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Abstract: The Mar Menor is a coastal lagoon threatened by the development of intensive agriculture in the surrounding areas. Large amounts of pesticides from these areas are discharged into El Albujón, a permanent watercourse flowing into the lagoon.

We have used a multi-biomarker approach to assess the biological effects arising in bivalve species affected by agricultural pollution. Biomarkers indicative of neurotoxicity (acetylcholinesterase, AchE), oxidative stress (catalase, CAT; glutathione reductase, GR and lipid peroxidation, LPO), phase II biotransformation of xenobiotics (glutathione S-transferase, GST) and physiological stress (scope for growth, SFG) were measured in clams transplanted to four sites of the lagoon (two reference sites and two sites affected by the dispersion of the effluent of the El Albujón), for exposure periods of 7 and 22 days.

The hazards of this effluent were also examined by simultaneously measuring up to 83 contaminants (pesticides, PCBs, PAHs and others) in samples of fresh water from the watercourse mouth and seawater from the deployed sites, as well as the bioaccumulation of organochlorinated compounds and PAHs in the transplanted animals.

Biomarker responses showed marked differences between reference and affected sites after 7 and 22 days. However it was only after 22 days that Principal Component Analysis (PCA) of the biomarker responses distinguished between clams deployed in sites affected by the dispersion of the effluent of the watercourse and those from the reference sites. The chemical analysis of water showed high concentrations of pesticides close to El Albujón watercourse mouth, with the greatest input flux corresponding to the organophosphate chlorpyrifos, followed by pendimethalin and naphthalene, and at lower levels acenaphthene, terbuthylazine-desethyl and chlorpyrifos-methyl. In this regard, PCA analysis showed that the biological effects of the mixture of pesticides in caged clams after 22 days were reduced levels of AchE and SFG and increased levels of GR and phase II GST activity. An Integrated Biomarker Response index was calculated from the combination of these biomarkers, proving useful for the assessment of the impact of agricultural pollution in caged clams.

Impact assessment of agricultural inputs into a Mediterranean coastal lagoon (Mar Menor, SE Spain) on transplanted clams (*Ruditapes decussatus*) by biochemical and physiological responses

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1. Introduction

Marine bivalves have been used as bioindicators to identify chemical pollutants in coastal environments. However, the concentration of contaminants in tissues alone provides no information on the biological significance and deleterious effects of environmental pollution on biological systems. The need to detect and assess the effects of contaminants has led to the development of markers of their effects (i.e. biomarkers). These biological-effect methods, which range from responses measured at subcellular level (e.g. oxidative stress and DNA adducts) to whole-organism responses (e.g. scope for growth or disease occurrence), can indicate links between contaminants and ecological responses and can be used to indicate the presence of harmful substances in the marine environment (Thain et al., 2008).

Acetyl cholinesterase (AchE) activity is considered a valuable biomarker of exposure to neurotoxic compounds in vertebrate and invertebrate species, such as organophosphorus (OPs) and carbamate compounds used in agriculture as pesticides (Boquené and Galgani, 1998; Cooper and Bidwell, 2006). The toxicity of OPs results from their inhibition of cholinesterase enzymes, which catalyze hydrolysis of the neurotransmitter acetylcholine after it is released at the nerve synapse.

Oxidative stress is a common pathway of toxicity induced by several classes of pollutants (Winston and Di Giulio, 1991) by which production of reactive oxygen species (ROS) is enhanced. ROS can be highly toxic to aquatic organisms as they often produce cellular damage such as lipid peroxidation (LPO) in membranes, altered pyridine nucleotide redox status and DNA damage (Lemaire and Livingstone, 1993). Protection against the toxicity of oxyradicals towards cellular targets is afforded by a complex defence system consisting of both low molecular-weight scavengers and antioxidant enzymes. Enzymatic activities for the detoxification of ROS and the degree of LPO have been proposed as biomarkers of oxidative stress in bivalves exposed to different types of pollutants (Fernández et al., 2010a; Tsangaris et al., 2007; Vidal-Liñan et al., 2010). Some of the more commonly used antioxidant biomarkers include catalase (CAT), glutathione transferase (GST), glutathione reductase (GR), and lipid peroxidation (LPO). CAT is responsible for the breakdown of hydrogen peroxide into water and oxygen, which may be produced during basal aerobic metabolism or after a pollution-enhanced oxyradical generation (Winston et al., 1990). Although glutathione reductase (GR) does not play a direct role in the elimination of oxygen radicals it can be regarded as an essential antioxidant enzyme since it reduces oxidised glutathione (GSSG) and maintains the GSSG/GSH balance under oxidative stress, essential for cellular homeostasis and the operation of other enzymes (Winston and Di Giulio, 1991). GST represent a major group of phase II detoxification isoenzymes whose ‘natural’ substrates range from molecules of foreign origin to by-products of cellular metabolism. GSTs primarily catalyse the conjugation of GSH to various electrophilic compounds, but they can also act as glutathione peroxidase, as isomerases, or simply as binding proteins sequestering hydrophobic molecules, and can therefore be regarded as playing an antioxidant role (Manduzio et al., 2005; Prohaska, 1980).

Scope for growth, SFG, is a biomarker at the individual/whole organism level of biological complexity with a high degree of ecological relevance and for this reason is eminently applicable for biomonitoring programs (SIME, 2007). This technique involves the calculation of the energy available for growth under standardized laboratory conditions. It consists of evaluating the energy acquired by an organism after absorbing the food it has ingested and that lost in the respiratory and excretory processes, the difference between them being the energy the organism has available for production (growth and reproduction). The presence of contaminants in the marine environment alters this energy balance, making SFG a marker for toxic stress. SFG has been successfully applied in programmes monitoring chronic pollution (Albentosa et al., 2012; Cotou et al., 2002; Halldorsson et al., 2005; Toro et al., 2003a; Widdows et al., 1995; Widdows et al., 2002), acute pollution associated with a spill (Fernández et al., 2010b ; Larretxea and Pérez Camacho, 1995) and in laboratory contaminant exposure studies (Kraak et al.; 1997, Sobral and Widdows, 1997; Wang and Chow, 2002; Widdows and Page, 1993).

Different studies have shown the usefulness of marine clams as sentinel organisms for the detection of the impact of environmental pollution in coastal waters through the application of different biomarkers (Bebianno et al., 2004; Nasci et al., 1999, 2000). These biomonitoring studies have employed native populations of bivalves or organisms that have been transplanted from a reference site to a polluted area (Rank et al., 2007; Tsangaris et al., 2010, 2011). This latter strategy, called active biomonitoring (ABM), is based on comparing chemical and/or biological properties of samples collected from one population that, after randomization and translocation, has been exposed to different environmental conditions at monitoring sites (Romeó et al., 2003). This approach avoids bias related to the age and the reproductive status of the organisms and allows for better control of the accumulation and biological effects of contaminants over a predetermined exposure period. In addition, comparisons of sites are feasible even if natural populations are scarce (Tsangaris et al., 2011).

Overall, coastal lagoon environments are characterized by being isolated from the open sea, which makes them highly vulnerable to impacts. The Mar Menor lagoon is a shallow coastal basin connected with the Mediterranean Sea principally through three sea channels that receives a wide variety of chemical pollutants associated with anthropogenic activities. Its ecological equilibrium is threatened by massive urban growth and intensive agricultural activity (Conesa and Jiménez-Cárceles, 2007). The lagoon receives water run-off from the coastal plain of Campo de Cartagena, where intensive agricultural activity has taken place since 1979. At the present time, El Albujón watercourse constitutes the main collector in the Campo de Cartagena drainage system (García-Pintado et al., 2007), maintaining a regular flux fed by groundwater (drainage of irrigated crops) that is only continuous in the last 3-8 km, depending on the season (Velasco et al., 2006).

In a previous study by our group (Moreno-González et al., 2013a), the seasonal input of all the organic pollutants to the Mar Menor lagoon through El Albujón watercourse (considering both regular and flash flood periods) was characterized. In the aforementioned study, 71 semi-volatile organic pollutants were detected by stir bar sorptive extraction followed by capillary gas chromatography coupled to mass spectrometry (SBSE/GC/MS). Results showed that pesticide concentrations varied significantly along the watercourse and a clear seasonal pattern was detected, with a predominance of insecticides during summer and of herbicides during winter. The most commonly detected analytes were propyzamide, triazinic compounds and chlorpyrifos.

The objective of this study was to assess the effect of the El Albujón watercourse on the water quality of the Mar Menor by means of the biological effects elicited in a characteristic bivalve of this lagoon. For this purpose, a multi-biomarker approach was applied in transplanted *Ruditapes decussatus* clams caged at two sites affected by the dispersion of the watercourse input, according to the main currents, and at a further two sites not affected directly by significant pesticide inputs, which act as reference sites. Biomarkers included biochemical measurements which represent important endpoints of particular chemicals or mixtures expected in the study area: AchE, CAT, GST, GR, LPO and bioenergetics such as SFG used to detect general stress effects on the health status of clams. Active biomonitoring is also evaluated for the assessment of the risk of environmental contamination in this coastal lagoon where natural populations of bivalves are scarce due to the deterioration of their ecosystems.

2. Materials and Methods

2.1. Study area and experimental design

The Mar Menor (SE Spain) is a hypersaline coastal lagoon located in the Mediterranean Sea with a superficial area of 135 km^2 (Maria-Cervantes et al., 2008). The salinity of its waters ranges from 42 to 46 psu showing a north–south gradient, except in areas close to the principal channels connecting with the Mediterranean Sea or to freshwater inputs. The general circulatory pattern along this axis makes it possible to differentiate three basins within the Mar Menor (See Figure 1): (1) the northern basin, with the lowest mean salinity values, which shows a higher Mediterranean influence and lower hydraulic residence time than other basins; (2) the southern basin, with the most saline waters, which is the most confined area; and (3) the central basin, with intermediate values and corresponding to the mixing area of Mediterranean and lagoon waters.

The central basin of the lagoon receives runoff from El Albujón watercourse, which is the main collector of residues from the agricultural products used in the Campo de Cartagena (García-Pintado et al., 2007), and of effluents from the urban wastewater treatment plant of the town of Los Alcázares.

