The standard UV dose measurement and calculation methods for

13 collimated beam experiments described by Bolton and Linden (2003) were followed. UV

14 exposures were completed with shallow (liquid depth = 0.34 cm) samples that were well mixed

15 without inducing vortices, using a 1 cm micro stir bar in an 8.5 cm Petri dish placed on a flat bed

16 collimator. The distribution of UV intensity across the exposure surface (*i.e.* along a 0.5 cm by

17 0.5 cm grid) was measured using an IL1700 radiometer equipped with an SUD240 sensor

18 (International Light, Newburyport, MA) and was incorporated into the average UV dose

19 calculation, as described in detail by Bolton and Linden (2003). The incident UV intensity was

20 approximately 0.19 mW cm^{-2} at the exposure surface.

21   UV absorbance data (λ = 254 nm) were collected with a spectrophotometer (model

22 CE3055, Cecil Instruments, Cambridge, England). UV absorbance measurements were

23 determined using a fixed 11° angle centre mounted integrating sphere accessory (Labsphere,

7

1   North Sutton, NH) to account for scattered light (Christensen and Linden 2003; Mamane and

2   Linden 2006). The instrument was zeroed before each set of measurements using Milli-Q® water.

3   The UV dose calculation method presented in Bolton and Linden (2003) was followed to

4   determine the UV exposure time in the collimated beam apparatus. Since this method considers

5   the sample UV absorbance in the dose calculation, it resulted in longer exposure times for the

6   samples with higher UV absorbance. For example, for a UV dose of 14 mJ cm^{-2}, the exposure

7   time for samples without humic matter was typically 82 seconds while the exposure time for

8   samples with 120 mg L^{-1} AHA was about 180 seconds. Therefore, any subsequent reduction in

9   UV disinfection performance could possibly be attributed to time-dependent factors not taken

10   into account in this UV dose calculation, such as surface-level interactions between UV-

11   absorbing humic matter and target bacteria. It is believed, however, that any such factors would

12   be minor.

13   *E. coli* and *B. subtilis* samples were exposed to UV doses of 5 and 14 mJ cm^{-2}. These UV

14   doses were selected to achieve approximately 1- and 5-log inactivation of *E. coli* and 1- and 4-

15   log inactivation of *B. subtilis*. The doses were selected to achieve an upper inactivation target of

16   at least 4-log inactivation in the humic-free samples as Templeton *et al.* (2006) found the impact

17   of humic-coating was more pronounced for 4-log inactivation compared with 2-log inactivation

18   of MS2 and T4 phages. UV inactivation trials were replicated 5 times (n = 5) for each

19   combination of bacteria (*E. coli* and *B. subtilis*) and humic matter (AHA and SR NOM).

20

21   **Filtration trials to distinguish attached and unattached humics**

22   An experiment was designed to confirm that humic matter would attach to the surfaces of *E. coli*.

23   The test involved filtering water samples containing humic matter and *E. coli* using a 0.45 μm

1   pore-size nitrocellulose filter (Fisher Scientific, Pittsburg, PA). Details of the experiment are

2   presented in the Results section. As a control step, each filter was rinsed with 50 mL of Milli-Q$^{®}$

3   water prior to sample filtration to ensure no UV absorbing material was rinsed from the

4   membrane into the filtrate.

5

6   **Microbial methods**

7   Cultures of *E. coli* (ATCC$^{®}$ 15597) and *B. subtilis* (ATCC$^{®}$ 6633) were purchased from the

8   American Type Culture Collection (Manassas, VA). Stock cultures were frozen at -70°C with

9   10% sterile glycerol, according to Standard Method 9224-B (APHA 2005). *E. coli* culturing

10   followed the protocol in Standard Method 9224-C (APHA 2005) by inoculating 10 mL of tryptic

11   soy broth with *E. coli* from a plate with an inoculating loop and incubating the culture for 16

12   hours at 37 ± 0.5^{o}C. A 2.0 mL aliquot of the 16 hr culture was aseptically transferred to 500 mL

13   tryptic soy broth in a 1 L Pyrex$^{®}$ container and incubated for 4 hours at 37 ± 0.5^{o}C. *B. subtilis*

14   was cultured and stored in a similar manner except nutrient broth (Becton, Dickinson and

15   Company, Sparks, MD) was used as the growth medium, 20 mL of broth was used for the 16 hr

16   culture and 250 mL for the stock growth, and the *B. subtilis* was allowed to shake at 200 rpm

17   using an orbital rotator in all stages of incubation. The final *B. subtilis* culture was grown for 7

18   hours at 37 ± 0.5^{o}C.

19   On the day of experiments, the frozen cell culture (*E. coli* or *B. subtilis*) was thawed and

20   aseptically transferred into sterile centrifuge tubes and centrifuged at 14,480g (Sorvall RC 5C

21   Plus, Kendro Laboratory Products, Newtown, CT) for 15 minutes (*E. coli*) or 20 minutes (*B.*

22   *subtilis*) at 4°C. This process was repeated, with the supernatant removed each time and the

23   pellet re-suspended in 10.5 ml of sterile Milli-Q$^{®}$ water by vortexing (Vortex Genie 2, Fisher

1 Scientific, Pittsburgh, PA) set at a speed setting of 6. After the final centrifugation step, the

2 cultures were re-suspended in 10.5 ml of sterile Milli-Q® water and used that day for

3 experiments. The resulting stock concentrations of *E. coli* and *B. subtilis* were 10^8-10^9 CFU mL^{-1}

4 and 10^7 CFU mL^{-1}, respectively.

5   A control test was conducted to ensure that the freezing/thawing process had negligible

6 impact on the results. In the control test, both fresh and thawed *E. coli* without AHA and with 50

7 mg L^{-1} of AHA were exposed to 10 mJ cm^{-2} of UV light. The fresh and thawed *E. coli* each

8 exhibited approximately 3-log inactivation without humic matter and approximately 2-log

9 inactivation with 50 mg L^{-1} AHA, with no statistically significant difference between the fresh

10 and thawed cultures. The control test therefore suggested that the humic coating effect was

11 independent of freezing/thawing.

