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Connectivity of larval and juvenile common sole at a small and large spatial scale

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DISSERTATION PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR IN SCIENCES (BIOLOGY)

JULY 2019

JULY 2019

CONNECTIVITY OF LARVAL AND JUVENILE COMMON SOLE AT A SMALL AND LARGE SPATIAL SCALE

CONNECTIVITEIT VAN LARVALE EN JUVENIELE TONG OP EEN KLEINE EN GROTE RUIMTELIJKE SCHAAL

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Sciences (Biology)

July 2019

Uitgegeven in eigen beheer, Sophie Delerue-Ricard, Bruxelles

The research presented in this dissertation was funded by a grant of the Research Foundation – Flanders (FWO)

“We have to remember that what we observe is not nature in itself but nature exposed to our method of questioning “

Werner Heisenberg (1958)

ACKNOWLEDGEMENTS

First of all, thanks to all the members of my PhD jury and evaluation committee. You made this PhD come through and I am grateful for your time and efforts. A true thank word to Filip who gave me the opportunity to realize my biggest childhood dream of working in Marine Sciences (quite surprising for a countryside girl, raised far from the Sea, and different from most of my primary school classmates who wanted to become farmers), and my latest grown up dream of tracing early-life stages using population genomics, otolith shape and microchemistry work, on this cool project that is B-FishConnect, alongside Léo, Andreas, Geneviève, Kris, Filip and Johan.

A PhD was a professional but also a personal journey. I am pretty sure that not everybody goes that deep into soul searching to face the stress such an achievement can represent, but I decided to go for it and it made great changes in my life. It required so much adaptability, changes, efforts, hard work, collaborations, team work, motivation, determination, persuasion, loneliness, hope, trust, love, relaxation, fight… It was a whole trip to new horizons and it ended up after 6 years because I always kept on being eager to learn. I started learning and practicing meditation (with my loving co-practitioners), reiki, taichi (with the amazing master and friend Rolande), yoga for beginners, therapy (with the amazing Christine), love, self-love, self-confidence, acceptance of my own weaknesses and strengths, improved my communication skills and kept on practicing my favorite sport: diving, trained by excellent Belgian divers (who can dive in cold, dark, rough waters!!). I am not pretending to be any better today than yesterday but I am trying and that is quite an interesting perspective.

Friendship and love made my Belgian adventure a fantastic journey, filled with new ideas, feelings and hopes. I am thankful for the people I met on my way and who care for making this life a better life.

I would have never made it without the friendship and team work of the LBEG team (actual and former) members. That lab created a small family over the years thanks to the skill of Filip in choosing people who really fit in the team spirit. I would like to mention all of you who made that PhD a great journey but I'm sure I will forget some people, I hope you will forgive me for that. I would like to thank a billion times the core family of my PhD's life span: Franz, Els, Henrik, Nele, Jasmien, Pascal, Anurag, Federico and his (sometimes friendly) sock puppets, Ilaria & Allan, Nellie & Manu for staying zen ;) and organizing THE most amazing barathon of Leuven's History, and my other very close lab mates outside of the LBEG group: Leticía for our deep connexions beyond all borders, Lynn and Marie for all the laughs and understanding during our French/Dutch lunches, Sharon, Anouk & Bjorn for the wonderful welcome in your lovely board games volunteering association, Jelle, Luna & Sascha for all the love and great food, Jelena for your amazingness and the great work, Uli, Edwin, Laurens, Nedim, Konrad, Sarah P. for your kindness and all your efforts to make the Kolen Museum a better place for Internationals, and my Brazilian family who warms my heart: Toshiro & Cintia, Monica, Amélie & Andros, and Gio, Sara & Tiago.

Hartelijk bedankt to my flatfish family: Eveline & Sara, my flatfish mums, Els C. (who I never met but carefully read!), Adriaan R., Henk VdW., Audrey G. & Richard N. (who I met and still carefully read), Kevin, Andreas, and Kris (last but not least to the B-FishConnect project), my ILVO mates.

Special thanks to all students who helped us during these six years, including the great collaborations with Nele, Hanna, and Joren. Conny you are the jewel of all secretaries. I understand that no one wants to let you go in the Kolen Museum. Bart, you are our Conny. Working by your side Bart was a great pleasure and you have no clue how much of a support is your smile in the morning… because Bart is almost always the first one to open the lab doors :D ! Note for the future lab mates: if Bart is not there, either it is a weekend... or it is a holiday and you didn't know about it! Damned! I have special warm thoughts for all the PhD students who are still working on their PhD these days, especially Léo, Henrik, Franz, Els, Quentin M., Io, Sarah, Thomas K., Tim and Anna. Remember that you are the most valuable help to each other and you will benefit from teaming up as much as you can. Believe in yourself, set yours goals and boundaries. You can do it! I believe in you.

I also would like to thank the grown up kids of the LBEG lab and close-by labs for making the family stronger and switching to English or French in group discussions (as often as possible): Greg, Joost R., Maarten L., Maarten VS., Maarten VH, Anton, Caroline, Pieter, and Till for the crazy fish(y) reward you once offered me to make the reward from my best student award talk come true!! Thanks to Gary C. for bringing molecular markers and evolutionary questions across my way during my Erasmus in 2010 in Bangor, thanks to the team of teachers and administrative supports of the Master of Oceanography of Paris, Alain & Co, with a special mention to the Roscoff team with Eric T., Nathalie S., Christophe D., Christophe S., and Thierry C. for his supervision during my master thesis. I am thankful to Kelig M. and Bruno E. from Ifremer, and Fabien M. who supported me a lot more than expected through the publication of my first paper! Thank you for making University a better place! I also have a very special though for the bioinformaticians of the HPC of Leuven – especially Ehsan and Geert Jan – who made it possible for me to run a fancy bioinformatic pipeline ingesting huge amounts of genomic data on the cluster and ensure them that I will finally stop bothering them with my biology oriented requests and sincere apologies for my perseverant, curious and investigative nature.

Dear Audrey G., I learnt a lot from our collaboration in Bergen in many professional and personal aspects. Thank you for all your support from far away and the very nice visits and brunches in Brussels. That counted a lot for me as a woman scientist and human being in search of guidance. A very kind thanks to the team in Bergen with a special mention to Julie, Christel, and Siv who made this intense work experience a lot more enjoyable. In general, I would like to thank and encourage all the women in Science for all the hard work and perseverance. I would also like to thank all men in Science who help making equality a reality. As Christine says, trust the strength of the Feminine in you (which works for both all gender). I wish a colorful, gender equal, supportive, diverse and representative University (will take time but) is possible. Let's start now! Nothing Is Never Too Late. Let's "be the change you want to see in the World" as Gandhi said.

Léo, you get a paragraph but that will never be enough to tell you all my gratitude for our collaboration on this co-PhD thingy within the B-FishConnect project. Des apéros corses aux répétitions de présentations de conf', en passant par les moments de déprime et de coups durs, en partageant la joie d'un beau Martí sur cette Terre et de tes lectures toutes les unes plus intéressantes que les autres, et j'en passe. Je te souhaite tout le meilleur à toi et ta famille si mignonne dans votre appart' tout beau (une fois que vous aurez fini de le rénover, avec l'aide

de quelques petites mains volontaires !). Je finirai par ces deux mots magiques qui nous ont unis dès le début de cette aventure grâce à nos racines : *aligot* - *saucisse*.

I would like to thank all my families: Delerue, Ricard, Sournac, Ardouin, Vic, Pichot, Monier, Percival. Vous faites partie de qui je suis.

Merci à mes sœurs qui sont de si belles femmes travailleuses, passionnées et aimantes. Je prends modèle sur vous depuis le début (en opposition ou en similitude, bien évidemment) ! Merci d'avoir accepté d'être mes sœurs au-delà de nos différences. Merci à mes jumelles, Anne et Laure, qui sont des soutiens complémentaires, vitaux et intemporels – toujours accompagnées de Irène Machtrouk, Boris et Jeanine Morillon. Merci à mes neveux et nièces de m'avoir donné le virus d'expliquer la Science et le fond des océans avec des mots simples. Un merci tout particulier à René, Paul P. et mon parrain, Christian, et ma marraine, Brigitte, qui ont su cultiver la curiosité et l'émerveillement pour la Vie, la Sagesse, l'écologie, et la spiritualité chez moi et tant d'autres. Et merci à mes ami-e-s de tous temps, Soizic, Fanny, Leticía, Charlotte et Carole, Cariiiine, Vincent, Franz et Felix, Uli, Ariane et Alexia pour votre amitié durable. Last but not least, a very special and warm thanks to the team Jean d'Ardenne and Las Amazonas, the strong, independent, lovely and free women that I got the pleasure to live with in these Belgian years of happiness and though hard work. Darlings, you know you all deserve to live your dreams, so go for it!!!! Un merci tout particulier à Annelies, Elena, Keti, Martina et Miriam qui ont vécu avec une Sophie en fin de thèse pendant 2 ans voire 3 ! Chapeau !

Merci Dimitri pour toute ta confiance, ta douceur, ton soutien, ta générosité, ton amour et tous les beaux moments de rires, de plongées et de belles choses à construire que nous avons partagé et allons partager. Hartelijk bedankt.

Merci pour la douceur, la confiance, les coups de pouces et toutes les erreurs d'éducation à Hervé et Marie, les parents que m'a donnée la Vie (cf. la citation de Khalil Gibran sur les enfants). Merci à la Vie et ses surprises !

Le sujet de cette thèse est intéressant à plus d'un niveau puisqu'il traite des origines et des connexions entre ADN, chimie, et compréhension de mouvements de migrations. Sommes-nous seulement définissables par nos racines biologiques ou bien pouvons-nous évoluer et devenir un être complexe et riche de diversité grâce à nos migrations, en lien ou pas avec nos racines ?

Je dédicace cette thèse aux enfants, femmes et hommes migrants et réfugiés de notre planète, qui ont bien souvent plus de difficultés à se déplacer pour trouver refuge que les poissons de nos océans qui s'exportent illégalement à travers le Monde, bien trop facilement à mon avis.

I would like to dedicate this PhD to migrant children, women and men, refugees of our planet, who have too often more troubles to move to reach a refuge than fish of our oceans that are illegally exported around the World, way too easily in my opinion.

Sophie, Brussels, 20th of June 2019, World Refugee Day


@VLIZ DAY 2016

SUMMARY

Population persistence depends on recruitment during early life. In marine populations, colonization, replenishment and resilience is influenced by connectivity between spawning and nursery grounds. Larval connectivity, i.e. the successful exchange of individuals between spawning and nursery grounds, is therefore a crucial factor for successful recruitment. Recruitment can be highly variable on small temporal and spatial scales. Yet, empirical evidence of connectivity between distinct spawning and nursery grounds is scarce. Connectivity of the early-life stages of marine organisms is complex to explore due to poor knowledge on larval sources and sinks, and sampling challenges. Understanding connectivity at the scale of larval dispersal is paramount to manage metapopulation persistence, prompting for the further development of multi-disciplinary approaches. Fisheries research is increasingly conceptually and technically benefiting from the integration of genetic and phenotypic studies to unravel the evolutionary mechanisms of population structure.

In this thesis, we developed and integrated novel knowledge on the early-life connectivity of a flatfish at the scale of dispersal (5-500 km) with a multi-disciplinary approach. An intensive sampling program on a small geographical scale was conducted to collect juvenile sole *Solea solea* in the Southern North Sea and English Channel. Adult sole from existing collections were reanalyzed. Local connectivity was estimated by otolith shape, elemental analysis and molecular markers. The information from each of these tools contributed to a specific aspect of the ecological and evolutionary understanding of dispersal throughout the life cycle. Molecular markers were used as individual tracers of movement and as population tracers of gene flow. Otolith shape is a reliable individual phenotypic trait and elemental analysis tracks an individual from close to birth to capture. But more important, the combination of markers sensitive to complementary ecological and evolutionary processes is most promising. The multi-disciplinary approach proved useful to account for the local dynamics involved in the connectivity between spawning and nursery grounds and on the nursery grounds.

Juvenile sole sampled on the nursery grounds of the Southern North Sea were markedly different in otolith chemistry. The differences in elemental composition (especially for the elements Sr, Mg and Zn) suggested that movement following settlement is limited on the nursery ground, while pre-settlement dispersal is likely restricted to about 100 km. Thus, our results suggested a longer dispersal distance before settlement than after settlement, with some but overall few juvenile migrants between nursery grounds. Each sampling region locally

recruited uneven proportions of juveniles (0-100%) from four natal sources. This result validated modeling outputs suggesting that a single spawning ground may contribute to several nursery grounds. Evidence for local recruitment, larval dispersal to several neighboring nursery grounds and limited connectivity between nursery grounds of sole can contribute to the validation of biophysical models.

Despite the limited connectivity between nursery grounds, the level of genetic and otolith shape differentiation of juvenile sole in the North Sea is low. The low genetic differentiation could be explained by limited but significant mixing between nursery grounds at the juvenile stage, but also by gene flow on the spawning aggregations (at the egg or adult stage). Just a few migrants per generation are needed to maintain a high level of gene flow. The low level of successful recruitment of migrants observed with elemental analysis is compatible with the homogeneous population structure of sole in the North Sea as revealed by genetic markers. Yet, there is no evidence for sole of genetic mixing between spawning aggregations.

Regardless of high connectivity within the Southern North Sea, some evidence pointed to local structuring of sole. Within nurseries, elemental composition separated the western and the eastern sides of the Belgian nursery which might be explained by inflow from the Western Scheldt influencing water elemental composition. Local differences in putative adaptive markers between cohorts may be linked to selective pressure acting on 0-group sole on the nursery grounds. However, a causal link between molecular diversity and cohort structure would require further research. Provided that a small proportion of adults successfully contributes to the next generation (as predicted by evidence of sweepstake recruitment), local genetic differences may originate from one panmictic spawning aggregation. In addition, kin structure (i.e. settlement of related individuals in the same area) has been detected on the Belgian nursery. Our results suggest, as expected, limited relatedness between individuals which will not influence the Wahlund effect. Testing for chaotic genetic patchiness, a common feature of marine organisms, would require additional sampling.

As highlighted by this study, elemental composition and putatively adaptive genetic markers are of considerable value to understand connectivity in species with extended larval dispersal dynamics and / or low levels of genetic differentiation as it is the case for sole in the North Sea. The findings are meant to calibrate and to be combined with biophysical models to contribute to stock management.

SAMENVATTING

Het in stand houden van vispopulaties is afhankelijk van rekrutering tijdens de eerste levensfases. Kolonisatie, toename en veerkracht van mariene vispopulaties worden beïnvloed door de connectiviteit tussen paaigebieden en kinderkamers. Larvale connectiviteit, d.w.z. de succesvolle uitwisseling van individuen tussen paaigebieden en kinderkamers, is daarom een cruciale factor voor een succesvolle rekrutering. Rekrutering kan zeer variabel zijn op kleine temporele en ruimtelijke schaal. Toch is empirisch bewijs van connectiviteit tussen verschillende paaigebieden en kinderkamers schaars. De connectiviteit van de eerste levensfasen van mariene organismen is moeilijk te onderzoeken vanwege de gebrekkige kennis over larvale bronnen en putten, en vanwege uitdagingen bij bemonstering. Inzicht in connectiviteit op de schaal van larvenverspreiding is van het grootste belang voor het in standhouden van metapopulaties. Dit vraagt om een verdere ontwikkeling van multidisciplinaire benaderingen. Visserijonderzoek profiteert in toenemende mate conceptueel en technisch van de integratie van genetische en fenotypische studies om de evolutionaire mechanismen van populatiestructuur te ontrafelen.

In dit proefschrift hebben we nieuwe kennis ontwikkeld en geïntegreerd over de vroege connectiviteit van een platvis op de schaal van verspreiding (5-500 km) met een multidisciplinaire aanpak. Een intensief bemonsteringsprogramma op kleine geografische schaal werd uitgevoerd om juveniele tong *Solea solea* in de zuidelijke Noordzee en Het Kanaal te verzamelen. Volwassen tong uit bestaande verzamelingen werd opnieuw geanalyseerd. De lokale connectiviteit werd onderzocht aan de hand van otolietvorm, elementaire analyse en moleculaire merkers. De informatie van elk van deze methoden droeg bij tot een specifiek aspect van het ecologische en evolutionaire begrip van de verspreiding over de hele levenscyclus. Moleculaire merkers werden gebruikt als individuele merkers van verplaatsing en als populatie merkers van genenstroom. Otolietvorm is een betrouwbare individuele fenotypische eigenschap gebruikt voor elementaire analyses waarbij het individu van geboorte tot vangst gevolgd wordt. Een veelbelovende aanpak is de combinatie van merkers die gevoelig zijn voor complementaire ecologische en evolutionaire processen,. De multidisciplinaire aanpak bleek doeltreffend voor het in kaart brengen van de lokale dynamiek die betrokken is bij de connectiviteit tussen paaigebieden en kinderkamers en op de kinderkamers.

De chemische samenstelling van de otolieten van bemonsterde juveniele tong op de kinderkamers van de Zuidelijke Noordzee was duidelijk anders. De verschillen in elementaire samenstelling (vooral voor de elementen Sr, Mg en Zn) suggereerden dat de verspreiding na

vestiging op de kinderkamers beperkt is, terwijl de verspreiding vóór de vestiging waarschijnlijk beperkt blijft tot ongeveer 100 km. Onze resultaten suggereerden dus een verdere verspreiding vóór de vestiging dan na de vestiging, met enkele, maar over het algemeen weinig juveniele migranten tussen de kinderkamers. Elke bemonsteringsregio rekruteerde lokaal ongelijke percentages juvenielen (0-100%) uit vier natale paaigebieden. Dit resultaat bevestigt de resultaten die voorspeld werden uit een model, en suggereren dat een enkele paaigrond kan bijdragen tot meerdere kinderkamers. Bewijzen voor lokale rekrutering, de verspreiding van larven naar verschillende naburige kinderkamers en de beperkte connectiviteit tussen de kinderkamers voor tong kunnen bijdragen tot de validatie van biofysische modellen.

Ondanks de beperkte connectiviteit tussen kinderkamers is het de genetische differentiatie en de differentiatie in otolietvorm van juveniele tong in de Noordzee klein. De lage genetische differentiatie kan verklaard worden door een beperkte, maar significante vermenging van de kinderkamers in de juveniele fase, maar ook door de genenstroom op de paaigebieden (vermenging van eieren of van het volwassen stadium). Er zijn slechts enkele migranten per generatie nodig om een hoog niveau van genenstroom in stand te houden. Het lage niveau van succesvolle rekrutering van migranten die met elementaire analyse zijn waargenomen, is verenigbaar met de homogene populatiestructuur van tong in de Noordzee, zoals aangetoond met genetische merkers. Toch is er geen bewijs in tong voor genetische menging tussen paai-aggregaties.

Ongeacht de hoge connectiviteit binnen de Zuidelijke Noordzee, zijn er aanwijzingen dat tong lokaal is gestructureerd. Binnen de kweekgebieden scheidde de elementaire samenstelling de westelijke en oostelijke zijden van de Belgische kweekgebieden. Dit verschil kan verklaard worden door instroom uit de Westerschelde die de elementaire samenstelling van het water beïnvloedt. Lokale verschillen in vermeende adaptieve merkers tussen cohorten kunnen verband houden met selectieve druk op de 0-groep tong op de kinderkamers. Een oorzakelijk verband tussen moleculaire diversiteit en cohortstructuur aantonen vereist echter verder onderzoek. Op voorwaarde dat een klein deel van de volwassenen met succes bijdraagt aan de volgende generatie (zoals voorspeld door bewijs van *sweepstake* rekrutering), kunnen lokale genetische verschillen voortkomen uit één panmictische paai-aggregatie. Bovendien werd op de Belgische kinderkamers de verwantschapsstructuur (d.w.z. de vestiging van verwante individuen in hetzelfde gebied) onderzocht. Onze resultaten suggereren, zoals verwacht, een beperkte verwantschap tussen individuen, die het Wahlund effect niet zal beïnvloeden. Het testen op

chaotische genetische *patchiness*, een gemeenschappelijk kenmerk van mariene organismen, in tong zou bijkomende bemonstering vereisen. .

Zoals benadrukt door deze studie, zijn elementaire samenstelling en vermeende adaptieve genetische merkers van grote waarde om connectiviteit te begrijpen bij soorten met een uitgebreide larvale verspreidingsdynamiek en/of lage niveaus van genetische differentiatie zoals het geval is voor tong in de Noordzee. De bevindingen zijn bedoeld om de biofysische modellen nauwkeuriger af te stellen en bij te dragen aan het beheer van de visbestanden.

LIST OF ABBREVIATIONS

A

AHC: Ascending Hierarchical Classification

APOA-I: Apolipoprotein A1

B

BSX: Brain-specific homeobox

C

CYP1B1: Cytochrome P450 1B1

D

DAPC: Discriminant Analysis of Principal Components

DNA: Deoxyribonucleic Acid

E

EBFM: Ecosystem-Based Fishery Management approach

EFDs: Elliptic Fourier Descriptors

F

FAO: Food and Agriculture Organization (United Nations)

FDR: False Discovery Rate

F_{IS}: inbreeding coefficient

FP: Fourier Power

G

GBS: Genotyping By Sequencing

GI: Gini Index

GWAS: Genome-Wide Association Study

H

H_e: expected heterozygosity

H_o: observed heterozygosity

HTS: High-Throughput Sequencing

HWE: Hardy Weinberg Equilibrium

I

IBD: Isolation By Distance

IBM: Individual-based Model

ICES: International Council of the Exploration of the Sea

L

LA-ICPMS: Laser Ablation Inductively-Coupled Plasma Mass Spectrometry

LD: Linkage Disequilibrium

LOD: Limit of Detection

M

MPA: Marine Protected Areas

MSY: Maximum Sustainable Yield

MSFD: Marine Strategy Framework Directive

N

NA: missing value

NCV: Non Communicated Value

NGS: Next Generation Sequencing

NIST: National Institute of Standards and Technology

NR1H4: Nuclear Receptor subfamily 1 group H member 4

P

PCA: Principal Component Analysis

PCoA: Principal Coordinate Analysis

PCR: Polymerase Chain Reaction

PCs: Principal Components

PLEKH5: Pleckstrin homology domain containing, family A member 5

pRDA: Partial Redundancy Analysis

Q

QTL: Quantitative Trait Locus

R

RAPDs: Randomly Amplified Polymorphic DNAs

RADseq: Restriction site Associated DNA Sequencing

RF: Random Forest

RRS: Reduced Representation Sequencing (RRS = GBS + RADseq)

S

SNP: Single Nucleotide Polymorphism

SNF: Similarity Network Fusion

STECF: Scientific, Technical and Economic Committee for Fisheries (EU)

SSB: Spawning Stock Biomass

SST: Sea Surface Temperature

T

TAC: Total Allowable Catch

TRIM8b: Tripartite Motif containing 8b

TSS: True Skill Statistics

TABLE OF CONTENT

GENERAL INTRODUCTION

One of the major attributes of many marine species is that they produce large numbers of small pelagic larvae that drift in the ocean for some time. Larvae may disperse up to hundreds of kilometers before reaching suitable nursery grounds (Palumbi 2003). However, our current understanding is that larval dispersal can be highly restricted through processes such as larval retention (Cowen et al. 2000, Jones et al. 2005, Pinsky et al. 2017). Establishing the degree to which different populations are connected by larval dispersal is a fundamental goal for researchers interested in understanding ecological and evolutionary processes. Assessing local and metapopulation dynamics, stability of community structure, patterns of local adaptation, maintenance of genetic diversity, sustainability of fisheries, and resilience of ecosystems to human exploitation and changing environmental conditions, all require some knowledge of rates and patterns of exchange among populations (Botsford et al. 2001, Carvalho & Hauser 1994, Gawarkiewicz et al. 2007, Kerr et al. 2017).

While sound progress has been made with the understanding of connectivity of older life stages, connectivity of larvae and juveniles remains largely unresolved for most marine populations (Ayram et al. 2016, Cowen et al. 2007, Treml et al. 2015). This lack of knowledge represents a fundamental obstacle to obtaining a comprehensive understanding of the population dynamics of marine organisms. Furthermore, a lack of spatial context that connectivity information would provide has limited the ability of ecologists to assess the efficiency of novel management strategies (Palumbi 2004). Effective management requires a good knowledge of the scale of larval dispersal and the size of discrete local populations (Berglund et al. 2012, Jones et al. 2007, Kritzer & Sale 2004).

Connectivity of early-life stages is key to many ecosystem processes, and numerous methods have been developed to explore and estimate connectivity within metapopulations (Cowen et al. 2006, Cowen & Sponaugle 2009, Jones et al. 2009). Because each method is often applied to a specific scale and is underlined by specific assumptions, the use of multiple methods at different temporal scales may be necessary to completely understand a system (Thorrold et al. 2002). The application of several methods in a given system should enable measures at different spatio-temporal scales and lead to a better knowledge of the crucial connections between populations.

The general aim of this PhD thesis is to account for the local dynamics involved in the larval connectivity between spawning and nursery grounds and the juvenile connectivity between nursery grounds. We chose the common sole *Solea solea* (Linnaeus 1758, hereafter called sole) to be the focal species of this study for its ecological and commercial interest. The reasons for studying population structure and connectivity in marine exploited populations will be detailed in the following sections.

1. Connectivity and population structure in demersal fish

1.1 Importance of dispersal during early-life stages

Movement in the broad sense may happen for various reasons, such as searching for the optimal nursery, avoiding predation or searching for mating opportunities. Dispersal is one of the key modes of movement by an individual to complete its life cycle, along with migration and foraging (Nathan et al. 2008). Dispersal has many ecological and evolutionary consequences such as influencing gene flow (i.e. the exchange of genes or alleles from one population to another), increasing the adaptive potential (i.e. the capacity to adapt to new conditions), maintaining local populations and the definition of the extent of a species range (Kokko & López-Sepulcre 2006). The primary mechanism of dispersal happens during the early-life stage for many marine fish (Gaines et al. 2007).

Larval dispersal represents the main vector to increase habitat range for many demersal fishes. Broadcast spawners release numerous gametes. Larvae may disperse up to hundreds of kilometers before reaching suitable nursery grounds (Palumbi 2003) but the current insight is that larval dispersal is highly restricted (Jones et al. 2005, Pinsky et al. 2017). Interestingly, larval dispersal is not synonymous with larval transport. Larval transport (also called advection) is defined as the spatial movement of larvae, generally only considered in its horizontal direction for simplification (Pineda et al. 2007). In contrast, larval dispersal goes beyond larval transport and refers to the movement of larvae from a spawning source to a settlement site (Lowe & Allendorf 2010). The distance between the spawning and nursery ground is the realized dispersal of the larva. Opposed to realized dispersal, the potential dispersal distance of a pelagic marine larva is simply the maximum duration that the larvae can swim multiplied with its dispersal

speed (which combines both swimming speed of the larva and the speed of advection by water currents). Potential dispersal is generally much larger than realized dispersal. Survival during the pelagic stage and settlement success are as important as larval transport for realized dispersal (Cowen & Sponaugle 2009). Successful settlement is constrained by the availability of suitable habitats.

1.2 Ecological and evolutionary metapopulations

One may consider the habitat range as forming metapopulations, a network of patchy local populations connected through migration (Ovaskainen & Hanski 2003). Some local populations are the source (donor) of individuals, whereas others function as sinks (recipients). Population networks are connected through movement of individuals either as larvae, juveniles or adults (i.e. connectivity, Palumbi 2003). The definition of connectivity encompasses larval dispersal but is also influenced by postsettlement mortality and condition from settlement to successful reproduction (Pineda et al. 2007). Nonetheless, larval dispersal is often considered as the main driver of connectivity, especially when the exchange is measured at the time of settlement, and is essential for the maintenance of local populations (Gaines et al. 2007). The number and frequency of spawning grounds contributing to one (or several) nursery ground(s) define the larval connectivity. Juvenile connectivity is the exchange of juveniles between nursery grounds. Connectivity influences colonization, replenishment and resilience of marine populations. Therefore information about connectivity is essential for a good understanding of ecosystem responses to changing environmental conditions (Gawarkiewicz et al. 2007) and must be taken into account for management solutions (Berglund et al. 2012).

Connectivity and dispersal can be considered over a short (ecological) or a long (evolutionary) time scales (Hendry 2017). Ecological connectivity can be defined as the movement of individuals between spatially discrete populations, and evolutionary connectivity implies both the movement of individuals between populations and establishment for long enough to contribute to the gene pool of the new population (Lowe & Allendorf 2010, Wright 1949). This definition of evolutionary connectivity implies gene flow. We distinguish connectivity from migration as it is used in the ecological literature to refer to the periodic and regular movement of a large number of individuals from one place to another (Lowe & Allendorf 2010).

A certain degree of connectivity is necessary for persistence of sink (recipient) populations, especially when very fragmented and close to extinction (e.g. the rescue effect, Brown & Kodric-Brown 1977). Fragmentation of natural habitats by human activity, such as separating nursery grounds into smaller and less connected habitats by dredging or mineral aggregate extraction for example, can be a major issue for the sustainability of natural populations (OSPAR 2000). Certain fishing gear, such as beam trawls, also cause lasting physical damage to habitats and benthic communities (Rabaut et al. 2008). Consequently, many native coastal marine species have been reduced due to habitat destruction (Mineur et al. 2012). Questions arising are at which level is fragmentation a problem and what level of genetic diversity is required to prevent deleterious alleles (i.e. genetic variants lowering the fitness) from being increasingly expressed in a metapopulation. Metapopulations are key for maintaining biodiversity and genetic diversity, and therefore improving resilience of ecosystems (Carvalho & Hauser 1994, Chapin et al. 2000, Kritzer & Sale 2004). Nevertheless, even when connectivity is necessary for persistence, benefits of connectivity to sink populations must be weighed against costs to source (donor) populations, risks of increased mortality and inter-specific competition in an occupied habitat patch for dispersers themselves (Lowe & Allendorf 2010).

The spatial configuration of the network and exactly where in the network the different types of habitat patches are located will affect the influence of particular populations on the dynamics of the metapopulation as a whole (Ovaskainen & Hanski 2003). The intensity of different processes depends on the spatial location of the population within the habitat range. Carrying capacity is the maximum number of organisms of a particular species that can be supported indefinitely in a given environment. Carrying capacity may constrain population growth more at the core of a habitat range than at the margins. At the core, there is little dispersal, little reproductive effort as there is much more intense intra-specific competition and limited resources (lower carrying capacity). In contrast, the habitat margins are much more favorable for mating and dispersal when colonizing a habitat with good biotic and abiotic conditions (Fronhofer & Altermatt 2015). Nevertheless, habitats might have more suitable environmental conditions at their core than at their margins and therefore may be able to support more individuals and have a higher carrying capacity.

1.3 Early-life stage challenges and life cycle dynamics

The contribution of larval dispersal to the population dynamics and structure of marine organisms has been recognized for more than a century (Hjort 1914, Thorson 1950). Hjort's pioneering work (1914) showed that variability in early life survival was a dominant cause of variation in recruitment. The role played by environmental factors in larval dispersal was identified 20 years later (Cushing 1969). Sinclair and Iles (1989) shared the view that the dispersal of larvae from their favorable area is very limited, as opposed to Secor (1999) who believed that individuals were able to migrate further depending on the energetic demands of the population. Nonetheless, individuals deviating too much from the population's trajectory get lost to the population. Hence, the spawning site is the major factor influencing recruitment. Moreover, larval feeding success is correlated to the spatio-temporal overlap between larvae and their zooplankton prey ("match-mismatch" hypothesis; Cushing 1969, 1990).

An alternative to the stationary view of Sinclair and Iles (1989) is that individuals may migrate between spatially distinct spawning grounds, nursery grounds and adult feeding grounds in a so called "Migration Triangle" (Harden-Jones 1968, Fig. I.1a) or the work of Secor (1999, Fig. I.1b) considering population size over time. Further developments of the migration triangle theory lead to Bakun's triad (1996) which is oriented towards the energy balance and food regulation. Bakun's triad distinguishes three phases in larval history: (1) enrichment of nutrients, (2) concentration of food particles, and (3) retention of larvae at the appropriate spot

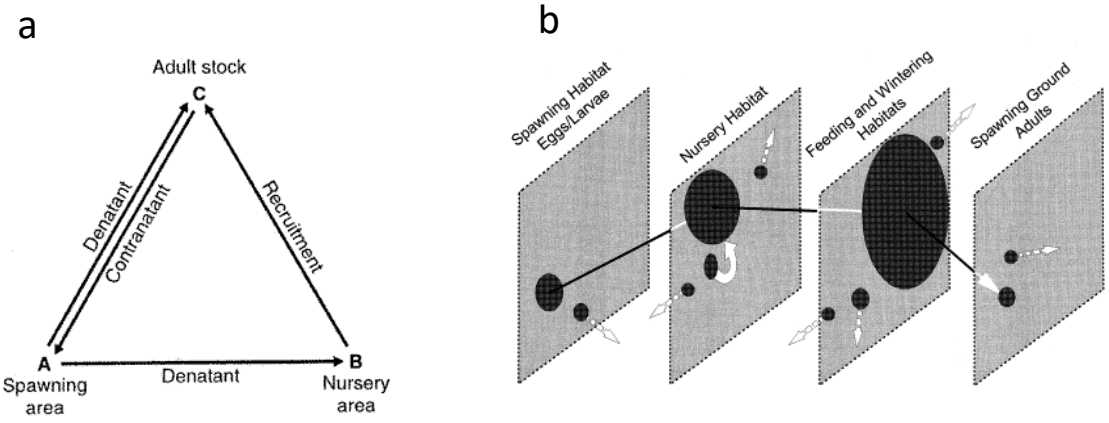


Figure I.1: (a) The migration triangle (Harden-Jones 1968) and (b) the more dynamic view of Secor (1999)

during the life cycle. Larval retention may happen through favorable water currents linked to the nursery location (Creutzberg et al. 1978, Fox et al. 2009, Rijndorp et al. 1985).

Survival during larval dispersal depends mainly on food availability, infection by viruses, bacteria and parasites, predation and habitat access. Three general concepts describe larval survival during the pelagic stage (reviewed in Anderson 1988): theoretically, survivors should be the larvae that (1) are larger at a given age ("bigger is better" hypothesis, Miller et al. 1988), (2) grow faster ("growth-rate" hypothesis, Bailey and Houde 1989) and/or (3) have shorter early stages ("stage-duration" hypothesis, Anderson, 1988). In other words, the most growth-efficient the larval phenotype is, the better the larval quality (Shima & Swearer 2009). Accordingly, Shima & Swearer (2010) postulate that long-distance dispersal drives a reduction in future fitness. The fitness of an individual is equal to the relative contribution of its genotype to the next generation compared to other genotypes (Hartl & Clark 2007). Longer-distance dispersers experience more food stress unless if they enter local hydrodynamic features such as eddies where food may be concentrated. Nonetheless, actual effects on dispersal kernels (i.e. the probability distribution of larvae dispersing to a specific distance) are likely to be context-dependent. Stamps (2006) suggested that longer dispersal times can lead to greater selectivity for settlement sites and only high quality dispersers can afford longer search in the water column where predation pressure is elevated. Thus, higher quality dispersers – often the larger individuals – may dominate habitats at further dispersal distances, resulting in a spatial heterogeneity of population structure and enabling colonization of new habitats.

Early-life stages experience a high mortality which leads to a large potential for selection towards the fittest genotype-phenotype (Hemmer-Hansen et al. 2014). In addition, early-life stages have a lower tolerance for habitat variability (Pörtner & Farrell 2008, Pörtner & Peck 2010, Fig. I.2). In the case of sole, the earliest life stages have a more narrow range in thermal tolerance than later (adult) life stages (Teal et al. 2012). Maturation refers to fish becoming sexually mature, prior to first spawning (Pörtner & Peck 2010). Hence, juveniles are very vulnerable to environmental change (Pimentel et al. 2014).

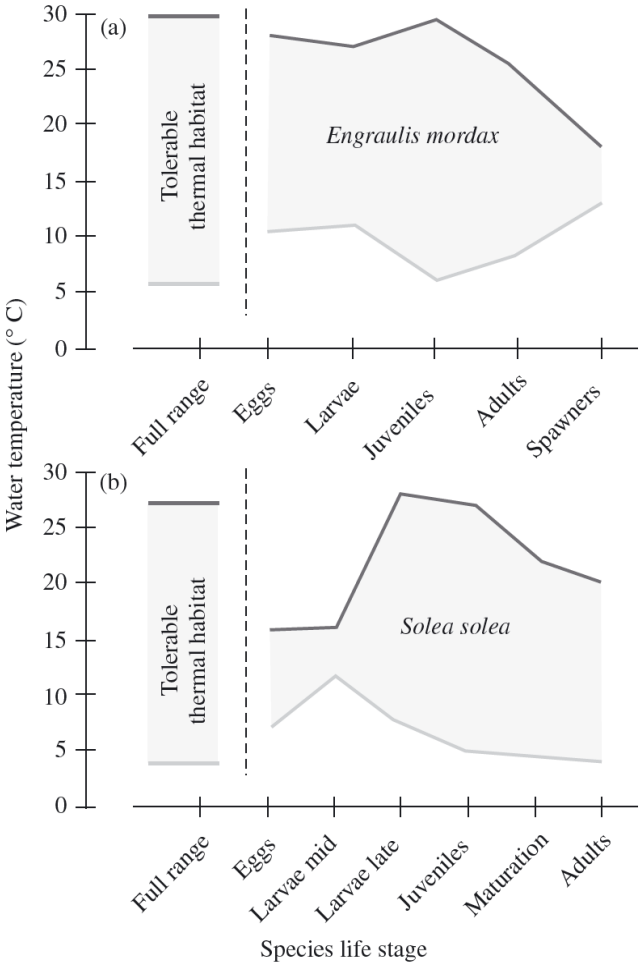


Figure I.2: Ontogenetic changes in thermal habitats or tolerance for *Engraulis mordax* (L.) in the California current (from Brewer 1976) and *Solea solea* in the North Sea (from Rijnsdorp et al. 2009 in Teal et al. 2012).

1.4 Genetic connectivity and evolutionary forces

In a given environment, favorable heritable traits will increase in frequency through generations by natural selection. Local adaptation refers to the process by which the fitness of individuals in their local habitat outperforms the fitness of immigrants due to natural selection (Carvalho 1993). In combination with natural selection, the balance between gene flow, which generally increases genetic diversity, and counteracting forces like mutation and genetic drift, may lead to population differentiation (Ward et al. 1994, Taylor & Hellberg 2003). In each generation, new alleles appear by spontaneous mutation due to mutagen-induced DNA damage and DNA replication errors or due to recombination (Ellegren & Galtier 2016, Pritchard et al. 2010). Genetic drift also increases genetic diversity by inducing a random fluctuation of allele frequency across generations and is higher in a small gene pool (see Ellegren & Galtier 2016 for a review). Processes at the population level are dependent on the effective population size (i.e. number of adults effectively reproducing) and the migration rate of new individuals (Waples & Gagiotti 2006, Fig. I.3). The Wright-Fisher genetic model of population structure assumes no selection, no mutation, no migration, non-overlapping generation times and random mating (Hartl & Clark

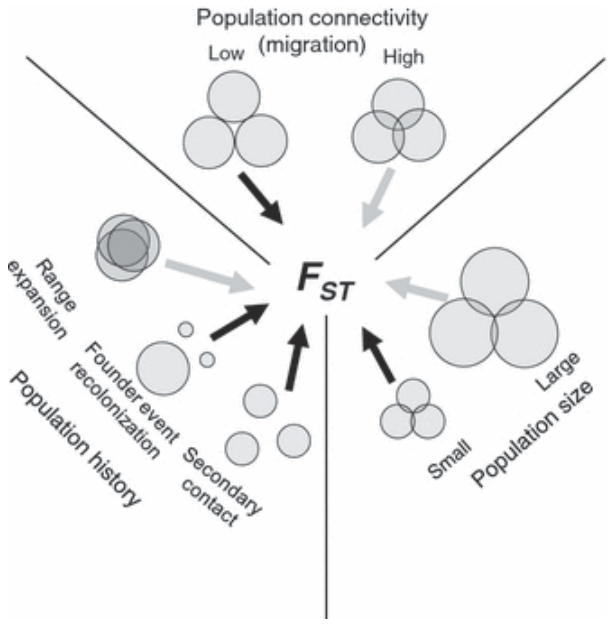


Figure I.3: Factors influencing genetic population differentiation if selection is not considered (Hauser & Carvalho 2008). Grey arrows indicate factors reducing differentiation whereas black arrows indicate factors promoting differentiation.

2007), therefore providing a null assumption for studying how more complex evolutionary forces such as selection can affect population structure.

The difference between the DNA sequence of two individuals forms the genetic diversity, known as polymorphism. Genetic diversity reflects the appearance and disappearance of genetic variants (i.e. alleles) over time and space. As already mentioned, on top of the random fluctuations of alleles induced by genetic drift, new alleles appear each generation through mutations. Interestingly, the mutation rate is not constant across the genome and across species. Beneficial mutations can be fixed rapidly and erase pre-existing genetic information at the selected locus and nearby loci on the chromosome (i.e. through a selective sweep). A selective sweep typically happens after an environmental change and reduces or eliminates genetic diversity in the neighborhood of the beneficial allele (through hitchhiking effects, Luikart et al. 2003). The beneficial allele was either already present in the population prior to the environmental change (i.e. a soft sweep due to large standing genetic variation, Barrett & Schulter 2008) or is a single new mutation (i.e. hard sweep). In addition, heritability of genetic traits may be quantitative (polygenic traits). The recent discovery of these processes has put emphasis on obtaining information about the position of loci in the genome structure (i.e. within or outside of functional genes) and in relation to other loci of interests. Linkage disequilibrium causes an association between loci that are not under selection and loci under selection (due to physical linkage on a chromosome but not always). Hence, linked loci will change similarly in allele frequency (Ellegren & Galtier 2016, Pritchard et al. 2010).

Moreover, spawning is non-random and may result in genetic patchiness through progeny issuing from very few parents (sweepstake effect, Hedgecock 1994). The signal of genetic patchiness is observable through genetic diversity and relatedness with closely related individuals dispersing together. Many marine species show a chaotic spatial structure with larger spatial differentiation on a small scale than at the scale of their range (chaotic genetic patchiness, Broquet et al. 2013, Eldon et al. 2016). This is linked to the variability of the reproductive success with a small number of individuals effectively reproducing (low effective population size, Nielsen et al. 2009a. In addition to the spatial structure, spawning might be divided through time, leading to a “cohort structure”. A short environmental window is available to enable successful larval development and later recruitment (see the “match-mismatch”

hypothesis, Cushing 1969, see *section I.1.2*). A cohort structure will lead to closely related individuals dispersing together.

Finally, both spawning time and location will impact dispersal pathways of larvae. Many species seem to move to specific locations to spawn and some, such as European plaice *Pleuronectes platessa*, return to their original spawning location to spawn (i.e. spawning site fidelity, Hunter et al. 2003). As dispersers compete with each other, the arrival order of individuals in a new habitat patch influences community dynamics and structure (i.e. the "priority effect"). Early migrants can suppress late arriving individuals either via a numerical advantage or via local adaptation (De Meester et al. 2016). In addition, another key feature leading to a competitive advantage of some dispersers against others and against resident species is the habitat quality of the natal patch compared to the new habitats. Because traits of individuals are altered by experiences in their natal habitat, differences in the natal habitat of dispersers can carry over when individuals disperse to new habitats and alter their fitness and interactions with other species (Allen & Rudolf 2016). In other words, migration from a high-quality habitat patch gives a competitive advantage to migrants in comparison to migrants originating from low-quality habitat patches. Therefore spawning ground location and dispersal pathways may impact larval pool quality and pass "carry-over" effects onto juvenile survival (Pineda et al. 2007, Shima & Swearer 2010). The legacy effects of dispersal pathways can be predominant over spawning ground origin. For example, a transplant experiment in reef fish showed that a better larval dispersal environment leads to a higher survival at adulthood (Shima & Swearer 2009). For species with spawning site fidelity, a similar spawning ground location means a higher chance to have a similar gene pool. A gene pool locally adapted to target nurseries gives a competitive advantage to "local" juveniles compared to migrants coming from further away. The identity and origin of dispersers is a key component to community structure and sustainability of populations.

1.5 Marine populations are structured at small spatial scales

Although the spatial scale of potential gene flow is clearly linked to the mobility of organisms, the observed population structure is not necessarily linked to the mobility of organisms (Hauser & Carvalho 2008). There are several reasons for that: (a) the presence of physical and ecological barriers which can be temporary (seasonal structures such as stratification fronts and upwelling, short term mesoscale processes such as eddies) and less visible from a terrestrial point of view (Caley et al. 1996, Manel et al. 2003); (b) active behavior in habitat choice as well as reproductive philopatry; (c) sex-biased dispersal patterns and low effective population size where only a minority of the population effectively reproduces; (d) the influence of natural selection through genetic adaptation; or (e) historical demographic events which have removed populations from being at an equilibrium between gene flow and genetic drift (Avise 1994, see *section I.1.3* for definitions). In fact, theoretical population models are used to predict the main forces acting on populations and better understand population structure. Most of these models (such as the Wright-Fisher model see *section I.1.3*) assume that populations are at migration-drift equilibrium. However, it is not clear whether such an equilibrium is ever met in marine populations (Manel et al. 2016). Hauser & Carvalho (2008) have summarized the main factors influencing genetic population differentiation in three categories if selection is not considered: population connectivity, population size and population history (Fig. I.3 page 8).

Contrary to former conceptions of marine larvae being passively transported over long distances leading to well-mixed populations, larvae stay close to the parental population through various adaptive behaviours and hence limit gene flow. Genetic differentiation and self-recruitment occur in marine populations at a much smaller spatial scale than expected. This observation represents a central paradigm shift in marine ecology (Swearer et al. 2002, Teacher et al. 2013). Local retention is an important factor in self-recruitment (Burgess et al. 2014). Self-recruitment is the fraction of recruits to a location that originate from local parents (= local recruits / total recruitment to a site, including larvae from other origins). Local retention is the fraction of local reproductive output retained at the focal site (= local recruits / total reproductive output of a site, Lett et al. 2015). Surprisingly, self-recruitment has been observed over small spatial scales. For example, one third of the panda clownfish larvae (9-12 days of pelagic larval duration) returned to their 0.5 km² natal site for spawning with many larvae settling less than 100 m away from their birth site (Jones et al. 2005).

In the marine ecosystem passive dispersal of larvae is more frequent than active dispersal (Burgess et al. 2016). Nevertheless, marine larvae can modify their horizontal distribution by swimming vertically and reaching different currents (Marchand & Masson 1989). The difference between individual-based models including larval behavior strategies or being passive underlines the presence of active processes of larval retention (Cowen et al. 2000). Larval swimming behavior, changes in buoyancy, and ontogenetic changes in vertical position influence horizontal transport of larvae (Lacroix et al. 2013). Additionally, many species can delay settlement, up to three weeks in the case of sole (*Solea solea* L.) (Marchand 1991). Larvae can orient themselves within a 10 km radius to select a settlement site based on terrestrial chemical cues and freshwater input (Dixson et al. 2011, Kerstan 1991). The diversity of marine larval behaviors and physical mechanisms acting over reduced spatial scales (< 10 km) make larval dispersal very difficult to measure and predict (Lacroix et al. 2013). Clearly, larval behavior plays an even more important role in larval dispersal as advection and diffusion. Nearshore physical processes tend to be complex and many operate including buoyancy driven flows, wind-forcing, surface and internal tides, large-amplitude internal waves, cross-shore and alongshore hydrodynamic processes, stratification, and boundary layer effects. Cross-shore movement is critical for species whose later developmental stages move offshore as adults to reproduce offshore and whose larvae must return to shallow water nurseries to grow. Meso- and large-scale processes, such as eddies, upwelling and the North Atlantic Oscillation (NAO) also affect larval dispersal. Most barriers to dispersal in the marine environment are not absolute and they act subtly, constraining movement in one direction compared to another or on a short temporal scale (Caley et al. 1996, Gaylord & Gaines 2000).

On top of individual behavior, group behavior plays a key role in recruiting successfully. Synchronous spawning will likely increase survival rate by decreasing predation pressure on individual larvae. Spawning time and pelagic larval duration will be influenced by seasonality because peak spawning and the rate of physiological development are temperature-dependent (Fonds 1979). Pelagic larval duration is shorter for larvae hatching later in the spawning season (provided that the sea water temperature is higher as is the case for spring spawners, Thia et al. 2018). Later spawning often means warmer waters but also different spawners. For example, older spawners may spawn later in the season, as shown in plaice (Simpson 1959). An optimal spawning timing will result in better food availability and thus in higher survival rate (Cushing 1969, 1990).

2. Tools to estimate marine connectivity

The weak structuring of marine populations necessitates the implementation of sensitive tools to assess the driving forces of connectivity (Palumbi 2004, Selkoe et al. 2008). Many tools are available nowadays to conduct model-based or field-based studies and address two central questions relevant to connectivity: (1) the complex recruitment patterns (Hjort 1914) and (2) a recent paradigm shift recognising extensive genetic population structure among many marine taxa, and extensive adaptive differentiation and biocomplexity (Hauser & Carvalho 2008). In the end, the combination of high resolution simulations and field observations could help tackle these two central questions.

Connectivity is expected to be high in the field for populations with evidence of weak (genetic) structure (Cuveliers et al. 2012, Nielsen et al. 2012). Nevertheless, direct experimental measures of connectivity (i.e. tagging and transplant experiments) are often not feasible due to the large population size, small body sizes, the production of too many small propagules and the logistical difficulties of sampling long-distance dispersal events (Lowe & Allendorf 2010). Thus indirect methods such as studying parasite diversity (Raeymaekers et al. 2013) and otolith (i.e. fish ear bone) shape (Campana & Casselman 1993, Mahé et al. 2016) or elemental fingerprints (Morat et al. 2014, Tanner et al. 2013, 2016) have been used to explore the composition of fish stocks and the connectivity between populations (Pawson & Jennings 1996). More recently, molecular markers (Bekkevold et al. 2011, Bravington et al. 2016) and modeling of larval transport (Koubbi et al. 2006) are frequently used to estimate connectivity (Palumbi 2003).

In the absence of clear genetic structure, other tools such as biophysical models may be useful. Based on empirical evidence, Individual-based Models (IBMs) allow us to simulate larval dispersal under past or future climate scenarios and better understand processes at stake (Hufnagl et al. 2013, Lacroix et al. 2013, 2018). This is a very helpful heuristic tool in order to quantify the role of physical and biological constraints on the recruitment process and the connectivity of exploited marine organisms. However, the lack of precise information on larval swimming capability hampers the parametrization of behavior routines of models (Lacroix et al. 2013). Poor knowledge of behavior but also other parameters such as pelagic larval duration and mortality lead to model uncertainties (Barbut et al. 2019).

In this PhD thesis, three different methods were investigated to study connectivity during the early life stages of a marine fish: otolith shape, otolith elemental composition and genetics (see detailed objectives in *section I.5*).

