

Article

Microspheres as Surrogate Helminth Eggs: A Comparative Labscale Sedimentation Study for Tap- and Wastewater

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Abstract: Re-use of water containing helminth eggs during irrigation for agricultural purposes poses health risks, and likewise during research, due to the potential of spreading on contact. Therefore, polystyrene latex microspheres could be used as surrogates for chemical or biological species during colloidal transport. The aim here is to compare the settling velocities of microspheres having varied surface coatings—that is, proteins A, G and A/G; with that of real helminth eggs obtained from literature. The settling velocities of the microspheres were experimentally determined in tap- and wastewater, as well as theoretically in tap water; which was found to be within the range of mean values for those experimentally determined. There were no differences amongst the microspheres types used for settling in wastewater (i.e., A = 0.072 ± 0.02 ; G = 0.060 ± 0.03 ; A/G = 0.053 ± 0.01 mm/s). The same applied for settling in tap water (i.e., A = 0.068 ± 0.02 ; G = 0.047 ± 0.004 ; A/G = 0.095 ± 0.02 mm/s), except for microsphere G being different from microsphere A/G. All three types of microspheres settled at velocities lower than that of the wastewater particles ($=0.118 \pm 0.03$). T-test analyses of settling velocities of microspheres in both tap- and wastewater, versus that from literature (i.e., *Ascaris*, *Trichuris* and *Oesophagostomum*), showed that microsphere A and A/G may surrogate for *Ascaris* in tap water, the same as A/G for *Oesophagostomum*. In wastewater however, both microspheres A and G are a good fit for *Trichuris*.

Keywords: helminthes; microspheres; sedimentation; wastewater

1. Introduction

The use of treated wastewater for agricultural irrigation represents an alternative to alleviate shortage of water in arid climatic zones of the earth [1]. However, both treated and non-treated wastewater use impose risks linked to pathogens present therein. In this regard, helminth eggs are one of the main concerns considering health and safety as they are highly infective. They are the most difficult biological parasites to inactivate in wastewater and sludge [2], due to their high resistance to environmental influences and disinfection. The presence of helminthes is also considered the highest risk of wastewater related disease transmission, due to their long latency periods, long persistence in the environment and low infective dose without any practical host immunity [3]. The main associated diseases recorded hereof are helminthiasis [4,5], due to the use of polluted water for agricultural irrigation in low-income regions. It is estimated that around a quarter of the total human population are infected with helminthiasis globally [6], with most transmissions by the

eggs via the human-water-soil-crop-human pathway [7]. Helminthes infections are generally long lasting, occasionally causing severe pathology, although major clinical symptoms are often absent [8]. Symptoms include: anemia, loss of appetite and weight. There is also an impact on the growth and development of children. A common example is Ascariasis, which is very common and endemic in Africa, Latin-America and the Far East [9]. *Ascaris* eggs are usually transmitted via unsafe food and water. Worse, the eggs that do survive can remain viable in the environment for up to at least 9 years (as cited in [10]). Therefore, *Ascaris* eggs have been used as indicators for testing the efficiency of wastewater and sludge treatment since they show high resistance towards many chemicals and physical conditions [11].

There are various ways of pathogen removal during wastewater or sludge treatment. Methods are usually dependent on the type of organism and their detention times throughout the process [12,13]. However, most removals could be attributed to sedimentation (although primary sedimentation processes for pathogens are highly variable) and adsorption or incorporation into the biological floc that forms [12]. Again, for removal via sedimentation and filtration, the sizes and weight of pathogens are key parameters to consider.

With the increased use of reclaimed wastewater for agricultural purposes, settling ponds are commonly employed in low income countries for sedimentation of wastewater particles before irrigational use. Sedimentation allows suspended solids that are heavier than water to settle by gravity, since there are differences amongst densities of particles and fluid. In this regard, helminthes properties, such as specific gravity, parasite dimensions and liquid density, can cause variations in sedimentation rates [12]. Also required for the sedimentation process is an undisturbed retention time, and at longer times some level of settling of helminth eggs in sedimentation ponds occurs [14].

