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– THE IMPORTANCE OF RAPID AND ACCURATE IDENTIFICATION OF *PHOTOBACTERIUM*
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1 **Full title: Fish photobacteriosis – the importance of rapid and accurate identification of**
2 *Photobacterium damsela* subsp. *piscicida*

3

4 **Running title: MALDI-TOF MS for *Ph. damsela* subsp. *piscicida***

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33 **ABSTRACT**

34

35 MALDI-TOF MS was tested for identification of *Photobacterium damsela* subsp. *piscicida* on
36 isolates grown on two media, cultured at three incubation times, applied on the target plate by the
37 direct sample spotting (DS), the on-target extraction (OTE) and by the full extraction (FE)
38 method, in triplicates. Identification of samples grown on blood agar (BA) outperformed
39 identification on tryptic soy agar (TSA) by 0.64% for DS and OTE. The OTE gave highest
40 scores in both culture media, all incubation times and replicates. Reliable 24 h species
41 identification were 61.54 %, 84.61 %, and 53.85 % for samples grown on TSA and identified by
42 DS, OTE and FE, respectively. For isolates grown on BA they were 76.92 %, 96.15 %, and
43 30.77 %, respectively. When identified by OTE, the 48 h identification was 93.58 %, but for 72 h
44 declined to 71.79 %. The reliable identification with the highest score from the first measurement
45 was 100 % only for OTE from BA (24 h), whereas OTE from TSA gave 84.61 % (24 h), 76.92
46 % (48 h), and 84.61 % (72 h). The reliable MALDI-TOF MS identification of *Ph. damsela*
47 subsp. *piscicida* is incubation time, media, target plate preparation, and replicate dependent.

48

49 **KEYWORDS**

50 *Photobacterium damsela* subsp. *piscicida*, MALDI-TOF MS

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53 **1. INTRODUCTION**

54

55 *Photobacterium damsela* subsp. *piscicida* is a bacterium causing a serious health condition of
56 both farmed and wild fish populations. It gives rise to a septicaemia with only few signs in the
57 acute phase. Typical skin lesions are manifested as granulomatous ulcerative dermatitis,
58 particularly in the region of the pectoral fin and caudal peduncle. Internally, granulomatous-like
59 deposits may occur on liver, kidney and spleen. The condition was termed pasteurellosis,
60 photobacteriosis and/or pseudotuberculosis due to the distinctive pathology (Austin and Austin,
61 2007). The disease has been responsible for heavy losses in farmed marine fish worldwide, and
62 has also spread to farmed and wild fish stocks in the Mediterranean area (Toranzo et al., 1991;
63 Magariños et al., 2003; Mladineo et al., 2006).

64 The organism may be isolated by inoculating swabs of kidney and/or spleen material onto
65 marine agar 2216E, tryptic soy agar (TSA), nutrient agar or blood agar supplemented with 1-2%
66 NaCl, with incubation at 22-25°C for 48–72 h. On conventional media, shiny, grey-yellow,
67 entire, convex colonies develop (Romalde, 2002; Austin and Austin, 2007). It may be identified
68 by phenotypic methods such as conventional plate and tube tests, by modified API 20E rapid
69 identification system (Topić Popović et al., 2007) with a typical profile number 2005004
70 (Romalde, 2002). It can also be identified by serological analysis with slide agglutination tests
71 (Magariños et al., 1992), and with ELISA tests (Bakopoulos et al., 1997). Various molecular
72 tools were applied for its identification and differentiation from the subspecies *damsela*,
73 including multiplex-PCR, AFLP or PCR-RFLP assays (Osorio et al., 1999; Osorio et al., 2000;
74 Kvitt et al., 2002, Zappulli et al., 2005; Amagliani et al., 2009).

75 Although some of the tests listed above are straightforward and highly reproducible in
76 identification of *Ph. damsela* subsp. *piscicida*, the complete identification frequently is time
77 consuming, some methods require specialized training, technical skills and support, often are

78 labour-intensive, while some require enrichment of target organism and costly reagents (Topić
79 Popović et al., 2017). Nevertheless, *Ph. damsela* subsp. *piscicida* outbreaks in fish require fast
80 and reliable identification in order to move to the next step of prescribing treatment and control
81 measures. To that effect, matrix-assisted laser desorption/ionization time of flight mass
82 spectrometry (MALDI-TOF MS) is the most promising method for bacterial identification. It is a
83 rapid and accurate proteomic method, detecting ribosomal protein fractions of bacteria to be used
84 for classification of the organisms (Lay, 2001). Further identification is based on the detection of
85 mass signals from proteins specific at genus, species or subspecies levels (Benagli et al., 2012).
86 MALDI-TOF MS analyses whole bacterial cells within minutes after cultivation, with high
87 throughput and low running costs (Topić Popović et al., 2017).

88 However, the identification requires a pure and intact colony, while the accuracy of the
89 method may be impacted by culture media, incubation time of the bacterium, and sample
90 preparation (Demirev et al., 1999; Saffert et al., 2011; Veloo et al., 2014). Therefore, the aim of
91 this work was to investigate the sensibility and reproducibility of MALDI-TOF MS for accurate
92 identification of *Ph. damsela* subsp. *piscicida*. This was tested by analysing clinical isolates
93 grown on two different media, cultured at three different incubation times, and applied on the
94 target plate by the direct sample spotting, the on-target extraction and by the full extraction method.

