On the effects of high intensity impulsive sound on young European sea bass *Dicentrarchus labrax*, with special attention to pile driving during offshore wind farm construction

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On the effects of high intensity impulsive sound on young European sea bass *Dicentrarchus labrax*, with special attention to pile driving during offshore wind farm construction

Over de effecten van intens impulsief geluid op jonge Europese zeebaarsen Dicentrarchus labrax, met extra aandacht voor het heien tijdens de bouw van offshore windmolenparken

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en zoeken het vinden waard

DANKWOORD

“kzin streke”

Een doctoraat doe je niet alleen, dat is wat je telkens hoort van ‘oud doctoraatstudenten’. Tijdens de voorbije vier jaar heb ik dit gelukkig ook mogen ervaren. Allereerst wil ik dan ook m’n promotoren bedanken. Professor Magda Vincx, bedankt voor het vertrouwen en geloof in me. Jij hebt mij voorgesteld aan de overige promotoren voor deze doctoraatsstudie, Dr Kris Hostens, Prof Dr Steven Degraer, Dr Sofie Vandendriessche en Prof Dr Dick Botteldooren. Het projectvoorstel op tafel omvatte vissen, windmolens, onderwatergeluid en zou onderzocht worden aan de hand van veld- en labo experimenten. Allemaal kernwoorden die als muziek in m’n oren klonken. Ik haalde de IWT beurs binnen dankzij de inhoudelijke en motiverende steun van jullie.
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“**tis oal gin oar snien”**

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"wienne wuk ziej antkraam"

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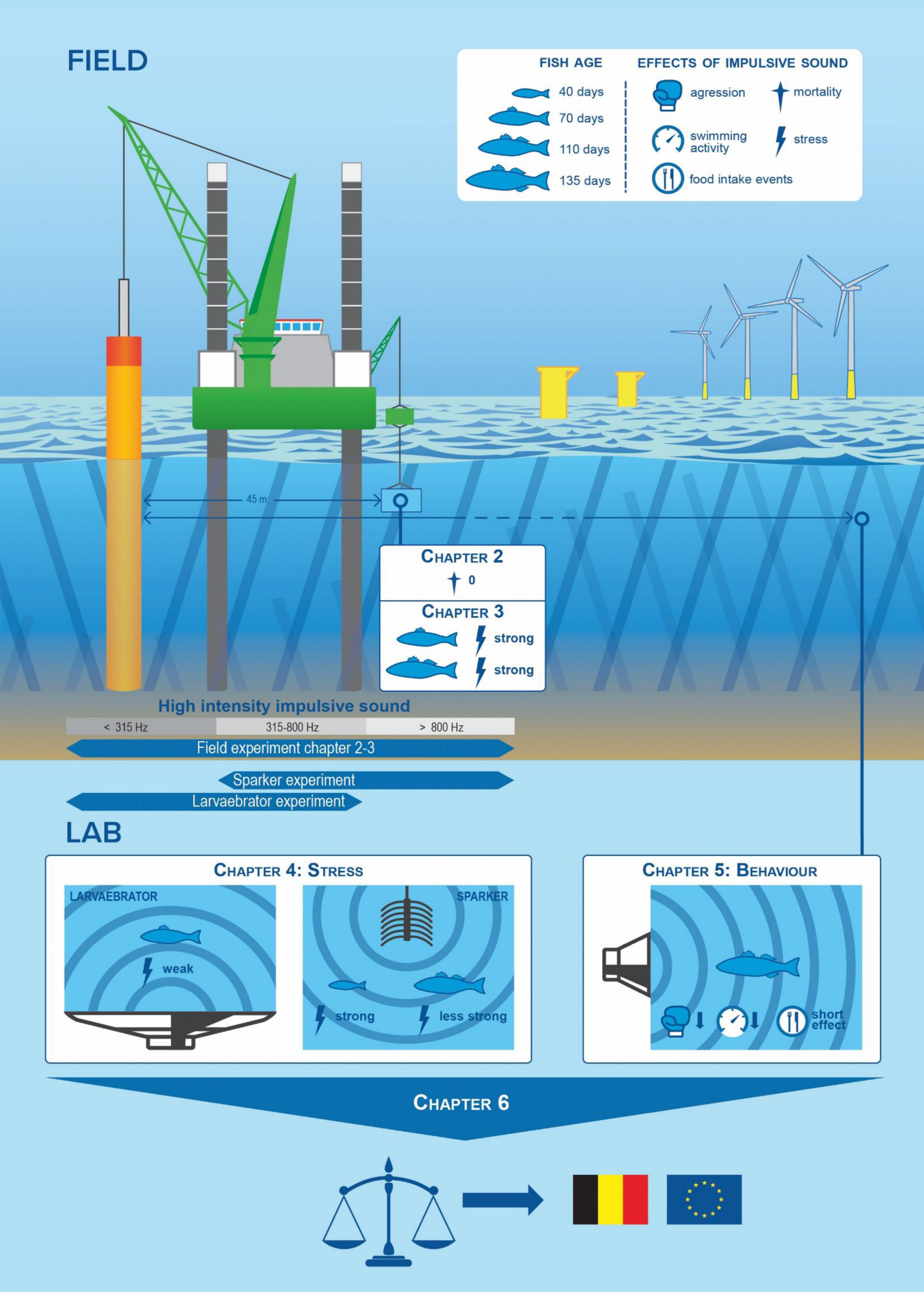
Elisabeth

Maart 2016



FIELD

FISH AGE

- 40 days
- 70 days
- 110 days
- 135 days

EFFECTS OF IMPULSIVE SOUND

- agression
- ✝ mortality
- swimming activity
- ⚡ stress
- food intake events

CHAPTER 2

✝ 0

CHAPTER 3

⚡ strong

⚡ strong

High intensity impulsive sound

< 315 Hz	315-800 Hz	> 800 Hz

Field experiment chapter 2-3

Sparker experiment

Larvaebrator experiment

LAB

CHAPTER 4: STRESS

LARVAEBRATOR

⚡ weak

SPARKER

⚡ strong

⚡ less strong

CHAPTER 5: BEHAVIOUR

↓ ↓ short effect

CHAPTER 6

SUMMARY

More than 25 years ago, a relation between man-made (anthropogenic) sound and its negative effects on marine mammals was established. Since then, marine mammals have dominated the bioacoustics research, although recently the focus has widened to fish, and to a lesser extent, also to invertebrates. The frequency range of man-made sound often overlaps with the hearing range of the fish. Consequently, underwater sound has the potential to cause auditory injuries, physiological stress and behavioural disturbance, and to mask biologically relevant sounds. In addition, sound pressure can influence the swim bladder volume which can result in (mortal) internal injuries. So, depending on the characteristics of the sound and the fish species, the impact of anthropogenic sound on fish can range from immediate death to no impact at all. Since fish are a vital component in most ecosystem food webs, and as many fish species have a high economic value, it is necessary to document the effects of sound and to define thresholds for different combinations of sound sources and fish species.

In this PhD study, we addressed sound effects related to pile driving during **offshore wind farm** (OWF) construction, an increasingly important human activity throughout the North Sea. Pile driving effects were assessed for young individuals of European sea bass *Dicentrarchus labrax*, a fish species with a closed swim bladder, so-called physoclists. The PhD started from the assumption made by a Dutch report in 2009 (Prins et al., 2009), which hypothesized a 100% mortality in fish eggs and larvae up to 1 km around a pile driving source. This assumption was based on modelled fish larvae distributions, mortality rate due to underwater explosions and back-calculated energy levels of underwater sound related to pile driving activities in the Dutch part of the North Sea.

The PhD study has a multidisciplinary approach, aiming to disentangle the effects of impulsive sound (produced by pile driving) on young fish, thereby focusing on the following research questions:

- **(I) Are young fish (larvae and juveniles) affected by impulsive sound, what are the effects, and at what level do they manifest, *e.g.* mortality, stress responses or behavioural responses?**
- **(II) Can the effects on young fish be linked to a specific sound-related metric or biological parameter? Can sound thresholds at which underwater sound negatively affects young fish be identified?**
- **(III) What is the ecological significance of the observed effects?**
- **(IV) How will the results from this PhD add to management and policy regulations in Belgium (and Europe), *i.e.* in order to minimise the environmental impact of pile driving activities in future offshore wind farms, and to achieve Good Environmental Status (GES) for Marine Strategy Framework Directive (MSFD) descriptor 11?**

The context of this multidisciplinary study on the impact of pile driving on European sea bass is outlined in **Chapter 1**. A general introduction to the underwater world of sound is given. In the North Sea, shipping, seismic surveys, underwater explosions and pile driving are identified as the main contributors to the anthropogenic sound energy. Sound plays an essential role in conveying environmental information to marine fauna (e.g. marine mammals, fish and invertebrates). Particularly in marine mammals, sound plays a key role in social and foraging behaviour. But of all vertebrates, fish exhibit the greatest diversity in hearing sensitivity and hearing structures. The frequency range of man-made sound largely overlaps with the hearing range of fish. In addition, high-intensity impulsive sound can cause (mortal) physical damage to marine mammals and fish. The exact impact, the underlying mechanisms and the ecological consequences of anthropogenic sound on marine life are not yet understood, especially for fish. In Europe, anthropogenic underwater noise was labelled as a pollutant within the MSFD of the European Commission. Consequently, the impact of underwater sound on marine life, generated by various anthropogenic sound sources, need to be evaluated in order to take appropriate measures. Throughout the North Sea, a new anthropogenic sound source, pile

driving, was recently introduced. It is the main method to install OWFs and will regularly be used during the next couple of years. Therefore, this PhD study took pile driving as the source of high intensity impulsive sound to study its impact on marine fish.

The next four chapters (**Chapter 2 – 5)** present the assessment of the impact of high intensity pile driving sound on acute and delayed mortality, acute and chronic physiological stress responses and the impact of lower intensity impulsive sound on the behaviour of young European sea bass. Also, the critical sound parameters of physiological stress responses are studied in detail.

Chapter 2 presents the results of an *in situ* experiment on board of a pile driving vessel, addressing acute and delayed mortality of juvenile (68 and 115 days old) European sea bass. It was the first field study to assess fish mortality as close as 45 m from an offshore pile driving source over complete pile driving sessions. Fish were exposed to 1739 up to 3067 pile driving strikes with a single strike sound exposure level (SEL_{ss}) between 181 and 188 dB re 1 $\mu Pa^2 \cdot s$, and a cumulative sound exposure level (SEL_{cum}) between 215 and 222 dB re 1 $\mu Pa^2 \cdot s$. No increased acute mortality was observed when we compared European sea bass (68 and 115 days old) exposed to pile driving with a control group exposed to ambient background sound levels in between the pile driving sessions. This study validates the results provided by other studies inside acoustically controlled chambers in the laboratory. Fish survival was further monitored in the lab for two weeks. At least under optimal laboratory conditions, we observed no delayed mortality caused by pile driving. This study rejected the 100% mortality hypothesis as stated by a Dutch report in 2009 (Prins et al., 2009). Moreover, if internal injuries were present, they were shown not to be mortal.

In **Chapter 3** the physiological stress response of juvenile sea bass (68 and 115 days old) to high intensity sound produced by pile driving was investigated. So far, this was not yet studied. During the same *in situ* study as described in Chapter 2, primary, secondary and tertiary stress responses were investigated during and after exposure to a complete pile driving session. As a primary stress response proxy, whole-body cortisol seemed to be too sensitive to ‘handling’ bias. However, a strong secondary stress response to pile driving was detected as significant

reductions in oxygen consumption rate (49 – 55%) and low whole-body lactate concentrations. In contrast to fish used on the first day of the experiment, the fish used on the second day had already been indirectly exposed to pile driving. Fish in the control group of that second day reduced their respiration by 34 to 40% compared to the control group on the first day. This may be indicative of a prolonged stress response or increased sensitivity towards new stressors. A tertiary stress response only manifests when homeostasis cannot be re-established. After 30 days in the laboratory, specific growth rate and condition of the exposed fish were not affected compared to unhandled fish, so a tertiary stress response was absent. Only a short-term reduction in metabolic rate was demonstrated while the long-term consequences of repeated impulsive sound exposure for fish in the field are yet to be determined.

Chapter 4 explores the critical sound parameters responsible for the acoustic physiological stress response observed in the field experiment. The primary and secondary stress responses of larval and juvenile European sea bass to strong impulsive sound were compared between two lab experiments using different sound sources (SIG sparker and larvaebrator). These results were then compared with the stress responses measured during an *in situ* pile driving study (Chapter 3). Both lab sound sources produced similar levels for the standard sound pressure metrics as the *in situ* pile driving, being zero-to-peak sound pressure level ($L_{z\text{-}p}$) of 208 dB re 1 µPa), SEL_{ss} of 181 dB re 1 µPa²·s and SEL_{cum} of 214 dB re 1 µPa²·s. However, the three sources differed in their sound frequency spectra. The whole-body cortisol results (a proxy for primary stress responses) confirmed the susceptibility of both juvenile and larval fish to handling stress. Still, the increased (or altered) whole-body cortisol levels indicated that high intensity impulsive sound evoked an acoustic primary stress response. Common ground between the field and two lab experiments was found at the high energy levels (SEL_{ss}) produced between the 315 and 630 Hz 1/3 octave bands. This frequency range covers the responsiveness range of European sea bass to sound, relating the primary stress response in juvenile sea bass to hearing. Reduced oxygen consumption rates of ~50% were observed in the juveniles in the field experiment and larvae in the sparker experiment, and to a lesser extent in the juveniles of the sparker experiment. Consequently, the secondary stress response can most likely be linked to high intensity sound produced at higher frequencies

(>800 Hz), above the responsiveness range of European sea bass. This secondary stress response may be associated with the pressure induced swim bladder oscillations. It may be clear that high intensity impulsive sound must cover a broad frequency range (similar to a real *in situ* pile driving) to evoke strong secondary stress responses, such as reduced oxygen consumption rate and reduced whole-body lactate levels in juvenile sea bass. This implies that lab results can not directly be translated to the real world, as some known (like frequency content) and unknown parameters may not be comparable. More studies on different life stages and on the role of non-standard sound parameters - such as particle motion - are needed to further clarify the triggering parameters and sound thresholds of the stress response of fish.

In **Chapter 5** the impact of underwater sound on fish behaviour is examined. Underwater sound has the potential to disturb the behaviour of fish even at lower sound pressure levels, resulting in a much wider impact range around the pile driving source than high sound pressure levels. Since functionally important behaviour, such as social interactions and foraging, can contribute significantly to the survival and reproduction of fish, any impact on functional traits can directly be translated into fitness consequences. However, so far only a couple of studies have tested the acute impact of anthropogenic sound exposure on fish behaviour. Consequently, in **Chapter 5,** fish behaviour was studied in response to impulsive sound on three consecutive days in a laboratory set-up. In this laboratory study, we tested the influence of pile driving sound on the swimming activity and aggressive behaviour of young juvenile European sea bass *Dicentrarchus labrax* before, during and immediately after the 25 min sound exposure period (1000 strikes, SEL_{ss} =146 dB re 1 μPa²·s, $L_{z\text{-}p}$ = 165 dB re 1 μPa; SEL_{cum} = 176 dB re 1 μPa²·s). We also tested the impact on feeding tendency and efficiency when fish were already exposed to the impulsive sound for 15 minutes. Juvenile sea bass interrupted their swimming activities and ceased any aggressive actions to conspecifics at the onset of the impulsive sound exposure. These behavioural effects returned to the pre-exposure baseline within the 25 minute exposure period. On the first day, a reduced number of food intake events were observed during and after the sound exposure, which can indicate an attention shift induced by the sound exposure. This attention shift was no longer clearly observed during the two following days of the experiment. Feeding efficiency was not affected by the sound exposure and illustrated that sea bass were alert to external stimuli under impulsive sound exposure. These findings indicate that the initial response does not persist but can progress over time or under repeated exposure. It remains to be tested whether this also applies to wild-ranging fish.

In **Chapter 6**, the technical and practical challenges for field and lab experiments in bioacoustics are discussed. In this chapter, the results of this PhD study are discussed in a broader perspective through comparison with literature on pile driving and other anthropogenic impulsive sound sources to provide an answer to the four research questions of this thesis.

The results of Chapter 2 to 5 allowed to answer research question I on the specific impact of high intensity or strong impulsive pile driving sound on European sea bass. Exposure to a complete pile driving session as close as 45 m from a pile driving activity did not result in acute or delayed mortality of juvenile European sea bass. It did lead to a strong physiological stress response limited to a relative short period of time, which can be extended by multiple sound exposures. Based on the field and lab results, the physiological stress responses found in larvae and juveniles could be related to the standard sound metrics (SEL_{ss}, SEL_{cum} and L_{z-p}) and the frequency range in which the highest energy was found. Furthermore, the primary and secondary stress response could be related to hearing and swim bladder oscillations, respectively. The studies mentioned above involve high intensity underwater sound found at close range from the pile driving source. At larger distances from the pile driving source, the impulsive sounds contain less energy but can still induce a behavioural response in juvenile European sea bass at the onset of the sound exposure. During the sound exposure, European sea bass were able to recover from the initial stress response, and repeated exposure had no clear effect on feeding. Combining these results with other data from literature reveals the interspecific variability of fish in their behavioural response to the same type of stressor. More species with varying life history strategies need to be studied before the results can be generalised with confidence.

To provide an answer on research question II, the study results are integrated with current knowledge. This allows us to make suggestions regarding sound thresholds for mortality, physiological stress and behavioural changes of young physoclistous fish. Since mortality was absent in our field study, the mortality threshold must lie above the measured sound parameters (SEL_{ss}> 188 dB re 1 µPa²·s; SEL_{cum}> 222 dB re 1 µPa²·s.; L_{z-p} > 210 dB re 1 µPa). This study is the first to propose a sound threshold range at which physiological stress responses in juvenile fish are evoked: high-intensity impulsive sound need to have at least a SEL_{ss} of 170 to 181 dB re 1 µPa²·s at frequencies higher than 315 Hz to evoke physiological stress. A threshold for behavioural disturbance linked to pile driving cannot yet be determined.

Additionally, consequences on an ecological level need to be evaluated (research question III). In other words, effects on an individual level need to be scaled up to population level, since individual effects in fish are subordinate to population effects from an ecological point of view. In order to do so, data on the presence of sound sources, sound propagation, individual impact, population size, distribution, and affected (sub)population are needed before the individual effect can be modelled into a population effect. This is not yet possible for fish, but given the results about the effects found on individual fish, it can be assumed that the ecological consequences of pile driving sound on fish health are subtle.

Evaluating the European and national legislation on man-made underwater sound is necessary to provide adequate advice to minimise the impact of pile driving activities on the marine Environment (research question IV). In Europe, the Marine Strategy Framework Directive (MSFD) defined a Good Environmental Status (GES) in which underwater sound needs to be at levels that do not adversely affect the ecosystem (Descriptor 11). A Technical Subgroup Noise (TSG Noise) has been commissioned to further develop the descriptor on underwater noise. This subgroup proposed the establishment of a sound register, to log all sound producing human activities. The subgroup also identified 'considerable displacement' of marine organisms as the most relevant impact of impulsive sound. Finally, an inventory of the pulse-block days in the EU regional seas can be modelled. This is based on the presence of anthropogenic sound sources that are producing sound levels above the threshold linked to the 'considerable displacement' in ¼ ICES rectangles[1]. A GES should be applicable to all marine

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[1] ICES rectangles provide a grid over an area between 36°N and 85°30'N and 44°W and 68°30'E. These rectangles are intervals of 30' (longitude) and 1° (latitude).

organisms, while the TSG Noise mainly based its advice on marine mammals. ‘Considerable displacement’ may not be the most relevant impact on fish. Fish are also neglected in the national legislation of the EU Member States. Based on this PhD, our management advice is that the effects of pile driving sound on fish are considered to be more subtle than anticipated and no stringent measures are needed ad hoc in Belgium or in other member states. However, more research is needed to support or reject the decision to exclude fish from management, thereby still ensuring GES for all marine fauna.

Finally, future research targets are identified to further unravel the impact of pile driving sound on fish. These are needed to progress towards an acoustically sound approach. The lack of particle motion data remains a big gap and needs to be addressed by future studies. The underlying critical sound parameters that evoke physiological stress and behavioural responses in fish need to be unravelled further. Furthermore, data is needed on the long-term impact of acoustic stressors in order to model the ecological consequences of pile driving at population level. Studying the fish in their natural environment with new technologies is a promising strategy. Finally, the impact of continuous sound that will be produced for the next 20 years of the operational OWFs on fish health need to be addressed.

SAMENVATTING

Meer dan 25 jaar geleden werd de link gelegd tussen geluid geproduceerd door menselijke activiteiten en de negatieve effecten van dat geluid op zeezoogdieren. Vanaf dat moment werd het bio-akoestisch onderzoek gedomineerd door de zeezoogdieren, hoewel meer recent de focus verruimd werd naar hun prooidieren, namelijk vissen en in mindere mate mariene invertebraten. Het frequentiebereik van geluid geproduceerd door menselijke activiteiten (of antropogeen geluid) overlapt in de meeste gevallen met het gehoorbereik van vissen. Dit heeft tot gevolg dat zowel gehoorschade, fysiologische stress als gedragsstoornissen kunnen worden veroorzaakt door onderwatergeluid. Daarnaast kunnen biologisch relevante geluiden worden gemaskeerd. Daarenboven kan de geluidsdruk het zwemblaasvolume beïnvloeden, wat op zijn beurt kan zorgen voor (dodelijke) interne verwondingen. Afhankelijk van de specifieke geluidsparameters en de vissoort kan de impact van antropogeen geluid variëren van onmiddellijke sterfte tot helemaal geen effect. Gezien vissen een vitale component vormen in de meeste voedselwebben en gezien de economische waarde van vele vissoorten, is het noodzakelijk om informatie te verzamelen over de potentiële effecten van dit onderwatergeluid. Bovendien moeten drempelwaarden vastgelegd worden voor verschillende combinaties van geluidsbronnen en vissoorten.

In dit doctoraatsonderzoek onderzochten we de geluidseffecten gerelateerd aan het heien van funderingspalen tijdens de constructiefase van **offshore windmolenparken** (OWP), een steeds belangrijkere menselijke activiteit. We onderzochten de effecten van het heien op jonge zeebaarzen (*Dicentrarchus labrax*), een vissoort met een gesloten zwemblaas, een physoclist. Deze doctoraatstudie is gestart vanuit de toenmalige assumptie uit het Nederlands rapport Prins et al. (2009), dat 100% van de viseieren en vislarven zouden sterven in een straal van 1 km rond de hei-activiteit. Deze hypothese was gebaseerd op de gemodelleerde verspreiding van vislarven, het sterftecijfer gerelateerd met onderwater explosies en de teruggerekende geluidsniveaus van onderwatergeluid gegenereerd door heiactiviteiten in het Nederlands deel van de Noordzee.

Dit doctoraatsonderzoek heeft een multidisciplinaire aanpak, met als doel inzicht te verwerven in de effecten van impulsief geluid (geproduceerd tijdens het heien) op jonge vissen. Daarbij werden volgende onderzoeksvragen naar voren geschoven:

(I) Worden jonge vissen (larven en juvenielen) beïnvloed door impulsief geluid, wat zijn de effecten en op welk niveau manifesteren die zich, bijvoorbeeld mortaliteit, stressreacties of gedragsveranderingen?

(II) Kunnen de effecten op jonge vissen in verband gebracht worden met een specifieke geluids-gerelateerde parameter of een biologische parameter? Kunnen we geluidsdrempelwaarden definiëren waarboven jonge vissen negatief worden beïnvloed door het onderwatergeluid?

(III) Wat is de ecologische relevantie van de waargenomen individuele effecten?

(IV) Hoe kunnen de resultaten van deze studie bijdragen aan het marien beheer en beleid in België (en Europa) zodat de milieu-impact van het heien in toekomstige offshore windmolenparken beperkt wordt en om een goede milieutoestand (GES) kan bekomen worden voor de 11de descriptor van de kaderrichtlijn Mariene Strategie (MSFD)?

De context van deze multidisciplinaire studie over de impact van heigeluid op zeebaars is weergegeven in **Hoofdstuk 1**. Eerst wordt een algemene inleiding gegeven tot de onderwaterwereld van geluid gegeven. Scheepvaart, seismisch onderzoek, onderwaterexplosies en hei-werkzaamheden werden geïdentificeerd als de belangrijkste antropogene geluidsbronnen. Geluid speelt een essentiële rol in de overdracht van informatie uit de omgeving over te brengen naar de mariene fauna, zoals zeezoogdieren, vissen en invertebraten. Vooral bij zeezoogdieren is geluid uiterst belangrijk en vormt het de basis voor hun sociaal- en foerageergedrag. Maar van alle vertebraten zijn het de vissen die de grootste diversiteit aan gehoorstructuren en gevoeligheid voor geluid vertonen. De frequentiebreedte van antropogeen geluid overlapt grotendeels met de frequenties die hoorbaar zijn voor de meeste vissen. Bovendien kan sterk impulsief geluid dodelijke (fysieke) schade toebrengen bij zeezoogdieren en vissen. De precieze impact, de onderliggende mechanismen en de ecologische gevolgen van antropogeen geluid op het mariene leven zijn nog niet goed gekend en al zeker niet met betrekking tot vissen. Binnen de Europese kaderrichtlijn Mariene Strategie wordt antropogeen onderwatergeluid officieel erkend als een vervuilende factor. Bijgevolg moet de impact van het gegenereerde geluid op de mariene fauna geëvalueerd worden, om zodoende maatregelen te kunnen nemen. Over de hele Noordzee werd recent een nieuwe antropogene geluidsbron, het heien, geïntroduceerd. Dit is de meest gebruikte methode om funderingspalen voor OWP te installeren en zal zeker de komende jaren nog veel worden toegepast. Daarom werd voor deze doctoraatstudie het heien gekozen als dé geluidsbron van sterk impulsief onderwatergeluid om de impact ervan op mariene vissen te onderzoeken.

In de volgende vier hoofdstukken (Hoofdstuk 2 tot 5) wordt een inschatting gemaakt van de impact van intens heigeluid op directe en indirecte mortaliteit en op directe en chronische fysiologische stress. Daarnaast wordt de impact van laag intensief impulsief geluid op het gedrag van jonge zeebaars nagegaan en worden kritieke geluidsparameters gerelateerd aan de fysiologische stress respons, in meer detail bestudeerd.

Hoofdstuk 2 geeft de resultaten weer van een *in situ* experiment aan boord van een heiplatform, waarbij zowel directe en uitgestelde mortaliteit bij juveniele zeebaars (68 en 115 dagen oud) werden onderzocht. Het was het eerste en voorlopig enige veldexperiment dat erin slaagde om vissterfte te bepalen gedurende een volledige heicyclus op een afstand van slechts 45 m van de hei-activiteit zelf. Vissen werden blootgesteld aan 1739 tot 3067 heislagen met een geluidsbelasting (‘*single strike sound exposure level’,* SEL_{ss}) van 181 en 188 dB re 1 µPa²·s per heislag, en een cumulatieve geluidsbelasting (‘*cumulative’* SEL_{cum}) van 215 tot 222 dB re 1 µPa²·s. De blootgestelde juvenielen (68 en 115 dagen oud) vertoonden geen verhoogde directe mortaliteit vergeleken met een controle groep die enkel aan het achtergrondgeluid in de zee werd blootgesteld. Hiermee werden de resultaten van andere labo-experimenten, die waren uitgevoerd in akoestisch gecontroleerde kamers, gevalideerd. Na het veldexperiment werden de vissen nog twee weken opgevolgd in het labo. Onder optimale labo-condities konden we ook geen uitgestelde mortaliteit vaststellen. Indien de vissen interne verwondingen opliepen tijdens de blootstelling aan het heigeluid bleken deze alleszins niet dodelijk te zijn. Deze studie verwerpt aldus de 100% mortaliteitshypothese die in 2009 in een Nederlands onderzoek naar voor werd geschoven.

In **Hoofdstuk 3** werd de fysiologische stressrespons van juveniele zeebaars (68 en 115 dagen oud) op intens heigeluid onderzocht. Dit werd tot nu toe nog niet eerder onderzocht. Tijdens hetzelfde *in situ* experiment als hierboven beschreven (Hoofdstuk 2), werden eveneens de primaire, secundaire en tertiaire stressreacties onderzocht tijdens en na blootstelling aan een volledige heisessie. Als maat voor de primaire stressrespons bleek ‘*whole-body’* cortisol heel gevoelig te zijn aan stress geassocieerd met het transport en het behandelen van de vissen voor en tijdens het experiment. Er werd echter een sterke secundaire stress reactie waargenomen, in de vorm van significante reducties in zuurstofconsumptie (49-55%) en lage *‘whole-body’* lactaat concentraties. In tegenstelling tot de vissen van dag één, waren de vissen die op de tweede dag aan het heigeluid werden blootgesteld, onrechtstreeks reeds blootgesteld aan de heisessie op de eerste dag. In de controle groep van dag twee verminderde de zuurstofopname met 34 - 40% ten opzichte van de controle groep op de eerste dag. Dit kan wijzen op een langdurige stressreactie of op een toegenomen gevoeligheid voor nieuwe stressfactoren. Tertiaire stressreacties manifesteren zich enkel wanneer de homeostase in de vis niet hersteld geraakt en konden niet waargenomen worden. Terug in het lab, werden na 30 dagen in optimale labo condities namelijk geen afwijkingen gevonden in specifieke groeisnelheid of algemene conditie vergeleken met de ‘niet-behandelde’ controle groep. Alleen een kortstondige vermindering in de conditie kon worden aangetoond. Gevolgen op lange termijn van herhaaldelijke blootstellingen aan sterk intens impulsief geluid voor vissen in het wild zijn tot nog toe niet onderzocht.

Hoofdstuk 4 onderzoekt de essentiële geluidsparameters die verantwoordelijk zijn voor de fysiologische stressreacties waargenomen in het veldexperiment tijdens het heien. De primaire en secundaire stressreacties van larvale en juveniele zeebaarzen op intens impulsief geluid werden enerzijds vergeleken tussen twee labo-experimenten met verschillende geluidsbronnen (SIG Sparker en Larvaebrator) en anderzijds met de stressreacties die werden waargenomen tijdens het *in situ* veldonderzoek (Hoofdstuk 3). De geluidsbronnen van beide labo-experimenten genereerden vergelijkbare geluidsniveaus voor de standaard geluidsdruk parameters als deze gemeten tijdens de veldstudie (*'zero-to-peak sound pressure level'* (L_{z-p}, 208 dB re 1 µPa), SEL_{ss}, 181 dB re 1 µPa²·s en SEL_{cum}, 214 dB re 1 µPa²·s). Daarentegen was het frequentiegebied waarin het geluid geproduceerd werd in de drie experimenten verschillend. '*Whole body'* cortisol (een proxy voor primaire stress reacties) bevestigde de gevoeligheid van juveniele en larvale vis voor behandelingsstress. Toch wijzen de toegenomen of gewijzigde '*whole-body'* cortisol waarden erop dat sterk impulsief geluid een primaire akoestische stressrespons kan veroorzaken. De geluidsniveaus van de drie experimenten overlapten qua SEL_{ss} tussen de 315 en 630 Hz 1/3 octaafbanden. Deze frequenties overlappen met het bereik waarin Europese zeebaars reageert op geluid. Hierdoor kan de primaire stressreactie gekoppeld worden aan het gehoor. Tot 50% afname in zuurstofverbruik werd waargenomen bij de juvenielen in het veldexperiment en bij de larven in het sparker experiment. Bijgevolg kan de secundaire stressrespons eerder gerelateerd worden aan de intensieve geluidsimpulsen geproduceerd in de hogere frequenties (>800 Hz). De secundaire stressrespons zou waarschijnlijk geassocieerd met de zwemblaas trillingen, geïnduceerd door de geluidsdruk. Het moge duidelijk zijn dat een sterk impulsief geluid een breed frequentiebereik moet overspannen (zoals gemeten tijdens het echte heien in het veldexperiment) om duidelijke secundaire stressreacties (zoals verminderd zuurstofverbruik en verlaagde lactaat concentraties) uit te lokken bij juveniele vissen. Dit betekent dat de labo-experimenten niet rechtstreeks kunnen geëxtrapoleerd worden naar de 'echte' wereld, omdat sommige gekende en ongekende geluidsparameters (bvb. frequentiebereik) mogelijks niet volledig vergelijkbaar zijn. Deze studie was een eerste stap in het ontrafelen van de onderliggende mechanismen die verantwoordelijk zijn voor het uitlokken van akoestische stressreacties. Verder onderzoek is nodig op de verschillende levensstadia van vissen en om de rol van andere niet-standaard geluidsparameters, zoals '*particle motion*' te bestuderen.

In **Hoofdstuk 5** wordt de impact van onderwatergeluid op het gedrag van vissen onderzocht. Onderwatergeluid kan mogelijks ook het gedrag van vissen verstoren, zelfs op een lager geluidsniveau, waardoor de risicozone rond de geluidsbron veel ruimer wordt. Functioneel gedrag, zoals sociale interacties en foerageergedrag, draagt significant bij tot het overleven en de reproductie van vissen. Daarom kan elke impact op die functionele eigenschappen direct vertaald worden naar gevolgen voor de fitheid van de vissen. Tot nu toe hebben slechts enkele studies vooral de acute impact van antropogeen geluid op het gedrag van vissen onderzocht. Daarom werd in **Hoofdstuk 5** het gedrag van vissen bij impulsief geluid opgevolgd over drie opeenvolgende dagen. In dit labo-experiment werd de invloed van heigeluid op de zwemactiviteit en uitingen van agressie van juveniele zeebaars getest, tijdens en direct na een dagelijkse 25-minuten durende blootstelling (1000 heislagen, SEL_{ss} = 146 dB re 1 µPa²·s, L_{z-p} = 165 dB re 1 µPa; SEL_{cum} = 176 dB re 1 µPa²·s). Daarnaast werd ook de impact op het eetgedrag en -efficiëntie van de juveniele vissen bestudeerd, nadat ze al 15 minuten waren blootgesteld aan het afgespeeld geluid. Elke dag opnieuw werd de zwemactiviteit onderbroken en stopten alle agressieve aanvallen naar hun soortgenoten bij aanvang van de geluidsblootstelling. Maar reeds gedurende de 25 minuten geluidsblootstelling vertoonden de vissen een herstel van hun zwem- en agressief gedrag zoals voor de geluidsblootstelling. Op de eerste dag veroorzaakte de blootstelling aan het impulsief geluid een aandachtswijziging bij de vissen. Dit resulteerde in een verminderd aantal voedselopnamemomenten tijdens en direct na de blootstelling. Deze aandachtswijziging werd niet langer duidelijk waargenomen op de 2de en 3de dag van het experiment. Verder werd bewezen dat de vissen alert bleven voor externe stimulansen tijdens de geluidsblootstelling. Deze resultaten tonen aan dat de initiële reactie van vissen op het geluid verder kan evolueren. Er moet echter nog onderzocht worden of deze resultaten ook van toepassing zijn voor wilde vissen en andere vissoorten.

In **Hoofdstuk 6** worden de technische en praktische uitdagingen behandeld die veld- en labo-experimenten in het bio-akoestisch onderzoek met zich meebrengen. De resultaten van deze studie worden besproken in een ruimer perspectief, door die te vergelijken met bestaande literatuur over heigeluid en andere antropogene impulsieve geluidsbronnen. Zo kan een antwoord gegeven worden op de vier onderzoeksvragen van deze studie. De resultaten uit de vorige hoofdstukken laten toe om de impact van intens impulsief heigeluid op Europese zeebaars te beschrijven (onderzoeksvraag I). Blootstelling aan het onderwatergeluid op een afstand van slechts 45 meter tijdens een volledige heisessie bleek niet dodelijk te zijn voor juveniele zeebaars, noch acuut noch uitgesteld. Het leidde wel tot fysiologische stress, al was die beperkt tot een relatief korte periode. Deze periode van stress kan wel verlengd worden door herhaalde geluidsblootstellingen. Gebaseerd op de resultaten van de veld- en labo-experimenten kon de fysiologische stressrespons van larven en juvenielen geassocieerd worden met de standaard geluidsparameters (SEL_{ss}, SEL_{cum} and $L_{z\text{-}p}$) en het frequentiebereik waarin de hoogste geluidsenergie werd genoteerd. Verder blijkt het geluid in te werken op het gehoor en op de zwemblaas wat de primaire en secundaire stressreactie veroorzaakt bij de vissen. Bovenstaande studies gebruiken impulsief geluid met hoge intensiteit, wat enkel gemeten wordt dichtbij de hei-activiteit zelf. Op grotere afstand van de hei-activiteit bevat het impulsief geluid minder energie, maar dit kan nog steeds een gedragsverandering induceren, althans bij het begin van de geluidsblootstelling. Tijdens de blootstelling vertoonden de juveniele zeebaarzen herstel van de initiële reactie. Herhaalde blootstellingen hebben bovendien geen duidelijk effect op het eetgedrag. Als we onze resultaten combineren met andere gedragsstudies kunnen we besluiten dat de gedragsreactie van vissen op een zelfde stressor (bvb. geluid) sterk afhangt van soort tot soort. Meer soorten met verschillende levensstrategieën moeten bestudeerd worden vooraleer we de resultaten kunnen veralgemenen.

Om een antwoord te bieden op de onderzoeksvraag II worden de resultaten geïntegreerd met de bestaande kennis waardoor geluidsdrempels voorgesteld kunnen worden i.v.m. mortaliteit, fysiologische stress of gedrag bij jonge vissen met een gesloten zwemblaas. Aangezien er geen sterfte optrad in het veldexperiment, moet de geluidsdrempel voor mortaliteit hoger liggen dan de gemeten geluidsparameters (SEL_{ss} >188 dB re 1 μPa^2.s; SEL_{cum} >222 dB re 1 μPa^2.s.; $L_{z\text{-}p}$ > 210 dB re 1 µPa). Deze studie is de eerste om een geluidsdrempel voor te stellen waarboven een fysiologische stressrespons bij juveniele vissen kan worden uitgelokt: intens impulsief geluid moet tenminste een SEL_{ss} van 170 tot 181 dB re 1 $\mu Pa^2 \cdot s$ bij frequenties hoger dan 315 Hz bereiken om fysiologische stress te induceren. Een geluidsdrempel voor gedragsveranderingen gerelateerd aan heigeluid kon niet worden bepaald.

Naast het toekennen van geluidsdrempels aan verscheidene effecten die vissen kunnen ondervinden tijdens het heien, moeten ook de ecologische consequenties geëvalueerd worden (onderzoeksvraag III). Met andere woorden: de individuele effecten moeten worden opgeschaald naar het niveau van een vispopulatie, want vanuit een ecologisch standpunt zijn de individuele effecten op vissen ondergeschikt ten opzichte van de effecten op een ganse populatie. Om deze oefening uit te voeren, zijn er gegevens nodig over de aanwezige geluidsbronnen, de voortplanting van het geluid, de individuele impact, de populatiegrootte, de verspreiding van de soort en de grootte van de getroffen (sub)populatie. Tot nu toe zijn er nog niet voldoende gegevens verzameld en kon de ecologische impact voor vissen nog niet worden ingeschat. Maar gelet op de resultaten die deze studie aantoonden voor de individuele vissen, kunnen we veronderstellen dat de ecologische gevolgen van heien en heigeluid op de gezondheid van een vispopulatie eerder subtiel te noemen zijn.

De Europese en nationale wetgeving moet geëvalueerd worden zodat advies kan gegeven worden om de impact van onderwater geluid op vissen verder te minimaliseren. Binnen de Europese kaderrichtlijn Mariene Strategie werd een goede milieutoestand gedefinieerd aan de hand van 11 kwantitatieve descriptoren. Hierin wordt gesteld dat de geproduceerde onderwatergeluidsniveaus geen nadelige gevolgen mogen hebben voor het mariene ecosysteem (Descriptor 11). Een technische werkgroep rond onderwatergeluid (TSG Noise) werd samengesteld om deze onderwatergeluid descriptor verder uit te werken. Deze werkgroep adviseerde om een geluidsregister op te zetten waarin alle antropogene geluidsbronnen opgenomen worden. Daarnaast werd 'aanzienlijke verplaatsing' van mariene organismen aangeduid als de belangrijkste impact van impulsief onderwatergeluid. Ten slotte stelde de werkgroep voor om 'geluidsverstoringsdagen' in alle Europese regionale zeeën te modelleren, aan de hand van de aanwezigheid van antropogeen onderwatergeluid in ¼ ICES kwadranten[1], waarbinnen het geproduceerde geluid boven de geluidsdrempel gaat die 'aanzienlijke verplaatsing' veroorzaakt. Een goede milieutoestand moet van toepassing zijn op alle mariene organismen, terwijl het advies van de TSG Noise werkgroep voornamelijk gebaseerd is op zeezoogdieren. Het is mogelijk dat 'aanzienlijke verplaatsing' niet de belangrijkste impact is bij vissen. Doorgaans worden vissen ook niet meegenomen in de nationale wetgeving van de EU lidstaten.

Gebaseerd op deze doctoraatstudie kunnen we nu de onderzoeksvraag IV beantwoorden. We kunnen besluiten dat de impact van heigeluid op vissen minder erg is dan aanvankelijk werd voorspeld. Daarom zijn er voorlopig geen strengere maatregelen nodig in België noch in de

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[1] ICES kwadranten vormen een rooster over het gebied tussen 36°N en 85°30'N en 44°W en 68°30'O. De kwadranten verschuiven per interval van 30' (longitude) en 1° (latitude).

andere lidstaten. Wel is er meer onderzoek nodig om de beslissing rond het niet opnemen van vissen in de wetgeving en het marien beheer wetenschappelijk te ondersteunen, en toch een goede milieutoestand voor alle mariene fauna te garanderen.

Tenslotte worden een aantal toekomstige en onbeantwoorde onderzoeksvragen opgesomd, die moeten beantwoord worden om een goede aanpak te garanderen van het menselijk gegenereerd onderwater geluid in de mariene omgeving. Het gebrek aan data en kennis over '*particle motion*' blijft een hiaat in de wetenschappelijke kennis dat dringend onderzocht moet worden. Ook de onderliggende geluidsparameters die fysiologisch stressreacties en gedragsveranderingen induceren, moeten verder onderzocht worden zodat deze gereduceerd kunnen worden. Bovendien zijn data nodig over de lange termijn impact van antropogeen impulsief geluid om de ecologische gevolgen van het heien en het heigeluid op populatieniveau te modelleren. Hierbij is het opportuun om de vissen in hun natuurlijke habitat te bestuderen, gebruik makende van nieuwe technologieën, zoals telemetrie. Daarnaast moet ook de impact van het voortdurend aanwezige geluid tijdens de operationele fase van de offshore windmolenparken op vissen worden nagegaan. Dit operationeel geluid zal zeker de komende 20 jaar continu geproduceerd worden en alleen diepgaand onderzoek hieromtrent kan een antwoord bieden op vragen rond de invloed van deze menselijke activiteit op de gezondheid en het welzijn van de visbestanden.

List of Abbreviations

Abbreviation	Description	Unit
ANOVA	Analysis of variance	
BCLAS	Belgian Council for Laboratory Animal Sciences	
BMM	Beheerseenheid van het Mathematisch Model van de Noordzee	
BPNS	Belgian part of the North Sea	
c	Sound of speed	$m \cdot s^{-1}$
dph	Days post hatching	days
EC	European Commission	
EEZ	Exclusive economic zone	
EU	European Union	
EWEA	European Wind Energy Association	
FEM	Finite element model	
FHWG	Fisheries Hydro-acoustic Working Group	
FWO	Flemish Fund for Scientific Research	
GBF	Gravity based foundation	
GES	Good environmental status	
IBL	Institute of Biology Leiden	
ICES	International Council for the Exploration of the Sea	
ILVO	Institute for Agricultural and Fisheries Research	
IMO	International Maritime Organization	
ISO	International Organization for Standardization	
IWT	Agency for Innovation by Science and Technology	
JNCC	Joint Nature Conservation Committee (UK)	
lme	Linear mixed-effects model	
$L_{p\text{-}p}$	Peak-to-peak sound pressure level	dB re 1 µPa
$L_{z\text{-}p}$	zero-to-peak sound pressure level	dB re 1 µPa
MARECO	Marine Biology and Management Section of OD nature	
MMC	Marine Mammals Commission	
MPA	Marine protected area	
MS	Member state	
MSFD	Marine strategy framework directive	
MSP	Marine spatial planning	
MSY	Maximum sustainable yield	

Abbreviation	Description	Unit
MW	Megawatt	
NMFS	National Marine Fisheries Service	
NRC	National Research Council	
OWF	Offshore wind farm	
OWP	Offshore windpark	
p	Sound pressure	Pascal
PCoD	Population Consequences of Disturbance	
RCMG	Renard Centre of Marine Geology (Ugent)	
RIA	Radioimmunoassay	
RWS	Rijkswaterstaat	
SD	Standard deviation	
SE	Standard error	
SEL_{cum}	Cumulative sound exposure level	dB re 1 $\mu\text{Pa}^2\cdot\text{s}$
SEL_{ss}	Single strike sound exposure level	dB re 1 $\mu\text{Pa}^2\cdot\text{s}$
SGR	Specific growth rate	
SL	Standard length	mm
SL_0	Initial standard length	mm
SL_t	Final standard length	mm
SPL	Sound pressure level	dB re 1 µPa
SVL	Sound velocity level	dB re 1 $\text{nm}\cdot\text{s}^{-1}$
TAC	Total allowable catch	
TIFF	Tagged image file format	
TNO	Toegepast-Natuurwetenschappelijk Onderzoek	
TSG Noise	Technical subgroup of MSFD	
TTS	Temporary hearing threshold	
UGCT	Centre for X-ray Tomography of Ghent University	
UNCLOS	United Nations Convention of the Law of the Sea	
v	Particle velocity	
VEL_{cum}	Cumulative sound velocity level	dB re 1 $(\text{nm}\cdot\text{s}^{-1})^2\cdot\text{s}$
VEL_{ss}	Single strike sound velocity level	dB re 1 $(\text{nm}\cdot\text{s}^{-1})^2\cdot\text{s}$
W_0	Initial wet weight	g
W_t	Final wet weight	g
WW	Wet weight	g
Z	Acoustic impedance	$\text{Pa}\cdot\text{s}\cdot\text{m}^{-3}$
λ	Wavelength	m
ρ	Density	$\text{kg}\cdot\text{m}^{-3}$

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CHAPTER 1

General introduction

Setting the scene

Several human activities introduce sound into the sea (*e.g.* shipping, offshore wind farms, seismic surveys). In the past decade, the interest in green energy at sea increased, *e.g.* through wind turbines, tidal turbines or wave power. Next to the increase of continuous sound, the increased impulsive sound related to pile driving for these wind turbines are of concern not only for sea mammals, but also for fish and other marine animals. Based on modelled fish larvae distributions, mortality rate due to underwater explosions and back-calculated energy levels of underwater sound related to pile driving activities in the Dutch part of the North Sea, Prins et al. (2009) assumed a 100% mortality in fish eggs and larvae up to 1 km around a pile driving source. This assumption circulated at the start of the PhD thesis. The introductory chapter will guide the reader through the world of underwater sound and its relation to marine fauna. Specifically, detection and interpretation of sound by fish is explained. Secondly, the potential impact of anthropogenic underwater sound on marine fauna is discussed, with special emphasis on fish. The current status of the legal framework around the anthropogenic sound is described. Finally, the research questions, the case study and the thesis outline are explained.

1.1 The underwater world of sound

1.1.1 Underwater sound characteristics

Underwater sound is a vibration of fluid particles that propagate as a longitudinal pressure wave away from the sound source. Although the sound wave causes fluctuations in particle velocity, fluid density, pressure, and even temperature, the strength of the sound is most often characterized by the sound pressure. **Sound pressure** is measured in Pascal, but often the sound pressure level is used, *i.e.* the effective value of the pressure wave expressed on a logarithmic scale. The sound pressure level (SPL) in water is calculated as 20 log ($P_{measured}/P_{ref\ water}$) with $P_{ref\ water}$ being 1 µPa (Au and Hastings, 2008). This reference value clearly differs from the reference pressure in air ($P_{\text{ref air}}$ = 20 µPa), which calls for some caution when comparing under water *versus* air sound levels.

The second sound parameter - **particle motion**[a] - is described as a vector with a magnitude and a direction (Casper and Mann 2006). Particle motion is complementary to sound pressure and is either calculated as particle displacement (m), velocity ($m.s^{-1}$) or acceleration ($m.s^{-2}$) (Andersson, 2011, Van der Graaf et al., 2012). All three measures of particle motion are related in a frequency dependent way (Nedelec et al., 2016)[b].

The speed of sound (*c*) is a constant and relates frequency (Hertz, cycles per sec) to wavelength (m). The speed of sound is directly related to material properties and is dependent on temperature and static pressure. Hence the deep sound channels that may exist in the ocean where the combination of temperature and pressure causes the lowest sound speed, serving as a waveguide (Walree, 2013). High frequencies have short wavelengths and low frequencies have longer wavelengths. In addition, absorption of the sound tends to increase with frequency which causes high frequencies to attenuate faster than low frequencies. Sound in water travels faster ($1500\ m \cdot s^{-1}$) and further compared to sound in air ($340\ m \cdot s^{-1}$) (Ainslie et al., 2009).

Underwater sound pressure is usually measured with a hydrophone, but measuring particle motion is less straightforward. In the far field of the source; that is at least several times the size of the source and several wavelengths away from the source, sound propagates as a plane wave[c]. The relationship between sound pressure (*p*) and particle motion (*v*) is then described by a (temperature dependent) constant, known as the **acoustic impedance** (*Z*), where $Z = p \cdot v^{-1}$ (Nedelec et al., 2016). In addition, under mild assumptions of the fluid, *Z* can also be described as ρc, *with* ρ being the density of the fluid. However, free field conditions are uncommon at sea - except in deep water and far from boundaries - for the entire frequency range. Hence, particle motion can usually not simply be calculated from sound (Nedelec et al., 2016) . In the far field, particle motion can be calculated from the pressure gradient measured between two hydrophones (MacGillivray and Racca, 2006, Zeddies et al., 2010). However, in the near field[d], pressure and particle motion decay at different rates (Zeddies et al., 2012). Moreover, particle motion is a vector and needs to be measured along three axes. So, accelerometers mounted in the x-y-z axes are a better option to measure particle motion (Sigray and Andersson, 2011).

Based on sound pressure and particle motion, a whole range of sound metrics can be calculated, which are needed to fully describe the underwater sound (TNO, 2011). The standardisation of these underwater sound parameters is currently being finalised (ISO/TC 43/SC 3[1]). Two types of sound are usually distinguished, continuous *vs.* transient sounds, and these are described by different metrics (De Jong et al., 2011). **Continuous sound** is characterised by average sound pressure level (SPL), average sound velocity level (SVL) and frequency spectrum. **Transient or impulsive sounds** are characterized by pulse duration, peak rising time, frequency content, duty cycle, zero to peak sound pressure level ($L_{z\text{-}p}$), zero to peak sound velocity level ($SVL_{zero\text{-}to\text{-}peak}$), sound energy per pulse (single strike sound exposure level, $SEL_{ss;}$ single strike velocity exposure level, VEL_{ss}), total energy received over time (cumulative sound exposure level, SEL_{cum}, cumulative velocity exposure level, VEL_{cum}). A full description and calculation of these metrics is given in Ainslie (2011).

1.1.2 Sources of underwater sound

Sound is omnipresent in the marine environment and can be either natural or human-induced (Figure 1) (Hildebrand, 2009). The combination of all the sounds creates the soundscape of the environment (Fay, 2009, Hastings and Sirovic, 2015). **Natural sound** arises from abiotic sources such as wind, rain, lightning, thermal sound, gravel sound, flow sound, wave-wave interactions, but also from biotic sources, such as actively emitted acoustic signals by sea mammals and snapping shrimps (*Alpheus heterochaelis*) (Ladich, 2004, Slabbekoorn, 2004, Ainslie et al., 2009, Popper and Hastings, 2009a). The primary natural sources of ambient sound in the ocean have frequencies between 1 and 50 kHz (Ainslie et al., 2009). Over the last decades, the presence of underwater sound in the marine environment by human activities increased considerably in the marine environment, both intentionally and unintentionally. These sounds vary substantially in their amplitude and frequency profile, but also in temporal and spatial patterns (Blickley and Patricelli, 2010, Slabbekoorn et al., 2010).

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[1] ISO/TC 43/SC 3: International Organization for Standardization for underwater acoustics.

