The use of environmental DNA (eDNA) in biological monitoring of the Natterjack toad (*Epidalea calamita*)

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Abstract

Environmental DNA (eDNA) is a promising new tool in the field of conservation management, which can contribute to non-destructive species determination as well as improved data on species occurrence. Still, it is important to gain knowledge on how eDNA works compared to conventional monitoring methods in order to evaluate the detection efficiency.

In this study, I have investigated the use of eDNA as a compliment to the traditional survey methods used in Sweden's national biogeographical monitoring of the red listed Natterjack toad (*Epidalea calamita*). *E. calamita* occurs in 4 different counties, Västra Götaland having a large viable population distributed on small islands in Kattegat. In Skåne, Blekinge and Halland, where the species is not considered common, it occurs in a much wider range of habitats such as coastal rock-pools, heath meadows and gravel pits. The difference in population status and county, have resulted in two slightly different survey and sampling methodologies.

The study demonstrates that eDNA proved to be a useful tool for species identification and species occurrence. The assurance of species identification and low amount of potential false positives and negatives, provide justification for the usefulness of eDNA in the national monitoring of *E. calamita*. However, difference in results between counties indicates that there is a need for site-specific protocols to assure the most reliable results for all counties.

Introduction

About a third of all amphibian species are classified as globally threatened, constituting the most threatened animal group compared with birds and mammals (Stuart et al 2004). Amphibians are, due to their permeable skin and their dependence of suitable aquatic and terrestrial habitats, sensitive to changes in the environment and can therefore be considered as "indicators of overall environmental health" (Collins and Storfer 2003). The severe decline in populations is thus of great concern. The global decrease is assigned to invasive species, landscape modification, climate change, contaminants and diseases (Collins and Storfer 2003). Various large-scale conservation efforts have been undertaken to counteract this, these include restoration of the aquatic and terrestrial habitats, translocations and intense monitoring programs (Nyström and Stenberg 2007).

Today, many amphibians are protected by global agreements and national laws, furthermore in Sweden all herptiles are protected and for most species also their habitats. Still a major challenge, when it comes to amphibians and population declines, is that in many cases information on what is causing these declines is lacking as well as information on local abundances, which is crucial in relation to effective conservation efforts (Thomsen and Willerslev, 2015). In Sweden, this is true for the Natterjack toad (*Epidalea calamita*), which is classified as vulnerable on the Swedish red list (Artdatabanken, 2015). The species has been declining in Sweden since the 1960s as a result of anthropogenic disturbances such as urbanization, eutrophication, habitat destruction and modification (Nyström and Stenberg 2007, Pröjts, 2012). Habitats that have become less optimal in combination with increased competition with the Common toad (*Bufo bufo*) are suggested to be important factors behind the national decline in Sweden, a situation also applicable to Britain (Pröjts 2012, Beebee 1977). In order to evaluate the status and local threats of different populations of *E. calamita*, monitoring of *E. calamita* should also include data on *B. bufo*, which is known to be a superior competitor during the tadpole stage (Bardsley and Beebee 1998, Bardsley and Beebee 2001:1, Bardsley and Beebee 2001:2). Both species coexist in some ponds in Sweden, which complicates traditional monitoring of tadpoles do to a large similarity between the species. *B. bufo* tadpoles are typically larger and have slightly lighter color compared to *E. calamita* tadpoles. However, the only way to truly tell the species' apart is to examine their mouthparts in a microscope, which would involve killing tadpoles. Subsequently, alternative and non-destructive methods to determine these species in the field need to be assessed.

Amphibian monitoring in Sweden

Sweden and other countries in Europe are to report the population status of *E. calamita* and 10 other amphibians every 6th year, since these species are included in the habitat directive (appendix 2 and 4) (Nyström et al 2016). Additionally, *E. calamita* is protected by the Species Protection Ordinance (SFS 2007:845 § 4, 5) and included in the Bern convention (appendix II) (Nyström et al 2016). The results of biological monitoring are essential for efficient conservation management and helping to improve the conservation efforts as it can reveal distribution and negative populations trends (Thomsen and Willerslev, 2015). In general, most amphibians are monitored during the reproductive period by visual observations and counting of individuals, such as estimating the number of calling males. Other types of monitoring

involve methods including kick-net sampling (Pilliod et al 2013) or dip-netting (Thomsen et al 2012) for catching larval stages. There are different factors that make amphibians a challenging group to monitor for example the semi-aquatic lifestyle, the relatively short reproductive period, and large natural fluctuations in breeding population sizes from year to year (Marsh and Trentham 2001). Additionally, there is a variation in detectability as a consequence of weather conditions. Surveys of calling male *E. calamita* optimally have to be at night, after heavy rain, as the males during dry periods tend not to be near the wetlands. Despite favorable weather conditions, these males' does not necessarily call (M. Stenberg pers. comm.). The monitoring of tadpoles of *E. calamita* is also complicated, as these cannot be distinguishable from *B. bufo* tadpoles in the field (Nyström and Stenberg 2016). Additionally, red listed and vulnerable species, such as *E. calamita*, stress the need for non-destructive methods of monitoring. Due to limited resources in conservation management there is a need to rationally choose the most efficient methods in regards to detection probability and funding. Funding is also limited in relation to the biogeographical monitoring period of 2013-2018. Until recently, the population size estimates of *E. calamita* in Sweden were typically based on counts of the number of calling males estimated during breeding season at three subsequent evenings. This procedure is found to be too time consuming in relation to the budget for the biogeographical monitoring period of 2013-2018. Thus, it has been suggested that this procedure should be replaced by estimations of tadpole density and notations of the presence or absence of *B. bufo* in the same waters (Nyström et al 2017). This procedure will limit the time needed (only one visit during daytime) and it is not weather dependent, contrary to the traditional method of counting calling males. A problem with this method, aside from the difficulties to do species identification in the field, is that it can be challenging to estimate tadpole densities in turbid or overgrown waters (Nyström and Stenberg 2016, Nyström et al 2017).

Analyses of environmental DNA (eDNA) from water samples could provide a valuable complement to the conventional field sampling, as it is a non-destructive method for species determination and less time consuming than most conventional methods (Nyström and Stenberg 2016). An eDNA sample can also reveal potential presence of a species, and therefore contribute to data on distribution where the conventional method might not. The application of eDNA in the field of conservation biology is still relatively new, but has shown promising results for detection of rare or invasive freshwater amphibians (Ficetola et al 2008, Goldberg et al 2011, Pilliod et al 2013, Thomas et al 2012, Dejean et al 2012, Thomsen and Willerslev 2015). The method is applicable in different types of habitats such as streams (Goldberg et al 2011 and Pilliod et al 2013) and ponds (Dejean et al 2012, Ficetola et al 2008, Thomas et al 2012). In the study of Thomas et al (2012), eDNA confirmed 91-100 % of the occurrences registered by conventional dip-netting of the Common spadefoot toad (*Pelobates fuscus*) and the Great crested newt (*Triturus cristatus*). Additionally, eDNA confirmed the presence of the species in 5 out of 8 ponds with historical records, where conventional methods did not. For this reason, eDNA is suggested to be more sensitive compared to conventional survey methods (Thomsen et al 2012, Pilliod et al 2013). The greater sensitivity compared to traditional monitoring were also evident in the study of Dejean et al (2012). Here, eDNA proposed the existence of the American bullfrog (*Lithobates catesbeianus*) in

additional wetlands compared to the conventional monitoring. Subsequently, more intense field surveys verified the presence of *L. catesbeianus* in 11 additional ponds, compared to the results of the conventional method.

However, there are different factors that influence the probability of detection, such as the presence of the species, the concentration of eDNA, sample interference (e.g. inhibitors such as humic substances), capture and extraction efficiency of DNA (Goldberg et al 2016, Thomsen and Willerslev 2015). Many of these variables can be correlated to the degradation time, which can vary from 1 day to 8 weeks (Goldberg et al 2016). Degradation can be effected by a variety of abiotic factors such as temperature, ultraviolet radiation and sediments, while other factors such as pH and salinity can mediate interactions between sediments and DNA (Barnes et al 2014, Goldberg et al 2016). The effect of some of the factors might be entirely context dependent, as many studies have shown divergent results (Barnes et al 2014). Thus, studies on the efficiency of eDNA to detect a species should evaluate the results with great care (Goldberg et al 2016), contemplating factors that can affect the result, such as the lab and field protocol including considerations of the possibility of false negatives or positives and optimization.

To my knowledge, no studies on the use of eDNA for detecting *E. calamita* is currently published. However, eDNA should be applicable for detection of *E. calamita* in breeding sites that are typically small and shallow, as the concentration of DNA should be detectable if the species is present or have been recently present in relation to time of sampling. The sampling size and amount of water needed should additionally be small compared to for example rare species in a stream environment, reducing the workload and theoretically have both a detectable concentration and good capture efficiency.

