



# Gastrointestinal Parasites and Bacteria in Free-Living South American Sea Lions (*Otaria flavescens*) in Chilean Comau Fjord and New Host Record of a *Diphyllobothrium scoticum*-Like Cestode

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Present study aimed to characterize gastrointestinal parasites and culturable bacteria from free-living South American sea lions (*Otaria flavescens*) inhabiting waters of Comau Fjord, Patagonia, Chile. Therefore, a total of 28 individual fecal samples were collected from sea lions within their natural marine habitat during several diving expeditions. Using classical parasitological techniques, study revealed infections with five different gastrointestinal parasite genera. In addition, bacterial cultures showed presence of at least 28 different bacterial genera. Referring to parasites, protozoan, and metazoan species were found with some of them bearing anthroponotic potential and/or pathogenic impact for these marine mammals. As such, four of identified parasite genera harbored zoonotic potential (i.e., *Entamoeba*, *Balantidium*, *Diphyllobothrium*, *Anisakis*) and one genus (*Parafilaroides*) represented a specific lungworm of marine pinnipeds. Proglottids from fecal samples showed high morphological homology to “*Diphyllobothrium*” *scoticum* (Rennie and Reid, 1912; Meggitt, 1924), which was found in Antarctic sea leopards (*Hydrurga leptonyx*; Phocidae), but contained eggs of smaller size. Molecular characterization revealed 97–100% identity to a new “*Diphyllobothrium*” species which was recently isolated from a Californian sea lion (*Zalophus californianus*; Otariidae) in San Francisco. As such, *O. flavescens* represents a new host record for this parasite species. Furthermore, potential zoonotic bacteria (i.e., *Clostridium*, *Escherichia*, *Vibrio*, *Yersinia*, *Salmonella*) were identified amongst others in *O. flavescens* indicating a reservoir role for these pinnipeds in marine ecosystem. Current data should be considered as a baseline study for future monitoring surveys on anthroponotic pathogens circulating in wild free-living sea lions and their possible impact on public health issues and marine wildlife.

**Keywords:** *Otaria flavescens*, *Diphyllobothrium scoticum*, *Balantidium*, *Entamoeba*, *Clostridium*

## INTRODUCTION

South American sea lions (*Otaria flavescens*) belonging to family Otariidae are common carnivorous pinnipeds living along Eastern and Western coasts of South America. They are endemic in Argentina, Peru, South Brazil, and Chile (Vaz-Ferreira, 1982; Crespo, 1988; Túnez et al., 2008; Aznar et al., 2012; Hernández-Orts et al., 2013; Pereira et al., 2013). Along Chilean coastal shores, including Southern and Northern Patagonian fjords (Haussermann et al., 2014), more than 200 colonies of free-living South American sea lions were described.

So far, abundant reports on ecology, feeding, behavior, reproduction, sightings, life history parameters, as well as population dynamics of this otariid species are available (Crespo et al., 1997, 1999; Lima and Páez, 1997; Alonso et al., 2000; Naya et al., 2002; Suarez et al., 2005; Haussermann et al., 2014). Several reports have further focused on endogenous helminth fauna and microbiota of South American sea lions, comprising single parasite and bacterial species records, taxonomy, and population studies on some of these parasitoses and bacterial diseases (Cattan et al., 1976; George-Nascimento and Carvajal, 1981; Zdzitowircki, 1986; Hernández-Orts et al., 2013). Nonetheless, scarce data have been reported on gastrointestinal protozoan parasites occurring in free-living South American sea lions within their natural marine environment (Hermosilla et al., 2016b). Same holds true for studies on marine mammal microbiota, despite their crucial ecological roles in oceanic ecosystem (Bik et al., 2016). Being apex predators, South American sea lions are likely to play a role as indicators of ocean health as reported elsewhere for other marine mammals (Hunt et al., 2013).

Although recent conservation efforts have allowed South American sea lion populations to slowly recover, they still are classified as least concern and would profit from a better understanding of acute, chronic or subclinical effects of parasitic and bacterial diseases for their long-term survival and preservation (Glad et al., 2010; Hermosilla et al., 2016b). In this context, pinniped-associated gastrointestinal microbiota (Glad et al., 2010) and parasites (Cattan et al., 1976; Hernández-Orts et al., 2013; Hermosilla et al., 2016b) are well-known to play a critical role in sea lion health, nutrition, differentiation of host tissue, reproduction, colonization resistance and adequate maturation of host innate and adaptive immune system (Bik et al., 2016). In healthy individuals, gut microbiota are robust and resistant to perturbations and maintain their composition in normal range to sustain homeostasis, symbiosis and proper immunity (Ohno, 2015).

The present study therefore aimed to investigate gastrointestinal parasite diversity and to identify culturable bacterial species in fecal samples being collected without any disturbance of animals during several diving expeditions at Comau Fjord of Northern Patagonia, Chile. Current findings expand knowledge on parasitic and bacterial diversity in these marine mammals and further discuss impacts of zoonotic pathogens on ocean and public health, since many of these pathogens most probably are also present in marine-derived

products, such as bivalves, gastropods, crustaceans, cephalopods and fishes, foreseen for human consumption.

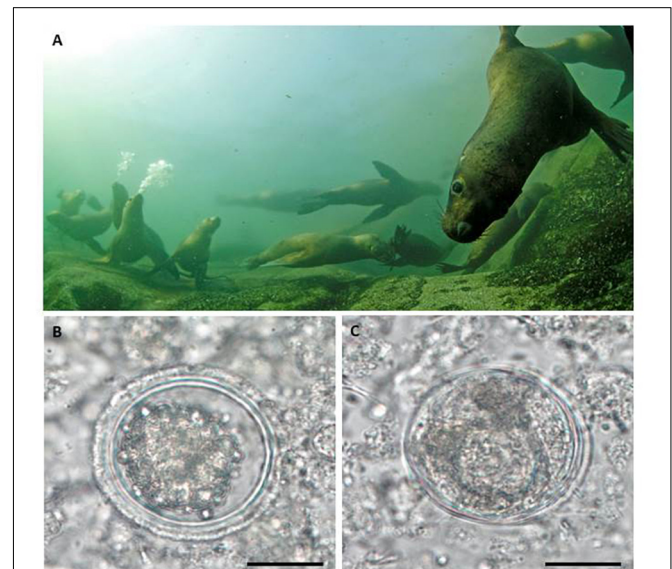
## MATERIALS AND METHODS

### Study Area, Sample Collection, Coproscopical, and Bacterial Analyses

South American sea lions (*O. flavescens*) were investigated in a colony allocated at 11th Region of Chile (Aysén) in Comau Fjord (42.39°S, 72.44°W), Northern Patagonia, Chile. The colony was composed of approximately 200 animals comprising males, females and puppies. General topography of study area was composed of rocky shores, where sea lion colony was allocated, surrounded by waters containing robust bull kelps (*Durvillaea antarctica*) as dominant seaweed in this fjord. Waters in front of colony were shallow and maximum depth in opened water was approximately 30 m.

