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Experimenteel onderzoek naar het akoestisch effect van de schroef op ongewervelden in het maritiem milieu

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Experimental research into the effect of anthropogenic sounds produced by the propeller of commercial ships on invertebrates in the marine environment

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Foreword

In one of the classes on ecology, the students, including myself, were introduced to the subject of noise pollution. It first seemed a bit of a laughing matter, but we soon discovered it to be a real problem threatening the marine environment. Then we were required to see the documentary "The death of the Oceans?" (BBC, 2010). This showed once more that noise is not something to be taken as a joke. It lead me to the question: what was the effect in terms of noise that the transition from sailing to motorised navigation had? It is a question that remains unanswered, but with this thesis I tried to provide some part of the answer.

This thesis did not come to be without the aid of a number of people, whom I would like to thank hence. First of all many thanks to Prof. Dr. Geert Potters, whom made me aware of the issue at hand and then supported me throughout my work as my promotor and a mentor in sciences I had no knowledge off, such as biochemistry. I am grateful to Dr. Kris Hostens, Dr. Daan Delbare and Dr. Elisabeth Debusschere, whom helped me upon arriving at the Institute for Agricultural and Fisheries Research (ILVO). My regards go to Dr. Elisabeth Debusschere in particular for guiding me through the experiment. I would furthermore like to thank David Vuylsteke for his technical support and Hans Hillewaert for his help with the microscope. I would also like to thank the library team at the Flemish Institute for the Sea (VLIZ), which readily satisfied my steady stream of requests for literature. Many thanks go to Benoit Versavel and P. C. Kerpel at VLOOT, for the help and information on RV Simon Stevin. My gratitude goes out to my mother and brother, for reading the raw material. Lastly I would like to thank my wife, Nathalie Mertens, for her relentless support in this endeavour.

Contents

Foreword....i
List of Pictures....v
List of Tables....vi
List of Abbreviations....vii
Summary....viii
1 Introduction....1
2 Aims and Hypothesis....3
3 Experimental ideas and the coming about the actual experiment....4
4 Theoretical background....6
4.1 Introduction....6
4.2 Acoustics....6
4.2.1 Introduction....6
4.2.2 General sound....7
4.2.3 Sound Pressure Level (SPL) and Intensity ratio....9
4.2.4 Marine acoustics vs. Airborne acoustics....9
4.2.5 Reflection....9
4.2.6 Propagation....13
4.2.6.1 Spreading loss....13
4.2.6.2 Chemical relaxation/absorption....14
4.2.6.3 Refraction....15
4.2.6.4 In shallow water....16
4.2.7 Sound muffling systems....18
4.2.8 Sound pressure vs. particle motion....20
4.2.9 Monopoles, dipoles and quadrupoles....20
4.2.10 Signal to noise ratio....21
4.2.11 Sound measuring....22
4.2.12 Sound production....23
4.2.12.1 The marine soundscape....23
4.2.12.2 Ship noise....28
4.2.12.3 In an experimental tank....39
4.2.12.4 Comparison....41
4.2.13 Reproducing sound....41
4.3 The marine environment....41
4.3.1 The Sea....41
4.3.2 Brown shrimp, an ideal test subject....42
4.3.2.1 The brown shrimp (Crangon crangon)....42
4.3.2.2 The common mussel (Mytilus edulis)....49
4.3.2.3 Brine Shrimp (Artemia salina)....49
4.3.2.4 Amphipoda (Bathyporeia pilosa)....49
4.3.2.5 Baltic macoma (Macoma balthica)....50
4.4 Anatomy of the hearing organs....50
4.4.1 Introduction....50
4.4.2 Hearing organs....50
4.4.2.1 Statocyst....51
4.4.2.2 The Dorsal Organ....53
4.4.2.3 Aesthetascs and setae....54
4.5 Impact of the environment on an individual: from stress to death....64

4.5.1 Mortality (acute ↔ delayed).....64
4.5.2 Injury.....65
4.5.3 Behaviour.....66
4.5.4 Stress.....68
4.6 Ecology.....83
4.6.1 Research basis.....83
4.6.2 Ecology.....84
4.6.3 Community.....87
4.6.4 Population.....87
4.6.5 Interaction.....88
4.6.6 Fitness.....89
4.6.7 Generalists vs. Specialists.....90
4.7 Doing an experiment on Marine Bioacoustics.....90
5 The experiment.....92
5.1 The setup.....92
5.1.1 Ideal setup of the initial plan.....92
5.1.1.1 Problems and why not.....93
5.1.1.2 Legal issues.....93
5.1.2 The actual experiment.....94
5.1.2.1 The audio playing setup.....96
5.1.2.2 The sound measuring setup.....98
5.2 Gathering the brown shrimps.....98
5.3 The design.....99
5.4 Sampling and data collection.....101
5.4.1 Observation.....101
5.4.1.1 Temperature and Acidity.....101
5.4.1.2 Salinity.....101
5.4.1.3 Mortality.....101
5.4.1.4 Sound pressure and particle motion.....102
5.4.2 Sampling.....103
5.4.3 Processing the data.....104
5.4.3.1 SPL and particle motion on computer.....104
5.4.3.2 Sample processing.....105
6 Results.....116
6.1.1 Physical environment (temperature, acidity and salinity).....116
6.1.2 SPL/ Particle motion.....116
6.1.3 Protein/HSP 70.....122
6.1.4 Other observations.....123
7 Discussion.....124
7.1.1 Physical Environment (temperature, acidity and salinity).....124
7.1.2 SPL/ Particle motion.....124
7.1.3 Protein.....126
7.1.4 Other observations.....128
7.1.5 General discussion.....128
7.2 Synthesis of the hypothesis.....130
7.3 What went wrong and what could be improved.....131
7.4 Future research.....131
8 Case study.....133
8.1 Sound levels on shrimps from RV Simon Stevin.....133

8.2 Discussion....................138
9 Conclusion....................139
Bibliography....................140

List of Pictures

Fig. 3.1: The initial experimental plan....5
Fig. 4.1: Traverse waves....7
Fig. 4.2: Longitudinal waves....7
Fig. 4.3: Reflection and transmission of an incident plane wave at a fluid - fluid interface....10
Fig. 4.4: Geometry for propagation with a surface-reflected component....11
Fig. 4.5: Theoretical Lloyd mirror propagation showing the interference and far field....12
Fig. 4.6: Chemical relaxation and its contributing aspects....15
Fig. 4.7: The near-field anomality and shallow water attenuation factor....17
Fig. 4.8: Cutoff frequencies for propagation of sound for different bottom types:....18
Fig. 4.9: Theoretical directivity patterns....21
Fig. 4.10: Different aspects of the ambient noise level....24
Fig. 4.11: Ambient noise by sea state....25
Fig. 4.12: Knudsen curves, thermal noise and the addition of shipping noise....26
Fig. 4.13: Propeller views and Bernoulli effect....29
Fig. 4.14: Typical cavitation types on a marine propeller....31
Fig. 4.15: Ship SELs....36
Fig. 4.16: Sound profiles....37
Fig. 4.17: Depth chart of the Belgian Exclusive Economic Zone....41
Fig. 4.18: Crangon crangon....42
Fig. 4.19: A caridean with its parts....43
Fig. 4.20: Mytilus edulis....49
Fig. 4.21: Artemia salina....49
Fig. 4.22: Bathyporeia pilosa....49
Fig. 4.23: Macoma Balthica....50
Fig. 4.24: Statocyst without statolith 53
Fig. 4.25: The statocyst is just visible under the eye of C. crangon....53
Fig. 4.26: Dorsal organ under EM....54
Fig. 4.27: Dorsal organ above the dorsal spine (white surface)....54
Fig. 4.28: B: Setae. C: Denticles. D: Setules....56
Fig. 4.29: Pappose setae under EM....57
Fig. 4.30: Plumose setae under EM....58
Fig. 4.31: Serrulate setae under EM....58
Fig. 4.32: Serrate setae under EM....59
Fig. 4.33: Papposerrate setae under EM....59
Fig. 4.34: Simple setae under EM....60
Fig. 4.35: Cupsedate setae under EM....60
Fig. 4.36: Chelae....62
Fig. 4.37: Setae on the general cuticle....63
Fig. 5.1: Bro Distributor....95
Fig. 5.2: Loud tanks....96
Fig. 5.3: Silent tanks....96
Fig. 5.4: Storage tanks....96
Fig. 5.5: Inside a tank with C. crangon....96
Fig. 5.6: Audio playing setup....97
Fig. 5.7: Silica gel granules....97
Fig. 5.8: Sound measuring setup....98
Fig. 5.9: Graphic sound in all tanks (1-10)....99

Fig. 5.10: Sprattus sprattus....................100
Fig. 5.11: Alitta virens....................101
Fig. 5.12: Displays with various menu's on the amplifier....................103
Fig. 5.13: Taking samples 104
Fig. 5.14: Taking samples....................104
Fig. 5.15: SDS-PAGE....................109
Fig. 5.16: SDS-PAGE....................109
Fig. 5.17: Gel-casette....................110
Fig. 5.18: Gel electrophorasis....................112
Fig. 6.1: Physical parameters....................116
Fig. 6.2: SPLs (left) and Particle Motion (right)....................118
Fig. 6.3: SPLs....................121
Fig. 6.4: Protein concentration: pure (left) and as fraction of dry mass (right)....................122
Fig. 7.1: Ship SELs for three types of tankers in 1/3 octave bands....................126
Fig. 8.1: Cutoff frequency....................134
Fig. 8.2: SELs....................135
Fig. 8.3: Chemical attenuation....................136
Fig. 8.4: Near-field anomaly and attenuation factor....................137
Fig. 8.5: SPL, TL, RL and fc....................138

List of Tables

Table 1: HSP families in Eukaryotes....................76
Table 2: Start and end positions of fishing runs on 18/02/2016....................99
Table 3: Separating gel compositions....................111
Table 4: Number of shrimp showing particular interest in food....................123
Table 5: Calculation data....................133
Table 6: Distance calculations....................134
Table 7: H....................137

List of Abbreviations

SONAR	Sound Navigation and Rangin
SPL	Sound Pressure Level
TL	Total Loss
ECA	Emission Control Area
SNR	Sound to Noise Ratio
rpm	rotations per minute
CIS	Cavitation Inception Speed
HSP	Heat Shock Protein
mRNA	messenger RNA
DNA	Deoxyribonucleic acid
ATP	Adenosine triphosphate
EM	Electrone Microscope
LW ratio	Length to Width ratio

Summary

Inleiding

Het begin van de Industriële Revolutie luidde het einde van het zeilschip in en de opkomst van de gemotoriseerde vaart. Sinds die tijd is de economie veranderd en is tijd belangrijk geworden. Dit wilt zeggen dat steeds meer en meer schepen te water gaan. Die schepen maken tevens hoe langer hoe meer kabaal in het water. Het is zo erg dat het scheepsgeluid een inherent onderdeel van het geluidslandschap is geworden, en wel dermate dat het dat landschap tegenwoordig domineert.

Jammer genoeg kwam de mens er pas heel laat (1948) achter dat mariene dieren ook geluiden maken. Pas veel later kwamen de wetenschappers dan tot het besef dat vele mariene dieren geluiden kunnen waarnemen en zelfs analyseren en interpreteren. Het zijn niet enkel bekende dieren, zoals walvissen, dolfijnen, haaien en sommige vissen, maar ook minder voor de hand liggende dieren, zoals vele schaaldieren (vb. *Crangon crangon*, Linnaeus 1758) en zelfs weekdieren.

De reden voor het veelvuldig gebruik van geluid door mariene dieren is dat in de zee (en vloeistoffen in het algemeen) geluiden zich heel gemakkelijk voortplanten (in lucht: 344m/s tegenover in zee: 1500m/s). Dat is vooral het geval voor lagere frequenties (20-200Hz).

Nu zijn er drie “toevalligheden” die net niet goed uitkomen. (1) Zonder het antropogene deel van de schepen is er een kleine dip in het natuurlijke spectrum van het geluidslandschap, nl. ronde 50-500Hz. (2) De meeste mariene dieren waarvan bekend is dat ze kunnen horen, zijn gespecialiseerd in de geluidsband tussen 10Hz en 1000Hz (mede omdat dit vroeger de 'vrije' frequentie was). (3) Het dominante scheepsgeluid bevindt zich tussen 50Hz en 500Hz. Dit wil dus zeggen dat dieren sowieso vatbaar zijn voor de belangrijkste frequentieband van het antropogene geluid. Het is bekend dat mariene zoogdieren en koppotigen (vb. inktvissen) schade oplopen aan de gehoororganen en stress krijgen onder invloed van dit geluid.

Dit onderzoek gaat na of ook *C. crangon*, een belangrijke diersoort in de Europese wateren, stress krijgt van scheepsgeluid. Dit kan onderzocht worden door observaties op verschillende vlakken:

- Biochemisch: door middel van bio-markers zoals HSP70
- Visuele waarnemingen van gedrag en interacties.
- Populatiefluctuaties die het succes van organismen weergeven.
- Scope for growth: de groei waarnemen.

Uit al deze opties blijkt dat de garnalen bij stress meer energie verbruiken en dus meer voedsel moeten opnemen of er minder moeten proberen verbruiken.

Onderzoeksvragen

Deze studie focuste zich op de volgende vragen:

1) Kunnen grijze garnalen (*C. crangon*) geluiden horen? (literatuur)
2) Zorgt het antropogene geluid, geproduceerd door de schroef van commerciële schepen voor stress bij grijze garnalen (*C. crangon*)?
3) Kan er geconcludeerd worden dat het antropogene geluid, geproduceerd door de schroef van commerciële schepen een impact heeft op de fitness van grijze garnalen (*C. crangon*)?

Tot dit doel werd een experiment uitgevoerd met levende garnalen in een experimenteel opzet, waarbij de wetenschappen van de mariene bio-akoestiek werden toegepast.

Materiaal en Methode

Op 18 februari 2016 werden grijze garnalen gevangen aan boord van het onderzoeksschip Simon Stevin. Er werd gevist op 6 aansluitende slepen met een kleine versie van een standaard treilnet. De garnalen waren continu voorzien van vers zeewater aan boord en werden in overlevingsbakken vervoerd op de rit van 5min tussen het schip en het laboratorium. In het laboratorium werden ze ondergebracht voor acclimatisatie in tanks van 60x90x30cm. Na een periode van 11 dagen werden ze overgebracht in de blootstellingsbakken (26x37x23cm), waar ze 3 dagen rust kregen voor het starten van het experiment.

Het experiment hield in dat over een periode van 14 dagen in vier bakken (1-4) telkens 24 garnalen werden blootgesteld aan een opname van het onderwatergeluid van het schip Bro Distributor (Fig. 5.1 p.95) d.m.v. twee exciters per bak. Simultaan waren vier bakken (7-10) met elk 24 garnalen vrij gehouden van dit geluid in de mate van het mogelijke. De bakken stonden in dezelfde ruimte, waren aangesloten op hetzelfde zeewater circulatie systeem en stonden op dezelfde tafel (Fig. 5.2-5.3 p.96). De temperatuur, de zuurtegraad en het zoutgehalte werden dagelijks gemonitord en bleken constant (T=8,4°C; pH=8,4; S=35‰). Het laboratorium was onderhevig aan een gestuurd verlichtingsregiment van 12D/12L. Het geluidsspectrum werd tevens regelmatig gecontroleerd. Er traden enkele problemen op met het glitchen van de computer (input-probleem), het oververhitten van de versterker (overload) en de luchtvochtigheid voor de elektronica. Deze problemen waren echter goed onder controle te houden en hadden vermoedelijk geen invloed op de resultaten. De

garnalen kregen een variatie aan voedsel 'op dag 7, dag 9, dag 11,dag 15 en dag 17. Er werden stalen genomen dag 1 (voor het starten van het afspelen van geluid), dag 2 (na 20uur geluids-blootstelling), dag 8 (na 6 dagen en 20uur), dag 15 (na 13 dagen 21uur en 45min) en 1 dag 22uur en 30min na het uitschakelen van het geluid, op dag 17. Bij elke staalname werden willekeurig 4 garnalen uit elke bak genomen. Deze garnalen werden vervolgens gemeten, gewogen, geschild en de rugspier (het deel dat tevens geconsumeerd word) apart gewogen en vervolgens snelgevrozen op -80°C en opgeslagen op -20°C. Nadien was het de bedoeling om de garnalen te onderzoeken op HSP70 waarden. Dit door een Markwell proteïne assay te doen, gevolgd door een SDS-PAGE (12,5%T) en een Coomassie Blue staining.

Resultaten

De resultaten van de metingen van het geluid in de bakken leverde een aantal interessante grafieken op. Uit Fig.6.2-6.3 op p.118-121 blijkt dat het geluid vrij constant was doorheen het experiment. Het moet wel gezegd worden dat de stille bakken wanneer er geen geluid speelde altijd luider waren dan de luide bakken.

Wegens tijdgebrek en logistieke problemen is de SDS-PAGE niet uitgevoerd en zijn daar dus geen resultaten van. De Markwell assays zijn wel uitgevoerd. Daarvan staan de resultaten in Fig. 6.4 op p.122. De resultaten zijn de gemiddelde proteïne concentraties, met standaarddeviatie, van de rugspieren van 1 garnaal per bak per dag (de overigen werden bewaard voor latere verwerking).

Het viel tevens op dat, wanneer de garnalen op dag 7 eten kregen, de garnalen uit de luide bakken veel meer geïnteresseerd waren in het eten, zoals zichtbaar is in Table 4 op p.123.

Discussie

Het geluid was redelijk constant gedurende het hele experiment. Om een onbekende reden is het geluid in bak 3 over het hele experiment ongeveer 10dB lager dan de andere luide bakken, maar dat had geen invloed op de proteïne concentraties van de garnalen in die bak. Bij de geluidsmetingen moet rekening gehouden worden met het feit dat door het ontstaan van staande golven er op korte afstanden grote geluidverschillen optreden in de bakken. Het is daarnaast onmogelijk om telkens de geluiden op te meten op exact dezelfde plaats om dat probleem aldus te omzeilen. Onregelmatige metingen kunnen daar aan gewijd worden.

De algemene tendens van de proteïne concentraties lijkt op het eerste gezicht raar. Maar rekening houdende met het verschil in geluidsterkte tussen stille en luide bakken en de evolutie daarvan, en

daarnaast met de voedermomenten, wordt het duidelijk dat het geluid waarschijnlijk gezorgd heeft voor het verschil in proteïne concentraties:

- Aanvankelijke was we bak met het meeste geluid de bak met de hoogste proteine concentratie in de garnalen, wat op stress kan wijzen.
- Het feit dat de garnalen uit de luide bakken meer interesse toonden in eten op dag 8 na een periode van 'uithongering' wijst op een verschil in energiehuishouding en dus stress.
- Het laagste niveau van proteïne concentratie was in de luide bakken na een periode van 'uithongering'. Daarna konden ze door regelmatige voeding hun concentraties geleidelijk terug aanvullen, maar ze konden maar volledig terug op gelijke hoogte komen (recupereren) wanneer het geluid weg was.

Daar er verder geen verschillen waren tussen de beide groepen bakken behalve het geluid en de individuele verschillen van de garnalen zelf, kunnen we een antwoord formuleren op de vragen.

1) Kunnen grijze garnalen (*C. crangon*) geluiden horen?

   Uit de literatuur en het feit dat er een verschil in proteïne concentraties was, kan afgeleid worden dat grijze garnalen (*C. crangon*) geluiden kunnen horen.

2) Zorgt het antropogene geluid, geproduceerd door de schroef van commerciële schepen voor stress bij grijze garnalen (*C. crangon*)?

   Uit de tendensen en de aangehaalde argumenten kan een sterk vermoeden afgeleid worden dat de grijze garnalen (*C. crangon*) stress ondervonden door het antropogene geluid, geproduceerd door de schroef van commerciële schepen.

3) Kan er geconcludeerd worden dat het antropogene geluid, geproduceerd door de schroef van commerciële schepen een impact heeft op de fitness van grijze garnalen (*C. crangon*)?

   Het kan niet geconcludeerd worden dat het antropogene geluid, geproduceerd door de schroef van commerciële schepen een impact heeft op de fitness van de grijze garnalen (*C. crangon*). Er is wel een vermoeden dat ze een negatieve invloed ondervinden.

1 Introduction

The end of the era of sailing was marked by the invention of the steam engine, which in time was replaced by the fuel combustion engine. To transform the power of these new engines into trust, Archimedes ancient invention of the propeller (albeit greatly improved) was adopted to trust ships through the water. Since the advent of the industrial era, the number of ships and marine anthropogenic activities have been increasing tremendously. Accordingly, the contribution of ships to the marine noise spectrum has been growing as well, resulting in an overall louder marine environment (Au and Hastings, 2008; Urick, 1984; Tucholski, 2006; McKenna et al., 2012; Okeanos, 2008). Additionally, it doesn't aid that the prime interest in profit does not care for sound emission (Baudin and Holger, 2015). The noise extends today to such levels that it has become inherent to the marine soundscape, where it dominates the lower frequencies (Okeanos, 2008).

It wasn't until the Second World War that, with the realisation of acoustic mines, the discovery was made of marine species' ability to produce and hear sounds (Urick, 1984). The link between the anthropogenic production of sounds (e.g. ship propulsion, SONAR, seismic surveys, pile driving) and the possible detrimental impacts on marine animals came only many years later. For marine vertebrates, the use of sound is essential in activities such as foraging, predator detection, mate attraction and habitat selection (Samson et al., 2014; Urick, 1984). Anthropogenic sounds, and ships noise in particular, can overpower these important animal acoustic cues easily and disrupt these activities (McKenna et al., 2012). Furthermore, ship noise is proven to result in stress in whales (Renilson et al., 2013) and may also lead to behavioural changes in them (Okeanos, 2008). For invertebtrates, knowledge on hearing abilities is still limited (Samson et al., 2014), though functional behaviour responses were observed in cuttlefish (Samson et al., 2014), crab (*Panopeus* spp.) (Hughes et al., 2014), fiddler crabs (Hughes et al., 2014) and lobster (Hughes et al., 2014). In four species of cephalopods (*Loligo vulgaris*, Lamarck, 1798; *Sepia officinalis*, Linnaeus, 1758; *Octopus vulgaris*, Cuvier, 1798; *Illex coindetii*, Vérany, 1839) damage causes by ship sound was experimentally proven (André et al., 2011). Crustaceans too are reported to be afected by anthropogenic sounds (Lovell et al., 2005).

Location is an important factor to the exposure level of the sound, as the levels decrease over a distance. For animals, location is everything in terms of fitness however (Campos et al., 2012). Coastal areas, e.g. estuaries (Menezes et al., 2006), are typically busy places for human activity. Unfortunately, that is equally true for marine animals, viz. many fish and crustaceans (Menezes et al., 2006). Their habitats are thus endangered by the noise of human activity (McKenna et al.,

2012), especially shipping.

In virtually all European seas, which are at times congested in shipping, crustaceans are present. The commercially important brown shrimp (*Crangon crangon*, Linnaeus, 1758) is particularly present in all these regions. The brown shrimp as an important part of many European ecosystems (Oh et al., 2001), plays a vital role in commercial fish species such as whiting and plaice and is an important member in species-poor, inhospitable regions such as the Baltic Sea (Sandberg et al., 1996). This makes it strange that so little interest has been shown in this species and makes it of particular interest for my study. As these are coastal species, living between the surf almost on the beach and a maximum depth of 300m, they seem particularly susceptible to shipping noise.

2 Aims and Hypothesis

This study focussed on the impact of the noise produced by ships on brown shrimp. For this purpose, an experiment was carried out in a laboratory setting similar to one performed by Hughes et al. (2014). In this study the applied sciences were the multidisciplinary marine bio-acoustics and bio-chemistry. The impact of medium to long time exposure to the sound of a ship was investigated, resulting in these three research objectives.

1) Are brown shrimp able to hear sounds? (literature)
2) Do anthropogenic sounds produced by the propeller of commercial ships cause stress to brown shrimps?
3) Can it be concluded that anthropogenic sounds produced by the propeller of commercial ships have an impact on the fitness of brown shrimp?

3 Experimental ideas and the coming about the actual experiment

When I first approached Mr. Potters for writing my thesis I had ideas on experiments which would not be possible. The initial idea was to put (at that point yet unspecified) fish into tree big tanks. Two tanks would be fitted with two types of ship propellers and one would serve as control group. The idea was to let the sound of these propellers have an impact on the fish for a prolonged period of time, viz. over one month, and then analyse the different effects of those propellers. After some deliberation, including the contemplation on some seagoing insects, the final concept was to put mussels (*Mytilus edulis*, L.) in hanging culture and on the bottom, brine shrimp (Artemia salina, L.) and langoustines (Nephrops norvegicus, L.) or brown shrimp (*Crangon crangon*) in 3 large 500-1000 l. tanks (one with a propeller with nozzle, one with a propeller but without a nozzle and a control group without any propeller). The idea was to have the propeller sound have an impact on the animals over a prolonged time. The tanks would be supplied with zooplankton and phytoplankton, air pumps for aeration and water supply to counter evaporation. In this manner a complete circle of waste material filterers, 'plants', herbivores and carnivores would be in place. As a natural cycle or small ecosystem with competition for food and a choice of prey this would enable us to measure the different interactions. The tank would be furthermore equipped with cameras to observe behaviour and interaction changes between the control group and both tanks with propellers. Biochemical changes, relative numbers of species (species success) and growth rates could amongst other be measured too. This would thus result in a study at the community/ecosystem level, comprising several relevant species. This would be a study which is lacking according to Madeira et al. (2012).

It soon became clear that this would not be manageable. First off all the, sheer number of observations would be impossible to process. Secondly, ordering a ship propeller and dimensioning the set-up accurately to the actual environment would be challenging to say the least. The third factor was time and financing such a project, as I was rather late to start everything up.

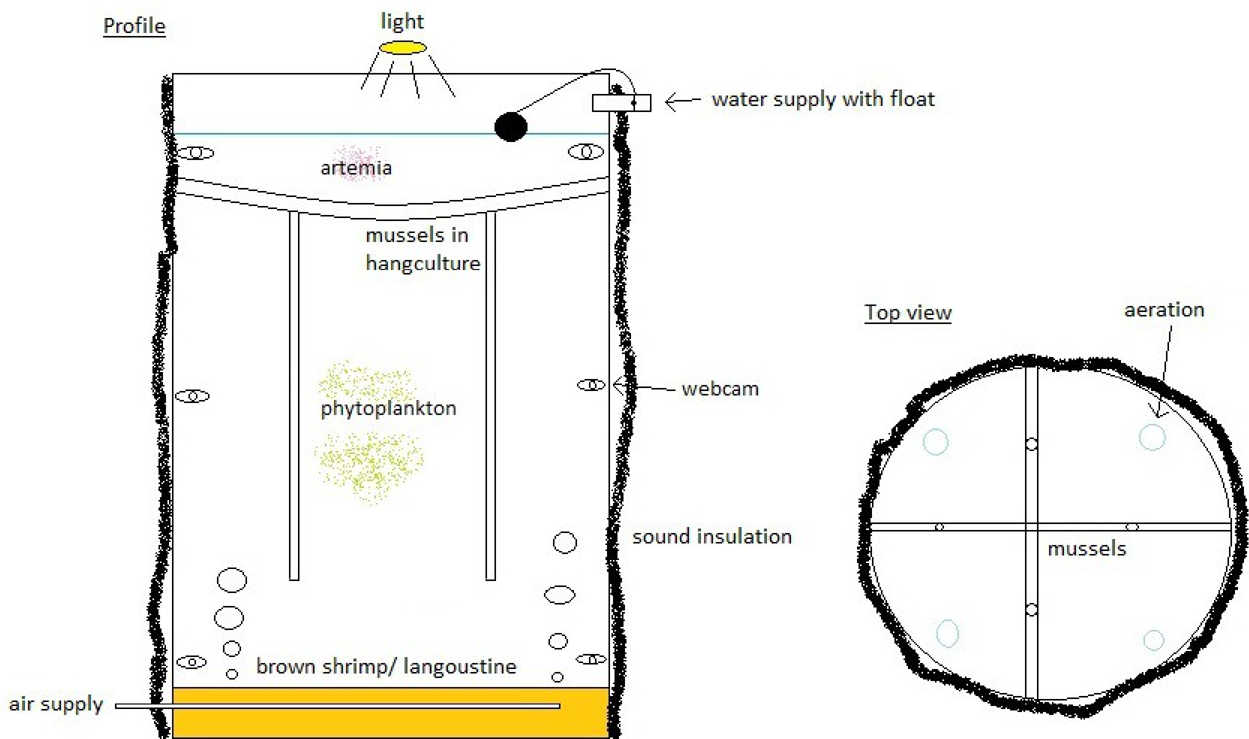


Fig. 3.1: The initial experimental plan.
(Left) in profile. (Right) top view
(Own collection)

In chapter 5 I will discuss briefly a concept of this experiment which, with less financial constrictions and time limits, would have been a 'workable' project.

In the end I was brought into contact with Miss. Debusschere and Mr. Hostens at the ILVO in Ostend where my goals of this research were brought to reason and an experiment was set up and carried out. In short, a set of shrimp (*C. crangon*) were put in a series of small tanks in an as sound-neutral environment as possible. A number of tanks were put under the influence of recorded sound of a ships propulsion and an equal number of tanks was left in silence. Gradually samples were taken and the presence of stress would be observed through the presence of HSPs, which is a widespread biomarker in aquatic toxicology (Feder and Hofmann, 1999a).

After providing a necessary theoretical basis in chapter 4 I will discuss the build and process of this experiment in chapter 5 more thorough.

4 Theoretical background

Materials and Methods (part 1)

4.1 Introduction

Marine bioacoustics is a multidisciplinary science that studies natural history through the eyes of acoustics. This means that by studying the sound in the ocean and making observations we will study its inhabiting animals in every facet of their life. Hence, the research becomes multidisciplinary, as we will need amongst others biology, psychology, physiology, veterinary medicine, mathematics and statistics, electrical engineering, ecology and computer sciences to get a handle on these various studies. Discussing all important theoretical issues in detail will lead us too far off-track for this dissertation. This being said, a brief discussion of acoustics, the local environment of the study and behavioural aspects are in order as well as a more thorough, elaborate investigation of auditory physiology and biology of the studied animal (*Crangon crangon*) and some particular biochemical aspects related to stress. I will then briefly address some legal aspects..

An example of bio-scoustics: A type of extremely territorial bat was divided into two different species as two couples usually seemed to inhabit the same habitat, because they used different frequency ranges.

4.2 Acoustics

A general analysis of marine acoustic theorems will be given for the general understanding of the study, with derived formulas as tools for both practical, personal use and to investigate the case as it unfolds. To illustrate the theory, the situation of the shrimps at a location of the capture area will be used in chapter 8.

4.2.1 Introduction

For us humans, of the five senses, sight is the most important, because it gives the fastest and most reliable information (so says the brain). Messing with this info (e.g. with optical illusions) will cause us problems although other senses may have easily been able to correct this info for us. On the other hand, when we lose our sight our brain will learn to rely on other senses. The reason sight is so fast and reliable is that light travels very fast through air (3.10^8 m/s) compared to sound (344 m/s) and that there is less ambient light to muffle the info we seek. Acoustic energy propagates in water more efficiently than almost any form of energy and can be utilized by animals for a variety

of purposes (Au and Hastings, 2008). Similarly to our two eyes making a very nice 3D picture, sound can make a very nice 3D picture for aquatic species.

Similarly to us humans, sound is used for social and environmental purposes (or simply to humour us in a good mood? (Urick, 1984)) by aquatic species too. All sounds, whether they are produced by a non-human biological source, a natural source (such as wind, rain, earthquakes, etc.) or humans, convey information and may be used by animals in their struggle for survival (Au and Hastings, 2008).

4.2.2 General sound

As described by (Au and Hastings, 2008).

Acoustic energy in water consists of molecular vibrations that travel at the speed of sound (Au and Hastings, 2008). This means that a requirement for sound is a material medium. The vibrations in water and other fluids (like air) form longitudinal waves, vibrating in the direction of propagation, while in inelastic materials they may form transverse waves, vibrating normal to the direction of propagation.

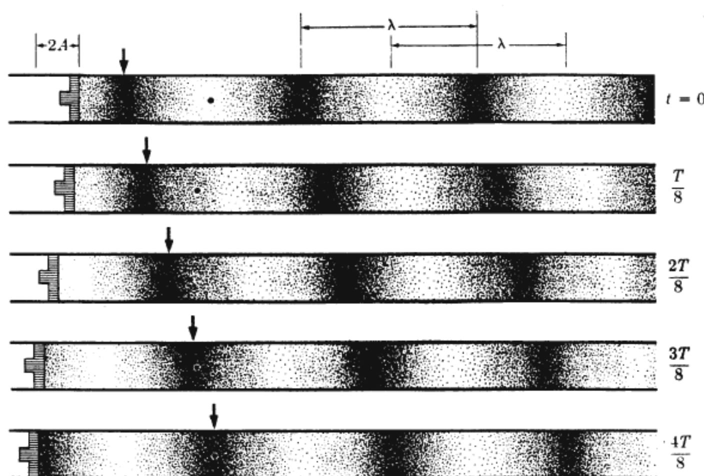


Fig. 4.1: Traverse waves (Au and Hastings, 2008)

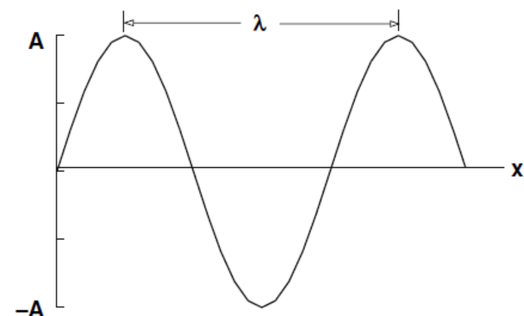


Fig. 4.2: Longitudinal waves (Au and Hastings, 2008)

In transverse waves, like the waves on the sea, molecules move up and down along the path. In longitudinal waves the movement is declined by 90° and molecules move forward and back, creating dense (compression, positive) and open (rarefaction, negative) areas, while on average the mass does not move. These compressions and rarefactions can be measured in **sound pressure level** (SPL). The forward (positive) and backward (negative) movements are called **particle motion**. They are the two variables of sound that can be observed.

For the ongoing explanation we will assume the sound wave to be a plane wave in which all properties will be the same in all directions of propagation. We will assume it to be a simple sine/cosine wave.

Assuming a small force to disturb a small area of fluid, a disturbance will be generated in terms of pressure and density (equation of continuity). Considering the law of conservation of mass[1], Newton's second[2] and third[3] laws, the equation of state[4] and assuming that sound propagates with such speed that temperature will remain the same we can derivate the following formulas:

$$p=(\frac{K}{\rho_0})\rho$$
$$c=\sqrt{\frac{K}{\rho_0}}=f.\lambda$$
$$p=A\cos(\omega t-kx)$$
$$k=\frac{\omega}{c}=2\frac{\pi}{\lambda}$$
$$\xi=\frac{A}{\omega\rho_0 c}\sin[\omega(t-\frac{x}{c})]$$

where: p = pressure; K = bulk modulus of the fluid; ρ = density; c = speed; f = frequency; λ = wavelength; u = particle velocity; ξ = particle displacement; A = amplitude; ω = radian frequency; t = time of event; x = distance from basemark; k = wavenumber.

Particle velocity and pressure run in phase while particle displacement is 90° out of phase, lagging behind.

Acoustic analogy with Electricity

Acoustic intensity is defined as the rate of flow of energy through a unit area normal to the direction of the wave propagation (Au and Hastings, 2008). This can be interpreted analogous to electrical circuit analysis. Acoustic intensity (I) compares to electrical power (P), pressure (p) to voltage (U) and particle velocity (u) to current (I).

$$I=pu=(\frac{p}{\rho c})p=\frac{p^2}{\rho_0 c} \quad \leftrightarrow \quad P=UI=RI^2=\frac{U^2}{R} \quad \text{with} \quad p=(\rho c)u \quad \leftrightarrow \quad U=RI$$

therfore $z=\rho c$

where: I = acoustic intensity; z = acoustic impedance

This is correct for plane waves. For spherical waves, pressure and particle velocity will no longer be

1 New mass cannot be created, nor can mass be destroyed.
2 F=m.a
3 When a body applies a force onto another body, the same force is applied to it by the other body
4 Fluid pressure is a function of temperature and density.

in phase and therefore its acoustic impedance will include both resistance and reactance (like electrical current).

In electronics, power is expressed as an average (P_{ave}) and voltage as an effective or root mean square value (U_{rms}). Similarly, acoustic intensity is expressed like the former (I_{ave}) and pressure like the latter (p_{rms}), though the subscripts are usually considered implicit. As such for a sinusoidal wave with given amplitude, frequency and period, the following is true:

$$p_{rms}=p=\frac{A}{\sqrt{2}}=0{,}707\,A$$

4.2.3 Sound Pressure Level (SPL) and Intensity ratio

Acoustic pressure, however nicely described above here is not an easy unit to work with as it is very small in comparison to ambient pressure. Acoustic pressure is expressed in µPa, while atmospheric pressure is expressed in hPa (with an average of 1,013hPa), being 10^{11} times larger. Therefor relative quantities are used, with dB unit: Sound Pressure Level (SPL) and Intensity ratio.

$$SPL=20\log\left(\frac{p}{p_0}\right) \qquad I\,dB=10\log\frac{I_1}{I_2}$$

where p_0 is a reference level of 1 µPa, which leads to the unit of SPL: dB re 1µPa (re being the abbreviation of referenced).

4.2.4 Marine acoustics vs. Airborne acoustics

In airborne acoustics for SPL a reference level of 20µPa is used, which means a difference of 26dB. Also, the density of water is much greater than of air, which means for a comparison in normal circumstances (t = 20°C, ρ_{sea} = 1,026kg/m³), SPL in water will be 35,6dB greater.

4.2.5 Reflection

Acoustic plane waves act as vectors with both direction and magnitude at an instant moment. This means that when two “different” mediums meet, the acoustic wave will act 'vectorial' (e.g. when two snooker balls hit one another).

An incident ray (p_i) hits a surface (between a medium with velocity c_1 and a medium with velocity c_2) , resulting in a reflected (p_r) and transmitted (p_t) part. Snell's Law dictates:

$$\frac{\cos\theta_i}{\cos\theta_t}=\frac{c_1}{c_2}=\frac{k_2}{k_1}=\frac{\lambda_1}{\lambda_2}$$ with angles relative to the surface

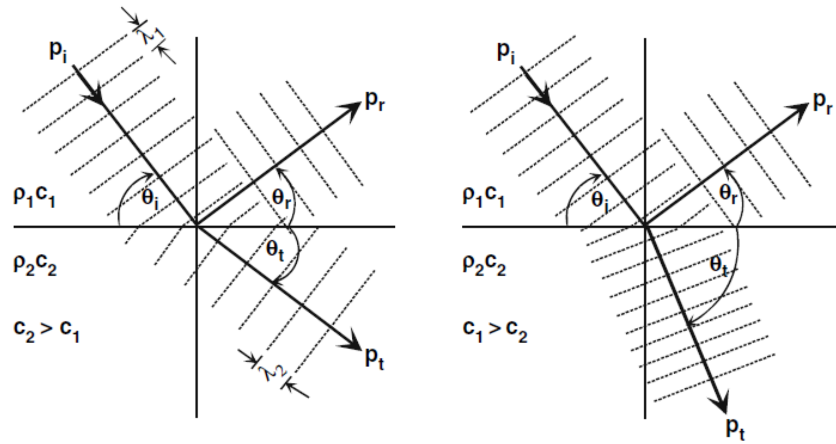


Fig. 4.3: Reflection and transmission of an incident plane wave at a fluid - fluid interface (Au and Hastings, 2008)

Two boundary conditions need to be true, which combined can be simplified as: at an infinitely small distance between the two mediums, variables on both side should be the same. This leads for *sound pressure* to:

$$p_i + p_r = p_t \Leftrightarrow 1 + R = T \text{ with } R = \frac{p_r}{p_i} \text{ and } T = \frac{p_t}{p_i}$$

where: R = refelection coefficient; T = transmission coefficient.

From Snell's law

$$\sin\theta_t = \sqrt{1 - (\frac{c_2}{c_1})^2 \cos^2\theta_i} = \sqrt{1 - \frac{\cos^2\theta_i}{\cos^2\theta_c}}$$

where: θ_c = critical angle.

Which leads to three possible results:

- $c_1 > c_2 \Rightarrow \theta_i < \theta_t$ → transmission wave towards lower velocity medium
- $\theta_i = \theta_t \Leftrightarrow \frac{c_1}{c_2} = \cos\theta_i$ → no transmission
- $c_1 < c_2 \Rightarrow \theta_i > \theta_t$ → transmission wave is imaginary and therefore the wave is fully reflected

As for *particle velocity*:

$$u_{n,t} = u_{n,i} + u_{n,r} \quad \rightarrow \quad \frac{\sin\theta_i}{\rho_1 c_1}(1-R) = \frac{T\sqrt{1 - \frac{\sin^2\theta_i}{\cos^2\theta_c}}}{\rho_2 c_2}$$

therefore when $\theta_i < \theta_c$ and z_1 and z_2 being the specific acoustic impedances

$$R = \frac{z_2 \sin\theta_i - z_1 \sin\theta_t}{z_2 \sin\theta_i + z_1 \sin\theta_t} \text{ and } T = \frac{2 z_2 \sin\theta_i}{z_2 \sin\theta_i + z_1 \sin\theta_t}$$

and when $\theta_i = 90°$

$$R = \frac{z_2 - z_1}{z_2 + z_1} \text{ and } T = \frac{2 z_2}{z_2 + z_1}$$

At the water surface (air medium meets water medium): $\rho_{marine} c_{marine} \gg \rho_{air} c_{air} \Leftrightarrow z_{marine} \gg z_{air}$ Then from water to air $R \approx -1$ and from air to water $R \approx 1$, meaning the wave is fully reflected, though from water to air 180° out of phase.

This is all true for a plane surface area. Wind generates rough sea surfaces however, which randomise and diminish this effect to a certain extend. Wave breaking under strong wind also produces entrained air-bubbles below the sea surface. Both wave form surface and trapped air bubbles scatter sound reflection and lead to surface reverberation (Zhang, 2005).

Lloyd Mirror Effect

The sea surface can have a profound effect on the acoustic field of a near surface source because of interference caused by the summation of a surface reflected signal and a direct signal. Given the following situation for a sinusoidal acoustic signal:

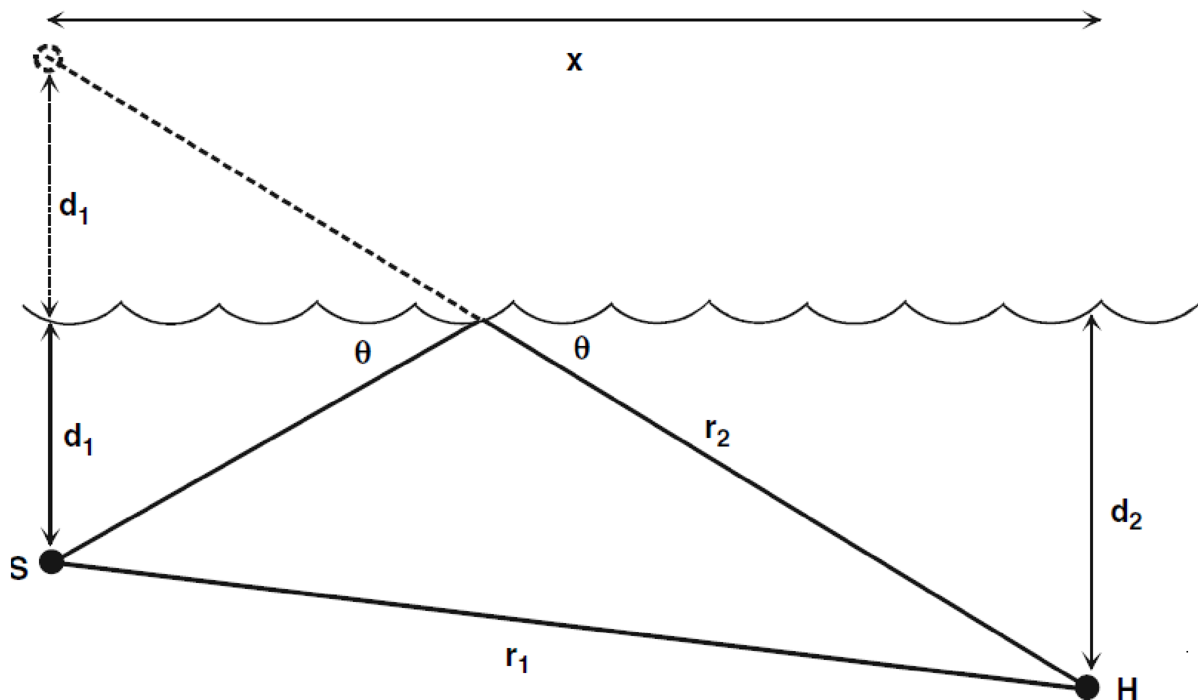


Fig. 4.4: Geometry for propagation with a surface-reflected component (Au and Hastings, 2008)

where S is sound producer and H a hydrophone receiver. For which is true:

$$p_1 = \frac{A}{r_1} sin2\pi f(t+\tau_1) \text{ and } p_2 = \frac{RA}{r_2} sin2\pi f(t+\tau_2)$$
$$r_1 = \sqrt{(d_1 - d_2)^2 + x^2} \text{ and } r_2 = \sqrt{(d_1 + d_2)^2 + x^2}$$
$$I = \frac{A}{\rho c} \frac{1}{T} \int_0^T \left(\frac{1}{r_1} \sin \omega t + \mu \frac{1}{r_2} \sin \omega (t+\tau) \right)^2 dt$$

The latter resulting in 3 parts of the acoustic field depending on the relation between r_1 and r_2:

- $r_2 \gg r_1$: The *near field*: (i.e. when the hydrophone is close to the source) the second term is negligible and as such the surface reflection, while intensity decays in a $1/r^2$ fashion.
- between $r_2 = 2r_1 \vee r_1 = 2\sqrt{d_1 d_2}$ and $r_0/r = 1$:The *interference field*: where reflected and direct signals alternatingly destroy and boost each other.
- $r_0/r \leq 1$: The *far field*: where the field decays in a smooth manner.

