1 **Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates**

2 **from the United Kingdom**

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13

16 **Abstract**

17 Routine application of antimicrobials is the current treatment of choice for rainbow trout

18 fry syndrome (RTFS) or bacterial coldwater disease (BCWD) caused by *Flavobacterium*

19 *psychrophilum*. In this study, the antimicrobial susceptibilities of 133 *F. psychrophilum*

20 isolates, 118 of which were from the UK, were evaluated by broth microdilution and disc

21 diffusion methods following VET04-A2 and VET03-A guidelines of Clinical and

22 Laboratory Standards Institute (CLSI), respectively. Isolates were categorised as wild

23 type (fully susceptible, WT) or non-wild type (NWT) using normalised resistance

24 interpretation (NRI) determined cut-off values (CO_{WT}). Broth microdilution testing

25 showed that only 12% of UK isolates were WT to oxolinic acid (MIC CO_{WT} ≤0.25 mg L^{-}

26 ^{1}) and 42% were WT for oxytetracycline (MIC CO_{WT} ≤0.25 mg L^{-1}). In contrast, all the

27 isolates tested were WT (MIC CO_{WT} ≤2 mg L^{-1}) for florfenicol, the main antimicrobial

28 for RTFS control in the UK. Disc diffusion-based CO_{WT} values were ≥51 mm for 10 µg

29 amoxicillin, ≥44 mm for 30 µg florfenicol, ≥30 mm for 2 µg oxolinic acid and ≥51 mm

30 for 30 µg oxytetracycline. There was a high categorical agreement between the

31 classifications of the isolates by two testing methods for florfenicol (100%),

32 oxytetracycline (93%), and oxolinic acid (99%).

33

34 *Keywords*: *Flavobacterium psychrophilum*, antimicrobial susceptibility, epidemiological

35 cut-off values, disc diffusion, broth microdilution, rainbow trout fry syndrome.

Introduction

Flavobacterium psychrophilum, a Gram-negative, filamentous, psychrotrophic bacterium, is the aetiological agent of rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (BCWD), which was first described in USA in 1946 (Borg 1948). *F. psychrophilum* infection has been found throughout North America, Europe and elsewhere in Turkey, Australia, Peru, Japan and Korea (Barnes & Brown 2011). A commercial vaccine against RTFS/BCWD is still not available (Gómez *et al.* 2014). Alhtough phage therapy (Stenholm *et al.* 2008; Kim *et al.* 2010a; Castillo *et al.* 2012) and the use of probiotic bacteria (StrömBesto & Wiklund 2011; Korkea-aho *et al.* 2011; Boutin *et al.* 2012; Burbank *et al.* 2012) have been suggested to be a promising alternative to the use of antibiotics in aquaculture, further studies are needed to prove the consistent effect of these green/blue technologies on preventing the infection of *F. psychrophilum*. Therefore, the use of antibiotics is currently the treatment of choice for controlling RTFS/BCWD outbreaks, resulting in a concern about the development of antimicrobial resistance by *F. psychrophilum* (Gómez *et al.* 2014). In the UK, three antibiotics (florfenicol, oxytetracycline and amoxicillin) are licensed for use in aquaculture by the UK Veterinary Medicines Directorate (VMD) (http://www.vmd.defra.gov.uk/).

Several studies have examined the antimicrobial susceptibility of *F. psychrophilum* isolated from the USA (Pacha 1968, Soule *et al.* 2005; Van Vliet *et al.* 2017), the UK (Rangdale *et al.* 1997; Verner-Jeffreys & Taylor 2015; Smith *et al.* 2016), Denmark (Lorenzen *et al.* 1997; Bruun *et al.* 2000; Dalsgaard & Madsen 2000; Schmidt *et al.*

58   2000; Bruun *et al.* 2003; Smith *et al.* 2016), France (Michel *et al.* 2003), Japan (Izumi &

59   Aranishi 2004), Turkey (Kum *et al.* 2008; Durmaz *et al.* 2012; Boyacıoğlu & Akar 2012;

60   Boyacıoğlu *et al.* 2015), Canada (Hesami *et al.* 2010), Spain (Del Cerro *et al.* 2010),

61   Norway (Nilsen *et al.* 2011), Chile (Henríquez-Núñez *et al.* 2012; Miranda *et al.* 2016)

62   and Finland (Sundell *et al.* 2013). However, differences in the medium and growth

63   conditions used in these studies and variations in the interpretive criteria used make

64   comparisons difficult. In addition, some of these studies included only a small number of

65   isolates, while others produced susceptibility data that was too diverse to allow any

66   estimate of cut-off values to interpret their meaning.

67   Smith *et al.* (2013) addressed the need for standardised and internationally

68   recognized protocols for laboratory *in vitro* susceptibility testing in monitoring and

69   surveillance programmes and the use of standardised methods to calculate

70   epidemiological cut-off values for interpretation of the meaning of the data collected in

71   such surveys.

72   The aim of the present study was to evaluate the antimicrobial susceptibility of 140

73   *F. psychrophilum* isolates, 125 of which were obtained within the UK, by the disc

74   diffusion and standardised broth microdilution methods following VET03-A (CLSI 2006)

75   and VET04-A2 (CLSI 2014a) guidelines respectively, as recommended by the Clinical

76   and Laboratory Standards Institute (CLSI) for aquatic bacteria with an optimal growth

77   temperature below 35ºC.

78

79   **Materials and Methods**

Bacterial isolates and growth conditions

A total of 140 *F. psychrophilum* isolates, previously described by Ngo *et al.* (2017) for genetic and serological diversity, were examined in this study. This collection comprised 125 isolates obtained within the UK during 2005-2015 and 15 isolates from other countries (France, Denmark, Finland, Ireland, Chile and the USA) (Table 1 and 2); 123 *F. psychrophilum* isolates were obtained from rainbow trout (*Oncorhynchus mykiss*), 16 from Atlantic salmon (*Salmo salar*) and one from coho salmon (*O. kisutch*). *F. psychrophilum* type strain NCIMB 1947^T (ATCC 49418^T) was included for comparative purposes. For all the experiments, the *F. psychrophilum* isolates were grown in Modified Veggietone (MV) medium [veggitones GMO-free soya peptone (Oxoid, UK), 5 g L^{-1}; yeast extract (Oxoid, UK), 0.5 g L^{-1}; magnesium sulphate heptahydrate (Fisher chemicals, UK), 0.5 g L^{-1}; anhydrous calcium chloride (BHD), 0.2 g L^{-1}; dextrose (Oxoid, UK), 2 g L^{-1}; agar (solid medium; Oxoid, UK), 15 g L^{-1}; pH 7.3] at 18°C for 72 – 96 h. Broth cultures were shaken at 140 rpm. Stock cultures were maintained at -70°C in tryptone– yeast extract–salts medium supplemented with glucose [FLP – tryptone (Oxoid, UK), 4.0 g L^{-1}; yeast extract, 0.4 g L^{-1}; anhydrous calcium chloride, 0.2 g L^{-1}; magnesium sulphate heptahydrate, 0.5 g L^{-1}; D(+)-glucose (Sigma, UK), 0.5 g L^{-1}; Cepeda *et al*. 2004] with 10% glycerol and on Protect-Multi-purpose cryobeads (Technical Service Consultants Ltd, UK).

