Effect of species-counting protocols and the spatial distribution of effort on rarefaction curves in relation to decision making in environmental-impact assessments

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Abstract Rarefaction Curves are frequently used in Environmental Impact Assessments to evaluate sampling sufficiency, but without clear guidelines of how to ensure that the assumptions of the methods are met. Infrastructure projects in the Brazilian Amazon and elsewhere often occupy extensive areas in remote locations with difficult access, and random sampling under such conditions is impractical. We tested the influence of sampling unit (sample or individual), and geographic distance between samples on rarefaction curve s, and evaluated the magnitude of errors resulting from the misuse of rarefaction curve in decision making, using frogs from four Amazonian sampling sites. Individual-based rarefaction curve were steeper than those generated by sample-based rarefaction curve. Geographic distance influenced the number of exclusive species in a predictable fashion only in one area, and not in the Environmental Impact Assessment site. Misuse of rarefaction curve generated large errors in the identification of vulnerable taxa. Because the rarefaction curve model is sensitive to the assumption of randomness and geographic distance can influence it unpredictably, we suggest that rarefaction curve should generally not be used to estimate sample completeness when making management decisions for environmental licensing purposes.

Key words: decision making, environmental impact assessment, environmental legislation, species rarefaction curve, species richness.

INTRODUCTION

A rarefaction curve is a model for estimating the number of species in any assembly defined in time and space. Rarefaction curves are used to predict the relationship between sample effort and species to be found, and it has been suggested that they should be used as criteria to determine sampling sufficiency in Environmental Impact Assessments involving diverse taxonomic groups, such as lizards in Australia (Thompson 2007; Thompson et al. 2007), troglobitic species in Europe (Trajano 2010) and fish in Amazon streams (Hambler & Canney 2013). Samples are considered sufficient when a predetermined proportion of the species estimated to be present is reached (Thompson et al. 2007), and the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), the Brazilian environmental agency responsible for licensing large civil-construction projects, began requiring rarefaction curve in Environmental Impact Assessments from 2007 (IBAMA 2007). However, there are assumptions for the valid use of rarefaction curve to estimate sampling adequacy that are unlikely to be met in Environmental Impact Assessments. As the legal requirements do not define the spatial arrangement of sampling, they do not guarantee that estimates of sampling sufficiency are realistic, and we show in this paper that their use can strongly bias management decisions.

The species accumulation curve describes the relationship between the number of species detected and their sequential accumulation via some unit of effort. This can be defined in terms of the number of individuals, or sample units that can be spatial (e.g. number of plots) or temporal (e.g. number of hours of observation, collection days) (Colwell & Coddington 1994). The shape of the species-accumulation curve changes depending on how the accumulated effort is ordered, and potentially the same group of samples can have several species-accumulation curves. A single descriptor of the relationship between the number of species and the accumulation of effort for a group of samples or individuals can be obtained with rarefaction curves (Magurran 2004).

Thompson (2007) suggested that the Australian Environmental Protection Authority adopt an rarefaction curve-based criterion of sample sufficiency using

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the number of individuals as the unit of effort. Similarly, Environmental Impact Assessments in Brazil have estimated the number of species with individual-based protocols (Eletrobras et al., 2009 – vol 14, p. 500). However, a key premise for the use of rarefaction curves is that the sample effort is applied consistently within the area of interest and that each data value is independent. In general, data independence can only be assured by random sampling, but if the starting position of a regular grid is chosen randomly, the resulting sampling array can effectively be considered random in most cases (Williams et al. 2002). In general, during fieldwork for environmental assessments, individuals are sampled collectively in samples and are not encountered randomly or individually in systematic sampling. Because it is often more effective to collect multiple individuals in sampling units (hereafter referred to as sample-based collecting), many authors recommend the adoption of collective samples as the unit of effort (Gotelli & Colwell 2001). When rarefaction curves for taxa collected in samples are based on individuals, the asymptote, and therefore the number of species in the area is underestimated (Gotelli & Colwell 2001). In Environmental Impact Assessments, this results in sampling being terminated prematurely, or overconfidence in estimates of biotic complementarity.

