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Cuticular Swabs and eDNA as Non-Invasive Sampling Techniques to Monitor *Aphanomyces astaci* in Endangered White-Clawed Crayfish (*Austropotamobius pallipes* Complex)

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Abstract: In endangered crayfish conservation projects, it is paramount to map the distribution of the causative agent of crayfish plague, *Aphanomyces astaci*, in native populations. Considering the inapplicability of the destructive cuticular sampling protocol for monitoring endangered populations, we explored the use of non-invasive sampling techniques to detect this pathogen with molecular assays. In the present study, we exploited environmental DNA (testing increasing water volumes combined with different filter porosities) and cuticular swabs to collect *A. astaci* DNA. In addition, we evaluated the impact of the storage method on DNA preservation during field activities. After the first evaluations performed on both highly infected *Austropotamobius pallipes* and carrier *Procambarus clarkii* specimens in laboratory conditions, these sampling techniques were applied to wild populations of white-clawed crayfish. Our findings highlight better results with the filtration of 5 L of water with filters of 2.7 µm porosity for eDNA analysis and demonstrate that cuticular swabbing is equally effective as the World Organisation of Animal Health's protocol. Storage in absolute ethanol proved to be the best solution to preserve swabs and filter samples for up to a week at room temperature. In conclusion, we suggest an integration of both sampling methods when monitoring *A. astaci* for conservation purposes.

Keywords: quantitative PCR; non-indigenous crayfish species; crayfish plague; monitoring project

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1. Introduction

The genus *Austropotamobius*, Skorikov, 1907 [1], hosts some of the largest freshwater crustaceans native to central and southern Europe, largely widespread in the past from the Iberian Peninsula to the Balkans and from the United Kingdom to the south of Italy [2]. Genetic studies performed by many research groups currently subdivide the genus into four species: *Austropotamobius torrentium* (von Schrank, 1803) [3]; *A. fulcisianus* (Ninni, 1886) [4]; *A. pallipes* (Lereboullet, 1858) [5], which is the principal exponent of the genus; and the recently described *A. bihariensis* (Pârvulescu, 2019) [6]. Moreover, taxonomical studies unravelled hidden biodiversity within the white-clawed crayfish *A. pallipes*, ranking it as a complex species [7]. The white-clawed crayfish is genetically distinct in two lineages, both present in the Italian peninsula: *A. pallipes sensu stricto* and *A. italicus*. The first is located in the north-western regions and surpasses the Alps spreading across Europe. The second is widespread in the rest of the Italian territory and encompasses four structured clades: *A. italicus carinthiacus*, *carsicus*, *italicus*, and *meridionalis* [8]. *Austropotamobius* spp. have nocturnal habits and are frequently found in creeks or brooks in mountainous areas within mid-high altitudes and abundant riparian vegetation in cool, clean and well-oxygenated

waters [9,10]. Nowadays, these populations constitute isolated reproductive units, often confined to single mountain streams or separate basins [11]. Like other crayfish, *Austropotamobius* spp. play a fundamental ecological role in the benthic region and are considered one of the keystone organisms in freshwater environments [12]. They control the freshwater community by feeding on benthic fauna and algae, contributing to sedimentation with their burrowing activities. Furthermore, the species are well integrated into the trophic chain, being predated by fish, birds and mammals [12,13]. Therefore, their sudden loss severely compromises freshwater ecosystems [13]. The distribution of the *A. pallipes* complex suffers from a rapid contraction and fragmentation. Indeed, several Italian regions and other European countries have documented a significant population decrease in white-clawed crayfish since the 1970s [14–17]. The decline of native crayfish has been attributed to multiple causes: the expansion of human settlements, causing habitat modification and water pollution; the invasion of alien species; and the spread of infectious diseases [14,17–19]. Furthermore, this condition is exacerbated by its slow growth rate and low fecundity compared to invasive crayfish [14,18]. Consequentially, *A. pallipes* is listed in the IUCN Red List of Threatened Species [20] and it is included in the primary conservation lists, in the annexes of the EU Habitats Directive and in several regional laws, which prohibit its capture, transport and trade.

The crayfish plague disease, caused by the oomycete *Aphanomyces astaci* Schikora, 1906, represents the main threat to European indigenous crayfish species (ICS). This water mould, belonging to the Leptolegniaceae family, infects crayfish by penetrating through the cuticle and, in susceptible species, proliferating into the dermis and underlying tissues. Zoosporangia are then produced on the outer cuticular surface and release biflagellate zoospores of about 8–12 µm in the water [21–23]. Zoospores are chemotactically attracted to crayfish cuticle and motile for up to 3 days. They can persist through cysts in a moist environment for several weeks and repeat the motile-resistance phases up to three times, thus extending their infectivity [22–24]. The spread of the zoospores can occur at any time during the year, but it is more likely in late spring and summer due to higher water temperatures that cause an increase in its metabolic activity [25]. This obligate parasite has co-evolved with North American crayfish species [26,27], which act as biological carriers, while ICS appear more susceptible. Since the second half of the 19th century, crayfish plague has caused the mass mortality of ICS throughout Europe, starting from the Lombardy region in Italy where the disease was first described [28]. Since 1960, the subsequent introduction for farming of non-indigenous crayfish species (NICS) from North America [29–31] has spread the pathogen to other European countries [32–38]. The studies carried out with different marker genes (RAPD, mitochondrial DNA, chitinase) [39–41] on *A. astaci* isolates reveal five genetic clusters (genotypes A–E) linked to specific host species [42]. Genotype A is related to its first introduction in Europe [43] and has been isolated from native crayfish *Astacus* sp. [44] and *Austropotamobius* sp. [45,46]. Furthermore, in some cases, this genotype exhibits a low pathogenicity, persisting with a low prevalence in ICS populations acting as carriers [39]. Further genotypes (B and C) have been isolated in Europe from imported signal crayfish (*Pacifastacus leniusculus* (Dana, 1852) [47]), while genotype D has been detected in red swamp crayfish (*Procambarus clarkii* (Girard, 1852) [48]) [43,49–51] and has been described as highly pathogenic in indigenous crayfish [52]. Lastly, genotype E has been found in an introduced population of spiny-cheek crayfish (*Faxonius limosus* (Rafinesque, 1817) [53]) [54]. Whereas NICS are co-adapted to *A. astaci* and often carry it on the cuticle without showing clinical signs of the disease [55], in white-clawed crayfish the infection often progresses in an outbreak with high mortality. Although resistance phenomena are documented in white-clawed crayfish populations, they are usually limited to genotypes with low pathogenicity (A) and mainly depend on the population's health status [46,52,56]. Therefore, given its high lethality and invasive potential, *A. astaci* is considered one of the 100 worst alien species in the world [57]. Furthermore, crayfish plague outbreaks are notifiable to the World Organisation of Animal Health (WOAH: formerly OIE—2019), which gives technical guidelines to monitor crayfish, and collect and

analyse samples (OIE 2019—Manual of Diagnostic Tests for Aquatic Animals). The WOAH disciplines the protocol on suspected outbreaks and states that the surveillance of wild crayfish populations is complicated, describing only the sampling of the cuticle performed on recently dead or sacrificed specimens [58].