Aims and hypotheses

This project is a part of Sweden's national biogeographical monitoring of *E. calamita* in 2016-2017, on behalf of the Swedish environmental protection agency. The aims of my study were to 1) compare the detection rate of eDNA with the detection rate of the conventional field method of counting tadpoles, spawn strings and, for Västra Götaland, adults, and 2) investigate if and to what extent *E. calamita* and *B. bufo* coexist in *calamita* ponds in Västra Götaland and the counties Skåne, Blekinge, Halland, encompassing the entire distribution range in Sweden. These aims generated the following hypotheses to be tested:

1. if the presence or absence of *E. calamita* and *B. bufo* is dependent on monitoring method
2. if both methods give the same result in detecting *E. calamita* and/or *B. bufo* in the same ponds/wetlands

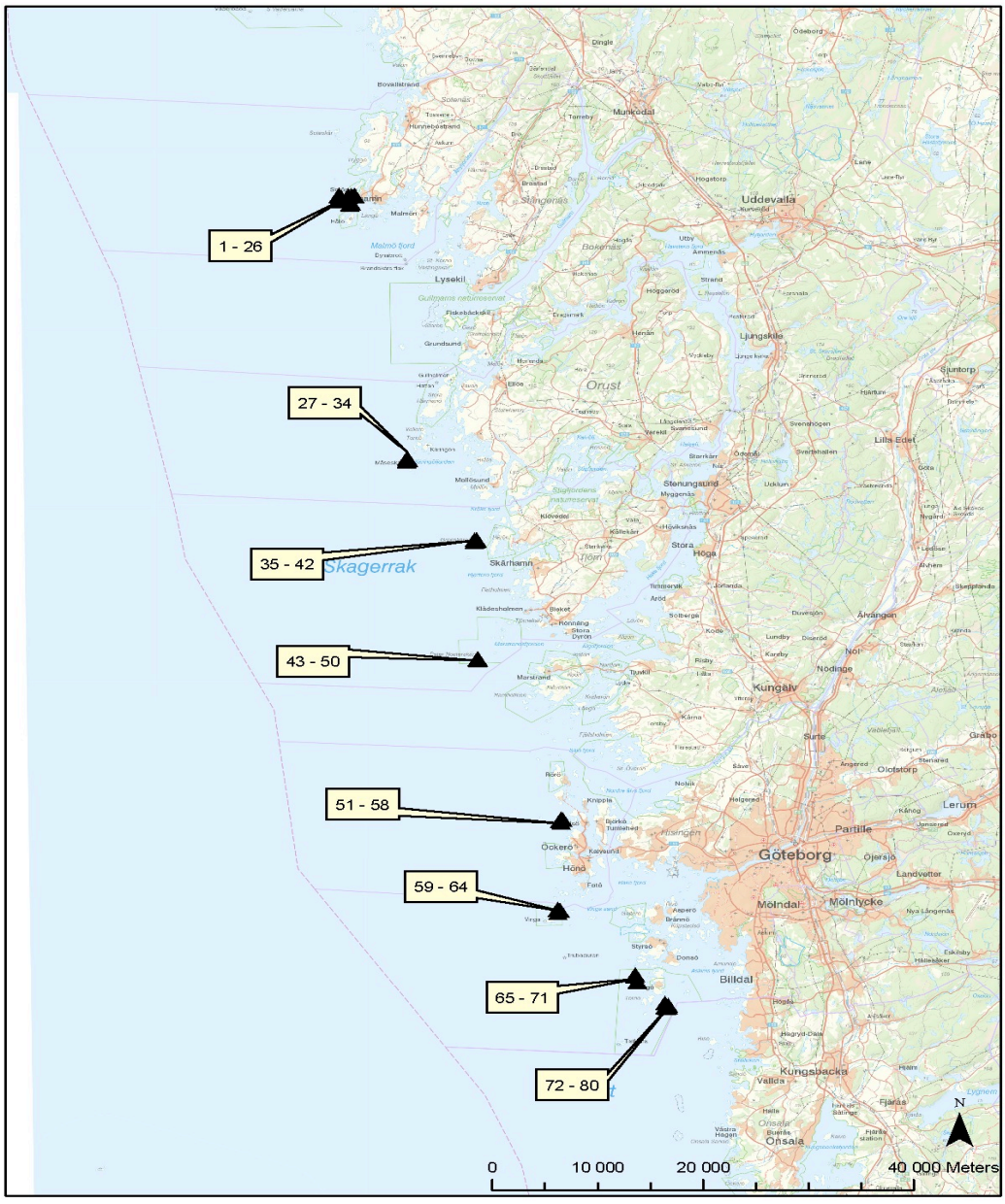
And if:

3. if *E. calamita* and/or *B. bufo* coexist in the same ponds/wetlands

Materials and methods

General approach and study sites

The distribution of *E. calamita* in Sweden is largely known since most findings are reported on "Artportalen" (Nyström et al 2016). Artportalen is a database where the public as well as professionals can report findings of different species (Artportalen 2017, Nyström et al 2016). Within the national monitoring program for *E. calamita* ("Biogeografisk uppföljning") there are two different approaches. In the county of Västra Götaland, the species is quite common. Therefore, only the largest populations are monitored, due to economical constraints. A total of 80 sites have been surveyed during 2016 and 2017 (map 1). In other parts of Sweden, the counties of Skåne (map 2), Blekinge (map 3) and Halland (map 4), all sites are monitored. In these latter counties a total of 149 sites with the *E. calamita* have been reported to Artportalen (Nyström et al 2016), of those 51 sites were surveyed in 2017.