95

96

97 **2. MATERIAL AND METHODS**

98

99 **Bacterial strains**

100

101 The cultures used in this study were the isolates of *Ph. damsela* subsp. *piscicida* from various
102 fish species. A total of 26 strains were used, originating from European sea bass (*Dicentrarchus*
103 *labrax*), gilthead sea bream (*Sparus aurata*), and striped bass (*Morone chrysops* x *M. saxatilis*),
104 farmed in land-based farms, in-shore and off-shore floating cages in Italy and Tunisia. All the
105 strains were initially cultured on TSA medium supplemented with 1.5% NaCl (Oxoid Ltd,
106 England UK). Their taxonomical position was established by morphological, physiological and
107 biochemical tests, namely standard plate and tube tests and API 20E panels (bioMerieux, Marcy
108 l'Etoile, France). API 20E tests resulted with the profile number 2005004 for all isolates. Before
109 MALDI-TOF MS analyses, all isolates were cultured at 22°C on TSA and Blood Agar, BA
110 (Certifikat doo, Croatia) enriched with 1.5% NaCl.

111 For the purposes of testing the method for differentiation between *Ph. damsela* subsp.
112 *piscicida* and subsp. *damsela*, nine strains of *Ph. damsela* subsp. *damsela* isolated from
113 gilthead sea bream farmed in land-based farms and off-shore floating cages in Italy and Albania
114 were used. They were cultured and prepared under the same conditions as the subsp. *piscicida*
115 strains.

116 Molecular identification was performed using multiplex PCR assay for *ureC* (subsp.
117 *damsela*) and 16S rRNA genes (Osorio et al., 2000).

118

119 **MALDI-TOF MS**

120

121 The application of the isolates on the plate was performed by the direct sample spotting, the on-
122 target extraction and by the full extraction method, after 24, 48, and 72 hours of incubation on
123 each growth medium (TSA and BA). All the procedures were performed in triplicate for each

124 individual strain. A total of 1404 measurements were conducted on *Ph. damselae* subsp.
125 *piscicida* and 486 measurements on *Ph. damselae* subsp. *damselae*.

126 Direct sample spotting: a single bacterial colony from each tested strain was smeared
127 onto a 96-spot polished steel target plate (Bruker Daltonik, Bremen, Germany). They were
128 allowed to air dry at room temperature (RT), after which 1 μ L of MALDI matrix was added to
129 each bacterial colony (saturated solution of α -cyano-4-hydroxycinnamic acid (HCCA) in 50%
130 acetonitrile and 2.5% trifluoroacetic acid (Bruker Daltonik, Bremen, Germany).

131 On-target extraction: a single bacterial colony from each tested strain was smeared onto a
132 96-spot steel target plate. Subsequently 1 μ L of 70 % formic acid (Kemika, Croatia) was added
133 to each bacterial colony. After drying, 1 μ L of MALDI matrix was added to each spot.

134 Full extraction: a loopful of a bacterial colony from each tested strain was suspended in
135 300 μ L of LC-MS-grade water (Fisher Chemical, St. Louis, MO) and immediately vortexed.
136 Further, 900 μ L of 100 % ethanol (Kemika, Croatia) was added to the suspension, vortexed and
137 centrifuged at 16 000 g for 2 minutes. The supernatant was discarded and the pellet
138 recentrifuged. After discarding the supernatant, the pellet was dried at RT and resuspended in 20
139 μ L of 70 % formic acid. The suspension was mixed by pipetting and 20 μ L of acetonitrile was
140 added, mixed and centrifuged at 16 000 g for 2 min. Before overlying with 1 μ L of MALDI
141 matrix, 1 μ L of supernatant was added to each plate spot and allowed to dry.

142 MALDI-TOF MS was performed with a Bruker Biotyper (Bruker Daltonik, Bremen,
143 Germany) system. The microflex LT mass spectrometer was calibrated with a bacterial test
144 standard (Bruker Daltonik). Spectra were acquired in the positive linear mode between 2 to 20
145 kDa of mass range using FlexControl software in automatic mode. Bruker Biotyper 3.0 software
146 (Bruker Daltonik) were used to analyse the spectra. Identification criteria were following: a log

147 score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to
148 2.299 indicated probable species identification, a score 1.700 to 1.999 indicated probable
149 identification to the genus level, while a score of < 1.700 was considered unreliable. In order to
150 minimize random effects, data obtained with replicate measurements were added to the
151 calculation.

152

153

154 **3. RESULTS**

155

156 **The impact of sample preparation on identification results**

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158

159 Three sample preparation protocols were tested for preparation of bacterial samples. The
160 application of the *Ph. damsela* subsp. *piscicida* isolates on the plate was performed by the direct
161 sample spotting, the on-target extraction and by the full extraction method. Samples prepared by
162 the on-target extraction were overall better identified than by the other two protocols. The on-
163 target extraction gave high log scores indicating highly probable species level identification, in
164 both culture media, in all incubation times and in all replicates, when compared to the direct
165 sample spotting, and to the full extraction method (Tables 1 and 2). Of 1,404 individual
166 measurements in total, the on-target extraction in total gave 85.47 % and 87.18 % highly
167 probable species level identifications for samples cultivated on TSA and BA, respectively. The
168 direct spotting resulted in 100 % identification to the genus level in all cases, while the full

169 extraction method identified to the species level 97.86 % and 97.01 % of strains cultivated on
170 TSA and BA respectively, all other factors comprised.

