Fish energy budget under ocean warming and flame retardant exposure

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Abstract

Climate change and chemical contamination are global environmental threats of growing concern for the scientific community and regulatory authorities. Yet, the impacts and interactions of both stressors (particularly ocean warming and emerging chemical contaminants) on physiological responses of marine organisms remain unclear and still require further understanding. Within this context, the main goal of this study was to assess, for the first time, the effects of warming (+5 °C) and accumulation of a polybrominated diphenyl ether congener (BDE-209, brominated flame retardant) through dietary exposure on energy budget of the juvenile white seabream (*Diplodus sargus*). Specifically, growth (G), routine metabolism (R), excretion (faecal, F and nitrogenous losses, U) and food consumption (C) were calculated to obtain the energy budget. The results demonstrated that the energy proportion spent for G dominated the mode of the energy allocation of juvenile white seabream (56.0-67.8%), especially under the combined effect of warming plus BDE-209 exposure. Under all treatments, the energy channelled for R varied around 26% and a much smaller percentage was channelled for excretion (F: 4.3-16.0% and U: 2.3-3.3%). An opposite trend to growth was observed to faeces, where the highest percentage (16.0±0.9%) was found under control temperature and BDE-209 exposure via diet. In general, the parameters were significantly affected by increased temperature and flame retardant exposure, where higher levels occurred for: i) wet weight, relative growth rate, protein and ash contents under warming conditions, ii) only for O:N ratio under BDE-209 exposure via diet, and iii) for feed efficiency, ammonia excretion rate, routine metabolic rate and assimilation efficiency under the combination of both stressors. On the other hand, decreased viscerosomatic index (VSI) was observed under warming and lower fat content was observed under the combined effect of both stressors. Overall, under future warming and chemical contamination conditions, fish energy budget was greatly affected, which may dictate negative cascading impacts at population and community levels. Further research combining other climate change stressors (e.g. acidification and hypoxia) and emerging chemical contaminants are needed to better understand and forecast such biological effects in a changing ocean.

Keywords: ocean warming; BDE-209; white seabream; energy budget; metabolism.

Ethical statement

Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University (ORBEA) and were conducted in accordance with legal regulations (EU Directive 2010/63).

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1. Introduction

Climate change is one of the greatest environmental challenges that cause profound and diverse impacts on marine organisms and ecosystems (IPCC, 2014). Anthropogenic activities are strongly influencing climate, primarily through fossil fuels, industrial, agricultural, and other land use emissions that alter atmospheric carbon dioxide (CO_2). Hence, one direct consequence of cumulative and continuing emissions of CO_2 since the industrial revolution is the increase of surface ocean temperatures (Doney *et al.*, 2012). According to the latest report of the Intergovernmental Panel on Climate Change (IPCC), the average temperatures on the Earth's surface are expected to rise between 0.3 °C and 4.8 °C by the end of the 21st century with strong local variations. Additionally, it is predicted that extreme high temperature events (heat waves) will become more extreme and frequent (IPCC, 2014). Ocean warming represents a major threat to many marine organisms deeply affecting their physiological responses, namely growth, feeding, acid–base balance, metabolism and behaviour in ways that often compromise species fitness and survival (Pörtner and Peck, 2010; Rosa *et al.*, 2012a). Thus, temperature is one of the main abiotic factors that can lead to significant changes on the energy budget of an organism (Yurista, 1999). The energy budget describes the energy intake and expenditure within the whole organism, leading to the partitioning of ingested energy into the major physiological components of the energy budget equation: C=G+R+F+U, where energy consumed in form of food (C) is the sum of energy retained in materials deposited during growth (G), energy spent for respiration (R), energy losses through faeces (F) and energy losses via nitrogenous excretory products (U; non-faecal) (Jobling, 1994). At optimal temperature conditions, an organism allocates a great part of its energy input into vital functions, as well as in the build-up of energy storage. In contrast, under stress (sub-optimal high temperatures), fish cannot consume enough food to meet, for example, the increasing metabolic costs, whereby energy is often limited and can only cover costs of essential functions, such as standard metabolism (Clarke and Johnston, 1999). Indeed, warming greatly affect metabolic rate of fish, since there is a dependent relation between the variables (temperature-oxygen) and oxygen supply becomes limited at high temperature borders (Pörtner and Peck, 2010; Rosa *et al.*, 2014). Consequently, all of biological processes are directly affected

by temperature, namely food intake, nutritional efficiency and whole-body composition (Burel *et al.*, 1996; Fang *et al.*, 2010). Zheng and their collaborators (2008) also reported that growth rate and faecal production increased in fish species at higher temperatures, while nitrogen excretion decreased, leading to a higher O:N ratio (ratio of oxygen consumed to nitrogen excreted). On the other hand, ocean warming impacts are also expected to directly influence the availability and toxicological effects of marine pollutants (Marques *et al.*, 2010). Recent studies, evidenced that marine fish species tend to accumulate emerging chemical contaminants in warmer conditions (Maulvault *et al.*, 2016, 2017). Such trend was demonstrated in our recent work with seabass (*Dicentrarchus labrax*) juveniles exposed to methylmercury (MeHg) via diet, with specimens exposed to warmer temperatures (22 °C) showing higher levels of MeHg in different tissues (muscle, liver and brain; Maulvault *et al.*, 2016). The contaminants of emerging concern are a subject of growing interest for the scientific community and regulatory authorities, since constitute a risk for human health and biota, and for which there is still insufficient scientific knowledge (Cruz *et al.*, 2015). Among these contaminants, polybrominated diphenyl ethers (PBDEs) are an important class of flame retardants (FRs) consisting of 209 congeners, which have been commercialized as penta-, octa-, and decabrominated mixtures. PBDEs are additive flame retardants, i.e. they are not bound to the polymer, but have been widely used in a variety of products (e.g. plastics, furniture, vehicles) to make them less combustible and to retard flames (Alaee *et al.*, 2003). These FRs are persistent in the environment, accumulate in food chains and show toxic effects on hormonal regulation and affect neuronal, thyroid and liver-related activities (Costa and Giordano, 2011). Hence, these substances are the subject of a wide range of toxicologic and ecotoxicologic studies. Due to these concerns of environmental persistence, bioaccumulation and toxicity, lower brominated PBDEs have been banned in several countries, but the congener decabromodiphenyl ether (BDE-209), primary constituent of deca-BDE, is still used. Nonetheless, it is worth noting that some European countries (e.g. Norway) or states in the U.S.A. (e.g. Maine) have recently banned their use (Costa and Giordano, 2011). Despite the lower levels of BDE-209 found in water, seafood has been identified as the major dietary source of this compound for human uptake, since it can be biotransformed to lower and more toxic congeners

via the food web (Kierkegaard *et al.*, 1999; Elliott *et al.*, 2005; Stapleton *et al.*, 2006). Several studies have addressed the effects of water temperature on energy budget of some fish species (e.g. Xie *et al.*, 2011), but the conclusions were not consistent among them, and others only focused on the effects of chemical contaminants under climate change (e.g. Maulvault *et al.*, 2016, 2017; Sampaio *et al.*, 2018) without ever reporting the effects on energy budgets. Yet, a small number of studies investigated the effects of BDE-209 dietary exposure only on growth of marine species (Kuo *et al.*, 2010; Zhang *et al.*, 2013; Sha *et al.*, 2015) and, to our knowledge, there are no studies that investigated the interactive effects of both stressors (ocean warming and the presence of emerging chemical contaminants) in the perspective of fish bioenergetics. In this context, the main goal of this study was to investigate, for the first time, the effects of ocean warming (+5 °C) and the dietary exposure to the emerging BDE-209 (60 ng g^{-1} dry weight, dw) on the energy budget of juvenile white seabream (*Diplodus sargus*), by measuring growth, routine metabolism, excretion and food consumption. This species was selected as a suitable biological model to evaluate these impacts since it generally inhabits coastal areas particularly susceptible to hydrographic changes and where climate change effects may certainly pose greater ecological and toxicological challenges (Marques *et al.*, 2010; Rosa *et al.*, 2012b). Additionally, this seabream species plays an important role in the coastal food web, as they link top predators with species occupying low trophic levels.

2. Materials and methods

2.1 Experimental design and sampling

Juvenile white seabream (*Diplodus sargus*) specimens from the same batch and with similar biometric characteristics (6.2±0.6 cm total length; 3.9±1.2 g total weight; mean ± standard deviation) were reared at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal) and transported in thermal isolated containers, with constant aeration, to Guia Marine Laboratory (MARE-FCUL, Cascais, Portugal). Upon arrival, fish were randomly distributed in 12 rectangular incubating glass tanks (98×33×24.7 cm; 100 L total capacity each), each with independent recirculating aquaculture systems (RAS). Each RAS

was equipped with biological filtration (model FSBF 1500, TMC Iberia, Portugal), physical filtration (protein skimmer; ReefSkimPro, TMC-Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal), independent and automatic temperature (Frimar, Fernando Ribeiro Lda, Portugal) and pH control (model Profilux 3.1 N, GHL, Germany) via solenoid valves system. Each incubation system (n=12) had independent pH measurements, by means of pH electrodes (n=24 in total), connected to the Profilux system apparatus. pH values were monitored every 2 s and lowered by injection of a certified CO_2 gas mixture (Air Liquide, Portugal) via air stones or increased by tank aeration with CO_2-filtered (using soda lime, Sigma-Aldrich) air. Seawater used in the RAS was filtered (0.35 μm) and UV sterilized (Vecton 600, TMC Iberia, Portugal). Ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels were monitored regularly, by means of colorimetric tests kits (Tropic Marin, USA) and kept below 0.05 mg L^{-1}, 0.20 mg L^{-1} and 2.0 mg L^{-1}, respectively. In order to avoid physiological stress related to high animal density, fish density was kept below 5 g body weight L^{-1} in each incubation tank. Daily, mortality was registered in each treatment and seawater was partially replaced (around 20%).