The 125 isolates from 27 sites within the UK in this study had been isolated between 2005 and 2015 with the majority (110 strains, 88%) being retrieved between 2011 and 2013. Among these isolates, 51 genotypes and 7 plasmid profiles were detected

102 (Ngo *et al.* 2017) (Table 1). However, within this set of 125 isolates, there were five

103 groups of two or three isolates that were recorded as having the same site, sampling time

104 point, genetic profiles and susceptibilities. In order to avoid the over-representation, 7

105 potential replicates were eliminated from the analysis and only 118 UK isolates were

106 included in the analyses. The epidemiological cut-off values were calculated from the

107 data obtained from 118 UK isolates and 15 isolates from other countries. The frequencies

108 of NWT phenotypes circulating the UK during 2005 – 2015 were estimated from the

109 analysis of 118 isolates.

110

111 **Minimum inhibitory concentration (MIC) testing**

112 The MICs for *F. psychrophilum* isolates were performed using Sensititre CMP1MSP

113 plates (Trek Diagnostic Systems; ThermoScientific.com/microbiology). These test plates

114 were 96-well, dry-form plates that contained twofold serial dilutions of the following

115 antimicrobial agents: ampicillin (AMP) 0.03–16 mg L^{-1}, amoxicillin (AMOX) 0.25–16

116 mg L^{-1}, erythromycin (ERY) 0.25–128 mg L^{-1}, enrofloxacin (ENRO) 0.002–1 mg L^{-1},

117 florfenicol (FFN) 0.03–16 mg L^{-1}, flumequine (FLUQ) 0.008–4 mg L^{-1},

118 ormetoprim/sulphadimethoxine (PRI) 0.008/0.15–4/76 mg L^{-1}, oxolinic acid (OXO)

119 0.004–2 mg L^{-1}, oxytetracycline (OTC) 0.015–8 mg L^{-1} and

120 trimethoprim/sulphamethoxazole (SXT) 0.015/0.3–1/19 mg L^{-1}.

121   The MIC assays were determined using the broth microdilution protocol

122 recommended for *F. psychrophilum* in the CLSI guideline VET04-A2 (CLSI 2014a).

123 Colony counts on inoculum suspensions were performed to ensure that the final inoculum

124 density was close to 5.0×10^5 colony-forming units (CFU) per millilitre.

125

126 **Disc diffusion testing**

127 Disc diffusion susceptibility of the *F. psychrophilum* strains was determined by the

128 protocol suggested in the guideline VET03-A (CLSI 2006) with modification on the agar

129 percentage of the culture medium. It should be noted, however, that this protocol has not

130 been formally accepted as a standard by CLSI. The test was performed on diluted

131 Mueller-Hinton medium (Sigma-Aldrich, UK; 3 g L^{-1}) containing 1.5% agar (Agar No.

132 1, LP0011, Oxoid, UK) (MHA) and 5% foetal calf serum (FCS; Gibco, Fisher

133 chemicals, UK) and plates were incubated at $15^{\circ}C$ for 68 – 72 h. Antimicrobial agent

134 discs (Oxoid, Basingstoke, UK) containing 10 µg amoxicillin ($AMOX_{10}$), 5 µg

135 enrofloxacin ($ENRO_5$), 30 µg florfenicol (FFN_{30}), 2 µg oxolinic acid (OXO_2), 30 µg

136 oxytetracycline (OTC_{30}) and 25 µg trimethoprim/sulphamethoxazole (SXT_{25}) were

137 employed.

138

139 **Quality control**

140 As specified in VET04-A2 (CLSI 2014a) the quality control strain *Escherichia coli*

141 NCIMB 12210 (ATCC® 25922) was included in every MIC test run and was assayed on

142 diluted CAMHB at $18^{\circ}C$ as described above. However, no quality control ranges have

143 been established for any disc diffusion protocol specifying these incubation conditions

144 (CLSI 2006). Therefore, the *F. psychrophilum* type strain NCIMB 1947[T] was also

145 included in every test run to monitor the performance of the method.

146

147 **Statistical analysis**

148 The antimicrobial susceptibility patterns of 133 *F. psychrophilum* isolates used in this

149 study were analysed by application of protocol and species-specific epidemiological cut-

150 off values (CO_{WT}). These values allow isolates to be categorised as fully susceptible

151 (wild type, WT) or manifesting reduced susceptibility (non-wild type, NWT). In this

152 work, CO_{WT} values were calculated for both the MIC and disc diffusion data by the

153 normalised resistance interpretation (NRI) method (Kronvall 2003; 2010). This NRI

154 method was used with permission from the patent holder, Bioscand AB, TÄY, Sweden

155 (European patent No 1383913, US patent No 7,465,559).

156   MIC distributions were analysed using the NRI method of Kronvall (2010). A fully

157 automatic Excel spreadsheet for performing these NRI analyses is available on-line

158 (http://www.bioscand.se/nri/). In data sets where a small percentage (<5 %) of the WT

159 observations were "below-scale", these observations were treated as having the MIC

160 value immediately below the limit of the plate quantitation. When the percentage of the

161 WT observations "below-scale" was >5%, the data set was considered as unsuitable for

162 NRI analysis (Smith *et al.* 2016).

163   The NRI analyses for zone histograms were performed using a modification of the

164 standardised protocol developed by Kronvall & Smith (2016). In this modification, the

165 peak values of the zone sizes for the putative WT isolates were established using 8-point

166 rather than 4-point rolling means.

167

168 **Terminology**

169 The acronyms ECV and ECOFF have been used by the CLSI and European Committee

170 on antimicrobial susceptibility testing (EUCAST) respectively for epidemiological cut-

171 off values set from data generated in multiple laboratories. In the present study, the term

172 CO_{WT}, as previously employed by Smith *et al.* (2016), was used to indicate

173 epidemiological cut-off values that have not been set by either of these international

174 agencies. It has been suggested that the terms resistant and sensitive should not be used to

175 refer to the categories identified by epidemiological cut-off values (Silley 2012).

176 Following this suggestion, when isolates are categorised by epidemiological cut-off

177 values, the terms wild type (WT) and non-wild type (NWT) should be used for fully

178 susceptible isolates and isolates exhibiting reduced susceptibility respectively.

179

180 **Results**

181 **Quality control**

182 The MIC values obtained with the quality control reference strain *E. coli* NCIMB 12210,

183 grown at 18°C for 72-96 h in diluted CAMHB, were within the acceptable range

184 published by CLSI in VET03/04-S2 guideline (CLSI 2014b). All the inoculum

185 suspensions used in MIC tests were confirmed to have the density ranging from 4.8 x 10^5

186 to 5.3 x 10^5 CFU mL^{-1} by colony counts.

187   *F. psychrophilum* type strain NCIMB 1947[T] was included in all disc diffusion tests

188   and the inhibition zone data of this strain were 56 – 72 mm for $AMOX_{10}$, 60 – 75 mm for

189    $ENRO_5$, 57 – 64 mm for FFN_{30}, 64 – 86 mm for OTC_{30}, 45 – 56 mm for OXO_2 and 16 –

190   44 mm for SXT_{25}. The mean of the ranges of these zone sizes for these six agents against

191   the *F. psychrophilum* type strain was 16.5 ± 7.6 mm.

192

193   **NRI analysis of susceptibility data**

194   The distribution of MIC values of 133 *F. psychrophilum* isolates for ten antimicrobial

195   agents is shown in Table 3 and 4. MIC-based CO_{WT} values of antimicrobial agents are

196   presented in Table 5. The distribution of disc diffusion zones of the isolates for six

197   antimicrobials is presented in Figure 1 and the zone data-based CO_{WT} values of

198   antimicrobial agents are shown in Table 6.

199

200   *Oxytetracycline*

201   MIC data for OTC showed a clear bimodal distribution (Table 3). The modal group with

202   lower MICs was assumed to represent the WT group. NRI analysis calculated the

203   standard deviation of the log_2 normalised WT distribution as 0.68 and a CO_{WT} value of

204   ≤ 0.25 mg L^{-1} (Table 5). Applying this cut-off, fifty-six (42%) of the 133 isolates analysed

205   were categorised as WT.