171

172 **The impact of culture media on identification results**

173

174 There was a difference regarding the culture media and successful acquisition of mass spectra
175 (Figure 1). Identification of samples grown on BA outperformed identification on TSA for
176 0.64% for the direct sample spotting and the on-target extraction methods, as the BA mass
177 spectra had the greater number of signals and the higher signal-to-noise ratio. However, when
178 performing the full extraction, all time-points considered, samples grown on BA had 13.68 % of
179 unreliable identifications and 11.11 % of no-identifications, compared to 5.13 % unreliables and
180 1.71 % no-identifications for samples grown on TSA. TSA medium enabled correct
181 identification of *Ph. damsela* subsp. *piscicida* samples against the Biotyper database as highly
182 probable species level identification in 49.57 % compared to 41.45 % for BA when performing
183 the full sample extraction.

184

185 **The impact of incubation time on identification results**

186

187 The *Ph. damsela* subsp. *piscicida* strains were incubated for 24, 48, and 72 h on each growth
188 medium. Samples incubated for 24 h gave better results over other incubation times as the
189 number of successful identifications to the species level was higher (Tables 1 and 2), as well as
190 the mass spectral quality sufficient for automatic acquisition. Reliable species identification (log
191 scores of 2.300 to 3.000 and 2.000 to 2.299), considering every measurement for each species,

192 after 24 hours were 61.54 %, 84.61 %, and 53.85 % for samples grown on TSA and isolated by
193 the direct sample spotting, the on-target extraction and by the full extraction method,
194 respectively. However, when compared with identification results of samples grown for 48 and
195 72 h and isolated by the on-target extraction, the results did not change significantly (83.33 %
196 and 87.18 % respectively). Reliable species identification (log scores of 2.000 to 3.000) after 24
197 hours were 76.92 %, 96.15 %, and 30.77 % for samples grown on BA and isolated by the direct
198 sample spotting, the on-target extraction and by the full extraction method, respectively. Again,
199 when identified by the on-target extraction, the results did not change significantly for 48 h
200 (93.58 %), but declined for isolates grown for 72 h (71.79 %).

201

202 All measurements were performed in triplicate for each individual strain. The identical
203 identification result for all three measurements was for: TSA medium and the direct sample
204 spotting 9.40 %, the on-target extraction 20.94 %, by the full extraction method 12.82 %; for BA
205 medium and the direct sample spotting 15.38 %, the on-target extraction 23.93 %, and by the full
206 extraction method 3.74 %. The on-target extraction, therefore, for both media gave the most
207 reliable results in terms of identical identification of replicates. The reliable identification with
208 the highest score (log scores of 2.000 to 3.000) from the first measurement amounted to 100 %
209 only for the on-target extraction from BA (24 h) (Figures 2, 3), and 92.31 % (48 h), whereas the
210 on-target extraction from TSA amounted to 84.61 % (24 h), 76.92 % (48 h), and 84.61 % (72 h).
211 If the first measurement was not reliable, the number of subsequent measurements needed for
212 probable to highly probable species level identification was the least for the direct method after
213 24 h of incubation on both media.

214

215 **The differentiation of *Ph. damsela* subsp. *piscicida* and subsp. *damsela* strains**

216

217 MALDI-TOF MS successfully differentiated the strains under evaluation, although with
218 variable identification results regarding the sample preparation, as presented in Table 3. We
219 found that the score values of the subspecies *damsela* and *piscicida* differed also regarding the
220 incubation time and the medium used. In all cases, the full extraction method was the method of
221 choice for identification of subsp. *damsela*, as on the TSA medium it gave 100 % of probable
222 species identification when incubated for 48 h, with an average score of 2.100, all strains
223 considered. When using BA, the average score for the full extraction (48 h) was 2.030, and
224 slightly increased to 2.067 for full extraction (72 h).

225

226

227 **4. DISCUSSION**

228

229 There are numerous challenges for identification of *Ph. damsela* subsp. *piscicida*, which
230 particularly concern discrimination between closely related strains and the need for rapid
231 identification in cases of disease outbreaks. Notwithstanding the existence of serological tests
232 and molecular tools for its identification and differentiation from the subspecies *damsela*
233 (Osorio et al. 2000), these challenges can be successfully tackled with the application of
234 MALDI-TOF MS (Topić Popović et al, 2017). The proper identification depends on the quality
235 of the acquired spectrum and the presence of the reference spectrum in the MALDI-TOF MS
236 database. For that purpose we tested *Ph. damsela* subsp. *piscicida* strains grown on two
237 different media, cultured at three different incubation times, and prepared for application on the

238 target plate by three protocols, in triplicates, with the intention to determine the optimal
239 conditions for its identification. At this time, there is no published work on the influence of those
240 parameters on the MALDI-TOF MS identification of *Ph. damsela* subsp. *piscicida*.

