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Co-inertia multivariate approach for the evaluation of anthropogenic impact on two commercial fish along Tyrrhenian coasts

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ABSTRACT

Aliphatic hydrocarbon levels were determined by the GC/MS technique in fish livers of *Engraulis encrasicolus* (*Ee*) and *Trachurus trachurus* (*Tt*), collected from a particular area of the Mediterranean Sea, called GSA 10, which is located exactly in Tyrrhenian Sea between Campania coast and North Sicily coast. The aim was to evaluate their potential use as specific bioindicators towards this class of contaminants. Both *Tt* and *Ee* are considered to be pollution monitoring bioindicators, due to their dominance in marine communities and economic fishing interest. *Ee* showed a higher tendency to bioaccumulate TAHs, due to the lower quantity of fatty acids in liver tissues with respect to *Tt*. The area under study has been characterised a) chemically with the acquisition of temperature, oxygen and salinity profiles along the water column, and b) ecologically with the determination of amino acid contents in fish eyes, in order to gain information on the adaptation to environmental changes. Moreover, specific activities of two hydrolytic enzymes, such as alkaline phosphatase and peroxidase in fish epidermal mucus, together with lactate in blood plasma and cortisol levels, have been investigated for the first time, in order to obtain insights into the effects of hydrocarbons on animal welfare. A multiple co-inertia analysis was also applied to chemical and environmental parameters, in order to explore any possible correlation between different variables. The multivariate approach showed a clear spatial distribution between environmental and chemical variables in *Ee*, whilst there was an absence of a spatial trend in *Tt*. Moreover, the chemometric analysis showed a very high correlation between amino acid profiles and environmental variables for both species, confirming the possibility of being used as ecological welfare indices for short-term environmental variations.

1. Introduction

The surveillance, monitoring and control of maritime traffic in the Mediterranean Sea has been strongly strengthened in recent years, thanks to the more rigorous enforcement of specific rules and regulations by the European Community (Ferraro et al., 2009). The Joint Research Centre of the European Commission has monitored oil pollution in the Mediterranean Sea in the long-term using satellites equipped with Synthetic Aperture Radar (SAR) (Ferraro et al., 2009); analysis of a large number of images highlighted oil spills in marine waters. Indeed, fishery resources and coastal ecosystems of the Mediterranean area are continuously stressed by the growing presence of oily contaminants. Different anthropogenic activities, such as coke and petroleum refining industries, accidental oil spills and leakages from ships and offshore platforms (Wolfe et al., 1994), aerial fallout (Simcik et al., 1996), rainwater runoff (Cardellicchio et al., 2007), vehicle traffic (Brož et al., 2000) and domestic heating can be considered potential sources of oil marine pollution. The major source of oil marine pollution is the deliberate and/or illegal discharge of tank washing residues, engine room wastes and fuel oil sludge from ships. Oily contaminants from crude oil are complex mixtures containing a wide number of petroleum-based compounds; among these, one interesting

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fraction is represented by Total Petroleum Hydrocarbons (TPH), which may form a thin film at the surface of marine waters and are separated from the heavier fraction accumulated in sediments (Wang et al., 2006). The total aliphatic hydrocarbons (TAH) are the major fraction of TPH, but their behaviour in marine waters and marine organisms is rarely monitored and the available data are not homogenous or complete for defining the ecotoxicological risk in aquatic environments. In a similar way to PAHs, the exposure of marine organisms such as shellfish and pelagic or semi-pelagic fishes to aliphatic hydrocarbons may result in bioaccumulation through the skin, gills or liver, due to their high lipid solubility and persistence in the marine environment, thus increasing the risk in the food chain and for human health (Enuneku et al., 2015). Several investigations have already suggested a close connection between the most common human cancers and dietary sources (Enuneku et al., 2015). The determination of hydrocarbons in water is not sufficient to establish the effective risk for the marine environment and humans, because their analytical concentrations may be lower than the operational limit reported for specific analytical methodologies (EN ISO 9377:2, 2000); moreover, they are strongly dependent on water flow and the intermittence of contaminant discharge, sometimes with drastic fluctuations. As a consequence, the pollutant concentrations in the water column are not representative parameters, neither are they able to describe the environmental conditions at the time of sampling. For this reason, a monitoring program based on the use of fishes as bioindicators can provide more accurate results to describe the petroleum contamination of marine environments. Already, some authors have appropriately applied “Systems Biology” in studies of the effects of petrochemical contaminants on caged marine species, in order to define the environmental health status (Cappello et al., 2017; Maisano et al., 2017; Fasulo et al., 2015). In order to choose efficient bioindicators of petroleum contamination in water systems (Enuneku et al., 2015) some parameters were taken into consideration (Abaunza et al., 2008): species abundance, spatial distribution throughout the Mediterranean Sea, tolerance to changes in salinity, resistance to stress and the ability to accumulate a wide range of pollutants (Cardellicchio et al., 2007). This led to the selection of two interesting commercial species from the Mediterranean as bioindicators for the evaluation of petroleum contamination on the aquatic fauna: *T. trachurus*, a semi-pelagic fish of the family Carangidae, and *E. encrasicolus*, a small pelagic fish of the family Engraulidae.

