

Non-lethal rapid biodiversity assessment



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ABSTRACT

For several animal taxa, non-lethal techniques that do not rely on collecting individuals are routinely used to assess biodiversity (e.g. point counts in birds). Identification often relies on the ability of the observer, are subjected to errors, but populations are not impacted. Thus, multiple counting sessions (MCS) that allow using robust analyses (e.g. unbiased Chao richness estimate) are available. However, for most species (e.g. arthropods), trap systems must be set up. Killed individuals are collected and later accurately identified in the laboratory, but unbiased MCS become unavailable. Environmental DNA bar-coding provides an alternative, yet it requires important technical support and is not designed for MCS. Lethal rapid biodiversity assessments (RBA), derived from classical trap surveys and based on less accurate identifications (morphospecies are used), have been successfully developed to relax technical constraints. In this study, we combined non-lethal and RBA approaches to address logistical, analytical and ethical issues. We tested five versions of a protocol to visually survey the macro-fauna of hedgerows. A large number of individuals were directly identified in the field, mostly arthropods but also vertebrates. Identification error varied with taxonomic level and lineage, but remained low at the morphospecies level. Importantly, estimates tended to reach asymptotes, suggesting that local richness was appropriately appraised. Like any technique, non-lethal RBA (NL-RBA) present both advantages and weaknesses, and may improve the toolbox to survey biodiversity.

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1. Introduction

Most biodiversity surveys that focus on small organisms (e.g. Invertebrates) are based on lethal techniques (e.g. pitfall traps); large numbers of individuals are collected, generally killed, and later identified in the laboratory. This raises conservation and ethical concerns. Especially because most animal populations decline, including common invertebrate species (Biesmeijer et al., 2006); and because recent researches demonstrated that invertebrates are subjected to pain (Sneddon, 2004; Elwood, 2011; Magee and Elwood, 2013). Furthermore, important numbers of individuals belonging to non-targeted species are accidentally killed (by-catch) including protected species (Pearce et al., 2005). More constraining regulations that will include invertebrates are expected in the future. Testing the usefulness of alternative non-lethal (or less destructive) methods to sample biodiversity is thus timely.

Several efficient non-lethal techniques are routinely used: notably point counts and visual sampling to monitor birds, anurans, and several invertebrate species (Roy and Sparks, 2000). Because

successive surveys can be performed without taking-off individuals from the environment, these techniques permit to implement multiple counting sessions (MCS) and thus to take into account species detectability, time and observer heterogeneity (Williams et al., 2002). Multiple counting sessions are essential to obtain robust estimates of species richness or abundance. Unfortunately, these techniques are currently limited to conspicuous or easily sampled taxa and to particular periods: diurnal butterflies, birds, and anurans during the breeding season notably. They remain inappropriate for the far more diverse array of cryptic organisms represented by various insects, arachnids, crustaceans, annelids or vertebrates for instance.

Environmental DNA bar-coding is an alternate technique that considerably increased the efficiency and span of field surveys (Hebert et al., 2003; Hajibabaei et al., 2007; Bohmann et al., 2014). Although highly effective in identifying species from potentially any taxonomic group, this technique offers presence/absence information (i.e. mitochondrial CO1 gene is poorly variable at the species level). It cannot provide reliable estimates of individual numbers and does not provide information regarding individual status (e.g. body size). Environmental DNA sampling is also a non-lethal technique; species are detected by the trace of their DNA on environmental samples and thus disturbance is minimized

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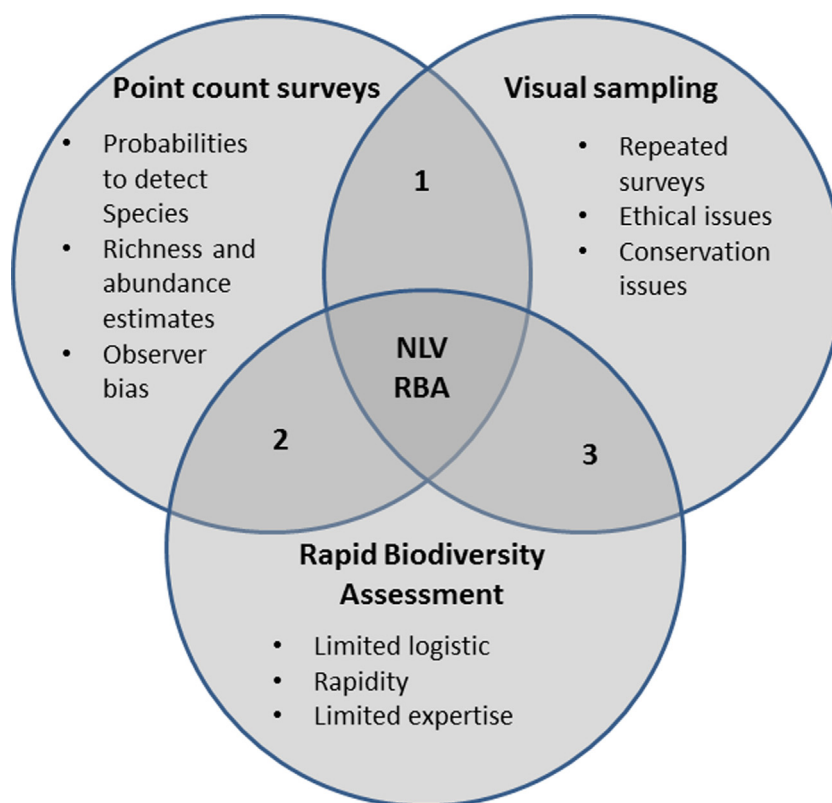