12   Sample enumeration for *E. coli* and *B. subtilis* was performed by spread plate technique

13 according to Standard Method 9215-C (APHA 2005), with plates of tryptic soy agar (Becton,

14 Dickinson and Company, Sparks, MD) and nutrient agar (Sigma-Aldrich Inc., St. Louis, MO),

15 respectively. Plates were incubated at 37 ± 0.5^oC for 20 to 24 hours prior to enumeration.

16 Concentrations of 10^7 CFU mL^{-1} of *E. coli* or 10^6 CFU mL^{-1} of *B. subtilis* were used for the UV

17 inactivation and filtration experiments. *B. subtilis* can exist as both an endospore and as a

18 vegetative cell. Since *B. subtilis* was used to represent Gram positive bacteria, it is the vegetative

19 cell needed for these experiments. Since the endospore is much more UV resistant than the cell, a

20 quality control step was followed to ensure that no spores were present in the *B. subtilis* stock.

21 On each day of experiments, the stock culture was confirmed to be free of *B. subtilis* endospores

22 via enumeration as outlined by Verhille *et al.* (2003) by heating the sample to 70^oC for 15

23 minutes prior to plating.

1

2 **Microscopy Imaging**

3 Images of *E. coli* and humic matter were collected using a Hitachi H7000 transmission electron

4 microscope (TEM) (Hitachi High-Technologies Canada Inc., Rexdale, ON). It was expected that

5 any affinity of humic matter for the surface of bacteria might be observed under magnification.

6 Samples were prepared for examination using the direct application method for negative staining

7 (Doane and Anderson 1987). A 5 µL aliquot of sample (*E. coli*, *E. coli* + 120 mg L^{-1} AHA, or *E.*

8 *coli* + 120 mg L^{-1} SR NOM) was placed directly onto a Formvar®-carbon coated copper grid of

9 400 mesh grating size. The sample droplet was allowed to sit on the grid for 120 seconds without

10 drying. The grid was then negatively stained by adding either a 10 µL aliquot of aqueous 2%

11 phosphotungstic acid (PTA) (pH 6.5) or a 10 µL aliquot of 2% aqueous uranyl acetate (UA)

12 directly onto the sample droplet. After 30 seconds for the PTA or 15 seconds for the UA, excess

13 fluid from the stain and sample droplet was drawn off the grid with filter paper, leaving a thin

14 film of stained sample on the grid surface which was left to dry. Once dry, the samples were

15 examined using TEM with an accelerating voltage of 75 kV and images captured using a charged

16 couple device.

17

18 **Data presentation and statistical analysis**

19 Student t-test comparisons ($\alpha = 0.05$) were made between the log inactivation data sets for the

20 trials with humic matter (*i.e.* AHA or SR NOM) versus the control trials (*i.e.* without humic

21 matter). A minimum of five replicate exposures were conducted for each humic condition at each

22 UV dose.

1   Box and whisker plots are used in this study to present the log inactivation data, in which

2   the upper and lower edges of the box represent the 75th and 25th percentile values of the data set

3   respectively, and the whiskers extend to the maximum and minimum values of the data set. This

4   displays the entire distribution of each data set instead of considering only mean values, which is

5   inappropriate for data sets that do not conform to a normal distribution.

6

7   **RESULTS**

8   **UV inactivation results**

9   The effect of AHA and SR NOM on inactivation of bacteria (*E. coli* and *B. subtilis*) at two UV

10   doses (5 and 14 mJ cm^{-2}) is reported in Figure 1 and Figure 2 for *E. coli* and *B. subtilis*,

11   respectively. For the *E. coli* without the addition of humic matter approximately 1- and 5-log

12   inactivation were observed for low-pressure UV doses of 5 and 14 mJ cm^{-2}. These results are

13   consistent with the UV-sensitivity of *E. coli* to UV light reported in literature (Hijnen *et al.*

14   2006). Since the UV inactivation kinetics of ATCC® 15597 *E. coli* and ATCC® 6633 *B. subtilis*

15   (vegetative cells) were not found in the literature, the kinetics are presented along with

16   regression data in Figure 3.

17   The log inactivation of both *E. coli* and *B. subtilis* achieved for a constant UV dose of 14

18   mJ cm^{-2} decreased with the addition of both AHA and SR NOM. Based on a Student t-test

19   comparison of means using a 95% confidence level ($\alpha = 0.05$), there was a statistically

20   significant difference between the log inactivation of *E. coli* for a UV dose of 14 mJ cm^{-2}

21   between the samples with no humic addition (0 mg L^{-1}) and both 50 and 120 mg L^{-1} of either

22   AHA or SR NOM. The results shown in Figure 1 indicate the magnitude of the reduction in UV

23   effectiveness increased with humic content suggesting the level of protection is concentration

1   dependent. A reduction in log inactivation in the presence of AHA is consistent with data

2   presented by Templeton *et al.* (2006) for inactivation of MS2 and T4 phages.

3   In addition to being statistically significant, the magnitude of the impact of humic matter

4   on the inactivation of *E. coli* and *B. subtilis* is dramatic. For the samples containing *E. coli*

5   exposed to 14 mJ cm^{-2}, only 2.5-log inactivation was achieved when the samples contained 120

6   mg L^{-1} of either AHA or SR NOM. This represents a 50% reduction in log inactivation when

7   compared to the 5-log inactivation of *E. coli* achieved for the same UV dose in the absence of

8   humic matter. Similarly, when comparing the inactivation of *B. subtilis* in the samples with and

9   without 120 mg L^{-1} AHA, a 50% reduction in log inactivation was observed (from 4-log without

10   AHA to 2-log with 120 mg L^{-1} AHA). In terms of the impact on log inactivation, SR NOM was

11   slightly less effective at protecting *B. subtilis* compared to AHA.