Over the years, the EU has funded conservation projects to protect and preserve white-clawed crayfish and their habitat through the financial instrument of the LIFE program. In Italy, these included the CRAINat (LIFE08 NAT/IT/000352), RARITY (LIFE10 NAT/IT/000239) projects and the ongoing LIFE-CLAW project (LIFE18 NAT/IT/000806), aimed at monitoring the presence of *A. astaci*, counteracting the dispersal of NICS and protecting and increasing the stocks of white-clawed crayfish populations through the reintroduction of juveniles produced in breeding facilities [59]. Health monitoring of ICS populations, for mapping *A. astaci* distribution and selecting sites for broodstock recruitment to prevent the introduction of carriers into farms, is paramount to increase the chances of conserving and strengthening the species. Furthermore, the circulation of low pathogenic genotype A in farmed *A. pallipes* can result in crayfish plague outbreaks when animals are stressed [56,60]. The protocols suggested in the WOAH manual require dead or sacrificed specimens, but their inapplicability in wild and healthy endangered populations compels the exploration of alternative, non-destructive, techniques, which should agree with the results achieved by destructive standard procedures.

Environmental DNA (eDNA) analysis has quickly become one of the most valuable techniques to assess the presence of multiple species [61–66]. Indeed, this convenient method is often applied for both ecological and conservation aims [67–69] and pathology/epidemiological studies [70–72]. Although several methodological studies have been carried out, protocols were often modified ad hoc, according to the experimental purposes and the environmental features (i.e., epidemiology/conservation; lentic/lotic environment) [73]. The eDNA analysis ensures a clear advantage in detecting biodiversity, such as relative species abundance in aquatic and terrestrial ecosystems [74–76], focussing the assay on the genetic material diluted in an environmental sample, often recovered by concentrating a volume of air, water, or soil [61,77]. The DNA released by organisms dispersed in the environment is found free, adherent to debris, in cell fragments, spores or other reproductive forms. For this reason, it is crucial to develop the correct sampling techniques and sample storage methods, while DNA extraction can rely on already established protocols or commercial kits [61,78]. The success of the analyses also depends on the target dilution and the presence of contaminants and/or inhibitors in soils and sediments, which may affect the quality of the extracted DNA and its downstream applications [79–81]. Hence, parallel sampling, applying more than one technique, should be considered at least in the initial stages.

Focussing on crayfish, two non-invasive sampling methods are described in the literature: swabbing via disposable swabs [60] and scrubbing with brushes [82] to collect the epibiotic communities. We prefer to test and optimise the first protocol due to its higher practicality on field sampling and considering it less prone to cross-contamination. The present work further investigates using practical and cheap storage solutions to preserve samples during field activities.

Therefore, the aims of the present work are to (i) compare the performance of detecting *A. astaci* DNA with invasive sampling (extraction from cuticle following WOAH methods) and non-invasive sampling (swabs and eDNA) in carriers and susceptible crayfish; (ii) define the best-storing solution to preserve field samples until laboratory processing; and (iii) test the performance of non-invasive sampling in a pilot study on the field to monitor the presence of *A. astaci* in selected watercourses.

2. Materials and Methods

2.1. eDNA Sampling Procedure

Environmental DNA sampling procedures were adapted from the method proposed by Strand [64,83] to monitor the presence of crayfish and *A. astaci* in watercourses through

eDNA. The equipment was designed to be portable and easy to use, even in mountainous and difficult-to-access areas [84,85]. The technical part of the sampling protocol comprised a peristaltic garden pump driven by a cordless driver drill to vacuum the water through a filtering system. The latter, connected to the pump via an acetate hose (φ = 10 mm), is composed of a replaceable fibreglass filter (Whatman—Merck KGaA, Darmstadt, Germany) plugged in a filter holder (Nalgene—Merck KGaA) (Figure S1a). After filtration, filters are rolled up using tweezers and inserted in 15 mL Falcon tubes. Tweezers and the filtering system are properly disinfected with sodium hypochlorite 0.06% *v/v* (after each sampling) [86]. In the initial assessments, filters of different porosity were considered (0.45, 1.6 and 2.7 µm). Assuming that all selected filters trap zoospores (due to their size of 8–12 µm) and DNA adherent to the debris, the number of *A. astaci* detected through molecular assays should be proportional to the volume of water filtered. Details regarding the water volumes filtered and the filter porosity and storage conditions selected are provided in the sections below.

2.2. Cuticular Swabbing Procedure

The swabbing procedure was adapted from the first evaluation of Manfrin and Pretto [60], and it was carried out on the cuticle of crayfish by swabbing the surface with a thin (φ < 5 mm) disposable cotton swab (Cultiplast®; Code: 112698). In particular, we focused on the joints of pereiopods, ocular peduncles, telson and uropods, which reported the highest chance of *A. astaci* detection [87], followed by general swabbing of the dorsal and ventral surface of the abdomen and cephalothorax. Then, each swab was cut to remove the excess metal rod and its tip was stocked in a 1.5 mL Eppendorf tube; details about the storage conditions are provided below.

2.3. Non-Invasive Sampling in Carrier Species

The trial was performed under laboratory conditions in the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) aquarium facility, setting up a 300-litre plastic tank with hollow bricks as refuges to host wild red-swamp crayfish (*P. clarkii*) (Figure S1b). The experiment aimed to test the presence of *A. astaci* in the water, even in the absence of mass mortality, and to determine the best combination of filter size and filtered volume to ensure good reliability. Specimens were collected near the facility in early July 2020. We housed 33 crayfish of different sizes (cephalothorax length spanned 33–62 mm) for 8 weeks in the tank, feeding them with commercial trout pellets and changing half of the water volume once a week. These conditions allow *P. clarkii* to increase their cuticular infestation and the release of zoospores in the water column. After the first two weeks of acclimatisation, eDNA was sampled through the filtering system every two weeks until the end of the experiment, before feeding (to reduce turbidity) and water change. During the laboratory trial, we considered filters of three sizes (porosity: 0.45, 1.6 and 2.7 µm) and we assessed two different volumes of sampled water for every porosity to evaluate the best combination and the limits of the method (Figure 1a) [73,88]. The highest volume was chosen accordingly to the maximum volume of water that could be filtered by each porosity, to simulate the clogging of the filter. As expected, clogging happened more quickly with the size 0.45 µm filter, for which it was difficult to filter up to 1 L; this porosity was taken as a reference to compare results in quantitative analyses of *A. astaci*. Considered volumes spanned between 0.5 and 5 L: 0.5 L and 1 L were filtered with filters of 0.45 µm; 1 L and 2.5 L were evaluated with filters of 1.6 µm; while 3 L and 5 L were tested with filters of 2.7 µm. This evaluation helped to understand the performance of the filtering system in natural lotic conditions, where the creeks transport suspended particulate matter (debris, cells, fragments of food and faeces) that could easily clog the filters.

