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Local connected fractal dimension analysis in gill of fish experimentally exposed to toxicants

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ABSTRACT

An operator-neutral method was implemented to objectively assess European seabass, *Dicentrarchus labrax* (Linnaeus, 1758) gill pathology after experimental exposure to cadmium (Cd) and terbuthylazine (TBA) for 24 and 48 h. An algorithm-derived local connected fractal dimension (LCFD) frequency measure was used in this comparative analysis. Canonical variates (CVA) and linear discriminant analysis (LDA) were used to evaluate the discrimination power of the method among exposure classes (unexposed, Cd exposed, TBA exposed). Misclassification, sensitivity and specificity, both with original and cross-validated cases, were determined. LCFDs frequencies enhanced the differences among classes which were visually selected after their means, respective variances and the differences between Cd and TBA exposed means, with respect to unexposed mean, were analyzed by scatter plots. Selected frequencies were then scanned by means of LDA, stepwise analysis, and Mahalanobis distance to detect the most discriminative frequencies out of ten originally selected. Discrimination resulted in 91.7% of cross-validated cases correctly classified (22 out of 24 total cases), with sensitivity and specificity, respectively, of 95.5% (1 false negative with respect to 21 really positive cases) and 75% (1 false positive with respect to 3 really negative cases). CVA with convex hull polygons ensured prompt, visually intuitive discrimination among exposure classes and graphically supported the false positive case. The combined use of semithin sections, which enhanced the visual evaluation of the overall lamellar structure; of LCFD analysis, which objectively detected local variation in complexity, without the possible bias connected to human personnel; and of CVA/LDA, could be an objective, sensitive and specific approach to study fish gill lamellar pathology. Furthermore this approach enabled discrimination with sufficient confidence between exposure classes or pathological states and avoided misdiagnosis.

1. Introduction

Gills are the largest piscine interface with the surrounding environment, being directly and permanently in contact with potential waterborne irritants and, as a result, are suitable markers for aquatic pollution (Mallatt, 1985; Bernet et al., 1999; Manera, 2013a). Their complex structure has been described both in physiological and pathological conditions (Mallatt, 1985; Wilson and Laurent, 2002). Moreover, the pivotal role of gills in maintaining fish homeostasis, by means of gas exchange, osmoregulation, acid-base regulation, and excretion is well documented (Evans, 1987; Evans et al., 2005; Brauner and Rombough, 2012). The pathology of gills as a result of exposure to waterborne toxins has been extensively studied under both experimental conditions and in the native environment. Gills respond to a wide range of environmental physico-chemical stimuli in a limited manner and accordingly gill responses should be treated as general, though sensible, biomarkers (Mallatt, 1985; Dezfuli et al., 2006; Giari et al., 2007; Gomes et al., 2012; Nascimento et al., 2012; Wolf et al., 2015; Manera et al., 2016).

Because of their peculiar architecture, gills are intrinsically "delicate" and subject to develop artifacts during manipulations for fixation and tissue processing. This fragility leads to possible false positive/type I errors, which however can be readily dismissed provided an adequate control is available (Mallatt, 1985). In contrast, false negative/type II errors cannot be as easily dismissed. To avoid this type of error specific guides are required to correctly assess both fish gill pathology and morphometry in order to evaluate

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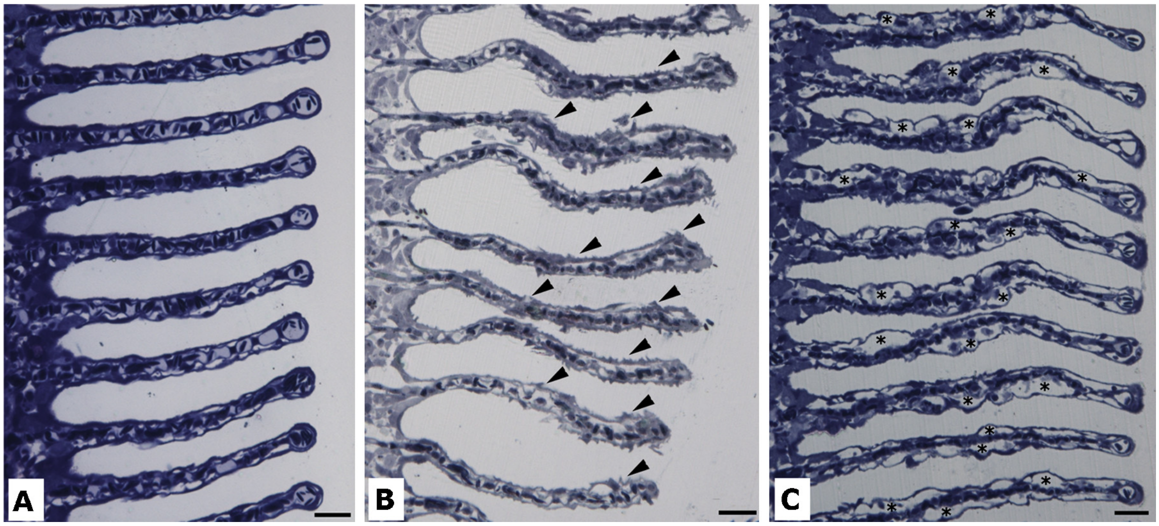

