

Interactive comment on “Ocean acidification dampens warming and contamination effects on the physiological stress response of a commercially important fish” by Eduardo Sampaio et al.

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Anonymous Referee #1

General comments

“This manuscript aims to address a topic of significant importance, namely the interaction between climate change stressors and contamination in coastal regions, and particularly its impact on species of commercial importance. This is certainly a topic of great interest and an area that has been identified as a significant knowledge gap in

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the field at present. Despite this potential and the undoubted requirement for such a study within the field, regrettably the manuscript presented here does not adequately address this question. As it stands there is insufficient detail presented throughout the methods to adequately appraise what has been done, there appear to be a number of methodological oversights that hamper the interpretation of the results and this has, to a large extent, led to many of the conclusions drawn not being supported by the data. Based on these factors I believe the manuscript at least requires major revisions to include this required detail, as well as restructure the conclusions to match what has actually been undertaken. It would then require re-review to appraise the manuscript in its new form. If it is not possible to include this required detail in full, in its current state the manuscript is not of sufficient quality to be published.”

Response: We thank the referee for his suggestions which have served to greatly improve the manuscript. We hope we have now provided sufficient detail on the methodologies employed in this work. We also hope to have clarified some misinterpretations throughout the text. Below, we reply to each comment in a point-by-point manner. Please note that Page and Line numbers now correspond to the marked up version of the manuscript.

Specific comments

Comment #1: Abstract, discussion and conclusions – Throughout the manuscript the authors suggest the reduced accumulation of mercury in tissues under combined exposure is due to metabolic depression, and a subsequent reduced apatite/ingestion of food, initiated by elevated CO₂. However, the authors do not measure any parameters in the current study that could confirm or counter this suggestion. There is no indication that these fish ingested less food so the conclusions, certainly as they are presented, are unfounded. Reduced accumulation could in fact be caused by a number of different mechanisms in the organism by which elevated CO₂ augmented Hg accumulation, either by metabolic depression, reduced appetite (could be caused by alternative mechanism), reduced digestive efficiency, reduced uptake across the gut epithelium,

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greater egestion of Hg or impacts of Hg transport and complexation in plasma to reduce delivery to measured tissues. All are potentially feasible and at present insufficient information is known about this to surmise it is metabolic depression. It is vital to indicate that whilst altered accumulation is noted, which differs between specific tissues, the mechanism is not known. Following this point, the authors have not cited two key references on ocean acidification and mercury contamination recently published (Li et al Scientific Reports 7:324 2017; and Wang et al ES&T 51:5820 2017). It is possible these were published after initial submission of the current manuscript, but in light of altered mercury accumulation under elevated CO₂ these two manuscripts are key as they support the current finding.

Response: The authors acknowledge that no additional parameters were measured to validate the conclusion that lower Hg accumulation under increased CO₂ was due to metabolic depression. Nonetheless, based on previous studies, there are reasons to believe this is the case. A wide range of organisms show metabolic decrease in response to increased extracellular acid–base stress (Kroeker et al., 2010), and especially to simultaneous occurrence of warming and acidification (Harley et al., 2006; Harvey et al., 2013; Rosa et al., 2013; Rosa and Seibel, 2008). Concerning CO₂, theoretically, the prioritization of acid–base regulation and ion regulatory enzyme machinery for CO₂ excretion (e.g. pyruvate kinase) may lead to lower metabolic activity in other enzymes (energy reallocation), as reported in other fish (Perry et al., 1988). As MeHg accumulation rates are positively correlated with metabolic rates (Dijkstra et al., 2013), these results would support the claim that acidification affects toxic compound accumulation rates (Schiedek et al., 2007). Given our simultaneous exposure to both warming and acidification, which has been shown to undeniably suppress metabolic rates directly (Christensen et al., 2011; Harley et al., 2006; Rosa et al., 2013; Rosa and Seibel, 2008; see also Harvey et al., 2013 and Kroeker et al, 2010) we still hold the conviction that metabolic processes may be at play. However, the authors also acknowledge that they were not aware of the recent research pointed out by the referee, which thoroughly picks apart the causes of behind these mechanisms. We thank