For sample collection, boat- and underwater-based surveys during scuba dives in front of sea lion colony were conducted in summer of 2014. This unique non-invasive research methodology not only allows underwater behavioral observations of sea lions (Figure 1A) which are otherwise impossible to monitor, but additionally provides easy access to sea lions' fecal material without any animal disturbance as demonstrated recently for scat collections of free-swimming dolphins (Kleinertz et al., 2014) and large baleen whales (Hermosilla et al., 2016a; de Vos et al., 2018).

In this study, a total of 28 individual fecal samples were collected during underwater survey activities. In total 7 scuba dives were performed (with durations of 15–20 min each) to



**FIGURE 1 | (A)** Scat collection of South American sea lions (*Otaria flavescens*) ( $n = 28$ ) was performed by scuba dives under water in front of sea lion colony of Comau Fjord, Patagonia, Chile, without disturbance of animals. **(B)** Illustration of an *Anisakis* spp. egg and **(C)** a *Balandium* spp. cyst (scale bar 20  $\mu\text{m}$ ) identified in sea lion fecal samples.

collect all scat samples. Whenever sea lion defecation occurred, scat samples were immediately collected in 15 ml plastic tubes (Falcon). Thereafter, a fecal swap was taken and transferred into special tubes containing Amies transport medium with charcoal (Nerbe, Winsen, Germany) for bacterial cultivation (Hermosilla et al., 2018) and rest of scat material was fixed in 70% ethanol for further parasitological diagnosis. All swap- as well as fecal-samples were stored at 4°C until further diagnosis within the vessel of Huinay Scientific Field Station (Chile) responsible for sea lion scat collection and thereafter transferred to Germany. Spontaneously shed helminth stages were collected during scuba dive episodes and fixed in 70% ethanol. Scat samples as well as helminth specimens were then transferred to Huinay Scientific Field Station, Puerto Montt, Chile, and stored at 4°C until further parasitic and bacteriological analyses. Parasitological as well as bacteriological analyses were conducted at the Institute of Parasitology and at the Institute for Hygiene and Infectious Diseases of Animals, respectively, both institutions belonging to Faculty of Veterinary Medicine of the Justus Liebig University Giessen, Giessen, Germany.

Coprocopical parasitic analyses included standard sodium acetate acetic acid formalin (SAF) technique according to Yang and Scholten (1977) for detection of parasite eggs, cysts, sporocysts, and oocysts within fecal samples as described elsewhere (Hermosilla et al., 2013). In addition, carbol fuchsin-stained faecal smears (CFS) were carried out for the detection of *Cryptosporidium* spp. oocysts according to Heine (1982). Moreover, a commercially available coproantigen ELISA (ProSpecT®, Oxoid) was performed for detection of *Giardia*- and *Cryptosporidium*-antigens in fecal samples. Parasitological identification of helminth species, cysts, oocysts, and eggs was based on morphological criteria referring to previous reports (Anderson, 1992; Kleinertz et al., 2014; Hermosilla et al., 2016a,b).

For bacterial analyses, fecal samples were streaked on blood agar containing 5% defibrinated sheep blood (E. Merck, Darmstadt, Germany) and water-blue metachrome-yellow lactose agar (acc. to Gassner, E. Merck). Plates were incubated at 37°C and analyzed after 24 and 48 h. Colonies were sub-cultured and pure cultures were identified using standard morphological and biochemical methods as well as with MALDI-TOF mass spectrometry using the Microflex LT/SH® instrument according to the manufacturer's instructions (Bruker Daltonics, Bremen, Germany) (Bisping and Amtsberg, 1988; Burkhardt, 1992; Carter et al., 1995). For *Clostridium* (*C.*) *perfringens* detection, samples were streaked on Zeissler agar (E. Merck, Darmstadt, Germany) and incubated at 37°C overnight under anaerobic conditions in a jar using AnaeroGen™ gas sachets (Oxoid, Wesel, Germany). After streaking on agar plates, fecal material was placed in Müller-Kauffmann Novobiocin Tetrathionate broth (E. Merck) with iodine as well as in Rappaport-Vassiliadis enrichment broth (Oxoid) which was incubated at 37 and 43°C, respectively. Ten µl of each broth was cultured on water-blue metachrome-yellow lactose agar as well as on Brilliance *Salmonella* agar containing *Salmonella* Selective Supplement (Oxoid, Wesel) after 24 and 48 h of broth incubation. Suspect *Salmonella* colonies were sub-cultured on water-blue metachrome-yellow lactose agar

and analyzed after 24 h of incubation by MALDI-TOF mass spectrometry. *Salmonella* serovars determination was performed at the National Reference Laboratory for the Analysis and Testing of Zoonosis (*Salmonella*) at the German Federal Institute of Risk Assessment (Berlin, Germany).

All fecal sampling procedures were conducted in accordance to Institutional Ethic Commissions of Huinay Scientific Field Station (Chile) and of Justus Liebig University Giessen (Germany), and in accordance to current Chilean Animal Protection Laws.

## Morphological Analyses and Staining of Cestodes

Two cestode strobilas – one collected from a rock of seal colony and another retrieved from a fecal sample (sea lion ID F07) – were investigated microscopically for further species identification. Strobilas preserved and shipped in 70% ethanol were hydrated for 1 day in water at 4°C with several changes. Complete fragments, sagittal, median sagittal and transverse sections of strobila were examined using stereomicroscopy (Leica) and were documented by a digital camera and CellSens® software (both Olympus). Some strobila fragments and thick transverse sections – cut with a razor blade – were stained with iron acetocarmine (Merck) following protocol of Georgiev et al. (1986) and mounted in DePeX. For a detailed analysis of reproductive organs, strobila fragments (1 cm) were fixed in 4% formaldehyde, embedded in paraplast and 10 µm sagittal and transverse sections were stained with hematoxylin and eosin (HE). HE staining was performed with Harris hematoxylin (Thermo Fisher Scientific) and 0.25% Eosin Y (Merck) in 80% ethanol following a xylene-free protocol which uses 1.7% dish washer solution at 90°C as deparaffinizing agent and over-drying at 60°C instead of dehydration (Negi et al., 2013). Microscopic measurements were performed using a digital camera and CellSens® software (Olympus).