Native *Ruditapes decussatus* clams (3.4 cm mean shell length) were collected from the clean area of Las Encañizadas located in the northern Mar Menor (October 2010), which shows the highest influence of the Mediterranean Sea. This site was chosen for its location far from agricultural, urban, and industrial influences. The clams were maintained, with no mortality, for 10 days in the laboratory using clean filtered seawater, after which they were placed in baskets used for oyster culture (120-125 specimens per basket) and put in stainless steel cages (3 baskets/cage) immersed at 4 sites (Figure 1), about 40-60 cm over the bottom sediment. Selected sites 3 (S3) and 4 (S4) were located close to the El Albujón watercourse, 0.5 and 1.5 km downstream from the wadi mouth, respectively. According to the main currents found in this area both sites are directly affected by the input from the said watercourse.

Two sites were used as reference sites, site 1 (S1) located in the northern basin close to Lo Pagán, and site 2 (S2) located upstream of the El Albujón watercourse mouth, near the Los Alcázares waterfront, which according to the lagoon's circulatory system are not affected by the dispersion of the effluent from the wadi.

Once the cages were immersed, a systematic sampling campaign was developed to characterize levels of pollutants in water, temperature, pH, dissolved oxygen and salinity at each sampling point and the watercourse mouth. For this purpose samples of surface water (5-25 cm depth) were collected twice a day (morning and evening) during the first eight days after transplantation from the points at which the cages were moored. At the same time measurements were made of the water flow, temperature, pH, dissolved oxygen, salinity and organic pollutant concentrations in water samples from El Albujón watercourse mouth, and pollutant fluxes were estimated for the first week of the study.

Clam samples were taken at 0, 7 and 22 days and analyzed for total body burdens of persistent OCs and PAHs, biomarkers and physiological status. One-hundred and twenty clams were randomly collected from the 3 baskets at each site in each sampling period. Twenty individuals were used for SFG, biochemical and condition index analysis and the remaining one hundred individuals for chemical analyses. It is important to note that physiological and biochemical determinations were carried out on the same individuals in order to improve the correlation between biochemical and physiological processes. For this purpose, after the SFG and biometric determinations, gills and digestive gland organs were dissected and frozen in nitrogen liquid until further biochemical analysis was performed.

Contaminant concentrations in the transplanted clams were measured in the soft tissues obtained after removing the flesh from their shells, which was then stored at -20ºC until analysis.

2.2. Water characterization

Temperature, pH, conductivity and dissolved oxygen were determined in situ using a portable multiparametric probe (VTW), and water samples were stored at -20ºC until their analysis. 14 PAHs, 7 PCB congeners, 13 triazines, 17 organophosphorus pesticides, 17 organochlorine pesticides and a further 12 organic pollutants were determined in surface and seawater samples by stir bar sorptive extraction and thermal desorption coupled to capillary gas chromatography-mass spectrometry (SBSE/GC/MS), applying the procedures proposed by Moreno-González et al. (2013a) and Moreno-González et al. (2013b), respectively. The applied procedure consisted of a stage of SBSE, after which analytes were desorbed from the stir bar at 280 ºC for 12 min, cryofocused in a PTV injector at 40 ºC and then analysed by GC/MS in full-scan mode.

2.3. Physiological parameters

The clams were acclimatised after transport for 24 h in tanks under controlled temperature and feeding conditions (19ºC and a diet of the microalgae *Isochrysis galbana*, clone T-ISO). Physiological measurements were taken the day after the clams arrived at the laboratory, to which end they were transferred to an open-flow system consisting of ten chambers, each containing two living clams, and two empty chambers used to represent the inflow of the chambers with clams. During the physiological experiment, clams were maintained under continuous standardized conditions (filtered 39 psu SW, 19ºC, and a standard algal ration of *I. galbana*, 0.8 mg organic matter AFDW L^{-1}), these being the conditions defined for the particular environmental conditions in the Mar Menor lagoon. Clearance rates (CR) were calculated from the difference in food concentrations at the inflow and outflow points of the flow-through system. The flow rate was adjusted in order to maintain a difference between inflow and outflow concentrations of less than 40% of the inflow. Ingestion rates (IR) were obtained from the clearance and food concentration rates, expressed as units of organic matter per litre. This was done by filtering inflow samples through previously rinsed, ashed and weighed Whatman GF/C filters. The filters were then rinsed with a 0.5 M ammonium formate solution, dried for 24 h at 100ºC and ashed at 450ºC for 1 h. The difference between dry weight and ashed weight was taken as organic weight.

The efficiency of the gills, as the organ responsible for filtering food, was estimated by means of clearance efficiency (CE), obtained from the ratio between the clearance rate and gill size, expressed as weight.

Absorption efficiency (AE) is the least sensitive parameter to pollution involved in SFG calculation (Honkoop et al., 2003), and therefore a constant AE was considered for all sites, which was measured before transplantation under the same standardized food conditions.

The energy consumption rate, as represented by the respiration rate (RR), was determined in sealed glass respirometers each containing a single clam with filtered sea water at 19ºC. Oxygen concentrations were measured using a YSI 52 DO instrument connected to a YSI 5905 self-stirring BOD probe. Physiological rates were standardised for a specimen of 1 g flesh dry weight using the allometric exponent b = 0.67, which relates the variation in physiological rates to animal size (weight) (Bayne and Newell, 1983). Physiological rates were converted to energy equivalents (J g^{-1} h^{-1}) in order to calculate their energy balance, using the energy equivalents referred to in Widdows and Johnson (1988). SFG was calculated by means of the energy balance expression: $I = F + R + P$ thence $P(SFG) = I - F - R = (I * AE) - R$, where I is the energy ingested, F the energy lost in faeces, AE the absorption efficiency, R the energy consumed in respiration and P the energy available for somatic and gonadal growth (SFG). Energy lost via excretion was not included in the above equation because it accounted for less than 5% of the acquired energy (Bayne and Newell, 1983).

2.4. Biometric measurements

After dissection the valves, gills, digestive gland and remaining tissues were wet weighed. Water content percentages for each organ were determined in the initial clams. Dry weights (dw) were calculated from these water percentages. The following anatomical indices were calculated: Condition Index (CI = soft body dw/total dw), Gill Index (GI= gill dw/total dw) and Hepato-somatic Index (HI = digestive gland dw/total dw).

2.5. Clam chemical analyses

PAHs (fluorene, phenanthrene, anthracene, benz[a]anthracene, fluoranthene, chrysene, benzo[b]fluoranthene, pyrene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, benzo[g,h,i]perylene, dibenzo[a,h]anthracene and benzo[e]pyrene) and organochlorinated compounds (OCs) (PCBs: CB28, CB52, CB101, CB105, CB118, CB138, CB153, CB156 and CB180; organochlorine pesticides (OCPs): op´- DDT, pp´-DDT, pp´- DDE, pp´-DDD, γ-hexachlorocyclohexane, α-hexachlorocyclohexane, hexachlorobenzene, trans-nonachlor, aldrin, dieldrin, endrin and isodrin) were extracted from clam samples using specific Soxhlet and purification procedures for each pollutant group, according to the analysis procedures proposed by Viñas et al. (2002) and by Fernández et al. (2010a), respectively. The final extracts were analyzed by HPLC with fluorescence detection for PAHs and by GC-ECD for OCs, using external calibration curves and 2-methyl-chrysene as the internal standard for PAHs (1-600 $\mu g\ L^{-1}$ for each compound) or CB155 for OCs (1-70 μg/L for each compound).

2.6. Neurotoxicity biomarker, AChE

The method of Bocquené and Galgani (1998) was used for the measurement of AChE activity in gills. Each sample was composed of gills of the two animals grouped previously for SFG determination. Tissues were homogeneized using a Potter-Elvehjem homogenizer in 1/2 w/v in 0.02 M phosphate buffer containing 0.1% triton X-100, pH 7. Extracts were then centrifuged at 10,000 g for 20 min and an aliquot of the supernatant was used in the assay. Acetylthiocoline was used as specific substrate. Absorbance at 412 nm was recorded for samples and blanks. AChE activity was expressed in nmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 13.6 mM^{-1} cm^{-1}.

2.7. Oxidative stress biomarker, CAT-GR-LPO

Each sample was composed of the digestive glands of the two specimens grouped previously in the SFG determination. Tissues were homogenised (1:4, w/v) in K-phosphate buffer 100 mM, pH 7.6 containing 0.15 M KCl, 1 mM DTT and 1 mM EDTA. After sequential centrifugations at 600 g for 15 min, 13,000 g for 20 min and 100,000 g for 60 min, the resulting microsomal pellet (microsomal fraction) was separated from the supernatant (cytosolic fraction) and resuspended in approximately 1 mL of microsomal buffer (50 mM Tris–HCl pH 7.6 containing 20 % glycerol, 1 mM DTT and 1 mM EDTA). Cytosolic fractions were used for enzyme determinations and microsomal fractions for LPO analysis.

CAT activity was measured at 240 nm ($\varepsilon = -0.04\ mM^{-1}\ cm^{-1}$) in an assay mixture that contained 50mM K-phosphate buffer pH 7.0 and 50 mM H_2O_2 (Claiborne, 1985).

GR activity was measured at 340 nm ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$) in an assay mixture that contained 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG and 0.06 mM NADPH (Ramos-Martínez et al., 1983).

LPO was quantified as thiobarbituric acid reactive substances (TBARS) at 535 nm estimating the aldehyde (malondialdehyde-MDA) formed using a standard of malonaldehyde bis-(dimethylacetal) (Buege and Aust, 1978).

2.8. Phase II Detoxification GST

GST activity was measured according to Habig et al. (1974) using chlorodinitrobenzene (CDNB) as substrate. The formation of S-2,4-dinitro phenyl glutathione conjugate was monitored following its absorbance at 340 nm (extinction coefficient, $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

In all cases, results were expressed in relation to the protein concentration of each subcellular fraction determined according to Lowry et al. (1951).

