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A metabarcoding approach for detecting protozoan pathogens in wild oysters from Prince Edward Island, Canada

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ABSTRACT

Food and waterborne protozoan pathogens including *Cryptosporidium parvum*, *Giardia enterica* and *Toxoplasma gondii* are a global concern for human public health. While all three pathogens have been detected in commercial shellfish, there is currently no standard approach for detecting protozoan parasites in shellfish. Common molecular and microscopic methods are limited in the number of pathogens they can simultaneously detect and are often targeted at one or two of these pathogens. Previously, we developed and validated a novel 18S amplicon-based next-generation sequencing assay for simultaneous detection of *Cryptosporidium* spp., *Giardia* spp. and *T. gondii* in shellfish. In this study, we applied the assay for protozoan pathogen detection in wild oysters from Prince Edward Island (PEI). Oysters were harvested from restricted and prohibited areas, classified by the Canadian government according to fecal coliform counts in surrounding waters, and different fractions (whole tissue homogenate and hemolymph) were analyzed. Protozoan DNA was detected using metabarcoding in 28%, of oysters tested ($N = 128$), and the pathogen read counts in oyster homogenate were considerably higher than those in hemolymph. Protozoan read count thresholds were established for classifying probable oyster contamination with pathogens to account for low levels of background protozoan reads detected in negative controls. Assay results showed protozoan contamination was not associated with harvesting site classifications, suggesting that using fecal indicators for ensuring food safety may be insufficient. Due to the complex matrix, an oyster DNA reduction step may further improve the pathogen detection sensitivity of the assay. Results from this study affirm that novel metabarcoding is a promising screening tool for detection of protozoan pathogens in shellfish.

1. Introduction

Food and waterborne protozoan pathogens are considered a global public health concern due to their ubiquity and ability to cause serious and potentially fatal disease in people (Fayer et al., 2004). *Cryptosporidium* spp., *Giardia* spp., and *Toxoplasma gondii* are among the parasites most commonly attributed to food- or waterborne illness (Bahia-Oliveira et al., 2017; Betancourt, 2019; Boarato-David et al., 2017). Cryptosporidiosis and giardiasis cause gastrointestinal disease that, while less serious in immunocompetent adults, can result in more severe symptoms and even death in children and immunocompromised individuals (DuPont, 2013; Khalil et al., 2018). While infection with *Toxoplasma* is typically asymptomatic or results in mild flu-like symptoms, in pregnant women, it may cause fetal loss or birth defects in the newborn. Toxoplasmosis in immunocompromised people can be lethal due to disseminated disease (Dubey, 2013).

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T. gondii and some species of *Cryptosporidium* and *Giardia* are zoonotic with possible animal-to-human transmission. *Cryptosporidium parvum* and *Giardia enterica* have many animal hosts, including wildlife and domestic animals such as livestock. Felids (domestic cats and wild felids) are the only known definitive host for *T. gondii* (Fayer et al., 2004). Transmission of *Giardia* cysts and *Cryptosporidium* and *T. gondii* oocysts (hereafter collectively referred to as (oo)cysts) commonly occurs fecal-orally through consumption of contaminated food or water (Dawson, 2005; Dorny et al., 2009). Rain can cause runoff from urban and rural land, transporting protozoan (oo)cysts into water used for recreation, aquaculture, and agriculture. In seawater, *C. parvum* and *T. gondii* oocysts can remain viable for up to 1 year and 2 years respectively (Hohweyer et al., 2013; Lindsay and Dubey, 2009). *G. enterica* cysts are thought to be somewhat resistant to salinity though their exact longevity in seawater requires further study (Fayer et al., 2004). All three parasites have been detected in filter-feeding bivalve shellfish worldwide (Hohweyer et al., 2013; Willis et al., 2013). The contamination of commercial shellfish such as oysters with protozoan (oo)cysts is a notable public health concern due to the tendency for shellfish to be eaten undercooked or raw. Cases of shellfish-borne protozoal illness are likely underreported due to failure to report mild cases, challenges in determining the source of foodborne outbreaks, and lack of standardized pathogen screening protocols (World Health Organization, 2010).

