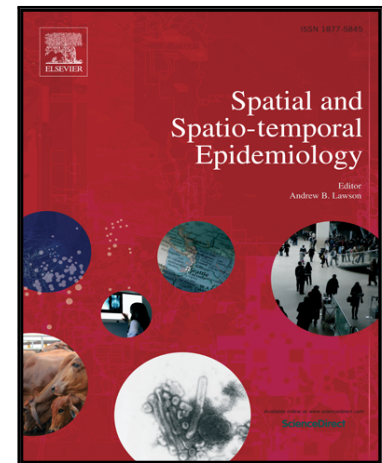


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Highlights

- The global clustering analysis suggests the response of *C. rogercresseyi* to pyrethroids is spatially structured and distance-dependent.
- The local clustering analyses indicate there were two areas, one located in northern Los Lagos region and the other in central Aysén region, where the post-treatment adult lice level attributed to the farm effect was significantly higher than in the rest of the study area.
- These spatial clusters remained even once we adjusted for environmental and management predictors, suggesting unknown factors were causing the clustering in these areas.
- One potential factor that may explain the clustering is resistance to pyrethroids of local sea lice populations. Further investigation should be carried out to confirm this finding.

A multivariable assessment of the spatio-temporal distribution of pyrethroids performance on the sea lice *Caligus rogercresseyi* in Chile

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Abstract

Synthetic pyrethroids have been widely used in Chile to control the sea lice *Caligus rogercresseyi*, a major ectoparasite of farmed salmon. Although resistance of *C. rogercresseyi* to pyrethroids has been reported in Chile, there is no information regarding the geographic extent of this problem. In this study we explored the spatial and temporal variation of *C. rogercresseyi*'s response to pyrethroids in Chile from 2012 to 2013. We modeled lice abundance one week after treatment with a linear mixed-effects regression, and then we performed spatial and spatio-temporal cluster analyses on farm-level effects and on treatment-level residuals, respectively. Results indicate there were two areas where the post-treatment lice counts were significantly higher than in the rest of the study area. These spatial clusters remained even once we adjusted for environmental and management predictors, suggesting unmeasured factors (e.g. resistance)

were causing the clustering. Further investigation should be carried out to confirm this hypothesis.

Keywords

Caligus; pyrethroids; multivariable analysis; spatio-temporal analysis

1. Introduction

The copepods *Lepeophtheirus salmonis* and *Caligus rogercresseyi*, commonly called sea lice, are considered the most important ectoparasites that affect farmed salmonids around the world (Burka et al., 2012; Costello, 2006). Although rarely life threatening, when sea lice are numerous on fish they can produce extensive skin damage, osmotic stress, and increase susceptibility to secondary infections (Costello, 2006; González et al., 2000; Johnson et al., 2004). Cost increases are related to reduction of growth, reduced feed-conversion efficacy of fish, delousing treatments, and reduced marketability of the final product (Costello, 2009). In regions where the salmon industry is located in areas with important populations of wild salmon such as British Columbia and Norway there is concern about the potential negative impact of sea lice from farmed salmon on wild stocks, although this question is under active debate (e.g. Brooks, 2005; Brooks and Stucchi, 2006; Krkošek et al., 2007; Krkošek et al., 2013).

The Chilean salmon industry represents an important economic sector at the provincial and national levels. In 2016, this industry produced 727,812 tons of farmed salmonids of which 73% was Atlantic salmon, 15% Coho salmon and 12% rainbow trout (Sernapesca, 2017). Projections for 2017 indicate the total production will surpass 800 thousand tons. In 2016, this industry

accounted for 5.8% of total exports of the country (DIRECON, 2017). The Chilean salmon industry consists of about 500 companies that employ more than 50,000 people, including salmon growing, salmon processing, and service companies. During 2016, more than 490 fish farms (fresh or sea water facilities) reported commercial activity (Sernapesca, unpublished data). Internationally, Chile accounts for about 18% of world production of farmed salmon (FAO, 2017).

The economic impact of *C. rogercresseyi* in Chile has been estimated in more than USD\$ 650 million per year, which includes losses due to decreased production performance and increased costs because of antiparasitic treatments (Bustos, 2007), becoming the more important parasitic disease for the Chilean salmon industry. *C. rogercresseyi* is subjected to both surveillance and control official programs since 2007 (Sernapesca, 2007).

Pharmacologic treatments are the most widely used strategy against sea lice infestations in farmed salmonids (Torrissen et al., 2013). The most commonly used drugs in the last decade are emamectin benzoate (EB), the synthetic pyrethroids deltamethrin and cypermethrin, and the organophosphate azamethiphos (Burridge et al., 2010). However, treatment failures have been reported in most of the larger salmon-producing regions of the world for many drugs (Bravo et al., 2008b; Bravo et al., 2013; Jones, 2012, 2013; Lees et al., 2008a,b; Sevatdal et al., 2005; Sevatdal & Horsberg, 2003).

One of the causes for treatment failures is the decrease of sensitivity of some sea lice populations towards several of these chemotherapeutants. So far, populations of *L. salmonis* have

demonstrated to be less sensitive to emamectin benzoate (Westcott et al., 2008; Whyte et al., 2013), deltamethrin (Sevatdal et al., 2005; Whyte et al. 2014), cypermethrin (Sevatdal et al., 2005), and azamethiphos (Fallang et al. 2004). A recently implemented program for monitoring sensitivity of *L. salmonis* to antiparasitic agents in Norway has revealed decreased sensitivity to deltamethrin and azamethiphos and substantial differences across regions (Jansen et al., 2016). Subsequent research has found that a single mutation in acetylcholinesterase (AChE) is a major condition that confers *L. salmonis* resistance to azamethiphos (Kaur et al., 2015), and that the proportion of the resistance genotype substantially varies along the Norwegian coast (Kaur et al., 2016).

In Chile, low sensitivity or resistance of the sea lice *C. rogercresseyi* has been reported towards EB (Bravo et al., 2008b; Bravo et al., 2010), and to deltamethrin (Helgesen et al., 2014). More recently, a large-scale study based on bioassays revealed the sensitivity of *C. rogercresseyi* to deltamethrin, cypermethrin and azamethiphos varies spatially at different scales (Marín et al., 2015).

Other causes of treatment failure are re-infestation after the procedure, especially in areas with intense salmon farming activity, and problems with drug administration which may include insufficient reduction of the pen volume during treatment resulting in dilution of the product, improper use of tarpaulin, and premature evaluation of treatment (i.e. post-treatment sample is taken too early) (SEARCH project, 2006).

Evaluation of treatment efficacy is a core activity in sea lice control, as it may help to early

detect sensitivity loss of local lice populations (Jackson et al., 2011; SEARCH project, 2006). Treatment success generally is evaluated by comparing the sea lice levels before and after treatment. When information about the possible drivers of treatment outcome is available, multivariable techniques may help to quantify the contribution of each predictor on the treatment result. Multivariable approaches have been used to evaluate the performance of treatments with EB in Scotland (Lees et al., 2008a,b), and New Brunswick, East Canada (Jones et al., 2012, 2013), and for treatments with pyrethroids in Chile (Arriagada et al., 2014).

The evaluation of spatial clustering of treatment performance is important because it may inform about the nature of factors that drive a spatial dependence. The combination of multivariable techniques and spatial statistics may help to generate hypotheses about factors affecting treatment performance and their distribution in space. The objective of this study was to explore the spatial and spatio-temporal variation of *C. rogercresseyi* response to pyrethroid treatments and to identify factors related to this variability.

