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Potential effect of pesticides currently used in salmon farming on photo and chemoautotrophic carbon uptake in central – southern Chile

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ABSTRACT

Aquaculture has become an important component of Chilean economy, especially in the southern region, where salmon farming is an active industry. However, high density in salmon cages can increase stress and susceptibility to parasitic outbreaks of the copepod *Caligus rogercresseyi*. The pesticides used against sea lice can have potential effects on non-target microbiota and on the structure and functioning of aquatic ecosystems. The objective of this study was to investigate the response of natural microbial communities to the addition of the anti-lice pesticides azamethiphos, deltamethrin and emamectin benzoate and their potential impact on photoautotrophic and chemoautotrophic carbon fixation in central-southern Chile (37°S to 42°S). The addition of pesticides on primary production samples was related to changes in carbon uptake, which were significant if a single pesticide was applied, mainly emamectin benzoate and azametiphos. In surface waters of central Chile, emamectin benzoate produced a 60–90% decrease for both photo and chemoautotrophic carbon fixation. Enhanced rates were also observed for in situ primary production as a result of azamethiphos addition in northern Patagonia. Such stimulation, although limited, was possibly related to the supply of nitrogen and phosphate for phytoplankton requirements by this organophosphate compound.

1. Introduction

During the last 20-years, aquaculture has grown steadily as an alternative to the use of fishery resources (Duarte et al., 2007; Fao, 2016). In southern Chile in particular, aquaculture has become an important component of the economy (Medina and Ramos-Jiliberto, 2009), mainly due to the development of salmon farming (Burridge et al., 2010).

Along with an increase in production, high susceptibility of cultured salmon to parasitic infections has been reported (Burridge et al., 2010). The copepod *Caligus rogercresseyi* is the most important parasite currently affecting salmon farms in Chile (Bravo et al., 2013). It causes economic losses that can reach US$0.30 per kg (Bravo, 2003; Carvajal et al., 1998; Costello, 2009). Chemical products are necessary to increase fish survival rates and controlling the development of sea lice outbreaks. Between 2000 and 2007, only emamectin benzoate was authorized for the treatment of *C. rogercresseyi* infections in Chile, and was applied as a food additive (Sevatdal et al., 2005; Stone et al., 2000). When administered at a dose of 50 μg kg fish^{-1} day^{-1} during seven days, it could reduce sea lice population (mainly as juvenile chalimus and adult stages) by 90% (Bravo et al., 2012). After observing resistance by *C. rogercresseyi* to emamectin benzoate, the use of deltamethrin (a synthetic pyrethroid applied as a bath treatment) was authorized (Burridge et al., 2010). Deltamethrin interferes with the transmission of nerve impulses, causing paralysis and the subsequent death of the parasite (Burka et al., 2012). In 2013, the organophosphate azamethiphos was also approved for use in salmon farming. This compound inhibits the activity of the acetylcholinesterase enzyme, therefore improving the control of sea lice (Bravo et al., 2015; Burridge et al., 2010; Kazemi et al., 2012).

Since the pesticides used for sea lice control were initially developed for the control of parasites in livestock, their effect on the aquatic environment was initially unknown (Burridge et al., 2010; Nash, 2003). The potential effect of these substances on non-target species (mainly metazoa) has received attention in recent years (Bhanu et al., 2011; Burridge et al., 2004; Canty et al., 2007; DeLorenzo et al., 2001; Johnson et al., 2004), but our understanding of potential impacts on the structure and functioning of aquatic ecosystems is still poorly understood (Burridge et al., 2010; Buschmann et al., 2006) and needs a microcosms and mesocosms approach (Medina et al., 2004).

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Carbon fixation into organic matter is essential to trophic interactions in marine ecosystems and is mainly associated to photoautotrophic phytoplankton (Eppley and Peterson, 1979; Field et al., 1998) and chemoautotrophic bacteria (Boschker et al., 2014; Li et al., 2012). Many factors have been suggested to limit microbial growth in coastal systems, including nutrient concentration and light intensity (Daneri et al., 2000; Falkowski and Davis, 2004; Iriarte et al., 2007).

The increase in aquaculture activities in southern Chile, as well as agriculture-influenced river runoff and the melting of glaciers (Pantoja et al., 2011), could generate non Redfield inputs of nutrients to aquatic systems, potentially changing phytoplankton structure and modifying carbon fixation fluxes (Beman et al., 2005; Iriarte et al., 2010; Labbé-Ibañez et al., 2015; Olsen et al., 2014). Some organic compounds can also add to the marine nutrient pool (as N or P) of the water column (Amon and Benner, 1994). Because of the nature of the anti-lice pesticides currently in use, it is fair to hypothesize that the nutrient pool can interact with such compounds.

The goal of our study was to investigate the potential effect of three pesticides used against *C. rogercresseyi*: emamectin benzoate, deltamethrin and azamethiphos on photoautotrophic and chemoautotrophic carbon fixation in two key regions of Chile.

2. Materials and methods

2.1. Study area and oceanographic survey

Incubations for primary production were conducted in two regions in central-southern Chile: Llico Bay, located south of Gulf of Arauco (37.1°S 73.5°W; Fig. 1A) and the Caucahue Channel located at Chiloé Island in Northern-Patagonia (42.1°S 73.4°W; Fig. 1B). Llico Bay was visited 5 times on board of R/V Kay Kay II (University of Concepción) between December 2014 and April 2016 (Table 1). The Caucahue Channel was visited twice on board L/M "Don José". The first cruise was carried out in June 2014 and a second cruise was performed in January 2015 (Table 1).

In order to describe the hydrographic variability of the water column, we used a CTD Minus X (AML Oceanographic, Canada) at three stations inside Llico Bay (BLL1, BLL2 and BLL3; Fig. 1, Table 1). At Caucahue, profiles were done using a CTDO data sensor (SAIV A/S, Norway). Stations Q1, Q2, Q3, Q5, Q6, Q9, Q10 and Qc are presented in Fig. 1B.

Chemical and biological sampling was carried out using Niskin bottles in every oceanographic cruise. Bacterioplankton and *Synechococcus* sp. abundances were determined by flow cytometry (Marie et al., 2000) at the Laboratory for Oceanographic Processes and Climate (PROFC; University of Concepcion, Chile). Nutrient samples were collected to determine nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3+}) concentrations. Samples were prefiltered by 0.7 µm and then stored at −20 °C until their colorimetric analysis using a Brann Luebbe autoanalyzer (Aminot and Kérouel, 2007). N/P ratio was estimated using the addition of NO_3^- and NO_2^- divided by PO_4^{3+} concentrations. Total Chlorophyll-*a* (Chl-*a*) concentrations were estimated using a Turner Design fluorometer (Holm-Hansen et al., 1965).

Table 1
Geographical location of the sampling stations at Llico Bay and Caucahue Channel.

Study area	Station ID	Latitude (°S)	Longitude (°W)	Depths of sampling (m)
Llico	BLL1	37.192	73.547	2, 4, 6
Llico	BLL2	37.159	73.563	2, 10, 15
Llico	BLL3	37.137	73.574	2, 10, 20
Caucahue	Q1	42.135	73.458	2, 10, 20
Caucahue	Q2	42.117	73.422	2, 10, 20, 30, 40
Caucahue	Q3[a]	42.102	73.409	2, 10, 20, 30, 50
Caucahue	Q5	42.108	73.366	2, 10, 20, 30, 50, 60
Caucahue	Q6[b]	42.130	73.365	2, 10, 20, 30
Caucahue	Q8	42.205	73.380	2, 10, 20, 30
Caucahue	Q9	42.165	73.429	2, 10, 20, 30, 50
Caucahue	Q10	42.151	73.446	2, 10, 30
Caucahue	Qc	42.028	73.325	2, 10, 20, 30, 50, 65, 80

[a] In situ Primary production experiment only in Caucahue summer 2015.
[b] In situ Primary production experiment only in Caucahue winter 2014.

2.2. In situ carbon uptake experiments

Rates of carbon uptake were estimated with the (^{13}C) stable isotope technique (Fernandez and Farías, 2012; Slawyk and Raimbault, 1995; Slawyk et al., 1977).

Seawater samples were distributed into 600 mL polycarbonate bottles (previously autoclaved) and inoculated with a 0.5 mL of Sodium bicarbonate C-13 solution ($NaHCO_3$ Icon Isotopes IC 4628; 3.6456 mg ^{13}C mL^{-1}; 0.5 µmol mL^{-1} final concentration). Incubations were done using an in situ mooring line deployed at sunrise and recovered 7 h later. At sunset, the bottles were recovered and filtered through 0.7 µm Whatman GF/F filters (precombusted at 450 °C, 4 h) using a vacuum pump. Filters were stored at −20 °C until analysis by isotope mass spectrometry at the Laboratory for Biogeochemistry and Applied Stable Isotopes (LABASI) of Pontificia Universidad Católica de Chile using a Thermo Delta V Advantage IRMS coupled with a Flash2000 Elemental Analyzer.

