A validation scale to determine the readiness of environmental DNA assays for routine species monitoring

Running title: eDNA assay validation scale

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Key words: digital PCR, eDNA, endpoint PCR, https://edna-validation.com, quantitative PCR, species-specific, species detection

Abstract

The use of environmental DNA (eDNA) analysis for species monitoring requires rigorous validation - from field sampling to the analysis of PCR-based results - for meaningful application and interpretation. Assays targeting eDNA released by individual species are typically validated with no predefined criteria to answer specific research questions in one ecosystem. Hence, the general applicability of assays as well as associated uncertainties and limitations, often remain undetermined. The absence of clear guidelines for assay validation prevents targeted eDNA assays from being incorporated into species monitoring and policy; thus, their establishment is essential for realizing the potential of eDNA-based surveys. We describe the measures and tests necessary for successful validation of targeted eDNA assays and the associated pitfalls to form the basis of guidelines. A list of 122 variables was compiled, consolidated into 14 thematic blocks, (e.g. "*in silico* analysis"), and arranged on a 5-level validation scale from "incomplete" to "operational" with defined minimum validation criteria for each level. These variables were evaluated for 546 published single-species assays. The resulting dataset was used to provide an overview of current validation practices and test the applicability of the validation scale for future assay rating. Of the 122 variables, 20% to 76% were reported; the majority (30%) of investigated assays were classified as Level 1 (incomplete), and 15% did not achieve this first level. These assays were characterised by minimal *in silico* and *in vitro* testing, but their share in annually published eDNA assays has declined since 2014. The meta-analysis demonstrates the suitability of the 5-level validation scale for assessing targeted eDNA assays. It is a user-friendly tool to evaluate previously published assays for future research and routine monitoring, while also enabling the appropriate interpretation of results. Finally, it provides guidance on validation and reporting standards for newly developed assays.

Key words: digital PCR, eDNA, endpoint PCR, https://edna-validation.com/, quantitative PCR, species-specific, species detection

1. Introduction

Determining the occurrence of species is essential for ecology and requires sensitive and accurate detection methods. Within the last decade, species detection from environmental DNA (eDNA; i.e. detection of extra-organismal DNA released by organisms into their environment) has shown great potential for routine species surveys (Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Goldberg et al., 2016; Deiner et al., 2017; Langlois, Allison, Bergman, To, & Helbing, 2020; Sepulveda, Nelson, Jerde, & Luikart, 2020). The interest in molecular species detection has fuelled the development of over 500 assays, reviewed herein, that utilise PCR to amplify DNA or RNA extracted from environmental samples. Generally, "targeted" eDNA assays must be specific to the species of interest and possess high sensitivity to allow detection at low densities, low DNA concentrations, and across spatiotemporal scales (Goldberg et al., 2016; MacDonald & Sarre, 2017).

A targeted eDNA assay encompasses the entire workflow used to detect a species' DNA from an environmental sample, inclusive of field sampling through to the interpretation of PCR-based results; it does not just consist of the primers and probes. Thus, adherence to workflows will determine the success or failure of an eDNA assay because methodological choices influence performance and sensitivity (e.g. Doi, Takahara, et al., 2015; Tsuji, Takahara, Doi, Shibata, & Yamanaka, 2019). In practice, assays are often validated within a specific system to answer a set question about the target species. Hence, applications beyond this initial development are hampered by the poor understanding of remaining uncertainties, such as the potential for false positives resulting from non-target amplification or contamination, or false negatives resulting from low sensitivity, sample degradation, low DNA yield protocols or inhibition (Goldberg et al., 2016; Lacoursière-Roussel & Deiner, 2019). In a management

context, false positives and false negatives may lead to misuse of resources (e.g. funds and personnel) for issues such as rare species protection and invasive species control. Both scenarios foster inaccurate interpretation of results, fuelling arguments against the routine use of eDNA detection for species monitoring (Jerde, 2019).

Aside from a few well-validated eDNA assays already incorporated into routine monitoring, the application of published assays is a minefield for end-users to navigate. We illustrate this through two examples. The assay for great crested newt (*Triturus cristatus* (Laurenti, 1768), a legally protected species in the UK [Natural England, 2015]) was one of the first eDNA assays validated in both laboratory and field trials against conventional tools, demonstrating its potential for routine monitoring (Thomsen et al., 2012; Rees, Bishop, et al., 2014). After successful validation, a national eDNA-based citizen science monitoring scheme was tested and showed that large-scale eDNA sampling can enable distribution modelling (Biggs et al., 2015). These initial studies paved the way for eDNA-based *T. cristatus* detection to inform new policies aimed at providing landscape-level species protection (L. R. Harper, Buxton, et al., 2019). Studies have since investigated optimal methods of eDNA capture, relative abundance and detection probability estimation, and the influence of seasonality as well as biotic and abiotic factors on *T. cristatus* eDNA detection and quantification (Buxton, Groombridge, & Griffiths, 2017; Buxton, Groombridge, Zakaria, & Griffiths, 2017; Buxton, Groombridge, & Griffiths, 2018a, 2018b). Due to these combined efforts, the assay has undergone exemplary validation and has been operational for management since 2014.

Conversely, no assays have been successfully applied to routine monitoring of invasive American crayfish. For example, there is a lack of consensus on a single assay for the signal crayfish (*Pacifastacus leniusculus* (Dana, 1852)). Larson et al. (2017) developed and tested an assay against conventional trapping, but five other assays have also been proposed with differing degrees of validation and divergent *in silico* and *in vitro* approaches (Agersnap et al., 2017; Dunn, Priestley, Herraiz, Arnold, & Savolainen, 2017; K. J. Harper, Anucha, Turnbull,

Bean, & Leaver, 2018; Mauvisseau et al., 2018; Robinson, Uren Webster, Cable, James, & Consuegra, 2018). The *P. leniusculus* assays were developed using a variety of strategies for eDNA sampling, capture, extraction, qPCR of different genetic markers, and applied across genetically diverse populations within the species range. Due to this substantial methodological variability, direct comparisons between results obtained from these assays are impossible. Therefore, the *P. leniusculus* assays represent a minefield for end-users, despite the need for accurate and sensitive tools to enable actionable species management of this invasive species in Europe, Japan, and California (USA).

These case studies exemplify how consensus and dissent in assay validation can influence the implementation of eDNA analysis for species monitoring. Developing guidelines to determine the suitability of eDNA assays for end-users will therefore ensure that ecological insights or management decisions are based on robust molecular analyses with quantifiable uncertainties and clear inference limits (Goldberg et al., 2016; MacDonald & Sarre, 2017; Nicholson et al., 2020; Sepulveda et al., 2020). Here, we describe the general validation process for targeted PCR-based methods and examine the extent of assay validation and reporting in the eDNA literature. We present an eDNA assay validation scale, which establishes criteria to enable the classification of assays based on their accuracy and sensitivity for single-species detection. To demonstrate the utility of the scale, we performed a meta-analysis of targeted eDNA assays published in 327 papers as of 11 April 2019 (546 assays). By placing an eDNA assay on the validation scale, end-users can determine the recommended scenarios for application and improve assay performance with further validation.