2.1 Otolith shape and microchemistry

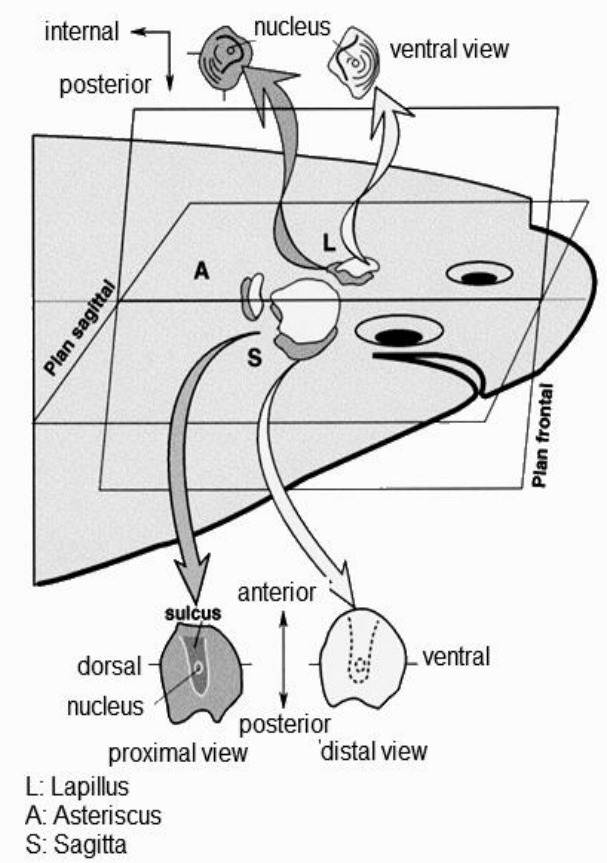


Figure I.4: Fish otoliths come in three pairs: sagittae, lapilli and asterisci (De Pontual et al. 2003)

Otoliths are made of calcium carbonate and protein, and located in the inner ears of teleost fish (Fig. I.4). Their function is hearing and balance. They develop before hatching during the egg stage (Campana 1999). At that time larvae develop from the fertilised egg and the nucleus of the otolith incorporates maternal material (Rut-tenberg et al. 2005). Otoliths grow incrementally throughout fish's life at a rate proportional to somatic growth (Campana 1999). They incorporate trace elements from the surrounding environ-ment, hence changing their chemical composition.

Fish that occupy different environments for part or all of their life show differences in otolith composition (Campana, 1999) and morphology (Cardinale et al. 2004). Consequently otoliths provide a permanent record of the fish's life history (Kennedy et al. 2002). Measurements of otolith shape, incremental patterns and chemical composition are used to characterize fish from different geographic locations and elucidate stock structure (e.g. Schaerlaekens et al. 2011). Growth patterns of otoliths have been used to retrace life-history traits such as somatic growth, reproduction and fisheries-induced evolutionary changes (Mollet et al. 2007, Sturrock et al. 2012). The ability of geochemical tags to track larval movement

depends upon substantial variation in the elemental composition of those tags among locations of interest (Thorrold et al. 2002). Especially estuaries and coastal regions provide the ideal variation in chemical and physical characteristics to generate significant differences in otolith composition (Thorrold et al. 2007). Applications, benefiting from intercalibration exercises, involve a wide range of fish taxa and are mostly carried out between estuaries/rivers or at a spatial scale larger than 1000 km (i.e. where chemical differentiation is expected to be strong). For example, microchemical profiles of juvenile otoliths differentiate among estuarine nursery use in eastern Australia (Gillanders & Kingsford 2003, Schilling et al. 2018). Similarly, most studies have compared intraspecific differences in otolith shape either over large distances (>500 km e.g. Vieira et al. 2014), across oceanographic barriers (Tuset et al. 2003) or between habitats (Morat et al. 2014, Vignon & Morat 2010). It is not yet clear what can be learned from otolith shape at small spatial scales and in the absence of strong oceanographic barriers.

Laser ablation inductively-coupled plasma mass spectrometry (LA-ICPMS) was used in this PhD to identify movement-related information in otoliths from spawning to settlement at the nursery ground i.e. time-resolved chemical data. One advantage of LA-ICPMS is that chemical data can be associated to an age as measured by the distance from the core of the otolith (i.e. egg stage, about 2-3 days after spawning). We chose to measure trace elements (and therefore to study “elemental composition” instead of isotopic ratio) because our research question was not linked to connectivity along a salinity gradient (as in lagoon-inhabiting species, such as sole in the Mediterranean Sea). Nevertheless, the downside of the laser ablation technique is that the material ablated is relatively small, limiting detection capabilities and therefore limiting the number of measured elements present in low concentration in otoliths. Although microchemistry is unquestionably one of the most powerful tool to study small spatial scale connectivity, it has three main disadvantages: microchemistry is (1) species-specific, (2) linked to physiology and (3) affected by strong (intra- and inter-annual) temporal variations in water chemistry (Chang & Geffen 2013, Reis-Santos et al. 2012).

Otolith shape has been used in this PhD eventhough it is known to be of a lower spatial resolution compared to microchemistry. The lower resolution power of otolith shape might be attributed to the relatively long time required for shape differences to become significant. Otolith shape has a rather complex nature and is influenced by genetic background, physiology (linked to ontogenetic development in juveniles) and environment (Cardinale et al. 2004, Hüssy

2008). In addition, the use of otolith shape represents a challenge to study juvenile fish connectivity, especially in flatfish. During the early-life stages, otoliths evolve from circular to more complex shapes (Lagardère & Troadec 1997, Hüssy 2008), which may limit the utility of otolith shape as stock marker for immature fish or fish of different age classes. Moreover, left and right otoliths may be different, i.e. directionally asymmetrical, particularly in flatfishes (Mille et al. 2015). Environmental and anthropogenic pressure may cause stress-induced changes such as increased levels of directional asymmetry (Gagliano & McCormick 2004), which is disadvantageous because it interferes with hearing and orientation (Anken et al. 2002, Lychakov & Rebane 2005). Nonetheless, otolith shape is a lot less complex to preserve and does not suffer from contamination. Thus, studying shape is more accessible and also less costly than microchemistry. Because of the above mentioned limitations of otolith based techniques, molecular markers have been used to complement the view on connectivity given by otolith.

2.2 Molecular markers and sequencing techniques

The use of molecular markers to trace fish origin and reveal the genetic basis of life-history traits under natural selection has been evolving over the past 50 years (Hauser & Carvalho 2008). Tracing of wild fish has made considerable progress through the introduction of high resolution markers such as microsatellites (Glover et al. 2008) and especially Single Nucleotide Polymorphism markers (SNPs, Nielsen et al. 2012). Individuals are assigned with high probability to their source population, provided that a map of the complete natural range is available. Microsatellites are made of simple repetitive motifs from 2 to 8 Deoxyribonucleic Acid (DNA) bases. They are common in eukaryotic DNA and show a high degree of polymorphism. Despite the success of microsatellites in the last decade, there are also some major limitations, namely: (1) homoplasy (parallel evolution of identical characteristics), (2) presence of null alleles, (3) complex mutation patterns, (4) frequent errors in genotyping, (5) difficult reproducibility between laboratories, and finally (6) microsatellites can be rare in some chromosomal regions or in some species. Recently, and partially because of these limitations, SNPs are increasingly used as an alternative to microsatellites. SNPs have become increasingly established in the field of molecular ecology (Morin et al. 2004), fisheries genetics (Hüssy et al. 2016) and aquaculture (Palti 2009). Unlike the commonly used microsatellites, SNPs possess many characteristics that make them attractive for population and evolutionary studies (Diopere et al. 2013, Helyar et al.

2011). SNPs are biallelic and based on the sequence change of one base. The main advantages of SNPs (Brumfield et al. 2003) are that: (1) they are abundant, (2) widely distributed across the genome, more often found than microsatellites in coding regions, (3) their mutation pattern is well known, (4) the risk of errors in genotyping is rather small, (5) they are more reliable and transferable across laboratories, and finally (6) genotyping of degraded material is possible. However, a major drawback of SNP markers is that because of the low information content per locus, a large number of loci must be screened. This represents a problem particularly for a non-model organism when there is no or little sequence information is available. However, Next Generation Sequencing (NGS) development is trying to overcome these problems while sequencing is becoming cheaper and faster (Bernatchez 2016, Hendricks et al. 2018). Bioinformatic mitigation strategies a fruitful area for future development (Campbell et al. 2017, Leigh et al. 2018, O'Leary et al. 2018, see *Chapter 3* and *General Discussion* for more details).

2.2.1 Detecting demographic and adaptive processes

Identifying appropriate genetic markers is an important challenge. Based on assumptions such as diversity, heterozygosity and differentiation, single SNP loci can address demographic (neutral) or adaptive (outlier) processes. "Neutral" means that the changes in the DNA will not affect the ability of an organism to survive and reproduce. Neutral genetic markers are helpful to clarify the population of origin. However, they might not be sensitive enough to detect subtle genetic differences between neighboring populations on a small spatial scale. Populations may undergo local selection (i.e. adaptation in response to selection that varies geographically) despite high rates of gene flow (Nielsen et al. 2009b). In order to detect fine scale genetic structure and the presence of local adaptation, high throughput genotyping methods detect thousands of SNP loci across the genome (Limborg et al. 2012, Nielsen et al. 2009a, Pujolar et al. 2014). Non-neutral genetic markers, also called "outlier" markers, have been reported to be very useful. F_{ST} outlier tests - tests that identify values of F_{ST} larger than expected by drift alone under Wright's model assumptions (i.e. the null distribution) - are popular to identify genes that are candidates for divergent selection among populations. F_{ST} is a standard measure of the variance of allele frequencies among populations (Wright 1949). Genetic drift tends to increase genetic differences among populations, while gene flow reduces average differences. Moreover, population structure that results from demographic histories will also affect the F_{ST} values of

neutral genes (Lotterhos & Whitlock 2014). In addition, selective forces can affect the distribution of F_{ST} values. Outlier markers are often linked to functional genes under selection. For this reason, outlier markers may be more relevant for management and traceability than neutral markers.

2.2.2 Bottom-up vs. top-down genomic approaches

Using an appropriate marker is one concern, but choosing the correct method to analyze the data is at least as important for finding signatures of selection in marine fish. Data analysis methods can be categorized in two approaches, namely top-down and bottom-up (Vasemägi & Primmer 2005). Top-down approaches, such as Quantitative Trait Locus (QTL) mapping and Genome-Wide Association Studies (GWAS), start from phenotypic variation and aim to identify the genetic basis of a phenotypic trait (i.e. functional genetic variation). QTL analysis aims to explain the genetic basis of variation in complex traits by linking quantitative phenotypic data (trait measurements) and genotypic data (molecular markers, Complex Trait Consortium 2003). Until recently, most population genetic analyses of local adaptation focused on identifying genes that may affect a phenotype (such as sexual dimorphism of Chinese tongue sole, Wang et al. 2018, or growth rate Tao & Boulding 2003). These studies have been valuable to elucidate the molecular mechanisms of adaptation but are not able to discover adaptive processes without prior knowledge of gene function. GWAS or QTL do not require that loci linked to functional genes are identified *a priori,* but these methods are mostly applied on model species, such as mice (Flint & Eskin 2012) or Atlantic salmon (Sodeland et al. 2013). QTL mapping requires a linkage map (based on the recombination frequency of markers) to identify quantitative traits of interest. GWAS, on the other hand, compares the DNA of two groups of individuals with alternative states of a trait to detect genetic markers associated to the trait. Both GWAS and QTL mapping can only point to genomic regions that may contain genes of functional importance without specifying causality.

Thanks to NGS and Reduced Representation Sequencing (RRS) genotyping methods, genomic resources are growing exponentially and allow a bottom-up approach (Ross-Ibarra et al. 2007). Bottom-up approaches, such as the candidate gene approaches and transcriptome/genome scans, target DNA polymorphisms and assess whether a particular locus has

been affected by selection as opposed to neutral evolutionary processes. If the neutral hypothesis is rejected, the target locus or a gene in close proximity might affect phenotypic variation and hence, the fitness of an organism. In the candidate gene approach, candidate genes of known function are screened for polymorphisms. Such an approach increases the chance of finding signatures of selection (Pampoulie et al. 2006) but it still requires a solid *a priori* functional knowledge. Genome scans, on the other hand, genotype a large number of randomly chosen markers at a high sequencing coverage (i.e. number of copies of the same genetic variant), which increases the chances of identifying loci that display high levels of genetic structuring (Lotterhos & Whitlock 2015). These loci might be directly subjected to selection or linked to another locus that is directly under selection (known as genetic hitch-hiking, Luikart et al. 2003). This approach may lead to identifying candidate genes and a better understanding of causality. During the initial phase of adaptive divergence, only a small fraction of the genome is subjected to genetic differentiation ("genomic islands", Nosil et al. 2009). Moreover, adaptive phenotype traits are most often linked to several genes ("polygenic", Bernatchez 2016). Therefore, the key is to screen as many markers as possible. Interestingly, continuous developments of the RRS technology has enabled delivery of genome-wide SNPs for both model and non-model organisms, with protocols especially adapted to non-model organisms (Toonen et al. 2013). However, data quality and the power of detecting a signal of selection is improved by physical linkage between SNP loci which is often lacking for non-model organisms. If a reference genome is not available because it is not a model organism, the use of a reference genome from a closely related species to map the genomic markers is highly recommended (Manel et al. 2016). Therefore potential candidate loci can be matched to functional regions and the gene function can be investigated.

2.3 Combining multiple tools to study connectivity

Dispersal and connectivity can be considered over a short (ecological) or a long (evolutionary) time scales (Hendry 2017). Recent genetic studies suggest that ecological and evolutionary estimates of marine dispersal may provide a congruent view of connectivity (Pinsky et al. 2017, Reis-Santos et al. 2018). Genetic diversity informs on the short-term ecological time scale of dispersal, but also on the long-term demographic processes and colonization history, whereas otolith shape and microchemistry inform on the short-term ecological dispersal (Fig. I.5).

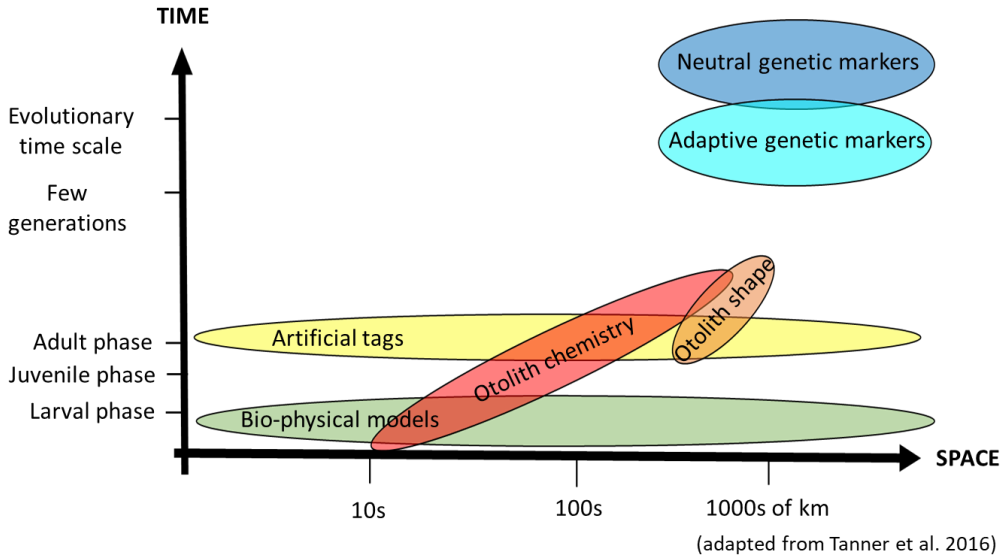


Figure I.5: Scheme representing the spatio-temporal scope of different traceability markers commonly used in stock delineation studies. X-axis represents spatial scale in 10s, 100s and 1000s kilometers and Y-axis represents temporal scale ranging from individual life history stages to evolutionary time scales. Artificial markers refer to both internal and external tags (Tanner et al. 2016).

Eventhough evolving genomic technologies make it increasingly easy to collect a large data set of genotypes, many marine species show subtle genetic differentiation (Pampoulie et al. 2008). Thus, genetic markers may be less suitable to study connectivity on ecological time scales. Otolith shape and elemental composition may complement genetic studies to grasp the different scales at which processes are happening (Campana & Thorrold 2001, Delerue-Ricard et al. 2018). Combining several data types that integrate information on the evolutionary and / or ecological time-scale will likely enhance discrimination power in detecting spatial and temporal structure (Table I.1 and Fig. I.5). Population assignment tests are conceptually similar to studies using chemical tags or the shape of fish otoliths (Thorrold et al. 2007). Both methods

result in assignment matrices that can be compared statistically. However, the various methods measure processes at different spatio-temporal time scales (Kaplan et al. 2017). In addition, different methods have different sensitivity. For example, physical tagging can trace short time scale processes but requires recapture and knowledge about dispersal (Hunter et al. 2003) whereas DNA is even available from historical samples (ancient DNA). The genetic signal requires some elapsed time since differentiation or local adaptation before it can be identified (see *section I.2.2.1*). Thus, otolith shape and microchemistry may complement genetic studies to grasp the different scales at which processes are happening (Campana & Thorrold 2001, Table I.1, Fig. I.5).

Table I.1: Temporal stability, effect of gene flow and use of the four methods applied in this thesis

	Otolith shape	**Elemental fingerprint**	**Neutral genetic markers**	**Outlier loci**
Used to	Assess stock structure in fisheries	Assess the environmental history of an individual	Infer population structure from demography and drift	Infer population structure potentially from natural selection
Influenced by gene flow among groups	**-**	**-**	**+**	**+/-**
Temporal stability	Influenced by major phenotypic and environ-mental changes	Year to year variation	Evolutionary time scale	Fast evolution

We believe that otolith applications combined with genetic tools have the potential to revolutionize our understanding of the integrity of fish populations and the management of fish stocks. One question that can be addressed by otolith shape, geochemical markers and genetic tools is the extent to which populations are differentiated.

3. Study system: the North Sea

3.1 Physical environment of the North Sea

The focal area of the PhD thesis is the North Sea, a shallow basin of over 700 000 km^2, with an average depth of 95 m and a maximal depth of about 700 m, along the Norwegian Trench (OSPAR 2000). The Southern North Sea is shallower than the Northern North Sea, with an average depth of 30 m. The salinity varies between 32 and 35 in most regions of the North Sea; inflow from the Baltic Sea and the many rivers dilutes the salinity locally. Mean Sea Surface temperature (SST) is 9.5 °C and is increasing progressively at a rate of 0.6 °C/100 years (OSPAR 2000). The residual circulation is anticyclonic (anticlockwise), due to the inflow of Atlantic Ocean waters passing out along the Scottish coast before reaching the Southern North Sea, then mixing with the outflow from the Baltic in the Skagerrak, dropping in salinity and passing out along the Norwegian coast. The English Channel connects the North Sea with Atlantic waters through a narrow passage with a strong tidal system. During the warmer months of the year (spring to autumn), a strong thermocline stratifies the North Sea in two parts (north and south of the Friesian front located around 54 °N, Fig. I.6). The thermocline has an effect on larval dispersal for species that spawn during the stratification, such as turbot (Barbut et al. 2019, Vandamme et al. 2014, Vandamme 2014). The climate of the North Sea is highly influenced by the inflow of water from the Atlantic Ocean (OSPAR 2000). The coastline around the North Sea is associated with a wide range of habitats such as sandbanks, mudflats, marshes and estuaries which are suitable nursery grounds for many marine fishes (Gibson 2015). The North Sea counts a large number of estuaries (n = 90), which drain the continent. The largest rivers are the Rhine and the Elbe, followed by the Scheldt, Meuse, Ijssel, Ems and Weser from South to North on the western side of the North Sea, and the Thames, Wash and Humber on the UK coast.

The North Sea is particularly sensitive to fragmentation as the activity levels in the North Sea are very high in view of an industrialized shoreline (OSPAR 2000). The North Sea is one of the most frequently travelled seas worldwide. There is increasing competition for the use of space for transport, exploitation of natural resources, fishing, tourism, conservation and these interests are not always compatible. Overexploitation, pollution, habitat loss and eutrophication have further modified overall ecosystem functioning and services (Allison et al. 2009, Doney et al. 2012). Ecosystems of the Northeast Atlantic Ocean are shifting towards a warmer status (Beaugrand et al. 2003). Such climatic changes are strongly affecting marine populations and

communities (Ottersen et al. 2010) and the prospects for the exploitation of natural resources (Cheung et al. 2009). An important and drastic consequence shaping the marine biota is the increasing frequency of regime shifts (Reid et al. 2001), which are abrupt and persistent changes in an ecosystem, usually after the transition of a tipping point. In response, international organizations have called for strong actions (FAO 2010, IPCC 2007). The significance of such changes in ecosystem functioning is largely reflected in the spatio-temporal heterogeneity in abundance and life history traits of organisms and populations (Selkoe et al. 2010).

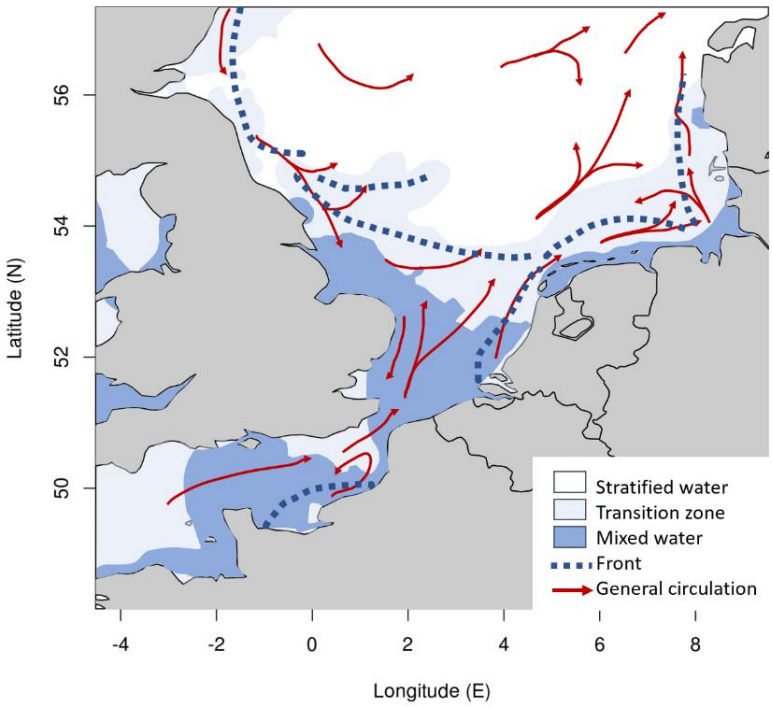


Figure I.6: Currents and fronts of the Southern North Sea (Barbut et al. 2019, modified from OSPAR 2000)

Substantial efforts are being made to integrate the objectives of the Marine Strategy Framework Directive (MSFD) in the new EU Common Fisheries Policy, as part of an Ecosystem-Based Fishery Management approach (EBFM). As a result, fisheries ecosystem plans have been developed for the North Sea and two other major European marine regions (Piet et al. 2011). Such efforts are also being made at the national level. For example, Belgium has been developing a coastal zoning plan (1999-2005), for which currently the Minister of the North Sea is developing spatial management plans. Currently, under pressure of the EU, the protected area covers 33% of the Belgian part of the Continental Shelf.

3.2 Variation in the chemistry of dissolved metals

The use of microchemistry to discriminate among fish stocks implies that their environments are chemically different and temporally sufficiently stable (Sturrock et al. 2012). However sampling all water masses might be unrealistic and may have limited added value because of the complexity of the incorporation of the microchemical signal (Morat et al. 2014). Element concentrations in the otoliths are not always correlated to the local water chemistry because of the physiological processes that regulate element intake, especially in natural habitats (Campana 1999). Nevertheless, while the relationships between incorporation of elements and environment is not always clear, there is no question that otolith composition is affected by environmental conditions (Elsdon et al. 2008). Therefore, microchemical studies increasingly integrate water samples to guide the choice of elements to be measured in the otoliths. Seawater elemental concentrations are influenced by natural (basin-specific signatures) and anthropogenic (pollution from continental and marine sources) inputs of elements.

Many areas along the North Sea coasts receive runoff water that carries the chemical signature of local river catchments (De Witte et al. 2016, Hamer et al. 2006, Van Alsenoy et al. 1993). The chemical composition of the European rivers draining in the North Sea each represents a drainage basin with a distinct geology (Mesozoic Alp geology for the Rhine, Eocene deposits of the Scheldt, Paleocene deposits for the Thames, Quaternary deposits for the Ems and Dollard, van Balen et al. 2005, Hartman et al. 2014, Preusser 2008, Royse et al. 2012, Yang & Nio 1989). Onshore-offshore gradients are observed in relation to the distance from riverine inputs for certain elements. Therefore, the area is particularly suited for otolith multi-elemental studies of fish spatial origin and movements. In addition to river signatures, influences come from the pollution history, and physiology and temporal variations in chemical signal. Although clean-up initiatives were carried out in most European estuaries and tended to decrease metal concentrations over the last decades (Emeis et al. 2015), the North Sea is one of the most polluted seas of the world (Halpern et al. 2008, Portman 1989) as most of Europe's largest ports are located by its coasts (OSPAR 2000). Heavy metals may originate from human activities such as dredging, dumping, shipping and the petroleum industry (OSPAR 2000) but also from natural inputs from rivers, the atmosphere, sediments and bio-geochemical processes (Scholten et al. 1998). The two main sources of riverine nutrient inputs are agricultural runoff and discharge of urban wastewater (OSPAR 2000).

In fish otoliths, each chemical element is indicative of specific processes or sources (Campana 1999, Sturrock et al. 2012). Low strontium (Sr) concentrations match with strong estuarine influences, linked to salinity gradients (Campana 1999, Leakey et al. 2009). For barium (Ba), environmental sources include salinity, terrestrial runoff, groundwater, pollution and remobilization from sediments (Hamer et al. 2006). However, otolith Sr and Ba concentrations may also be influenced by intrinsic factors such as genetics, diet, condition and ontogeny and additional extrinsic factors such as the ecological niche (Izzo et al. 2018), supporting former evidence (Barnes & Gillanders 2013, Sturrock et al. 2012). The Wadden Sea shows high manganese (Mn) concentrations that reflect enrichment in dissolved and particulate Mn compared to the surrounding water masses and/or hypoxic conditions (Dellwig et al. 2007, Limburg et al. 2015). The Belgian coastal zone is strongly influenced by the Scheldt, Rhine and Meuse rivers (Lacroix et al. 2004). Dissolved and particulate metal concentrations (Cadmium - Cd, Copper - Cu, Lead - Pb, Zinc – Zn which are associated with pollution) decreased significantly except for Cd in the Scheldt in the period 1978-1995 (Baeyens 1998). Four distinct areas were distinguished based on dissolved Ni, Cd, Cu, Zn and Fe and particulate Mn: the Rhine estuary, the Dutch coastal waters, the center of the Southern Bight of the North Sea and the English coastal waters (Nolting 1986). Based on a reduced set (Cd, Cu and Zn), the differences between the central Southern Bight and the coastal region pointed to the Rhine and the Scheldt as the main sources of trace elements (Duinker & Nolting 1982). The Belgian coast is influenced by the highly industrialized Scheldt Estuary, with actual and past traces of heavy metal pollution (Halpern et al. 2008, Portman 1989) whereas the inner Thames Estuary has been rehabilitated (Andrews & Rickard 1980, Attrill & Thomes 1995). Another interesting element, Rubidium (Rb), is associated to ingested plastic pollution (Lavers & Bond 2016). Overall, the reported chemical differences in seawater are promising for the application of otolith chemistry for population assignment. Despite the promising spatial patterns of chemical variation, one must keep in mind the presence of inter- and intra-annual differences in water chemistry affecting otolith chemical fingerprints (Chang & Geffen 2013, Elsdon et al. 2008, Reis-Santos et al. 2012, Sturrock et al. 2012).

4. European sole as a model species

Flatfish are not only economically but also ecologically important as they act as predators and also constitute an important source of food for other species in the wild (van der Veer et al. 2011). Sole (*Solea solea* L.) offers a perfectly suitable case to study adaptive evolution and connectivity. The good knowledge of sole biology, the well documented population genetics at the North Atlantic and Mediterranean scale for adult sole, available models of the connectivity and the latest developments in genotyping and trace element analyses, justify a study on local connectivity and population genetics of sole early-life stages.

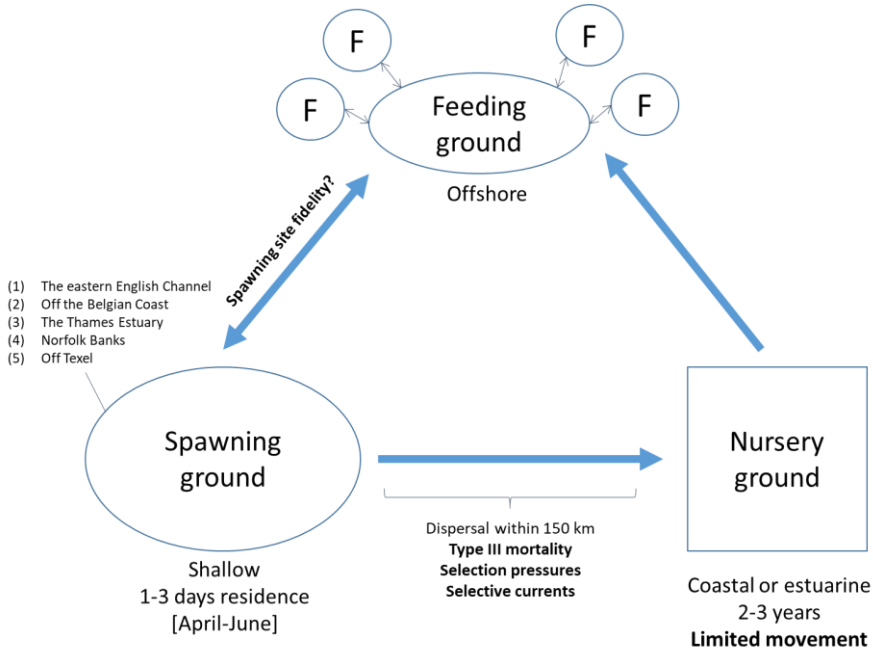


Figure I.7: Life cycle of sole (adapted from Gibson 2015)

4.1 The life cycle of sole

Flatfish typically occupy different, spatially non-overlapping, habitats during their life cycle: (1) spawning grounds, where both spawning adults and eggs remain only for short time, (2) nursery grounds, where juvenile fish typically reside for two to three years, and (3) feeding grounds, where adult fish reside when they are not reproducing (Gibson 2015, Fig. I.7).

There are five main sole spawning grounds in the Southern North Sea: (1) the Eastern English Channel, (2) the Thames Estuary, (3) off the Belgian coast, (4) the Norfolk Banks and (5) off Texel (Rijnsdorp et al. 1992, fig. I.8a). Spawning grounds are shallower than 30 m depth. Eggs are batch spawned in spring during a period of 3 to 4 months at temperatures above 10°C (Gibson 2015). Spawning peak is May-June in the North Sea, but spawning is earlier in warmer springs (Rijnsdorp et al. 1992). Pelagic eggs hatch after 7 to 8 days depending on temperature. Upon resorption of the yolk sac, the pelagic larvae feed on copepod nauplii and dinoflagellates (Russell 1976, Fig. I.9). The mortality of planktonic stages is high due to predation, the lack of prey, infections and unsuitable environmental conditions (Dinis et al. 1999). Mortality significantly drops with age following a type III survival curve (Gibson 2015). Postlarvae settle in nurseries at 7-10 mm length (van der Veer et al. 2001). The duration of the pelagic larval phase depends on water temperature and food availability but lasts four weeks on average (Rijnsdorp et al. 1992). Estimates of larval dispersal range between 80 km (Dorel et al. 1991) and 300 km depending on the spawning ground considered (Lacroix et al. 2013), with an average dispersal distance of about 150 km as estimated by biophysical modeling and genetic markers (Kotoulas et al. 1995, Lacroix et al. 2013). Nursery grounds are located in shallow sandy or muddy coastal areas with reduced salinity (mostly less than 10 m deep, Rijnsdorp et al. 1992). The distribution of nurseries is almost continuous along the North Sea coasts (Ellis et al. 2012, Rijnsdorp et al. 1992, see Fig. I.8b). Koustikopoulos et al. (1991) observed late stage larvae offshore in the Bay of Biscay and concluded that they had settled 20-90 km from the nursery grounds. One explanation could be the offshore location of spawning grounds in the Bay of Biscay compared to in the North Sea. A mix of complex currents and behavior drive larvae into favorable nursery grounds (Marchand & Masson 1989). Juveniles are adapted to the demersal habitat where they find small sized food items such as *Corophium* crustaceans. After two to three years on the nursery grounds, older individuals progressively move offshore to feed on polychaetes, mollusks and small crustaceans (Dorel et al. 1991). Adults feed mostly at night (Horwood 2001). Maturity is reached at about 3 years of age and 25 cm (i.e. the minimum landing size). One female produces an average of 300 000 eggs per spawning event (Horwood 2001). Intensive selective fishing has shortened the historical generation time of 8 years to 4.5 years (Cuveliers et al. 2011) and reduced size-at-age (Mollet et al. 2007). Adults migrate annually between feeding and spawning grounds. Tagging evidence support a strong “homing” behavior in some flatfish species who, once recruited to a spawning ground, will continue to return to spawn there (Hunter et al. 2003). However it is not known whether sole recruits return to the spawning

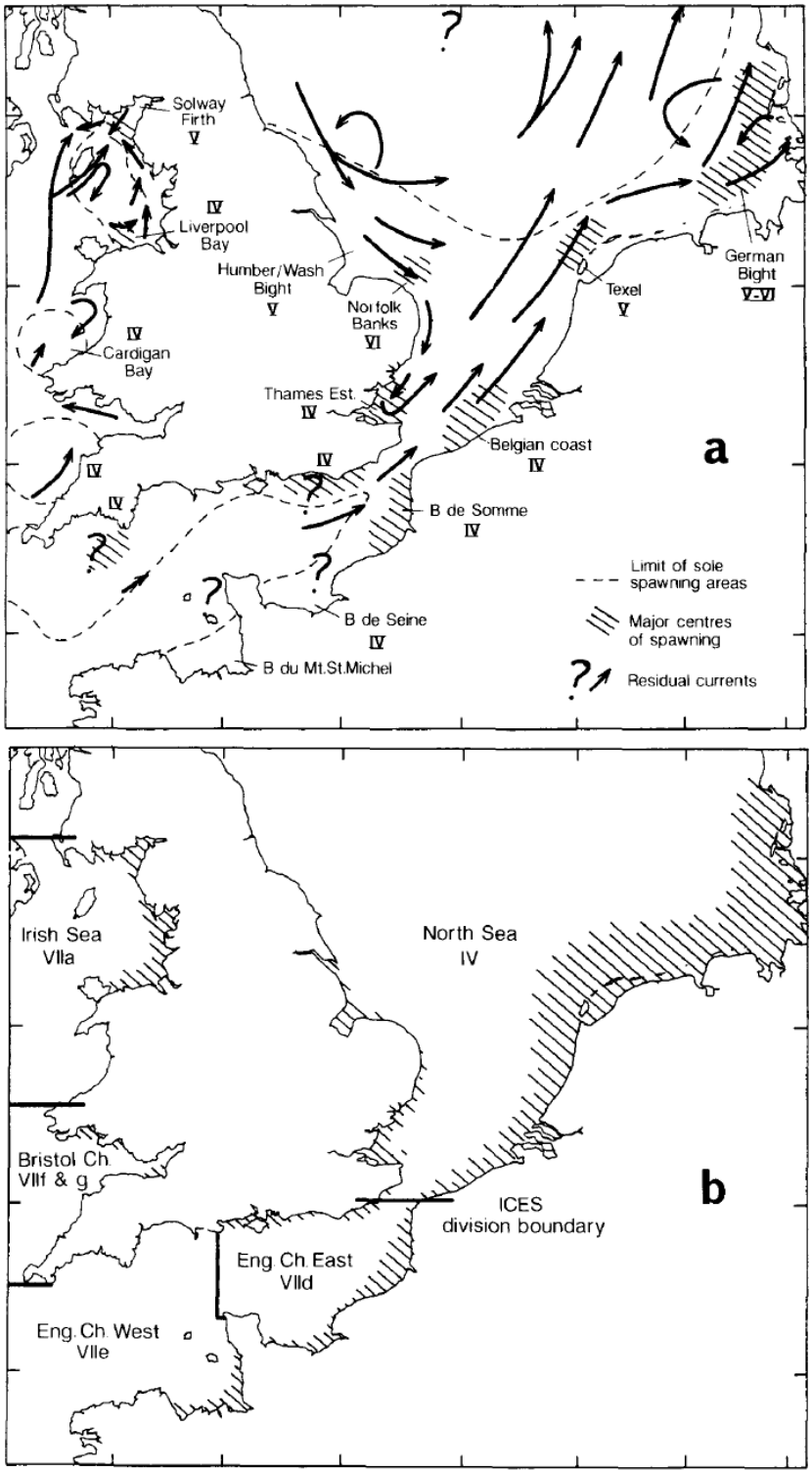


Figure I.8: Spawning ground (a) and nursery ground location (b) of sole of the Southern North Sea and local currents (Rijnsdorp et al. 1992). Updated maps from 2010 show similar high density patches (Ellis et al. 2012).

ground where they have been spawned (“natal homing”, Horwood 2001). There is a distinct difference between natal homing and homing. Natal homing would be supported by fixed population structure or adult aggregation on the spawning grounds through time.

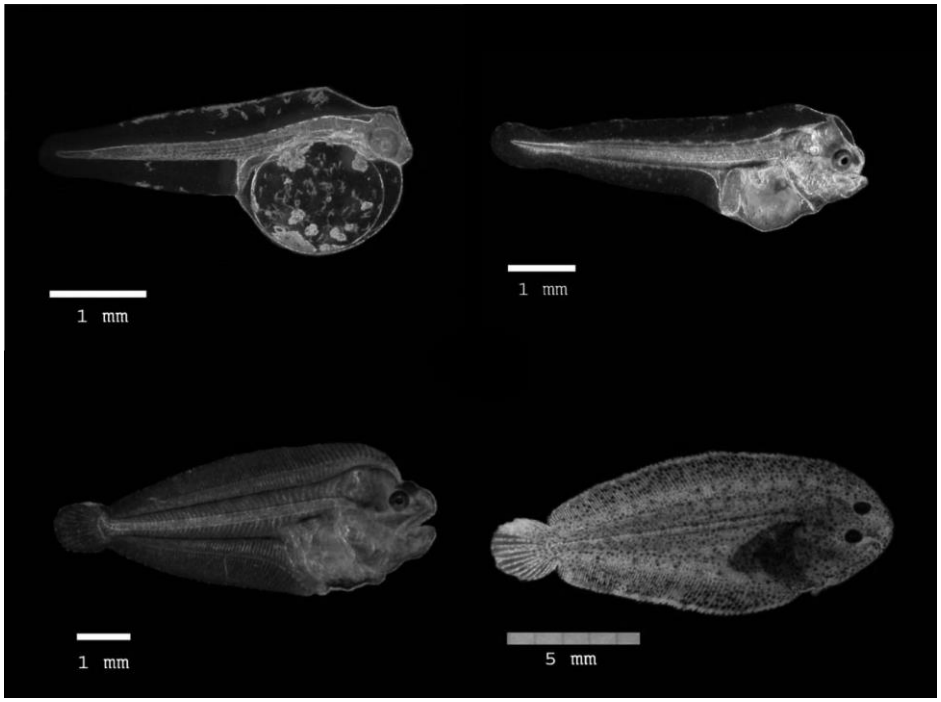


Figure I.9: Metamorphosis in *Solea solea* after Palazzi et al. (2006). Upper left: pre-larva on the day of hatching. Upper right: larva 9 days after hatching. Lower left: larva 20 days after hatching. Lower right: juvenile (metamorphosis completed) 28 days after hatching.

4.2 Stock status, fisheries and aquaculture

In the North East Atlantic Ocean, sole is managed in ten stocks (ICES 2018; Fig. I.10). According to the assessment models, five stocks, namely the Baltic Transition Zone, North Sea, Western English Channel, Eastern English Channel, and the northern and central Bay of Biscay, are performing well. Although the Baltic transition zone and the North Sea are fished above the

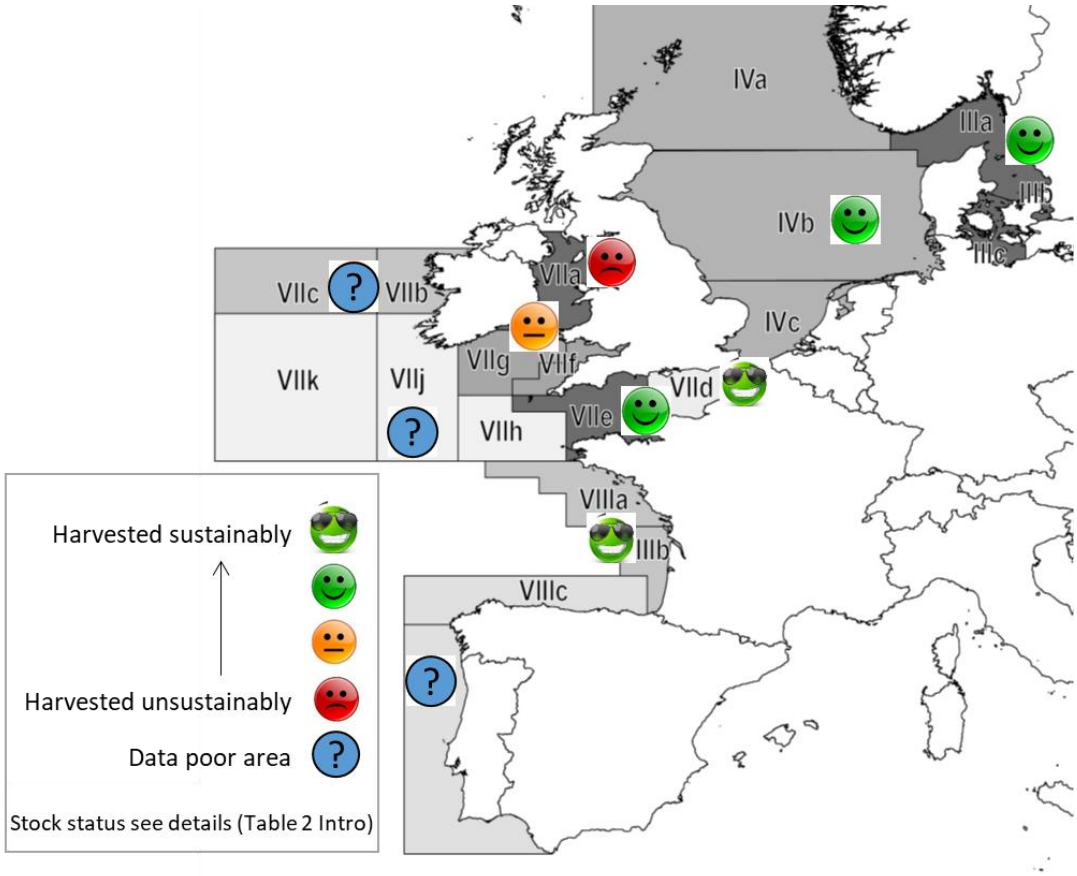


Figure I.10: ICES management units of sole. The ten management units are indicated in different shades of grey (some of them comprise more than one ICES area): 1. Baltic transition zone (IIIabc), 2. North Sea (IVabc), 3. Eastern English Channel (VIId), 4. Western English Channel (VIIe), 5. Bristol Channel and Celtic Sea (VIIfg), 6. Irish Sea (VIIa), 7. West of Ireland (VIIbc), 8. Celtic South and southwest of Ireland (VIIhjk), 9. northern and central Bay of Biscay (VIIIab), and 10. Cantabrian Sea and Atlantic Iberian waters (VIIIc and IXa).

Maximum Sustainable Yield (MSY), the North Sea outnumbers other stocks in size and also has the largest Total Allowable Catch (TAC). The Bristol Channel and Celtic Sea also have a Spawning Stock Biomass (SSB) that is sufficiently large to ensure an optimal use in the long term (full reproductive capacity). It is fished within safe biological limits, but there is an increased risk for stock collapse in the long term (i.e. fishing mortality is higher than the precautionary level). The

status of the Irish Sea stock, on the other hand, is less positive. The Irish Sea stock has the lowest SSB, which is below the level to maintain the MSY. Finally, the status of the stocks of the Celtic Sea South and southwest of Ireland, west of Ireland and, the Cantabrian and Atlantic Iberian waters, is unknown because of a lack of data. Nevertheless, the Celtic Sea South and southwest of Ireland stock seems to be doing relatively fine compared to an estimated trend.

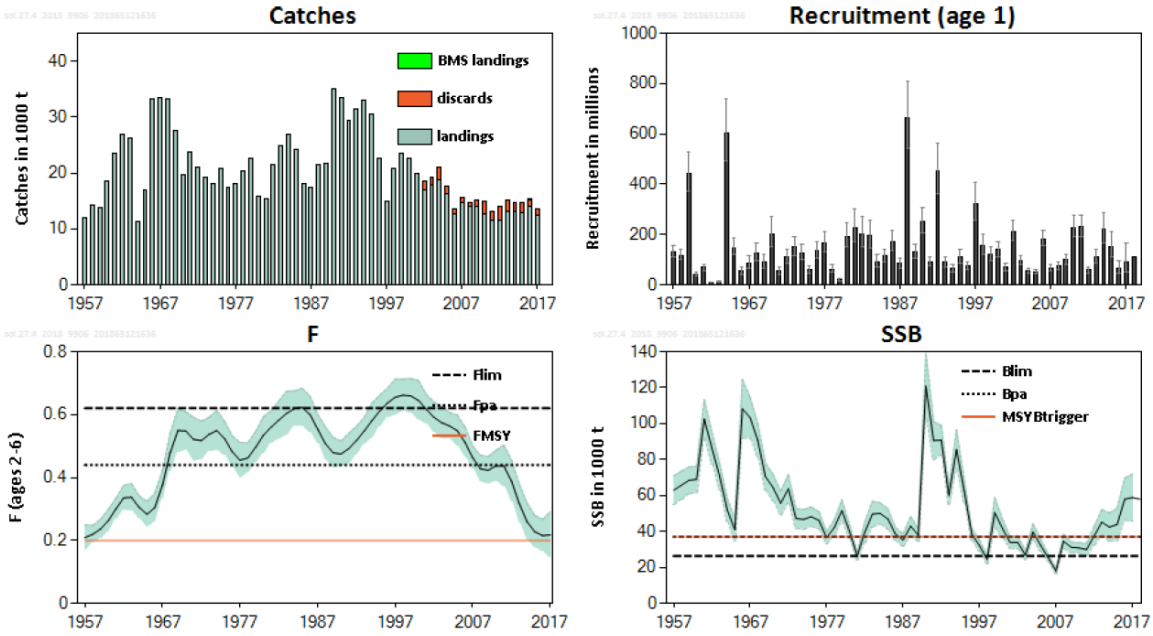


Figure I.11: sole stock assessment in the North Sea (ICES subarea IV) of the catches, recruitment (R), mortality (F) and spawning stock biomass (SSB). Estimates of discards are only available since 2002. Shaded areas (F, SSB) and error bars (R) indicate approximately 95% confidence intervals (ICES 2018).

The Belgian fishing fleet is highly specialized in beam trawling (Anon 2012, Lescrauwaet et al. 2013) focusing primarily on flatfish. The catch represents a total value of 80 million €, of which 80% are sold in Belgian ports. Sole represents the second highest value of landings in the EU and 25% of the landings are sold in Belgium (Anderson et al. 2012). Being one of the most traditional dishes in Belgium, sole generates on its own 30% of the current total value of fisheries in Belgium representing almost 28 million € (STECF 2018). Worldwide landings of wild caught sole amount to 33,000 tonnes, with aquaculture only contributing 100 tonnes per year. Belgian landings amount to 2,200 tonnes, originate mostly from the North Sea and Western English Channel, and represent 10% of all Belgian fish landings in weight (STECF 2018).

As mentioned before, the sole stock (55 000 ton) is exploited sustainably in the North Sea (ICES 2018, STECF 2018, Fig. I.11). Nevertheless, fishing mortality in the North Sea has been

above the precautionary reference limit of 0.4 during the period 1967-2007 and has been decreasing during the last few years but remains above the maximum sustainable yield limit (0.2, ICES 2018). Consumer demand for fish is high (especially for sole in Belgium) while the resource is becoming scarce across Europe. ILVO accidentally discovered some samples sold as cod proved to be cheaper species in fillets and in prepared food meals (ILVO 2014). Therefore sole is a potential target for tracebility issues. A sample of fresh sole from 27 Belgian fish shops demonstrated erroneous labelling in a few cases (Renders & Sas 2014). Despite its high market value (in Belgium a fillet costs 27€ on average), sole was not identified as a major target for substitution, except for a recent study conducted on the German seafood market (with a 50% substitution rate, Kappel & Schröder 2016). More recently, Christiansen et al. (2018) also reported mislabeling of sole (11.1%), which was substituted with other flatfish species or catfish. Despite a high European demand for validated and cost-effective tools for tracking stocks, intraspecific traceability is considered to be in its infancy (Nielsen et al. 2012).

Due to the high consumer demand and the depletion of many fish stocks, sole is also an interesting species for aquaculture. However, habitat shifts throughout the life cycle complicate management. Aquaculture research has some tradition (Howell et al. 1997, Imsland et al. 2003) and is based on the easier to breed and closely related species Senegalese sole *Solea senegalensis* L. (Tinti & Piccinetti 2000). Experimental selective breeding initiatives of sole (Blonk et al. 2010) have stimulated the development of genomic tools. The genome size of both species is around 700 Megabases (Mb) and covers 22 chromosomes (Libertini et al. 2002). Cerdà & Manchado (2013) advocated in a review on flatfish genomics for the use of NGS technologies to gather a profound knowledge of the biology. This has been implemented in research by Dotti do Prado et al. (2017), and Diopere et al. (2018). Also a linkage map was developed for sole based on transcriptome markers (Diopere et al. 2014). When comparing resources of six flatfish species used in aquaculture (Robledo et al. 2017), a common set of ~2500 orthologues and ~150 common miRNAs were identified in flatfish transcriptomes, likely reflecting their evolutionary diversification. More recently, Manchado's team has been assembling a reference genome for *S. senegalensis* and granted us preliminary access to call our genetic markers (**Chapter 3**). The reduced genetic diversity of aquaculture populations in *S. solea* (Exadactylos et al. 1999) and *S. senegalensis* (Sánchez et al. 2012) may threaten natural populations in case of repeated escape events (Bylemans et al. 2016, Exadactylos et al. 2007).

