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Negative effects of the sea lice therapeutant emamectin benzoate at low concentrations on benthic communities around Scottish fish farms

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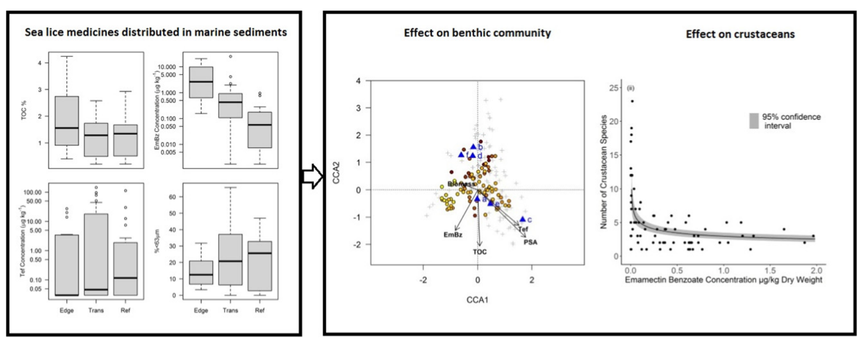
HIGHLIGHTS

- Emamectin benzoate (EmBz) was widely detected in benthic sediment during the survey.
- Benthic community composition was secondarily ordinated along a gradient of EmBz.
- EmBz had the biggest effect on benthic crustacean abundance and richness.
- The distribution of EmBz beyond fish farms was linked to impacts on benthic ecology.

GRAPHICAL ABSTRACT



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ABSTRACT

Emamectin benzoate is used as an in-feed treatment for the control of sea lice parasites in all of the main farmed Atlantic salmon (*Salmo salar*) facilities worldwide (Norway, Chile, Scotland and Canada). Investigations into its effect on non-target benthic fauna resulting from its excretion from farmed fish and uneaten feed have been limited. This paper presents the findings from a study that intended to assess the impact of emamectin benzoate on benthic fauna using a new low detection method for emamectin benzoate. Eight fish farms in the Shetland Isles, Scotland were surveyed, with sediment sampled along transects radiating from the farms analysed for benthic ecology, sediment chemistry and sediment veterinary medicine residues (analysed for emamectin benzoate and teflubenzuron). Canonical Correspondence Analysis (CCA) and Generalised Linear Mixed Modelling (GLMM) were used to assess which environmental parameters observed during the survey had the biggest effect on benthic community composition and abundance, and more specifically crustacean abundance and richness. Emamectin benzoate was found in 97% of samples, demonstrating widespread dispersion in the sediments sampled. The CCA showed that species composition was predominantly ordinated along a gradient of particle size, with a secondary axis dominated by a change in emamectin benzoate and organic carbon enrichment. Peaks in abundance of crustacean species were predicted to be organised along a gradient of emamectin benzoate concentration. The GLMM corroborated this by showing that emamectin benzoate had the strongest negative effect on total crustacean abundance and species richness, though there was some degree of collinearity with organic

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carbon, that had a smaller effect. Overall, this study shows that, following its use as an in-feed treatment for sea lice, emamectin benzoate residues are more widely distributed in the benthic environment than previously thought, and have a statistically significant effect on benthic ecology at the concentrations observed in this study.

1. Introduction

Marine finfish aquaculture producers in Scotland aim to increase annual production to between 300,000 and 400,000 t by 2030 (Scotland Food and Drink, 2016). Atlantic Salmon (*Salmo salar*) will form the largest proportion of this annual output where fish are grown in open water pen nets predominantly on the West Coast, Western Isles, Orkney and Shetland (Marine Scotland, 2009).

One of the biggest fish health issues the Scottish aquaculture industry has to contend with is parasitic sea lice (Costello, 2009). Sea lice are copepods of the genera *Caligus* and *Lepeophtheirus* that feed on the blood, skin and mucus of the salmon by attaching to the flesh of the fish. The biggest impacts are seen when lice induced lesions become infected. However, there are also physiological impacts related to stress, osmoregulation, changes to blood composition and impaired swimming performance that make fish husbandry difficult (Finstad et al., 2000; Torrissen et al., 2013).

Fish farm operators use therapeutants as one of the tools to control sea lice numbers. Government trigger levels of three female lice per fish are used to initiate a site-specific action plan (Marine Scotland, 2017), whilst a lower industry code of good practice trigger level of 0.5–1 female louse per fish is often used to prevent lice infestation (SSPO, 2018). There are two main medicine administration types used to control lice numbers, dosage via a bath treatment or via in-feed treatment. The latter is the focus of this study. Only in-feed treatments using the active substance Emamectin Benzoate (herein referred to as EmBz) are licensed for use in Scotland; treatments using the active substance Teflubenzuron (herein referred to as Tef) ceased in 2015 and Diflubenzuron (herein referred to as Dif), used in Norway, is not consented for use in Scotland. In-feed EmBz treatment provides longer-term protection against sea lice (up to 62 days, Stone et al., 2000), as EmBz is absorbed by the gut and distributed to tissues within the fish. Subsequently, it is metabolised by the fish and excreted in faeces (Kim-Kang et al., 2004), therefore being released into the environment via faeces and uneaten food pellets.

The long degradation half-life (>120 days in marine sediment, EFSA, 2012) and hydrophobic nature (log KoW of 5 at pH 7 and 23 °C, US EPA, 2009) of EmBz means that it could persist in marine sediments underneath and around fish farm cages, resulting in a high risk of exposure to benthic organisms. The chemical action is non-targeted, therefore species of the same sub-phylum as sea lice (crustacea) are subject to the same mode of action (e.g. Willis and Ling, 2003), with impacts on larger crustacean species also documented (e.g. Veldhoen et al., 2012). Benthic crustaceans contribute to ecosystem processes such as bioturbation, bioengineering and biodeposition (Bertics et al., 2010; Kristensen et al., 2012; Coates et al., 2016), which enhance biodiversity. Therefore, it is important to understand the potential for these organisms to be affected by EmBz. As such, the Scottish Environment Protection Agency (SEPA) regulates the use of EmBz and set standards to protect non-target species in marine sediments.

The current Environmental Quality Standard (EQS) for EmBz is set at 0.763 μg/kg wet weight sediment at a distance of 100 m from the cage, whilst a cage edge trigger level is set at 7.73 μg/kg wet weight to protect sediment reworker species. The standard was set in 1999 and was derived from a Maximum Acceptable Toxicant Concentration (MATC) for the most sensitive species tested, *Arenicola marina*, a sediment dwelling polychaete. An assessment factor of 100 was applied to this value to derive the EQS. However, this standard may no longer be applicable given the methodology for deriving the standards has changed since the EQS was set (EC, 2011) and the test species is unlikely to be the most sensitive given the toxic effect of EmBz. Furthermore, the use of EmBz in Scottish Aquaculture has increased, with more frequent treatments at more locations (Murray, 2015).

Studies from other countries have reported levels of EmBz above the Scottish EQS in marine sediment around finfish cages e.g. Canada (Park, 2013), Norway (Langford et al., 2014), and Chile (Tucca et al., 2017). However, the only study to find possible links between EmBz sediment concentration and impact on a benthic crustacean species was from Park (2013) who demonstrated a reduction in Spot prawn (*Pandalus platyceros*) abundance and size immediately following treatment compared to two months later.