Presently, there is no standard protocol for detection of protozoa in shellfish (Hohweyer et al., 2013). Two common methods are direct fluorescent antibody (DFA) tests and polymerase chain reaction (PCR) (Hohweyer et al., 2013). DFA tests detect protozoa using (oo)cyst-specific monoclonal antibodies; however, there is limited specificity when distinguishing between species with high structural similarities (for example, *Cryptosporidium* spp.). Additionally, DFA tests cannot be used to detect *T. gondii* because specific monoclonal antibodies have not yet been optimized (Hohweyer et al., 2013). PCR (simplex or multiplex) is often used as either a primary detection technique or in addition to DFA tests and involves amplifying target protozoan gene(s) for species and/or genotype identification. Simultaneous multispecies identification using current PCR methods is limited (Hohweyer et al., 2013). Recently, we validated a novel metabarcoding based assay for simultaneous detection of *Cryptosporidium*, *Giardia*, and *T. gondii* in shellfish (DeMone et al., 2020). Metabarcoding can provide powerful high throughput taxonomic identification of multiple species within a sample by using universal primers to target a "barcode" marker sequence with high interspecies variability and low intraspecies variability (Porter and Hajibabaei, 2018). Rapid, sensitive and simultaneous detection of protozoan pathogens in shellfish could serve as an additional tool in current shellfish safety procedures. The objective of this study was to apply this novel metabarcoding method to test for the presence of protozoan pathogen contamination in wild oysters harvested from seawater off the coast of Prince Edward Island, Canada. Assay results were intended to provide insights into protozoan DNA read levels detected in oyster hemolymph (oyster circulatory fluid) and tissue homogenate as well as shellfish contamination from different harvesting classification zones.

2. Materials and methods

2.1. Oyster harvesting and sample preparation

Eastern oysters (*Crassostrea virginica* $N = 128$) were collected from Charlottetown harbour, Hillsborough River, North River and West River systems, PEI (Canada). Sampling sites included 4 areas classified as 'Prohibited' for shellfish harvesting and 4 areas classified as 'Restricted' (Fig. 1). Harvesting areas are classified by the Canadian Shellfish Sanitation Program (CSSP) based on chemical and biotoxin levels (Canadian Food Inspection Agency, 2019). Following collection, oysters were placed in the refrigerator in the laboratory and processed within 96 h at the Atlantic Veterinary College in PEI. Hemolymph was obtained through aspiration using a sterile needle and syringe inserted into the adductor muscle. The remaining oyster tissue was shucked, enzymatically digested using pepsin, and mechanically homogenized (Robertson and Gjerde, 2008). DNA from hemolymph and homogenate samples were extracted separately using the DNeasy Blood and Tissue Kit (Qiagen, CA, USA) as previously described (Shapiro et al., 2019). In brief, 100 µl of sample was combined with 180 µl of ATL buffer and subjected to one freeze and thaw cycle (4 min each in liquid nitrogen and in boiling water). Proteinase K (40 µl) was added to the sample and incubated overnight at 56 °C. The nucleic acids were eluted with 50 µl 10% AE buffer. Due to time and labour limitations, only oysters from Restricted Site 1 were tested individually. Oysters from Sites 2–8 were batched into pairs such that each hemolymph and homogenate 'batch' represented DNA from two oysters from the same site.

