Quantitative Metabarcoding for Invertebrate Pest Monitoring and Management

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Abstract

Invertebrate pests are one of the most significant threats to global agriculture. To monitor these pests, invertebrate trapping methods are commonly used to collect a representation of the diversity of pest species present in the ecosystem. Assessing and monitoring such diversity is key to inform pest management strategies, but due to the complexity of bulk trap samples from non-selective trapping methods (hundreds or even thousands of individuals per sample), it can be difficult to obtain this information in a timely manner. Metabarcoding is a promising tool for shortening the time required to determine what agriculturally significant pests are present in trap samples, while providing more precise identifications (i.e. to species level). However, this method currently only provides semi-quantitative relative abundance data, instead of the accurate absolute abundance information required to effectively monitor and manage pest populations. There is a need to increase the capacity for quantitative metabarcoding, which is a rapidly growing avenue of research in similar fields of study, such

as medical research and environmental DNA research. This review summarises existing advances in quantitative metabarcoding, highlighting key sources of bias, emerging correction methods, and studies that have driven progress toward truly quantitative applications in bulk invertebrate samples from agricultural systems. Through consolidating insights from across a diverse range of ecological applications of metabarcoding, we present a practical roadmap for improving the quantitative outputs and interpretation of metabarcoding results and integrating these novel methods into agricultural pest monitoring and management.

Keywords

Quantitative metabarcoding; Bias; Invertebrates; Sequencing; Relative abundance; Absolute abundance

Background

Invasive invertebrate pests present a major global threat to food security and profitability (Cook et al. 2011, Paini et al. 2016). These pests can lower the overall yield of agricultural crops by not only directly consuming plant material – e.g. mites (Umina et al. 2004), locusts (Wright 1986), budworms and armyworms (Wilson 1981) – but also by acting as vectors of bacterial, fungal and viral pathogens (Pimentel et al. 2000) – e.g. mites (Stenger et al. 2016), and aphids (Smith and Sward 1982, Milne and Delves 1999). Bradshaw et al. (2016) estimated the global impacts of invasive insects to be over US$70 billion per year to goods and services, with US$25 billion directly attributed to agriculture. The rise in global temperatures due to climate change, as well as increased global trade, is also increasing the frequency of invertebrate pest invasions and establishment by broadening their suitable geographic ranges (Skendžić et al. 2021). A clear example of this is the rapid spread of aphid

47 species across the world (Bell et al. 2015, Sheppard et al. 2016), which are some of the most
48 destructive and costly agricultural pests (Hoffmann et al. 2008, Van Emden and Harrington
49 2017).

50 Systematic monitoring of pest abundances and seasonal phenology is critical for forecasting
51 outbreaks and enacting timely management actions before significant crop damage can occur.
52 Standard practice involves capturing individual pests in traps, identifying them
53 taxonomically, then quantifying their abundance – or change in abundance – to predict
54 potential damage to the crop or ecosystem (Suckling 2016). A myriad of trapping methods are
55 routinely deployed, each varying in cost, sensitivity, and taxonomic breadth (Yi et al. 2012,
56 McCravy 2018, Montgomery et al. 2021), but regardless of design, a single non-selective trap
57 can often yield hundreds to thousands of specimens that must be sorted, identified and
58 counted. Processing these bulk catches by morphology alone is increasingly untenable, with
59 taxonomic expertise in global decline (Drew 2011, Paknia et al. 2015, Engel et al. 2021,
60 Hochkirch et al. 2022), and the sheer volume of invertebrates per sample can become
61 overwhelming and labour intensive (Piper et al. 2019). Consequently, many monitoring
62 programs narrow their focus to a handful of target species, with traps often subsampled, and
63 non-target specimens identified only to higher taxonomic ranks or not at all. This approach
64 risks potentially overlooking rare or unexpected species and precluding any community-level
65 analyses of pest interactions with other species, such as predators or parasitoids.

66 Molecular (i.e. genetic) diagnostic approaches offer reliable, species-level identifications
67 without requiring specialist taxonomic expertise and can reveal cryptic diversity that is
68 missed by morphological identification (Loxdale et al. 2016). For instance, many pest aphids
69 form complexes of morphologically indistinguishable species that differ in host preferences
70 and economic impact, yet can frequently be distinguished using molecular methods
71 (Raymond et al. 2001, Coeur d'Acier et al. 2004, Loxdale and Lushai 2007, Jean and Jean-

72 Christophe 2010, Blackman and Eastop 2017, Loxdale et al. 2017). Like traps, molecular
73 diagnostic methods can be either targeted or untargeted. Targeted methods, such as real-time
74 or quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays, can
75 provide rapid and accurate identification of one or a few species, but require specific assay
76 development and optimisation for each target species (Piper et al. 2019). Alternatively, DNA
77 barcoding is a generic method that amplifies and sequences a standardised genetic locus –
78 most commonly a fragment of the mitochondrial *Cytochrome Oxidase* subunit I (COI) gene –
79 from a single individual to generate a species-specific "barcode" (Hebert et al. 2003). As
80 barcode libraries accumulate, and curated sequences are linked to taxonomically identified
81 voucher specimens, this enables accurate assignment of field-collected individuals to known
82 species, while simultaneously delineating representatives of undescribed taxa (Hebert et al.
83 2003). The COI gene is favoured as a barcode marker for many animal taxa because its
84 substitution rate provides sufficient interspecific resolution to differentiate species, while
85 remaining largely conserved within species (Huang et al. 2008), and being a multi-copy
86 mitochondrial gene, it is readily amplified from even crude DNA extracts. Widespread
87 adoption of COI DNA barcoding has led to the public availability of millions of DNA
88 barcodes, representing a sizeable proportion of described invertebrate taxa (Porter et al. 2014,
89 Porter and Hajibabaei 2018), including many pest species (Piper et al. 2019). Yet the one-
90 specimen-at-a-time workflow of DNA barcoding becomes a major bottleneck when
91 surveillance and monitoring programs routinely collect hundreds or even thousands of
92 specimens per trap (Geiger et al. 2016, Karlsson et al. 2020, Hobern 2021).

93 DNA metabarcoding (Fig. 1B) overcomes this limitation through high-throughput sequencing
94 (HTS) of barcode amplicons *en masse* from bulk samples of mixed organisms, dramatically
95 increasing both the number of invertebrates that can be processed and capturing far greater
96 taxonomic breadth than traditional methods (Fig. 1A) (Taberlet et al. 2012, Gaither et al.

2022). Initially developed for broad-scale biodiversity surveys (Taberlet et al. 2012), metabarcoding has already demonstrated a number of agricultural applications, including profiling insect communities in environmental DNA (eDNA) samples (Song et al. 2025), reconstructing the diets of beneficial predatory insects from faecal and gut samples (Kim et al. 2022), tracking seasonal dynamics of target species (Martoni et al. 2023 B, Burgess et al. 2024), and detecting and monitoring invasive pests (Batovska et al. 2021, Young et al. 2021) and beneficial species (e.g. pollinators, predators and parasitoids (Martoni et al. 2023 A, Mustafa et al. 2025)). Studies like Petsopoulos et al. (2024) are beginning to utilise the potential of metabarcoding for long-term monitoring of invertebrates in agroecosystems, allowing for the accumulation of community-wide datasets to be correlated with various environmental factors, including seasonality and weather, to forecast outbreaks and develop time-sensitive management strategies (Martoni et al. 2023 B). By identifying specimens previously discarded as bycatch, metabarcoding also enables community-level analyses, revealing novel associations between pests and their natural enemies that could inform biological control strategies (Lue et al. 2023). Beyond surveillance and monitoring, metabarcoding can also support experimental studies where robust species identification is required, including contrasting pest and beneficial assemblages across crop types, management regimes, and treatment durations (Qian et al. 2024, Habel et al. 2025), benchmarking different trap designs to determine which (alone or in combination) best represents site level diversity (Li et al. 2023, Martoni et al. 2023 A), or determining the impacts of agricultural stressors on the environment (Beentjes et al. 2022).

Despite its undeniable utility and increasing uptake, metabarcoding still faces one major challenge: HTS read counts do not directly reflect the number of individuals for a given species within a sample. The core of this issue is that metabarcoding and other molecular assays count DNA molecules, not individuals, and the number of molecules contributed per

122 individual depends on a complex interplay between species-specific morphological and
123 molecular traits interacting with each stage of the metabarcoding workflow, the overall
124 outcome of which are taxonomic biases (Fig. 2; Box 1) (Sipos et al. 2007, Gonzalez et al.
125 2012, Pawluczyk et al. 2015, Piñol et al. 2015, McLaren et al. 2019, Piñol et al. 2019, Shelton
126 et al. 2023). Furthermore, metabarcoding and other HTS assays provide compositional data
127 (also referred to as relative, or proportional, data), where the DNA molecule counts returned
128 for each taxon are conditionally dependent on the counts of all other taxa within the sample
129 (Fig. 3A) (Brooks et al. 2015, Gloor et al. 2017). Therefore, if the representation of molecules
130 from one taxon increases due to taxonomic bias the measured counts of other taxa will appear
131 to decrease, even if their absolute abundances in the original sample remain unchanged (see
132 Box. 2 and Fig. 3B). Consequently, attempts to equate individual read numbers for specific
133 species from metabarcoding data to abundance and biomass estimates have produced only
134 weak or system-specific relationships (Yu et al. 2012, Yates et al. 2019, Stoeckle et al. 2021,
135 Rourke et al. 2022), as reviewed by Lamb et al. (2019). A lack of viable quantitative data
136 from metabarcoding and/or an understanding of how to control or correct for bias within
137 assays means that questions regarding trends or comparisons of target pest species absolute
138 abundances cannot yet be answered, nor comparisons be made to historical datasets generated
139 using traditional methods. Thankfully, a new generation of emerging methods – ranging from
140 novel PCR approaches, to new statistical methods, to complementary imaging approaches
141 (explored below; Fig. 4) – offer new avenues for improving quantitative metabarcoding;
142 however these methods are yet to see widespread validation or adoption in invertebrate
143 biomonitoring or agricultural contexts.

