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Streptococcus phocae, AN EMERGING PATHOGEN FOR SALMONID
CULTURE

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29 **Abstract**

30

31 This work describes the characterization of the causal agent of disease outbreaks that,
32 from 1999, occurred repeatedly during the summer months (temperatures higher than
33 15°C) in Atlantic salmon (*Salmo salar*) cage-farmed in Chile affecting both smolts
34 and adult fish cultured in estuary and marine waters, reaching in some occasions a
35 cumulative mortality up to 25% of the affected population. Diseased fish showed
36 exophthalmia with accumulation of purulent and haemorrhagic fluid around eyes, and
37 ventral petechial haemorrhages. At necropsy, haemorrhage in the abdominal fat,
38 pericarditis, and enlarged liver, spleen and kidney are common pathological changes.
39 Gram-stained smears revealed the presence of Gram-positive cocci, α -hemolytic,
40 negative for oxidase and catalase tests. Although biochemical characterization of the
41 isolates using the miniaturized system rapid ID 32 Strep suggested their assignation to
42 genus *Gemella*, sequencing and RFLP analysis of the 16S rRNA revealed that
43 bacteria associated with the mortalities belong to *Streptococcus phocae*. Serological
44 studies demonstrated that all the salmon isolates are antigenically homogeneous,
45 which can facilitate the development of preventive measures and, although sharing
46 some antigenical determinants, they belong to a different Lancefield group than the
47 type strain isolated from seals. On the basis of these facts, we conclude that the
48 species *S. phocae* is an emerging pathogen for salmonid culture in Chile, and it should
49 be included as a new member of the warm water streptococcosis.

50

51 **Key words:** *Streptococcus phocae*, Atlantic salmon, Emerging pathogen

Introduction

Fish streptococcosis, is currently considered as one of the main limiting factors in the aquaculture industry, due to the significant economic losses (higher than \$ 150 million) that these infections cause in different cultured fresh- and seawater fish species worldwide (Austin and Austin, 2007; Ringø and Gatesoupe, 1998; Romalde and Toranzo, 2005). The first description of a streptococcal infection causing rainbow trout (*Oncorhynchus mykiss*) mortalities dates from 1956 (Hoshina *et al.*, 1958). Since then, the disease has increased its host range, including yellowtail (*Seriola quinqueradiata*), salmonids, Japanese eel (*Anguilla japonica*), tilapia (*Oreochromis* spp.), striped bass (*Morone saxatilis*) and turbot (*Scophthalmus maximus*), among others, as well as its geographical distribution (Austin and Austin, 2007; Ringø and Gatesoupe, 1998; Romalde and Toranzo, 2005).

There has been an important controversy about the number and the nature of the bacterial species involved with streptococcosis (Austin and Austin, 2007), probably due to the difficulty of an accurate identification at species level within this bacterial group when using conventional microbiological criteria. With the development of molecular taxonomic techniques, it was possible to more accurately determine the precise taxonomic status of many isolates. Currently, there is general acceptance of the division of streptococcosis into two forms according to the virulence of the agents involved at high or low temperatures (Ghittino, 1999). “Warm water” streptococcosis, causing mortalities at temperatures higher than 15°C, typically involves species such as *Lactococcus garvieae* (synonym *Enterococcus seriolicida*), *Streptococcus iniae* (synonym *S. shiloi*), *S. agalactiae* (synonym *S. difficilis*) or *S. parauberis*. On the other hand, “cold water” streptococcosis is caused by *Vagococcus salmoninarum* or *L. piscium* and occurs at temperatures below 15°C.

Streptococcus phocae, a beta-hemolytic streptococcal species, was first isolated from clinical specimens of harbor seal (*Phoca vitulina*) affected by pneumonia (Skaar *et al.*, 2003). Further studies described isolations of this species from other marine mammals including Cape fur seal (*Arctocephalus pusillus pusillus*), ringed seal (*Phoca hispida*) and harbor porpoise (*Phocoena phocoena*) and gray seal (*Halichoerus grypus*) and cetaceans (Henton *et al.*, 1999; Lawson *et al.*, 2004; Raverty and Fiessel, 2001; Raverty *et al.*, 2004; Vossen *et al.*, 2004), associated in general to respiratory infections.