In Scotland, Black (2005) conducted one of the first investigations following EmBz authorisation and concluded that, whilst the fish farms had an impact on benthic assemblages, it was difficult to separate this from the likely impact of organic enrichment and/or the natural variability of the marine environment. Similarly, Telfer et al. (2006) concluded that there were no significant impacts on benthic assemblages from a single treatment at one farm, with observed impacts instead attributed to organic enrichment. A more recent study by Wilding and Black (2015), that used the data returns submitted by operators to SEPA, found differing results however. They used Generalised Linear Mixed Effect modelling to demonstrate an impact of EmBz use on crustacean abundance and richness. However, the study used data collected for compliance purposes and not for understanding widespread environmental impacts. Concurrent sediment EmBz concentrations and ecology data were unavailable so the authors modelled crustacean response to EmBz treatment data. This means that measured EmBz concentrations and, therefore, exposure were not considered in their analysis.

Given that concentrations have been observed above the EQS in other countries (Langford et al., 2014, and Tucca et al., 2017), and that EmBz use has been linked to impacts on benthic crustacea within the EQS limits (Park, 2013; Wilding and Black, 2015) there is scope for an investigation that assesses the widespread impact of EmBz using concurrently collected concentration and benthic ecology data. This paper presents the findings from the first study to collect data on EmBz concentrations and benthic ecology simultaneously. The objectives of the paper are to (i) determine the concentrations and distribution of in-feed sea lice medicines (EmBz and Tef) in the benthic marine environment (ii) assess the impact of observed in-feed sea lice medicine residues (EmBz and Tef) on overall benthic community composition and (iii) assess the impact of observed in-feed sea lice medicine residues (EmBz and Tef) on benthic crustacea.

2. Material and methods

2.1. Field methodology

Eight salmon marine cage fish farms were surveyed in Shetland, Scotland from 31/05/2017 to 22/06/2017 (Fig. 1). A cross-section of farms were selected from a range of different sediment types, current flows, water body sizes, history of EmBz use and fish farm operators. At each fish farm, three transects were sampled, with transect length and direction selected according to the modelled impact footprint of the fish farm using the autoDEPOMOD model (Cromey et al., 2002).

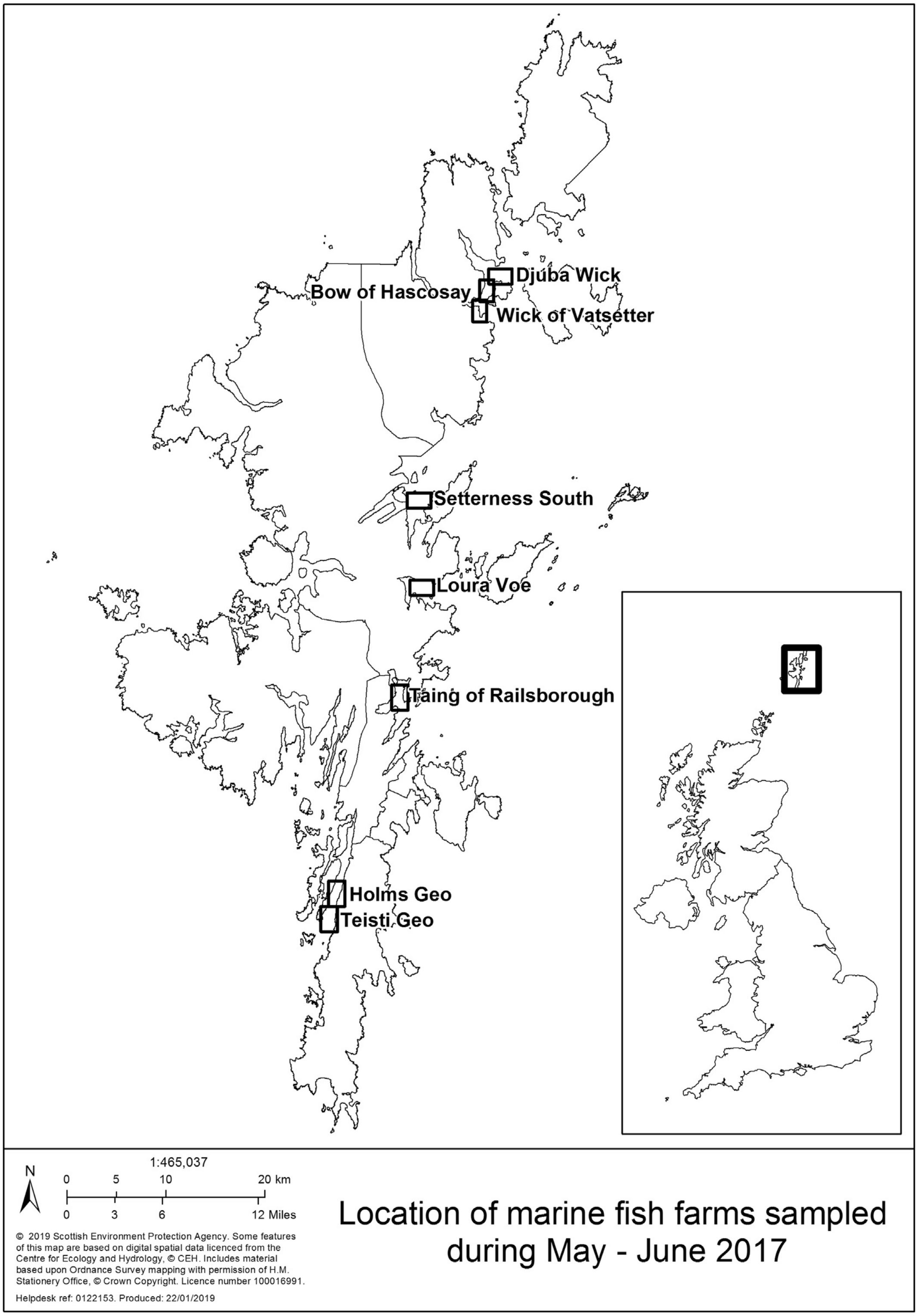

Fig. 1. Location of the eight fish farms surveyed in the Shetland Isles during May and June 2017 with inset image of the study area within the context of the United Kingdom.

Three samples were taken along each transect for both chemical and ecological analysis: one at the cage edge, one at a distance to represent the edge of the modelled impact and one beyond the modelled impact of the fish farm. A minimum of two reference stations were also selected at each site, where no impact from the fish farm was expected to have occurred according to the autoDEPOMOD modelled footprint. GPS units were used to collect accurate location information for the survey boat at each sampling station.

Sediment samples were collected from the seabed for both chemical and ecological analysis using a 0.045 m^2 Van Veen grab sampler attached to a winch from a small survey vessel. At each sample location, three separate replicate grab samples were taken for chemical analysis as per EC (2010). From each of these three grab samples, two cores were taken to a depth of 5 cm using a stainless steel corer: one for sea lice medicine residue analysis and one for supporting variables (Table 1). All samples were frozen on the day of collection and

Table 1
Parameters included in the GLMM and CCA modelling approaches.