For Diphyllbothriidea species identification, keys and checklists (Dailey, 1975; Delyamure et al., 1985; Felix, 2013; Hernandez-Orts et al., 2015; Kuchta and Scholz, 2017), expedition reports on cestode collections from pinnipeds (Fuhrmann, 1921; Markowski, 1952b; Yurakhno and Maltsev, 1997) and primary literature on individual *Diphyllbothrium* species from seals (Yurakhno and Maltsev, 1994; Hernández-Orts et al., 2015) were reviewed. Species classification follows most recent revision of Diphyllbothriidae family with polyphyletic *Diphyllbothrium* species considered *incertae sedis* indicated by inverted commas (Waeschenbach et al., 2017).

## Molecular Analyses of Cestodes

To identify sea lion cestodes sequence data of ribosomal DNA region – 18S, ITS1, 5.8S, ITS2, partial 28S – and complete mitochondrial cytochrome oxidase subunit 1 gene (*cox1*) were generated and compared to GenBank database entries. Genomic DNA was isolated from cestode proglottids using DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The rDNA region was amplified using primer combinations NF1 (Porzinska et al., 2009)/D3A (Nunn, 1992) and ZX-1 (Waeschenbach et al., 2007)/L2230 (Lockyer et al., 2003). The

*cox1* gene was amplified using following primer combinations: Cox1F/Cox1R (Wicht et al., 2010), COI Dice1F/Dice11R (Van Steenkiste et al., 2015), and Diphyllnad/Diphyllrrnl (Mercado et al., 2010). PCRs were performed in 50  $\mu$ l reactions using HOT FIREPol Blend Master Mix 7.5 mM MgCl<sub>2</sub> (Solis BioDyne, Tartu, Estonia), 200 nM of forward and reverse primers each, and 100 ng of worm DNA under the following conditions: 15 min 95°C initial denaturation, 35 cycles 20 s 95°C, 30 s 54°C, 2.5 min 72°C and 5 min 72°C final extension. For amplification of partial *cox1* with primers Dice1F and Dice11R a touchdown-protocol was performed (Van Steenkiste et al., 2015). Amplicons were gel-purified, cloned, and sequenced by an external service provider (LGC Genomics GmbH, Berlin, Germany). Complete sequences of rDNA and mitochondrial region were assembled from overlapping amplicons and analyzed by BLAST search against GenBank database. Sequences are available under accession numbers KY945917 (rDNA, gravid proglottid), KY945922 (*cox1*, gravid proglottid) and MF893274 (*cox1*, immature proglottid fecal sample F07), respectively.

For phylogenetic analysis, a BLAST search in GenBank database was conducted with amplified sequences and a dataset of high-scoring Diphyllbothriidae taxa was chosen. For each taxon, homologous sequences of 3'-region of 18S rDNA and 5'-region (D2-D3) of 28S rDNA were concatenated and aligned by MAFFT 7 (Katoh and Standley, 2013). Only those taxa were considered with both sequence regions available from GenBank (Supplementary Table S1). Aligned sequences corresponded to nucleotides 1451-1650 (3' 18S) and nucleotides 3851-4700 (5' 28S D2-D3) of rDNA sequence from gravid proglottid (KY945917). A phylogenetic tree was constructed using Bayesian analysis (MrBayes 3.2) and TreeDyn from Phylogeny.fr website (Dereeper et al., 2008).

## RESULTS

### Parasite Infections

Parasitological analyses of South American sea lion fecal samples revealed five different parasite taxa comprising two protozoan and three metazoan taxa. Metazoan parasites consisted of cestodes (one species) and nematodes (two species). Neither acanthocephalan nor trematode eggs were detected in current survey. A list with parasite stages and their respective prevalence is represented in Table 1. Overall, most prevalent metazoan parasitic stages found in sea lion colony were eggs of Diphyllbothriidae gen. sp. (44.8%), followed by eggs of *Anisakis* spp. (34.5%; Figure 1B) and at much lower prevalence *Parafilaroides* spp. larvae (3.4%). Most prevalent protozoan parasitic stages found in sea lion samples were cysts of *Balantidium* spp. (13.8%; Figure 1C) followed by cysts of *Entamoeba* spp. (3.4%). Neither oocysts/antigens of *Cryptosporidium* nor cysts/antigens of *Giardia* were found. Referring to parasite genus level, these parasitological findings include one new host records, i.e., *Entamoeba*, for *O. flavescens*.

Important to note is that some of gastrointestinal endoparasites detected in South American sea lions bear