2.9. Statistical analyses

Results of biometric (weights, CI, GI, HI), SFG and biochemical (AchE, CAT, GR, LPO, GST) parameters were reported as mean ± S.D. The Shapiro–Wilk and Levene's tests were applied to test normal distribution and homogeneity of variance, respectively. The variation of each parameter between sites at each time of sampling was tested by one way analysis of variance (ANOVA). Pairwise comparisons (LSD test) were made to determine which values differ significantly when a significant overall ANOVA was found ($p < 0.05$). PCA analysis was performed to discriminate sampling sites according to biochemical and physiological responses (AchE, CAT, GR, LPO, GST and SFG), using the individual data obtained for these biomarkers in each organism after 22 days. Those biomarkers which discriminate between reference sites and El Albujón-impacted sites according to the PCA were integrated in the IBR (Integrated Biomarker Response) described in Beliaeff and Burgeot (2002) by means of star plots of the biomarker data. Star plots were used to represent the scores (standardized data) of the above-mentioned biomarkers for each site. IBR index is the star plot area. As this area depends on the position of each biomarker in the star plot, we consider the use of SS (sum of scores) instead of the sum of areas (IBR in Beliaeff and Burgeot) to reflect the biomarker responses more accurately. Nevertheless, both indices have been included in the results. These indices have been used in order to determine the overall biological effect of the El Albujón watercourse on the transplanted clams.

Statistical analyses were performed with the statistical package SPSS 11.0 and the significance level was set at $\alpha = 0.05$.

3. Results

3.1. Chemical characterization of water and clams

3.1.1. Surface and seawater samples

Mean values of the hydrological parameters measured during the first 8 days in the deployed sites are shown in Table 1. The environmental parameters show a marked similarity between all four sites where the cages were moored: 41 psu, 9 mg $O_2 \cdot L^{-1}$ and 17 ºC.

The mean, maximum, minimum and median concentrations of detected organic pollutants in El Albujón watercourse mouth for the first eight days of clam exposure are shown in Table 2. Higher mean concentrations were detected for chlorpyrifos (1,829 ng L^{-1}), naphthalene (415 ng L^{-1}) and pendimethalin (499 ng L^{-1}), showing maximum concentrations higher than 2,000 ng L^{-1} in the three pollutants. Significant daily variations in concentration were observed for all analytes, with only chlorpyrifos, flutolanil and pendimethalin being found in all samples.

The organic pollutant concentrations detected in the surrounding seawater of the cages (1-4) immersed in the Mar Menor lagoon are shown in Table 3. Between 27-32 pollutants were detected in the 4 sampling areas, including PAHs, triazines and organophosphorus pesticides (OPs), amongst others. More than 8 compounds displayed maximum concentrations higher than 20 ng L^{-1} in S2, S3 and S4, but only 3 in S1. Mean concentrations of PAHs and triazines were similar between all four areas, although important differences were observed for the OPs detected. In this case, chlorpyrifos and chlorpyrifos-methyl were the most commonly found. Mean concentrations of chlorpyriphos in S3 and S4 (52.1 and 26.6 ng L^{-1}, respectively) were higher than in sampling areas S1 and S2 (7.5 and 10.2 ng L^{-1}, respectively). A wide range of chlorpyrifos concentrations was detected in S3 and S4, 3.0-199.3 and 1.5-86.5 ng L^{-1}, respectively. Chlorpyrifos-methyl concentrations were also higher in S3 and S4 (13.3 and 10.1 ng L^{-1}, respectively) than in the other two areas, but with values significantly lower than those of chlorpyrifos. Chlorpyrifos-methyl concentrations also displayed a substantial degree of daily variation, reaching maximum values in excess of 28 ng L^{-1} in S2, S3 and S4. In this regard, it is worth noting that the mean concentration of these OPs is between 30-50% higher in S2 than in S1, but also that maximum values recorded for S3 and S4 were 100-600% higher than in the latter. Pendimethalin concentrations were also higher in S3 and S4 than in S1 and S2, reaching maximum levels of 21.9 and 33.5 ng L^{-1}, respectively. Similar concentrations were also detected for propyzamide, tributhylphosphate and chlortal dimethyl in all 4 sites.

With regard to triazines, terbuthylazine-desethyl, terbuthylazine and propazine were detected in the majority of water samples from all sites, with maximum concentrations ranging between 16.5-28.1, 7.0-12.5 and 8.2-12.4 ng L^{-1}, respectively. The remaining triazines (simazine, atraton, atrazine, prometryn, prometon) were only detected in a small percentage of water samples.

3.1.2 Clam samples

Exposure to PAHs, PCBs, pp'DDT and its metabolites was studied by analysing their concentrations in the soft tissues of the transplanted clams (see Table 4). PCB and pp' DDE levels in transplanted clams from sites S3 and S4 increased lineally over time, which was not the case in clams taken from sites S1 and S2. Maximum concentrations were reached in S4, where after 22 days the concentration of the sum of the 7 PCBs analysed and of p,p'DDE were 0.52 and 1.29 ng g^{-1}, respectively. Levels of pp' DDD y pp' DDT were always lower than the detection limits. In the case of the bioaccumulation of PAHs, levels increased from the initial value of 1.57 ng g^{-1} recorded for all four sites, the highest concentrations being found in site S1, where PAH levels doubled over the 22 day immersion period.

The concentration of the rest of the chemical pollutants analyzed in the clam tissues, not included in Table 4 (aldrin, dieldrin, isodrin, endrin, lindane, a-hexachlorocyclohexane, lindane and hexachlorobenzene), were below the detection limit.

3.2. Biochemical analyses

Biochemical biomarker results, together with those for ANOVA and post hoc analysis, are shown in Table 5. After 7 days of exposure significant differences were recorded for all oxidative stress markers, depending on the site from which the clams were taken (ANOVA, $p < 0.05$). The only biochemical response that showed no significant differences (ANOVA, $p > 0.05$) between sites at the end of the first week was AChE.

The effect of exposure on AChE activity was observed after 22 days in sites S3 and S4, where levels were lower than in sites S1 and S2 ($p < 0.05$). In addition, AchE levels showed a slight but significant relationship with two parameters which defined the filtration capacity of the clams, such as the clearance rate CR ($r = 0.4375$, $p < 0.01$, $n = 40$) and the clearance efficiency CE ($r = 0.5590$, $p < 0.001$, $n = 40$).

The lowest CAT levels recorded after 7 days were in clams from S4, although they were only significantly lower than in those from S3. After 22 days, however, activity levels at sites S2, S3 and S4 were significantly lower than at S1 ($p < 0.05$). Furthermore, CAT levels at S3 were significantly lower than those found at S2 and S4.

The results for GR, an enzyme related with the elimination of ROS and the cellular metabolism of glutathione, showed a very different pattern, with a high degree of variability. After 7 days activity levels were broadly similar, with no significant differences between sites S1, S2 and S4, although those from S3 were significantly higher than those from S1 and S4. After 22 days, however, mean GR levels detected in clams from sites S3 and S4 were significantly higher than those recorded for sites S1 and S2 ($p < 0.05$).

LPO levels were significantly higher in clams from S2, S3 and S4 than in those from S1 after 7 days' exposure. At the end of the 22- day period, however, although LPO levels remained higher in the three sites located in the central area of the lagoon than in the northernmost one, this difference was only significant in the cases of S2 and S3.

With regard to GST levels, during the first exposure period the only differences compared to those recorded at S1 were found at S3, whilst after 22 days the mean values for S3 and S4 were higher than those for S1 and S2, although only statistically significant in the case of S4.

3.3. Biometric clam measurements

The condition index (Table 6) of transplanted animals showed no statistical differences between sites after the first 7 days. After 22 days, however, CI levels recorded in clams from sites S2, S3 and S4 were significantly higher than those from site S1. In addition to these quantitative differences in CI, slight qualititative differences were also found between the relative weights of organs at the end of the same period. Thus, the GI of clams from S1 was higher than that of specimens from S2 and S3 ($p < 0.05$), whilst the relative weight of the remaining portion, RI, was significantly lower in animals from S1 as compared to those from S4. In the case of the digestive gland, HI did not show any significant difference between sites.

3.4. Physiological clam measurements

Generally speaking, the physiological rates determined under standardised laboratory conditions were similar ($p > 0.05$) between sampling sites at 7 days after transplantation. After 22 days, however, significant differences became apparent (see Table 7).

Dual standardisation (weight and size) was carried out for the purpose of analysing clearance rates, the standards being taken as 1 g flesh dry weight and 50 mm, respectively. Individual, or size-standardised clearance rates were significantly lower in clams from S4 than in those from the other three sites. When clearance rates were expressed by unit of weight, they were not only lower in clams from S4, but also in those from S3. In the case of clams from S2, CR was slightly lower than in those from S1, but the differences were not significant.

When the efficiency of the clearance process (CE), i.e. clearance rate per unit of gill weight, was analysed, it was found to be significantly higher in clams from S1 and S2 than in those from S3 and S4.

In contrast to clearance rates, in the case of respiration rates no significant differences ($p < 0.05$) were found between sites, indicating that the consumed energy fraction in the energy balance equation was similar at all 4 sites.

SFG estimations mirrored those described above for CR, namely a significantly lower SFG value for sites S3 and S4 (12.10 y 8.76 J ind^{-1} h^{-1}, respectively) as compared to that for site S1. This was particularly true for the clams from S4, which showed a 40% decrease in SFG in comparison with those from S1 (14.8 J ind^{-1} h^{-1}). Furthermore, the 25% negative difference in SFG values between S4 and S3 is also significant, ($p<0.05$). Finally, differences in the value of this biomarker were also found between clams from sites S2 (12.68 J ind^{-1} h^{-1}) and S1, although in this case they were not significant.

3.5. Principal component analysis

PCA performed on biomarker data extracted two main factors which explained 63.7% of the total variance. Factor 1 (39.95% of total variance) is characterized by high loadings of AchE and SFG (0.87 and 0.57, respectively) and low loadings of GR and GST (-0.79 and -0.61, respectively); Factor 2 (24.96 % of total variance) by high loadings of CAT (0.76) and low loadings of LPO (-0.76).

Positions assigned in the PCA to each analyzed organism along the two main factor axes appear in Figure 2 (Factor 2 vs Factor 1). Clams from S1 and S2 were clearly differentiated from the organisms collected from S3 and S4 by their location on the positive side of Factor 1, as a result of their high levels of AchE and SFG, and low levels of GR and GST activity. On the contrary, S3 and S4 were located on the negative side of Factor 1 due to their low AchE and SFG and high levels of GR and GST. These biomarkers thus make it possible to discriminate the effects of the input of pesticides from the watercourse on the clams deployed at S3 and S4. A second group consisting of most of the clams from S1 and S4 is located in the positive section of Factor 2, an axis which was characterized by high CAT and low LPO loadings. In contrast, the majority of clams from S2 and those collected from the nearby S3 are to be found in the negative section of Factor 2.

