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Estimation of the number of *Anisakis* larvae in commercial fish using a descriptive model based on real-time PCR

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ABSTRACT

BACKGROUND

Seafood parasitization by *Anisakis* (Anisakidae) larvae has been reported in most of the oceans and seas worldwide. The presence of these nematodes in commonly consumed fish represents a potential hazard for consumers as they can provoke gastrointestinal symptoms and allergic reactions. In the present work, the capacity of a SYBR Green qPCR protocol to quantify *Anisakis* larvae in commercial fish was evaluated using experimentally spiked samples with different numbers (0-50) of *A. simplex* third-stage larvae (L3). To verify the agreement of the obtained results, 25 naturally infected fish specimens of Atlantic blue whiting underwent a parallel visual inspection.

RESULTS

The logarithmic behavior of the Cq data obtained from the experimentally spiked samples allowed the development of a descriptive mathematical model that correlates the Cq value with the number of *Anisakis* larvae ($R^{2=}$0.9908, CV=2.37%). In the commercial blue whiting specimens there was a high correlation between the results of the molecular technique and the visual inspection (R^2=0.9912); the Bland-Altman analysis showed that 94% of the differences were within the limits of agreement (-4.98 and 6.68), indicating the reliability of the descriptive mathematical model based on the SYBR Green qPCR technique.

CONCLUSION

The descriptive function presented based on the SYBR Green qPCR assay is promising as a sensitive and accurate tool for measuring the *Anisakis* larval load in commercial fish, with a potential application not only in the food industry but also in prevention programs for public health.

KEYWORDS

Anisakis

Foodborne parasites

Food safety

Larval quantification method

Molecular technique

1. INTRODUCTION

Foodborne parasites are considered important pathogens as they can cause disease and economic loss worldwide; humans can be exposed to these parasites through the food supply chain (Trevisan et al., 2019). Seafood parasitation by larvae of anisakid nematodes has been reported in most oceans and seas worldwide (D'amico et al., 2014). The presence of these parasites in commercial fish is a potential hazard for consumers as they can provoke gastrointestinal symptoms and allergic reactions. The sibling species *Anisakis simplex* sensu stricto and *A. pegreffii*, which belong to the *A. simplex* sensu lato complex, are the most common causative agents of human clinical cases of anisakiasis and allergic reactions in Europe and Asia (Mattiucci et al., 2018). In addition, clinical cases of *Pseudoterranova* spp. and *Contracaecum* spp. have been reported in the Southern hemisphere (Shamsi and Butcher, 2011; Weitzel et al., 2015). Other ascaridoid larvae highly prevalent in fish are the non-pathogenic *Hysterothylacium* species (Raphidascarididae), which can be misidentified as *Anisakis* spp. because of morphological similarities (Simsek et al., 2018).

The clinical manifestations of gastro-intestinal anisakiasis, caused by living larvae, include epigastric pain, nausea, vomiting and diarrhea (Baird et al., 2014). Allergic reactions, caused by live and dead larvae, are characterized by urticaria, angioedema, bronchospasm and even anaphylactic shock (Audicana et al., 2002). Studies suggest *A. simplex* (s.l.) is a major hidden producer of allergic incidents associated with food (Anıbarro et al., 2007).

Although *Anisakis* larvae mostly infect visceral organs, they can also penetrate the musculature and accumulate in edible parts of fish. The presence of these nematode larvae in fish flesh is important not only from the health point of view but also because of its economic implications, since it decreases fish quality and provokes consumer rejection (Cipriani et al., 2016). Visual inspection is currently mandatory in fish quality control and several types of techniques have been applied to improve the visual detection of these parasites, including candling, pressing, digestion and UV illumination, (Gómez-Morales et al., 2018; Guardone et al., 2016; Levsen et al., 2005). Enzyme-linked immunosorbent assays (ELISA) have been developed for the quantification of *Anisakis* proteins using polyclonal antibodies (Werner et al., 2011). More recently, proteomics methods based on liquid chromatography tandem mass spectrometry (LC–MS/MS) have been applied to detect *Anisakis* proteins in fish and fish matrices (Fæste et al., 2016). Parallel reaction monitoring (PRM) mass spectrometry has also been used to detect anisakid-specific peptide biomarkers in commonly consumed fish (Carrera et al., 2016).