Fish were acclimated to laboratory conditions during two weeks before the start of the experiment at the following abiotic conditions: dissolved oxygen (DO) above 5 mg L^{-1}; temperature=19±0.4 °C; pH=8.06±0.10; salinity=35±1‰ (WTW handheld Meter Multi 350i, Germany) and photoperiod of 12 h light and 12 h dark (12L:12D). Five days before initiating the climate change exposure scenarios, seawater temperature was slowly raised (1 °C per day) until reaching 24 °C in tanks simulating warming conditions, in order to allow specimens to acclimate at this temperature before the beginning of the trial. No mortality occurred during the acclimation period. Fish were exposed to four scenarios during 56 days to understand future ocean changes expected for 2100 (i.e. seawater warming, Δ=5 °C according to IPCC projections scenario RCP8.5; IPPC, 2014): i) Scenario 1 (S1) – 19 °C_Control, i.e. seawater temperature set at 19 °C (control; current conditions used in juvenile seabream rearing in Iberian Peninsula) and animals fed with the control diet; ii) Scenario 2 (S2) – 24 °C_Control – seawater temperature set at 24 °C (warming scenario) and animals fed with the control diet; iii) Scenario 3 (S3) – 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; and iv)

Scenario 4 (S4) – 24 °C_BDE-209 exposure – seawater temperature set at 24 °C (warming scenario) and animals fed with BDE-209 contaminated diet. Each treatment comprised three independent replicate tanks (n=17 animals per replicate/tank, i.e. 51 animals per treatment). During the experimental period, specimens were fed with 2% of the average body weight (divided in two meals per day) with the respective diets (non-contaminated for control treatments and during acclimation period for all treatments, and contaminated with BDE-209 for contaminated treatments during the trial). Individual weekly food rations were adjusted according to fish size and biomass in each tank throughout the experiment. Before each sampling, fish were fasted for 24 hours and anaesthetized using tricaine methanesulfonate (70 mg L^{-1} of MS-222; Sigma-Aldrich, St Louis, MO, USA) buffered with sodium bicarbonate ($NaHCO_3$; Sigma-Aldrich) using a ratio of 1:1 to reduce fish stress.

In the beginning and end of the experiment (T0 and T56), all fish were fasted for 24 h to allow evacuation of faeces before bulk-weighing. Additionally, fish were weighed weekly to adjust feed rations in all treatments. Before weighting, fish were anaesthetized using tricaine methanesulfonate (MS222 solution; 2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ and 1 g of MS222 per litre of seawater). Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University and conducted according to legal regulations (EU Directive 2010/63). Five specimens per replicate/tank (n=15 per treatment) were randomly weighted and sampled at the beginning and end of the experiment and frozen at -20 °C for chemical analysis and assessment of energy content. All samples (whole fish) were freeze-dried for 48 h, at -50 °C with low vacuum pressure (approximately 10^{-1} atm; Power Dry 150 LL3000, Heto, Czech Republic), homogenized and kept at -80 °C until further analyses. Also, biometric data (total weight and total length) was registered at T0 and T56 (Table 2).

2.2. Feed

Non-contaminated (control) and contaminated dry inert pellets (BDE-209-spiked) with the same nutritional composition were manufactured by a specialized feed producing company (SPAROS

Lda, Olhão, Portugal), considering the nutritional requirements for juvenile white seabream (detailed formulation and chemical composition can be consulted in Table A.1). Briefly, all ingredients were grinded (below 200 µm) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Ingredients and fish oil were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain), and further humidified with 25% deionized water at room temperature. The diet was extruded at 3.0 mm by means of a low-shear extruder (P55, Italplast, Italy). Upon extrusion, feed pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). A batch of this control diet was contaminated with BDE-209. A contaminant stock solution was prepared beforehand, by solubilising the contaminant in a selenium solution (250 µg/mL) and further diluted with acetone, in order to achieve a BDE-209-enriched feed with a nominal concentration of 60 ng g^{-1} on a dry weight basis (dw). This stock solution was further mixed in fish oil (total volume of 200 mL) and this solution was top-coated to the pellets with a pressurized spraying container (standard flat-fan nozzle; size 250 µm; pressure 3.1 bar). Contaminated diet was stored in a dark and cool place during its usage (about 1.5 month). Contamination level of this PBDE congener was systematically assessed during the experiment, by quantification of BDE-209 content in both feeds, using a gas chromatography-mass spectrometry (GC-MS; Agilent 7890B GC coupled to an Agilent 7000C triple quadrupole mass spectrometer), as described in detail by Cruz *et al.* (2017). The concentration of BDE-209 in control and contaminated diets were below limit of detection (LOD) and 60.52±1.55 ng g^{-1} dry weight (dw; mean±standard deviation, n=3), respectively. The concentration of BDE-209 in the contaminated diet was considered to be ecologically relevant and representative of high levels (i.e. ~10x higher the average concentrations) of this congener contamination found in marine species inhabiting contaminated coastal areas (e.g. Noyes and Stapleton, 2014; Airaksinen *et al.*, 2015; Cruz *et al.*, 2015).

2.3. Biochemical analyses

Dry matter, ash, total lipids, crude protein and gross energy were determined in the diet and fish body according to the Association of Official Analytical Chemists methods (AOAC, 2005). The

contents of crude protein and gross energy were also determined in the faeces. Briefly, moisture was determined by drying the sample overnight at 105 °C (lab heater, P-Selecta 207, Portugal); ash by incineration in a muffle furnace at 500 °C for 16 h (Heraeus Hanau, TYP. MR170, Germany); and total lipids were determined with the Soxhlet extraction method using ethyl ether. Crude protein (N×6.25) was analysed using the Dumas method, in a nitrogen analyser (LECO, Model FP-528, St Leco Corporation, St. Joseph, MI, USA) calibrated with EDTA. Gross energy was determined by combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany) calibrated with benzoic acid. All chemical analyses were carried out in duplicate.

2.4. Excretion

2.4.1. Determination of faecal losses

During the 56 days of each experiment, faeces were collected daily by siphoning before feeding, oven-dried at 65ºC, weighted, homogenized and stored at -20°C for analysis of energy and nitrogen content. The faeces collection from each tank were pooled (n=3 per treatment) and measured by combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany).

2.4.2. Determination of nitrogenous losses (non-faecal excretion)

Energy loss through excreted nitrogenous compounds (ammonia-N) was determined at the end of the experiment using 5 fish enclosed in 500 mL glass bottles for 1 h for each treatment. Controls without fish (two blanks per treatment) were also used and ammonia levels were measured to evaluate the potential loss of nitrogenous compounds through bacterial action or diffusion in the experimental tanks. The bottles were filled with oxygen saturated seawater and sealed without any air bubbles. Trials were carried out in the same conditions (temperature, pH and salinity) of each treatment. The seawater was filtered and frozen in 20 mL plastic flasks for ammonia quantification. Ammonia concentration was determined according to Berthelot method (Grasshoff, 1983). Samples were treated with alkaline citrate, sodium hypochloride and fenol in

the presence of sodium nitroprussiate which catalyses the reaction. The blue colour formed by indofenol plus ammonia reaction was measured at 630 nm. Ammonia excretion rate (AER; expressed as mg kg^{-1} dw L^{-1} h^{-1}) was calculated as follows:

$AER=\Delta[NH_4^+]\times VH_2O/DW/\Delta T$,

where $\Delta[NH_4^+]$ is the difference between the ammonia levels in the sample and blank, VH_2O is the volume of water (L) in the glass bottle, DW is the fish dry weight, and ΔT is the time duration of the trial (h).

2.5. Metabolism

Oxygen consumption rates (routine metabolic rates; RMRs) were determined according to previously established methods (Rosa and Seibel, 2008, 2010; Anacleto *et al.*, 2014) with some modifications. At day 56 of the experiment, respiration trials were conducted in an intermittent flow-through respirometer system to measure oxygen consumption rates and to determine RMRs. Respiration chambers were filled with seawater from the experimental tank to ensure the correct temperature, pH and dissolved oxygen levels and placed in thermostable water baths (Lauda, Lauda-Königshofen, Germany) in order to control the temperature. The seawater parameters were controlled using a WTW hand-held Meter Multi 350i compact (WTW, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) in order to ensure that conditions were maintained under the correct parameters levels. Oxygen levels within the respirometers remained above 70% air saturation at all times. Juvenile fish were fasted overnight before being placed in the respirometers and allowed to acclimate (10 min.) before data collection for stabilisation of oxygen uptake rates. Individual fish from each experimental tank were individually weighted (range: 5.3–13.4 g wet weight; ww) and placed into separate cylindrical respirometers (7.1 cm diameter, 15 cm length, 15 cm width, 14 cm depth, 545 mL volume; Loligo Systems, Tjele, Denmark), with a removable lid for introduction and removal of the fish. One of the chambers was used as control for each treatment and the oxygen consumption observed in this blank chamber was then subtracted from the values obtained in chambers containing fish. Six fish were used from each experimental tank/treatment. Respirometers were connected to an automated flush pump that

flushed the respirometers with air-saturated seawater until attaining initial oxygen levels. The concentration of dissolved oxygen in the respirometers were monitored and recorded with an fibre-optic system and contactless oxygen sensor spots (FireSting O_2, PyroScience, GmbH, Aachen, Germany) using the Pyro Oxygen Logger software. Continuous monitoring of oxygen concentration in the chambers was performed visually through real-time graphics on the computer monitor. The respirometers with fish were covered with a black bag to reduce light levels and disturbance. Calibrations were performed with seawater containing 100% air saturation representing full oxygen saturation. Oxygen consumption rates were determined according to the decline in oxygen concentration in the respirometers between flush cycles. Intermittent flow enables measurements to be taken over an extended period of time without depleting dissolved oxygen concentrations in the respirometer below normoxic concentrations. Fish were returned to their respective holding tanks following respirometry. Respirometers were cleaned regularly to ensure that background microbial respiration remained negligible. Metabolic data were analysed after importing the text file from the FireSting O_2 software into Excel 2013. Linear regressions between water oxygen concentration and time were made for each measurement period and the slopes derived from the regressions were used to calculate routine metabolic rate (RMR, mg O_2/kg body wet weight/h) according to the following equation:

$$RMR = (b \times V \times t)/W$$

where b is the slope of the oxygen reduction curve (minus the blank control), V is the volume of the respirometer minus the volume of the fish assuming that 1 kg of seawater is equivalent to 1 kg of fish (in L), t is the time interval over which O_2 is assessed (in hours) and W is the wet weight of fish (in kg). Additionally, O:N ratios were calculated by dividing the amount (atoms) of oxygen consumed by the nitrogen excreted, being used to estimate what substrates (carbohydrate, protein and lipid) the organisms use for metabolism (Babarro *et al.,* 2000):

$$O{:}N = (RMR/16)/\,(AER/17)$$

2.6. Data calculation

2.6.1. Survival and animal fitness

Survival rate (SR, %) was calculated according to the following equation:

$$SR = final\ number\ of\ fish/initial\ number\ of\ fish \times 100$$

The Fulton's condition index (K) was directly calculated from the biometric data to determine fish condition, according to the formula (Ricker, 1975):

$$K = TW/TL^3 \times 100$$

where TW is the fish total weight (g wet mass) and TL is the total length (cm).