241 The direct spotting and the full extraction methods in this work were inferior to the on-
242 target extraction at all time-points. On-target extraction gave high log scores from both culture
243 media, and in all replicates. Although the direct spotting is an acceptable practice, in general it
244 gives lower identification rates, and in such cases additional manual analysis is required
245 (Anderson et al, 2012). For differentiating subspecies of *Ph. damsela*, Pérez-Sancho et al.
246 (2016) proceeded with the protein extraction protocol with formic acid, skipping the step of the
247 direct spotting. Indeed, in this work, subsp. *damsela* was best identified when using a full
248 extraction method, however, as opposed to subsp. *piscicida* strains, only after a prolonged
249 incubation (48 and 72 h), and with a slightly better overall identification score when using the
250 TSA medium.

251 We expected that *Ph. damsela* subsp. *piscicida* would be identified successfully from
252 both media used in this study. Despite being cultured on different media, bacteria should be
253 identifiable by MALDI-TOF MS, as a set of low-molecular-weight constitutively expressed
254 proteins form ions in the specific signature regardless of the type of medium. The housekeeping
255 functions of such genes are always required although various media change metabolic needs of
256 bacterium under cultivation (Valentine et al, 2005). Also, potential culture dependency of some
257 mass signals might be excluded from the software analysis (Sauer et al, 2008). Mazzeo et al.
258 (2006) found that representative signal pattern for *Ph. damsela* subsp. *piscicida* was always
259 present in mass spectra regardless of the medium used, and MALDI-TOF MS correctly identified
260 all *Ph. damsela* clinical isolates grown on blood agar in the work of Pérez-Sancho et al. (2016).

261 However, in this work *Ph. damsela*e was slightly better identified when grown on blood agar, but
262 only for the direct sample spotting and the on-target extraction methods. For the full sample
263 extraction protocol, isolates grown on TSA were better isolated by 8 %. It is not clear why there
264 are differences in identification success between TSA and BA media, but similar results were
265 obtained by Walker et al. (2002) with *Staphylococcus aureus*. They observed that colonies from
266 blood agar produced more spectral peaks, attributable to blood components.

267 In order to obtain good quality spectra, it is preferred to pick the bacterial cells in their
268 log phase of growth, since MALDI-TOF MS recognizes mostly 16S ribosomal proteins (Veloo et
269 al, 2014). We have thus tested the impact of the incubation time on the identification result. Also,
270 all our isolates were grown at 22°C, as Austin and Austin (2007) recommend that the
271 temperature of incubation for *Ph. damsela*e be maintained at 22-25°C for optimum growth.
272 Various incubation temperatures of gram-negative bacteria in the work of Ford and Burnham
273 (2013) did not result in misidentifications, and we therefore speculate that a slight increase of the
274 incubation temperature, if so set, would not influence the quality of the spectra obtained. In all
275 cases, the strains were correctly identified after 24 h of incubation to a reliable species
276 identification level. The identification rate was not enhanced by prolonging the incubation time,
277 however in cases of the on-target extraction, it mostly maintained on the same level after 48 h,
278 for isolates from both growth media, and even increased by 4 % after 72 h for isolates grown on
279 TSA. Thus, MALDI-TOF MS procedure might be conducted as soon as the adequate growth is
280 noted, or in case of *Ph. damsela*e subsp. *piscicida* after 24 h of incubation. Although some
281 authors (Balážova et al, 2014) report the issues with sample preparation after longer cultivation,
282 due to cells firmly attached to the growth medium, and the residues of the medium thus possibly
283 interfering the identification results, we did not have such problems with the growth of *Ph.*

284 *damselae* subsp. *piscicida*. Colonies grown at 72 h and later were convex, entire and easily
285 detachable from the medium. It is reported that pleomorphism of the bacterium is pronounced in
286 older cultures, and cells display Gram-variability in young 12-18 h cultures (Austin and Austin,
287 2007), features which did not impact the identification in this work.

288 The frequency of appearance or reproducibility gives each peak its significance as it is
289 measured from replicate spectra used for the reference signature (Valentine et al, 2005).
290 Reproducibility of the identification procedure is of great importance since strain differentiation
291 (for example subsp. *damselae* vs. subsp. *piscicida*) is based on limited number of peaks. Thus, a
292 decrease in the mass spectral quality could diminish the MALDI-TOF MS features which govern
293 strain differentiation (Balážova et al, 2014).

294 The influence of a number of repeated measurements on overall identification success is
295 rarely mentioned in the literature. In this work, the identical identification result for all three
296 repetitions ranged from 9.40 % to 20.94 % (TSA, all sample preparation protocols) and from
297 3.74 % to 23.93 % (BA, all sample preparation protocols). Again, the on-target extraction had
298 the most reliable results in terms of identical identification of replicates.