As a simple example for the interference field we will assume $r_1 \approx r_2$; then:

$$I = \frac{I_0}{r^2} 2(1 - \cos \frac{\pi r_0}{r}) \text{ and } I_0 = \frac{A}{\rho c}; r_0 = \frac{4 f d_1 d_2}{c}$$

where: I_0 = the reference intensity at 1m; r_0 = a reference range.

Hence from the former, when r_0/r = 2,4,.. then I will be a minimum and when r_0/r = 3,5,.. then I will be a maximum.

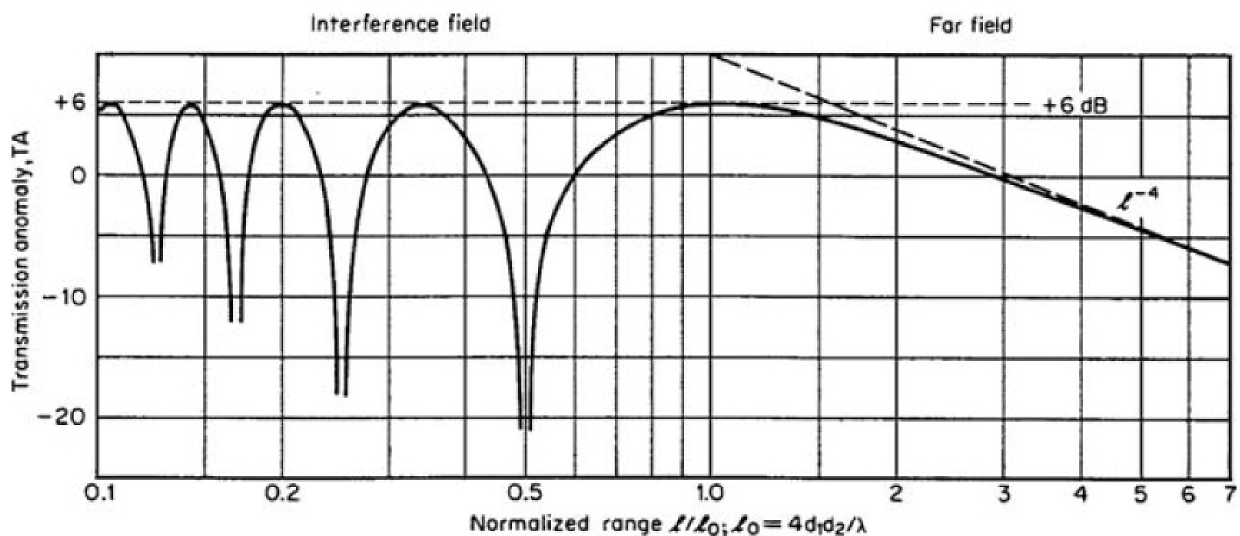


Fig. 4.5: Theoretical Lloyd mirror propagation showing the interference and far field (Au and Hastings, 2008)

4.2.6 Propagation

Sound propagation in all simplicity is limited firstly due to the *spreading* of the energy as it propagates in all directions. Secondly it is limited due to *absorption* and *attenuation.* Sound energy is converted into heat. Absorption losses in seawater are caused by the effect of shear and volume viscosity (above 100kHz), ionic relaxation of magnesium sulfate ($MgSO_4$) (intermediate frequencies up to a few 100 kHz), molecules and a complicated boric acid ionization process (low frequencies up to a few kHz)(Au and Hastings, 2008; Ainslie and McColm, 1998). For this reason, SPL and intensity ratio are expressed at a distance of 1m, making the complete unit notation: dB re 1µPa at 1m. Total Loss (TL) is the combination of both losses and can be expressed as:

$$TL = 10\log\frac{I_0}{I_1} = 20\log\frac{p_0}{p_1} = \textit{Spreading Loss} + \textit{Absorption}$$

For this part a plane wave form is used, but no longer necessarily a constant sine/cosine wave. This means that it is a wave of constant pressure, propagating (longitudinally) in the direction perpendicular to the plane of constant pressure, in any repetitive function of time.

4.2.6.1 *Spreading loss*

The simplest form of *spreading loss* is spherical. This means that sound propagates radially from a single central source, forming spherical surfaces of constant pressure. In this case the following is true at a distance R_1 of the source:

$$\textit{Spreading Loss}[\text{dB re } 1\mu\text{Pa at } 1\text{m}] = 20\log R_1$$

and

$$p(r,t) = \frac{f(t - r/c)}{r}$$

indicating that pressure loss as function *f* is solely dependent on distance.

For particle velocity:

$$\vec{u}(r,t) = \left[\frac{p(r,t)}{\rho_0 c} + \frac{\int_{-\infty}^{t} p(r,\tau)\,d\tau}{\rho_0 r}\right]\vec{e}_r$$

Indicating particle velocity has two major parts, one caused by the flexibility and the other the bulk flow near the source. In the “far field” (r >> λ) the second part approaches 0 and particle velocity acts as a plane wave. In the near field the second part becomes important.

The impedance now becomes complex having both resistance and reactance:

$$Z=\frac{P}{u_r}=\frac{\rho_0 c\left(1+j/kr\right)}{1+\left(j/kr\right)^2}$$

4.2.6.2 Chemical relaxation/absorption

Chemical relaxation can be split up into three main terms (Fig. 4.6):

$$\alpha=\alpha_{H_3BO_3}+\alpha_{MgSO_4}+\alpha_{H_2O}$$

The chemical absorption due to boric acid is described by Fransois and Garison through empirical observation in Mohite-Patil et al. (2010) as:

$$\alpha_{H_3BO_3}=\frac{A_1 P_1 f_1 f^2}{f_1^2+f^2}$$

where

$$A_1=\frac{8{,}86}{c}10^{(0{,}78\,pH-5)}\left[\frac{dB}{km\,kHz}\right]$$

$$P_1=1$$

$$f_1=2{,}8\sqrt{\frac{S}{35}}10^{\left(4-\frac{1245}{273+T}\right)}[kHz]$$

$$c=1412+3{,}21\,T+1{,}19\,S+0{,}0167\,D\left[\frac{m}{s}\right]$$

Similarly the chemical absorption due to magnesium sulfate is given by Fransois and Garison in Mohite-Patil et al. (2010) as:

$$\alpha_{MgSO_4}=\frac{A_2 P_2 f_2 f^2}{f_2^2+f^2}$$

where

$$A_2=21{,}44\frac{S}{c}(1+0{,}025\,T)\left[\frac{dB}{km\,kHz}\right]$$

$$P_2=1-1{,}37.10^{-4}D+6{,}2.10^{-9}D^2$$

$$f_2=\frac{8{,}17.10^{\left(8-\frac{1990}{273+T}\right)}}{1+0{,}0018(S-35)}[kHz]$$

and: T = temperature [°C]; f = frequency

Lastly the chemical absorption due to the pure water component is as by Fransois and Garison in Mohite-Patil et al. (2010) as:

$$\alpha_{H_2O} = A_3 P_3 f^2$$

$$P_3 = 1 - 3{,}83.10^{-5} D + 4{,}9.10^{-10} D^2$$

for $T \leq 20\,°C$

$$A_3 = 4{,}937.10^{-4} - 2{,}59.10^{-5} T + 9{,}11.10^{-7} T^2 - 1{,}5.10^{-8} T^3 \left[\frac{dB}{km\,kHz^2}\right]$$

for $T > 20\,°C$

$$A_3 = 3{,}964.10^{-4} - 1{,}146.10^{-5} T + 1{,}45.10^{-7} T^2 - 6{,}5.10^{-10} T^3 \left[\frac{dB}{km\,kHz^2}\right]$$

with T = temperature [°C]; S = salinity; D = Depth

Ainslie and McColm (1998) developed a simplified version based on the original formulas proposed by Francois R.E. and Garrison G.R. in 1982, by fixing temperature, salinity and acidity, which results in a relative accuracy error of 10%.

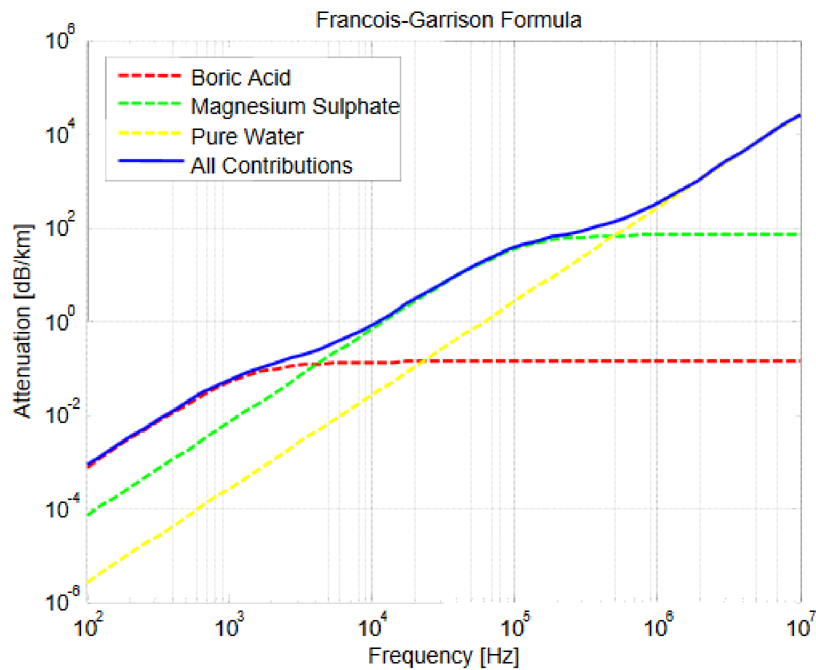


Fig. 4.6: Chemical relaxation and its contributing aspects (Kraus, 2016)

4.2.6.3 Refraction

Refraction of sound is bending of the wave caused by a change in sound speed at an interface or a sound speed gradient as a function of depth, temperature, salinity and pressure (hydrostatic) (Au and Hastings, 2008). This interface will not be constant.

The sound-velocity profile of the ocean by depth is divided in a surface layer (local daily changes by surface variables), seasonal thermocline (temperature deceases with depth and is affected by seasonal changes), main thermocline (only decreases with depth) and deep isotherm layer (constant

temperature of 3,88°C). At a depth of 1000m sound velocity will reach a minimum and the difference between surface and -1000m will only be 25m/s.

There are however various simple expressions for sound velocity as function of temperature, salinity and depth. The following is given by Medwin:

$$c(z) = 1449{,}2 + 4{,}6\,T - 0{,}055\,T^2 + 0{,}00029\,T^3 + (1{,}34 - 0{,}01\,T)(S - 35) + 0{,}016\,z$$

where: T = temperature [°C] (0°C <T<35°C); S = Salinity (>45ppt); z = depth (<1000m)

We assume furthermore that sound velocity does not change horizontally so that the ocean can be stratified horizontally.

Keeping this in mind 3 different models were developed over time to predict acoustic propagation.

- Ray theory: threating sound as light rays
- Normal mode: using a wave guide approach
- Parabolic equation (PE) approximation: numerically solved using an iterative approach

The latter is used by most ocean propagation software. As the depths involved in this research do not exceed 40m, it would be beyond the scope of this work to elaborate on these models.

4.2.6.4 In shallow water

Acoustic propagation in shallow water is a very complex situation, because the sound will bounce up and down between bottom and surface, scattering in all directions at every point. Sea state, bottom type and landscape and water depth make it very difficult to put a general descriptive function in place. Marh and Schulkin found 3 empirical formulas after about 100'000 measurements depending on the relation between r and an arbitrary H, for which

$$H = \sqrt{\frac{1}{8(D+L)}}$$

where D = water depth [ft]; L = layer depth [ft.] and H [kiloyard]

$$r < H: \quad TL = 20\log r + \alpha r + 60 - k_L$$

$$H \le r \le 8\mathrm{H}: \quad TL = 15\log r + \alpha r + \alpha_T\left(\frac{r}{H} - 1\right) + 5\log H + 60 - k_L$$

$$r > 8\mathrm{H}: \quad TL = 10\log r + \alpha r + \alpha_T\left(\frac{r}{H} - 10\right) + 10\log H + 64{,}5 - k_L$$

where: TL = Total Loss; r = distance [kyd.]; α = absorption coefficient [dB/kyd.]; k_L = "near-field anomaly"; α_T = shallow water attenuation coefficient.

The near-field anomaly is a "bouncing" coefficient in function of frequency, that depends on bottom

type and sea state. A graphical representation is given in Fig. 4.7. The shallow water attenuation coefficient is in function of frequency and depends on bottom type and sea state. A graphical representation is given in Fig. 4.7.

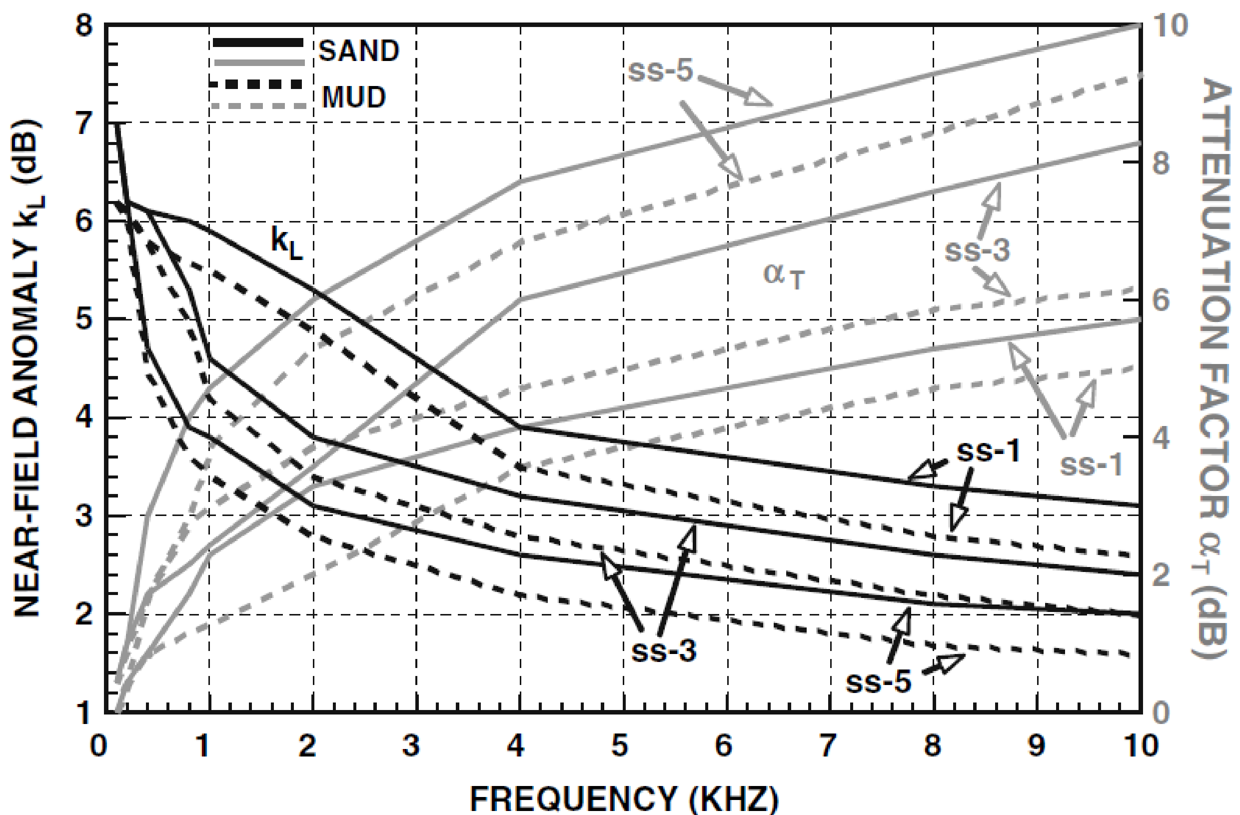


Fig. 4.7: The near-field anomality and shallow water attenuation factor (Au and Hastings, 2008)

The long-range transmission loss is rather like a cylindrical loss, while the short distance is more like a regular spherical transmission loss, taking the bouncing signal into account.

The decay in sound intensity is determined by geometric spread, the reflection loss at the sea boundaries and the additional absorption and scattering of sound in the water column, however the dominant one is reflection loss at the bottom and the surface (Abakumove, 2008).

Cutoff frequency

The propagational characteristic of sound waves in shallow water can best be approached from a normal mode perspective (Au and Hastings, 2008). This is a complicated matter which will not be discussed here. It is however interesting to know that there is a cutoff frequency f_c, which means that no sound can propagate for a certain depth and bottom type below this frequency. It can be calculated or read on the chart (Fig. 4.8).

$$f_c = \frac{c_W / 4D}{\sqrt{1 - c_W^2 / c_s^2}}$$

where: c_W = sound speed in water; D = water depth; c_s = sound speed of the bottom.

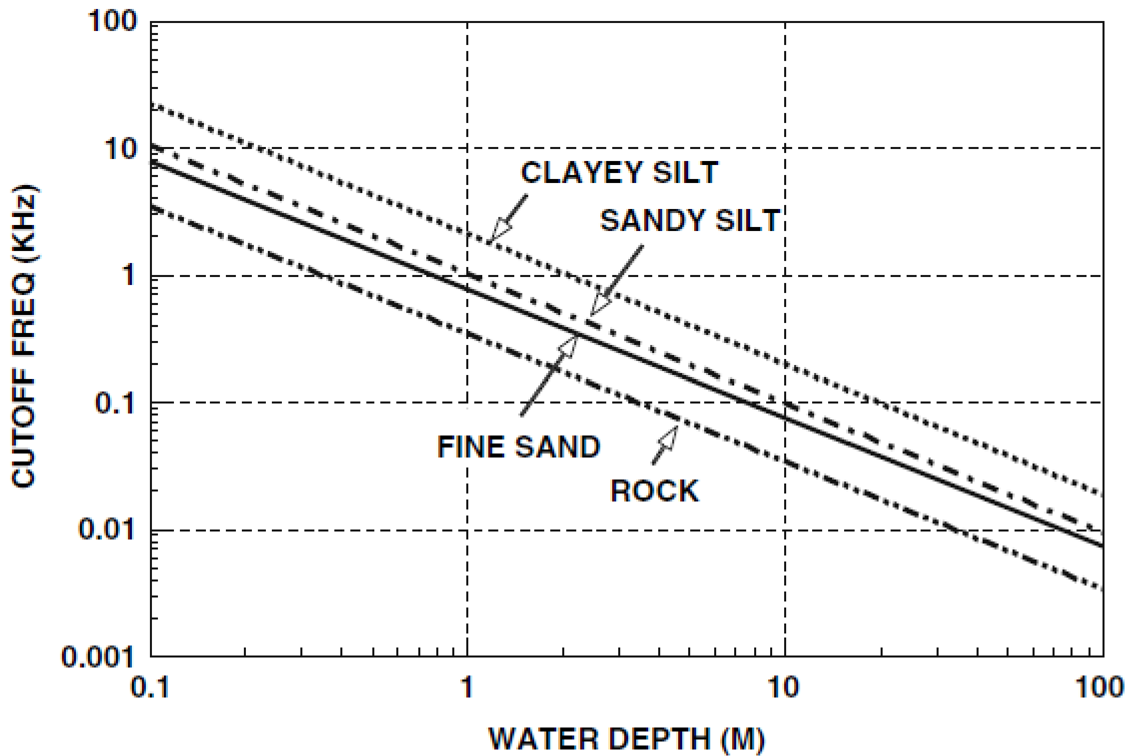


Fig. 4.8: Cutoff frequencies for propagation of sound for different bottom types: sound will not propagate in shallow water below the cutoff frequency (Au and Hastings, 2008)

4.2.7 Sound muffling systems

Many systems exist that muffle sound production of underwater anthropogenic sources. On ships many concepts have been developed to improve efficiency. These concepts generally decrease Sound Emission Levels (SEL's).

A common use to mask sounds of pile driving associated with the construction of wind farms is a bubble curtain. It creates a bubble screen around the development site which effectively muffles high frequency sounds, while being ineffective for lower frequencies (Debusschere, 2016). It is however not practically feasible for ships.

Installation of **ECA's** (Emission Control Area) in certain areas has resulted in some ships avoiding that area altogether, which leads to a local decrease in sound pollution (Renilson et al., 2013). This can be a positive impact locally, in particular for especially fragile local ecosystems. Overall this actually increases sound input in the soundscape however, as sound propagates easier in deep areas compared to shallow coastal areas where ECA's are usually installed.

To improve sound emission from merchant ships it might be interesting to look at **naval technologies** for such purposes. In modern naval architecture sound emission is important to avoid passive sonar detectability and hence many techniques have been developed and standard practices have been refined. While some of these techniques improve fuel efficiency, more often the contrary is true. Secondly the development is often expensive, not to mention “top secret”. This makes this path a though one for commercial adaptation (Renilson et al., 2013).

Modern non-toxic antifouling **coating** systems make the hull too slippery for fouling to stick. As an extra benefit these decrease the ship resistance, thus improving efficiency (e.g. in medium size tankers of 100000 dwt by up to 6%). As local imperfections (e.g. dents, cracks) significantly affect local cavitation (see 4.2.12.2), these coatings are expected to decrease SEL's too (Renilson et al., 2013). Purposely developed sound obscuring coatings, often containing rubber, often decrease efficiency however.

Ships are **design**ed and calibrated for optimum conditions, which in reality are rarely encountered. This means that designing it to be silent doesn't necessarily make it silent. A better solution, both in exploitational and sound pollution perspective, might be designing the ship to perform optimal for a broad set of environmental circumstances.

Improving the wake field and thus the water flow around the propeller decreases cavitation and thus SEL's and furthermore improves efficiency (Renilson et al., 2013):

Air lubrication systems improve ship efficiency in excess of 5% and according to Tinsley (2016) dampen propeller excitation. This reduces noise and vibration levels.

Propellers exist specially designed to minimise cavitation/vibration and thus reduce SEL's (e.g. High Skew Propellers; Contracted and Loaded Tip (CLT) propellers; Kappel propellers; and New Blade Section (NBS) propellers) (Renilson et al., 2013).

Hub caps are an aspect of consideration. Their proper design will reduce vortex cavitation and improve propeller efficiency, particularly for controllable pitch propellers (Renilson et al., 2013).

Propeller-rudder interaction is a considerable aspect of interest where vortexes easily form. Concepts, such as twisted rudders and rudder fins, are designed to improve efficiency, but will likely improve sound emission levels too. The Costa Propulsion Bulb (CPB), a concept where the propeller is integrated hydrodynamically with the rudder by fitting a bulb to the rudder in line with the propeller shaft, eliminates the hub vortex. Loading at the inner radii should thus be increased. It is claimed to reduce noise levels by 5 dB. Propeller Cap Turbines and Propeller Boss Cap Fins (PBCF) reduce this vortex cavitation (see 4.2.12.2)(Renilson et al. 2013).

In some cases, 'slow-steaming' decreases cavitation and thus reduces noise pollution. This is however not the case for ships fitted with variable pitch propellers, for which this will increase SEL's. Certain sound sources on board are independent of ship speed however, which limits the noise reduction (Okeanos, 2008).

4.2.8 Sound pressure vs. particle motion

Particle motion and sound pressure are different expressions of sound. Sound pressure follows the general patterns of the medium as an entity, showing alternating compression and rarefaction, which indicates potential energy. Particle motion follows the path of the molecules, moving back and forth, accelerating and decelerating, which is an indication of kinetic energy.

Practically and in general, sound pressure relates to the magnitude of sound or the loudness experienced by the receiver (and produced by the source), while particle motion relates to its sense and direction.

While sound pressure oscillations are measured by observing a vibratable surface, particle motion requires an accelerometer of some kind. The back-and-forth hydrodynamic flow can be sensed by tiny hairs, which thus function as an accelerometer. This form is found in many species, even without auditory specialization (see 4.4.2.3)(Samson et al., 2014).

4.2.9 Monopoles, dipoles and quadrupoles

(based on the work by Russell et al. (1999))

Any sound source whose dimensions are much smaller than the wavelength of the sound it produces, will act as a **monopole**. This means sound is radiated equally in all directions and at every point at distance *r* from the source the produced pressure is the same. This generates a circular directivity pattern (Fig 4.9a). Monopole power is proportionate to the square of the frequency ($\Pi \sim \omega^2$).

A **dipole** is formed by combining two monopoles with 180° phase difference at a distance preventing a standing wave at eigenfrequency to occur in between. As a result, the fluid between the sources moves back and forward in between. The directivity pattern is no longer circular, as no sound is produced on the perpendicular between both sources and a maximum is realised on the line between both sources (Fig. 4.9b). The dipole power is proportionate to ω^4 ($\Pi \sim \omega^4$). This means a dipole is a less efficient sound source, compared to a monopole.

There are two types of **quadrupoles**, which are formed by four monopoles: a lateral quadrupole

(sources form a square, 180° phase difference in neighbouring sources) and a longitudinal quadrupole (sources on a line, outside sources have a 180° phase difference with inner sources). The latter results in a more significant dipole, where both sides of the directivity pattern are allmost circular (Fig. 4.9d). The former results in a Clover shaped directivity pattern with two dead sound lines perpendicular between the sources, their complementary directions resulting in maxima (Fig.4.9c). The power of a quadrupole is proportionate to ω^6 ($\Pi \sim \omega^6$). This means a quadrupole is an even less efficient sound source.

Besides the clear reduction in efficiency, the type will also deform the wave pattern when plotting SPL against frequency (reducing SPL at certain frequencies, while maintaining SPL at other frequencies).

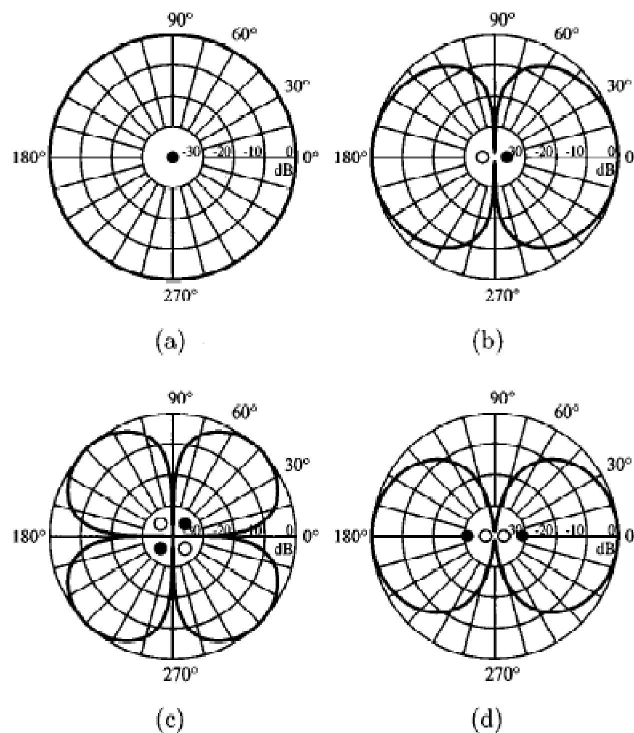


Fig. 4.9: Theoretical directivity patterns for far-field SPLs radiated from (a) monopole, (b) dipole, (c) latheral qudrupole, and (d) longitudinal quadrupole. (Russell et al., 1999)

4.2.10 Signal to noise ratio

Signal to noise ratio (SNR) it the ratio of the power of a signal and the power of the background noise. In dB it gives the added SPL to the background noise.

$$SNR = \frac{P_{signal}}{P_{noise}} \quad \Rightarrow SNR_{dB} = P_{signal,dB} - P_{noise,dB}$$

4.2.11 Sound measuring

Measuring and transmitting acoustic signals underwater is done mechanically with electroacoustic transducers. The most well-known systems are SONAR (**so**und **n**avigation **a**nd **r**anging) systems, which can be both transmitting and receiving (listening) or only receiving (passive sonar). Both are used in the merchant marine (depth measuring), fishing industry (finding fish) and military (ship detection). A problem, which is of particular magnitude to the latter, is that animals make many noises too, and distinguishing these biologic sounds from anthropogenic sounds is paramount for ship detection. Between WW2 and the mid-1970's, the US Navy has undertaken intense research in this respect (Au and Hastings, 2008). Sadly, this knowledge is still considered too sensitive for global access, as it would allow much insight into marine wildlife.

For scientific purposes, precise and otherwise specialised equipment is used, though based on the same principles. Electroacoustic transducers consist of elements that are able to convert electrical energy into acoustic energy (transmitting) and acoustic energy into electrical energy (listening). This acoustic energy in this case is pure sound pressure. Underwater listening transducers are called hydrophones (microphones in air), while transmitting transducers are called projectors. Four types are distinguished, based on the used material:

- Piezoelectric transducers: Certain crystalline substances possess piezoelectric properties, viz. quartz, ammonium dihydrogen phosphate and Rocelle salt. This means that when pressure is applied an electric charge between some crystalline surfaces is formed or when voltage is placed across, they will acquire stress, viz. they vibrate.
- Electrostriction transducers: These exhibit the same properties as the piezoelectric transducers. The difference is that the electrostriction tranducers are made of polycrystalline ceramics which need to be properly polarised. The advantage of these is that they can be easily shaped to the demand as the name suggests. They are also called piezoelectric ceramics and therefore are actually of the same type as the former. Examples of materials exhibiting these properties are barium titanate (BaTi) and lead zirconate titanate (PZT).
- Magnetostriction transducers: Materials used in this type of transducers (e.g. Terfenol-D and cobalt) change dimensions when placed in a magnetic field. Conversely, when deformed under elastic strain (such as sound waves) the flux density changes and the conductor surrounding it, measures an electromotive force.
- Electrodynamic transducers: This third type operates like air-speakers, but are adapted for aquatic/marine use. They are particularly well equipped for low frequency sound generation.

- Piezoelectric plastics and rubbers: These more modern materials have become more popularl.

As shown above all transducers convert material stress into some form of electric current. As the motion of the material under stress is very small, so is the generated current, which reflects the need of an amplifier to enhance the output and make readings possible.

As the detailed internal and mathematical workings of hydrophones and speakers serves no point in the understanding of this study, they are considered beyond its scope.

The measuring of particle motion is still a very different thing and a development still in its infancy. A method exists with two hydrophones and measuring the difference in sound pressures, but this method is found to be inaccurate at best. The method used for this study is a composition of three pressure measuring devices fitted in three orthogonal directions on a titanium block. It is based on the theory that on a fixed free point, the difference in pressures in three directions results in the direction and magnitude of fine motion of water particles and thus particle motion. Although this is theoretically very nice, practical accuracy is somewhat uncertain.

When describing data in function of frequency, three types of frequency axis can be used. It can be done either in narrowband (e.g. with 1Hz intervals), in 1/3 octaves or in 1/n octaves. Usualy narrowband or 1/3 octaves are preferred (Baudin and Holger, 2015).

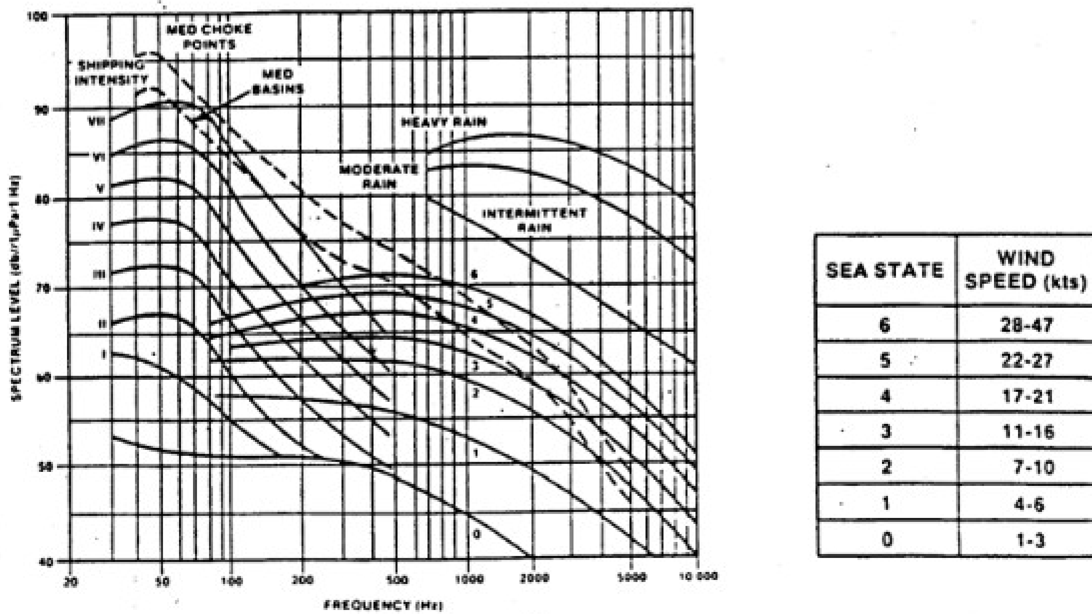
4.2.12 Sound production

Noise in the environment is produced by many things. The soundscape of the sea forms the background noise for all that lives in it (see 4.2.12.1). As will become clear, the part produced by ships is of particular interest and significance (see 4.2.12.2). For the experiment it is important to have some understanding of both and its significance towards the playing of sounds in a laboratory environment in a tank (see 4.2.12.3). In 4.2.12.4 a comparison will be given.

4.2.12.1 The marine soundscape

When listening to the environment under the water level a large soundscape is observable. By listening carefully, the different aspects can be distinguished. Degraer S. (2009) could distinguish distant shipping and the construction noise from the Thornton windfarm from the Knudsen sound (see High Sonic Band) during his recordings at Bligh Bank. Seismic surveys executed by the petroleum industry (253 dB (re 1 μPa at 1 m)) and underwater volcanic eruptions (in excess of 255 dB (re 1 μPa)) are among the louder sounds, making them easily distinguishable (Lovell et al., 2005). Overall there is a standard graphic (Fig. 4.10), which predicts the soundscape more or less

accurately.



SEA STATE	WIND SPEED (kts)
6	28-47
5	22-27
4	17-21
3	11-16
2	7-10
1	4-6
0	1-3

NOTE: ADD 2 dB TO SHIPPING NOISE LEVELS FROM JANUARY THROUGH MARCH AND SUBTRACT 2 dB FROM SHIPPING NOISE LEVELS FROM JULY THROUGH SEPTEMBER.

AMBIENT NOISE LEVEL VERSUS FREQUENCY

Fig. 4.10: Different aspects of the ambient noise level.
(Tucholski, 2006)

As is clearly visible in Fig. 4.11, the dominant source at lower frequencies is shipping noise, which propagates well over long distances and is unafected by the seastate (Renilson et al., 2013; Wittekind, 2014; Okeanos, 2008; McKenna et al., 2012). At moderate frequencies, the sea-state is dominant, while in the extremely high-frequency band, thermal noise takes the upper hand (Haelters J. et al. 2009).

The soundscape in the ocean is generally divided into two groups: continuous sound and intermitted sound. This is a somewhat strange dividing, because sea-state and ships are considered continuous sounds, while rain and marine animals are considered intermittent. Sea-states come and go as do rains, while ship presence is as frequent as animal presence (for a given location). For the purpose of this study, this dividing will remain however.

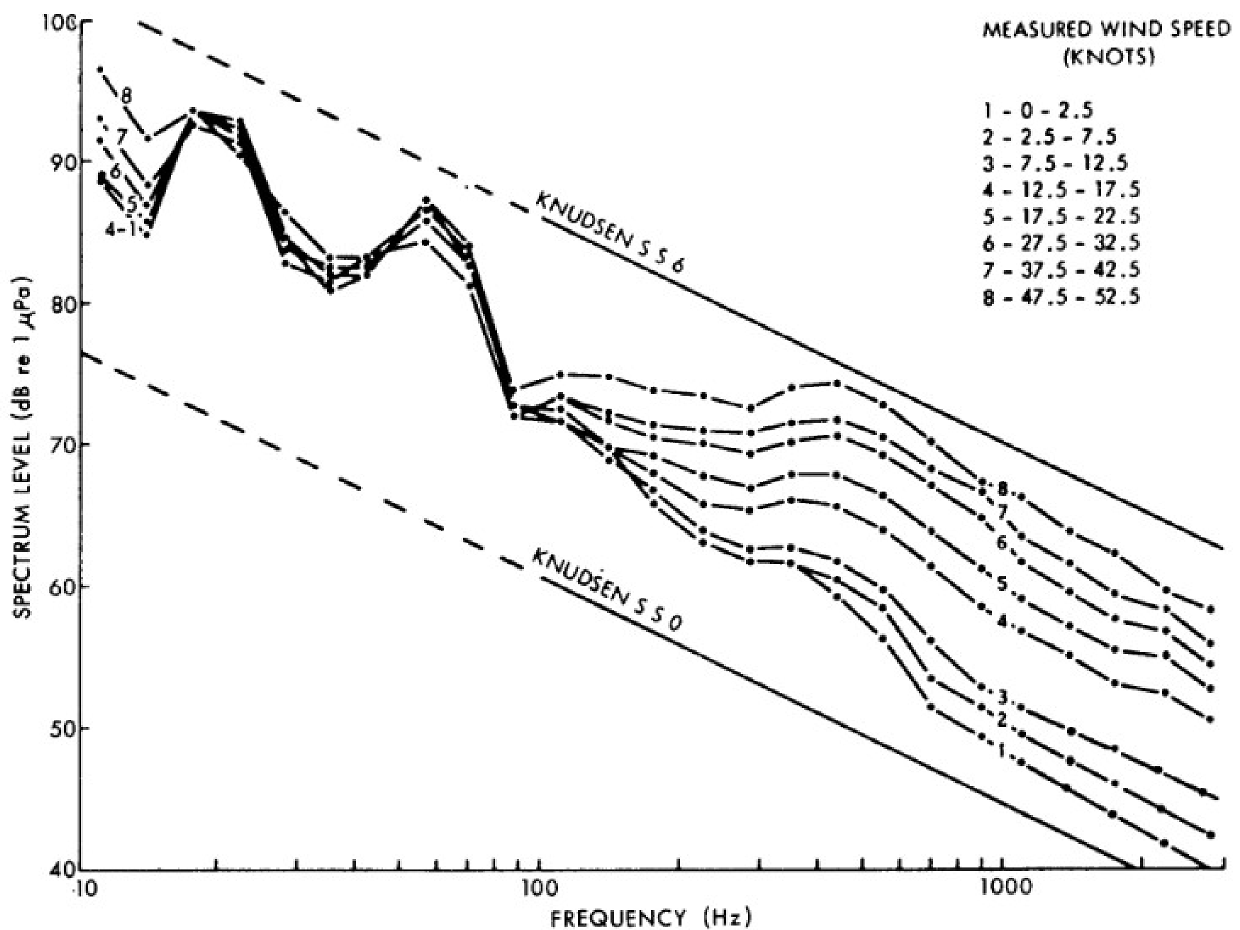


Fig. 4.11: Ambient noise by sea state
The ambient noise frequency spectrum is dominated by shipping and virtually independent of the sea state.
(Urick, 1984)

Continuous sound

Ultra-low band (<1 Hz)

This band is of little significance as the wavelengths are so large. Contributers are seismic activity and tides (Urick, 1984). Tucholski (2006) considers seismic activity sound to be present up till 10Hz and tidal and turbulence sounds until 100Hz, though he too considers them of insignificant magnitude.

Infrasonic band (1 to 20 Hz)

This band contains the strong blade-rate fundamental frequency of propeller-driven vessels, plus one or two of its harmonics (Urick, 1984).

Additionally, pseudo-noise (noise produced by interaction between hydrophone and currents and temperature variations), flow-noise (caused by boundary layer turbulence and vortexes) and cable-strumming (caused by cable interactions in case a flexible cable is used) form part of the self-noise

of the operator (Urick, 1984). In the absence of any of these noises and shipping noise, environmental noises become greater than electrical noise, which indicates this in itself does not interfere with measurements. This environmental noise then becomes only subject to the sea state and precipitation.

Low sonic band (20 to 200 Hz)

This band is dominated by shipping (Urick, 1984; Tucholski, 2006). In its absence, sea state and percipitation dominate this band (Fig. 4.12).

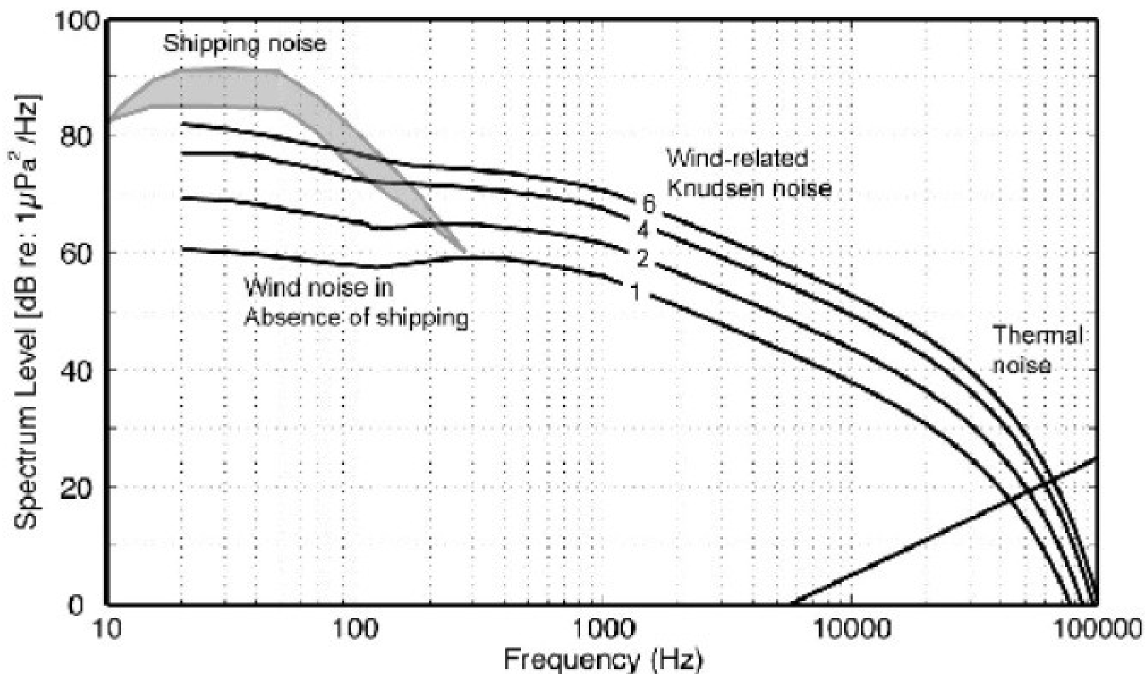


Fig. 4.12: Knudsen curves, thermal noise and the addition of shipping noise (Okeanos, 2008)

High sonic band (200 to 50,000 Hz)

This frequency band was first studied by Knudsen V.O. during the Second World War. The result of his measurements were the Knudsen curves (lines on a logaritmic graph)(Fig. 4.12)(Urick, 1984). These curves very accurately predict ambient sound as a factor of the sea state (and thus wind). There is only some error when below 1 kHz in deep water, where the line flattens. In shallow water this does not, because the waves break on the seabed (Urick, 1984). Wind and sea-state are thus the dominant factor in the High Sonic Band (Tucholski, 2006; Urick, 1984).

