Waterborne protozoan pathogens in environmental aquatic biofilms: Implications for water quality assessment strategies

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Abstract

Biofilms containing pathogenic organisms from the water supply are a potential source of protozoan parasite outbreaks and a general public health concern. The aim of the present study was to demonstrate the simultaneous and multi-spatial occurrence of waterborne protozoan pathogens (WBPP) in substrate-associated biofilms (SAB) and compare it to surface water (SW) and sediments with bottom water (BW) counterparts using manual filtration and elution from low-volume samples. For scenario purposes, simulated environmental biofilm contamination was created from *in-situ* grown one-month-old SAB (OM-SAB) that were spiked with *Cryptosporidium parvum* oocysts. Samples were collected from the largest freshwater reservoirs in Luzon, Philippines and a University Lake in Thailand. A total of 69 samples (23 SAB, 23 SW, and 23 BW) were evaluated using traditional staining techniques for *Cryptosporidium*, and immunofluorescence staining for the simultaneous detection of *Cryptosporidium* and *Giardia*. In the present study, WBPP was found in 43% SAB, 39% SW, and 39% BW samples tested with SAB results reflecting SW and BW results. Further, the potential and advantages of using low-volume sampling for the detection of parasite (oo)cysts in aquatic matrices were also demonstrated. Scanning electron microscopy of OM-SAB revealed a naturally-associated testate amoeba shell, while *Cryptosporidium* oocysts spiked samples provided a visual profile of what can be expected from naturally contaminated biofilms. This study provides the first evidence for the simultaneous and multi-spatial occurrence of waterborne protozoan pathogens in low-volume

environmental aquatic matrices and warrants SAB testing along with SW and BW matrices for improved water quality assessment strategies (iWQAS).

Capsule

Analysis of biofilms along with surface water and sediments in environmental aquatic systems leads to improved detection and isolation of waterborne protozoan pathogens.

Keywords: Asia; biofilms; *Cryptosporidium*; *Giardia*; lakes

1. Introduction

Cryptosporidium and *Giardia* are protozoan agents of diarrheal outbreaks worldwide (Baldursson and Karanis, 2011; Efstratiou et al., 2017a; Karanis et al., 2007; Mac Kenzie et al., 1994). Cryptosporidiosis is the second leading global cause of infantile mortality next to rotavirus infection (Checkley et al., 2015), while giardial infections are reported to be at more than 200 million cases annually worldwide. Both *Cryptosporidium* and *Giardia* are listed in the 'Neglected Diseases Initiative' by the World Health Organization (Savioli et al., 2006). The immunocompromised population is at highest risk in contracting these waterborne protozoan pathogens (WBBP) with low infectious dose; ingestion of as few as 10 (oo)cysts have been reported to cause morbidity even in the immunocompetent population (Dupont et al., 1995; Okhuysen et al., 1999; Ortega et al., 1997; Steiner et al., 1997).

Biofilms are formed from the transition of planktonic cells to sessile cells, which leads to the accumulation and establishment of stable interactions between pure and multi-species communities (Matos et al., 2017; Suba and Masangkay, 2013). The complexity of the biofilm

68 community is brought about by the introduction of other unicellular organisms like protozoans
69 (Murphy et al., 2018; Waiser et al., 2016; Xu et al., 2014), which can facilitate the interspecies co-
70 evolutionary processes as recently reported and discussed in the biofilm inhabitation of
71 *Acanthamoeba* spp. infected with bacteria containing bacteriophages with the possibility of
72 virophages as well (Bekliz et al., 2016; Masangkay et al., 2018).

73 Biofilms are potential reservoirs of human and zoonotic pathogens in environmental
74 aquatic systems and contribute to the persistence and transmission of waterborne protozoan
75 pathogens and other microorganisms (Ryan et al., 2016). The biofilm roughness contributes to the
76 attachment of protozoan (oo)cysts (DiCesare et al., 2012a; Wolyniak et al., 2009; 2010) and the
77 biofilm mass as a whole provides UV protection for (oo)cysts trapped within its matrix which
78 contributes to the environmental persistence of potentially-pathogenic protozoans (DiCesare et al.,
79 2012b).

