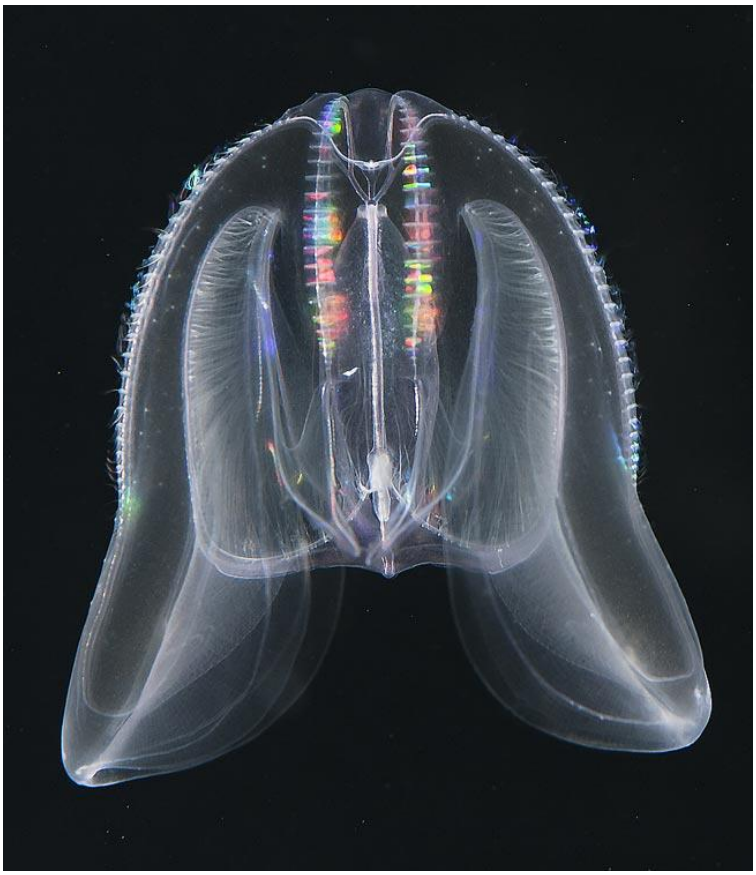
Mnemiopsis leidyi gut content extraction for the purpose of molecular analysis



ABSTRACT

For ecological impact assessments of invasive species accurate dietary analyses are required. In the case of diet assessments on *Mnemiopsis leidyi* discrepancies between the assumed generalistic diet and the environment was found. Previous studies relied on visual identification of prey species, and soft bodied prey types were underrepresented or absent in the diet while numerous in the environment. This study develops a molecular diet analysis to study if selection takes place or if visual identification is hampered by visual diet assessments. This was tested with bivalve veligers as they are easy to identify both visually and molecularly. It was found that almost all *Mnemiopsis leidyi* stomachs contained bivalve DNA while only in 70% veligers were identified visually. It was concluded that molecular analysis of gut contents can be used to aid in the strengthening of dietary impact assessments.

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INTRODUCTION

The lobate ctenophore *Mnemiopsis leidyi* (A. Agassiz, 1865) is a species with high potential of invading foreign ecosystems with large effects on the stability of these systems (Shiganova, 1998). This ctenophore is native to estuaries at the North and South Atlantic coast of the American continent. The abiotic conditions in these areas vary throughout the year. Sea temperature is low during winter and increased fresh water input in spring and summer result in low salinity. Adaptations to this variation make *M. leidyi* tolerant to a wide range of temperature and salinity (Burrell and van Engel, 1976). In addition, *M. leidyi* is a hermaphroditic organism that can self-fertilize (Kremer, 1976) and that easily are transported to new areas through the ballast water of ships. Thus, the flexibility in life history in combination with a high tolerance to environmental fluctuations means that *M. leidyi* has a high potential for colonizing and establishing in non-native ecosystems.

Mnemiopsis leidyi is a generalistic feeder that filters the water column by creating a current towards the oral lobes and onto the tentillae. The created current is difficult to detect by high motile prey, and is high enough to enable *M. leidyi* to filter relatively large amounts of water (Colin *et al*. 2010). When favorable conditions occur in the summer months, these ctenophores can form extensive blooms in a short period of time. These blooms can exert heavy predatory pressure on the zooplanktonic community. During some days predatory impact (the removal of zooplanktonic standing stock) can be as high as 100% (Purcell *et al* 2001, McNamara *et al* 2010).