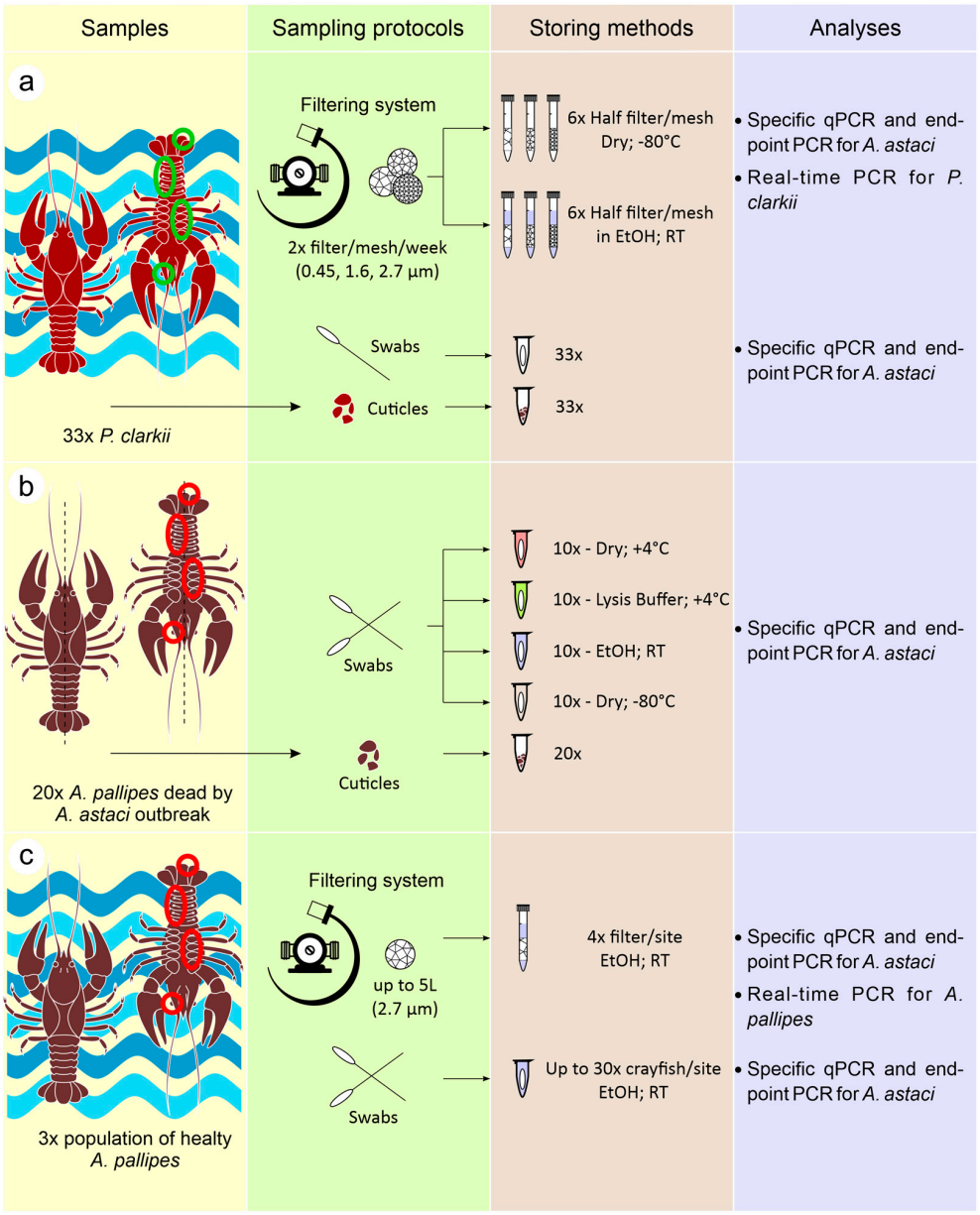

Figure 1. Experimental design. Graphical overview of the experimental design presented in the current work to summarise samples (*P. clarkii*, *A. pallipes*, water), sampling protocols (eDNA, swabs, cuticles), number of specimens and storing method (solution, temperature) and performed analyses: (**a**) Non-invasive sampling in carrier species; (**b**) Non-invasive sampling in susceptible species; (**c**) In-field tests using 3 wild populations.

The eighteen filters collected (6 per each porosity size) were cut in half and stored in 15 mL Falcon tubes, with each half stored either dry at −80 °C or in absolute ethanol (10 mL) at room temperature. At the end of the trial, total DNA was extracted and processed for molecular investigations. *Procambarus clarkii* were analysed specimen-by-specimen to compare the performance of cuticular swabbing and destructive cuticle sampling methods in carrier species. The crayfish were first swabbed (as described in Section 2.2) and then sacrificed by freezing (−80 °C for 48 h). Frozen specimens were thawed and dissected, collecting cuticle portions from pereiopod joints, telson and uropods, ocular peduncles and melanised spots (30–50 mg for each specimen). Both swabs and cuticular fragments were immediately extracted following the WOAH protocol summarised below. Finally, extracts were stored at −20 °C until further processing.

2.4. Non-Invasive Sampling in Susceptible Species

We investigated the reliability of non-invasive sampling and different conservation solutions to detect the presence of *A. astaci* in susceptible species, compared with the results obtained from testing cuticles (standard WOAH method). This test was performed on white-clawed crayfish (*A. pallipes* complex) collected during a crayfish plague outbreak in a restocking facility located in Friuli Venezia Giulia Region—Northern Italy, in December 2013 (LIFE RARITY project) [56,60]. Moribund or freshly dead specimens with gross signs of crayfish plague (loss of limbs, ataxia, paralysis and cuticular melanisation) were collected from tanks arranged with a recirculating water system. Crayfish specimens stored frozen at −20 °C since the plague episode were assumed as equally infested. Twenty specimens were thawed at room temperature and were virtually longitudinally divided by swabbing each half separately with a sterile cotton swab. Then, the 40 swabs were randomly mixed by simple random sampling strategy [89] and stored for a week with different strategies: ten in absolute ethanol at room temperature (EtOH 100%, RT), ten in the lysis buffer of QIAamp DNA Mini Kit (ATL buffer, +4 °C), and ten dry (+4 °C). The last group of ten swabs was kept dry at −80 °C, which is considered one of the best ways to preserve DNA samples [90–92]. Following the guidelines available at WOAH, fragments of abdominal cuticle, joints of pereiopods, telson and ocular peduncle of the 20 white-clawed crayfish were collected as previously described and immediately extracted and processed as described below (Figure 1b).

2.5. In-Field Tests

In order to ensure the applicability of non-invasive sampling methods, we selected three white-clawed crayfish populations from different creeks in the Tuscan-Emilian Apennine National Park, located between 800 and 1500 m above sea level. The watercourses were fordable creeks with a mean depth of 40 cm and mean width of 390 cm and were characterised by different flow rates: fast in A and slow in C and B (with the presence of litter in the second one). Considering the endangered status of the species *A. pallipes* and the continuing decline of populations [20], we prefer not to provide the exact coordinates of the populations, but we are willing to share them for research purposes.