New strategies for the fast detection of fish-borne parasites are molecular-based techniques that can also be applied to processed food products regardless of thermal treatments, such

as canning and freezing, or mechanical handling, such as sieving and chopping. Polymerase chain reaction (PCR) has been used to identify anisakid species isolated from commonly consumed fish; the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear ribosomal DNA have proved to be useful for genotypic identification (Mattiucci et al., 2018). Real-time PCR (qPCR) has been used to detect *Anisakis* DNA in experimentally inoculated fish (Lopez and Pardo, 2010) and naturally infested commercial fish samples, as well as in different types of commercial fish-derived food, providing highly sensitive results (Mossali et al., 2010). The quantitative capacity of qPCR methods applied in food with probes or fluorescent dyes such as SYBR Green (Rodríguez-Lázaro and Hernández, 2013), avoiding the use of expensive labeled probes, can facilitate the detection and quantification of *Anisakis* larvae in fish and fish-derived food. In the present work, we evaluated the ability of an *Anisakis* specific SYBR Green qPCR protocol to accurately quantify *Anisakis* larvae in commercial fish, presenting a descriptive model that can express the relationship between molecular results and the number of larvae present.

2. MATERIALS AND METHODS

2.1 Experimental fish samples

A total of 570 *A. simplex* (s.l.) third-stage (L3) larvae isolated from viscera and belly flaps of specimens of Atlantic blue whiting (*Micromesistius poutassou*), purchased in retail stores in Barcelona, were used for contaminating fish samples. Larvae were identified morphologically (Koie, 1993; Petter and Maillard, 1988) and stored in physiological solution at 4 °C until use.

Fillets of hake approximately 80 g in weight (*Merluccius merluccius*), purchased in retail stores, were checked for *Anisakis* absence by visual inspection, which was performed by dissection and observation under a stereomicroscope (Leica MZ6) using incident and transmission light. The hake specimens came from the Mediterranean Spanish coast, an area of low parasitation by *Anisakis* (Barcala et al., 2018). In addition, fillets from the fish tail were used, in which the parasite is not as likely (Roca-Geronès et al., 2020). Samples of 15 g of *Anisakis*-free fillets were experimentally contaminated with different numbers of *A. simplex* (s.l.) L3 larvae (0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50); four fillets for each larval number tested were used. Samples were homogenized in a Stomacher-400 (Seward, UK) with 100 ml of lysis solution (100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, and 80 mg/L proteinase K) for 60 min. The samples were kept rotating at 350 rpm for 60 minutes at 55° C.

2.2 Commercial fish samples analyzed

Twenty-five specimens of Atlantic blue whiting, purchased in retail stores in Barcelona, were studied. Each fish was divided in four parts: visceral organs including mesenteries, and anterior ventral, anterior dorsal and posterior musculature. Fish parts were dissected under a stereomicroscope, and all the larvae observed were morphologically identified and counted. Each fish part, together with the larvae detected in it, was homogenized as described for the experimental *Anisakis*-spiked samples, and in the case of viscera 50 ml of lysis solution was added.

2.3 DNA extraction

Genomic DNA purification was performed with the Wizard Genomic DNA Purification Kit (Promega, Spain). DNA was extracted from 200 µL fish homogenates. Samples were mixed with 600 µL of Nuclei Lysis Solution and 17.5 µL of 20 mg/ml Proteinase K (Roche Diagnostics, Spain**)** and incubated overnight at 55°C. DNA was precipitated with ethanol at 70% (Sigma-Aldrich, Spain), and resuspended in the rehydration solution (10mM Tris-HCl, 1mM EDTA). Two independent DNA extractions were performed for each fish homogenate. The quality of the DNA extraction was assessed by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, USA). Purified DNA was stored at -20°C until qPCR analysis.