2.2. NGS library preparation and sequencing

Next generation sequencing (NGS) library preparation was performed using an adaptation of the Illumina 16S Metagenomic Sequencing Library Preparation guide (Illumina, 2013) as described in DeMone et al. (2020). In a two-step PCR process, the V4 region of the

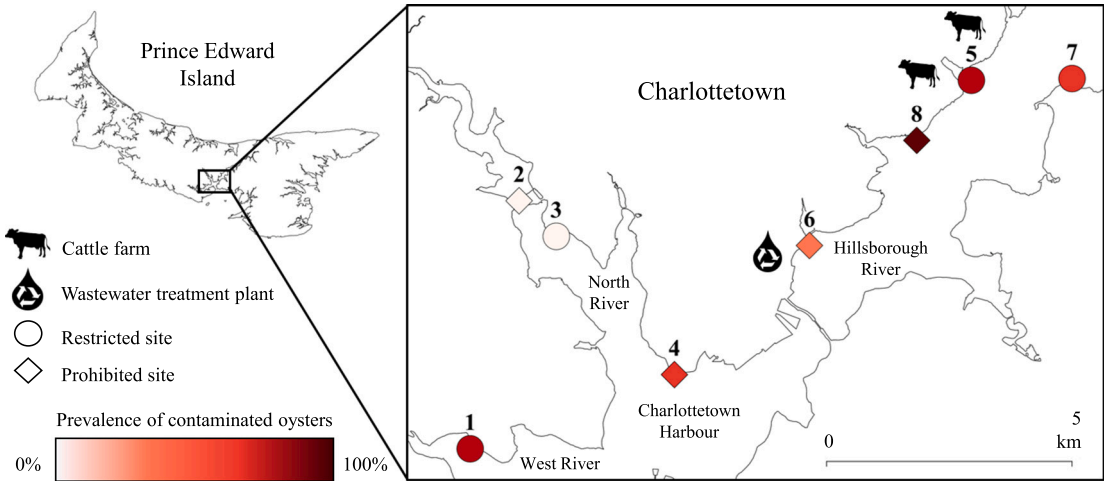

Fig. 1. Map depicting prohibited (diamonds) and restricted (circles) oyster harvesting sites near Charlottetown, Prince Edward Island, Canada. The shaded color inside the sampling symbol corresponds with the prevalence of oysters that were contaminated with *Toxoplasma gondii, Cryptosporidium* spp. or *Giardia* spp. DNA.

18S rRNA gene was amplified by target PCR (PCR 1; Table S3) and indexed with Nextera XT v2 Index 1 and 2 primers (PCR 2). Library quantification, normalization, and 2 × 250 bp Illumina MiSeq (v2 kit, PE500) sequencing were performed by the University of Guelph's Agriculture and Food Laboratory (Guelph, ON, Canada).

2.3. Data quality assurance and control

A cocktail of 1000 *T. gondii*, *G. enterica* and *C. parvum* (oo)cysts was prepared as a positive control and DNA extracted as described above. A negative control consisted of only PCR reagents. For all samples except the reagent blank controls, 500 copies of each parasite gBlocks were added to all samples to address possible PCR amplification bias and to discriminate possible laboratory contamination. Parasite gBlocks were synthetic sequences designed with assistance from Integrated DNA Technologies (IDT) Inc. (Coralville, IA) with identical primer binding sites and %GC but sufficient internal nucleotide variation for differentiation from target protozoan amplicons.

2.4. Bioinformatic analysis

Sequence data was processed and analyzed using the pipeline described in DeMone et al. (2020). Forward and reverse reads were assembled into contigs using Paired End reAd mergeR (PEAR) (Zhang et al., 2014). Synthetic gBlocks were identified with Basic Local Alignment Search Tool (BLAST) and subsequently separated from the other contigs before further processing (Altschup et al., 1990). Contig trimming, alignment, chimera removal and classification were performed using software package "mothur", following a modified version of the Illumina MiSeq 16S rRNA standard operation procedure (Kozich et al.,

