Ecological Indicators 106 (2019) 105537

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Evaluation of C-reactive-like protein in *Mytilus galloprovincialis*

Lorena Franco-Martínez[a], Asta Tvarijonaviciute[a,*], Sandra V. Mateo[a], José J. Cerón[a], Diego Romero[b], Miguel Oliveira[c], Mariana Teles[d], Silvia Martínez-Subiela[a]

[a] Interdisciplinary Laboratory of Clinical Analysis INTERLAB-UMU, Regional Campus of International Excellence Mare Nostrum, University of Murcia, Espinardo, Murcia 30100, Spain
[b] Department of Toxicology, Regional Campus of International Excellence Mare Nostrum, University of Murcia, Espinardo, E-30071, Murcia 30100, Spain
[c] Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal
[d] Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain

ARTICLE INFO

Keywords:
Acute phase response
Biomonitoring
C-reactive protein
Hypoxia
Pollution

ABSTRACT

C-reactive protein (CRP) is an acute phase protein of the innate immune system with an important role as biomarker in many biological processes in mammals. However, there is a lack of information regarding CRP in molluscs and there is no data about the presence of CRP-like protein in mussels. In the present work, CPR-like protein was identified and purified from the digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. In addition, it was demonstrated that CRP-like protein can be measured accurately and precisely by a high-throughput automated method. Finally, higher levels of CRP-like protein in mussels exposed to hypoxia and nanoplastics were detected when compared to controls. The results obtained indicate that CRP-like protein could be a suitable novel biomarker of inflammatory response in mussels for biomonitoring programs being economic, easy and rapid to determine.

1. Introduction

In marine environments, oxygen availability, pollutants, overfishing and acidification are among the most important ecological concerns (Bijma et al., 2013). Oceanic oxygen availability around the world has decreased drastically in the last few decades as a consequence of important factors related to climate change such as decreased oxygen solubility and enhanced respiratory oxygen demand at elevated temperatures (Bijma et al., 2013; Diaz, 2001). The impact of reduced oxygen availability is also increased by pollution and other anthropogenic activities (Bijma et al., 2013). As pollutants, plastics are among the most important hazards to aquatic ecosystems (Yu et al., 2018). Nanoplastics (< 100 nm) are pollutants of high relevance since they are capable of passing through biological barriers, can bioaccumulate, and be transferred to other organisms through the food chain (Rossi et al., 2014; Kaloyianni et al., 2009; Chae et al., 2018).

For coastal environments, bivalves are considered as ideal organisms for biomonitoring programs since they are easy to sample, sedentary, filter-feeding, robust to adverse conditions, and represent important routes of entry of contaminants into humans (Kimbrough et al., 2008; Sericano et al., 2014; Widdows et al., 2002; Brandts et al., 2018). *Mytilus galloprovincialis* (Mediterranean mussel) is widely used as bioindicator (Franco et al., 2016), as recommended by the International Council for the Exploration of the Sea (Bråte et al., 2018). Since bivalves do not have an adaptive immune system, their innate immunity is crucial for defending against pathogens and inflammation (Castellanos-Martínez et al., 2014; Campos et al., 2015). Therefore, traditional biomarkers of innate immune response in other species could be presented in mussels and may be useful for the evaluation of the water state in biomonitoring programs.

The acute phase response (APR) is a key component of the innate immune response to tissue injury, pollution exposure, or immunological disorders, with the purpose of restoring homeostasis and removing the cause of its disturbance (Ebersole and Cappelli, 2000; Whicher and Westacott, 1992). APR is very fast, in terms of hours, showing a non-specific response being considered as one of the earliest markers for any pathologic process or disease that cause inflammation (Cerón et al., 2008). In fish and invertebrates, APR is viewed as being more robust than in mammals as compensation for a less-evolved adaptive immune system (Armstrong and Quigley, 1999). As part of this innate immune response in vertebrates, concentrations of certain proteins vary as a consequence of an inflammatory reaction of the host that occurs shortly after any tissue injury. These proteins are known as acute phase proteins (APP) and have received increasing attention in human and

* Corresponding author.
E-mail address: asta@um.es (A. Tvarijonaviciute).

https://doi.org/10.1016/j.ecolind.2019.105537
Received 11 January 2019; Received in revised form 26 June 2019; Accepted 1 July 2019

veterinary medicine in recent years due to their role in the diagnosis, response to treatment, and prognosis of the patients' outcome (Cerón et al., 2008). Therefore, in the last few decades, much information about the APR and APP has become available in mammals; however, the information about APP in bivalves is limited to serum amyloid A, heat shock proteins, or metallothioneins.

C-reactive protein (CRP) is the most studied APP in a number of species including humans and fish (Eckersall and Bell, 2010). Furthermore, in humans, CRP is the best characterized biomarker of inflammation (Devaraj et al., 2010) and it is considered an exquisitely sensitive systemic marker of inflammation (Pepys and Hirschfield, 2003). This protein is mainly produced in the liver, and it has proven value as a biomarker of inflammation in a wide range of conditions including systemic inflammation, vasculitis, infections or cancer (Pepys and Hirschfield, 2003; Bahar et al., 2007; Tvarijonaviciute et al., 2016). CRP-like proteins are characterised by a calcium-dependent binding affinity for monoesters of phosphorylcholine, which is a major constituent of fungal and bacterial capsules (Kolberg et al., 1997; Volanakis and Kaplan, 1971). CRP has been successfully used as biomarker of pollution (Genser et al., 2015; Ghosh and Bhattacharya, 1992; Viehmann et al., 2015; Hennig et al., 2014) and hypoxia in different species (Genser et al., 2015; Ghosh and Bhattacharya, 1992; Viehmann et al., 2015; Hennig et al., 2014; Padhy et al., 2013; Shang et al., 2014) since these conditions are known to cause inflammation (Shang et al., 2014; Rückerl et al., 2007). In fishes, studies revealed that CRP change after 12 h of exposure to nonlethal doses of xenobiotics, or after 24 h of heavy metals exposure (Ghosh and Bhattacharya, 1992). In bivalves, sialic acid-specific lectins from the *Crassostrea gigas* oyster and from *Modiolus modiolus* mussels has been proposed to as CRP since they precipitate *Streptococcus pneumonia* polysacarid C and bind to phosphorylcholine in calcium presence (Olafsen, 1995). Besides, in invertebrates, it seems that lectins concentration can stay constant while its activity increases in the form of conformational or structural changes, including modifications in its subunits (Olafsen, 1995). However, to the best of the authors' knowledge, there is a lack of reports regarding the presence and behaviour of CRP-like protein in *M. galloprovincialis*.

