Exploring changes in bacterial communities to assess the influence of fish farming on marine sediments

Esther Rubio-Portillo, Adriana Villamor, Victoria Fernandez-Gonzalez, Josefa Antón, Pablo Sanchez-Jerez

PII: S0044-8486(18)32115-X
DOI: https://doi.org/10.1016/j.aquaculture.2019.03.051
Reference: AQUA 634009

To appear in: *aquaculture*

Received date: 3 October 2018
Revised date: 28 January 2019
Accepted date: 23 March 2019

Please cite this article as: E. Rubio-Portillo, A. Villamor, V. Fernandez-Gonzalez, et al., Exploring changes in bacterial communities to assess the influence of fish farming on marine sediments, aquaculture, https://doi.org/10.1016/j.aquaculture.2019.03.051

Exploring changes in bacterial communities to assess the influence of fish farming on marine sediments

Esther Rubio-Portillo[1], Adriana Villamor[2*], Victoria Fernandez-Gonzalez[2], Josefa Antón[1], Pablo Sanchez-Jerez[2]

1. Department of Physiology, Genetics and Microbiology. University of Alicante; Sant Vicent del Raspeig. 03690 Alicante (Spain)
2. Department of Marine Science and Applied Biology. University of Alicante; Sant Vicent del Raspeig. 03690 Alicante (Spain)

* Current address: International Council of the Exploration of the Sea, H.C. Andersens Blvd. 44-46 Copenhagen, Denmark

Abstract

Changes in bacterial assemblages along an environmental gradient determined by the distance to aquaculture installations were analysed, using denaturing gradient gel electrophoresis to assess the influence of fish farming on marine sediments. Our findings show that changes in the structure of the bacterial community are a useful indicator for determining the environmental impact of aquaculture farms, due to the rapid response to changes in nutrient load, and could be an alternative strategy for monitoring programmes. Delta and Epsilonproteobacteria linked to the sulphur cycle were detected in the sediments beneath the cages. Since these groups were not found in the sediments at control stations, they serve as indicators for assessing the impact of the organic load from fish farming on marine sediments.

Keywords: Fish farming, DGGE, Mediterranean, Environmental impact, 16S rRNA

INTRODUCTION

Marine fish farming impacts the marine environment, principally due to the input of a substantial amount of organic matter and nutrients, resulting from uneaten food and fish excretions, which are released into the environment and accumulate in the underlying sediments (Holmer et al., 1991;

Karakassis et al., 1998). The sea bed below fish cages is where the contaminant build-up and low-oxygen conditions caused by fish farming activities are normally most critical. This increase in organic matter may result in eutrophic and anoxic conditions in the sediments, with a consequent reduction in the oxygen available for benthic assemblages, in addition to higher levels of toxic products, including sulphides and ammonium (Holmer and Kristensen, 1992; Holmer and Frederiksen, 2007)

Because of this potential environmental impact, as part of proper sustainable development, the monitoring of aquaculture activities in coastal areas is a very effective tool (FAO, 2009), particularly with regard to the generally accepted need to detect and correct negative environmental impacts. Benthic eukaryotic communities have been extensively used as quality indicators because they live in close association with the substratum (Fernandez-Gonzalez et al., 2013; Tomasetti et al., 2016). Indeed, during recent decades, the analysis of direct changes in benthic assemblages ("taxonomy-based metrics"), linked to the development of benthic biotic indexes ("autecology-based metrics"), has been extensively applied to monitor the environmental quality of European water bodies (Van-Hoey et al., 2010; Birk et al., 2012). In spite of this widespread use, the application of these indexes poses several problems (Quintino et al., 2006; Dauvin et al., 2012; Labrune et al., 2012; Aguado-Giménez et al., 2015). For instance, identifying fauna is a time-consuming process, subject to error and requiring taxonomic expertise, and could result in a "taxonomic impediment", as well as being a labour-intensive task.

Considering that one gram of marine subsurface sediment may harbour up to 10 billion microorganisms of possibly thousands of different species (Whitman et al., 1998), prokaryotic microbial community biomass and diversity are much higher than that of eukaryotes. Furthermore, it is well known that microbial populations respond rapidly to environmental changes, meaning they could be useful for assessing alterations in benthic conditions, i.e., the effects of fish farming on the underlying sediments (Danovaro, 2000; Vezulli et al., 2002; La Rosa et al., 2004; Bissett et al., 2006; Luna et al., 2012).

An understanding of ecosystem responses to anthropogenic impacts like fish farming is required to ensure the sustainability of coastal environments, and it is necessary to establish techniques that facilitate an accurate and easy interpretation of the differences between disturbed and control areas. Here, we have investigated the effects of fish farming on microbial assemblages in the sediment along an environmental gradient determined by the distance to the aquaculture installations. For this purpose, we used a molecular approach, namely denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified partial 16S

rRNA gene from environmental DNA. Our findings show that there are no differences in microbial diversity under the cages but there are changes at bacterial community structure level, mainly after intense feeding periods.

MATERIAL AND METHODS

Study area and sampling procedure

Two coastal fish farms (for gilthead sea bream, *Sparus aurata*, and sea bass, *Dicentrarchus labrax*), exhibiting different environmental conditions, were chosen for this study. The farms are located on the southeastern coast of Spain: Farm 1 (latitude: 38 °05' 15.6" N, longitude: 00°35' 46.8" O); and Farm 2 (latitude: 38° 07' 13.4" N, longitude: 00° 35' 46.7" O). Farm 1 is situated at a depth of 25 m and Farm 2 at 30 m. For each farm, we tested the influence of coastal aquaculture, including three sampling stations at increasing distances from the fish cages, downstream of the direction of the main current: the farm itself (directly below the cages); an intermediate point (at the edge of the farm facilities, as defined by the concession delimitation buoys); and a control point (more than 1 km from the fish farm).

The sampling was carried out between 2009 and 2010, in two distinct seasons: the first was at the end of a light fish-feeding period (March); and the second was after an intense feeding period (October). The magnitude of the seasonal effect was determined by the changes in the control locations. These samples were previously used to study amphipod assemblages and for sediment analysis (Fernandez-Gonzalez et al., 2013), in addition to polychaete assemblages (Martinez-Garcia et al., 2013).