2. Methods

We evaluated the adult lice abundance one week after a pyrethroid bath treatment, while controlling for other factors that affect sea lice abundance, such as pre-treatment levels, environmental, and management variables. We subsequently evaluated whether this adjusted treatment performance was clustered in space and time within the Chilean salmon industry.

2.1. Data

All data for the analyses were obtained from the Chilean salmon industry's Sea Lice Monitoring

Program, administered by the Instituto Tecnológico del Salmon (Intesal). Each participating farm reports *C. rogercresseyi* counts of juvenile (chalmus I to IV), mobile adults (male and non-gravid females) and gravid female lice, from a 10-fish sample each drawn from four pens on a weekly basis (40 fish in total). Sampling followed the protocols of the Official Caligidosis Surveillance and Control Program (Sernapesca, 2012). Participating farms also report delousing treatments, including the drug used, and start and end dates of the procedure at the farm level. In addition, fish production and environmental information, are also reported on a weekly basis. Our study was restricted to data collected from January 2012 to September 2013, due to a change in the treatment reporting scheme before that period.

2.2. *Inclusion criteria*

We selected delousing treatments performed with synthetic pyrethroids, either deltamethrin or cypermethrin, as these were largely the most used antiparasitic agents during the study period (Sernapesca, 2015), and from Atlantic salmon or rainbow trout production cycles whose onset was dated within our study period. We discarded single treatment procedures that were reported over a period greater than 16 days, because longer treatments are not encouraged by the Chilean authority. According to the legislation in force during 2012 and 2013, we assumed that bath treatments were applied to all cages on the farm. In most cases, a single farm contributed more than one treatment during the study period.

2.3. *Predictors*

The selection of predictors for multivariable analyses was based on our *a priori* knowledge of the factors that impact treatment performance at both farm and local area levels. Among the on-

farm predictors, we included the juvenile and adult lice mean abundance observed one week before the treatment, fish-related variables (i.e. mean weight, total biomass, stocking density, and species), and the week of the production cycle. We also evaluated the time (in days) that the procedure took to treat all pens in the farm, and the time (in weeks) since the previous treatment, because recent treatments could select for tolerant lice and, thus, undermine the current treatment's success. All these factors have been described as having an impact on *C. rogercresseyi* levels (Arriagada et al., 2014; Kristoffersen et al., 2013; Yatabe et al., 2011; Zagmutt-Vergara et al., 2005). We included a categorical variable indicating previous treatments used during the four-week period before the treatment in evaluation, as Arriagada et al. (2014) found that previous treatments may impact the performance of the current treatment. Water temperature and salinity, recorded in the week of treatment, were also included as they are known to impact development and survival of adult *C. rogercresseyi* (Bravo, 2010; Bravo et al., 2008a; González & Carvajal, 2003).

In order to account for the external infectious pressure effect (Jansen et al., 2012; Kristoffersen et al., 2013) on the treatment outcome, we included in our model the reproduction potential of lice in the neighbouring farms (NRP) within a 30-km seaway distance of the farm of interest, two weeks before the treatment event. This variable was expressed as the sum of gravid female mean abundances ($GF_{j,l(g)-1}$) at neighbouring farms (excluding farms rearing Coho salmon) (j) two weeks before the treatment (g) and within 30 km seaway distance of the treatment farm (i), each of those also weighted by seaway distance and a Gaussian kernel density ($w_{30k}(d_{i,j})$). The neighbouring farm's reproductive potential (NRP_g) was therefore calculated as follows:

$$\text{NRP}_g = \sum_{j \in A_i} \text{GF}_{j,t(g)-2} w_{30k}(d_{i,j}) \quad , \quad i = \text{farm}(g), \quad (\text{Eq. 1})$$

where A_i is the area included within the 30-km seaway distance from the treatment farm (i).

The Gaussian kernel weights ($w_{30k}(d_{i,j})$) for each neighbouring farm (j) relative to the farm of interest (i) were calculated as follows:

$$w_{30k}(d_{i,j}) = \frac{1}{\sqrt{2\pi\tau}} e^{-\frac{1}{2\tau^2}d_{ij}^2} \quad (\text{Eq. 2})$$

where d_{ij} is the seaway distance between the farm of interest (i) and the neighbouring farm (j), and the bandwidth τ given as one-fourth of the total distance plus one, i.e. $\tau=(2*30+1)/4=15.25$ km, a default setting for kernel functions in the R language.

Our study was performed over an 87-week period. We included calendar time in the model building process, expressed as 21 monthly time periods (January 2012 to September 2013), in order to capture seasonal trends of sea lice levels. We assessed this predictor in linear, quadratic and cubic forms, in order to capture sea lice variations over time.

2.4. Descriptive analysis

We calculated the mean, median, 90% range and number of observations (treatments) for the adult lice mean abundance one week after treatment for various levels of the predictors included in the study. In order to describe the spatial distribution of treatment performance, we built a map

depicting mean lice abundance one week after treatment calculated at the neighbourhood level for each of the 54 salmon farming neighbourhoods as defined by the Subsecretary of Fisheries and Aquaculture (Subpesca, 2011), according to five categories defined by cut points of 3, 6 and 9 adult lice mean abundance (Fig. 1).

2.5. Multivariable analyses

We built a linear mixed model to account for the factors that influence the treatment performance. The outcome for this model was the adult lice mean abundance, one week after treatment. Adult lice included female (gravid and non-gravid), and male adult lice. We chose adult *C. rogercresseyi* for the outcome because pyrethroids are authorized for this life stage in Chile (SAG, 2013) and it has been reported these drugs are more effective on adult lice (Arriagada et al., 2014). The adult lice mean abundances were log transformed, as suggested by a Box-Cox analysis, in order to better meet model assumptions. In order to permit the log transformation of zero values, we added 0.3 to the adult lice abundances. This value was chosen from a range between 0.0001 to 1, using a Box-Cox procedure as suggested by Venables & Ripley (1999). Because, in most cases, we had several treatments per farm, we included farm random effects in order to estimate each farm's contribution to the outcome. The model equation for our final model could be expressed as:

$$\ln(Y_g + 0.3) = X_g\beta + Z_gb_{farm(g)} + \varepsilon_g \quad (\text{Eq. 3})$$

where Y_g is the adult lice mean abundance at treatment (g), X_g is the vector for fixed effects, β is the corresponding coefficient vector, while $Z_gb_{farm(g)}$ represents all random terms at the farm

level, including the random intercept. Errors (ε_g) were assumed to follow an exponential correlation structure due to repeated observations in time on each farm that were not evenly spaced.

2.5.1. Model building and validation

We initially built a linear mixed model including company and farm as random effects. Random effects were kept only if the variance estimate was different from zero, based on full maximum likelihood (ML) estimation. Inference for fixed effects was based on the Wald test and for random effects we used the likelihood ratio test (LRT). Variables with the highest p -value were dropped one at a time, until all predictors were significant at $p < 0.05$. Non-significant potential confounders ($p > 0.05$) were kept in the model if their removal induced a change of 20% or greater on any other predictor's coefficient. Collinearity was reduced by retaining one predictor in cases where two or more were highly correlated ($|\rho| > 0.7$). When no further variables were dropped from the model, we tested biologically-plausible two-way interactions between fixed effects. In addition, predictors that were considered to potentially affect the farm effect were included as random slopes at the farm level. Assumptions of normality and homoscedasticity of the random effects and residuals were explored visually using Q-Q plots and plotting standardized residuals vs. fitted values. In addition, residuals were plotted against continuous predictors to assess the assumption of linearity. Extreme observations (standardized residuals numerically > 3) were excluded in a sensitivity analysis from the final model in order to evaluate their impact on the coefficients; however, they were considered in the subsequent analyses. Statistical modelling was performed with the statistical package Stata, version 13 (StataCorp LP).