the referee for this useful comment and we have altered our interpretation, changing the text: “However, such effect may be offset by CO₂-linked decreases in mercury accumulation (Sampaio et al., 2016; Schiedek et al., 2007; Wang et al., 2017)” (Page 2, Lines 29-31) “Instead, our results support recent studies demonstrating that hypercapnia dampens Hg accumulation in marine organisms (Li et al., 2017; Sampaio et al., 2016; Wang et al., 2017). There are several possible reasons which may underpin such an interaction, encompassing digestive (reduced digestive efficiency, reduced uptake through the gut membrane, reduced appetite, increased Hg depuration) and molecular (competition between Hg and H⁺ ions for binding sites, impacts on Hg plasma transport, lower phospholipidic membrane permeability) mechanisms (Li et al., 2017). A recent study has also found that the lysosome-autophagy pathway was up-regulated by combined exposure to Hg and increased CO₂, enabling better animal fitness which may potentially reduce Hg accumulation and toxicity (Wang et al., 2017). In addition, taking into account that the occurrence of both warming and acidification changes physiological thresholds (Christensen et al., 2011; Harley et al., 2006; Rosa et al., 2013; Rosa and Seibel, 2008), a degree of metabolic depression may also play a role on decreasing HgT accumulation (Dijkstra et al., 2013; Sampaio et al., 2016).” (Page 11, Lines 4-16) “In general, warming conditions enhanced MeHg accumulation but CO₂-linked impacts countered this effect.” (Page 12, Lines 28-29)

References Dijkstra, J. A., Buckman, K. L., Ward, D., Evans, D. W., Dionne, M. and Chen, C. Y.: Experimental and Natural Warming Elevates Mercury Concentrations in Estuarine Fish, PLoS One, 8(3), 1–9, doi:10.1371/journal.pone.0058401, 2013. Harley, C. D. G., Hughes, A. R., Kristin, M., Miner, B. G., Sorte, C. J. B. and Carol, S.: The impacts of climate change in coastal marine systems, Ecol. Lett., 9, 228–241, 2006. Harvey, B. P., Gwynn-Jones, D. and Moore, P. J.: Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming., Ecol. Evol., 3(4), 1016–1030, doi:10.1002/ece3.516, 2013. Kroeker, K. J., Kordas, R. L., Crim, R. N. and Singh, G. G.: Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms., Ecol. Lett.,

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Comment #2: Abstract, discussion and conclusions – Similarly to the point above, the authors repeatedly suggest elevated H⁺ impacts mercury accumulation/toxicity at a molecular level, but no acid base measures were made. Also it is a common misunderstanding that elevated CO₂ results in chronic acidosis in fish plasma, this is not the case. Elevated CO₂ results in acute acidosis which is rapidly compensated for by an elevation in bicarbonate, returning the plasma H⁺ to normal levels. Therefore the suggestion that elevated H⁺ impacts on mercury toxicity/accumulation is not supported,

especially as acid-base parameters are not presented that counter this common response noted in acid-base compensating species such as fish. The authors need to again re-interpret data and re-write conclusions to better reflect the demonstrated results and not make broad unsupported conclusions, pinned loosely on previously published literature that has been misinterpreted/misunderstood.

Response: We would like to point out that we never said that acidosis was present in a long-term perspective, nor did we assume that fish are not able to acid-base compensate, a mechanism that is already extensively described (Brauner and Baker, 2009; Heuer and Grosell, 2014; Michaelidis et al., 2007; among many more). In fact, besides some logistical and time constraints, that was the main reason why no acid-base measurements were performed. Having said that, acid-base compensation occurs mainly by increasing bicarbonate (HCO_3^-) levels in both blood and cellular, which in turn leads to a normalization of intracellular and extracellular pH (Heuer and Grosell, 2014; Michaelidis et al., 2007). The chemical equation that underpins this reaction is as follows: $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$