4.3 Evidence of population structure under a regime of high gene flow

The population structure of sole in the Northeast Atlantic Ocean has been a source of debate over the last two decades. On the one hand, some authors reported broad-scale panmixia of sole populations using either allozymes (Exadactylos et al. 1998, Kotoulas et al. 1995), Randomly Amplified Polymorphic DNAs (RAPDs)(Exadactylos et al. 2003), or exon-primed intron-crossing (Rolland et al. 2007). On the other hand, other authors suggested isolation-by-distance (Kotoulas et al. 1995), and spatially structured regions at a small scale such as the Bay of Biscay (Guinand et al. 2008) or between continental Europe (Biscay/German Bight) and the British Isles (Eastern Coast of England/Irish Seas) (Exadactylos et al. 2003) using allozymes and RAPD. The most recent genetic studies based on adult sole (Cuveliers et al. 2012, Diopere et al. 2018, Nielsen et al. 2012) identified four subpopulations: the Bay of Biscay, the North Sea and eastern English Channel, the Baltic Sea transition zone, and the Irish and Celtic Sea. Cuveliers et al. (2012) organized the most extensive sampling design which covered almost the entire Atlantic distribution. The authors confirmed the isolation-by-distance pattern suggested by Kotoulas et al. (1995). However, they focused on neutral processes such as gene flow and drift. Despite the low neutral genetic differentiation due to high gene flow and the lack of random genetic drift, local adaptation might still characterize subpopulations (as highlighted by Diopere et al. 2018). Moreover, most studies focused on adult stages whereas early-life stages of sole are experiencing high selection pressure due to high mortality, partly responsible for the high variability in recruitment (Rijnsdorp et al. 1992). Except for Guinand et al. (2008) former studies did not consider how genetic drift, selection, and gene flow might affect colonization of nursery grounds by larvae, and might reflect patterns of gene flow across nursery grounds and/or local selective processes during the juvenile stage. Genetic studies In the Bay of Biscay have suggested temporal stability of the genetic structure between age-0 and -1 sole but significant differences among sub-adults (Exadactylos et al. 1998, Guinand et al. 2008, Rolland et al. 2007). Differential selection on the nursery grounds might be responsible for the differences observed between subadults (Guinand et al. 2008). In the case of fish juveniles, local genetic variability among cohorts may exceed genetic variation at wider geographical scales (as seen in Rolland et al. 2007). Finally, local genetic differences might have been missed by large scale studies (such as Cuveliers et al. 2012 and Diopere et al. 2018).

Defining subpopulations is crucial because failure to account for multiple subpopulations within a management unit may lead to the potential collapse of the less productive subpopulations and a loss of resilience to environmental change (Carvalho & Hauser 1994, Hutchinson 2008, Kerr et al. 2017). The North Sea stock shows evidence of weak population structure (Cuveliers et al. 2012, Nielsen et al. 2012), which can be attributed to a combination of the large population size and strong connectivity (Hauser & Carvalho 2008, see Fig. I.3, Cuveliers et al. 2012). However, spawning is non-random and might result in patterns of genetic patchiness through progeny issuing from a few parents (Hedgecock 1994). Therefore, independent subpopulations may exist within the North Sea, although stock boundaries are not well defined and are not considered in stock assessment. The management tools of sole are mostly targeting ecological short-term demographic independence, as opposed to evolutionary long-term demographic independence (Waples et al. 2008). They were not originally developed in relation to the biology of the species but rather to geopolitical interests (Reiss et al. 2009).

5. Objectives and outline of the PhD thesis

The overall objective of this thesis was to provide new insights in the ecological and evolutionary processes linked to the connectivity of early-life stages of a flatfish inhabiting the North Sea. We looked at three spatial scales: (1) within and (2) between nursery grounds of the Southern North Sea and (3) across the Northeast Atlantic Ocean. The general research question was: What is the spatio-temporal variation of connectivity of early-life stages between and within the spawning and nursery grounds in sole? More specifically, we focused on the five following key hypotheses:

Hypothesis 1: The population structure of adult and juvenile sole is subtle

Hypothesis 2: Self-recruitment is a common feature of sole

Hypothesis 3: Several spawning grounds contribute to a single nursery ground of sole

Hypothesis 4: Juvenile sole constitute the progeny of a single genetically homogeneous spawning aggregation

Hypothesis 5: Juvenile sole show limited connectivity between nursery grounds

The connectivity of the early-life stages of sole has been studied using otolith shape, microchemistry, genomic markers and/or an integrated holistic approach. Three methods (resulting in seven marker types), each providing complementary information, were investigated: (left and right) otolith shape and elemental fingerprints (larval, settlement and nursery ground signatures) were used to assign individual fish to their spawning and nursery grounds while high-resolution genotyping was used for identifying relatedness between individuals and (neutral and outlier) population structure (see *section I.2.2.1* for definitions).

In **Chapter 1** we focus on the potential of otolith shape to understand the biology of juvenile sole. A single otolith of adult fish is commonly used to discriminate between fish stocks. However, adult flatfish have asymmetrical otoliths (i.e. difference between left and right otoliths). We test for the presence of asymmetry in early-life stages and assess the sensitivity of otolith shape for the recognition of populations on a small spatial scale (5 - 500 km; *Hypothesis 5*).

In **Chapter 2** we look at differences in otolith elemental fingerprints throughout the early life stage. Few studies offer a dynamical view of connectivity but rather provide a screen-shot of population structure at a given stage (e.g. juveniles on the nursery grounds). Moreover, most studies focus on large spatial scales of hundreds of kilometers and compare estuarine habitats. We cover this knowledge gap for sole in the North Sea by looking at differences in otolith chemistry between open coastal environments from hatching to sampling. Significant differences in otolith chemistry between nursery grounds would allow us to trace potential migrants at the juvenile stage (*Hypothesis 5*). We also assess the number of potential spawning ground origins and the frequency of self-recruitment in sole (*Hypothesis 2*, *3* and *4*).

Local adaptation may occur when selection overrides both random genetic drift and the homogenizing effect of gene flow between populations. Sole is a high gene flow species and the occurrence of local adaptation is expected to be occasional. However, its large effective population size should enhance the response to selection because of the large standing genetic variation (Barrett & Schulter 2008). Sole experience diverse environments due to its wide distribution area. A seascape analysis on the Northeast Atlantic scale identified winter seawater temperature, food availability (measured as chlorophyll water concentrations) and coastal currents as the main drivers of geographical distribution of genetic diversity (Diopere et al. 2018). However, selective pressures at the local scale may be substantial, especially for early-

life stages due to extremely high mortality (following type III survival curve, Gibson 2015). In **Chapter 3** we look in detail into the potential effect of connectivity on the large and small scale evolutionary patterns of sole using hundreds of molecular markers. The neutral markers allow to monitor changes in neutral genetic diversity, the presence of cohort structure and sweepstake effects, while the markers putatively under selection (i.e. outliers) allow to better understand genetic basis of location adaptation (*Hypothesis 3* and *4*). Additionally, we integrate adults from sole populations outside of the North Sea (resequenced from Diopere et al. 2018) and distinguish genetic signatures between life stages (juvenile vs. adult; *Hypothesis 1*).

In the **General Discussion and Perspectives** I summarize the results of the three chapters and put them into perspective with the literature. We assess the convergence between evolutionary and ecological connectivity through the integration of otolith shape, elemental signature, neutral and outlier genetic markers. We speculate that combining evolutionary and ecological methods may provide us with an improved accuracy to detect spawning ground signature and nursery ground signature, thus with a new understanding of early-life connectivity at the individual level. To identify the spawning ground origin of a single juvenile, one may use the larval elemental signature of the otolith, which is influenced by the spawning ground chemistry, and genetic markers, which are linked to the parental gene pool. To distinguish nursery ground signatures, otolith shape and sampling elemental signatures on the nursery can be combined. In addition, we suggest new lines for future research and improvements to be made to optimize tools to better understand connectivity such as: developing and validating biophysical models in a context of evolutionary models, developing genomic knowledge to better understand the basis of adaptation, monitoring temporal variability in microchemistry tools, and refining criteria and assumptions of methods to detect and define adaptive markers. Ultimately, evidence from the field and models should be integrated into management strategies. Finally, global change will clearly be a central criteria to take into account to predict the future of environmental and fisheries research.

Chapter 1

Size-effect, asymmetry and small-scale spatial variation in otolith shape of juvenile sole in the Southern North Sea

Sophie Delerue-Ricard, Hanna Stynen, Léo Barbut, Fabien Morat, Kelig Mahé, Pascal I. Hablützel, Kris Hostens, Filip A.M. Volckaert

Abstract

While otolith shape analysis can provide a valuable tool for discriminating between fish populations, factors which may influence otolith shape, such as the effect of size, directional asymmetry in growth, and local environmental conditions, are often unknown. Here, we analyzed differences in otolith shape across three size classes of age-0 common sole *Solea solea L.* from nursery grounds off the Belgian coast and in the Wadden Sea. Across size classes, form-factor decreased and roundness remained consistently high in both nursery grounds, while ellipticity increased in the Belgian nursery. Directional asymmetry between left and right otoliths measured by Fourier coefficients accounted for 0.96% and 7.2% of the variance when comparing otoliths overall, and for each size class, respectively. Within the Belgian nursery, results were consistent across sampling years and locations. In addition, otolith shape was marginally different between nursery grounds, but highly variable within nursery grounds. A small divergent group, which seems partly related to fish size, was noted at both spatial and temporal scales. Based on these results and before embarking on a study of population structure using otolith shape in age-0 common sole, we recommend testing for directional asymmetry and fish size effects across the entire region of interest.

Published online in Hydrobiologia (24 August 2018)

1. Introduction

Coastal ecosystems play a key role as nursery grounds for juvenile fish (Costanza et al. 1998) because of their high productivity. However, coastal nursery grounds have a discontinuous distribution, and experience increasing fragmentation due to anthropogenic pressures, which can result in changes of metacommunity diversity and dynamics (Jung et al. 2017). In addition, habitat heterogeneity within nursery grounds may influence the spatio-temporal dynamics of fish populations on a small spatial scale (Le Pape et al. 2003).

Various indirect methods are available to assess the spatial population dynamics of the early-life stages of marine fish, such as modeling of larval transport, comparison of parasite communities, analysis of genetic differentiation, chemical composition and shape of fish otoliths, or tagging of the late juvenile stages (Cadrin et al. 2014, Koubbi et al. 2006, Pawson & Jennings 1996, Neves et al. 2018). Despite the diversity of methods, population structure and connectivity patterns between and within nursery grounds remain challenging to evaluate (Kaplan et al. 2017). Moreover, each tool may integrate information at specific, yet different spatial and temporal scales. Biophysical modeling of larval transport and otolith shape variation focus on “ecological” time scales (Lacroix et al. 2013, Thorrold et al. 2001), while genomic tools have been applied to measure population structure over both short “ecological” and longer “evolutionary” time scales (Pinsky et al. 2017). Yet, advanced genomic tools work best with extensive genomic background information on the species of interest and well-preserved DNA.

Otoliths are calcified structures residing in the inner ear of fish, growing with the constant deposition of successive calcium carbonate layers. As they grow, otoliths incorporate time-delimited information that can be used to describe the development and ambient environmental conditions experienced by the individual. In addition, otolith shape is a useful and well-established tool to discriminate between species and stocks (Campana & Casselman 1993). However, ontogenetic development affects otolith shape through changes in growth and metabolism, especially during sexual maturity (Cardinale et al. 2004, Hüssy 2008, Mérigot et al. 2007). During the early-life stages, otoliths evolve from circular to more complex shapes (Lagardère & Troadec 1997, Hüssy 2008), which may limit the utility of otolith shape as stock marker for immature fish or fish of different age classes. Moreover, left and right otoliths may be different, i.e. directionally asymmetrical, particularly in flatfishes (Mille et al. 2015). Environmental and anthropogenic pressure may cause stress-induced changes such as increased

levels of directional asymmetry (Gagliano & McCormick 2004), which is disadvantageous because it interferes with hearing and orientation (Anken et al. 2002, Lychakov & Rebane 2005).

Most studies have compared intraspecific differences in otolith shape either over large distances (>500 km e.g. Vieira et al. 2014), across oceanographic barriers (Tuset et al. 2003) or between habitats (Morat et al. 2014, Vignon & Morat 2010). It is not yet clear what can be learned from otolith shape at small spatial scales and in the absence of strong oceanographic barriers. Here we used otolith shape analysis to investigate small-scale nursery structure of the flatfish sole *Solea solea* (Linnaeus, 1758; Soleidae) in the shallow subtidal of the Southern North Sea. Sole is less abundant than European plaice (*Pleuronectes platessa* L.) or dab (*Limanda limanda* L.*)* in the study area, but it has a high commercial value, contributing to important regional and local demersal fisheries. To date, larval connectivity patterns between spawning and nursery grounds remain unclear (Burt & Millner 2008, Lacroix et al. 2013). In the Southern North Sea, sole displays peak spawning from April to June. After hatching the pelagic larvae drift for ~1 month in the water column before settling in a nursery (Russell, 1976; van der Land, 1991). Genetic data from adult sole suggest isolation by distance along the Atlantic coast, and weak population structure within the Southern North Sea (Cuveliers et al. 2012, Diopere et al. 2018), with a mean dispersal distance estimated at 150 km (Kotoulas et al. 1995, Lacroix et al. 2013). Within the nursery grounds, age-0 sole travel shorter distances, between 10 and 30 km (Le Pape & Cognez 2016). Nevertheless, small-scale spatio-temporal variation in connectivity between and within nursery grounds has, to the best of our knowledge, not been investigated empirically. The degree of isolation between and within nursery grounds may be estimated from the variation in otolith shape. Being able to delineate the smallest level of spatial resolution is an important step to measure connectivity and to better understand recruitment patterns.

In the current study we addressed three key questions to determine whether otolith shape can be used to assess small-scale spatial patterns in juvenile flatfish: (1) what is the effect of fish size on otolith shape; (2) what is the effect of directional asymmetry between left and right otoliths, and (3) whether is there spatio-temporal variation in the otolith shape of age-0 sole.

2. Material and methods

We investigated variation in otolith shape over two years and at two spatial scales within the dispersal range of sole larvae, to better understand the resolution of otolith shape variation between and within nursery grounds in the Southern North Sea. Juvenile sole were sampled at the regional scale (between nursery grounds, 500 km distance) from the Belgian and Wadden Sea nursery grounds in 2014 (NL2014 and BE2014). Juvenile sole were sampled at the local scale (within nursery) from the eastern and the western shallow subtidal coastal zones of the Belgian nursery in 2013 and 2014 (BE2013 and BE2014, 40 km distance between coastal zones). The discriminatory power of otolith shape was tested using Fourier coefficients and shape indices (see further). In addition, asymmetry between left and right otoliths and correlations between the shape indices and fish size were estimated using the combined data from all fish from the three datasets (BE2013, BE2014, NL2014).

2.1 Sample collection

Juvenile sole were sampled off the Belgian coast from late August to late September in 2013 and from mid-September to mid-October in 2014 (Fig. 1.1; Table 1.1) and at two stations in the Wadden Sea in September 2014. At each site, specimens were collected by beam trawling either on board of RV *Simon Stevin* (B-FishConnect project campaign), RV *Belgica* (Belgian Demersal Young Fish Survey, DYFS) or RV *Stern* (Dutch DYFS). Sea surface temperature was measured at each site at the time of collection. Each fish was measured to the nearest mm (Standard Length, SL). Sagittal otoliths were extracted from a total of 314 individuals ranging from 52 to 102 mm SL. Finally, each otolith was cleaned, sonicated, and then stored dry in plastic vials. For this study, age-0 sole were used. Fish age was confirmed by the absence of an annual ring in the otolith. To assess variation in otolith shape associated with fish size, the 314 individuals were divided into three standard length size-classes, L1 (52-76 mm, n = 105), L2 (76-82 mm, n = 105) and L3 (82-102 mm, n = 104).

Table 1.1: Number of fish analyzed per station and per nursery (Belgian coast and Wadden Sea), including the date of capture, GPS coordinates, name of sampling survey (Demersal Young Fish Survey, DYFS, and B-FishConnect) and the sea surface temperature at the time of sampling are given

Year	Area	Coast side	Station	Sample size	Sampling date	GPS coordinates	Sea surface temperature (°C)	Survey
2013	Belgium	West	OOST	14	28/08/2013	51.23 N, 2.80 E	19.3	B-FishConnect
			ST16	30	10/09/2013	51.19 N, 2.70 E	18.8	DYFS
		East	ST09	33	09/09/2013	51.35 N, 3.00 E	19.5	DYFS
			ST05	33	12/09/2013	51.45 N, 3.01 E	17.7	B-FishConnect
			ST37	29	12/09/2013	51.48 N, 3.14 E	17.8	DYFS
			ST06	14	13/09/2013	51.38 N, 2.85 E	17.7	DYFS
			FT02	20	24/09/2013	51.43 N, 3.31 E	16.9	DYFS
			Total	173				
2014	Belgium	West	ST23	30	15/09/2014	51.13 N, 2.70 E	18.1	DYFS
		East	ST09	27	16/09/2014	51.35 N, 3.00 E	18.5	DYFS
			FT01	23	10/10/2014	51.35 N, 3.10 E	16.9	B-FishConnect
			Total	80				
	Wadden Sea		NL01	34	16/09/2014	53.48 N, 6.49 E	18.2	DYFS
			NL02	27	23/09/2014	53.35 N, 6.97 E	18.1	DYFS
			Total	61				

2.2 Otolith shape indices and Fourier coefficients

Left and right sagittae were placed on a microscope slide with a black background, positioned with the *sulcus acusticus* oriented towards the observer and the posterior side oriented to the top. External transmitted light sources were used and adjusted to illuminate the otoliths. High-contrast images were produced using an Olympus ColorView digital microscope camera linked to an Olympus BX51 microscope (20x magnification). Images were then processed with the TNPC 7 software ('Digital Processing for Calcified Structures'; www.tnpc.fr) to extract the following morphometric parameters: surface area of the otolith (A_o); otolith perimeter (P_o); maximum length (L_o); and width (O_w) to the nearest 10^{-2} mm. Based on these measurements, form-factor,

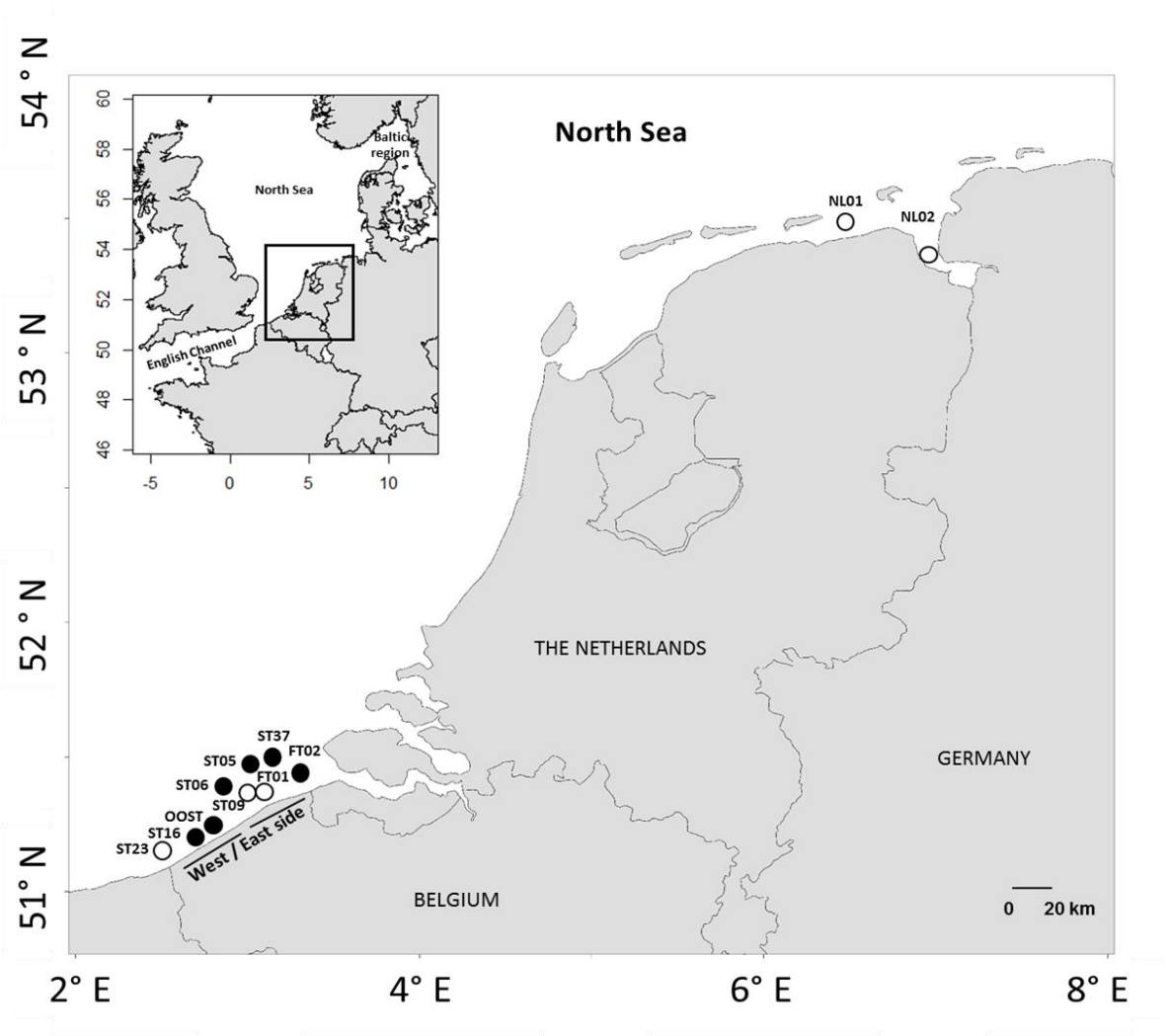


Figure 1.1: Map of the sampling stations of age-0 sole on the Belgian and the Wadden Sea nursery grounds in 2013 (full circle) and 2014 (empty circle)

roundness, circularity, rectangularity, and ellipticity were calculated as in Tuset et al. (2003). Form-factor estimates surface area irregularity and has a maximal value of one in the case of a perfect circle. Roundness and circularity describe the proximity of shape to a circle and have minimal values of one and 12.57, respectively. The closer both indices approach the minimal value, the closer the shape of the otolith is to a perfect circle. Rectangularity gives the proportion of the length and width with respect to the area and has a maximal value of one in case of a perfect square. Ellipticity describes the proportion of change in the different axes (Tuset et al. 2003).

In addition to the use of shape indices, otolith contours were described by Elliptic Fourier Descriptors (EFDs) which were obtained with TNPC 7 software. For each otolith, the first 99 elliptical Fourier harmonics were extracted and normalised with respect to the first harmonic. Hence, they were invariant to otolith size, rotation and starting point of the contour description (Kuhl & Giardina 1982). To determine the number of elliptical Fourier harmonics required to reconstruct the otolith outline, the Fourier Power (FP) spectrum was calculated for each individual otolith. For the n^{th} harmonic, Fourier Power is given by the equation:

$$FP_n = ({A_n}^2 + {B_n}^2 + {C_n}^2 + {D_n}^2)/2,$$

where A_n , B_n , C_n and D_n are the Fourier coefficients of the n^{th} harmonic.

Cumulative Fourier Power (FP_c) was calculated by summing the Fourier power of each harmonic:

$$FP_c = \sum_1^n FP_n$$

The number of harmonics was chosen such that the mean cumulated FP reached 99.99%; hence shape was reconstructed at 99.99% (Mérigot et al. 2007, Gonzalez-Salas & Lenfant 2007). The first harmonic was not considered for further analysis (except for reconstructing average shape), because it had already been used for normalization, and because it would dominate shape reconstruction and mask the information derived from the other harmonics (Crampton 1995).

2.3 Statistical analysis

First, a pilot experiment was conducted to assess the consistency of our methodology with regard to otolith position and lighting. Ten randomly chosen otoliths were repositioned and four replicate pictures were taken. A dendrogram analysis was performed to test the extent of differences between images of the same otolith and differences between images of different otoliths.

Multi-collinearity between shape indices was assessed by Pearson correlation. Only form-factor, roundness and ellipticity were kept for further analyses. Shape indices were compared between length classes, the two nursery grounds, and sampling years. Differences in the mean

values of shape indices between each size class and each dataset were assessed using non-parametric *k*-sample Anderson-Darling tests under the null hypothesis that all samples originated from the same distribution.

Before analyzing asymmetry levels and spatio-temporal variation in the Fourier coefficients, Principal Component Analysis (PCA) was applied to the Fourier coefficients to avoid collinearity of shape descriptors, and to reduce the number of dimensions while retaining the majority of the variance (Rohlf & Archie 1984). Only PCs with eigenvalues higher than the mean of all eigenvalues were retained to remove Principal Components (PCs) associated with noise. Fourier coefficients were significantly correlated with fish size. Residuals of the PCs of Fourier coefficients have been used to remove the effect of fish size, as in Mahé et al. (2016).

Partial Redundancy Analysis (pRDA) tests were performed with the PCs of the Fourier coefficients as response variables to explore the effect of otolith side, between and within-nursery spatial variation, year, coastal region and Sea Surface Temperature (SST). Redundancy analysis is an extension of multiple regression analysis to multivariate response data (Legendre & Legendre, 2012). In all pRDAs, SL was used to correct for fish size, and a permutation test was used to assess the significance of the explanatory variables. A pRDA was performed to test the effect of otolith side (left/right) on shape. To visualize and quantify differences between left and right otoliths, the average otolith shape of each side was also built by outline reverse Fourier transformation, as in Mille et al. (2015).

Dendrograms were produced by Ascending Hierarchical Classification (AHC) to verify the PCA clustering of individuals at the regional and local scales. AHC maximizes the similarities within clusters and differences between clusters of similar otolith shapes. To compute the AHC, the function 'HCPC' based on Ward's distance was used.

pRDA was also used to determine regional and local scale (between and within nursery) annual differences in otolith shape. We used multiple linear regressions to test at the regional scale the effect of nursery, sampling date, and SST on the Fourier coefficient PCs, and to test at the local scale the effect of year, coastal zone (eastern/western), and SST on the same PCs. We determined the variance explained by each explanatory variable. Only the left otoliths were used in the RDAs. Distribution of standard length was compared using a randomization test. All

statistical analyses were performed using the *ade4*, *FactoMineR*, *vegan* and *stats* packages in the statistical environment R (R Development Core Team 2018).

The outlines of the mean Fourier coefficients (prior to size correction) were plotted as an overlay image to visualize differences in otolith shape between left and right otoliths. The same overlay was used to visualize differences between left otoliths of the Belgian and Wadden Sea nursery grounds, and between left otoliths of the two PCA clusters (Mille et al. 2015).

3. Results

Cluster analysis showed that Fourier coefficients extracted from replicated images of the same otolith were consistently grouped together and our measures were accurate (Suppl. Fig. 1.1). Otolith form-factor, ellipticity and roundness were not redundant (Pearson correlation test, $P > 0.05$) and were kept for further analysis. For each dataset (BE2013, BE2014 and NL2014), form-factor significantly decreased with increasing standard length (Fig. 1.2a; Table 1.2). Only in the Belgian nursery ellipticity was significantly different between length classes for both sampling years (Fig. 1.2b). Roundness was generally close to one and did not vary significantly between size classes or nursery grounds but varied between sampling years in the Belgian nursery (Suppl. Fig. 1.2). In addition, otoliths sampled in 2014 off the Belgian coast were significantly more circular than in the Wadden Sea, and also significantly more circular and rounder than otoliths sampled in 2013 on the Belgian nursery as shown by form-factor variations (Table 1.2).

The Fourier coefficients differed significantly between left and right otoliths ($n = 314$, $P < 0.01$). This directional asymmetry explained almost 6% of the variance (Table 1.3) and was spread homogeneously across the otolith outline (Fig. 1.3a). Although the overall average directional asymmetry was quite small when all datasets and size classes were pooled (0.96% overall), asymmetry was much higher when each dataset and size classes were examined separately (mean asymmetry for each dataset was 7.2%). Left otoliths of sole (blind side) were larger than right otoliths (eyed-side) and were thus kept for further analysis.

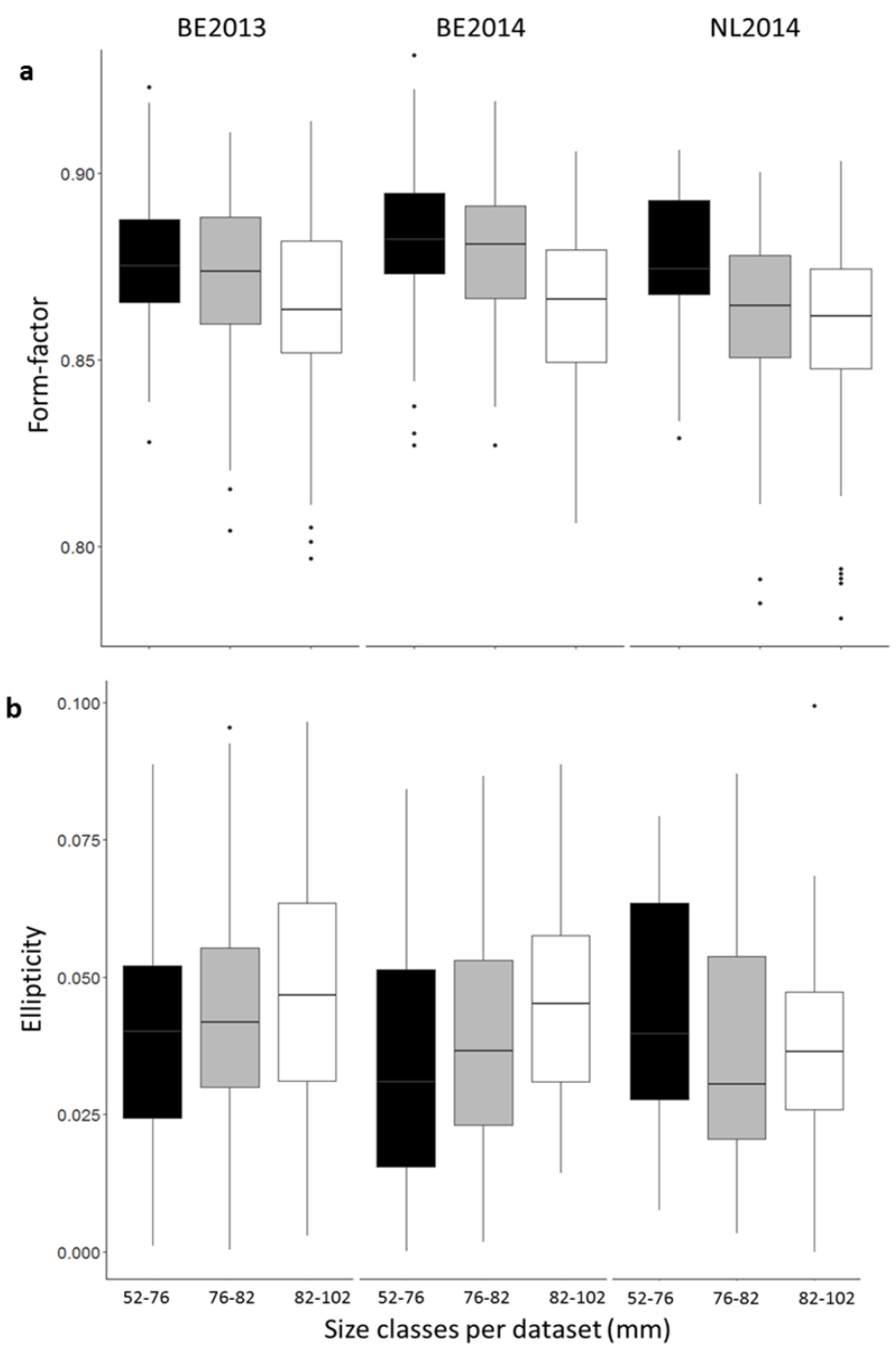


Figure 1.2: Boxplot of form-factor (a) and ellipticity (b) for 314 age-0 sole juveniles of sole of three size classes for each dataset colored from darker to lighter according to size classes (n = 173 for BE2013, n = 80 for BE2014, n = 61 for NL2014)

Table 1.2: Summary of the mean value of the shape indices (form-factor, ellipticity and roundness) per size class for each dataset (n = 173 for BE2013, n = 80 for BE2014, n = 61 for NL2014) and comparisons of each shape index between the three different size classes (L1, L2 and L3) for each dataset and for the comparisons of all age-0 sole together at the regional (Belgian vs. Wadden Sea nursery) (n = 141) and at the local scale (Belgian 2013 vs. 2014) (n = 253), including the significance level

Shape indices	Datasets	L1 (52-76 mm)	L2 (76-82 mm)	L3 (82-102 mm)	L1-L2-L3 *P* value	All sizes	*P* value
Form-factor	BE2013	0.876	0.871	0.864	< 0.001	NL vs BE 2014	< 0.001
	BE2014	0.883	0.879	0.865	< 0.001		
	NL2014	0.874	0.860	0.855	< 0.01	BE 2013 vs 2014	< 0.01
Ellipticity	BE2013	0.039	0.043	0.048	0.016	NL vs BE 2014	0.723
	BE2014	0.034	0.038	0.047	0.012		
	NL2014	0.042	0.036	0.037	0.122	BE 2013 vs 2014	0.042
Roundness	BE2013	0.947	0.946	0.939	0.354	NL vs BE 2014	0.807
	BE2014	0.961	0.960	0.943	0.081		
	NL2014	0.946	0.958	0.953	0.102	BE 2013 vs 2014	0.005

Table 1.3: Summary of partial redundancy analysis (pRDA) for asymmetry (n = 314) and for spatio-temporal variations at the regional scale (Belgian vs. Wadden Sea nursery) (n = 141) and at the local scale (eastern vs. western coastal zones of the Belgian nursery and 2013 vs. 2014) (n = 253). Degrees of freedom (df), significance values and the variance explained by each variable (R^2) are included

Hypothesis	Factors	df	*P* value	R^2 adjusted (%)
Asymmetry: all otoliths	Otolith_side	1	0.01	5.8
Regional differences: NL-BE 2014	Nursery	1	0.04	0.6
	Sampling date	1	0.41	< 0
	SST	1	0.49	< 0
Local differences: BE 2013-2014	Year	1	0.08	< 0
	Sampling date	1	0.34	< 0
	East / West coastal regions	1	0.70	< 0
	SST	1	0.19	< 0

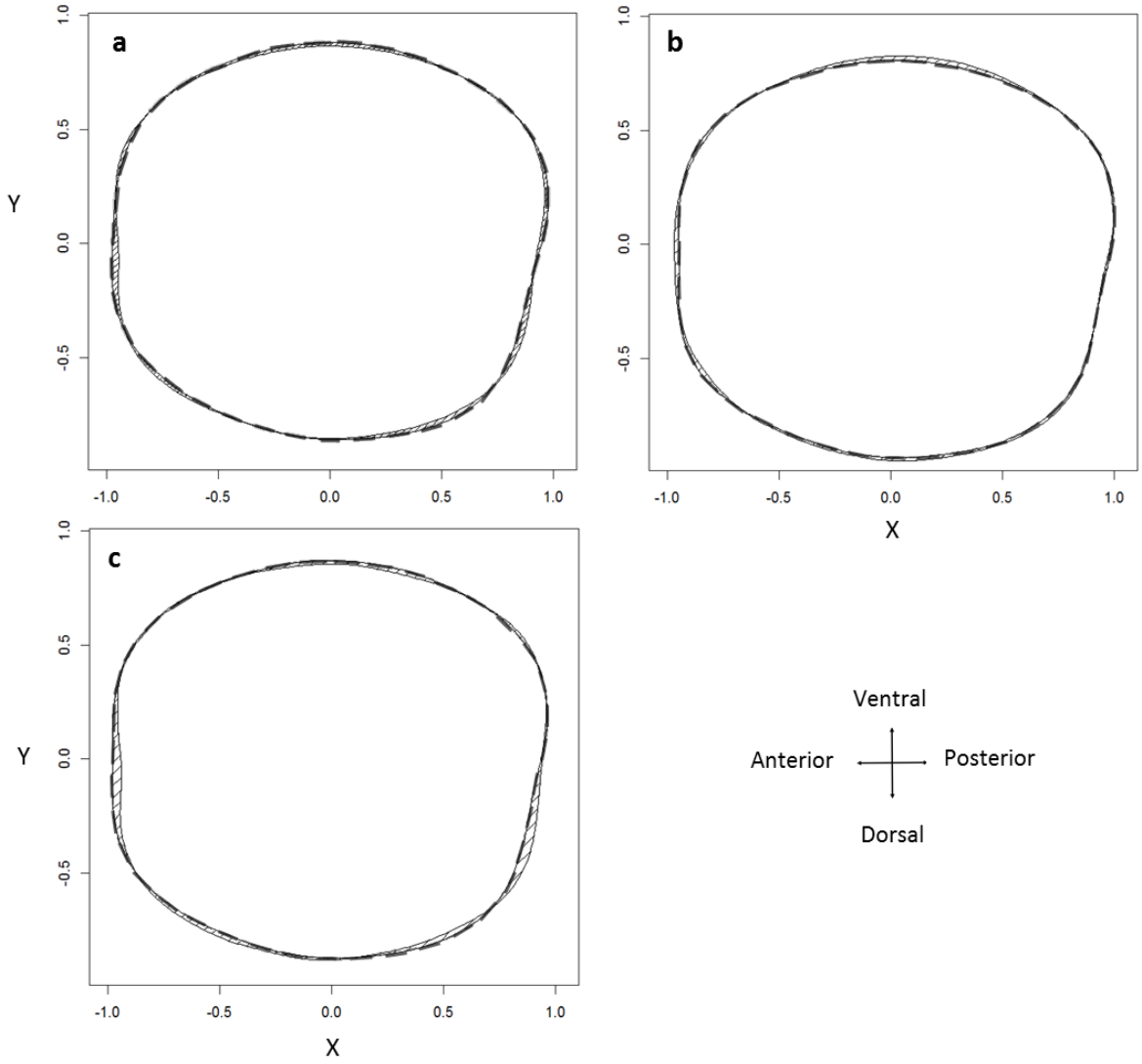


Figure 1.3: Reconstruction of average sagittal otolith shape for 314 age-0 sole based on Elliptic Fourier Descriptors as a function of (**a**) otolith side (left dotted vs. right full line); (**b**) geography (Belgian nursery dotted vs. Wadden Sea full line); and (**c**) PCA clustering (Cluster 1 dotted vs. Cluster 2 full line see Figure 4 for the clusters). Non-overlapping regions of the otolith have been crosshatched to show differences between otolith shape

Among the 99 Fourier harmonics extracted to describe the left otolith contour, the first 22 harmonics explained more than 99.99 % of the variance and were thus retained for multivariate analyses. Small differences in average shape (1.2 % overall) were visible between the Belgian and Wadden Sea nursery grounds based on the reconstructed average otolith shape (Fig. 1.3b). The variance in otolith shape explained by fish size (P = 0.086 and P = 0.004 for NL-BE 2014 and BE 2013-2014, respectively) was removed (see Material and methods section Statistical analysis for more details). The first and second axes from a PCA comparing the Belgian and Wadden Sea nursery grounds in 2014 accounted for 30.2 and 28.6 % of the total variance, respectively. At the local scale (within the Belgian nursery) in 2013 and 2014, the first and second PCs accounted for 31.8 and 28.6 % of the total variance, respectively. Ten PCs had eigenvalues above the mean eigenvalue, and accounted for 91.0 % of the variance when comparing shapes at both nursery grounds in 2014, and 90.6 % when comparing shapes in the Belgian nursery between years. Both PCA plots showed considerable variation in otolith shape both at the regional and local scale, and it was not possible to detect PCA clusters by nursery or by year (Fig. 1.4a and 1.4b). However, two distinct clusters were observed at both geographical scales and during both sampling years based on the first two PCs. These clusters were also supported in both cases by clustering dendrograms (Suppl. Fig. 1.3). A small number of fish, belonging to both the Belgian and Wadden Sea nursery grounds (Fig. 1.4a) and both 2013 and 2014 Belgian samples (Fig. 1.4b), clearly diverged in otolith shape. This divergent group of fish consisted of approximately 10% of the individuals from NL2014, 10% of BE2014 and 16% of the BE2013, independent of standard length size class. Shape diverged in two areas on both the posterior or anterior side of the otolith and accounted for 11.5% of shape variation (Fig. 1.3c). For the 2014 dataset, fish from the divergent group were significantly longer (SL: 72 to 95 mm) than those from the majority cluster (SL: 57 to 93 mm) (Fig. 1.5; randomization test for NL-BE 2014: n = 141, W = 1221, P = 0.041). In the Belgian nursery, the average SL of the divergent group was not significantly longer than that of the majority cluster for the local dataset (Fig. 1.5; randomization test for BE 2013-2014: n = 253, W = 4258, P = 0.525).

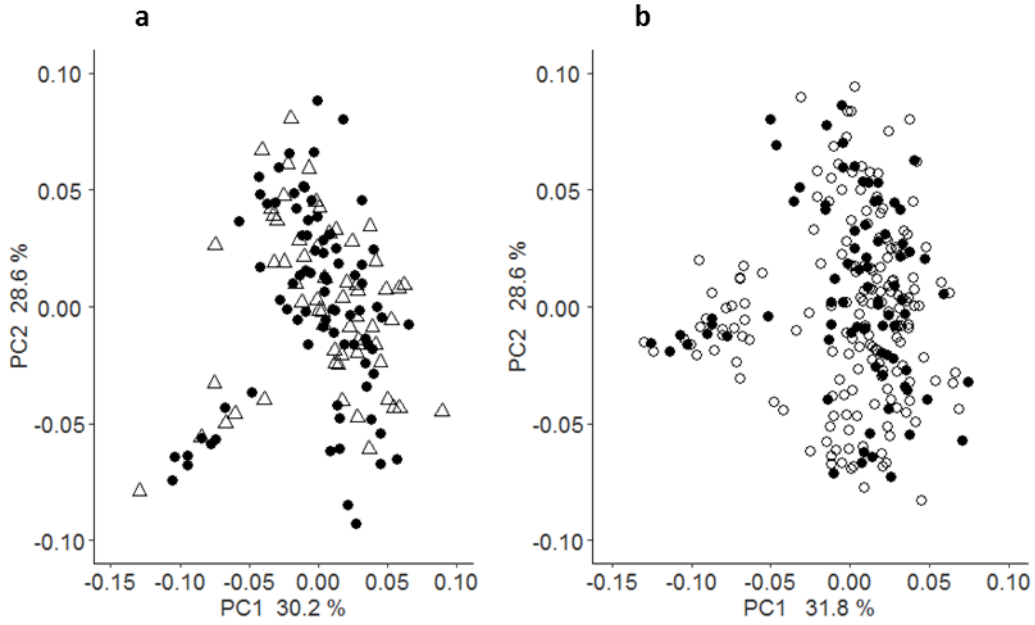


Figure 1.4: Principal Component Analysis of Fourier coefficients of otoliths of juvenile sole at (**a**) the regional scale (Belgian coast (●) vs. Wadden Sea nursery grounds (Δ)), and (**b**) at the local Belgian scale for the year 2013 (○) vs. 2014 (●)

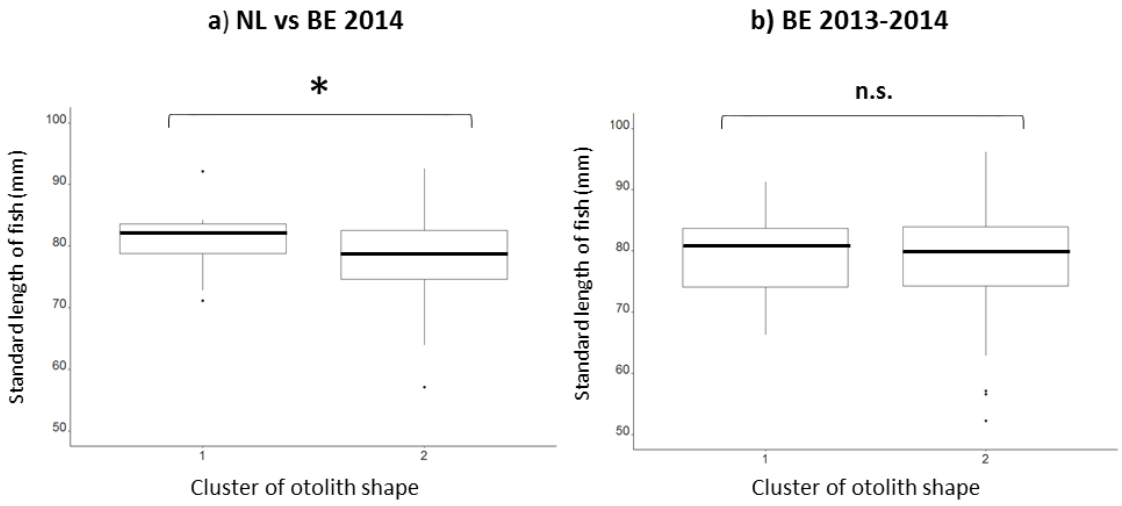


Figure 1.5: Size distribution of the standard length (in mm) of the two clusters of sole sampled in 2014 on the Wadden Sea and Belgian nursery grounds (a) and on the Belgian nursery in 2013 and 2014 (b). The average standard length was significantly different between the two clusters (as indicated by the star in a) for the nursery comparison while it was not significantly (n.s.) different between the two clusters on the Belgian nursery (b)

Redundancy analysis (RDA) was used to correlate the observed pattern of otolith shape with potential explanatory variables (Table 1.3). At the regional scale, otolith shapes in the Belgian and Wadden Sea nursery grounds differed significantly, even though that difference was only marginally significant (P = 0.04) and explained less than 1% of the variance. At the local scale of the Belgian nursery, neither an effect of sampling year nor of coastal zone (eastern/western) on otolith shape was detected. Both SST and sampling date did not significantly explain variation in otolith shape, irrespective of the spatial scale considered.

4. Discussion

Analysis of otolith shape on age-0 sole collected in the Southern North Sea revealed five interesting results. Otolith shape, as described by shape indices and Fourier coefficients, was influenced by fish size. Weak but significant directional asymmetry in otolith shape was observed between the left and right otoliths. Among-site variation in otolith shape was small but significant at the regional scale (Belgian vs. Wadden Sea nursery grounds, 500 km distance). Such variation was absent at the local scale (eastern vs. western coastal zones of the Belgian nursery, 40 km distance) and between two consecutive years (2013 vs. 2014).

Impact of fish size and directional asymmetry on shape in age-0 sole

Several confounding factors were taken into account before considering which factors drive spatial and temporal variation of otolith shape. First of all, a single age group (age-0 sole) was considered. Secondly, we corrected Fourier coefficients for fish size (Campana & Casselman 1993; Cardinale et al. 2004, Mérigot et al. 2007). Thirdly, variation due to methodology and individual variability was successfully addressed in a pilot experiment focusing on positioning and lighting methods of the photography.

Adult otoliths tend to be more complex in shape than juvenile otoliths (Capoccioni et al. 2011, Morat et al. 2017). Directional asymmetry in shape, mass and chemical composition have been observed between the left and right otoliths in adult and juvenile flatfish but never quantified in juvenile sole (Mille et al. 2015, Mérigot et al. 2007). This study demonstrates that

directional asymmetry is already present in age-0 flatfish. Our results suggest that asymmetry builds up over time because of differential growth between the larger otolith on the blind side vs. the smaller otolith on the eyed side. Still, the limited variation observed between both otolith sides supports Mille et al. (2015) suggestion of the presence of an evolutionary selection pressure against asymmetry to avoid negative effects on fish hearing and equilibrium. Our study establishes that, similarly to adults (Mille et al. 2015), left and right otoliths should not be used interchangeably in shape analyses of juvenile sole. This lack of interchangeability is likely for all other analyses carried out on otoliths, such as chemical asymmetry (Morat et al. 2014). Moreover, fish size should always be accounted for because it affects otolith shape even in age-0 fish. Our results are consistent with Mapp et al. (2017), who showed that including size information increases the power of stock discrimination based on juvenile otolith shape. The form-factor decreased with increasing size in both nursery grounds and during both sampling years, while ellipticity increased in the Belgian nursery. Roundness did not vary significantly either with fish size or between nursery grounds. The only difference in roundness we could detect was in the Belgian nursery, where roundness was lower in 2013 than in 2014. Overall, roundness was higher than observed in Mediterranean samples (Mérigot et al. 2007), which might be due to higher growth rates in the Mediterranean Sea compared to the North Sea sole (Morat 2011). In summary, we show that otoliths of age-0 sole in the Southern North Sea were relatively round, directionally asymmetrical, and that area irregularity increased during the first year of life.

Small differences between nursery grounds

Our results show that we can detect variation in otolith shape at relatively small spatial scales. Overall, the average shape differed more between nursery grounds than between otolith sides, although these differences disappear when size classes are taken into account and asymmetry measured within each. This result suggests that differences between nursery grounds are size related. Assuming comparable environmental conditions at the Belgian and Wadden Sea nursery grounds (OSPAR, 2000), one might expect a comparable nursery ground environment impact on otolith shape, which we do not observe. Thus other factors, such as genetic background, spawning location and dispersal may also be important in influencing otolith shape (Cardinale et al. 2004, Capoccioni et al. 2011, Lombarte et al. 2003, Mapp et al. 2017). This observation is

supported by the similar levels of variation in otolith shape between nursery grounds and between the two successive sampling years. Genetic levels are expected to vary only to a limited extent within the Southern North Sea, because sole exhibit an almost homogeneous population structure (Cuveliers et al. 2012, Diopere et al. 2018). At the same time, earlier microchemical studies point to a reduced mobility and site fidelity of juvenile sole in the Southern North Sea (Cuveliers et al. 2010, Le Pape & Cognez 2016). Differences in otolith shape and microchemistry between two 'adjacent' nursery grounds support some degree of natal site fidelity, which is not unexpected given the temporally stable location of the spawning grounds of sole.

A divergent group of age-0 sole at both nursery grounds

We noticed two distinct groups of otolith shapes in both PCA analyses and clustering dendrograms. This result raises the question as to whether these individuals, representing 10 and 16% of all age-0 sole screened respectively, experienced a somewhat different environment or have a different origin. Fish of the divergent group exhibited a larger size than most other fish. However, the divergent group was sampled across sampling dates (spanning over one month), suggesting that shape differences are not linked to age differences between individuals.

The environmental conditions experienced during juvenile dispersal depend on spawning ground and planktonic drift. According to the biophysical model of Lacroix et al. (2013), larvae arriving at the Belgian and Wadden Sea nursery grounds originate from the spawning grounds off the Belgian coast, the Eastern English Channel and the Thames Estuary. Between-year connectivity levels are comparable, although the relative proportions of source populations vary. Building on the results of the present study, other approaches would be required to identify larval origin. For example, spawning time can be estimated from otolith microstructure and used to back-calculate larval origin (Amara et al. 1993). Shape differences might be related to residence time on the nursery ground (Delerue-Ricard et al. *submitted*). Additionally, otolith microchemistry is effective in resolving small-scale spatial differences. Based on otolith microchemistry, Cuveliers et al. (2010) were able to distinguish nursery grounds of sole from the Thames Estuary, Belgian coast, and Wadden Sea. However, chemical signatures of spawning grounds of sole in that area have yet to be explored. In summary, complementary information on otolith chemistry, biophysical modeling or a combination using the

aforementioned techniques, should reveal the origin of spawning, and the dispersal pathways (Campana 2005, Morat et al. 2014). Such information would contribute to a better understanding of the connectivity patterns of sole between spawning and nursery grounds.