The relationship between fish total weight and the respective organ weight was calculated for the liver (i.e. hepatosomatic index, HSI) and the viscera (i.e. viscerosomatic index, VSI), in order to provide information on liver and viscera condition, using the following equations:

$$HSI\ (\%) = liver\ wet\ weight/total\ fish\ wet\ weight\ (g) \times 100$$

$$VSI\ (\%) = viscera\ wet\ weight\ (g)/total\ fish\ wet\ weight\ (g) \times 100$$

2.6.2. Growth performance

Relative growth rate (RGR, %), specific growth rate in terms of protein (SGRp, % day^{-1}) and energy (SGRe, % day^{-1}), feed intake (FI, % body weight day^{-1}) and feed efficiency (FE) were calculated according to the following equations (Fang *et al.*, 2010):

$$RGR = [(W2 - W1)/W1]] \times 100$$

$$SGRp = [(lnP2 - lnP1)/t] \times 100$$

$$SGRe = [(lnE2 - lnE1)/t] \times 100$$

$$FI = [F/(t \times (W2 + W1/2))] \times 100$$

$$FE = (W2 - W1)/F$$

where W2 and W1 were the final and initial body weight of the fish, respectively, P2 and P1 were final and initial protein content, respectively, E2 and E1 were final and initial energy content, respectively, t was the duration of the experiment (days) and F was the dry weight of feed intake during the experimental period (g).

2.6.3. Energy budgets

Energy budgets were calculated according the model proposed by Carfoot (1987):

C=G+F+U+R,

where C is energy consumed, G is energy accumulated for growth, F is the energy loss in faeces, U is the energy loss as ammonia excretion; and R is the energy loss through respiration. Value of G in the budget equation was calculated as follows:

G=(W2×E2)–(W1×E1)

where W2 and W1 are the final and initial wet body weights of fish (g), respectively; and E2 and E1 are final and initial energy contents of the fish (J g^{-1}), respectively.

The energy values of F were determined using an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). For determination of value of U, ammonia-N levels (AER) were converted into energy by multiplying the coefficient of 24.83 kJ g^{-1} N (Elliott, 1976). The oxygen consumption was converted to energy (value of R) using a conversion factor of 13.84 J mg^{-1} O_2 (442.88 J $mmol^{-1}$ O_2) (Guinea and Fernandez, 1997).

The assimilation efficiency (K_1, %) and net growth efficiency (K_2, %) were calculated using the following equations (Ye *et al.*, 2009):

$$K1 = [(G + R)/(G + R + U)] \times 100$$

$$K2 = [G/(G + R)] \times 100$$

2.7. Statistical analysis

All data were checked for normality of distribution and homogeneity of variances using Kolmogorov–Smirnov and Levene's tests, respectively. Two-way analysis of variance (ANOVA) was used to analyse the effects of temperature and exposure to dietary BDE-209 on energy budget parameters. One-way ANOVA, followed by post-hoc tests (Tukey HSD) or non-parametric multiple comparison tests (Dunn's) were performed to analyse differences in indices (K, HSI, VSI, SR, RGR, SGR, FI and FE) and energy budget parameters (C, G, F, U and R) among treatments. The correlation between variables (energy budget parameters and other indexes, temperature and BDE-209 contaminated diet) was tested by Pearson's correlation coefficients.

Differences were considered significant at a probability levels of $p<0.05$, using STATISTICA™ software (Version 10.0, StatSoft Inc., Tulsa, Oklahoma, USA). All data were expressed as mean±standard deviation (mean±SD).

3. Results

3.1. Survival and fitness

Throughout the 56-day experiment, the survival rate of juvenile white seabream ranged between 88.2% and 94.1% in treatments 19 °C_BDE-209 exposure and 24 °C_BDE-209 exposure, respectively (Table 1). There was no significant differences in survival rate between the four treatments ($p>0.05$). The mortality rate observed in all treatments was mainly caused by cannibalism as this species demonstrated a normal aggressive behaviour.

Insert Table 1

Despite no significant differences were observed in Fulton's condition (K) and hepatosomatic index (HSI) between the four treatments, the viscerosomatic index (VSI) was significantly changed by temperature (two-way ANOVA, $F=6.04$, $p<0.05$; Table A.2), i.e. fish exposed to control conditions (i.e. 19 °C_Control) showed significantly higher VSI (6.87±0.81) than fish subjected to warmer temperatures (i.e. 24 °C_Control; 5.57±1.10; $F=3.06$, $p<0.05$). Comparing to the initial values at the beginning of the experiment, significant differences were also observed for K at 24 °C_BDE-209 exposure ($p<0.05$), HSI at 24 °C_Control and VSI at 19 °C_Control ($p<0.01$; Table 1).

3.2. Biochemical composition

Moisture, protein, fat, ash and gross energy contents in the body of juvenile white seabream exposed to the different treatments are listed in Table 2. Fish body protein content was affected by both temperature and BDE-209 contaminated diet (two-way ANOVA, $F=6.23$, $p<0.05$ and $F=45.95$, $p<0.001$, respectively; Table A.3) as well as fish fat content ($F=44.86$, $p<0.001$ and $F=14.65$, $p<0.01$, respectively). Fish ash was only affected by temperature ($F=22.83$, $p<0.01$). No

significant differences were observed between moisture and gross energy of the fish between the different treatments ($p>0.05$). Yet, there was a significant interaction between temperature and BDE-209 contaminated diet in gross energy and also in protein contents ($F=6.03$, $p<0.05$ and $F=50.50$, $p<0.001$, respectively). In comparison with initial values of biochemical composition, significant differences were detected after 56 days of experiment ($p<0.05$), except for moisture in both treatments at 19 °C, for protein in treatment 24 °C_Control and for ash in treatment 24 °C_BDE-209 exposure. Moisture, protein and ash contents significantly decreased after 56 days of experiment in each treatment, whereas fat and gross energy significantly increased ($p<0.05$).

Insert Table 2

3.3. Growth

Table 1 shows that significant differences were registered in the initial and final body weights of animals among treatments. Fish subjected to warming conditions and BDE-209 exposure (i.e. 24 ºC_BDE-209 exposure) obtained the highest body weights ($Wi=5.95\pm1.25$ g, $F=4.67$, $p<0.01$; $Wf=10.26\pm2.99$ g, $F=3.67$, $p<0.05$), whereas fish exposed to control temperature and BDE-209 exposure (i.e. 19 ºC_BDE-209 exposure) showed the lowest total length ($TL=7.23\pm0.44$ cm, $F=3.93$, $p<0.05$). A strong positive correlation was found between fish total weight and length ($r=0.95$; $p<0.001$; data not shown). The two-way ANOVA analysis also showed that temperature significantly affected final fish weights ($F=5.23$, $p<0.05$) and a significant interaction was registered between temperature and BDE-209 contaminated diet in the initial body weight ($F=9.83$, $p<0.01$; Table A.2).

Feed intake (FI) and specific growth rate in terms of energy and protein (SGRe and SGRp, respectively) did not show significant differences within treatments (Table 1 and Figs. 1B and 1C). However, FE was significantly affected by temperature and BDE-209 contaminated diet (two-way ANOVA, $F=71.16$, $p<0.001$ and $F=23.18$, $p<0.001$, respectively; Table A.2) and RGR was only significantly affected by temperature ($F=27.71$, $p<0.01$; Table A.2). Moreover, a significant interaction between temperature and BDE-209 contaminated diet occurred only for FE

(F=24.52, p<0.001; Table A.2). Fish exposed to warmer temperatures and contaminated diet (i.e. 24 ºC_BDE-209 exposure) revealed significantly higher FE (1.47±0.32; Table 1) and RGR levels (107.1±18.0; Fig. 1A).

Insert Fig. 1

3.4. Excretion and metabolism

Ammonia excretion rate (AER) was significantly affected by temperature and BDE-209 contaminated diet (two-way ANOVA, F=5.34, p<0.05 and F=22.30, p<0.001, respectively; Table A.2). Indeed, fish exposed to warming conditions (i.e. 24 °C_Control) showed significantly higher AER (0.076±0.021 mg NH_4 g^{-1} dw L^{-1} h^{-1}), whereas fish subjected to contaminated diet (i.e. 19 °C_BDE-209 exposure and 24 °C_BDE-209 exposure) presented significantly lower AER (0.084±0.007 and 0.111±0.023 mg NH_4 g^{-1} dw L^{-1} h^{-1}, respectively; F=9.22, p<0.01; Fig. 2A).

Insert Fig. 2

Regarding routine metabolic rates (RMRs), fish subjected to control temperature and BDE-209 exposure displayed significantly lower levels (464±76 mg O_2 kg^{-1} ww h^{-1}; Fig. 2B) than the other treatments (p<0.01). The two-way ANOVA showed that RMRs of juvenile white seabream were significantly affected by both temperature and BDE-209 contaminated diet (F=15.96, p<0.001 and F=17.60, p<0.001; Table A.2) and there was also a significant interaction between temperature and BDE-209 contaminated diet (F=6.48, p<0.05).

