1 Applications and limitations of measuring environmental DNA as indicators of the presence of

2 aquatic animals

3 Helen C. Rees[1], Kevin C. Gough[2], David J. Middleditch[3], James R. M. Patmore[4], & Ben C. Maddison[1].

4 [1]ADAS UK Ltd, School of Veterinary Medicine and Science, The University of Nottingham, Sutton

5 Bonington Campus, Leicestershire, LE12 5RD.

6 [2]School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington

7 Campus, Leicestershire, LE12 5RD.

8 [3]ADAS UK Ltd, ADAS Boxworth, Boxworth, Cambridgeshire, CB23 4NN.

9 [4] REC Ltd, Osprey House, Pacific Quay, Broadway, Manchester, M50 2UE.

10

11 Emails: helen.rees@adas.co.uk, Kevin.Gough@nottingham.ac.uk, david.middleditch@adas.co.uk,

12 jpatmore@recltd.co.uk, ben.maddison@adas.co.uk.

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19 Corresponding Author: Helen C. Rees, ADAS UK Ltd, School of Veterinary Medicine and Science, The

20 University of Nottingham, Sutton Bonington Campus, Leicestershire, LE12 5RD. Email:

21 helen.rees@adas.co.uk. Telephone: +44 (0)115 951 6747. Fax: +44 (0)115 951 6415

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Summary

1. In Rees *et al*. (2014) we reviewed the current status of eDNA to monitor aquatic populations. Our aim was to focus on discussion of methodologies used, application of eDNA analysis as a survey tool in ecology, and include some innovative ideas for using eDNA in conservation and management.
2. Roussel *et al*. (2015) claim that analysis of Rees *et al*. (2014b) and other publications highlights the downsides of the method and they suggest that some conclusions should be toned down. Many of their arguments were covered in our original paper (Rees *et al.* 2014b) however, they make the point that modelling approaches should be encouraged and we fully agree with this suggestion.
3. Roussel *et al*. (2015) also claim that we neglected to recognise that there are two sources of imperfect detection (at the field level and the laboratory level), we would dispute this fact.
4. *Synthesis and applications*. Roussel *et al.* (2015) re-iterate many of the points made in the original paper but do cover some additional areas that improve the debate on the use of eDNA. Both the comment (Roussel *et al.* 2015) and our reply clearly highlight that detailed laboratory protocols and rigorous field sampling design are crucial factors which require sufficient reporting in the literature to allow for experimental comparison and replication. Any development of a new method for eDNA detection should be compared directly with established "gold standard" methods for the detection of the species/habitat under investigation. None of the issues raised in Roussel *et al*. (2015) would alter our main conclusions.

Keywords

eDNA, false positive, limit of detection, PCR, sampling design, species detectability, study comparison, water sampling

Introduction

eDNA analysis in the context of monitoring aquatic and semi-aquatic species via species-specific DNA from water bodies is still in its relative infancy. Because of this there is much still to be done in terms of optimising and improving eDNA analysis. Our review intended to discuss the state of both the field sampling and laboratory analysis of these samples and to suggest areas which required improvement, we also discussed innovative ideas for how eDNA can be applied to conservation and management. It was not our intention to produce an exhaustive review of every aspect of the field, we aimed instead to produce a review with specific emphasis on environmental water sampling, the PCR analysis itself, and then the specific areas where this technique has been applied: eDNA persistence; biomass estimation; species composition; and invasive and rare or threatened species; along with a comparison of eDNA versus traditional methods. There was no intention to neglect what are deemed to be important areas of this type of work such as the use of modelling for sampling design. In this respect we welcome the endeavour of Roussel *et al*. (2015, hereafter '*Roussel*') in providing comment on our review paper (Rees *et al.* 2014b). We respond to the five main points below.

Reply to Roussel

The first point made by *Roussel* states that it is 'premature to imply that eDNA will be an efficient tool for detection all aquatic animal species'. We agree with this statement and also that from Ficetola *et al*. (2008) who stated that eDNA is 'useful for studying secretive aquatic or semi-aquatic species, which release DNA into the environment through mucus, faeces, urine and remains'. In our review we do not imply that eDNA can be used for 'all' aquatic species we merely point out that: 1)