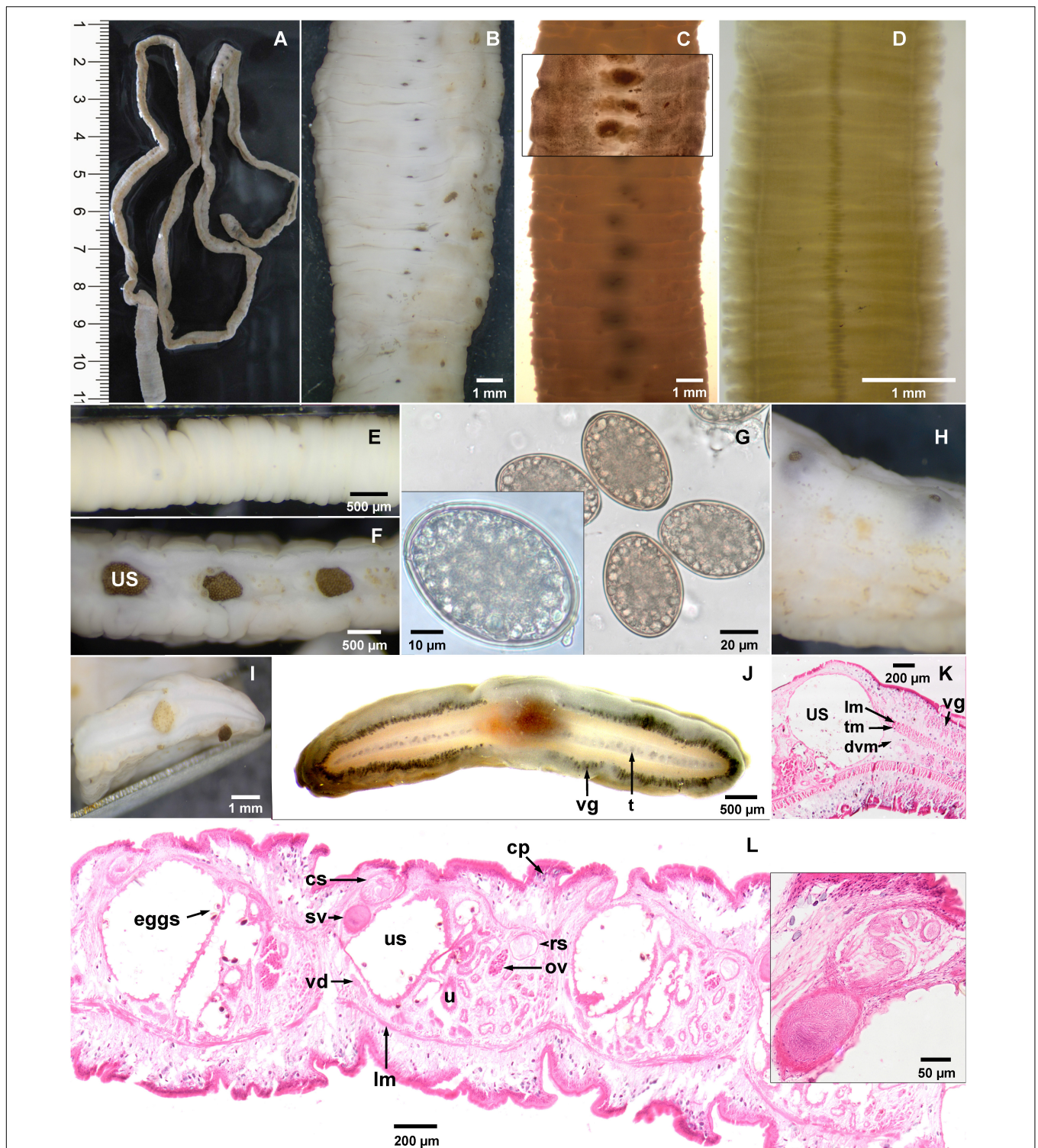
**TABLE 1** | Gastrointestinal parasites identified in South American sea lion (*Otaria flavescens*) fecal samples with respective percentages.

		Stage	Percentage (number of animals)
Protozoan	<i>Entamoeba</i> spp.	Cysts	3.4 (1)
	<i>Balantidium</i> spp.	Cysts	13.8 (4)
Metazoan	<i>Anisakis</i> spp.	Eggs	34.5 (10)
	Diphyllbothriidae gen. sp.	Eggs	44.8 (13)
	<i>Parafilaroides</i> spp.	Larvae	3.4 (1)

anthropozoonotic potential, such as *Balantidium*, *Entamoeba*, Diphyllbothriidae (*Adenocephalus*) and *Anisakis*.

### Morphology of “*Diphyllbothrium*” Proglottids

Two scolex-less “*Diphyllbothrium*” strobilas, a large one (length 50 cm) with gravid proglottids from a rock near colony (Figure 2A) and a short one with immature proglottids isolated from a fecal sample (Figure 2D) were collected. Sequencing *cox1* gene of both strobilas confirmed that these belong to same species and proved that large strobila also originated from sea lions. Both strobilas were segmented, slightly craspedote (Figure 2C) with proglottids wider than long. Gravid proglottids had a mean width of 7233  $\mu$ m and a length of 1037  $\mu$ m (min-max: 7077–7418  $\mu$ m  $\times$  825–1385  $\mu$ m;  $n = 11$ ); immature proglottid was much smaller with a mean width of 2500  $\mu$ m. Gravid strobila was fleshy thick (1.5 mm; Figures 2E,I; Immature: Figure 2E) with a striking bulged, pocket-like appearance on ventral surface around uterine openings (Figure 2H). As shown in unstained longitudinal sections, these pockets correspond to the large distal uterine sac, densely packed with hundreds of eggs (Figure 2F). Eggs liberated from uterus were unembryonated, oval, thick-walled, operculated and with a knob on opposite pole (Figure 2G). Their mean size was 57.4  $\mu$ m  $\times$  40.9  $\mu$ m (min-max: 54.1–61.3  $\mu$ m  $\times$  38.5–44.1;  $n = 22$ ). Uterine openings were slit-like and situated in a transverse groove, irregularly alternating lateral to midline of strobila (Figures 2B,C). Vitelline glands (max. size 175  $\mu$ m  $\times$  30  $\mu$ m) were seen in cortical, lateral parts of proglottids in iron acetocarmine stained strobila fragments and thick transverse sections, leaving a free space around median region of uterus openings and genital pores (Figures 2C,J). Numerous calcareous corpuscles (18  $\mu$ m  $\times$  9  $\mu$ m, pale violet stained) were seen in dorsal and ventral cortical parenchyma (Figure 2L). Testes were located between well-developed (100  $\mu$ m thick) dorsal and ventral longitudinal muscles (Figure 2K) arranged in a single layer with 15–20 testes seen in transverse sections on each side of segment (Figure 2J). The ovary in posterior part of proglottid was followed by a tube-like uterus, which made 5–6 loops on each side (Figure 2C) terminating in a large uterine sac lined with villous tissue (Figure 2L). In HE-stained sagittal sections, eggs have been lost during processing but a few numbers of bile-colored eggs and their pattern in villous tissue