As described above, two major categories of **anthropogenic sounds** can be discerned: (1) continuous sounds which are generally low in intensity but remain in the marine environment for a long time, and (2) transient, short-lived sounds, which can be either impulsive or non-impulsive. Impulsive sounds are characterized by sudden, very high intensity broadband impulses, while non-impulsive transient sounds, like underwater construction sounds, lack these sudden outbursts of sound (Popper et al., 2014). Impulsive sound sources include some forms of active sonar, but are mainly related to explosions, seismic airgun shots and pile driving. In the North Sea, shipping, seismic surveys (airguns), underwater explosions and pile driving are the main contributors to the total anthropogenic sound energy (Ainslie et al., 2009)

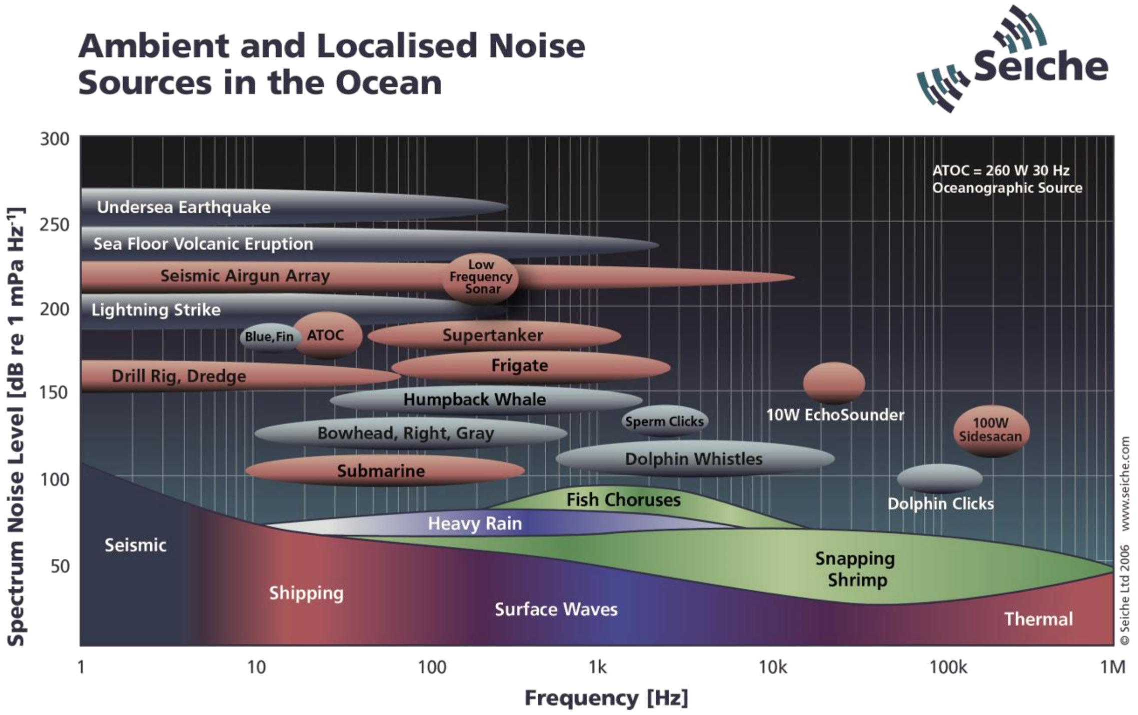

Figure 1. Spectral density of anthropogenic activities and abiotic and biotic sound sources measured at 1 m. Figure taken from Coates (2002)© Seiche Ltd. 2006.

Continuous sound raises the background sound level and can either be tonal or broadband. Examples are sounds from shipping, the operation of offshore wind turbines, dredging activities, and some active sonar systems. Most energy of underwater sound due to shipping is found below 1 kHz (De Jong et al., 2011, McKenna et al., 2012). Increased sound background levels are of particular importance in aquaculture facilities, where they are caused by aerators, pumps, water circulation, feeding, maintenance machinery, and room ventilation (Bart et al., 2001, Davidson et al., 2007, Craven et al., 2009). **Active sonar** is used to explore and map the ocean. It can be continuous or impulsive and may vary in its operational frequency, from ≤1 kHz (low frequency) over 1 to 10 kHz (mid frequency) to ≥10 kHz (high frequency) (Popper et al., 2014).

Explosions are single impulsive sounds and produce spherical shock waves which travel faster than the speed of sound in water (Popper et al., 2014). Seismic airgun shots and pile driving pulses consist of repeated impulsive sounds, and differ in waveform and energy distribution. Like active sonar, **seismic airguns** are used to acoustically explore the substrate. They have a strong peak level without lower order oscillations in the waveform. The peak energy is found between 20 – 50 Hz up to 200 Hz but can range up to 1000 Hz (Ainslie et al., 2009). Seismic airguns are usually towed behind a vessel in an array, containing many airguns that are fired at the same time. Interaction between airguns causes vertical and horizontal directivity patterns in the far field, resulting in different particle motion and sound pressure fields in each measurement direction. There is no direct contact with the sediment, but reflections (‘surface ghost’) from the seafloor can contribute to the waveform (McCauley et al., 2000, Sertlek and Ainslie, 2015). It is difficult to determine the SEL_{cum} of the seismic airgun sound that ‘hits’ an animal, because the SEL_{ss} of each shot from a moving vessel is different, and the distance to the animal changes as well (Popper et al., 2014).

Pile driving also generates a repeated impulsive sound, but unlike airguns, pile driving is a stationary sound source that is used for the construction of bridges, offshore oil and gas rigs, and wind turbines. Pile driving sound is characterized by a rapid rise time to the sound pressure peak level and a subsequent decay period with oscillating maximum and minimum SPL. Consequently, the $L_{z\text{-}p}$ alone is not sufficient to describe pile driving sound, and additional information, *e.g.* on SEL_{ss} (which accounts for the total energy of that sound pulse) is required (Caltrans, 2007, Popper et al., 2014). The size of the hammer, the diameter of the monopiles and the seafloor characteristics determine the source energy level (Madsen et al., 2006). When the pile driving hammer hits the pile, an impulse sound is created, which propagates through the air and down the pile (transient stress wave) into the seabed (see subsection

1.5.2; Figure 8). Hence, sound is radiated from the pile into the air, the surrounding water and the sediment. Waves in the sediment can bounce back to the water column at a distance from the pile, thus creating localized zones of very high or low sound pressure and acoustic particle motion. Consequently, sound levels can be higher in certain areas further from the pile than close to it (Caltrans, 2001, Hastings and Popper, 2005, Popper and Hastings, 2009a). Pile driving sound close to the source (up to 2 km distance) is mostly broadband, with peak sound energy stretching from 100 to 2000 Hz, but with considerable energy up to 10 kHz (Stockham et al., 2010). Bailey et al. (2010) showed that high frequencies (>5 kHz) are almost completely attenuated at 4 km distance, yet pile driving sound could still be discriminated from background levels at a distance up to 70 km (Ainslie et al., 2009).

1.1.3 Importance of sound for marine fauna

Sound plays a key role in conveying environmental information to marine organisms. Sound travels faster and further in water than in air, whereas visual information is often limited (Popper et al., 2003, Hastings and Popper, 2005). Marine ecosystems host a wide variety of species and sound is used to a varying extent at several trophic levels, from small invertebrates, to fish and marine mammals (Kiszka et al., 2015). Sound through hearing, is essential for communication, mate selection and predator-prey interactions (Myrberg, 1972, Ketten, 1998). Especially in **marine mammals**, the key role of sound has been repeatedly demonstrated, be it the production of low frequency sounds, for example by humpback whales, or the production of clicks for echolocations by dolphins and porpoises (Amundin, 1998, Southall et al., 2007). Sound is known to be used for social interactions, such as reproduction and maintenance of group structure, but also for orientation, foraging and in response to predators (Wartzok and Ketten, 1999, Southall et al., 2007, Clark et al., 2009).

Much less knowledge exists on the hearing and use of sound by **marine invertebrates**. Having ciliated hair cells, they are able to sense water movements or particle motion close to a sound source (Budelmann, 2010, Mooney et al., 2012). In cephalopods and crustaceans, the statocyst has been recognized as the primary sound detection organ, which acts as an accelerometer in response to particle motion (Samson et al., 2014). Different species of squid, crab, lobster and sea urchins are known to produce sound (Iversen et al., 1963, Montgomery et al., 2006). The loudest marine invertebrate is the snapping shrimp (*Alpheus heterochaelis*), which uses the snapping to stun or even kill small prey. This snapping sound is a dominant sound source in tropical and sub-tropical waters, and it increases the ambient sound by 20 dB (Herberholz and Schmitz, 1998). Another example was given by Stanley et al. (2012), who found a correlation between the settlement of five coastal crab species (*Hemigrapsus sexdentatus, Cyclograpsus lavauxi, Cymo andreossy, Schizophrys aspera, Grapsus tenuicrustatus*) and habitat-related underwater sound cues. This demonstrates the importance of sound for the survival of marine invertebrates.

1.1.3.1 In the dark, fish see by hearing

Fish are the most ancient group of vertebrates and with more than 30,000 species they represent half of all known vertebrates (Kisia, 2003, Froese and Pauly, 2015). Due to their ecological adaptability, they show an incredible diversity in ecology and in the habitats they occupy. Although no external ear structures or holes are visible, all fishes studied to date are able to detect sound (reviewed in Slabbekoorn et al., 2010).

Both biotic and abiotic sound sources create an auditory scene for the fish, which provides information on the environment, and gives cues for orientation, navigation, communication, mate selection and larval settlement (Montgomery et al., 2006, Clark et al., 2009, Slabbekoorn et al., 2010, Holles et al., 2013). However, in contrast to marine mammals, which mostly perceive hearing through sound pressure, the main hearing component for fish is **particle motion** (Southall et al., 2007). All fish are able to detect particle motion by one or two sensory systems with sensory hair cells (similar to those in the mammalian ear), i.e. the lateral line and the inner ear with the otolithic organs. These can both convert particle motion into electrical signals triggering the nervous system (Slabbekoorn et al., 2010).

The **lateral line** picks up signals that originate close to the fish, *i.e.* within one or two body lengths, while the inner ear detects signals in the near and far field (Popper and Carlson, 1998). The lateral line plays a primary role in sensing water motion and low frequencies, ranging from less than 1 Hz up to several hundred hertz (Karlsen and Sand, 1987, Popper and Carlson, 1998, Engelmann et al., 2000).

Unlike any other vertebrate group, there is an incredible diversity in **ear anatomy** (Popper and Fay, 1993, 2011). Not only the anatomy, but also the hearing process varies across fishes, and the extreme variation in hearing bandwidth is unseen in vertebrates (Popper and Fay, 2011). The majority of fish are sensitive to low frequencies of 30 to 1000 Hz, but special adaptations stretch the upper limit to 5000 Hz in some species and even up to 180 kHz (Slabbekoorn et al., 2010). Fish larvae and young juveniles are known to hear in the same frequency range as the adults (Egner and Mann, 2005, Zeddies and Fay, 2005, Wright et al., 2011).

BOX 1. The primary hearing organ of a fish: the inner ear

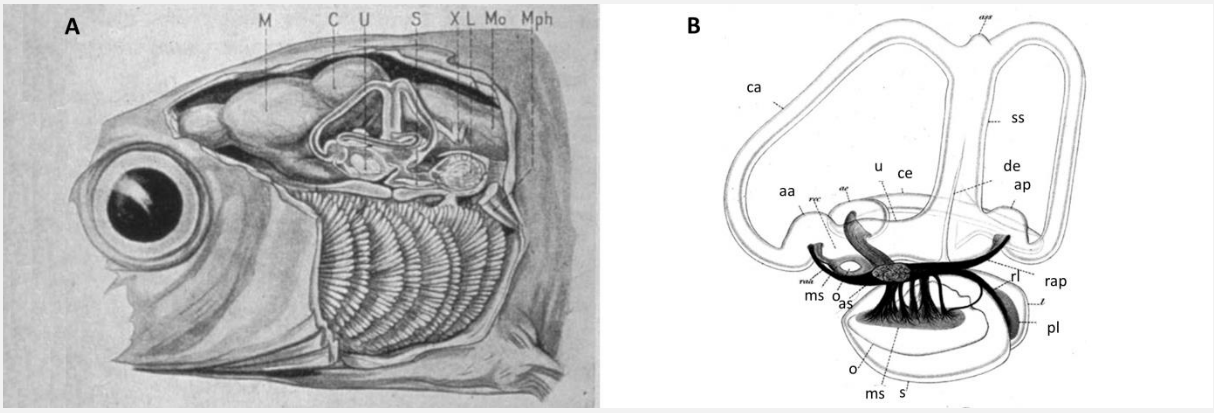

Figure 2. (A) Lateral view of the head of a minnow (*Phoxinus laevis*) (taken from Hastings and Popper, 2005). This picture shows the location of the inner ear. U – utricular otolithic end organ; S – saccule; L – Lagena; M – medulla of brain; C – Cerebellum of brain; X – 10th cranial nerve. **(B)** Medial view of the right inner ear of Atlantic salmon (*Salmo salar*) (adapted from (Popper and Fay, 2011). aa, ap: ampullae of semicircular canals; ac: 8th cranial nerve to ear; ca, cp, ce: semicircular canals; de: ductus endolymphaticus; mn: macula neglecta; ms: saccular epithelium (macula sacculus); o: otolith; pl: lagena epithelium (papilla lagena); rac, rap: rami of eighth cranial nerves to semicircular canals; rl: lagena branch of 8th nerve; rs: saccular branch of 8th nerve; ru: utricular branch of 8th nerve; s: saccular chamber; ss: common canal; u: utricular chamber.

The inner ear consists of three **otolith organs**: the saccule, lagena and utricle (Figure 2) (Nedwell et al., 2004). Although all three otolithic organs are to some extent involved in hearing, the saccule plays a more prominent role and shows the greatest interspecific variation in this function (Popper and Fay, 2011). The basic structure of the otolithic organs closely resembles the function of an accelerometer (Popper and Fay, 2011). Each otolithic organ exists of three components: the otolith, the sensory epithelium with hair cells and an otolithic membrane. An otolith is three times denser than the rest of the body, the latter having a density similar to water (Sand and Enger, 1973). In response to particle motion, the fish body and sensory epithelia move with the same amplitude and phase as the water particles, while the denser otolith moves at a different amplitude and phase (Popper and Fay, 2011). The ciliary bundles in the hair cell experience a shearing force and transduce the perceived acoustic information to the nervous system. Since the hair cells are arranged in orientation groups, they also gather information about the directivity of the particle motion. Together with the relative output levels of both ears and the information of the hair cell orientation groups, the fish perceives a direction of particle motion (Fay and Edds-Walton, 2008, Popper and Fay, 2011).

In addition to the direct stimulation of the ear by particle motion, some fish are also able to indirectly detect sound pressure through the **swim bladder** (Fay and Popper, 1974, 1975, Popper and Lu, 2000). Other than buoyancy control, the swim bladder is also involved in sound production, respiration and hearing (Popper et al., 2014). The perception of sound pressure allows the fish to detect higher frequencies over longer distances, resulting in broader frequency ranges and lower hearing thresholds (Sand and Enger, 1973, Radford et al., 2012, Voellmy, 2013). Swim bladders or other gas filled organs are less dense and more compressible than the fish body or the surrounding water. As a result, the gas filled organs undergo volume changes that transduce the pressure into particle motion, which is then perceived by the inner ears. There is, however, a quick attenuation, so the distance between the inner ear and the swim bladder is a limiting factor.

In different fish groups, several mechanisms have evolved to cope with this limitation (Popper and Lu, 2000). On the basis, Popper and Fay (2011) proposed a **continuum of pressure sensitivity** (Figure 3), thereby abandoning the rather simplistic concept of “hearing generalists” and “specialists”, which divided fish into those species hearing solely through direct stimulation of the inner ear and species hearing through direct and indirect stimulation (Popper and Fay, 2011). The position of fish species along this continuum depends on the relative contribution of pressure detection and the efficiency of transmitting the information to the inner ear (Popper and Fay, 2011). The most advanced ‘hearing’ structures are the **Weberian ossicles**. These are a series of four bones forming a chain between the swim bladder and the spinal medulla, assisting in the transmission of oscillations. This structure can be found in carps, minnows, catfishes, electric eels and characins (all belonging to the Superorder Otophysi) (Diogo, 2009, Lechner et al., 2011, Popper and Fay, 2011). Another type of specialization is the air-filled **suprabranchial chamber** next to the inner ear, as can be found in labyrinth fishes (Anabantoids) (Wysocki et al., 2006). A similar specialization is the **air-filled bullae** close to the inner ear in herring (*Clupea harengus*) (Kastelein et al., 2008)

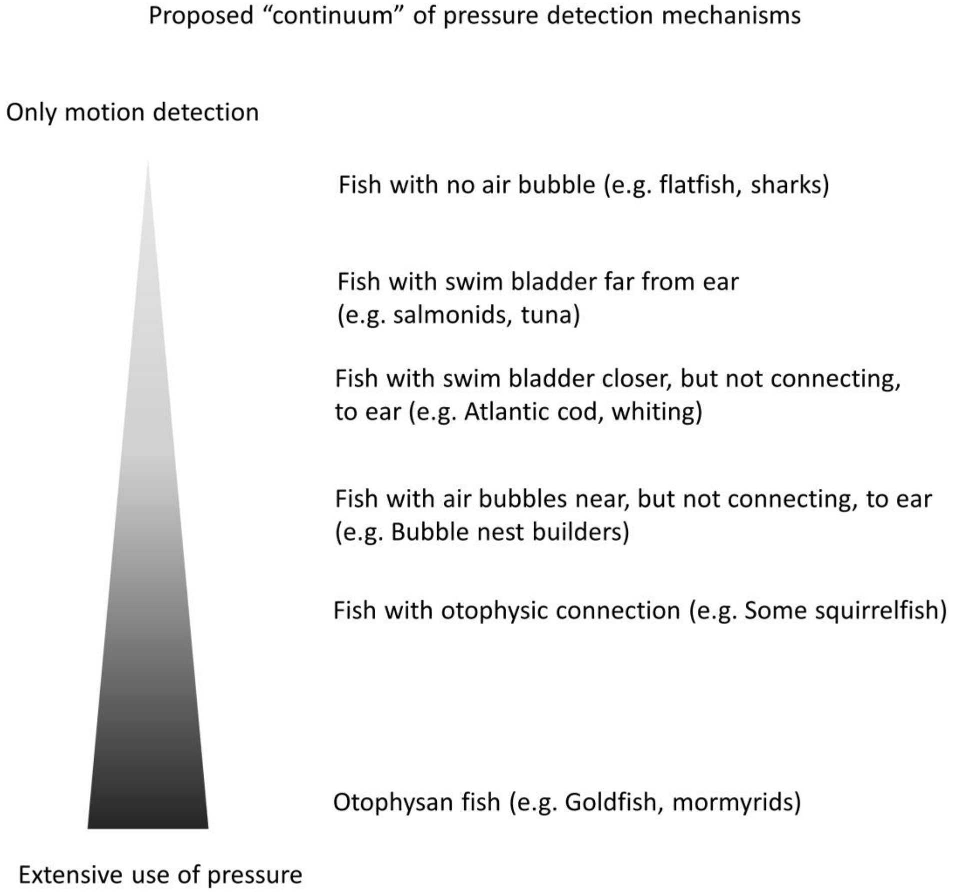

Figure 3. Graphic representation of the hearing capabilities continuum of fish, adapted from Popper and Fay (2011). The continuum depends on the relative contribution of particle motion and pressure and the presence of specialized structures enhancing the perception of sound pressure.

Next to hearing, more than 800 fish species from 108 families are known to actively produce sound (Kaatz, 2002, Rountree et al., 2006, Slabbekoorn et al., 2010). Some fish species are able to produce sound already as larvae (Staaterman et al., 2014). The majority of these **biologically produced sounds** are broadband signals with most energy below 500 Hz (Slabbekoorn et al., 2010). The temporal and spectral sound characteristics depend on species, gender, population, size, and motivation of the fish (Kihslinger and Klimley, 2002, Amorim, 2006, Kasumyan, 2008, Slabbekoorn et al., 2010). The sounds that fish produce are used for reproduction, including courtship and spawning, but can also be an expression of aggression or a tool in territoriality and defence (Myrberg, 1997, Popper and Carlson, 1998, Radford et al., 2015). These biologically produced sounds contribute to the marine soundscape, which will not be further discussed in this thesis (Fay, 2009, Hastings and Sirovic, 2015).

1.2 Impacts of anthropogenic sound on marine fauna

In 1991, a report was published that linked sonar manoeuvres to mass strandings of Cuvier's beaked whales (Simmonds and Lopezjurado, 1991). In the following years, more 'atypical' strandings were reported, which led to the hypothesis that anthropogenic sound may harm **marine mammals**. Consequently, marine mammals became the key interest group for bioacoustics research. Exposing marine mammals to human-induced sound can lead to physical injury, temporary or permanent hearing loss, and changes in foraging behaviour or habitat-use (Gordon et al., 2003, Madsen et al., 2006, MMC, 2007).

More recently, the bioacoustics research has expanded also to the prey species of marine mammals; such as fish and invertebrates. For **invertebrates**, the focus mainly lies on the impact of continuous shipping sound (Morley et al., 2014). Several studies have shown that shipping sound affects the foraging and anti-predator behaviour, but it also causes stress in shore crabs (*Carcinus maenas*), and impairs the embryonic development leading to increased mortality in sea hares (*Stylocheilus striatus*) (Wale et al., 2013a, b, Nedelec et al., 2014). Exposure to low frequency and high intensity sound resulted in modification in the sensory hair cells of the statocysts, which are responsible for balance and position (Andre et al., 2011). Since most invertebrates are slow swimmers, it is difficult for them to escape from harmful environmental conditions, so they are more at risk when exposed to human-induced sound.

1.2.1 Impact of anthropogenic sound on fish

The anatomy, physiology, behaviour and ecology of fish are much more diverse than in marine mammals. Several studies have shown that fish make use of acoustic cues for different functions, and different species show a diverse sensitivity to sound (Hastings and Sirovic, 2015, Ruppe et al., 2015). Therefore, anthropogenic sound is related to a wide impact range on fish. The frequency of human-induced underwater sound largely overlaps with the range of **biologically relevant sound** for fish and marine mammals (Figure 4) (Hastings and Popper, 2005, Slabbekoorn et al., 2010). The impact of anthropogenic sound on fish ranges from immediate death to no effect, and depends on a wide variety of acoustic and biotic factors, including sound type, intensity, duty cycle, temporal structure, duration, predictability, frequency content, fish species, life stage, and life history (table 1) (Hastings 2005, Slabbekoorn 2010).

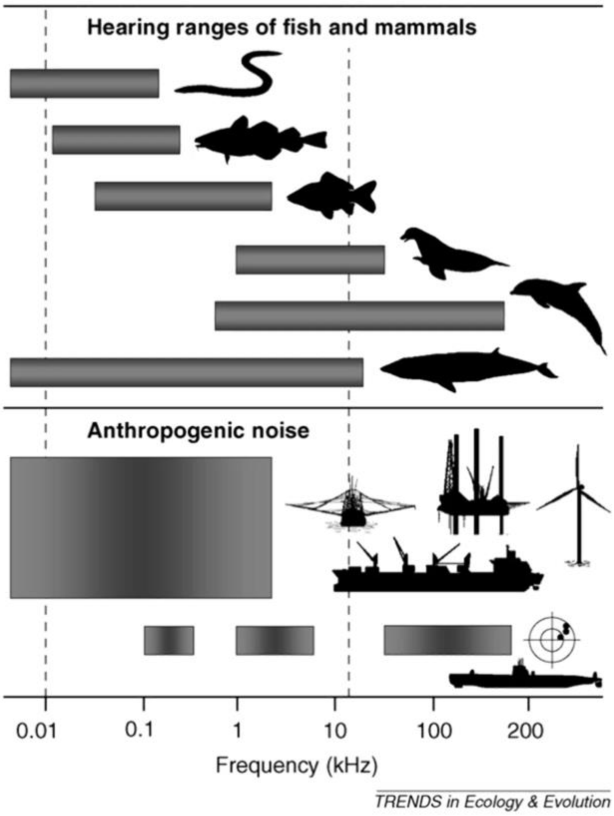

Figure 4. Hearing ranges of fish and sea mammals in relation to the frequency range of sound generated by anthropogenic activities. The dotted line indicates the hearing range of humans. At the bottom, the frequency ranges are given for low-frequency, mid-frequency and high-frequency sonar. Figure adapted from Slabbekoorn et al. (2010).

Intuitively, strong or high intensity impulsive sounds are thought to be more detrimental than less intense continuous sound. Although it is unlikely that continuous sound can cause immediate mortality or sublethal injuries, mortality can still occur as a result of long-term stress, reduced foraging performance, diseases or a higher susceptibility to predation (Purser and Radford, 2011, Halvorsen et al., 2012b, Voellmy, 2013). Furthermore, anthropogenic sound can also cause hearing loss and evoke behavioural effects (Figure 5) (Smith et al., 2004).

Hair cells and the auditory nerve of the inner ear may suffer damage from both exposure to low sound levels for a long period of time and exposure to higher sound levels for a shorter period (Scholik and Yan, 2002, McCauley et al., 2003, Smith et al., 2006, Popper et al., 2007, Popper and Hastings, 2009b). Hearing loss does not always occur after exposure to high intensity impulsive sound. It happens primarily in fishes with hearing specializations, and it is not permanent (Popper et al., 2007, Kane et al., 2010). Hearing loss is accompanied by a fitness reduction in terms of increased vulnerability to predators and reduced foraging success, and the inability to communicate and sense the acoustic environment (Oestman et al., 2009, Popper et al., 2014). Unlike other vertebrates, fish can replace or repair damaged sensory hair cells (Meyers and Corwin, 2008). Consequently, spontaneous repair limits the effects associated with hair cell damage (Smith et al., 2006, Smith et al., 2011, Casper et al., 2013b). The magnitude of the temporary hearing loss is affected by sound pressure level, frequency, duration of the sound, and the size, health and life stage of the fish.

Table 1. Potential negative effects of anthropogenic sound on fish.

Impact	Type of effect
Mortality	Acute and delayed mortality
Barotrauma	Swim bladder rupture, haemorrhages, gas embolism
Auditory damage	Damage to inner hair cells
	Temporary hearing threshold (TTS)
Stress	Primary, secondary and tertiary stress responses
	Hypertension, immune function
Behavioural disturbance	Immediate response (e,g, startle responses, vertical diving, increased group cohesion)
	Decreased foraging efficacy, disturbed anti-predator response disturbed territorial behaviour
	Avoidance of the area (short-term or long-term?)
	spawning?
Masking	Communication with conspecifics
	Biologically relevant sounds
Fitness	Increased egg mortality, reduced larval growth

Mortality associated with explosions has been reported for all life stages, from larval to juvenile and adult fish (Yelverton et al., 1975, Keevin and Hempen, 1997, Govoni et al., 2008). The severity of the injury following an explosion is correlated with the type of **swim bladder** (Caltrans, 2009). In **physostomous** fishes, the bladder is directly connected to the oesophagus, enabling the fish to efficiently adjust bladder volume. In **physoclistous** fishes, the connection with the oesophagus disappears in the early life stage. In these cases, changing swim bladder volume is done by secreting gas through a gas gland on the swim bladder wall (Prosser, 1973). This process is much slower, so these fish are unable to quickly reduce the tension on their swim bladder during exposure to high impulsive sound pressure (Halvorsen et al., 2012a).

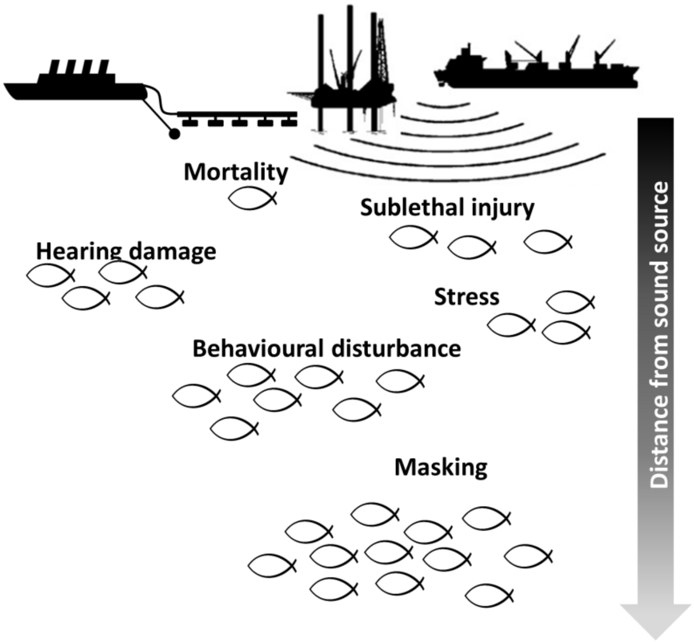

Figure 5. Potential acute impact of impulsive (left: airgun and pile driving) and continuous (right: shipping) anthropogenic sound on fish. Figure modified from Slabbekoorn et al. (2010) and Thomsen et al. (2006).

Sublethal injuries are mostly related to swim bladder compressions as a result of rapid pressure changes, which can injure both the swim bladder itself and the adjoining organs (**barotrauma**) (Stephenson et al., 2010, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a, Casper et al., 2013b). The severity is correlated to the type of swim bladder, as described above (Oestman et al., 2009). Damage to the swim bladder can implicate hearing loss for the fish (Popper et al., 2014). Moreover, a rapid pressure change may cause dissolved gasses in the blood to come out of solution. These bubbles can migrate through the body causing hematomas and capillary dilation (Halvorsen et al., 2012b).

Human-induced sound may also affect **fish behaviour** (Hastings and Popper, 2005, Slabbekoorn et al., 2010). Any disturbance of behaviour may reduce the individual fitness of the fish and, may lead to mortality (Purser and Radford, 2011). There is a clear interspecific variability in behavioural responses (Voellmy et al., 2014a, Voellmy et al., 2014b, Shafei Sabet et al., 2015). **Startle responses** have been observed in several fish species following seismic airgun shooting (Pearson et al., 1992, Santulli et al., 1999, Wardle et al., 2001, Hassel et al., 2004). Similarly, decreased commercial catches have been reported in commercial line and trawl fisheries during and after seismic surveys, suggesting **active avoidance** (Skalski et al., 1992, Engas et al., 1996, Hirst and Rodhouse, 2000, Slotte et al., 2004, Lokkeborg et al., 2012b, a). Changes in depth distribution and swimming pattern can also occur (Pearson et al., 1992, Slotte et al., 2004, Fewtrell and McCauley, 2012). Attention shifts due to the presence of anthropogenic sound could change anti-predator behaviour and **foraging behaviour** (Purser and Radford, 2011, Bracciali et al., 2012, Voellmy et al., 2014a, Voellmy et al., 2014b, Simpson et al., 2015). Continuous sound sources such as boat sound were found to induce avoidance reactions, changes in swimming and **shoaling behaviour** (Schwarz and Greer, 1984, Vabo et al., 2002, Handegard et al., 2003, Mitson and Knudsen, 2003, Sara et al., 2007). Finally, **breeding fish** differed in their response to boat sound when eggs were present or absent, and nest-digging behaviour and antipredator defence were reduced (Bruintjes and Radford, 2013, 2014). Such a disturbance can lead to the abandonment of good feeding and spawning grounds or can impede the migration to better feeding and spawning areas (Popper and Hastings, 2009b).

Behavioural responses can co-occur with **physiological stress responses** (Neo et al., 2014). Stress can be induced by both natural (*e.g.* predator sound) and anthropogenic sound (Voellmy, 2013), and can be defined as “a condition in which the dynamic equilibrium of the animal organism – the so-called **homeostasis** - is threatened or disturbed, due to intrinsic or extrinsic stimuli, commonly defined as stressors” (Chrousos and Gold, 1992). An individual will try to adapt to the stressor by re-allocating the energy of growth and reproduction towards

restoring processes (Selye, 1974, Bonga, 1997, Barton, 2002). This involves a cascade of biochemical and physiological pathways which can be combined and described as primary, secondary and tertiary stress responses. The primary stress response is situated at the brain and involves the initial neuroendocrine responses. The chromaffin tissue releases catecholamines and the hypothalamic-pituitary-interrenal (HPI) axis is activated and releases corticosteroids (Bonga, 1997). These hormones mediate the secondary response at blood and tissue level which involves immediate physiological adjustments to re-establish homeostasis (Bonga, 1997, Barton, 2002). The tertiary response is only observed when the animal fails to re-establish homeostasis. The health and fitness are negatively affected, for example by causing **hypertension** or cardiovascular and immune system impairments (Sapolsky, 1990, Wright et al., 2007, Kight and Swaddle, 2011). In contrast to stress perceived by chemical pollutants and social factors, little is known about stress induced by noise pollution (e.g. Adams et al., 1989, Barton and Iwama, 1991, Fox et al., 1997, Haddy and Pankhurst, 1999, Sakakura and Tsukamoto, 1999, Barreto and Volpato, 2006, Farombi et al., 2007, Dube and Hosetti, 2010, Naik and Patil, 2010).
Impulsive sounds, *e.g.* from seismic air gun shooting, led to the release of primary stress hormones (cortisol and adrenaline) in Atlantic salmon *Salmo salar* and European sea bass *Dicentrarchus labrax* (Sverdrup et al., 1994, Santulli et al., 1999). Continuous sound, such as boat sound or predator foraging sounds can elevate plasma cortisol, but overall no long-term physiological stress was noted in different fish species (Smith et al., 2004, Remage-Healey et al., 2006, Wysocki et al., 2006, Wysocki et al., 2007). Likewise the behavioural responses, interspecific variation in stress responses to anthropogenic sound have been reported as well (Smith et al., 2004, Wysocki et al., 2006, Wysocki et al., 2007).

Biologically relevant sounds may be **masked** by anthropogenic sound at any distance from the sound source where sound levels are above the background level. Biologically relevant sounds include communication in function of reproduction, the location of prey and the avoidance of predators. For example, vocalization - an effective tool for territorial defence used by red-mouthed goby (*Gobius cruentatus*) – can be masked by boat sound (Sebastianutto et al., 2011). Any problem with the detection or recognition of these biologically relevant sounds (the so-called ‘masking effect’) will translate into lower fitness of the fish (Rheindt, 2003, Slabbekoorn et al., 2010).

The vulnerability and severity of the above described effects can also depend on the **life stage** of the fish. Eggs, larvae and young juveniles are assumed to be at higher risk since they are passively transported by the currents and are unable to actively swim away from the sound exposure (Popper et al., 2014). Nonetheless, little information is available on the impact of sound on the early life stages. Increased egg and fry mortality have been reported in relation to seismic sound (Kostyunchenko, 1973, Dalen and Knutsen, 1987). Exposure to air gun shooting lead to variable survival rates in post-larval fish (Booman et al., 1996). Decreased egg viability and larval growth have been recorded for sheepshead minnow (*Cyprinodon variegatus*) when background sound levels increased with 15 dB, while longnose killifish (*Fundulus similis*) were not affected (Banner and Hyatt, 1973). Under boat noise, hatching success and post-hatching larval growth of a cichlid fish (*Neolamprologus pulcher*) were unaffected (Bruintjes and Radford, 2014). While under regular and random noise, the growth, behaviour and development of larval cod (*Gadus morhua*) were affected. Furthermore, survival-related characteristics were compromised by the timing of the acoustic exposure (Nedelec et al., 2015).

1.3 Legal framework

Anthropogenic activities that introduce sound into the marine environment are still increasing globally (Frisk, 2012), but the main cause of increased underwater sounds is related to the increasing demand for (green or blue) energy. The exhaustion of fossil fuels, problems with radioactive nuclear waste storage and a necessary decrease in greenhouse gas emissions, loudly call for renewable alternatives. In Europe, the turn towards renewable energy started in 2001, by enforcing indicative targets for every member state for 2010 (European Directive 2001/77/EC). By 2020, the overall European **renewable energy sources** should contribute 20% of the total energy consumption (Renewables Directive 2009/28/EC). This 20% target can be reached by investing in ‘green’ technologies such as solar energy, wind power, hydropower or biofuels. Competition for space, contrasting opinions on aesthetic values and the “not in my backyard” phenomenon resulted in the development of offshore regions for producing renewable energy.

Offshore wind farms (OWFs) are popular in all countries along the North Sea (Breton and Moe, 2009). The potential impact of construction sound on marine mammals - and to a lesser extent on fish - has triggered the labelling of man-made underwater sound as a pollutant within the European Commission Marine Strategy Framework Directive (MSFD). A similar route was already followed in the U.S. by the National Marine Fisheries Service (NMFS) (Oestman et al., 2009, Erbe, 2013). Within the MSFD, underwater noise is incorporated as the **11th descriptor for Good Environmental Status** (GES), which states that “the introduction of energy, including underwater noise, should be at levels that do not adversely affect the marine environment” (Directive 2008/56/EC). Sound is defined as “biologically adverse” when normal activity and behaviour is disturbed, or when growth, survival and reproduction are compromised (NRC, 2005, Popper et al., 2014).

Two indicators for underwater noise have been proposed so far, concerning (1) low and mid frequency impulsive sound and (2) continuous low frequency sound (ambient noise) (Commission Decision 2010/477/EU). The indicator 11.1.1 covers pile driving, use of sonars, and air gun shooting, while indicator 11.2.1 covers shipping, dredging and energy installations (see Chapter 6). However, as empirical data are lacking, no thresholds are yet assigned for the two indicators, and each EU country is free to assign its own sound thresholds (Van der Graaf et al., 2012). The harbour porpoise in particular, and marine mammals in general, are the target species in the legal measures concerning impulsive sound taken by the EU member states (Van der Graaf et al., 2012). The only country that incorporated fish into their policy to date was The Netherlands. The underlying motivation was the role of fish as a food source for marine mammals and birds. Consequently, pile driving was banned in the first half of the year between 2011 and 2015, due to the high abundance of fish larvae (van Ginkel and Tach, 2014). In the United States, pile driving is not yet allowed in marine areas due to strict legislation on the disturbance of marine mammals, but it is allowed in rivers and lakes (Popper et al., 2014).

In 2009, the US Fisheries Hydro-acoustic Working Group developed non-auditory tissue damage ‘interim criteria’ for fish, but these were based on a limited amount of information (Oestman et al., 2009). The SEL_{cum} threshold for fish larvae <2 g wet weight (WW) was set lower than for young fish >2 g WW (SEL_{cum} of 183 *vs.* 187 dB re 1 µPa²·s, respectively), while the $L_{z\text{-}p}$ of 206 dB µPa was the same for both groups.

Several mitigating measures have already been proposed, developed or even obliged within the EU to reduce the amount of sound energy released into the marine environment, such as ‘silent’ ships (Mitson, 1995), acoustic deterrent devices (RWS, 2015), ramp-up procedures (Rumes et al., 2015), bubble curtains (Diederichs et al., 2014), marine mammal observers (JNCC, 2010), ‘days of disturbance’ (Heinis et al., 2015) (see Chapter 6). However, our knowledge of the potential impact of impulsive sound on marine life related to the construction of OWFs is still limited. Consequently, we urgently need better knowledge on the specific impacts on marine life, on the underlying sound parameters, and on the choice of safe sound thresholds. Only then, new concessions can be approved conform GES norms in the EU marine waters.

1.4 Research questions

With ever increasing anthropogenic activities at sea, the identification of the pressures they exhibit on the marine fauna and the marine ecosystem is urgently needed. Many pressures have already been identified, *e.g.* physical disturbance due to fisheries and sand extraction; chemical disturbance due to contamination, acidification and litter; hydrological disturbance due to climate change; biological disturbance due to introduced species and removal of species; electromagnetic changes; etc. (Defra, 2015). However, little knowledge exists on the potential effects of human-induced underwater sound on the different ecosystem levels: individual, population, community and ecosystem.

Ideally, the impact of different kinds of anthropogenic sound (continuous and impulsive sound) on different ecosystem components should be determined. Marine mammals have so far received most attention in bioacoustics research, while considerably less in known about the effects of underwater sound on fish. Fish are a vital component in most food webs, and they significantly contribute to different biogeochemical nutrient cycles (Holmlund and Hammer, 1999). Many fish species have a high economic value, as they are fished or cultured, and they serve as one of the main protein sources for humans (Naylor et al., 2000).

During the coming years, pile driving will be omnipresent in the North Sea: thousands of offshore wind turbines will be built (EWEA, 2014). The construction of the OWFs since 2008 in the Belgian part of the North Sea (BPNS) created a unique opportunity to investigate the impact of impulsive sound related to pile driving activities on the well-being of marine fish.

This is a multidisciplinary thesis, aiming to disentangle the effects of impulsive sound (produced by pile driving) on young fish, thereby focusing on the following research questions:

- **(I)** **Are young fish (larvae and juveniles) affected by impulsive sound, what are the effects, and at what level do they manifest, *e.g.* mortality, stress responses or behavioural responses?**
- **(II)** **Can the effects on young fish be linked to a specific sound-related metric or biological parameter? Can sound thresholds at which underwater sound negatively affects young fish be identified?**

(III) What is the ecological significance of the observed effects?

(IV) How will the results from this PhD add to management and policy regulations in Belgium (and Europe), *i.e.* in order to minimise the environmental impact of pile driving activities in future offshore wind farms, and to achieve Good Environmental Status (GES) for Marine Strategy Framework Directive (MSFD) descriptor 11?

1.5 Case study: young seabass and the offshore wind farm Northwind in the Belgian part of the North Sea

For this PhD study, the effects of human-induced impulsive sound on young European sea bass *Dicentrarchus labrax* were investigated, in relation to pile driving activities during the construction of Northwind, the third offshore wind farm in the BPNS.

1.5.1 Offshore wind farms

To comply with EU renewable energy rules (Renewables Directive 2009/28/EC[2]), the overall production target for the whole North Sea by 2020 (all EU countries bordering the North Sea) has been set at more than **32 200 Megawatt** (MW) (EWEA, 2014). Offshore wind farms (OWFs) are a relatively new concept, but rapidly became the leading offshore energy producing system in Europe. By the end of 2013, OWFs already provided 8343 MW renewable energy.
For Belgium, at least 13% of its total energy needs from renewable energy sources by 2020 to comply with this EU Directive 2009/28/EC. By means of the Royal Decree of 20 December 2000[3], amended by the Royal Decree of 17 May 2004[4], and again by the Royal Decree of 3 February 2011[5], a single OWF area of **238 km²** (7% of the BPNS) was designated to host eight

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[2] Directive 2009/28/EC on the promotion of the use of energy from renewable sources and amending and subsequently repealing Directives 2001/77/EC and 2003/30/EC.

[3] Koninklijk besluit betreffende de voorwaarden en de procedure voor de toekenning van domeinconcessies voor de bouw en de exploitatie van installaties voor de productie van elektriciteit uit water, stromen of winden, in de zeegebieden waarin België rechtsmacht kan uitoefenen overeenkomstig het internationaal zeerecht.

[4] Koninklijk besluit tot wijziging van het koninklijk besluit van 20 december 2000 betreffende de voorwaarden en de procedure voor de toekenning van domeinconcessies voor de bouw en de exploitatie van installaties voor de productie van electriciteit uit water, stromen of winden, in de zeegebieden waarin België rechtsmacht kan uitoefenen overeenkomstig het internationaal zeerecht.

[5] Koninklijk besluit tot wijziging van het koninklijk besluit van 20 december 2000 betreffende de voorwaarden en de procedure voor de toekenning van domeinconcessies voor de bouw en de exploitatie van installaties voor de

(originally seven) concessions (Figure 6) (Belpaeme et al., 2011, Brabant et al., 2013). The whole OWF area is located in the North-eastern part of the BPNS (www.4coffshore.com). The area borders the Dutch EEZ, at a distance **between 22 and 57 km** from the Belgian shoreline, and at a depth between 5.9 and 40 m. The sediment bed is generally characterised by sandy sediments.

In 2008, the first wind turbines were built in the C-Power concession area. At present (dd. December 2015), three wind farms are operational (C-Power, Belwind and Northwind). These comprise 182 turbines, each having a capacity of 3 to 6 MW. Five other domain concessions (Rentel, Norther, Seastar, Northwester 2 and Mermaid) have been permitted in the meantime. With more than 300 turbines up and running, it is expected that the OWFs will account for 43% of the Belgian 2020 targets for renewable energy (Degraer et al., 2013). The domain concessions are granted for 20 years, encompassing **a construction phase, an operational phase and a decommissioning phase**. The construction phase usually starts with preparing the seabed, followed by the installation of the foundations (in most cases by means of pile driving). Afterwards, a transition piece and the turbine itself are fixed on to the pile (Brabant et al., 2013). In the meantime, cables are laid between the foundations (in-field cables) and between the OWF and the power stations on land (high voltage power cables). In most cases, a scour protection system is installed around the foundation to prevent seabed erosion and sediment transport (Whitehouse et al., 2011). During the operational phase, the OWF produces electricity and is continuously maintained. At the end of the concession phase, the entire OWF officially needs to be decommissioned, including the turbines, the erosion protection layers and cables.

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productie van elektriciteit uit water, stromen of winden, in de zeegebieden waarin België rechtsmacht kan uitoefenen overeenkomstig het internationaal zeerecht

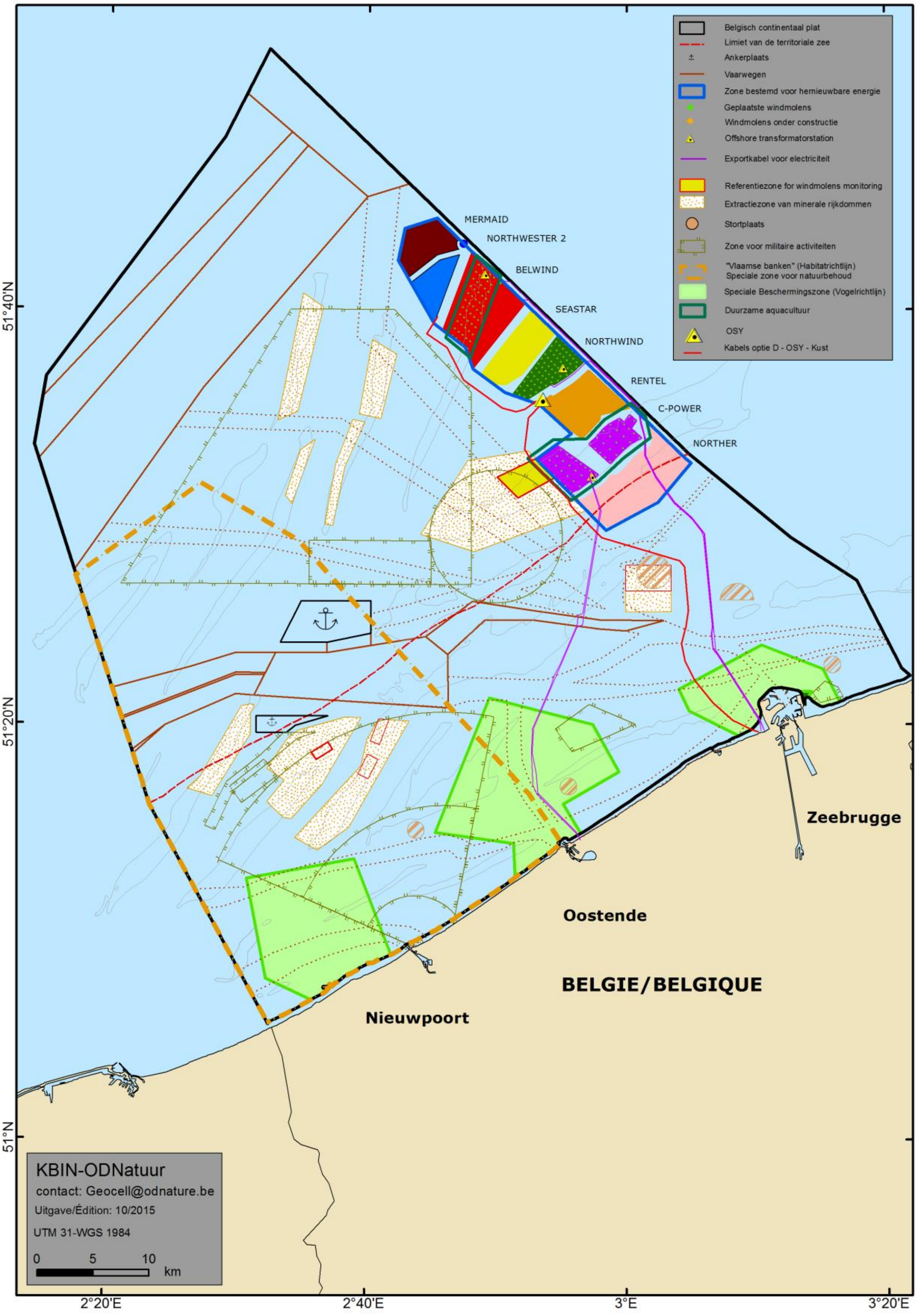

Figure 6. Overview of the offshore wind farms within the concession zone of the Belgian part of the North Sea.

So far, three different foundation types have been used in the BPNS, including **gravity based foundations (GBFs), jacket foundations and monopile foundations** (Figure 7). The decision to use a certain foundation type depends on long-term predictions of the dune mobility and possible seabed levels, water depth, the presence of geological formations of the seafloor, and production and installation costs. During the installation of the foundations, sound is introduced in the sea. The installation of the GBFs can be considered as the "silent" option, elevating the background sound level with 5 to 25 dB. This is similar to sound levels produced by different ships (Haelters et al., 2009). Pile driving of pin piles (~1.4 m diameter, jacket foundations) and monopiles (~5 m diameter) is more commonly used. This generates strong impulsive sound with an equal amount of SEL_{cum} of 196 dB re 1 µPa².s and a SEL_{ss} of 145 to 168 dB re 1 µPa².s at 750 m from the sound source. The time needed to drive the foundations into the seabed is longer and more strikes are needed for four pin piles (ca. 319 min, 9476 strikes) than for one monopile (120 min, 2982 strikes) (Norro et al., 2013).

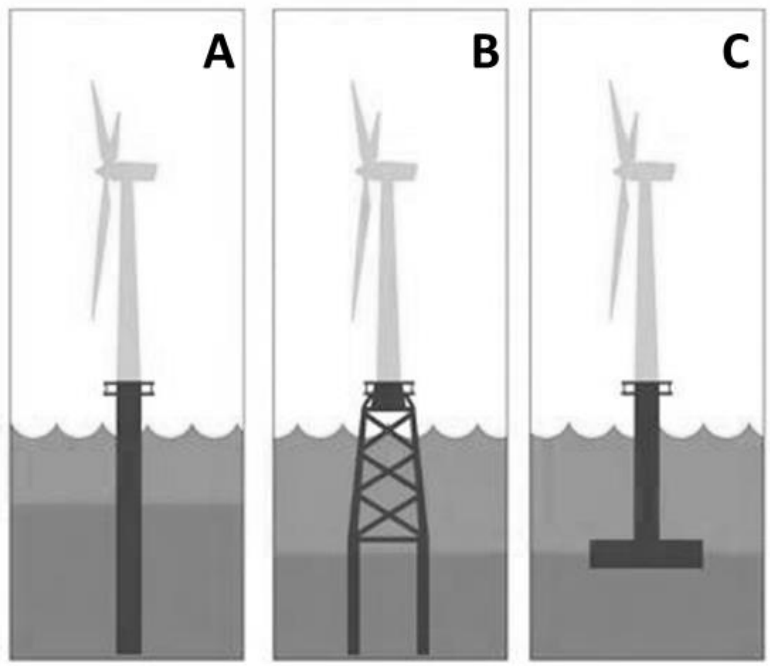

Figure 7. Foundation types for offshore wind turbines used in the Belgian part of the North Sea: (A) monopile, (B) jacket and (C) gravity based foundation. Figure modified from Czyzewski (2012).

1.5.2 Pile driving acoustics

In order to predict the ecological impacts of pile driving, the acoustic source mechanisms and propagation characteristics must be understood. When driving a pile into the sediment, the hammer releases energy (between 100 and 1200 kJ) each time it hits the pin pile or monopole foundation (Norro et al., 2013). About 0.5% of that energy is transformed into acoustic energy which is released into the marine environment (Dahl et al., 2015).

Both simple and complicated models are used to calculate the sound propagation of pile driving in the sea water. One of the simple approaches is to start with a field measurement at a given distance and to calculate the sound field at other distances, based on a **simple spherical spreading law** (Nehls et al., 2007). This model features a point source and an infinite domain without boundaries, within which sound waves can freely propagate. Consequently, interference with the surface and bottom, and volume attenuation (*e.g.* conversion of acoustic energy into heat) are not taken into account. The use of the spherical spreading loss is not very accurate, since offshore pile driving is done in relatively shallow water and the sound source acts as a conical wave (Mach cone) rather than being a point source (see following paragraph).

The more complicated approaches take the monopile source level as a starting point. Three methods can then be used to calculate the waveguide propagation of pile driving sound: (1) parabolic equation, (2) wave number integration, and (3) normal mode. All three models are able to solve the wave equation in a frequency domain. Only the '**normal mode' method** will be discussed in more detail (Nijhof et al., 2015), to illustrate the principles behind a complex and accurate propagation model.