Flocculation enhances sedimentation of particles and as indicated by [15], the amount of helminth eggs removed during chemical assisted sedimentation were comparable to plain sedimentation, although achieved at a shorter retention time. Likewise, [14] showed that, natural sedimentation for 3 h reduced helminth eggs by approximately 24% (i.e., from 14–10.6 eggs/L), whereas with *Moringa oleifera* as treatment, helminth eggs reduction was exponential to less than 1 egg/L for an optimum reduction attained within 2–2.5 h after which no further reduction was recorded.

An experiment to determine the settling velocities of helminth eggs (*Ascaris suum*, *Trichuris suis*, and *Oesophagostomum* spp.) and wastewater particles in tap- and wastewater using Owen tubes [16] indicated that helminth eggs in wastewater were incorporated into particle flocs with different settling. Ref. [17], again aimed to determine the erodibility (erosion rate and erosion threshold) and settling velocity of *Ascaris* and *Trichuris* eggs at different time points after incorporation into sediment. They found that:

- Interaction between eggs and bulk sediment manifested in an increased settling velocity of suspended eggs when sediment was present in the suspension as compared to the situation of settling in clean water.
- Also, incorporation into the sediment bed and aggregation with sediment particles decreased the mobility of both helminthes egg types.

They therefore suggested that helminth eggs should not be viewed as single entities in water systems when modeling their distribution, since the mobility of the sediments present in the water stream are used to determine both erodibility and settling velocity of the eggs.

Ref. [18] documented the surface properties of *Ascaris lumbricoides* var. *suum* to be an outer protein layer whose margin may be smooth or mammillated. Invariably, helminthes have negatively charged surfaces for the purpose of parasite-host interactions, physiological functions and immune response, as well as a layer covering called glycocalyx which consists of carbohydrates conjugated as glycoproteins, glycolipids and mucopolysaccharides [19].

Research experiments with helminth eggs in the lab pose potential risks health and safety-wise. One would have to consider effective disinfection methods for contaminated skin, eyes, lab equipment,

consumables, bench tops, laboratory floors, and also spill hazards. Even though good practices and maximum care could be employed to avoid contamination, there is also the possibility of fluorescent polystyrene microspheres being utilized as surrogates for other pathogens, including the protozoan parasite *Cryptosporidium* spp., viruses, and bacteria [20–23]. Moreover, polystyrene latex microspheres are widely used as surrogates for bio-colloid transport in porous media, even though relatively few studies directly compare microsphere transport with that of the microorganism it is intended to represent, particularly at the field scale [24]. It would therefore be interesting to determine that, microspheres can also be used given the characteristics of helminthes eggs in terms of size, density and consistency.

Fluorescent microspheres can be adapted as surrogates to mimic the surface and important physical/chemical properties of helminthes. They can also be utilized in wastewater to determine flow or movement of chemicals, as well as biological species. The use of latex microspheres in controlled tracer tests has proven to be a useful tool for investigating the phenomena of preferential transports [24] and they have the advantage of equal reactivity. Fluorescent microspheres are non-hazardous and also do not decay. Nevertheless, there is the issue of sensitivity of signals to pH and temperature.

This study therefore aims to experimentally determine the settling velocity of microspheres with varied surface coating types (protein A, G and A/G) in tap- and wastewater in comparison to real helminth eggs from literature. How do the selected microspheres differ from each other? And how different is the settling velocity of the microspheres in water, compared with the use of Stokes' law as a predictive model?

2. Materials and Methods

2.1. Microspheres

Choice of microspheres was based on size, shape and outer-layer covering comparative to real helminth eggs, which are common worldwide as well as being of medical importance. Typical helminthes properties are shown in Table 1.

The microspheres were spherically shaped (coated fluorescent Particles, Yellow, 1% w/v, $\approx 40.2 \mu\text{m}$; ordered from—Lake Forest, IL, USA). Microspheres of choice had same shape and size but varied in surface coatings (i.e., Protein A, G and A/G). Thus:

- Protein A: surface protein originally found in the cell wall of the bacterium *Staphylococcus aureus*.
- Protein G: an immunoglobulin-binding protein expressed in group C and G *Streptococcal* bacteria much like Protein A, but with differing specificities.
- Protein A/G: recombinant fusion protein that combines IgG-binding domains of both Protein A and Protein G. This fusion protein is expressed in *E. coli*.

