Running Page Head: Pinna nobilis Quantitative PCR

Identification of potential recruitment bottleneck in larval stages of the giant fan mussel *Pinna nobilis* using specific quantitative PCR.

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Abstract

Pinna nobilis is an endangered species of fan mussel found along coastal Mediterranean waters requiring special attention for conservation. Populations are restricted in number generally, due to anthropogenic disturbances, disease, and in some areas, low rates of recruitment. To date, the difficulties associated with the identification of planktonic stages have prompted the use of benthic collectors as a proxy for quantifying larval supply, despite important information being lost regarding planktonic processes. Herein, we present evidence of spawning utilizing a qPCR assay developed for detecting genomic DNA of *P. nobilis* to enable specific identification of planktonic stages of this bivalve species. This tool could augment the knowledge of the life history of this species throughout the Mediterranean. In the Ebro Delta (Catalunya, Spain) it has been used to better understand what might be limiting their reproduction locally. We demonstrate the ability to differentiate DNA of *P. nobilis* from other bivalve mollusks and distinguish between fertilized and unfertilized eggs of *P. nobilis*, which may be a crucial point in future studies for understanding the low level of recruitment seen in this natural population of *P. nobilis*. We also show evidence of larval presence during the expected spawning period although abundances were so low that they pose new questions about factors controlling planktonic availability.

Keywords: shellfish larvae; Mediterranean; Pinna; qPCR

Introduction

The endangered fan mussel *Pinna nobilis* L. is the largest and one of the more emblematic marine Mediterranean bivalves, reaching sizes of up to 120 cm, and 20 yrs age (Zavodnik et al., 1991; Butler et al. 1993). Its endemic nature prompted its inclusion within the endangered Mediterranean species list at the Barcelona convention (Protocol ASPIM Annex 2), and the European Union has also strictly prohibited the capture of animals (Habitats Directive Annex IV EEC 1992). The European Council Habitats Directive 92/43/EEC, on conservation of natural habitats and the wild fauna and flora, proclaims that *P. nobilis* is strictly protected by the above named Annex IV of the EEC, and all forms of deliberate capture or killing of fan mussel specimens are prohibited by law (Centoducati et al. 2007). Yet, poaching activities continue (e.g., Siletic & Peharda 2003), and an indirect threat is still imposed by a multiplicity of anthropogenic stressors such as trawling fisheries, eutrophication, sea level rise and temperature increase among other causes (Marbà et al., 1996), which may also trigger mass mortalities. For instance, a significant large-scale mortality occurred during the summer of 2016 along the Spanish Mediterranean coast and the Balearic Islands, possibly associated with outbreaks of a haplosporidan parasite (Darriba 2017). In certain areas such as the Mljet lakes (Croatia), and the Mar Grande Basin (Italy) *P. nobilis* populations have been reported to experience some increase (e.g., Siletic & Peharda 2003, Centoducati et al., 2007), although common abundances do not usually exceed one individual per 100 m^2 (Guallart & Templado 2012).

Recruitment rates of *P. nobilis* within benthic collectors are often used as a proxy for larval availability (e.g., Kersting & García-March 2007), but planktonic abundances *per se*, have never been evaluated in any region. To distinguish

bivalve species by larval morphology is not easily achieved as the veliger larval morphology reflects more ancestral states than adult morphology and therefore makes identification to species level of the plankton stages unlikely (Malchus & Sartori 2013). Yet, the abundance of planktonic stages of *P. nobilis* in open waters is not necessarily the same or has the same patterns of scale of abundances as in the benthos, and the pelagic-benthic coupling may not affect all species equally (Ebert 1983). For instance, it has been shown that large-scale environmental factors (e.g., currents, nutrients, temperature, etc.) were the most influential on larval availability, whereas benthic abundances were determined by the complexity of spatial distribution of preferential substrates for settlement, predation and migration occurring at smaller spatial scales (Prado et al. 2012).

Alfacs Bay (Ebro Delta, Catalonia) has been reported to contain the second largest population of *P. nobilis* in the Mediterranean with an estimated number of over 90,000 individuals, with peak densities of up to 20 individuals per 100 m^2, and mean densities of 1.61 m^2 (Prado et al., 2014). The population is characterized by an extremely superficial distribution of individuals growing over extensive meadows of the seagrass *Cymodocea nodosa*, at depths from 20 cm down to 130 cm (Prado et al., 2014). Although this is close to the shallow distribution limit established for the species (ca. 0.5 to 60 m; Butler et al., 1993), it usually displays density peaks at depths from ca. 3-4 m to 14-15 m (Garcia-March and Kersting 2006; Katsanevakis 2007; Coppa et al., 2013) and those inhabiting depths less than 1 m are only rarely reported (see Zakhama-Sraieb et al., 2011). The origin of the Alfacs Bay population is uncertain, and was apparently reduced to a few isolated individuals during the 1990's (J. Romero, pers. comm.), until a major recruitment event occurred, although larval supply

from other nearby locations such as the Sierra de l´IRTA, Columbretes Islands, or L'Ampolla de Mar is also possible. Currently, the population displays a very homogeneous size structure (between 40 and 60 cm) and lacks individuals smaller than 20 cm, suggesting an important limitation on the availability of larvae and/ or high mortality during the processes of benthic settlement and recruitment (Prado et al., 2014). In fact, the distribution of individuals in the bay is highly variable, including large unpopulated areas, presumably due to changes in habitat and/ or the physicochemical conditions of the medium (e.g., agricultural runoff from extensive rice cultivation in the northern shore of the bay, and salt evaporation ponds along the inner part of the southern shore; Prado et al., 2014). Therefore, developing an adequate tool for evaluating larval availability and fertilization success will help assessing the factors driving the demographic of the population within the bay (but also applicable to other regions). Such information could be very helpful for the implementation of conservation programs that take into account the effect of local factors such as agricultural runoff and/ or adult densities in planktonic processes.