12   In contrast to the results at the higher UV dose, when the lower UV dose was applied (5

13   mJ cm^{-2}), humic matter had no statistically significant impact on inactivation for either *E. coli* or

14   *B. subtilis*, although a slight trend is observed in Figure 1 and 2. One possible explanation is that

15   the impact of humic matter on the inactivation of *E. coli* or *B. subtilis* was smaller than the

16   variation within the data set for UV dose of 5 mJ cm^{-2} when only about 1-log inactivation was

17   achieved. An alternative explanation is that only a small portion (*e.g.* < 10%) of the bacteria

18   were affected by the humic coating and therefore no appreciable impact was seen for 1-log

19   inactivation. Note that there was little variation in the microbial enumeration for the controls (0

20   mJ/cm^2), indicating that most of the variability observed in Figures 1 and 2 was due to exposure

21   of the organisms with UV.

22   The *E. coli* log inactivation results for varying sample pH are presented in Figure 4.

23   There was no statistically significant difference ($\alpha = 0.05$) between log inactivation of *E. coli* for

1   a UV dose of 14 mJ cm^{-2} with change in pH (5, 7, and 9) for samples with AHA or SR NOM at

2   50 mg L^{-1}.

3

4   **Humic washing trials**

5   A further inactivation trial was completed to determine whether the humic coating and observed

6   reduction in UV effectiveness can be reversed by washing the humic matter from the bacteria. *E.*

7   *coli* and 120 mg L^{-1} AHA were mixed and the inactivation using 10 mJ cm^{-2} of UV light was

8   determined. A parallel sample without UV exposure was centrifuged at 14,480*g* for 15 minutes

9   at 4°C. The pellet was re-suspended in sterile Milli-Q$^{®}$ water. This process was repeated 3 times.

10   Aliquots of the washed sample were then exposed to 10 mJ cm^{-2}. The log inactivation for each

11   sample condition is reported in Table 1 (loss of viable bacteria during the washing was limited to

12   < 10% of the initial counts. These losses were accounted for in subsequent calculations of log

13   inactivation). Standard deviations are reported for replicate (n = 5) exposures. A 2.8-log

14   inactivation of *E. coli* was observed in the absence of humic matter, which was consistent with

15   the earlier experiments (Figure 3). When comparing the level of inactivation of *E. coli* in the

16   sample containing 120 mg L^{-1} AHA with the AHA-free sample, a nearly 2-log decrease in the *E.*

17   *coli* reduction for a dose of 10 mJ cm^{-2} was observed (*i.e.* from 2.8 log to 1.1 log). After washing

18   the parallel *E. coli* / AHA mixture, the level of inactivation increased to 2.1 log, partially back to

19   the original 2.8 log inactivation.

20   It was clear from visual inspection of the *E. coli* pellet after centrifugation that most, but

21   not all, of the AHA was removed from the *E. coli* during washing. In the absence of humic

22   matter, the *E. coli* pellet appears opaque. With the AHA in the sample a dark spot in the middle

23   of the pellet was evident. So while most of the humic matter was removed by centrifugation

1   there was some AHA that remained even after a third rinse by centrifugation. It was also clear

2   from the UV absorbance of the re-suspended samples (see Table 1) that nearly all the AHA was

3   removed by the washing process. The *E. coli* itself typically contributed to a UV absorbance of

4   0.3 cm^{-1}; however, with the addition of humic material the UV absorbance typically reached 2.7

5   cm^{-1}. After washing, the sample absorbance decreased to 0.4 cm^{-1}. While it is not clear what

6   effect centrifugation has on the humic-cell interaction, it is clear that a small amount of humic

7   matter (*i.e.* the amount left in the washed sample that caused a slight increase, $\sim 0.1 \text{ cm}^{-1}$, in the

8   sample UV absorbance) is able to have an appreciable impact on the effectiveness of UV light

9   for disinfection; in this case, representing an average of 0.7-log less inactivation. It may be that a

10   small fraction of the humic matter is strongly attached to the cell, unless centrifugation

11   confounded this assessment by altering the humic-cell attachment. This implies that even though

12   the earlier results showed statistically significant ($\alpha = 0.05$) protection at only the highest

13   concentrations of humic matter (*i.e.* 50 and 120 mg L^{-1}), it may be possible for humic matter at

14   lower concentrations to accumulate on a cell's surface and offer protection from UV light. This

15   evidence is important in the context of drinking water treatment. While most waters contain

16   much lower concentrations of humic matter than used in this study, it is hypothesized that over

17   time, microorganisms in the environment could accumulate and concentrate humics on their

18   surface which might offer more protection from UV light than would be predicted based on the

19   bulk water humic concentration. Future work should explore this hypothesis.

20

21   **Effect of humic matter on particle size**

22   The UV inactivation experiments did not involve a coagulation step, nor was coagulation desired

23   in order to isolate the hypothesized surface-coating phenomenon from particle interferences. To

1   determine if the addition of AHA or SR NOM resulted in any coagulation or aggregation of

2   cells, particle size analysis was undertaken. Particle size analysis was completed using a

3   Multisizer 3 particle analyzer (Beckman Coulter Canada, Mississauga, ON) for samples with and

4   without humic matter. The samples without humic acid were virtually free of particles > 3 μm (<

5   0.5% of particles by number). Similarly with 120 mg L^{-1} of either AHA or SR NOM, there was

6   little, if any, aggregation of *E. coli* with the addition of humic matter (< 0.5% of particles by

7   number > 3 μm). The purpose of this exercise was to verify that *E. coli* was dispersed and that

8   the addition of AHA or SR NOM did not create humic particles or aggregates of *E. coli*. This

9   was an important control step demonstrating that the protection from UV light was not due to

10 particle-related shielding or protection due to cell aggregation.

11

12 **Filtration trials**

13 A separate experiment was conducted to test the theory that humic matter could attach to the

14 surface of *E coli*. The *E. coli* was mixed with dissolved humic matter and then filtered through a

15 0.45 μm filter that would capture all *E. coli* but allow dissolved humic matter to pass through.