We hypothesized that CRP-like protein could be presented in *M. galloprovincialis*, and could be used as an inflammation biomarker in this species. Thus, the main objectives of this study were: (1) to determine if CRP-like protein is present in mussels, (2) to validate an automated assay for its measurement, and (3) to evaluate CRP-like protein behaviour in the digestive gland in two situations in which mussels are classically employed as bioindicators such as hypoxia and pollutant exposure. For this, we attempted to establish a method for the purification of mussel CRP-like protein by using an affinity column coupled with o-phosphorylethanolamine-agarose (PEAD-agarose). Our results were verified with SDS page and western blotting. Thereafter, an automated method for human CRP measurement was analytically validated for the measuring of CRP-like protein in the digestive gland of mussels, and the behaviour of this protein was analysed in surplus samples from two different trials previously made and published, in which two different stressor agents were applied: (1) one producing an oxidative stress secondary to hypoxia (Franco-Martínez et al., 2018), and (2) the second consisting of nanoplastics exposure that was performed for other studies (Brandts et al., 2018).

2. Material and methods

For the present study, 59 mussels were employed: 15 mussels were used in purification and validation studies, 20 in hypoxia experimental set-up, and 24 mussels were used in experimental exposure to nanoplastics.

2.1. CRP-like protein purification and detection

For the purification and detection of CRP-like protein, six wild mussels *(M. galloprovincialis)* (length, 4.3 ± 0.37 cm; weight, 9.3 ± 0.35 g) from El Gorguel, Murcia (Spain), were employed. Digestive glands were carefully extracted from each animal, processed and analysed immediately as described elsewhere (Franco et al., 2016). Briefly, sample preparation consisted of mechanical homogenization using an automatic homogenizer (Precellys Evolution, Bertin Technologies, Saint-Quentin, Yvelines, France) in 1:4 w/v potassium buffer (Franco et al., 2016). After centrifugation (30 000*g*, 90 min, 4 °C), the supernatant was transferred to Eppendorf tubes and used in further analysis.

CRP-like protein was purified using affinity chromatography. For purification purposes, six digestive glands were extracted and processed as described above. The digestive gland homogenates were then pooled, filtrated with 1.2 µm filters (Minisart® Syringe Filters, Sartorius) and concentrated using Amicon 3 K (Millipore), until a final volume of 4 mL was obtained. The affinity chromatography column was prepared by the following method described elsewhere (Pontet et al., 1978; Onishi et al., 1994) with some modifications: 2.5 g of Epoxy- activated Sepharose 6B (GE Healthcare) were suspended in ultrapure water and washed on a sintered-glass filter for one hour with 200 mL of ultrapure water. Then, 0.24 g of ligand O-phosphoryletanolamine (GE Healthcare) were dissolved in 200 mM $NaHCO_3$ + 500 mM NaCl pH 11. Coupling was performed by gently mixing the suspension in a stoppered vessel for 16 h at 30 °C. The gel was left in contact with 1 M ethanolamine pH 8 buffer for 4 h in order to block any remaining active group. Then, the gel was washed 3 times alternating 0.1 M acetate buffer + 500 mM NaCl pH 4 and 100 mM Tris-HCl buffer containing 500 mM NaCl, pH 8. The gel was packed in a glass column (XK16, GE Healthcare). Elution operations were performed at 1 mL/min flow rate, monitored at 280 nm using an automated liquid chromatography system (ÄKTA pure, GE Healthcare) and collected in fractions of 1 mL. Eluted fractions from the absorbance peak were then pooled, concentrated using Amicon 3 K (Millipore), and used for SDS-PAGE and western blotting.

For CRP-like protein verification, western blotting assay for the CRP-like protein purificate was performed using a Mini Trans-Blot device (Bio-Rad) and employing nitrocellulose membranes. Briefly, transference was performed for 1 h 25 V. Afterwards, membrane was blocked with 5% roti blocking buffer (B6429, Sigma Aldrich) for 16 h, followed by incubation for 2 h at room temperature with 1:500 diluted primary antibody (goat anti human CRP, Olympus® CRP OSR 6147 Olympus Life and Material Science Europe GmbH) and for one hour 1:16000 diluted secondary antibody (anti-goat-IgG, A5420-1 mL, Sigma Aldrich) conjugated with HRP. Blots probed for CRP-like protein were developed using ECL™ Select Western Blotting Detection Reagent (GEHealthcare) and ImageQuant LAS 500 (GE Healthcare) scanner.

2.2. Biochemical measurements and analytical validation

2.2.1. Biochemical measurements

CRP-like protein concentration was measured using a human immunoturbidimetric test (CRP OSR 6147 Olympus Life and Material Science Europe GmbH).

Total protein content (PT) was measured colorimetrically with a commercial kit (OSR6132, Beckman Coulter), as indicated by the manufacturer.

An automated biochemistry analyser (Olympus AU600, Germany) were employed for all determinations. The results in terms of biochemical data were expressed before and after correcting according to each sample's protein content, as reported in previous studies (Franco-Martínez et al., 2018).

2.2.2. Analytical validation

The analytical validation of the automated immunoturbidimetric assay for CRP-like protein determination was performed in nine digestive glands of mussels collected from el Gorguel (Murcia). For this, the precision, accuracy and lower limit of detection were evaluated according to previous studies in the mussels' tissues (Franco et al., 2016).

Precision was evaluated by calculating the intra- and inter-assay coefficient of variation (CV) after the analysis of two samples with high and low concentrations of CRP-like protein. The samples were analysed five times in a single assay run for intra-assay CV, and in five separate runs carried out on five consecutive days for inter-assay CV. The CV of the assay was calculated as the standard deviation (SD) divided by the mean value of the analysed replicates × 100%. A spiking recovery was performed by mixing two samples in different ratios, and calculating the recovery percentage by a comparison of the expected and the observed results. Recovery was evaluated thrice. Linearity under dilution was analysed by the effect of serial dilutions of three samples with ultrapure water (without dilution and diluted 1:2; 1:4; 1:8; 1:16; 1:32; and 1:64). The obtained results were compared to the expected values, and linear equation and correlation coefficients were calculated. The lower limit of detection was assessed by the lowest concentration of CRP-like protein that could be distinguished from a specimen of zero value in each assay. For this, potassium buffer was measured 10 times and the lower limit of detection was calculated as the double of the standard deviation plus the mean of the measurements.

2.3. Experimental set-up

2.3.1. Hypoxia

For the hypoxia experimental set-up, 20 mussels (length, 4.5 ± 0.3 cm; weight, 9.4 ± 0.36 g) were collected from the Gorguel (Murcia) and processed as described in the "2.1. CRP-like protein purification and detection" section.

The experimental hypoxia set-up was performed as described previously (Franco-Martínez et al., 2018). Briefly, twenty mussels were acclimated for two days under controlled conditions of pH (8.03 ± 0.07), osmolarity (1086.3 ± 28.39 mmol kg^{-1}), temperature (19 ± 1 °C), continuous aeration, and natural photoperiod (14 h light: 10 h dark) into twelve-litre tanks. Afterwards, the animals were randomly divided into two groups: hypoxic and control. Animals (n = 10) from the control group were kept under the same acclimation period conditions for 96 h. The inter-valvar water of mussels of the hypoxic group (n = 10) was removed, and the animals were air-exposed at room temperature for 96 h. Two animals from the hypoxic group died during the air-exposure period and were eliminated from the study. No feeding was provided during the experimental set-up. These samples were previously used for oxidative stress evaluation in mussels (Franco-Martínez et al., 2018).