Both A and G can be described as binding proteins and bacterial antibodies; although Protein G is known to have a broader specificity for antibodies than Protein A [25].

Table 1. Some helminthes properties [18,26–28].

Species	Shape	Diameter (μm)	Surface
<i>Ascaris lumbricoides</i>	Elliptical	45–75 \times 35–50	Protein coated
<i>Trichuris trichiura</i>	Barrel shaped; Hyaline polar plug at each end	50–55 \times 22–24	smooth; yellow-brown color
<i>Taenia solium</i>	Spherical	31–43	
<i>Toxocara canis</i>	Nearly spherical	80–85 \times 75	
<i>Oesophagostomum</i>	Ovoid	69–78 \times 41–48	Thin-shelled

2.2. Sedimentation

Sedimentation experiments in both tap and waste-water were conducted with suspensions of ≈ 200 – 600 counts of each type of microsphere in a volume of 380 cm^3 . The range of microsphere counts in the suspensions was due to difficulty in quantification of the microspheres. Experiments were done using Owen tubes (Figure 1) made of acrylic plastic, and with an internal diameter of 2.2 cm. The experimental method is similar to that described by [29]. Ref. [30] reported that, the small diameter of the Owen tube does not interfere with sedimentation since the upward flow of displaced fluid does not significantly hinder the sedimentation if the particle is smaller than approximately $1/45$ of the column diameter.

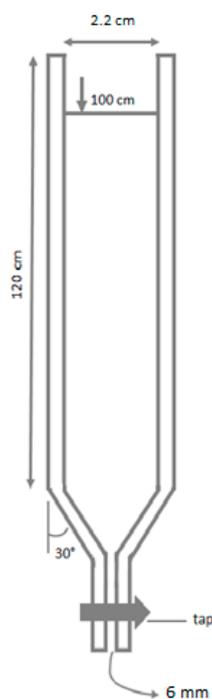


Figure 1. Bottom withdrawal tube.

The Owen tube was rinsed well with 0.01% Tween20 (VWR International S.A.S., Fontenay-sous-Bois, France) solution prior to the start of the experiment to minimize adhesion to its wall. About 100 mL of suspensions of either tap- or wastewater plus microspheres was poured into the Owen tube. The tube was thereafter filled up to the 100 cm mark. To ensure the suspension was thoroughly mixed, the top end of the tube was closed and then repeatedly turned upside down, with the air bubble acting as a mixing device. As soon as the air bubble had left the lower end of the tube, after the last inversion, the stopwatch was started. The tube was thereafter fixed in a vertical position and at predetermined time intervals (i.e., 4, 8, 15, 30, 60, 120, 180, 240, 300, 300+), successive withdrawals were made from the tap at the bottom of the Owen tube into centrifuge tubes. This was done individually for each type of microsphere.

The collected samples were thereafter centrifuged (Heraeus Multifuge X3 FR) at 4700 rpm for ≈ 7 min. The supernatants were then discarded leaving about 10 mL of microsphere sample suspension in the bottom of each tube. These were then filtered onto filter paper and the microsphere counts were thereafter quantified by observation under the confocal laser scanning microscope (Leica Microsystems, TCS SP8) with filter tube N2.1 for green excitation.

Data for microsphere counts had to be normalized, since withdrawals of successive samples are from a progressively smaller volume of the initial suspension. The same applied for the time of withdrawal of samples, because the distance settled was shorter for later the samplings. In this

instance, the depth factor is used to correct the cumulative weights of sediment (beginning from the bottom) and times required for a full 1 m height.

$$\text{Depth factor} = \text{Total suspension height (cm)}/\text{suspension fall distance (cm)}$$

Therefore,

$$\text{Normalized sediment in suspension} = \text{Cumulative sediment} \times \text{depth factor}$$

$$\text{Normalized time} = \text{Sampling time} \times \text{depth factor}$$

2.3. Experimental Settling Velocity of Microspheres and Statistical Analyses

The median settling velocity was calculated from experimental data using the Oden curve method [31,32], for both microspheres as well as wastewater particles. The number of microspheres per each sub sample was used for the calculation, whereas dry weights were used for wastewater particles. One-way analysis of variance (ANOVA) was conducted using Origin Pro, for the comparative analysis of the three microsphere types in tap- and wastewater separately in order to determine if there were differences in their settling velocities. Multiple group comparisons were thereafter utilized for additional comparisons of means. Additionally, comparisons of the settling velocities were also conducted for each microsphere type against those of helminth eggs obtained from the literature.