The use of quantitative polymerase chain reaction (qPCR) has proven to be useful for detection of low abundance and cryptic species in aquatic environments (Ficetola et al., 2008), or where morphological characteristics cannot be used for species discrimination (Andree et al. 2011). More recently, qPCR has been shown to be an effective and sensitive tool for the detection and quantification of planktonic organisms (e.g., Vadopalas 2006; Endo et al., 2010), whose identification through classical observational methods was extensive and arduous. More relevantly, qPCR has been used for the detection and quantification of larvae in other species of commercial bivalve such as abalone

(Vadopalas et al. 2006) and invasive alien species such as zebra and golden mussels (Frischer et al. 2002; Endo et al. 2009). For these purposes planktonic abundances are estimated by plotting threshold cycle (Ct) values on the standard curve obtained from template DNA extracted from serial dilution of eggs/ larvae (see for instance, Endo et al., 2010). Hence, the limitation to the development of the method relies on the availability of planktonic material of the target species for construction of a proper calibration curve. Moreover, the interpretation of results requires some consideration be given to the chromosome endowment of the analytic sample (fertilized or non-fertilized egg or larva), and the copy number of the genomic region being targeted for the assay.

In the particular case of *P. nobilis*, the apparent short period of larval planktonic life, between five and ten days (De Gaulejac & Vicente 1990), may help to reduce variability in the number of larval cells among individuals collected at different time points. Hence, it was considered that quantitative PCR could be applied for the efficient detection and quantification of *P. nobilis* eggs and larvae, and has lead us to the following specific objectives: 1) to identify a species-specific marker for a qPCR assay; 2) design primers for specific amplification of *P. nobilis* DNA, which are well conserved at species level, but with high interspecific variability to avoid false positives in screening environmental samples (Andree et al., 2011; Dejean et al., 2011; Wilcox et al., 2013); 3) to establish a useful calibration curve for fertilized and unfertilized eggs and larvae of *P. nobilis* that allow interpretation of Ct values from field samples; and 4) to determine a field sampling method most effective for detecting and quantifying the presence of *P. nobilis* larvae or gametes. Meeting these objectives will provide the techniques for more expansive evaluations in other Mediterranean regions hosting *P. nobilis*.

Materials and Methods

Sample collection

Tissue samples were collected from living *P. nobilis in situ* by pinching off a small piece of mantle using a 15 cm long hemostat inserted between semi-opened valves, and preserving in 70% ethanol until later DNA extraction (Fig. 1). For the purpose of obtaining DNA sequence data, samples were initially collected from two sites: Alfacs Bay (n= 21) [40º 35´ 40.59´´N; 0º 39´ 37.36´´E] and to the south, outside of the bay, in the port of Las Casas de Alcanar (n= 9) [40º 33´ 09.32´´N; 0º 31´ 53.64´´E] (Fig. 2).

Initially (from 06/01/16 to 07/01/16), only 500 ml water samples (n= 3 per site) were collected immediately adjacent to individual adult animals *in situ* and later filtered through a 10 µm nylon mesh. The mesh was rinsed with lysis buffer to collect material for extraction and kept frozen until further processing. In June, when the first 500 mL samples were collected, they were also examined in the lab by light microscopy and bivalve larvae identified and counted. However, the observation of single larvae in this volume was rare, and from 07/15/16 to 10/01/16, sampling was conducted every 15 days using a plankton net having an open aperture of 0.2 m diameter and 30 µm size mesh (sufficient for catching eggs and larvae). Samples were collected as before in 3 sites (n= 3 replicates per site) by walking ca. 30 m over the seagrass bed of *Cymodocea nodosa*, equivalent to a filtration volume of 3.77 m^3. Samples were preserved in 70% ethanol and separated into different size fractions: 40 µm (egg sizes ranging from 40 to 70 µm and larval sizes from ca. 80 to 110; Trigos et al. 2017), 125 µm and 250 µm (to check for additional entangled material) using sieve nets. A

preliminary observation of each fraction and sample was also conducted in order to determine the abundance of eggs and larvae and the feasibility of their separation. For eggs, given the large abundance and the absence of identification keys that could aid the distinction from any other marine invertebrates, separation was considered unfeasible and not conducted. However, all bivalve larvae observed were collected. When the number was too large for accurate counting (hundreds to thousands of individuals), fractions were divided into smaller subsamples (1/2, 1/4, or 1/8 depending on the number), and later the total number of larvae in the sample calculated from these subsample values. Individual larvae were separated under the dissecting microscope (NIKON SMZ1500) using a zooplankton “micro-spoon” fabricated from a thin piece of platinum wire to which a loop of ca. 80 µm diameter was formed. All separated larvae from each replicate sample were kept in 1.5 ml vials with 30 µl of Qiagen AE buffer (to ease larvae release from the spoon) and stored at -80ºC until later DNA extraction (see below).

For the construction of calibration curves we used ethanol preserved D-veliger larvae (all of these ca. 85 µm in length), and fertilized and unfertilized egg samples of *P. nobilis* (Fig. 3) from an *in vitro* reproduction study performed previously in another institute (Trigos et al. 2017) (Fig. 4). Also, eggs produced in the lab were used for creating pseudo-field samples to test the likelihood of collecting eggs from seawater and detecting the presence of *P. nobilis* DNA. This was done by collecting 10 eggs or 1 egg and placing them into different volumes of filtered seawater (15, 50, or 500 mL) that were filtered through a 10 µm nylon mesh for subsequent DNA extraction and testing by qPCR.

DNA extraction, amplification, sequencing and specific qPCR amplification

Extraction of DNA utilized the Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA, U.S.A.) and for all samples the $A_{260/280}$ ratios were examined by spectrophotometry to evaluate purity. Seawater samples were filtered using 10 µm mesh nylon filters and the filtrate treated the same as eggs and larvae samples using the same protocol. DNA concentrations for all tissue samples were normalized to 100 ng/ µL for PCR. For all other sample types (eggs, larvae, or seawater) 1µL of DNA, from a total eluate of 30 µL, was added directly to the reaction or (see below) diluted 1:10.