Fig. 1. Schematic position of non-lethal visual RBA (NL-RBA) relative to three approaches to survey biodiversity respectively represented with a circle. Several important features are listed. Point count surveys are limited to few taxa (e.g. birds, anurans); they allow multiple count sessions (MCS) to take into account probabilities of detection for instance. Visual samplings have been used on different taxa (e.g. butterflies), however not yet to survey cryptic terrestrial species; they do pose ethical problem. Rapid Biodiversity Assessments offer logistical advantages and are usually based on lethal trap sampling, especially to study cryptic species. Approaches are not exclusive: e.g. participative surveys (e.g. butterfly crowd survey) occupy the overlapping area #3. In this study we combined for the first time the characteristics of the three approaches, notably to survey cryptic species (central area) without capturing and killing individuals.

compared to other approaches. However, to have species names on environmental barcoding data reference, DNA barcoding library is required (Cristescu, 2014). It also entails important laboratory work, and thus necessitates substantial funds and access to relatively sophisticated technical resources. In addition, although vertebrate species are often accurately identified, name/species assignment is more problematical in other taxonomic groups (Funk and Omland, 2003; Meyer and Paulay, 2005). Thus, Environmental DNA bar-coding is currently considered as complementary to other classical approaches (DeSalle, 2006; Hajibabaei et al., 2007; Valentini et al., 2009). Using a given taxon as a surrogate to estimate other groups species richness is another alternative (Cardoso et al., 2004); but studies that incorporated a wide range of taxa (e.g. vertebrates, insects and plants) failed to support the surrogacy principle and suggested that multiple surveys are more reliable (Van Jaarsveld et al., 1998). Overall, there is no ideal sampling method to estimate biodiversity and lethal trapping is often inevitable for accurate identification of species.

Nonetheless, different studies demonstrated that, depending upon the question addressed, identification at the species level is not compulsory. Rapid biodiversity assessment (RBA) based on lethal trap systems but where individuals are assigned to morphospecies through rapid visual inspection have been successfully used in different taxa (Oliver and Beattie, 1993, 1996; Cardoso et al., 2004; Ward and Larivière, 2004; Biaggini et al., 2007; Obrist and Duelli, 2010; Braga et al., 2013). RBA are fundamentally less accurate in terms of taxonomic information compared to classical laboratory methods; they nonetheless provide useful data to picture biodiversity and they are considerably less constraining in terms of logistic. The major advantages of RBA are represented by

the low cost/efficiency ratio and the relatively low level of expertise required: it is usually easier to identify individuals at the family than at the species level.

Three other potential but currently untested advantages of RBA can be listed: (1) adopting non-lethal approach, notably to survey cryptic species; (2) performing wide taxonomic surveys, e.g. monitoring invertebrates and vertebrates; (3) implementing MCS to calculate unbiased richness estimates. In this study, we combined for the first time these three potential advantages (Fig. 1). We tested five different versions of a visual protocol to survey the macrofauna in the hedgerows of cultivated fields: this type of habitat is important for biodiversity but is subjected to strong anthropogenic pressures (Baudry and Jouin, 2003; Midgley, 2012; Hooper et al., 2005). We notably focused on cryptic species (i.e. species that are difficult to observe in the field due to their camouflage, secretive lifestyle, etc.). For instance, instead of (lethal) traps we used corrugated concrete slabs positioned in the field to attract and spot cryptic animals (Bonnet et al., 1999; Ballouard et al., 2013). We also explored natural refuges (e.g. stones) and performed classical visual transects using different walking speeds. In all cases, we relied on visual determinations directly in the field and without capture. Importantly, we did not focus specifically on a particular taxonomic segment of the fauna (e.g. spiders); instead we attempted to include a wide range of taxa. To examine the usefulness of this non-lethal approach, two main issues were assessed.

- (1) Does non-lethal RBA allows for the observation of sufficient numbers of morphospecies and individuals? Richness estimate analyses based on MCS were used to examine this issue.

- (2) What is the degree of accuracy when assigning individuals to a morphospecies? We used digital pictures and multiple assessments to evaluate this question.

We emphasize that our goal was not to examine the costs and benefits respectively associated with lethal versus non-lethal techniques (e.g. performing parallel sessions using barber traps vs visual sampling). These approaches are fundamentally different and cannot be directly compared: e.g. MCS and associated analyses are not possible using lethal technique whereas the taxonomic precision of lethal trap-sampling cannot be reached using crude visual identification. Our main objective was to test for the first time a non-lethal RBA technique, and to broaden the application of the point count approach, notably to survey cryptic species.

2. Material and methods

2.1. Study sites

We sampled hedgerows in two agro-ecosystems in central-western France, respectively situated near Chizé (CHZ, Deux-Sèvres 79-District; 46°06'59.4" N, 0°21'01.0" W) and near Dompierre-Sur-Mer (DSM, Charente 16-District; 46°10'34.6" N, 1°03'07.2" W). In both areas, fields are traditionally bordered by a network of hedgerows (i.e. forming a bocage landscape) with native trees and shrubs sometimes associated to small stone walls and/or earth banks. We selected 69 hedges (61 in CHZ and 8 in DSM) to encompass a wide gradient of hedge types and situations ranging from residual hedges (both vegetal cover and bank almost totally erased) to intact hedges with abundant vegetal cover and large banks. The study sites included different type of crops (meadow, fallow, corn, etc.), agricultural practices (e.g. in terms of pesticide uses, organic versus classical practices), or connectivity (dense hedge network versus isolated hedge). However, in the current study we did not aim to examine the consequences of this diversity; instead we focused on the potential usefulness of non-lethal RBA.