Tt species can be considered as sentinels of pollution monitoring, within offshore and coastal environments, due to their dominance in marine communities and economic fishing interest (Marigómez et al., 2006; Bodiguel et al., 2009; Martínez-Morcillo et al., 2019). On the contrary, small fish species such as *Ee* play a fundamental ecological role, because they belong to a lower trophic level and act as carriers in the marine food chain of pollutants with a higher trophic level (Martínez-Morcillo et al., 2019), in the biomagnification processes. In this work, samples were collected in a particular area of the Mediterranean Sea, called GSA 10, during summer 2016. Analytical determinations of hydrocarbons in fish livers were performed by the gas chromatograph/mass spectrometry (GC/MS) technique after solvent extraction. The considerable ability of this soft tissue to accumulate relevant concentrations of several classes of contaminants, including hydrocarbons, is well known from the literature (Varanasi and Stein, 1991). For each sampling site along the water column, both chemical-physic parameters and ecological data were collected by mean determinations of temperature, salinity, dissolved oxygen and the amino acid profiles of fish eyes (Falco et al., 2016), respectively. Moreover, since the response to environmental stress could affect the physiological and metabolic behaviour of marine organisms (Wendelaar Bonga, 1997; Sapolsky et al., 2000; Ross et al., 2000; Guardiola et al., 2014a), blood lactate and cortisol levels (Stephens et al., 1997; Marentette et al., 2013) were quantified, while the specific activities of two hydrolytic enzymes (alkaline phosphatase and peroxidase) (Valavanidisa et al., 2006; Guardiola et al., 2014b) were determined in fish epidermal mucus. Indeed, one of the first responses of the skin epithelium to a stressful situation is the increased release of mucus by goblet cells (Iger and Abraham, 1997; Berntssen et al., 1997). This represents the main mechanism of adaptation to environmental stress conditions and exposure to contaminants (Karami et al., 2012), showing an ability to induce antibacterial activities, skin regeneration, and immune stimulation (Rai et al., 2013). Finally, a multiple co-inertia analysis was applied to couple chemical parameters, environmental peculiarity, stress indicators, amino acid profiles and contamination levels in fish. This symmetrical approach (Dray, 2003, Gallego-Álvarez and Fernández-Gómez, 2016; Min et al., 2019) is a very general and flexible way to explore any possible correlation between different variables (Min et al., 2019). These peculiarities make this analytic approach a straightforward method to evidence possible correlations that have rarely been investigated in previous studies, as in our case.

2. Materials and methods

2.1. Study area and sampling operations

The fish samples of *Ee* and *Tt* together with environmental data (temperature, oxygen and salinity) were collected during an oceanographic campaign named “Evatir 2016” carried out with the oceanographic ship “*G. Dalla Porta*” in the summer season of 2016, from July 18th to August 16th. The sampling operation was carried out in the geographical area located in the central - southern Tyrrhenian Sea (GSA 10). The "EVATIR 2016″ oceanographic campaign is an integral part of the Project "Extension of the Medias acoustic campaign (Mediterranean International Acoustic Survey) in the geographical sub-area GSA 10 (Central and southern Tyrrhenian Sea), funded by the Ministry of Agricultural, Food and Forestry Policies (Mipaaf) under the European Fund for Maritime Affairs and Fisheries (FEAMP). The research program has been jointly developed in cooperation with the Institute of Coastal Marine Environment - National Research Council. Sampling map and detailed information on sampling sites are reported in Fig. 1 and Table 1, respectively.

A pelagic net system “volante in barca”, including acoustic data system (Simrad ITI), has been used to capture fish (length 78 mt, depth 22 mt, mesh 18 mm).

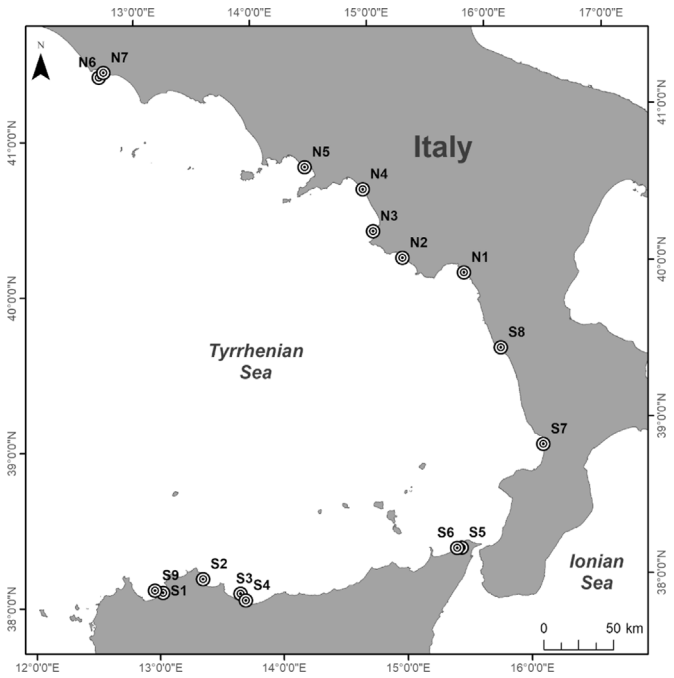

Fig. 1. Sampling sites map of *E. encrasicolus* and *T. trachurus* species.

Table 1
Sampling site, spatial coordinates, number of hauls per site and environmental parameters.