206   The disc diffusion zone sizes for OTC_{30} showed considerable diversity at the high

207   zone end (Figure 1A). However, NRI analysis of these data identified a high zone modal

208   group with a standard deviation of 7.44 mm. If this modal group was assumed to

209   represent zones obtained from WT isolates, a provisional CO_{WT} value of ≥51 mm could

210   be calculated (Table 6). Applying this cut-off, sixty-five (49%) of the 133 isolates

211   analysed were categorised as WT.

212   The categorisation of isolates resulting from applying the cut-off of ≤0.25 mg L^{-1} to

213   the MIC data agreed with the categorisation resulting from applying the disc zone cut-off

214   of ≥51 mm to the zone data for 93% of the 133 isolates studied (Figure 2A).

215

216   *Amoxicillin and ampicillin*

217   For AMOX, 98 observations (100% of the lower MIC modal observations) and for AMP,

218   24 observations (24% of the lower MIC modal observations) were recorded as "below-

219   scale" (Table 3). On this basis, neither of these data sets was considered suitable for NRI

220   analysis.

221   As in MIC data set for AMP there was a clear separation of the low MIC and high

222   MIC modal groups, this data set was considered suitable for estimating CO_{WT} by visual

223   examination. The estimated value generated by this subjective method was ≤0.125 mg L^{-1}

224   for AMP. A scatterplot of the paired MIC data for these two beta-lactam agents (Figure

225   3A) suggested a high correlation between them and also demonstrated that AMOX might

226   have the same distribution as AMP.

227   The disc diffusion zone sizes for $AMOX_{10}$ were also bimodal (Figure 1B). NRI

228   analysis of these data calculated a standard deviation of the normalised WT distribution

229   of 5.2 mm and a CO_{WT} value of ≥56 mm (Table 6). A scatterplot of the paired MIC

230   values vernus inhibition zone sizes for amoxicillin suggested a high correlation between

231   them (Figure 2B).

232

233   *Florfenicol*

234   MIC data for FFN showed a clear unimodal distribution (Table 3). This modal group was

235   assumed to represent the WT isolates. NRI analysis calculated a standard deviation of the

236     $\log_2$ normalised WT distribution of 0.68 and a CO_{WT} value of ≤ 2 mg L^{-1} (Table 5).

237   The disc diffusion zone sizes for FFN$_{30}$ were also unimodal (Figure 1C). NRI

238   analysis of these data calculated a standard deviation of the normalised WT distribution

239   of 5.6 mm and a CO_{WT} value of ≥ 45 mm (Table 6).

240   Applying the cut-off of ≤ 2 mg L^{-1} to the MIC data and the disc zone cut-off of ≥ 41

241   mm to the zone data categorised 100% of the 133 isolates studied as WT (Figure 2C).

242

243   *Oxolinic acid, Flumequine and Enrofloxacin*

244   The MIC values of OXO, FLUQ and ENRO were bimodally distributed (Table 3). NRI

245   analysis calculated the standard deviation of the $\log_2$ normalised WT distribution as 0.67,

246   0.57 and 0.74 for OXO, FLUQ and ENRO respectively. The MIC CO_{WT} values

247   calculated from these data were ≤ 0.25 mg L^{-1} for OXO, ≤ 0.125 mg L^{-1} for FLUQ and

248   ≤ 0.032 mg L^{-1} for ENRO (Table 5). When these CO_{WT} values were applied, 21 (16%), 20

249   (15%) and 20 (15%) of the 133 isolates were categorised as WT with respect to OXO,

250   FLUQ and ENRO respectively.

251   Scatterplots of the MIC data for OXO against those for FLUQ and ENRO (Figure

252   3B and 3C) demonstrated a high (>97.7%) categorical agreement in both cases. This

253   suggests that it would be safe to accept MIC data for OXO as a predictor of reduced

254   susceptibility to the FLUQ and ENRO (Smith *et al.* 2016). Adoption of this proposal

255   would reduce the cost of routine susceptibility testing.

256   The disc diffusion zone sizes for OXO_2 were bimodal (Figure 1D). NRI analysis of

257   these data calculated a standard deviation of the normalised WT distribution of 8.5 mm.

258   This high standard deviation is probably a result of the fact that high zone modal group

259   was diverse and composed of only a few observations. This suggests that the disc CO_{WT}

260   value calculated by NRI analysis of ≥30 mm (Table 6) should only be treated as a

261   provisional value. Applying the cut-off of ≤0.25 mg L^{-1} to the MIC data for OXO and the

262   disc zone cut-off of ≥30 mm to the zone data resulted in 99% agreement in the

263   categorisation of the 133 isolates studied (Figure 2D).

264   The disc diffusion zone sizes for FLUQ were not determined and those for ENRO

265   did not show any visually obvious high zone modal group and were not subject to NRI

266   analysis (Figure 1E).

267

268   *Erythromycin*

269   MIC values of ERY had a unimodal distribution. NRI analysis calculated a standard

270   deviation of the log_2 normalised WT distribution of 0.98 and the CO_{WT} value was

271   calculated as ≤8 mg L^{-1} (Table 3 and 5). This value determined that all 133 *F.*

272   *psychrophilum* isolates analysed were WT for ERY.

273

274 *Ormetoprim/Sulphadimethoxine and Trimethoprim/Sulphamethoxazole*

275 The distributions of the MIC values for these two potentiated sulfonamide agents were

276 diverse but appeared to be unimodal (Table 3). NRI analysis generated provisional CO_{WT}

277 values for PRI and SXT of ≤ 320 mg L^{-1} and ≤ 160 mg L^{-1}, respectively. However, the

278 standard deviations calculated for the normalized distribution of these putative WT

279 observations, 1.39 $\log_2$ mg L^{-1} and 1.67 $\log_2$ mg L^{-1} for PRI and SXT respectively, were

280 higher than those recorded for all other agents in this work (Table 5). Therefore, the

281 validity of these CO_{WT} values was questionable.

282 The disc diffusion zone sizes for SXT did not show any visually obvious high zone

283 modal group and were not subject to NRI analysis (Figure 1F).

284

285 **Discussion**

286 **Data precision**

287 *Precision of MIC data sets*

288 The precision of any CO_{WT} value is a function of the precision of the observational data

289 used to calculate it. Smith *et al.* (2012) demonstrated that the standard deviations of the

290 normalised distributions of the $\log_2$ WT observation calculated by the NRI analysis could

291 provide a proxy measurement of precision. The median of the standard deviation

292 calculated for 22 *F. psychrophilum* data sets published by Michel *et al.* (2003), Smith *et*

293 *al.* (2016) and Van Vliet *et al.* (2017) was 0.70 $\log_2$ mg L^{-1}. In this work, the median

294 value of standard deviations calculated for ENRO, ERY, FFN, FLUQ, OTC and OXO

295   (Table 5) was 0.72 $\log_2$ mg L^{-1}. This suggests that the MIC data sets obtained in this work

296   for these agents were of an acceptable level of precision and were of sufficient quality

297   that they could be used to calculate CO_{WT} values.

298   The standard deviations calculated for potentiated sulphonamide MIC data**,** 1.39

299     $\log_2$ mg L^{-1} and 1.61 $\log_2$ mg L^{-1} for PRI and SXT respectively in this work, were,

300   however, considerably larger and were taken to indicate significant imprecision. Smith *et*

301   *al.* (2016) and Van Vliet *et al.* (2017), who used the same testing protocol as was used in

302   this work also reported very low precision in the MIC data they obtained for these agents

303   (mean 1.43 $\log_2$ mg L^{-1}). Due to their low precision, it was considered that valid CO_{WT}

304   could not be established for PRI and SXT data obtained in this work.