Thus, despite pH being normalized by balancing the ratio between $\text{H}^+ + \text{HCO}_3^-$ and H_2CO_3 (it generally stabilizes at $\sim 0.05/0.1$ units lower than in normocapnic conditions), it is important to note that H^+ levels in the organism are still increased relatively to basal levels, especially in long-term acclimations to hypercapnia- where there is a constant influx of H^+ ions (Heuer and Grosell, 2014; Michaelidis et al., 2007). Moreover, due to cell prioritization, intracellular and extracellular pH often display significantly different values: the former is up-regulated to normocapnic levels or higher, while the latter generally stabilizes at lower pH (ΔpH can reach $\sim 0.3-0.7$) (Brauner and Baker, 2009; Heuer and Grosell, 2014). This also partially contributes to increased H^+ levels. As our reasoning is grounded on molecular interactions (both oxidative stress-inducing and ROS-mitigating) of increased H^+ chemical reactions (see also Dean, 2010), it does not imply for fish acid-base compensation to fail. In light of the new recent studies mentioned by the referee, we have introduced some new considera-

tions to our Abstract/Discussion/Conclusion. The reason we do not believe that the lysozyme-autophagy pathway (Wang et al., 2017) is solely responsible for the antagonistic relationship between stressors is that it does not account for hypercapnia-induced oxidative stress and chaperone activation. Within this context, we have rephrased our interpretations and changed the text accordingly:

In the Abstract: “Together with CO₂-promoted removal of damaged proteins and enzymes, we argue that simultaneous increase in hydrogen (H⁺) and reactive oxygen species (e.g. O₂⁻) radicals is partially compensated through chemical reaction equilibrium balancing.” (Page 1, Lines 26-29)

In the Discussion: “Increased CO₂ (co-occurring with Hg contamination) may elicit the up-regulation of the lysosome-autophagy pathway, which is responsible for removing damaged proteins and organelles, effectively reducing oxidative stress (Wang et al., 2017). This mechanism may contribute to alleviate not only Hg induced stress, but also warming-related oxidative stress. We also argue that this antagonistic relation can be partially explained by a CO₂-related increase of H⁺ ion concentrations in the blood and cellular surroundings, counterbalanced by bicarbonate increase (acid-base compensation) to normalize pH levels (Heuer and Grosell, 2014; Michaelidis et al., 2007). By itself, the presence of excessive H⁺ ions activates free radical neutralizing defenses (Tiedke et al., 2013), which is in line with the present findings when hypercapnia was the sole stressor. However the production of O₂⁻ and further complementary ROS radicals (e.g. OH⁻) by other stressors may result in facilitated H₂O and H₂O₂ formation, due to chemical reactions balancing equilibrium (e.g. H⁺ + OH⁻ → H₂O), thus eliminating free radicals and decreasing activity of antioxidant enzymes to basal standards.” (Page 11/12, Lines 31/1-10) “More so than for oxidative stress, the enhanced removal of damaged proteins and enzymes indirectly promoted by increased CO₂ (via up-regulated lysosome-autophagy) may have especially contributed to subside protein chaperone production. Given that Hsp70 production can also be stimulated by high ionic (e.g. H⁺) concentrations (Feder and Hofmann, 1999), we reason that the same

additional mechanism by which hypercapnia potentially modulates oxidative stress can be applied for heat shock response” (Page 12, Lines 18-22)

In the Conclusions: “In fact, despite negative effects prompted as a sole stressor, acidification consistently elicited antagonistic responses to temperature and contamination effects on oxidative stress (including heat shock response), which may be explained by stimulated removal of damaged proteins and organelles (Wang et al., 2017). Moreover, we also argue that the mechanistic interactions found are coadjuvanted by the coinciding increase of hydrogen (H⁺) and radical reactive oxygen species (e.g. O₂⁻, OH⁻), which subsequently nullify each other due to the spontaneous equilibrium of chemical reactions (e.g. H⁺ + OH⁻ → H₂O).” (Page 12/13, Lines 29/1-6)

References Brauner, C. J. and Baker, D. W.: Patterns of Acid–Base Regulation During Exposure to Hypercarbia in Fishes, in Cardio-Respiratory Control in Vertebrates, edited by M. L. Glass and S. C. Wood, pp. 1–546, Springer Berlin Heidelberg., 2009. Dean, J. B.: Hypercapnia causes cellular oxidation and nitrosation in addition to acidosis: implications for CO₂ chemoreceptor function and dysfunction., J. Appl. Physiol., 108(6), 1786–95, doi:10.1152/japplphysiol.01337.2009, 2010. Heuer, R. M. and Grosell, M.: Physiological impacts of elevated carbon dioxide and ocean acidification on fish, AJP Regul. Integr. Comp. Physiol., 307(9), R1061–R1084, doi:10.1152/ajpregu.00064.2014, 2014. Michaelidis, B., Spring, A. and Pörtner, H. O.: Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*, Mar. Biol., 150(6), 1417–1429, doi:10.1007/s00227-006-0436-8, 2007.