2.2. Non-lethal protocols

Five slightly different protocols were tested. Individuals were not collected but directly identified and/or photographed in the field. We also implemented a technique designed to study cryptic fauna such as reptiles for instance: corrugated slabs were placed in the field and regularly inspected (e.g. Bonnet et al., 1999). Three protocols (1–3) were relatively similar as they relied on visual searching of the fauna without exploring shelters; the two others (4–5) attempted to target more particularly sheltered individuals.

- (1) Rapid visual transect (RVT). The observer walked along the side of the hedge most exposed to the sun (i.e. south in our study area) and attempted to identify the animals observed. Walking speed was approximately set at 1 m s^{-1} on a standardized 40 m distance. This method was expected to target relatively conspicuous species, mobile and colourful animals, notably large flying insects (Dennis et al., 2006). The surface surveyed per hedge represented approximately 100 m^2 .
- (2) Slow visual transect (SVT). Walking speed was approximately set at 0.2 m s^{-1} (slow walking speed) on a standardized 20 m distance. The observer scrutinized the ground in front of him. In comparison to the RVT, this protocol was assumed to improve the observation of less conspicuous species. The respective total durations to run a RVT or a SVT were relatively similar but varied approximately from 1.5 to 2.5 min; fluctuations were caused by varying amount of times devoted to record and/or photography individuals.

- (3) Focal observation (FO). The observer stopped at a random point of the hedge, remained immobile during one minute and recorded all animals visible (i.e. point-count survey). When no animal was observed, the observation ceased (e.g. under cool windy conditions). When at least one animal was observed, the observation period was extended to five minutes.
- (4) Active searching (AS). The observer randomly selected a spot and actively explored the vegetation and shelters (e.g. lifting stones, small logs) during 1 min, corresponding approximately to 10 m of hedge examined. As above, when at least one animal was observed, searching was extended to five minutes. This protocol was designed to find individuals sheltered beneath stones, barks, or litter.
- (5) Corrugated slabs (CS). In each hedge, three fibrocement corrugated slabs ($1.25 \text{ m} \times 1.00 \text{ m}$, corrugation wave $15 \text{ cm} \times 10 \text{ cm}$) were placed using a 20 m interval. The slabs were placed in the hedgerows in winter at least 4 months prior to the beginning of the study (hence no vegetation developed under cover, as under natural shelters like stones). The slabs were lifted and the observer attempted to identify and count all the animals under cover. This protocol was specifically designed to observe a wide range of cryptic species, including small and relatively large organisms (e.g. woodlice to snakes). The total surface surveyed per hedge was approximately of 4 m^2 .

2.3. Taxonomic identification

Individuals were identified and assigned to a given morphospecies by the observers directly in the field to the finest taxonomic level possible. Rapid visual morphospecies identification was based on simple criteria: broad morphology, body size, colour, peculiarities. For various reasons, taxonomic accuracy varied greatly with lineages and/or development stages. For instance, as only one species of hedgehog (*Erinaceus europaeus*) occurs in our study area, and because this species cannot be confounded with another one, all individuals were accurately assigned at the species level. Similarly, many remarkable species belonging to different taxa were easily determined (e.g. *Iphiclides podalirius*, *Argiope bruennichi*, *Dysdera crocata*, all reptiles, all large mammals). Yet, in many cases individuals were described at a broader taxonomic level: for instance many spiders were identified at the family or genus level. In RBA these taxonomic complications are taken into account; for instance, an individual identified at the species or family level is assigned to a morphospecies (Oliver and Beattie, 1996).

In practice, most individuals were easily assigned to a morphospecies (see results), and individuals that could not be allocated into at least a broad taxonomic group (e.g. most small flying insects) were discarded from analyses. In case of uncertain assignment, the observer photographed the individual with a digital camera for later identification. A total of 5976 pictures were collected in the field (most individuals were pictured more than once). Identifications based on pictures were performed in the laboratory using different image sources such a field guides or Internet data bases (e.g. Jones et al., 1990; galerie-insecte.org/galerie; arachno.piwigo.com); a method employed in previous studies (Oliver and Beattie, 1993, 1996; Kerr et al., 2000; Cardoso et al., 2004; Biaggini et al., 2007). If the identification problem persisted, pictures (mainly spiders) were sent to specialists. Henceforth, individuals not easily identified in the field were referenced as problematic morphospecies.

The visual method we used was potentially subjected to important identification mistakes. In order to limit identification errors, each observer could use in the field a set of colour plates with the most common morphospecies found in our study area. In addition, to better gauge identification error rate, we randomly pictured 100 individuals in the field and compared the identification

provided by the observer in the field against careful photo-identification performed independently in the laboratory by well-trained observers. To assess more specifically the identification error of the problematic morphospecies, we performed three independent identifications on 456 randomly selected photographs.

Visual identification of morphospecies relied on the agility of the observers. Eight different observers were involved in the surveys. Only one was experienced at the beginning of the study (e.g. senior author); therefore the other observers received a 4-days training before field surveys.