Sampling Site	Longitude	Latitude	Collections per site	T[a] (°C)	S[a] (‰)	D.O[a] (mg/L)
S_1	12° 59′ 38.292″ E	38° 6′ 37.980″ N	5	24.85	37.8	4.69
S_2	13° 26′ 45.132″ E	38° 7′ 16.572″ N	7	25.49	37.9	4.69
S_3	13° 42′ 4.741″ E	38° 3′ 27.792″N	8	25.06	38.0	4.72
S_4	13° 49′ 3.972″ E	38° 0′ 49.068″ N	9	26.01	37.9	4.66
S_5	15° 25′ 51.089″ E	38° 15′ 20.448″ N	13	25.56	38.1	4.71
S_6	15° 26′ 8.848″ E	38° 15′ 44.561″ N	14	26.30	38.0	4.63
S_7	16° 11′ 19.32″ E	38° 51′ 30.312″ N	15	24.16	38.0	4.88
S_8	15° 54′ 31.86″ E	39° 29′ 48.959″ N	17	23.63	38.0	5.10
S_9	12° 59′ 38.292″ E	38° 6′ 37.980″ N	30	22.50	38.1	5.28
N_1	15° 38′ 47.040″ E	40° 0′ 56.988″ N	19	24.23	38.0	4.96
N_2	15° 8′ 34.321″ E	40° 7′ 15.359″ N	20	23.94	38.0	5.01
N_3	14° 47′ 32.940″ E	40° 18′ 58.428″ N	21	24.59	38.0	4.93
N_4	14° 48′ 14.688″ E	40° 33′ 6.228″ N	22	24.12	38.0	4.97
N_5	14° 21′ 27.061″ E	40° 40′ 51.359″ N	24	22.79	37.9	5.17
N_6	12° 39′ 52.74″ E	41° 21′ 48.06″ N	27	24.07	37.8	5.19
N_7	12° 39′ 53.316″ E	41° 21′ 47.815″ N	31	22.89	38.1	5.17

[a] Mean values in the column water.

Among the captured animals, *Ee* and *Tt* specimens were selected which showed a good vital activity, in order to minimise the variations in stress levels. A small number of each species, generally 4–5 fishes, has been stocked in tanks with a volume of 24 L, containing oxygenated seawater.

2.2. Environmental data collection

Temperature, oxygen and salinity data were acquired along the water column, using the Seabird CTD probe (SBE 9/11 plus), and processed according to the Mediterranean and Ocean Data Base directions, in order to obtain profiles from the upper layer of the water column to a depth of 20 m. Samples of water were collected and stored at −20 °C.

2.3. Sampling procedure of biological tissues

From each individual, the liver, eyes, mucus and blood were collected, immediately removed and stored at an appropriate temperature. Livers were sampled immediately upon death for TAH determinations, inserted into special test tubes and placed into liquid nitrogen. Subsequently, samples were stored at −80 °C up to the analytic determination of aliphatic hydrocarbons. Skin mucus was collected by gently scraping the dorso-lateral surface of specimens using a cell scraper, taking care to avoid contamination with blood and/or urinogenital and intestinal excretions. The mucus samples were homogenised with equal volumes of sterile seawater, vigorously shaken and then centrifuged (2000 rpm, 10 min, 4 °C). The supernatant was stored at −80 °C. Blood samples were collected from the caudal vein with an insulin syringe and left to clot at 4 °C for 4 h. The serum was collected after centrifugation (10.000 × g, 5 min, 4 °C) and stored at −80 °C. The eyes of both fish species were sampled by means of special scalpels, taking care not to lose the optic liquid and avoiding also collecting the optic nerve.

2.4. Procedure for the extraction and quantitative determination of TAH by GC-MS analysis

All solvents and reagents used in our experiments (e.g. water, hexane, acetone, sodium sulphate anhydrous, and silica gel flash 60 mesh) were purchased from Sigma Aldrich (Germany); their purity was suitable for GC/MS determinations. The hydrocarbon standards obtained from Ultra Scientific (Italy) and used for the calibration curves were the following: C-11 n-undecane (CAS: 001120-21-4), C-13 n-tridecane (CAS: 00069-50-5), C-15 n-pentadecane (CAS: 000629-62-9), and C-17 n-eptadecane (CAS: 000629-78-7). The stable isotope-labelled internal standard compounds were n-dodecane ^{2}H 26 (CAS: 016416-30-1) and n-exadecane ^{2}H 34 (CAS: 015716-08-2). Each liver sample, consisting of about 100 mg, was spiked with 25 µL of ^{2}H-labelled internal standards mixture (at 100 ppm, for each component), allowed to equilibrate for 5 min and added with 1 mL of H_2SO_4 solution (10% w/w). The spiked samples, placed into sealed glass tubes, were vortexed for 1 min and sonicated for 5 min. Then, the samples were digested in this acidic environment, at controlled temperature (60 °C) for 1 h. The following liquid-liquid extraction was carried out using an aliquot of 5 mL of a solvent mixture (acetone-hexane, 40:60 v/v). After the addition of this mixture, the glass tube was vortexed and sonicated for 1 and 5 min, respectively. In order to separate the organic phase from the aqueous one, each sample was centrifuged for 5 min at 4000 rpm using a Thermo Fisher Scientific SL 16 centrifuge. By means of a Pasteur pipette, the organic solvent portion was removed and easily separated from the water-organic solvent interface. The recovered extract was treated with 300 mg of anhydrous sodium sulphate (Na_2SO_4) to remove water residues. For the removal of fatty acids and triacylglycerol (TAG) contamination, 1 g of silica flash (60 mesh) was weighed and added to the dried organic phase in each sample. In the following step, each sample, vortexed for 1 min, was sonicated for 5 min and finally centrifuged at 4000 rpm for 5 min. The organic layer, removed by a Pasteur pipette, has been concentrated to 500 µL under a gentle nitrogen stream using a Turbovap device. Finally, prior to instrumental analysis, each extract was filtered by 45 µm filters and the filtrate was collected in screw cap vials. During the development of a sample treatment method to extract hydrocarbons from fish livers, different protocols were used in order to maximise the efficiency of the extraction process and reduce the contaminants in the final extract. The optimised method was tested on fish liver samples in either glass or plastic tubes. An unexpected and massive contamination of samples occurred if the extraction procedure was carried out in plastic tubes. These tubes released hydrocarbons with even and odd numbers of carbon atoms in their alkyl chain. As a result of this evidence, all sample treatments were performed in glass tubes. In marine water samples, hydrocarbons were determined by means of a Purge and Trap Autosampler. Specifically, each sample of marine water was diluted with mineral water (1:5), prior to the purging procedure. The instrumental setup for the analysis performed on Atom X (Tekmar) Purge and Trap device was the following: valve oven temperature 200 °C, transfer line temperature 249 °C, sample mount temperature 90 °C, sample heater temperature 90 °C, pre-sweep time 0.25 min, purge time 15 min, purge flow 40 mL/min (N_2 99.9995), purge temperature 22 °C, dry purge time 0.50 min, dry purge flow 100 mL/min, dry purge temperature 20 °C, desorb pre-heat temperature 245 °C, desorb time 1.00 min, desorb temperature 300 °C, drain flow 300 mL/min, and adsorbing cartridge matrix Carboxen™ 2000.