2.6. Spatial analyses

The spatial cluster analysis was performed on farm-effect predictions from different multivariable models, estimated as best linear unbiased predictors (BLUPs). Spatial clustering was evaluated using both global and local clustering methods (Pfeiffer et al., 2008).

2.6.1. Global cluster analysis

Global cluster analysis was performed on farm-effect predictions estimated from the final full linear mixed model, which included calendar time and production time. Global clustering was quantified by the Moran's I statistic (Pfeiffer et al., 2008), in OpenGeoDa software version 1.2.0 (www.geodacenter.asu.edu). Moran's I was calculated using spatial weights with threshold distances, based on non-Euclidean (i.e. seaway) distances, and it was estimated for five 10-km distance bands (0-10, 10-20, 20-30, 30-40, and 40-50 km) in order to explore the extent of the spatial clustering. The hypothesis of no clustering was tested through Monte Carlo hypothesis testing based on 999 permutations of the farm effect predictions across farms in our study area. The Moran's I coefficient was calculated as follows:

$$I = \frac{n}{\sum_i \sum_j \omega_{ij}} \frac{\sum_i \sum_j \omega_{ij} (x_i - \bar{x})(x_j - \bar{x})}{\sum_i (x_i - \bar{x})^2} \quad (\text{Eq. 4})$$

where n is the number of farms, x_i and x_j are the farm-effect predictions at locations i and j , and ω_{ij} is a weight based on the seaway distance between locations i and j . The farm-effect predictions mean (\bar{x}) was assumed equal to zero.

In models including random slopes the variance is not constant, but rather a function of the fixed predictor for the random slope (Dohoo et al., 2009). In our context, this means variance of farm effects may change along the production cycle and, therefore, the cluster detection could be affected. In order to explore this possibility, we estimated Moran's I coefficients at week 10 and 60 in the production cycle, in addition to the analyses described in the previous section corresponding to production cycle week 34 (i.e. the mean value)¹.

2.6.2. Local cluster analyses

The local cluster analysis was performed in order to detect clusters of high values for farm-effect predictions using the Normal model of the scan spatial analysis (Kulldorff, 1997), implemented in the package SaTScan, version 9.1.1. (www.satscan.org), and based on non-Euclidian distances as described in Kulldorff et al. (2009). Clusters of low values for farm effects were also explored. The local cluster analysis was limited to a spatial window up to 40 km from the farm of interest, as previous research has found *C. rogercresseyi* might spread up to 30 km (Kristoffersen et al., 2013). For these analyses, a “null” model (containing only the fixed effects for production week and calendar time, as well as the farm level random intercept and slope) served as baseline to compare with the full model, which included significant farm-level predictors. The final full model predictions were compared to the null model predictions to explain those spatial clusters observed from the null model. We use the term “null” model because it lacks all the predictors related to sea lice abundance contained in the set of full models.

2.7. Spatio-temporal analyses

¹ For the sake of simplicity, we use the term ‘farm effect prediction’ for the farm effects predicted at the mean value of the production cycle week (i.e. 34). Farm effects predicted at other values of the production cycle (i.e. 10 or 60), are explicitly indicated.

For the spatio-temporal analysis, residuals at the treatment level were calculated from “reduced” models, defined as those models without temporal predictors. As such, the two reduced models included in this analysis were: 1) the final model as described in section 2.5, excluding calendar time (the full-reduced model), and 2) the null model as described in section 2.6.2, also without calendar time (the null-reduced model). Calendar weeks were excluded from these analyses in order to leave the temporal effect within the residuals, allowing the spatio-temporal analyses to capture potential interactions between space and time, as were observed in the descriptive analyses. Clustering of residuals was explored using the scan spatial analysis, implemented in the package SaTScan, version 9.1.1., and based on non-Euclidian distances. The scan spatial window was limited to 40 km seaway distance, while the temporal window was set at 20% of the total time.

3. Results

3.1. Descriptive analysis

A total of 1,090 treatment events met the inclusion criteria of our study. These treatments were performed on 218 farms spread over 45 neighbourhoods in Los Lagos and Aysén regions. There were reported, on average, 5 treatments per farm (range: 1-17) during the study period. Eighty-eight percent of treatments were performed on Atlantic salmon, while 12% on rainbow trout. During the study period the average weight of Atlantic salmon and rainbow trout was 2.03 kg (SD=1.21) and 1.73 kg (SD= 0.71), and the water temperature averaged 10.8° C (SD=1.4).

The abundance of adult *C. rogercresseyi* one week after a pyrethroid treatment had a mean of 6.9 (SD=14.8) lice per fish, although it varied considerably across neighbourhoods (Figure 1). The

adult lice mean abundance one week before the treatment was 8.7 (SD=11.2).

Associations between predictors and our outcome were appreciable (Table 1). Among the on-farm predictors, pre-treatment sea lice levels, fish-related variables, such as weight and fish biomass, and the duration of treatment exhibited positive relationships with the levels of lice after the procedure. The salmon species showed a positive association with the post-treatment levels, with rainbow trout showing a slightly greater mean abundance than Atlantic salmon. The post-treatment adult lice levels seemed to increase as time goes by in the production cycle (Table 1). The Spearman correlation coefficient (ρ) between the adult lice abundance and production cycle week was 0.44 ($p<0.001$). Calendar time also appeared to be associated with the post-treatment sea lice levels ($\rho=0.37$, $p<0.001$). In the case of environmental variables, water temperature exhibited a low, but significant positive association with our outcome ($\rho=0.08$, $p<0.011$), while water salinity showed no relation with the post-treatment levels ($\rho=-0.01$, $p=0.638$). Finally, the number of neighbouring farms within a 30-km seaway distance showed a positive pattern with our outcome, but only at low and medium values of adult lice levels.

3.2. Multivariable analyses

The final model (Table 2) contains all factors that showed significant associations to the result of antiparasitic treatments using synthetic pyrethroids in Chile between January 2012 and September 2013. Factors exhibiting a positive relationship with the post-treatment lice levels were the pre-treatment adult and juvenile lice levels, water salinity, production cycle week, the time it took to complete the treatment for the whole-farm, and the distance-weighted gravid female mean abundance at neighbouring farms two weeks before the treatment. Calendar time

also presented a positive association to post-treatment; however, its strength varied over the study period (i.e. association was not linear). All these factors showed highly significant associations (i.e. $p < 0.001$).

The inclusion of a random slope for production cycle week significantly improved the model fit, suggesting that the increment of sea lice along the production cycle significantly varied across farms. Regarding the treatment procedure, the time it took to complete the treatment for the whole-farm was also positively associated with the outcome ($p < 0.001$). Previous delousing treatments in the same farm during 4 weeks before the treatment in evaluation did not have any significant impact ($p=0.099$) on the adult lice levels; therefore, it was removed from the final model.

The unexplained variance remained at both the farm and treatment levels in the full model. The proportion of the variance at the farm level (i.e. intra-class correlation coefficient, ICC) varied as a function of the production cycle week: higher values of ICC were reached at the start and finish of the cycle (ICC start: 0.49; ICC finish: 0.43), while the lowest value was around the middle of the cycle (ICC 0.25). This means that treatment performance depends on both farm- and treatment-level factors not included in the model, whose proportion varies over the production cycle. Farm-level factors not included in the model seem of lesser importance than treatment-level factors, specially in the mid section of the cycle when they represented only the 25% of the unexplained variance.