O:N ratios of the juvenile white seabream subjected to different treatments are shown in Fig. 2C. The O:N ratios were significantly lower in control diet treatments (15.23±1.52 and 15.83±1.04 in 19 °C_Control and 24 °C_Control, respectively) than in contaminated diet treatments (19.35±1.10 and 21.22±0.90 in 19 °C_BDE-209 exposure and 24 °C_BDE-209 exposure, respectively; F=22.07, p<0.001). The two-way ANOVA revealed that O:N ratio of juvenile white seabream was only significantly affected by BDE-209 exposure (F=60.34, p<0.001; Table A.2).

3.5. Energy budget

Energy intake from feed and energy allocation in percentage of total energy intake is presented in Figs. 3A and 3B. Energy intake showed significant differences within treatments ($p<0.001$): warming conditions and BDE-209 contaminated diet exposure (i.e. 24 °C_BDE-209 exposure) promoted higher energy acquired from feed (992±32 J g^{-1} day^{-1}), whereas treatments with control temperature (i.e. 19 °C_BDE-209 exposure and 19 °C_Control) showed the lowest levels of energy intake (562±5 and 854±2 J g^{-1} day^{-1}, respectively).

Insert Fig. 3

Regarding energy distribution, the highest fraction of energy was allocated to growth (G) in all treatments (between 56.0 and 67.8%; Fig. 3B), followed by respiration (R; around 25-26%) and excretion through faeces (F; between 4.3 and 16.0%) and ammonia (U; between 2.3 and 3.3%). Growth energy was significantly higher under warming and BDE-209 exposure via diet (i.e. 24 °C_BDE-209 exposure=67.8±1.4%; Fig. 3B), compared to the control temperature and BDE-209 exposure via diet (i.e. 19 °C_BDE-209 exposure; 56.0±1.4%; $F=29.85$, $p<0.01$). On the other hand, energy loss via faeces (F) exhibited an opposite trend to growth, with the latter treatment showing the highest percentage (19 °C_BDE-209 exposure=16.0±0.9%; Fig. 3B), while significant lower percentage was observed under 24 °C_BDE-209 exposure (4.3±0.1%; $F=201.47$, $p<0.0001$). Non-significant differences between treatments were observed for energy lost through ammonia excretion and respiration ($p>0.05$).

In general, energy intake and energy distribution were significantly influenced by temperature and emerging BDE-209 exposure via diet ($p<0.05$; Table A.3), except respiration that was not influenced by temperature ($p>0.05$). Concerning assimilation and net growth efficiencies (K_1 and K_2, respectively), no significant differences in K_2 were detected between any treatment ($p>0.05$), whereas K_1 was only significantly lower under 19 °C_BDE-209 exposure (88.9%; $F=109.1$, $p<0.001$; Fig. 3C).

4. Discussion

Several studies have assessed the effects of temperature on the energy budget of different fish species, whereas others only focused on the effects of chemical contaminants under a climate

change context, without ever reporting the effects on energy budgets. Moreover, the interactive effects of these environmental stressors on fish bioenergetics are poorly known and require further understanding. Therefore, this study aimed to attenuate this knowledge gap by providing the first empirical evidence of the combined impacts of warming and contamination on fish energy budget. Since temperature is recognized as the most important abiotic factor affecting physiological functioning of most marine (poikiolothermic) organisms (i.e. body temperature varies over time with environmental temperature), its role on fish energy budget has also been intensively investigated, as in *Sparus aurata* (Requena *et al.*, 1997), *Trematomus bernacchii* (Sandersfeld *et al.*, 2015), *Cynoglossus semilaevis* (Fang *et al.*, 2010), *Oncorhynchus tshawytscha*, *Salvelinus namaycush*, *Oncorhynchus mykiss* (Kao *et al.*, 2015), *Oreochromis niloticus* (Xie *et al.,* 2011), *Rachycentron canadum* (Sun *et al.*, 2006a; Sun and Chen, 2009) and *Gadus morhua* (Kreiss *et al.*, 2015). However, the conclusions on the influence of temperature on energy budget of species were not consistent among these studies.

When an environmental change occurs (ex. warming), an organism needs to perform metabolic adjustments, which will be stressful and have direct consequences on its fitness that is influenced by the energy budget. Here, despite survival, fish condition (K) and hepatosomatic index (HSI) were not significantly affected by temperature, viscerosomatic index (VSI) decreased significantly with warming (Table 1), which is correlated with fat content. Indeed, other authors demonstrate that VSI and fat content increase at low temperature, which may be linked to a deposition of liver glycogen and lipids (Couto *et al.*, 2008; Sandersfeld *et al.*, 2015; Huang *et al.*, 2016). This finding it is not surprising taking into account that temperature can influence fish body composition, by affecting the stability of structural components, particularly lipids and proteins (Van Ham *et al.*, 2003; Fang *et al.*, 2010). Similar results were reported by Brodte *et al.* (2006) for lipid content in the Antartic eelpout. Such response to warming may be led by a shift from lipid- to carbohydrate-based metabolism (Windisch *et al.*, 2011). One can also argue that the lower content of fat in fish under warming (Table 2) maybe also a consequence of enhanced metabolic processes associated to growth (García-Guerrero *et al.*, 2003). In fact, protein and ash contents increased with warming in juvenile white seabream. It is worth noting that the use of the

protein fraction as an energy source for the maintenance of animal metabolic needs only takes place when carbohydrate and lipid reserves have already been greatly depleted (Barber and Blake, 1981). Proteins have an important physiological role in the supply of structural elements and in the catalysis of metabolic reactions (Dittrich, 1991). Regarding gross energy, warming and BDE-209 exposure did not affect the content of *D. sargus*.

Despite no differences were observed in specific growth rate in terms of protein and energy (SGRp and SRGe, respectively; Figs.1B and C), temperature had a significant positive effect on fish growth, namely in terms of an increase in wet weight and relative growth rate (Table 1 and Fig. 1A). It is well known that temperature can affect fish growth directly by controlling feed consumption, nutrient requirements and food passage time (Smith, 1989). While increasing temperature may elicit negative effects on growth due to higher energy cost for maintenance metabolism, may also elicit positive ones due to higher food consumption and digestion rates (Xie and Sun, 1992, 1993). Sandersfeld *et al.* (2015) observed a reduction in mass growth (84%) in Antartic fish *Trematomus bernacchii* at 2 and 4 ºC when compared with the control group at 0 ºC, which can be explained by reduced food assimilation rates at warmer temperatures. Within a suitable temperature range, growth rate increases with increasing temperature, but when the temperature reaches the upper extreme of the tolerated range, usually the opposite happens (Hasan and Macintosh, 1991). Regarding *D. sargus*, its wide continuous distribution range in temperate/subtropical areas indicates that this species tolerates water temperatures until 34 ºC, but the preferred temperatures range between 15 and 17 ºC, which corresponds to the spawning period that generally occurs between March to June (Morato *et al.*, 2003).

Concerning feed efficiency (FE), there was a significant increase under warming (Table 1), which is in accordance with Guerreiro *et al.* (2012) that also obtain higher FE at 22 ºC than at 16 ºC, suggesting that this temperature can be nearer the optimum temperature for the species as FE is usually maximized at temperatures near the optimum for maximum growth of the species (Árnason *et al.*, 2009). Although it is well established that fish eat to meet their energetic demands (Bureau *et al.*, 2002) within limits, they may also adjust feed intake to meet protein requirement (Peres and Oliva-Teles, 1999). On the other hand, our results are contradictory with other authors

that demonstrated a decrease in FE when temperature increased (e.g. *Rachycentron canadum*; Sun *et al.*, 2006a). Other study performed on young grass carp (Cui *et al.*, 1995) showed that temperature did not significantly affect FE. This discrepancy in the results probably resulted from interspecific differences, or from the reason that the designed temperature in the experiment of a given species was not beyond its suitable temperature range.

In relation to the excretion and metabolism rates (AER and RMR, respectively), our results also showed that both increased with warming conditions in juvenile *D. sargus*. The higher levels of AER observed with increased temperature (0.176±0.021 mg NH_4 g^{-1} dw L^{-1} h^{-1}; Fig. 2A) are consistent with previous findings for other fish species (Tidwell *et al.*, 2003; Leung *et al.*, 1999). Also, Forsberg and Summerfelt (1992) have shown that the AER in *Stizostedion vitreum vitreum* juveniles varies between groups exposed to 20 and 25 ºC (30.1 and 45.2 mg NH_4 kg^{-1} h^{-1}, respectively). In comparison to *D. sargus*, AER was slightly higher, varying between 41.7 and 50.0 mg NH_4 g^{-1} h^{-1} at 19 and 24 ºC, respectively (Fig. 2A). This can be due to the fact that at higher temperatures, increases in metabolic rates are partially attained via the transamination and deamination of the amino acids ingested in the diet, resulting in the excretion of ammonium and the release of left-over carbon used as an energy source (Forsberg and Summerfelt, 1992).

The increase in RMR of juvenile *D. sargus* with warming was observed in both control and contaminated treatments, although was only significant under exposure to the flame retardant (i.e. 463.8±75.8 mg O_2 kg^{-1} ww h^{-1} at 19 °C to 634.6±99.5 mg O_2 kg^{-1} ww h^{-1} at 24 °C; Fig. 2B and Table A.2). Since temperature directly affects the rate of all biological processes, this positive trend between the two variables is expected and consistent with the results of previous studies for other fish species, such as *Sparus aurata* (Requena *et al.*, 1997). Indeed, each species has a thermal optimum at which it functions are the best, and increasing temperatures beyond that point will cause metabolic rates to decline (Pang *et al.*, 2015). For example, Chen *et al.* (2006) found that between 6 and 34 ºC, RMR of juvenile common carp drastically decreased above 30 ºC. Moreover, species also has an upper and lower lethal temperatures or critical thermal maxima or minima (CT_{max} and CT_{min}, respectively), beyond which metabolic functions cannot continue. Therefore, the concept of oxygen and capacity-limited thermal tolerance (OCLTT) demonstrated

by Pörtner and Knust (2007) aims to explain this effect of temperature on body functioning. With water temperatures gradually rising as climate change progresses, temperatures may move farther away from species' thermal optima and closer to their thermal maxima, creating unfavourable environmental conditions for many species (Iannucci, 2015). In this study, it is apparent that the temperature have not reached maximum enough (at least 24 °C) to make their metabolic damage to juvenile *D. sargus*.