2.4. Searching effort

Field sessions ($N=92$) were carried out during the day, from March to September (2011 and 2012), at air temperatures ranging between 15 °C and 32 °C but not during rainfalls. The five protocols were run in a random order in each hedge and during each field session (i.e. all types of protocols were run on the same days). On average 2–3 people were involved during each survey, and each observer participated to 21 days of field work. Cumulated field effort represented 226 days (days \times observers) representing a total of 550 h of survey. Each protocol was tested between 5 and 11 times in each hedgerow (average = 6.95 ± 0.74 times). In total, the observers surveyed 2760 m of hedgerow during rapid visual transects and 1380 m during slow visual transects; focal observations required 34 h of field work; active searching required 36.5 h; finally 201 slabs [two hedge were not fitted with slabs] were inspected seven times (average = 7.44 ± 0.16 times).

2.5. Richness estimates

Because successive surveys were performed in each hedge without collecting (i.e. removing) individuals, we compared the biodiversity sampled by each protocol with the unbiased variant, Chao 1 richness estimators notably (Colwell & Coddington, 1994). Chao estimator can take into account the fact that different morphospecies have different probabilities of being observed (e.g. rare versus common species), and that many remain unseen or unidentified during surveys. Consequently, Chao estimator tends to provide higher species numbers than a raw count of morphospecies. We also provided cumulative raw counts and performed rarefaction analyses for comparisons and because they are widely used estimators. Although rarefaction analyses are particularly useful for comparisons (they tend to reach rapidly asymptotes), they should be employed with caution because their application relies on generally unverified assumptions (e.g. random spatial distribution of individuals), and thus they tend to produce markedly skewed curves. These issues were out of the scope of the current study however; indeed we did not aim to perform comparisons among habitats or techniques for instance, but we focused on the possible utilization of non-lethal RBA as a novel sampling method. Richness estimates were performed using Vegan package (Oksanen et al., 2012).

Several individuals may have been counted more than once. Our protocols do not permit taking into account such effect (like most point-counts). However, this effect was likely limited because the time elapsed between two surveys in a given hedge was generally greater than one month. Thus Chao richness estimates provided information essentially associated with the detectability of each morphospecies over time rather than individual detectability.

2.6. Proportion of predators

Predators are considered as useful bio-indicators (Burger, 2006; Sergio et al., 2009); therefore we examined the proportion of

predators. We also considered the proportion of vertebrate predators because they represent the largest top predators; and hence they provide a crude integrative index of the underlying trophic levels.

Analyses were performed with R (R Development Core Team, 2012).

3. Results

3.1. Numbers of individuals and morphospecies

A total of 62,382 observations (possibly a smaller number of individuals) were observed and assigned to 521 morphospecies. Most of the morphospecies and individuals sampled were arthropods (89.4%) with an overwhelming proportion of insects and spiders (Fig. 2). Insects provided 31,722 observations (50.9% of the total) and 388 morphospecies (74.3% of the total); spiders provided 8382 observations (13.4%) and 63 morphospecies (6.9%). Vertebrates provided 1318 observations (2.1%) and 40 morphospecies (7.7%). For several other lineages (e.g. annelids) few observations were recorded and therefore they were not considered.

3.2. Taxonomic accuracy and identification errors

The efficiency and taxonomic accuracy associated with morphospecies assignment were variable. Most of the individuals observed were easily assigned to a morphospecies ($N=59,469$; 95.4%); the number of problematical individuals that required picture identification in the laboratory was thus relatively low ($N=2902$; 4.6%). Among easily identified morphospecies, 44.5% of the individuals were assigned to a species, 23.1% were identified at the genus level, 28.0% at the family level, and 3.7% at the order level. A small proportion of individuals (0.7%) could only be assigned to a very broad group (e.g. 'spiders').

Consequently, in this study, rapid visual identifications of morphospecies performed in the field included first a large proportion of individuals assigned to a species (42.4% [0.954×0.445]), then lower proportions of individuals assigned to a genus (22.0%) or to a family (26.7%), and finally a small proportion of individuals assigned to an order (3.5%). The taxonomic accuracy of the morphospecies ranged essentially from species to family levels (~90% of the observations).

Among easily identified morphospecies, identifications performed directly in the field versus in the laboratory using randomly selected pictures ($N=100$) revealed that 6% of the identifications were incorrect at the species level. Error rate decreased with broader taxonomic accuracy: 0% of the genus or family identifications were incorrect. In the problematic group specifically, the three independent identifications revealed substantial error rate: 33.3% at the species level, 11.4% at the genus, 4.4% at the family, and 0.7% at the order levels (+0.4% undetermined). However, these errors impacted only a small proportion of the total number of individuals sampled (<5%).

Overall, taking into account both easily identified and problematical morphospecies, the identification error rate associated with the assignment of individuals to a morphospecies was modest (0.6%). This low error rate reflects the fact that the observers did not overestimate their discrimination capacity. For example they prudently determined less than 50% of the individuals at the species level and often retained genus or family levels to assign individuals to a given morphospecies.