3.2.1. Model fit

Residuals at the treatment level were approximately normally distributed, with only 4 (0.4%)

observations with standardized residuals greater than 3. In addition, residuals appeared to be homoscedastic as similar variances were observed across fitted values. Assumption of linearity between the outcome and continuous predictors was met. Predictions for the farm effect, as well as random coefficients, did not show any obvious departure from normality and appeared to be homoscedastic.

3.3. Spatial analyses

3.3.1. Global cluster analysis

The Moran's I indicated that the farm-effect predictions from the full model (estimated at week 34 in the production cycle) exhibited a positive spatial autocorrelation and this correlation depended on the distance band used (Figure 2). Moran's I coefficient observed in the 0 to 10 km distance band for weeks 10, 34 and 60 were 0.326 ($p=0.001$), 0.267 ($p=0.001$) and 0.086 ($p=0.127$), respectively. This means that farms with more or less lice than expected after treatment (based on the predictors of the final model) were located closer together in space, but only during the first half of the production cycle. In the second half, treatment outcomes were no longer similar even between farms located in the proximity. Generally, the similarity among treatment performances appeared to diminish as distance between farms increased; however, this trend was not linear over distance bands (Figure 2).

3.3.2. Local cluster analyses

The purely spatial scan statistic performed on the farm effect predictions from the null model found two significant clusters of high values. The most significant cluster (cluster 1, $p=0.001$) was located in central Aysén region, including all farms from neighbourhood 20 and farms in

neighbourhoods 18b, 18c, 18d, 19b and 21b (Figure 3a). This cluster consisted of 29 farms. The mean adult lice level at farms within this cluster was 1.55 times greater than in the rest of the study area (Table 3). The second significant cluster (cluster 2, $p=0.002$) consisted of 4 farms located in the neighbourhood 3a, in northern Los Lagos region (Figure 3a). In this case the, the relative ratio of adult lice counts was 2.79 in favour of farms within the cluster (Table 3).

When we ran the local cluster analysis on the farm effect predictions from the full model, practically the same two significant clusters were found; however, the cluster 1 ($p=0.011$) expanded to the west including new farms from neighbourhoods 19a, 19b and 20, but excluding farms from neighbourhoods 18c and 18d in the east (Figure 3b). Adult lice levels inside the cluster were 1.22 greater than those outside the cluster, and this cluster included 28 farms. Cluster 2 ($p=0.024$) included the same four farms as the analysis based in the null model; the relative ratio in adult lice counts decreased to 1.60 (Table 3). In other words, farms with treatments that performed worse than expected (according to predictors in the full model) were closer to each other than to farms with better-than-expected treatments. No significant clusters of low values for farm effects or for random coefficients were detected (results not shown).

3.4. Spatio-temporal analyses

The spatio-temporal analyses performed on the residuals from the reduced-null model detected three significant clusters. In these areas, the observed sea lice counts after the treatment were higher than predicted by the final model (Figure 4 and Table 4). The most significant cluster was located in neighbourhood 18a (northern Aysén region), during March, April and May 2013, and consisted of 7 farms. During that period of time, adult lice levels inside the cluster were 3.69

times greater than in the rest of the study area. The second significant cluster ($p=0.002$) was observed in central Aysén region between February and April 2013, and involved 16 farms from neighbourhoods 20, 19b, 19a and 21b. In this case, the relative ratio of adult lice counts for the second cluster was 1.94. A third significant cluster ($p=0.041$) was detected in northern Los Lagos region during March and April 2013, and consisted of four farms from neighbourhood 3a. Adult lice levels inside the cluster were 2.57 times greater than outside the cluster. When the same analysis was carried out using the residuals from the reduced-full model, these three clusters were no longer significant, and no other clusters were found. No significant clusters of low values for residuals were detected.

4. Discussion

Our analysis demonstrated some clustering of the performance of pyrethroids treatments in space in Chile between January 2012 and September 2013. When the spatial clustering was analysed at the farm level (farm effect predictions), we found two significant clusters: cluster 1 in central Aysén region (neighbourhoods 18b, 18c, 18d, 19b, 20 and 21b) and cluster 2 in northern Los Lagos region (neighbourhood 3a) (Figure 3a). Salmon farming neighbourhoods for the study area are depicted in Figure S1. This agrees with the descriptive analysis, where it was observed that the worst treatment results were in these neighbourhoods (Figure 1). These two clusters remained when we controlled for on- and off-farms and environmental variables (Figure 3b), suggesting that other unmeasured factors were driving the spatial dependence of treatment performance across farms. One of these factors might be the development of resistance to pyrethroids by local sea lice populations.

The spatio-temporal analysis performed on residuals at the treatment level revealed three clusters, two in the same area as the clusters detected in the purely spatial analysis, plus a third one located in northern Aysén region (Figure 4). These three clusters were no longer significant after controlling for all predictors in the full model, suggesting that the clustering was driven by the predictors included in the final model, and that these predictors also clustered in space and time.

The global spatial cluster analysis performed on the farm effect predictions from the full model found that treatment outcomes were more similar at shorter distances between neighbouring farms. Because the models accounted for the most important predictors, these findings might be the result of resistance development of sea lice to pyrethroids, or due to factors related to the treatment procedure. It is important to mention that we did not have access to any information related to the sensitivity of sea lice to pyrethroids (i.e. bioassay results), or details regarding the treatment modality (i.e. tarpaulin, well boat) or drug administration procedures; therefore, their potential effects remain in the model's unexplained variability (i.e. farm effects and residuals).

It is well known that spread of resistance to chemotherapeutants in sea lice is mediated by the flow of resistance genes between sea lice metapopulations (i.e. farms) (Denholm et al. 2002; Tully & Nolan, 2002). A recent study has revealed that resistance of *L. salmonis* to azamethiphos is conferred by a mutation in the acetylcholinesterase (AChE) gen (Kaur et al., 2015), and that the resistant genotype has spread along the Norwegian coast (Kaur et al., 2016), confirming the hypothesis of resistance gene flow. In Chile, research indicates that sensitivity of *C. rogercresseyi* to pyrethroids is widespread, with several problematic areas (Marín et al., 2015);

however, the genetic mechanisms of lice low sensitivity remain unknown.

Our study modeled the spatial distribution of the unexplained variability of antiparasitic treatments outcomes, after controlling for the effect of environmental and productive factors. A potential factor responsible for driving this unexplained variability is the resistance of *C. rogercresseyi* to pyrethroids, which has already been documented in Chile. The high spatial variability of our farm effects, their global clustering and their aggrupation in local clusters in southern Chile, is consistent with the hypothesis of resistance gene flow and with the observed behavior of *C. rogercresseyi* sensitivity in Chile and *L. salmonis* in Norway, studied by bioassays in both cases. Therefore, we believe that our epidemiological approach complements the research carried out so far and constitutes an alternative method for regions where sensitivity data is not available.

We also found that the similarity of treatment performance in space depended on the stage of the production cycle when it was evaluated. Specifically, the treatment performance clustered in the first half of the production cycle (i.e. weeks 10 and 34), but not in the second half (i.e. week 60). Consistently, the spatial scan statistic found two significant clusters at week 10 in the production cycle, but none at week 60 (data not shown). This trend was observed in the full model, which contained calendar time suggesting that treatment outcomes were similar in space at a particular week of the production cycle, regardless of the actual time at which the cycle was.

The lack of spatial clusters towards the end of the production cycle weakens the hypothesis of sensitivity to pyrethroids as the driver of the spatial dependence among farm effect predictions,

because resistance should be maintained and even increased in the sea lice population over time. However, it may also indicate that other factors play a role in the observed spatial variability, making the treatment performance more dissimilar among neighbouring farms later in the production cycle. Such factors should most likely to be related to the age or size of fish, because these variables behave in a similar manner across farms, regardless of the calendar time.