Ultrasonic band (> 50 kHz)

In these frequencies, depending on sea state, thermal noise begins to dominate (Urick, 1984). Thermal noise is the noise of molecular bombardment. It is the analogue of the Nyquist electrical noise, which limits the use of a hydrophone.

Intermitted sound

Intermitted sound can be seen as significantly contributing, but not as omnipresent as continuous sound. It is divided into two groups: biologic and non-biologic noises.

Biologic noise

These sounds are produced by marine animals. They comprise of audible noises such as whoops, grunts, whines, moans and chirps. Most of these sounds are produced by crustacea, marine mammals and certain species of fish. Though not all species of fish produce noises in their natural environment and conditions, nearly all produce some sounds when subjected to outside stimuli (e.g. electric shocks). Though these sounds only occur within the habitats of these species, these sounds are loud and clearly identifiable. Examples are snapping shrimp snaps (*Alpheus lottini*, Fabricius 1798), harbour porpoise clicks (*Phocoina* phocoina, Linnaeus, 1758) and the croaking of Atlantic croakers (*Micropogonias undulatus*, Linnaeus, 1766).

Non-biologic noise

Rain (or, more gernerally speaking, precipitation) is an important source of non-biologic noise as it occurs frequently and can last for a while. The noise produced by rain is due to three factors:

- The impact itself: the main factor for regular rain.
- The oscillations of the object after impact: more important with snow.
- The oscillations of the air taken along under the surface: it is the most important factor when firing a gun in the water.

The size of the rain droplets and the intensity of the rainfall (drizzle vs. downpour) are the main factors for rain-noise.

Explosions caused by the off-shore petroleum industry is an anthropogenic source of significant magnitude (+20-30dB re 1 µPa).

Seismic activities (**seaquakes**) were observed to raise noise levels at least 20 dB re 1 µPa in 5-35Hz frequency range. Volcanic eruptions are observable below 100Hz with a peak in the 10 Hz range.

Measurements in 90m deep water, 8,5km offshore in Montery Bay, California showed 10dB re 1 µPa SPL increases due to **surf**[5] in the 100-700Hz range. The narrow bandwidth is due to the propagation limits.

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[5] Surf is the crashing of sea waves on the shore.

4.2.12.2 Ship noise

Ships are a major contributor to ambient underwater background noise. Ships generate a lot of noise over variable parts of the spectrum during normal operation (McKenna et al., 2012). The fact that this is during normal operation, means that this noise is rather inevitable. At best, attempts can be made to diminish sound generation.

Note that especially those vessels equipped with dynamic positioning (DP) systems might radiate strong underwater noise. The reason is threefold: (1) they remain in place for a while; (2) they often use multiple propellers at the same time; and (3) the propellers barely operate at an efficient, silent regime (Baudin and Holger, 2015).

Sources of shipsound

Not all factors attributing to ship noise generation are well understood (Renilson et al., 2013). The most prominent ones are however well known. There are three main causes of ship-generated noise (Renilson et al., 2013; Wittekind, 2014; Okeanos 2008):

- the propeller(s) in both low frequencies and medium to high frequencies;
- the machinery (main engine(s) and auxiliaries) in medium and high frequencies; and
- the movement of the hull through the water.

Generally, the propeller is the most dominant of these three sources, but depending on ship type this may vary (Renilson et al., 2013). If the noise level of one component is 10dB above the others, it becomes dominant and the others become irrelevant (Renilson et al., 2013).

Ships can generate noise from design “flaws” and because some sounds are inevitable. Imperfections and small damages (e.g. scratches, corrosion) can however negatively affect local cavitation, resulting in increased hydro-acoustic noise (Renilson et al., 2013; McKenna et al., 2012).

It stands to reason that sound emission from ships is asymmetrical. The level of the stern aspect is 5 – 10 dB re 1 μPa louder than bow aspect on average (McKenna et al., 2012).

Propeller

Noise of the propeller can be viewed as a monopole in some aspects (see 4.2.9)(Wittekind, 2014).

A ship and its main propulsion propeller(s) are designed for a certain speed, viz. the design speed. This design speed is supposed under certain operating conditions, which are however unrealistic. Firstly, full power conditions are assumed, which are actually unachievable, as wear and tear will limit the actual power output and the hull will quickly sustain speed impeding imperfections.

Secondly, the propeller is designed for a wake distribution at this design speed under calm sea conditions (viewed from scale models and/or digital models), while sea state varies significantly, influencing the wake distribution. Thirdly, full load conditions are assumed, which in reality is not always the case (Renilson et al., 2013).

Propellers work on three basic propelling forces/principles (Dokkum, 2006):

- Pressure: the aft side of the blade pushes the water aft;
- Suction: the for side of the blade sucks the ship forward with a low pressure area; and
- Bernoulli principle: the blades of a propeller are shaped like an airplane wing, creating the aforementioned pressure and suction, hence enhancing these effects (Fig. 4.13[6]).

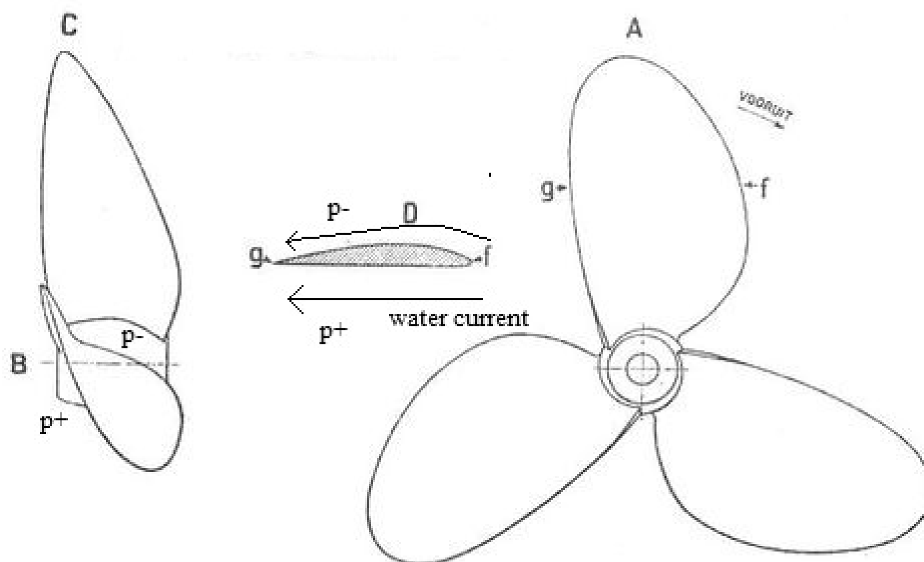


Fig. 4.13: Propeller views and Bernoulli effect
(Own collection)

The propeller efficiency depends largely on the flow/wake field of the propeller, which depends on (Dokkum, 2006):

- The underwater shape of the ship (block coefficient C_B[7]): If the aft part of the largest beam of the ship ends abruptly a low pressure area will form. A large block coefficient implies this and results in a bad wakefield. The ship will in any case always disturb the flow-field to the propeller and create a non-uniform inflow (Okeanos, 2008).
- The power delivered to the propeller and its angular velocity (rotations per minute or rpm):

6 'VOORUIT' = forward

7 Block coefficient is the ratio of ship underwater volume to the volume of a block with diameters: length between perpendiculars, beam moulded (inside the shell plating) and draught (van Dokkum, 2006; Wittekind, 2016)

If the water doesn't run into the propeller fast enough, it might slip excessivly. Additionally if the pressure around the propeller is to low, it will have difficulty sucking water into it. High thrust loading means that a large amount of thrust is required to propel a ship with a comparatively small propeller (Okeanos, 2008) and is a problem as the large amount of energy is exerted on the relatively small surface.

- The number of blades and their shape and smoothness: The number of blades distributes the power of the propeller better on the water and decreases vibration. The smoothness of the blades effects the aforementioned propeller forces, particularly the Bernoulli effect. (Okeanos, 2008)
- The propeller diameter: With a larger propeller diameter, a greater force on the water (and thus transition of the engine power to the water) is achieved and more of the wake water is used. Thus with a greater diameter less rpm is needed for an equal engine power (Dokkum, 2006).
- The ship's speed: the ship speed directly translates in the flow field, as it creates a water current through the propeller. At a certain speed however, the low pressure area at the stern will start to counter this flow.
- Possible appendages are designed to improve wake field (Wittekind, 2014).
- The number of propellers can influence the wake field. Twin propeller arrangements have generally more homogenous wake fields and lower trust loading when compared to single propellers (Okeanos, 2008). Aditionally, they will form a dipole in the near field.
- The eigenfrequency of the propeller (Neugebauer et al., 2008).

When pressure in a liquid decreases below the vapour-pressure, vapour bubbles form, which, when pressure increases again, implode. This process is called **cavitation**. Around the propeller this is a frequently occurring process, because of the intended pressure zones to propel the ship and unintended vortexes. The water pressure is thus important in respect of both propulsion and cavitation to opposite effects, viz. low pressure means low propeller efficiency and high speed, but high cavitation levels and vice versa.

Sadly though, no matter how well a propeller and ship are designed, above a certain relative water approach velocity (= ship's speed - wake speed) cavitation will always occur. Above a certain critical ship speed the pressure aft will drop to such an extent that the added low pressure around the propeller will result in cavitation. This is critical speed is called the Cavitation Inception Speed

(CIS) (Renilson et al., 2013). CIS depends on inflow speed variation, propeller loading, propeller submergence, and quality of the propeller design (Wittekind, 2014). Additionally, this cavitation noise is spread over the frequency spectrum, making it impossible not to have some part of the ship exciting at its eigenfrequency (Arndt et al., 2015). More so, while speeding up, the major cavitation frequency will at some point align with the ships or propellers eigenfrequency, which is to be avoided for a prolonged time to avoid damage (Arndt et al., 2015). It is for this reason that commercial ships have fixed speed regimes (e.g. dead slow ahead, full ahead).

The propeller itself makes noise (by singing), but the low frequency cavitation noise is far more loud and the most important factor in ship sound emission, dominating all other hydroacoustic noise the ship produces (Renilson et al., 2013; Okeanos, 2008). Thus, above the CIS, which is always lower than the design speed, cavitation noise will always exist. This cavitation noise will occur primarily in the tonals of the blade rate. Cavitation will always occur first around the 12 o'clock position, where it stands to reason that pressure is at its lowest in the aft field (Renilson et al., 2013; Chakraborty, 2016). Every time a blade passes this position this will produce a cavitation sound shock, thus creating sound at the rhythm of the passing blades. These appear as tonals at multiples of the blade rate, viz. rpm times the number of blades.

$$f_h = \frac{rpm * \#_{blades}}{60}$$

where: f_h = harmonic frequency; $\#_{blades}$ = the number of blades

The first harmonic will then be formed in function of these factors around 10Hz (below for large ships, above for smaller craft) (Wittekind, 2014). This type of cavitation is known as sheet cavitation. Together with tip vortex cavitation, it is responsible for most vibration on board (Arndt et al., 2015). With increasing rpm speed above CIS, SEL will increase (Wittekind, 2014).

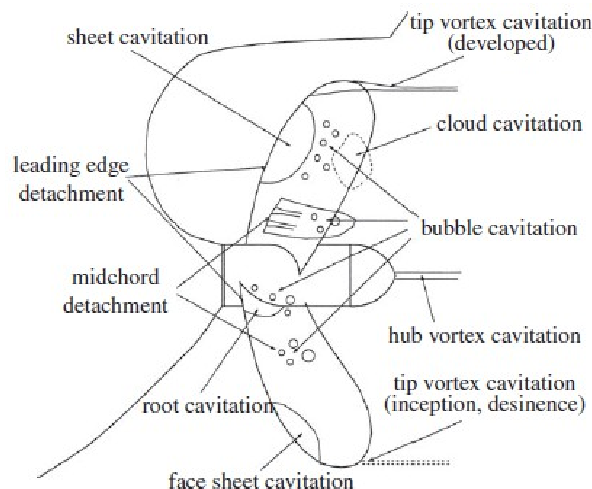


Fig. 4.14: Typical cavitation types on a marine propeller. (Arndt et al., 2015)

Cavitation will often happen even before CIS however, but it is only from CIS that it will have a non-negligible effect on speed and hence sound and vibrations. When the propeller is working inefficiently cavitation will increase and apparant CIS may decrease (CIS will happen at a lower speed)(Renilson et al., 2013; Wittekind, 2014).

'Slow steaming' philosophies to reduce fuel consumption, result in a less efficient use of the propeller, particularly for variable pitch propellers, which in turn can lead to increased cavitation (Renilson et al., 2013). When speed is reduced below CIS however, overall hydroacoustic noise levels may be considerably reduced in fixed propellers, but not over the entire frequency-spectrum. This speed is however likely to be too slow to be practicable for most merchant ships, hence noise from cavitation is to be expected in general and certainly when the economy flourishes (Renilson et al., 2013).

Fig. 4.14 shows the many known forms of propeller cavitation, though when talking of propeller cavitation, sheet cavitation is generally meant.

The singing vortex is often mentioned in literature. It is the result of tip vortex cavitation. This cavitation is amongst others the result of inertia on the tip of the propeller blades. This cavitation constantly forms bubbles, which form a vortex behind each blade tip. The interaction of this cavitation stream with the rudder blade (usually hanging behind the popeller), can result in heavy damage to the rudder. These flattened cavitation vortexes spin around their own axes (in both directions), have a flow away from the propeller and towards the propeller, and expand and contract as a result of pressure variations. These movements follow frequencies depending on many variables. When all variables fall in line this vortex will move at its eigenfrequency resulting in a massive dominating singing vortex (Arndt et al., 2015).

The propeller hub generates efficiency-reducing **vortices** which are prone to cavitation (Renilson et al., 2013). These vortices are particularly common for variable pitch propellers[8] (Renilson et al., 2013). The propeller-rudder interaction is significant for propulsive efficiency and if improperly designed result in detrimental vortices too (Renilson et al., 2013).

In absence of cavitation, the dominant low frequency noise will result from blade vibrations (sheet cavitation). This is however at a negligible level (it is very low and machinery noise will overpower it).

Above CIS broad band high frequency noise will be generated because of the now constant propeller cavitation (not only around the 12 o'clock position which will still dominate) and the

[8] In variable pitch propellers the blade pitch or attack angle of the blade is changed with varying propulsion demands, while maintaining a constant rpm (Dokkum, 2006).

vortex cavitation (Wittekind, 2014).

An unknown mechanism results in a constant broad band spectrum sound peaking around 40–50Hz in most ships, which increases in SEL according to speed, but doesn't vary in frequency. It is a sound that transmits well in the water, but doesn't penetrate well in the ship (Wittekind, 2014).

It can be concluded that small increases in efficiency in the propeller, may reduce cavitation and hence propeller-generated noise, thus decreasing the dominant ship noise contributions (Renilson et al., 2013). It is worth mentioning that it is not always feasible to design the propeller for the highest efficiency, due to propeller size and/or engine bottom speed limitations, thus negatively affecting sound emission (Wittekind, 2014).

Another important facet of propeller cavitation lies in its ability to cause damage. If the vapour bubbles do not implode away from ship structure it will create deep pits and result quickly in serious damage (Arndt et al., 2015).

From special propulsion systems such as pump-jets, Voith-Schneider propellers, waterjets, super-cavitating propellers and surface piercing propellers noise generation has been limitedly studied (Okeanos, 2008). For this reason and their limited use in commercial shipping these will not be included in this study.

Engines

Three main types of carbon fuel engines are widely used in commercial shipping that contribute to a ship's sound field:

1) **Diesel generators**: Commercial ships are usually fit with three diesel generators for auxiliary power supply, being mainly electricity (Wittekind, 2014; Okeanos, 2008). Their sound is characterized by tonals at multiples of half the rotational speed. The typical electric frequency on board is 60Hz, thus engines running usually at 720 rpm. Therefore a 720Hz a diesel generator has tonals at 720/60/2 = 6Hz and its multiples up to kHz range (Wittekind, 2014).

2) **Main low speed engines** (diesel or heavy fuel): Main engines of this type can be found on all modern and large commercial ships. They are vast slow rotating (usually 70-120 rpm) power plants, usually linked directly without gearbox to the propeller. Their slow two-stroke revolution speed and few actions make them relatively quiet. Their sheer size and the fact that this means they are hard-mounted[*] makes them loud. They have no clear sound

[*] Hard-mounted indicates that the item is fitted to the main structure without any vibration-limiting appliances (a soft tissue support). Resilient-mounted items are thus isolated. For this isolation rubber fittings are used which can

signature; therefore, their contribution is somewhat obscure (Wittekind, 2014).

3) **Main medium speed engines** (usually diesel): These often four-stroke engines are used on smaller ships and usually operate at 514rpm. This means a reduction gear box is needed for the main propulsion. Usually an electric generator is also linked to the engine which requires this predictable constant rpm. For this reason these ships are usually fit with a variable pitch propeller system. Medium speed engines are usually resilient-mounted, making their sound contribution equivalent to those of diesel generators (Wittekind, 2014).

To enable the engine to produce sound in the surrounding water, the sound vibrations are transmitted through the hull, which has a large surface. For this reason, it cannot be seen as a monopole source (Wittekind, 2014; Okeanos, 2008). Machinery noise usually dominates the medium to high frequency spectrums and in absense of cavitation also the lower frequency ranges (Okeanos, 2008).

Hull

The hull itself produces little to no sound by itself. It could be argued that certain extremities, viz. stabilising fins, could create vortex noise. Additionally, corrosion and fouling as well as certain efficiency boosting upgrades, viz. air lubrication, might result in some noise. There is no data on these subjects however and its sound is undoubtedly inferior to propeller cavitation and engine noise.

The hull functions however as the “speaker” for the engine as it transfers the engine noise into the sea as mentioned above.

The study by McKenna et al. (2012) clearly shows that there is a distinct irregular emission pattern for ships. The fore and aft part would muffle sound emission levels (SEL) by 3-5 dB. SELs received from the stern may even be 5-10dB higher. Data from a small vessel show a muffling effect of 12 dB by the bow and 9 dB by the stern. In all cases the highest SELs were from the broad sides. This is due to the bow-wave and the bulbous bow at the bow, and the bubble wake at the stern, which block some of the noise. Additionally, the broad side has a large surface which efficiently transmits noise into the surrounding ocean (McKenna et al., 2012). The larger differences in small craft might be due to the lower efficiency in the propeller, resulting in more bubbles and a bigger, less efficient bow wave.

reduse sound emission by 15-20 dB (Okeanos, 2008).

Shiptypical

The signature sound emission of ships depend predominantly on its design. General formulas for ship sound emission will be discussed in this study which show variations not exceeding 10-15dB above 100Hz (Wittekind, 2014). McKenna et al. (2012) states however that a relatively accurate description should incorporate the ship type. The ship type will inevitably enforce some limitations and requirements for efficient and legal exploitation on the hull shape and hence the ship speed (e.g. bulk carriers will be more box like and thus slow, while container ships will have a more streamlined body and hence higher speeds). It can be assumed that cavitation levels will be similar in freight carrying commercial ships, thus the dominating cavitation sound will generally not differ more than 10 dB (Wittekind, 2014). While it is sometimes stated (e.g. by Okeanos (2008)) that SELs depend on ship speed, Wittekind (2014), Renilson et al. (2013) and McKenna et al. (2012) contradict this with their respective data. Renilson et al. (2013) found it to be true when comparing ships of the same type. SELs are also independent of ship dimensions to some extent.

The seven ship types as designated by the World Shipping Encyclopedia from Lloyd's Registry of Ships are (McKenna et al., 2012):

1) Bulk Carriers
2) Container Ships
3) Crude Oil Tankers
4) Chemical Tankers
5) Product Carriers
6) Vehicle Carriers
7) Open Hatch Cargo Ships:

   As general cargo ships (open hatch cargo ships) are disappearing, these are usually quite loud because of their age (Wittekind, 2014).

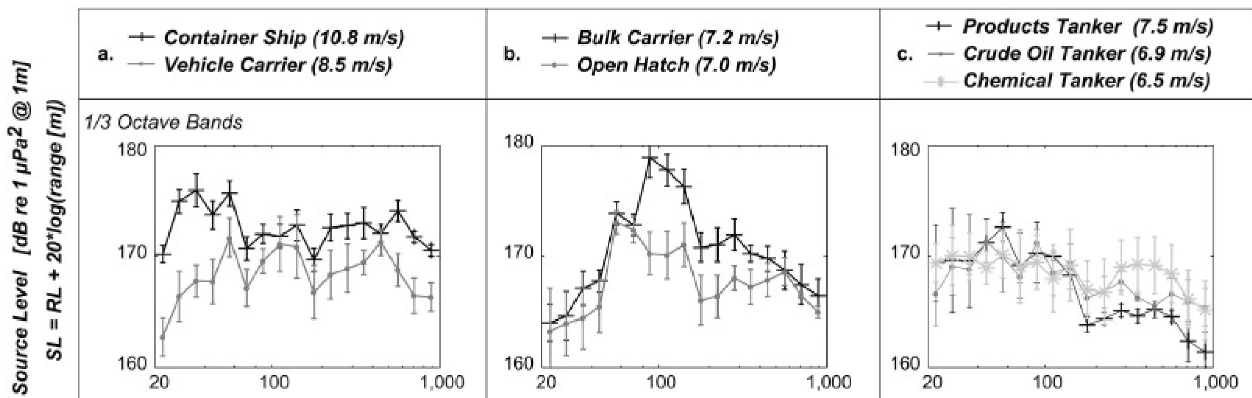


Fig. 4.15: Ship SELs
(a) container ships and vehicle carriers, (b) bulk carriers and open hatch cargos, and (c) three types of tankers in 1/3 octave bands
(McKenna et al., 2012)

These ship types show distinct patterns of noise production, but average broad band SELs (20-1000Hz) vary only between 180 and 186 dB re 1 µPa at 1m, with bulk carriers and container the loudest (186 dB) and chemical tankers the most silent (180 dB)(McKenna et al., 2012; Renilson et al., 2013). Dominant frequencies with bulk carriers are around 100Hz, while container ship and tanker noise is dominant below 40Hz. In general, it can be seen as predicted before that frequencies above 300Hz contain less acoustic energy (Fig. 4.15)(McKenna et al., 2012).

Though these ship type signatures hold merit, major individual deviations should be expectable (Wittekind, 2014). Additionally, because of measuring inaccuracies, model-inaccuracies and environmental uncertainties (exact soil type, precise salinity and temperature gradients, etc.) overall inaccuracy within 10-15dB is to be expected in the model (Wittekind, 2014).

When attempting to predict noise impact of shipping in a region it is thus usefull to have an estimate of the amount of each ship type passing by. These figures will give a better insentive towards SEL-frequency distribution than the overall number and according broad band SELs alone. Another reason is lastly, that one ship type typically navigates faster than another and hence influences the total amount of acoustic energy input (McKenna et al., 2012).

Load condition

In propeller sound the Lloyd Mirror effect has an important effect (Renilson et al., 2013; Okeanos, 2008).

If the ship is under light loading condition (e.g. in ballast conditions) the propeller sits much shallower into the water (possibly partly surfaced) as opposed to under full loading condition, where the ship sits at its deepest. A shallow propeller will have a lower CIS (due to a lower pressure

around the propeller) and thus a higher level of cavitation at the same speed (Renilson et al., 2013). The reflection and the Lloyd Mirror effect on deep propellers usually overcomes this increase in sound however (Renilson et al., 2013). It can thus be said that SEL increases with increased draught (Renilson et al., 2013). Wittekind (2014) states a difference of 3dB between ballast and full loading condition.

Some ships are rather independent of their loading condition, as their load capacity is more dependent on volumetric capacity (e.g. vehicle carriers) instead of their deadweight capacity (e.g. bulk carriers). The former will always have a relatively shallow propeller.

In 3D graphics when SELs are set out against frequency and position, U-shaped interference patterns can be observed. These are a result of the creation of a dipole situation between the actual source (propeller) and the reflecting the surface. This is the Lloyd Mirror effect (Fig. 4.16) (McKenna et al., 2012).

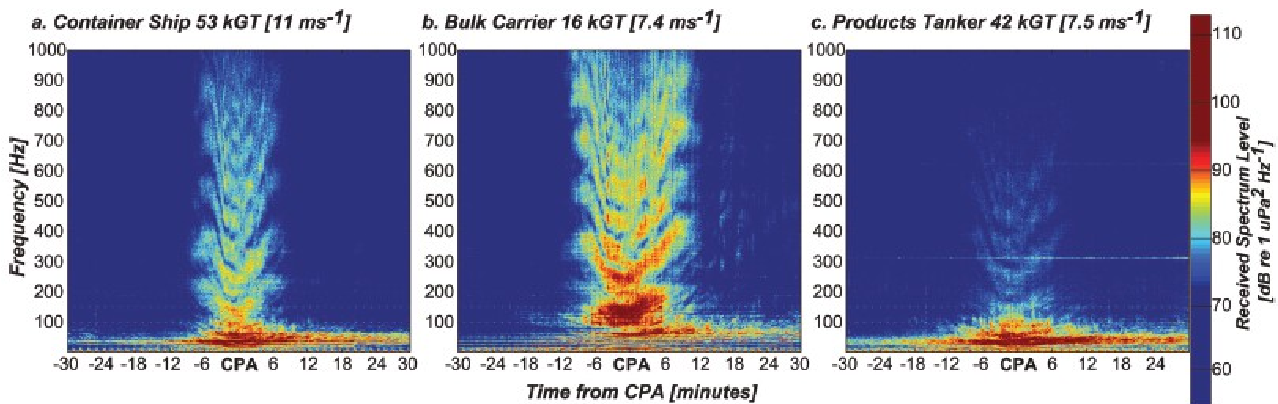


Fig. 4.16: Sound profiles
Received sound levels during 1-h passages of three different ship-types: (a) Container ship. (b) Bulk carrier. (c) Product tanker. Figures are centered at CPA of the ship to the HARP. Negative CPA is bow aspect; whereas positive values are sternaspect. Top figure series shows the received levels as color (dB re 1 µPa^2/Hz)
(McKenna et al., 2012)

In a formula

The main parameters included in these formulas are:

- Displacement
- Speed in relation to CIS
- Block coefficient to indicate wake field variations
- Mass of the diesel generator engine and its mounting (resilient/rigid)

The first three relate to the absent propulsive power. These formulas are proposed for ships with

fixed propellers and variable pitch propellers at design pitch (Wittekind, 2014).

Three main contributors are considered: low-frequency cavitation noise F_1, high-frequency cavitation noise F_2 and diesel engine noise F_3. Averaged SPLs in third octaves will be used from each contributer and then added to form SELs.

$$SEL=10\log\left(10^{\frac{F_1}{10}}+10^{\frac{F_2}{10}}+10^{\frac{F_3}{10}}\right)$$

The main difference between F_1 and F_2 is that the former is considered a monopole and the latter a dipole, which means propagation loss follows 40log(distance) rather than 20log(distance) parallel to the surface (Wittekind, 2014). The limit between those is given by:

$$f=\frac{cr}{8z_s z_r}$$

where: c = speed of sound (m/s); r = slant distance from source to receiver; z_s = source depth; z_r = receiver depth.

Low frequency cavitation noise F_1 is then depicted as:

$$F_1=2{,}2.10^{-10}f^5-2.10^{-7}f^4+6.10^{-5}f^3-8.10^{-3}f^2+0{,}35.f+125+A+B$$

$$A=80\log\left(4c_B\left(\frac{v}{v_{CIS}}\right)\right)$$

$$B=10\log\left(\frac{\Delta}{\Delta_{ref}}\right)^{\frac{2}{3}}$$

where: f = frequency [Hz]; c_B = block coefficient; A = speed and C_B factor; B = displacement factor; v = speed [kn]; v_{CIS} = CIS [kn]; Δ = displacement [t]; Δ_{ref} = reference displacement = 10,000 t

This results in a curve of showing SELs in third octaves in dB re 1µPa at 1m in function of frequency, peaking at 40Hz (Wittekind, 2014).

High frequency cavitation noise F_2 is expressed as follows:

$$F_2=-5\ln(f)-\frac{1000}{f}+10+B+C$$

$$C=60\log\left(\frac{v}{v_{CIS}}.1000.C_B\right)$$

where: C = speed and C_B modelling factor

which has stronger dependence on ship shape. The laws to describe this factor are unknown (Wittekind, 2014). It is artificially made to fit the result.

Diesel generator engine noise F_3 is represented in the following function. It is an empirical function assuming a medium speed four-stroke engine. E is a state parameter, equalling 1 if rigidly

mounted and 0 is resiliently mounted.

$$F_3 = 10^{-7} f^2 - 0{,}01 f + 140 + D + 15 E$$
$$D = 15 \log(m) + 10 \log(n)$$

where: D = generator number and mass factor; m = generator mass [t]; n = number of generators operating at the same time.

Predicting sound emission levels

Although Okeanos (2008) states that current prediction procedures and test models from on-board noise and vibration limitation requirements could easily be adapted to predict underwater noise emissions, McKenna et al. (2012) found that the basis for these procedures and models are based on old small ships and that the ship's type is an import and absent factor in those. Additionally, ship size and power have significantly increased over time.

When comparing scale model sound and sound in real life ships, important features of the sound spectrum match well (Wittekind, 2014). This signifies that scale models could give reliable predictive indications on sound emission for the finished ship.

While older simulations of overall sound emission from ships show rather conservative emission levels, modern simulations and prediction models show even higher contributions to the total background noise (Okeanos, 2008).

4.2.12.3 In an experimental tank

In an experimental lab set-up tanks are used to recreate the environment of the test subjects. These tanks are small in comparison to the open sea. Because of the long acoustic wave lengths in water many standing waves may form at frequencies called resonant frequencies. These resonant frequencies have a wavelengths equal to multiples of the eigenfrequency of the tank (Akamatsu et al., 2002). Because particle velocity is directly proportional to the pressure gradient, extremely high and near zero values may exist from point to point in a tank. This can result in random-like figures and directions of particle velocity and steep gradients in the pressure pattern all over the tank where velocity and pressure are no longer in phase and there is no more free field and plane wave. To avoid this, following guideline should be taken into account if possible:

- Use as large and as deep a tank as possible.
- Use a directional transducer or sound projector.
- The directional transducer should be located close to or against a wall of the tank and the

subject located directly in front of the transducer about 2–3m away (Au and Hastings, 2008), because at close range the SPLs will vary too much on too short a distance.

- The subject should be far away from the wall directly behind it to take advantage of spreading loss of the reflecting sound of the back wall (Au and Hastings, 2008).
- Reduce reflected components if possible, by using bafflers (screens at the surface and bottom to cancel bottom and surface reflection). This will only work however if the frequency is high enough.
- Use short pulse signals to avoid the formation of standing waves.

A (too) low frequency, which will result in standing waves occurs when the wave length equals the shortest dimension of the tank.

It is furthermore noteworthy when calculating resonance frequencies from the eigenfrequency that plastic and glass walls of aquaria are easily bent by sound waves. This means that they act as antinodes and not nodes (Akamatsu et al., 2002).

Small experimental tanks

In small experimental tanks (1-2m length/width), wavelengths often exceed the dimensions of the tank. Additionally, the constant sound wave results in a combined sound of the original at time *t* and multiple reflections of that sound at time *t+ax+by+cz* (*ax*, *by* and *cz* being multiples of reflections form the 3 pairs of surfaces)(Akamatsu et al., 2002). In such cases the previous pointers become useless. In these tanks the signal usually reaches the wall of the tank before its end. These reverberations are a major problem and the multiple reflections can cause standing waves where multiple resonances may occur. For a rectangular tank the resonance frequency is given by:

$$f_{lmn}=\frac{c}{2}\sqrt{\left(\frac{l}{L}\right)^2+\left(\frac{m}{W}\right)^2+\left(\frac{n}{D}\right)^2}$$

where: L = length; W = width; D = water height

l, *m* and *n* are integers that specify the mode of interest. The lowest resonance frequency occurs when *l=m=n=1*

With more distance from the sound source, the initially produced sounds SPL decreases quickly (more rapidly than for spherical spreading) and the SPL of the resonance frequency becomes dominant (as it does not vary in strength, because it is a standing wave).

4.2.12.4 Comparison

Small tanks are limited in their use, as low frequencies have difficulty to perform in particle motion, while standing waves occur at larger frequencies. Careful choosing of the tank dimensions is therefore required.

4.2.13 Reproducing sound

Sound can be reproduced underwater by piezoelectric transducers (see 4.2.11 Measuring sound). Another group is the moving-coil transducers, which are basic air speakers adapted for underwater use. Alternatively, exciters can be used. These are fixed to a 'flexible' side (glass or plastic) of the tank and make the entire side vibrating and producing the noise.

4.3 The marine environment

The marine environment comprises all aspects, comprising physical aspects, viz. wind, rain, water, as well as biological aspects, viz. the animals living in, on and underneath it. They are the source and the purpose for this study.

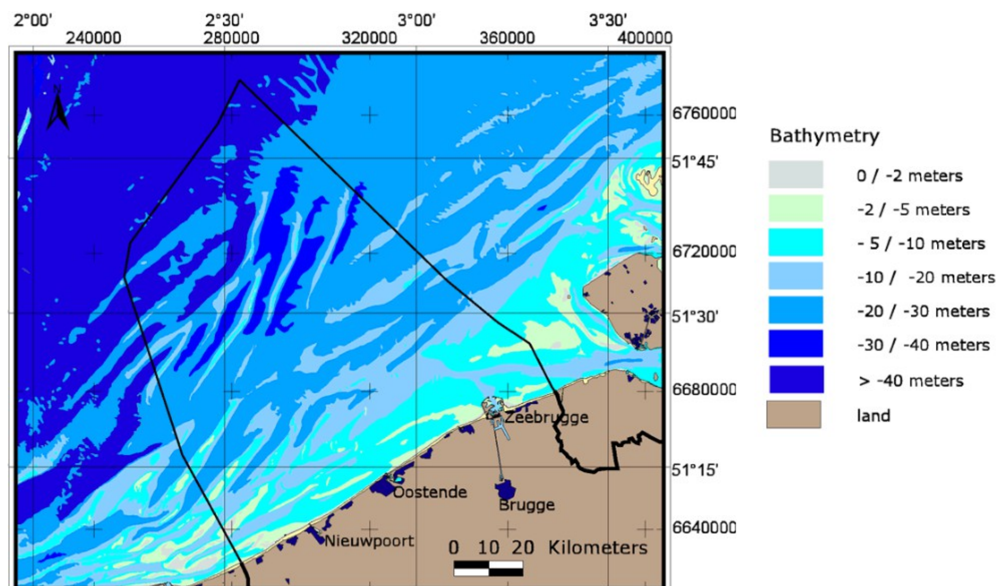


Fig. 4.17: Depth chart of the Belgian Exclusive Economic Zone (MUMM, 2016)

4.3.1 The Sea

The Belgian part of the North Sea is a shallow sea with an average depth of around 20m and a tide differences on average between 2,8m and 4,9m. While covering 3454km², the Belgian part consists of barely a half percent of the total area of the North Sea. The sandy bottom forms a large sand bank system parallel to the coast (Fig. 4.17). During very low tides the tops may submerge. The current runs parallel to the coast, turning with the tide between SW and NE. Water temperatures range

smoothly between 4-9°C by the end of February and 16-19°C mid-August. Salinity is relatively stable around 35‰ with local decreases near river mouths. Winds are dominantly SW, while other wind directions equal in frequency on other occasions. The wind speed averages around 8m/s. (MUMM, 2016)

The dominant NE current allows for mixing of North Atlantic and North Sea water, providing oxygen and nutrient rich water. These conditions allow for a thriving marine fauna. Besides phyto- and zooplankton, the North Sea supports amongst others a variety of invertebrates (e.g. crustaceans, bivalves) and vertebrates (e.g. fish, marine mammals and birds). These species (besides their right to exist) are important to the economy, culture and folklore and tourism.

Economically sole, plaice, tarbot, lemon sole, anglerfish, cod, whiting, brown shrimp, brill, squid, dab and ray and skate are, in order of significance, important to Belgian fisheries. Additionally, though not always from the Belgian part of the North sea, these fishes as well as some bivalves are a part of the Belgian eating culture and Burgundian life style viz. mussels, oysters and flatfish. Horseback brown shrimp fishers at Ostend are part of the Belgian cultural heritage and its folklore. Harbour porpoise and especially seals are sights for tourists. (Economie, 2015)

4.3.2 Brown shrimp, an ideal test subject

C. crangon is an ideal test species for several reasons (Menezes et al., 2006). Its biology is reasonably well known. There is decent knowledge about, amongst others at the ILVO. It is a species that is small and easily manipulated, while its size makes it ideal for quantitative research, yet visible enough for qualitative aspects.

4.3.2.1 The brown shrimp (Crangon crangon)

As based on (Smaldon, 1993)

Parent: Linnaeus, 1758
Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Infraorder: Caridea
Family: Crangonidae
Genus: Crangon

Fig. 4.18: Crangon crangon (Hillewaert, 2005)

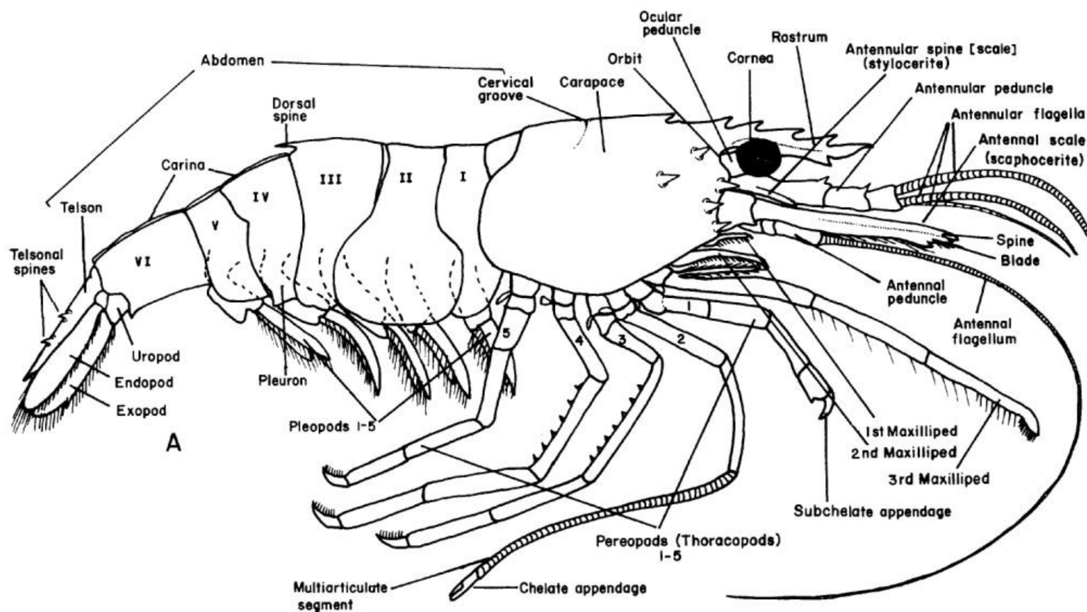


Fig. 4.19: A caridean with its parts (McLaughlin, 1980)

Arthropoda

The most obvious defining adaptive character in arthropods is that they are clad in an exoskeleton, the cuticle, which offers both protection and mechanical support (Garm, 2004).

Decapoda

The Crustacea is home to the order of the Decapoda (10 legged ones), which is a large group, comprising shrimps, prawns, lobsters, crayfish and crabs. Decapods are mainly marine, though some live in fresh water and even fewer have amphibious habits, spending most of their life on land only to breed in the water.

Decapods have several peculiarities, which rather define this taxon. They have maxillipeds, which are three pairs of thoracic limbs that accessorise the mouthpart. They also have five pairs of **pereiopods**, which are leg-like limbs, which lie posterior to the latter and give them their name.

Decapods are generally divided into two major groups: the Natantia and the Reptantia. The Natantia comprise all prawns and shrimps (though these are not distinctly discernable). Although they are all swimmers, some are entirely pelagic, while others have adopted a more benthic way of life. The bodies of Natantia are normally somewhat laterally flattened. The exoskeleton is quite weak, which allows for a fair amount of flexibility. Of the usually long and slender "legs", pereiopods, the first two or three out of five pairs carry pincers or **chelae**, with one pair being often more prominent. At the front often a spine, the **rostrum**, is detected protruding between the eyes as well as a flattened scale, the **scaphocerite**, by the second **antenna**, which acts

as a stabilizer during swimming. All six abdomen segments, **pleonites**, are usually fairly equal in length and expand downwards into rounded armoring, the **pleura**. Some abdomen segments have broad leaf-like appendages, the **pleopods**, that propel the animal during swimming. The Reptantia include crayfish, lobsters, hermit crabs and true crabs, besides some smaller more obscure groups, like the mud- or ghost-shrimps. Reptants are crawlers and therefor their body design is generally not build for swimming, though some have adapted slightly in order to swim. Reptant species generally show a distinctively different build: a small first abdomen segment, smaller pleopods (no swimming), a small or no rostrum, a smaller or no scaphocerite and sturdy pereiopods for crawling, of which the first two or three pairs carry pincers or chelae, with the foremost always being larger and heavier. Though some reptant species may show fairly broad pleopods, they use them for a different reason: to ventilate their burrow.

Caridea

The Natantia hold three groups: Caridea, Stenopodia and Penaeidea. They can be distinguished based on gill structure, chelae pairs, abdominal features and reproduction habits. The Caridea have overlapping lateral edges, the **pleura**, both forward and afterward on the second segment and always carry only two pair of chelae.

A typical specimen of the Caridea can be divided into three parts: head, **thorax** and **abdomen** or pleon. Contrary though, the head and first three segments of the thorax have fused and head and thorax are covered under one shield: the **carapace**. The carapace covers the back all the way down to the top of the pereiopods and subsequent **gills**, the antennae, the antennules, the maxillae and the maxillipeds. The carapace and protruding rostrum may carry several spines, grooves and ridges (carinae). The **compound eyes**, existing out of a cornea and a two segment stalk, lay in the orbit. The five pairs of pereiopods are used for feeding and locomotion. They are typically shaped in seven segments: coxa, basis, ischium, merus, carpus, propus and dactil; although sometimes pereiopod 2 may have more segments. Some forms have exopods on the basis, which are remnants of their former biramous nature. Some carry epipods on the coxae and possibly maxillipeds. Natants may have any of three types of gills depending on where they protrude: pleurobranches are dorsal, arthrobranches on the joint between body and maxilliped and/or pereiopod and podobranches on the epipods. All types are covered by the carapace forming a branchial chamber. The pair of **antennules** exist of a three segment peduncle topped by usually two flagellae. The formost segment may have a **stylocerite** extending. The **antennae** consist of a peduncle, one flagellum and the **scaphocerite**, which may carry one or two spines. Both antennae and antennules have sensory functions. The mouth is comprised of a pair of “tooth” (**mandibles**, which may carry palp and may have a moular

and incisor function), two pairs of **maxillae** and three pairs of **maxillipeds**. The second pair of maxilla bears a **scaphognathite**, which creates a water current over the gills. The six segmented pleon is complemented at the end by the tail (**telson**), which, with the **uropods**, forms a fan-like structure for quick escapes. The first five pleonites each carry a pair of pleopods, existing of an endopod and an exopod. In some species the pleopods of the three middle pleonites carry a small appendage (**appendix interna**), which connect the pleopods to improve synchronization for swimming. On males the formost appendix interna is different and is called the appendix masculina; it is used during sex. The testes lie in the thorax and pleon, with ducts leading to openings on the coxa of the fifth pereiopods. In females the first pleopods are normally broader and more leaf-like. The ovary lies in the thorax, sometimes extending into the pleon, leading by ducts to openings on the coxa of the third pereiopods.

During growth, shrimps and prawns need to **moult**, because of their exoskeleton. They moult often in their first year, because of the rapid growth into their mature form. In later years they generally moult less frequently. The frequency depends a lot on age, reproductive position and temperature, with some don't moult at all during winter colds.