16 The UV absorbance of the *E. coli* retained on the filter was determined (denoted as "particulate

17 UV absorbance" in Figure 5), with the theory that filtered *E. coli* that had first been exposed to

18 humic matter would have a higher UV absorbance than filtered *E. coli* that had not been exposed

19 to humic matter in a control sample, thereby demonstrating that the humic matter would attach to

20 the surface of the *E. coli*. (Note that in practice, it was not possible to measure the UV

21 absorbance of the filtered *E. coli*. Instead, the absorbance of the "filtered *E. coli*" was calculated

22 as the difference between the absorbance of the initial sample containing *E. coli* plus humic

23 matter and the absorbance of the filtered sample).

1   The results of this experiment are shown in Figure 5. Control samples containing only

2   120 mg L^{-1} AHA or SR NOM (no *E. coli*) experienced a reduction in UV absorbance of

3   approximately 0.08 to 0.02 cm^{-1} across the filter, presumably due to absorption or entrapment of

4   colloidal humic matter by the filter. "Clean" *E. coli* alone (*i.e.* humic matter concentration of 0

5   mg L^{-1} in Figure 5) had a UV absorbance of approximately 0.10 cm^{-1}, which was completely

6   removed when passing the sample through the filter. Therefore, the total UV absorbance lost

7   across the filter due to "clean" *E. coli* plus humic matter not attached to cells could be predicted

8   to be in the order of 0.12 to 0.18 cm^{-1}. However, when *E. coli* was first mixed with 120 mg L^{-1}

9   AHA, the absorbance lost across the filter averaged 0.3 cm^{-1}, suggesting that some humic matter

10 was attaching to the cells and being removed. In contrast, for the 50 mg L^{-1} AHA sample and all

11 samples containing SR NOM, there was no conclusive demonstration that humic matter was

12 removed across the filter by being attached to the *E. coli* cells, since the loss in absorbance

13 across the filter was no greater than the 0.2 cm^{-1} observed for the controls.

14

15 **Microcopy imaging of *E. coli***

16   TEM images of *E. coli* are presented in Figure 6. The *E. coli* (*e.g.* Figure 6 (a)) was easily

17 identified by its shape and typical flagella and pili in samples with and without humic matter.

18 Humic matter (both AHA and SR NOM) stained with PTA produced distinct grainy capsules that

19 were not observed for *E. coli* in the absence of humic matter (*e.g.* Figure 6 (b) and (c) vs. (a)). As

20 shown in Figure 6 (b) and (c) these capsules tended to interact with *E. coli*.

21

22 **DISCUSSION**

23 **Impact of humic matter on UV inactivation**

1   The inactivation results show that the AHA coating phenomenon reported for viruses (MS2 and

2   T4) (Templeton *et al.* 2006) can also be observed for bacteria (*E. coli* and *B. subtilis*). In

3   addition, the humic matter surface-coating phenomenon is not restricted to commercial humic

4   acids (AHA) but was also found for SR NOM. Both AHA and SR NOM were found to offer

5   statistically significant ($\alpha = 0.05$) protection of both *E. coli* and *B. subtilis* at concentrations of 50

6   and 120 mg L^{-1} for a UV dose of 14 mJ cm^{-2}. For samples exposed to 14 mJ cm^{-2}, up to a 50%

7   decrease in log inactivation was observed for samples with 120 mg L^{-1} of humic matter

8   compared to samples with no humic matter added.

9

10   **Correlating humic content to reduction equivalent dose**

11   Visual inspection of inactivation data for the samples exposed to 14 mJ cm^{-2} in Figure 1 and

12   Figure 2 suggests the impact of surface coating on UV disinfection depends on the concentration

13   of humic matter in the sample. This relationship was explored by regressing humic concentration

14   with reduction equivalent dose (RED) for the samples exposed to 14 mJ cm^{-2}. RED is a single

15   value for UV dose that is determined by entering the log inactivation measured during UV

16   exposure into the UV dose-response curve for the specific microorganism. The RED for each of

17   the inactivation data sets presented in Figure 1 and Figure 2 was determined using the dose-

18   response data presented in Figure 3 for both *E. coli* and *B. subtilis*. The correlations were based

19   on percent decrease in RED with humic matter so that the trends might be separated from the

20   UV-sensitivity of *E. coli* and *B. subtilis*. The results are presented in Figure 7. In addition to

21   inactivation data from this study, MS2 and T4 data for UV doses of 80 and 7 mJ cm^{-2},

22   respectively, from Figures 1 and 2 in Templeton *et al.* (2006) were converted to RED and

23   included for comparison. The linear correlation coefficients (r^2) for all but one condition (*B.*

1   *subtilis* in SR NOM) were > 0.95, indicating a strong linear relationship between the RED and

2   concentration of humic matter in the sample. Based on the regression, for an increase of 10 mg

3   L^{-1} of either AHA or SR NOM, a decrease in RED between 3% and 4% is expected.

4   The bacteria and viruses used in these studies have different surface properties and

5   sensitivity to UV light. The surrogate bacteria used in this study were selected to represent both

6   Gram positive and negative bacteria; the viral surrogates used by Templeton *et al.* (2006) were

7   selected from separate categories based on adsorption to soil particles. In terms of UV

8   sensitivity, 4-log inactivation of *E. coli*, *B. subtilis*, MS2 and T4 is achieved for a UV dose of

9   approximately 11, 14, 80, and 7 mJ cm^{-2}, respectively. Therefore, the trend observed in Figure 7

10   appears independent of both microbial surface properties and UV sensitivity. The relationship

11   between RED and humic content, which suggests that a 1% decrease in RED can be expected for

12   every 3 mg L^{-1} humic matter, might therefore be broadly applicable to many microorganisms.

13

14   **Impact of pH on *E. coli* inactivation in the presence of humic matter**

15   Bacterial surfaces contain functional groups (*e.g.* carboxyl, phosphate, and phenolic) that change

16   charge with pH as protons are gained and lost (Harden and Harris 1953). It was hypothesized

17   that the humic-cell surface interactions and therefore the level of surface-coating and protection

18   from UV light might depend on the surface characteristics of the cells (which are pH dependent).