At the end of July 2020, for each population up to 30 crayfish were swabbed and measured for the cephalothorax length. The swabs were stocked in absolute ethanol and then kept at room temperature until further processing. One month later, after the results had been obtained from both the in-house experiment and the swabs analyses, the same creeks were selected to test the eDNA protocol described above. The samplings were performed using suitable porosity (2.7 µm) and filtering water in 4 points per site, up to 5 L or until filter clogging (Figure 1c) as ascertained during in house experiment. The sampling points were set a few metres downstream from the crayfish populations (<10 m), where the creek creates pools with slow water current. Negative controls to detect possible contamination through air or aerosol were obtained by leaving a swab and a filter funnel exposed with a 0.45 µm filter plugged in during the sampling activities. The filters were stocked in absolute ethanol at room temperature until molecular analysis. Then, the results between eDNA and swabs were compared.

2.6. DNA Extraction

Total DNA was extracted from both swabs and cuticles, processing them separately, with the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Cuticles were ground with TissueLyser II (Qiagen GmbH) at 30 HZ for 30 s before the lysis step and then processed according to the manufacturer's protocol. Swabs stored in different storage solutions were treated similarly, modifying the first step for samples stored in ethanol since it can inhibit extraction and downstream applications. In this case, samples (swabs and filters) were removed from tubes discarding the ethanol. Then, the fixative residue was evaporated, leaving the samples for 10–15 min under a fume hood before the extraction. Eppendorf tubes of 1.5 mL were used during the lysis step to completely submerge the swabs with

200 µL of lysis buffer (ATL + proteinase K), as indicated by the kit protocol. DNA from filters was extracted using CTAB 2% protocol as described by Strand [64] and Rusch [85]. This protocol was applied in order to avoid the loss of small DNA fragments retained in the filter matrix or adsorbed to soil debris, and overcome the possible effect of inhibitors during the lysis and washing steps that could occur with a commercial kit. CTAB protocol was carried out by splitting the lysis volumes into two subsamples (a and b) and using 1.5 mL Eppendorf tubes during the precipitation and centrifugation steps instead of 2 mL Eppendorf tubes, to increase the visibility of the pellet during the wash steps. The volumes of the reagents from the published protocol were scaled proportionally to fit in 1.5 mL tubes. The final elution was performed in 50 µL of elution buffer for all the subsamples to avoid excessive dilution of the extract.

DNA integrity and quality were assessed respectively with agarose gel (0.8%) and NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), while DNA quantity was estimated with Qubit 4 Fluorometric (Thermo Fisher Scientific Inc.).

2.7. Molecular Assays

In the present manuscript, we referred to quantitative PCR (hereafter: qPCR) when the starting quantity of the molecular target in the samples was estimated using a standard curve in a Real-Time PCR (i.e., the absolute quantification performed with the assay for the detection of *A. astaci* DNA). Conversely, we used the more generic term "Real-Time PCR" when a qualitative assay (presence/absence) was performed (i.e., the molecular detection of crayfish species DNA) [93].

DNA extracted from cuticles, swabs and water samples were evaluated for the presence of *A. astaci* DNA using the qPCR protocol published by Vrålstad et al. [94]. The amplification protocol was modified in the annealing temperature, according to Rusch et al. [85] (Table S1), to minimise the possibility of false positives due to the presence of other *Aphanomyces* species in the watercourses (i.e., *Aphanomyces fennicus* [95]). Each qPCR was calibrated with 10-fold dilutions of synthetic DNA produced in-house, encompassing the ITS portion detected by the assay [94], to estimate the starting concentration in copies/µL (analogous to PCR Forming Units/µL) [96,97]. Quantification of the pathogen expressed in "agent levels" (A0–A6), as well as Limit of Detection (LoD: representing A1–A2 edge = 5 copies/µL) and Limit of Quantification (LoQ: A2–A3 edge = 50 copies/µL), follow Vrålstad et al. [94]. To summarise, we considered samples negative for *A. astaci* when no amplification was detected (A0) and positive when quantification levels were ≥5 copies/µL (≥A2). We chose to re-test samples with quantification levels between 0.1 and 5 copies/µL (A1) or without sigmoid curves to evaluate possible inhibition or non-specific products. This classification considers the presence of multiple copies of the ITS region along the oomycete genomes [98–100]. In particular, around 138 ITS-target region per zoospore was estimated in *A. astaci*; thus, the LoD of 5 copies/µL corresponds to 0.02–0.04 zoospore/µL. In addition, to avoid false positive results, we considered positive for *A. astaci* samples in which both replicates scored results ≥ LoD, with comparable estimation (meaning ranging in the same agent level). Furthermore, positive samples were subjected to end-point PCR and sequencing to confirm the results. End-point PCR was carried out with specific primers for *A. astaci* (primers 42/640) [87]. Direct amplification was considered with a quantification > 500 copies/µL (>A3 level). In contrast, samples with lower quantity (range LoD-LoQ = A2 level) were pre-amplified through nested PCR, using universal primers for the ITS region (primer ITS 1/ITS 4) in the first reaction [87,101,102]. All samples with suitable amplicons of *A. astaci* were subjected to Sanger sequencing to confirm the result via NCBI.

During the in-field tests, we investigated the inhibitions in Real-Time PCR with eDNA samples calculating the difference in cycle threshold (ΔC_t) between undiluted and 5-fold diluted templates [98,103]. Furthermore, the amplifiability of samples was assessed through Real-Time PCR and end-point PCR. For eDNA samples from the in-field and in-house experiment, published Real-Time PCR protocols to detect the presence of

A. pallipes and *P. clarkii* DNA were used to verify the DNA extraction efficacy [104,105]. The techniques targeted mitochondrial DNA genes, focussing on the fragment of *small subunit ribosomal RNA* (*16S rRNA*) and the barcoding region of *cytochrome c oxidase subunit I (cox1)*, respectively.

End-point and qPCR were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the conditions summarised in the Supplementary Materials (Table S1). Negative and positive controls were included in all amplifications, and when necessary, end-point PCR products were visualised by electrophoresis in 1% agarose gel.

2.8. Statistical Analyses

The data collected were validated and investigated using the software Stata 17.0 [106]. Main statistical measures, such as mean, standard deviation, median and quartiles, were calculated for quantitative variables to summarise the results from sampling types and volumes. In addition, Kruskal–Wallis nonparametric test for two or more independent samples (after the evaluation of its applicability through Levene's test), Wilcoxon signed-rank test on matched-pairs data and the Spearman rank correlation test were performed to evaluate possible differences between sampling types or volumes. If Kruskal–Wallis nonparametric test was significant, then Pairwise Comparisons were performed through Dunn's test. For the statistical analyses, a 5% level of significance was considered.