Solution/suspension of isolated constituents of wastewater (Cl-salts, soap/tenside and wastewater particles) was also utilized in further sedimentation experiments. Here, synthetic wastewater constituents as per [33] for Cl-salts and soap were used. Wastewater particles were isolated via centrifugation and then decantation afterwards. Cl-salt solution = (30 mg NaCl + 7 mg KCl + 7 mg CaCl₂·2H₂O)/L; soap solution = (50 mg soap)/L.

2.4. Theoretical Settling Velocity of Microspheres

Stokes' law was employed in the calculation of the settling velocity of microspheres in water. Latex microspheres do not interfere with each other and their settling velocity under creeping flow conditions (Reynolds number less than 1) in a Newtonian fluid can be described using the Stokes law [34]. The fluorescent microspheres under observation are uniform in material composition, discrete spheres, and contain smooth surfaces per the manufacturer's description. Thereby the equation:

$$V_s = g d^2 (\rho_p - \rho_l) / 18 \mu, \quad (1)$$

where V_s is settling velocity (m/s), g is gravitational acceleration ($=9.81 \text{ m/s}^2$), d is microsphere diameter ($=0.00004 \text{ m}$), ρ_p is specific density of microsphere ($=1060 \text{ kg/m}^3$), ρ_l is specific density of the liquid ($=1000 \text{ kg/m}^3$), and μ is dynamic viscosity of the liquid ($=0.001 \text{ kg/m}\cdot\text{s}$).

Tap- and wastewater properties:

Characteristics of tap water used are as follows: pH = 7.6; conductivity = 526 $\mu\text{S/cm}$.

The wastewater was obtained from the grit chamber of a nearby municipal wastewater treatment plant. Characteristics are as follows: pH = 7.4; conductivity = 1244 $\mu\text{S/cm}$; density = 1.01 g/cm^3 ; Total suspended solids = 154 mg/L ; chemical oxygen demand (COD) = 317 mg/L

Microsphere characteristics are as follows:

They are spherically shaped; approximately 38–44 μm in diameter; surface coatings of either Protein A, G or A/G; density = 1.06 g/cm^3 .

3. Results and Discussion

3.1. Settling Velocities of Microspheres in Tap- and Wastewater

The results of the sedimentation experiments of microspheres with different outer coatings (A, G and A/G) in tap- and wastewater are shown in Figure 2A,B. The mean settling velocities in tap water, based on four replications for each microsphere type, were: A = 0.068 mm/s \pm 0.0197 (\pm sd); G = 0.047 mm/s \pm 0.0043; and A/G = 0.095 mm/s \pm 0.0216. There was very little variation in the four experimental replicates for microsphere G compared with that of microspheres A and A/G. A one-way between subjects ANOVA was conducted with Origin Pro 2017, to compare the settling velocities of the three microspheres in question. There was a significant difference at 0.05 level for the three microsphere types [F(2, 9) = 8.13, p = 0.001]. Post hoc comparisons using the Tukey test further showed that, the mean settling velocity for microsphere G was lower than that of microsphere A/G.