Comment #3: Discussion – The authors suggest the Fulton condition may diminish under mercury contamination. Whilst AIC indicates the best fit model as slightly negative the statistic (p-Value) clearly indicates no significant effect and therefore suggesting this is not the case may mislead readers to interpret a result that is not supported statistically, even if it may support a previous publication.

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Response: Following the reviewer's instructions, we have removed any mentioning of negative effects on the Fulton condition. We have changed the introductory paragraph of the Discussion to: "The present study showed that Hg contamination, ocean warming and acidification interactively affected fish physiology at sublethal levels, i.e. zero mortality and also no effects on Fulton condition were registered. The fact that the meagre (*A. regius*) is a very resilient species and easily adapts to environmental alterations (Monfort, 2010) may explain the absence of deleterious effects at an organism level, after 30 days of exposure." (Page 10, Lines 14-19)

Comment #4: Intro and methods - The justification of mercury, and methylmercury, in fish from coastal regions is insufficient. There is no quantification of levels within the environment from different regions globally, how coastal compares to open ocean and how this then translates into a burden for fish populations. As it stands this is not adequate for a contaminant manuscript, and belies the statement that an environmentally relevant concentration was used, as stated in the methods. What is an environmentally relevant concentration, where does the level chosen fit with measured environmental levels from different regions globally, and even just within the region the study was undertaken in. Finally, if the route of uptake is solely dietary for fish then how do environmental levels correspond to burdens in prey species and thus exposure in the experimental organism? Is the level chosen a typical contaminant level in prey species in an impacted environment or the level in water/sediment? This needs clarifying, and fully justifying in relation to existing literature and levels previously used.

Response: Mercury (originating mainly from industrial residue) accumulates in the sediments of river basins and estuaries (Mason, 2001). Posteriorly, it is transported to the open ocean via particulate and dissolved sediments in water currents and accumulated within animals, but in much less quantity (Guentzel et al., 1996). Thus, it is logical that fish which often make use of estuaries are more vulnerable to mercury accumulation, as we have stated in the Introduction of the manuscript (Page 2, Lines 18-20 and Page 3, Lines 21-22). As the referee correctly inferred, the concentrations of mercury used

for this study were based on levels of contamination found in contaminated coastal areas (specifically the extensively studied, contaminated estuary of Aveiro, Portugal) for species that are natural prey of the meagre (e.g. Cardoso et al., 2014; Nunes et al., 2008). These mercury concentrations can also be found in other areas globally, e.g. Florida, USA (Kannan et al., 1998). We have changed the text in order to provide a more comprehensive picture: “Given our dietary option, ecologically relevant MeHg concentrations were chosen based on levels (low contamination, ~ 0.12 mg kg⁻¹ wet weight (ww); and high contamination, ~ 1.6 mg kg⁻¹ ww found in common *A. regius* prey species from contaminated coastal areas (Cardoso et al., 2014; Kannan et al., 1998; Nunes et al., 2008). The pellets given to fish allocated to non-contaminated and contaminated treatments had approximately 0.60 ± 0.01 mg kg⁻¹ dry weight (dw) and 8.02 ± 0.01 mg kg⁻¹ dw of MeHg, respectively, which were considered to mimic the concentrations found in the field (see Maulvault et al., 2016, 2017). Feed composition, manufacturing and MeHg spiking processes were executed as described by Maulvault et al. (2016).” (Page 4/5, Lines 30-32/1-6)