Primers used for amplification of the ribosomal DNA internal transcribed spacer region (ITS-1, 5.8S, ITS-2) were DinoE 5´ CCKSTTCAYTCGCCRTTAC 3´ and 18dKA 5´ CACACCGCCCGTCGCTACTACC 3´ (Andree et al. 2011) and included at a final concentration of 0.6 µM each. Additionally, each 25 µL reaction also contained 800 µM dNTP´s, 2 mM $MgCl_2$ and 5% DMSO. The thermal cycler program used was 40 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 1.5 min, preceded by 5 min at 95 ºC, and followed by 10 min at 72 ºC. The resulting genomic fragment was approximately 1200 bp. Each PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.), then sent to a private company for bi-directional sequencing using the same primers as those in the original amplification (Sistemas Genomicos, Valencia, Spain). The resulting sequence data was edited and trimmed using BioEdit (Hall 1999) to remove terminal primer sequence artifacts and cleaned of any aberrant base-calling before aligning the forward and reverse reads to construct a consensus sequence representative of this population of the species. The sequence was further subjected to analysis using BLAST to confirm the identity of the sequence obtained, as well as identify what species might have sequences already

described that are similar. In this way, species-specific primers can be designed using an alignment containing sequences from the extant species. The *P. nobilis* sequence was used for designing the species-specific primers that avoided the repeat regions to be applied in the qPCR assay, with four primer pairs designed and tested (data not shown). The primer pair Pin ITSF3 5´ GTACCTGTGCCGAGTTCTCTCG 3´and Pin ITSR2 5´ CGCCGTTGGATGAAACCGTACG 3´ resulted in the strongest and more specific amplification producing a 180 bp product, and this pair was chosen for use in assay optimization. The primer BLAST application (within GenBank NCBI) was used to test *in silico* the specificity of the primers and validate the absence of any other priming site within sequences currently in the NCBI database. After testing various annealing temperatures, the final qPCR program consisted of 45 cycles of 95ºC for 20 sec, 66ºC for 30 sec, preceded by 5 min at 95 ºC, and followed by dissociation stage for determining the melt curve. All reactions contained 1X SYBR Green mix (Ref# 4364344, Thermo Fisher Scientific, USA) and 0.5 µM of each primer. Samples were analyzed in triplicate, and each plate (96 wells) contained no-template controls and positive controls of genomic DNA from adult tissues to confirm validity of the reactions. For all positive results, comparison of melt curves of unknowns from all collection sites (Las Casas de Alcanar, Alfacs Bay, and southeast of France) were made to melt curves of our known positive control from adult tissues; this was performed in lieu of sequencing the amplified products to confirm positives.

Calibration curves, specificity, and assay optimization

To confirm specificity of the assay primers were tested using 50 ng of genomic DNA from the three most abundant commercially produced shellfish species:

Crassostrea gigas, Cerastoderma edule, and *Mytilus galloprovincialis*. Further, the species-specific qPCR was tested on different numbers of eggs and larvae produced by captive spawning (as mentioned above) from individuals located in the Southeast of France and preserved in 70% ethanol (Trigos et al. 2017). Specific numbers of fertilized and unfertilized eggs, and larvae from these lab samples (n= 5, 10, 20 and 50; with two replicates of each), were used for the purpose of establishing a correlation between the Ct values obtained and the number of individuals (fertilized/ unfertilized eggs or larvae); ie. - a calibration curve. Further, genomic DNA obtained from adult tissues were also tested for calibration and used to evaluate the overall efficiency of the assay. Additionally, a subset of DNA extracts was serially diluted (1:10, 1:20, 1:50, 1:100) to remove/dilute potential PCR inhibitors that may have originated in either the egg matrix, or seawater as has been seen previously (Andree, et al. 2011). DNA extractions were performed as before with the Qiagen Blood and Tissue Kit, but with the additional step of bead-beating using a BeadBeater-8 (BioSpec Products, Bartlesville, OK, U.S.A.). All DNA extracts were eluted in a final volume of 30 µL. Linear regression of the calibration curve established a slope that was to be used for the calculation of amplification efficiency (E%) following the equation: $E\% = 10^{(-1/slope)} - 1$.

Results

During analysis of the intergenic transcribed spacer (ITS) rDNA sequences, there were found multiple repeat regions (short poly-A, poly-G and poly-C stretches) with one poly-adenine repeat [$A_8(GCAC)A_5$] that was problematic for sequencing

to properly clarify the number of repeats. Aside from the repeat regions the remaining sequence from all 30 isolates provided a clear consensus sequence. The two longest unambiguous sequences obtained have been uploaded to GenBank under accession numbers **KX101234** and **KX101235**. Of the 447 bivalve mollusk ITS ribosomal DNA sequences currently in GenBank, results from BLAST analysis found only one species (other than *P. nobilis*), *Perna perna* that was a near match with a similarity index of 96.4%. No primer binding of the designed primers to the *Perna perna* sequence was evident *in silico*. Testing the different primer sets for *P. nobilis*, demonstrated each of the primer pairs tested had distinct melt curves (data not shown). The primer pair chosen amplified its respective target more robustly as compared to the other primer sets, as evidenced by the lower Ct value obtained when using the same quantity of DNA. Further, no amplification was seen when using genomic DNA from *C. gigas, C. edule, or M. galloprovincialis,* confirming in part, the specificity of the assay. Optimization of the assay using the DNA from adult tissues established an E%= 99.8% (R^2 = 0.997). This was similar for fertilized (97.6%; R^2= 0.89) and unfertilized (99.8%; R^2= 0.98) eggs although the *y* intercepts were 50.33 and 54.23, respectively. The slope obtained when all egg samples were included indicated efficiency of 100.9% (Fig. 4).

Results of the calibration curve using larvae samples gave erratic results with no correspondence between extracted DNA and specific numbers of larvae per sample. We did obtain a standard curve using larvae however, the slope obtained was -3.73 indicating a E% = 85.4%. Another assay was conducted using seven replicates of only one larvae. The mean of the Ct values from these was 36.9. Using the standard curve for diploid eggs a Ct of 36.9 is approximately 17,844

300 diploid cells. This value was used to correct for all the other calculations made
301 from larvae isolated from field samples. Thus, the Ct value obtained from one
302 larva was used to normalize the quantity of *P. nobilis* larvae within plankton net
303 field samples, giving a range from 0.2 to 32.4 (i.e., up to 8.6 larvae·m^{-3}).

304 Field samples of 500 mL water collected in Alfacs Bay were all negative. Tests
305 performed to establish possible losses of eggs and larvae during the filtration
306 process were evaluated using pseudo-field samples (filtered seawater samples
307 spiked with either ten eggs or one egg) showed that samples with one egg in
308 more than 50 mL were not consistently detectable.