Map 1. Sites monitored in Västra Götaland 2016 and 2017. Numbers refers to ID in appendix 1
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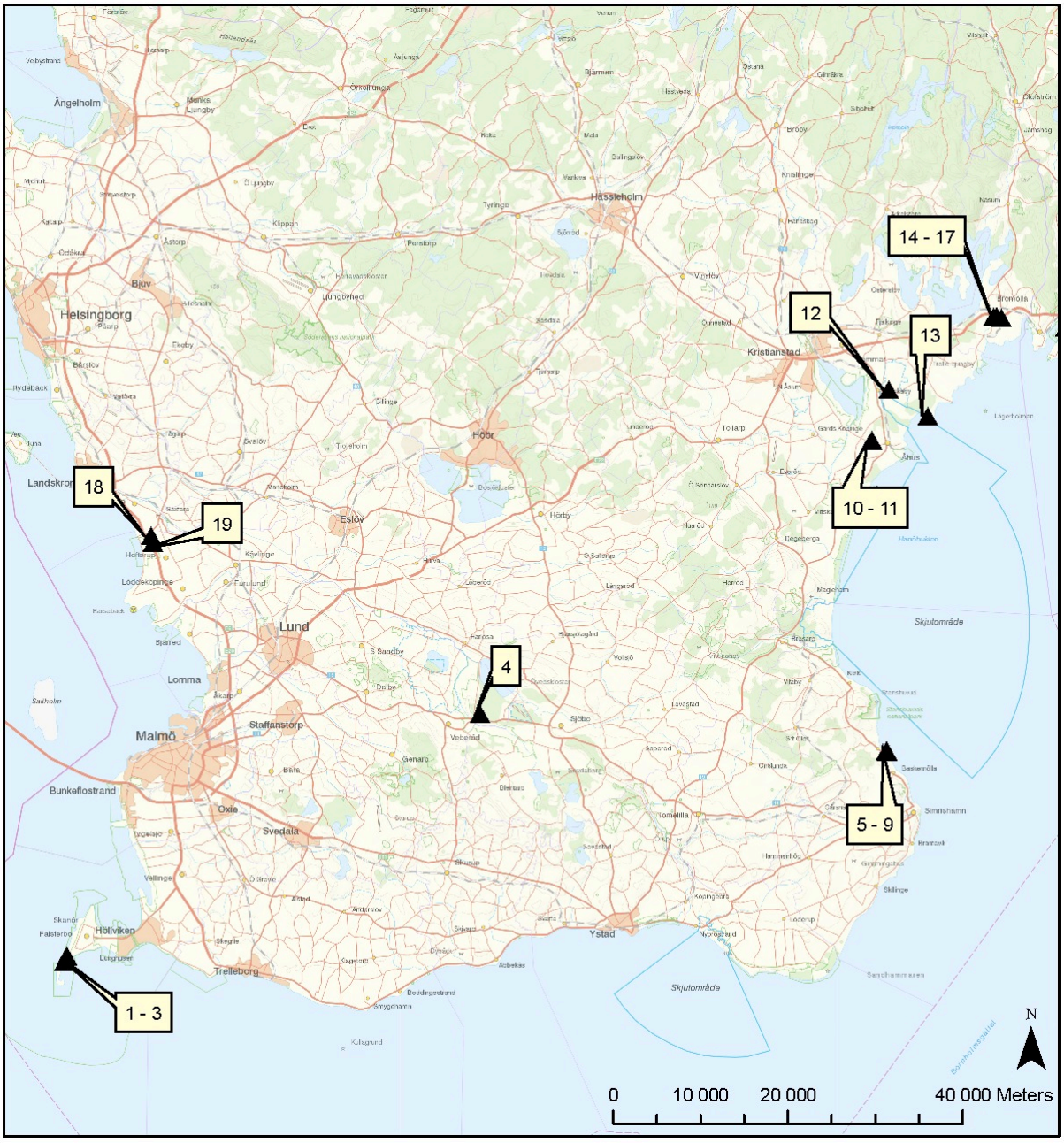
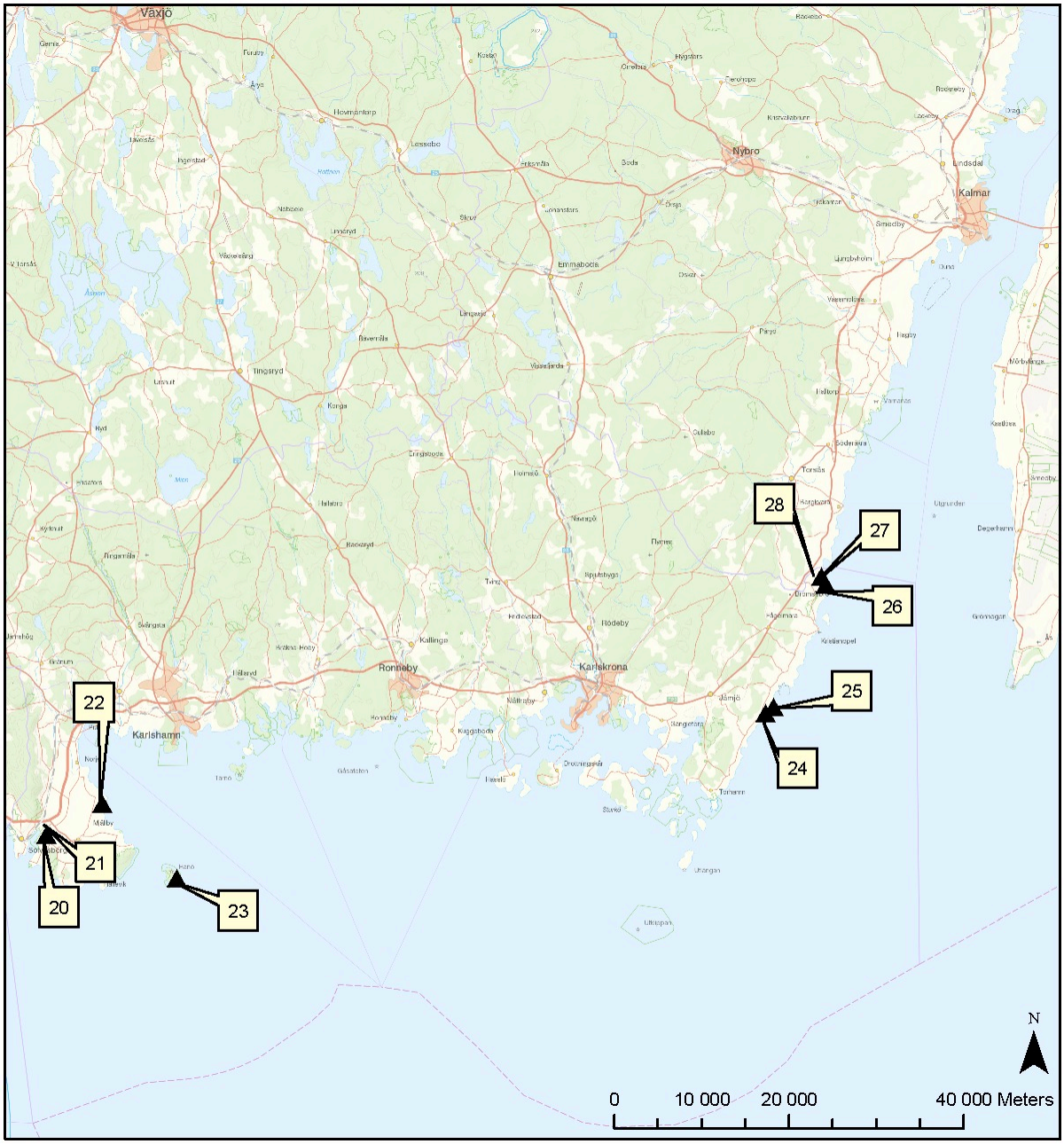
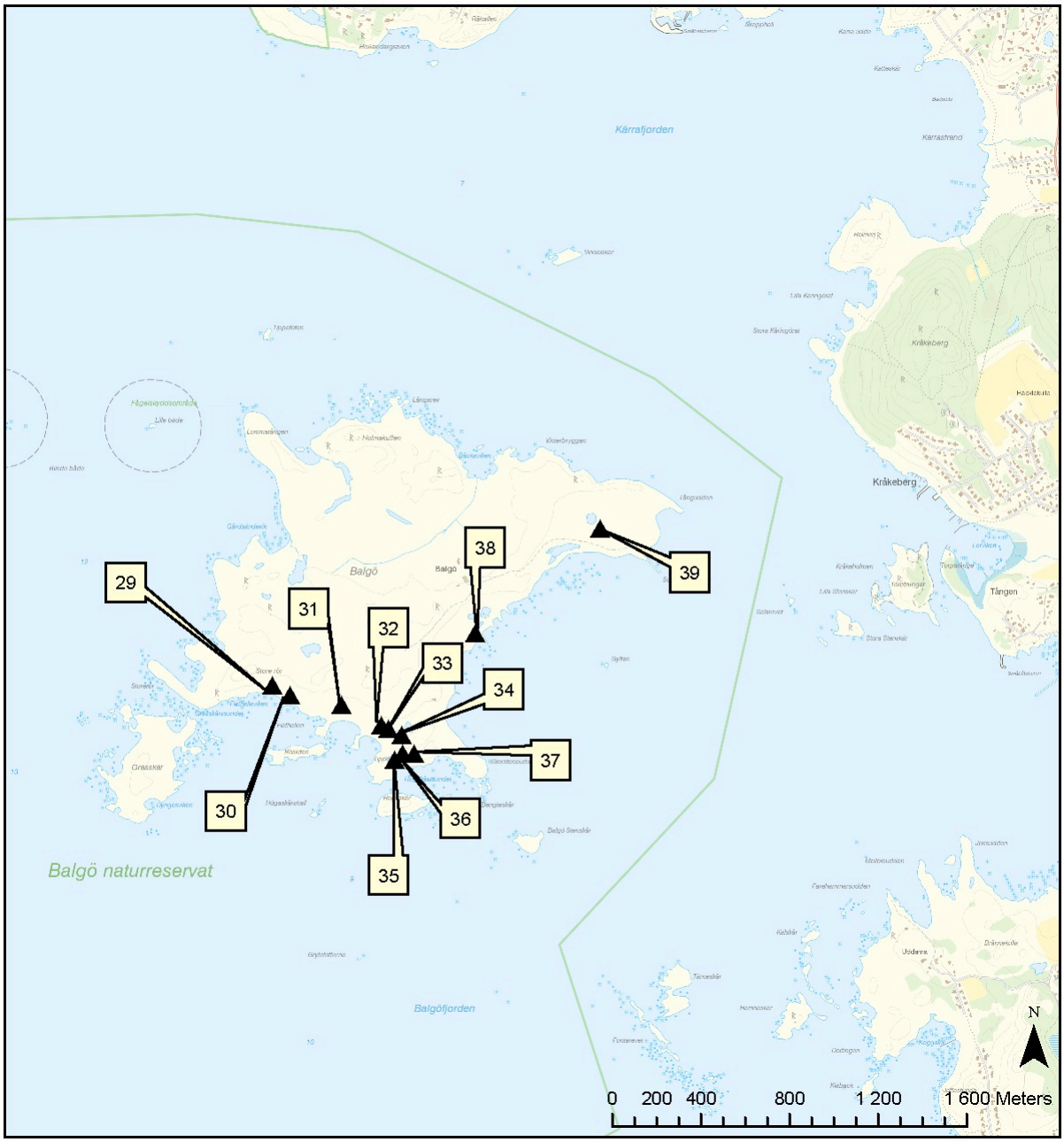


Map 2 Sites monitored in Skåne 2017. Numbers refers to ID in appendix 2 © Lantmäteriet.



Map 3. Sites monitored in Blekinge 2017. Numbers refers to ID in appendix 2 © Lantmäteriet.



Map 4. Sites monitored in Halland 2017. Numbers refers to ID in appendix 2 © Lantmäteriet.

In Västra Götaland the 80 sites included are all rock-pools situated on islands. Due to the difference in environment characteristics and the survey methodology (described in the following section), the data from Västra Götaland is analysed separately from the other counties.

In Skåne the 19 sites included are composed of a wide range of habitats such as coastal rock-pools, heath meadows and ponds in forested environments. In Blekinge the habitats were mainly gravel pits, a total of 9 sites were included. In Halland samples from 11 sites were included and all consisted of shallow ponds in coastal meadows on the island of Balgö. In order to gain more knowledge on the habitat use by *E. calamita* and potential site-specific challenges for the two monitoring methods I visited four different sites in October

2017. The sites selected, encompassed the entire range of habitat types, rock-pools, gravel pits and lakes.

Survey method in Västra Götaland

The county administrative board of Västra Götaland conducted the surveys during the summer of 2016 (25 May-22 June) and the summer of 2017 (31th of May) following the method and protocol developed in collaboration with the county administrative board of Skåne, the county of Västra Götaland and Ekoll AB (Anderson and Nilsson 2016). Each site was visited twice during the same day. Estimations of tadpole density were done during the first visit, at daytime. The second visit was conducted at nighttime between 10 pm - 3 am the same day. During this visit, inventories of adult *E. calamita* were done by estimating number of calling males in addition to notations of other observed non-vocal adults. In total 80 sites were visited, 66 sites in 2016 and 14 in 2017. The 14 sites monitored in 2017 differ in methods from the surveys performed in 2016, because these surveys only included data on tadpole density obtained during one visit. These sites are therefore disregarded when reporting densities of spawn strings and adults, but included in all statistical analyses.

Water samples were taken from all rock-pools to analyze for eDNA from both *E. calamita* and *B. Bufo*. The samples were taken during the second visit the first day in 2016 and the first visit in 2017.

Survey method in Skåne, Blekinge and Halland

Ekoll AB and the county administrative board of Blekinge conducted the surveys during the summer of 2017 (9-28 June), following the method and protocol suggested in Nyström et al (2016). Inventories of *E. calamita* were done by estimating the number of tadpoles during daytime in end May-July. One spawn string is assumed to be equivalent to approximately 1000 tadpoles. Depending on the predictability of the spawning season, each site should be visited 1-2 times, if the first visit proves to be too early in the season (Nyström et al 2016, Nyström et al 2017).

Of the original 51 sites, a total of 39 sites were included in this study. The 12 sites were excluded because they were either dried out or not possible to visit and take water samples. All 39 sites have information on tadpole density and water samples taken for eDNA analysis (presence of *E. calamita* and *B. bufo*).

Analysis of eDNA from water samples

Water samples for eDNA extraction were collected in new and clean water bottles. All bottles were labeled in order to be coupled with the results from the field survey (tadpole counts, spawn strings and presence of adults). At Västra Götaland 50 mL were taken from each water body, regardless of the size, at one site within the wetland. In Skåne, Blekinge and Halland a sample of 100-500 mL was collected from each site. Furthermore all water bodies included in this survey had an area of less than 1000 m^2. In habitats with an area of 100m^2 of less, a sample of 100 mL was assumed to be enough. The samples should be representative for the sites, and water was therefore taken from several places within the sampled site. The samples were then kept cool and frozen during the same day. Samples were analyzed in October 2017-February 2018.

The analysis of eDNA was done by the Museum of Natural History in Sweden ("Naturhistoriska riksmuseet"). The filtering followed the procedure of Agarsnap et al (2017), the Danish method, except that a 0.45 µm Sterivex-filter was used instead of 0.22 µm. The DNA extractions were done by KingFisher™ Duo Prime Purification System and the KingFisher™ Cell and Tissue DNA Kit. PCR procedure followed the procedure of Thomson et al (2012) with one positive control and negative control on each PCR-plate. For details on primers and probes, see appendix 1. Three subsamples from each sample were analyzed for DNA, and considered positive if at least ⅓ were positive. If all three subsamples were negative for a wetland, in which tadpoles were observed, three new subsamples were analyzed for DNA.