80 The aim of this study was to demonstrate the presence of *Cryptosporidium* and *Giardia*
81 (oo)cysts in environmental aquatic substrate-associated biofilms (SAB) and compare its results
82 against surface water (SW) and bottom water with sediment (BW) counterparts through manual
83 filtration and elution of low-volume samples and to introduce the significance of biofilms as a
84 biological reservoir for WBPP.

85

86 **2. Materials and Methods**

87 *2.1. Study sites*

88 The University Lake of Nakhon Si Thammarat, Thailand (Fig. 1.A) was chosen as one of
89 the study sites due to its recreational use and its importance as a habitat for local amphibians,
90 reptiles, small mammals, livestock, and aquatic birds. Fig. 1.B illustrates the other study sites in

91 the Philippines (Laguna de Bay, Taal Lake, Pantabangan watershed and The Seven Lakes of
92 Laguna, and Ipo watershed), four of which were tested for the presence of WBPP. The Ipo
93 watershed site was selected for the one-month-old SAB (OM-SAB) *in-situ* culture experiment.
94 Laguna de Bay is the largest lake in the Philippines and is the major site for freshwater aquaculture
95 in Luzon (Guzman, 2006; Israel, 2007). Taal Lake is the third largest lake in the country and the
96 primary source of freshwater fish in the surrounding provinces (Martinez, 2011). The Pantabangan
97 watershed was constructed by flooding a town and erecting a dam structure for hydroelectric
98 power. The Seven Lakes of San Pablo Laguna is a system of crater lakes, where Sampaloc and
99 Bunot Lake are used for aquaculture. Palakpakin Lake connects to a river system and Mohicap
100 Lake is enclosed by natural surroundings that offer tourists and local residents a breath-taking view
101 of the area. The remaining Yambo, Pandin, and Kalibato Lakes are mainly used for picnic and
102 water activities. The Ipo watershed has been created by a river system that drains the larger Angat
103 dam, which provides the majority of the water supply to Manila and its nearby metropolis.
104

105 *2.2. Sample collection*

106 Surface water samples (SW) were collected in 50 mL sterile polyethylene containers by
107 collecting no more than 30 cm below the water surface from each sampling area (Table 1). Bottom
108 water with sediments (BW) were collected from a water depth of one meter along the shoreline
109 that was composed of one-part sediments and four-parts bottom water. Substrate-associated
110 biofilms (SAB) were harvested from aquatic plants whenever present. Short segments of small
111 aquatic plants were collected and washed with sterile distilled water to remove non-adherent cells,
112 cut into smaller portions in order to fit loosely inside 50 mL sterile polyethylene containers with a
113 final volume of 50 mL sterile distilled water. In the absence of aquatic plants, adherent biofilms

(approximately 2 g) were scraped from rocks no more than 30 cm below the water surface. Samples were transported to the laboratory and processed within 48 hours after collection. The 50 mL samples in this study aimed to provide initial data on the capability and feasibility of detecting pathogenic (oo)cysts from environmental aquatic matrices using a low-volume sampling technique. The low-volume 50 mL sample was elected to represent the hypothetical volume of a water sample that could be accidentally ingested or inhaled by an individual during water activities, which could lead to the infection of *Cryptosporidium* and/or *Giardia* if present in the water source.

2.3. Sample processing

Each sample was vortexed for one minute to dislodge adherent cells from any larger organic substances and debris in order to distribute the bio-colloids evenly throughout the sample matrix and left to stand for 5 min to settle heavier solids. The SW, BW and SAB sample suspensions were each manually filtered through a 1.2 µm glass microfiber filter fitted inside a 50 mL sterile disposable syringe (Masangkay et al., 2016). Glass microfiber filters were recovered and placed in sterile disposable polyethylene plates, where the filtered sediments were scraped using a sterile inoculating loop and 5 mL sterile distilled water as eluent. The 5 mL eluates were transferred to sterile test tubes and centrifuged at 1500 g for 15 minutes (US EPA Method 1623, 2005) where 3 mL of the supernatants were discarded and the remaining 2 mL and pellet were mixed to form a suspension and subsequently transferred and stored in microcentrifuge tubes for smearing within 24 hours.