144 This review aims to: (i) synthesise current knowledge on quantitative metabarcoding; (ii)
145 catalogue the major sources of bias across the workflow—from field sampling to
146 bioinformatics; (iii) summarise emerging methods that have been developed to detect,

147 mitigate, and correct those biases; and (iv) evaluate key studies that have pushed toward truly
148 quantitative metabarcoding, highlighting their strengths, limitations and relevance to bulk
149 invertebrate samples from agricultural systems. By consolidating insights from across the
150 diverse range of ecological applications to which metabarcoding has been applied, we outline
151 a practical roadmap for improving the quantitative outputs and overall interpretation of
152 metabarcoding results and how these novel methods can be integrated into agricultural pest
153 monitoring and management.

154 Box 1: Qualitative and quantitative bias

155 In this review, bias is defined as any process that causes metabarcoding results to diverge
156 from the underlying biological reality—either in which taxa are detected or in how their
157 abundances are represented.

158 Qualitative bias affects taxon detection (presence/absence) and the apparent community
159 composition within a given site or ecosystem. In bulk invertebrate sampling, it can arise when
160 the sampling method is not equally likely to capture all taxa present at a site and therefore can
161 affect both morphological and molecular assessments of bulk samples. In metabarcoding,
162 qualitative bias can also occur downstream during laboratory or bioinformatic steps if, for
163 example, some taxa: fail to amplify; are missing from reference databases; or are lost through
164 stochastic subsampling (dilution, amplification, and subsampling through library preparation)
165 when starting DNA is rare. Thus, qualitative bias primarily concerns false negatives/false
166 positives and whether the species list recovered is representative of the true community.

Quantitative bias, on the other hand, affects estimations of abundance for taxa that are detected. It arises due to the interaction between species-specific biological variables and each step of the metabarcoding protocol, causing some taxa to be preferentially sequenced over others, distorting the relationship between the number of individuals in the original samples and the number of DNA sequences observed after sequencing. In extreme cases of quantitative bias, taxa can be reduced to the point that they fall below detection thresholds, effectively creating qualitative bias.

Box 2: Absolute vs. relative abundance

In the context of quantitative metabarcoding, relative abundance describes taxa in proportion to one another within a sample, whereas absolute abundance refers to the unit-scaled amount of each taxon within a sample (e.g. number of individual invertebrates of each species, or an equivalent measure such as biomass or DNA molecules) (McLaren et al. 2022). Relative abundance can address questions framed against the rest of the community, for example whether a focal taxon is increasing or decreasing relative to another taxon or the entire assemblage. However, relative abundance alone cannot reliably answer questions that require an absolute scale, such as "how many individuals of species X are there in this sample?" or "what is the total population size of species X at this site?" (Stämmler et al. 2016, Vandeputte et al. 2017).

Converting relative abundance to absolute abundance requires an external measurement that anchors proportions to a total sample load. For example, if metabarcoding provides for taxon i in sample j a relative proportion P_{ij}, and an independent estimate T_j of the total amount of biological material in j is available (e.g. total insect counts, total biomass, or total marker copy number from qPCR/ddPCR), then absolute abundance of i in j can be estimated as $A_{ij} = P_{ij} \times T_j$ (Fig. 4D). In the absence of an external measurement, compositional data analysis

(CoDA) methods enable valid cross-sample comparison of community composition by analysing taxa in terms of ratios (e.g. log-ratios) rather than raw proportions (for more information see (Greenacre 2021, Greenacre et al. 2023)). This avoids the "constant sum constraint" where increases in one taxon cause apparent decreases in others even if their absolute abundances are unchanged (Fig. 3B). Importantly, by scaling to absolute abundance, CoDA transformations both improve interpretability and statistical validity for relative data, however they do not on their own resolve taxon-specific quantitative biases introduced by the assay (Table 1). These biases can still distort abundance estimates unless they are reduced experimentally or corrected analytically (see below).

Main text

Considerations and Limitations for Quantitative Metabarcoding

Sampling

Quantitative metabarcoding is affected by biases introduced throughout the entire workflow, beginning with the initial collection of individuals from the field. Trap type and deployment strategy strongly shape the community observed, because taxa differ in their likelihood of capture by different methods (Yi et al. 2012). For example, malaise traps preferentially collect flying Diptera and Hymenoptera (deWaard et al. 2019, Skvarla et al. 2021), pitfall traps target ground-dwelling species (Southwood and Henderson 1978), light traps favour nocturnal taxa such as moths (Bowden and Church 1973), and window traps are most effective with certain groups of beetles (Boiteau 2000). While there is an extensive variety of invertebrate trapping strategies (Yi et al. 2012, McCravy 2018, Montgomery et al. 2021), key design variables include trap selectivity, spatial layout, trap height, and the collection period/frequency.

Trap selectivity lies on a continuum, shaped by both the capture mechanism and the degree to which a trap attracts particular taxa. At one end, “passive” devices, such as Malaise, pan, or sticky traps, capture a broad cross-section of the community by intercepting or retaining species that naturally encounter the trap. At the other end, “active” or “attractant-driven” traps use visual cues (e.g. light, or trap colour), food baits, or semiochemicals (e.g. pheromones) that exploit insect behaviour or physiological state to increase capture efficiency for particular targets, often at the cost of reduced taxonomic breadth (Greenslade and Greenslade 1971, Ballard and Gold 1982, Hughes et al. 1998, Meagher Jr and Mitchell 2001, Yu et al. 2006, Whitfield et al. 2019). Because attractant-driven approaches rely on species-specific sensory and behavioural responses, they generally require extensive prior knowledge of the target’s visual preferences (e.g. coloured traps (Williams 1973, Brødsgaard 1989, Atakan and Canhilal 2004), bait trapping (Greenslade and Greenslade 1971, Ballard and Gold 1982, Hughes et al. 1998, Yu et al. 2006), or pheromones (Meagher Jr and Mitchell 2001, Whitfield et al. 2019)). As a result, they may be less suitable for broad-scale biomonitoring without clear targets, and, if not carefully considered, can introduce systematic biases, including sex- or life stage-specific differences in attraction and dispersal (Bell et al. 1972, Riedl et al. 1976, Mullen 1992, Turchin and Odendaal 1996, Walker et al. 2003). Between these extremes are powered traps (e.g. suction traps), which – like passive traps – can sample a relatively broad range of alate or airborne insects, but use power to increase capture rate within a limited sampling radius. Many pest taxa lack reliable lures, and others (notably aphids) have no known effective attractants. In such cases, non-selective traps – typically suction or sticky traps – must be used (Congdon et al. 2019A, Congdon et al. 2019B, Congdon et al. 2019C, Martoni et al. 2023 B), with several countries deploying extensive suction trap networks to monitor pest aphid abundances (Halbert and Pike 1990, Harrington et al. 2001, Teulon and Scott 2006, Lagos-Kutz et al. 2020).

The spatial arrangement and number of traps deployed within a site affects both abundance and composition because trapping is inherently stochastic and communities vary even over short distances. Malaise traps separated by tens of metres can share substantially fewer taxa than more closely spaced traps (Steinke et al. 2021), and catches often differ between crop edges and interiors (Evans et al. 2016, Nguyen and Nansen 2018), or with surrounding vegetation context (Fahrig and Jonsen 1998, Isaacs et al. 2009). For pitfall trapping, wider spacing (5-10 metres) has been shown to increase the likelihood of detecting rare taxa, compared with 1 metre spacing (Ward et al. 2001), demonstrating that simply adding traps without considering arrangement and spacing may yield limited gains in detected diversity. Sample complexity (high richness and uneven abundances) can also interact with later steps of the metabarcoding workflow, potentially leading to preferential amplification and sequencing of highly abundant species, reducing the accuracy of community recovery with metabarcoding (Creedy et al. 2019) with a greater likelihood for stochastic drop-outs of low abundance species.

Trap height and interception area also influences captures (Gillespie and Vernon 1990, Atakan and Canhilal 2004, Teulon et al. 2004, de Souza Amorim et al. 2022). Communities can shift with height as short-range dispersers typically occur lower in the wind column than long-range dispersers (Hodges et al. 2024). In a multi-height malaise trap comparison (ground, 8, 16, 24, and 32 metres tall), de Souza Amorim et al. (2022) found strong vertical stratification in community composition, with Diptera, Hymenoptera, and Coleoptera being most abundant at ground level, while Lepidoptera and Hemiptera were more abundant in taller traps. Additionally, of the 856 Diptera species collected, 61.6% of those species were not collected at ground level, highlighting the importance of potentially including traps of varying heights to provide a more evenly collected species diversity within a given environment. An additional factor to increase the diversity of species captured is to increase

the surface area of the traps used for collections (Gressitt and Gressitt 1962, Work et al. 2002). Apparent deviations between metabarcoding and morphological identifications can be caused by the presence of trace DNA of prey species targeted by the focus taxa (Toju and Baba 2018, Ershova et al. 2023), as well as airborne eDNA transmitted into traps via wind (Johnson et al. 2021), which is likely linked to the surface area of the trap opening.

Finally, collection frequency and timing influences both taxonomic recovery and the ability to infer trends in abundance. More frequent collections (i.e. hourly or daily) improve temporal resolution and reduce the likelihood of DNA degradation in comparison to samples collected less frequently (i.e. weekly or monthly) (Krehenwinkel et al. 2018), but also increase logistical and equipment costs and necessitates processing and sequencing of a larger number of smaller samples compared to less frequent collections. Sampling schedules may also need to account for diel activity to distinguish between species dispersal during the day vs. at night (Green 1999).