For the three models, it is necessary to get a clear idea on how the hammer energy is translated into acoustic energy by the steel pile, and how this acoustic energy is then transmitted into the surrounding water (Figure 8). When the impact hammer strikes the pile foundation, it causes a deformation of the pile wall, which travels down along the pile (a so-called Poisson effect). The resulting swell or bulge of the pile acts as a sound source and radiates a wave front in the water and sediment. All wave fronts sent down the pile combine into a Mach cone (Zampolli et al., 2013, Dahl et al., 2015). The Mach cone corresponds to energy radiating at a certain angle, determining the energy contribution of each pile strike to each mode. When hitting the bottom of the pile, the propagation bulge encounters an impedance mismatch between the pile and the sediment, which reflects the propagation bulge upwards in the sediment (Reinhall and dahl, 2011). Back at the water-air interface, another reflection causes the bulge to propagate downwards again, with continuous energy loss. Each reflection causes a small angle shift between the pile and the Mach front.

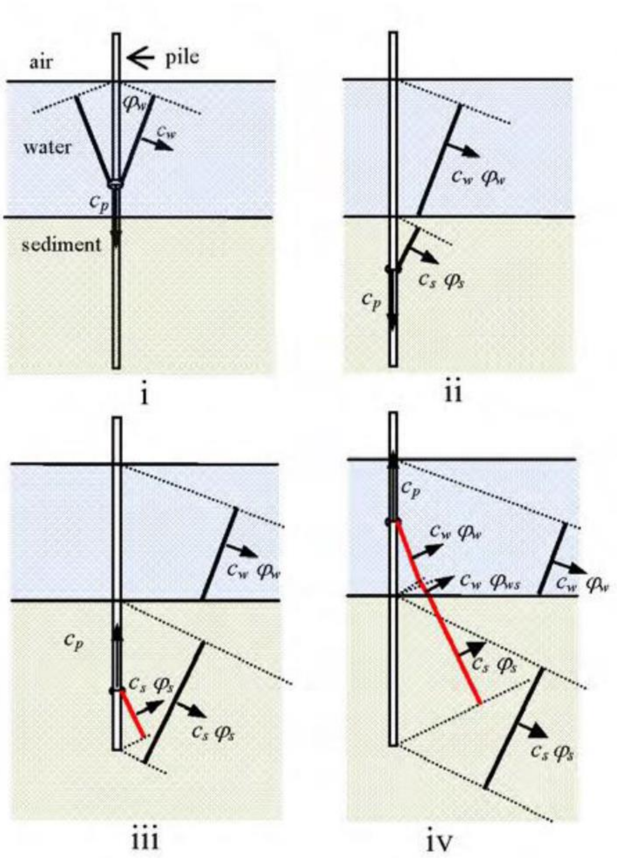

Figure 8. Presentation of the Mach cone wave front radiated from steel pile due to pile compression. i – ii illustrate the wave front travelling down along the steel pile while penetrating the sediment. iii – iv illustrate the reflection of the wave front at the bottom of the steel pile causing the wave front to travel upwards along the steel pile. Θ_{W} is the angle of the Mach cone in water and Θ_{S} in the sediment. Figure taken from (Reinhall and dahl, 2011).

The 'normal mode' approach uses the force of the hammer blow as a function of time (**hammer strike pulse**) as input source for a linear, axisymmetric structural acoustic frequency domain **finite element model** (FEM model) (Zampolli et al., 2013). The FEM model calculates the acoustic pressure at a short distance (near field, for example 1 m) from the pile over the entire water column. The near field solution is then decomposed into modal contribution factors that serve as an input to a normal mode propagation model, *in casu* the hybrid piling source and propagation model developed by TNO (Nijhof et al., 2015). Water depth and properties, sediment type and roughness of the sea surface are also required. This 'normal mode' propagation model computes the decay of the acoustic pressure up to large ranges, taking into account the effect of the environment on the acoustic propagation (Figure 9).

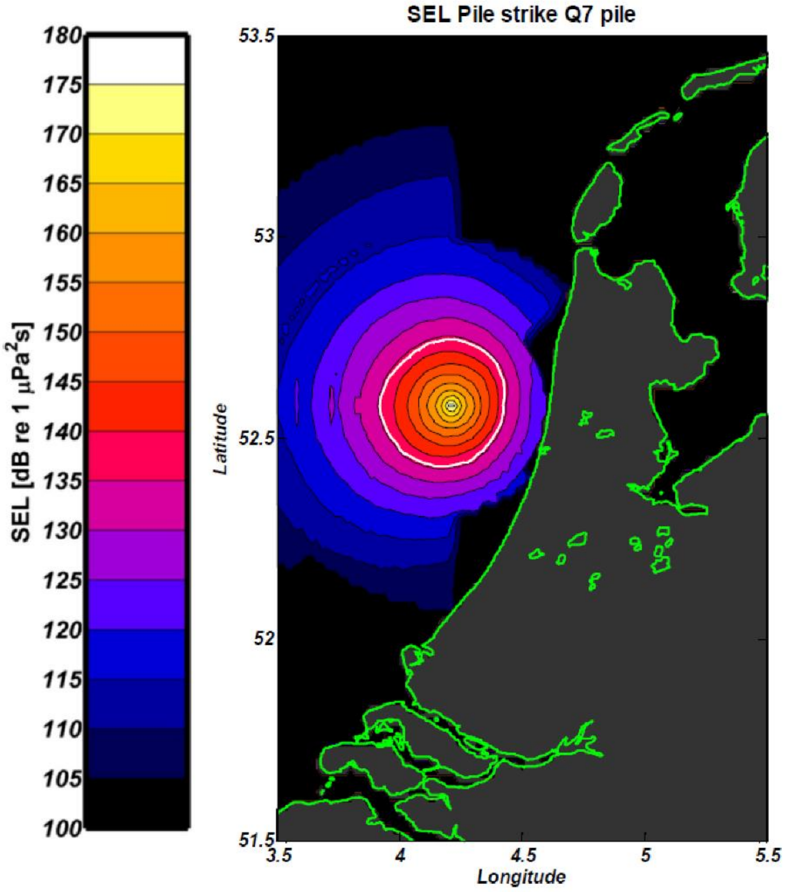

Figure 9. Output of the Aquarius 2 model, a normal mode propagation model. The modelled propagation of pile driving sound is represented as the unweighted SEL$_{ss}$ at 1 m above the bottom. Figure taken from (Nijhof et al., 2015).

1.5.3 Study species: European sea bass

In this PhD study, we focused on European sea bass, ***Dicentrarchus labrax*** (Linnaeus, 1758) (Figure 10). This is an economically important fish species for both commercial and recreational fisheries and aquaculture. Since 2012, the North East Atlantic stock is in rapid decline. The fishing mortality is four times higher than the number to ensure maximum sustainable yield (MSY) (Council Regulations (EU) 2015/960)[6]. ICES called for an accurate stock assessment (ICES, 2004, Pawson et al., 2005). Today, European sea bass fisheries are not managed by means of the EU Total Allowable Catch (TAC) quota (Council Regulation (EU) 2015/104)[7] but monthly boat limits for vessels are assigned according to their gear (Council Regulations (EU) 2015/960)[6]. A minimum landing size of 42 cm needs to be taken into account (EU 2015/1316)[8]. Recreational fishing accounts for a large portion (~25%) of the sea bass catches and is now limited to three fish per day per angler (EU 2015/523)[9]. In aquaculture, European sea bass is year-round available and is therefore frequently used in experiments.

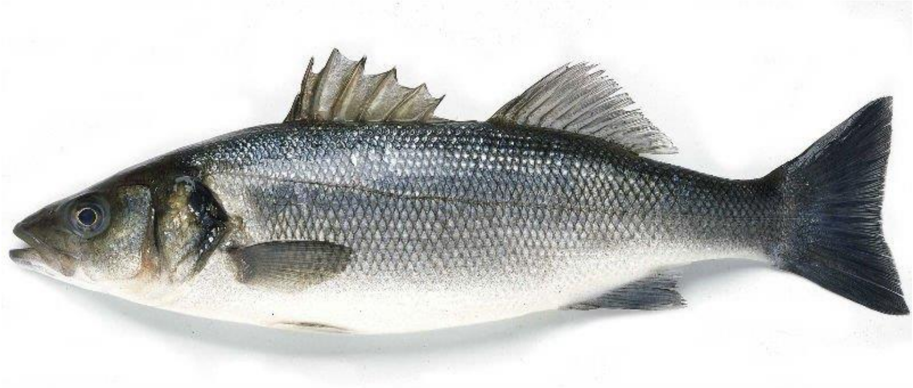

Figure 10. Adult European sea bass *Dicentrarchus labrax.*

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[6] Council Regulations (EU) 2015/960 of 19 June 2015 amending Regulation (EU) 2015/104 as regards certain fishing opportunities.

[7] Council Regulation (EU) 2015/104 of 19 January 2015 fixing for 2015 the fishing opportunities for certain fish stocks and groups of fish stocks, applicable in Union waters and, for Union vessels, in certain non-Union waters, amending Regulation (EU) No 43/2015 and repealing Regulation (EU) No 779/2014.

[8] Council Regulation (EU) 2015/1316 of 30 July 2015 derogating from the Council Regulation (EC) No 850/98, as regards the minimum conservation reference size for sea bass (*Dicentrarchus labrax*)

[9] Council Regulation (EU) 2015/523 of 25 March 2015 amending Regulations (EU) No 43/2014 and (EU) 2015/104 as regards certain fishing opportunities.

Biology

European sea bass belongs to the Class Actinopterygii, Order Perciformes, Family Moronidae. European sea bass is characterized by two separate dorsal fins, the first with spiny rays, and the second with soft rays (Figure 10). European sea bass is a slow growing, long lived species. It is a **demersal** species, present in a wide variety of habitats, including estuaries, lagoons, coastal waters, rivers and the offshore marine environment (to 100 m water depths). Schooling is common for young fish, while adults appear to be less social (Froese and Pauly, 2015). Juvenile and adult sea bass are voracious **opportunistic predators**, with a diet including small fish and a large variety of invertebrates, such as prawns, crabs, and cuttlefish (Kelley, 1987). Similar to their parents, larvae are equally opportunistic predators, but feed on smaller prey (Fritsch et al., 2007). Sea bass are slow growing and mature late and show site fidelity, which makes them vulnerable to site-specific anthropogenic pressures. Physiologically, European sea bass is a **physoclist** with no connection between the swim bladder and inner ear. The swim bladder of the European sea bass is not close to the inner ear and these fish lack specialized structures to make them more sensitive to sound pressure (Bouton et al., 2015). Testing the behavioural responsiveness of sea bass (adults) to sound pressure showed that sound between 100 and 700 Hz induced a reaction (Kastelein, 2008).

Distribution

Sea bass is a **eurythermal** (2 – 32 °C) and **euryhaline** (fresh water to hypersaline) marine teleost fish species (Varsamos et al., 2001). The species is distributed across the North Atlantic from Senegal to Norway, the Mediterranean and the Black Sea, and has been reported in Iceland (Fritsch et al., 2007). Sea bass individuals show a high site fidelity to summer feeding grounds (Carroll, 2014). Furthermore, sea bass individuals are ectothermic organisms and therefore highly dependent on temperature, for example growth, sexual differentiation, spawning and hatching success (Vinagre et al., 2009). Under recent climate change, sea bass is moving northwards (Pawson et al., 2007).

Life cycle

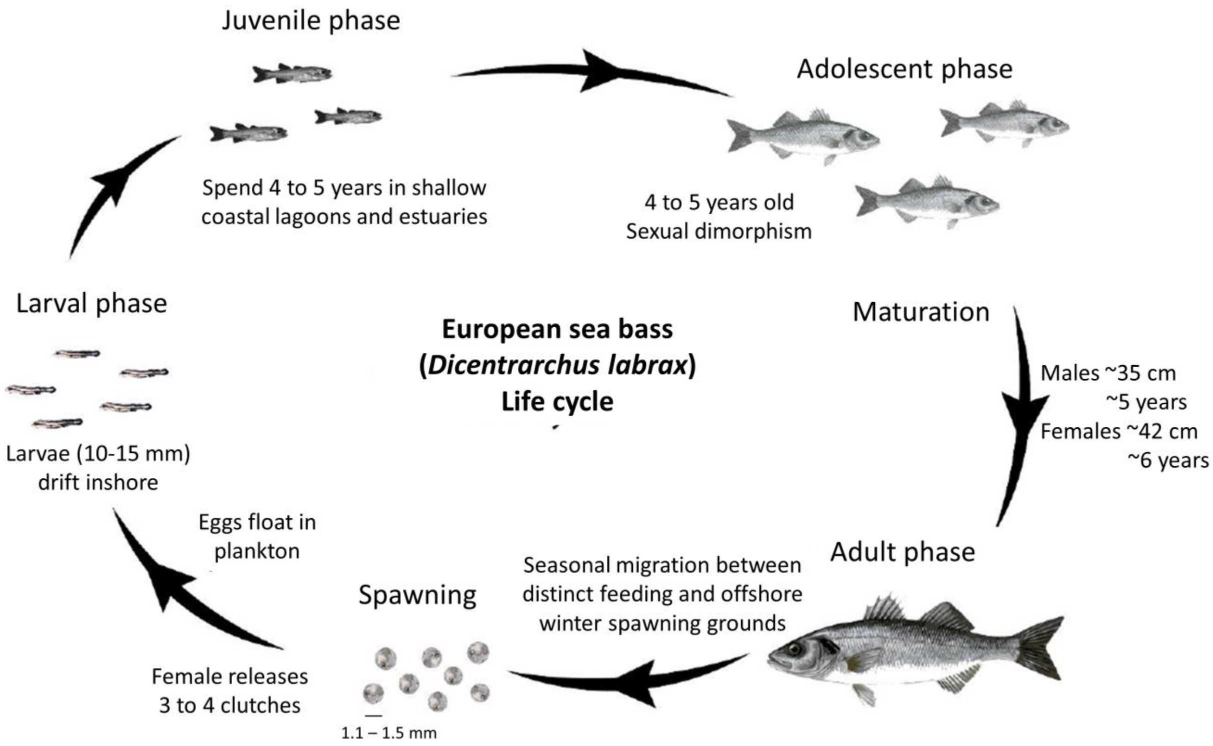

Figure 11. Life cycle of the slow growing European sea bass (*Dicentrarchus labrax*), taken from Carroll (2014). In this thesis, larvae and juveniles were the target life stages.

The sea bass life cycle consists of eggs and larvae, juveniles, adolescents, and adults (Figure 11). Maturity is reached between 4 and 7 years and sea bass can live up to 20 years (Pawson et al., 1987). Females reach maturity at older age and bigger sizes compared to males, called sexual dimorphism (Kennedy and Fitzmaur, 1972). Maturity is reached at younger age in warmer waters (Mediterranean) compared to the North Sea. **Adults** migrate offshore to spawn in open sea, in winter (December to March) in the Mediterranean and up to June in the Atlantic Ocean (Pawson et al., 2007). European sea bass is an **r-strategist**, with females producing 0.25 to 0.5 million eggs per kilogram body weight. Eggs are 1.1 – 1.5 mm in diameter and hatch into larvae at 3 mm. Larval development takes about 40 days (up to 15 mm)[e]. **Eggs and larvae** are pelagic and are dispersed over large distances during the first three months towards the coastal nursery areas (Jennings and Pawson, 1992, Pickett and Pawson, 1994, Varsamos et al., 2001). The onset of sex differentiation starts at 200 days post hatching (dph), and is highly influenced by temperature. High temperatures favouring male development

(Koumoundouros et al., 2002). The **juveniles** mainly reside in nearshore shallow areas (Cattrijsse et al., 1994). In the adolescent phase, the subsequent difference in growth rate between the sexes becomes apparent at ~35 cm (sexual dimorphism), with females being larger (Kennedy and Fitzmaur, 1972, Diaz et al., 2013). Sexual maturation in the North Atlantic occurs from the fourth to the seventh year for males (23 – 30 cm) and in their fifth to eighth year for females (31 – 40 cm), when they weigh about 2 kg WW. Maximum sizes are 60 cm for males and 70 cm for females (Carroll, 2014).

1.6 Outline of the PhD thesis

For this PhD study, a stepwise and multidisciplinary approach was used to formulate an answer to the four research questions. Ecological, physiological, biochemical and acoustical aspects are combined throughout the different research steps. Moreover, this study is the first to combine field and lab experiments to unravel the impact of high intensity impulsive sound on larval and juvenile life stages of a physoclistous fish species. Next to the general introduction (**Chapter 1**) and the general discussion (**Chapter 6**), the PhD thesis comprises four A1 research papers, being either published, under revision, submitted to a peer reviewed journal or in preparation. These chapters are stand-alone units and can be read separately.

First, the immediate and delayed **mortality** of pile driving sound on young fish are assessed when exposed to high intensity sound pressure levels. In the *in situ* study **(Chapter 2, Debusschere et al., 2014)** juvenile sea bass were exposed to high intensity impulsive sound pressure levels ($L_{z\text{-}p}$ max of 210 dB re 1 µPa $L_{z\text{-}p}$) during a complete pile driving session as close as 45 m to the pile driving activity, to investigate immediate mortality. We predicted that exposure to a complete pile driving session would cause high mortality in young fish. The surviving individuals were subsequently monitored for two weeks in the lab to investigate delayed mortality, which we also estimated to be high.

Then, the focus lies on the potential **stress responses** in larval and juvenile fish evoked by three different impulsive sound sources. In **Chapter 3 (Debusschere et al., 2016)** we investigated whether *in situ* generated impulsive sound during offshore pile driving induces acoustic stress in juvenile fish. The *in situ* experiments with sea bass were the same one as for Chapter 2, carried out during 'real' pile driving activity at 45 m from the pile driving location. Acute primary and secondary stress responses and long-term tertiary responses were linked to the condition of the fish. We predicted that the pile driving exposure would lead to strong acute stress responses in the fish but with minimal consequences on the long-term.

In **Chapter 4 (Debusschere et al., submitted)**, lab experiments were carried out with larval and juvenile sea bass to investigate the potential impact of different high intensity impulsive sound sources on both primary and secondary stress parameters. The sound sources (sparker and larvaebrator) had similar standard pressure metrics compared to the *in situ* pile driving recordings, although they differed in frequency spectra. Stress responses were determined in juvenile sea bass, and compared to the *in situ* results to determine which sound metrics are really important. In addition, stress responses in sea bass larvae were compared with the responses of juvenile, to assess life stage sensitivity to impulsive sound. We predicted that the stress responses in larvae would be much stronger compared to juveniles and also that the three different sound sources can evoke different intensities of stress responses.

Further, the **behavioural responses** to impulsive sound in juvenile fish are described. Impulsive sound has the potential to disturb fish behaviour, even at moderate sound pressure levels. In **Chapter 5 (Debusschere et al., in prep.),** we tackled the impact of impulsive sound exposure on the swimming activity, aggressive behaviour, and feeding tendency and efficiency of juvenile sea bass. This study was based on a lab experiment in which fish were exposed to play-back impulsive sound during three consecutive days. Consequently, habituation to the sound was also investigated. The outcome was linked to possible consequences related individual fitness.

Finally, the acquired knowledge is integrated by means of a general discussion (**Chapter 6**), thereby focusing on practical challenges of the experiments, the obtained results, the link between effects and sound parameters and thresholds. Also, the ecological relevance of the obtained data is discussed. Suggestions for offshore wind energy management and renewable energy policy are made in order to minimize the environmental impact of pile driving activities in future offshore wind farms and to achieve a good environmental status for underwater noise, being the 11th MSFD descriptor. Finally, some perspectives and recommendations for future research are presented.


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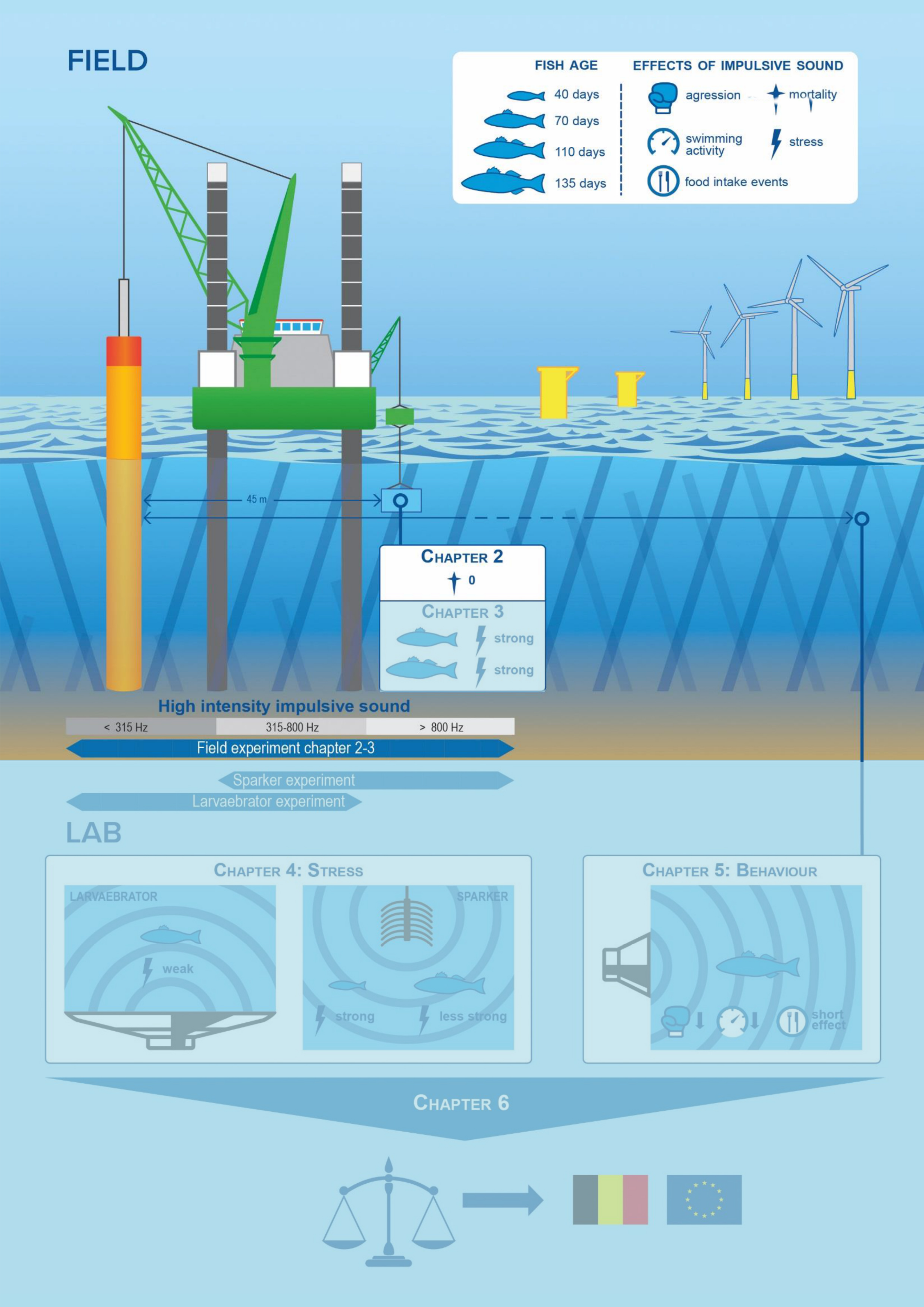
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CHAPTER 2
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CHAPTER 3
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CHAPTER 4: STRESS
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weak
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strong
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CHAPTER 5: BEHAVIOUR
short effect
CHAPTER 6

CHAPTER 2

IN SITU MORTALITY EXPERIMENTS WITH JUVENILE SEA BASS *DICENTRARCHUS LABRAX* IN RELATION TO IMPULSIVE SOUND LEVELS CAUSED BY PILE DRIVING OF WINDMILL FOUNDATIONS

Adapted from:

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Abstract

Impact assessments of offshore wind farm installations and operations on the marine fauna are performed in many countries. Yet, only limited quantitative data on the physiological impact of impulsive sounds on (juvenile) fishes during pile driving of offshore wind farm foundations are available. Our current knowledge on fish injury and mortality due to pile driving is mainly based on laboratory experiments, in which high intensity pile driving sounds are generated inside acoustic chambers. To validate these lab results, an *in situ* field experiment was carried out on board of a pile driving vessel. Juvenile European sea bass (*Dicentrarchus labrax*) of 68 and 115 days post hatching were exposed to pile driving sounds as close as 45 m from the actual pile driving activity. Fish were exposed to strikes with a sound exposure level between 181 and 188 dB re 1 µPa².s. The number of strikes ranged from 1739 to 3067, resulting in a cumulative sound exposure level between 215 and 222 dB re 1 µPa².s. Control treatments consisted of fish not exposed to pile driving sounds. No differences in immediate mortality were found between exposed and control fish groups. Also no differences were noted in the delayed mortality up to 14 days after exposure between both groups. Our *in situ* experiments largely confirm the mortality results of the lab experiments found in other studies.

Keywords

Dicentrarchus labrax, pile driving, offshore wind farms, piling vessel, field experiment, mortality

1. Introduction

The increasing demand for renewable energy has led to innovative techniques and numerous ambitious projects. Offshore wind energy is particularly popular across the North Sea bordering countries. However, the construction of offshore wind farms - and the pile driving activities in particular - involve strong impulsive sounds, which are potentially harmful to marine fishes and more specifically to the early life stages of fishes, i.e. eggs, larvae and young juveniles (Southall et al., 2007; Popper and Hastings, 2009a; Popper and Hastings, 2009b; Van der Graaf et al., 2012). These early life stages are important as prey for pelagic fishes and for the recruitment to the adult fish populations, which stresses the need to understand how underwater sounds affect their fitness. Remarkably, many offshore wind farms are being installed or planned without extensive quantitative data on the physiological impact of strong impulsive pile driving sounds on fishes (Popper and Hastings, 2009a; Popper and Hastings, 2009b).

In light of environmental impact control, it is necessary to establish sound level thresholds for pile driving at which fishes don't get injured. A first step in this process is to assess the sound level range causing immediate or delayed mortality (Popper and Hastings, 2009a). Such assessments have been performed for Chinook salmon (*Oncorhynchus tschawytscha*), hybrid striped bass (white bass *Morone chrysops* x striped bass *Morone saxatilis*) and common sole (*Solea solea*), through lab experiments, using different methods to generate high-intensity 'pile driving' sounds in acoustically controlled chambers (Bolle et al., 2012; Halvorsen et al., 2012b; Casper et al., 2013). Juvenile Chinook salmon of 103 mm ± 8.75 (SD) exhibited mortal injuries, which include any mortality or injuries that can lead to mortality, at relatively high single-strike sound exposure levels (SEL_{ss}) of 187 dB re 1 µPa²s, leading to a cumulative sound exposure level (SEL_{cum}) of 220 dB re 1 µPa²s for 1920 strikes (Halvorsen et al., 2012b). The hybrid striped bass of two different sizes; average 1.3 g and 17.2 g, exhibited mortal injuries at a SEL_{ss} of 180 dB re 1 µPa².s for 960 strikes, resulting in a SEL_{cum} of 210 dB re 1 µPa².s (Casper et al., 2013). For common sole larvae that were exposed to a SEL_{ss} of 186 dB re 1 µPa².s for 100 strikes, leading to a SEL_{cum} of 206 dB re 1 µPa².s, no difference in mortality was found between control and exposed groups up to 7 days after exposure (Bolle et al., 2012). Since these laboratory experiments have not yet been verified in the field, there is a need to expose juvenile fishes to the sound exposure levels present in the immediate vicinity of the pile driving activity in order to examine direct or delayed mortality.

In the current study, a 'worst-case scenario' *in situ* field experiment was carried out to fill the gaps in the establishment of sound level thresholds for young fishes. Sound pressure was measured alongside a piling platform (45 m from the pile) and immediate and delayed mortality in young sea bass (*Dicentrarchus labrax*) were assessed.

2. Material and Methods

2.1 Piling vessel and study location

To examine the impact of pile driving, this field study was performed on board of the jack-up piling vessel Neptune DP2 (GeoSea). Location was the Lodewijckbank, Belgian Part of the North Sea (Figure 1). This field experiment is part of the scientific research within the zone for renewable energy issued by the Belgian Ministry for the North Sea as described in the environmental permit NV Eldepasco (De Sutter and Volckaert, 2008).

Access to the deck side opposite to the pile driving activity was granted to perform the experiment, as close as 45 m from the monopile. Each monopile was designed for its specific position in the wind farm and varied in length, diameter and weight. Two monopiles were installed per trip using a hydraulic piling hammer (IHC Hydrohammer® B.V.). Monopiles C8 (Lat N 51.637648, Long E 2.9003345, WGS84) and B3 (Lat N 51.629995, Long E 2.926765) were driven into the seabed during the first trip, monopiles G7 (Lat N 51.60667, Long E 2.881473) and G8 (Lat N 51.602782, Long E 2.877282) during the second trip (Table 1, Figure 1).

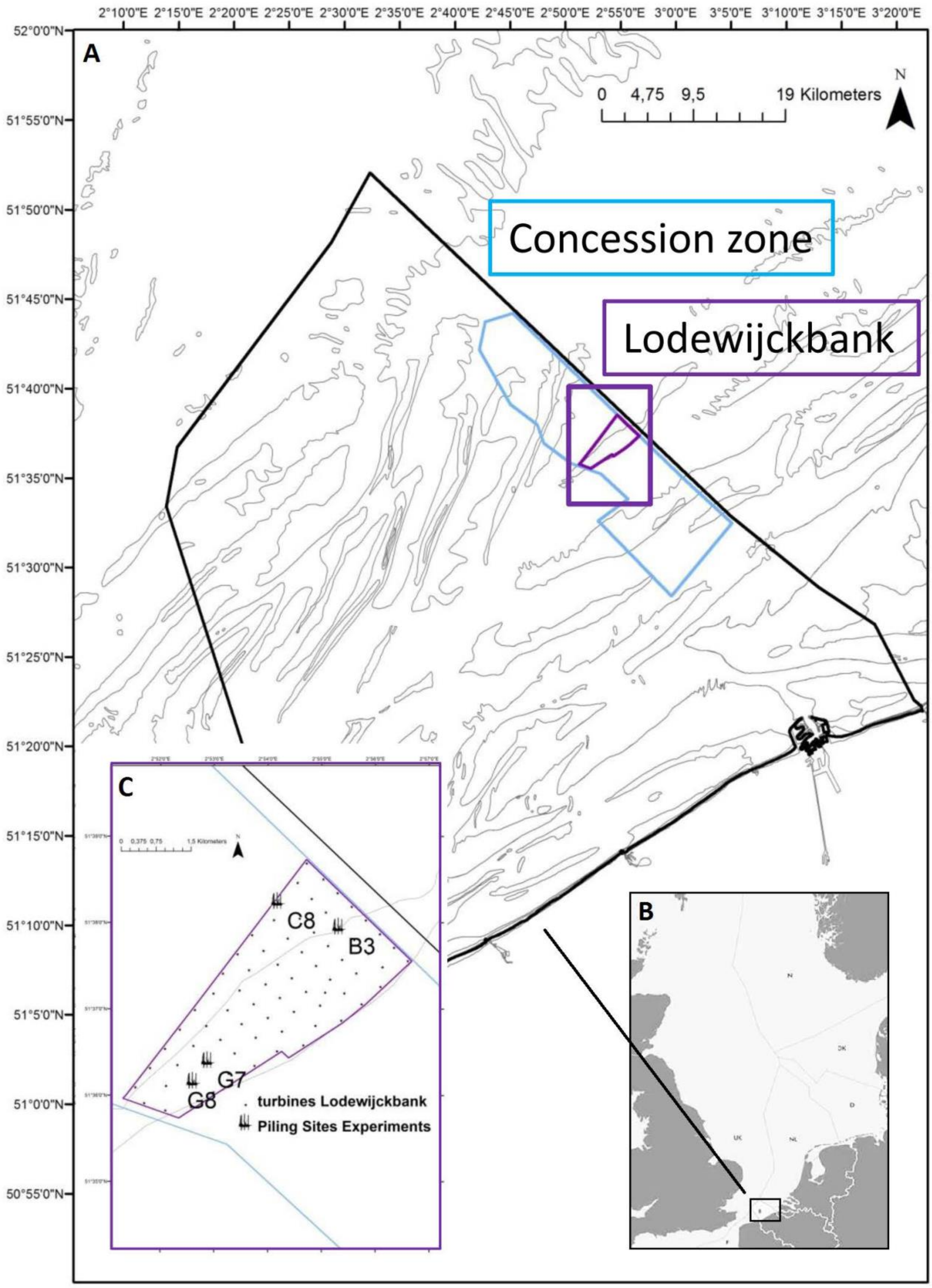

Figure 1. The offshore renewable energy zone in the Belgian part of the North Sea (a). North Sea exclusive economic zones (b). The Northwind concession on the Lodewijckbank includes 72 monopiles of ~ 5 m diameter (c). The experiment was repeated at four monopiles (C8, B3, G7, G8).

Table 1. Characteristics of the four monopiles and number of hammering strikes, energy and time of pile driving for each monopile.

Monopile number	C8	B3	G7	G8
Trip	1	1	2	2
Date	19/06/2013	19/06/2013	27/08/2013	28/08/2013
Time of day (h)	01:47	18:38	22:39	11:15
Weight (tons)	471	357	346	346
Diameter (m)	5	5	5.2	5.2
Steel thickness (mm)	50	50	50	50
Length (m)	62	57	56	56
Depth in seafloor (m)	33	32	30	33
Total strikes	2282	2331	3249	2964
Maximum energy/strike (kJ)	1173	867	1069	1162
Total energy (kJ)	2333436	2276948	2333240	2526331
Total pile driving time (h)	1:29	1:14	1:45	1:23
Net hammering time (h)	0:57	0:59	1:17	1:10

2.2 Fish characteristics and preparation

European sea bass (*Dicentrarchus labrax*) is commercially exploited in the Southern North Sea and Mediterranean Sea, both through fisheries and aquaculture. Sea bass is a well-studied species, especially concerning larval growth, development and skeletal formation (Roncarati et al., 2001; Koumoundouros et al., 2002; Sfakianakis et al., 2006; Zouiten et al., 2011; Sfakianakis et al., 2013). Swim bladder formation in sea bass larvae starts 6 **d**ays **p**ost **h**atching (dph) and is complete at 16 days. Sea bass is a physoclist round fish with an opening between the mouth and swim bladder only during the first days of the swim bladder formation (van der Kooij et al., 2007). Thereupon, buoyancy is controlled by gases retrieved from the blood, hence volume changes cannot be performed abruptly (Halvorsen et al., 2012a).

As sea bass eggs and larvae are available year round in the Ecloserie Marine de Gravelines (France), this largely facilitates the use of this species in experiments. This study was carried out in accordance with the Belgian Council for Laboratory Animal Science (BCLAS) guidelines. The experimental protocol was approved by the ethical committee of the Institute for

Agricultural and Fisheries Research (ILVO) (Permit Number: 2012/178). The field study did not involve endangered or protected species.

Sea bass of 45 dph (hatched at the Ecloserie Marine de Gravelines) were incubated in cylindro-conic linear low-density polyethylene (LLDPE) containers of 9.5 L at ILVO. They were provided with aeration and a flow through of UV-sterilized sea water on a half-closed recirculation system. The fingerlings were kept in this aquaculture unit until the experiment could take place on board of the piling vessel. They were fed twice a day with Aglonorse (300-500 µm) or MariCo Start 1.5 mm (Coppens). The water temperature in the cultivation aquaria was 20.1 °C ± 0.5, with a salinity of 30.2 ppt ± 0.1 and a pH of 8.1 ± 0.1.

Fingerlings of 68 dph were used for the first *in situ* experiments on board of the piling vessel, while young sea bass of 115 dph from another batch were used during the second trip. Sea bass fingerlings were transported to the piling vessel during the first trip in 2 L containers and the second trip in 10 L buckets, each provided with oxygen tablets (JBL). After 3.5 hours, the seawater in the containers was renewed on board and a continuous air supply was provided. The sea bass were fed with Aglonorse (300-500 µm) or MariCo Start 1.5 mm (Coppens). Debris and dead fish were removed from the container and seawater was renewed on a daily basis to ensure good water conditions. Fish were checked twice a day and if they were in poor condition (showing illness, stress or decreased activity) they were not used in the experiments. Humane endpoints were incorporated if the fish would display behavioural abnormalities or haemorrhage. The fish would be humanely sacrificed by transferring them into an overdose anaesthetic ((2ml of 5g benzocaine dissolved in 25 ml acetone) / 1 L seawater).

2.3 Acoustic equipment

Sound pressure was measured using a Brüel & Kjaer hydrophone (type 8104, voltage sensitivity 47.7 µV.Pa^{-1}, charge sensitivity 0.391 pC.Pa^{-1}, 10 m cable). The hydrophone was connected to the charge channel of a Brüel & Kjaer portable amplifier (Nexus type 2690-0S). The measurement chain was completed with a multi-channel portable recorder (Tascam DR-680). The signal was recorded in 1-channel WAVE format (.wav) on Compact Flash cards of 16 GB (SanDisk Ultra) with a sampling rate of 44 100 Hz at 24 bit.

2.4 Experimental setup and treatment

The experimental unit existed of two parts: a stainless steel frame holding a case with the field recorder and amplifier; and a similar frame holding the six 500 mL vials with the fish (Figure 2). The vials were made of poly 4-methyl, 1- pentene (PMP) with an acoustic impedance of 1.84 Rayl, which is as close as possible to the impedance of seawater (1.56 Rayl) (Bradley and Wilson, 1966; Thompson, 1990). The hydrophone was attached to the stainless steel cables holding the frames in such a way that it was hanging unobstructed 0.3 m above the vials. The whole experimental unit was lowered with a crane submerging the lowest frame to a depth of 2.5 m below the water surface.

Dead sea bass release toxins which negatively affect the survival of the others. To counteract this phenomenon, each treatment group (exposed and control) was subdivided into six vials per experiment. The number of fingerlings in the vials was based on their oxygen demand and on oxygen availability in the seawater in the vials, furthermore they were randomly assigned to a vial and group. During the first trip, each vial had a density of 20 individuals (68 dph; wet weight 42.8 ± 15.8 mg; standard length 16.8 ± 1.5 mm), whilst the density during the second trip was reduced to 2 individuals per vial (115 dph; 1613.3 ± 472.5 mg; 47.9 ± 4.5 mm). After transferring the fish into the vials, each vial was filled with care until a bubble of seawater would form on top of the vial. Then, the screw cap was screwed on the vial with a small overspill of seawater as a consequence. Each vial was checked for air bubbles before it went into the sea. After ~1.5 hours (approximate duration of 1 monopile hammering event) the vials were inspected for immediate mortality, after which the fish were put together per two vials into a 2 L container with air supply. The same procedure was followed with the control group in the absence of pile driving and repeated for the following exposed and control groups, resulting in 4 experiments each. The total sample size was 528 individuals with 120 fish in each treatment group in the first two experiments and 12 fish per group in the third and fourth experiment.

After three days on board of the piling vessel, the fingerlings were transported back to the laboratory (see 2) and placed in cylindro-conical aquaria (9.5 L, first trip) or rectangular aquaria (30 L, second trip) per treatment and experiment. Delayed mortality was monitored twice a day during the following 14 days after exposure. 72 fish and 4 fish were followed for delayed mortality for each group respectively from the first and second trip, while the other 48 and eight of each group were stored in liquid nitrogen to analyse sub-lethal effects, and more precisely effects on stress hormone levels(Debusschere et al., 2016). At the end of the monitoring period, all fish were humanely euthanized.

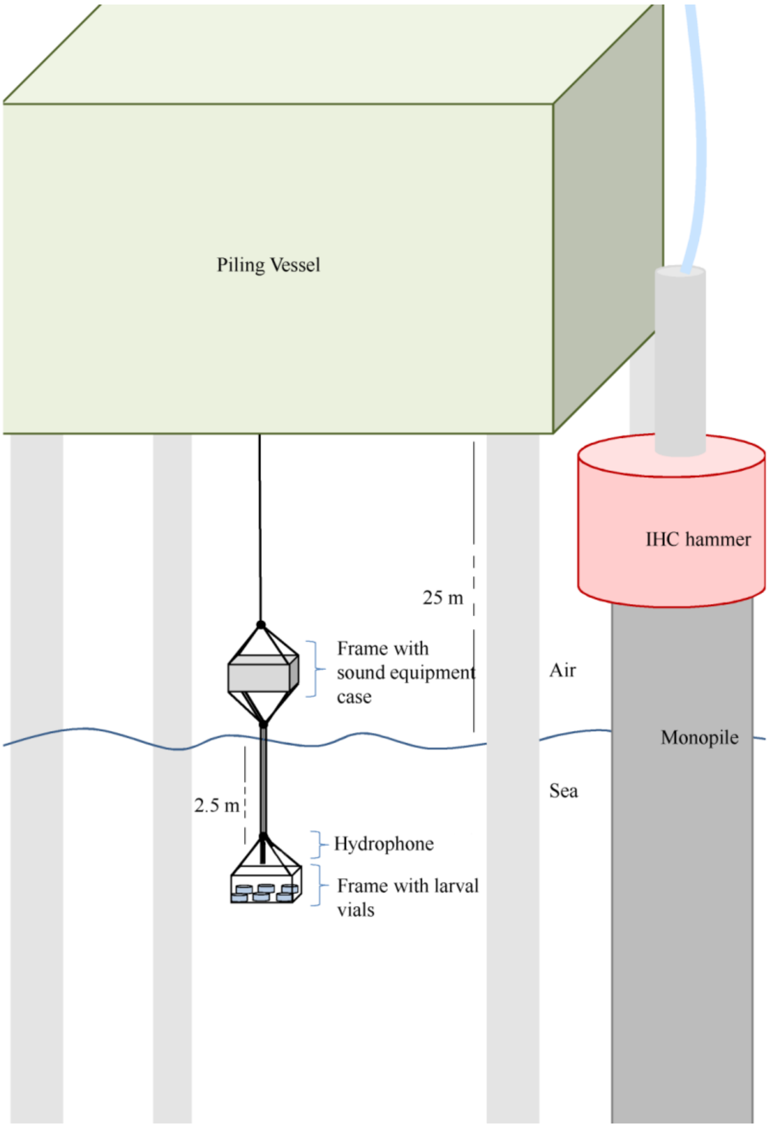

Figure 2. Experimental unit at the side of the piling vessel opposite to the monopile and hammering unit. The piling vessel is jacked-up 25 m above the sea surface on four steel piles. The upper frame above the sea surface holds the sound recording equipment. The lower frame holds the fish in vials and is 45 m away from the pile driving activity. The hydrophone is attached between the steel cables of the lower frame.

2.5 Sound analyses

Metrics for the sound pressure *p* were calculated using Matlab R2012b (version 8.0).
The combination of single strike sound exposure level, cumulative sound exposure level and the total number of impulse events can be successfully correlated to the severity of fish injuries (Casper et al., 2012; Halvorsen et al., 2012b). In addition to these sound pressure metrics, the peak sound pressure level and the 1/3-octave band containing most energy were also calculated.

- The sound pressure level (SPL) is defined as the logarithmic measure of the instantaneous sound pressure within a given time interval. The unit is dB re 1µPa.
- The peak sound pressure level ($L_{z\text{-}p}$) is defined as the level associated to the maximum absolute value of the instantaneous sound pressure within a given time interval. The unit is dB re 1µPa.
- The single strike sound exposure level (SEL_{ss}) is defined as the level associated to the integral of the squared sound pressure over the duration of a single impulse event. The unit is dB re 1 µPa^2.s. Impulse events were detected on the basis of the SPL time series. The detection threshold was set to 170 dB, and single events were selected using a temporal window around the times that the threshold is exceeded, 0.20 s before the threshold and 0.50 s after. The minimum time between two events was set to 0.50 s. These optimal values were set after visual inspection of the recordings.
- The cumulative sound exposure level (SEL_{cum}) is defined as the decibel sum of the sound exposure level over a number of individual impulse events. The time between the individual impulse events is excluded using this procedure.

  Given the average SEL_{ss} and the number of impulse events, the cumulative levels can be more practically (p) calculated (Bolle et al., 2012; Halvorsen et al., 2012b) as:

$$SEL_{cum,p} = SEL_{ss} + 10 \log_{10} (\text{number of impulse events}) \qquad [1]$$

2.6 Statistical analyses

The Plymouth Routines In Multivariate Ecological Research (PRIMER) programme, version 6.1.12 with PERMANOVA add-on software, was applied for statistical analyses (Clarke and Gorley, 2006). A significance level of $p < 0.05$ was used in all tests. The univariate permutational ANOVA's (PERMANOVA) were carried out with a 2 - factor design including treatment (T) and age (A) to analyse immediate mortality, while the delayed mortality over 14 days was analysed with a 3 - factor design, including treatment (T), age (A), and days after exposure (D) analysed. Each group (exposed and control) per experiment is one replicate. The data were not transformed and Euclidean distance similarity matrices were applied. The permdisp assumption was met for the acute mortality and delayed mortality (respectively $p_{perm} = 0.14$ and $p_{perm} = 0.5697$). Due to the low replicate number the Monte Carlo P-value was preferred over the permutation P-value (Anderson, 2005). A simple linear regression analysed the potential linear relationship between total pile driving energy (Table 1) and SEL_{cum} (Table 2) using R version 2.15.1. Non-parametric Spearman rank tests were carried out to examine a correlation between the monopile characteristics (weight, length, penetration depth) and the pile driving characteristics (total energy, total strikes and maximum energy per strike). Furthermore, a non-parametric Spearman rank test was performed with penetration depth at each strike and energy per strike.

3. Results

3.1 Sound parameters

No correlation was found between the monopile characteristics (weight, length, penetration depth, Table 1) and the pile driving characteristics (total energy, total strikes, and maximum energy per strike). Also, no linear relationship was found between the cumulative sound exposure level calculated for the total number of strikes ($SEL_{cum,p}$) (Table 2) and the total energy necessary for one monopile ($R^2 = 0.14$). On the other hand, the energy needed per strike was positively correlated with the penetration depth, which means that the type of sediment layers that have to be penetrated forms a key factor in terms of energy requirements (Spearman's rank correlation coefficient 0.94 (C8); 0.90 (B3); 0.94 (G7); 0.92 (G8)).

The pile driving sound levels that were measured during the four experiments at 2.5 m below the water surface reached on average SEL$_{ss}$ of 181 - 188 dB re 1 µPa²s, rose to SPL_{peak} of 210 - 211 dB re 1 µPa², and led to SEL$_{cum}$ of 215 - 222 dB re 1 µPa².s, with 1739 up to 3067 strikes per monopile (Table 2). The L$_{z\text{-}p}$ was constant across the four monopiles (see example in Figure 3), while B3 (experiment 2) had higher SEL$_{ss}$ and SEL$_{cum}$ values compared to the other three monopiles. The dominant energy during exposure (SEL$_{ss}$) was present at 125-200 Hz, although no steep decline was recorded towards the higher frequencies (Figure 4a). For the experiments with control groups that were carried out in the absence of pile driving activities on the piling vessel, only sound pressure level could be measured. The SPL varied between 127 and 145 dB re 1 µPa (Figure 4b).

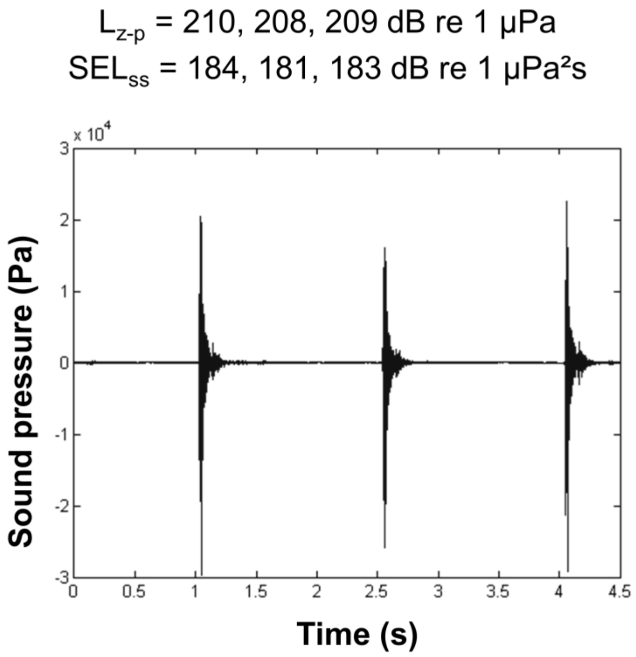

Figure 3. Detail of three consecutive piling strike signals. Detail of three consecutive piling strike signals recorded in the field for underwater sound pressure during experiment 3 (G7) (strike number 3005 – 3007 at 4892 – 4896.5 s).

Table 2. Sound pressure metrics measured at 45 m during pile driving of the four monopiles and for the control groups of each experiment.

Monopiles	**C8**	**B3**	**G7**	**G8**
Trip number	1	1	2	2
Sound metrics to which the fish were exposed to				
total strikes exposed to*	1739	2312	3067	2959
time between peaks (s)	1.49	1.49	1.39	1.41
SELss mean (dB re 1 µPa^2.s)	183	188	181	183
SELss max (dB re 1 µPa^2.s)	185	191	185	187
SELss min (dB re 1 µPa^2.s)	160	159	173	157
L_{z-p} (dB re 1 µPa)	210	210	211	211
SELcum (dB re 1 µPa^2.s)	215	222	217	218
SELcum,p (dB re 1 µPa^{2}s)	215	222	216	218
1/3 octave band with most energy (Hz)	200	160	125	200
Control replicate	**1**	**2**	**3**	**4**
SPL (dB re 1 µPa)	138	128	145	136
1/3 octave band with most energy (Hz)	31.5	25	40	100

* Different from total number of strikes in table 1 as net time of exposure is less than total hammering time

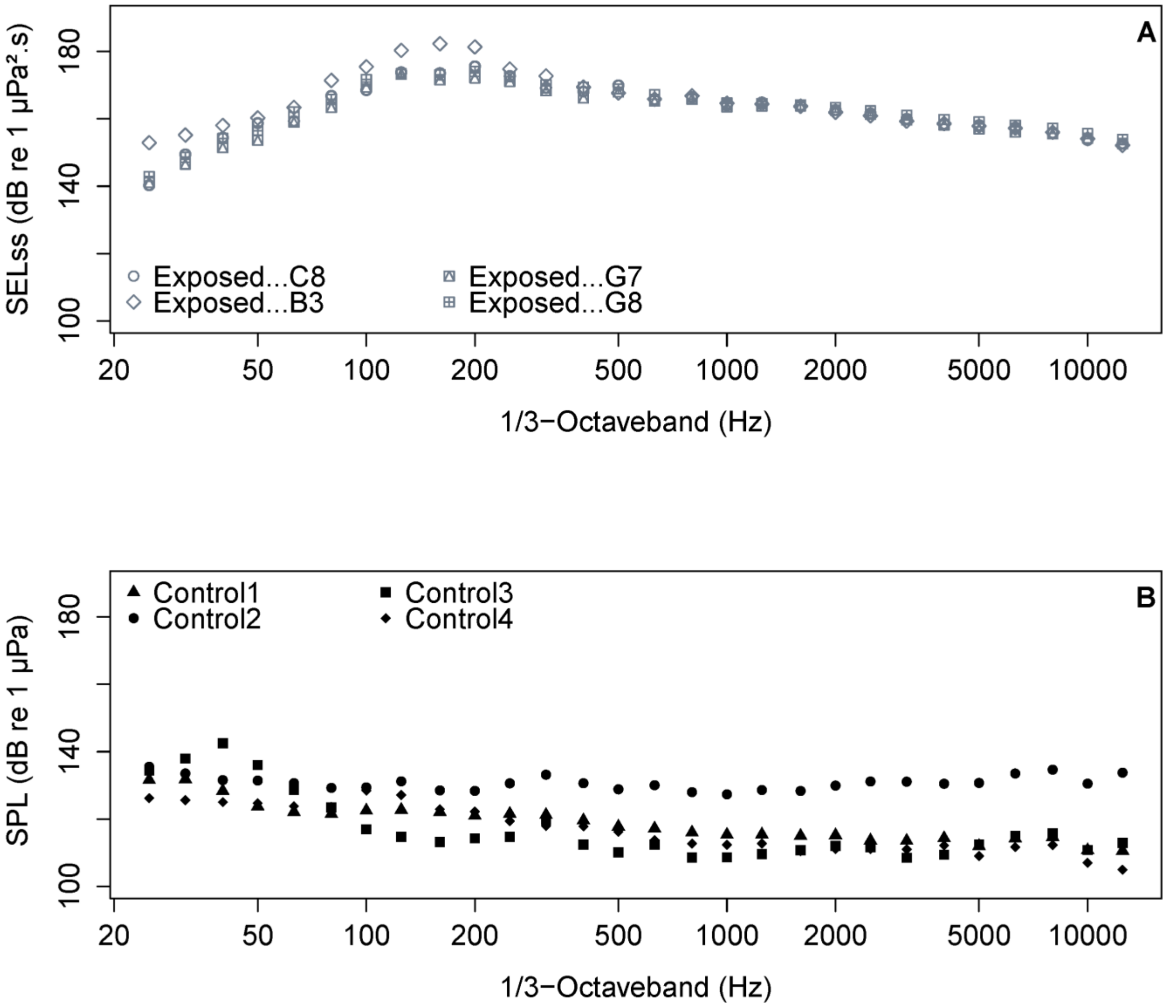

Figure 4. Measured frequency spectra in the presence and absence of pile driving. Mean SEL$_{ss}$ (A) of the total recorded piling strikes versus 1/3 octave bands and SPL (B) of the control groups versus 1/3 octave bands. C8, B3, G7, G8 are the groups exposed to pile driving; Control 1 – 4 are the control groups.