Sediment characterisation

Sediment particle size was determined using the wet sieve method (Buchanan, 1984), with the finest fraction (< 0.063 mm) selected as a variable for statistical analysis. Total free sulphide (TFS) content was measured using a sulphide antioxidant buffer solution and ascorbic acid, with a silver/sulphide half-cell electrode, following the method described by Wildish et al. (1999). Total sulphur (TS), total nitrogen (TN), and total organic carbon (TC) were determined with a CHNS auto-analyser (elemental auto-analyser LECO 932); the ^{15}N isotope composition was measured using an EA-IRMS (Thermo Finnigan) analyser in continuous flow configuration joined to a stable ratio mass spectrometer (Delta Plus). The ^{15}N

isotopic composition was expressed as: $\delta^{15}N(‰) = [(Rsample / Rstandard) - 1] \times 10^3$, where $R = {}^{15}N/{}^{14}N$, atmospheric N_2 being the standard and 0.1‰ the analytical precision.

DNA extraction and chain reaction amplification of 16S rRNA genes

Community DNA was extracted from frozen sediment samples using the UltraClean Soil DNA Isolation Kit (Mo BIO; Carlsbad, CA), according to the manufacturer's instructions. The genomic DNA extracted was used for the PCR amplification of partial bacterial 16S rRNA genes, using the specific primer 341f-GC (Muyzer et al., 1993) and 518R (Sánchez et al., 2007). Each PCR mixture contained: 5 µl of 10× PCR reaction buffer (Invitrogen), 2.5 µl of 50 mM $MgCl_2$, 1 µl of a 10 mM dNTP mixture, 1 µl of 10 µM (each) primer, 1 unit of Taq polymerase, at least 60 ng of the extracted DNA, and sterile MilliQ water up to 50 µl. The PCR programme for bacteria was: 94ºC for 5 min, 65ºC for 1 min, 72ºC for 3 min, and 9 touchdown cycles of: 94ºC for 1 min, 65ºC (with a decrease of 1ºC in each cycle) for 1 min, 72ºC for 3 min, followed by 20 cycles of: 94ºC for 1 min, 55ºC for 1 min, and 72ºC for 3 min (Muyzer et al., 1993). During the final cycle the extension step was increased to 30 min to minimise double band formation (Janse et al., 2004) and eliminate heteroduplexes; 5 µl of PCR products were used as templates for a 5-cycle reamplification (65°C and 55°C annealing temperature for bacteria and eukaryotes, respectively), using fresh reaction mixture, as described by Thompson et al. (2002).

Analysis of bacterial community composition using denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using a DCode System (Bio-Rad, Hercules, CA). PCR products (400-600 ng) were separated by electrophoresis at 100 V for 16 h in a linear gradient from 40% to 65% for bacteria (where 100% of the denaturant consisted of 7 M urea and 40% formamide) in a 6% (w/v) polyacrylamide gel (acrylamide–bisacrylamide gel stock solution 37.5:1; Bio-Rad), in 1×TAE buffer (40 mM Tris, pH 8.0; 20 mM acetic acid; and 1 mM EDTA). The DGGE gels were stained for 30 min with SYBR Green, visualised under UV light, and photographed using a Typhoon 9410 (Amersham Biosciences) system.

Bands of interest were excised from the DGGE gels using sterile scalpel blades and soaked overnight in 20 µl of MilliQ water. Two µl of each band was then reamplified with the same primer set and checked again by DGGE to ascertain whether these corresponded to single bands and confirm that the correct band of interest had been isolated. PCR products were purified using the GeneJET PCR purification kit (Fermentas, EU), and 50 ng were sequenced with primer 341f-GC (bacteria) using an ABI 3730xl

sequencer (Applied Biosystems). The sequences obtained were compared with reference sequences using the BLAST (Basic Local Alignment Search Tool) software from the National Centre of Biotechnology Information website (http://www.ncbi. nlm.nih.gov/).

Statistical analysis

The DGGE images were analysed using PyElph 1.4 software (Pavel and Vasile, 2012). The presence/absence of individual bands in each sample was used to construct a binary matrix representing the banding patterns. Multivariate analyses were used to compare the composition and diversity of the bacterial communities associated with the different samples. The effect of fish farming on the bacterial assemblage as a whole was tested using PERMANOVA analysis based on the Bray-Curtis dissimilarities of the untransformed data (Anderson 2001, McArdle & Anderson 2001), and ANOVA analysis for differences in diversity indexes. The data was analysed according to a 3-factor model, where the main factors were: 'distance' with 3 levels (farm, intermediate and control); 'intensity of fish production', with 2 levels (light and intense); and 'location', with 2 levels (Farm 1 and Farm 2).

To assess the relationship between the environmental variables and the bacterial community a Redundancy Analysis (RDA) was employed (lengths of DCA gradients < 2). The significance of each variable was tested with a Monte Carlo permutation test (999 unrestricted permutations), and variables were considered significant if the permutation test value was $p \leq 0.05$. The RDAs were performed using the program CANOCO, version 4.52 (ter Braak et al., 2002).

RESULTS

In this work a total of 36 sediment samples, taken around two fish farms after two different fish-feeding periods, were used to assess changes in the bacterial communities related to aquaculture activities using DGGE analysis. A total of 41 different bands were observed in DGGE gels, and 18 diagnostic bands were selected for sequencing based on their presence in sediments from farm installations or control sites (see example in Fig. 1). Although a 200 bp sequence length may be considered too short for a phylogenetic analysis, it can still be used for diagnostic purposes, as confirmed by the fact that our sequences were closely identified with bacteria that have previously been reported from marine sediments (Table 1).

The Shannon diversity index ranged from 2.662 ± 0.03 to 3.025 ± 0.08 in sediments beneath the cages, and from 2.793 ± 0.02 to 2.944 ± 0.03 in control sediments (Table 2). The three-way ANOVA revealed that there is no significant effect on bacterial diversity ($p > 0.05$). However, the bacterial community composition analysis, carried out using multivariate PERMANOVA, detected significant differences in the main factors, in addition to significant interactions between these factors (Table 3). For this reason, the effects of the main factors were not interpreted (Underwood, 1997). These differences were also evidenced in the MDS ordination, which showed a clear segregation of the bacterial community composition according to the fish farming installation (Fig. 1). Differences were also detected in the bacterial community composition in October, in both installations; however, no changes relating to sampling time were detected in either the control or intermediate zones, which cluster together, suggesting that the differences detected under the cages could be related to the increased feeding and consequent nutrient load (Fig. 1).