The importance of the spatial scale

Understanding local population dynamics is crucial, especially for coastal habitats, which are key nursery grounds for many marine species. Over the last decades changes in food webs and sheltering conditions provided by structuring benthos have dramatically affected nursery function along the North Sea coast (Jung et al. 2017, Rabaut et al. 2013, Van der Veer et al. 2011). We examined otolith shape across the Southern North Sea at a scale close to the average dispersal distance of sole (150 km, Kotoulas et al. 1995). Small, but significant differences were identified between two adjacent nursery grounds. Within each nursery ground, no differences in otolith shape were found, despite differences in habitat characteristics between the eastern and the western coastal zones of the Belgian nursery, with mostly muddy to sandy sediments in the east and sandy to muddy sediments in the west (Van Hoey et al. 2004). Each sediment type harbors a specific macrobenthic assemblage (Degraer et al. 2008, Rabaut et al. 2013), with a distinct prey spectrum for sole and potential influence on otolith shape. However, starvation experiments suggest that food quantity could be more important than food type to determine otolith shape. Thus, if juvenile sole would be restricted to one coastal nursery ground or region within the Belgian nursery, the limited effect of food type on otolith shape could partially explain the absence of variation within the Belgian nursery (Cardinale et al. 2004, Gagliano & McCormick 2004, Hüssy 2008).

In conclusion, small scale differences in otolith shape can be diagnostic and may play a role in population dynamic studies. During the first year of life of sole, otolith shape evolves from round to a more complex shape. Directional asymmetry between left and right otoliths is already present in age-0 sole and has therefore to be taken into account in flatfish juveniles and adults alike. Despite a large variation in otolith shape, a subtle but significant difference was observed at a scale of 500 km, i.e. bigger than the dispersal scale of sole, which is a promising finding for the study of small-scale spatial patterns. At the local scale (within nursery grounds), no

significant shape differences were noted between the eastern and the western coastal zones of the Belgian nursery, nor between 2013 and 2014. However, a number of samples showing a divergent otolith shape, probably linked to fish size, was found on both nursery grounds in both successive years. Complementary studies on the integration of high resolution genotyping, elemental analysis of otoliths and biophysical modeling of early-life stages, are expected to provide additional evidence on the dispersal dynamics between spawning and nursery grounds.

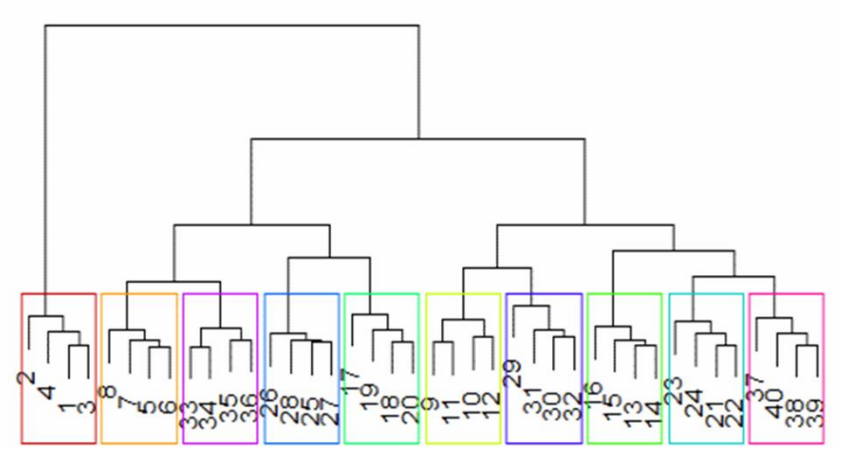
Acknowledgments

Special thanks to K. Vanhalst (Institute for Agricultural and Fisheries Research, ILVO), the crew of RV *Simon Stevin* and RV *Belgica*, L. Bolle (Wageningen Marine Research), the crew of RV *Stern* and the B-FishConnect team for sampling. We are grateful to E. De Keyser, H. Christiansen, F. M. Heindler, F. Calboli (KU Leuven), B. Ernande (Ifremer), G. Lacroix (Royal Belgian Institute of Natural Sciences, RBINS), A. Vanden Bavière, J. Robbens (ILVO) and M. R. Siskey (Stony Brook University) for constructive comments. The B-FishConnect project was funded by the Research Foundation – Flanders (project number G.0702.13N). Thanks also to three anonymous reviewers, who provided many helpful comments.

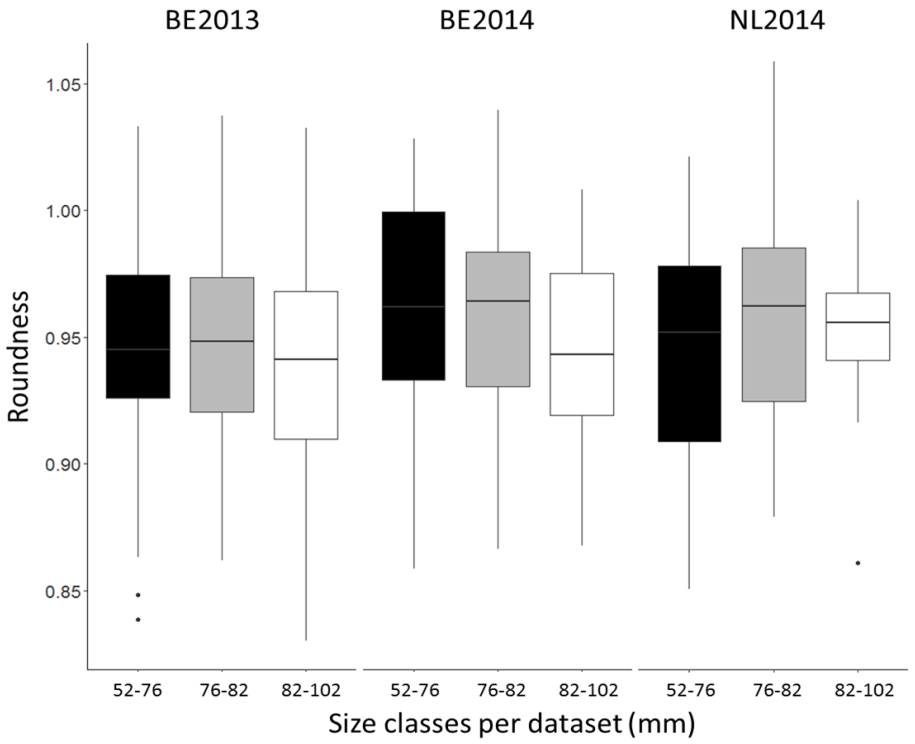
Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

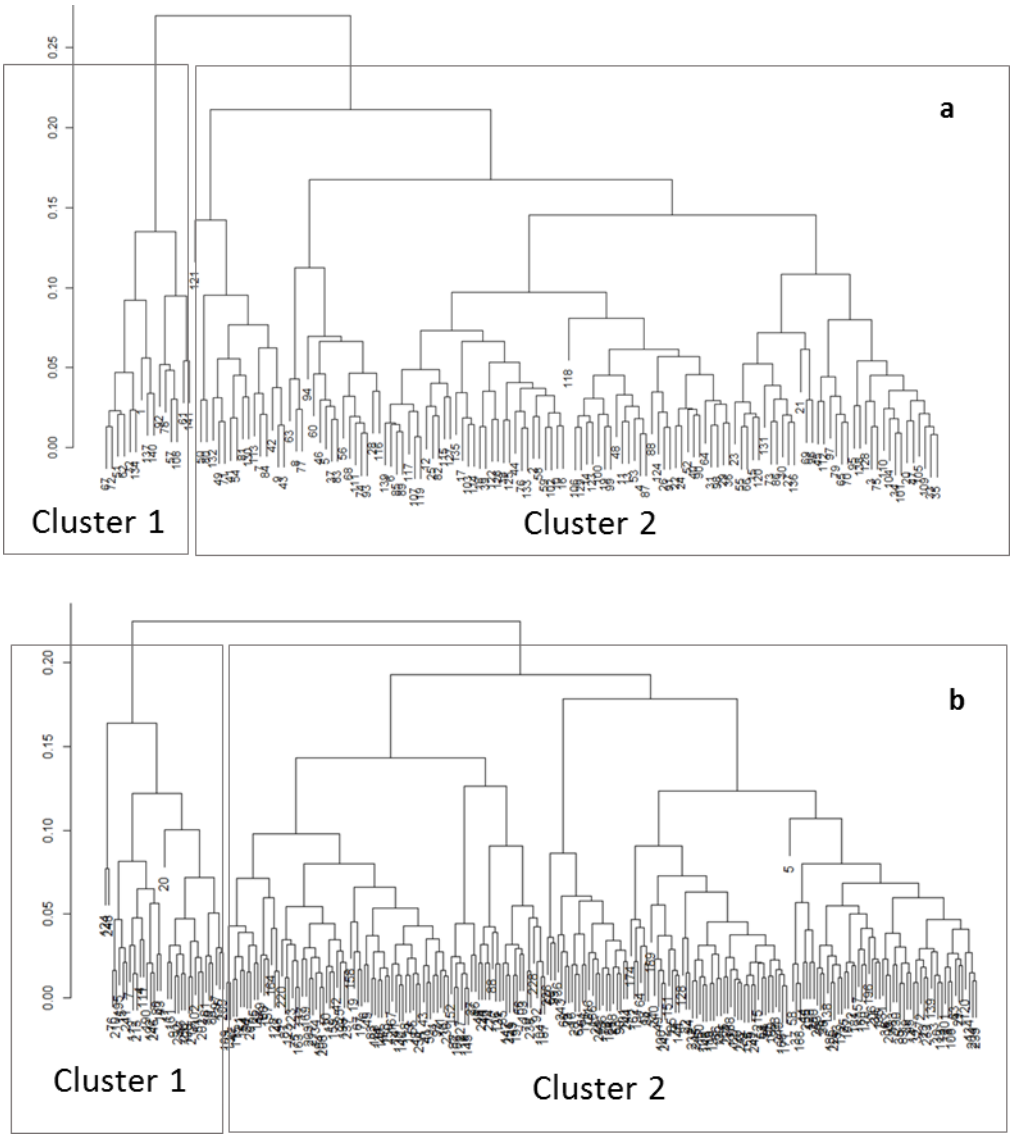
Supplementary material



Supplemenatry Figure 1.1: Cluster dendrogram of the similarity distances of the Fourier coefficients of ten randomly chosen otoliths of juvenile sole, based on Ward's distance. Picture numbers range from 1 to 40, with four consecutive pictures (e.g. 1-4, 5-8, etc.) being from the same otolith



Supplementary Figure 1.2: Boxplot of roundness for 314 age-0 sole juveniles of sole at three size classes for each dataset colored from darker to lighter according to size classes (BE2013, BE2014 and NL2014)



Supplementary Figure 1.3: Cluster dendrogram of the similarity distances of the Fourier coefficients of the juvenile sole sampled at the Belgian and Wadden Sea nursery grounds in 2014 (**a**) and at the Belgian nursery in 2013 and 2014 (**b**) using a complete hierarchical clustering method, based on Ward's distance

Chapter 2

Restricted small-scale dispersal of larval and juvenile sole between spawning and nursery grounds

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Abstract

In marine organisms, connectivity between spawning and nursery grounds influences colonization, replenishment and resilience of populations. Connectivity rate, measured as the exchange of individuals between spawning and nursery grounds, is therefore a crucial determinant of stock size. However, connectivity of early-life stages is hard to explore due to sampling limitations and insufficient knowledge on potential larval sources. Here we present new insights into pre- and post-settlement dispersal of the common sole (*Solea solea* L.) at a spatial scale of 5-500 km in the Southern North Sea. Marginal multi-elemental signatures in the otoliths of 213 juvenile soles from this area indicated that otolith chemistry could be used to successfully discriminate (80% of overall accuracy) the juvenile habitats along the UK, the Belgian and the Dutch coastlines. Larval signatures of the same fish indicated that these four regions most likely recruit uneven proportions of juveniles (0-100%) from four chemically distinct natal sources. Differences in coastal water composition among regions (especially for Mg, Mn and Zn) suggest that sole migration following settlement is limited in the Southern North Sea. Pre-and post-settlement dispersal both seem to be largely restricted to 100 km. The results contribute to the calibration of biophysical models of larval drift and to decision making for both fisheries management and marine spatial planning at the national and European levels.

Submitted to Journal of Sea Research (3rd of November 2018, in review since 13th of May 2019)

1. Introduction

Connectivity, i.e. the exchange of larvae, juveniles or adults among geographically separated groups (Palumbi 2004), drives colonization, enables replenishment and promotes resilience of populations to disturbances (Botsford et al. 2001, Cowen et al. 2007). In marine organisms with a planktonic larval phase, connectivity between spawning and nursery grounds is one of the main drivers of recruitment (Harden-Jones 1968, Rijnsdorp et al. 1992). Spawning ground origin and dispersal pathways may impact the quality of the larval pool and thereby modulate juvenile survival (Pineda et al. 2007, Shima & Swearer 2010). In addition, anthropogenic activities or environmental changes of the spawning grounds may modify connectivity and, therefore, impact supply to the nursery grounds (Lacroix et al. 2018). Therefore, understanding connectivity at the scale of dispersal is paramount to apply adapted management measures for metapopulation persistence (Batista et al. 2015, Burgess et al. 2014, Krueck et al. 2017). Connectivity may be more important than habitat quality for the design of Marine Protected Areas (MPAs, Berglund et al. 2012, OSPAR 2013) so increasing calls are made for prioritizing locations that are self-replenishing, inter-connected, and/or important larval sources (Krueck et al. 2017). However, while the efficiency of MPA positioning depends on the quality of input data regarding fish dispersal (Batista et al. 2015), data on fish movements at the larval and juvenile stages are still difficult to acquire.

The distribution and dispersal of flatfishes have been investigated extensively, both through sampling and tagging studies (Burt & Millner 2008, Dorel et al. 1991, Ellis et al. 2012, Gibson 2015) and through the use of biological tags like otolith microchemistry (Darnaude & Hunter 2018, Hunter et al. 2003). The ecology of the common sole (*Solea solea* L., 1758; Soleidae) has been particularly well studied, especially at the adult stage (Cuveliers et al. 2012, Diopere et al. 2018, Exadactylos et al. 2003). The species mainly spawns from March to June along the shores of the North East Atlantic Ocean (Ellis et al. 2012, Rijnsdorp et al. 1992). After hatching, the pelagic larvae drift for about one month in the water column before settling in a shallow coastal or estuarine nursery (Russell 1976, van der Land 1991). Estimates of larval dispersal range between 80 km and 300 km depending on the spawning ground considered, with an average drift of about 150 km as estimated by biophysical modeling and genetic markers (Dorel et al. 1991, Kotoulas et al. 1995, Lacroix et al. 2013). Yet, empirical evidence of connectivity between sole spawning and nursery grounds is scarce (Morat et al. 2014). Similarly,

little is still known about the movement of juvenile soles in the months following settlement. Population structuring among the nursery grounds and the adult feeding grounds of sole has been characterized through otolith chemistry along the coasts of the Atlantic Ocean and the Mediterranean Sea (Cuveliers et al. 2010, Leakey et al. 2009, Morat 2011, Tanner et al. 2012). However, this information is still largely lacking in the North Sea. The few studies conducted so far showed that the distribution of sole nurseries is rather continuous along the North Sea coasts (Ellis et al. 2012, Rijnsdorp et al. 1992) and biophysical modeling suggested a mixed contribution of several spawning grounds to most juvenile habitats (Lacroix et al. 2013, Savina et al. 2010, 2016). However, a recent shift was observed in the distribution of North Sea sole (Engelhard et al. 2011), attributed to a shift in the distribution of its spawning and nursery grounds (Ellis et al. 2012), as well as to a longer larval dispersal due to colder temperatures experienced by early hatched larvae (Lacroix et al. 2018). These recent changes, probably caused by both climatic and fishing pressures, call for a focus on the early-life connectivity of sole in the area. Understanding the patterns of dispersal and their causes is of primary importance because climate change modeling at the level of the North Sea predicts an increase in larval recruitment for some fish species although with strong regional differences among nursery grounds (Lacroix et al. 2018).

This study represents a first attempt to measure connectivity between the spawning and nursery grounds of sole in the Southern North Sea using otolith microchemistry. Otoliths are calcified structures in the inner ear which grow with regular increments and incorporate time-delimited information (Campana 1999). For example, when juvenile fish settle on the nursery ground, the high metabolic impact of the metamorphosis is recorded in the otolith growth (De Pontual et al. 2000), allowing to separate the growth bands deposited during the larval drift at sea from those formed after the benthic settlement. Revealing chemical differences between the pre- and post-settlement regions of the otolith can inform about dispersal at the larval stage but also about fish movements within and between nursery ground areas. Indeed, the chemical composition of otoliths has already been proven effective to infer differences in spawning origin and to discriminate among nursery grounds (e.g., Gibb et al. 2017, Lazartigues et al. 2017, de Pontual et al. 2000). Although small-scale migration patterns in coastal zones remain challenging to trace using otolith microchemical signals because of the short residence time of water (Tanner et al. 2012), estuaries often carry strong chemical signals, at large and small spatial scales (Gillanders & Kingsford 2003, Di Franco et al. 2012, de Pontual et al. 2000).

Although clean-up initiatives were carried out in most European estuaries and tended to decrease metal concentrations over the last decades (Emeis et al. 2015), the North Sea is one of the most polluted seas of the world (Halpern et al. 2008, Portman 1989). Many areas along its coasts receive runoff water that carries the chemical signature of local river catchments (De Witte et al. 2016, Hamer et al. 2006, Van Alsenoy et al. 1993). Onshore-offshore gradients are observed in relation to the distance from riverine inputs for certain elements. Therefore, the area is particularly suited for otolith multi-elemental studies of fish spatial origin and movements. In this work, we focused on seven coastal areas used as nursery grounds by sole in the Southern North Sea, along the UK, Belgian and the Dutch coastlines, separated by 5 to 500 km. We tested whether the otoliths of juvenile soles from these sampling locations have discriminable multi-elemental signatures. Based on the composition of the larval part of the otoliths, we also estimated the number of chemically distinct spawning grounds (further referred to as natal sources) that supply them with larvae. We then quantified the extent of local (<100 km) pre- and post-settlement dispersal in the species by comparing the elemental signatures from three key periods of fish life: the larval phase, the settlement phase, and the last few weeks on the nursery ground before capture.

2. Material and methods

2.1 Sample collection

Age-0 and age-1 sole belonging to five cohorts, hereafter referred to as settled juveniles, were collected between 2006 and 2016 on 11 major Southern North Sea nursery grounds (Ellis et al. 2012, Rijnsdorp et al. 1992) spread across four 'sampling regions' (Fig. 2.1 and Table 2.1): (1) the UK coast (sampled near the Sizewell power station and in the Thames Estuary), (2) the Belgium coast (sampled at four locations: B1, B2, B3 and B4) (3) Balgzand_Zandvliet (sampled at Zandvliet in the Scheldt Estuary and Balgzand in the Dutch part of the Wadden Sea) and (4) the Ems-Dollard Estuary. At all sites, a 3 m beam trawl (the mesh size of the cod end was 10 mm) was used to catch the fish. Unfortunately, the sampling occurred over several years but only two nursery grounds (B03 and B06) were sampled in consecutive years (in 2013-2014). At all sites, juvenile soles were sampled between the end of May and October, except for the Sizewell power station sample collected in March. Water temperature was measured on site on the day of

sampling. All fish were measured and weighted (standard length in mm and total weight in g; Suppl. Fig. 2.1) and kept frozen until dissection.

In order to have a sufficient number of fish per site to enable discrimination, nearby nursery grounds in each region were grouped, resulting in seven geographically distinct nursery areas, hereafter referred to as 'sampling locations'. Balgzand and Zandvliet sampling locations were grouped together based on preliminary results, low sample size and similarities in otolith chemical signatures. The closest sampling locations in this work (along the Belgian coast) are 5 km apart, the furthest being 500 km apart. Therefore, patterns occurring at a scale of less than 100 km (i.e. the full extent of the Belgian coastline) were considered local, whereas the patterns occurring at more than 100 km (i.e. among the four sampling regions) were considered regional.

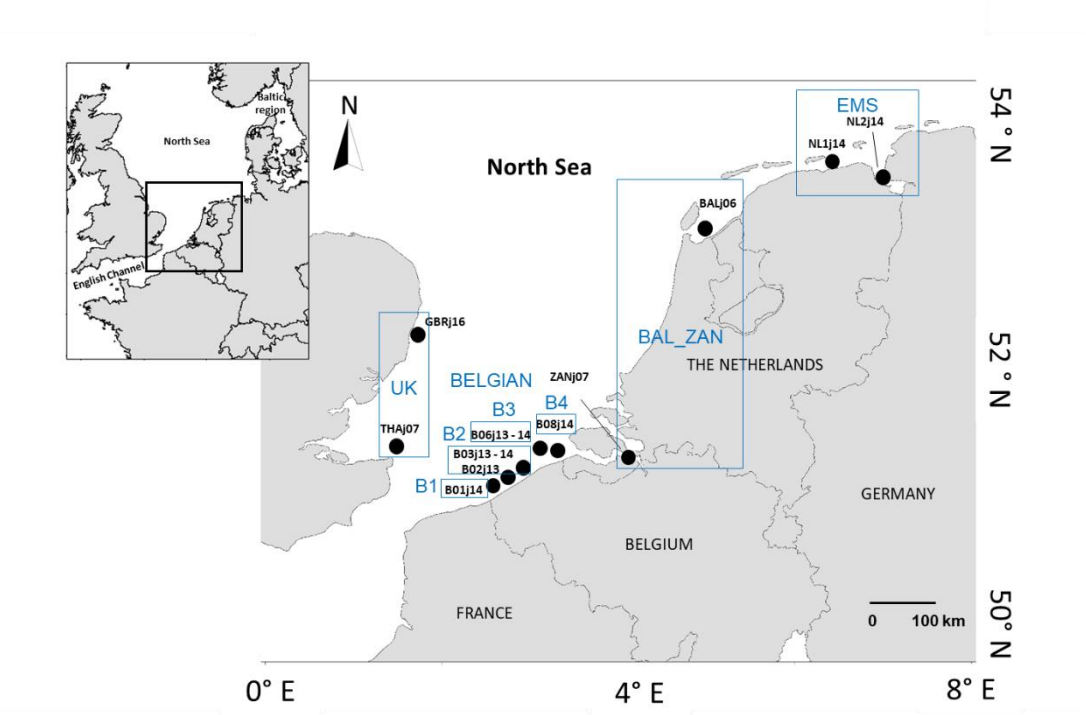


Figure 2.1: Map of the sampling locations of juvenile sole (n = 213) off the UK, Belgian and Dutch coasts. Sample codes are built with the station name (e.g. “B02” for “B02j13”), the life stage of the fish (“j” for juvenile) and the year of sampling (“13” stands for 2013).

Table 2.1: Number of sole juveniles utilized for otolith analysis per sampling location and region (UK, Belgian and Dutch coasts), including the date of sampling, geographical coordinates and the sea surface temperature at the time of sampling (NA = Not Available).

Region	Sampling location	Station	Sample size	Sampling date	GPS coordinates	Sea surface temperature (°C)
UK coast	UK	GBRj16	19	14/03/2016	52.21 N, 1.63 E	NA
Total 32		THAj07	13	01/08/2007	51.42 N, 1.40 E	17.7
Belgian coast	B1	B01j14	16	15/09/2014	51.13 N, 2.70 E	18.1
	B2	B02j13	15	10/09/2013	51.19 N, 2.70 E	19.3
		B03j13	21	28/08/2013	51.23 N, 2.80 E	18.8
		B03j14	10	26/05/2014	51.23 N, 2.80 E	15.6
Total 114	B3	B06j13	18	09/09/2013	51.35 N, 3.00 E	19.5
		B06j14	17	16/09/2014	51.35 N, 3.00 E	18.5
	B4	B08j14	17	10/10/2014	51.35 N, 3.10 E	16.9
BAL_ZAN (Balgzand_ Zandvliet)	Zandvliet	ZANj07	8	01/10/2007	51.40 N, 4.19 E	16
Total 22	Balgzand	BALj06	14	01/08/2006	52.96 N, 4.95 E	18
EMS	Ems-Dollard estuary	NL1j14	16	16/09/2014	53.48 N, 6.49 E	18.2
Total 37		NL2j14	21	23/09/2014	53.35 N, 6.97 E	18.1

2.2 Otolith extraction and preparation

All equipment for otolith extraction and handling was acid washed in a 10% nitric solution prior to use. Because the chemical composition, shape and mass of otolith is asymmetric in flatfish (Mille et al. 2015, Mérigot et al. 2007), only the left sagittal otoliths were used in this study. For all fish, they were extracted and weighed to the nearest 0.005 mg and fish age-class (0 or 1) was determined by macroscopic examination. To remove any surface contamination, the otoliths were rinsed with distilled water and 0.1% nitric acid, and sonicated in vials filled with ultrapure water. Otoliths were then mounted in molds, covered with epoxy resin and transversely cut through the nucleus using diamond blades mounted on an ISOMET Buehler precision saw. Thin sections were obtained by polishing the otoliths cuts to expose the nucleus on one side. They were all sonicated again in ultrapure water, left to dry for 24 h under a class 100 laminar flow hood and randomly glued on a glass slide for later analysis.

2.3 Otolith elemental analysis

Otolith elemental composition was determined by Laser Ablation Inductively-Coupled Plasma Mass Spectrometry (LA-ICPMS) using a Nu Instruments ATTOM ES High resolution ICP-MS coupled to a Resonetics RESOlution M-50 193 nm excimer laser ablation system with helium as carrier gas. Otolith material was ablated along the same transect for all fish from the core to the dorsal edge. Laser beam diameter for this was set at 64 µm, and analyses were made with an energy of 90 mJ (Attenuated 50%), a frequency of 7 Hz and at a speed of 5 $\mu m.s^{-1}$. A total of 26 isotopes were measured (Suppl. Table 2.1). For some elements, two isotopes were initially quantified to test for possible mass interference. Precision and accuracy were measured using National Institute of Standards and Technology (NIST) 610, FEBS and NIES standards of known composition. Standards were measured at least every 15 measures for accuracy and precision calculations. NIST 612 was used for calibration. The chemical signal was processed with Iolite (Paton et al. 2011). Data below the Limit of Detection (LOD = 3 x standard deviation of the blanks, Anstead et al. 2015) were set to zero (average missing data per element was 2.1 ± 3.7%; Suppl. Table 2.1). After visual inspection of the data for outliers, missing values, interference between isotopes and precision and accuracy (Suppl. Table 2.2), the nine most reliable elements (^{23}Na, ^{26}Mg, ^{55}Mn, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{86}Sr, ^{137}Ba, ^{208}Pb, see Panfili et al. 2002) were kept for further

analyses. All results were normalized to ^{43}Ca (the internal standard) and expressed in µmol mol^{-1} for Mg/Ca, Mn/Ca, Cu/Ca, Zn/Ca, Rb/Ca, in mmol mol^{-1} for Na/Ca, Fe/Ca and Sr/Ca, and in pmol mol^{-1} for Pb/Ca

2.4 Selection of early life history zones and signal processing

Primary increments have been validated as daily in larval and juvenile sole and characteristic marks represent hatching, first feeding and metamorphosis (Amara et al. 1998, Lagardère 1989, Lagardère & Troadec 1997). Three zones along the transect analyzed were retained for this work: (1) the 'otolith edge', i.e. the portion laid down during the last weeks before capture and reflecting the signature of the sampling location, (2) the 'post-settlement' portion just after the metamorphosis mark reflecting the signature of the nursery ground colonized at benthic settlement, and (3) the 'larval' area just outside the core, reflecting the signature of the fish natal source (Fig. 2.2). On average, the three zones were all 77 ± 5 µm long, which corresponds to 2-3 weeks of signal integration (Lagardère & Troadec 1997). They were respectively positioned at 50 µm from the edge of the otolith, 30 µm from the settlement mark and 50 µm from the primordium (as the core area is 40 ± 10 µm long for sole, Lagardère et al. 1995). Measures on the otoliths for this were taken with a Leica M125 microscope (objective 10x).

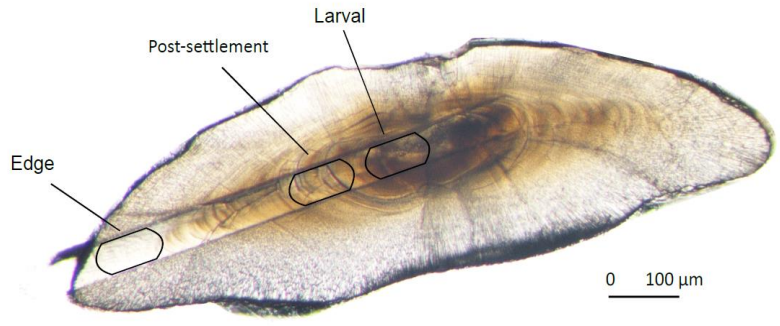


Figure 2.2: Picture of otolith of juvenile sole (transmitted light, x80 magnification) showing the positions of the larval, settlement and sampling signatures retained for this study along the laser sampling transect.

2.5 Data analysis

2.5.1 Spatio-temporal variation in elemental signatures on the nursery grounds

The concentrations of the nine elements retained for this study did not meet assumptions of normality and homoscedasticity (Shapiro-Wilk and Bartlett tests) even after $\log_{10}$ transformation. Therefore, the temporal stability in the chemical signal was tested for the two sites sampled in 2013 and 2014 (B03 and B06) using several Wilcoxon tests and correcting significance levels using a Benjamini-Hochberg correction for multiple testing. Spearman correlations between fish length and the elements within regions or locations. Effect of fish length was investigated using spearman correlation for each element within each sampling region and location. Spatial variation in otolith signature was investigated using the Kruskal Wallis test (for single elements) followed by a *post hoc* comparison (Dunn test) to determine which elements contributed to the differences among sampling locations. For this, we used the chemical signatures recorded on the edge of the otolith for all fish, as we were sure they reflected the final site of capture.

Spatial discrimination success among sampling locations (also based on otolith edge signatures) was assessed using the Random Forest (RF) algorithm (Breiman 2001) because it requires no *a priori* on the distribution of the data and allows to significantly improve discrimination power when using otolith multi-elemental data (Mercier et al. 2011). Details of the method as applied in this study are provided in Mercier et al. (2012) and Tournois et al. (2017). First, all the possible combinations of elements out of the nine retained in this work were tested to identify that allowing to achieve the best discrimination accuracy using RF. For this, data were centered and reduced to give the same weight to all elements in the spatial discrimination. To avoid circular reasoning, 75% of the signatures at sampling location were randomly selected to build and “train” each possible combination of elements ('RF classifier') while the remaining 25% were used to test its reliability in re-assigning signatures to the correct origin. In each case, 500 classification trees and 100 iterations were used and minimum, maximum and average overall discrimination accuracies were calculated to assess the value of each RF classifier for spatial discrimination as in Tournois et al. (2017). Once the list of elements leading to the best discrimination (i.e. the optimal RF classifier) were identified, classification

accuracies and the True Skill Statistics (TSS, Allouche et al. 2006) were calculated for each sampling location. The accuracy represents the total number of fish correctly reclassified to their sampling location while TSS also accounts for true negative prediction. TSS ranges from -1 to +1, where +1 represents 100% of correct prediction of presence or absence in a given habitat, 0 indicates random predictions and -1 indicates 100% incorrect predictions. Finally, the contribution of each element to the spatial discrimination was assessed by calculating the mean decrease in global Gini Index (GI, Breiman 2001) after its removal from the optimal classifier. GI ranges from 0 (when all elements equally contribute to the total discrimination) to 100 (when a single element contributes to 100% of the total discrimination). The higher the decrease in GI when one element is removed from the classifier, the more that element is essential to the discrimination.

2.5.2 Investigation of potential migration after settlement

Once validated based on otolith edge signature (see *section 2.5.1* of this chapter), the optimal RF classifier (500 trees) was used to identify the nursery area most likely occupied by all juvenile soles just after metamorphosis, thereby allowing detection of migration among sampling locations after the benthic settlement. For this, the optimal RF classifier trained with all otolith edge signatures was used to assign each post-settlement otolith signature to its most likely location of origin (among the seven sampled). For each fish, 100 iterations were made to allow calculation of the percentage of assignment of the post-settlement signature to each sampling location, including that of capture.

2.5.3 Natal sources

A K-means clustering analysis was performed on the region of the otolith corresponding to the larval phase (see *section 2.4* of this chapter for details) to gain insight into the number of larval origins contributing to the focal nursery grounds and connectivity patterns between them. Larval clusters were defined based on the material outside the core area as the core is potentially under maternal influence and enriched in some elements associated to physiological changes rather than with environmental signals (e.g. Brophy et al. 2003, Ruttenberg et al. 2005). Larval clusters were defined by unsupervised clustering (i.e. without taking sampling location into

account). The optimal number of clusters was determined using the *NbClust* package (Charrad et al. 2014) which compares 30 different types of clustering methods. In addition, data was centered and reduced. Centering and reducing data is interesting because k-means clustering as a sphericity assumption and tends to give more weight to large clusters and is less likely to identify small larval sources. K-means clustering was used to assign a number of potential natal sources based on the larval signatures (see *section 2.5.4* of this chapter).

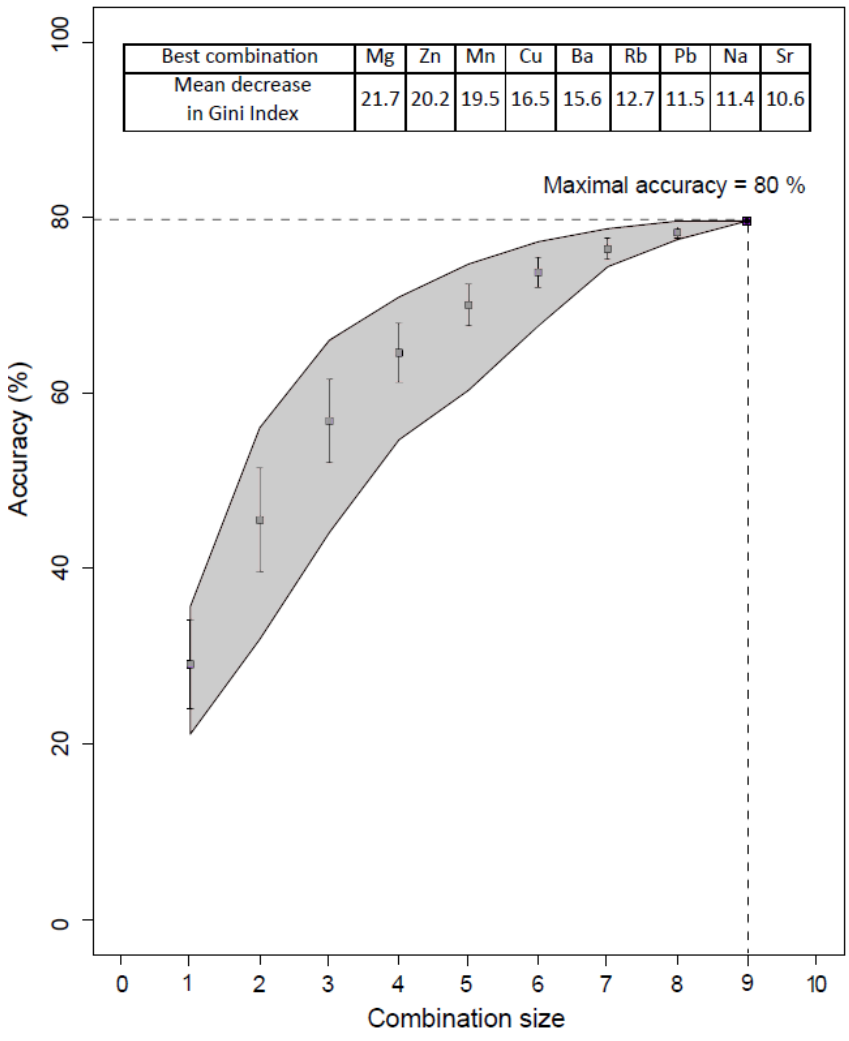


Figure 2.3: Effect of elemental combination size (number of elements included in the otolith signatures) on the discrimination accuracy among the seven sampling locations obtained using the Random Forest (RF) approach. The gray area shows the interval between the minimum and maximum accuracies for each combination size, while the mean accuracy (± SD) is depicted by the white squares. The table shows the list of elements included in the optimal RF classifier ranked by the corresponding mean decrease in the Gini index (importance for spatial discrimination).

3. Results

Otolith signatures were successfully measured for 213 juvenile soles, from all sampling locations and regions (Table 2.1). The mean percentage of data below the LOD was ≤ 4% for all elements, except for Li (57%), Co (61%), Cd (96%) and Pb (11%), which were excluded from further analyses. Most elements had a relative standard deviation for NIST 612 ≤ 6, with exception of Li (15), Na (8), Mg (7) and Fe (10). The recovery rates and relative standard deviations of other standards are listed in Suppl. Table 2.2. Nine elements (Na, Mg, Mn, Cu, Zn, Rb, Sr, Ba, Pb) were regularly detected at all sampling locations with good precision and accuracy. Therefore they were retained for statistical analyses (Suppl. Table 2.3a). Spearman correlation analysis showed that correlations between fish length and the elements within regions or locations vary in no particular direction and are rarely significant after correction for multiple testing (Suppl. Table 2.4).

3.1 Spatial variation in the elemental signature at the sampling location

Discrimination accuracy among all sampling locations was maximal (80%) when all nine elements (Na, Mg, Mn, Cu, Zn, Rb, Sr, Ba and Pb) were included in the RF classifier. This indicates that all elements are informative for discriminating among the seven nursery areas sampled in the Southern North Sea (Fig. 2.3). Using this optimal RF classifier, the accuracy of correct reassignment for otolith edge signatures was >80% for all sampling locations except for those along the Belgian coast where average success was 78.1 ± 9.2% (Table 2.2). TSS were all positive, maximal for Balgzand-Zandvliet (0.93) and high (> 0.70) for UK, Ems-Dollard and for all locations in Belgium except for B3 (0.60). Overall classification success to sampling region was very high for all regions (89.3% on average), especially for Balgzand-Zandvliet (100%) and Ems-Dollard (92.4%, Table 2.3). Belgian sampling locations were sometimes confounded with each other %due to similarities in the concentrations for Rb (B1-B2-B3-B4), Mn (B2-B4), Pb (B2-B3), or Na, Cu, Zn, Ba, and Pb (B1-B4, Fig. 2.3). Overall, most regions were found to be chemically distinct. However, a limited number of the otolith edge signatures recorded on the UK nursery grounds resembled the B3 sampling location due to similarities in Na, Cu, Rb, and Ba concentrations (Table 2.3).

Table 2.2: Classification accuracies and True Skill Statistics (TSS) for all sampling location when using the optimal RF classifier for spatial discrimination of sole nurseries.

	UK	B1	B2	B3	B4	BAL_ZAN	EMS
Classification accuracy (%)	80.4	88.3	79.0	65.9	79.3	93.2	85.7
TSS	0.75	0.88	0.72	0.60	0.77	0.93	0.82

Table 2.3: Accuracy in re-assignment to sampling location based on multi-elemental otolith signatures. Each cell shows the percentage of individual sole assigned to each sampling location (see Table 2.1) by the optimal Random Forest (RF) classifier (see Fig. 2.3). Numbers on the diagonal indicate individuals successfully re-assigned to their sampling location. Boxes refer to individuals belonging to the same sampling region.

Sampling location \ Assigned location	UK	B1	B2	B3	B4	BAL_ZAN	EMS	Region	Average success
UK	71.5	0.0	4.0	19.0	0.0	0.0	5.5	71.5	
B1	0.0	83.4	0.0	0.0	16.6	0.0	0.0	100.0	79.0 % per sampling location
B2	5.7	0.0	77.7	5.7	4.4	0.0	6.5	87.8	
B3	15.6	0.0	11.4	68.7	0.0	0.0	4.2	80.2	
B4	0.0	12.4	21.7	0.0	59.3	0.0	6.7	93.3	
BAL_ZAN	0.0	0.0	7.6	0.0	0.0	100.0	0.0	92.4	89.3 % per sampling region
EMS	0.0	0.0	0.0	0.0	0.0	0.0	92.4	100.0	

Table 2.4: Assignment of otolith settlement signatures to sampling locations (see Table 2.1) using the elements selected by the optimal Random Forest (RF) classifier based on sampling location discrimination. Numbers on the diagonal indicate individuals successfully re-assigned to sampling location as defined in Table 2.1. Boxes refer to individuals belonging to the same sampling region.

Settlement signature at sampling location \ Assigned location	UK	B1	B2	B3	B4	BAL_ZAN	EMS	Region	Average assignment
UK	36.5	0.0	8.5	42.6	7.5	2.5	2.5	36.5	
B1	0.0	40.1	7.9	18.8	33.3	0.0	0.0	100.0	46.9 % to sampling location
B2	0.0	21.7	63.8	10.2	4.3	0.0	0.0	100.0	
B3	2.9	22.9	14.9	41.4	18.0	0.0	0.0	97.1	
B4	5.9	0.1	29.4	23.5	41.1	0.0	0.0	94.1	
BAL_ZAN	5.6	8.2	36.9	2.7	3.7	77.4	3.3	28.1	76.2 % to sampling region
EMS	1.9	13.0	4.3	0.0	0.0	14.7	28.1	77.4	

Contributions to nursery discrimination varied among elements. Mean decrease in Gini index indicated that Mg, Zn and Mn were the three most important elements for classification accuracy, providing on their own an accuracy of 57% (Fig. 2.3). While Mg and Mn concentrations were significantly lower in UK compared to Balgzand-Zandvliet, Zn was lower in UK and Belgium. Otolith edges, representing the chemical signatures of the sampling locations, showed significant differences in Na (KW = 60.87, 13, $p < 0.001$), Mg (131.24, 6, $p < 0.001$), Mn (95.77, 6, $p < 0.001$), Cu (91.20, 6, $p < 0.001$), Zn (119.77, 6, $p < 0.001$), Rb (67.92, 6, $p < 0.001$), Sr (66.587, 6, $p < 0.001$), Ba (113.00, 6, $p < 0.001$) and Pb (65.27, 6, $p < 0.001$). For instance, Mg and Mn concentrations were significantly lower on UK nursery grounds than in the other regions, while the nursery of Balgzand_Zandvliet showed significantly higher Ba and Mn average values and a greater variability in Mn concentrations (Fig. 2.4). The Ems-Dollard estuary was characterized by the significantly lower average Cu concentrations.

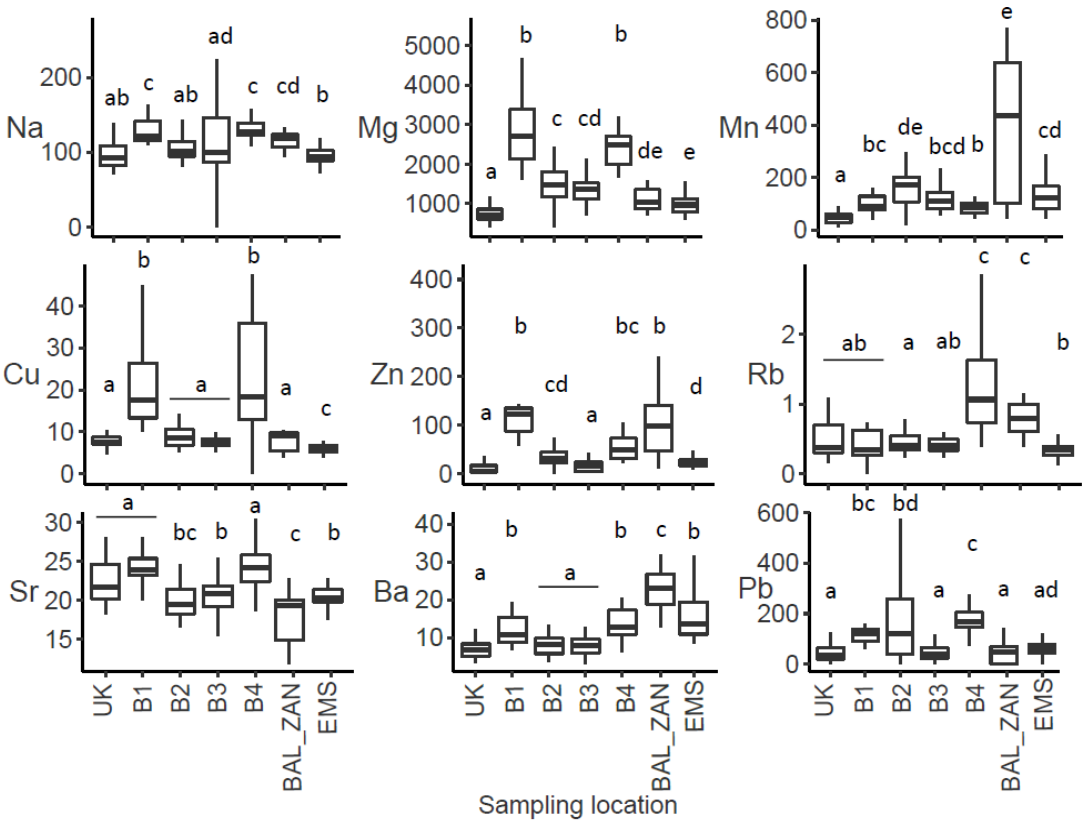


Figure 2.4: Box plots of the elemental composition (expressed as ratios to Ca) of juvenile sole otoliths at all sampling locations (see Table 2.1). For each sampling location, the solid line and the box correspond to the median and the interquartile range, respectively. Letters indicate groups of significantly different signatures based on Dunn's post hoc test ($p < 0.05$).

3.2 Temporal variation in elemental signatures on the nursery grounds

Inter-annual variation in otolith edge signatures were observed at both the B03 and B06 sampling sites. Differences between 2013 and 2014 signatures were significant ($p < 0.001$) at both sites for four elements (Mg, Mn, Cu and Pb; Mg (207, $p < 0.001$), Mn (172, $p < 0.01$), Cu (31.5, $p < 0.01$), and Pb (38.5, $p < 0.01$) for B03; Mg (253, $p < 0.001$), Mn (265.5, $p < 0.001$), Cu (54, $p < 0.001$), and Pb (272, $p < 0.001$) for B06.) out of nine. Other elements were not significantly different between years: Zn (72.5, $p = 0.176$), Rb (163.5, $p = 0.014$), Sr (154, $p = 0.040$), Ba (106, $p = 0.983$) for B03; Na (W = 84, $p = 0.023$), Rb (129, $p = 0.438$), Sr (98, $p = 0.072$) for B06. Additionally, significant inter-annual variations in Na signatures (W = 192.5, $p < 0.001$) were observed at B03, while the concentrations at B06 showed significant variations in Zn (W = 306, $p < 0.001$) and Ba (W = 279.5, $p < 0.001$).

3.3 Prediction of movement after settlement

For most juveniles, post-settlement signatures were assigned to a sampling location close to the capture location (76.2 %), or even to the capture location itself (46.9 %), indicating reduced movements for juvenile sole after arrival on the nursery ground (Table 2.4). However, in some fish, post-settlement signatures were assigned to a region totally different from the capture location. For example, 42.6% of the fish sampled along the UK coast had post-settlement signatures assigned to sampling locations in Belgium (B3, Table 2.3). These reassignments could be explained by similarities in Na, Cu, Rb, and Ba concentrations at the Belgian and UK sampling locations (Fig. 2.4). On the other hand, assignment errors based on otolith concentrations were limited or inexistent between the sampling locations at the Belgian coast and Balgzand_Zandvliet or Ems-Dollards (Table 2.3). Therefore, the fish sampled in Balgzand-Zandvliet (13%) and Ems-Dollard (36.9%) that had post-settlement signatures assigned to Belgium sampling locations could be putative migrants exchanged between sampling regions after benthic settlement (because sampling location signatures are spatially distinct as shown in Table 2.3). The post-settlement otolith signatures assigned to Belgium sampling locations (B1) in fish captured on Balgzand-Zandvliet were high in Mg and Zn (Fig. 2.5), similarly to those recorded in the otolith edges of the fish from B1. Similarly, some post-settlement signatures from the fish captured along the Ems-Dollard were assigned to Balgzand-Zandvliet, due to the

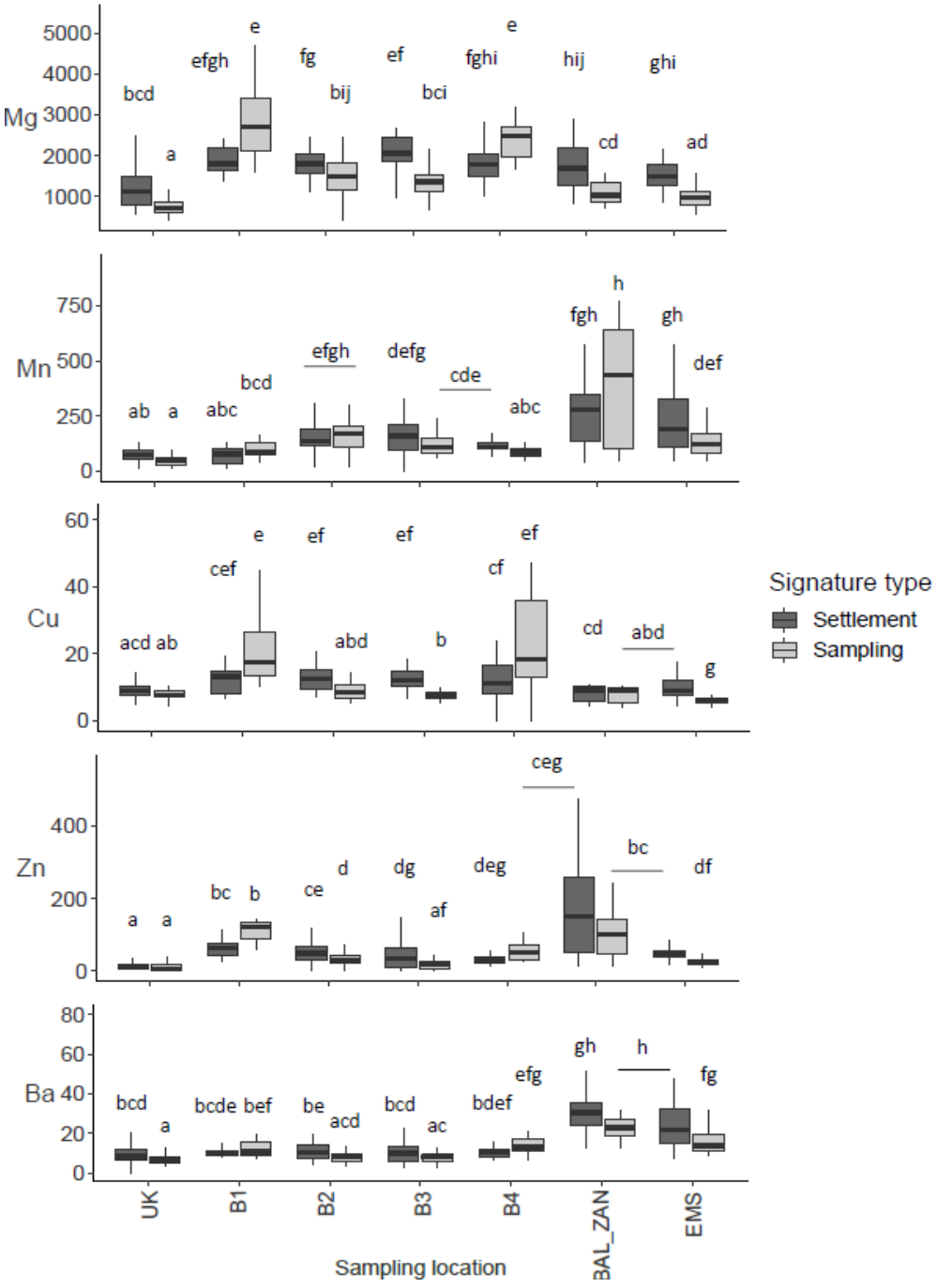


Figure 2.5: Box plots of the elemental composition (expressed as ratios to Ca) of juvenile sole otoliths at settlement (dark grey) and sampling locations (light grey, see Table 2.1). For each sampling location, the solid line and the box correspond to the median and the interquartile range, respectively. Letters indicate groups of significantly different signatures based on Dunn's post hoc test ($p < 0.05$).

high Mn concentrations. Nevertheless, if movements among nursery grounds are confirmed, they would happen for a reduced number of fish (about 20 fish of the 213 fish analyzed here).