Before moulting calcium is removed from the old exoskeleton, resulting in white spots appearing on the body, which is a useful indicator. When the moulting process starts, the animal extends its pleopods and pereiopods downwards and the exoskeleton splits backwards between carapace and pleon. The cephalothorax is slowly withdrawn first, followed by the pleon and other limbs by flicking the pleon vigorously. This takes only 9-25 seconds. Now the animal is immobile on its side, because the body is completely soft. It is now at its weakest and susceptible to predation. Within minutes the pleopods harden, allowing it to swim. Within the hour the pereiopods are hardened, allowing running. The remaining hardening of the moult takes about a further two days.

Immediately before **copulation** the female moults into a “breeding dress” in which ovigerous setae appear on the pleopods among other modifications for egg carriage. During copulation, which differs between species and in natants lasts only a few seconds, the male ejects spermatophores under the plion of the female. Depending on the species, the female spawns thousands of eggs anywhere from 2 h to 48 h after copulation. At this point the eggs run past the spermatophores, becoming fertilised. Unfertilised eggs fall off within several days. Zoea larvae, hatching from the eggs, moult several times into rather different forms, eating plankton. The duration of the larval stage varies. Some are kept at the adult pleopods, while others are free-living and others hatch into advanced forms. During the carriage the female never moults, but after “releasing” the offspring she loses here “breeding dress”.

Life expectancies vary from two to over eight years, sexually maturing after about the end of their first year.

Some 2000 species of natant Decapoda inhabit the European seas, of which about 1650 are Caridea, making them by far the most dominant group. They vary widely in habitat, from intertidal areas, through the shallow sublittoral down to the abyssal depths. Some live in estuaries or saltmarshes, while others inhabit freshwater areas.

Crangonidae

Crangonidae are limited to soft bottom littoral and sublittoral areas of cold and temperate regions of the Northern Hemisphere, where they have developed a large temperature tolerance in lower and upper limits. Some species have developed such low temperature tolerances that they are considered Arctic boreal species. In the Northeast Atlantic, i.e., in the European waters, only two species occur: *C. crangon* and *C. allmanni*. *C. allmanni* inhabits deeper areas (20-360 m) offshore within the brown shrimp's distribution range, yet limited to the south to the Bay of Biscay. In the Northwest Atlantic, only one species is known: *C. septemspinosa*. The Northeast Pacific inhabits 8 taxa and the Northeast Asian Pacific hosts 7 species. In total there ought to be 20 different species, but due to hybridization in some species and lack of genetic research no certainty exists in the matter (Campos et al., 2012).

The genus Crangon itself probably originated in the North Pacific. The divergence between Pacific and the Atlantic species was at least 6,6 million years ago, suggesting a trans-arctic migration during the opening of the Bering Strait, after which time they have become isolated (Campos et al., 2012).

A peculiar feature common to most species is their role as both predator and prey of flatfish, viz. large *Crangon* sp. preying on juvenile flatfish and adult flatfish preying on *Crangon*. All *Crangon* sp. are considered opportunistic and calculated hunters, investing the least energy for the most result. They prey on what is readily available and abundant, only being selective in size when there is enough choice (Oh et al., 2001). Morphologically Crangonidae are characterized by the sub-chelated first pereiopods (ambulatory thoracopods) and a short rostrum (Campos et al., 2012).

Most taxa are dioecious; some are protandric hermaphrodites with primary females; *C. crangon* appears to be facultative. Another habit seems to be the spawning in deeper and saline regions, possibly to provide more stable temperature to enhance development (eggs and larvae can have a narrower tolerance for temperature and salinity and less stress means better/faster development) and to avoid predation of immature juveniles, which linger in shallower regions. This migration habit

however has been suggested to be a relic of a past relevant movement too, which nowadays seems to make no sense in the current environmental conditions (Campos et al., 2012).

***Crangon crangon*, Crangon vulgaris**

The brown shrimp (grijze garnaal, *C. crangon*) is sexually dimorphic in size, with females reaching a length of up to 90mm and males only 75mm. They are transparent with pigment cells which can change its colour from brownish to grey and a sand-like speckled camouflage pattern. They carry an unarmed small triangular rostrum of about half the length of the eyes. They have one spine on top and two pairs on the sides: one at the lower front (**pterygostomian spine**) and one on its “cheeks” (**hepatic spine**). A pointed stylocerite is present, about half the length of the antennular peduncle. The third maxilliped is as long as the scaphocerite with protrusive apical spine and bares an arthrobranch and exopod. The mandible has only the molar process even though it is very sharply pointed. The first pereiopod is broad with strong chelae, while the second and third are comparatively underdeveloped, being less long and strong. The sixth pleonite and telson are smooth dorsally and grooveless, the latter baring two pairs of small lateral spines. All but the foremost pleopods are two-segmented and have no appendix interna.

Ovigerous females occur on different periods, depending on location. In some regions spawning occurs twice a year, though usually only once. In some regions ovigerous females can be found all year long. On average egg-bearing females can be found from December until August and population peaks are during January-February and July-August. *C. crangon* **copulates** while the female is still soft after moulding into her “breeding dress”. They carry up to 15000 eggs, which develop depending on temperature (e.g. 10 weeks at 6°C and 3-5 weeks at 15°C). The planctonic larval stage takes about five weeks and equally much stages. These planctonic larvae accordingly swim freely and can be found near the surface and in the middle area, before settling in the post larval stage on the bottom (Campos et al., 2012). Males live only 2 years on average, while the female usually lasts about another year.

C. crangon is known to be an active voracious **predator** and eats small amphipods, cumaceans, mysids, meiofaunal crustaceans, small bottom dwelling fish, copepods, polychaetes, molluscs, bivalve spat and siphons, dead animals, young ones of its own species and others of its own species while moulting (cannibalism takes up 20% of its diet, depending on the season (Oh et al., 2001; Campos et al., 2012)). In short, brown shrimp eat almost every bottom-dwelling species. They are also a relevant predator for young plaice, especially in groups of juveniles. Prey size preference increases with growth, starting off with the zoea larvae preying on meiofauna (zooplankton) and

finishing with the adults which take on even small fish. They are supposed to feed mainly after dusk and before dawn, giving them a diel activity pattern. The most important factor affecting the diet however is its spatial and temporal availability.

Sandberg et al. (1996) proves Campos et al. (2012) that they also alter eating habits according to environmental inputs and prey abundancy, playing a vital role in fitness and hence evolution of prey species.

On the other hand, *C. crangon* is itself a **prey** for many species. Though mankind may seem its most important predator, fish still predate more on them, for example rockling and smelt for juveniles and flatfish (brown shrimp makes up respectively 10%, 20% and 30% of the diet of plaice, dab and turbot), rays and gadoids (inlcuding bib, cod and whiting) for adults (Campos et al., 2012). Whiting can occasionally severely decimate brown shrimp populations (Campos et al., 2012). As benthic predators spider crabs, hermit crabs and the brittle-star Ophiura ciliaris are named. From the air they have to fear greenshank, redshank, avocet, spoonbill, divers, grebes and certain diving ducks, though this is mostly in estuaries and shallow water areas.

These animals live on soft bottom areas from mud and sandy shores to gravel (Campos et al., 2012), where they cover themselves with sand to avoid predators. They **inhabit** areas of less than 2m depth up to depths of 150m, though some suggest even down to 300m. *C. crangon* migrates between shallow and deeper regions, hatching in the shallower and migrating in winter to the deeper areas (Campos et al., 2012).

Four well distinguished phylogeographic groups with large concentrations exist both by genetic and morphometric standards in the Northeastern Atlantic, the Mediterranean, the Adriatic, and the Black Seas (Campos et al., 2012). Hence they are found in all European seas: on the Atlantic coast from the White Sea and the The Islandic shores in the North through the North Sea, into the Baltic sea, all the way South to the South of the Moroccan shores, in the Mediterranean and in the Black Sea. In these epibenthic communities they are dominant species, making up between 90 and 60% of biomass and numbers, especially in bare sand areas with no vegetation. In the Mediterranean their numbers are in decline (Campos et al., 2012).

C. crangon is listed as being closely related to the Northeast Atlantic taxon *C. septemspinosa* (Say, 1818), both sharing an estuarine occurrence, to the Northeast Pacific *C. alaskensis* (Lockington 1877), and to *C. affinis* (De Haan, 1849) from Northeast Asia. However, this is not certain; they might be subspecies of a single species or even full synonyms of each other. Also, the south European forms inhabiting the Mediterranean and the Black Sea have sometimes been considered to

be a subspecies (Campos et al., 2012).

Fig. 4.20: Mytilus edulis
(De Wulf, 2005)

4.3.2.2 The common mussel (Mytilus edulis)

Parent: Linnaeus, 1758
Kingdom: Animalia
Phylum: Mollusca
Class: Bivalvia
Subphylum: Pteriomorphia
Order: Mytilida
Superfamily: Mytiloidea
Family: Mytilidae
Genus: Mytilus

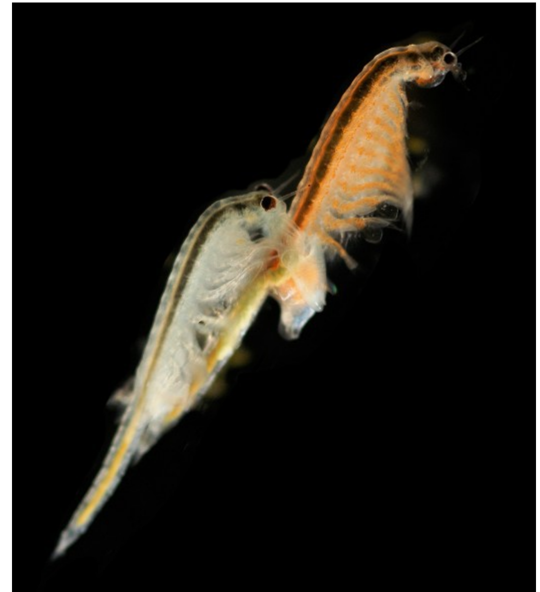


Fig. 4.21: Artemia salina
(Hillewaert, 2010)

4.3.2.3 Brine Shrimp (Artemia salina)

Parent: Linnaeus, 1758
Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Branchiopoda
Subclass: Sarsostraca
Order: Anostraca
Family: Artemiidae
Genus: Artemia

4.3.2.4 Amphipoda (Bathyporeia pilosa)

Parent: Lindström, 1855
Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Superorder: Peracarida
Order: Amphipoda
Suborder: Senticaudata
Infraorder: Gammarida
Parvorder: Gammaridira
Superfamiliy: Gammaroidea
Family: Bathyporeiidae
Genus: Bathyporeia

Fig. 4.22: Bathyporeia pilosa
(Hillwaert, 2002)

4.3.2.5 Baltic macoma (Macoma balthica)

Fig. 4.23: Macoma Balthica (Decleer, 2010)

Parent:	Linnaeus, 1758
Kingdom:	Animalia
Phylum:	Mollusca
Class:	Bivalvia
Subphylum:	Heterodonta
Superorder:	Imparidentia
Order:	Cardiida
Family:	Tellinidae
Genus:	Macoma

4.4 Anatomy of the hearing organs

4.4.1 Introduction

Particle motion is the most likely stimulus for marine animals without compressible air cavities (Samson et al., 2014). At least one species of crustacea is sensitive to particle motion (Lovell et al., 2006). Cephalopods are found to have accelerometer-like auditory systems, like many fish (Samson et al., 2014). Many crustaceans have shown to posses auditory sytems too.

4.4.2 Hearing organs

In this part the focus will be on the hearing abilities of the prawn *C. crangon*, as it is the subject of this study, while the hearing abilities of all subjects of the initial experimental idea will not be discussed.

The hearing abilities of Crustacea have been proven by several studies (Campos et al., 2012; Lovell et al., 2005). For hearing in the strictest sense to be attributed to an organism, the physiological response to sound should be initiated by a specialised receptor mechanism (organ)(Lovell et al., 2006). Since arthropods are clad in a chitin exoskeleton, the cuticle, these receptors need to be specially modified to have some meens of passage for the signal through it. To this purpose several organs are both internally and externally present, though these are all mechanoreceptors (to some extent, if not entirely).

A dorsal organ is present in most Crustacea (Laverack and Crombie, 1988). Monteclaro et al. (2010) suggests a lateral line exists in crustaceans, such as can be found in cephalopods (Samson et al., 2014) and certain sonic and general fish (Akamatsu et al., 2002). Heinsich and Wiese (1987) and Garm (2004) found mechanoreceptor clusters all over the carapace, pleon and telson as well as the entire cuticle in several crustaceans. These and other numerous setae and the aesthetascs form the external mechanoreceptors and chemoreceptors. Internally, a pair of stathocysts exist in all species

of Malacostraca, which is quite similar to those in generalist fish and uses mechanosensory hairs. The mandibles show two peculiar mechanosensory structures too and though they are included it is not proven that they are used for hearing in the strictest sence.

Lovell (2006) proved hearing in *P. serratus*, a European crustacean, between 30 Hz and around 3000Hz with the statocyst similar to general fish. Campos (2012) proved furthermore for *C. crangon* hearing capabilities with tail setae between 10Hz and 300Hz. This suggests that *C. crangon* can hear sounds in the frequency range between 10Hz and 3000Hz. Directional hearing in *C. crangon* was proven by Campos et al. (2012). Samson et al. (2014) suggests this to be true in larval invertebrates too and suggests the possibility for cephalopods.

These common mechanoreceptors have proven to be using scolopidiae (a basic mechanosensory organ that detects pressure and vibrations)(Kouyama et al., 1981; Lovell et al., 2005; Monteclaro et al., 2010), which would prove convergence in evolution with vertebrates. A further common bandwidth in hearing frequency supports this assumption.

4.4.2.1 Statocyst

The basic statocyst is a balancing organ, much like the mammalian inner ear. It is formed in its essence by an innervated sac filled with liquid (with lower density than the rest of the body) or air, which by its buoyancy gives the direction of "up", thus telling the fish in which position it is upright. The ready innervation and flexibility of the membrane allows for observation of outside pressure such as depth and arguably sound pressure levels. Some species (e.g. cephalopods) developed it into the format as described under "*C. crangon*" of this section (albeit with a single solid statolith). It functions similarly to a gyroscope: the inertia of the solid object (statolith) in the centre of the sac makes it lag behind the surrounding substance, giving directionality to the motion, which in this case is a pressure wave (sound) and hence it reads particle motion.

The statocyst has been found in the Anaspidacea, Amphipoda, Isopoda, Mysidacea and Decapoda (Dendrobranchiata, some Caridea, Astacidea, Palinura and Brachyura), although some species of the Caridea are devoid of the organ. Apart from these crustacean taxa, the only taxon in which the statocyst has been found is Malacostraca (Sekiguchi and Terazawa, 1997). Similar to generalist fish, in cephalopods it is considered the primary sound detection organ (Samson et al., 2014).

In crustaceans, the statocyst is located either at the anterior end of the animal in the basal segment of each antennule, or posteriorly within the uropods, abdomen or telson. It has been well-established that the crustacean statocyst functions as an equilibrium organ by initiating corrective movements to maintain the animal's position in the water column (Lovell et al., 2006), analogous to

the mammalian ear. From an anatomical perspective, cephalopod statocysts could support directional hearing (Samson et al., 2014), which is equally true for crustaceans.

C. crangon

The two statocyst organs found in *P. serratus* and *C. crangon* lie opposit to one another with medial symmetry, in the basal peduncle segment of the oppositing antennules (fig. 4.24). The statocyst is innervated by the otic ganglion, which emanates from a bed of peripheral nerve fibres lying under the mound directly beneath the receptor array (Lovell et al., 2006).

The statocyst opens onto the dorsal surface of the basal segment; the cavity is oval and runs in a booth shape downwards, with its long axis parallel to that of the antennule, and it narrows towards the front. The chitin exoskeleton of the puerulus is very thin except around the opening of the statocyst and the cuticle overhangs the anterior part of the opening. Two rows of plumose setae flank the opening, forming a mesh, net-like structure (Sekiguchi and Terazawa, 1997; Shen, 1934), thus effectively closing the statosyst to the external environment (Lovell et al., 2005).

The epithelium of the statocyst contains a number of mechanosensory hairs or setae embedded in a highly innervated sensory epithelium or cushion. The hairs support a dense statolith structure in the lumen set in a gelatinous medium and functions similarly to the otolith structures found in the vertebrate vestibule (Lovell et al., 2005). It is kept in the lumen of the sac by a net of fine hairs that grow on these setae.

The statolith is composed of large grains of sand deposited mostly in the posterior portion of the sac. Unlike statoliths observed in other decapods such as the Astacidea and Brachyura, they are not cemented together to form a mass. As the prawn grows and concequently moults, so does the statocyst. Each time the prawn moults, the statocyst is cast off with the old moult. A new larger statocyst with larger and more setae then surfaces with the new moult, which hence requires a larger statolith (Lovell et al., 2005). As the sac has a large dorsal opening the sand grains (new statolith) are readily taken in through it (Shen, 1934). Depriving *C. Crangon* and *P. serratus* (and possibly other crustaceans) from sand during moulting, deprives them as such from a statolith and renders the statocyst useless for hearing purposes.

The setae are set in a crescent around the back of the epithelium, reaching from one side to the other, along about 75% of the circumference forming the sensory ridge. These club like setae are set in a single row, inclined downward into the lumen (fig 4.23).(Shen, 1934)

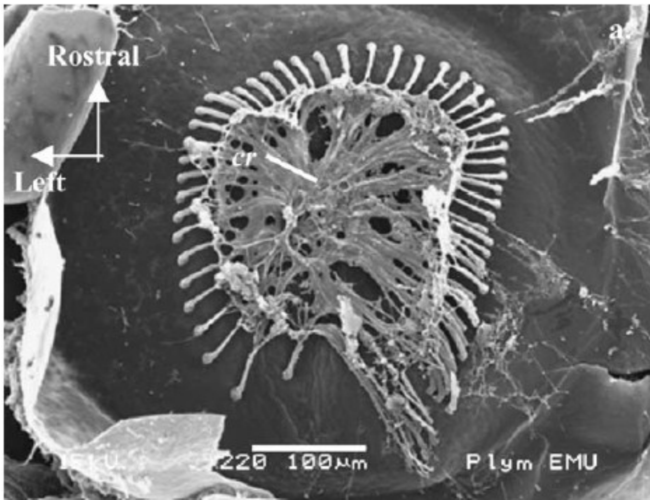


Fig. 4.24: *Statocyst without statolith (Lovell et al., 2005)*

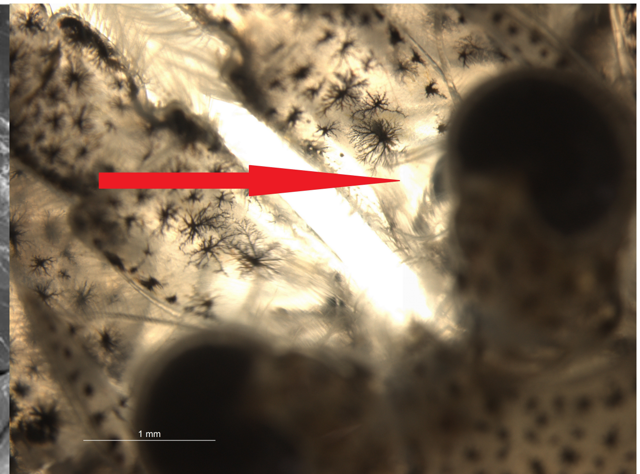


Fig. 4.25: The statocyst is just visible under the eye of C. crangon (Own collection)

4.4.2.2 The Dorsal Organ

The function of the organ is yet unestablished, but it is suggested to have a excretory or absorptive, and/or mechanosensory function (Laverack and Crombie, 1988).

The dorsal organ has been described for trilobites and euphausids (Laverack and Crombie, 1988). Furthermore, its presence has only been described for reptant crustaceans and three species of natantia: C. *crangon, Pandalus montagu*i and *Thoralus cranchii* (Laverack and Crombie, 1988). Reptant decapoda show these organs only in larval and sometimes in juvenile stages, while natant species show them in all stages.

The dorsal organ in *C. crangon* is located near the base of the anteriorly-directed spine immediately posterior to the rostrum (Fig. 4.27). Occasional abnormalities in position occur and the organ may appear flat or anterior to the spine. In one exceptional case two large central pores have been seen. There is no significant or consistent difference, except in overall size, between small immature and larger adults (Laverack and Crombie, 1988). In *Pandalus montagui*, the organ is positioned to the rear of the posterior-most tooth on the dorsal carapace (Laverack and Crombie, 1988).

In *C. crangon* the dorsal organ is an elliptically-shaped structure, which is essentially an island of thinner epicuticle, consisting of outer folds surrounding a slightly depressed area. The flexible area is lozenge shaped, about 70 µm long, with the papillae (pore-like structures) disposed in a row down the axis, two on either side of the central complex (Fig. 4.29)(Laverack et al., 1996). The central complex has an invagination of epicuticle that forms a thin-walled, blind-ending tube running down through the integument. A single large cell, which has its microvilli and membranes penetrated by folds of epicuticle, but which lacks any enervation, surrounds the tube (Laverack et al., 1996).

The four papillae each lay in a pit and are each innervated by four dendrites. The organ has thus sixteen neurons in total. This gives them a typical build for a crustacean mechanisensor.

The overall flexible structure of the very thin outer cuticle suggests that the mechanoreceptors are monitoring the position or movements of the papillae or the whole surface of the sensory dorsal organ (Laverack et al., 1996). In all pressure (depth) meters, compression of a gas chamber is measured. This could be similar here, where the central gland around the dendrites may contain spongy material or a non-gaseous material with different compressibility from water (Laverack and Crombie, 1988). Additionally, chemosensory functions of the dorsal organ are excluded, because of a lack of innervation of the central complex (Laverack and Crombie, 1988).

In all other caring species, the domes (papillae) are positioned in a square arrangement around the central pore (Laverack and Crombie, 1988), making the overall assembly more round shaped.

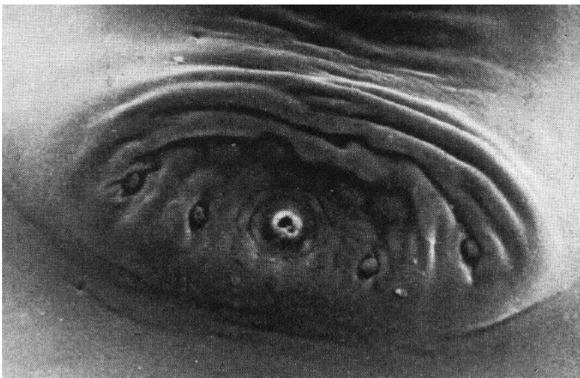


Fig. 4.26: Dorsal organ under EM (Laverack and Crombie, 1988)

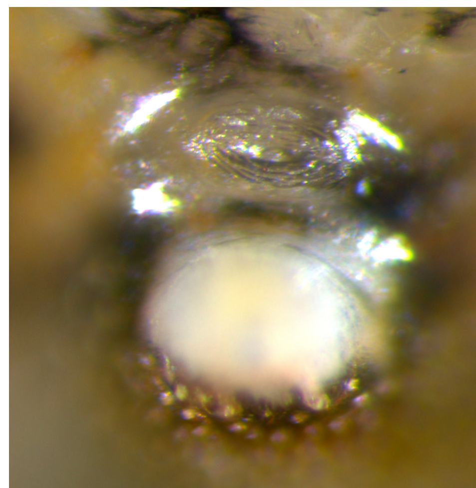


Fig. 4.27: Dorsal organ above the dorsal spine (white surface)
(Own collection)

4.4.2.3 Aesthetascs and setae

The cuticle and almost all body parts of Crustacea bear a large number of specialized cuticular structures, differing in shapes, sizes, basis, etc. They have various functions or none at all, though those we are interested in are mechanoreceptors (and bimodal ones which may also be chemoreceptors). This large group consists of elongate, hair-like projections that normally have a distinct articulation at the base, making them flexible. It is believed that these structures are homologous within Crustacea and probably also with other arthropods. These are called setae, sensilla, bristles or even 'hairs' (Garm, 2004).

Garm (2004), among others, defines setae based on homology and evolution. More specifically, they look at the basal articulation with the general cuticle, the presence of annulus and of articulated outgrowths on the setal shaft (setules). This is not an ideal basis as most types of setae will have

many if not all of these characteristics and the outgrowths occur on general cuticle too. In contrast, the functionality of the setae may be a much more interesting way of categorizing them, as functionality leads to evolution and homology, but adequate information on their functionality is not present for all setae (Garm, 2004). A combination of both being off course an even better classification system.

Large concentrations of highly diverse setae can be found around and on the mouthparts (Garm, 2004; Geiselbrecht and Melzer, 2014), the antennules and antennae (Monteclaro et al., 2010; Heinisch and Wiese, 1987) and Heinisch and Wiese (1987) found them on carapace, walking legs, subchelate legs, abdominal tergites (the belly), telson, and inner and outer uropods too.

It needs mentioned that these definitions and descriptions are generalized brief descriptions and actual ad hoc setae may differ somewhat as these definitions are based on studies of a select number of species.

Though we called all of them setae so far, there are actually three or four different types of outgrowths according to Garm (2004), from which I will mention the fourth only later:

1) *Setae*

   They can be found on various places, though primarily on limbs and body edges. They are large (50µm-2mm) and always circular in the basal region. They may have outgrowths on themselves, an articulation with the general cuticle in a socket area, an annulus and/or a pore. They always have a continuous lumen containing semi-circular sheath cells basally along with sensory cilia (they are innervated).

2) *Denticles*

   They can be found on some of the setae, but also on some other parts, like the general cuticle. They are the smallest of all (1-30µm) and are smooth, flat and pointed. They have no articulation, no outgrowths, no pore and no lumen. They are by all means solid cuticle. They occur on the setae in two parallel rows, always orientated along the setal shaft and pointing distally. Denticles are always found distal to the annulus.

3) *Setules*

   They can be found on many of the setae and some other parts. They are slender, long (2-150µm), tapered and usually round at the basis and furthermore flat. They are serrated along the distal edge and have an articulation in a socket. They have no pore or associated cells. They may have a distal lumen, though never to the full extend. They are made of cuticle. On setae they always stand perpendicular on the shaft at the basis and tend to bend distal.

Long setules can be found along the full extend while small ones only distal from the annulus. They can form rows, but usually are randomly distributed along the shaft.

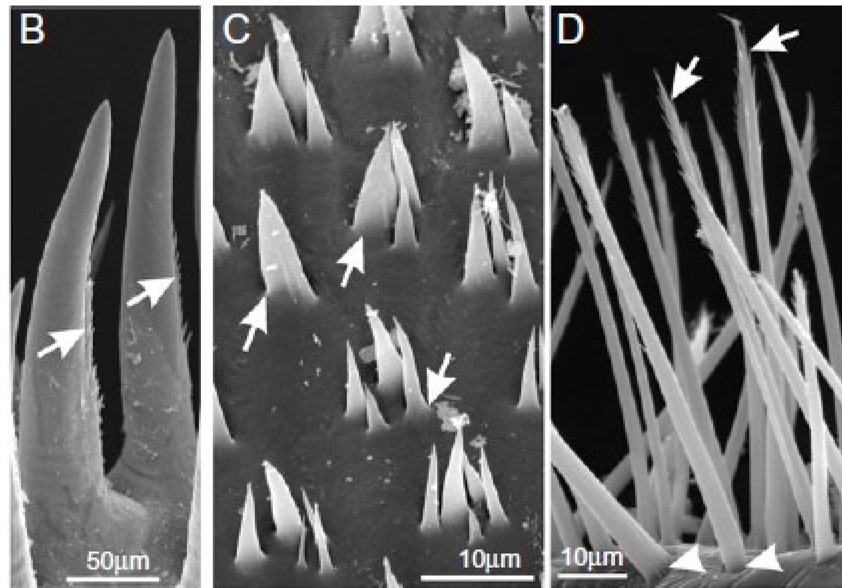


Fig. 4.28: B: Setae. C: Denticles. D: Setules (Garm, 2004)

Though the latter two are usually very distinct, sometimes setules gradually grow into denticles along the same seta (from base to tip). From an evolutionary point of view the opposite may however be equally true, setules being modified versions of denticles or vice versa. Furthermore outgrowths with a non-circular base are never setae (and usually setules)(Garm, 2004).

Attachment to the cuticle may occur in three manners: infracuticular **articulation** (the outgrowth is attached through (a) cell(s), which lays entirely below the cuticle, with the body, making it seem as though the outgrowth sits in a pit), supracuticular articulation (the outgrowth is attached through (a) cell(s), which protrudes outside the cuticle, with the body) and without any transition (the outgrowth rises smoothly from the cuticle)(Garm, 2004).

All setae have an **annulus** on the proximal half. It is a remnant of the ontogeny in an invaginated state. During the intermoult the annulus may completely disappear or become difficult to distinguish by the process of thickening, stretch and wear.

The **pore**-bearing setae may have the pore in two different positions: terminal and subterminal pores. Subterminal pores are less common.

During development and after moulting the **sheath cells** form the cuticular parts of the setae. Another function of these important cells is to support and protect the **dendrites**, which are the core of innervation and sensory functions and often run through the continuous lumen all the way to the tip and their **celia** (Garm, 2004). Mechanoreceptors dual innervation in shrimp and crayfish is

similar to those in lateral line of teleost fish. This dual innervation with opposite polarity might be to remain functional while they are deflected (i.e. when swimming) (Heinisch and Wiese, 1987). The neurons of the setae are connected to a limited number of neurologic pathways. Like in computers and the human eye many receptors connect to a limited number of multimodal fibres, but result in a detailed picture (Heinisch and Wiese, 1987).

Generally, seven types of setae can be distinguished, though more are described at times and intermediate forms certainly exist too, because denticles and setules are not always easily distinguished. These however are the seven most commonly described:

Pappose setae

Pappose setae are usually very long and slender (LW ratio >15), never displaying a pore and infracuticularly articulated. They have flexible, long (50–150 μm), well defined, serrated setules scattered randomly along the entire length of the shaft, projecting 45-90° to the shaft from an infracuticular articulation, shortening distally. A wide membranous area around the socket makes them very flexible.

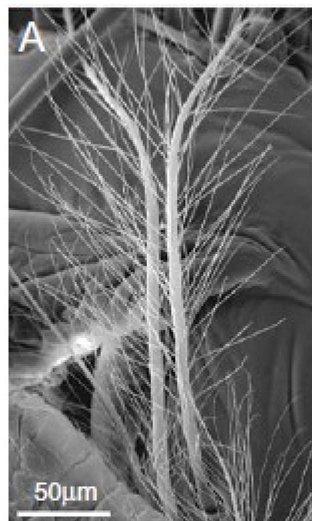


Fig. 4.29: Pappose setae under EM (Garm, 2004)

Plumose setae

Like pappose setae, plumose setae have long setules along the entire shaft, but they are arranged in two strict rows on opposite sides of the seta, giving them a feather-like appearance. However, the setules are never serrated, weakly articulated and often situated in a groove. Plumose setae have a supracuticular articulation, making them extremely flexible and never carry a pore. There are two “subtypes”: smooth ones and annulated ones (more than once) which are extra flexible. Monteclaro et al. (2010) specifies two subtypes, viz. procumbent and standing, though it is

doubtful their angle gives any need for differentiation.

Fig. 4.30: Plumose setae under EM (Garm, 2004)

Serrulate setae

Serrulate setae are slim and have small setules (< 15 µm long) distal to the annulus, randomly or in rows (usually three). The setules diminish gradually in size towards the tip and articulation is often difficult to detect. They lean towards the tip of the seta (<45°). Setule shapes differ, from usually serrated-leaf-like to the less common square-shape with toothed distal edge. Serrulate setae have an infracuticular articulation and come in two forms: very pointed, with the tip formed by setules and without pore (most common); and bent, with very small scale-like setules and a terminal pore.

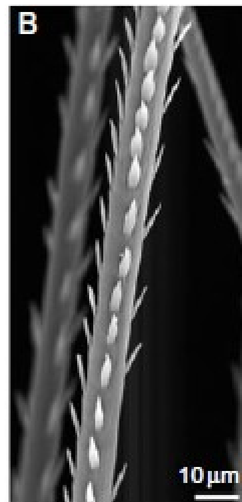


Fig. 4.31: Serrulate setae under EM (Garm, 2004)

Serrate setae

Serrate setae have two rows of densely packed denticles with 120°-180° between them distal to the annulus, diminishing in size towards the tip of the seta. On the opposite side between a few and several hundred toothed setules may lay against the shaft, being equal in size to the denticles. Serrate setae have infracuticular articulation and its tips come in two forms. Firstly, there are the

pointed, with the tip formed by the denticles and possibly with a subterminal pore (most common). Secondly, there are the bent serrate setae, where the denticles stop a little off the tip, with the outer side covered by very small scale-like setules and a terminal pore. The bent setae are normally longer than the pointed. Modifications in the denticles occur. There is an often narrow membranous area around the socket.

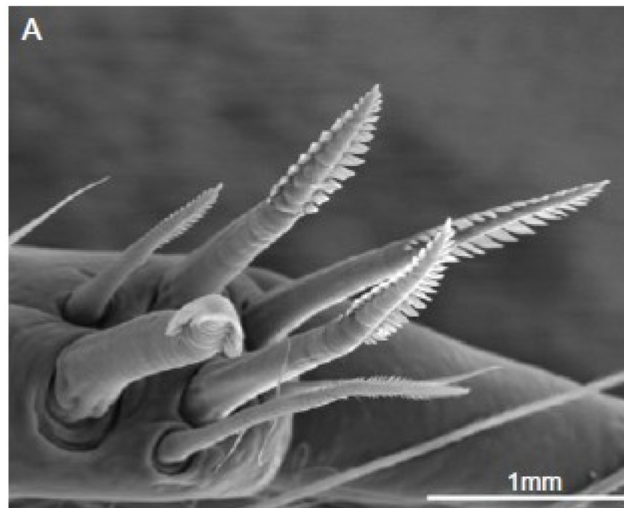


Fig. 4.32: Serrate setae under EM
(Garm, 2004)

Papposerrate setae

Papposerrate setae are long and slender. On the proximal 50-66% of the shaft they have long, randomly arranged setules like pappose setae, complemented distal by two rows of denticles like serrate setae. This is often a gradual transition rather than a sudden change. On the opposite side of the denticles there may be small setules, similar in form to the proximal ones. Papposerrate setae have an infracuticular articulation with a well-developed membranous area and are not very common. This type of setae is often named differently in different literature and could be considered an intermediate type.

Fig. 4.33: Papposerrate setae under EM
(Garm, 2004)

Simple setae

Simple setae are long, slender, pointy and completely naked (making them simple) except for possibly a terminal pore. They have an infracuticular articulation. They are the more abundant setal type. Simple setae are often specified by being large, medium or small.

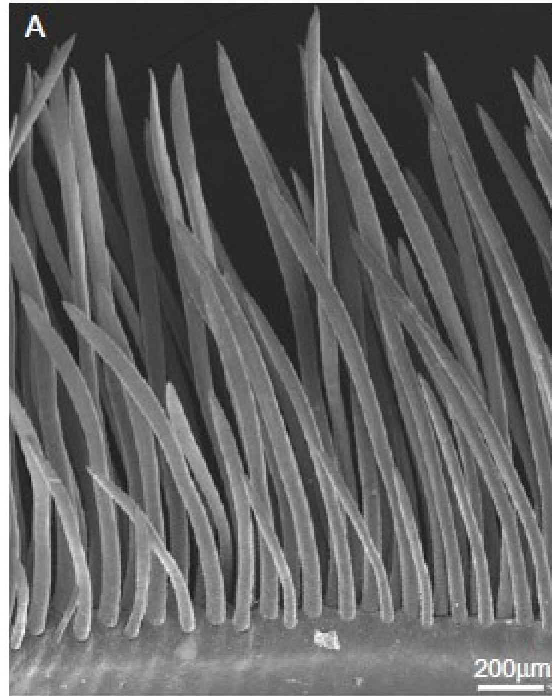


Fig. 4.34: Simple setae under EM (Garm, 2004)

Cuspidate setae

Cuspidate setae are very robust (LW ratio < 8), broad at the base, tapering gradually towards the somewhat rounded tip. There might be a subterminal pore and they are usually naked. Sometimes two rows of small denticle-like structures lay on the mid-section, though below the distal third. In this case the seta is usually slightly bent, with the outgrowths on the outside. Usually infracuticular articulation is observed at the basis with a very weak membranous area but sometimes there is no articulation at all. At any rate the seta is set fixed in position. This seta can be found all over the cuticle of *C. crangon*.

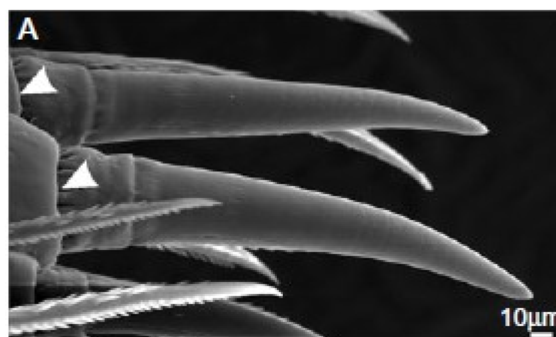


Fig. 4.35: Cupsedate setae under EM (Garm, 2004)

An eighth type of seta might be **ooseta**, brush seta, seta type A 20 or seta type U (all synonyms). They are naked proximal to the annulus and have long setules distally. Monteclaro et al. (2010) mentions guard setae and companion setae, and Heinisch and Wiese (1987) mention fringe hairs. More thorough discussion transcends the current study however.

Setae (with round basis) have been found on many fossils, making them an “old” development. Loss of articulation probably happened regularly during evolution to encompass mechanical functions requiring a very robust and steady seta (Garm, 2004).

The aformentioned fourth type of outgrowth is the aesthetasc. These seem to be restricted to the lateral flagellum of the antennule and are considered putative unimodal chemoreceptors, though they may also be bimodal, viz. being mechanoreceptors too. Since Garm (2004) does not consider functionality however they are considered simple setae in his work. They are however considered the primary olfaction organs. In all other works read concerning setae the presence of a pore is linked to chemoreceptory functions, making these statements somewhat contradictory. Monteclaro et al. (2010) supports this by stating that there are probably other chemoreceptors too.

Some of the various locations where setae are found on *C. crangon* will be treated in the following part.

Antennules

The biramous antennule, viz. a lateral and medial flagellum, are bimodal. Plumose, cuspidate, simple setae (of various forms) and aesthetascs arm both flagellum, making them both mechanosensory and chemosensory. Kouyama (1981) found particular innervation to the seta on the anterio-dorsal edge of the basal segment. Monteclaro et al. (2010) believes that the medial and lateral flagellum are used for different purposes. The lateral flagellum is adapted more for olfactory functions (smelling) in pursuit of prey, sex and social interaction, while the medial flagellum is adapted more for acoustic functions, acting as a hydrodynamic detector. When either one gets lost however, the other is believed to take over the function of the lost one.

In *P. clarkii*, responses to particle velocity between 5Hz and 200Hz, with a minimum at 50Hz were recorded by Monteclaro et al. (2010). These results are to be explained by the setae lengths and their inertia (to fast movements cannot be followed and to large movements go unnoticed). It is however to be expected that *C. crangon* uses its flagella in a similar manner, though auditory limits will vary somewhat.

Giving their position, they can be assumed to share a function with the frontal position of eyes in

other animals: locking in on prey and predators ahead. For some reason (possibly to improve chemoreception), some crustacea regulary flicker their antennules, but *C. crangon* rarely does so (Monteclaro et al., 2010).

The medial flagella carry densely packed longitudinal rows of plumose setae on both sides (Heinisch and Wiese, 1987) and spread over the cuticle, simple setae are visible. The lateral flagella seem naked, apart from tight groups of 5 aesthetascs in one base, located medial distal one group per segment, forming a row on the ventral side (Heinisch and Wiese, 1987).

The tailfan

The inner and outer uropods of the tailfan carry plumose seta. On the inner uropods the setae are more numerous and form several longitudinal rows, whereas on the outer uropods one single row runs parallel to the central ledge. Heinisch and Wiese (1987) found the uropods to be strongly directional in their response, showing response especially when movement (sound) is intercepted directly ahead, behind or on its sides. Heinisch and Wiese (1987) proved that *C. crangon* can hear sounds in its tailfan between 10-300Hz (no higher observations made) with peak results around 170Hz and particle acceleration around 83cm/s. *C. crangon* chooses to ignore sounds below 50Hz however.

The claws

The claws of *C. rangon* carry a few sets of simple setae probably for feeling what it holds, besides a membranous area.

Fig. 4.36: Chelae
(Own collection)

The pereiopods

At the propodite-carpopodite joint of the subchela leg, serrate setae were found (Heinisch and Wiese, 1987). Along the walking legs pappose setae are found randomly distributed (Heinisch and Wiese, 1987). If these serve any auditory function or even any function at all is unclear.

Scaphocerite and scaphognathite

Both structures show fringe hairs, which Heinisch and Wiese (1987) claims not to be innervated, making them setules with no sensory function.

The mouthparts

The mouthparts, viz. mandibles, maxillipeds, maxilla and moular process (the actual "tooth"), carry many different types of setae, some with chemosensory, some with mechanosensory and some with bimodal functions (Geiselbrecht and Melzer, 2014). It can be assumed though that these are all for tasting and feeling what goes into the mouth, rather than hearing in the strictest sence. One exception are the pappose setae, found only laterally, especially on the exopods of the maxillipeds, where they have the least contact with food, leading to the conclusion that these might actually have auditory purposes.

The general cuticle

The general cuticle is littered with small simple setae. Though their function is unclear, these might be similar to hair lines in general fish (Heinisch and Wiese, 1987) and cephalopods.

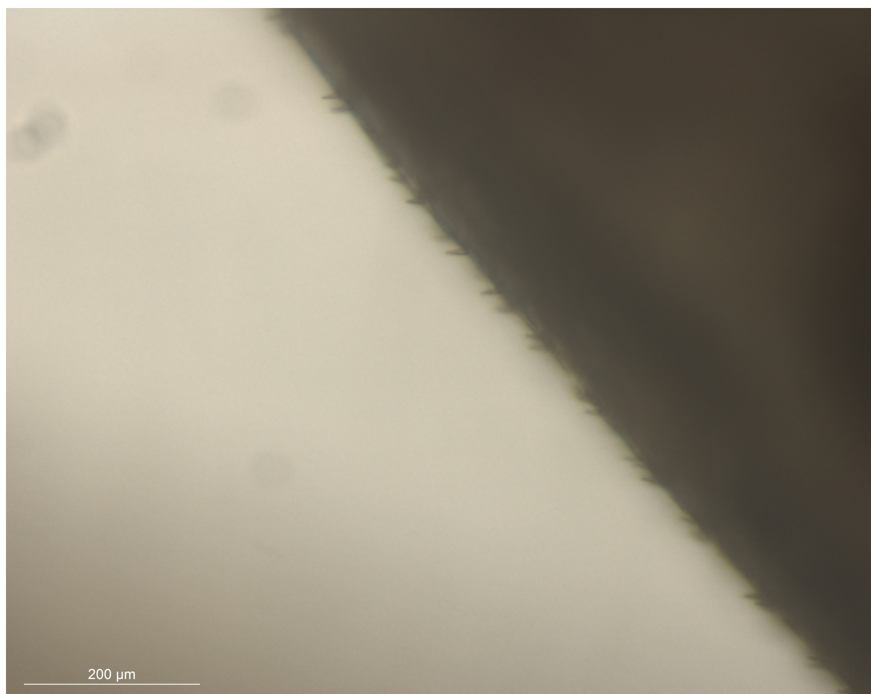


Fig. 4.37: Setae on the general cuticle.
(Own collection)

Lacinia mobilis

The lacinia mobilis is a most peculiar structure amongst the various appendages arming the gnathal edges of the moular process of mandibles in Peracarida (possibly in Decapoda too, certainly in their larval forms). It is an odd looking organ, with a distinct movable socket through clear articulation and innervation, giving it a mechanosensory function.

It is found in different forms on either side of the same animal. The **left** one is much bigger and has five dendrites in two clusters. The smaller **right** one has a proximal pore, being thus presumed chemosensory too and has two dendrites in one cluster reaching into the organ. Both are believed to have a different origin too. The latter from the setal row accompanying the moular process and the former from the incisor process directly. This makes it however somewhat unlikely for the left one to be present in *C. crangon* (as it has no incisor process).

Technically it could be seen as a seta, but because of its irregular shape and left-right difference I put it under a separate part. It is highly unlikely that this is an auditory organ, but for the completion it was included in this work.