2. Criteria and principles of validation

2.1. General requirements for an eDNA laboratory

All laboratory activities are subject to error. In order to have confidence in results, quality standards and good practices are required in diagnostic laboratory environments (e.g. World Health Organization, 2011; Halling, Schrijver, & Persons, 2012). Although few eDNA-processing laboratories will employ 'ancient DNA' practices (e.g. full body suit and positive air pressure with HEPA filtered inflow), all laboratories conducting eDNA analysis should utilise a unidirectional workflow where pre-PCR steps are performed in separate laboratories dedicated to low DNA quality and quantity (Goldberg et al., 2016). Completely standardised laboratory environments are rare and the use of proficiency tests (as conducted by UK laboratories participating in *T. cristatus* monitoring) can help end-users understand the quality of results obtained among different laboratories. Even results obtained from an extensively validated assay can be questionable when they are not produced within a suitable laboratory environment (Goldberg et al., 2016).

2.2. Reporting standards for in silico*,* in vitro *and* in situ *validation of assays*

Targeted eDNA assay validation is a multi-step process. It can be divided into *in silico* validation (i.e. computer-based tests for primer specificity), *in vitro* validation (i.e. laboratory tests with reference tissue samples) and *in situ* validation (i.e. field tests with eDNA samples) (see Goldberg et al., 2016; MacDonald & Sarre, 2017; Langlois et al., 2020; So, Fong, Lam, & Dudgeon, 2020). Understanding the utility of an assay requires both knowledge of the context in which it has been designed, and a broader understanding of how it was developed. Here, we give a brief overview of what key steps comprise assay validation, with a focus on troubleshooting steps that may be necessary when applying previously published assays to new locations or with modified laboratory practices (Table 1).

169 The first step is *in silico* assay validation, the goal of which is to determine assay
170 specificity based on known sequence diversity. Sequence diversity has three categories,
171 including sequences from: (i) closely related and co-occurring species, (ii) closely related but
172 geographically distinct species, and (iii) distantly related but co-occurring species (i.e.
173 sequences that could co-amplify and produce false positive results for a target species). By
174 checking primer specificity from available sequences, the geographic area of applicability for an
175 assay can be maximised through identifying and removing potential issues of co-amplification.
176 Typically, public or custom databases are used for performing *in silico* amplification (e.g.
177 ecoPCR [Boyer et al., 2016]; primerBlast [Ye et al., 2012]; PrimerTREE [Cannon et al., 2016];
178 PrimerMiner [Elbrecht & Leese, 2017]). While reference sequence libraries are often far from
179 complete and many of the factors influencing successful PCR amplification cannot be simulated,
180 *in silico* testing provides a first impression of primer performance and should be conducted.
181 The essential components of *in vitro* assay validation are optimisation, specificity, and
182 sensitivity. Tests with varying PCR chemistry, reaction volume, primer/probe concentration,
183 cycling conditions and technical replication will ensure optimal, standardised and error-free
184 target DNA amplification (Bustin et al., 2009; Wilcox, Carim, McKelvey, Young, & Schwartz,
185 2015; Goldberg et al., 2016). The assay must then be tested against closely related and co-
186 occurring non-target taxa to ensure specificity, which is not automatically guaranteed after
187 successful *in silico* testing (Goldberg et al., 2016; So et al., 2020). Ideally, tissue-derived DNA
188 samples from multiple individuals spanning a defined geographic area are tested to ensure the
189 assay is robust to genetic variants of target and non-target species. Amplicons should be
190 Sanger sequenced to confirm species identity (Goldberg et al., 2016), although short fragments
191 (<100 bp) have limited sequence length available for species determination (Meusnier et al.,
192 2008). Next, the Limit of Detection (LOD) must be determined to assess assay sensitivity, and
193 the Limit of Quantification (LOQ) determined, if the measurement of eDNA quantity is desired.
194 Generally, these values are obtained using a dilution series of quantified DNA amplicons or

synthesized gene fragments (e.g. IDT gBlocks™ Gene Fragment) based on public or *de novo* reference sequences (Langlois et al., 2020). The LOD and LOQ have various definitions in the eDNA literature, but were recently standardised by Klymus et al. (2019), where LOD is the lowest standard concentration at which 95% of technical replicates amplify and LOQ is the lowest standard concentration for which the coefficient of variation (CV) value is <35%. Unfortunately, the existence of past definitions requires the final LOD and LOQ to be reported as well as the definition used. We note that these metrics apply directly to the assay as developed and assume no interference during PCR from the rest of the species' genome (i.e. if a gBlock is used), other genetic material, or inhibitory compounds.

Finally, the assay must be validated *in situ* by surveying sites with and without the target species (Goldberg et al., 2016). It must be tested against conventional tools for presence/absence detection and tests for estimation of relative abundance/biomass are advisable. Assays are deemed successful if eDNA and conventional detections concur at occupied sites and no eDNA detections are observed at definitively unoccupied sites. Sanger sequencing of eDNA amplicons can provide additional evidence but cannot distinguish sample contamination from true detections (Goldberg et al., 2016). Besides screening for the target species, negative eDNA samples (or all eDNA samples if quantification is necessary) should be tested for inhibition. This requires an Internal Positive Control (IPC) assay for synthetic DNA (e.g. ThermoFisher) or an assay for non-target species using exogenous or endogenous DNA (Goldberg et al., 2016; Veldhoen et al., 2016; Doi et al., 2017; Furlan & Gleeson, 2017).

Advanced *in situ* validation may investigate the influence of biotic (e.g. abundance, biomass, life stages, microbial activity) and abiotic (e.g. temperature, pH, ultraviolet light, salinity) factors influencing eDNA origin, state, fate, and transport (Barnes & Turner, 2016; Lacoursière-Roussel & Deiner, 2019; Wang et al., 2021). Assays that account for spatial (e.g. shoreline versus offshore) and temporal (e.g. summer versus winter) variation in eDNA distribution and abundance due to the ecology of a species can be implemented with greater confidence (de

Souza, Godwin, Renshaw, & Larson, 2016; Lawson Handley et al., 2019). Occupancy modelling using eDNA data is desirable as it accounts for detection probability while estimating site occupancy, even if all field samples from a site return negative. Hierarchical models that incorporate eDNA occupancy and detection probabilities at site, sample, and technical replicate levels are most accurate and can be implemented in software such as R (e.g. package "eDNAoccupancy" [Dorazio & Erickson, 2018]) or PRESENCE (MacKenzie et al., 2002). However, model assumptions regarding false positives should be carefully considered.

3. Types and trade-offs of targeted eDNA detection methods

Amid the processing chain (i.e. sampling to data analysis) for a targeted eDNA assay, PCR warrants extra consideration as the technological spectrum and potential for variation is enormous. Previous publications have typically defined an assay as the primers (and probe) required for DNA amplification and associated visualisation (e.g. agarose gel electrophoresis, qPCR instrumentation). However, differences between the multiple detection instruments and chemistry used in combination with species-specific primer (and probe) sets can fundamentally change the sensitivity and specificity of targeted eDNA assays. Table 2 provides an overview of amplification types and their associated trade-offs.