In order to assess the influence of environmental variables on the bacterial community a RDA analysis was carried out. The results of this RDA showed that the first two axes together explained 69.5% of the variability between the bacterial community and the environmental data. The biplot of the first two RDA axes (Fig. 2a) indicated that the differences between the two farming facilities correlated strongly with the depth and organic matter content of the sediments. Farm 1 presented a greater quantity of organic matter in its sediments, due to its shallower location. Differences between distances to the cages were more pronounced in Farm 1, where $\delta^{15}N$ and total free sulphide (TFS) increased from the control to the farm, while the Total Organic Carbon (TC) content was lower beneath the cages than in the intermediate zones. Seasonal effects were not detected in the bacterial communities from the sediments, but in samples from below the cages of Farm 1 the highest values of $\delta^{15}N$ were recorded in October, after a more intense feeding period (Fig. 1a).

Eight bands (3, 7, 9, 10, 12, 13, 14, and 16) were identified as the most important for distinguishing the bacterial community from the sediments beneath the cages, since these were not found in sediments from control stations (Fig. 1b). Five bands were detected exclusively in sediments under the cages, belonging to Deltaproteobacteria (bands 9 and 10), Gammaproteobacteria (12), and Epsilonproteobacteria (Band 13 and 14). The Deltaproteobacteria were closely related to the Desulfobulbaceae and Desulfobacteraceae families, which are sulphate-reducing bacteria (SRB), while the Epsilonproteobacteria, including the *Sulfuvorum* and *Arcobacter* genera, are sulphide-oxidising (SOB).

The other three bands corresponded to *Spirochaeta* spp. (Bands 3, 7 and 16), and were detected either in sediments under cages or in intermediate stations (Figure 2b).

DISCUSSION

Fish farming in coastal areas affects sediment quality status, as observed previously in the same facilities studied in this work (Fernandez-Gonzalez et al, 2013). The higher values of $\delta^{15}N$ detected in the sediments beneath the cages reflect an increase in organic matter deriving from fish farming (Tomassetti et al., 2016); this generates anoxic sediments, confirmed by an increase in the TFS (Hargrave et al., 1997). Here, we have shown that molecular approaches are a feasible tool for ascertaining changes in the microbiota resulting from the environmental impact of aquaculture facilities.

Changes in the microbial community, especially bacteria linked to the sulphur cycle, have been detected related to the impact of fish farming. Potential SOB species of Epsilonproteobacteria, and potential SRB species of Deltaproteobacteria, which could be involved in sulphur cycling, were only detected in sediments from under the cages, which presented anoxic characteristics. SRB were represented by the Desulfobulbaceae and Desulfobacteraceae families, which are typically associated with regular organic loads (Saravanakumar et al., 2012) and anoxic sediments (Pjevac et al., 2014). Additionally, sulphide-oxidising Epsilonproteobacteria (SOB) from the genus *Sulfuvorum* and *Arcobacter* were recovered only from beneath the cages, where higher levels of TFS were also recorded. These two genera are frequently retrieved from marine sulphidic habitats, such as hydrothermal sediments (Teske et al., 2002), but have also been recovered from marine sediments (Cifuentes et al., 2000), including sediments from beneath a salmon farm (Aranda et al., 2010). These results are in line with previous studies that showed how the sulphur cycle becomes more active in sediments under fish farms (Asami et al., 2005). In sediments strongly enriched in organic matter and depleted in oxygen, as found under fish farms, carbon mineralisation fluxes become completely driven by anaerobic bacteria, which increase the accumulation and emissions of sulphide (Hargrave et al., 2008), and sulphur reduction rates can be up to 10 times higher in fish farm sediments than coastal sediments (Holmer and Kristensen, 1992). This study therefore confirms the fact that sulphate-reducing bacteria could serve as indicator for assessing the impact of the organic load from fish farming on the marine sediment, as was previously proposed by Asami et al. (2005).

Bands related to the *Spirochaeta* and *Vibrio* genera, which are ubiquitous inhabitants of aquatic environments, were also important for distinguishing the bacterial community in sediments from beneath the cages. *Spirochaeta* includes free-living anaerobic and anaerobic facultative species that are common in sediments affected by aquaculture facilities (Lin et al., 2015; Verhoeven et al., 2016), and species belonging to the *Vibrio* genus are heterotrophic bacteria that respond rapidly to increases in available nutrients and changing environmental conditions (Hagstrom et al., 2001). The presence of these two genera beneath the cages is, therefore, also related to the increased organic load in the sediments produced by fish farming activities. Furthermore, certain *Vibrio* species have been recognised as significant pathogens in marine fish farming (Pujalte et al., 2003; Austin and Austin, 2012), and some are able to survive in marine sediments (Enger et al., 1989). Hence, the sediments beneath fish farms could be an important reservoir for *Vibrio* fish pathogens, potentially disseminating the pathogens across large geographic areas, in addition to being infection vectors.

Our findings show that, due to their rapid response to changes in nutrient load, changes in bacterial community structures are a useful indicator for determining the environmental impact of aquaculture farms, and could be an alternative strategy for monitoring programmes, as previously suggested by various authors (Danovaro, 2000; Fodelianakis et al., 2015; Stoecka et al., 2018). Our results show that Delta and Epsilonproteobacteria, linked to the sulphur cycle and which were detected in sediments from beneath the cages but not control station sediments, could also be used as indicators for assessing the impact of the organic load from fish farming on marine sediments. However, the assessment of microbial diversity could be improved by using pyrosequencing platforms that allow a greater diversity assessment of dominant and rare members of the microbial community (Sogin et al., 2006). This would enable us to determine the best bacterial species to be used as indicators for assessing the impact of the organic load from fish farming on marine sediments.

Acknowledgment

This study forms part of the 'Selección de indicadores, determinación de valores de referencia, diseño de programas y protocolos de métodos y medidas para estudios ambientales en acuicultura marina' project funded by the 'Spanish National Plans of Aquaculture' (JACUMAR). The research was supported by Spanish Ministry of Economy and Competitiveness project CLG2015_66686-C3-3 (JA), which was also supported by financing from the European Regional Development Fund (FEDER). ERP thanks the Generalitat Valenciana for a postdoctoral grant (APOSTD-2016-091). We are grateful to the staff of the fish farms that gave us access and help during the study.

Figure captions

Figure 1. Non-metric multidimensional scaling (NMDS) ordination where the distances between samples are based on the ranks of the Bray-Curtis dissimilarities between 16S DGGE fingerprints from the sediment samples. The figure shows axis 1 and 2 of a 3-dimensional ordination (stress = 0.054, R2 = 0.98).

Figure 2. a) Biplot of RDA axes 1 and 2 for bacterial community samples and environmental parameters. b) Each bacteria band sequenced is represented by a circle divided into different sections according to the proportions in the sampling sites, according to the distance from the fish farming facility. The bacterial bands are labelled as in Table 1.