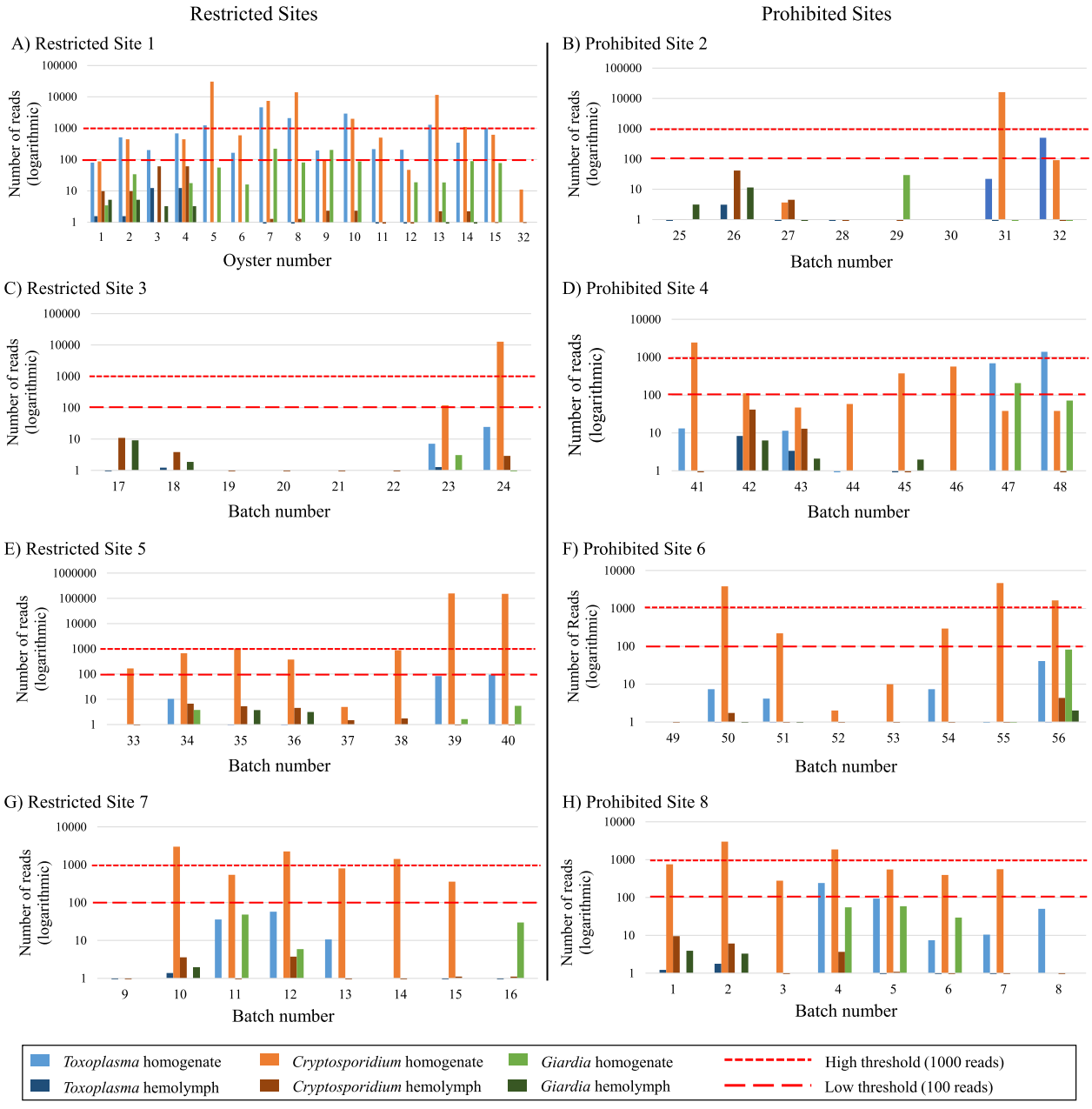

Fig. 2. Parasite read counts that were classified in oyster homogenate and hemolymph samples. Oysters were harvested from 8 restricted and prohibited sites near Charlottetown, PEI, Canada. (A) Each sample consisted of a single oyster. (B–H) Samples consisted of batches of two oysters. For each color, the lighter shade shows parasite counts found in the homogenate while the darker shade corresponds with parasite reads in the hemolymph.