305

306   *Precision of disc diffusion data sets*

307   Smith & Kronvall (2015) analysed zone data for reference control strains *E. coli* ATCC

308   25922 and *Aeromonas salmonicida* ATCC 33658 and demonstrated a reduction in

309   precision as the incubation temperature decreased and time increased. Analysis of the

310   data obtained from the reference strain *F. psychrophilum* NCIMB 1947^T and from the test

311   isolates in this work suggest a similar effect of temperature and time on precision of zone

312   size data.

313   The mean of the ranges of zone sizes for the control reference strain *E. coli* NCIMB

314   12210 provided in the guideline VET03-A (CLSI 2006) for tests performed at 35°C,

315   28°C and 22°C were 7.7 ± 0.8 mm, 8.0 ± 1.5 mm and 11.8 ± 2.0 mm respectively (Smith

316 and Kronvall 2015). In this work, the mean range obtained at 15°C for six agents against

317 the control strain *F. psychrophilum* NCIMB 1947^T was 16.5 mm ± 7.6 mm

318   The mean of standard deviations of the 19 zone data sets obtained at 28°C in studies

319 of *Edwardsiella tarda* and *Vibrio harveyi* was 2.53 mm (Lim *et al.* 2016). For 13 data

320 sets of *A. salmonicida* obtained at 22°C, the mean was 3.9 mm (Miller & Reimschuessel

321 2006; Smith *et al.* 2007). In this work, the disc diffusion assays were performed at 15°C

322 and the mean standard deviation of the normalised distributions of the four disc data sets

323 was 6.7 mm.

324   These comparisons suggest that the low precision of the zone data sets obtained in

325 this work was most probably a function of the inherent property of this type of assay

326 rather than any laboratory specific errors in the performance of the assays. However, the

327 low level of precision suggests that any CO$_{WT}$ calculated from these zone data should be

328 treated as only provisional estimates.

329

330 **Categorical agreements**

331 With the calculated MIC CO$_{WT}$ and provisional disc diffusion-based CO$_{WT}$ of FFN, OXO

332 and OTC, it is possible to calculate the percentage agreement between the categorisation

333 of the 133 isolates obtained by analysing the observed MIC measures and the zone size

334 data. The values of these categorical agreements were 100% for FFN, 99% for OXO and

335 93% for OTC. This high level of categorical agreement raise the possibility that, although

336 the disc diffusion protocol used in this work generated data of low precision, the

337   provisional CO_{WT} calculated from them may have some value in detecting isolates of

338   reduced susceptibility.

339   It should, however be noted that Smith & Kronvall (2015) demonstrated that

340   reduced temperatures and prolonged incubation time increased not only the level of intra-

341   laboratory variation but also the level of inter-laboratory variation in the data generated.

342   High inter-laboratory variation of the data will have the consequence that although any

343   provisional disc CO_{WT} calculated in one laboratory may have some value in interpreting

344   zone data produced in that laboratory, it may be misleading if applied to zone data

345   obtained in another laboratory. In other words, the CO_{WT} values for MIC data calculated

346   in this work are probably laboratory-independent and of general or 'universal'

347   applicability. However, it is probably safer to treat the CO_{WT} values for inhibition zone

348   data generated in this work as only of local applicability. As a consequence, each

349   laboratory using this protocol to perform disc diffusion assays would have to generate

350   their own CO_{WT} values. The disc diffusion test protocol used in this work has not been

351   accepted as a standard by CLSI. It is possible that further optimisation such as using a

352   higher incubation temperature ($18^{\circ}C$) may lead to a protocol with increased precision.

353

354   **Comparison of CO_{WT} values calculated for MIC measures determined by**

355   **standardised broth microdilution protocols of CLSI for *F. psychrophilum***

356   The values for any CO_{WT} are protocol-specific. It is, therefore, legitimate to compare the

357   CO_{WT} calculated in this work with those published by Smith *et al.* (2016) and Van Vliet

358   *et al.* (2017), who also used the standardised broth microdilution protocol (CLSI 2014a)

359   and NRI method to calculate CO_{WT} for *F. psychrophilum* from MIC data. This

360   comparison can be made with respect to three agents (FFN, OXO and OTC). For FFN

361   and OXO, the same CO_{WT} values (≤ 2 mg L^{-1} and 0.25 mg L^{-1} respectively) were

362   calculated from all three studies. For OTC, Smith *et al.* (2016) and Van Vliet *et al.* (2017)

363   calculated a CO_{WT} of ≤ 0.125 mg L^{-1} compared to the 0.25 mg L^{-1} calculated in this work.

364   It should, however, be noted that in this work, no isolates were recorded as manifesting

365   an MIC of 0.25 mg L^{-1} for OTC (Table 3) and the categorisation of the 133 isolates

366   studied here would be the same if either CO_{WT} value was applied to them. This

367   agreement in the CO_{WT} values calculated illustrates the value of the use of standardised

368   test protocols and statistically based interpretive criteria and suggests that it should be

369   possible for CLSI to set internationally applicable, laboratory-independent ECVs for this

370   species.

371

372   **Frequencies of UK *F. psychrophilum* isolates with reduced susceptibility**

373   Applying the CO_{WT} values calculated or, in the case of AMP, estimated in this work

374   (Table 5) to the MIC data from these 118 UK *F. psychrophilum* isolates, the frequencies

375   of those with reduced susceptibility were 92% for FLUQ, 90% for ENRO, 88% for OXO,

376   58% for OTC, 32% for AMP and no isolates were recorded with reduced susceptibility

377   for FFN and ERY. However, as noted by Smith *et al.* (2016), ERY, a drug whose primary

378   value is in treating infections by gram-positive bacteria, has never been recommended for

379   the control of *F. psychrophilum* infections of aquatic animal. There have been two earlier

380   studies of NWT frequencies in UK *F. psychrophilum* isolates. Rangdale *et al.* (1997)

381 investigated the susceptibility of 47 *F. psychrophilum* isolates, 36 of which were

382 collected in the UK. However, their MIC data sets were of very low precision (mean log2

383 standard deviation for FFN, OTC and OXO of 3.24 $\log_2$ mg L^{-1}) and therefore, reliable

384 estimates of NWT frequencies could not be assessed. In a smaller study (27 UK *F.*

385 *psychrophilum* isolates) that used the same testing protocol and statistically based

386 interpretive criteria as used in this work, Smith *et al.* (2016) reported NWT frequencies

387 similar to those reported here. When the isolates studied here are combined those studied

388 by Smith *et al.* (2016), the frequency of NWT phenotypes in the 145 UK isolates

389 obtained during 2005 – 2015 were 85%, 59% for OXO and OTC respectively, and no

390 NWT phenotypes were reported for FFN.

391

392 **Antibiotic use in UK rainbow trout farming**

393 Verner-Jeffreys & Taylor (2015) reported the use of four agents (FFN, OXY, AMOX and

394 OXO) in attempts to control RTFS in the UK. The survey revealed that FFN was the

395 treatment of choice in the industry. These FFN treatments were generally considered very

396 effective. Where other antimicrobials (OTC, OXO or AMOX) were used, the therapeutic

397 response was reported as either mixed or poor.

398   These anecdotal reports of comparative treatment efficacies reflect closely the

399 frequencies with which isolates of reduced susceptibility were detected in this work. This

400 in turn suggests that routine susceptibility testing, associated with appropriate

401 interpretation of these data obtained, would be cost-effective and an essential element in

402 the prudent use of antibiotics in aquaculture.