4.5 Impact of the environment on an individual: from stress to death

The impact of a certain source and nature on an organism or group of organisms can present itsself in different ways. The most obvious and deleterious way is **mortality** (which is also very easy to observe). Another way, which may be(/is) less harmful but immediately more difficult to attribute and for the latter observe is **delayed mortality** and **injury**. **Stress** is another one and seemingly the least harmful. Stress can be measured in several ways. It can be measured directly by observing the **behaviour** visually (e.g. a human sweating as a result of heat). This does leave some room for interpretation and subjectivity. Stress can also be measured more objective with **biomarkers**. Such biomarkers can amongst others be: acetylcholinesterase (AchE; involved in neurologic synapses), lactate dehydrogenase (LDH; an enzyme for rapid, large energy demand), glutathione S-transferase (GST; detoxicating enzymes) and the aforementioned HSPs; the latter of which I will discuss in detail. All have been used often for numerous studies on a large variety of species in many different scenarios (Menezes et al., 2006). Another method is the observation of the organisms' environment. absorption or excess of nutrients, minerals etc. may increase or decrease in response to stress and in aquatic environments may thus be easily measured.

4.5.1 Mortality (acute ↔ delayed)

Mortality is the most severe consequence an agent (i.e. a chemical, physical or biological

disturbance) can have. It is very cruel and doing tests based on mortality requires a lot of living resources and results in limited information without through biochemical post-mortem analysis, which requires specialized knowledge and large financial backing.

Common practices to study the toxicollogy of a (usually chemical) agent are LD50 (median lethal dose) and LT50 (median lethal time) tests. In LD50 tests, several sets of species (in standard toxicology usually lab rats or mice) are given a dose of an agent. Lethality is recorded and from this data a statistical curve is calculated. From that curve the LD50 is the point at which 50% of the individuals in a species will die. NOAEL (No Observed Adverse Effect Level) is the point on the curve where no statistical relevant impact in frequency and severity of severe effects occures and LOAEL (Limited Observed Adverse Effect Level) is a little higher. Because of inter- and intraspecies differences extrapolation to other species should be avoided.

Alternatives to the LD50 and LT50 exist with similar results towards lethality, decreasing the lives spent.

Two types of mortality are furthermore defined. Acute mortality happens instantly or almost immediately as a direct cause of the agent. Delayed mortality has a link to the agent but usually occurs due to complications (which may be a direct consequence of the agent). Acute mortality does not necessitate post-mortem analysis, while delayed mortality should probably be followed by post-mortem analysis, certainly if one works with captured specimens. When delayed mortality occurs it is interesting to know if the species succumbed to the agent directly, as a result of energy depletion caused by the consequent stress or that perhaps a stress cascade caused another latent agent to result in its death (Stentiford et al., 2005).

For this study, mortality is only a factor if it occurs on a statistical significant level. In this case LT50 would be of interest. By recording the time at which species in the studie die and then calculating the medial point would result in a time at which 50% of the species would survive exposure to the noise or LT50 for a given sound sample.

4.5.2 Injury

Injury can take several forms and results.

First of all external and internal injuries are distinguished. External injuries are usually easy to spot, while internal injuries are much harder to identify. Additionally, in small species, viz. *C. crangon*, specialised equipment is required, such as a light microscope or an electron microscope of some sort. Internally these injuries might also be visible on a cellular level and not be clearly observable

(e.g. discolouration, tears).

Secondly, injuries might be able to mend and be fully restored or only partly or not at all. An abrasion may fully restore, while a chemical burn may cause a lasting wound. If a human loses a leg, the wound will heal, while in salamanders, entire limbs may regrow over time. Thirdly fitness needs to be put in perspective (see 4.6.6). An injurie may have some or no impact on fitness.

4.5.3 Behaviour

Behaviours in animals come by in many forms, e.g. herding, morning coffee, a certain preference in food. These behavioural features are very interesting for experiments and observations, as they are observable in living species and changes are not immediately life threatening. The use of behavioural paradigms to obtain audiological information from aquatic animals is a methodology favoured by a number of authors, though they often use conditional training or conditioning of the heart rate by electroshocks (Lovell et al., 2005). I am however not acquainted with training of shrimps in any way, which makes this approach rather undesireable. A drawback of this method is however the fact that animals can ignore certain frequencies and may be able to learn to ignore certain stimuli (Heinisch and Wiese, 1987) and regard them as irrelevant, allowing for a refocusing of sensory mechanisms (Samson et al., 2014).

In *C. crangon* a number of behavioural patterns are interesting and useful for observations in the initial experimental idea. I will briefly discuss a few of them.

- Flight or fight response:

  Samson et al. (2014) used sound pericles on cuttlefish and observed well defined response behaviours in them, to analyse their hearing capabilities. These sounds were to represent predator signals for the animals. Similarly, *C. crangon* has a few well defined escape behaviours. First, it buries itself to protect and hide itself. Secondly, it can swim steered, fast forward. Thirdly, it has a sudden backflip response, which shoots him upwards and backwards by flexing its body from an overstretched into a foetus-like position in a rather uncontrolled fashion. When approaching *C. crangon* from behind and above they will always swim very fast forward and never backflip, unless in acute distress (eg. when in the net) and when approaching them from any direction but behind, they will always swim very fast and use the backflip. Heinisch and Wiese (1987) supports my observations that different directional observations of *C. crangon* result in the different escape reactions. Samson et al.

(2014) found that escape patterns were more severe when sound levels were higher.

- Startle:

  The startle response has been described for several taxa, though mostly vertebrates and insects, and is provoked by an intense and unexpected stimulus (Samson et al., 2014). It is an automatic reaction, which can be difficult to observe, as it is an unconrolled motion that erects the animal in a state of vigilance.

- Flicking:

  Flicking is the back and forward motion of the antennae/antennules. Monteclaro et al. (2010) oberved flicking behaviour in *P. clarkii* by applying jet streams to the cephalothorax (water motion stimulus), tapping the walls of the tank (sound stimulus) and adding food extracts (chemical stimulus). *P. clarkii* is known to be a species that often flicks, *C. crangon* however is not (Monteclaro et al., 2010), therefore this might be a less interesting behaviour.

- Eyestalk-positioning:

  Animals look around when a disturbance is observed. *C. crangon* has facet eyes, which make it difficult to observe where they look. Also, *C. crangon*'s eyes are positioned on a stalk so it can protect its eyes somewhat with the carapace. When disturbed however, the shrimp will either protect its eyes, or protrude them in order to see more (as was observed in crayfish *Procambarus* by Heinisch and Wiese (1987)). As *C. crangon* is rather small, this might be a difficult thing to observe easily and/or require large resolution observation material.

- Feeding behaviour:

  C. crangon, being a voracious opportunistic predator is an active 'forager' (also hunter). Sandber et al. (1996) demonstrated however that when brought under severe stress its feeding pattern changed from actively hunting *Bathyporeia pilosa* to siphon cropping *Macoma balthica*.

- Diel living:

  C. crangon is a night active animal, hunting after dusk and before dawn (Sandberg et al., 1996), giving it a diel activity pattern. When stressed more energy is spent (see Stress), which may cause it to hunt during the day too, changing its diel behaviour and predation rate. Additionally, if a more dominant night hunter would arrive in its habitat, a similar change in behaviour may evolve.

- Respiratory and cardiac activity:

  Hunter and Uglow (1993) stated that heart rate and scaphognatite activity increases when handling and the environment changes. These are typical facets to observe in a lot of stress research, though the size of *C. crangon* makes this as difficult as doing the eyestalk-positioning observations.

- Danger evasion:

  In *C. crangon* the low tolerances to severe hypoxia/anoxia is compensated by its high mobility (and thus ability to avoid such circumstances) and ability to sense such upcoming situations (Hagerman and Vismann, 1995). When faced with stressful situations (physical danger e.g. high temperature) *C. crangon* will be able to migrate away from the source. This could be observed in a tank by there average, relative position to the sound source.

The fly *Calliphora* was observed to turn quickly in reponse to observed movement while moving fast (flying), but not while moving slowly (walking). This could indicate that locomotion movements might be correlated to more obvious orientation movements. Which could be the case with fast swimming, slow swimming and walking off *C. crangon* (Heinisch and Wiese, 1987).

As changes in the physical environment alter the behaviour of the physiologically affected organisms, the ecological balance between predator and prey, for example, might be disturbed. (Sandberg et al., 1996)

4.5.4 Stress

Stress in its strictest form, is a response of an organism to a deviation from homeostasis (the natural equilibrium state or optimum condition) (Kregel, 2002) and is thus linked to change (Seaward, 2013). Selye (1980) defined stress as the nonspecific response of the body to any demand made upon it. These changes or demands can be presented in many different forms. We can firstly distinguish anthropogenic causes (e.g. cadmium pollution by industrial effluents) and natural causes (e.g. summer heat). More specifically a stressor may be physical (e.g. cold and heat), chemical (e.g. formalin and ether), or psychologic in nature (Szabo et al., 2012). Tkáčová et al. (2012) distinguishes phsychological (conflict, frustration, unhealthy lifestyle, mental fatigue, pain, worry, interpersonal conflicts, work overload and others) and somatic (environmental physical effects and somatic pathologies) stressors. The demand is however nonspecific; it requires adaptation to a problem, regardless of what that problem may be (Selye, 1973b). In its origin it was believed that the stress response was identical for both positive and negative demands (pleasure and pain). It now

is clear that stress responses are very similar to a variety of demands and Selye's definition was altered accordingly: Stress is the inability to cope with a perceived (real or imagined) threat to one's mental[9], physical[10], emotional[11], and spiritual[12] well-being, which results in a series of physiological responses and adaptations (Seaward, 2013).

In different species a different expression of stress is possible (Napierska and Malgorzata, 1998; Kregel, 2002). In all cases, on a cellular level said deviation results always[13] in the production of stress proteins (Feder and Hofmann, 1999a), of which the HSPs (Heat Shock Proteins) are the most popular (and the best known). In an organism stress will result in a difference in substance balance, which may then result in excess or absorption of certain substances (e.g. glucose, ammonia)(Hunter and Uglow, 1993). Field measurements of filtration rates of the common mussel *Mytilus edulis* were significantly reduced by even very little manipulation (Hunter and Uglow, 1993), while *C. crangons* amonia excretion rate intensifies by handling (Hunter and Uglow, 1993).

Under normal circumstances, a body's metabolism is expected to be a well-balanced system, aimed at maintaining organismal homeostasis (Smith and Selye, 1979). Stress is usually considered as those environmental factors disturbing this homeostasis. Some stress is however essential for the well being (Selye, 1980; Smith and Selye, 1979). Stress is furthermore present even under normal situations, which are experienced as homeostasis, for regular bodily upkeep (Selye, 1973b). As such, physiologically speaking, stress can be defined as the rate of wear and tear on the body (Selye, 1980).

Stress as a process

First of all there are three kinds of stress: eustress, neustress and distress (Seaward, 2013). Eustress relates to positive circumstances (kissing your girlfriend or animals finding food). Neustress are inputs that are considered neither good nor bad (the notion a 1-point drop of the Dow Jones to a donkey). Distress is always negative and is usually abbreviated stress. It can be acute (sudden and intense) or chronic (prolonged and lingering) (Seaward, 2013). The different ways of experiencing stress are personal and depend on exogenous (adaptation, such as experience) and endogenous (evolution through genetics, sex, etc.) conditions (Selye, 1980; Selye, 1976).

The whole physiological process of responding to changes or stressors lead to the general

[9] Mental refers to intellectual abilities (reasoning, identifying, etc.)(Seaward, 2013).
[10] Physical refers to bodily health (Seaward, 2013).
[11] Emotional refers to the control of emotions (e.g. anger and fear)(Seaward, 2013).
[12] Spiritual well-being requires a consciousness of ones self and it is about the appreciation of that, both socially and personally (Seaward, 2013).
[13] Reduced sets of chaperones exist in a variety of species (Richter et al., 2010).

adaptation syndrome (GAS). This process takes three stages.

The first is the alarm stage, which prepares the body for whatever may come. This first reaction to a stressor is referred to as the the fight-or-flight response, which is a general bodily response to prepare the organism to do either one. Fight is thought to be linked to anger and results in a short burst for energy and power viz. short, intense anaerobic work. Flight on the other hand is linked to fear and results in preparation for prolonged energy demand for a retreat and flight. An additional option is the freeze response, which makes us remain as fixed as possible and is often linked to post traumatic stress disorders in humans. Most mammals show to have developed to prepare for both fight and flight. They are however not developed for a heavily stress induced life. When an organism endures any stress, the body interprets this to some degree as a danger and therefore results in the preparation as the fight-or-flight response, with the accompanying bad and good effects of it. It does however aid little to nothing for any psychic issues (mental to some degree, emotional and spiritual) (Seaward, 2013).

The second stage is resistance, thus the prepared reaction is executed in order to return the organism to a stage of homeostasis. When facing stress that requires systematic adaptation, the organism can respond in three distinctly different manners: nervous, hormonal, and immunologic and phagocytic (Selye, 1973). There are two basic mechanisms which help us put up with the aggressor: endure it (syntoxic) or elliminate it (catatoxic) (Selye, 1973b).

The third stage is exhaustion: when the stressor remains, inevitably the organism will induce some form of damage and ultimately death may occur. This does not mean that the organism cannot succomb during the other two stages (Selye, 1973b).

This stress will have both beneficial and deleterious effects.

Non-lethal stress improves the tolerance for future stress and thus allows an organism to cope more easily with similar stress and to cope with otherwise lethal stress, thus leading to adaptation (Kregel, 2002). An already stressed organism however will be worse affected by additional stressors (e.g. having cold making a human more susceptible to a cold)(Hagerman and Vismann, 1995). In essence then, one might say that health and homeostasis are greatly dependent upon adaptation or the ability to cope with the stresses of life (Smith and Selye, 1979).

It is excluded that HSPs alone are responsible for this adaptation (of improved tolerance). Feder and Hofmann (1999a) suggests that stress proteins in general may even have little to do with this process.

Richter et al. (2010) states that in more recent research “cross protection” is proven, where stress

caused by one stressor can lead to protection against stress from other stressors in an organism. This would be logic seeing that cells have limited reactions to stressors and that only a limited number of stress proteins exist to provide protection against numerous stressors (see molecular/cell level). Exercising leads to stress, as it creates several 'stressors', such as a sudden need for oxigen and energy and the creation of heat. Regular exercising allows us thus to cope better with stress and as such exercising itself and thus generates stamina (Kregel, 2002). Richter et al.'s (2010) statement would also suggest that it could provide better resistance in a broader sense too.

Putting the HSPs into the perspective: As said above stress leads to the production of stress proteins, in particular for this study: HSPs. These HSPs are a part of the protein quality control system, conferring stress tolerance (Madeira et al., 2012) and functioning as cell chaperones in normal conditions, excecuting “husbandry” tasks (see molecular/cell level). Although species differ in form, looks and DNA, the genes encoding HSPs are highly conserved and occur in every species in which they have been sought (Feder and Hofmann, 1999a). Depending on their geographic habitat, organisms in nature risk exposure to temperatures ranging from −100° to more than 100°C, and comparable extremes of chemical and gas concentration, food and water availability, hydrostatic pressure, radiation, and toxic substances of human origin. It makes sense that this makes the HSP expression, as well as other stress protein expression pattern species-specific (Napierska and Malgorzata, 1998). It is even population- and animal-specific with significant polymorphism (Madeira et al., 2012). This intraspecific variation might be due to genetic variation among individuals, health and nutritional status, reproductive status, sex, age, antioxidant status of the individuals, HSP mRNA stability, pre-existing pool of heat shock transcription factors (HSF) and HSPs, differential microhabitat use, past thermal history, parental (epigenetic) effects, and natural environmental conditions.

HSPs can be seen as adaptations maintained via natural selection, if there are intraspecific variations with a genetic basis and if effects on individual fitness occur (Madeira et al., 2012). With small DNA differences in species, small differences in HSP expression arise in response to stress. The animals being the most succesful in coping with the stress (most appropriate HSP expression) will survive. Hence stress (e.g. in response to varying environmental temperatures), experienced throughout their lives, including on molecular, physiological, organismal and behavioural levels impacts the organism's performance and fitness which in result has an impact on Darwinian evolution, leading to adaptive changes (Madeira et al., 2012).

The aforementioned adaptation means on a cellular level that an amount of stress proteins (such as HSPs) is maintained in the cell, which thus anticipates future stress. This means that the cell now

'knows' how to deal with such stress. In extreme situations it can even be an adequate buffer to survive situations which might otherwise have killed it.

It would seem that organisms frequently produce HSPs in nature, but nature has found ways to “cheat”. Hence species do not evolve tolerance for certain environmental stressors, because of their abillity (and evolution) to run away from it (e.g. *C. crangon* from anoxia). Their biochemical specialization or their ability to eliminate the stress-factor keeps their equilibrium stable(e.g. wearing a coat in winter prevents humans from developing cold resistance or fur)(Hagerman and Vismann, 1995; Feder and Hofmann, 1999b). Some tolerance however is always necessary as equable environments can contain HSP-inducing microhabitats (Feder and Hofmann, 1999a) and variotion in physical characteristics (i.e. temperature, pH, chemical concentrations, etc.).

Off course, if HSP expression were purely beneficial, natural selection should have maximised it. By contrast, the HSP expressing genes did not evolve towards unlimited amplification in the genome, and HSPs have grown to be subject to stringent autoregulation by multiple molecular mechanisms. These findings imply that HSPs can have both positive and negative impacts on fitness, and that natural selection probably balanced these impacts in setting a(n advantagious) level of HSP expression. In *Drosophila* for instance, small to moderate increases in HSP70 levels enhance inducible thermotolerance, while large increases in HSP70 levels decrease thermotolerance; in between levels of HSP70 are thus the logic outcome of evolution (Feder and Hofmann, 1999a).

The negative effects could be explained by at least two things: First, high concentrations of HSPs could be downright toxic, directly interfering with other cell processes (by binding other proteins too tenaciously), or could change in function, having a deleterious effect on the metabolism and physiology of the cell. Second, HSP synthesis and degradation could deplete a cell’s or organism’s nutrient and energy stores beyond tolerable levels and/or engulf the cells synthetic/catabolic apparatus to such an extend that it interferes with the processing of other essential biomolecules (Feder and Hofmann, 1999a). Amongst others cell growth and division could thus be decreased (Madeira et al., 2012). Also, this energy cannot be used for other purposes anymore, impacting fertility/fecundity, develoment, energy budget and survival, which in turn impacts fitness.

Stress proteins with emphasis on HSP70

General

HSPs were first discovered in 1962 in *Drosophila melanogaster* larvae that were exposed to a “heat shock”, hence the name heat shock proteins (HSPs). Later studies lead to the discovery of a series

of proteins in the 70-kDa range and beyond, that were expressed by heat shock and that other stressors also induced their expression. Additional research demonstrated strong cytoprotective effects, involvement in regulatory pathways and molecular chaperoning functions for other cellular proteins (Kregel, 2002). Furthermore, HSPs are involved as cytokines in the immune mechanism (Tkáčová and Angelovičová, 2012) and are associated with tolerance development to a variety of stresses, including hypoxia, ischemia, acidosis, energy depletion, cytokines such as tumor necrosis factor-alpha (TNF-α), and ultraviolet radiation in short and longer term (Kregel, 2002).

Some stressors enhance, some inhibit production of HSPs (Napierska and Malgorzata, 1998). Some HSPs show rapid expression (after 10-15min), while others peak somewhat later (after 20min), and still others do not show expression but after 2 hours (Richter et al., 2010). It can be said at this point that the cell responds in a variety of complex ways (Kregel, 2002).

Causes?

As mentioned before, all deviation from homeostasis leads to stress and consequently stress protein expression. A few of these stressors are as follows.

Cadmium (and other heavy metals (Feder and Hofmann, 1999a)) result in expression of stress proteins in fish and invertebrates (Napierska and Malgorzata, 1998). As humans are known to gain cancer from these, it is safe to say that it causes stress to us too (even by just hearing we might be comming into direct contact with it, causes us stress). Tkáčová and Angelovičová (2012) states that heavy metals result in HSP expression in all species expressing HSPs.

Handling of species can cause stress too, though this may not always be visible and the manner of handling is an important factor too (e.g. when a person handels its dog causes different stress than when an unknown veterinarian would) (Hunter and Uglow, 1993). As stress responses are not always expressed immediately, they can also have apparently “no effects” when tested (Madeira et al., 2012).

Variable environmental *temperatures* cause internal stress too (Madeira et al., 2012). These can be normal (e.g. winter-summer (Feder and Hofmann, 1999a), the sun) or caused by antropogene sources (direct e.g. by cooling water or indirect e.g. by global warming) and acute viz. with a strong inclination/declination gradient or latent viz. slow increase.

The *chemical and physical* environment (including the aforementioned temperature) induce stress within species in a similar manner as it would induce stress in objects, but in a much more complicated way (Menezes et al., 2006).

Other examples on cellular level of causes are cellular *energy depletion*, and extreme concentrations of *ions*, other *osmolytes*, *gases*, *desiccation* and various *toxic substances* (Feder and Hofmann, 1999a; Kregel, 2002). According to Kregel (2002) the following (and many more) stimuli lead to HSP70 expression as well: *hypoxia*, *acidosis*, *ischemia-reperfusion*, *reactive oxygen species* (ROS), *reactive nitrogen species* such as nitric oxide, and *viral infection*. Tkáčová and Angelovičová (2012) adds the following causes: *cold*, *UV radiation*, *bacterial infections*, *pesticides* and *changes in transcription and translation*.

In which animals and how?

The main effectors of the heat shock response (and thus HSPs) are found in both prokaryotic and (higher and lower) eukaryotic cells (Kregel, 2002; Richter et al., 2010; Feder and Hofmann, 1999a). Apparently, they are universal (Richter et al., 2010) and one of the most ancient and highly conserved groups of all proteins. HSPs are a clear proof and example of Darwin's theory of evolution, as they can be retraced (with some modifications) to all predecessors at the levels of gene sequence, genomic organization, regulation of gene expression, and protein structure and function (Feder and Hofmann, 1999a). Independently of species and habitat, the mechanism will be activated in all organisms by a small temperature increase (Richter et al., 2010). All animals having a minimum lower and upper temperature threshold value for expression and a maximum value of expression. By concequence the typical habitat temperature range usually defines the threshold temperatures and ranges for HSP induction, thermophilic species (from warm environments) having higher thresholds than psychrophilic species (from colder environments) (Feder and Hofmann, 1999a). For example *F. cataglyphis* continues to express HSP up to 45°C, although theoretically HSPs stop working above 42°C, *P. cartilagineum* has a threshold value of 5°C for HSP70 and ubiquitin transcription and some *Archaea* have a threshold value in excess of 100°C.

Types of stress proteins

Not all stress proteins are HSPs. HSPs are traditionally classified into different classes according to their molecular weight (Madeira et al., 2012; Kregel, 2002; Feder and Hofmann, 1999a; Tkáčová and Angelovičová, 2012) and sequence homology (Feder and Hofmann, 1999a). The suffix dictates the approximate weight in kDa (1Da = 1 g/mol; Da = Dalton) of the HSP[14]. We generally distinguish the following families: 10, small, 40, 60, 70, 90, 100 and 110. Small HSPs are those between 12-43 kDa (Tkáčová and Angelovičová, 2012). There are also some which are named with a full name instead of a “kDa-name”, including proteases, ubiquitin and dehydrins (Tkáčová and

[14] The molecular mass of HSP70 is 70000g/mol (in comparrison for water 18g/mol; sugar 342g/mol).

Angelovičová, 2012). Sometimes more families are defined and used. In eukaryotes these families house several members with possibly differing function, inducibility and intracellular localization, making their independent properties sometimes hard to establish (Feder and Hofmann, 1999a). They are all defined by their own genomes in the DNA, which may incidently vary in location intraspeciewise (Feder and Hofmann, 1999a).

HSP70s and HSP90s appear to have an even broader stress-response, while others are more specific (Napierska and Malgorzata, 1998). The HSP70 family comprises at least four distinct proteins with a common sequence, which are synthesised because of different stimuli: HSP72, HSP73, HSP75 and HSP78; all having redundant and confusing acronyms (Kregel, 2002).

The following table gives a short review of most families by molecular weight, giving their location and biological function.

Protein	Phenotype	
Hsp10	Cellular:	Tolerance of ischemia (no phenotype) tolerance of ischemia when co-expressed with Hsp60
Hsp27	Cellular:	Resistance to chemotherapeutic drugs; resistance to hydrogen peroxide; resistance to hydrogen peroxide (no phenotype); resistance to ultraviolet radiation (no phenotype); resistance of tumorcells to monocytes; sensitivity to lymphokine-activated killer cells (no phenotype); tolerance of hyperthermia; resistance to tumor necrosis factor; tolerance of ischemia; resistance of actin polymers to cytochalasin; accelerated nuclear protein aggregation; accelerated decline of thermal radiosensitization
Crystallin	Cellular:	Tolerance of hyperthermia; tolerance of ischemia; resistance to tumor necrosis factor; resistance to hydrogen peroxide
Hsp60	Cellular:	Tolerance of hyperthermia (no phenotype); tolerance of ischemia (no phenotype); tolerance of ischemia when co-expressed with Hsp10
Hsp65	Cellular:	Tumor regression; loss of tumorigenicity
	Tissue/organ:	regression of malignant tumors
Hsp70	Cellular:	Tolerance of hyperthermia; tolerance of ischemia/hypoxia; recovery from translational and transcriptional inhibition following heat shock; regulation of heat-shock response; tolerance of endotoxin; reduced protein denaturation upon heat exposure; tumorigenicity; cell proliferation; resistance to hydrogen peroxide; resistance of tumor cells to monocytes; sensitivity to lymphokine-activated killer cells (no phenotype); escape from drug-induced cell cycle arrest; protein glycosylation; tolerance of ultraviolet radiation; apoptosis; resistance to apoptosis (no phenotype)
	Tissue/organ:	Recovery of contractility after ischemia; reduction in myocardial infarct size; reduction of hyperthermic damage to midgut; resistance of heart to ischemic injury; resistance of hippocampus to ischemic injury
	Organismal:	Tolerance of hyperthermia; growth and development; regulation of heat-shock response; persistence in nature (no phenotype)
Hsc70	Organismal:	Tolerance of hyperthermia
Hsp72	Cellular:	Apoptosis (no phenotype); protection against heat-induced nuclear protein aggregation; protection against hypoxia; protection against thermal radiosensitization
	Tissue/organ:	Reduction in myocardial infarct size
Grp78	Cellular:	Protein secretion
Hsp90	Cellular:	Tolerance of hyperthermia; tolerance of ischemia (no phenotype); apoptosis; apoptosis (no phenotype); cell proliferation and cell cycle control; glucocorticoid receptor function
Hsp100	Organismal:	Host infection in Leishmania
Hsp101	Cellular:	Tolerance of hyperthermia
Many Hsps	Cellular:	Recovery of cell proliferation after heat shock; recovery from chromosome damage after heat shock; tolerance of hyperthermia; tolerance of ischemia
HSF	Organismal:	Oogenesis and development; thermotolerance

Table 1: HSP families in Eukaryotes
(Feder and Hofmann, 1999a)

Molecular/cell level

HSPs are part of the protein quality control system, conferring stress tolerance. HSPs have cytoprotective effect, act on regulating pathways (translocation of proteins across membranes), help stabelise the cytoskeleton and maintaining other proteins, primarily by dealing with denaturation (Kregel, 2002; Tkáčová and Angelovičová, 2012). This means HSPs and other stress proteins act in non-stressed situations too. Some HSPs work as chaperones, stabilizing denatured polypeptides and nascent proteins, regulating their folding and preventing their interaction and the occurrence of cytotoxic aggregations and even repairing or at times destroying them (Madeira et al., 2012; Richter et al., 2010; Feder and Hofmann, 1999b); repairing is found to be more energy efficient than destroying and building it anew, but sometimes proteins become "pair total" and as such they need to be destroyed (Richter et al., 2010). These problems are found to be ancient and ubiquitous, explaining the antiquity of HSPs and their presence in various organelles.

Stress proteins protect against immediate stress too but also conduct cellular repair processes after stress occurred (Napierska and Malgorzata, 1998; Kregel, 2002; Richter et al., 2010). A common consequence of stress is the unfolding of proteins and non-native conformations, which are fixed by the chaperoning functions of HSPs (Feder and Hofmann, 1999a). "Invasive" molecules, such as oxidised proteins, free radicals and heavy metals are dealt with too, by minimizing their inappropriate interactions (Feder and Hofmann, 1999a). Another primary function during stress is the protection of native protein integrity and maintain their translation (Kregel, 2002).

HSPs are also involved in apoptosis (natural suicide mechanism of cells, triggered by the organism itself) and inflammation (Kregel, 2002).

Production process

The activation of various intracellular signalling pathways induces HSP expression. All known stresses, if sufficiently intense, result in HSP expression (Feder and Hofmann, 1999a), though technically they come down to a simple few: foreign molecules, viz. unfolded, denatured or otherwise damaged native proteins and invasive molecules e.g. heavy metals; energy depletion, viz. glucose exhaustion and/or oxidative stress (Tkáčová and Angelovičová, 2012); and cytoskeletal or membrane damage viz. their rigidity or fluidity (Richter et al., 2010). In short stress means a disruption of protein homeostasis in cellular terms (Richter et al., 2010). Increase in temperature results typically in anomalies in the native proteins and damage to the cytoskeleton and membrane, thus the actual temperature does not result in HSP expression, but rather the consequences of the temperature (Tkáčová and Angelovičová, 2012).

As a result of stress the cytoskeleton becomes defective, organelles shift to incorrect places and intracellular transport processes break down. The Golgi system and the endoplasmic reticulum (ER) become fragmented and the number of mitochondria and lysosomes decreases. The uncoupling of oxidative phosphorylation and the loss of mitochondria are connected to a dramatic drop in ATP levels during heat stress. As for the nuclear processes RNA splicing is strongly affected. Nucleoli, where ribosome is assembled, swell, and large granular depositions of incorrectly processed ribosomal RNAs and aggregating ribosomal proteins are formed. In the cytosol, stress granules are formed, viz. large RNA-protein structures, containing nontranslating mRNAs, translation initiation components, and other proteins affecting mRNA function. This results in a global decrease in translation. The membrane gets damaged too and the resulting increase in permeability lowers the cytoplasmic pH and alters the ion consentration. All these consequences obviously lead to problems for the cell cycle and abilities. Accumulation of consequences possibly results in death (Richter et al., 2010).

The actual initiation of the expression of HSPs happens when a specific protein binds to the heat shock element (HSE) of the DNA and initiates the assembly of the transcription machinery. In homeostasis a heat shock factor (HSF; HSF1, σ32) is bound with the chaperones (HSP70, HSP90, HSP40), when the number of unfold proteins increases, the HSPs will automatically disconnect to fold them (Tkáčová and Angelovičová, 2012) and the HSFs will bind to the HSE, activating the transcription amongst others (Richter et al., 2010). There are several more steps, requirements and regulatory functions to be fullfilled in order to actually produce HSP70 in a cell, but a more thorough explanation is beyond the scope of this research (Kregel, 2002).

Functional groups

Functionally, stress-inducible proteins can be grouped into seven classes: 1) molecular chaperones; 2) proteolytic system (clear misfolded or irriversibly aggregated cells); 3) curing non-physiological covalent modifications of nucleic acid (repair DNA and RNA); 4) metabolic enzymes; 5) regulatory proteins; 6) sustaining cellular structures; 7) transport-, detoxifying and membrane-modulating proteins (Richter et al., 2010). This does not mean that individual HSPs cannot have pleiotropic effects, interacting with multiple systems in diverse ways (Feder and Hofmann, 1999a) or that different groups should not work together in various ways (Richter et al., 2010). Additionally, some proteins have been proven to shift between chaperone and protease functions depending on the temperature (Richter et al., 2010).

Another functional grouping system is listed below:

a) **Molcular chaperones**: This group contains HSP100s, HSP90s, HSP70s, HSP60s, and small HSPs (sHSPs) and several other heat-inducible molecular chaperones, like HSP33. A constant need for chaperone assistance is required firstly during the folding of novo proteins, their transport through membranes and their integration into the various organelles. Secondly they have an important task in refolding of non-native polypeptide chains, as the stability of the cellular proteins is low and aggregation competes with productive folding even at physiological temperatures. All molecular chaperones interact promiscuously with a broad range of unfolded proteins (Richter et al., 2010; Tkáčová and Angelovičová, 2012).

b) **Chaperonins**: These are ring-shaped chaperones that encapsulate non-native proteins in an ATP-dependent manner (Richter et al., 2010).

c) **HSP70**: The prokaryotic version, called DnaK, shares about 60% sequence identity with eukaryotic HSP70s in the cytosol and organelles. Under physiological conditions, HSP70s are involved in the de novo folding of proteins, and under stress they prevent the aggregation of unfolding proteins and can even refold aggregated proteins. HSP70 consists of two domains, an ATPase domain and a protein binding domain (Richter et al., 2010).

d) **HSP90**: They are found in very high concentrations in the cytosol under physiological conditions, and it is further upregulated under stress. It has not as broad a spectrum as HSP70 and it does not bind unfolded proteins, but rather nativelike proteins. HSP90s work together with a large cohort of cochaperones that associate in a defined order during the chaperone cycle, making it a complex and sophisticated apparatus (Richter et al., 2010).

e) **HSP100**: The HSP100 group are AAA ATPases. HSP100 enables proteins to become refolded and supports protein disaggregation (Richter et al., 2010).

f) **sHSPs**: These protect proteins from irreversible aggregation. They are ATP-independent chaperones. By binding to unfolded proteins, they form a sort of storage units of these, which can later be refolded by HSP70 and/or HSP100 (Richter et al., 2010).

Multicellular

In multicellular organisms HSPs have purposes both intracellular and extracellular. Extracellular HSP70 is known to facilitate antigen presentation e.g. in macrophages and dendrites. Macrophages and lymphocytes produce inflammatory cytokines in response to external HSP70. HSP70 on the surface of tumor cells possibly allow natural killer (NK) cells to recognise the neoplastic cells. HSPs on the cellular surface or in its vicinity have an immune-stimulating response in certain

conditions (e.g. viral infection or necrotic cell death) (Kregel, 2002).

HSPs may also have a role in tissue (as a whole) stress tolerance in multicellular eukaryotes (Feder and Hofmann, 1999a), which may in turn have additional levels of regulation for its expression (Richter et al., 2010).

Growth and development (pregnancy)

Many species exhibit characteristic and distinctive patterns of HSP expression (or nonexpression) during the various stages of development, including gametogenesis, embryogenesis, and metamorphosis (Feder and Hofmann, 1999a), and play roles in particular during segmentation, DNA synthesis, transcription, translation, and protein rolling and their transport through membranes (Tkáčová and Angelovičová, 2012). Often these HSP expressions are restrained, as high levels of HSPs are especially detrimental to developmental stages during rapid cell growth and division. Many animal species have been proven not to mount a heat-shock response during early stages of embryogenesis, when protein synthesis may be particularly profound (Feder and Hofmann, 1999a). In *Drosophila* for example larvae transformed with extra copies of the HSP70 gene showed greater larva-to-adult mortality and slower development (Feder and Hofmann, 1999a).

Stress and HSPs can thus be detrimental to developing cells, though HSPs can also be beneficial in minimizing resulting defects of said stress. In this case parental provision of HSPs or HSP mRNAs may override gametic or embryonic absence of HSP expression, presumably as their absense in the face of stress can have lasting (negative) effects on development (e.g. phenocopying of genetic defects) and even result in death (Feder and Hofmann, 1999a).

The age factor

Young organisms have sometimes an overexpression of HSPs, while adults have a relatively normal expression and aged organisms an underexpression (Kregel, 2002). This can be explained because protein damage accumulates with age, resulting thus in the aforementioned weakening expression, as well as a lower tolerance for stress (Feder and Hofmann, 1999a). Exercise, causing mediocre cellular stress, results in a long-term improvement of the age dependant decrease of HSP expression (Tkáčová and Angelovičová, 2012) and thus an improved stress resistance. This means theoretically that an aging organisms adaptability is at least partly countered by its rate of HSP expression and tolerance or vice versa organisms may have evolved to build up resistance to counter its decreasing tolerance and ability to express HSPs.

The accumulating protein damage does however inevitably result in cell death and consequently at

some point the death of the organism, affecting thus its lifespan and senescence. Reducing the protein damage and increasing HSP expression could thus prolong life according to Feder and Hofmann (1999).

Physical vs psychological stress in multicellular organisms

While physical stress (in a pure molecular/energetic sense) seems confirmed in all organisms, psychic stress seems plausible only for multicellular organisms. It can even be stated that it would be limited to animals.

In any case, there is no evidence yet as to how stress tolerance and adaptation in that direction is linked to HSPs in multicellular eukaryotes and their comprising tissue (Feder and Hofmann, 1999a). It is known however that stress tolerance is improved by improving it in the weakest organ (Kregel, 2002). It is apparent though that the nervous systems and blood-borne chemicals in the form of hormones (and HSPs) transfer stress through the organism. These can then be linked to both physiological and psychological aspects, though further elaboration surpasses the scope of this study.

Importance of stress proteins

The variable response capabilities and functions of stress proteins and more specifically HSPs make them excellent for application in biomonitoring and environmental toxicology, hence their popular use in aquatic toxicology (Feder and Hofmann, 1999a).

Stressful conditions can work as evolutionary forces through stress proteins, leading to adaptive changes in populations that can then lead to new species (Madeira et al., 2012). The learning process of animals and their cell can counter this evolution too. When species get used to a certain stressor which does not proof dangerous, they can learn to ignore it (Samson et al., 2014), while they do not evolve to overcome it. Thus stress proteins form an excellent medium to study adaptation, evolution and learning processes in species.

Through a ‘stress cascade’ one stressor can lead to aggravated effects of another stressor, making the second one (which might be a dormant infection) much more dangerous. It is also well known in aquaculture that stress under the livestock can lead to diseases (Stentiford et al., 2005).

Feder and Hofmann (1999b) state that the failure of HSPs to execute their tasks is thought to underlie numerous and important human diseases (Feder and Hofmann, 1999a). Furtheron HSPs are of interest in many diseases (e.g. cancers, bacterial infections) (Kregel, 2002).

According to Feder and Hofmann (1999b) specific combinations of heavy metals can induce such

distinctive patterns of HSP expression in soil nematodes that these patterns can become diagnostic fingerprints for specific toxicants in soils. Such applications can potentially give important information on contamination without the need for digging into the soil (e.g. leakage of the buried mustard gas shells at the coast could be traced by monitoring such species).

Things to keep in mind when analysing measurements

When measuring stress one needs to be aware that different stressors may act upon the testsubject, resulting in incorrect and strange results (e.g. a certain amount of subjects may unknowingly have a disease, while some may not, resulting in stress in itself).

One must also realise that while in a laboratory numerous stressors are taken out of the equation. In nature often many stressors act together, making the realistic equation more complicated. Additionally, we may be attributing the results to the wrong stressor or one stressor may enhance tolerance to another (Feder and Hofmann, 1999a). Stress response is not influenced by acclimatisation in the lab, but the lab environment may not result in the same 'state of stress' as it would be in nature (Hunter and Uglow, 1993).

The temperature threshold values for HSP expression for a species can show seasonal variation, meaning that base values may differ, but also sensitivity to stressors may vary intraspecieswise depending on the season of capture (e.g. *G. mirabilis*' temperature threshold value is significantly higher in summer than in winter (Feder and Hofmann, 1999a)). Also the concentration of HSPs present in an organism can vary because of their chaperoning role in warmer and colder periods (e.g. dealing with protein denaturation because of the heat)(Madeira et al., 2012).

Another uncertainty is the influence of the capture area. Organisms from a species living in a modestly stressful environment may have a different stress response than organisms from said species in a highly stressful environment (e.g. *C. crangon* caught near the beach vs. *C. crangon* caught at 40m depth between sand banks or in a calm estuary). Equally so it may be of importance where the organisms were caught in relation to their geographical distribution. In the centre these may be 'at ease', while evolution and constant battles against stress may change HSP expression at its frontier (Feder and Hofmann, 1999a).

According to Madeira et al. (2012) handling stress does not affect HSP levels instantly. When waiting too long, these will obviously be affected.

Precision must be acertained, so that multiple HSPs (or HSP groups) are not mistaken for a single one, as this my obscure phenomena of grave importance significantly (Feder and Hofmann, 1999a).

4.6 Ecology

Everything and everyone is intertwined. Some species have a more central role in communities than others.

Some definitions

Community ecology:	The study of patterns in the diversity, abundance, and composition of species in communities, and the processes underlying these patterns
Community dynamics:	Changes over time in the relative abundances of species in a specified area, including extinctions and species additions via dispersal or speciation
Species composition	For a given community, a state defined by the abundances of all species
Species relative abundance	The proportion of all organisms in a given area that are of a given species; equivalent to species frequency.
Species density	The number of organisms of a given species per unit of space
Community size	The total number of organisms in a community
Absolute fitness	The quantity of offspring produced by an individual organism per unit of time, including survival of the organism itself
Relative fitness	The absolute fitness of a given organism divided by the mean absolute fitness across all individuals in the community
Species fitness (absolute or relative)	The mean fitness (absolute or relative) across all individuals of a given species in the community; for absolute fitness, this is equivalent to the species per capita population growth rate.

(Vellend, 2010)

4.6.1 Research basis

In order to understand the interaction and intricate processes at hand in an ecological study or any scientific study a model (a set of laws: mathematic, logic or otherwise) needs to be formed, based on previous knowledge. Baskett (2012) states that this model is based on two of three aspects, on which the study will focus, while ignoring the third.

The three aspects are:

- generality: as opposed to specificity, relates to a broad field of the model
- realism: relates to the closeness of its applicability to the real world
- precision: relates to numerical accuracy of mathematical calculations

When (1) focusing on realism and precision at the expense of generality, potentially testable predictions for a specific situation are formed. When (2) focusing on generality and realism at the expense of precision on the other hand, qualitative predictions about different possible outcomes can be made. Lastly (3) when focusing on generality and precision at the expense of realism, null-type models are build. When gently adding realism to the latter, the effects on the model's overall dynamics can be studied (Baskett, 2012).

Putting it simple the following can be stated for a study: realism is reflected in emperical numbers, precision in mathematical laws and generality in logical conclusions and theories.

Combining all three aspects is theoretically possible, but practically making such a model would require a large list of complex differential formulas, which practically would not be managable and still come short to a degree in realism.

In ecology, the third type is usually used, as it will practically allow the researcher to test his thesis on reality. Haski et al. (1991) agrees that progress can be made efficiently by testing simple hypotheses with new data.

4.6.2 Ecology

Ecology is the science that studies living beings in relationship to its environment. This environment can be viewed from a physical point of view (i.e. light, temperature, water,..) or a biotic point of view (i.e. interaction between different species) (Potters, 2014). Factors such as time (evolution, duration,..) and human impact (as a source or a subject) are often important aspects in ecological studies.

Several specialisations have developed over the years: molecular ecology, behaviour ecology, population ecology, autecology (study of all interactions of one species), ecosystemecology, paleo-ecology and community ecology are some examples.

Vellend (2010) defines community ecology as the study of patterns in the diversity, abundance, and composition of species in communities, and of the processes underlying these patterns. He furthermore states that ecological patterns are powered by “only” four distinctive processes: selection, drift, speciation, and dispersal. These four prosesses are very analogous to the “big four”

of population genetics: selection, drift, mutation, and gene flow. Genetics and ecology are in fact closely related (as exemplified by the family of the HSPs as well): selection drives genetic evolution.

Allthough the main attempt of ecology is to gain a broad view in natural patterns, most studies (not unlike this) focus on single processes. While these hold much valuable information it is more important to focus on a proven pattern, rather than a proven quantitative process in a specific set-up (Vellend, 2010). This means that focussing on generalism and realism should be key. By doing enough research while focussing on realism and presicion, generalism is added though. Mechanistic functional responses that describe organism–environment interactions are integrated into basic community and evolutionary ecological models (Baskett, 2012) and tested with observed data.

The four processes can be shortly described as follows: speciation converges more or less to a temporal factor, drift to randomness, selection to environmental interaction and dispersal to the spacial factor. A more thorough explanation is however in order.

Selection

Vellend (2010) describes selection as the determenistic outcome of local interactions among species and between functionally distinct species and their environments. In other words, selection occurs when variants in a population prove more succesfull than others (they have better fitness than the others). Three forms of selection are distinguished:

- Constant selection happens constant and continuisly in time and space and is independent of species density.
- Frequency- or density-dependent selection depends on the density of the species itself or other species. It depends on both the qualitative (interaction) and the quantitave ecological relationship between the populations.
- Spatially- or temporally-variable selection enables species that otherwise could not have lived together to coexist. Spatiotemporally varying selection allows the same species to evolve at different rates and/or locations to evolve in different species.

