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Chemometric data analysis application to *Sparus aurata* samples from two offshore farming plants along the Apulian (Italy) coastline

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ABSTRACT

The levels of polychlorodibenzo-*p*-dioxins (PCDDs), polychlorodibenzofurans (PCDFs), dioxin-like polychlorobiphenyls (DL-PCBs), non-dioxin-like polychlorobiphenyls (NDL-PCBs), and polybromodiphenyl ethers (PBDEs) in fish collected from two marine offshore farming plants were determined. Each sample was constituted by specimens of the same size collected at the same time in four different seasons along the farming year. The feeds given were of industrial origin and the plants were positioned in two different sites respectively exposed to different environmental characteristics. A chemometric approach was applied to interpret the subtle differences observed in fish body burdens across the three chemical groups taken into consideration. The approach consisted in a stepwise multivariate process including a hierarchical cluster analysis (CA) and a linear discriminant analysis (DA). The two main clusters determined by CA were subjected to the canonical DA, backward and forward selection procedures to select the best discriminative functions. A clear temporal and spatial discrimination was found among the samples. Across the three chemical groups, the monthly separation seemed to depend on the growth process and the main exposure was due to the feed. In addition, the two plants differed significantly from the environmental point of view and the most important discriminating group of chemicals were the NDL-PCBs. The approach resulted really effective in discriminating the subtle differences and in individuating suggestions to improve the quality of culturing conditions.

1. Introduction

Worldwide, aquaculture is more and more becoming the main source of fishery products, thus playing a significant role in creating employment opportunities in local coastal communities (FAO, 2006). Within the enlarged European Union, the aquaculture industry accounts for a total of 1.3 million tonnes of fishery products a year (http://ec.europa.eu/fisheries), with fish species-specific differences among the areas dedicated to farming, depending on the environmental characteristics of the sea basin. In particular, the sea bream is the most important farmed species is in the Mediterranean area.

It is generally acknowledged that fish farming may increase the intake of omega-3 and omega-6 fatty acids in general population, thus helping to prevent the occurrence of cardio-vascular diseases, and in general promoting a good health status. However, such dietary benefits may be challenged by the levels of pollutants accumulated by fish, such as polychlorodibenzo-*p*-dioxins (PCDDs), polychlorodibenzofurans (PCDFs), dioxin-like polychlorobiphenyls (DL-PCBs), non-dioxin-like polychlorobiphenyls (NDL-PCBs), and polybromodiphenyl ethers (PBDEs). Their occurrence in fish, with respect to other foods of animal origin, can differ of a factor 10 in concentration, thus representing the most contributing factor to the daily intakes of the aforesaid contaminants (Hites et al., 2004). One way to maximize the farmed fish intake benefits, is to reduce their contamination levels by a sound use of feed items and the choice of an appropriate site for fish farming.

This work is aimed to characterize the differences in the accumulated PCDDs, PCDFs, DL-PCBs, NDL-PCBs and PBDEs in two farming plants, possibly due to different feed regimes and the site choice. To this end, a chemometric approach was used to analyze the data set consisting of farmed sea bream samples. The two offshore farms considered were in the southern Adriatic sea and were managed according to a Good Agricultural Practice (GAP) regime.

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This, in turn, means that all the factors possibly inducing a quality reduction in the fish products are taken into consideration and possibly eliminated. Therefore, the fish contaminant body burden would be expected to be under control. However, during the farming period, there is a turn-over of feeds for optimal sustenance of fish growth, along to a seasonal variation in feed consumption as a consequence of the diverse water temperature between winter and summer. Additionally, the two plants are exposed to different environmental factors possibly including a chemical impact from the surrounding environment.

2. Materials and methods

2.1. Sampling and analysis

Sea bream was chosen for this study mostly because of its commercial relevance and its leading role in the Mediterranean aquaculture scenario. Fish samples were collected from two offshore farming plants respectively called Giovinazzo and Mattinata situated along the Apulia coastline in southern Italy. The Giovinazzo plant is set at 3 miles from the coast at a site characterized by an 80 m depth and a fast sea stream. The Mattinata plant is relatively close to the coast at a depth not exceeding 10 m. Sampling was performed between August 2006 and April 2007, in the months of August, November, February, and April. Fish of similar size were collected at each sampling month and ten fillets, each one taken from a single fish, were pooled together to form the analytical sample. The fillets were excised from the fish body adopting the prescriptions of the EC Regulation 1883/2006 (EC, 1883/2006). This procedure remained unchanged in spite of the fish weight increase due to growth (from 100 to 350 g). The above-mentioned operations were performed immediately after sampling and each individual analytical sample was then stored separately at –20°°C until pre-treatment.