2.4 SYBR Green qPCR

A pair of primers, targeting a fragment of the mitochondrial cytochrome *c* oxidase subunit II gene were used, which have been previously validated as specific for *Anisakis* (Lopez and Pardo, 2010).The molecular analysis was performed using a high sensitive SYBR Green qPCR assay (Godínez-González et al., 2017). Reaction mixtures were prepared in 384 optical well plates containing 5 µL of 1× SYBR Green Master Mix (Roche Diagnostics, Spain), 0.6 µL of 300 nM of forward and reverse primers, and 2.5 µL of DNA. *A. simplex* (*s.l.*) larval DNA and molecular biology-grade water (Sigma-Aldrich, Spain) were used as positive and negative controls, respectively. Amplifications were carried out in the thermocycler LightCycler 480 Instrument II (Roche Diagnostics, Germany), programmed to hold 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 65 °C for 1 min. The melting curve analysis was as follows: 95 °C for 1 min, 40

°C for 1 min, 65 °C for 1 s and then an increase of 1 °C/s to 95 °C for 30 s. All analyses were performed in duplicate.

Results were expressed as quantification cycle (Cq) values; all samples with a Cq ≥ 35 were considered negative. The specificity was ascertained by comparing the melting temperatures (Tm) of the amplification products from studied samples to that of the positive control (Tm=76°C).

2.5 Statistical analysis

A univariate analysis of variance (ANOVA) was used to obtain different groups of Cq values associated with a determinate number of *Anisakis* larvae in the experimentally spiked samples. A Bland-Altman analysis was performed to assess the agreement between the two quantitative detection methods, visual and SYBR Green qPCR (Bland and Altman, 1999). Limits of agreement were calculated using the mean and standard deviation of the differences between the two measurements. All statistical analyses were carried out using SAS 9.4 software (SAS Institute, USA).

3. RESULTS

3.1 *Anisakis* SYBR Green qPCR study on experimentally contaminated fish samples

The molecular analysis of experimentally spiked hake samples revealed a logarithmic relationship between the number of *A. simplex* (s.l.) larvae present and the Cq values, with results ranging from 24.3 to 17.7, corresponding to 1 and 50 larvae, respectively. These findings allowed us to develop a descriptive function expressing the experimental results ($Cq = -1.529x + 24.109$; $R^2=0.9908$) (Figure 1). According to the analysis of variance of the Cq

values, six distinct groups (A to F) related to the number of larvae were discernable ($P<0.05$): group A, with a Cq of 35, corresponded to the absence of *Anisakis* larvae in the sample; group B corresponded to the presence of 1-3 larvae; group C, 4-10 larvae; group D, 11-25 larvae; group E, 26-40 larvae; and group F to more than 40 larvae. The assay precision, expressed as the coefficient of variation (CV), ranged from 0.95% to 2.37%, indicating the technique was highly reproducible (Table 1).

3.2 Visual inspection and SYBR Green qPCR study on commercial fish

The visual inspection of 25 Atlantic blue whiting resulted in the detection and identification of *Anisakis* and *Hysterothylacium* larvae, coinfection being observed in three specimens and exclusive *Hysterothylacium* infection in two. A total of 834 *Anisakis* larvae were identified as *A. simplex* (s.l.), ranging from 0 to 112 with a mean intensity of 41.7 per fish. On the other hand, 29 *Hysterothylacium aduncum* larvae were observed, ranging from 2 to 10 with a mean intensity of 5.8 larvae per fish infected with this parasite.

The *Anisakis* load in the different parts of fish was studied in parallel by visual inspection and with SYBR Green qPCR. The molecular results expressed as the number of *Anisakis* larval equivalents calculated using the descriptive function (NLE) and the number of larvae observed by visual inspection (NLV) are indicated in Table 2. The correlation analysis showed a high concordance between both methods regarding the number of larvae in the analyzed samples ($R^2=0.9912$) (Figure 2A). Although experimentally the largest number of larvae tested was 50, in naturally infested fish the descriptive function was able to estimate a much larger number (up to 100) of larval equivalents, as in the case of the visceral organs

of one of the analyzed specimens (NLE=119; NLV=112) (Table 2). Furthermore, Bland-Altman analysis showed that 94% of the differences were within the limits of agreement (-4.98 and 6.68) (Figure 2B). In the two specimens of blue whiting infected only by *Hysterothylacium*, the Cq value for each fish part was 35, showing that the presence of this raphidascaridid did not interfere with the quantification of *Anisakis* larvae.