2.4. Microscopy of Cryptosporidium and Giardia (oo)cysts

For all samples, 25 µL of the pellet suspension was made into a 1 cm diameter smear on a clean glass slide in duplicate. Screening for *Cryptosporidium* oocysts was performed by staining with modified Kinyoun's (MK), modified Safranin Methylene Blue (SMB), and Auramine (Aura). Microscopic confirmation of *Cryptosporidium* spp. and *Giardia* spp. (oo)cysts were performed by Direct Antibody Fluorescent Testing (IFT) using the Aqua-GloTM G/C Direct Comprehensive Kit (Waterborne Inc. USA) according to the manufacturer's instructions. Light microscopy of MK and SMB smears was performed by examining 200 oil immersion fields using a Nikon Model Eclipse E100LED light microscope. An upright epifluorescence incident light excitation trinocular UB microscope with a three-megapixel camera was used to examine 200 high power fields of the Aura and IFT smears. Suspected *Cryptosporidium* and *Giardia* (oo)cysts were compared to stained positive controls (A100FLR-1X Aqua-Glo G/C Direct positive control, Waterborne Inc. USA), where MK and SMB stained *Cryptosporidium* (oo)cysts stood out as round bodies measuring 4 to 6 µm in diameter with occasionally visible sporozoites that are bright red against a blue background. Aura and IFT stained *Cryptosporidium* oocysts fluoresced bright apple-green against a black background (CDC DPDx Cryptosporidiosis). *Giardia* cysts stained with IFT were ovoid, measuring 10 to 14 µm, and fluoresced bright apple-green against a black background (CDC DPDx Giardiasis).

2.5. OM-SAB grown *in-situ*

Naturally grown *in situ* one-month-old substrate-associated biofilms (OM-SAB) were produced from the Ipo Watershed using glass coverslip substrates that were secured to 20 X 3 cm plastic panels held together by a 20 X 5 cm Styrofoam body in the horizontal and vertical

orientation on either side (Supplementary 1 material 1). The constructed substrates were immersed at least 30 cm below the surface of the water from October 1, 2018 to November 1, 2018.

2.6. OM-SAB microscopy panel

To be able to determine the presence of naturally-associated *Cryptosporidium* and *Giardia* (oo)cysts in OM-SAB, coverslip panels with OM-SAB were collected and washed with sterile distilled water to remove non-adherent cells and individually placed into 50 mL sterile polyethylene containers, transported to the laboratory, and harvested by scraping the coverslip substrate-side (in contact with the water column) with sterile scalpel blades, and prepared into 2 mL microcentrifuge tube suspensions using sterile distilled water. OM-SAB suspensions were vortexed for one minute and left to stand for five minutes to settle the heavier particles. 25 µL of the OM-SAB suspension was aspirated and prepared in duplicate into 1-cm diameter smears on clean glass slides and stained with MK, SMB, Aura, and IFT (Aqua-GloTM G/C Direct Comprehensive Kit) according to the manufacturer's directions.

2.7. Scanning electron microscopy (SEM) of OM-SAB

To be able to provide SEM visualization of the natural architecture and composition of an environmental aquatic substrate-associated biofilm, horizontal and vertical OM-SAB grown on glass coverslips substrates were fixed with absolute methanol and allowed to dry for 24 hours. The coverslip substrates were gently broken into shards of approximately 3 X 3 mm with care not to disrupt the OM-SAB, then attached to a carbon tape secured on the SEM (TM3000 Hitachi Tabletop SEM) metal platform for examination at the University of Santo Tomas, Thomas Aquinas Research Centre.