Hydrocarbon analysis in liver samples was performed using a gas chromatograph Trace GC1310 (Thermo Fisher Scientific, San Jose, CA) connected to a triple quadruple mass spectrometer (GC/MS/MS TSQ 8000) (Thermo Fisher Scientific, San Jose, CA) equipped with an auto sampler (Triplus RSH) (Thermo Fisher Scientific, San Jose, CA). The injector (PTV) program was the following: after injection, the temperature was increased from 75 °C to 250 °C, at 10 °C/min, held for 1 min and then increased to 320 °C at 14.5 °C/min; after this, it was held for 20 min, and finally reduced to 75 °C prior to the next injection. The injection volume was 1 μL. Hydrocarbons were separated by a chromatographic column (Thermo Scientific TG-5SILMS length: 60 m; id: 0.25 mm; film thickness: 0.25 μm). The column oven temperature program was the following: initial temperature 70 °C (2 min-hold) followed by a 10 °C/min ramp up to 270 °C (15 min-hold), followed by a 15 °C/min ramp up to 320 °C (10-min hold). The data were acquired operating in positive ion Selected Reaction Monitoring (SRM) mode. The electron beam energy was set at 50 eV (nominal value). Source temperature was set at 280 °C and the transfer line at 300 °C. Ultra-pure (99.9995%) helium was used as a carrier gas and the flow rate was maintained at 1 mL/min. The most representative ions have been opportunely chosen, in order to generate a selected ion monitoring (SIM) method, which has been employed for the quantitative determination of hydrocarbons. Calibration for hydrocarbons was carried out with four hydrocarbons at odd carbon number atoms (C-11, C-13, C-15 and C-17). The calibration curve, using a mix standard solution at 100 ppm for each component, covers the concentration ranges from 0.5 to 50 mg/kg. The concentration level for each hydrocarbon was 0.5, 1, 2, 5, 10, 25 and 50 mg/kg. Correlation coefficients (R^2) of the calibration curves were in the range from 0.98 to 0.99. The limit of detection (LOD) and the limit of quantitation (LOQ) values, calculated on the basis of directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry, were as follows: C-11:0.11 mg/kg (LOD) and 0.35 mg/kg (LOQ); C-13: 0.05 mg/kg (LOD) and 0.15 mg/kg (LOQ); C-15: 0.003 mg/kg (LOD) and 0.01 mg/kg (LOQ) and C-17: 0.018 mg/kg (LOD) and 0.06 mg/kg (LOQ).

Several blank samples (solvent blanks spiked with internal standard mix and reagents blanks) were acquired before and during each experimental session in order to determine any eventual contamination and its level. Each sample was injected three times.