The first observation of *M. leidyi* as an introduced species occurred in the Black Sea in 1982 (Vinogradov, 1989). Trophic imbalance from overfishing and eutrophication caused an excess of zooplankton, and in combination with the lack of a natural predator this led to massive *M. leidyi* blooms in 1989. Intra-guild predation by *M. leidyi* (through consuming fish eggs and larvae) and resource competition resulted in a collapse of the fish stock (Gucu 2002, Bilio 2004). Further transportation of individuals from the Black Sea population through ballast water introduced *M. leidyi* to the Caspian Sea. *M. leidyi* blooms coincided with a 5-fold density and 6 fold biomass decrease of zooplankton in the Caspian Sea compared to months when *M. leidyi* was absent (Shiganova *et al*. 2001). Some *M. leidyi* populations in the Caspian Sea were calculated to have predatory impacts of up to 62% on the total biomass of the zooplanktonic standing stock. (Finenko 2006, Shiganova *et al* 2001). Recently, successful populations have established in the North Sea, Wadden Sea and Baltic Sea (Faasse & Bayha 2006, Boersma *et al*. 2007, Javidpour *et al.* 2006). Microsatellite analysis revealed that these populations originated from a different native habitat than the Black Sea population further underlining the invasive potential of *M. leidyi* (Reusch *et al*. 2010).

The first observations of *M. leidyi* in the Wadden Sea caused concerns that the impact the introduction would cause major effect on the stability of this highly diverse ecosystem. Bivalves are an important class in the Wadden Sea, both from ecologic and economic perspective. Many bird species that winter in this area rely on the high abundance of bivalve species located on the mudflats (van Roomen *et al.* 2012). Bivalves are also an important part of the diet of several fish species that use the Wadden Sea as a nursery (Tulp *et al*. 2010). *Mytilus edulis* spat is harvested in the Wadden Sea to sustain the mussel beds near Zeeland and it is transferred to culture lots for grow-out for commercial use. *M. leidyi* is known to consume vast amounts of bivalve veligers. This can be as large as 75% of their diet (Purcell *et al*. 1991). On some days they can consume nearly 100% of the bivalve veligers present in the water column (McNamara *et al*. 2010). Many of the bivalve species found in the Wadden Sea spawn in the periods that high abundance of *M. leidyi* can be expected (Cardoso *et al*. 2009, De Vooys 1999, Philippart *et al*. 2003). If *M. leidyi* blooms occur during major bivalve spawning events, this could potentially have large effects on bivalve recruitment. This study attempts

to calculate the predatory impact the *M. leidyi* population has on the bivalve veliger abundance in the Wadden Sea.

Impact assessments rely heavily on visual analysis of *M. leidyi* stomach contents. However, due to the high gut clearance rates and low digestion times visual counts can result in an underestimation of the number of prey items ingested. Furthermore, visual identification of bivalve veliger species is difficult, especially in the earlier stages of development (Hendriks *et al*. 2005). DNA analysis has the potential to identify bivalve species and can detect prey items that are only ingested in low numbers or rapidly digested.

In this study we will attempt to develop a stomach content extraction procedure that can be used molecular species identification in the diet of *M. leidyi*. By comparing the results of the visual counts to the DNA analysis of stomach contents it is possible estimate the effectiveness of both techniques. Molecular analysis of the stomach content can potentially be used in other studies to test for missing species in the stomach content.

MATERIALS AND METHOD

Stomach content extraction and zooplankton sampling

Sampling was performed at high tide at the Napoleon dam area near Den Helder (the Netherlands). A flownet was used to limit damage to *M.leidyi*, which could result in regurgitation of stomach content inside the net. *M. leidyi* stomach contents were extracted by injecting 10 ml. of sterile filtered seawater into the stomach cavity. The filtered seawater was obtained by filtering natural seawater over a 0.2 µm filter and sterilizing the water with UV radiation. The stomach contents were extracted on board the research vessel right after biomass sampling. The method of stomach extraction was tested prior to field sampling under a dissection microscope. Samples for determining the *in situ* zooplankton composition and present bivalve species were obtained by filtering 2 l of seawater over a 50 µm sieve and rinsed out with filtered seawater. Both the zooplankton samples and stomach contents were either preserved in 2 % formaline for visual analysis or vacuum filtered on 20 µm nylon filters and stored at -80 °C for molecular analysis of bivalve species in diet.