The results obtained from the different parts of the fish analyzed were classified among the six groups (A-F) according to the number of larvae of *Anisakis* (Table 2). The visceral organs were the most parasitized, with 20/25 infected fish (80% $_{95\%}$CI 60-92); the larvae being distributed in all groups (A-F), with high percentages in groups E (24%, $_{95\%}$CI 12-43) and F (20% $_{95\%}$CI 9-39), those with the highest parasite load. The anterior ventral musculature showed 18/25 parasitized fish (72% $_{95\%}$CI 52-86) with a great variability: 28% ($_{95\%}$CI 14-28) of the samples corresponded to group A, 28% ($_{95\%}$CI 14-28) to group B, 16% ($_{95\%}$CI 6-35) to group D, 12% ($_{95\%}$CI 4-29) to group C, and 16% ($_{95\%}$CI 6-35) to group E. The anterior dorsal musculature presented *Anisakis* in only 8/25 fish (32% CI 17-52), containing a low number of larvae: 68% ($_{95\%}$CI 48-83) of the samples were classified as group A, 24% ($_{95\%}$CI 12-43) as group B and 8% ($_{95\%}$CI 1-26) as group E. The posterior musculature, with 6/25 parasitized fish (24% $_{95\%}$CI 11-44) showed the lowest larval presence, as *Anisakis* were detected only in 24% ($_{95\%}$CI 12-43) of the samples belonging to group B.

DISCUSSION

The European Food Safety Authority reported approximately 20,000 clinical cases of anisakiasis worldwide up to 2010, with more than 90% from Japan. In Europe, Spain is

considered to have the highest incidence of this disease (EFSA, 2010). The two sibling species *A. simplex* (s.s.) and *A. pegreffii* have been described as the main etiological agents of human anisakiasis and allergic reactions; however, some studies have reported the possible risk of invasive infection caused by *A. physeteris* and *A. paggiae* (Romero et al., 2013). These data highlight the need to validate reliable techniques for the detection and quantification of *Anisakis* in consumed fish and fish-derived food and thus help prevent anisakiasis and allergic incidents.

The qPCR technique is a promising tool to determine the presence of foodborne pathogens because of its precision, speed, cleanliness and safety (Rodríguez-Lázaro and Hernández, 2013). In this study, the descriptive function obtained after SYBR Green qPCR assays allowed to quantify the number of larvae of *A. simplex* (s.l) in naturally infected fish, which would include the sibling species *A. simplex* (s.s.) and *A. pegreffii.* Other species of *Anisakis* such as *A. physeteris* could also be detected using this qPCR technique, although its different amplification behaviour, with two-log Cq lateness from the two sibling species, would not led to apply the proposed descriptive function (Godínez-González et al., 2017).

In the present work, the application of the descriptive function obtained allowed an estimation of the number of *Anisakis* larvae in the analyzed blue whiting specimens, and the results were closely correlated with those obtained by visual inspection. In the fishery sector, over the last 50 years, the visual inspection has been widely used, but the results obtained may vary according to the operator training and lighting conditions or because of low standardization (Guardone et al., 2016; Llarena-Reino et al., 2012). Currently, the artificial enzymatic digestion procedure by CODEX (STAN 244-2004) is the recommended

procedure for anisakids detection and counting in certain fish species and commercial displays (Llarena-Reino et al., 2013). Alternative techniques such as UV-press (Karl and Leinemann, 1993) are increasingly carried out; however, samples should be analyzed after thawing and factors as the presence of skin and the fat content can reduce the efficiency and sensitivity (Yang et al., 2013). The molecular technique described in our study has great sensitivity and specificity and allows the detection of *Anisakis* larvae embedded in the muscle, as well as in the two previous techniques, which may go unnoticed in a visual inspection (Stormo et al., 2007). Sample processing is simple and is done without removing the fish skin, which does not interfere in the results. The specificity for *Anisakis* of the primers used ensures that the presence of larvae from other common and non-pathogenic species in the same area, such as those of *Hysterothylacium* that are morphologically similar, does not interfere with the results. The possibility of automatizing the molecular process, would allow the analysis of large amount of samples in a short period of time and with reduced staff.