In order to avoid contamination in the laboratory, all bottles and equipment was sterilized using chlorine and/or UV-light. Furthermore, researchers were wearing lab coats, gloves etc. and are not allowed to enter other parts of the laboratory during their work with eDNA. The laboratory is further secured by air-locks between the other laboratories and the windows are air-tight.

Data analysis

Chi-square test of independence was performed to test if:

1. the presence or absence of *E. calamita* and *B. bufo* is dependent on monitoring method
2. *E. calamita* and *B. bufo* coexists in the same ponds/wetlands.

And sign tests were used to test if:

3. both methods give the same result in detecting *E. calamita* and *B. bufo* in the same ponds/wetlands

Results

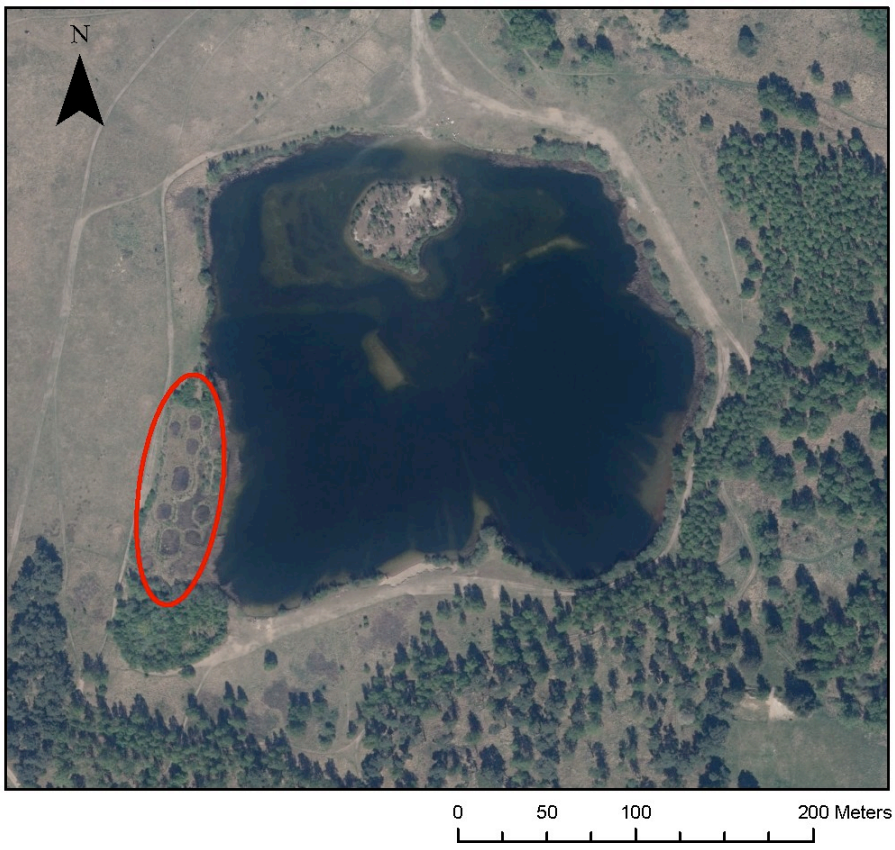
Visited sites and descriptions

The selected sites encompassed the entire range of habitat types favored by the *E. calamita*; rock-pools, gravel pits and lakes. Some of the areas were constructed as a conservation effort for *E. calamita*, some were a result of human activities and two were natural. I visited four sites in October 2017, which were surveyed during the summer of 2017. These visits were done in order to gain more knowledge on the habitat use of *E. calamita* and potential site-specific challenges for the two monitoring methods. Area specific details and results from the inventories are gathered in appendix 2 and 3.

In Järavallen forest area (Swedish: "Järavallen skogsområde"), the sites were located in the proximity of two lakes, both sites were overgrown by trees and reeds, and both sites were dried out in October. The northern site (picture 1, site ID 18 in map 2) was a dry overgrown area. The southern site (picture 2, ID 19 in map 2 and map 5) was composed of several dried out pools created for the *E. calamita* in year 2003 in the proximity of a large lake.

Picture 1. Järavallen forest area, ID 18 in map 2. Picture taken 26.10.17.

Picture 2. Järavallen forest area, ID 19 in map 2 and map 5. Picture taken 26.10.17.



Map 5. Ponds for *E. calamita* at Järavallen. © Lantmäteriet.

In Flommen, the site was composed of multiple water bodies on a golf course, three of these were included in the biological monitoring in 2017. The surrounding habitat was composed of short grass, sandy areas, heath and beach habitats, with trees and bushes in the proximity. The first site was a canal with an abundant submerged vegetation. The canal was shallow, but not enough to dry out (picture 3, ID 3 in map 2). The second and third sites were relatively deep, with sparse submerged vegetation (picture 4, ID 1 in map 2).

Picture 3. Flommen golf course, ID 2 in map 2. Picture taken 25.10.17.

Picture 4. Flommen golf course, ID 1 in map 2. Picture taken 25.10.17.

In Vik, the habitat was composed of several shallow rock-pools, with minor submerged vegetation (Picture 5, ID 5 in map 2). The sites were located a few meters from the Baltic sea and deciduous forest.

Picture 5. Vik, Prästans badkar, ID 5 in map 2. Picture taken 27.10.17.

In Blekinge, the sites were located in a gravel pit. The surrounding habitat was characterized by sand, gravel, trees and two lakes. The pools were shallow but not shallow enough to dry out. Emergent vegetation covered most part of both pools (Picture 6, ID 20 in map 2).

Picture 6. Blekinge gravel pit, ID 20 in map 2 Picture taken 19.10.17.

Comparison of detection by conventional methods and eDNA

E. calamita in Västra Götaland

In the total of 80 sites that were surveyed, 5 sites were excluded from further analysis do to inhibitors.

27 sites had tadpoles that occurred in densities between 10 – 4000. 25 sites had observations of adults, numbers between 1 – 17 and lastly 5 sites had observations of spawn strings with numbers between 1 – 20. The occurrence of *E. calamita* is shown in figure 1.

Chi-square test of independence was performed to examine the relation between the frequency distribution and the monitoring methods. The relation between these variables was significant ($\chi^2 = 3.87$, d.f. = 1, $P < 0.050$). There was a difference in the species frequency distribution obtained by the two methods of monitoring (figure 1).

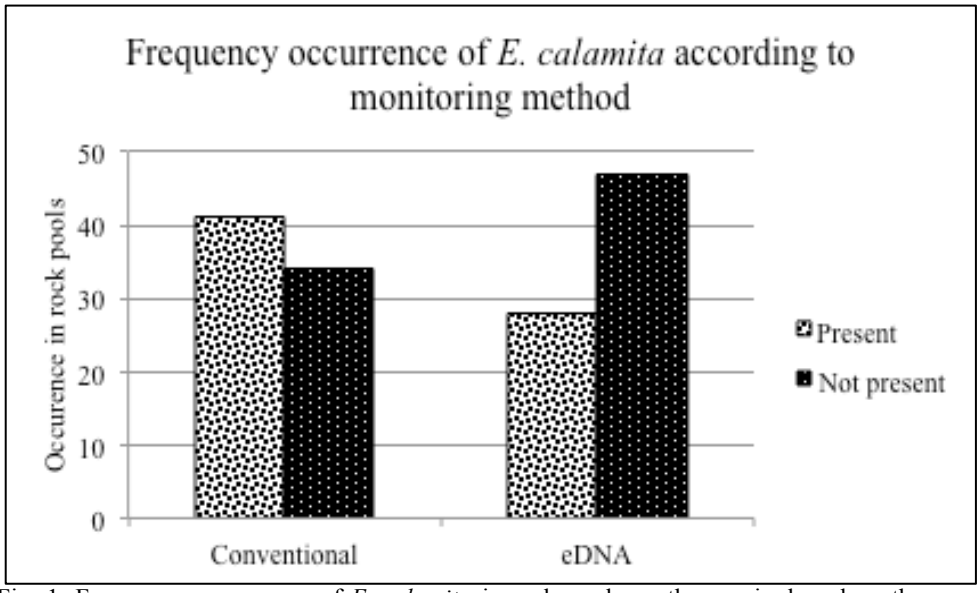


Fig. 1. Frequency occurrence of *E. calamita* in rock pools on the y-axis, based on the presence of spawn strings, tadpoles and/or adults. Type of method, conventional (visual, auditory and tadpole counting) and eDNA on the x-axis. Each method consist of two groups, representing the number of times *E. calamita* where either considered present or not present with the respective methods.

A sign test was performed to examine the relation between the results (presence or absence of *E. calamita*) and the monitoring method (conventional or eDNA). The results showed a significant difference between the variables (P =0.012). The presence or absence by *E. calamita* is significantly different between monitoring method and the results are thus dependent on the type of monitoring (conventional or eDNA) (table 1).

Table 1. Detection of *E. calamita* using conventional monitoring methods and eDNA.

	Presence of eDNA	**Absence of eDNA**
Presence by conventional method	24	16
Absence by conventional method	4	31

Note: The number in each cell represents the frequency occurrence of *E. calamita* according to monitoring method.

There was consistency between approximately 73 % of occurrences (presence and absence) between the two monitoring methods. Furthermore, there was DNA from *E. calamita* in 4 additional waters, which did not have any field observations of the species. 16 field observations of *E. calamita* could not be confirmed by the subsequent eDNA analysis (table 1). The field observations in tadpole densities in the pools ranged between 10-1000, 1-3 adults and up to 7 spawn strings. In four of these cases, eDNA confirmed the presence of *B. bufo* instead of *E. calamita* (field observations ranged from 1-3 adults and in one case, estimated 300tadpoles).