299 Our data show that MALDI-TOF MS protein fingerprinting is capable of identification of
300 *Ph. damsela* subsp. *piscicida* from the database for this bacterium, irrespective of culture media
301 and incubation time. However, there are nuances in those parameters as their optimization should
302 be developed for a greater log score of identification results, and for the reduction of the data
303 variation between laboratories. Recognizing the need for standardization of sample preparation,
304 and for the purpose of obtaining good quality spectra and probable to highly probable species
305 level identification of *Ph. damsela* subsp. *piscicida*, the authors suggest that MALDI-TOF MS
306 be performed after 24 h of incubation, from fresh cultures. Also, we established that although the

307 on-target extraction yields the best results regarding sample preparation, the direct spotting
308 should be performed as a first step in order to potentially minimize additional manual sample
309 extraction. Sample preparation definitely plays a major role in overall result, and is crucial in
310 distinguishing the subsp. *piscicida* from subsp. *damselae*. According to the result of this study
311 and the relatively low number of identical readings in all replicates, we suggest that three
312 measurements be conducted for every sample preparation protocol in order to obtain
313 reproducible fingerprints allowing reliable identification. MALDI-TOF MS is already
314 established as a confident method for identification of environmental aquatic bacteria. Upon
315 further updating of the databases, and optimization of protocols for identification of bacteria
316 directly from tissues, it will certainly become an indispensable tool in aquatic organisms'
317 microbiology.

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433 **Table 1.** Identification results regarding sample preparation of *Ph. damsela* subsp. *piscicida*
 434 strains cultured on Tryptic soy agar (TSA) supplemented with 1.5% NaCl (as log score). The
 435 samples were applied on the target plate by the direct sample spotting (A), the on-target
 436 extraction (B) and by the full extraction method (C). Results presented are the average of three
 437 measurements.
 438

Strain ID	24 h incubation			48 h incubation			72 h incubation		
	A	B	C	A	B	C	A	B	C
177/04	2.009	2.088	2.067	1.947	2.046	2.165	2.001	2.035	2.048
235/04	2.040	2.044	2.047	1.905	1.965	2.109	2.003	2.032	1.905
299/C/04	2.048	1.999	1.953	1.951	2.084	2.038	1.999	2.062	1.888
319/04	2.020	2.072	2.045	1.931	2.017	2.051	2.035	1.994	2.006
79/05	1.993	1.989	2.073	1.866	2.051	2.086	1.948	1.896	1.758
114/05	1.974	2.042	2.040	2.024	2.047	2.071	1.959	2.045	1.947
189/A/05	2.016	2.028	2.071	1.889	2.097	2.050	1.979	2.025	1.954
189/C/05	2.054	1.956	2.063	1.944	2.041	1.967	1.993	2.071	2.065
256/05	1.994	2.019	2.030	2.008	2.066	2.021	2.035	1.955	1.823
314/05	2.029	1.978	2.011	2.026	2.110	2.112	2.006	2.068	1.952
328/05	2.003	2.124	2.093	2.039	1.993	2.029	2.051	2.028	2.120
82/06	2.008	2.058	2.037	2.085	2.088	1.923	2.006	2.093	1.787
262/08	1.850	2.030	1.933	2.005	1.948	2.116	1.914	1.996	1.351
243/10	2.078	2.086	1.938	2.019	2.028	2.018	2.004	2.011	2.037
97/14	2.017	2.032	1.814	2.003	2.054	2.065	1.973	2.067	1.599

325/C/14	2.032	2.065	1.939	2.101	2.105	1.570	2.045	2.061	2.063
352/B/14	1.925	2.092	2.079	1.738	2.052	1.686	1.948	2.054	1.911
395/14	1.836	2.058	1.912	1.864	2.070	1.807	1.995	2.044	1.851
396/14	1.882	2.046	1.850	1.957	1.995	1.910	2.045	2.031	2.072
399/14	2.016	2.040	1.931	1.863	2.085	1.643	1.983	2.045	1.960
277/A/15	2.015	2.076	1.757	1.979	2.037	2.003	1.934	2.077	1.950
278/16	1.898	1.998	1.975	1.925	2.043	2.019	1.914	2.046	2.029
305/15	1.815	2.085	1.931	1.829	2.041	1.919	1.994	2.049	1.856
335/15	2.041	2.033	1.859	1.898	2.123	1.921	1.969	2.011	2.074
340/16	2.015	2.133	1.809	1.955	2.031	1.952	2.039	2.046	2.039
342/A/16	2.043	2.153	1.918	1.915	2.062	2.071	1.951	2.034	1.998

% strains identified to genus/species with highly probable/probable level identification:

genus	100	100	100	100	100	88.46	100	100	92,31
species	65.38	80.76	46.15	34.61	84.61	61.54	42.31	84.61	38.46

439

440

441

442 **Table 2.** Identification results regarding sample preparation of *Ph. damsela* subsp. *piscicida*
443 strains cultured on Blood agar (BA) supplemented with 1.5% NaCl (as log score). The samples
444 were applied on the target plate by the direct sample spotting (A), the on-target extraction (B)
445 and by the full extraction method (C). Results presented are the average of three measurements.
446