85 In this paper, the biochemical, serological and genetic characterization of the
 86 streptococcal strains isolated during several years from salmon showing septicemic
 87 disease is described. In addition, we present data on their implication in the
 88 appearance and progression of the disease in Chile from its first observation in 1999,
 89 the possible origins, as well as the clinical significance for the Chilean salmon
 90 industry.

91

92 **Materials and Methods**

93 **Bacterial strains.** A total of 34 strains isolated from internal organs (kidney, spleen
 94 or brain) or from ulcers of diseased cage-cultured Atlantic salmon in Chile were
 95 studied (Table 1). In addition, the type strain DSMZ 15635 of *Streptococcus phocae*,
 96 isolated from seal in Norway, was also analyzed for comparison. The bacteria were
 97 routinely grown on Columbia sheep blood agar (CBA)(Oxoid Ltd., Basingstone,
 98 England) and on tryptone soy agar supplemented with 1% NaCl (TSA-1) (Difco
 99 Laboratoires, Detroit, Mich.) under aerobic conditions at 22-25°C for 48h. Stock
 100 cultures were maintained frozen at -70°C in criobille tubes (AES Lab., France) or in
 101 tryptone soy broth (TSB-1) with 15% glycerol.

102 **Phenotypic tests.** All the strains were subjected to taxonomical analysis by standard
 103 morphological, physiological, and biochemical plate and tube tests (Facklam, 2002;
 104 McFaddin, 1983; Schleifer, 1986). The ability of the strains to grow at 4, 10, 25, 30
 105 and 37°C was tested in TSA-1 over a period of 10 days. Growth at different salinities
 106 was determined in basal medium (neopeptone 4g/L, yeast extract 1g/L, agar 15g/L)
 107 supplemented 0, 0.5, 3, 6.5 and 8% NaCl after 1 week incubation.

108 The commercial Rapid ID 32 Strep and API ZYM miniaturized systems (bioMeriux,
 109 Madrid, Spain) were used following the manufacturer's instructions except for the
 110 temperature of incubation which was set at 25°C. Standardized inocula were prepared
 111 by suspending cells in tubes containing sterile distilled water and adjusted
 112 spectrophotometrically to D.O of 1.0 (A_{580})(Ravelo *et al.*, 2001).

113 The drug sensitivities of the strains were determined by the disc diffusion method on
 114 Muller-Hinton agar (Oxoid) with 1% NaCl. The following chemotherapeutic agents
 115 (micrograms per disc) were used: oxolinic acid (10), trimethoprim-sulfamethoxazole
 116 (23.75/1.25), amoxycillin (25), oxytetracycline (30), erythromycin (15), and
 117 florfenicol (30).

Genetic characterization.

Genetic characterization by sequencing of the 16S rRNA gene was also carried out. Isolation of DNA from pure cultures was performed using the “Instagene” matrix (Bio-Rad), following manufacturer’s recommendations. 16S rRNA genes were amplified by PCR with universal primers pA and pH (Hutson *et al.*, 1993). Primers (Funke *et al.*, 1995) corresponding to internal conserved regions of this gene were used for the sequencing reactions in an Eppendorf Mastercycler Personal (Eppendorf, Germany), with the CEQ-Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, USA). Sequencing products were analyzed using a CEQ 8000 Sequencer (Beckman Coulter, USA). Comparative sequence analyses were conducted using FASTA3 program. Sequences of the closest relatives were retrieved from Gene bank/EMBL, and the alignments and phylogenetic analysis were performed by the Neighbour-joining method using the ClustalX (Thompson *et al.*, 1997). Bootstraps were calculated with 1000 replications, and *Streptococcus ferus* (AY058218) was used as out-group sequence. Phylogenetic tree was drawn using the software NJPlot (Perrière and Gouy, 1996).

In addition, adscription of the salmon isolates to the species *S. phocae* was also confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene using the restriction enzymes *EcoRI* and *HincII* (BioLabs, Schalbach, Germany) as described by Vossen *et al.* (2004).

Serological assays

Antiserum against the salmon isolate 2857 was prepared by intravenous injections of rabbits with formalin-killed cells suspended in saline as previously described (Magariños *et al.*, 1992). This protocol was approved by the Ethics Committee for Animal Experiments of the University of Santiago. Whole cell preparations of the isolates were employed in all the serological assays performed. Slide agglutination tests were conducted following the procedures of Toranzo *et al.* (1987) and the dot blot assays were performed as described by Cipriano *et al.* (1985).