Many assays - especially those published in earlier years - use endpoint PCR. However, most assays to date employ real-time quantitative (q)PCR allowing for greater sensitivity and quantitative data. More recent publications have used digital (d)PCR for absolute quantification. Alternatively, LAMP and CRISPR have been shown to be suitable for eDNA applications, decreasing the requirements of in-field testing equipment (M. R. Williams et al., 2017; M. A. Williams et al., 2019). A few publications use alternative methods such as PCR combined with restriction fragment length polymorphism (RFLP). All amplification types (Table 2) enable distribution and occupancy modelling, provided enough biological and technical replication is

247 employed (Hunter et al., 2015; Goldberg, Strickler, & Fremier, 2018; Wilcox et al., 2018).
248 However, endpoint PCR in combination with agarose gels is the only type where a detection
249 limit cannot be set objectively (low sensitivity) and which does not provide estimates of DNA
250 copy number (Doi, Uchii, et al., 2015; Yamanaka & Minamoto, 2016; Hunter et al., 2017;
251 Thalinger, Wolf, Traugott, & Wanzenböck, 2019). Depending on the management context and
252 study size, the optimal detection instrument can vary, albeit technological advances and
253 required accuracy will continue to shift the focus away from endpoint PCR.
254
255 **4. Evaluating the current status of assay validation**
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257 To understand current validation practices for targeted eDNA assays, we generated an
258 extensive list of variables deemed important for assay validation by 35 experts in the field of
259 targeted eDNA detection whom convened at a DNAqua-Net EU COST Action (Leese et al.,
260 2016) workshop held on 26-27 March 2018 at the University of Innsbruck, Austria. The variables
261 in this list consist of 18 categorical variables (e.g. species identity, target gene, sample type)
262 and 104 binomial variables directly associated with the eDNA processing chain, from primer
263 design to interpretation of field study results (SI1).
264 A comprehensive literature database for targeted eDNA assays was built in three steps.
265 First, we included all papers listed on the 'eDNA assays' web page
266 (https://labs.wsu.edu/edna/edna-assays/) as of 10 April 2019. Second, we conducted a Web of
267 Science literature search on 11 April 2019, including the search terms "environmental DNA" and
268 "eDNA" but excluding terms associated with microbial organisms and metabarcoding (SI2A).
269 Third, the resulting 660 Web of Science entries were manually checked for suitability (i.e.
270 macrobial target organism, targeted eDNA detection intended) leading to a combined database
271 of 327 papers. For each of the assays contained in these papers, the 122 variables were
272 recorded in a checklist by one of the authors. Before data entry, all authors validated the same

four papers (Deiner & Altermatt, 2014; Rees, Bishop, et al., 2014; Thalinger et al., 2016; L. R. Harper, Griffiths, et al., 2019) to ensure recorder standardisation. Validation efforts were classified as 1 for “tests done, or parameter reported”, 0 for “variable not reported, or no testing done”, and NA in cases where the respective variable did not match the assay type (e.g. filter type when samples were precipitated). When an assay was used in multiple papers, all validation efforts were summarised in one entry and the literature database was extended with the papers reporting primer sequences or other methodological aspects. As the type of amplification is important for assay validation, primer pairs used on multiple detection platforms were given separate database entries per amplification type. However, because most assays developed were presented in one publication, we did not account for slight variations in other aspects of the workflow (e.g. different extraction method, different filter type). After recording the values for each eDNA assay using the validation checklist (SI1), each author scored the assay intuitively based on a preliminary version of the validation scale (see section 5). The resulting database of 122 variables for each assay was the basis for all further analyses using R (R Core Team, 2020) and associated packages (SI2B).

Altogether, 546 assays from 327 papers were assessed. Of these assays, 227 were designed to detect fish species and 74 were designed to detect amphibian species; hence, it is unsurprising that ~80% of assays utilised water sampling. Fourteen percent of the assays were tested on tissue only and few assays were optimised for other sample types such as aerosol, sediment, snow or soil. More than 80% of assays were reported in only one paper, and most were designed for qPCR (~60%) or endpoint PCR (~35%) platforms. The cytochrome c oxidase subunit I (*COI*) gene was the most popular (>40%) genetic marker, followed by the cytochrome b (*cytb*) gene (~23%) (SI3).

5. The 5-level assay validation scheme

To enable standardised assay validation and reporting in the future, we assigned the assay-specific variables to 14 thematic blocks such as "*in silico* analysis", "PCR", or "extensive field testing of environmental samples" (Table 3, Fig. 1). Each of the variable blocks contains between three and 26 variables (SI1). Some of the variable blocks summarize basic practices (e.g. "*in silico* analysis") while others describe advanced assay validation (e.g. "detection probability estimation from statistical modelling"). To simplify the reporting of an assay's validation level in the future, the blocks were placed on a five-level scale enabling the categorisation of assays from Level 1 (incomplete) to Level 5 (operational), and the interpretation of associated field study results (Fig. 1). The scale is additive, which means for an assay to be placed at Level 3, it must fulfil the reporting requirements of Levels 1, 2, and 3. Level 1 (incomplete) summarizes assays for which basic *in silico* analysis, target tissue testing and general information regarding PCR were reported. Level 2 (partial) – assays were characterized by comprehensive reporting of PCR conditions and *in vitro* testing on closely related non-target species. For assays placed at Level 3 (essential), the target organism was successfully detected from an environmental sample and the specifics of DNA extraction and concentration of eDNA from the environmental sample (i.e. filtration, precipitation) were reported. The LOD, extensive field testing and *in vitro* testing on co-occurring non-target species were the variable blocks specifically associated with Level 4 (substantial). At Level 5 (operational), the assay has been subjected to comprehensive specificity testing, detection probability estimates from statistical modelling and investigations of ecological and physical factors potentially influencing eDNA in the environment (Table 3, Fig. 1).

The placement of assays on this 5-level scale is not straightforward, since each of the 14 variable blocks contains variables associated with either rudimentary or substantial validation and reporting. For instance, the thematic block "concentration of eDNA from environmental sample" contains the variable "volume/weight of environmental sample", which was reported for

almost all assays, but also contains “pressure used for filtration”, which was rarely reported and/or measured. Therefore, a minimum criterion was introduced for each variable block functioning as proof of validation and ensuring standardized placement of assays on the validation scale. For example, “detection from an environmental sample” was used as evidence that some validation had been undertaken in the block “detection obtained from environmental samples” (Table 3). To reach a level on the validation scale, an assay must fulfil all minimum reporting criteria for that level and any preceding levels.

Based on this classification, the results (detected vs. not detected) obtained from eDNA assays become directly interpretable: when Level 1 or Level 2 assays are applied to environmental samples without any further validation steps, it is impossible to tell whether the target species is present or absent independent of the PCR result. Amplifications with a Level 3 assay can be interpreted as “target is likely present”; however, non-amplifications are inconclusive. When Level 4 and Level 5 assays do not lead to amplification, the target is likely absent. Positive PCR results at Level 4 and 5 mean that the target species is almost certainly present (Fig. 1).

For a quantitative analysis of reporting practices, we calculated a total scoring percentage and a block scoring percentage for each assay. The total scoring percentage was defined as the proportion of the 104 binary variables which were reported. For each of the 14 blocks, the block scoring percentage was calculated by dividing the number of variables tested/reported by the complete set of variables associated with the block. Both calculations included only variables relevant to the applied methods (e.g. for assays using filtration, precipitation variables were omitted; see Box 1 for example assays).