References

Aguado-Giménez F, Gairín JI, Martinez-Garcia E, Fernandez-Gonzalez V, Ballester Moltó M, Cerezo-Valverde J, Sanchez-Jerez P (2015) Application of "taxocene surrogation" and "taxonomic sufficiency" concepts to fish farming environmental monitoring. Comparison of BOPA index versus polychaete assemblage structure. Marine Environ Res 103: 27-35.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56: 1919-1925.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Aust Ecol 26: 32–46.

Aranda C, Paredes J, Valenzuela C, Lam P, Guillou L (2010) 16S rRNA gene-based molecular analysis of mat-forming and accompanying bacteria covering organically-enriched marine sediments underlying a salmon farm in Southern Chile (Calbuco Island). Gayana 74(2): 125-1235.

Asami H, Aida M, Watanabe K (2005) Accelerated Sulfur Cycle in Coastal Marine Sediment beneath Areas of Intensive Shellfish Aquaculture Accelerated Sulfur Cycle in Coastal Marine Sediment beneath Areas of Intensive Shellfish Aquaculture. Appl Environ Microbiol 71(6): 2925-2933.

Austin B, Austin DA (2012). Bacterial fish pathogens (p. 652). Heidelberg, Germany: Springer.

Birk S, Bonne W, Borja A, Brucet S, Courrat A, Poikane S, Solimini A, van de Bund W, Zampoukas N, Hering D (2012) Three hundred ways to assess Europe's surface waters: An almost complete overview of biological methods to implement the Water Framework Directive. Ecol Indic 18: 31-41.

Bissett A, Bowman J, Burke C (2006). Bacterial diversity in organically-enriched fish farm sediments. FEMS Microbiol Ecol 55(1): 48-56.

Boucher G, Lambshead PJD (1995) Ecological biodiversity of marine nematodes in samples from temperate, tropical, and deep-sea regions. Conserv Biol 9: 1594-1604.

Buchanan JB (1984) Sediment analysis. In: Holme NA, McIntyre AD (eds) Methods for the study of marine benthos. Blackwell Scientific Publications, Oxford, p 41-64.

Cifuentes A, Antón J, Benlloch S, Donnelly A, Herbert RA, Rodríguez-Valera F (2000) Prokaryotic diversity in *Zostera noltii* colonized marine sediments. Appl Environ Microbiol 66(4): 1715-1719.

Cook EJ, Black KD, Sayer MDJ, Cromey CJ, Angel DL, Spanier E, Tsapakis M (2006) The influence of caged mariculture on the early development of sublittoral fouling communities: a pan-European study. ICES Mar Sc 63(4): 637-649.

Danovaro R. (2000). Benthic microbial loop and meiofaunal response to oil-induced disturbance in coastal sediments: a review. Int J Environ Pollut 13(1-6): 380-391.

Dauvin JC, Alizier S, Rolet C, Bakalem A, Bellan G, Gomez Gesterira JL, Grimes S, de-la-Ossa-Carretero JA, Del-Pilar-Ruso Y (2012) Response of different benthic indices to diverse human pressures. Ecol Indic 12:143-153.

Dorigo U, Volatier L, Humbert JF (2005). Molecular approaches to the assessment of biodiversity in aquatic microbial communities. Water Res *39*(11):2207-2218.

Duplisea DE, Hargrave BT (1996). Response of meiobenthic size-structure, biomass and respiration to sediment organic enrichment. Hydrobiologia 339: 161-170.

Enger O, Husevåg B, Goksøyr J (1989) Presence of the fish pathogen Vibrio salmonicida in fish farm sediments. Appl Environ Microbiol 55(11): 2815-2818.

FAO (2009) Environmental impact assessment and monitoring in aquaculture. *FAO Fisheries and Aquaculture Technical Paper.* No. 527. Rome, FAO. 2009. (pp 57). (http://www.fao.org/docrep/012/i0970e/i0970e00.htm). Accessed 9 September 2016.

Fernandez-Gonzalez V, Aguado-Giménez F, Gairin J I, Sanchez-Jerez P (2013). Exploring patterns of variation in amphipod assemblages at multiple spatial scales: Natural variability versus coastal aquaculture effect. Aquacul Env Interac 3(2): 93-105.

Fitridge I, Dempster T, Guenther J, de Nys R (2012). The impact and control of biofouling in marine aquaculture: a review. Biofouling 28(7): 649-669.

Fodelianakis S, Papageorgiou N, Karakassis I, Ladoukakis ED (2015) Community structure changes in sediment bacterial communities along an organic enrichment gradient associated with fish farming. Ann Microbiol 65(1): 331-338.

Grego M, De Troch M, Forte J, Malej A (2009) Main meiofauna taxa as an indicator for assessing the spatial and seasonal impact of fish farming. Mar Pollut Bull 58(8): 1178-1186.

Hagstrom A, Pinhassi J, Zweifel UL (2001) Marine bacterioplankton show bursts of rapid growth induced by substrate shifts. Aquatic Microbial Ecology 24: Pujalte 109-115.

Hargrave BT, Holmer M, Newcombe CP (2008) Towards a classification of organic enrichment in marine sediments based on biogeochemical indicators. Mar Pollut Bull 56: 810-824.

Hargrave BT, Phillips GA, Doucette LI, White MJ, Milligan TG, Wildish DJ, Cranston RE (1997) Assessing benthic impact of organic enrichment from marine aquaculture. Water Air Soil Pollut 99: 641−650.

Holmer M (1991) Impacts of aquaculture on surrounding sediments: generation of organic-rich sediments. In: De Pauw N, Joyce J (eds) Aquaculture and the environment. Eur Aquacult Soc Spec Publ 16:155-175.

Holmer M, Kristensen E, 1992. Impact of fish farming on metabolism and sulfate reduction of underlying sediments. Mar Ecol Progr Ser 80: 191-201.

Holmer M, Frederiksen M S (2007) Stimulation of sulphate reduction rates in Mediterranean fish farm sediments inhabited by the seagrass *Posidonia oceanica*. Biogeochemistry 85: 169e184.

Janse I, Bok J, Zwart G (2004) A simple remedy against artificial double bands in denaturing gradient gel electrophoresis. J Microbiol Methods 57: 279-281.

Karakassis I, TsapakisM, Hatziyanni E (1998) Seasonal variability in sediment profiles beneath fish farm cages in the Mediterranean. Mar Ecol Prog Ser 162: 243−252.

La Rosa T, Mirto S, Mazzola A, Maugeri T L (2004). Benthic microbial indicators of fish farm impact in a coastal area of the Tyrrhenian Sea. Aquaculture 230: 153-167.