The Lancefield streptococcal grouping (Lancefield, 1933) for the fish isolates was confirmed using Diagnostic Reagents (Oxoid Ltd., Basingstone, England) according to the manufacturer’s protocol. The strains *Streptococcus agalactiae* S/246 (serogroup B), group G *Streptococcus* S/299 and *Streptococcus mitis* S/290 were included for quality control in every test run and were grown as described above. These strains were obtained from the Instituto de Salud Pública, Chile.

Virulence for fish and mice

In order to determine the virulent capacities of the isolates, infectivity trials were conducted using different fish species and mice. Representative isolates (Au15, 2857, and C4) were intraperitoneally (i.p.) inoculated in Atlantic salmon (20 g) and rainbow trout (15 g). Prior to challenge, fish were randomly sampled to be subjected to microbiological analysis which indicated that they were free of *S. phocae* and other pathogens. Groups of 15 fishes were inoculated (0.1 µl) with different bacterial doses ranging from 10^4 to 10^9 cells/fish. Control fish received 0.1 µl of saline solution (0.85% NaCl). In addition, one group of 15 Atlantic salmon (20 g) was subcutaneously inoculated (0.1 µl/fish) with of two doses (10^5 and 10^6 cells/fish) of the isolate Au15. Fish were maintained in seawater tanks at $19 \pm 1^\circ\text{C}$ with aeration during the course of the experiments (21 days) making a daily 50% water renovation. Fish mortality was daily checked during this period. Dead fish were microbiologically analyzed for re-isolation of the inoculated strain. The degree of virulence was expressed as lethal dose 50% (LD₅₀) calculated as described by Reed and Muench (1938).

To determine the pathogenic potential for homoiotherm animals, a mouse virulence assay was performed including three isolates from Atlantic salmon and the *S. phocae* type strain DSMZ 15635^T. Briefly, groups of 5 BALB/C mice (10-12 weeks old, 21-25 g) were inoculated i.p. with bacterial dose of 10^7 cells (0.1 µl/mouse). Mortalities were recorded daily during a period of 10 days. Strain CDC 2227-81 of *Photobacterium damsela* subsp. *damsela*, previously described as pathogenic for mice (Osorio *et al.*, 2000) was included in the assay as positive control.

The Ethics Committee for Animal Experiments of the University of Santiago approved the protocols of this study.

Results

From 1999, disease outbreaks occurred repeatedly during the summer months (temperatures higher than 15°C) in Atlantic salmon (*Salmo salar*) farmed in Chile affecting both smolts and adult fish cultured in floating cages in estuary and marine waters. The disease presents a rapid time course with appearance of clinical signs in 1 or 2 days, although in acute cases fish can not show any symptom. Isolates studied in this work were isolated from 28 independent outbreaks, showing cumulative mortalities between 3 and 25% of the affected population. From its first appearance

186 until 2003, the disease has only been detected in waters of the Reloncaví estuary (X
187 Chilean Region), being further spread to other Chilean Regions, in some cases
188 associated to fish with origin in Reloncaví but sold and transported as smolts.

189 Diseased fish showed exophthalmia with accumulation of purulent and haemorrhagic
190 fluid around eyes, ventral petechial haemorrhages, skin abscesses and, in some cases,
191 muscle liquefaction with formation of deep ulcerative areas (Fig. 1). At necropsy,
192 haemorrhage in the abdominal fat, pericarditis with accumulation of a white fluid in
193 the pericardic cavity, and enlarged liver (showing a yellowish colour), spleen and
194 kidney are common pathological changes (Fig. 1). Gram-stained smears revealed the
195 presence of Gram-positive cocci in all cases.

196 The microbiological analysis of internal organs (kidney, spleen and brain) and ulcers
197 revealed the presence on CBA, in pure culture or as major colony type, of pinpoint
198 white colonies (0.5 mm diameter). The isolated bacteria were Gram positive chain-
199 forming cocci (0.6-0.9 μm diameter), beta-haemolytic, negative for the catalase and
200 aesculin hydrolysis tests, growing at 25 and 37°C and at pH 9.6 but not with 6.5%
201 NaCl or 40% bile salts. These results indicated that the bacteria could be assigned
202 presumptively to the Genus *Streptococcus*.