Deltamethrin and azamethiphos were added to primary production

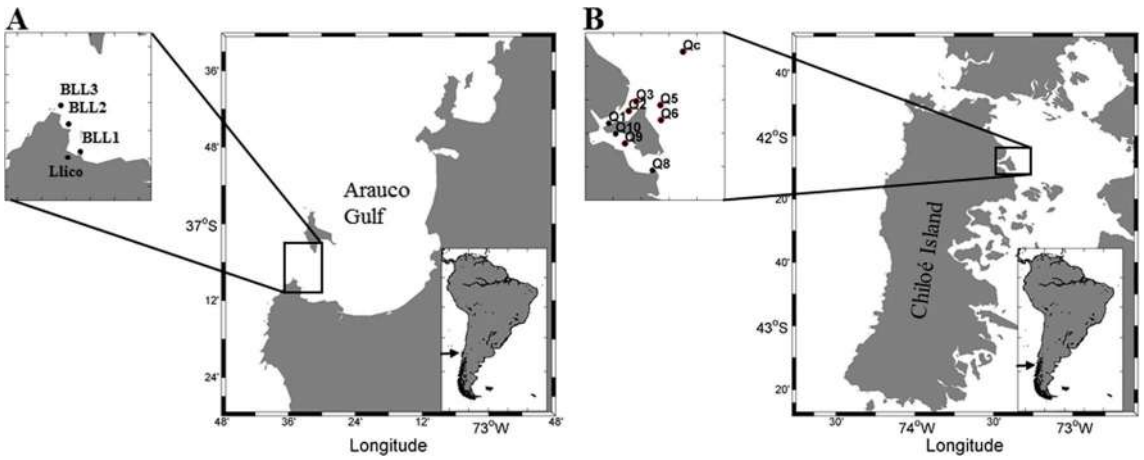

Fig. 1. Study areas and sampling stations in situ primary production experiments. A) Llico Bay (Gulf of Arauco; central Chile). Stations BLL1, 2 and 3 were visited for in situ experiments. The coastal point represents the site of on deck experiments. B) Caucahue channel and the inner sea of Chiloé, Northern Patagonia. Overlapped red circles indicate sampling points for in situ primary production experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of in situ primary production incubations during the Llico and Caucahue cruises.

Location	Dates	Season	Stations of in situ primary production	Pesticides solution concentration and isotopes
Caucahue	18–30/06/2014	Winter 2014	Q2, Q5, Q6, Q9, Qc	^{13}C (0.5 μmol mL^{-1}), azamethiphos (1.2 μmol L^{-1})
Caucahue	20–30/01/2015	Summer 2015	Q2, Q3, Q5, Q9, Qc	^{13}C (0.5 μmol mL^{-1}), azamethiphos (1.2 μmol L^{-1})
Llico	01–05/12/2014	Spring 2014	BLL1, BLL2, BLL3	^{13}C (0.5 μmol mL^{-1}), azamethiphos (1.2 μmol L^{-1}), deltamethrin (3 μmol L^{-1}), azamethiphos (1.2 μmol L^{-1}) + deltamethrin (3 μmol L^{-1})
Llico	21–25/07/2015	Winter 2015	BLL1, BLL2, BLL3	^{13}C (0.5 μmol mL^{-1}) + azamethiphos (1.2 μmol L^{-1}), deltamethrin (3 μmol L^{-1}), azamethiphos (1.2 μmol L^{-1}) + deltamethrin (3 μmol L^{-1})
Llico	21–25/10/2015	Spring 2015	BLL1, BLL2, BLL3	^{13}C (0.5 μmol mL^{-1})
Llico	05–09/01/2016	Summer 2016	BLL1, BLL2, BLL3	^{13}C (0.5 μmol mL^{-1})
Llico	21–29/04/2016	Autumn 2016	BLL1, BLL2, BLL3	^{13}C (0.5 μmol mL^{-1}), azamethiphos (1.2 μmol L^{-1}), deltamethrin (3 μmol L^{-1}), azamethiphos (1.2 μmol L^{-1}) + deltamethrin (3 μmol L^{-1})

incubations at doses determined according to concentrations used in sea lice treatments: 3 μg L^{-1} for deltamethrin (Siwicki et al., 2010) and 0.2 mg L^{-1} for azamethiphos (Burridge et al., 2010; Canty et al., 2007; Davies et al., 2001). The dose for emamectin benzoate was estimated in order to obtain the highest possible experimental concentration by dividing the standard into two equal parts (Table 2).

Incubation depths at Llico Bay and Caucahue channel were determined from the compensation depth (~2.7 times the depth of view of the Secchi disk) where the light availability allows phytoplankton growth and the oxygen produced by photosynthesis is equal to that consumed by respiration. At Llico Bay, profiles were sampled at 2 and 4 m (BLL1) and 2 and 10 m (BLL2 and BLL3). Profiles at Caucahue channel included 2, 10 and 30 m depth. Integrated primary production was estimated by using the trapezoid method and using the depth of the photic zone (Ze) as integration level (average value 10.5 ± 3 m for Llico Bay and 30.9 ± 3.2 m for Caucahue). As BLL1 station was only 5 m depth we did not estimated integrated carbon uptake rates for that station. Ze was estimated by using measuring incident solar radiation and light penetration in the water column with a Secchi disk.

2.3. On deck carbon uptake experiments

In order to simultaneously study photo and chemoautotrophic carbon uptake, several on deck experiments using stable isotopes (^{13}C) were performed at Llico Bay (BLL2; Fig. 1) with surface water (2 m). These experiments were carried out in winter 2014, spring 2015, and summer and autumn 2016 (Table 1; Fig. 2).

In order to simulate solar radiation, we performed atmospheric measurements of incident Photisynthetically Active Radiation (PARWm^{-2}; 400–700 nm) using a portable radiometer (RM-21 Dr. Gröbel, Germany). Photoautotrophic samples were incubated under a combination of two cutoff filters: 0.3 Neutral density 209 (47% of average transmittance between 400 and 700 nm, Lee filters®) and a Steel Blue 117 (Lee filters®). Dark treatments were put in a closed incubator. Temperature conditions throughout the experiment were maintained with a continuous flow of water.

Seawater (580 mL) was poured into Duran Schott bottles and amended with a ^{13}C solution (0.5 mL at 0.5 μmol mL^{-1} final concentration) and 1 mL of pesticide solutions as described in Table 2. The pesticide treatments used were azamethiphos (^{13}C + A), deltamethrin (^{13}C + D) and emamectin benzoate (0.66 mmol L^{-1}, Dr. Ehrenstorfer GmbH; ^{13}C + B). Combined treatments were also used: azamethiphos and deltamethrin (^{13}C + AD) and the combination of azamethiphos, deltamethrin and emamectin benzoate (^{13}C + ADB). During spring 2014 at Llico Bay, no combined treatments were used. We chose to test the effect of combined pesticides because it is likely that different pesticides co-occur in the water column during coordinated (or synchronized) treatments which are commonly used in order to improve treatment performance (Arriagada et al., 2017). Moreover, as seen in previous studies (Wang et al., 2016), toxicity can be increased using combination of compounds compared to the use of single chemicals (Laetz et al., 2009). All incubations were done in duplicate.

2.4. Estimating carbon uptake rates

Carbon uptake rates (mg C m^{-3} t^{-1}) were estimated according to Slawyk and Raimbault (1995) and Fernandez and Farías (2012) following Eq. (1):

$$\rho DI^{13}C = [((\%R_{POC} - 1.112) * (POC * 1000/12 * V_f))/\%R_{DIC}] * 12/1000 \quad (1)$$

where V_f is the filtrated volume, POC represents the amount of particulate carbon obtained by mass spectroscopy (μg) and $\%R_{POC}$ is the enrichment in ^{13}C in the GF/F filter after the incubation. $\%R_{DIC}$ is the excess of enrichment of ^{13}C after the inoculation (T_0) computed according to Eq. (2):