3.4 Natal sources

The K-means clustering approach identified four clusters of chemically distinct larval signatures (Fig. 2.6), suggesting four different natal sources for the Southern North Sea nursery grounds. All natal sources were shown on the figure but, for visualization purposes, contribution percentages were only labeled for sources contributing to more than 20% to a nursery region. Sources 1 and 2 were the main contributors to the Belgian nursery grounds, and were characterized by the highest concentration of Mn, Zn, and Ba for source 1, and Cu Rb, and Pb for source 2 (Fig. 2.6, Suppl. Table 2.3b). The UK nursery grounds primarily received larvae from sources 3 and 4, and the Ems-Dollard Estuary from sources 1 and 3. Both Balgzand and Zandvliet received larvae from a single source. However the main origin of larvae was source 3 for Zandvliet and source 1 for Balgzand (detailed results not shown).

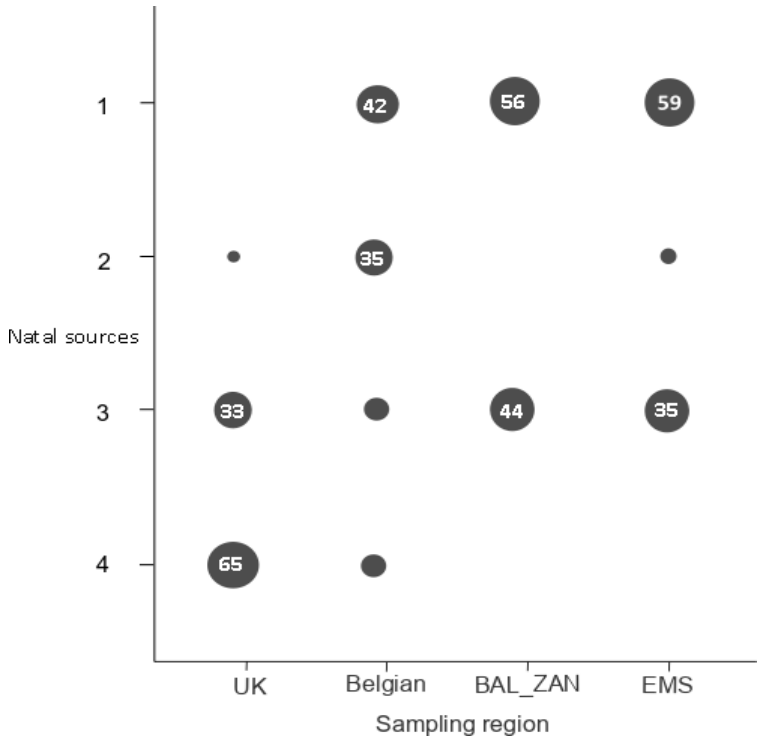


Figure 2.6: Assignment (%) of juvenile sole to the four natal sources identified based on the chemical variation in larval otolith signatures. For each sampling region, the percentage contribution of each natal source (cluster 1: n = 85, cluster 2: n = 43, cluster 3: n = 47, cluster 4: n = 38) is listed in the corresponding circle. Dots without labels represent contributions below 20%.

4. Discussion

Spatial differences in otolith chemistry clearly discriminated juvenile sole from nursery grounds along the Belgian and Dutch coast and indicated some overlap between the UK and Belgian coast. The high discrimination power between sampling regions is consistent with the restricted movement of sole following settlement. Overall, re-assignment based on settlement signatures was highest to the closest sampling location. This result suggests a limited movement after settlement on the nursery grounds for most juvenile sole. Nevertheless, the signatures of some juveniles sampled on the Balgzand_Zandvliet and the Ems-Dollard nurseries were more similar to fish sampled in Belgium. In addition, four distinct chemical natal sources contributed to the three nursery grounds of sole investigated.

Strong chemical differentiation in the nursery grounds

Despite the challenge of working at a small spatial scale (5 km between the closest sampling locations) and in the coastal area, the chemical signatures of eight elements provided high nursery-specific assignment levels for the juvenile sole. Assignment rates to the nursery grounds within the same region (89.3%) are comparable with previous studies (Cuveliers et al. 2010: 88%, Tanner et al. 2012: 71-80%). Nine elements were selected by the optimal RF classifier to achieve the highest accuracy but the first five elements already achieved an accuracy of 70%. The high assignment success might be related to the chemical composition of the European rivers draining in the North Sea as each represents drainage basin with a distinct geology (Mesozoic Alp geology for the Rhine, Eocene deposits of the Scheldt, Paleocene deposits for the Thames, Quaternary deposits for the Ems and Dollard, van Balen et al. 2005, Hartman et al. 2014, Preusser 2008, Royse et al. 2012, Yang & Nio 1989). In addition to river signatures, influences come from the pollution history, and physiology and temporal variations in chemical signal (see below). The North Sea is one of the most polluted seas world-wide due to the high level of industrialization (Halpern et al. 2008, Portman 1989). Despite a decreasing trend in metal concentrations due to strict regulations over the last decades (Emeis et al. 2015), local concentrations in sediments and suspended matter can remain high (De Witte et al. 2016, Van Alsenoy et al. 1993). Sr concentrations were lowest in Balgzand_Zandvliet and Ba concentrations were highest in locations under strong estuarine influence (i.e. Zandvliet, Balgzand and the Ems-

Dollard Estuary). The patterns of otolith Sr and Ba matched these salinity gradients (Campana 1999, Leakey et al. 2009). Zandvliet is situated in the brackish part of the Scheldt Estuary where salinity reaches about 6 (Limburg 1995). Similarly, Balgzand and the Ems-Dollard Estuary are low salinity nursery grounds. Additional sources of Ba include terrestrial runoff, groundwater, pollution and remobilization from sediments (Hamer et al. 2006). Furthermore, a recent meta-analysis showed that otolith Sr and Ba concentrations may be influenced by intrinsic factors such as diet, condition and ontogeny and additional extrinsic factors such as the ecological niche (Izzo et al. 2018), supporting former evidence (Sturrock et al. 2012). Balgzand and the Ems-Dollard Estuary exhibited similar concentrations for most elements although they are located in different tidal basins of the Wadden Sea and sampled eight years apart. For example, both locations exhibited high Mn concentrations that may reflect enrichment of the Wadden Sea in dissolved and particulate Mn compared to the surrounding water masses and/or hypoxic conditions (Dellwig et al. 2007, Limburg et al. 2015). Pb, Cu and Zn, which are associated with pollution, were higher in sole otoliths from the Belgian coast compared to the Thames Estuary, while Rb -which is associated to ingested plastic pollution (Lavers & Bond 2016)- levels were similar. The Belgian coast is influenced by the highly industrialized Scheldt Estuary, with actual and past traces of heavy metal pollution (Portman 1989, Zwolsman et al. 1996) whereas the inner Thames Estuary has been rehabilitated (Andrews & Rickard 1980, Attrill & Thomes 1995). Despite the distance between the two UK sampling locations, the chemical similarities are supported by the regional water circulation which moves in a northeast direction (Portman 1989).

Pre- versus post-settlement dispersal

Random Forest (RF) classification of the settlement signatures reassigned about 10% of the fish analyzed in this study from one region to another sampling region. This is reasonable, when taking the spatial variability of accuracy of the optimal RF classifier into account. For example, fish that settled in Belgium could have moved after settlement and be sampled off the Dutch coast. Nevertheless, the assignment of settlement signatures across sampling regions may be related to chemical changes in the ambient environment (i.e. more movement before than after settlement). One to three months have passed between the settlement and sampling signatures. Overall, our results suggest that the main drivers of otolith chemistry are operating

at a scale of approximately 100 km as supported by the assignment units. This finding is consistent with an average dispersal of about 150 km based on biophysical modeling and genetic markers (Kotoulas et al. 1995, Barbut et al. 2019). Field surveys reported comparable dispersal distances between spawning and nursery grounds (80-100 km, Dorel et al. 1991).

Importantly, microchemistry is not only affected by geography but also by time and physiological differences (Chang & Geffen 2013, Reis-Santos et al. 2012). For example, higher Sr levels are associated with higher growth rates during metamorphosis (de Pontual et al. 2000). We therefore intentionally looked at elements outside the nucleus and outside the metamorphosis zone (after the Sr peak) to avoid measuring chemical variation associated with ontogenic events. However, temporal variability in the microchemical signal may be driven by seasonal or inter-annual variation (Tanner et al. 2012). Although most studies found a significant effect of season or year on the microchemical signal, temporal variation did not hinder spatial discriminatory power, as partially confirmed by the present study (Cuveliers et al. 2010, Reis-Santos et al. 2012, Tanner et al. 2012).

Self-recruitment, a common feature of marine organisms (Swearer et al. 2002, Christie et al. 2010), appeared to be the predominant pattern of connectivity. Most juveniles remained on their nursery grounds after settlement (Burt & Millner 2008, Le Pape & Cognez 2016). Although most individuals recruited locally, our results revealed the presence of putative juvenile migrants that recently entered another region. Differences in the microchemistry profile between individuals sampled within the same region attest to the recent movement of juveniles between regions and validate the biophysical models of Lacroix et al. (2013). Because just a few migrants per generation support a high level of gene flow (Slatkin 1993), our findings seem compatible with the homogeneous population structure of sole in the North Sea (Cuveliers et al. 2012, Diopere et al. 2018). In addition to the realized dispersal between nursery grounds, the source of the larvae contributing to each nursery ground is key for understanding the dynamics of population structure.

Identification of four chemically distinct natal sources

The nine most reliable elements to discriminate between juvenile sole also identified four natal clusters. The similar signatures between recently settled juveniles and juveniles captured later on in the nursery ground favors the local recruitment hypothesis. Nevertheless, most nursery grounds were populated with juveniles from several natal sources. The three sampled nursery grounds recruited uneven proportions of settlers (0-100%) from four natal sources. Two of the four natal clusters did not contribute juveniles to the nursery ground along the UK coast. Since the UK nursery ground is characterized by high Mn, Cu, Zn, Rb, and Pb concentrations, it suggests that the settled juveniles have originated from a distinct natal source. This finding is consistent with biophysical models that predicted mixed input from several spawning grounds to most North Sea nursery grounds, except for the mostly self-recruiting UK nursery ground (Lacroix et al. 2013).

One of the natal clusters, cluster 3, was characterized by high Ba, which can be an estuarine signature (Hamer et al. 2006). The presence of some individuals with a natal cluster 3 signature on the nursery grounds near the UK, Balgzand_Zandvliet, and the Ems-Dollard Estuary suggest that there is some dispersal from the UK coast to the east. The English Channel spawning ground seems to be the main contributor to the Belgian nursery ground (Lacroix et al. 2013) facilitated by the residual current flowing north-east. Zandvliet and Balgzand, two locations under strong estuarine influence and pooled for the discrimination analyses because of the similarities in elemental concentrations and the low sample size, were each supplied by a single natal source. The isolated signatures of Zandvliet and Balgzand could be an artefact of low sample size because the K-means clustering method used is sensitive to sample size and less likely to identify small larval clusters. Indeed, densely sampled areas seem to have a more diverse set of natal sources. Natal cluster 1, characterized by high Mn, Zn and Ba, potentially under influence of freshwater, contributed highly to Balgzand_Zandvliet but also to Belgium and the Ems-Dollard Estuary. Freshwater sources are the estuaries of the Thames, Scheldt, Meuse, Rhine and Ems-Dollard. However, this study was not able to confirm whether the four chemical clusters reflected geographically distinct natal sources.

Connectivity research based on microchemistry requires the establishment of an extensive chemical baseline from putative sources (i.e. larvae in the spawning grounds and juveniles in the nursery grounds) to re-assign individuals of unknown origin. Establishing an

extensive baseline is unrealistic due to sampling limitations and knowledge gaps. However, statistical tools incorporating uncertainty and enabling assignment to missing sources provide an alternative. For example, Bayesian models provide uncertainty of assignment estimates (Reis-Santos et al. 2018). Estimating the potential number of sources would help to proceed during the validation step (i.e. know better how many spawning sources to sample) and allow hypothesis-testing of contribution of different spawning grounds to focal nursery grounds using other methods such as genetic markers (Reis-Santos et al. 2018). Nevertheless, using Bayesian models does not eliminate the need to validate the contribution of the spawning grounds to the nursery grounds. Determining the chemical signal of larvae on the spawning grounds is key to the validation of connectivity patterns.

An additional sampling challenge is the shift in location of the spawning and nursery grounds, and spawning season of many fish species and plankton communities in the North East Atlantic Ocean over the last 20 years (Beaugrand et al. 2002, Edwards & Richardson 2004, Cheung et al. 2013, Munday et al. 2013). The spawning season of sole has shifted to earlier in the year (Fincham et al. 2013) and the distribution of adults has shifted southward (Engelhard et al. 2011). Spawning and nursery ground locations have not shifted, however modeling suggests a potential for geographical shifts in larval recruitment at the nursery grounds and connectivity due to climate change (Lacroix et al. 2018).

Biophysical individual-based models are arguably one of the best tools to explore transport dynamics of flatfish larvae (Hufnagl et al. 2013) as they allow predictions over a large spatial extent and a high temporal frequency, far more than can be realized empirically. However, the lack of precise information on key parameters for larval survival such as swimming capability, mortality and pelagic larval duration hamper the parametrization of behavior routines of individual-based models. Therefore the behavior scenarios of recent models remain to be validated (Hufnagl et al. 2013, Lacroix et al. 2013, Savina et al. 2010). Based on the results of the present study, we confirm the model predictions that several spawning grounds contribute to the nursery grounds of the Southern North Sea and estimates of dispersal distance.

Transport mechanisms of flatfish larvae to the nursery grounds

Our results suggest limited movement of juveniles between and within nursery grounds post settlement, which is compatible with a genetic pattern of isolation by distance (i.e. the further geographically remote samples are, the more genetically distinct they are) (Cuveliers et al. 2012, Diopere et al. 2018). Despite a potential for long distance dispersal, species tend to settle locally (Iacchei et al. 2013, Pinsky et al. 2017). Plaice larvae also move less than might be expected from passive drifters. Larvae are retained on the spawning grounds due to nycthemeral migrations (i.e. day and night differences in migration behavior) and selective tidal transport (Creutzberg et al. 1978, Fox et al. 2009, Rijnsdorp et al. 1985). Nonetheless, our results suggest that for North Sea sole mixed natal sources contribute to several nursery grounds, contributing to the genetic homogeneity of the sole of the North Sea (Cuveliers et al. 2012, Diopere et al. 2018).

Based on a limited number of repeated sampling locations, the variation in chemical signature between successive years at two Belgian sampling locations was smaller than the spatial variation. This suggests that settlement is site-specific. Biophysical models point at considerable inter-annual variation of larval connectivity, which correlates with inter-annual recruitment variability (Lacroix et al. 2013). Although recruitment variability has been attributed to winter mortality in sole (Rijnsdorp et al. 1992), overall variation in recruitment remains poorly understood. Therefore future research should focus on integrating several measures of connectivity (such as biophysical modeling, genetics and otolith chemistry) across cohorts to consider the temporal persistence of elements (Reis-Santos et al. 2018).

The location of spawning grounds is tightly linked to nursery grounds. In the North Sea, the spawning grounds of sole are located more inshore and in shallower waters compared to other flatfish spawning in the same season (Rijnsdorp et al. 1992). Flatfish optimize settlement success in two ways. Firstly, successful spawning ground locations have been selected over time and flatfish have a high fidelity to their spawning ground (Hunter et al. 2003). One of the most important requirements of a successful spawning ground is the presence of suitable hydrographic conditions to transport eggs and larvae to the nursery ground (Symonds & Rogers 1995). Secondly, settlement can be delayed up to three weeks (Marchand 1991 for common sole). Factors triggering the delay are linked to terrestrial and benthic chemical cues, and freshwater input (Dixson et al. 2011, Freckelton et al. 2017, Kerstan 1991).

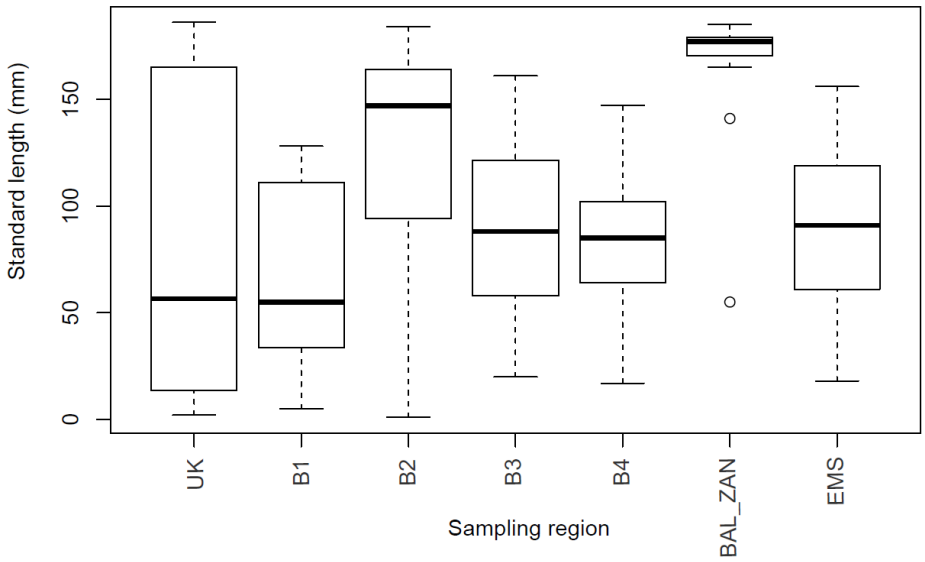
Conclusions

Recruitment success appears to be determined before the end of the first year in common sole (Rijnsdorp et al. 1992). This study brings new evidence on the connectivity dynamics of the early-life stages because the realized dispersal is assumed to take place during this period (Batista et al. 2015, Burgess et al. 2014, Krueck et al. 2017). The excellent spatial resolution of the microchemical signal of the sole otolith enabled us to identify several natal sources and connectivity patterns in the southern North Sea, consistent with the results of biophysical models of larval dispersal (Barbut et al 2019, Lacroix et al. 2013, Savina et al. 2016). Hence elemental analysis is a very valuable tool for species with extended larval dispersal and low genetic differentiation. To implement a network of MPAs as advocated in management (OSPAR 2013), understanding the directionality of flow between larval sources (i.e. spawning grounds) and sinks (i.e. nursery grounds), and connectivity patterns is crucial (Botsford et al. 2003, Palumbi 2004). Overall, inter-regional connectivity of sole larvae was limited. What remains to be assessed is the relative contribution of each life stage and inter-annual variation to dispersal.

Acknowledgements

Special thanks to K. Vanhalst (Institute for Agricultural and Fisheries Research, ILVO), the crew of RV *Simon Stevin,* RV *Stern,* RV *Cefas Endeavour* and RV *Belgica*, L. Bolle (Wageningen Marine Research, NL), J. Smith (Cefas, UK) and the B-FishConnect team for sampling, and to two anonymous reviewers for insightful comments. Research was funded by the B-FishConnect research project (G.0702.13N) and the Scientific Research Network 'Eco-evolutionary dynamics in natural and anthropogenic communities' (grant W0.037.10N), both funded by the Research Fund - Flanders (Belgium).

Supplementary material



Supplementary Figure 2.1: Box plots of the standard length of the juveniles sampled for each of the four sampling regions. The solid line and the box correspond to the median and the interquartile range, respectively.

Supplementary Table 2.1: Limit Of Detection (LOD = 3 x standard deviation of the blanks) and percentage of missing values for each element after setting values below LODs to zero. Concentration of each element was normalized to ^{43}Ca (the internal standard) and expressed in µmol mol^{-1}, except for Na/Ca, Fe/Ca and Sr/Ca (in mmol mol^{-1}) and and Pb/Ca (in pmol mol^{-1}). Only the elements in bold were retained for final analyses.

Isotope	Mean concentration (see legend for units)	Mean LOD	Missing value (%)
Li^{7}	0.4 ± 0.5	0.4 ± 0.4	57.0
Na^{23}	**2624.5 ± 635.8**	**4.3 ± 16.4**	**4.1**
Mg^{25}	38.9 ± 20.9	1.0 ± 10.7	0.6
Mg^{26}	**39.5 ± 17.2**	**0.8 ± 0.3**	**0.1**
Cr^{52}	8.6 ± 2.6	0.7 ± 0.4	1.0
Mn^{55}	**7.9 ± 6.7**	**0.1 ± 0.2**	**0.2**
Fe^{57}	82.6 ± 80.5	11.8 ± 10.9	0.1
Co^{59}	0.7 ± 3.3	0.2 ± 0.4	60.7
Ni^{60}	2.6 ± 1.6	0.2 ± 0.1	0.1
Cu^{63}	**0.7 ± 0.4**	**0.0 ± 0.0**	**0.9**
Zn^{64}	4.8 ± 4.4	0.2 ± 0.1	0.1
Cu^{65}	0.8 ± 1.4	0.1 ± 0.1	24.1
Zn^{66}	**3.7 ± 4.6**	**0.1 ± 0.4**	**0.6**
Rb^{85}	**0.1 ± 0.0**	**0.0 ± 0.0**	**3.6**
Sr^{86}	**1805.4 ± 255.4**	**0.3 ± 0.2**	**0.1**
Sr^{88}	1813 ± 248.1	0.1 ± 0.4	0.1
Cd^{111}	0.0 ± 0.0	0.0 ± 0.0	97.7
Cd^{114}	0.0 ± 0.0	0.0 ± 0.0	96.3
Ba^{137}	**2.0 ± 1.5**	**0.0 ± 0.3**	**0.4**
Ba^{138}	2.0 ± 1.5	0.0 ± 0.1	0.9
Pb^{204}	0.7 ± 0.5	0.5 ± 0.5	97.2
Pb^{206}	0.0 ± 0.1	0.1 ± 1.0	22.1
Pb^{207}	0.1 ± 0.1	0.0 ± 0.3	27.9
Pb^{208}	**0.0 ± 0.1**	**0.1 ± 0.6**	**10.8**

Supplementary Table 2.2: Accuracy and precision of the ICPMS internal standards (FEBS, NIST610, NIES) for measuring the concentrations of all the elements measured in this study (NA = missing value, NCV = Non Communicated Value). Recovery rate is the ratio of the measured concentration to the certified or reference concentration (in %). Relative standard deviation is the ratio of the standard deviation to the average concentration (in %). Elements in bold were retained for final analyses.

Isotope	Recovery rate			Relative standard deviation		
	FEBS	NIST610	NIES	FEBS	NIST610	NIES
Li^{7}	108	121	NCV	69	38	45
Na^{23}	**93**	**215**	**92**	**15**	**391**	**36**
Mg^{25}	123	134	136	18	18	62
Mg^{26}	**129**	**129**	**135**	**13**	**15**	**24**
Cr^{52}	NCV	93	NCV	20	7	34
Mn^{55}	**NCV**	**105**	**NCV**	**30**	**6**	**30**
Fe^{57}	NCV	109	NCV	43	18	37
Co^{59}	NCV	119	NCV	70	7	50
Ni^{60}	7160	105	NCV	26	7	58
Cu^{63}	**23**	**80**	**103**	**36**	**12**	**20**
Zn^{64}	NCV	108	476	11	8	20
Cu^{65}	28	111	104	44	8	33
Zn^{66}	**NCV**	**106**	**212**	**27**	**7**	**27**
Rb^{85}	**NCV**	**115**	**NCV**	**31**	**9**	**63**
Sr^{86}	**NCV**	**99**	**99**	**10**	**7**	**8**
Sr^{88}	NCV	105	99	11	7	8
Cd^{111}	4783	107	2652	NA	9	70
Cd^{114}	NaN	111	1725	NA	10	52
Ba^{137}	**95**	**106**	**95**	**13**	**8**	**13**
Ba^{138}	94	111	94	12	8	12
Pb^{204}	85	106	2892	9	10	44
Pb^{206}	73	119	1551	35	41	32
Pb^{207}	67	115	1819	35	23	29
Pb^{208}	**71**	**112**	**1779**	**38**	**10**	**27**

Supplementary Table 2.3: (a) Log transformed mean elemental signature (± standard deviation) for each sampling location (see Table 1, otolith sampling location signature). (b) Log transformed mean elemental signature (± standard deviation) of the four natal clusters identified by K-means clustering based on the larval signature (cluster 1: n = 85, cluster 2: n = 43, cluster 3: n = 47, cluster 4: n = 38). For the list of sampling locations see Table 1.

a

	Mg	Mn	Cu	Zn	Rb	Sr	Ba	Pb
THAj07	6.6 ± 0.2	3.6 ± 0.6	2 ± 0.2	3.3 ± 0.6	-0.1 ± 0.4	3 ± 0.1	1.9 ± 0.3	4.4 ± 0.7
GBRj16	6.6 ± 0.3	3.8 ± 0.6	2.1 ± 0.2	1.5 ± 0.5	-1.2 ± 0.3	3.1 ± 0.1	1.9 ± 0.4	3.7 ± 0.7
B01j14	7.9 ± 0.3	4.5 ± 0.5	2.9 ± 0.4	4.8 ± 0.5	-0.7 ± 0.8	3.2 ± 0.1	2.5 ± 0.4	4.9 ± 0.6
B02j13	7.3 ± 0.2	5.1 ± 0.3	2.0 ± 0.2	3.3 ± 0.4	-0.9 ± 0.3	3.0 ± 0.1	2.3 ± 0.5	4.2 ± 0.6
B03j13	7.4 ± 0.2	5.1 ± 0.3	2.1 ± 0.3	3.5 ± 0.3	-0.7 ± 0.3	3.0 ± 0.1	1.9 ± 0.4	4.8 ± 0.9
B03j14	6.6 ± 0.3	4.3 ± 0.7	2.5 ± 0.2	3.7 ± 0.4	-1.0 ± 0.3	2.9 ± 0.1	1.9 ± 0.4	5.7 ± 0.4
B06j13	7.3 ± 0.2	4.9 ± 0.3	1.9 ± 0.1	3.2 ± 0.3	-0.9 ± 0.2	3.0 ± 0.1	2.2 ± 0.2	4.2 ± 0.4
B06j14	7.0 ± 0.2	4.4 ± 0.3	2.1 ± 0.2	1.5 ± 0.3	-0.9 ± 0.3	3.1 ± 0.1	1.8 ± 0.3	4.0 ± 0.8
B08j14	7.8 ± 0.3	4.4 ± 0.3	3.1 ± 0.6	3.9 ± 0.5	0.0 ± 0.6	3.2 ± 0.1	2.6 ± 0.4	5.1 ± 0.4
ZANj07	6.8 ± 0.2	4.5 ± 0.4	1.6 ± 0.2	3.6 ± 1.1	-0.6 ± 0.2	2.6 ± 0.1	3.0 ± 0.3	4.0 ± 0.4
BALj06	7.1 ± 0.2	6.3 ± 0.4	2.3 ± 0.1	4.8 ± 0.4	-0.1 ± 0.3	3.0 ± 0.1	3.2 ± 0.3	4.4 ± 0.4
NL1j14	6.8 ± 0.2	4.9 ± 0.5	1.8 ± 0.2	3.2 ± 0.6	-1.3 ± 0.4	3.0 ± 0.1	2.5 ± 0.3	4.1 ± 0.4
NL2j14	6.9 ± 0.3	4.8 ± 0.5	1.8 ± 0.3	3.1 ± 0.5	-1.0 ± 0.2	3.0 ± 0.1	2.8 ± 0.4	4.1 ± 0.4

b

	Mg	Mn	Cu	Zn	Rb	Sr	Ba	Pb
1	7.7 ± 0.3	5.2 ± 0.6	2.7 ± 0.4	4.8 ± 0.6	-0.6 ± 0.4	3.1 ± 0.1	2.9 ± 0.5	4.6 ± 0.4
2	7.8 ± 0.3	4.5 ± 0.5	3.0 ± 0.6	4.1 ± 0.7	-0.1 ± 0.6	3.1 ± 0.2	2.3 ± 0.4	5.6 ± 0.6
3	7.3 ± 0.4	4.5 ± 0.5	2.1 ± 0.3	3.5 ± 0.5	-0.5 ± 0.5	3.0 ± 0.2	2.8 ± 0.6	4.3 ± 0.5
4	7.3 ± 0.4	4.2 ± 0.5	2.2 ± 0.3	1.7 ± 0.5	-0.8 ± 0.4	3.1 ± 0.1	2.0 ± 0.5	3.8 ± 0.6

Supplementary Table 2.4: Spearman correlations (upper table) between body size and the elements, and associated p values (lower table) within each sampling region (a) and each sampling location (b). Significant values (alpha level 0.05) are reported before (in bold) and after (underlined) Bonferroni correction for multiple testing.

a

	Na	Mg	Mn	Cu	Zn	Rb	Sr	Ba	Pb
UK	0.131	-0.037	-0.057	-0.179	0.694	0.680	-0.508	-0.047	0.180
B1	-0.352	-0.475	-0.124	0.381	0.094	0.103	-0.046	0.194	-0.100
B2	-0.426	-0.494	-0.241	0.244	0.066	-0.115	-0.210	-0.112	0.400
B3	0.005	0.073	0.158	0.039	0.097	-0.124	-0.113	-0.171	0.012
B4	0.315	-0.020	0.043	-0.548	-0.038	0.156	-0.296	-0.131	-0.256
BAL_ZAN	-0.134	-0.151	-0.473	-0.381	-0.530	-0.470	-0.533	-0.251	-0.122
EMS	0.160	0.095	0.091	-0.160	0.146	0.223	0.168	-0.070	0.050

	Na	Mg	Mn	Cu	Zn	Rb	Sr	Ba	Pb
UK	0.420	0.819	0.725	0.269	**0.000**	**0.000**	**0.001**	0.774	0.265
B1	0.182	0.063	0.648	0.145	0.730	0.704	0.867	0.470	0.712
B2	**0.003**	**0.000**	0.106	0.102	0.664	0.447	0.161	0.458	**0.006**
B3	0.976	0.674	0.364	0.825	0.576	0.478	0.519	0.326	0.948
B4	0.219	0.940	0.870	**0.023**	0.885	0.551	0.249	0.615	0.322
BAL_ZAN	0.543	0.493	**0.023**	0.073	**0.009**	**0.024**	**0.009**	0.249	0.580
EMS	0.345	0.575	0.593	0.345	0.389	0.184	0.319	0.679	0.768

b

	Na	Mg	Mn	Cu	Zn	Rb	Sr	Ba	Pb
THAj07	0.440	-0.054	0.432	-0.797	-0.320	-0.099	-0.145	-0.234	-0.272
GBRj16	-0.126	-0.187	0.074	-0.041	0.181	0.090	-0.324	-0.058	0.068
B01j14	-0.352	-0.475	-0.124	0.381	0.094	0.103	-0.046	0.194	-0.100
B02j13	0.256	0.191	-0.111	-0.089	-0.360	0.330	-0.091	-0.252	0.137
B03j13	-0.145	-0.135	0.065	-0.194	0.103	0.306	0.176	-0.116	0.290
B03j14	-0.006	0.370	0.839	-0.030	0.030	0.267	-0.091	0.479	-0.285
B08j14	0.315	-0.020	0.043	-0.548	-0.038	0.156	-0.296	-0.131	-0.256
B06j13	-0.306	0.191	0.284	0.331	0.503	0.289	0.291	0.334	0.327
B06j14	0.297	0.162	0.164	-0.252	-0.044	-0.537	-0.470	-0.652	-0.010
ZANj07	0.830	0.565	0.753	-0.051	-0.358	0.038	0.308	-0.230	-0.183
BALj06	0.503	0.618	0.322	0.647	-0.309	-0.036	0.032	0.099	0.163
NL1j14	0.088	-0.041	0.174	-0.224	0.305	0.200	0.097	-0.109	0.194
NL2j14	0.133	0.125	0.065	-0.166	-0.037	0.266	0.298	-0.057	0.003

	Na	Mg	Mn	Cu	Zn	Rb	Sr	Ba	Pb
THAj07	0.132	0.860	0.141	**0.001**	0.286	0.748	0.637	0.442	0.369
GBRj16	0.530	0.351	0.711	0.841	0.365	0.657	0.100	0.773	0.737
B01j14	0.182	0.063	0.648	0.145	0.730	0.704	0.867	0.470	0.712
B02j13	0.357	0.495	0.694	0.751	0.187	0.229	0.746	0.365	0.626
B03j13	0.531	0.558	0.780	0.399	0.657	0.177	0.445	0.617	0.203
B03j14	0.987	0.296	**0.002**	0.946	0.946	0.455	0.802	0.166	0.427
B08j14	0.219	0.940	0.870	**0.023**	0.885	0.551	0.249	0.615	0.322
B06j13	0.217	0.446	0.253	0.179	**0.035**	0.244	0.241	0.176	0.185
B06j14	0.247	0.534	0.528	0.327	0.869	**0.028**	0.057	**0.006**	0.970
ZANj07	**0.011**	0.144	**0.031**	0.904	0.385	0.928	0.457	0.584	0.664
BALj06	0.056	**0.014**	0.242	**0.009**	0.262	0.899	0.911	0.726	0.562
NL1j14	0.745	0.880	0.519	0.405	0.251	0.456	0.720	0.689	0.470
NL2j14	0.567	0.588	0.780	0.471	0.873	0.244	0.189	0.806	0.991

Chapter 3

Small-scale population genomic patterns of various life stages of the flatfish sole in the Northeast Atlantic Ocean

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Abstract

Investigating spatio-temporal genomic diversity of marine fishes is relevant for sustainable management. Large effective population sizes and complex life cycle dynamics, however, may lead to low overall differentiation and complicate the detection of adaptive variation. We used reduced representation (ddRAD) sequencing of 136 adult and 295 juvenile individuals of sole (*Solea solea*) to investigate genomic differentiation and adaptation of this highly exploited commercial flatfish. We compare large scale differentiation in the Northeast Atlantic Ocean with small-scale differentiation within the North Sea, the center of the distribution range of sole. Neutral genetic markers (1,378 Single Nucleotide Polymorphisms - SNPs) resolved subtle large scale genetic structure across the sampled distribution, from off the Iberian peninsula (Spain) to Skagerrak (Denmark). However, even a large sampling effort at a small-scale using 34 outlier SNP loci and powerful assignment methods, wasn't able to unambiguously resolve within North Sea differentiation. Instead, the limited significant pairwise differentiation between adult and juvenile sole points to chaotic genetic patchiness and high levels of mixing on small spatio-temporal scales. These results were further corroborated through 34 outlier loci, several of them linked to brain and sensing/immunological genes, that show putative signatures of ongoing selection, but no pronounced spatial pattern. Genetic connectivity of sole seems sufficiently large to support current fisheries management units, although adaptive genomic variation by life stage might play an as far poorly documented contribution.

1. Introduction

In the marine realm, broadcast spawning species are often characterized by high connectivity (i.e. the exchange of individuals among geographically separated groups) through advection during the planktonic larval phase. Together with the large effective population size this leads to usually low levels of genetic differentiation between populations (Cuveliers et al. 2011, Hare et al. 2011, Hauser & Carvalho 2008). Nonetheless, subtle temporal and spatial genetic variation among marine populations is measurable and biologically relevant (Lamichhaney et al. 2017, Limborg et al. 2012). Genetic population structure and the associated connectivity levels are crucial factors for the persistence and organization of metapopulations (Batista et al. 2015, Burgess et al. 2014, Krueck et al. 2017). Widely distributed marine species typically occur in large metapopulations that may be shaped by complex underlying patterns, including source-sink dynamics, local extinction and recolonization, and demographic effects (Hanski, 1998, Sale et al. 2006). Within this framework, organisms may also experience geographically variable selection pressures and maintain locally adapted subpopulations concurrent with high rates of gene flow (Nielsen et al. 2009, Pinho & Hey 2010). Deciphering and distinguishing signals of neutral and adaptive genetic variation through detailed analysis of molecular diversity and genetic linkage is necessary to understand potentially subtle, yet complex metapopulation structures. Overall, few variant sites in a species' genome may contribute to adaptation to the local environment (e.g. Berg et al. 2015, Lamichhaney et al. 2017). These so-called "genomic islands of divergence" (Nosil et al. 2009) include loci directly under selection or hitch-hiking through linkage with loci under selection. Given the high rates of change in marine environments (Munday et al. 2013, Pinsky et al. 2019) it is important to assess whether and how adaptation confined to few genomic regions is achieved in marine species with high connectivity and highly structured life cycle.

The early life stages of bentho-pelagic flatfishes typically occupy different, spatially non-overlapping habitats: (1) spawning grounds, where both spawning adults and eggs remain only for a short time, (2) nursery grounds, where juvenile fish typically reside for two to three years, and (3) feeding grounds, where adult fish reside outside the spawning period (Gibson 2015). Within this "migration triangle" (Harden-Jones 1968) flatfishes also switch from benthic to pelagic (eggs and larvae) and back (settling juveniles). Furthermore, eggs and larvae of broadcast spawners are especially subject to very high, temporally variable mortality rates causing large

variance in reproductive success (Hedgecock 1994). Consequently, only few adults contribute to the next generation (Hedgecock 1994, Hedgecok & Pudovkin 2011). This sweepstake recruitment process can lead to a pattern of chaotic genetic patchiness, where temporal variation among cohorts and/or spatially localized differentiation occurs (Johnson & Black 1982). On the other hand, a few migrants per generation may be sufficient in marine fishes with small ratio of effective to census population size to maintain high levels of gene flow and low genetic differentiation (Hauser and Carvalho 2008, Slatkin 1993).

The different components of the life cycle of broadcast spawners are affected by a range of processes that have the potential to influence long-term (i.e. evolutionarily significant) structure. First, planktonic life stages are restricted in their dispersal through physical and biological retention, such as ocean current patterns, seasonality or vertical migration (e.g. Barbut et al. 2019, Lacroix et al. 2013). The subsequent settlement process is then again determined by both intrinsic (e.g. age) and extrinsic (e.g. salinity) conditions (Dixson et al. 2011, Freckelton et al. 2017, Kerstan 1991). Limited movement of juveniles on the nursery grounds (e.g. Le Pape & Cognez 2016) should increase genetic differentiation between localities. The same holds for directed, spatial behavior such as natal homing (e.g. Bonanomi et al. 2016, Darnaude & Hunter 2018). Together, these properties may lead to increased differentiation at few genomic loci despite high gene flow. Overall, one may distinguish between large scale (hundreds of kilometers) and small-scale (tens of kilometers) to characterize the spatio-temporal population genetics patterns of marine fish.

High density characterization of the genome is required to resolve small-scale genetic structure and local adaptation (Andrews et al. 2016, Baird et al. 2008, Nielsen et al. 2009, Paris et al. 2018). Earlier studies of local adaptation commonly investigated candidate loci (i.e. putatively under selection) by focusing on phenotypic variation to identify the genetic basis of a specific trait (Chatziplis et al. 2007, Diopere et al. 2018, Gutierrez et al. 2012, Sciarra et al. 2018). While these studies have been invaluable to elucidate the molecular mechanisms of adaptation, they required a top-down approach where loci of interest are determined *a priori*. With high-throughput sequencing (HTS) a bottom-up approach is now feasible (Ross-Ibarra et al. 2007), even in non-model species (Peterson et al. 2012, Toonen et al. 2013). Using a genome of the target or a related species is beneficial to map Single Nucleotide Polymorphisms (SNPs) and assess linkage (Manel et al. 2016). Candidate loci showing indications of ongoing selection and adaptation can be matched to genes or genomic regions in order to eventually understand

functional links (Tiffin & Ross-Ibarra 2014). Among the many studies on marine species applying HTS to investigate genetic structure and adaptation (Andrews et al. 2016, Hendricks et al. 2018, Hohenlohe et al. 2010), few have made a comparison between life stages (but see Sato et al. 2018). It is thought that genetic structure is more pronounced in early life stages than in adults due to high mortality rates (Houde 1997). Especially small-scale patterns may be overlooked when only assessing adult specimens. For example, genotyping of eggs of Atlantic cod *Gadus morhua* revealed that an inshore stock had potential for recovery (Svedäng et al. 2018).

Common sole (*Solea solea*, Linnaeus 1758; Soleidae; hereafter called sole) is abundant and of great commercial importance in the Northeast Atlantic Ocean. The species plays a key role in the benthic food web as prey (juveniles) or predators (adults) (Gibson 2015). Large scale genetic studies using microsatellites or gene-linked markers identified four sole populations: the Baltic Transition Zone, the Bay of Biscay, the North Sea/Eastern English Channel, and the Irish and Celtic Sea (Cuveliers et al. 2012, Diopere et al. 2018). In addition, highly targeted SNP panels can be used to assign individual (adult) specimens back to their source population (Nielsen et al. 2012). However, so far not any study has genotyped SNPs throughout the sole genome to investigate small-scale structure and genomic islands of divergence that may contribute to local adaptation. The North Sea might be especially important for the entire sole metapopulation due to the many spawning and nursery grounds, and large spawning biomass. Five main subtidal spawning grounds have been identified in the Southern North Sea (Ellis et al. 2012): (1) the eastern English Channel, (2) off the Belgian coast, (3) in the Thames Estuary, (4) on the Norfolk Banks, and (5) in the inner German Bight. Peak spawning occurs from April to June and after hatching pelagic larvae drift for about one month, covering an average of 150 km as estimated by biophysical modeling (Lacroix et al. 2013). Subsequently, juveniles settle in a shallow coastal or estuarine nursery (Russell 1976, van der Land 1991). Nursery grounds are distributed almost continuously along the North Sea coast (Ellis et al. 2012, Rijnsdorp et al. 1992). After transition to a benthic life style and settling on the nursery ground, the amount of spatial exchange is low (Le Pape & Cognez 2016, Delerue-Ricard et al. *in review*). Accordingly, genotypes of 0- and 1-group sole from the Bay of Biscay are temporally stable, while sub-adults show temporal differences (Exadactylos et al. 1998, Rolland et al. 2007, Guinand et al. 2008). This pattern could be caused by high, and spatially variable selection on eggs and larvae as emphasized in the sweepstake hypothesis on the one hand and higher amounts of mixing at the feeding and/or spawning grounds on the other hand (Koutsikopoulos et al. 1995). There are presently no

indications of natal homing for sole and, based on adult specimens, the entire North Sea is genetically recognized as one population (Cuveliers et al. 2012, Diopere et al. 2018). Unlike in the Bay of Biscay, recruitment in the North Sea is determined by the first year of life (Rijnsdorp et al. 1992) and can also be seasonally variable (Hovenkamp et al. 1991). The exact dynamics of the life cycle of sole within a relatively small spatial area such as the Southern North Sea are incompletely understood. Apparent homogeneity of the adult stages might be underlain by relevant genetic variability of the early life stages that is only detected with high resolution genotyping.

In this study we aim at assessing (1) the large scale genetic population structure of sole in the Northeast Atlantic Ocean, (2) the small-scale genetic population structure of sole in the North Sea and (3) whether life stage specific processes affect neutral and adaptive genomic diversity of sole. In a bottom-up approach we genotype hundreds of adult and juvenile sole and identify candidate loci that might be linked to ongoing selection.

2. Material and Methods

2.1 Sample collection

In total, 136 adult and 295 juvenile sole were collected at 18 sampling sites. Adults were collected by Diopere et al. (2018) and resequenced with ddRAD sequencing (see below). These samples were collected between 2007 and 2017 from five regions: Bay of Biscay, Celtic Sea, English Channel, North Sea and Baltic Transition Zone (Fig. 3.1). In addition, age-0 and -1 sole, hereafter referred to as juveniles, were collected in the North Sea between 2006 and 2016 (Fig. 3.1, Table 3.1). Juveniles were caught off the UK coast (Sizewell power station and Thames Estuary), the Belgian coast, Scheldt Estuary (Zandvliet), and the Wadden Sea (Balgzand off Texel, and Ems-Dollard Estuary), all areas that represent major nursery grounds (Rijnsdorp et al. 1992, Ellis et al. 2012). All samples were collected by beam trawling with research vessels; the closest stations were five km apart. Patterns observed at a scale of <100 km (i.e., the full extent of the Belgian nursery) were considered local and small-scale, whereas patterns observed at a scale of >100 km were considered regional. Fish were frozen until dissection. In the laboratory, fish were thawed, measured for standard length to the nearest mm and fin clips were taken and immediately stored in absolute ethanol.

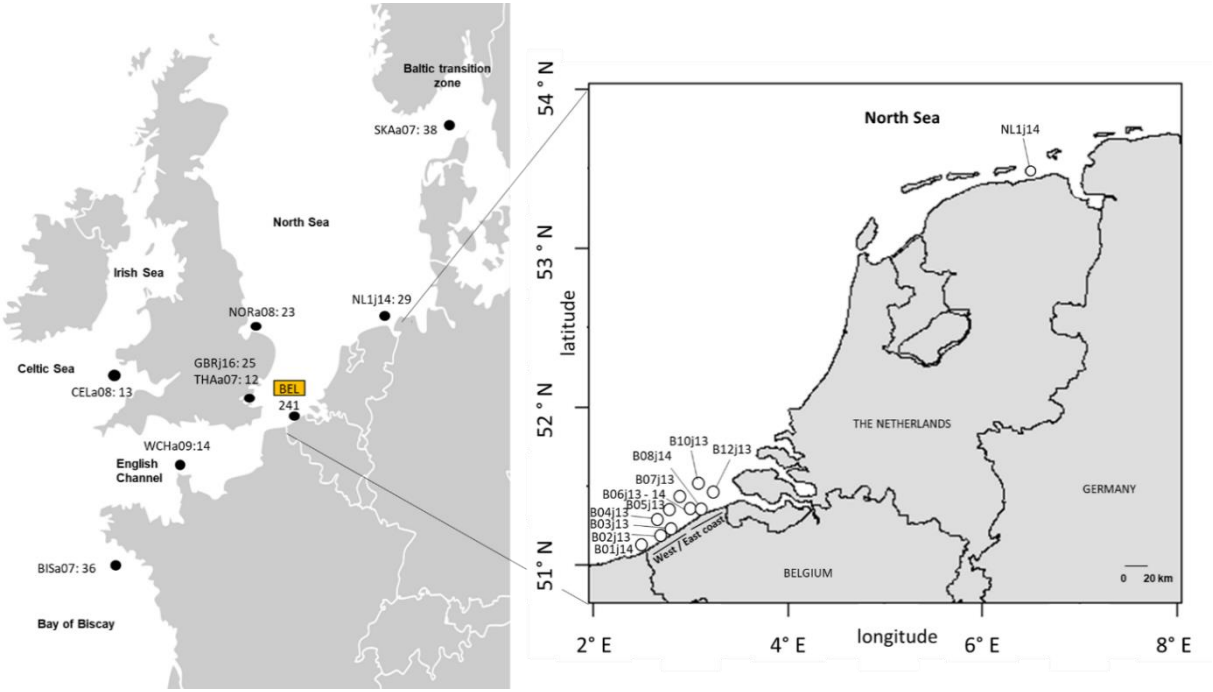


Figure 3.1: Sampling locations in the Northeast Atlantic Ocean and North Sea where juvenile (location codes containing a "j" and empty circles) and adult (location codes containing an "a" and full circles) specimens of sole, were sampled between 2008 and 2017 using beam trawls (further sampling details in Table 3.1).

2.2 DNA extraction, genomic library preparation and sequencing

Genomic DNA was extracted using a standard salt-extraction protocol (Cruz et al. 2017) and RNA was removed with the Riboshredder RNase Mixture (Epicenter, Madison, USA). DNA quantity was measured with the QuantIt Picogreen dsDNA assay (Thermo Fisher Scientific, Waltham, USA) and DNA quality was checked on a 1% agarose gel. Seven double digest restriction-site associated DNA (ddRAD, Peterson et al., 2012) libraries were prepared using the *SbfI* and *SphI* enzymes as described in detail in Palaiokostas et al. (2015), but with the following modifications. Libraries consisted of 96 to 141 individuals. In the first library, DNA from all specimens was pooled after enzyme digestion and adapter ligation, and then used for size selection (320-590 bp cut from the gel with a blade) and PCR amplification (with 18 cycles) as in Palaiokostas et al. (2015). For the six other libraries, DNA from all specimens was individually PCR amplified (18-21 cycles) and then standardized to a concentration of 7 ng/μL before pooling. This strategy was

found to positively affect the homogenous distribution of reads across specimens. Size selection of these libraries was furthermore conducted on a Pippin Prep (Sage Science, Beverly, USA), selecting fragments between 300-600 bp. Libraries were sequenced on an Illumina HiSeq2500 ® platform in paired-end mode at the Genomics Core of the KU Leuven (www.genomicscore.be, KU Leuven, Belgium).

2.3 Sequence quality control, genotype calling and filtering

Raw reads were checked for overall sequencing quality using FastQC (Andrews 2010, Fig. 3.2). Demultiplexing was done with the 'process_radtags' module of STACKS (Catchen et al. 2011, 2013). All SNP genotyping steps were carried out using dDocent v2.2.19 (Puritz et al. 2014), which is specifically designed to detect informative SNP markers in paired-end reads (Puritz et al. 2014). dDocent is a bash wrapper that relies on CD-HIT to assemble reads (Li et al. 2001), Burrows-Wheeler Alignment to map reads (Li & Durbin 2010), SAMtools to convert SAM to BAM files (Li et al. 2009), and FreeBayes to call SNPs (Garrison & Marth 2012). dDocent was run with the reference genome of the Senegalese sole *Solea senegalensis* (Manchado et al. in prep) and using largely default settings.