3. Results

3.1. Non-Invasive Sampling in Carrier Species

The qPCR recorded positive results for *A. astaci* in all eDNA samples (Figure 1a) and showed concordance between all replicates (standard deviation < 0.35). Considering that no difference between the two storing methods or sampling date was observed, the results were merged according to the filter size (Table S2 and Figure 2). The findings showed a significant positive correlation between the amount of *A. astaci* detected and the increase in filtered volumes ($n = 18$, $\rho = 0.7175$, p-value < 0.05). The highest average quantity was acquired by filtering 5 L of water with filters of 2.7 µm porosity, recording an A3 level and the results being significantly different, with higher values than those detected with lower porosities (Dunn's Pairwise Comparison test: p-value < 0.05). The amounts retrieved using 2.7 µm filters combined with 5 L (2.44×10^2 copies/µL $\pm$ 3.90×10^1) were comparable to results obtained by filtering 3 L (Dunn's Pairwise Comparison test: p-value > 0.05), and they recorded an initial level of *A. astaci* similar to those found by cuticular swabs (2.17×10^2 copies/µL). Considering filters of 0.45 µm and 1.6 µm, the detected levels of *A. astaci* set in the A2–A3 range were not significantly different (Dunn's Pairwise Comparison test: p-value = 1) among the different volumes tested. Instead, 1 L × 1.6 µm or 0.5 L × 0.45 µm combinations exhibited values of *A. astaci* quantification distributed over a wider range.

The *P. clarkii*-specific Real-Time PCR assay (Table S1) proved the lack of inhibitors in the eDNA samples, confirming the good quality of the extracts. Furthermore, the similar C_t values (22–24) retrieved from the filters indicated a comparable amount of starting genetic material. This finding suggests that the DNA shed by the red swamp crayfish was easily retained by the filters regardless of their porosity, probably because it was bound to debris and cell fragments [107].

At the end of the eDNA trial, 33 swabs and cuticle samples were collected from stabulated *P. clarkii* and directly extracted. The extracts were submitted to *A. astaci*-specific molecular assays (Figure 1a), amplifying the same template volumes despite the differences in quality and quantity of the DNA obtained from the two sampling protocols. The quantification through qPCR shows a substantial difference between the two sampling methods (Figure 3), detecting a considerable presence of *A. astaci* in 20 samples from swabs. While considering cuticles, only seven samples were undoubtedly positive. Thirteen specimens were entirely negative in both cuticle samples and swabs, probably due to

a recent moult or because they were in the early stages of infection. The discrepancy between swab and cuticle sampling methods becomes more evident considering the agent level scale, which placed swabs mainly in the A3 range (average quantity = 2.16 × 10^2 copies/µL ± 1.85× 10^2), while cuticles were in the A1–A2 range (average quantity = 4.29 × 10^0 copies/µL ± 3.09 × 10^0). Specific *A. astaci* end-point PCR was applied on the 7 positive samples from cuticles and on the 20 positive samples from swabs retrieving amplicons in 3 and 16 samples, respectively (Table S3). All negative samples were tested using the PCR amplification of the *16S rRNA* fragment, obtaining positive results and thus excluding the presence of inhibitors in the extract (Table S1).

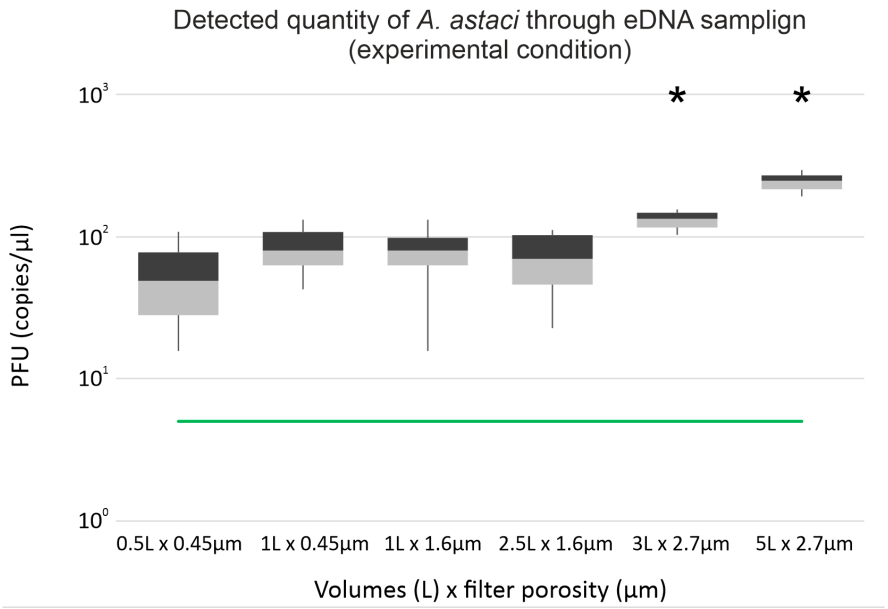

Figure 2. Comparison between filtered volumes and filter size carried out on the water of *P. clarkii* stabulated in the IZSVe aquarium facility. During the experiment, six filters per volume were collected every two weeks. The absolute quantifications are expressed as PCR Forming Units (copies/µL) in qPCR. The green line indicates the LoD level (5.00 copies/µL). *, indicate comparable results.

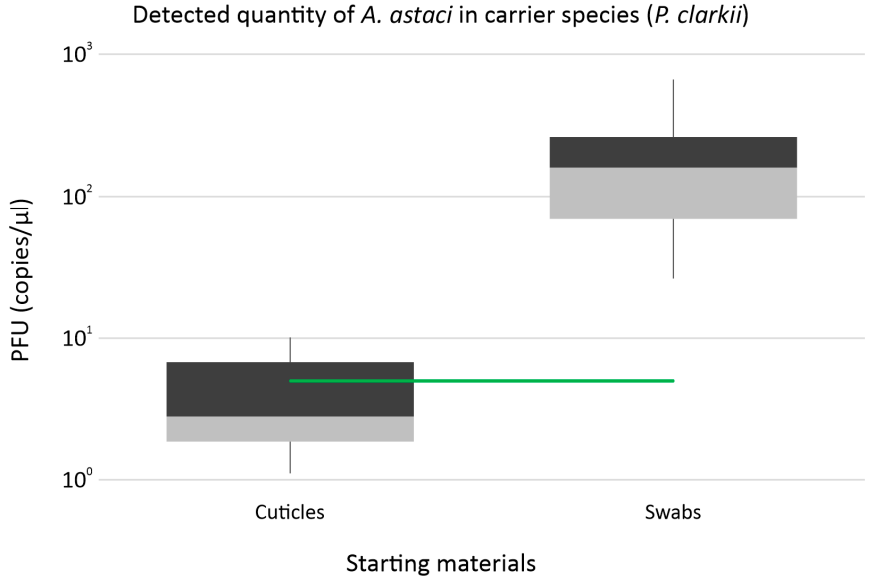

Figure 3. Comparison between sampling protocols carried out in *P. clarkii* after 8 weeks stabulation. The absolute quantifications are expressed as PCR Forming Units (copies/µL) in qPCR. The green line indicates the LoD level (5.00 copies/µL).