After 96 h, all animals were sacrificed, and their digestive glands were extracted and processed individually as described above.

2.3.2. Exposure to nanoplastics

For the exposure to nanoplastics experimental set-up, 24 mussels (length, 4.4 ± 0.19 cm, weight, 8.8 ± 0.38 g) from Aveiro, Portugal were used. Mussels were acclimated for 10 days in 80 L glass tanks containing aerated and filtered artificial seawater under controlled laboratory conditions of pH (7.9 ± 0.06), temperature (19 ± 0.24 °C), salinity (35 ± 0.0), and natural photoperiod (14 h light: 10 h dark). Afterwards, mussels were randomly distributed into 2 experimental groups (three replicate tanks per experimental condition), containing 4 animals in 3.5 L of experimental media. Animals were exposed for 96 h to the experimental conditions: control, 0.005, 0.05 and 0.5 mg/L polystyrene nanoplastics (PSNP). During this period, water conditions were as previously described for the acclimation period. These samples had previously been used for nanoplastic effect evaluation on metabolic and antioxidant systems in mussels (Brandts et al., 2018).

Digestive glands were dissected, immediately frozen in liquid nitrogen individually, and then stored at −80 °C until further processing. Digestive glands were homogenized in 1:4 (weight/volume) in potassium-phosphate buffer using an ultrasound homogenizer (Sonifier 250, Branson), incubated for 30 min at room temperature, and then centrifuged (2 000*g*, 10 min, 4 °C). Supernatant was centrifuged at 4 °C consecutively (600*g*, 15 min; 13 000*g*, 20 min; and 30 000*g*, 90 min), and the supernatant was collected in Eppendorf tubes, stored at −80 °C and delivered to University of Murcia for analysis.

2.4. Statistical analysis

The intra-CV, inter-CV, lower limit of detection and linearity under dilution were calculated using Excel 2010 (Micro- soft Corp., Redmond, WA).

A post hoc power analysis was conducted using the values obtained to verify the null hypothesis. By using the mean and standard deviation of the CRP-like protein for each group, and a power of 80% with at 5% level of significance, the number of individuals for each group in both experimental set-ups were calculated. The data analysis was done using ClinCal statistic analyser software (available at https://clincalc.com/stats/samplesize.aspx) (Rosner, 2011).

For biochemical measurements, CRP-like protein concentrations were reported as median (25 – 75% percentile) in mg/L (mg/g when data were corrected by protein content), unless otherwise stated. The D'Agostino & Pearson omnibus normality test was employed to check the distribution of data, and the non-parametric statistical Mann Whitney *U* test was used to compare between groups, since data were not normally distributed. In all cases, a value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed with the statistical software GraphPad Prism 6.0.

3. Results

3.1. CRP-like protein purification and detection

Elution profiles of CRP-like proteins from mussels using affinity chromatography on o-phosphorylethanolamine-agarose (PEAD-agarose) column are shown in Fig. 1. Fractions 9 to 11 were selected, pooled and processed as described in "2.1. CRP-like protein purification and detection" section. Fig. 2 shows SDS-PAGE and western blotting of the pooled processed fractions eluted from column and purified porcine CRP, showing a multimeric band pattern that was similar to porcine CRP purified except for the lack of a band of 20 kDa.

CRP-like protein from the digestive glands of mussels was eluted from an PEAD-agarose column in one absorbance peak of 60 mAu. Western blot analysis using purified CRP-like protein confirmed that the antibody recognized the mussel's CRP-like protein, showing a triple band pattern (bands of around 60, 33 and 29 kDa) (Fig. 2).

3.2. Analytical validation

Detailed data obtained for spiking recovery, accuracy, and linearity studies are shown in Tables 1 and 2 and Fig. 3. Recovery ranged from 94 % to 103 %, intra-assay CV was < 2 %, while inter-assay CV was > 39 %. Dilution of the samples resulted in a linear regression equation with correlation coefficients higher than 0.98 in all cases. The lower limit of detection was 2.63 mg/L (mean, 2.1 mg/L; SD, 0.27 mg/L).

3.3. Experimental set-up

3.3.1. Hypoxia

Results of the hypoxia experiment are summarized in Fig. 4. The CRP-like protein concentration was significantly higher in mussels

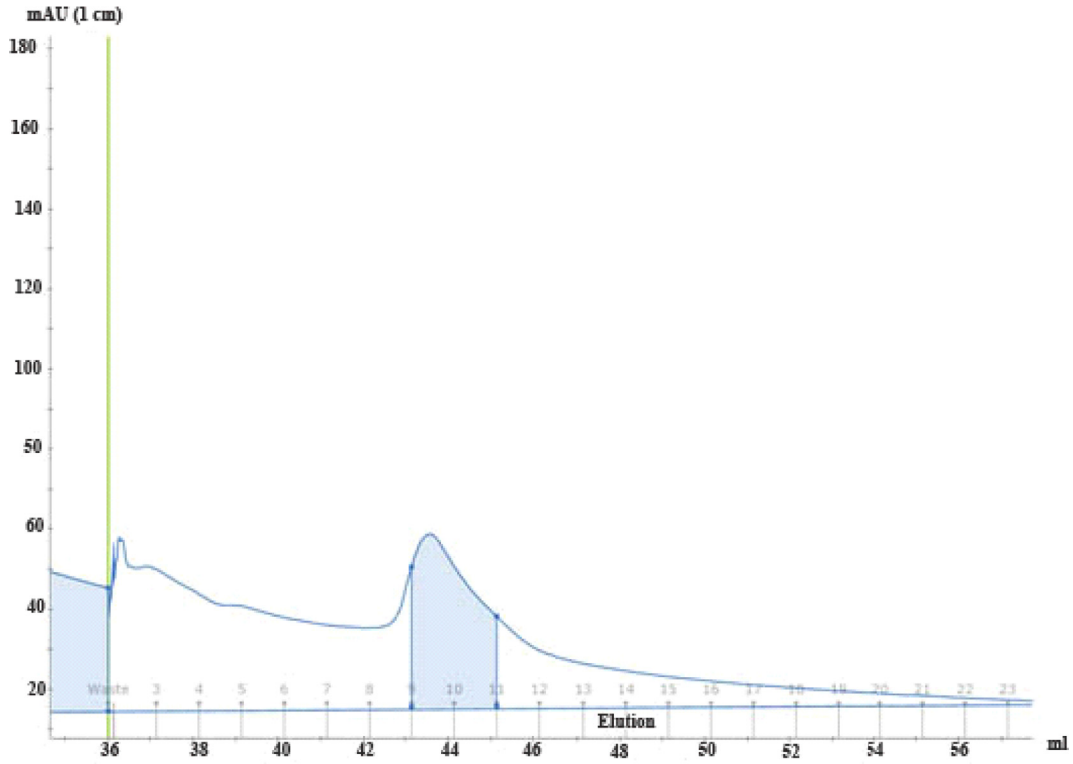

Fig. 1. Elution profile of digestive gland homogenates from *M. galloprovincialis* CRP-like protein from PEAD-agarose column.