3.2 Sea bass survival

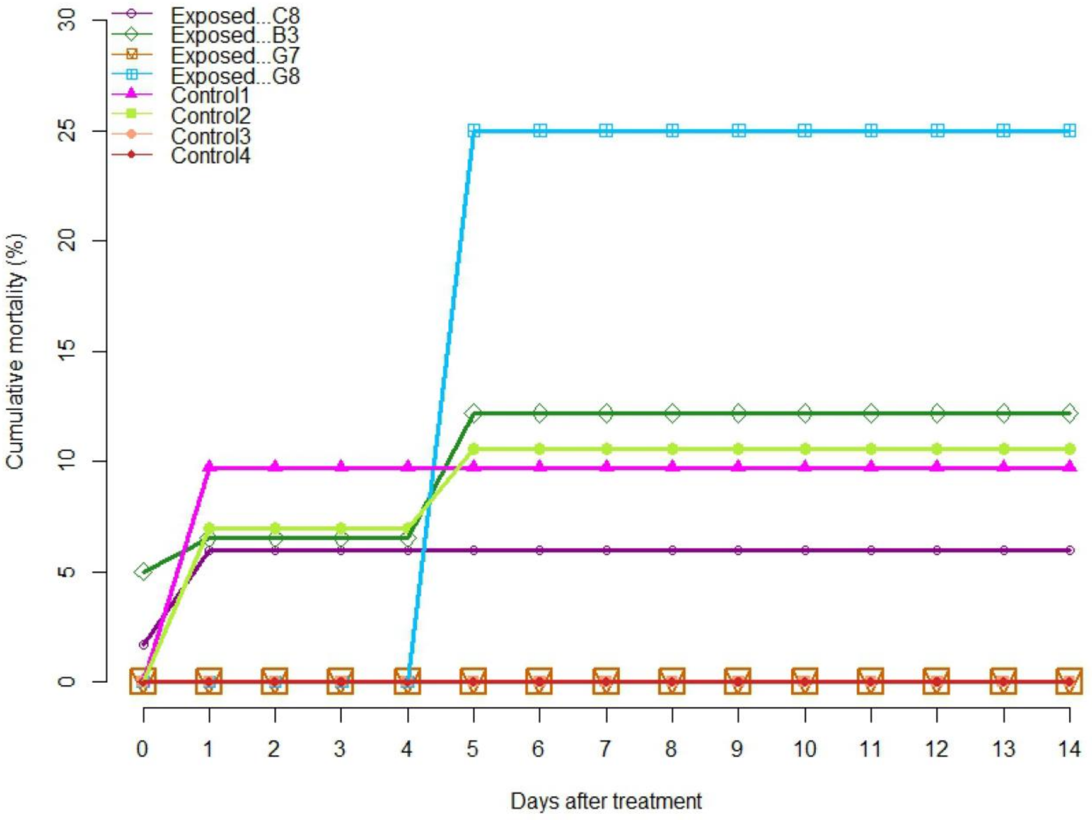

Figure 5. Cumulative mortality during 14 days after the experiment. Trip 1 (C8, B3, Control 1 - 2) and trip 2 (G7, G8, Control 3 - 4). All fish were transported back to the laboratory on day three.

During the first trip with the 68 dph fish (120 fish per treatment group), no immediate mortality was observed for the control groups, whereas a mortality of 1.7 and 5% was found for the exposure experiments 1 and 2, respectively (Figure 5). The dead fish were only seen in one of the six vials in experiment 1 and in two vials in experiment 2. During the second trip (experiments 3 and 4) with the 115 dph fish (12 fish per treatment group), no mortality was observed immediately after the experiments. The difference in immediate mortality (Monte Carlo p-value = 0.11) between control and exposed groups was not statistically significant. Transportation back to the lab did not cause direct mortality. At the end of the 14-day monitoring period of trip one, 9% of the exposed fish and 10.1% of the control fish died[1]. As

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[1] This experiment had an additional treatment consisting of ‘unhandled’ fish per experiment that stayed in the lab during the two weeks. These were not exposed to handling or transport and showed a mortality rate of 13.3 ± 4.7% for the 68 dph fish and 0% for the 115 dph fish over the 14 days.

only four fish per group of the second trip were followed for delayed mortality, a dead fish caused a mortality increase of 25%. One fish of the exposed group died on day four and one fish of the control group on day 13, the latter jumped out of the aquarium and was not included in the statistical analysis. Delayed mortality did not differ significantly between the control and exposed groups of both trips (Monte Carlo p-value = 0.39).

4. Discussion

4.1 Field exposure

As far as we know, this work on the effects of sound on fish presents the first *in situ* field study carried out as close as 45 m from offshore pile driving of monopiles. As such, our study is very valuable to validate the mortality results of laboratory experiments presented in other studies (Bolle et al., 2012; Casper et al., 2012; Halvorsen et al., 2012a; Halvorsen et al., 2012b). Although, the underwater sound levels during the experiments with the control groups were above the background levels in the North Sea (Haelters et al., 2009), due to sound radiation through the four jack-up piles of the piling vessel and the presence of the working vessels, these sound levels were unlikely to cause immediate or delayed mortality (Wysocki et al., 2007; Popper and Hastings, 2009b; Neo et al., 2014).

Both ages of sea bass that were used in our experiments are considered new fingerlings, but juveniles of 68 dph seem to be more sensitive in general (higher mortality) compared to juveniles of 115 dph. Still, both age groups largely survived the exposure to the high sound pressure levels that were exhibited over a complete piling session.

Exposure in the field to such sound levels did not cause significantly increased mortality during the first 14 days after exposure of sea bass (*Dicentrarchus labrax*) of 68 and 115 dph. This result strengthens the trend seen in the recent lab studies with the survival of common sole larvae (*Solea solea*) (Bolle et al., 2012), juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) (Halvorsen et al., 2012b), lake sturgeon (*Acipensen fulvescens*), Nile tilapia (*Oreochromis niloticus*), hogchoker (*Trinectes maculatus*) (Halvorsen et al., 2012a), and hybrid striped bass (white bass *Morone chrysops* x striped bass *Morone saxatilis*) (Casper et al., 2013). The recorded sound pressure levels (SEL_{ss}, SEL_{cum}, $L_{z\text{-}p}$) were comparable to the values measured in laboratory experiments using a high intensity controlled impedance fluid filled wave tube

(HICI-ft) (Casper et al., 2012; Halvorsen et al., 2012a; Halvorsen et al., 2012b; Casper et al., 2013) or a larvaebrator (Bolle et al., 2012). Accordingly, our sound measurements (SEL_{ss}, SEL_{cum}, L_{z-p}) confirm the sound pressure levels that have been used in the lab to mimic real time pile driving sound levels. Bearing in mind the expensive and challenging logistics to execute field experiments in addition to the difficulties to control all environmental parameters, laboratorial studies are a good approach.

4.2 Sound pressure thresholds

Exposure to sound pressures can lead to internal injuries. Sound pressure thresholds for the onset of injury in juvenile Chinook salmon (*Oncorhynchus tshawytsch*) were recommended as: SEL_{cum} of 210 dB re 1 µPa².s derived from 960 strikes, each strike having a SEL_{ss} of 180 dB re 1 µPa².s (Halvorsen et al., 2012b). These values have been exceeded during pile driving of each monopile in our experiments. In an experiment using a HICI-ft, it was shown that after exposure to pile driving sounds, more individuals of a physoclist fish species were injured and the injuries per fish were more severe compared to a physostomous fish and a flatfish species, the latter lacking a swim bladder (Halvorsen et al., 2012a; Halvorsen et al., 2012b). These observations were strengthened by another experiment in the HICI-ft with the physoclist fish, the hybrid striped bass (white bass *Morone chrysops* x striped bass *Morone saxatilis*) (Casper et al., 2013). Physoclist species are slower to change the volume of their swim bladder, which increases their vulnerability to high sound pressure levels (Mann et al., 2007). Furthermore, more severe injuries and a higher number of injuries were reported in the larger size group of hybrid striped bass (white bass *Morone chrysops* x striped bass *Morone saxatilis*) physoclist fish (mean size 17.2 g) compared to smaller size group (mean size 1.3 g) (Casper et al., 2013). This is in contrast to the distinction made by the National Marine Fisheries Service (NMFS) in the interim criteria for pile driving. This states that fishes less than 2 g are more susceptible to injury than larger fish when exposed to impulsive pile driving (Stadler and Woodbury, 2009; Casper et al., 2013). Internal sublethal injuries could also be present in the physoclist sea bass we used in our experiments, but this was beyond the scope of the present study. There was no time on board of the pile driving vessel to dissect the fish to determine the injury level. Another experiment with the HICI-ft showed that fishes can heal from injuries as post-exposure time increased, after they were exposed to 960 strikes of 187 dB re 1 µPa².s (SEL_{cum} of 217 dB re 1 µPa².s) (Casper et al., 2012; Casper et al., 2013). However, internal injuries can also lead to mortality (Halvorsen et al., 2012b). Mortal injuries in physoclistous hybrid striped bass of 1.3 g appeared at energy exposures with SEL_{ss} of 180 dB re 1 µPa².s and SEL_{cum} of 210 dB re 1 µPa².s for 960 strikes (Casper et al., 2013). Similar levels did not result in increased mortality in our experiments. The mortality we noted, in any of the experiments, is more likely to be natural than to be attributed to handling stress or internal injuries since the delayed mortality rates of the unhandled treatment[1] were similar to both *in situ* treatments. The sound pressure threshold causing mortality in juvenile sea bass less than 2 g lies above L_{z-p} of 211 dB re 1 µPa, SEL_{cum} of 222 dB re 1 µPa².s derived from 2312 strikes, and SEL_{ss} of 188 dB re 1 µPa².s.

4.3 Acoustic particle motion

As shown above, the presence of sublethal or lethal injuries is correlated with the presence and type of a swim bladder, and sound 'pressure' seems to be the main sound component that induces the injuries. Nevertheless, the other underwater sound component 'acoustic particle motion' may also have an impact on the fishes, e.g. hearing damage, behavioural and masking studies (Popper and Hastings, 2009a; Tasker et al., 2010). Although it is unlikely that particle motion has a direct effect on mortality, which is the topic of this paper, it is also influenced by pile driving and literature is very scarce on particle displacement, velocity and acceleration in the ocean. Particle motion is a highly directional quantity and fishes may be able to determine the sound source direction (Popper and Fay, 2011). While only those fishes that have evolved towards an acoustic coupling between their swim bladder (or other gas-filled structures) and their ear, can sense sound pressure, all fishes are able to sense particle motion (Hastings and Popper, 2005; Wysocki et al., 2009).

Shallow water acoustics are characterized by propagation complexities, like direct transmission, reflection and re-radiation (Ainslie et al., 2009; Oestman et al., 2009). Therefore, potential effects of changes in the acoustic particle motion will only be seen in the near field of a sound source. Hence, it is recommended to measure both sound components in assessing all the effects of underwater sound.

4.4 Underwater sound in a wider perspective

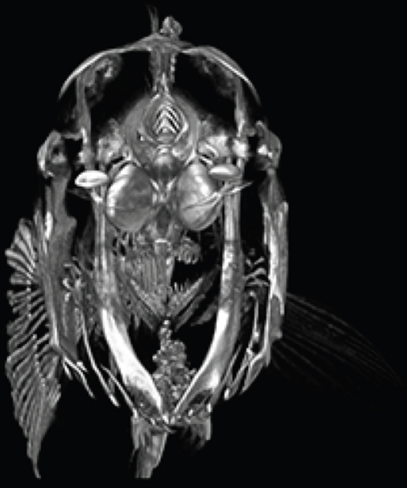
The Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC) aims at a good environmental status (GES) by 2020 for all European waters. One of the eleven descriptors concerns the impact of anthropogenic underwater noise. At present, there is insufficient knowledge on the impact of impulsive sounds to establish proper sound level thresholds for the marine environment. The US Fisheries Hydro-acoustic Working Group (FHWG) formulated interim criteria for maximum sound levels fishes could be exposed to without causing non-auditory tissue damage. The maximum SEL_{cum} for fishes weighing <2 gram was set at 183 dB re 1µPa².s and for fishes >2 gram at 187 dB re 1 µPa².s (Oestman et al., 2009). Also, SEL_{ss} and the number of strikes should be taken into account, and the importance of particle motion should be explored further to develop sound criteria.

The sound pressure levels that were measured in this field study only represent a snapshot of the acoustic near field, with acoustic characteristics measured at a depth of 2.5 m below the sea surface and 45 m away from the sound source. Moreover, these *in situ* experiments can be seen as a 'worst case' scenario in terms of exposure time (number of strikes) and sound levels. In the real world, young sea bass will be drifting with the currents through the wind farm construction zones, which influences the residence time in these zones. Most likely, the encounter time to very high sound levels (pressure and particle motion) will be too short to induce any physiological effects, except during slack tide when the water currents decrease to a minimum.

This *in situ* field study confirms and validates the mortality results found by laboratory experiments and contributes to our general understanding of the effects of pile driving sounds, a first step in the assessment process to establish sound criteria.

ACKNOWLEDGEMENTS

The authors would like to thank Northwind NV and its contractor GeoSea NV for their collaboration and support during the field experiments. Pieter Tomas, Luc Dekoninck, David Vuylsteke, Fernand Delanghe, and Jan Ranson are acknowledged for their technical support.



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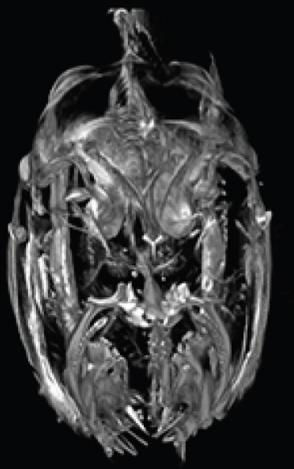

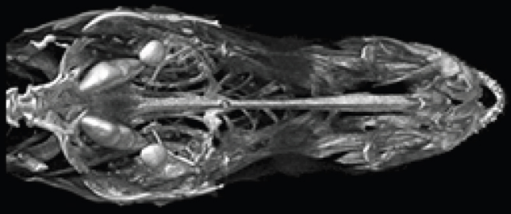

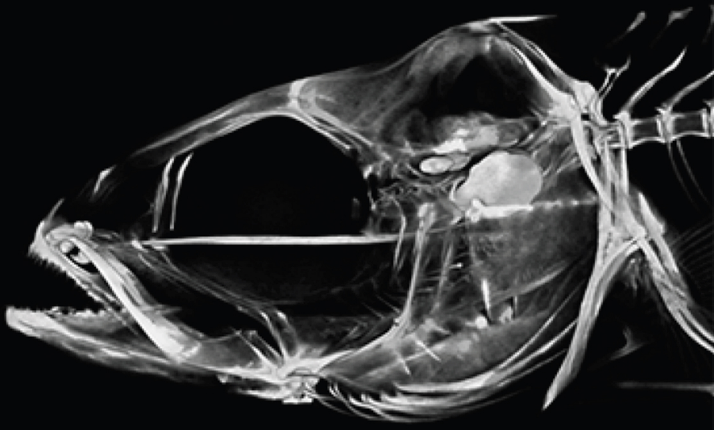

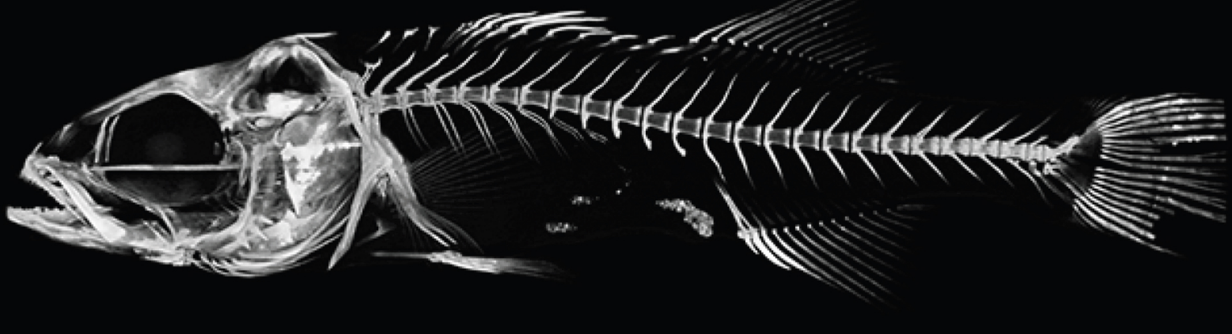

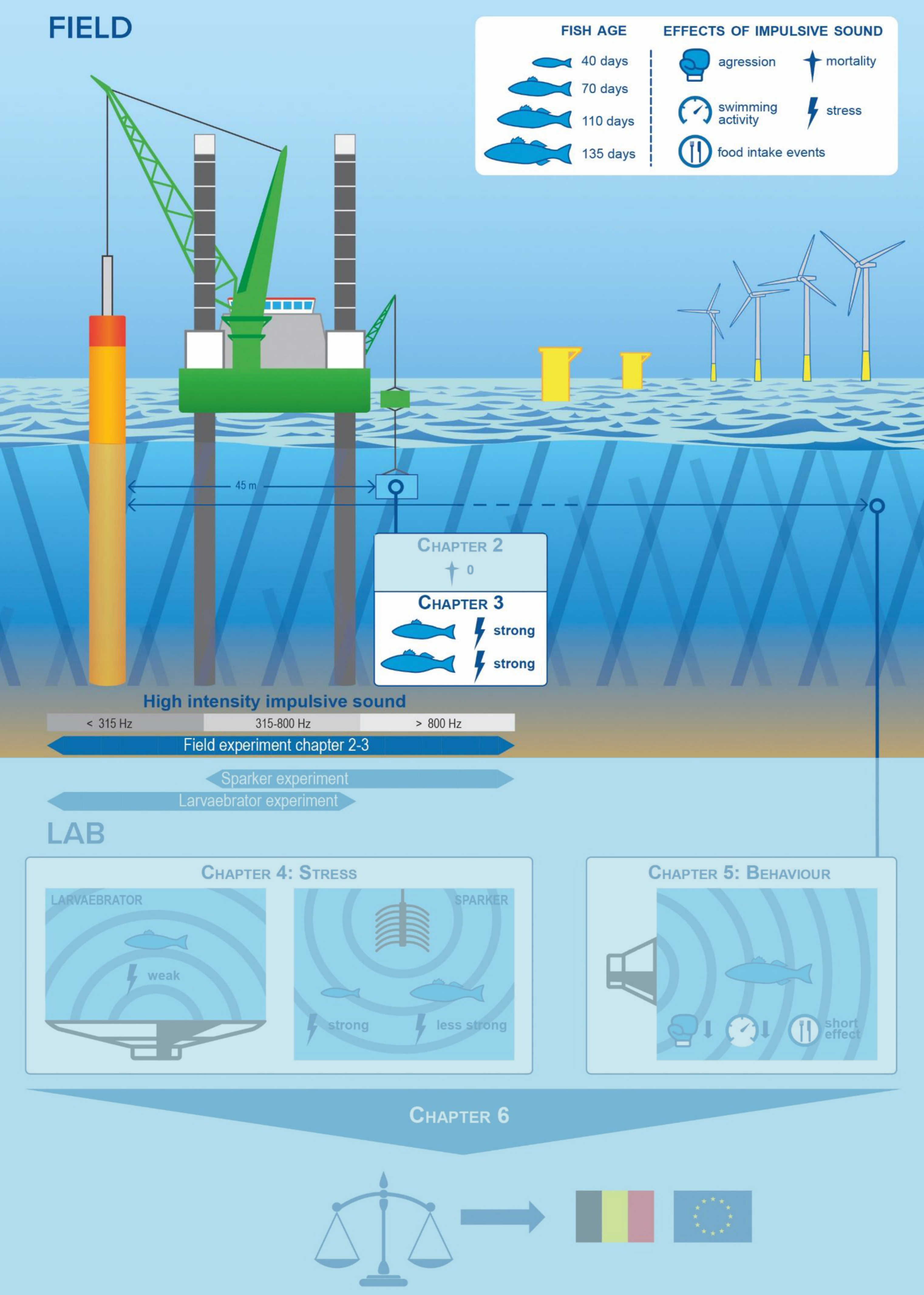
FIELD
FISH AGE
40 days
70 days
110 days
135 days
EFFECTS OF IMPULSIVE SOUND
agression
mortality
swimming activity
stress
food intake events
45 m
Chapter 2
0
Chapter 3
strong
strong
High intensity impulsive sound
< 315 Hz
315-800 Hz
> 800 Hz
Field experiment chapter 2-3
Sparker experiment
Larvaebrator experiment
LAB
Chapter 4: Stress
LARVAEBRATOR
weak
SPARKER
strong
less strong
Chapter 5: Behaviour
short effect
Chapter 6

CHAPTER 3

ACOUSTIC STRESS RESPONSES IN JUVENILE SEA BASS *DICENTRARCHUS LABRAX* INDUCED BY OFFSHORE PILE DRIVING

Adapted from:

Debusschere E, Hostens K, Adriaens D, Ampe B, Botteldooren D, De Boeck G, De Muynck A, Sinha AK, Vandendriessche S, Van Hoorebeke L, Degraer S, 2016. Acoustic stress responses in juvenile sea bass Dicentrarchus labrax induced by offshore pile driving. Environmental Pollution 206, Part B, 747-757.

Abstract

Underwater sound generated by pile driving during construction of offshore wind farms is a major concern in many countries. This paper reports on the acoustic stress responses in young European sea bass *Dicentrarchus labrax* (68 and 115 days old), based on four *in situ* experiments as close as 45 m from a pile driving activity. As a primary stress response, whole-body cortisol seemed to be too sensitive to 'handling' bias. On the other hand, measured secondary stress responses to pile driving showed significant reductions in oxygen consumption rate and low whole-body lactate concentrations. Furthermore, repeated exposure to impulsive sound significantly affected both primary and secondary stress responses. Under laboratory conditions, no tertiary stress responses (no changes in specific growth rate or Fulton's condition factor) were noted in young sea bass 30 days after the treatment. The long-term consequences of repeated impulsive sound exposure for fish in the field are yet to be determined.

Keywords

Impulsive sound, *in situ* experiments, juvenile fish, acute stress responses, condition

1. Introduction

Anthropogenic sound research has mostly been conducted in the terrestrial environment, although aquatic environments also suffer from noise pollution (Slabbekoorn et al., 2010, Kight and Swaddle, 2011, Broucek, 2014). Marine organisms rely on sound for survival, communication, detection of prey and predators, individual recognition, orientation, navigation, mate selection, shoaling, and even larval settlement (Wartzok and Ketten, 1999, Slabbekoorn and Bouton, 2008, Caltrans, 2009, Clark et al., 2009, Slabbekoorn et al., 2010, Stanley et al., 2012, Popper et al., 2014). During several decades, a variety of anthropogenic sounds have been introduced into the marine environment. Sound is generated from shipping, seismic surveys, sonar equipment, underwater explosions and offshore construction. In addition to these anthropogenic noises, sound is naturally present in the ambient marine environment and marine animals also produce sounds. This mixture of biotic and abiotic noise creates a highly complex acoustic environment (De Jong et al., 2011) that affects marine organisms in different ways (Popper and Hastings, 2009b). Underwater noise travels faster and further from the sound source compared to air-borne sound, and the frequency of anthropogenic underwater sound largely overlaps with the range of biologically relevant sounds (Hastings and Popper, 2005, Slabbekoorn et al., 2010). This combination makes artificial sound a potential threat to marine life. Human-generated underwater sound has been classified as a pollutant worldwide and must therefore be monitored. Within the EU Marine Strategy Framework Directive (MSFD) (2010/477/EU European Commission Decision), two indicators have been proposed, *i.e.* impulsive and ambient (continuous) noise. Empirical data to assign thresholds are however lacking (Van der Graaf et al., 2012).

To meet renewable energy targets, the number of constructed and planned offshore wind farms (OWFs) is rapidly increasing in the North Sea, with pile driving as the most commonly used method to anchor the piles. This activity is characterized by high intensity impulsive sounds, with sound pressure levels (from zero to peak, L_{z-p}) up to 210 dB re 1 µPa (e.g. Debusschere et al., 2014). Although OWFs are being constructed all over the North Sea, quantitative data on the impact of pile driving on fish are largely lacking. Recently, a number of acoustically controlled chamber experiments have been carried out, mainly focusing on mortality and injuries (Bolle et al., 2012, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a). These studies found that after exposure to pile driving sound levels, fish without a swim bladder (flatfish) are not susceptible to barotrauma injuries. In contrast, injuries were quite often noticed and were more severe in physoclistous fish (closed swim bladder) compared to physostomous fish (open swim bladder), with most of the injuries noted in tissues close to the swim bladder. These studies also revealed that most injuries did not lead to mortality under optimal lab conditions. Mortal injuries in round fish were observed, but only after exposure to sound level thresholds above 180 to 187 dB re 1 $\mu Pa^2 \cdot s$ for SEL_{ss} (single strike sound exposure level) and 210 to 220 dB re 1 $\mu Pa^2 \cdot s$ for SEL_{cum} (cumulative sound exposure level).

The behavioural responses in fish related to acoustic stress induced by pile driving have received much less attention than physical injury or mortality (Barton, 2002, Popper and Hastings, 2009a). Pile driving noise can be categorized as a ‘type 1’ stressor: it leads to spatially localised stress that is detectable in individual organisms (Shuter, 1990). To re-establish homeostasis after being exposed to a stressor, three main compensatory physiological stress responses are of importance in fish (Wedemeyer et al., 1990). The primary response is a combination of stimulating the sympathetic nervous system, a release of catecholamines and activation of the hypothalamic-pituitary-interrenal axis, which incites the release of steroid glucocorticoid hormones such as like cortisol (Barton, 2002, Schulte, 2014). The corticosteroids and catecholamines mediate a secondary response, covering an adjustment of the physiological metabolism (glucose, lactate, adenylate energy charge), haematological and immune features, and changes in respiration (Pickering, 1981, Rotllant and Tort, 1997, Iwama, 1998, Simontacchi et al., 2008). The tertiary response is related to whole-animal performance, including growth, condition, behaviour, fecundity, disease resistance and survival (Wedemeyer et al., 1990), and is only observed when the initial responses failed to re-establish homeostasis (Pavlidis et al., 2011).

In Debusschere et al. (2014) the results on mortality in juvenile sea bass *Dicentrarchus labrax* after exposure to pile driving sounds were presented based on a number of *in situ* experiments. To date, no other offshore *in situ* experiments have been published, mainly because of the logistical challenges related to conducting experiments that combine fish and sound near OWF construction sites. The aim of the current study was to determine whether pile driving is perceived as an acoustic stressor in fish, more specifically in young sea bass, based on the same four *in situ* field experiments. The primary response level was determined by the biochemical parameter whole-body cortisol; the secondary response level by respiration (oxygen consumption rate) and whole-body lactate; the tertiary response level by growth, weight, condition and skeletal deformation.

2. Materials and Methods

2.1 Model organism and in situ location

2.1.1 Juvenile sea bass

For this experimental study young European sea bass *Dicentrarchus labrax* were used as model organism. Sea bass is an important fish species, both for commercial fisheries and in aquaculture. It is a euryhaline and eurythermic species, distributed throughout the North Atlantic, Mediterranean and Black Sea, and inhabiting several demersal (benthic) habitats down to 100 m (Varsamos et al., 2001). Young sea bass of 45 days post hatching (dph) were collected from the Ecloserie Marine de Gravelines (France) and incubated in cylindro-conic containers of 9.5 L at the Institute for Agricultural and Fisheries Research (ILVO, Ostend). The sea bass were fed twice a day with Aglonorse (300–500 mm) or MariCo Start (1.5 mm) (Coppens). This study was carried out in accordance with the Belgian Council for Laboratory Animal Science (BCLAS) guidelines, and the experimental protocol was approved by the ILVO's Ethics Committee (Permit Number: 2012/178).

2.1.2 In situ location

The *in situ* field experiments were performed aboard the jack-up pile driving vessel Neptune DP2 (GeoSea NV) during OWF construction work on the Lodewijckbank, 30 km off the Belgian coast, in the Belgian Part of the North Sea. This set-up permitted us to monitor the stress responses of fish related to impulsive sounds as close as 45 m from real-time pile driving. Four experiments were carried out during two trips. Per trip, two monopile bases were driven into the seabed using a hydraulic hammer on two consecutive days (IHC Hydrohammer® BV):

monopiles C8 (N51.638° - E2.900°, WGS84) and B3 (N51.629° - E2.927°) during the first trip (18-20 June 2013), and monopiles G7 (N 51.607° - E2.881°) and G8 (N51.603° - E2.877°) during the second trip (26-28 August 2013). For detailed information on the monopiles themselves, see Debusschere et al. (2014).

2.2 Experimental design

2.2.1 Treatments

The four experiments consisted of three treatments each: (1) lab control, where fish were subjected to background sound in the lab, but were not subjected to transport or other handling effects ('unhandled' fish); (2) *in situ* control, where fish were subjected to handling and transportation to and from the pile driving vessel and were subjected to ambient background sound while submerged into the sea before or between two pile driving events; and (3) *in situ* exposure, where fish were subjected to handling and transportation as described above, plus exposure to underwater sound of pile driving during 1.5 hour (~ duration of one pile driving event) at a distance of 45 m from the hydraulic hammering activity.

2.2.2 Handling and transportation

For the lab control treatments, 2 * 120 cultured sea bass fingerlings of 68 dph (experiments 1 and 2) and 2 * 12 juveniles of 115 dph (experiments 3 and 4) were kept in separate aquaria (closed, aerated; volume = 9.5 L and 30 L, respectively). For the *in situ* treatments, a total of 480 and 48 fish on trip 1 and on trip 2, respectively, were transported to the pile driving vessel. The fish were pooled per 30 in 2 L containers for trip 1 (experiments 1 and 2) and per four in 10 L containers for trip 2 (experiments 3 and 4). Oxygen tablets (JBL) were added to the seawater and the containers were completely filled to minimize movement of the water within the container. Transportation lasted about 3 hours in total. Due to logistical restrictions, all fish containers had to be embarked at the start of each trip on board of the pile driving vessel and no sound isolation could be provided. This brought about that fish used on the first day (experiments 1 and 3) of each trip were still 'sound naïve', whereas fish used on the second day (experiments 2 and 4) were already indirectly exposed to pile driving sound through vibrations of the pile driving vessel on the previous day. The lack of sound isolation on the pile driving vessel may have affected the biochemical, physiological and morphological responses.

Once aboard the pile driving vessel, the sea water in the containers was aerated and refreshed daily. For the *in situ* treatments the fish were placed in closed 500 mL vials (composed of poly 4-methyl, 1- pentene), with an acoustic impedance of 1.84 Rayl, which was as close as possible to the impedance of seawater (1.56 Rayl) (Bradley and Wilson, 1966, Thompson, 1990). Each treatment consisted of nine 500 mL vials: six filled with fish and seawater and three filled with only seawater to measure bacterial respiration. The number of fingerlings per vial was based on oxygen demand and oxygen availability in the seawater. For the first two experiments, 20 fingerlings of 68 dph were used per 500 mL vial (in total 120 fish per treatment). Because the fish used in experiments 3 and 4 were older and bigger, only two individuals were placed together per 500 mL vial (in total 12 fish per treatment).

After submerging, the vials were brought aboard and a number of fish were euthanized and stored in formaldehyde or liquid nitrogen (also for the lab control, see analyses below), while the rest of the fish were transferred per 24 or per 2 in 2 L or 10 L aerated containers of each treatment per experiment during trip 1 and 2, respectively. At the end of each trip (three days per trip) the remaining live fish were transported back to the lab (see above) and transferred into cylindro-conical aquaria (9.5 L, trip 1) or rectangular aquaria (30 L, trip 2) per experiment and per treatment for another 30 days. Figure 1 gives a schematic overview of the experimental design and flow of the analyses.

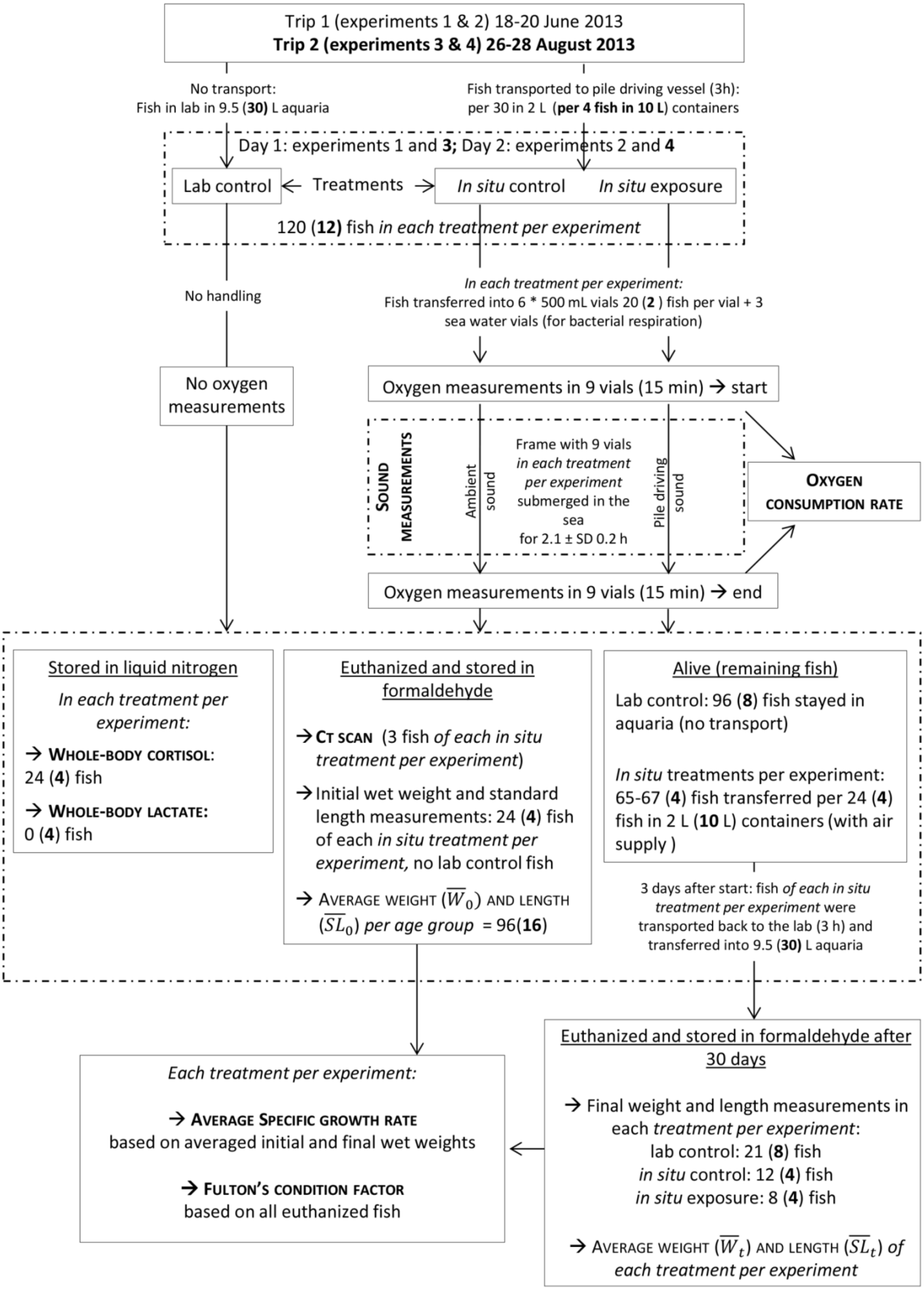

Figure 1. Schematic diagram of the experimental design and flow of the analyses. During trip 2, the exact same procedures as trip 1 were followed, but with fewer fish (between brackets and in bold).

2.2.3 Field construction and sound recording

The *in situ* construction consisted of two parts: the upper frame containing the sound recorder equipment (not submerged) and the lower frame (hanging 3.5 m lower) holding the vials (nine per treatment), the latter being submerged to 2.5 m below the sea surface (Figure 2). The vials were firmly attached to the stainless steel construction with cam straps. The *in situ* construction was operated with a crane at a distance of 45 m from the pile driving activity, *i.e*. the other side of the pile driving vessel. During the *in situ* treatments (*in situ* control and exposure) the underwater sound pressure (ambient background noise and pile driving) was recorded for 1.5 hour (~ duration of one pile driving event). Sound pressure was recorded using a hydrophone (Brüel & Kjaer type 8104, voltage sensitivity 47.7 $\mu V \cdot Pa^{-1}$, charge sensitivity 0.391 $pC \cdot Pa^{-1}$, 10 m cable), hanging unobstructed below the sea surface at 0.3 m above the vials. The hydrophone was connected to a Brüel & Kjaer portable amplifier (Nexus type 2690-0S) and recorded on a multi-channel portable recorder (Tascam DR-680) as a one channel waveform audio file format (.wav) with a sampling rate of 44 100 Hz at 24 bit. The sound pressure metrics, zero-to-peak sound pressure level (L_{z-p}), single strike and cumulative sound exposure level (SEL_{ss} and SEL_{cum}), were calculated using Matlab R2012b (version 8.0). A detailed description of the sound pressure parameters can be found in Debusschere et al. (2014).

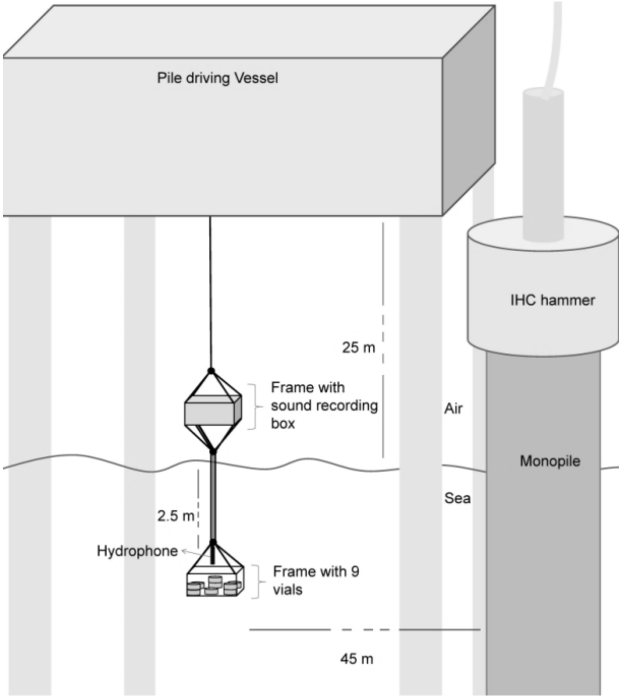

Figure 2. Experimental construction used at the pile driving vessel (adapted from Debusschere et al., 2014). The pile driving vessel was jacked-up 25 m above the sea surface. The *in situ* experiments were carried out at 45 m from the pile driving activity.

2.3 Physiological measurements

2.3.1 Oxygen consumption

Oxygen consumption could not be measured for the lab control treatment as this would have necessitated handling the fish. At the beginning of each *in situ* control and exposure treatment, the nine vials were carefully filled to the rim until a bubble of seawater formed at the top of the vial. Oxygen ($\mu mol \cdot L^{-1}$), temperature (°C) and salinity (ppt) were measured before closing the lid. Oxygen concentration was measured with an oxygen microsensor 100 µm (Unisense) connected to a pico-ammeter, temperature by means of an IKS temperature sensor connected to an IKS aquastar, and salinity using a refractometer. On average 15 minutes were required to complete the nine oxygen measurements. At the end of each treatment, the oxygen level in the nine vials was measured again in the same order, and following conversion steps were executed. The pico-amp values were standardized to $\mu mol \cdot L^{-1}$, based on measurements in 100% oxygen saturated seawater and a 0% oxygen solution (sodium ascorbate and sodium hydroxide dissolved in fresh water) at similar temperature as the seawater in the vials. Bacterial respiration was accounted for by subtracting the average reduced oxygen concentration in the three vials containing only seawater (n = 3 per treatment) from the values (start minus stop concentrations) measured in the six fish-filled vials (n = 6 per treatment) (Table 1). Oxygen consumption (µmol·L^{-1}) was further corrected for total fish wet weight and water volume per vial, and standardized per hour (µmol·g^{-1}·h) to calculate fish oxygen consumption rates.

Table 1. Overview of the data used in the statistical analyses, per treatment (3), experiment (4) and trip (2).

	Trip 1			Trip 2		
	Experiment 1 (similar numbers for experiment 2[d])			Experiment 3 (similar numbers for experiment 4[d])		
	Lab control	*In situ* control	*In situ* exposure	Lab control	*In situ* control	*In situ* exposure
Physiological measurements						
Bacterial respiration		n = 3	n = 3		n = 3	n = 3
Oxygen consumption rate		n = 6	n = 6		n = 6	n = 6
Biochemical analyses						
Whole-body cortisol	n = 3[a]	n= 3[a]	n = 3[a]	n = 4	n = 4	n = 4
Whole-body lactate[b]	-	-	-	n = 4	n = 4	n = 3
Morphological analyses						
Initial SL and WW[c]	-	n = 4 * 24		-	n = 4 * 4	
Final SL and WW[d]	n = 21(10)	n = 12(10)	n = 8(14)	n = 8(8)	n = 4(3)	n = 4(3)
Specific growth rate [e]	n = 1	n = 1	n = 1	n = 1	n = 1	n = 1
Fulton's condition factor[d]	n = 21(10)	n = 12(10)	n = 8(14)	n = 8(8)	n = 4(3)	n = 4(3)
Skeletal deformations (CT scan)	-	n = 3	n = 3	-	n = 3	n = 3

[a] biomass of seven fish pooled per treatment for experiments 1 and 2

[b] not enough biomass left for whole-body lactate analyses for experiments 1 and 2; same individuals used for whole-body lactate as for whole-body cortisol in experiments 3 and 4

[c] initial standard length (SL, mm) and wet weight (WW, g) based on 4 * 24 and 4 * 4 fish taken together from both in situ treatments and both experiments for trip 1 and trip 2, respectively

[d] numbers for experiments 2 and 4 between brackets. These numbers differ because at each of three moments during the 30 day period, 12 fish of trip 1 (experiments 1 and 2) were taken out of the aquaria to monitor their growth (these data were not used further), thus reducing the final number of fish.

[e] based on averaged initial and final wet weights, leading to one value per treatment

2.4 Biochemical analyses

Immediately after the oxygen measurements at the end of the *in situ* control and exposure treatments, 24 fish (experiments 1 and 2) or 4 fish (experiments 3 and 4) were randomly collected from the 500 mL vials and frozen in liquid nitrogen for whole-body cortisol and whole-body lactate analyses (this only for experiments 3 and 4). A similar procedure was followed in the lab for the lab control treatments.

2.4.1 Whole-body cortisol

On average 0.3 g of fish biomass (wet weight, WW) were needed for cortisol analysis, therefore seven fish (68 dph) of the same treatment were pooled to determine whole-body cortisol levels in experiments 1 and 2, resulting in 3 samples (n = 3) per treatment and experiment (Table 1). For trip 2, the biomass of each 115 dph fish was enough to measure individual cortisol concentrations (n = 4 per treatment). Whole-body cortisol was quantified following a combination of published extraction methods with slight modifications (Dejesus et al., 1991, Ramsay et al., 2006, Sink et al., 2007, Egan et al., 2009, Bertotto et al., 2011). After pulverization in liquid nitrogen, the fish powder (0.3 g WW) was suspended into 1.5 mL of phosphate buffer (pH 7.2) in a glass test tube of 15 mL on ice, and subsequently extracted twice with 3 mL diethyl ether. Each sample was vigorously vortexed for 30 s and centrifuged at 3500 rpm for 5 min at 4 °C to separate the aqueous and ether layers, after which the ether layer was transferred into a new glass tube of 5 mL by freezing the aqueous layer for 15 min at -80°C. After evaporation for at least 12 h, the dried samples were resuspended with 250 µl saline phosphate-buffer with 0.1% gelatine, vortexed for 30 s and stored for 24 hours at 4 °C before measuring the cortisol concentration.

The ImmuChem^{TM} Coated Tube Cortisol ^{125}I RIA kit (radio immune assay, MP Biomedicals, LLC) was used to determine whole-body cortisol levels by means of a Perkin Elmer Wallac 1480 Wizard 3” Gamma Counter (Zaventem, Belgium). The values of all cortisol samples were corrected for extraction efficiency, dilution and fish weight. Extraction efficiency was measured by comparing nine duplicate samples from extra lab cultured fingerlings, i.e. three samples of three 63 dph fish pooled together (0.10 ± SD 0.08 g WW) and six 110 dph individuals (1.21 ± SD 0.15 g WW), where 7.5 ng cortisol·mL^{-1} (MPBio Diagnostics) was added before and after diethyl ether extraction (Yeh et al., 2013). An average extraction efficiency of 73 ± SD 21% of whole-body cortisol from juvenile sea bass was calculated, similar to extraction efficiencies obtained by other studies (Dejesus et al., 1991, Ramsay et al., 2006).

2.4.2 Whole-body lactate

The measurement of whole-body lactate followed a simpler protocol. On average 0.25 g of fish biomass were needed per sample for whole-body lactate analyses, which was not possible from the limited number of 68 dph fish left after whole-body cortisol analyses (Table 1). Therefore, whole-body lactate could only be analyzed for the 115 dph fish (experiments 3 and 4) based on the same fish used as for whole-body cortisol (n = 4 per treatment). After pulverizing in liquid nitrogen, 0.25 g WW of the pulverised fish powder was suspended into 0.5 mL of 8% perchloric acid at 4 °C and centrifuged. The supernatant was neutralized in 1N $KHCO_3$ and vortexed. Subsequently whole-body lactate concentrations were measured by colorimetric assays using commercial enzymatic lactate assay kits (R-Biopharm AG, Darmstadt, Germany).

2.5 Morphological analyses

After each *in situ* treatment, 24 fish were randomly collected from the six vials per treatment in experiments 1 and 2 (4 fish per treatment in experiments 3 and 4) and euthanized by an overdose of benzocaine/acetone solution and fixed in 7% buffered formaldehyde. From these subsets (n = 96, resp. n = 16) the standard length (SL, mm) and wet weight (WW, g) were measured, and the average length and weight per age class ($\overline{SL}_0$ and $\overline{W}_0$) were calculated. Furthermore, three individuals (n = 3) of each *in situ* treatment per experiment were checked for skeletal deformations.

All of the surviving fish (five to seven fish died per treatment on trip 1) were further handled and transported back to the lab as described above (65 - 67 fish per *in situ* treatment in experiments 1 and 2; four fish per *in situ* treatment in experiments 3 and 4). At the lab, all fish of each treatment per experiment were transferred into cylindro-conical aquaria (9.5 L, experiments 1 and 2, trip 1) or rectangular aquaria (30 L, experiments 3 and 4, trip 2). Water temperature was 20.1 ± SD 0.5 °C and salinity 30.2 ± SD 0.1 in the lab aquaria. After 30 days a number of fish (different numbers for each treatment and experiment, Table 1) were euthanized, measured and weighed (SL_t and W_t) to determine growth and condition, and subsequently stored in 7% formaldehyde solution.

2.5.1 Specific growth rate

As fish could not be followed individually in the experiments, the average length and weight after 30 days ($\overline{SL}_t$ and $\overline{W}_t$) were calculated in each treatment per experiment (n = 1 per treatment) (Table 1). The average specific growth rate (SGR, %·day^{-1}) was calculated for each treatment per experiment as $\overline{SGR} = (\ln \overline{W}_t - \ln \overline{W}_0)$ / Δt where $\overline{W}_t$ and $\overline{W}_0$ were the final and initial average wet weights of the fish (g) respectively, and Δt was the time between W_t and W_0 (Handeland et al., 2008).

2.5.2 Overall condition

The health or condition of the fish in each treatment per experiment was measured by means of Fulton's condition factor K, which was defined as $K= W_t/SL_t^3 \times 100$, where W_t was the wet weight (g) and SL_t the standard length (cm) of the fish after 30 days in the lab (Table 1).

2.5.3 Skeletal deformations

The three individuals (n = 3) that were randomly selected per *in situ* treatment for potential irregularities in their skeletal structure were scanned at the Centre for X-ray Tomography of Ghent University (UGCT) (Table 1). The fish of trip 1 were scanned with the HECTOR micro-CT scanner (Masschaele et al., 2007), fish of trip 2 with a micro-CT scanner (Masschaele et al., 2013). All projection images were reconstructed using the Octopus-package developed at UGCT[f] (Vlassenbroeck et al., 2007). Image stacks in TIFF-format were uploaded in Amira (version 5.5) and volume rendered 3D images were generated using the Volren module to visualize the fish skeletal. Virtual cuts were generated to allow for a detailed screening of the cranial and postcranial internal skeleton structures.

2.6 Statistical analyses

A linear mixed-effects model was applied to the measured variables (oxygen consumption rate, whole-body cortisol, specific growth rate and Fulton's condition factor), with treatment and age as fixed effects and experiments 1 to 4 ($trip_{(1-2)}\_day_{(1-2)}$) as the random effect. Whole-body lactate was also analysed with a linear mixed-effects model with treatment as the fixed factor and experiments 3 – 4 as the random effect. The number of samples per analysis is given in Table 2. The analysed data were considered normally distributed, based on the graphical evaluation (histogram and Q-Q plot) of the residuals, which allowed for ANOVA type III analyses on the different linear mixed-effects models. Significant differences (p <0.05) were further tested through *post-hoc* pairwise comparisons with least-square means and p-values corrected by means of Tukey-Kramer adjustment for multiple comparisons. All analyses were performed using R-Studio for Windows.

3. Results

3.1 Sound pressure

Detailed results on the measured sound pressure parameters during the four *in situ* experiments have been published in Debusschere et al. (2014). In summary, the ambient background SPL (as measured during the *in situ* control treatments) ranged from 128 to 145 dB re 1µPa. The impulsive sound pressure generated during the pile driving activity itself (*in situ* exposure treatment) was much higher, with SEL_{ss} ranging from 181 to 188 dB re 1 µPa²·s, L_{z-p} of 210 dB re 1µPa, the number of strikes during one pile driving session ranging between 1739 and 3067 strikes, and SEL_{cum} reaching 215 up to 222 dB re 1µPa²·s. The dominant energy during exposure (SEL_{ss}) was found at a frequency of 125-200 Hz, slowly decreasing toward the higher frequencies. The sound pressure levels (SPL) measured in the cylindro-conical and rectangular aquaria were respectively, 128.7 ± 3.2 dB re 1 µPa and 130.2 ± 2.3 dB re 1 µ Pa.

3.2 Whole-body cortisol

No significant differences between the treatments and age groups could be shown ($F_{2,34}$ = 1.78, p = 0.183). The whole-body cortisol levels of the 'unhandled' fish (lab control treatment) in both 68 and 115 dph age groups were low, respectively 3.37 ± SD 0.98 $ng \cdot g^{-1}$ fish and 5.51 ± SD 4.16 $ng \cdot g^{-1}$fish (Figure 3A). The sea bass that were used for the *in situ* control treatments showed elevated whole-body cortisol levels of 41.91 ± SD 17.27 $ng \cdot g^{-1}$ fish in experiments 1, 2 and 3, with increased values on the second day of each trip (*i.e.* experiments 2 and 4), and reaching 128.36 ± SD 111.47 $ng.g^{-1}$ fish in experiment 4. No significant differences were noted between the *in situ* control and *in situ* exposure treatments in each of the four experiments. Only the *in situ* exposed fish of the third experiment (trip 2, day 1) showed a trend towards elevated whole-body cortisol levels of 88.3 ± SD 28.9 $ng \cdot g^{-1}$ fish compared to the whole-body cortisol levels of 41.8 ± SD 18.6 $ng \cdot g^{-1}$fish for the respective *in situ* control treatment.