Figure 3 shows the IBR values (and their corresponding sums of scores) calculated from the 4 biomarkers selected on the basis of PCA (AchE, SFG, GR, GST), which make it possible to differentiate between the sites affected by the outflow from the El Albujón watercourse (S3 and S4) from those that were not (S1 and S2). The highest IBR values correspond to sites S3 (IBR=5.60) and S4 (IBR=13.15), which are the most heavily affected by the El Albujón outflow. It is also worth noting that this index is considerably higher for S4 than for S3.

4. Discussion

The clam *Ruditapes decussatus* has been proposed as a bioindicator species of chemical pollution and the measure of the biomarkers in their tissues as a promising approach to monitor the effects of contaminants in the marine environment (Bebianno et al., 2004). In this field study we have used a multi-biomarker approach for environmental risk assessment in clams transplanted at sites of a coastal lagoon influenced by the input of a wide variety of pollutants derived from agricultural activities.

4.1. Surface watercourse chemical inputs

In previous studies carried out in El Albujón watercourse (Moreno-González et al., 2013a) more pesticides were detected in autumn than in other seasons, and for this reason autumn was the season chosen to evaluate the impact of El Albujón watercourse inputs on clam biology. A daily characterization of water pollutant levels was performed in our study, showing a significant range of variation in all cases (Table 2). A continuous input of PAHs and pesticides through El Albujón watercourse to Mar Menor lagoon was detected, with the greatest input flux corresponding to the organophosphate chlorpyrifos, followed by pendimethalin and naphthalene, and at lower levels acenaphthene, terbuthylazine-desethyl and chlorpyrifos-methyl. These pollutants were also previously detected in 2009 in a spot sampling performed in this watercourse in autumn (Moreno-González et al., 2013a). The concentrations detected were of the same order of magnitude in the majority of analytes in both studies, except for PAHs, chlorpyrifos, chlorpyrifos-methyl and pendimethalin, which showed significantly higher concentrations and fluxes in the present study, and for terbumeton and myclobutanil, which were detected at higher levels in 2009 (Moreno-González et al., 2013a). As an example, chlorpyrifos and pendimethalin inputs were 290.05 g week^{-1} and 110.07 g week^{-1} respectively in this study, being significantly higher than those obtained in 2009 (13.31 and 17.35 g week^{-1}, Moreno-González et al., 2013a). However in other cases lower levels of contaminants were recorded, for example terbuthylazine-desethyl, with a flux of 12.44 g week^{-1} as compared to the 58.61 g week^{-1} referred to in the same study, for autumn 2009. To sum up, both studies reveal the enormous variability in inputs of this nature, with large variations being recorded on a daily or weekly basis. These points to the need for intensive sampling, such as that performed in this study (various times a day over a period of several days) or the one referred to above (various weeks at different times of the year, especially during flash flood events).

The concentrations of PAHs and pesticides in surface waters from El Albujón watercourse were lower than Environmental Quality Standards (EQS) (Directive 2008/105/EC), except in the case of chlorpyrifos. Both mean (1,829 ng L^{-1}) and maximum (23,017 ng L^{-1}) concentrations detected for this insecticide were significantly higher than the EQSs established for annual average and maximum allowable concentration for inland surface waters: 30 y 100 ng L^{-1}, respectively. Consequently, high spot concentrations of organic pollutants in this watercourse may be affecting biota in the area. In fact juvenile fish (mullet and sand smelt) mortality was detected twice at El Albujón watercourse mouth during the study period, but specific studies are required to evaluate the origin of these events.

As well as the compounds referred to above, a large number of pesticides were detected at lower levels (see Table 2), which in spite of their low levels of concentration may make a significant contribution to the toxicity of the waters flowing from the El Albujón watercourse into the Mar Menor lagoon. Furthermore, the intense analytical effort made in this study notwithstanding, there are many other compounds used in agriculture, industry or the home, such as carbamates, surfactants, pharmaceuticals or phthalates, amongst others, which may also be affecting the ecosystem. This is the reason why in this study of the biological effects of pollutants from the El Albujón watercourse it was decided to use general biomarkers with the ability to reflect the toxicity of the complex mixtures of contaminants present in the marine environment.

In general terms, the majority of pesticides flowing through El Albujón watercourse were also detected in seawater samples at the different sampling sites. Pesticide concentrations were highest at sampling sites S3 and S4 due to the direct influence of inputs from the watercourse on these sites. Indeed, the most prevalent compounds entering the lagoon through the El Albujón watercourse, such as chlorpyrifos and pendimethalin, were those that registered the highest mean and absolute concentrations at both sites (see Table 3). Chlorpyrifos concentrations were markedly higher than those described in the literature for coastal ecosystems close to areas of intensive agricultural activity, such as Chesapeake Bay (McConell et al., 1997). The maximum concentration recorded at S3, 199.3 ng L^{-1}, is 100 times higher than the maximum values described in the aforementioned study, and is also higher than the maximum allowable concentration proposed by the EU Directive on Environmental Quality Standards (Directive 2008/105/EC) in other surface waters.

Pesticide concentrations at S2, despite the site's proximity to the El Albujón watercourse mouth, are noticeably lower than at S3 and S4, because it is not affected by this input as a consequence of the prevailing current in that area (according to the general circulatory pattern proposed by Pérez-Ruzafa et al., 2005). This current flows southwards and mingles with the input from the watercourse, directly affecting sites S3 and S4 (see Fig. 1).

In the case of p,p' DDE and PCBs, their concentrations in the tissues of transplanted clams from sites S3 and S4 doubled initial values at the end of the experimental period, indicating that although these pollutants were not detected in the samples of water from the watercourse in this study, there is an input via this route into the lagoon, as has been revealed in the case of other bivalves (León et al., 2013). However, the bioaccumulation of PCBs and DDE in the transplanted clams was low in comparison with that reported in bivalves from other coastal areas (Carro et al., 2010; Fernández et al., 2010a). In the case of PAHs, their concentrations in water were similar in all four sites (Table 3), and bioaccumulation in clams was only detected in S1 and S2, but not in S3 and S4 (Table 4). Consequently, PAHs should not be responsible for the biological alterations observed in these sites, especially in view of their low concentrations found in clams.

4.2. Biochemical responses

It is always very difficult to ascertain the effects of pollution on animal health from the body burden or concentration in water alone. AChE inhibition has been widely used as a biomarker of the neurotoxic effects of organophosphate and carbamate pesticides (Fulton and Key, 2001). In this sense the results of this study support the use of this biomarker in transplanted clams to evaluate their exposure to organophosphates, since after 22 days these organisms showed a significant degree of AChE inhibition at sites S3 and S4, where numerous pesticides were detected in the water, of which the most prevalent was chlorpyrifos, with concentrations ranging from 1.5 to 199.3 ng L^{-1} (Table 3). These results are in accordance with previous studies carried out in clams *R. decussatus* and mussels *M. galloprovincialis*, which also demonstrated an inhibition of AChE in areas characterized by intensive agricultural activity where pesticides and biocides were frequently used (Dellai et al., 2011; Escartín and Porte, 1997). However, AChE activity in aquatic organisms may also be inhibited by other contaminants including heavy metals, PAHs, hydrocarbons, surfactants, phytotoxin and other industrial pollutants (Bebianno et al., 2004; Choi et al., 2011). More recently, Matozzo et al. (2012) have shown, in the clam *R. philippinarum*, the capacity of triclosan, a chlorinated byphenyl ether widely used as antimicrobial and antifungal agent in soaps, shampoos and cosmetics, etc., to significantly reduce AChE gill activity at environmentally realistic concentrations. According to all the above-mentioned studies, AchE inhibition has been suggested as indicative of general stress.

The study of the relationship between AchE and higher-level biomarkers in bivalves, such as feeding rate, is indispensable if we wish to translate the inhibition of AchE activity induced by pollutants into an ecological perspective, as suggested by other authors (Yaqin et al., 2011). In marine invertebrates ciliary movement of the gills is controlled by acetylcholine, dopamine and serotonine (Cooper and Bidwell, 2006; Yaqin et al., 2011). Bivalves use their gills not only as a respiratory apparatus but also as a filter feeder organ, and cilia are responsible for moving water (and particulate matter). Therefore OP and carbamate pesticides inhibit cholinesterase activity which may lead to severe physiological impairment of marine animals such as a reduction in the feeding efficiency of marine mussels (Yaqin et al., 2011). The significant correlations found in this study between AChE levels and the clearance rate after 22 days ($r=0.4375$, $p<0.01$, $N=40$), and thus with SFG levels ($r=0.4130$, $p<0.01$, $N=40$), may indicate that lower levels of CR are a consequence of the suppressed AchE activity. Moreover, a higher correlation coefficient was observed when clearance rate is expressed by gill size, in other words what now refer to as clearance efficiency CE ($r= 0.5590$, $p<0.001$, $N=40$). In fact the PCA analysis performed on the biochemical and physiological measurements after 22 days grouped the clams from S3 and S4, which were characterized by having the lowest AchE and SFG levels and the highest GR and GST levels (Fig. 2). This relationship is an important consideration for the application of AChE as a biomarker of ecological risk assessment in the coastal zone. In this sense, effects on ecologically-relevant parameters such as survival, growth or behavior have been studied and related in marine organisms to the reductions of cholinesterase enzymes (Cooper and Bidwell, 2006). For example, Kumar and Champman (1998) determined that the chronic exposure of the eastern rainbow fish (*Melanotaenia duboulayi*) to sublethal levels of profenofos resulted in a 70 % reduction in AChE activity with associated decreases in growth rates, food consumption rates and food conversion efficiency.