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182 *2.8. Spiking of OM-SAB for Aura, IFT, and SEM*

183 In order to simulate environmental contamination of OM-SAB with *Cryptosporidium*
184 oocysts, 25 µL of OM-SAB suspensions were spiked with 10 µL of *C. parvum* oocysts in 1 X 10^6
185 cells / mL concentration (P102C @ 1x10/6, *Cryptosporidium parvum* oocysts, 1 million, in 4 mL,
186 Waterborne Inc. USA) and prepared in duplicate into 1 cm diameter smears on clean glass slides
187 and stained with Aura and IFT. For SEM analysis of spiked OM-SAB, one vertical and one
188 horizontal OM-SAB were fixed with absolute methanol, allowed to dry for 24 hours, then
189 manually broken into shards of approximately 3 X 3 mm with care not to disrupt the OM-SAB,
190 then spiked with 5 µL of *C. parvum* oocysts, and allowed to dry for 24 hours for visualization
191 through SEM.

192

193 **3. Results**

194 Table 1 outlines the coordinates (geographical location), WBPP, and contributory
195 contamination sources for each sampling area. The majority of the samples tested positive for
196 waterborne protozoan pathogens in Thailand (3/3) and the Philippines (15/20) sampling areas.
197 Overall, 78% (18/23) of the aquatic sample matrices tested in this study contained at least one
198 WBPP in at least one sample matrix. Table 2 highlights the natural association of *Cryptosporidium*
199 and *Giardia* (oo)cysts in SAB relative to SW and BW matrices tested in this study. IFT confirmed
200 22% (5/23) *Cryptosporidium* and 4% (1/23) *Giardia* (oo)cysts in SAB which collates with 39%
201 positivity from both SW and BW counterparts. Table 3 shows a 33% (3/9) agreement between SW
202 and SAB samples positive for WBPP and the increased detection capacity of WBPP by 50% (7/14)
203 as compared to SW negative for WBPP. The comparison of BW and SAB demonstrated similar

results. Table 4 provides a panel of microscopy results for OM-SAB, where horizontal and vertical samples were negative for naturally-associated WBPP. The absence of *Giardia* cysts was consistent in both natural and spiked OM-SAB after staining with Aura and IFT, which confirms its absence from the samples tested in the study. Fig. 2. demonstrates positive controls of *Cryptosporidium* and *Giardia* (oo)cysts that were used as a reference for microscopic identification. Fig. 3. shows the microscopic detection of *Cryptosporidium* and *Giardia* (oo)cysts in SW, BW, and SAB using the elected staining techniques. Fig. 3.a-c demonstrates typical *Cryptosporidium* oocysts stained with MK and SMB as round (oo)cysts with internal structures that stained red by carbol fuchsin, while Fig. 3.d-f demonstrates apple-green fluorescence attributed to Auramine and IFT stains. All *Cryptosporidium* oocysts (Fig. 3.a-e) fit within the 4 to 6 µm diameter range and had morphologic characteristics similar to the positive control of *C. parvum* (as shown in Fig. 2.a-d). Fig 3.f demonstrates the oval to ellipsoid morphology and size range of 10 to 14 µm of *Giardia* cysts, similar to the *G. lamblia* positive control found in Fig. 2.e. Fig. 4. shows pictures of OM-SAB grown *in situ* in Ipo watershed on glass coverslip substrates with microscopic architecture and associated structures visualized through SEM. Thick networks of exopolysaccharide matrix were ~~ob~~served in Fig. 4.d which was res~~p~~onsible for binding the contents of the biofilm microcosm. A naturally-associated testate amoeba shell (Fig. 4.e) was incidentally identified in a vertical OM-SAB. Fig. 4.g demonstrates simulated environmental contamination by spiking with *C. parvum (*oo)cysts for scenario purposes and the relative comparison of the size of *C. parvum* oocysts against contents of the OM-SAB. Evidence of the temporal accumulation and the diversity of organic materials in the biofilm matrix, mainly, freshwater diatoms (Fig. 4.f), contributed~~s~~ to the variable surface roughness and porosity which can potentially facilitate attachment and trapping of pathogenic (oo)cysts and other organic

materials. Horizontal substrates and substrates immersed at 122 cm~~.2 meter~~ depth had denser biofilm growth compared to vertical and 30 cm depth counterparts which were observed by measuring the biofilm mass through light absorbance at optical density of 590 nm using a spectrophotometer (Supplementary~~l~~ material 2) while~~and~~ dry weight expressed in grams was measured using an analytical balance (Supplementary material -3). All staining methods detected 0-1 (oo)cyst per 25 µL smeared suspension thereby providing an approximated maximum (oo)cyst load of 2,000 (oo)cysts per 50 mL of sample matrix in the present study or 40,000 (oo)cysts per 1 L of the sample matrix. On very rare occasions, 0-2 (oo)cysts per 25 µL were observed.