**FIGURE 2 |** Morphology of *Diphylobothrium scoticum*-like strobila collected from South American sea lions (*Otaria flavescens*) of Comau Fjord, Chile. **(A–C, F–L)** gravid strobila, **(D, E)** immature strobila. **(B–D)** Ventral view of gravid **(B, C)** and immature strobila **(D)** showing variation in size and shape and irregularly alternating uterine openings. **(E)** Lateral (immature strobila) and **(F)** median sagittal view. **(G)** Eggs with operculum and knob. **(H)** Ventral view of species-specific pocket-like terminal uterine sac. **(I, J)** Transverse thick sections: **(J)** showing 15–18 testes in single layer on each side and vitelline glands (iron acetocarmine staining). **(K)** Transverse section showing vitelline glands and muscle layers (HE staining). **(L)** Median sagittal section showing male and female genital organs; eggs in uterine sac were lost during processing. lm, tm, dvm longitudinal, transverse, dorso-ventral muscles; cp, calcareous particles; t, testes; vd, vas deferens; sv, seminal vesicle; cs, cirrus sac; ov, ovary; rs, receptaculum seminis; u, uterus; us, uterine sac; vg, vitelline glands.

were still seen. The *receptaculum seminis* seen in median longitudinal sections was filled with sperms (Figure 2L). Male genital organs were located anterior from uterine sac. The *vas deferens* led to a muscular seminal vesicle completely filled with sperms followed by cirrus-sac with coiled cirrus (Figure 2L). Size and shape of genital apparatus was of taxonomic value according to Markowski (1952b). Cirrus-sac wall appeared less muscular than wall of seminal vesicle. In all median sagittal sections, cirrus-sac was longer than seminal vesicle.

However, two differences of present specimens compared to *D. scoticum* are obvious. Firstly eggs obtained from gravid proglottids from *O. flavescens* are considerably smaller, and secondly cirrus sac and seminal vesicle have a reverse size ratio (Table 2). For further detailed characterization we performed additionally molecular analysis.

### Molecular and Phylogenetic Analyses of “*Diphyllbothrium*” *Scoticum*-Like Strobilas

In order to confirm species identity of two strobilas, we amplified and compared a fragment of mitochondrial genome from both specimens including complete *cox1*- and partial *nad3*- and *16S*-sequences. Complete amplicons were 3017 bp for both strobilas with an identity of 99.4% (2999/3017 bp) –

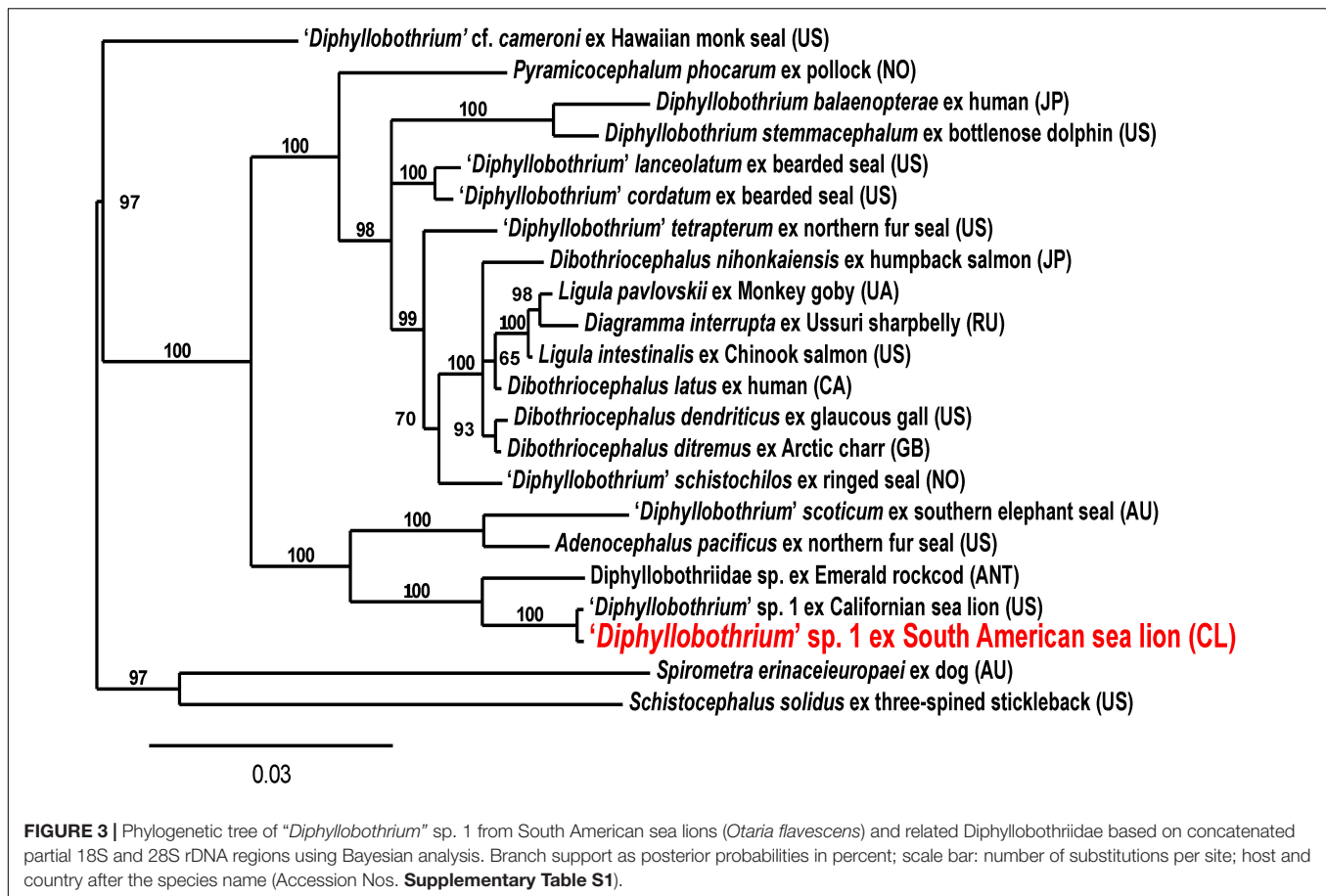
clearly beyond the threshold of 9% determined for *cox1* in delimitation of platyhelminth species (Hebert et al., 2003; Vilas et al., 2005) – confirming that both were haplotypes of same species. A BLAST search of this sequence toward GenBank resulted in a closely related sequence (KY552890) of 97% identity (1519/1566 bp) belonging to an unidentified *Diphyllbothrium* sp. 1 from a California sea lion (Waeschenbach et al., 2017), whereas sequences from other morphologically identified diphyllbothriidean species exhibited a lower homology of 89% identity or less (e.g., *Dib. dentriticus* KY552847: 89%, 841/949 bp; *Dib. latus* AB269325: 86%, 2586/3022 bp; *A. pacificus* KR269744: 83%, 1295/1566 bp). Thus, the two “*Diphyllbothrium*” *scoticum*-like strobilas found were supposed to be conspecific with *Diphyllbothrium* sp. 1.