Reads were then filtered using VCFtools (Danecek et al. 2011). From the raw 1,520,000 SNPs obtained after demultiplexing, we retained 990,000 bi-allelic SNPs. We subsequently filtered for minimum allelic depth of four and allelic balance between 0.25 – 0.75 (971,600 SNPs remaining), minimum minor allele frequency of 5% (311,500 SNPs remaining) and excluded loci with >10% missing data over all individuals (1,489 SNPs remaining).

Finally, with these 1,489 SNPs we performed some last filtering steps in R (R development Core Team 2018) using adegenet (Jombart 2008, Jombart and Ahmed 2011). All loci with observed heterozygosity >0.5 and expected heterozygosity >0.6 were removed. The *poppr* package (Kamvar et al. 2014) was used to assess the distribution of missing data across sampling locations and specimens and calculate linkage disequilibrium between loci. Hardy-Weinberg Equilibrium was assessed using the *pegas* package (Paradis 2010*)*. Loci that were not in Hardy-Weinberg Equilibrium in 2/3 of the populations (after 5,000 Monte Carlo permutations) and loci in Linkage Disequilibrium (LD > 0.7) were excluded after correcting p-values (alpha = 0.05) for multiple testing following Benjamini and Hochberg (1995).

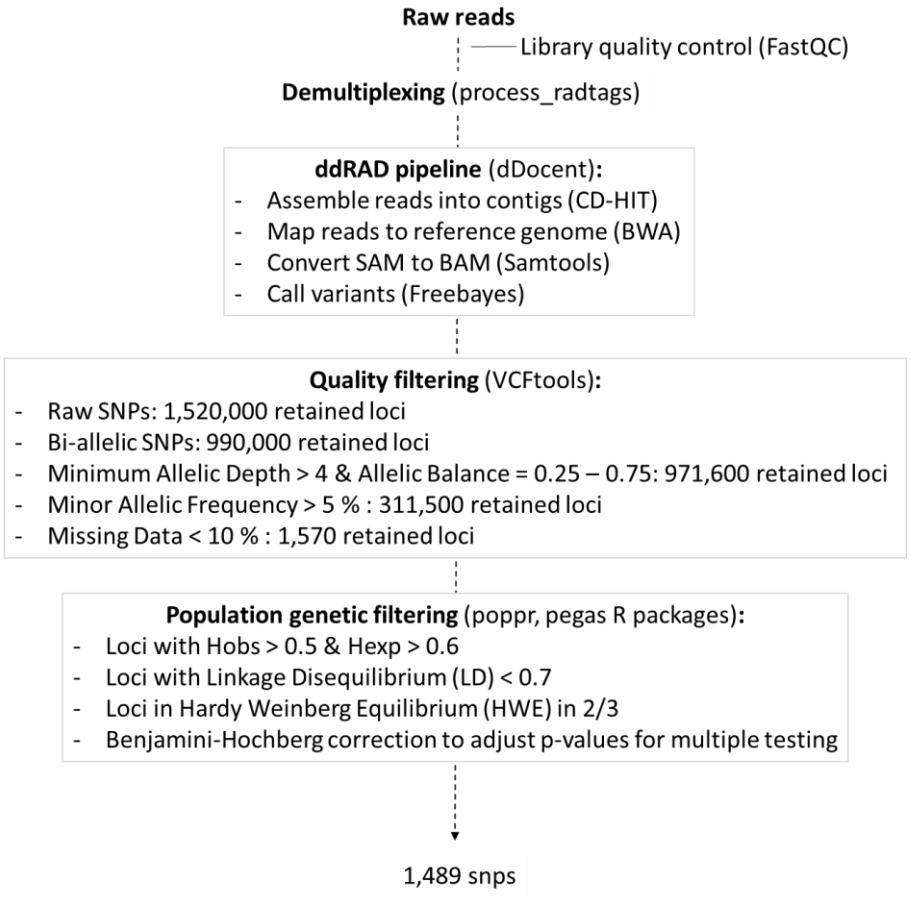


Figure 3.2: Bioinformatic pipeline followed to retain trustworthy SNP loci from raw sequencing reads

Table 3.1: Sampling details for sole, collected in the Northeast Atlantic Ocean and North Sea, including sample code (including abbreviated location, adult/juvenile, year), name of sampling region, latitude and longitude details in decimal degrees, life stage (juvenile (J) , adult (A), adult sampled during spawning season (A*)), sampling time (year-month), sample size (N), average expected (He) and observed (Ho) heterozygosity, and inbreeding coefficient (FIS) per location as calculated with evolutionarily neutral genetic markers.

Location code	Sampling region	ICES area	Latitude	Longitude	Stage	Year-month	N	H_e	H_o	F_{IS}
BISa07	Bay of Biscay	VIIIa	45.92	-1.69	A*	2009-03	36	0.27	0.31	-0.10
CELa08	Celtic Sea	VIIg/f	50.81	-5.01	A*	2008-04	13	0.28	0.32	-0.12
WCHa09	Western English Channel	VIIe	49.66	-2.13	A	2009-09	14	0.26	0.22	0.14
NORa08	Southern North Sea	IVc	52.92	2.24	A	2008-08	23	0.25	0.24	0.04
GBRj16	Southern North Sea	IVc	52.21	1.63	J	2016-03	25	0.26	0.24	0.06
THAa07	Southern North Sea	IVc	51.47	1.33	A	2007-08	12	0.26	0.24	0.08
B01j14	Southern North Sea	IVc	51.13	2.70	J	2014-09	30	0.26	0.24	0.06
B02j13	Southern North Sea	IVc	51.19	2.70	J	2013-09	27	0.26	0.24	0.07
B03j13	Southern North Sea	IVc	51.35	3.00	J	2013-08	22	0.26	0.24	0.08
B03j14	Southern North Sea	IVc	51.35	3.00	J	2014-05	23	0.26	0.24	0.06
B04j13	Southern North Sea	IVc	51.24	2.82	J	2013-09	25	0.26	0.23	0.08
B05j13	Southern North Sea	IVc	51.32	2.89	J	2013-09	12	0.26	0.23	0.08
B06j13	Southern North Sea	IVc	51.35	3.00	J	2013-08	21	0.26	0.23	0.08
B07j13	Southern North Sea	IVc	51.38	2.85	J	2013-09	13	0.26	0.23	0.08
B08j14	Southern North Sea	IVc	51.35	3.10	J	2014-10	31	0.26	0.24	0.07
B10j13	Southern North Sea	Ivc	51.46	3.12	J	2013-09	21	0.26	0.23	0.08
B12j13	Southern North Sea	IVc	51.43	3.31	J	2013-09	16	0.26	0.23	0.08
NL1j14	Wadden Sea	IVc	53.48	6.49	J	2014-09	29	0.26	0.24	0.07
SKAa07	Baltic Transition Zone	IIIa	57.16	11.65	A	2017-11	38	0.27	0.28	0.00

2.4 *Genome scans to detect outlier loci*

After the final filtering steps 1,412 loci across 431 individuals were retained and subsequently used in three genome scan methods to detect outlier loci putatively under selection (i.e. loci that have allele frequencies outside the general distribution of all alleles). We used pcadapt (Luu et al. 2016) and OutFLANK (Whitlock & Lotterhos 2015) to complement the widely used BayeScan (Foll & Gaggiotti 2008), because both methods provide a lower False Discovery Rate (FDR) than BayeScan in a situation such as in our study system: a divergence model with admixture (i.e. there is a hierarchical population structure where differentiation varies between populations, cf. Figure 3 from Luu et al. 2016).

Five kb of genome sequence both upstream and downstream of the outlier loci was extracted using Bedtools for gene annotation (Quinland & Hall 2010). This strategy was used, because outlier loci may be prone to hitchhiking with a neighboring gene (Manel et al. 2016). The sequences of outlier loci were compared with the tongue sole *Cynoglossus semilaevis* genome and the annotation revised. These sequences were compared with the zebrafish *Danio rerio* transcriptome to identify closely related genes for functional analysis. The functional analysis was done under relaxed conditions using ClueGo (Bindea et al. 2009). Finally, the revised annotations were linked to large functional categories that might be used to build a logical framework. They were BLASTED onto the new draft of *Solea senegalensis*.

2.5 Genomic diversity and population structure

To estimate genomic diversity within and among populations, allelic diversity indices including average observed (H_o) and expected heterozygosity (H_e) were calculated, together with inbreeding coefficient (F_{IS}) and pairwise F_{ST} estimates, using the R package *Hierfstat* (Goudet 2005). Broad-scale population structure was visualized with Principal Coordinate Analysis (PCoA) based on pairwise F_{ST} distance for evolutionarily neutral and outlier SNP loci. Small-scale population structure was investigated with Principal Component Analysis (PCA) and subsequent Discriminant Analysis of Principal Components (DAPC) using the *adegenet* package (Jombart 2008, Jombart et al. 2010). DAPC was performed on neutral and outlier SNP loci separately, and an α score optimization was conducted to determine the number of principal components to re-

tain. In addition, Isolation By Distance (IBD) was assessed as the correlation between over water distance (x axis) and F_{ST} / 1 – F_{ST} (y axis, see Rousset 1997). Over water distances were calculated using the *marmap* package (Pante & Simon-Bouhet 2013).

Similar to Bayesian clustering programs, the sNMF function of the *LEA* package (Frichot & François 2015, Frichot et al. 2014) was used to estimate individual admixture coefficients from the genotypes. Assuming the number of ancestral populations, this function provides least-squares estimates of ancestry proportions. The quality of fit of the statistical model to the data is provided by a cross-validation, creating the entropy criterion. The lowest entropy corresponds to the number of ancestral populations that best explains the genotypic data (Frichot et al. 2014). An analysis with STRUCTURE (Pritchard et al. 2000) was applied through the *ParallelStructure* package (Besnier & Glover 2013) to compare ancestry coefficient estimates from different methods.

To assess more specifically whether life stage specific processes affect the neutral and adaptive genetic structure of sole, we distinguished juvenile and adult populations in the previously mentioned PCoA and pairwise F_{ST} analyses. In addition, genetic assignment of juveniles to adult populations from the Bay of Biscay, Celtic Sea, Western English Channel, Thames, Norfolk, and Skagerrak was performed with the software GENECLASS2 (Piry et al. 2004) following the procedure applied in Pukk et al. (2016). The reassignment test was applied to the adult populations only to assess the ability of our baseline dataset to correctly self-assign adult fish to their sampling location. Assignment success was calculated as the percentage of individuals correctly re-assigned to their sampling location with a probability above 95%. GENECLASS2 calculates the likelihood of individual fish belonging to a reference population which was compared with the distribution of likelihoods of 1000 genotypes simulated from each adult population with a frequentist method (Paetkau et al. 2004) using the criterion of Paetkau et al. (1995).

2.6 Relatedness between juveniles

Relatedness between pairs of individuals was calculated with the final data set of 1,412 SNPs. The advantage of kinship analysis compared to parentage analysis is that it requires no *a priori*

knowledge of relationships among genotyped individuals. Pairwise relatedness was calculated using the Wang estimator, while allowing for inbreeding and using the default settings as implemented in the *related* R package (Pew et al. 2015). This R package was developed based on the COANCESTRY software (Wang 2011). The idea behind this method is that two related individuals share a common ancestor which can be discerned from the mean number of alleles shared between individuals. In marine populations, few adult individuals may contribute to the next generation because of sweepstake effects (Hedgecock 1994, Hedgecock & Pudovkin 2011). A large pairwise relatedness of two individuals suggests a non-random population structure and could be the sign of sweepstake effects and/or cohort structure. To enhance the chances of detecting a sweepstake effect or cohort structure, individuals should be sampled at the same time. One important factor that confounds kinship likelihood is LD, which often causes relationships to be overestimated and increases false positives (Sun et al. 2016). Loci in LD (see *section 2.3* of this chapter for details) were removed prior to kinship analysis. Values of the relatedness coefficients detected by the *related* package were confirmed by the R package *Demerelate* (Kraemer & Gerlach 2017).

3. Results

3.1 Genomic diversity

Prior to the calculation of genomic diversity, 61 loci in linkage disequilibrium and 3 loci not in Hardy-Weinberg Equilibrium were removed from the data (after correction for multiple testing). Observed (H_O) and expected (H_E) heterozygosity per sample for all remaining 1,412 SNP loci ranged between 0.22 and 0.32 and 0.25 and 0.28, respectively (Table 3.1). The Celtic Sea and Bay of Biscay population samples showed considerably larger observed heterozygosity (0.32 and 0.31, respectively), with the sample of the Baltic Transition Zone showing intermediate (0.28) and all other samples comparably lower H_O (0.22 - 0.24). In contrast, expected heterozygosity was relatively uniform among samples, with Baltic Transition Zone , Celtic Sea, and Bay of Biscay only slightly larger (0.27 - 0.28) than all other samples (0.25 – 0.26). Similarly, patterns of F_{IS} varied between the Celtic Sea and Bay of Biscay (-0.10 - -0.12), Baltic Transition Zone (0.00) and remaining samples (0.04 - 0.14).

3.2 Genome scans for putative selection

Pcadapt detected 133 loci putatively under selection (after thinning, i.e. removing SNP in potential LD, Benjamini-Hochberg correction for multiple testing and with q = 0.05), whereas OutFLANK and BayeScan detected 58 and seven outlier loci (alpha = 0.05), respectively. Thirty-four loci were detected by at least two of the methods and retained. All loci that were not flagged by either method, i.e. 1,378 SNPs, were retained as a putatively evolutionary neutral data set. Twenty three outlier loci could be matched to genes within 5 kb up/downstream (Suppl. Table 3.1). We proceeded with all 34 outlier loci to maximize information for subsequent population genetic structure analyses.

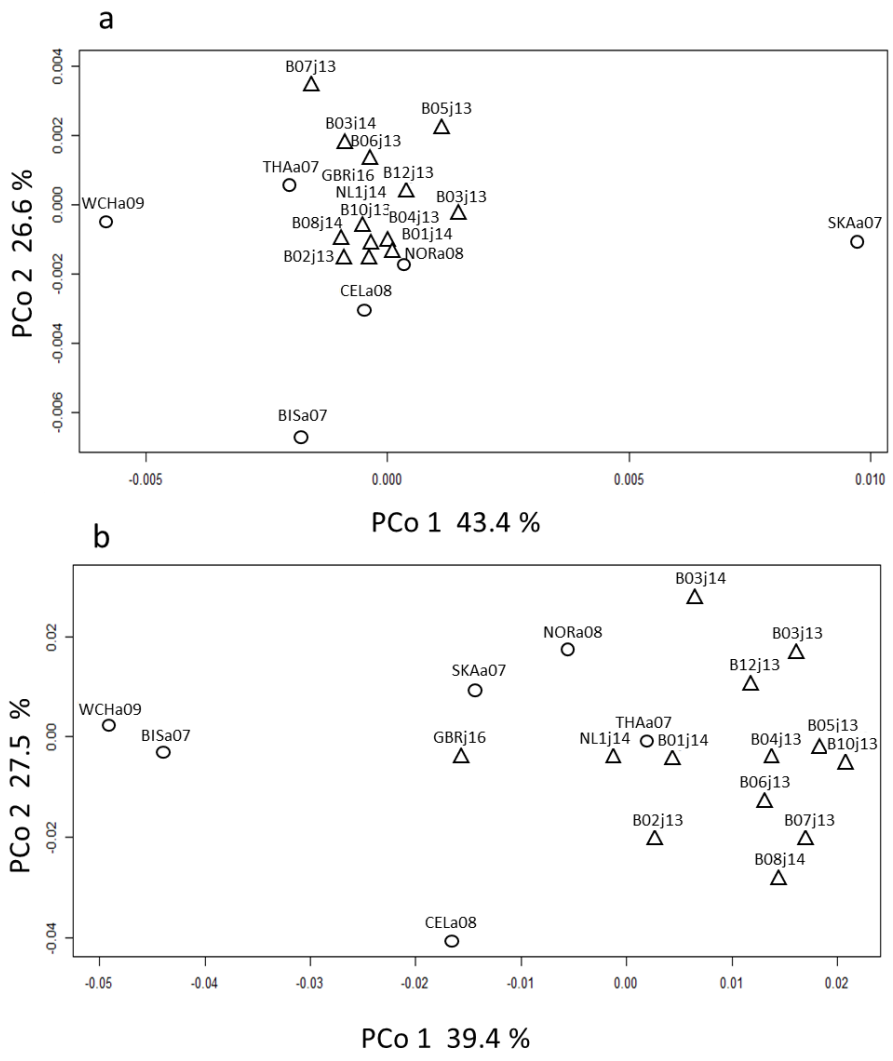


Figure 3.3: Spatial genetic differentiation based on F_{ST} values between sole samples in the Northeast Atlantic Ocean and North Sea as inferred from (a) neutral and (b) putatively adaptive genetic markers and visualized by principal coordinate analysis (PCoA). Shape represents juvenile (△) and adult (○) populations. The variance explained is indicated in % on each axis. Sampling locality codes as in Table 3.1.

Among the 34 outlier loci, 31 were assigned to a putative gene on the *Cynoglossus semilaevis* genome and 21 to a putative gene on the phylogenetically less related zebrafish genome (Suppl. Table 3.1). Among these, 18 genes were assigned through gene ontology to a function. Genes linked to the brain and sensing/immunology were the most important categories identified.

3.3 Neutral, adaptive population structure and relatedness

Pairwise F_{ST} values based on neutral loci ranged from -0.002 to 0.016. Among 176 pairs of sites, 105 were statistically differentiated ($p < 0.05$), 18 of which involved samples from the Baltic Transition Zone and another 18 involved samples from the Bay of Biscay (Table 3.2). In contrast, most within North Sea comparisons were not differentiated. This pattern of subtle, albeit significant spatial structure between larger geographic provinces, was confirmed with PCoA analyses (Fig. 3.3). Most prominent genetic differentiation was observed along axis one between the Baltic Transition Zone (SKAa07) and the Western English Channel (WCHa07) and the Bay of Biscay (BISa07) samples in the South-East (Fig. 3.3a). The North Sea samples clustered centrally between geographically distant samples. Accordingly, physical geographical distance correlated significantly with genetic divergence suggesting an IBD pattern ($r = 0.040$, $p = 0.001$, Fig. 3.4a and b). Individual-based methods broadly supported the large scale neutral genetic structure as

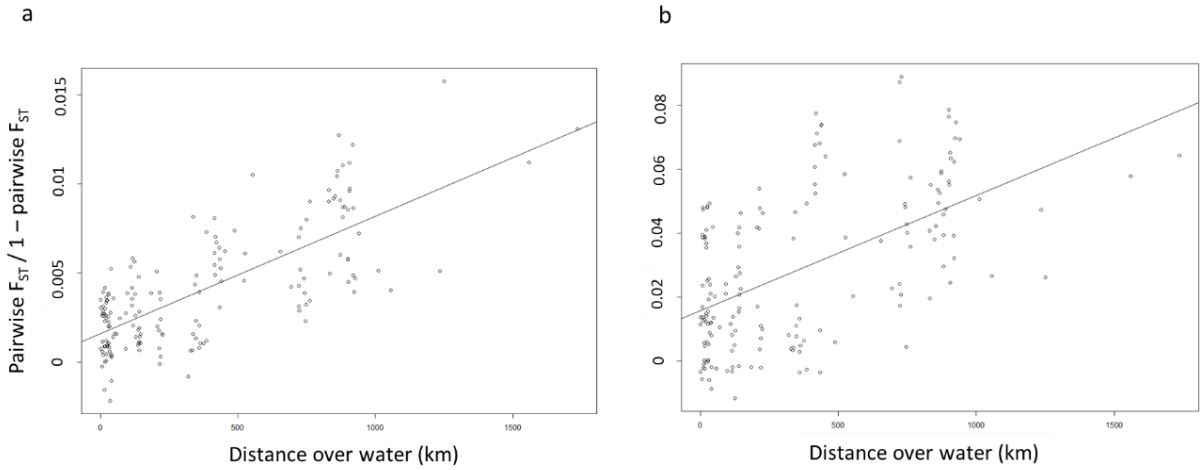


Figure 3.4: Spatial genetic differentiation between sole samples in the Northeast Atlantic Ocean and North Sea as inferred from (a) neutral and (b) outlier genetic markers based on the correlation of genetic (measured as pairwise F_{ST} / 1- pairwise F_{ST}) and physical distance (measured over water).

observed with F_{ST} tests and PCoA. North Sea samples were largely similar while the other geographic regions diverged from them. Principal coordinate analysis revealed that the Celtic Sea diverged from the North Sea (along PC1) and from the Baltic and English Channel samples (along PC2; Fig. 3.5a). This pattern was partly confirmed with DAPC. Specimens of the Baltic Transition Zone diverged from North Sea individuals along axis one, while sole from the Bay of Biscay, the English Channel, and the Celtic Sea differentiated along axis two corresponding to a South-North gradient (Fig. 3.5b). The two plots are almost identical but differ in that the PCA shows only two PCs while the DAPC shows two DAs (summarizing 100 PCs). It explains why the variation is more concentrated in the DAPC. Bayesian clustering approaches as implemented in STRUCTURE were not able to detect population clusters (results not shown), as expected due to the low F_{ST} values. SNMF analysis on the other hand identified $K = 4$ as the most likely number of groups, but the purported clusters did not correspond to geographical sampling groups.

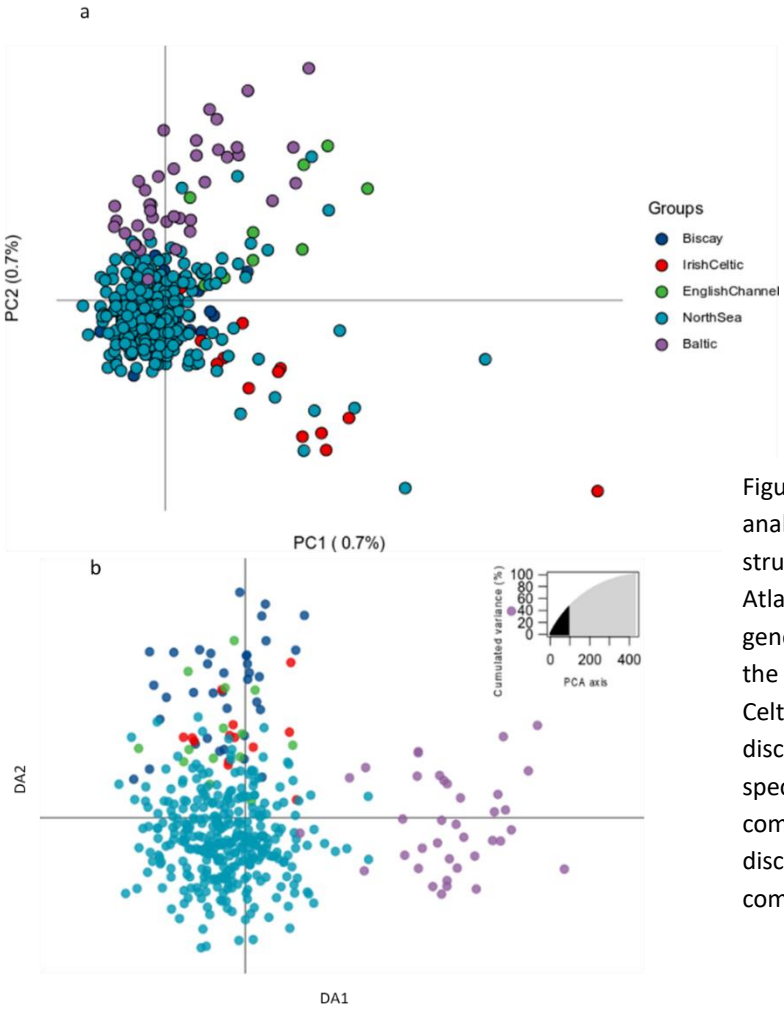


Figure 3.5: Individual-based analyses of genetic population structure of sole in the Northeast Atlantic Ocean based on neutral genetic markers. Individuals from the Baltic Sea, English Channel, Celtic Sea, and Bay of Biscay can be discriminated from North Sea specimens in (a) principal component analysis (PCA) and (b) discriminant analysis of principal components (DAPC).

Table 3.2: Pairwise F_{ST} values of the neutral (above the diagonal) and outlier loci (below the diagonal) in juvenile (J, light green cell color) and adult (A, dark blue cell color) populations, ordered from West to East (see Table 3.1 for abbreviations). Values in bold are significant.

		BISa07	CELa08	WCHa09	B08j14	B12j13	B03j14	B03j13	B07j13	B02j13	B06j13	B04j13	B01j14	B10j13	B05j13	THAa07	GBRj16	NORa08	NL1j14	SKAa07
		A	A	A	J	J	J	J	J	J	J	J	J	J	J	A	J	A	J	A
BISa07	A		**0.005**	**0.007**	**0.004**	**0.007**	**0.008**	**0.006**	**0.011**	**0.004**	**0.009**	**0.006**	**0.006**	**0.005**	**0.010**	**0.009**	**0.005**	**0.005**	**0.005**	**0.013**
CELa08	A	0.06		**0.008**	0.002	0.003	**0.007**	**0.004**	**0.005**	**0.003**	**0.005**	**0.003**	**0.004**	**0.003**	**0.007**	**0.006**	**0.004**	**0.005**	**0.004**	**0.011**
WCHa09	A	0.01	0.04		**0.005**	**0.006**	**0.006**	**0.008**	**0.007**	**0.005**	**0.006**	**0.005**	**0.007**	**0.005**	**0.007**	**0.004**	**0.006**	**0.006**	**0.008**	**0.016**
B08j14	J	**0.07**	0.00	**0.07**		0.000	**0.003**	0.001	**0.003**	0.001	0.000	0.001	**0.002**	0.001	0.001	**0.003**	0.001	0.001	0.001	**0.010**
B12j13	J	**0.07**	0.05	0.06	0.04		0.000	-0.001	-0.002	0.001	0.000	0.000	**0.002**	-0.002	0.002	**0.005**	0.001	0.000	-0.001	**0.009**
B03j14	J	**0.05**	0.08	0.05	0.05	0.00		**0.003**	**0.003**	**0.003**	**0.004**	**0.003**	**0.004**	**0.004**	**0.003**	**0.004**	0.002	**0.004**	**0.004**	**0.011**
B03j13	J	**0.07**	0.06	**0.06**	0.05	-0.01	0.00		0.002	0.001	0.000	0.001	0.001	**0.002**	0.000	**0.004**	0.001	**0.002**	**0.002**	**0.008**
B07j13	J	**0.06**	0.02	**0.07**	0.00	0.02	0.04	0.04		**0.003**	0.001	**0.004**	**0.005**	0.002	0.004	**0.005**	0.001	**0.005**	0.001	**0.013**
B02j13	J	**0.05**	0.02	**0.05**	-0.01	0.04	0.04	0.04	0.01		**0.003**	**0.003**	0.001	0.000	0.003	0.002	0.002	0.002	0.001	**0.009**
B06j13	J	**0.06**	0.05	**0.06**	-0.01	0.04	0.03	0.04	0.00	0.00		0.001	**0.004**	**0.003**	0.003	**0.006**	0.001	0.002	0.002	**0.011**
B04j13	J	**0.07**	0.02	0.06	0.02	0.01	0.01	0.01	0.01	**0.02**	0.01		**0.002**	0.001	0.003	**0.003**	0.001	0.000	0.001	**0.009**
B01j14	J	0.06	0.02	0.05	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.00		0.002	0.003	**0.004**	**0.003**	0.002	0.001	**0.010**
B10j13	J	0.07	0.04	0.07	0.02	0.01	0.03	0.02	0.01	**0.02**	0.01	0.01	0.00		0.003	**0.004**	0.001	0.002	0.001	**0.009**
B05j13	J	**0.06**	0.08	**0.07**	0.00	0.05	0.05	0.05	0.00	0.00	0.00	0.01	0.01	0.01		**0.006**	0.002	**0.004**	**0.005**	**0.009**
THAa07	A	0.04	0.04	0.04	0.01	0.00	0.01	0.00	0.01	0.01	0.00	0.00	0.00	-0.01	0.00		0.003	**0.004**	**0.003**	**0.012**
GBRj16	J	**0.03**	0.05	0.01	0.03	0.04	0.04	0.03	0.03	0.02	0.02	0.02	0.02	0.04	0.03	0.02		0.001	0.001	**0.010**
NORa08	A	**0.05**	0.05	0.04	0.05	0.00	0.01	0.00	0.04	0.04	0.04	0.01	0.01	0.02	0.05	0.00	0.02		0.002	**0.009**
NL1j14	J	**0.05**	0.03	0.04	0.00	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.01		**0.010**
SKAa07	A	**0.06**	0.05	0.03	**0.05**	0.04	**0.04**	**0.04**	0.05	**0.05**	**0.05**	**0.03**	0.02	0.04	**0.06**	0.04	**0.02**	**0.03**	0.02	

After scanning the genome for loci putatively under selection (n = 34), the outlier SNPs were used to investigate small scale patterns of potential adaptive population structure. Pairwise F_{ST} comparisons with this data were characterized by larger genetic differentiation with values up to 0.07. Fewer comparisons (30 of 176) than in the neutral data set were statistically significant ($p < 0.05$), of which 10 and 13 pairs involved samples from the Baltic Transition Zone and the Bay of Biscay, respectively (Table 3.2). The differentiation of Baltic fish was somewhat less pronounced in the PCoA visualization of outlier loci. Samples from the Bay of Biscay (BISa07) and the Western English Channel (WCHa09) grouped together, clearly separated from the other samples. The Celtic samples (CELa08) on the other hand were more divergent along the second axis and more distinct than in the neutral data set (Fig. 3.3b and 4b). Individual-based analyses (PCA and DAPC) of outlier loci showed no clustering that corresponded with to geographical structure (Suppl. Fig. 3.1a and b).

The PCoA plot of genotypes showed that populations from a wider geographical area (adult populations) were more differentiated than central North Sea populations (juvenile populations) for neutral markers (Fig. 3.3a), unlike the markers putatively under selection (Fig. 3.3b). The pairwise F_{ST} values of juvenile and adult populations (Table 3.2) supported this pattern. Pairwise F_{ST} comparisons based on neutral markers where more significant between adult populations than between juvenile populations. More comparisons between juvenile and adult populations were significant based on neutral than on outlier markers. The same holds true for comparisons between juvenile populations. More interestingly, outlier loci highlighted significant genetic differentiation between juvenile populations of the Belgian coast and adult populations of the Bay of Biscay, the western English Channel, and the Baltic transition zone, whereas pairwise F_{ST} values of adult populations were in most cases insignificant. In addition, none of the pairwise F_{ST} comparisons of the adult population of the Thames to other adult populations were significant based on outlier loci, whereas most pairwise F_{ST} comparisons of North Sea juveniles to adult populations were significant and of a higher F_{ST} value (> 0.04). Finally, the accuracy of re-assignment of our data was assessed based on adult populations using GENECLASS2. Self-assignment of adult populations revealed a very low discrimination power. Re-assignment success was maximal for the Biscay population (success of 17.6%), while none of the other adult populations were able to re-assign any adult correctly (success of 0%, results not shown). Due to the low re-assignment success, the assignment of juveniles to adult populations couldn't be trusted and was therefore not presented here.

Three pairs of juveniles showed a pairwise relatedness between 0.96 and 0.98. Two pairs were sampled on the same date and site (site B06j13 on 09/09/2013 for fish number 9996 vs. 9997 and site B04j13 on 19/09/2013 for fish number 9295 vs. 9309), and one pair was sampled on a different date and site (site B12j13 on 24/09/2013 for fish number 1128 vs. site B07j13 on 13/09/2013 for fish number 6931, results not shown).

4. Discussion

Sole populations of the Northeast Atlantic Ocean show high levels of gene flow and connectivity. Our results confirm the large scale spatial population structure in the Northeast Atlantic Ocean, North Sea and Baltic Transition Zone (Cuveliers et al. 2012, Diopere et al. 2018) and add a new perspective on the small-scale distribution of young life-stages. Large scale genetic differentiation is proportional to geographical distance (isolation by distance). Gene flow is extensive in the Southern North Sea with evidence for clustering of related individuals. There are additional indications that adaptive processes shape the population pattern of sole. Markers putatively under selection linked to the brain and sensing/immunology highlight better differences than neutral markers, especially between juvenile and adult populations.

Challenges in the population genomics of non-model species

SNPs scored with Reduced Representation Sequencing such as RADseq are of particular interest for non-model species as it allows for the identification of adaptive processes without specifying loci of interest *a priori* (bottom-up approach). Notwithstanding, our ability to identify selectively important traits is constrained by the limited knowledge of gene function in fish in general (Tiffin & Ross-Ibarra 2014). An alternative strategy is to specifically target genomic regions with a larger likelihood of being under selection or linked to genes (top-down approach). Diopere et al. (2018) detected stronger signals of local adaptation than in the present study, which might be attributed to SNP loci sourced from a muscle transcriptome of sole (Nielsen et al. 2012). Outlier loci have been linked to growth related genes and skeletal muscle (Diopere et al. 2018). However, the present study identified 34 outlier loci that were not picked up by transcriptome derived markers. Therefore both approaches seem to reveal complementary aspects of

adaptation in the sole genome. Also, Diopere et al. (2018) detected a higher ratio of outlier loci compared to the total number of loci than our results (19/426 or 4.5% vs. 34/1,412 or 2.4%, respectively). The bottom-up approach chosen in this study may underestimate processes compared to a top-down approach (Ross-Ibarra et al. 2007). Nevertheless, some processes linked to non-coding regions of the genome, such as regulating gene expression, may be key to local adaptation and therefore important. In addition, Reduced Representation Sequencing only reveals an estimated 1% of the genome. If one wants to understand the full adaptive scope of a genome, approaches would include Quantitative Trait Locus (QTL) mapping and genome-wide association studies (GWAS, Vasemägi & Primmer 2005). However, QTL is mostly applied on model species, such as mice (Flint & Eskin 2012) and Atlantic salmon (Sodeland et al. 2013), while GWAS depends on phenotyping (Brennan et al. 2018). Regardless of the sequencing approach used to identify candidate loci potentially under selection, independent validation of functional importance of these loci is key to change the status of genes from candidates to causative (Tiffin & Ross-Ibarra 2014).

Neutral and adaptive patterns from large- to small-scales

Our study makes use of the largest SNP panel for sole so far. It supports previously identified subpopulations and confirms four major clusters as proposed by Diopere et al. (2018). Differentiation between each cluster is subtle but biologically meaningful. First, the strongest genetic differentiation was found between the Baltic Transition Zone and the other regions. Sole populations live in a transition zone along a salinity gradient, which may have influenced local adaptation (Nielsen et al. 2004, Limborg et al. 2012). The Baltic transition zone is geographically and hydrodynamically isolated (Johannesson & André 2006) and represents an edge population. In our study, heterozygosity levels observed in the Baltic Transition Zone were higher than in the North Sea which conflicts with low expectations of genetic diversity in edge populations (Eckert et al. 2008) and the low spawning stock biomass compared to the North Sea (ICES 2018). However, high genetic diversity may characterize the range edge (Assis et al. 2013). Second, our results confirmed that the Celtic Sea sole population is genetically differentiated from the North Sea (Diopere et al. 2018). Spawning sites in the Celtic and Irish Sea may be relatively isolated geographically and hydrographically as observed in dab *Limanda limanda* (Fox et al. 2000, Tysklind et al. 2013). Tidal coastal flow in the Celtic and Irish Sea has been implicated in isolating

populations in the Celtic Sea by favoring dispersal in a western direction (Coscia et al. 2013) and retaining flatfish juveniles on the local nursery grounds (Fox et al. 2006). Third, the genetic profile of the Western English Channel was transitional with evidence of admixture between the Bay of Biscay and the North Sea. The English Channel represents a physical boundary between the southern (warm Lusitanian) and the northern (cold Boreal) biogeographical provinces (Ayata et al. 2010). Finally, the Bay of Biscay is the most southern population of this study and temperature might be the most important factor driving structure between the Bay of Biscay and the other populations as detailed in the seascape analysis of Diopere et al. (2018).

The clusters described above are positoned along a latitudinal gradient, which has been linked to minimum winter temperature (Rijnsdorp et al. 1992, Diopere et al. 2018) and does not represent a signature of recolonization from refugia after the Last Glacial Maximum (Maggs et al. 2008, Diopere et al. 2018). The latitudinal gradient in temperature influences life-history traits such as initiation of spawning (Fonds 1979, Rijnsdorp & Vingerhoed 1994), peak spawning (Fincham et al. 2013, Rijnsdorp et al. 1992) and the duration of larval development (Fonds 1979). Temperature differences may be reinforced by climate change as environmental impacts differ regionally (Lacroix et al. 2018). In this study, we identified 34 outlier loci based on the reference genome of the Senegalese sole. The function of most outlier loci related to brain and sensing/immunology. We highlight some of their characteristics below. APOA-I is linked to omega 3 fatty acid assimilation (Pan et al. 2004, Pizzini et al. 2017). PLEKH5 (pleckstrin homology domain containing, family A member 5) is involved in brain development in humans (De Toledo et al. 2001). BSX (brain-specific homeobox) is an essential factor for neuronal neuropeptide Y and agouti-related peptide function and locomotor behavior in the control of energy balance in humans (www.uniprot.org). TRIM8b (tripartite motif containing 8b) is involved in the immune system and shows elevated protein turnover in thermally stressed rainbow trout (Verleih et al 2015). NR1H4 (nuclear receptor subfamily 1 group H member 4) is involved in nutrient sensing (Preidis et al. 2017). CYP1B1 is one of the 18 families of Cytochrome P450 and is involved in detoxification (Uno et al. 2012).

Isolation by distance (IBD) has been found in sole based on allozyme markers (Kotoulas et al. 1995), mitochondrial markers (Cuveliers et al. 2012) and nuclear markers (Cuveliers et al. 2012, Diopere et al. 2018). Hence, the population structure of sole combines the impact of environmental and physical distances between populations (Diopere et al. 2018). Seascape

analysis can be particularly useful to point at the drivers of the spatial genetic patterns at a large spatial scale (see Selkoe et al. 2016 for a review). A seascape analysis on sole of the Northeast Atlantic Ocean identified winter seawater temperature, food availability (measured as chlorophyll water concentrations) and coastal currents as the main drivers of geographical distribution of genetic diversity (Diopere et al. 2018). However, understanding small-scale patterns might benefit from a redundancy analysis because in such case not only subtle genetic structure is taken into account but also the environmental factors. Environmental factors such as a lower salinity or the presence of a front can be determining for small-scale connectivity, especially for early-life stages, as has been shown for other fish species including flatfish (Barbut et al. 2019, Lamichhaney et al. 2017, Vandamme et al. 2014). Identifying the effect of subtle environmental gradients remains challenging, especially because sole lacks the genomic resources of a model species.

Juvenile versus adult genetic differentiation and cohort dynamics

Spatial genetic structure may vary between life stages. In the Northeast Atlantic Ocean, most genetic differentiation was found between adult populations as shown by the pairwise F_{ST} comparisons and former studies (Cuveliers et al. 2012, Diopere et al. 2018). Most studies on flatfish focus on adult population structure (e.g. Cuveliers et al. 2012, Diopere et al. 2018, Exadactylos et al. 1998, Vandamme et al. 2014) or on juveniles (e.g. Guinand et al. 2011, 2013), with some exceptions studying juveniles and adults (Guinand et al. 2008, Hoarau et al. 2002, Rolland et al. 2007). Nevertheless, the few studies comparing juvenile and adult sole used allozymes or microsatellites, which are less powerful than large SNP datasets derived from RADseq and developed to detect subtle genetic differentiation across the genome (see Bernatchez 2016 for a review). On the one hand, our results support previous studies on the North Atlantic scale as subtle differences in population structure were observed. On the other hand, at the North Sea scale, outlier loci highlight a clear differentiation between juveniles and adults. The differentiation between juveniles and adults could be strengthened and confirmed by increasing the overlap in sampling sites and timing between the juvenile and adult samples and the aging of the adults. Cohort structure of the adults is confounded while juveniles belong to a few well documented cohorts. Genetic studies in the Bay of Biscay and the English Channel suggest that selection is acting on sub-adults but not on age-0 and 1 sole (Guinand et al. 2008,

2011, Rolland et al. 2007). This could be a specificly linked to regional features. In the Bay of Biscay and English Channel, access to benthic habitat, the main bottleneck constraining recruitment, might explain higher selective pressure on sub-adults than on juveniles (Le Pape et al. 2003). In the North Sea, however, recruitment is determined during the first year of life (Rijnsdorp et al. 1992). Processes influencing juvenile population structure, such as larval behavior, hydrodynamic conditions, nycthemeral migration and selective tidal transport (Creutzberg et al. 1978, Fox et al. 2009, Koutsikopoulos et al. 1991) vary locally. For example, sole inhabiting the English Channel show little connectivity between the English coasts, the Western and Eastern French coasts (Rochette et al. 2012) whereas sole of the North Sea seem to be well connected between the Belgian coast, UK coast and the Wadden Sea according to our results. Genetic differentiation might be more pronounced between cohorts due to the variable selection pressure and mortality rates of eggs and larvae (type III survival curve, Gibson 2015, Houde 1997). In addition, adult fish may be more mobile and undertake directed migrations, e.g., to specific spawning areas (Jansen et al. 2013). This may lead to the mixing up of populations. Moreover, few adult individuals contribute to the next generation, because of sweepstake effects (Hedgecock & Pudovkin 2011). The skewed reproductive rate in conjunction with drastic mortality rates of planktonic eggs and larvae is likely reflected in patterns of selection and local adaptation. The relatedness of two juveniles sampled a few days apart could support sweepstake effects and non-random mating but this result needs to be confirmed with more samples. Kin structure reveals another dimension of population structure and qualifies some of the genetic homogeneity found in sole and many other flatfish species at the scale of the Northeastern Atlantic Ocean (Cuveliers et al. 2012, Diopere et al. 2018, Hoarau et al. 2002, Rolland et al. 2007). Genetic homogeneity might be a direct consequence of the mixing of eggs at the spawning grounds as observed by Koutsikopoulos et al. (1995) or the mixed supply of spawning grounds to several nursery grounds as suggested by microchemistry studies (Delerue-Ricard et al. *in review*). Despite a high genetic homogeneity, this study might be able to detect relatedness using a high number of polymorphic loci compared to previous studies.

Management implications

Spatially variable exploitation rates may severely impact local populations (Allendorf et al. 2008, Fogarty et al. 1991). The exact match between biological populations and management units is of primary importance to ensure efficient conservation measures. It is advisable that management and conservation measures incorporate evolutionary information of spatial structure and its interconnections even in a single species assessment context (Ovenden et al. 2015, Barbosa et al. 2018). Accordingly, connectivity may be more important than habitat quality for the effective design of MPA (Berglund et al. 2012, OSPAR 2013).

The actual boundaries of management units have been mostly defined according to management concerns, such as geo-political boundaries and harvesting location, and to a lesser extent represent scientific judgement (Waples & Gaggiotti 2006, Reiss et al. 2009). Fisheries management assessments, however, examine sole living in the Northeast Atlantic Ocean in ten stocks, (ICES 2018, Fig. I.10). Our study suggests four key genetic clusters in the Northeast Atlantic Ocean. Hence the management units are more finely structured than the evolutionary population units. That type of mismatch between management and evolutionary population units is of a lesser concern than when complexity is underestimated (Reiss et al. 2009, Kerr et al. 2017). However, sole stocks have been historically under strong selective pressure due to high exploitation rates (Lescrauwaet et al. 2013). This has led to measurable impact on the phenotype and life history traits (Mollet et al. 2013, Fincham et al. 2013). Although fishing mortality has been decreasing in the past few years in some stocks, regional differences remain and many sole stocks are fished at unsustainable levels (ICES 2018). Our results tend to support that sole should be managed in larger units for evolutionary reasons than what is actually implemented by ICES. Nonetheless, tradeoffs to define management units have to be carefully evaluated to meet both economic and ecological standards (Pilling et al. 2018).

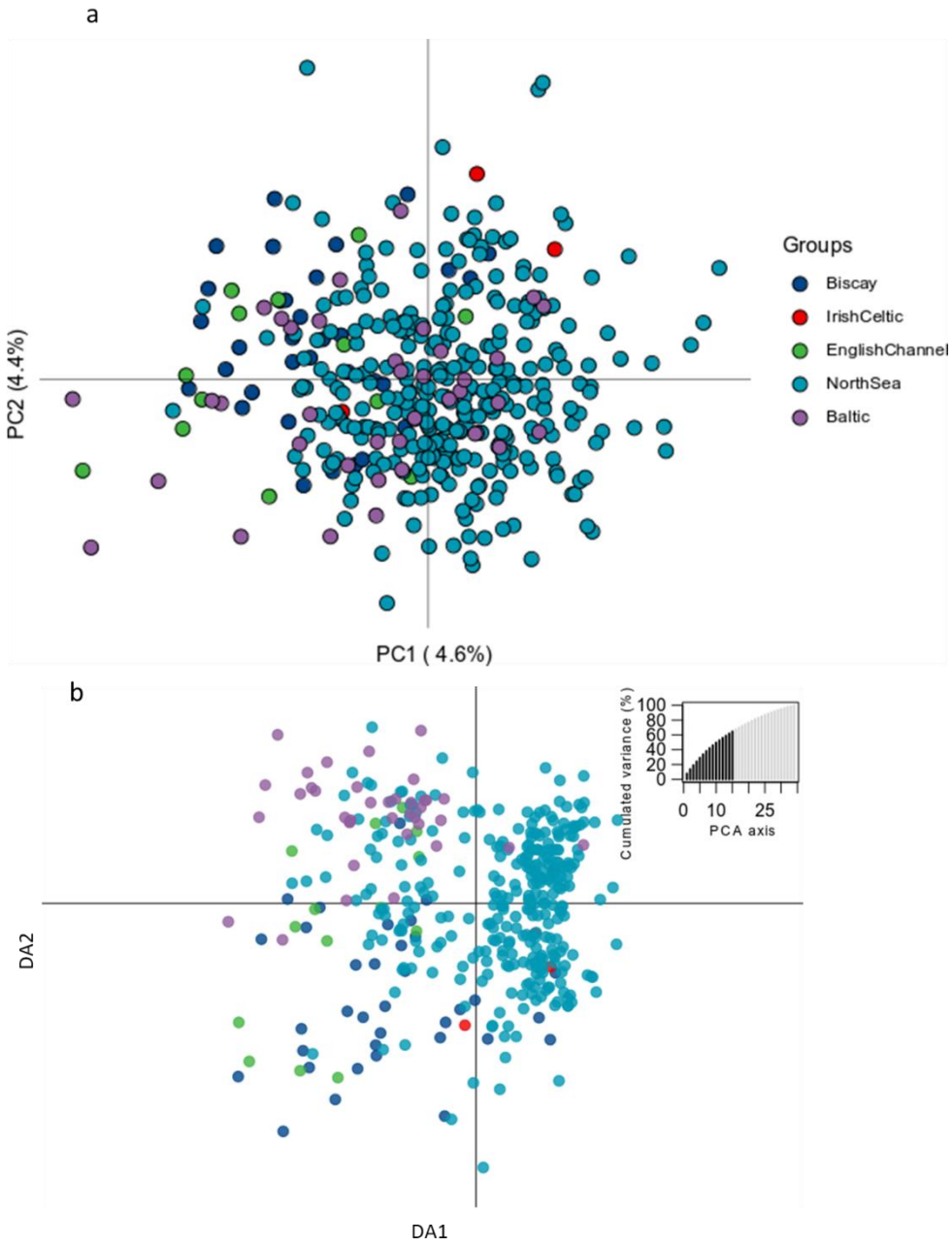
In conclusion, we confirmed and provided further evidence for subtle, but biologically relevant genetic structure in sole of the Northeast Atlantic Ocean. A combination of neutral and adaptive processes shaped sole population structure at a small spatial scale differently in adults compared to juveniles. Overall, a panel of 1,489 SNPs detected four genetic clusters organized in a pattern of isolation by distance, while at the scale of tens of kilometers gene flow is counteracting selection and genetic drift. Combining genetic markers with other genetic and

phenotypic markers that integrate information on the ecological time-scale are likely to enhance discrimination between subpopulations.

Acknowledgements

Special thanks to K. Vanhalst and A. Vanden Bavière (Institute for Agricultural and Fisheries Research, ILVO), the crew of RV *Simon Stevin,* RV *Stern,* RV *Cefas Endeavour* and RV *Belgica*, L. Bolle (Wageningen Marine Research, NL), J. Smith (Cefas, UK) and the B-FishConnect team for sampling. We are grateful to P. Hablützel (Flanders Marine Institute, VLIZ) for constructive comments. Research was made possible by the B-FishConnect research project (G.0702.13N) and the Scientific Research Network 'Eco-evolutionary dynamics in natural and anthropogenic communities' (grant W0.037.10N), both funded by the Research Fund - Flanders (Belgium).

Supplementary material



Supplementary Figure 3.1: Individual-based analyses of genetic population structure of common sole (*Solea solea*) in the Northeast Atlantic Ocean based on outlier loci. Individuals from the North Sea, Baltic Sea, English Channel, Celtic Sea and Bay of Biscay are differentiated by color in (a) principal component analysis (PCA) and (b) discriminant analysis of principal components (DAPC).

Supplementary Table 3.1: Thirty-four outlier loci identified in sole, populations in the Northeast Atlantic and North Sea, that are putatively under selection and could be linked to genes with a function.

Locus ID	Gene	Molecular function
SNP548_26650	cell wall integrity and stress response component 3-like	Immune system
SNP637_17347	Unknown	
SNP987_61121	FH1/FH2 domain-containing protein 3-like	Cytoskeleton
SNP1243_50137	tripartite motif containing 8	Immune system
SNP1425_15774	disks large homolog 2	transmission of neuronal signals
SNP1657_54859	DLG associated protein 5	transmission of neuronal signals
SNP2483_92212	junctional cadherin complex regulator, brain specific homeobox	
SNP2483_92272	junctional cadherin complex regulator, brain specific homeobox	Pineal development
SNP3101_19478	Unknown	
SNP3103_53070	zinc finger RANBP2-type containing 3	Endocrine, Food control, metabolic rate and immune function
SNP3141_50920	homer scaffolding protein 1	Olfaction
SNP3266_7259	ATPase phospholipid transporting 10A	
SNP3330_16790	nucleophosmin-like	myeloid cell numbers
SNP3546_48923	Unknown	Immune system
SNP5967_8914	pleckstrin homology domain-containing family A member 5-like	Brain development
SNP7548_17404	Unknown	
SNP8995_55124	IQ motif and SEC7 domain-containing protein 2	
SNP9140_53057	Unknown	
SNP9372_162120	Unknown	
SNP9640_37977	Unknown	
SNP9701_8269	voltage-dependent T-type calcium channel subunit alpha-1I-like	transmission of neuronal signals
SNP9734_21162	Unknown	

SNP9803_7259	lymphoid enhancer binding factor 1	Immune system
SNP10620_4869	Unknown	
SNP10643_18529	dnaJ homolog subfamily A member 3, mitochondrial-like	
SNP13044_121567	nuclear receptor subfamily 1 group H member 4	Nutrient-sensing
SNP13079_30743	leucine-rich repeat-containing protein 30-like	--
SNP17651_77986	Ribonucleoside-diphosphate reductase large subunit	nucleotide metabolism
SNP17919_143818	sodium-dependent phosphate transport protein 2A-like	phosphate homeostasis and mineralization
SNP21995_1085	apoA-I gene for apolipoprotein A-I	
SNP54285_1406	Unknown	
SNP80698_2077	cytochrome P450 1B1-like	Immune system
SNP122682_341	Unknown	
SNP245772_479	attractin (atrn)	Protection against UV irradiation

GENERAL DISCUSSION AND PERSPECTIVES

Establishing the degree to which the local populations of a metapopulation are connected is fundamental to understand ecological and evolutionary processes (Botsford et al. 2001). Coastal ecosystems play a key role as nursery grounds for juvenile fish (Costanza et al. 1998) because of their high productivity. However, connectivity of natural populations is increasingly disturbed by habitat loss and fragmentation, especially at coastal nursery grounds under high anthropogenic pressures (Jung et al. 2017, Mineur et al. 2012, OSPAR 2000). Dispersal controls the reorganization of ecological networks, especially under changing environmental conditions (Thompson & Gonzalez 2017, *section D.4.2* of the **General Discussion**). In addition, the spatial heterogeneity of environmental pressures may be reinforced by climate change as environmental impacts differ regionally (Lacroix et al. 2018, *section D.4.3*). All this puts populations at great risk of losing resilience due to the reduced genetic diversity (Pinsky et al. 2013, Ruzzante et al. 2006). In order to adapt management strategies to preserve genetic and ecological (bio)diversity, knowledge on the connectivity and dispersal patterns of early-life stages between spawning and nursery grounds of fish is essential (Hemmer-Hansen et al. 2014, OSPAR 2013).