2013; Schloss et al., 2009). The reference database used for contig alignment and classification was a customized 18S V4 version of Silva (v 132) (Quast et al., 2013). Raw read counts were adjusted for the amplicon dilution step that was performed during normalization and prior to sequencing. Read counts were also adjusted using gBlock read counts based on the known starting copy number (500) in all samples.

Two sequence-read contamination threshold levels were estimated based on the average background protozoan read counts detected in the PCR reagent negative control (Fig. S1). Low levels of protozoan DNA (<10 reads) were detected in negative controls, likely due to low levels of protozoa DNA present in the laboratory environment that were subsequently detected using the highly sensitive NGS approach (no visible bands were present on gel electrophoresis for these blank reagent controls). Samples with protozoan reads above the established "threshold" were considered contaminated. The 'low threshold' was established at 100 reads and the 'high threshold' at 1000 reads, which correlates with approximately 10× and 100× the average protozoan reads detected in the negative controls, respectively.

Univariate analyses were performed in R to test for associations between protozoan parasite contamination and harvest site classification (restricted and prohibited), as well as co-contamination with more than one protozoan pathogen (R Development Core Team, 2019). Analyses

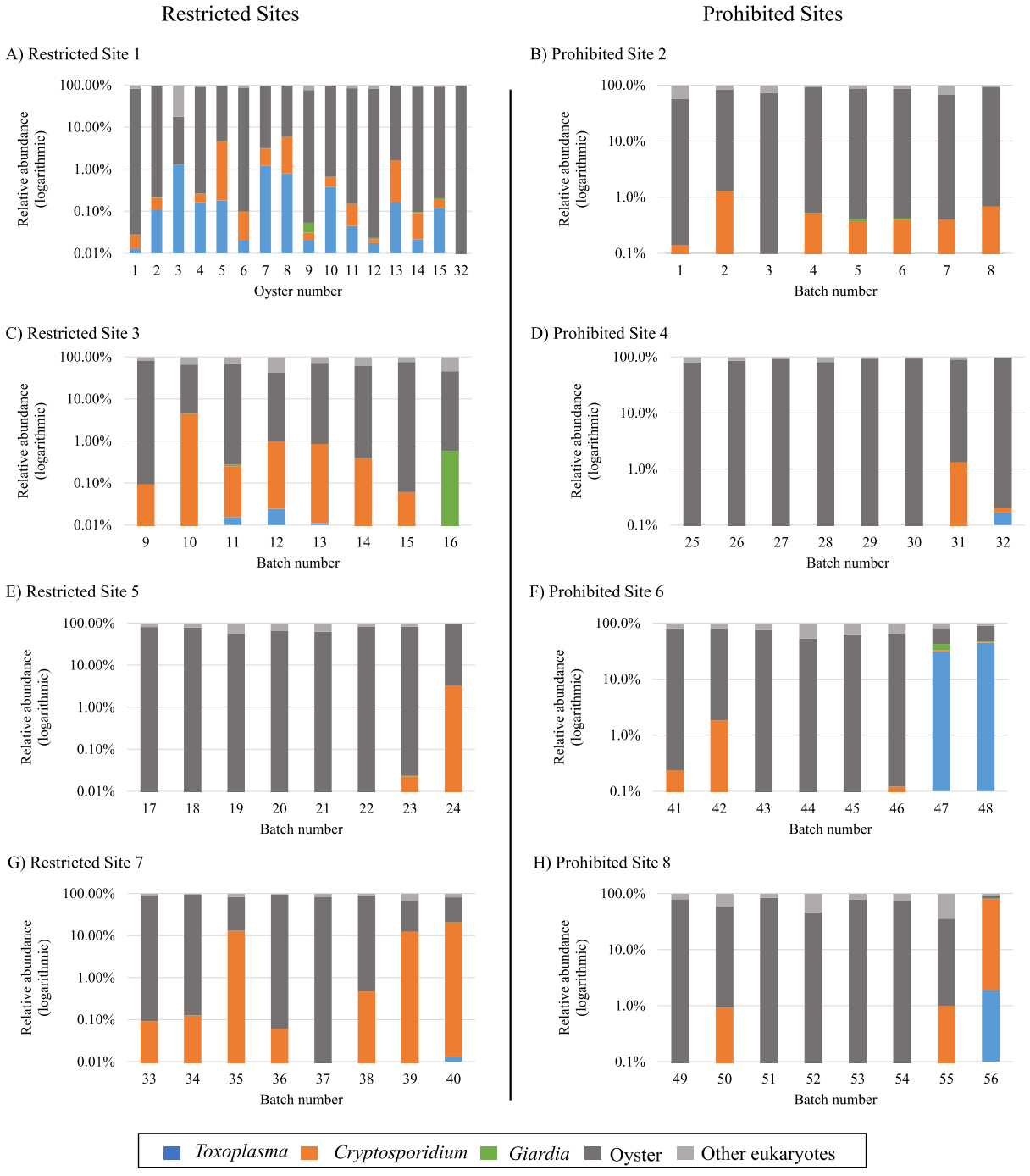

Fig. 3. Relative read abundance classified in whole tissue homogenates from oysters harvested from 4 Restricted and 4 Prohibited sites near Charlottetown, PEI, Canada. (A) Each sample consisted of a single oyster. (B–H) Samples consisted of batches of two oysters. Hemolymph results were not depicted due the very low levels of parasite reads in these samples.

were done using both low and high threshold values. A Fisher's exact test was used for analysis of samples with less than 5 counts in any outcome cells of a 2 × 2 contingency table, while the Chi Square test was used for samples with at least 5 counts in all outcome cells. Associations were considered significant when $P \leq 0.05$.