309 Larvae from the plankton net samples were collected manually using a micro-
310 spoon under a dissecting microscope and used in a pool as DNA extraction
311 material. From among these samples, several (15/62) were identified as
312 containing *P. nobilis* (Table 1). The number of positives increased slightly from
313 June – July to August- September. From the total number of positive samples the
314 mean Ct value was 36.41.

315 Further, although DNA was obtained from both field samples and preserved
316 lab-cultured samples, there were some doubts about possible PCR inhibition
317 since the expected amplification at Ct values lower than 25 were not observed.
318 Serial 1:10 dilutions of some samples were performed to eliminate potential
319 inhibitors, but the expected reduction in Ct values was not observed. Yet, we
320 continued using 1:10 dilutions in order to increase the quantity of sample
321 available for experimentation.

322

323 **Discussion**

The present study constitutes the first approach conducted for quantifying the abundance of *P. nobilis* larvae in the water column, and proved to be effective in detecting *P. nobilis* DNA from larvae obtained from field samples. The assay did not amplify DNA of other mollusk and non-target bivalve species as confirmed by comparison to the melt-curve profiles from known positive samples. Further, the few positives detected among the larvae collected from plankton net samples, (as well as the low number of "hits" seen by BLAST analysis), strongly suggest this assay has a high specificity. In addition, it worked well with *P. nobilis* DNA from three different populations (Alfacs Bay, Las Casas de Alcanar and Southeast of France), evidencing its broad applicability for similar quantitative assessments in other Mediterranean regions.

The documented ITS rDNA region used in qPCR assays was rich in homopolymer and repeat sequences (areas under less selective genetic pressure) that likely caused the difficulty in getting equivalent amplification from all primer sets initially tested despite their being designed to avoid these regions. Yet, studies of genetic diversity may find this region useful since regions rich in repeat sequences, such as those found here, frequently expand or contract during the course of evolution (Read et al. 2004); this being the basis for microsatellite analysis. The high rate of mutation during evolutionary time scales in ITS regions of the rDNA cistron, as compared to coding regions, means that there is less probability of encountering a similar sequence in closely related species to which species-specific primers can bind, and in some cases may assist in population marker development (Wren et al. 2000). Previous phylogenetic studies related to this genus have used more standard genetic markers such as cytochrome oxidase (Sanna et al. 2013), microsatellite DNA (Gonzalez-

Wanguemert et al. 2014), or nuclear and mitochondrial ribosomal genes (Lemer et al. 2014). In the case of the latter study, cryptic species were identified within the family Pinnidae. Hence, utilizing the ITS region of the rDNA cistron might reveal still more genetic diversity, adding new insights into variability among different populations.

Only few *P. nobilis* larvae were present during the expected summer spawning period (Kersting et al. 2007). All samples displayed high Ct values (between 31.9 and 39.6 with mean value of 36.41), indicative of very low abundance of planktonic stages, yet still supporting the efficiency of the species-specific qPCR as a detection tool. The highest number of larvae visually observed among the samples that tested positive by qPCR was 3,237 collected from zone 3 on Oct. 1, and given the Ct value obtained of 37.49, it is possible that only one larvae among those could be considered as being *P. nobilis*. If the recruitment bottleneck resided with ecological processes occurring in the benthos then a paucity of developmental stages would be expected in the substrata while there would be an abundance of planktonic stages in the water column. However, the low abundance of planktonic stages observed suggests some local processes are affecting the water column, rather than the benthos, and could be interfering with the production of viable larvae. Although during the study we chose three sites where population densities of *P. nobilis* were significantly different (Prado et al. 2014), the low Ct values observed prevented determination of a clear relationship between each zone and their larval abundance. Although, among potential causes of larval supply limitation at the broad spatial scale of the Alfacs Bay, endocrine disrupting organo-phosphates discharged from rice fields during the summer period might be causing alterations in the proper functional

development of the reproductive system (Terrado et al. 2007; Frye et al, 2012). Several such compounds have been identified in environmental compartments and wildlife of the Ebro Delta, and reproductive impacts on water fowl (Mañosa et al. 2001), as well as alterations of acetylcholinesterase (AChE) activity in *Mytilus galloprovincialis* (Escartin and Porte 1997) have been reported. In addition, lower salinity in Northern areas of the Bay due to persistent discharge of agricultural water also affect *P. nobilis* reproduction, and ultimately may account for differences in adult abundances among the three zones (Prado et al. 2014).

In all, only 15 samples were identified as containing *P. nobilis* DNA from mid-July to the beginning of October 2016. However, there were 30% more positive samples in September/October (n= 9) than in July/August (n= 6). In addition, the Ct value of larval samples from Sept-Oct was ca. 2.7 times lower than in the July-Aug period, suggesting an increase in larval abundance, or in size of individual larvae. Yet, according to our calibration results for fertilized eggs, there were few *P. nobilis* in the water column (an average of $12.4 \cdot m^{-3}$), which indicates very low availability of larvae. Although the number of *P. nobilis* larvae has never been quantified in previous studies and is impossible to establish common abundance ranges, the very low occurrence of juveniles in the area (Prado et al. 2014) suggests that they are abnormally low. This pattern is also confirmed by local recruitment data for 2016, which registered a total absence of individuals (Prado et al., in prep) whereas in other areas nearby such as the Columbretes Islands, located only 100 km south from Alfacs Bay, recruitment has been clearly demonstrated (Kersting et al. 2007). In other species of bivalve such as the brown mussel *Perna perna* and the clam *Ruditapes decussatus* larval peaks of