3.3. Richness estimates

Cumulative counts of morphospecies, either using estimates (Chao 1 richness estimators; rarefaction analyses) or raw counts

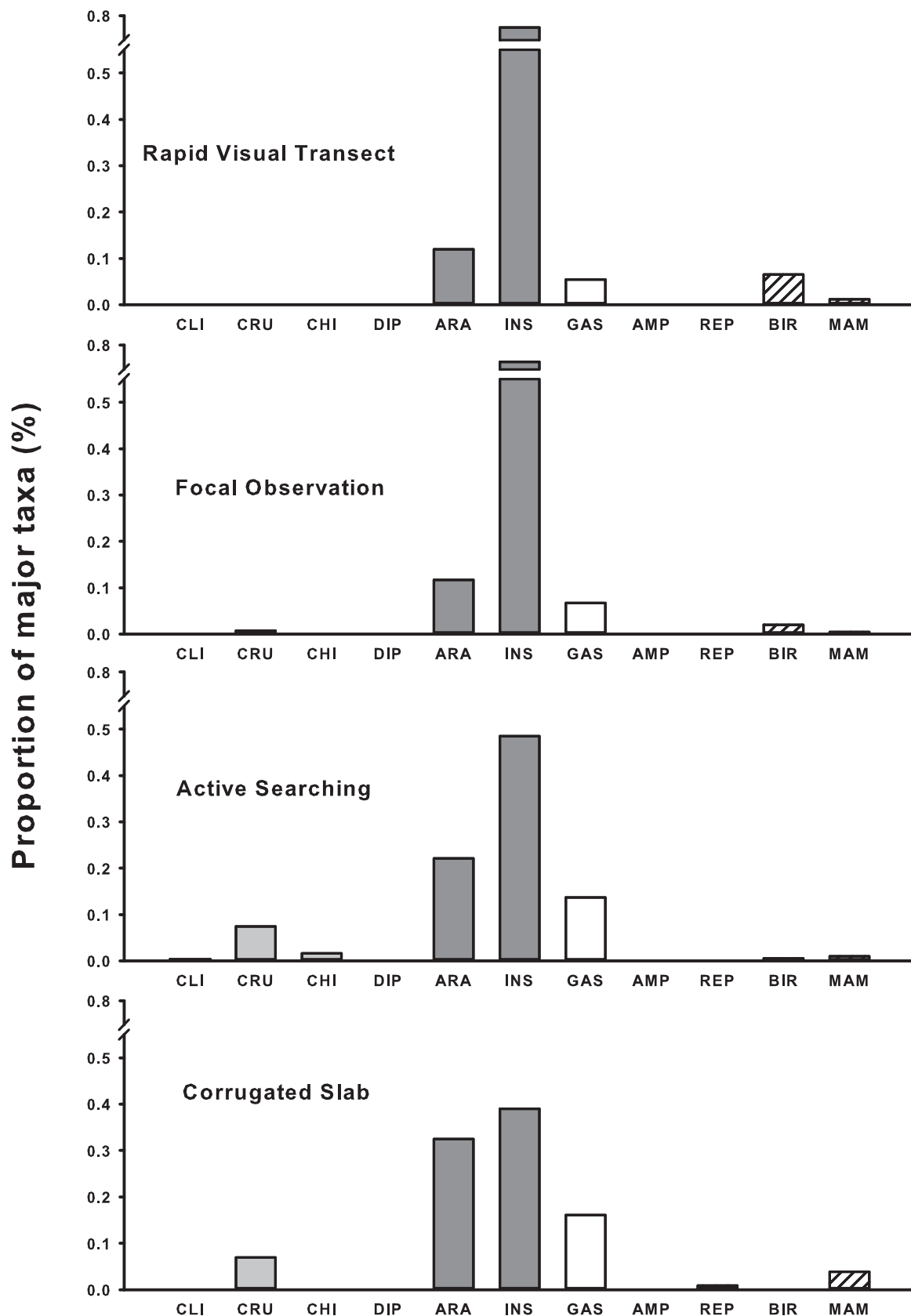


Fig. 2. Proportion of individuals sampled in major taxonomic groups observed in hedgerows using 4 protocols (see text for details; SVT data were very similar to RVT and hence are not displayed). CLI=Clitellata; CRU=Crustaceans; CHI=Chilopoda; DIP=Diplopoda (all these groups are indicated with light grey bars); ARA=Arachnids (dark grey bars); INS=Insects (dark grey bars); GAS=Gastropoda (white bars); AMP=Amphibians; REP=Reptiles; BIR=Birds; MAM=Mammals (all vertebrates are indicated with hatched bars).