Model parameter	Parameter code	Parameter type	Description	Transformation	In GLMM after parameter selection?	In CCA after parameter selection?
Crustacean abundance	ab_Crust	Response	Count of the number of individual crustaceans observed		Y	N
Crustacean species richness	no_Crust	Response	Count of the number of different crustacean species observed		Y	N
Benthic community composition		Response	Count of all individual for each species identified	Square root transformed	N	Y
Emamectin benzoate dry weight concentration	EmBz	Fixed predictor	Dry weight concentration of emamectin benzoate (ng/kg)	Log transformed and mean centred	Y	Y
Teflubenzuron dry weight concentration	Tef	Fixed predictor	Dry weight concentration of teflubenzuron (μg/kg)	Log transformed and mean centred	N	Y
Total organic carbon	TOC	Fixed predictor	Percentage organic carbon in sediment (%)	Log transformed and mean centred	Y	Y
Particle size <63 μm	PSA	Fixed predictor	Percentage of sediment with particle size <63 μm	Mean centred	Y	Y
Sediment moisture content	Mois	Fixed predictor	The percentage moisture content of the sampled sediment	Mean centred	N	N
Abundance of enrichment polychaetes	ab_Poly	Fixed predictor	Count of the number of individual enrichment polychaetes observed	Log transformed and mean centred	N	N
Emamectin benzoate mass	EmBz_Mass	Fixed predictor	The predicted mass of emamectin benzoate remaining following treatments and degradation over time	Mean centred	Y	N
Biomass at time of sampling	Biomass	Fixed predictor	The fish biomass at each farm at the time of sampling (tonnes)	Mean centred	Y	Y
Depth	Depth	Fixed predictor	Depth of water from which sample was collected	Mean centred	Y	Y
Bed speed	bed_speed	Fixed predictor	The average flow speed at the sea bed collected over a two week period when site was licensed	Mean centred	N	N
Within predominant flow direction	InFlow	Fixed predictor	A 0 or 1 value that represents whether the sample is along a transect within the main direction of flow from the fish farm cages. 0 = not in main flow direction, 1 = within main flow direction	Mean centred	Y	Y
Fallow period	Fallow	Fixed predictor	A 0 or 1 value that represents whether the sample belongs to a farm within a fallow period. 0 = fallow period, 1 = active	Mean centred	N	N
Distance from centre of cage group	Distance	Fixed predictor	The distance of each sampling point from the centre of the cage group	Rescaled to [0,1] range	N	Y
Site	Site	Random predictor	Name of each fish farm site		Y	N
Observation level parameter	ObsID	Random predictor	Observation level parameter added to account for mode overdispersion		Y	N

maintained frozen below −18 °C before being sent to the SEPA laboratory in North Lanarkshire for analysis.

For benthic ecology samples, two 0.045 m^2 grab samples were taken at each location and sampled for macrofaunal benthic ecology as per ISO 16665:2014. Samples were washed through a 1 mm sieve in the field, with any macrofauna left on the sieve mesh carefully extracted using forceps. Samples were preserved in a buffered formosaline solution (4% formaldehyde). A third grab sample was taken and sampled for Particle Size Analysis (PSA) using a 5 cm plastic corer following National Marine Biological Analytical Quality Control Scheme guidelines (NMBAQC: Mason, 2016).

2.2. Laboratory methodology

Samples for chemical analysis were analysed for the residues of the sea lice medicines EmBz and Tef, as well as for supporting parameters including particle size fraction below 63 μm (PSA), percentage loss ln lgnition (LOI), percentage total organic carbon (TOC) and percentage moisture content.

Particle size analysis was undertaken using laser granulometry to determine the fraction of the sample below 63 μm that constitutes 'fine' material. LOI followed the British Standard method BS EN 15169:2007, using a drying temperature of 105 °C and an ignition temperature of 550 °C. The method for determining percentage organic carbon was compliant with British Standard BS EN 13137:2001 and uses a dynamic flash combustion of the sample (following acid digestion to remove carbonates) from which the proportion of organic carbon in the sample was calculated after combustion gases have been detected.

Tef was extracted from the sediment using an Accelerated Solvent Extraction (ASE) technique. Following clean up, the sample was passed through a Liquid Chromatograph with Tandem Mass Spectrometric detection (LC-MS/MS) that separates, identifies and quantifies Tef. The Limit of Detection (LOD) for this method was 0.05 μg/kg. The method was accredited to ISO/IEC 17025 by the United Kingdom Accreditation Service (UKAS). A more detailed outline of the method, including quality control and assurance, is provided in the supplementary material.

A detailed outline of the analytical method for EmBz is presented in SEPA (2019). A simplified outline of the methodology is presented here and in the supplementary material. Sediment was extracted for EmBz using a manual Quick, Easy, Cheap, Rugged, Effective, Rugged and Safe (QuEChERS) method (Anastassiades et al., 2003) with acetonitrile solution and a magnesium sulphate drying agent. Following SPE clean up, the extract was analysed by liquid chromatography with high resolution mass spectrometric detection to separate, identify and quantify EmBz concentrations. The LOD for the method is 0.0034 μg/kg dry weight. The method used was accredited to ISO/IEC 17025 by UKAS.

Ecology samples were rinsed on 1 mm sieves to remove the formaldehyde and aqueous Rose Bengal dye was added for 20 min to stain all the macrofauna contained within the sample residue to aid detection. The residue was rewashed to remove excess dye and then the sample poured into white trays and spread out to allow all the macrofauna to be picked out with forceps and placed in vials with preservative (industrial methylated spirit). All the macrofauna specimens were identified and counted with the aid of stereo and compound microscopes and standard taxonomic identification literature. The procedure for analysing macrofauna samples follows guidance laid down by the NMBAQC Scheme (Worsfold & Hall, 2010).

2.3. Statistical methodology

Two different statistical approaches were applied to the dataset; (i) Canonical Correspondence Analysis (CCA) to assess the impact of environmental variables on overall benthic species community and (ii) Generalised Linear Mixed Modelling (GLMM) to assess the impact of environmental variables on benthic crustacean metrics (abundance and species richness).

Table 1 details the response and predictor variables used in each analysis, with any data transformations undertaken to meet model assumptions. Samples with missing predictor variables were removed from the analysis. A single value for each chemistry parameter at each sample location was calculated using the mean of the three replicates.

Predictor variables were scaled to have zero mean and unit variance, and EmBz, Tef and TOC were log transformed to account for strong positive skews in the distributions. Distance from the fish farm was rescaled to 0–1 for the CCA. Within both approaches, Variance Inflation Factors (VIFs) were used to assess collinearity between the predictor variables. Variables with VIFs >5 were deemed collinear and removed from the analysis (detailed in Table 1), the process was repeated until all remaining variables had VIFs <5.

All statistical procedures were conducted using the R software package (R Core Team, 2018).

2.3.1. Canonical Correspondence Analysis (CCA)

Benthic invertebrate species abundance tends to have a unimodal distribution along a gradient of disturbance (Rosenberg, 2001). Detrended Correspondence Analysis (DCA) confirmed that a unimodal approach is most appropriate in this case as the community gradient spanned 5.66 standard deviations (Lepš and Šmilauer, 2003). Canonical Correspondence Analysis (CCA) was therefore used to investigate the benthic community response to disturbance around farms in Shetland.

CCA is vulnerable to inaccurately modelling the points of highest abundance of infrequently recorded species so a minimum species observation threshold of 10 was chosen to build the model, after testing the number of species and proportion of total sample abundance retained at thresholds between 2 and 25 (Supplementary material A3).