399 hundreds to thousands of individuals per m^{-3} have been reported and result in
400 high rates of recruitment (Chícharo and Chícharo 2001, Porri et al. 2006).
401 Since the number of *P. nobilis* larvae in any sample was small (mean Ct value
402 for all positives of 36.41), most of the observed larvae were likely from other
403 locally abundant species such as oysters, mussels, clams and/or cockles. For
404 instance, in the case of the sample with the highest recorded abundance of larvae
405 (15,272), collected on Sept. 15th, no *P. nobilis* DNA was detected. At present, the
406 identification of morphometric characters requires extreme expertise and
407 specialization in ontogenetic bivalve development (e.g., Malchus and Sartori
408 2013), so this information was not collected in this work. Yet, future
409 multidisciplinary studies may also include this type of analysis prior to DNA
410 extraction and qPCR.
411 In the use of 500 mL water samples, none tested positive. Although when using
412 the plankton net protocol for sampling , the estimated volume of water filtered (ca.
413 3.77 m^3) was an increase of over 7000 fold. Yet, negative results for seawater
414 samples of 500 mL is coherent with low Ct values and scarce positive results
415 from tests performed with collected larvae stages of *P. nobilis*. This was
416 surprising given that the bay harbors the second largest population of this species
417 in the Mediterranean (Prado et al., 2014). Possible reasons for the reduced
418 number of positive results could be dilution of target species planktonic stages
419 within the large dimensions of the Alfacs Bay (ca. 49 Km^2 and average depth of
420 3.13 m). This dilution effect due to the large volume of the bay might be further
421 enhanced by the continual renewal rate of the bay´s water, even though tidal
422 changes are low in the Mediterranean. This is in contrast to similar works in which
423 water from ponds with little or no water renewal were analyzed by qPCR for the

presence of frog (*Rana catesbeiana*) DNA (Ficetola et al., 2008) where sloughed epithelial cells contained in mucus from the frogs skin might accumulate in the absence of continual water renewal. Further, in this study, when testing "pseudo-field samples" only 15 and 50 mL volumes containing one egg of *P. nobilis* consistently detected positive signals, whereas 500 mL volumes used resulted in a negative. However, all volumes were positive when ten eggs were added. This hints at some possible underestimation of DNA contents in field water samples of 500 mL, although this volume was still preferred to maximize the potential of capturing multiple planktonic stages. Additional factors that may have contributed to the absence of positive water samples include low rate of adult mortality (a cause of release of necrotic tissue and cells from moribund animals), reduced spawning and larval abundance, or any combination of these factors.

Although the bay is also the site for intensive shellfish culture (*C. gigas,C. edule* and *Mytilus galloprovincialis*) as well as artisanal harvest of other natural shellfish populations (*Cerastoderma* sp., carpet shell clam *Ruditapes decussatus* and the razor clam *Solen marginatus*) no false positives were detected, as would be evident from the melt-curve profiles.

Calibration curves of fertilized and unfertilized eggs, and adult tissue (data not shown) showed very similar slopes, but differed in their y-intercept an indication of their cells being haploid or diploid. There was significant deviation among replicates that might have been due to artifacts such as free DNA in the aqueous fraction that was transferred with the eggs into the subsample tubes, and/or free DNA adherent to the surface of the eggs via DNA-binding cell surface receptors such as nucleolin (Bennett et al. 1985; Chen et al. 2008). Moreover, the eggs and larvae used were from experimental captive spawning in which all of the larvae

449 produced eventually died. Therefore, it can be presumed that the larvae used
450 may not have been in optimal condition, and if in a moribund state in which cells
451 were undergoing apoptosis the quality of the genetic material obtained may not
452 have been consistent among animals within a sample, or cells within an individual
453 animal. Additionally, ribosomal DNA copy number is likely to increase post-
454 fertilization, by regulatory mechanisms that implicate genes like *fob1* or similar
455 (Joshzuka et al. 2002), that would also contribute to variation between individual
456 eggs and egg groups. Given that the absence or low rates of fertilization might
457 be indicative of some type of infertility among adult individuals (e.g., Lewis and
458 Ford 2012), this ability to differentiate between fertilized and unfertilized eggs
459 might be a useful tool in future studies aimed at establishing the causes of larval
460 and recruitment failure.

461 To conclude, the development of analytical tools such as qPCR to assess the
462 availability of planktonic stages of *P. nobilis* and the ability to differentiate
463 between fertilized and non-fertilized eggs should help to address possible
464 bottlenecks related to gamete viability, clarify concerns of agro-chemical impacts
465 and species-specific sensitivity, and determine the influence of large scale
466 environmental factors on the larval recruitment of this species. Future research
467 efforts need to focus on using qPCR tools to monitor larval abundances in the
468 Alfacs Bay and in other Mediterranean areas, in order to address some of these
469 questions to aid in understanding better how to improve management of wild
470 stocks of this emblematic Mediterranean bivalve.

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473 **Acknowledgements**

The authors wish to thank the Zoo Barcelona Foundation for an Antoni Jonch grant 2015, which provided the economic support necessary to accomplish this work. Authors are very grateful to Dr. Miguel Alonso García-Amilivia for the construction of the zooplankton micro-spoon for the collection of *P. nobilis* eggs and larvae. Also, authors would like to thank Pep Cabanes, Lluis Jornet, and David Mateu for technical assistance during periodical fieldwork sampling in Alfacs Bay.

Conflict of Interest: The authors declare that they have no conflict of interest.

References

Andree, K. B., Quijano-Scheggia, S., Fernández, M., Elandaloussi, L. M., Garcés, E., Camp, J., & J. Diogene, 2011. Quantitative PCR Coupled with Melt Curve Analysis for Detection of Selected *Pseudonitzschia* spp. (Bacillariophyceae) from the Northwestern Mediterranean Sea. Applied and Environmental Microbiology 77: 1651-1659.

Bennett, R. M., Gabor, G. T., & M. M. Merritt, 1985. DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. Journal of Clinical Investigation 76: 2182–2190.

Butler, A., Vicentem, N., & B. De Gaulejac, 1993. Ecology of de Pterioid bivalves *Pinna bicolor* Gmelin and *P. nobilis* L. Marine Life 3: 37-45.

Chícharo, L. & M. A. Chícharo, 2001. A juvenile recruitment prediction model for *Ruditapes decussatus* (L.)(Bivalvia: Mollusca). Fisheries Research 53: 219-233.

Centoducati, G., Tarsitano, E., Bottalico, A., Marvulli, M., Lai, O. R., & G. Crescenzo, 2007. Monitoring of the endangered *Pinna nobilis* Linné, 1758 in the Mar Grande of Taranto (Ionian Sea, Italy). Environtal Monitoring and Assessment 131: 339-347.

Chen, X., Kube D. M., Cooper, M. J., & P. B. Davis, 2008. Cell Surface Nucleolin Serves as Receptor for DNA Nanoparticles Composed of Pegylated Polylysine and DNA. American Society of Gene Therapy 16: 333-342.