E. calamita in Skåne, Halland and Blekinge

E. calamita tadpoles were observed in 13 of the 39 sites in Skåne, Blekinge and Halland (figure 2) at densities between 2-2.500. Eight sites also had field observations of *B. bufo* tadpoles. In four of the eight sites *B. bufo* was observed in the field with *E. calamita* (figure 2). Chi-square test of independence was performed to examine the relation between the frequency distribution and the monitoring methods. The relation between these variables was not significant for either *E. calamita* ($\chi^2 = 0.0$ d.f. = 1, P = 1) or *B. bufo* ($\chi^2 = 0.35$, d.f. = 1, P = 0.55), there was no difference in the species' frequency distribution (number of ponds with *E. calamita* or *B. bufo*) obtained by the two methods of monitoring (figure 2).

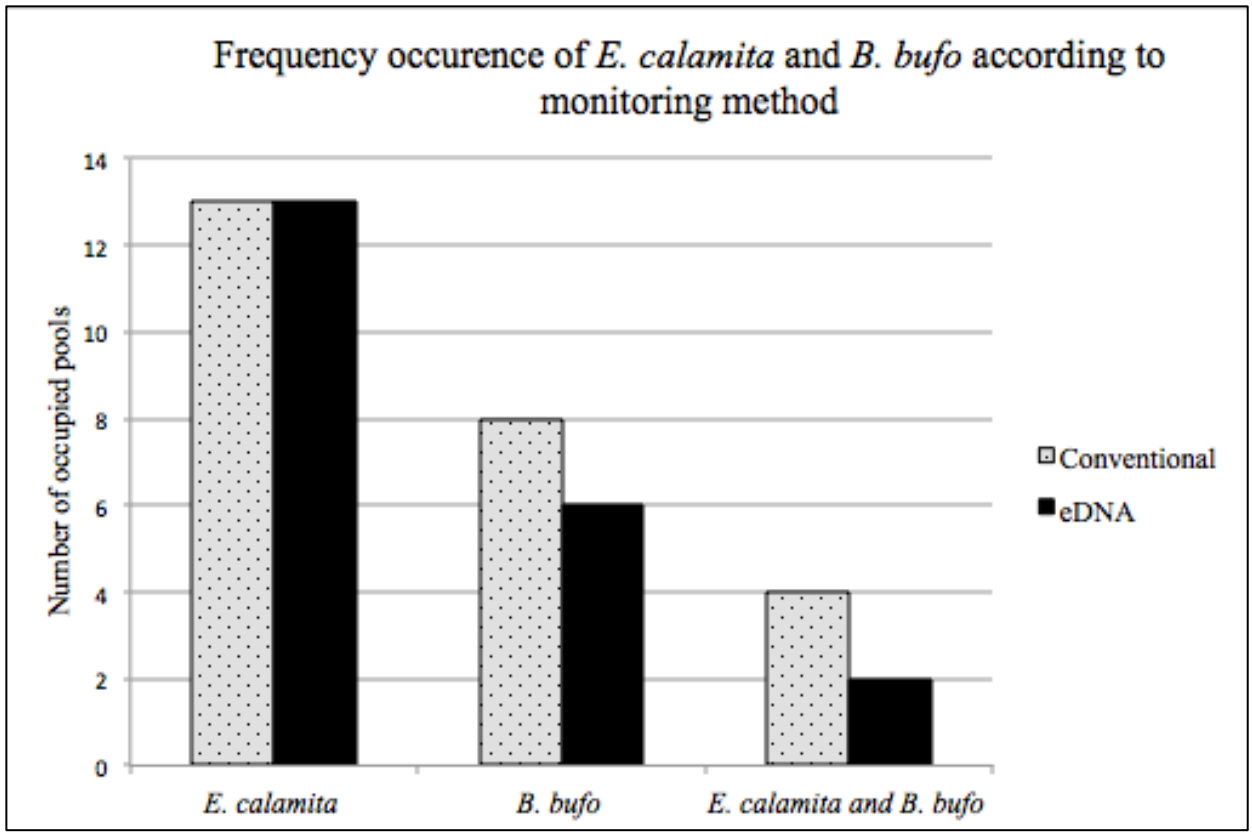


Fig. 2. Frequency occurrence of *E. calamita* and *B. bufo* according to method. Number of occupied pools on the y-axis and the total occurrence of *E. calamita* and *B. bufo* on the x-axis. *B. bufo* was observed in the field with *E. calamita* in 4/8 sites based on conventional methods and 2/6 based on the eDNA analysis. Each cluster consisting of two columns representing either conventional method (counting of tadpoles) or eDNA.

A sign test was performed to examine the relation between the results (presence or absence of *E. calamita* and *B. bufo*) and the monitoring method (conventional or eDNA). The results were not significant (P = 1.0 for *E. calamita* and P > 0.68 for *B. bufo*). However, as there are only a few numbers of paired observations for *B. bufo* (total of 8 field observations and 6 positive eDNA results), the data on *B. bufo* does not fulfill the requirements for a robust analysis (figure 2 and table 3). The data on *E. calamita* fulfill the requirements (total of 13 field observations and 13 positive eDNA results, figure 2). The presence or absence by *E. calamita* is not significantly different between monitoring method and the results are thus not dependent on the type of monitoring (conventional or eDNA) (table 2).

There was consistency between approximately 79.5 % of occurrences (presence and absence) between the two monitoring methods. Furthermore, there was DNA from *E. calamita* in 4 additional waters, which did not have any field observations of the species (table 2). In two of these cases, *B. bufo* tadpoles were observed, but not confirmed by the subsequent eDNA analysis. For one of these samples, the eDNA analysis was not entirely conclusive, which entails that the amount of DNA was very low and that the positive results should be interpreted with caution. There was 4 field observations of *E. calamita* there was not confirmed by the eDNA analysis (table 2). Two of these water pools had low tadpoles densities (between 150-200), both had visual observations of *B. bufo* but eDNA confirmed only one of these observations. The third water pool that was not confirmed by eDNA had 10 spawn strings of *E. calamita*. The fourth had 1200 tadpoles.

Table 2. Detection of *E. calamita* using conventional monitoring methods and eDNA.

	Presence of eDNA	**Absence of eDNA**
Presence by conventional method	9	4
Absence by conventional method	4	22

Note: The number in each cell represents the frequency occurrence of *E. calamita* according to monitoring method.

There was consistency between approximately 85 % of occurrences (presence and absence) of *B. bufo* between the two monitoring methods. Furthermore, there was DNA from *B. bufo* in 2 additional waters, which did not have any field observations of the species (table 3). There was 4 field observations of *B. bufo* there was not confirmed by the eDNA analysis (table 3). In two of these cases there were field observations of *B. bufo* tadpoles and no *E. calamita*, but subsequent eDNA analysis revealed the presence of *E. calamita* and no *B. bufo*. In one water pool, tadpole *E. calamita* was observed together with larger tadpoles which was assumed to be *B. bufo*, this assumption could not be confirmed by the subsequent eDNA analysis.

Table 3. Detection of *B. bufo* using conventional monitoring methods and eDNA.

	Presence of eDNA	**Absence of eDNA**
Presence by conventional method	4	4
Absence by conventional method	2	29

Note: The number in each cell represents the frequency occurrence of *B. bufo* according to monitoring method.

Coexistence of E. calamita and B. bufo

In Västra Götaland 6 out of 75 samples had DNA from *B. bufo*, of these, non had DNA from *E. calamita* (figure 3). Chi-square test of independence performed to examine the relation between the occurrence of *E. calamita* and *B. bufo*, did confirm a significant relation between the variables ($\chi^2 = 3.89$, d.f. = 1, $P < 0.050$). However, as more than 20 % of the expected values are less than 5, this data does not fulfill the requirements for a robust analysis. The low expected frequencies are presumably a result of the few samples with DNA from *B. bufo* (figure 3).

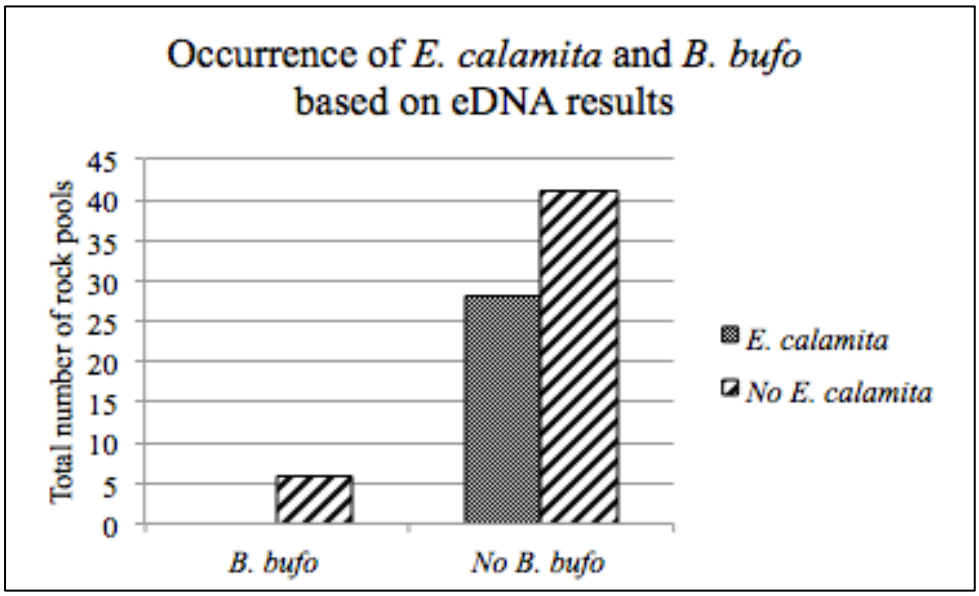


Fig. 3. The occurrence of *E. calamita* and *B. bufo* based on eDNA results from Västra Götaland. The total number of rock pools on the y-axis and the presence or absence of *B. bufo* on the x-axis. Each group consists of two clusters, representing the number of times *E. calamita* where either present or not present according to results of the eDNA analysis.