Of the 546 assays analysed, the majority (30%) were classed as Level 1. Of the remainder, 15% ($N = 83$) did not fulfil the minimum criteria necessary to reach Level 1, and no assay reached Level 5 (Fig. 1 and Fig. 2A). Newer assays published after 2016 were more likely to reach Level 4, and the percentage of assays failing to reach Level 1 gradually declined since

2014 (Fig. 2B). Generally, the total scoring percentage for all variables in a level increased from Level 0 to Level 4, but its variation was non-uniform with most outliers observed at Level 2 (Fig. 2C). Assays not reaching Level 1 exhibited scoring percentages between ~20% and ~55%, clearly showing the difference between incomplete and partially validated assays, which did not achieve higher levels due to one or several missing validation step(s). Generally, variables associated with lower levels on the validation scale were more likely to be reported or tested. Nevertheless, some variables (e.g. “haplotypes of target tissue” or “pressure used for filtration”), were addressed by fewer than 10% of assays (Fig. 2D).

The rigour of the minimum criteria was evaluated by tallying the not achieved/reported cases. Specifically, 62 assays did not reach Level 1 because the targeted species sequence(s) used in primer design were not reported, which is a prerequisite for this level. Eight assays did not report the primer sequence. The lack of target detection from an environmental sample and omission of filter type or precipitation chemicals were the most restrictive criteria and were not fulfilled by 80 and 43 assays respectively, most of which were exclusively used for tissue tests (Fig. 2E). For assays ranked at Levels 2 and 3, there was agreement between the intuitive assay rating provided by the recorder and that assigned by the objective criteria. For assays placed at Level 1 following objective criteria, authors tended to be more liberal and rated them one or two levels higher (SI4). Finally, a classification tree analysis (De’Ath & Fabricius, 2000) was carried out to identify common characteristics of assays placed at each level of the validation scale (SI5). Most assays failing to reach Level 1 showed distinctly low levels of *in silico* validation. This was also true for most Level 1 assays (N = 103), albeit these showed higher levels of target tissue validation (Fig. 3). On the other end of the spectrum, Level 4 assays were characterised by substantial testing or reporting for *in vitro* testing, field sample processing, LOD determination, PCR, and field testing.

6. Conclusions and outlook

The validation scale and reporting standards developed here are an inclusive set of guidelines for targeted eDNA assays. They can facilitate communication between the scientific community, commercial providers and government agencies, and provide guidelines regarding the application of previously published assays or the development and publication of new assays (Sepulveda et al., 2020). One needs to acknowledge that a strict standardisation of eDNA assays will not be possible due to applications for manifold taxa in diverse ecosystems combined with technological advances. By checking which of the 122 validation variables have been addressed, it is possible to identify both available and missing information needed to successfully develop or reuse an assay. The level of validation required for successful routine species monitoring will also differ on a case-by-case basis. For example, the *T. cristatus* assay was not placed on Level 5 of the validation scale (in part due to lack of reporting) but was still approved as an official survey method in the UK. As a general recommendation, authors should report as much information as possible on the conducted validation steps, either in the main text or in the supplementary material. Additionally, developers should consult existing guidelines for best practices along the validation workflow prior to assay design and fieldwork (Bustin et al., 2009; Goldberg et al., 2016; MacDonald & Sarre, 2017; Klymus et al., 2019). To make the validation process accessible, we provide a checklist in SI1. Furthermore, a website (https://edna-validation.com/) was created to summarise the cornerstones of the validation process and the validation scale. This website will function as a curated repository for newly developed assays and authors are encouraged to enter all 122 variables and the minimum criteria to rank assays and calculate their scoring percentages on the validation scale. The website will also serve as a living document when improvements in technology and/or our understanding of eDNA in the environment advance.

The 5-level validation scale designed here provides an overview of the capabilities and uncertainties of targeted eDNA assays. However, the binary data entry system cannot replace a close check of previous publications as it does not always allow a qualitative assessment. Details for validation variables are often spread across different sections in a publication or ambiguously displayed. Thus, the checklist can be used as standard reporting guidelines for targeted eDNA assays. It should be emphasised that for specific research questions and associated publications, minimal validation efforts may be sufficient. Nevertheless, thorough validation is needed to reduce uncertainties and overcome the limitations associated with eDNA-based species monitoring. Furthermore, it is important that practitioners consider how an assay can be modified (e.g. using different PCR reagents) and whether this changes its validation level.

The successful application of targeted eDNA assays for routine species detection and monitoring largely depends on the scientific community and the industry providing eDNA services. Laboratories participating in ring tests such as that proposed for metabarcoding (Blackman et al., 2019) can facilitate consensus on analysis standards. For now, assay developers must respond to queries and help troubleshoot reproducibility issues. Such engagement will facilitate the application of targeted eDNA assays by other users and outside their original geographic scope or academic context. Finally, it is necessary for both the scientific community and the commercial laboratories to communicate realistic applications and limitations to end-users, as often an assay is not bad *per se*, but simply unsuitable.

Data availability

The literature database, the validation checklist compiled from it, the R code, and seven datasets derived from the checklist and used for analyses are available on Figshare at

https://doi.org/10.6084/m9.figshare.12184860.v1 The literature database, the validation scale, and the full checklist are also available at https://edna-validation.com/.

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Conflict of interest

KB is the co-founder and CEO of Nature Metrics Ltd., a for profit company dedicated to the analysis of environmental DNA. HCR manages environmental DNA services for RSK ADAS Ltd., a for profit environmental consultancy. DS and MT are co-founders of Sinsoma GmbH., a for profit company dedicated to the analysis of environmental DNA.

Author's contributions

KB conceived the original idea, which was developed by all co-authors. BT compiled the literature database and BT, KD, LRH, HCR, RCB, DS, MT, and KB collected the data. BT analysed the data and led the writing of the manuscript for which first drafts of sections were provided by LH, RCB and KD. All authors contributed critically to the drafts and gave final approval for publication.

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Tables and Figures

Table 1: A guideline for troubleshooting at different stages of the validation process.