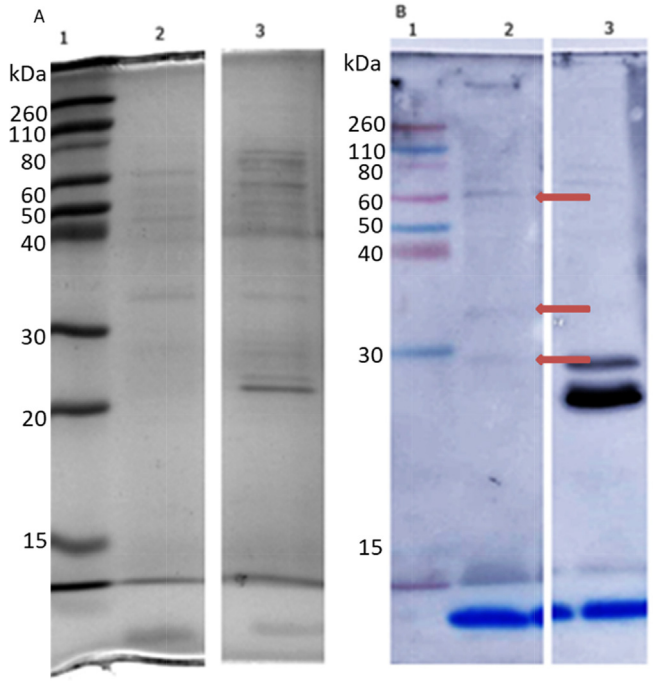

Fig. 2. Polyacrylamide gel electrophoresis (15% SDS-PAGE) of purified mussel CRP-like protein (A) and Western blot probing for mussel CRP-like protein (15% SDS-PAGE transferred on to a nitrocellulose membrane) by goat anti human CRP antibodies (B). Line 1: Novex® pre-stained SDS-PAGE molecular weight markers (kDa). Line 2: purified mussel CRP-like protein from digestive gland homogenate (10 µL). Lane 3: purified porcine serum CRP (10 µL).

exposed to hypoxia for 96 h (43.82 [36.42–63.52] mg/L) when compared to the control group (27.19 [22.43–28.62] mg/L) ($p \leq 0.001$). Significantly higher CRP-like protein concentrations were also observed in mussels subjected to hypoxia when compared to controls, after correcting each sample by total protein content (11.37 [9.08–13.62] and 18.73 [14.37–23.09] mg/g for the control and hypoxic groups, respectively). A power analysis test indicates that 10 subjects were required for each group in order to obtain a power of 80 % with a 5 % level of significance.

Table 1
Representative spiking recovery. Two samples with different amounts of CRP-like protein were mixed in different ratios and recovery, expressed as a percentage, was calculated for each dilution for comparison of expected versus measured concentrations.

Ratio A	Ratio B	Observed	Expected	Recovery (%)
100	0	82.96	82.96	100
75	25	91.49	90.25	101.38
50	50	99.85	97.53	102.38
25	75	107.99	104.82	103.03
0	100	112.10	112.10	100

Table 2
Intra- and inter-assay coefficients of variation (CV) of CRP-like protein in digestive gland homogenates of *M. galloprovincialis*.

Comparison	Pool	Mean	SD	CV (%)
Intra	1	56.48	0.29	0.516
	2	44.64	0.55	1.24
Inter	1	35.08	13.73	39.15
	2	10.05	1.98	19.7

3.3.2. Exposure to nanoplastics

CRP-like protein concentrations in mussels exposed to nanoplastics for 96 h and control group are summarized in Fig. 5. CRP-like protein concentration was significantly higher in mussels exposed to 0.05 and 0.5 (18.4 [26–14.2] and 15 [19.95–14.2] mg/L, respectively) in comparison with the control group (7.05 [3.7–8.8] mg/L) ($p < 0.05$). CRP-like protein concentration in mussels exposed to 0.005 (11.3

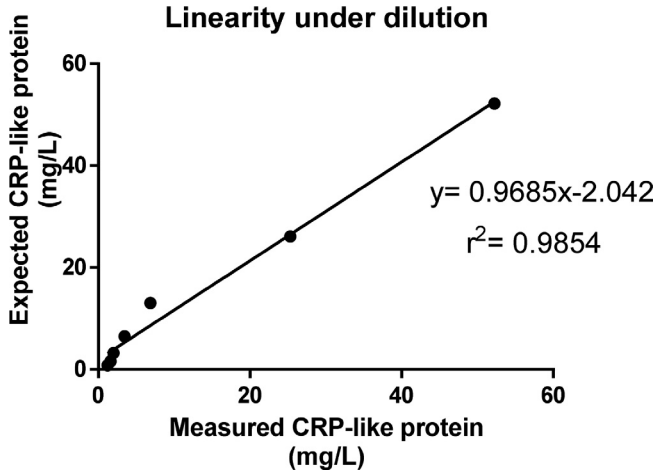

Fig. 3. Representative graph of linearity under dilution of CPR-like protein of *M. Galloprovincialis* digestive gland.

[13.12–9.19] mg/L PSNP) was higher than that observed in the controls, although this difference was not of statistical relevance. When data was corrected by total protein content, statistically higher CRP-like protein concentrations were observed in animals exposed to 0.05 and 0.5 mg/L PSNP (16.21 [21.13–12.37] and 15.92 [19.69–14.08] mg/g, respectively) ($p < 0.01$) when compared to the controls (6.87 [7.57–5.58] mg/g). The power analysis test indicates that 4 mussels were required for each group for a power of 80 % with a 5 % level of significance.

4. Discussion

This is the first study in which CRP-like protein was detected, characterized and measured by a high-throughput validated assay in *M. galloprovincialis* mussels. The protein was purified by affinity chromatography from the digestive glands of mussels, and a western blotting was performed in order to verify the findings. Finally, the usefulness of CRP-like protein for biomonitoring purposes was evaluated by the exposure of mussels to two of the most pertinent threats to marine ecosystems: hypoxia and pollutants (Bijma et al., 2013). The identification of this CRP-like protein not only increases our knowledge of the immune system of mussels, but it can also be useful in biomonitoring programs and the management of altered oxygen pathophysiologies.