In Skåne, Blekinge and Halland *E. calamita* and *B. bufo* shared pools in up to four cases (figure 2), chi-square test of independence performed to examine the relation between the occurrence of *E. calamita* and *B. bufo*, did not confirm any significant relation between the variables (field: $\chi^2 = 1.26$, d.f. = 1, P = 0.26 and eDNA: $\chi^2 = 0$, d.f. = 1, P = 1). The presence or absence of the species seems to be independent of the presence or absence of the other species.

Discussion

It is important to gain knowledge on how eDNA works compared to conventional monitoring methods, in order to evaluate it as a compliment to traditional field surveys. In this study, eDNA was evaluated as a compliment to the biogeographical monitoring of *E. calamita* in its entire distribution range in Sweden. In Västra Götaland, the habitat consists of rock-pools distributed on small islands in Kattegatt. In Skåne, Blekinge and Halland, a much wider range of habitats were included; coastal rock-pools, heath meadows, gravel pits, ponds in forested environments and shallow ponds in coastal meadows on the island of Balgö. Besides differences in habitats, two slightly different methodologies in survey and sampling were applied. The results from Västra Götaland and Skåne, Blekinge and Halland were also different, demonstrating the importance of assessments on the efficiency of eDNA before implementation and the need for precautions when evaluating the results.

Comparison of detection by conventional methods and eDNA – frequency of occurrence

In Västra Götaland there was a significant difference in the species' frequency distribution (number of ponds with *E. calamita*) obtained by the two methods of monitoring. In contrast, the results from Skåne, Blekinge and Halland suggest that there was not a significant difference between the frequency distribution and the monitoring methods. In Västra

Götaland conventional monitoring showed occurrence of *E. calamita* in 53 % of the visited pools whereas eDNA only showed 37 %. In Skåne, Blekinge and Halland both methods showed occurrence of *E. calamita* in 33 % of the visited wetlands. The difference in results between counties could be a result of difference in methodology e.g. sample sizes and the water sample collecting procedure. In Skåne, Blekinge and Halland the sample sizes varied from 100-500 mL depending on the size of the wetland, and the water was collected at different locations within the wetland. In Västra Götaland one sample of 50 mL (at one location) was taken from each rock-pool. The low frequency occurrence by eDNA in this county, could be affected by sampling method. Sample sizes in other studies of eDNA have ranged from 15 mL - 10 L depending on the size of the water. Small waters or mesocosms having the lowest sample size and streams the largest (Rees et al 2014). Studies similar to this, have successfully used small volumes of 15 mL, but have taken 3 samples per water body at different locations (Thomsen et al 2012, Dejean et al 2012, Ficetola et al 2008). Rees et al (2014) argue that three samples for water bodies such as ponds should be standardized methodology. This might not be possible in all cases with *E. calamita*, as the species typically exists in small and shallow wetlands, thus collecting the samples from multiple locations instead could ensure a reasonable detection probability as it did in Skåne, Blekinge and Halland.

I suggest the following modifications of the protocol to accommodate the low frequency occurrence in Västra Götaland:

Increasing the sample size to 100 – 500 mL dependent on the size of the rock-pools, in addition to collecting the sample from different locations within the rock-pool. This methodology worked well in Skåne, Blekinge and Halland, where the frequency occurrence was equal between the monitoring methods. Alternatively, three samples pooled from different locations in the water body could be used, as this approach has been used successfully in other studies (Thomsen et al 2012, Dejean et al 2012, Ficetola et al 2008). Collecting multiple water samples in individual containers' makes it possible to see how many replicates needed for verification of field observations by eDNA (S. Bensch pers. comm.). If the two methods of monitoring would result in the same frequencies, then eDNA surveys could be an effective tool, in relation to the bigogeographical monitoring, for comparing occurrence frequencies between years, emphasizing that "eDNA, like any other monitoring approach, will only detect a proportion of the total sites occupied by a given species" (Thomsen and Willerslev 2015).

Comparison of detection by conventional methods and eDNA – site specific occurrence

In Västra Götaland, there was a significant difference between the results obtained by the two monitoring methods. The presence or absence of *E. calamita* in this county, is thus dependent on the type of monitoring (conventional or eDNA). In Skåne, Blekinge and Halland, there was not a significant difference between the results obtained by the two monitoring methods. It seems reasonable that presence or absence of a given species to some extent will depend on the monitoring method, as a result of wetland environment and the species' use of that particular wetland. Characteristics such as overgrown wetlands or water with high humic content, makes it difficult to do conventional monitoring, and some tadpoles may be missed. Furthermore, some wetlands might not be used for breeding or it might be to

early in the season. The species might also exist in very low densities or has already left the water for the time of inventory. In addition, abiotic factors such as warm water and UV radiation, could be factors that would result in a rapid DNA degradation, thus leading to different results in presence compared to conventional monitoring (Barnes et al 2014, Goldberg et al 2016, N. Gyllenstrand, pers. comm.). The overall detection success by eDNA verified by field surveys was approximately 73 % for *E. calamita* in Västra Götaland, 79.5 % for *E. calamita* and 85 % for *B. bufo* in Skåne, Blekinge and Halland. These findings are comparable to the study of Thomsen et al (2012) that had 91-100 % detection success with other amphibians. In total, there were 8 wetlands with DNA from *E. calamita* (4 in Västra Götaland and 4 in Skåne, Blekinge and Halland), which did not have any field observations of the species. Overall, it seems like a true result, due to occurrence of *E. calamita* on the islands of Västra Götaland and possibly ”wrong” species identification in Skåne, Blekinge and Halland. Moreover, one site had eDNA results that was not entirely conclusive, which entails that the amount of DNA was very low and that the positive results should be interpreted with caution. However, positives originating from the presence of dead animals or transferred by a predator (Rees et al 2014) cannot be rejected. In this study, it seems less likely that potential false positives could be a result of “low specificity of the primers and probes, and non-target template competition” (Rees et al 2014), as this would have resulted in a more general problem. Other studies have suggested that additional samples positive for DNA could be a result of the greater sensitivity of eDNA compared to conventional methods (Thomsen et al 2012, Dejean et al 2012 and Pilliod et al 2013), which seems plausible in this study as well.

Overall, there were 20 cases where field observations of *E. calamita* were not confirmed by the presence of eDNA. 5 cases could be due to ”wrong” species identification as DNA *B. bufo* were present instead of *E. calamita*. The field observations ranged from 1-3 adults and estimated 150-300 tadpoles. The remaining 15 cases of field observations that were not confirmed by the presence eDNA can possibly be assigned to false negatives. In Skåne, Blekinge and Halland, there were three observations of *E. calamita* in the field (10 spawn strings and 200-1200 juveniles) that were not confirmed by eDNA analysis. In Västra Götaland there were 12 observations of *E. calamita* in the field (1-3 adults, 7 spawn strings and 10-1000 tadpoles) that were not confirmed by eDNA analysis.

I hypothesized that eDNA should work well for detection of *E. calamita* in wetlands that are small and shallow, as these characteristics would ensure a high concentration of DNA if the species is present or have been recently present in relation to time of sampling. However, these characteristics might also work in favor of false negatives as these type of waters are typically warm and would receive high inputs of UV-radiation, which can result in a rapid DNA degradation (Barnes et al 2014, Goldberg et al 2016). Furthermore, the chemical composition in all rock pools in Västra Götaland is highly affected by the surrounding Kattegat in terms of salinity and dilution (Pröjt, 2012). According to Barnes et al (2014) salinity can mediate reactions between DNA and the sediment. The rate of dilution in the rock-pools could also result in DNA amounts below a detectable threshold (Rees et al 2012). 5 samples in Västra Götaland were excluded due to inhibitors. Inhibitors could be humic acids or humic substances (Thomsen and Willerslev 2015).

I suggest three possible modifications to accommodate the number of false negatives in the biogeographical monitoring:

First, increase the number of PCR replicates. In this study three PCR replicates per sample were analyzed for each sample. If all three subsamples were negative for a wetland, in which tadpoles were observed, three new subsamples were analyzed for DNA. Other studies have analyzed three to six PCR replicates per sample, besides having three samples per waterbody, resulting in up to 15 PCR replicates (Thomsen et al 2012, Dejean et al 2012, Ficetola et al 2008, Goldberg et al 2011). Ficetola et al (2008) further differentiated between the number of ponds with positive water samples and the number of positive PCRs. The study showed that the amplification success was significantly higher in ponds with high density of *L. catesbeianus* compared to the amplification success when the density was low. When the relative density of *L. catesbeianus* was low, number of positive water samples varied between 2/3-3/3 and the number of positive PCRs between 2/9-6/9. "Variation in eDNA shedding rates among species, sexes, ages, seasons and habitat characteristics" can also influence species eDNA concentration in a sample (Goldberg et al 2016) and it is unknown how much DNA *E. calamita* release into the environment. This needs to be experimentally verified. Furthermore, these types of water environments are more unstable compared to lakes or oceans in physical and chemical parameters, which can affect the quality and amount of the DNA (N. Gyllenstrand, pers. comm.). These uncertainties can motivate increasing the number of PCR replicates. Thus, increasing the number of PCR replicates, could lead to an increase in positive samples, where the DNA potentially could be present because of e.g. feces deposited by a predator (Rees et al 2014). Thus, in relation to the biogeographical monitoring, these types of positives are preferred over false negatives, as it could still provide some knowledge of presence of the species in an area.

Second, using a smaller filter size. In this study the samples were filtered in the laboratory through a 45 μm filter similar to Goldberg et al (2011). The length of the mitochondrial DNA (mtDNA) is approximately 3 μm, as this degrade, it breaks into smaller pieces that may be missed. Using a 22 μm filter, would potentially increase the amount of DNA captured during the filtering process (N. Gyllenstrand, pers. comm.). Though considering that humic acids or humic substances can clog filters and function as inhibitors in PCR reactions, it might not be optimal to implement smaller mesh sizes in the monitoring (Goldberg et al 2016, Thomsen and Willerslev 2015). Rather than using the same approach to all waters, using smaller mesh sizes could be implemented in Västra Götaland, as it seems the problem with false negatives are more pronounced here. However, using smaller mesh sizes could increase the risk of false positives (e.g. the species is not currently present or the DNA could originate from predators feces), as smaller DNA fragments are tolerated. Still, in relation to the biogeographical monitoring, false positives are preferred over false negatives.