General issues	*In silico* validation	*In vitro* validation	*In situ* validation
Applying assay to a new geographic location	Test non-target sequences available on public/custom databases from this location	Test tissue from non-target species present at this location, including species present at original location if populations are genetically diverse	Test assay at sites where new, potentially problematic non-target species are known to occur
Sparse reference data	Where possible, Sanger sequence tissue DNA from target and non-target species to generate reference sequences	Extensively test assay on tissue available for target and non-target species, i.e. test extracts from multiple individuals	Extensively test assay at sites where target species co-occurs with non-target species lacking reference sequences, and at sites where only non-target species lacking reference sequences occur

In vitro validation issues	Troubleshooting guidelines	*In situ* validation issues	Troubleshooting guidelines
Poor amplification efficiency	Optimise reaction volume	**Expected species presence/absence not confirmed**	Survey sites with conventional tools and eDNA metabarcoding if possible
	Optimise cycle number		
	Optimise primer-probe concentration	**False positives**	Discard samples corresponding to contaminated controls; consider lack of specificity; Sanger sequence results
	Optimise annealing temperature using gradient PCR		
	Optimise technical replication		Resample with more stringent decontamination measures in place
	Check pipetting accuracy		
Poor specificity	Increase annealing temperature	**False negatives**	Check for inhibition and treat samples if inhibited; consider timing and spacing of sampling
	Add hydrolysis probe		
	Perform melting curve analysis		Increase technical replication
	Try other enzymes (e.g. EMM)		Resample and increase biological replication and volume of water collected
	Consider redesign		
Low sensitivity (see also Klymus et al., 2019)	Ensure enough replication of fresh standards used when establishing the Limit of Detection	**Poor quality Sanger sequencing**	Purify amplicons prior to sequencing
	Use TE buffer and tRNA to make standard dilutions, not molecular grade water		Concentrate amplicons prior to sequencing
	Use low retention tubes and pipette tips when preparing standards to prevent adsorption to plastic	**Inhibition (determined by failed/skewed amplification of IPC or non-target assay)**	Use a DNA extraction kit that includes an inhibitor removal step (e.g. mu-DNA, Qiagen QIAamp DNA Stool Mini Kit, Qiagen DNeasy PowerWater Kit, Qiagen DNeasy PowerSoil Kit)
	If possible, switch to PCR platform with greater sensitivity		Apply an inhibitor removal kit (e.g. OneStep Inhibitor Removal Kit, Zymo Research Corp.) to samples
	Consider redesign		Use PCR reagents designed to handle inhibition (e.g. TaqMan Environmental Master Mix, Bovine Serum Albumin)

657 **Table 2:** Detection methods and their trade-offs used for targeted eDNA assays. Abbreviations
658 are as follows: polymerase chain reaction (PCR), capillary electrophoresis (CE), quantitative (q),
659 digital (d), intercalating dye (ID), loop-mediated isothermal amplification (LAMP).

Trade-offs	Endpoint PCR-gel	Endpoint PCR-CE	qPCR-ID	qPCR-probe	dPCR- ID	dPCR-probe	LAMP
Quantification	no	yes (limited precision)	yes	yes	yes	yes	yes, with real-time monitoring
LOD (sensitivity)	medium - high DNA concentration (depending on which intercalating dye)	low - medium DNA concentration (with a well-designed assay)	low DNA quantities	low DNA quantities	absolute DNA quantities	absolute DNA quantities	medium DNA concentration
Specificity	medium	medium	medium	high	medium	high	high
Cost reagents	low	medium	high	high	high	very high	medium
Cost equipment	low	medium	medium	medium	high	high	low - medium (depending on LAMP cycler)
Multiplex possible	yes, if different sizes are used	yes, if different sizes or fluorophores are used	difficult	yes, if different fluorophores are used	difficult	yes, if different fluorophores are used	yes
Time (PCR to data)	slow	slow	medium	medium	slow	slow	fast (15-30 min)
Assay type transferable	yes	yes	yes	yes	yes, to qPCR	yes, to qPCR	not easily
Effort to design assay	low - medium	low - medium	high	high	high	high	high
Specialised software	advisable	advisable	advisable	required	advisable	required	required
Sequencing confirmation	yes	yes	yes	yes	limited	limited	limited

660 **Table 3:** The thematic variable blocks of the 5-level validation scale and their respective

661 minimum criteria.

Validation level	Variable blocks	Minimum criteria
Level 1	*in silico* analysis	target species
	target tissue testing	target tissue
	target tissue PCR	primer (and probe) sequence
Level 2	comprehensive reporting of PCR conditions	DNA extract volume in PCR
	in vitro testing on closely related non-target species	any in vitro non-target testing
Level 3	extraction method performed on eDNA samples	method of extraction
	concentration of eDNA from environmental sample	filter type or precipitation chemicals
	detection obtained from environmental samples	detection from an environmental sample (artificial or natural habitat)
Level 4	Limit of Detection (LOD)	LOD determined
	extensive field testing of environmental samples	multiple locations or multiple samples
	in vitro testing on co-occurring non-target species	any advanced *in vitro* testing
Level 5	comprehensive specificity testing	non-co-occurring/closely related species checked from *in silico*
	detection probability estimation from statistical modelling	any effort made towards detection probability estimation
	understanding ecological and physical factors influencing eDNA in the environment	any factor influencing eDNA in the environment tested

662 **Figure 1:** An overview of the 5-level validation scale. For each of the levels (incomplete to
663 operational), the main accomplishments in the validation process and an appropriate
664 interpretation of results are provided.
665
666 **Figure 2:** The main outcomes of the meta-analysis based on 546 assays from 327 publications
667 are presented in panels A to E. Assay classification is based on the minimum criteria presented
668 in Figure 1. Level 0 codes for assays that did not reach Level 1 on the validation scale. The
669 colour coding is consistent for all panels: Level 0 (grey), Level 1 (dark purple), Level 2 (blue),
670 Level 3 (turquoise), Level 4 (green), and Level 5 (yellow). Panel A shows the distribution of
671 assays across levels of the validation scale. Panel B displays the percentage of assays (*N* =
672 546) rated Level 0 to 4 that have been published each year since 2003. Panel C summarises
673 variable reporting per assay level. Panel D shows the percentage of assays reporting a specific
674 variable (colour-coded according to level). Panel E shows the minimum criteria necessary to
675 reach each level of the validation scale, and the percentage of Level 0 to 4 assays that did not
676 report these. All variable abbreviations are listed in SI1.
677
678 **Figure 3:** Classification tree analysis identifying the criteria distinguishing assays at different
679 levels of the validation scale. The conditions along the branches show the criteria on which the
680 dataset is split. Numbers in coloured leaves show the validation level of the assays in the
681 respective leaf. Numbers below the leaves represent the number of assays per validation level,
682 summarised inside an individual leaf. The displayed percentage is the proportion of assays
683 summarised per leaf.
684
685 **Box 1:** Examples of assays rated at Levels 0 to 4. The vertical tile plot shows which of the
686 minimum criteria the assay fulfils (yellow tiles), and the bar chart gives the scoring percentage
687 (i.e. the proportion of variables that were tested or reported) for each of the variable blocks.

