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Identification of *Cryptosporidium parvum* and *Blastocystis hominis* subtype ST3 in Cholga mussel and treated sewage: Preliminary evidence of fecal contamination in harvesting area

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ABSTRACT

Cryptosporidium parvum and *Blastocystis hominis* are foodborne parasites known for causing diarrhea. They accumulate in mussels grown on contaminated water bodies, due to the discharge of treated sewage from sewage treatment plants (STP). Despite this, some countries like Chile do not include these parasites in the control or monitoring of sewage water. The objective of this research was to evaluate the contamination of *C. parvum*. and *B. hominis* from treated sewage (disinfected by chlorination) and Cholga mussels in a touristic rural cove from the bay of Concepción. Cholga mussels from commercial stores and a treated sewage sample were analyzed. *Cryptosporidium* spp. was identified by Ziehl-Neelsen-Staining (ZNS) and *C. parvum* by direct-immunofluorescence assay (IFA) from ZNS-positive samples. *Blastocystis hominis* was identified by PCR using locus SSU rDNA. *C. parvum* and *B. hominis* subtype ST3 were found in 40% and 45% of Cholga mussel samples, respectively, and both parasites were identified in the treated sewage. *Blastocystis hominis* SSU rDNA gene alignment from Cholga mussels and treated sewage showed 89% of similarity, indicating that could be the same parasite in both samples. We describe the first evidence of possible contamination with these parasites from treated sewage to Cholga mussel suggesting an environmental contamination with high human risk. Based on these results, further studies will consider all the rural coves and STP from the bay to prevent possible contamination of these parasites.

1. Introduction

Cryptosporidium parvum and *Blastocystis hominis* are two parasites transmitted by fecal contamination (Karanis et al., 2007;

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Thompson et al., 2016). They can cause intestinal diseases in people with diverse degrees of severity and chronicity (Torgerson et al., 2015). In some cases, they can cause death in people due to severe dehydration (Zahedi and Ryan, 2020).

Cryptosporidium spp. and *B. hominis* are zoonotic microorganisms, transmitted by feces, that resist disinfectants and adverse environmental conditions (Martín-Escolano et al., 2023) being persistent in the environment (Zahedi and Ryan, 2020). They are recognized as waterborne since they are transmitted mainly through water contaminated with feces (WHO, 2016). Moreover, *Cryptosporidium* spp. and *B. hominis* represent 63% and 8.1%, respectively, of all the outbreaks produced worldwide (Efstratiou et al., 2017).

One of the sources of water contamination is sewage treatment plants (STP) (Vera et al., 2014). The envelope of these parasites presents resistance to certain disinfection treatments, producing differences in removal efficiencies (Nasser, 2016; Suarez et al., 2022). It has been estimated that the prevalence of *Cryptosporidium* spp. in treated sewage and raw sewage was 25.7% and 40.1%, respectively (Darei et al., 2021). Therefore, there is a potential risk of parasite transmission through the discharged treated sewage (WHO, 2016). Australia, the United States, and New Zealand have monitoring programs for *Cryptosporidium* spp. in discharged treated sewage as a preventive control, which have reduced infectious outbreaks in the population by avoiding environmental contamination (King et al., 2017; Zahedi and Ryan, 2020; Petterson et al., 2021).

In Chile, no outbreaks related to these parasites have been reported, but they are the most frequent parasite causing diarrhea in children under five years old (Epidemiology Department, Ministry of Health Chile, 2020). In addition, *B. hominis* had an estimated prevalence of 30.4%, where subtypes 3 and 4 were the most frequent (Peña et al., 2020). Therefore, the detection of these parasites in the treated sewage is required since they can reach water sources (eg.: rivers, lakes, and sea), and irrigation systems contaminating vegetables, fruits, and seafood. (Zahedi and Ryan, 2020). Moreover, the current regulations for discharges of treated sewage to water bodies consider only the evaluation of fecal or thermotolerant coliforms (Supreme Decret 90, 2000).