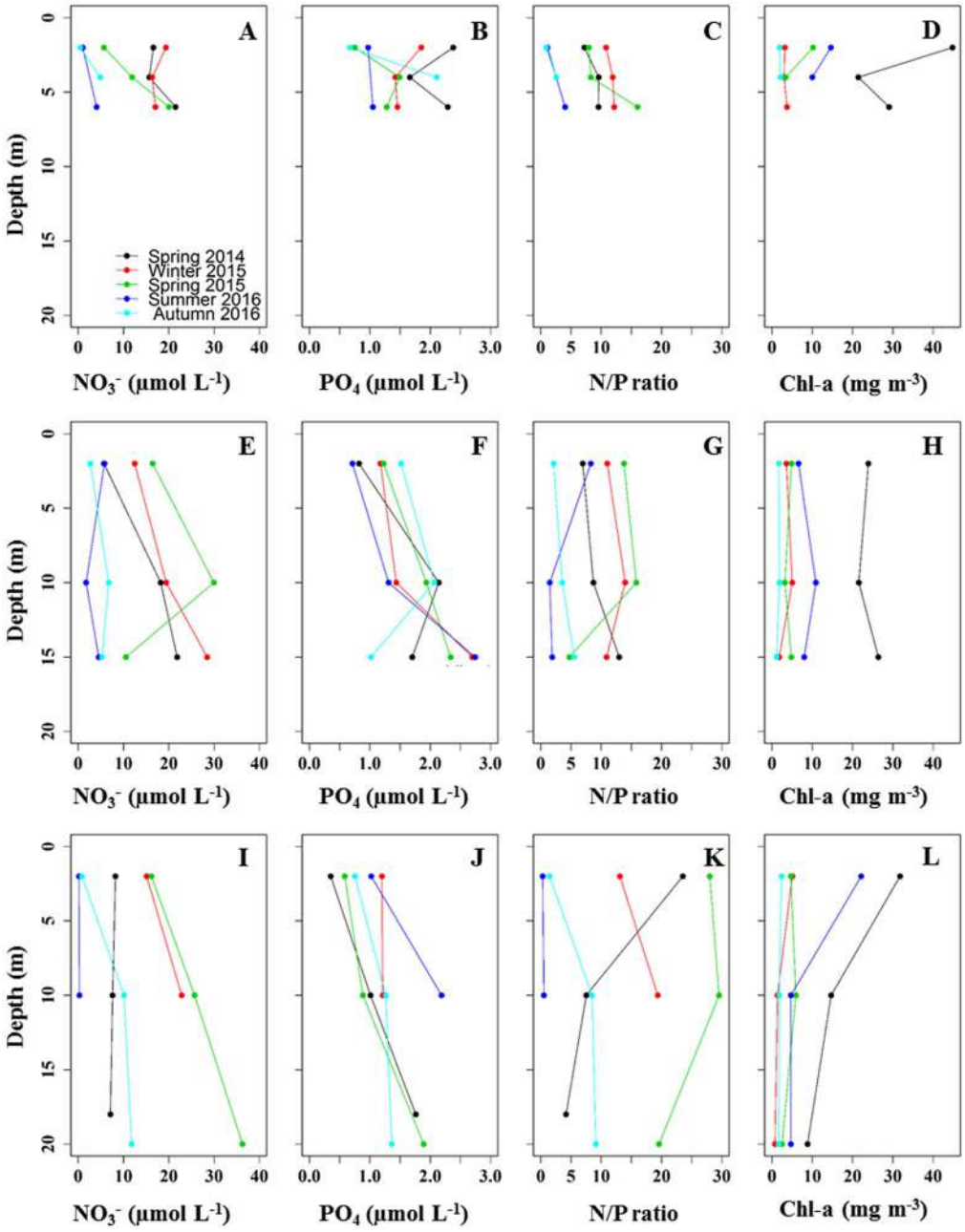

Fig. 2. Profiles of nitrate (µmol L^{-1}), phosphate (µmol L^{-1}), N/P ratio and Chl-*a* (mg m^{-3}) at Llico Bay stations from spring 2014 to autumn 2015. A, B, C) BLL1 D, F, G) BLL2 H, I, J) BLL3.

$$\%R_{DIC} = \frac{((^{13}C * {}^{13}DIC/V_b) + (DIC_i * 0.0112))}{(DIC_i - (^{13}C * {}^{13}DIC)/V_b)} \quad (2)$$

where ^{13}C is the volume of isotopic solution, ^{13}DIC represents the concentration of the ^{13}C added inoculums. The term 0.01112 represents the natural abundance (average) of ^{13}C. DIC_i represents the concentration of DIC in the sample before the addition of tracer. The values used in this study were 26 mg C L^{-1} for Llico bay and 25.1 mg C L^{-1} in the case of Caucahue channel (Alarcon et al., 2015; Jantzen et al., 2013). For in situ incubations, hourly rates were multiplied by 12 in order to obtain daily carbon fixation values (mg C m^{-3} d^{-1}).

2.5. Statistical analysis

For Llico (duplicate) and Caucahue (triplicate) data, paired *t*-tests were performed in order to compare treatments with respect to control conditions. To determine if there were significant variations between carbon fixation experiments among treatments, a 2-way ANOVA was computed with the "ANOVA.2way.R" function (Legendre, 2007). Homogeneity of variance was tested by using the Bartlett's test. Whenever data did not pass the test, a log transformation was implemented. Whenever a significant difference existed, a multiple t-test with a Bonferroni correction was performed. All statistical analyses were performed using the software R (https://www.r-project.org/).

3. Results

3.1. Seasonal environmental variability in central Chile (Llico Bay)

Temperature and salinity in Llico Bay varied between seasons, although similar trends were observed among stations (Supplementary Fig. 1). Thermal stratification was registered during summer and spring with maximum surface values at BLL1 and BLL2 (~16 °C), whereas in winter profiles showed lower temperature and salinity values at BLL1 and BLL2 (31.5). Nitrate showed higher concentrations in spring 2014 and winter 2015 compared to spring 2015, summer 2016 and autumn

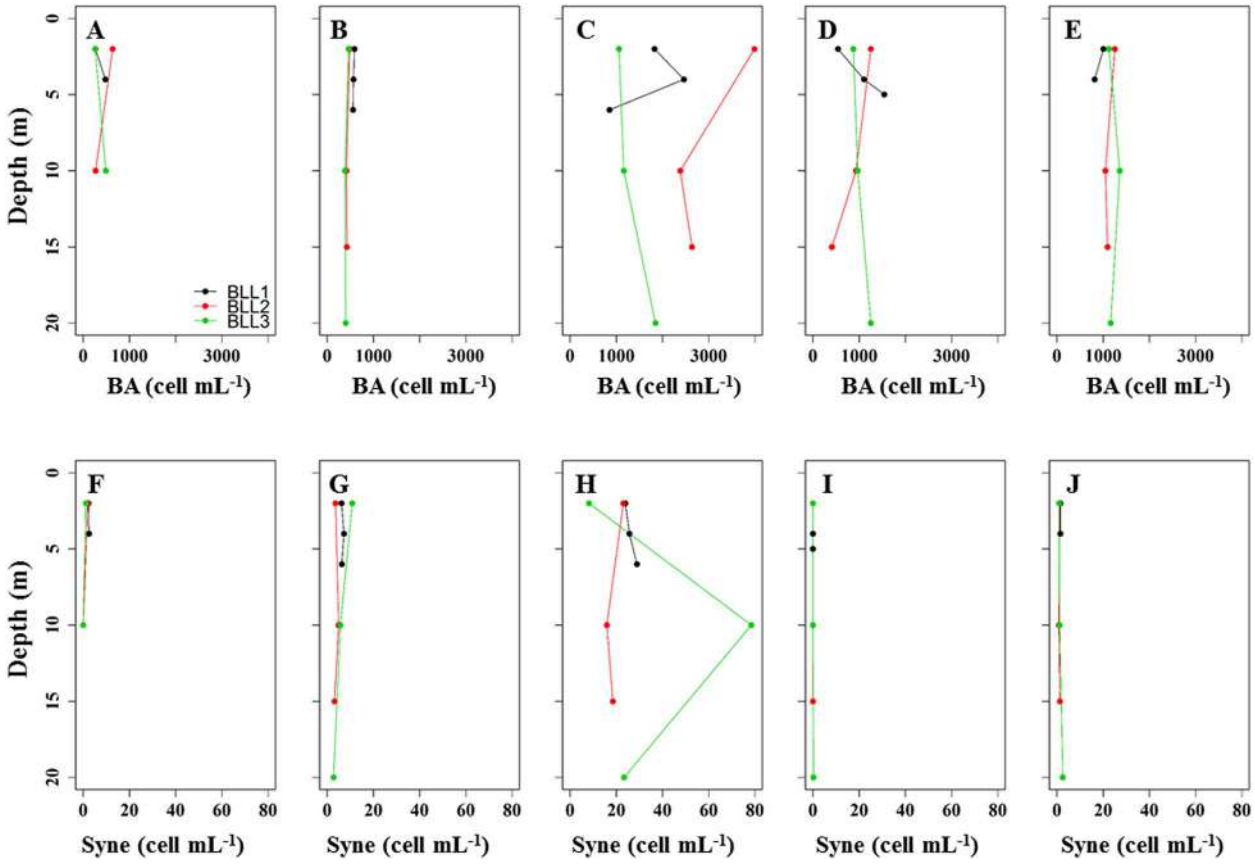

Fig. 3. Profiles of bacterioplankton and *Synechococcus* sp. abundances (10^3 cell mL^{-1}) at BLL1, BLL2 and BLL3 stations. A, F) spring 2014 B, G) winter 2015 C, H) spring 2015 D, I) summer 2016 E, J) autumn 2016.

2016 (Fig. 2). No seasonality was observed in phosphate concentrations. Station BLL1 had an average concentration of 1.491 ± 0.555 µmol L^{-1} of PO_4^{3+} while values were 1.656 ± 0.644 and 1.190 ± 0.521 µmol L^{-1} for stations BLL2 and BLL3, respectively. The N/P ratio varied between seasons with lower values estimated during summer 2016 at BLL3 (0.31 and 0.5, for 2 and 10 m respectively). Also, station BLL3 presented the maximum N/P ratios during spring 2015 at 2 and 10 m (27.98 and 29.51, respectively; Fig. 2). The highest concentrations of Chl-*a* were observed in spring 2014, specifically at BLL1 (44.82 mg m^{-3}), followed by summer 2016 and spring 2015 (Fig. 2D). The lowest Chl-*a* value was observed in winter (0.72 mg m^{-3}).

Incident PAR radiation showed a marked seasonality. Values were highest during spring 2015 (median of 350 W m^{-2} at 13 h) and lowest in autumn 2016 (median of 70 W m^{-2} at 12 noon) (Fig. 5A–D).

Bacterioplankton abundances (BA) showed small changes among stations with the exception of the spring 2015 (Fig. 3), when maximum values were observed at BLL2 (3990 10^3 cell mL^{-1}). The cyanobacteria *Synechococcus* sp. showed abundances generally below 10 10^3 cell mL^{-1}, but increased to an average of 27 ± 20 10^3 cell mL^{-1} and a maxima of 78 10^3 cell mL^{-1} at station BLL3 during spring 2015.