19   The effect of pH (5, 7, and 9) on AHA and SR NOM protection of *E. coli* from low-pressure UV

20   disinfection was tested for a UV dose of 14 mJ cm^{-2} (*i.e.* 4-log inactivation with no humic

21   matter) using samples with 50 mg L^{-1} of AHA or SR NOM.

22   No change in absorbance of UV light at 254 nm by humic matter (AHA or SR NOM)

23   was observed for change in sample pH between pH 5 and 9. This is an observation consistent

1   with other publications (Weishaar *et al.* 2003). This is important as a change in UV absorbance

2   of the humic matter with pH would have confounded the assessment of the impact of pH on the

3   humic-coating and subsequent protection of *E. coli* cells.

4   While pH had no statistically significant effect on the level of inactivation, this is not a

5   strong conclusion due to the scatter in the data. Notably, the boxes for the SR NOM data in

6   Figure 4, which represent the 75th and 25th percentile values of the data set, are relatively broad

7   (up to 1.5-log) compared to the AHA data (< 0.5-log). In this data, any true pH effect would be

8   less than ~1-log.

9   Fein *et al.* (1999) report that the absorption of Aldrich[®] humic acid to *B. subtilis* cells

10   decreased with pH (between 2 and 12) with greater than 90% adsorption at pH < 5 and negligible

11   adsorption at pH > 9. Apparently this phenomenon did not impact protection of *E. coli* from UV

12   light under the conditions of this experiment. Parallel experiments on *B. subtilis* were not

13   conducted.

14

15   **Humic-bacteria interactions**

16   Two approaches were considered to examine the humic-bacteria interactions. The first was a

17   series of filtration trials to qualitatively assess the presence of humic-bacteria attachment, and the

18   second was imaging of samples via transmission electron microscopy (TEM) to examine the

19   interactions on a microscopic scale.

20   The filtration study (Figure 5) showed that in once instance, when mixing *E. coli* with

21   120 mg L^{-1} AHA, humic matter might attach to the cells to a degree that was measured by

22   observing an increase in the UV absorbance of the cells trapped by the 0.45 μm filter. While all

23   other conditions using SR NOM and lower AHA concentrations did not provide results that

1 could be distinguished above the background variability in the measurements, the evidence with

2 the 120 mg L^{-1} AHA suggests that this mechanism of protection from UV disinfection is

3 plausible.

4   To gather more evidence for this mechanism of protection, TEM images were collected

5 of representative samples. However, these images are not of aqueous samples and since the

6 impact of fixing and staining on the humic-cell interactions is not known, only very general

7 inferences from these images can be made regarding aqueous humic-bacteria interactions.

8 Scanning of the sample using the TEM indicated nearly all *E. coli* were interacting with humic

9 matter but not all humic matter was near the *E. coli*. These results suggest there is an affinity

10 between humic matter and *E. coli* surfaces when fixed and stained on the Formvar®-carbon

11 surface. These images do not necessarily reflect the aqueous humic-cell interactions, but they do

12 suggest that an affinity exists in principle between *E. coli* and humic matter.

13   Since the stain used to collect the TEM images (*i.e.* PTA) might affect the interaction

14 between humic matter and cells, a second stain was used. The *E. coli* and humic matter in UA

15 stained samples appeared quite different from the PTA stained images. One difference was the

16 absence of the humic capsule around the cell (see Figure 6 (d)) that was characteristic with the

17 PTA stained samples containing humic matter (*i.e.* Figure 6 (b) and (c)). At first glance, this

18 suggests no humic-cell interaction when the UA stain was used; however, anecdotal evidence of

19 humic matter interacting with the *E. coli* surface is present. In the samples with humic matter

20 stained with UA, a tear in the Formvar®-carbon was observed around most *E. coli* cells (*i.e.* the

21 white ring around the *E. coli* in Figure 6 (d)). Formvar®-carbon tearing is not uncommon in TEM

22 analysis and typically occurs in regions of the grid that were damaged during drying or that are

23 heavily loaded. In the UA stained *E. coli* samples that did not contain humic matter, little tearing

1   was observed, even when 2 or 3 cells were in close proximity. This suggests that for samples

2   containing humic matter, each of the *E. coli* cells had considerably more mass than the *E. coli* in

3   the humic-free sample, due possibly to humic matter interacting with the cell but not being

4   stained by the UA.

5   These images, therefore, indicate that there is an affinity between humic matter and *E.*

6   *coli* surfaces. The extent to which the fixing and staining process impacted the images is unclear.

7   The filtration trials suggest that in aqueous samples this interaction is either weak enough to

8   allow separation during filtration or limited to small amounts of humic material that are difficult

9   to measure by conventional spectroscopy. Since the washing trial results (Table 1) indicated that

10 about 0.7-log reduction in UV effectiveness was observed for humic matter contributing to a UV

11 absorbance of only 0.1 cm^{-1}, a relatively small fraction of the humic matter might be responsible

12 for the protective effect observed. Furthermore, since efforts to wash humic matter from bacteria

13 were partially successful, most of the humic-cell interactions may be weak or reversible.

14   To summarize, the following conclusions can be drawn from this study:

15 1. Both AHA and SR NOM offered statistically significant ($\alpha = 0.05$) protection of both *E. coli*

16   and *B. subtilis* (gram positive and gram negative bacteria) at concentrations of 50 and 120 mg

17   L^{-1} for a UV dose of 14 mJ cm^{-2}.

18 2. A linear correlation was observed between decrease in RED and humic content (pooling data

19   from this study and the literature). A decrease in RED of 1% was observed for every 3 mg L^{-}

20   ^{1} of humic matter. This relationship was largely independent of microorganism and humic

21   type.

22 3. The level of protection from low-pressure UV light offered by AHA or SR NOM was not

23   sensitive to pH between 5 and 9.