203 When the miniaturized system Rapid ID 32 Strep was employed, 30 out of 34 Atlantic
204 salmon isolates rendered the profile 040000000000, showing a positive reaction only
205 for the phosphatase alkaline test. Four strains rendered the same profile than the type
206 strain, 04000001000, being positive also for the utilization of maltose. With both
207 profiles, strains were classified according to the API database as belonging to the
208 Genus *Gemella* (*G. haemolysans* or *G. morbillorum*). On the other hand, all the
209 isolates and the type strain showed the same enzymatic patterns in the API ZYM
210 system, being positive for the production of alkaline and acid phosphatases and
211 leucine arylamidase.

212 All the isolates showed the same pattern of resistance/susceptibility to
213 chemotherapeutic agents *in vitro*, being susceptible to amoxycillin, oxytetracycline,
214 erythromycin and florfenicol and resistant to flumequine, oxolinic acid and
215 trimethoprim-sulfamethoxazole.

216 Sequencing analysis of the 16S rRNA gene revealed that all the Atlantic salmon
217 isolates studied were identical, and that the sequences obtained showed similarities
218 higher than 98.18% with *S. phocae* (98.18 and 100 % identity with sequences
219 AF235052 and AJ621053, respectively)(Fig. 2). The next relatives were *S. pyogenes*

and *S. agalactiae* with similarities slightly higher than 93%. Moreover, in the RFLP analysis all the isolates rendered the expected fragments employing either *HincII* or *EcoRI* endonucleases. Restriction with the enzyme *HincII* restriction yielded two characteristic fragments with sizes of 180 and 1230 bp (Fig. 3), whereas *EcoRI* rendered three DNA fragments with sizes of 170, 380, and 840 bp (data not shown). Serological studies using different techniques, such as slide agglutination and dot blot, with specific serum raised against the Atlantic salmon isolate 2857, demonstrated that all the salmon isolates are antigenically homogeneous, sharing antigenic determinants with the type strain isolated from seals (Table 1). However, when the commercial Lancefield streptococcal grouping kit was employed, the isolates from fish were classified as group G, although showing also a weak cross-reactivity with serum from antigenic group A, while the type strain DSMZ 15635^T was assigned to serogroup F. The three isolates inoculated were pathogenic for Atlantic salmon with LD₅₀ values ranging from 1.9×10^5 and 5.6×10^5 bacteria per fish but not for rainbow trout when i.p. inoculated. Mortalities began two days after inoculation, being observed a dramatical increase by day 6. No mortalities were observed in the control groups. Inoculated bacteria could be recovered from dead fish which showed in all cases the typical necrotic lesions observed in the natural outbreaks. On the other hand, subcutaneously inoculated salmon showed a gross inflammation in the injection area, which progressed to necrosis with caseous material and liquefaction. Mortalities started 4 days after inoculation reaching 80 and 100% for the low and high dose respectively. None of the *S. phocae* isolates, including the type strain, were pathogenic for mice, being observed no mortalities during ten days. Three out of the 5 BALB/C mice inoculated with the strain of *P. damsela* subsp. *damsela* utilized as positive control, died during the same period.

Discussion

Streptococcus phocae, a member of the pyogenic streptococcal group (Köhler, 2007) has been described as an organism associated with disease and mortality of different seal species in Norway, German North and Baltic seas and South Africa (Henton *et al.*, 1999; Skaar *et al.*, 1994; Vossen *et al.*, 2004). More recently Gibello *et al.* (2005), during the development of a PCR protocol for *Streptococcus iniae*, employed some streptococcal strains from the bacterial collection at the FRS marine Laboratory in

253 Aberdeen, Scotland, originally isolated from Atlantic salmon in Chile which after
 254 their genetic characterization were also assigned to the species *S. phocae*. These
 255 authors indicated the necessity to determine the real clinical significance of this
 256 bacterial species for the Chilean salmon industry. In this work, a collection of 34
 257 strains of *S. phocae* isolated from diseased Atlantic salmon were biochemical,
 258 serological and genetically characterized and data on the appearance and progression
 259 of the disease in Chile from its first observation in 1999 were presented, in order to
 260 get a better understanding of this pathology.