One of the main seafood consumed in Chile is bivalves (11.9 kg per capita per year), like Cholga mussel, which enhance the gastronomic tourism and economy from different rural zones (National Fisheries and Aquaculture Servicie, Ministry of Economy, Develpoment and Tourism, Chile, 2021). The health authority recommends the consumption of cooked Cholga, but in practice, it is at the discretion of the consumer. For example, products such as mollusk salads are offered as raw seafood in the coves.

The Cholga, like other mussels, tends to accumulate diverse types of contaminants, such as parasites, due to their feeding system (filtration of nutrients in water) (Giangaspero et al., 2019; Li et al., 2023). In this context, *Cryptosporidium* spp. was detected in different species of mussels harvested from Mangaratiba city (Rio de Janeiro state, Brazil) with a prevalence of 26.7% to 38.9% (Mariné Oliveira et al., 2016), and mussels from Malta Lake (Poland) with a prevalence of 5.1% for *B. hominis* (Słodkowicz-Kowalska et al., 2015).

Due to the high prevalence in Chile of these parasites, it is necessary to evaluate and monitor *C. parvum* and *B. hominis* in the environment, especially, in harvesting zones of bivalves where treated sewage is discharged. Moreover, the current regulation does not include these parasites in the microbiological analysis of the treated sewage (Supreme Decret 90, 2000). Therefore, this research evaluates the contamination of *C. parvum*. and *B. hominis* from treated sewage (disinfected by chlorination) and Cholga mussels in a touristic rural cove from Chile.

2. Materials and methods

2.1. Parasite in mussel

2.1.1. Sampling

A total of 73 Cholga mussels were obtained from three commercial stores (CS-1, CS-2, and CS-3) from a rural cove located in Concepcion Bay, 28.6 km from the city of Concepción, Chile. The Cholga mussels were purchased two times during the spring (October and November 2022). The samples were transported on ice to the Parasitology Laboratory (University of Concepcion). The identification of Cholga mussels (*Aulacomya ater* species) was based on their morphological characteristics (Aldea and Valdovinos, 2005). These mussels were grouped according to their shell size (12–10 cm, 3 mussels per group; <10 cm, 5 mussels per group). Finally, a total of 5, 7, and 8 groups from CS-1, CS-2, and CS-3, respectively, were obtained.

2.1.2. Processing

The Cholga mussels were washed externally with sterile distilled water. Valves were removed and the hepatopancreas and gills were extracted (Cazeaux et al., 2022). Both organs were homogenized in phosphate buffer saline (PBS) with a porcelain mortar and pestle. The homogenized organs were filtered through a strainer. The filtrate was allowed to settle in a 50 mL tube (12 h at 4 °C), and the sediment was dissolved in 70% (*v*/v) of ethanol and stored for 12 h at 4 °C.

2.2. Parasite in treated sewage

2.2.1. Sampling

Twelve liters of the treated sewage were taken from the disinfected effluent. The treated sewage was transported to the Parasitology laboratory (Faculty of Biological Science, University of Concepcion) in sterile glass bottles with screw caps on ice (4 °C). The supplementary material S1 describes the sewage treatment plant (STP).

2.2.2. Processing

For the parasite extraction, the treated sewage was filtered through a cellulose membrane (3.0 μm pore size). Then, a volume of 25 mL PBS with 1% of Tween (PBS-T) was added to the membrane, and subjected to ultrasound extraction (40KHz ultrasonic bath, 10 min

at 25 °C) to release the parasite. The solution was centrifuged (1500 ×*g*, 15 min at 4 °C). The supernatant was discarded, and the pellet was divided into two equal parts to detect *C. parvum*. and *B. hominis*.

2.3. Detection of C. parvum

The detection of *C. parvum* oocysts in Cholga mussel groups was performed by the modified Ziehl-Neelsen Stain method (ZNS) (Mead and Arrowood, 2020) combined with an immunofluorescence assay (IFA) according to Giangaspero et al. (2005). Only positive groups for ZNS were analyzed with the IFA. For the IFA, an anti-*Cryptosporidium parvum* antibody conjugated to FITC (J27E, from Thermofisher, USA) was used according to the manufacturer's instructions. An inverted fluorescence microscope was used at 40× magnification (Olympus, IX81) to visualize *C. parvum* oocysts, and the images were processed with the Cell-IR software version 2.0.