Strain ID	24 h incubation			48 h incubation			72 h incubation		
	A	B	C	A	B	C	A	B	C
177/04	2.057	2.057	1.911	2.053	2.010	2.065	1.849	2.016	2.057
235/04	2.001	2.065	1.755	1.980	2.008	2.031	1.876	2.067	2.045
299/C/04	2.025	2.057	1.945	1.990	2.065	1.984	1.878	2.053	2.089
319/04	2.022	2.052	1.966	2.018	2.062	2.058	1.806	1.950	2.105
79/05	2.018	2.060	1.926	1.970	2.049	2.080	1.752	2.021	2.066
114/05	2.030	2.131	1.947	1.986	2.044	2.083	1.793	2.037	2.112
189/A/05	2.072	2.121	1.704	2.029	2.103	2.026	1.867	2.038	2.136
189/C/05	2.072	2.063	1.543	2.030	2.129	2.088	1.775	2.089	2.106
256/05	2.031	2.028	1.975	1.985	2.111	2.054	1.987	1.976	1.877
314/05	2.013	2.111	1.889	1.956	2.188	1.808	2.081	2.048	1.654
328/05	2.034	2.183	1.997	2.029	2.208	1.954	2.033	2.093	1.955
82/06	1.965	2.107	1.940	1.981	2.177	1.843	2.042	2.140	1.634
262/08	2.025	2.080	1.872	1.984	2.108	1.858	2.041	1.975	1.951
243/10	2.028	2.130	1.943	2.106	2.054	1.478	2.024	2.057	1.989
97/14	2.053	2.134	2.086	1.950	2.125	1.860	1.983	1.994	1.924
325/C/14	2.031	2.138	2.153	2.046	2.087	1.909	1.870	1.951	1.793

352/B/14	2.079	2.096	1.772	2.041	2.114	1.986	1.956	2.008	1.953
395/14	2.048	2.110	1.959	2.057	2.102	1.434	2.036	2.097	1.989
396/14	2.076	2.181	1.784	2.090	2.189	2.076	2.012	2.075	2.023
399/14	2.079	2.107	1.906	2.026	2.079	1.497	2.110	2.011	1.900
277/A/15	1.926	2.101	1.958	2.056	2.036	1.751	2.087	2.029	2.093
278/16	2.125	2.119	1.793	2.119	2.071	1.695	2.042	2.034	2.049
305/15	2.116	2.102	1.676	2.047	2.100	1.818	2.120	2.102	2.083
335/15	2.014	2.056	1.922	2.030	2.080	1.973	2.074	2.071	1.698
340/16	1.999	2.100	1.884	2.041	2.038	1.862	2.051	2.024	1.951
342/A/16	2.009	2.111	1.598	2.023	2.130	1.819	2.021	2.119	1.716

% strains identified to genus/species with highly probable/probable level identification:

genus	100	100	92.3	100	100	96.15	100	100	96.15
species	88.46	100	7.69	65.38	100	34.61	53.84	80.77	46.15

447

448

449 **Table 3.** Identification results regarding sample preparation of *Ph. damsela* subsp. *damsela*
 450 strains cultured on Tryptic soy agar (TSA) and Blood agar (BA) supplemented with 1.5% NaCl
 451 (as log score). The samples were applied on the target plate by the direct sample spotting (A), the
 452 on-target extraction (B) and by the full extraction method (C). Results presented are the average
 453 of three measurements.

454

TSA									
Strain ID	24 h incubation			48 h incubation			72 h incubation		
	A	B	C	A	B	C	A	B	C
308/14	1.769	1.881	2.062	1.865	2.038	2.052	1.961	1.983	2.129
33/E/15	1.903	1.943	2.122	1.920	2.022	2.136	1.895	2.011	2.118
204/16	1.985	1.840	1.814	1.865	2.008	2.108	1.893	2.019	2.029
231/16 E	1.821	1.793	2.049	1.911	2.046	2.200	1.948	1.983	1.920
164/10	1.683	1.786	1.915	1.782	1.786	2.093	2.019	1.936	1.832
201/15	1.823	1.820	2.044	1.773	1.917	2.026	1.904	1.779	2.052
204/A/15	1.773	1.902	2.118	1.835	1.912	2.127	1.936	1.858	2.181
236/16	1.769	1.897	1.999	1.840	1.776	2.011	1.842	1.809	1.926
150/B/15	1.970	1.879	1.999	1.984	2.020	2.146	1.872	2.035	2.088

BA									
Strain ID	24 h incubation			48 h incubation			72 h incubation		
	A	B	C	A	B	C	A	B	C

308/14	1.794	2.014	1.981	1.865	2.020	2.050	1.908	1.969	2.088
33/E/15	1.836	2.149	2.064	1.903	1.924	2.190	1.925	1.982	2.163
204/16	1.784	1.943	1.842	1.914	2.008	1.944	2.055	2.049	2.161
231/16 E	1.828	1.817	1.904	1.899	1.722	2.127	2.017	2.122	2.011
164/10	1.880	1.732	1.939	1.683	1.675	1.999	1.961	1.852	1.878
201/15	2.009	1.990	1.971	1.871	1.925	1.943	1.905	2.020	2.079
204/A/15	1.975	1.732	1.981	1.936	1.650	2.119	2.035	1.881	2.161
236/16	1.697	1.844	2.062	1.823	2.043	1.989	1.932	1.978	2.080
150/B/15	2.002	1.981	1.796	1.871	1.772	1.912	1.971	1.848	1.970

455

456

457 **Figure 1.** Representative spectra (m/z 2.000 to 14.000) for *Ph. damsela* subsp. *piscicida* for
458 each culture medium enriched with 1.5% NaCl: tryptic soy agar, blood agar.