I will first address the five key hypotheses on the connectivity patterns of sole that are tested in this PhD thesis (*section D.1*). Secondly, I will present new evidence and discuss whether a multi-disciplinary approach to connectivity may improve knowledge on the population dynamics of sole at the scale of ecological dispersal (*sections D.2*). Thirdly, I will summarize the major findings of my PhD thesis on the larval and juvenile connectivity of sole in regards to the state-of-the-art (*section D.3*). Finally, the management implications of our research and suggestions of perspectives for future research on connectivity are formulated (*section D.4*).

1. Connectivity of sole in the Northeast Atlantic Ocean

Sole provides an excellent model for studying population connectivity in the Northeast Atlantic Ocean. First of all we can count on extensive background knowledge on its biology and environment (Cuveliers et al. 2012, Robledo et al. 2017). Secondly, sole is a key player in the ecosystem. Thirdly, intensive bottom trawling in the North Sea has modified the habitat and the exerted a high selection pressure (Lescrauwaet et al. 2013, Mollet et al. 2013, Diopere 2014). Fourthly, the large rivers entering the North Sea with their natural and pollution based chemical signatures represent an interesting case for elemental analysis (Emeis et al. 2015). Yet, empirical evidence of connectivity between spawning and nursery grounds at the spatial scale of dispersal (i.e. in the order of magnitude of 100 km) is scarce. The general aim of the PhD thesis was to account for the local dynamics involved in the connectivity between spawning and nursery grounds and on the nursery grounds. We examined early-life stages of sole at the "large" scale of the Northeast Atlantic Ocean to address the population structure "context" and at the "small" regional scale of the Southern North Sea to address local dynamics at the scale of ecological dispersal.

This PhD thesis investigated several approaches to measure ecological and evolutionary connectivity. Initially, we assessed the power of natural tags such as otolith shape (**Chapter 1**) and microchemistry (**Chapter 2**) to reveal connectivity in juvenile sole on a small-scale between and within nursery grounds. Otolith shape was also used to detect the signal of asymmetry in juveniles as differences between left and right otoliths have implications for the detection of stocks (**Chapter 1**). Elemental analysis of the otoliths was used to trace pre- and post-settlement dispersal and estimate the number of potential spawning ground as sources of the nursery grounds in the Southern North Sea and Wadden Sea (**Chapter 2**). In **Chapter 3**, connectivity patterns were compared at the scale of the Northeast Atlantic Ocean and the North Sea by using neutral and highly differentiated (outlier) genomic markers. Finally, otolith shape, otolith elemental analysis and genomic information of connectivity were integrated at the individual level using fused networks to find evidence of local connectivity (*section D.2.3* of the **General Discussion**). Based on the results of the aforementioned chapters, summarized in Figure D.3 and D.4, we propose the following hypotheses in relation to the dispersal and connectivity of sole.

Hypothesis 1: The population structure of adult and juvenile sole is subtle

There are four major genetic clusters at the largest spatial scale, namely the Bay of Biscay, Western English Channel and North Sea, Celtic Sea, and the Baltic Transition Zone (**Chapter 3**). Our SNP set supported previously identified populations (Diopere et al. 2018). The clusters are located along a latitudinal gradient which might be linked to an environmental gradient (such as winter temperature). It is less likely that it represents a signature of recolonization from refugia after the Last Glacial Maximum (Maggs et al. 2008). The latitudinal temperature gradient leads to differences in life-history traits such as initiation of spawning (Fonds 1979) and peak spawning (Fincham et al. 2013, Rijnsdorp et al. 1992). The strongest genetic differentiation was found between the Baltic Transition Zone and the more southern populations. The Skagerrak and Kattegat populations were located in a transition zone along a salinity gradient which may have influenced local adaptation (Limborg et al. 2012). The transition zone is geographically and hydrodynamically isolated (Johannesson & André 2006) and represents an edge population with reduced genetic diversity (Eckert et al. 2008). However, there is recent evidence for high genetic diversity at the edge range (Assis et al. 2013). In our study, heterozygosity levels observed in the transition zone were higher than in the North Sea, possibly pointing to the regular arrival of migrants from various source populations. The transition sole population has a low census population size compared to the North Sea and experiences a high mortality (ICES 2018). The Celtic Sea sole population is genetically well differentiated from the North Sea populations (Diopere et al. 2018). Celtic and Irish Sea populations may be isolated through discrete spawning sites (Fox et al. 2000) as observed in dab *Limanda limanda* and turbot (Tysklind et al. 2013, Vandamme et al. 2014). Tidal coastal flows in the Celtic and Irish Sea favor dispersal in a western direction (Coscia et al. 2013) and retention of flatfish juveniles on local nursery grounds (Fox et al. 2006). The genetic profile of the third cluster, the Western English Channel was also picked up by Diopere et al. (2018): a transitional group with evidence of admixture between the Bay of Biscay and the North Sea. The English Channel represents a physical boundary between the warm southern (Lusitanian) and cold northern (Boreal) biogeographical provinces (Ayata et al. 2010). Finally, the Bay of Biscay is the southernmost population of the Northeast Atlantic. Throughout the range, isolation by distance has been evidenced for sole based on allozymes (Kotoulas et al. 1995), mitochondrial markers (Cuveliers et al. 2012) and nuclear markers (Diopere et al. 2018).

Against our expectations, the neutral genetic diversity of adult and juvenile sole was similar (**Chapter 3**). This could be due to the unbalanced sampling design: we are missing juveniles from the locations where we sampled adults and vice-versa. Therefore, our results on the comparison between juvenile and adult gene pool has to be interpreted with caution. Yet, the absence of differences between the juvenile and adult gene pool could be due to mixing of spawning aggregations as supported by observations of sole eggs mixing on the spawning grounds in the Bay of Biscay (Koutsikopoulos et al. 1995). Similar data would be valuable for the North Sea. In addition, natal philopatry might also influence greatly the level of genetic structure, leading to more stable genetic structure and a lower gene flow. However, despite tagging evidence for spawning area fidelity (i.e. philopatry) of plaice (Darnaude & Hunter 2018, Hunter et al. 2003), there is no evidence of philopatry for sole sole yet. Moreover, philopatry is not a proof for well-structured populations as plaice indicates quite some panmixia which persists on the spawning grounds.

Outlier genetic markers showed small-scale spatial structuring and an order of magnitude higher genetic differentiation within the Belgian nursery compared to neutral markers (**Chapter 3**, *section D.2.3*). Genetic differentiation might be more pronounced in early-life than in adults due to the high selection pressure linked to the high mortality rate of eggs, larvae and juveniles (type III survival curve, Gibson 2015, Houde 1997). In addition, adult fish may be more mobile and undertake directed migrations, e.g., to reach specific spawning areas (Jansen et al. 2013). Moreover, few adult individuals contribute to the next generation, because of sweepstake effects (Hedgecock & Pudovkin 2011). This skewed reproduction rate in conjunction with drastic mortality rates of eggs and planktonic larvae is likely reflected in patterns of selection and local adaptation. The high relatedness of two juveniles sampled a few days apart could support sweepstake effects and non-random mating.

Hypothesis 2: Self-recruitment is a common feature of sole

Unique to previous studies on sole, we explored dispersal during the early life cycle and compared pre- and post-settlement connectivity. Contrary to the traditional view, marine populations may not be as "open" as previously thought. Despite the potential for long distance dispersal, our results show that local recruitment is common for sole in the North Sea (within a 100 km scale, **Chapter 2**). Self-recruitment is common among marine organisms (Swearer et al. 2002, Christie et al. 2010). In **Chapter 2**, most juvenile sole remained on the nursery ground after settlement as is the case for most flatfish species (Le Pape & Cognez 2016). Our results are consistent with a tagging study of sole which shows a high level of site fidelity as juvenile sole recruited mainly to the adult population of the same area (Burt & Millner 2008).

Although most individuals recruited locally, our results revealed differences in microchemical profile between individuals sampled within the same region (**Chapter 2**). This could be the result of putative juvenile migrants that recently entered a new region or due to the temporal variabililty in the microchemical signal. Temporal stability of the otolith elemental signature influences the reconstruction of individual migration histories. In marine fish, temporal variability in the microchemical signal is driven by seasonal or inter-annual variation (Tanner et al. 2012). Although most studies found a significant effect of season or year on the elemental signal, temporal variation did not hinder spatial discriminatory power, as confirmed by several studies (Cuveliers et al. 2010, Reis-Santos et al. 2012, Tanner et al. 2012). Nevertheless, a better understanding of the temporal stability of the microchemical signal might benefit from experimental studies in controlled environments or through transplant experiment in the field. Knowledge on the incorporation of elements might help answering the question of the temporality of the signal. To mitigate ignorance, ideally the study design should integrate temporal replicates to assess the consistency of microchemical signals. Although temporal variability in the microchemical signal cannot be excluded, the observed differences in microchemical profile between individuals sampled within the same region (**Chapter 2**) could attest the occurrence of recent movements of juveniles between regions and support long distance connections (> 150 Km) at the larval stage revealed by the biophysical model of Lacroix et al. (2013).

Differences in coastal water composition among regions were especially strong for three elements: Mg, Mn and Zn. Temperature and growth rate appeared to be correlated with

variations in otolith Mg/Ca and Mn/Ca (Sturrock et al. 2015). Mn is gaining increasing popularity as an environmental marker (Limburg et al. 2015); it is especially interesting in our study because the Wadden Sea is enriched in dissolved and particulate Mn, which may point to hypoxic conditions (Dellwig et al. 2007, Limburg et al. 2015). While variations in Zn/Ca are associated to pollution levels in the environment (Baeyens 1998), they may also be associated to hormonal changes in female fish (Sturrock et al. 2015).

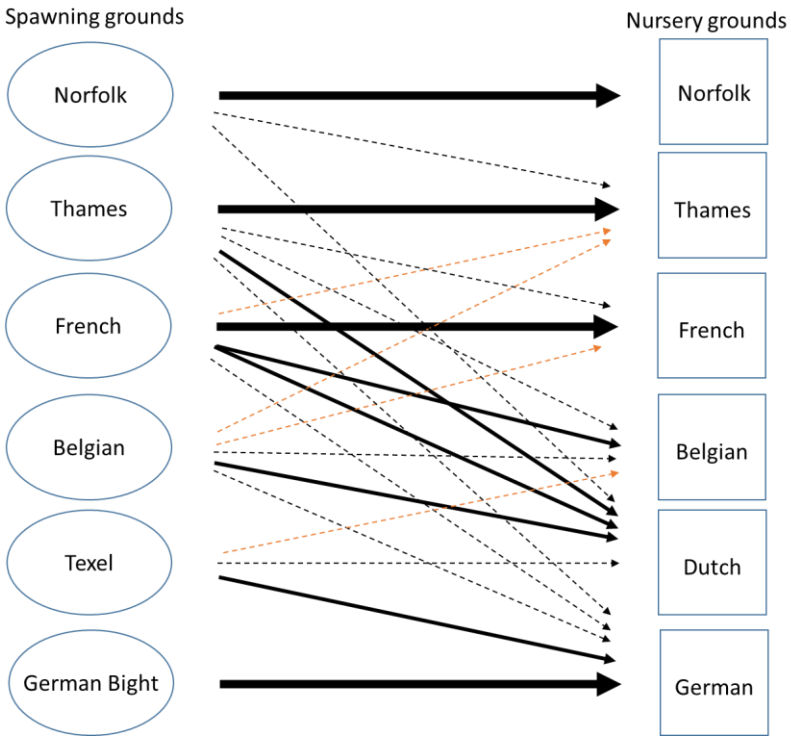


Figure D.3: Summary of the connectivity between spawning and nursery grounds as assessed by the Individual Based Model of Lacroix et al. (2013) and Barbut et al. (2019), simulated over a 12-year period (1995 – 2006). This figure contributes to address *Hypothesis 3*. The strength of the modeled connections is expressed by the thickness of the arrow. Black arrows point to dispersal along the residual current while orange arrows point to dispersal against the residual current.

Hypothesis 3: Several spawning grounds contribute to a single nursery ground of sole

For most marine fish, the pelagic phase before settlement represents a critical period in dispersal, shaping connectivity patterns between populations (Burgess et al. 2014, Di Franco et al. 2012). Thus, knowledge about species dispersal characteristics (e.g. number of natal origins, dispersal distances) is crucial for understanding connectivity and fish population dynamics. Otolith near-core chemistry has been used to infer potential number of natal origins (Di Franco et al. 2012, Calò et al. 2016, Wright et al. 2018). A significant challenge remains however to disentangle the signature of common origin from that of similar water chemistries or physiological overprinting (maternal effect or linked to ontogenetic changes, Brophy et al. 2003, Ruttenberg et al. 2005, Sturrock et al. 2012).

The otolith near-core elemental composition of sole suggest the contribution of several spawning grounds of sole to a nursery ground (**Chapter 2**). The finding is consistent with biophysical models that predicted input from several spawning grounds to most nursery grounds in the North Sea, except for the mostly self-recruiting UK nursery ground (Savina et al. 2010, Lacroix et al. 2013, Barbut et al. 2019). The level of connectivity between each nursery ground varies between years, although most nursery grounds are seeded by one main spawning ground (Lacroix et al. 2013, fig. D.3). As shown by Lacroix et al. (2013), local recruitment was the main source of larvae for the French (English Channel), Thames, Norfolk, German (Bight) nursery grounds and the Dutch (off Texel) nursery to a lower extent. The Belgian spawning ground was the main supplier of larvae to the Dutch nursery ground. The French spawning ground supplies the Belgian and Dutch nursery grounds, while the Dutch spawning ground contributes to the German nursery ground.

The spawning and nursery ground are tightly linked. Flatfish have optimized settlement success in two ways: Firstly, successful spawning ground locations have been selected over time (Symonds & Rogers 1995) and flatfish have a high fidelity to the spawning ground (Hunter et al. 2003). One of the most important requirements of a successful spawning ground is the presence of suitable hydrographic conditions to transport eggs and larvae to the nursery grounds (Symonds & Rogers 1995). After hatching, flatfish larvae are retained close to the spawning grounds hydrodynamically and disperse locally due to a combination of nycthemeral migration (i.e. day and night differences in migration behavior) and selective tidal transport (Cowen et al. 2000, Creutzberg et al. 1978, Fox et al. 2009, Rijnsdorp et al. 1985). Secondly, settlement can be

delayed up to three weeks (Marchand 1991 for sole) in response to terrestrial chemical cues and freshwater runoff (Dixson et al. 2011, Gerlach et al. 2007, Kerstan 1991). This increases the chances of a reproducible settlement pattern over time. Moreover, because settlement is influenced by spawning ground location, spawning ground fidelity might be favored as shown by tagging experiments in other flatfish (Hunter et al. 2003). Natal homing behavior (as observed in plaice, Darnaude & Hunter 2018, or cod, Bonanomi et al. 2016) means that the same individuals reproduce repeatedly at the same location, thus limiting exchange of adults from different gene pool between spawning grounds. Yet, natal homing has not been demonstrated in sole.

Our findings are consistent with an average modeled larval dispersal of about 150 km (Barbut et al. 2019, Lacroix et al. 2013). Field surveys reported comparable dispersal distances between spawning and nursery grounds (80-100 km, Dorel et al. 1991). Modeling of larval dispersal showed that connectivity between the different zones of the North Sea is considered moderate (Savina et al. 2010) but might be sufficiently high to explain genetic homogeneity within the North Sea (**Chapter 3**, Slatkin 1993). Moreover, according to the model of Lacroix et al. (2013), connectivity at the nursery grounds varies between years and is expected to be influenced by global change (Lacroix et al. 2018). Therefore, although most nursery grounds receive larvae from several spawning grounds, both studies from 2010 and 2013 suggest the presence of subpopulations in the Eastern English Channel, the Southern North Sea and the Wadden Sea.

We learn from the results of this PhD thesis that complementary approaches are needed to identify larval origin. For example, spawning time can be estimated from otolith microstructure and used to back-calculate larval origin (Amara et al. 1993). Chemical signatures of sole larvae drifting from the spawning grounds have yet to be explored and compared to juvenile otoliths to assess the contribution of each spawning grounds to the nursery.

Hypothesis 4: Juvenile sole constitute the progeny of a single genetically homogeneous spawning aggregation

Despite the limited empirical connectivity between nursery grounds (**Chapter 2**, see *Hypothesis 5*) and biophysical models suggesting juvenile subpopulations (Lacroix et al. 2013, Savina et al. 2010), the level of genetic differentiation of juvenile sole in the North Sea is low (**Chapter 3**). Just a few migrants per generation are required to maintain a high level of gene flow (Hauser & Carvalho 2008). Thus, the low level of successful recruitment of migrants observed in **Chapter 2** is compatible with the homogeneous population structure of sole in the North Sea as revealed by genetic markers (Cuveliers et al. 2012, Diopere et al. 2018, **Chapter 3**). The low genetic differentiation might be explained by limited but significant mixing between the nursery grounds at the juvenile stage, but also by adult gene flow or mixing of eggs on the spawning aggregations (Koutsikopoulos et al. 1995, see *Hypothesis 1*). Hoarau et al. (2002) suggested that spawning aggregations of plaice consist of sub-groups under a model of panmixia. Panmixia contrasts with the genetic differentiation that is expected as a result of sweepstake reproductive success (Hedgcock 1994). There is some evidence of egg mixing on the spawning aggregations for sole in the Bay of Biscay (Koutsikopoulos et al. 1995), but not yet for adults as shown in Atlantic mackerel *Scomber scombrus* (Jansen et al. 2013).

Nevertheless, outlier loci show some structure at the scale of the Belgian nursery when one zooms in on the small spatial scale (< 100 km) (*section D.2.3*). Selective pressure on age-0 sole on the nursery grounds is high due to high mortality rates (Houde 1997). The genetic differences observed between sampling events might be linked to cohort structure but our results need more spatial and temporal replicates before a firm conclusion is made. Guinand et al. (2008) did not observe genetic differentiation among juvenile sole (age-0 and -1) but among sub-adults. In the Bay of Biscay, access to benthic habitat is the main bottleneck to recruitment and might explain higher selective pressure on sub-adult rather than on juvenile stages (Le Pape et al. 2003). A bottleneck to recruitment in the North Sea is survival during the first year of life; it is partly linked to winter water temperature and access to nursery area (Rijnsdorp et al. 1992). Therefore selection pressure in the North Sea is stronger during the first year of life. Mismatch between egg and larvae production and favorable environmental conditions may lead to a low recruitment success (Cushing 1990) which would cause low local effective population size and favor genetic drift, leading to an increase in spatial genetic isolation observed among adults. This

might lead to chaotic genetic patchiness originating from one panmictic spawning aggregation, provided that a small proportion of adults successfully contribute to the next generation (as predicted by sweepstake effects, Hedgecock 1994). In years with a larger local effective population size, drift will be lower, and genetic differentiation among nurseries less obvious.

In addition, kin structure (i.e. settlement of related individuals in the same area) has been detected on the Belgian nursery with two individuals being more related to each other than the other juveniles (**Chapter 3**). If many individuals were locally related, the local inbreeding coefficient would increase and a Wahlund effect might be observed (Hoarau et al. 2005). In contrast, our results suggest relatedness between very few individuals. Additional intensive sampling at a small scale is required to better estimate 'temporal connectivity' in order to test the relatedness and chaotic genetic patchiness hypotheses.

The absence of strong differences between North Sea sole spawning aggregations might reflect a high level of gene flow as is the case for pelagic species such as herring *Clupea harengus* (Limborg et al. 2012, Lamichhaney et al. 2012) and Atlantic cod *Gadus morhua* (Nielsen et al. 2009b, Heath et al. 2014) and for other flatfishes such as plaice *Pleuronectes platessa* (Hoarau et al. 2002), turbot *Scophthalmus maximus* (Vandamme et al. 2014) and flounder *Platichthys flesus* (Hemmer-Hansen et al. 2007). A low level of population differentiation might result from the lack of a migration-drift equilibrium due to recent divergence of large populations (Nielsen et al. 2009a, Spies et al. 2018, Waples 1998) or from a large effective population size (as measured in sole with Ne higher than 1 000, Cuveliers et al. 2012). A fourth explanation might be that larvae arriving at the nursery ground originate from several spawning grounds (as discussed in detailed in *Hypothesis 2*). Disentangling these four explanations still remain a challenge for sole of the North Sea.

Hypothesis 5: Juvenile sole show limited connectivity between nursery grounds

Juvenile sole from four nursery grounds in the Southern North Sea had a distinct elemental fingerprint (especially for Mg, Mn and Zn) using state-of-the-art laser ablation ICPMS (Nu Attom). With an overall high self-assignment rate per sampling location (79%) and per sampling region (89%), discriminant analyses confirmed the strong spatial differences in otolith microchemistry (**Chapter 2**). Hence the nursery signature was identified with confidence on a local and regional scale (5-500 km), which is quite challenging in coastal nurseries with a relatively low water residence time. Many elemental studies of nurseries focused on migrations between chemically contrasting environments such as estuarine migration or dispersal on large spatial scales (Di Franco et al. 2012, Gillanders & Kingsford 2003, Morat et al. 2014). Sole might have a microchemical discriminatory power above average due to its lower mobility and association with local river-specific fingerprints (Leakey et al. 2009). The limited juvenile movement on the nursery grounds fits well with other studies. Burrows et al. (2004) conducted a transplant experiment of tagged juvenile plaice and showed long-shore site fidelity. Once settled, juvenile flatfish remain localized on the nursery ground (Le Pape & Cognez 2016), thus favouring strong chemical signatures of their otolith. Closer to our focal area, Cuveliers et al. (2010) distinguished nursery grounds of sole from the Thames and Scheldt Estuary, and the Wadden Sea, although molecular markers failed to observe differences (Cuveliers et al. 2012). Temporal variability in the microchemical signal may be driven by seasonal or inter-annual variation (Tanner et al. 2012). Although season or year have an impact on the elemental fingerprint, this does not hinder spatial discriminatory power (Cuveliers et al. 2010, Reis-Santos et al. 2012, Tanner et al. 2012). Nevertheless, temporal variability should be monitored and accounted for. Otolith microchemistry yielded high assignment success to estuarine nursery grounds in the Bay of Biscay, suggesting nursery fidelity (de Pontual et al. 2000).

Otolith shape had a lower assignment success, which might be attributed to the relatively long time required for shape differences to become significant. Otolith shape has a rather complex nature and is influenced by genetic background, physiology (linked to ontogenetic development in juveniles) and environment (Cardinale et al. 2004, Hüssy 2008). Thus, other factors such as spawning ground location and dispersal route may influence shape (Capoccioni et al. 2011, Lombarte et al. 2003, Mapp et al. 2017).

Physical tagging of sole in the North Sea and English Channel also confirmed the restricted movement of juveniles (Burt & Millner 2008). Coastal and estuarine nursery habitats are valuable habitats as they provide shelter and food. The high concentrations of juveniles can be explained by the high abundance of food (Amara 2004, van der Veer et al. 2000), resulting in enhanced growth. Higher growth rates are associated with a lower vulnerability to predators (Bailey and Houde 1989).

2. Integration of different connectivity measures

2.1 Different tools measure different processes

Overall, otolith shape and microchemistry seem to be the best tools to trace back the origin of adult sole at a regional spatial scale where molecular markers might fail due to a lack of power (Cuveliers 2012, Sturrock et al. 2012). Microchemical concentrations tend to be species-specific. However phylogenetically related species tend to have similar microchemical variations (Reis-Santos et al. 2008, Swearer et al. 2003). This makes sense because microchemistry is governed by growth rate, physiology, genetics all of which are under influence of selection (Sturrock et al. 2012). Leakey et al. (2009) investiageted differences between juvenile sole, seabass and whiting, and concluded that sole had the highest discriminatory power. This might be attributed to its lower mobility and close association with the sediment. The handling of otoliths has to be done with care to avoid contamination. Geffen et al. (2013) conducted a cross validation exercise to compare laboratories and LA (Laser Ablation) vs. SB (Solution Based) ICPMS. Interestingly, some elements are more prone to drift than others. They highlighted the need for careful standardization between methods and laboratories, and further comparison studies. Moreover, a traceability tool can only be useful if the temporal variation of the signal is less than the spatial variation. The latter has to be further investigated for sole, since we tested this hypothesis on too few samples (**Chapter 2**). Shape morphometrics have the advantage to be fairly easy to implement and at a lower cost than genomics and microchemistry. In addition, otolith shape does not require the strict standardization procedures that are required by microchemistry standards and geomic markers. However, the resolution does not reach the level of microchemical analysis. Microchemistry traces the contribution of nursery grounds to adult stocks as a marker “through time” (i.e. the chemical trace remains stable in the otolith while the shape continuously changes) and differences imply shorter periods of geographic separation

than otolith shape (Sturrock et al. 2012). In this PhD thesis, otolith microchemistry was the most powerful traceability tool for identifying juvenile sole in the North Sea due to the strong signature observed at the nursery grounds (**Chapter 2**), but also within the Belgian nursery ground (*section D.2.3*). In addition, microchemistry is of considerable value for species with extended larval dispersal and / or low level of genetic differentiation, as it is the case for sole in the North Sea.

Yet, genetic markers are more promising for tracing young stages such as eggs and juveniles. The main requirement for the successful implementation of genetic traceability is the availability of many molecular markers and a database for the development of validated operational genetic tests. Our ability to develop and use genome-wide approaches to DNA analysis has been largely based on the recent, rapid advances in high-throughput Next Generation Sequencing technologies (NGS). The genome-wide identification, characterization and selection of DNA markers provide the best approach to develop traceability protocols that deliver a maximum amount of information at a minimal cost. The cornerstone of this strategy is that, within the genome, a small proportion of genetic markers are unique to the population (Nielsen et al. 2012). Recently genetic tagging has been streamlined by several new methods that reduce the sequencing effort and screening to a few thousand genetic markers at a highly reduced cost compared to whole genome sequencing (e.g. Bernatchez 2016). Despite the low level of genetic differentiation, highly differentiated (outlier) genomic markers, several of them linked to brain and sensing/immunological genes, identified clusters in the western and the eastern coast of the Belgian nursery, which might be attributed to local adaptation (**Chapter 3**, *section D.2.3*, Rijnsdorp et al. 1992).

2.2 Why connectivity measures may benefit from integration

As mentioned in the previous section, a wide range of methods have been developed to explore and estimate connectivity within metapopulations (Cowen et al. 2006, Cowen & Sponaugle 2009, Jones et al. 2009). Because each method is often applied to a specific scale and is underlined by specific assumptions, the use of multiple methods at different temporal scales may be necessary to completely understand a system (Thorrold et al. 2002). To identify the spawning ground origin of a single juvenile, one may use the larval elemental signature of the otolith, which is initially influenced by the spawning ground chemistry, and genetic markers,

which are linked to the parental gene pool. To distinguish nursery ground signature, otolith shape and sampling elemental signatures on the nursery can be combined. Combining several types of markers that integrate information on the evolutionary and / or ecological time-scale, such as otolith shape, elemental composition and genetic markers, are likely to enhance discrimination power in detecting spatial and temporal structure.

As shown by biophysical models (Lacroix et al. 2013), the Belgian and Wadden Sea nursery grounds may be receiving larvae from several shared or isolated spawning grounds (as discussed in *Hypothesis 3 section D.1*). We aim at documenting and quantifying individual dispersal between the Southern North Sea spawning and nursery grounds by combining information from seven types of markers: otolith shape (left and right otolith), elemental composition (larval, settlement and sampling location) and (neutral and outlier) genetic markers. Specifically, we tested congruence between otolith elemental composition of the larval phase and genetic signatures to unravel potential spawning ground origin. We also tested whether the elemental signature at the sampling location coupled to otolith shape improved the quality of assignment to the nursery ground. Finally, the third aim of this study was the quantification of congruence between the different methods to measure ecological and evolutionary connectivity of juvenile sole with a novel statistical approach designed to find consensus patterns. We applied a network fusion analysis to several methods in our study system in an attempt to measure connectivity at different spatiotemporal scales and lead to a better knowledge of the crucial connections between populations. We focused on sole inhabiting the Southern North Sea, including the Scheldt Estuary and the Wadden Sea and sampled on a spatial scale between 5 and 500 km (see Fig. A.1 and Table A.1 in Appendix for sampling details). Ninety-two age-0 sole provided seven types of markers that were interested in. These juveniles are a subset of the full dataset analyzed in chapter 1, 2 and 3: otolith shape (left and right otolith), elemental composition (larval, settlement and sampling location) and (neutral and outlier) genetic markers (see Appendix 1 for the full *material and methods section*).

2.3 Results on the integration of connectivity estimates

2.3.1 Otolith shape analysis

Otolith shape analyses pointed to differentiation between the Belgian and the Wadden Sea nursery grounds (Suppl. Fig. A.1). The first and second principal components accounted for 10.9 and 10.6% in the left otolith and 10.5 and 10.2% in the right otolith, respectively. None of the explanatory variables (nursery, site or fish length) had a significant effect on otolith shape (Table D.1).

2.3.2 Otolith elemental analysis

The first two PC axes of the larval and the sampling location signatures explained more variance than the settlement signature (52.7%, 66.4% and 37.4%, respectively, Suppl. Fig A.1). All elemental signatures were significantly explained by spatial variation between and on the nursery grounds, but not by fish length (Table D.1). For the larval signature, two pairs of sites differed significantly from each other between the Belgian and Wadden Sea nursery grounds (NL1j14 vs. B03j13 and NL1j14 vs. B06j13) and one pair within the Belgian nursery (B03j13 vs. B01j14) after correcting for multiple testing. For the settlement signature only one pair of sites was significant between the two nursery grounds (NL2j14 vs. B01j14) and two pairs of sites within the Belgian nursery (B08j14 vs. B01j14 and B06j14 vs. B01j14). Both larval and settlement signatures suggest a subtle isolation of B01j14, the most western site on the Belgian nursery.

Sampling location signature was the most differentiated marker. Both nursery grounds seemed well isolated and chemically distinct. However, a limited number of individuals sampled in one nursery ground chemically resembled the other nursery ground, indicating inter-regional connectivity or overlap in chemical signatures. Putative migrants were sampled in the Wadden Sea (fish number 11803, 11817, 11892, 11894 and 11902) but were assigned to Belgium, while some Belgian individuals were assigned to the Wadden Sea (fish number 3933, 5913 and 5914).

Ten pairs of sites were significantly different between the two nursery grounds and within the Belgian nursery ground (Suppl. Table A.1). The westernmost site on the Belgian nursery ground, B01j14, was significantly different from all other Belgian sites. The easternmost site

Table D.1: Permutational multivariate analyses of variance of otolith shape, elemental and genetic markers of sole. Nursery = Belgian nursery vs. Wadden Sea nursery, SL = standard length, DF = degrees of freedom, SS = sum of squares, MS = mean square, F = f statistics, R2 = variance explained. Significant p-values (α = 0.05) are indicated in bold.

Data	Effect	DF	SS	MS	F	R^2	*P value*
Left otolith shape	Nursery	1	4.780	4.781	0.472	0.005	0.92
	Site	6	62.610	10.436	1.030	0.069	0.43
	SL	1	1.390	1.392	0.137	0.002	1
Right otolith shape	Nursery	1	14.580	14.584	1.483	0.016	0.119
	Site	6	76.080	12.680	1.289	0.084	0.054
	SL	1	3.060	3.064	0.312	0.003	0.974
Larval microchemistry	Nursery	1	75.010	75.011	11.863	0.103	**0.001**
	Site	6	122.840	20.474	3.238	0.169	**0.002**
	SL	1	5.330	5.327	0.843	0.007	0.456
Settlement microchemistry	Nursery	1	79.410	79.410	12.176	0.109	**0.001**
	Site	6	93.800	15.633	2.397	0.129	**0.001**
	SL	1	13.470	13.475	2.066	0.019	0.062
Sampling location microchemistry	Nursery	1	56.810	56.809	11.929	0.078	**0.001**
	Site	6	272.720	45.454	9.545	0.375	**0.001**
	SL	1	3.210	3.205	0.673	0.004	0.59
Neutral genetic markers	Nursery	1	2915.000	2915.300	1.062	0.012	0.053
	Site	6	17152.000	2858.700	1.042	0.068	**0.011**
	SL	1	2944.000	2944.000	1.073	0.012	0.053
Outlier genetic markers	Nursery	1	73.200	73.233	1.101	0.012	0.287
	Site	6	503.800	83.964	1.262	0.081	**0.009**
	SL	1	89.700	89.735	1.349	0.015	0.101

on the Belgian nursery ground, B08j14, was significantly different from all other Belgian sites, while being the only site that was not significantly different from the two Wadden Sea sites. Elemental composition explained the best amount of among-site and among-nursery variation (R^2 = 1.3 – 3.4% as opposed to < 1% for other markers; Table D.1).

2.3.3 Neutral and outlier genetic variation, and relatedness

Genetic diversity of juvenile sole was comparable at all eight sites. The observed and expected heterozygosity (H_o and H_e) per sample for all 1,412 SNP loci ranged between 0.23 and 0.24 and 0.25 and 0.26, respectively (Table D.1). Pairwise genetic differentiation among sites was not statistically significant in most cases for both neutral and outlier markers (Suppl. Table A.2). Exceptions were two pairs of between nursery grounds comparisons (B08j14 vs. NL1j14 and B02j13 vs. NL1j14) and two pairs of within nursery ground comparisons with B08j14, the most eastern Belgian nursery site (B08j14 vs. B01j14 and B08j14 vs. B02j13) for neutral genetic markers. Absolute values of differentiation ranged from -0.002 to 0.005 for neutral markers and -0.010 to 0.048 for outlier markers (as measured by F_{ST}). Two individuals sampled at site B06j13 showed a pairwise relatedness as large as 0.97 (fish number 9996 vs. 9997).

2.3.4 Within- and between spawning and nursery ground signature

Outlier SNP loci had low re-assignment percentages, ranging from 0.0 to 41.5% (average: 39.6%; Table D.2). Exceptions were B01j14, B03j13 and NL2j14, with re-assignment percentages of 66.7, 66.7 and 61.5%, respectively. Re-assignment success of the neutral genetic markers and larval elemental signature was highly variable (8.3 to 80.0% with an average of 42.8% for neutral genetic markers; 0.0 to 76.5% with an average of 51.6 for larval elemental signature). In contrast, otolith shape resulted in re-assignment to sampling sites with an overall average to high accuracy for (38.5 to 66.7%; average: 50.5%) and settlement elemental signature (33.3 to 82.4%; average: 56.7%). Exception was site B06j13 with the lowest re-assignment success (average B06j13 over all methods: 23.8%). Most of the individuals of B06j13 were re-assigned to B06j14, its temporal replicate. The highest re-assignment success was achieved for sampling site elemental signature (40.0 to 88.2%; average: 67.9%). In three out of five cases sampling location shows a stronger signal than settlement signature. In one case (B02j13) this is the reverse.

Table D.2: Re-assignment percentages of individual sole to sampling location for different markers. Sampling locations are defined in Table A.1.

Site	Larval elemental signature	Settlement elemental signature	Sampling location elemental signature	Neutral genetic markers	Outlier genetic markers	Left and right otolith shape	Mean over sites
B01j14	60.0	33.3	86.7	60.0	66.7	46.7	**58.9**
B02j13	53.3	80.0	60.0	40.0	40.0	46.7	**53.3**
B03j13	66.7	50.0	75.0	8.3	66.7	66.7	**55.6**
B06j13	0.0	28.6	28.6	14.3	28.6	42.9	**23.8**
B06j14	75.0	62.5	87.5	50.0	12.5	50.0	**56.3**
B08j14	76.5	82.4	88.2	58.8	41.2	52.9	**66.7**
NL1j14	20.0	40.0	40.0	80.0	0.0	60.0	**40.0**
NL2j14	61.5	76.9	76.9	30.8	61.5	38.5	**57.7**
Mean over markers	**51.6**	**56.7**	**67.9**	**42.8**	**39.6**	**50.5**	**51.5**

Table D.3 : Redundancy Analysis (RDA). DF = degrees of freedom, F = f statistics. Significant p-values ($\alpha = 0.05$) are indicated in bold.

Data set 1	Data set 2	DF	Variance	F	R^2	*P value*
Larval elemental signature	Neutral genetic markers	2	0.125	1.352	0.029	**0.001**
Larval elemental signature	Outlier genetic markers	2	0.126	1.364	0.030	**0.001**
Left and right otolith shape	Neutral genetic markers	2	0.036	0.384	0.009	**0.001**
Left and right otolith shape	Outlier genetic markers	2	0.073	0.780	0.017	**0.001**

Only the elemental analyses showed a clear differentiation in a PCA plot. The two nursery grounds differentiated on the first axis for larval, settlement and sampling location elemental (Suppl. Fig. A.1). Nevertheless, both types of genetic markers were significantly correlated to larval elemental markers (Table D.3). Likewise, both type of genetic markers were also significantly correlated to left and right otolith shape (Table D.3).

2.3.5 Similarity Network Fusion analysis

Similarity networks were run to assess connectivity for each marker at two geographical scales: first we looked at connectivity between the Belgian and Wadden Sea nursery grounds and then within the Belgian nursery ground (Fig. D.1). At the North Sea scale, the neutral genetic markers and otolith shape revealed a high level of connectivity between both nursery grounds with Similarity Network Fusion (SNF). On the other hand, connectivity was less extensive based on outlier loci and elemental signatures. Similarly, within the Belgian nursery, SNF revealed a high level of connectivity for the neutral genetic markers and otolith shape. Despite the small spatial scale, SNF highlighted significant differences between sites based on the elemental composition and outlier genetic markers. SNF identified an elemental cluster on the eastern side of the Belgian nursery (B03j13 - B08j14), while an outlier genetic cluster was identified on the western side of the Belgian nursery (B01j14 - B02j13).

In addition, genetic markers and larval elemental signature were combined to test for discrimination between the spawning grounds (Suppl. Fig. A.2a and b). Otolith shape and sampling location elemental signature were also combined to test for discrimination between the nursery grounds (Suppl. Fig. A.2c). In all cases connectivity was strengthened between both nursery grounds and within the Belgian nursery ground, for both neutral and outlier genetic markers. The clusters initially spotted with elemental analysis disappeared.

Moreover, to visualize whether genetic markers were explained by the spawning or nursery ground origin, heat maps of genetic markers were plotted according to clusters based (1) on the larval elemental signature for the spawning ground origin (Fig. D.2a) and (2) on the sampling location elemental for the nursery ground origin (Fig. D.2b). The initial aim was to

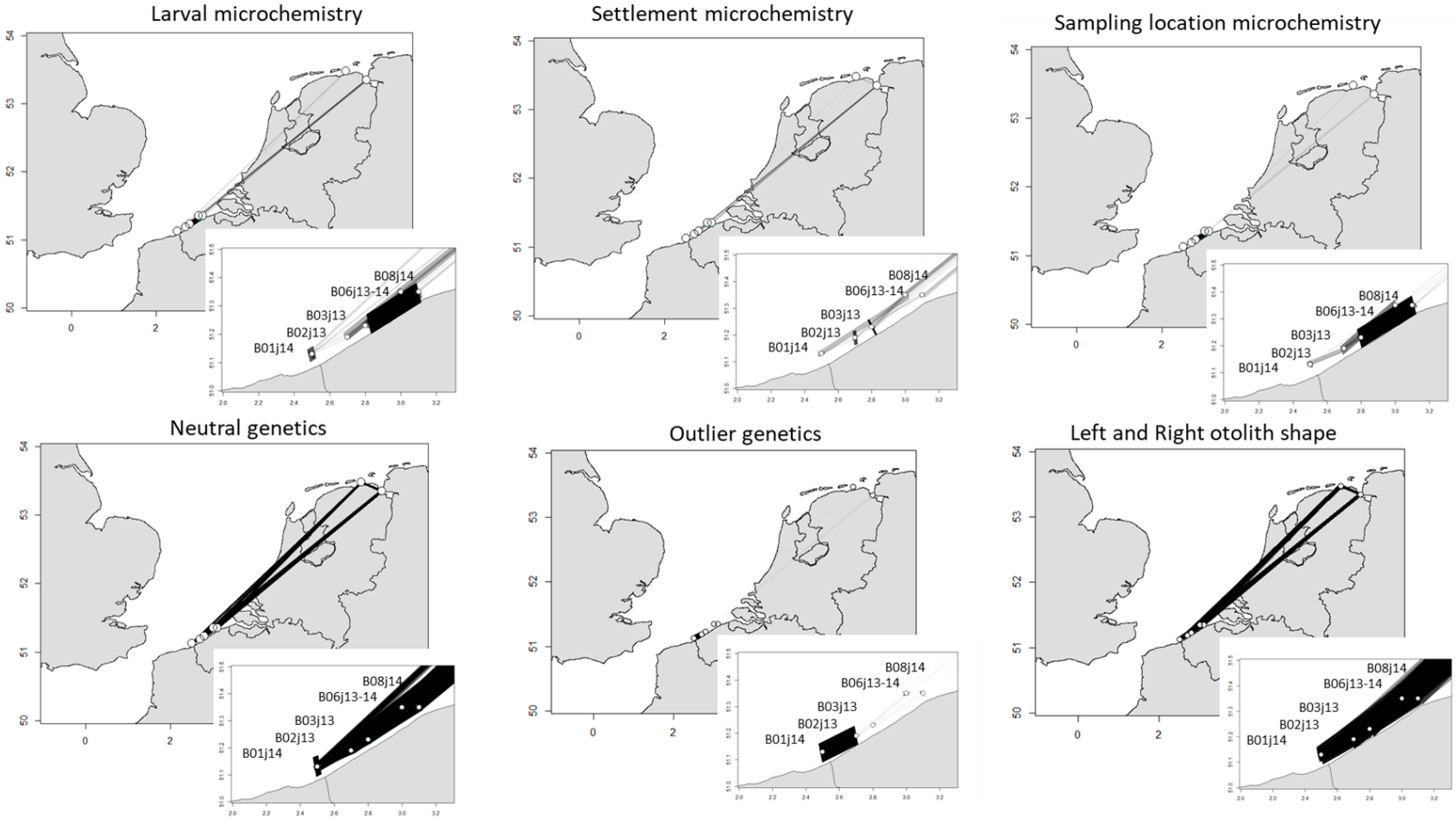
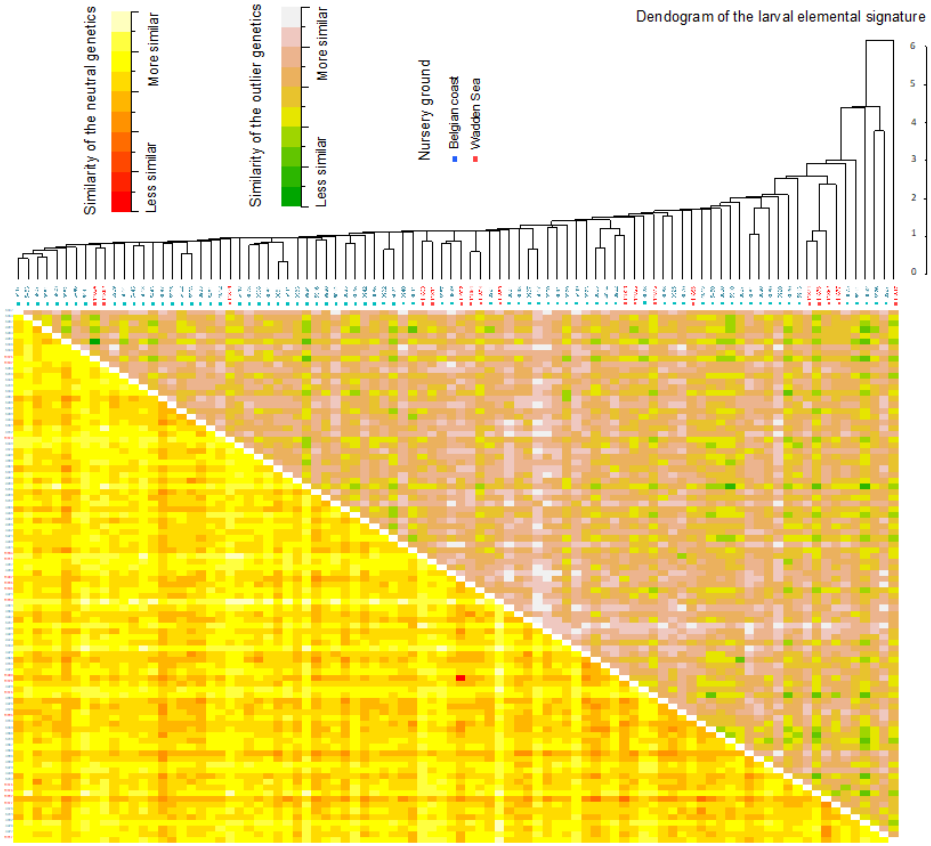


Figure D.1: Fused similarity networks among eight sampling sites of sole based on otolith shape, otolith elemental signatures of larvae, at settlement and capture, and neutral and outlier genetic markers.

a



b

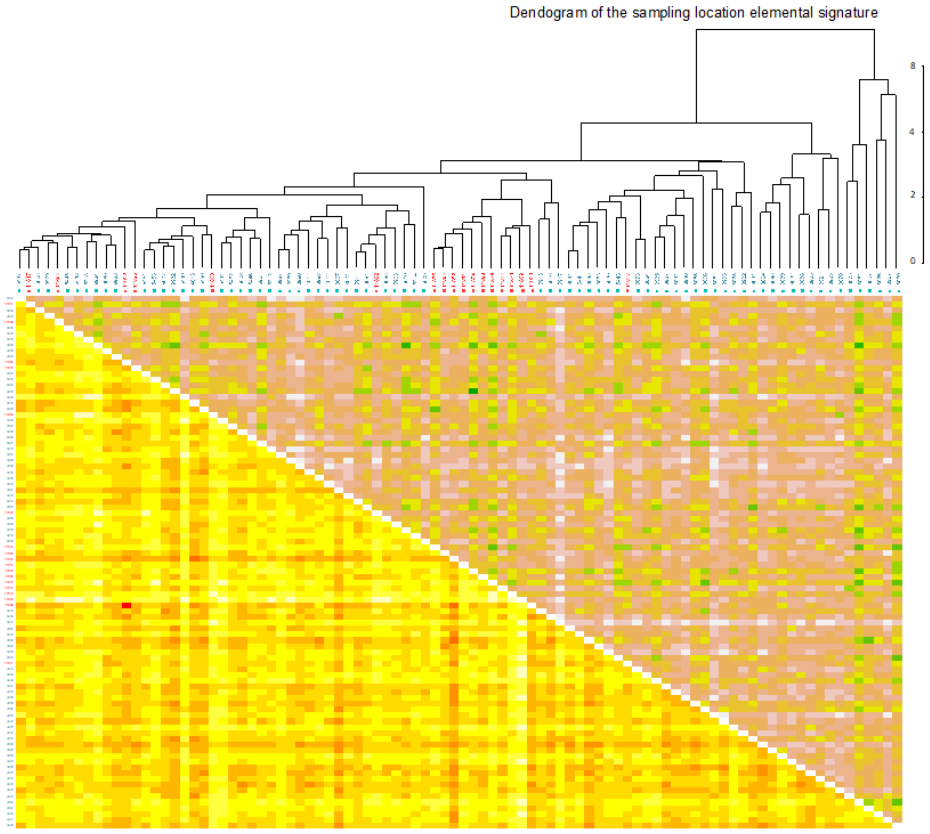


Figure D.2: Heat map of the outlier loci (upper matrix) and neutral genetic markers (lower matrix) as explained by (a) larval or (b) sampling location elemental composition. The gradient of colors points to the genetic similarity and range from the more similar (darker) to the less similar (lighter). Sample names are colored by sampling region (blue for Belgian nursery and red for the Wadden Sea nursery).

identify a link between the genetic and the elemental signature of putative migrants between nursery grounds. Heat maps revealed genetic clusters based on elemental composition of larval and sampling location signatures that matched with outlier genetic markers but not with neutral genetic markers. Genetic clusters were more apparent based on sampling location than based on larval elemental signature (judging by the dendrogram shape). Genetic clusters did not seem to be linked to nursery ground identity.

2.4 Discussion on the integration of connectivity estimates

We inferred connectivity and population structure of age-0 sole collected in the Southern North Sea by integrating data on otolith shape, elemental composition and genetic markers. While each marker revealed the population structure of juvenile sole differently, a consensus pattern emerged after fusion of the networks with SNF. Overall connectivity is high, but there is some evidence for local structuring. While neutral genetic markers showed little differentiation, the Belgian and the Wadden Sea nursery were well-characterized chemically by elemental analysis of sampling location. Within the Belgian nursery, the eastern and western side of the Belgian nursery ground are distinct based on the nursery signature (sampling location), but also based on the spawning ground signature (larva). In addition, relatedness analysis picked up one highly related pair of juveniles.

Spatial differentiation

Overall, 92 individuals yielded data for otolith shape, elemental composition (larval, settlement and sampling location) and (neutral and outlier) genetic markers. The sample size of this study lays in between previous multi-disciplinary studies on connectivity and traceability (Higgins et al. 2010 for cod n = 482, Marengo et al. 2017 for the common dentex *Dentex dentex* n =79). Previous studies looked at adult differentiation regionally (Marengo et al. 2017) or at a large spatial scale (Higgins et al. 2010). Most multi-disciplinary studies considered the population level because sample sizes were considerably constrained by keeping only individuals that contain all types of information. Nevertheless, the detection of subtle population structure benefits from combining various markers at the individual level.