261 The taxonomical analysis of the Atlantic salmon isolates revealed a great
 262 homogeneity, regardless the year of isolation or geographical area of origin, and that
 263 they were nearly identical to the type strain of this bacterial species. Such
 264 homogeneity has been previously described by other authors for *S. phocae* (Gibello *et*
 265 *al.*, 2005; Henton *et al.*, 1999; Skaar *et al.*, 1994; Vossen *et al.*, 2004). Although the
 266 preliminary characterization indicated their assignation to the genus *Streptococcus*,
 267 when the miniaturized Rapid ID 32 Strep system was employed, the profiles obtained
 268 corresponded to good identification of members of the genus *Gemella*, mainly *G.*
 269 *haemolysans*. However, although it has been reported that differentiation of some
 270 streptococcal groups and *Gemella* strains is not easy (Lee *et al.*, 2004), these later
 271 organisms are alpha-haemolytic Gram variable cocci forming pairs or tetrads,
 272 properties completely different from those of the salmon isolates. Moreover,
 273 misidentifications of *S. phocae* strains using this miniaturized system were also
 274 reported by Gibello *et al.* (2005), although these authors observed profiles typical
 275 from *Gardnerella vaginalis*. A reason for these misidentifications can be that *S.*
 276 *phocae* is not included in the API Database, which is focused mainly in human
 277 pathogens.

278 The development and application of molecular techniques have greatly facilitated the
 279 identification and classification of streptococci (Bentley and Leigh, 1995; Romalde
 280 and Toranzo, 2005; Tapp *et al.*, 2003), having the advantages of time-saving and
 281 accuracy. The use of molecular methods, such as sequencing or RFLP analysis of the
 282 16S rRNA gene, in the identification of the Atlantic salmon isolates unequivocally
 283 demonstrated that all of them belong to the species *S. phocae*.

284 Serological typing is an effective approach in the epidemiological study of various
 285 Gram-positive bacteria. The results from the Lancefield typing, using reagents of the

different antigenic groups, showed that the fish isolates could be assigned to antigenic group G although displaying also a weak reaction with antiserum A. *S. phocae* strains from seals have been described as belonging to Lancefield's groups C and F (Skaar *et al.*, 1994; Vossen *et al.*, 2004). In fact, the reference strain (DSMZ 15635^T) from seal included in the study belonged to the Lancefield's group F. Therefore, the Atlantic salmon isolates represent a distinct serological group within *S. phocae*. On the other hand, all isolates, including the type strain, reacted with the antiserum raised in rabbit against the strain 2857, indicating the existence of some common antigenic components among salmon and seal isolates of *S. phocae*, probably in the cell envelope.

From the data obtained, it seems clear that movement of fish, in particular smolts, between farms located in different geographical areas has played an important role in the spreading of the disease across Chile. More difficult is to find the origin for the first appearance of the disease in the Reloncaví estuary. We can speculate that a possible origin can be the numerous colonies of marine mammals present in these Chilean regions (Schlatter and Huckle-Gaete, 1999; Sielfeld, 1999). Marine mammals capture cultured salmon through the cage nets, and also cause a great stress with subsequent appetite losses and high susceptibility to diseases (Arnold, 1992). Further studies are needed to confirm this hypothesis.

Although some chemotherapeutants were effective *in vitro*, oral treatments with oxytetracycline (80-120 mg/kg fish for 15-20 days), erythromycin (50-70 mg/kg fish for 7-14 days) or florfenicol (15-20 mg/kg fish for 15 days) reduced mortalities only in part and during the treatment period when applied in the fish farms. The low efficacy of the chemotherapy in controlling this streptococcal disease in Atlantic salmon has led to an effort in developing preventive measures, including the use of auto-vaccines which together with an early detection of clinical signs, maintenance of fish at low densities, cleaning of the cage nets, etc., helped to decrease the impact of *S. phocae* in the cultured fish.

In summary, and on the basis of the facts presented, we conclude that the species *S. phocae* is an emerging pathogen for salmonid culture in Chile, and it should be included as a new member of the warm water streptococcal syndrome.