Visual analysis of diet

The zooplankton samples were washed in a 20µm sieve and transferred to a counting tray, specifically made for counting zooplankton. Under a bright field dissection microscope, the taxonomic category of the zooplankton was determined and the number of individuals per category was counted. The samples containing the stomach content of *M. leidyi* were directly poured into the counting trays to avoid sample loss in the washing step.

Pilot experiment for molecular analysis

Multiple pilot experiments for testing the extraction and preservation methods for molecular analysis were performed. *M. leidyi* specimens used in these experiments were caught *In situ* with the flow net and transferred into a Kreisel aquarium for acclimatization to 15°C and fed with *Artemia* nauplii. Experiments were performed in 20 L aquaria with *Mytilus edulis* larvae densities averaging 50 ind./liter as ambient veliger density in the Wadden Sea varies between 10-100 ind./L (excluding spawning peaks) during the periods with high *M. leidyi* abundance(Bos *et al* 2006). Feeding times were set at 30 min. In the first set of experiments, the extracted stomach contents were either preserved in 70% ethanol or at -80°C. The second set of experiments tested tested the vacuum filter method used for *in situ* sampling.

Molecular analysis of bivalve species in gut content

The DNA of the prey items present in the zooplankton and gut samples was extracted according to the standard protocol using the Mo-Bio lab. Inc. Powersoil® DNA isolation kit. After extraction, the quality of the DNA was assessed by gel electrophoresis on a 1% Agarose gel. This step was added to examine the quality and length of the DNA fragments present in the samples. Primers for six bivalve species (*Crassostrea gigas, Mythilus edulis, Cerastoderma edule, Mya arenaria, Macoma balthica, Ensis directus*) were used in the PCRs for species determination. The PCR products were analysed on

2% Agarose (75V., 50 Min.). The staining of the DNA fragments with ethyl bromide was performed at a separate stage. When the staining was completed, photos of each gel were taken under UV radiation. The samples were then scored 1 (bivalve species present) or 0 (bivalve species absent).

Table 1: Reagents and PCR protocol used for molecular analyses (*annealing temperature for *C. gigas* primers was 52.5 °C)

Reagent	Total reaction vol. 25	ul	PCR Protocol Time	Temperature	
PCR water	16.875	ul	2.00'	94 °C	
10X PCR buffer	2.5	ul	0.30'	94 °C	
dNTP's (2,5 mM)	2.5	ul	0.30'	*50 °C	36x
F Primer (50 uM)	0.25	ul	1.00'	72 °C	
R Primer (50 uM)	0.25	ul	07.00'	72 °C	
BSA	0.5	ul	05.00'	4 °C	
Biotherm⁺ Taq	0.125	ul	pauze	15 °C	
DNA	2	ul			

RESULTS

Visual analysis of stomach contents and zooplankton abundance

A total of 45 extracted stomach contents were used in this analysis. 13 stomach contents contained no traces of any prey type. The 6 most abundant prey types found in the zooplankton samples were copepod juveniles (copepod nauplii and copepodites), adult copepods of multiple species, balanid nauplii, balanid cyprids, bivalve veligers, and polychaete larvae (table 1). Polychaete larvae were the most abundant species in the zooplankton samples, while balanid cyprids were only found in low densities.

Table 2: Average (ind. L^{-1}) and relative abundance (%) of zooplankton *In situ* and in *M. leidyi* diet

	Average counts		Relative abundance		Absent in diet
	Zooplankton	*Diet*	*Zooplankton*	*Diet*	
Copepod nauplii	4.97	4.03	2.75	17.65	11
Copepod adult	31.7	13.91	17.54	60.88	7
Balanid nauplii	5.04	0.97	2.79	4.24	18
Balanid cyprid	0.14	1.09	0.07	4.79	22
Bivalve veliger	2.46	2.84	1.36	12.45	7
Polychaete larvae	136.40	0.00	75.48	0.00	32

Feeding experiments

The first series of feeding experiments to investigate the possible preservation techniques gave no positive results. It was not attempted to do a PCR analysis as Nanodrop analysis showed no DNA was present in the samples after the extraction. The next feeding experiment was performed using the new gut content and zooplankton preservation method (i.e. Micropore vacuum filter). In addition, the DNA extraction method was changed to the "Mobio lab. Powersoil" extraction kit. These samples appeared to contain no DNA after gel electrophoresis, except for the positive control. Nonetheless, a PCR analysis was performed despite the apparent lack of DNA because these could simply be included in the *in situ* molecular analysis and if small amounts of DNA would be present, PCR could still be successful. However, no PCR product was found. Attempts to alter the experimental set-up used in the pilot experiments were unsuccessful.