Our full model showed that the sea lice trends over the production cycle varied significantly across farms, ranging from marked increase to light decrease of lice levels (data not shown). If we consider that in the final model we controlled for different external and internal infectious pressures, and environmental factors as well, differences in on-farm production conditions remain as potential drivers for such patterns. Some production factors that showed an important variability in our dataset and that have been demonstrated to impact sea lice levels are number of stocked fish, fish biomass, fish mean weight and stocking density.

Generally speaking, the purely spatial and the spatio-temporal analysis could be picking up two different components of clustering. The spatial analysis, based on farm effect predictions, represents unknown factor(s) of slow development or somewhat constant in time, while the spatio-temporal analysis performed on residuals is related to unknown factor(s) which vary between treatments within a farm.

It is worth noting that when we controlled for predictors (full model), clustering of farm effect predictions was marginally affected (i.e. the same number of clusters between the null and full models were found), while clustering for residuals disappeared completely. This suggests that the

predictors mostly explained factors operating at the treatment level, which seems logical since the majority of the fixed effects included in the final model (with the exception of water salinity) showed a larger variation between treatments compared to between farms (see farm ICC in Table 1). Consequently, it can be presumed that farm-level predictions are representing farm-level factor(s) more constant in time. Furthermore, we learnt these farm-effect predictions were clustered in space. A potential factor that fits the behaviour of farm-level predictions is the resistance of sea lice to pyrethroids.

It is interesting to observe the different effect that controlling of predictors had on the two clusters detected in the spatial analysis. When we controlled for predictors in the purely spatial analysis, cluster 1 expanded to a larger area, instead of shrinking, as it would be expected, although the total number of farms reduced from 29 to 28. We think that happened because the new farms included in the cluster 1 with the full model were more distant, and SaTScan does not take into account the actual seaway distance between farms, but a rank of “closeness”. In the case of cluster 2, controlling for predictors did not have any impact on the size/location of the cluster. This may have happened because farms within cluster 2 were relatively isolated from the rest of the farms in the area (Figure 3b).

The three clusters detected in the spatio-temporal analysis (based on the reduced-null model) were consistently present during the last months of the farms’ production cycles. Descriptive data shows that post-treatment lice levels increased at the end of the production cycle (Table 1), and, in many cases, these peaks occurred at similar calendar time, which seems logical due to coordination of the production cycle within neighbourhoods in Chile. When we controlled for

pre-treatment levels and external infectious pressure (NRP), these clusters disappeared which suggests these variables accounted for exchange of sea lice between neighbouring farms. In other words, the spatio-temporal clusters observed in the reduced-null model were due to sea lice exchange at the end of the production cycle, and they were more prone to be detected in areas where the production cycles were coordinated in time.

Other unknown factors that may impact sea lice levels among the last months of the production cycle, such as the level of compliance of treatment procedures, might also explain this variation; however, as clustering disappeared after controlling for predictors, we can presume these unknown factors would not show spatial dependence among farms. This is consistent with our interpretation of results from the spatial cluster analysis (farm effect predictions), because these unknown factors that could explain the lack of cluster in the spatio-temporal cluster analysis, may be the same as those responsible for the lack of clustering in the second half of the production cycle (purely spatial analysis).

The short time frame defined for this study (relative to the salmon production cycle), meant we were able to, in many cases, include only a portion of the production cycle. That may affect the cluster detection, especially in the spatio-temporal analyses. Future research should consider longer study periods, ideally including at least 2 production cycles, in order to better explore spatio-temporal clusters.

The methodology used in this study may be of particular interest to policy makers interested in evaluating reasons for antiparasitic treatment failures in regions where assessments of sea lice

sensitivity have not been conducted. In Chile and other regions, the only way to assess the sensitivity of lice to specific drugs is by means of bioassays, which, besides being expensive, lack reproducibility. For this reason, characterizing the sensitivity level of sea lice in a region is an arduous and expensive task. The multivariate approach we presented here allows to adjust the observed treatment efficiency by environmental, management and productive factors that influence the parasitic loads or that exhibits a spatial structure, leaving factors such as the level of resistance within the unexplained variability, whose spatial characteristics can be studied.

5. Conclusion

We found that the performance of pyrethroids was clustered in space and time in Chile between January 2012 and September 2103. There were two areas where the response to treatments on farms were more similar for farms in close proximity, even after controlling for environmental and management factors. The reason for the presence of spatial clustering of poor treatment responses is unknown, but it appears that there may be areas that are more problematic for sea lice control. Further research is required to confirm the patterns observed in this study and determine their cause, including the possibility of treatment resistance.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Farm-level intra-class correlation coefficients (ICC) and descriptive statistics for the adult *Caligus rogercresseyi* mean abundance one week post-treatment at different predictor levels included in the model building process.

Variables	Farm ICC ¹	Levels	Mean	Median	90% range	n
Adult lice mean abundance one week before treatment	0.156	< 4 ≥ 4 to < 8 ≥ 8	2.82 4.97 11.67	1.83 3.95 5.90	0.13 – 10.93 0.58 – 9.05 1.48 – 65.18	204 507 379
Juvenile lice mean abundance one week before treatment	0.293	< 2 ≥ 2 to < 6 ≥ 6	2.75 5.31 13.70	1.95 4.03 6.93	0.15 – 8.08 0.75 – 9.58 2.08 – 66.83	303 488 299
Fish biomass within farm during treatment (tons)	0.281	< 1,000 ≥ 1,000 to < 2,000 ≥ 2,000	4.68 6.00 10.66	2.98 4.03 5.38	0.23 – 9.05 0.38 – 12.15 1.48 – 45.38	361 417 312
Fish mean weight at treatment (kg)	0.185	< 1.5 ≥ 1.5 to < 3 ≥ 3	4.17 6.74 12.05	2.88 4.20 5.88	0.20 – 8.78 0.48 – 13.53 1.85 – 57.60	423 430 237
Stocking density during treatment (kg/m ³)	0.298	< 4 ≥ 4 to < 7 ≥ 7	3.63 6.80 10.17	2.88 4.03 5.45	0.23 – 8.58 0.40 – 12.15 1.18 – 45.38	366 349 375
Species		Atlantic salmon Rainbow trout	6.82 7.44	4.16 3.35	0.38 – 17.88 0.28 – 29.43	954 136
Production cycle week	0.141	< 30 ≥ 30 to < 50 ≥ 50	3.68 5.59 16.05	2.78 4.40 6.55	0.23 – 8.65 0.73 – 11.88 2.03 – 68.28	422 455 213
Calendar time (months)	0.527	Jan-12 to Jun-12 Jul-12 to Nov-12 Dec-12 to Apr-13 May-13 to Sep-13	2.56 3.91 11.26 7.45	1.91 3.46 5.18 5.09	0.15 – 7.35 0.29 – 8.30 0.85 – 57.60 1.10 – 16.60	156 340 358 236
Duration of the treatment procedure in the farm (days, limited to 16 days)	0.202	< 3 ≥ 3 to < 6 ≥ 6	5.54 6.99 8.56	2.96 4.36 4.80	0.20 – 12.05 0.58 – 15.88 0.83 – 32.83	316 546 228
Time since last treatment (weeks)	0.371	< 5 ≥ 5 to < 8 ≥ 8	8.92 6.58 4.05	5.10 4.25 2.63	0.80 – 29.43 0.48 – 19.45 0.20 – 11.43	479 305 306
Water temperature (°C)	0.042	< 10 ≥ 10 to < 12 ≥ 12	4.16 7.38 9.07	3.73 4.63 3.79	0.50 – 8.60 0.55 – 16.6 0.23 – 45.98	321 471 298
Water salinity (ppt)	0.850	< 30 ≥ 30 to < 32 ≥ 32	4.05 6.76 7.85	3.25 4.53 3.85	0.35 – 8.60 0.78 – 19.35 0.30 – 22.65	147 440 503
Number of neighbouring farms within a 30 km seaway distance, NRP	0.034	< 6 ≥ 6 to < 12 ≥ 12	3.66 7.72 6.97	3.08 3.90 4.73	0.23 – 8.58 0.28 – 26.88 0.75 – 18.8	141 514 435

¹ Intra-class correlation coefficient (ICC) estimated from a null linear mixed effects model with the variable as the outcome and with farm random effect as the only predictor.