DNA Extraction

DNA extractions from bulk samples can introduce substantial taxonomic bias because species differ in how much DNA they contribute during lysis and how efficiently that DNA is recovered. During extraction – due to differences in body size and structure (soft or hard-bodied exoskeleton) (Martoni et al. 2022), genome size (Morgan et al. 2010, Pornon et al. 2016, McLaren et al. 2019), and DNA-per-cell yield – certain taxa will produce greater quantities of DNA than others. The extraction approach used interacts with these factors, with non-destructive methods (e.g. gentle lysis of whole specimens that remain intact after extraction) tending to produce read proportions that reflect specimen surface area (Marquina et al. 2019, Martoni et al. 2022), while destructive methods (homogenising specimens) more closely track specimen biomass (Iwaszkiewicz-Eggebrecht et al. 2023). While a more

accurate relationship between read counts and biomass has some benefits for quantification, the DNA released from large-bodied invertebrates can dominate the sequencing data and limit the detection of smaller species. To limit this distortion, particularly for destructive extractions, studies often separate and organise samples by size (i.e. separate large invertebrates from small ones) prior to DNA extraction (Elbrecht et al. 2017, Deagle et al. 2018, Elbrecht et al. 2021). Alternatively, increasing sequencing depth can improve detection of smaller taxa (Elbrecht et al. 2021), however this does not remove biomass-driven biases in relative abundance. Non-destructive DNA extractions have the added benefit of retaining intact specimens for post-hoc confirmation of species detections using morphological and single-specimen barcoding, which is particularly valuable in regulatory contexts such as biosecurity surveillance (Batovska et al. 2021, Iwaszkiewicz-Eggebrecht et al. 2023, Martoni et al. 2023 A, Martoni et al. 2023 B).

The specific DNA extraction protocols and chemistries used can also introduce quantitative biases, as certain lysis buffers perform better with certain species depending on biomass (Schiebelhut et al. 2017, Marquina et al. 2022, Iwaszkiewicz-Eggebrecht et al. 2023). Lysis duration also has an effect, particularly for non-destructive methods, as longer lysis periods tend to favour larger individuals over smaller ones, as the former naturally have more cells while the latter may have already been fully lysed earlier in the reaction (Iwaszkiewicz-Eggebrecht et al. 2023). The method used to clean lysed DNA can also have an impact. Silica-column extraction methods are capable of becoming “saturated” with DNA extracted from large mixed samples, effectively imposing a non-linear relationship between the biomass/number of insects and the amount of DNA extracted, contributing to the overall “compositional” nature of the resulting data. Magnetic bead-based extraction methods have similar limitations, with the amount of total DNA extracted limited by the number of magnetic beads present in the solution (Marquina et al. 2022). Simple extraction methods that

313 omit any cleanup step are liable to produce more unpredictable variation in performance
314 across extractions due to potential PCR inhibitors not being removed (Martoni et al. 2022,
315 Fowler et al. 2023).

316 Amplification

317 One of the most widely recognised sources of metabarcoding bias arises during PCR
318 amplification, where mismatch between primers and the diverse template molecules released
319 from a mixed sample create species-specific differences in binding strength and amplification
320 efficiencies (Sipos et al. 2007, Gonzalez et al. 2012, Pawluczyk et al. 2015, Piñol et al. 2015,
321 Piñol et al. 2019). This can result in certain taxa outperforming others during PCR
322 amplification and consequently becoming overrepresented in sequencing libraries and read
323 datasets (Shelton et al. 2023), due to competition for reagents and the compositional state of
324 the resulting data produced. This presents a particular problem for the widely adopted COI
325 barcode region, where there are no strictly conserved nucleotide sites to design truly universal
326 primers (Deagle et al. 2014, Liu et al. 2023) – as it is a protein coding gene, many amino
327 acids have a fully redundant 3rd position, allowing for significant variation to occur for
328 nucleotides within this position with little consequence on amino acid sequence and protein
329 structure. Primer mismatches have an especially negative impact on amplification when
330 occurring at the primer 3' end (Bru et al. 2008, Boyle et al. 2009, Stadhouders et al. 2010,
331 Piñol et al. 2019, Liu et al. 2023), due to disruption of the polymerase active site (Beard et al.
332 2004, Johnson and Beese 2004). The option to completely forego PCR and instead sequence
333 non-amplified DNA using methods such as meta/mitogenomics has been explored and shown
334 to have relative success in producing accurate quantitative data (Zhou et al. 2013, Bista et al.
335 2018, Ji et al. 2020, Garrido-Sanz et al. 2022), however this requires high-quality DNA (Ji et
336 al. 2020) and involves more costly and complex laboratory, sequencing, and analysis
337 workflows, which could limit its applications to large monitoring and surveillance programs.

Another potential factor that can contribute to amplification bias between species is differences in gene copy number, often termed Copy Number Variation (CNV) (Kembel et al. 2012, Krehenwinkel et al. 2017). For nuclear markers, CNV reflects varying numbers of gene copies within the nuclear genome (e.g. the tandem repeat structure of the rRNA operon, or autosome ploidy), while for mitochondrial markers, it reflects differences in the number of mitochondria per cell and/or the number of mitochondrial genomes per mitochondrion, which can vary across tissues and species (Wiesner et al. 1992). The ratio of nuclear to mitochondrial DNA likely differs greatly across species and cannot be easily determined without methods that quantify nuclear and mitochondrial DNA separately. As mitochondrial CNV and the factors that influence it are not currently well understood across invertebrates (Chen et al. 2020, Calogero et al. 2023), this warrants further investigation given its potential to significantly impact metabarcoding quantification.

The DNA polymerase used during PCR can also bias abundance data due to certain polymerases having amplification preferences for GC content, amplicon length, and certain sequence motifs (Dabney and Meyer 2012, Pan et al. 2014, Nichols et al. 2018). Degenerate primers that utilise inosine bases (which pair with cytosine, uracil and adenine) can negatively impacted PCR when using polymerases that are not tolerant of inosine (Knittel and Picard 1993, Zheng et al. 2008, Elbrecht et al. 2019, Zheng et al. 2024). Additionally, erroneous or artefactual sequences introduced during PCR amplification and later sequencing steps, such as chimeras, can lead to incorrect classifications when not removed during filtering steps (Tedersoo et al. 2018).

Sequencing

Sequenced DNA reads are only a subset of the total amplified DNA produced from a given sample, creating compositional data with a sum that does not vary based on the number of

362 individual invertebrates present in the original sample (otherwise known as the “constant sum
363 constraint”; Fig. 3C) (Gloor et al. 2017, Quinn et al. 2018, Harrison et al. 2021, Shelton et al.
364 2023). Essentially, sequencing data is constrained by the library size offered by the
365 sequencing method used, and will provide compositional estimates of reads per species
366 relative to every other species present within the sample, preventing meaningful across-
367 sample comparisons of read counts (Quinn et al. 2018). It is also possible that quantitative
368 estimates can differ due to the sequencing platform and chemistry used (Porazinska et al.
369 2010), as certain chemistries have a preference for particular GC compositions (Qin et al.
370 2023) (particularly relevant for targeting mitochondrial DNA barcodes as they commonly
371 have lower GC content (Saccone et al. 1999)), and can differ significantly in per-base error
372 rate (i.e. Illumina vs. Nanopore) (Wang et al. 2021). For long read technologies,
373 homopolymer-associated errors can potentially reduce sensitivity for taxa whose barcodes
374 contain longer homopolymeric regions, introducing additional bias (Tedersoo et al. 2021).

375 Bioinformatics

376 After sequencing, raw reads must be processed into biologically meaningful units and
377 assigned taxonomic names. In metabarcoding, this “bioinformatics” step effectively
378 determines what is considered a species detection, how sequencing and PCR errors are
379 handled, and how reads are translated into taxonomic labels—so choices made here can
380 directly alter both the species list recovered and the relative abundance patterns inferred
381 (Plummer et al. 2015). A key decision is whether to summarise sequence reads as Amplicon
382 Sequence Variants (ASVs) or Operational Taxonomic Units (OTUs). ASV approaches (i.e.
383 DADA2; (Callahan et al. 2016)) treat each unique sequence as the biological unit and use
384 statistical models of abundance and co-occurrence to attempt to distinguish true biological
385 sequences from PCR and sequencing errors. These inferred sequences are then taxonomically
386 annotated using a reference database and, where needed, aggregated to the desired rank (e.g.

387 species, strain, or higher level) to estimate relative abundance. OTU approaches, in contrast,
388 attempt to define biological units directly by clustering reads using a fixed similarity
389 threshold intended to approximate a “barcode gap” at a given rank (e.g. commonly 97%
390 nucleotide identity for species-level clustering of COI barcodes), and a single representative
391 sequence from each cluster is then taxonomically annotated. A key limitation of OTU
392 methods is that no single similarity threshold is universally appropriate across all taxonomic
393 groups, which can cause closely related species to be merged or intraspecific haplotypes to be
394 split when the clustering threshold is poorly matched to the taxonomic groups contained in a
395 mixed sample (Pauvert et al. 2019). In addition, OTU definitions can be dataset-dependent
396 (i.e. the clusters obtained depend on the set of sequences and clustering procedure used),
397 which reduces comparability across studies or when samples are processed in separate
398 batches (Callahan et al. 2017). For these reasons, sequence-resolved ASV workflows are
399 often considered more amenable to quantitative interpretation than OTUs (Sickel et al. 2023).
400 However, ASV-based approaches can also split genuine within-species variation into multiple
401 sequence variants that—if reference matches are incomplete or ambiguous—may be assigned
402 to different or higher taxonomic ranks and therefore not collapsed together, effectively
403 diluting the estimated abundance of that species (Estensmo et al. 2021).

404 Using COI and other mitochondrial markers has the disadvantage of potentially mis-
405 amplifying Nuclear Mitochondrial (NUMT) pseudogenes, which are regions of the nuclear
406 genome where mitochondrial DNA has been incorporated (Bensasson et al. 2001, Leite 2012,
407 Hebert et al. 2023). NUMTs are well documented in certain taxa, such as locusts (Liu et al.
408 2024) and tephritid fruit flies (Morrow et al. 2015, Krosch et al. 2020). As NUMTs are no
409 longer under the same selective constraints as the functional mitochondrial gene, they can
410 accumulate substitutions and frameshifts independently over time. As a result, unintentional
411 amplification of these NUMTs can result in multiple highly divergent sequences from the

same individual, that could be misinterpreted as unique species (Song et al. 2008, Wang et al. 2018, Krosch et al. 2020, Hebert et al. 2023). Furthermore, in some cases NUMTs can be preferentially amplified over a species' true mitochondrial sequence, potentially lowering relative abundances for certain species, while inflating abundances of "fake" species (Song et al. 2008). As NUMT sequences do not represent the actual target region, they should be filtered from metabarcoding datasets based on the presence of frameshifts and early stop codons (Andújar et al. 2021, Schultz and Hebert 2022) that would not occur in a functional mitochondrial gene; however not all NUMTs carry easily distinguishable features (Song et al. 2008, Shokralla et al. 2014, Creedy et al. 2020).

