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Influence of incubation time on antimicrobial susceptibility testing of pathogenic *Vibrio anguillarum* and *Vibrio vulnificus* isolated from fish

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ABSTRACT

A multi-laboratory study was performed to investigate the most suitable incubation time for susceptibility tests of fish pathogens *Vibrio anguillarum* and *Vibrio vulnificus* performed at 28 °C. An isolate set consisting of 30 *V. anguillarum* and 26 *V. vulnificus* was used by four participating laboratories in Denmark, France, Sweden, and the Netherlands. Inhibition zone diameters were determined by agar disc diffusion for eight agents and Minimum Inhibitory Concentration (MIC) values were determined for seven agents using the standard CLSI testing protocols for non-fastidious organisms that specify 24-28 h incubation. In this work an additional set of readings was made after 48 h incubation. In total, 1120 paired zone sizes and 399 paired MIC observations were made at the two incubation times. Examination of the data demonstrated that incubation time had a small but statistically significant effect on the numerical values of susceptibility measures. However, the effects of incubation time on the precision of the data sets and the categorisation of isolates based on the application of epidemiological cut-off values were slight and statistically non significant. These analyses suggest that the susceptibility of these *Vibrio* species could be established using protocols that specify either 24-28 h or 44-48 h incubation.

This study does not provide evidence that prolonged incubation to 48 h improves the quality of data generated by the tests. Therefore, it is recommended that the existing standard CLSI protocols with 24-28 h at 28 °C should be adopted for susceptibility testing of *V. anguillarum* and *V. vulnificus*.

1. Introduction

Vibrio anguillarum is a serious pathogen of aquatic animals (Haenen et al., 2014) and *Vibrio vulnificus* may cause disease in both aquatic animals and humans (Austin, 2010; Dalsgaard et al., 1999). Therefore, both these species should be included in programmes for monitoring and surveillance of antimicrobial susceptibility of aquatic organisms that are recommend in the OIE Aquatic Animal Health Code (OIE, 2019). The OIE Aquatic Code recommends that susceptibility should be established using internationally-harmonized and standardised testing protocols. Turner and Ashley (2019) and Schwarz et al. (2010) have commented that non-compliance with standardised testing protocols appears to be relatively common in the published literature on the susceptibility of human and veterinary pathogens. Schwarz et al. (2010) have commented that these standardised protocols provide strict rules and minor modifications of the testing conditions are not acceptable. Smith and Egan (in press) reported that such modifications of standard protocols were common in published studies of *Vibrio* spp. susceptibility. Therefore, the issue of whether the currently available standardised testing protocols published by CLSI (2006, 2014) without any

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modification are suitable for these two *Vibrio* species needs to be addressed.

Smith and Egan (2018), following an extensive review of the published literature on susceptibility testing of *Vibrio* spp., suggested that it was safe to recommend that the antibacterial agent susceptibility of *Vibrio alginolyticus, V. anguillarum, V. harveyi, V. parahaemolyticus* and *V. vulnificus* could be established using protocols that specify the use of Mueller-Hinton media without additional NaCl. The review further suggested that *V. alginolyticus, V. harveyi, V. parahaemolyticus* and *V. vulnificus* could be tested at 35 °C or 28 °C. However, they found no studies that had tested *V. anguillarum* at temperatures above 28 °C and recommended that this species could be tested at 28 °C but not at 35 °C. The only internationally standardised susceptibility testing protocols that specify the use of unmodified Mueller-Hinton media and incubation at 28 °C are those given in the guidelines VET03-A (CLSI, 2006) and VET04-A2 (CLSI, 2014). Both these protocols specify that results obtained at 28 °C should be read after 24-28 h incubation.

An essential component of any standard CLSI guideline is the inclusion of mandatory quality control (QC) procedures. A central component of these QC procedures is the demonstration of compliance with the acceptable ranges for reference strains. With respect to the protocols for non-fastidious organisms at 28 °C (CLSI, 2006, 2014) the acceptable ranges have been set for two reference strains (*Escherichia coli* ATCC 25922 and *Aeromonas salmonicida* ATCC 33658), recorded after 24-28 h incubation. Acceptable ranges for reference strains after 44-48 h incubation at 28 °C incubation have not been set. Until such ranges are set, tests that generate data obtained after 44-48 h cannot claim to have been performed using a standard CLSI protocol. Nor, importantly, is it legitimate to apply any internationally harmonized interpretive criteria developed for data recorded after 24-28 h incubation to any data recorded after 48 h incubation. Essentially a protocol that specifies a prolonged incubation period must be considered a new susceptibility testing protocol.