For settling velocities of microspheres in wastewater (see Figure 2B), the means are as follows: A = 0.072 mm/s \pm 0.021; G = 0.060 mm/s \pm 0.027; A/G = 0.053 mm/s \pm 0.008. Also, the wastewater particles settled at a rate of 0.118 mm/s \pm 0.027. Here the least variation was observed for the replications of microsphere A/G. There was no difference among the settling velocities of the three microsphere types when compared statistically. However, all three microsphere types in wastewater had lower settling velocities than that of wastewater particles (see Table 2), contrary to the findings of [16], where wastewater particles settled at a slower rate than helminth eggs involved in their experimentation. The faster settling velocity of wastewater particles than microspheres could be attributed to probable aggregation of the variably charged particles in wastewater, leading to the formation of bigger and heavier particles. There is also the possibility of likely impediment caused by the wastewater particles within the sedimenting suspension to slow down the rate of settling of microspheres. As indicated by [35], sludge particles are not spherical and a large number are present in the fluid.

Also notable is the obvious drop in settling velocity of microsphere A/G in wastewater in comparison to tap water, when wastewater particles, on the other hand, are settling at a much faster rate. According to [36], settling did not contribute to *E. coli* removal within sedimentation experiments, as approximately 50%, 20% and 90% of the bacteria were “free floating” or associated with particles <5 μ m in size for domestic septic tank effluent, treated wetland effluent and domestic wastewater. In their study they also pointed out the level of variability in *E. coli* removal processes that could be observed within different wastewater, and wetland environments. In general, the sedimentation method allows an adequate disinfection process for the generated water and not necessarily a decrease in the bacteria content at more than one logarithmic unit. One could therefore correspond the inconsistency of settling of microsphere A/G in tap- and wastewater with the explanations by [36]. It could further be reasoned as indicated by [37] that, protein A/G being a fusion protein and containing the combined properties of both protein A and G, provides excellent binding potential toward the widest variety of antibody species and subclasses. Also, as indicated by [38], the surfactant nature of naturally occurring organic matter suggests the possibility of micelle and hemimicelle formations, although surfactants may enhance the mobility of pesticides if concentrations are low enough to preclude micelle formation [39]. It may therefore be hypothesized that microsphere A/G may have likely been hindered by the effect of surfactants present in wastewater when aggregation occurs during settling.

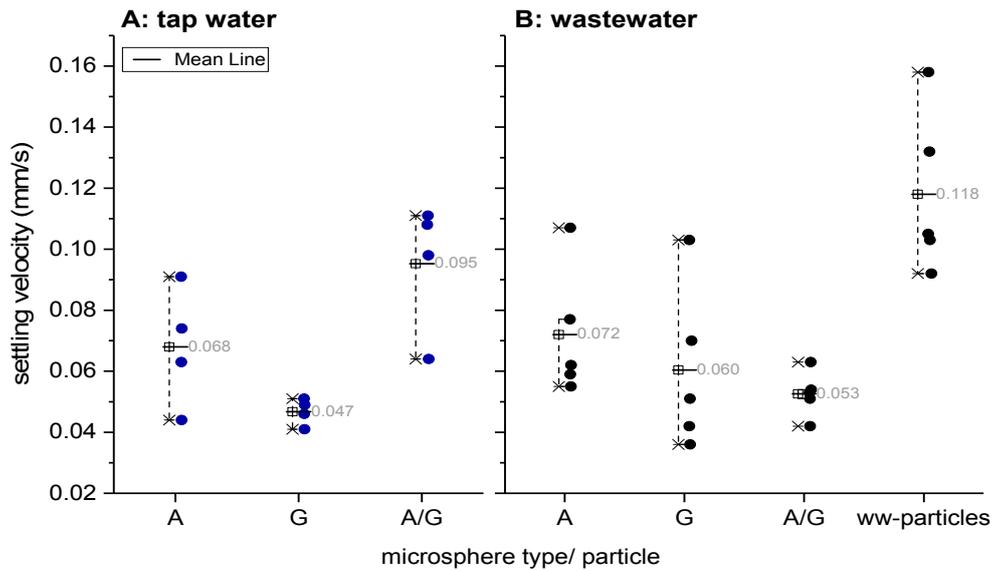


Figure 2. Settling velocity of microspheres with different surface coatings (protein A, G and A/G) in tap water (A) and wastewater (ww) (B).