References Cardoso, P. G., Pereira, E., Duarte, A. C. and Azeiteiro, U. M.: Temporal characterization of mercury accumulation at different trophic levels and implications for metal biomagnification along a coastal food web, *Mar. Pollut. Bull.*, 87(1), 39–47, doi:10.1016/j.marpolbul.2014.08.013, 2014. Guentzel, J. L., Powell, R. T., Landing, W. M. and Mason, R. P.: Mercury associated with colloidal material in an estuarine and an open-ocean environment, *Mar. Chem.*, 55(1–2), 177–188, doi:10.1016/S0304-4203(96)00055-2, 1996. Kannan, K., Smith Jr., R. G., Lee, R. F., Windom, H. L., Heitmuller, P. T., Macauley, J. M. and Summers, J. K.: Distribution of Total Mercury and Methyl Mercury in Water, Sediment, and Fish from South Florida Estuaries, *Arch. Environ. Contam. Toxicol.*, 34, 109–118, doi:10.1007/s002449900294, 1998. Mason, R. P.: The Bioaccumulation of Mercury, Methylmercury and Other Toxic Elements into Pelagic and Benthic Organisms, in *Coastal and Estuarine Risk Assessment*, edited by N. Newman, M. Roberts jr, and R. Hale, pp. 127–149, Lewis Publishers, Boca Raton, FL, USA., 2001. Nunes, M., Coelho, J. P., Cardoso, P. G., Pereira, M. E., Duarte, A.

C. and Pardal, M. A.: The macrobenthic community along a mercury contamination in a temperate estuarine system (Ria de Aveiro, Portugal), *Sci. Total Environ.*, 405(1–3), 186–194, doi:10.1016/j.scitotenv.2008.07.009, 2008.

Comment #5: Methods - Following this, the total amount of mercury (mg per kg of food), is higher than the content of methylmercury added as an additive (8.02 MeHg, 8.28 HgT). This is not possible. Also how were these levels measured (or is it nominal)? **Response:** We assure the reviewer that this is standard for all scientific works where, being the most bioaccumulated form of mercury in the environment, methylmercury (MeHg) is used (Maulvault et al., 2016, 2017; Sampaio et al., 2016; Wang et al., 2013, 2017b). On top of naturally occurring demethylation, higher total mercury concentration is due to the ubiquity of mercury, under several (organic and inorganic) forms, in the natural environment. A standard feed diet is composed of fish meals, oils and other compounds, which already contain a certain quantity of mercury (not only methylmercury, but also in its other chemical forms). Naturally, a control feed, where no spiking is performed, contains trace levels of mercury. Thus, when spiking a diet with MeHg, adding these facts, it is common for the total amount of mercury to be higher than that of methylmercury (e.g. see studies referenced). Lastly, the procedure for the measurement of MeHg is similar to HgT, explicit in section “2.2 Total mercury and Methylmercury accumulation” (Page 6, Lines 5-18). For the sake of clarity, we rephrased: “Afterwards, HgT (all samples) and MeHg (feed samples) were determined (10-15 mg for solids or 100-200 μ l for liquids) by atomic absorption spectrometry (AAS), following EPA (2007) by means of an automatic Hg analyser (AMA 254, LECO, USA) with a detection threshold of 0.005 mg kg⁻¹ ww.” (Page 6, Line 5-8) And added: “Feed composition, manufacturing and MeHg spiking processes were executed as described by Maulvault et al. (2016). Fish were fed two to three times a day and total feed quantity provided per day was approximately 1% (standard calculation for aquaculture) of animal weight (at the end of 30 days, each fish was given approximately 0.0106 mg of HgT). Selected feed quantity also minimized food remains, which, in case of existing, were siphoned together with fish faeces after feeding.” (Page 5, Line 5-12)

References Maulvault, A. L., Custodio, A., Anacleto, P., Repolho, T., Pousao, P., Nunes, M. L., Diniz, M., Rosa, R. and Marques, A.: Bioaccumulation and elimination of mercury in juvenile seabass (*Dicentrarchus labrax*) in a warmer environment, *Environ. Res.*, 149, 77–85, doi:10.1016/j.envres.2016.04.035, 2016. Maulvault, A. L., Barbosa, V., Alves, R., Custódio, A., Anacleto, P., Repolho, T., Pousão Ferreira, P., Rosa, R., Marques, A. and Diniz, M.: Ecophysiological responses of juvenile seabass (*Dicentrarchus labrax*) exposed to increased temperature and dietary methylmercury, *Sci. Total Environ.*, 586, 551–558, doi:10.1016/j.scitotenv.2017.02.016, 2017. Sampaio, E., Maulvault, A. L., Lopes, V. M., Paula, J. R., Barbosa, V., Alves, R., Pousão-Ferreira, P., Repolho, T., Marques, A. and Rosa, R.: Habitat selection disruption and lateralization impairment of cryptic flatfish in a warm, acid, and contaminated ocean, *Mar. Biol.*, 163(10), 217, doi:10.1007/s00227-016-2994-8, 2016. Wang, R., Feng, X. Bin and Wang, W. X.: In vivo mercury methylation and demethylation in freshwater tilapia quantified by mercury stable isotopes, *Environ. Sci. Technol.*, 47(14), 7949–7957, doi:10.1021/es3043774, 2013. Wang, X., Wu, F. and Wang, W.-X.: In Vivo Mercury Demethylation in a Marine Fish (*Acanthopagrus schlegelii*), *Environ. Sci. Technol.*, (May), acs.est.7b00923, doi:10.1021/acs.est.7b00923, 2017b.