Thirty *ortho*-substituted NDL-PCBs, 17 PCDD and PCDF congeners, eight mono-*ortho* DL-PCBs, the non-*ortho*-substituted PCBs 77, 81, 126 and 169, and PBDE 17, 28, 47, 66, 99, 100, 138, 153, 154, 183 and 209 were determined. The analytical procedure was adapted from the USEPA Method No. 1613 (1994) for PCDD and PCDF determination. An adaptation of such procedure was described by De Felip and Miniero (1999).

Upon delivery to the laboratory for pretreatment the analytical samples were allowed to thaw and rinsed with distilled water. Then, they were added with ^{13}C-labeled standards, allowed to rest for hours, and homogenized with anhydrous Na_2SO_4. An aliquot of each sample was extracted using a Soxhlet apparatus with a 50% mixture of acetone and *n*-hexane. The extract was concentrated to 20 mL using a rotary evaporator; a 2 mL aliquot was used for lipid content determination by a gravimetric method. Clean-up was carried out by filtration through Extrelut impregnated with concentrated sulfuric acid, resting on a silica gel layer (di Domenico et al., 1992). The extract was then analyzed for PCDDs, PCDFs, DL-PCBs, NDL-PCBs and PBDEs, with three different Power-Prep separation programs.

Quantification was performed by high-resolution gas chromatography coupled with low-resolution mass spectrometry (HRGC-LRMS) used in the selected ion monitoring mode (SIM) for NDL-PCBs and PBDEs. HRGC-HRMS(SIM) was employed to determine PCDDs, PCDFs, and DL-PCBs operating at 10,000 mass resolution. A procedural blank was run together with three to five samples. Reliable measurements were allowed above the limit of determination with a repeatability in the order of |±10%| (extended uncertainty, |±20%|). The recovery rates of labeled ISs were accepted within 40–120%; values outside this range led to specific evaluation, possibly rejection of trial.

2.2. Data treatment

Most multivariate methods require variables to conform to a normal distribution, thus the normality of the distribution of each variable was checked by analyzing kurtosis and skewness before multivariate statistical analysis. The original data were demonstrated to be almost normally distributed and positively skewed with standardized Kurtosis coefficients greater than zero ($p = 0.005$). After data log-transformation, all skewness and kurtosis values were significantly reduced, therefore log-transformation was adopted for data analysis. For each sample, congener concentrations were normalized against the pertinent cumulative analytical concentration, the latter obtained by adopting the medium bound approach when dealing with limits of determination. After data validation, congeners with a high incidence (>40%) of non-detects throughout the samples were removed to minimize the non-detect impact on the statistical approach (Miniero et al., 2007).

2.3. Chemometric approach

The levels of the above-mentioned chemicals were analyzed by a stepwise statistical approach including a hierarchical cluster analysis (CA) and a linear discriminant analysis (LDA). The CA uses iterative approaches to find structure in data by identifying their natural groupings whereas the LDA procedure is designed to distinguish between two or more groups of data based on a set of p observed quantitative variables. It does so by constructing discriminant functions that are linear combinations of the variables. The discriminant scores, a single new composite variable, are calculated by these functions which have the form

$$D = w_1Z_1 + w_2Z_2 + w_3Z_3 + \ldots w_pZ_p$$

where D is the discriminant score, w_p the weighting (coefficient) for variable p, Z_p is the standardized coefficient for variable p.

An essential requirement of the LDA procedure is the normally distributed data (Fielding, 2007). The goal of this approach is to display the most significant patterns, looking for possible groupings and sources of data variation, as well as for their temporal and spatial distributions, through resolution and modeling of data (Goncalves et al., 2006; Zhou et al., 2007). For CA, a squared Euclidean distance was always used as the interval measure for clustering using the following distinct linkage methods: between-group linkage, within-group linkage and Ward's methods. The groups of variables individuated by the CA were singly evaluated by the canonical, forward (FS) and backward (BS) LDA, with the objective to distinguish the ones which have driven the formation of the groups of closed related samples. The forward and backward DA procedures adopted to construct the best discriminating functions took into account the statistical significance of the variables and in the data evaluation a critical F value of 4 was used (F-to-enter and F-to- remove). The two classification factors considered, the sampling month (August, November, February, May) and the fish plant (Giovinazzo, Mattinata) were known as possible sources of data variation due to the influence of different feed types and of different environmental characteristics.

3. Results and discussion

3.1. Data description

The lipid-base data on PCDD, PCDF, DL-PCBs NDL-PCB, and PBDE congeners are reported in Table 1. Among the PCDD and PCDF congeners the most important ones defining the sample-specific profile are D1, D2, F1, F2, F3, F4, and F5. The highest