In the case of the treated sewage, before using ZNS and IFA techniques, the *Cryptosporidium* spp. oocysts were isolated by immunomagnetic separation (IMS) according to the manufacturer's instructions (Dynabeads™ anti-Cryptosporidium kit from Thermofisher, USA).

2.4. Detection of B. Hominis

2.4.1. Conventional PCR

The SSU rDNA gene was used to identify *B. hominis* in the treated sewage and Cholga mussel groups by conventional PCR according to Böhm-Gloning et al. (1997). Briefly, the genomic DNA extraction was performed with the DNeasy Powersoil® DNA extraction kit (Qiagen, USA) according to the manufacturer's instructions. The conventional PCR was performed using the DreamTaq DNA polymerase (Thermofisher, USA). The PCR conditions were as follows: 95 °C for 4 min; 40 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and a final extension of 72 °C for 5 min. The PCR products ϵ 500 pb) were visualized in electrophoresis agarose gel (1.5%).

2.4.2. Sequencing of PCR product

The PCR products were cut off from the agarose gel and sent to the AUSTRAL-omics (Universidad Austral, Chile) laboratory for agarose DNA extraction and sequencing. The sequences were analyzed in the Bioedit Sequence Alignment Editor 7.05.3 program (Hall, 1999). A BLASTn was performed to identify the subtype of *B. hominis* by comparing the PCR product sequence with the subtypes available on the GenBank as described by Santín et al. (2011). All hits below an alignment length of 400 bp were removed. Subtypes were determined by an exact match or closest similarity (>85%) with sequence data from known *Blastocystis* subtypes (*BLAST® Command Line Applications User Manual [Internet]*, 2008).

2.5. Physicochemical and microbiological characterization of the raw sewage and treated sewage

The raw sewage and treated sewage were physiochemically and microbiologically characterized according to the Standard Methods protocols (Baird and Bridgewater, 2017). For this, 200 mL of raw or treated sewage was taken for analysis. In both samples, the temperature, pH, and turbidity parameters were determined in situ with an OAKTON multiparameter (model PC650–4800485). Also, the residual chlorine concentration was determined in situ only for the treated sewage by the Checker®HC colorimetric meter (model HI701). For determination of total suspended solids (TSS), volatile suspended solids (VSS), and chemical oxygen demand (COD), the samples were transported on ice and analyzed in the Environmental Engineering and Biotechnology Group (GIBA) laboratory at the University of Concepcion.

For the quantification of total and fecal coliforms, a volume of 100 mL of raw sewage and treated sewage samples were transported on ice in <2 h to the laboratory. Total and fecal coliforms were determined by the most probable number method. The samples were cultured in aerobic lauryl sulfate lactose medium at 35 °C for 18 h.

Table 1
Positive sample for *C. parvum* and *B. hominis* in Cholga mussel from different commercial store and in treated sewage from sewage plant.

Sample	Total sample	*Cryptosporidium* sp.	*Cryptosporidium parvum*	*Blastocystis hominis*
		ZNS[2]	IFA[2]	PCR[2]
CS-1[1]	5	3 (60.0%)	1 (20.0%)	0 (0.0%)
CS- 2[1]	7	4 (58.0%)	3 (43.0%)	4 (57.0%)
CS-3	8	6 (75.0%)	4 (50.0%)	5 (62.5%)
Commercial store (Total)	**20**	**13(65%)**	**8 (40%)**	**9 (45%)**
Treated sewage	**12 L**	**positive**	**positive**	**positive**

[1] Sample taken in the same day.
[2] Detection method. ZNS: Ziehl Neelsen staining; IFA: Immunofluorescences Assay; PCR: Polymerase Chain Reaction.