Labrune C, Romero-Ramírez A, Amouroux JM, Duchêne J C, Desmalades M, Escoubeyrou K, Buscail R, Gremare A (2012) Comparison of ecological quality indices based on benthic macrofauna and sediment profile images: a case study along an organic enrichment gradient off the Rhône River. Ecol Indic 12:133-142.

Lin R, Lin X, Guo T, Wu L, Zhang W, Lin W (2015) Metaproteomic analysis of bacterial communities in marine mudflat aquaculture sediment. World J Microb Biot Verhoeven.

Luna GM, Corinaldesi C, Anno A D, Pusceddu A, Danovaro R (2012). Impact of aquaculture on benthic virus e prokaryote interactions in the Mediterranean Sea. Water Res 47(3): 1156-1168.

Martinez-Garcia E, Sanchez-Jerez P, Aguado-Giménez F, Ávila P, Guerrero A, Sánchez-Lizaso JL, Collado C (2013) A meta-analysis approach to the effects of fish farming on soft bottom polychaeta assemblages in temperate regions. Mar Pollut Bull 69(1–2): 165–171.

Mazzola A, Mirto S, Danovaro R (1999) Initial fish-farm impact on meiofaunal assemblages in coastal sediments of the Western Mediterranean. Mar Pollut Bull 38(12): 1126-1133.

Mazzola A, Mirto S, La Rosa T, Fabiano M, Danovaro R (2000) Fish-arming effects on benthic community structure in coastal sediments: analysis of meiofaunal recovery. ICES Mar Sc 57(5): 1454-1461.

McArdle BH, Anderson MJ (2001) Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecol 82: 290–297.

Mirto S, La Rosa T, Gambi C, Danovaro R, Mazzola A (2001) Nematode community response to fish-farm impact inthe western Mediterranean. Environ Pollut 116: 203-214.

Mirto S, Bianchelli S, Gambi C, Krzelj M, Pusceddu A, Scopa M, Holmer M, Danovaro R (2010) Fish-farm impact on metazoan meiofauna in the Mediterranean Sea: analysis of regional vs. habitat effects. Marine Environ Res 69: 38e47.

Moreno M, Ferrero TJ, Gallizia I, Vezzulli L, Albertelli G, Fabiano M (2008) An assessment of the spatial heterogeneity of environmental disturbance within an enclosed harbour through the analysis of meiofauna and nematode assemblages. Estuar Coast Shelf S 77: 565-576.

Muyzer G, De Waal EC, Uitterrlinden AG (1993) Profiling in complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695-700.

Pavel AB, Vasile CI (2012) PyElph-a software tool for gel images analysis and phylogenetics. BMC bioinformatics 13(1): 13-19.

Pjevac P, Kamyshny A, Dyksma S, Mussmann M (2014) Microbial consumption of zero-valence sulfur in marine benthic habitats. Environ Microbiol 16: 3416-3430.

Pujalte MJ, Sitja-Bobadilla A, Maclan MC, Belloch C, Alvarez-Pellitero P, Perez-Sanchez J, Uruburu F, Garay E (2003) Virulence and molecular typing of Vibrio harveyistrains isolated from cultured dentex, gilthead sea bream and European sea bass. Syst Appl Microbiol 26: 284-292.

Quintino V, Elliott M, Rodrigues AM (2006) The derivation, performance and role of univariate and multivariate indicators of benthic change: case studies at different spatial scales. J Exp Mar Biol Ecol 330: 368-382.

Riera R, Sanchez-Jerez P, Rodriguez M, Monterroso O, Ramos E (2012) Long-term monitoring of fish farms: Application of Nematode/Copepod index to oligotrophic conditions. Mar Pollut Bull 64(4): 844-850.

Sánchez N, Herranz M, Benito J, Pardos F (2012). Kinorhyncha from the Iberian Peninsula: new data from the first intensive sampling campaigns. Zootaxa, 3402(1): 24-44.

Sánchez O, Gasol JM, Massana R, Mas J, Pedrós-Alió C (2007) Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. Appl Environ Microbiol 73(18): 5962-5967.

Saravanakumar C, Dineshkumar N, Alavandi SV, Salman V, Poornima M, Kalaimani N (2012) Enrichment and identification of large filamentous sulfur bacteria related to Beggiatoa species from

brackishwater ecosystems of Tamil Nadu along the southeast coast of India. Syst Appl Microbiol 35: 396-403.

Semprucci F, Losi V, Moreno M (2015) A review of Italian research on free-living marine nematodes and the future perspectives on their use as Ecological Indicators (EcoInds). Mediterr Mar Sci 16(2): 352-365.

Sogin ML, Gunderson JH (1987). Structural diversity of eukaryotic small subunit ribosomal RNAs. Ann NY Acad Sci 503: 125-139.

Sørensen MV, Pardos F (2008) Kinorhynch systematics and biology an introduction to the study of kinorhynchs, inclusive identification keys to the genera. *Meiofauna Mar* 16: 21-73.

Stoecka T, Frühea L, Forstera D, Cordierb T, Martinsc CIM, Pawlowski J (2018) Environmental DNA metabarcoding of benthic bacterial communities indicates the benthic footprint of salmon aquaculture. Mar Pollut Bull 127:139-149.

Sutherland TF, Levings CD, Petersen SA, Poon P, Piercey B (2007) The use of meiofauna as an indicator of benthic organic enrichment associated with salmonid aquaculture. Mar Pollut Bull 54(8): 1249-1261.

Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E (2012). Towards next- generation biodiversity assessment using DNA metabarcoding. Mol Ecol 21(8): 2045-2050.

ter Braak CJF, Smilauer P (2002) CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5). Microcomputer Power: Ithaca, NY, USA, p 500.

Teske A, Hinrichs KU, Edgcomb V, de Vera Gomez A, Kysela D, Sylva SP, Jannasch HW (2002). Microbial diversity of hydrothermal sediments in the Guaymas Basin: evidence for anaerobic methanotrophic communities. Appl Environ Microbiol 68(4): 1994-2007.

Theron J, Cloete TE (2000). Molecular techniques for determining microbial diversity and community structure in natural environments. Crit Rev Microbiol 26(1): 37-57.

Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res 30(9): 2083-2088.

Tomassetti P, Gennaro P, Lattanzi L, Mercatali I, Persia E, Vani D, Porrello S (2016) Benthic community response to sediment organic enrichment by Mediterranean fish farms: Case studies. Aquaculture 40: 262-27.