The work reported here was designed to investigate whether 24-28 h incubation at 28 °C and the use of Mueller-Hinton media without additional NaCl was adequate for the susceptibility testing of *V. anguillarum* and *V. vulnificus* or whether the quality of the data generated would be sufficiently improved by an additional 24 h incubation to justify the development of a new susceptibility testing protocol.

2. Materials and methods

2.1. Laboratories

The participating laboratories were those of the Mycoplasmology-Bacteriology and Antimicrobial Resistance Unit of Ploufragan-Plouzané-Niort Laboratory (ANSES), the Unit for Fish and Shellfish Diseases at DTU Aqua (Technical University of Denmark, National Institute of Aquatic Resources), Kgs Lyngby, Denmark (DTU), National Reference Laboratory for fish at the National Veterinary Institute, Uppsala, Sweden (SVA) and National Reference Laboratories for Antimicrobial Resistance and for Fish Diseases, Lelystad, the Netherlands (WBVR).

2.2. Isolates

The isolate set analysed was composed of 30 *V. anguillarum* and 26 *V. vulnificus* isolates. The *V. anguillarum* set of isolates consisted of 10 isolates each from archive collections of SVA, DTU, and WBVR, respectively. For *V. vulnificus*, seven isolates were provided by SVA, 10 by DTU, and nine by WBVR. Identification at the species level was performed by each laboratory on their own isolates using MALDI-TOF (Bruker). All isolates had been collected from diseased fish between 1980 and 2017 (see Table S1).

2.3. Disc diffusion assays

Disc diffusion assays were performed in three laboratories (ANSES, DTU and WBVR). Each laboratory performed tests on the same set of 56 isolates using the range of agents routinely used by them in their national diagnostic and/or monitoring programmes. The conditions for the disc diffusion assays were based on those provided in the CLSI guideline VET03-A for susceptibility testing of non-fastidious organisms at 28 °C (CLSI, 2006). This protocol specifies the use of Mueller Hinton agar without any additional NaCl and the recording of zones after 24-28 h incubation. It should be noted that Mueller-Hinton agar may contain some NaCl deriving from the meat extract and caesin peptone used in its manufacture. However the concentrations of NaCl from these sources is likely to be < 1% (Ron Miller, pers. comm). In this work this protocol was modified by the recording of zone sizes after 24 h and also after 48 h incubation.

The agents and content of the discs used were 25 μg amoxicillin (AMO), 10 μg ampicillin (AMP), 30 μg chloramphenicol (CHL), 30 μg florfenicol (FFN), 2 μg oxolinic acid (OXO), 30 μg oxytetracycline (OXY), 30 μg tetracycline (TET) and 1.25/23.75 μg trimethoprim/sulfamethoxazole (TRS). The abbreviations adopted for the antimicrobial agents were those recommended in the EUCAST System for Antimicrobial Abbreviations (http://www.eucast.org/ast_of_bacteria/guidance_documents/). The discs were obtained from various manufacturers listed in Supplementary Table S2.

2.4. Minimum inhibitory concentration (MIC) assays

Microdilution MIC assays were performed in two laboratories (ANSES and SVA). The conditions for these assays were based on those provided in the CLSI guideline VET04-A2 for microdilution susceptibility testing of non-fastidious organisms at 28 °C (CLSI, 2014). This protocol specifies the use of cation adjusted Mueller Hinton broth (CAMHB) without any additional NaCl and the recording of results after 24–28 h incubation. In this work this protocol was modified by the recording of results after 24 h and 48 h incubation.