403   Verner-Jeffreys & Taylor (2015) reported that within the UK most batches of

404   rainbow trout were treated with FFN at least once during every production cycle. Thus,

405   given the relatively high frequency of NWT phenotypes detected with respect to the

406   alternative agents available (OXO, OTC and AMOX), it would appear that, as it currently

407   operates, the UK rainbow trout industry is critically dependent on the continued clinical

408   efficacy of FFN. Some concern must be expressed about the long-term sustainability of

409   an industry that would be affected by the emergence of strains of *F. psychrophilum* that

410   were clinically resistant to this agent.

411   As FFN is the agent of choice to treat *F. psychrophilum* infection in many countries,

412   it is reasonable to postulate that this critical dependence of the continued clinical efficacy

413   of FFN is not unique to the UK but is wide-spread in the global trout farming industry.

414   The global situation with respect to FFN susceptibility of *F. psychrophilum* can be

415   assessed from a number of studies that have been published. Studies that have employed

416   standard MIC testing protocols and that generated data of adequate precision have been

417   reported from Denmark and the UK (Smith *et al.* 2016), Chile (Miranda *et al.* 2016) and

418   USA (Van Vliet *et al.* 2017). Studies of the antimicrobial susceptibility of Danish (Bruun

419   *et al.* 2000) and French (Michel *et al.* 2003) *F. psychrophilum* isolates that used non-

420   standardised agar dilution protocols have also been published Combining the data

421   presented in these studies with the data generated in this study provides a total of 829

422   measurements of FFN susceptibility, of which only two (0.2%), both collected in Chile,

423   were categorised as NWT with respect to FFN. Recently the presence of a region

424   containing resistance genes to florfenicol (*floR*), tetracycline (*tet*X), streptothricin and

425   chloramphenicol acetyltransferase gene was detected in *Chryseobacterium* spp. from

426   rainbow trout (Verner-Jeffreys *et al.* 2017). However, this resistance gene cassette was

427   not widely distributed in Flavobacteriaceae isolates (Verner-Jeffreys *et al.* 2017).

428   These data would indicate, rather surprisingly, that the selective pressure that must

429   have resulted from the use of this agent during the 25 or more years since the introduction

430   of the antibiotic to aquaculture (Smith 2008) has not yet resulted in any significant

431   emergence of strains of *F. psychrophilum* strains with reduced susceptibility to this agent.

432   However, it cannot be automatically assumed that this situation will continue. Given the

433   significance of FFN to the global trout farming industry, and as recommended by the

434   World Animal Health Organisations (http://www.oie.int/international-standard-

435   setting/aquatic-code/access-online), it is essential that programmes designed to detect any

436   emergence of isolates of *F. psychrophilum* with reduced susceptibility to this agent are

437   implemented as a matter of urgency by all relevant authorities.

438

439   **Conclusions**

440   Interpretation of MIC data by NRI analysis for *F. psychrophilum* generated by the

441   standardised microdilution protocol (CLSI 2014a) provided an overview of the

442   frequencies of isolates manifesting reduced susceptibility in 118 UK isolates. There was a

443   general agreement between the frequencies of isolates manifesting WT phenotypes for

444   the agents FFN, OXO and OTC, observed in this work, and the reports of their clinical

445   success when used in commercial farms in the UK. On the basis of this, it is strongly

446   recommended that, in order to ensure rational and prudent use of antibiotics to control *F.*

447 *psychrophilum* infections, susceptibility testing using standardised methods should be

448 performed in association with all on-farm administrations of the antibiotics.

449 The Aquatic Animal Health Code of the World Animal Health Organisation

450 recommends that all relevant authorities should implement programmes for the

451 monitoring and surveillance of the susceptibility of aquatic animal pathogens to

452 antibiotics used in their areas. As the UK trout farming industry is critically dependent on

453 the continued efficacy of FFN in the control of RTFS, the implementation of such a

454 programme with respect to *F. psychrophilum* would appear to be a priority for the UK.

455 The standardised MIC susceptibility testing protocol of CLSI and the epidemiological

456 cut-off values developed in this work would provide the analytical methods for such a

457 programme.

458

467

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663

Table 1. Summary of 118 UK *F. psychrophilum* isolates analysed in this study

Location	Host source	No. of sites	Site	Year of isolation	No. of sampling times	No. of strains	No. of genotypes (*)	No. of plasmid profiles (*)
Scotland	*RT(93)/ AS(14)*	*20*		*2005-2015*	*46*	*102*	*47*	*7*
			Scot I	2005-2014	16	25	11	4
			Scot II	2013	1	1	1	1
			Scot III	2011-2015	4	13	9	4
			Scot IV	2013	1	5	2	2
			Scot V	2013-2015	4	27	16	5
			Scot VI	2009	1	1	1	1
			Scot VII	2007	1	1	1	1
			Scot VIII	2005	1	1	1	1
			Scot IX	2006	1	1	1	1
			Scot X	2011-2013	2	3	2	1
			Scot XI	2015	1	4	3	1
			Scot XII	2010	1	1	1	1
			Scot XIII	2005	1	1	1	1
			Scot XIV	2013	1	2	1	1
			Scot XV	2013	2	3	2	1
			Scot XVI	2014-2015	4	9	4	3
			Scot XVII	2007	1	1	1	1
			Scot XVIII	2009	1	1	1	1
			Unknown (2)	2009-2012	2	2	2	2
England	*RT*	*6*		*2007-2015*	*8*	*13*	*5*	*2*
			Eng I	2013	3	8	4	2
			Eng II	2015	1	1	1	1
			Eng III	2015	1	1	1	1
			Eng IV	2015	1	1	1	1
			Eng V	2007	1	1	1	1
			Eng VI	2007	1	1	1	1
Northern Ireland	*RT*	*1*	*N Ire I*	*2013*	*2*	*3*	*2*	*2*
Total	**RT(111)/ AS(14)**			**2005-2015**	**56**	**118**	**51**	**7**

665 RT, rainbow trout; AS, Atlantic salmon

666 (*) Genotypes and plasmid profiles of *F. psychrophilum* isolates determined by Ngo *et al.* (2017).

667

668

669   **Table 2.** Fifteen *F. psychrophilum* isolates from outside the UK used in this study

670

Countries	Host source	Year of isolation	No. of strains	No. of genotypes[a]	No. of plasmid profiles[a]
Chile	RT	1995-1997	2	2	2
Denmark	RT	1990-1994	3	3	1
Finland	RT	1996	2	2	1
France	RT	unknown-2013	3	2	1
Ireland	AS	2006	1	1	1
USA[b]	RT(3)/CS(1)	unknown - 2004	4	4	2
Total	**RT(13)/AS(1)/CS(1)**	**unknown - 2013**	**15**	**13**	**3**

671   RT, rainbow trout; AS, Atlantic salmon; CS, coho salmon

672   [a] Pulsotypes and plasmid profiles of *F. psychrophilum* isolates determined by Ngo *et al.* (2017).

673   [b] including the *F. psychrophilum* type strain NCIMB 1947[T]

674

675

676 **Table 3.** MIC values (mg L^{-1}) determined for 133 *F. psychrophilum* isolates

	Off scale	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	Off scale
β lactams																
AMOX	93										19	13	8			
AMP	24					66	3				15	17	8			
Macrolides																
ERY	4									4	24	93	8			
Phenicols																
FFN							1	9	69	54						
Quinolones																
ENRO			7	10	3	3	51	17	14	28						
FLUQ					3	16	1	1	3	41	13	13				42
OXO						7	13	1	1	6	47					58
Tetracyclines																
OTC					12	40	4		2		7	33	32			3
	Off scale	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	Off scale

677 Shaded areas indicate MIC values could not be determined using Sensititre CMP1MSP plates.

678 Off scale indicates the number of strains whose MIC lay outside of the range that could be determined using these plates.