1   This is a proof of concept study to assess, under controlled conditions, the impact of

2   humic matter on UV disinfection. The concentration of humic matter found to have a statistically

3   significant effect on UV disinfection (*i.e.* 50 and 120 mg L^{-1}) is not typical of most natural

4   waters. Further study is needed to understand the implications of the surface coating and

5   subsequent protection of bacteria and viruses for waters with humic content at concentrations

6   common to surface water. For instance, since the washing trials showed that appreciable

7   protection (*i.e.* 0.7 log) was observed for a small amount of humic matter remaining attached to

8   the cells, high humic concentrations may not be a necessary condition for coating and subsequent

9   protection. The possibility that prolonged exposure of microorganisms to humic concentrations

10 typical of most surface water (*e.g.* ~3 mg L^{-1}) may result in similar protection due to a gradual

11 accumulation and concentration of humic matter, is worth future study. The potential for humic

12 coating and subsequent protection of *Cryptosporidium* oocysts and *Giardi*a cysts from UV light

13 also warrants investigation as UV light is often installed in a treatment train to target these

14 chlorine-resistant pathogens.

15   The potential for humics to decrease the effective RED might be especially significant in UV

16 systems with elevated organic content. One example is wastewater applications that are only

17 targeting coliform bacteria and might apply a UV dose of only 20 mJ cm^{-2}. Since wastewater and

18 grey water contain higher levels of organics, a reduction in RED might be more pronounced

19 when compared to a surface water low in organics.

20

1 of the Faculty of Medicine, University of Toronto, for assistance in the TEM analysis, and

2 Carole Baxter of the Drinking Water Research Group (University of Toronto) for her assistance

3 in the laboratory.

4

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3   and Reactivity of Dissolved Organic Carbon. *Environ Sci Technol* **37**, 4702-4708.

1   **Table 1.**   Impact of washing on *E. coli* inactivation and sample UV absorbance.

2

Sample	Log Inactivation ± SD	UV absorbance (cm^{-1})
E. coli (no humics)	2.8 ± 0.2	0.3
E. + 120 mg L^{-1} AHA	1.1 ± 0.2	2.7
Washed *E. coli*	2.1 ± 0.4	0.4

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16 **Fig. 1.**   *E. coli* log inactivation by low-pressure UV in the presence of AHA or SR NOM
17   at pH 7 (n = 5).

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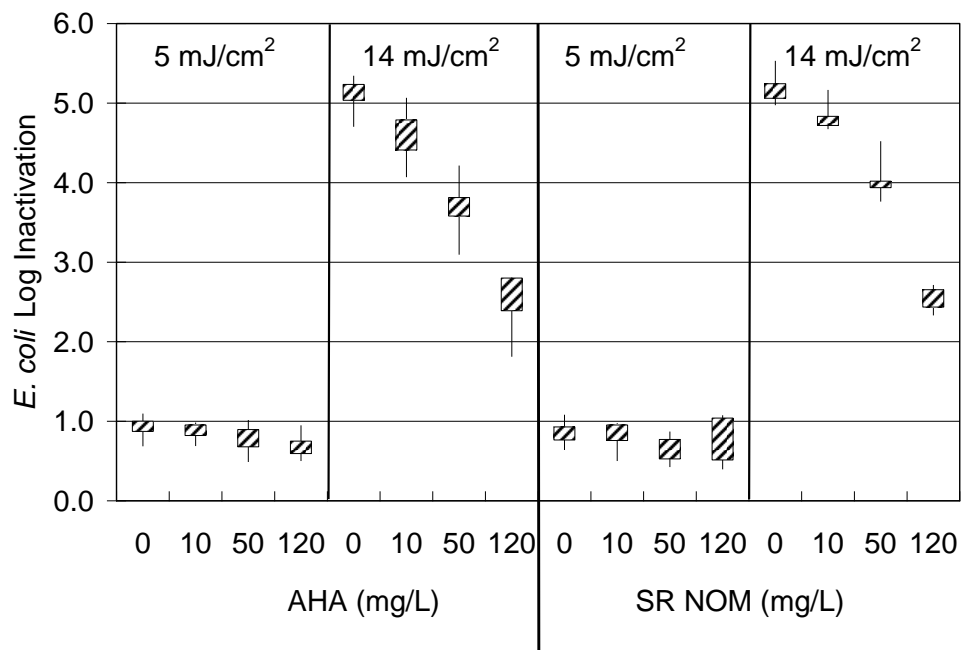
Fig. 2.   *B. subtilis* (vegetative cells) log inactivation by low-pressure UV in the presence of AHA or SR NOM at pH 7 (n = 5).