The layout of the ninety-six well microtiter plates used by the laboratories who performed MIC were those used by them in their routine diagnostic and/or monitoring programmes. The ANSES laboratory made their plates in their own laboratory using a Biomek automated liquid handler (Beckman Coulter, Paris, France). The range of concentrations in these plates included AMO (0.06–128 mgL^{-1}), FFN (0.031–128 mgL^{-1}), OXO (0.002–16 mgL^{-1}) TRS (0.016/0.3–32/608 mgL^{-1}) and TET (0.016–128 mgL^{-1}). The SVA laboratory used commercially available plates (VetMIC Aquatic Art. No. E395128), a panel developed for antimicrobial susceptibility testing of bacteria isolated from aquatic animals (SVA, Section of Substrate Production). The range of concentrations in these plates included AMP (0.06–8 mgL^{-1}), FFN (0.12–8 mgL^{-1}), OXO (0.008–1 mgL^{-1}) and OXY (0.03–4 mgL^{-1}) and TRS (0.03/0.6–4/76 mgL^{-1}).

2.5. Normalised resistance interpretation (NRI) analysis

Normalised resistance interpretation (Kronvall, 2003, 2010) provides an objective method calculating epidemiological cut-off values (CO_{WT}) that allow the categorising of isolates as either fully susceptible wild type (WT) members of their species or as non-wild-type (NWT) isolates that manifest a susceptibility significantly lower that that of the WT isolates. The inhibition zone and MIC data NRI analyses were performed using the automatic Excel spreadsheets available at http://www.bioscand.se/nri/.

2.6. Statistical analyses

All statistical analyses, including Mann-Whitney, Student *t* and Wilcoxon's matched-pairs signed-rank tests, were performed using

InStat 3.1a (GraphPad Software, Inc.).

3. Results and discussion

The three laboratories which performed disc diffusion assays reported that the growth of all of the 30 *V. anguillarum* and 26 *V. vulnificus* isolates on unmodified Mueller Hinton agar without additional NaCl was sufficient to allow zone size measurements to be made after 24 h and after 48 h incubation at 28 °C. The two laboratories who performed microdilution tests also reported that the growth of all of these isolates in unmodified CAMHB was sufficient to allow MIC values to be determined after incubation at 28 °C for 24 h and 48 h.

Zone sizes were determined for four agents (FFN, OXY, OXO and TRS) in three laboratories and for a further four agents (AMO, AMP, CHL and TET) in two laboratories. Each laboratory recorded zone sizes manifest by the 56 isolates after 24and 48 h incubation. In total, 1120 pairs of zone sizes, recorded after these two incubation times, were generated.

The MIC values were determined for FFN, OXO and TRS in two laboratories and for AMO, AMP, OXY and TET in a single laboratory and again each laboratory recorded MIC values for the 56 isolates after 24 h and after 48 h incubation. The vast majority (72%) of the MIC values with respect to TRS for both species and all the MIC values for AMP against *V. anguillarum* could not be quantified as they appeared to lie outside the range provided for in either of the plate designs used. Therefore, these data sets were excluded from any subsequent analysis. In addition, a further 22 MIC values for various other agents against some isolates appeared to lie outside the range that could be determined by the microplates used in this study and were therefore also excluded. In total, 399 paired, quantitative MIC values were generated.

The paired measures of susceptibility (zone sizes or MIC values) were examined to answer three questions. Was there a quantitative difference in the measures recorded after 24 h incubation and those recorded after 48 h? Was there a difference in the precision of data obtained at the two times? Was there a difference in the categorisation of isolates made from analysis of the data obtained at the two times?

3.1. Effect of time on quantitative measures of susceptibility

3.1.1. Zone size data

For each of the 1120 separate assays (analysing laboratory, isolate and agent tested) the difference in the zone sizes recorded at 24 h and after 48 h incubation was calculated. A frequency plot of these differences showed a normal distribution with a mean of 0.08 mm and a standard deviation (SD) of 1.9 mm (Fig. 1). For 93% of the paired data sets the differences were less than 3 mm. This indicates that, when the data for the two species were taken together, incubation time exerted no significant or consistent effect on zone size. However, when the data for each species were treated separately, a species-specific significant difference was apparent. The mean of the zone sizes recorded for *V. vulnificus* after 24 h incubation was 1 mm smaller than those recorded after 48 h incubation. In contrast for *V. anguillarum* the mean of the zones recorded after 24 h incubation was half a mm larger. Mann-Whitney analysis of the 600 paired data for *V. anguillarum* and the 520 paired data for *V. vulnificus* demonstrated that the difference in the effect of time for each species was significant ($p < .0001$).