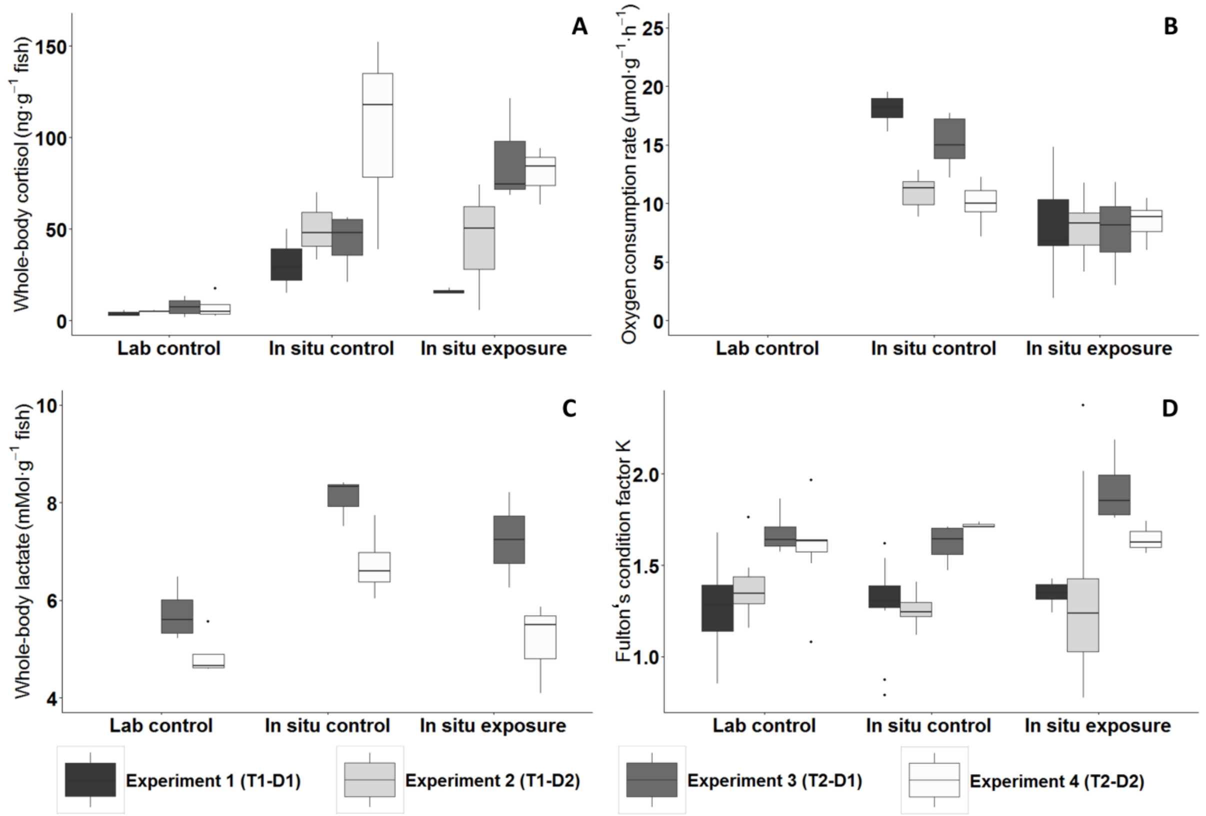

Figure 3. Stress responses of juvenile European sea bass *Dicentrarchus labrax*, based on four experiments (trip 1-2; day 1-2) and three treatments each: no handling/no exposure (lab control), exposed to ambient sound (*in situ* control), and exposed to impulsive sounds during a complete pile driving session at 45 m from the pile driving activity (*in situ* exposed). Fish were 68 days old (dph) at the start of experiments 1 and 2 and 115 dph in experiments 3 and 4. (A) Whole-body cortisol (ng·g^{-1} fish); (B) Oxygen consumption rate (µmol·g^{-1}·h^{-1}); (C) Whole-body lactate (mMol·g^{-1} fish, no data for experiments 1 and 2); (D) Fulton's condition factor K measured after 30 days.

3.3 Oxygen consumption rate

At the start of experiments 1 and 2 (trip 1), the temperature and salinity measured in the vials were 17.4 ± SD 1.4 °C and 33.3 ± SD 0.6 ppt, respectively, and 14.6 ± SD 0.7 °C and 33.5 ± SD 0.4 ppt, respectively, at the end. At the start of experiments 3 and 4 (trip 2), the temperature and salinity in the vials were 20.3 ± SD 0.5 °C and 36 ± SD 0.7 ppt, respectively, and at the end, 19.9 ± SD 0.3 °C and 36 ± SD 0.7 ppt, respectively. The bacterial respiration under ambient underwater sound (*in situ* control treatment) was 3.2 ± SD 9.4 µmol·L^{-1} and -2.5 ± SD 4.5 µmol·L^{-1} under pile driving underwater sound (*in situ* exposure treatment), which was not significantly different.

A clear reduction in oxygen consumption was observed between the *in situ* control and exposure treatments in the four experiments, irrespective of the age of the sea bass ($F_{1,42}$ = 13.08, p = 0.0008) (Figure 3B). The fish used on the first day of both trips (experiments 1 and 3) showed a decrease in oxygen consumption rates of 55% and 49%, respectively, after the exposure to pile driving sound relative to the respective *in situ* control treatments. A similar oxygen consumption rate was measured in the fish on the second day of both trips (experiments 2 and 4) after the exposure to pile driving sound, although the difference with the respective *in situ* control treatments was smaller. For the *in situ* control treatments of experiments 2 and 4 the oxygen consumption rate was already reduced to 34 – 40% compared to those in experiments 1 and 3.

3.4 Whole-body lactate

Whole-body lactate levels could only be measured for the 115 dph fish used in experiments 3 and 4 (trip 2). Significant differences were noted between treatments ($F_{2,17}$ = 16.6, p = 0.0001), with significantly higher whole-body lactate levels for the *in situ* control treatment *vs*. the lab control treatment for both experiments (*post-hoc* test: p = 0) (Figure 3C). The whole-body lactate levels in the *in situ* control treatments were also higher than those in the respective *in situ* exposure treatments of both experiments (*post hoc* test: p-value = 0.0036).

3.5 Growth and condition

At the moment of the *in situ* experiments 1 and 2, the average individual wet weight ($\overline{W}_0$) of the 68 dph fish was 0.0428 ± SD 0.0158 g and the average standard length ($\overline{SL}_0$) has 16.8 ± SD 1.5 mm. For the 115 dph fish (experiments 3 and 4) $\overline{W}_0$ was 1.613 ± SD 0.473 g and $\overline{SL}_0$ 47.8 ± 4.4 mm. After 30 days, the average individual weight ($\overline{W}_t$) and standard length ($\overline{SL}_t$) varied between 0.14 and 0.29 g, respectively, *i.e.* between 21.9 and 28.4 mm for the smallest age class and between 4.65 and 6.05 g; and 64 and 71.5 mm for the older age class.

No significant differences were found in SGR between the different treatment groups after 30 days ($F_{2,4}$ = 0.55, p = 0.61). On average, SGRs were slightly higher in 68 dph fish (experiments 1 and 2) than in 115 dph fish (experiments 3 and 4) across all treatments ($F_{1,2}$ = 6.52, p = 0.12). The SGR of fish used on the first day of each trip (experiments 1 and 3) were also slightly higher compared to the fish used on the second day (experiments 2 and 4) (Table 2).

Table 2. Mean specific growth rates (SGR) in juvenile sea bass for four experiments and three treatments after 30 days. Fish were 68 days post hatching (dph) at the start of trip 1 and 115 dph for trip 2.

	Trip 1 (68 dph)		**Trip 2 (115 dph)**	
Treatment/Experiment	**Exp 1 (day 1)**	**Exp 2 (day 2)**	**Exp 3 (day 1)**	**Exp 4 (day 2)**
Lab control	0.046	0.050	0.043	0.043
***In situ* control**	0.062	0.054	0.046	0.037
***In situ* exposed**	0.071	0.053	0.039	0.035

Under optimal lab conditions, no significant differences were noted 30 days after the start of the experiment between the three treatments (lab control, *in situ* control and *in situ* exposure) for Fulton's condition factor K ($F_{2,97} = 0.75$, $p = 0.47$) (Figure 3D). Fulton's K was higher for the older fish (on average 1.67 ± SD 0.18 for trip 2) than for the younger age class (on average 1.24 ± SD 0.24 for trip 1) ($F_{1,2} = 16.95$, $p = 0.05$). Only the *in situ* exposed fish of experiment 3 showed higher values (although not significantly different) for Fulton's condition factor compared to the respective lab and *in situ* control treatments.

3.5 Skeletal deformation

Examination of the 3D X-ray images did not reveal any skeletal abnormalities in fish from the *in situ* control and exposure treatments in both length/age groups (Figure 4).

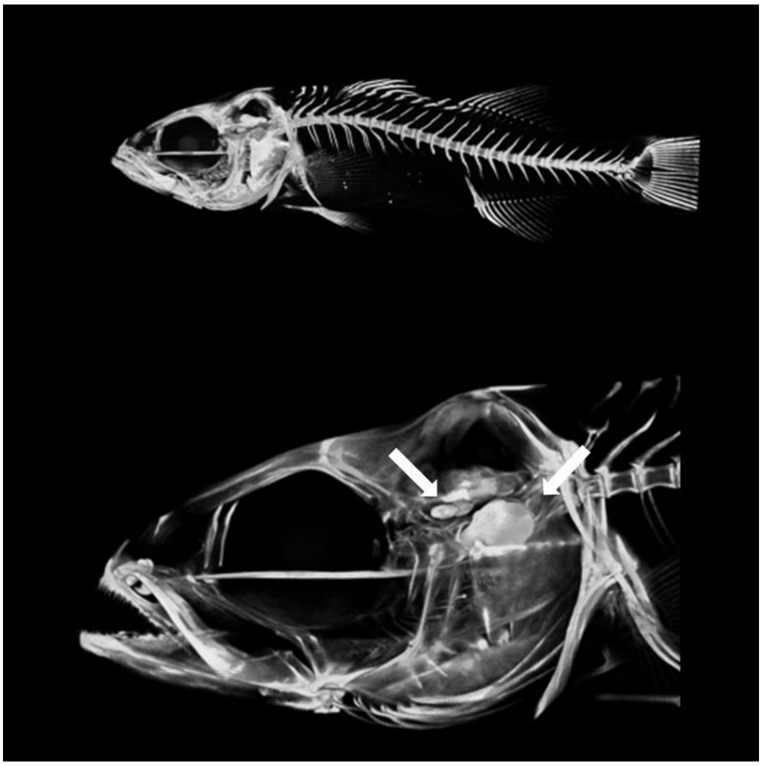

Figure 4. CT scan of a 68 dph juvenile sea bass (from trip 1) after *in situ* exposure to offshore pile driving sound, with a close-up of the head. The arrows point to the otoliths.

4. Discussion

Debusschere et al. (2014) based on the same *in situ* experiments, showed that impulsive sound generated by pile driving did not invoke significant increases in immediate or delayed mortality in young sea bass *Dicentrarchus labrax*. Furthermore, various lab studies have revealed that under optimal lab conditions strong impulsive sound may cause fish injuries, but these normally do not lead to mortality (e.g. Bolle et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a, Casper et al., 2013b). The assessment of stress responses in fish related to impulsive sounds has received much less attention. Santulli et al. (1999) reported primary and secondary stress responses in sea bass after exposure to air gun impulses as shown by increased cortisol and lactate serum levels, and reduced levels in the energy transfer nucleotides and in glucose serum. In the current study, the importance of pile driving as a stressor for juvenile sea bass *Dicentrarchus labrax* at 45 m from an offshore pile driving activity could be evaluated by means of physiological, biochemical and morphological analyses. In general, juvenile fish exposed to *in situ* pile driving sound exhibited strong secondary stress responses during the exposure, but no long-term condition consequences could be noted after exposure when fish were kept under optimal lab conditions.

4.1 Experimental design and the need for naïve fish

Physiological and morphological responses to environmental stressors are a reaction to maintain or re-establish homeostasis in the fish body. The cascade of induced events acts on all systems of an animal, referred to as an ‘integrated’ stress responses (Wedemeyer et al., 1990, Bonga, 1997, Schulte, 2014). However, it is not always clear whether the responses are induced by the investigated stressor - in this case pile driving - or by another factor. The values for both whole-body cortisol and lactate were significantly higher in the *in situ* control treatment *vs.* the lab control (‘unhandled’) treatment. As such, it can be stated that handling and transport of the fish clearly influenced the biochemical responses. Several studies already noted the effect of handling stress on the biochemical reactions in fish (Barton et al., 1985, Barton et al., 1986, Hemre et al., 1991, Ruane et al., 1999, Barton, 2002), which makes *in situ* experiments rather challenging. The handling stress did not lead to additional stress since the mortality rates of all three treatments were similar over a period of two weeks (Debusschere et al., 2014)

The second problem is related to non-deliberate exposure to a certain stressor. The ambient background sound levels (as measured during the *in situ* control treatments) were already high, due to the noise generated by the pile driving vessel and the working vessels. Furthermore, fish sampled on the second day of each trip already indirectly experienced effects of the pile driving activity through vibrations of the vessel. This is clearly reflected in the differences in the physiological and biochemical parameters measured on the second day (experiments 2 and 4) compared to the first day (experiments 1 and 3) in both trips and age groups. The frequency at which discrete stressors elicit a stress response, the combination of multiple stressors and acute *vs.* continuous stressors all play important roles in the response process (Barton, 2002). These results emphasize the need to work with ‘fresh’ or ‘stress-naïve’ fish to measure their unbiased ‘real’ response to pile driving. This was not the case in the *in situ* experiments on the second day, as all fish had to be embarked at the beginning of each trip.

4.2 Primary stress responses

This study also demonstrated the necessity of choosing robust parameters according to fish species and experimental design (*e.g.* lab versus *in situ* experiments). Cortisol is a widely accepted stress indicator of the primary stress response (Pickering, 1993, Bonga, 1997, Ramsay et al., 2006, Schulte, 2014). Cortisol is responsible for the redistribution of energy flows to handle stressors at the cost of reproduction, growth and immunity in the long-term (Bonga, 1997, Pavlidis et al., 2011). Measuring whole-body cortisol levels is a good alternative to plasma cortisol when the blood volume is insufficient (Dejesus et al., 1991, Ramsay et al., 2006, Pavlidis et al., 2011), which was surely the case for the small 68 dph fish.

For the ‘unhandled’ juvenile sea bass (lab control treatment), the whole-body cortisol values were low and can be considered as a baseline for both weight/age classes (68 and 115 dph). On the other hand, the whole-body cortisol method seemed to be inadequate to quantify *in situ* primary stress responses in fish. The results for the *in situ* treatments were not uniform and a clear trend could not be observed, because whole-body cortisol levels were too sensitive and not resilient enough for the *in situ* experiments. Prolonged exposure to a certain stressor desensitizes the fish and attenuates the neuroendocrine and metabolic responses, thereby compromising the homeostatic mechanisms (Reid et al., 1998, Kight and Swaddle, 2011), which results in ‘normal’ cortisol levels (Barton, 2002). Fish used on the second day of each trip were indirectly subjected to pile driving on the first day, which might have influenced their primary stress response. Moreover, on the first day of trip 1 a technical problem occurred during the jack-up of the pile driving vessel, with increased noise and vibrations for a couple of hours right next to the place where the fish were held. This again might have led to whole-body cortisol levels appearing as ‘normal’ in the *in situ* exposure group (comparable to the *in situ* control group) of the first experiment.

Recently, a new method for cortisol determination has been proposed, using a standardized portion of the caudal peduncle (Guest et al., 2015). This new method seems to be more robust for young fish and might be more suitable to determine primary stress responses in *in situ* experiments. However, for experiment 3 (first day of trip 2) significantly higher whole-body cortisol levels were measured in the *in situ* exposure group compared to the *in situ* control group of this experiment. Because fish were still stress naïve (apart from the 'handling' bias) and no technical problems were encountered during that experiment, these are the only values representing a 'real' whole-body cortisol response to pile driving sound, without interference of other strong stressors.

4.3 Secondary stress responses

In contrast to whole-body cortisol, the secondary stress response to pile driving was clearly represented by changes in oxygen consumption rate and whole-body lactate levels. Respiratory activity is commonly used as an indicator to evaluate animal stress and metabolic changes under environmental deterioration (Dalla Via et al., 1998, Chebbi and David, 2010). Several studies showed that physical stress normally elevates the oxygen consumption, whilst toxic reagents affect respiration in either way (Barton and Iwama, 1991, Brown et al., 2005, Patil and David, 2008, Naik and Patil, 2010). Oxygen consumption showed a clear decrease (49 - 55%) when juvenile sea bass were exposed to impulsive sounds at 45 m from the pile driving activity (*in situ* exposure treatments) compared to the *in situ* control treatments. The reduction in oxygen intake might point towards a metabolic depression to minimize energy investments and to save energy stores under stressful conditions. This can also suggest a reduced mobility related to strategic freezing, which is a typical 'anxiety' reaction (Blaser et al., 2010, Cachat et al., 2010, Barbosa et al., 2012, Kalueff et al., 2013). Such an anxiety reaction probably occurred in the *in situ* control fish on the second day of each trip (experiments 2 and 4), related to the indirect exposure to pile driving sounds through the pile driving vessel when the first monopile was driven in the sea bed. To investigate potential restraints in movement and feeding due to pile driving sound, another series of 'lab' experiments is needed, as this type of behavioural stress responses cannot be investigated by means of *in situ* experiments. On the other hand, an increase in ventilation rate of eels (*Anguilla anguilla*) was observed at lower sound pressure levels during a playback of shipping noise (Purser et al., 2016). Consequently, anthropogenic noise can affect respiration either way dependent on the added sound is perceived by the organism.

Whole-body lactate also proved to be a good indicator of the secondary stress response in juvenile sea bass. Again, transport, handling and high ambient noise on the pile driving vessel influenced the whole-body lactate levels, as the *in situ* control values were much higher than the ‘unhandled’ lab control values. These increased whole-body lactate values were most probable related to sudden accelerations directly after the transfer of the fish into the vials and swimming activity in the vials (Buscaino et al., 2010). On the other hand, the significantly lower whole-body lactate levels in the *in situ* exposure treatment reflected a quick transition to freezing after juvenile sea bass were exposed to impulsive sounds, as was already suggested by the metabolic depression related to the lower oxygen consumption rates of the exposed fish. The whole-body lactate data strengthen the hypothesis that pile driving truly is perceived as an acoustic stressor in juvenile fish.

4.4 Tertiary stress responses

Except for the differences between the two sea bass age groups, no differences in specific growth rates or Fulton’s condition factor K could be found over a 30-day period between lab control, *in situ* control and *in situ* exposure treatments in the four experiments. At least under optimal laboratory conditions, the tertiary stress response appeared to be absent, meaning that juvenile fish, *in casu* European sea bass, are able to re-establish homeostasis without any implications on growth or condition when exposed to an acoustic stressor.

This might not be the case in the wild, as a reduced oxygen consumption rate and associated reduced mobility likely delays the foraging start. In addition, stressed fish often have a reduced appetite and postpone feeding (Barton et al., 1987, Bernier and Peter, 2001, Bernier, 2006, Leal et al., 2011), leading to a lower energy level. Neo et al. (2014) showed that exposure to impulsive sound prolonged the behavioural recovery time compared to a continuous sound exposure. Overall, the general condition in wild fish will decrease if reduced oxygen consumption rates correspond to reduced general alertness and reduced anti-predator response (Brown et al., 2005).

4.5 Sound metrics

In this *in situ* study sound pressure was measured as one of the main sound metrics. Due to technical limitations, no data could be collected on particle motion, the second main sound component. Since particle motion is the main driver in hearing (Popper and Fay, 2011), it can be assumed to play an important role in the stress response. However, up until now data on particle motion are almost non-existent in literature. Therefore, it cannot be distinguished whether sound pressure, particle motion or a combination is the dominant stressor for fish.
Wysocki et al. (2006) also noted the importance of amplitude and frequency fluctuations with regard to stress responses in fish. Furthermore, the strength of the stress response depends on duration, intensity, amplitude predictability, and temporal and spectral frequency of the exposure sound (Kight and Swaddle, 2011). The acute stress response may be strong close to the sound exposure and is expected to decrease as exposure distance increases or when the impulsive sound fades out. For example, Neo et al. (2014) noted that sea bass exposed to impulsive sounds ($L_{z\text{-}p}$ 165 dB re 1μPa) showed startle responses, bottom diving, increased swimming speed and group cohesion. Buscaino et al. (2010) showed increased lactate and haematocrit levels in sea bream (*Sparus aurata* L.) and sea bass when exposed to a sound pressure of 150 dB rms re 1 μPa in a 0.1 – 10 kHz linear sweep. Accordingly, both fish species showed higher activity levels at these lower sound pressure levels. Future studies are needed to disentangle the importance of the various sound characteristics on the stress response of fish.

4.6 Implications for pile driving activities

Although the *in situ* experiments provided evidence that pile driving may be perceived as an acoustic stressor by juvenile sea bass, it remains unclear as to what extent the stress responses invoke a negative effect on the condition of the fish in the wild. The temporarily reduced mobility during sound exposure may render the fish more vulnerable to predation. Moreover, chronic or repeated exposure to a stressor can turn a mild stress response into detrimental consequences, like decreased immune functioning and problems with reproduction or feeding (Kight and Swaddle, 2011, Nedelec et al., 2014). The artificial hard substrate of the already installed OWF foundations attracts juvenile fish, where they benefit from food and shelter availabilities (Reubens et al., 2011). Perversely, the OWFs may become an ecological trap, as pile driving in the vicinity of existing OWFs will affect a high number of fish that might not be able to actively escape due to their anxiety reaction to impulsive sounds generated during pile driving. Within the current MSFD descriptor, mitigation measurements mainly focus on reducing sound exposure and sound peak levels (SEL_{ss} and $L_{z\text{-}p}$) with regard to sea mammals (Betke, 2014). The attention for fish is country dependent (Faijer, 2014), and other animals are even completely ignored. Also, the potential impact of other sound metrics (especially particle motion) is not taken into account yet. A major concern is that the results for the studied species (juvenile sea bass) cannot easily be transposed to other fish species or age classes, mainly due to differences in physiological status and species specific differences in response to certain stressors (Barton, 2002). Therefore, additional field studies with other fish species (both physoclistous and physostomous fish) and other age classes are required.

5 Conclusions

Although the *in situ* experiments on the pile driving vessel were logistically demanding, they provide important insights into the acoustic stress response in juvenile fish. Strong and acute secondary stress responses were revealed when young sea bass *Dicentrarchus labrax* were exposed to impulsive sounds as close as 45 m from a pile driving activity. Especially oxygen consumption rate, and to a lesser extent whole-body lactate proved to be robust parameters. The stress reaction seems to be anxiety related, causing a temporary decrease in metabolic rate during sound exposure. Under optimal lab conditions, the acute stress responses did not reveal negative consequences on growth or condition of the juvenile fish in the long-term, although questions remain on the impact of this short term reduced condition in the wild. Whether repeated exposure to impulsive sound, related to the successive construction of offshore wind farms, or other sound metrics, *in casu* particle motion, will amplify the stressor effect, remains to be studied.

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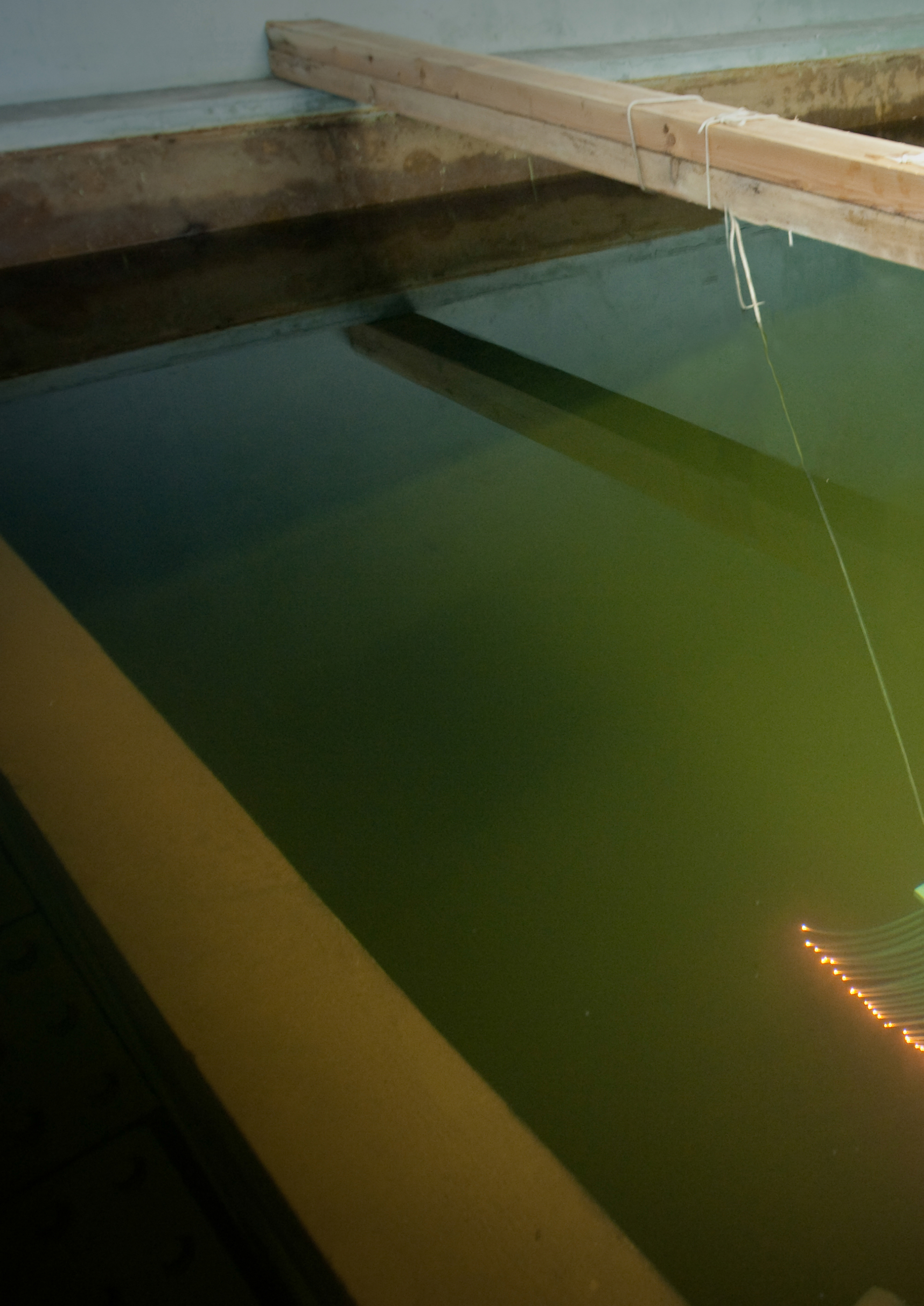

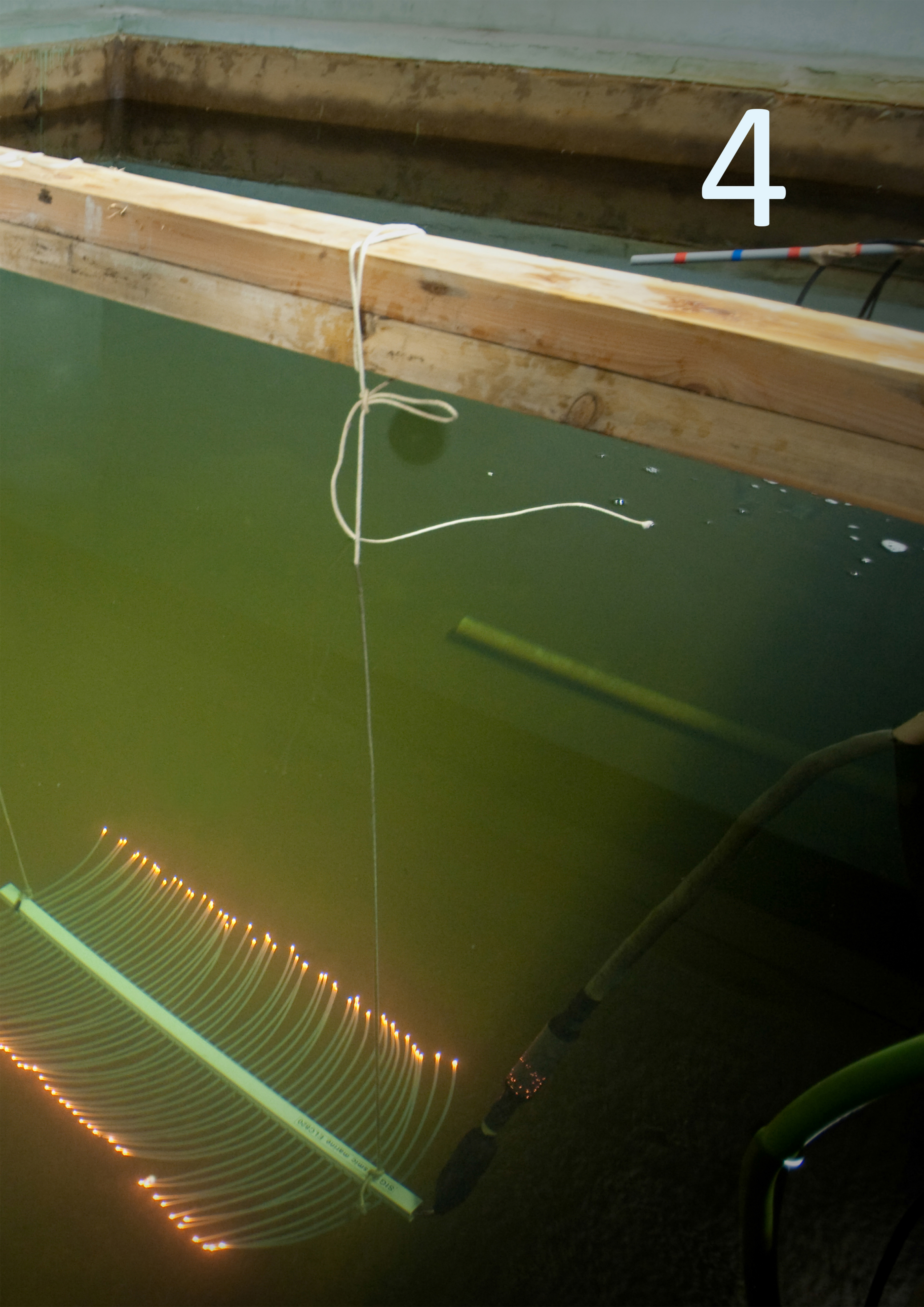

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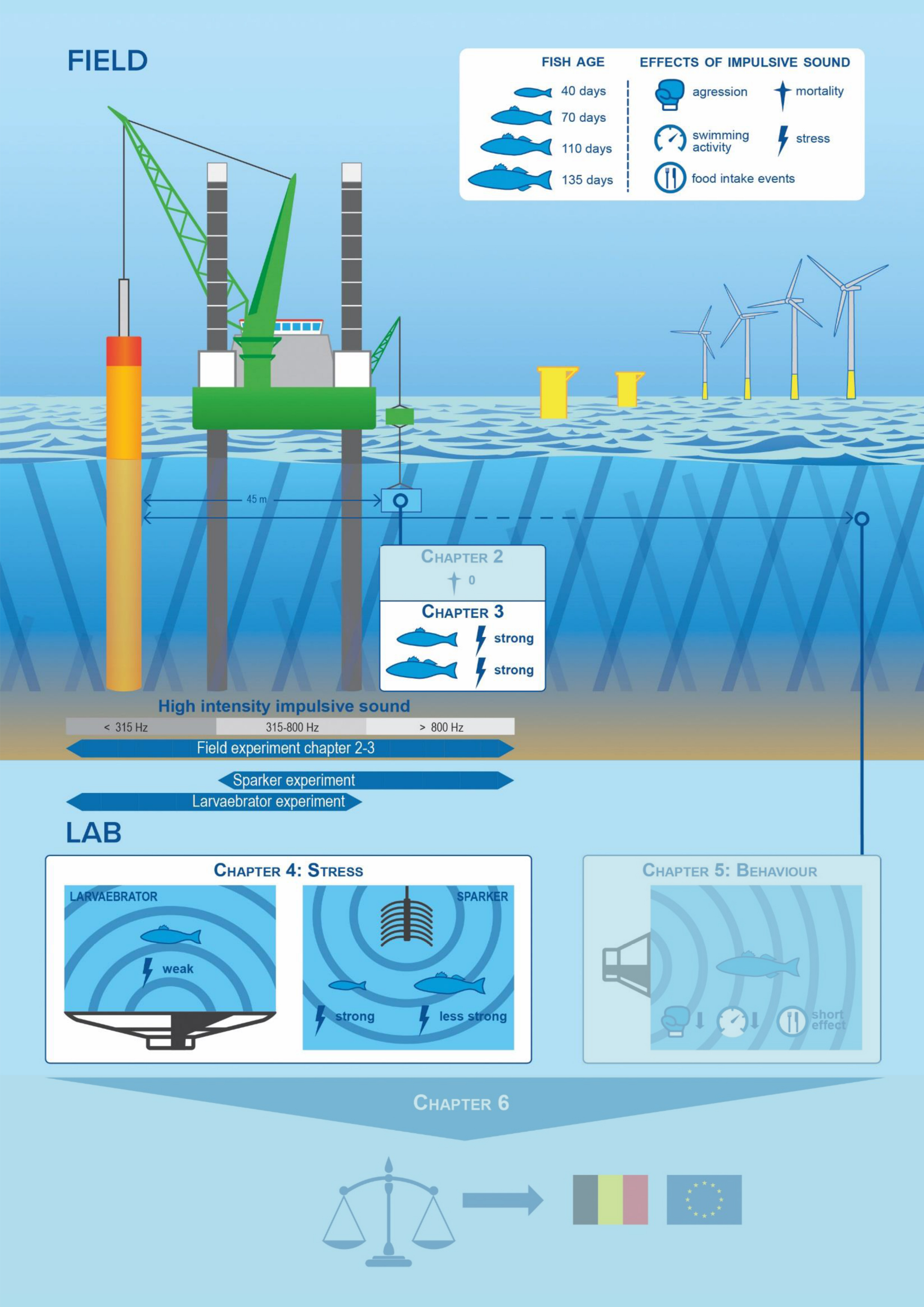
FIELD
FISH AGE
40 days
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EFFECTS OF IMPULSIVE SOUND
agression
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CHAPTER 2
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CHAPTER 3
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strong
High intensity impulsive sound
< 315 Hz
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Field experiment chapter 2-3
Sparker experiment
Larvaebrator experiment
LAB
CHAPTER 4: STRESS
LARVAEBRATOR
weak
SPARKER
strong
less strong
CHAPTER 5: BEHAVIOUR
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CHAPTER 6

CHAPTER 4

HIGH INTENSITY IMPULSIVE SOUND EVOKING STRESS RESPONSES IN JUVENILE FISH: LAB VERSUS *IN SITU* FIELD EXPERIMENTS

Adapted from:

Debusschere E, Sinha AK, De Coensel B, Blom E, Bolle L, Botteldooren D, De Boeck G, Degraer S, De Jong C, Vandendriessche S, van Rooij D, Vincx M, Wessels P, Winter HV, Hostens K, Submitted. High intensity impulsive sound evoking stress responses in juvenile fish: lab versus in situ field experiments. Journal of experimental marine biology and ecology.

Abstract

Pile driving generates high intensity sound in the marine environment. This may negatively affect fish health and behaviour. Based on lab experiments, high intensity sound thresholds related to barotrauma injuries in fish have already been identified. For physiological stress responses, such thresholds do not exist. In the present study, the stress responses of larval and juvenile European sea bass (*Dicentrarchus labrax*) to high intensity impulsive sound were evaluated through lab experiments using a SIG sparker and larvaebrator, and compared to stress responses recorded in a recently conducted field study close to a real pile driving event. Both lab sound sources produced similar levels for the standard sound pressure metrics as the *in situ* pile driving, being zero-to-peak sound pressure level ($L_{z\text{-}p}$, 208 dB re 1 µPa), single strike exposure level (SEL_{ss}, 181 dB re 1 µPa²·s) and cumulative sound exposure level (SEL_{cum}, 214 dB re 1 µPa²·s). However, the three sources differed in their sound frequency spectra.

The whole-body cortisol results (a proxy for primary stress responses) confirmed the susceptibility of both larval and juvenile fish to handling stress. Still, the increased (or altered) whole-body cortisol levels indicate that high intensity impulsive sound evoked an acoustic primary stress response. Common ground between both lab and field experiment was found at the high energy levels (SEL_{ss}) produced between the 315 and 630 Hz 1/3 octave bands. This range falls within the frequency range to which European sea bass react to sound. Therefore, the primary stress response in juvenile fish can be related to hearing. Reduced oxygen consumption rates (a proxy for secondary stress) in larvae and to a minor extent in juveniles could be related to high intensity sound produced at higher frequencies (>800 Hz). Still, high intensity impulsive sound covering a broad frequency range (like a real *in situ* pile driving) is needed to evoke strong secondary stress responses (*e.g*. reduced oxygen consumption rate and whole-body lactate levels) in juvenile sea bass. This implies that lab results can not directly be translated to the real world, since known or unknown parameters (such as frequency content) can differ. More studies on different life stages and on the role of non-standard sound parameters such as particle motion are needed to further clarify the triggering parameters and sound thresholds of the stress response of fish.

Keywords

Impulsive sound, lab experiments, fish, stress responses, frequency content, hearing, and swim bladder movement

1. Introduction

Renewable energy production by offshore wind farms is popular in Europe (Ho, 2015). Wind farm construction usually involves pile driving, which generates high intensity impulsive sound, potentially affecting marine fauna, like marine mammals, fish and invertebrates (Popper et al., 2014). Studies on the impact of pile driving on marine fish are mainly based on laboratory experiments, as field experiments are impeded by logistical challenges, such as safety perimeters, unpredictability of the pile driving activity, and problems related to fish transport and housing (e.g. Halvorsen et al., 2011, Bolle et al., 2012, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a, Casper et al., 2013b). Laboratory studies have the advantage that the impact variables, in this case sound pressure, can be manipulated, the environment can be controlled, and the experiments can be repeated, which is not that straightforward in the field (Calisi and Bentley, 2009). However, the lack of field observations is a handicap when interpreting lab experiments or to validate and extrapolate lab results to the real world (Levitt and List, 2007). For example, based on lab simulations a combination of three sound metrics, single strike sound exposure level (SEL_{ss}), cumulative sound exposure level (SEL_{cum}) and total number of strikes, has been proposed to predict barotrauma related to high level impulsive sound (Halvorsen et al., 2012b, Casper et al., 2013b). However, such ‘threshold’ values have not yet been validated in a real *in situ* field situation.

The novelty of the present study is the comparison of two laboratory studies with the results of an *in situ* study. This allowed for the evaluation of the combined zero-to-peak sound pressure level (L_{z-p}), SEL_{ss} and SEL_{cum}, next to other variables like frequency, to explain dose-response relationships between stress in juvenile fish and high intensity impulsive sound pressure. The *in situ* study has been performed by Debusschere et al. (2014, 2016), exposing young European sea bass (*Dicentrarchus labrax*) of 68 and 115 days old to pile driving generated impulsive sound as close as 45 m from a real-time pile driving event. Mortality and the primary, secondary and tertiary stress responses were investigated[1]. The results showed no acute or delayed mortality (Debusschere et al., 2014), but the fish showed strong secondary stress responses, manifested by a 50% decrease in oxygen consumption rate and lower whole-body lactate levels compared to the control group (Debusschere et al., 2014, Debusschere et al., 2016). Both stress responses suggest an anxiety-based reduced activity (Cachat et al., 2010, Teulier et al., 2013), and this acute response to high intensity impulsive sound did not result in a tertiary stress response since the condition of the fish was unaffected 30 days after the pile driving exposure under optimal lab conditions.

In the present study, the *in situ* results for juvenile sea bass were compared to the stress responses in two lab experiments, using other sources of high intensity impulsive sounds than pile driving, but with similar standard sound metrics (L_{z-p}, SEL_{ss}, SEL_{cum}). One lab experiment was also performed with fish larvae to compare stress responses between different life stages (larvae and juveniles). Primary stress response was assessed through differences in whole-body cortisol levels and secondary stress responses through differences in oxygen consumption rate and whole-body lactate levels.

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[1] More information on the stress responses can be found in Chapter 1 section 1.2.1

2. Material and methodology

Two different sound sources were used for the lab experiments. The first lab experiment was based on sound produced by a SIG sparker seismic device[g], the second lab experiment on sound produced in an acoustically controlled chamber, the so-called 'larvaebrator' (Bolle et al., 2012). Both sound sources had comparable values for the standard sound metrics ($L_{z\text{-}p}$, SEL_{ss}, and SEL_{cum}) that are commonly used to describe impulsive sound pressure. Total number of strikes, time between the consecutive strikes, and SEL_{ss} over the 1/3 octave bands (frequency) were also reported for both sources.

Acute stress responses to impulsive sound were investigated in both experiments for juvenile European sea bass, and also for larvae in the sparker experiment. European sea bass (*Dicentrarchus labrax*) is a physoclistous round fish, with individuals behaviourally reacting to sound signals between 0.1 and 0.7 kHz (Kastelein et al., 2008). Sea bass juveniles and larvae were provided by the Ecloserie Marine de Gravelines in France. The experiments were carried out in accordance with the Belgian Council for Laboratory Animal Science (BCLAS) guidelines, and were approved by the ethical committee of the Institute for Agricultural and Fisheries Research (ILVO) under permit numbers 2015/251 (sparker) and 2014/216 (larvaebrator).

2.1 Impulsive sound generation

2.1.1 The sparker

A SIG sparker (type ELC820) is designed for seismic research. Its multi-tip sound source produces pulses of 300 up to 500 joules, shooting at one pulse per second. A circle of air stones was installed around the fish vials (see further) to reduce the sound exposure of the 300 joules (-8 dB for $L_{z\text{-}p}$; -7dB for SEL_{ss}, instead of 2dB). The sparker was deployed in a 10 000 L seawater reservoir at 2 m under the water surface. The experimental vials (500 mL, poly-4-methyl, 1-pentene) were submerged at 1.6 m below the water surface and at 1.2 m distance from the sparker. A hydrophone was hanging freely at 0.1 m above the vials, inside the air circle for the 'minimum energy' treatment.

Sound pressure was measured using a Bruël & Kjaer hydrophone (type 8104, voltage sensitivity 47.7 $\mu V \cdot Pa^{-1}$, charge sensitivity 0.391 $pC \cdot Pa^{-1}$, 10 m cable), connected to the charge channel of the B&K portable amplifier (Nexus type 2690-0S). The signal was recorded by a portable recorder (Tascam DR-680) on a Compact Flash card (SanDiskUltra) in a 1-channel WAVE format (.wav) with a sampling rate of 44 100 Hz at 24 bit. The sound pressure metrics were calculated using custom made Matlab scripts (R2012b, version 8.0).

2.1.2 The larvaebrator

The larvaebrator was specifically designed for controlled exposure of small fish to impulsive sound under laboratory conditions (Bolle et al., 2012). It consists of an underwater sound projector (LFPX-4) and a rigid-wall (28 mm thick steel) test chamber (160 mm high, Ø 110 mm). The dimensions of the test chamber (1.25 L) ensure a homogeneously distributed sound pressure field. The larvaebrator produced a single pulse per second. The playback sound is comparable to the sound measured at 100 m from a windmill pile driving event in the North Sea (Ainslie et al., 2009). Sound pressure was measured by four pressure transducers (PCB 116A02, nominal sensitivity 8 $\text{pC}\cdot\text{psi}^{-1}$). The signals were recorded (at sample frequency 65 536 Hz) on a multichannel analyser (B&K PULSE-3160-A-042 and B&K PULSE-3050-A-060) and a laptop computer with B&K PULSE software (LABSHOP, version 17.1).

2.2 Experimental design

2.2.1 The sparker experiment

The experiment was performed with sea bass juveniles (109-111 dph; wet weight WW 1.21 ± SD 0.15 g; standard length SL 43.7 ± SD 1.6 mm) and larvae (37-39 dph; WW 0.008 ± SD 0.003 g; SL 12.60 ± SD 0.98 mm). The fish were acclimated for six days to 15.6 ± 0.4 °C (at salinity 37 and pH 8.1) in nine aquaria of 30 L (six with juveniles, three with larvae). Every hour 50% of the seawater was replaced. The juveniles were fed with MariCo Start 1.5 mm (Coppens); the larvae were fed twice a day with Aglonorse 200-300 µm.

Fish were randomly assigned to one of four treatments: (1) lab control, *i.e.* subjected to background sound in the lab, with no handling or transport effects (‘unhandled’ fish); (2) reservoir control, *i.e.* undergoing handling (transfer into 500 mL vials) and transport, next to submersion in the 10 000 L reservoir, but only subjected to ambient background sound in the reservoir; (3) minimum exposure, *i.e*. similar to treatment 2, but exposed to the sparker shooting at 300 joules with the air circle active; (4) maximum exposure, *i.e.* exposed to the sparker shooting at 500 joules.

Each treatment was replicated three times. For each replicate of treatment 1, four juveniles and 60 larvae were taken from the aquaria, and directly transferred into liquid nitrogen. For treatments 2 to 4, the larvae and juveniles were transferred with a net (mesh size 500 µm) from the 30 L aquaria to 500 mL vials. No anaesthetics were used as this required recovery time and could influence fish behaviour and physiology (Mylonas et al., 2005). For each replicate of treatments 2 to 4, six vials were filled with two juveniles each, three vials with 30 larvae each and three vials with seawater only to measure bacterial respiration. Treatment 2 was exposed to the ambient background sound in the seawater reservoir for 45 minutes. Treatments 3 and 4 were exposed for 45 minutes to the sparker sound, resembling the total number of strikes received during a complete pile driving session of a monopile in the field (Table 2) (Debusschere et al., 2014). At the end of each treatment, four juveniles and 60 larvae were stored in liquid nitrogen. The remaining fish were used for other analyses not reported in this study.

2.2.2 The larvaebrator experiment

Sea bass juveniles (57 dph; WW 0.105 ± SD 0.083 g; SL 20.55 ± SD 2.47 mm) were acclimated for six days to 18 ± 0.16 °C (at salinity 26 and pH 7.97) in 15 aquaria of 32 L. Every hour, 80% of the aquarium volume was replaced. The fish were fed three times a day with Aglonorse 300 – 500 µm. Due to logistic problems, no larvaebrator experiments could be conducted with fish larvae. The experiment consisted of three treatments: (1) lab control, *i.e.* fish subjected to background sound in the lab, but no transport or other handling effect ('unhandled' fish); (2) test chamber control, *i.e.* fish transferred into the test chamber of the larvaebrator and subjected to ambient background sound within the test chamber; (3) impulsive exposure, *i.e.* fish exposed to 999 strikes inside the test chamber of the larvaebrator. The impulsive sound exposure was limited to 999 pulses in 17 minutes to guarantee a constant temperature in the test chamber.

Each treatment was repeated five times, with 30 juveniles per replicate in treatment 1 ('lab control') and 50 juveniles per replicate for treatments 2 and 3 (more juveniles required for oxygen consumption measurements). Juvenile sea bass were first transferred with a net (mesh size 100 µm) from the 32 L aquaria into a 500 mL glass beaker (no anaesthetics used), and then transferred into liquid nitrogen (treatment 1) or the larvaebrator (treatment 2 and 3). Bacterial respiration was measured after 17 minutes in the test chamber (filled with seawater alone) at three separate moments. At the end of each treatment, 15 juvenile fish were transferred into liquid nitrogen. The remaining fish were used for other analyses not reported in this study.

2.3 Physiological and biochemical analyses

2.3.1 Oxygen consumption rate

Stress responses in fish were assessed by measuring the oxygen consumption rate during exposure to either background ambient sound or impulsive playback sound. For the lab control treatments (unhandled fish) of both experiments, no oxygen consumption measurements were performed, as this would have evoked handling stress.

For each replicate of the other treatments, all vials in the sparker experiment (treatments 2 - 4) and the test chamber in the larvaebrator experiment (treatments 2 - 3) were completely filled with seawater and air bubbles removed. At the beginning and end of each replicate, oxygen concentration ($\mu mol \cdot L^{-1}$), temperature (°C) and salinity were measured, respectively using a Unisense oxygen sensor of 100 µm connected to a pico-ammeter, an IKS temperature sensor and a refractometer Atago type S/Mill-e. Calibration of the oxygen sensor data was done using 100% oxygen saturated seawater and a 0% oxygen solution (sodium ascorbate and sodium hydroxide dissolved in freshwater) at the same temperature as the seawater. Bacterial respiration was accounted for by calculating the difference in oxygen concentration at the beginning and end in the vials, resp. the test chamber, that only contained seawater, and subtracting this averaged value from the start-stop differences in oxygen concentration measured in the vials and test chamber containing the fish. Oxygen consumption ($\mu mol \cdot L^{-1}$) was subsequently corrected for total wet weight of the fish (g WW) and water volume (L) in the vials, respectively test chamber, and standardized to oxygen consumption per hour ($\mu mol \cdot g^{-1} \cdot h^{-1}$).

2.3.2 Whole-body cortisol and lactate levels

For whole-body cortisol analyses at least 0.3 g of fish biomass (WW) was needed, whereas whole-body lactate samples needed at least 0.25 g WW. Therefore, 30 larvae of 38 dph (sparker), respectively three juveniles of 63 dph (larvaebrator) needed to be pooled per replicate for the whole-body cortisol analyses. The biomass of the 110 dph juveniles in the sparker experiment was sufficient to measure whole-body cortisol and lactate levels per individual fish for each replicate. As all available larvae from the sparker experiment were completely used for the cortisol analyses, no whole-body lactate analyses could be performed on the larvae in this experiment. For the larvaebrator replicates, the juveniles needed to be pooled per two to measure whole-body lactate (Table 1). The number of samples per replicate varied in the larvaebrator due to practical problems during analysis.

Whole-body cortisol was extracted as described in Debusschere et al. (2016). Each sample of 0.3 g WW of fish tissue was pulverized in liquid nitrogen and suspended into 1.5 mL phosphate buffer (pH 7.2) in a glass test tube of 15 mL on ice. The whole-body cortisol was extracted twice with diethyl ether. Whole body cortisol concentrations were determined with the ImmuChemTM Coated Tube Cortisol 125I RIA kit (MP Biomedicals, LLC) and measured with the Perkin Elmer Wallac 1480 Wizard 3" Gamma Counter (Zaventem, Belgium). The values of all cortisol samples were corrected for extraction efficiency, dilution and fish weight. Extraction efficiency was measured by comparing 13 duplicate samples where 7.5 ng cortisol mL^{-1} (MPBio Diagnostics) was added before or after diethyl ether extraction (Yeh et al., 2013). An overall average of the extraction efficiency of 73 ± SE 9% of whole-body cortisol was calculated.

Whole-body lactate analyses were performed on 0.25 g of pulverised fish powder suspended into 0.5 ml 8% perchloric acid at 4 °C and centrifuged. The supernatant was neutralized in 1N $KHCO_3$ and vortexed. Whole-body lactate levels were measured by colorimetric assays, using the commercial enzymatic lactate assay kit (R-Biopharm A.G., Darmstadt, Germany).

Handling stress was quantified by comparing whole-body cortisol and whole-body lactate levels in fish in the 'unhandled' lab control replicates (treatment 1) *vs.* the fish from treatment 2 (respectively reservoir control in sparker and test chamber control in larvaebrator experiment).

2.4 Comparison with in situ results

The *in situ* study that was published in Debusschere et al. (2016) exposed juvenile sea bass to impulsive sound related to pile driving as close as 45 m to a real pile driving event. This study consisted of two trips with each two pile driving sessions, carried out on two consecutive days (= 4 replicates). The fish were 68 and 115 dph, respectively on trip 1 and 2, which were comparable to the fish used in the larvaebrator (63 dph) and the sparker experiment (110 dph). The study comprised three treatments: (1) lab control, *i.e.* subjected to background sound in the lab, but not to transport to the pile driving vessel or other handling effects (‘unhandled’ fish); (2) *in situ* control, *i.e.* submerged from a pile driving platform at 2.5 m below sea surface and subjected to ambient background sound alone; and (3) *in situ* exposure, *i.e.* submerged from the pile driving platform and exposed to impulsive sound during a complete pile driving session (~1.5 h) at 45 m from the pile driving activity. Oxygen consumption rate and whole-body cortisol and lactate levels were determined conform the above described methods (detailed information can be found in Debusschere et al., 2016). Details on the sound metrics (L_{z-p}, SEL_{ss}, SEL_{cum} and SEL_{ss} over the 1/3 octave bands) measured during a complete pile driving session can be found in Debusschere et al. (2014). For this comparison, only the results for the first day of each trip (replicates 1 and 3) were used to eliminate the effect of fish not being ‘sound naïve’, which was the case on the second day of this *in situ* study (Debusschere et al., 2016).

2.5 Statistical analyses

An overview of the number of samples and replicates per analysis is given in table 1. Linear mixed-effects models were applied for the variables whole-body cortisol, oxygen consumption rate and whole-body lactate levels, with ‘treatment’ as fixed effect and ‘replicate’ as random effect. Oxygen consumption rate of the juveniles in the sparker experiment and whole-body cortisol data in both lab experiments were log-transformed to meet parametric assumptions. Based on a graphical evaluation (histogram and Q-Q plot), the data were considered normally distributed and ANOVA type III F-test analyses were performed. Significant differences ($p < 0.05$) were further tested through *post hoc* pairwise comparisons with least-square means, with p-values (p_{adj}) corrected by Tukey-Kramer adjustments for multiple comparisons. All analyses were performed in R-studio for Windows. For the *in situ* study similar statistical analyses have been performed (Debusschere et al., (2016).