For further molecular analysis we amplified rDNA region – 18S, ITS1, 5.8S, ITS2 and partial 28S – from the gravid strobila; 5994 bp. The 18S sequence was 100% identical with the Californian isolate (KY552794; 1994 bp) and partial 28S sequence was almost identical having only a 4 bp deletion (KY552829; 1481/1485 bp). This confirms supposed species identity based on *cox1* sequence, which showed more variation (97% identity) because mitochondrial genes evolve faster than nuclear ones. In conclusion, “*D.*” *scoticum*-like strobilas from Chilean South American sea lions were identified as “*Diphyllbothrium*” sp. 1.

To investigate taxonomic position and relationship to other Diphyllbothriidae genera and species a concatenated

**TABLE 2 |** Morphometric data of “*Diphyllbothrium*” sp. 1 from *Otaria flavescens* compared to “*Dip*”. *scoticum* from *H. leptonyx* based on literature [µm; (mean)].

Identified as	“ <i>Diphyllbothrium</i> ” sp. 1 n = 1	<i>Dibothriocephalus</i> <i>scoticus</i> n = 1	<i>Diphyllbothrium</i> <i>scoticum</i> n = ?	<i>Diphyllbothrium</i> <i>scoticum</i> n = 21
Source	Present study	Rennie & Reid 1912 Fuhrmann, 1921	Markowski 1952	Yurakhno 1994, 1997
Host	<i>O. flavescens</i>	<i>H. leptonyx</i>	<i>H. leptonyx</i>	<i>H. leptonyx</i>
Location	Comau Fjord Chile	South Orkney Isl. Antarctica	Graham Land Antarctica	Balleny Isl. Antarctica
<b>Strobila</b>				
length	>500 mm	133–290 mm	520–1300 mm	56–420 (231) mm
width	2.5 mm (immature) 7 mm (gravid)	2.5–6.8 mm	5–18 mm	3.6–11.5 (6.4) mm
<b>Longitudinal Muscle Layer (lm)</b>				
Thickness	90–100	70–120	150	58–147 (99)
Uterine-sac (us)	+	+	+	+
Egg size	54–61 × 39–44	70–80 × 44–48	76–79 × 56	68–76 × 50–53
<b>Testes (t) In Transverse Section</b>				
Diameter	150	80–100	150	143
No. each side	15–20	18–25	14–15	12–19
<b>Seminal Vesicle (sv)</b>				
Length	160–250	–	285	182–374 (259)
Width	100–160	–	180	100–224 (137)
Wall thickness	6–14	–	17	–
<b>Cirrus-Sac (cs)</b>				
Length	203–340	200	231	154–274 (224)
Width	125–200	120	142	66–108 (84)
Wall thickness	3–8			



sequence of 18S 3'-region and 28S 5'-region was used for phylogenetic analysis. In inferred phylogenetic tree (Figure 3) the “*Diphyllobothrium*” sp. 1 species clusters with *A. pacificus*, “*D.*” *scoticum* collected from a Southern elephant seal (*Mirounga leonina*) of Macquarie island (island between Tasmania and Antarctica) and another unidentified *Diphyllobothriidae* gen. n. sp. of a plerocercoid from an Antarctic fish (*Trematomus bernacchii*). The latter two and “*D.*” *scoticum* were new sequences recently added to GenBank (Waeschenbach et al., 2017) for which no morphological descriptions were reported at time of writing this manuscript.

## Bacterial Findings

Bacteriological analyses of fecal samples of South American sea lions revealed a wide bacterial diversity. A complete list with bacterial species and their respective frequencies is illustrated in Table 3. At least 45 different bacterial species could be cultured from sea lion samples with *Escherichia coli* (82.1%) and *Clostridium perfringens* (64.3%) being most predominant ones. Of six detected *Salmonella* (S.) isolates, four were identified as serotype S. Cerro and two as S. Pensacola. To authors knowledge no report exists, so far, on serotype Pensacola in marine mammal sources. Furthermore, *Yersinia enterocolitica* could be detected in one fecal sample in this study.

## DISCUSSION

The usefulness of current applied non-invasive collection method, which clearly left free-swimming sea lions unmolested in their natural environment, was reinforced (Hermsilla et al., 2015; de Vos et al., 2018). Overall, five different parasite taxa were here found in individual sea lion samples thereby covering a rather narrow range of parasites when compared to other studies (Hernández-Orts et al., 2013; Hermsilla et al., 2016b). Eggs of Diphyllobothriidae gen. sp. showed highest prevalence with 44.8%. Based on present analyses of cestode strobilas, these eggs most probably belong to “*Diphyllobothrium*” sp. 1 and *A. pacificus* – the latter being quite abundant in South American sea lions (Torres et al., 2012; Hernández-Orts et al., 2013, 2015). So far, three diphyllobothriidean tapeworms were reported in Chile: two freshwater species [*Dibothriocephalus latus* (syn. *Diphyllobothrium latum*) and *Dib. dendriticus* (syn. *Dip. dendriticum*)] and one marine species [i.e., *Adenocephalus pacificus* (syn. *Dip. pacificum*)] (Torres et al., 2012) originating from stranded sea lions from Patagonia (Hernández-Orts et al., 2013) and Valdivia (Hermsilla et al., 2016b) but at lower prevalences of 26.8 and 13%, respectively.

Several morphological features used to differentiate diphyllobothriidean species are intraspecific variables, which necessitate molecular characterization for species verification

**TABLE 3 |** Bacterial species isolated from South American sea lion (*Otaria flavescens*) samples with respective percentages.