Within the antioxidant system, CAT normally acts on the H_2O_2 produced in the reduction of $O_2^{\cdot-}$; this enzyme efficiently prevents lipid peroxidation by neutralizing oxyradicals. Nevertheless, when this oxidative stress increases, an inhibition of CAT activity has been found (Regoli and Principato, 1995). Several studies conducted with transplanted mussels have demonstrated a decrease in CAT activities at polluted sites in addition to a reduced capability for neutralizing ROS and an increased susceptibility to oxidative stress (Pampanin et al., 2005; Tsangaris et al., 2010). In the present study the clams deployed in the central basin (S2, S3 and S4) showed significantly lower levels of CAT than those from S1 after 22 days (Table 5). A deficiency in these defence mechanisms indicates a toxic effect of ROS, and the organisms become more sensitive to oxidative stress (Bebianno et al., 2004). Thus, low levels of CAT activity in clams transplanted at these sites were linked with higher LPO levels at S2, S3 and S4 than at S1, although the LPO differences were only statistically significant for S2 and S3. CAT levels in clams sampled at this time correlated significantly with LPO levels ($r= -0.429$, $p<0.006$, $N=40$). Therefore the organisms deployed at these sites within the central basin could be exposed to pollutants which decrease CAT activity and induce oxidative stress, in contrast to the clams from S1. It is known that environmental pollutants could produce an inhibition of CAT by different mechanisms, for example by the presence of reactive oxygen species $O_2^{\cdot-}$. When the antioxidant system works correctly, superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. But, when the quantity of $O_2^{\cdot-}$ is too great, they become catalase inhibitors (Geret et al., 2002; Schreck et al., 2008). Indeed, analysis of organic contaminants in water samples collected simultaneously from the same sites detected a compound such as 4-nonylphenol, a detergent present in urban waste water, mainly in samples of water from site S2 (where it ranged from 100 to 250 ng L^{-1}) (V. León, unpublished results), which induces a significant inhibition of SOD in exposed clams (Matozzo et al., 2004). The same is true of phthalates, capable of inhibiting SOD (Orbea et al., 2002), which were detected in water samples analysed in this study, but not quantified. Other compounds could inhibit this enzyme directly, one example being the non-selective herbicide aminotriazole, not analyzed in this work but broadly used, which inhibits catalase via the binding of iron atoms in its active site (Lushchak et al., 2011).

OP toxicity in clams implies more than AChE inhibition; for example, chlorpyrifos and fenthion induce in vitro and in vivo generation of ROS, such as H_2O_2, superoxide ($O_2^{\cdot-}$) and the hydroxyl radical ($HO^{\cdot}$) and also increase lipid peroxidation (Bagchi et al., 1995). The metabolism of OPs is connected with glutathione consumption and this may trigger oxidative stress. Peña-Llopis et al. (2002) demonstrated that two marine bivalves exposed to fenitrothion showed depletion of reduced and oxidized glutathione in the digestive gland, gills and muscle. In the presence of oxidative stress, GSH oxidizes to GSSG by glutathione peroxidase or non-ezymatically to remove ROS and hydroperoxides. This GSSG is then reduced to GSH by GR at the expense of oxidizing NADPH. GR enzyme is therefore essential for the maintenance of the GSH/GSSG ratio and the cellular redox status, protecting cells against oxidative damage (Fernández et al., 2010a). In this study the high levels of GR detected in clams from sites S3 and S4 after 22 days, as well as the inclusion of GR levels in Factor 1 of the PCA (together with AchE, GST and SFG) may reflect an increase in the capacity for recycling GSH and protecting against pesticide toxicity in these clams affected by agricultural pesticides levels capable of reducing AchE. In addition, this may indicate a coordinated enzymatic regulation with GST enzymes to restore the GSH consumed by these enzymes.

The GST isoforms are involved in the metabolism of OP and OCPs and have been used as biomarkers of these substances in molluscs (Hoarau et al., 2004). However, GST can also act as a non-SeGPs and can therefore be regarded as playing an antioxidant role (Prohaska, 1980). High levels of GST could thus be regarded as an activation of the second phase of detoxification processes of the digestive gland or antioxidant action against chemical pollutants. Previous studies in clams *R. decussatus* showed that this activity increased significantly in gills after exposure to organochlorine compounds whereas it remained unchanged in hepatopancreas (Hoarau et al., 2004). In field studies with clams (*R. philippinarum*) transplanted to a gradient of chemically polluted sites in Hong Kong, CAT and GST in hepatopancreas correlated significantly with OCPs and PCBs (De Luca-Abbott et al., 2005). However, very few studies have investigated the effects of OP on these enzymes in aquatic organisms (Kristoff et al., 2008). In the present study, after 22 days of *in situ* exposure, the levels of GST in clams showed a moderate and significant increase at S3 and S4, respectively. In addition, GST showed a significant negative correlation with AChE ($r = -0.418$, $p < 0.007$, $N = 40$). These results suggest that the increase in GST in clams may be attributed to their exposure to pesticides. This is in accordance with previous studies, which have shown that OP pesticides induce this activity in selected tissues of certain fish species, examples being the two-fold increase in GST activities in kidneys of *Cyprinus carpio* and *Oreochromis* *nilocitus* (Ozcan Oruc et al., 2004) or in the oligochaete *Lumbriculus variegatus* and the gastropod *Biomphalaria glabrata* (Kristoff et al., 2008).

4.3. Physiological response

It is generally believed that biochemical changes due to pollution occur more quickly than those at a higher biological level, e.g. physiological responses (Wu et al., 2005). This would appear to be the case in this study, where no differences in physiological rates were observed after the first 7 days of exposure whilst biochemical markers began so show some effects after the same period. After 22 days of exposure, however, physiological indicators displayed a similar result to that obtained from biochemical markers, namely a quantifiable impact of the presence of contaminants on transplanted clams from sites S3 and S4. The exposure period used in this study, almost one month, seems to be of sufficient length to observe the effect of pollutants coming from the El Albujón watercourse on the physiological parameters of the clams analysed. Extending this exposure time to a period of 1 to 6 months, for example, would not lead to any increase in the physiological response to the presence of contaminants, according to Tsangaris et al. (2007).

SFG values display a negative gradient associated with proximity to the mouth of the watercourse: S1>S2>S3>S4. Although SFG in clams from site S2 was lower than that of clams from S1, the differences were not significant. Just as was observed in the case of biochemical markers, the effect of pollutants on SFG is more pronounced in the mooring sites located south of the watercourse mouth (S3 and S4) than in S2, to the north of the same watercourse. This would be a consequence of the movement of water within the Mar Menor lagoon, which circulates from the north to the south, carrying with it the input from the watercourse towards sites S3 and S4. The greater impact observed on the energy balance of clams from S4 as compared to those from S3 may be related to the greater availability of particulate matter at the former, where recorded TPM values were twice as high as those for the latter (unpublished data). The integration of the 4 biomarkers selected by PCA into the IBR also reveals a greater impact on the transplanted clams from S4 than on those from S3. An increase in pollutant toxicity as a result of the addition of particulate matter (i.e. food) has previously been described in laboratory studies (Björk and Gilek, 1996; Okay et al. 2000, 2006). This greater toxicity could be associated with the increase in the clearance rate that occurs when there is an increase in the concentration of food in the environment, within a range of low concentrations (Riisgard and Randlov, 1981). A higher clearance rate implies a greater exposure to the toxins present in the water. An alternative explanation for the greater toxicity of hydrophobic organic compounds, such as those detected in the input from the El Albujón watercourse, would be their greater bioavailability when particulate matter, to which they can adhere, is present in the environment (Chu et al., 2000, 2003). A third explanation for the greater impact on clams from S4 than on those from S3 could be related to the higher concentrations of dissolved contaminants in the water column at the latter site (Table 3), which may have led to a reduction in the clearance rate or even the temporary closure of the animals’ valves during the experimental period, the consequence being a reduction in the impact of this increased presence of pollutants on the general physiological condition of the clams (Cooper & Bidwell, 2006). This third possibility could have occurred at certain peak moments during the continuous flow of contaminants into the lagoon, when maximum values of up to 200 ng L^{-1} (4 times higher than the mean) were recorded for chlorpyrifos, for example,at S3 (Table 3). However, in all probability the greater impact of the presence of contaminants on the condition of clams from S4 as compared to those from S3 is not solely due to one of the above-mentioned factors, but rather to a combination of them all.

A further point to note is that the SFG of clams from site S2, although not significantly different from that of clams from site S1, is nevertheless almost 15% lower. Biochemical biomarkers such as LPO also reveal an impact on clams from S2, which may be related to the existence of other compounds not analysed in this study but used in agricultural activities in the area (see previous paragraph). In this context it should be pointed out that El Albujón watercourse receives wastes not only from agricultural areas but also from an urban wastewater treatment plant, which may lead to the presence of pollutants other than those analysed in this study that could have an effect on the levels of the biomarkers determined in clams, such as surfactants, pharmaceuticals, phthalates and/or personal care products.

The physiological rate with the greatest influence on the energy balance equation, and thus on the estimation of SFG, is the clearance rate, which determines the energy gain under the temperature and feeding conditions used in this experiment. Furthermore, the clearance rate has been shown to be the most sensitive of all physiological rates to the presence of pollutants (Honkoop et al., 2003; Kraak et al., 1997; Widdows et al. 1982), as a result of which its use has been proposed as an alternative to SFG as a physiological biomarker (Toro et al. 2003b). In the present study involving transplanted clams SFG values obtained from the different sampling sites were shown to reflect the CR values obtained from the same specimens (Table 7). According to Filgueira et al. (2008) and Iglesias et al. (1996) clearance rates should be standardised by length rather than by weight when differences in condition index (CI) are observed. Albentosa et al. (2012), in an extensive study of the use of SFG in mussels as an indicator of the presence of pollutants, have observed that the high degree of variability in the CI of the mussels sampled conditions the SFG values obtained under standardised laboratory conditions as a result of the customary standardisation of these parameters by weight. In the case of the study reported here CI was higher in clams from sites S2, S3 and S4 than in those from S1, a finding that would be related to the greater availability of food at the former (unpublished data). CR was thus standardised by length as well as by weight, and the length-standardised CR of clams from site S4 was significantly lower than that of clams from any of the other three mooring sites, between which no significant differences were observed. Standardisation by length is based on the fact that the clearance rate is proportional to gill area (Jones et al., 1992), which is more a function of the specimen's length than of its weight (Iglesias et al., 1996). However, studies of oysters have revealed a direct relation between gill area and gill weight (Honkoop et al., 2003), meaning that both of these metrics can be indicative of gill size. Given this premise, it is our opinion that clearance rates should be standardised by gill weight instead of by total weight. Thus, if we consider clearance rate by gill unit (CE) we see that the clearance rates of clams from both S3 and S4 are significantly lower than that of the reference clams.