Settling velocity values resulting from microsphere sedimentation in isolated wastewater constituents' suspension are shown in Figure 3. This was done to further explain the differing trend of velocities between tap- and wastewater. Here, similar trends of sedimentation can be observed for sedimentation of microspheres in wastewater (Figure 2B) and that in salts and soap solution (Figure 3A,B). There were no differences observed amongst the microspheres in Cl-salts and soap/tenside solutions, as well as wastewater particles' suspension. Likewise, no differences were observed for comparisons of each microsphere sedimentation in the various solutions. Microsphere A/G again shows lower settling velocities for Cl-salts and soap solutions ($p = 0.034$ and 0.035 respectively) when compared with that in tap water. It can therefore be argued that Cl-salts and tenside may tend to slow down the settling capacity of microsphere A/G.

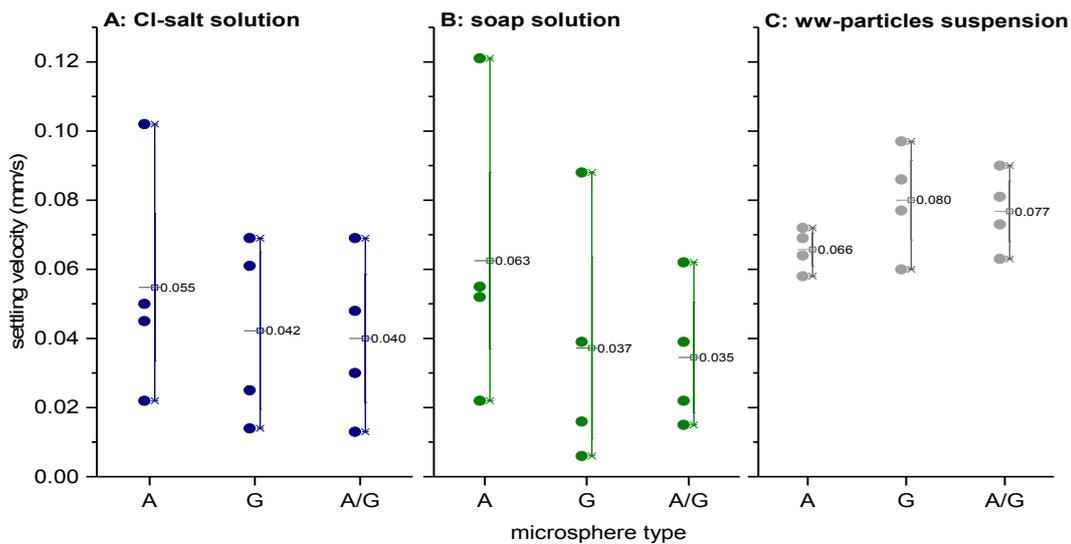


Figure 3. Settling velocity of microspheres with different surface coatings (protein A, G and A/G) in Cl-salt solution (A), soap solution (B) and wastewater particles suspension (C).

Table 2. Tukey test results of mean comparisons of settling velocities of microspheres and wastewater particles.

	<i>p</i> -Value (2-Tailed)
ww particles vs. microsphere A	0.0216 **
ww particles vs. microsphere G	0.0040 *
ww particles vs. microsphere A/G	0.0013 *

ww = wastewater; * $p < 0.01$; ** $p < 0.05$

3.2. Theoretical Settling Velocity in Tap Water

The theoretical settling velocities, following Stokes' law (Equation (1)) were calculated for microsphere sedimentation in tap water. Considering that all three types of microspheres had the same particle diameter and density, we can therefore assume that their settling velocities are also the same. The result obtained for the theoretical settling velocity after calculation was 0.052 mm/s, which falls within the range of that determined experimentally (i.e., 0.047–0.095 mm/s). Contrary however, this calculated result is lower than those indicated by [40] (see Table 3) and [41] for *Ascaris* and *Trichuris* (i.e., 20 mm/min [=0.333 mm/s] and 16 mm/min [=0.267 mm/s] respectively). On the other hand, Ref. [34] calculated the settling velocity of latex beads with diameter 90 μm to be 13.7 mm/min (=0.228 mm/s) which they indicated to be close to that of the helminth eggs per the study of [41]. The difference in settling velocities may be attributed to the different relative densities, shapes and sizes of particles under observation. In this instance, the density of microspheres ($\approx 1.06 \text{ g/cm}^3$) compared to *Ascaris* and *Trichuris* as per Table 3 is lower; same for the smaller size of microspheres in relation to the study of [41].