Isolate or group	percentage (number of animals)
<i>Achromobacter</i> spp.	10.7(3)
<i>Acinetobacter</i> spp.	7.1(2)
<i>Aeromonas</i> spp.	35.7(10)
<i>Alcaligenes faecalis</i>	7.1(2)
<i>Arthrobacter</i> <i>protophormiae</i> /spp.	10.7(3)
<i>Buttiauxella</i> spp.	3.6(1)
<i>Citrobacter brakii</i> /freundli group/ <i>gillenii</i> / <i>koser</i> /spp.	32.1(9)
<i>Clostridium perfringens</i> /spp.	67.9(19)
<i>Comamonas</i> spp.	3.6(1)
<i>E. coli</i> / <i>E. coli</i> var. <i>haemolytica</i>	82.1(23)
<i>Edwardsiella tarda</i>	32.1(9)
<i>Enterobacter</i> <i>aerogenes</i> / <i>amnigenus</i> / <i>asburiae</i> / <i>cloacae</i> /spp.	53.6(15)
<i>Enterococcus</i> <i>aquamarinus</i> / <i>faecalis</i> /spp.	21.4(6)
<i>Escherichia hermannii</i>	3.6(1)
<i>Hafnia alvei</i>	7.1(2)
<i>Klebsiella pneumoniae</i>	10.7(3)
<i>Kluyvera</i> spp.	10.7(3)
<i>Moraxella</i> spp.	50.0(14)
<i>Neisseria</i> spp.	3.6(1)
<i>Proteus mirabilis</i> / <i>vulgaris</i> /spp.	14.3(4)
<i>Pseudochrobactrum</i> <i>asaccharolyticum</i>	3.6(1)
<i>Pseudomonas</i> spp.	57.1(16)
<i>Raoutella</i> <i>ornithinolytica</i> / <i>planticola</i> / <i>terrigena</i> /spp.	35.7(10)
<i>Salmonella</i> spp.	21.4(6)
<i>Serratia fonticola</i>	3.6(1)
<i>Vagococcus fluvialis</i>	7.1(2)
<i>Vibrio</i> spp.	3.6(1)
<i>Yersinia enterocolitica</i>	3.6(1)
$\alpha$ -haem. <i>Streptococci</i>	39.3(11)
$\gamma$ -haem. <i>Streptococci</i>	10.7(3)

as discussed for *A. pacificus* by Hernández-Orts et al. (2015). Surprisingly, morphology of strobilas from *O. flavescens* was almost identical to “*Dip.*” *scoticum* previously only identified in sea leopards of Antarctica. “*Dip.*” *scoticum* is characterized by a species-specific uterine sac also present in species from *O. flavescens*. The only obvious differences were the smaller size of eggs and size proportion of cirrus sac versus seminal vesicle. Diphyllbothriid species egg size was previously shown to be intraspecific and host-related highly variable (Hernández-Orts et al., 2015; Leštínová et al., 2016; Yamasaki et al., 2016). However, maximum length of eggs from “*Diphyllbothrium*” sp. 1 (54–61  $\mu$ m) was clearly shorter than minimal length of “*Dip.*” *scoticum* eggs (68–79  $\mu$ m). Suspected findings of *D. scoticum* outside of Antarctica were reported, interestingly in *O. flavescens* [Baylis and Hamilton (1934) and Markowski (1952a) from

Falkland islands; Hernández-Orts et al. (2011); Hernández-Orts et al. (2013) from Argentina Northern Patagonia], but later these cases were identified as *A. pacificus* or remained undefined (Baer et al., 1967; Hernández-Orts et al., 2015). In order to exclude misidentification we also performed a molecular characterization of two proglottids. During preparation of this publication a sequence from a diphyllbothriidean species of *Zalophus californianus* from San Francisco was published by Waeschenbach et al. (2017) with 100% identity to present isolates, but apart from scolex no digitals/drawings were included. Additionally, a sequence assigned to “*Dip.*” *scoticum* was published by same authors and a second new species not assigned to a genus. However, unexpected to our morphological findings, sequence identity to “*Dip.*” *scoticum* sequence was not higher than to other diphyllbothriids (cox1: 84%, 462/551; KY552883); but were more closely related in phylogenetic analysis. According to authors, “*Dip.*” *scoticum* was collected from a Southern elephant seal (*M. leonina*) of sub-antarctic Macquarie island (Tasmania, Australia) (Johnston, 1937). More importantly, diphyllbothriasis still represents a neglected fish-borne zoonosis not only in Chile (Mercado et al., 2010; Torres et al., 2012) but also worldwide (Curtis and Bylund, 1991; Chai et al., 2005; Scholz et al., 2009; Kuchta et al., 2015). Humans become infected by ingestion of raw or undercooked fish carrying plerocercoid-stages of *Diphyllbothrium*. In summary, morphology of proglottids of present “*Diphyllbothrium*” species were most consistent with descriptions of *D. scoticum*. This Antarctic cestode species was firstly collected by Rennie and Reid (1912) during Scotia Scottish National Antarctic expedition (1902–1904) and described as *Dibothriocephalus scoticus* from leopard seals (*Hydrurga leptonyx*) and later renamed as *Diphyllbothrium scoticum* by Meggitt (1924). Most detailed descriptions are given by Fuhrmann (1921) from collections of Scotia, Markowski (1952b) from several worms found in four leopard seals of British Graham Land expedition (1934–37) and Yurakhno and Maltsev (1994) with data summarized from 21 specimens from leopard seals of Balleny island. Johnston (1937) also found these cestodes in sea leopards in the Aurora collection of the Australasian Antarctic expedition (1911–14) but did not provide a further description. Uterine sac is a typical species-specific feature of this species but larger than in species *D. lobodoni* from crabeater seal (*Lobodon carcinophagus*) of the Pacific region of Antarctica.

Anisakidae gen. sp. eggs were also found at a high prevalence of 34.5%. Based on a reported high frequency of occurrence in South American sea lions (George-Nascimento and Carvajal, 1980; Hernández-Orts et al., 2013), these eggs most probably originated from the anthropozoonotic ascarid nematodes *Contracaecum*, *Pseudoterranova*, or *Anisakis*. These marine ascarids usually parasitize stomach and small intestine, either freely within the gastrointestinal lumen or firmly attached to gut mucosa thereby inducing focal inflammation (Young and Lowe, 1969; Geraci and St Aubin, 1987; Hermosilla et al., 2016b). Occasionally, deep mucosal penetration by larval or adult stages can lead to severe ulcers, gastritis, enteritis and even intestinal wall perforation (Young and Lowe, 1969; McClelland, 1980; Geraci and St Aubin, 1987). In humans, allergic reactions



against *Anisakis simplex* major allergen were recently reported (Martinez-Aranguren et al., 2014; Garcia Alonso et al., 2015).