3.2. In situ carbon uptake in central Chile (Llico Bay)

Carbon uptake rates were variable among stations (Fig. 4) but were generally higher in surface compared to deeper levels. Station BLL1 (Fig. 4A–E) showed constant levels of carbon fixation (398 ± 114 mg C m^{-3} d^{-1}) in surface waters, except in autumn 2016 when the lowest rates were obtained (48 ± 9 mg C m^{-3} d^{-1} and 57 ± 3 mg C m^{-3} d^{-1}, 2 and 4 m depth respectively). At 4 m depth, average rates were close to 340 ± 88 mg C m^{-3} d^{-1}. Station BLL2 showed maximum rates at 2 m in spring 2014 and summer 2016 (1215 mg C m^{-3} d^{-1} and 571 mg C m^{-3} d^{-1}, respectively; Fig. 4F and I) and minimum values in winter 2015 and autumn 2016 (283 mg C m^{-3} d^{-1} and 298 mg C m^{-3} d^{-1}, respectively; Fig. 4G and J). At 10 m depth, average carbon uptake decreased to an average value of 42 ± 88 mg C m^{-3} d^{-1}. Carbon uptake rates at BLL3 were variable in surface waters with maximum values of 513 mg C m^{-3} d^{-1} in autumn 2016 and minimum values of 174 mg C m^{-3} d^{-1} in winter 2015. Rates also showed a subsurface maximum at 10 m in spring 2014 and 2015 (496 ± 15 mg C m^{-3} d^{-1} and 217 ± 12 mg C m^{-3} d^{-1}, respectively; Fig. 4K and M).

Integrated primary production at stations BLL2 and BLL3 showed high and similar primary production levels during summer (3.69 and 3.80 g C m^{-2} d^{-1}, respectively). Winter values of integrated primary production rates reached 0.65 g C m^{-2} d^{-1} for BLL2 and 0.43 g C m^{-2} d^{-1} for BLL3.

After addition of pesticides, rates of carbon uptake showed variable values compared to the control (^{13}C only) at all stations and seasons (Fig. 4O). Results for paired *t*-test per station (Table 3) confirmed different responses of carbon assimilation rates to the addition of pesticides and combinations of pesticides. During spring 2014, lower rates were observed with ^{13}C + D (specifically at BLL1 station; paired t–test, $p < 0.001$) compared to the control. The reduction accounted for 23 and 30% at 2 and 4 m, respectively. For winter 2015, only station BLL3 showed increased C fixation in the presence of ^{13}C + D (paired *t*-test, $p < 0.003$). The increase was close to 6 and 54% at 2 and 10 m, respectively. Finally, no significant differences were found for pesticide additions at any station during the autumn season 2016 (Fig. 4E, J, O).

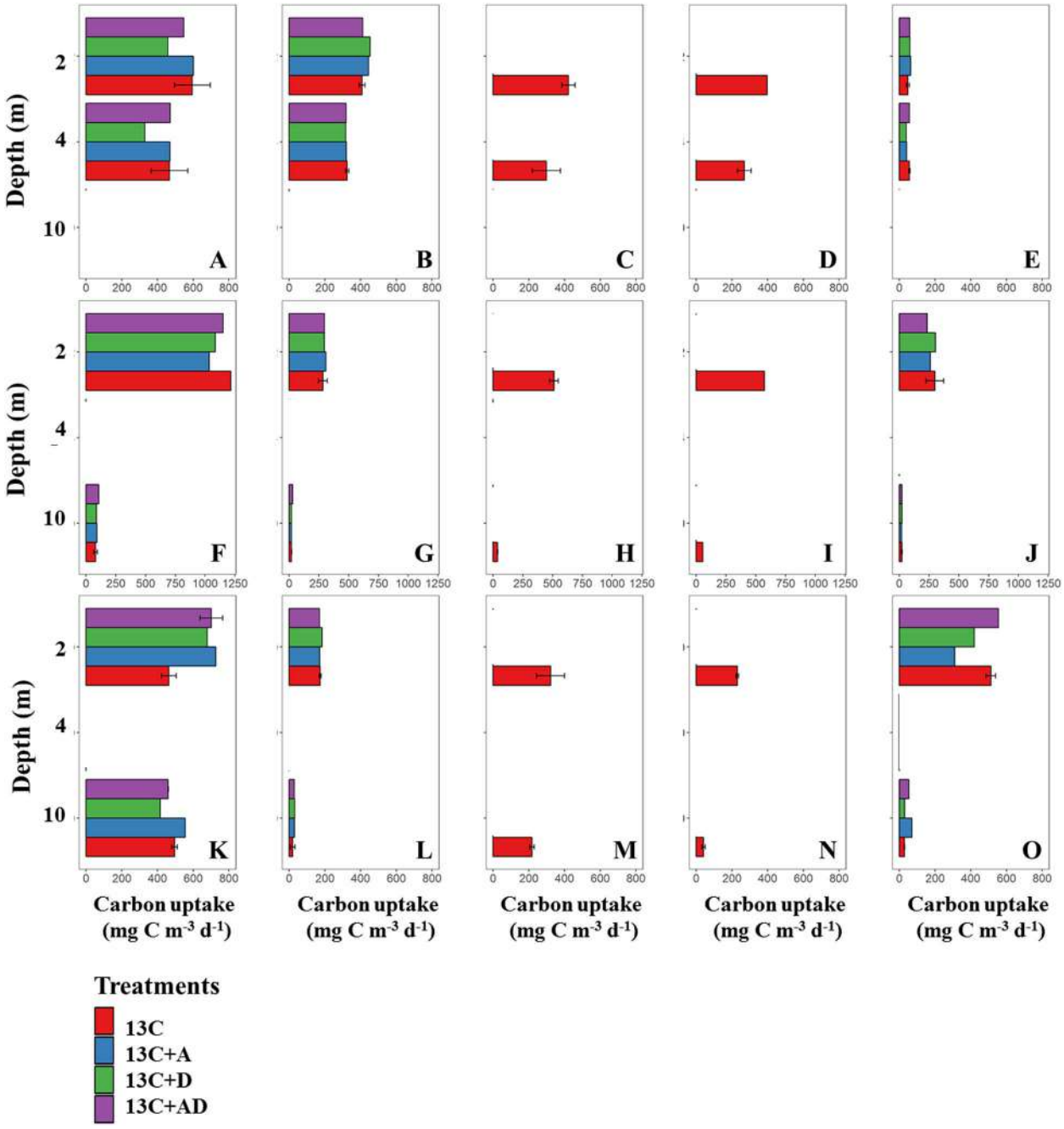

Fig. 4. Carbon uptake rates (mgC m^{-3} d^{-1}) per depth at BLL1 (upper panels), BLL2 (middle panels) and BLL3 (lower panels) stations (Llico Bay). A, F, K) spring 2014 B, G, L) winter 2015 C, H, M) spring 2015 D, I, N) summer 2016 E, J, O) autumn 2016. Error bars represent standard deviation (duplicate).

3.3. Pesticide effect on surface photoautotrophic and chemoautotrophic carbon uptake

Time course experiments showed that after 2 h of incubation, photoautotrophic carbon uptake (Fig. 5E–H) was higher in spring and summer (37.55 and 45.99 mg C m^{-3} h^{-1}, respectively) compared to autumn (7.89 m C m^{-3} h^{-1}) and winter (24.01 mg C m^{-3} h^{-1}). Dark C fixation rates were high at all seasons with maximum values (26.55 and 29.39 mg C m^{-3} h^{-1} after 2 and 6 h of incubation, respectively) in spring 2015 (Fig. 5I–L). In the experiment carried out in spring 2015, chemoautotrophic uptake represented 70%–74% of photoautotrophic activity at 2 and 4 h of incubation. In contrast, winter chemoautotrophic activity represented only 5 to 20% of photoautotrophic carbon fixation. A 2-way ANOVA for dark incubations showed significant differences in each experiment (Table 4). Only in spring 2015 the experiment showed a significant effect of pesticides on dark carbon uptake ($p = 0.001$) and also in the interaction of the factors (Pesticides per Incubation time, $p = 0.028$). These carbon uptake rates were significantly different ($p < 0.05$) in the treatments $^{13}C + B$ and $^{13}C + ADB$ compared to the rest of the treatments and the control both at 2 and 6 h of incubation (Table 4).

Results of a 2-way ANOVA under light conditions showed significant differences for all the experiments. Carbon uptake rates for spring and winter 2015 experiments under light conditions showed significant differences between the ^{13}C control and the treatments $^{13}C + A$ and $^{13}C + B$ ($p = 0.012$ and $p < 0.001$, respectively). After 6 h of incubation, only significant differences between $^{13}C + A$ and $^{13}C + B$ ($p = 0.042$) were found. Phototrophic carbon uptake rates

Table 3
Results from pairwise *t*-test for the on deck carbon fixation experiments performed in Llico Bay.

Season	Light Condition	Incubation time	Incubation time	2						6				
			Pesticides	13C	13C + A	13C + AD	13C + ADB	13C + B	13C + D	13C	13C + A	13C + AD	13C + ADB	13C + B
Winter 2015	Light	2	13C + A	+										
			13C + AD	na	na									
			13C + ADB	na	na	na								
			13C + B	*	+	na	na							
			13C + D			na	na	*						
		6	13C		+		na	na	+					
			13C + A	+			na	na	+					
			13C + AD	na	na	na	na	na	na	na	na			
			13C + ADB	na	na	na	na	na	na	na	na	na		
			13C + B	*	+	na	na		*	+	+	na	na	
			13C + D	+		na	na	+				na	na	+
Spring 2015	Dark	2	13C + A											
			13C + AD											
			13C + ADB	+	+	+								
			13C + B	+	+	+								
			13C + D				+	+						
		6	13C				+	+						
			13C + A				+	+						
			13C + AD				+	*						
			13C + ADB	*	*	*			*	*	*	*		
			13C + B	*	*	*			*	*	*	*		
			13C + D				+	*					*	*
Spring 2015	Light	2	13C + A											
			13C + AD											
			13C + ADB	*	*	*								
			13C + B	*	*	*								
			13C + D				*	*						
		6	13C				*	*						
			13C + A				*	*						
			13C + AD				*	*						
			13C + ADB	*	*	*			*	*	*	*		
			13C + B	*	*	*			*	*	*	*		
			13C + D				*	*					*	*
Summer 2016	Light	2	13C + A	na										
			13C + A + D		na									
			13C + ADB		na									
			13C + B	na	na	na	na							
			13C + D		na			na						
		6	13C	na	na	na	na	na	na					
			13C + A		na			na		na				
			13C + AD		na			na		na				
			13C + ADB		na			na		na				
			13C + B		na			na		na	+	+		
			13C + D		na	+	+	na		na			+	+

+: Represents $p < 0.05$. *: Represents $p < 0.001$. na: No comparison.