4. Discussion

4.1 Microscopy panel and low-volume sampling

The results of this study show that traditional staining methods of MK and SMB are cost-effective but provided lower detection compared to fluorescence techniques. The low-cost MK staining method has been reported to exhibit 66% sensitivity and up to 88% specificity (Elsafi et al., 2014; Johnston et al., 2003), and only requires a light microscope (Current and Garcia, 1991). In the present study, only 4% (1/23) SAB tested positive for *Cryptosporidium* (oo)cysts using MK, which collates with SW and BW results (Tables 1 and 2). In similar studies, MK detected only 2% (3/135) *Cryptosporidium* (oo)cysts from water samples in Turkey, which were identified as *C. parvum* after polymerase chain reaction (Aslan et al., 2012). In addition, MK presented with 71% (53/75) positivity for *Cryptosporidium* oocysts in a metropolitan watershed in the Philippines, which suggests heavy parasite contamination during the three-day sampling period (Masangkay et al., 2016). In the present study, the staining characteristics of *Cryptosporidium* were identical for both MK and SMB, where 7% (2/23) positivity for SMB was relatively close to the results obtained

from MK but lower than Aura with a 39% (9/23) screening positivity. These results suggest variability in the power of (oo)cysts detection depending on the microscopic method and the utility of Aura as a convenient screening method for detection of *Cryptosporidium* oocysts in water samples (Ahmed and Karanis, 2018; Hanscheid et al., 2008; Smith et al., 1989). In the present study, IFT findings were positive for *Cryptosporidium* and *Giardia* (oo)cysts at 22% and 4%, respectively but IFT kits were expensive and required a fluorescence microscope. However, the cost may be justified by the high degree of sensitivity (99%) and specificity (100%) for *Cryptosporidium* and 96% to 100%, respectively, for *Giardia* (Adeyemo et al., 2018; Pacheco et al., 2013). Researchers in different parts of the globe have extensively contributed to establishing the importance of water analysis methodologies in the effective detection of WBPP (Estratiou et al., 2017a; 2017b; Plutzer and Karanis, 2016). Likewise, the establishment of cost-effective concentration methods for WBPP in water samples like flotation and flocculation being applied to large and lower volume water samples have gained traction over the past two decades (Gallas-Lindemann et al., 2013; 2016; Karanis et al., 2006; Karanis and Kimura, 2002; Koloren et al., 2016; 2018; Kourenti et al., 2003; Kourenti and Karanis, 2004; 2006; Ma et al., 2019; Tsushima et al., 2001; 2003a; 2003b). Low-volume water sampling for the detection of *Cryptosporidium* and *Giardia* is not routinely performed but a number of studies have already confirmed its benefits as reported in the PCR positivity of *Cryptosporidium* in 50 mL raw river water samples in China (Xiao et al., 2012), and a study in the Philippines that reported both MK and PCR confirmation and sequence identification of *C. hominis* directly from 50 mL samples (Masangkay et al., 2016; 2019). These methods are cost-effective alternatives to Method 1623 or other methodologies, providing effective detection of *Cryptosporidium* and *Giardia*, particularly in high-turbidity aquatic samples (Bilung et al., 2017; Efstratiou et al., 2017b). In the present study, low-volume