Van Hoey G, Borja A, Birchenough S, Buhl-Mortensen L, Degraer S, Fleischer D, Kerckhof F, Magni P, Muxika I, Reiss H, Schröder A, Zettler M L (2010) The use of benthic indicators in Europe: From the Water Framework Directive to the Marine Strategy Framework Directive. Mar Pollut Bull 60(12): 2187-2196.

Vanreusel, A. (1990). Ecology of the free-living marine nematodes from the Voordelta (Southern Bight of the North Sea). 1. Species composition and structure of the nematode communities. Cah Biol Mar 31: 439-462.

Verhoeven J, Salvo F, Hamoutene D, Dufour S (2016) Bacterial community composition of flocculent matter under a salmonid aquaculture site in Newfoundland, Canada. Aquacult Env Interac 8: 637-646.

Vezzulli L, Chelossi E, Riccardi G, Fabiano M (2002) Bacterial community structure and activity in sea farm sediments of the Ligurian sea (Western Mediterranean). Aquacul Int 10: 123-141.

Vincx M, Meire P, Heip C (1990) The distribution of nematodes communities in the Southern Bight of the North Sea. Cah Biol Mar 31: 107-129.

Warwick RM (1987). Meiofauna: their role in marine detrital systems. In Detritus and microbial ecology in aquaculture. ICLARM conference proceedings 14: 282-295.

Whitman WB, Coleman DC, Wiebe WJ (1998). Prokaryotes: the unseen majority. P Natl Acad Sci USA *95*(12): 6578-6583.

Wilding TA, Cromey CJ, Nickell TD, Hughes DJ (2012) Salmon farm impacts on muddy-sediment megabenthic assemblages on the west coast of Scotland. *Aquacult Env* Interact 2:145-156.

Wildish DJ, Akagi HM, Hamilton N, Hargrave BT (1999) A recommended method for monitoring sediments to detect organic enrichment from mariculture in the Bay of Fundy. Can Tech Rep Fish Aquat Sci 2268: 1-31.

Williams GC (2011) The global diversity of sea pens (Cnidaria: Octocorallia: Pennatulacea). PLoS ONE 6: e22747.

Table 1. Bacterial 16S rRNA sequences of selected DGGE bands.

and	Sequence length (bp)	Phylogenetic group	Best hit in NCBI source (% sequence identity, accesion no.)	Closest type strain (% sequence identity, accesion no.)
1	163	Gammaproteobacteria	Uncultured *Methylobacter* Lake sediments (97, AY007295)	*Methylosarcina lacus* (97, AY007296)
2	150	Deltaproteobacteria	Uncultured delta proteobacterium clone Marine sediments (99, KR086704)	*Desulfomonile tiedjei* (89, AM086646)
3	166	Spirochaetes	Uncultured *Spirochaeta sp*. Clone Ocean water (100, KF758585)	*Spirochaeta perfilievii* (98, AY337318)
4	154	Deltaproteobacteria	Uncultured deep sea bacterium Sediments (97, AM997367)	*Sandaracinus amylolyticus* (86, HQ540311)
5	163	Gammaproteobacteria	Uncultured Gammaproteobacteria North Yellow Sea sediment (98, FJ545453)	*Thioprofundum lithotrophicum* (94, AB468957)
6	160	Epsilonproteobacteria	Uncultured *Campylobacter* Fecal sampel (96, FJ681903)	*Campylobacter volucris* (96, FM883694)
7	164	Spirochaetes	Uncultured *Spirochaeta sp*. Clone Ocean water (99, KF758585))	*Spirochaeta perfilievii* (97, AY337318)
8	167	Epsilonproteobacteria	Hydrothermal event (98, JN874032)	*Nitratifractor salsuginis* (98, AB091292)
9	161	Deltaproteobacteria	Uncultured *Desulforhopalus* acidic fen soil (97, GU127732)	*Desulforhopalus vacuolatus* (97, L42613)
10	167	Deltaproteobacteria	Uncultured Deltaproteobacteria Sediment sample (97, FM242377)	*Desulfosarcina variabilis* (98, M34407)
11	170	Epsilonproteobacteria	Uncultured Nitratifractor Hydrothermal event (99, JN874032)	*Nitratifractor salsuginis* (99, AB175500)
12	166	Gammaproteobacteria	*Uncultured Vibrionaceae* ottlenose dolphin forestomach (99, JQ193457)	*Vibrio sagamiensis* (99, AB428909)
13	168	Epsilonproteobacteria	Uncultured *Sulfuvorum* sediment from anoxic fjord (99, JF495273.1)	*Sulfurovum lithotrophicum* (99, AB091292)
14	169	Epsilonproteobacteria	Uncultured *Arcobacter* Ocean water (98, FJ155006)	*Arcobacter venerupis* (98, HE565359)
15	161	Gammaproteobacteria	Uncultured *Shewanella* Marineinvertebrate gut (98, KF798447)	*Shewanella sediminis* (98, CP000821)
16	167	Spirochaetes	*Spirochaeta litoralis* Marine sediments (100, KC261848)	*Spirochaeta litoralis* (97, FR733665)
17	159	Gammaproteobacteria	*Uncultured Gammaproteobacteria Marine sediments* (98, JX138875)	*Thioalkalivibrio sulfidiphilus* (93, EU709878)
18	159	Actinobacteria	Ucultutred bacterium (97, KU713387)	*Rhodococcus yunnanensis* (97, AY602219)

Table 2. Values of the Shannon index in the investigated sediments from the two fish farming installations. Reported are the average ± standard deviation (n [2)..

Distance	Intensity of fish production	Location	Shannon diversity
Control	Light M	Farm 1	2.944 ± 0.03
		Farm2	2.852 ± 0.02
	Intense O	Farm 1	2.793 ± 0.02
		Farm2	2.833 ± 0.03
Intermediate	Light	Farm 1	2.889 ± 0.04
		Farm2	2.995 ± 0.04
	Intense	Farm 1	2.961 ± 0.02
		Farm2	2.813 ± 0.03
Farm	Light	Farm 1	2.662 ± 0.03
		Farm2	2.961 ± 0.02
	Intense	Farm 1	2.859 ± 0.15
		Farm2	3.025 ± 0.08

Table 3. Results of 3-factor multivariate PERMANOVA based on Bray-Curtis similarity of bacterial assemblages. The factors that were analyzed were distance, period of fish production and location, fixed and orthogonal.