In the present study, CRP-like protein was purified from digestive gland homogenates of *M. galloprovincialis*, showing a peak at 60 mAU. Since PEAD-agarose columns showed higher binding affinity than affinity columns coupled with phosphorylcholine as a ligand in other species (Onishi et al., 1994), a PEAD-agarose column was employed. Analysis of the SDS-PAGE of purified CRP-like protein from digestive gland showed a multimeric pattern. This pattern of CRP-like protein in mussels is very similar to that observed for purified CRP from porcine sera employed in the present study and reported elsewhere (Martinez-Subiela et al., 2007). Western blotting of purified CRP-like protein confirmed that the antibody recognizes a CRP-like protein that showed a triple band pattern of around 60, 33 and 29 kDa. The bands of 29 and 33 kDa could be compatible with CRP-like protein monomers, while the 60 kDa band – of approximately twice the molecular weight - suggests the presence of CRP-like protein dimeric components. These results are compatible with that previously described in other species, such as 27.5 and 25.5 kDa bands from pooled carp sera CRP purificate (MacCarthy et al., 2008), or from *C. gigas* lectins composed by 21, 22.5 and 33 kDa CRP-like protein subunits (Olafsen, 1995). As suggested in some studies, we hypothesize that differences found in the molecular weight of the purified CRP-like protein may be due to glycosylation in some of the subunits of CRP-like protein molecules (MacCarthy et al., 2008; Muñoz Prieto et al., 2017; Parra et al., 2006).

The automated assay used for the measurement of CRP-like protein in the digestive gland of mussels showed an adequate analytical validation according with the limits for good method functionality (Cossio et al., 2012; CVM, 2001) in terms of intra-assay precision (with an intra-assay CV lower than 5 %), recovery (with all ranges between 120 % and 80 %) and linearity (with correlation coefficients under dilution close to 1). However, since the inter-assay was above the acceptance range, it is recommended to analyse all the samples in the same run, as performed in the present study. This observed high inter-assay CV could be attributed to the presence of a high number of proteases and microorganisms present in mussels' digestive glands, which may cause degradation and alterations in CRP-like protein composition through time. Thus, further studies should be made in order to test strategies for the improvement of the stability of CRP-like protein in mussels, such as the use of antiproteases.

In the present study, the biochemical results of CRP-like protein are shown before and after being corrected by total protein concentration because, although digestive gland protein content was not statistically different between the groups of each experimental set-up, the convenience of correcting the data by protein content has been suggested in order to reduce the possible bias related to sample dilution for the homogenization process (Franco-Martínez et al., 2018) or alterations in protein content due to desiccation during an experimental hypoxia set-

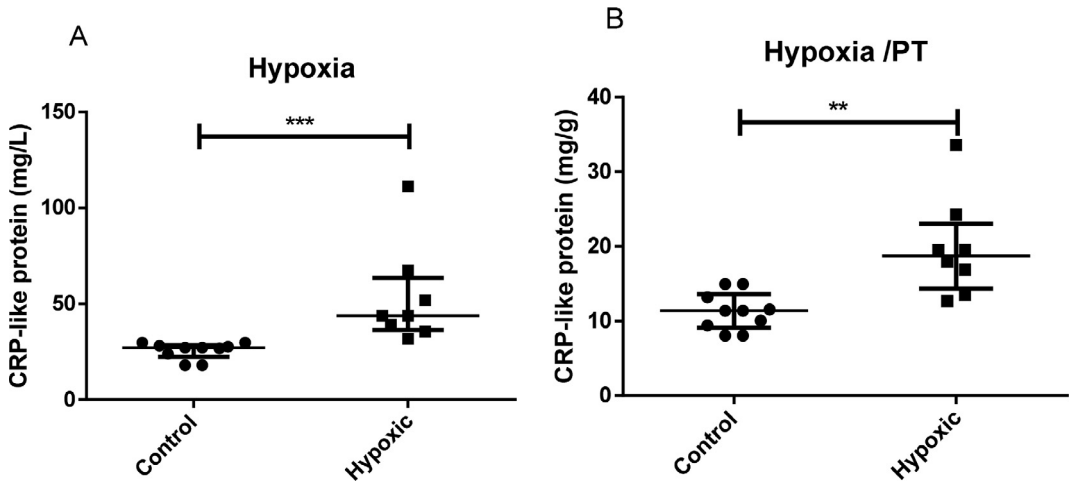

Fig. 4. CRP-like protein concentrations in controls (n = 10) and hypoxic (n = 8) mussels before (A) and after (B) correcting data by their total protein content. ** $P < 0.01$; *** $P < 0.001$ according to Mann Whitney Test.

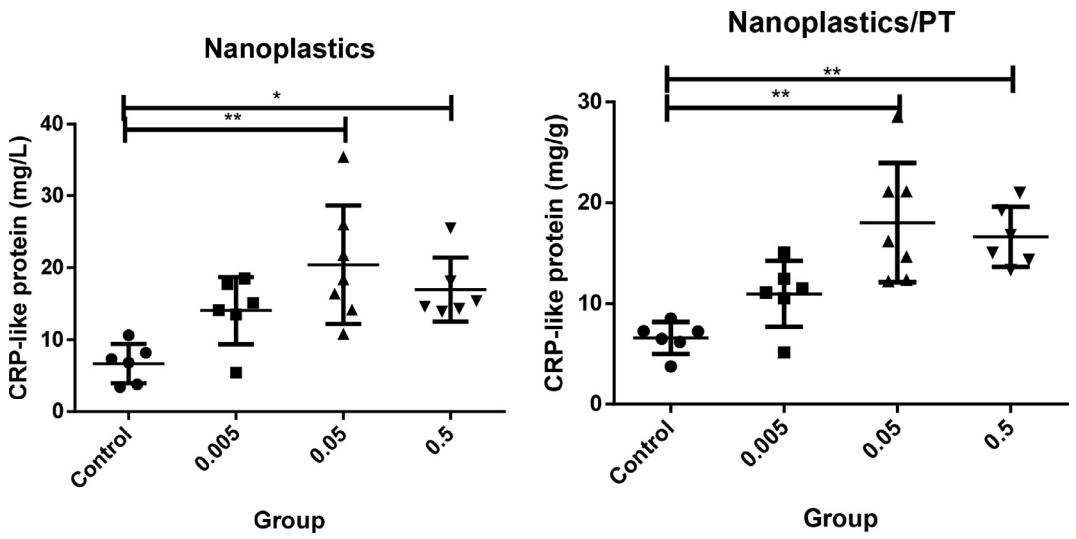

Fig. 5. CRP-like protein concentrations in controls and mussels exposed to nanoplastics before (A) and after (B) correcting data according to their total protein content. Mann Whitney Test were performed, and asterisks indicate statistically significant difference between the groups (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

up. In both cases, CRP-like protein was statistically significantly higher in hypoxic and pollutant-exposed mussels. Therefore, further studies would be desirable in order to clarify the need for correcting by protein content for CRP-like protein measurements.