Models were selected using stepwise deletion of variables to minimise AIC and the number of axes to include was determined through inspection of a scree plot (Supplementary material A3). Robustness of the model configuration to input data was tested by inspecting the linear constraint scores of each model term on CCA1 and CCA2 using subsets of the community dataset with the species observation thresholds mentioned earlier. Model, variable and axis significance was tested with the anova.cca function in the package vegan (Oksanen et al., 2018).

Because of the nature of the dataset, spatial autocorrelation had the potential to influence species optima. Therefore, three spatial covariates related to location (Depth, InFlow, Distance) were partialled out of the CCA and the residual sample scores were plotted on a map to check for any spatial patterns (Supplementary material A3). Taking spatial variables as covariates acknowledges there are many unmeasured pressures associated with fish farming that are likely to decrease linearly with distance from the farm; or that may be more or less important at different depths and tidal flow regimes. By requiring the model to separately account for spatial variables, the effect of other environmental predictors is attributable to variation in those environmental predictors over and above any variability within those predictors that is confounded with space. Partialling covariates to understand the effects of variables of interest in thus way is a well-established method (Legendre and Legendre, 1998).

2.3.2. Generalised Linear Mixed Models (GLMMs)

Both response variables (crustacean abundance and species richness) represent ecological count data, therefore a Poisson GLMM with a log link function was selected as the most appropriate model type (Zuur et al., 2010). Model overdispersion was assessed by determining the ratio between residual deviance and degrees of freedom. A ratio of 1.5 was used as the threshold for overdispersion. If the models were overdispersed an object level random effect was added to model extra-Poisson variation in the response variable (Harrison, 2014). A random effect was added at the farm level (Site) to account for localised environmental variables that were not explicitly included in the analysis as fixed effects.

The model selection process for determining the best fitting GLMM was to first create a 'global' model with all predictor variables included (after removing collinear variables using VIFs) using the lme4 package (Bates et al., 2015). Fish farm 'Site' was included as a random effect variable in all models. A multi-model inference approach was then used to select the best fitting model using the 'dredge' function from the MuMIn package (Barton, 2018). A second order Akaike Information Criteria (AICc) was used to assess model fit. ΔAICc, the difference between the AICc of each proposed model and the model with the lowest AICc was used to select the best fitting models; and all models with ΔAICc<2 were considered. In the first instance, model parsimony was preferred over model averaging. Therefore, where one of the best fitting models was nested within all the others, a likelihood ratio test (LRT) was used to check that the additional variables did not significantly improve model fit at the 95% confidence level. If there was significant improvement in model fit then parameters were averaged across all models identified as having significantly improved fit by the LRTs. In all cases, model assumptions were checked using diagnostic plots.

The 'effects' package (Fox, 2003) was used to simulate the fixed effect of individual predictor variables within the best fitting model.

3. Results

3.1. Survey results

In total, 83 of 90 data points were suitable for inclusion in the statistical analysis. Missing data points were a result of missing depth data or failed ecology grab samples where the seabed was not suitable for a benthic sediment grab. This was primarily due to the physical nature of seabed dominated by calcareous algae (*Lithothamnion* sp.) restricting closure of the grab sampler and impacting on the volume of the sample collected. Whole survey chemical results by location with respect to fish farm cages are presented in Fig. 2 (additional plots by fish farm are shown in Supplementary section A2).

Of the chemistry replicate samples collected, 97% had a detection of EmBz above the LOD (0.004 μg/kg dry weight), with detections at all farms surveyed. Concentrations generally followed a spatial gradient linked to distance from the cages, with the highest concentrations found in the immediate vicinity of the cages (Fig. 2). Approximately 7% of the samples >100 m from the cages (where the EQS applies) were above the EQS (0.763 μg/kg wet weight), whilst 17% of cage edge sample were above the cage edge trigger value (7.630 μg/kg wet weight).

Tef was detected at three of the eight farm sampled during the survey, with 36% of samples taken >100 m from the cages detected above the EQS. Where it was detected, Tef concentrations were generally higher away from the cages (Fig. 2 and Supplementary material A2).

TOC generally followed the same pattern as EmBz, with percentage TOC highest under the cages and decreasing with distance from the cages (Fig. 2). There was some observed variability around this general trend however, with slight increases in TOC observed along the northern transects of Holms Geo and Bow of Hascosay (Fig. 1).

There was a slight spatial gradient in particle size observed at a number of the sites with sediments increasing in fineness with distance from

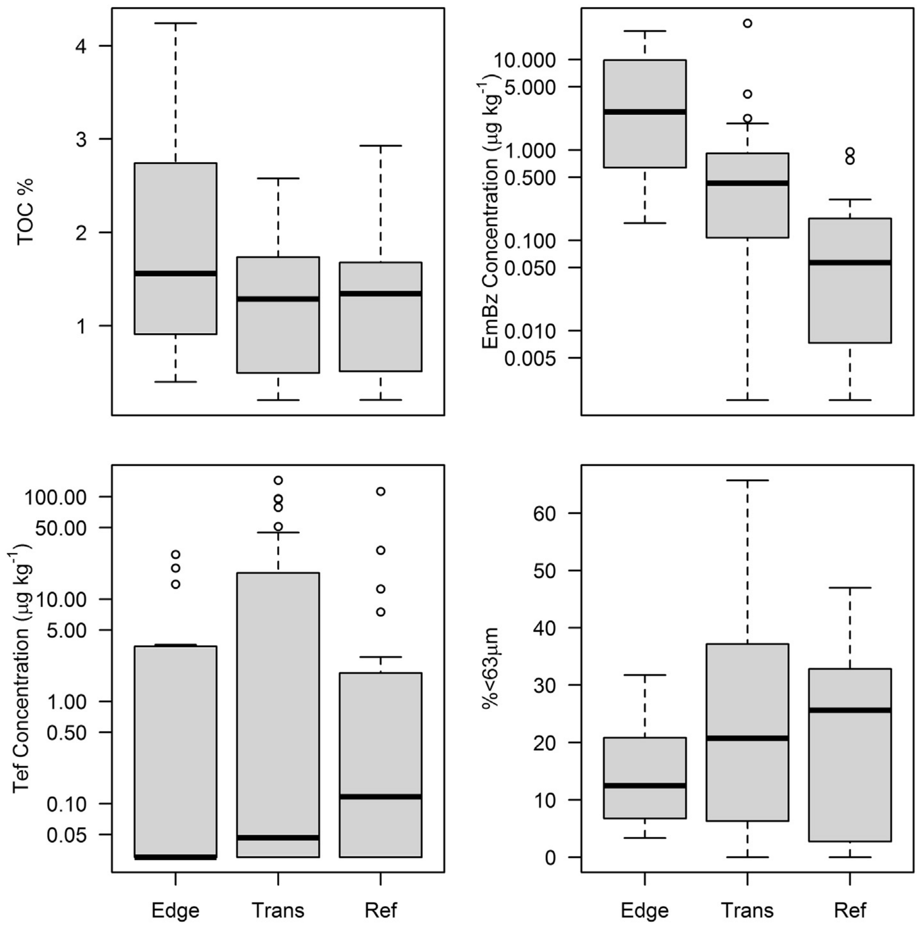

Fig. 2. Box plots of chemistry parameters for the whole survey by sample location group (cage edge = at the edge of the fish farm cage 0 m, transect = samples along the transect >0 m and reference = reference sites a minimum of 500 m from the fish farm). Raw data can be found in the Supplementary material.

cage at Taing of Railsborough and Loura Voe, with the opposite pattern observed at Djuba Wick. Particle size predominantly varied between sites with some consisting of coarser sediment more associated with sandy sediments (e.g. Bow of Hascosay, Djuba Wick and Wick of Vatsetter) and others of finer sediment associated with sandy mud (e.g. Holms Geo, Loura Voe and Taing of Railsborough). Variability in particle size was generally lower under the cages than out along the transects and at reference stations (Fig. 2). This may be reflective of the closer spatial proximity of cage edge stations when compared to transect and reference stations.