Selection can be seen as Darwinian evolution, hence these are some of the important underlying factors: response to abiotic factors, disturbance regiment, species interaction, species densities, specialization degree and limiting resources (Vellend, 2010).

Drift

In all simplicity drift is the random factor. The most obvious stochastic elements of are finite numbers of birth, death and procratrination. For an event to be purely ecological drift, species need to be demographically identical (Vellend, 2010). Practically this presents an empirical nightmare, given the variety of differences between species. Additionally, “random” events/empirical data is usually attributed to some reason or other. Nonetheless, Vellend (2010) states that many empirical studies exist in which a compelling case can be made for drift as an important process underlying community dynamics. In fact, everyone has run in to some form of randomness at one point in its life. It needs said though that drift usually is a marginal factor in models.

Speciation

Speciation litteraly translates in the formation of new species, but also in their extinction. These changes of species composition have an important impact of the community model (Vellend, 2010). The easiest way to show the effect and result is the difference in community sizes in identical environments or alternatively in different environments while species had an equal amount of time to evolve in it.

There is a proven linear impact of the size of the regional species-pool on the local species-pool. At regional spatial scales, the rate of speciation can enter mathematical models of local communities directly as a key determinant of community dynamics, though speciation on a local scale may be neglegible (Vellend, 2010).

Dispersal

Dispersal is the movement of species. The important factor is their rate of movement or spreading (Vellend, 2010). Species ability to move between communities is an important factor in dynamical models both for themselves, e.g. to escape stressful environements, and for species whit whom they interact, e.g. the annual migration of swallows on insect populations. The ultimate purpose is to increase individual fitness through an optimal exploitation of resources (van Moorter et al., 2013).

The population size of the dispersers and recipient community compositions affect the impact of dispersal on dynamics. Two types of dispersal patterns are relevant: mainland-island models (on-way dispersal from an infinite size to a small local community) and island models (link of small local communities called metacommunities) (Vellend, 2010). Dispersal is the link that connects separate communities by moving species.

Dispersal can interact with selection or drift to influence community patterns at regional and local

scale (Vellend, 2010). Movement is a primary adaptation especially in dynamic resource landscapes (van Moorter et al., 2013). They are generally put in function of frequency and distance, as resourse availability is a spatiotemporal variable – the more frequent and shorter movements are usually considered of less ecological significance. The triggers for these movements are of particular interest.

In a long term perspective, movement also allows conquering of new terrritories, weather these happen at a slow or fast phase, and can result in the development of new species (e.g. *C. crangon* spread through all European seas and the Black Sea and the Mediterranean populations have no use for low temperature resistance, which may lead to their development into seperate species).

4.6.3 Community

Vellend (2010) describes a community as a group of organisms representing multiple species living in a specified place and time.

Regardless of specification, communities are of central interest in ecology, independent of their coherence and integrity (Vellend, 2010). Every community-model and by extend ecological-model is build from any of the formentioned processes or a combination thereof (except from only speciation and or dispersal). This means that 12 models can be build of these processes (Vellend, 2010).

A historically important debate is about the “balance of nature”, viz. whether or not communities reach an equilibriumstate (Vellend, 2010). This gives rise to particular frameworks: equalizing vs. stabilizing (species evolving towards being equal vs. balancing each other out), local vs. regional and metacommunity (Vellend, 2010). Two additional influential concepts are worth mentioning: metabolic theory of ecology and ecological stoichiometrie. Both see everything as physical entities with functional chemical and energy flows with specific efficiencies (Vellend, 2010).

4.6.4 Population

A population is a group of organisms of the same species that live in the same area at the same time (this means that several populations of the same species can live spread over the world) (Potters, 2014).

Populations are considered to evolve on their own, because of small individual differences between the species organisms. Stressfull conditions, leading to adaptive changes (Madeira et al., 2012) are specific for each population. HSPs can be seen as such adaptations maintained via natural selection (Madeira et al., 2012).

Within community-models, mechanistic functional models describing population growth rates and fitness can be a binding factor between ecological and evolutionary dynamics (Baskett, 2012). This can be translated in a web-based view, popular in ecology.

4.6.5 Interaction

Vellend (2010) discerns three distinct kinds of interaction, allthough some have subsidiary forms. They are competition, predation and mutualism. The basis of these interactions is linked directly to species fitness. Their purposes in this sense are predominantly trophic and procreational. These ways of coexistence results in population-density fluctuations over time and ultimately selection. The outcome of the interactions however also depends on the environmental conditions (Vellend, 2010).

It can thus be said that interactions and environment go hand in hand in shaping evolutionary trajections and community dynamics (Cothran, 2015).

An interaction often skipped, which may also be an important driver of ecological relevance is reproductive interference or anisogamy: interspecific reproduction adversely affecting fitness (Cothran, 2015). Commonly known results are mules and hinnies, but it is an interaction found between *C. crangon* and *C. allemani* too.

Competition

Competition is the interaction where multiple organisms compete over available resources (Potters, 2014). These resources can take many forms: food, sexual mates, light, water and habitat are only a few examples. The competition can be intraspecieswise (e.g. male stags competing for does) and interspecieswise (e.g. caracaras and vultures competing for a cadaver). Despite the competition lowering fitness for the loser, stable combinations of coexistance with benefiting aspects for both often exist. According to Vellend (2010), competitive exclusion on the other hand is often a result of environmental conditions favouring the conquerer, thus via selection, one forces the other to move (dispersal) or go extinct (speciation). In the event two species are competetively equivalent enough, drift takes on a dominant role (Vellend, 2010).

Two types of competition are identified: interference, viz. direct competition for resources, and exploitation, viz. each organism using as much available resources as possible and as such denying them to the competition.

Predation

Predation is the interaction where two or more organisms directly interact and one or more organisms eat the other (Potters, 2014).

Several types of predation are distinguished: **true predation**, viz. a hunter eats its prey, **grazing**, viz. organisms eat plant life, **parasitism**, viz. a parasite lives in or on its host, while consuming it (partly), and **parasitoidism**, viz. organisms using others as hatchery for their offspring.

The succes and as such rate of true predation and grazing, while being primarily dependant on "competitive" equality, depends to some extend to environmental factors (Baskett, 2012; Sandberg et al., 1996) and size difference of predator, prey and possible third parties (Yamaguchi and Kishida, 2016). Predation rate models are typically based on one of three types as Baskett (2012) describes. In general it can be said that the model starts from a linear function and for each additional factor taken into account a degree is added. These models are relatively phenomenological, while adding factors such as temprature or grazing rate are founded in metabolic theories of ecology and ecological stoichiometrie.

Mutualism

Mutualism is the interaction between two species, where both species benefit (Potters, 2014). Intraspecieswise the term cooperation is used. Symbiosis is often wrongfully used as a synonym. Symbiosis is theoretically an "interaction" where both organisms live in close relation to each other, being either mutualistic or parasitic.

A well known example is between sharks, turtles or whales and remoras or pilot fish.

4.6.6 Fitness

The fitness of a species is its ability to thrive in a certain biotic and abiotic environment and produce generations of offspring (Potters, 2014). Each organism has its own fitness (except clones), which propagate evolution. The fitness lays in the organisms genotype or DNA and fenotype or physical being. Besides appearance and strength (Potters, 2014; Yamaguchi and Kishida, 2016), amongst others behaviours (Hanski et al., 1991; van Moorter et al., 2013), the ability to move, adaptability (Madeira et al., 2012;Feder and Hofmann, 1999a), reproductive succes (Baskett, 2012) and intelligence (Samson et al., 2014) are vital modifiers.

Some species use a "rainbow" strategy (using multiple aspects) and are sometimes called generalist species (e.g. *C. crangon*), while others are specialists, focussing on one particular aspect (e.g. tortoise focussing on a strong shell). The key to succes and fitness it seems however is efficiency

under a variety of environmental conditions.

4.6.7 Generalists vs. Specialists

The terms generalists and specialists usually applies to two types of predators, though it is sometimes used in other terms too.

Specialists are predators that highly specialize in on one species or a limited number of prey (depending on the degree of specialization) such as panda's and koala's. It has been demonstrated by theoritical studies that specialist predators drive a predator-prey limit cycle with steep gradients (Hanski et al., 1991).

Generalists are predators that use a wider range of prey, depending on which prey species are currently most abundant, e.g. humans and *C. crangon*. They are often opportunistic, preying on what is readily available. Generalist predators with relatively stable populations have a stabelizing effect on the population dynamics of prey species, as they will crop of the tops of prey population densities (Hanski et al., 1991).

Interestingly, both can have an opposite effect too. Populations of highly mobile specialists preying on high concentrations of prey only, have an effect akin to the stabilizing effect of generalists on prey population dynamics (Hanski et al., 1991). Generalists with limited mobility may locally become functionally specialists when one prey species is dominantly available throughout the year. An abundance of generalist predators may replace the function of specialists and remove their effect on population dynamics all together (Hanski et al., 1991).

It is furthermore noteworthy that the number of generalist predators decrease with increasing latitude and altitude (Hanski et al., 1991). This makes sense as the number of species decreases alltogether and the environment becomes more harsh, necessitating specialization. *C. crangon* may thus act as generalist predator in lower latitudes and as specialist in higher latitudes, giving rise to the possibility of speciation between populations.

The therm generalist is sometimes applied to animals whom have not focussed their abilities on one single sense.

4.7 Doing an experiment on Marine Bioacoustics

The **European Union**'s (EU's) Marine Strategy Framework Directive (2008/56/EC), aiming at sustainable human use by improving the conditions of the European seas, has set objectives in eleven Descriptors of Environmental Status. The eleventh is: "Introduction of energy, including

underwater noise, is at levels that do not adversely affect the marine environment”. In response to this directive the EU started two main programs to investigate underwater acoustics. They are “Achieve Quieter Oceans by Shipping Noise Footprint Reduction” (AQUO) and “Suppression of Underwater Noise Induced by Cavitation” (SONIC). AQUO and SONIC as a result published FP7 – Grant Agreement no. 314227 “Guidelines for Regulation on UW Noise from Commercial Ships” in collaboration with Bureau Veritas and DNV GL.

The **International Organisation for Standardization** (ISO) works on fascilitating international corporation. In the field of marine bioacoustics, the workgroup ISO/TC 43/SC 3 Underwater Acoustics has produced a document which standardizes the measurements of ship sounds in ISO 17208-1:2016 “Underwater acoustics -- Quantities and procedures for description and measurement of underwater sound from ships -- Part 1: Requirements for precision measurements in deep water used for comparison purposes”.

Besides the ISO's and EU's work on enhancing research, steps are taken towards the protection of animals in marine environments against noise pollution. For the law, noise is split into two parts: the part that affects humans on board (both noise and vibrations) and the part that is put into the sea and affects the marine environment.

The International Maritime Organisation (IMO) has agreed on guidelines in its Sub-Committee, viz. Marine Environmental Protection Committee (MEPC), on their 66th session as part of the “tier III“ standards for MARPOL. These guidelines contain recommendations to address the adverse impacts on maritime life, caused by commercial shipping. They are a first step into international regulation.

5 The experiment

Material and methods (part 2)

5.1 The setup

5.1.1 Ideal setup of the initial plan

For an optimal acoustic situation, a low and wide tank is ideal. In such a case a 40-50cm deep tank of an arbitrary 3m length and breadth should be ideal to be both manageable and sufficiently large. Taking no account for what is practically feasible, the tank should in length and breath be larger than the longest wavelength of an audible sound for marine animals, which is of 10 Hz frequency. With a theoretical speed of sound in salt water of 1500m/s this results in:

$$\lambda = \frac{v}{f} \Leftrightarrow \lambda = \frac{1500\, m/s}{10\, Hz} = 150\text{m}$$

A more suitable depth for such a tank would then be 2 meters. This is however completely unrealistic.

In the more realistic setup of a 3x3x0,4m tank, there would then be a choice between two benthic filter species (bivalves): either mussels (*Mytilus edulis*) or macomas (*Macoma balthica*). Either choice would work, though macomas are to be preferred as they bury themselves in sand and use a siphon to function, while mussels hold firm on top of the bottom layer. *Mytilus* would thus alter the soundfield (as they would become the top layer of the bottom). Of course, this would not be a huge problem as mussels tend to form large 'fields' and as such would alter the soundscape as they would do in a natural setting. On the other hand, mussels would block sand access for *C. crangon*, thus denying them shelter and more importantly: new statoliths if they should mould. A second problem is the fact that it is unknown wether shrimps are equipped to harvest mussels while sifting.

A second species would then be the brown shrimp (*Crangon crangon*), as it is kept alive easily, while langoustines *(Nephrops norvegicus*) are not.

A third species presents another choice, though with a rather easy outcome when taking realism into account. The choice is between brine shrimp (*Artemia*) and an amphipod (*Bathyporeia pilosa*). Brine shrimp are easily kept and well undrestood animals, but they are not indigenous to the marine environment, but rather to salt lakes and salt marshes. The amphipod *B. pilosa* is a well-known prey for *C. crangon* and should survive well in a laboratory setup. The convincing factor in the choice is however that *B. pilosa* is indigenous to the North Sea and marine environments.

Besides species to observe and a bottom layer of sand (at least 5cm), the tank should be fitted with a single sound producer (marine speaker). In a comparative study including several tanks, sounds of different ship types could be used to make a comparison. For monitoring purposes, each tank should be permanently fitted with a hydrophone. For observations the tank should be fitted with a number of cameras to allow a complete covering of the tank. A continuous water refreshing system should be fitted to ensure constant water characteristics.

Observations of the community are proposed to be made over a prolonged period of time to make long term prognoses. Such a period would be in terms of several weeks to a month.

Fitting a 3x3x0,4m tank with brown shrimp, amphipods and macomas (as Sandberg et al. [1996] did) would allow for observations in a community setting. Possibly, the individual populations could show altered behaviours, but additionally the interactions could alter between species within the community, which according to Lovell et al. (2005) should be promising. Besides imagery observations, numbers of each species could be counted to show species succes and predation rates. Additionally samples could be taken in numbers relative to the community composition to observe size, weight and stress through biomarkers viz. HSP70, CEA and fat content.

5.1.1.1 Problems and why not

Such a study was deemed unfeasable as it presented a couple of problems.

- Making a vast amount of photo/video imagery during a one month period requires a lot of time to process, which simply would at least stretch the time for a master's thesis.
- The size of the tanks and alocating them to a somewhat soundneutral area was not possible within the budget.
- Further smaller problems which are identical for the setup discussed in 5.1.2 will be discussed in 5.1.2.1

The first two issues were ample reason not to take this approach.

5.1.1.2 Legal issues

A second questing entails the use of fishes or other vertebrate species. Due to Belgian law, vertebrates can only be used in experiments under strict supervision of a certified research scientist, well trained in decent use of animal test subjects, and of an ethical commission. Details can be found in:

- Royal resolution of the 30th November 2001 on prohibition of certain animal tests

- Law of the 14th August 1986 on the protection and well-being of animals
- Royal resolution of the 29th of May 2013 on the protection of test animals

Furthermore there is one directive from the EU:

- Directive 2010/63/EU of the European Parliament and of the Council of the 22nd September 2010 on the protection of animals used for scientific purposes.

Since the Antwerp Maritime Academy does not have the required personnel, nor the ethical commission to deal with animal welfare during experimentation, we were limited to the use of invertebrates.

5.1.2 The actual experiment

A set of ten tanks, numbered 1 to 10, were available in a laboratory at ILVO to work with. The use of five tanks with sound (1-5) and five tanks without sound (6-10) was intended, but proved not to be possible (Fig. 5.9). Preliminary testing showed that sound from tank nr. 5 was very audible in tank nr. 6 because of their proximity. This meant that only two sets of four tanks would be used, viz. tank nr. 1-4 with sound (Fig. 5.2) and tank nr. 7-10 (Fig. 5.3) without sound. The tanks were simple plastic boxes, which can be bought at a hardware store. Outside dimensions are 40x30x30cm, while inside wet dimensions are 37x27x23cm (length, breadth and depth respectively), making them 23l tanks. The entire facility at ILVO, including the laboratory is subject to a lightning system of 12L/12D, simulating day and night. The tanks were plugged into the seawater recirculation system, which is a continuous half-open recirculation system, with UV filter and bio filter. Water was injected under the surface to avoid the constant sound of water inflow. There were no air diffusers installed as the water recirculation system would provide sufficient oxygen. The laboratory was kept as silent as possible, because of the nature of the experiment. Preliminary tests with an isolating mounting (a synthetic fibre panel under a tank) proved them to be unsuitable, as they only marginally improved sound isolation.

Each tank was equipped with two speakers (setup in a manner described under 5.1.3.1), fixed above each other, the lower one 3cm above the bottom, centered to the middle of the side of the tank. Each speaker was screwed on a polypropylene plate (60x60x4mm), that was glued to the tank wall with Tec7 cement. The speakers were somewhat protected from condensation by a plastic flap taped over the speakers. All speakers played the same recording of a ship's underwater noise for the duration of the experiment. Together with the tank wall these made excellent speakers (Akamatsu et al., 2002).

The recordings were taken at 200m distance at Plymouth Harbour from the ship Bro Distributor

when making 10kn (Wale et al., 2013). The ship is described by the following details (Fig. 5.1) (Lindström, 2009):

IMO: 9313113

MMSI: 219261000

Call Sign: OZGI2

Flag: Denmark

Vessel Type: Product/Chemical-tanker

GT: 11344t

Deadweight: 14907t

LOA/BOA: 146,8m/22,03m

Year Built: 2006

Fig. 5.1: Bro Distributor
(Lindström, 2009)

Fig. 5.2: Loud tanks (Own collection)

Fig. 5.3: Silent tanks (Own collection)

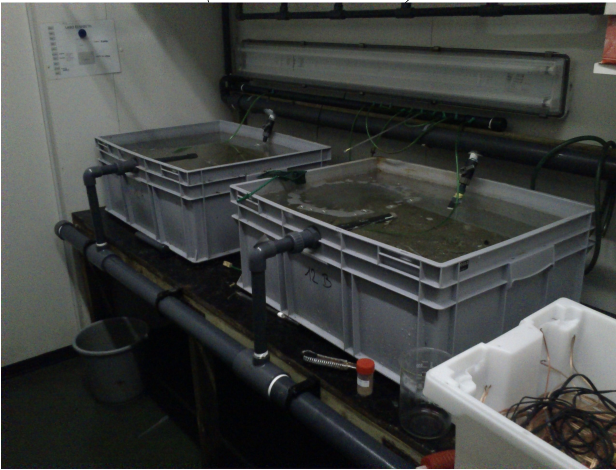


Fig. 5.4: Storage tanks (Own collection)

Fig. 5.5: Inside a tank with C. crangon (Own collection)

5.1.2.1 The audio playing setup

The sound producing system was setup in the manner here described (Fig. 5.6):

Two speakers a tank: RED plug on the striped wire end was connected to the big speaker connector. BLEU plug on the plain wire end was connected to the small speaker connector.

Amplifier: (OSD MX-1260) The striped wire was connected to the anode (+) The plain wire was connected to the cathode (–)

The sound is mono viz. monophonic or monaural or one channel. This means that a speaker could be connected on the R(ight) channel and a speaker on the L(eft) channel. This implies that every tank is designated one output location on the amplifier.

External audio card: (Esi U46XL USB audio interface) Each ouput location of the U46XL is

double (left and right channel for stereo sound). Each output channel was split with a simple splitting domino into two input channels (R and L) on the amplifier, and hence fed one tank. This means that two output locations with two channels each would provide for the four tanks.

The input from the U46XL comes directly from the computer through a USB cable.

Computer: The shipsound was played in a loop on Sound Forge Pro 10. The sound recording was in the ".wav" format and altered from a mono, one channel sound into a six channel sound.

The speakers were exitors (60x60mm, Visaton EX 60 S), fit with 2 connectors: a big positive one and a small negative one. The audio wires between the speaker and amplifier were standard audio cables (rip cord twin flex cable). The wires between amplifier and U46XL were standard audio wires, commonly used in home environments.

All systems were 'protected' against the humidity in the laboratory by placing a cardboard box over the computer and a cardboard box over amplifier and U46XL. Under these boxes a cup with silica gel granules was placed to absorb the moisture close to the appliances (Fig. 5.7). Because the box covering impeded cooling, the boxes had to be removed once a day for 15' to permit cooling. This was not a big issue as it would permit the replacing of orange granules, which are saturated with water.

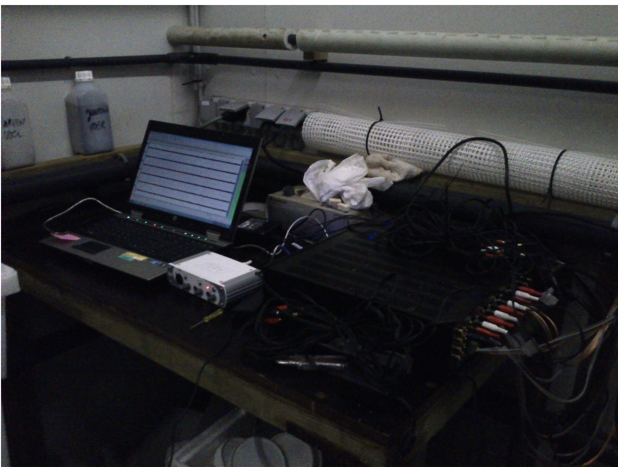


Fig. 5.6: Audio playing setup (Own collection)

Fig. 5.7: Silica gel granules (Own collection)

5.1.2.2 The sound measuring setup

The sound measuring system with hydrophone and particle motion meter/accelerometer (Fig. 5.8):

- A **hydrophone** (Bruël & Kjaer, type 8104, 10m cable) was connected to the charge-channel (nr. 1) on the amplifier.
- A **particle motion meter** was built with three independent accelerometers (Bruël & Kjaer, type 5958-A-010, 10m cable), fixed on a stainless steel block in three directions to form an orthogonal setup. Each of these were plugged into the amplifier on its deltatron channels (nr. 2, 3 and 4).
- The four output channels of the **amplifier** (Nexus, type 2690-OS) were connected to the four input channels of the recorder.
- Sounds were recorded onto an SD-card (SanDisk Ultra 16Gb) with a **recorder** (Tascam, type DR-680) at 24bit-rate and 44kHz in a wav-file. The recorder showed the file name on the display. Each channel was recorded into a separate file.

All equipment is portable and allows for use on board to make in situ recordings if chosen.

Fig. 5.8: Sound measuring setup
(Own collection)

5.2 Gathering the brown shrimps

The brown shrimp were collected with the marine research vessel RV Simon Stevin with a small version (3m) of the standard trawlnet (8m) on 6 consecutive hawls outside the harbour of Ostend (Table 2) on February 18th 2016. The trawling did not result in big hauls, as the fishing season is between April and November, with a peak during the months of September and October. The caught brown shrimp were kept in life tanks both on board and during transportation by car to the ILVO in

Ostend, Western-Flanders, Belgium. On board the life tanks were provided with a run-through system of seawater taken directly from the sea. At ILVO, they were transferred into two large tanks (90x60x30cm) in the exposure room/laboratory for acclimatisation (tank nrs. 11 and 12, Fig. 5.4). Both tanks were equipped with a seawater recirculation system. A 3cm bottom layer of sand was provided to allow the brown shrimp some cover.

	Start/End	Time	Latitude	Longitude
Tow 1	Start	09:20	N 51°15'11,4"	E 002°50'50,2"
	End	09:35	N 51°14'65,7"	E 002°49'70,5"
Tow 2	Start	09:45	N 51°14'15,6"	E 002°48'84,0"
	End	10:30	N 51°15'73,8"	E 002°51'69,0"
Tow 3	Start	10:40	N 51°16'00,0"	E 002°52'07,0"
	End	11:25	N 51°14'51,0"	E 002°49'34,5"
Tow 4	Start	12:00	N 51°14'16,0"	E 002°48'93,0"
	End	12:45	N 51°15'60,1"	E 002°51'43,0"
Tow 5	Start	13:05	N 51°16'03,0"	E 002°52'03,0"
	End	13:50	N 51°14'62,0"	E 002°49'49,0"
Tow 6	Start	14:10	N 51°14'13,0"	E 002°48'87,0"
	End	14:55	N 51°15'51,0"	E 002°51'56,0"

Table 2: Start and end positions of fishing runs on 18/02/2016
(Own collection)

5.3 The design

Test phase Start and end positions of fishing runs on 18/02/2016

On February 23 the complete sound setup was tested. The tanks were subjected to sound from the fitted speakers and a control record was made of the resulting noise to see if the noise production was similar within the two sets of tanks. The results did not seem satisfactory at first, but after consideration, they were considered good as tanks nr. 5 and nr. 6 were left out (Fig. 5.9).

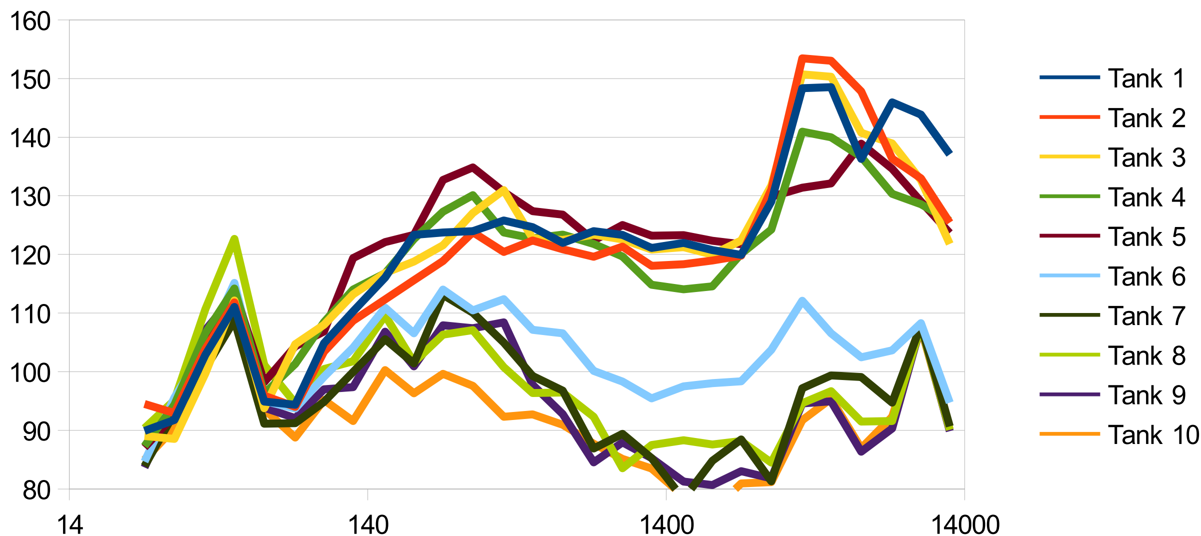


Fig. 5.9: Graphic sound in all tanks (1-10)
(Own collection)

Acclimatisation

On February 29 tank nr. 1-4 were fitted with speakers and all tanks that would be used for the experiment (nr. 1-4 and 7-10) were supplied with brown shrimp. Twenty animals were put in each individual tank, viz. ten from tank nr. 11 and ten from tank nr. 12. The following day, 4 more shrimps (two from respectively nr. 11 and nr. 12) were put in each tank, totalling the amount of shrimps per tank to 24. This was done because already some shrimps had died overnight due to cannibalism after moulting.

Experiment

On march 2 the experiment was started. The sound producing system was setup, after which the first set of shrimp samples was taken as reference. Then the sound was started up, playing a two minute recording of the underwater noise from Bro Distributor. Twenty hours after starting the sound, a set of shrimp samples were taken a first time during the subjection period. Another six days later another set of shrimp samples was taken. Seven days and 1h45min later a fourth set of shrimp samples were taken after which sound was switched of and the sound producing setup removed. One day and 22h30min after stopping the sound, a last set of shrimp samples was taken. This concluded the experiment.

At intervals, sound measurements were taken to ensure the sound would remain more or less the same for the duration of the experiment. The first sound measurements were taken before the first sampling (during absolute silence) and again after the sound was started up for initial observation. On the 3rd, 7th, 10th, 13th and 15th day sound measurements were taken for monitoring purposes.

The shrimp were fed with three types of dead species to keep them alive and prevent cannibalism to a certain extend. Each tank was fed one day before the start of the sound treatement with one mussel (*Mytilus edulis*, Fig. 4.20). On day 7 they were fed another mussel. On day 9 they were fed a king ragworm (*Alitta virens*, Fig. 5.11) as well as on day 11. The 15th day day were fed another mussel and finally on day 17 they were fed with equal amounts of European sprat (*Sprattus sprattus*, Fig. 5.10).

Fig. 5.10: Sprattus sprattus
(Own collection)

Fig. 5.11: Alitta virens
(Own collection)

5.4 Sampling and data collection

The effect of physical manipulation (capture and handling) on animals is a key consideration when making in situ or laboratory measurements of physiological performance or parameters. For example, Vismann (1990) showed that field measurements of filtration rates of mussel *Mytilus edulis* were significantly reduced by minimal manipulation. (Hunter and Uglow 1993)

5.4.1 Observation

We followed the suggestion of Menezes et al. (2006) that chemical and physical variables in the environment, which induce biomarker responses, are easily checked and as such should be. To this purpose, observations were carried out preferably during 'daylight' to minimize disturbances.

5.4.1.1 Temperature and Acidity

Temperature and acidity were checked daily with the readings on the monitoring system present at ILVO, which employed IKS aquastar sensors. The monitoring system is linked to the filtration system which is centralized for the site, but has several zones. The acidity and temperature are linked to daily fluctuations in the North Sea.

5.4.1.2 Salinity

Salinity was checked once with a refractometer (Atago, type S/Mill-e) on the first day. It is assumed to be constant as the system is large enough to be considered stable.

5.4.1.3 Mortality

Mortality was visually checked daily. Dead shrimp were removed with a simple net as soon as possible in order to ensure they didn't becoming a meal for the surviving ones. Disturbance was kept at a minimum however: only local lighting by mobile phone was used to count the living ones

and remove the dead.

5.4.1.4 Sound pressure and particle motion

To start recording, the amplifier and recorder need to be started and following protocols need to be followed. First off all though, the hydrophone and 3D accelerometer need to be put into the water. It is important to make sure that: both hydrophone and 3D accelerometer are completely submerged in a position and depth which is the same every time in every tank, are free from each other and the tanksides, and are not positioned in front of the water inflow; that the amplifier and recorder are unplugged and run on batteries or that the earthing is plugged in; and that cables make as little contact as possible (certainly with the audio cables to the speaker).

On the amplifier (Fig. 5.12):

- Switch on the amplifier
- Starting screen:
  nr. 1 (channel) measures pressure from the hydrophone in *mV/Pa*
  nr. 2 – 4 measures particle-acceleration from the accelerometer in V/ms^{-2}
- To access the main menu: press home
- Choose “Amplifier setup” to alter sensitivity, which must always be recorded for calculations, when prosessing the data:

  nr. 1 was set to 10 *mV/Pa*

  nr. 2 – 4 were set to 3,16 V/ms^{-2}

  These settings are chosen to get an audible output (of sufficient strength to be 'heared' by the recorder), but to such a level as not to damage the amplifier. Each led next to the screen would light up when the signal was to strong.
- Choose “Trandsducer test / Reference signal” to play a reference signal on the amplifier with a known value that can be offset in Hz for calibration of the recorded signal:

  nr. 1 was at 159 *Hz*

  nr. 2 – 4 were set at 1 *kHz*

Fig. 5.12: Displays with various menu's on the amplifier (Own collection)

On the recorder:

- Switch on the recorder
- The main screen shows the following info: bit-rate, frequency-depth, file type, file number and a timer
- To record press “pause”, then press “record” and to stop recording press “stop”
- Each recording should include a short reference signal to allow the user to compare different measurements

5.4.2 Sampling

Brown shrimp (*C. crangon*) were subjected to the sound for a prolonged time. At time intervals samples of the shrimp were taken to test for protein concentration and HSP70 expression.

Sampling was performed during 'daylight' hours. While sampling red lightning was switched on to improve visibility for the fishing, while limiting the disturbance for the shrimps, and switched off again in between.

Equipment

The following equipment was needed for the sampling of the shrimps: gloves, liquid nitrogen, a petri dish, a scalpel and pincette, a ruler, a jar, some seawater from the tanks, a balance, cryo vials, a special marker for -80°C, a pencil and paper for data recording and tissues.

Procedure

The jar was filled with some seawater for temporarily housing of the samples. Labels with identification marks were put on the cryo vials. A set of 4 shrimps from a single tank was taken and put in the jar.

In the lab the balance was tared to the weight of the petri dish. A shrimp from the jar was pat dry on

a tissue and put it on the petri dish. The length of the shrimp was measured from the tip of the tail (telson) to the eyes (not including the latter) using the ruler (Laverack and Crombie 1988). The shrimp was weighed including the petri dish on the tared balance. Now the shrimp was killed with a steady stab in the carapace using the scalpel and the abdomen (plion) was cut away from the rest of the body where the carapace meets the first pleonite. Then the balance was tared to the weight of a cryo vial. Using the scalpel the pleura, pleopods and tail (uropods and telson) were removed. The remaining tissue (abdominal musculature) was put in the designated cryo-vial, which then weighed using the balance. The cryo vial was then snap-frozen in liquid nitrogen.

This process was repeated for each shrimp and each tank. The processing of each set of 4 samples took 20-30min. Each time sampling took a little less time, from 4h the first time to 2h55min the last time.

When the shrimp tissue was sufficiently frozen the cryo-vials were put in the freezer (-20°C) for preservation (Madeira et al. 2012).

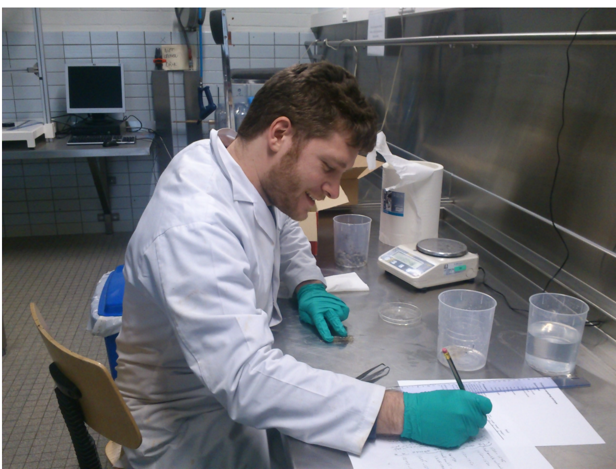


Fig. 5.13: Taking samples (Own collection)

Fig. 5.14: Taking samples (Own collection)

5.4.3 Processing the data

5.4.3.1 SPL and particle motion on computer

To process the recordings on the computer the following steps were taken:

- Using Sound Forge Pro 10 all sounds (from the four channels) were combined into one file using copy-paste. This means that 4 single channel (mono) .wav-files need to be pasted into 1 four-channel .wav-file.
- The 4 reference signals from the individual channels were transferred into separate calibration wav-files (ex. cal1.wav)

- All excess recordings that were not from the produced sound period were removed from the track.
- All created files were collected into one map for each tank and the Matlab program script (calibration files, 4 channel sound recording, script) was included.
- The script was run in Matlab with all settings adjusted to requirements (sensitivity, etc.), and the sound data were processed by pressing play.
- The results appeared in the folder in a .txt-file (standard notebook file). These results could then be easily copy-pasted into a .xls (excel-sheet) and put into a graph.

5.4.3.2 Sample processing

The samples, which were frozen at -20°C need to be processed, because the point is to know the HSP70 protein concentration. In order to do this, all proteins need to be extracted from the sample (see Protein extraction). Then the total amount of proteins present in each sample will need to be established (see Protein analysis). With this information the different proteins can be told apart and the amount of HSP70 can be found (see SDS-PAGE (Laemmli)). The proteins can then be fixed and coloured with a Coomassie Blue staining (see Stabilizing the visual result).

All chemical products are produced by Acros (unless otherwise stated) and delivered by Filter Service in Eupen, Belgium.

Protein extraction

Equipment

The following items and substances were required to extract the proteins from the samples: a mixer (Ultraturrax; from Filter Service, Eupen, Belgium), containers/tubes which fit into the centrifuge disk and allow the samples to be mixed in, a centrifuge (Eppendorf centrifuge 5430F with F-35-6-30 rotor and FA-45-30-11 rotor), pipettes of different sizes, Eppendorf tubes, freezer, acidity-meter, beaker, stirring rod, mannitol, EDTA, HEPES, ascorbate, demineralized water

Procedure

First of all, the extraction buffer needed to be prepared. The extraction buffer or homogenisation buffer was a solution/mixture of 0,3 M mannitol; 1 mM EDTA; 30 mM HEPES; and 4 mM ascorbate in demineralized water. After making this mixture, Tris-HCl was added until an acidity of 7,5pH was reached. The extraction buffer is used to separate the proteins from the other substances and keep the proteins in a stable form at the same time.

For every sample the following steps had to be taken:

Samples were selected from the stock in the freezer and kept at 4°C (which means that they are to be kept in ice water). Keeping the samples at 4°C stopped protein activity. The sample was put into a workable tube, which fitted in the centrifuge rotor and allowed for the mixing of the sample tissue. A manageable amount of extraction buffer was then added to the sample (in 10ml cryovails 2000µl was added and in the smaller tubes (3ml, made to fit in the centrifuge disk) 500µl was added), which would allow for a liquid homogenous result, which didn't spill over when mixing. Now the sample tissue was mixed. After mixing, the remainders on the mixer were rinced into the tube with an additional 500µl extraction buffer. The mix was kept at 4°C at all time. The next step was to put the homogenates into the centrifuge. This was done paired, placing two tubes in opposite locations to keep the centrifuge stable. The homogenates were centrifuged at 7200g at 4°C for 20'.

After centrifugation the supernatant was stored into eppendorf tubes and temporarily frozen at -20°C awaiting further processing.

Protein analysis

Based on (Potters, 2005; Noble and Bailey, 2009; Klarbring, 2011).

There are three types of protein assays that are popular in modern science, they are: BCA protein assay, Bradford assay and Markwell assay. The fourth Lowry assay is older, but still holds some users.

The **Lowry assay** (Lowry et al., 1951), being the oldest one, forms the base for the others. The first step of the assay is a biuret reaction in which proteins reduce Cu^{2+} to Cu^{1+} in an alkaline environment (which obviously contains Cu^{2+} in the first place) due to peptide bonds. Active constituents in the now added Folin and Ciocalteau reagent are then reduced by the newly formed Cu^{1+}, which colours blue. As the amount of Cu^{1+} is proportionate to the amount of proteins, the resulting blue colour can then be measured at 750nm.

The **BCA assay** (Smith et al., 1985) uses bicinchoninic acid (BCA). The first step is the same biuret reaction as in the Lowry assay, but BCA is added to the mix. Four BCA-peptides bind to one Cu^{1+}-ion, forming a complex with an absorbance at 562nm, which can then be read as proportion to the amount of proteins.

The **Bradford assay** (Bradford, 1976) uses the binding features of Coomassie Brilliant Blue G-250. This substance, which is coloured red, changes in colour to blue when it binds with proteins. This

means that when monitoring the change from 465nm to 595nm, the amount of proteins can be deduced.

The **Markwell assay** (Markwell et al., 1978) is more or less the same as Lowry's method. The big difference is that Markwell added sodium dodecilsulphate (SDS) to the mixture. SDS allows for faster solubilisation, compared to Lowry's overnight reaction requirement. The Markwell essay is read at 660nm however.

When choosing between these assays there are a number of things to take into account. Accuracy is obviously the most important one, but sometimes other aspects take preference. When a quick result is required with not to much accuracy to get a general sense of protein contents the Bradford assay is to be chosen, as it can be performed fast and easily. The disadvantage of the BCA method is that several reduction agents can interfere with the Cu^{2+} and thus alter the results. The Lowry assay has similar problems, which are somewhat overcome in the Markwell method. The added SDS prevents the precipitation of non-ionic and cationic detergents and stabilises the soluble copper content in the medium, denying these from binding to sucrose.

As there was more familiarity with the Markwell assay, it was chosen for this study. The following protocol was to be followed for the results of this study (Potters, 2005).

All colour measurements are done using a spectrophotometer.

Equipment

Beakers, erlenmeyers and containers for the reagent, stirring rod and automatic stirring apparatus, material to perform tasks at 4°C (au bain marie; small vessels, water and ice supply; cold bench), cuvettes tailor made for the spectrophotometer, a spectrophotometer, an adequate set of pipettes, timer, Na_2CO_3, NaOH, Na-tartrate, SDS, demineralized water, $CuSO_4.5H_2O$, Folin-Ciocalteu reagent

Procedure

First of all several reagent needed to be prepared (of which A and B could be kept for years at room temperature). Another required reagent is the Folin-Ciocalteu reagent, which was bought commercially. The Folin-Ciocalteu reagent is to be diluted with demineralized water a ratio 1:1 however.

Reagent A consists of 2% Na_2CO_3; 0,4% NaOH; 0,16% Na-tartrate; 1% SDS and is completed until 100% with demineralized water.

Reagent B consists of 4% $CuSO_4.5H_2O$ and 96% demineralized water.

Reagent C is to be made at last minute before use and is the mix of reagent A and B at a ratio 100:1.

To be able to determine the protein concentration by colour intensity, a calibration line is required. This is done with a solution of 1mg/ml chicken serum albumin (a simple protein that can be commercially purchased) in the same extraction buffer as the sample homogenates. This means that a series of cuvettes were prepared to contain 0, 10, 20, 40, 60, 80, 100, 150 and 200µl of the albumin solutions diluted to 1ml with demineralized water. This calibration was made in duplo. From every sample homogenate a similar cuvette with 10, 20 and 40µl was made and diluted to 1ml with demineralized water. The next step was to add reagent C (freshly mixed and the same mix for every sample). With 30" intervals 2ml (2000µl) reagent C was added to every cuvette. This was allowed to sit overnight (a minimum of 30' is required). Then 200µl Folin-Ciocalteu reagent was added at 30" intervals to each cuvette. After setting for 45' a stable colouring should have become which was then read at 660nm (at 30" intervals) using the spectrophotometer. These results could then be interpreted in comparison to the calibration curve from the albumin solutions.

SDS-PAGE (Laemmli)

Based on (Potters, 2015; Caprette, 2005a, 2005b, 2012, 2015b; He, 2011)

SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis is the process of separating macromolecules in an electric field. In the case of SDS-PAGE or Laemmli method (Laemmli, 1970), the proteins are run through a discontinuous polyacrylamide gel and the sodium dodecyl sulfate (SDS) is used to denature the proteins.

Step 1

The first step of the method is the preparation of the protein sample for the SDS-PAGE. This means that the sample has to be mixed with a treatment solution for denaturation, adjusting viscosity, colouring, correcting acidity, uniforming electrical charge and adjusting acidity. Unfortunately, not all these things can be done by a single molecule. Denaturing, as mentioned before, is the deformation of a protein. For the purpose of this procedure, the protein needs to be relieved from primary (acid sequence of the polypeptide), secondary (interactions with water leading to local 2D structure, including helices, pleated sheets and turns), tertiary (hydrogen bonding, hydrophobic amino acids, Van der Waal's forces, and disulfide bonding all together lead to a 3D structure) and quaternary structure (covalent or non-covalent bounds to molecules making them functional proteins). To this purpose SDS, DTT (dithiothreitol) and heat are added. SDS molecules will link up to the primary structure (functionally removing it), giving the protein a negative charge and adding significant weight (the ratio of SDS to proteins is about 1,4g:1g). As equal charges repel, the protein

will become a linear structure (1D) and thus remove the secondary and tertiary structure. Some proteins will however be linked to strong hydrophobic molecules (such as lipids). By adding heat, these will be shaken loose to allow the SDS to do its work. Sulfhydryl-groups will bound together to form covalent disulfide bounds. These strong bounds require a strong reducing agent id est DTT. Tris is concentrated HCl and as buffer is used to modify the acidity. Glycerol adjusts the viscosity, protecting the sample from floating out of the well. The dye adds colouring for visual effects.

Procedure of step 1

Dilute SDS from the bottle to 10%. Make the 2x treatment solution: a solution of 4% SDS 10%; 20% glycerol; 0,2M DTT; 0,02% bromophenol blue; Tris HCl pH 6,8 and demineralized water. Prepare the smaples with extraction buffer to all contain the same concentration of proteins. Now mix 1 volume of the prepared samples with 1 volume of 2x treatment solution and heat for about 10min (boiling is not needed). A final protein concentration of 2mg/ml should be optimal.