The O:N ratio is an effective index for assessing the contribution of protein catabolism to total metabolism and can provide indices of balance between the catabolism rates of proteins, carbohydrates, and lipid substrates in animal tissues (Jadhav *et al.*, 2012). This parameter is affected by both endogenous physiological status and exogenous environmental factors (Rocha *et al.*, 2005). High O:N ratio (>30) points out catabolism of carbohydrates and lipids (Bayne, 1976), while low O:N ratio (<30) rather points out protein catabolism, i.e. conditions of alimentary deficiency (Mace and Ansell, 1982). In this study, results showed a prevalence of protein-dominated catabolism in all treatments (between 15.22±1.52 and 21.22±0.90; Fig. 2C). Similar values of O:N ratio were found by Lemos *et al.* (2006) and Rocha *et al.* (2005). On the other hand, Zheng *et al.* (2008) observed a change of O:N ratio from 13.12-20.91 at temperatures of 6, 10, 15 and 20 ºC to 51.37 at 25 ºC for juvenile *Miichthys miiuy*, which is indicative of the change in the metabolic substrate (lipids and carbohydrates instead of proteins) that can be related to lower nitrogen excretion. Within this point of view, it is more cost-effective to catabolize fat rather than protein (1 g will release approximately 9.5 and 4.5 kcal of energy, respectively; Cook *et al.*, 2000), being an adaptive strategy of fish with greater thermal tolerance. Interestingly, O:N ratio in the present study was only affected by BDE-209 exposure and not by warming (Table A.2), whereby fish subjected to BDE-209 contaminated diet exposure presented higher O:N ratio (19.35±1.10 and 21.22±0.90 at 19 and 24 ℃, respectively; Fig. 2C).

In this study, we obtained the following energy budget equations for juvenile white seabream for respective treatments: 100C=62.6G+9.4F+2.5U+25.5R (19 °C_Control); 100C=56.0G+16.0F+2.6U+25.4R (19 °C_BDE-209 exposure); 100C=62.7G+7.4F+3.3U+26.5R

(24 °C_Control); and 100C=67.8G+4.3F+2.3U+25.6R (24 °C_BDE-209 exposure; Fig. 3B). In general, the higher proportion of food energy was allocated to growth (56.0-67.8%) and metabolism (25.4-26.5%), which is in accordance with several studies (e.g. Tang *et al.*, 2003; Sandersfeld *et al.*, 2015), but in inverse order of magnitude, i.e. higher energy channelled for metabolism, followed by growth. Generally, in most studies performed with fish, metabolism costs usually make up 50% of total energy expenditure and growth around 30% (Fang *et al.*, 2010; Xie *et al.*, 2011). Nevertheless, the animals used in this experiment were juvenile fish, which means that species do not reproduce before having reached a certain size. It is known that this species reaches sexual maturity at 2 years, with an approximate size of 17 cm, whereby the energy expenditure of studied animals is only channelled to growth and not yet to reproduction. Therefore, factors influencing energy allocation and thereby growth in this species are likely to have far-reaching consequences for life history. The obtained results also contradict research conducted by Tang *et al.* (2003) regarding to the excretion parameters, i.e. higher energy allocated to faeces (4.3-16.0%), followed by ammonia (2.3-3.3%; Fig. 3B). According to Sun *et al.* (2006a), faeces and nitrogenous excretion only account for a small portion of food energy in carnivorous fish species and do not greatly influence the proportion of food energy allocated to growth, so it is the metabolism that plays an important role in influencing the proportion of energy intake allocated to growth. Other authors also detected opposite findings in relation to the percentages of energy allocation for juvenile fish species, namely *Cynoglossus semilaevis* (Fang *et al.,* 2010), *Rachycentron canadum* (Sun *et al.*, 2006a) and *Trematomus bernacchii* (Sandersfeld *et al.*, 2015). Besides temperature, other factors can influence the energy budget of an organism, such as feed type or composition, ease of finding and consuming food, experimental method, fish body size, current fish life stage, and environment-specific stressors such as predator density (Jobling, 1994; Xie *et al.*, 1997; Tang *et al.*, 2003; Yang *et al.*, 2003). For example, Fang *et al.* (2010) indicated that the highest proportion for growth could be obtained at a ration below satiation or at satiation. Concerning the flame retardant studied, BDE-209 is the most commonly used commercial PBDE, and is of particular concern due to its bioaccumulation and biomagnification capacity via aquatic food web (Noyes *et al.*, 2011). Moreover, this abundant congener can be metabolised into lower

brominated congeners with higher toxicity to aquatic organisms (Kuo *et al.*, 2010). Studies have associated BDE-209 exposure to liver toxicity, reproductive toxicity, endocrine toxicity and potentially carcinogenic effects (Du *et al.*, 2008). However, a limited number of published studies investigated the effects of BDE-209 dietary exposure only on growth of marine species. Zhang *et al.* (2013) observed acute toxic effects on the growth and inter-specific competition of marine bloom-forming microalgae. Sha *et al.* (2015) also detected a significant influence of this congener on the population growth and reproduction parameters of rotifer, *Brachionus plicatilis*. Other authors showed that BDE-209 through dietary exposure during 30 days even at low concentrations (2 $\mu g\ g^{-1}$) affected negatively juvenile fish growth rates (*Coregonus clupeaformis*) by measuring the sagittal otolith increment width (Kuo *et al.*, 2010). To our knowledge, this study was the first that measured all the energy budget parameters to evaluate the potential effects of this contaminant uptake. The presented results revealed that BDE-209 exposure during 56 days significantly affects the energy budget parameters (C, G, F, U and R) as well as the assimilation efficiency (K_1; Table A.3). The combined conditions (i.e. 24 °C_BDE-209 exposure) promoted higher energy acquired from food and also higher energy was particularly channelled to growth, whereas lower energy was conducted to excretion via faeces and ammonia losses (Figs. 3A and 3B).

Conclusions

In conclusion, the present study indicated that warming and BDE-209 contaminated diet exposure had significant effects on the energy budget of juvenile white seabream. The energy partitioned for metabolism varied around 26% under all treatments, and warmer temperatures generally promoted higher wet weight, RGR, protein and ash contents as well as lower VSI, while under flame retardant exposure promoted higher O:N ratio. Nevertheless, the combined effects of both stressors led to higher FE, AER and RMR and lower fat content. Further research combining other climate change stressors (e.g. acidification and hypoxia) and emerging chemical contaminants are needed to better understand and forecast the biological responses in the ocean of tomorrow.

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Competing interests

The authors declare that they have no actual or potential competing interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Figure captions

Fig. 1. A) Relative growth rate (RGR, %) and B,C) specific growth rate in terms of energy and protein (SGRe and SGRp, respectively, % day^{-1}) in juvenile white seabream (*Diplodus sargus*) after 56 days of exposure to warming and contaminant (mean±SD; n=9). Different letters indicate significant differences between treatments (ANOVA test, $p<0.05$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

Fig. 2. Effects of warming and BDE-209 contamination on A) ammonia excretion rate (AER; mg NH_4 g^{-1} dw L^{-1} h^{-1}), B) routine metabolic rate (RMR; mg O_2 kg^{-1} ww h^{-1}) and C) O:N ratio of juvenile white seabream (*Diplodus sargus*) after 56 days of exposure to warming and contaminant (mean±SD; n=6). Different letters indicate significant differences between treatments (ANOVA test, $p<0.01$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

Fig. 3. Effects of warming and BDE-209 contamination on A) energy intake from feed (C; J^{-1} g^{-1} day^{-1}), B) energy allocation (%), C) assimilation efficiency (K_1) and net growth efficiency (K_2) of juvenile white seabream (*Diplodus sargus*) after 56 days of the experiment. Values (mean±SD; n=6) are represented as the percentage of dry body weight. Different letters indicate significant differences between the treatments (ANOVA test, $p<0.05$). Abbreviations: C - energy intake; G - energy deposit for growth; F - energy loss through faeces; U - energy loss through ammonia excretion; R, energy loss through respiration; K_1 - assimilation efficiency; K_2 - net growth efficiency; 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

Table captions

Table 1. Survival, initial wet weight, final wet weight, total length, Fulton's condition index, hepatosomatic and visceral indices, feed intake and feed eficiency of juvenile white seabream (*Diplodus sargus*) in the four treatments after 56 days of experiment.

Values (mean±SD; n=9) with different letters (a-b) in the same row indicate significant differences between treatments (ANOVA test, $p<0.05$). Asterisk (*) represent significant differences between initial K, HSI and VSI and each treatment (t-student, $p<0.05$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet; Wi – Initial wet weight; Wf - Final wet weight; TL – Total length; K – Fulton's condition index; HSI – hepatosomatic index; VSI – viscerosomatic index; FI – Feed intake; FE – Feed eficiency.

Table 2. Effects of warming and BDE-209 contamination on biochemical composition of juvenile white seabream (*Diplodus sargus*) after 56 days of the experiment.

Values (mean±SD; n=3) correspond to the percentage of dry body weight. Different letters in the same row indicate significant differences between the four treatments (ANOVA test, $p<0.05$). Asterisk (*) represent significant differences between initial biochemical composition and each treatment (t-student, $p<0.05$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

Appendix A

Table A.1. Formulation, ingredients and proximate chemical composition (% dry weight, dw) of the experimental diets (control and contaminated) used for juvenile white seabream (mean±standard deviation, n=3). Different letters indicate significant differences between the diets (t-student test, $p<0.05$).