3. Results

3.1. C. parvum and B. Hominis in Cholga

The detection of *C. parvum* and *B. hominis* in the Cholga mussel sample is presented in Table 1 and Fig. 1. These samples were acquired from three different Commercial Stores in the same rural cove.

Cryptosporidium spp. were identified in Cholga mussels from the three commercial stores. This parasite was detected in 65% (13 groups) of the Cholga mussel groups by the ZNT, where 40% (8 groups) correspond to the zoonotic *Cryptosporidium parvum* species detected by IFA.

B. hominis SSU rDNA gene was detected in CS-2 and CS-3 stores (Table 1). For CS-2, from a total of 7 groups of samples, 4 were positive. In the CS-3, 8 groups were analyzed and 5 were positive. Overall, 45% (9 groups) of Cholga mussel sample groups were positive for *B. hominis*. From these 9 groups, the PCR product of one representative sample was sequenced. The sequence analysis by BLASTn matches with high identity (99%) and query cover of 98% to the sequence deposited in GenBank (LC413891.1) corresponding to the subtype ST3.

3.2. C. parvum and B. Hominis in treated sewage

The treated sewage from a STP that discharged into a rain channel up to the beach near the Cholga harvesting areas, was analyzed to determine the possible source of the fecal contamination (**Fig. S1**).

The physicochemical and microbiological (fecal coliforms) parameters of this treated sewage sample (12*L*) agreed with the Chilean discharge norm (Table 2; Supreme Decret 90, 2000). The treated sewage was positive for *Cryptosporidium* spp. by ZNS. In addition, the IFA positivity indicates that the zoonotic *C. parvum* species was present in the treated sewage.

For *B. hominis*, the SSU rDNA gene was positive in the analyzed treated sewage by PCR. The PCR product analyzed by BLASTn matches with 89% of identity and a query cover of 84% with the sequence deposited in GenBank (MK801389.1), which corresponds to the subtype ST3. Moreover, the sequence alignment between *Blastocsytis hominis* SSU rDNA PCR product from Cholga mussels and treated sewage presented 89% of identity (*E*-value $= 2 \times 10^{-132}$), suggesting a possible relationship between Cholga mussels and treated sewage *Blastocystis hominis* contamination (Fig. 2).

The identification positive parasite of *Cryptosporidium* spp. and *Blastocystis hominis* indicate that there is a parasite contamination in the treated sewage from the STP, which is discharged to the sea near the Cholga mussel harvesting area. These parasites accumulate in Cholga mussels due to their feeding system (filtration of nutrients suspended in water). Therefore, there is a high risk of ingesting these parasites through consuming raw Cholga mussels, a common practice in some countries like Chile.