Step 2

The second step is the actual protein separation by means of the PAGE. There are two types: continuous and discontinuous. The continuous method uses one gel, while the discontinuous uses two gels with a different buffer (Fig. 5.16). Though the continuous is easier te make, the discontinuous gives more accurate results and should therefore be preferred.

The complete construction of the PAGE consists of a gel box, one to four gels between two plates (0,75mm; 1mm; or 1,5mm apart), power supply (anode and cathode), an electrode buffer and a comb. In Fig. 5.15 the complete assembly can be seen. The complete setup can be commercially bought, but they can be made in a lab too. The procedure of making the gel is described below.

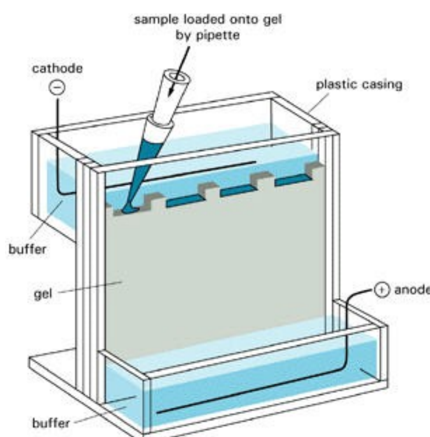


Fig. 5.15: SDS-PAGE (Lane, 2001)

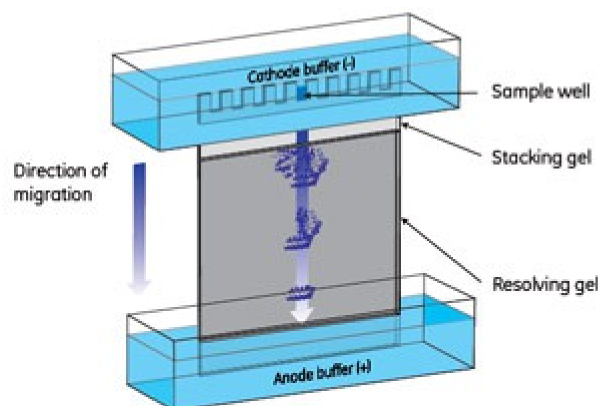


Fig. 5.16: SDS-PAGE (Own collection)

The PAGE method is based on the molecular mass, the size and electric charge of the proteins. The

protein samples are pipetted into the wells (small gates that are left after removing the comb), which are submerged by the electrode buffer. The samples will come to lay on the bottom of each well. One well needs to be used for a calibration solution. As visible in Fig. 5.16 the two gels are on top of each other: the top gel is the stacking gel and has large pores; and the bottom gel is the resolving or separating gel and has much smaller pores. When applying a current between the electrodes, the negatively charged proteins will first travel through the stacking gel towards the anode. These will stack in order of molecular mobility upon meeting the separating gel. After migrating into the stack the proteins will start to travel down the separating gel. The pre-ranked proteins travel through the much smaller pores in this gel, which means difference between the larger (heavier) proteins above and the smaller (lighter) proteins below will be magnified in distance travelled. The migration distance (R_f) is thus negatively proportionate to the log of the mass of the protein. The calibration solution will give a logarithmic scale of molecular mass. When the pure die line reaches the bottom of the separating gel, electrophoresis is complete and the current is switched off. At this point the proteins stop migrating and are "fixed" in place in order of molecular mass (M). The thickness and colouring of the band will give an indication of the abundance of proteins with that mass.

Procedure step 2

- Making the gels:

First a gel cassette needs to be assembled. It is a setup that holds two glass plates separated by a (teflon) spacer on both sides, sitting in a three-sided box. The spacers should be as thick as the gels should be and work with the comb, because the gel will be made in between. A gel casette can hold several gel sandwitches by placing them against one another and it is sealed by an additional transparent plate. The setup is made waterproof by sealing the rims on both sides and bottom (e.g. with clay). Once this is done, temporarily insert a comb into the foremost space and make a mark about 1cm below the bottom of the comb. This line will mark the top level of the separating gel (Fig. 5.17). Remove the comb again.

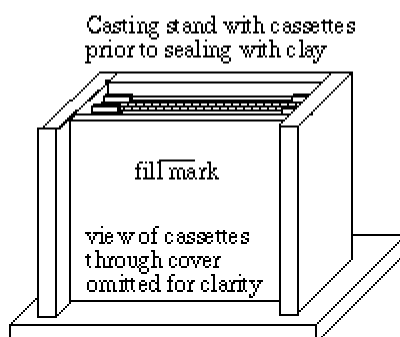


Fig. 5.17: Gel-casette (Caprette, 2012)

The next step is preparing the solutions needed to make the gel (both gel mixes will be described in detail below). These are the solutions:

- Acrylamide solution 30%: 29,2% Acrylamide (FW 71.08) and 0,8% bisacrylamide (FW 154.17) in demineralized water. THIS IS A VERY DANGEROUS PRODUCT
- Separating gel buffer: 1,5M Tris HCl; pH 8,8; 200ml
- Stacking gel buffer: 0,5 M Tris HCl; pH 6,8; 50ml
- Ammonium persulphate: 10% Ammonium persulphate (FW 228.2) in demineralized water.
- Water saturated n-butanol: 50ml n-butanol to 5ml demineralized water. Mix well and allow phases to separate. Only the top layer is the required solution. Separating gel compositions
- Separating gel mix (10ml): The different percentages are for the varying concentration of acrylamide (T%). The choice depends on the min/max mass of molecules that should be observable: high concentration and density allows for small molecules and vise versa. A 12,5% concentration should work according to Napierska (1998).

		5,0%	7,5%	10,0%	12,5%
Acrylamide 30%	ml	1,67	2,50	3,33	4,17
Separating gel buffer	ml	2,50	2,50	2,50	2,50
SDS 10%	ml	0,10	0,10	0,10	0,10
Demineralized water	ml	5,83	5,00	4,17	3,33
TEMED	µl	5	5	5	5
Amonium persulphate	mg	10	10	10	10

Table 3: Separating gel compositions (Own collection)

- Stacking gel mix 4,5% (10ml): 1,5ml acrylamide 30%; 2,5ml stacking gel buffer; 0,1ml SDS 10%; 6ml demineralised water; 10µl TEMED; and 10mg amonium persulphate

The making of the gel mixes should be done carefully, because the purpose is a polymerisation. First the separating gel mix is made. The acrylamide 30%, the separating gel buffer and the SDS 10% should be diluted in the demineralised water in an erlenmeyer. Stopper the erlenmeyer and swirl for about 5' to deaerate the mixture. Now add the TEMED and amonium persulphate gently and swirl again without creating bubbles to mix. As soon as the mixing is adequate (will not take long), the now ready separating gel mix should be poored between the plates using a pipette up to the marked line. If multiple gels are made, all of them should be filled. Small differences in level will settle after a while. Top the gel mix with ±0,5cm of water saturated n-butanol, by gently pipetting it angled at about 45° in the corners near the sides. If after pipetting in the corners the first time, the level is not adequate, first progress to the next gels. This will allow the water saturated n-butanol to settle and prevents it from pushing the separating gel mix up in the other gels. The

topping off and blocking of the air, and deaerarion is necessary because the separating gel mix will polymerize in absense of oxigen. n-butanol is used, because water would mix with the gel. The additional benefit is that it will level the gel, showing a very clear separation line after 15-20' if succesfull. The added water will prevent the gel from drying out.

After 1h the gel should be completely polymerized. At that point the water saturated n-butanol can be removed again, using filter paper and/or by tipping it sideways and pipetting it out. Rince with some demineralised water and dry with filter paper and/or pipette. Now the stacking gel mix can be made, which is done in the same manner as the separating gel mixture. Pooring it on top of the separating gel can be done with the comb in place. Otherwise poor in enough (on sight) and place the comb (with pincers down) in the gel, ensuring there is no air trapped underneath. For the stacking gel, deaeration is not important, because uniformity is not required. The proteins merely need to run through.

When the stacking gel mix has polymerised completely (1h), the gels can be taken out of the casette with the glass plates and spacers attached, ready for use. The excess material can be cut of.

- Setting the PAGE up:

One solution needs to be prepared: the electrode buffer consisting of 25mM Tris; 192mM glycine; and 0,1% sodium dodecyl sulfate.

Fix the gels in the gel box, as depicted in Fig. 5.18. Top and bottom get a separate 'bath', the top one fixed with the cathode and the bottom one with the anode. The top of the gel with the wells, which were created by the comb, should be completely submerged (see fig. 5.16). In the form in Fig. 5.18, two gels are run simultanious. The inner glass plates being shorter than the outside. Both the anode and cathode chamber are filled with an adequate amount of electrode buffer. Inserting the electrodes and covering the gel box completes the setup.

- Using the PAGE:

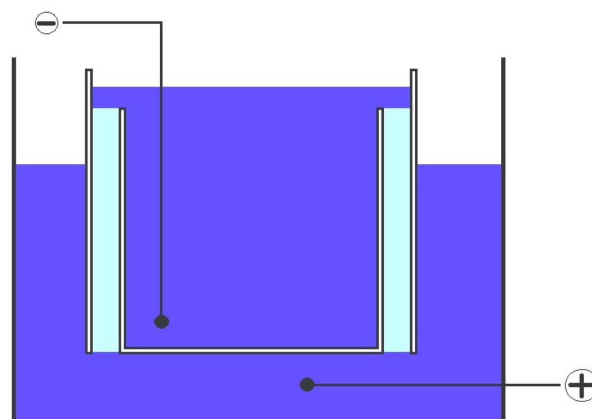


Fig. 5.18: Gel electrophorasis
(Own collection)

The powersource is connected to the alectrodes. The prepared protein samples are gently pipetted into the wells, making sure the tip is inside the well on the good side of the glass plates. The samples will settle on the bottom of the well. Use one of the outer wells to put in a calibration sample with known molecular masses. Now switch on the current: 150V should suffice; the current should be sufficiently high, but not to high as to cook the gels. After 10-20' the proteins should have stacked on the deviding line. Then the proteins should start travelling through the separating gel. When the lowest colour front reaches the bottom (preferably a little earlier), the power should be switched off. The proteins will start diluting into the gels when the power is switched off. The next step should be done as quickly as possible.

Stabilizing the visible result

There are several options to keep the results visible after SDS-PAGE. Those most common used are: Coomassie Blue staining or the silver staining. Coomassie Blue results in a stable picture of the presence of different proteins by size and their abundance to some extent. Though Coomassie Blue is not very exact it should provide the sought answers. For this reason Coomassie Blue is chosen to give the results.

Procedure

Four solutions need to be mixed before beforehand (some before the end of the SDS-PAGE). They are these:

- Fixing solution: 50% methanol; 10% glacial acetic acid; and 40% demineralized water.
- Staining solution: 0,1% Coomassie Brilliant Blue R-250; 50% methanol; 10% glacial acetic acid; and 39,9% demineralized water. (also Fixing solution and 0,25% Coomassie Brilliant Blue R-250 by weight)
- Destaining solution: 40% methanol; 10% glacial acetic acid; and 50% demineralized water.
- Storage solution: 5%glacial acetic acid; and 95% demineralized water.

When the power of the SDS-PAGE is switched off, the proteins will start diluting into the gel. This means it is paramount that the gel is quickly transferred into the fixing solution. It should be left there for at least 1h to overnight. After 1h the fixing solution should be changed once. The fixing solution causes the proteins to precipitate (become insoluble). The next step is to add colouring. Put the gel into the staining solution. According to the sources there are now two options: Sinica (2005) suggests putting it in for 20' with gentle agitation, while Haynes (2016) instructs to leave it in the solution on a shaker for 4h to overnight at room temperature. The gel can then be destained: the

excess colouring will be removed using the destaining solution. The gel is to be put in it and rinced, either using a shaker (Haynes, 2016) or rincing it in the solution (Sinica, 2005). The destaining solution should be replenished several times, until all excess dye is removed. The dye will bind to the proteins and not to the acrylamyde. Rincing will thus remove the unbound dye, while the proteins remain bound to the die. The final result will give a clear blue pattern of proteins. The gel can be stored in the storage solution.

Spectrophotometrics

A spectrophotometer is an instrument, consisting of two main parts: the spectrometer (which is a light with an adjustable wavelength, viz. colour) and the photometer (which measures the intensity of light). The cuvette with liquid is placed between spectrometer and photometer. The photometer can thus measure the intensity of light passing through the cuvette, which is proportionate to the light not passing through the liquid filled cuvette which is abdsobed by the liquid. The result is a displayed voltage.

If a colour intensity in a solution is proportionate to the concentration of the searched product, then measuring this intensity will result in a concntration. The Markwell assay results in a blue colouring proportionate to the protein concentration. This means that blue light passes through the best. Green and red light are thus absobed most. Measuring the red light at 660nm wavelength will thus give a good result.

In a formula, the following is correct (Beer's law):

$$I = I_0 * 10^{-kcl}$$

$$\frac{I}{I_0} = 10^{-kcl} = T$$

$$-\log T = \log A = \log \frac{I}{I_0} = kcl$$

where: I = transmitted light through pure solvent; I_0 = transmitted light through solution; k = constant depending on the wavelength and product; c = concentration of the coloured product; l = distance travelled by the light through the liquid; T = the transmittance of the solution; A = the absorption of the solution. (Caprette, 2015a)

The cuvettes are made 1cm wide, thus l equals 1cm. The constant k is a known number. This means that the concentration c is directly proportionate to the absorption A on the constructed logaritmic calibration scale.

$$\log A = c \,.\, cte$$

6 Results

6.1.1 Physical environment (temperature, acidity and salinity)

The temperature fluctuated between 8,0°C and 8,7°C, with an average of 8,4°C (stdev <0,09). Acidity fluctuated between 8,36pH and 8,41pH, with an average of 8,39pH (stdev <0,01). Salinity was constant at 35‰ throughout the experiment (Fig. 6.1).

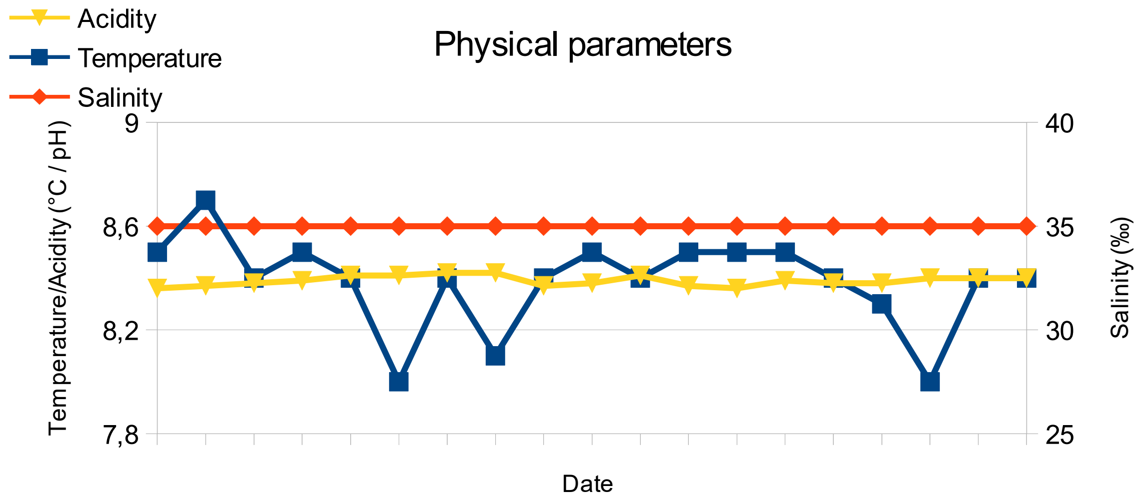


Fig. 6.1: Physical parameters
(Own collection)

6.1.2 SPL/ Particle motion

Sound measurements on the different days of each tank resulted in the graphics above the line (Fig. 6.2). In each graphic, each tank is displayed in a separate curve with a colour constant through all graphics depicted. The left-hand graphics display SPLs in dB re 1 µPa as a function of frequencies in third octaves[15]. The righthand graphics show particle motion in function of frequencies in third octaves.

The graphics show more or less constant SPL graphics for all tanks but one, and a differing measuring in tank nr. 1 on day 3. The tank which shows some difference in the graphics is tank nr. 3. The particle motion graphic for day 13 is inexplicably different from the others, not taking into account a different axis offset that was applied.

It is clearly observable that there was some influence from the noise from the loud tanks (nr. 1-4) on the silent tanks (nr. 7-10) for SPLs. This is especially true near the 250Hz frequency rise. When examining the graphics of particle motion, no influence was noticeable though.

[15] 1/3 octaves are: 25; 31,5; 40; 50; 63; 80; 100; 125; 160; 200; 250; 315; 400; 500; 630; 800; 1000; 1250; 1600; 2000; 2500; 3150; 4000; 5000; 6300; 8000; 10000; and 12500 Hz

When no sounds were played, peaks in SPL are observable around 50Hz and 10000Hz in all tanks. This peak is only observable at 10000Hz in particle motion though. Below 50Hz SPL was barely increased by the played noise, while above SPLs clearly rose, with a plateau between 250Hz and 2500Hz and a peak around 5000Hz. For particle motion, levels only start rising above 125Hz, with clear elevation above 200Hz.

The following graphics below the line (Fig. 6.2) show the daily measurements of each tank. The left-hand graphics display SPLs in dB re 1 µPa as a function of frequencies in third octaves. The right-hand graphics show particle motion in function of frequencies in third octaves. It is clearly visible that the SPLs and particle motion stayed very constant in each tank. Once again one measurement in tank nr. 1 on day 3 stands out and was considered erroneous. Because of the strangeness of particle motion measurements and differenct offset on day 13, these were not included in these graphics.

Averages

The figures in the table in Fig. 6.3 are average sound levels (all in dB re 1 µPa). The first section are averages by day of each tank measured. The figures on the right are averages of the former figures, in the first column of all the data when sound was on (day 1-14), followed by the average of the data when sound was off (day 1 and day 16). On the extreme right the SNR is calculated as the difference between the two columns on its left.

The data below the daily averages are the daily averages of the loud an silent tanks and their standard deviation. The bottom data represent the average of all data combined: Off is the average of all data when there were no sounds playing (121,2 dB; stdev: 1,51); Loud on is the average of all data of the loud tanks when sounds were playing (160,6 dB; stdev: 1,79); Silent on is the average of all data of the silent tanks when sounds were playing (127,8dB; stdev: 1,63).

The graphic in Fig. 6.3 gives a visual impression of those figures. It can be noted that the sounds in tank nr. 3 were significantly lower (5-10dB), resulting in high standarddeviations. On day 2 in tank nr. 1, on day 1 with sound in tank nr. 4 and on day 13 in tank nr 2 sounds seem abnormally high too. There also seem to be rather big differences between measurements of the silent tanks on daily basis.

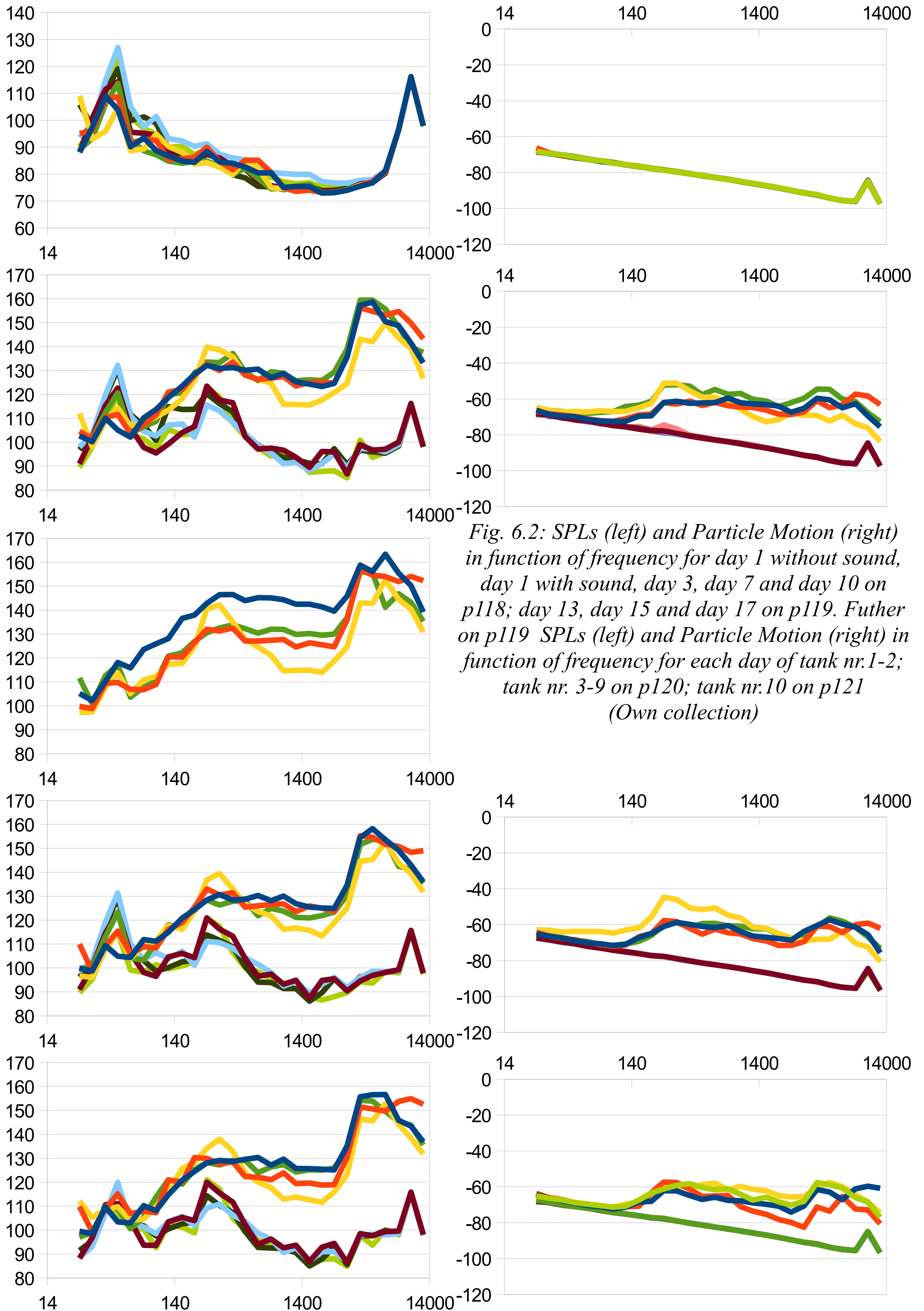
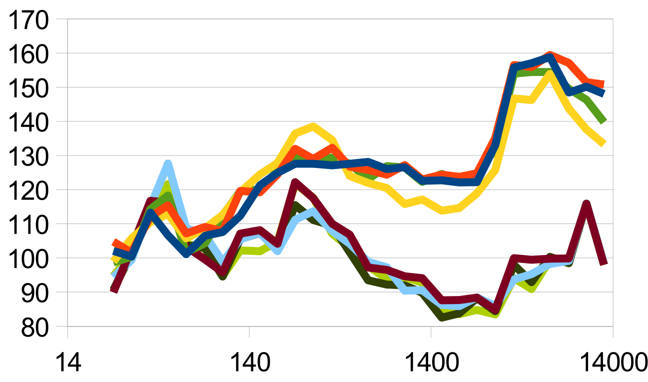


Fig. 6.2: SPLs (left) and Particle Motion (right) in function of frequency for day 1 without sound, day 1 with sound, day 3, day 7 and day 10 on p118; day 13, day 15 and day 17 on p119. Futher on p119 SPLs (left) and Particle Motion (right) in function of frequency for each day of tank nr.1-2; tank nr. 3-9 on p120; tank nr.10 on p121 (Own collection)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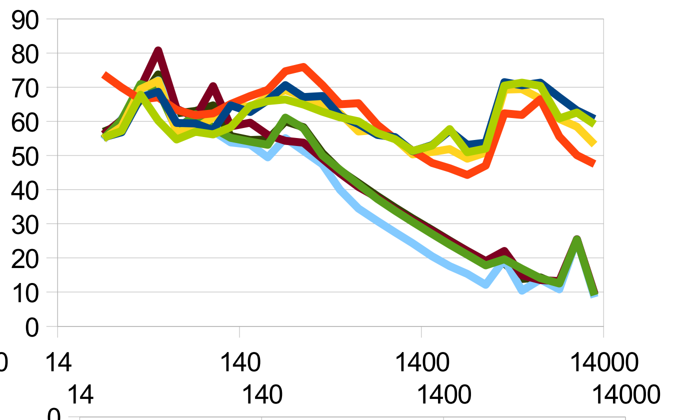
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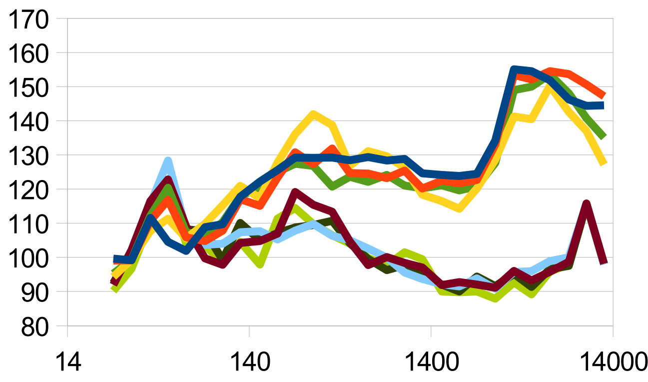
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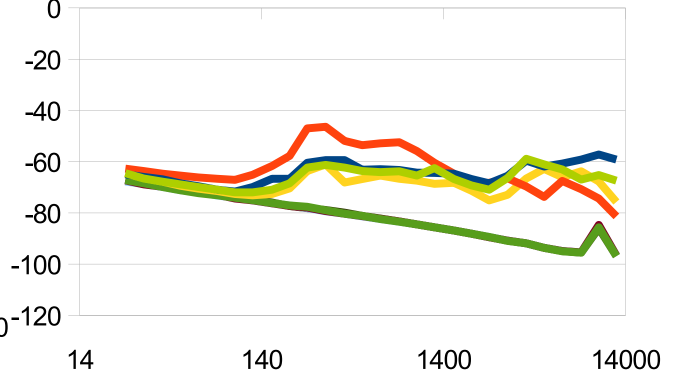
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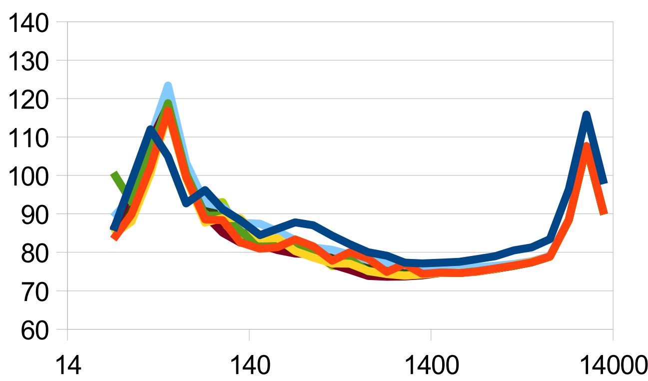
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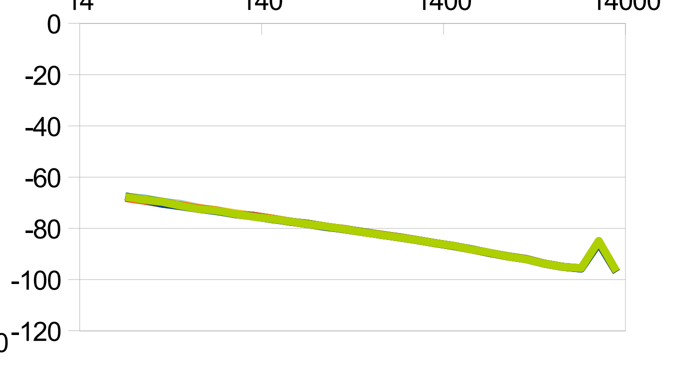
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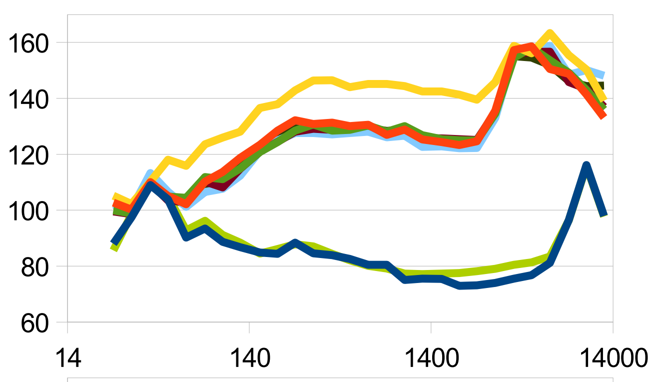
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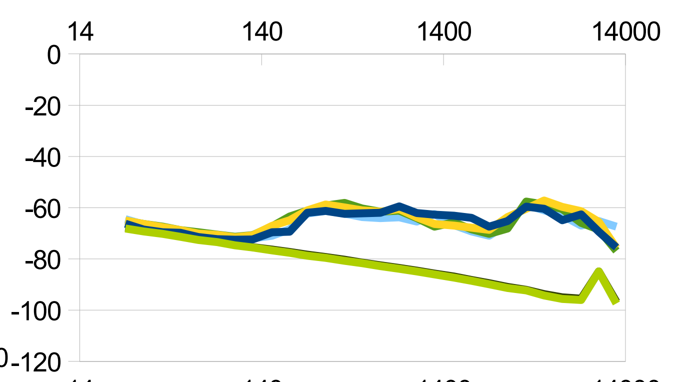
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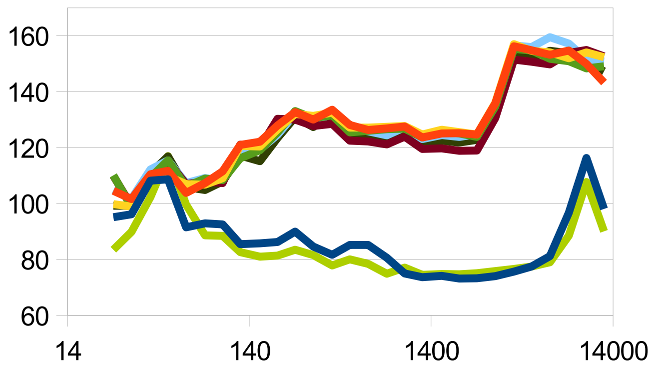
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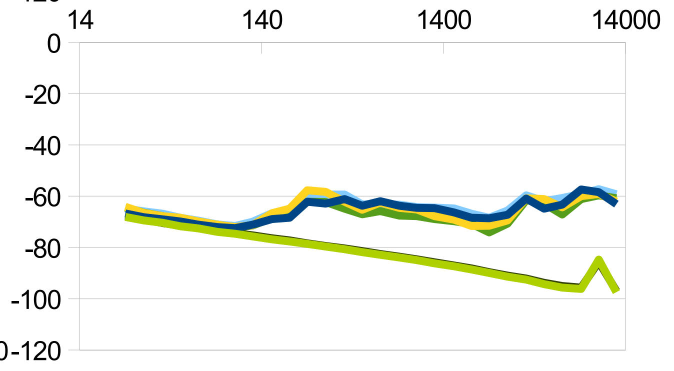
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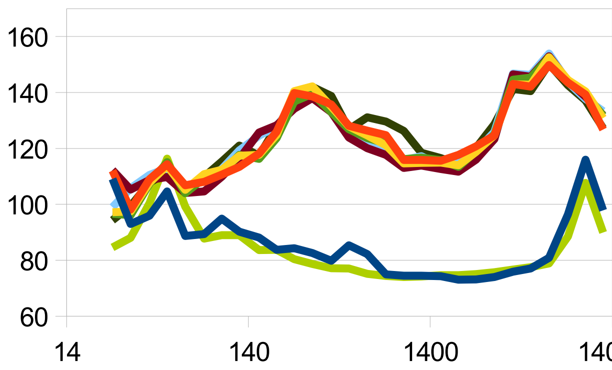
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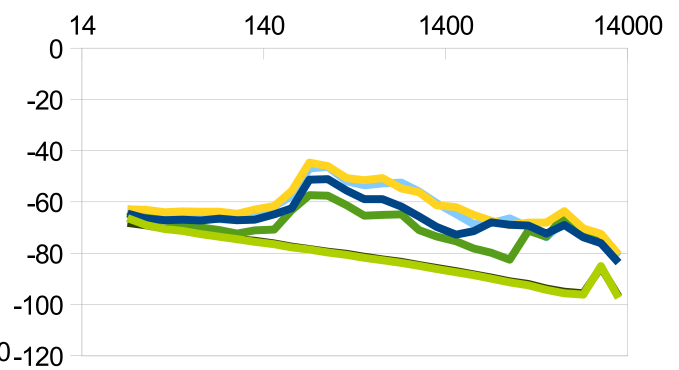
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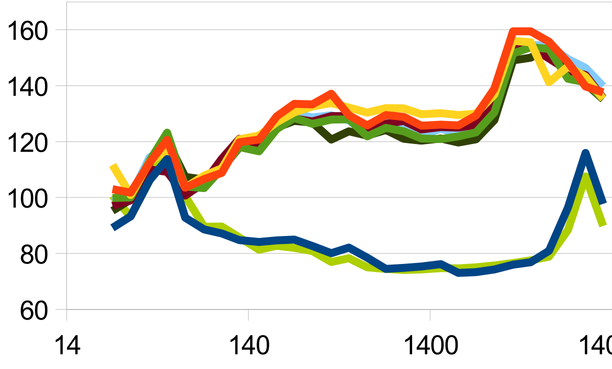
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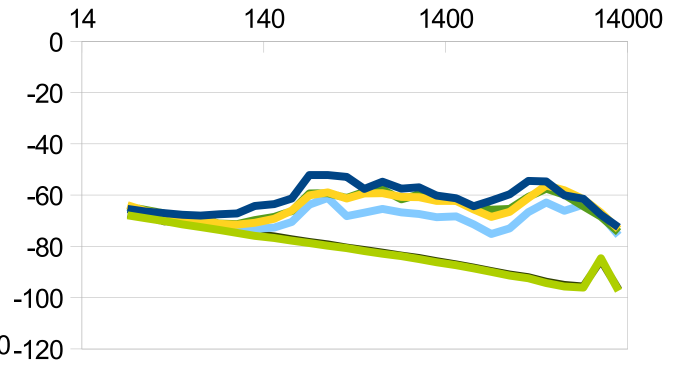
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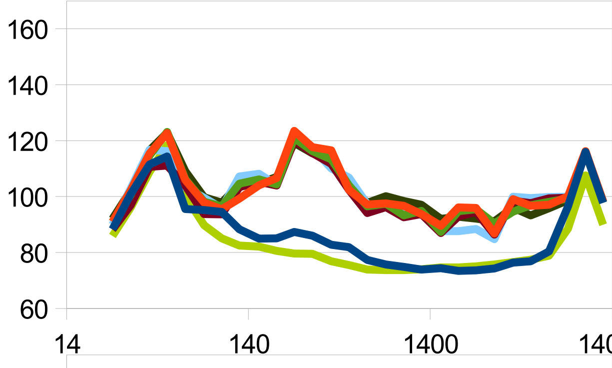
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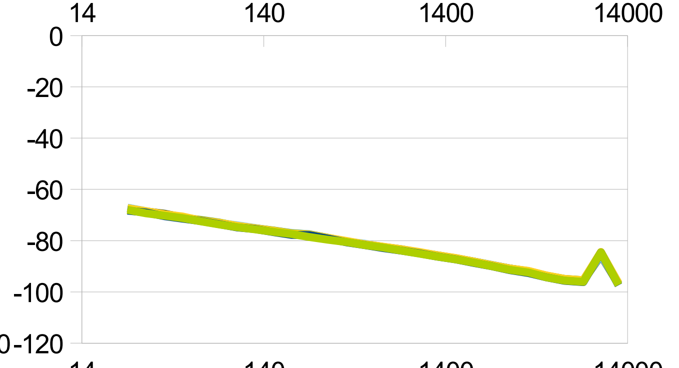
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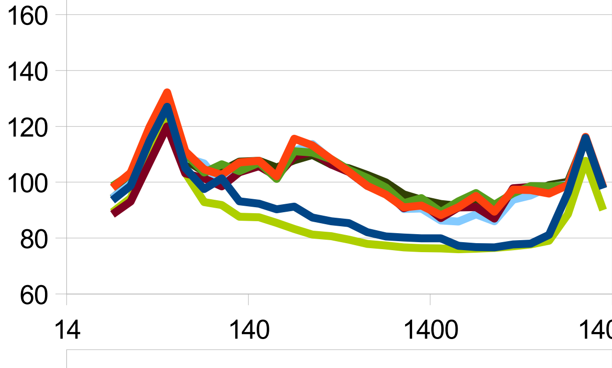
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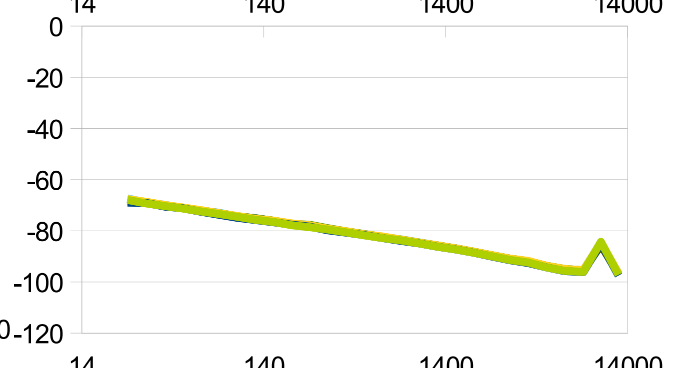
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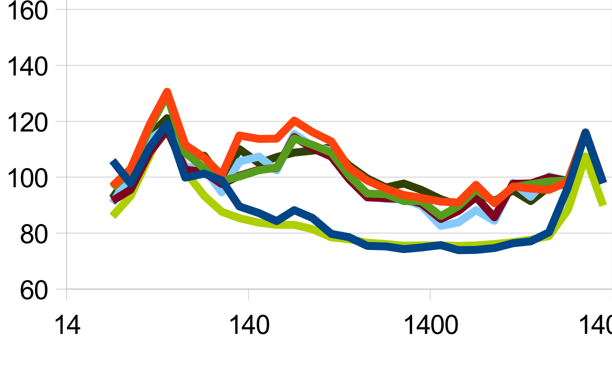
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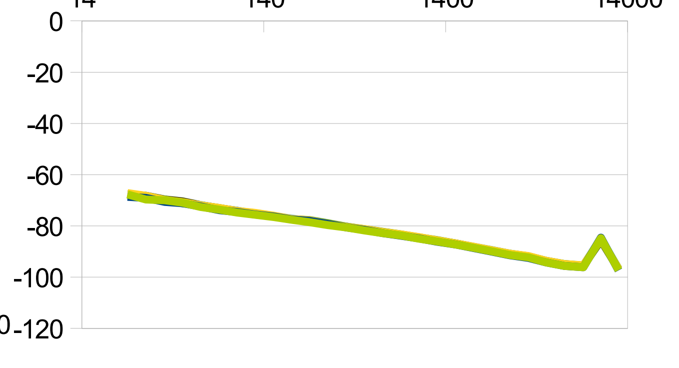
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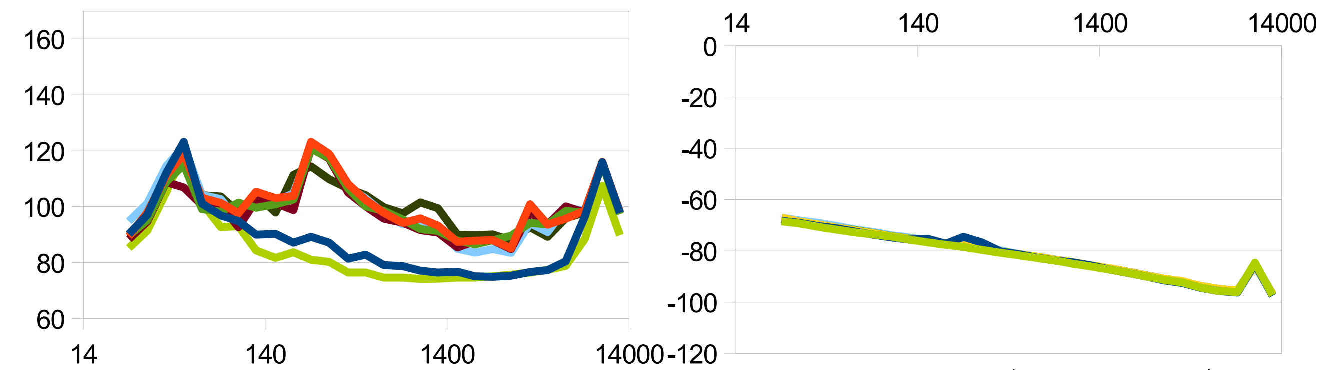


	Day by day averages for each tank								Average for each tank		
	03-03-16 off	03-03-16 on	04-03-16	08-03-16	11-03-16	14-03-16	16-03-16	18-03-16	Sound on	Sound off	SNR
1	117,2955	161,6877	166,1479	161,1034	161,374	162,7663	159,3877	117,8105	162,638153	117,560629	45,077523456
2	117,6634	161,2907	162,1031	160,2487	160,3504	164,0016	160,3261	117,5945	161,615077	117,629087	43,985990484
3	117,2545	152,7175	154,6603	154,7228	155,1003	155,8444	152,6937	117,1317	154,444927	117,193534	37,251393453
4	118,3845	163,4885	159,3688	157,9947	158,3068	159,8289	156,9191	119,5006	159,896105	118,978305	40,917799224
7	119,2824	127,8699		126,8127	123,638	125,9255	126,4735	119,1808	126,352365	119,231897	7,1204677741
8	127,6203	132,7923		131,9263	122,6703	128,6536	129,1355	123,7484	130,168816	126,102267	4,0665489078
9	121,4758	131,8419		130,1505	121,8547	123,1322	124,2681	121,2235	128,047727	121,351482	6,6962447489
10	124,2616	126,4565		124,1915	123,8354	126,523	124,3139	119,8165	125,226171	122,58454	2,6416307907

gemiddelde	Averages by day								
	Loud	117,6737458	161,1733461	162,3673343	159,1315892	159,3663061	161,57533	158,1696259	118,1068984
	St Dev	0,26168671	2,407494816	2,412308707	1,424692953	1,383204896	1,81368962	1,704769049	0,516860549
	Silent	124,2738813	130,4969161		129,2116996	123,0704697	126,4824218	126,5337373	121,369291
	St Dev	1,802315872	1,527884296		1,723917419	0,458743819	1,137659327	1,150682879	1,012981513

samen	General average		St Dev
	Off	121,2041183	1,511721976
	Loud on	160,5507925	1,788684964
	Silent on	127,857662	1,63214251

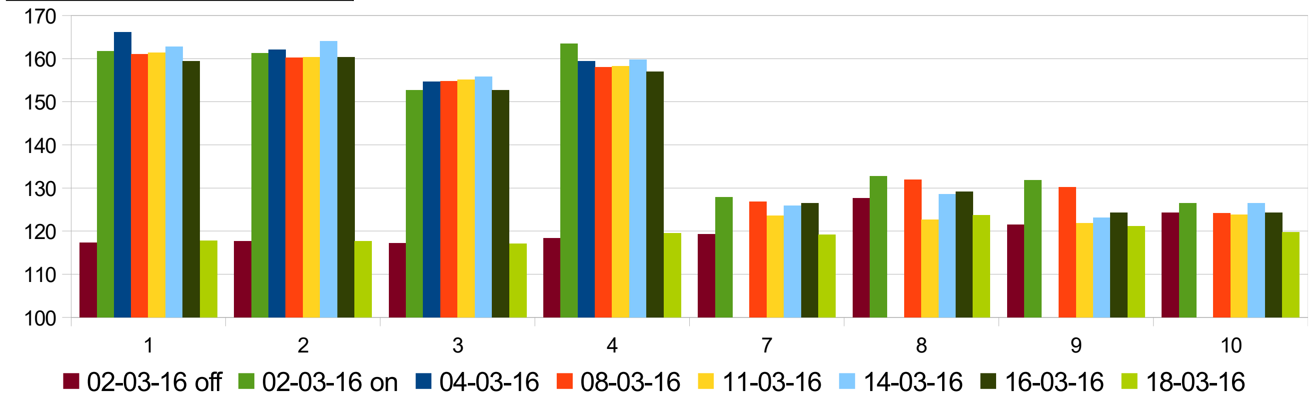


Fig. 6.3: SPLs

Table on top: figures with daily averages (loud and silent), averages a tank, and average with sound (silent and loud) and without sound

(Own collection)

For corrective purposes the first resonance frequency in the tank needs to be calculated. This is done using the following formula:

$$f_{lmn} = \frac{c}{2}\sqrt{\left(\frac{l}{L}\right)^2 + \left(\frac{m}{W}\right)^2 + \left(\frac{n}{D}\right)^2}$$

With the dimensions of the tank: $L = 0{,}37m$; $W = 0{,}27m$; and $D = 0{,}23m$; the sound speed: $c = 1484{,}29\ m/s$; for the first resonanse frequency: $l = m = n = 1$.