Macrofaunal analysis recorded 777 taxa across all samples. 20 phyla and 37 classes were represented in the dataset. Polychaete worms were the most diverse group, followed by molluscs and crustaceans (summarised in Table 2). The results highlighted a gradient of community impact response with distance from the cages, with the greatest impact observed under the cages. The metrics presented in Fig. 3 demonstrate this with the Infaunal Quality Index (IQI; UKTAG, 2014), Infaunal Trophic Index (ITI; Codling and Ashley, 1992), species richness, Shannon H′, Pielou J′ and Simpson 1-λ′ indices all lowest under the cages, and increase with distance from the fish farm cages.

Table 2
Taxonomic breakdown of community dataset. Numbers in parentheses are how many taxa were retained for CCA analysis.

Phylum	Number of taxa
Annelida	283 (68)
Mollusca	157 (28)
Arthropoda	154 (7)
Bryozoa	61 (−)
Echinodermata	48 (5)
Cnidaria	33 (1)
Others	41 (9)

3.2. Canonical Correspondence Analysis

At a minimum observation threshold of 10 sampling locations, 117 benthic species were retained for analysis with CCA. The model selected had five constraining explanatory variables: Biomass, EmBz, TOC, PSA and Tef. In addition to this were the three spatial conditioning variables: Depth, InFlow and Distance. The CCA model was:

$$\text{ShetlandBenthosSpecies} \sim \text{Biomass} + \text{EmBz} + \text{Tef} + \text{TOC} + \text{PSA} + \text{Condition}(\text{Depth} + \text{InFlow} + \text{Distance}) \quad (1)$$

where ‘ShetlandBenthosSpecies’ was the benthic community data matrix, and the variables in the ‘Condition’ parentheses are partialled out from the ordination. The constrained axes explained 21.6% of total inertia. Where the first two constrained axes, CCA1 and CCA2 explained 81.1% of constrained inertia. Partialled out inertia associated with spatial variables explained 15.4% of total inertia. The first constrained axis (CCA1) represented a gradient of sediment particle size and Tef, which had a strong positive loading. Biomass and EmBz had a moderate negative loading on CCA1. The second constrained axis (CCA2) represented a gradient of TOC, EmBz, Tef and PSA. The biplot demonstrated community composition organised along predominantly the axis of variation of PSA/Tef and EmBz (Fig. 4). Crustacean species retained in the reduced

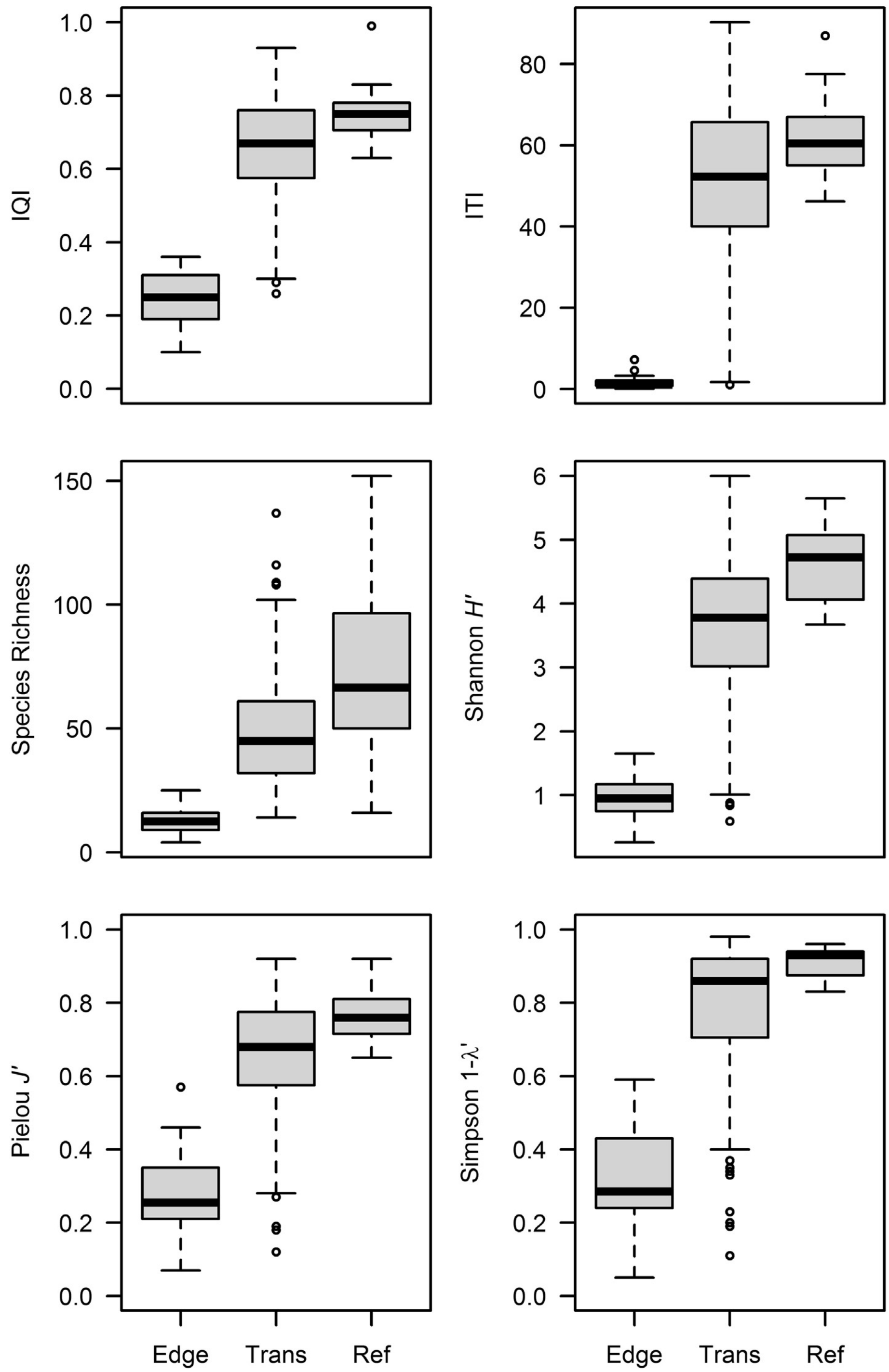

Fig. 3. Box plots of ecology parameters for the whole survey by sample location group (group (cage edge = at the edge of the fish farm cage 0 m, transect = samples along the transect >0 m and reference = reference sites a minimum of 500 m from the fish farm)). Raw data can be found in the Supplementary material.

dataset were divided into three groups: three species found only in coarse sediment, low TOC, low EmBz conditions (*Ampelisca typica*, *Urothoe elegans*, *Pariambus typicus*), two species found in mixed conditions (*Ampelisca tenuicornis*, *Tanaopsis graciloides*) and one species found in fine sediments (*Pagurus cuanensis*) (Fig. 4).