Using sample-based rarefaction curves is only valid if the samples are distributed randomly in the area of interest. Sampling plots in only a subset of the area results in a smaller number of species estimated to be present than when plots are distributed across the whole area (Chiarucci et al. 2009). The degree of bias in estimates of the number of species present, and therefore in sampling sufficiency, depends on the difference between the area sampled and the area in which inferences will be made, and on the biological group being investigated. Taxa that have clumped distributions (high beta diversity) will be subject to greater bias in estimates than taxa in which all species occupy the whole area relatively homogeneously. When the areas to be surveyed are large, remote and difficult to access, such as those in the Amazon basin, and many other regions where large infrastructure projects have been proposed, the placement of samples in truly random arrays becomes unfeasible. The choice of individuals as the unit of effort can further confuse the interpretation of rarefaction curves, as the bias of rarefaction-curve methods is influenced by two types of clumping – that between individuals and that between sampling units. Another difficulty with extracting relevant information from large areas is that the sampled area is often much smaller than the area of interest. In general, studies for hydroelectric-dam Environmental Impact Assessments in Brazil survey only a small portion of the area that would be affected (e.g. CHESF et al. 2009 Vol. 2 Sect. 2 p. 6-36).

Most conservation decisions are based on the concept of complementarity (Margules & Pressey 2000), which evaluates the number of species that are not in common between potential management units. Differences among sampling units are expected as a simple effect of distance (Hubbell 2001). If differences depend only on distance, it may be possible to correct estimates for clumped sampling. Use of rarefaction curves assumes that sampling is done only within relatively homogeneous habitats, in which case distance should have little effect, and any such effects should be logarithmically related to the distance between samples (Hubbell 2001). When species are clumped, the distance-decay relationships for assemblage similarity can be much more complicated (Morlon et al. 2008). Therefore, we also evaluated the effect of clumped sampling on the number of species unique to one or other management unit in comparisons between management units.

Despite the known biases, the effects of species clustering are strongly influenced by spatial scale (Réjou-Méchain et al. 2011) and may not always represent a serious problem in the calculation of rarefaction curves or evaluation of complementarity. In this paper, we evaluate the magnitude of the bias caused by these common practices in the construction of rarefaction curves in environmental assessments, using as a case study large-scale surveys of frogs in the Santo Antônio hydroelectric-dam site in Brazil, and studies at three other localities covering a smaller area that used similar methods.

MATERIALS AND METHODS

Study area and experimental design

Similar sampling protocols were used at the four localities for which we obtained data. Three, all in Amazonas State, were created mainly for academic studies (Uatumá Biological Reserve [Uatumá – 59W15′07″, 15S48′25″] and Fazenda Experimental of the Universidade Federal do Amazonas [UFAM – 60W05′48″, 2S38′38″], both occupying approximately 25 km², and Reserva Florestal Adolpho Ducke [Ducke – 59W55′48″, 2S57′40″] with 64 km²). The fourth, the Santo Antônio hydroelectric dam, with sample plots spread over an area of 2529 km², was located around the Madeira River, Rondônia State, Brazil (64W30′, 9S09′54″). Sampling followed the RAPELD (Rapid Assessment Protocolo de Estudos de Longa Duração, PELD is acronym for LTER Long-Term Ecological Research) system at all four sites. This system uses parallel straight transects, separated from one another by 1 km, with 250 m-long plots that follow a single local contour positioned every 1 km on each transect (for further details, see Magnusson et al. 2005, 2013). The number of transects, and hence plots varies between localities, and a single set of transects and plots is called a sampling module. To compare individual-based rarefaction curve and sample-based rarefaction curve we...
used all data available with plots as sampling units. For comparisons based on complementarity we used standard modules as the sampling unit so that effort was equal in all samples. A standard module was defined as a set of two transects of 5 km with their corresponding plots. The Santo Antonio locality has seven standard modules spaced 15–49 km from each other along the river that runs through the study area. At the other localities, the standard modules were close to and parallel to each other so that they covered the whole area of interest. Uatumã has a grid with six transects separated from one another by 1 km (three modules). Ducke has a grid with nine transects, each with eight plots and UFam has four transects with eight plots each. We standardized the sampling intensity by using standard modules based on two 5 km transects, each with five plots in each locality. To evaluate the effect of clumping on complementarity, we used small modules consisting of five plots distributed along a single 5 km transect, hereafter referred to as transects. In the last simulation, we used four standard modules at Santo Antônio to calculate the bias caused by clumping of plots on identification of vulnerable species. The tables of data and geographic localization are provide in Supporting Information section (Tables S1, S2 and S3 in Appendix S1).

At each locality, frogs were sampled in three nocturnal surveys using two standardized sampling methods: visual sampling and auditory survey. Each plot was walked by two observers who recorded the number of individuals of each calling species, and searched visually on the leaf litter and vegetation to a height of about 2 m. All frog sightings and/or all calls heard to a distance of approximately 10 m on either side of the centre line were counted. Sampling began at the end of twilight and was terminated around 23:00. Sampling was carried out during the rainy season (November to June) when frogs are easier to detect. Plots were surveyed from November 2002 to May 2004 at Ducke with 72 plots, November 2007 to May 2008 at Uatumã with 30 plots, November 2008 to May 2009 at UFAM with 32 plots, and February 2010 to June 2011 at Santo Antônio, with 70 plots.