688 Bars are coloured according to the score obtained from a block with dark purple coding for “no
689 validation” and yellow coding for “comprehensive validation”.
690

Figure 1

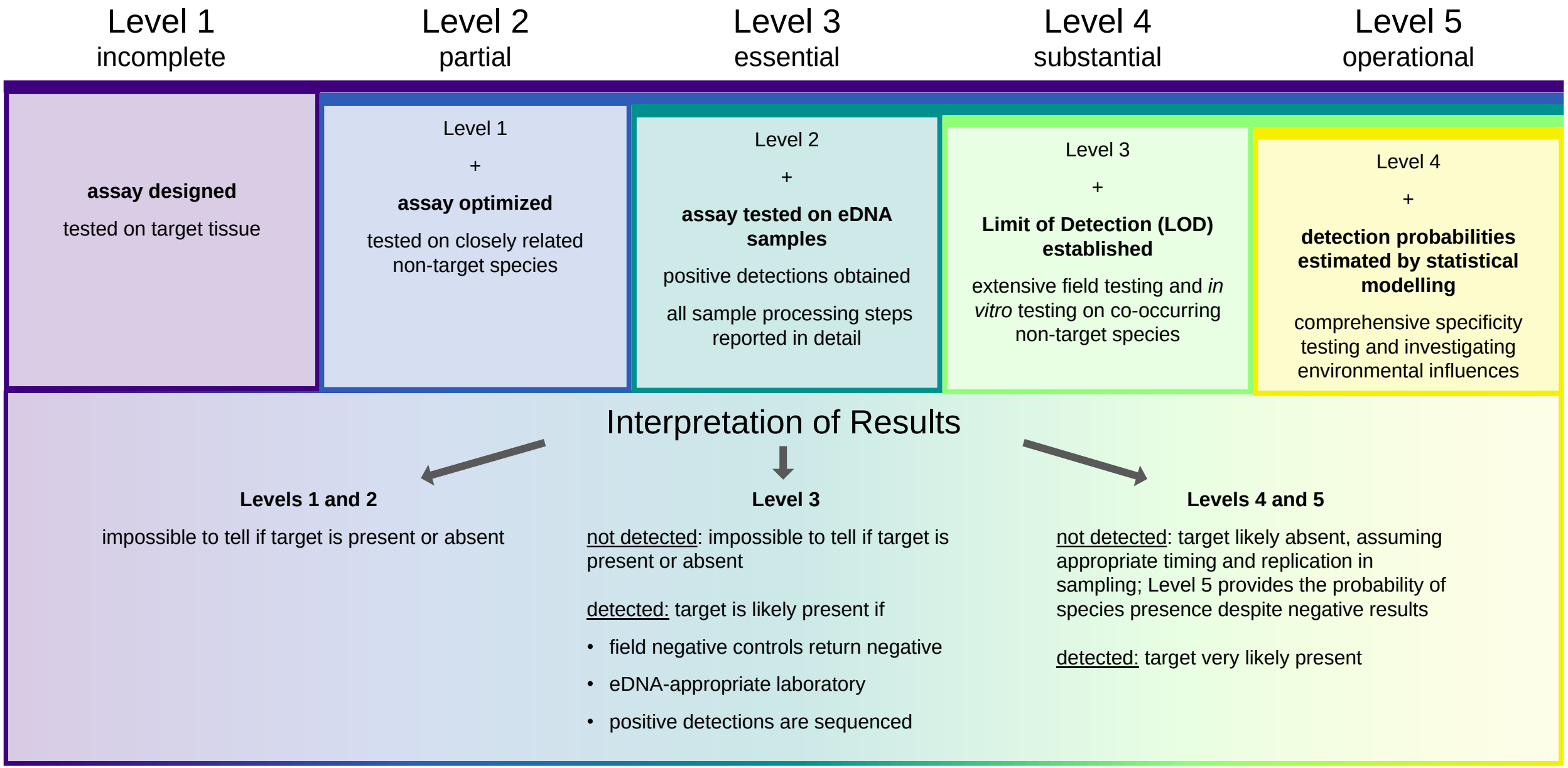
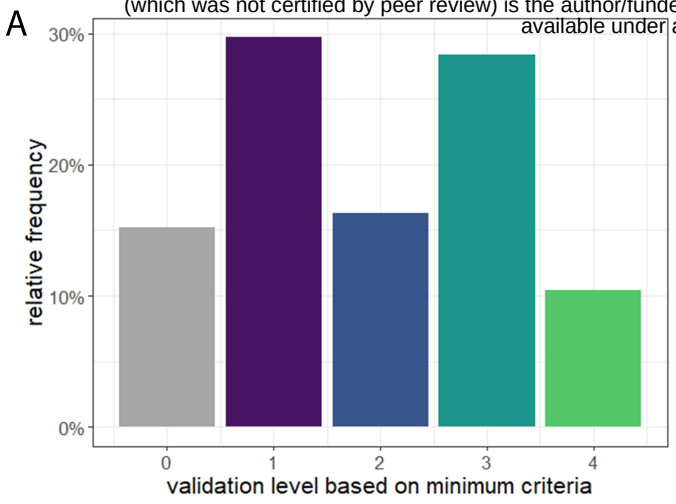
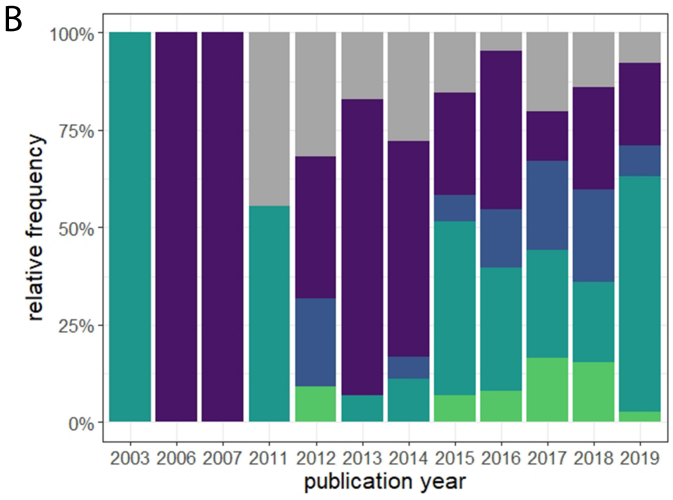
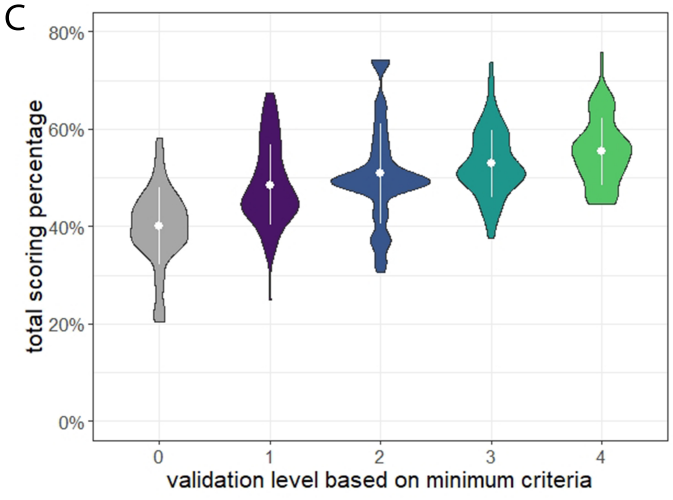
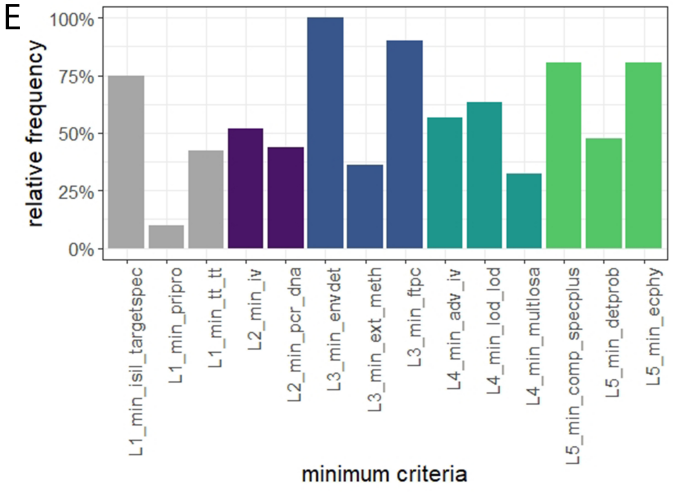