273 water sampling offered the advantages of ease of collection and transport, multiple matrix
274 sampling (SW, BW, and SAB), multiple area sampling per study site, reproducibility, and
275 significantly lower test cost. The (oo)cyst load of approximately 2,000 (oo)cysts per 50 mL or
276 40,000 (oo)cysts per 1 L sample matrix indicated high contamination of the sampling areas with
277 WBPP. These estimates, however, should not be taken as absolute counts as (oo)cysts can be
278 unevenly dispersed and associated with bio-colloids, thereby further complicating its' non-
279 homogeneous distribution in environmental aquatic matrices. The variation of positive and
280 negative results per staining method across each 50 mL sample may have been influenced by the
281 non-homogeneous distribution of (oo)cysts in the aquatic sample matrices.
282
283 *4.2 Spatial distribution of WBPP in aquatic matrices*
284 Spatial distribution of *Cryptosporidium* and *Giardia* in sample matrices as shown in Table
285 1 and Table 2 demonstrate that BW and SW, and in particular SAB are all suitable environmental
286 aquatic matrices for the detection of WBPP. Out of the 23 combined sampling areas (3 in Thailand
287 and 20 in the Philippines), 78% (18/23) were positive for *Cryptosporidium* and/or *Giardia* in at
288 least one sample matrix, where 39% (9/23) of both SW and BW, and 43% (10/23) SAB, tested
289 positive. Results of this study on the presence of *Cryptosporidium* and/or *Giardia* in environmental
290 drinking and recreational waters can be supported by the investigations done in 2016 in the La
291 Mesa Watershed in the Philippines, where a high incidence of *Cryptosporidium* and *Cyclospora*
292 was documented over a three-day period (Masangkay et al., 2016). Similarly, a study in Malaysia
293 reported 100% (24/24) of SW to be positive for *Cryptosporidium* and *Cyclospora* (Bilung et al.,
294 2017), with *Giardia* cysts reported to contaminate SW as well (Lass et al., 2017; Ramo et al.,
295 2017). Studies investigating pathogenic protozoans from BW are rare; one exemplary study for

296 BW was reported in 2017 from the Yunlong Lake in China where 47% (28/60) BW tested positive
297 for *Cryptosporidium* (Kong et al., 2017). Although *in vitro* extracellular excystation of
298 *Cryptosporidium* has been elaborated in *Pseudomonas aeruginosa* aquatic biofilms (Koh et al.,
299 2013; 2014), there has been no documented case of the natural-association of *Cryptosporidium* in
300 environmental aquatic biofilms until the first report of *C. hominis* in SAB that was isolated from
301 a freshwater sponge in the Philippines in 2019 (Masangkay et al., 2019). In the present study, the
302 value of SAB as a supplemental sample matrix provided close agreement with both SW and BW
303 positivity and increased reporting of WBPP as compared to analyzing SW alone (Table 1 and
304 Table 3). The possible contributory factors for contamination of the sampling areas and the cycling
305 of WBPP between humans and the environment can come from many sources (Table 1) including
306 anthropogenic activities, communities with poor sanitary and living conditions, and improper
307 domestic wastewater sanitization procedures (Adamska, 2014; Bhattachan et al., 2017; Masangkay
308 et al., 2016). The presence of wildlife, domestic, and farm animals in the surroundings of lakes
309 and other water reservoirs plays a significant role in parasite transmission to other animals and
310 water sources thereby contributing to zoonotic transmission of *Cryptosporidium* and *Giardia* (Gil
311 et al., 2017; Wells et al., 2019; Zahedi et al., 2016). In addition, birds, fish, amphibians, and small
312 mammals have been tested and reported to be positive for *Cryptosporidium* and *Giardia* as well
313 (Hublova et al., 2016; Karanis et al., 1996; Ryan, 2010; Yang et al., 2015). Soil run-off, mixed
314 with animal and human excreta can contribute to source water contamination with WBPP (Dai and
315 Boll, 2003; Norman et al., 2013). Aquatic plants and immersed substrates, as demonstrated in this
316 study, can harbor pathogenic (oo)cysts through the temporal accumulation of (oo)cysts in SAB.
317 These contaminating factors are best exemplified in the University Lake of Thailand, which
318 simulates anthropogenic, zoogenic, and environmental cycling of WBPP in a small water