3.1.2. MIC data

As the MIC values were obtained using a two-fold dilution series they were $\log_2$ transformed and the difference in the $\log_2$ MIC values recorded at 24 h and 48 h was calculated for the 399 paired observations. In contrast to the difference recorded for zone sizes, the distribution of the differences in $\log_2$ MIC showed a skewed distribution (Fig. 2). There was no difference in the MIC determined at the two times in 235 pairs (59%) but for 146 (37%) pairs the MIC determined after an additional 24 h incubation was 1 dilution higher. The MICs determined after the longer incubation were two and three dilutions higher in 13 pairs (3%) and 3 pairs (1%) respectively. However, for only one pair was the MIC recorded at 48 h smaller (by 1 dilution) than that recorded at 24 h. When the data from both species were combined, analysis by Wilcoxon's matched-pairs signed-rank test demonstrated that the increase in the $\log_2$ MIC determined after the longer incubation time was statistically significant ($p < .0001$). This suggests that MIC values generally increase as the time of incubation is extended.

As with the disc zone data, when the MIC data for each species were treated separately, a significant species-specific difference was apparent. In the case of the $\log_2$ MIC data the effect of incubation time was greater for *V. anguillarum* (mean difference 0.61 $\log_2$ mg L^{-1}) than for *V. vulnificus* (mean difference 0.31 $\log_2$ mg L^{-1}). Mann-Whitney analysis of the 201 paired data for *V. anguillarum* and the 198 paired data for *V. vulnificus* demonstrated that the difference between the extent of the increase in the MIC with the longer incubation time between the two species was significant ($p < .0001$).

In comparing the effects of incubation time on zone sizes and MIC values it is important to remember that there is a difference in the sensitivity of the two methods. In their foundation work on *in vitro*

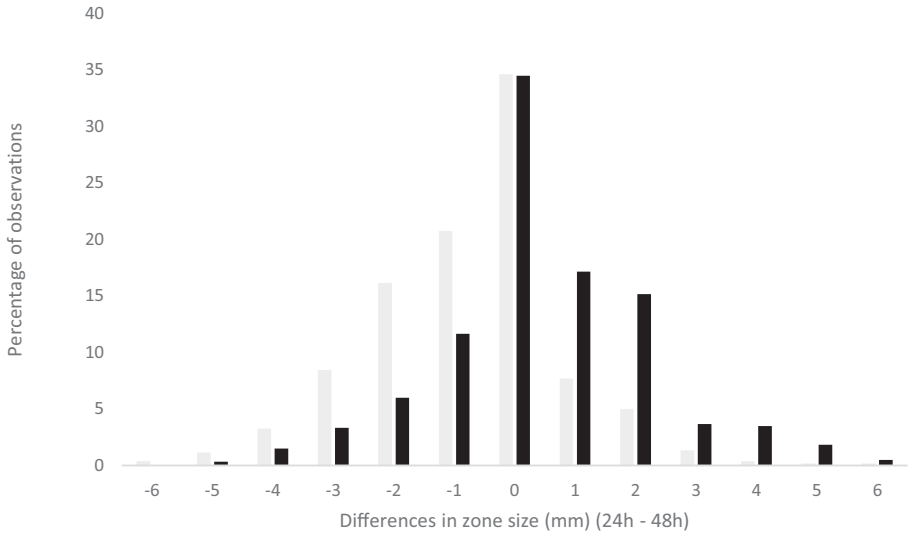

Fig. 1. Distribution of difference (mm) between zones measured in the agar diffusion assay after 24 h and 48 h incubation. Grey bars indicate distribution of 600 difference for *V. anguillarum* and black bars indicate distribution of 520 difference for *V. vulnificus*.