Comment #6: Methods (page 4 lines 1-7) - The description of the conditions, and particularly their maintenance is not sufficient. It states ammonia, nitrate and nitrite were regularly monitored and kept within recommended levels. How was this tested, what were the accepted levels and what were the levels measured within the experiment? Also how were high levels mitigated against and how often? Furthermore, it mentions salinity was kept at 35.0 ± 1.0 g/l NaCl? The probe listed is a conductivity probe so does not measure in g/l of NaCl but gives a conductivity measure or salinity as a psu. Also how was salinity maintained? i.e. is this addition of deionised water to compensate for evaporation? Or addition of additional NaCl? The description is confusing, and could be interpreted as additional NaCl addition. Any further addition of NaCl would significantly alter osmolality thus this needs clarifying to explain if input water fluctuated in salinity. A better description of this process is therefore required.

Response: We apologize for not having provided more detail on these matters, but we have been said to be overzealous with these descriptions in recent publications. However, it is our pleasure to fill the gaps the referee points out in this section. Ammonia ($\text{NH}_3/\text{NH}_4^+$), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Colorimetric kits, Aquamark, Germany), and kept below detectable levels (i.e. $\text{NH}_3/\text{NH}_4^+ < 0.25 \text{ mg l}^{-1}$; $\text{NO}_2^- < 0.10 \text{ mg l}^{-1}$; $\text{NO}_3^- < 0.2 \text{ mg l}^{-1}$). Salinity was not measured through a conductivity probe, we apologize for the omission. We opted instead for a refractometer (V2, TMC Iberia, Portugal) and took daily measurements as with temperature and pH. Salinity was also incorporated in the calculation of seawater carbonate chemistry (Table S1). We acknowledge the mistake (g/l) and have removed salinity units (see below). The addition of deionised water or any kind of water except sea water would modify carbonate chemistry and render our pH manipulation useless (Cornwall and Hurd, 2015). All potential fluctuations in both these parameters were solved by the seawater flux, and in the case of nutrients, by the biological filter described (Page 4, Lines 2-5). As detailed in the Methods section (Page 4, Lines 5-9), each experimental unit (or recirculatory aquatic system, RAS) was a semi-closed system with a constant seawater flux (complete turnover rate in 24h) precisely to maintain parameters such as salinity and nutrients. Thus, the mitigation of potential problems was done a priori and no additional action was needed during the course of the experiment. We have added the pertinent information in the text: “To prevent fluctuations in environmental parameters, each RAS worked as a semi-closed system, with constant low flow external water input (flux $> 2 \text{ l h}^{-1}$; 50 l tank turnover rate = 24 h). Consequently, ammonia ($\text{NH}_3/\text{NH}_4^+$), nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were daily checked (Colourimetric kits, Aquamark, Germany), and kept below detectable levels (i.e. $\text{NH}_3/\text{NH}_4^+ < 0.25 \text{ mg l}^{-1}$; $\text{NO}_2^- < 0.10 \text{ mg l}^{-1}$; $\text{NO}_3^- < 0.20 \text{ mg l}^{-1}$), and salinity was kept at 35.0 ± 1.0 (V2 Refractometer, TMC Iberia, Portugal). Temperature and pH (multiparametric probe, Multi3420 SET G, WTW) were measured daily, directly in the holding tanks. Photoperiod was fixed at 12 h light : 12 h dark.” (Page 4, Lines 5-14). References Cornwall, C. E. and Hurd, C. L.: Experimental design in ocean acidification

research: problems and solutions, ICES J. Mar. Sci., 73, 572–581, 2015.