3. Results

Sequence quality control metrics calculated by the Illumina BaseSpace sequence hub showed an average %Q30 of 82.03% and a %PF of 89.25%. Approximately 40.6 million contigs were assembled and 23.1 million contigs were successfully classified. Fig. 2 illustrates the protozoan read counts detected in oyster homogenate and hemolymph samples harvested from four prohibited and four restricted shellfish harvesting sites. Contamination of hemolymph with protozoan DNA was not detected at either the high or low detection threshold levels (Fig. 2). In all homogenate samples, reads representing oyster DNA had the highest relative abundance (Fig. 3). Table 1 summarizes the prevalence of protozoan DNA detected in oyster homogenate samples at both threshold levels. Using the low contamination threshold, *T. gondii* was detected in oyster homogenate from Restricted Site 1 and Prohibited Sites 2, 4 and 8. At the higher threshold, *T. gondii* was detected in homogenate samples from Restricted Site 1 and Prohibited Site 4. *Cryptosporidium* spp. DNA was detected in homogenates from all harvesting sites at both low and high threshold levels. *Giardia* spp. were only detected in homogenate samples from Restricted Site 1 and Prohibited Site 4 at the low threshold level. *T. gondii* and *Giardia* spp. were the most prevalent of three targeted parasites in oysters harvested from Restricted Site 1 (87.5% and 12.5%, respectively) and *Cryptosporidium* spp. contamination was most prevalent at Prohibited Site 8 (100.0%). Using the low threshold, the odds of *T. gondii* detection were significantly higher in oysters from restricted sites than from prohibited sites [odds ratio (OR) 3.70, 95% confidence interval (CI) 0.99–17.46, $P = 0.03$]. At the low threshold, the odds of detecting *T. gondii* in oysters appeared to be greater in oysters that were also contaminated with *Giardia* (OR ∞, 95% CI 1.32–∞, $P = 0.01$), however the large CI interval should be noted with confidence of this association being low. At the high threshold, *Cryptosporidium* spp. detection was more likely in oysters contaminated with *T. gondii* (OR 17.64, 95% CI 1.78–889.60, $P = 0.004$) though this association also yielded a large CI.