2.5. Experimental procedure for the determination of enzymatic activities analysis and amino-acid contents

The phosphatase activity was measured in a solution prepared by dissolving 30 μL of the skin mucus with 170 μL p-nitrophenyl phosphate liquid 4 mmol/L (by Sigma-Aldrich, Germany) in ammonium bicarbonate buffer containing 1 mmol/L $MgCl_2$ at pH 7.8 (Ross, 2000). The peroxidase activity in skin mucus samples was measured from 30 μL of skin mucus sample diluted with 120 μL of Hank's buffer using 50 μL of TMB 20 mmol/L and H_2O_2 5 mmol/L as substrates (Quade, 1997). One unit was defined as the amount producing an absorbance change of 1 and the activity expressed as U/mg of mucus proteins. The concentrations of total cortisol were measured in the plasma sample using a commercially available kit (Intermedical Diagnostics srl) according to the manufacturer's instructions. The lactate plasma levels were determined using the Accutrend Plus Kit (Roche Diagnostic, Germany). Amino acids were evaluated in fish eyes by means of high performance liquid chromatography (HPLC), using the Shimadzu RF-10 AXL apparatus (Falco, 2016), equipped with an autosampler SIL-20ACHT and a fluorescence detector. Protein hydrolysis in eyes was performed with some modification, with respect to the method described in the literature (Dai, 2014). Dissected eyes were placed in a tube containing 1 mL of HCl 6 mol/L, and N_2 was insufflated for 1 min. Then, the capped tubes were warmed in an oven at 110 °C for 2 h. The samples were gently shaken to ensure that the sample was completely dissolved in the solution. After 24 h, the solution, dried carefully under N_2, was suspended in 1 mL of HPLC grade water and stored at 4 °C. A pre-column derivatisation with *o*-phathaldialdehyde (OPA) was performed according to the procedure in the literature (Dai, 2014). The following amino acids were determined: aspartate plus asparagine (Asp + Asn), glutamate plus glutamine (Glu + Gln), serine (Ser), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), tyrosine (Tyr), valine (Val), lysine (Lys), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), and methionine (Met).

2.6. Statistical Analysis Procedure

The variables considered in this study, i.e. environmental and biological parameters and TAH concentrations, were grouped in six different data-frames (DFs): 1) DF containing the composition of all 15 amino acids in each specimen; 2) DF containing the environmental variables (Env) measured in each haul (sea surface temperature; salinity and dissolved oxygen); 3) DF containing biochemical indicator of stress (Cortisol, Lactate, Alkaline phosphatase and Peroxidase activity); 4) DF containing the amount of hydrocarbons measured (TAH) in the livers; 5) haul spatial coordinates (Latitude and Longitude); and 6) Individual length (Leng). In order to compare different DFs containing different types of data and measures, all variables were standardised using the Z-scores method. With the aim of characterising each data frame and then making useful associations between them, we first carried out a Principal Component Analysis for each data frame. The correlation between each pair of DFs was measured through random variable "RV" coefficients (Escoufier, 1973). The RV coefficient is a multivariate generalisation of the squared Pearson correlation coefficient, and range between 0 and 1, where 0 means no association and 1 means perfect association. The significance of the RV coefficient was estimated by means of a permutation test (Heo, 1998). Successively, we performed a multiple co-inertia analysis (Chessel, 1996), applied to all PCA results. This is a multivariate method for coupling k data frame ($k > 2$), sharing the same rows (individuals or variables). Statistical analyses were performed with R software; the *FactoMineR package* (Kostov, 2013) was used for PCA and *ade4* (Dray, 2003) for multiple co-inertia analyses.

3. Results and discussion

3.1. Experimental results

Temperature, oxygen and salinity data acquired along the water column in the area under study, as reported in Table 1, have been processed according to the Mediterranean and Ocean Data Base directions. The mean values of profiles obtained from the upper layer of the water column to a depth of 20 m for each site are reported in Table 1, and used in the next step of statistical calculations. TAH levels, calculated as sum of n-undecane (C-11), n-tridecane (C-13), n-pentadecane (C-15), and n-eptadecane (C-17) concentrations in *Ee* and *Tt* liver samples, have been reported in Table 2. Contaminant concentration levels in *Ee* and in *Tt* livers range from 4.7 (S9) to 35.2 mg/kg (N1) and from 0.7 (S4) to 3.9 (S8) mg/kg, respectively, with a standard error always less than 10%. The largest quantities detected in both specimens in the sites located near Salerno's gulf, i.e. from N1 and N5 sites (Table 1), can be explained considering that, in this area, the human activities contribute to strongly implement the level of contaminants, due to the high amount (from 23.1 to 2670.4 ng/L) of hydrocarbons discharged from the Sarno River to the Tyrrhenian in offshore and coastal areas (Qu, 2018). Generally, the average hydrocarbon values measured show relevant differences ($p < 0.01$) in the liver samples of two species, and the different tendency to accumulate organic contaminants can be related to the lipid content of the species (Shriadah, 2001). *Ee* shows an average lipid content of 17% of the total composition with respect to wet weight, whereas *Tt* is characterised by a lower lipid content, i.e. 2% (Orban, 2011). Our results, in terms of absolute

Table 2
Amount (mg/kg) of total aliphatic hydrocarbons, expressed as average concentration for collection site, in fish livers.

Site	Average Concentration[a] (mg kg^{-1})		BAF	
	Ee	*Tt*	*Ee*	*Tt*
S1	8.7 ± 2.8	–	48	–
S2	14.1 ± 1.6	1.5 ± 0.9	77	8
S3	6.9 ± 2.6	3.9 ± 1.0	38	22
S4	–	0.7 ± 0.1	–	4
S5	8.2 ± 1.7	–	45	–
S6	–	0.8 ± 0.4	–	4
S7	–	0.8 ± 0.1	–	4
S8	–	3.8 ± 1.8	–	22
S9	4.7 ± 0.1	–	26	–
N1	35.2 ± 14.5	–	194	–
N2	–	3.0 ± 1.0	–	17
N3	5.1 ± 0.7	–	28	–
N4	8.3 ± 0.6	–	46	–
N5	14.3 ± 1.1	–	79	–
N6	29.6 ± 1.5	–	164	–
N7	–	1.6 ± 0.7	–	9

[a] ± std. error.