319

320 **Nucleotide sequence accession number.**

321 The nucleotide sequence corresponding to the 16S rRNA gene of *S. phocae* 2857 was
322 deposited in the GenBank/EMBL Database as representative of the Atlantic salmon
323 isolates, and it has been assigned the accession number EF599165.

324

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Figure Legends

Fig. 1.- Clinical signs observed in the diseases Atlantic salmon. a) hemorrhages around the eye; b) ventral petechial haemorrhages; c) ventral skin abscesses; d) deep ulcers with muscle liquefaction; e) pericarditis with accumulation of a white fluid (arrow) in the pericardic cavity; f) haemorrhage in the abdominal fat and cavity; g) enlarged liver and spleen.

Fig. 2.- Phylogenetic tree of 16S rRNA sequence for the Atlantic salmon isolates and the type strains of the closest streptococci. *Streptococcus ferus* (AY058218) was employed as outgroup. Horizontal branch lengths are proportional to evolutionary divergence. Significant brootstrap values of 1000 replicates appear next tyo the corresponding branch. The bar represents 1% sequence divergence. Sequences from relative species were obtained from GeneBank Database, and their accesion numbers are indicated after the species name.

Fig. 3.- Restriction of the amplified 16S rRNA gene with endocnuclease *HincII*. Lanes: M, molecular size marker (50-2000 ladder; Sigma); 1 and 2, amplicon and correspondent restriction from *S. phocae* DSMZ 15635^T; 3 to 7, amplicons and correspondent restrictions from Atlantic salmon isolates 2857, C-4 and PF-157, respectively. Numbers on the left indicate the position of molecular size marker in bp. Numbers on the right indicate the size of the specific amplified products and restriction fragments in bp.

Table 1.- Origin and serological characterization of the *Streptococcus phocae* isolates used in this study.

Isolate	Origin	Year of Isolation	<u>Reaction with serum against isolate 2857</u> Lancefield group	
Atlantic salmon isolates				
7053-1	Reloncaví Estuary, Chile	1999	+	G
9547-1	Reloncaví Estuary, Chile	1999	+	G
2743-1	Reloncaví Estuary, Chile	2001	+	G
2857	Reloncaví Estuary, Chile	2001	+	G
3385	Reloncaví Estuary, Chile	2001	+	G
3618	Reloncaví Estuary, Chile	2001	+	G
3770	Reloncaví Estuary, Chile	2002	+	G
344	Reloncaví Estuary, Chile	2004	+	G
751	Reloncaví Estuary, Chile	2004	+	G
2880	Reloncaví Estuary, Chile	2004	+	G
5070	Reloncaví Estuary, Chile	2004	+	G
C-4	Reloncaví Estuary, Chile	2004	+	G
Au11	Herradura, Chile	2004	+	G
Au13	Hualaihué, Chile	2004	+	G
Au14	Reloncaví Bay, Chile	2004	+	G
Au15	Curaco de Lin Lin, Chile	2004	+	G
PF-132	Chaitén, Chile	2004	+	G
PF-133	Herradura, Chile	2004	+	G
PF-136	Reloncaví Bay, Chile	2004	+	G
PF-137	Reloncaví Estuary, Chile	2004	+	G
PF-140	Calbuco, Chile	2004	+	G
PF-142	Comau Fiord, Chile	2004	+	G
PF-143	Reloncaví Estuary, Chile	2004	+	G
PF-145	Marimelli, Chile	2004	+	G
PF-146	Marimelli, Chile	2004	+	G
PF-148	Quillaípe, Chile	2004	+	G
PF-153	Quillaípe, Chile	2004	+	G
PF-155	Calbuco, Chile	2004	+	G
PF-030	Punta Venet, Chile	2005	+	G
PF-056	Castro, Chile	2005	+	G
PF-058	Castro, Chile	2005	+	G
PF-059	Castro, Chile	2005	+	G
PF-134	Chaitén Chile	2005	+	G
PF-157	Reloncaví Estuary, Chile	2005	+	G
Reference strain				
DSMZ 15635 ^T	<i>Phoca vitulina</i> , Norway	1994	+	F

Fig. 1.- Romalde et al.

Fig. 2.- Romalde et al.

Fig. 3.- Romalde et al.