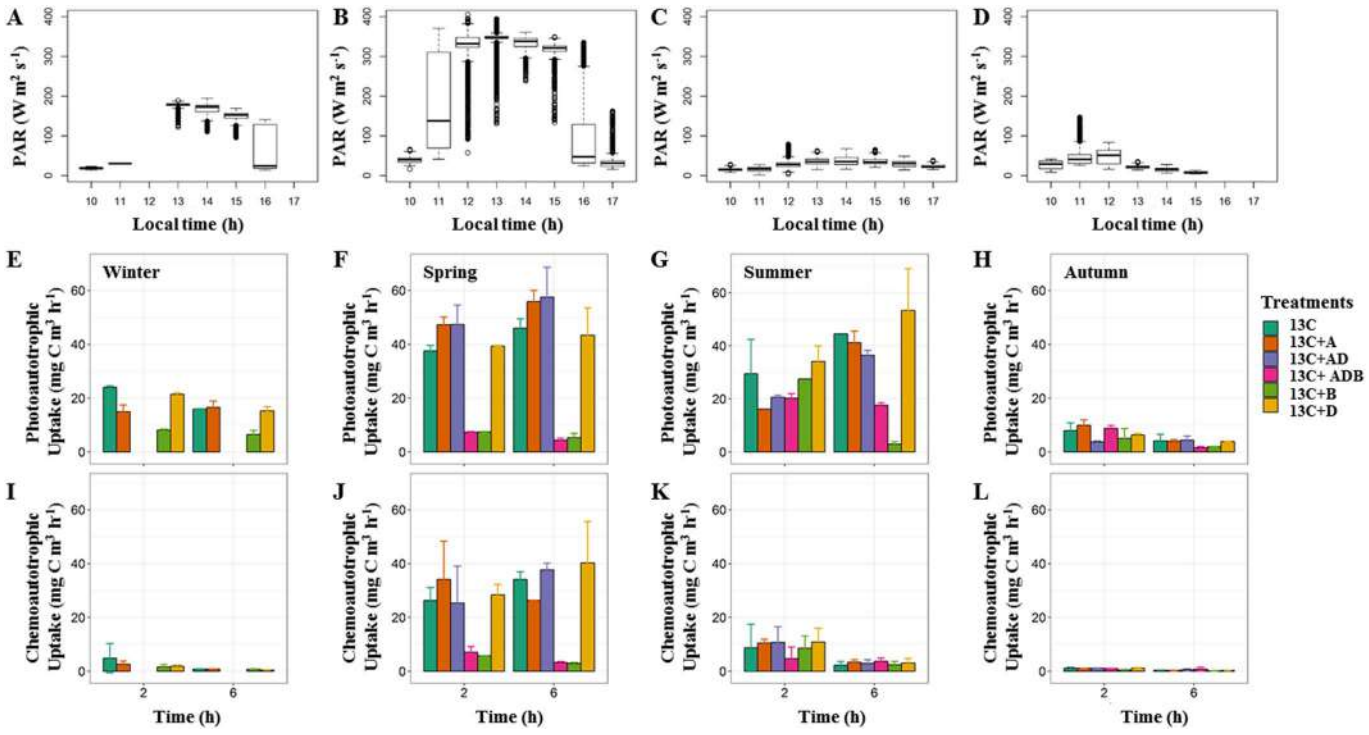

Fig. 5. Boxplot of PAR radiation (Wm^{-2}) and photoautotrophic and chemoautotrophic carbon uptakes ($mg\ C\ m^{-3}\ h^{-1}$) obtained during on deck experiments under light and dark conditions with the addition of ^{13}C (control) and pesticides. Experiments were performed with samples of station BLL2 at Llico Bay during four field campaigns. A, E, I) winter 2015 B, F, J) spring 2015 C, G, K) summer 2016 D, H, L) autumn 2016. Bars represent mean of duplicate values. Error bars show standard deviation.

Table 4
Results from 2-way ANOVA examining the effect of pesticides and the time of incubation on the rates of carbon uptake under dark and light conditions at Llico Bay.

Season	Date	Source of variation	d.f	Dark		d.f	Light	
				F	P		F	P
Winter 2015	July 205	Pesticides	3	0.516	0.688	3	59.800	**0.001**
		Time	1	14.213	**0.007**	1	24.921	**0.001**
		Pesticides × time	3	0.134	0.957	3	9.152	**0.010**
		Residuals	8			8		
Spring 2015	October 2015	Pesticides	5	56.720	**0.001**	5	216.539	**0.001**
		Time	1	0.917	0.357	1	0.479	0.548
		Pesticides × time	5	4.058	**0.029**	5	4.800	**0.028**
		Residuals	12			12		
Summer 2016	January 2016	Pesticides	5	0.320	0.899	5	4.944	**0.008**
		Time	1	14.099	**0.007**	1	3.453	0.078
		Pesticides × time	5	0.406	0.829	5	3.291	**0.031**
		Residuals	12			12		
Autumn 2016	April 2016	Pesticides	5	1.901	0.157	5	2.125	0.138
		Time	1	26.395	**0.004**	1	26.380	**0.001**
		Pesticides × time	5	0.787	0.568	5	2.482	0.097
		Residuals	12			12		

Significant values are presented in bold.

during spring 2015 were significantly different ($p < 0.05$) after 2 and 6 h of incubation in treatments amended with ^{13}C + B and ^{13}C + ADB in comparison with all other pesticide treatments and the control (Table 4). Light treatments during summer 2016 did not show significant differences with respect to the control after 2 h of incubation ($p > 0.05$). However, the treatments ^{13}C + B and ^{13}C + ADB were significantly different to all the treatments after 6 h of incubation ($p < 0.05$). No significant differences were found between ^{13}C + D and ^{13}C + AD ($p = 0.670$).

3.4. Seasonal environmental variability in Caucahue channel (Chiloé)

Caucahue Channel showed seasonal variability with lower temperature values during winter (11 °C) in the first 30 m of the water column compared with summer (Supplementary Fig. 2). Salinity on the other hand showed stronger vertical variations in winter compared to summer. During winter, profiles showed the influence of freshwater restricted to the first 2 m and values then remained stable with depth.

Nitrate concentrations in winter and summer were generally below 5 μmol L^{-1} with the exception of Q2, Q6 and Q5 which showed maximum values (Fig. 6A) in surface and 20 m (Q2), 20–30 m (Q6) and 20 and 50 m depth (Q5). Phosphate concentrations during summer did not increase with depth showing average concentrations of 0.5 μmol L^{-1} in the water column with the exception of stations Q5 and Q6. However values increased with depth in winter reaching ~2 μmol L^{-1} at 60 m depth at station Qc (Fig. 6B, F). The N:P ratio varied with depth and

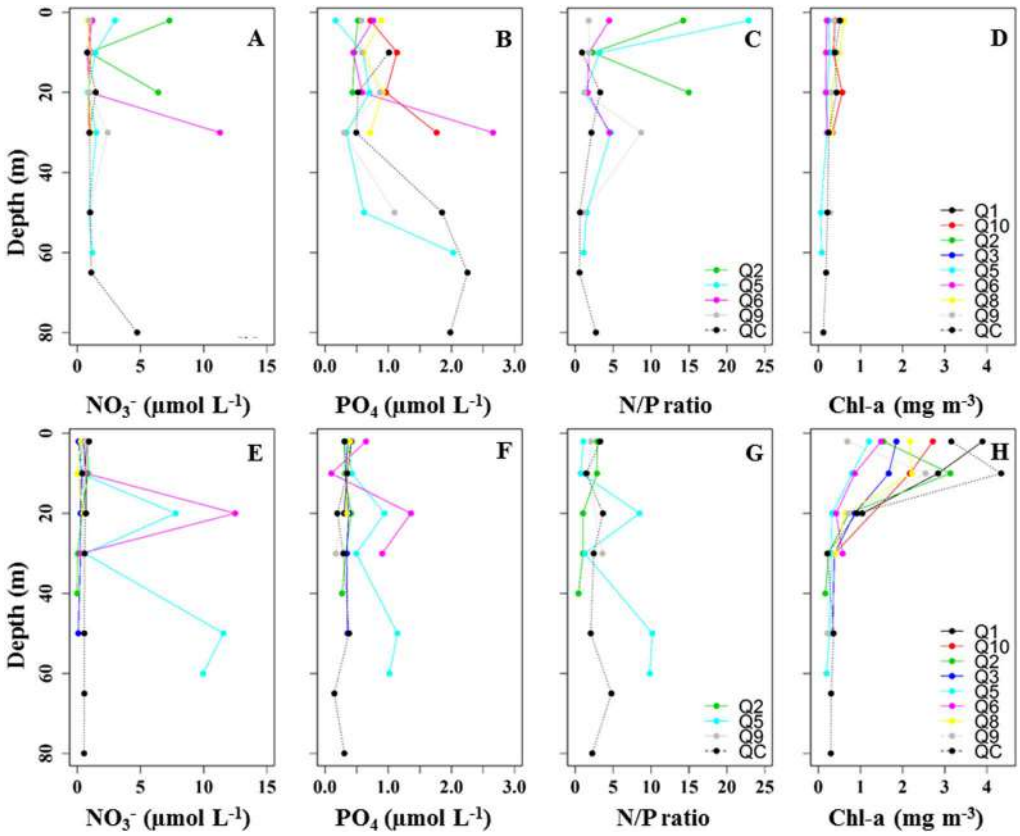

Fig. 6. Vertical profiles for nitrate (µmol L^{-1}), phosphate (µmol L^{-1}), N/P ratio and Chl-*a* (mg m^{-3}) for stations located in Caucahue. Winter 2014: of A) NO_3^-, B) PO_4^{3+}, C) N/P ratio and D) Chl-*a*. Summer 2015 of E) NO_3^-, F) PO_4^{3+}, G) N/P ratio and D) Chl-*a*.

between stations as well as seasonally (Fig. 6C, G). Station Qc showed the lowest average N/P ratio (1.71 ± 1.17, 2.84 ± 1.14 in winter and summer, respectively) while the highest values were obtained at stations Q2 and Q5, particularly in winter (14.95 and 22.84, at 20 and 2 m respectively).