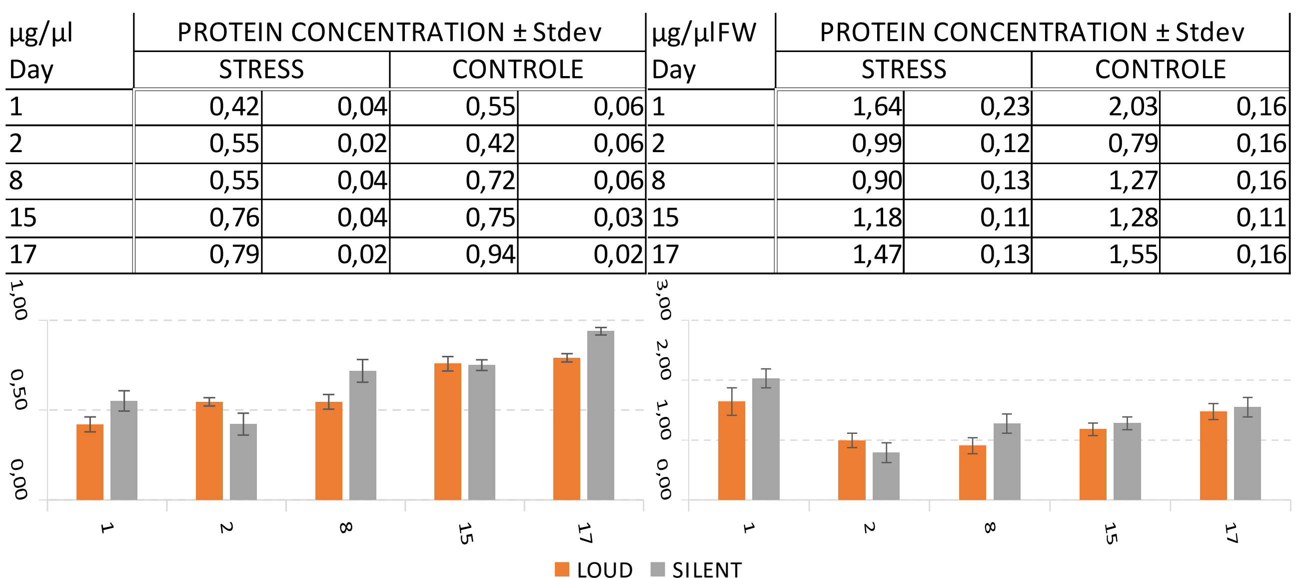
The resulting first resonanse frequency is then $f_r = 4689{,}37\ Hz$.

Problems

There were some problems with the sound. On several occasions the sound would just stop playing and constantly keep repeating the same short burst of sound, resulting in a sound comparable to an alarm that keeps beeping in one long monotone whistle. This was attributed to the long uninterrupted playing of the sound, while the computer was not otherwise engaged. These events were called “glitching of the sound setup”. This glitching occured on day 7, day 12 and day 13. On day 12 the amplifier got into overload on one channel and the sound stopped playing altogether. For the latter there is no explanation; the former could be due to the fact that the humidity and salt in the environment caught up with the amplifier.

6.1.3 Protein/HSP 70

It was impossible due to time restrains and problems with planning to perform the SDS-PAGE and find the desired HSP 70 concentrations. The measuring of protein concentrations was performed with the Markwell method, giving the following results.



µg/µl	PROTEIN CONCENTRATION ± Stdev			
Day	STRESS		CONTROLE	
1	0,42	0,04	0,55	0,06
2	0,55	0,02	0,42	0,06
8	0,55	0,04	0,72	0,06
15	0,76	0,04	0,75	0,03
17	0,79	0,02	0,94	0,02

µg/µlFW	PROTEIN CONCENTRATION ± Stdev			
Day	STRESS		CONTROLE	
1	1,64	0,23	2,03	0,16
2	0,99	0,12	0,79	0,16
8	0,90	0,13	1,27	0,16
15	1,18	0,11	1,28	0,11
17	1,47	0,13	1,55	0,16

Fig. 6.4: Protein concentration: pure (left) and as fraction of dry mass (right) (Own collection)

The tables and graphics on top show the results for the measurements of total amounts of proteins. The left-hand table shows the results in pure concentration, while the right-hand side takes the starting mass of the tissue into account. This means that the right-hand figures are more representative, although the results are less accurate because of the inaccuracy of the weighing of the tissue. The right-hand table will be used as such. The measurements before noise was played show that the shrimps in tanks nr. 7-10 are higher than those in tanks nr. 1-4. The protein concentrations lowered from day 1 to day 2 in all shrimps, though more so in the shrimps in the

silent tank (-1,24 µg/µgFW) compared to the loud tank (-0,65 µg/µgFW), the latter of which now having higher protein concentrations: loud tanks at 0,99 µg/µgFW compared to silent tanks at 0,79 µg/µgFW. On day 8 protein concentrations were still low compared to the initial concentrations. The concentrations in the loud tanks decreaced further to 0,90 µg/µgFW, while the concentrations in the silent tanks bounced back to 1,27 µg/µgFW. On day 15 concentrations rised in the loud tanks (1,18 µg/µgFW), while remaining level in the silent tanks (1,28 µg/µgFW). Once sounds were turned of, a two-day period of rest resulted in the reaugmentation of the protein concentrations to a similar level in both loud (1,47 µg/µgFW) and silent tanks (1,55 µg/µgFW) on day 17.

6.1.4 Other observations

Mortality within the populations of the tanks, both in the experimental tanks (nr. 1-4 and 7-10) and the acclimatisation/storage tanks (nr.11 and 12) was present. In the experimental tanks an average of 1,5 (stdev 0,46) animals died in every tank during the entire experiment, spread equally between both silent and loud tank sets.

When the shrimp were given food the first time during the subjection period, viz. on day 7, an interesting number of shrimps were quickly gone to eat in each tank (Table: 4). When given food on the other days, no noteworthy amounts of shrimp were showing interest in the provided food.

tank	1	2	3	4	7	8	9	10
# eating shrimp	4	3	4	1	0	1	1	0

Table 4: Number of shrimp showing particular interest in food (Own collection)

7 Discussion

7.1.1 Physical Environment (temperature, acidity and salinity)

The physical environment showed small changes, which are the result of daily fluctuations in temperature and acidity, as well as the difference in hour when taking measurements. It was not possible to take these measurements at the same time every day.

7.1.2 SPL/ Particle motion

The measurement of tank nr. 1 on day 2 was different to such an extent that these have to be considered faulty. Which part of the information flow erred is unclear however.

Results from tanks nr. 4 on day 1 with sounds playing and tank nr. 2 on day 13 deviate from the otherwise stable measurements for an unknown reason. A possible explanation could be that the hydrophone hing closer to the water inflow.

The particle motion measurements on day 13 gave very different results. It is probable that something went wrong during the measuring due to malfunctions of the equipement.

The data from the tanks show there is defenitly influence on the SPLs of the silent tanks from the loud tanks (average SNR = 5,01dB) especially around the 250Hz frequency. Since this difference is not translated into particle motion, since the difference between loud and silent tanks is still greater by far (SNR of averages = 32,70dB) and considering the varying nature of the silent measurements (stdev = 1,63) this influence can be neglected. These influences were caused by the physical proximity of these tanks to one another and the fact that they are located on the same long table. Another reason is that the pipings that both provide and remove water are connected and may stransfer some sounds. The fact that the tank most distant from the loud tanks has the least influence is telling, while the fact that the largest influence is not in the closest one migh be ofputting. It needs to be taken into account that tank nr. 8 and nr. 9 seemed to pick up more environmental sounds during measurements with no sounds too. Lastly, initial tests had showed no big impact was to be expected.

Tank nr. 3 showed generally lower SPLs. On day 13 at 1630 the upper exciter fell from tank nr. 3, but was soon reapplied, though behind its former location, 6cm from the bottom. It is possible that it had not been glued as good as the others and possibly be somewhat free, and was thus more susceptible to the humidity and salt. This could eplxain the lower SPLs, thoug it might be more likely that one of the speakers was a little damaged. Using them for a longer while made them heat

up, which could then result in damages.

Surprisingly, when no sound was playing a peak was visible in the 50 Hz region and the 10000 Hz region. What the actual reason is, can only be guessed at. A calculated guess would dedicate the 50Hz peak, which is present in the SPL graphics, but not the particle motion graphics, to a hydrophone characteristic, id est its self-noise. The 10000Hz peak, visible in both graphics, might be the frequency of the internal clock of the amplifier and/or the recorder. These could then result in a false peak on the results.

When playing sounds, no additional sound is visible in the 50Hz region on the SPL graphics and the particle motion graphics. This could mean that the frequency is too low to show up in the tanks or that there is a difference, but because of its relative small increase by the ship sound and because of the small variations in the results, it is not noticeable. In any case it matters little, because it is not represented in the particle motion curves either and it is assumed that this is what *C. crangon* picks up.

The visible sable increased sound levels in both SPL graphics and particle motion graphics between 250 and 2500Hz range is to be expected, as it is the typical band for high frequency cavitation noise from ships.

The large peak around the 5000Hz frequency, which is visible on both SPL graphics and particle motion graphics, falls in line with the first resonance frequency of the tank and while it is to be ignored when comparing it to the actual noise of a chemical tanker, the shrimps were still exposed to it. It is thus an unrealistic part in the experiment.

When considering all graphics and ignoring the inconsistencies due to measuring faults, it is clear that the sound levels were reasonably stable, certainly in the loud tanks. It should be obvious though, that these loud tanks are much less susceptible to small noises from the environment or small differences in placing the hydrophone, compared to the silent tanks. The thing that made the silent tanks even more susceptible to fluctuations was the fact that they were closest to the door. Additionally when considering the graphics from the loud tanks and ignoring the 5000Hz peak, and comparing them to the demonstration graphic from the chemical tanker in 4.2.12.2 “Shiptypical” as displayed below, the likeness is rather satisfactory (Fig. 7.1).

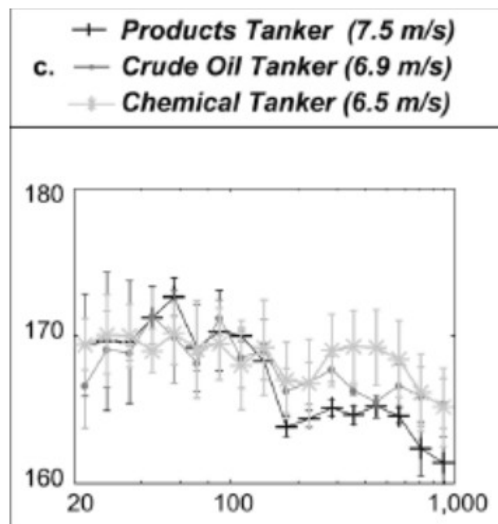


Fig. 7.1: Ship SELs for three types of tankers in 1/3 octave bands (McKenna et al., 2012)

7.1.3 Protein

The higher protein concentration in tanks nr. 7-10 before noise was played could result from the fact that samples were taken from tank nr. 10 to tank nr. 1 in decreasing order. This means that shrimps in the high number tanks could have shown an initial fast protein production peak as a result of handling stress, while the later samples were more used to the activity in the laboratory. Another explanation could be that the sounds in tanks nr. 7-10 without sounds playing, were considerably louder than in tanks nr. 1-4 (SNR = 6,60dB re 1µPa), possibly resulting in an onset of stress in the former, predating the actual start of the experiment. This last explanation is supported by the fact that the highest protein concentrations are seen in the loudest tanks. These sound differences are not represented in the particle motion part though, were no differences were visible. Another explanation could be that *C. crangon* is supposedly predatory active around dusk and dawn. The first sampling was done in the advent of dusk, meaning that the shrimp could be chemically preparing for increased activity. Yet other factors could have provided the full explanation, e.g. temperature fluctuations, gender, size or moulting stage, meaning this difference could be entirely unrelated to stress.

A clear lowering of protein concentration after 20h of sound induction is visible in the samples from day 2. The concentration lowered the most in the now silent tanks nr. 7-10 (-1,24 µg/µgFW) below the level of tanks nr. 1-4 (difference: -0,65 µg/µgFW). The difference with higher concentrations in shrimps from the loud tanks compared to the silent tanks could be explained by the fact that the loud tanks are now in fact the loud tanks (161,17 dB re 1 µPa) and the silent tanks the silent tanks (130,50 dB re 1 µPa), as is backed by the particle motion graphics. Additionally the increase in sound was comparatively major in the loud tanks (+43,50 dB re 1 µPa) opposed to the silent tanks

(+6,22 dB re 1 µPa). The higher protein concentration could thus indicate stress. The general lower concentrations could be explained by the fact that night passed and the shrimp have settled more to their environment and unstressed a little. The fact that the concentrations have lowered to a lesser extend could then be linked to the lingering sounds played in tanks nr. 1-4, which might induce stress in these. Another explanation for the general lower concentrations could be the sampling time in the early afternoon (as was the case for all further sampling moments 2-5). At this point of the day, shrimp are supposed to be less active, which could explain the low protein concentrations. Another explanation could be found in the fact that there were now less shrimp in the tanks, leading to less stress and thus less proteins. Individual difference caused by other factors could have been the whole reason, e.g. gender, size or moulting stage, meaning this difference could be entirely unrelated to stress.

Halfway the experiment on day 8 protein concentrations have lowered by 10% in the loud tanks(-0,9 µg/µgFW), while they have risen over 50% in the silent tanks (+0,45 µg/µgFW). The silent tanks now clearly having higher concentrations. The shrimp were fed a day prior to the sampling. The shrimp in the loud tanks could have started to succumb to the stress caused by the constant sound, resulting in decreased protein concentrations as they started to run out of energy and food for the production thereof. The shrimp in the silent tanks, not being stressed, would have been able to keep up their protein concentrations without difficulty in the absence of stress caused by noise. As with all measurements, differences in concentrations can be caused by individual differences and factors which were not incorperated in the experiment or had nothing to do with the experiment whatsoever.

After this period, feeding became rather regular. Data from samples on day 15 shows that the concentrations in the loud tanks rose with 31% to 1,18 µg/µgFW, while the silent remained constant at 1,28 µg/µgFW. These results support the explanation that the shrimp were initially succumbing towards day 7 while now having regularly restored nutrition and energy allows for protein management. The constant sound could cause stress, resulting in an inefficient protein production, resulting in the relative lower protein concentration in the loud tanks. Alternatively, the prolonged sound treatment could have resulted in trauma to the hearing organs of the shrimp in the loud tanks. This could then result in relatively higher protein concentrations in the shrimps in the silent tanks, as these can still hear the softer noise. This theory is however slightly undermined by the fact that there was still no significant particle motion detectable in the silent tanks.

After this prolonged period of exposure to the ship sounds, the sound was silenced on day 15. Samples from about two days later on day 17 show concentration increases to similar levels of 1,47

µg/µgFW and 1,55 µg/µgFW in the loud and silent tanks respectively. The remaining small difference could once again be explained by the louder tanks being once again tanks nr. 7-10, where thus the stress is generated. The lingering stress caused by the sound and the shrimps inability to recuperate (on such short notice) could also be given as a reason for the difference. The difference is in any case very small and could even be considered negligible or due to an unnamed other source, causing small differences within populations. In that case it could be said that the protein levels have settled and are now on a regular level, though there is no comparison for this data.

When overviewing the general trend, differences are observable over the time period (not including the difference between the samples from day 1 and the other measurements, which may be explained to the difference in sampling time). The only difference in the shrimps' environment was the sound played. This could indicate that there was some effect caused by the played sound.

7.1.4 Other observations

Within the populations of all tanks, both in the experimental tanks (nr. 1-4 and 7-10) and the acclimatisation/storage tanks (nr.11 and 12) animals died off, though not at the expected rate. In the experimental tanks an average of 1,5 (stdev 0,46) animals died, spread over the entire duration of the experiment, and spread equally between both silent and loud tank sets. This means that on average barely 0,09 shrimps died every day in each tank. Both the low amount and the even spread indicate that this result is not significant for this research.

The specific heightened interest in food on day 7 in the loud tanks compared to the feeble interest of the silent tanks could be explained by stress. As stress consumes energy and nutrients, the stress could cause the shrimps in the loud tanks to require more after a prolonged period of exposure with no food availability. This stress could only be caused by the played sound (there is a possibility there was other stress, but as that is not known it is considered unlikely at best). This could be a firm indicator that the sound causes stress in the shrimp.

7.1.5 General discussion

As there seemed not to be a significant difference in protein concentration between the shrimps in tank nr. 3 and those in the other tanks, it is probable that the difference in sound was not significant either. The fact that the peaks in the 50Hz frequency range was omnipresent, albeit loud, is considered to be of no importance, because there were clear differences in protein concentrations between the shrimps from silent and loud tanks. As such, this frequency cannot have dominated the audible noise for the shrimp. The same can be said about the 10000Hz peak during silence, which

became part of the overal soundscape in the loud tanks when subjected to the recordings. Their similar nature during the entire experiment did not result in the same protein concentrations in all tanks.

The stable sound results are indicative for the success of the playing of the sound in the tanks. Nothing went absolutely wrong during the subjection periods, except for the glitches and the falling off of one speaker. The latter did not show in the results of the protein measurements, while the former might be ignored. The reason why the glitching might be ignored, lies in the fact that it may be easier for the shrimp to learn to ignore a narrow band of sound frequencies compared to a broader band of frequencies. This might be contradictive, but the learning process is something that would ease the coping with the noise and thus increase the lowering of stress in the shrimp in the loud tanks. This was not observed (as major differences remained) and the glitching can thus be considered not relevant.

The major differences in soundscapes between the silent and loud tanks were in the plateau between 250Hz and 2500Hz and the peak around 5000Hz. The plateau was clearly caused by the recordings and find their origin in the high frequency cavitation sound. The 5000Hz peak unfortunately is a result of the first resonance frequency and not representative to the real nature.

C. crangon has hearing capabilities, which are assumed to range between 10Hz and 3000Hz and certain between 10Hz and 300Hz (Campos et al., 2012). This means that the 5000Hz peak from the first resonance frequency did not affect the shrimps. Furthermore, since the 50Hz peak was proven to be of no significance, it is the playback of the high frequency cavitation noise (≥250Hz) which probably caused the differences in protein concentrations between the shrimps in the loud and the silent tanks.

Although there is no reference to know wether stress in *C. crangon* would result in higher or lower protein concentrations, there were clear changes in protein concentrations both in time and between tanks. Even when taking the large difference from the first day into account and out of the equation this is apparent. Taking them out of the equation still shows significant differences in concentrations between the populations of the loud and silent tanks. Taking into account the measurements of the first day, shows that the shrimps in the silent and in the loud tanks changed places when comparing concentrations between day 1 and day 2 and once again between day 2 and day 8. This means that it is very likely that something happened to the shrimps during the subjection period that was caused by the high frequency cavitation noise. The consequence (if present) here is stress. Since injury to the hearing organs leads in acute cases to stress and over a longer period of time is caused by stress, both roads lead to stress. The concentration differences between day 2 and day 8, both in magnitude

and sense of change support the theory of the stress accumulating to famine in the shrimps in the loud tanks. This is supported even more by the increased interest in food in the loud tanks. The fact that these ship sounds influence these shrimp, means certainly they hear them, which in turn means that they might not be able to be vigilant on all the necessary sound frequencies[16].

The resulting concentrations of the shrimps on day 17 after a break from the noise are to be seen as inconclusive, though it is likely that these measurements point to recovery. This holds the most merit because of the evenness between both concentrations, which points towards a renewed equal situation for both populations (albeit that the surviving shrimp from the loud tanks should now be more resilient if stress did indeed occur in them again (Kregel, 2002)).

There is off course no complete certainty in the matter, because these results might be caused by differences in the individual test subjects for reasons such as age, sex, size, genetic abilities and personal history (Richter et al., 2010) or by drift.

Though the effective frequency falls within the range normally occupied by the natural noise of wind and sea, these sounds would definitly overpower it with SPLs when comparing the magnitude to the Knudsen curves (Fig. 4.11 on p.25). As they have been heard, they have a role in the life of *C. cangon* as well as for cephalopods (Samson et al., 2014).

7.2 Synthesis of the hypothesis

With the research now completed, the questions can now be answered.

In response to the first question, weather brown shrimps (*C. crangon*) are able to hear sounds, the answer is definitely positive, as is proven by Campos et al. (2012) and this study.

As to the second question, wether anthropogenic sounds produced by the propeller of commercial ships cause stress to brown shrimps (*C. crangon*) there can as of yet be no conclusive answer. It is however probable that these sounds, being heared, cause stress.

The answer on the second question does already exclude to some extend a decisive answer on the third question, whether anthropogenic sounds produced by the propeller of commercial ships have an impact on the fitness of brown shrimp (*C. crangon*). As it is probable that the anthropogenic sounds produced by the propeller of commercial ships causes stress to the brown shrimp, it is equally probable that these sounds have an impact on the fitness of brown shrimp, but it cannot be concluded.

[16] Samson et al. (2014) suggests this to be true for cuttlefish.

7.3 What went wrong and what could be improved

Several things did not go entirely as planned. Most of those were possibly foreseeable, but as is apparent: I did not. The first issue lies in the moulting and consequent cannibalism of the shrimp. It is possible to force the shrimp to moult, by a sudden change in physical environment. This ensures all shrimps are then in the same stage of moulting (Lovell et al., 2006).

A second problem which was expected to arise, but for which precautions were taken as much as possible, was the hostile humid sea air in the laboratory. The previously described construction proved effective, but the heating might have contributed to the problems with the computer regarding sound playing. The easiest solution for this problem would be to put the audio playing setup in an adjoining room and run the cables inside. To ensure the continue playing of the sound a server should be used however, as these are built to run programs for a very long time without interruption.

Thirdly there is the size of the tanks (Samson et al., 2014). Though the size was not a major issue, a tank with calculated dimensions and empirical proof of efficacy with slightly larger tanks might have served better. As it is, the tanks used were available and finding another room and time to build a complete new set of tanks from plastic slabs was not possible. Additionally, there was the price to be kept in mind too.

A fourth issue, which might have served both efficiency and accuracy was the selection of brown shrimp by size. In the first place the use of a shrimp sieve while catching them on board, might have saved time and kept size differences limited. A further measuring and selecting of shrimp by size could then have homogenised the populations in each tank some more. This would then decrease the incertainty of the result.

The fifth item is not as much a problem as it would have been help if it were applied. Besides the afore mentioned size measuring, weighing the shrimp in advance would have helped too. If they would have then been starved, a change in weight and mortality in extreme cases would have provided additional and more conclusive indicators towards stress.

7.4 Future research

Further research is definitely necessary into this matter. Firstly, the experiment needs to be concluded and could be expanded to some degree: carbohydrate and fat contents and other stress proteins could be checked, which would give more conclusive answers to the hypothesis. Secondly, it is always wise to repeat an experiment (albeit equally wise to improve certain steps of the

process). This would greatly improve the conclusiveness of the answers of the hypothesis, while it would allow to ask more questions and gather more data (e.g. weight, size, influence of gender, physical trauma (André et al., 2011)). By taking samples on more locations from different populations, the influence of the local DNA might also be incorporated into the research (Campos et al., 2012). Furthermore, there might be periodic variation in their capacity to cope with stressors. The introduction of cameras to observe the interest of the shrimps in their food and general behaviour would also provide much information.

Additional research into the sound profile of different ships and their types would furthermore greatly improve answers to these questions as they would allow to check the accuracy of the playbacks of the recordings and result in better predictions for the marine sound scape (McKenna et al., 2012). Research into the different aspects and specifications of propellers with respect to produced noise is another interesting and important issue for future law makers (Okeanos, 2008).

The execution of the original experiment might give insight in the social and ecologic effects and the severity of the stress caused by the anthropogenic sounds produced by the propeller of commercial ships. Another approach would be a test with visual and auditory predators and prey in the presence of ships noise (Samson et al., 2014).

Auditory perception has as yet to be defined behaviourally, indicating a promising area for future psychoacoustic research. The use of behavioural paradigms to obtain audiological information from aquatic animals is a methodology favoured by a number of authors (Lovell et al., 2005).

8 Case study

To put the theory into practice, a calculation will be presented here that will show the calculated SPL of RV Simon Stevin. A day with sea state 1 (good weather) will be expected for the ship sailing out of the port of Ostend to the starting point of the third fishing tow on the 18th February 2016. The point from where RV Simon Stevin acts as the sound source is at the entrance of the harbour (N 51°14'16,0" E 002°55'18,6"), while the brown shrimp at the start of the run are the receivers of the sound. The next involves the calculation of the total absorption of the sound by the sea. The concluding calculation results in the received sound levels by the shrimp. The physical environmental factors for these calculations were approximations to those measured in the tanks during the experiment. For the purpose of this calculation the bottom is supposed to be straight. As no accurate data concerning the bottom depths and the thickness of the sand bottom were available, these were guessed to some degree. Furthermore, the following data was gathered to be used:

	Unit	Symbol	Data
Seawater emperature	°C	T	8,4
Acidity	pH	A	8,4
Salinity	ppt or ‰	S	35
Depth	m	z or D	10
Sound speed	m/s	c	1484,2911
Horizontal distance	m	r	6582,5394
Source depth	m	Z,s	2,4
Reciever depth	m	Z,r	10
Block Coefficient		C,B	0,6225
Velocity	kn	v	12
CIS	kn	v,CIS	9,1
Displacement	t	Δ	597
Engine mass	t	m	4,75
# engines		n	3
Layer depth	ft	L	3
Depth ft	ft	D	32,808
Horizontal distance kyd	kyd	r	7,1987526

Table 5: Calculation data
(Own collection)

Noise of the propeller can be viewed as a monopole because the cavitation bubbles. For determination of the exact position, we would need the draft of the ship, the size of the propeller, the position where cavitation occurs, and the height of the stern wave created by the ship itself. (Wittekind, 2014)

8.1 Sound levels on shrimps from RV Simon Stevin

The first thing that needs to be calculated is the distance (*z*) between source and receiver using the

Meridional parts method and sound speed (c) in seawater on the day of measuring, using the following formula:

$$c = 1449{,}2 + 4{,}6\,T - 0{,}055\,T^2 + 0{,}00029\,T^3 + (1{,}34 - 0{,}01\,T)(S - 35) + 0{,}016\,z$$

then

$$c = 1484{,}2911\,m/s$$

	latitude	meridional par	longitude
Source	N 51°14'16,0”	3573,274	E 002°55'18,6'
Shrimp	N 51°16'00,0”	3576,03	E 002°52'07,0'
difference	0°01'44,0” N	2,756	0°03'11,6' W
Direction (rad)	0,8587737555		
Direction (°)	49,204111749		
Distance (')	3,5542869343		
Distance (m)	6582,5394023		

Table 6: Distance calculations
(Own collection)

The next step is to check the cut-off frequency on Fig. 8.1. Making the calculation was not possible as no data was at hand to do this and making calculated guesses were not possible.

With a water depth of 10m and a fine sand bottom the cut-off frequency is found at 0,07 kHz or 70 Hz.

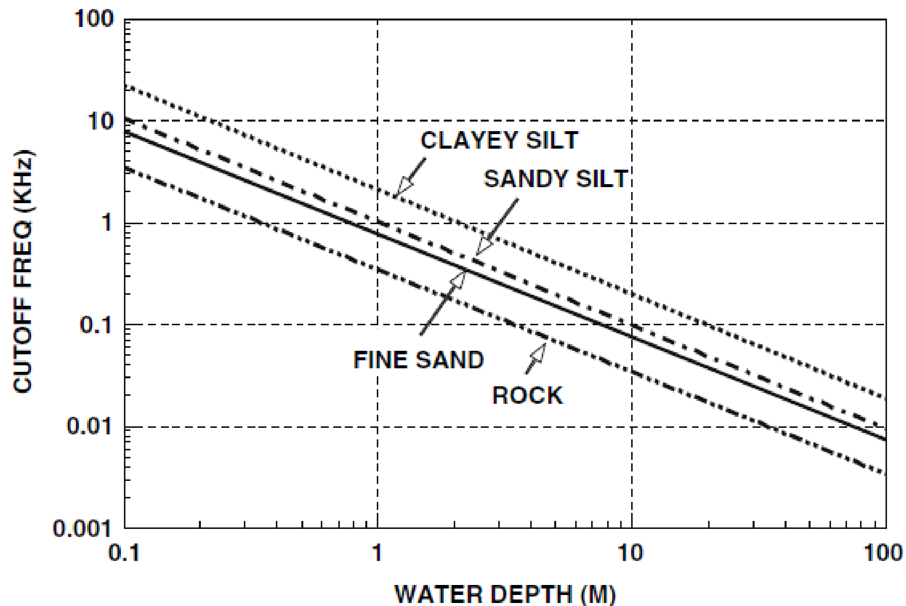


Fig. 8.1: Cutoff frequency
(Au and Hastings, 2008)

Now it is time to calculate the produced sound (SEL):

$$SEL = 10\log\left(10\frac{F_1}{10} + 10\frac{F_2}{10} + 10\frac{F_3}{10}\right)$$

where F1 is low frequency cavitation noise; F2 is high frequency cavitation noise; F3 diesel

generator noise.

$$F_1 = 2{,}2.10^{-10} f^5 - 2.10^{-7} f^4 + 6.10^{-5} f^3 - 8.10^{-3} f^2 + 0{,}35.f + 125 + A + B$$

$$A = 80\log\left(4c_B\left(\frac{v}{v_{CIS}}\right)\right)$$

$$B = 10\log\left(\frac{\Delta}{\Delta_{ref}}\right)^{\frac{2}{3}}$$

$$F_2 = -5\ln(f) - \frac{1000}{f} + 10 + B + C$$

$$C = 60\log\left(\frac{v}{v_{CIS}}.1000.C_B\right)$$

$$F_3 = 10^{-7} f^2 - 0{,}01 f + 140 + D + 15E$$

$$D = 15\log(m) + 10\log(n)$$

These calculations result in sound levels in function of frequency, as is shown in Fig. 8.2.

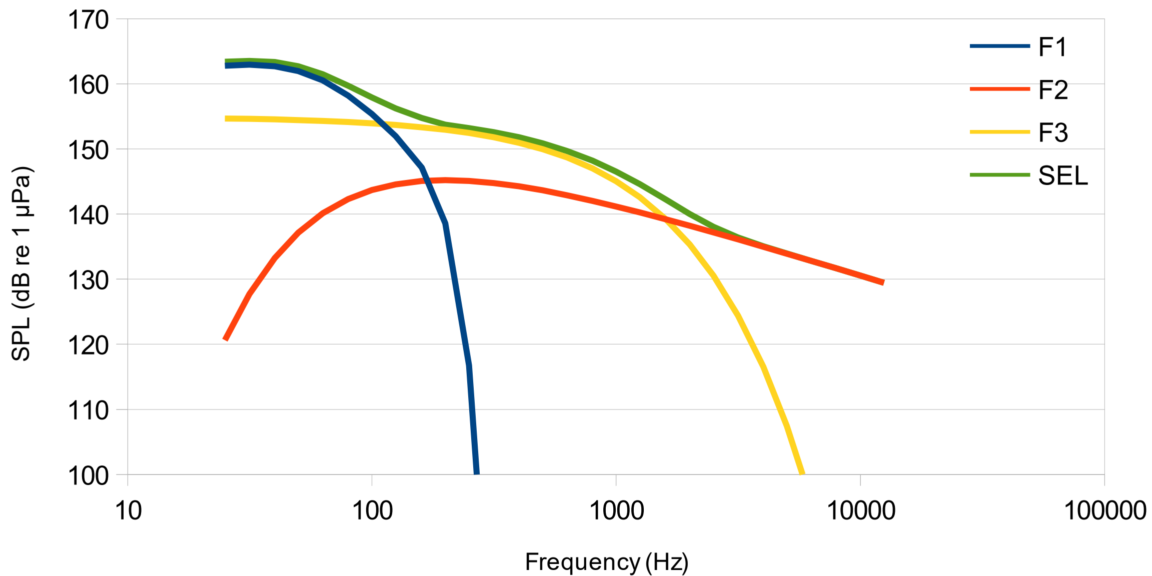


Fig. 8.2: SELs
(Own collection)

The following step is to calculate the absorption of sound by the sea, using the following formulas:

$$\alpha = \alpha_{H_3BO_3} + \alpha_{MgSO_4} + \alpha_{H_2O}$$

where the terms are attenuation by boric acid (H_3BO_3), magneseum sulfate ($MgSO_4$) and pure water (H_2O) respectively.

$$\alpha_{H_3BO_3} = \frac{A_1 P_1 f_1 f^2}{f_1^2 + f^2}$$

where

$$A_1 = \frac{8{,}86}{c} 10^{(0{,}78\, pH - 5)} \left[\frac{dB}{km\, kHz} \right]$$

$$P_1 = 1$$

$$f_1 = 2{,}8 \sqrt{\frac{S}{35}}\, 10^{\left(4 - \frac{1245}{273+T}\right)} [kHz]$$

$$c = 1412 + 3{,}21\, T + 1{,}19\, S + 0{,}0167\, D \left[\frac{m}{s} \right]$$

$$\alpha_{MgSO_4} = \frac{A_2 P_2 f_2 f^2}{f_2^2 + f^2}$$

where

$$A_2 = 21{,}44 \frac{S}{c} (1 + 0{,}025\, T) \left[\frac{dB}{km\, kHz} \right]$$

$$P_2 = 1 - 1{,}37.10^{-4} D + 6{,}2.10^{-9} D^2$$

$$f_2 = \frac{8{,}17.10^{\left(8 - \frac{1990}{273+T}\right)}}{1 + 0{,}0018 (S - 35)} [kHz]$$

$$\alpha_{H_2O} = A_3 P_3 f^2$$

where

$$P_3 = 1 - 3{,}83.10^{-5} D + 4{,}9.10^{-10} D^2$$

for $T \leq 20\,°C$

$$A_3 = 4{,}937.10^{-4} - 2{,}59.10^{-5} T + 9{,}11.10^{-7} T^2 - 1{,}5.10^{-8} T^3 \left[\frac{dB}{km\, kHz^2} \right]$$

for $T > 20\,°C$

$$A_3 = 3{,}964.10^{-4} - 1{,}146.10^{-5} T + 1{,}45.10^{-7} T^2 - 6{,}5.10^{-10} T^3 \left[\frac{dB}{km\, kHz^2} \right]$$

These are also frequency dependent and result hence in Fig. 8.3.

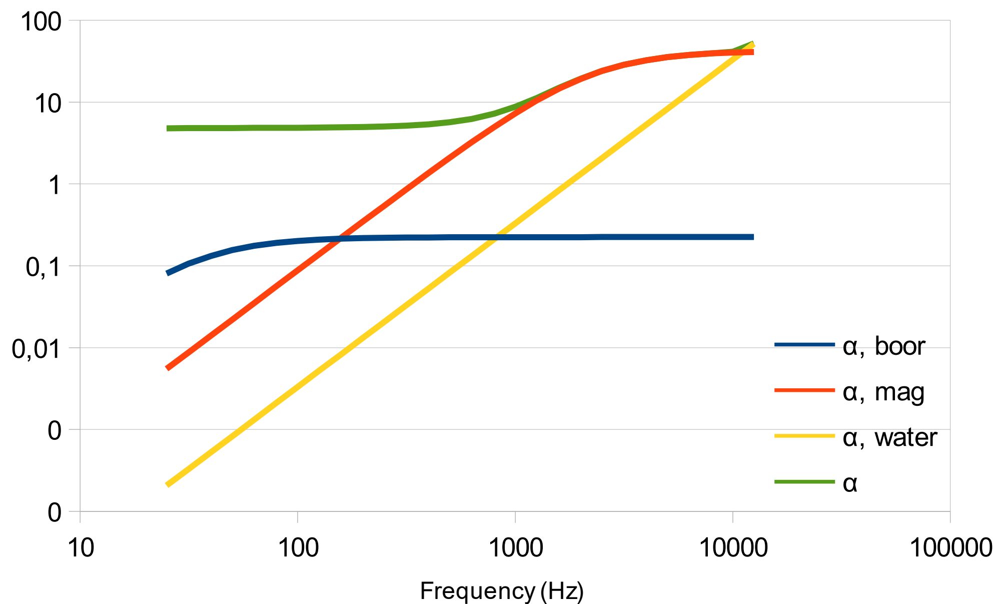


Fig. 8.3: Chemical attenuation
(Own collection)

The near field anomality (k_L) and the shallow water attenuation coefficient (α_T) can be read furthermore on Fig. 8.4:

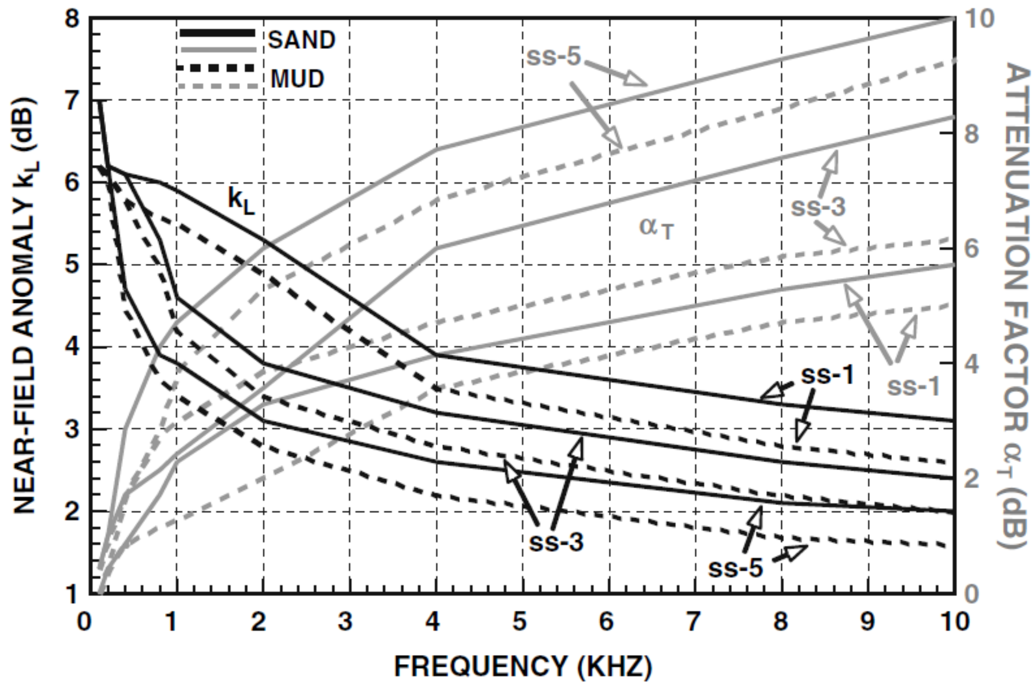


Fig. 8.4: Near-field anomaly and attenuation factor (Au and Hastings, 2008)

Then factor H is required to know which formula is to be used for the calculation of the total loss (TL) using the following formula:

$$H=\sqrt{\frac{1}{8(D+L)}}$$

Then

H	0,0590833313
8H	0,4726666503
r>8H	

Table 7: H (Own collection)

and the last formula is to be used:

$$r<H: \quad TL=20\log r+\alpha r+60-k_L$$

$$H\leq r\leq 8H: \quad TL=15\log r+\alpha r+\alpha_T(\frac{r}{H}-1)+5\log H+60-k_L$$

$$r>8H: \quad TL=10\log r+\alpha r+\alpha_T(\frac{r}{H}-10)+10\log H+64{,}5-k_L$$

With the results *TL(f)* and *SEL(f)*, now *RL* can be calculated:

$$Recieved\ Level(RL)=Produced\ Level(SEL)-Transmission\ Loss(TL)$$

Resulting in Fig. 8.5:

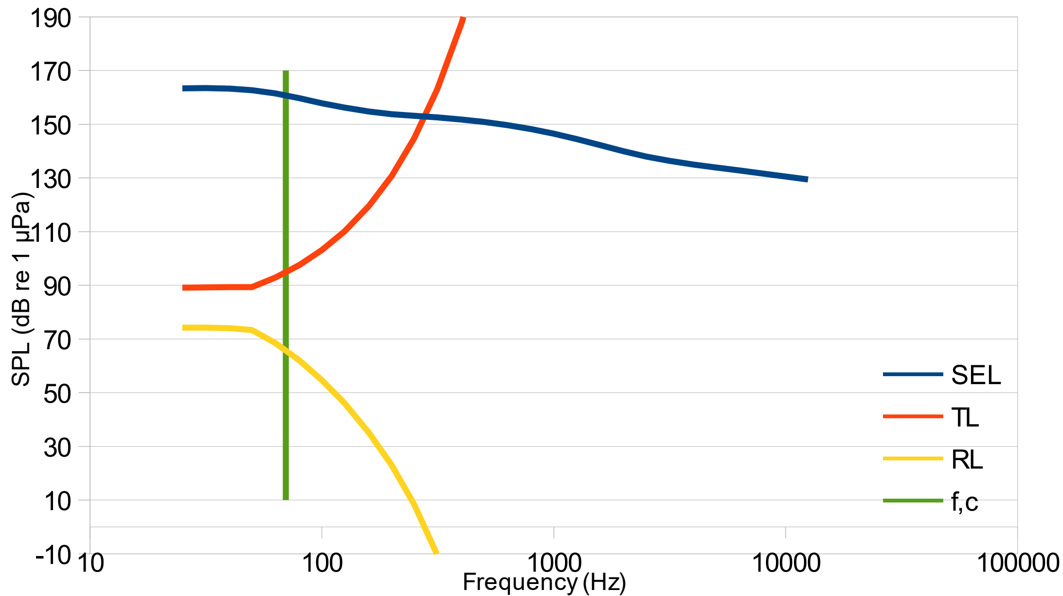


Fig. 8.5: SPL, TL, RL and f_c
(Own collection)

8.2 Discussion

When looking at the results from these calculations, the graphics are the most important. From them we can derive a lot of information. First off all, when comparing the SEL graphic (Fig. 8.2) to the graphics of the measurements from the tanks during the experiment (Fig. 6.2), analogy is clear. It is off course important to ignore the peak around 5000Hz due to the first resonance frequency in the tanks in this case. Secondly, the curves in Fig. 8.3 gives satisfactory results when compared to those in Fig. 4.6 on p.15 by Kraus D. (2016). Lastly, the final resulting graphic in Fig. 8.5 on this page shows the received sound levels by the shrimp at a distance 3,5' from the source. The cut-off frequency indicates that no sound is received below 70Hz, while the total loss keeps the received sound under 250Hz. This means an overall good result was become.

One needs to keep in mind that RV Simon Stevin is not a completely adapted ship to put the theory to the test, as it was designed for low sound emission.

When comparing these results to the sound spectrum of the sea, it is clear that this is a realistic result, while it inversely proves that only the low frequencies propagate over a reasonably long distance. The other peculiarity about this result and the fact that the/this ships noise fills in the gap where otherwise little sound would be, is that it is exactly at these frequencies that amongst others *C. crangon* tunes in to listen to its environment.

9 Conclusion

When the wind blows across the sea, waves, clouds and rain are created. Together the wind and waves and before humans came, marine life would make up the continuous sound spectrum of the marine acoustic sound scape. There is a dip in the sound produced by the elements in the sea (Urick, 1984), where most marine animals, including whale, crustaceans, fish and cephalopods seem to be specialised in listening too. Unfortunately, shipping has now taken that place as being the third dominant sound source in the marine soundscape, outbidding on many places gale winds and freak waves.

Unsurprisingly, many marine species experience negative effects from those sounds. Whales have been proven to be caused stress and physical trauma. Cephalopods have been proven to have sustained physical trauma due to sounds, even though their senses are primarily focused on sight. Crustaceans, and in particular *Crangon crangon*, are potentially affected by anthropogenic noise (Lovell et al., 2005), which this study supports.

Much research is however still needed to fully comprehend the consequences of our actions in this aspect. This research should not be limited to the studying of marine animals proven to make sounds themselves, because a mute is not necessarily deaf. It is only through research that lawmakers are able to make sound decisions and that the IMO will transform guidelines into MARPOL.

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