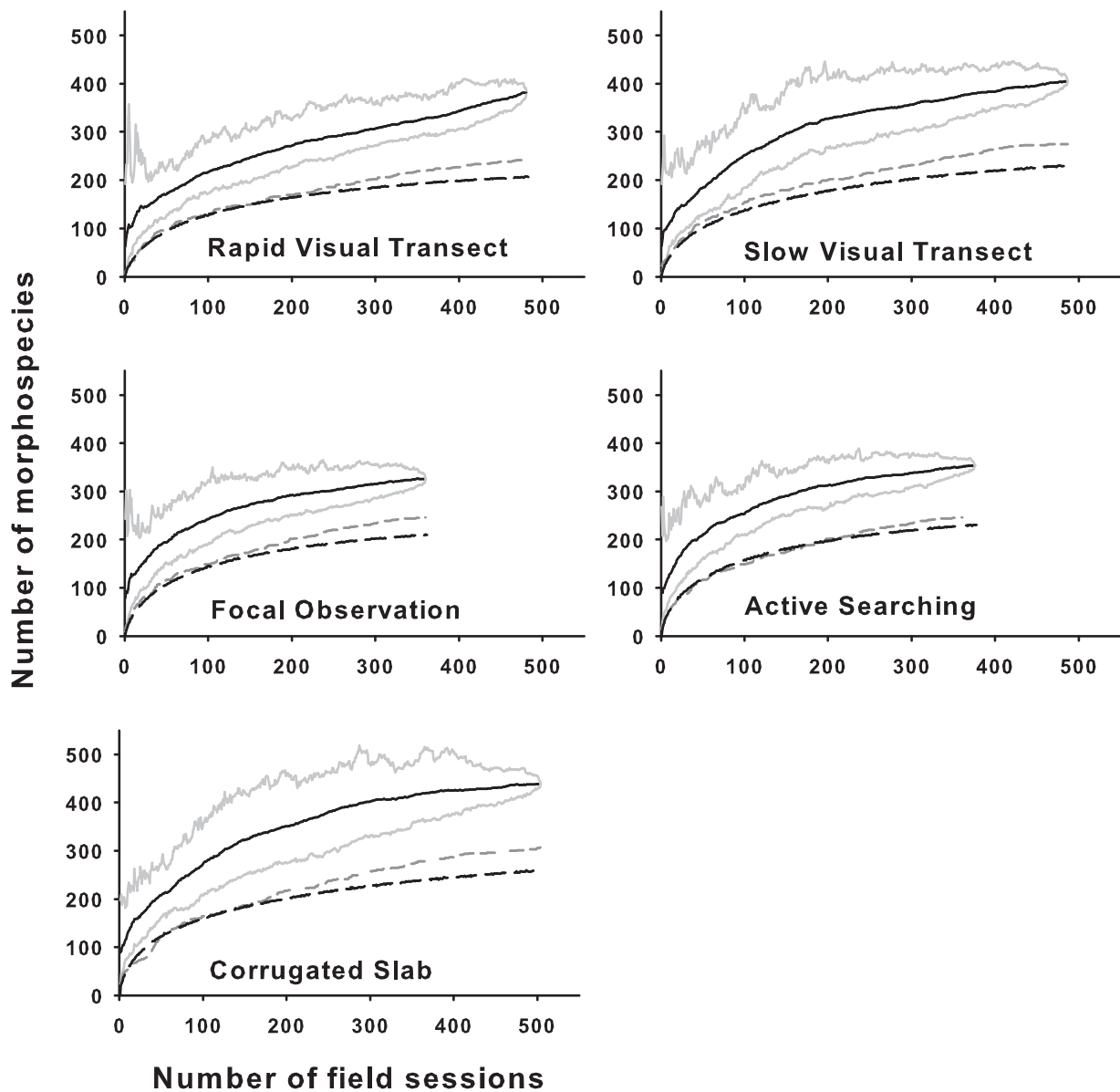


Fig. 3. Morphospecies richness (Y-axis) as a function of increasing sampling effort (X-axis, number of surveys) was analyzed using three methods: (1) raw counts, (2) rarefaction analyses, and (3) unbiased Chao 1 unbiased estimates. Raw counts are indicated with grey dashed lines; rarefaction analyses with black dashed lines; Chao estimates and 95% confidence intervals with continuous black and grey lines. Five visual protocols were tested (see text for details) corresponding to the five panels. Three protocols (RVT, SVT, FO) targeted animals visible in the open. Two protocols (AS, CS) targeted cryptic species that typically remain sheltered under stones or logs. Sampling effort represents the cumulative number of sessions (i.e. MCS) for each protocol performed in the 69 hedgerows surveyed in the course of the two years study.

tended to follow asymptotic trajectories with increasing sampling effort (Fig. 3). Raw counts and rarefaction analyses provided very similar trends both in terms of curve shape and values (e.g. asymptotic values); yet, rarefaction analyses tend to slightly underestimate specific richness. Although the five protocols yielded relatively similar results, several differences were detected. Focusing on unbiased Chao estimates (thereby taking into account the probability to detect rare morphospecies and considering confidence intervals), the specific richness provided by the RVT did not reach a clear plateau (Fig. 3). For the other protocols, SVT, FO, AS and CS, specific richness curves tended to follow asymptotes (Fig. 3). CS provided the highest estimated number of morphospecies (438) and the highest estimated maximal value (>500). This later value was close to the actual absolute total number of morphospecies observed combining the results from the five protocols ($N=521$). As expected, raw counts and rarefaction analyses provided lower

values. For example using CS, an absolute number of 309 morphospecies were counted.

3.4. Proportion of predators

Morphospecies belonging to three taxa that are exclusively or mostly represented by predators (i.e. spiders, Carabids, reptiles) were regularly observed (Table 1, Fig. 2) and provided most of the predator observations (51.1%). Various morphospecies from diverse taxa (e.g. Reduviidae bugs; centipedes; birds) provided the rest of predator observations. The overall proportion of predators sampled by each protocol varied significantly ($\chi^2 = 18.07$, $df = 4$, $P < 0.001$). Samples collected using protocols based on visual transect and focal observation contained approximately 27.6% of predators (respectively 25.7% for RVT, 29.8% for SVT, and 26.9%

Table 1
Comparison between the mean numbers of species identified and mean numbers of individuals counted, in randomly selected published studies versus current study (range indicated in brackets). In the current study, morphospecies were used whereas other studies used species. The taxonomic groups retained in this table correspond to the most commonly arthropods sampled using barber traps; notably spiders, several major insect groups (e.g. Apioidea; Hemiptera), and arthropods as whole. Sources: 1: [Dippenaar-Schoeman et al. \(2009\)](#), 2: [Duelli et al. \(1999\)](#), 3: [Galle et al. \(2010\)](#), 4: [Richman and O'Keefe \(2012\)](#), 5: [Banaszak-Cibicka and Zmihorski \(2011\)](#), 6: [Hostetler and McIntyre \(2001\)](#), 7: [Ngo et al. \(2013\)](#), 8: [Patin and Michez \(2007\)](#), 9: [Koivula \(2002\)](#), 10: [Richard \(2004\)](#), 11: [Azeria et al. \(2012\)](#), 12: [Magagula \(2003\)](#), 13: [Wardhaugh et al. \(2012\)](#), 14: [Heiden et al. \(2012\)](#), 15: [Laamari et al. \(2009\)](#), 16: [Fauvel \(1999\)](#), 17: [Khruleva and Vinokurov \(2007\)](#), 18: [Bambaradeniya and Edirisinghe \(2008\)](#), 19: [Bird et al. \(2000\)](#), 20: [Haddad et al. \(2009\)](#), 21: [Perfecto et al. \(1997\)](#), 22: [Rataux et al. \(2011\)](#), 23: [Rudgers and Clay \(2008\)](#), 24: [Symstad et al. \(2000\)](#).