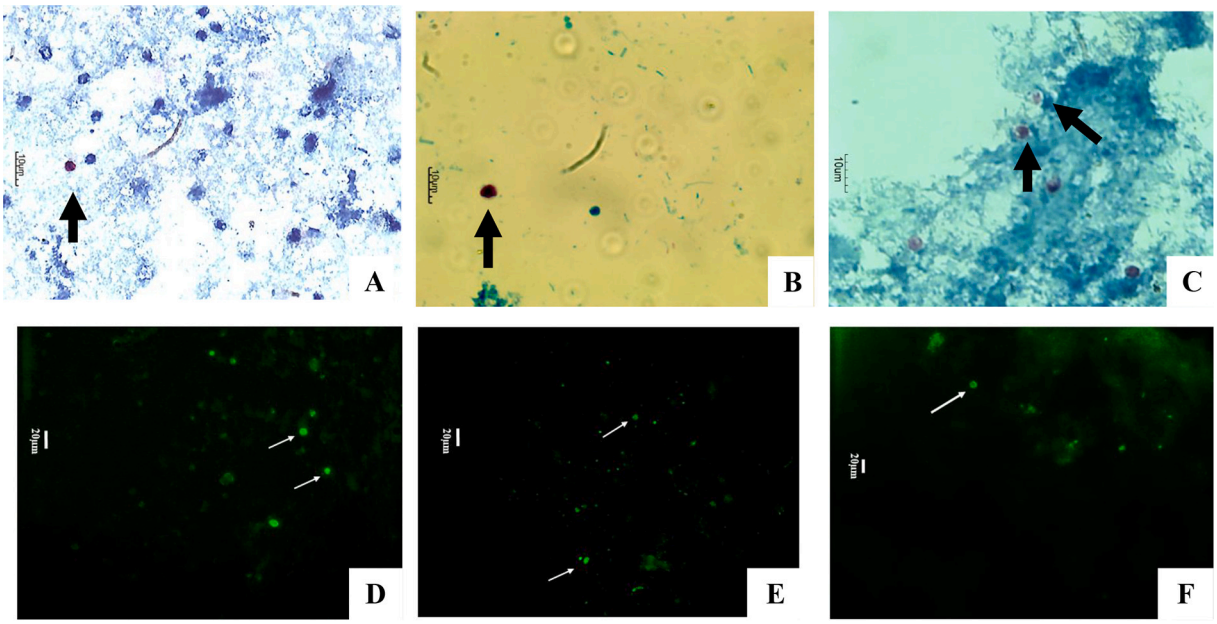
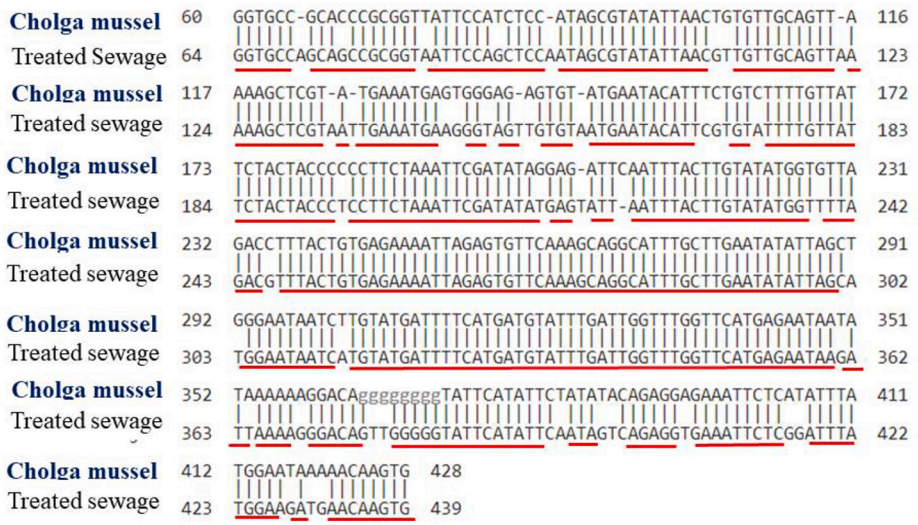


Fig. 1. Microscopic observation of *Cryptosporidium* spp. oocyst in Cholga mussel, treated sewage and control (feces). Optical Microscopic by ZNS (A) Cholga, (B) treated sewage and (C) Feces (black arrow). In Fluorescence Microscopic by IFA (D) Cholga, (E) treated sewage and (F) feces (white arrow).

Table 2
Physicochemical and microbiological characterization of raw sewage and treated sewage from sewage plant.

Parameters	Raw sewage	Treated sewage
Temperature (°C)	16.1	15.4
Turbidity (NTU)	326.0	30.5
pH	7.95	3.85
TSS (mg L^{-1})	613.0	37.0
VSS (mg L^{-1})	533.0	30.0
COD (mg L^{-1})	624.0	143.7
Residual chlorine (mg L^{-1})	–	0.61
Total Coliforms (CFU/100 mL)	3.1×10^7	1×10^0
Fecal Coliforms (CFU/100 mL)	6.4×10^6	1×10^0

TSS: Total Suspended Solid; VSS: Volatile Suspended Solid; COD: Chemical Oxygen Demand.