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France;
[4] Chlorella powder: 62.5% CP, 9.2% CF, ALLMICROALGAE, Portugal.
[5] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[6] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain;
[7] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[8] SAVINOR UTS, Portugal
[9] LECICO P 700IPM, LECICO GmbH, Germany.
[10] Guar gum HV109, SEAH International, France.
[11] OceanFeed: 10.2% CP, Ocena Harvest Technology, Ireland.
[12] Paramega PX, Kemin Europe NV, Belgium
[13] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy.
[14] Rapeseed-derived crude glycerol, IBEROL, Portugal.
[15] PREMIX Lda, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.

[16] Calculated as, total carbohydrates (starch, free sugars, cellulose) = 100-(protein+fat+ash).

Table A.2. Results of two-way ANOVA evaluating the effects of temperature (T) and BDE-209 contaminated diet (CD) on survival, initial wet weight (Wi), final wet weight (Wf), total length (TL), Fulton's condition index (K), hepatosomatic and viscerosomatic indices (HSI and VSI, respectively), feed intake (FI), feed efficiency (FE), relative growth rate (RGR), specific growth rate in terms of energy and protein (SGRe and SGRp, respectively), ammonia excretion rate (AER), routine metabolic rate (RMR) and O:N ratio of juvenile white seabream (*Diplodus sargus*). Asterisks indicate significant differences ($p<0.05$; *df*=10).

Table A.3. Results of two-way ANOVA evaluating the effects of temperature (T) and BDE-209 contaminated diet (CD) on biochemical composition, energy budget parameters (C, G, F, U and R), assimilation and net growth efficiencies (K_1 and K_2, respectively) of juvenile white seabream (*Diplodus sargus*). Asterisks indicate significant differences ($p<0.05$; *df*=10).

Table A.4. Results of two-way ANOVA evaluating the effects of temperature (T) and BDE-209 contaminated diet (CD) showing the results of normality and homogeneity of variances tests (p-values).

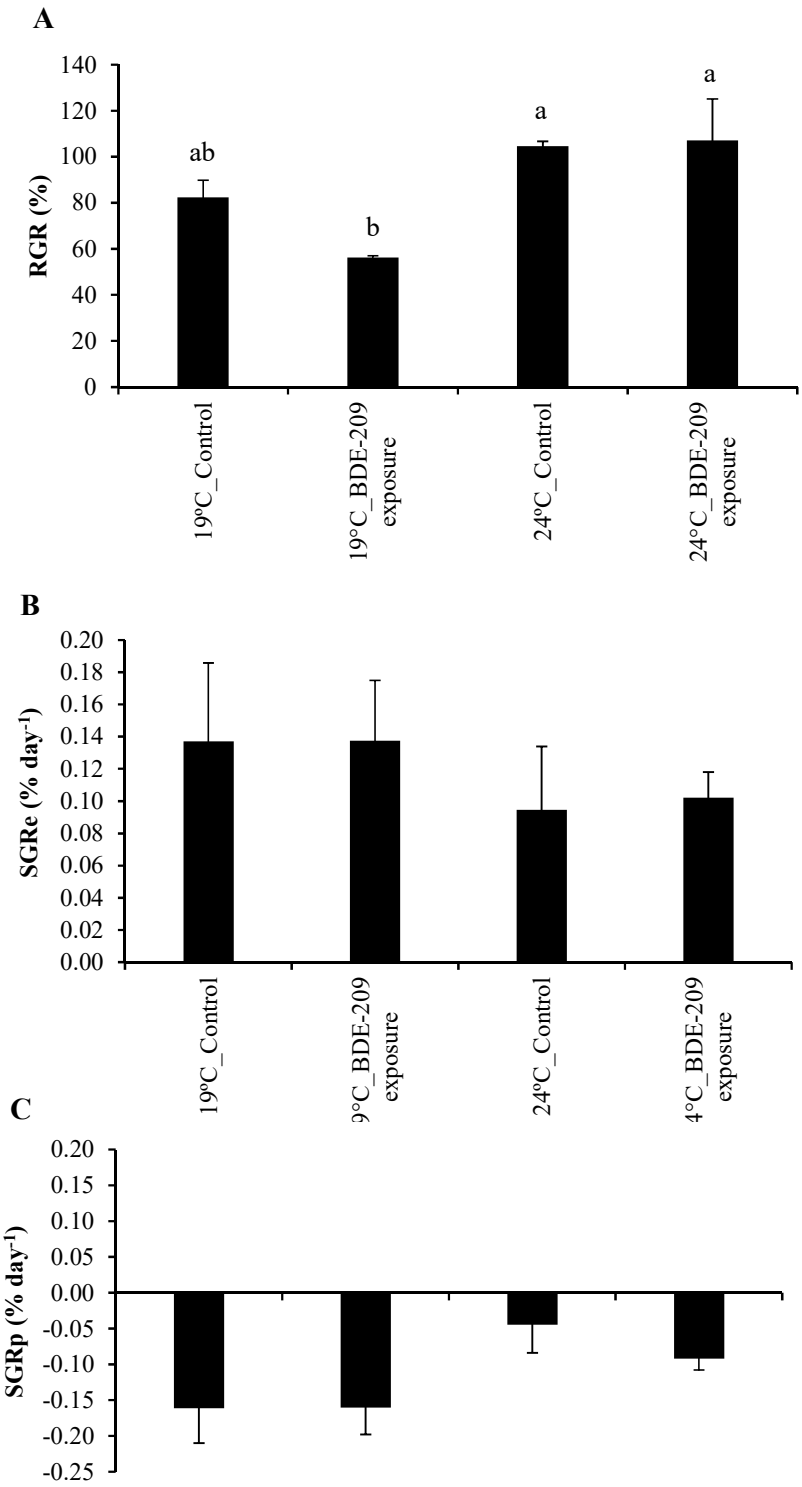


Fig. 1. A) Relative growth rate (RGR, %) and B,C) specific growth rate in terms of energy and protein (SGRe and SGRp, respectively, % day^{-1}) in juvenile white seabream (*Diplodus sargus*) after 56 days of exposure to warming and contaminant (mean±SD; n=9). Different letters indicate significant differences between treatments (ANOVA test, $p<0.05$). Abbreviations: 19 °C_Control

– seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

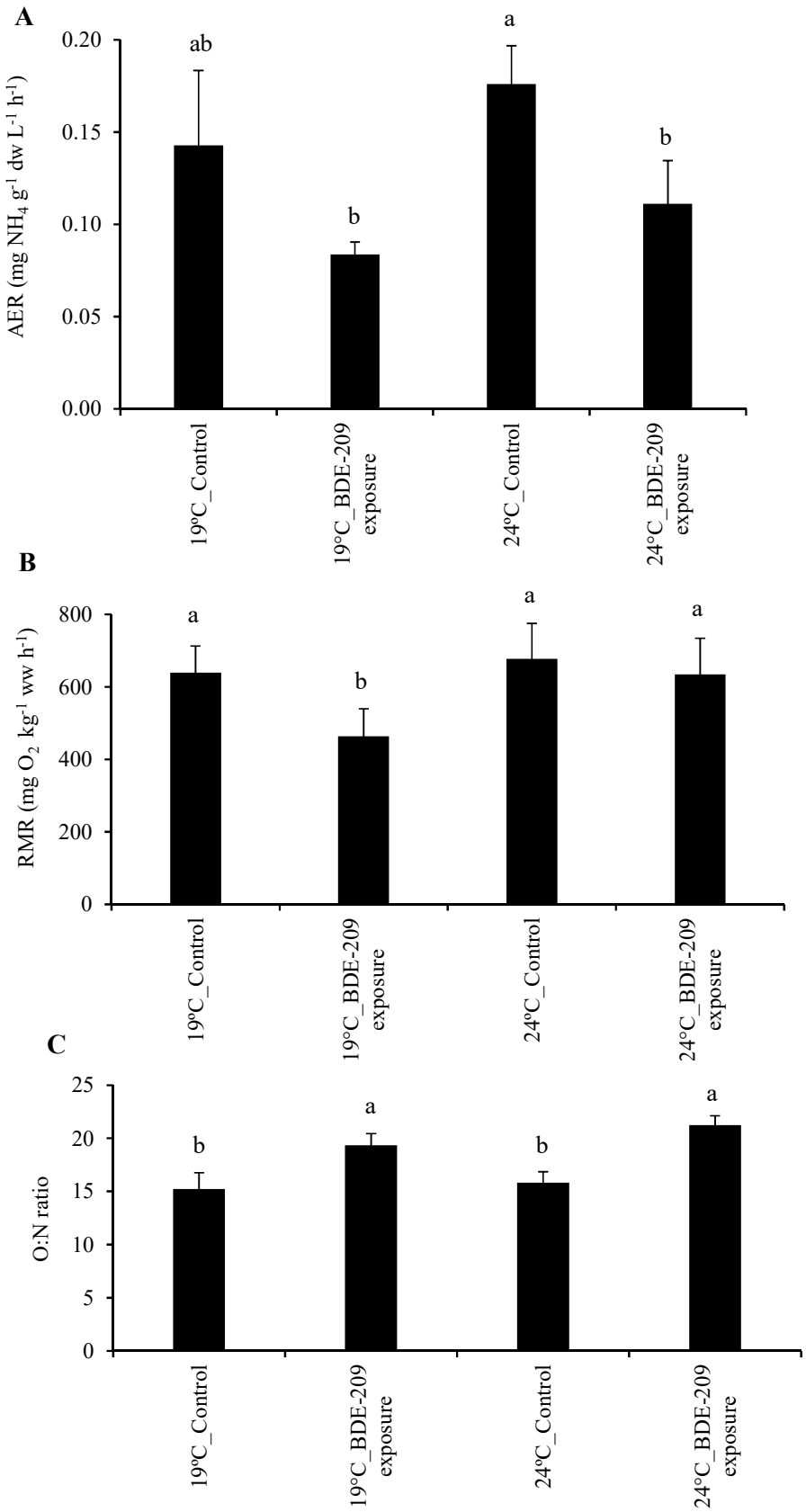
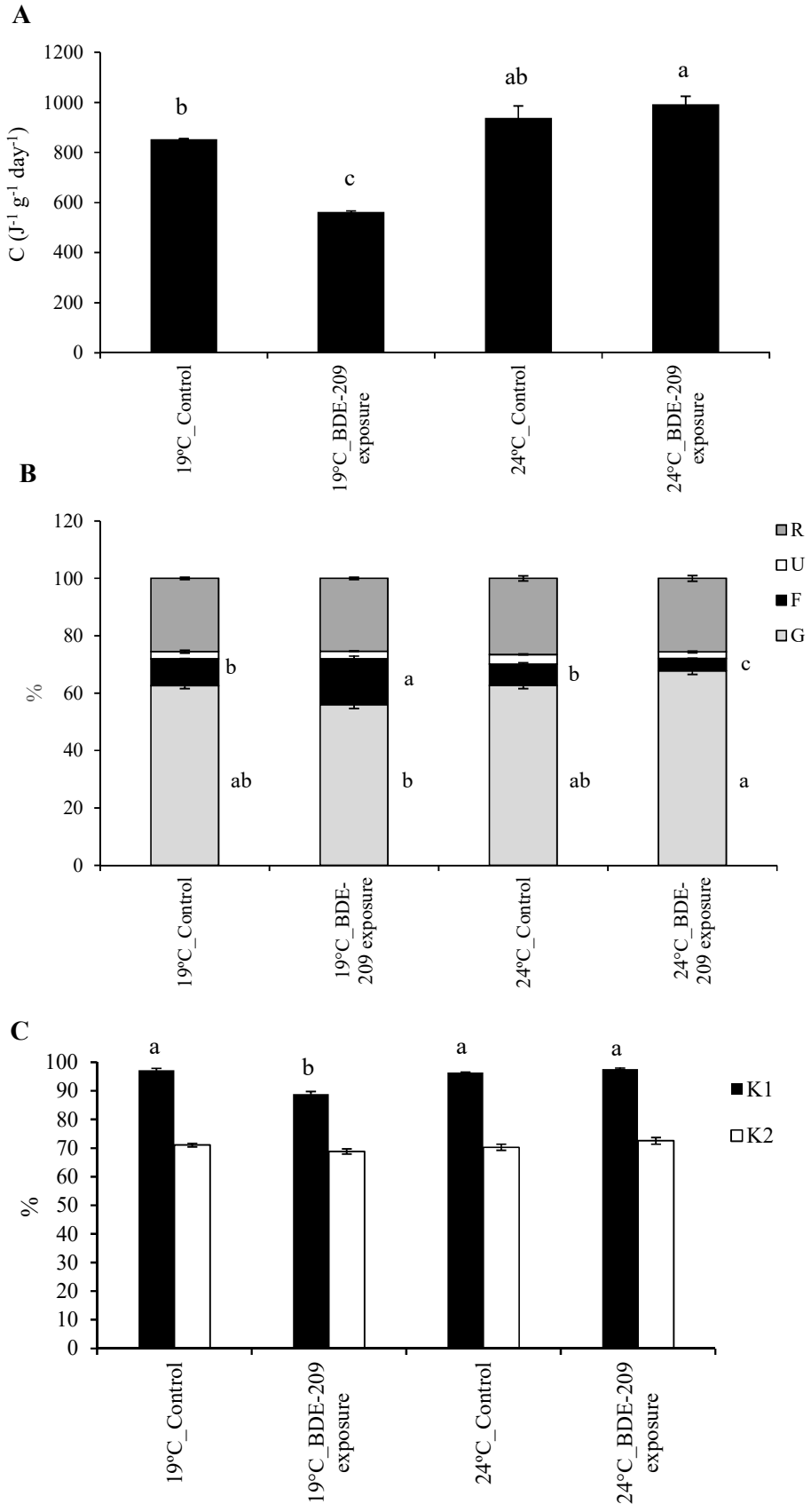

A
AER (mg NH4 g-1 dw L-1 h-1)
0.20
0.15
0.10
0.05
0.00
ab
b
a
b
19ºC_Control
19°C_BDE-209 exposure
24ºC_Control
24°C_BDE-209 exposure
B
RMR (mg O2 kg-1 ww h-1)
800
600
400
200
0
a
b
a
a
19ºC_Control
19°C_BDE-209 exposure
24ºC_Control
24°C_BDE-209 exposure
C
O:N ratio
25
20
15
10
5
0
b
a
b
a
19ºC_Control
19°C_BDE-209 exposure
24ºC_Control
24°C_BDE-209 exposure

Fig. 2. Effects of warming and BDE-209 contamination on A) ammonia excretion rate (AER; mg NH_4 g^{-1} dw L^{-1} h^{-1}), B) routine metabolic rate (RMR; mg O_2 kg^{-1} ww h^{-1}) and C) O:N ratio of juvenile white seabream (*Diplodus sargus*) after 56 days of exposure to warming and contaminant (mean±SD; n=6). Different letters indicate significant differences between treatments (ANOVA test, $p<0.01$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.


A
1200
1000
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C (J-1 g-1 day-1)
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19ºC_Control
19°C_BDE-209 exposure
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24°C_BDE-209 exposure
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K1
K2
19ºC_Control
19°C_BDE-209 exposure
24ºC_Control
24°C_BDE-209 exposure

Fig. 3. Effects of warming and BDE-209 contamination on A) energy intake from feed (C; J^{-1} g^{-1} day^{-1}), B) energy allocation (%), C) assimilation efficiency (K_1) and net growth efficiency (K_2) of juvenile white seabream (*Diplodus sargus*) after 56 days of the experiment. Values (mean±SD; n=6) are represented as the percentage of dry body weight. Different letters indicate significant differences between the treatments (ANOVA test, $p<0.05$). Abbreviations: C - energy intake; G - energy deposit for growth; F - energy loss through faeces; U - energy loss through ammonia excretion; R, energy loss through respiration; K_1 - assimilation efficiency; K_2 - net growth efficiency; 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

Table 1. Survival, initial wet weight, final wet weight, total length, Fulton's condition index, hepatosomatic and visceral indices, feed intake and feed eficiency of juvenile white seabream (*Diplodus sargus*) in the four treatments after 56 days of experiment.

	19 °C_Control	**19 °C_BDE-209 exposure**	**24 °C_Control**	**24 °C_BDE-209 exposure**
Survival (%)	90.0±0.0	88.2±10.2	90.2±9.0	94.1±5.9
Wi (g)	5.07±1.41^{ab}	4.50±0.80^{ab}	3.93±1.09^{b}	5.95±1.25^{a}
Wf (g)	9.61±2.29^{ab}	7.05±1.54^{b}	9.88±2.07^{ab}	10.26±2.99^{a}
TL (cm)	8.04±0.66^{a}	7.23±0.44^{b}	8.03±0.55^{a}	8.03±0.74^{a}
K	1.82±0.11	1.84±0.14	1.88±0.08	1.93±0.14*
HSI	1.30±0.56	1.25±0.33	0.90±0.15*	1.21±0.29
VSI	6.87±0.81^{a,*}	6.27±0.91^{ab}	5.57±1.10^{b}	6.05±0.87^{ab}
FI (g g^{-1} day^{-1})	0.47±0.10	0.48±0.04	0.52±0.10	0.43±0.09
FE	0.72±0.23^{b}	0.70±0.33^{b}	1.15±0.14^{b}	1.47±0.32^{a}

Values (mean±SD; n=9) with different letters (a-b) in the same row indicate significant differences between treatments (ANOVA test, $p<0.05$). Asterisk (*) represent significant differences between initial K, HSI and VSI and each treatment (t-student, $p<0.05$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet; Wi – Initial wet weight; Wf - Final wet weight; TL – Total length; K – Fulton's condition index; HSI – hepatosomatic index; VSI – viscerosomatic index; FI – Feed intake; FE – Feed eficiency.

Table 2. Effects of warming and BDE-209 contamination on biochemical composition of juvenile white seabream (*Diplodus sargus*) after 56 days of the experiment.

	Initial	19 °C_Control	19 °C_BDE-209 exposure	24 °C_Control	24 °C_BDE-209 exposure
Moisture (%)	72.67±0.23	70.81±1.45	72.05±1.39	71.64±0.26*	71.46±0.59*
Protein (%)	54.78±1.63	50.85±0.39^{b,*}	50.98±0.65^{b,*}	54.63±0.93^{a}	49.17±0.16^{b,*}
Fat (%)	19.90±0.06	26.10±0.62^{a,*}	24.66±0.13^{ab,*}	23.45±1.10^{bc,*}	21.63±0.38^{c,*}
Ash (%)	18.55±0.39	16.66±0.34^{b,*}	17.03±0.15^{ab,*}	17.84±0.40^{ab,*}	18.30±0.48^{a}
Gross energy (kJ g^{-1})	18.87±0.39	20.48±0.33*	20.21±0.24*	19.70±0.18*	20.29±0.32*

Values (mean±SD; n=3) correspond to the percentage of dry body weight. Different letters in the same row indicate significant differences between the four treatments (ANOVA test, $p<0.05$). Asterisk (*) represent significant differences between initial biochemical composition and each treatment (t-student, $p<0.05$). Abbreviations: 19 °C_Control – seawater temperature set at 19 °C and animals fed with the control diet; 19 °C_BDE-209 exposure – seawater temperature set at 19 °C and animals fed with BDE-209 contaminated diet; 24 °C_Control – seawater temperature set at 24 °C and animals fed with the control diet; 24 °C_BDE-209 exposure – seawater temperature set at 24 °C and animals fed with BDE-209 contaminated diet.

Appendix A

Table A.1. Formulation, ingredients and proximate chemical composition (% dry weight, dw) of the experimental diets (control and contaminated) used for juvenile white seabream (mean±standard deviation, n=3). Different letters indicate significant differences between the diets (t-student test, $p<0.05$).