Level 1
incomplete
Level 2
partial
Level 3
essential
Level 4
substantial
Level 5
operational
assay designed
tested on target tissue
Level 1
+
assay optimized
tested on closely related non-target species
Level 2
+
assay tested on eDNA samples
positive detections obtained
all sample processing steps reported in detail
Level 3
+
Limit of Detection (LOD) established
extensive field testing and in vitro testing on co-occurring non-target species
Level 4
+
detection probabilities estimated by statistical modelling
comprehensive specificity testing and investigating environmental influences
Interpretation of Results
Levels 1 and 2
impossible to tell if target is present or absent
Level 3
not detected: impossible to tell if target is present or absent
detected: target is likely present if
• field negative controls return negative
• eDNA-appropriate laboratory
• positive detections are sequenced
Levels 4 and 5
not detected: target likely absent, assuming appropriate timing and replication in sampling; Level 5 provides the probability of species presence despite negative results
detected: target very likely present

Figure 2:









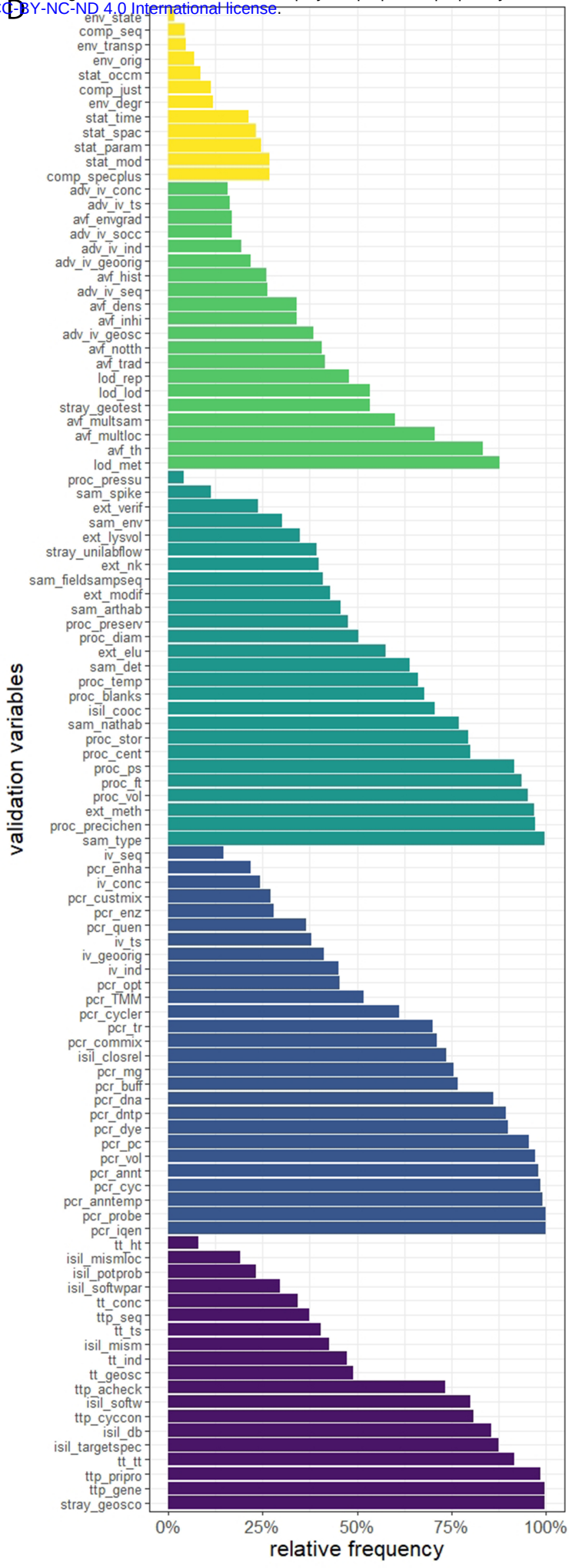
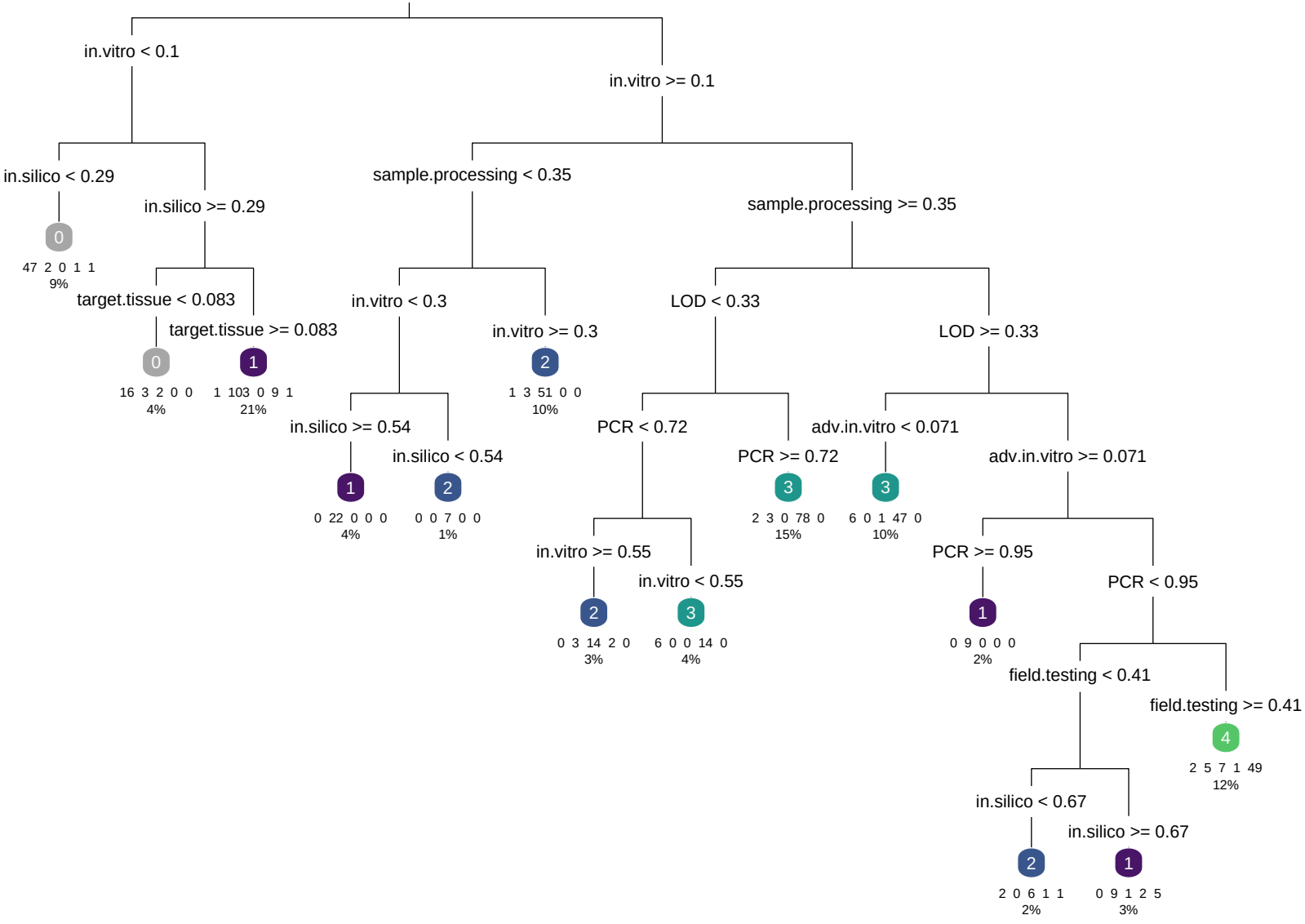
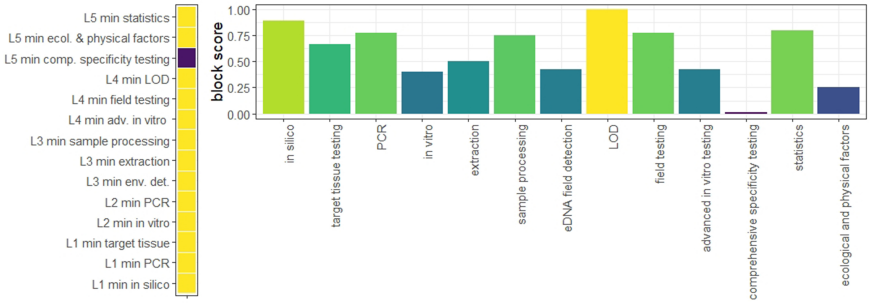