Winter concentrations of Chl-*a* were on average 4 times lower than summer values (Fig. 6D, H). In winter, stations Q8 and Qc showed the highest concentrations with surface levels around 0.613 mg Chl-*a* m^{-3} and 0.516 mg Chl-*a* m^{-3}, respectively. In summer the stations with highest concentrations were Q1 (3.894 mg Chl-*a* m^{-3}), Q2 (1.544 mg Chl-*a* m^{-3}), Q3 (1.855 mg Chl-*a* m^{-3}) and Qc (3.155 mg Chl-*a* m^{-3}).

Microbial abundances in the Caucahue Channel varied between stations and seasons. In winter, bacterioplankton was more abundant at Q6, Q8, Q9 and Q10 compared to Qc (Fig. 7A, B). In summer, the abundance of bacterioplankton was 6-times higher than winter (Fig. 7C). The maximum abundances were observed at stations Q9 (1837×10^3 cell mL^{-1}), Q10 (1632×10^3 cell mL^{-1}) and Q1 at 10 m depth (1736×10^3 cell mL^{-1}; Fig. 7D). Station Qc presented abundances lower than 1000×10^3 cell mL^{-1}. Overall, abundances of *Synechococcus* sp. were 50 times higher in summer compared to winter (Fig. 8). Higher values were observed at stations Qc, Q8 and Q9 ($\sim 3 \times 10^3$ cell mL^{-1}) in winter 2014 (Fig. 8A, B). Summer 2015 showed the highest abundances at stations Q9, Q10 and Q1 located at the southern area of the channel.

3.5. Primary production in Caucahue channel

Primary production estimated by in situ incubations showed high variability throughout the channel and between seasons (Figs. 9 and 10). During winter, carbon uptake reached 10 mg C m^{-3} d^{-1} at 2 m and decreased with depth. At station Qc the surface uptake rate was the highest observed (20 mg C m^{-3} d^{-1} at 2 m). Pesticide additions resulted in an increase in C fixation rates, only for the treatment ^{13}C + A which was only significant at station Qc (paired *t*-test, p = 0.028). Rates presented an increase of 8, 23 and 49% at 2, 10 and 30 m, respectively. Treatment ^{13}C + D and the combined treatment A + D did not show significant changes in in situ primary production rates. Integrated primary production for Q2 and Q9 reached 0.13 g C m^{-2} d^{-1} and 0.22 g C m^{-2} d^{-1}, while station Qc reached levels of 0.44 g C m^{-2} d^{-1}.

During summer the magnitude of carbon uptake in surface waters (2 m) varied from 90 mg C m^{-3} d^{-1} at station Q9 to 170 mg C m^{-3} d^{-1} at station Q2. Station Qc showed high rates compared to the channel, reaching 378 mg C m^{-3} d^{-1}. On the other hand, the treatments inoculated with pesticides did not show significant differences with their respective controls at any station (paired *t*-test, p > 0.05). Estimations of integrated primary production showed the highest values at Qc (7.44 g C m^{-2} d^{-1}) for the summer season (Table 5). Stations Q9 and Q2 reached 3.36 g C m^{-2} d^{-1} and 2.59 g C m^{-2} d^{-1}, followed by Q3 (1.45 g C m^{-2} d^{-1}). Finally, station Q3 presented the lowest magnitude in the Caucahue channel with 0.76 g C m^{-2} d^{-1}.

4. Discussion

Our study was conceived as a contribution to the understanding of the potential responses of photoautotrophic and chemoautotrophic carbon uptake to the presence of the anti-lice pesticides azamethiphos, deltamethrin and emamectine benzoate. We reported carbon uptake rates using both in situ and on deck experimental approaches in two economically important areas in central (~37°S) and southern (~42°S) Chile. It is the first study oriented specifically to microbial communities involved in primary production, with implications for the local carbon biogeochemical cycle.

Seasonal and spatial environmental variability was clear both in Llico Bay and Caucahue channel with the highest TChl-*a* concentrations during the spring-summer productive periods. Primary production rates

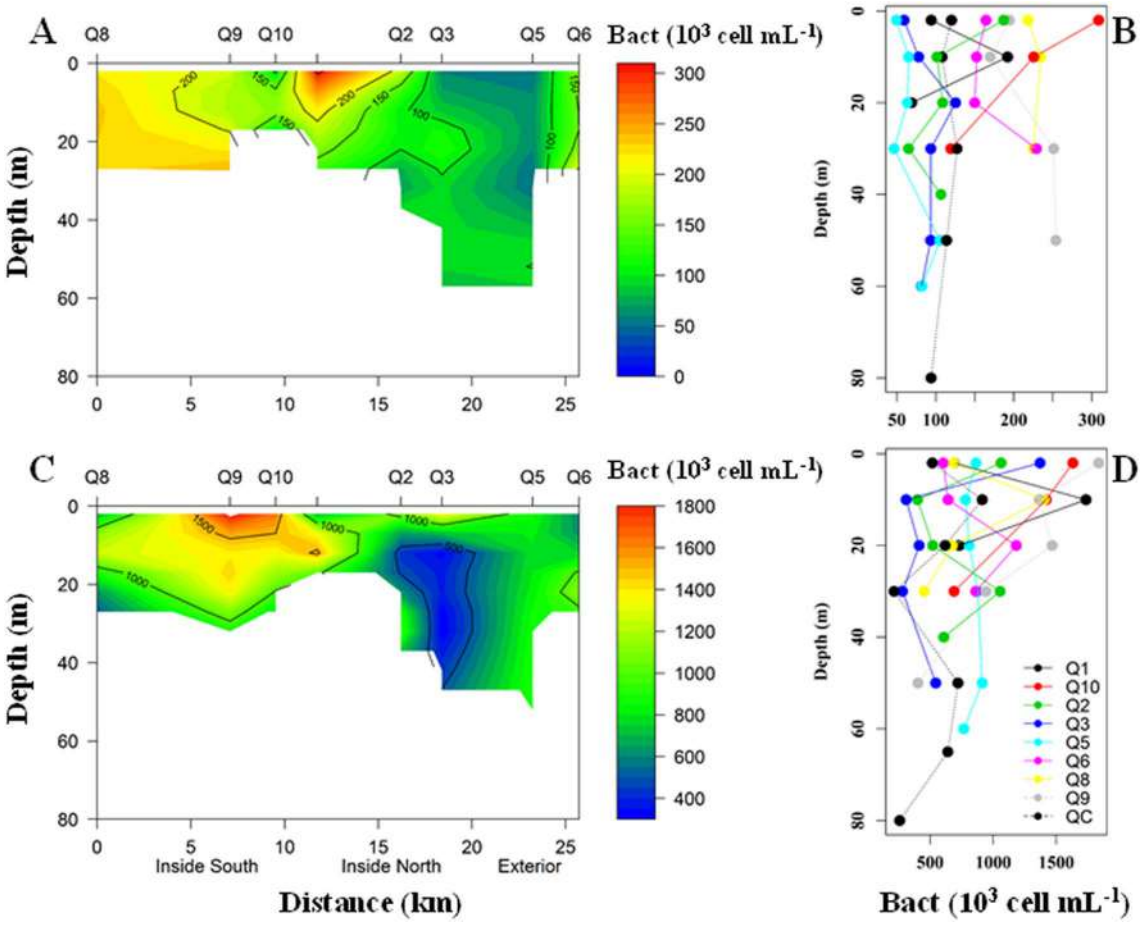

Fig. 7. Spatial distribution and vertical profiles of bacterioplankton abundance (10^3 cell mL^{-1}) at Caucahue channel. A, B) winter 2014 C, D) summer 2015.

at Llico Bay were lower than gross primary production values previously reported for the Humboldt Current System (Daneri et al., 2000; Montero et al., 2007; Vargas et al., 2007). However, they fall within the range of carbon fixation reported for central Chile (Montero et al., 2007) in winter and spring-summer (Table 6). Coastal station BLL2 showed maximum values exceeding 1 g m^{-3} d^{-1} in summer 2016. Integrated PP was also high near the coast, reaching 3.8 g C m^{-2} d^{-1}.

These values for Llico Bay exceed those estimated for stations inside the Caucahue channel but are close to values from control station Qc which was the most productive station sampled even compared with stations inside the Caucahue Channel and previous gross primary production estimations for the Inner sea of Chiloé (Gonzalez et al., 2010; González et al., 2010; Iriarte et al., 2016). Also, summer primary productivity registered at station Qc was similar to previously published data for the region (Aracena et al., 2011; Cuevas et al., 2004; González et al., 2010; Jacob et al., 2014) and also for other Chilean Patagonian fjords (González et al., 2010). Integrated primary production showed differences of 15 and 20 fold between summer and winter at stations inside the Caucahue channel while the highest integrated primary production was registered at st Qc (Gonzalez et al., 2010; González et al., 2010; Iriarte et al., 2016). Consequently, we believe the conditions encountered during our study are representative of the annual patterns of variability in central-southern Chile for photoautotrophic carbon fixation.