The connectivity of flatfish larvae is thought to be high as revealed by biophysical models of larval dispersal (Lacroix et al. 2013) and low genetic differentiation (Cuveliers et al. 2012). However, most juvenile sole remained on the nursery ground they settled on. This observation matches with most flatfishes (Le Pape & Cognez 2016, Delerue-Ricard et al. submitted, **Chapter 2**). Overall, SNF results indicated higher connectivity for larvae than for juveniles between the Belgian and Wadden Sea nursery grounds, especially for otolith shape and neutral genetic markers, in accordance with former studies (Cuveliers et al. 2012, Diopere et al. 2018). Nevertheless, individual sampling sites revealed some differences between markers as witnessed by the fluctuating connections between local sampling sites. From all seven markers, elemental composition on the nursery of capture and outlier loci showed the highest power of differentiation, even within a small nursery off the Belgian coast.

Mixed contribution of spawning grounds

To identify the original spawning ground of a single juvenile, one may use the larval elemental signature of the otolith which is influenced by the spawning ground chemistry and genetic markers which are linked to the parental gene pool. Interestingly, the larval elemental signature identified three natal clusters. The larval elemental signature of the eastern side on the Belgian nursery showed more similarity with the Wadden Sea nursery than with the western side of the Belgian nursery. This result might indicate a shared spawning ground between the eastern side of the Belgian nursery and the Wadden Sea nursery (as discussed in *Hypothesis 3*). From the biophysical model of Lacroix et al. (2013) we learned that larvae settling at the Belgian and Wadden Sea nursery grounds originated mainly from spawning grounds in the Eastern English Channel, off the Belgian coast, and in the Thames Estuary. However, the contribution of each source populations to one nursery ground may vary between years. Our results support the existence of different spawning ground origins between the western and the eastern sides of the Belgian nursery ground. Nevertheless, sole has an average dispersal of about 150 km as estimated by biophysical modeling, physical tags and otolith microchemistry (Burt & Millner 2008, Barbut et al. 2019). Field surveys also reported comparable dispersal distances between spawning and nursery grounds (80-100 km, Dorel et al. 1991). Thus, with an average dispersal distance (< 150 km) less than half the distance separating the Belgian and Wadden Sea nursery

grounds (> 300 km), the shared contribution between spawning grounds must be limited and relate to rather exceptional dispersal events favored by unique advection events.

In this study, neutral genetic markers showed an homogeneous population structure. This result is compatible with the high (expected) level of gene flow (Cuveliers et al. 2012, Diopere et al. 2018, **Chapter 3**) because few migrants are needed to impact gene flow (Hauser & Carvalho 2008, Slatkin 1993) and lead to a low population structure. Sole has also a large effective population size which limits population differentiation (Carvalho & Hauser 1994, Cuveliers et al. 2011, discussed in *Hypothesis 4*). The question remains which life stage(s) contribute most to gene flow. A low genetic differentiation could be attributed to limited but significant mixing between the nursery grounds at the juvenile stage, but also by gene flow on the spawning aggregations of adults. The high level of local recruitment and the low level of successful recruitment of migrants observed in this study (and on a larger dataset in **Chapter 2**) is compatible with the homogeneous population structure of sole in the North Sea as revealed by genetic markers (Cuveliers et al. 2012, Diopere et al. 2018). Our results do not support a local pattern of isolation by distance (i.e. the further geographically apart the samples are, the more genetically differentiated they are) as evidenced using genetic markers at larger spatial scales (Cuveliers et al. 2012, Diopere et al. 2018). The limited distance between the Belgian coast and the Wadden Sea, and the reduced number of nursery grounds sampled in this study might be a limiting factor to pick up a signal of isolation by distance.

Despite the absence of significant pairwise F_{ST} comparisons, outlier genetic markers showed small-scale spatial structuring and an order of magnitude higher genetic differentiation within the Belgian nursery compared to neutral markers (as discussed in *Hypothesis 1*). Moreover, a skewed reproduction rate (linked to sweepstake effects, Hedgecock & Pudovkin 2011) in conjunction with drastic mortality rates of eggs and planktonic larvae is likely reflected in patterns of selection and local adaptation. The high relatedness of two juveniles sampled a few days apart could support sweepstake effects and non-random mating. In addition, genetic differentiation at the small spatial scale within the Belgian nursery may be linked to the presence of a genetic structure linked to cohort dynamics (as discussed in *Hypothesis 1*).

Residency on the nursery ground

Otolith elemental composition has proven to be effective in resolving small-scale spatial differences (as discussed in *Hypothesis 5*). Despite the challenges of working at a small spatial scale (5 km between the closest sampling locations) and in the coastal area, the chemical signatures of nine elements provided high nursery-specific assignment levels of sole. Regional assignments to the nursery grounds (68% in this chapter, 89% in **Chapter 2**) are comparable with previous studies (Cuveliers et al. 2010: 88%, Tanner et al. 2012: 71-80%). Our results support a reduced mobility and site fidelity of age-0 individuals. Site B08j14 was significantly different from all other Belgian sites, while being the only site that was not significantly different from the two Wadden Sea sites. This might be explained by a later sampling date (10th of October 2014) which is also the year the Wadden Sea samples were collected. Another explanation relates to the position of B08j14 on the most eastern site on the Belgian nursery, under the influence of the Scheldt inflow (i.e. a distinct freshwater mass). For example, otolith Sr concentrations may match salinity gradients (Campana 1999, Leakey et al. 2009). Nonetheless, elemental composition is not only affected by geography but also by time and physiological differences (as discussed in *Hypothesis 2 and 5*, Chang & Geffen 2013, Reis-Santos et al. 2012).

The otolith shape of juvenile fish may also serve as an indicator of the nursery ground signature (Delerue-Ricard et al. 2018). However, most differentiation in otolith shape appears to be acquired later on in life after reproduction, or is incremental over time (Cadrin et al. 2014). Otolith shape analyses of the 92 juvenile sole pointed to very limited differentiation, in contrast to the more pronounced differences based on a larger data set of 314 juveniles which split otolith shapes in two groups (Delerue-Ricard et al. 2018, **Chapter 1**).

In summary, differences in ecological connectivity between two 'adjacent' nursery grounds can be more effectively traced using elemental composition than otolith shape and neutral genetic markers. In addition, complementary information on the larval elemental composition and pelagic larval drift estimates may both provide reliable information on the origin of spawning and the dispersal pathways of juveniles (Campana 2005, Morat et al. 2014). In a context of high gene flow, outlier loci may be especially useful to trace cohort structure. Such information contributes to a better understanding of the connectivity patterns of sole between spawning and nursery grounds.

3. General conclusions and perspectives

The contribution of this PhD thesis to the knowledge on the connectivity within and between spawning and nursery grounds of sole is summarized in Fig. D.4. Our results confirm four major clusters with subtle differentiation in the Northeastern Atlantic Ocean: the Bay of Biscay, North Sea and the English Channel, Celtic Sea and Baltic Transition Zone (Diopere et al. 2018, **Chapter 3**). Spawning aggregations are not fully discrete but follow an isolation by distance pattern from south to north (**Chapter 3**). A single spawning ground contributes mainly to just one nursery ground; some secondary spawning grounds contribute in some years and not in others (Lacroix et al. 2013 see Fig. D.3, **Chapter 2**). Juveniles recruit locally and connections between nursery grounds are limited, which results in strong elemental fingerprints of each nursery (**Chapter 2**). Within the North Sea, the genetic differentiation of juveniles based on neutral markers is low due to high level of gene flow (eggs or adults mixing on the spawning grounds), large effective population size and the lack of a migration-drift equilibrium. However, kin structure in juvenile groups might be maintained to a limited extent, favoured by sweepstake recruitment. In addition, outlier loci showed evidence of natural selection at the Belgian nursery (*section D.2.3*). Selection likely impacts the first year of life through high mortality rates removing juveniles the least adapted to the local environment. The genetic basis for such selection could be linked to brain and sensing/immunological genes (**Chapter 3**) and muscle related genes (Diopere et al. 2018). Whether adults from different nursery grounds recruit to a common feeding ground and later return on the same spawning ground year after year remains an open question. Tagging suggest site fidelity of sole with juveniles recruiting mainly to the adult population of the same area (Burt & Millner 2008). Strong evidence for natal philopatry has been shown in co-occurring populations of plaice (Darnaude & Hunter 2018, Hunter et al. 2003). We therefore suspect that most exchanges among populations happen during the pelagic larval phase or between spawning aggregations. Based on our results using otolith shape and elemental composition, and genomic markers, we propose the following connectivity model for sole between and within the spawning and nursery grounds in the North Sea (Fig. D.5).

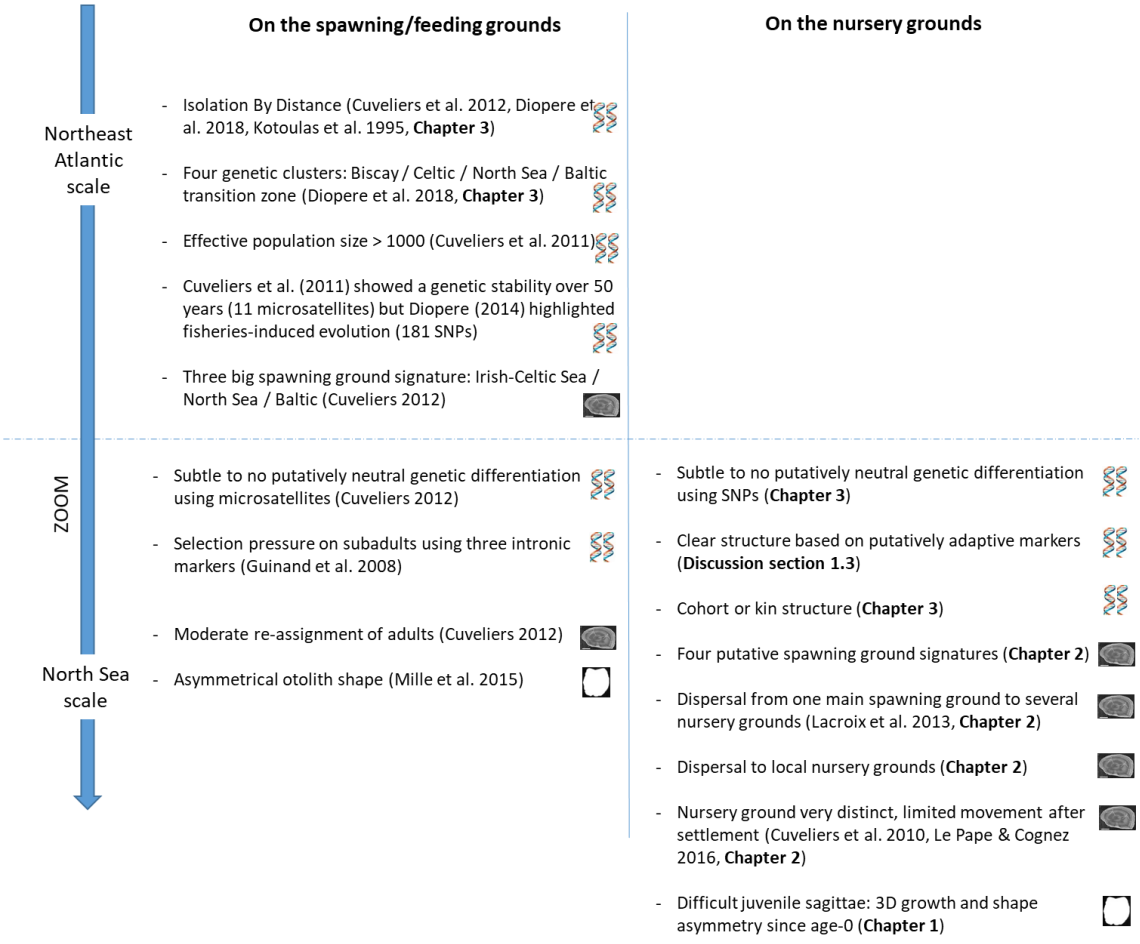


Figure D.4: Summary of the inferences from the otolith and molecular markers regarding adult sole (left panel) and juvenile sole (right panel) for the Northeast Atlantic Ocean (upper panel) and North Sea (lower panel). This figure addresses *Hypothesis 1 and 5* and is an attempt to visualy clarify the differences between juveniles and adults, and the differences between the Northeast Atlantic Ocean and the North Sea scale.

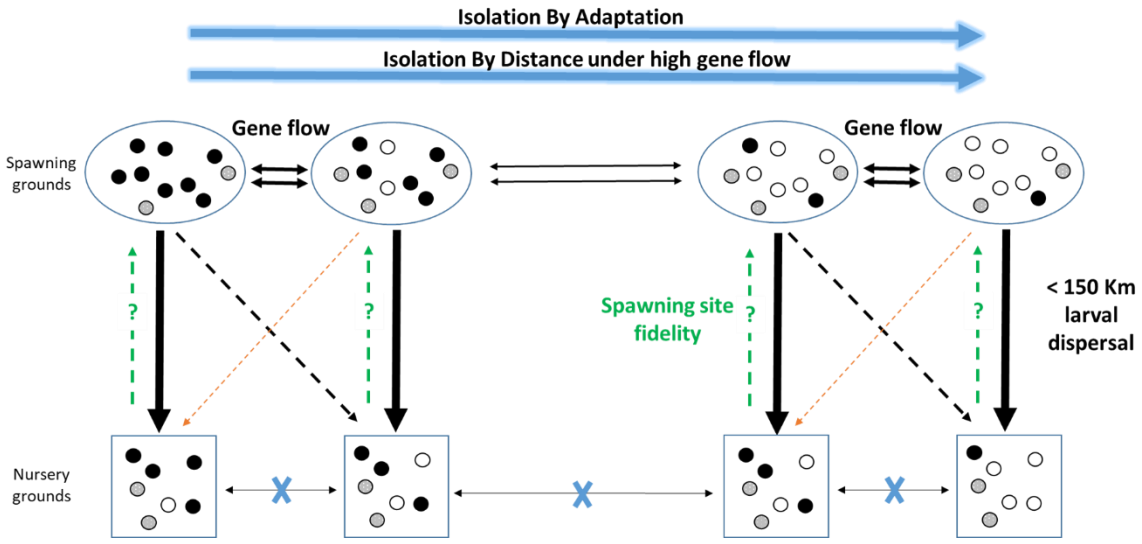


Figure D.5: Migration model of sole in the North Sea. Arrows indicate the direction of movement, the thickness of the arrows indicate the intensity of the connections. Black arrows indicate dispersal along the residual current while orange arrows indicate dispersal against the residual current. Green arrows with question marks indicate uncertainties about spawning site fidelity. Dots represent the gene pool. Adjacent spawning grounds are genetically similar because of gene flow evolves into a pattern of Isolation by Distance (IBD).

4. Research perspectives

Optimized tools will improve our understanding of connectivity. Therefore it is essential to refine the methodological criteria to detect and better understand adaptation (*section D.4.1*), and to validate and develop biophysical and community models in an evolutionary context (*section D.4.2*). In addition, global change clearly plays a central role to take into account the future of environmental and fisheries research (*section D.4.3*). Ultimately, several complementary aspects of field based evidence should be integrated into management strategies (*section D.4.4*).

4.1 Tracing the genetic basis of adaptation

A wide range of statistical methods are available to discriminate evolutionarily neutral and adaptive processes. The advantages and disadvantages of outlier methods relate to the many assumptions (e.g., Helyar et al. 2011, Flanagan & Jones 2017, François et al. 2016), which makes the method and threshold to determine outliers quite subjective. Many methods designed to detect outlier loci assume a specific model of demographic history (Whitlock & Lotterhos 2015). For example, one of the most common genetic methods to detect outliers (*Arlequin,* Excoffier & Lischer 2010) assumes an island model, hence assuming a narrow range of expected F_{ST} values. The consequence is that a high number of false positives may appear. Flanagan & Jones (2017) recommend a Bayesian maximum likelihood approach (instead of *Lositan* (Beaumont & Nichols 1996) for example) in the case of small populations and a low migration rate to account for non-independence of populations. In addition, a stringent false discovery criterion (FDR) set at 5% reduces the likelihood of false positives. However, useful adaptive information might be lost if weak outlier loci end up in the neutral dataset. Evolutionary changes induced by fishing, for example, are expected to be gradual, thus producing weak outliers. To increase the power of rejecting neutrality, François et al. (2016) advocate the combination of several methods. In **Chapter 3** of this PhD thesis, we used a combination of methods because OutFLANK (Whitlock & Lotterhos 2015), and pcadapt (Luu et al. 2016) were shown to provide a lower FDR than BayeScan (Foll & Gaggiotti 2008) in a situation that is likely in our study system: a divergence model with admixture (i.e. there is a hierarchical population structure where differentiation varies between populations). However, using several methods and retaining only the outlier loci that are shared between these methods reduces the number of outlier loci. A limited number

of loci might lower our ability to resolve population structure at the scale of the North Sea. More and more the value of a genome-wide approach consisting of several thousands of markers is appreciated to resolve small spatial scale structures (Hohenlohe et al. 2010, Manel et al. 2016).

RADseq has its limitations to reveal subtle patterns of local adaptation. RADseq underestimates diversity due to nonrandom haplotype sampling linked to polymorphism in restriction sites (potentially targeting repetitive and low coding regions (Arnold et al. 2013). In addition, lower sampling depth leads to more missing data, in turn leading to higher rates of false positives or negatives in genome selection scans. Important here is to restrict the analysis to loci with a high read depth (> 10X). Targeting less genomic fragments is one way to ensure a better coverage and to be conservative in selecting reliable loci. The resulting high read coverage has advantages for accuracy (i.e. lower probability of erroneously including genotyping error) but on the other hand lower marker density is achieved as the number of fragments is inversely proportional to coverage. High SNP densities, however, are important to effectively scan for loci putatively under selection (Lowry et al. 2016). Therefore we advise to include an optimization step (via *in silico* digestion and lab optimization) to target the desired number of SNPs. This will guarantee a reliable coverage for a maximum number of SNPs (e.g. O'Leary et al. 2018). In addition to the desired coverage, the two other key parameters to optimize before library preparation are the selection of the restriction enzyme (a duo of enzymes in the case of ddRAD) and the size selection of fragments. An important consideration is that these three key parameters (coverage, enzyme restriction sites and size selection) may vary due to technical bias between sequencing runs and sequencing machines which is referred to as 'library effect'. Potential solutions to library effects are increasingly considered and bioinformatic mitigation strategies are worth looking into as suggested by Campbell et al. (2017), Leigh et al. (2018) and O'Leary et al. (2018). Finally, linkage disequilibrium has to be accounted for because SNP loci in the same haplotype block are likely to be redundant. However, not all loci will be in complete linkage disequilibrium within the same haplotype block, because new mutations and recombination may arise within one block (Lowry et al. 2016).

Genome-wide genotyping methods enable the identification of adaptive traits without specifying loci of interest *a priori*. This bottom-up approach is known as genome wide association studies (Hendricks et al. 2018). However, the explanation for selectively important traits is limited to the putative gene function provided by annotation analysis. We called upon an

automated annotation of the outlier loci identified in the southern North Sea population of sole based on the unpublished reference genome of Senegal sole *Solea senegalensis* (**Chapter 3**, Tiffin & Ross-Ibarra 2014). The function of genes involved in various developmental and physiological processes of teleost fish in general and more specifically in *Solea solea* and *S. senegalensis* sole is increasingly documented (Cerda & Manchado 2013, Ferraresso et al. 2013, Benzekri et al. 2014, Alves et al. 2016, Robledo et al. 2017). Although it is beyond the scope of this PhD thesis to characterize genes and provide insight in the physiological pathways of flatfish, knowledge of the genes involved in response to stress (e.g. predation, temperature, pollutants, hypoxia) would provide essential insights into the genetic bases of adaptation (Guinand et al. 2008). Such knowledge is important not only for improving our basic understanding of human-induced and natural evolutionary processes, but also for predicting future trajectories of biodiversity and for setting conservation priorities (Nielsen et al. 2009a).

4.2 Capturing field based evidence with evolutionary biophysical models

Coupled hydrodynamic individual-based models have matured to the extent that they simulate drift of larvae in conjunction with biological features such as growth, condition and behavior (e.g. Lacroix et al. 2013, Miller 2007). This is a very helpful heuristic tool in order to quantify the role of physical and biological constraints on the recruitment process and the connectivity of exploited marine flatfish. In the context of climate change and fisheries management, models complement field studies to test for future scenarios (as discussed in *section D.4.3*). On the one hand, biophysical individual-based models are arguably one of the best tools to explore transport dynamics of flatfish larvae (Hufnagl et al. 2013) as they allow predictions over a large spatial extent and a high temporal frequency, far more than can be realized empirically. On the other hand, the lack of precise information on key parameters for larval survival such as larval swimming capability, mortality and pelagic larval duration hampers the parametrization of behavior routines of individual-based models. Therefore recent models include several behavior scenarios that remain to be validated (Hufnagl et al. 2013, Lacroix et al. 2013, Savina et al. 2010). Hence the need for field based validation of model and the integration of both approaches in the same study design. Based on the results of the present study (**Chapter 2 and 3**), we confirm model predictions that several spawning grounds contribute to the nursery grounds of the Southern North Sea and modeled estimates of dispersal distance.

Metacommunity theory develops contexts for how complex networks, such as food webs or metapopulations, will reorganize under environmental change. Thompson & Gonzalez (2017) modelled habitat colonization dynamics taking species interactions into account at the metacommunity level. The authors claim that dispersal ensures that species associations are maintained as they shift in space; hence networks retain similar composition and structure. On the other hand, some species might have higher or lower dispersal rates, and this could locally change network structures. In any case, the key role of dispersal reinforces the need to manage habitat connectivity to sustain species and interaction diversity into the future (Gawarkiewicz et al. 2007). This is particularly interesting since biophysical models predicted an increase in recruitment and longer larval dispersal at the North Sea scale, with nursery-specific regional differences. For example recruitment is predicted to decrease for Belgian nursery (Lacroix et al. 2018). Such increase in recruitment and dispersal distance may also increase the rate of longer dispersal. An increase in longer dispersal could also mean a decrease in local shorter dispersal. However, our results support the predominance of self-recruitment in sole, as has been shown in many marine populations (Swearer et al. 2002, Christie et al. 2010, **Chapter 2**).

An aspect to consider in evolutionary models is the arrival order of individuals in a new habitat patch because it influences community dynamics and structure. Priority effects suggest that early migrants can suppress late arriving individuals either via a numerical advantage or via local adaptation (De Meester et al. 2016). Habitat quality of the natal patch compared to the new habitats is another key feature leading to a competitive advantage of some dispersers against others and against resident species. Because traits of individuals are altered by experiences in their natal habitat, differences in the natal habitat of dispersers can carry over when individuals disperse to new habitats and alter their fitness and interactions with other species (Allen & Rudolf 2016, Pineda et al. 2007, Shima & Swearer 2010). Priority effects and competitive advantages linked to natal habitat quality reinforce the importance of better understanding arrival date on the nursery and natal origin of dispersers to better understand local dynamics.

4.3 Impact of climate change

Population dynamics and connectivity are important to predict effects of environmental change. The genetic differentiation of sole between the Baltic Transition Zone and the rest of the North Sea suggests that connectivity is restricted between regions at the edge of the species range. Thus if sole would disappear from the Baltic Transition Zone, a substantial amount of genetic diversity would be lost and recolonization from the North Sea would be uncertain. Nonetheless, an overfished population may be replaced quickly by a more common population, as has happened with Atlantic cod from the Flamborough Head population through enhanced migration of the North Sea population (Hutchinson et al. 2003). Ecosystems of the Northeast Atlantic Ocean are gradually becoming warmer (Beaugrand et al. 2003). Temperature is one of the most important environmental factors influencing growth rate and mortality. Evidence suggests that climate change will impact fish distribution and productivity in a complex way at the local scale. Climate change could have local positive effect on larval recruitment. Climate change modeling has shown an increase in larval recruitment at the North Sea scale (9%) but with strong regional differences between nursery grounds (Lacroix et al. 2018). Spawning and larval dispersal will be affected by the rising seawater temperature and change in wind speed and direction. Spawning time initially depends on a latitudinal gradient related to the minimum winter temperature (Fonds 1979, Rijnsdorp et al. 1992, Rijnsdorp & Vingerhoed 1994) but local hydrodynamics may overrule the latitudinal trend (Fincham et al. 2013, Vinagre et al. 2008). In the North Sea, strong year classes of sole occurred after a strong winter, while severe winters induce additional mortality (Rijnsdorp et al. 1992). With rising temperature, the availability of shallow winter habitats would increase in the southern North Sea (Dulvy et al. 2008). To summarize, climatic and anthropogenic effects are the main drivers of so far a southward shift in the spatial distribution of sole (Dulvy et al. 2008, Engelhard et al. 2011), and an earlier spawning with longer pelagic larval duration due to colder temperatures experienced by early hatched larvae (Fincham et al. 2013, Lacroix et al. 2018). Metabolic constraints on the physiology of sole are an important driver of the niche shifts (Teal et al. 2012). Also pelagic species such as Atlantic cod are shifting their distribution (Dulvy et al. 2008, Engelhard et al. 2014). In addition, the food web might be impacted as predation and competition increase. Higher average temperatures and milder winters will produce a northward shift of competitor species such as the solenette *Buglossidium luteum* (van Hal et al. 2010). It is currently unclear whether climate impacts predominantly fish distribution through processes that occur during the early life-

history stages (as suggested by Rindorf & Lewy 2006), or through temperature tolerances/preferences during the adult phase. Nevertheless, what is clear is that climate change will impact fish distribution and productivity in a complex way at the local scale. Hence, knowledge on dispersal patterns of each life stage at the local scale between each grounds takes a critical role in implementing an effective management plan.

4.4 Implications for fisheries management

The match between biological populations and management units is of primary importance to ensure the efficiency of conservation measures. However, the actual boundaries of management units have been largely answered to management concerns, such as political boundaries and harvesting location, and to a lesser extent represent scientific judgement (Waples & Gaggiotti 2006, Reiss et al. 2009). Mismatches between biological and management units have been documented in several species of the Northeast Atlantic Ocean (Kerr et al. 2017, Reiss et al. 2009). Failure to account for multiple components within a management unit may lead to the potential collapse of the less productive component and a loss of resilience to environmental change (Carvalho & Hauser 1994, Hutchinson 2008). Improving the current understanding of stock structure is a decisive step to ensure that stock assessment and management is properly designed and robust to uncertainties. Moreover knowledge on the proportional contribution of stocks to the exploitable population contributes to sustainable exploitation.

Evidence of putatively neutral genomic markers of sole does not point to a mismatch between biological and management units (**Chapter 3**). On the contrary, the number of biological units is lower than the number of management units. Putatively neutral genomic markers detected four main clusters: the Bay of Biscay and North Sea, English Channel, Celtic Sea, and the Baltic Transition Zone. However, given that not all adults sampled were caught on the spawning ground, we cannot exclude that our results underestimated the number of genetic subpopulations. Nevertheless, ignoring complex population structure and connectivity by splitting (or lumping) a population in different “substocks” is not advised either as it gives false estimates of population size and potential for resilience, which can translate in a suboptimal use of the resource (see Kerr et al. 2017 for a review on how to reconcile management and biological units). In contrast with neutral genetic markers and otolith shape, differences based on outlier

genetic markers and microchemistry were more pronounced at small spatial scales. Yet, while otolith microchemistry may point to groups of fish with shared environmental histories, these groups may mix on the spawning grounds and be best managed as a single unit. Thus defining which level of differentiation is sufficient for groups of fish to be managed as separate stocks is difficult and depends on a number of factors specific to one system (Campana 2005). Finally, to be able to combine economic and ecological objectives, fishery objectives have to be explicitly defined to evaluate trade-offs between risk to fish stocks, yield, employment, and other social objectives (Pilling et al. 2008).

An increasingly recognized way to protect stocks is the implementation of no catch zones and marine protected areas (MPAs) within a biogeographic region. The MPAs are supposedly connected through larval dispersal and juvenile or adult migration (IUCN 1994, NRC 2000). Monitoring studies show that networks of MPAs considering subpopulations in networks (i.e. metapopulations) are a key instrument for conservation, including fishery resources, through the replenishment of young stages, the increase in total biomass and abundance, the rebuilding of population structure and the strengthening of community diversity (Halpern 2003). Sound management of MPAs requires a good knowledge of metapopulation structure (Botsford et al. 2003, Palumbi 2004). One of the key parameter to implement network of MPAs is to understand the flow (direction and intensity) between larval sources (i.e. spawning grounds) and sinks (i.e. nursery grounds). However, few studies of the nursery grounds follow individual movement throughout life but most rather provide a temporally fixed screenshot of population structure at a given stage (e.g. juveniles on the nursery grounds) and work on larger spatial scale of thousands of kilometers. Understanding the exchange of larvae and juveniles between habitats at the scale of dispersal is paramount to design appropriate management tools for metapopulation persistence (Batista et al. 2015, Burgess et al. 2014, Krueck et al. 2017). The efficiency of MPAs relates to the quality of input data (Batista et al. 2015). Connectivity may have a higher priority than habitat quality for the design of MPAs (Berglund et al. 2012, OSPAR 2013). Krueck and co-authors (2017) recommend to prioritize locations that are self-replenishing, inter-connected, and/or important larval sources. As a matter of fact, the benefits of an area closed to fishing extends beyond the range of the reserve itself through the immigration of juvenile and adult fish or through spillover of eggs and larvae (Gell & Roberts 2003). Another key parameter to consider is that all species cannot be taken into account for designing an MPA (Jenkins & Stevens 2018). This PhD thesis focused on the connectivity patterns

of a single focal species. However, our approach may be extended to other flatfish species with similar life history traits (Barbut et al. 2019). Sedentary species will probably benefit more from an MPA than mobile species (Gell & Roberts 2003). Nevertheless, highly migratory species might benefit from protection of key habitats for their life cycle, such as nursery grounds (Gell & Roberts 2003). In addition, the community will indirectly benefit from closure to fishing through the positive impact on benthic prey and a more balanced size-frequency spectrum. On the other hand, MPAs should be designed for resilience without favoring a specific trophic level but to achieve a natural balance.

Conclusion

Understanding the origin and connections of larval and juvenile dispersers between spawning and nursery grounds, which is the focus of this PhD thesis, is a key component of community structure and crucial for the sustainability of populations. Recent developments in biophysical models, genomics, otolith shape and microchemistry clearly enhance traceability power and our ability in distinguishing evolutionary and ecological processes behind population structure.

APPENDICES

Appendix 1. Material and methods

A.1.1 Sample collection

Ninety two age-0 sole were sampled off the Belgian coast from late August to mid-September in 2013 and from mid-September to mid-October in 2014 (Fig. A.1; Table A.1) and at two stations in the Wadden Sea in September 2014. At each site, specimens were collected by beam trawling either with RV *Simon Stevin* (B-FishConnect project campaign), RV *Belgica* (Belgian contribution to the ICES Demersal Young Fish Survey, DYFS) or RV *Stern* (Dutch contribution to the ICES-DYFS).

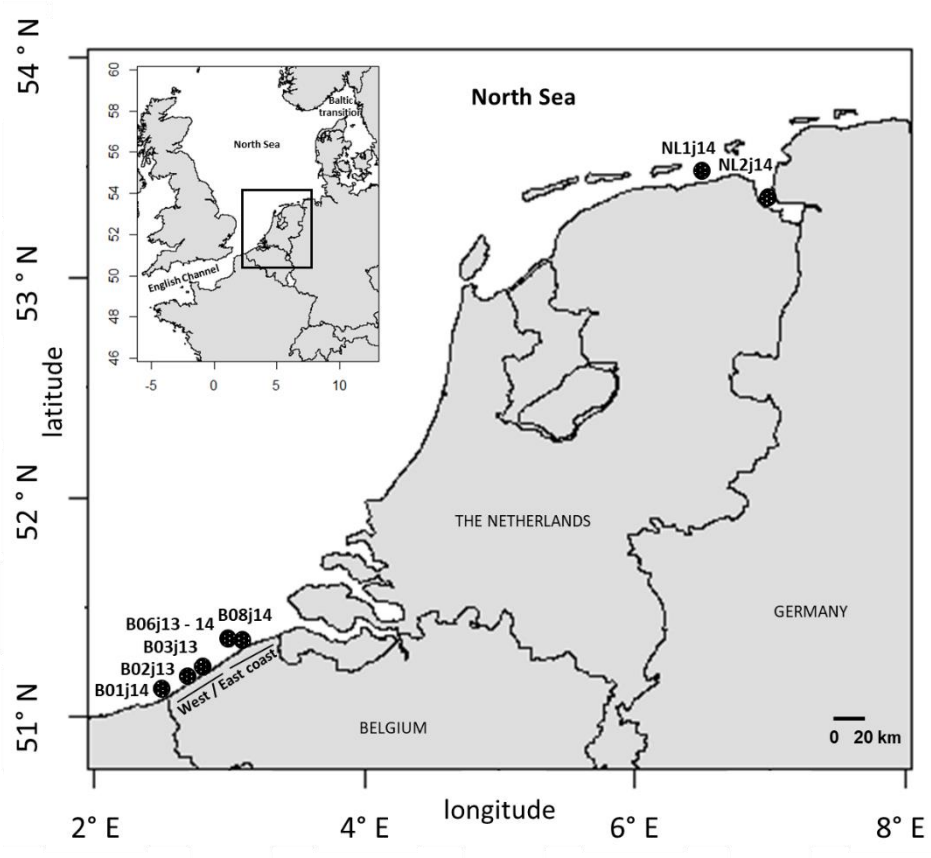


Figure A.1: Map of the sampling locations of settled sole in the North Sea and the Wadden Sea (n = 92)

Fish were frozen on site. In the laboratory, fish were thawed, measured for standard length to the nearest millimeter, fin clips were taken and immediately stored in absolute ethanol. Otoliths were extracted with acid washed non-metallic tweezers to avoid contamination, cleaned, sonicated, and then stored dry in plastic vials. All sole were age-0, which was confirmed by the absence of an annual ring in the otolith.

Table A.1: Sampling details of sole collected in the Southern North Sea, including the codes of the sampling sites ordered from West to East, sampling nursery ground (Belgian or Wadden Sea), latitude and longitude (in decimal degrees), sampling date (day/month/year), sample size (N), expected (He) and observed (Ho) heterozygosity, and inbreeding coefficient (F_{IS}). The codes of the sampling locations include the first three letters of the sampling site, the letter "j" for juveniles, and the sampling year.

Location	Nursery ground	Latitude	Longitude	Date	N	H_e	H_o	F_{IS}
B01j14	Belgian coast	51.13	2.70	15/09/2014	15	0.26	0.24	0.06
B02j13	Belgian coast	51.19	2.70	10/09/2013	15	0.26	0.24	0.06
B03j13	Belgian coast	51.35	3.00	28/08/2013	12	0.26	0.24	0.08
B06j13	Belgian coast	51.35	3.00	09/09/2013	7	0.26	0.23	0.08
B06j14	Belgian coast	51.35	3.00	16/09/2014	8	0.26	0.24	0.06
B08j14	Belgian coast	51.35	3.10	10/10/2014	17	0.25	0.24	0.06
NL1j14	Wadden Sea	53.48	6.49	16/09/2014	5	0.26	0.24	0.08
NL2j14	Wadden Sea	53.48	6.49	23/09/2014	13	0.26	0.24	0.08

A.1.2 Otolith shape analysis

Left and right sagittae of juveniles were analyzed following a procedure similar to Delerue-Ricard et al. (2018, **Chapter 1**).

A.1.3 Otolith elemental analysis

Thin sections of otoliths were transversally cut as close as possible to the nucleus as detailed in Delerue-Ricard et al. *in review* and **Chapter 2**. Three zones along the transect analyzed were retained for this work: (1) the 'otolith edge', i.e. the portion laid down during the last weeks before capture and reflecting the signature of the sampling location, (2) the 'post-settlement' portion just after the metamorphosis mark reflecting the signature of the nursery ground colonized at benthic settlement, and (3) the 'larval' area just outside the core, reflecting the signature of the fish natal source (Fig. 2.2).

A.1.4 SNP genotyping, genetic variation and relatedness

Genomic DNA was extracted from juveniles and analyzed as detailed in **Chapter 3**.

A.1.5 Within- and between spawning and nursery ground signature

We estimated the effect of nursery, site and standard length of fish using a permutational multivariate analyses of variance (permanova) performed on distance matrices as implemented in the *adonis* function of the *vegan* package (Oksanen et al. 2013). When the site explanatory variable was significant, the *pairwise.adonis* function captured which pairs of sites were significantly different. Significance levels were corrected for multiple testing using Benjamini-Hochberg correction.

We used Principal component analysis (PCA, *stats* R package) for each marker to visualize differentiation among individuals and sampling sites. The significance of the differentiation for each marker was tested using linear discriminant analyses (LDA, White & Ruttenberg 2007, *MASS* package) to quantify the probability of re-assigning individuals to sampling location. LDA quantified the number of individuals sampled in one site and assigned to another site. The misassigned individuals could be identified.

We used Mantel tests and redundancy analyses (RDA) to test for significance of the correlations between markers. Both approaches are complementary: Mantel tests assess the significance of linear relationships between distance matrices while RDA is more powerful when looking at variables relationships and also detects non-linear relationships (Legendre & Fortin 2010). Correlations of distance matrices were tested using a Mantel test (*mantel.rtest* function of the *ade4* package) with 9999 permutations (Dray & Dufour 2007). RDA (*vegan* package, Oksanen et al. 2013) was used to investigate the covariation between (1) the elemental signature of the larval phase and genetic markers (representing the spawning ground signature), (2) elemental signature of sampling location and otolith shape (representing the nursery ground signature). Each RDA was based on the first two PCs of each marker. To visualize the combined effect of markers to estimate spawning and nursery ground signatures, heatmaps of similarities

were created using the *cheatmaps* package. Model selection was applied to test for the best clustering method ('ward.D', 'single', 'complete', 'average', 'mcquitty').

A.1.6 Similarity Network Fusion analysis

The availability of multiple marker types emphasizes the need for integrative methods to identify congruent patterns. However, combining different markers remains a major bottleneck due to three computational challenges: (1) the differences in scale, collection bias and noise in each data set; (2) the small number of samples compared to the amount of measurements (as in genome-scale genetic markers); (3) the redundant nature of the information provided by different markers (Wang et al. 2014). The common approach is to analyze separately different markers and to qualitatively estimate the difference and similarity in patterns. This approach implicitly assumes a hierarchy between markers such that morphological data that are time efficient and less expensive may be prioritized compared to other more expensive techniques such as molecular markers. It constitutes an over-simplification as subtle patterns of diversity (linked to putatively adaptive loci or not) are less likely to be detected. Moreover, cases where different markers are seemingly conflicting are less likely to be published.

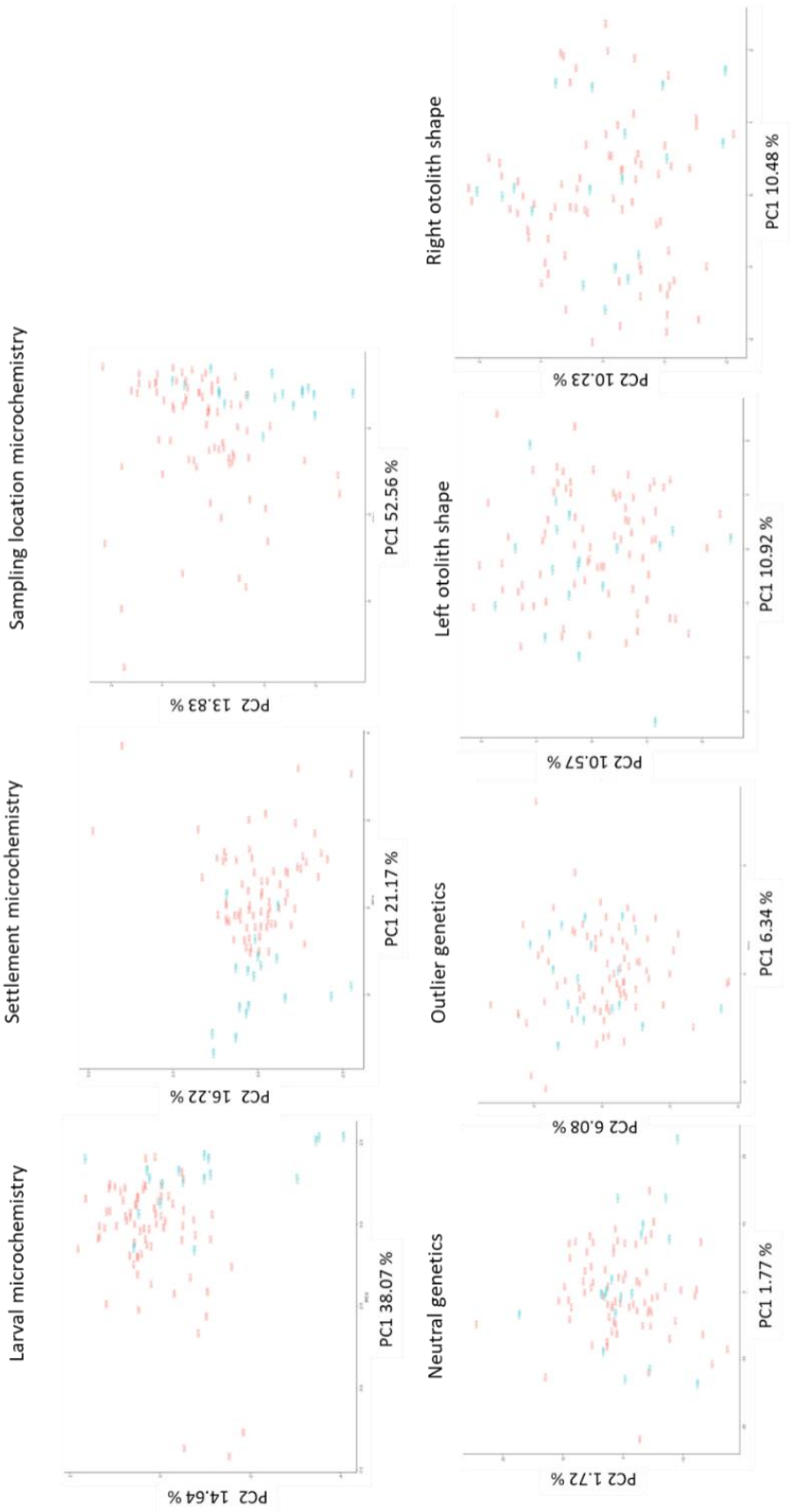
The simplest way to combine biological data is to concatenate normalized measurements. To reduce the number of variables, ordination techniques such as principal component analysis (PCA) are commonly used. However, concatenation of markers may decrease the signal-to-noise ratio (Wang et al. 2014). In the present study, we applied a different approach to combine multiple markers which does not lead to the loss of complementary information. Similarity Network Fusion (SNF) is robust to noise and data heterogeneity and can derive useful information even from a small sample size (Wang et al. 2014).

SNF consists of two steps: first, SNF converts each type of markers into a network based on similarity matrix of the normalized data. Once each marker is in comparable formats, it then fuses all networks into a single network. Common or complementary patterns of all or a subset of the data are retained into the fused network. The fused network is updated with each additional marker in a non-linear iterative process. Similarities among objects will become more pronounced while networks supported by only one type of marker will lose importance. The proof of concept was conducted in cancer research (Wang et al. 2014) and has been applied to

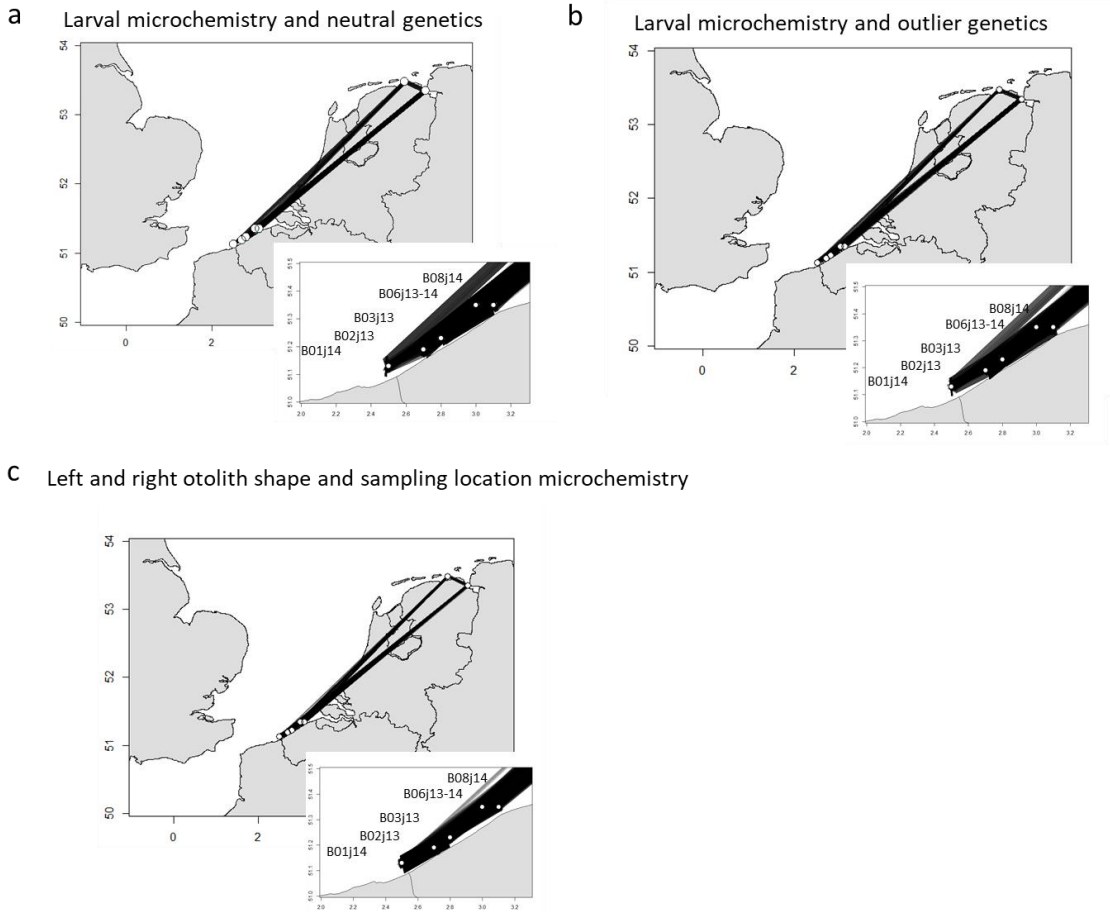
diabetes and neurology medical research (Dhifallah & Rekik 2018, Li et al. 2015) but the method can be adapted to any area of science, and especially to natural sciences, where consensus on clusters of individuals or populations would gain by being identified based on the combination of different markers (Wang et al. 2014).

Euclidian distance matrices were calculated for all seven markers as follows: on 21 individual-derived Fourier harmonics for left and right otolith shape, on individual-derived otolith elemental signatures of eight chemical elements for larval, settlement and sampling location, on individual allele frequencies for 1738 neutral and 34 outlier SNP loci. Distance matrices were converted into affinity matrices using the *affinityMatrix* function of the *SNFtool* package (Wang et al. 2014). We set the hyperparameter $\mu = 0.3$, which is within the range recommended by the author of the package (0.3 – 0.8). Due to the high similarity between neighboring sites, the nearest number of neighbors (KNN) was set low (KNN = 5) to favor high similarities compared to low similarities to emphasize local patterns of geographical structure. The analysis was repeated 20 times for each marker.

Appendix 2: Supplementary material



Supplementary Figure A.1: PCA of the nursery signatures of sole based on all seven markers. Color represent the nursery ground (red for the Belgian and blue for the Wadden Sea nursery grounds)



Supplementary Figure A.2: Fused similarity networks among eight sites of sole characterizing spawning ground based on larval elemental and (a) neutral or (b) outlier genetic markers and, characterizing nursery ground based on (c) left and right otolith shape and sampling location elemental signature.

Supplementary Table A.1: Significant pairwise comparisons of otolith elemental signatures between sampling location between the Belgian and the Wadden Sea nursery grounds and within the Belgian nursery ground, with sampling locations within the Belgian nursery ground ordered from West to East. F = f statistics, R^2 = variance explained, P values were adjusted after correcting for multiple testing with the Benjamini-Hochberg correction. See table 1 for the list of site abbreviations.

Spatial scale	**Site 1 vs. site 2**	**F**	**R^2**	***P value***	***P value adjusted***
Belgian vs. Wadden Sea nursery ground	NL1j14 vs. B01j14	39.724	0.688	0.001	0.004
	NL2j14 vs. B01j14	29.027	0.537	0.002	0.006
	NL1j14 vs. B02j13	16.305	0.475	0.001	0.004
	NL2j14 vs. B02j13	10.174	0.289	0.002	0.006
	NL1j14 vs. B03j13	8.293	0.356	0.009	0.022
	NL1j14 vs. B06j13	17.344	0.634	0.005	0.013
	NL2j14 vs. B06j13	6.550	0.278	0.013	0.028
	NL1j14 vs. B06j14	9.892	0.473	0.011	0.025
Belgian nursery ground	B01j14 vs. B02j13	14.260	0.337	0.001	0.004
	B01j14 vs. B03j13	21.523	0.463	0.001	0.004
	B01j14 vs. B06j14	15.994	0.432	0.002	0.006
	B01j14 vs. B06j13	14.363	0.418	0.002	0.006
	B01j14 vs. B08j14	71.788	0.705	0.001	0.004
	B08j14 vs. B02j13	28.493	0.487	0.001	0.004
	B08j14 vs. B03j13	13.326	0.330	0.001	0.004
	B08j14 vs. B06j14	14.070	0.380	0.001	0.004
	B08j14 vs. B06j13	21.006	0.488	0.001	0.004

Supplementary Table A.2: pairwise F_{ST} values of the neutral (above the diagonal) and outlier SNP loci (below the diagonal) of sole. Significant pairwise F_{ST} values ($\alpha = 0.05$) are indicated in bold.

	B01j14	B02j13	B03j13	B06j13	B06j14	B08j14	NL1j14
B01j14		0.002	0.002	0.003	0.000	**0.003**	0.003
B02j13	0.018		0.000	0.003	0.002	**0.005**	**0.003**
B03j13	0.020	0.037		-0.001	0.000	0.003	0.002
B06j13	0.008	-0.010	0.048		-0.002	0.004	0.003
B06j14	0.015	0.012	0.016	0.004		0.002	-0.002
B08j14	0.023	-0.002	0.037	-0.002	0.007		**0.003**
NL1j14	0.014	0.008	0.003	0.002	0.006	0.010	

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LIST OF PUBLICATIONS

Delerue-Ricard, S., Stynen, H., Barbut, L., Morat, F., Mahé, K., Hablützel, P.I., Hostens, K., Volckaert, F.A.M., 2018. Size-effect, asymmetry, and small-scale spatial variation in otolith shape of juvenile sole in the Southern North Sea. Hydrobiologia xx: 1-14. https://doi.org/10.1007/s10750-018-3736-3

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Connectivity of larval and juvenile common sole at a small and large spatial scale

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JULY 2019