Mussels show a high display of evolutionary adaptation when dealing with hypoxia since they often habitat in inter-tidal areas (Giannetto et al., 2015). In fact, most coastal biomonitoring programs rely on mussels that habitat in those inter-tidal zones instead of subtidal areas due to their easier collection (Vidal-Liñán and Bellas, 2013). CRP-like protein levels were observed to be higher in mussels challenged with hypoxia. Hypoxic mussels showed 1.61-fold higher CRP-like protein concentrations than the controls. The increase was 1.64-fold when the results were corrected by each sample's protein content, being in concordance with studies that report higher levels of CRP in humans exposed to hypoxia (Shang et al., 2014; Masuda et al., 2008). These reports suggested that severe hypoxia causes inflammation and subsequent increases in CRP levels. In previous studies (Franco-Martínez et al., 2018; Mosca et al., 2013), mussels exposed to hypoxia showed oxidative stress, which has been related to acute inflammation (Shang et al., 2014; Yilmaz Avci et al., 2017); although CRP-like protein may be increased due to ROS production or the oxidative stress itself. Therefore, further studies are desirable in order to discern the mechanisms that promoted the increase of CRP-like protein in mussels exposed to hypoxia. However, to the best of the authors' knowledge, this is the first study evaluating CRP-like protein response in mussels due to hypoxia.

CRP-like protein concentrations were higher in mussels exposed to nanoplastics when compared with the controls (2.07, 2.61, and 2.12-fold for 0.005, 0.05, and 0.5 groups, respectively). These increases were similar when data were corrected by total protein content, with 1.6 (0.005), and 2.35 (0.05), and 2.31-fold (0.5) higher CRP-like protein concentrations than in the control group. A previous study revealed that nanoplastics increased the oxidative status and caused peroxidative damage in the digestive gland (Brandts et al., 2018), which can cause inflammation. Our results showed higher CRP-like protein concentrations in the animal exposed to the most toxic mixture, suggesting that this protein could be useful, not only as biomarker of exposure, but also of severity. Similar findings were also observed in the case of *Labeo rohita* fish, in which different levels of CRP were induced, depending on the degree of damage *in vivo* (Sinha et al., 2001). Similarly, different increases in CRP concentrations were described in mammals, depending on the severity of disturbance (Gabay and Kushner, 1999; Martínez-Subiela et al., 2002).

This preliminary study has some limitations such as the relatively small sample size, and therefore individual variations could have influenced the predictive power of the CRP-like protein in the experimental set-ups. Secondly, no glycosylation studies were carried out, although differences in CRP glycosylation among species have been reported in fish (Sinha et al., 2001; Pepys et al., 1982) and other lectins are glycosylated in mussels. Lastly, although even shorter acclimation periods are used in literature and, by definition, the acute phase response only lasts a few days (Cerón et al., 2005), it cannot be assured that the two days of acclimation period in both groups of the hypoxia experimental set-up were sufficient to grant normalisation of baseline CRP-like protein levels. Ideally, the experimental procedures should have been repeated in order to determine the reproducibility of the results. Therefore, further studies are needed in order to confirm our observations. In addition, although in our model, CRP-like protein increases after hypoxia and pollutant exposure, the protein is not specific of these conditions, and it could potentially increase after any situation that could produce an injury to the mussels, which may be of utility for screening studies for different ecological conditions. Lastly, differences between the CRP-like protein levels were observed between the different experimental set-ups. These differences could be explained by different processing protocols, or due the existence of confounding factors such as sampling site or seasonality, among others. However, this limitation could be solved by the use of a control group for each experiment.

5. Conclusions

CRP-like protein is present in *M. galloprovincialis* digestive glands and can be measured acutely, precisely and economically by high-throughput automated assays. In addition, the increase observed in mussels exposed to two important ecological concerns (hypoxia and nanoplastics) suggest that CRP-like protein may potentially be considered as a suitable biomarker for any environmental condition that may cause direct or indirect inflammation to mussels. Thus, the measurement of CRP-like protein in mussels may have a great potential value in biomonitoring programs.

Acknowledgements

LFM was granted with predoctoral contract 'FPU' of University of Murcia, Spain. AT has a post-doctoral fellowship "Juan de la Cierva Incorporación" supported by the "Ministerio de Economía y

Competitividad", Spain. MT has a post-doctoral fellowship from FCT (SFRH/BPD/109219/2015) supported by the European Social Fund and national funds from the "Ministério da Educação e Ciência (POPH – QREN – Tipologia 4.1)" of Portugal. MO had financial support of the program Investigador FCT, co-funded by the Human Potential Operational Programme and European Social Fund (IF/00335-2015).

Declaration of Competing Interest

The authors report no other conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Armstrong, P.B., Quigley, J.P., 1999. Alpha2-macroglobulin: an evolutionarily conserved arm of the innate immune system. Dev. Comp. Immunol. 23, 375–390 DOI: S0145-305X(99)00018-X [pii].

Bahar, G., Feinmesser, R., Shpitzer, T., Popovtzer, A., Nagler, R.M., 2007. Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile. Cancer 109, 54–59. https://doi.org/10.1002/cncr.22386.

Bijma, J., Pörtner, H.O., Yesson, C., Rogers, A.D., 2013. Climate change and the oceans - what does the future hold? Mar. Pollut. Bull. 74, 495–505. https://doi.org/10.1016/j.marpolbul.2013.07.022.

Brandts, I., Teles, M., Gonçalves, A.P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., Martins, M.A., Soares, A.M.V.M., Tort, L., Oliveira, M., 2018. Effects of nanoplastics on Mytilus galloprovincialis after individual and combined exposure with carbamazepine. Sci. Total Environ. 643, 775–784. https://doi.org/10.1016/j.scitotenv.2018.06.257.

Bråte, I.L.N., Blázquez, M., Brooks, S.J., Thomas, K.V., 2018. Weathering impacts the uptake of polyethylene microparticles from toothpaste in Mediterranean mussels (M. galloprovincialis). Sci. Total Environ. 626, 1310–1318. https://doi.org/10.1016/J.SCITOTENV.2018.01.141.

Campos, A., Apraiz, I., da Fonseca, R.R., Cristobal, S., 2015. Shotgun analysis of the marine mussel Mytilus edulis hemolymph proteome and mapping the innate immunity elements. Proteomics 15, 4021–4029. https://doi.org/10.1002/pmic.201500118.

Castellanos-Martínez, S., Diz, A.P., Álvarez-Chaver, P., Gestal, C., 2014. Proteomic characterization of the hemolymph of Octopus vulgaris infected by the protozoan parasite Aggregata octopiana. J. Proteomics. 105, 151–163. https://doi.org/10.1016/j.jprot.2013.12.008.

Cerón, J.J., Eckersall, P.D., Martínez-Subiela, S., 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet. Clin. Pathol. 34, 85–99. https://doi.org/10.1111/j.1939-165X.2005.tb00019.x.

Cerón, J.J., Martinez-Subiela, S., Ohno, K., Caldin, M., 2008. A seven-point plan for acute phase protein interpretation in companion animals. Vet. J. 177, 6–7. https://doi.org/10.1016/j.tvjl.2007.12.001.

Chae, Y., Kim, D., Kim, S.W., An, Y.J., 2018. Trophic transfer and individual impact of nano-sized polystyrene in a four-species freshwater food chain. Sci. Rep. 8. https://doi.org/10.1038/s41598-017-18849-y.