An additional factor that may contribute to inaccurate abundance estimates is the presence of mitochondrial DNA heteroplasmy within certain taxa, which can lead to multiple unique sequences being sequenced from the same individual (Rubinoff et al. 2006). Li et al. (2021) displayed in a study on fig wasps how the presence of mitochondrial heteroplasmy in these taxa caused a simulated metabarcoding test to overestimate the number of OTUs by 3.2X the number of actual morphological species present. In contrast to NUMTs, in cases of species where mitochondrial heteroplasmy is known, reference databases may need to include all possible variants to ensure all potential species are included in per-species abundance estimates.

Taxonomic assignment is another step where bias can be introduced. Because reference libraries are incomplete, particularly for understudied taxa or geographic regions, many metabarcoding sequences will lack an exact database match and must instead be placed using taxonomic assignment algorithms that infer the most likely classification. For quantitative metabarcoding, it is critical to minimise both over-classification (collapsing sequences from multiple real taxa into a single taxon) and under-classification (assigning taxa, or within-taxon variants, only to higher ranks), as both can distort abundance estimates. Errors are more

likely when relying on uncurated public databases that contain an unknown proportion of mislabelled or low-quality records (Nilsson et al. 2006), or when reference sets are dominated by ecologically irrelevant taxa that increase the chance of misleading matches (Mugnai et al. 2023). Database choice therefore involves a trade-off: smaller databases may lack representation, while larger databases may include mislabelled or irrelevant records, and these aspects can further interact with the taxonomic assignment method and confidence thresholds used (for a more thorough discussion of taxonomic assignment methods for metabarcoding (see (Hleap et al. 2021)) (Piper et al. 2019, Mugnai et al. 2023). Hence, it is recommended to use curated "in-house" databases that incorporate sequences from local or relevant taxa, and if data from large public repositories are used, this should be filtered to remove mislabelled and low-quality sequences (Rodgers et al. 2017, Axtner et al. 2019, Mugnai et al. 2023).

Steps Towards Improving Quantification in Metabarcoding

Primer design and amplification

Previous studies aiming to solve bias-related issues in metabarcoding have primarily focused on primer design in order to improve the amplification of the target gene, and in turn, the quantitative accuracy of relative abundance data (Piñol et al. 2019), unfortunately with limited success. The use of degenerate primers can partially compensate for primer-template mismatches between species (Elbrecht and Leese 2017, Krehenwinkel et al. 2017, Elbrecht et al. 2019), as well as circumnavigate a balance between accurate species identification and consistent abundance estimations (Elbrecht and Leese 2017). However, primers that rely too heavily on degenerate bases have increased risk of off-target amplification of taxa outside the studies scope (e.g. fungal or bacterial sequences) or pseudogene loci (Leese et al. 2021). An alternative approach for invertebrates is to use barcode genes that contain more conserved

regions for placing universal primers (i.e. 16S, rRNA), which amplify evenly without the need for degenerate bases (Marquina et al. 2019). For example, Liu et al. (2023) showed more accurate estimations of invertebrate biomass with 16S primers when compared to COI primers. Nevertheless, COI still dominates public reference sequence databases (Elbrecht et al. 2016, Liu et al. 2020), and the slower evolutionary rate of 16S can reduce resolution between closely related species (Andújar et al. 2018). Several studies have recommended using multiple primer sets targeting several genomic regions simultaneously (e.g. COI and 16S primers together (Valente Penner et al. 2024)) (Piñol et al. 2015, Hajibabaei et al. 2019, Thomsen and Sigsgaard 2019, de Kerdrel et al. 2020). However, in mixed-community metabarcoding, sequences from different loci cannot be confidently linked to the same specimen (Axtner et al. 2019), and because different loci can recover different subsets of taxa or assign the same taxa to different ranks (Alberdi et al. 2018), integrating multi-locus outputs into a single consensus abundance estimate per-taxon remains challenging.

Irrespective of the primers or target marker, optimising PCR conditions can help mitigate amplification bias. Using fewer PCR cycles where possible (Polz and Cavanaugh 1998, Yang et al. 2021) and a lower annealing temperature (Sipos et al. 2007, Yang et al. 2021) can reduce the compounding effect of differential amplification bias that accumulates throughout the PCR process. This can also reduce "drop-outs" of sequences with lower starting DNA quantities (Piñol et al. 2015). It is recommended to include a minimum of three PCR replicates per sample, as stochasticity in PCR amplification can create differences in diversity and abundance between replicates of the same sample (Alberdi et al. 2018, Grey et al. 2018), which can then be used to identify under- or over-estimation of relative abundance (Shelton et al. 2023). Additionally, amplicon sequence errors introduced by PCR can be reduced by using high-fidelity polymerases (Lee et al. 2016, Potapov and Ong 2017, Nagai et al. 2022), however they are generally more expensive, increasing the cost-per-sample, and they may

introduce more quantitative bias in preference of higher GC sequences compared to lower-fidelity polymerases (Nichols et al. 2018).

Unique molecular identifiers

One strategy to measure and reduce amplification-driven bias is to tag each input DNA molecule with a Unique Molecular Identifier (UMI) sequence prior to PCR amplification (Kivioja et al. 2012). This is achieved by incorporating a short string of degenerate bases into the design of one or both primers (Karst et al. 2021, Luo et al. 2023), creating a complex cocktail of many thousands of unique oligonucleotides when synthesised. A Single Primer Extension (SPE) (Hoshino and Inagaki 2017, Hoshino et al. 2021), or a small number of PCR cycles (i.e. 2 cycles), is used to incorporate UMIs into the initial amplicons produced from each starting molecule, followed by a second PCR step to exponentially amplify the tagged molecules to obtain sufficient sequencing quantities. After sequencing, reads sharing the same UMI combination (i.e. produced by PCR from the same starting DNA molecule) can be clustered to reconstruct a consensus sequence and count the number of original molecules present before any PCR amplification bias was introduced (Fig. 4A) (Lundberg et al. 2013, Hoshino and Inagaki 2017, Hoshino et al. 2021, Çiftçi et al. 2023, Luo et al. 2023).

In practice, UMI-based metabarcoding workflows introduce their own design constraints. The UMI sequence spacer (i.e. number of unique combinations of degenerate bases) must be sufficiently larger than the number of input molecules to minimise UMI collisions (multiple input molecules sharing the same tag; see Figure 4 of (Clement et al. 2018)). Furthermore, the number of tagged molecules carried into the second PCR must be balanced against sequencing depth: it must be low enough to ensure multiple reads are obtained for every input molecule—enough to build a consensus sequence—while also generating sufficient total sequencing to sample most UMIs present in the library. If too many tagged molecules are

carried forward relative to sequencing depth, many UMIs will be observed only once or not at all, and the number of input molecules cannot be reliably estimated. Errors within the UMI sequence itself can further bias estimates by inflating the apparent number of unique molecules or causing different input molecules to be incorrectly grouped together (Karst et al. 2021, Yan et al. 2023). To mitigate this, some UMI protocols incorporate partially "structured" UMIs (e.g. Y or R nucleotides), rather than completely degenerate (N) nucleotides, enabling detection and filtering of UMI sequencing errors; however, this reduces the available sequence space for a given UMI length.

Incorporating UMIs into amplicon sequencing protocols has been increasingly adopted in medical and forensic genomics to increase sensitivity, resolve PCR duplicates, and correct for allelic bias (Kivioja et al. 2012, Sun et al. 2024), and it has also been used to increase the accuracy of Nanopore amplicon sequencing in a metabarcoding context (Karst et al. 2021). Use of UMIs for correcting amplification bias in metabarcoding, however, remains limited. In principle, UMI-derived input molecule counts should better reflect the relative proportions of starting DNA among species (Kivioja et al. 2012, Hoshino et al. 2021), and allow measurement and correction of any deviations introduced by PCR bias. In practice, Luo et al. (2023) noted that UMIs alone were insufficient for reliable quantification without the use of a spike-in, or other method that provides an external measurement for translating relative to absolute abundances (Box 2). Hoshino et al. (2021) reported moderate success using a UMI-based approach (termed qSeq) to quantify eDNA collected from tanks containing five fish species. This was, however, a relatively simple mock community, and expanding this to a natural aquatic habitat containing the cumulative DNA of a larger number of fish species (and other groups of organisms that could be amplified depending on the specificity of the primers used) with varying sizes and levels of DNA shedding may be more challenging. Yu et al. (2022) noted when attempting to utilise the UMI method to monitor fish species from open

ocean eDNA samples, the adapted primers actually resulted in decreased detection of species when compared to the results from the standard primers, resulting in a trade-off between detection and quantification goals.

Correcting for bias using mock communities

Mock communities ("mocks") – artificial mixtures of selected taxa assembled with known input quantities – can be used to measure the amount of bias introduced throughout a metabarcoding protocol by comparing the relationship between expected and observed abundances (Martoni et al. 2022). If bias acts consistently across samples, bias estimates from mock communities can be used to infer taxon-specific amplification efficiencies and derive correction factors to adjust abundance estimates in samples of unknown composition (Fig. 4B) (Ershova et al. 2023, Gold et al. 2023, Moinard et al. 2023, Shelton et al. 2023). For instance, Ershova et al. (2023) used species-specific conversion factors estimated from zooplankton mock communities to correct for biomass and PCR bias, showing an overall increase in the Pearson correlation between relative biomass and sequence counts from 0.57 to 0.77.