Table 1. Overview of the number of samples (n) per replicate used in the statistical analyses for the sparker and larvaebrator experiments.

Experiment	**Sparker experiment[e]**				**Larvaebrator experiment[e]**		
Treatment	Lab control	Reservoir control	Minimum exposure	Maximum exposure	Lab control	Test chamber control	Impulsive exposure
Treatment number	1	2	3	4	1	2	3
	3 replicates per treatment				5 replicates per treatment		
Bacterial respiration[a]	-	n = 3	n = 3	n = 3	-	n = 3	
Oxygen consumption rate[b]	-	n = 6 | 3	n = 6 | 3	n = 6 | 3	-	n = 1	n = 1
Extraction efficiency	n = 13 (taken from different treatments/experiments/replicates)						
Whole-body cortisol[c]	n = 3 | 2	n = 3 | 2	n = 3 | 2	n = 3 | 2	n = 4,2,3,1,2	n = 3,3,3,2,3	n = 3,3,3,4,4
Whole-body lactate[d]	n = 3 | -	n = 3 | -	n = 3 | -	n = 3 | -	n = 2,2,3,3,2	n = 3,3,3,2,3	n = 3,3,3,4,4

[a] Sparker experiment: bacterial respiration measured in three vials filled with seawater alone (for each replicate and treatment). Larvaebrator experiment: bacterial respiration measured three times at random in a test chamber filled with seawater only

[b] Sparker experiment: oxygen measured in six vials with juveniles and three vials with larvae at beginning and end for each replicate per treatment. Larvaebrator experiment: oxygen measured once at beginning and end in the test chamber with juveniles for each replicate per treatment.

[c] Sparker experiment: number of samples for juveniles and larvae separated by a bar; one juvenile used per sample and 30 larvae pooled per sample per treatment and replicate. Larvaebrator experiment: three juveniles used per sample per treatment; different number of samples per replicate due to analysis problems

[d] Sparker experiment: the same juvenile individuals are used for whole-body lactate as for whole-body cortisol; not enough larval biomass for whole-body lactate analyses. Larvaebrator experiment: two juveniles used per sample per treatment; different number of samples per replicate

[e] The remaining fish of each replicate per treatment were used for analyses not discussed in this manuscript, and either kept alive or euthanized and stored in 7% formaldehyde or liquid nitrogen

3. Results

3.1 Sound pressure metrics

As was expected, the L_{z-p}, SEL_{ss} and SEL_{cum} generated by the sparker at 500 joules ('maximum exposure') and the larvaebrator were similar to the *in situ* metrics (Table 2). The number of strikes and exposure period were kept lower in the larvaebrator experiment to ensure a constant temperature in the test chamber. In both laboratory experiments, the time between the pulses was a little shorter than in the field. The sharpness of the sparker signal was higher compared to the signal produced by the larvaebrator or *in situ* pile driving, which were more similar (Figure 1).

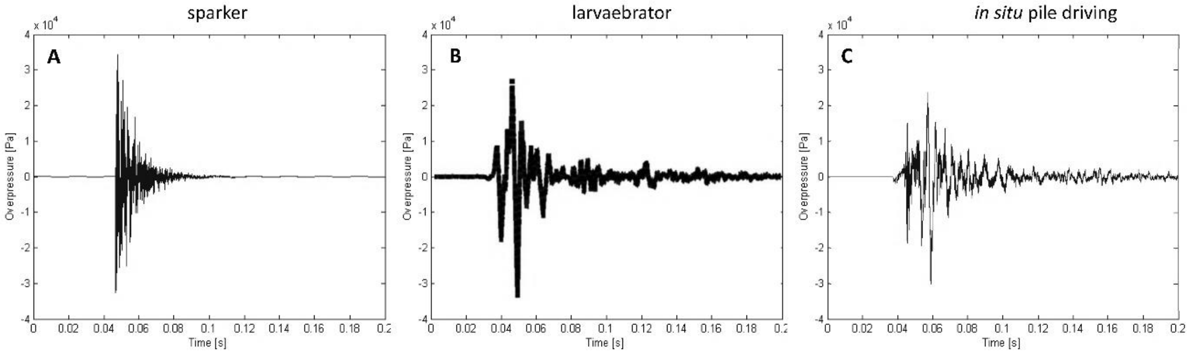

Figure 1. Signal shape produced by the (A) SIG sparker (at 500 joule; SEL_{ss} = 181.5 dB re 1 µPa²s); (B) Larvaebrator (SEL_{ss} = 185 dB re 1 µPa²s; figure taken from Bolle et al. (2012)) and (C) *in situ* pile driving (monopile C8, $SEL_{s}s$ = 183.5 dB re 1µPa²s).

The dominant energy during *in situ* exposure (SEL_{ss}) was present at 125-200 Hz, although no steep decline was recorded towards the higher frequencies (Debusschere et al., 2014). Compared to the *in situ* study, the sparker at maximum energy (500 joules, treatment 4) only reached similar high SEL_{ss} levels from 315 Hz onwards, while above 1600 Hz the SEL_{ss} rose permanently above the *in situ* levels (Figure 2). At minimum energy (300 joules with the air circle active, treatment 3), the sparker SEL_{ss} were consistently lower than the *in situ* levels throughout the 1/3 octave band. In treatment 3, the larvaebrator produced similar SEL_{ss} levels as in the *in situ* experiment from 63 Hz onwards, reaching a higher peak at 200-250 Hz. However, at frequencies >800 Hz the sound energy in the larvaebrator rapidly decreased far below the *in situ* levels. The sparker (500 joules, treatment 4) and larvaebrator (treatment 3) overlapped in the 315 to 630 Hz 1/3 octave band.

Table 2. Standard sound metrics (mean ± SD or range) measured at 1.2 m from the sparker in the 10 000 L seawater reservoir and measured within the test chamber of the larvaebrator. For comparison, the measurements at 45 m from a real *in situ* pile driving event (Debusschere et al., (2014) are given as well. The ambient background sound pressure level (Leq) is given for the reservoir in the sparker experiment, the lab control and the ambient SPL in the North Sea of the *in situ* experiment.

			SIG sparker		Larvaebrator		*In situ* study	
	Lab control	Reservoir control	Minimum exposure	Maximum exposure	Impulsive exposure	lab control	*In situ* control	*In situ* exposure
SELss (dB re 1 µPa²·s)			170 ± 4	181 ± 0,5	186 ± 1			184 ± 3
SPLz-p (dB re 1 µPa)			203 ± 4	211 ± 0,3	210 ± 1			210.5
SELcum (dB re 1 µPa²·s)			201-209	214-215	215-216			215-218
Peak 1/3 octave band (Hz)			50	50 and 315	125 and 200			125-200
Total number of strikes			2790-2834	2702-2734	999			2282-3249
Time between strikes (sec)			1	1	1			1.3
Total exposure period (min)			46-48	45	17			57-77
Leq (dB re 1 µPa)	130 ± 2.3	160 ± 0.2				129 ± 2.8	136 ± 12	

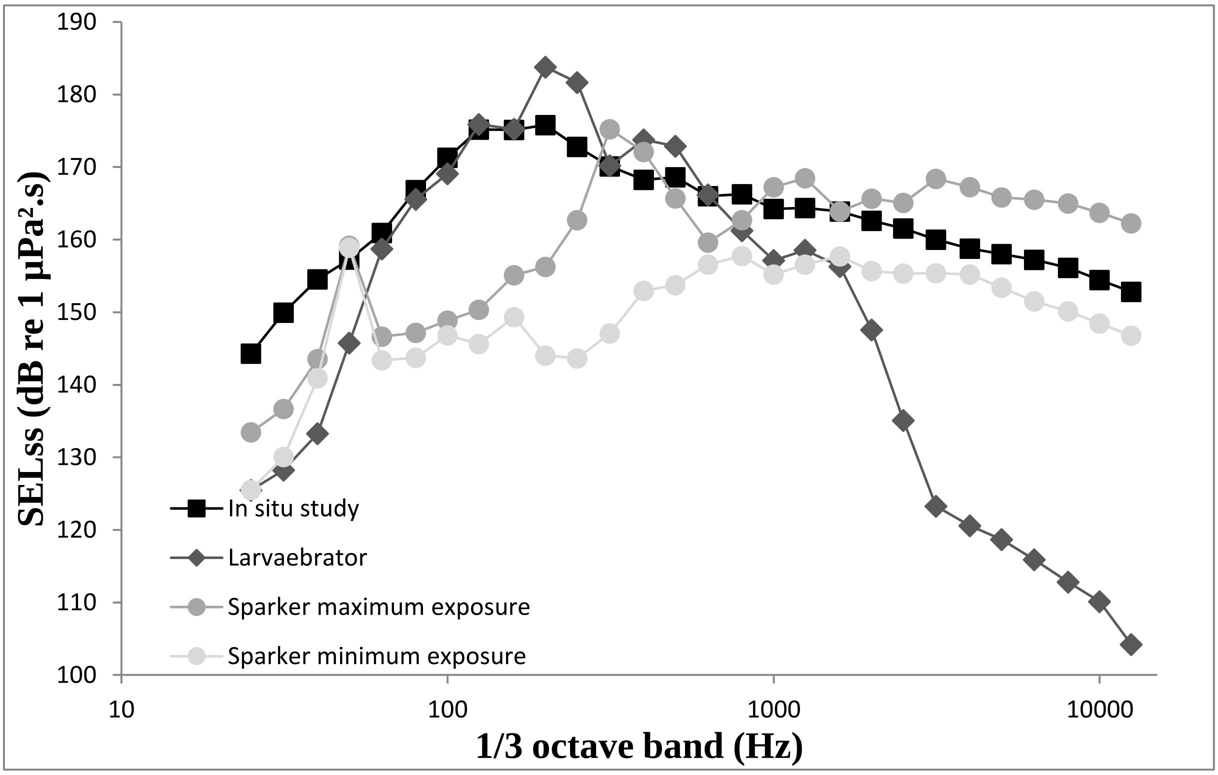

Figure 2. The measured frequency spectra of the sparker and larvaebrator experiments compared to the *in situ* experiment, showing the mean single strike sound exposure levels (SEL_{ss}) in the 1/3 octave bands (SD not shown for reasons of comparison).

3.2 Whole-body cortisol

There was a clear handling effect in all experiments. Both juveniles (110 dph) and larvae (38 dph) in the sparker experiment had elevated whole-body cortisol levels in treatments 2 to 4 (*i.e.* transferred into the 500 ml vials and lowered in the seawater reservoir) compared to the lab control ('unhandled') treatment (juveniles: $F_{3,8} = 21.12$, $p = 0.0004$, $p_{adj} <0.001$ in all *post hoc* comparisons; larvae: $F_{3,8} = 12.79$, $p = 0.002$; $p_{adj} <0.001$) (Figure 3A). Also the 63 dph juveniles in treatments 2 and 3 (*i.e.* fish in test chamber) of the larvaebrator experiment had significantly higher whole-body cortisol levels compared to the lab control fish ('unhandled') treatment ($F_{2,26} = 36.33$, $p <0.0001$, $p_{adj} <0.001$ in both *post hoc* comparisons) (Figure 3B). The same holds true for the comparison between the *in situ* control and exposure treatments vs. the lab control treatment in both age groups of the *in situ* experiment ($F_{2,34} = 8.02$, $p = 0.0014$, $p_{adj} <0.01$ for the *post hoc* comparisons) (Figure 3C).

Next to handling stress, the larvae (38 dph; sparker exp.) and juvenile age classes (63 dph; larvaebrator exp. and 115 dph; *in situ* exp.) showed a non-significant trend towards elevated whole-body cortisol levels under high intensity impulsive sound exposure (treatments 3 and 4 in the sparker and treatment 3 in the larvaebrator experiment) compared to the respective treatments 2 (Figure 3A-C). For the 110 and 68 dph juveniles (respectively sparker and *in situ* experiment) a more or less opposite trend in whole-body cortisol levels was shown.

3.3 Oxygen consumption

The bacterial respiration was -3.07 ± SD 7.60 $\mu mol \cdot L^{-1}$ in the sparker experiment and -2.99 ± SD 7.17 $\mu mol \cdot L^{-1}$ in the larvaebrator experiment. In the sparker experiment, the oxygen consumption rate of the 110 dph juveniles was slightly lower (although not significant) during the 'maximum exposure' treatment *versus* the 'reservoir control' and 'minimum exposure' treatments (10.39 ± SD 1.54 $\mu mol \cdot g^{-1} \cdot h^{-1}$ *vs*. 11.8 ± SD 3.18 $\mu mol \cdot g^{-1} \cdot h^{-1}$ and 12.78 ± SD 2.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$) ($F_{2,6} = 3.59$, $p = 0.09$) (Figure 3D). For the sea bass larvae (38 dph) in the sparker experiment, the difference in oxygen consumption was more prominent ($F_{2,6} = 5.44$, p-value = 0.04): larvae exposed to the maximum energy of the sparker (treatment 4) respired ~45% less (15.9 ± SD 8.2 $\mu mol \cdot g^{-1} \cdot h^{-1}$) compared to the larvae in the 'reservoir control' and 'minimum exposure' treatments (29.0 ± SD 3.9 $\mu mol.g^{-1}.\ h^{-1}$, $p_{adj} = 0.01$ in both post-hoc comparisons).

In the larvaebrator experiment, the oxygen consumption rate for the 63 dph juveniles was slightly (although not significantly) higher during impulsive sound exposure (21.1 ± SD 6.3 μmol·g^{-1}·h^{-1}) compared to the 'test chamber control' treatment (18.9 ± SD 6.3 μmol·g^{-1}·h^{-1}) ($F_{1,8}$ = 0.31, p = 0.59) (Figure 3E).

Exposure to a real pile driving session at 45 m from the sound source induced a ~50% decrease in oxygen consumption rate in the two juvenile sea bass age groups (18.04 ± SD 1.29 μmol g^{-1} h^{-1} to 8.02 ± SD 4.42 μmol·g^{-1}·h^{-1} for the 68 dph fish, and 15.22 ± SD 2.22 to 7.76 ± SD 3.2 μmol·g^{-1}·h^{-1} for the 115 dph fish) (Figure 3F).

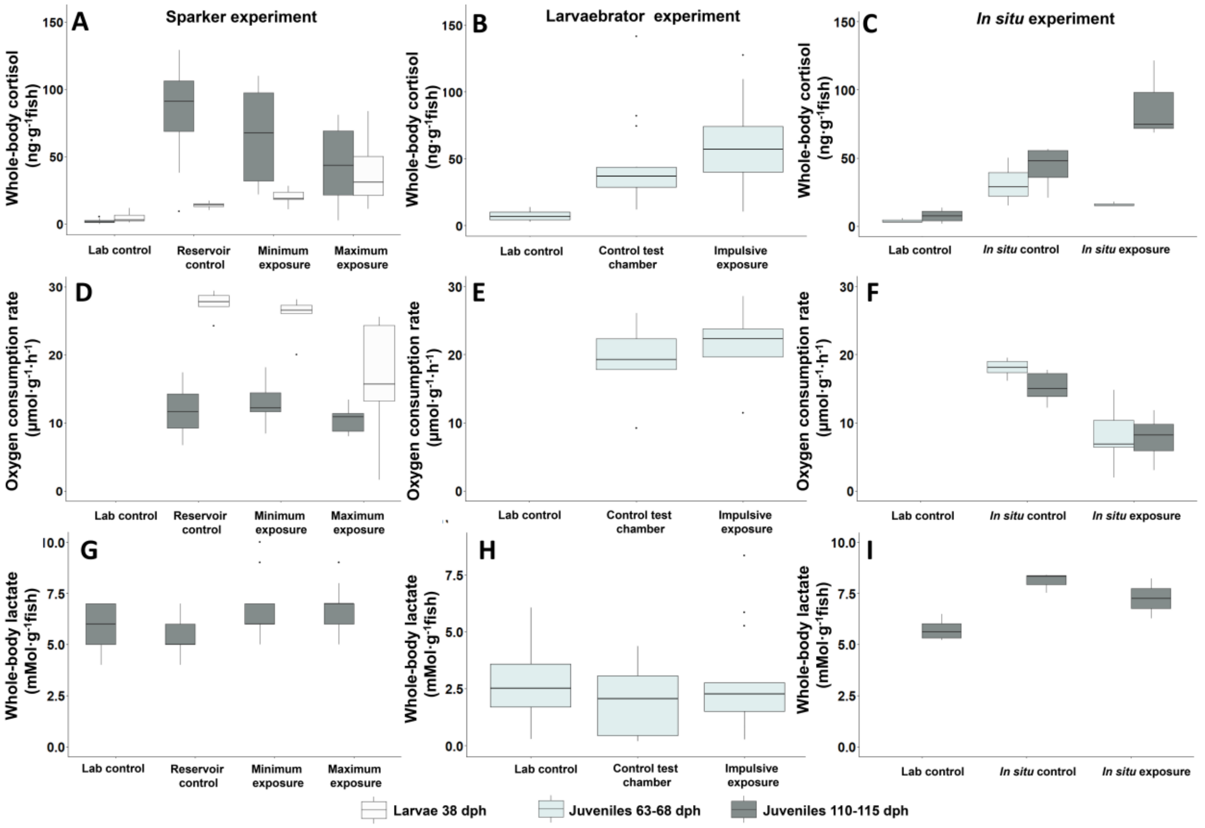

Figure 3. Biochemical and physiological stress responses of fish to high intensity impulsive sound in two lab experiments (sparker and larvaebrator experiment) and one field experiment (*in situ* experiment adapted from Debusschere et al. (2016). The experiments were performed with European sea bass larvae (38 dph, sparker experiment) and juveniles (110 dph in sparker experiment, 63 dph in larvaebrator experiment, 68 and 115 dph in *in situ* experiment). (A-C) whole-body cortisol levels, (D-F) Oxygen consumption rate, (G-I) whole-body lactate levels. The Box-and Whisker plots represent the median between the 25 and 75% percentiles of the box, outliers are plotted as individual points.

3.4 Whole-body lactate

In both lab experiments, no significant differences were observed in whole-body lactate levels between all treatments (sparker experiment $F_{3,8} = 2.0$, $p = 0.19$; larvaebrator $F_{2,9} = 0.43$, $p = 0.66$), respectively with 110 and 63 dph juveniles (Figure 3G,H). Overall, whole-body lactate levels were lower in the younger juvenile age class (larvaebrator experiment). Only for the *in situ* experiment, whole-body lactate levels for the 115 dph juveniles were lower during '*in situ* exposure' compared to the '*in situ* control' treatment (Figure 3I).

4. Discussion

4.1 Stress responses to high intensity impulsive sound

4.1.1 Primary stress response

Whole-body cortisol is a primary stress response indicator. It illustrated the important effect of handling stress in both lab experiments (sparker and larvaebrator), as was also seen in the *in situ* experiment (Debusschere et al., 2016). The handled fish in treatment 2 ('reservoir control', 'test chamber control', '*in situ* control') showed higher whole-body cortisol levels compared to the respective 'lab control' treatments (treatment 1). Additionally, the high whole-body cortisol levels of the juvenile fish during treatments 2 to 4 of the sparker experiment could also be a stress response to the high ambient sound levels generated by the water pumps in the 10 000 L water reservoir, which could not be switched off during the experiments. Other than being sensitive to handling, whole-body cortisol levels also depend on the individual fish response. This was reflected by the variability between replicates, which indicated the different potential of each individual fish to cope with stress, and might have masked the specific stress response to the sound (Overli et al., 2007, Silva et al., 2010).

Next to the above described variation, the data showed an increasing trend in whole-body cortisol levels in response to high intensity impulsive sound exposure in larvae of the sparker experiment, juveniles (63 dph) of the larvaebrator and juveniles (115 dph) of the *in situ* experiment, although this trend was not significant in all cases. In contrast, a decreasing trend was observed in the juveniles of the sparker (110 dph) and juveniles of the *in situ* experiment

(68 dph). The underlying mechanism is yet to be determined, but it is clear that the high intensity impulsive sound treatments (‘*in situ* exposure’, ‘maximum exposure’ and ‘impulsive exposure’) were perceived differently compared to the ‘*in situ* control’, ‘minimum exposure’ and ‘test chamber control’ in the three experiments, respectively.

The sparker and larvaebrator, *i.e.* the sound sources used in the lab experiments were selected because of their similar standard sound pressure metrics (SEL_{ss}, $L_{z\text{-}p}$ and SEL_{cum}) compared to the levels measured during the *in situ* experiment. However, the frequency spectra showed that the high energy produced in the three experiments only overlapped between the 315 and 630 Hz 1/3 octave bands. This is probably the common ground for the observed primary stress response in juvenile and larval fish when exposed to high intensity impulsive sound. The common ground is situated within the responsiveness range of European sea bass (100 – 700 Hz) (Kastelein et al., 2008). The lower frequencies of this responsiveness range (100 – 315 Hz) were also present at high SEL_{ss} in the larvaebrator and the real pile driving event. This indicates that a primary stress response was evoked by hearing the high intensity impulsive sound. If hearing is involved in the primary stress response, then particle motion will be important as well. Particle motion is the main sound component involved in fish hearing, acting on the inner ear and lateral line (Popper and Fay, 2011, Radford et al., 2012).

4.1.2 Secondary stress response

Hearing of impulsive sound in the lower frequency range might have increased the whole-body cortisol levels in juvenile fish, but it is not necessarily the trigger for a secondary stress response, as indicated by the oxygen consumption rate. According to Dube and Hosetti (2010), any variation in the respiration rate reflects stress. Overall, the oxygen consumption data for larvae and juvenile fish were in line with the inverse relationship between oxygen consumption ($\mu mol \cdot g^{-1} \cdot h^{-1}$) and body weight (g) (Segovia et al., 2012). In relation to high intensity sound exposure, the respiration rate decreased in most experiments, except for the larvaebrator experiment. The main difference in the measured sound parameters between the latter and the field and sparker experiments, was the rapid decrease of SEL_{ss} at frequencies >800 Hz in the larvaebrator experiment. Sound pressure is known to influence the swim bladder volume (Dalecki, 2008, Casper et al., 2013a). These swim bladder oscillations can even lead to the rupture of the swim bladder above a certain amplitude (Dalecki, 2008). The threshold for injury may be the lowest at the resonance frequency of the swim bladder which is situated in the higher frequency range (Bolle et al., 2013). Injuries can be accompanied by physiological stress responses (Baker et al., 2013), therefore, these strong oscillations within the fish body could potentially intensify the stress response of the fish to the sound exposure. Consequently, the acoustic stress response in fish does not need to be associated exclusively with hearing. The importance of the amplitude was illustrated by the respiration rate of larvae in the sparker experiment, which was significantly reduced under high amplitude impulsive sound (maximum exposure) compared to the lower amplitude impulsive sound (minimum exposure). Moreover, the oxygen consumption rate also decreased for juveniles under the 'maximum exposure' in the sparker experiment, although this response was less pronounced compared to the *in situ* juveniles. The combined results suggest that the higher frequencies (>800 Hz), in combination with high standard sound metrics levels, are important in evoking a secondary stress response. Judging from the difference between the minimum and maximum exposure treatments in the sparker experiment, the SEL_{ss} threshold for this secondary stress response in larvae and juvenile fish probably ranges between 170 and 180 dB re 1 $\mu Pa^2 \cdot s$ from 315 Hz onwards. Furthermore, the sharpness of the signal (rise time and speed) was excluded as the main triggering sound parameter for a secondary stress response in juveniles. Since the signal of the sparker experiment was much sharper compared to the signal of the larvaebrator and *in situ* pile driving.

Another indicator of a secondary stress response is whole-body lactate. Overall, the whole-body lactate levels measured in the juvenile fish followed the positive relationship between age and lactate concentration, as described by (Goolish, 1989). However, in contrast to the *in situ* experiments, whole-body lactate levels in juveniles were similar across the treatments in each lab experiment, and did not increase due to handling or decrease due to high intensity sound exposure. Together with the less pronounced reduction in respiration rates, this indicates that the standard sound metrics alone are not sufficient to explain this secondary stress response in juvenile fish. Overall, a high SEL_{ss} over a broad frequency range, as in the *in situ* experiments, seems to be necessary to induce strong primary and secondary stress responses in juvenile sea bass.

4.2 Ecological validity of the experiments

Laboratory experiments are a cornerstone in science (Levitt and List, 2007). They are indispensable to answer fundamental questions as they are conducted in a controlled environment and enable serial measurements on single individuals (Calisi and Bentley, 2009). However, creating a complex sound field in the lab representative for the wild proved to be extremely challenging. A number of compromises are in most cases unavoidable. Therefore, the impact shown in lab experiments can be either underestimations or overestimations of the potential impact in the field. On the other hand, the differences in the sound field between the three sound sources revealed that non-standard sound metrics, such as frequency spectra (and probably particle motion) are also important, which should be taken into consideration when designing bioacoustic lab experiments. In addition, other acoustic stress factors need to be considered, such as the temporal structure of the sound: pattern of occurrence, predictability and duty cycle. Temporal structure is thought to affect behavioural responses (Neo et al., 2014, Neo et al., 2015, Neo et al., submitted).

Still, for an optimal interpretation of the impact of high intensity impulsive sound on stress responses, lab experiments need to be complemented with *in situ* field experiments, as this is the only sound that really matters in the real world. Experiments in the field with larvae and eggs are still to be conducted, which will be practically even more challenging than with juvenile fish. Also, bioacoustic stress studies with adult fish both in the lab and the field are largely lacking. During the *in situ* experiment, adult whiting (*Merlangius merlangus*) were observed belly-up at the sea surface during offshore pile driving (personal observation 27/07/2013). These fish were probably in distress due to swim bladder problems. Further research on this matter is necessary in order to obtain a complete picture of stress responses to high intensity impulsive sound across different life stages and fish species.

4.3 Conclusion

High intensity impulsive sounds, characterized by similar basic sound metrics L_{z-p} of 210-211 dB re 1 µPa; SEL_{ss} of 181-186 dB re 1 µPa²·s and SEL_{cum} of 214-218 dB re 1 µPa²·s evoked a stress response in fish larvae and juveniles. However, the specific characteristics of the stress response did vary, depending on the sound source. Our results indicate that the high energy at the lower frequencies (315 – 630 Hz) is probably important for causing a primary stress response through hearing. Further, the higher frequencies (>800 Hz) are likely to cause a reduction in oxygen consumption rates in larvae. However, to induce clear secondary stress responses in juvenile sea bass sound producing high energy over a broad frequency range seems to be needed. This study was a first step in elucidating the underlying mechanisms of acoustic stress responses. This process should receive more attention. The lack of knowledge on the proper measurement and assessment of particle motion, the 'non-standard' second sound component, urges for a better understanding of fundamental underwater acoustics and of the importance of different sound metrics in fish physiology and biochemistry.

ACKNOWLEDGEMENTS

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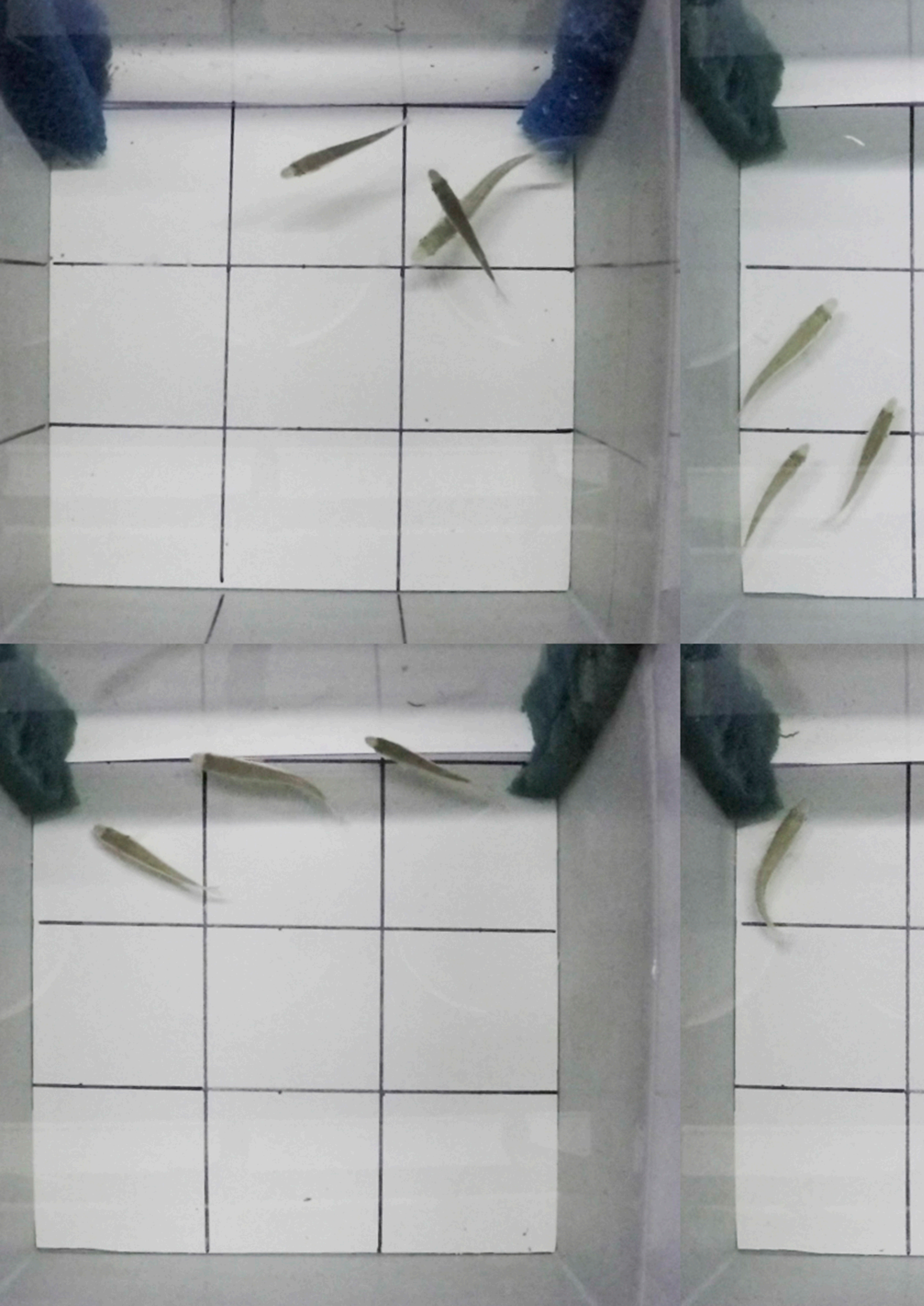

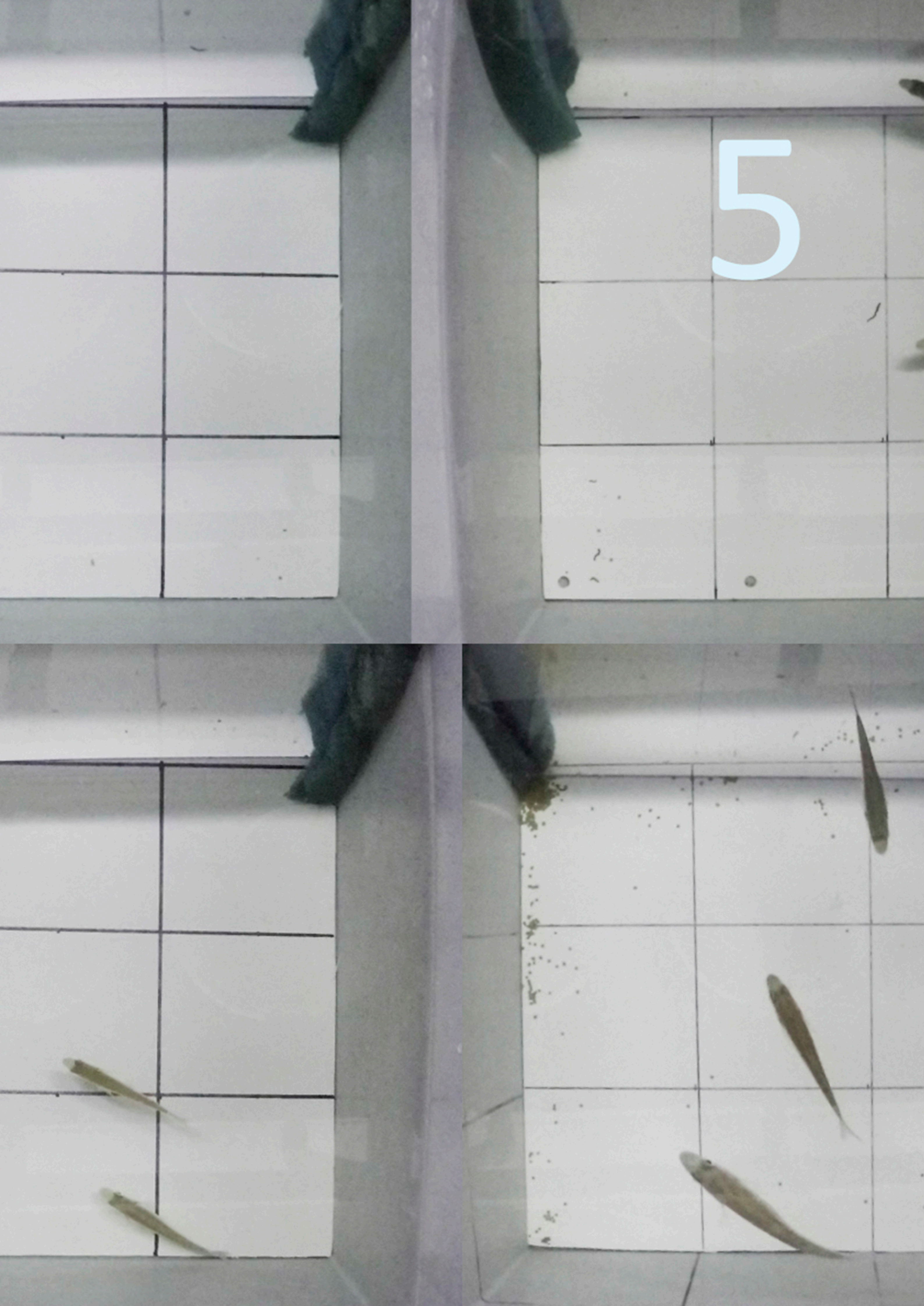

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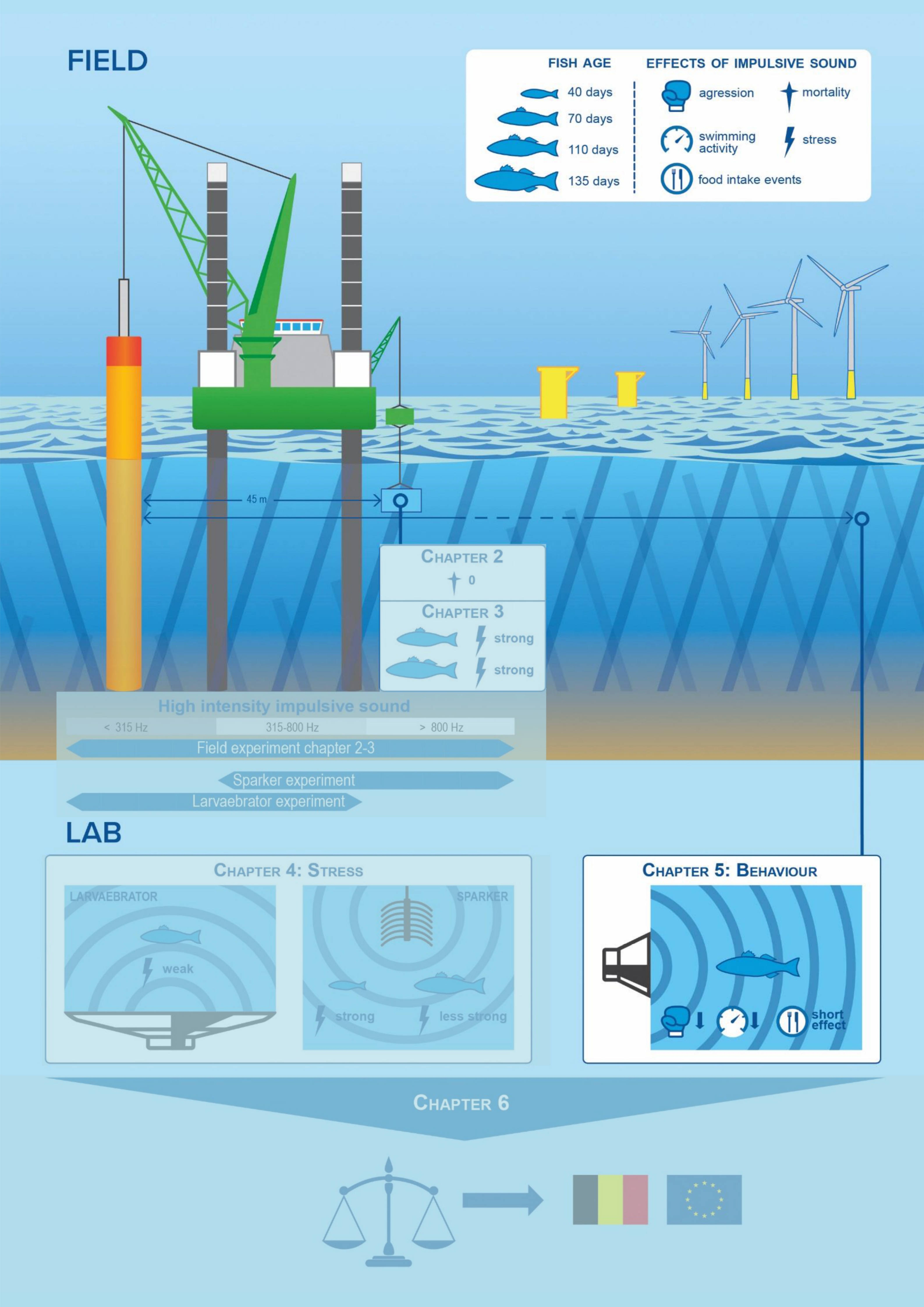
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EFFECTS OF IMPULSIVE SOUND
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Field experiment chapter 2-3
Sparker experiment
Larvaebrator experiment
LAB
CHAPTER 4: STRESS
LARVAEBRATOR
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CHAPTER 5: BEHAVIOUR
short effect
CHAPTER 6

CHAPTER 5

IMPACT OF IMPULSIVE SOUND ON ACTIVITY, SWIMMING AND FEEDING BEHAVIOUR OF JUVENILE EUROPEAN SEA BASS

Adapted from:

Debusschere E, Vercauteren M, Slabbekoorn H, Ampe B, De Coensel B, Botteldooren D, De Boeck G, Thomas P, Vandendriessche S, Degraer S, Vincx M, Hostens K, In preparation. Impact of pile driving playback on behaviour and feeding tendency in juvenile European sea bass.

Abstract

The increasing presence of man-made sound in the marine environment has the potential to impact fish behaviour, even at large distances from the sound source. Some behavioural traits, such as social interactions and foraging behaviour significantly contribute to the fish survival and reproduction. Little is known about the impact of sound exposure on the behaviour of fish and the few existing studies are limited to the evaluation of an acute impact. There has been even less attention for delayed effects during or after prolonged or repeated sound exposure. In this laboratory study, the influence of playback pile driving sound on the swimming activity and aggressive attacks on conspecifics was investigated. Juvenile European sea bass *Dicentrarchus labrax* were used as a model organism. Secondly, the impact on feeding tendency and efficiency during and directly after sound exposure was investigated. Juvenile sea bass interrupted their swimming activities and ceased all aggressive attacks on conspecifics at the onset of impulsive sound exposure. The behavioural effects of sound exposure showed recovery to the pre-exposure baseline within the 25 minute exposure period. On the first day, a reduced number of food intake events were observed during and after the sound exposure, which can indicate an attention shift induced by the sound exposure. This attention shift was no longer clearly observed during the two following days of the experiment. Feeding efficiency was not affected by the sound exposure and showed that sea bass were alert to external stimuli under impulsive sound exposure. These results indicate that the initial response does not persist but can progress over time or under repeated exposure. It remains to be tested if a similar response will be exhibited by wild-ranging sea bass.

Keywords

Feeding behaviour, swimming activity, aggression, recovery, attention shift, pile driving, European sea bass

1.Introduction

The introduction of anthropogenic sound into the marine environment can affect the well-being of fish and other marine organisms. High intensity pile driving for example is known to cause auditory injury, barotrauma and physiological stress in fish (Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013, Popper et al., 2014, Debusschere et al., 2016). Sound at high and low intensity levels can also affect fish behaviour, if there is spectral overlap with the hearing range (Slabbekoorn et al., 2010). Typically, pile driving sound is louder than the background noise in the sea, and audible to fish up to tens of kilometres away from the source (Smith et al., 2004, Bailey et al., 2010). Such artificial and audible sounds can mask biologically relevant signals from conspecifics or environmental cues, and potentially interrupt, disturb and deter the fish present in the ecosystem (Slabbekoorn et al., 2010, Popper et al., 2014).

Several studies started exploring sound impact on fish behaviour. There are convincing but anecdotal reports on fish responses to noisy activities in the field (e.g. Engas et al., 1996, Sara et al., 2007, Hawkins et al., 2014). Also, a growing number of reports examined behavioural responses to sound exposure in fish in captivity, in order to determine critical parameters for acoustic sensitivity in fish (e.g. Kastelein et al., 2008, Voellmy et al., 2014a, Neo et al., 2015). For example, fish have been shown to startle, speed up or slow down, change schooling behaviour, or shift down the water column in response to anthropogenic sound exposure field (e.g. Engas et al., 1996, Sara et al., 2007, Hawkins et al., 2014). Neo et al. (2014) reported initial startle responses, closer aggregation and diving down to the bottom for adult seabass (*Dicentrarchus labrax*) exposed in an outdoor basin.

The impact of behavioural changes in response to sound exposure depends on the eventual fitness consequences. Linking a fitness consequence to a behavioural response is quite straightforward for functional traits, such as foraging, anti-predator responses, territorial defence, distinct dispersal or migratory decisions and courtship behaviour (Picciulin et al., 2010, Purser and Radford, 2011, Sebastianutto et al., 2011, Popper et al., 2014, Voellmy et al., 2014a, Voellmy et al., 2014b, Simpson et al., 2015). Any disturbance of such behaviour may have an immediate impact on body condition, survival or reproductive success (Radford et al., 2014). However, the anxiety-related acoustic responses observed in swim behaviour as described by Neo et al. (2014), are more difficult to interpret, as they do not translate easily into survival or reproductive value.

A few indoor lab experiments with captive fish species addressed the acute impact of short-term sound exposure on foraging behaviour. Both three-spined sticklebacks (*Gasterosteus aculeatus*) and European minnows (*Phoxinus phoxinus*) were negatively affected during sound exposure (Purser and Radford, 2011, Voellmy et al., 2014a). Sticklebacks showed noise-dependent discrimination and handling errors in attacking and swallowing water fleas (*Daphnia magna*), while minnows appeared to eat less. Also, zebrafish (*Danio rerio*) showed more handling errors under both continuous and impulsive sounds (Shafei Sabet et al., 2015). The explanation for the sound impact in the latter three studies was either a temporary interruption of the activities related to an increase in perceived predation risk (Bonga, 1997, Barbosa et al., 2012, Kalueff et al., 2013), or a performance decline due to noise-induced attention shifts (Mendl, 1999, Chan et al., 2010).

All studies so far rather focused on short, acute impacts on fish behaviour. It remains to be tested whether the negative impact on foraging behaviour remains after or during a prolonged exposure to high intensity impulsive sound (Bejder et al., 2009). In the current study, we investigated the immediate behavioural impact and potential habituation and recovery in juvenile European sea bass, in relation to 25 minutes of impulsive sound exposure repeated on three consecutive days. We investigated the impact on the activity (duration) and on the number of aggressive encounters between two social companions. We also tested whether prolonged sound exposure (15 minutes or more) affected feeding tendency and efficiency during sound exposure and directly after cessation of the sound exposure.

2. Methods

2.1 Ethical note

The experiments were carried out in accordance with the Belgian Council for Laboratory Animal Science (BCLAS) guidelines. The ethical committee of the Institute for Agricultural and Fisheries Research (ILVO) approved this experiment under permit number 2014/235. The fish were allowed to acclimate for three days in their test set-up before performing the experiment. At the start of the test, all fish showed normal foraging behaviour. After the experiment, all fish were humanely sacrificed by transferring them into an overdose of anaesthetic (5 g benzocaine dissolved in 25 ml acetone and 1 L seawater).

2.2 Study species and aquarium conditions

The European sea bass is a commercially important fish species, for both fisheries and aquaculture. It is a eurythermic and euryhaline marine fish inhabiting a wide variety of habitats, including estuaries, lagoons, coastal waters, rivers and the marine environment down to 100 m water depth (Kottelat and Freyhof, 2007). The species is a physoclist, meaning that the fish only has a mouth – swim bladder connection during the first days of swim bladder formation. European sea bass were shown to react to sound signals between 0.1 and 0.7 kHz (Kastelein et al., 2008). For this experiment, four months old juveniles were obtained from the Ecloserie Marine de Gravelines in France. Six aquaria (30 L) were provided with aeration and a continuous flow of UV-sterilized seawater from a semi-closed recirculation system (Figure 2). The water temperature was 18.3°C with a salinity of 28 ppt and a pH of 8.6. The juveniles were fed once a day with MarciCo start 1.5mm (Coppens) and were kept in a 12:12 light dark-regime.

2.3 Experimental design

The experiment consisted of six aquaria (30 L), each containing one focal fish (136 days post hatching (dph), standard length (SL) = 74.7 SD ± 3.9 mm; wet weight (WW) = 7.4 SD ± 1.1 g), and two slightly smaller companions (136 dph, SL = 59 SD ± 0.6 mm; WW = 3.7 SD ± 0.5 g). The results were limited to the behaviour of the focal fish. The two companion fish were only present to facilitate normal behaviour of the focal fish, as juvenile sea bass are social fish (Froese and Pauly, 2015). All three fish were swimming freely and were thus in olfactory and physical contact with each other. The distinct size of the focal fish allowed to recognize it on the video images without external tagging.

Three days before the start of the experiment, the focal and companion fish were distributed over the six experimental aquaria (30 L). The experiment was executed for three consecutive days, where fish were exposed daily to 25 min. of impulsive sound, corresponding to 1000 strikes per day. Each day, fish behaviour was recorded continuously for 85 min., starting at 10h00 a.m., with 30 min. before, 25 min. during and 30 min. after sound exposure. Based on the daily recordings, fish behaviour was analysed three times (three consecutive days) for four periods: (1) 'before exposure', the first 10 min. of the 30 min. before the sound exposure; (2) 'start exposure', the first 10 min. of the sound exposure; (3) 'mid-exposure', 10 min. starting at 15 min. after sound exposure started; (4) 'after exposure', first 10 min. directly after sound exposure ceased (Figure 1).

In addition, the experiment had a fully crossed design with three feeding moments (one per day) carried out in different order in the six aquaria over the three consecutive days, relative to the sound exposure period (Table 1). The three feeding moments were: (1) FEEDpre: fish were fed 30 min. before sound exposure; (2) FEEDdur: fish were fed 15 min. after the start of sound exposure; (3) FEEDpost: fish were fed immediately after sound exposure ceased. Feeding behaviour was only analysed for the first 10 min. after food pellets were offered (Figure 1).

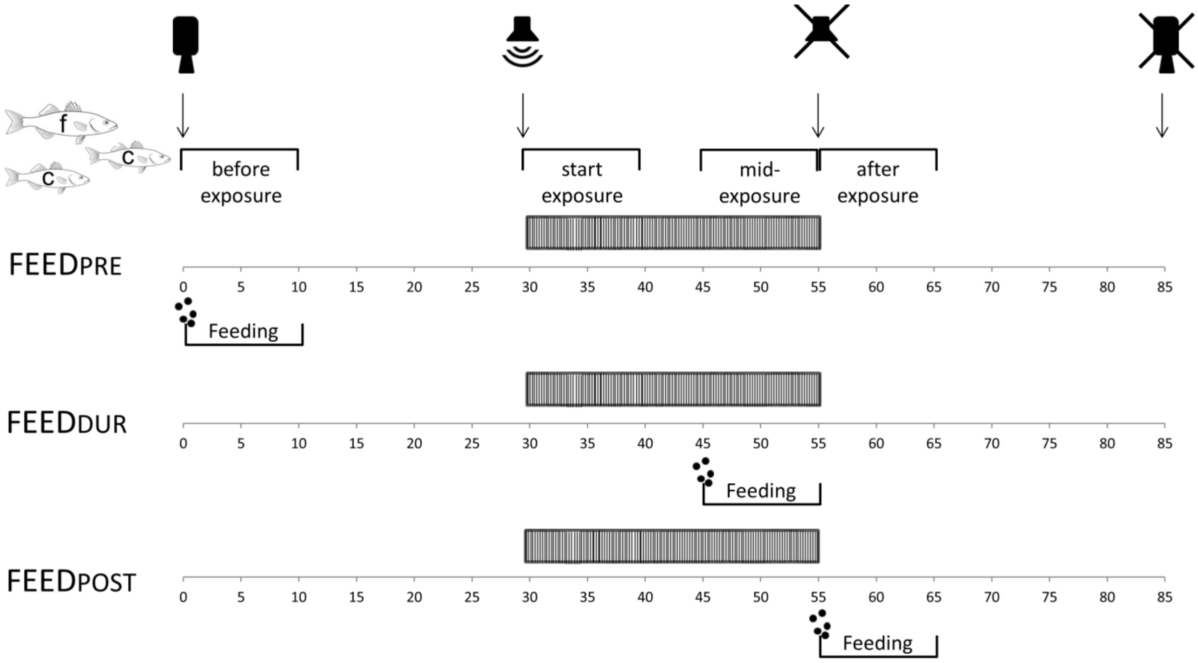

Figure 1. Schematic representation of the experimental periods during each day. The four periods (before exposure, start exposure, mid-exposure, after exposure) in which the behaviour of the focal fish was analysed, were the same each day in each experimental aquarium. The timeline per feeding moment (FEEDpre, FEEDdur, FEEDpost) represents the entire recording of the fish for one day (85 min.), the feeding moment, the 10 min. period in which feeding behaviour was analysed and the impulsive sound exposure period (25 min.). This experimental design was repeated on three consecutive days; each day a different feeding moment was applied in the six aquaria (see table 1).

Table 1. Experimental design. The moment of feeding (FEEDpre, FEEDdur, FEEDpost) in each of the six aquaria (focal fish 1 to 6) varied in a different order over the three consecutive days. * Missing data due to camera failure.

Focal fish	Day 1	Day 2	Day 3
1	FEEDdur	FEEDpre	FEEDpost
2	FEEDpost	FEEDpre	FEEDdur*
3	FEEDpost	FEEDdur	FEEDpre
4	FEEDdur	FEEDpost	FEEDpre
5	FEEDpre	FEEDpost	FEEDdur
6	FEEDpre	FEEDdur	FEEDpost

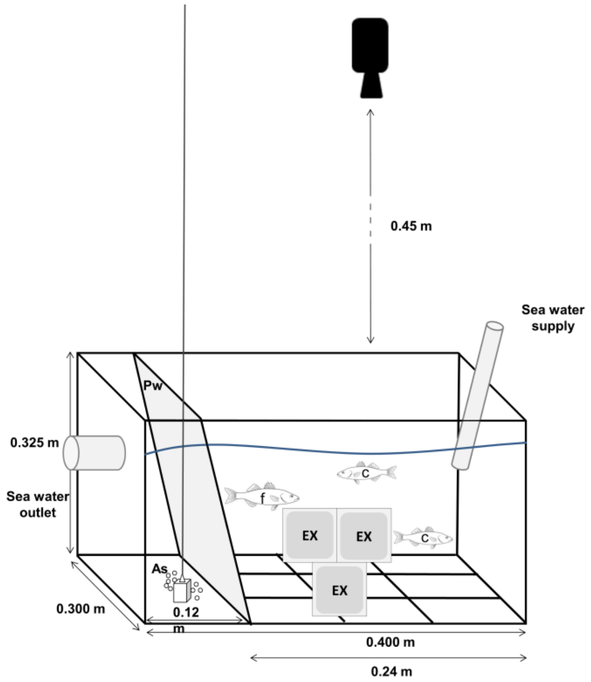

Figure 2. Experimental set-up in one of the six aquaria, each with one focal (f) and two companion (c) fish. Continuous sea water flow and air supply (As) were foreseen in each aquarium. Three exciters (EX) were screwed together to one aquarium wall to produce the impulsive sound. A camera was installed above the aquarium to film fish behaviour. The plexi wall (Pw) kept the focal and companion fish out of the shaded area where fish were not visible for the camera.