The Uatumã, Ducke and UFAM localities are separated by up to 140 km, and are covered by relatively homogeneous old-growth tropical forest and therefore meet the assumption of a single community type being sampled. The Santo Antônio sampling modules are separated by up to 90 km, and one of the objectives of the surveys was to determine whether the area was covered by relatively homogeneous forest or had distinct habitats that might merit specific conservation actions.

Statistical analysis

**Individual-based protocol versus sample-based protocol**

We used the rarefaction program to generate individual-based rarefaction curves and the exact method for sample-based rarefaction curve using the R package ‘vegan’ (Oksanen et al. 2013). We used the specaccum package, which implements Gotelli & Colwell’s algorithm (Colwell et al. 2004). The method for samples is compatible with the rarefaction method by individuals because both estimate the average number of species not found previously by sampling effort. The rarefaction curve result is the average number of species not previously found in a sample with one less effort unit, which in this case is a sampled area. Here, abundance is not used, but only presence/absence (Colwell et al. 2004). To plot rarefaction curve when the effort is measured by individuals, specaccum uses the rarefaction method, which calculates the average number of species not previously found for each number of individuals. The integration of the two rarefaction curves (individual-based and sample-based) occurs because the rarefaction method calculates the average number of individuals per sample and plots this number with number of samples. We show the number of individuals and number of samples together on graphs as in Gotelli and Colwell (2001). The relative difference between individual- and sample-based rarefaction curves was calculated as the difference between individual-based rarefaction curve and sample-based rarefaction curve for a given effort divided by the estimated sample-based rarefaction curve value. This provided the relative difference between the rarefaction curves for a given effort, which is a measure of the bias introduced by the use of individual-based rarefaction curves when the individuals were collectively sampled. The average difference for all possible numbers of samples was used to represent the differences between the curves.

**Bias in conservation decisions due to not meeting the assumptions of rarefaction curves**

Decisions about the effects of environmental impacts are sometimes made on the basis of comparisons of numbers of species, prioritizing conservation of areas with more species, and sometimes on the basis of complementarity between areas to be impacted and areas to be conserved. The latter type of decision has been widely used in recent decades with the objective of protecting areas that have more exclusive species (those not found in other areas within the protected-area system). Although we do not agree with the practice of making decisions based only on the number of species, we investigated the effects of bias induced by the inappropriate use of rarefaction curves for both decision criteria.

**Effect of clumped sampling on complementarity.** The effect of clumped sampling on the complementarity of sampling modules was investigated by comparing the number of exclusive species between transects (number of species that occurred in one but not in the others) and their geographic distance. As rivers are known geographic barriers, at the Santo Antonio locality we used only transects on the left side of the river.

Most transects were close together in the academic localities. To avoid giving undue weight to localities with more transects, within these sites, we chose one transect randomly and its nearest neighbour to be used in analyses. As the same transect was involved in more than one distance, observations are not independent and probabilities were obtained by comparing the observed statistic to the statistics calculated for 1,000 random permutations of the distances.

**Effect of clumped sampling on overestimation of sampling adequacy for identification of vulnerable species.** To illustrate
the effect of inappropriate sampling, we compared the results based on only one standard module from the Santo Antonio site with those based on the use of all four modules. We refer to the use of one module with two transects as the reduced set. As this reduces the geographic coverage and all individuals are collected within a radius <5 km, we considered this as clumped sampling. We refer to the four modules, each with two transects at the Santo Antonio locality as the complete set because the area they covered was similar to that of the whole area of interest. In each module there were five transects and a total of ten plots. Within the reduced set, four plots closer to water were within the area to be impacted and six were in areas that would not be directly impacted by the dam. In the complete set, 16 plots, four in each module, were within the area to be inundated and 24 were outside the impact area (Fig. 1).

We used rarefaction curves based on plots combined with jackknife estimates of the total number of species present to determine adequacy of sampling and the magnitude of possible errors in ascribing species to the list found only in the area to be affected. Thompson et al. (2007) suggested that collection of more than 80% of the species estimated to present in a locality indicated adequate sampling in both uniform and heterogeneous environments.