459

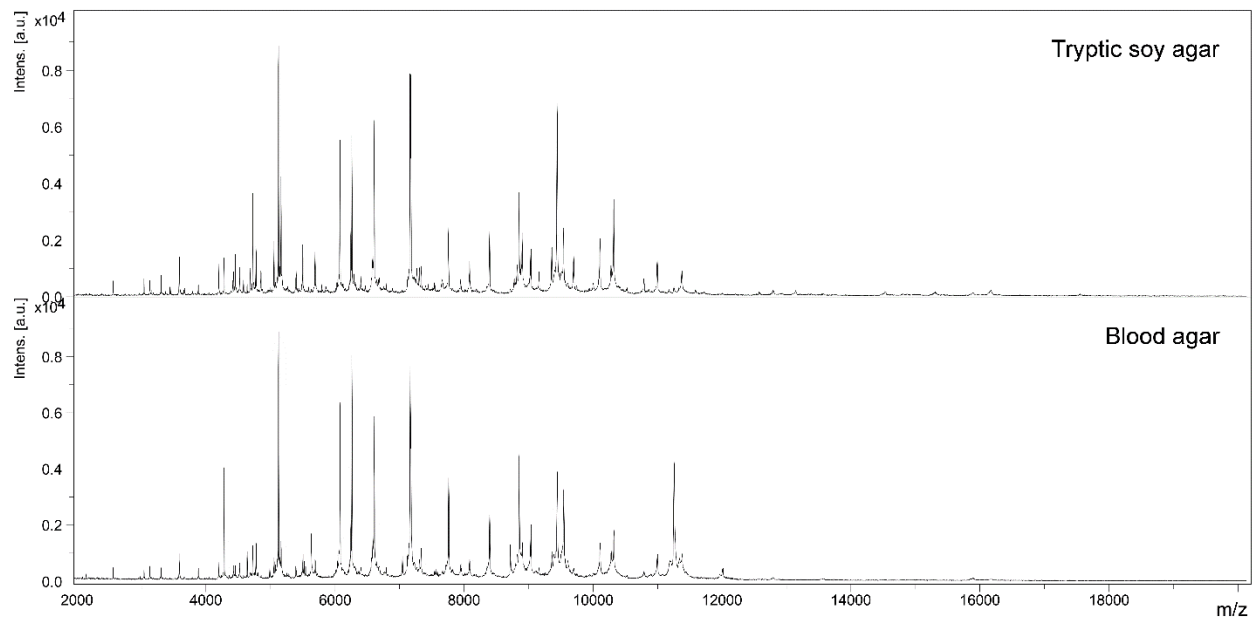
460 **Figure 2.** The gel view of raw spectra for 26 isolates of *Ph. damsela* subsp. *piscicida* (24h, on-
461 target extraction, blood agar enriched with 1.5% NaCl)

462

463 **Figure 3.** PCA clustering results as 3D and 2D plots (scores plots and loading plots) of the
464 spectra of tested *Ph. damsela* subsp. *piscicida* (24 h, on-target extraction, blood agar enriched
465 with 1.5% NaCl). The isolates are clustered, demonstrating the homogeneity of the protein
466 spectra among 26 isolates. Each dot represents the spectrum of one isolate.

467

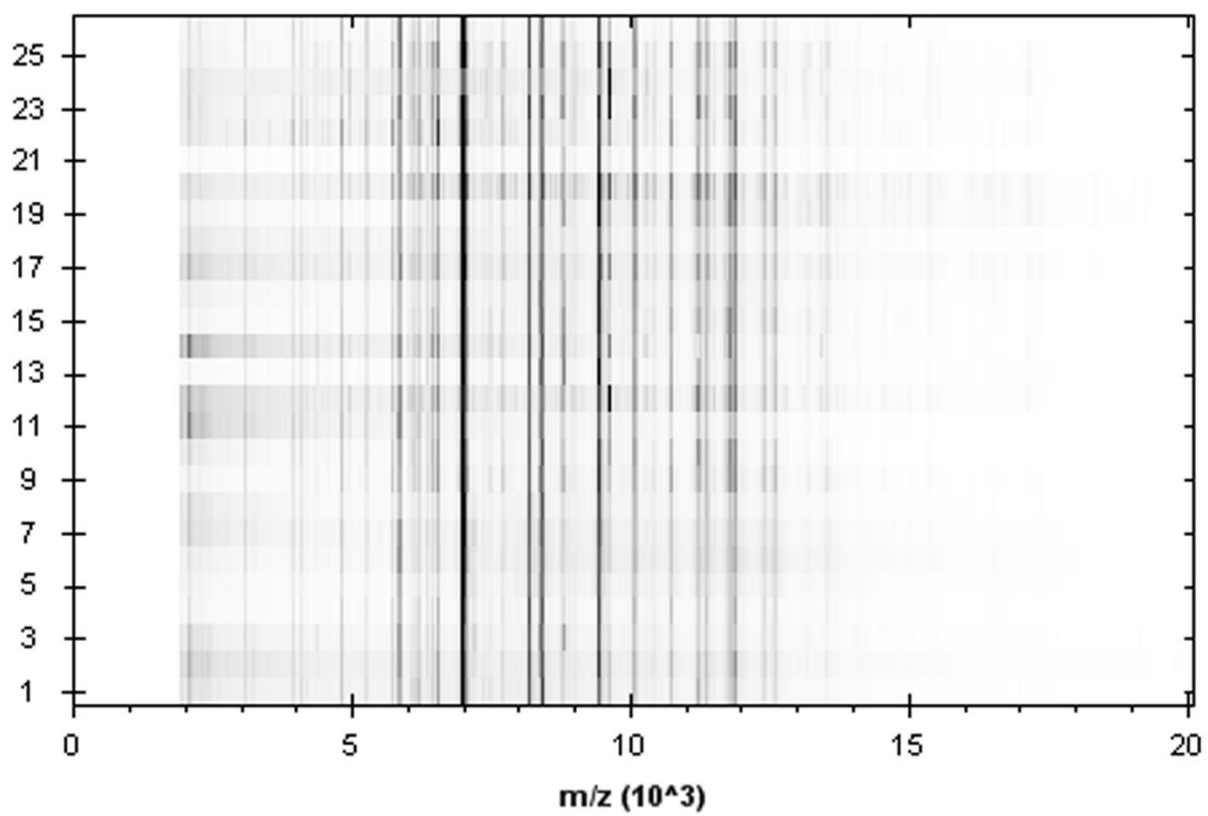
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470 Fig. 1