Source	df	MS	P value
Distance	1	0.24462	0.001
Period of fish production	1	0.11238	0.003
Location	1	2.35003	0.001
Distance x Period of fish production	2	0.20256	0.028
Distance x Location	2	0.11682	0.001
Period of fish production x Location	1	0.06432	0.001
Distance x Period of fish production x Location	2	0.15391	0.001
Residual	24	0.01633	
Total	35		

Changes in bacterial communities from sediments beneath aquaculture installations were detected using denaturing gradient gel electrophoresis.

Changes in bacterial community stuctures, due to their rapid response to changes in nutrient load, are a useful indicator for determining the environmental impact of aquaculture farms,

Delta and Epsilonproteobacteria linked to sulfur cycle are a useful indicator for assessing the impact of fish farming over the marine sediment.

Sediments beneath fish farms could be an important reservoir for *Vibrio* fish pathogens, potentially disseminating the pathogens across large geographic areas, in addition to being infection vectors

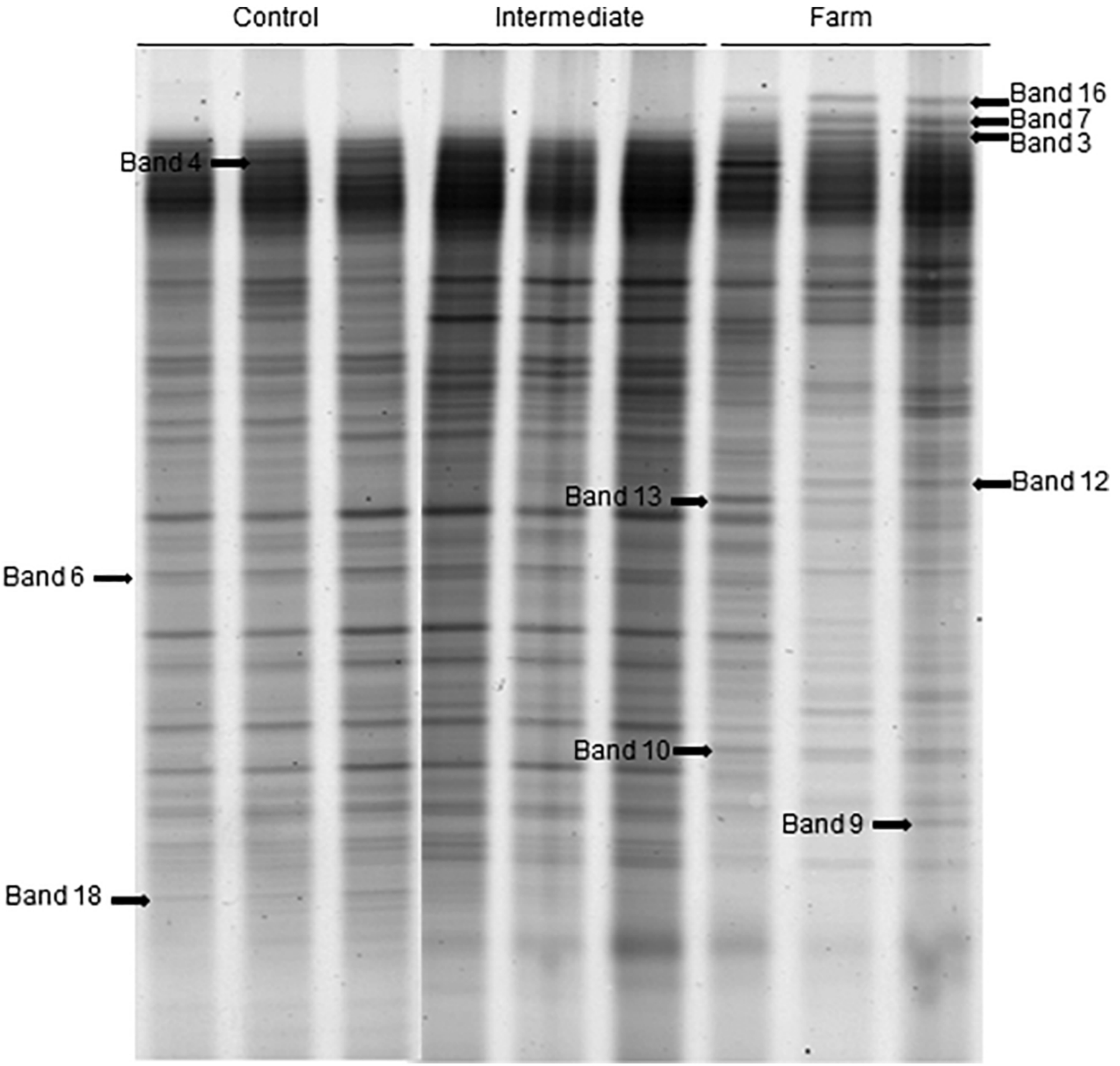
Control
Intermediate
Farm
Band 16
Band 7
Band 3
Band 4
Band 12
Band 13
Band 6
Band 10
Band 9
Band 18