Cossio, M.L.T., Giesen, L.F., Araya, G., Pérez-Cotapos, M.L.S., VERGARA, R.L., Manca, M., Tohme, R.A., Holmberg, S.D., Bressmann, T., Lirio, D.R., Román, J.S., Solís, R.G., Thakur, S., Rao, S.N., Modelado, E.L., La, A.D.E., Durante, C., Tradición, U.N.A., En, M., Espejo, E.L., Fuentes, D.E.L.A.S., De Yucatán, U.A., Lenin, C.M., Cian, L.F., Douglas, M.J., Plata, L., Héritier, F., 2012. Schalm's veterinary hematology 6th ed, doi:10.1007/s13398-014-0173-7.2.

C. for V.M. (CVM). U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Guidance for Industry Bioanalytical Method Validation, 2001. https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf (accessed November 7, 2017).

Devaraj, S., Valleggi, S., Siegel, D., Jialal, I., 2010. Role of C-reactive protein in contributing to increased cardiovascular risk in metabolic syndrome. Curr. Atheroscler. Rep. https://doi.org/10.1007/s11883-010-0098-3.

Diaz, R.J., 2001. Overview of Hypoxia around the World. J. Environ. Qual. 30, 275. https://doi.org/10.2134/jeq2001.302275x.

Ebersole, J.L., Cappelli, D., 2000. Acute-phase reactants in infections and inflammatory diseases. Periodontology 2000 (23), 19–49. https://doi.org/10.1034/j.1600-0757.2000.2230103.x.

Eckersall, P.D., Bell, R., 2010. Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. Vet. J. 185, 23–27. https://doi.org/10.1016/j.tvjl.2010.04.009.

Franco, L., Romero, D., García-Navarro, J.A., Teles, M., Tvarijonaviciute, A., 2016. Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of Mytilus galloprovincialis exposed to pollutants: analytical validation and effects evaluation by single and mixed heavy metal exposure. Mar. Pollut. Bull. 102, 30–35. https://doi.org/10.1016/j.marpolbul.2015.12.010.

Franco-Martínez, L., Romero, D., Rubio, C.P., Tecles, F., Martínez-Subiela, S., Teles, M., Tvarijonaviciute, A., 2018. New potential biomarkers of oxidative stress in Mytilus galloprovincialis: Analytical validation and overlap performance. Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 221–222, 44–49. https://doi.org/10.1016/j.cbpb.2018.04.006.

Gabay, C., Kushner, I., 1999. Acute-phase proteins and other systemic responses to inflammation. N. Engl. J. Med. 340, 448–454. https://doi.org/10.1056/NEJM199902113400607.

Genser, B., Teles, C.A., Barreto, M.L., Fischer, J.E., 2015. Within- and between-group regression for improving the robustness of causal claims in cross-sectional analysis. Environ. Heal. 14, 60. https://doi.org/10.1186/s12940-015-0047-2.

Ghosh, S., Bhattacharya, S., 1992. Elevation of C-reactive protein in serum of Channa punctatus as an indicator of water pollution. Indian J. Exp. Biol. 30, 736–737. (accessed 28/11/2017). http://www.ncbi.nlm.nih.gov/pubmed/1459653.

Giannetto, A., Maisano, M., Cappello, T., Oliva, S., Parrino, V., Natalotto, A., De Marco, G., Barberi, C., Romeo, O., Mauceri, A., Fasulo, S., 2015. Hypoxia-Inducible Factor α and Hif-prolyl Hydroxylase Characterization and Gene Expression in Short-Time Air-Exposed Mytilus galloprovincialis. Mar. Biotechnol. 17, 768–781. https://doi.org/10.1007/s10126-015-9655-7.

Hennig, F., Fuks, K., Moebus, S., Weinmayr, G., Memmesheimer, M., Jakobs, H., Bröcker-Preuss, M., Führer-Sakel, D., Möhlenkamp, S., Erbel, R., Jöckel, K.-H., Hoffmann, B., Heinz, I.G., 2014. Nixdorf recall study, association between source-specific particulate matter air pollution and hs-CRP: local traffic and industrial emissions. Environ. Health Perspect. 122, 703–710. https://doi.org/10.1289/ehp.1307081.

Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, A., Koutsogiannaki, S., Alamdari, H., Koliakos, G., Dimitriadis, V.K., 2009. Oxidative effects of inorganic and organic contaminants in haemolymph of mussels: utility of recently- developed methods for measuring total antioxidant capacity and protein carbonylation. In: 9Th Symp Oceanogr. Fish. pp. 1324–1329.

Kimbrough, K.L., Lauenstein, G.G., Christensen, J.D., Apeti D.A., (2008). An assessment of two decades of contaminant monitoring in the Nation's Coastal Zone., (2008). http://aquaticcommons.org/2232/1/MWTwoDecades.pdf (accessed 30/09/2016).

Kolberg, J., Høiby, E.A., Jantzen, E., 1997. Detection of the phosphorylcholine epitope in streptococci, Haemophilus and pathogenic Neisseriae by immunoblotting. Microb. Pathog. 22, 321–329. https://doi.org/10.1006/mpat.1996.0114.

MacCarthy, E.M., Burns, I., Irnazarow, I., Polwart, A., Greenhough, T.J., Shrive, A.K., Hoole, D., 2008. Serum CRP-like protein profile in common carp Cyprinus carpio challenged with Aeromonas hydrophila and Escherichia coli lipopolysaccharide. Dev. Comp. Immunol. 32, 1281–1289. https://doi.org/10.1016/j.dci.2008.04.004.

Martinez-Subiela, S., Eckersall, P.D., Campbell, F.M., Parra, M.D., Fuentes, P., Cerón Madrigal, J.J., 2007. A time-resolved immunofluorometric assay for porcine C-reactive protein quantification in whole blood. Luminescence. https://doi.org/10.1002/bio.945.

Martínez-Subiela, S., Tecles, F., Eckersall, P.D., Cerón, J.J., 2002. Serum concentrations of acute phase proteins in dogs with leishmaniasis. Vet. Rec. 150, 241–244. https://doi.org/10.1136/VR.150.8.241.

Masuda, T., Murata, M., Honma, S., Iwazu, Y., Ogura, M., Onishi, A., Shimada, K., Kusano, E., Asano, Y., 2008. Nocturnal hypoxia is associated with elevated C-reactive protein in dialysis patients. Clin. Kidney J. 1, 470–471. https://doi.org/10.1093/ndtplus/sfn156.

Mosca, F., Narcisi, V., Calzetta, A., Gioia, L., Finoia, M.G., Latini, M., Tiscar, P.G., 2013. Effects of high temperature and exposure to air on mussel (Mytilus galloprovincialis, Lmk 1819) hemocyte phagocytosis: Modulation of spreading and oxidative response. Tissue Cell 45, 198–203. https://doi.org/10.1016/j.tice.2012.12.002.