Taxonomic group	Mean number of morphospecies identified		Mean number of individuals counted		Source
	Other studies	This study	Other studies	This study	
Araneae	133.3 (20–412)	64	7518 (1599–16,951)	8382	1–4
Apoidea	97.2 (54–164)	9	1553.2 (677–1871)	2201	5–8
Carabidae	57.6 (25–108)	42	5351.8 (330–17,720)	502	2, 9, 10
Coleoptera	116.7 (35–372)	111	23,366.3 (320–81,171)	3365	4, 11–13
Hemiptera	58.6 (18–120)	82	449.5 (223–667)	3607	4, 14, 15
Heteroptera	99.2 (29–225)	72	3135 (893–5389)	2097	3, 16, 17
Arthropod	406 (49–789)	467	40,778 (901–112,238)	46,690	4, 18–24

for FO). The two other protocols sampled greater proportions of predators: 35.4% for AS and 42.4% for CS.

3.5. Proportion of vertebrate predators

The proportion of vertebrate predators (essentially reptiles and shrews; 68.4% of the observations and 42.1% of the predator morphospecies) sampled using different protocols varied significantly ($\chi^2 = 13.39$, $df = 4$, $P < 0.001$). Samples collected using protocols based on visual transect and focal observation contained approximately 38.5% of vertebrate predators (respectively 34.3% for RVT, 44.8% for SVT, and 36.5% for FO). The two other protocols sampled a greater proportion of vertebrate predators: 70.7% for AS and 51.4% for CS.

4. Discussion

This study reports the first attempt to use non-lethal RBA, notably to survey various cryptic species of the terrestrial macrofauna that are generally sampled with lethal techniques. Crude rapid visual identifications associated with RBA (lethal or not) present both advantages and limitations; inevitably our results reflect this duality.

The first interest of RBA is to rapidly collect large sample size ([Oliver and Beattie, 1993, 1996](#)). Because, most tasks are rapidly performed directly in the field, the total amount of time required to “process” individuals was considerably lower compared to classical surveys that involve many steps in the field and in laboratory: notably collecting trap content, cleaning traps, replacing chemicals, transport and storage of samples to the laboratory, sorting targeted taxa, carefully examining each individual, placing identified items into new vials + tags. Published studies do not provide mean values regarding the total amount of time necessary to process individual (field + laboratory work) precluding comparison with RBA. In the current study, approximately 100 individuals (observations) were identified and recorded per hour (including the small proportion of problematical individuals that required additional laboratory work). Surveys were rapidly performed with limited logistical support; more than 60,000 observations were collected. Several groups of arthropods have been frequently sampled in previous studies (e.g. Carabids; [Pearce and Venier, 2006](#)), providing a fulcrum for a crude indirect comparison with the current study, limited to the order of magnitude in terms of sample sizes ([Table 1](#)). Non-lethal RBA enabled us to observe 467 arthropods morphospecies versus 406 species on average (range 49–789) using other inventory techniques. We collected 46,690 observations (possibly less individuals) versus 40,778 individuals on average in previous studies (range 901–112,238). Although this comparison is

limited (morphospecies are not identical to species), it suggests that non-lethal RBA permit to collect reasonable amount of information.

The second interest of the non-lethal RBA tested was to collect information on individuals belonging to a wide range of taxa, including various invertebrate and vertebrate morphospecies; thereby providing a multi-group assessment ([Van Jaarsveld et al., 1998](#)). Combining five protocols, approximately 500 morphospecies were observed; essentially insects and spiders identified at various taxonomic levels, but cryptic vertebrates (e.g. snakes) and various other invertebrates (e.g. myriapods) were also regularly recorded.

However, to be of some utility, the two advantages presented above must be examined in the light of the inherent limitations associated with the use of morphospecies. In the current study, most morphospecies observations could be associated to a species, and the taxonomic accuracy remained relatively narrow, ranging from species to family levels. Nonetheless, the term morphospecies has little taxonomic accuracy (i.e. species, genus and family are considered at the same level) and thus cannot be employed to obtain lists of species. Because biodiversity assessments do not necessarily rely on the acquisition of comprehensive lists of species, RBA have been successfully tested and used on different taxa; including in field studies using a very broad taxonomic level ([Cardoso et al., 2004](#); [Biaggini et al., 2007](#); [Braga et al., 2013](#)). Reviewing the legitimacy of the morphospecies concept to perform surveys is out of scope of this study (see [Oliver & Beattie, 1993, 1996](#)). Instead, we examined a generally overlooked question: identification error rate. Random comparisons using photographs and repeated identifications suggested that identification error rate was low, notably at the family and/or genus levels; likely because it is relatively easy to discriminate individuals of the macro-fauna at these taxonomic levels ([Biaggini et al., 2007](#); [Gaston and Williams, 1993](#)). Identification error rate relied on the assumption that easily identified individuals were correctly assigned to a morphospecies. This assumption could not be fully verified because individuals were not collected (no voucher specimen available) although many were photographed and identified repeatedly. However, we note that repeated identification of collected/observed individuals (even preserved in alcohol) is usually not performed and thus error values were not available (not estimated/or reported) in previous studies. Interestingly, point count surveys can take into account identification error; thus, a possible improvement for non-lethal RBA that permit MCS would be to set up protocols with two (or more) observers ([Williams et al., 2002](#)).