679

680

681 **Table 4.** MIC values (mg L^{-1}) determined for potentiated sulphonamide drugs against 133 *F. psychrophilum* isolates

682

	Off scale	0.008/0.15	0.015/0.30	0.03/0.59	0.06/1.19	0.12/2.38	0.25/4.75	0.5/9.5	1/19	2/38	4/76	Off scale
PRI						1	2	15	28	43	29	15
SXT					1	11	37	30	48			6

683

684 Shaded areas indicate MIC values could not be determined using Sensititre CMP1MSP plates.

685   Table 5. Cut-off values (CO$_{WT}$) calculated using NRI from 133 MIC observations

Agent	Number of WT observations	Standard deviation [a] (log$_2$ mg L^{-1})	CO$_{WT}$ (mg L^{-1})
AMP[b]	93 (70%)	ND[c]	≤ 0.125
ENRO	20 (15%)	0.74	≤ 0.032
ERY	133 (100%)	0.98	≤ 8
FFN	133 (100%)	0.68	≤ 2
FLUQ	20 (15%)	0.57	≤ 0.125
OTC	56 (42%)	0.68	≤ 0.25
OXO	21 (16%)	0.67	≤ 0.25
PRI	133 (100%)	1.39	≤ 16/304
SXT	133 (100%)	1.61	≤ 8/152

686   [a] Standard deviation of the normalised distribution of MIC values for WT strains.

687   [b] CO$_{WT}$ value of AMP was estimated by visual examination.

688   [c] ND: not determined

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691   Table 6. Provisional cut-off values (CO$_{WT}$) calculated using NRI from 133 inhibition zone
692 observations

Agent	Number of WT observations	Standard deviation* (mm)	CO$_{WT}$ (mm)
AMOX$_{10}$	90 (68%)	5.20	≥56
FFN$_{30}$	133 (100%)	5.61	≥45
OTC$_{30}$	65 (49%)	7.44	≥51
OXO$_2$	20 (15%)	8.50	≥30

693   * Standard deviation of the normalised distribution of MIC values for WT strains.