The type of bias captured by a mock community-derived correction factor depends on how the mock community is constructed. For instance, a DNA mock (pooled DNA from multiple single-species extractions) captures biases arising from amplification, as well as subsequent library preparation and sequencing steps, but will not account for any biases introduced in earlier steps, such as DNA extraction. In contrast, specimen-based mocks (mixed whole individuals) will capture biases introduced through DNA extraction and all downstream steps, and can therefore better reflect whole-protocol distortion (Kembel et al. 2012, Thomas et al. 2016, Krehenwinkel et al. 2017, Liu et al. 2023). By comparing bias estimates from mock designs that include or exclude particular stages, total protocol bias can be partitioned into

559 step-specific components (Martoni et al. 2022). For practical application, specimen-based
560 mocks are preferable for measuring and correcting for protocol-introduced bias, but it is often
561 challenging to obtain enough individual specimens to assemble complex and comprehensive
562 mocks that realistically reflect the composition of field samples. DNA mocks are easier to
563 construct as individual DNA extracts can be obtained from single specimens and mixed in
564 controlled proportions. When creating DNA mock communities, quantification should ideally
565 target the template DNA region of interest (i.e. mitochondrial copies for COI), rather than
566 total genomic DNA, to avoid confounding the estimates through differences in
567 mitochondrial-copy number across species (Shaffer et al. 2025). Neither approach, however,
568 will account for any biases incurred prior to lab processing, such as different DNA
569 degradation rates between species or selectivity of sampling methods for different taxa. These
570 effects can be modelled or incorporated into calibration in some contexts, but doing so may
571 reduce transferability if field samples differ in trapping conditions or degradation state.

572 A central limitation of mock-based correction is transferability. The most obvious challenge is
573 that correction factors cannot be calculated for taxa that were not included in the mock
574 community (Shelton et al. 2023, Sickel et al. 2023). Because field samples, particularly from
575 passive traps, can contain highly variable and unpredictable assemblages, constructing mocks
576 that capture the complete diversity of real communities is likely impossible, and correction
577 factors cannot be derived for novel species that are either being detected for the first time, or
578 simply haven't been accounted for in mock community designs (Sickel et al. 2023). In
579 practice, this means correction will only be applicable for only a subset of the taxa in each
580 sample. Nevertheless, mock-based calibration could still be viable for more targeted analysis,
581 where correction factors are developed just for species of interest, with the remaining species
582 uncorrected or excluded from analysis. There is also the potential for correction factors to be
583 extrapolated to closely related species as bias-causing factors such as body size, exoskeleton

hardness, and sequence composition are often phylogenetically conserved (Krehenwinkel et al. 2017, Liu et al. 2023). For example, Liu et al. (2023) examined the relationship between read abundance, biomass, and primer mismatches in beetle communities using both COI and 16S primer sets, finding both bias patterns and their potential explainer (primer mismatches) to be phylogenetically conserved between related species. However, since this evidence comes from only a single taxonomic group, broader validation across more diverse taxa will be required to determine how reliably phylogenetic patterns can support extrapolation of correction factors at larger scales.

Because mock-derived correction factors can typically be applied to only a subset of taxa in a sample, an important open question is whether taxon-specific bias is truly independent of community context and therefore transferable from a simplistic mock to much more diverse and variable field samples. McLaren et al. (2019) showed that when abundances were analysed using CoDA log-ratio transformations (i.e. as ratios to another taxon or to the sample geometric mean), estimated bias and the resulting correction factors for each taxon were independent of the overall sample composition. However, these results were obtained using relatively simple communities, and context dependence remains plausible for more diverse samples. For example, if a taxon with very high PCR efficiency is present in the mock communities used to derive the correction factors, but absent from the field samples to which those corrections are applied, competitive dynamics during PCR amplification may shift, increasing the effective amplification of other taxa and causing their realised bias to deviate from the mock-derived expectations (Shelton et al. 2023). Practical aspects of mock construction can further affect transferability. Lab-reared invertebrates may differ from trap-caught invertebrates due to reduced intraspecific diversity (especially in clonal taxa such as aphids) or systematic differences in body sizes and tissue compositions driven by developmental temperature and nutrition (Chakraborty et al. 2025). Storage conditions and

specimen age prior to mock assembly could also introduce unforeseen variation via DNA degradation, altering DNA yield and template quality relative to contemporary trap samples that will likely provide more degraded DNA yields than perfectly preserved specimens (often used in mocks). These issues emphasise the need for independent evaluation of correction factors. Assessing performance on the same mock communities used to derive the factors is likely to overestimate the accuracy. So using cross-validation (Shelton et al. 2023) and/or testing on held-out "test sets" of mock communities or independent field samples that were not used to generate the correction factors could provide a more realistic assessment of whether correction factors meaningfully reduce bias under operational conditions.

Correcting for bias without mock communities

Several studies have introduced equations that allow for the estimation and correction of bias that has been introduced to sequencing data via the various steps taken throughout library preparation in the absence of mock communities (Darby et al. 2013, Silverman et al. 2021). For example, Silverman et al. (2021) describe using a "calibration sample", created by pooling DNA from all samples together, which is then split into multiple aliquots and amplified across a range of PCR cycles before being sequenced alongside the experimental samples. A log-linear regression is then used to model the relationship between PCR cycle number and the relative abundance of each species using the calibration samples, from which PCR efficiency factors can be estimated and used to correct PCR bias in the experimental samples. The authors do, however, note the challenge of capturing the earliest PCR cycles (where primer-template mismatches have the greatest impact) in the calibration samples, as low cycle numbers do not produce sufficient template for sequencing. Gimpel et al. (2025) describe utilising a mixed pool of synthetic oligos with varying sequence features and motifs to identify factors driving amplification bias and measure PCR efficiencies, this could in turn be applied to developing correction factors for sequences with known features. Darby et al.

(2013) describe an alternative strategy to develop correction factors without the use of mock communities, instead using a "guess-and-test" approach, which utilises an algorithm that can generate potential correction factors and by comparing the predicted specimen counts generated from applying those correction factors to metabarcoding data against a reference dataset of known specimen counts and known species-specific copy numbers, optimal correction factors can be selected (Darby et al. 2013, Darby et al. 2020). A key limitation, however, is that these methods focus on just the amplification stage and do not address upstream sources of bias, such as those introduced during DNA extraction.

Obtaining absolute abundance measures

As discussed in Box 2, absolute abundance is significantly more informative than relative abundance for many surveillance and management questions (Box 2), but it cannot be obtained directly from metabarcoding data without additional external measurements that anchor read proportions to a total sample load. The choice of external measurement determines the unit of measurement of the resulting dataset, so it should ideally match the quantity of interest for downstream inference (e.g. total insect counts or biomass).

A strategy that has seen some uptake in eDNA and microbial studies is the addition of Internal Standards (ISDs) or "spike-ins" of known quantities into each sample to improve quantitative interpretation and comparability across samples (Fig. 4C) (Harrison et al. 2021). Spike-ins can take several forms, including biological specimens (exotic specimens or DNA extracts) that are not expected to be found in the study area (Ji et al. 2020, Iwaszkiewicz-Eggebrecht et al. 2023, Luo et al. 2023) or synthetic DNA constructs such as Callio-synth and tp53-synth (Tourlousse et al. 2017, Palmer et al. 2018, Marquina et al. 2021, Iwaszkiewicz-Eggebrecht et al. 2024). When added at the same quantity to each sample, the spike-in provides a common reference that makes technical variation in extraction, library preparation,

658 and sequencing depth easier to detect and adjust for. In practice, read counts can be
659 normalised to the spike-in (e.g. by scaling taxon counts relative to spike-in counts), enabling
660 more consistent between-sample, within-taxon comparisons and, potentially, more direct
661 comparisons across independent projects if standardised synthetic spike-ins are adopted
662 (Iwaszkiewicz-Eggebrecht et al. 2024). However, spike-ins do not resolve species-specific
663 biases within samples (e.g. differential extraction or amplification efficiency), meaning
664 relative differences among taxa within a community can remain distorted even when
665 between-sample scaling is improved. Spike-ins also face practical limitations similar to
666 mock-community calibration: the most accessible standards (synthetic DNA constructs or
667 extracted DNA) are typically added post-extraction, meaning they cannot capture extraction-
668 related loss or inhibitor effects. Furthermore, calculating absolute abundance using spike-ins
669 of synthetic DNA constructs or extracted DNA calibrates the data to DNA molecule numbers,
670 whereas the quantities of interest in many surveillance contexts are counts of individual
671 organisms or biomass, which still require additional information or modelling to link DNA
672 copies to organism abundance. Using biological spike-ins of physical specimens circumvents
673 many of these limitations, but they do not account for variation among samples due to
674 conditions encountered before reaching the lab, and care must be taken to ensure the species
675 is truly alien to the study environment Iwaszkiewicz-Eggebrecht et al. (2026).

676 An alternative to spike-ins is to measure total sample load directly—for example, by counting
677 the total number of individuals (or weighing total biomass) prior to processing—and then use
678 this value to rescale metabarcoding-derived read proportions to an absolute abundance scale
679 (see Box 2; Fig. 4D). While much of the existing quantitative metabarcoding literature has
680 focused on microbes, where counting individuals often requires specialised equipment such
681 as cell sorters (Jian et al. 2021), trap-based monitoring of invertebrates has a key advantage:
682 it provides a physical bulk sample that can be counted or weighed relatively easily, providing

683 an external reference for abundance and biomass before extraction—and, when using non-
684 destructive methods, potentially re-examined after sequencing as well. Therefore, obtaining
685 absolute abundance information for invertebrates can be as simple as counting individuals in
686 a mixed sample, or by measuring wet or dry mass for individuals, groups of morphologically
687 similar taxa, or the entire sample at once. Comparable approaches have been used in other
688 contexts, such as microscopy-derived counts and biomass estimates to scale metabarcoding
689 proportion in phytoplankton (Mikhailov et al. 2025). This approach has the benefit of
690 anchoring metabarcoding outputs to more relevant and interpretable units (e.g. counts or
691 biomass), but adding manual counting or weighing to processing workflows can add
692 substantial workload in large-scale programs processing hundreds of trap samples.