Coppa, S., de Lucia, G. A., Magni, P., Domenici, P., Antognarelli, F., Satta, A., & A. Cucco, 2013. The effect of hydrodynamics on shell orientation and population density of *Pinna nobilis* in the Gulf of Oristano (Sardinia, Italy). Journal of Sea Research 76: 201-210.

De Gaulejac, B., & N. Vicente, 1990. Ecologie de *Pinna nobilis* (L.) mollusque bivalve sur les côtes de Corse. Essais de transplantation et expériences en milieu contrôlé. Haliotis 10: 83-100.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & C. Miaud, 2011. Persistence of Environmental DNA in Freshwater Ecosystems. PLoS ONE 6:e23398

Ebert, T. A. 1983. Recruitment in echinoderms. Echinoderm Studies 1: 169-203.

Endo, N., Matsumura, K., Yoshimura, E., Odaka, Y., & Y. Nogata, 2010. Species-specific detection and quantification of common barnacle larvae from the Japanese coast using real time PCR. Biofouling 26: 901-911.

Endo, N., Sato, K., & Y. Nogata, 2009. Molecular based method for the detection and quantification of larvae of the golden mussel *Limnoperna fortunei* using real-time PCR. Plankton Benthos Research 4: 125–128.

Ficetola, G. F., Miaud, C., Pompanon, F., & P. Taberlet, 2008. Species detection using environmental DNA from water samples. Biology Letters 4: 423–425.

Frischer, M. E., Hansen, A. S., Wyllie, J. A., Wimbush, J., Murray, J., & S. A. Nierzwicki- Bauer, 2002. Specific amplification of the 18S rRNA gene as a method to detect zebra mussel (*Dreissena polymorpha*) larvae in plankton samples. Hydrobiologia 487: 33-44.

Frye, C., Bo, E., Calzà, L., Dessì-Fulgheri, F., Fernández, M., Fusani, L., Kah, O., Kajta, M., Le Page, Y., Patisaul, H. B., Venerosi, A., Wojtowicz, A. K., & G. C. Panzica, 2012. Endocrine Disruptors: A review of some sources, effects, and mechanisms of action on behavior and endocrine systems. Journal of Neuroendocrinology 24 :144–159.

García-March, J. R., & D. K. Kersting, 2006. Preliminary data on the distribution and density of *Pinna nobilis* and *Pinna rudis* in the Columbretes Islands Marine Reserve (Western Mediterranean, Spain). Organisms Diversity and Evolution 6: 06-16.

Gonzalez-Wanguemert, M., Costa, J., Basso, L., Duarte, C. M., Serrao, E. A., & I. Hendriks, 2015. Highly polymorphic microsatellite markers for the Mediterranean endemic fan mussel *Pinna nobilis*. Mediterranean Marine Science 16: 31-35.

Guallart, J., & J. Templado, 2012. *Pinna nobilis*. In: VV. AA., Bases ecológicas preliminares para la conservación de las especies de interés comunitario en España: Invertebrados. Ministerio de Agricultura, Alimentación y Medio Ambiente. Madrid, p 81.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Johzuka, K., & T. Horiuchi, 2002. Replication fork block protein, Fob1, acts as an rDNA region specific recombinator in *S. cerevisiae*. Genes to Cells 7: 99–113.

Katsanevakis, S. 2007. Growth and mortality rates of the fan mussel *Pinna nobilis* in Lake Vouliagmeni (Korinthiakos Gulf, Greece): a generalized additive modelling approach. Marine Biology 152: 1319-1331.

Kersting, D. K., & J. R. García-March, 2007. Preliminary data on *Pinna nobilis* larval recruitment using mesh collectors in Columbretes Islands Marine Reserve, NW Mediterranean. In: European Symposium on Marine Protected Areas. 25th-28th September 2007 Murcia, Spain.

559 Lemer, S., Buge, B., Bemis, A., & G. Giribet, 2014. First molecular phylogeny of
560 the circumtropical bivalve family Pinnidae (Mollusca, Bivalvia): Evidence
561 for high levels of cryptic species diversity. Mol Phylo Evol 75:11–23
562 Lewis, C., & A. T. Ford, 2012. Infertility in male aquatic invertebrates: a review.
563 Aquatic Toxicology 120: 79-89.
564 Malchus, N., & A. F. Sartori, 2013. Part N, Revised, Volume 1, Chapter 4: the
565 early shell: ontogeny, features, and evolution. Treatise Online 61: 1–114.
566 Mañosa, S., Mateo, R., & R. Guitart, 2001. A Review of the Effects of Agricultural
567 and Industrial Contamination on the Ebro Delta Biota and Wildlife.
568 Environmental Monitoring and Assessment. 71: 187–205.
569 Marbà, N., Duarte, C.M., Cebrián, J., Gallegos, M.E., Olesen, B., & K. Sand-
570 Jensen, 1996. Growth and population dynamics of *Posidonia oceanica* on
571 the Spanish Mediterranean coast: elucidating seagrass decline. Marine
572 Ecology Progress Series 137: 203-213.
573 Porri, F., McQuaid, C. D., & S. Radloff, 2006. Spatio-temporal variability of larval
574 abundance and settlement of *Perna perna*: differential delivery of mussels.
575 Marine Ecology Progress Series 315: 141-150.
576 Prado, P., Caiola, N., & C. Ibáñez, 2014. Habitat use by a large population of
577 *Pinna nobilis* in shallow waters. Scientia Marina 78: 555-565.
578 Prado. P., Tomas, F., Pinna, S., Farina, S., Roca, G., Ceccherelli, G., Romero,
579 J., & T. Alcoverro, 2012. Habitat and scale shape the demographic fate of
580 the keystone sea urchin *Paracentrotus lividus* in Mediterranean
581 macrophyte communities. PloS ONE 7:e35170
582 Read, L. R., Raynard, S. J., Rukść, A., & M. D. Baker, 2004. Gene repeat
583 expansion and contraction by spontaneous intrachromosomal