concentration, have been compared with data reported for fish livers of the same specimens (Shriadah, 2001; Enuneku, 2015), showing that our measurements were consistent with the literature. In particular, our hydrocarbon values fall in the concentration range from 0.17 mg/kg (Shriadah, 2001), determined on *Sardinella aurita* in the Gulf Region, to 73 mg/kg (Enuneku, 2015), determined on *Tt* in Nigeria. On the basis of this evidence, we observed that health risks for specimens living in the area investigated in this campaign cannot be considered negligible. Thus, it is important to consider that *Tt* and *Ee* are well known to accumulate a higher concentration of hydrocarbons in other soft tissues, such as the gills, muscles and kidney (Enuneku, 2015), showing a relevant connection between the bioaccumulation of contaminants and the biomagnification process along the trophic chain. In order to better understand their potential use as bioindicators with respect to this class of contaminants, the TAH concentrations in water samples and the Bioaccumulation Factor (BAF) in liver tissues of *Tt* and *Ee* were evaluated. TAH concentrations in water samples were always below the limit of detection of the GC/MS method. TAH and BAF for liver samples are summarised in Table 2. The higher BAF values that were generally calculated for *Ee* are probably due to the most relevant lipid content being in liver tissues. Even though a toxicological profile for TPH has been available since 1999 from the U.S. Department of Health and Human Services and several reports have highlighted the negative effects of TAH on marine organisms and human beings (Enuneku, 2015; American Chemistry Council n-Alkane VCCEP Consortium, 2004), only a few, incomplete pieces of information on regulatory reference limits are available for this class of substance, as well as due to the controversial debate on the relationship between their ecotoxicological risks and the negative impact on human health. On the basis of these considerations, the acquired BAF data represent a useful indication for the interpretation in the fish of the risk factors associated with the presence of hydrocarbon substances in the Tyrrhenian Sea.

The composition of eyes amino acids relatively to *Ee* and *Tt* has been determined as a total concentration and reported in Table 3. The results obtained represent a mean value of 39 samples of *Ee* and 68 samples of *Tt*.

The results reported show a higher amino acid composition, and a generally more relevant variability in *Ee* than in *Tt*. Considering the quite different concentration obtained for the species investigated, data were successively analysed by one-way Permutational Multivariate ANOVA (PERMANOVA), performed on a triangular matrix based on Bray-Curtis similarity, in order to verify whether the two species may be considered homogeneous with respect to the composition of amino

Table 3
Concentration profiles for each amino acid (AA) in the two considered species.

AA	Average Concentration [a] (mg/kg)	
	Ee	*Tt*
Asp + Asn	54.2 ± 3.6	33.7 ± 2.5
Glu + gln	90.3 ± 6.2	48.1 ± 4.1
Ser	52.8 ± 4.5	20.1 ± 2.0
His	22.7 ± 1.4	17.9 ± 1.2
Gly	108.6 ± 9.7	45.2 ± 3.8
Thr	34.5 ± 2.9	15.4 ± 1.2
Arg	102.4 ± 7.9	55.6 ± 4.2
Ala	35.1 ± 4.4	26.4 ± 2.1
Tyr	67.7 ± 8.7	31.0 ± 2.4
Met	32.8 ± 2.2	18.7 ± 1.2
Val	26.8 ± 1.6	16.1 ± 1.2
Phe	34.9 ± 2.1	22.5 ± 1.6
Ile	24.0 ± 1.6	13.7 ± 1.0
Leu	27.7 ± 2.2	17.7 ± 1.4
Lys	30.1 ± 2.1	17.0 ± 1.3

[a] ± std. error.

acids. Statistical parameters, obtained from multivariate analysis (d.f. = 1; F = 23.214; $p < 2.2\ e^{-16}$), confirmed that the composition of amino acids from the eyes of the two investigated pelagic fishes are species-specific (Riveiro, 2011). These results suggested the need to perform the following determinations for the two species separately.

In Table 4, as a representative example of both species and all sampling sites, we reported the stress indicator results as average values of a congruent data set ($N > 30$) for *Ee* and *Tt* in the N_1 sampling site. Complete calculation results, concerning all sites (see Table 1), were obviously taken into account for the data evaluation, performed by co-inertia multivariate approach.

It is crucial to point out that the data were acquired on biological samples (plasma, mucus) recovered in a short time, i.e. immediately after the species capture phase, since an evaluation of short-term stress indicators allowed us to make an assessment of anthropogenic impacts that were very close to the real ones. Mucus alkaline phosphatase and peroxidase enzymatic activities were measured for the first time in these species, showing that values in *Tt* were always higher than in *Ee*; the high variance of *Ee* could be explained by the difficulty recovering mucus from the thin and soft skin of this species. Generally, for all sites, the data showed a greater concentration of cortisol with respect to other stress indicators (Vazzana, 2002; Cammarata, 2012) for both species. As can be seen in the example data reported (Table 5), we observed a mean value of 251.27 (U.L/mg protein) in *Ee* and 196.7 (U.L/mg protein) in *Tt*. Consequently, we cannot exclude short-term stress alteration mechanisms, which could be in part caused by the fishing conditions. This consideration could be supported by the low values of lactate, which generally needs more time to increase its levels in the plasma (Begg, 2004). A preliminary statistical analysis performed on alkaline phosphatase, peroxidase enzymatic activities, lactate and cortisol data highlighted the significant species-specific differences between *Ee* and *Tt* (d.f. = 1; F = 18.23; $p < 2.52\ e^{-11}$).