Figure 3:

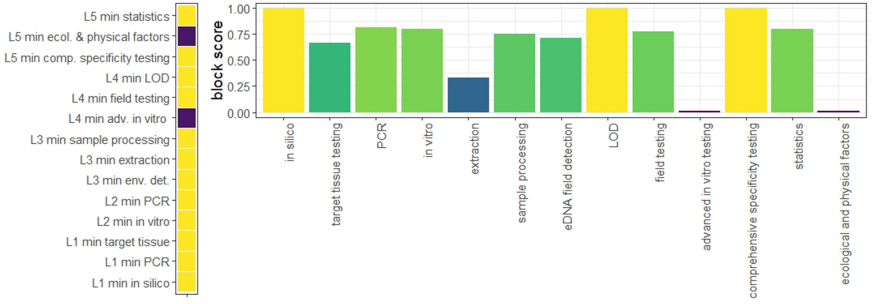

in.vitro < 0.1
in.vitro >= 0.1
in.silico < 0.29
in.silico >= 0.29
sample.processing < 0.35
sample.processing >= 0.35
0
47 2 0 1 1
9%
target.tissue < 0.083
target.tissue >= 0.083
in.vitro < 0.3
in.vitro >= 0.3
LOD < 0.33
LOD >= 0.33
0
16 3 2 0 0
4%
1
1 103 0 9 1
21%
2
1 3 51 0 0
10%
in.silico >= 0.54
in.silico < 0.54
PCR < 0.72
PCR >= 0.72
adv.in.vitro < 0.071
adv.in.vitro >= 0.071
1
0 22 0 0 0
4%
2
0 0 7 0 0
1%
3
2 3 0 78 0
15%
3
6 0 1 47 0
10%
in.vitro >= 0.55
in.vitro < 0.55
PCR >= 0.95
PCR < 0.95
2
0 3 14 2 0
3%
3
6 0 0 14 0
4%
1
0 9 0 0 0
2%
field.testing < 0.41
field.testing >= 0.41
4
2 5 7 1 49
12%
in.silico < 0.67
in.silico >= 0.67
2
2 0 6 1 1
2%
1
0 9 1 2 5
3%

Box 1:

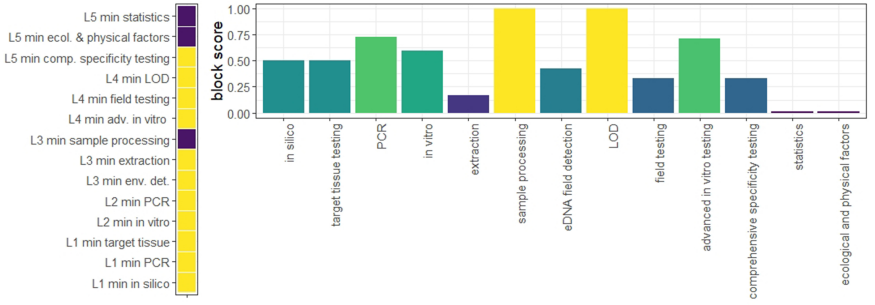
Level 4: Assay no. 165 detecting brook trout, *Salvelinus fontinalis*. Assay information was published in paper nos. 291, 12, 145, 295, 144, with latest publication in 2018. Despite low validation efforts for *in vitro* testing and field detection, the only minimum criterion that prevented this assay attaining Level 5 was “comprehensive specificity testing”.



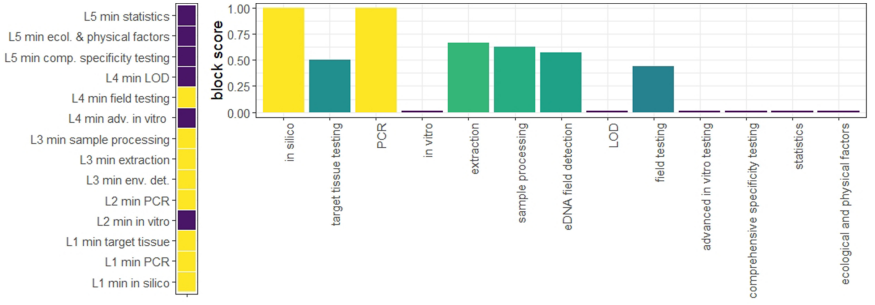
Level 3: Assay no. 473 detecting the crucian carp, *Carassius carassius*. Assay information was published in paper no. 119 in 2019. No advanced *in vitro* testing was conducted for this assay, but validation efforts were substantial for most thematic blocks, especially “*in silico* testing”, “LOD determination”, and “comprehensive specificity testing”.



Level 2: Assay no. 270 detecting common eelgrass, *Zostera marina*. Assay information was published in paper no. 117 in 2018. Despite low validation efforts in many thematic blocks, only the minimum criterion of “sample processing” prevented this assay from reaching Level 4 on the validation scale



Level 1: Assay no. 315 detecting the waterlouse, *Asellus aquaticus*. Assay information was published in paper no. 163 in 2015. With additional *in vitro* testing, this assay would have attained Level 3. A clear decrease in validation efforts is visible for variable blocks associated with higher levels of the validation scale.



Level 0: Assay no. 343 detecting *Daphnia magna*. Assay information was published in paper no. 243 in 2018. Despite some advanced validation, this assay does not fulfill the minimum criterion of “*in silico* testing” and “target tissue testing”. Therefore, it could not be classed as Level 1 on the validation scale.