Comment #7: Methods - There is no measure (or data presented) of methylmercury or total mercury in experimental water. This is a major omission, and gives no indication as to what proportion of the contaminant leaches from food into water, particularly if any food remains uneaten and in the tank for any time. It also prevents the discussion of amounts of methylmercury that egested immediately into the water by this fish, not being taken up or bioaccumulated.

Response: Our previous study showed that, contrary to inorganic mercury, the quantity of methylmercury leached from the feed to the water was below detection levels, making water measurements irrelevant (Maulvault et al., 2016). In other words, although measurements in the water are important when working with inorganic mercury, methylmercury is a more strongly lipophilic and hydrophobic molecule. It preferentially adheres to sediment and accumulates in the tissues of animals (i.e. fish) via prey (Mason, 2001). Moreover, the quantity of food administered (1 % fish weight per fish) is standard for aquaculture and has been calculated so that remains are minimum. In the rare occasions food was not ingested, it was immediately siphoned together with fish faeces.

We added this information in the text: “Feed composition, manufacturing and MeHg spiking processes were executed as described by Maulvault et al. (2016). Fish were fed two to three times a day and total feed quantity provided per day was approximately 1% (standard calculation for aquaculture) of animal weight (at the end of 30 days, each fish was given approximately 0.0106 mg of HgT). Selected feed quantity also minimized food remains, which, in case of existing, were siphoned together with fish faeces after feeding.” (Page 5, Line 5-12)

References Maulvault, A. L., Custodio, A., Anacleto, P., Repolho, T., Pousao, P., Nunes, M. L., Diniz, M., Rosa, R. and Marques, A.: Bioaccumulation and elimination of mercury in juvenile seabass (*Dicentrarchus labrax*) in a warmer environment, Environ. Res.,

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149, 77–85, doi:10.1016/j.envres.2016.04.035, 2016. Mason, R. P.: The Bioaccumulation of Mercury, Methylmercury and Other Toxic Elements into Pelagic and Benthic Organisms, in Coastal and Estuarine Risk Assessment, edited by N. Newman, M. Roberts jr, and R. Hale, pp. 127–149, Lewis Publishers, Boca Raton, FL, USA., 2001.

Comment #8: Throughout - Given the commercial importance of the species, one surprising oversight is the fact that no discussion on different tissue burdens were made with respect to human consumption and climate change impacts. The only place this is alluded to is in the title! This is particularly relevant given the possibility that elevated CO₂ reduces Hg accumulation possibly reducing transfer of hg into humans directly via consumption of muscle tissue, which could be an important result. This would provide some wider context in which to place the importance of this study generally, as well as contaminant/climate changes studies more generally. Response: We thank the referee for this thoughtful comment and have introduced considerations on this matter: “From a consumer perspective, our study showed that the counter-acting CO₂ effect (hampering warming-stimulated Hg accumulation) was consistent in the muscle, the main tissue ingested by human population. Since this is the most relevant tissue for commercialization, such results constitute an important finding in the area of seafood safety, worthy of further research.” (Page 11, Line 16-20) “Further knowledge on climate change and contamination impacts on fish ecophysiology (and biochemical stress-coping mechanisms) will help towards better comprehension of future fish stocks’ health condition and tissue-dependent contaminant accumulation, consequently forecasting socio-ecological consequences in the oceans of tomorrow. Another pertinent knowledge gap that has been scarcely addressed is how oxidative stress and lipid peroxidation modify the nutritional value and general palatability of seafood, particularly fish. Thus, further multi-stressor studies on seafood safety and biochemical changes should be performed with the intent of helping stakeholders and regulatory authorities define future consumption recommendations and legislation.” (Page 13, Line 8-15)

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Technical corrections:

Technical correction #1: Page 1, Line 18-19 – Sentence beginning “Despite the more than likely co-occurrence...” is weak and doesn’t read well. Needs stronger justification (see above) to enable stronger conviction in abstract, as well as explicitly highlight that contaminant/climate change stressor interactions are largely overlooked, rather than just “these stressors”.