3.2. Non-Invasive Sampling in Susceptible Species

The qPCR analyses showed the presence of *A. astaci* in almost all tested specimens (Figure 1b), which exhibited a low standard deviation between replicates (Ct standard deviation < 0.2). Pairwise comparison between cuticles and swabs did not detect significant differences (Wilcoxon single-rank, *p*-value = 0.625), suggesting at least an equivalent ability of the two sampling methods to detect *A. astaci*, with pathogen levels between the A4 and A6 ranges (Table S4). Analyses on cuticles and swabs preserved in absolute ethanol showed comparable results considering the agent levels scale (*p*-value = 0.16), with a higher average quantification found in the latter, concurrently showing a significant negative strong correlation (n = 10, ρ = −0.6364, *p*-value = 0.0479). In contrast, samples stored at +4 °C (in lysis buffer and dry) displayed a drop in the average level among the A2–A3 range, with significant differences in comparison to results from cuticles (*p*-values < 0.05) (Figure 4). Furthermore, within the group of swabs stocked dry at refrigerating temperature, for a sample the absolute quantification was estimated slightly below the LoD level (A1 = 4.98 copies/µL). In the other two examined crayfish from the same group, a higher standard deviation between replicates (>0.5) was detected. Finally, all samples were tested through end-point PCR with the *A. astaci*-specific primers to investigate the reliability of non-invasive sampling and storage methods. These results showed marked amplicons in all cuticle samples, in eight swabs stored at −80 °C and in nine swabs fixed in absolute ethanol at room temperature (Table S4), while in refrigerated samples, amplifications mainly failed.

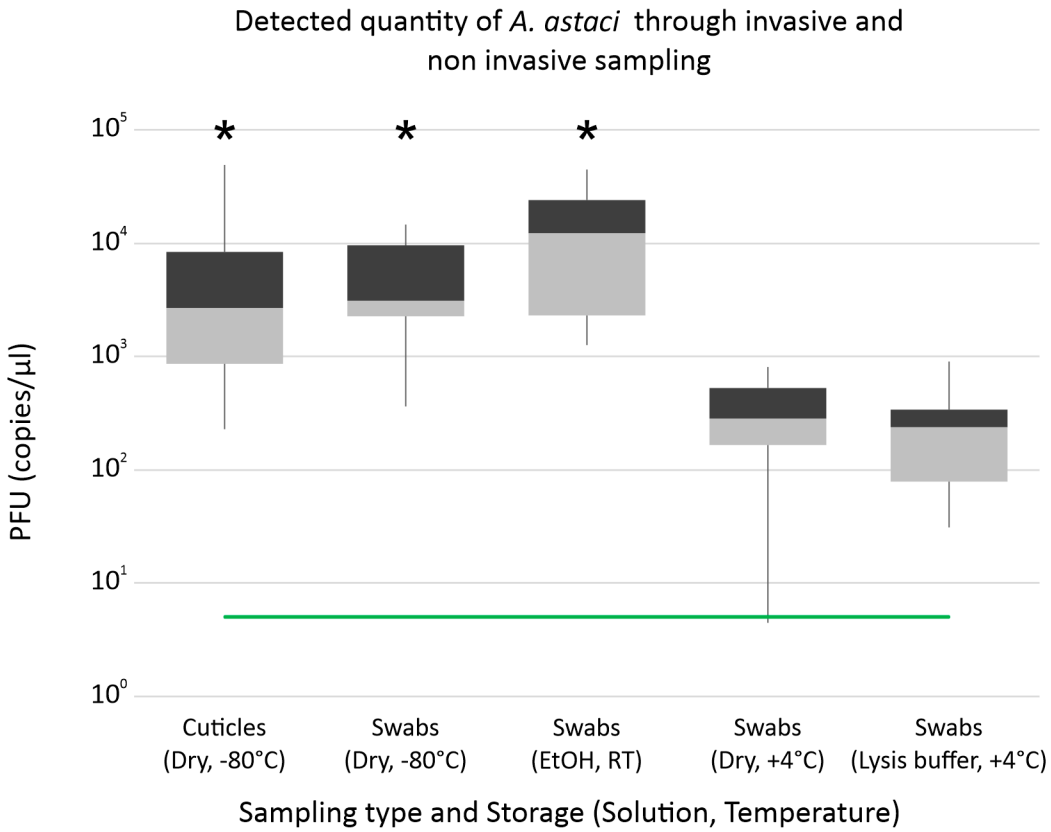

Figure 4. Comparison between storing methods (solution and temperature) in dead and moribund *A. pallipes* collected during the *A. astaci* outbreak in Friuli Venezia Giulia. The absolute quantifications are expressed as PCR Forming Units (copies/µL) in qPCR. The green line indicates the LoD level (5.00 copies/µL). *, indicate comparable results.

3.3. In-Field Tests in Susceptible Species

The non-invasive sampling methods (swabs and eDNA) were applied in three creeks among Emilian Apennines (Figure 1c). After visual confirmation of white-clawed crayfish presence along the streams, sampling occurred during the night, swabbing up to 30 specimens per creek. At the end of the survey, 30 samples were collected from sampling site A, 19 from site B, and 20 from site C. Samples from each creek were extracted and analysed separately. These samples showed quantifications of *A. astaci* spanning from 5.09×10^0 to 1.49×10^4 copies/µL, with only seven negative samples: two swabs in site A, one in site B and four in site C.

eDNA samples collected one month later (four filters for each creek) showed positive results for *A. astaci* in all three sampling sites. Regarding sites A and C, this analysis (amplifying undiluted template) showed that only two filters gave positive results with concordant replicates. Conversely, the other two filters resulted in a high standard deviation between replicates, or were negative. In order to avoid the inhibition effect, samples were diluted 1:5 and re-tested. They resulted all positive with average A2 levels: $7.63 \times 10^1 \pm 6.38 \times 10^1$ copies/µL in site A and $5.03 \times 10^1 \pm 2.72 \times 10^1$ copies/µL in site C (Figure 5). In contrast, watercourse B, characterised by lower flow and where decomposing plant material was present on the bottom, initially yielded results below or slightly above the LoD level. However, after diluting the samples 1:5 and re-testing, the results were above the LoD and concordant between replicates in three filters, with an estimated initial number of $1.99 \times 10^1 \pm 2.21 \times 10^1$ copies/µL (Figure 5; Table S5) setting them in the A2 range. The other filter showed a high C_t standard deviation between replicates, with results both above and below the LoD and, thus, discarded. The *A. pallipes* DNA was detected only in 1:5 diluted samples from creeks A, B and C using the protocol on the *16S rRNA* fragment proposed by Manfrin [105], with C_t ranging between 38 and 40.