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20 **Fig. 3.** UV dose-response curve for *E. coli* (ATCC[®] 15597) and *B. subtilis* (ATCC[®] 6633)
21 vegetative cells by low-pressure UV light (pH = 7).
22
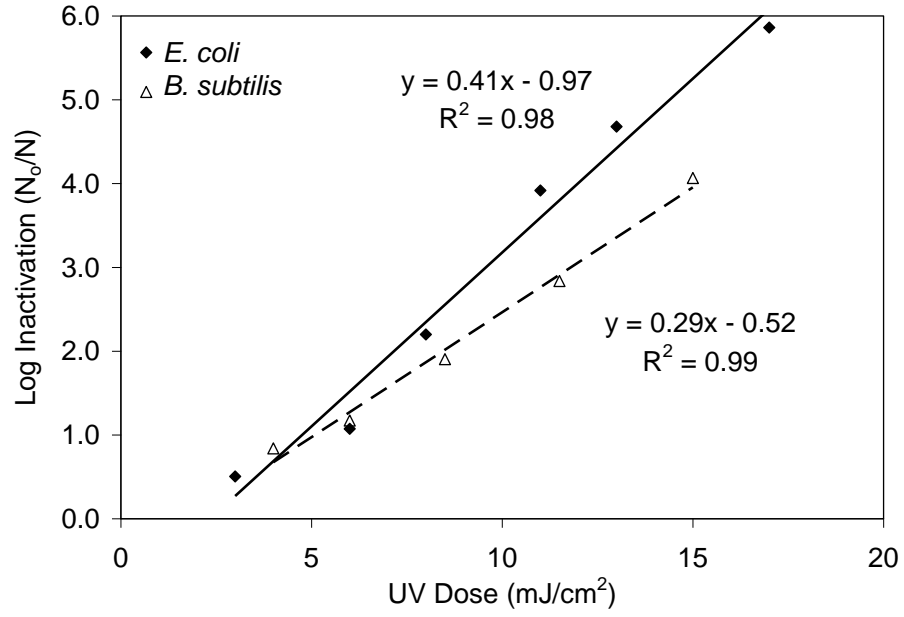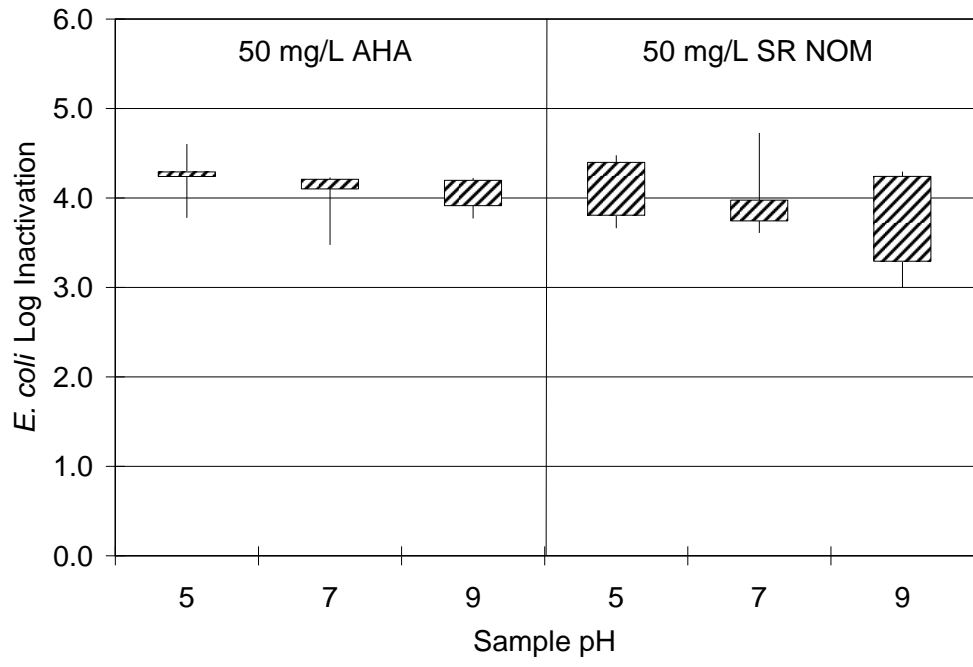
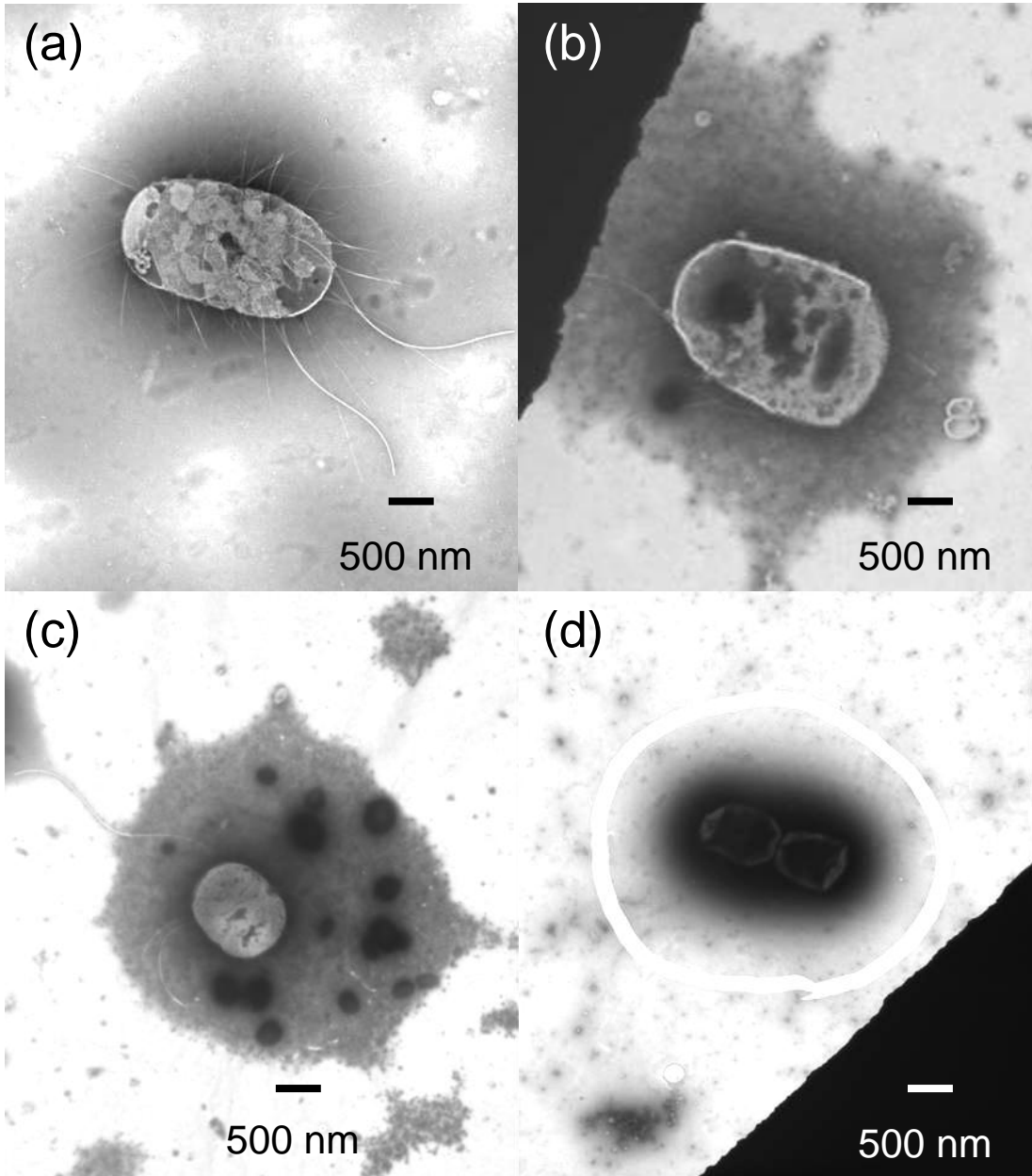**Fig. 4.** *E. coli* log inactivation by a low-pressure UV dose of 14 mJ cm^{-2} in the presence of AHA or SR NOM at pH 5, 7, or 9 (n = 5).
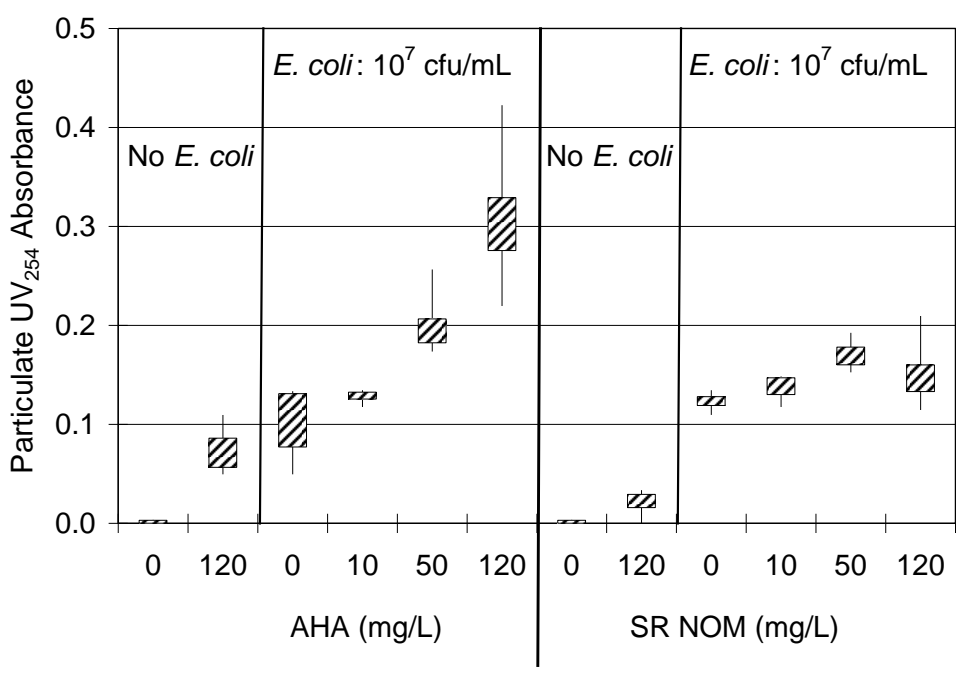
Fig. 5. Absorbance of UV light at 254 nm due to *E. coli* and humic acids (AHA or SR NOM) calculated to be retained on 0.45 μm filters at pH 7 (n = 5).