4. Discussion

Commercial shellfish aquaculture is a key industry for Canada's coastal provinces with PEI being the largest producer (Statistics Canada, 2019). Using a novel metabarcoding-based assay, DNA from three important zoonotic, foodborne pathogens, *Cryptosporidium* spp., *Giardia* spp. and *T. gondii*, was detected in wild oysters harvested off the coast of PEI. The results from this assay were used to compare protozoan read levels in different shellfish fractions (hemolymph and whole tissue homogenate) as well as protozoan contamination of shellfish in different harvesting areas. This is the first time metabarcoding has been used to analyze a commercially important shellfish species for zoonotic, foodborne pathogens.

Due to the high sensitivity of NGS, this assay also detected low levels of background protozoan contamination in all samples, including negative controls containing reagents only. Thus, we implemented a minimum protozoan read threshold for analyzing results from field-collected oysters. This was done to more reliably discriminate between true presence of protozoan parasites in wild oysters and low numbers of target pathogen reads that are inadvertently introduced into reaction mixtures in laboratory spaces that commonly work with these microorganisms (DeMone et al., 2020). For optimal data interpretation, this threshold should be chosen based on both background contamination levels found in experimental negative controls and the desired diagnostic sensitivity and specificity of the assay. Two threshold levels were established for this study based on experimental negative control levels of <10 reads for each parasite (Fig. S1): a "low threshold" of 100 reads and a "high threshold" of 1000 reads.

The CSSP categorizes shellfish harvesting areas into three main categories based on water chemical levels and fecal coliform counts: Approved, Restricted, and Prohibited (Canadian Food Inspection Agency, 2019). Shellfish harvested from Approved sites (median fecal coliform most probable number (MPN) < 14/100 mL) do not require any processing whereas shellfish from Restricted areas (median fecal coliform MPN <88/100 mL) must undergo depuration prior to entering the market. Shellfish from Prohibited areas (areas near sewage and industrial outfalls, marinas, and floating living accommodations) are not permitted to be harvested for consumption. A notable finding from this study was that *Cryptosporidium* spp. and *Giardia* spp. contamination in oysters was not associated with harvesting site classifications, and this finding was consistent at both the low and high threshold detection levels (Table S1). This lack of association reaffirms previous findings that *Cryptosporidium* spp. and *Giardia* spp. in shellfish do not correlate

Table 1
Prevalence of oysters contaminated with *Toxoplasma gondii*, *Cryptosporidium* spp. and *Giardia* spp. DNA that were detected using metabarcoding. Oysters were obtained from restricted and prohibited harvesting sites near Charlottetown, PEI, Canada. Presence of parasite reads was interpreted using low and high thresholds as described in materials and methods. At the low threshold, samples with parasite read levels >100 reads were considered contaminated, and at the high threshold, samples were considered contaminated if parasite reads >1000.

	Batches tested[a]	Number of contaminated oysters (prevalence)					
		Low threshold			High threshold		
		Toxoplasma	*Cryptosporidium*	*Giardia*	*Toxoplasma*	*Cryptosporidium*	*Giardia*
Restricted sites							
Site 1	16[b]	14 (87.5%)	11 (68.8%)	2 (12.5%)	5 (31.3%)	6 (37.5%)	0
Site 3	8	0	2 (25%)	0	0	1 (12.5%)	0
Site 5	8	0	7 (87.5%)	0	0	2 (25.0%)	0
Site 7	8	0	6 (75%)	0	0	3 (37.5%)	0
Total	40	14 (35.0%)	26 (65.0%)	2 (5.0%)	5 (12.5%)	12 (30.0%)	0
Prohibited sites							
Site 2	8	1 (12.5%)	1 (12.5%)	0	0	1 (12.5%)	0
Site 4	8	2 (25.0%)	4 (50.0%)	1 (12.5%)	1 (12.5%)	1 (12.5%)	0
Site 6	8	0	5 (62.5%)	0	0	3 (37.5%)	0
Site 8	8	1 (12.5%)	8 (100%)	0	0	2 (25.0%)	0
Total	32	4 (12.5%)	18 (56.3%)	1 (3.1%)	1 (12.5%)	7 (21.9%)	0