The impact of pesticides on carbon uptake processes was variable and significant effects were detected in the treatments with a single pesticide at Llico Bay (emamectin benzoate and deltamethrin), and at Caucahue Channel (azamethiphos). However, combined treatments did not have a significant impact on photoautotrophic primary production. The use of incubations to assess the in situ responses of primary production to pesticides revealed an increase in rates related to the addition of the organic phosphorous compound azamethiphos during the winter Caucahue campaign, and this effect was local (observed only at station Qc; Fig. 9). These findings are in contrast with the only field study on the effect of azamethiphos on microorganisms (Burridge et al., 2010). In that study, no changes in dissolved oxygen and Chl-*a* levels were observed during or after pesticide addition, suggesting neutral effects on primary production. Although we did not follow Chl-*a* concentrations during our incubations, our PP fluxes suggest a potentially significant effect of pesticides in the study areas with the addition of single-pesticide treatments. Although combined treatments did not show significant changes in carbon fixation rates, our results with single pesticide amendments have implications for the synchronization of pesticide treatments in neighboring salmon farming facilities.

At station Qc, the highest primary production co-occurred with the highest *Synechococcus* sp. abundance, Chl-*a* concentrations and the lowest N/P ratio of all field campaign (Fig. 6D, H). The low N/P ratios found in Caucahue and particularly at station Qc are on the range of previous results obtained for the Inner Sea of Chiloé, suggesting the presence of waters with nitrogen deficiency compared to P (Iriarte et al., 2007; Olsen et al., 2017; Olsen et al., 2014). Under these conditions, the use of the organophosphate insecticide azamethiphos (Mayor et al., 2008), a highly soluble compound (Burridge et al., 2010; Canty et al., 2007) could potentially represent a source of P and N in periods of deficiency in zones with high productivity and low nutrients availability. However, little information has been reported of nitrogen enrichment in the water column at the inner Sea of Chiloé as a result of aquaculture activity (Soto and Norambuena, 2004).

Emamectin benzoate produced a 60 to 90% decrease in photoautotrophic primary production during the experiments performed in

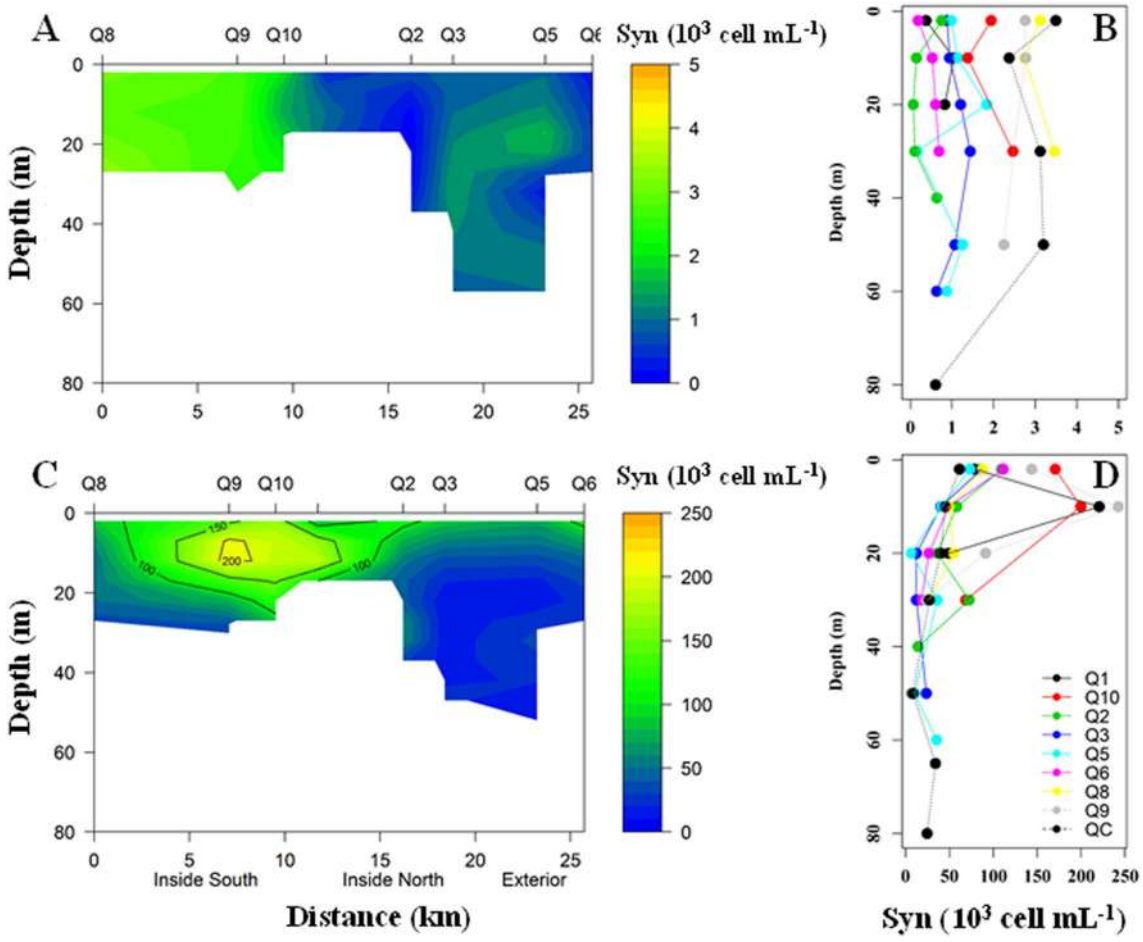

Fig. 8. Spatial distribution and vertical profiles of *Synechococcus* sp. abundance (10^3 cell mL^{-1}) at Caucahue channel. A, B) winter 2014 C, D) summer 2015.

Llico Bay. This effect was observed in spring, summer and even in winter but no changes were observed in autumn. However, chemoautotrophic primary production was only decreased during the spring season (70–80%). The avermectin emamectin benzoate is a semi-synthetic derivative produced by *Streptomyces avermetilis* (Bravo et al., 2008; Cárcamo et al., 2011; Stone et al., 2000). The absence of

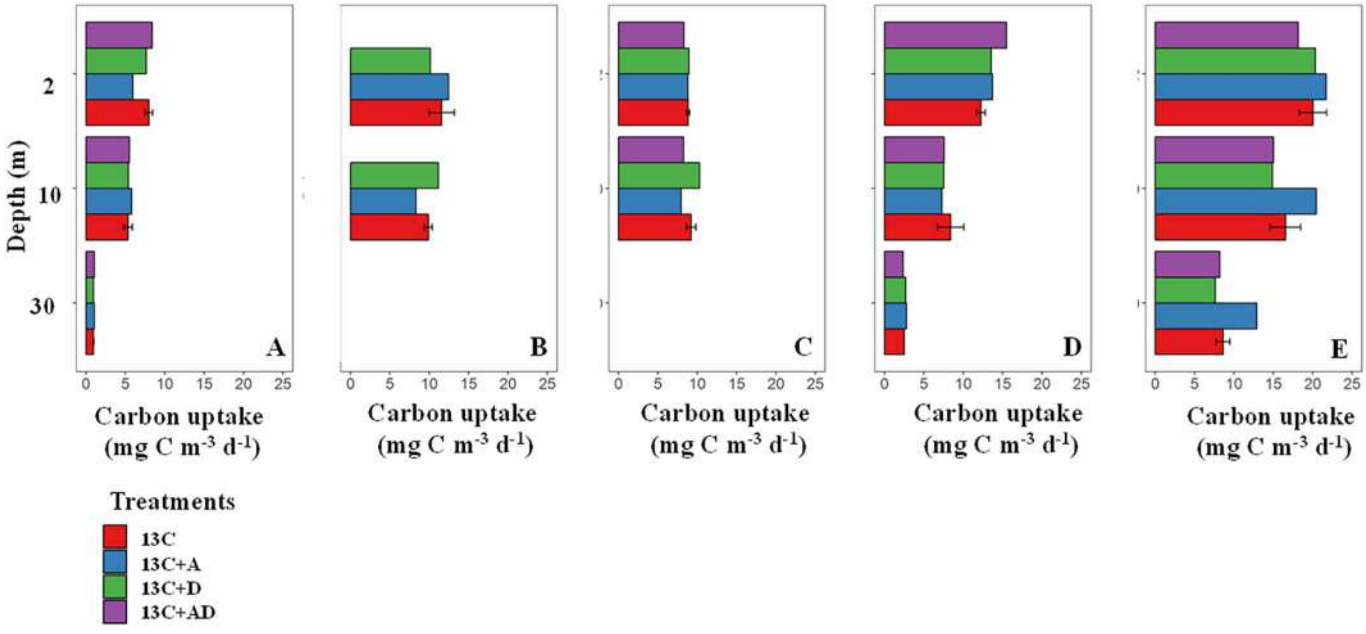

Fig. 9. Carbon uptake rates (mg C m^{-3} d^{-1}) with the addition of pesticides at Caucahue Channel during winter 2014. A) Q2, B) Q5, C) Q6, D) Q9 and E) Qc. Error bars represent standard deviation (duplicate).