Figure 1

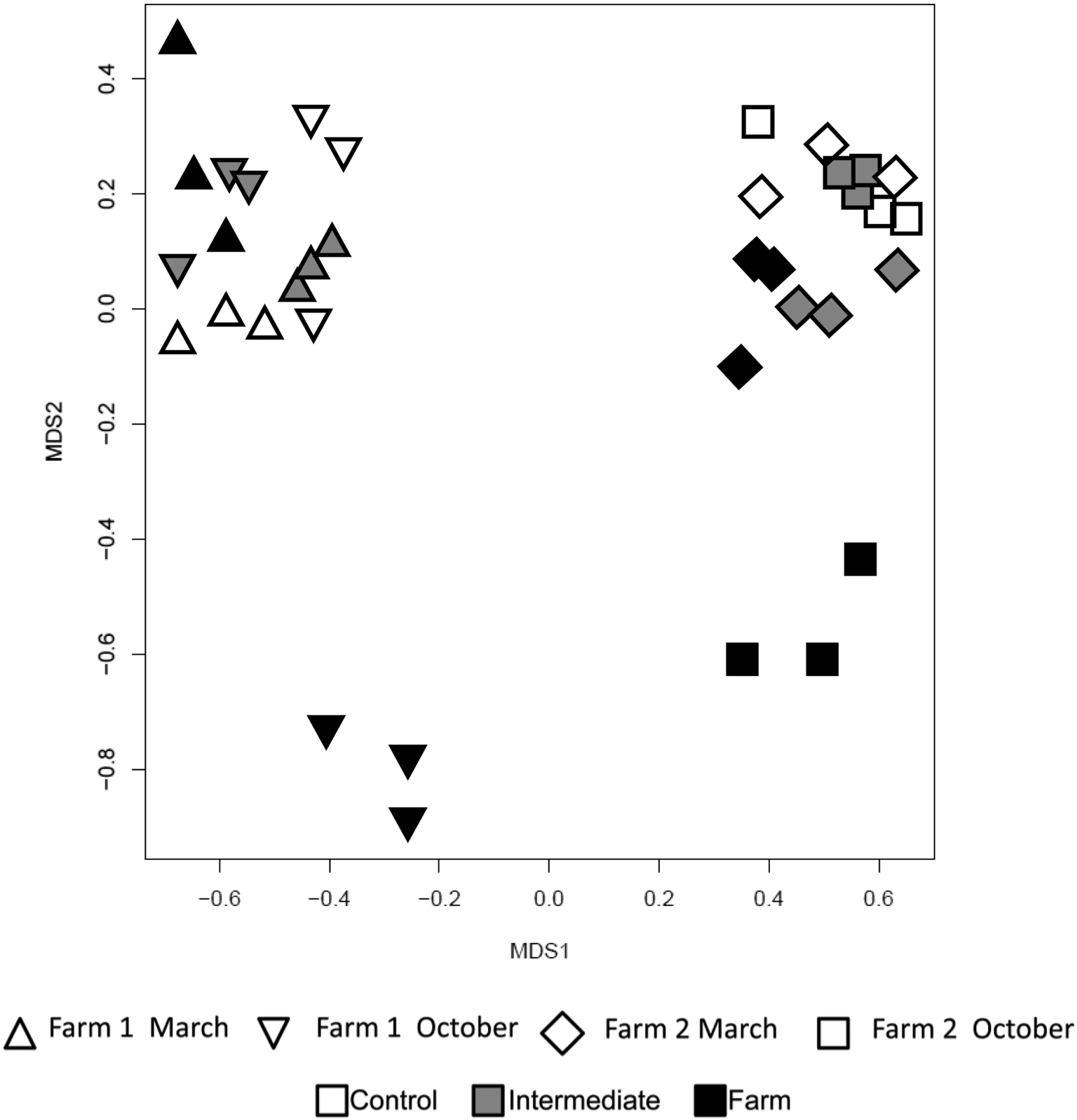
MDS2
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6
MDS1
Farm 1 March
Farm 1 October
Farm 2 March
Farm 2 October
Control
Intermediate
Farm

Figure 2

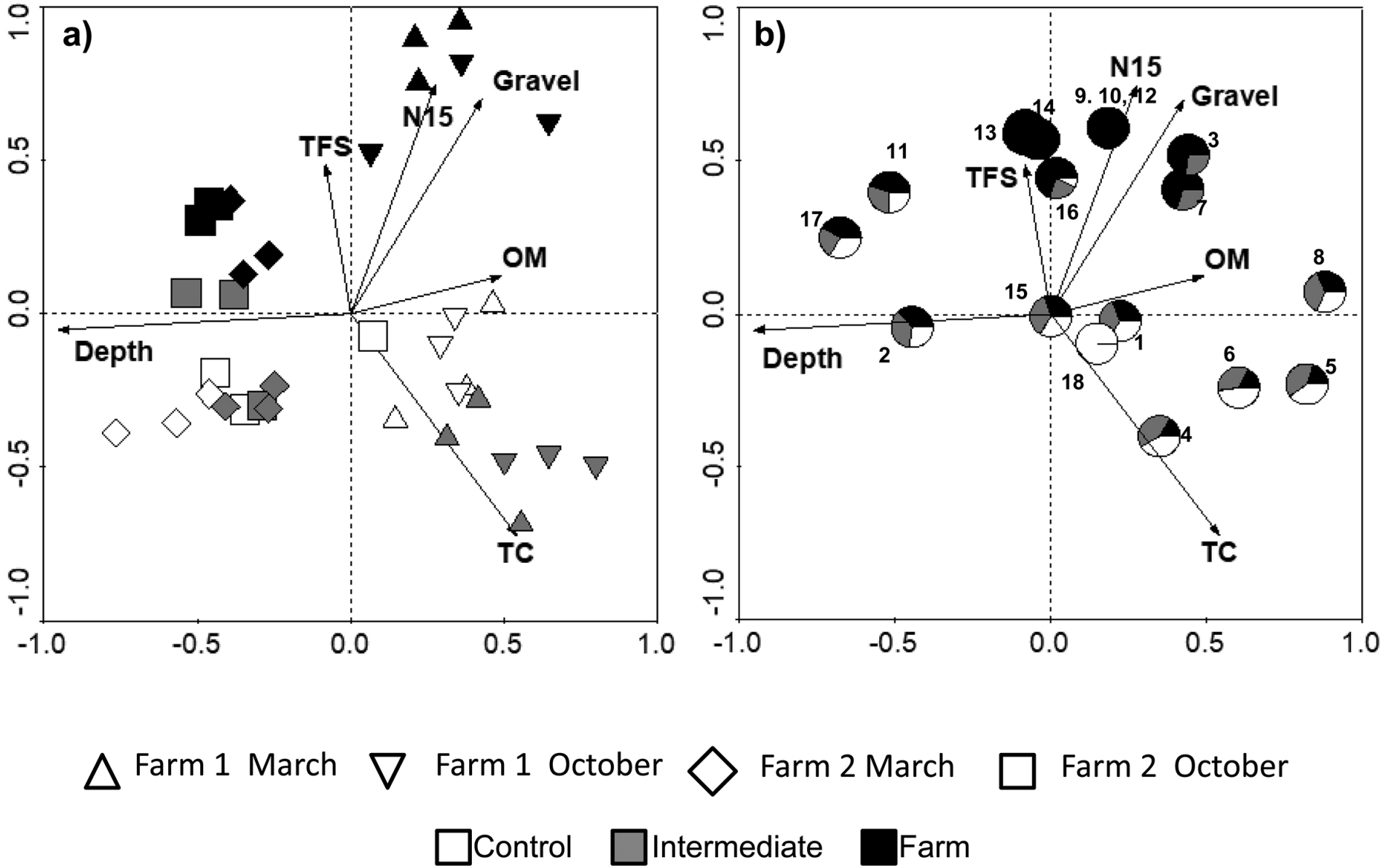
a)
b)
N15
Gravel
TFS
OM
Depth
TC
9. 10, 12
14
13
11
3
16
17
7
8
15
1
2
6
5
18
4
Farm 1 March
Farm 1 October
Farm 2 March
Farm 2 October
Control
Intermediate
Farm

Figure 3