Molecular analysis

19 samples of zooplankton and 16 *M. leidyi* gut contents were analyzed for the presence of DNA of six bivalve species. The samples were taken in the months June, July and August. Table 2 shows the number of positives for each species of bivalve. All zooplankton samples contained at least one species of bivalve, while two *M. leidyi* gut samples contained no bivalve DNA. The analysis with primers for *Mya arenaria* gave the most positives (15 positives for zooplankton and 14 positives for the gut contents), while *Macoma balthica* was present in none of the samples.

Table 3: Number of samples where bivalve DNA was present in *in situ* zooplankton and *M. leidyi* diet per bivalve species.

	Total	Ensis	Mytilus	Cerastoderma	Crassostrea	Macoma	Mya
Zooplankton	16	12	10	10	9	0	15
Gut	19	6	4	0	6	0	14

DISCUSSION

The feeding experiments, which were performed to test the different preservation techniques were unsuccessful. No DNA was found after extraction in the first two experiments designed to test the preservation techniques. The samples (with either 10 ml of water 10 ml of ethanol) were too large to extract DNA directly. Therefore, the samples had to be centrifuged. Usually the target DNA would aggregate in the pallet and the supernatant can be drained without significant loss of the sample. However, in our experiments it was observed, that long strands of material assumed to be of the *M. leidyi* stomach walls remained suspended in the supernatant, even after maximal advised centrifuge speed and duration. Removal of the supernatant was therefore not possible without losing much of this material. It is likely that this resulted in loss of target species DNA, which might have been attached to the strands. Therefore, the preservation technique was unsuitable for this study.

During microscope examination of the *M. leidyi* stomachs directly after the experiments, it appeared that no bivalve veligers were ingested in the second set of experiments. A few factors might account for the lack of feeding by *M. leidyi* in the pilot.
The experimental setup consisted of 25 l aquaria without circulation in the water column. As bivalve veligers are planktonic (Wildish & Kristmanson, 1997), they have possibly sunk to the bottom of the experimental tank. Other possibilities were that the *M. leidyi* individuals in the experiments were more motile and might therefore not have been in contact with the provided prey or that the *M. leidyi* simply did not feed during the experimental time period.
Several attempts to alter the experimental setup to solve these problems were investigated. The most promising was creating small Kreisel aquaria to overcome the sinking of the veligers. However, due to time constraints and operational problems it was not possible to pursue this. Thus, feeding rates and digestion times needed to investigate in situ predatory impact of *M. leidyi* could not be calculated.

Visual analysis of diet

Initially, the visual analysis of the diet compared to the zooplankton standing stock was set up to estimate the predatory impact of *M. leidyi*. When no feeding was observed in the pilot experiments, it was determined that the estimation of predatory impact would not be a priority. Still, the visual dietary analysis was of importance for the assessment of the stomach content extractions, and for recommendations regarding future predatory impact assessments based on the conclusions of the molecular analysis.

The visual analysis revealed a result found in similar dietary studies of *M. leidyi* (McNamara *et al.* 2010). Polychaete larvae in both this and our study were the most abundant species found in the zooplankton standing stock, but they were not found to be ingested by the ctenophore. This is surprising, as it is assumed that *M. leidyi* is a generalistic feeder. Although they are more likely to consume fast moving prey over slower moving prey (Waggett and Costello, 1999), the high density of polychaete larvae largely increases the encounter rate. Therefore, large quantities of polychaete larvae were expected to be present in the stomach contents. One explanation is very rapid digestion of polychaete larvae due to their softer outer shell (compared to bivalves and copepods), making visual identification of these larvae almost impossible. Most samples contained heavily digested material that might potentially be of polychaete larvae, but this could not be positively concluded. In

studies performed on stomach contents of fish it was found that especially larval prey items are difficult to observe after a short time of digestion (e.g. Schooley, 2008; Legler et al., 2010). Molecular analysis of the stomach contents could potentially provide insights in whether *M. leidyi* avoids polychaete larvae or if polychaetes are indeed being consumed.