RESULTS

Individual-based protocol versus sample-based protocol

Sample-based rarefaction curves had shallower slopes and underestimated the number of species encountered for a given sampling effort (Fig. 1). The medium difference between individual-based rarefaction curve and sample-based rarefaction curve was highest at Santo Antônio (25.7%). At the UFAM and Uatumã localities, the differences were similar to each other (10.0% and 9.6%, respectively). The smallest difference was found in Ducke (3.1%) (Fig. 2).

Effect of clumped sampling on complementarity

For Ducke, Uatumã and UFAM, the number of species found in only one transect (exclusive species – excl) in comparisons among transects (Fig. 2) had an asymptotic (power function) with distance (dist) (excl = 6.23 × dist^{0.356}), permutation test with 9999 repetitions: $P = 0.001$). However, at the Santo Antônio locality, the relationship was not asymptotic and could be represented by a parabola ($20.38 + 0.321 × \text{dist}^2$, permutation test with 9999 repetitions: $P = 0.041$). The dissimilarity among sampling units (transects) increased monotonically up to distances of about 40 km, but sampling units separated by large distances had less exclusive species than sampling units separated by intermediate distances (Fig. 3).

Effect of clumped sampling on estimation of sampling adequacy for identification of vulnerable species

A total of 22 species were found in the impacted area within the reduced set (partial coverage of the area of interest), and the jackknife estimate based on samples indicated that this represented about 85% of the species in the impacted area. Of these, ten were not found in the adjacent area and would be considered species of conservation concern to maintain local biodiversity.

The same analyses using the complete set indicated that there were about 49 species in the area to be impacted, and 16 of these were not found outside the impacted area. The 16 species exclusive to the impacted area and therefore relevant to conservation, in the complete set included only three of the species identified in the reduced set as of conservation concern, and included another 13. These results are not concordant with the rarefaction curve analysis of data from the impacted area, which indicated that only six species had not been detected. Therefore, besides giving biased estimates for the number of unique species, the rarefaction curve analysis of sampling adequacy gave erroneous confidence in those estimates.
DISCUSSION

**Individual-based protocol versus sample-based protocol**

The difference between sample-based and individual-based rarefaction curve was evident at all sites, but the maximum difference was higher at the Santo Antônio site (33.4%), which covered a larger area.

The degree of clumping of species influences the difference between slopes of individual and sample-based rarefaction curves. The difference between individual- and sample-based rarefaction curves can be used as an index of clumping, but it does not indicate at what scale the clumping occurs (Gotelli & Colwell 2001). The magnitude of the difference can only be used to evaluate the degree of clumping in the whole area if the collective sampling units were distributed randomly within the area. In some cases,
inadequate local sampling might cause a sharp increase in number of species with the number of individuals just because only the common widespread species were found and this increases evenness (Peterson 1975). As evenness depends on the number of individuals within sample units and the distribution of the sample units, there is no simple way of relating evenness to degree of clumping. Therefore, use of individual-based rarefaction curves for sample-based collection leads to erroneous conclusions about sampling completeness in terms of the proportion of species estimated to be in the area that was encountered for a given level of effort, and the bias is inherently unpredictable.

Effect of clumped sampling on complementarity

The positive relationship found between the number of exclusive species and geographic distance in the sites in relatively homogeneous forest near Manaus corroborates the results of other studies (Arrhenius 1921; Chiarucci et al. 2009). However, at Santo Antônio, the number of exclusive species had a more complex relationship with geographic distance. Sites further apart had fewer exclusive species than sites at intermediate distances. This probably reflects patchily distributed habitats, with the most distant sites being more similar in terms of environment. This is a useful result for conservation planning, but means that the assumption that only one community type was sampled was not met and the proportions of different habitats sampled will affect the rarefaction curves. If sampling had been random and intensive, the resulting species-accumulation curve could still be representative of the area, if not of a particular community. However, as the sampling was clumped, and the distribution of different communities within the area is unknown, the rarefaction curves are not interpretable.

Effect of clumped sampling on estimation of sampling adequacy for identification of vulnerable species

In Brazil, the proposed Environmental Impact Assessment for the Riacho Seco hydroelectric dam on the São Francisco River included clumped...
surveys covering only about 7.1% for the total area of potential impacts (http://licenciamento.ibama.gov.br/Hidrelétricas/UHE%20Riacho%20Seco/TEXTOS/Vol%201%20/Anais%20EIA%20RL%20-%202007.pdf). The Environmental Impact Assessment for the Santo Antônio do Jari hydroelectric dam used more plots, but presented an rarefaction curve only for individuals (http://licenciamento.ibama.gov.br/Hidrelétricas/Santo%20Antônio%20%28Rio%20do%20Jari%29/TEXTOS/Resultados%20EIA%20RL%20-%202007.pdf, pages 46 and 52). In the case of Santo Antônio do Jari, the consultants used rarefaction curve to imply that there were more species in areas outside the area to be impacted than in it, and concluded that no species were endangered in the impacted area. In fact, estimates from rarefaction curves cannot be used to determine whether any or all species in the area to be impacted are in the surrounding areas. If there were more species in the impacted area, it would be a reasonable assumption that some must not occur in the surrounding areas, but this would be a very weak and indirect assessment.