75 eDNA analysis has been used as an accurate indicator of species presence in a range of
76 environments; 2) that techniques such as eDNA analysis are of great interest in the management and
77 conservation of vulnerable species and would be of great use in these areas; and 3) that eDNA is
78 particularly useful for those species which are difficult to detect using conventional methods. We go
79 on to state that the success rate of eDNA analysis is not always 100% illustrating that it is not always
80 an efficient tool for the detection of aquatic animal species.
81 *Roussel* rightly chose one of their own publications (Treguier *et al.* 2014), which was not available at
82 the time of writing Rees *et al.* (2014b), as an example of eDNA being an inefficient tool on its own for
83 the detection of the red-swap crayfish, *Procambarus clarkia*. They showed that when crayfish
84 abundance was estimated to be low by conventional trapping, eDNA detection efficiency was poor
85 illustrating a limitation of eDNA detection for this species/habitat combination. However, *Roussel*
86 did not mention that this same study found that eDNA had a better overall detection efficiency than
87 conventional trapping (73% versus 65% respectively) and that experimental evidenced suggested
88 that false positives were unlikely in this case so would not account for the higher detection
89 efficiency. This result would suggest that in this case eDNA was more efficient than traditional
90 survey. A further example of an invertebrate which has been successfully detected using eDNA
91 analysis is the tadpole shrimp, *Lepidurus apus* (Thomsen *et al.* 2012) which was detected at 100%
92 success rate in 10 ponds known to contain this species. Both studies illustrate that detection of
93 invertebrate species is possible and that with continuing developments in PCR technologies and
94 sampling methods the detection efficiency of invertebrate species could be improved making eDNA
95 a more efficient tool for their detection.
96 In Rees *et al*. (2014b) we stated that 'The probability of detection will vary for the chosen target
97 species and will be dependent on the taxa, the density of organisms, and also the type of water
98 body.' therefore, we completely agree with *Roussel* that when studying a new target species,
99 preliminary tests to estimate the detectability of the target DNA should be a pre-requisite for
100 surveying. We would go further and suggest that any such development of a new method for eDNA

101 detection should be compared directly with any established "gold standard" method for the
102 detection of the species/habitat under investigation. As mentioned in the original review, given the
103 developmental stage of applying eDNA to ecological surveys of aquatic animals it is vital that new
104 emerging methodologies are correlated directly with the established survey methods for a particular
105 species/habitat type, enabling a robust comparison and assessment of new eDNA survey methods.
106
107 The second point made by *Roussel* is again correct in that it is often perceived that eDNA analysis is
108 more efficient that traditional surveys. As we stated in Rees *et al*. (2014b) eDNA has 'the potential of
109 greater sensitivity over traditional survey methods' we do not state that it is more efficient. eDNA
110 analysis has been shown to be more efficient in a few studies (Dejean *et al.* 2012; Takahara,
111 Minamoto & Doi 2013) and latterly (Treguier *et al.* 2014) but our review points out that the eDNA
112 success rate was not always 100% when the species was known to be present (Thomsen *et al.* 2012)
113 illustrating that it is not always more efficient.
114 *Roussel* is again correct to state that the abundancies of the species in question is not always stated
115 within the literature and that it is therefore 'difficult to understand how the method might perform
116 across a gradient of target species density' an important point which does add to our review. We
117 agree that each survey method (eDNA versus traditional) is imperfect and we devoted a whole sub-
118 section in Rees *et al*. (2014b) to 'eDNA versus traditional survey methods' where we compared the
119 use of eDNA analysis and traditional survey methods. We agree that there should be more
120 comparisons between eDNA and traditional census methods, so much so that this is exactly what we
121 did in a recent study on great crested newts, *Triturus cristatus* in the UK (Rees *et al.* 2014a). Likewise
122 we agree that it would be very useful for method comparison if species abundance were included in
123 these studies and feel that this is an important point which has not necessarily been mentioned
124 elsewhere.
125

126 The third point made by *Roussel* concentrates on the reporting of experimental details, and false
127 positives. We agree with the comments made on reporting of experimental details and in fact the
128 point should be made here that consideration of the MIQE guidelines (Bustin *et al.* 2009) (Minimum
129 Information for Publication of Quantitative Real-Time PCR Experiments) should be done before
130 publication of any study involving real-time PCR a point which both our Rees *et al*. (2014b) review
131 and *Roussel* neglected to make. The background to these guidelines is that there is very little
132 consensus on 'how best to perform and interpret real time PCR (qPCR) experiments' and that this
133 problem is exacerbated by the fact that little experimental information is included in published
134 studies. Not only do these guidelines encourage the inclusion of the complete
135 reaction/thermocycling conditions and limits of detection as suggested should be included in
136 publications by *Roussel*, but it also covers many other items that should be reported to allow for
137 useful comparison or replication of experiments (provided in the form of a useful checklist). Also
138 encouraged is the correct use of nomenclature which can often be confused in publications.
139 In terms of false positives *Roussel* makes the point that we also made in that false positives 'occur
140 when there is no target DNA in the sample'. *Roussel* is correct that the term 'false positive' should be
141 carefully and unambiguously defined and of course in terms of positive PCR signals you need to
142 consider the possibility, as was stated the review, that 'there is target DNA in the sample but no
143 viable/live organisms in the system'. During the peer-review process of our review paper (Rees *et al.*
144 2014b) we were asked to be careful about the use of the wording 'false positive'. We therefore
145 suggested that a 'false positive' could also be termed a 'false detection'. Perhaps our definitions
146 should have been made more clearly to state that a 'false positive' occurs when there is no target
147 DNA in the sample and a 'false detection' occurs when there is target DNA in the sample but no
148 viable/live organisms in the system. It will be important for future publications to stipulate whether
149 they are referring to a 'false positive' or a possible 'false detection'. *Roussel* goes on to state that
150 'false detections' need more attention if we want to understand what an eDNA detection
151 represents. We would like to emphasise the point that in some species that are transiently present