2.4 Experimental arena

Six aquaria were used in this experiment in which impulsive sound was introduced by means of three exciters per aquarium. Three polypropylene plates (60 x 60 x 4 mm), each holding an exciter (Visaton EX 60S), were mounted on the long side of the aquarium (30 L grey polypropylene boxes, 400 x 300 x 325 mm). Additionally, a camera (Go Pro 3) was mounted at 0.45 m above each aquarium to record fish behaviour. A plexi-plate was installed inside the aquarium at 120 mm from the short side to keep the fish out of the small shaded area where fish were not visible for the camera (Figure 2). To optimise video quality, the seawater recirculation system and aeration were switched off during video recording.

2.5 Acoustical set-up

For this experiment, the sound recording of a real pile driving event was played. The sound recording was made on June 19th, 2013 when the monopile B3 (5 m diameter) was driven into the seabed (IHC Hydrohammer B.V.) on the Lodewijckbank (Lat N 51.629995, Long E 2.926765) in the Belgian part of the North Sea. For the recording, a hydrophone (Bruël & Kjaer type 8104, voltage sensitivity 47.7 $\mu V \cdot Pa^{-1}$, charge sensitivity 0.391 $pC \cdot Pa^{-1}$, 10 m cable) was positioned at 45 m from the pile driving source and 2.5 m below the sea surface (Debusschere et al., 2014). A random sequence of ten consecutive strikes (15 sec.) was selected for playback, with a mean single strike sound exposure level (SEL_{ss}) of 188 ± SE 0.07 dB re 1 $\mu Pa^2 \cdot s$ and zero-to-peak sound pressure level ($L_{z\text{-}p}$) of 210 ± SD 0.01 dB re 1 µPa.

The playback track was played from a pc in a loop for 25 min. through an external audio card (Esi U46XI USB audio interface) connected to a 12-channel amplifier (OSD audio model MX-1260). Each aquarium had two exciters in parallel connection to a channel and one exciter in direct connection to a channel of the amplifier. As such, the three exciters were acting as one sound source.

Prior to the experiment, the ambient sound pressure (without playback of sound) and the impulsive underwater sound pressure during playback of the pile driving track were both recorded at nine points in each aquarium (in a 80 x 80 mm grid, Figure 2) with a Bruël & Kjaer hydrophone (Type 8104, 10m cable) connected to the charge channel of a Bruël & Kjaer portable amplifier (Nexus type 2690-0S). A multi-channel portable recording (Tascam DR-680) was used to measure the different sound components. The recorded sound was saved on Compact Flash cards of 16 GB (SanDisk Ultra) in a WAVE format (.wav) with a sampling rate of 44 100 Hz at 24 bit. The sound files were imported and analysed in Matlab and exported to Microsoft office Excel 2007 for further analysis of SEL_{ss}, cumulative sound exposure level (SEL_{cum}) and $L_{z\text{-}p}$.

2.6 Behavioural observation & analyses

The behaviour of the focal fish was analysed for all 10 min periods specified above, *i.e.* before exposure, start exposure, mid-exposure and after exposure in relation to the different feeding moments on 3 consecutive days. Behavioural features of interest were startle response, swimming activity, aggression, influence of food presence on swimming activity, number of food intake events and food pellet uptake efficiency. These features were manually scored using Observer XT (version 8.0 Noldus). A startle response was defined as a brief and sudden movement causing the fish to accelerate. Aggression was counted as tail biting of the focal fish to the companion fish. Both behavioural features are counted as number expressed by each focal fish. The swimming activity of the fish was scored as the duration the fish were swimming calmly relative to the total time of each recording (%). Sudden accelerations or increased swimming activity because of aggressive behaviour were not taken into account. Feeding behaviour was only analysed for the first 10 minutes after food pellets were offered. Successful food intake events were logged as the number of actual food intake events without spitting out the food. The food pellet uptake efficiency of the fish was described as the number of successful attacks on food pellet relative to the total food pellet attacks (%).

2.7 Statistics

The number of startle responses was analysed with ‘period’ and ‘day’ as fixed factors in a linear mixed-effect (LME) model. Focal fish was introduced as random effect in all LME models to account for repeated measures. Swimming activity of the fish was first compared between ‘period’ and ‘day’ in a LME model. Secondly, the LME model was repeated with an extra factor ‘influence of food presence (present *vs* absent)’ in addition to the ‘period’ and ‘day’, on swimming activity. For this analysis, the ‘start exposure’ periods were not taken into account, as no food was offered during that period (Table 2). Aggressive behaviour was only seen in three of the six focal fish and the aggressive attacks were insufficient for a statistical test. The data were visually inspected for their dependence of ‘period’, ‘day’ and ‘food presence (FP)’. Feeding behaviour analyses were restricted to those periods of the three days in which fish were fed (Figure 1). The total number of successful food intakes was again analysed by means of an LME model, with ‘feeding moment’ and ‘day’ as fixed factors and ‘focal fish’ as random factor. Food pellet uptake efficiency was analysed in a similar way, but averages were used instead of total sums.

Normal distribution of the residuals in each model was assumed based on a graphical evaluation of the histogram and Q-Q plot of the residuals. There was a slight deviation from normality but this will probably only have a negligible effect on the fixed factors of the LME model with a random effect. ANOVA type III analyses were then performed with significance levels set at 0.05. *Post hoc* pairwise comparisons were performed with least-square means, and p-values were corrected by means of Tukey-Kramer adjustment for multiple comparisons. All analyses were performed with the LME-function in R 3.2.2 (R foundation of Statistical Computing, Vienna, Austria). The statistical power of this study is low since the experiment could only be repeated once due to technical problems with the amplifier during the second repetition.

3. Results

3.1 Sound characteristics of the playback impulsive sound

The playback of pile driving sound simultaneously produced by the three exciters generated a $L_{z\text{-}p}$ of 165.4 (mean) ± SD 3.6 dB re 1 µPa and a SEL_{ss} of 145.9 ± SD 2.5 dB re 1 $\mu Pa^2 \cdot s$. The sound pressure level in each quadrant was not constant but the sound field can be considered homogeneous due to the small differences. These values were respectively 44.6 and 42.1 dB lower than the originally recorded *in situ* pile driving strikes. Comparing the SEL_{ss} over the 1/3 octave bands between the original and playback, showed that the difference increased above 45 dB between the 250 Hz and 3150 Hz 1/3 octave band (Figure 3A). The mean ambient sound pressure level (SPL) of the aquaria in the absence of impulsive sound playback was 113.2 ± SD 1.2 dB re 1 µPa and most energy was found in the lower frequencies and in a peak at the higher frequencies (Figure 3B).

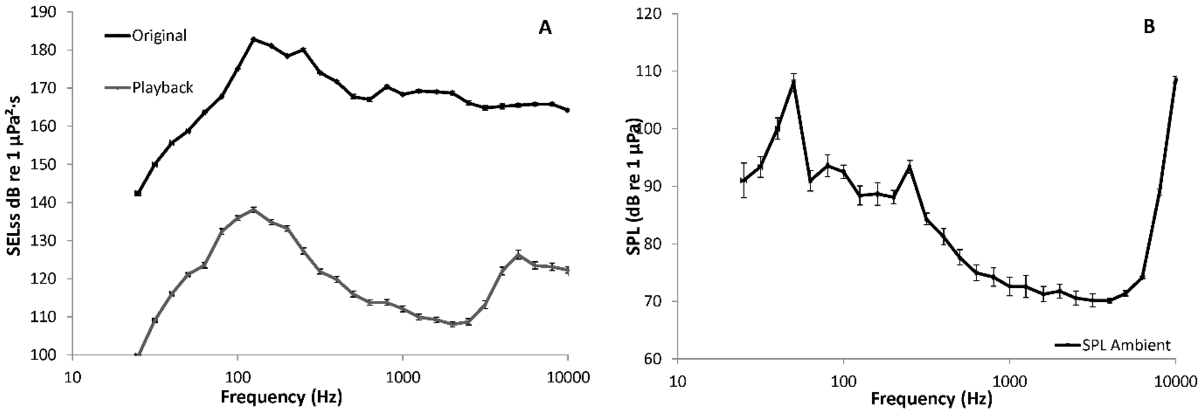

Figure 3. Measured frequency spectra of (A) the single strike sound exposure level (dB re 1 µPa²·s) (mean ± SE) over the 1/3 octave bands of the original recorded 10 consecutive pile driving strikes and the playback of the original recording in the experimental aquaria; (B) the ambient sound pressure level (dB re 1 µPa) (mean ± SE) over the 1/3 octave bands.

Table 2. Results of the linear mixed-effect model (ANOVA, type III) for the tested parameters. Interaction terms were given when significant.

	Period	**Day**		
Startle response	$F_{3,57} = 3.29$	$F_{2,57} = 2.23$		
	p = 0.03	p = 0.12		
Activity	$F_{2,57} = 7.00$	$F_{2,57} = 1.08$		
	p = 0.0004	p = 0.34		
Aggression	-	-		
	Period*	**Day**	**Food presence (FP)**	**FP:Day**
Activity	$F_{2,38} = 1.17$	$F_{2,38} = 2.58$	$F_{1,38} = 8.76$	$F_{2,38} = 6.38$
	p = 0.32	p = 0.089	p = 0.005	P = 0.004
Aggression	-	-	-	-
	Feeding moment	**Day**		
Food intake**	$F_{2,7} = 1.36$	$F_{2,7} = 5.01$		
	p = 0.32	p = 0.446		
Efficiency**	$F_{2,7} = 0.62$	$F_{2,7} = 0.13$		
	p = 0.13	p = 0.88		

* Excluding the 'start exposure' period in which food was never offered. ** only taking into account the periods in which food was really offered

3.2 Fish activity and interaction with companion fish

A trend of more startle responses was observed at the start of the impulsive sound exposure (start exp) compared to the other periods (before exp, mid-exp, after exp) (*post hoc* $p_{adj} < 0.1$) (Table 2). Aggressive attacks towards the companion fish were only observed in three of the six focal fish. This aggressive behaviour completely disappeared at the start of sound exposure (start exp), but re-appeared already to some extent (most obvious on the second day) in the last 10 min. of sound exposure (mid-exp) (Figure 4A). After sound exposure (after exp), the number of aggressive attacks was more or less comparable to the 'before exposure' period on all three days. Visual inspection of the data revealed an interaction effect between food presence and period on the aggressive behaviour. While aggressive attacks decreased in the presence of food, they disappeared in the presence of food and sound (Figure 4B).

The swimming activity (the percentage of time spent swimming) of the six focal fish significantly decreased each day at the beginning of sound exposure (start exp) compared to the other three periods (before exp, mid-exp and after exp; *post hoc* $p_{adj} < 0.05$ for all tests). In the periods (before exp, mid-exp and after exp), the swimming activity was comparable between the periods and between the consecutive days (Figure 4C). No significant interaction effect was found between the food presence and period for swimming activity ($F_{2,28} = 0.31$; $p = 0.73$). On the other hand, the swimming activity significantly increased when food was present on the second and third day (*post hoc* $p_{adj} < 0.05$ for both tests), but this was not the case on the first day (Figure 4D; Table 2).

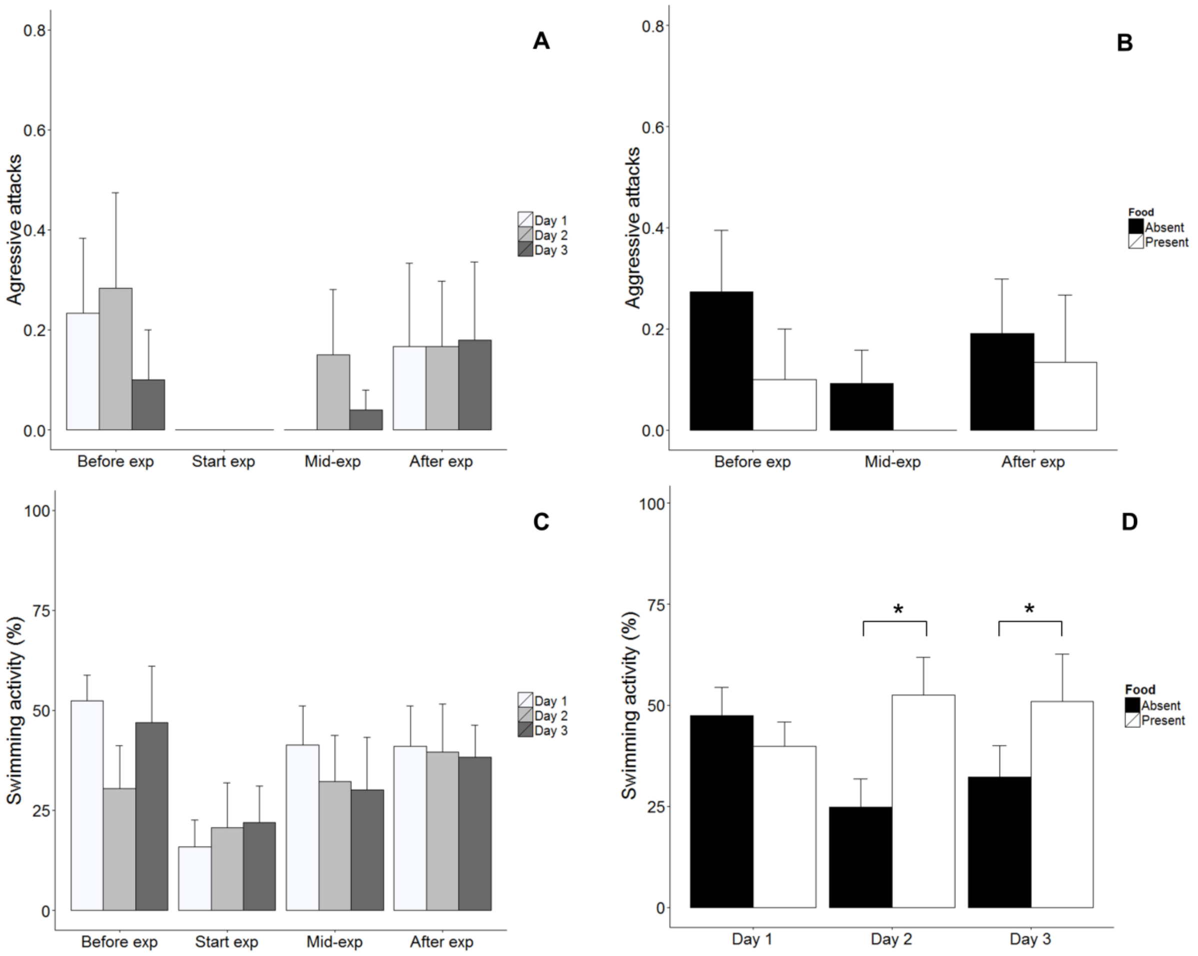

Figure 4. Aggressive attacks (mean ± SE) and swimming activity (mean ± SE) of the focal fish: (**A** and **C**) during the first 10 minutes of each of the four periods (before exp, start exp, mid-exp and after exp); **B** grouped per period and **D** grouped per experimental day in the presence and absence of food (the start-exposure period is excluded in both **B** and **D**, as food was never offered at the beginning of sound exposure). Swimming activity was significantly reduced in start exposure (**C**) and at day 2 and day 3 influenced by the presence of food, indicated by * (**D**).

3.2 Feeding behaviour

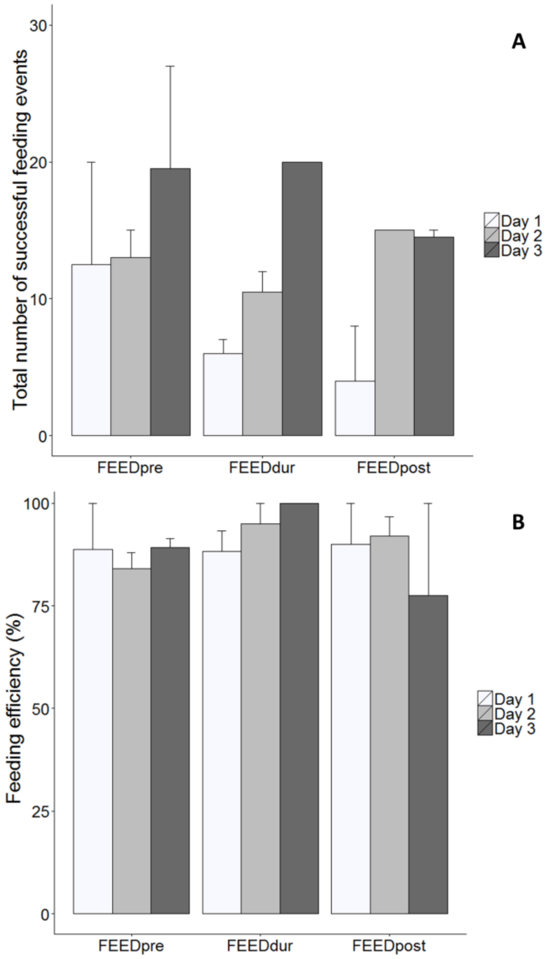

Figure 5. Feeding behaviour of the focal fish for each feeding moment, i.e. before (FEEDpre), during (FEEDdur) and after (FEEDpost) sound exposure on three consecutive days. (A) Total number (sum ± SE) of successful feeding events in 10 minutes after food was offered. (B) Feeding efficiency (%) during 10 minutes after food was offered (mean % ± SE).

Although no significant differences were found in the number of successful food intake events between the three feeding moments (FEEDpre, FEEDdur and FEEDpost), food intake by the focal fish seemed to be less successful on the first day when they were offered food during (FEEDdur) or after (FEEDpost) sound exposure, compared to before (FEEDpre) (Figure 5A). Overall, the focal fish seemed to eat more on day 3 compared to day 1, independent of the feeding moment (Table 2). The feeding efficiency of the focal fish, *i.e.* the ability to actually ingest the attacked food pellet was not affected by sound exposure or by feeding moment (Figure 5B).

4. Discussions

The aim of this study was to investigate the potential impact of impulsive sound exposure on swimming activity, aggressive and feeding behaviour in juvenile sea bass, before, during and after sound exposure over three consecutive days. Swimming activity and aggressive attacks significantly decreased at the start of sound exposure. However, both behavioural parameters showed recovery to pre-exposure levels already during or directly after the sound exposure period. The number of food intake events was lower on the first day during and after the sound exposure than on the next two days. This can indicate the presence of a temporary attention shift on the first day which faded on day two and three. Feeding efficiency was not significantly by the impulsive sound exposure.

4.1 Impact of impulsive sound playback on fish behaviour

The present study confirms the fact that fish often startle as a first response at the onset of sound exposure (Wardle et al., 2001, Neo et al., 2014, Voellmy et al., 2014a). After this brief initial startle response, the juveniles reduced their activity level. This behaviour was most likely anxiety-driven, as it occurs when fish perceive a mild stressor (Malavasi et al., 2008, Ranaker et al., 2012). Decreased activity was also reported for European minnow to playback shipping noise (Voellmy et al., 2014a), and was suggested to occur in juvenile European sea bass during a high intensity pile driving event (Debusschere et al., 2016).

On all three consecutive days, recovery to the initial swimming behaviour was observed already during sound exposure, as the activity levels of the fish in mid-exposure were similar to the levels before and after sound exposure. Similarly, Neo et al. (2014) reported behavioural recovery to a pre-exposure baseline during underwater sound exposure for adult European sea bass. Recovery time may be delayed by the temporal structure (e.g. intermittency and pulse regularity) (Rankin et al., 2009, Neo et al., 2014); the intensity of the stimuli (Rankin et al., 2009); and the perceptual ability of the species (Greggor et al., 2014). In the present study, the recovery during impulsive sound exposure suggests that the juvenile fish evaluated impulsive sound as no life threatening stressor quite rapidly. Repeated exposure can either result in long-term habituation or in a higher sensitization of the fish to the external stimuli. The latter will normally lead to an increased response and will aggravate the impact with time (Rankin et al., 2009). As the swimming activity decreased each day at the start of the sound exposure and returned to normal levels after exposure, neither long-term habituation nor sensitization was observed in terms of swimming activity over the three days of the experimental period. This is more likely to occur on a larger time scale or after more repeated exposures (Barton et al., 1987, Neo et al., in prep.).

Another behavioural trait observed in juvenile sea bass was the aggression towards subordinates. In the present study, the impulsive sound exposure did interrupt the aggressive behaviour in juvenile sea bass for a brief period of time, but aggression re-appeared during and definitely after sound exposure. This was also observed during a pilot study (Vercauteren, 2014). Due to the low number of aggressive attacks, this could not be statistically supported in this study. Nevertheless, it can indicate that fish were briefly distracted by the sound exposure and by extension, also by the presence of food, resulting in a reduction of aggressive attacks. Social dynamics and intragroup interactions can be affected differently by different sound types (Bruintjes and Radford, 2013) and can vary among fish species. For example, Bruintjes and Radford (2013) showed that the number of aggressive attacks towards subordinates increased under playback of boat noise at SPL of 127 dB re 1 µPa in the cooperatively breeding cichlid species *Neolamprologus pulcher*.

4.2 Impact of impulsive sound on feeding behaviour

All studies that already tackled the impact of sound exposure on feeding behaviour of fish only captured the initial acute feeding response of fish during sound exposure (Purser and Radford, 2011, Voellmy et al., 2014a, Shafei Sabet et al., 2015). The current study looked beyond that initial feeding response. Food pellets were either added when fish were already exposed to the impulsive sound for 15 min. or immediately after 25 min. of sound exposure. The first 15 min. of sound exposure allowed the fish to assess the threat level of the acoustic stressor. On the first day of the experiment, a trend was observed of reduced food uptake by the focal fish during and after sound exposure, which probably coincided with an attention shift. Due to the low sample size, this trend could not be statistically supported. Similar attention shifts were found to be responsible for the reduced foraging performance of three-spined stickleback and the food handling problems of zebrafish under sound exposure (Purser and Radford, 2011, Shafei Sabet et al., 2015). On day two and three of this experiment, the total number of food intake events by juvenile sea bass was not significantly affected during or after the impulsive sound exposure period and the presence of food induced an overall higher swimming activity. The feeding moment during the sound exposure corresponded to the timing of the behavioural recovery in swimming activity and aggression. This can most likely be attributed to short-term habituation of the fish to the impulsive sound stressor. The feeding efficiency was unaffected throughout the experimental period. In contrast to the living prey (*Daphnia sp.*) used in other laboratory experiments (Purser and Radford, 2011, Voellmy et al., 2014a, Shafei Sabet et al., 2015), food pellets were used in the present experiment. As less effort is needed to consume food pellets, the 'real' foraging performance of juvenile sea bass might be somewhat overestimated. Overall, this study showed that fish were able to respond to external stimuli (presence of food) under impulsive sound exposure, which demonstrates their alertness.

4.3 Ecological implications

Translating lab-based results to the 'real world' is not easy and must be done with great caution (Calisi and Bentley, 2009). The selection of exposure stimuli is always a compromise between biological relevance and adequate replication for external validity (Slabbekoorn and Bouton, 2008). In this study, we focused on a proof-of-concept case study with the laboratory experimental setting using a fish species and one single recording source (pseudoreplication) related to our complementary *in situ* pile driving study (Debusschere et al., 2014). This means that extrapolation to other species, other *in situ* conditions and other impulsive exposure conditions in general, requires further testing. At this stage, the lab results cannot be generalised but only give an indication of the expected impact in the real underwater world. Based on the recent literature, it can be stated that behavioural responses are species-specific, as most species vary in foraging and life history strategies, and are partly related to the type of sound playback (King and McFarlane, 2003, Purser and Radford, 2011, Voellmy et al., 2014a, Voellmy et al., 2014b, Shafei Sabet et al., 2015). Furthermore, the behavioural response to the acoustic stressor will probably also be more pronounced in wild fish compared to hatchery-reared fish (Malavasi et al., 2008). The results showed that the response of European sea bass changed over time. Therefore, caution is needed when making extrapolations based on initial acute responses.

4.4 Conclusions

This study demonstrated that juvenile sea bass showed an initial response each time the fish was exposed to the acoustic (playback pile driving) stressor. The fish already showed recovery during the sound exposure to pre-exposure levels, as was observed in swimming activity and aggression. Signs of an attention shift were noted on the first day, such as a decrease in food intake events, but were not observed during repeated sound exposure. The results suggest that acute behavioural responses expressed by fish at the start of the sound exposure period do not persist. Hence the response is different when the fish are allowed to assess the threat-level of the newly introduced stressor.

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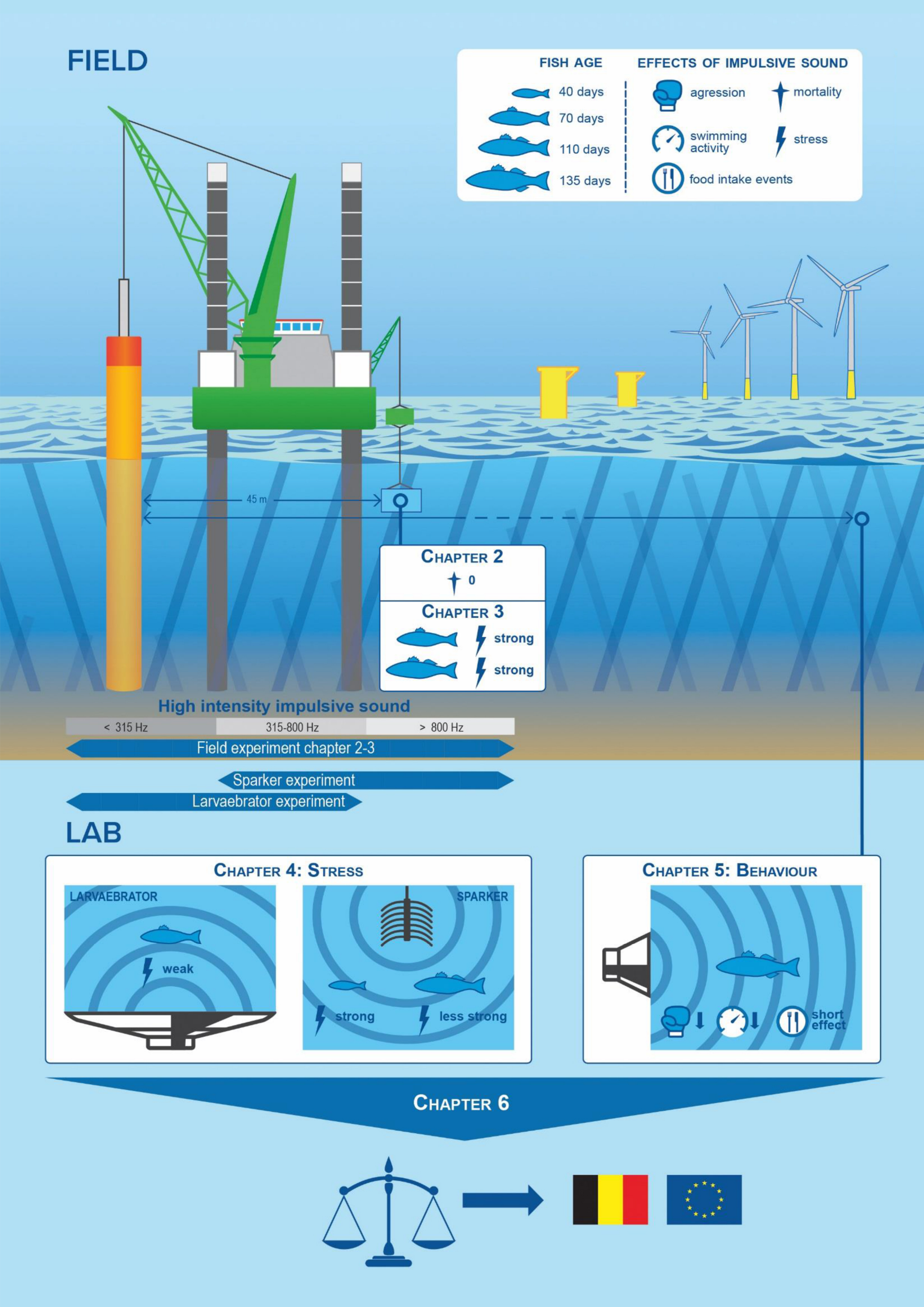
FIELD
FISH AGE
40 days
70 days
110 days
135 days
EFFECTS OF IMPULSIVE SOUND
agression
mortality
swimming activity
stress
food intake events
45 m
CHAPTER 2
0
CHAPTER 3
strong
strong
High intensity impulsive sound
< 315 Hz
315-800 Hz
> 800 Hz
Field experiment chapter 2-3
Sparker experiment
Larvaebrator experiment
LAB
CHAPTER 4: STRESS
LARVAEBRATOR
weak
SPARKER
strong
less strong
CHAPTER 5: BEHAVIOUR
short effect
CHAPTER 6

CHAPTER 6

IMPACTS OF UNDERWATER SOUND: GENERAL DISCUSSION

1 Context, aims and value of this study

Offshore construction activities such as the construction of offshore wind farms lead to increasing levels of underwater sound in the marine environment. However, information on the environmental impact of both the construction and operational sound was scarce at the onset of this study. Marine mammals were already known to be affected by high intensity impulsive sounds and were consequently the species of concern in this research field. Other ecosystem components were thought to be affected by the increasing underwater sound as well, but proof was unavailable at the time. This thesis therefore elaborates on the impact of sound on young fish. European sea bass (*Dicentrarchus labrax*) was used as a model species.

First, this study aimed to investigate if there was an effect, and if so, at what level it manifested. A unique opportunity presented itself to perform a field study on board of a pile driving vessel. This represented a 'worst-case' scenario: fish were exposed at close range to a complete pile driving session, during which high intensity impulsive sound was generated. Short-term and long-term mortality and stress levels were assessed. The results of this field experiment were then linked to the results of two lab experiments, to identify the critical sound parameters. Finally, the pile driving impact on fish behaviour was assessed at lower sound pressure levels, which corresponded to a larger distance from the pile driving sound. The results of Chapter 2 to 5 show that the actual impact of anthropogenic underwater sound on young fish is less acute than previously hypothesized.

This discussion starts with a critical analysis of the used methodologies. Then, the main results of the separate studies are integrated with current knowledge. Important sound parameters and sound thresholds are considered if they can be assigned to specific effects. Subsequently, the observed effects are discussed from an ecological perspective. A review on international directives and national legislation related to the generation of underwater sound is provided, with policy recommendations based on this study. Finally, a general conclusion of this study is made by answering the four research questions and future research topics are proposed.

2 Investigating sound impacts: not a piece of cake

2.1 Building blocks of the experiments

Choice of fish

A study on the impact of pile driving on young fish should ideally be performed with a very sensitive species that is present at the offshore wind farm (OWF) zone, omnipresent in the North Sea, or in need of hard substrates, such as cod (*Gadus morhua*) (Reubens et al., 2013a). However, working with wild cod or wild fish in general was practically not feasible as a high number of larval and juvenile wild fish of the same age class and length were required. Therefore, hatchery fish had to be considered. European sea bass *Dicentrarchus labrax*, is a commercially important species in the North Sea and in contrast to cod, cultured close by, at the Ecloserie Marine de Gravelines in France. The constant supply of European sea bass and the vicinity of the hatchery facilities were a great added value to this PhD.

European sea bass *Dicentrarchus labrax* is frequently used in experimental studies (Malavasi et al., 2004, Malavasi et al., 2008, Pavlidis et al., 2011, Zouiten et al., 2011, Tsalafouta et al., 2015), including studies in bioacoustics (Neo et al., 2014, Neo et al., 2015b, Neo et al., in prep., Neo et al., submitted). Although it is a hatchery species, European sea bass is a stress sensitive species, especially at first feeding and when all fins are formed (Tsalafouta et al., 2015), but also as an adult (Fanouraki et al., 2011). Furthermore, it has a closed swim bladder (physoclist), and is consequently part of the highest risk group for barotrauma under high intensity impulsive sounds (Halvorsen et al., 2012a). It should be kept in mind that the behavioural responses of hatchery reared fish can deviate from their wild conspecifics. Such a deviation was found in the anti-predator response of European sea bass, where individuals from the hatchery responded weaker compared to wild individuals (Malavasi et al., 2004, Malavasi et al., 2008, Benhaim et al., 2012). Therefore the obtained results may be an underestimation of the real response.

Complexity of underwater sound measurements and reproduction

The sound field close to the sound source and sea surface is very complex, so measurements only represent a snapshot of that sound field. In the field experiment, the experimental cage holding the vials and hydrophone during the field experiment was submerged at 2.5 m below sea surface. This depth was determined by the length of the experimental setup and the technical limitations of the crane on board of the pile driving vessel. However, the standardized depth to measure underwater sound pressure was defined between ½ and ¾ of the total water column (Robinson et al., 2014). Since the spatial distribution of the sound pressure is depth dependent, lowering the fish and hydrophone deeper into the water would have exposed them to higher sound pressure levels (Robinson et al., 2014).

In the lab, playback of sound into the water is a major challenge. Acoustically controlled chambers have been developed to reproduce a homogeneous high intensity pile driving sound field (Bolle et al., 2012, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a). But even these highly technological devices have limitations in terms of frequency range, water content and salinity, and the size of the fish they can accommodate. (Bolle et al., 2012, Halvorsen et al., 2012b). To reproduce the sound field inside an open aquarium, one can also use underwater speakers (Mueller-Blenkle et al., 2010, Voellmy et al., 2014b, Neo et al., 2015b, Shafei Sabet et al., 2015). We used exciters attached to the aquarium wall that uniformly distributed the sound field (Chapter 5).

In each of these approaches, the created sound field is an approximation of the original sound, since the equipment has limitations in terms of sound level and frequency. The dimensions and features of the aquarium also have an impact on the complexity of the created sound field in terms of absorption, reflection and resonance (Carr et al., 2007). Since the lower frequencies (<1 kHz) are important studying behavioural disturbance (Chapter 5), standing waves of these frequencies need to be avoided. This was achieved by selecting aquarium dimensions (< ¼ λ) which cannot host standing waves smaller than 1 kHz.

Particle motion in an open aquarium cannot be controlled and needs to be measured. In this PhD, particle velocity was measured by three accelerometers which were mounted in the x-y-z axis on a stainless steel block, making the device negatively buoyant. Due to a technical defect of one of the accelerometers on board of the pile driving vessel, particle motion data could not be acquired. Unfortunately, further data problems with the three-axis accelerometer implied that we had to exclude the remaining results on particle motion from the PhD as well. Little data are available on particle motion but interest has grown over the last years. The measuring equipment has also only recently been made widely available. Therefore, future studies need to incorporate particle motion into this type of experiments since all fish perceive particle motion, including fish without a swim bladder. Its role in the effects of impulsive sound in fish should be clarified.

External stressors in the field study

The performed field experiment on board of the pile driving vessel (Chapter 2 and 3) was a unique chance to study a 'worst-case' scenario of pile driving on juvenile fish. Important results were obtained but the experiment implied high handling and transportation stress for the fish. Additionally, installing sound isolation as well as a sea water recirculation system was not feasible on board of the pile driving vessel. Also, all fish needed to be embarked at the beginning of the trip. Consequently, fish used to sample the second monopile of that trip already endured indirect vibrations of the first pile driving session. Our data showed the necessity to work with sound naïve fish. Therefore, fish should ideally be embarked between pile driving sessions.

2.2 From lab and field *experimental data to real impact assessment*

Lab, field, and natural experiments

Every experimental approach is chosen based on the research question under investigation, but obviously, each approach has its advantages and disadvantages. Three main experimental types were distinguished and considered for this study: (1) **laboratory-based** experiments, (2) **field** experiments and (3) **natural** experiments.

Lab experiments are a useful tool to investigate direct cause-effect relationships under controlled conditions (Calisi and Bentley, 2009). Repeating experiments under similar conditions is easier than in the field and fish can fully acclimatize to the artificial environment. Laboratory experiments can also be used to study the impact of specific sound characteristics on fish in detail.

Field experiments in bio-acoustics are carried out in various degrees of real-world conditions. In most cases, fish are held in some type of enclosure. The sound source is preferably an actual anthropogenic activity (Debusschere et al., 2014, Debusschere et al., 2016), although anthropogenic sound can also be played back by a loudspeaker in the field (Holles et al., 2013, Neo et al., submitted). The unique collaboration with the offshore wind industry allowed for performing a field experiment on board of the pile driving vessel. In our field experiment fish were held in a confined space and exposed to a complete pile driving session without an option to swim away, therefore representing the 'worst-case' scenario. This way, mortality, barotrauma and stress can be studied. The stress levels of the fish could be influenced by the lack of escape opportunities, which are always present in the wild. Field experiments are practically and technically challenging and cannot answer each question in detail. If they can be performed, they provide valuable and potential new insights in the concerning impact that may not be detected in the lab.

Natural experiments use wild fish in their natural environment. This type of experiment is the way forward to study the 'real' natural response of fish to anthropogenic sound (Hawkins et al., 2014, Radford and Simpson, 2014, Hawkins et al., 2015). Just like field experiments, they are subject to logistical challenges (Radford and Simpson, 2014). The sound source can either be a real human activity or a loudspeaker (Holles et al., 2013, Hawkins et al., 2014, Nedelec et al., 2014). In calm habitats such as enclosed bays or tropical coastal waters, sedentary fish behaviour can be filmed or observed by scuba divers. Echosounders can also be used to monitor school behaviour and even the individual behaviour within the school (Hawkins et al., 2014). Passive (*e.g.* acoustic telemetry) or active acoustics (*e.g.* echosounder) can be used to determine the behaviour of free-ranging fish to the sound exposure in the wild (Hawkins et al., 2015). These techniques are also suitable for rough and dynamic systems. Acoustic telemetry, for example has proven to provide valuable long-term information on fish behaviour in the North Sea (Reubens, 2013). These techniques offer new opportunities for future research and vary in the resolution of obtained data, at the individual level (Reubens et al., 2013b, Nedelec et al., 2014) or fish school and population level (Hawkins et al., 2014).

Extrapolation to the real world

Extrapolating experimental results to the real world and generalising results must always be done with great caution. In terms of fish species, the vulnerability of each studied species to the underwater sound must be assessed and its position on the vulnerability scale should be determined. In this study, a hatchery-reared physoclistous fish was used, meaning that they have a closed swim bladder. Consequently, sea bass belongs to the highest risk group for barotrauma due to its closed swim bladder, while the swim bladder itself plays only a minor role in increasing the hearing range and sensitivity since they do not have specialized hearing structures (Bouton et al., 2015). Therefore, European sea bass has not the highest hearing sensitivity possible in fish and stronger physiological and behavioural responses can be expected in fish with a higher hearing sensitivity such as herring (*Clupea harengus*) (Kastelein et al., 2008). In terms of ecological validity of the lab-based results, the establishment of a field-lab link proved to be very valuable. Such a link also allows to identify robust indicators and to extract detailed information on the results from the lab experiments (Debusschere et al., submitted, Neo et al., submitted). The results from the lab experiment evaluating the impact of impulsive sound underestimated the 'real' stress response elicited in the field by real pile driving (Chapter 3 and 4). In terms of sound type, the sound used in the studies must be representative for the real sound source in order to obtain meaningful data. For example, fish did not show a stress response or disturbed feeding when exposed to continuous Gaussian noise but they did under continuous shipping sound at a similar sound pressure level (Wysocki et al., 2006, Purser and Radford, 2011, Holles et al., 2013, Voellmy et al., 2014a). In our study, representativeness was achieved by using real anthropogenic sound sources (*e.g.* pile driving and SIG sparker), a high intensity acoustic chamber. Only the playback of real pile driving sound was less representative since it was a loop of one single pile driving session instead of multiple pile driving sessions. In terms of behavioural responses, it is essential to consider and, if possible, determine inter- and intraspecific variation. In this study, we focussed on one species, so integrating the results with other (future) studies is a prerequisite for a reliable extrapolation.

3 Are young fish affected by impulsive sound and to what extent?

3.1 Pile driving sound does not kill young fish

In this PhD study, we were able to perform a unique experiment on board of a pile driving vessel, where we exposed juvenile European sea bass to a complete pile driving session at 45 m from the sound source. No acute or delayed mortality was found due to pile driving (Chapter 2).

Airguns can induce mortality depending on the fish species and life stage, and on the distance to the sound source (Booman et al., 1996). For explosions, a negative relationship was found between fish size and mortality (Yelverton et al., 1975). The lethality of explosions was found to be directly related to their detonation velocity (Keevin and Hempen, 1997). Based on the potential of airguns and explosions to cause mortality in fish, a 100% mortality of fish larvae in a range of 1 km around the pile driving source was assumed in a Dutch impact study (Prins et al., 2009). In this study, no acute mortality was found as close as 45 m away from the pile driving activity. Both our data and the laboratory results performed in acoustically controlled chambers (*i.e.* the larvaebrator and the High Intensity Controlled Impedance Fluid filled wave Tube, HICI-ft), therefore strongly **contest this assumption** (Bolle et al., 2012, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a, Debusschere et al., 2014, Bolle et al., submitted-b). Within these experiments, acute mortality was tested on larvae, post-larvae and juveniles of physostomous and physoclistous round fish, and flatfish. The main difference with airguns and explosion studies was their higher measured zero-to-peak sound pressure levels ($L_{z\text{-}p}$ = 220 – 242 dB re 1 µPa) (Yelverton et al., 1975, Booman et al., 1996, Keevin and Hempen, 1997, Govoni et al., 2008, Bolle et al., submitted-b). Accordingly, pile driving could induce fish mortality at sound pressure levels that exceed the ones measured in the above-mentioned experiments (single strike sound exposure level (SEL_{ss}) = 183 dB re 1 µPa²·s; $L_{z\text{-}p}$ = 210 dB re 1 µPa and cumulative SEL (SEL_{cum}) = 216 dB re 1 µPa²·s).

High intensity impulsive sound can influence the swim bladder and can induce internal injuries, which could lead to delayed mortality (Halvorsen et al., 2012b). Barotrauma injuries in physoclistous fish, which are the most sensitive species, were seen at a minimum SEL_{ss} of

177 dB re 1 µPa²·s for 960 strikes yielding a $\mathrm{SEL}_{\mathrm{cum}}$ of 207 dB re 1 µPa²·s (Halvorsen et al., 2012a, Halvorsen et al., 2012b). These levels were exceeded during our field experiment and although internal injuries were not examined in this field study, we did not observe delayed mortality under optimal laboratory conditions (Debusschere et al., 2014). In addition, studies with the HICI-ft and larvaebrator suggested that fish could recover from internal injuries resulting from pile driving exposure, at least under laboratory conditions (Halvorsen et al., 2011, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a, Casper et al., 2013b, Bolle et al., submitted-a, Bolle et al., submitted-b). Delayed mortality can still occur in field situations, but is more likely to occur due to associated negative consequences of swim bladder buoyancy problems, such as a temporary inability to avoid predators, than from the injuries itself.

3.2 Impulsive sound induces a short-term stress response in juvenile fish

This PhD study was the first to study the physiological stress responses of fish under pile driving exposure and found that high intensity impulsive sound caused a stress response in juvenile fish which led to a temporary metabolic reduction.

A primary stress response was found in larval and juvenile European sea bass under high intensity impulsive sound (Chapter 3 and 4). Whole-body cortisol was used as indicator for the **primary stress response**, and proved to be very sensitive to handling, transportation, vibrations from the surroundings and high ambient sound. These confounding factors can easily mask the acoustically-induced primary stress response in fish (Chapter 3 and 4). A more clear primary stress response (*i.e.* serum cortisol) to airgun impulses was obtained in caged adult European sea bass, which also exhibited a secondary stress response (Santulli et al., 1999). In our field and lab experiment, the manifestation of a clear strong **secondary stress response** depended on the life stage and sound characteristics (discussed in subsection 4.3.). Especially oxygen consumption rate proved to be a robust indicator for the secondary acoustic stress response. On average, a 50% decrease in oxygen consumption rate was measured in juveniles during pile driving exposure and in larvae during sparker impulses, while the decrease in respiration rate in juveniles during sparker impulses was much more subtle. Since physiological responses are often expressed by a behavioural change (Barton, 2002, Martins et al., 2012), we can argue that a respiratory reduction of 50% will most likely be accompanied by a reduced activity level (Chapter 3). Such an anxiety-related reaction can even cause 'freezing' (Egan et al., 2009, Cachat et al., 2010, Barbosa et al., 2012, Kalueff et al., 2013). Freezing is also a behaviour shown in the presence of a predator (Vehanen, 2003, Malavasi et al., 2008) and leads to less predator-prey interactions (Ranaker et al., 2012). Fish that reduce their activity or freeze under high intensity impulsive sound will not actively escape from the sound source and will be exposed longer to higher sound levels compared to fish that can escape. This reduction in activity is however only induced when a certain amplitude of sound pressure is exceeded, as was indicated by the secondary stress response of larval and juvenile fish under minimum and maximum exposure of the sparker (Chapter 4). Consequently, if fish are scared away before the start of pile driving by using for example acoustic deterrents, that would probably diminish the number of freezing fish.

On the second day of the field study on board of the pile driving vessel, fish had already been subjected to indirect pile driving sound through vibrations of the vessel the day before (Debusschere et al., 2016). These fish showed a strong secondary stress response both under ambient and pile driving sound. The extent of the response was comparable to the acoustic stress response of the fish on day 1. This observation either indicates a prolonged stress response or a higher sensitivity to stress once exposed to pile driving sound.

Nevertheless, no long-term consequences were observed after being exposed directly and indirectly to a pile driving session (Chapter 3). The tertiary stress response was measured by changes in specific growth rate and Fulton's condition index after 30 days under optimal lab conditions. Similar to the observations in the pile driving study, physiological recovery was also observed in adult sea bass within ~72 h after airgun impulses (Santulli et al., 1999). No long-term stress responses were observed in goldfish (*Carassius auratus*) after 21 days continuous sound exposure (Smith et al., 2004) .

3.3 Disturbance of fish behaviour involves a broad spatial impact range around the sound source

Playback of pile driving sound briefly interrupted swimming and aggressive behaviour of juvenile sea bass, but the fish showed already recovery during the sound exposure. An attention shift caused a reduced number of food intake events on the first day, while this effect faded during the sound exposure on the two consecutive days (Chapter 5).

Several studies have started to explore the sound impact on fish behaviour of both continuous and impulsive sound. Field data showed reduced commercial catch rates of cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) after seismic surveys (Engas et al., 1996) and changed swimming patterns of Bluefin tuna (*Thunnus thynnus*) under boat noise (Sara et al., 2007). Recently, these field studies have been complemented by laboratory studies, which studied the response of fish to anthropogenic underwater sound in detail (Mueller-Blenkle et al., 2010, Neo et al., 2014, Neo et al., 2015b, Neo et al., in prep., Neo et al., submitted). Our behavioural study showed, in accordance with other lab studies, that fish showed a startle response at the onset of the sound exposure (e.g. Neo et al., 2014, Voellmy et al., 2014a, Debusschere et al., in prep.). The initial startle response is followed by a second behavioural response, most likely dependent on the acoustic stressor perceived by the fish. In our study, juvenile sea bass reduced their swimming activity and ceased aggressive attacks under pile driving playback (SEL_{ss} of 146 dB re 1 µPa²·s). Another study with European adult sea bass, showed increased swimming activity and bottom diving under impulsive sound playback (SEL_{ss} of 156 – 167 dB re 1 µPa²·s) (Neo et al., 2015a). At high intensity impulsive sound levels, as recorded during the field study (Debusschere et al., 2016), juvenile European sea bass decreased their respiration rate by 50%, which is most likely associated with a strong reduction in activity levels (freezing). In addition, the response of European sea bass to the acoustic stressor was stronger during the night than during the day (Neo et al., in prep.) In the lab experiments, juvenile and adult sea bass were able to recover to their pre-exposure behaviour during the sound exposure (Neo et al., 2014, Debusschere et al., in prep.). This recovery is most likely the result of a threat assessment performed by the fish, during which the acoustic stressor is considered harmless. This judgement can allow the fish to habituate to the sound during the sound exposure (Bejder et al., 2009) (also see BOX 1). Furthermore, fish were distracted by the sound exposure and by the presence of the food, resulting in a lower number of aggressive attacks.

Such an attention shift can also reduce the foraging performance of fish, including decreased food consumption and increased food handling errors, as observed during the acute impact studies of ~6 min. sound exposure (Purser and Radford, 2011, Voellmy et al., 2014a, Shafei Sabet et al., 2015). Under prolonged playback of pile driving (15 min. or more), the attention shift was also present on the first day of the behavioural study, as it reduced the number of food intake events during and after the sound exposure (Chapter 5). This effect faded on the second and third day and feeding was no longer affected. The ability to discriminate between food and non-food items was not investigated but this is more likely to occur in selective feeders (three-spined stickleback *Gasterosteus aculeatus*) than more active and opportunistic feeders (*e.g.* European minnow *Phoxinus phoxinus*, zebrafish *Danio rerio*, European sea bass) (Frost, 1943, Grant and Kramer, 1992, Volckaert et al., 2008, Matthews et al., 2010, Purser and Radford, 2011, Shafei Sabet et al., 2015). In addition, feeding efficiency rates were unaffected under prolonged sound exposure, but might be an underestimation since food pellets instead of living prey were used.

Whether there was sound or not, fish started to eat when food was added to the aquaria. This demonstrated that fish were still alert during and after the sound exposure. This could imply that they are also alert to other external stimuli, such as predators. However, our study provided no insight in the alertness of the fish at the onset of the sound exposure. Short-term lab studies combining ship noise and predators, found that juvenile eels (*Anguilla anguilla*) were less startled and caught more frequently by predators than under background sound, while three-spined stickleback had an increased response speed and the European minnow showed an unchanged anti-predator response (Voellmy et al., 2014b, Simpson et al., 2015). Still, to obtain an accurate view on the predator-prey interaction under anthropogenic noise, a real predator should be used since they can also be affected. The anti-predator response should also be studied under prolonged exposure.

BOX 1. Fish can assess the potential threat of a stressor (*i.e.* anthropogenic sound), resulting in sensitization, habituation or tolerance to the acoustic stressor.

Sensitization is the process where fish increase their responsiveness, and learn that prolonged or repeated exposure will have significant consequences for their wellbeing. **Habituation** is the opposite, fish show a persistent weakening in their responsiveness to the acoustic stressor until fish experience little to no effect.
Tolerance occurs when the intensity of the disturbance does not provoke a response in the fish

The behavioural responses of fish are likely to differ between species since not all fish detect the same range, amplitude or frequency (Popper and Carlson, 1998). In addition, the high variety of intrinsic characteristics (*i.e.* history, motivation, sex, size, coping style) are likely to influence their behavioural response (Overli et al., 2004, Silva et al., 2010, Martins et al., 2012, Voellmy, 2013). This makes extrapolations more difficult (Purser and Radford, 2011, Voellmy et al., 2014a, Shafei Sabet et al., 2015).

In conclusion, the results showed that juvenile fish can recover from the initial behavioural response over a brief period. Fish are able to habituate to the sound, at least under a limited number of repeated exposures (Neo et al., 2014, Debusschere et al., in prep., Neo et al., in prep.). However, short-term habituation does not necessarily apply to the long-term. For example, some fish, do not habituate to the seasonal boat noise but shift their foraging activity to the quiet periods of the day and week (Bracciali et al., 2012). Data on long-term habituation, sensitization or tolerance to repeated exposures is not yet available but is important to know the consequences on their distribution range and reproduction.

4 Are the effects related to specific sound-related or biological parameters and can sound thresholds be assigned?

4.1 The undefined difference between life stages

The National Marine Fisheries Service (NMFS) developed interim criteria for pile driving and distinguished **between fish <2 g and >2 g,** based on the positive relationship for mortality between impulse strength and body weight data from underwater explosions (Yelverton et al., 1975). Similarly, other studies show that early life stages of fish are more sensitive to toxicants (Azad, 2013) and possibly to other external stressors. In the case of pile driving, most studies are limited to one age group and are not comparing the effects of the same sound source on different life stages. In our study, no mortality was observed, so a difference in vulnerability for different size classes could not be analysed for this effect parameter (Chapter 2). Casper et al. (2013a) showed that juveniles (17 g) of hybrid striped bass sustained more injuries than juveniles (<2 g) under pile driving. In Chapter 4, we showed that the larvae exhibited a stronger secondary stress response under the maximum exposure of the sparker compared to the juveniles, although the stress response of the larvae was comparable to the stress response in juveniles exposed to pile driving. Therefore, more research is needed on the specific triggering sound parameters in the different life stages. Given the current limited amount of data, no such distinction can yet be supported or refuted for pile driving sound (Bolle et al., 2012, Halvorsen et al., 2012a, Casper et al., 2013a, Debusschere et al., 2014, Debusschere et al., 2016, Bolle et al., submitted-a, Bolle et al., submitted-b).