Respiration rates, unlike clearance rates, were not affected by the pollutants in the input from the El Albujón watercourse. The relation between this physiological parameter and the presence of toxic compounds is by no means clear. In some cases it has been shown that oxygen consumption increases in the presence of exposure to toxins as a result of the increased energy consumption deriving from detoxification processes (Sobral and Widdows, 1997; Widdows and Donkin, 1991; Widdows and Page, 1993). Other studies, however, demonstrate a reduction in oxygen demand associated with the decrease in the feeding rate that normally ensues from the presence of toxic compounds (Naimo et al., 1992). Whichever the case, energy acquisition processes, and fundamentally the ingestion rate, have a greater impact on the estimation of SFG than consumption processes such as the respiratory rate, and are also more pollution-sensitive (Widdows and Johnson, 1988).

Conclusion

The results of this study reveal the considerable input of a great variety of pesticides used in the nearby agricultural area through El Albujón watercourse to the Mar Menor lagoon. The application of a substantial set of biochemical and physiological biomarkers in clams transplanted in the lagoon has made it possible to characterize the environmental hazards of these effluents after their dilution in the sea water in the lagoon. In this regard, PCA analysis summarised the results and demonstrated that the clams deployed around the mouth of the watercourse, although exposed to concentrations of pesticides dissolved in water lower than those specified by EQS (Directive 2008/105/EC) for surface water (except for chlorpyrifos), nevertheless showed a high degree of stress, with reduced levels of AchE, SFG, CE and increased levels of GR and phase II GST activity. Chlorpyrifos and other pollutants with lower concentrations detected in this work, together with other pollutants present in the environment but not analysed in this study, may be acting simultaneously, provoking a significant impact on clams. In addition the results show that active biomonitoring, using clams as an indicator organism, could be a useful strategy for assessing the impact on biota in the Mar Menor of the chemicals used in the nearby Campo de Cartagena, characterized by intensive agricultural practices.

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Table1

Table 1. Hydrological parameters (S, salinity, DO, dissolved oxygen and T, temperature) of Mar Menor seawater during the first 8 days in the four transplantation sites and the wadi mouth.

Site	Parameters		
	S psu	DO mg L^{-1}	T ºC
S1	40.96±0.40	9.13±1.07	17.5±1.1
S2	41.61±0.25	8.70±0.73	17.7±1.0
S3	41.26±0.65	9.58±1.82	17.7±0.6
S4	41.01±0.88	9.65±1.54	17.2±0.9
Wadi mouth	10.48±0.29	9.51±1.57	18.7±1.1

Table2

Table 2. Organic pollutant concentrations (ng L^{-1}) and fluxes (g $week^{-1}$) found in surface water samples from El Albujón watercourse mouth for eight days in the autumn of 2010.

	Concentration (ng·L^{-1})					**N**	**Flux**
	Mean	**S.D.**	**Minimum**	**Maximum**	**Median**		(g $week^{-1}$)
p,p-DDE	n.d.	-	n.d.	0.9	n.d.	17	-
Dieldrin	n.d.	-	n.d.	11.1	n.d.	17	-
Endrin	n.d.	-	n.d.	2.9	n.d.	17	-
pp DDD	n.d.	-	n.d.	3.0	n.d.	17	-
Naphthalene	415.4	831.3	n.d.	2633.8	75.3	17	96.61
Acenaphthylene	15.2	36.6	n.d.	138.4	n.d.	17	4.03
Acenaphthene	51.1	107.2	n.d.	437.3	18.6	17	13.44
Fluorene	12.6	23.8	n.d.	73.6	1.3	17	3.22
Anthracene	3.5	6.6	n.d.	18.5	n.d.	17	0.75
Fluoranthene	0.7	0.9	n.d.	2.7	0.3	17	0.18
Pyrene	0.8	0.8	n.d.	2.6	0.6	17	0.18
Simazine	n.d.	-	n.d.	12.5	n.d.	17	-
Propazine	2.9	7.6	n.d.	28.2	n.d.	17	0.19
Atrazine	b.q.l.	-	n.d.	7.4	1.5	17	0.32
Terbutryn	1.5	1.0	n.d.	4.3	b.q.l.	17	0.26
Prometon	n.d.	-	n.d.	8.2	n.d.	17	-
Terbuthylazine	11.2	8.0	n.d.	25.5	9.6	17	2.31
Terbumeton	4.8	5.5	n.d.	17.6	4.9	17	0.73
Chlorpyrifos	1828.7	5485.7	18.9	23017.1	404.7	17	290.05
Fenthion	n.d.	-	n.d.	23.4	n.d.	17	-
Chlorpyrifos-methyl	45.7	163.4	n.d.	679.2	2.7	17	6.56
Terbuthylazine-desethyl	53.2	53.8	n.d.	210.0	37.0	17	12.44
Flutolanil	16.8	10.2	4.0	40.7	15.5	17	3.18
Tributhylphosphate	4.9	10.4	n.d.	32.3	n.d.	17	0.81
Propyzamide	9.0	12.9	n.d.	56.0	5.7	17	2.00
Pendimethalin	499.4	610.4	7.0	2201.1	312.2	17	110.07
Boscalid (nicobifen)	b.q.l.	16.4	n.d.	67.8	n.d.	17	0.63
Myclobutanil	n.d.	-	n.d.	b.q.l.	n.d.	17	0.63
Oxyfluorfen	n.d.	-	n.d.	b.q.l.	n.d.	17	0.14
Chlorthal-dimethyl	1.2	0.9	n.d.	3.5	1.1	17	0.06
Cyprodinil	b.q.l.	-	n.d.	3.5	b.q.l.	17	0.22

n.d.: not detected

b.q.l.: below the quantification limit

Table3

Table 3. Organic pollutant concentrations (ng L^{-1}) found in the water samples collected in the four clam sites deployed for eight days in the autumn of 2010. Two samples were collected every day at different times (N 16).

	S1			S2			S3			S4		
	Mean±S.D.	**Minimum**	**Maximum**	**Mean±S.D.**	**Minimum**	**Maximum**	**Mean±S.D.**	**Minimum**	**Maximum**	**Mean±S.D.**	**Minimum**	**Maximum**
CB 28	n.d.	n.d.	n.d.	n.d.	n.d.	1.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CB 52	n.d.	n.d.	n.d.	0.4±1.7	n.d.	6.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
p,p-DDE	n.d.	n.d.	0.4	n.d.	n.d.	0.4	n.d.	n.d.	0.4	n.d.	n.d.	0.3
Endrin-aldehyde	n.d.	n.d.	n.d.	2.8±9.1	n.d.	36.2	2.1±8.5	n.d.	35.0	n.d.	n.d.	3.5
Naphthalene	18.6±10.3	b.q.l.	36.0	23.9±30.6	b.q.l.	110.7	19.4±15.4	b.q.l.	64.1	16.4±19.6	b.q.l.	87.1
Acenaphthylene	2.2±2.8	n.d.	9.6	1.5±2.0	n.d.	7.4	1.3±1.8	n.d.	5.6	0.7±1.4	n.d.	5.3
Acenaphthene	0.3±0.8	n.d.	3.1	1.4±3.7	n.d.	12.7	0.5±1.7	n.d.	7.2	1.3±3.3	n.d.	11.2
Fluorene	2.5±1.7	0.7	7.9	3.7±6.2	n.d.	23.2	2.7±2.7	0.6	12.3	3.1±6.2	0.6	26.1
Phenanthrene	4.6±2.9	1.5	10.3	3.1±1.7	0.8	6.4	3.6±1.9	1.1	8.9	2.7±1.7	1.0	7.5
Fluoranthene	1.3±0.8	0.3	2.7	0.6±0.3	n.d.	1.2	0.6±0.4	n.d.	1.4	0.6±0.4	0.2	1.4
Pyrene	1.2±0.9	0.3	3.4	0.5±0.3	n.d.	1.0	0.6±0.4	n.d.	1.4	0.4±0.2	0.3	0.7
Chrysene	n.d.	n.d.	1.0	n.d.	n.d.	0.8	n.d.	n.d.	0.8	n.d.	n.d.	0.6
Benzo(e)pyrene	0.3±0.3	n.d.	1.2	0.3±0.3	n.d.	1.2	0.2±0.2	n.d.	0.8	0.2±0.2	n.d.	0.6
Benzo(b)fluoranthene	n.d.	n.d.	0.6	n.d.	n.d.	0.6	n.d.	n.d.	0.3	n.d.	n.d.	0.5
Benzo(k)fluoranthene	n.d.	n.d.	0.4	n.d.	n.d.	0.8	n.d.	n.d.	0.5	n.d.	n.d.	0.6
Benzo(a)pyrene	n.d.	n.d.	0.4	n.d.	n.d.	0.6	n.d..	n.d.	n.d.	n.d.	n.d.	0.5
Benzo(ghi)perylene	n.d.	n.d.	0.4	n.d.	n.d.	0.5	n.d.±n.d.	n.d.	n.d.	n.d.±0.2	n.d.	0.7
Simazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.±3.3	n.d.	13.4	n.d.	n.d.	n.d.
Atraton	n.d.±1.4	n.d.	5.9	1.4±5.2	n.d.	21.5	n.d.	n.d.	3.0	1.4±5.1	n.d.	20.5
Propazine	4.3±4.3	n.d.	12.2	3.4±4.1	n.d.	10.3	3.8±3.4	n.d.	8.2	2.9±3.6	n.d.	9.3
Atrazine	n.d.	n.d.	n.d.	n.d.±1.5	n.d.	6.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Prometryn	n.d.±0.6	n.d.	2.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.3±1.1	n.d.	4.3
Prometon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.±1.2	n.d.	5.0
Terbuthylazine	3.9±2.0	0.8	7.0	4.4±2.5	n.d.	8.4	5.5±3.2	1.0	12.5	4.4±2.9	0.9	10.3
Diazinon	0.5±1.3	n.d.	4.9	n.d.±0.2	n.d.	1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlorpyrifos	7.5±4.2	1.2	15.5	10.2±7.6	0.8	31.3	52.1±48.7	3.0	199.3	26.6±26.0	1.5	86.5
Tokution (prothiofos)	1.1±4.4	n.d.	18.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
m-parathion	n.d.	n.d.	n.d.	n.d.	n.d.	0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chlorpyrifos-methyl	4.7±3.3	n.d.	11.9	7.6±8.0	n.d.	33.7	13.0±8.3	2.0	28.1	10.3±8.0	1.1	28.2
Terbuthylazine-desethyl	n.d.±5.5	n.d.	18.7	n.d.±4.9	n.d.	16.5	6.8±9.7	n.d.	28.1	5.3±7.2	n.d.	23.6
Flutolanil	n.d.	n.d.	n.d.	n.d.	n.d.	0.8	0.5±0.5	n.d.	1.4	0.6±0.5	n.d.	1.6
Tributhylphosphate	11.4±7.0	2.2	26.8	9.4±7.2	n.d.	22.7	12.2±7.6	2.1	22.9	7.6±5.2	2.2	21.3
Propyzamide	15.1±9.9	n.d.	33.6	15.3±12.2	n.d.	38.4	23.9±15.7	n.d.	47.0	16.5±15.1	n.d.	47.1