Response: We rephrased: “Future interactive effects between contaminants and climate change stressors are still largely unknown, even though such interactions will play a key role in shaping the ecophysiology of marine organisms.” (Page 1, Lines 16-19)

Technical correction #2: Page 1, Line 29 – should read mechanisms not mechanism

Response: Changed.

Technical correction #3: Page 2, Line 2 (and throughout) – should be CO₂ sub-scripted, this error occurs in a number of positions throughout manuscript, also sometimes is sub-scripted so inconsistent.

Response: Corrected.

Technical correction #4: Page 2, Line 4-5 – I would argue greenhouse gas effect is increasing global temperatures, and this is resulting in projected further increase (already increased by 0.76 °C from pre-industrial) in surface ocean temperature of . . . by end of the century.

Response: Changed to: “Moreover, conjointly with other “greenhouse” gases, increased CO₂ has triggered a continuous rise in mean ocean temperatures (nowadays increased by 0.76°C from pre-industrial values), and predictions point to a further 0.3-4.8 °C increase by the end of the century (IPCC, 2014).” (Page 2, Lines 2-6)

Technical correction #5: Page 2, Line 22 – Should read “. . . Sampaio et al., 2016) and

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ultimately mortality (Coccini et al., 2000).”

Response: Changed.

Technical correction #6: Page 3, Line 7 – protein not proteins

Response: Changed.

Technical correction #7: Page 3, line 11 – responses not response

Response: Changed.

Technical correction #8: Page 3, Line 16 – remove the before estuaries

Response: Removed.

Technical correction #9: Page 4, Line 10 – should be pH controllers not controller

Response: In this case, although multiple pH probes were used, all were connected to a single pH controller, i.e. a Profilux system (± 0.1 , Profilux 3.1N, GHL). However, we have changed phrasing for the sake of clarity: “We used a Profilux system (± 0.1 , Profilux 3.1N, GHL) as pH controller, connected to each tank by individual pH probes.” (Page 4, Lines 17-18)

Technical correction #10: Page 5, Line 5 – Length³ should be super-scripted

Response: Changed.

Technical correction #11: Page 5, Line 19 – remove with before nitric acid

Response: Removed.

Technical correction #12: Page 5, Line 23 – should be gill not gills

Response: Changed.

Technical correction #13: Page 5, Line 25 – remove posteriorly Response: Removed.

Technical correction #14: Page 5, Line 26 – rewrite as “..response concentrations,

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quantified” removing were

Response: Changed.

Technical correction #15: Page 6, Line 18 – assume is potassium periodate not potassium per iodate

Response: Corrected.

Technical correction #16: Page 6, Line 23 (and page 7, line 16) – mg-2 needs superscripting

Response: Done.

Technical correction #17: Page 6, Line 25 – insert space before Superoxide

Response: Done.

Technical correction #18: Page 7, Line 5 – is the % inhibition of SOD activity calculated as maximum inhibition, average inhibition at each 5 minute time point or from initial and final, just measured every 5 minutes over 25 minutes so potentially have different rates of inhibition and total overall inhibition over this time course

Response: It is the average inhibition from initial to final (25 minutes, 5 minute readings are used to create the slope). We included the information: “... , which allowed the assessment of inhibition percentage per minute (averaged from 25 minutes),...” (Page 7, Line 28-29)

Technical correction #19: Page 7, Line 23 – insert space before and

Response: Done.

Technical correction #20: Page 8, Line 2 – insert space in mg-1 total

Response: Done.

Technical correction #21: Page 9, Line 19 (and other places) – *A. regius* needs italicisation

ing

Response: Corrected throughout the manuscript.

Technical correction #22: Page 10, Line 2 – notoriously is an odd choice of words, suggest just removing as reads fine without replacing

Response: Changed according to referee's suggestions.

Technical correction #23: Page 10, Line 23 (and page 11, line 15) – H2O needs subscripting

Response: Done.

Technical correction #24: Page 18 – Why is the x-axis reversed on figure 1, d, compared to b and c. This confuses comparisons.

Response: Indeed, we apologize for the mistake and have corrected it. See new Figure 1 in the marked manuscript.

Please also note the supplement to this comment:

<https://www.biogeosciences-discuss.net/bg-2017-147/bg-2017-147-AC1-supplement.pdf>

Interactive comment on Biogeosciences Discuss., <https://doi.org/10.5194/bg-2017-147>, 2017.

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