Cholga sequence large	Treated sewage sequence large	Score	*E*-value	Identities	% identities	Gaps
428pb	439pb	455 bits (246)	$2x10^{-132}$	335/377	88.96%	9/377 (2%)

Fig. 2. Alignment sequence of SSU rDNA gene of *Blastocystis hominis* from Cholga mussel and treated sewage. Underlined in red shows the coincidence between the sequences. Below is the summary table of the alignment results (Blastn). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Cryptosporidium parvum. and *B. hominis* are foodborne cause intestinal disease in the population, mainly acute and chronic diarrhea when food is not well cooked or sanitized (Badparva and Kheirandish, 2020; Bouzid et al., 2013). Nevertheless, the source of transmission to seafood, such as Cholga mussels, has not been reported in Chile. On the other hand, these parasites have been detected in treated sewage worldwide (WHO, 2016) representing a contamination source to Cholga mussels and consequently to humans (Fayer et al., 2003; Lucy et al., 2008).

In this work, both parasites were detected in Cholga mussels. This mussel can concentrate microorganisms from the seawater due to their filtration feeding process (Ben-Horin et al., 2015), but they can depurate them from their system when the water body is without or in low levels of these parasites (Willis et al., 2013; Ben-Horin et al., 2015). Nevertheless, the presence of these parasites in Cholga mussels indicates water contamination. In addition, the climate change generated in the Bay of Concepcion a low seawater renewal rate due to a change in the marine currents (González-Saldía et al., 2019), therefore, increasing the probability of parasite bioaccumulation (Rose et al., 2001; Willis et al., 2013; Ligda et al., 2020).

These results are in agreement with Giangaspero et al. (2005), who identified *C. parvum* in 23 groups of *Chamelea gallina* clams taken from the coasts of the Adriaco Sea (Abruzzo region). Słodkowicz-Kowalska et al. (2015) identified in *Anodonta anatine* and *Unio tumidus* mussels, harvested from the municipal reservoir of Malta Lake (Poland), *Cryptosporidium* spp. and *B. hominis* in 15.4% and 5.1% of the samples, respectively, among other parasitic agents. Lucy et al. (2008) identified *Cryptosporidium* and other parasites

through IFA and FISH techniques in shellfish samples from Ireland's coast. Their results suggest a relationship between the presence of an STP, which discharges in the harvesting area, with the presence of *Cryptosporidium* spp. oocysts (up to 22.3 oocysts g^{-1} of shellfish). In addition, shellfish contamination with parasites could be a long-term phenomenon (Lucy et al., 2008). Recently, Ligda et al. (2020) detected the presence of *Cryptosporidium* spp. in three treated wastewaters, corresponding to 22% of positive samples, which are discharged near the sea farm of *Mytilus galloprovincialis* mussel on the Thermaikos Gulf (North Greece). But *Cryptosporidium* spp. was not detected in the mussel samples, nevertheless, the high detection limit of the IFA ($5.0–5 \times 10^3$ oocyst mL^{-1} Kuczynska et al., 2003; O'Leary et al., 2021) and the low contamination pressure were attributed to this result.

Gomez-Couso et al., 2003 analyzed samples of shellfish such as mussels, clams, and oysters grown under a culture system on the coast of Galicia (Spain) and imported from other European countries. In this study, *Cryptosporidium* spp. oocyst was detected by IFA in 34.4% of the samples (total 203). They also identified the presence of fecal coliforms (by the most probable number method) in the samples but were under the human risk limit according to the current Spain regulation (<300 coliforms per 100 g of shellfish). Nevertheless, the consumption of raw shellfish with *Cryptosporidium* spp. can represent a health problem for people and its monitoring should be considered in the regulation norm (Gomez-Couso et al., 2003). Recently, Srisuphanunt et al. (2023) detected in 17.5% of commercial oyster samples the presence of *Cryptosporidium* spp. by IFA. The oysters were harvested from two shellfish aquaculture sites (Gulf of Thailand coast) close to several sewage discharge areas.

The above research studies recommend parasite detection in mollusk to prevent public health problems by studying the harvesting area, and increasing quality control detection (Gomez-Couso et al. (2003); Giangaspero et al., 2005; Lucy et al., 2008; Słodkowicz-Kowalska et al., 2015; Ligda et al., 2020; Srisuphanunt et al., 2023).