Third, changing the filtration procedure to filtration at site. Filtration at site would further reduce the degradation time and secure immediate preservation (Goldberg et al 2016). However, there have been divergent results of the benefits of different filtration procedures (Pilliod et al 2013, Yamanaka et al 2016). The study of Pilliod et al (2013) suggested that different filtration procedures have the same probability of detection which contrast to the results of Yamanaka et al (2016). The study of Yamanaka et al (2016) showed that on-site filtration and direct preservation of the filters on ice, had higher DNA concentration,

compared to transportation of samples on ice and transportation of sample at ambient temperature followed by filtration process in the laboratory. Yamanaka et al (2016) suggest on-the-road filtration of the samples, to reduce time in the field. However, in relation to the bigogeographical monitoring this methodology would be impractical, both in relation to the transportation of ice in the field but also due to the time it takes to filtrate samples. Filtration at site would further increase the time in the field, thus decreasing the number of sites visited during one day and thus, increasing the cost of monitoring which is not optimal.

Coexistence of E. calamita and B. bufo

Data from all counties suggest that the presence or absence of the *E. calamita* is independent of the presence or absence of *B. bufo*. However, there were few field observations and samples with *B. bufo*. *B. bufo* was only present in 6 rock-pools in Västra Götaland, in which *E. calamita* did not occur. In Skåne, Blekinge and Halland *B. bufo* were present in 6-8 wetlands, of which there where only 2 - 4 cases where *B. bufo* shared pools with *E. calamita*.

Based on this study design, it is not possible to explain the pattern of occurrence by the two species as there are many other factors unaccounted for that can affect the occurrence of *E. calamita*, such as conductivity (Stenmark and Segerlind, 2015). Though, based on the small occurence of *B. bufo* in these areas, the presence does not seem to be a problem for *E. calamita* from a conservation management perspective.

Conclusions and future studies

The overall goal of this study was to 1) compare the detection rate of eDNA with the detection rate of the conventional field method of counting tadpoles, spawn strings and, for Västra Götaland, adults, and 2) investigate if and to what extent *E. calamita* and *B. bufo* coexist in *calamita* ponds in Västra Götaland and the counties Skåne, Blekinge, Halland, encompassing the entire distribution range in Sweden.

There was a high detection success by eDNA verified by field surveys - approximately 73 – 79.5 % for *E. calamita* and approximately 85 % for *B. bufo*. In this study eDNA proved to be a useful tool for species identification and species occurrence, even for the common species *B. bufo*. The assurence of species identification and low amount of potential false positives and negatives, provide justification for the usefullness of eDNA in the national monitoring of *E. calamita*. Though, the fact that the results were different between Västra Götaland and Skåne, Halland and Blekinge, suggest that eDNA should not just be used without precautions. Besides the possibility of bias due to different personal, there was also a difference in the methodology and the wetland environments, that could have resulted in the high amount of false negatives from Västra Götaland. Overall, these results indicate that there is a need for site specific protocols to assure the most reliable results for all counties.

Furthermore, the data suggest that *E. calamita* in these areas coexist with *B. bufo* in some wetlands. Though, based on the small occurrence of *B. bufo* in these areas, the presence does not seem to be a problem for *E. calamita* from a conservation management perspective.

Amphibians are one of the most threatened animal groups worldwide and many are, like *E. calamita*, rare and threatened. Subsequently, conventional monitoring of such species

encompasses difficulties and ethical considerations. New tools in conservation management are therefore meet with appreciation, eDNA being one of these. The potential and future prospects of this tool are vast, but precautions should be taken when interpreting the results. This study have contributed to the knowledge of the benefit using eDNA as a compliment to traditional monitoring for better species identification and improved data on occurrence of species. Despite an overall high detection success, the amount of false negatives in this study also demonstrate that eDNA should not stand alone, as it could lead to false conclusions about species absence. Still, there is need for more research on which effect water chemistry, and other abiotic and biotic factors can have on the degradation of DNA (Thomsen et 2012, Goldberg et al 2016) and, site specific protocols need to be implemented.

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Appendix 1. Primers and probes, specification

Bufo bufo cytb	Seq 5'-3'
F-primer	CGGAACCGAACTTGTTCAG
R-primer	ATGAAGAAAAAGAAGGTGGAGT
Probe	[6FAM]CTCAGTAGATAACGCAACCCTGACACG[BHQ1]
Length	136 bp

Bufo calamita 16S	Seq 5'-3'
F-primer	TTACTTCACCAAGCAATATGACTATA
R-primer	TGTGTTGATGCTTAGATGCG
Probe	[6FAM]CACAATGTAACCTCCACGCTGAAAGAA[BHQ1]
Length	121 bp

Appendix 2. Västra Götaland

ID	Date (y/m/d	Name of island	North (Sweref99TM)	East (Sweref99 TM)	Number of calling males	Number of males that did not call	Number of females	Total number of adults	Number of spawn strings	Estimated number of tadpoles	Field observations (0=absent, 1=present)	eDNA *E. calamit*	eDNA *B. bufo*
1	160612	Norra Buskär	6475031	277523	0	1	0	1	0	0	1	Positiv 3/3	Negativ
2	160612	Norra Buskär	6475041	277510	0	0	0	0	0	0	0	Negativ	Negativ
3	160612	Norra Buskär	6475152	277444	0	0	0	0	0	0	0	Negativ	Negativ
4	160612	Norra Buskär	6475103	277414	0	0	1	1	0	0	1	Negativ	Negativ
5	160612	Norra Buskär	6475040	277355	0	0	1	1	0	1000	1	Positiv 3/3	Negativ
6	160612	Norra Buskär	6474968	277486	0	0	1	1	0	200	1	Negativ	Negativ
7	160612	Södra Buskär	6474853	277627	0	0	0	0	0	4000	1	Positiv 3/3	Negativ
8	160612	Södra Buskär	6474777	277686	0	0	0	0	0	1500	1	Positiv 3/3	Negativ
9	160612	Södra Buskär	6474500	277469	0	0	1	1	0	0	1	Negativ	Negativ
10	160612	Södra Buskär	6474486	277427	0	0	0	0	0	0	0	Negativ	Negativ
11	160612	Södra Buskär	6474523	277354	0	0	0	0	0	0	0	Negativ	Negativ
12	170531	Holländareberget	6474939	278304						0	0	Negativ	Negativ
13	170531	Holländareberget	6474960	278317						700	1	Positiv 3/3	Negativ
14	170531	Holländareberget	6475066	278293						200	1	Negativ	Negativ
15	170531	Holländareberget	6475084	278608						200	1	Negativ	Negativ
16	170531	Holländareberget	6475096	278598						0	0	Negativ	Positiv 3/3
17	170531	Smögenön	6475017	278936						0	0	Negativ	Negativ
18	170531	Smögenön	6475088	278867						0	0	Negativ	Negativ
19	170531	Smögenön	6475106	278859						0	0	Negativ	Negativ
20	170531	Smögenön	6475134	278876						0	0	Negativ	Negativ
21	170531	Smögenön	6475130	278908						0	0	Negativ	Negativ
22	170531	Kleven	6474043	278471						600	1	Inhibitor	Inhibitor
23	170531	Kleven	6474048	278460						400	1	Inhibitor	Inhibitor
24	170531	Kleven	6474167	278461						0	0	Inhibitor	Inhibitor
25	170531	Kleven	6474146	278466						0	0	Inhibitor	Inhibitor
26	170531	Kleven	6474240	278601						0	0	Inhibitor	Inhibitor
27	160525	Måseskär	6445188	283955	4	0	1	5	20	50	1	Positiv 2/3	Negativ
28	160525	Måseskär	6445188	283968	2	0	0	2	7	10	1	Negativ	Negativ
29	160525	Måseskär	6445227	283907	1	1	2	4	5	20	1	Positiv 2/3	Negativ
30	160525	Måseskär	6445255	283868	0	0	0	0	0	0	0	Negativ	Negativ
31	160525	Måseskär	6445146	283976	0	0	0	0	0	0	0	Negativ	Negativ
32	160525	Måseskär	6444961	283852	1	8	8	17	0	0	1	Positiv 3/3	Negativ
33	160525	Måseskär	6444936	283854	1	5	4	10	5	0	1	Positiv 3/3	Negativ
34	160525	Måseskär	6445025	283828	0	4	2	6	1	0	1	Positiv 3/3	Negativ
35	160611	Altarholmen	6436090	290241	0	0	0	0	0	0	0	Negativ	Negativ
36	160611	Altarholmen	6436095	290252	0	3	0	9	0	150	1	Positiv 1/3	Negativ
37	160611	Altarholmen	6436056	290227	0	0	0	0	0	10	1	Positiv 2/3	Negativ
38	160611	Altarholmen	6436071	290445	0	0	0	2	0	0	1	Negativ	Negativ
39	160611	Altarholmen	6436060	290463	0	0	0	0	0	1000	1	Negativ	Negativ
40	160611	Altarholmen	6436027	290519	0	2	1	3	0	100	1	Positiv 2/3	Negativ