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Table 2. Coefficient estimates, standard errors and *p*-values for explanatory variables in the final model for the log-transformed adult *C. rogercresseyi* mean abundance one week after an immersion treatment using synthetic pyrethroids on farmed Atlantic salmon and rainbow trout, from January 2012 to September 2013 in Los Lagos and Aysén regions in Chile (n=1,090).

Variable name	Estimate	Standard error	<i>p</i> -value	95% confidence interval	
Fixed effects parameters					
Intercept	-1.6882	0.3577		-2.3893	-0.9871
Log of adult mean abundance one week before treatment	0.2116	0.0365	<0.001	0.1401	0.2832
Log of juvenile mean abundance one week before treatment	0.2269	0.0261	<0.001	0.1757	0.2781
Water salinity (ppt)	0.0447	0.0105	<0.001	0.0242	0.0652
Production cycle week (centered at 34, rescaled by 10)	0.1289	0.0205	<0.001	0.0887	0.1689
Duration of the treatment procedure in the farm (days)	0.0333	0.0080	<0.001	0.0176	0.0490
Calendar time (weeks, rescaled by 10)	0.2362	0.0520	<0.001	0.1343	0.3381
Calendar time (weeks, rescaled by 10) (quadratic term)	-0.0169	0.0046	<0.001	-0.0258	-0.0079
Neighbourhood reproduction potential two weeks before treatment (30 km seaway distance)	0.2910	0.0331	<0.001	0.2261	0.3559
Random effects parameters					
Farm					
var: intercept	0.1130	0.0221		0.0770	0.1658
var: beta (production cycle week)	0.0105	0.0040		0.0050	0.0220
covar: intercept, production cycle week	-0.0104	0.0068		-0.0238	-0.0030
Residual					
var: error	0.3121	0.0165		0.2815	0.3461

Table 3. Spatial clusters of high farm effect predictions (at production week 34) from the null and final linear mixed models for adult lice abundance one week after a single treatment with pyrethroids, from January 2012 to September 2013 in Los Lagos and Aysén regions in Chile.

Model	Cluster	Number of farms	Neighb. involved	Furthest distance ¹ (km)	Log adult lice		RR ² adult lice count	P
					Mean (SD) inside	Mean (SD) outside		
Null	1	29	18b, 18c, 18d, 19b, 20, 21b	67.2	0.379 (0.339)	-0.056 (0.429)	1.55	0.001
	2	4	3a	6.2	1.008 (0.657)	-0.019 (0.417)	2.79	0.002
Final	1	28	18d, 19a, 19b, 20, 21a, 21b	76.6	0.176 (0.168)	-0.025 (0.249)	1.22	0.011
	2	4	3a	6.2	0.459 (0.214)	-0.009 (0.242)	1.60	0.024

¹ Seaway distance (km) between the two most distant farms within a cluster.

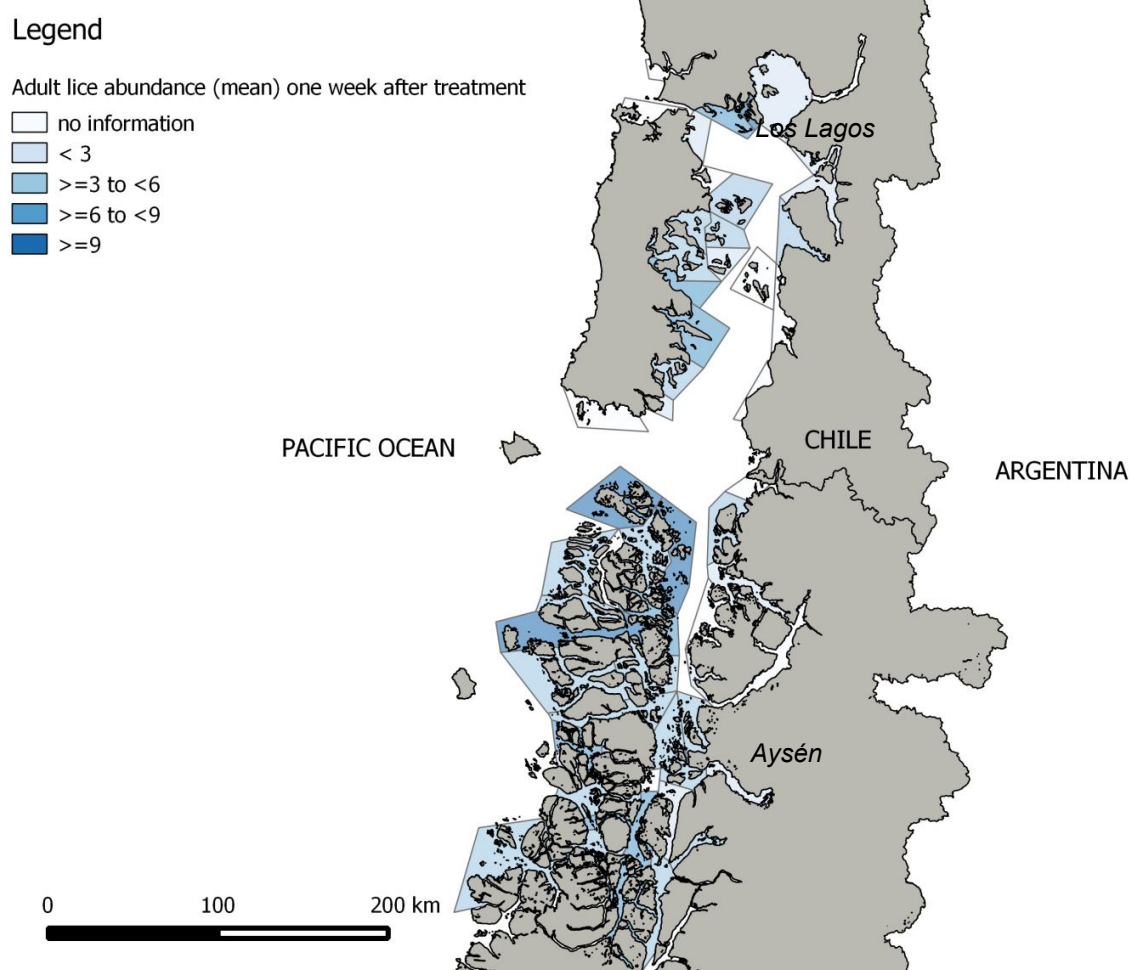
² Relative ratio of adult lice counts (inside cluster:outside cluster).

Table 4. Spatio-temporal clusters of high residuals at the treatment level from the reduced-null linear mixed models for adult lice abundance one week after a single treatment with pyrethroids, from January 2012 to September 2013 in Los Lagos and Aysén regions in Chile.