4 **Fig. 6.**   TEM images of (a) *E. coli* bacteria (no humic matter) stained with PTA, (b) *E. coli*
5   and AHA stained with PTA, (c) *E. coli* and SR NOM stained with PTA, and (d) *E.*
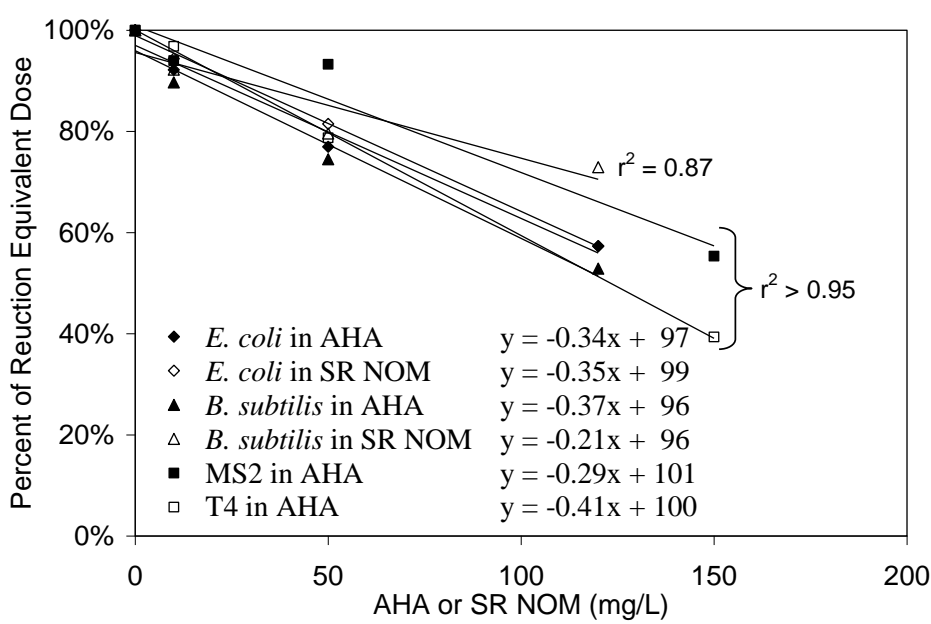6   *coli* and AHA stained with UA.
7

Fig. 7. Percent of reduction equivalent dose of *E. coli*, *B. subtilis*, MS2 or T4 in the presence of AHA or SR NOM. MS2 and T4 data adapted from Templeton *et al.* (2006). (The MS2 and T4 inactivation data was converted to RED using MS2: $y = 0.047x + 0.22$; T4: $y = 0.68x$ where y is log inactivation and x is UV dose.)