Although visual analysis of diet is indeed possible with extracted stomach contents, the sample size of this analysis remains very small. To avoid severe degradation of the samples, only a small number of individuals could be extracted per haul due to the long handling time during the extraction process. It became evident that a large variance in the abundance of species present in the stomachs made the sample size is too small to draw accurate conclusions, certainly when this technique would be applied in determination of in situ clearance rates.

Molecular analysis

In the molecular analysis of the stomach contents of *M. leidyi,* 89.5% of the stomach contents contained DNA from at least one species of bivalve. If that percentage is compared to the visually analyzed samples (where only 55.6% of the stomach contents contained at least one bivalve veliger) it appears that the molecular analysis has a significantly higher detection probability. It was previously stated, that in the visual analysis there was a high variance of prey items in stomach contents. However, the results of the molecular analysis indicate that a significant proportion of present bivalve veligers in the diet were overlooked when using only the visual analysis data.

However, sample selection does not account for the difference in the presence/absence of bivalve veligers between the samples used in the molecular analysis and the 32 visually analyzed samples that contained prey items. When the empty stomach content samples are excluded from the analysis, still only 78.1% of the samples contained at least one bivalve veliger.

The visual analysis relies on the presence of the veliger's shell. This does not necessarily apply to the molecular analysis. It is possible that the shell of the bivalve veliger is already egested by the predator but strands of DNA are still present in the gut when the content is extracted. This would give a positive result in the molecular analysis but would be missed in the visual analysis. It is arguable whether this particular bias is of importance for the estimation of predatory impact. Some may argue that for the simplification of the estimation it should only rely on the presence of the veligers shell. Furthermore, the molecular analysis is not a quantitative measure, thus estimating the implications of the found bias for predatory impact analysis is difficult. Although some research has been carried out where the amount of DNA is extrapolated to relative prey density (e.g. Njestgard *et al.* 2008), this might not be applicable yet for our intended purpose. It also requires labour intensive calibration by testing DNA detection limits and concentrations for various digestion times and temperature ranges (King *et al.* 2008), but it points to underlying problems in estimating predatory impact based on visual counts. Moreover, the visual analysis of the stomach contents is a very labour intensive procedure. Samples often contain large numbers of prey items, and heavily digested material clump together in the samples, making it easy to overlook the smaller prey items. With an average of only 2.8 bivalve veligers per sample in the visual analyses it is likely that some veligers were overlooked, thus creating the difference between the two analyses. If it is assumed that this underestimation of smaller prey species is present in all samples and is of importance for not only bivalve veligers, considerable biases towards larger prey species can occur in predatory impact studies (Feller *et al.*, 1985)

General conclusions

In this study it was demonstrated that molecular tools are useful to determine prey composition of predatory comb jellyfish. Although the pilot experiments were not successful, the *in situ* samples contained high grade DNA. Therefore, it can be concluded that this technique can be used for determination of prey species when visual identification is not possible.

However, it is not advisable to use the extraction protocol for all dietary studies of *M. leidyi*. Whether or not this procedure can be applied, relies on intended research goal. If the stomach contents are used for visual analyses for the estimation of predatory impact it might not be of high importance to have extracted stomachs. The handling time per individual is simply too long to create a decent sample size. In that case, using this technique would make it impossible to make significant conclusions.

The stomach content extraction method for molecular analysis appeared to function very well. Almost all stomach samples contained at least some bivalve DNA of one of the 6 species tested. Five out of the six species tested was found in at least one stomach samples. Only *Macoma balthica* was found in no stomachs, however, was neither found in any ambient water samples.

Molecular analyses have the potential to identify which prey types are underestimated. It was shown that visual analysis of *M. leidyi* diet could results in an underestimation of the contribution of some prey types to the true dietary composition. Analyses to determine predatory assessments rely heavily on visual identification of the diet, and could therefore be affected by the bias created. When future refinement of molecular techniques has the potential to give quantitative estimations, the molecular analysis of diet and thus the necessity to extract the stomach contents will be a valuable addition to dietary assessment and predatory impact studies.

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