584 homologous recombination in mammalian cells. Nucleic Acids Research
585 32:1184-1196.
586 Sanna, D., Cossu, P., Dedola, G. L., Scarpa, F., Maltagliati, F., Castelli, A.,
587 Franzoi, P., Lai, T., Cristo, B., Curini-Galletti, M., Francalacci, P., & M.
588 Casu, 2013. Mitochondrial DNA Reveals Genetic Structuring of *Pinna*
589 *nobilis* across the Mediterranean Sea. PloS ONE 8(6):e67372
590 Siletic, T., & M. Peharda, 2003. Population study of the fan shell *Pinna nobilis* in
591 Malo and Veliko Jezero of the Mljet National Park (Adriatic Sea). Scientia
592 Marina 67: 91-98.
593 Escartin, E., & C. Porte, 1997. The use of cholinesterase and carboxylesterase
594 activities from *Mytilus galloprovincialis* in pollution monitoring.
595 Environmental Toxicology and Chemistry 16: 2090–2095.
596 Terrado, M., Kuster, M., Raldúa, D., Lopez de Alda, M., Barceló, D., & R. Tauler,
597 2007. Use of chemometric and geostatistical methods to evaluate
598 pesticide pollution in the irrigation and drainage channels of the Ebro river
599 delta during the rice-growing season. Anal Bioanal Chem. 387:1479–1488
600 Trigos, S., Vicente, N., Prado, P., & Espinós, F. J, 2017. Adult spawning and early
601 larval development of the endangered bivalve *Pinna nobilis*. Aquaculture.
602 Vadopalas, B., Bouma, J. V., Jackels, C. R., & C. S. Friedman, 2006. Application
603 of real-time PCR for simultaneous identification and quantification of larval
604 abalone. Journal of Experimental Marine Biology and Ecology 334: 219–
605 228.
606 Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley,
607 A. R., & M. K. Schwartz, 2013. Robust Detection of Rare Species Using

608 Environmental DNA: The Importance of Primer Specificity. PLoS ONE
609 8:e59520
610 Wren, J. D., Forgacs, E., Fondon III, J. W., Pertsemlidis, A., Cheng, S. Y.,
611 Gallardo, T., Williams, R. S., Shohet, R. V., Minna, J. D., & H. R. Garner,
612 2000. Repeat Polymorphisms within Gene Regions: Phenotypic and
613 Evolutionary Implications. American Journal of Human Genetics 67: 345–
614 356.

615 Zavodnik, D., Hrs-Brenko, M., & M. Legac, 1991. Synopsis on the fan shell *Pinna*
616 *nobilis* L. in the eastern Adriatic Sea. In: Boudouresque, C. F., Avon, M.,
617 & V. Gravez, (Eds.), Les Espèces Marines à Protéger en Méditerranée.
618 GIS Posidonie, Marseille, p 169-178.
619 Zakhama-Sraieb, R., Sghaier, Y. R., Omrane, A., & F. Charfi-Cheikhrouha, 2011.
620 Density and population structure of Pinna nobilis (Mollusca, Bivalvia) in the
621 Ghar El Melh lagoon (N-E Tunisia). Bulletin de l'Institut National des
622 Sciences et Technologies de la Mer de Salammbô 38: 65-71.
623

Figure1. – Non-lethal sample collection *in situ* from *P. nobilis*, using a hemostat.

Figure 2. - Map indicating the location of each sampling zone within the study site (Z1 = zone of high abundance; Z2 = zone of intermediate abundance; Z3 = zone of low abundance). Dotted lines indicate the irrigation canals used for managing water flows through the rice fields in the Ebro delta.

Figure 3. – Exemplars of *P. nobilis* eggs (left) and larvae (right) produced in the laboratory using artificial gamete induction/fertilization.

Figure 4. Calibration curve demonstrating distinct results for fertilized and unfertilized eggs of *P. nobilis*. Squares = fertilized egg samples. Circles = unfertilized egg samples. Linear regression of results for fertilized or unfertilized eggs (solid lines) indicate efficiencies of amplification of 99.8% and 98.6%, respectively. Efficiency of assay calculated from the slope of the linear regression obtained from all egg samples was 100.9% (dotted line).

Table 1. Summary of qPCR results from larvae collected between mid-July and September. The number of larvae shown in “Subsample” is the number of larvae in the extracted DNA samples tested. “Total N” is the number extrapolated from counting the subsample. All positives recorded had high Ct values between 31.9 and 39.6 (mean = 36.41). Larvae number are extrapolated from the calibration curve obtained from diploid eggs. All values larger than 0.5 were rounded to the nearest whole number. (* indicates values normalized using the Ct value obtained from qPCR with one larvae: 37.5)

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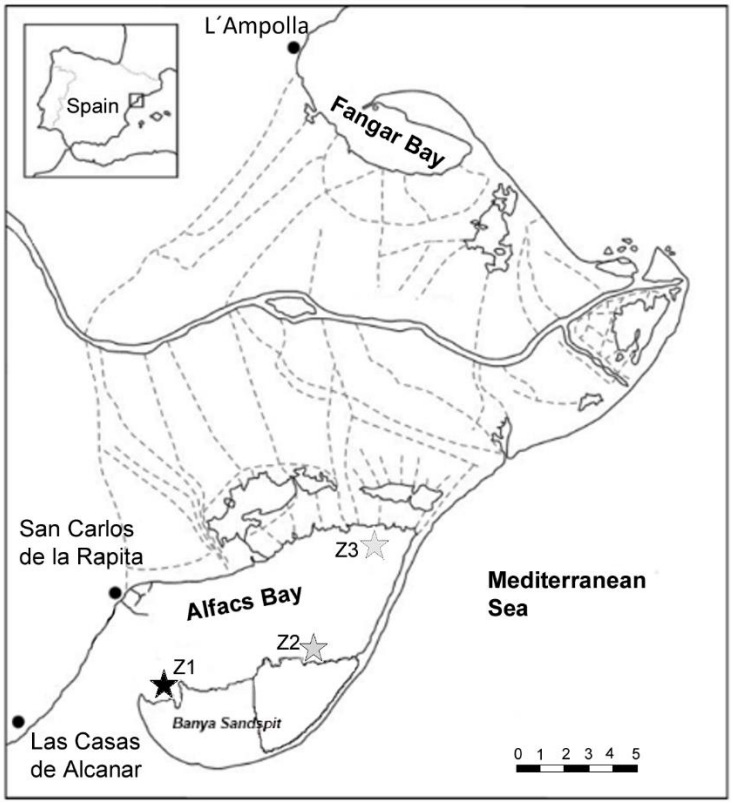