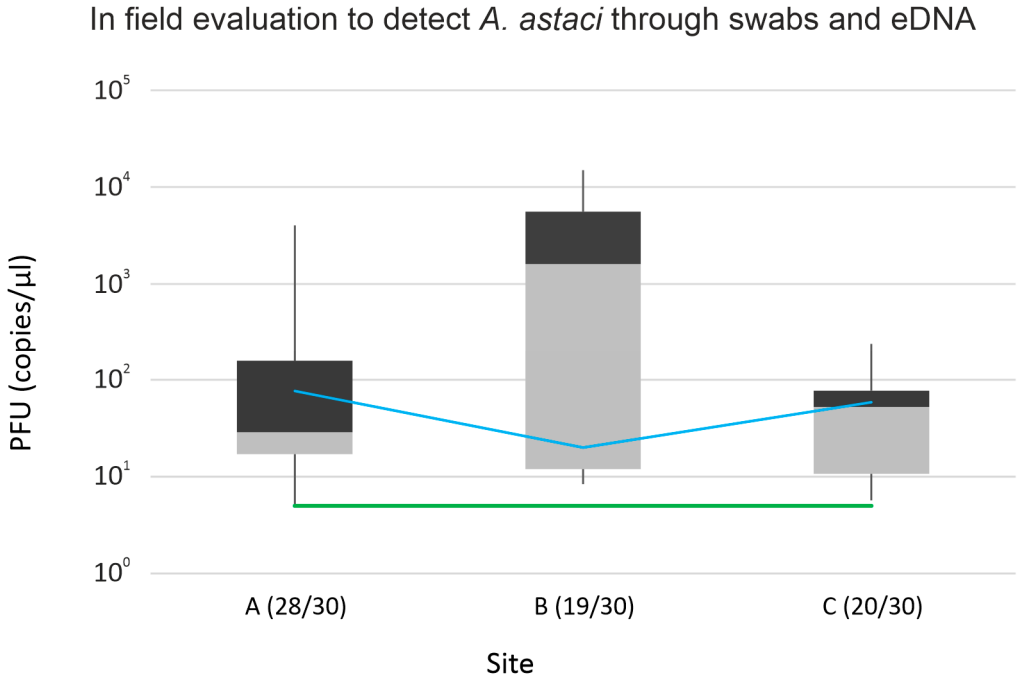

Figure 5. Results of the experimental trial performed in sites A, B and C with the *A. pallipes* complex. The boxplot represents the variability of *A. astaci* in swabs through qPCR. The numbers in brackets indicate positive samples on specimens tested (30). The absolute quantifications are expressed as PCR Forming Units (copies/µL). The light blue line indicates the average level of *A. astaci* in eDNA samples; the green line indicates the LoD level (5.00×10^0 copies/µL).

4. Discussion

eDNA has proven to be an effective technique for several monitoring studies of *A. astaci* and for assessing NICS and ICS presence in various European countries [64,85,108,109]. Its applicability to lentic environments (lakes and marshes) has been extensively evaluated, whereas studies performed on lotic environments with fast-streaming water (as in the present work) are less frequent [25,85,108]. The successful identification of the circulation of the oomycete *A. astaci* in NICS or ICS populations can be affected by the applied methods, timing of sampling (season/mean temperature) and preservation of samples during field activities. Therefore, in order to take advantage of the best environmental conditions, we planned to collect carrier and susceptible species in mid-late summer, since the median temperature increases, leading to higher crayfish activities, which are correlated to increased production/release of *A. astaci* zoospores and crayfish DNA shed due to moult and higher metabolic rate [25].

During experiments performed at the IZSVe aquarium facility with the eDNA sampling methods, all filter porosities were theoretically inadequate to trap free DNA, which is only about 1.2–2.2 nanometres wide [110]. However, their size was small enough to retain and capture zoospores and a fraction of dissolved debris bound to DNA fragments during filtration [21,25,107,111]. In our study, the 5 L of water filtered with 2.7 µm porosity represented the best combination between volumes and filter size, allowing the detection of amounts of *A. astaci* DNA comparable with those obtained by analysing swabs (Figures 2 and 3). Indeed, the experimental conditions (high concentration of crayfish in a confined water volume with limited water replacement) were designed to facilitate the detection of *A. astaci* by filters, inducing the increase in zoospores in the water without causing mortality in *P. clarkii* carriers. However, this circumstance could also occur in wild populations of *A. pallipes*, where a low-pathogenic strain of *A. astaci* (putative genotype A) circulates [39,52] in the absence of mortality events.

According to our results, the ability to identify *A. astaci* in carrier animals by swabbing appears to be 10 to 100 times higher than by diagnostic sampling (WOAH) (Figure 3). Considering that in carrier species, an efficient immune response with melanisation of infected areas forces *A. astaci* hyphae to grow on the outer portion of the cuticle, the difference between the two sampling methods (cuticles and swabs) observed in *P. clarkii* could be related to the low concentration of sub-cuticular hyphae present in this crayfish [112]. This discrepancy could also be increased by the removal of a portion of the external hyphae or zoospores during swabbing. In addition, swabs can test all of the crayfish surfaces and easily reach the invaginations and cuticle cavities without needing to focus the sampling on melanised spots, as occurs when analysing cuticles. This consideration could be extended to *A. pallipes*, also. In this regard, during the last years, an equivalent strong immune reaction against *A. astaci* has also been reported in resistant populations of white-clawed crayfish [52]. However, our findings indicate that it is necessary to sample several specimens to represent the population because about one-third of the red swamp crayfish tested were entirely negative with both cuticular and swabs sampling. From this perspective, it is undeniable that swabs offer the possibility of improving yields by testing more cuticle surfaces and performing the sampling on live and healthy animals that do not need to be sacrificed [60]. Therefore, aware of the simplifications in the model used, testing 30 specimens per population, focussing on large-size crayfish, which moult less frequently (prolonged anecdysis stage), may provide the best chances of success [113].

On the contrary, the swabs and cuticles stored at −80 °C obtained from highly infested *A. pallipes* specimens scored comparable results. Pavić et al. [82] obtained similar results comparing brush and cuticles from dead *Astacus leptodactylus* specimens (experimentally infected by *A. astaci* zoospores). These findings are probably due to both the large concentration of zoospores and extra-cuticular hyphae and a similarly high number of hyphae in the cuticle layer and immediately underlying tissues, as often occurs in dead or moribund crayfish of susceptible species during crayfish plague outbreaks [114]. In addition, the

dramatic reduction in the *A. astaci* DNA observed in samples stocked dry and in lysis buffer at room temperature was due to the storage method and not the sampling protocol.

Alternative and practical methods to store samples during fieldwork activities were evaluated to overcome the use of expensive, hazardous and/or polluting solutions (i.e., RNA-later, liquid nitrogen). In our experiment with *P. clarkii*, comparing the filters stored dry at −80 °C and in absolute ethanol at room temperature did not highlight differences, retrieving comparable results during extractions and qPCR analysis. Similar results were observed in susceptible species with a high level of infestation, comparing quantifications from swabs stored dry at −80 °C and in absolute ethanol at room temperature. We also considered lysis buffer and dry storage at refrigerated temperatures (Figure 4), but they showed a relevant reduction in the investigated target. Thus, absolute ethanol offers a practical and economical conservation method that allows the sample to be kept at room temperature (Figure 4). Ethanol has been used historically with success in stocking sample tissues during field activities [115–117], where the immediate freezing was often inapplicable [92,118]. However, several studies show considerable DNA degradation when samples are stored in a solution of 70% ethanol [119]. According to our experience, considering the limited amount of water retained during the swabbing and filtration in the samples, the efficacy of absolute ethanol was confirmed to store both swabs and glass microfibre filters for up to a week at room temperature, beyond which we recommend storing samples at a refrigerated temperature [120].