Third most prevalent parasite found in sea lions was *Balantidium* (13.8%). Within genus *Balantidium*, *B. coli* is the only species nowadays considered as pathogenic for diverse terrestrial mammals including humans (Ponce-Gordo et al., 2011; Hassell et al., 2013; Yin et al., 2015; Zanzani et al., 2016). *Balantidium* infections were recently recorded in free-swimming fin whales from Azores Archipelago, Portugal (Hermosilla et al., 2016a), and in wild South American sea lions in the city of Valdivia, Chile (Hermosilla et al., 2016b), indicating that *Balantidium* is most probably circulating in different oceanographic areas. Interestingly, *B. coli* infections were also reported in Chilean humans and pigs (Palomino and Donckaster, 1971; Letonja et al., 1975).

Referring to current bacterial findings, *Mycobacterium pinnipedii* (tuberculosis), *Edwardsiella tarda* and *Clostridium* sp. infections were already demonstrated to circulate in wild populations of South American sea lions (Konagaya et al., 2006; de Amorim et al., 2014; Fernandez et al., 2014). *Clostridium* spp., especially *C. perfringens*, was one of most abundant bacteria isolated in this study. Clostridiales were identified as part of microbiota of different marine mammal species before assuming that they may be involved in chitin degradation (Konagaya et al., 2006; Greig et al., 2014; Delpont et al., 2016; Medeiros et al., 2016; Soverini et al., 2016). *C. perfringens* is also regarded as a common microorganism in marine environments (Miller et al., 2006). Overall, high abundance of Clostridiales might be due to environmental and dietary consistence. However, different case reports also indicate their pathogenic potential in marine mammals. As such, *C. perfringens*-septicemia in a stranded common dolphin in California (Danil et al., 2014), *C. perfringens*-induced colonic rupture in a captive Californian sea lion (Van Bonn, 1995) and cases of myositis and skin lesions in different marine mammals (Greenwood and Taylor, 1978; Buck et al., 1987; Lang et al., 2014) were documented. Furthermore, the potential of Clostridiales to causing severe wound infections in humans is also well known (Stevens et al., 2012).

*Edwardsiella tarda*, which was detected in 32.1% of current samples, was described as a constituent of marine mammal microbiota (González-Fuentes et al., 2010). There is no data on its pathogenic potential for marine mammals, but *E. tarda* worldwide is an economically important fish pathogen (Park et al., 2012). As zoonotic opportunistic pathogen, it might causes fatal human water- or food-borne infections leading to gastroenteritis, endocarditis, empyema, hepatobiliary infections, peritonitis, intra-abdominal abscesses, osteomyelitis, wound infections, meningitis, or bacteremia (Hirai et al., 2015).

In line to other marine mammal species (Buck et al., 2006; Lockwood et al., 2006; Schaefer et al., 2009; Greig et al., 2014; Delpont et al., 2016), most abundant bacterial species in this study was *E. coli*. This bacteria forms part of physiological intestinal microbiota (Greig et al., 2014) but is also described as an opportunistic pathogen causing septicemia (Steiger et al., 1989; Higgins, 2000; Carrasco et al., 2011) and wound or umbilical infections (Lockwood et al., 2006; Lang et al., 2014). Pathogenic *E. coli* O157:H7 serotypes were not yet detected in marine

mammals but opportunistic water-borne human infections have to be considered.

As zoonotic pathogens, *Salmonella* serotypes (serotype Cerro and Pensacola) were isolated from six animals (21.4%) in this study. Several *Salmonella* serotypes from a variety of marine mammal species were described before (Gilmartin et al., 1979; Thornton et al., 1998; Fenwick et al., 2004; Stoddard et al., 2008; Davison N. et al., 2010; Davison N. J. et al., 2010; Carrasco et al., 2011; Berardi et al., 2014; Baily et al., 2016), but serotype S. Cerro was only once reported in sea lions from New Zealand (Fenwick et al., 2004). Whilst serotype S. Cerro is frequently isolated from dairy cows, serotype Pensacola is infrequently described in human samples (Edwards et al., 1948). Past studies revealed that cross infections between marine and terrestrial mammals might occur, especially based on pinniped amphibious lifestyle in contrast to cetaceans. Thus, detection of same serotypes in humans and pinnipeds might result from shared coastal environments. Due to public health concerns, incidence of this pathogen should be monitored in future.

Another microorganism of zoonotic risk, *Yersinia enterocolitica*, was only detected in one animal. Infrequent isolation of this pathogen was also described in dolphins (Buck et al., 2006). Human infections are usually food-borne causing a wide range of clinical symptoms, commonly gastrointestinal disorders (Bancerz-Kisiel and Szweida, 2015). The marine bacteria *Vibrio* spp. were as well isolated from one animal in current study. Most members of this genus are non-pathogenic but a few species are able to cause wound infections or gastrointestinal disease in humans (Colwell, 2006). In line to other studies (Higgins, 2000; Buck et al., 2006; Lockwood et al., 2006; Morris et al., 2011), *Pseudomonas* spp. (other than *P. aeruginosa*) were also here detected. However, to reveal pathogenic potential of *Y. enterocolitica*-, *Vibrio* spp.-, and *Pseudomonas* spp.-isolates, further analyses on species identification, serotyping and molecular detection of virulence genes are necessary. Similarly, further investigations are required to reveal importance of all described bacteria in health status of marine mammals.

## CONCLUSION

In conclusion, this survey adds data on new anthropozoonotic parasite and bacterial records to wild South American sea lions of Patagonia, Chile, and calls for more integrated research to avoid exposure of humans and pinnipeds to these circulating invasive pathogens. Regular monitoring programs are required to identify variations in incidence or prevalence of pathogens, in order to detect on time emergent diseases before observing consequences at population level. It will be of particular importance not only to include national authorities for public health issues but also biologists/ecologists responsible for preservation of these threatened marine mammal species in South America.

## AUTHOR CONTRIBUTIONS

CH, EP-B, CE, SP, and AT designed the project, coordinated and planned field studies. VH and GF collected sea lion

scat samples in Patagonia, Chile. CH, JH, LS, SS, and EP-B carried out parasitological- and bacteriological-analyses. JH performed molecular characterization and phylogeny analysis of *Diphyllobothrium* sp. 1. CH, JH, LS, EP-B, CE, and AT prepared the manuscript. All authors reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00459/full#supplementary-material>

**TABLE S1** | Data set of 18S and 28S sequences used for phylogenetic analysis (if not indicated, sequences refer to Waeschenbach et al., 2017).

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