All selected explanatory variables were statistically significant predictors of community composition and CCA1 and CCA2 were both statistically significant linear combinations of these explanatory variables (Table 3). Positive scores on CCA1 indicated finer sediments with a higher Tef concentration, moderately low EmBz concentration and lower fish farm biomass. Positive scores on CCA2 indicated low EmBz and TOC concentrations and moderately coarse sediments. Model residuals did not have an obvious structure when mapped to sample location coordinates (Supplementary material A3). Sensitivity analysis of species indicated a stable order of loadings on CCA1 and CCA2 at a minimum species observation frequency of 10 (Supplementary material A3).

Because the majority of crustacean species were observed fewer than 10 times, the CCA model was used to predict species scores on CCA1 and CCA2 for all taxa observed between 2 and 9 times (these were excluded from the initial analysis). Predicted optima of infrequently sampled crustacean taxa were associated with coarse sediment

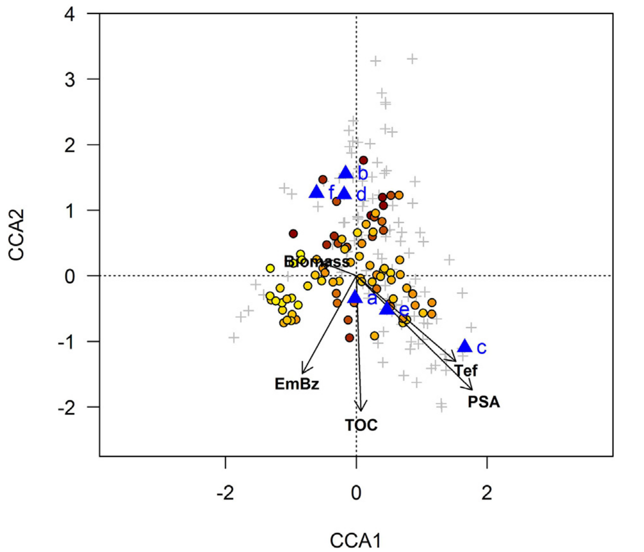

Fig. 4. CCA biplot. Filled circles are sample scores coloured by the ratio of EmBz to TOC at each sampling point. Blue triangles are crustacean species (a = *Ampelisca tenuicornis*, b = *Ampelisca typica*, c = *Pagurus cuanensis*, d = *Pariambus typicus*, e = *Tanaopsis graciloides* and f = *Urothoe elegans*). Grey crosses represent all other non-crustacean taxa. Arrows are scaled by loadings on each axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

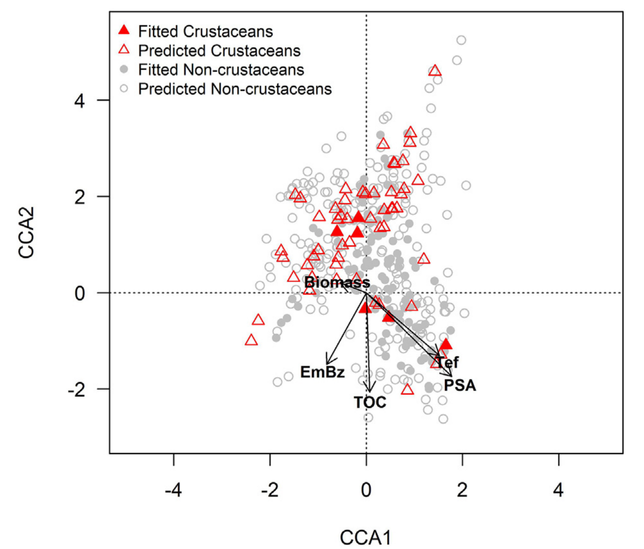

Fig. 5. CCA biplot with predicted species scores. Fitted scores (filled symbols) are species scores of species used in configuration of the CCA. Predicted scores (open symbols) are species scores for species observed between 2 and 9 times, estimated from CCA linear combination scores.

and tended to have negative CCA1 scores but a range of CCA2 scores associated with varying in EmBz concentrations (Fig. 5).

3.3. Generalised Linear Mixed Model

Using VIFs the parameters removed from the model selection process due to multilinearity were: Fallow, bed_speed and Mois. The parameters used in the global model are shown in Table 1.

3.3.1. Crustacean abundance

The model selection process generated three models combinations of four predictor variables: InFlow, EmBz, Biomass and Depth. A model including only EmBz and InFlow was nested in all of the best fitting models and none of the more complex models significantly improved model fit. Therefore, a parsimonious model with two fixed effects was deemed the best fitting model:

$$\text{ab\_Crust} \sim \log(\text{EmBz}) + \text{InFlow} + (\text{Site}) + (\text{ObsID}) \tag{2}$$

where the brackets represent random effects parameters. The coefficients for the fixed effects in the best fitting model are shown in Table 4, they show that EmBz had a significant ($p < 0.001$) negative effect on crustacean abundance. The effect of InFlow was weaker (but still significant) and shows that crustaceans were more abundant when samples were taken in the predominant flow direction from the cages.

A plot demonstrating the effect of EmBz on crustacean abundance from the best fitting model is shown in Fig. 6.

Residual plots for the best fitting model do not show any obvious patterns and are shown in Supplementary material A3.

There was some observed collinearity between EmBz and TOC, although not enough to be removed in the VIF process. When EmBz was removed from the model selection process TOC was a significant predictor of crustacean abundance, but had less explanatory power than EmBz and was less significant ($p = 0.01$).

3.3.2. Crustacean species richness

The model selection process generated three models using five different predictor variables including: EmBz, InFlow, Biomass, Depth and TOC. All of the three models contained a nested model containing the parameters EmBz, Biomass and InFlow. Using LRT, the addition of

Table 3
Permutation test of significance of variance explained by CCA model versus chance.

Permutation test for cca under reduced model				
Number of permutations: 999				
Model: sqrt(ShetlandBenthosSpecies) ~ Biomass + EmBz + Tef + TOC + PSA + Condition(Depth + Inflow + Distance)				
Full model				
	D.F.	χ^2 distance	F	p-Value
Model	5	0.76261	5.0819	<0.001***
Residual	74	2.22096		
Model terms				
Biomass	1	0.08138	2.7116	<0.001***
EmBz	1	0.27215	9.0480	<0.001***
TOC	1	0.13711	4.5684	<0.001***
PSA	1	0.21559	7.1833	<0.001***
Tef	1	0.05697	1.8981	0.029*
Residual	74	2.22096		
Model axes				
CCA1	1	0.35402	11.7957	<0.001***
CCA2	1	0.26472	8.8203	<0.001***
CCA3	1	0.06418	2.1384	0.023*
CCA4	1	0.04843	1.6136	0.097•
CCA5	1	0.03126	1.0416	0.374
Residual	74	2.22096		

*** = significant $p < 0.0001$, ** = significance $p < 0.001$, * = significant $p < 0.05$, dot = close to 0.05.

Table 4
Parameter estimates, standard error, z score and associated p value of fixed parameters in GLMMs.