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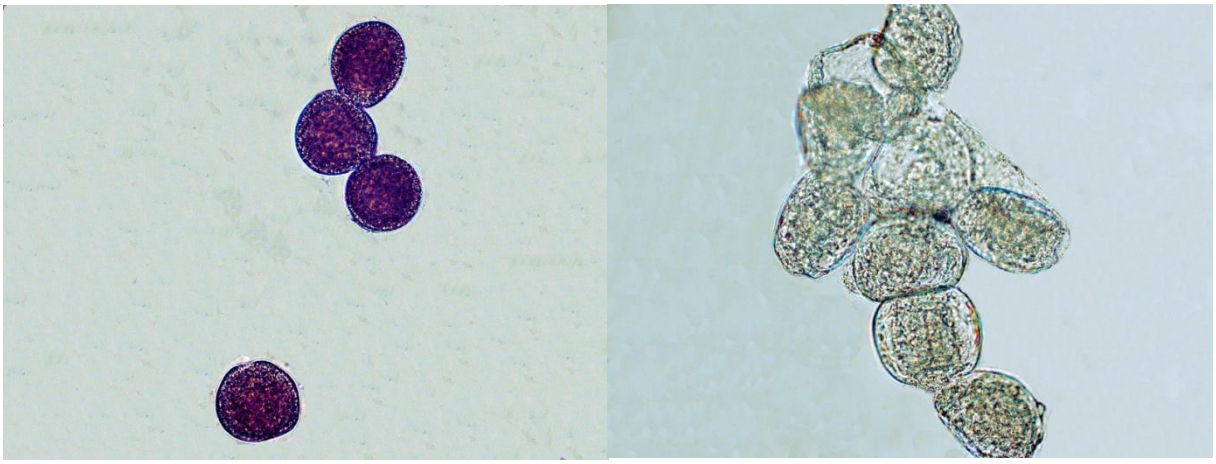

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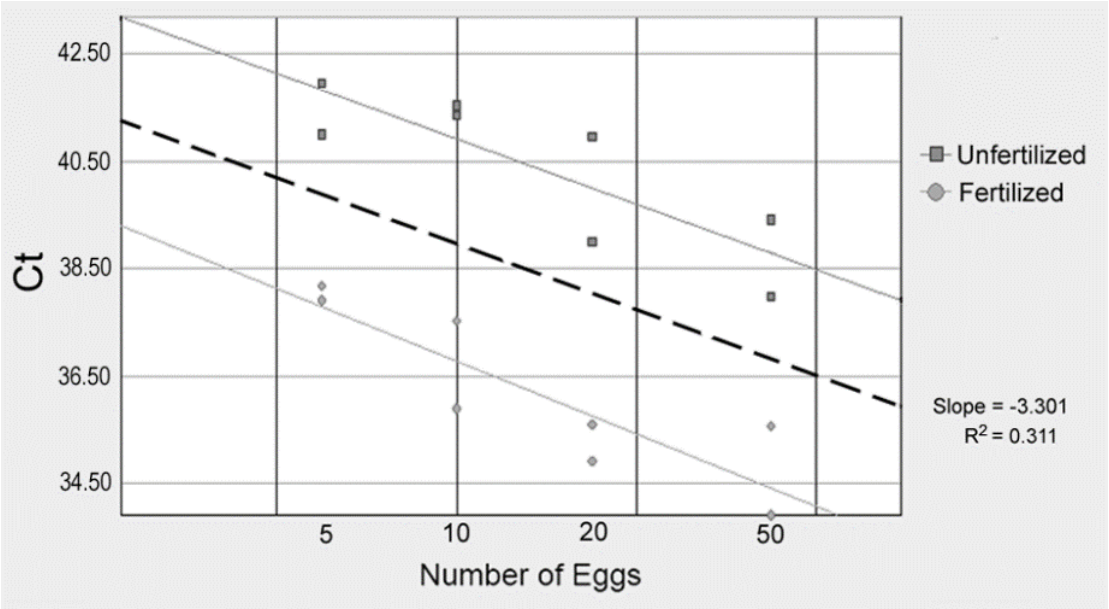

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9 Table 1

	Larval Counts from Plankton Net Samples				qPCR		
Date	**Site**	**Replicate**	**Subsample**	**Total N**	**+/-**	**Mean Ct**	**# Larvae ***
07/15/2016	Zone 1	R1	174		-		
		R2	129		-		
		R3	141		-		
	Zone 2	R1	0		-		
		R2	3		+	38,39	0.4
		R3	154		-		
	Zone 3	R1	14		+	38,04	0.5
		R2	11		+	36,19	1.6
		R3	102		-		
08/01/2016	Zone 1	R1	102		-		
		R2	126		-		
		R3	99		-		
	Zone 2	R1	50		-		
		R2	49		-		
		R3	104		-		
	Zone 3	R1	156		-		
		R2	365		-		
		R3	446	1279	-		
08/15/2016	Zone 1	R1	30		+	35,85	2.1
		R2	37		+	39,57	0.2
		R3	52		+	38,59	0.3
	Zone 2	R1	262	1368	-		
		R2	141	566	-		
		R3	116	233	-		
	Zone 3	R1	750	2149	-		
		R2	851	3143	-		
		R3	472	1905	-		
09/01/2016	Zone 1	R1	22		+	36,11	1.7
		R2			-		
		R3			-		
	Zone 2	R1	33		-		
		R2	29		+	37,32	0.8
		R3	56		+	31,86	32.4
	Zone 3	R1	30		-		
		R2	302		-		
		R3	39		+	37,76	0.6
09/15/2016	Zone 1	R1	54		+	32,06	28.1
		R2	116		-		
		R3	271	797	-		
	Zone 2	R1	175		-		
		R2	185		-		
		R3	262		-		
	Zone 3	R1	306	686	-		
		R2	363	580	-		
		R3	526	15272	-		
10/01/2016	Zone 1	R1	118		+	35,02	3.6
		R2	321	924	-		
		R3	250	3237	+	37,49	8.6
	Zone 2	R1	11		+	35,50	2.6
		R2	68		-		
		R3	53		-		
	Zone 3	R1	25		-		
		R2	40		+	36,39	1.4
		R3	20		-		

Cover Letter

Graphic Summary