The prevalence of *Anisakis* larvae and infection intensity in wild fish vary according to species, fishing area, season and individual characteristics of the fish (Mattiucci et al., 2018; Roca-Geronès et al., 2020). As in the vast majority of marine fish, blue whiting tend to accumulate *Anisakis* larvae in their visceral organs (Madrid et al., 2012; Roca-Geronès et al., 2020), which were the most parasitized part of the fish in this study. However, larval migration to the flesh also constitutes an important source of infection, reflected in the high number of larvae detected in the anterior ventral musculature. The parasitic load in the musculature decreases towards the posterior end of the fish, and the anterior dorsal and posterior musculature are considered the safest parts for human consumption because of the

absence or low number of larvae detected (Cipriani et al., 2015). Other studies using visual inspection have reported similar *Anisakis* larval distribution in this host species in specimens from different fishing areas （Chía et al., 2010; Gómez-Mateos et al., 2016, Roca-Geronès et al., 2020).

Several studies worldwide have shown that sensitization to *Anisakis* allergens varied widely in prevalence (up to 81%) in patients that have presented allergic reactions to seafood (Mazzucco et al., 2018). There is a risk of nonviable *Anisakis* material inducing allergic reactions (Moneo et al, 2005). Different techniques that destroy the larvae cannot provide effective protection against allergic reactions, as residual parasite presence remains after applying food processing treatments such as cooking, canning or freezing (Rodríguez-Mahillo et al., 2010; Tejada et al., 2015). The SYBR Green qPCR technique used to optimize the model described amplifies a small fragment of the target region, which is stable under thermal food processing. Thereby, extremely fragmented DNA could anneal the primers, permitting the analysis of highly processed samples (Godínez-González et al., 2019).

Authorities in food safety and fishing facilities currently employ detection methods for *Anisakis* control; however, measurement scales of parasitic load have not been established, and rejection criteria and limits of detection have not been standardized for use by food handlers (Commission Regulation (EC) No 2074/2005; FDA 2011). The function described could be applied to optimize an automated molecular platform for the control of a large number of samples and batches of commercial fish, as well as fish-derived foods. The classification of batches of fish according to their parasitic load by molecular methods

could orientate decision-making regarding usage, thus reducing the risk of accidental ingestion of *Anisakis* larvae. Finally, the descriptive function presented based on the SYBR Green qPCR assay is promising as a sensitive and accurate tool for measuring the *Anisakis* larval load in commercial fish, with a potential application not only in the food industry but also in prevention programs for public health, helping to improve the safety and quality of fishery products and consumer protection.

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Table1. Results of SYBR Green qPCR applied to hake samples experimentally contaminated with determinate numbers of *Anisakis* larvae.

Number of *Anisakis* larvae	**Cq[*†] (95% CI)**	**CV (%)**
0	35.0 (33.9-35.6)[A]	1.53
1	24.3 (23.4-25.2)[B]	2.37
2	22.9 (22.4-23.7)[B]	2.14
3	22.5 (22.3-22.7)[B]	0.97
4	21.4 (21.1-21.6)[C]	1.24
5	21.5 (21.3-21.9)[C]	1.41
10	21.7 (21.2-21.9)[C]	1.66
15	20.0 (19.7-20.3)[D]	2.05
20	20.2 (19.9-20.6)[D]	1.07
25	19.8 (19.7-20.1)[D]	0.95
30	19.1 (18.9-19.3)[E]	1.73
35	18.7 (18.4-19.0)[E]	0.96
40	18.5 (18.2-18.8)[E]	1.64
45	17.6 (17.2-17.9)[F]	1.55
50	17.7 (17.4-17.8)[F]	1.13

Cq, mean quantification cycle value; CI, confidence interval; CV, coefficient of variation
Letters A-F indicate statistical groups detected relating Cq values to a determinate number of larvae.
[*] Four DNA extractions were amplified by qPCR in duplicate
[†] Values with the same letter are not significantly different (Tukey, P<0.05)

Table 2. Comparison of the number of *Anisakis* larvae obtained by the descriptive function and visual inspection in the 25 naturally infected blue whiting analyzed and distribution of the different fish parts among the groups (A-F).