ID	Date (y/m/d	Name of island	North (Sweref99TM)	East (Sweref99 TM)	Number of calling males	Number of males that did not call	Number of females	Total number of adults	Number of spawn strings	Estimated number of tadpoles	Field observations (0=absent, 1=present)	eDNA *E. calamita*	eDNA *B. bufo*
41	160611	Altarholmen	6435929	290393	0	0	0	0	0	0	0	Positiv 2/3	Negativ
42	160611	Altarholmen	6435867	290282	0	0	0	0	0	0	0	Negativ	Negativ
43	160608	Hamneskär	6422593	290534	0	0	0	0	0	3000	1	Positiv 2/3	Negativ
44	160608	Hamneskär	6422598	290544	0	0	2	2	0	3000	1	Positiv 1/3	Negativ
45	160608	Hamneskär	6422587	290523	0	0	0	0	0	0	0	Negativ	Negativ
46	160608	Hamneskär	6422620	290561	0	0	0	0	0	30	1	Negativ	Negativ
47	160608	Hamneskär	6422618	290578	0	0	0	0	0	0	0	Negativ	Negativ
48	160608	Hamneskär	6422520	290564	0	0	0	0	0	0	0	Negativ	Negativ
49	160608	Hamneskär	6422592	290623	0	0	0	0	0	0	0	Negativ	Negativ
50	160608	Hamneskär	6422637	290612	0	0	0	0	0	0	0	Positiv 1/3	Negativ
51	160622	Lindholmen	6404373	298632	2	1	3	6	0	0	1	Positiv 1/3	Negativ
52	160622	Lindholmen	6404508	298502	0	0	0	0	0	100	1	Positiv 1/3	Negativ
53	160622	Lindholmen	6404513	298484	1	1	1	3	0	300	1	Positiv 1/3	Negativ
54	160622	Lindholmen	6404411	298397	0	0	0	0	0	0	0	Negativ	Negativ
55	160622	Lindholmen	6404290	298351	0	0	0	0	0	0	0	Positiv 1/3	Negativ
56	160622	Lindholmen	6404267	298356	0	0	0	0	0	200	1	Positiv 2/3	Negativ
57	160622	Lindholmen	6404065	298506	0	0	0	0	0	0	0	Positiv 3/3	Negativ
58	160622	Lindholmen	6404072	298524	3	0	0	3	0	0	1	Positiv 3/3	Negativ
59	160609	Södra In-Vinga	6394188	298139	0	0	0	1	0	0	1	Negativ	Positiv 1/3
60	160609	Södra In-Vinga	6394072	298324	0	0	0	0	0	1500	1	Positiv 3/3	Negativ
61	160609	Södra In-Vinga	6394069	298379	0	0	0	0	0	100	1	Positiv 3/3	Negativ
62	160609	Södra In-Vinga	6394077	298439	0	0	0	1	0	3000	1	Positiv 3/3	Negativ
63	160609	Södra In-Vinga	6393897	298214	0	0	0	0	0	0	0	Negativ	Negativ
64	160609	Södra In-Vinga	6394228	298178	0	0	0	0	0	0	0	Negativ	Negativ
65	160601	Lökholmen	6386001	305758	0	0	0	0	0	0	0	Negativ	Negativ
66	160601	Lökholmen	6386049	305729	1	0	1	2	0	0	1	Positiv 3/3	Negativ
67	160601	Lökholmen	6386074	305718	0	0	0	0	0	0	0	Negativ	Negativ
68	160601	Lökholmen	6386622	305495	0	0	0	0	0	0	0	Negativ	Negativ
69	160601	Lökholmen	6386644	305503	1	1	1	3	0	0	1	Negativ	Positiv 1/3
70	160601	Lökholmen	6386809	305503	1	1	1	3	0	0	1	Negativ	Negativ
71	160601	Lökholmen	6386805	305549	0	0	0	0	0	0	0	Negativ	Negativ
72	160601	Valö	6383152	308302	0	0	0	0	0	0	0	Negativ	Positiv 2/3
73	160601	Valö	6383105	308513	2	0	0	2	0	1000	1	Negativ	Negativ
74	160601	Valö	6383111	308508	0	0	0	1	0	300	1	Negativ	Positiv 2/3
75	160601	Valö	6383125	308553	0	0	0	0	0	0	0	Negativ	Negativ
76	160601	Valö	6383674	308334	0	0	0	0	0	0	0	Negativ	Negativ
77	160601	Valö	6383368	308674	0	0	0	0	0	0	0	Negativ	Negativ
78	160601	Valö	6383180	308668	0	0	0	0	0	0	0	Negativ	Negativ
79	160601	Valö	6383319	308680	0	0	0	0	0	500	1	Negativ	Negativ
80	160601	Valö	6383329	308674	0	0	0	0	0	100	1	Negativ	Positiv 2/3

Appendix 3

Number	Site name	County	East (Sweref 99TM)	North (Sweref 99TM)	Date (y/m/d)	Estimated number of tadpoles	Number of spawnstrings	Water sample (mL)	eDNA *E. calamita*	Field observation of E. calamita (0=absent, 1 = present)	eDNA *B. bufo*	Field observation of B. bufo (0=absent, 1 = present)	Additional observations
1	Flommen södra Damm 8	Skåne	361813	6139851	170628			500	Negativ	0	Negativ	0	Tadpoles of edible frog
2	Flommen södra Nabben, 2 dammar	Skåne	361496	6139190	170628	1100		500	Positive (1/3)	1	Negativ	0	Juveniles of edible frog, Lissotriton vulgaris,
3	Flommern södra, Damm 7 kanalen	Skåne	361808	6139930	170628			500	Negativ	0	Negativ	0	
4	Vomb SO	Skåne	409049	6167657	170622			500	Negativ	0	Positive (3/3)	1	Common toad and Smooth newt
5	Huvudlokal Vik, Prästens badkar	Skåne	455631	6163317	170609		1	100	Positiv (3/3)	1	Positiv (3/3)	0	
6	Huvudlokal Vik	Skåne	455580	6163354	170609	200		100	Negativ	1	Negativ	1	
7	Huvudlokal Vik	Skåne	455555	6163378	170609	150		100	Negativ	1	Positiv (3/3)	1	
8	Huvudlokal Vik	Skåne	455584	6163352	170609		2	100	Positiv (2/3)	1	Negativ	0	
9	Huvudlokal Vik	Skåne	455598	6163336	170609	150		100	Positiv (3/3)	1	Negativ	0	
10	Åhus, 90-16, norra grustaget	Skåne	453852	6199090	170609			500	Positiv (3/3)	0	Negativ	1	Tadpole *B. bufo*. Site overgrown with reed
11	Horna grushåla, SO	Skåne	453897	6198891	170609		10	500	Negativ	1	Negativ	0	
12	Rinkaby skjutfält, Lokal 90-10, Pers damm	Skåne	455855	6204804	170615			100	Negativ	0	Negativ	0	
13	Huvudlokal Landön-Hammaren	Skåne	460319	6201805	170614			100	Positiv (1/3)	0	Negativ	0	
14	Dammen, rondellen Bromölla	Skåne	468773	6213049	170614			500	Negativ	0	Negativ	0	
15	Huvudlokal 4 nya dammarna norr E22 Brom	Skåne	467826	6213151	170614			500	Positive (1/3)*	0	Negativ	0	
16	Huvudlokal 4 nya dammarna norr E22 Brom	Skåne	468103	6213119	170614			500	Negativ	0	Negativ	0	No amphibians observed, but appropriate water for *E. calamita*
17	Huvudlokal 4 nya dammarna norr E22 Brom	Skåne	468217	6213113	170614			500	Positive 1/3	0	Negativ	1	Tadpole *B. bufo* (?), a lot of vegetation, does not seems appropriate as habitat for *E. calam*
18	Järavallen, norra sjön	Skåne	371431	6188063	170628			200	Negativ	0	Negativ	0	
19	Järavallen, södra sjön	Skåne	371557	6187313	170628			500	Negativ	0	Positive 1/3	1	Common toad and Smooth newt
20	Sölve västra	Blekinge	476029	6211930	170519			500	Negativ	0	Negativ	0	Smooth newt
21	Sölve östra	Blekinge	476088	6211919	170519	250		500	Positiv (1/3)	1	Negativ	0	Smooth newt
22	Lörby grustag	Blekinge	482488	6215478	170519			500	Negativ	0	Negativ	0	Smooth newt
23	Hanö, Vindhalla 1	Blekinge	491011	6206942	170519	1200		500	Negativ	1	Negativ	0	Smooth newt and Great crested newt
24	Attanäs, NV om, ursprungliga vattnet 1	Blekinge	558347	6225765	170515			500	Negativ	0	Negativ	0	
25	Krogsnäs	Blekinge	564762	6241456	170515			500	Negativ	0	Positive (2/3)	0	
26	Bredaviks udde, nya dammen	Blekinge	565309	6240557	170515			500	Negativ	0	Negativ	0	
27	Bredaviks udde, ursprunglig damm	Blekinge	565247	6240503	170515			500	Negativ	0	Negativ	0	
28	Gullholma 1 (nygrävt)	Blekinge	559292	6226502	170515			500	Negativ	0	Negativ	0	
29	Strandpöl Balgö	Halland (Baljö)	328344	6338738	170619			500	Negativ	0	Negativ	0	Smooth newts
30	Strandpöl Balgö	Halland (Baljö)	328424	6338694	170619	50		500	Positiv (1/3)	1	Positive (1/3)	1	Tadpoles mainly from Common toad
31	Strandpöl Balgö	Halland (Baljö)	328658	6338648	170619			500	Negativ	0	Negativ	0	
32	Strandpöl Balgö	Halland (Baljö)	328838	6338558	170619	2		100	Positiv (1/3)*	1	Negativ	0	
33	Strandpöl Balgö	Halland (Baljö)	328870	6338540	170619			100	Negativ	0	Negativ	0	
34	Strandpöl Balgö	Halland (Baljö)	328929	6338511	170619			500	Negativ	0	Negativ	0	Common frog tadpoles
35	Sandbank på västra Balgö	Halland (Baljö)	328898	6338403	170619			500	Negativ	0	Negativ	0	Larvae of Smooth newt
36	Strandpöl Balgö	Halland (Baljö)	328932	6338430	170619			100	Negativ	0	Negativ	0	
37	Strandpöl Balgö	Halland (Baljö)	328985	6338428	170619			500	Negativ	0	Negativ	0	
38	Sandbank på västra Balgö	Halland (Baljö)	329261	6338971	170619	1000		500	Positiv (2/3)	1	Negativ	1	Larger tadpoles presumebly from Common toad, observations of Smooth newt
39	Strandpöl Balgö	Halland (Baljö)	329823	6339441	170619	2500		100	Positive (1/3)	1	Negativ	0	