Cluster	Number of farms	Months	Neighb. involved	Furthest distance ¹ (km)	Log adult lice		RR ² adult lice count	P
					Mean (SD) inside	Mean (SD) outside		
1	7	Mar-13 to May-13	18a	12.2	1.290 (0.396)	-0.016 (0.534)	3.69	0.001
2	16	Feb-13 to Apr-13	20, 19a, 19b, 21b	55.9	0.651 (0.609)	-0.013 (0.541)	1.94	0.002
3	4	Mar-13 to Apr-13	3a	6.2	0.936 (0.383)	-0.007 (0.546)	2.57	0.041

¹ Seaway distance (km) between the two most distant farms within a cluster.

² Relative ratio of adult lice counts (inside cluster:outside cluster).



1.

Figure 1. Mean abundance of adult *C. rogercresseyi* one week after treatment by salmon farming neighbourhood during January 2012 and September 2013 in the study area.

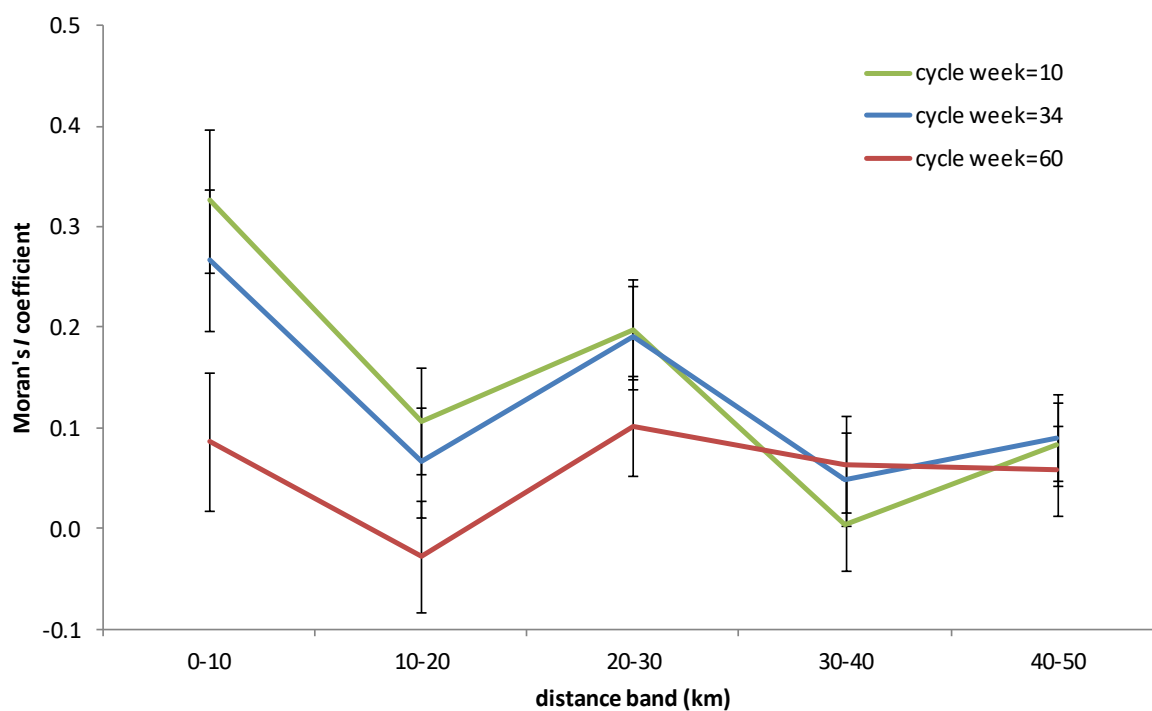


Figure 2. Moran's I coefficients with simple standard error bars for farm effects at production cycle weeks 10, 34 (random intercept), and 60 from the final model at different seaway-distance bands.

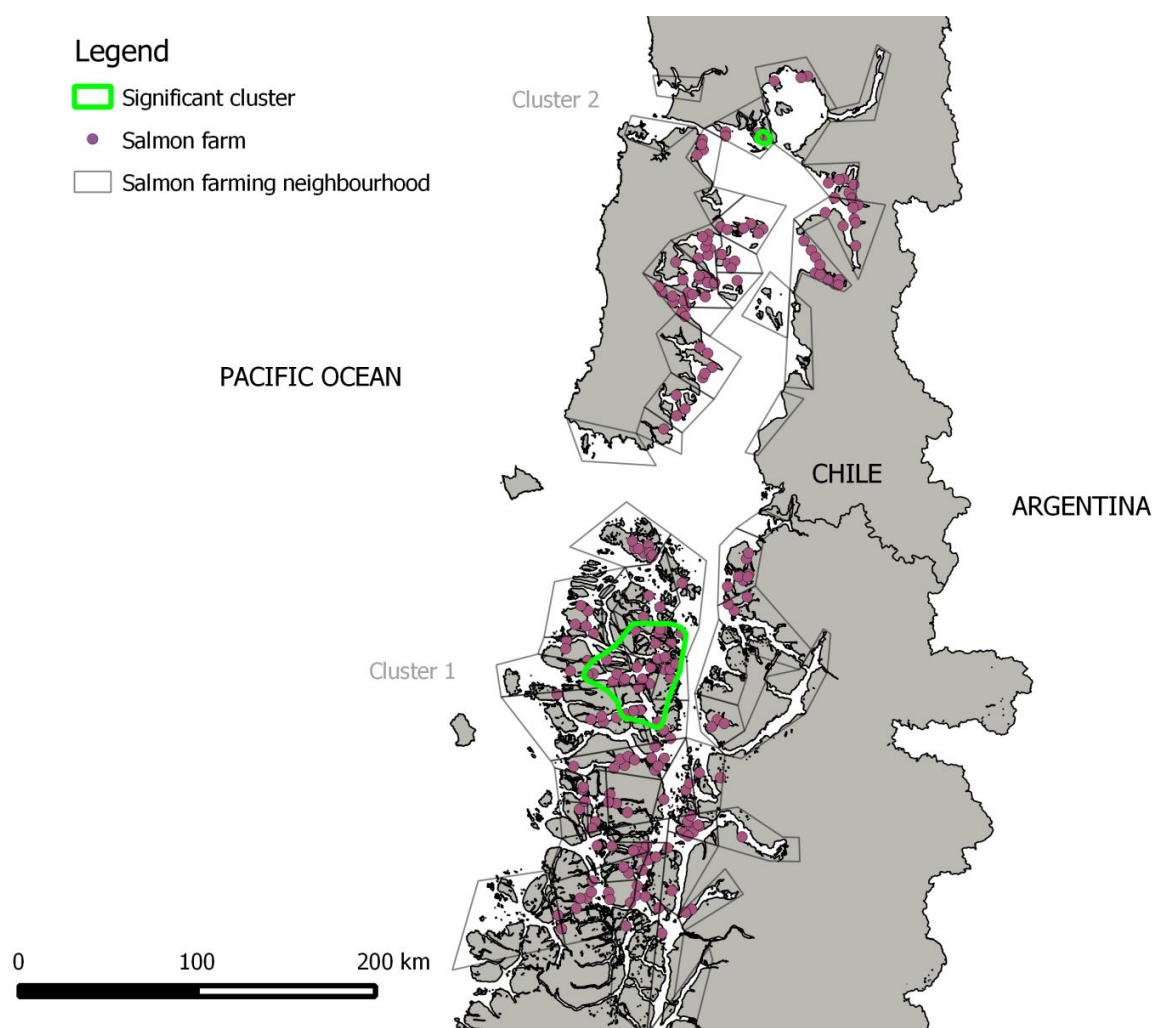


Figure 3a. Significant clusters of high farm effect predictions from the null model at week 34 detected in the purely spatial cluster analysis.