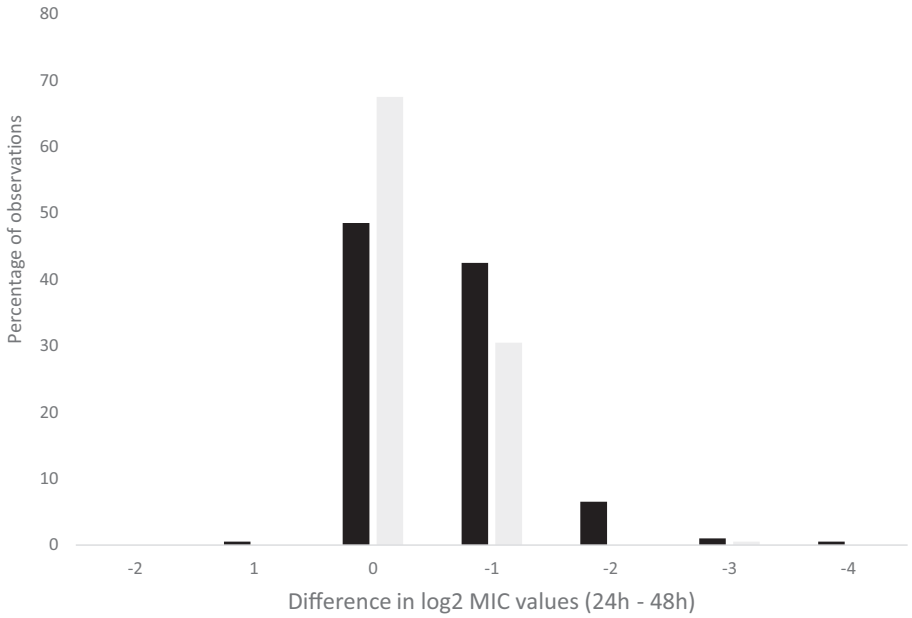

Fig. 2. Distribution of difference between $\log_2$ MIC values measured after 24 h and 48 h incubation. Grey bars indicate distribution of 201 difference for *V. anguillarum* and black bars indicate distribution of 198 difference for *V. vulnificus*.

susceptibility, Ericsson & Ericsson and Sherris (1971) demonstrated that disc diffusion was both more precise and more sensitive than MIC determinations. They calculated that the mean slopes of the linear regression of paired $\log_2$ MIC values and disc zones for 50 antimicrobial agent/species combinations was 2.84 mm $\log_2 mg^{-1}$ $L^{-0.1}$. If this conversion factor was applied to the MIC data above it would suggest that the mean difference for *V. anguillarum* of 0.61 $\log_2$ mg L^{-1} would be equivalent in size to a difference of 1.7 mm in disc zone data. Similarly, the mean difference for *V. vulnificus* of 0.31 $\log_2$ mg L^{-1} would be equivalent in size to a difference of 0.9 mm in disc zone data.

3.2. Effect of time on precision of measures of susceptibility

It was considered possible that, if growth of these isolates was incomplete after 24 h, this might lead to an increased difficulty in obtaining accurate susceptibility measurements. In turn this might lead to a reduction in precision of the data sets recorded at this time. Smith and Kronvall (2014) have argued that the standard deviation of the normalised distributions of WT observations as calculated by NRI analysis (SD) provides a proxy measure of the relative precision of data sets. Therefore, the data obtained in this work were examined to establish whether there were significant differences in the SD values of the data sets recorded at the two times.

3.2.1. Zone size data

NRI analysis was performed on the combined zone size data generated by all laboratories for each bacterial species and antimicrobial agent at each temperature, and their SD values were calculated. In two of the species/agent data sets, those of AMO and AMP for *V. anguillarum*, all isolates were categorised as NWT. Therefore, for these data sets normalised distributions of WT observations could not be calculated. When the data from both species were combined, the mean of the SD for the other 14 data sets was 2.91 mm when the 24 h observations were analysed and 3.05 mm when the 48 h observations were analysed. Analysis with the paired *t*-test demonstrated that the difference between the SD values obtained at the two times was not significant ($p = .84$).

There was an indication that there might be a slight difference in the effect of time on the distributions of the SD values for the two species. The mean SDs for the *V. anguillarum* sets were slightly smaller for the 24 h sets (3.08 mm) than those for the 48 h sets (3.51 mm). For *V. vulnificus* the equivalent values were 2.78 mm and 2.64 mm. However, for neither species were the differences in SD values significant ($p > .5$). Overall this suggests that incubation time exerted no significant or consistent effect on zone data set precision.