[a] Oysters from Sites 2–8 were tested in 8 batches of 2 oysters each (128 oysters were tested in total).
[b] Oysters ($n = 16$) from Site 1 were initially tested individually to set up the sample library preparation workflow in our laboratory.

with fecal coliform counts in surrounding waters, the metric used to categorize shellfish harvesting areas (Canadian Food Inspection Agency, 2019; Willis et al., 2013). At the low pathogen detection threshold, the odds of detecting *T. gondii* in oysters from Restricted harvesting sites was 3.7 times more likely than in oysters from Prohibited sites, though the 95% confidence interval (0.99–17.46) and relatively small sample size ($N = 128$) should be considered (Table S1). These results support previous findings that *T. gondii* contamination in shellfish was found to be independent of fecal coliforms in surrounding water (Shapiro et al., 2018).

Both hemolymph and whole tissue homogenate preparations of oysters were analyzed in this study; however, hemolymph samples did not contain large enough numbers of reads to be considered contaminated, while homogenate samples from the same oysters contained protozoa read numbers that exceeded both contamination thresholds (Fig. 2). While hemolymph is a less complex matrix type than tissue homogenate, and can be more quickly prepared for downstream analysis, it does not reflect all contaminants (including (oo)cysts) within an oyster. Protozoan (oo)cysts are known to concentrate not only in the hemolymph of shellfish but also the gills and digestive tract (Hohweyer et al., 2013). The low levels of protozoan reads found in the hemolymph fraction of contaminated oysters indicates that analysis of only hemolymph may not be sufficient for effective oyster surveillance using this assay.

The density and complexity of the oyster tissue matrix has been a challenge throughout the assay development process. Most samples evaluated in this study were batches of two oysters (DNA from either hemolymph or homogenate samples representing two oysters). The batched processing for library preparation and Illumina sequencing was performed due to financial and personnel time constraints. While this technique allowed for more oysters to be tested, targeted V4 18S amplification of (oo)cyst DNA may have been less efficient due to the comparatively large amount of background oyster DNA. This is further supported by the observation that total read numbers from the targeted protozoan pathogens (Fig. 2) and their relative abundance (Fig. 3) in unbatched oysters (Restricted Site 1) were noticeably higher than batched oysters from the other sites.

In term of relative read abundance, oyster reads vastly outnumbered protozoan reads regardless of whether the samples were batched or unbatched (Fig. 3). An oyster DNA reduction step in the laboratory workflow would likely increase the sensitivity of this assay. Earlier work from our team has described enzymatic and bleach pre-treatment of oyster tissues in an attempt to reduce the strength of oyster DNA signal, however results were inconsistent (DeMone et al., 2020). Several other methods have been proposed for reducing the interference of high background DNA levels with detection of target organisms: Depletion of Abundant Sequences by Hybridization (DASH), blocking primers, and target enrichment sequencing. DASH is a technique that employs a Crispr-Cas9 system to target and cleave unwanted DNA sequences (Gu et al., 2016). Blocking primers are oligonucleotides used to target and bind to unwanted sequences and prevent amplification (Vestheim and Jarman, 2008). Target-enrichment is a technique that involves capturing target sequences through their hybridization with “probe” sequences and washing away unwanted background DNA prior to sequencing (Gaudin and Desnues, 2018).

In conclusion, applying a novel metabarcoding assay enabled the successful detection of protozoan parasites in wild oysters. While this method performed best on whole oyster tissue homogenate, the complex matrix contains high levels of background DNA that hinder efficient amplification of target pathogens. This method has promising applications for screening shellfish for zoonotic protozoan pathogens and for routine surveillance that can be improved with the implementation of a background DNA reduction step. The lack of association between oyster contamination with protozoan pathogens and harvesting site classifications further demonstrates that fecal coliform counts in surrounding water is not an effective metric for predicting the presence of protozoan pathogen contamination. Direct testing of shellfish such as oysters for zoonotic parasites should thus be considered as an additional tool for ensuring safe seafood products to consumers.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109315.

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