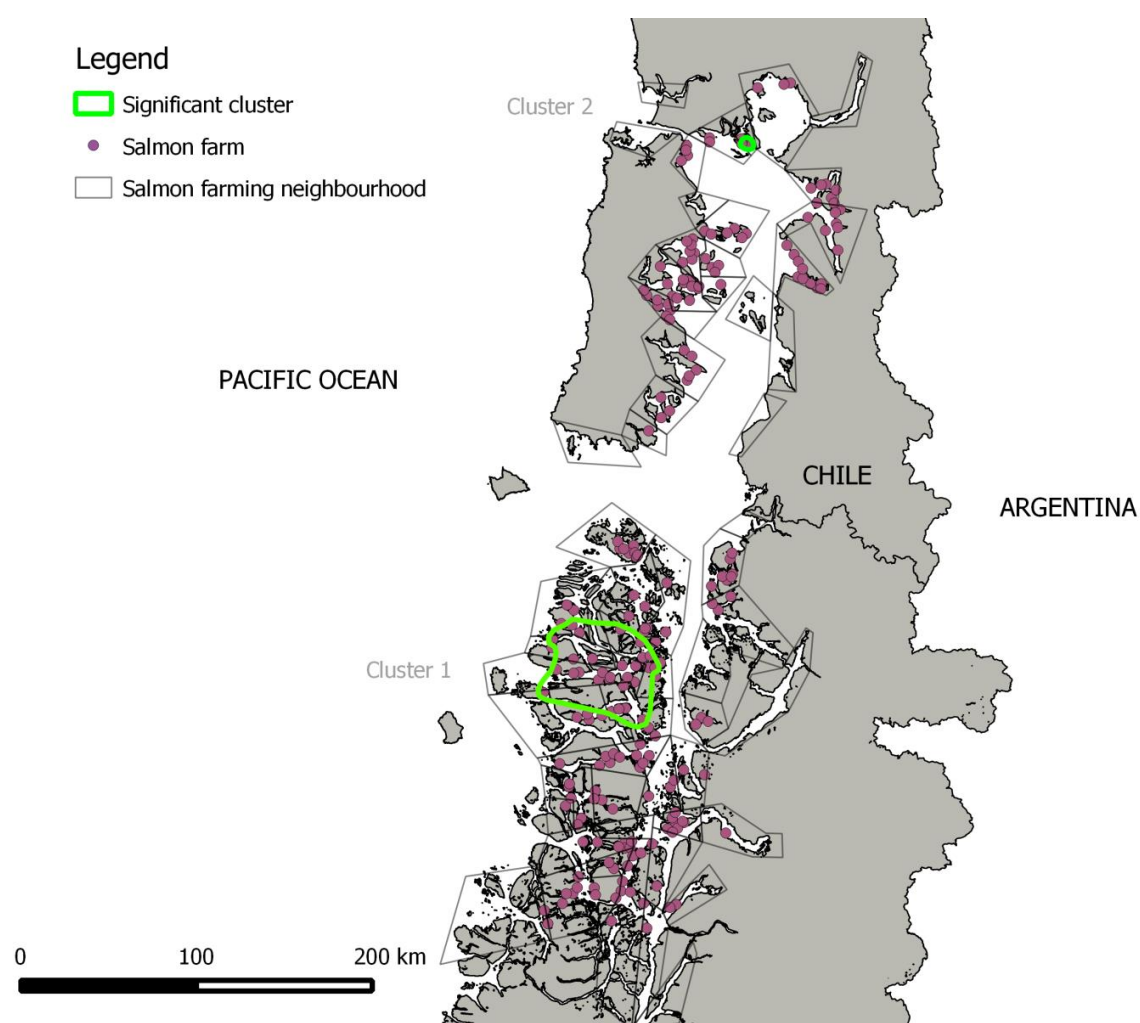


Figure 3b. Significant clusters of high farm effect predictions from the full model at week 34 detected in the purely spatial cluster analysis.

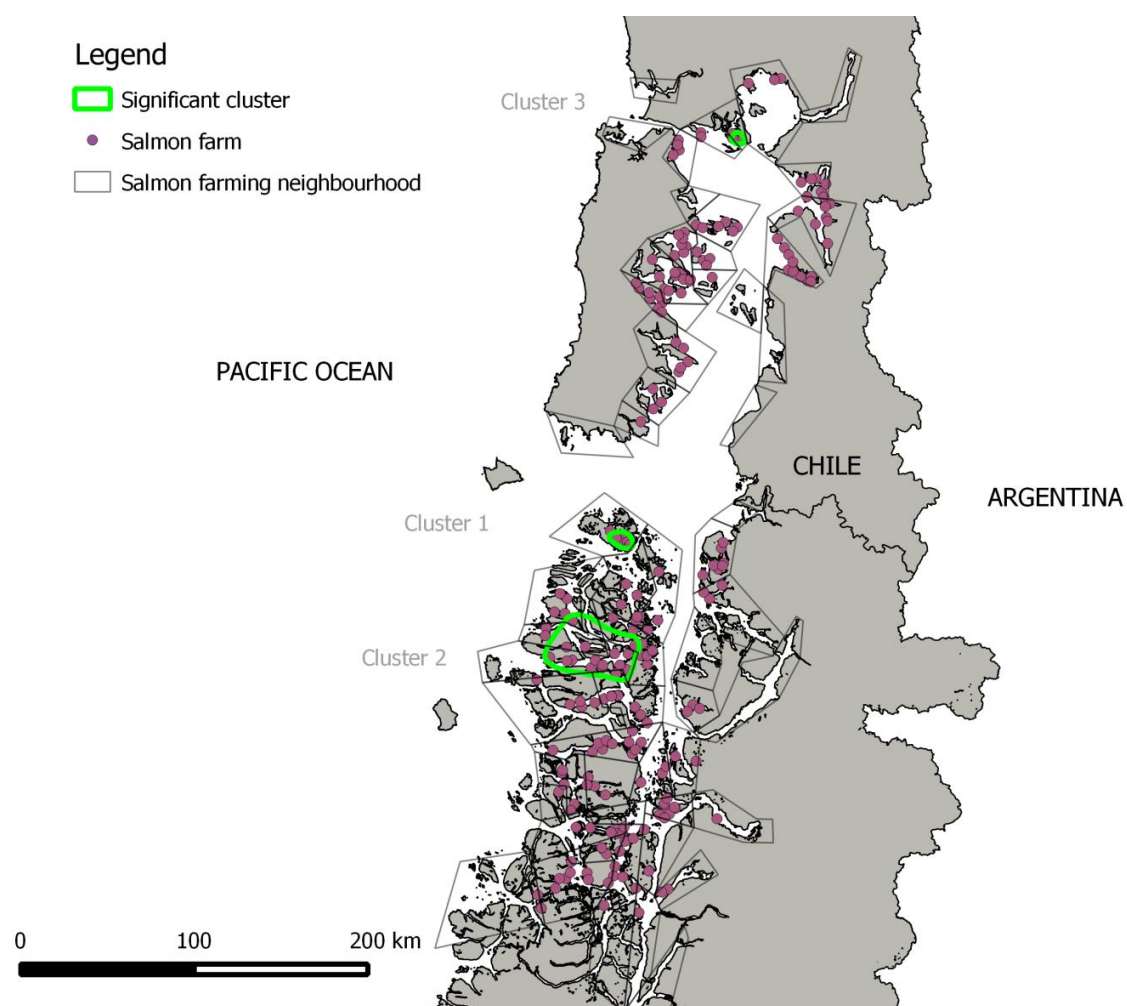


Figure 4. Significant clusters of high treatment level residuals from the reduced-null model detected in the spatio-temporal cluster analysis.