152 in water and for example move between ponds, an eDNA detection would still be valid whether the
153 species was present or not at the time of sampling especially when trying to define species
154 distribution. Of course, as suggested by *Roussel* consideration should be made to the fact that DNA
155 in a water sample could originate from predator faeces or dead animals which we also stated in Rees
156 *et al* (2014b). We agree with *Roussel* that care should be taken to avoid the inclusion of sedimentary
157 material when taking water samples as sediment could contain eDNA from past site occupancy
158 rather than recent (dependent on eDNA degradation rates) or present occupancy. We would also
159 point out that in our experience the presence of sediment can act to inhibit the PCR reactions. The
160 use of an inhibition control (Biggs *et al.* 2014) can help to monitor for this and other inhibitors which
161 may be present in a sample. In fact, the inclusion of an inhibition control is now carried out as
162 standard in our laboratories for every sample being tested.
163
164 The fourth point made by *Roussel* relates to sampling design, and we agree that rigorous sampling
165 design is important and that tools such as site occupancy models for example the program
166 PRESENCE version 6.4 [available from http://www.mbr-pwrc.usgs.gov/software/presence.html
167 (MacKenzie *et al.* 2002)] can be used. *Roussel* stated that in Rees *et al*. (2014b) we failed to
168 recognise that there are two sources of imperfect detection: at the field level and in the laboratory.
169 We would dispute this comment as we have a large section devoted to sampling in which we discuss
170 the numerous different methods employed for sampling in the literature. We discuss how groups
171 have improved the sampling coverage of the water systems and noted that methods for sample
172 collection will vary depending on the type of water body. Additionally we recognised that the
173 probability of detection will depend on several factors including the taxa, the density of organisms,
174 and the type of water body. We also note that there is considerable variation in the processing of
175 the water samples prior to the extraction step for which we state that 'a direct comparison of these
176 methods to monitor the effects of different processing methods on eDNA extraction and detection
177 would be useful'. This implies that there are sources of imperfect detection at the field level,

178 although we may not have stated so in so many words, the citations in *Roussel* that list the studies

179 (Schmidt *et al.* 2013; Ficetola *et al.* 2014) which illustrate this imperfect detection are useful and

180 should be considered when designing sampling regimes.

181

182 The fifth point made by *Roussel* deals with the application of eDNA detection to running water, an

183 area in which there has been few studies (*Roussel* indicates 9 papers have been published on this

184 subject) . We chose to discuss the sampling regimes used so far and concluded that 'methods for

185 sample collection will vary' depending on the environment which is to be studied. We did not discuss

186 the issue of downstream transportation of DNA as there was little information on this within the

187 published studies. *Roussel* is correct that the implications of discharge variation and dendritic

188 organization will need to be considered when studying these environments along with DNA

189 degradation (which was discussed within Rees *et al*. (2014b)). This again goes back to the point that

190 *Roussel* made about the importance of the study design and the use of modelling approaches such

191 that all these factors are taken into consideration prior to study commencement.

192

193 Conclusions

194 For the most part we agree with the comments of *Roussel* and in several instances the comments

195 made do not correspond to what was said in our review paper but may correspond to the other

196 papers as analysed by *Roussel*. We note that many of the points raised were already covered in our

197 original review paper although there are some additional valid considerations, which are discussed

198 above. Additionally, we dispute the fact that we neglected to discuss the potential for errors in the

199 field sampling although we did not included discussion on the use of modelling during the study

200 design phase, which was an important omission. We feel that our original conclusions are valid:

201 eDNA analysis is fast becoming an important tool in the study of aquatic species; alternative

202 methods of PCR or novel methodologies could be used to provide rapid on-site detections; the fact

203 that there does not appear to be 'a single study which could be used as a template for

204 standardization of this technique' due to the very different natures of the environments being
205 studied, the taxa, the density of organisms, and the disparity in the way samples are scored as
206 positive; the fact that next generation sequencing of eDNA samples could become a powerful tool
207 for monitoring species diversity; and finally that the technique has been used as an 'accurate
208 indicator of the presence of species in a range of aquatic environments'.
209 In agreement with the conclusions of *Roussel*, we feel that eDNA analysis is a 'promising method',
210 that more investigations are required, and as with any method users should be aware of potential
211 weaknesses within its use. *Roussel* concludes that non-detection of target species DNA does not
212 mean the absence of that species, nor does a positive detection mean that the species is present.
213 This is of course true for the reasons already discussed above. However, it is also the case that
214 traditional field survey methods are not always accurate when recording the absence of a particular
215 species, particularly in cases where a secretive or elusive species is being surveyed. This points to the
216 fact that neither field survey nor eDNA analysis is perfect, which supports a statement made in the
217 original review and is our concluding remark here, that 'eDNA should not be used to replace or
218 disregard the knowledge and expertise of experienced field ecologists and taxon specialists, but
219 should become an important tool to enhance limited conservation resources'.
220

221 ## Acknowledgments

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