319 catchment (Fig. 3.a-b and Table 1), where all sample matrices were positive for *Cryptosporidium*
320 oocysts and TS1A3 SW was simultaneously positive for both *Cryptosporidium* and *Giardia.*
321
322 *4.2. Aquatic biofilms: implications for water quality assessment strategies (WQAS)*
323 Biofilms in relation to WQAS have been limited to tap water systems (Wingender and
324 Flemming, 2011; van der Kooijet al., 2017; Zhou et al., 2017), while biofilms in natural freshwater
325 resources are not screened for *Cryptosporidium* and *Giardia* contamination. The natural-
326 association of a testate amoeba shell suspected of being *Difflugia* (Qin et al., 2011) on the OM-
327 SAB surface (Fig. 4.e) demonstrates the potential for interactions between SAB and bio-colloids
328 like *Cryptosporidium* and *Giardia* (oo)cysts (Luo et al., 2016; Searcy et al., 2006). Parasite load
329 and temporal accumulation contribute to the presence of WBPP in SAB as can be observed from
330 Ipo watershed where OM-SAB grown *in-situ* were negative for *Cryptosporidium* and *Giardia*
331 (Table 4) seemingly because of low parasite load where only one *Cryptosporidium* oocyst was
332 detected from all SW and BW from all four sampling sites (results not shown). Fig. 4.c and 4.d
333 demonstrate biofilm roughness and porosity and a network of the exopolysaccharide matrix which
334 can trap *Cryptosporidium* and *Giardia* (oo)cysts (Wolyniak et al., 2010). This is in agreement with
335 the observations presented in Fig. 4.g, where *C. parvum* oocysts spiking experiment revealed the
336 relative size comparison between *Cryptosporidium* oocysts and the size of channels and spaces on
337 the OM-SAB surface, which can potentially permit the temporal accumulation and trapping of
338 WBPP, including larger testate amoeba shells among other bio-colloids. Further, the abundance of
339 organic debris in the OM-SAB permits UV radiation protection, which further contributes to the
340 maintenance of (oo)cyst viability (DiCesare et al., 2012b). As shown in Table 3, additional analysis
341 of SAB generated 33% agreement with positive SW results and enhanced detection of WBPP to

342 50% by not declaring the water samples as not contaminated based on negative SW results alone
343 with similar results observed from SAB and BW comparisons. As opposed to the real-time nature
344 of contamination from SW and BW matrices, SAB is a matrix, which submits results based on the
345 temporal accumulation of WBPP similar to reports of temporal accumulation of *Cryptosporidium*
346 oocysts in marine shellfish (Pagoso and Rivera, 2017) and freshwater sponge in the Philippines
347 (Masangkay et al., 2019). The results of this study support the hypothesis of the natural-association
348 of WBPP in SAB that can be exploited to detect *Cryptosporidium* and *Giardia* (oo)cysts from
349 environmental freshwater resources and lead to improved water quality assessment strategies
350 (iWQAS). The formulation of screening initiatives for the simultaneous testing of SW, BW, and
351 SAB in multiple sampling areas per study site can also provide important data for limiting the
352 exposure of humans and animals to WBPP, stimulate government responses, regulatory actions,
353 and improved accessibility to screening protocols.
354

355 **5. Conclusions**

356 Screening biofilms for waterborne protozoan pathogens has been an underappreciated tool
357 in the detection and mitigation of waterborne infections and outbreaks. The results of this study
358 provide evidence that analysis of aquatic substrate-associated biofilms leads to improved water
359 quality assessment strategies. Employing more than one microscopy method for the detection of
360 waterborne protozoan pathogens in low-volume samples can improve *Cryptosporidium* and
361 *Giardia* detection from surface water, bottom water with sediments, and substrate-associated
362 biofilms. Biofilms can act as biological reservoirs for waterborne protozoan pathogens by
363 associating and protecting (oo)cysts from UV exposure within their matrices and surfaces.
364

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Conflict of interests

The authors declare no conflict of interest.

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HIGHLIGHTS

- Analysis of aquatic biofilms increased the detection of protozoan (oo)cysts.
- Biofilms acted as biological reservoirs for *Cryptosporidium* and *Giardia* (oo)cysts.
- *Cryptosporidium* and *Giardia* (oo)cysts were detected in low-volume samples of 50 mL.
- 50 mL sampling permits multiple sampling, reproducibility, and lower test cost.
- 50 mL is the lowest water volume reported for the detection of *Cryptosporidium*.

Graphical abstract