Another limitation of using morphospecies as surrogate of species is that it automatically underestimates actual species numbers, especially in the richest taxa that require careful laboratory

inspection (e.g. most Coleoptera and spiders). For example, in the current study, 64 spider morphospecies were identified whereas the mean number of spider species identified in previous studies was 133 (Table 1). On the other hand, the number of bug morphospecies (order Hemiptera) observed ($N=82$) was greater compared to the mean number found in published studies ($N\sim 59$); likely because most bugs are relatively slow moving and easily characterized at the species or genus levels through visual inspection. More generally, the protocols tested did not permit to survey very small species and most insects when flying, thereby missing several major groups. Thus, an unknown proportion of key and threatened species is overlooked. Further, our sampling targeted individuals visible during daytime. We also ignored many inconspicuous animals like terricolous or xylophagous species. However no survey technique is immune from major biases: for instance Barber pitfall traps are not designed for repeated surveys, but they are also inappropriate to survey flying insects, and spiders sitting in their web, and identifying species on larval stages is often complicated.

The taxonomic limitation of RBA was partly compensated in our study by the non-lethal component of the protocols that allowed us to encompass a wide array of taxa. In the current study, sampling simultaneously most of the reptilian diversity (only one semi-aquatic snake species present in the surveyed area was not observed) with various invertebrate taxa as recommended for multi-group biodiversity assessments was useful to obtain a gross picture of the biodiversity (Haila and Margules, 1996; Van Jaarsveld et al., 1998). Mixing in the analyses morphospecies belonging to a wide taxonomic range (e.g. insect versus mammals) may generate spurious results because the respective taxonomic diversity and life history traits of arthropods and vertebrates are not easily comparable. This issue was not a major problem in our study as Chao estimator take into account detectability and abundance biases. Further, sampling a wide range of lineages does not mean that all species must be retained simultaneously in a single analysis. For instance, reptiles and spiders could be treated separately providing multiple parallel assessments (Van Jaarsveld et al., 1998).

Importantly, the light logistic and non-lethal approach permitted to perform repeated sampling (MCS) and to obtain asymptotic curves. Asymptotes are desired in biodiversity surveys, but they are rarely obtained. Because the five protocols were run simultaneously and all provided asymptotic cumulative trajectories, it was possible to compare richness outputs straightforwardly. Focal observation (FO) and transects (RVT & SVT) derived from classical protocols (Laake et al., 2004) mostly allowed identifying conspicuous organisms such as diurnal butterflies, wolf spiders or mammals for example. They provided similar values in terms of specific richness (~ 250 morphospecies counted; ~ 300 to ~ 400 morphospecies estimated). RVT included morphospecies transitorily present in the hedges (e.g. roe-deer, raptors) but that actually use other habitats (e.g. nearby forests); richness estimators failed to clearly reach a plateau (Fig. 3). Two less classical protocols, AS and CS, were more efficient to detect cryptic species. They notably allowed observing well sheltered species (e.g. under stones) and revealed an important proportion of important top predators belonging to a wide range of species and body sizes. In addition, many micro-mammals (e.g. voles, wood-mice; 95.2% of mammalian observations, 36.4% of mammalian morphospecies) were observed under slabs (Fig. 2). Lifting slabs (CS) was a standardized, fast, and precise method. This method allowed us to detect particularly secretive top predators (i.e. snakes, many spiders). Identifications were facilitated through slab lifting because individuals generally remained motionless for a brief period before fleeing away. It surveys small areas (25 times smaller compared to RVT); thus slabs placed in precise habitats are well-designed to setup occupancy modelling studies (Nichols et al., 2007).

5. Conclusions

We acknowledge non-lethal RBA (NL-RBA) approach is in its very early stages of development; many validation and improvements are required. For instance we doubt that NL-RBA can be easily applied in species-rich contexts (e.g., tropical and subtropical forests) where the taxonomic richness is very high but where the number of individuals observed per species is low. Further, it is difficult to identify rapidly morphospecies, especially for a neophyte, in such rich contexts. However, NL-RBA offers several promises. The taxonomic diversity sampled partly compensates for the lack of taxonomic accuracy and provides a wide (although biased) picture of the biodiversity that can be used for comparative studies regarding habitats or the impact of anthropogenic disturbances for example. Our results suggest that various cryptic animals can be sampled, especially important predators (e.g. reptiles). Moreover MCS can be implemented, enabling to rapidly accumulate large samples, to obtain asymptotic curves, and to use robust analyses (Williams et al., 2002). Observing and picturing animals through non-lethal RBA do not require specific permits, is likely to be approved by ethic committees and by the public. Non-lethal RBA can thus be used for educational purposes and in strictly protected areas. There is no comprehensive or ideal technique to assess biodiversity; non-lethal RBA may improve the toolbox to survey biodiversity, at least to address specific questions and without worrying about ethical issues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecolind.2015.06.004>

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