Model	Fixed effects	Estimate	Std error	z	p-Value
Crustacean abundance	Intercept	1.81	0.13	13.8	<0.0001***
	EmBz	−0.86	0.12	−6.6	<0.0001***
	InFlow	0.34	0.13	2.6	0.009**
Crustacean richness	Intercept	1.19	0.08	15.2	<0.0001***
	EmBz	−0.54	0.08	−6.80	<0.0001***
	Biomass	0.19	0.08	2.46	0.01*
	InFlow	0.19	0.08	2.37	0.02*

*** = significant $p < 0.0001$, ** = significance $p < 0.001$, * = significant $p < 0.05$.

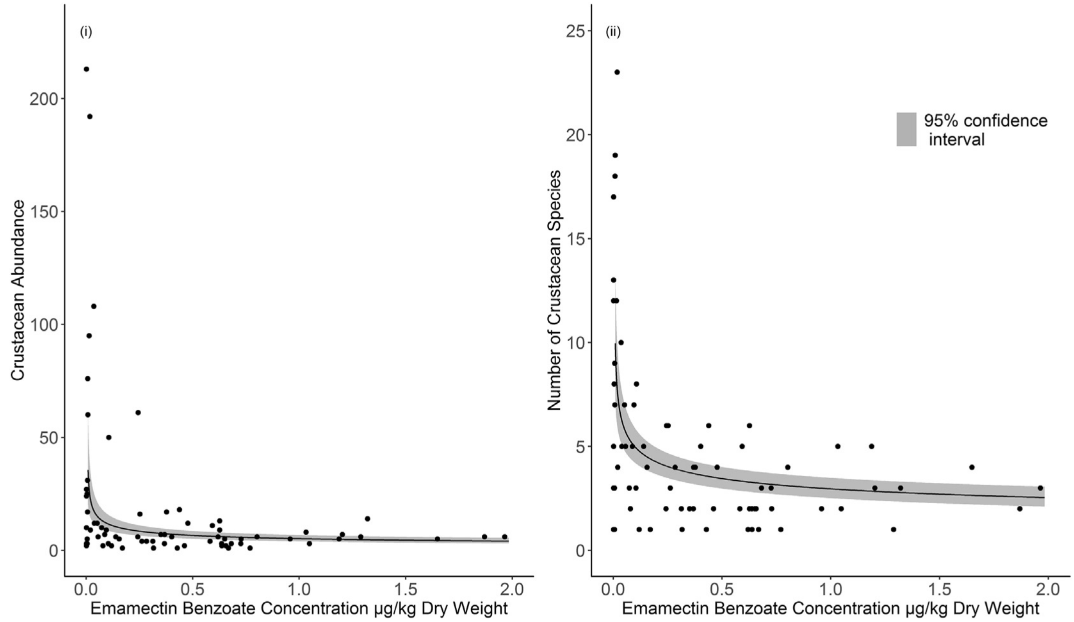

Fig. 6. EmBz effect on (i) Crustacean abundance and (ii) crustacean species richness.

the parameters Depth and TOC were not significant and therefore the three-parameter parsimonious model was deemed the best fitting.

$$\text{no\_Crust} \sim \log(\text{EmBz}) + \text{InFlow} + \text{Biomass} + (\text{Site}) + (\text{ObsID}) \quad (3)$$

Model coefficients for the averaged model are shown in Table 4, demonstrating that EmBz had a significant ($p < 0.001$) negative effect on crustacean species richness. A weaker, but still significant ($p < 0.05$) positive effect was also present for InFlow and Biomass.

A plot for the effect of EmBz on crustacean species richness is shown in Fig. 6.

The model selection process was rerun without EmBz to assess the potential collinear effect of TOC as per the crustacean abundance model process. TOC again had a significant effect on crustacean abundance, but explanatory power was not as large as EmBz.

4. Discussion

4.1. Sea lice medicines in the environment

The results demonstrated a widespread detection (97% of samples) of EmBz in the sediments sampled from the survey. Such ubiquitous distribution across multiple sample locations at various distances from the cages (including reference stations) has not been documented in previous studies and surveys (e.g. Black, 2005; Telfer et al., 2006). This finding suggests that, at least in the study area, EmBz is distributed much more widely in the environment than previously observed. Such a finding is potentially attributable to a number of factors.

EmBz has been licensed for use in Scotland as a treatment for sea lice in caged finfish for almost 20 years (SEPA, 1999). Over this time, usage has increased (Murray, 2015), which suggests accumulation in sediment is likely given its known persistence. This has been demonstrated with other persistent sea lice medicines e.g. Tef (Samuelsen et al., 2015; this survey). In addition, the areas surveyed in Shetland are predominantly representative of dynamic hydrographic conditions, with a high likelihood of sediment dispersal and entrainment across large areas. As a result, sediments with bound EmBz may be distributed more widely under these conditions than in areas with more sluggish flow.

This is further demonstrated by the results for Tef, which has not been used at any of the fish farms surveyed since December 2013 and was found at three of the eight sites. The fact that concentrations are still being detected (with no other uses in the marine or terrestrial environment in the UK), highlights its persistence. Although evidence for an impact on benthic invertebrates could not be separated from the effect of particle size in this study, its presence in marine sediments collected during this survey demonstrates that legacy substances can persist in the environment long after use.

The method used to analyse sediments for EmBz in this survey had a Limit of Detection (LOD) an order of magnitude lower than the previous best method (SEPA, 2019 and Supplementary material). The expanded range of detectable concentrations showed that 24% of sample locations had an observed EmBz concentration within the range of the previous best LOD (0.108 μg/kg dry weight) and the LOD of the new low detection method (0.0034 μg/kg dry weight). This highlights the importance of having low-level detection methods when attempting to understand the extent of persistent substances in the marine sediment environment.

EmBz concentrations were arranged along a gradient, with the highest concentrations observed at the cage edge, decreasing with distance from cage. This is again reflective of the deposition, distribution and degradation of EmBz over time. The same spatial gradient was observed for benthic ecology in Fig. 3 and TOC in Fig. 2. These patterns are similar to those observed in other studies on fish farm impacts (Brown et al., 1987; Hall-Spencer and Bamber, 2007; Mayor et al., 2010) and TOC enrichment from fish farms (Carroll et al., 2003; Kutti et al., 2007; Norði et al., 2011).

4.2. Impact of environmental parameters on benthic community composition

The CCA results showed that benthic community composition was most strongly affected by PSA and Tef, and secondarily by TOC and EmBz. The strong effect of particle size on benthic community composition is well established (Rhoads and Germano, 1986; van Hoey et al., 2004). Tef was found in high concentrations in mainly finer sediments, therefore it was not possible to distinguish the effect of Tef from the effect of particle size on the benthic community. This may be attributable to the high affinity of Tef to bind to fine organic particulate (K_{oc} of 21,139–32,556 mL/g in soil, EFSA, 2008). The combined effect of both constrained axes resulted in organisation of the community across all samples, which followed the disturbance pattern laid out in Pearson and Rosenberg (1978).

4.3. Impact of environmental parameters on crustacean taxa

EmBz had the single biggest negative effect on both crustacean abundance and crustacean species richness in the GLMMs and was the environmental parameter most closely aligned to the distribution of crustacean species optima in the CCA.