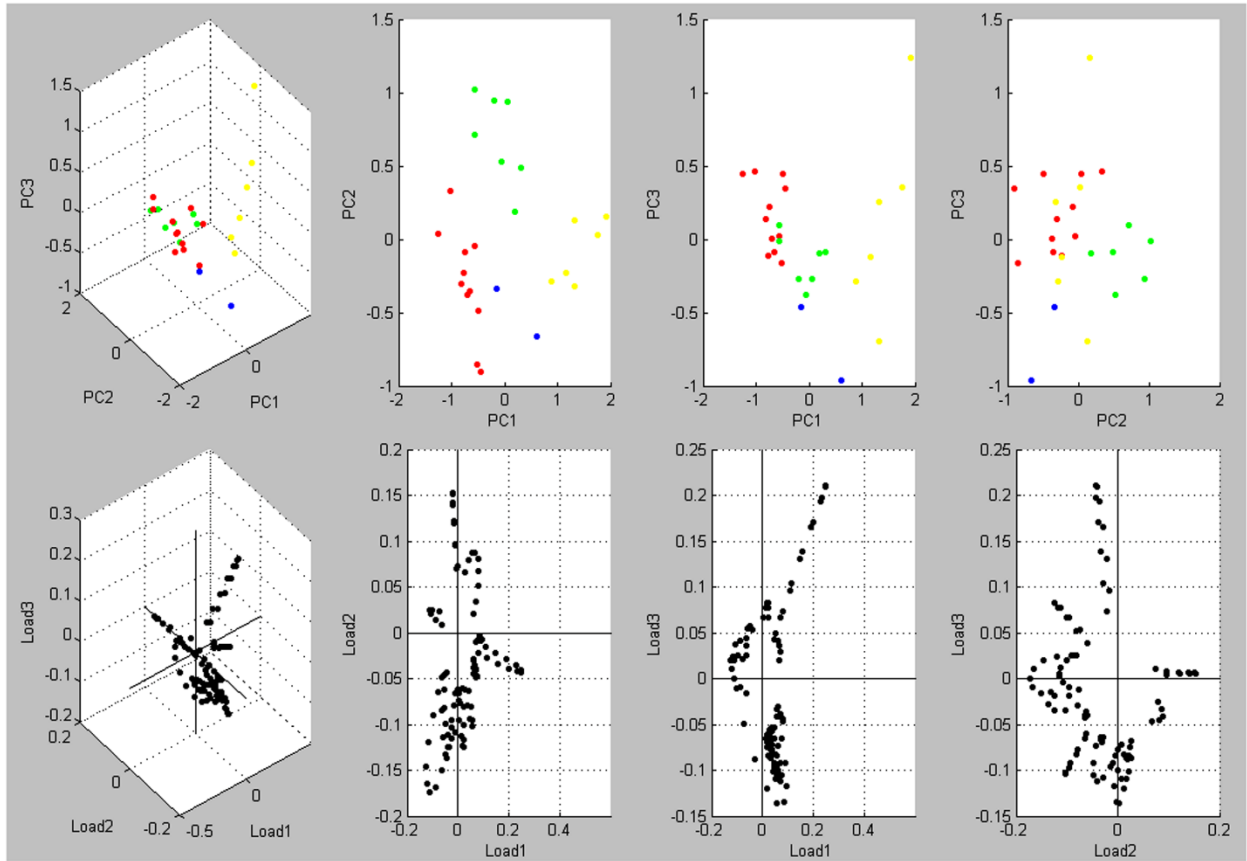
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472

473 Fig. 2

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475

476 Fig. 3

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