Fig. 1. European seabass. Unexposed (A), Cd exposed (B), TBA exposed (C) secondary gill lamellae. Shrinkage/curling of epithelial cells (black arrow heads) are clearly visible in Cd exposed lamellae, whereas epithelial lifting (black asterisks) occurred mainly in TBA exposed lamellae. Semithin sections. Toluidine Blue. Scale bar = 20 μm.

subtle gill pathological responses. False negative/type II errors, which are a greater concern than false positive/type I error should be adequately controlled by means of proper screening test sensitivity (Mallatt, 1985; Manera, 2013b,c; Szczypinski et al., 2014; Manera et al., 2016).

Fish gill assessment is currently used in environmental and ecotoxicological studies, despite the absence of any standardized method to quantify gill changes. Thus gill lesions are assessed only in a qualitative or semi-quantitative manner (Mallatt, 1985; Pawert et al., 1998; Pandey et al., 2008; Abdel-Moneim et al., 2012; Gomes et al., 2012; Nascimento et al., 2012). Nevertheless, efforts have been made to apply metrics, indices, scores and also stereological approaches to evaluate fish gill status in environmental and toxicological studies (Pinkney et al., 1989; Lease et al., 2003; Pane et al., 2004; Alvarado et al., 2006; Monteiro et al., 2009; Agamy, 2013; Hawkins et al., 2015). Indeed, these methods rely mainly on cell counts, additional tissue elements, or reaction pattern recognition. All of these approaches are time consuming tasks and require trained personnel for operation and supervision. Not surprisingly, operator-dependent errors can arise and need to be recognized and corrected accordingly. Furthermore, to date no attempt has been made both to assess the sensitivity and the specificity of the adopted methods, and to validate the results. The authors have recently characterized branchial lamellar pathology in European seabass [*Dicentrarchus labrax* (Linnaeus, 1758)] experimentally exposed to Cadmium (Cd) and terbuthylazine (TBA), and compared the findings with unexposed cases. Guided expert quantitative and fractal analysis were performed on selected images (the same as in the present study) from resin embedded, semithin sections to test possible differences according to exposure class and the discrimination power (in term of misclassification and false positive/type I and false negative/type II errors) of the two methods, both with original and cross-validated cases, in order to obtain the respective sensitivity and specificity. Guided expert quantitative analysis was confirmed to be a reliable method to objectively characterize fish gill pathology with specific regard to toxicological trials, thus ensuring standardization and reproducibility (Manera et al., 2016). In contrast, fractal analysis alone did not approach the discrimination power that was found for guided expert analysis. Nevertheless, the authors concluded that fractal analysis deserved further investigation, because local connected fractal dimension (LCFD) analysis might prove useful in evaluating possible local variations in complexity, as opposed to the global fractal dimension utilized in that study (Manera et al., 2016). In effect, the mean global fractal dimension measurement previously used may have masked local variations in complexity, which might have been better evidenced using the LCFD analysis as proposed by Landini et al., 1995.

Fractal analysis, as an extension of conventional Euclidean geometry, constitutes a reliable method to objectively describe and summarize object complexity and heterogeneity. Fractal analysis comprises two main measures: fractal dimension, the measure of an object's complexity or its "roughness"; and lacunarity, the measure of its rotational and translational invariance or its heterogeneity and texture (Mandelbrot, 1982; Kenkel and Walker, 1996; Smith et al., 1996; Losa, 2011; West, 2013; Manera et al., 2014, 2016). As a simplification, fractal analysis relies on the estimation of the self-similarity properties of a fractal object, in which a portion of the same looks like the whole at different scales (Mandelbrot, 1982).

The aim of the present research was to implement an operator unbiased, objective method to comparatively assess European seabass gill pathology after exposure to Cd and TBA. The method relies on an algorithm-derived LCFD frequency measure, as opposed to a global fractal dimension measure previously performed on the same experimental material (Manera et al., 2016). Furthermore misclassification, sensitivity and specificity, both with original and cross-validated cases, were computed.

2. Materials and methods

The present study was based on image analysis of photomicrographs taken from a selection of semithin sections obtained in previous experimental trials (Dezfuli et al., 2006; Giari et al., 2007). In particular, all the previous histological sections compatible with the adopted image analysis, in term of tissue orientation and covered area, were included in this survey. Consequently the experimental design is only briefly summarized here and readers are referred to the cited literature.

2.1. Experimental fish and acute exposure

D. labrax specimens (mean total length, 124.4 mm; mean mass, 18.8 g; n = 45), previously acclimated for two weeks (22‰ salt water; mean temperature, 19.9 °C; 12 h daylight photoperiod), were exposed to four incremental doses (4.47 mg l^{-1} [0.0398 mM],