The best-fit GLMM for crustacean abundance contained only the fixed effects EmBz, InFlow and a random effect to account for site. Whilst, the best-fit model for crustacean species richness contained the parameters EmBz, InFlow and Biomass. Removing EmBz from the GLMM model selection process for both crustacean abundance and species richness demonstrated a significant, albeit weaker effect of TOC. This demonstrates a degree of collinearity between EmBz and TOC, which to some extent is expected. Both were concurrently sourced from the same fish farm effluent, meaning they both decrease with distance from the cages, as shown in Fig. 2. In addition, EmBz has a very high affinity to bind to organic carbon with K_{oc} values ranging from 28,363 mL/g to 728,918 mL/g in the literature (USEPA, 2009; EFSA, 2012), meaning higher TOC levels in sediment increases the likelihood of EmBz adsorption. Both have demonstrable effects on crustaceans. EmBz interferes with the gamma aminobutyric acid and chloride channels in crustaceans, which causes a loss of cell function and paralysis (Burridge et al., 2004). Organic enrichment alters oxygen availability and increases sulphide concentrations within sediment (Pearson and Rosenberg, 1978; Sutherland et al., 2007), impacting on sensitive crustacean species inhabiting the sediment surface and subsurface (Sutherland et al., 2007). Results from the GLMM reinforce the findings of the CCA by demonstrating that EmBz was the main predictor of crustacean abundance and species richness in the dataset. This corroborates the key finding of Wilding and Black (2015), who linked widespread crustacean impacts to the use of EmBz using national operator returns data.

Small crustaceans respond negatively to fish farm impact, however, disentangling the attribution of impact to organic enrichment or EmBz effects has previously proven difficult (Telfer et al., 2006; Hall-Spencer and Bamber, 2007). The majority of crustaceans found in this study were amphipods, which are well known to respond to disturbance differently according to lifestyle (Pezy et al., 2018; Poggiale and Dauvin, 2001; De-la-Ossa-Carretero et al., 2012; Wilding et al., 2017). This was clear from the correspondence of lifestyle to species scores on the CCA ordination. Tube-building amphipods had optima in moderate EmBz concentrations, which may reflect the ability of this lifestyle to control the microenvironment in the tube (De-la-Ossa-Carretero et al., 2016). In this group, *Tanaopsis graciloides* and *Ampelisca tenuicornis* are able to facultatively switch between feeding on suspensions and deposits (De-la-Ossa-Carretero et al., 2012; Shojaei et al., 2015; Guerra-García et al., 2014; Wilding et al., 2017) which potentially alters vulnerability of these species to EmBz depending on levels in these two media. The only crustacean that seemed to be insensitive to EmBz was not an amphipod: the epifaunal hermit crab *Pagurus cuanensis.* Epifauna do not respond to organic discharges in the same way as infauna, and are able to move into areas of impact to feed opportunistically before withdrawing (Pearson and Rosenberg, 1978; Hall-Spencer et al., 2006). The species with the greatest affinity for low EmBz concentration in this study tended to have a number of characteristics: interstitial burrowing, obligate deposit feeding detritivory, and low mobility (Connor et al., 2004; Guerra-García et al., 2014; Queirós et al., 2013; Pezy et al., 2018; Shojaei et al., 2015; De-la-Ossa-Carretero et al., 2012). Interestingly one of these was *Pariambus typicus*, which has been shown to respond positively to moderate organic enrichment around fish farms (Fernandez-Gonzalez et al., 2013; Guerra-García and García-Gómez, 2005), inviting the conclusion that EmBz acted to prevent this expected response in this study.

These effects have the potential to influence the rate of recovery of fallowed marine fish farm sediments. Bioturbation can release EmBz from sediments (Stomperudhaugen et al., 2014). However, the slow and shallow bioturbation of cage edge opportunist organisms like *Capitella* under conditions of food enrichment potentially limits the rate of this process (Przeslawski et al., 2009). EmBz, therefore, has the characteristics to be a long-term barrier to the participation in succession, particularly of crustaceans that perform important bioturbating and bioengineering roles (Coates et al., 2016). The extent to which this has occurred due to the toxic effects of consuming sediment with a high EmBz load, versus the chronic effect of consuming sediment denuded of interstitial crustacean meiofauna that make up a proportion of the diet of vulnerable species (Guerra-García et al., 2014) requires improved understanding of how EmBz can affect meiofauna (Bright et al., 2004).

The predominant flow direction (InFlow) had a weak but significant positive effect on both crustacean abundance and richness. Hall-Spencer and Bamber (2007) found negative impacts along transects in the predominant direction of flow but did not look at samples perpendicular to this for comparison. This effect may be attributable to the rise in diversity associated with moderate enrichment (Pearson and Rosenberg, 1978), or a factor not considered in this study. It is not possible to draw conclusions on this finding from the data presented in the study. It must be emphasised however that the effect was small, with abundance and richness dominated by the negative effect of EmBz/TOC.

Farmed fish biomass at the time of sampling also had a weak but significant positive effect on benthic crustacean richness. Whilst variation in salmon biomass between farms could have been expected to have a strong effect on benthic communities (Forrest et al., 2007), further investigation into this effect within the data from the survey showed that sites with high biomass were on the coarsest sediments. We therefore suggest this effect is a result of particle size rather than biomass, as suggested by the dominance of particle size on community composition in the CCA in Section 3.2. The role of stocking biomass compared to other environmental variables would need to be further resolved with investigation across a wider range of sediment types.

4.4. Further work

Results from the CCA demonstrated that benthic community composition was strongly related to particle size, however, the Shetland Islands typically have relatively coarse sediments compared to some other areas of Scotland, especially the west coast where sediments are muddier (JNCC, 2016). An investigation encompassing more of the sediment particle size spectrum to demonstrate the wider applicability of the findings of this study is desirable, especially as aquaculture impacts have been demonstrated to vary depending on sediment granulometry (Fernandez-Gonzalez et al., 2013).

EmBz has been used in the study area for around 20 years suggesting there may be some degree of resilience in the benthic community sampled. In addition, the near ubiquity of EmBz detected in samples taken means that there were no truly undisturbed reference conditions sampled. A survey of this nature at a site that has never used EmBz would be beneficial so that the effects of EmBz and organic enrichment can be compared to just the effects of organic enrichment.

As discussed in Section 4.3, the exposure of a species to sediment-associated chemicals is a function of its mobility, burrowing, reproductive and feeding behaviour (Wilding et al., 2017). The conclusions of this paper are, therefore, not readily extrapolated to crustacean species that do not exhibit the same behaviours as the crustaceans impacted in this study. Previous studies have highlighted the potential impacts of EmBz on more transient, larger crustacean species, such as Waddy et al. (2010) who demonstrated that EmBz impacted on the molt cycle of American Lobsters (*Homarus americanus*). Further investigation using surveillance is therefore required to understand if the effects demonstrated in this study are applicable to economically important crustacean species.

5. Conclusions

EmBz was detected at almost every location sampled in the survey and Tef was detected in half of the locations surveyed nearly 5 years after the cessation of use. Such widespread occurrence of EmBz in the environment has not been observed in previous studies and suggests residues may be distributed more widely than previously thought. In addition, analysis of the data demonstrates an effect of EmBz on abundance, diversity and community structure of benthic ecology at the concentrations observed during the survey. Within crustaceans, low mobility taxa with a burrowing and detritivorous lifestyle were identified as particularly vulnerable to EmBz. These findings demonstrate effects on crustacea below the level of the current EQS (0.763 μg/kg wet weight).

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Appendix A. Supplementary data

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