		Group A	Group B	Group C	Group D	Group E	Group F
Visceral organs	Cq±SD*	35.0±0.0	23.37±0.4	21.1±0.6	19.5±0.2	19.0±0.1	17.1±1.1
	NLE	0(0-0)	3(2-3)	(6-10) †	21(21-25)	29(27-33)	43(43-119)
	NLV	0(0-0)	2(2-3)	(8-11) †	23(22-24)	28(26-35)	45(45-112)
	Nº of fish	5	3	2	4	6	5
Anteroventral musculature	Cq±SD*	35.0±0.0	23.6±0.9	21.9±0.3	20.25±0.2	19.1±0.3	
	NLE	0(0-0)	1(1-3)	4(4-6)	15(12-15)	28(28-40)	
	NLV	0(0-0)	1(1-3)	4(3-5)	12(12-16)	26(25-39)	
	Nº of fish	7	7	4	3	4	
Anterodorsal musculature	Cq±SD*	35.0±0.0	23.5±0.3			19.3±0.02	
	NLE	0(0-0)	1(1-2)			(26-27)†	
	NLV	0(0-0)	1(1-2)			(25-28) †	
	Nº of fish	17	6			2	
Posterior musculature	Cq±SD*	35.0±0.0	23.9±0.6				
	NLE	0(0-0)	1(1-2)				
	NLV	0(0-0)	1(1-2)				
	Nº of fish	19	6				

Group A-F indicate the groups described by SYBR Green qPCR related to the number of larvae: A, no larvae presence; B, 1-3; C, 4-10, D; 11-25; E, 26-40; F, >40

NLE, number of *Anisakis* larval equivalents calculated using the descriptive function; NLV, number of larvae observed by visual inspection; Cq, mean quantification cycle value; SD, standard deviation.

Number of larvae expressed as Mode (Range).

* Two DNA extractions from each sample were amplified by qPCR in duplicate

† Bimodal group

Figure 1. Descriptive function obtained by plotting the mean Cq values obtained by SYBR Green qPCR with respect to the number of *Anisakis* larvae (from 0 to 50), expressed as natural logarithm (Ln), in experimentally contaminated fish samples. The correlation coefficient (R^2) is indicated.

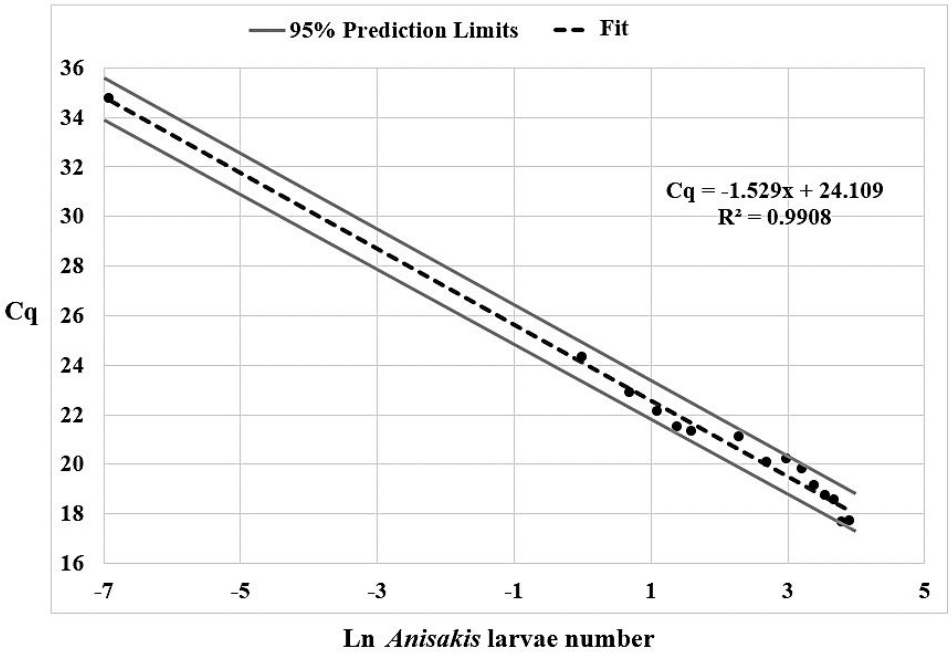

Figure 2. Correlation (A) and Bland–Altman (B) plots showing the concordance between the results obtained by the descriptive function and visual inspection for *Anisakis* larval quantification in the 25 naturally infected blue whiting analyzed. The correlation coefficient (R^2) and 95% limits of agreement (LoA) are indicated.