Table 4
Alkaline phosphatase and peroxidase activity, cortisol and lactate mean values of samples in N_1 sampling site, from *Ee* and *Tt*.

Stress Indicator	Average value[a] (U.L./mg protein)	
	Ee	*Tt*
Alkaline P.	5.2 ± 3.8	41.7 ± 15.4
Peroxidase A.	5.3 ± 4.7	16.1 ± 3.2
Cortisol	251.3 ± 53.5	196.7 ± 46.5
Lactate	2.3 ± 0.7	2.6 ± 0.7

[a] ± std. error.

Table 5
p-value of correlation for *Ee* (a) and *Tt* (b).

(a)	*Ee*					
	AA	Env	Sp C	Stress Ind	TAH	Leng
AA	1	< 0.001	< 0.001	0.027	0.238	0.09
Env	_	1	< 0.001	0.001	0.014	< 0.001
Sp C	_	_	1	0.004	< 0.001	< 0.001
Stress Ind	_	_	_	1	0.414	0.569
TAH	_	_	_	_	1	< 0.001
Leng	_	_	_	_	_	1
(b)	*Tt*					
	AA	Env	Sp C	Stress Ind	TAH	Leng
AA	1	< 0.001	< 0.001	0.899	0.054	0.207
Env	_	1	< 0.001	0.074	0.006	0.033
Sp C	_	_	1	0.673	0.108	0.914
Stress Ind	_	_	_	1	0.06	0.594
TAH	_	_	_	_	1	0.175
Leng	_	_	_	_	_	1

3.2. Statistical evaluation

According to the previously reported multiple co-inertia approach (See § *2.6 Statistical Analysis Procedure*), and considering all previously identified data [amino acids (AA), environmental (Env), hydrocarbons (TAH), stress indicators (Stress Ind.), spatial coordinates (Sp C), and length of fishes (Leng)], in Table 5 we reported p-values of correlations for *Ee* (a) and *Tt* (b).

Values in Table 5 could be more easily explained by considering a correlation circle plot, obtained through a Multiple Factor Analysis (MFA) and drawn in Fig. 2a and b for *Ee* and *Tt*, respectively. The multiple co-inertia space was composed of two axes, accounting for 30% and 15% for *Ee*, and for 29% and 17% for *Tt* of the variance, respectively.

Both graphs generally showed that some parameters, such as spatial coordinates (longitude, latitude) and environmental parameters (temperature, oxygen), have high statistical meaningfulness; moreover, temperature and oxygen are strictly correlated with axes 1 ($p < 0.001$), even if these parameters showed a negative correlation, with high temperature corresponding to lower levels of dissolved oxygen.

A few interesting evaluations can be made for *Ee*, on the basis of biological indicators and TAH concentration results, reported in Fig. 2a and Table 5a. Multiple co-inertia analysis evidenced a spatial trend between the variables, allowing us to make a separation between the northern and southern locations. Amino acids determined in the eyes of *Ee* were significantly correlated with the environmental parameters and spatial coordinates. Specifically, leucine (Leu), glutamate (Glu) and alanine (Ala) showed a positive correlation with higher temperature values, which is distinctive of sampling sites falling within the coastal area between the S_1–N_1 locations (see Fig. 1); high values of dissolved oxygen are mainly correlated with individuals coming from sites located at high altitude, i.e. N_2–N_7 (see Fig. 1), which showed major contents of tyrosine (Tyr) and methionine (Met). Moreover, Ala and Leu showed a positive correlation with peroxidase and alkaline phosphatase activities, whilst Tyr and Met were strongly correlated with cortisol. This relation between amino acids and stress factors is a very important novelty for biological knowledge and opens up possible applications in stress evaluation responses. The highest levels of cortisol were found at high latitude, whilst peroxidase and alkaline phosphatase in the area were related to an increase in temperature. According to the correlation circle plot, hydrocarbons probably accumulated more abundantly in the livers of *Ee* with higher individual lengths (adult specimens), which are the predominant population in coastal areas (higher longitude values). However, co-inertia analysis has shown no relevant correlation between stress biomarkers and TAH contamination levels for *Ee* specimens, even