Muñoz Prieto, A., Tvarijonaviciute, A., Escribano, D., Martínez-Subiela, S., Cerón, J.J., 2017. Use of heterologous immunoassays for quantification of serum proteins: The case of canine C-reactive protein. PLoS One 12. https://doi.org/10.1371/journal.pone.0172188.

Olafsen, J.A., 1995. Role of lectins (C-reactive protein) in defense of marine bivalves against bacteria. Adv. Exp. Med. Biol. 371A, 343–348. (accessed November 30, 2017). http://www.ncbi.nlm.nih.gov/pubmed/8525940.

Olafsen, J.A., 1995. Role of lectins (C-reactive protein) in defense of marine bivalves against bacteria. Adv. Mucosal Immunol. 343–348. https://doi.org/10.1007/978-1-4615-1941-6_72.

Onishi, T., Shimizu, T., Kajikawa, T., 1994. Simple and efficient purification of C-reactive protein from canine serum. J. Vet. Med. Sci. 56, 417–419. (accessed May 16, 2018). http://www.ncbi.nlm.nih.gov/pubmed/8075242.

Padhy, G., Sethy, N.K., Ganju, L., Bhargava, K., 2013. Abundance of plasma antioxidant proteins confers tolerance to acute hypobaric hypoxia exposure. High Alt. Med. Biol. 14, 289–297. https://doi.org/10.1089/ham.2012.1095.

Parra, M.D., Tuomola, M., Cabezas-Herrera, J., Cerón, J.J., 2006. Analytical and clinical validation of a time-resolved immunofluorometric assay (TR-IFMA) for canine C-reactive protein in serum. Vet. Res. Commun. 30, 113–126. https://doi.org/10.1007/s11259-006-3201-z.

Pepys, M.B., Hirschfield, G.M., 2003. C-reactive protein: a critical update. J. Clin. Invest. 111, 1805–1812. https://doi.org/10.1172/JCI200318921.

Pepys, M.B., De Beer, F.C., Milstein, C.P., March, J.F., Feinstein, A., Butress, N., Clamp, J.R., Taylor, J., Bruton, C., Fletcher, T.C., 1982. C-reactive protein and serum amyloid P component in the plaice (Pleuronectes platessa L.), a marine teleost, are homologous with their human counterparts. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 704, 123–133. https://doi.org/10.1016/0167-4838(82)90139-X.

Pontet, M., Engler, R., Jayle, F., 1978. One step preparation of both human C-reactive protein and CIt. FEBS Lett. 88, 1–4.

Rosner, B., 2011. Fundamentals of Biostatistics. MA: Brooks, Boston.

Rossi, G., Barnoud, J., Monticelli, L., 2014. Polystyrene nanoparticles perturb lipid membranes. J. Phys. Chem. Lett. 5, 241–246. https://doi.org/10.1021/jz402234c.

Rückerl, R., Greven, S., Ljungman, P., Aalto, P., Antoniades, C., Bellander, T., Berglind, N., Chrysohoou, C., Forastiere, F., Jacquemin, B., von Klot, S., Koenig, W., Küchenhoff, H., Lanki, T., Pekkanen, J., Perucci, C.A., Schneider, A., Sunyer, J., Peters, A., 2007. AIRGENE study group, air pollution and inflammation (Interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. Environ. Health Perspect. 115, 1072–1080. https://doi.org/10.1289/ehp.10021.

Sericano, J.L., Wade, T.L., Sweet, S.T., Ramirez, J., Lauenstein, G.G., 2014. Temporal trends and spatial distribution of DDT in bivalves from the coastal marine environments of the continental United States, 1986–2009. Mar. Pollut. Bull. 81, 303–316. https://doi.org/10.1016/j.marpolbul.2013.12.049.

Shang, Y., Mu, L., Guo, X., Li, Y., Wang, L., Yang, W., Li, S., Shen, Q., 2014. Clinical significance of interleukin-6, tumor necrosis factor-α and high-sensitivity C-reactive protein in neonates with hypoxic-ischemic encephalopathy. Exp. Ther. Med. 8, 1259–1262. https://doi.org/10.3892/etm.2014.1869.

Sinha, S., Mandal, C., Allen, A.K., Mandal, C., 2001. Acute phase response of C-reactive protein of Labeo rohita to aquatic pollutants is accompanied by the appearance of distinct molecular forms. Arch. Biochem. Biophys. 396, 139–150. https://doi.org/10.1006/abbi.2001.2592.

Tvarijonaviciute, A., Aznar-Cayuela, C., Rubio, C.P., Ceron, J.J., López-Jornet, P., 2016. Evaluation of salivary oxidate stress biomarkers, nitric oxide and C-reactive protein in patients with oral lichen planus and burning mouth syndrome. J. Oral Pathol. Med. https://doi.org/10.1111/jop.12522.

Vidal-Liñán, L., Bellas, J., 2013. Practical procedures for selected biomarkers in mussels, Mytilus galloprovincialis - Implications for marine pollution monitoring. Sci. Total Environ. 461–462, 56–64. https://doi.org/10.1016/j.scitotenv.2013.04.079.

Viehmann, A., Hertel, S., Fuks, K., Eisele, L., Moebus, S., Möhlenkamp, S., Nonnemacher, M., Jakobs, H., Erbel, R., Jöckel, K.-H., Hoffmann, B., 2015. Heinz Nixdorf Recall Investigator Group, Long-term residential exposure to urban air pollution, and repeated measures of systemic blood markers of inflammation and coagulation. Occup. Environ. Med. 72, 656–663. https://doi.org/10.1136/oemed-2014-102800.

Volanakis, J.E., Kaplan, M.H., 1971. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. Exp. Biol. Med. 136, 612–614. https://doi.org/10.3181/00379727-136-35323.

Whicher, J., Westacott, C., 1992. The acute phase response. London In: Biochem. Inflamm. pp. 243–272.

Widdows, J., Donkin, P., Staff, F.J., Matthiessen, P., Law, R.J., Allen, Y.T., Thain, J.E., Allchin, C.R., Jones, B.R., 2002. Measurement of stress effects (scope for growth) and contaminant levels in mussels (Mytilus edulis) collected from the Irish Sea. Mar. Environ. Res. 53, 327–356. https://doi.org/10.1016/S0141-1136(01)00120-9.

Yilmaz Avci, A., Avci, S., Lakadamyali, H., Can, U., 2017. Hypoxia and inflammation indicate significant differences in the severity of obstructive sleep apnea within similar apnea-hypopnea index groups. Sleep Breath. 21, 703–711. https://doi.org/10.1007/s11325-017-1486-5.

Yu, X., Ladewig, S., Bao, S., Toline, C.A., Whitmire, S., Chow, A.T., 2018. Occurrence and distribution of microplastics at selected coastal sites along the southeastern United States. Sci. Total Environ. 613–614, 298–305. https://doi.org/10.1016/j.scitotenv.2017.09.100.