The overall extent to which the misuse of rarefaction curves is affecting decisions in environmental-impact studies is hard to quantify because most results are published only in the grey literature, and very often the spatial distribution of sampling is not sufficiently documented to allow critical evaluation of the results. However, the frequent recommendation to use rarefaction curves to evaluate sampling adequacy in Environmental Impact Assessments in places as diverse as Australia (Thompson 2007; Thompson et al. 2007), Europe (Trajano 2010) and Brazil (IBAMA 2007) indicates that the problem is widespread.

Whereas the limitations of using species richness as a criterion for conservation decisions are well known (Margules & Pressey 2000), the problems with using algorithms based on complementarity in hyper diverse regions where sampling is likely to be inadequate are less well understood (Magnnsson et al. 2013: 70-72). If rarefaction curves indicate that sampling was adequate when it was not, complementarity analyses may indicate the wrong set of species for analyses. In the case of our simulation of reduced sampling in the impact area of the Santo Antônio hydroelectric dam, inadequate spatial coverage combined with rarefaction curves identified ten species as being potentially extirpated from the local area by the dam, whereas the more complete analysis indicated that seven of those species did not merit special attention, and a further 13 species not identified in the first analysis were potentially endangered by the dam. The magnitude of this error (20 species misclassified) could not be predicted from the first rarefaction curve analysis, which indicated that only about six species had been overlooked in the impact area. Obviously, being found only in the impacted area is not the only criterion for listing species as being of concern, but it is the first step in selecting species that are priorities for further research. For example, Carneiro et al. (2016) used complementarity of species in impacted and adjacent areas to identify species of conservation concern that merited subsequent analyses using species-distribution models.

The effort (area sampled and researcher hours) of fieldwork at Santo Antônio was the greatest ever made for Environmental Impact Assessment-based biodiversity sampling in the Amazon rainforest (at least up to the year of its completion in 2010). Nevertheless, the rarefaction curves for anurans still did not stabilize. Major infrastructure programs in other biologically diverse areas also cover enormous areas. For instance, the Three Gorges Dam on the Yangtze River in China flooded 1084 km² (Xie 2003) and the Altai gas pipeline from Russia to China will run 2800 km through some of the remotest areas in the world (Söderbergh et al. 2010).

The fact that the rarefaction curve could not estimate the number of species in the impact area of the Santo Antônio dam may not be a great problem for evaluation of the Environmental Impact Assessment, as use of species richness for decision making is questionable (Margules & Pressey 2000). Finding species that are restricted to the area to be impacted, or which are more abundant there, is a more appropriate criterion for decision making with Environmental Impact Assessments (Koblitiz et al. 2011). Adequate coverage of the area of interest, with sampling distributed throughout the study area should be a recommendation for Environmental Impact Assessments in any future large infrastructure-construction projects. However, because of logistical and financial restrictions, random sampling may not be feasible and decisions should be made on estimates of complementarity that take into account the possibility of false absences rather than on biased and uninformative estimates of the number of species in the area.

If it is decided that it is necessary to use rarefaction curves in environmental-impact studies, and they remain as a legal requirement, the recommendations of the academic literature should be put into operational practice. It is common in Environmental Impact Assessments for rarefaction curve to be estimated from samples from only a portion of the area of interest, or using rarefaction curves based on individuals as sample units. While such methods are attractive, because they require less effort, they are not short-cuts to rarefaction curves that provide an accurate estimate of the number of species present. As truly randomized sampling over large areas and achieving rarefaction curve stability for megadiverse
groups appears impractical (e.g. Longino et al. 2002), we suggest that stabilization of rarefaction curves should not be used routinely to evaluate the adequacy of sampling in environmental impact studies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. The geographic coordinates of all plot in this work.
Table S2. Table with species data collection of Reserva Ducke, Rebio Uatumá and Reserva Ufam localities.
Table S3. Species data collection of Santo Antonio locality.
Appendix S1. Species data collection and geographic coordinates of plots.