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A

B

C

D

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723 E
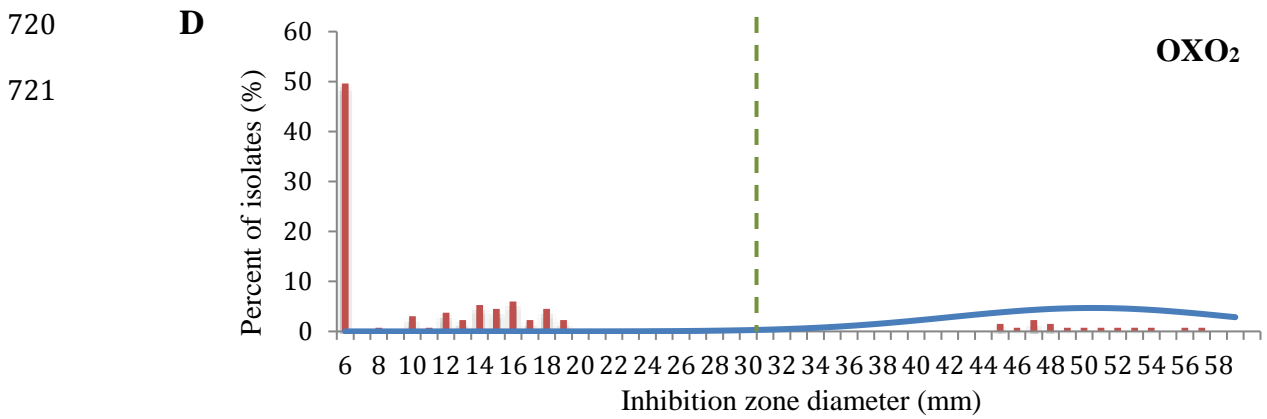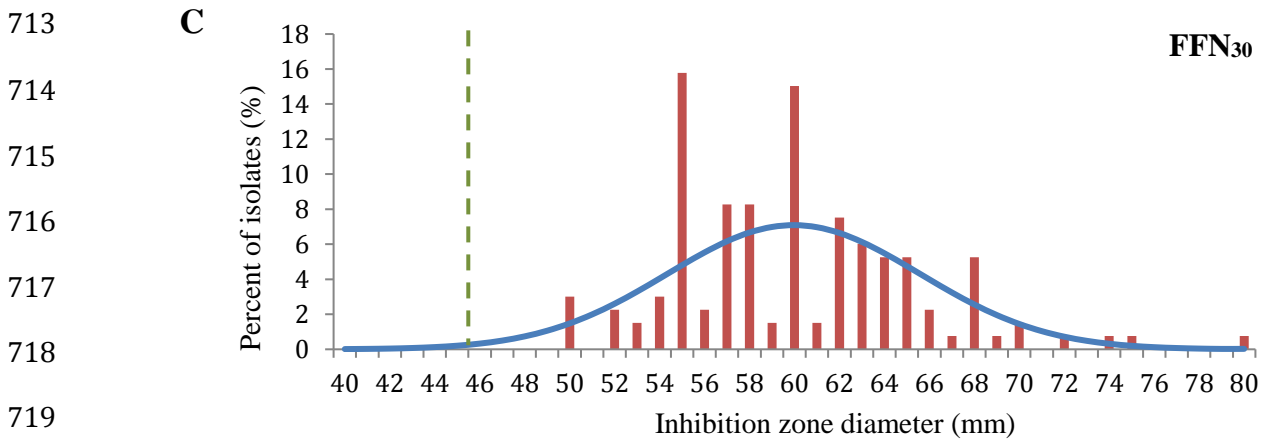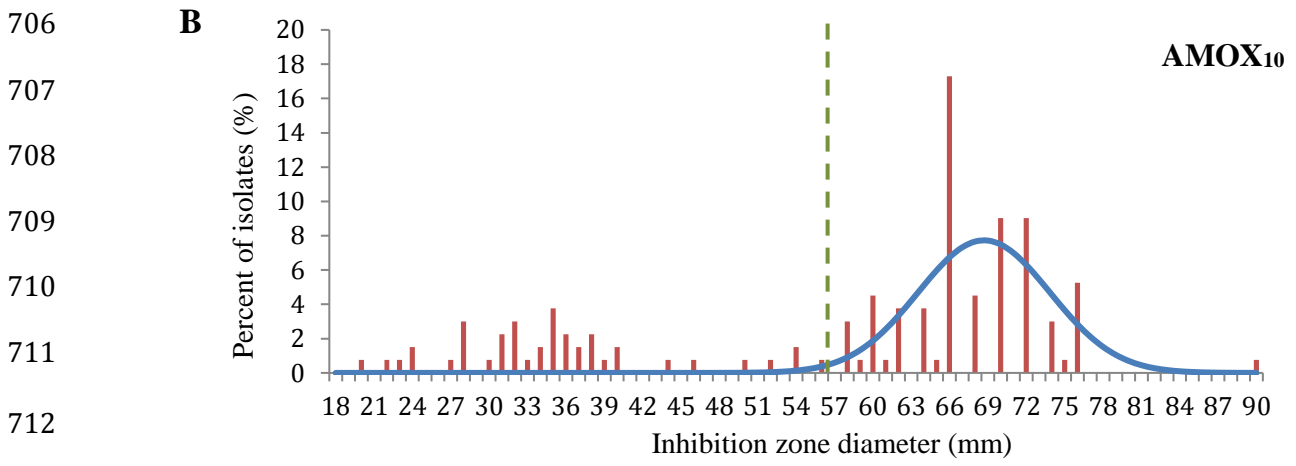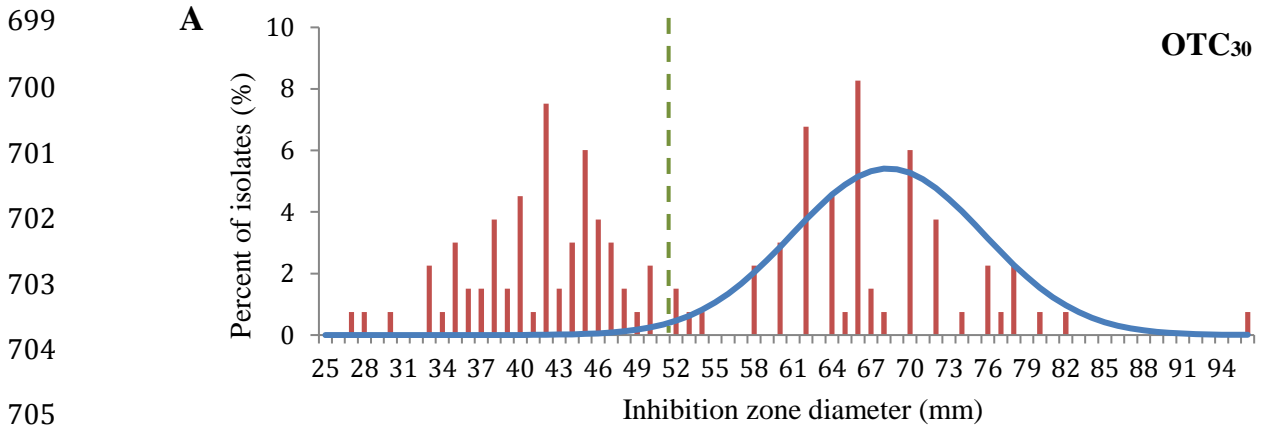
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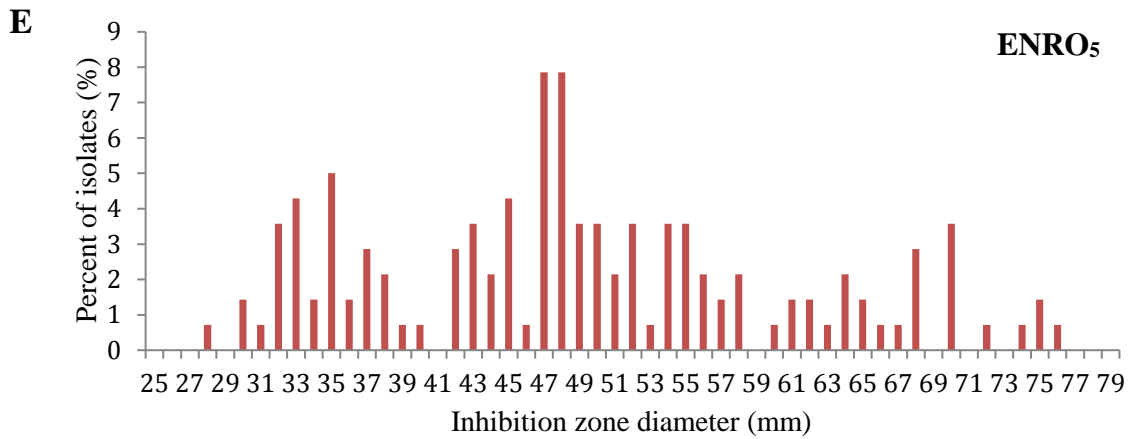
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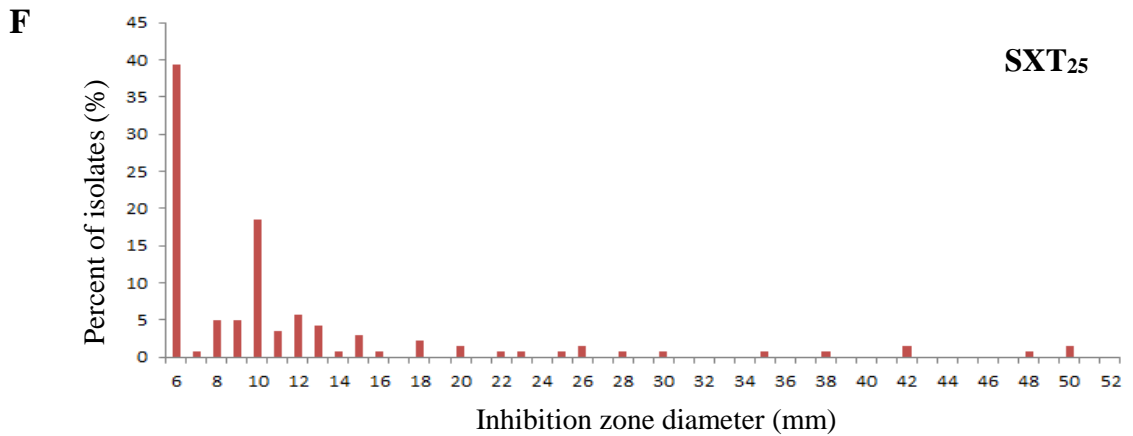
Figure 1. Distribution of 133 *F. psychrophilum* strains according to inhibition zone diameters generated by disc diffusion method for 30 µg oxytetracycline (A, OTC_{30}), 10 µg amoxicillin (B, $AMOX_{10}$), 30 µg florfenicol (C, FFN_{30}), 2 µg oxolinic acid (D, OXO_2), 5 µg enrofloxacin (E, $ENRO_5$) and 25 µg trimethoprim/sulphamethoxazole (F, SXT_{25}). The continuous line represents the 8 point rolling means, the vertical dashed line represents the calculated disc diffusion-based cut-off value.

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745

A

Panel A — MIC (mg L⁻¹) vs Inhibition zone diameters (mm)

MIC	26-30	31-35	35-40	41-45	45-50	51-55	56-60	61-65	66-70	71-75	76-80	81-85	86-90	91-95	96-100
>8		2	1												
8			6	11	11	3			1						
4		1	1	6	12	10			1						
2					2	2	2	1							
1															
0,5							1		1						
0,25															
0,125							1	3	1						
0,063							4	12	15	4	3	1			1
0,031							2	2	2	2	5				
0,016															

Inhibition zone diameters (mm)

B

Panel B — MIC (mg L⁻¹) vs Inhibition zone diameters (mm)

MIC	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85-89	90-94
4		4	3		1										
2		1		6	5	1									
1			2	5	8	2	1	1							
0,5															
0,25															
<0.25							3	6	17	30	28	8			1

Inhibition zone diameters (mm)

C

Panel C — MIC (mg L⁻¹) vs Inhibition zone diameters (mm)

MIC	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84
>2										
2										
1				7	22	17	8			
0,5				6	25	25	9	3		1
0,25					1	5	2		1	
0,125								1		

Inhibition zone diameters (mm)

D

Panel D — MIC (mg L⁻¹) vs Inhibition zone diameters (mm)

MIC	6-9	10-14	15-19	20-24	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64
>2	58												
2	9	18	20										
1		2	4										
0,5			1										
0,25			1										
0,125										5	3	4	1
0,063										4	2	1	
0,031													

Inhibition zone diameters (mm)

Figure 2. Plot of 133 paired MIC values versus disc diffusion zone diameters for oxytetracycline (A), florfenicol (B) and oxolinic acid (C). A continuous thick line presents the calculated cut-off line of the microbial agent.