Ingredients (%)	Diets	
	Control and BDE-209 contaminated	
Fishmeal LT70[1]	40.0	
Fish protein concentrate[2]	7.8	
Squid meal[3]	10.0	
Chlorella[4]	2.0	
Soy protein concentrate[5]	4.0	
Soybean meal 48[6]	4.0	
Wheat meal[7]	6.0	
Fish oil[8]	12.0	
Soy lecithin[9]	2.0	
Guar gum[10]	1.2	
Macroalgae[11]	5.0	
Antioxidant[12]	0.5	
Monocalcium phosphate[13]	1.0	
Glycerol[14]	3.5	
Vitamin and mineral premix[15]	1.0	
Proximate chemical composition (dw)	**Control**	**BDE-209 contaminated**
Moisture (%)	4.55 ± 0.03[b]	7.30 ± 0.03[a]
Protein (%)	57.71 ± 0.82	58.11 ± 0.62
Fat (%)	16.89 ± 0.31	17.22 ± 0.27
Total carbohydrates[16]	16.17 ± 1.07	14.22 ± 0.72
Ash (%)	9.24 ± 0.02[b]	10.45 ± 0.04[a]
Gross energy (kJ g^{-1})	21.86 ± 0.15[a]	21.58 ± 0.04[b]

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France;
[4] Chlorella powder: 62.5% CP, 9.2% CF, ALLMICROALGAE, Portugal.
[5] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[6] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain;
[7] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[8] SAVINOR UTS, Portugal
[9] LECICO P 700IPM, LECICO GmbH, Germany.
[10] Guar gum HV109, SEAH International, France.
[11] OceanFeed: 10.2% CP, Ocena Harvest Technology, Ireland.
[12] Paramega PX, Kemin Europe NV, Belgium
[13] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy.
[14] Rapeseed-derived crude glycerol, IBEROL, Portugal.
[15] PREMIX Lda, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.
[16] Calculated as, total carbohydrates (starch, free sugars, cellulose) = 100-(protein+fat+ash).

Table A.2. Results of two-way ANOVA evaluating the effects of temperature (T) and BDE-209 contaminated diet (CD) on survival, initial wet weight (Wi), final wet weight (Wf), total length (TL), Fulton's condition index (K), hepatosomatic and viscerosomatic indices (HSI and VSI, respectively), feed intake (FI), feed efficiency (FE), relative growth rate (RGR), specific growth rate in terms of energy and protein (SGRe and SGRp, respectively), ammonia excretion rate (AER), routine metabolic rate (RMR) and O:N ratio of juvenile white seabream (*Diplodus sargus*). Asterisks indicate significant differences ($p<0.05$; *df*=10).

Parameters	MS	F	p	Parameters	MS	F	p
Survival				**FE**			
Temperature (T)	27.71	0.506	0.497	*Temperature (T)*	3.923	71.16	<0.001*
BDE-209 contaminated diet (CD)	3.489	0.064	0.807	*BDE-209 contaminated diet (CD)*	1.278	23.18	<0.001*
T x CD	24.25	0.443	0.524	*T x CD*	1.352	24.52	<0.001*
Error	54.79			*Error*	0.055		
Wi				**RGR**			
Temperature (T)	0.225	0.154	0.697	*Temperature (T)*	2679	27.71	0.006*
BDE-209 contaminated diet (CD)	4.499	3.077	0.087	*BDE-209 contaminated diet (CD)*	278.50	2.880	0.165
T x CD	14.37	9.828	0.003*	*T x CD*	411.40	4.255	0.108
Error	1.462			*Error*	96.69		
Wf				**SGRe**			
Temperature (T)	27.27	5.230	0.029*	*Temperature (T)*	0.003	2.074	0.209
BDE-209 contaminated diet (CD)	10.69	2.051	0.162	*BDE-209 contaminated diet (CD)*	0.000	0.022	0.888
T x CD	19.45	3.730	0.062	*T x CD*	0.000	0.017	0.901
Error	5.215			*Error*	0.002		
TL				**SGRp**			
Temperature (T)	1.400	3.784	0.060	*Temperature (T)*	0.017	6.776	0.060
BDE-209 contaminated diet (CD)	1.480	4.000	0.054	*BDE-209 contaminated diet (CD)*	0.001	0.427	0.549
T x CD	1.480	4.000	0.054	*T x CD*	0.001	0.453	0.538
Error	0.370			*Error*	0.002		
K				**AER**			
Temperature (T)	0.057	3.909	0.057	*Temperature (T)*	0.003	5.338	0.043*
BDE-209 contaminated diet (CD)	0.013	0.893	0.352	*BDE-209 contaminated diet (CD)*	0.013	22.30	<0.001*
T x CD	0.002	0.110	0.742	*T x CD*	0.000	0.048	0.832
Error	0.014			*Error*	0.001		
HSI				**RMR**			
Temperature (T)	0.426	3.232	0.082	*Temperature (T)*	121017	15.96	<0.001*
BDE-209 contaminated diet (CD)	0.149	1.127	0.296	*BDE-209 contaminated diet (CD)*	133430	17.60	<0.001*
T x CD	0.292	2.214	0.146	*T x CD*	49152	6.482	0.015*
Error	0.132			*Error*	7583		
VSI				**O:N ratio**			
Temperature (T)	5.222	6.045	0.020*	*Temperature (T)*	5.229	4.069	0.071
BDE-209 contaminated diet (CD)	0.032	0.037	0.848	*BDE-209 contaminated diet (CD)*	77.53	60.34	<0.001*
T x CD	2.680	3.102	0.088	*T x CD*	1.391	1.083	0.323
Error	0.864			*Error*	1.285		
FI							
Temperature (T)	0.000	0.001	0.978				
BDE-209 contaminated diet (CD)	0.018	2.534	0.121				
T x CD	0.020	2.789	0.105				
Error	0.007						

Table A.3. Results of two-way ANOVA evaluating the effects of temperature (T) and BDE-209 contaminated diet (CD) on biochemical composition, energy budget parameters (C, G, F, U and R), assimilation and net growth efficiencies (K_1 and K_2, respectively) of juvenile white seabream (*Diplodus sargus*). Asterisks indicate significant differences ($p<0.05$; *df*=10).

Parameters		MS	F	p
Biochemical composition	**Moisture**			
	Temperature (T)	0.106	0.082	0.781
	BDE-209 contaminated diet (CD)	0.142	0.111	0.748
	T x CD	3.121	2.424	0.158
	Error	1.287		
	Protein			
	Temperature (T)	2.577	6.234	0.041*
	BDE-209 contaminated diet (CD)	18.99	45.95	<0.001*
	T x CD	20.87	50.50	<0.001*
	Error	0.413		
	Fat			
	Temperature (T)	21.52	44.86	<0.001*
	BDE-209 contaminated diet (CD)	7.030	14.65	0.006*
	T x CD	0.097	0.201	0.667
	Error	0.480		
	Ash			
	Temperature (T)	2.988	22.83	0.009*
	BDE-209 contaminated diet (CD)	0.345	2.636	0.180
	T x CD	0.004	0.027	0.878
	Error	0.131		
	Gross energy			
	Temperature (T)	0.288	3.932	0.095
	BDE-209 contaminated diet (CD)	0.057	0.774	0.413
	T x CD	0.442	6.027	0.049*
	Error	0.073		
Energy budget	**C**			
	Temperature (T)	111442	128.20	<0.001*
	BDE-209 contaminated diet (CD)	19025	21.89	0.009*
	T x CD	46018	52.94	0.002*
	Error	869		
	G			
	Temperature (T)	82423	64.96	<0.001*
	BDE-209 contaminated diet (CD)	13704	10.80	0.022*
	T x CD	58558	46.15	0.001*
	Error	1269		
	F			
	Temperature (T)	1645	344.21	<0.001*
	BDE-209 contaminated diet (CD)	154.33	32.29	0.005*
	T x CD	689.03	144.17	<0.001*
	Error	4.78		
	U			
	Temperature (T)	90.51	5.12	0.047*
	BDE-209 contaminated diet (CD)	389.90	22.04	<0.001*
	T x CD	0.192	0.011	0.919
	Error	17.69		
	R			
	Temperature (T)	3066	3.22	0.090
	BDE-209 contaminated diet (CD)	5724	6.00	0.025*
	T x CD	5424	5.69	0.028*
	Error	953.24		
	K_1			
	Temperature (T)	31.12	98.92	<0.001*
	BDE-209 contaminated diet (CD)	25.68	81.62	<0.001*
	T x CD	46.21	146.88	<0.001*
	Error	0.315		
	K_2			

Temperature (T)	4.456	4.768	0.094
BDE-209 contaminated diet (CD)	0.003	0.003	0.961
T x CD	10.11	10.82	0.030*
Error	0.935		

Table A.4. Results of two-way ANOVA evaluating the effects of temperature (T) and BDE-209 contaminated diet (CD) showing the results of normality and homogeneity of variances tests (p-values).

Parameters		Normality	Homogeneity of variances
Indices	**Survival**	0.395	0.064
	Wi	0.092	0.583
	Wf	0.086	0.297
	TL	0.513	0.562
	K	0.270	0.228
	HSI	0.326	0.051
	VSI	0.474	0.976
	FI	0.109	0.094
	FE	0.123	0.288
	RGR	0.622	0.534
	SGRe	0.859	0.423
	SGRp	0.558	0.254
	AER	0.140	0.148
	RMR	0.483	0.216
	O:N ratio	0.615	0.578
Biochemical composition	**Moisture**	0.513	0.062
	Protein	0.176	0.118
	Fat	0.507	0.062
	Ash	0.865	0.543
	Gross energy	0.973	0.666
Energy budget	**C**	0.088	0.063
	G	0.117	0.139
	F	0.192	0.062
	U	0.178	0.140
	R	0.134	0.064
	K_1	0.871	0.065
	K_2	0.971	0.072