In our study, the evaluation of the STP that discharges treated sewage to the beach near the harvesting zone of Cholga mussels was analyzed to identify a point source of contamination (**Fig. S1**). It is well known that the STP contributes to the environmental contamination of both parasites (WHO, 2016; Ligda et al., 2020). The STP from the studied zone consists of primary (grid), secondary (vermifilter), and disinfection (chlorination) processes. The STP recollects the raw sewage from 200 inhabitants. It is worth mentioning that the studied sector is an isolated area (5 km from the urban city across the hill), where the main economic activity is the harvesting of marine products, mainly mollusk and fishing, and there is no presence of important livestock activities. Furthermore, the harvest and purchase day of the Cholga mussel was performed during the spring season, which is characterized by a decrease in the level of precipitation (the average in October and November were 33 mm and 14 mm, respectively) (CR2, Center for Climate and Resilience Research, Chile, 2020, Chile) and the level of gastronomic tourism increase.

Our results agree with the reported disinfection efficiency (< 3.2%) of *Cryptosporidium* spp. (Suarez et al., 2022). In addition, *C. parvum* was identified by IFA in treated sewage and Cholga mussel suggesting a transmission pathway. In addition, we suggest that the species of *B. hominis* identified in treated sewage and Cholga mussel samples. For this subtype determination, we used the *B. hominis* SSU rDNA barcoding, which is the most representative gene for subtype identification (Santín et al., 2011). Moreover, this subtype is the most prevalent in South America and Chile (Stensvold et al., 2009; Jimenez et al., 2019; Peña et al., 2020).

The chlorination disinfection process is not efficient for the elimination of *C. parvum* and *B. hominis*, as was shown. In addition, the discharged sewage could be contaminating the environment near the harvesting area of the Cholga mussel. This result agrees with other studies (Fayer et al., 2003; Lucy et al., 2008). The most efficient disinfection process to lower *Cryptosporidium* spp. is ultrafiltration combined with chlorination or UV radiation (Nasser, 2016). However, the monitoring of the treated sewage in the discharges must consider microorganisms such as *Cryptosporidium* spp. and *B. hominis*, that are resistant to these disinfection processes.

In countries like the United States, New Zealand, Australia, and England, *Cryptosporidium* sp. waterborne outbreaks in people were reported (Ma et al., 2022; Garcia and Hayman, 2023). Therefore, in those countries, *Cryptosporidium* spp. is included in the microbiological water quality regulation, mainly in drinking water (EPA, 2001; Noke, 2008; Health Canada, 2019). The regulations established the monitoring of drinking water, surface water, and even groundwater (EPA, 2001; Noke, 2008; Health Canada, 2019). When *Cryptosporidium* sp. is above the allowed limits, as occurred by chlorination disinfection, the reduction efficiency must be continuously monitored up to values below the 3log (Noke, 2008; Health Canada, 2019). Moreover, most countries do not have regulations considering the presence of these parasites in mussel, clams, and shellfish harvesting areas, and only fecal coliforms are considered as a water quality parameter (EPA, 2001; Gomez-Couso et al., 2003; Noke, 2008; Health Canada, 2019).

We suggest that there could be contamination of *C. parvum* and *B. hominis* from the treated sewage to Cholga mussel. These parasites and other fecal contamination microorganisms resistant to disinfection treatment be monitored corrective actions on the STP. They also be considered in sewage-treated discharge regulations. In future studies, we will extend sample zones, consider seasonal variation during the sampling, and as well as parasites confirmatory assays.

5. Conclusion

The presence of *C. parvum* and *B. hominis* in treated sewage and Cholga mussels suggests contamination. These results are preliminary, we suggest that might be from the discharge of the STP to the Cholga mussel harvesting area. Further investigation should be performed to verify this contamination along the Bay. Also, it is relevant to for discharged into the surface ecosystem.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2023.e00214.

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