771   **A**

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AMP MIC (mg L^{-1})

```
8
4
2                                      2       7
1                          4      12      0
0.5                       15
0.25
0.125        3
0.063       66
0.031
<0.31       24
        <0.25   0.25   0.5    1    2    4    8
```
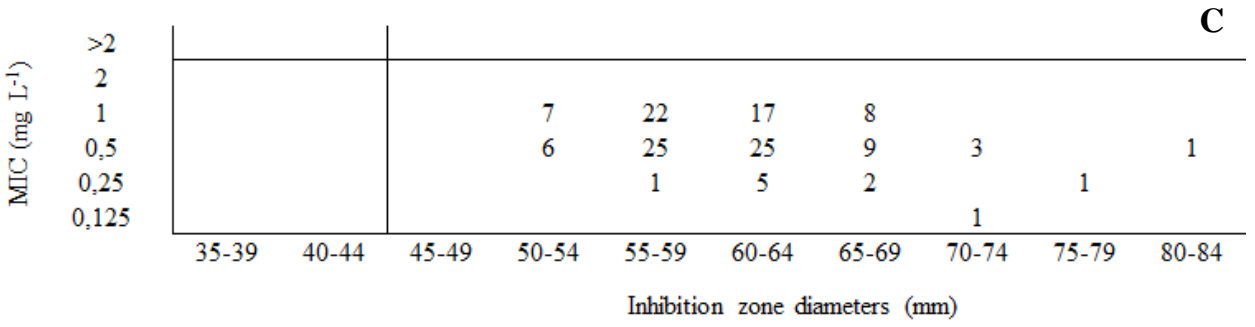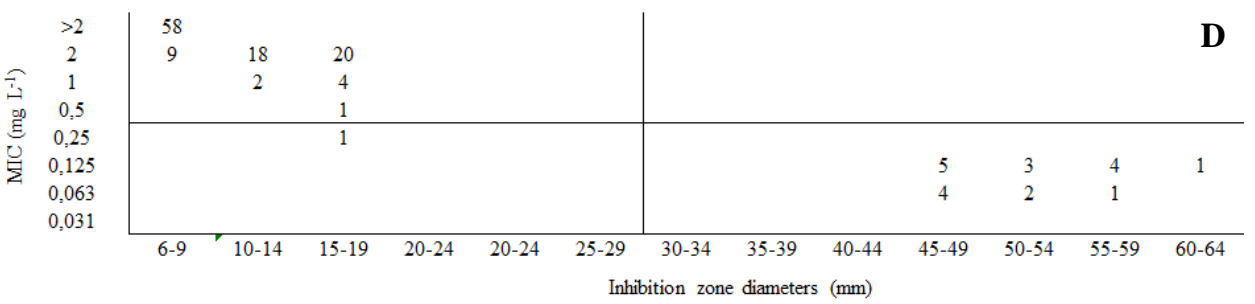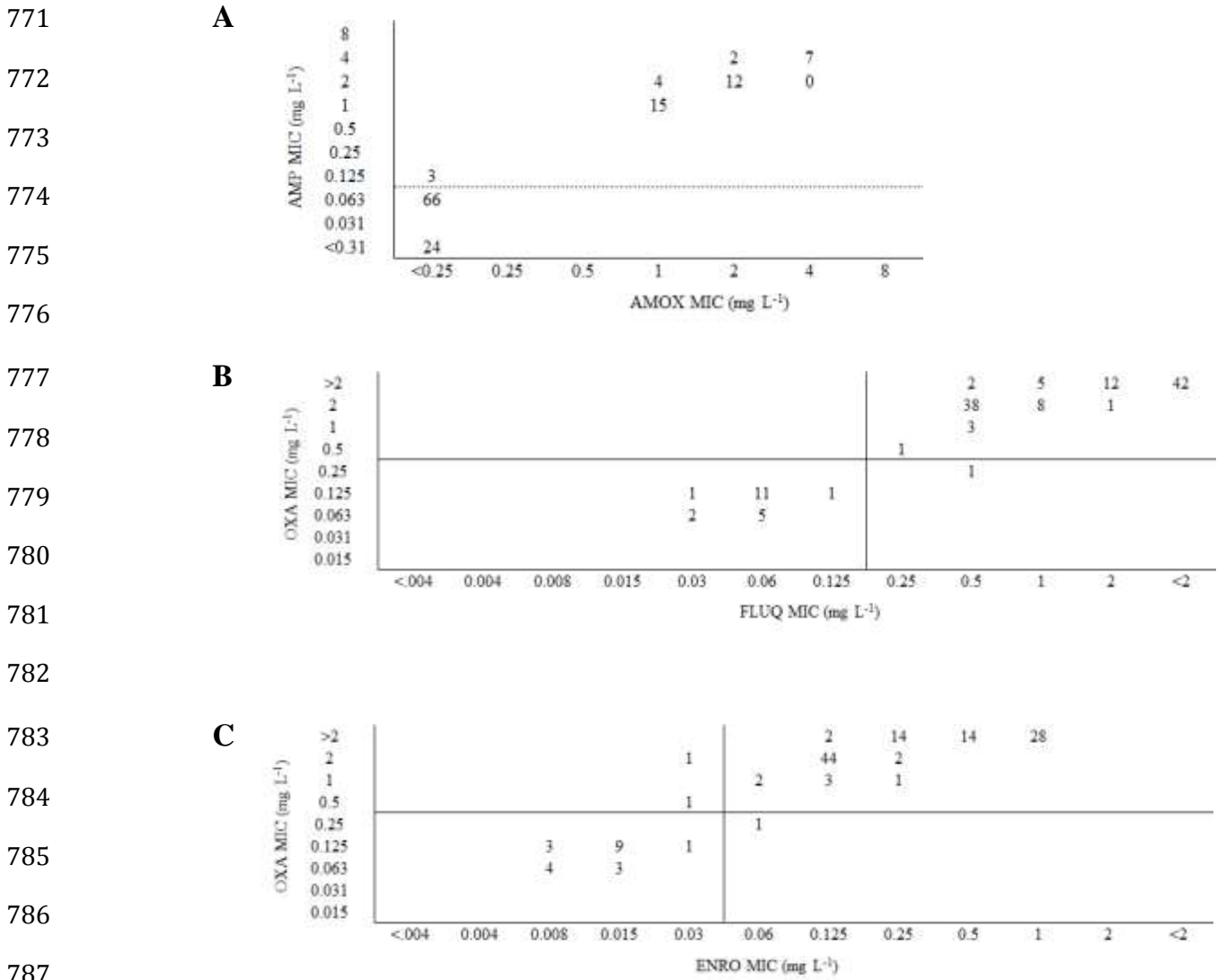
AMOX MIC (mg L^{-1})

777   **B**

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OXA MIC (mg L^{-1})

```
>2                                              2     5    12   42
2                                               38    8
1                                               3
0.5                                        1
0.25                                            1
0.125               1     11    1
0.063               2     5
0.031
0.015
       <.004  0.004  0.008  0.015  0.03  0.06  0.125  0.25  0.5   1    2    <2
```

FLUQ MIC (mg L^{-1})

783   **C**

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OXA MIC (mg L^{-1})

```
>2                                         2     14    14   28
2                              1           44    2
1                          2     3     1
0.5                    1
0.25                       1
0.125            3     9     1
0.063            4     3
0.031
0.015
       <.004  0.004  0.008  0.015  0.03  0.06  0.125  0.25  0.5   1    2    <2
```

ENRO MIC (mg L^{-1})

788 **Figure 3.** Plot of 133 paired MIC values between antimicrobial agents within beta-

789 lactam group (A: ampicillin and amoxicillin) and quinolone group (B: oxolinic acid

790 and flumequine; C: oxolinic acid and enrofloxacin). A continuous thick line presents

791 the calculated cut-off line of the microbial agent. A dashed line presents an estimated

792 cut-off value.