3.2.2. MIC data

NRI analysis was performed on the combined MIC data generated by all laboratories for each bacterial species and antimicrobial agent for both incubation times and the SD values of the normalised WT observations were calculated. Again, as with respect to AMO and AMP all *V. anguillarum* isolates were categorised as NWT, SD values could not be calculated for these data sets. The mean of the SD for the other 12 data sets was 0.756 $\log_2$ mg L^{-1} when the 24 h observations were analysed and 0.687 $\log_2$ mg L^{-1} when the 48 h observations were analysed. Analysis with the paired *t*-test demonstrated that the difference between the SD values obtained at the two times was not significant ($p = .41$). There were too few data sets to allow any valid statistical evaluation of any species effects.

3.3. Effect of time on the calculation of the frequencies of NWT

A major aim of susceptibility testing is to categorise isolates as either WT or NWT. Therefore, the data sets were examined to establish whether there were differences in the categorisation of isolates based on measurements of their susceptibility made by individual laboratories at the two times.

3.3.1. Zone size data

For each species/agent combination NRI analysis was used to calculate epidemiological cut-off values (CO_{WT}) from the combined zone size data of all laboratories who reported zone sizes after 24 h incubation. Again, as all isolates of *V. anguillarum* were presumed to be NWT with respect to AMO and AMP, these data sets were not included in these analyses. This process was repeated for the data recorded after 48 h incubation. The mean of the difference between the 14 paired CO_{WT} calculated after the two incubation times was 0.21 mm and for 85% of them the difference was less than 4 mm.

Isolates were then categorised by application of the relevant species- and agent-specific CO_{WT} value to the zones recorded by individual

laboratories for them at the two times. The differences between the categorisations generated from the 24 h data and those generated from the 48 h data were calculated. In total 1000 comparisons were made and in 973 (97.3%) there was agreement in the categories established at the two times. Overall this suggests that incubation time exerted no significant or consistent effect on the categorisation of isolates based on their zone sizes, always provided that the CO_{WT} values were obtained under the same incubation conditions as the observational data they were applied to.

3.3.2. MIC data

For each species/agent combination NRI analysis was used to calculate epidemiological cut-off values (CO_{WT}) from the combined data of all laboratories who reported quantitative MIC values after 24 h incubation. Again, as all isolates of *V. anguillarum* were presumed to be NWT with respect to AMO and AMP these data sets were not included in these analyses. This process was repeated for the data recorded after 48 h incubation. For six of the ten data sets the CO_{WT} values calculated after the two times were identical, for three CO_{WT} calculated after 48 h were one dilution higher and for one it was two dilutions higher. Thus, the effect of incubation time on CO_{WT} determination reflects the effect it has on MIC values.

The agreement between the categorisations of isolates based on analysis of the 24 h data and those based on the 48 h data were calculated. In total, 388 comparisons were made and in 381 (98.2%) there was agreement in the categories established at the two times.

4. Conclusions

In this work performed at four laboratories in a cooperation of five countries, both incubations for 24 h and 48 h generated susceptibility data of adequate quality. The additional 24 h incubation time resulted in only minor and largely statistically non significant differences in disc zone data and small but statistically signifiant increases in MIC data, and had little effect on the precision of the data obtained by either method. To a very large extent (> 97%), there was agreement between the categorisation of isolates based on the data obtained after the two incubation times.

The currently available standard testing protocol for non-fastidious organisms such as *V. anguillarum* and *V. vulnificus* at 28 °C specifies an incubation period of 24 h–28 h (CLSI, 2006, 2014). Adopting 48 h as a standard incubation time for these species would necessitate the development of a new standard method with its appropriate quality control requirements. To establish such a new protocol would require time, money and effort. It follows that any argument in favour of adopting 48 h incubation as a standard for these species must demonstrate that the resultant gains, either in precision of data generated or in the improvement in the categorisation of isolates, would be sufficient to justify undertaking the work that would be involved. It is argued that the differences recorded in this work do not provide sufficient grounds for such action and that existing standard testing protocols (CLSI, 2006, 2014) are adequate for the susceptibility testing of *V. anguillarum* and *V. vulnificus*.

It should also be noted that adopting a protocol with a longer (48 h) incubation time would result in a 24 h hour delay in providing results to veterinarians but would not improve the quality of those results.

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Disclaimer

Daniela Ceccarelli is currently employed by the Research Executive Agency. The views expressed are purely those of the author(s) and may not in any circumstances be regarded as stating an official position of the European Commission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735258.

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