4.2 Not all fish are equal

Fish vary greatly in their vulnerability to stressors and response to the stressor. Anthropogenic sound is classified as a physical stressor, just as fishing. The physical stress caused by beam trawling is of little impact to skates (rays and sharks), while most whiting, herring and pouting are dead by the time fish are sorted on board of the fishing vessel. An intermediate sensitivity to beam trawling was seen in flatfishes, such as sole (*Solea solea*) and plaice (*Platessa platessa*), or in round fishes, such as cod (*Gadus morhua*) (Depestele et al., 2014). A comparable species-dependent sensitivity can be assumed with regard to anthropogenic underwater sound. Sound pressure influences the gas volume of the swim bladder, which can cause injury to the organs surrounding the swim bladder, rupture to the swim bladder itself, or induce physiological stress (Halvorsen et al., 2012a, Debusschere et al., 2016, Debusschere et al., submitted). During our first trip on board the pile driving vessel, adult whiting (*Merlangius merlangus*) were observed floating at the sea surface during the pile driving while no buoyancy/swim bladder problems were observed for European sea bass (personal observation June 18th, 2013).

Next to perceiving sound pressure with their swim bladder, fish can also really hear anthropogenic sound and that hearing can cause physiological stress and behavioural responses when sounds become loud noise (Hawkins et al., 2014, Voellmy et al., 2014a, Debusschere et al., in prep., Debusschere et al., submitted). Just as the responses to natural stressors, such as predator presence, the response to anthropogenic noise is characterised by inter- and intraspecific variations (Metcalfe et al., 1987, Barbosa et al., 2012, Voellmy et al., 2014b). The exact response of the individual fish or species may vary according to their hearing sensitivity and range, life history, fight-or-flight strategy, genetic build, body armour and threat assessment (Bonga, 1997, Popper and Carlson, 1998, Silva et al., 2010, Voellmy et al., 2014b). Most studies use hatchery-reared species, since it is difficult to obtain and maintain wild fish (DeTolla et al., 1995). Species suitable for aquaculture can endure some kind of suboptimal period and are inherently not the most sensitive species (Bartley et al., 2000). After a couple of generations, aquaculture species may differ in their behaviour or response, which can become slightly weaker than in their wild conspecifics (Ruzzante, 1994, Malavasi et al., 2004, Malavasi et al., 2008, Benhaim et al., 2012). When interpreting the results, the latter must be taken into account. We can also try to study the wild and more sensitive species by using non-invasive methods, such as active and passive acoustics. Nevertheless, the impact assessment studies need data based on species that vary in a variety of characteristics and over a variety of contexts and approaches.

4.3 Critical sound parameters triggering impact yet unclear

The results suggest that the frequency range of the high intensity sound is important for physiological stress responses, in which the lower frequencies (63 – 630 Hz) causing a primary stress response and the higher frequencies (>800 Hz) also a secondary stress response.

High intensity pile driving sound as close as 45 m did not induce mortality but induced a strong secondary stress response in juvenile European sea bass (Debusschere et al., 2016). As the field study described in this PhD thesis was the first one to tackle stress responses by pile driving, no information on the underlying triggering sound parameters was available. Therefore, two lab experiments were set up that could produce high intensity sound pressure levels comparable to the field experiment (SEl_{ss} of 181 – 186 dB re 1 $\mu Pa^2 \cdot s$; $L_{z\text{-}p}$ of 210 dB re 1 μPa; SEL_{cum} of 215-218 dB re 1 $\mu Pa^2 \cdot s$) but differing in their frequency content containing the highest sound energy (Chapter 4). So, both experiments partly overlapped with the measured field frequency spectrum (**field-lab link**). The combination of the SEL_{ss} and SEL_{cum} was sufficient to predict a stress response in European sea bass. The same combination of parameters was used to describe barotrauma (Halvorsen et al., 2012b). This correlation does not necessarily imply a causal relationship.

Still, the specifics of the stress response could not be derived from the combination of these standard sound pressure parameters (SEL_{ss}, $L_{z\text{-}p}$ and SEL_{cum}). All fish showed a primary stress response in all three experiments. Other than the similar levels of the SEL_{ss}, $L_{z\text{-}p}$ and SEL_{cum}, a shared overlap in high SEL_{ss} in 1/3 octave bands (315 – 630 Hz) was also found and is situated in the responsiveness range of European sea bass (0.1 – 0.7 kHz) (Kastelein et al., 2008). Therefore, we can conclude that the primary stress response could be triggered by hearing the high intensity impulsive sound. European sea bass, like most fish species hear in this frequency range that also corresponds with the major energy content of pile driving (Madsen et al., 2006, Tougaard et al., 2009, Slabbekoorn et al., 2010). A factor that is also important in hearing, but of which we still do not have enough data, is particle motion.

The oxygen consumption rate of the juveniles and larvae was decreasing under the high intensity impulsive sound exposure, except in the larvaebrator experiment. The main difference in sound characteristics was the higher frequency sound content in the sparker experiment (> 800 Hz), compared to the larvaebrator. Since the majority of the energy was located in the higher frequencies outside the hearing range of European sea bass, the impact of sound pressure could be critical, for example by causing swim bladder oscillations (Dalecki, 2008). This study indicated that swim bladder oscillations became stressful above a certain amplitude, the corresponding SEL_{ss} were between 170 and 181 dB re 1 $\mu Pa^2 \cdot s$ in the frequencies higher than 315 Hz. Under these sound characteristics larvae were more sensitive to the impulsive sound exposure than juveniles. The less pronounced secondary stress response of the juveniles in the lab experiment compared to the field experiment suggest that high intensity impulsive sound over a broader frequency range, as measured in the field, is needed to induce a strong stress response in juveniles, or that other sound parameters are important. Other studies regarding the stress responses of fish under continuous sound at much lower sound pressure levels reported the importance of the amplitude fluctuations of sound (Smith et al., 2004, Wysocki et al., 2006). Frequency fluctuation is also important: a difference in stress response and feeding behaviour was observed between boat sound and white sound[1] (Wysocki et al., 2006, Purser and Radford, 2011). Temporal structure (*i.e.* pulse repetition interval) in addition to SEL_{ss} was important in terms of behavioural disturbance (Neo et al., 2014, Neo et al., 2015b). Habituation to the sound may be delayed or disturbed by a change in sound characteristics and unpredictability of the sound (Rankin et al., 2009, Mueller-Blenkle et al., 2010).

There is a need for more in depth studies to identify the triggering sound parameters and to select biological characteristics that can be used in management.

4.4 Can sound thresholds be defined for fish?

Sound thresholds associated with specific impacts in fish need to be based on a variety of studies. Therefore, an overview of ISI catalogued papers that focused on the impact of pile driving on fish (in preparation, submitted, under revision or published dd 29 November 2015) is given in Table 1 to illustrate the available data at this time. All studies are recent, focus on impact at species level, and address the acute impact of one or multiple short-term sound exposure events. Studies have not yet tackled the long-term effects of sound exposure. The vast majority of these studies are laboratory experiments, and use hatchery-reared fish (except for Bolle et al., submitted-b). The studies focus on sound pressure whereas particle motion is not structurally included in the papers.

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[1] White (Gaussion) sound is a random signal with a flat power spectrum.

Table 1. Overview of the papers in preparation, submitted, under revision or published on the impact of pile driving on fish. Field experiments are indicated by a grey background color. Values of the sound pressure parameters are given to which fish were exposed in each study.

Fish species	Life stage	$\mathrm{SEL_{ss}}$ dB re 1 µPa²·s	$\mathrm{L_{z\text{-}p}}$ dB re 1 µPa	$\mathrm{SEL_{cum}}$ dB re 1 µPa²·s	SPL dB re 1µPa	Total number of strikes	References
Mortality							
Sole	larvae	186	210	206		999	Bolle et al. 2012
European sea bass	juveniles < 2 g	187	210	215-222		2282-3249	Debusschere et al. 2014
European sea bass; herring	larvae	186	210	216		999	Bolle et al. Submitted-b
Barotrauma injury							
Chinook salmon	juveniles	171 - 187	199-213	215-222		960 or 1960	Halvorsen et al. 2012a
Nile tilapia; lake sturgeon; hogchoker	juveniles	186		216		960	Halvorsen et al. 2012b
Chinook salmon	juveniles	180 and 187		210 and 217			Casper et al. 2012
hybrid striped bass; Mozambique tilapia		180-183-186		210-213-216		960	Casper et al. 2013a
hybrid striped bass	<2 g and 18g juveniles	186		216			Casper et al. 2013a
European sea bass	juvenile	185	209	215		999	Bolle et al. submitted-a
Inner ear damage							
hybrid striped bass; Mozambique tilapia		180-183-186		210-213-216		960	Casper et al. 2013a
Physiological stress							
European sea bass	juveniles < 2 g	187	210	215-222		2282-3249	Debusschere et al. 2015
European sea bass	larvae, juveniles < en >2 g	170-181-186	203-211-210	204-215-2016		999-2832-2723	Debusschere et al. Subm.
Behavioural responses							
European sea bass	adults				165		Neo et al. 2014
zebrafish	adults				120-140	450	Neo et al. 2015
zebrafish	adults				122	180	Sabet et al 2015
European sea bass	adults	156-167	180-192			1800	Neo et al. Subm.
European sea bass	adults	156-167	180-192			~1800	Neo et al. In prep.
European sea bass	juveniles	156	175	186		1000	Debusschere et al. In prep.

None of these studies found **acute fish mortality** related to pile driving. This does not mean that pile driving is harmless and that mortality thresholds are unnecessary: mortality can still occur at very close range (<45 m) of the pile driving activity (Popper and Hastings, 2009, Debusschere et al., 2014). Associated mortality can for example occur when sensitive species (*e.g.* whiting, see above) suffer from buoyancy problems and are eaten by predators. Popper et al. (2014) proposed sound thresholds for mortality and potential mortal injuries. Three categories based on sensitivity were distinguished for juveniles and adults: (1) fish without a swim bladder; (2) fish with a swim bladder that is not involved in hearing; and (3) fish with a swim bladder that is involved in hearing. Such a sensitivity based distinction could not yet be made for larvae (Bolle et al submitted; Debusschere et al 2014; Bolle et al 2012). Consequently, an $L_{z\text{-}p}$ of 207 dB re 1 µPa²·s was set for all larvae and for the most sensitive group of the juveniles and adults, *i.e.* the fish having a swim bladder involved in hearing. The proposed SEL_{cum} was 210 dB re 1 µPa²·s for larvae and at 207 dB re 1 µPa²·s for juveniles and adults (Popper et al., 2014). Our field and lab studies indicate that these values are still an underestimation of the real mortality thresholds (Chapter 2 to 4).

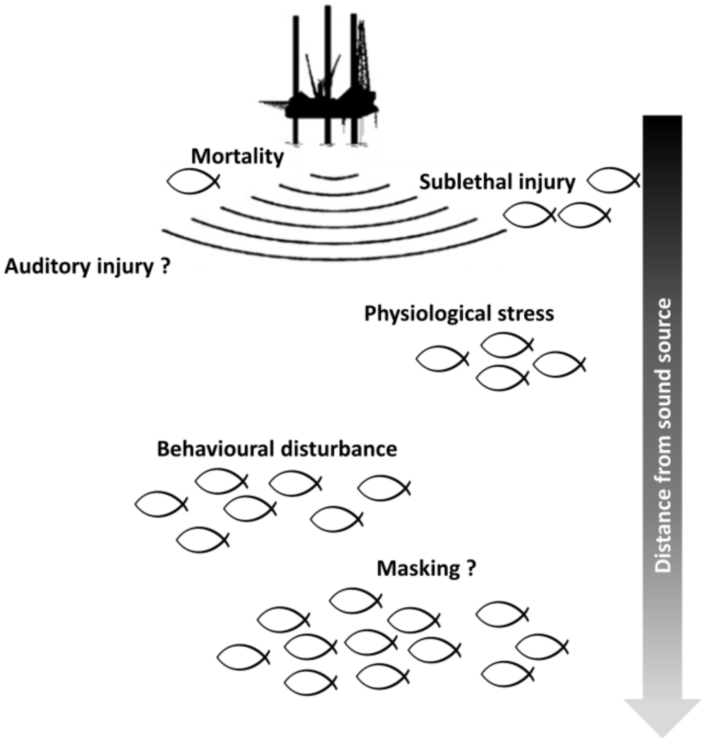

Figure 1. Visual presentation of the potential impacts fish can suffer from pile driving at a certain distance from the pile driving source. Figure modified from Slabbekoorn et al. (2010) and Thomsen et al. (2006).

The **physiological responses** of fish to pile driving sound are largely unknown. The primary, secondary and tertiary stress responses of European sea bass were investigated for the first time within the framework of this PhD (Debusschere et al., 2016, Debusschere et al., submitted). High intensity impulsive sounds, characterized by similar basic sound metrics $L_{z\text{-}p}$ of 210-211 dB re 1 µPa; SEL_{ss} of 181-186 dB re 1 µPa²·s and SEL_{cum} of 214-218dB re 1 µPa²·s evoked a stress response in larvae and juveniles of European sea bass. Although the specifics of the exhibited stress responses vary depending on frequency content and other unknown sound parameters. European sea bass showed a stress response between SEL_{ss} of 170 – 181 dB re 1 µPa²·s spread over frequencies higher than 315 Hz (Chapter 4). These should be seen as interim thresholds since this is based on one fish species. Additional studies on particle motion, frequency content of the sound, the swim bladder morphology and discomfort of the fish species need to be studied in depth in order to extract the combination of critical sound components and biological parameters.

Behavioural changes can occur at a wider range from the pile driving source since pile driving overlaps with the hearing range of most fish and is audible up to tens of kilometres from the source (Bailey et al., 2010, Slabbekoorn et al., 2010). Short-term responses (*e.g.* startle responses and intensified swimming behaviour (*e.g.* zebrafish) can be evoked by pile driving sound as low as sound pressure level (SPL) of 122 dB re 1 µPa (Neo et al., 2015a). Due to the high variation in the underlying intrinsic characteristics of fish species, assigning behavioural thresholds will be extremely difficult. To which behavioural response the sound threshold should be linked is still under debate. This can be to the initial response, the disturbance of a functional trait immediately affecting fitness, the level above habituation to the sound is unlikely, or to a number of repeated exposures to which the fish cannot habituate.

In conclusion, research papers on the impact of pile driving are all recent and much is yet to be explored in order to obtain a full assessment of the potential short- and long-term impact of pile driving on fish, either in single or multiple events. More data on a variety of fish species is required to establish general sound thresholds (BOX 2). In depth studies unravelling the critical sound parameters and biological characteristics are needed as well.

BOX 2. Sound thresholds for barotrauma and auditory injury in fish under pile driving

Barotrauma injuries have been studied in six species covering physoclistous fish, physostomous fish and flatfish (Halvorsen et al., 2011, Casper et al., 2012, Halvorsen et al., 2012a, Halvorsen et al., 2012b, Casper et al., 2013a, Casper et al., 2013b). These studies targeted juveniles and/or adults, not fish larvae. The onset of barotrauma corresponded to SEL_{ss}, number of strikes and SEL_{cum}. These values were drastically higher than the current interim criteria made by NMFS for barotrauma injury (*i.e.* 187 dB re 1 µPa²·s SEL_{cum} to 207 dB re 1 µPa²·s, generated by 960 strikes of a SEL_{ss} of 177 dB re 1 µPa².s (Oestman et al., 2009). So, Popper et al. (2014) proposed a new threshold for recoverable injury in the 'most sensitive' species, *i.e.* the ones having a swim bladder involved in hearing. This threshold was set at SEL_{cum} of 203 dB re 1 µPa²·s or L_{z-p} of 207 dB re 1 µPa. Barotrauma was thought to be related to the total energy impacting an individual, without considering SEL_{ss} and total number of strikes (Stadler and Woodbury, 2009). This so called '**equal energy hypothesis**' **was rejected** during this process of determining thresholds (Halvorsen et al., 2012b). It is now generally accepted that a threshold needs to incorporate both a SEL_{ss} and a SEL_{cum} parameter. This supports the idea of dual criteria since L_{z-p} and SEL_{ss} are closely related (Carlson et al., 2007). Still, the underlying mechanisms of barotrauma following underwater sound are not yet fully understood. There is for example, a variety in observed injuries between physoclists that cannot be explained (Halvorsen et al., 2012a). There are however indications that swim bladder morphology and frequency content of the sound could play a major role, but further research is needed to confirm this hypothesis (Halvorsen et al., 2012a, Halvorsen et al., 2012b, Popper et al., 2014).

Impact assessments of different source types on **auditory injury** in fish have shown that a **temporary threshold shift** can occur, most likely related to inner ear hair cell damage (Smith et al., 2006). Fish have the ability to recover from hair cell damage (Popper et al., 2005, Popper et al., 2007, Kane et al., 2010, Casper et al., 2013b). Only one study dealt with auditory and tissue injury due to the high intensity sound of pile driving (Casper et al., 2013b). At least for hybrid striped bass (white bass *Morone chrysops* x striped bass *Morone saxatilis*) barotrauma occurred more frequently than inner ear damage at these high intensity sound exposure levels (SEL_{ss} = 180 – 186 dB re 1 µPa²·s; SEL_{cum} = 210 -216 dB re 1 µPa²·s). A threshold to auditory injury cannot be assigned based on one study but it is highly likely that auditory injury will be covered by the thresholds assigned to prevent barotrauma injury.

5 Are pile driving effects on fish ecologically significant?

Determining thresholds is an important step in implementing acquired knowledge into daily practice. However, an analysis of the ecological significance of an observed impact will determine whether these thresholds must be translated into mitigation measures to be taken during offshore activities. So, are pile driving effects on fish ecologically significant?
Although the fish will most likely experience multiple events in their life, data are still limited to short-term individual responses (Erbe, 2013). The individual impact can provide the basis for a population impact assessment (Bejder et al., 2009). From an **ecological and conservation point of view**, individual effects in fish are subordinate to (sub)**population effects**. Consequently, population effects due to sound exposure need to be addressed.

5.1 Physiological stress impacts are negligible, yet long-term behavioural impacts remain unclear.

Mortality, barotrauma and most likely also auditory injury can occur in individual fish at high sound exposure levels found at close range of the pile driving source (see 6.4.4). Such small-scale effects are highly unlikely to cause a significant effect at population level. However, in some situations, such as the co-occurrence of pile driving at spawning grounds or at the migration route between spawning and nursery grounds, the population recruitment may be immediately affected. The acute stress response in juvenile fish to a pile driving session did not translate into long-term stress consequences and is therefore limited to a short-term metabolic reduction. This can be associated with acute vulnerability to other treats in the environment, such as diseases or predators (Bonga, 1997). However, these predators can also be affected by the pile driving sound.
Since young fish are attracted to the hard substrate around offshore wind farm foundations (OWFs) (Reubens et al., 2011), the construction of new OWFs close to operational OWFs will

affect a much higher number of fish and may involve serious ecological effects. Even though the measured effects are moderate and temporary, the altered physiological stress responses in the fish used on the second day of the field experiment indicate that cumulative effects are possible. Furthermore, repeated exposure to a stressor can turn a mild stress response into adverse effects, decreased immune system functioning, and disturbed foraging behaviour and reproduction (Kight and Swaddle, 2011, Nedelec et al., 2014, Simpson et al., 2015). Hence, caution is needed not to minimize small-scale individual impacts too far.

Behavioural disturbance can occur at lower sound pressure levels, implying a wide impact range around the pile driving source and consequently a larger portion of the fish populations that can be affected (Popper et al., 2014). At the moment, knowledge is limited to the acute effects on behaviour while no long-term data are available. These are needed to assess the ecological consequences of behavioural responses. Acute behavioural responses to pile driving, such as changes in swimming activity are difficult to translate into fitness consequences (Chapter 5). On the other hand, functional traits, such as foraging success and predator-prey interactions, are directly connected to the individual fitness (Shafei Sabet et al., 2015). The current studies suggest subtle recoverable effects on behaviour, but even the most subtle effects might turn out to be important. Short-term habituation does not give information on the long-term effects. Fish can habituate or sensitize to (repeated) exposure to anthropogenic impulsive sound, which will moderate or amplify the effects (Rankin et al., 2009). Recovery from their initial response during the sound exposure was observed in the behavioural study, but habituation over the three days was unclear (Chapter 5). Repeated exposure may involve long-term changes in behaviour, distribution and reproduction. A reduced spatial distribution for a prolonged period of time, may for example result in a reduced exchange of genes among subpopulations (Popper et al., 2014). On the long-term, anthropogenic sound might be considered as a selective force, favouring the individuals or species that are less sensitive to the sound exposure, with the potential to change species interactions (Slabbekoorn and Halfwerk, 2009, Voellmy, 2013). Natural experiments (using acoustic telemetry) are an ideal tool to study these impacts.

5.2 Impact at population level not clear?

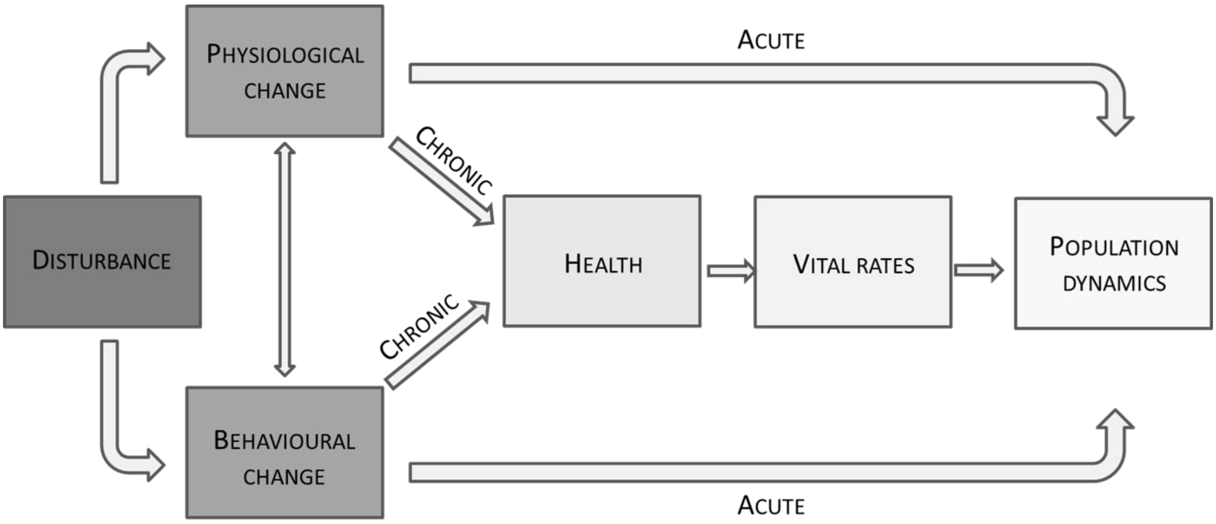

Figure 2. Population Consequences of Disturbance (PCoD) framework, adapted from Heinis et al. (2015).

Translating individual effects to a population level is a difficult task. However a roadmap has been developed to assess the effects of multiple impulsive underwater sound sources on the population level of marine mammals, via the population consequences of disturbance (PCoD) framework (NRC, 2005, Heinis et al., 2015). The same general principles can be applied to fish populations. The PCoD model has already been used to calculate the impact of pile driving and seismic sources on harbour porpoise (*Phocoena phocoena*) population in The Netherlands (Heinis et al., 2015). The following steps cover abiotic and biotic factors and serve as the required input data for the population consequences of disturbance (PCoD) model (step 6) (Figure 2):

Step 1. Quantify the impulsive sound sources (*e.g.* duty cycle, frequency, duration, sound levels) in time and space in the relevant area inhabited by the fish populations (*e.g.* Figure 3). Model the propagation of the sound of one or multiple sound sources (*e.g.* pile driving and seismic sources). The choice of an accurate propagation model is vital to obtain the best model which reflects the real situation. The model also needs to incorporate multiple sources which can be difficult. For example, using a simple geometric spreading model can underestimate the sound levels up 3 to 40 dB in the 0.1 – 10 kHz range of cumulative sound sources (Pine et al., 2014).

Step 2. Decide which effect of impulsive sound on fish individuals is the most relevant and can negatively influence their survival, maturation, reproduction (vital rates).

Step 3. Assign a sound level threshold to the unwanted effect, chosen in step 2.

Step 4. Calculate the area of disturbance (x km^2) around the sound sources, based on the sound propagation model (step 1) and sound threshold of the unwanted impact (step 3). Within this area, the number of individuals affected by the sound can be estimated if the abundances in the area are known. This group is labelled as the 'vulnerable sub-population'. Furthermore, the duration of the disturbance needs to be estimated and can be expressed as 'days of disturbance'.

Step 5. Translate the individual effects to the population level based on estimated population size.

Step 6. Feed the data to the PCoD model to calculate the consequences of the acoustic disturbance on a population level. Population effects are population growth and structure, transient dynamics, sensitivity, elasticity and extinction probability. The PCoD model calculates a relationship between the 'days of disturbance' and the population reduction.

At this stage, the model cannot yet be applied to fish since the data at individual level are still insufficient. In the model used on harbour porpoise, behavioural disturbance (*e.g*. avoidance) was assigned as the meaningful impact in step 2 (Heinis et al., 2015). This could also be the impact chosen for fish since mortality, barotrauma, and auditory injury are only occurring at close range. However, data on behavioural responses are insufficient to support this decision, especially on the long-term consequences. Therefore, quantifying the temporal and spatial scale of the impact on fish is not yet possible. Furthermore, the impact on K-strategists (marine mammals, elasmobranchs) will more easily translate into a population reduction compared to r-strategists (most fish) (Adams, 1980).

In the near future, the impact assessment should proceed from single species level to multispecies level, assessing the consequences of pile driving for the trophic food web (predator-prey interaction). In addition, the cumulative impact of other pressures present in the marine environment (*e.g.* fisheries, pollution, shipping noise) (Scheffer et al., 2005, Vasas et al., 2007, Popper et al., 2014) and repeated exposure to pile driving needs to be evaluated for fish on both short-term and long-term. Finally, the impact of noise pollution should be assessed at ecosystem level.

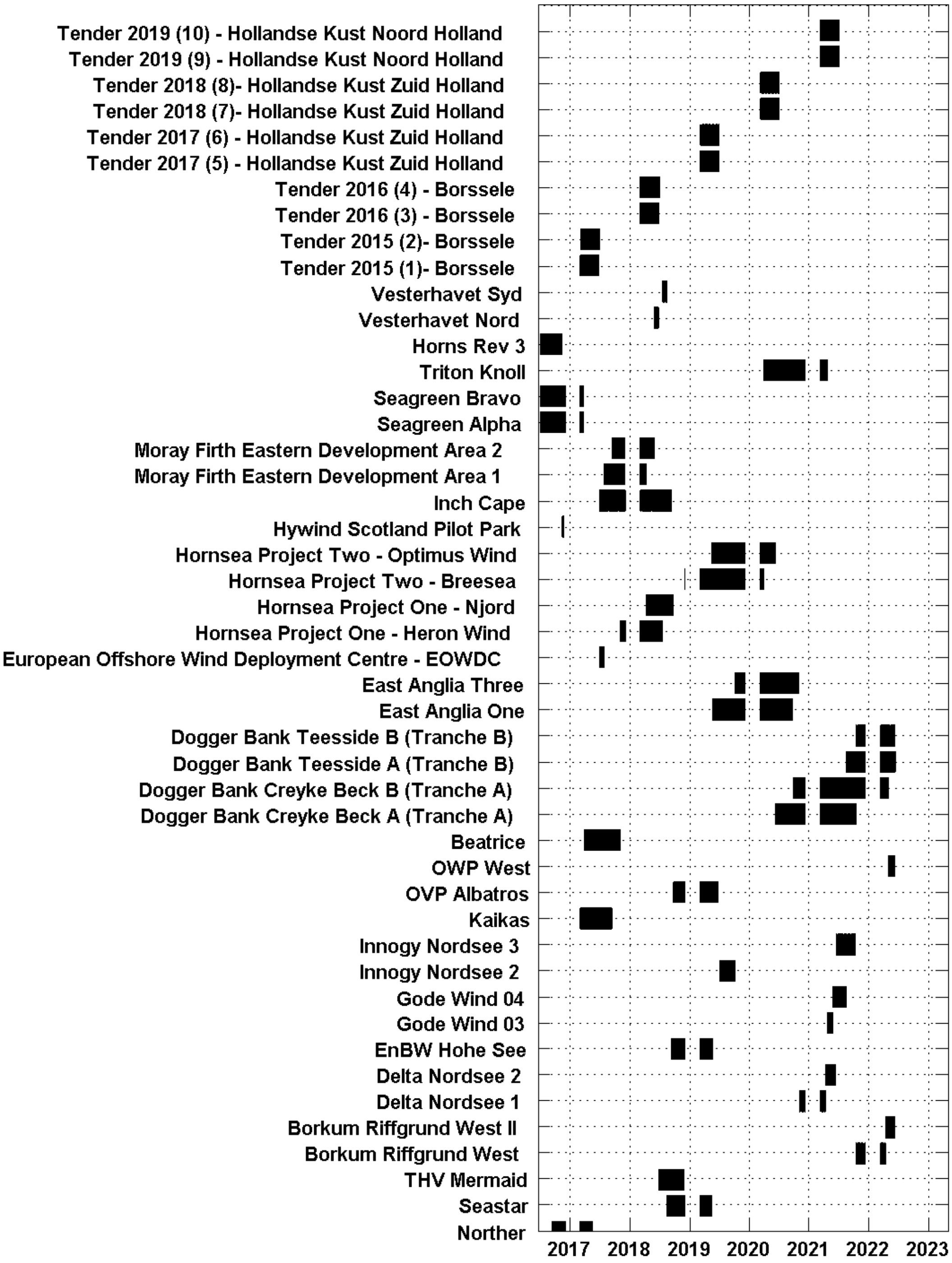

Figure 3. Example of the pile driving planning of the next couple of years in the North Sea to construct offshore wind farms. Figure taken from Heinis et al. (2015).

6 Do regional and national policies with regard to impulsive sound need to be refined?

6.1 Marine Strategy Framework Directive (MSFD): descriptor 11

The impact of pile driving on the health and wellbeing of marine life is a global concern (Williams et al., 2015). Anthropogenic noise can legally be described as a “transboundary pollutant”, since it travels across legal boundaries (McCarthy, 2004) and meets the definition of marine pollution by the United Nations Convention of the Law of the Sea (UNCLOS) (United Nations Division for Ocean Affairs and the Law of the Sea, 1982). The management of underwater sound is regulated at national level in the US and Australia. In Europe, a more international holistic approach is taken to regulate underwater sound, in the form of the Marine Strategy Framework Directive, MSFD (Erbe, 2013). The overall aim of MSFD is to achieve a good environmental status (GES) by 2020 of European marine waters, described by 11 descriptors (BOX 3).

BOX 3. Eleven qualitative descriptors to describe a good environmental status as giving with the MSFD framework (Directive 2008/56/EC).

Descriptor 1. Biodiversity is maintained.
Descriptor 2. Non-indigenous species do not adversely alter the ecosystem
Descriptor 3. The population of commercial fish species is healthy
Descriptor 4. Elements of food webs ensure long-term abundance and reproduction
Descriptor 5. Eutrophication is minimised
Descriptor 6. The sea floor integrity ensures functioning of the ecosystem
Descriptor 7. Alteration of hydrographical conditions does not adversely affect the ecosystem
Descriptor 8. Concentrations of contaminants do not affect the ecosystem
Descriptor 9. Contaminants in seafood are within safe levels
Descriptor 10. Marine litter does not cause harm
Descriptor 11. Introduction of energy (including underwater noise) does not adversely affect the ecosystem

A Technical Subgroup on underwater noise and other forms of energy (TSG Noise) was commissioned to further develop Descriptor 11. Two general indicators of underwater sound were agreed upon: (1) indicator 11.1.1 low and mid frequency impulsive noise; and (2) indicator 11.2.1 ambient noise (Commission Decision 2010/477/EU) (Van der Graaf et al., 2012). Within these indicators, marine mammals and fish are the species of concern (see BOX 4).

BOX 4. Indicators defined for the 11th descriptor of GES:

Indicator 11.1.1 on low and mid frequency impulsive sounds

The proportion of days and their distribution within a calendar year over areas of a determined surface, as well as their spatial distribution, in which anthropogenic sound sources exceed levels that are likely to entail significant impact on marine animals measured as Sound Exposure Level (in dB re 1 $\mu Pa^2 \cdot s$) or as peak sound pressure level (in dB re 1 µPa peak) at one metre, measured over the frequency band 10 Hz to 10 kHz.

Indicator 11.2.1 on continuous low frequency sound (ambient noise)

Trends in the annual average of the squared sound pressure associated with ambient noise in each of two third octave bands, one centred at 63 Hz and the other at 125 Hz, expressed as a level in decibels, in units of dB re 1 µPa, either measured directly at observation stations, or inferred from a model used to interpolate between or extrapolate from measurements at observation stations.

The MSFD targets the **ecosystem level** and addresses **the cumulative impact** of activities. Consequently, the indicator is insufficient to manage individual events. These must be regulated at national level, by environmental impact assessments (EIAs) (Dekeling et al., 2014). As most anthropogenic activities are licensed and executed under regulated conditions, an opportunity is created to initiate a coordinated and coherent protocol for measuring high intensity impulsive sounds.

The first step proposed by the TSG Noise is to create a sound register of the temporal and spatial scale of all impulsive sound sources. The goal is to establish the current level and trend of these impulsive sounds. The targeted impulsive sound sources for this sound register are airguns, pile driving (Figure 3), sonar and explosions and by extension sparkers, boomers and scientific echo sounders (Dekeling et al., 2014). TSG Noise suggests to work with spatial coverage of ¼ of the ICES rectangles and to register the sound levels at 1 m from the sound through back-calculations (SEL_{ss} or $L_{z\text{-}p}$) from the measured distance. Preferably extra information is logged in the register, *e.g.* time and date, gps position, source level, duration, duty cycle, frequency content, directivity, source depth, speed of sound source. This information needs to be shared on a regional sea level (Dekeling et al., 2014). This register can for example be used in the first step of the PCoD model.

The next step, comparable to step 2 of the PCoD model, is to choose the most relevant impact of impulsive sound on marine organisms. The TSG Noise decided upon ‘considerable displacement’ which may cause population effects. ‘Considerable displacement’ means displacement of a significant proportion of individuals for a relevant time period and at a relevant spatial scale” (Van der graaf 2012). The spatial distribution of this displacement can vary significantly depending on the species. The behavioural disturbance range around the pile driving source or other sources is much wider compared to the strong acute impact of (sub)lethal injuries. This results in a number of affected individuals, that is several orders of magnitude higher than the individuals with clear acute injuries (Mooney et al., 2009).

Cumulative exposures to different sound sources can also be modelled and these data are in fact an inventory of the **pulse-block days** in the EU’s regional seas (Van der Graaf et al., 2012). A pulse-block day is assigned to a ¼ ICES rectangle when a certain threshold is exceeded (step 4 of the PCoD model). These pulse-block days can be used in **marine spatial planning** to reduce cumulative impacts (Dekeling et al., 2014). Cumulative effects of repeated sound exposures and synergetic effects of other anthropogenic pressures need to be modelled as well, but the precise method is not yet fully understood (Van der Graaf et al., 2012, Erbe, 2013).

Overall, indicator 11.1.1 shows some serious shortcomings when considering fish (and marine invertebrates):

- The indicator covers all impulsive sound sources but it is not yet understood if fish and marine mammals respond in a similar way to all impulsive sound sources. At close range, the responses may differ between the sound sources, but as the sound signal changes with distance, behavioural responses may be the same at larger distance (Hermannsen et al., 2015). If behaviour responses are anxiety-driven, similar responses across impulsive sound sources can be expected.
- The indicator uses ‘displacement’ as the most relevant impact to cause population effects. While this suits marine mammals, it does not necessarily fit for fish. As shown in Chapter 3, and 5; fish do not necessarily swim away but may reduce their activity or even freeze during sound exposure, which can lead to a reduced distribution range. As the acute behavioural effects might be less ecologically relevant in fish compared to marine mammals under impulsive sound, more long-term data are needed to underpin the minor role of fish for this indicator.
- The indicator is a sound pressure indicator. Sound pressure is the main sound component involved in hearing in marine mammals, but fish and invertebrates mainly “hear” particle motion. At the moment, no causality or even a clear correlation between behavioural impacts of fish and sound pressure has been established. More research on the triggering sound parameters is necessary.
- The indicator proposes to work with SEL_{ss} and L_{z-p}, without any consideration of frequency content. As showed in Chapter 4, frequency content might be important causing stress in fish and consequently behavioural responses.
- The indicator requires that impulsive sound sources are indicated by a sound level at 1 m from the sound source. As measurements at 1 m are impossible, accurate sound propagation models are needed. In order to maximize comparison, an international agreement on the appropriate model is required.

6.2 Implementation of MSFD at member state level

In accordance with the MSFD, each member state (MS) is required to set its own ‘critical sound level’ for its marine waters. A review of EIA’s and legislation regarding underwater sound in EU countries (see BOX 5), demonstrates the country-dependent priorities in terms of species protection, associated mitigation and procedure to tackle the sound issue. This led to a disjoint and disparate management for the same region and ecosystem (Erbe, 2013). In each EU country, marine mammals are at the centre of underwater sound legislation and mitigation projects. The Netherlands was the only member state to include fish into their decision making process in the period 2011 to 2015, but it recently excluded the fish from the permit requirements (RWS, 2015). Overall, legislation in the member states is still at the basic level of single project management, except in The Netherlands. In the UK, there are no sound restrictions, and Belgium and Germany have a fixed sound level threshold. A fixed level is strict and may require expensive sound mitigation measures, going up to 22 million euro on mitigation for one OWF (Erbe, 2013) or it may results in a higher total number of strikes at reduced energy level per strike. The Netherlands advocate a new dynamic and flexible system assigning a ‘sound budget’ to the user. That sound budget is based on the number of ‘days of disturbance’ as suggested by the TSG Noise, a population impact assessment (PCoD model) and a percentage of acceptable population reduction over a number of years (RWS, 2015). A sound budget allows flexibility and is based on the number, diameter of piles, predicted decibel level and number of pile driving days. It does however require both a population estimation and a sound propagation model for multiple sound sources. In the UK, Germany and The Netherlands, individual marine renewable energy projects are already required to model the emitted sound. This can be used to establish the noise register proposed by the TSG Noise and to infer potential impacts on marine life.

BOX 5. Current legislation enforced by some member states

Belgium

Pile driving has to start with a ‘ramp-up’ procedure, slowly raising the energy per hammer blow (strike). Only after 10 min, can the maximum energy per blow be reached. This leaves an escape window for marine mammals to leave the pile driving area. The impulsive sound level at 750 m from the sound source should not exceed 185 dB re 1 µPa ($L_{z\text{-}p}$) (Rumes et al., 2015).

The Netherlands

The Netherlands chose to apply the precautionary approach and banned pile driving from January till June. In this period, the highest abundances of fish larvae are recorded. Pile driving at that time was expected to result in an unacceptable reduction of food availability for birds and marine mammals (van Damme et al., 2010, Boon, 2012, van Ginkel and Tach, 2014). After four years of monitoring (2011-2015), a consensus was reached that pile driving was not as deadly to fish larvae as expected in 2009 (Prins et al., 2009). This was based on lab experiments with the larvaebrator (Bolle et al., 2012, Bolle et al., submitted-b) and on other experiments (e.g. Halvorsen et al., 2012a, Debusschere et al., 2014). Priorities shifted and fish were excluded from monitoring priorities. The ban on pile driving was lifted and sound thresholds protecting marine mammals were established, varying between SEL_{ss} of 160 and 172 dB re 1 µPa²·s at 750 m from the sound source (RWS, 2015). The exact threshold is determined per case based on the total number of monopiles to be constructed and on the season. In all cases, a ramp-up procedure must be applied and underwater sound must be measured at each monopile. Fish are assumed to be protected by the rules enforced for marine mammals, since these are more sensitive to pile driving (RWS, 2015). Frequency content was not included into the sound threshold as the threshold would become too complex.

Germany

Germany decided on a limit for sound production of 160 dB re 1 µPa².s SEL_{ss} and 190 dB re 1 µPa peak-to-peak pressure level ($L_{p\text{-}p}$) at 750 m from the pile driving source (Betke, 2014). Prior to pile driving, marine mammals have to be scared away with deterrent devices. The onset of pile driving should follow a ramp-up procedure. During pile driving, the noise should be monitored at various distances to check the effectiveness of the mitigation measures (*e.g.* big bubble curtain) (Dähne et al., 2014).

UK

Currently, the UK does not set sound threshold levels for offshore pile driving activity, but developers are required to carry out project specific noise modelling as part of the application process. During the construction of monopile foundations, acoustic monitoring of a number of pile driving sessions is often required, but the measured levels do not influence the construction schedule. Mitigation measures in the UK include a ramp-up procedure and a combination of marine mammal observers and passive acoustic monitoring. If areas host resident seals, the Joint Nature Conservation Committee (JNCC) can ask to use acoustic deterrent devices (JNCC, 2010).

Mitigation method application varies between EU member states (see BOX 6). Mitigation measures aim to scare the marine fauna away or to reduce the overall decibel level emitted into the marine environment, but they are not necessarily reducing the sound pressure equally over the entire frequency range (Diederichs et al., 2014). Bubble curtains for example, are more effective in the higher frequency range (> 1kHz), which is optimal for marine mammals but not necessarily for fish, since most fish hear < 1 kHz. Still, fish are assumed to benefit from the legal frameworks installed to protect marine mammals (Diederichs et al., 2014, RWS, 2015). The ramp-up-start procedure can prolong the pile driving time and its effectivity is unclear. Furthermore, it is possible that fish are attracted rather than scared away (Cato, 2015, Neo et al., submitted).

BOX 6. Sound impact mitigations

The aim of sound impact mitigation is to reduce the impact of the emitted underwater sound on marine life. A variety of methods can be applied based at different stages of the sound production and transmission (overview see Verfuss, 2014):

- Deter marine life away from the pile driving zone (*e.g.* seal scarers, ramp-up procedure)
- Change foundation type (gravity based, suction bucket foundation)
- Change installation technique (blue piling, vibratory piling (not yet approved), offshore foundation drilling)
- Modify the hydraulic hammer (ramp-up procedure, piling cushion)
- Reduce the sound transmission in the water (bubble curtain, small, big, double, triple); hydro sound damper, casings)
- Pile driving period cannot concur with the most sensitive period for marine life (marine mammals, fish or invertebrates).

6.3 A responsible way forward

Given the increasing number of offshore activities generating impulsive sound on a transboundary scale, international collaboration and an agreement on how to proceed, are essential (figure 3). The Directive follows **an adaptive management approach**, so any new information on the consequences of sound on fish (or marine mammals) can be incorporated. In 2012, the marine strategies were set out for the first time and they will be reviewed every six years.

Based on the results of this PhD study, suggestions can be formulated for adaptations of policy at the regional and national level:

Suggestions at European level:

- An agreement on the definitions and aims of a GES is needed among the member states of Europe, in order to align legislation.

- The knowledge gaps should be identified and prioritized by the member states of Europe in order to improve baseline and specialized knowledge *e.g.* short-term and long-term behavioural impact and cumulative effects of multiple pressures and sound sources. The research should be spread over the member states to accelerate the data generation. Scientific proof is needed to support the exclusion of fish in management.
- The precautionary approach should be taken for important habitat, such as feeding, spawning and nursery grounds. Since these ensure recruitment, they are in need of special restrictions on time, duration and area of the impulsive activities (Nowacek et al., 2015).
- Regional and international alignment on sound propagation models, sound metrics and measuring methods will simplify the exchange of data on sound.
- Continuous acoustic monitoring of impulsive sounds needs to be carried out, registered and shared across the countries. Then, the sound register should be used in the coordination of the timing of impulsive sound activities in the same region, assigning sound budget per country or per project can minimize the ecological effects (IMO, 2001, Van der Graaf et al., 2012, Erbe, 2013). This will result in a compatible marine spatial planning among member states.

Suggestions at national level

- Environmental impact assessment should move from a single-source regulatory approach to the incorporation of multiple sources.
- National legislation should evolve towards the ecosystem approach, taking into account the most sensitive species into their policy. In sound pollution policy, marine mammals have been the number one target group from the start. At the moment, this seems ecologically justified as acute mass mortality of fish did not occur (see Chapter 2). Nevertheless, a GES applies to all marine fauna and caution is advised since long-term behavioural changes in fish are not yet studied.

7 General conclusions

Using a multidisciplinary approach, we examined the pile driving impact on young European sea bass (*Dicentrarchus labrax*) step by step. The results of this study largely contributed to the understanding of the impact of pile driving on fish. As a conclusion, the research questions of the PhD are answered:

(I) Are young fish (larvae and juveniles) affected by impulsive sound, what are the effects, and at what level do they manifest, *e.g.* mortality, stress responses or behavioural responses?

Young physoclistous fish are impacted by pile driving but the impact is more subtle than originally expected. No acute or delayed mortality due to pile driving was observed for young European sea bass (<2 g wet weight) as close as 45 m from the pile driving source. Based on these results, we reject the 'worst-case' scenario of 100% mortality of fish larvae in a radius of 400 - 1000 m around the pile driving (Debusschere et al., 2014). Under real-time and playback high intensity pile driving sound larvae and juveniles expressed a primary stress response (Debusschere et al., 2016, Debusschere et al., submitted). Dependent on the frequency content of the impulsive sound, also a secondary stress response was observed in larvae and juveniles. These stress responses involved a short-term metabolic reduction as no long-term (tertiary) stress response was observed one month after the real pile driving exposure. Nevertheless, repeated exposures to pile driving or other stressors might prolong or decrease re-establishment to homeostasis. At lower impulsive sound levels, fish showed a short-term acute behavioural response, expressed as decreased activity level and aggressive behaviour. Recovery of these behavioural features was observed during the sound exposure. During the first sound exposure, fish showed also signs of a noise-induced attention shift, which reduced the number of food intake events. This was no longer observed during repeated sound exposure. Fish remained alert under the sound exposure (Debusschere et al., in prep.).

(II) Can the effects on young fish be linked to a specific sound-related metric or biological parameter? Can we identify sound thresholds at which underwater sound negatively affects young fish?

Through the field-lab link in this PhD study, a stress response in larvae and juveniles could be linked to the combination of sound pressure parameters SEL_{ss}, SEL_{cum} and $L_{z\text{-}p}$ (Debusschere et al., submitted). Primary stress response is more likely to be caused by hearing (lower frequency range) while the secondary stress response is most likely also associated with swim bladder oscillations. The secondary stress response was linked with the higher frequencies, especially for larvae, while juveniles required high sound energy over a broader frequency range. This study is the first to propose a sound threshold range at which physiological stress is induced in fish. High intensity impulsive sound is needed at SEL_{ss} levels between 170 and 181 dB re 1 $\mu Pa^2 \cdot s$ spread over frequencies higher than 315 Hz (Debusschere et al., submitted).

(III) What is the ecological significance of the observed effects

From an ecological viewpoint, individual short-term effects are less important compared to long-term effects at population level. Since no acute or delayed mortality were found and the physiological stress responses were limited in time, these acute effects are unlikely to be of any ecological significance. Behavioural responses are only studied during and shortly after the impulsive sound exposure. In this specific study, the observed effect was rather mild. However, based on the diversity of behavioural responses, more research is required on behavioural disturbance and to decide on its ecological relevance (Chapter 6).

(IV) How will the results from this PhD add up to management and policy regulations in Belgium (and Europe), *i.e.* in order to minimise the environmental impact of pile driving activities in future offshore wind farms, and to achieve GES for MSFD descriptor 11?

No dramatic ecological impact or collapse due to impulsive sound on young fish was found. The effects on fish are more subtle than anticipated and therefore, no stringent measures are needed ad hoc in Belgium or other member states. However, valuable suggestions are made with regard to national and European regulations.

(1) More long-term data and targeted detailed research studies are needed to study the ecological consequences of the more subtle behavioural effects. This will provide more extensive scientific evidence in support of or against the decision to exclude fish from management and the statement that fish are protected under the marine mammal sound thresholds set by national law.

(2) It is yet not clear how and if a stressful event at fish egg or larval stage will have an effect later in their life. Therefore, the precautionary approach should apply for spawning grounds of fish and taken into account during the planning of the offshore construction phase.

(3) A suggestion to the current regulations in Belgium is the monitoring and modelling of the emitted underwater sound of the pile driving in the Belgian Part of the North Sea. This can directly be used in the proposed register of TSG Noise.

8 Remaining challenges and opportunities for future research

Impact assessment of pile driving is a relatively new chapter in the bioacoustics research (Table 1) and therefore, several questions remain. Mortality and barotrauma have been addressed by a number of studies, so the attention has moved to physiological and behavioural responses to impulsive sound. We started this PhD with a doomsday scenario. Four years of bioacoustics research have scaled back the potential impacts on fish but also demonstrated the complexity and variety of responses to impulsive sound. This PhD was executed on a single species to understand the basic responses. The following challenges need to be investigated to advance our knowledge on the impact of man-made sound on fish, to feed population models and to progress **towards an acoustically sound approach** in terms of human activities.

(1) **Particle motion** remains a big gap in this research field. Particle motion is the most important sound component involved in hearing of fish (and marine invertebrates) and consequently, it is the dominant component acting at behavioural level. Despite our efforts, we did not succeed in obtaining qualitative data on particle motion in our experiments. Very recently, particle motion is gaining more attention and the equipment and analysing method is being developed and tested (Nedelec et al., 2016). Particle motion needs to be measured in all lab set-ups and in the near field. Then, the role of particle motion in the negative effects encountered by fish under impulsive sound should be clarified. This will lead to a more accurate and complete assessment. In case particle motion is ecologically relevant, it must be included in the legal framework (EIAs) and monitoring.

(2) The quest for **critical sound parameters** ($L_{z\text{-}p}$, SEL_{ss}, SEL_{cum}, single strike particle velocity exposure level (VEL_{ss}), cumulative VEL (VEL_{cum}), zero-to-peak particle velocity level ($V_{z\text{-}p}$)) and sound characteristics (*e.g.* duration, amplitude, spectral and temporal frequency, predictability, rise time) that induce the effects in fish needs to be continued further. Based on the findings, meaningful suggestions for conservation and management strategies will follow. Furthermore, they will allow to determining whether the sound thresholds established to protect marine mammals also protect fish.

(3) The current studies are based on single species level and should evolve towards **multispecies level**. However, single species studies form the basis of all further research and the specific individual impact needs to be thoroughly assessed, based on an integrative framework, combining biochemical, genetic and physiological processes. Therefore, this research needs a variety of species, preferably with a larger share of wild fish, which vary in age class, life history and strategy, swim bladder morphology, and which are studied in a variety of contexts. Both short-term and long-term data need to be gathered and in order to determine the **ecological consequences** of the pile driving on fish, targeted research is needed on:

- the impulsive sound impact on fish eggs
- the impulsive sound impact on larval settlement
- the long-term consequences of the sound exposure during early life stages of fish
- the impulsive sound impact on adult fish (stress responses, behavioural disturbance, reproductive behaviour, communication)
- the impact of repeated sound exposures on the functional traits (*e.g.* foraging behaviour, anti-predator behaviour) of fish. The biological consequences on the individual fish relate to the impact severity of one sound event, the recovery ability between two sound exposures and the total number of sound exposure events. Here, multiple species at different levels of the food web can be introduced
- the synergetic effects of other pressures (*e.g.* pollution, fisheries, climate change) during the sound exposure
- the fish distribution during and after multiple sound exposure events in the wild. Will they leave good spawning and feeding grounds and are their fitness consequences on the long-term

(4) An extrapolation can be made to the **population and community level** based on the data combined with the sound register. In case of deleterious effects, the impact on the **food web** (multispecies) needs to be assessed. Answering these research questions will require a combination of lab, field and natural experiments and a good collaboration with the offshore wind industry. Experiments exploiting new technologies (*e.g.* active acoustics, tagging) at an appropriate spatial and temporal scale and for a variety of sound sources should

be encouraged. Especially studies tackling the behavioural impact require detailed observations of the individuals and schools in the natural environment.

(5) Last but not least, once the OWF is installed, the operational phase starts and continuous sound will be generated during at least the following 20 years (Norro et al., 2011). It raises the background sound pressure level with ~20 dB in the whole frequency range (Norro et al., 2011). This sound overlaps with the hearing range of fish (Slabbekoorn et al., 2010) and is categorized under the second indicator of the TSG Noise (Indicator 11.2.1, see BOX 4) (Van der Graaf et al., 2012). In some countries (*e.g.* Belgium and The Netherlands), the OWFs are a fisheries free zone and serve as a refugium for marine life (Reubens, 2013, van Ginkel and Tach, 2014). Fish and in particular juveniles are attracted to the hard substrates that host a rich invertebrate epifauna that can serve as a food source (Reubens et al., 2011, Reubens et al., 2014). The impact of the continuously raised sound levels needs to be investigated. These levels are unlikely to scare fish away but they can potentially disturb the behaviour, cause chronic stress, mask communication and orientation signals and decrease the overall fitness and survival of the individual fish (Wahlberg and Westerberg, 2005). Studies should focus on the impact of the juveniles and the ecological relevance at population level. At the moment, no long-term studies have yet been performed, even though OWFs are the perfect site for a natural set-up experiment.

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