The applicability of non-invasive methods to field activities was further tested during the first suitable season since the start of the LIFE-CLAW project, choosing creek hosting populations of *A. pallipes*. The sampling with a convenient and manageable procedure to collect eDNA was applied to creeks. These lotic environments represent conditions where eDNA detection should be easier than in lentic waters, due to the higher filtration capacity before clogging and the reduced degradation phenomena in water [25,64,121,122]. The three streams were characterised by clear and oxygenated waters with different flow rates (A fast and B, C slow), or with the presence of litter in the stream (B), ideally representing the variability that will be addressed during the monitoring steps planned in the project. The application of both methods, swabs and eDNA, returned concordant overall results on *A. astaci* presence in the three sampling sites, even though the variability among the data was quite heterogeneous (Figure 5).

The specific molecular assay to detect the target can be affected by the total amount of extracted DNA, which could inhibit the reaction or disguise the amplification of the fragment of interest, producing nonspecific amplicons [123,124]. In addition, as observed in previous studies, the presence of inhibitors can affect the PCR reaction and is mainly related to the presence of humic acids that are co-extracted during eDNA extraction steps [81,125,126]. Conveniently, in our experiment, serial dilutions of the samples made it possible to bypass the inhibition effect due to the excess of the DNA template and the presence of co-precipitants, managing to obtain positive results for *A. astaci*, albeit at a lower level. The same dilution of the eDNA samples was successfully applied to detect the presence of *A. pallipes* through specific real-time PCR, avoiding the inhibition effect also in this assay. The discordance observed in the results obtained using an undiluted eDNA template suggests caution in the interpretation of samples that appear negative, without a sigmoid curve in the amplification chart or lower than the A2 level of *A. astaci*. Swab analysis on selected samples can easily overcome these limitations.

Finally, during our experiments, cuticles, swabs and eDNA results indicated that a percentage of samples, under natural conditions, might have borderline levels (A0–A1) of *A. astaci*, which can be easily interpreted as a false negative (i.e., site B). This is especially problematic when populations are selected as broodstock donors and crayfish are transferred to reproductive facilities [56] or when populations need to be relocated upstream or to nearby areas. This situation might be related to the life cycle of the pathogen and the number of spores present or released into the water. Therefore, it is critical to test a sufficient number of samples in order to assure a reliable monitoring of the

population [127,128]. Thus, considering a low prevalence (i.e., 10%) of *A. astaci* in carrier populations [56,60] and the high specificity (100%) and sensibility (95%) of the molecular assay (qPCR) used, the WOAH aquatic manual indicates at least 30 samples/population to achieve 95% confidence [129]. In addition, the status of *A. pallipes* populations that are often limited to a few individuals and/or fragmented along the watercourse, and the time needed to perform the sampling through swabs, should be considered.

Regarding eDNA, it is necessary to test multiple sampling points in order to increase sample diversity and to avoid false negative results (i.e., four sampling points/site), possibly collecting them at different stages during the summer [64,85,130].

5. Conclusions

The eDNA method used in this study proved suitable to detect both *A. astaci* and the presence of crayfish DNA in confined water volumes (e.g., ponds or farms) and streams. We also successfully applied swabs to detect *A. astaci* in both susceptible and carrier species; the swabbing procedure is more straightforward than eDNA analysis, allowing in-field sampling without the need for dedicated equipment to be used. However, for monitoring and reintroduction programs, it is advisable to apply an integrated approach, swabbing part of the population while simultaneously collecting eDNA samples. To prevent the degradation of field samples during sampling campaigns, our results support the use of absolute ethanol as a storage solution, confirming its applicability in both cotton swabs and microfibre glass filters. Currently, for the monitoring purposes of the LIFE-CLAW project, we are successfully applying the non-invasive methods described to evaluate the infection levels and distribution patterns of *A. astaci*-positive crayfish populations. In conclusion, the proposed methods should be considered a useful, convenient and effective tool when classic diagnosis based on destructive cuticular sampling is not viable (i.e., an endangered ICS population without evidence of mortality). Considering the recently documented population of white-clawed crayfish resistant to, or with, chronic infection of *A. astaci* [46,52,82,131,132], these non-invasive methods can complement the diagnostic protocol, exhibiting good reliability to detect *A. astaci* even when applied to healthy carrier specimens for monitoring purposes.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15020279/s1, Figure S1: (a) eDNA filtering system during field activities; (b) *P. clarkii* tank during in-house experiment; Table S1. Primers and probes used for both end-point PCR (ep PCR) and quantitative PCR (qPCR) and Real-Time PCR to test the reliability of the methods proposed [87,94,101,104,105,133]. Table S2. Results of the *A. astaci* qPCR analysis on eDNA samples collected during *P. clarkii* housing at the IZSVe aquarium facility. The absolute quantification was expressed in PFU (copies/µL) and was reported in scientific notation. Estimated quantification below LoD level was reported in brown; N/A means no detected amplification or discordance between replicates. *, indicates samples sequenced after *A. astaci* end-point PCR. Table S3. Results of the comparison between sampling protocols (cuticle and swab) in *P. clarkii* after their housing at the IZSVe aquarium facility. The absolute quantification expressed in PFU (copies/µL) and indicated in scientific notation was reported only for positive samples (1–20); samples from 21 to 33 were completely negative. Estimated quantification below LoD level was reported in brown; N/A means no detected amplification or discordance between replicates. End-point PCR outcomes were expressed as positive (+) and negative (−) results. * Indicates faint amplification. Table S4. Results of fourth different storing methods to preserve samples during in-field activities. Highly infested *A. pallipes* specimens were collected during a crayfish plague outbreak in the restocking facility in Friuli Venezia Giulia Region-Northern Italy in December 2013 (LIFE RARITY project). The absolute quantification was expressed in PFU (copies/µL) and noted was in scientific notation. Estimated quantifications below LoD level or with average standard deviation > 0.40 were reported in brown; N/A means no detected amplification or concordance between replicates. End-point PCR outcomes were reported as positive (+) and negative (−). *, indicates faint amplification. Table S5. Results from non-invasive sampling methods carried out in creeks with wild *A. pallipes* population. The absolute quantification was expressed in PFU (copies/µL) and was reported in scientific notation. Estimated

quantifications below LoD level were reported in brown, N/A means no detected amplification or concordance between replicates; *, indicates samples sequenced after *A. astaci* end-point PCR.

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Institutional Review Board Statement: Regarding the trial with *Procambarus clarkii*, ethical approval is not required. Since 2016, the List of Invasive Alien Species of the Europe Union (Regulation EU 2016/1141) includes *P. clarkii*, considering it an invasive species. According to Regulation (EU) no 1143/2014 on the prevention and management of the introduction and spread of invasive alien species, its eradication is promoted from the territory. Furthermore, the Italian Legislative Decree no. 26/2014 regulating the use of invertebrate animals in experiments is related only to cephalopods and is not applied to crayfish.

Data Availability Statement: Data are available from the authors upon reasonable request.

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