Table 3. Settling velocities of some helminth eggs in water at 20 °C (as cited in [40]).

Parasite Egg/Cyst	Average Size (μm)	Relative Density	Velocity (m/h)
<i>Ascaris</i> –fertile	60 × 45	1.11	0.77 (=0.214 mm/s)
<i>Ascaris</i> –infertile	90 × 40	1.2	3.15 (=0.875 mm/s)
<i>Trichuris</i>	50 × 22	1.15	0.73 (=0.203 mm/s)

3.3. Comparative Analyses of Microspheres with Real Helminth Eggs

The results of comparative *t*-test analyses of settling velocities of microspheres in tap- and wastewater versus that obtained by [16] for helminth eggs (i.e., *Ascaris*, *Trichuris* and *Oesophagostomum*) are shown in Table 4. Some significant differences were observed for microspheres in comparison to aforementioned helminthes. In tap water, the settling velocities of microspheres A and A/G are not different from *Ascaris* (mean = 0.0612 mm/s) at 0.05 significance level. Then again, microsphere A/G is observed not to be different from *Oesophagostomum* (mean = 0.1262 mm/s). Furthermore, the settling velocities of microspheres A and G in wastewater were both not different from that of *Trichuris* (mean = 0.0866). It can therefore be proposed that the comparable microspheres could be employed experimentally in instances of substitution.

As indicated by [16], the settling velocity and behavior of *Trichuris* and *Oesophagostomum* eggs in wastewater is determined by the presence of particles in water, but for this experiment, wastewater particles were observed to settle faster than the microspheres. This outcome could again be said to be due to aggregate formation of the microspheres, which in turn is hindered by surfactant presence in the wastewater [38,39].

Table 4. *p*-Values (2-tailed) of settling velocities of microspheres A, G and A/G compared with *Ascaris*, *Trichuris* and *Oesophagostomum*.

Parasite Egg	A	G	A/G
Tap water:			
<i>Ascaris</i>	0.540 *	0.007	0.051 *
<i>Trichuris</i>	0.004	0.00002	0.016
<i>Oesophagostomum</i>	0.010	0.00005	0.064 *
Wastewater:			
<i>Ascaris</i>	0.001	0.001	0.00001
<i>Trichuris</i>	0.199 *	0.096 *	0.0005
<i>Oesophagostomum</i>	0.023	0.019	0.0001

* $p \geq 0.05$; the settling velocity of microsphere and helminthes are not significantly different.

4. Conclusions

This study indicates that Stokes' law is a good predictive model for the settling of microspheres in tap water, given that the theoretical value is within the range of experimental values obtained. Settling velocity of particles in wastewater is dependent on their size, shape, density and roughness, as well as existing hydraulic conditions, such as the viscosity of water and Reynolds number. Odd as it may seem, wastewater particles were documented to settle faster than microspheres during wastewater sedimentation, and this occurrence is contrary to the outcome in published literature of real helminth eggs settling in wastewater. Notwithstanding, suspensions of isolated constituents' of wastewater were utilized to further explain the differing trend of velocities of protein A/G in tap- and wastewater, and also for suspended solids; but similar trends of sedimentation were still observed. Therefore, more research is needed to find an exact explanation for this anomaly in observed settling velocities. Pertaining to the level of fit of surrogacy for real helminth eggs during experimentation with tap water, microsphere A and A/G could be used in place of *Ascaris*. Likewise, microsphere A/G could also be used for *Oesophagostomum*. Contrarily, both microsphere A and G can substitute for *Trichuris* in wastewater. These findings imply that although microspheres could in some instances substitute as helminth eggs, one would have to be mindful of size and surface coating, as well as other properties.

The study could be furthered by assessing how other available protein-coated microspheres, as well as different sizes, compare with real helminth eggs during sedimentation. Different types of particles and concentrations of suspended materials could also be factored in, determining their influence on settling velocity.

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