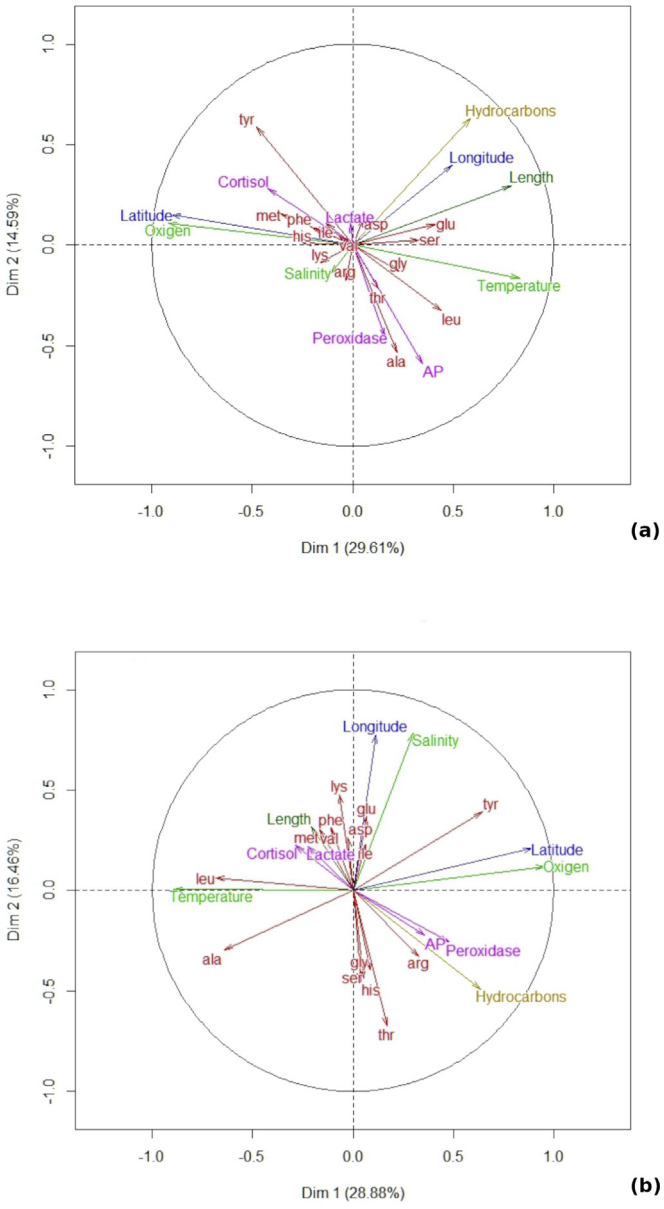

Fig. 2. Correlation circle plot of Multi Factor Analysis for *Ee* (a) and *Tt* (b).

though the concentration of hydrocarbons determined in livers is not negligible, as reported in Table 3. Probably, the chosen biomarkers were not appropriate to give useful answers with respect to the exposure of TAH, probably because they were not selective for this class of chemicals, or the amount determined in sampled tissues was too small (Table 4) to obtain suitable biomarker responses. However, we considered that the failed responses of biomarkers to xenobiotic exposures, in ecotoxicology and ecological risk assessment, is a controversial question, which has been frequently discussed but never completely solved (Forbes, 2006).

Considering the correlation circle plot for *Tt* data frame (Table 5b, Fig. 2b), we can observe a strong connection between latitude and dissolved oxygen, even if it is impossible to recognise any spatial trend between variables using this plot, as in the case of *Ee*. Amino acid composition was significantly affected by the environmental parameters, mainly temperature and dissolved oxygen values. Specifically, leucine (Leu) and alanine (Ala) showed a good correlation with temperature, while tyrosine (Tyr) was correlated with high value of dissolved oxygen and salinity. The differences in correlation responses in

the two examined species are obviously related to the different taxonomic position between the Carangidae *T. trachurus* and the Clupeiformes *E. encrasicolus*. The lifestyle and reproduction processes could also influence these differences; in particular, the fishing period (August) is the full phase of reproduction for *Ee*, which starts in April and has a peak at the end of September. On the contrary, the reproduction process for *Tt* starts in December and is completed before the end of June. In addition, the European anchovy (*Ee*) is euryhaline, being able to live in water with a salinity between 5 and 41. Peroxidase and alkaline phosphatase activities exhibited a quite good correlation with different amino acids, such as arginine (Arg) threonine (Thr), histidine (His), glycine (Gly) and serine (Ser), and interesting connections with TAH determined in livers. If we consider higher values of peroxidase and alkaline phosphatase activities for *Tt* compared to those for *Ee* (Table 4), we can conclude that they worked more appropriately as stress indicators for *Tt*, probably due to the smaller values of TAH in the latter species, as can be observed in Table 3. For both species, according to the literature (Alreshidi et al., 2016), data analysis has confirmed the fundamental influence of environmental conditions (and/or chemical pollutants) on the cytoplasmic levels of some amino acids, providing insights into the adaptability mechanisms of fishes.

4. Conclusions

The multiple co-inertia approach proved to be an excellent tool for the interpretation of any possible correlations between the different chemical, environmental and biological parameters considered here, giving us the possibility to evidence relevant relationships, which have been rarely investigated in previous monitoring studies. This approach also showed a clear spatial distribution between variables in *Ee*, whilst the absence of a spatial trend in *Tt*. Moreover this analysis confirmed the opportunity to use two commercial fish species as a biomonitoring probe, in order to evaluate the effect of oily pollutants on the living organisms in their habitat. Cortisol levels, mucus alkaline phosphatase and peroxidase enzymatic activities values have been detected for the first time in these species. Amino acid profiles were highly dependent on chemical-physical and environmental properties for both species, confirming the possibility of being used as ecological welfare indices, for short-term environmental variations. Nevertheless, the relevant differences in amino acid profiles of the two species have been explained by the different taxonomic position between the Carangidae *T. trachurus* and the Clupeiformes *E. encrasicolus*. TAHs are more highly accumulated in *Ee*, collected in the northern coastal areas, due to the presence of rivers, which spill off more hydrocarbon substances; however, no significant correlations were found between TAH levels in *Ee* and biochemical indicators (peroxidase and alkaline phosphatase). *Tt* always exhibited a lower tendency to bioaccumulate TAHs, due to the lower quantity of fatty acids in liver tissues, which is strictly related to biochemical indicators. In both cases, since the risks of bioaccumulation in these pelagic fish, which are widely consumed, could not be neglected, we intend to select more appropriate biomarkers, in order to give useful answers with respect to the oily contaminant exposure.

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