Pendimethalin	n.d.	n.d.	1.7	1.5±1.6	n.d.	6.6	5.1±6.2	0.4	21.9	5.3±8.3	n.d.	33.5
Chlorthal-dimethyl	1.4±0.9	n.d.	3.0	1.6±1.1	n.d.	3.8	2.3±1.3	0.5	4.0	2.1±1.6	0.4	6.4
Cyprodinil	n.d.	n.d.	0.5	n.d.	n.d.	0.5	n.d.	n.d.	0.6	n.d.	n.d.	0.8
Piperonyl butoxide	n.d.±1.1	n.d.	4.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected b.q.l.: below the quantification limit

Table4

Table 4. Concentrations of contaminants on a wet weight basis (ng g^{-1}) detected in the whole soft tissues of field-deployed clams at four Mar Menor sites, determined at 0, 7 and 22 days (PAH: Sum of polycyclic aromatic hydrocarbons, PCBs: polychlorinated biphenyls (sum of 7 congeners nº 28, 52, 101, 118, 138, 153 and 180).

Pollutants	Time	Sites			
	days	S1	S2	S3	S4
PCBs	0	0.15	0.15	0.15	0.15
	7	0.24	0.15	0.29	0.30
	22	0.22	0.24	0.44	0.52
p,p’ DDE	0	0.08	0.08	0.08	0.08
	7	0.26	0.13	0.70	0.69
	22	0.20	0.29	0.93	1.29
PAHs	0	1.57	1.57	1.57	1.57
	7	1.86	1.13	2.06	2.00
	22	3.04	2.72	2.31	2.06

Table5

Table 5. Mean values (±standard deviations) of acetylcholinesterase (AchE) measured in gills and catalase (CAT), glutathione reductase (GR), lipid peroxidation (LPO) and glutathione transferase (GST) measured in glands from *R. decussatus* clams transplanted to four sites (S1-S4) around the Albujon wadi mouth after 7 and 22 days of transplantation. Results of the multiple range test (LSD test) are also shown. Data with the same superscript indicate the absence of any significant difference between them (p=0.05).

	Neurotoxicity	Oxidative stress			Phase II-Detoxification
	AchE nmol $min^{-1}mg^{-1}$	CAT µmol $min^{-1}mg^{-1}$	GR nmol $min^{-1}mg^{-1}$	LPO nmol $min^{-1}mg^{-1}$	GST nmol $min^{-1}mg^{-1}$
Initial	1.76±0.44	283.8±55.1	45.32±9.29	0.589±0.157	773.9±114.5
7 days					
S1	1.66±0.41 ^{a}	221.4±43.0 ^{ab}	36.84±14.38 ^{a}	0.523±0.219 ^{a}	690.3±85.9 ^{a}
S2	1.55±0.29 ^{a}	225.4±94.9 ^{ab}	45.29±19.51 ^{ab}	0.695±0.129 ^{b}	745.1±156.8 ^{ab}
S3	1.63±0.33 ^{a}	276.2±59.7 ^{b}	57.39±16.38 ^{b}	0.690±0.129 ^{b}	838.7±140.6 ^{b}
S4	1.75±0.29 ^{a}	185.5±35.1 ^{a}	32.87±13.17 ^{a}	0.746±0.168 ^{b}	777.1±68.4 ^{ab}
22 days					
S1	1.89±0.38 ^{b}	227.9±38.0 ^{c}	29.95±5.51 ^{a}	0.722±0.093 ^{a}	833.8±94.1 ^{a}
S2	2.29±0.26 ^{c}	167.1±36.9 ^{b}	26.38±8.78 ^{a}	1.283±0.181 ^{b}	825.1±108.9 ^{a}
S3	1.35±0.22 ^{a}	123.8±31.4 ^{a}	39.78±8.40 ^{b}	1.399±0.202 ^{b}	954.1±298.1 ^{ab}
S4	1.13±0.16 ^{a}	182.3±30.5 ^{b}	45.91±10.94 ^{b}	0.943±0.201 ^{a}	1055.3±136.0 ^{b}

Table6

Table 6. Biometric measurements in *R. decussatus* clams transplanted to four sites (S1-S4) around the Albujon wadi mouth after 7 and 22 days of transplantation. Biological indices estimated were CI, condition index, GI, gill index, HI, hepatosomatic index and RI, rest index. Results of the multiple range test (LSD test) are also shown. Data with the same superscript indicate the absence of any significant difference between them (p=0.05).

	Length	Soft tissues				Biological indices			
		Total	Gill	Digestive Gland	Rest	CI	GI	HI	RI
	mm ind^{-1}	mg DW ind^{-1}							
Initial	33.94±0.77	304.5±28.9	39.0±5.7	31.8±4.7	233.7±25.3	8.4±1.2	12.8±1.5	10.5±1.8	76.7±2.8
7 days									
S1	34.01±1.20^{a}	291.9±38.0^{a}	41.0±7.5^{a}	35.3±7.3^{a}	215.6±32.3^{a}	8.3±1.0^{a}	14.1±2.0^{a}	12.2±2.7^{a}	73.7±4.4^{a}
S2	33.50±1.21^{a}	287.4±46.7^{a}	39.0±5.2^{a}	32.2±6.3^{a}	216.3±39.2^{a}	8.5±0.8^{a}	13.7±1.7^{a}	11.2±1.6^{a}	75.1±2.2^{a}
S3	33.06±1.61^{a}	290.5±37.6^{a}	38.5±5.6^{ab}	31.5±5.0^{a}	220.5±33.1^{a}	9.3±1.5^{a}	13.3±2.0^{a}	10.9±1.7^{a}	75.7±2.5^{a}
S4	33.78±1.26^{a}	311.3±33.5^{a}	45.9±8.6^{b}	37.9±7.2^{a}	227.5±32.7^{a}	8.8±1.3^{a}	14.8±2.4^{a}	12.3±2.9^{a}	72.9±4.8^{a}
22 days									
S1	34.77±1.13^{c}	287.9±40.3^{a}	43.7±5.2^{a}	33.9±4.2^{a}	210.2±34.3^{a}	7.3±1.0^{a}	15.3±1.5^{b}	11.9±1.6^{a}	72.7±2.5^{a}
S2	33.27±1.10^{a}	307.8±36.9^{ab}	42.5±5.3^{a}	35.7±7.9^{a}	229.6±27.5^{ab}	10.7±1.5^{b}	13.9±1.1^{a}	11.6±1.7^{a}	74.6±1.8^{ab}
S3	34.51±1.38^{bc}	370.4±39.6^{c}	51.4±6.6^{b}	43.3±8.1^{b}	275.6±30.9^{b}	10.1±1.8^{b}	13.9±1.3^{a}	11.7±1.7^{a}	74.4±1.9^{ab}
S4	33.58±1.24^{ab}	340.3±33.7^{bc}	48.7±4.0^{b}	35.5±4.7^{a}	255.9±29.0^{ab}	10.2±1.8^{b}	14.4±1.4^{ab}	10.4±1.0^{a}	75.2±1.8^{b}

Table7

Table 7. Physiological rates (mean values ± standard deviations) of clams kept under standardized laboratory conditions (17ºC, filtered seawater at 38 ppm, 0.78 mg l^{-1} of algal cells). Data with the same superscript indicate the absence of any significant difference between them at 95% level (LSD multiple range test, ANOVA performed between sites for each time). CR, clearance rate values are shown without standardization (CR_{ind}) and weight-standardized for 1 g clam dry weight (CR_w) or length-standardized for a clam of 50 mm (CR_l). CE, clearance efficiency calculated as the proportion of CR per gill size (measured as gill weight). RR, respiration rates and SFG, scope for growth, are shown without and with weight standardization.

	Clearance Rate				Respiration Rate		SFG	
	CR_{ind}	CR_w (1 g dw)	CR_l (50 mm)	CE	RR_{ind}	RR_w (1 g dw)	SFG_{ind}	SFG_w (1 g dw)
	L $ind^{-1}h^{-1}$				mg O_2 $ind^{-1}h^{-1}$		J ind^{-1} h^{-1}	
Initial	0.74±0.13	1.65±0.64	1.41±0.21	19.1±3.0	0.159±0.055	0.387±0.138	5.69±1.27	12.95±3.21
7 days								
S1	0.63±0.09 a	1.44±0.21 a	1.21±0.17 a	15.7±3.5 a	0.135±0.043 a	0.343±0.101 a	4.82±0.93 a	10.62±2.29 a
S2	0.66±0.19 a	1.52±0.39 a	1.30±0.35 a	17.0±4.5 a	0.151±0.051 a	0.389±0.143 a	4.94±1.75 a	10.82±3.57 a
S3	0.64±0.14 a	1.49±0.35 a	1.30±0.23 a	16.9±3.3 a	0.115±0.039 a	0.291±0.097 a	5.32±1.06 a	11.89±2.65 a
S4	0.66±0.15 a	1.44±0.30 a	1.27±0.26 a	14.8±4.1 a	0.135±0.023 a	0.327±0.060 a	5.14±1.57 a	10.84±3.17 a
22 days								
S1	0.80±0.10 b	1.85±0.19 c	1.48±0.14 b	18.4±2.4 b	0.141±0.025 a	0.362±0.071 a	6.63±0.88 b	14.80±1.47 c
S2	0.77±0.09 b	1.71±0.25 bc	1.54±0.13 b	18.4±2.9 b	0.167±0.052 a	0.401±0.109 a	5.95±1.06 b	12.78±3.19 bc
S3	0.77±0.08 b	1.51±0.22 ab	1.45±0.19 b	15.2±2.5 a	0.139±0.019 a	0.295±0.037 a	6.31±0.91 b	12.10±2.25 b
S4	0.63±0.17 a	1.31±0.37 a	1.24±0.33 a	12.9±3.2 a	0.166±0.044 a	0.377±0.121 a	4.43±1.30 a	8.76±2.68 a

Figure Captions

Figure 1. Locations in the Mar Menor coastal lagoon for clams field exposure and circulatory patterns inside the lagoon.