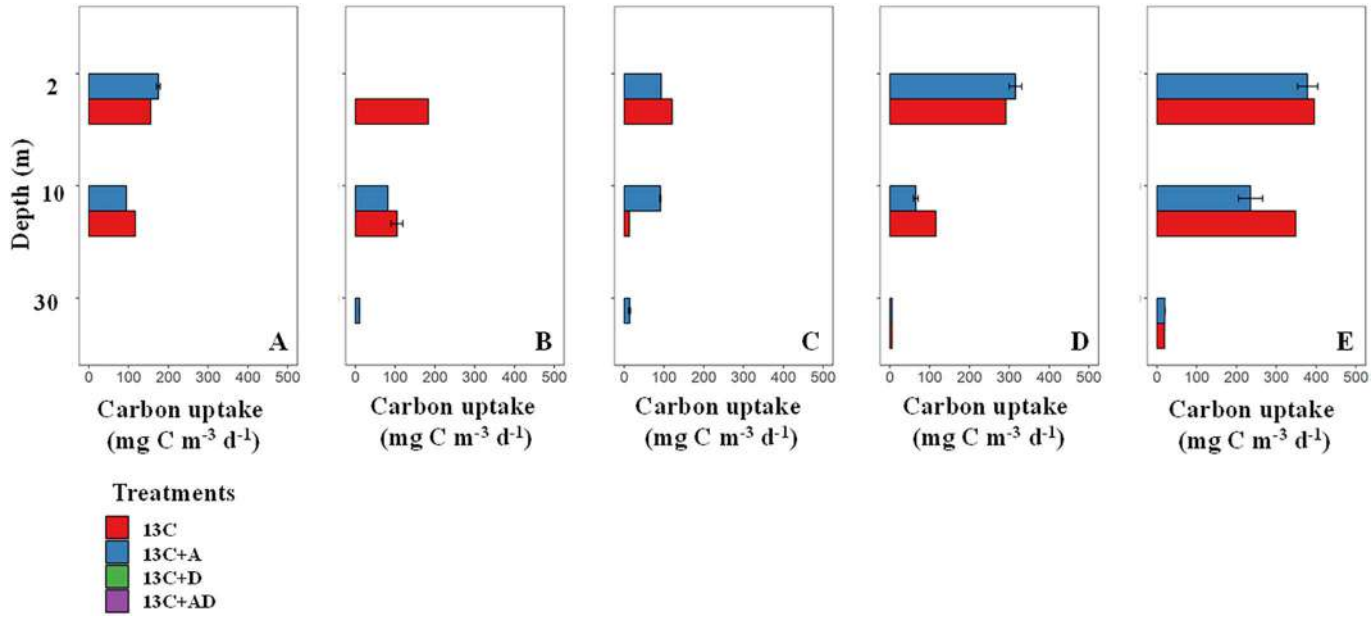

Fig. 10. Carbon uptake rates (mgC m^{-3} d^{-1}) with the addition of pesticides during summer 2015. A) Q2, B) Q3, C) Q5, D) Q9 and E) Qc. Error bars represent standard deviation (duplicate).

Table 5
Summary of daily integrated primary production in the Caucahue channel.

Station	Winter 2014 (gC m^{-2} d^{-1})	Summer 2015 (gC m^{-2} d^{-1})
Q2	0.13	2.59
Q3		3.34
Q5	0.31	1.03
Q6	0.28	
Q9	0.22	3.36
Qc	0.44	7.44

significant effects of the commercial product emamectin benzoate (SLICE®) on to non-target species have been reported by many authors, suggesting that this compound is not toxic to organisms at the recommended concentrations (Burridge et al., 2010; Burridge et al., 2004; Willis and Ling, 2003) or that the effect is limited to a small number of organisms (Waddy et al., 2002). A particularly toxic effect has been described in different species of mammals (Olsvik et al., 2008; Yen and Lin, 2004), fishes (Horsberg, 2012), invertebrates (Burridge et al., 2004; Grant, 2002; Ioriatti et al., 2009) and even acute intoxication in humans (Yen and Lin, 2004). It therefore has the potential to produce a negative impact altering the structure and diversity within the indigenous organisms at different trophic levels (Burridge et al., 2010; Johnson et al., 2004). Given the available evidence for bioaccumulation, our results imply a potential impact for herbivores as well as at higher trophic levels in the water column.

For microorganisms, toxic or inhibitory effects of pesticides could depend on the microbial species exposed and community composition (DeLorenzo et al., 2001). A recent study suggests that changes in diversity can impact the appearance of resistance to chemical treatments (Becker and Liess, 2015). As this is (to the best of our knowledge) the first study providing evidence of negative effects of emamectin benzoate on natural carbon fixing microbial communities, our results can potentially affect local residence time and cycling of organic matter by modifying community composition and fluxes. More studies are needed to accurately tackle this issue.

Table 6
Comparison of Integrated Primary Production estimations obtained during this study compared with published values reported for the same area.

Location	Season	Primary productivity	Source
Chilean Patagonian fjord	Winter	0.2–0.3 g C m^{-2} d^{-1}	Gonzalez et al., 2010
	Spring	5.1 g C m^{-2} d^{-1}	
Inner Sea of Chiloé	Spring	4.5 g C m^{-2} d^{-1}	Jacob et al., 2014
Inner Sea of Chiloé	Spring-summer	2.4–5.8 g C m^{-2} d^{-1}	Aracena et al., 2011
Northern Patagonia		1.6–2.6 g C m^{-2} d^{-1}	
Inner Sea of Chiloé	Winter-spring	0.1–3.2 g C m^{-2} d^{-1}	Gonzalez et al., 2010
Inner Sea of Chiloé	Winter	0.4 g C m^{-2} d^{-1}	This study
	Summer	7.4 g C m^{-2} d^{-1}	
Caucahue Channel	Winter	0.1–0.2 g C m^{-2} d^{-1}	This study
	Summer	2.5–3.3 g C m^{-2} d^{-1}	
Concepción Bay	Spring-early autumn	1–25.8 g C m^{-2} d^{-1}	Montero et al., 2007[a]
	Autumn-early spring	0–0.8 g C m^{-2} d^{-1}	
Concepción Bay		0.5–5.5 g C m^{-2} d^{-1}	Vargas et al., 2007
Concepción Bay	Winter	0.3–0.5 g C m^{-2} d^{-1}	Cuevas et al., 2004
	Spring	1.2–8.7 g C m^{-2} d^{-1}	
Concepción Shelf	Spring	1.2–5.9 g C m^{-2} d^{-1}	Daneri et al., 2000
Llico Bay	Autumn-winter	0.2–0.6 g C m^{-2} d^{-1}	This study
	Spring-summer	1.9–3.8 g C m^{-2} d^{-1}	
Iquique and Antofagasta		3–9 g C m^{-2} d^{-1}	Thiel et al., 2007

[a] Reported one of the highest value for the HCS.

Addition of deltamethrin resulted in a variety of responses of primary production in Llico Bay but effects were absent in the Caucahue channel. Decreasing rates were detected in spring 2014 for station BLL1, where carbon uptake dropped by 23–30%. However, increasing rates were also observed in winter 2015 for station BLL3 in which primary production increased between 6 and 54%. Deltamethrin is a synthetic pyrethroid (Bhanu et al., 2011; Leboulanger et al., 2009; Pavan et al., 1999) that has shown low toxicity for mammals (Burridge et al., 2010; Rehman et al., 2014) and high toxicity to arthropods (Knapp et al., 2005). There are no reports of a direct effect on microorganisms but Knapp et al. (2005) explained that the addition of deltamethrin could cause a decrease of zooplanktonic arthropods. The subsequent release of nutrients via reduced grazing pressure on phytoplankton and bacterial populations, could therefore temporarily allow phytoplankton growth. A similar situation was described for fenvalerate, a pyrethroid insecticide which addition was associated to non-permanent grow on phytoplankton abundance and a decrease in zooplankton abundance, generating a nutrient input from the deceased zooplankton to the system (DeLorenzo et al., 2001). In both studies, just the pesticide addition did not produce an effect in phytoplankton abundance, but rather an increased supply of nutrients and/or light was necessary for a long-term stimulation. This could explain the temporal variability of our results. From the data obtained in our study, it appears that the use of anti-lice treatments can modify carbon fixation fluxes and therefore have an impact on local biological productivity. These effects can eventually co-occur with nutrient inputs from aquaculture that can potentially exacerbate the changes observed on carbon fluxes.

In summary, the response of microbial communities exposed to the addition of different pesticides can be variable, depending on the compound used and the seasonal variability of the target area. In some cases, the effect may not be direct and can require special conditions in the system to produce an impact, such as light intensity levels, nutrients concentration or zooplankton abundance (predation pressure). These diverse responses of the environment to the use of chemicals in the salmon farming, evidence the need of more studies in order to understand which environmental factors can influence the effects of pesticides on primary production and therefore on the local carbon cycle. Considering that primary production sustains the marine food chain and others economic activities, such as bivalve culture and fisheries, unraveling the variability of pesticide effect is relevant to determining the overall impact of aquaculture on natural ecosystems.

5. Conclusion

In conclusion, the use of pesticides in marine waters can produce changes on microbial photo and chemoautotrophic carbon uptake. Although variable, these effects show significant alterations of carbon fixation fluxes if a single pesticide is applied as opposed to a combination of two or more compounds. Emamectin benzoate can potentially act as a depressor of carbon fixation while azamethiphos can stimulate primary production in conditions of nutrient limitation or deficiency. The effect of pesticides may be related to the magnitude of primary production and favorable conditions for phytoplankton activity. It is also related to nutrient deficiency as observed for azamethiphos in the Caucahue channel. Considering the increasing global importance of aquaculture, our study provides important evidences on the fate and microbial utilization of pesticides in marine environments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2017.12.048.

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