693 An alternate method for counting invertebrate samples involves image analysis, which can be
694 paired with machine learning to identify individuals ideally to species level, or at least to a
695 higher taxonomic rank (Høye et al. 2021, Tannous et al. 2023). Even without relying on
696 machine learning for species identification, there is clear potential for producing insect counts
697 per sample from images, then pairing it with species identification data generated from
698 metabarcoding, to produce an equivalent insect count/abundance per species per sample. This
699 could be incorporated into modern lab protocols by taking images of bulk samples or sticky
700 traps and applying an image segmentation process in software like ImageJ (Schneider et al.
701 2012) or more advanced segmentation models (i.e. Segment Everything Model (Kirillov et al.
702 2023)) to separate out and count individuals. In recent years, there has been an increase in the
703 use of traps equipped with cameras, which show promise for remote trap monitoring to
704 decrease user interaction and input into sampling efforts (López et al. 2012, Pegoraro et al.
705 2020, Preti et al. 2021). More recently, automated laboratory platforms such as the
706 DiversityScanner (Wührl et al. 2022) can extract and sort specimens from bulk samples, then
707 image and prepare them for DNA extraction; it is hoped that one day these devices will also

be able to automatically taxonomically identify invertebrates using pre-established barcodes associated with imagery (Valan et al. 2019, Ärje et al. 2020, Wührl et al. 2022, Meier et al. 2024). In the interim, a practical hybrid approach may be to combine imaging-derived counts or biomass estimates (Bereciartua-Pérez et al. 2022, Kargar et al. 2025) with metabarcoding-derived identifications and taxon proportions to determine approximate species abundances of invertebrates, however this is yet to be tested experimentally. This hybrid approach may be particularly useful for morphologically similar species that cannot be reliably differentiated from an image alone and requires genetic discrimination, which can be provided through metabarcoding analysis.

Abundance inferred from presence-absence data

In the absence of being able to measure abundance directly and reliably from metabarcoding read counts, abundance may instead be indirectly inferred from presence-absence data collected across a sufficient spatial and temporal scale of sampling. Taxa that are more abundant in the environment are generally expected to be detected in more sampling units (i.e. spatially distributed traps) and more consistently through time. This creates a pattern in which sites with frequent detections of a particular species can be inferred to have higher abundance than sites with only occasional detections. Ecological models such as the Royle-Nichols model and related approaches (Royle and Nichols 2003) formalise this concept by inferring abundance from repeated detection/non-detection data. Bush et al. (2023) demonstrates applying this concept in a metabarcoding context using a case-study of river macroinvertebrate monitoring, finding patterns of abundance across collection sites inferred using the Roye-Nichols model reflected both seasonal changes and predator exclusion when comparing caged and open collection sites. In a similar vein, Brandão-Dias et al. (2026) discussed the potential of using within-sample haplotype frequencies to estimate abundance from eDNA samples; however, thus far this method has only been tested with simulated data

and hinges heavily on the quality of the marker region selected and its capacity for measuring intraspecific variation across numerous species (if applied in a multi-species context). Although these approaches remain in their infancy, and rely on a number of assumptions about detection, dispersal, and population structure, they offer a promising way to extract ecologically meaningful abundance signals from metabarcoding datasets where read counts themselves are not quantitatively reliable. They may be particularly valuable for historical datasets in which no experimental corrections were included to account for the numerous methodological biases discussed in this review.

Conclusions

Throughout this review, we have demonstrated that transforming metabarcoding from a surveillance and biodiversity discovery tool into a reliable and statistically robust quantitative approach for invertebrate population monitoring within agriculture requires three complementary approaches: (i) optimising protocols to minimise the introduction of taxon-specific bias; (ii) incorporating calibration (e.g., mock communities or related approaches) to estimate and correct for residual taxonomic bias; and (iii) pairing metabarcoding with an external measurement (e.g., total counts, biomass, or marker copy number) to anchor relative read proportions to an absolute-abundance scale. Although substantial challenges remain in applying these approaches across the full diversity of species detected by metabarcoding, there is still clear value in achieving consistent abundance estimates for a small number of species, or even just one species, across multiple samples. Even where metabarcoding data is known to be biased, a growing number of studies indicate that these biases can remain sufficiently consistent to allow reliable inference of relative changes in abundance. This is especially relevant in agricultural systems, where monitoring efforts are often focussed on

particular groups of interest, where even moderate changes in relative abundance may be sufficient to inform management decisions.

The current time period marks an important transition point in this field, as metabarcoding becomes ubiquitous in invertebrate biomonitoring, as there is still much room for improvement for its adoption specifically within agricultural pest monitoring and management. While bias remains pervasive and is still inadequately addressed in many studies, awareness of these limitations is growing, and novel methods for identifying, quantifying, and correcting bias are being rapidly proposed and adopted. At the same time, alternative high-throughput monitoring technologies, including imaging, artificial intelligence, and smart traps, are developing quickly and can provide accurate insect counts, albeit often without the taxonomic resolution needed to distinguish cryptic or morphologically similar species. The future of quantitative invertebrate monitoring may therefore lie in the integration of multiple complementary approaches, combining the species-level resolution and biodiversity discovery potential of metabarcoding with the accurate counting of imaging-based methods. Realising this potential will require stronger collaboration across disciplines to address these methodological challenges and to develop robust monitoring systems for agriculture and beyond.

Data Availability

Not applicable

Abbreviations

16S: 16 Svedberg units; ASV: amplicon sequence variant; BSA: bovine serum albumin; CoDA: compositional data analysis; COI: *cytochrome oxidase* subunit I; CNV: copy-number variation; DADA2: Divisive Amplicon Denoising Algorithm 2; ddPCR: digital droplet PCR;

DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; eDNA: environmental DNA; GC: guanine-cytosine; HECTOR: parallel multistage homopolymer spectrum based error corrector; HTS: high-throughput sequencing; ISD: internal standard; LAMP: loop-mediated isothermal amplification; NUMT: Nuclear Mitochondrial Pseudogene; OTU: operational taxonomic unit; PCR; polymerase chain reaction; qPCR: quantitative PCR; rRNA: ribosomal ribonucleic acid; SPE: single-primer extension; UMI: unique molecular identifier.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

L.J.G. and A.M.P. conceptualised the manuscript. L.J.G. drafted the manuscript with input from J.S., F.M., M.J.B., B.C.R., and A.M.P. All authors read and approved the final manuscript.

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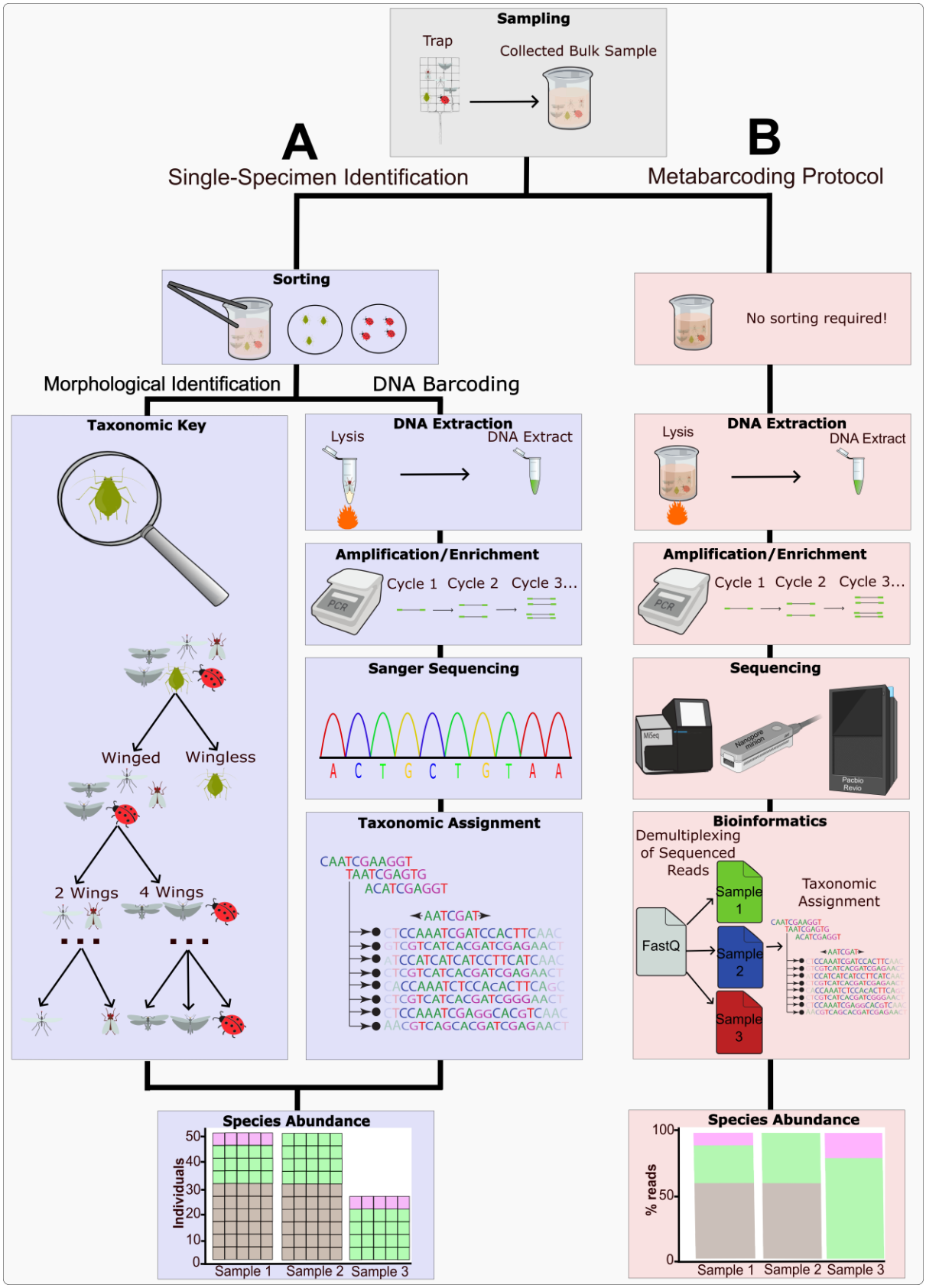

Sampling
Trap
Collected Bulk Sample
A
Single-Specimen Identification
B
Metabarcoding Protocol
Sorting
No sorting required!
Morphological Identification
DNA Barcoding
Taxonomic Key
Winged
Wingless
2 Wings
4 Wings
DNA Extraction
Lysis
DNA Extract
Amplification/Enrichment
PCR
Cycle 1
Cycle 2
Cycle 3...
Sanger Sequencing
A C T G C T G T A A
Taxonomic Assignment
CAATCGAAGGT
TAATCGAGTG
ACATCGAGGT
AATCGAT
Sequencing
Bioinformatics
Demultiplexing of Sequenced Reads
FastQ
Sample 1
Sample 2
Sample 3
Taxonomic Assignment
Species Abundance
Individuals
Sample 1
Sample 2
Sample 3
% reads

Figure 1. Representation of arthropod identification workflows using (A) traditional single-specimen identification and (B) High throughput DNA metabarcoding. Some images used in this figure were created in BioRender, Martoni, F. (2026) https://BioRender.com/k7xp1jp and https://BioRender.com/1byxv9q.