Figure 2. Results of PCA of the two main Factors produced by biomarkers (AchE, CAT, GR, LPO, GST and SFG) in clams, *Ruditapes decussatus*, caged at 4 sites (S1, S2, S3 and S4) in the Mar Menor lagoon for 22 days.

Figure 3. Integrated Biomarker Response (IBR) of those biomarkers which discriminate between reference sites and El Albujón-impacted sites according to the PCA: AchE, GR, GST and SFG. SS, sum of scores data calculated to obtain the IBR index are also shown for each site.

Figure1

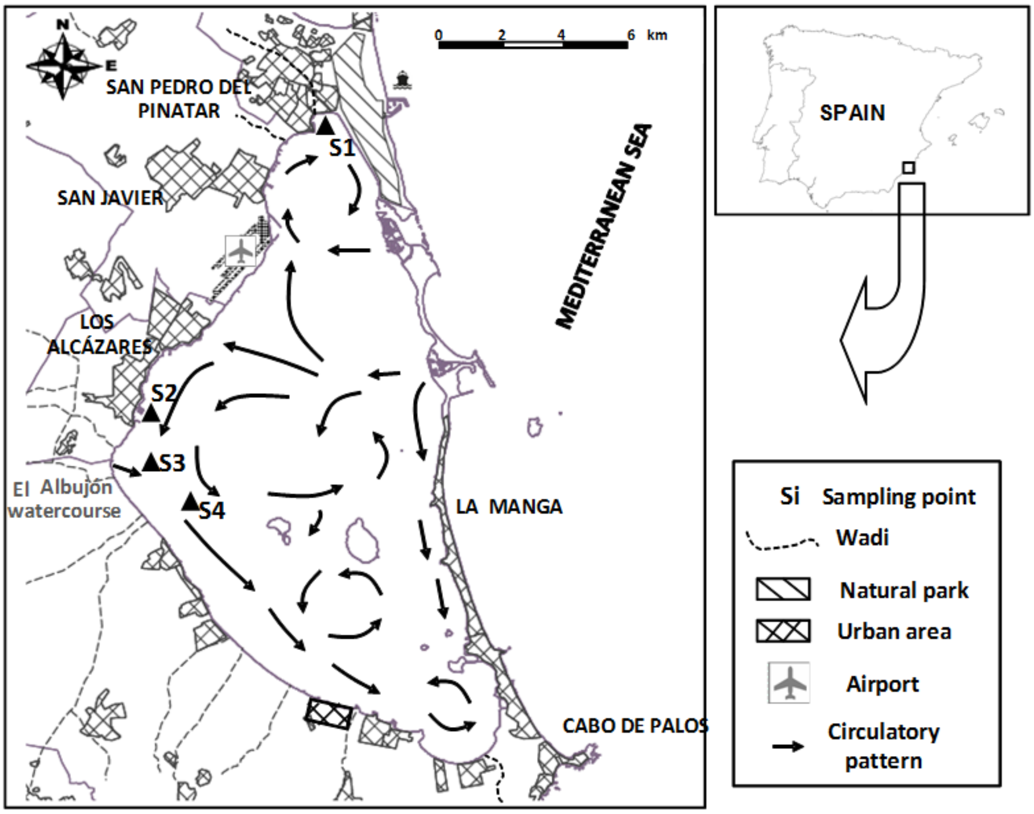
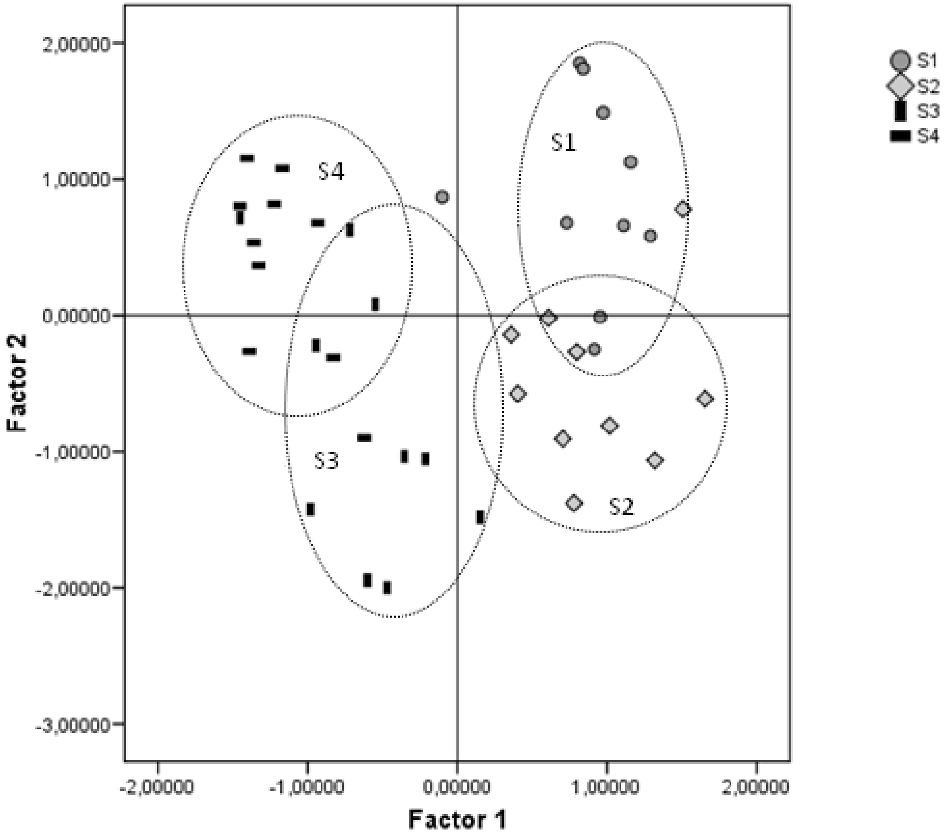
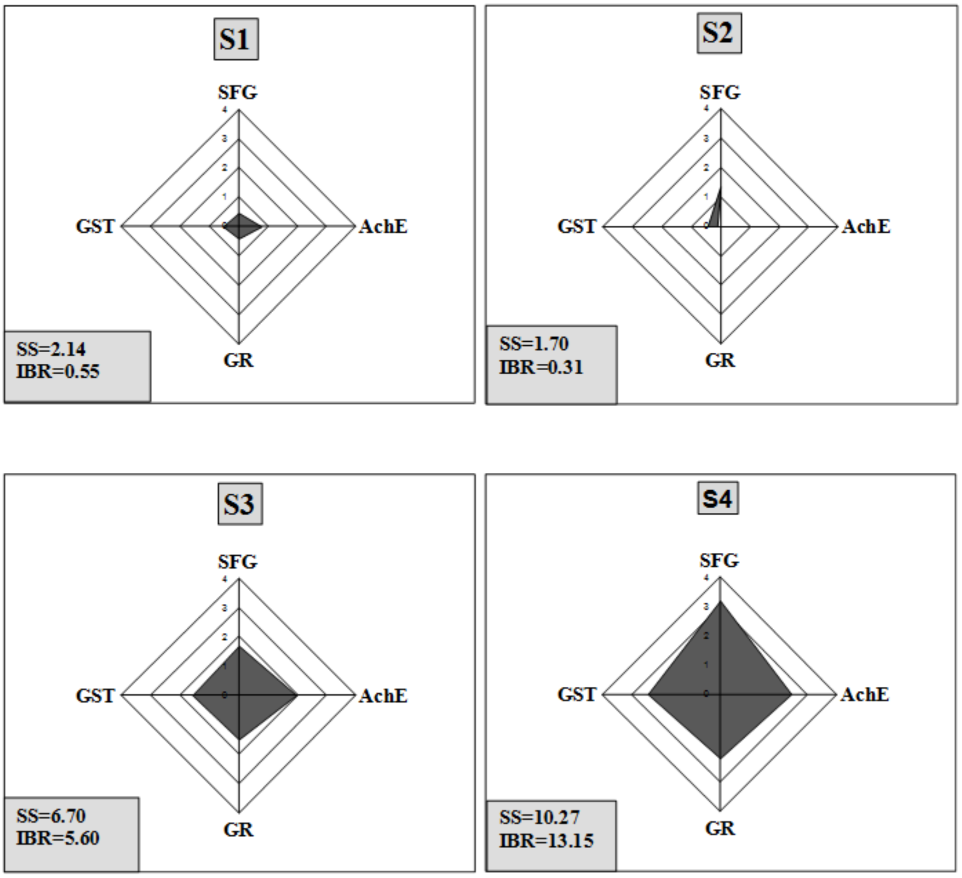

N
E
0 2 4 6 km
SAN PEDRO DEL PINATAR
SAN JAVIER
LOS ALCÁZARES
El Albujón watercourse
S1
S2
S3
S4
MEDITERRANEAN SEA
LA MANGA
CABO DE PALOS
SPAIN
Si Sampling point
Wadi
Natural park
Urban area
Airport
Circulatory pattern

Figure2



S1
S2
S3
S4
2,00000
1,00000
0,00000
-1,00000
-2,00000
-3,00000
-2,00000
-1,00000
0,00000
1,00000
2,00000
Factor 1
Factor 2

Figure3


S1
SFG
GST
AchE
GR
SS=2.14
IBR=0.55
S2
SFG
GST
AchE
GR
SS=1.70
IBR=0.31
S3
SFG
GST
AchE
GR
SS=6.70
IBR=5.60
S4
SFG
GST
AchE
GR
SS=10.27
IBR=13.15

***Highlights (for review)**

Highlights

- Clam caging approach is a useful tool to assess agricultural pollution effects
- Oxidative stress, neurotoxic (AchE) and physiological (SFG) effects were evaluated
- PCA analysis of the biomarkers differentiate between exposed and reference clams
- We adopted a multi-biomarker approach-integrated biomarker response (IBR)