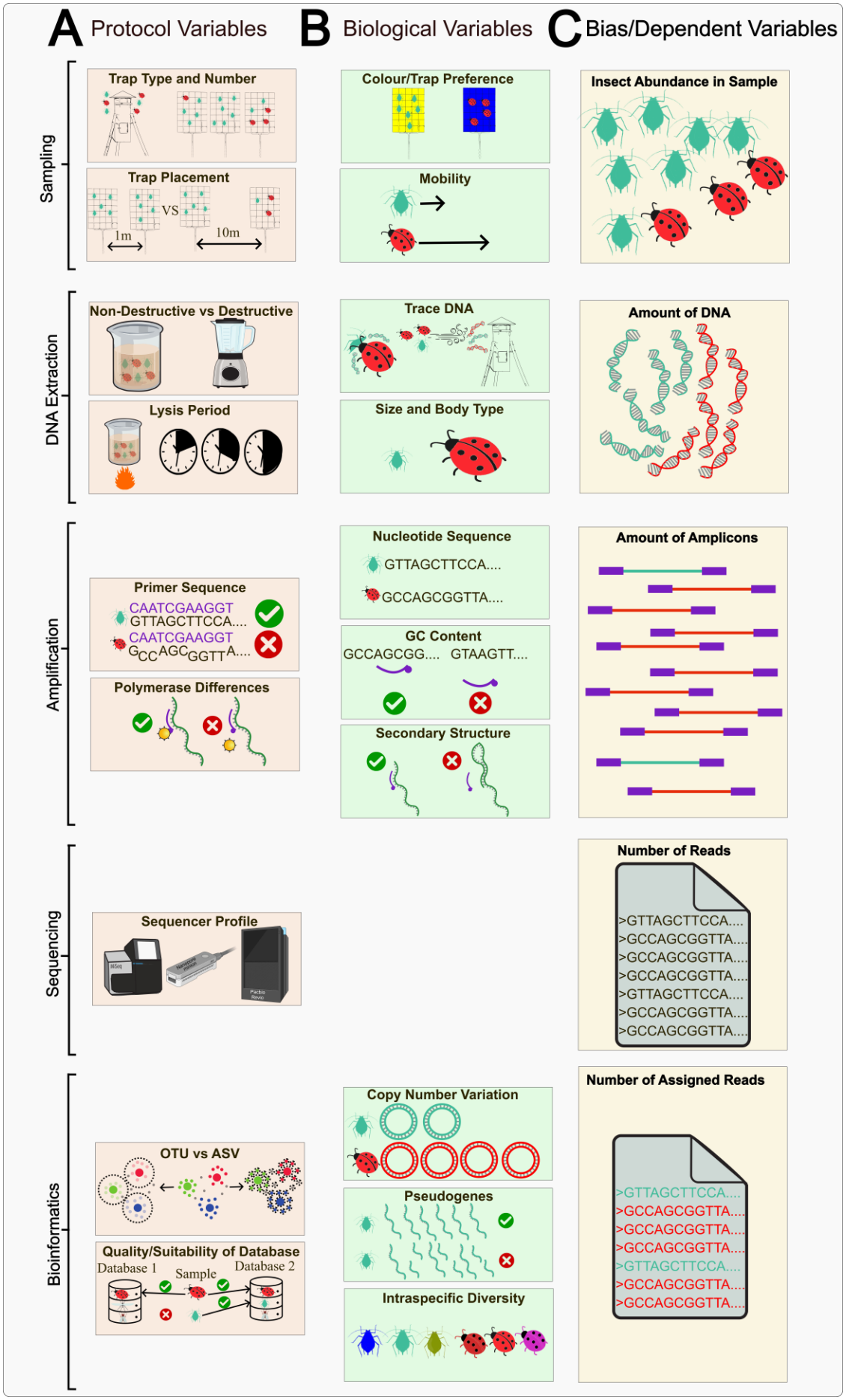

A Protocol Variables
B Biological Variables
C Bias/Dependent Variables
Sampling
DNA Extraction
Amplification
Sequencing
Bioinformatics
Trap Type and Number
Trap Placement
VS
1m
10m
Colour/Trap Preference
Mobility
Insect Abundance in Sample
Non-Destructive vs Destructive
Lysis Period
Trace DNA
Size and Body Type
Amount of DNA
Primer Sequence
CAATCGAAGGT
GTTAGCTTCCA....
CAATCGAAGGT
GCCAGCGGTTA....
Polymerase Differences
Nucleotide Sequence
GTTAGCTTCCA....
GCCAGCGGTTA....
GC Content
GCCAGCGG.... GTAAGTT....
Secondary Structure
Amount of Amplicons
Sequencer Profile
Number of Reads
>GTTAGCTTCCA....
>GCCAGCGGTTA....
>GCCAGCGGTTA....
>GCCAGCGGTTA....
>GTTAGCTTCCA....
>GCCAGCGGTTA....
>GCCAGCGGTTA....
OTU vs ASV
Quality/Suitability of Database
Database 1
Sample
Database 2
Copy Number Variation
Pseudogenes
Intraspecific Diversity
Number of Assigned Reads
>GTTAGCTTCCA....
>GCCAGCGGTTA....
>GCCAGCGGTTA....
>GCCAGCGGTTA....
>GTTAGCTTCCA....
>GCCAGCGGTTA....
>GCCAGCGGTTA....

Figure 2. The combinatory relationship between (A) protocol variables and (B) biological variables which produce (C) Bias. Some images used in this figure were created in BioRender, Martoni, F. (2026) https://BioRender.com/k7xp1jp and https://BioRender.com/1byxv9q.

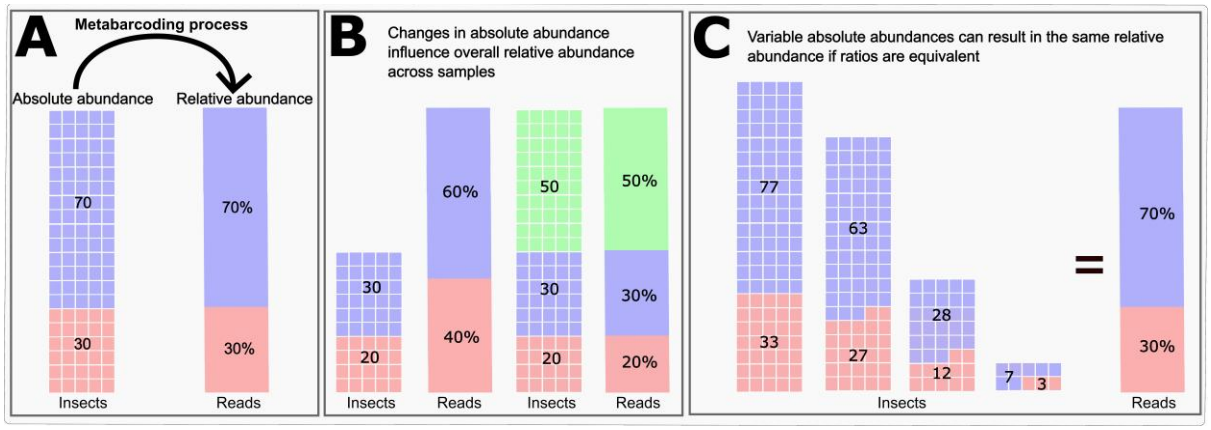
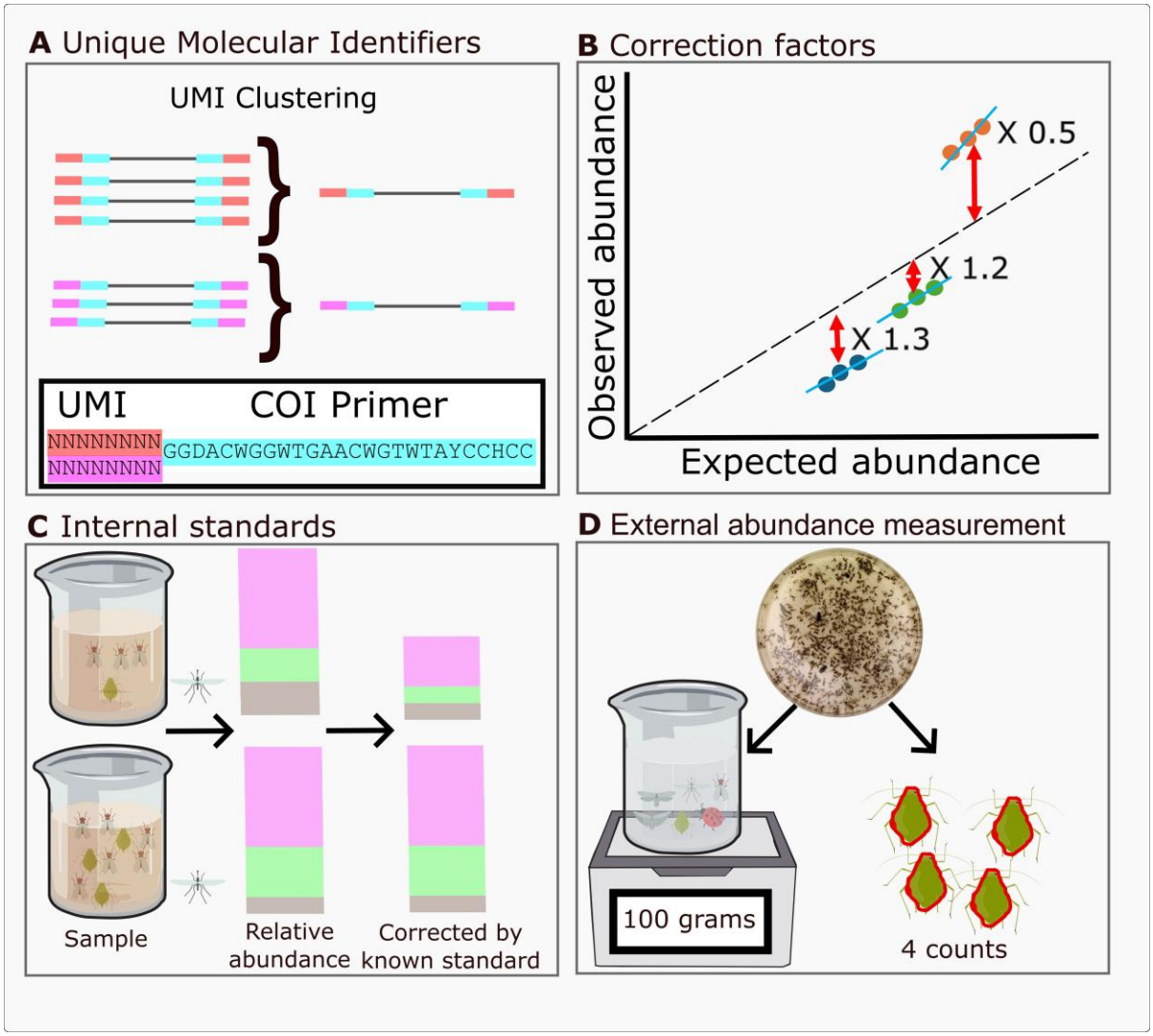


Figure 3. Difference between relative abundance and absolute abundance across the metabarcoding protocol and the resulting issues caused when trying to interpret relative abundance data. (A) The transition from absolute to relative abundance caused by metabarcoding, (B) a visualisation of how the total absolute abundance of a sample can cause the same quantity of insects to produce wildly different relative abundances, and (C) another consequence of constant sum constraint is the difficulty of relying purely on relative abundance to determine absolute abundance when multiple absolute abundances can produce the same relative abundance.



1473

1474 Figure 4. Potential avenues for mitigating quantitative bias. (A) Unique molecular identifiers
1475 to counteract amplification bias, (B) species-specific correction factors generated from mock
1476 communities to remove overall bias, (C) Including an internal standard in equal amounts to
1477 all samples to correct for cross-sample variation, and (D) an external measurement used to
1478 retrieve absolute abundance from relative abundance.

Step	Potential source of bias	Bias explanation	Potential mitigation
Sampling	Trap type	Difference in diversity of species collected (Yi et al. 2012).	Use trap types appropriate to research/surveillance question and target species. Factors to consider may include the substrate in which the target dwells (e.g. water, soil, air), which may vary depending on seasonality and/or biological cycle.
	Trap colour	Can attract or repel different groups of invertebrates (Williams 1973, Disney and Erzinclioglu 1982, Brødsgaard 1989, Dafni et al. 1990, Aguiar and Sharkov 1997, Leong and Thorp 1999, Kitching et al. 2001, Atakan and Canhilal 2004).	Choose trap colours appropriate for target species.

	Attractant used	Attractants (i.e. lures, light, pheromones, or bait) are designed to attract specific taxa, species and/or sexes (Greenslade and Greenslade 1971, Bell et al. 1972, Bowden and Church 1973, Riedl et al. 1976, Ballard and Gold 1982, Mullen 1992, Turchin and Odendaal 1996, Walker et al. 2003, Yi et al. 2012).	Use attractants appropriate for the diversity of target species and ensure that combinations of two or more attractants do not modify their characteristics.
	Trap placement and replication	Trap sampling is stochastic and depends on dispersal of target species, this can cause rare or short-dispersing species to be missed if not enough traps are deployed (Evans et al. 2016, Nguyen and Nansen 2018, Steinke et al. 2021).	Ensure number of trap replicates is sufficient to allow rare species capture, reaching the desired sampling effort for the required degree of sensitivity.

	Trap height	Different species disperse at different heights and may not be caught by traps not placed at these heights (Gillespie and Vernon 1990, Atakan and Canhilal 2004).	Consider dispersal behaviour of target species, which may be impacted by seasonality, environmental factors (e.g. strong winds), and biological cycle. Use multiple traps of varying height within a site if information is scarce or target species may be present at different heights.
DNA Extraction	Trace DNA	Predator invertebrates can be contaminated with DNA of prey species; airborne eDNA from invertebrates not physically present in the sample may be collected by traps (Toju and Baba 2018, Johnson et al. 2021, Ershova et al. 2023).	Retain specimens after non-destructive DNA extractions to allow assessment of the presence of species present in low read quantity.
	DNA extraction method	Different methods will have different levels of efficiency for extracting DNA from species based on physical characteristics (Schiebelhut et al. 2017, Marquina et al. 2022, Iwaszkiewicz-Eggebrecht et al. 2023).	Choose a standard method of extraction for all samples across a study, which works best for target species.

	Lysis period	Longer lysis periods can result in large-bodied invertebrates overwhelming samples with DNA, but shorter lysis periods can cause hard-bodied invertebrates to be underrepresented (Iwaszkiewicz-Eggebrecht et al. 2023).	Determine a lysis period that allows for invertebrates of variable size and body-type to be equally represented in a sample. Alternatively, invertebrates can be partitioned by size to prevent the impact of uneven extraction of DNA based on lysis length.
	Size and structure of organisms	Larger and softer-bodied invertebrates can release more DNA during extractions. These differences can also be present within species (e.g. sex-specific differences) (Martoni et al. 2022).	Partition specimens based on size and body type and process separately.
Amplification	Primer mismatch	Species with sequences that more closely match PCR primer sequences will be amplified better than other species (Deagle et al. 2014, Piñol et al. 2015, Ashfaq and Hebert 2016, Piper et al. 2019, Liu et al. 2023).	Design primers with degenerate bases to lower the chance of primer mismatches in target species.

	Polymerase	Certain polymerases can preferentially amplify species' sequences based on specific GC content (Beard et al. 2004, Johnson and Beese 2004, Dabney and Meyer 2012, Nichols et al. 2018).	Choose a polymerase that is better at amplifying a diversity of sequences. For example, Nichols et al. (2018) has shown some polymerases are less impacted by differences in GC content.
	Copy Number Variation (CNV)	Species that have a higher initial copy-number of the marker of interest will be present in a larger quantity within the final amplicon pool (Wiesner et al. 1992, Kembel et al. 2012, Angly et al. 2014, Krehenwinkel et al. 2017).	Account for CNV across species of interest in estimates of relative abundance in analysis. For nuclear genes, this may require counting copies in a high-quality genome assembly, while for mitochondrial genes this may require comparing ratios of mitochondrial-to-nuclear DNA (i.e. using qPCR, or WGS).
	DNA secondary structure	Sequences with a higher secondary structure energy can hinder primer annealing and have a lower amplification efficiency (Korvigo et al. 2022).	Adding BSA and DMSO to the PCR master mix can reduce secondary structure formation (Farell and Alexandre 2012).

	Homopoly-mers	Barcode sequences with homopolymeric regions may be incorrectly genotyped on some sequencing platforms, which can have disadvantageous carry-on effects during bioinformatic steps (Tedersoo et al. 2021).	When working with species with known homopolymeric regions within target barcode regions, design primers that avoid these regions. If homopolymeric regions are unavoidable, there are homopolymer error-correction methods, such as HECTOR (Wirawan et al. 2014), that can be included into bioinformatic analysis workflows.
Sequencing	Sequencer used	Quantity and quality of sequences produced by different sequencing platforms and kits can differ, impacting barcode sequence inference, taxonomic assignment and relative abundance estimates (Porazinska et al. 2010).	Standardise the sequencing platform used within each study. When this is not feasible, include the same control samples across different sequencing platforms to allow for detection of sequencer-specific batch effects.

Bioinformatics	Nuclear Mitochondrial Pseudogenes (NUMTs)	NUMT sequences can be taxonomically assigned as divergent, unidentified species, causing relative abundance measures to be impacted for certain species. They can also potentially be preferentially amplified over real sequences (Song et al. 2008, Wang et al. 2018, Hebert et al. 2023).	Use filtering metrics that remove sequences with frameshifts and premature stop codons (common in NUMTS and should not be present in protein coding genes). When targeting a non-coding gene region, high-quality nuclear and mitochondrial reference genomes may be required to ensure no paralogs or pseudogenes are being amplified .
	Sequence delineation (OTU vs. ASV)	Inappropriate metrics used for forming OTUs can cause closely related species to be combined into the same OTU (Pauvert et al. 2019, Fasolo et al. 2024).	Explore effects of varying OTU thresholds on results to ensure it is not biasing relative abundance, or analyse sequences as ASVs so only identical sequences are grouped together.
	Constant sum constraint	Sequence reads are proportional to the subset of DNA molecules that survive extraction, amplification, and sequencing, rather than to the absolute number of starting individuals (Gloor et al. 2017, Harrison et al. 2021, Shelton et al. 2023).	Use an external measurement of total sample abundance to differentiate relative abundances across samples, i.e. counts, biomass, or spike-ins.

	Bioinformatics pipeline	Different software algorithms and parameters can result in variation in the number and diversity of species detected across samples (Plummer et al. 2015).	Use a standardised pipeline and parameters across all samples to maintain consistency and avoid batch effects within an experiment.
	Reference database	Smaller databases with either a lack of representative sequences or inappropriate sequences can cause species to be either misidentified or not identified to species level (Nilsson et al. 2006, Mugnai et al. 2023).	Confirm all target species are included and differentiable based on the target region. Consider creating a custom database only from sequences from trusted sources. Additionally, if target species either do not have existing sequences available, or have not been properly validated, it may be necessary to generate new sequences from voucher specimens.

1479 Table 1. Potential sources of bias in each step of the metabarcoding workflow and methods for mitigation.